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RESEARCH PAPERS

Pruning practices influence infection and dissemination of *Calosphaeria pulchella*, the cause of Calosphaeria canker of sweet cherry

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Summary. Calosphaeria canker of sweet cherry, caused by *Calosphaeria pulchella*, is a limiting factor for sweet cherry production, but the role of pruning practices on pathogen dissemination remains unknown. Three experimental treatments were compared during summer and winter seasons, to assess their effects on pathogen transmission. The treatments were: i) using disinfected pruning shears; ii) pruning shears used to cut through diseased branches before each subsequent cut (non-disinfected pruning shears); and iii) artificial inoculation of fresh pruning wounds with *C. pulchella*. Six months after pruning, branches were cut from trees for disease assessment and fungal isolation. Pruning with non-disinfected pruning shears increased disease incidence and severity, compared with the use of disinfected shears. Artificially inoculated branches gave the greatest disease incidence and severity. Results from the various treatments were consistent for both winter and summer pruning. These confirm that frequent disinfection of pruning tools is advised for the effective management of Calosphaeria canker of sweet cherry.

Key words: pathogen dissemination, pruning shears, *Prunus avium.*

Introduction

Calosphaeria pulchella (Pers.:Fr.) J. Schröt (anamorph: *Calosphaeriophora pulchella* Réblová, L. Mostert, W. Gams & Crous), a fungus in the Calosphaeriales (Barr, 1985; Réblová *et al*., 2004; Damm *et al*., 2008; Réblová, 2011; Réblová *et al*., 2015), is the causal agent of Calosphaeria canker of sweet cherry (*Prunus avium*) (Trouillas *et al*., 2012).

Characteristic symptoms of this disease in sweet cherry trees include branch and main scaffold dieback, as well as wood cankers and vascular necroses. In general, cankers, starting from a wound, initiate around the pith and progressively develop into the xylem, cambium, phloem, and cortical tissues. In the late stages of infection, cankers are noticeable in older scaffold branches and trunks, and are usually associated with symptoms of branch dieback and leaf desiccation (Trouillas *et al*., 2012). *Calosphaeria pulchella* can produce circinate groups of perithecia beneath the periderm of infected branches and trunks (Trouillas *et al*., 2012).

Calosphaeria pulchella has been reported on sweet cherry in Australia, California, France and Italy (Trouillas *et al*., 2010; 2012), and more recently in Spain (Berbegal *et al*., 2014). This fungus also occurs on trees of peach (*Prunus persica*) (Adaskaveg *et al*., 1993), nectarine (*P. persica*) (Trouillas *et al*., 2012), and almond (*P. dulcis*) (Arzanlou and Dokhanchi, 2013), although the relevance of the pathogen on these other *Prunus* spp. remains unexplored.

Trouillas *et al*. (2012) suggested that ascospores of *C. pulchella* may constitute the main inoculum for the disease, and that pruning wounds and sunburn lesions, as well as scars left by the abscission of leaves near buds, could be the primary infection sites. In addition, spore trapping studies by Trouillas *et al*.,

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(2012) revealed that the aerial concentration of *C. pulchella* spores was greater in rainy periods or during sprinkler irrigation events occurring in the spring and summer months. These authors indicated that further research investigating the infection process by *C. pulchella* was required to provide better understanding of the epidemiology of Calosphaeria canker of sweet cherry.

In Spain, Calosphaeria canker has been found only in the Alicante province, but since its first detection in 2012 in a few orchards, the disease has become more widespread, and is currently considered a limiting factor for sweet cherry production (Berbegal *et al*., 2014). In this region, sweet cherry orchards are drip irrigated and hand pruned twice each year: in winter during dormancy and in late summer (Coque and Díaz, 2005). Many aspects of Calosphaeria canker epidemiology, such as the role of pruning practices on pathogen dissemination, remain unknown. Thus, the objectives of this study were to: (i) determine the role of pruning shears in the dissemination of *C. pulchella* in sweet cherry orchards; and (ii) compare the effect of winter vs. summer pruning on pathogen infection.

Materials and methods

Experimental sites

The experiments were conducted in two separate sweet cherry orchards (Field 1 and Field 2) with histories of Calosphaeria canker, located at Villena (Alicante province, eastern Spain). Both orchards contained trees of cv. Marvin, with between tree plant spacings of 4 m between trees and 3 m between rows, and the trees were drip irrigated. The trees were 25 years old in Field 1 and 26 years old in Field 2. Prior to the experiments, no fungicides had been used to attempt management of Calosphaeria canker.

Pruning experiments

In each orchard, three different pruning treatments were compared, both during summer (August) and winter (December) of 2014. The treatments were: i) pruning shears disinfected before each pruning cut by immersion for 1 min in a 1.5% sodium hypochlorite solution and washed twice with sterile distilled water; and ii) non-disinfected pruning shears, which were used to cut through a diseased branch affected by Calosphaeria canker before each subsequent pruning cut. These treatments were compared with artificial inoculation of fresh pruning cuts with *C. pulchella*. In this case, pruning shears were disinfected as described above before each pruning cut and fresh pruning wounds were inoculated immediately with mycelial agar plugs (diam. = 5 mm) taken from a 20-d-old colony of *C. pulchella* grown on potato dextrose agar (PDA) (Biokar-Diagnostics) at 25 ºC in darkness and obtained from a diseased sweet cherry tree.

In each orchard a factorial experimental design was used with ten repetitions. Pruning treatment and time (winter or summer) of pruning were the experimental factors. Each factor combination occurred once per tree. In each tree, two separated young sweet cherry branches (3 ± 0.5 cm diam.) were randomly selected, and pruned at its distal end with one of the treatments described above. At inoculation, no lesions were observed in any of the branches, and wounds were immediately sealed with Parafilm (Bemis Company Inc.).

Six months after pruning, the branches were cut from the trees for disease assessment and fungal isolation. The branches were sectioned longitudinally from the site of inoculation, and these sections were surface disinfected for 1 min in 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Isolation from each branch was conducted by plating seven small pieces of necrotic tissue from the edge of each developing lesion, or just below the area of inoculation if no lesion was visible, onto malt extract agar (MEA) (Difco Laboratories), supplemented with 0.5 g L^{-1} streptomycin sulphate (MEAS) (Sigma-Aldrich Laboratories). Plates were incubated for 10–15 d at 25 ºC in the dark. *Calosphaeria pulchella* colonies were identified based on morphology (pink to red colonies with white margins, which produced hyaline, allantoid to oblong-ellipsoidal conidia) (Barr, 1985), and confirmed by DNA sequence comparison in GenBank database using the internal transcribed spacer region (ITS1 - 5.8S - ITS2) of the rDNA, as described by Berbegal *et al*. (2014).

Statistical analyses

Percent Calosphaeria canker incidence was calculated based on isolation results, with a branch being considered infected when *C. pulchella* was recovered from at least one of the seven isolated wood pieces. Percent Calosphaeria canker severity was estimated as the number of positive isolations (wood pieces infected with *C. pulchella*) relative to the total number of wood pieces cultured. Data were subjected to factorial analysis of variance performed with the General Linear Model (GLM) using Statistical Analysis System software SAS (version 9.0, SAS Institute Inc.). Factors considered for the model were: type and time of pruning, and their interaction. Mean values were compared using the Fischer´s least significant difference (LSD) procedure at P=0.05.

Results

The effects of the different pruning treatments on Calosphaeria canker incidence are demonstrated in the data in Table 1. No statistically significant differences were detected between winter and summer pruning (Field 1, P=0.4549; Field 2, P=1). However, significant differences (P< 0.05) were found among the different pruning treatments in both fields, and the interactions between the experimental factors were not significant (Field 1, P=0.5709; Field 2, P=0.8625). In both fields and for both pruning times, the lowest percentage of infected branches (from 10 to 15%) was obtained when disinfected shears were used. The use of non-disinfected shears resulted in infection percentages ranging from 40 to 50%, and the greatest percentages of infected branches were obtained for wounds that were artificially inoculated (80 to 95%).

Results of Calosphaeria canker severity (Figure 1) were consistent with those obtained for disease incidence. In both fields, there were no significant differences between winter and summer pruning (Field 1, P=0.6718; Field 2, P=0.1231), and there was no interaction between time and pruning treatment (Field 1, P=0.0710; Field 2, P=0.4213). However, significant differences (P<0.05) were found between the different pruning treatments. In Field 1, there were no significant differences between using disinfected shears (mean incidence = 10.7% for winter pruning and 5.4% for summer) or non-disinfected shears (6.8% for winter pruning and 20.4% for summer). Inoculated wounds gave the greatest of disease severities (mean = 56.1% for winter pruning and 41.4% for summer), with these values being from those for other two pruning treatments. In Field 2 in winter, there were significant differences for Calosphaeria canker severity values obtained from the three pruning treatments, which were less when disinfected shears were used (mean = 2.9%), moderate when non-disinfected shears were used (17.5%) and greatest for the artificially inoculated wounds (37.1%). In Field 2 in summer, Calosphaeria canker incidence was significantly different between disinfected shears (mean = 2.9%) when compared with non-disinfected shears (33.6%) and inoculated wounds (44.3%), but there were no significant differences between the values from the non-disinfected shears and the inoculated wounds.

Discussion

This is the first study to examine the spread with pruning shears of *C. pulchella*, causal agent of Calosphaeria canker of sweet cherry. Our results confirm that pruning with non-disinfected shears increases the incidence and the severity of the disease in branches of sweet cherry trees, when compared with disease parameters in trees pruned with disinfected shears.

The role of contaminated pruning tools favouring infection and spread of fungal pathogens has been

Table 1. Percent (%) Calosphaeria canker incidence on branches of sweet cherry trees 6 months after application of the different pruning treatments

Pruning type	Field 1		Field 2	
	Winter	Summer	Winter	Summer
Disinfected shears	15 ± 7.5[x] a[y]	10 ± 6.5 a	10 ± 6.5 a	10 ± 6.6 a
Non-disinfected shears	40 ± 9.8 b	50 ± 10.4 b	40 ± 9.8 b	45 ± 11.5 b
Inoculated wounds	85 ± 7.5 c	95 ± 4.9 c	85 ± 7.5 c	80 ± 10.9 c

[x] Mean ± standard error percentages are based on ten replicates per treatment and Field.
[y] Means followed by the same letter are not significantly different (P<0.05) as indicated by least significant difference tests.

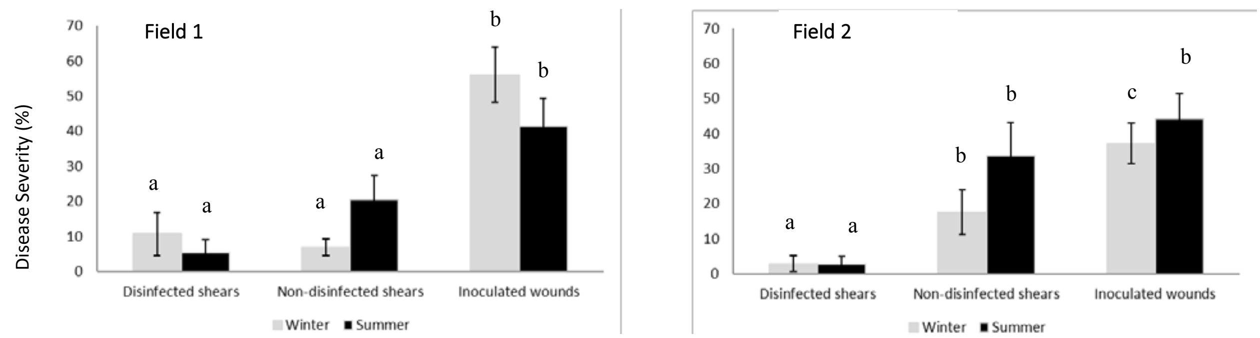

Figure 1. Percent (%) Calosphaeria canker severity in branches of sweet cherry trees 6 months after application of different pruning treatments. Mean percentages are based on ten replicates per treatment. Within each season, means accompanied by the same letter are not significantly different (P> 0.05). Bars represent the standard errors of the means.

demonstrated for different host-fungus combinations, such as *Seiridium cardinale* and *S. cupressi* which cause cypress (*Cupressus macrocarpa*) canker (Hood *et al*., 2009), and *Cadophora luteo-olivacea*, *Diplodia seriata*, *Eutypa lata*, *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora* (the causal agents of grapevine trunk diseases (Agustí-Brisach *et al*., 2015)). A similar study conducted on sweet cherry trees concluded that *Pseudomonas syringae* pv. *syringae*, which causes bacterial canker of stone fruit trees, was not transmitted after cutting through active cankers and immediately using the same pruning tool to make heading cuts on healthy trees (Spotts *et al*., 2010).

In the present study, the effects of the different pruning treatments and artificial inoculation were consistent between winter and summer pruning. Greatest Calosphaeria canker incidence and severity in branches resulted from the artificial inoculation of pruning wounds, followed by the use of non-disinfested pruning shears and disinfected pruning shears. These results also emphasizes the ability of *C. pulchella* to successfully infect pruning wounds during winter and summer seasons. This result is in contrast with the study of Barakat and Johnson (1998), who inoculated fresh pruning wounds on sweet cherry trees with *Leucostoma cinctum* (which causes Leucostoma canker) at different months during a full year. These authors found that infection by this pathogen can occur throughout the year, with greatest proportions of wounds becoming infected during spring and summer months. Moreover, inoculation with conidia placed directly on wounds, and infection resulting from pruning shears contaminated with conidia and mycelia, resulted in similar incidence and severity of cankers.

In *C. pulchella*, Trouillas *et al*. (2012) reported ascospores as the most likely inoculum for the disease. These are released from perithecia mainly during autumn, winter and spring rains, and during summer in orchards using sprinkler irrigation. To date in Spain, perithecia of *C. pulchella* have not been observed in diseased sweet cherry trees (Berbegal *et al*., 2014). Further studies are therefore required to determine the geographical range of the perithecial stage of *C. pulchella*, as well as environmental conditions favouring the production of perithecia. In the present study, low proportions of *C. pulchella* infections were detected in branches that had received treatment with disinfected pruning tools, where wounds were sealed immediately after pruning. This suggests the existence of latent infections of the pathogen in branches, and additional possible infection pathways.

Current recommendations for cultural practices for the management of Calosphaeria canker in sweet cherry trees include avoiding the use of sprinkler irrigation, as well as avoiding pruning during rain or irrigation events, which can favour the release and dispersal of pathogen spores (Trouillas *et al*., 2012). In addition, our results emphasize the need for frequent disinfection of all pruning tools during pruning operations. These cultural practices should be adopted, together with protection of pruning wounds using registered fungicides, as an integrated approach to management of Calosphaeria canker of sweet cherry.

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