INFECTION OF WOUNDS IN YOUNG *EUCALYPTUS NITENS* BY ASCOSPORES AND CONIDIA OF *ENDOTHIA GYROSA*

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ABSTRACT

A shadehouse-based technique to inoculate seedling stems with ascospores and conidia was developed. Seventeen-month-old seedlings from three different localities of the Toorongo provenance of *Eucalyptus nitens* (Deane et Maiden) Maiden were inoculated with the ascospores, conidia, and mycelium of each of three *Endothia gyrosa* (Schw.:Fr.) Fr. isolates. The lesions produced from the inoculation of the different forms of each isolate were compared. Both ascospores and conidia of *E. gyrosa* were able to initiate and establish infections through wounds, and results suggest that both are able to play a role in dissemination of the pathogen.

Keywords: inoculation; infectivity; ascospores; conidia; *Endothia gyrosa*; *Eucalyptus nitens*.

INTRODUCTION

Endothia gyrosa or its anamorph *Endothiella gyrosa* Sacc. (*sensu* Barr 1978) is ubiquitous on many forest trees including *Acer*, *Fagus*, *Quercus*, *Eucalyptus* (Sankaran *et al.* 1995). It has been reported from Australia (Walker *et al.* 1985; Old *et al.* 1986; Davison & Coates 1991; Yuan & Mohammed 1997), South Africa (van der Westhuizen *et al.* 1993), North America (Roane *et al.* 1974; Appel & Stipes 1984), and Europe (up to five cultures from *Castanea* and *Quercus* in Italy have been deposited in the Centraalbureau voor Schimmelcultures, Netherlands).

Endothia gyrosa is common in south-east Australia (Old *et al.* 1986). In Tasmania *E. gyrosa* is the most frequently encountered fungal species associated with stem cankers of several eucalypt species (Yuan & Mohammed 1997). This fungus has usually been considered a non-aggressive pathogen of vigorously growing eucalypt trees within Australia (Old *et al.* 1990), although recently significant damage to young (2–3 years old) *Eucalyptus globulus* Labill. has been observed in Western Australia (C.Shedley, pers. comm.). In a 16-

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year-old mixed provenance trial of *E. nitens* in north-west Tasmania near Tewkesbury, numerous severe cankers associated with *E. gyrosa* were found on non-stressed and vigorous trees (Yuan & Mohammed 1997; Wardlaw in press). In addition, a high incidence of stem decay in the infected trees originated from damage caused by these severe cankers (Yuan & Mohammed unpubl. data). In South Africa *E. gyrosa* can have a severe impact on eucalypt plantations (van der Westhuizen *et al.* 1993).

Artificial inoculation techniques with *E. gyrosa* have been well established in several studies but most inoculations involved insertion of small amounts of mycelial inoculum into wounds (Roane *et al.* 1974; Appel & Stipes 1984; Old *et al.* 1990; van der Westhuizen *et al.* 1993; Shearer 1994). Inoculations by Old *et al.* (1986) using conidia of *E. gyrosa* resulted in the production of kino veins on pole-sized *E. delegatensis* R.T.Baker. However, little is known about the relative infection ability or infectivity (defined as capacity to infect host plants, *sensu* Garrett 1966) of ascospores and conidia, or their respective roles in the dissemination and disease cycles of ascomycetous canker fungi such as *E. gyrosa*. Conidia may be particularly important in disease dissemination in Western Australia where the teleomorph of *E. gyrosa* has not been found, although its asexual *Endothiella* stage is widespread (Shivas 1989; Shearer 1994). The outbreak of severe cankers associated with *E. gyrosa* in north-west Tasmania and the abundance of the teleomorph may be associated with more virulent forms of the fungus (Yuan & Mohammed unpubl. data). Since only the teleomorph was observed in this particular site, subsequent re-infections were most likely initiated by ascospores (Yuan & Mohammed 1997; Wardlaw in press).

The objectives of this study were to develop a technique for inoculating with the different spore types and to compare cankers resulting from inoculating *E. nitens* seedlings with ascospores, conidia, and mycelium.

MATERIALS AND METHODS

Seedlings

Seventeen-month-old potted *E. nitens* seedlings (mean diameter approx. 12 mm and c. 1 m tall) of the Toorongo provenance from three different localities (NE-13 Upper Thompson; NE-14 Toorongo Plateau; NE-15 Mt Erica, Victoria) were used for the study. After inoculation the seedlings were kept in a shadehouse and watered automatically from an overhead system.

Source of Isolates

Three *E. gyrosa* isolates (designated TAS12, TAS13, and TAS14) were used for inoculations. TAS12 and TAS13 were collected from a *E. nitens* plantation with severe stem cankers associated with *E. gyrosa* (as previously mentioned). TAS14 was collected from a large (c. 30 cm long) canker on a tree in a *E. regnans* F. Muell plantation at Westfield, south-central Tasmania. All three isolates were single spore isolates recovered from the ascospores.

Preparation of Inoculum

Freshly collected cankered bark with *E. gyrosa* perithecia was cut into small pieces (c. 2 mm^3) and soaked in 50 ml sterile water for 20 minutes. After being macerated in a blender

("Ronson", model 8344) for 20–30 seconds, the mixture was filtered through gauze to remove the larger pieces of bark, and then 2–3 times through tissue paper. The filtered solution was centrifuged at a speed of 1000–2000 rpm to collect spores which were then diluted with sterile water to either 2×10^7 spores/ml or 0.5×10^7 spores/ml. The estimation of spore concentration was carried out using a Neubauer counting chamber (hemacytometer) (Booth 1971).

Pycnidia were obtained from cultures growing on 3% MEA incubated at 22°C for 10 days under cycles of alternating 12 hours fluorescent light (36 W) and 12 hours dark. Conidial suspensions were prepared by washing the plates with sterile water, followed by filtering and centrifugation as above. Conidia collected were diluted with sterile water to 2×10^8 spores/ml and 0.5×10^8 spores/ml. The spore concentration was estimated as above.

Mycelial inoculum was prepared on wheat and rice bran medium using the method of Old & Kobayashi (1988).

Inoculation Tests

Seedling stems for spore inoculation were wounded to the depth of the cambium using a cork-borer (3 mm in diameter) which was surface-sterilised with 95% ethanol before each use. A drop of spore suspension (1 µl) was applied to a wound using an automatic pipettor. The two concentrations prepared resulted in either 5000 or 20 000 ascospores or either 50 000 or 200 000 conidia being inoculated in each wound. Three wounds were made along stems of each seedling at a height of 10–20 cm above soil level, leaving 10–20 cm between each wound. The upper two wounds were inoculated with the two different concentrations of spore-suspension and the lowest one received sterile water (1 µl) as a control. As soon as they were applied the drops of spore-suspension were completely absorbed into the plant tissue. The inoculated wounds were wrapped with plastic film.

Seedlings were inoculated with the mycelium-mixed wheat/rice bran as described by Old & Kobayashi (1988). Two wounds were made on the stem of each seedling; the upper one was inoculated with small amounts (just enough to fill the wound) of the mycelium-mixed bran and the lower with autoclaved fungus-free bran as a control.

Each treatment (e.g., isolate × locality × inoculum type) was replicated five times and was distributed in five separate blocks. Within each block, the seedlings were randomly arranged based on a table by Green (1968). Altogether 135 seedlings (3 localities × 3 isolates × 3 inoculum types × 5 replicates) were inoculated.

The experiment was carried out in spring (November 1996). The wounds were observed over a period of 4 months and the final lesion measurements were carried out 4 months after inoculation; in previous seedling inoculation tests with the mycelium of various isolates of *E. gyrosa*, differences in symptom expression were detectable after 4 months (Yuan & Mohammed unpubl. data)

The stem canker evaluation included assessment of: (1) presence or absence of callus around or over the wound; (2) fungal sporulation in bark surrounding the inoculation point; (3) extent of longitudinal and tangential spread of lesions; (4) internal longitudinal spread of discoloration in xylem (assessed by cutting through the lesions longitudinally); (5) ability to re-isolate the fungus.

Lengths of external lesions were used as the response variable for all treatments. Data were analysed using the Minitab statistical package for analyses of variance (ANOVA), and LSD were used to test the significance of differences between treatment means.

RESULTS

When inoculated into wounds, ascospores and conidia were both able to produce lesions significantly longer ($p \leq 0.05$) than controls (Fig. 1). Lesions produced by conidia and ascospores were ellipsoid to fusoid in shape, and dark brown to black (Fig. 2a,b). Four months after inoculation, the maximum mean lesion lengths produced by conidia and ascospores of TAS14 on NE-13 were respectively 12 mm and 14 mm. Tangential spread of the lesions produced by both ascospores and conidia was small (less than 95.3° and 104.7° respectively).

Internal discoloration by ascospores and conidia was highly correlated ($r=0.89, p < 0.001$) with the external lesion length (Fig. 2d,e). The maximum lengths were 35 mm and 30 mm for ascospore and conidial inoculation respectively.

There were no significant differences between spore inoculation type (ascospore *v.* conidia) although lesions produced by ascospores tended to be longer than those produced by conidia (Fig. 1).

All lesions produced by mycelium were significantly longer ($p \leq 0.05$) than those produced by ascospores and conidia of the same isolates, spreading longitudinally far beyond inoculation points externally and internally (Fig. 2c,f). Tangential spread was up to 180° for most of the lesions. They were different in shape and size from the lesions produced by conidia or ascospores and were often sunken or flat at the centre with longitudinal cracks.

When harvested, 90% of the lesions (81 out of 90) produced by ascospores had completely occluded. No fruiting bodies were observed on the surface of any lesions whether occluded or not.

Only 62% of the lesions induced by conidia had occluded after 4 months. Twenty-six percent of all lesions resulting from conidial infection, produced conidiomata. They all remained open at 4 months.

When harvested at 4 months, all lesions resulting from mycelial inoculation were still open and covered with abundant conidiomata, although callus tissue was observed around some of the lesions.

All attempts at re-isolating *E. gyrosa* from lesions induced by conidia or mycelium were successful, with a 100% fungal recovery (Fig. 2g). Re-isolations from lesions induced by ascospores were only slightly less successful (90%). Cultures isolated from stem pieces excised from the margins of lesions originating from either ascospores or conidia produced conidiomata after 2 weeks.

The two different spore concentrations used for each ascospore and conidial inoculum both resulted in infection. Lesions produced with the more concentrated spore suspension were slightly longer than those resulting from the lower concentration of spores, although there were no significant differences ($p > 0.05$) in lesion length between the two concentrations within each spore type.

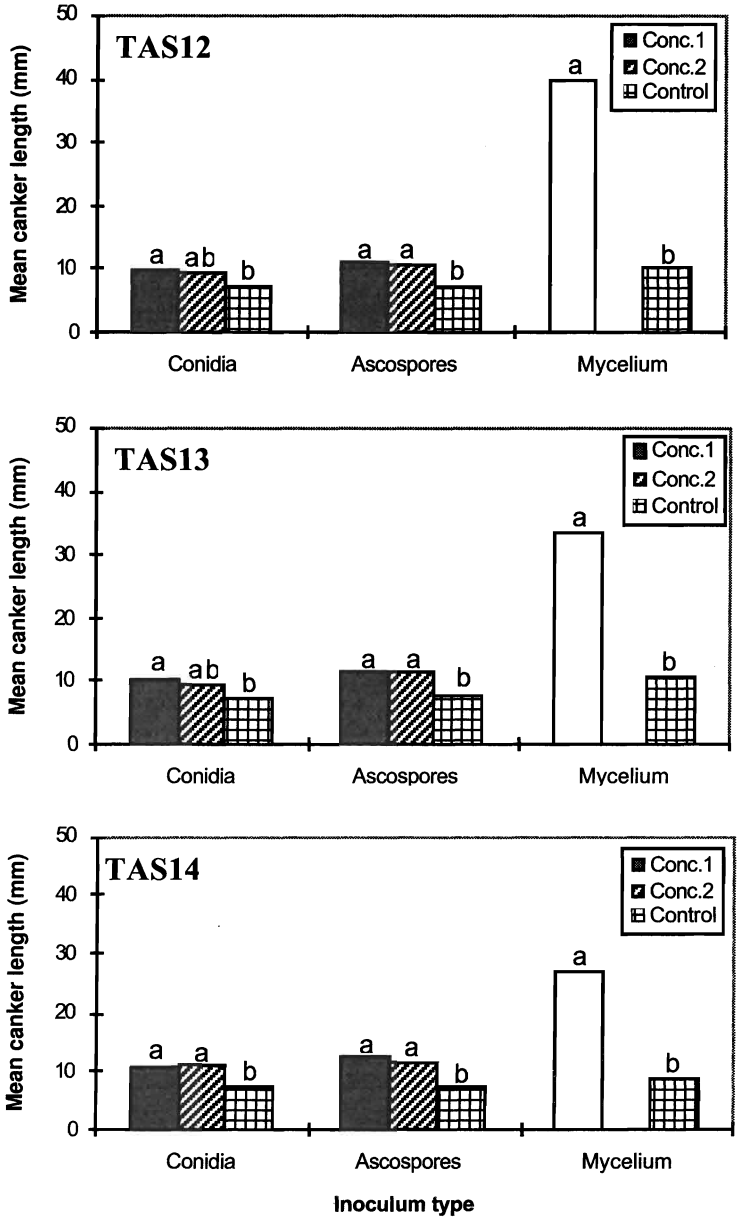

FIG. 1–Mean length of external lesions on seedling stems of *Eucalyptus nitens* inoculated with conidia, ascospores, and mycelia of three *Endothia gyrosa* isolates—TAS12, TAS13, and TAS14; each bar represents the mean from 15 lesions (3 different localities × 5 replicates).
Bars with the same letter(s) are not significantly different within conidial and ascospore (LSD 5% = 2.68), and within mycelial (LSD 5% = 10.03) inoculum type.
Conc.1 = Concentration 1 (0.5×10^7 spores/ml for ascospores and 0.5×10^8 for conidia); Conc.2 = Concentration 2 (2×10^7 for ascospores and 2×10^8 for conidia); the same quantity of mycelium-mixed wheat/rice bran was used for all mycelial inoculations.

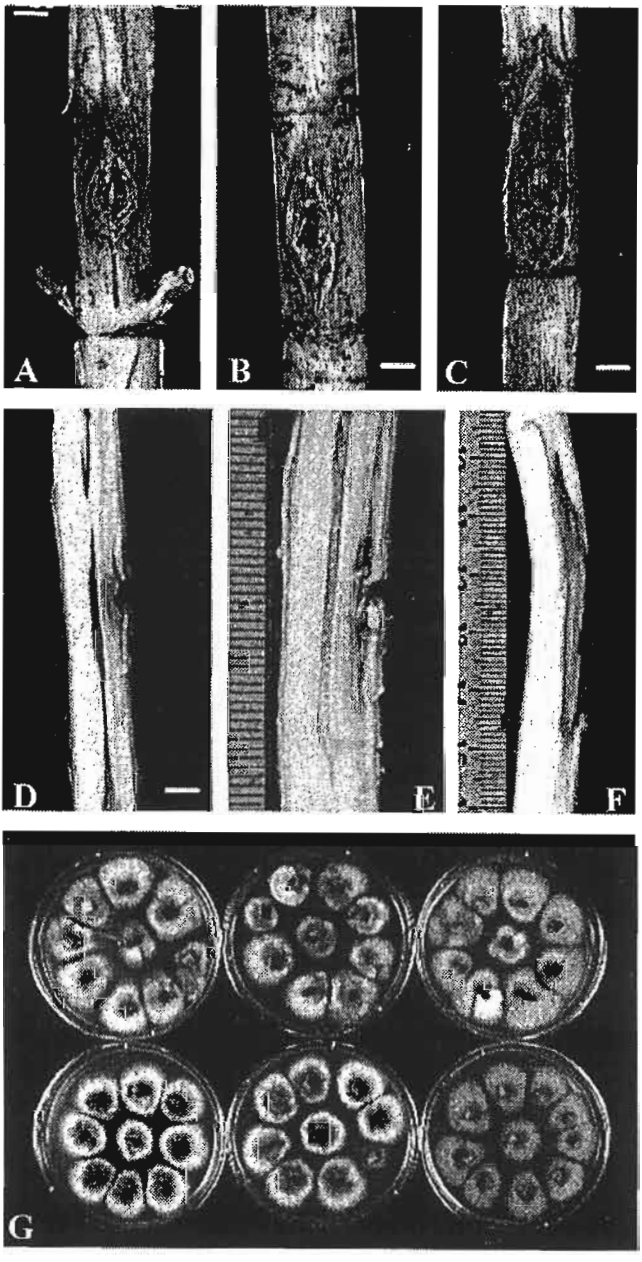

FIG. 2–Inoculation of 17-month-old seedlings of *Eucalyptus nitens* with conidia, ascospores, and mycelia of three *Endothia gyrosa* isolates.
A–C = lesions produced by (A) conidia, (B) ascospores, and (C) mycelium 4 months after inoculation; **D–F** = internal discoloration of wood associated with inoculation of (D) conidia, (E) ascospores, and (F) mycelium; **G** = re-isolation of *E. gyrosa* from lesions inoculated with conidia (left pair of plates), ascospores (middle pair), and mycelium (right pair). Bar = 4 mm for **A** and **C**; 3 mm for **B;** 5 mm for **D**.

Significant differences in lesion length were found between the *E. nitens* seedlings from different localities within spore ($p \leq 0.01$) and mycelial ($p \leq 0.05$) inoculation type.

DISCUSSION

Wound-inoculated ascospores and conidia can give rise to cankers of similar length, indicating that they can act as infective propagules for dissemination of the pathogen in natural conditions. There did not appear to be any self-inhibition of spores at the higher concentration tested.

Research has shown that fungal ability to sporulate on the surface of lesions, the number of spores produced, and variations in the period for which spores are latent in plant tissue are important factors that condition both pathogenicity and epidemic development (Johnson & Taylor 1976; Rotem 1978; Zadoks & Schein 1979). Bright-orange conidiomata of *E. gyrosa* are usually produced abundantly on the surface of induced cankers in inoculation tests, although perithecia have never been found on artificial cankers (Old *et al.* 1986; Yuan 1989; van der Westhuizen *et al.* 1993). Lesions resulting from both conidial and mycelial inoculation produced typical condiomata. The fact that there was no sporulation on any lesions of seedlings inoculated with ascospores in this test is somewhat unusual compared with conidial and mycelial inoculations. Re-isolations from the lesions induced by ascospores and conidia produced conidiomata after 2 weeks in culture, suggesting that mycelium developed from ascospores in wood tissue can produce conidiomata and conidia. It may have been possible to observe sporulation on surface of the lesions produced by ascospores if the length of the experiment had been extended.

The absence of sporulation and the higher occlusion rate in lesions resulting from ascospore inoculation may, however, indicate that lesions were slower to establish. In many ascomycetes the ascospore is predominantly for survival and for colonisation of new sites, and spores may have a dormant period. In contrast, conidia are most often dispersal spores with only a moderate capacity for dormant survival and germinate readily in the presence of nutrients (Carlile & Watkinson 1994). The fact that single ascospore *E. gyrosa* isolates were obtained by the authors without recourse to pre-treatment, e.g., heat at 50°–60°C as for *Neurospora* ascospores (Carlile & Watkinson 1994), may mean that ascospore dormancy cannot explain the observed differences in lesion development between spore types.

Since mycelium is not a natural inoculum, it could be argued that conidia or ascospores should be used as inoculum for pathogenicity tests for canker fungi. However, as demonstrated in this study, lesions were far more extensive with inoculated mycelium than with spore suspensions.

Ascospores of *E. gyrosa* cannot be obtained *in vitro*. Pronczuk & Messyasz (1991) found that spore inoculum of *Microdochium nivale* (Fr.) Samuels & Hallett was unable to cause disease in *Lolium perenne* L. while mycelial inoculum incited severe disease. They also concluded that spore inoculum requires longer incubation than mycelial inoculum and that the latter is more useful for screening plants for resistance.

Standard conditions are critical for consistent disease expression and comparison between isolates and host species. This is easier to achieve with mycelial inoculum which in the most situations can be easily produced in the required quantities in the laboratory.

This preliminary inoculation study provides the technique and basis for further investigation of the role and importance of conidia and ascospores in the disease cycle of *E. gyrosa*.

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