

## PERSISTENCE AND SURVIVAL OF SAPROPHYTIC FUNGI ANTAGONISTIC TO *BOTRYTIS CINEREA* ON KIWIFRUIT LEAVES

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### ABSTRACT

*Alternaria*, *Epicoccum* and *Ulocladium* spp. are potential competitors to *Botrytis cinerea*. The persistence and survival of these saprophytic fungi on the upper and underside of kiwifruit leaves were investigated in a field trial at Motueka from January to May 1996. Spore numbers and germination were measured as indicators of persistence and survival respectively. Spore number declined significantly on both leaf surfaces with time. Although germination declined significantly over time, after 16 weeks, the germination rate remained > 50% for all three genera. No major differences in spore number and germination between leaf surface nor genus were observed. The effects of UV radiation (254 nm) on spore germination *in vitro* for nine saprophytic isolates in comparison with 12 *B. cinerea* isolates are presented.

**Keywords:** *Alternaria*, *Epicoccum*, *Ulocladium*, UV radiation, germination.

### INTRODUCTION

Stem end rot of kiwifruit caused by *Botrytis cinerea* Pers.Fr. is the most important disease of stored kiwifruit in New Zealand. In the last three years, direct losses caused by *B. cinerea* have cost the industry up to \$16.3 million/annum (Garnham 1995). Necrotic areas on kiwifruit leaves have been identified as a major source of *B. cinerea* inoculum and a correlation was established between the level of necrotic tissue in the kiwifruit canopy, *B. cinerea* population size, contamination of the fruit surface with *B. cinerea* spores and stem end rot (Elmer *et al.* 1995; 1997; Pennycook *et al.* 1995). In 1994, a collaborative research programme between HortResearch and IPO-DLO, Wageningen, The Netherlands, was established to investigate the use of saprophytic fungi as biological control agents (BCAs) for control of *B. cinerea*.

Köhl and Fokkema (1994) stated in reference to wounds on leaves, "when man-made wounds have to be protected, the use of BCAs is promising because the target and the optimum application time are clearly defined and protection is required for only a few days". However, necrotic kiwifruit tissue is present throughout most of the growing season and optimum application timing may be difficult to identify. When applying a BCA in the field, the majority of antagonist inoculum will be deposited on healthy tissue. If this inoculum persists and survives, it would be available for colonisation of developing necrotic kiwifruit leaf tissue and could suppress *B. cinerea* sporulation. The number of BCA applications required over a season will partly depend on the survival rate of the BCA concerned.

Environmental factors such as moisture, temperature and radiation have a large influence on the survival of fungal spores (Caesar and Pearson 1983; Stevenson and Pennypacker 1988) and interact to give each leaf or leaf-part a unique micro-climate that is dependent upon its position with the plant canopy (Burrage 1971). Canopy position was shown to influence fungal survival (Caesar and Pearson 1983; Elmer and Kohl 1998). Although short wavelength UV radiation (250-270 nm) is a very small component of solar radiation, it is the main fungicidal element of solar radiation

(Rotem *et al.* 1985). In nature, most fungi are exposed during a portion of their life cycle to short wavelength UV radiation (Leach 1971). In this work we studied the persistence and survival of three genera of saprophytic fungi previously evaluated for their ability to suppress *B. cinerea* sporulation (Walter *et al.* 1996a and b). Fungi were exposed to UV short wavelength irradiation in the laboratory and to natural field conditions for a 16 week period on the upperside and underside of kiwifruit leaves.

## MATERIALS AND METHODS

### Isolates and inoculum

Saprophytic fungi evaluated were *Alternaria*, *Epicoccum* and *Ulocladium* spp. (Table 1). Fungi were maintained on MEA slopes (30 g maltex, 20 g agar/litre) at 4°C. Spores of the saprophytes and *B. cinerea* were obtained from four week and two week old cultures respectively, growing on oatmeal agar (30 g oatmeal, 20g agar/litre), at ambient temperature (20°C - 24°C) with a 12 photoperiod. Cultures were flooded with sterile distilled water plus 0.01% Tween 80 (Tween water) and the resultant suspension was filtered through a double layer of lens tissue (Whatman 105) to remove mycelial fragments. Suspensions were centrifuged at 3000 rpm for two minutes, the supernatant decanted and the spore pellet resuspended in Tween water to remove excess nutrients. This procedure was repeated twice. Spore suspensions were then counted with a haemocytometer and adjusted to 10<sup>6</sup> spores/ml for UV survival tests and 1.5 x 10<sup>6</sup> spores/ml for field survival tests. For field studies, each genus was applied separately as a mixture of equal proportions of the three isolates to investigate trends between genera rather than within a genus.

### UV survival

The time taken for UV radiation to reduce spore germination to 50% of the non-exposed values (LT<sub>50</sub>) was determined for nine saprophytic isolates in two replicate experiments and for 12 *B. cinerea* isolates. For each fungal isolate, the spore suspension (20 ml) was irradiated in a laminar flow cabinet equipped with a germicidal UV lamp (Philips TUV 30W/G30 T8) producing a wavelength of 254 nm. The spore suspension was placed in the bottom half of a sterile petri dish, on a magnetic stirrer and gently agitated at low speed using a sterile metal paper clip. The distance between the UV source and the magnetic stirrer was 22 cm. An aliquot of 100 µl was removed at regular intervals for up to 120 minutes and three 30 µl droplets pipetted onto potato dextrose agar (Difco). Germination (%) was assessed after 24 hours incubation in the dark at 20°C.

### Field survival

On 16 January 1996 spore suspensions of either *Alternaria*, *Epicoccum*, *Ulocladium* spp. or a water control (tap water plus 0.01% Tween 80) were sprayed to run-off with a 2 litre hand-held pressure sprayer on the under or upper surface of kiwifruit leaves growing on a T-bar system in a Motueka orchard. The eight treatments ( three genera and control x two leaf surfaces) were applied in a split-plot design with genus as the main factor and with five replicate vines per treatment. An untreated buffer vine was used between vines of differing genera to minimise cross contamination. No pesticides were applied to the vines for the duration of the experiment.

### Measurements

Spore number and percentage germination were measured as an indicator of persistence and survival of spores respectively, within the kiwifruit canopy. Leaf samples were taken one day after spore application (t<sub>0</sub>), then weekly for four weeks and then fortnightly until harvest (16 weeks post application). At each sample date, five 4.9 cm<sup>2</sup> samples were taken with a cork borer from each of three leaves per plot. These were shaken vigorously by hand for two minutes in 30 ml of Tween water and a 10 ml aliquot centrifuged at 3000 rpm for two minutes. The supernatant was decanted off and the pellet resuspended in 1 ml of Tween water. The number of spores of the genus applied to each plot was counted with a haemocytometer (except four and six weeks after application). In the control treatments, the number of spores of each of the three genera was counted. At all sample times, spore germination (%) was measured by pipetting three 30 µl droplets of the concentrated washing solution onto 2% water

agar plates (Davis) amended with streptomycin sulphate (200 mg/litre) to limit bacterial growth. These were incubated at 20°C in the dark for 24 hours, after which spore germination was stopped by the application of Lactophenol Cotton Blue. Spore germination was recorded for 150 single spores or after five minutes microscopic observation if 150 spores could not be found.

### Statistical analysis

In the UV irradiation studies, because of low replication, isolates of each genus were used as replicates and Kruskal-Wallis analysis of variance and Mann-Whitney comparisons on median values were used to detect differences between genera.  $LT_{50}$  values were calculated from non-linear regression equations. For the field survival studies, data were normalised by logarithmic transformation and analysis of variance was performed to detect differences between treatments within and among sample times. Inconsistent trends over time made it inappropriate to fit regression lines to the data.

## RESULTS

### UV survival

$LT_{50}$  values differed significantly ( $P < 0.05$ ) between genera. *B. cinerea* isolates were the most sensitive to short wavelength UV radiation with a median  $LT_{50}$  of three minutes, followed by *Alternaria* spp. isolates with a median  $LT_{50}$  of nine minutes and *Ulocladium* spp. isolates with a median  $LT_{50}$  of 17 minutes. *Epicoccum* spp. isolates were the least sensitive with a median  $LT_{50}$  of 41 minutes. The mean  $LT_{50}$  value of each isolate is presented in Table 1.

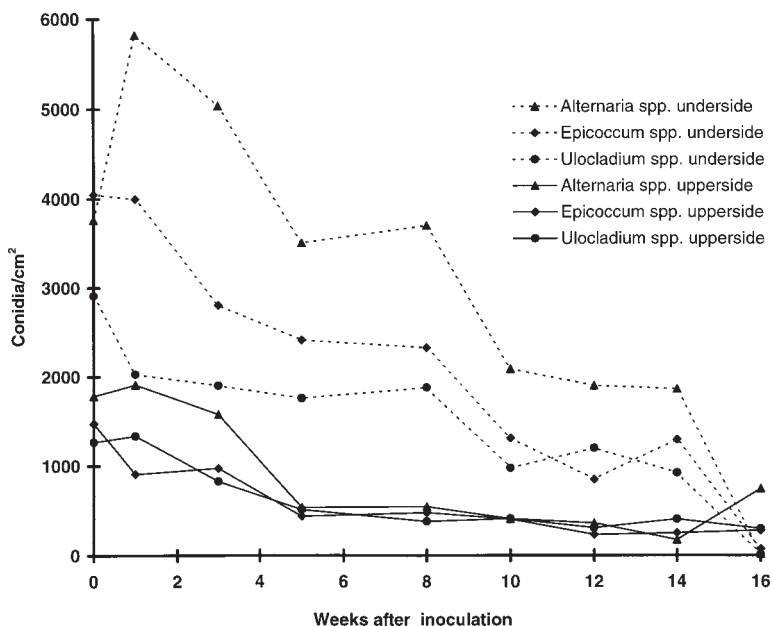
**TABLE 1:  $LT_{50}$  values (minutes) of isolates exposed to short wavelength UV radiation.**

| Genus                          | Isolate No. | Crop and Source                      | $LT_{50}$ <sup>1</sup> | Curve Type  | R <sup>2</sup> |
|--------------------------------|-------------|--------------------------------------|------------------------|-------------|----------------|
| <i>Alternaria</i>              | Aa1         | grapes, HortResearch                 | 9 <sup>2</sup>         | sigmoid     | 0.97           |
| <i>Alternaria</i>              | Aa3         | grapes, HortResearch                 | 13                     | sigmoid     | 0.99           |
| <i>Alternaria</i>              | Aa5         | grapes, HortResearch                 | 9                      | sigmoid     | 0.97           |
| <i>Epicoccum</i>               | B2          | kiwifruit, Lincoln University        | 51                     | logarithmic | 0.98           |
| <i>Epicoccum</i>               | Ep2         | kiwifruit, HortResearch              | 25                     | logarithmic | 0.98           |
| <i>Epicoccum</i>               | Ep3         | kiwifruit, HortResearch              | 41                     | logarithmic | 0.91           |
| <i>Ulocladium</i>              | 1142        | cucumber, ICMP                       | 18                     | logarithmic | 0.95           |
| <i>Ulocladium</i>              | 1145        | cucumber, ICMP                       | 17                     | logarithmic | 0.97           |
| <i>Ulocladium</i>              | 5634        | soybean, ICMP                        | 15                     | logarithmic | 0.75           |
| <i>B. cinerea</i> <sup>3</sup> |             | kiwifruit, HortResearch and Landcare | 3                      | sigmoid     | 0.998          |

<sup>1</sup> time taken to reduce germination to 50% of the non-exposed value; <sup>2</sup> mean germination of three droplets; <sup>3</sup> mean of 12 isolates.

### Field survival

Numbers of saprophytic spores recovered from the controls did not change significantly ( $P > 0.05$ ) over time. When averaged for all three genera over time for leaf upperside and underside, the spore number on the controls was 34 conidia/cm<sup>2</sup> with a maximum of 163 conidia/cm<sup>2</sup>. Eight weeks after application, the number of *Alternaria* spp. spores recovered from the leaf upperside had declined significantly ( $P < 0.05$ ), while numbers on the leaf underside had not declined significantly ( $P > 0.05$ ) after 16 weeks. After five weeks the number of *Epicoccum* spp. spores recovered from the leaf underside and leaf upperside started to decline significantly ( $P < 0.05$ ) when compared to spore number at  $t_0$ . *Ulocladium* sp. spore number declined significantly when compared to spore number at  $t_0$  after 16 weeks on the leaf underside and after eight weeks on the upperside (Figure 1).



**FIGURE 1: Mean number of conidia recovered from kiwifruit leaves. Note: 78% and 89% of cases, no effect of leaf surface nor genus respectively. Individual transformed replicates varied by no more than 40% of the mean.**

Spore germination declined significantly over the 16 week period for all genera, except *Epicoccum* sp. on the leaf underside (Figure 2). Trends as demonstrated in Figures 1 and 2 show no major differences in spore number and germination between leaf surface or genus.

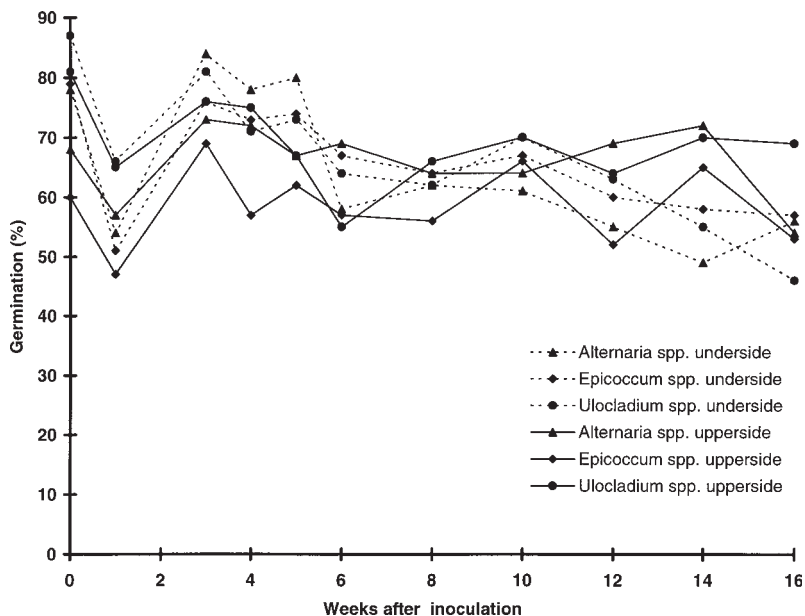
## DISCUSSION

Differences in sensitivity to short wavelength UV radiation between genera were observed in this study; *B. cinerea*, with its lighter pigmentation and thin cell walls was more sensitive to UV short wavelength radiation than the dark-walled *Alternaria*, *Epicoccum* and *Ulocladium* spores. These results confirm the findings of English and Gerhardt (1946) who found that *Alternaria* spp. were highly resistant to UV short wavelength radiation and *B. cinerea* mildly susceptible. They suggested that the effects of short wavelength UV radiation on spores diminished as pigmentation increased.

The differences in spore survival between genera in the laboratory were not observed in the field, presumably because leaves were sufficiently shaded by other leaves to provide protection to spores from damaging UV radiation.

Although all genera exhibited some decline in spore germination over time, after 16 weeks germination still ranged from 47 - 69%. The near equivalent germination on both leaf surfaces once again suggests that leaf surfaces were relatively protected from short wavelength UV radiation.

When compared to spore numbers at  $t_0$ , a significant decline in spore number/cm<sup>2</sup> occurred only after five weeks for the *Epicoccum* sp., eight weeks for the *Alternaria* sp. and *Ulocladium* sp. on the leaf upperside and after longer periods on the leaf underside. This indicates that spores have the ability to persist in the field for several



**FIGURE 2: Mean germination of conidia recovered from kiwifruit leaves. Note: 81% and 83% of cases, no effect of leaf surface nor genus respectively. Individual transformed replicates varied by no more than 13% of the mean.**

weeks, especially on the leaf underside. Elmer and Köhl (1998) suggested that densities of *Ulocladium atrum* spores on *Lillium* spp. leaves of greater than 4000/cm<sup>2</sup> should be maintained to ensure effective suppression of naturally occurring saprophytes. The relationship between efficacy and spore density of BCAs will need to be taken into account when applying BCAs and timing their re-application.

The higher spore number on the leaf underside compared to the upperside may be accounted for by the higher initial retention of spores on the leaf underside. This may be due to the hairy nature of the leaf underside or increased run-off on the shiny upper leaf surface. Spore number on the leaf underside exhibited a greater percentage decline than on the leaf upperside and after 16 weeks spore numbers/cm<sup>2</sup> on both leaf surfaces were nearly at the same level.

Spore number on the controls did not change significantly over time suggesting that no new spores (or numbers below a detectable threshold) of the genera studied entered the system. Although saprophytes may have colonised necrotic leaf tissue, no sporulation on the leaves sampled was observed. Therefore, we have assumed that the spores studied were those applied.

We conclude that spores of the saprophytic antagonists *Alternaria*, *Epicoccum* and *Ulocladium* spp. have the ability to persist and survive within a kiwifruit canopy for at least 16 weeks, in this case from early fruit development to harvest.

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