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Optimisation of an *in vitro* antifungal protein assay for the screening of potential antifungal proteins against *Leptosphaeria maculans*

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ABSTRACT

Canola is second only to soybean as the most important oilseed crop in the world. The global production of canola is forecast to continue to increase and as a result the canola industry will continue to flourish. However, it is threatened by several fungal diseases that affect canola and cost producers hundreds of millions of dollars a year in reduced yield and quality. Blackleg is the most common and devastating disease of canola and is caused by the fungus *Leptosphaeria maculans*. The fungus can infect any part of the plant at all growth stages and is a serious threat to the canola industry. Novel and more efficient antifungal agents which interfere with fungal growth and development are clearly needed to control this pathogen. This paper reports the establishment of a simple functional assay system for the screening of antifungal proteins against a virulent strain of *L. maculans*.

1. Introduction

Market demand for seed high in oil content combined with superior health traits has allowed canola trade to expand rapidly. Canola (*Brassica napus* L.) is the second most important oilseed crop in the world, after soybean (Raymer, 2002). During the past 20 years, canola has surpassed peanut, sunflower and, most recently, cottonseed in worldwide production. Canola production is scattered among 50 countries worldwide but is produced extensively in Europe, Canada, Asia, Australia and, to a lesser extent, in the United States (US) (McNew and Bixley, 2001; Raymer, 2002). In Australia, canola is now the largest oilseed crop representing 57 per cent of Australian oilseed production over the past 5 years (Australian Oilseeds Federation, 2008). A report from the Australian Bureau of Agriculture and Resource Economics expects production to reach 2.2 million tons by 2010.

The canola industry is influenced by several positive factors, including the high yield and profitability of the crop, the growing consumer acceptance of and demand for canola, heightened demand for the meal, and the importance of canola in winter crop rotations as a break crop. It is therefore not difficult to foresee that the global production of canola will continue to increase. As a result, the canola industry will continue to flourish. However, several fungal diseases that affect canola jeopardize this industry and cost producers hundreds of millions of dollars a year in reduced yield and quality. Blackleg is the most common and devastating disease of canola, especially in Australia, and is caused by the fungus *Leptosphaeria maculans* (Barbetti, 1994; Howlett et al., 2001). Blackleg isolates in Australia appear to be more virulent than those in other countries (Howlett et al., 1999) due to environmental conditions in Australia being more conducive to disease development. The fungus can infect any part of the plant at all growth stages and is a serious threat to the canola industry. Transformation of the plant genome with antifungal proteins is a potential way of safely controlling fungal pathogens. This strategy relies on these proteins interfering with fungal growth and development and has been demonstrated to be effective in several cases (Jha et al., 2009; Mackintosh et al., 2007; Coca et al., 2004; Wang et al., 1999; Grison et al., 1996; Broglie et al., 1991).

Different types of fungal growth inhibition assays have been developed for the identification and quantification of antifungal activity of natural or synthetic substances. Most of these assays, including the mycelial radial growth bioassay that is commonly used to determine antifungal activity against *L. maculans* (Pedras et al., 2006, 2007, 2009; Pedras and Jha, 2006; Pedras and Suchy, 2006), suffer from serious limitations (Broekaert et al., 1990). Microplate bioassays to measure the activity of such proteins and other compounds against fungal pathogens have been identified as the most promising *in vitro* bioassays for quantifying antifungal activity (Pryor et al., 2007). The simplest and most reliable quantitative assay for fungal growth inhibition is the automated microtitre plate broth assay developed by Broekaert et al. (1990). In this assay fungi are grown in wells of microtitre plates and their growth is monitored by

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measuring the turbidity of the microcultures with a microplate reader. By correlating culture absorbance with dry weight measurements, Broekaert et al. (1990) showed that the microplate reader could be used as a reliable tool for the monitoring of fungal biomass of various filamentous fungi. This assay for fungal growth inhibition has several advantages: fast and easy handling of large numbers of samples with the use of 96-well microplates, a microplate reader and adapted multichannel pipetting equipment; only small amounts of test substances and fungal spores are required; the assay can be adapted to non-sporulating fungi, in which case the mycelial fragments are used for inoculum; and it is highly reproducible (Broekaert et al., 1990; Ludwig and Boller, 1990). It should be noted, however, that the assay conditions reported by Broekaert et al. (1990) were for *Pycomyces blakesleeanus, Fusarium culmorum, Trichoderma hamatum, Septoria nodorum* and *Rhizoctonia solani* and therefore needed to be further optimised for *L. maculans*. This paper reports the establishment of a functional assay system for the screening of antifungal proteins against a virulent strain of *L. maculans*.

2. Materials and methods

2.1. Materials

Ace-AMP1 was obtained from Prof. Bruno Cammue (Katholieke Universiteit Leuven, Belgium). *L. maculans* (strain V4) culture was obtained from Dr Barbara Howlett (School of Botany, The University of Melbourne, Victoria, Australia). High purity type 1 water (Milli-Q water) was obtained by purification with a four-stage filtration system (Millipore). General laboratory reagents were obtained from Sigma-Aldrich (New South Wales, Australia) and were of analytical or molecular biology grade.

2.2. Media preparation

2.2.1. Preparation of cleared V8 juice

Cleared V8 juice was prepared by centrifuging 500 ml of Campbell's V8 juice (purchased locally) at 1000×*g*. The supernatant was decanted into a clean container and then filtered through glassfibre filter (GF/A) paper (Whatman) using a Büchner funnel. Excess cleared V8 juice was stored at −20 ± 1 °C.

2.2.2. Preparation of V8 juice broth (5%)

A 50 ml aliquot of cleared V8 juice was added to 0.1 g of $CaCO_3$ and made up to a volume of 1 l with Milli-Q water. The pH was checked and recorded to be 5.3. The V8 juice broth was then sterilised by autoclaving at 121 ± 1 °C for 30 min.

2.2.3. Preparation of V8 juice agar (10%)

A 100 ml aliquot of cleared V8 juice, 0.1 g of $CaCO_3$ and 20 g of Bacto agar was made up to a volume of 1 l with Milli-Q water. This was then sterilised by autoclaving at 121 ± 1 °C for 30 min and then allowed to cool to 60 ± 1 °C before pouring into 9 cm Greiner petri dishes (to $^1/_3$ the height of the dish) under aseptic conditions.

2.2.4. Potato dextrose broth (half strength)

Half strength potato dextrose broth (PDB; Difco) was prepared by dissolving 12 g of the powder in 1 l of Milli-Q water and then sterilised by autoclaving at 121 ± 1 °C for 30 min.

2.3. Culturing of L. maculans

L. maculans was subcultured by taking small pieces of agar containing pycnidia from sporulating cultures and transferring them onto two V8 juice agar plates. The cultures were incubated at 25 ± 0.5 °C under white fluorescent lights until spores were produced (usually within 1 to 2 weeks).

2.4. Isolation of spores from L. maculans cultures

Spores were isolated from well sporulated *L. maculans* cultures by adding 10 ml of sterile 0.05% Tween 20 to the plate and scraping the surface gently with a sterile disposable loop. The spore suspension was centrifuged at 1000×*g* for 15 min at room temperature. The supernatant was decanted and the pelleted spores were washed with 10 ml of Milli-Q water. The centrifugation was repeated and the supernatant was again decanted. The pelleted spores were resuspended in 0.5 ml of sterile 10% glycerol. The spore concentration was determined with the use of a haemocytometer (Neubauer type), and spores were then aliquoted and stored at −20 ± 0.5 °C. The isolation procedure was carried out under aseptic conditions.

2.5. Preliminary evaluation of in vitro fungal growth and growth inhibition using a previously reported quantitative 96-well plate assay

The growth of *L. maculans* in the absence and presence of a known antifungal inhibitor, sodium benzoate (Broekaert et al., 1990), was evaluated using the microtitre plate assay of Broekaert et al. (1990), with minor modifications. The assay was carried out in sterile, flat-bottomed, polystyrene, 96-well microtitre plates fitted with lids (Nunc) and performed under aseptic conditions in a laminar flow cabinet. Spores were added in an 80 µl aliquot in half strength PDB to give concentrations of 800, 1000, 2000 and 5000 spores/well into the appropriate wells of the microtitre plate. Sodium benzoate was added to the appropriate wells in a 20 µl aliquot to give final concentrations of 100, 200, 300 and 400 µg/ml. Sterile Milli-Q water was added to the appropriate wells as a control and a PDB blank was also included. Four replicate wells were set up for each treatment per experiment. The microtitre plates were then shaken on an orbital shaker for 1 min to mix the spores with the test solution. The plates were allowed to stand for 30 min at 22 ± 0.5 °C to allow the spores to sediment before the absorbance was measured at 595 nm using a Spectramax 250 microplate reader (Molecular Devices). The plates were incubated in the dark at 22 ± 0.5 °C, and absorbance readings were taken every 24 h over a time course of 15 days. The absorbances read with the microplate reader were viewed using the Softmax Pro Software (Version 1.2.0, Molecular Devices, USA), and the raw data were manipulated with use of this software. After processing the raw data, the absorbance was plotted against time using the GraphPad Prism Software package (Version 4.03; GraphPad Software Inc., USA), and growth of the fungi in the presence of test solutions were compared to the control to determine if there was any inhibition of growth.

2.6. Preliminary evaluation of assay conditions for the establishment of an in vitro antifungal protein assay specifically for L. maculans

The growth assay was performed as described above but different growth conditions (with variables such as spore density, temperature and growth medium) were tested. This was done by plating out spores in V8 juice broth at densities of 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000 and 10,000 spores/well in 4 replicate 96-well plates. This was repeated using half strength PDB as growth medium. The assay volume was 100 µl. Plates containing the different growth media were then incubated at 18 ± 0.5, 25 ± 0.5 and 30 ± 0.5 °C in the dark (wrapped in foil) and at 25 ± 0.5 °C under fluorescent lights for 15 days. Absorbance readings were taken at 24 h intervals.

2.7. Effect of growth medium on L. maculans growth

Spores were plated out at densities of 2000, 5000, 10,000, 20,000, 40,000, 60,000, 80,000, 100,000 and 150,000 spores/well in V8 juice broth in duplicate 96-well plates. V8 juice broth without spores served as a negative control. Eight replicate wells were set up for each treatment per experiment. This was repeated using half strength PDB as growth