WATER AND INOCULATION METHOD AFFECT GERMINATION AND PENETRATION BY *CIBORINIA CAMELLIAE*

V. VINGNANA SINGAM¹, P.G. LONG¹ and R.E. ROWLAND²

¹*Institute of Natural Resources, Massey University, Palmerston North*

²*Institute of Molecular BioSciences, Massey University, Palmerston North*

Corresponding author: vvingnan@massey.ac.nz

ABSTRACT

Possible interactions between free water and methods of inoculating camellia petals with *Ciborinia camelliae* were investigated. Spore suspensions were applied by pipette or atomiser, while non-aqueous applications were made with a paintbrush or gravitational deposition from an apothecium. Most spores germinated and penetrated the cuticle with pipetted spore suspensions or with gravitational deposition from an apothecium. Brush and spray applications resulted in less germination and fewer infections. Drying petal tissue before incubation in humid chambers reduced germination, penetration and development of symptoms. If water was added to petals with brush applied spores then multiple germ tubes and swollen hyphae were common and many germ tubes grew across the petal surface without penetrating.

Keywords: germination, penetration, free water, inoculation method, *Ciborinia camelliae*.

INTRODUCTION

Free water maintained continuously at the interface between the inoculum source and the surface of the inoculated tissue is essential for germination of many fungal spores, e.g. ascospores of *Venturia inaequalis* on apple (MacHardy 1996) and of *Whetzelinia sclerotiorum* on bean (Abawi & Grogan 1975). Ascospores of *Botryosphaeria obtusa* showed a relatively higher percentage germination and germ tube elongation with free water than without (Arauz & Sutton 1989), as did ascospores of *Mycosphaerella citri* (Whiteside 1974). There are reports of plant infection by dry-inoculated spores under conditions of high humidity. Examples are apple with ascospores of *Venturia inaequalis* (Clayton 1942) and rose petals with conidia of *Botrytis cinerea* (Williamson et al. 1995).

The effect of free water on germination of *Ciborinia camelliae* ascospores has not been studied. It is important to understand the effect of the presence of free water on spore germination and penetration in order to adopt appropriate inoculation methods in infection studies and screening for resistance to *C. camelliae*. The objectives of this work were to investigate the role of free water in ascospore germination and to assess the success of a range of inoculation methods for achieving high infection rates.

MATERIALS AND METHODS

Apothecia collected from Wellington Botanic Gardens were used as a source of inoculum. Four methods were used to apply ascospores of *C. camelliae* to camellia petals, which were then incubated 'wet' or 'dry'.

1. Pipette inoculation

Ascospore suspension (2 µl, 5x10⁴ spores per ml) was placed at four sites on each of four flower petals using a micro-pipette. Immediately after inoculation, half of the petals were transferred to a humid box and incubated at 20°C for 36 h. The remaining

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petals were air dried in a laminar flow cabinet for 1-2 h before placing them in humid boxes and incubating as described above.

2. Spray inoculation

A spore suspension at the same concentration as above was sprayed on four camellia flower petals using a fine atomiser. Immediately after spraying, half of the petals were placed in a humid box, the other half was air-dried and both sets were incubated as described above.

3. Gravity inoculation

Sclerotia with the stipes of their apothecia were wrapped with a wet facial tissue and suspended in an inverted position about 4-5 cm above six petals in a humid plastic container for 2 h. After inoculation, the petals were grouped into three sets. One set was incubated immediately and the other two sets were sprayed with sterile distilled water (SDW) using a fine atomiser. One SDW-sprayed set was incubated immediately, and the other was air dried and incubated as described above.

4. Brush inoculation

A paintbrush with fine bristles (15 mm long) was gently touched two or three times onto apothecial hymenia to pick up ascospores. The ascospores on the bristles were then inoculated on to six petals by gently touching the petal surface two to three times. After inoculation, the petals were grouped into three sets, which were treated as explained in the gravity inoculation method.

Microscopic observation

Pieces of tissues (about 25 mm²), were cut from inoculated petals (two pieces of tissue from each of the two petals in each inoculation x time treatment) at 6 h, 12 h and 24 h after inoculation. They were stained with 0.05% trypan blue in lactophenol on a glass slide over a Bunsen flame for a few seconds until the solution just boiled. The stained specimens were mounted in 0.1% FeCl₃ in 50% glycerol and observed under a compound microscope at 400 times magnification. The numbers of germinated and non-germinated spores together with germ tube penetration were counted, and germ tube length was measured. For each treatment, assessments were made from two fields of view at each of two sites on two petals. There were up to 52 spores per site and all hyphae observed were measured.

Symptom measurement

All petals were observed for symptom development, which was measured and assessed on a 0 to 4 scale (0 - No lesions; 1 - Slightly visible yellowish brown dots; 2 - Visible yellowish brown pin head size lesions; 3 - 1-2 mm size of brown lesions; 4 - Fully developed >5 mm size of brown lesions).

Each treatment was replicated three times and the experiment was conducted twice.

Statistical analysis

The experiment was analysed as a split plot design by a generalized linear model using the SAS computer programme.

RESULTS

Maximum spore germination of *C. camelliae* and germ tube penetration was reached at the 12 h incubation assessment in all treatments except the brush inoculation where germination was low at this time (Figs 1a-1h). Both the pipette and gravity inoculations resulted in almost 100% spore germination and penetration. If spore suspensions were allowed to dry before incubation in a humid chamber then germination and subsequent penetration were dramatically reduced (Figs 1a, 1b, 1c & 1d).

When spores were released from an apothecium and allowed to settle on a petal, the addition of water as a fine spray did not affect percentage germination or penetration (Figs 1e and 1f). If the water was allowed to dry before incubation then germination was reduced at 6 and at 12 h but by 24 h there was no effect on germination. Some spores were allowed to settle on glass slides and microscopic examination showed a small amount of liquid associated with each spore.

A subsequent spray of water enhanced germination of spores transferred from an apothecium to petals with a brush before incubation but if it was allowed to dry before incubation then there was very little germination or penetration (Figs 1g and 1h).

FIGURE 1: Percent germination and penetration of *C. camelliae* ascospores 6 h, 12 h and 24 h after incubation. (a) germination and (b) penetration from pipette inoculation, (c) germination and (d) penetration from spray inoculation, (e) germination and (f) penetration from gravity inoculation, and (g) germination and (h) penetration from brush inoculation. The SEM is shown on each bar.

Typically, ascospores of *C. camelliae* germinated on the petal surface and the germ tubes penetrated the cuticle almost immediately (Fig. 2a). In two treatments (pipetted spore suspension and brush applied spores after applying a water spray) where free water was present on the surface of the petals, approximately 10% of the germ tubes grew more than 6 μm over the leaf surface and such germ tubes did not penetrate the cuticle (Fig. 2b). These germ tubes averaged 16 μm in length in the pipette application and 7.4 μm in the wetted brush application with a maximum of 36 μm . In the latter treatment, these longer germ tubes were usually thicker and there were multiple germ tubes from each ascospore (Fig. 2c and 2d).

Symptoms appeared on petals inoculated by the gravity and brush methods within 24 h and by 36 h disease was extensive in the gravity treatment (Fig. 3a). When water was added to petals inoculated by the gravity method, fewer symptoms had appeared by 24 h but by 36 h the maximum disease rating had been reached (Fig. 3b). Symptom appearance in the brush treatment was similar whether water was sprayed on the petal or not. When spore suspensions were applied by pipette, symptom development was not as extensive as in the “dry” or “wet” gravity treatments, while a spray application of the suspension reached the same disease rating as the gravity method by 36 h (Fig 3b). Where water was used to

FIGURE 2: *Ciborinia camelliae* ascospore germination and germ tube growth. (a) Typical penetration and growth of germ tube underneath cuticle. (b) Surface germ tube growth after pipette inoculation. (c) Thicker germ tubes after brush inoculation. (d) Multiple spore germination after brush inoculation.

make spore suspensions or where “dry” methods were used and then water added, a subsequent drying of the inoculated petal surface both delayed the appearance of, and resulted in a reduced severity of, symptoms (Fig. 3c).

DISCUSSION

Ciborinia camelliae infects camellia flowers during July–November in New Zealand, a time when the weather is usually cool and humid. The petals on which ascospores land could be dry or have a coating of water from rain or dew. The presence of free water on

FIGURE 3: Symptom development on camellia flower petals 12 h, 24 h and 36 h after inoculation and incubation (a) without free water, (b) with free water and (c) after drying previously wet petal surface. The SEM is shown for each point.

the infection surface can have a marked effect on the germination behaviour of some spores as shown by Cole et al. (1996) for conidia of *Botrytis cinerea*. The effect of free water also has important implications for inoculation studies since inoculum loading is easily controlled in spore suspensions but not in air deposition methods.

Presence or absence of abundant free water was not critical for germination since percentage germination and disease development from spores settling on petals after release from an apothecium were similar to those of the spore suspensions. However, fewer spores transferred by brush germinated than did those obtained direct from an apothecium. Spores released from an apothecium had a small amount of liquid associated with them and this could have provided a reservoir of moisture that was critical for the first hour of germination and which could have been absent from the brush transferred spores. Alternatively, spores transferred by brush could have been damaged by the bristles or could have contained a higher proportion of immature spores released from asci ruptured by the bristles of the brush. Adding water to the petals immediately after transfer of spores by brush increased percentage germination and penetration after 12 h incubation but this effect had disappeared by 24 h.

Although addition of free water to spores inoculated by brush or gravity did not have a significant effect on numbers of spores germinating, there was some evidence that it could affect the subsequent growth of germ tubes and penetration. When brush inoculated petals were sprayed with water, germ tubes grew across the surface of the petal and those that grew more than 6 mm did not penetrate the cuticle. They were frequently swollen and many spores produced multiple germ tubes. Cole et al. (1996) found that germ tubes from conidia of *Botrytis cinerea* penetrated bean leaves immediately in the absence of free water but grew across the surface if water was present. They also found that some conidia stood on end in the absence of free water but there was no evidence of this behaviour with ascospores of *C. camelliae*. When germ tubes grow across the surface of the petals there is a greater probability of them contacting fungicide deposits than when penetration is adjacent to the spore. The germ tubes of *C. camelliae* usually penetrate adjacent to the spore and this could be one reason why fungicides have failed to give satisfactory control of this disease. The multiple germ tubes in the brush inoculated treatment could result from minor damage to the ascospore walls by the brush bristles while the extended germ tube growth, lack of penetration and swollen hyphae could result from fungitoxic material released from micro-damage to the petal surface by the brush.

The difference in spore germination and penetration between the pipette and spray ascospore applications is difficult to explain as the same spore suspension was used in each. Possibly the ascospores are delicate and some were damaged by the stress of being forced through a small aperture under pressure.

Overall, either the pipette application of spore suspensions or the use of apothecia suspended over petals would appear to give comparable results when used for inoculation of petals in experimental work. In the field, our results would suggest that there would be little difference between spores landing on a petal with no free water under high humidity conditions and spores landing on a petal wet with dew or rain. However, if the petal dries before penetration has taken place then the number of infections would be reduced.

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