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Colletotrichum acutatum occurs asymptomatically on sweet cherry leaves

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Abstract Leaves of sweet cherry, exposed to either paraquat or freezing to quickly senesce the leaf tissue, were incubated in about 100% RH at 25°C for 6 d. Sporulating colonies of *Colletotrichum acutatum*, the cause of anthracnose, developed on up to 100% of the paraquat-treated and frozen leaves, and on none of the untreated controls. Number of leaves and leaf area containing *C. acutatum* on naturally infected leaves increased over time from May to September. Mean incidence of *C. acutatum* on leaf blades on fruit spurs and vegetative shoots from eight orchard/year samplings were 41 and 33%, respectively. Secondary conidiation (formation of short hyphae and new conidia) from conidia applied to detached leaves took place 6 h after inoculation, but only up to 3% of the conidia formed new conidia. It may be concluded that asymptomatic sweet cherry leaves frequently host *C. acutatum* and may be a potential inoculum source for cherry fruit.

Keywords Bitter rot · Epidemiology · *Prunus avium*

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Colletotrichum acutatum Simmonds ex. Simmonds is the causal agent of anthracnose (also known as bitter rot) on sweet cherry (*Prunus avium* L) fruit in Norway. *C. acutatum* is widespread in Norwegian fruit production and is found in all commercial fruit crops including pome fruit, stone fruit and small fruit (Stensvand et al. 2006). Symptoms of the disease on sweet cherry have been observed only on aborted fruit and on ripe, normally developed fruit. Additionally both aborted and normally developed fruit may have asymptomatic infections of *C. acutatum* at the green fruit stage (Børve and Stensvand 2004). Bud scales on sweet cherry trees may harbour *C. acutatum* and are thus a possible inoculum source in spring (Børve and Stensvand 2006a). Likewise in sour cherry, fruit and fruit peduncles infected the previous year and still attached to the tree, may be an additional inoculum source (A. Stensvand and J. Børve, unpublished data). Asymptomatic infections of *C. acutatum* on leaves have been reported to occur on citrus (Zulfiqar et al. 1996), strawberry (Leandro et al. 2001; Mertely and Legard 2004), mulberry (Yoshida and Shirata 1999), and apple (Crusius et al. 2002), but the relative importance of such infections in yearly epidemics in those crops was not determined. On apple trees in

Brazil more inoculum of *C. acutatum* was found on mummified fruit than on leaves (Crusius et al. 2002). On sweet cherry trees, generative buds had a higher incidence of *C. acutatum* infections than vegetative buds (Børve and Stensvand 2006a). The objectives of these experiments were to find: i) to what extent healthy appearing sweet cherry leaves may harbour *C. acutatum*, ii) if the frequency of asymptomatic infections on leaves changes as the season progresses, and iii) if secondary conidiation can take place on the surface of sweet cherry leaves.

Materials and methods

Sweet cherry leaves were collected from experimental orchards at the Ullensvang Research Centre located in southwestern Norway and from nearby commercial orchards.

Stimulation of sporulation

Leaves with petioles were picked from fruit spurs at three different times on three different trees of cv. Kristin at Sekse (commercial orchard), which had a history of anthracnose on fruit. Asymptomatic colonization of apparently healthy leaves by *C. acutatum* was confirmed as follows. The leaf samples were divided in three subsamples, and two subsamples were surface sterilized in 0.5% NaOCl for 30 s, in 70% ethanol for 1 min, and rinsed in sterile water for 2 min. The subsamples were then laid on metal grids in sealed plastic boxes, with wet paper towels underneath the grids to maintain high humidity. One subsample was frozen for 2 h at −18°C (kept in the boxes during freezing). Leaves in the third subsample were surface sterilized in 0.5% NaOCl for 4 min, rinsed in tap water, dipped in 0.7% paraquat dichloride solution (Gramoxone, 28% a.i., Syngenta Crop Protection, Cambridge, U.K.) for 1 min, and rinsed in tap water, all according to a standard procedure for detection of *C. acutatum* on strawberry transplants (Cook 1993; EPPO 2004), and finally sealed in plastic boxes as described above. Leaves from all treatments were then incubated at 25°C in continuous artificial light. Each treatment was replicated three times, and each replicate consisted of six leaves. Number of leaves with visible sporulation of *C. acutatum* (conidia oozing out of acervuli in typical orange horn-like structures, Fig. 1) was recorded by viewing the leaves under a stereo microscope and confirmed by examining the conidia with microscopy after 6, 10, 14 and 20 d. The experiment was performed 3 times in June-July 2004.

In order to find the optimal time of freezing needed to stimulate sporulation of *C. acutatum*, fruit spur leaves of sweet cherry cv. Kristin from the Sekse orchard were surface sterilized as described above and then laid separately in the above mentioned moisture chambers and frozen at −18°C for 1, 2, 3, 4 or 5 h. Each freezing time was replicated three times with six leaves in each replicate. Following freezing, leaves were incubated at 25°C in continuous light for 6 d. Number of leaves with visible sporulation of *C. acutatum* was recorded. The experiment was performed three times in August 2004.

Seasonal differences in naturally infected leaves

Leaves from fruit spurs were sampled at 2-weeks intervals, starting at the end of May and ending in mid September from cvs. Kristin and Van in 2005 and Kristin in 2006. The trees of cv. Van were in a commercial orchard (Opedal), but not treated with

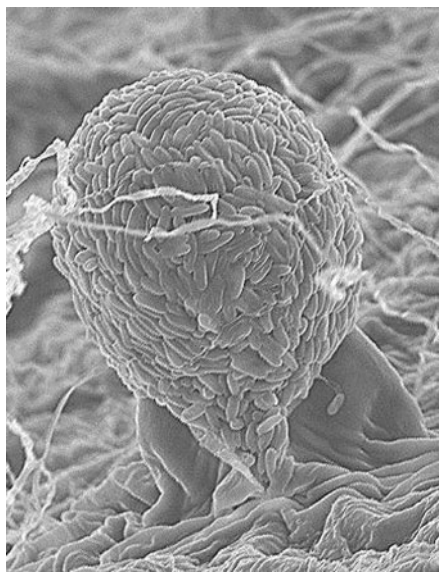

Fig. 1 Conidial mass of *C. acutatum* emerging from a surface sterilized and frozen (5 h at −18°C) sweet cherry leaf; observed by scanning electron microscopy after incubation for 6 d in saturated air, 25°C, and continuous light

fungicides. The trees of cv. Kristin (Kvitavoll orchard) were part of a cultivar collection treated regularly with fungicides. The orchards had a history of anthracnose on the fruits. The following spray program (product name, manufacturer, % active ingredient and amount product per hectare in parentheses) was performed at Kvitavoll both years: Fenhexamid (Teldor, Bayer CropScience AG, 50%, 2.25 kg) at early bloom, thiophanate-methyl (Topsin Granulat, Nisso Chemical Europe GmbH, 70%, 1.0 kg) at full bloom, bitertanol (Baycor 25 WP, Bayer Crop Science AG, 25%, 1.0 kg) at petal fall. Additionally in 2005 fenhexamid and iprodione (Rovral 75WG, BASF Agro BV, 75%, 2.25 kg) were applied at green fruit, and fenhexamid at yellow fruit. In 2006, dithianon (Delan WG, BASF Agro BV, 70%, 1.5 kg) was applied at green fruit, bitertanol and fenhexamide at green fruit, and fenhexamide at yellow fruit. All fungicides were used in rates as recommended by the manufacturers. Each time three replicate samples of six leaves were collected arbitrarily on fruit spurs in the trees. Leaves were surface sterilized, frozen at −18°C for 5 h, and incubated at 25°C in continuous light and saturated air for 6 d, all as described above. Number of leaves with visible sporulation of *C. acutatum* was recorded as described above, and surface area of the sporulating lesions as a percentage of the total leaf area was estimated to the nearest 5% on each leaf blade (Fig. 2) and petiole.

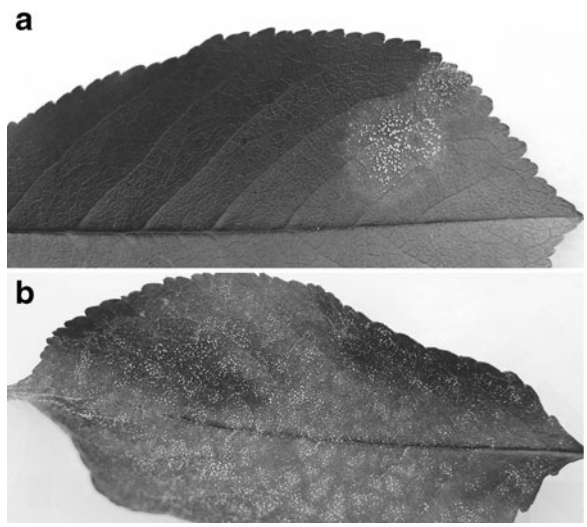

Fig. 2 Sporulation of *C. acutatum* on surface sterilized and frozen (5 h at −18°C) sweet cherry leaves incubated for 6 d in saturated air at 25°C and continuous light; sparse (**a**) and severe (**b**) sporulation

Leaf position

From each of three trees six leaves were collected from current season vegetative shoots (third leaf from shoot basis) and fruit spurs, respectively, treated as above with paraquat in 2004 and 2005, and frozen for 5 h in 2006 in order to stimulate sporulation of *C. acutatum* (as described above). The leaves were incubated as described above for 6 d, then number of leaves with visible infections (incidence) was counted, and the surface area of the sporulating lesions as a percentage of the total leaf area (severity) was assessed as described above. Leaves were collected four times in 2004 (June 28, July 7, August 8 and August 18), four times in 2005 (June 15, June 22, July 6 and August 3) and three times in 2006 (August 22, September 12 and September 26). In 2004, leaves were collected from cv. Kristin in the Sekse orchard and cv. Lapins from the experimental orchard Storåkeren. In 2005 and 2006, leaves were from cv. Kristin in the experimental orchard Kvitavoll and from cv. Lapins in the Storåkeren orchard and from the commercial orchard Bjotveit. All orchards had a history of anthracnose.

Secondary conidiation and appresorium formation

Detached sweet cherry leaves of cv. Lapins were inoculated with 100 (first two times) or 50 µl drops (third time) of conidial suspension (10^5 conidia ml^{-1}) or sterile water on each leaf and incubated for 0, 6 or 24 h at high humidity at 20°C. Experiments were performed three times with three leaves in each treatment. From each leaf three disks were cut out with a hole puncher. Leaf disks (1 cm diameter) were cleared in a mixture of 266 ml distilled water, 210 ml 90% ethanol and 14 ml 20% lactic acid. The leaf disks in the solution were first heated to the boiling point and then left standing for 24 h. Cleared leaf disks were then stained with lacto-fuchsin and rinsed in 20% lactic acid before they were mounted on a microscope slide. On each leaf disk number of conidia, conidia with hyphal growth, secondary conidia and conidia forming appressoria were recorded on two crossing diameters in a light microscope (400x).

Weather data

Weather data for the seasons with bi-weekly sampling of leaves (2005 and 2006) were obtained from a meteorological station located at Ullensvang research centre. Distance from the meteorological station to the experimental fields was 0–1,000 m, except from the Sekse (10 km apart) and Bjotveit (21 km apart) orchards.

Statistical analysis

All data were statistically analyzed with the GLM procedure of SAS (SAS Institute, Cary, NC), and differences between means were separated by Student Newman Keuls method at α=0.05. Incidence data were arcsine-square root transformed prior to analysis. All data presented are non-transformed means. Data from each experiment were analyzed separately. Data for seasonal progress of disease incidence and severity were probit transformed before pooled data for both years and both cultivars in one year were analyzed by the REG procedure of SAS.

Results

Stimulating sporulation

Naturally infected leaves treated with paraquat or freezing turned brown, and *C. acutatum* sporulated abundantly after 6 d (Table 1). On leaves incubated without pre-treatment, *C. acutatum* was first observed after 10 or 12 d, but the incidence was lower than for pre-treated leaves (data not shown). As mean of the three experiments the incidence on non-treated leaves was 25% after 10 d and 50% after 20 d (data not shown). There was no difference in the mean incidence of sporulating leaves between the freezing and paraquat methods after 6 d (Table 1). The sporulation and conidial shape and size were typical for *C. acutatum* after all incubation methods.

Leaves were brown 6 d after start of incubation when they had been frozen for 3 h or more. Less than 3 h freezing gave no change in leaf colour or only partial browning of the leaves. The incidence of leaves containing sporulating lesions of *C. acutatum* increased from 1 to 5 h freezing time, but there were no significant differences among 3, 4 or 5 h (mean of three experiments) (Fig. 3).

Weather data

Precipitation was higher in May 2005 than in May 2006 (100 and 37 mm, respectively), but in June it was similar (50 and 51 mm). Monthly mean temperature was higher in 2006 than in 2005 both in May (11.1°C and 9.5°C, respectively) and June (14.4°C and 11.5°C, respectively) (data not shown).

Seasonal differences

Incidence and severity on petioles was generally much lower than on blades, with a mean incidence of 3% on petioles and 28% on blades, and a mean severity of the infected leaves of 11% on petioles and 54% on blades, respectively. There were no differences among the different sampling times for the petioles (data not shown). The first observation of *C. acutatum* on leaf blades was June 1 in 2005 and July 18 in 2006. The mean number of leaves (incidence) and area of sporulation on each blade (severity) increased over time during the season (Fig. 4), both following a sigmoid pattern. Probit transformed data (pooled for 2005 and 2006) from leaf blades regressed against time (biweekly collection of leaves from May to September) for incidence and severity yielded the equations: $Y = 0.86X - 5.3$ and $R^2 = 0.90 (P = 0.0001)$, and $Y = 1.4X - 7.95$ and $R^2 = 0.89 (P = 0.0001)$, respectively.

Leaf position

C. acutatum was found on naturally infected leaves from both fruit spurs and current season vegetative shoots from all sites/years (Table 2). The overall mean incidence and severity on leaf blades and stalks from fruit spurs was significantly higher than on leaves from vegetative shoots. However, differences between leaf material coming from fruit spurs and shoots were not great and non-significant for several of the single site-year observations.

Secondary conidiation and appresoria formation

In the first experiment, 834 and 509 conidia were examined after 6 and 24 h, respectively. Formation of secondary conidia on the leaf surface was observed for two conidia (0.24%) 6 h after inoculation and for another two conidia (0.39%) 24 h after inoculation. In

Table 1 Percentage of naturally infected sweet cherry cv. Kristin leaves containing *Colletotrichum acutatum* after treatments with paraquat or freezing in 2005

	Incidence of sporulation (%)			
Experiment number (date started)	1 (June 8)	2 (June 22)	3 (July 1)	Mean
Nontreated	0[a]	0 b	0 b	0 b
Paraquat[b]	44.4	100 a	94.4 a	79.6 a
Freezing[c]	16.7	88.9 a	94.4 a	66.7 a
P-value	0.13	0.0003	0.01	0.0001

[a] Values denoted with different letters are significantly different according to Student Newman Keuls test at $P=0.05$. Mean of three replicate samples of six leaves in each

[b] Paraquat-treated and surface sterilized before incubation for 6 d in saturated air at 25°C and continuous light

[c] Surface sterilized and frozen for 2 h at −18°C before incubation for 6 d in saturated air at 25°C and continuous light

the second experiment, no secondary conidia were found, and in the third 7 of 228 (3.07%) were observed 24 h after inoculation. The mean percentages of conidia forming germ tubes and appressoria after 24 h in the three experiments were 7.7, 20.0, and 64.5%, respectively. Conidia were not found on control leaves inoculated with sterile water.

Discussion

The present investigation clearly documented that symptomless sweet cherry leaves are frequently colonized by *C. acutatum*. Sporulation of *C. acutatum* was induced on naturally infected leaves collected from early June until the end of September, and there was a build-up in incidence and severity over time. Earlier, we showed that bud scales are an important site for winter survival of *C. acutatum* in sweet cherry (Børve and Stensvand 2006a) and thus may be an important source for early infections of leaves and flowers. The proximity of the leaves hosting the fungus in the latter part of the season and young susceptible buds increases the likelihood of bud infection. In spring, new tissue emerges from these buds hosting the fungus. Thus the fungus secures a continuous supply of inoculum ready to attack flowers and fruit. Furthermore, we showed in the present work that secondary conidiation may occur after only 6 h on sweet cherry leaves, and even if produced in low numbers, under more favorable conditions than in nature (continuously 20°C and 100%RH) this indicates the potential *C. acutatum* has to reproduce in asymptomatic leaf tissue. This ability to survive and multiply in the absence of symptoms may explain why *C. acutatum* often causes unexpected crop losses in sweet cherry and points to an important factor to be explored in future work in developing an advisory system to manage this disease.

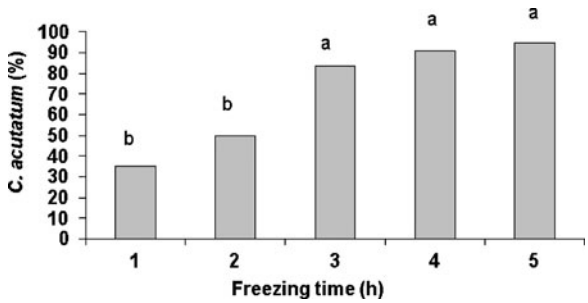

Fig. 3 Percentage naturally infected sweet cherry leaf blades showing conidial masses of *C. acutatum* after freezing for 1–5 h followed by incubation for 6 d in saturated air at 25°C and continuous light. *Bars* denoted with different letters are significantly different according to Student Newman Keuls test at $P=0.05$. Mean of three replicated experiments, with three replicate samples of six leaves in each

C. acutatum was found both on leaves located on fruit spurs and on shoots from the present season's growth, but the incidence was generally higher on leaves collected from spurs. Also, in an earlier study buds located on fruit spurs were more frequently colonized by *C. acutatum* than buds on vegetative shoots (Børve and Stensvand 2006a). Leaves on fruit spurs emerge earlier than leaves on vegetative shoots and are thus exposed to inoculum for a longer time. Furthermore, the proximity of diseased fruit to leaves and buds on fruit spurs compared to leaves and buds on vegetative shoots may be a reason for the higher incidence on leaves on generative shoots. Conversely, the proximity of the

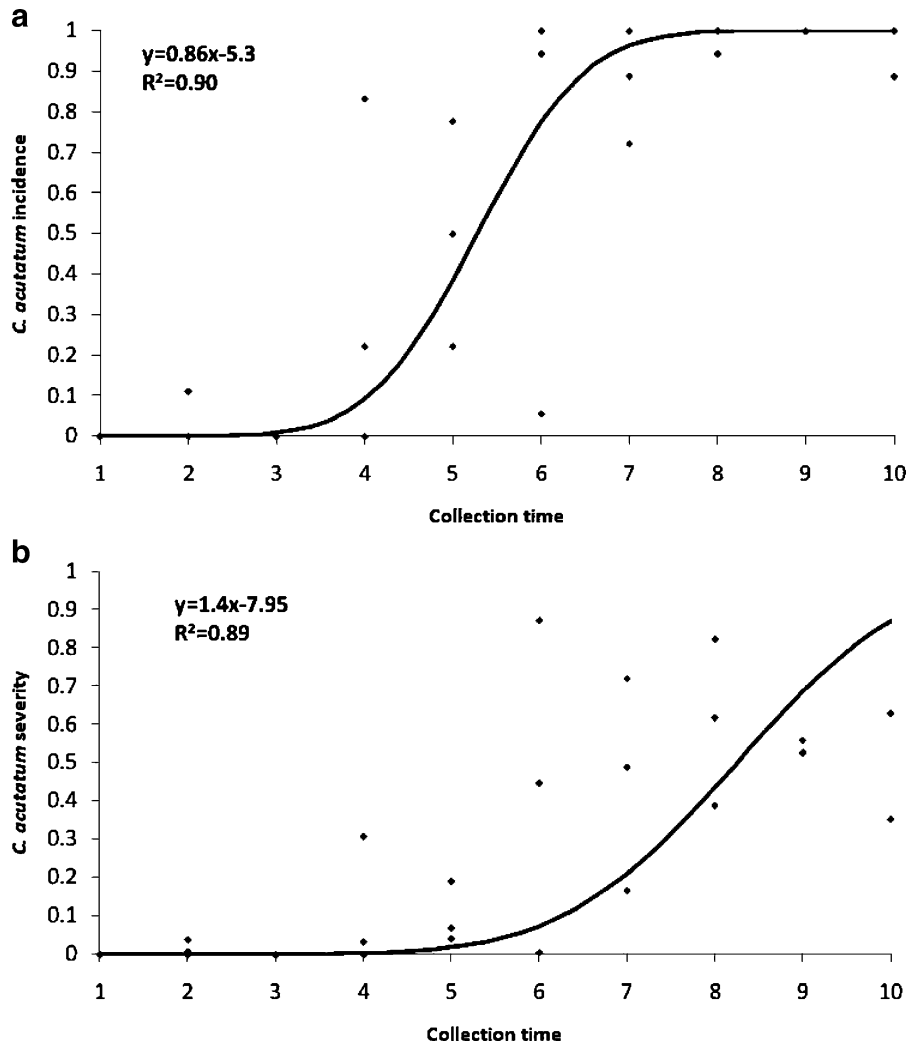

Fig. 4 Incidence (**a**) and severity (leaf area covered) (**b**) of *C. acutatum* sporulation (%) on naturally infected sweet cherry leaf blades collected biweekly from the end of May (week 1) until the end of September (week 10). Leaves were frozen at −18°C for 5 h followed by incubation for 6 d in saturated air at 25°C and continuous light before assessment. Numbers are from two cultivars (Kristin and Van) in 2005 and one (Kristin) in 2006, presented as mean of 3 replicate samples of six leaves for each data point. Regression equations of probit transformed data are presented in each graph

higher populations on spur leaves places them immediately adjacent to young susceptible fruit.

C. acutatum has been reported to produce symptoms on leaves of almond (Adaskaveg and Hartin 1997), blueberry (Yoshida and Tsukiboshi 2002), and pistachio (Ash and Lanoiselet 2001). On almond leaves, necrosis of host tissue appeared 72 h after inoculation (Dieguez-Uribeondo et al. 2005). Even when we observed a high number of appressoria forming on artificially inoculated leaves, necrotrophic lesions or any other symptoms due to infections of *C. acutatum* were never observed on either artificially inoculated or naturally infected leaves. Also in strawberry leaves it has been demonstrated that *C. acutatum* can produce appressoria and secondary conidia without causing any symptom development (Leandro et al. 2001, 2003a, b).

The increase in the incidence of natural infections of *C. acutatum* on leaves over time may be partly due to weather conditions. The first observed sporulating lesions of *C. acutatum* came later in 2006 than in 2005, and that may be because of the higher rainfall in May 2005 compared to May 2006. Another explanation for later detection in 2006 may be the fungicides used. In 2005, the only fungicide used with known effect against *C. acutatum* was thiophanate-methyl (Wharton and Diéguez-Uribeondo 2004), while in 2006 dithianon also was used at the green fruit stage. Dithianon is known to exhibit high efficacy against *C. acutatum* in sweet cherry (Børve and Stensvand 2006b).

The present experiments showed that freezing was an effective method to stimulate sporulation of *C. acutatum* on sweet cherry leaves, without the risk of

Table 2 Percent leaves (incidence) and leaf area (severity) of naturally infected sweet cherry leaves from fruit spurs and current season vegetative shoots sporulating with *Colletotrichum acutatum* (leaf material treated with paraquat (2004–2005) or freezing (2006) and incubated for 6 d at 25°C in continuous light)

Year, cultivar, orchard	Leaf location	Incidence (%)		Severity (%)	
		Blade	Petiole	Blade	Petiole
2004, Kristin, Kvitavoll	Fruit spur	93.1[a]	80.6	–	–
	Veg. shoot	59.7	26.4	–	–
	P-value	0.003	0.0001	–	–
Lapins, Storåkeren	Fruit spur	11.1	6.9	–	–
	Veg. shoot	8.4	1.4	–	–
	P-value	0.6	0.2	–	–
2005, Kristin, Kvitavoll	Fruit spur	23.6	13.9	2.6	2.7
	Veg. shoot	20.8	1.4	1.7	0.07
	P-value	0.8	0.02	0.6	0.02
Lapins, Storåkeren	Fruit spur	8.3	2.8	0.07	0.14
	Veg. shoot	1.4	0	0.63	0.07
	P-value	0.06	0.3	0.06	0.6
Lapins, Bjotveit	Fruit spur	13.9	18.1	1.9	1.7
	Veg. shoot	12.5	1.4	1.0	0.07
	P-value	0.4	0.003	0.5	0.002
2006, Kristin, Kvitavoll	Fruit spur	79.6	31.5	56.2	13.5
	Veg. shoot	63.0	3.7	30.0	3.0
	P-value	0.1	0.02	0.0001	0.01
Lapins, Storåkeren	Fruit spur	74.1	1.8	19.6	8.6
	Veg. shoot	59.3	1.8	14.2	0.9
	P-value	0.1	1.0	0.2	0.001
Lapins, Bjotveit	Fruit spur	53.7	0	14.4	0
	Veg. shoot	50.0	0	6.7	0
	P-value	0.7	–	0.03	–
Mean	Fruit spur	41.2	20.3	13.0	4.0
	Veg. shoot	33.0	4.8	8.7	0.6
	P-value	0.004	0.0001	0.001	0.0001

[a] Mean of three (2006) or four (2004 and 2005) replicated samplings, each with three samples of six leaves each

toxic or environmental hazards entailed in the use of paraquat. Maximum sporulation was obtained after freezing for 5 h. Freezing only for 2 h was a good way to stimulate sporulation of *Colletotrichum* spp. on strawberry petioles (Mertely and Legard 2004), but strawberry leaves were preferably frozen overnight (Leandro et al. 2003a). Mertely and Legard (2004) suggested surface sterilization after freezing to avoid saprophytic contamination. However, this was not carried out in the present investigation and other fungi were only occasionally found, and in small amounts. Most commonly observed were sterile mycelia (unidentified), *Monilinia laxa*, *Botrytis cinerea* and *Cladosporium* spp. Freezing provide an inexpensive and sensitive means to detect *C. acutatum* on foliage of sweet cherry.

Anthracnose may cause severe crop losses in sweet cherry, and efforts to lower the inoculum density may reduce the need for fungicide applications. Using disease-free planting material could potentially delay the introduction and severity of the disease in orchards. The European plant protection organization, EPPO, recommends the paraquat test (Cook 1993; EPPO 2004) when investigating presence of *C. acutatum* in certified strawberry planting material. There are no regulations for *C. acutatum* in production of fruit trees, and thus no control measures are undertaken in nurseries to avoid the fungus. By incubating buds (Børve and Stensvand 2006a) and using the freezing and incubation test of leaves, nurseries might also reduce the risk of distributing fruit trees contaminated with *C. acutatum*. Further-

more, these test methods could be developed for scouting of potential inoculum in cherry orchards and to subsequently adapt fungicide strategies to actual inoculum levels.

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