INTRODUCTION

The family Sclerotiniaceae in the phylum Ascomycota includes two important plant pathogens, *Botrytis cinerea* and *Sclerotinia sclerotiorum*, that infect numerous phylogenetically unrelated plant species and significantly affect crop production (Navaud et al., 2018). In the same family, *Ciborinia camelliae* causes a similar but distinct disease because it infects only the floral organs of ornamental plants of the genus *Camellia*, causing petal blight disease. Infection starts with a short asymptomatic stage, followed by the rapid browning and fall of *Camellia* blooms (Denton-Giles et al., 2013). The disease affects many ornamental *Camellia* cultivars (e.g., *C. japonica*, *C. reticulata*) and can also infect the commercially important tea crop, *C. sinensis* (Denton-Giles et al., 2013).

Regardless of their lifestyle, fungi produce a wide range of secreted proteins. Proteins produced by pathogenic fungi help to circumvent physical barriers in their plant hosts, degrade complex organic compounds to acquire nutrients (e.g., cutinases, cellulases, endopolygalacturonases; Kubicek et al., 2014), and manipulate host responses (e.g., toxins and immune suppressors; Frías et al., 2011; Zhu et al., 2013). The composition of proteinaceous secretomes is linked to fungal lifestyle. Symbiotic and biotrophic fungi, which...
obtain nutrients from living host tissues, extensively employ a sophisticated machinery of small secreted proteins (SSPs) that allows them to manipulate host cell responses (Kim et al., 2016). In contrast, necrotrophic fungi, which exploit plant defences to facilitate infection, tend to secrete more carbohydrate-active enzymes (CAZymes) than biotrophic fungi (Zhao et al., 2014) and the role of these enzymes in host specificity is still not known.

Empirical genomic, transcriptomic, and proteomic approaches, combined with bioinformatic analysis, can predict and identify fungal secreted proteins. The fungal secretome of S. sclerotiorum was predicted using the fungal genome sequence and a bioinformatic pipeline that detects genes that encode secreted proteins (Guyon et al., 2014). Transcriptomic analysis on Dothistroma septosporum identified important fungal secreted proteins, including CAZymes and cysteine-rich SSPs, based on their temporal expression patterns during a time course of a pine needle blight infection (Bradshaw et al., 2016).

While genomic and transcriptomic approaches only predict possible compositions of secretomes, empirical proteomic methods directly purify, detect, and quantify the proteins themselves. The quantitative proteome analysis of Xanthomonas oryzae, which causes rice blight, identified a secreted cysteine protease XoCP, which contributes to virulence of the pathogen (Wang et al., 2017). An analysis of protein–protein interactions in the rice leaf apoplast during infection by Magnaporthe oryzae, combined with subsequent transient protein expression studies, showed that fungal glycosyl hydrolases can function as apoplastic pathogenicity effectors (Kim et al., 2013). Proteomic methods also allow purification of proteins for further functional studies. For example, a fast protein liquid chromatography (FPLC) method was used to purify and characterize necrosis-inducing proteins in B. cinerea and Zymoseptoria tritici culture filtrates (Ben M’Barek et al., 2015).

Here, we describe an empirical proteomic analysis of the highly host-specific Camellia petal blight infection. We tested the in vitro and in planta secretomes of C. camelliae and compared their protein composition with the related broad-host pathogen B. cinerea. Fungal culture filtrate and apoplastic washes of infected plants induced necrosis in both host and nonhost plants, thus indicating that secreted proteins contribute to the virulence of C. camelliae, notably including previously unrecognized nonhost and non-organ-specific factors.

2 | MATERIALS AND METHODS

2.1 | Plant material

Camellia ‘Nicky Crisp’ (C. japonica × C. pitardii var. pitardii) (Kilmarnock Nurseries) and Camellia lutchuenensis (Wairere Nursery) shrubs were grown in the Massey University Plant Growth Unit glasshouse (40°22′41.22″S, 175°36′48.73″E) at temperatures ranging from 1 to 20°C (natural conditions in New Zealand) with air inlets covered to limit the entry of airborne C. camelliae ascospores. Fully open blooms were picked from shrubs immediately prior to fungal infection or infiltration experiments.

Wild-type Nicotiana benthamiana plants were grown in a Biosyn Series 6000 plant growth chamber (Contherm Scientific) at 21°C, 12-h/12-h light/dark period and 180 μmol·m−2·s−1 light intensity. Four- to 6-week-old plants were used for the infiltration experiments.

2.2 | C. camelliae collection and infection assay

Collection of C. camelliae apothecia, isolation of ascospores, and infection of Camellia petals was performed according to the previously published methodology of Kondratev et al. (2020). The estimated final coverage of sprayed petals was 4.5–5 × 10^4 ascospores/cm². Control petals were sprayed with the same volume of sterile Milli-Q water. Treated petals were kept in trays covered with plastic wrap in a Biosyn Series 6000 plant growth chamber at 21°C, 12-h/12-h light/dark period and 180 μmol·m−2·s−1 light intensity.

2.3 | Collection of C. camelliae culture filtrate

C. camelliae strain ICMP 19812 (International Collection of Microorganisms from Plants) was grown in the dark at 20°C on potato dextrose agar (PDA; Difco) plates. To obtain C. camelliae culture filtrate, 25–500 ml of 24 g/L potato dextrose broth (PDB; Difco) was inoculated with an agar plaque of size 10 × 10 mm obtained from the edge of a 1-week-old colony of C. camelliae. Cultures were grown at 20°C in the dark for up to 1 month in airtight 50- to 1000-ml bottles closed with a plastic lid. Culture filtrate was harvested by centrifugation of the cultures at 3000 × g for 5 min and the resulting supernatant was sterilized through a 0.2-μm filter attached to a 50-ml sterile syringe. Aliquots of the culture filtrate were stored at −20°C and defrosted when needed.

2.4 | Collection of apoplastic washes from Camellia ‘Nicky Crisp’ petals

Mock-inoculated and inoculated Camellia ‘Nicky Crisp’ petals were collected at 48 h postinoculation (hpi) and fully infiltrated with sterile Milli-Q water using a 1-ml syringe with a needle. Subsequently, three infiltrated petals were wrapped in dry Miracloth (Merck) and inserted into a 50-ml plastic tube. The tubes were covered with lids to clamp the edges of Miracloth and suspend the petals at the top of the tube, and were centrifuged at 400 × g for 15 min and 1000 × g for 5 min. The liquid was collected from the bottom of the 50-ml tubes (c. 300–500 μl), sterilized using 0.2-μm filters attached to 10-ml syringes, and transferred to 1.5-ml tubes to be stored at −20°C. The petals were inspected to ensure the absence of any mechanical damage caused by the procedure. This method is shown in Figure S1.
2.5 | Extraction of proteins

A quarter volume of 100% (wt/vol) trichloroacetic acid (TCA; VWR BDH Prolabo) was added to the sample, gently mixed by inversion and incubated for 30 min at 4°C. Tubes were centrifuged at 17,000 × g for 5 min and the supernatant was removed. Resulting pellets were washed twice with 200 μl of cold acetone centrifuging at 17,000 × g for 5 min between washes. Then, pellets were dried to remove the acetone in a 50°C heat block for 10 min.

2.6 | SDS-PAGE analysis

For Laemmli SDS-PAGE analysis, a Mini-PROTEAN (Bio-Rad) system was used with 12% acrylamide/bis-acrylamide (37.5:1, Bio-Rad) gels (Laemmli, 1970). PageRuler (Thermo Fisher Scientific) was added as a molecular weight marker. Gels were stained using the Pierce Silver Stain Kit (Thermo Fisher Scientific), according to the manufacturer’s protocol.

2.7 | LC-MS/MS analysis

Proteins were extracted from 400 ml of culture filtrate of 2-week-old C. camelliae liquid culture (one biological replicate) and 20 μg of total protein were reduced with 10 mM dithiothreitol at 56°C for 15 min, alkylated with 50 mM iodoacetamide at room temperature in the dark for 20 min, digested with trypsin (Promega) for 1 h at 45°C, then analysed on an Eksigent 425 nanoLC chromatography system (Sciex) connected to a TripleTOF 6600 mass spectrometer (Sciex). The sample was desalted and separated using a 0.3 × 10 mm trap column packed with Reprosil C18 media (Dr Maisch) and a 0.075 × 200 mm picofrit column (New Objective) packed in-house with Reprosil C18 media. The following gradient was applied at 250 nl/min using a NanoLC 400 UPLC system (Eksigent): 0 min 1% B; 2 min, 1% B; 105 min, 35% B; 110 min, 98% B; 115 min, 98% B; 116 min, 1% B, 120 min, 1% B (A, 0.1% formic acid in water: B, 0.1% formic acid in acetonitrile).

Mass spectrometry was performed on TripleTOF 6600 quadrupole-time-of-flight mass spectrometer (Sciex) scanning from 350 to 1600 m/z for 150 ms; the 30 most abundant multiply charged peptides were analysed by 40 ms MS/MS scans (m/z 100–1600) for a total cycle time of c. 1.5 s.

The resulting MS/MS data were searched against a database (110,542 entries in total) composed of the abovementioned C. camelliae sequences (Denton-Giles et al., 2020), Camellia ‘Nicky Crisp’ proteins (Kondratev et al., 2020) and common contaminant sequences. Protein sequences of Camellia ‘Nicky Crisp’ were previously predicted using Transdecoder v. 5 from the de novo plant transcriptome (NCBI Bio-Projects PRJNA518146), which was assembled using Trinity v. 2.2.0 (Haas et al., 2013). The search was performed using ProteinPilot v. 5.0 (Sciex) with the following search parameters: Sample Type, Identification; Search Effort, Thorough; Cys Alkylation, Iodoacetamide; Digestion, Trypsin.

The Analyst TF v. 1.7 software package (Sciex) was used to control the mass spectrometer and HPLC system.

2.8 | Necrotizing activity test

Necrosis-inducing activity of the culture filtrate and apoplastic fluids was tested on at least two petals or leaves. Liquids were infiltrated into Camellia petals using a 1-ml syringe with a needle, and without a needle into N. benthamiana leaves. One infiltration volume was approximately 50–100 μl.

2.9 | Temperature and proteinase K treatment

To check the influence of temperature on necrosis-inducing activity of the culture filtrate, 0.5 ml aliquots were kept for 4 h at 37, 50, or 80°C. To perform a proteinase treatment, 25 μl of proteinase K (Roche) stock solution (20 mg/ml) was added to 475 μl of the culture filtrate to a final concentration of the enzyme of 1 mg/ml and the mix was kept at 37°C for 4 h.

2.10 | Bioinformatic and statistical analysis

Sequences of proteins detected in C. camelliae culture filtrate and apoplastic fluids of Camellia ‘Nicky Crisp’ petals were imported into
Blast2GO v. 5.1 (Conesa et al., 2005) and searched against the NCBI nonredundant protein database (nr) using the Blastp algorithm with default settings (Altschul et al., 1990). Proteins were manually assigned into functional groups based on their best Blastp hits. Krona was used to visualize the resulting annotation of plant apoplastic proteins (Ondov et al., 2011). χ² independence tests of count tables were performed using base R v. 3.5 (R Core Team, 2020). Protein localization prediction was performed using WoLF PSORT (Horton et al., 2007).

3 | RESULTS

3.1 | C. camelliae culture filtrate has necrosis-inducing activity

Fungal culture filtrate that had been infiltrated into the apoplastic space of petals of the susceptible Camellia cultivar Nicky Crisp led to rapid browning around the infiltration area (Figure 1a), suggesting that the filtrate contained necrosis-inducing compounds. Infiltration with sterile PDB caused no necrosis.

3.2 | Necrogenic factors of C. camelliae culture filtrate are proteinaceous

To determine whether the necrogenic agents were secondary metabolites or proteins, the C. camelliae culture filtrate was treated with heat and proteinases. Proteins are expected to be heat-sensitive and disabled by proteinases. Heat treatment at 37°C slightly decreased the activity of the culture filtrate, as shown by the delayed development of necrosis (Figure 1b, left halves of petals on the first row). In contrast, addition of proteinase K at the same temperature completely stopped the necrosis-inducing activity (Figure 1b, right halves of petals on the first row). High temperature treatments at 50 and 80°C fully inactivated the culture filtrate (Figure 1b, second and third rows, respectively). Taken together, these observations suggested that the necrogenic agents of C. camelliae culture filtrate were proteinaceous.

3.3 | C. camelliae culture filtrate contains known fungal proteins

To identify the secreted proteins, the pellet resulting from TCA-acetone precipitation of a liquid culture was analysed using LC-MS/MS. In total, 94 fungal proteins were detected by comparison against the fungal proteome previously predicted using bioinformatic analysis of the C. camelliae genome sequence (Denton-Giles et al., 2020). These proteins were annotated using Blastp against the NCBI nonredundant protein database (Table S1). Out of 94 fungal proteins detected, 90% (85) were predicted by the WoLF PSORT tool to be extracellular, which was much higher than the 5% secreted proteins of the total C. camelliae proteome predicted by Denton-Giles et al. (2020). The most numerous protein groups were CAZymes, oxidoreductases, and proteases. The list of proteins obtained from C. camelliae culture filtrate is subsequently referred to as the in vitro secretome.

3.4 | C. camelliae infection induces changes in the apoplastic proteome of the host

After passing the petal cuticle and epidermis, C. camelliae continues its growth into the intercellular space of the petal mesophyll (Denton-Giles et al., 2013). The presence of the pathogen would be expected to induce changes in the plant apoplast and affect the apoplastic proteome. To test this hypothesis, we compared the proteomic composition of apoplastic fluids from mock-treated and infected susceptible Camellia ‘Nicky Crisp’ petals at 48 hpi. SDS-PAGE analysis of the proteins extracted from apoplastic fluids showed that the infected petals contained considerably less
protein than the mock-treated petals (Figure 2a); many protein bands were absent in the infected samples and the intensity of the remaining bands was lower.

To conduct a qualitative comparison between the proteomes of mock-treated and infected petal apoplasts, the proteins extracted from apoplastic fluids were also analysed using LC-MS/MS. Bioinformatically predicted *Camellia* 'Nicky Crisp' and *C. camelliae* proteomes were used to identify plant and fungal proteins in the mass spectrometry data. As a result, 164 plant proteins were identified in the mock-treated sample and 98 proteins in the infected sample. All detected proteins were annotated using Blastp against the NCBI nonredundant protein database.

Mock-treated and infected samples had 48 plant proteins in common (Figure 2b). The full list of detected proteins and their annotations is presented in Table S1. Based on their annotation, plant proteins were assigned into functional groups. Counts of individual proteins in these groups further highlighted protein frequency differences between mock-treated and infected samples. The two count tables differed significantly ($\chi^2 = 17.352, \text{df} = 8, p = 0.027$; Table S2) confirming that *C. camelliae* infection affects the frequency of many proteins secreted by the host. The mock-treated sample had a higher proportion of enzymes associated with plant cell wall biosynthesis and modification. Oxidoreductases, pathogen-resistance and signalling proteins were more frequent in the infected sample. Furthermore, two normally cytosolic proteins—ubiquitin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)—were detected in the infected sample only. Diagrams showing the functional groupings of these enzymes are given in Figures S2 and S3. In summary, SDS-PAGE and LC-MS/MS analyses showed that the petal apoplastic proteome of susceptible *Camellia* 'Nicky Crisp' undergoes significant changes as a result of infection with *C. camelliae*.

### 3.5 Secreted *C. camelliae* proteins are detected in infected petal apoplast

During infection of the petal apoplast, *C. camelliae* is expected to secrete proteins that facilitate fungal growth and virulence. Indeed, 55 fungal proteins were detected in infected petal apoplasts by reference to the *C. camelliae* genome sequence (Denton-Giles et al., 2020). All detected proteins were annotated using Blastp matches against sequences from the NCBI nonredundant protein database. This set of proteins is referred to as the in planta secretome (Table S1). Out of these proteins, 69% (38) were annotated as extracellular according to the WoLF PSORT tool. This is significantly higher than the 5% of the total *C. camelliae* proteome that was predicted to be secreted (Denton-Giles et al., 2020). Similar to the in vitro secretome, CAZymes, oxidoreductases, and proteases constituted the majority of detected proteins.

### 3.6 CAZymes are the main components of the *C. camelliae* in vitro and in planta secretomes

Comparison of the in vitro and in planta secretomes of *C. camelliae* shows that they share 25 fungal proteins, while 69 and 30 proteins were unique to the culture filtrate and infected plant apoplast, respectively (Figure 3). Based on their annotations, all proteins were classified into five functional groups: CAZymes, hypersensitive response elicitors (HR elicitors), oxidoreductases, proteases, and other proteins (see Table S1 for a full list of in vitro and in planta fungal secreted proteins). The percentage of CAZymes was higher in planta than in vitro, while the culture filtrate contained more oxidoreductases and proteases. Surprisingly, HR elicitors
were detected only in the culture filtrate. Nevertheless, the number of proteins in these functional groups did not differ significantly between the in vitro and in planta secretomes \((\chi^2 = 1.952, \text{df} = 4, p = 0.745; \text{Table S3})\). Moreover, protein counts also did not differ significantly from the in vitro secretomes of two \(B. \ cinerea\) strains (Table 1) (González- Fernández et al., 2014). In summary, secreted \(C. \ camelliae\) proteins were detected in the fungal culture filtrate and the apoplast of infected petals, and these protein sets had similar functional distributions, with CAZymes being the major group.

3.7 | Culture filtrate and apoplastic fluids cause necrosis in both susceptible and resistant plants

The observed similarity of host-specific \(C. \ camelliae\) versus broad-host \(B. \ cinerea\) secretomes led us to hypothesize that the necrogenic activity of \(C. \ camelliae\) secreted proteins may not be exclusive to its host plant. To test this, fungal culture filtrate and apoplastic fluids of infected petals were infiltrated into host \(C. \ camelliae\) petals and non-host \(N. \ benthamiana\) leaves (Figure 4). Two-week-old sterile PDB and apoplastic fluids of mock-treated petals were used as control infiltrations and no response of necrosis in petals or leaves was observed.

As expected, susceptible \(C. \ camelliae\) ‘Nicky Crisp’ petals developed necrosis in response to both fungal culture filtrate and apoplastic fluids of infected petals; however, both fluids also caused necrosis in petal blight-resistant \(C. \ lutchuensis\) petals and nonhost \(N. \ benthamiana\) leaves. This demonstrates that necrosis-inducing proteins from \(C. \ camelliae\) could cause necrosis in both host and nonhost plants.

4 | DISCUSSION

Using LC-MS/MS, together with in silico predicted plant and fungal proteomic data sets, we characterized the secreted proteome of the host-specific fungal pathogen \(C. \ camelliae\) and its \(C. \ camelliae\) plant host. Fungal proteins secreted in vitro and in planta demonstrated necrogenic activity against both host and nonhost plants, indicating a broader range of virulence activity than may be expected for this host-specific pathogen.

The comparison of lists of proteins detected in samples from fungi and petals treated in different ways resulted in only small overlaps...
that specifically target plant PR-1 proteins and mediate necrosis in vitro fungal secretomes. As predicted by the in silico approach in distribution of proteins did not differ between the in planta and in petal apoplasts and culture filtrates. Interestingly, the functional (Dickman & de Figueiredo, 2013).

well-known characteristic of the necrotrophic stage of the infection petal blight had caused leakage of the cytoplasm, which is another of cytosolic ubiquitin and GAPDH in the plant apoplast suggests that wheat (Breen et al., 2016; Lu et al., 2014). Moreover, the appearance of plant apoplastic proteins. It is unclear whether this is due to side effects of treatment, sampling bias or large variance between replicates, which are generally high for LC-MS/MS-based proteomic analysis (Tabb et al., 2010). Nonetheless, LC-MS/MS observations have proven informative in other settings (Escobar-Tovar et al., 2015; Shah et al., 2009; Wang et al., 2011), especially when considering the group-level distribution of proteins among functional groups, as undertaken here.

We found that petal blight infection is associated with a dramatic decrease in plant apoplastic proteins. It is unclear whether this is the result of the proteolytic activity of the fungal secretome (Jashni et al., 2015) or a host-driven decrease of plant protein production caused by the biotic stress of the pathogen. In agreement with the necrotrophic nature of the disease, the apoplast of infected petals had a higher percentage of many PR-proteins, including PR-1, which facilitates the propagation of pathogen-induced necrosis. For example, the necrotrophs S. nodorum and P. nodorum produce effectors that specifically target plant PR-1 proteins and mediate necrosis in wheat (Breen et al., 2016; Lu et al., 2014). Moreover, the appearance of cytosolic ubiquitin and GAPDH in the plant apoplast suggests that petal blight had caused leakage of the cytoplasm, which is another well-known characteristic of the necrotrophic stage of the infection (Dickman & de Figueiredo, 2013).

Fungal secreted proteins were detected both in the infected petal apoplasts and culture filtrates. Interestingly, the functional distribution of proteins did not differ between the in planta and in vitro fungal secretomes. As predicted by the in silico approach in

FIGURE 4 Necrogenic activity of Ciborinia camelliae culture filtrate and apoplastic fluids of infected susceptible Camellia 'Nicky Crisp' petals. Approximately 50–100 μl of liquid were infiltrated into petals of susceptible Camellia 'Nicky Crisp', resistant Camellia lutchuensis, and leaves of nonhost Nicotiana benthamiana. The first column shows the results for infiltrations of the culture filtrate, where sterile potato dextrose broth (PDB) was used as a control. The second column shows the infiltration results of apoplastic fluids from infected Camellia 'Nicky Crisp' petals extracted at 48 h postinoculation (hpi). Apoplastic fluids of mock-treated petals extracted at 48 hpi were used as a control. The photographs show representatives of at least two biological replicates [Colour figure can be viewed at wileyonlinelibrary.com].

of these sets. Some of the observed differences in proteome composition may be due to side effects of treatment, sampling bias or large variance between replicates, which are generally high for LC-MS/MS-based proteomic analysis (Tabb et al., 2010). Nonetheless, LC-MS/MS observations have proven informative in other settings (Escobar-Tovar et al., 2015; Shah et al., 2009; Wang et al., 2011), especially when considering the group-level distribution of proteins among functional groups, as undertaken here.

We found that petal blight infection is associated with a dramatic decrease in plant apoplastic proteins. It is unclear whether this is the result of the proteolytic activity of the fungal secretome (Jashni et al., 2015) or a host-driven decrease of plant protein production caused by the biotic stress of the pathogen. In agreement with the necrotrophic nature of the disease, the apoplast of infected petals had a higher percentage of many PR-proteins, including PR-1, which facilitates the propagation of pathogen-induced necrosis. For example, the necrotrophs S. nodorum and P. nodorum produce effectors that specifically target plant PR-1 proteins and mediate necrosis in wheat (Breen et al., 2016; Lu et al., 2014). Moreover, the appearance of cytosolic ubiquitin and GAPDH in the plant apoplast suggests that petal blight had caused leakage of the cytoplasm, which is another well-known characteristic of the necrotrophic stage of the infection (Dickman & de Figueiredo, 2013).

Fungal secreted proteins were detected both in the infected petal apoplasts and culture filtrates. Interestingly, the functional distribution of proteins did not differ between the in planta and in vitro fungal secretomes. As predicted by the in silico approach in

Denton-Giles et al. (2020), CAZymes had the highest abundance in both the in vitro and in planta secretomes, in line with the necrotrophic nature of C. camelliae (Bellincampi et al., 2014). Homologs of three small necrosis-inducing proteins—cerato-platanin (Frias et al., 2011), NLP2 (Schouten et al., 2008), and the 22 kDa glycoprotein EC91 (UniProt A0A166VV93)—were detected only in the culture filtrate (sequence IDs au3244, au2222, au4906 in Table S1, respectively). Whether these proteins are not produced in planta or were simply not detected in the apoplast remains unclear. Importantly, however, the in vitro and in planta secretomes had three fungal endopolygalacturonases in common (sequence IDs au4598, au9824, au774 in Table S1). These are homologs of important virulence factors in B. cinerea, which are recognized as invasion patterns in Arabidopsis thaliana (Kars et al., 2005; Zhang et al., 2014).

The necrogenic activity of infected apoplastic fluids and fungal culture filtrate was demonstrated by their infiltration into plant tissues. Interestingly, necrotic responses were observed in all tested plants, which included highly susceptible Camellia 'Nicky Crisp', resistant C. lutchuensis, and nonhost N. benthamiana. All of the controls were negative, as expected (Figure 4). Similarly, in the wheat-Z. tritici pathosystem, the pathogen culture filtrate induced necrosis in both susceptible and resistant plant lines (Ben M’Barek et al., 2015). In contrast, only susceptible wheat cultivars demonstrated a necrosis response to S. nodorum culture filtrate (Liu et al., 2004). In our experiment, the C. camelliae culture filtrate and infected apoplastic washes contained a necrotrophic repertoire of secreted proteins, which shares multiple conserved protein groups with the broad-host pathogen B. cinerea and S. sclerotiorum (Denton-Giles et al., 2020). This probably explains the observed effect on resistant and nonhost plants. This is important because it is consistent with the hypothesis that plant susceptibility and resistance to C. camelliae, and presumably host specificity, are defined by interactions occurring at the early stages of the infection (Kondratev et al., 2020).

Small cysteine-rich effector proteins have a highly stable molecular structure and have been shown to keep their necrogenic activity even after exposure to high temperatures (Liu et al., 2012). In contrast, the activity of CAZymes can be inhibited by heat treatment; for example, a secreted xylloglucanase of B. cinerea, BcXYG1, has a heat-sensitive tertiary structure, which is important for the induction of necrosis (Zhu et al., 2017). Moreover, plant host recognition of B. cinerea proteins in their native conformation (e.g., polygalacturonases [Zhang et al., 2014] and xyylanase 11A [Noda et al., 2010]) is required for full fungal virulence. Thus, matching observations with C. camelliae culture filtrate, we speculate that heat-sensitive CAZymes may be among the main secreted necrogenic components. For infected apoplastic washes, the necrogenic activity may be caused not only by fungal proteins, but also by damage-associated molecular patterns (DAMPs) that are released in the course of cell wall destruction (Benedetti et al., 2015).

The establishment of host specificity in a group of pathogens is a complex process, which requires the development of multiple
molecular mechanisms by the pathogen (Li et al., 2020). In the case of C. camelliae, it remains unclear if a secreteme that is highly similar to related broad-host necrotrophic pathogens is involved in actually establishing host specificity. Research on three Microbotryum species demonstrated that host specialization can be associated with changes in the amino acid sequence of secreted proteins, rather than gain or loss of secretome components (Beckerson et al., 2019). A similar mode of action may be occurring here, and comparative analysis of host-specific activity of detected candidate-effectors from B. cinerea and C. camelliae may help to explain the host specificity of the petal blight pathogen. Alternatively, there may be components within the mixed secretome that do affect specificity but they are masked by the other components that cause necrosis on nonhost plants.

Overall, we demonstrate the necrotrophic nature of the petal blight disease. C. camelliae produces a Sclerotiniaceae-like secreteme, which appears to contribute to the virulence of the fungus, but does not have a role in determining its host specificity. The recognition that the necrotrophic factors are proteins and most have broad-host action suggests that other factors cause host specificity.

ACKNOWLEDGEMENTS
The authors thank the New Zealand Camellia Memorial Trust for funding this research.

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

ORCID
Rosie E. Bradshaw https://orcid.org/0000-0001-5228-2685
Paul P. Dijkwel https://orcid.org/0000-0002-1432-2209

REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of the article at the publisher’s website.