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Cultural and infection studies on *Pyrenophora semeniperda*, a possible bioherbicide for annual grass-weeds

MATHEW A. CAMPBELL[1], RICHARD W. MEDD[2] and JOHN F. BROWN[1]

1 *Department of Botany, University of New England, Armidale, NSW 2351, Australia*
2 *NSW Agriculture, Agricultural Research and Veterinary Centre, Forest Road, Orange, NSW 2800, Australia*

Abstract. In Australia, wheat is the principal crop export and the greatest yield losses come from competition with weeds. Most broadleaf weeds can be satisfactorily controlled with a wide range of inexpensive herbicides. Grass-weed control in cereal crops has improved in recent decades with the development of many selective pre- and post-emergence herbicides. However, herbicides for use against grasses are expensive and control is still limited because of the close relationship of grass-weeds to cereals, the build-up of herbicide resistance, and increases in weed seed-banks despite herbicidal control. One strategy for the control of annual grass-weeds in cereal crops may be the inundative application of endemic pathogens as mycoherbicides. In this context, the use of seed-borne pathogens may be an extremely attractive prospect given that seeds are the only means by which these weeds carry-over and re-infest during the following growing season. In this paper we explore the potential of the seed-borne pathogen *Pyrenophora semeniperda* for bioherbicidal control of grasses in cereal crops. Particular reference will be made to growth and sporulation requirements, the infection process and the environmental factors influencing infection.

Introduction

Weeds are one of the limiting factors to *Triticum aestivum* L. (wheat) production in Australia. Of the weeds affecting cereal crops, it is the grasses that are generally more difficult to control because of their similarity in morphology, physiology and ecology to the crop species (Gill and Blacklow 1984). Losses from the competitive effects of these weeds can be as high as 50-75% of the potential weed-free yield (Leys and Dellow 1986; Combellack 1992).

A feature common to annual grasses is their prodigious seed production, which is required for survival, multiplication and invasion (Medd 1992). Seed-banks of annual grass-weeds in Australian cereal cropping systems are generally of a transient type. Wild oats (*Avena* spp.) are generally thought to persist in seed banks (Combellack 1992). However, there is mounting evidence that wild-oat populations persist because of the input of new seed (Medd *et al.* 1992, 1995). Medd (1990) reported that annual rates of wild oat seed-bank decline was in the vicinity of 70% per annum (i.e. a half-life of about six months). It would seem therefore, that tactics which reduce the input of seed would improve long-term control of wild oats.

The increasing number of weed species which are tolerant or resistant to the use of herbicides (*Bromus, Vulpia, Lolium* and *Avena* species) (Leys and Dellow 1986; Powles and Howat 1990; Gill 1995) re-emphasizes the need for biological control methods to be developed. One strategy for biologically controlling seed production of annual grasses in cereal crops may be the inundative application of seed-borne pathogens as bioherbicides.

Much of the research published on *Pyrenophora* species has been related to the effects of the pathogens on the winter cereals; for example, *Pyrenophora teres* Drechsler, that causes net blotch disease of barley and *Pyrenophora tritici-repentis* (Died.) Drechsler that causes yellow leaf-spot of wheat (Sivanesan 1987). By contrast, little research has been published on the bioherbicidal potential of *Pyrenophora* species. Wilson (1987) assessed the potential of the seed-borne species *Pyrenophora avenae* Ito and Kurib. for biocontrol of wild oats. Wilson and Hall (1987) concluded that although primary inoculation of seed did have some effect, the inundative application to seedling foliage showed the most bioherbicidal promise.

Pyrenophora semeniperda (Brittlebank & Adam) Shoemaker is a seed-borne pathogen that causes several symptoms in infected plants. The most striking symptom is the production of vegetative fungal stromata on infected seeds, which can lead to a reduction in the proportion of seeds that germinate or a decrease in seedling vigour. Elliptical eyespots are the typical lesions occurring on foliage infected by the pathogen. In general, these eyespots cause little damage to the host, but in severe infestations eyespots may coalesce to form large necrotic areas.

In this paper we explore the potential of the seed-borne pathogen *P. semeniperda* for bioherbicidal control of annual grasses. Particular reference will be made to growth and sporulation requirements, the infection process and environmental factors influencing infection.

Materials and methods

The growth and sporulation parameters of *P. semeniperda* tested were culture media, temperature, pH, photoperiod, and light quality. For details of the experimental procedures refer to Campbell (1996) and Campbell *et al.* (1996).

The infection process of *P. semeniperda* was examined on seedling- and adult-leaf-tissue of wheat and *Bromus diandrus* Roth. (bromegrass) and on floral- and seed-tissue of wheat, because it could be easily cultured and examined. Host tissues were inoculated, incubated for a 24 h dew-period at 20 ±1°C and prepared for microscopic examination with either light or electron microscopy, as detailed in Campbell (1996).

The environmental factors tested, which influence infection of wheat leaves and florets and *B. diandrus* leaves, were dew-period temperature, dew-period duration, light requirements during the dew period and physiological age of the host. The optima for the parameters tested were assessed and calculated using the techniques outlined in Campbell (1996).

Results

Radial growth and sporulation (expressed as conidia cm^{-2} and conidia $colony^{-1}$) were optimal on modified alphacel medium (MAM) (Campbell *et al.* 1996). Growth occurred over the temperature range 5-34°C and was optimal at 23.2±0.5°C. Sporulation occurred over the range 10-30°C and was optimal at 19.2±0.05°C when expressed as conidia $colony^{-1}$, and 18.3±0.3°C when expressed as conidia cm^{-2}. The pH for maximal growth was 4.7±0.5, while that for maximal sporulation was 5.4±0.2 when expressed as conidia $colony^{-1}$, and 5.7±0.1 when expressed as conidia cm^{-2}. *Pyrenophora semeniperda* required an alternating light/dark and temperature sequence for good sporulation. Growth was enhanced by light of wavelengths longer than 500 nm, while sporulation was enhanced by light of wavelengths shorter than 500 nm. Growth in liquid culture was greatest in V8 broth, malt-extract broth and modified alphacel broth, however, no sporulation was observed in these media. Sporulation was enhanced by mycelial wounding and illumination by near-ultra violet (NUV) light. Sealing of Petri plates with Parafilm® had a inhibitory effect on sporulation.

Germination of conidia generally exceeded 99% on leaves from seedlings of both plant species while on adult leaves only 20-30% germinated. Appressoria were formed as an essential component of the infection process on leaf material and were formed over the anticlinal epidermal cell walls of wheat and stomata of *B. diandrus*. Infection of leaf material from seedlings was observed to occur following inoculation with conidiophores and hyphal fragments. Very few sites of fungal penetration were observed in adult leaf tissue. Haloes were formed in response to infection by *P. semeniperda* and papillae were formed within the leaves as a resistance mechanism. The first post-penetration structures formed were intracellular vesicles with infection hyphae which ramified through the intercellular spaces of the mesophyll. Cellular disruption in advance of infection hyphae was observed. Ovarial infection of wheat was observed to occur either with or without the formation of an appressorium. Infection of the ovaries also occurred through cracks and wounds without an appressorium. Infection hyphae, formed within the developing caryopsis of wheat, grew intercellularly within the confines of the epidermis and the integuments. Infection of the developing embryo was not observed.

The optimum dew temperature for lesion development was 20.6±0.3°C (Fig. 1). However, the optimum for conidial germination was slightly higher (24±0.5°C). The optimum temperature for the production of infection structures was close to that for lesion development (21.3±0.2°C). The optimum dew temperature for floret infection was 23.6±0.6°C.

The effect of dew-period length on disease development followed a logistic growth-curve. The maximum number of lesions per leaf developed after a

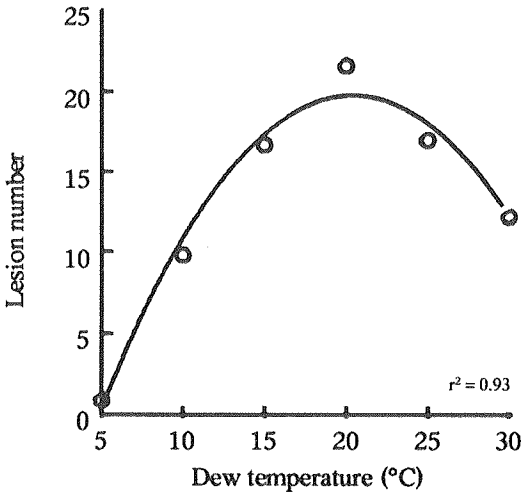

Fig. 1. The effect of dew-period temperature on the number of lesions formed by *P. semeniperda* (isolate DAR 71761) on leaves of wheat seedlings after 24 h incubation in the dark. Data are means of duplicate experiments, each of five replicates with five observations. The regression equation is $Y = -15.5(\pm2.1) + 3.3(\pm0.3)X - 0.08(0.01)X^2$ where Y = the number of lesions at seven days after inoculation and X = temperature of incubation during the 24 h dew-period.

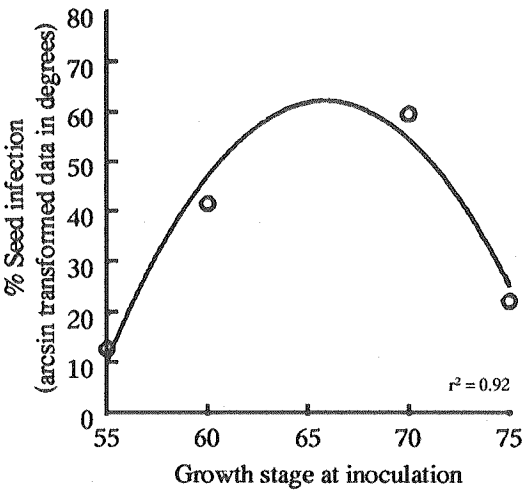

Fig. 2. Infection of mature wheat seeds by *P. semeniperda* (isolate DAR 71761) inoculated at four different stages of development according to the scale of Zadoks *et al.* (1974). Data are means of 10 replicates each with 100 observations. The regression equation is $(\text{arcsin } \sqrt{Y}) = 1.8x10^3(\pm87) + 58(\pm2.7)X - 0.44(\pm0.02)X^2$, where Y = proportion of mature seeds infected by *P. semeniperda* and X = growth stage of plants at inoculation.

21 h dew-period and did not increase with increasing duration of dew. Seed infection increased with increasing duration of dew up to 48 h after inoculation, when some 50% of seeds were infected. An initial dark phase during the dew period was a requirement for infection of wheat and *B. diandrus* leaves by *P. semeniperda*. No infection was observed on leaves that had been incubated with an initial light phase during the dew period. The reduction in infection due to light could be attributed to a decrease in conidial germination and subsequent development of infection structures. Infection of wheat seeds occurred at all stages of inflorescence-development that were tested. However, the maximal proportion of seed-infection occurred when inflorescences were inoculated at the end of anthesis (GS 70, Zadoks *et al.* 1974). The optimal growth-stage for inoculation of inflorescences calculated from a polynomial regression equation was the middle of anthesis (GS 66±1.4) (Fig. 2).

The growth and sporulation requirements for *P. semeniperda* were similar to those reported for other species of *Pyrenophora* (Campbell *et al.* 1996). It is postulated that the cardinal temperatures for growth of *P. semeniperda* are: minimum, 5°C; optimum, 23.2±0.5°C; maximum, between 30 and 35°C. For routine production of conidial inoculum, cultures were grown on MAM under NUV for seven days at laboratory temperature (15-25°C). After seven days colonies were wounded with a cork borer (3 mm) or with a scalpel blade. After a further seven days, incubation conidial suspensions could be made. Conidial densities of up to $4.5x10^6$ conidia colony^{-1} were regularly obtained using this method.

Discussion

Daniel *et al.* (1974) reported that the first major criterion a potential bioherbicidal candidate must fulfill is the ability to produce abundant and durable inoculum in artificial culture. The results from the present study elucidated conditions which enabled the production of conidial inoculum on solid media. Whilst these techniques enabled the production of adequate amounts of inoculum for the experimental treatment of plants in the field and glasshouse, they are not necessarily suitable for commercial production. Although *P. semeniperda* grew prolifically in liquid-shake culture, it could not be induced to sporulate. Thus, it may be possible to produce mycelial fragments, using fermentation, and to transfer these to other substrates for the mass-production of conidial inoculum, as a two-step production system (Walker

and Riley 1982). The use of mycelial fragments as infective propagules, for direct application to weeds, may be an attractive alternative because of the ease of culturing under commercial fermentation conditions. In the infection-process studies, hyphal fragments of *P. semeniperda* were observed to germinate and produce infection structures within leaf pieces. Further evidence that hyphae of *P. semeniperda* can be used as inoculum was obtained in field trials where infection of some 15% (data not presented) of *B. diandrus* seed was obtained. Investigations to promote the infectivity of mycelium and to examine methods for preserving mycelial inoculum, to enhance storage longevity, would be worthwhile. A common laboratory practice when culturing fungi is to seal Petri plates with plastic wrap (such as Glad Wrap® or Parafilm®) to reduce contamination and, or, water loss of the culture medium. In the present study, sealing of Petri plates with Parafilm® resulted in a reduction of conidial yield. Sealing of Petri plates may lead to a build-up of metabolic by-products or of staling-compounds such as CO_2, or entrap ozone encountered when using lamps which emit far-UV and NUV light, which reduced the ability of the fungus to sporulate. Harding (1968) and Rich and Tomlinson (1968) both reported that ozone reduced the ability of *Penicillium* species and *Alternaria solani* Ellis & Gibson to sporulate.

The results from the infection-process studies clearly demonstrated that *P. semeniperda* was not suited to foliar infection of adult leaves. This effectively eliminates the interesting possibility of using this organism as a mycoherbicidal agent that can infect leaves (thus reducing the weeds competitive ability) from which secondary inoculum may be produced to infect the weed florets. Infection of florets was observed to occur directly through the ovary walls. This confirmed the mycoherbicidal potential of *P. semeniperda* as a true seed-pathogen.

Infection of seeds of wheat by *P. semeniperda* occurred from the time of the emergence of the heads until the milky-dough stage of development. Although later growth stages were not tested it appears that some infection could occur. More than 40% of wheat seeds were found to be infected at the beginning of anthesis (GS 60) and seeds were most susceptible at around mid-anthesis (GS 64-65). The infectivity of *P. semeniperda* at different stages of inflorescence development corresponds with the infection-process results which showed that *P. semeniperda* could penetrate the ovary directly when the glumes are in an expanded position, allowing the entry of air-borne inocula. The glumes of the wheat inflorescence are only expanded at anthesis and when the filling-wheat-grain forced the glumes apart (Southwell *et al.* 1980). Similarly, internal seed-infection of barley grains by *P. teres* has been found to occur only when inoculations were made at flowering, although *P. teres* survived on the surfaces of seeds inoculated at maturity (Youcef-Benkada *et al.* 1994). Whilst wheat was chosen for these studies for experimental convenience, its susceptibility to *P. semeniperda* does not necessarily preclude the fungus as a bioherbicidal candidate (as discussed by Medd and Campbell in this Volume). However, translation of the results obtained on wheat, which has relatively uniform development, to infection of annual grass-weeds, which characteristically have asynchronous development, presents a considerable challenge. For example, floral development in *B. diandrus* may be staggered over two weeks. The limitations of the timing of susceptibility in weed populations may be overcome with sequential applications of inoculum or a delivery system that presents inoculum over a protracted period.

As is the case with many potential bioherbicidal candidates, the dew-period requirements of *P. semeniperda* for successful infection appear daunting. However, in preliminary field trials, infection of up to 73% of *B. diandrus* seeds was obtained with relatively simple formulation and application techniques (see Medd and Campbell this Volume). Seeds of several other grasses were less severely infected. It is obvious from the studies reported here that critical research is likely to be fruitful on the formulation of inoculum of *P. semeniperda* for inundative application. Factors to consider when testing different formulations are not only those which enable the pathogen to infect the greatest number of florets, but also those which enable the product to be stored, transported, tank-mixed and applied effectively.

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