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Production of *Diplodia scrobiculata* and *Diplodia pinea* pycnidia on ground Austrian pine needle agar medium

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Summary. The *in vitro* production of fruiting structures represents an important tool for the morphological identification of fungal genera or species. It is also important for the controlled production of spores to be used in experiments. However, some fungal species do not readily sporulate in pure culture. In the present study we induced the production of pycnidia of *Diplodia scrobiculata* and *D. pinea*, two species recalcitrant to sporulation in pure culture, by growing them on two media containing ground Austrian pine needle. The two fungal species grew equally rapidly on both media and pycnidial primordia were produced on the medium surface after only 4 days at room temperature. Conidia matured in less than two weeks and their germination rate at 25°C was about 96%, indicating high viability.

Key words: fungi, pycnidia, sporulation, *Pinus nigra*, *Sphaeropsis*.

Introduction

Sphaeropsis sapinea (Fr.: Fr.) Dyko & Sutton in Sutton (= *Diplodia sapinea* [Fr.] Fuckel, *Diplodia pinea* [Desm.] Kickx) is a pathogenic fungus that causes shoot blight and stem canker in several conifer species (Capretti, 1956; Swart and Wingfield, 1991; Stanosz *et al.*, 1996; de Wet *et al.*, 2000). This species was divided into two morphotypes, A and B, until recently, when the two morphotypes were designated as two distinct species: *D. pinea* and *D. scrobiculata* J. de Wet, B. Slippers & M.J. Wingf., respectively (de Wet *et al.,* 2003). These two species differ in a number of phenotypic traits: *D. scrobiculata* tends to be less aggressive (e.g. Blodgett and Bonello, 2003), produces fewer conidia than *D. pinea* (Smith and Stanosz, 1995), and its conidia are generally smaller (Cheng-Guo *et al.*, 1985). Some nucleic acid differences, e.g. in the mitochondrial small subunit ribosomal gene sequences (mt SSU rDNA), have also been found and have been exploited to develop species-specific primer pairs (Smith and Stanosz, 2006).

In studies in which the artificial inoculation of trees is required, e.g. to determine features of the host-pathogen interaction, a typical procedure is to wound the host and apply a plug of inoculum to the wound (e.g. Bonello and Blodgett, 2003; Luchi *et al.*, 2005b). However, in some cases it might be preferable to use a spore suspension to test specific hypotheses, such as whether different pine species are susceptible to inoculation of intact needles (e.g. Blodgett and Stanosz, 1997). Collection of conidia from naturally infected trees for this purpose is possible: both fungal species sporulate on the sheath of needle fascicles and on cone scales. However, while it is fairly easy to collect large amounts

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of spores from the cone scales, it is more difficult to collect them from the needle fascicle sheaths, due to the relatively low sporulation rate on the latter. Furthermore, it is never known whether those spores represent one or more than one genotype of the fungus, or even the same species. Thus, procedures for the *in vitro* production of conidia from single-genotype isolates have been used. The usual method to produce conidia is to inoculate whole pine needles overlaid on water agar (pine needle agar, or PNA) with a monoconidial isolate, wait for the production of the pycnidia, and then scrape each needle individually to break the pycnidia and release the conidia (Blodgett and Stanosz, 1997). This procedure, however, is very time-consuming and the spore yield is often inadequate for even medium-scale inoculation experiments.

In this study we tested the hypothesis that incorporation of powdered Austrian pine (*Pinus nigra* Arn.) needles, or a needle extract, into water agar would result in induction of *D. scrobiculata* and *D. pinea* pycnidia directly on the agar surface. If the hypothesis is correct, this method would simultaneously alleviate two problems: it would allow the production of conidia for identification purposes and also for the purpose of generating useful quantities of spore inoculum. Austrian pine is particularly susceptible to infection by *D. pinea* and *D. scrobiculata* (Blodgett and Bonello, 2003), is known to support pycnidial production *in vitro*, and therefore represents a good substrate to test this hypothesis.

Materials and methods

Media preparation

Green needles collected from 5-yr-old Austrian pine trees were ground to powder in liquid nitrogen using a mortar and pestle. Two kinds of agar media were prepared using the needle powder:

a) Ground pine needle agar (GPNA): needle powder was added to 2% (w:v) water agar (Difco Laboratories, Detroit, MI, USA), at a rate of 80 g l^{-1};

b) Filtered ground pine needle agar (FGPNA): 80 g of needle powder was added to one litre of distilled water and boiled on a magnetic stirrer/hot plate for 10 min with continuous mixing. The medium was then filtered through Whatman No.1 filter paper using a vacuum pump. Finally, 20 g agar l^{-1} of solution were added to solidify the medium.

As a control we used 2% water agar (WA). The pH of both media was measured before autoclaving (pH_{GPNA}=pH_{FGPNA}=4.20; pH_{WA}=6.58), and each medium was poured into 90 mm Petri dishes.

Fungal inoculation

Six-millimetre diameter mycelial plugs were taken from the margins of actively growing, seven-day-old WA cultures of monoconidial isolates of *D. scrobiculata* (isolate B2, Blodgett and Bonello, 2003) using a flame-sterilized cork borer, and were placed onto the various media. In order to have a term of reference for the development of *D. scrobiculata*, *D. pinea* (isolate 3AP, Blodgett and Bonello, 2003) was grown using the same procedure, but only on GPNA.

Eleven replicates per medium were used. All plates were incubated at 24°C under constant fluorescent light. Radial growth was measured, and pycnidial production was assessed daily. In each Petri dish, the number of pycnidia was counted under a dissecting microscope in a randomly selected, standardized area of the plate covered with a microscope slide cover-slip (3.24 cm^2).

Characterization of fruiting structures

In order to evaluate the viability of conidia produced by pycnidia arising from the various media, a conidial suspension produced by crushing pycnidia in water (1 ml of total water volume), was dispensed on the surface of one 90 mm Petri dish containing 1.5% WA, and incubated at 25°C for 5 hours. The percentage of germinated conidia (N=100) (i.e. conidia producing a germ tube or germ tube initial within 5 hours) was calculated for each fungal species. Moreover, the size of the pycnidia and the conidia produced by the two species in this experiment were determined by measuring the length and width of 10 pycnidia and 50 conidia per species using a compound microscope fitted with a micrometer.

Statistics

Effects of culture medium on numbers of pycnidia per reference area, and of species on pycnidial and conidial length and width, were determined using ANOVA of raw or square root-transformed

data (to satisfy ANOVA's homoscedasticity assumption). Means were separated by the least significant difference (LSD) method. All statistical analyses were conducted using SPSS 11.0 for Machintosh.

Results

Diplodia scrobiculata colonies grew faster on GPNA and FGPNA than on WA. Growth rates were similar to those of *D. pinea* on GPNA (Fig. 1). GPNA and FGPNA inoculated with *D. scrobiculata* began to turn brown from the central part of the plate after 3 days. GPNA plates inoculated with *D. pinea* showed the same browning effect, but only after 5 days. WA cultures did not exhibit browning in the same time frame.

Pycnidial production was observed for *D. scrobiculata* after 7 days on GPNA and FGPNA plates. GPNA plates inoculated with *D. pinea* showed the formation of white pycnidial primordia after 4 days; these primordia changed from white to brown 3 days later. After 13 days the pycnidia became brown and contained mature (i.e. melanized) conidia. The size of pycnidia and conidia of the two species are shown in Table 1.

Table 1. Size (length [± SEM] × width [± SEM]) of pycnidia and conidia produced in GPNA and FGPNA media for each species (one isolate per species). All measurements are in μm and were taken 13 days after inoculation of the plates.

Character	*D. scrobiculata*[a]	*D. pinea*[a]
Pycnidia	457 A (±15)×324 a (±11)	648 B (±33)×429 b (±18)
Conidia	27 A (±0.33)×14 a (±0.16)	36 B (±0.37)×13 b (±0.14)

[a] Different letters in the same row denote significantly different means: upper case for length, lower case for width (P<0.001 in all cases).

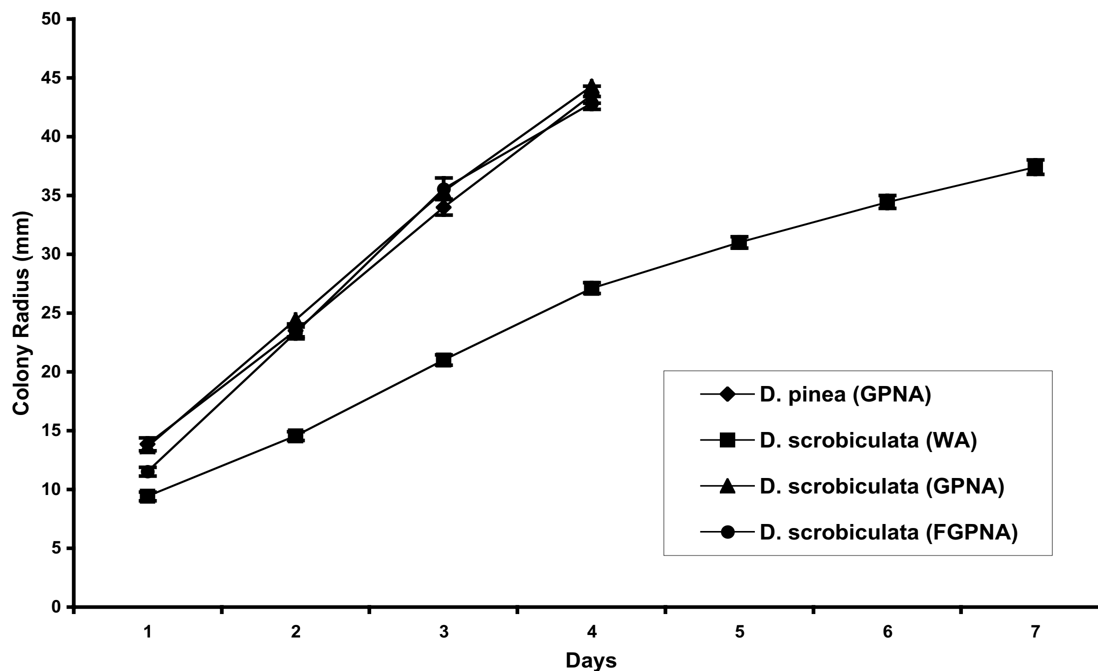

Fig. 1. Radial growth curves for *D. scrobiculata* and *D. pinea* on different substrates. *D. scrobiculata* grew at the same rate as *D. pinea* on substrates containing Austrian pine needle material and covered the plates in four days (plate radius = 45 mm). Standard error bars are masked by the treatment symbol in most cases.

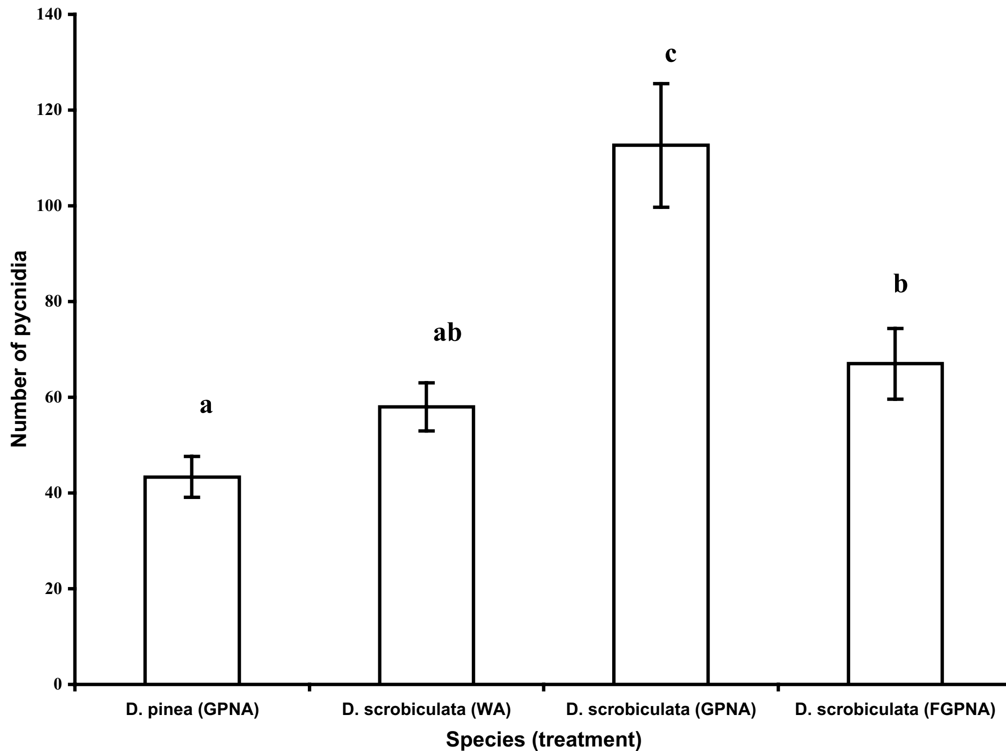

Fig. 2. Pycnidial production by *D. scrobiculata* and *D. pinea* on different substrates 13 days post-inoculation. Frequencies represent the number of pycnidia counted in a randomly selected 3.24 cm^2 area of the plate. Bars represent standard errors and different letters represent significantly different means by the LSD method.

Both needle media induced higher number of pycnidia than WA. However, a significantly higher number of pycnidia was produced on GPNA than on FGPNA. On GPNA, *D. pinea* produced fewer pycnidia than *D. scrobiculata* (Fig. 2).

In a germination test, 96.0% of conidia were viable for *D. scrobiculata,* and 97.0% for *D. pinea*.

Discussion

In this study we show that induction of pycnidia and viable conidia of *D. scrobiculata* and *D. pinea* is possible in pure culture if host tissue is incorporated into the medium, particularly when needle fragments are used, as in the case of GPNA. From both quantitative and temporal perspectives this approach is a significant improvement on the method of producing conidia by inoculating whole, sterilized pine needles (Smith and Stanosz, 1995). When needle powder was used, *D. pinea* and *D. scrobiculata* produced pycnidial primordia after only 4 and 7 days respectively, and mature conidia after only 13 days, compared with 4–5 weeks when intact Austrian pine needles were used (personal observation). The method of producing pycnidia on intact needles is common. We hypothesize that the difference in the pycnidial production rates between ground and intact needles may be due to the cuticle and epidermis of the intact needles, which represent barriers that the fungus must first overcome in the course of the invasion, prior to actual sporulation. Grinding the needles made the substrate immediately available to the fungi, and pycnidia were produced both on the needle fragments and on the agar surface. It is also possible that sporulation levels were tied to the nutrient content of the needles, which can vary over the seasons. This hypothesis could be tested by growing the fungi on needles collected at different seasons and might provide insights into the factors inducing sporulation, by correlating fruitbody production with levels of specific

nutrients. Such information may then be useful to devise better culture media to induce sporulation of these as well as of other recalcitrant fungal species.

Besides facilitating the production of conidia to be used in inoculation studies, the needle agar media also made it easier to measure them, since the pycnidia were not embedded in the needle, but grew on the agar surface from where they could be more easily excised and measured. Easy removal of pycnidia and conidia from their substrate is a significant improvement for the measurement of these structures, which can be quite useful in identification. Indeed, this procedure has been used successfully for the identification of *D. pinea* isolated from cedar (*Cedrus atlantica* (Endl) Manetti ex Carr.) (Luchi *et al.,* 2005a) and from Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (unpublished data). The measurements shown in Table 1 do not *per se* indicate species differences, since they were based on only one isolate per species. However, the very small standard errors suggested that pycnidia produced in this way were rather homogeneous in size, and this should facilitate species separation when based on several isolates per species.

From a phenotypic perspective, two points seem of interest. Firstly, on GPNA the growth rate of *D. pinea* and that of *D. scrobiculata* overlapped. This is a noteworthy departure from what is usually observed in more or less defined media, such as potato dextrose agar or WA, on which *D. pinea* always grows significantly faster than *D. scrobiculata*. Indeed, this difference in growth rate is usually taken to be a reflection of the higher aggressiveness of *D. pinea* (Palmer *et al*., 1987). Our data suggest that this phenotypic trait should not be used to assess aggressiveness, but may still be practically useful in separating congeneric species such as *D. pinea* and *D. scrobiculata*.

Secondly, *D. scrobiculata* discolored the GPNA and FGPNA media faster than *D. pinea*. Browning of the substrate is likely to be due to the oxidation of phenols and polyphenols in the needle extracts. This suggests that *D. scrobiculata* has a stronger oxidative enzymatic machinery than *D. pinea*, a metabolic property that may enhance host recognition and defense responses, reflected in the lower aggressiveness observed with *D. scrobiculata* in Austrian pine (Blodgett and Bonello, 2003). Investigation of these factors may contribute to our understanding of the chemical processes whereby resistance is induced in this and other conifer species.

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