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Steam thermotherapy strongly reduces *Botrytis* in strawberry transplants with no or minor negative effects on plant growth and yield

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Abstract The effect of steam thermotherapy on *Botrytis* spp. populations in strawberry transplants was evaluated. Tray plants rooted in 0.2 L peat plugs of seasonal flowering cvs. Falco, Sonsation, and Soprano, and everbearing cvs. Favori and Murano were pre-treated with steam at 37 °C for 1 h, followed by 1 h at ambient temperature and air humidity, and then 2 or 4 h steam treatment at 44 °C. Except for one cultivar with a slight reduction in yield, there were no negative effects on plant performance. Compared to untreated transplants, mean incidence of *Botrytis* on the five cultivars was reduced by 43 and 86% with the 2 and 4 h treatments, respectively. Within cultivars the reduction was significant in 2 and 3 experiments following the 2 and 4 h treatments, respectively. Sclerotia from four different isolates of *Botrytis* were subjected to treatment including 4 h of steam thermotherapy and subsequently tested for viability. Following 14 days of incubation, 90 to 100% (mean 97%) of treated sclerotia failed to produce mycelial growth compared with untreated sclerotia, which all germinated and produced mycelia. *Botrytis* isolates recovered from both treated and untreated strawberry transplants were tested for resistance to seven fungicides, including boscalid, fenhexamid, fludioxonil, fluopyram, pyraclostrobin, pyrimethanil and thiophanate-methyl. Multiple fungicide resistance was common; 35.5% of isolates were resistant to fungicides from at least three FRAC groups. Results indicate that steam thermotherapy treatment strongly reduces populations of *Botrytis* spp., including fungicide-resistant strains, in strawberry transplants with negligible negative impacts on the transplants.

Keywords Fungicide resistance · Grey mould · Heat treatment · Multiple fungicide resistance · Sclerotia

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Introduction

Grey mould, caused by *Botrytis* spp., is one of the most important fungal diseases in strawberry production, and in Norway up to 70% yield reduction has been reported if no fungicide treatments were carried out (Eikemo et al., 2013). Infection often remains asymptomatic initially, followed by tissue colonization and sporulation gradually increasing

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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, so healthy-appearing transplants produced under certified guidelines can become a primary source of inoculum when fields are established (Oliveira et al., 2017)

In commercial strawberry production, transplants are produced in plant nurseries and then shipped to production areas. Previous studies have demonstrated that strawberry transplants are a source of fungicide-resistant *Botrytis* spp. inoculum (Nielsen et al., 2022; Oliveira et al., 2017; Weber & Entrop, 2017). The introduction of resistant strains with planting material increases the risk of grey mould disease control failure in strawberry fruit production.

Current management of grey mould relies on the use of fungicides (Petrasch et al., 2019; Wedge et al., 2007). In addition to causing latent infections, *Botrytis* spp. have a polycyclic life cycle, so appropriate timing and frequency of fungicide applications are vital for effective disease control (Mertely et al., 2002; Powelson, 1960). However, increasing fungicide resistance in *Botrytis* populations threatens the efficacy of chemical control measures (Fernández-Ortuño et al., 2014; Hahn, 2014; Leroch et al., 2013). *Botrytis cinerea* is among *Botrytis* species categorized as high-risk pathogens for developing fungicide resistance due to high genetic variability, a polycyclic life cycle, short generation time, abundant sporulation and dispersal potential (Atwell et al., 2015; FRAC, 2019; Hahn, 2014; Leroux et al., 2002; Lucas et al., 2015; Veloukas et al., 2014). With the increasing threat of fungicide resistance, non-chemical approaches to reduce this pathogen population are crucial.

Thermotherapy, primarily warm water dipping of strawberry transplants, is an established method to control numerous phytopathogenic microbes and pests (Goheen & McGrew, 1954; Miller & Stoddard, 1956; Smith & Goldsmith, 1936; Staniland, 1953). Studies have nonetheless revealed variation in how well different cultivars tolerate thermotherapy. Mellor and Fitzpatrick (1961) found strawberry plants tolerated heat treatments better if they had well-developed roots and soil moisture was low. In another study, storing plants for two weeks at 0 °C prior to treatments increased hot water tolerance (Goheen et al., 1956). However, Turechek and Peres (2009) observed the opposite effect; cold stored plants of cv. Ventana tolerated heat treatments poorly, but when freshly dug plants were used, this cultivar was among those which performed best post treatment and tolerated 48 °C for up to 2 h. The cv. Strawberry Festival tolerated warm water treatments of 44 °C for up to 4 h well regardless of whether it was cold stored or not (Turechek & Peres, 2009). In other trials, non-dormant runner plants of seven cultivars all tolerated treatments killing endoparasitic nematodes (Staniland, 1953).

Brown et al. (2016) developed a technique for warm water dipping of strawberry transplants in Florida. It starts with a pre-conditioning treatment at 37 °C to induce heat shock proteins that increase heat tolerance in the strawberry plants. This is followed by exposing plants to ambient conditions for 1 h, and finally a 4 h treatment at 44 °C to eradicate pathogens. This protocol has been adapted to steam thermotherapy treatment of transplants to eradicate several strawberry pathogens, including *B. cinerea*, *Colletotrichum acutatum*, *Phytophthora cactorum*, and *Xanthomonas fragariae* (Baggio et al., 2021; Da Silva Jr et al., 2019; Turechek et al., 2021; Wang et al., 2017; 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).

Objectives of this study were to: i) test the effect of steam thermotherapy treatment of rooted plug strawberry transplants on yield and quality parameters after planting; ii) test the effect of steam thermotherapy on latent *Botrytis* infections; iii) assess viability of *Botrytis* sclerotia subjected to steam thermotherapy treatment; and iv) test *Botrytis* isolates obtained from strawberry transplants for fungicide resistance.

Materials and methods

Strawberry transplants

Five cultivars of imported strawberry transplants were used for experiments. Everbearing cultivars included Favori, Murano and Soprano and seasonal flowering cultivars included Falco and Sonsation. All transplants were imported from the Netherlands as tray plants rooted in 0.2 L peat plugs and stored at -1.5 °C during winter. Transplants were transported

to the research facilities at Ås in south-eastern Norway in spring, where they were kept outdoors (0 to 10 °C) for 1–2 days prior to experiments.

Steam thermotherapy treatment of strawberry transplants

The cold stored tray plants were treated with steam thermotherapy according to a protocol developed in Florida (Brown et al., 2016; Turechek et al., 2013, 2021). The protocol entailed pre-treatment at 37 °C, followed by 1 h in ambient conditions (approximately 20–25 °C and 30–50% RH), and then 2 or 4 h treatments at 44 °C. The cabinet used for aerated steam treatments was constructed by the company Myhrene AS (Lier, Norway), currently named Moleda AS. The cabinet structure and function may be further studied elsewhere (Baggio et al., 2021; Johansen et al., 2022; Turechek et al., 2021).

For cv. Soprano, each experiment included 120 strawberry transplants arbitrarily divided into three equal groups: non-treated control, 2 h and 4 h treatment at 44 °C. Transplants to be treated with steam thermotherapy 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 in close vicinity to the plants in the centre of the tray. After treatment, aerial parts of half of the transplants in each group (20 plants) were removed and stored at -20 °C until used for analysis of *Botrytis* incidence. The other half of the transplants were used for analysis of growth parameters. The experiment with cv. Soprano was conducted twice during April 2020.

For the remaining four cultivars, steam thermotherapy treatments were conducted in two runs of the aerated steam chamber in late April and early May 2020, respectively. The first run was with cvs. Favori and Murano (54 plants of each) and the second was with cvs. Falco and Sonsation (64 plants of each). Transplants of each cultivar were arbitrarily divided into three treatment groups as previously described. After treatment, plant parts including whole leaves and leaflets were collected from the transplants and stored at -20 °C for analysis. The crown and two or more green leaves were still attached, and all plants were then brought to a high plastic tunnel research facility (a polyethylene covered, ventilated but not heated greenhouse) for further growth and yield assessments.

Effect of steam thermotherapy on *Botrytis* in transplants

Transplants from the control and steam thermotherapy treatment groups were analysed for the presence of *Botrytis* infections. Plant material was removed from freezer storage, thawed and rinsed in running tap water. Plant material was then dried and placed in 14 cm diameter plates with 12.5 cm diameter Whatman™ filter papers moistened with 2 ml autoclaved water to maintain high humidity. The plates were sealed in plastic bags and incubated at ambient temperature. After seven days, plant parts were examined under a stereo microscope (14×) for the presence of *Botrytis* conidiophores and conidia.

In each of the two experiments with cv. Soprano, four replicates included five transplants in each. For each transplant, three to four leaves were incubated to assess *Botrytis* incidence. Mean incidence (%) per plant was calculated by dividing number of leaves with *Botrytis* by 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. Incidence (%) was calculated as described above, and, in addition, the area of each leaf unit covered with *Botrytis* was used to calculate mean severity (%) per replicate.

Isolates of *Botrytis* were obtained from cvs. Falco, Favori and Murano, including 23, 24 and 15 isolates from untreated, 2 h or 4 h treated transplants, respectively (Supplementary Table 1). Conidia were transferred directly from infected plant material to 9 cm diameter plates containing acidified potato dextrose agar (APDA, Difco™ Potato dextrose agar amended with 0.2% w/v tartaric acid). After three days, plates were moved to continuous light to induce sporulation, and conidia from each bulk spore isolate were subsequently collected and stored at -20 °C in a 20% glycerol solution until used to start new plates for production of conidia for fungicide resistance testing.

Effect of steam thermotherapy on plant growth and yield

Transplants of cv. Soprano from the treatment groups (control, 2 h, 4 h) were potted in 3.5 L pots filled with fertilized and limed peat soil for growth and yield assessments. The potted transplants were placed in a randomized block design with five plants in each of four replicates in each treatment. Pots were arranged

on wooden pallets approximately 15 cm above ground in open air between two greenhouses. The plants were overhead irrigated with a shower hose as needed and fertilized once a week from the sixth week after potting. 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.

Transplants of cvs. Favori, Murano, Falco, and Sonsation 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, plant height, number of fruits, fruit weight, above ground fresh and dry weight, and root dry weight. The duration of the assessment period was 14, 16, and 23 weeks from potting to final assessment for cv. Soprano (dry weight recorded two weeks later), cvs. Falco and Sonsation, and cvs. Favori and Murano, respectively.

Steam thermotherapy treatment of sclerotia of *Botrytis* spp.

Sclerotia from four single-spore *Botrytis* isolates obtained from strawberry in Norway were treated with steam thermotherapy. The four isolates included were identified as *Botrytis* group S (96/16–16.4 and 96/16–19.10, referred to as BS1 and BS2), one *B. cinerea* sensu stricto (96/16–15.3, referred to as Bc), and one *B. pseudocinerea* (96/16–18.5, referred to as Bp). The isolates were characterized and examined for the presence of fungicide resistance-conferring mutations in a previous study (Nielsen et al., 2022). The BS1, BS2 and Bc isolates had been stored as conidia suspensions in a 20% glycerol solution at -20 °C, and the Bp isolate had been stored as dry sclerotia at -80 °C. For each isolate, either 5 µl of conidia suspension or a single sclerotium was transferred to a 4 cm diameter plate containing APDA and incubated at 20 °C. Once the mycelial growth approached the periphery of the plate, mycelium was transferred to new 9 cm PDA plates for each isolate 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 from sclerotia by rubbing with filter paper and rinsing under tap water in a Retsch stainless steel sieve (CISA, Spain). Rinsed sclerotia were placed on top of the stainless-steel sieve with filter paper and allowed to dry at room temperature. One hundred arbitrarily selected sclerotia from each isolate were weighed. Sclerotia from each isolate were then distributed into groups of seven and placed in 5×5 cm^2 muslin cloth bags closed with wire twist ties.

Sclerotia were treated with steam thermotherapy in the aerated steam chamber as described above for the 4 h treatment at 44 °C. For each of the four isolates, 16 bags containing sclerotia were divided into four replicates of four. Bags belonging to different replicates were suspended in four different IFCO trays, and each tray was equipped with a Tinytag TGP-4500 logger. Equivalent sets of 16 bags of sclerotia per isolate were prepared to remain outside the steam chamber as the control treatment. After treatment, both treated and control groups of sclerotia were brought to the laboratory and surface sterilized in a 1% NaOCl solution for one minute and then submerged twice in distilled water. Six sclerotia from each bag were transferred individually to wells containing 1 ml PDA in 24-well plates with lids (Nunclon™ Delta Surface, Thermo Fisher Scientific, Denmark). The plates were incubated with lids in the growth chamber described above at 20 °C with a 12 h light/dark cycle and 85% RH. Germination was assessed using a stereo microscope (14×) on the 14th day following incubation. The experiment was conducted twice.

Mycelial growth assay to test for fungicide resistance

Isolates of *Botrytis* (not single-spore) obtained from transplants were tested for fungicide resistance with a mycelial growth assay adapted from Fernández-Ortuño et al., (2014). The isolates were tested for resistance 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). Stock solutions were made for each fungicide with liquid media and used to amend growth media to obtain discriminatory concentrations (Table 1), and 1.5 ml of amended media was

pipetted into wells of 24-well plates (Nunclon™ Delta Surface, Thermo Scientific, Denmark).

A total of 62 *Botrytis* isolates obtained from different transplant cultivars which had been included in different thermotherapy treatment groups were selected for testing with the mycelial growth assay. Isolates were removed from freezer storage, and the spore solutions were transferred to new PDA plates. Once the isolates sporulated, conidia were transferred with sterile toothpicks to the centre of the growth medium in each well in the 24-well plates. After conidial transfer, plates were incubated at 20 °C in the dark for four days. Resistance categories used for assessment were based on diametric growth of mycelium in relation to 15 mm diameter agar wells; sensitive (no mycelium growth), low resistant (<20%), moderately resistant (20–50%) and resistant (>50%) (Fernández-Ortuño et al., 2014). Visual assessment was done with a stereo microscope (14×).

Statistical analysis

R version 4.0.4 (2020–06-22) was used for statistical analyses. Data obtained from the experiments were analysed with either one- or two-way analysis of variance (ANOVA). 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 an effect of isolate on sensitivity of sclerotia to thermotherapy treatment. Post-hoc analysis of the Chi-square test was done with Fisher's Exact Test.

Results

Temperature profiles

The temperature was reached around 20 min 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 h of treatment was followed by a slight but very brief drop in temperature, i.e., a drop of less than 2 °C for 3–4 min.

Growth and yield of strawberry transplants

There were marginal differences in values for registered growth and yield parameters among the cultivars for the different treatments (Table 2). However, for cv. Falco, yield was significantly reduced by 22% for the transplants which received the 4 h treatment at 44 °C compared to the non-treated. There were no significant differences in yield between the treated and the non-treated for the other cultivars.

Effect of steam thermotherapy on *Botrytis* in strawberry transplants

The mean *Botrytis* incidence in strawberry transplants in six experiments was reduced by 43 and 86% for transplants treated for 2 or 4 h at 44 °C, respectively (Fig. 1, $P < 0.001$). For specific cultivars, *Botrytis* incidence in the 2 h treatment was not significantly lower than the control, except for cv. Soprano. In the 4 h treatment, the reduction in *Botrytis* incidence varied between 71 and 100%

Table 1 Fungicides and discriminatory concentrations used for mycelial growth assay

Fungicide	Discriminatory concentration (mg/L)	FRAC code	Growth medium
Control	-	-	Czapek-Dox agar
Boscalid	75	7	Yeast Bacto acetate agar
Fenhexamid	50	17	Malt extract agar
Fludioxonil	0.5	12	Malt extract agar
Fluopyram	10	7	Yeast Bacto acetate agar
Pyraclostrobin[a]	10	11	Malt extract agar
Pyrimethanil	4	9	Czapek-Dox agar
Thiophanate-methyl	100	1	Malt extract agar

[a]Salicylhydroxamic acid dissolved in methanol was added to obtain a final concentration of 100 mg/L in the amended medium

Table 2 Days to anthesis and growth and yield performance per plant of strawberry transplants after steam thermotherapy treatments

Cultivar	Treatment[a]	Days to anthesis[b]	No. of runners	No. of leaves	No. of crowns	Yield (g)	Plant height (cm)	Fresh shoot weight (g)	Dry shoot weight (g)	Dry root weight (g)
'Falco'	0 h	33.5 a	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]Treatments included untreated control (0 h) or pre-treatment at 37 °C in aerated steam chamber, 1 h in ambient air, and then either 2 h or 4 h at 44 °C in an aerated steam chamber

[b]Values 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]Was not examined

compared to the control, but the reduction was significant only for cv. Murano ($P = 0.018$) and the two experiments with cv. Soprano ($P < 0.001$). The mean severity of *Botrytis* for cvs. Falco, Favori, Murano and Sonsation was reduced by 32% and 84% for the 2 and 4 h treatments, respectively, but it was significant only for the latter (Table 3).

Effect of steam thermotherapy on viability of *Botrytis* sclerotia

Results from the two experiments were combined for analysis. The size of sclerotia varied greatly among the isolates. Mean weights per sclerotium were 1.6, 12.6, 15.3, and 10.4 mg, for isolates BS1, BS2, Bc,

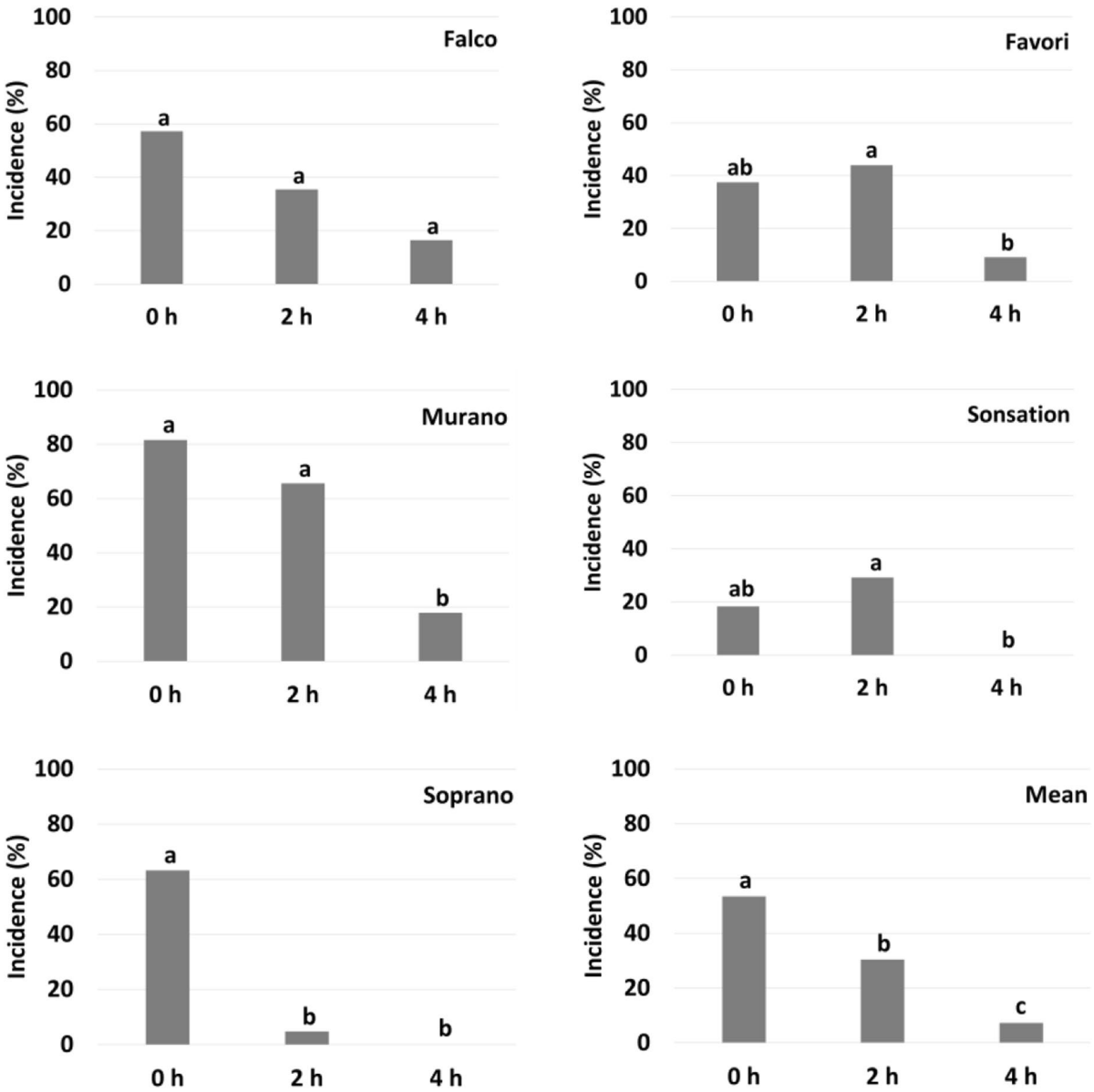

Fig. 1 Incidence (%) of *Botrytis* spp. observed on plant tissue of five strawberry cultivars; mean of six experiments, two with cv. Soprano and one each with cvs. Falco, Favori, Murano, and Sonsation. Plants were either not treated (0 h) or pre-treated at 37 °C in aerated steam, 1 h in ambient air,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

Table 3 Severity (%) of *Botrytis* spp. on leaves detached from 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 or 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

and Bp, respectively. All 768 untreated sclerotia produced mycelial growth in the 24-well agar plates. Of 192 sclerotia of each isolate that underwent steam thermotherapy, 0, 2, 3, and 18 sclerotia were found to be viable for isolates BS1, BS2, Bp and Bc, respectively. Therefore, 90 to 100% (mean 97%) of the treated sclerotia failed to produce mycelial growth in the 14 days following steam thermotherapy treatment. A 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, $P < 0.001$, and a 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 resistance in *Botrytis* from strawberry transplants

A total of 62 *Botrytis* isolates were tested for fungicide resistance with the mycelial growth assay. There was resistance to all fungicides tested among *Botrytis* isolates obtained from untreated strawberry transplants, and resistance was detected to all fungicides except fluopyram in isolates obtained from steam thermotherapy treated transplants (Table 4). There was a trend for resistance frequencies to be lower for isolates from transplants in the 4 h treatment group compared to the control for all fungicides except fludioxonil and pyraclostrobin. Multiple fungicide resistance was common. Isolates obtained from untreated transplants were resistant to up to five different fungicide groups as classified by FRAC, the Fungicide Resistance Action Committee (FRAC, 2022), and those from treated transplants were resistant to up to four FRAC groups (Table 5). Percentages of isolates resistant to fungicides from three or more FRAC groups were 56.5% and 23.1% for isolates obtained from untreated and steam thermotherapy treated transplants, respectively.

Resistance frequencies and multiple fungicide resistance also varied depending on the strawberry

Table 4 Fungicide resistance frequencies for *Botrytis* isolates obtained from strawberry transplants. Values are presented for the same 62 isolates both by treatment and by cultivar from which isolates were obtained

		Fungicide resistance frequencies[a]						
	n[b]	Bos	Fen	Flud	Fluo	Pyra	Pyri	Thio
Treatment[c]								
0 h	23	56.5	26.1	4.3	4.3	78.3	47.8	52.2
2 h	24	37.5	8.3	0.0	0.0	95.8	33.3	29.2
4 h	15	26.7	13.3	6.7	0.0	100.0	20.0	46.7
Cultivar								
Falco	16	62.5	25.0	0.0	0.0	93.8	12.5	31.3
Favori	19	21.1	0.0	0.0	0.0	78.9	26.3	15.8
Murano	27	44.4	22.2	7.4	3.7	96.3	55.6	66.7

[a]A mycelial growth assay was used to determine resistance to boscalid (Bos), fenhexamid (Fen), fludioxonil (Flud), fluopyram (Fluo), pyraclostrobin (Pyra), pyrimethanil (Pyri), and thiophanate-methyl (Thio). Results are for isolates categorized as resistant (R), except for boscalid, for which moderately resistant (MR) is included in addition

[b]Number of isolates tested

[c]Transplants were either untreated (0 h) or pre-treated at 37 °C in an aerated steam chamber, exposed to ambient conditions for 1 h, and then treated for either 2 h or 4 h at 44 °C in the aerated steam chamber

Table 5 Multiple fungicide resistance in 62 *Botrytis* isolates presented by both steam thermotherapy treatment and by strawberry cultivar from which isolates were obtained

		Multiple fungicide resistance[a]						
	n[b]	% 0R	% 1R	% 2R	% 3R	% 4R	% 5R	% 6R
Treatment[c]								
0 h	23	13.0	8.7	21.7	26.1	17.4	13.0	0.0
2 h	24	4.2	25.0	45.8	12.5	12.5	0.0	0.0
4 h	15	0.0	20.0	60.0	6.7	13.3	0.0	0.0
Cultivar								
Falco	16	6.3	31.3	31.3	6.3	12.5	12.5	0.0
Favori	19	15.8	31.6	47.4	5.3	0.0	0.0	0.0
Murano	27	0.0	0.0	40.7	29.6	25.9	3.7	0.0

[a]A mycelial growth assay was used to determine resistance and results are for isolates categorized as resistant (R), except for boscalid, for which moderately resistant (MR) is included in addition. Percentage of FRAC groups to which isolates were resistant is indicated by 0-6R. FRAC groups tested with the mycelial growth assay included 1 (thiophanate-methyl), 7 (boscalid and fluopyram), 9 (pyrimethanil), 11 (pyraclostrobin), 12 (fludioxonil), and 17 (fenhexamid)

[b]Number of isolates tested

[c]Transplants were either untreated (0 h) or pre-treated at 37 °C in an aerated steam chamber, exposed to ambient conditions for 1 h, and then treated for either 2 h or 4 h at 44 °C in the aerated steam chamber

cultivar from which isolates were obtained (Tables 4 and 5). There was notably less resistance detected in isolates obtained from cv. Favori transplants; no resistance was detected for fenhexamid, fludioxonil, or fluopyram, and resistance and the resistance frequency for boscalid was lower than for the other two cultivars. All isolates which were MR or R to fluopyram in the mycelial growth assay were also MR or R to boscalid.

Discussion

The results of this study demonstrate the potential for steam thermotherapy treatment to reduce *Botrytis* in strawberry transplants with negligible negative consequences for subsequent growth and yield. Rooted plug strawberry transplants used in experiments were imported from commercial nurseries, and they carried latent *Botrytis* infections, from which isolates were obtained and found to be resistant to up to five fungicides. Steam thermotherapy treatment in an aerated steam chamber, including 4 h at 44 °C, effectively suppressed *Botrytis* in transplants, which can contribute not only to better grey mould disease control but also fungicide resistance management. *Botrytis* sclerotia were also treated with steam thermotherapy, and a mean of only 3% produced mycelial growth afterwards.

Assessments of growth and yield parameters from our study corroborate previous findings from Florida and Norway, which demonstrated compelling effects of steam thermotherapy treatments for several pathogens in strawberry transplants without negative growth and yield effects (Da Silva Jr et al., 2019; Turechek et al., 2021; Wang et al., 2019; Zuniga, 2018; Zuniga & Peres, 2017). Strawberry transplants may vary in tolerance of heat treatments (Goheen et al., 1956; Mellor & Fitzpatrick, 1961; Turechek & Peres, 2009). 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 cultivars used in this study tolerated steam thermotherapy treatments well, except cv. Falco, for which yield was reduced by around one fifth.

Although the effect varied by cultivar, the overall reduction of *Botrytis* in transplants was significant for both the 2 and 4 h steam thermotherapy treatments. Reduction in incidence was not statistically significant in the cases of cvs. Favori and Sonsation, but there was still marked reduction, and these cultivars also had lower incidence of *Botrytis* in the untreated transplants, which may have contributed to the lack of statistical significance between the values. 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 temperatures, but high temperatures may hamper disease development and viability of perennating structures (Droby & Lichter, 2007; Jarvis, 1977; Oliveira et al., 2017). Steam treatment has also been demonstrated to halt *Botrytis* mycelial growth *in vitro* (Lagopodi et al., 2009). Although water on the plant surface should rapidly conduct heat into the plant during steam thermotherapy treatment, to some extent and for a period after start of the treatment, the inner plant tissue containing *Botrytis* structures may have been protected from the high temperature during steaming. It has also been demonstrated that heat exposure can increase production of heat shock proteins in *B. cinerea*, and this could contribute to heat tolerance (Lichter et al., 2003).

We also chose to examine the effect of steam thermotherapy on sclerotia in this study. In *Botrytis*, sclerotia production is triggered by darkness and low temperatures (Jarvis, 1977), conditions to which strawberry transplants are exposed during storage and transport. In addition, these structures can often survive more severe conditions than spores or mycelium (Willetts, 1971). Sclerotia in dead plant material or soil can serve as inoculum (Strømeng et al., 2009) and, as rooted plug transplants are accompanied by soil, it is relevant to examine how steam thermotherapy treatment affects sclerotia. In this study, steam thermotherapy treatment including 4 h at 44 °C strongly reduced viability of sclerotia produced by four *Botrytis* isolates. In a study in Florida, zero to 44% of sclerotia survived when immersed in a 44 °C water bath for 4 h, and sclerotia could also survive 4 h at 48 and 52 °C (Zuniga, 2018). In the latter experiments with sclerotia, there was great variability among isolates in susceptibility to heat treatments, but there was no indication provided for this discrepancy. There could be several reasons for the differences in effect of steam thermotherapy on sclerotia from the four isolates used in the present study. We observed that none of the treated sclerotia of BS1 produced mycelium, and these were by far the smallest sclerotia. Conversely, sclerotia of isolate Bc were the largest and had the most sclerotia able to produce mycelium following treatment, so size may have been a contributing factor. *Botrytis* is known for its high genetic variability, so other unseen factors may have affected difference in heat tolerance between the isolates (Atwell et al., 2015; Garfinkel, 2021; Walker, 2016). One of the isolates used to produce sclerotia was *B. pseudocinerea*, and two of the *B. cinerea* isolates were group S. Also, sclerotia were only assessed for 14 days. Perhaps a longer assessment period could have resulted in higher values for viability. Smith (1923) observed that heat exposure could lead to delayed conidia germination in *B. cinerea*. In addition, the sclerotia suspended in the aerated steam chamber in these experiments may have been more exposed to the heat and moisture from the steam thermotherapy treatment than if they had been partly embedded in plant tissue or in the soil, and this could have contributed to low viability after treatments.

Botrytis' high genetic variability has also contributed to its adeptness at developing resistance to fungicides. Planting material is known to harbour latent *Botrytis* infections, and the results from this study provide yet another example of how fungicide-resistant *Botrytis* is distributed and enters production systems in this manner (Nielsen et al., 2022; Oliveira et al., 2017; Weber & Entrop, 2017). Nurseries are known to use multiple sprays of products containing pyraclostrobin with boscalid and cyprodinil with fludioxonil in strawberry transplant production (Weber & Entrop, 2017), and the high frequency of fungicide resistance favours selection of resistant strains of *Botrytis* spp. (Fernández-Ortuño et al., 2012; Hu et al., 2016; Weber & Entrop, 2017). Among isolates obtained from the imported strawberry transplants in this study, resistance frequencies were high for several fungicides and multiple fungicide resistance was common. By not using single-spore isolates for testing, there is a chance that resistance frequencies were overestimated with the mycelial growth assay since sensitive conidia transferred together with resistant conidia would have been overgrown and registered as resistant in the assay (Fernández-Ortuño et al., 2014). However, resistance frequencies were in several cases far above the high-risk threshold of 20% (Schnabel et al., 2015). Chemical options for grey mould control in strawberry in Norway are limited to single-site fungicides from FRAC groups 7, 9, 11, 12, and 17. Growers have no multisite fungicide options, and some active ingredients are only available in combination (FRAC groups 7 and 11). These levels of resistance in the pathogen entering a perennial production system threaten the efficacy of chemical control of grey mould.

Considering our fungicide resistance results combined with high *Botrytis* incidence detected in transplants, there is urgent need for *Botrytis* control and resistance management even before the plants reach the field. Steam thermotherapy treatment addresses both issues. Steam thermotherapy treatment reduced *Botrytis* incidence in strawberry transplants. Latent infections are an inoculum source, and inoculum reduction contributes to better disease control and may reduce the need for chemical control. In addition, any tactics which reduce the pathogen population without fungicides also limit selection for fungicide resistance and can contribute to resistance management (Brent & Hollomon, 2007). Despite a limited sample size, resistance frequencies for isolates obtained from transplants which received the treatment with 4 h at 44 °C were similar to or lower than frequencies for isolates from the untreated transplants for all fungicides except pyraclostrobin. There were also more isolates resistant to fungicides from three or more FRAC groups from untreated versus treated transplants. Therefore, in this study, steam thermotherapy treatment reduced the pathogen population in the transplants, and the survivors had, in general, lower frequencies of fungicide resistance and less multiple fungicide resistance. Determining whether this trend holds true over time and for different cultivars in general requires more research.

In the future, steam thermotherapy with the use of an aerated steam chamber may also be used to produce healthy planting material of nursery stock of other dormant deciduous fruit, berry and ornamental crops. Steam thermotherapy has the potential to strongly reduce the need for fungicide control after planting and increase the longevity of important fungicides used for the control of *Botrytis* and other pathogens.

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Data availability The datasets generated during the current study are not publicly available, but may be obtained from the corresponding author on request.

Declarations

Compliance with ethical standards The presented results are not fabricated or manipulated. The manuscript is original, it is not published elsewhere, and it is not submitted to another journal. Information and statements by others are carefully credited. The research did not involve humans or animals as research objects.

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