CHAPTER ONE

INTRODUCTION

Charles Wilson (1969) wrote, 'The idea of using plant pathogens to control weeds is almost as old as the science of plant pathology itself.....Seeds of the idea to use plant pathogens to kill weeds have lain dormant since their sowing. There has been an occasional sprout to indicate survival. Dormancy has not been due to infertile seed but to the lack of cultivation by plant pathologists.'

The period of some 26 years since Wilson (1969) made the above statement has seen not only the seeds break their dormancy and germinate, but the seedlings have grown into adult plants. However, these adult plants have generally failed to produce the fruits of success. Although the past 26 years has seen widespread cultivation by plant pathologists (evidenced by the mounting body of literature and commercial products), the successful establishment of biological weed control has in many instances been nullified by financial and other constraints.

The science of biological weed control has been divided dichotomously into two distinct control strategies: classical biocontrol and inundative (bioherbicidal) biocontrol. This Ph.D. study was initiated to explore the potential of utilising the inundative approach to control seed production of annual grass weeds in winter cereal crops. This chapter reviews literature on the topic under four parts.

i) The science of biological control is discussed with particular emphasis on the use of the bioherbicidal approach and the integration of control technologies.

ii) A review of annual grass weeds in winter cereal crops, their potential for biocontrol, and the potential of seed pathogens as bioherbicidal candidates for these weeds.

iii) The bioherbicidal candidate *Pyrenophora semeniperda* (Brittlebank & Adam) Shoem. (anamorph *Drechslera campanulata* (Lèv.) B.Sutton) is reviewed.

iv) The specific objectives of this study are outlined.

1.1 Biological Control of Weeds with Plant Pathogens

1.1.1 The classical approach.

The classical approach to biological weed control involves the importation and release of one or more pathogens that attack the plant in its native habitat, into areas where the plant has invaded in the absence of natural enemies and has become established as a weed (Watson, 1991). By introducing some of the natural enemies, pressure is exerted on the weed and its competitive ability is reduced. This hopefully will lead to a reduction in the weed population (Mortensen, 1986). Classical biocontrol is a method whereby a long term and often permanent control can be provided with limited or no further inputs after the agent is introduced (Watson, 1992). Plant pathogens for classical biocontrol can only be used if there is sufficient evidence that they will not harm native or economically important flora (Hasan, 1988). The requirement of host specificity generally dictates the use of obligate parasites for classical biological control. Pathogens used in this approach are generally rusts and other obligate fungi capable of self-dissemination by air-borne spores, which cause epidemics after initial release (Mortensen, 1986).

The primary example of the introduction of an exotic plant pathogen in the biocontrol of a weed was the successful introduction of the rust fungus *Puccinia chondrillina* Bub. & Syd. for control of *Chondrilla juncea* L. (skeleton weed) in Australia in the early 1970's (Hasan, 1974; 1988). *C. juncea* was a serious weed of wheat growing areas in south-eastern Australia. Strains of the rust fungus were collected in the area of co-evolution with the host (the Mediterranean) and strongly pathogenic strains were tested for host specificity and stability before being released in Australia in 1971 (Hasan, 1988). After release, the rust established itself and spread rapidly with weed infestations dramatically reduced by more than 99 % (Cullen & Hasan, 1988). It has been suggested that the control of skeleton weed in Australia will be worth savings of $25.96 million per annum when the biocontrol system reaches equilibrium (Cullen, 1985). These savings will result from increased yields and reduced herbicide use (Evans & Ellison, 1990).

Another successful classical biocontrol project was the introduction into Chile of the rust fungus *Phragmidium violaceum* (Schultz) Winter to control *Rubus fruticosus* L. (blackberries) (Watson, 1991). Heliotrope rust (*Uromyces heliotropii* Sredinski) has recently

been introduced into Australia to control *Heliotropium europeaum* L. (common heliotrope) a weed of fallow land and pastures (Hasan, 1992).

Classical biological control of weeds is an option in areas such as dryland pastures, where it may be too inefficient or costly to use chemical herbicides. The cost of implementing classical biological control is generally borne by governments because there is no financial gain for chemical companies or private investors (Mortensen, 1986; Evans & Ellison, 1990). Evans & Ellison (1990) stated that classical biocontrol of weeds using plant pathogens was a neglected discipline and had tended to have been given a low priority due to a combination of quarantine and financial barriers.

1.1.2 The inundative approach.

In contrast to the classical approach, the inundative or bioherbicidal approach involves the artificial increase of a plant pathogen which is applied to the target weed in a manner similar to that of chemical herbicides (Templeton, TeBeest & Smith, 1979; Mortensen, 1986). Any plant pathogen can be considered for evaluation as a bioherbicide. However, fungi belonging to the form Class Deuteromycetes have received major attention because they are generally facultative and can be easily grown on artificial media which facilitates the mass production of infective propagules (Hasan, 1988). An aqueous suspension of infective propagules, either spores, mycelial fragments or both, is applied to produce disease and hence to control or suppress growth of a weed species (Hasan & Ayres, 1990; Watson, 1992). A bioherbicide using a fungal plant pathogen is often termed a mycoherbicide (Templeton *et al.*, 1979). Many of the natural constraints on disease development are overcome by the use of massive doses of virulent inoculum (Templeton, 1982; Mortensen, 1986; Watson, 1992). The application of an inundative dose of inoculum at the correct time shortens the lag period for inoculum buildup and pathogen distribution essential for natural disease epidemics (Daniel *et al.*, 1973; Charudattan, 1991). Furthermore, applications of bioherbicides can be timed to take advantage of favourable environmental conditions and the most susceptible stage of plant growth (Watson, 1992).

Epidemiologically, a disease reaches a threshold by two means: a high number of primary infections or a high rate of secondary infection (Yang & TeBeest, 1993). Bioherbicides are currently aimed at establishing a high number of primary infections through the application of massive amounts of inoculum. The compound interest equation *sensu*

Vanderplank (1963) illustrates the dynamics of a disease caused by a polycyclic pathogen in relation to three factors:

$$X = X_0 e^{rt}$$

where X is the current disease intensity, X_0 is the initial level of disease, e is the base for natural log, r is the rate of development and t is the duration of the interaction time (Vanderplank, 1963; Shrum, 1982). A bioherbicide may be effective if one of the 3 factors is manipulated. The disease intensity X may be increased with an increase in the initial inoculum (X_0), i.e. the application of large doses of inoculum. The disease intensity may be increased by increasing the rate of disease development (r), either by using a more aggressive pathogen and/or applying inoculum at a favourable period for infection (i.e. susceptible growth stage of weed or favourable environmental conditions). The time of the disease interaction (t) may also be altered if the pathogen is applied at a favourable time for infection. However, the greatest concern with time may be the length of time until the weed is brought under the economic threshold. Current thinking has illustrated the importance of secondary infections to the overall control efficacy of bioherbicides. Yang & TeBeest (1993) demonstrated that when low levels of initial infection occurred due to poor environmental conditions after application of Collego™, control must have resulted from the dispersal of the pathogen and subsequent secondary infections.

Exotic and indigenous species of plant pathogens have the potential to be used as bioherbicides. However, a pathogen which is endemic to an area and incites disease from year to year may be the most attractive prospect (Charudattan, 1991). This may be so from both a biological and a financial viewpoint. Biologically, endemic pathogens are primarily being assessed for bioherbicidal use because of the concerns of introducing an exotic pathogen which may be detrimental to native or economically important flora. Financially, it is much cheaper to search for candidate organisms in the country where the plant is a weed, rather than expensive surveys of the area of the plants evolutionary origin. However, although host specificity may be a greater constraint for introduced pathogens than endemic ones, endemic pathogens must still be sufficiently specific so as not to harm crops in which the target weed is to be controlled (Mortensen, 1986) or crops/native flora which may be in the vicinity of the proposed bioherbicidal application.

Both introduced and indigenous weed species can be controlled by mycoherbicides, particularly those in annual crops where specificity, immediacy and completeness of control

are paramount. In these agroecosystems, classical biocontrol may operate too slowly to reduce the weeds to below the economic threshold (Templeton *et al.*, 1979).

Several examples of bioherbicides which have been registered for use exist. The first bioherbicide was DeVine®, a formulation of the fungus *Phytophthora palmivora* (Butler) which was registered in 1981 for control of *Morrenia odorata* Lindl. (milkweed/stranglervine) in Florida citrus groves (Mortensen, 1986; Charudattan, 1991). The mycoherbicide consisted of a liquid concentrate of chlamydospores of the pathogen which was applied as a post-emergent spray and had a shelf life of about 6 weeks (Kenney, 1986). The market for DeVine® was quite small, specialized and concentrated on the citrus growing areas of Florida USA (Charudattan, 1991). Kenney (1986) reported that the only problem that existed with DeVine® was that it was too efficacious. A single treatment was still providing control some 8 years after application.

The second bioherbicide was Collego™, a formulation of the fungus *Colletotrichum gloeosporoides* (Penz.) Sacc. f.sp. *aeschynomene* which was registered in 1982 for control of *Aeschynomene virginica* (L.) B.S.P. (northern jointvetch) in rice and soybean crops (Bowers, 1986). Collego™ is supplied as a wettable powder formulation of dried spores produced by liquid fermentation and has a shelf life of about 18 months (Bowers, 1986). It is capable of killing both seedlings and adult northern jointvetch plants and has been widely accepted by rice and soybean growers (Bowers, 1986; Charudattan, 1991).

More recently, the bioherbicide BioMal®, a formulation of the fungus *Colletotrichum gloeosporoides* f.sp. *malvae* which causes an anthracnose of *Malva pusilla* Sm. (round-leaved mallow) was registered in 1992 (Makowski & Mortensen, 1992). Another bioherbicide in use is a formulation of *C. gloeosporoides* f.sp. *cuscutae* which is used in China for control of *Cuscuta* spp. (dodders) and is known as Luboa 2 (Watson, 1992).

Bioherbicides are being developed primarily for use where chemical herbicides are unsatisfactory for environmental, financial or biological reasons. The commercialisation of microbial herbicides thus far has required the developmental expertise and financial backing of private industry. This is because, bioherbicides require mass production of infective propagules, and application and storage techniques which may be similar to chemical herbicides (Mortensen, 1986).

However, the involvement of private industry in the process of commercialisation can have serious drawbacks. Chemical companies are generally interested in products with a large

market, so that the costs of development can be recovered. The market for bioherbicides may be too small to attract a financial response from many private companies. This may be despite the fact that the proposed bioherbicide can achieve weed mortality rates in excess of those achieved with chemical herbicides. For example, DeVine® which was marketed solely for use by Florida citrus growers is no longer commercially available (Charudattan, 1994). Collego™ was also aimed at a limited market and is no longer registered for use. Freeman & Charudattan (1985) reported that the commercialisation of *Cercospora rodmanii* Conway did not occur for marketing reasons on the part of a private company. Furthermore, bioherbicides are expected to provide control as fast, economical, and predictable as chemical herbicides. Unfortunately, this may not always be the case, because bioherbicides are involved with biological systems. Many potential bioherbicides may never be available for use commercially because of economic reasons, not because of product inadequacies. For bioherbicides to become widely accepted it would require the development of: a) bioherbicides which are formulated from several pathogens capable of controlling a large number of weeds simultaneously (Boyette, Templeton & Smith, 1979); b) pathogens which are able to be applied with other chemical herbicides; or c) bioherbicides based on broad host range fungi which can provide total weed control in a multi-weed system. For example the development of bioherbicides utilising the pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary which is capable of killing over 30 weed species has been proposed by Sands & Miller (1993). The possibility also exists for the use of bioherbicides which are comprised of 2 pathogens which may affect weeds utilising a synergistic interaction (Morin, Auld & Brown, 1993a,b). Morin *et al.* (1993b) reported synergy between the rust fungus *Puccinia xanthii* Schw. and the fungus *Colletotrichum orbiculare* (Berk. & Mont.) v. Arx on *Xanthium occidentale* Bertol. (Noogoora burr or cockleburr) in Australia. They reported that *C. orbiculare* alone caused only hypersensitive flecking, but interacted synergistically with the rust fungus to produce extensive lesions which on some occasions killed plants. Hallett & Ayres (1992) reported a similar synergistic interaction which resulted in death of *Senecio vulgaris* L. (groundsel) occurring between the rust fungus *Puccinia lagenophorae* Cooke and other secondary pathogens. Clearly, synergistic interactions that occur between facultative parasites would be more beneficial as a bioherbicidal strategy.

Although only a few bioherbicides have made it through the commercialisation process, many other candidates are currently being investigated and the prospects for further

commercialisation are encouraging. This may be the case especially when one considers the increasing importance of the environmental lobby in world politics. Bioherbicides fit neatly into the 'greening' policy of the agrochemical companies because of the absence of adverse effects on man, animals and the environment (Templeton, Smith & TeBeest, 1986) and furthermore, they can be marketed by them (Evans & Ellison, 1990). However, it is still important to foster the concept amongst both the scientific and agricultural communities.

1.1.3 Integration of bioherbicides with conventional control measures.

The term, integrated control describes the combined use of different types of agent for weed control (Hasan & Ayres, 1990) Such practices as the use of fertilizers, herbicides and pruning may alter the action of biocontrol agents and it is therefore necessary to take into account the whole agroecosystem for the management and control of weeds (Hasan, 1988). Both beneficial and detrimental interactions between commonly used pesticides and pathogens are important (Quimby & Walker, 1982). In practice integrated control has meant the combination of fungal pathogens with, arthropods, chemical herbicides and pesticides.

A serious deterrent for the commercialisation of bioherbicides has been the high specificity for one weed problem and the resultant low market potential (Templeton, 1986). The deterrent has been overcome with the integration of bioherbicidal pathogens and chemical herbicides in a number of experimental cases (Scheepens, 1987; Grant *et al.*, 1990a,b; Beste *et al.*, 1992). Furthermore, these experiments have shown that mixing of bioherbicides and chemicals may not only be effective in extending the number of weeds controlled, but also the efficacy of the bioherbicide itself. However, improved formulation of existing chemical herbicides may be required to take full advantage of these interactions because many of the petroleum based solvents used in formulating chemical pesticides are toxic to fungi, although the active ingredients are not (Templeton, *et al.*, 1986).

Extensive research has been done on the integration of the fungus in Collego™ and chemical pesticides. For example, Collego™ can be tank mixed with acifluorfen to control both northern jointvetch and *Sesbania exalta* (Raf.) Rydb. ex A.W. Hill (hemp sesbania) in rice fields (Smith, 1986). Klerk, Smith & TeBeest (1985) used other chemical pesticides to control insects and fungus diseases while still using Collego™ to control the northern jointvetch. They found that application of the systemic fungicide benomyl, reduced the efficacy of Collego™ when applied one week after the bioherbicide. Khodayari & Smith

(1988) further reported that benomyl reduced the efficacy of Collego™. Benomyl tolerant mutant strains of *C. gloeosporoides* have been reported which may be effective in reducing the inhibitory effects described above (TeBeest & Templeton, 1985).

With respect to synergy between chemical herbicides and bioherbicides, Scheepens (1987) demonstrated that *Cochliobolus lunatus* Nelson & Haasis could control *Echinochloa crus-galli* (L.) Beauv. (barnyard grass) when in combination with a sub-lethal dose of atrazine as low as 2.5 mg m^{-2}. This may be a particularly interesting prospect given that atrazine was used to control broad-leaved weeds in maize (Scheepens, 1987). Applications of BioMal® mixed with bromoxynil plus MCPA, imazethapyr, metribuzin and sethoxydim enhanced control of round-leaved mallow compared to inoculation with BioMal® alone (Grant *et al.*, 1990b). However, other chemical herbicides mixed at the recommended rates inhibited spore germination, appressorial formation and subsequent disease development (Grant *et al.* 1990a). They also reported inhibition of fungal growth by a range of fungicides and surfactants.

The mechanisms by which synergism may occur between bioherbicides and chemical herbicides are unknown (Hasan & Ayres, 1990). However, Hasan & Ayres (1990) postulated that synergism may be accounted for by two different processes. Firstly, the pathogen may influence the uptake or transport of the chemical. Secondly, if the chemical works by regulating plant growth, the chemical may inhibit the plants ability to resist or grow away from the effects of infection. Grant *et al.* (1990b) concluded that a synergistic interaction occurred because of a reduction in time requirements for a dew period, resulting in more infection by the BioMal® fungus.

A negative aspect of the integration of chemical pesticides with biological control, may be the effect of chemicals on the agents themselves. For example, when *Eichhornia crassipes* (Mart.) Solms (waterhyacinth) was controlled using large applications of chemical herbicides, resulting in a large foliar mortality over a short period, a large population of biocontrol arthropods died of starvation (Charudattan, 1986). More recently, Hoffmann & Moran (1995) described the reduction in control of *Sesbania punicea* (Cav.) Benth. when insecticide drift from citrus orchards increased the mortality of the introduced biological control agent *Trichapion lativentre* (Beguin Billecocq) (apionid weevil) in South Africa. Although the above examples refer to arthropod agents, there appears to be a real possibility that the same could occur for fungal plant pathogens after the application of fungicides,

particularly when one considers the effects of benomyl on Collego™ (Klerk *et al.*, 1985) and benomyl, mancozeb and other fungicides on the efficacy of BioMal® (Grant *et al.* 1990a).

The principle of mixing bioherbicidal pathogens with chemical herbicides has been demonstrated experimentally. This may help overcome the general constraint to commercialisation (particularly in the eyes of chemical companies) posed by high specificity of virulent pathogens (Templeton, 1986). Other possibilities also exist for companies searching for chemical herbicides. Chemicals may have increased weed control spectra when mixed with a fungus, or chemicals that are unmarketable alone, could have definite potential when combined with a fungus (Templeton, 1986).

1.2 Annual Grass Weeds in Cereal Crops

Triticum aestivum L. (Wheat) is harvested, somewhere in the world every month of the year (Zimdahl, 1992). About 231 million hectares of land is used for wheat production worldwide, which equates to 526 million metric tonnes of wheat produced per annum (Evans, 1992; Nalewaja, 1992). The greatest yield losses in cereal crops come from competition with weeds (Nalewaja, 1992). It is not surprising therefore, that weed control in wheat and other cereal crops is an enterprise of unequivocal importance. The world market for crop protection chemicals in 1990 was estimated at US$26.4 billion (Evans, 1992). Of this, 44 % was accounted for by herbicides. Perhaps the easiest weeds to control in cereal crops are annual broadleaf weeds which can be controlled with growth regulatory herbicides. Grass weed control has improved with the development of selective post-emergent herbicides, such as the lipid biosynthesis inhibitors, for example, diclofop. However, control is still limited because of the close relationship between cereals and these weeds, the build up of herbicide resistance (Powles & Howat, 1990), and increases in seed bank populations (Medd, 1990). Despite chemical herbicide control the annual grass weeds, *Avena fatua* L. (wild oats) and *Bromus tectorum* L. (downy brome) continue to be the 2nd and 3rd most important weeds of wheat in the USA respectively (Zimdahl, 1992).

1.2.1 Annual grass weeds and the Australian winter cereal industry.

Australia is one of the world's top 10 wheat producing nations and one of the top 5 wheat exporters (Australian Wheat Board, 1994). Some 8 to 10 million hectares are used for

wheat production annually with a monetary value for exported grain of US$2.14 billion from the 1993-94 crop (Australian Wheat Board, 1994). A further 4-5 million hectares of land are sown to the other cereal species (oats, *Avena sativa* L.; barley, *Hordeum vulgare* L.and triticale *Triticum* x *Secale*). Wheat is grown in all states of Australia except the Northern Territory and is largely confined to the regions where annual rainfall averages between 300 and 600 mm per annum (Simmonds, 1989). Because of the largely Mediterranean climate (i.e. mild, wet winters and hot, dry summers) in the wheat growing areas of Australia, nearly all wheat planted is of the so-called 'spring' wheat type. Some 'winter' wheat is grown in areas which are particularly prone to frost and may also be used for stock grazing. Wheats grown in Australia range from the soft grained varieties which are characteristically low in protein (8 to 10 %) and used primarily for cake and biscuit flour, to the hard grained varieties which are characterised by a high protein (13 to 15 %) content and used for bread and pasta flour (Campbell, 1991).

Weeds are one of the limiting factors to cereal 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 & Blacklow, 1984).

The most important grass weeds in the Australian cereal industry are *Avena* spp. (wild oats), *Lolium rigidum* Gaud. (annual ryegrass), *Bromus diandrus* Roth. and *Bromus mollis* L. (annual brome grasses), *Phalaris minor* Retz. and *Phalaris paradoxa* L. (annual phalaris species), *Hordeum* spp. (barley grasses) and *Vulpia bromoides* (L.) S.F.Gray (silver grasses) (Amor, 1985; Medd, 1987b; Poole & Gill, 1987). Although these grasses are weeds under cereal cropping, some are also considered to be important pasture species, for example, annual ryegrass and some brome species (Poole & Gill, 1987). Cereal yield losses from the competitive effects of these weeds can be as high as 50 to 75% of the potential weed-free yield (Leys & Dellow, 1986; Combellack, 1992) although yield losses under less severe weed infestations were typically in the order of 10 to 25% (Poole & Gill, 1987) (Table 1).

Annual grass weeds may also harbour disease organisms and act as sources of inoculum for infection of cereal crops. Further monetary inputs are required to control disease and lessen the subsequent effects on yield. Harris & Moen (1986), reported that several important soil-borne cereal pathogens can be isolated from grass weeds, including: *Gaeumannomyces graminis* (Sacc.) v. Arx & Oliver the causal agent of take-all disease;

Bipolaris sorokiniana (Sacc.) Shoem. (foot and root rot of cereals); *Fusarium graminearum* Schwabe (root rot) and *Rhizoctonia solani* Kühn the causal agent of many cereal diseases ranging from damping-off to root rots. Additionally, grass weeds can be infected by several seed and air-borne foliage pathogens that are also pathogenic on cereals. For example, *Pyrenophora teres* Drechsler (net and spot forms of net blotch of barley), *Puccinia graminis* Pers. (stem rust), *Pucinnia striiformis* Westend. (stripe rust) and *Leptosphaeria nodorum* E. Müller (glume blotch) (Sivanesan, 1987; Holliday, 1989). There have also been reports of an increase in iatrogenic disease with expanding use of herbicides in conservation tillage systems. For example, Harris & Moen (1986) reported that chlorsulfuron residues prediposed a wheat crop to early acute infection by the take-all fungus.

Table 1. Yield loss in wheat in southern Australia due to competition with some important annual grass weeds at a density of 100 weeds m^{-2}. Adapted from Poole & Gill (1987).

Weed	Percentage Loss
Wild oats	32
Brome grass	30
Barley grass	24
Ryegrass	8-20

Weed seed contamination of harvested grain can occur. Dockage penalties for grain produce with weed seed contamination can apply if certain requirements are not met. For example, the Australian Wheat Board has allowable wild oat seed limits of 50 seeds per half litre sample of wheat grain (Simmonds, 1989). Once this limit is exceeded dockage penalties apply. Mock (1987) reported that seed contamination of barley grain by 30 seeds per sample of brome grass resulted in a quality downgrading and the grower receiving \$25 t^{-1} less payment (1987 rates). Contamination of grain produce with some other contaminants can result in total exclusion of that particular harvest. For example, grain contaminated with seeds of *Eucalyptus* spp. or *Allium vineale* L. (wild garlic) (Simmonds, 1989).

In a recent study on soil structural stability, Caron, Kay & Perfect (1992) demonstrated a decrease in soil stability following the establishment of *Bromus inermis* Leyss. onto a poorly structured soil. They concluded that the decrease in stability was caused

by the physical fragmentation of aggregates by root penetration and/or by weakening of aggregates through enhanced wetting and drying. Although *B. inermis* is naturalised in the wheat growing areas of New South Wales, it is not considered a prominent weed of cereal crops. However, the possibility exists that other *Bromus* species may have a similar effect on soil stability. This may be of particular importance in cropping areas after cultivation when soil structure may be fragile. Soil degradation may occur if *Bromus* spp. predominate in fallowed paddocks.

With the advent of conservation tillage practices, (i.e. practices that eliminate some or all operations involving soil tillage) in the growing of broadacre cereal crops, a greater reliance on herbicides for the control of weeds has resulted (Medd, 1987b; Burgess, 1988). Poole (1986) estimated that in Western Australia in 1985, about $40 million was spent on grass weed control. Combellack (1989) estimated the financial losses for Australia due to weeds in all agricultural crops to be in the vicinity of $1870 million. Losses of $710 million were attributed to cultivation and $303 million for the purchase of herbicides and their application. In a study on the cost of *Avena* spp. to the Australian wheat industry, Medd & Pandey (1990a) reported a conservative estimate of a $42 million loss annually. This resulted from $30 million expenditure on herbicides and their application, and $12 million due to lost wheat yield from the competitive effects of wild oats within the crop. Herbicide sales for the control of grass weeds in cereal crops has been estimated to be in the order of $110 million in 1990 (Medd, 1992b).

1.2.2 Competitive effects of annual grass weeds.

Cereal yield losses occur because of the competitive effects of grass weeds growing within crops. Final yield of a crop is the summation over time of all the events and processes that served to increase or decrease the growth of the crop (Cousens *et al.*, 1991). Time and speed of emergence of both weeds and crops influence the relative competitive abilities. Both weeds and crops that emerge rapidly are likely to be more competitive (Medd, 1987a; Cousens *et al.*, 1991). Furthermore, early emerging seeds are more likely to survive and produce large numbers of seeds which can be added to the seed bank for seedling recruitment in future seasons (Peters, 1984). For example, Reeves (1976) demonstrated that ryegrass was more competitive in later sown crops and that competition occurred well before tillering was completed. Peters (1984) showed that wild oats that emerged early had a greater competitive

advantage over barley than those that emerged later. Peters (1984) also reported that the early emerging wild oat plants gave rise to between 91 % and 100 % of the seed shed. In another study, wild oats that emerged at a similar time to wheat were shown to be more competitive due to better root growth (Martin & Field, 1988). This was presumably because wild oats have a larger root system than wheat (Pavlychenko, 1937). Anderson (1986), demonstrated that *B. diandrus* is a stronger competitor than wheat in the first month of growth and that the early aggressiveness of the species allows the seedling to become quickly established. In contrast, Wilson & Wright (1990) showed that species which have a delayed emergence or slow initial growth rate may later dominate a mixture. The number of fertile tillers that cereal crops produce in early phases of the life-cycle influences the yield of that crop. Various stresses, including weed competition, during early growth reduces tillering and subsequent crop yield (Medd, 1987a). Reeves (1976) and Williams *et al.* (1986) described a reduction in fertile tiller production by wheat in response to competition by ryegrass. The number of fertile tillers produced by wheat was markedly reduced by competition with great brome when weed density was 400 plants m^{-2} (Gill and Blacklow, 1984). A similar finding was reported by Poole, Holmes & Gill (1986) when weed density was 270 plants m^{-2}.

Nutritional deficiencies during early crop growth may slow growth of the crop and allow weeds more opportunity to exploit the available resources (Medd, 1987a). Reduction in fertile tiller production may be linked with nutrient deficiency in the crop species due to competition with grass weeds. For example, ryegrass has been shown to compete with wheat for nitrogen early in its growth and reduce the number of fertile wheat tillers as a result (Smith & Levick, 1974; Reeves, 1976; Williams *et al.*, 1986). *B. diandrus* has been shown to compete with cereals for both nitrogen and phosphorous which results in large losses in grain yield (Gill & Blacklow, 1984). They suggested that a reduction in the number of wheat ears m^{-2} could be attributed to death of some potentially ear-bearing tillers due to nutrient stress. Anderson (1986) concluded, that *B. diandrus* is able to prevent the cereal from making full use of available nutrients. Peters (1984) also reported that the presence of wild oats during grain formation diminished grain protein levels due to competition for nitrogen. However, in some of his experiments, severe competition was actually found to increase nitrogen content of barley grain. Peters (1984) concluded that this could be the case because of a larger number of embryos within a given weight of sampled grain. In fertilised plots infested with wild oats, wheat grain yield decreased whilst the number of fertile panicles of wild oats

increased (Combellack, 1992). The addition of fertilisers to a crop that is infested with weeds may complicate the weed problem if weeds compete more efficiently for nutrients at developmental stages (Medd, 1987a).

Competition for soil moisture may also be a critical factor in determining the relative competitive abilities of cereal and grass weeds, particularly at anthesis and grain filling. Grain size in wheat is very sensitive to water stress during ear emergence and anthesis (Fischer, 1973 cited in Gill & Blackow, 1984). Radford *et al.* (1980) found that on the black earth soils of the Darling Downs in Queensland, which had large amounts of stored water, the rate of seeding wheat influenced its competitive ability against wild oats. They reported that in plots with low wild oat infestation relatively low seedling rates were required to obtain optimum yield. Conversely, in plots with high wild oat numbers a greater rate of wheat seeding was required. However, the combination of higher wheat densities with a wild oat population could deplete soil moisture reserves faster, resulting in water stress at anthesis and grain filling. Ryegrass may also be more competitive with high amounts of soil moisture (Williams *et al.*, 1986; Poole & Gill, 1987). Gill & Blacklow (1984) showed that great brome reduced the size of wheat grain by competition for water during grain filling.

Poole & Gill (1986) indicated that the order of competitiveness in terms of yield loss among the most prevalent annual grass weeds in Australian cereal crops was: wild oats = brome grass > barley grass > ryegrass.

1.2.3 Control of annual grass weeds in cereal crops - a brief account.

The most common methods of weed control in crops are the use of herbicides and/or cultivation (Combellack, 1989). Other methods commonly employed to reduce the competitive effects of grass weeds include: the sowing of more competitive cereal cultivars, optimizing seeding rates and sowing times, rotating crop species of both winter and summer crops, and 'spray-topping' to reduce seed set.

The traditional method of controlling weeds is the use of tillage. In recent times, the use of conservation tillage has become more widespread. Conservation tillage practices involve the use of fewer tillage operations, greater reliance on herbicides for weed control and the retention of crop residues on the soil surface. They are designed to conserve soil structure, fertility and moisture and reduce the risk of erosion (Burgess, 1988). Conventional tillage practices involve cultivating the land a number of times prior to sowing the crop to remove

weeds and to stimulate germination of weed seeds thus, depleting the seed bank (Medd, 1987a; Combellack, 1989; Morgan, 1989, 1992). Leys & Dellow (1986) reported that the most efficacious method of controlling *Hordeum* spp., *Bromus* spp. and *Vulpia* spp. was pre-sowing cultivation. Pre-emergent herbicides such as trifluralin and triallate may be incorporated into the soil with a final cultivation to control wild oats and annual ryegrass (Mullen & Dellow, 1993). Weeds that emerge during the crop phase are removed by post-emergent selective herbicides and/or further cultivation, particularly of the inter-row areas. Morgan (1992) suggested the use of chain harrows, knives and scarifiers to minimize the soil disturbance often encountered with extreme inter-row cultivation practices.

In conservation tillage systems the number of tillage operations are reduced by a substitution of herbicides and/or crops are sown by direct drilling (Combellack, 1989). Conservation tillage practices have caused a change in the spectrum of weeds and their relative importance (Burgess, 1988). For example, *B. diandrus* has increased in importance in cereal cropping in Australia despite ecological studies which suggest it should be easily controlled by tillage (Gill & Blacklow 1985). It appears that the reason for this upsurge in its importance is due to the introduction of reduced tillage cropping systems and particularly to the use of selective herbicides on its main competitors, ryegrass and wild oats (Poole & Gill, 1987). *Hordeum glaucum* Staud. (barley grass) has also risen to prominence as a weed of cereals since the advent of conservation tillage practices (Leys & Dellow, 1986) *Bromus* spp. and *Hordeum* spp. are relatively tolerant of the herbicides currently used in cereal cropping systems (Leys & Dellow, 1987; Mullen & Dellow, 1993). Consequently, there is no economical method available for the control of *Bromus* spp. in winter cereal crops (Mock, 1987). Similarly, there is no currently recommended selective herbicide for barley grass in cereal crops (Mullen & Dellow, 1993). Although *L. rigidum* can be adequately controlled by selective herbicides, ryegrass competition has been shown to be more severe in direct drilled plots (Williams *et al.*, 1986). Williams *et al.* (1986) postulated that this was due to improved nitrogen status of wheat grown under conventional cultivation. Pre-emergence herbicides are not compatible with conservation systems which has led to an increased reliance on post-emergence herbicides (Medd, 1987b). Many competition studies between cereals and grass weeds suggest that to minimize the effects of competition, post-emergent herbicides should be applied as early as the 2-3 leaf stage of growth (Streibig *et al.*, 1989). For example, Martin & Field (1988) concluded that emergent wild oat seedlings should be controlled within three

weeks of drilling a wheat crop. Reeves (1976) found that to minimize substantial losses occurring from ryegrass competition in wheat, control measures needed to be effective before the 3-leaf stage of the crop. Peters (1984) suggested the early removal of wild oats in a barley crop was necessary to minimize competition and the number of seeds set by the weeds.

Herbicides are costly and the decisions of when and how much to use are often imprecise. The decision as to whether to spray often takes place a long time before the weeds have had a harmful effect on the crop (Streibig *et al.*, 1989). Many studies on weed competition with cereal crops (yield loss) and economic analysis of that competition have been published (eg. Poole & Gill, 1987). Lybecker, Schweizer & King (1988) suggested that the weed management system which optimized economic return was one which was less herbicide-intensive.

An adverse consequence of repeated applications of herbicides is the build up of resistant weed populations (Powles & Howat, 1990). In 1992 there were one hundred weed biotypes resistant to herbicides in about 40 countries (Jutsum & Shaner, 1992). The problem of weed populations developing resistance to herbicides could become a major obstacle to the adoption of conservation tillage practices (Burgess, 1988). Several resistant weed biotypes have been found in Australia including: wild oats, barley grass and ryegrass (Tucker & Powles, 1988; Powles & Howat, 1990; Gill, 1995). Of most concern however, has been the development of cross resistant biotypes of ryegrass with resistance to a number of herbicide types. Weed plants in conservation tillage systems have minimal opportunities to backcross with plants from earlier generations that were less heavily selected for herbicide resistance. It is important therefore, to rotate herbicides used to minimize the chances of selecting for resistance in weed populations (Cussans, 1992). The use of herbicide mixtures may also help (Combellack, 1989).

There are a wide range of selective grass herbicides for use within broad-leaved crops such as *Lupinus angustifolius* L. (lupins), *Brassica napus* L. (canola) and *Pisum sativum* L. (field pea) (Mullen & Dellow, 1993). A method for suppressing annual grass weeds is to rotate crop species, and use grass selective herbicides such as fluazifop (e.g. Fusilade®) to control them within the crop. Conservation tillage has resulted in a wider range of crop species being grown in southern Australia (Medd, 1987b). In Victoria, the area sown to field peas in the Wimmera and Mallee districts increased by 82 % in one year after the introduction of fluazifop herbicides which could selectively control grasses (Mock, 1987). Rotation with

broadleaf crops is also recommended for the control of *Hordeum, Phalaris* and *Vulpia* spp. (Ley & Dellow, 1986). An advantage of crop rotation is that rotation with summer crops allows a winter fallow period. Alternative herbicides can be used during the winter fallow which decreases the selection pressure for resistant biotypes and may reduce the economic impact of a failed crop due to weed competition.

An effective indirect method of controlling annual grass weeds in cereal crops is to increase the seeding rate when a crop is sown. Radford *et al.* (1980) showed that increased wheat density reduced the dry weight of wild oats and decreased the production of wild oat seed especially when wild oat densities were low. Medd *et al.* (1985) suggested that an increase in density of wheat could reduce the competitive effects of ryegrass. Increasing seeding rates increased the grain yield of wheat and barley grown in brome grass infested plots, although the strategy appeared to have limited practical use (Zaicou & Gill, 1992). They also reported that increased seeding rates reduced the seed output of brome grass, particularly when grown in barley crops. The effects of increased crop density may also be enhanced by planting cereal cultivars which themselves are more competitive. Lemerle & Cousens (1992) reported that the wheat cultivar Olympic reduced dry matter of wild oats by 76 % and that the cultivar Owlet reduced the dry matter of wild oats by 48 % alone or 70 % when a low rate of diclofop-methyl herbicide was also used.

A feature of annual grass weeds is their ability to produce large amounts of seed with which to carry-over to following seasons. Therefore, the build-up of grass populations can be attributed to the increase in seed-bank populations. A novel method by which seed production and thus seed banks may be decreased is the use of 'selective spray-topping' (Medd, McMillan & Cook, 1992). This method utilised selective post-emergent herbicides applied when wild oats were at the elongation/booting stage of their development. Flamprop-methyl and fenoxaprop-ethyl reduced emergence of inflorescences by up to 99 % and seed production by up to 96 % (Medd *et al.*, 1992). Spray-topping using non-selective herbicides has also been used for *Hordeum* and *Vulpia* spp. (Leys & Dellow, 1986).

1.2.4 Seed kill as a strategy to control annual grass weeds in cereal crops.

Most agricultural soils contain large numbers of weed seeds. These seed banks provide a source of weed seedlings even when seed reinfestation is successfully prevented (Karssen & Bouwmeester, 1992). A feature common to annual grasses is their prodigious

seed production, which is required for survival, multiplication and invasion (Medd, 1992b). They are characterized by their low level of dormancy, rapidly diminishing seed banks in the absence of seed production and rapidly increasing populations if uncontrolled (Medd, 1992a). Furthermore, they behave as winter annuals and reproduce within the life span of the crop, adjusting reproductive yield according to the resources available (Medd, 1987b). Herbicides have generally contained the competitive effects of grass weeds on cereal crops. However, annual grass weeds continue to be a problem in cereal crops. Particularly when one considers that the change in cropping systems to conservation tillage has changed the relative importance of some species (e.g. *Bromus* and *Hordeum* spp.). The magnitude and persistence of seed banks are key factors in the perpetuation of weeds and the continuing need for weed control (Medd, 1987a).

Seed banks of annual grass weeds may be transient or persistent. Weed species which form transient seed banks, produce seeds that persist long enough only to re-establish a population in the following growing season when conditions become favourable for germination. That is, prior to seed production at the end of the growing season, the banks are empty due to massive germination or seed death (Karssen & Bouwmeester, 1992). Weed species which form persistent seed banks, produce seed which are able to survive for long periods in the soil before germination. Persistence of seeds is generally due to dormancy. Dormancy is defined by Medd (1985) as the inability of living embryos to grow when given favourable conditions, due to various physical or chemical constraints. Persistence of soil seed banks depends primarily on the control of germination. The depletion of seed banks is primarily due to germination and subsequent seedling emergence. Cultivation of soils may stimulate germination, thereby decreasing the seed bank. Seed banks may also be depleted due to the removal of seeds, and death due to rotting and predation by animals and microorganisms (Medd, 1987a). A third cause of seed bank depletion may be fatal germination (Karssen & Bouwmeester, 1992). That is, germination that is not followed by seedling emergence.

Seed banks of annual grass weeds in Australian cereal cropping systems are generally of the transient type, although some species may persist for some time. Wild oats are generally thought to persist in seed banks due to seed longevity in the soil (Combellack, 1992). However, there is mounting evidence that wild oat populations persist because of the input of new seed (Medd *et al.*, 1992). Evidence for this suggestion is the excellent control of

wild oats after a one or two year crop rotation with sorghum (Purvis, 1990; Jones, 1992). This rotational practice would not work if seeds persisted in the soil due to dormancy. Furthermore, Medd (1990) reported that the annual rates of wild oat seed bank decline was in the vicinity of 70 % per annum. It would seem therefore, that tactics which reduce the input of seed would improve long term control of wild oats. Selective spray-topping as described by Medd *et al* (1992) is one such way of reducing seed bank inputs by wild oats. Medd & Ridings (1989) used a demographic simulation model to explore the potential population effects of seed kill to control annual grass weeds. They concluded that destroying greater than 25 % of seeds produced would lead to marked reductions in population size and was therefore a biologically reasonable tactic. In an economic study, Medd & Pandey (1990b) demonstrated that the tactic of killing 60 % of wild oat seeds in combination with existing weed management practices generated \$12 ha^{-1} in additional profits. Medd & Pandey (1993) using a bioeconomic model investigated the economic benefits of seed kill. They concluded that herbicide applications to control seedlings in conjunction with 70 % seed kill contained wild oat populations and that this would lead to lucrative economic benefits. In a study of the competitive effects of wild oats on barley, Peters (1985) demonstrated that wild oat plants that were derived from large seeds were more competitive than those derived from smaller seeds. Thurston (1956) reported that larger primary seeds of wild oats are less dormant than the smaller secondary ones. Peters (1985) concluded that if seed was not permitted to return to the seed bank (i.e. seed kill or removal), primary seeds would decline and less vigorous secondary seeds would remain. Martin & Field (1988) concluded that in order to obtain long-term benefit from wild oat control, it was necessary to prevent the return of wild oat seeds to the soil.

Seed kill tactics would also be relevant for other grass weeds in cereal crops. For example, Gill & Blacklow (1985) demonstrated that *B. diandrus* lacked dormancy and concluded that a rapid reduction of the species could be achieved by decreasing the number of seeds in the soil and preventing seeds from returning. Similarly, Cheam (1986; 1987) suggested that control of *B. diandrus* could be affected by prevention of seed production. The standard advice for control of *Bromus* spp. in Great Britain (which occurs on 42 % of cropped fields) is to plough rather than use non-inversion tillage systems (Cussans *et al.*, 1994). It appears that the increase of *Bromus* spp. in Britain can be attributed to the same reasons as the build-up in Australia. Killing of brome seeds by mould-board ploughing should achieve

complete kill (Cussans *et al.*, 1994). Therefore, other methods by which seed kill can be expected could also be utilised. Seed kill tactics have also been used on *L. rigidum* (Wallace & Maling, 1992), *Hordeum* spp. and *Vulpia* spp. (Leys & Dellow, 1986).

Medd & Ridings (1989), Medd *et* al (1992) and Pandey, Medd & Lindner (1992) demonstrated that control of seed production by annual grass weeds in cereal crops had a greater impact on population control than can be achieved through control of plant numbers. Consequently, research on control measures which concentrate on seed kill is warranted.

1.2.5 The potential of seed-borne pathogens as biocontrol agents.

Winter cereal crops represent a major market for weed control products in Australia (Medd, 1992a). The increase of weed species that are tolerant or resistant to herbicides (*Bromus, Vulpia, Lolium* and *Avena* spp.) (Leys & Dellow, 1986; Powles & Howat, 1990; Gill, 1995) emphasises the potential of biological control methods to control these grassy weeds. Classical biocontrol does not hold much promise for the control of annual grasses because of their close relationship to cereal crops (Wapshere, 1990). It seems unlikely that agents for classical biocontrol would receive clearance from the quarantine service. If this is the case, the only other option is the development of bioherbicides using organisms which are already present in Australia on the weeds or their close relatives (Wapshere, 1990). The use of control methods aimed at destroying seeds and thus reducing the seed banks in agricultural soils has considerable potential for annual grass weeds. The following discussion outlines the potential of seed-borne pathogens for use as bioherbicidal agents to control annual grass weeds.

Bioherbicide research has largely been restricted to foliar fungal pathogens of broadleaf weeds such as: *A. virginica* (northern jointvetch) in the United States (TeBeest and Templeton, 1985); *Xanthium spinosum* L. (Bathurst burr) and *X. occidentale* (Noogoora burr) in Australia (McRae and Auld, 1988; Auld, *et al.*, 1990; Nehl and Brown, 1992); and *M. pusilla* (round-leaved mallow) in Canada (Makowski and Mortensen, 1992). In contrast, research on potential pathogens which reduce seed set, seed viability and/or seedling establishment for use in a bioherbicidal context has received little attention. Research that has been undertaken, has either not been reported in the literature, or has failed to draw convincing conclusions as to the efficacy of the tactic. Thurston & Cussans (1976) surveyed possible biocontrol agents of wild oats and concluded that only seed infesting fungi were

worth further study, especially *Phoma hibernica* Grimes, O'Connor & Cummins, however, no further research was published. Massion & Lindow (1986) reported that teliospores of the smut fungus *Sphacelotheca holci* Jack. infected inflorescences of the perennial grass species, *Sorghum halapense* (L.) Pers. (Johnson grass) completely eliminating seed set which could potentially reduce the weed seed bank. The fungus also decreases plant biomass and rhizome length of infected johnsongrass plants (Massion & Lindow, 1986). Wilson (1987) and Wilson & Hall (1987) demonstrated that *Pyrenophora avenae* Ito & Kurib. was capable of infecting seeds of wild oats and causing 75 % mortality of seedlings. However, they concluded that *P. avenae* would be better applied to wild oats as a foliage pathogen. The fungus could be used to control wild oats in wheat and barley crops but not oat crops due to its infectivity on cultivated *Avena* spp. (Wapshere, 1990).

The tactic of utilising pathogens of seeds for the biocontrol of annual grass weeds in winter cereal crops has potential providing a suitable pathogen is found (Medd and Ridings, 1989). Ungerminated seeds are hard to kill with chemicals. However, a diverse pathogenic mycoflora exists on and within many grass seeds (Hyde and Galleymore, 1951; Kiewnick, 1963;1964; Mortensen and Hsiao, 1987). Possible control of these weeds may be afforded by manipulation of these seed-borne pathogens and using them as mycoherbicides.

Seed-borne organisms produce a variety of effects, including stimulation or inhibition of germination, protection of desirable seeds from harmful microorganisms, and loss of seed viability (Charudattan, 1988). "Seed" pathogens which show potential as bioherbicidal agents fall into 4 broad categories.

1) Pathogens that infect the seed on the developing inflorescence. For example, *Ustilago tritici* (Pers.) Rostrup (the causal agent of loose smut of wheat), *Pyrenophora graminea* Ito & Kurib. (barley leaf stripe), *P. teres* (net blotch of barley) *P. semeniperda* (milo spot of grasses) (Shipton, Kahn and Boyd, 1973; Agarwal and Sinclair, 1987; Medd, 1992c). Pathogens in this category may reduce the viability of seed produced or result in seedling infection. Kiewnick (1963) reported that wild oat plants attacked by *Fusarium culmorum* (W.G.Sm.) Sacc. produced sterile seeds. Smith (1966) suggested that the seed rot caused by *P. semeniperda* may play an important role in limiting the seed production of some grass species.

2) Pathogens that adhere to the surface of mature seed, either in the inflorescence or in the soil, infecting the seedling upon germination of the seed. For example, *Tilletia tritici*

(Bjerk.) R.Wolff (Common Bunt), *Cochliobolus sativus* (Ito & Kurib) Drechsler ex Dastur (foot rot of wheat), *P. avenae* (oat leaf stripe) fit in this category (Agarwal and Sinclair, 1987; Wilson, 1987). Pathogens in this category may produce seedling blight and damping-off symptoms.

3) Pathogens that replace the seed in the inflorescence with fungal propagules or which prevent flowering. These include pathogens such as *Claviceps purpurea* (Fr.) Tul. (ergot of cereals), *T. tritici*, *S. holci* and some fungal endophytes belonging to the family Clavicipitaceae tribe Balansiae (Siegel, Latch and Johnson, 1987; Clay, 1989). The effect of these pathogens on their hosts is that they reduce the numbers of viable seeds produced.

4) Fungi that are either pathogenic or saprophytic on seeds lying in the soil. *Fusarium culmorum* and *Fusarium solani* (Mart.) Sacc. and damping-off fungi such as species of *Pythium,* and *Phytophthora* fit into this category (Kiewnick, 1963; Agrios, 1988). These pathogens may cause seeds to lose viability, increase dormancy or cause seedling mortality.

Pathogens that may be useful as bioherbicidal agents generally must fulfil the following criteria as outlined by Daniel, *et al.* (1973).

i) The pathogen must be able to produce abundant and durable inoculum in artificial culture.

ii) The pathogen must be genetically stable and specific to the target weed.

iii) The pathogen must be able to infect and kill the target weed in environments over a reasonably wide latitude.

Most research on bioherbicidal agents has been restricted to pathogens which are endemic. However, an exotic parasite could be equally effective as a bioherbicide if it possesses the necessary epidemiological attributes (Charudattan, 1991) and can pass the quarantine procedure.

The ability of a pathogen to sporulate in artificial culture may not be an obligatory trait as proposed by Daniel *et al.* (1973). Recently, obligate parasites have been used to initiate epidemics in a manner similar to the inundative strategy. Phatak (1992) reported control of yellow nutsedge (*Cyperus esculentus* L.) with the nutsedge rust (*Puccinia canaliculata* (Schw.) Lagerh. Control of yellow nutsedge was obtained by initiating an epidemic of the rust fungus by releasing uredospores over crops at the rate of 5g/ha. This approach was designed to augment naturally occurring inoculum, raising levels high enough to sustain a

seasonal epidemic. The fungus is to be marketed under the tradename "Dr. Biosedge" (Phatak, 1992).

An important question when discussing the use of "seed" pathogens as potential bioherbicidal agents is where does one start looking for potential candidates? Pathogens that kill the seed or prevent seed set would seem to have the most attractive prospects. Many potentially devastating agents such as the smuts are ecologically obligate parasites and do not sporulate readily on artificial media. As mentioned earlier, this need not be a limiting factor if an augmentative rather than inundative approach is pursued. The possibility of using smut fungi would be enhanced if methods of getting them to produce abundant spores on artificial media were defined. For example, the classical biological control agent *Entyloma compositarum* Farlow is able to be produced on artificial media (Trujillo, Aragaki & Shoemaker, 1988).

The use of "seed" pathogens in the soil also shows some promise. However, studies on the prospect of utilising hyperparasites of soil-borne fungal diseases (such as sclerotinia wilt of sunflower) have shown that large amounts of inoculum (approx. 1.8 t. ha^{-1}) must be added to the soil to give adequate control (Huang, 1980). This may also be the case with bioherbicidal control of weed seed. Furthermore, a bioherbicidal agent incorporated into the soil would require a high competitive saprophytic ability or possess other survival mechanisms such as the production of resting structures such as chlamydospores or sclerotia to compete with other organisms in the soil. The agent should also be an aggressive parasite that actively seeks out weed seed within the soil. Transmission of "seed" pathogens applied to soil would require extra research into application technology. This contrasts with aerially applied bioherbicides which may utilise already existing technologies.

Pathogens that either infect the seed while in the inflorescence or reduce the numbers of seed set also require special considerations. Since populations of annual grass weeds stagger their phases of reproduction, it would be appropriate to search for a pathogen that can infect seed at a range of different plant growth stages. For example, it may be possible to utilise a pathogen that sporulates on the host's leaf surfaces and produces airborne inoculum which subsequently infects the inflorescence (e.g. *P. teres* (Shipton, *et al.*, 1973)). A pathogen that only infects at a certain growth stage of the weed may be difficult to use as a bioherbicide. An agent that infects seed during flowering would require an inundative inoculation of the weed at the time of anthesis. Since anthesis is often staggered over several

weeks, high levels of seed infection would require spraying of the bioherbicide over the corresponding time interval. Furthermore, if environmental conditions were not conducive to infection at that time, application of the bioherbicide may be wasted. It might not be possible to correct the situation by reapplication, since the 'infection window' may be closed. This problem may be overcome with the use of inoculum applied in a pelletised form (such as sodium alginate pellets) to the surface of the soil. The inoculum may then be available for infection over a protracted period (Walker and Connick, Jr, 1983; Boyette, *et al.*, 1991).

Since some cereal crops are used as forage the bioherbicidal agent should have no toxic effects on livestock. Furthermore, cereal grain for human consumption must also be non-toxic. This therefore eliminates the use of fungi such as *C. purpurea* due to their production of toxic ergot alkaloids. Some species of *Pyrenophora* and *Fusarium* also produce mammalian toxins (Agrios, 1988; Scott, 1988).

Annual grass weeds might be effectively controlled by bioherbicidal agents that attack the seed either directly or indirectly. The most promising pathogens would appear to be those that give a "two pronged" effect. That is, those pathogens which infect both the foliage and the seed. The reasons for this are twofold. Firstly, foliage pathogens may give an early competitive edge to the crop and weaken or possibly kill the weed. Secondly, the production of secondary inoculum on leaves and the subsequent infection of seed may reduce the viability and/or number of seeds set. This would decrease the numbers of weed seed available for recruitment in the next growing season.

A further advantage of foliage/seed pathogens over those pathogens that only infect the seed may be the less stringent timing of bioherbicide application (depending on the pathogen).

1.3. *Bromus diandrus* and the Seed Pathogen *Pyrenophora semeniperda* as a Model to Study the Potential of Seed-Borne Pathogens for Control of Annual Grasses

1.3.1 The use of Bromus diandrus *as a tool for research of annual grass weeds.*

B. diandrus belongs to the family Poacae, sub-family Pooideae, tribe Poeae. It is an annual plant that can grow to 1 m tall. It is characterised amongst the genus *Bromus* by the presence of long awns (4 to 6 cm) and lemmas over 2 cm long (Wheeler, Jacobs & Norton, 1990). Weedy members of the genus include: *B. inermis*, *Bromus molliformis* F.E.Lloyd,

Bromus sterilis L. and *B. tectorum*. Other members of the genus are generally considered to be desirable pasture species.

Most of the work reported in this thesis concentrates on wheat and the weed species *B. diandrus*. *B. diandrus* was selected for the following reasons. *B. diandrus* is an annual grass weed with increasing importance to the winter cereal crop industry in Australia and may be as competitive as wild oats (Leys & Dellow, 1986). The increase in importance of *B. diandrus* can probably be attributed to the increase in non-till and minimum tillage cultivation systems which are used in most of the Australian cereal cropping regions. Furthermore, there are no currently recommended herbicides for the control of *B. diandrus* in winter cereal crops (Mullen & Dellow, 1993). The use of winter rotations with non-cereal crops such as field peas is often recommended for the control of *B. diandrus*. Herbicides which are effective against *B. diandrus* in grain legume crops include members of the herbicide group aryloxphenoxyproprionates (e.g. fluazifop-P, Fusilade®) (Mullen & Dellow, 1993). *B. diandrus* seed lacks dormancy and germination is synchronised (Poole & Gill, 1987). Thus from a practical point of view, *B. diandrus* is easier to work with than *A. fatua* especially in the field, because of its lack of dormancy and even germination. It has been suggested by workers both in Australia and overseas that control of brome grasses should be concentrated on reducing seed production (Peeper, 1984; Cheam, 1987; Cussans *et al.*, 1994). The purpose of the studies reported in this thesis was to explore the potential of using seed-borne pathogens for bioherbicides to prevent seed production and kill seeds. Since control of *B. diandrus* is aimed at reducing seed production, this species was considered to be useful for this research. Furthermore, preliminary observations provided convincing evidence of the ability of seed-borne pathogens to destroy seeds and deplete seed banks of *B. diandrus* by up to 90 % in trials at Wagga Wagga, NSW Australia (Dr R. W. Medd, personal communication).

1.3.2 Nomenclature and distribution of Pyrenophora semeniperda.

P. semeniperda (anamorph *D. campanulata*) was first described in Versaille, France in 1841 from seed of *B. inermis* (Léveillé, 1841 cited in Medd, 1992c). The anamorph was first described by Léveillé as *Angiopoma campanulatum*. According to the literature it has not been recorded in France since this original description. The anamorph has since been variously described under the following binomials: *Podosporiella verticillata* by O'Gara in

1915 from wheat kernels collected in Utah, USA; *Helminthosporium cyclops* by Drechsler in 1923 from material collected from leaf spot lesions found on *Danthonia spicata* (L.) Beauv. in Maine, USA; *Bipolaris cyclops* (Drechsler) Sprague in 1962; *Drechslera verticillata* (O'Gara) Shoemaker in (1966); and *D. campanulata* (Lév.) B.C. Sutton in 1976. A taxonomic description is given by Sivanesan (1987).

The teleomorph, which is rarely encountered (Medd, 1992) has only been described under three binomials: *Pyrenophora horrida* H. Sydow in 1924 from material collected in Stellenbosch, South Africa; *Pleosphaeria semeniperda* Brittlebank & Adam in 1924 from wheat and oats collected in Victoria, Australia; and *P. semeniperda* by Shoemaker in 1966.

P. semeniperda is widespread throughout the temperate and mediterranean-type climates of the world and has been reported in North America, New Zealand, South Africa and Australia (Medd, 1992c). In North America, *P. semeniperda* has been found in both the USA and Canada on a wide range of hosts including several *Bromus* species (Kreitlow & Bleak, 1964; Sivanesan, 1987). In South Africa, *P. semeniperda* has been isolated from leaf spots on oats and has been linked with a mycotoxicosis of grazing animals (van der Westhuizen, 1985; Scott, 1988). Medd & Jones (1992) found that *P. semeniperda* is widespread throughout the southern wheat growing areas of Australia on both crops and weed species although it was not found north of Lat. 32° S in eastern Australia. It has not been reported as a disease of any significance (O'Gara, 1915; Brittlebank & Adam, 1924; Wallace, 1959; Smith, 1966).

1.3.3 Host range and symptoms of infection

Medd (1992c) reviewed the world distribution and host range of *P. semeniperda*. *P. semeniperda* is unique amongst the graminicolous species of *Pyrenophora* because its host range also includes dicotyledonous genera. *P. semeniperda* has primarily been isolated from seeds and leaf lesions on over 70 species of grasses including all winter cereals (see Medd, 1992c; Medd & Jones, 1992). The fungus has also been isolated from 6 dicotyledonous genera including: *Daucus, Goodenia, Hedypnois, Hypochoeris, Pisum* and *Tragopogon.*

The most striking symptom of infection by *P. semeniperda* is the formation of stromata on infected seeds (Fig. 1.1). Stromata may arise from either the endosperm end or the embryo end of infected seeds. Generally, if stromata are formed in embryo tissue the seed will not germinate. Stromata on seeds may act as synnemata with conidia formed on the

apices of stromatal bristles (Fig. 1.2a). *P. semeniperda* can be easily identified in culture by the prolific formation of stromata (Fig 1.2b)

The fungus can also infect leaves and produce leaf spot lesions. The lesions appear as elliptical 'eyespots' due to the chlorotic halo surrounding necrotic tissue (Fig. 1.3). In severe cases, the spots may coalesce to form large areas of necrotic tissue (Fig. 1.3).

1.3.4 Objectives of this Study

The objectives of the studies reported in this thesis were to examine the potential of the seed-borne pathogen *P. semeniperda* as a bioherbicidal agent for use in winter cereal crops to control annual grass weeds. The specific objectives of the studies were:

1) To examine growth and sporulation of *P. semeniperda* on artificial media to determine the environmental conditions needed to produce large quantities of inoculum for field and glasshouse studies. An examination of morphological stability of isolates after storage was also made.

2) To examine and describe the infection process of *P. semeniperda* on seedling and adult plants of wheat and *B. diandrus* using light and electron microscopy.

3) To define the optimum environmental conditions required for infection of seedling and adult plants of wheat using both microscopic and macroscopic parameters.

4) To evaluate the potential of *P. semeniperda* to control *B. diandrus* by reducing seed production under field conditions using different types of inocula and timing of inoculation.

5) To investigate the possible production of metabolites by *P. semeniperda* and to examine the effects of any toxins on seedling and adult plants of wheat and *B. diandrus*.

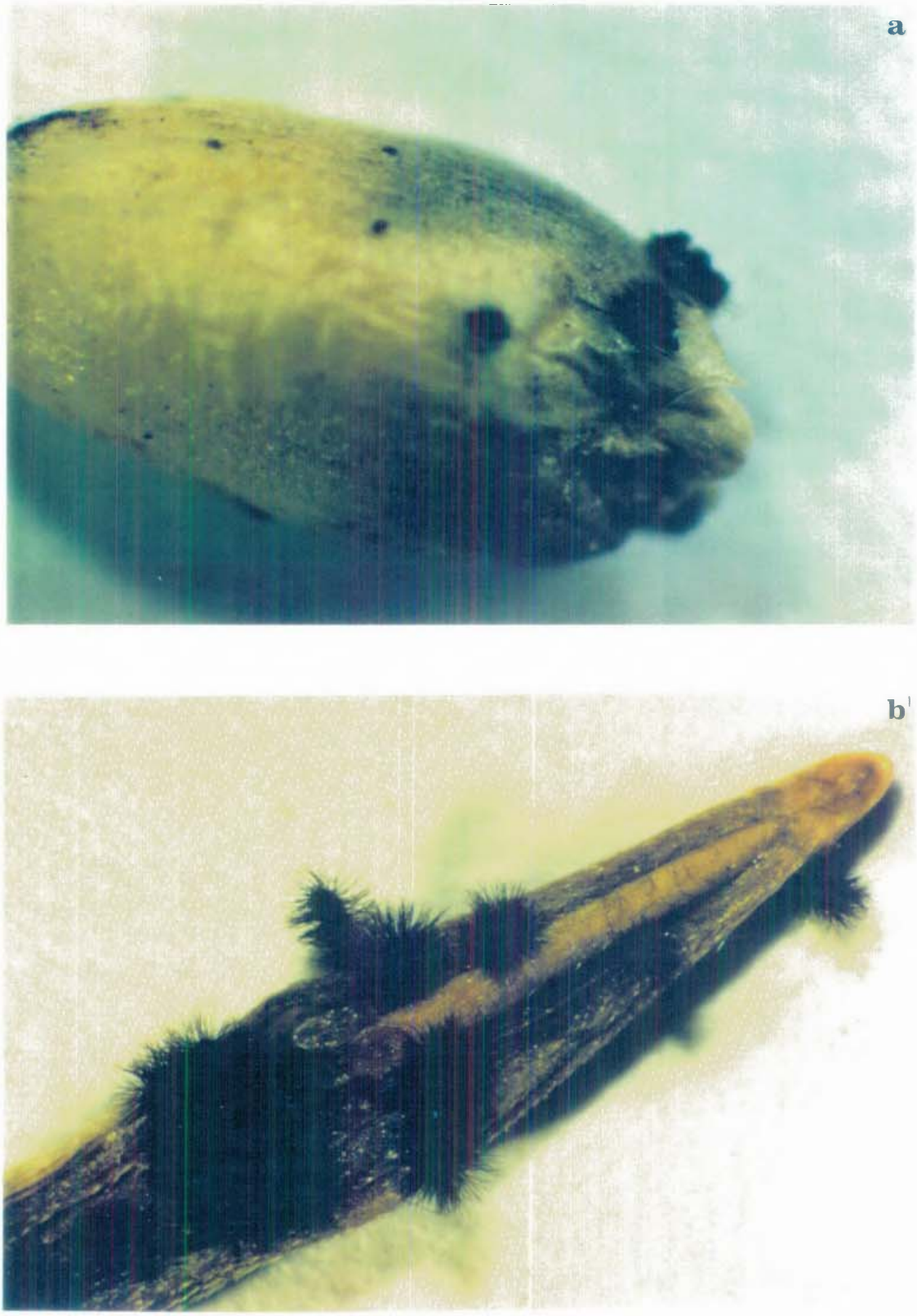

Fig. 1.1. Seeds of wheat (a) and *B. diandrus* (b) displaying characteristic stromatal development of natural infection by *P. semeniperda*. Approximately 50 X magnification.

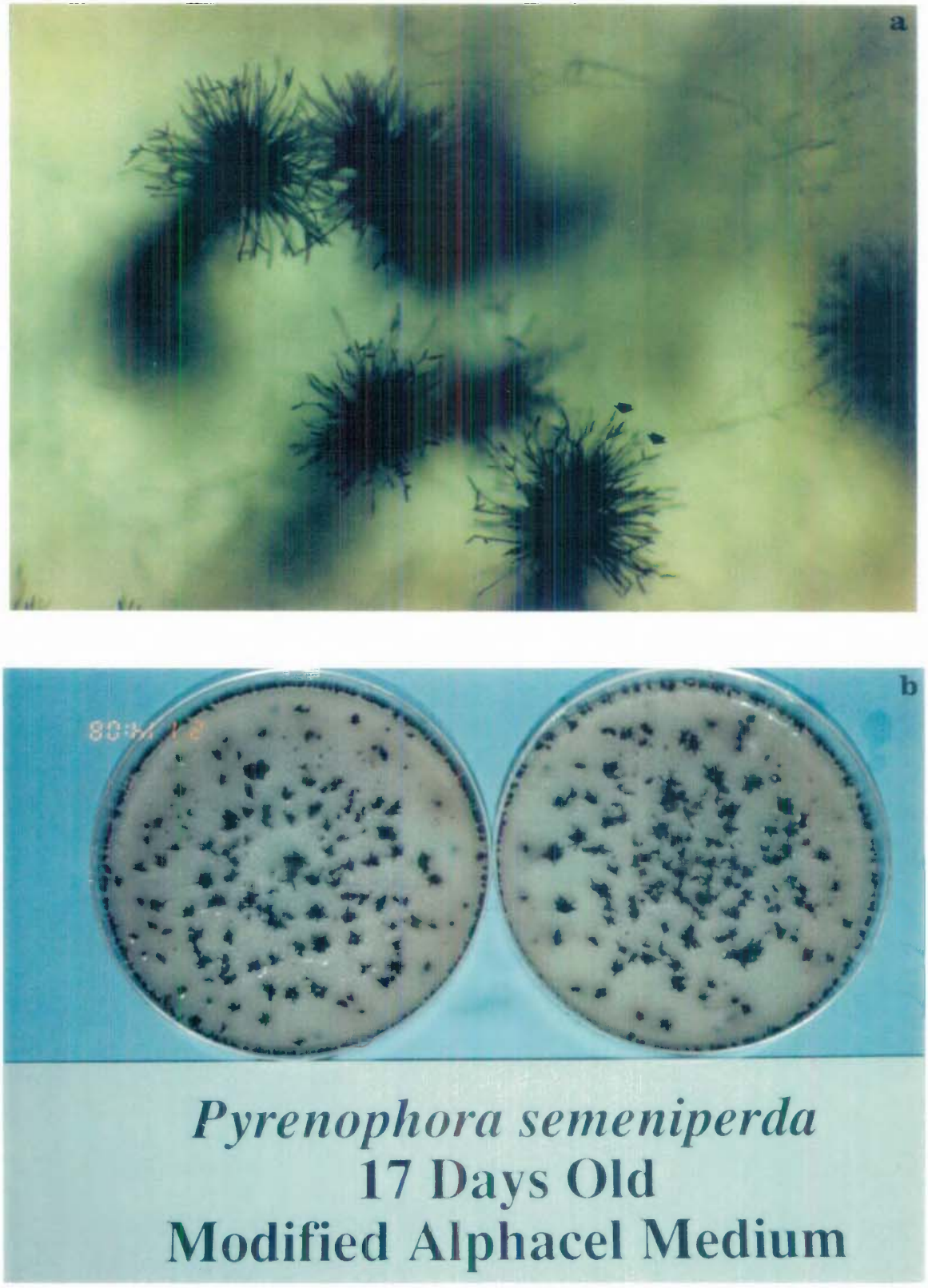

Fig. 1.2. a) Photomacrograph of conidia formation on apices of stromatal bristles of *P. semeniperda*. Magnification approx. 100 times.

b) Typical stromatal growth by *P. semeniperda* on culture medium containing oatmeal (X 0.7).

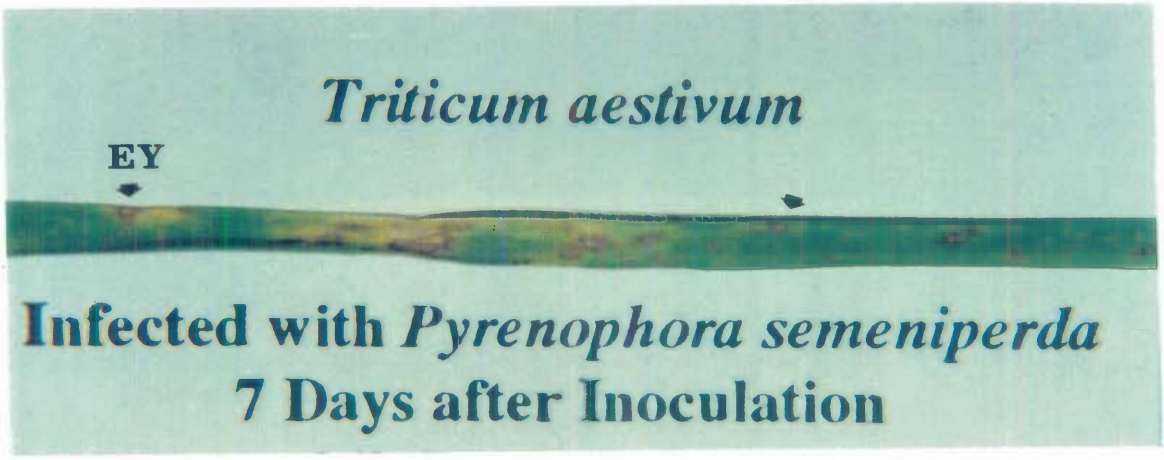

Fig. 1.3. Typical symptoms of leaf infection on wheat by *P. semeniperda*. Note the elliptical 'eyespots' (EY) and the large area of necrotic tissue.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Maintaining Cultures of Pathogens

2.1.1 Source, storage, maintenance and identity of isolates.

Isolates of *P. semeniperda* used in the studies reported in this thesis were obtained from the culture collection held at New South Wales Agriculture's, Agricultural Research & Veterinary Centre, Orange NSW, Australia. Isolates of *P. teres* were obtained from the Queensland Department of Primary Industry, Plant Pathology Culture collection held at Brisbane Qld, Australia (Table 2.1).

Table 2.1: Details of isolates of *P. semeniperda* and *P. teres* used in the studies reported in this thesis.

Species	Isolate no.	Host plant	Place of collection	Date
P. semeniperda	580681	*Avena ludoviciana*	Wagga Wagga, NSW	25/11/89
P. semeniperda	580129	*Vulpia bromoides*	Angaston, SA	8/11/84
P. semeniperda	580148	*Avena sativa*	Coonalpyn, SA	9/11/84
P. semeniperda	580170	*Horaeum vulgare*	Donald, SA	9/11/84
P. semeniperda	580520	*Chloris truncata*	York, WA	15/8/86
P. semeniperda	580534	*Triticum aestivum*	Corrigon, WA	15/8/86
P. semeniperda	580584	*Lolium perenne*	Cervantes, WA	22/8/86
P. teres	W.I.1535	*Horaeum vulgare*	Allora, Qld	12/4/87
P. teres	W.I.8712	*Horaeum vulgare*	Brisbane, Qld	9/6/77

P. semeniperda isolates were received in glass ampoules, and the *P. teres* isolates were received in small glass slants. On receipt of isolates, the ampoules were opened and the fungal material was placed on Petri plates containing potato dextrose agar (PDA). After 14 days incubation under laboratory conditions (15 to 25 °C), 20 three mm cores of fungus and PDA were cut from the growing edge of each different isolates' colony. The cores were

placed in ampoules and freeze-dried under partial vacuum. They were then sealed with a gas blow torch. A sample of each freeze-dried isolate was re-plated onto PDA to establish that the fungal material was still viable after undergoing the freeze-drying process. Isolates were freeze-dried to provide a backup of original isolate material. Ampoules were stored in the dark at room temperature.

Monoconidial isolates of each accession were maintained as stock cultures on modified alphacel medium (MAM, Appendix 1) at room temperature (15 to 25 °C) with a 12 hour photoperiod under near ultra-violet light (320-420 nm, 36.5 μmol m^{-2} s^{-1}) supplied by 2 x 40 W Osram cool white and 2 x 40 W Hitachi black light fluorescent tubes positioned 40 cm above the Petri plates. Light irradiance was measured with a Li-Cor LI189 Photometer. In an attempt to reduce genetic variability, stock cultures were periodically sub-cultured using a single conidium as inoculum (see Chapter 3). Wheat seedlings were regularly inoculated with conidia derived from stock cultures and *P. semeniperda* was re-isolated from leaf spot lesions in an endeavour to maintain pathogenicity of isolates. In the majority of studies isolate 580681 was used because a preliminary investigation showed it to be the most stable in terms of growth and sporulation after serial mass transfer. Other isolates were used for comparative studies when necessary.

The specific identities of all isolates used in the studies were ascertained by comparing conidial characteristics of isolates grown on MAM with those published by Sivanesan (1987). The characteristics of *Drechslera virreganensis* Wallwork, Lichon & Sivanesan which closely resembled *P. semeniperda* (Wallwork, Lichon & Sivanesan, 1992) were also compared with the isolates used in the present study. Isolates with characteristics which did not clearly match those of *P. semeniperda* were not used.

2.1.2 Preparation of media and cultures.

All culture media used were prepared immediately prior to use. Media used in all studies were autoclaved for 30 minutes at 103 kPa (121 °C). Unless otherwise stated, 15 ml portions of agar media were dispersed into 90 mm diameter plastic Petri plates using a repetitive syringe (Becton, Dickinson & Co., USA). Portions (125 ml) of liquid culture media were dispensed into 250 ml capacity borosilicate screw top bottles (Schott, Germany) using a Multiflex® digital peristaltic pump (Cole Parmer, USA) prior to autoclaving. Petri plates and liquid culture bottles were always inoculated with a 3 mm diameter piece of mycelium and agar excised from the growing edge of 7 day old monoconidial stock culture. Petri plates were sealed with Parafilm® (American Can Company, USA) to reduce water loss and to prevent contamination.

2.2 Maintenance of Host Material

2.2.1 Source of seed.

Seeds were obtained from the Department of Botany, Seed Collection, University of New England. Seeds of the spring wheat cultivar 'Cook' were used in all experiments unless otherwise stated. Seed of both wheat and *B. diandrus* was increased on plants grown in pots in the glasshouse (February to April 1992), and in field plots grown at the University of New England's farm "Laureldale" (see Chapter 7) in the winter season of 1992. The seed produced by plants grown in the field plots was used in all the experimental work requiring host material after 1992.

2.2.2 Plant Production.

All seeds were sown into a potting mix consisting of equal amounts (v/v) of sieved (4 mm aperture) river sand and vermiculite contained in one of three different pot types. For experiments requiring seedling leaf material, five seeds were sown into potting mix contained in 240 ml volume polystyrene cups (Castaway™, Australia) which had a 5 mm diameter drainage hole drilled out of the bottom. For experiments requiring older leaf material and plants in flower, three seeds were sown into potting mix contained in a 1000 ml volume plastic container (16.5 x 12 x 7 cm) which had a 5 mm diameter drainage hole drilled out of the bottom. Eleven g of slow release fertilizer (Nutricote®,Chisso Asahi Fertiliser Co., Japan) was added to the surface of each pot. The plants grown in 1000 ml capacity pots were thinned to one per pot at approximately 21 days after sowing. For field experiments, seeds were sown into potting mix contained in 64 cell (8 x 8, Kwik Pots®, Rite Gro, Australia) seedling trays. After sowing, pots were immediately watered to approximate field water holding capacity. Pots were watered every two days after seedling emergence. In addition, a liquid fertiliser was applied once a week (Aquasol™, Hortico Australia). All plants were grown in a glasshouse where temperatures ranged from 8 to 15 °C minimum and 15 to 25 °C maximum.

2.3 Inoculum Production

Isolates of *P. semeniperda* were grown on MAM and were initiated by inoculating Petri plates from monoconidial stock cultures. Petri plates were not sealed with Parafilm® (see Chapter

3). Petri plates were incubated under the same conditions as described in section 2.1.1. Seven days after inoculation, the colonies grown on Petri plates were wounded either by cutting through the mycelium and agar with a sterile stainless steel 3 mm diameter cork borer or by slashing through the mycelium and agar with a sterile scalpel (wound size ranged from 3 to 7 mm between slashes). This procedure was repeated until the whole colony had been wounded. The wounded colonies in Petri plates were then replaced under the same incubation conditions for a further 7 days.

After the full 14 day incubation period, conidial suspensions were prepared using one of the following methods. Conidial suspensions were always prepared immediately before use unless otherwise stated and kept refrigerated to prevent conidial germination. The first method was developed because it allowed for the maximal removal of conidia from the conidiophores when large volumes of inoculum was required for field studies. The second method was used to produce smaller volumes of conidial suspension for glasshouse based studies.

Method One: Five wounded cultures and their agar medium were removed from the Petri plates and placed into a 500 ml capacity screw top bottle containing 250 ml of refrigerated sterile distilled water. The bottle containing the cultures was shaken vigorously for two to three minutes by hand to remove conidia from their conidiophores. The resultant suspension was then filtered through a series of stainless steel laboratory sieves to a final size of less than 63 µm to remove agar and most mycelial fragments. The number of conidia in the suspension was determined by counting the conidia in 50 cell units (100 x magnification light microscopy) within a Sedgewick Rafter counting cell (Graticules Limited, England) and multiplying the result by 20 to give a final concentration in conidia ml^{-1}. Dilutions of suspensions were made when high densities of conidia reduced the accuracy of the technique.

Method Two: Twenty to 30 ml of refrigerated sterile distilled water was added to each wounded culture contained in Petri plates. The whole wounded colony was scraped gently with the blunt side of a scalpel blade and the resultant suspension was filtered through a series of stainless steel laboratory sieves to a final size of less than 63 µm to remove agar and mycelial fragments. The number of conidia ml^{-1} in the suspension was determined using a Sedgewick Rafter counting cell and the wetting agent Pulse® (Monsanto, USA) was added to the suspension (0.01% by volume).

2.4 Inoculation and Incubation Techniques

Plants were removed from the glasshouse just prior to inoculation. Inoculum was atomised onto host plants by a hand operated applicator (Canyon®) until run-off. Wheat and *B. diandrus* flowers were inoculated by dipping the spikelets into a conidial suspension. After inoculation, plants were immediately placed into a chamber housed within a controlled environment growth cabinet and fitted with an ultrasonic humidifier to provide a dew period. In some experiments, an environment of high humidity was created for inoculated wheat and *B. diandrus* spikelets by placing a plastic bag over the flowering head and fastening it with a paper clip. In some experiments, adult leaves were inoculated by placing a 100 µl drop of conidial suspension on a leaf held between two cover slips (22 x 22 mm) and secured with a paper clip.

All field inoculations were carried out at night after 2100 hours. The reasons for this were three-fold: firstly, the temperatures were cooler and the chances of the inoculum becoming desiccated were slight; secondly, the chances of 'spray drift' were lower since the wind was usually negligible after sunset; and thirdly, on most inoculation nights dew formation had already occurred, thus ensuring a post-inoculation dew period. Field plots were sprayed using a pressure sprayer (Solo, 10L) or a hand-held CO_2 propelled spray gun (Badger Air Brush Co., Illinois, USA).

2.5 Disease Assessment

2.5.1 Lesion development.

The level of disease that developed on leaves inoculated with a conidial suspension was assessed visually in two ways. Firstly. the number of lesions that had developed was counted on each leaf assessed, and secondly, the necrotic area of each lesion was given a size rating of between 1 and 4 based on the scale shown below:

1. Less than 1 mm in diameter
2. Between 1 and 5 mm in diameter
3. Between 5 and 10 mm in diameter
4. Greater than 10 mm in diameter

The level of damage that developed in leaves following infiltration with toxic filtrates was assessed visually and allocated a number between 1 and 5 based on the damage rating scale shown below:

1. No symptoms
2. Faint chlorosis
3. Chlorosis and slight necrosis
4. Necrosis and extensive chlorosis
5. Extensive necrosis only

The latent period for damage expression was also noted, although all damage assessments took place at 7 days after inoculation.

2.5.2 Seed infection.

A standard technique for assessing seed infection was as follows: Seven hundred and fifty ml of sterilised vermiculite was placed into a 1000 ml volume plastic food container (dimensions 17.5 x 12 x 7 cm). A plastic template with 20 holes spaced evenly throughout was placed onto the surface of the vermiculite and the seeds to be assessed were sown one per hole in each of the template holes. A further 250 ml of vermiculite was placed over the top of the seeds so that the seeds were sown at a depth of between 2 to 4 cm. The seeds were watered after sowing with 125 ml of sterile distilled water. A 500 ml volume plastic food container (dimensions 17.5 x 12 x 3.5 cm) was inverted over the top of the 1000 ml container and the two containers sealed together using 3 cm width plastic wrap (Glad Wrap®). The assay chamber was then placed in a dark incubator at a temperature of 15 °C for a 7 day incubation period.

After incubation, all the assay chambers were placed in a cool room (4 °C) to stop plant growth prior to assessment. Each assay chamber was then assessed for: seedling emergence, length of the coleoptile for each germinated seed, germinated seed, total seed germination, and the number of seeds with vegetative stromata produced by *P. semeniperda*. The position of the stromata on the seed was also recorded. A modification of this technique which included measuring the length of the longest radicle for each seedling was also used to assay the effects of culture filtrates of *P. semeniperda* on plant development. This technique will be called the Coleoptile/ Radicle length (CR) assay in other chapters of this thesis.

2.6 Statistical Analyses

The computer program Systat (Wilkinson *et al.*, 1992) was used to analyse all data reported in this thesis unless otherwise stated. All experiments were performed at least twice at different times unless otherwise stated. Results from duplicate experiments were pooled when homogeneity of variances was detected using Bartlett's test (Snedecor & Cochran, 1989). Data sets consisting of percentage values were arcsine transformed prior to analysis of variance (Angle= Arc Sin (Proportion)$^{1/2}$, Snedecor & Cochran, 1989). Other data sets were transformed to ensure normality of distribution and homogeneity of variance accordingly. Analysis of variance was performed on all data to test for treatment differences. Pairwise comparisons of treatment means were established with Tukey's HSD test (α=0.05 or 0.01) (Day & Quinn, 1989). Experiments which required measurements of the same parameter over time were analysed using univariate repeated measures analysis of variance, and treatment means separated by repeated measures contrasts. Linear or curvilinear functions were fitted to individual data points (not means), by generating linear or polynomial regression equations. Pairwise comparison of slopes of linear functions was made using t-tests between the various treatments. The error figures reported are: i) standard error of the mean and ii) standard error of co-efficient for regression equations. Experiments were either completely randomized or set up in completely randomized block designs unless otherwise stated.