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Pathogenicity of *Colletotrichum* species to *Protea* cultivars

C. M. Lubbe[A,C,F], *S. Denman*[B,C], *S. C. Lamprecht*[C,D] *and P. W. Crous*[C,E]

[A]ARC—Fynbos Unit, Private Bag X1, Elsenburg 7607, South Africa.
[B]Forest Research, Alice Holt Lodge, Farnham, Surrey G4 10LH, United Kingdom.
[C]Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa.
[D]ARC—PPRI, Private Bag X5017, Stellenbosch 7599, South Africa.
[E]Centraalbureau voor Schimmelcultures (CBS), Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.
[F]Corresponding author. Email: lubbec@arc.agric.za

Abstract. *Colletotrichum* species cause a wide range of diseases on Proteaceae. Four *Colletotrichum* species, *C. acutatum*, *C. acutatum* f. sp. *hakea*, *C. boninense* and *C. gloeosporioides*, have been associated with diseased Proteaceae in South Africa. In this study, the pathogenicity of these taxa was evaluated on three *Protea* cultivars. The relative aggressiveness of the isolates and the effect that wounding had on the host response were compared. Results showed that *C. boninense* and *C. acutatum* f. sp. *hakea* did not cause lesions significantly different from those on the controls. Isolates of *C. acutatum* and *C. gloeosporioides* originating from *Protea* were the primary pathogens associated with Colletotrichum leaf necrosis. Furthermore, *C. acutatum* was the main cause of anthracnose and stem necrosis of the selected *Protea* cultivars tested.

Introduction

The Proteaceae are indigenous to Australia, South Africa, Central America, South America, South-east Asia and the southwest Pacific Islands (Rebelo 1995). Certain species of the Proteaceae are commercially valuable and sought after as cut-flowers on several international markets. Consequently, they are being increasingly cultivated as the demand for fresh cut-flowers and germplasm grows in global trade. One of the factors limiting commercial production of the Proteaceae is damage caused by pests and diseases (Wright and Saunderson 1995; Crous *et al*. 2004). Some pathogens cause significant losses in the field and in nurseries, while others damage the appearance of blooms and, although they are not debilitating pathogens, they reduce the aesthetic value of the flowers. Pathogens may also pose a threat to natural ecosystems and crops in other countries and, therefore, international trade in Proteaceae might be restricted by quarantine regulations.

Among the most devastating fungal pathogens of members of the Proteaceae is *Colletotrichum gloeosporioides*, which causes damping-off, shepherd's crook (anthracnose), pruning wound dieback, leaf necrosis and stem dieback (Knox-Davies 1981; Knox-Davies *et al*. 1986; Von Broembsen 1989). Disease outbreaks in cultivated fields tend to be sporadic and are dependent upon climatic conditions suitable for disease development and high inoculum levels. Moderate temperatures (20–25°C) and humid conditions favour successful infection of proteas (Forsberg 1993). Young tissues are most affected, often displaying the shepherd's crook symptom or leaf necrosis. In nurseries, where conditions are often conducive to disease development, and young plants are susceptible to infection, annual losses are common.

Until recently *C. gloeosporioides* was the only *Colletotrichum* species known to affect Proteaceae and has been reported from most areas where these plants are cultivated. Hosts include species of *Banksia*, *Grevillea*, *Leucospermum*, *Leucadendron*, *Protea*, *Serruria* and *Telopea* (Greenhalgh 1981; Benic 1986; Knox-Davies *et al*. 1986; Von Broembsen 1989; Forsberg 1993; Moura and Rodrigues 2001; Taylor 2001). A recent molecular study demonstrated that isolates of *Colletotrichum* from members of the Proteaceae represent four taxa (Lubbe *et al*. 2004). In this study, the pathogenicity of representatives of each taxon currently identified as *C. acutatum*, *C. boninense* and *C. gloeosporioides* from *Protea* spp., and *C. acutatum* f. sp. *hakea* from *Hakea sericea*, were tested on cultivars of *Protea*. Although the final taxonomic positions of the two forms of *C. acutatum* have not yet been resolved, they are distinct ecological groups and are, therefore, treated separately. The relative aggressiveness of the taxa was also compared and the effect that wounding had on the host response was investigated. Since it was difficult to induce disease development under conditions of artificial inoculation (pilot trials, unpublished data) a wound treatment was included to introduce the pathogen directly into plant tissues. Furthermore, leaf wounds artificially simulate insect damage

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and stem wounds represent pruning wounds, which are both common occurrences in commercial plantations.

Methods

Isolates

The following species–isolate combinations were used for testing: *C. acutatum* from *Protea magnifica* and *P. repens* (two isolates viz. CBS 112992, CBS 113002), *C. acutatum* f. sp. *hakea* from *Hakea sericea* (CBS 112760), *C. boninense* from *Eucalyptus grandis* (CBS 110779) and *C. gloeosporioides* from *P. cynaroides* (CBS 113001). Only isolates originating in South Africa were used to avoid quarantine complications. For this reason, the isolate of *C. boninense*, which was obtained from *Eucalyptus* in South Africa, was used instead of isolates from *Protea* growing in Australia and Zimbabwe. Isolates were obtained from the following sources: the University of Stellenbosch (STE-U 194 = CBS 110779, STE-U 4454 = CBS 113001), the Biocontrol Unit of the Plant Protection Research Institute—Agricultural Research Council, South Africa (CBS 112760) and from diseased material sampled from orchards at Elsenburg in the Western Cape, South Africa (CBS 112992, CBS 113002).

Plant material

Rooted cuttings of the *Protea* cvv. 'Cardinal' (*P. eximia* × *P. susannae*), 'Carnival Too' (*P. compacta* × *P. neriifolia*) and 'Rubens' (*P. repens*) were planted into 20-cm diameter plastic pots containing a mixture of sterilised sand, peat and polystyrene (1 : 1 : 1). The plants were 2–4 months old and had one or two 10–15-cm long newly flushed shoots per plant.

Inoculum

Isolates were cultured on carnation leaf agar (CLA) (Fisher *et al*. 1982) and incubated for 4 weeks at 25°C under near-UV and cool white light with a 12 h photoperiod. The contents of three Petri dishes (9-cm diameter) with one carnation leaf per dish were used per isolate. Thus, three leaves (covered in conidial masses) were immersed in 10 mL sterile distilled water to produce spore suspensions of each isolate. The spore suspensions were filtered through four layers of sterile muslin to remove residual mycelia and carnation leaf tissue. The final spore concentration was adjusted to 10^6 spores/mL using a haemocytometer. A drop of Tween 20 (Holpro Analytics, Johannesburg, South Africa) was added to the spore suspension of each isolate.

Inoculation

Different plant parts were inoculated (leaves or stems) which were treated by either wounding or non-wounding. Wounds were made by pricking each leaf with a cork in which five insect mounting needles were inserted. Shoots were wounded by removing the top 1 cm. Each plant was inoculated with 10 mL spore suspension (wetting plants to run-off). Control plants were sprayed with sterile water.

Incubation

Plants were covered with plastic bags and incubated in the laboratory at 23−25°C for the first 48 h, during which they received only low light intensities emitted by fluorescent tubes. Thereafter, the bags were removed and the plants were transferred to a controlled environment incubation room (Conviron) at 25°C ± 2°C with a 12 h photoperiod for 12 days. The air was humidified using a Goldair GUH-852 home humidifier set at full capacity.

Re-isolation

At the end of the experiment, assessments were made and leaves and stems of plants were surface disinfested with 1% NaOCl for 2 min, 70% alcohol for 1 min and then rinsed in sterile water (filtered with activated carbon filters). Isolations were made from every leaf (whether necrotic or not) and from each stem tip of the most recent growth flush. Entire leaves were placed onto potato dextrose agar (PDA, Biolab, Midrand, South Africa), irrespective of necrosis, and were incubated for 14 days at room temperature (25°C ± 2°C). One piece of stem tissue from each of the stems was placed onto PDA. Where stem necrosis was evident, the tissue was taken from the margin between healthy and diseased tissue. Where no necrosis was evident, the tissue was selected at random from the inoculated stem tip.

Assessments

Four parameters were measured:

i) *Incidence of leaf lesions*. The percentage of current flush leaves with lesions was calculated as a proportion of the total number of current flush leaves per plant.
ii) *Incidence of stem lesions*. The presence of stem lesions was scored as being present or not. The data were then expressed as the percentage of stems that developed lesions.
iii) *Incidence of leaf infections*. Percentage leaf infection was based on the number of current flush leaves from which the pathogen was re-isolated out of the number of inoculated current flush leaves per plant.
iv) *Incidence of stem infections.* The percentage of infected stems was calculated as the proportion of inoculated stems from which the pathogen was re-isolated.

Statistical procedures

The experiment was set out in a completely randomised, four-factor factorial design. The factors were cvv. ('Cardinal', 'Carnival Too' and 'Rubens'), species (*C. acutatum* (double replication because two isolates were used), *C. acutatum* f. sp. *hakea*, *C. boninense*, *C. gloeosporioides* and a control), plant parts inoculated (leaves and stems) and treatments, wounding or not. There were two replicates giving a total of 144 plants tested. For the leaf inoculations, there were 12 plants (five isolates plus a control, and either wounded or not) for each of the three cvv. per replicate, giving a total of 72 plants used for leaf tests. The same treatment structure applied to stems.

Analyses. Combined analyses of variance for the two experiments were carried out after tests for homogeneity of variance indicated that the data were comparable. The ANOVA was done on the species data (i.e. five levels for the four species and a control, not forgetting that *C. acutatum* had a double replicate because two isolates were used) and the other factors (leaf or stem inoculated) and treatments (wounded or not). The analyses were carried out on the leaf lesion data, the percentage infected leaves and the infected stem data using SAS version 8.2 (SAS 1999) statistical software. The Shapiro-Wilk test was performed to test for non-normality (Shapiro and Wilk 1965) on all data used in the ANOVA tests. Student's t was calculated at the 5% confidence level to compare treatment means in the ANOVAs. The stem lesion data were subjected to 2 × C contingency tables, with C levels for each factor, and Chi Square tests were performed, where the 2 level of classification was whether or not stem lesions were present.

Results

Leaf lesions data

Significant interactions occurred for cultivar × treatment (wound/non-wound) ($P = 0.0321$) and plant part × treatment (wound/non-wound) ($P = 0.0021$) data. Since there were no interactions involving the species data, only the main effects of species were considered. Inoculation with *C. acutatum* and *C. gloeosporioides* caused 36.3% and 25.3%, respectively, of the leaves to develop lesions (Table 1). Although all the

Table 1. Percentage of *Protea* leaves with lesions and infected leaves after inoculation with *Colletotrichum* species

Colletotrichum species	Leaves with lesions (%)[A,B]	Infected leaves (%)[A,C]
C. acutatum	36.34a	37.55a
C. acutatum f. sp. *hakea*	11.91bc	46.73a
C. boninense	15.82bc	31.47a
C. gloeosporioides	25.32ab	46.94a
Control (sterile water)	5.98c	0.40b

[A]Means followed by the same letter in a column are not significantly different ($P = 0.05$).
[B]Leaves with lesions (%) = current flush leaves with lesions as a portion of the total number of current flush leaves.
[C]Infected leaves (%) = current flush leaves infected as a portion of the total number of current flush leaves from which isolations were made.

Table 2. Effect of wounding on the percentage of leaves with lesions after inoculating *Protea* cultivars with different *Colletotrichum* species

Cultivar	Leaves with lesions (%)[A,B]	
	Wounded	Non-wounded
Cardinal	14.20bc	4.64c
Carnival Too	45.62a	19.67bc
Rubens	21.94b	24.32b

[A]Means followed by the same letter are not significantly different ($P = 0.05$).
[B]Leaves with lesions (%) = current flush leaves with lesions as a portion of the total number of current flush leaves.

Table 3. Effect of wounding on disease expression (percentage leaves with lesions) and the proportion of infected leaves on the different plant parts inoculated

Plant part treated	Leaves with lesions (%)[A,B]		Infected leaves (%)[A,C]	
	Wounded	Non-wounded	Wounded	Non-wounded
Leaf	42.40a	16.20b	57.66a	28.51b
Stem	12.11b	16.54b	25.38b	33.35b

[A]Means followed by the same letter are not significantly different ($P = 0.05$).
[B]Leaves with lesions (%) = current flush leaves with lesions as a portion of the total number of current flush leaves.
[C]Infected leaves (%) = current flush leaves infected as a portion of the total number of current flush leaves from which isolations were made.

Colletotrichum species caused leaf lesions, *C. acutatum* f. sp. *hakea* and *C. boninense* did not cause lesions significantly different from those on the controls.

The cultivar × treatment (wound/non-wound) interaction is shown in Table 2. Wounding significantly increased the number of leaves with lesions only in 'Carnival Too'. Leaf susceptibility was greatly increased by wounding relative to stem susceptibility (Table 3). Overall, wounded leaves developed significantly more lesions than leaves that were not wounded and then inoculated (with the exception of cv. 'Rubens').

Infected leaves data

There was no four-way or three-way interaction, but there was significant ($P = 0.0034$) two-way interaction between treatments (wound/non-wound) × plant parts. The species and cultivars were not involved in interaction and can be interpreted as main effects. There were significant differences ($P = 0.0020$) between the species tested and the control, but not between the cultivars ($P = 0.2544$).

All the *Colletotrichum* species tested caused significantly higher percentages of leaf infection than the control (Table 1). In some species, a greater proportion of leaves were infected (*C. acutatum* f. sp. *hakea*, *C. boninense*, *C. gloeosporioides*) than those that showed symptoms. Relatively high infection levels (>30%) were obtained for all the species tested, but there were no differences among the species in levels of infection obtained. For leaf-inoculated plants, those with wounded leaves developed significantly higher levels of leaf infection (Table 3) than those with unwounded leaves.

Stem lesion data

The Chi square tests demonstrated that there were highly significant differences ($P < 0.0001$) amongst *Colletotrichum* species in their ability to form stem lesions. Stem lesions developed on 42% of plants inoculated with *C. acutatum*, whereas the other species caused less than 10% of stems to develop lesions (Fig. 1). There were also significant differences ($P = 0.0240$) amongst cultivars with the most stem lesions formed on 'Carnival Too' (29.2%), compared with 'Rubens' (12.8%) and 'Cardinal' (8.5%) (Fig. 1). The treatments (wounding or non-wounding) also significantly ($P = 0.05$) affected stem lesions and wounding more than doubled the number of stems with lesions (24%, Fig. 1), compared with unwounded stems (10%).

Stem infection data

There were significant differences between the species tested ($P = 0.0043$) sand between the treatments (wounded or

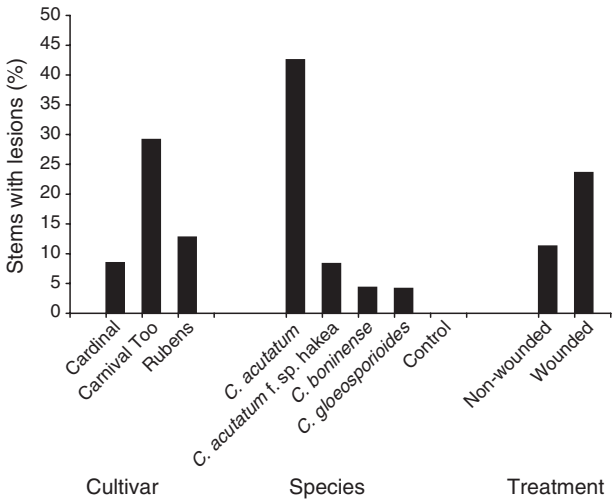

Fig. 1. Effect of cultivar, *Colletotrichum* species and wounding on the percentage of *Protea* stems with lesions. Stems with lesions (%) = current flush stems with lesions as a portion of the total number of current flush stems.

Table 4. Percentage of *Protea* stems infected after inoculation with *Colletotrichum* species

Colletotrichum species	Infected stems (%)[A,B]
C. acutatum	60.82a
C. acutatum f. sp. *hakea*	54.17a
C. boninense	37.68a
C. gloeosporioides	45.83a
Control (sterile water)	0.00b

[A]Means followed by the same letter are not significantly different ($P = 0.05$).
[B]Infected stems (%) = stems infected as a portion of the total number of stems from which isolations were made.

non-wounded) ($P = 0.0121$). Inoculation of the plants with any of the *Colletotrichum* species resulted in significantly more stem infections than the control plants (Table 4). However, at the 95% confidence level, the differences in ability of the various species to infect stems were not significant. Wounded plants developed significantly higher proportions of infected stems (57.2%) than plants that were not wounded (36.7%).

Discussion

The primary aim of this study was to establish the pathogenicity of four selected *Colletotrichum* taxa associated with diseased proteas. Although only a selection of isolates was used in the pathogenicity tests, they were representative of the species based on previous molecular results (Lubbe *et al*. 2004) and pilot pathogenicity tests. The results show that each species can infect *Protea* leaves and stems. *Colletotrichum acutatum* and *C. gloeosporioides* caused significantly more leaf necrosis than the control, whereas *C. acutatum* f. sp. *hakea* and *C. boninense* did not. The most aggressive species appears to be *C. acutatum*, because it could cause the greatest lesion damage on both leaves and stems of proteas. *Colletotrichum gloeosporioides* was as aggressive on leaves of proteas as *C. acutatum*. However, it was unable to cause major stem damage and, hence, should be regarded as a serious leaf pathogen of proteas, but probably being less involved with anthracnose, shepherd's crook and stem canker. This is also supported by isolation data obtained from diseased Proteaceae (Lubbe *et al*. unpublished data). Leaf infections were not always coupled with leaf necrosis and, in some cases, isolates were obtained from surface disinfested, asymptomatic material. This has implications for quarantine regulations in that plant material exported or imported cannot only be visually inspected.

The *C. boninense* isolate used in this study originated from *Eucalyptus grandis*. Its less aggressive pathogenicity might, therefore, be attributed to it not being inoculated onto the natural host. The same explanation may apply to the isolate of *C. acutatum* f. sp. *hakea*. There is little known about the pathogenicity of *C. boninense*, since Moriwaki *et al*. (2003) only recently described this species from Japan. *Colletotrichum boninense* was found on diseased proteaceous plants in Australia and Zimbabwe, and might be capable of causing disease on members of the Proteaceae in South Africa under certain environmental conditions. Further testing using isolates from *Proteaceae* is, therefore, necessary.

The three *Protea* cultivars tested were equally infected by the different *Colletotrichum* species, but demonstrated differences in susceptibility through disease expression (necrosis). Although statistical interactions made the interpretation of results difficult, 'Carnival Too' developed a high proportion of leaf and stem necrosis, whereas 'Cardinal' had the lowest levels of leaf and stem necrosis. This suggests that 'Cardinal' is less susceptible to *Colletotrichum* than 'Carnival Too'. Results were less clear with 'Rubens', which had high levels of leaf infection but lower levels of stem infection. This probably means that 'Rubens' is more likely to develop leaf blight than anthracnose, stem necrosis and shepherd's crook. However, at this stage, it is unknown whether this is the case under field conditions. It is suspected that the variation in susceptibility is not only the result of differences in cultivar resistance, but might also be due to variation in the leaf surface characteristics (Lubbe 2004).

Wounding generally increased the susceptibility of the *Protea* cultivars to both infection and lesion formation. Wounding is known to predispose plants to *Colletotrichum* infection (Muimba-Kankolongo and Bergstrom 1992; Shaw 1995). Muimba-Kankolongo and Bergstrom (1992) attributed the higher levels of anthracnose stalk rot of wounded maize plants to the availability of sucrose to

the pathogen. Sucrose causes an increase in conidium germination of *C. graminicola* (Bergstrom 1978). Wounding the *Protea* plants might have led to sucrose leaking from the wounded cells and, therefore, resulted in a higher percentage of germination. The fact that leaf wounding had a more significant effect than stem wounding is possibly due to the nature of the tissue, leaf tissue being less sclerophyllous.

Colletorichum acutatum was isolated from leaves that had lesions on them as well as from some that did not. It, therefore, seems that the pathogen is able to reside in the plant material in a latent form. Freeman *et al*. (2002) also found that symptomless material could yield the pathogen when they inoculated pepper, tomato and bean plants with a *C. acutatum* isolate originating from strawberry. They found that the pathogen survived and proliferated in these plants over a 3 month period without causing any symptoms, except on strawberry.

A factor that plays a role in the pathogenicity of *Colletotrichum* on *Protea*, and which was not measured in this study, is the phenological state of the host material. Pilot trials that were conducted prior to this study showed that lesions did not develop when material was inoculated during winter (results not shown). The *Protea* shoots of the most recent flush hardened off at the onset of winter and leaves were probably at their most resistant, and no symptoms developed on plants inoculated during the winter months.

This study may be used for developing a protocol for screening *Protea* cultivars for resistance to *Colletotrichum*. It is apparent that in screening procedures, all the pathogenic *Colletotrichum* species will have to be tested. If a single species was to be used in pathogenicity tests, a universal conclusion would not be possible on the susceptibility of proteas to *Colletotrichum*. Furthermore, different plant parts show different susceptibilities to the different species and this must also be taken into account. The difficulties experienced in obtaining infection and disease expression under artificial conditions also emphasise the important effect of the environment on the pathogen.

Finally, from the results obtained in this study, it is concluded that *C. acutatum* and *C. gloeosporioides* are the primary pathogens associated with Colletotrichum leaf necrosis, and that *C. acutatum* is the main cause of anthracnose and stem necrosis of the selected *Protea* cultivars in South Africa. A more comprehensive survey is necessary to determine the significance of the different *Colletotrichum* taxa on members of the Proteaceae.

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