CHAPTER FIVE
THE PRODUCTION OF TOXIC METABOLITES BY *PYRENOPHORA SEMENIPERDA IN VITRO*, AND POSSIBLE ROLES IN PATHOGENESIS

5.1 Introduction

Fungal pathogens of plants often produce disease symptoms by elaborating one or more phytotoxic compounds (Sugawara, *et al.*, 1987). Virtually all fungi are presumed to produce toxic compounds or plant growth regulators during pathogenesis. These are considered to contribute to disease development (Templeton, *et al.* 1986).

Yoder (1980) classified toxins produced by plant pathogens as "pathogenicity factors" and "virulence factors". A pathogenicity factor is required for disease to occur, whereas a virulence factor is not required for disease initiation, but its presence may determine the extent of disease expression (Yoder, 1980; 981; Hasan & Ayres, 1990). For example, isolates of *Cochliobolous carbonum* Nelson Race 1 collected from the field may or may not be pathogenic to *Zea mays* L. depending on whether or not they produce toxins (Pringle & Scheffer, 1964; Yoder, 1980). Pathogenicity results when a toxin is produced by *C. carbonum* and is therefore classified as a factor required for pathogenicity.

Toxins may also be classified as host-specific (selective) or non-specific (Yoder, 1980; Hasan & Ayres, 1990; Walton & Panaccione, 1993). Host-specific toxins have a high biological activity towards only the hosts of the producing pathogen, whereas non-specific toxins may affect plants in general. Hasan & Ayres (1990) suggested that the distinction between the two types of toxin specficity was clouded, because with some toxin systems, sufficiently high concentrations of host-specific toxin will affect plants that do not normally react to the pathogen. For example, the host-selective toxin, bipolaroxin which was produced by the *Cynodon dactylon* (L.) Pers.(couch or bermuda grass) pathogen *Bipolaris cynodontis* (Marignoni) Shoem. was host selective at a concentration of 0.038mM, but at 3.8mM the toxin could affect several species including: *Saccharum officinarum* L.(sugarcane), *Z. mays* and *A. fatua* (Suguwara *et al.*, 1985). In contrast, culture filtrates of *P. tritici-repentis* showed

remarkable specificity when applied to disease resistant and susceptible cultivars of wheat (Tomás & Bockus, 1987; Lamari & Bernier, 1989). Filtrates derived from isolates that produced disease symptoms on susceptible plants, produced similar symptoms when culture filtrates were applied to susceptible cultivars, but not to cultivars normally resistant to the pathogen. Lamari, Ballance & Bernier (1993) have since reported host-selectivity of purified and characterised *P. tritici-repentis* toxin. Furthermore, Sugawara *et al.* (1987) reported the production of a host-selective toxin by *B. maydis* which only affected corn cultivars bearing Texas-male-sterile cytoplasm. They concluded that it was plausible that the toxin had a role in the 1970 southern corn leaf-blight epidemic in the USA. Many host non-specific toxins are produced by fungi belonging to the genus *Alternaria* (Templeton, 1972). For example, several *Alternaria* species produce the toxin zinniol (Barash *et al.*, 1981; Cotty, Misaghi & Hine, 1983; Cotty & Misaghi, 1984). More recently, Maeiro, Bean & Ng (1991) have reported that *A. solani* produced several toxins in culture which may have included zinniol and alternaric acid. The non-specific tentoxin, a cyclic tetrapeptide is also produced by several species of *Alternaria* (Duke, 1992).

Fischer & Bellus (1983) listed 3 ways by which phytotoxicity can be caused in a host-parasite situation: 1) plants can be deprived of photosynthetic assimilates and hormones, resulting in slow metabolic breakdown of plant parts; 2) the pathogen can elicit a biochemical reaction in the plant, resulting in the production of phytoalexins which may be responsible for local toxicity; or 3) microorganisms often produce phytotoxins to break down plant defences and to prepare favourable conditions for interactions with the host plant. Symptoms of the production of phytotoxins include: chlorosis, necrosis, wilting, growth inhibition and hormonal disorders (Wheeler, 1981; Fischer & Bellus, 1983; Hasan & Ayres, 1990). Certain toxins may be transported for long distances in the xylem stream of the plant and can be particularly damaging (Hasan & Ayres, 1990). Toxins may cause cellular collapse, cellular leakiness, accumulation of secondary metabolites or ultrastructural alterations in organelles (Walton & Panaccione, 1993). For example, the toxin produced by *P. tritici-repentis* caused symptoms of either necrosis or extensive chlorosis (Lamari & Bernier, 1989). Smedegård-Peterson, (1977) reported that barley leaves which were affected by toxins isolated from *P. teres* showed increased respiratory rates compared to unaffected leaves. Pappu & Deshpande (1983) described induced leakage of electrolytes and carbohydrates from wheat seed affected by toxic culture filtrates of *Drechslera avenacea* (M.A. Curtis ex Cooke) Shoem. Walton &

Panaccione (1993) concluded that regardless of the action of toxins, their simple reason for being, is that they kill or metabolically compromise sensitive cells and turn the plant tissue into a nutrient medium.

Several bioassays for testing the effects and modes-of action of fungal phytotoxins have been reported (Yoder, 1981). These assays include inhibition of seedling root growth, lesion production on leaves, induction of electrolyte leakage (Yoder, *et al.* 1977) and sensitivity of plant protoplasts to phytotoxic compounds (Breiman & Galun, 1981; Strange, Pippard & Strobel, 1982). However, as stated by Walton & Panaccione (1993), 'an inappropriate bioassay will not manifest a toxin even if it is there.' Initial studies into the roles of toxins in pathogenesis have generally used either crude or partially-purified culture filtrates (e.g. Smedegård-Peterson, 1976; 1977; Graniti & Porta-Puglia, 1984; Deadman & Cooke, 1986; Tomás & Bockus, 1987; Hammouda, 1988; Pena-Rodriguez, Armingeon & Chilton, 1988; Lamari & Bernier, 1989). Although, the use of culture filtrates do not elucidate the chemical structure of a toxic compound or give quantitative data on toxin production, assays using filtrates are warranted before more complex and time consuming studies to purify and characterise the putative toxin/s can be initiated (Smedegård-Peterson, 1976). However, bioassays using crude filtrates (particularly those that have not been de-salted) may give misleading results if staling compounds are present (Smedegård-Peterson, 1976; Scheffer & Briggs, 1981).

Many *Pyrenophora* and related species have been reported in the literature to produce toxins as an integral part of their pathogenesis. Toxins have been purified and characterised for *P. tritici-repentis, P. teres* and *P. avenae* (Ballance, Lamari & Bernier, 1989; Tomás *et al.*, 1990, Smedegård-Peterson, 1976; Bach *et al.*, 1979; Friis, Olsen & Møller, 1991; Sugawara & Strobel, 1986). Furthermore, Lamari, Ballance & Bernier (1990) have also isolated a toxin from intercellular washing fluids recovered from seedlings inoculated with conidia of *P. tritici-repentis*, providing further evidence of its role in pathogenesis. Evidence for the existence of toxins in the host-parasite interactions of *P. graminea* (Hammouda, 1988), and *P. semeniperda* (Wallace, 1959) have also been reported.

As previously indicated in this thesis there is a growing interest in the biological control of weeds. One environmentally sensitive approach may be to use phytotoxic compounds produced by plant pathogens for direct application to weeds species. Alternatively, a study of the chemistry of these phytoxins may provide information for the

synthesis of novel herbicides (Robeson, *et al.*, 1984; Templeton *et al.*, 1986). The discovery of unique fungal toxins may also serve to locate vulnerable biochemical targets in the plant because pathogens have had millennia to co-evolve with their hosts and devise biochemical strategies to either kill or influence their host's physiology (Fischer & Bellus, 1983; Strobel, Kenfield & Bunkers, 1991). The discovery and use of toxic compounds as naturally derived herbicides may be attractive to agrochemical companies who can satisfy environmental requirements of non-harmful chemicals whilst satisfying their own fiscal policies. This has been the case particularly since mid-1980's when the United States Environmental Protection Agency relaxed requirements for pesticide registration (Freeman & Charudattan, 1985). In addition, given the concerns held by national quarantine authorities regarding the importation of organisms for both classical and inundative biological control, restrictions would not apply to the importation of a selective phytotoxin as a natural herbicide (Wapshere, 1990). This makes the exploration for phytotoxins as biorational herbicides a particularly engaging prospect. TeBeest (1993) has also suggested that the host specificity of mycoherbicides may be expanded by genetically transforming fungal pathogens of weeds with genes encoding toxins, such as phaseolotoxin and tabtoxin. This approach might overcome some of the constraints chemical companies have with regards to pouring research monies into the development of bioherbicides.

Several phytotoxins which are produced by fungi that attack weeds have been reported (Strobel *et al.*, 1991). For example, a toxin isolated from a *Bipolaris* pathogen of *S. halepense* (Johnson grass) (Pena-Rodriguez, 1988), bipolaroxin from *B. cynodontis* infecting *C. dactylon* (Sugawara *et al.*, 1985), and (-)-dihydropyrenophorin from *D. avenae* toxic to *S. halepense* (Sugawara & Strobel, 1986). An interesting use of a phytotoxin was reported by Jones, Lanini & Hancock (1988) who used a toxin derived from a non-pathogenic fungus, *Gliocladium virens* Miller, Giddens & Foster to control several important weed species. The toxin, viridiol, was produced when the fungus was grown on a medium of peat moss amended with nutrients. The medium was applied directly to the soil and emergence of most weeds was reduced by > 90 %. The use of a non-pathogenic fungus in this manner was advantageous because their was little likelihood of it becoming pathogenic to some untested host (Jones *et al.*, 1988). The only known commercial successes of herbicides originating from microbial products have been derived from non-pathogens (Duke, 1992). Two of these

are bialophos and glufosinate. Glufosinate was derived from the actinomycete *Streptomyces* sp. (Fischer & Bellus, 1983).

Wallace (1951; 1959) reported that the adverse effects of *P. semeniperda* on seed germination could have been caused by a toxin. He tested this hypothesis by placing seeds on filter paper which was 'watered' with crude culture filtrates. He found that shoot length was significantly reduced by the presence of the culture filtrates. However, when he 'watered' seeds in soil with filtrates, no effect on germination or seedling growth was observed. Evidence for the involvement of toxin/s in the disease of wheat and *B. diandrus* caused by *P. semeniperda* was observed in studies reported in Chapter 6 of this thesis. Firstly, infection of wheat with conidial inoculum resulted in the formation of lesions with extensive necrosis and chlorosis and secondly, microscopic studies showed that infection hyphae of *P. semeniperda* grew without penetrating cells and cell death occurred in advance of the hyphae. The studies reported in this chapter were initiated to assess the production of toxic metabolites by *P. semeniperda* and to investigate the role these metabolites played in the symptomatology of the disease. An examination of the sensitivity of wheat seedlings to toxic filtrates was correlated with susceptibility of wheat seedlings to inoculation with conidia. Isolates of the toxin producing pathogen, *P. teres*, were also used in some comparative studies.

5.2 Materials and Methods

The following method for culture filtrate preparation was used unless otherwise stated. All isolates of *P. semeniperda* and *P. teres* were cultured in the liquid medium of Tomás & Bockus (1987) (appendix 1) using the methods described in section 2.1.2. Inoculated culture bottles were incubated under laboratory lighting conditions with a temperature range of 17 to 25 °C and shaken on an orbital shaker set at 130 rpm. After 14 days incubation, mycelium was removed by vacuum filtration through Whatman No. 1 filter papers and concentrated under partial vacuum at 75 °C (Büch Re-121 Rotavapor) to one-fifth of the original volume and adjusted to pH 6.5 with 10N KOH. After refrigeration overnight (4 °C), salts and other precipitates were removed by centrifugation. The culture filtrates were then passed through a sterile 0.45 µm nitro-cellulose filter membrane (Sartorius, England) using a vacuum filtration system (Nalge Co.,USA). For experiments using bioassay chambers, concentrated culture

filtrates were diluted to their original volume with sterile distilled water. Filtrates were stored under refrigeration until use. Generally, filtrates were used within 3 days of preparation.

One of the following three methods was used to assess the effects of culture filtrates on plant development:

1) The coleoptile/radicle (CR) length bioassay described in section 2.5 was used except that seeds were 'watered' with the culture filtrates prepared in the manner described above.

2) Preliminary studies of the effects of concentrated culture filtrates on seedling leaves of wheat and *B. diandrus* were undertaken using the leaf infiltration device and method described by Hagborg (1970). However, it was found that infiltration of leaf material using the device was somewhat inconsistent, presumably because of leakage and irregularities in uptake of solutions. A method was therefore devised which overcame these problems while still allowing for a quick turnover of leaf samples. The wetting agent Pulse® was added to the concentrated culture filtrates (0.01 % by volume). Seedling leaves of intact plants were inoculated with a 10 µl drop of culture filtrate dispensed with a micro-pipette (Eppendorf, Germany). The plant was then placed in a bell jar and a vacuum was applied until the drop of filtrate had infiltrated the leaf. This process usually took about 30 sec. per leaf.

3) For experiments requiring leaves of older plants, a leaf puncture assay was used. The location on the leaf to be inoculated was punctured once with a fine hypodermic syringe. A 10 µl drop of culture filtrate was then placed on the puncture wound.

In all assays, controls consisted of sterile water and sterile concentrated, uninoculated culture media.

The level of damage that developed on leaves following infiltration of toxic filtrates was assessed visually as described in section 2.5.1.

In those experiments where two species were tested using the CR bioassay, interaction of species was not analysed because heterogeneity of variances ($P<0.01$) was detected between coleoptile and radicle lengths of wheat and *B. diandrus*.

5.2.1 Initial investigations.

To ascertain whether toxic compounds may play a role in the disease caused by *P. semeniperda* initial studies were undertaken using the CR bioassay. For these initial studies,

culture filtrates used were not concentrated under partial vacuum or de-salted. Both wheat and *B. diandrus* seeds were 'watered 'with crude filtrates derived from cultures of isolate 580681 grown on an orbital shaker or grown in a stationary position. In a separate study, the toxicity of filtrates after heat treatment was tested by comparing culture filtrates that were autoclaved (121°C, 103 kPa) for 15 min and those that were not. Five replicates were used per treatment and the experiments were performed twice. Contrasts were used to analyse relationships among the treatment means for these experiments.

5.2.2 Toxicity of culture filtrates derived from different isolates of P. semeniperda.

Seven isolates of *P. semeniperda* were examined using the CR bioassay to determine whether different levels of filtrate toxicity were produced by different isolates. The 7 isolates tested were: 580681; 580534; 58020; 580170; 580584; 580129 and 580148. Four replicates were used for each isolate and the experiment was performed twice.

5.2.3 Correlation between disease severity in the glasshouse and reaction to toxic filtrates.

The reactions of wheat seedlings to infiltration with toxic filtrates was compared to the disease severity of wheat seedlings inoculated with the conidia of five different isolates. The isolates used in this study were 580681, 580534, 580170, 580520 and 580148. The reaction of wheat (cv. Cook) seedlings to infiltration with toxic filtrates was evaluated using the vacuum assay method described above. Seedling leaves were inoculated using the cover-slip method with 500 conidia ml^{-1}. A low concentration of conidia was used so that disease reactions could be assessed without the confounding effects of lesion coalescence. The level of disease that developed on the wheat seedlings that were inoculated with conidial inoculum was assessed the same way as the toxic reactions. Both filtrate application and conidia inoculation were performed on the same day and plants were returned to the glasshouse and arranged in a randomized block design Five replicates were used, with each replicate consisting of five seedling leaves per pot. Correlations between, reaction to toxic filtrates in seedling wheat leaves, disease severity after inoculation with conidia and the results of the CR bioassay from section 5.2.2 were analysed using Pearson's correlation with Bonferroni-adjusted probabilities (Wilkinson, *et al.,* 1992).

5.2.4 Toxin production in relation to culture age.

To determine when toxin production was maximal in relation to the age of the culture filtrate, filtrates from isolate 580681 were harvested after different periods of incubation. The first harvest occurred after six days and thence every three for a total of 30 days. At each harvest, the pH of the filtrate was evaluated and the dry weight of the mycelium produced was ascertained. The CR bioassay with wheat and *B. diandrus* seeds was set up using sterile, concentrated and re-diluted filtrates. The experiment consisted of four replicates and was repeated once. The correlations between filtrate pH, mycelia dry weight and coleoptile length were analysed using the methods described above.

5.2.5 Relationship between filtrate toxicity and pH.

The results from the study in 5.2.4 suggested that the pH of culture filtrates decreased markedly over time. It is possible that the toxic factor may be pH related. Therefore, to determine whether the toxicity in filtrates was related to pH, the pH of inoculated and uninoculated filtrates were adjusted correspondingly. Cultures of isolate 580170 were grown for 12 days and harvested. The pH of both inoculated and uninoculated bulked filtrates (after concentration and dilution) were measured and gave readings of pH 5.0 for inoculated filtrates and pH 6.5 for uninoculated filtrates Treatments were prepared by removing half of each filtrate type and adjusting the pH using 10N KOH and 2N HCl so that the following treatments were tested using the CR bioassay with wheat: i) inoculated filtrate pH 5.0; ii) inoculated filtrate pH 6.5; iii) uninoculated filtrate pH 5.0; iv) uninoculated filtrate pH 6.5 and v) sterile distilled water. Treatments had five replicates and the experiment was not repeated.

5.2.6 Effect of filtrate concentration on toxic reaction type.

Five dilutions of concentrated culture filtrates were tested for their effect on reaction type in infiltrated seedling leaves of wheat and *B. diandrus*. Sterile culture filtrates from isolate 580170 that were concentrated to 1/5 of their original volume were denoted as 1X stock filtrates. From these stock filtrates, dilutions were made resulting in, 1/5X, 1/10X, 1/100X, and 1/1000X the concentration of the 1X stock filtrates. The same dilutions were made for uninoculated culture filtrates. Five replicates (each consisting of five seedling plants) were used for each treatment and the experiment was repeated once. Filtrates were applied using the vacuum assay method.

5.2.7 Effect of filtrates at different plant growth stages.

The effect of culture filtrates on plants at different developmental stages was assessed using the leaf puncture assay. Concentrated culture filtrates from isolate 580170 were applied to wheat plants at four different growth stages according to the decimal code for cereal growth stages (Zadoks, Chang & Konzak, 1974): seedling (GS 13); 1st node detectable (GS 31); first awns visible (GS 49) and beginning of anthesis (GS 60). The three top leaves at each growth stage were inoculated at each of three leaf positions: base, middle and tip. For each treatment there were 10 replicate plants. The treatments were all sown simultaneously. Filtrate application and disease assessments occurred over several months. The experiment was repeated once with five replicates in the second experiment.

5.2.8 Host specificity of culture filtrates.

The host specificity of culture filtrates was evaluated by applying filtrates of isolate 580170 to the leaves of several plant species using the vacuum infiltration assay. Plants of the following species were tested: wheat, *B. diandrus*, *Lablab purpureus* (L.) Sweet (Lablab), *Gossypium hirsutum* L.(cotton), *Helianthus annuus* L.(sunflower), *X. occidentale* (Noogoora burr). Prior to sowing, *X. occidentale* fruits were cut at the distal end to speed up seed germination. Seeds of the species tested were sown into vermiculite contained in seedling trays (36 x 30 x 3 cm). After 7 days growth, seedlings were transplanted into polystyrene cups as described in section 2.2.2, except that only one seedling was planted per cup. After a further 7 days, the first true leaves of all species were inoculated. Ten replicates were used for each treatment and the experiment was repeated once.

5.2.9 A comparison of the toxicity of filtrates produced by P. semeniperda and P. teres.

A comparison of the effects of culture filtrates from both *P. semeniperda* and *P. teres* was made using the CR bioassay technique. This was done because *P. teres* is not regarded to be a pathogen of wheat. However, it is considered to be a pathogen of *Bromus* spp. (Sivanesan, 1987). This study was made to determine the relative toxicities on wheat and *B. diandrus* of culture filtrates from *P. teres* and from *P. semeniperda*.

Cultures of isolates 580681 and 580170 of *P. semeniperda*, and isolates WI1535 and WI8712 of *P. teres* were established as described in section 5.2. Five replicates of each treatment were used and the experiment was repeated once.

5.3 Results

In all experiments seed germination was not affected by inoculated filtrates compared to controls.

5.3.1 Initial investigations.

Culture filtrates from *P. semeniperda* (isolate 580681) had a marked negative effect on coleoptile and radicle length of wheat and *B. diandrus* (Table 5.1). Inoculated filtrates that were produced while being incubated on a rotary shaker were significantly more toxic to coleoptiles and radicles of wheat and coleoptiles of *B. diandrus* than inoculated filtrates produced under static conditions ($P<0.05$). Filtrates significantly reduced radicle lengths of both wheat and *B. diandrus* compared with the application of water ($P<0.05$). Inoculated filtrates were consistently more toxic to wheat than *B. diandrus*.

Table 5.1. The effect of filtrates derived from *P. semeniperda* (isolate 580681) produced either while being incubated on a rotary shaker or unshaken, on coleoptile and radicle lengths of wheat and *B. diandrus* after 7 days growth in a bioassay chamber. Data are means of duplicate experiments.

	Wheat		***B. diandrus***	
Treatment*	**Coleoptile length (mm)**	**Radicle length (mm)**	**Coleoptile length (mm)**	**Radicle length (mm)**
Shaken I	23 ±1	35 ±3	71 ±0.9	89 ±1.5
Unshaken I	100 ±1.2	122 ±2.3	84 ±0.8	92 ±1.1
Shaken U	103 ±1.8	118 ±2.9	91 ±1	91 ±1.4
Unshaken U	94 ±1.6	98 ±3	86 ±0.9	88 ±1.2
Water	103 ±1.4	150 ±2.1	87 ±0.9	101 ±1.1

*Treatments were either inoculated filtrates (I) or uninoculated filtrates (U).

Filtrates retarded coleoptile and radicle growth of both wheat and *B. diandrus* regardless of whether or not they were autoclaved (Table 5.2). Uninoculated filtrates significantly reduced the length of wheat coleoptiles and radicles and *B. diandrus* radicles compared with the application of water ($P<0.05$).

5.3.2 Toxicity of culture filtrates derived from different isolates of P. semeniperda.

Filtrates derived from isolate 580170 reduced coleoptile and radicle lengths of both wheat and *B. diandrus* more than any other isolate tested (Fig. 5.1). Filtrates from 3 isolates (580170, 580681, 580148) reduced coleoptile lengths of wheat seedlings by more than 50 %

Table 5.2. The effect of autoclaved and unautoclaved filtrates from *P. semeniperda* (isolate 580681) on coleoptile and radicle lengths of wheat and *B. diandrus* after 7 days growth in a bioassay chamber. Data are means of duplicate experiments

	Wheat		***B. diandrus***	
Treatment*	**Coleoptile length (mm)**	**Radicle length (mm)**	**Coleoptile length (mm)**	**Radicle length (mm)**
autoclaved I	23 ±1	29 ±2	39 ±1.7	39 ±1.9
unautoclaved I	21 ±1.3	27 ±1.6	51 ±1.5	39 ±2.1
autoclaved U	63 ±2.4	54 ±2.4	81 ±2.6	50 ±2
unautoclaved U	65 ±2	47 ±2.9	83 ±2.9	43 ±2.5
Water	78 ±2.5	102 ±2.3	92 ±1.3	86 ±1.7

*Treatments are either inoculated filtrates (I) or uninoculated filtrates (U).

compared to the controls and were not significantly different from each other (P>0.05). Only filtrates from isolates 580170 and 580681 reduced coleoptile lengths of *B. diandrus* by more than 40 %. However, filtrates from isolate 580170 reduced coleoptile and radicle lengths of wheat and *B. diandrus* by more than 50 %. Filtrates from isolates 580584 and 580520 did not reduce coleoptile lengths of wheat relative to the controls (P>0.05). In addition, filtrates from isolate 580520 did not reduce coleoptile lengths of *B. diandrus* relative to controls (P>0.05). Application of uninoculated filtrates did not significantly reduce coleoptile lengths of wheat or *B. diandrus* (P>0.05) compared to water (Table 5.3).

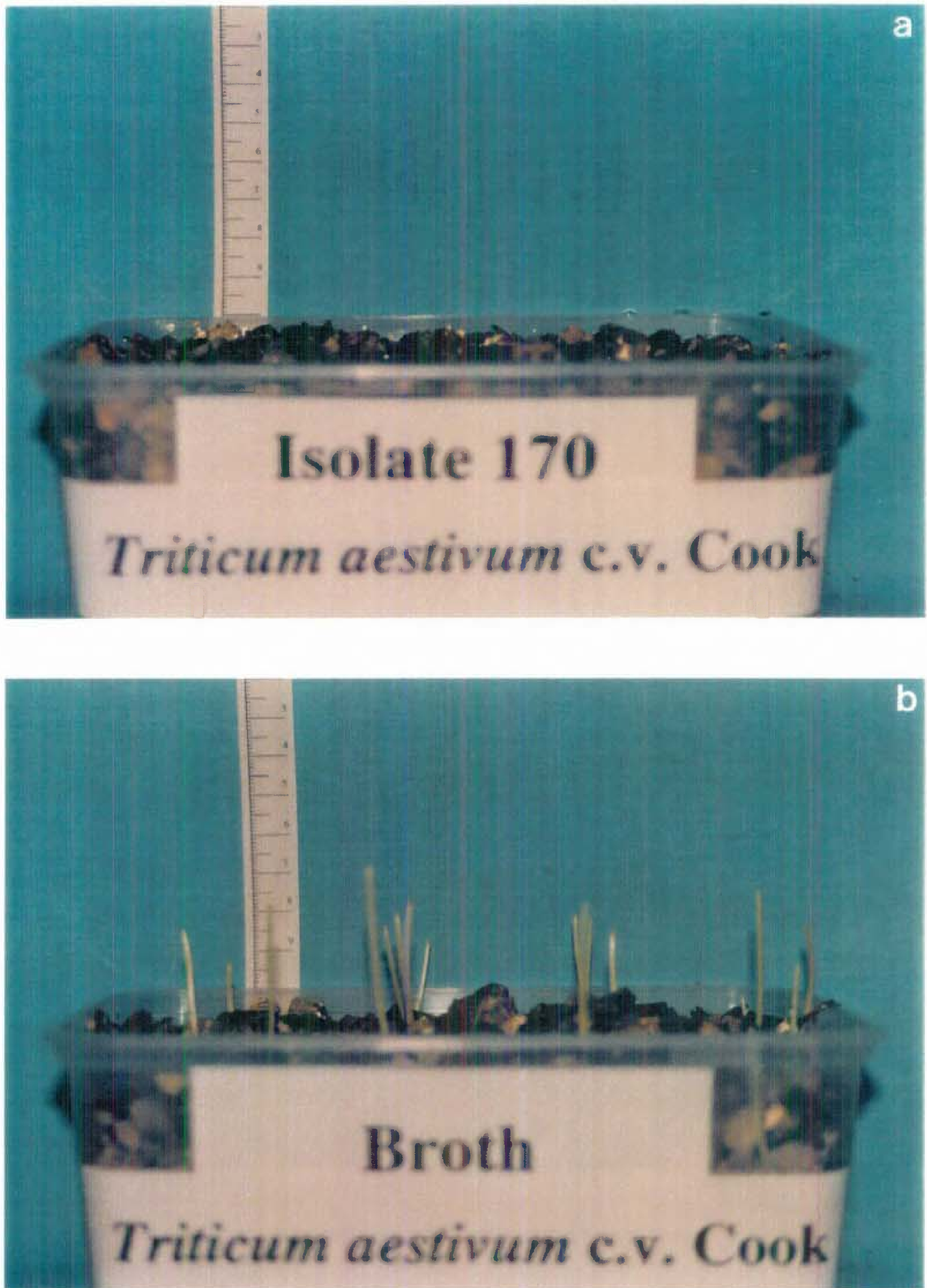

Fig. 5.1. The relative effect of filtrates derived from isolate 580170 of *P. semeniperda* (a) and uninoculated culture media (b) on coleoptile length of wheat in bioassay chambers.

Table 5.3. Effect of filtrates produced by 7 different isolates of *P. semeniperda* on coleoptile and radicle lengths of wheat and *B. diandrus* after 7 days growth in a bioassay chamber. Data are means of duplicate experiments. Data within the same column followed by a common letter are not significantly different according to Tukey's HSD test (P>0.05).

	Wheat		***B. diandrus***	
Isolate	**Coleoptile length (mm)**	**Radicle length (mm)**	**Coleoptile length (mm)**	**Radicle length (mm)**
Water	56 ±0.9**d**	104 ±5.1**d**	71 ±6**e**	80 ±8**d**
Uninoculated	55 ±3.8**d**	73 ±**c7.8d**	75 ±2.8**e**	61 ±4.1**c**
580170	13 ±1.1**a**	18 ±2.1**a**	18 ±2.7**a**	33 ±5.9**a**
580681	24 ±1.4**ab**	34 ±3.4**b**	44 ±3.3**b**	44 ±3.1**b**
580129	41±4.5**c**	51 ±9.9**c**	60 ±6.1**c**	56 ±4.7**c**
580148	20 ±0.9**a**	35 ±3.5**b**	65 ±1.5**cd**	61 ±4.3**c**
580584	50 ±3.1**d**	59 ±4.3**c**	67 ±4.2**d**	58 ±2.4**c**
580534	31 ±2.9**b**	34 ±3.1**b**	67 ±8.8**d**	54 ±6.1**c**
580520	45 ±5.6**cd**	54 ±7.7**c**	74 ±5.2**e**	61 ±6.5**c**

5.3.3 Correlation between disease severity of inoculated plants and reaction to toxic filtrates.

The symptoms of infiltration of filtrates into wheat leaves were very similar to those formed after infection with conidial inoculum (Fig. 5.2). Chlorotic halos and necrotic tissue were the primary symptoms. The level of disease severity that developed in seedling wheat plants inoculated with conidia of *P. semeniperda* was closely correlated with the reaction of seedling wheat plants to infiltration with culture filtrates (Table 5.4, Fig. 5.3). Disease severity and reaction to filtrate was highest when plants were inoculated with conidia and filtrates derived from isolates 580681 and 580170 (P<0.05). Significantly less severe disease and lower toxic reaction was observed when wheat seedlings were inoculated with conidia or filtrates from isolate 580520. A significant correlation was observed between disease severity (from conidial inoculation) and reaction to filtrates after 7 days (P<0.01). A significant negative correlation was observed between the coleoptile lengths of wheat seedlings after filtrate application and growth in a bioassay chamber for 7 days and reaction to filtrates in

wheat seedling leaves after 7 days ($P<0.01$). Similarly, a significant negative correlation was observed between disease severity and coleoptile lengths of wheat seedlings after filtrate application and growth in a CR bioassay chamber for 7 days ($P<0.01$). Correlations of lower magnitude were observed between wheat radicle length after filtrate application and growth in a CR bioassay chamber for 7 days and disease severity, reaction to filtrates and coleoptile length.

Fig. 5.2. Symptoms produced by (a) application of filtrate derived from *P. semeniperda* (isolate 580170) to wheat leaves at 24 h after infiltration and (b) inoculation of wheat leaves with conidia of isolate 580170 at 7 days after inoculation.

Table 5.4. Glasshouse disease severity and reactions of wheat to filtrates produced from 5 isolates of *P. semeniperda*. Data are of means of duplicate experiments. Data in the same column followed by a common letter are not significantly different according to Tukey's HSD test ($P>0.05$).

Isolate	Glasshouse disease severity	Reaction to filtrate
580681	3.6 ±0.1**a**	3.5 ±0.1**a**
580170	3.5 ±0.2**ab**	3.9 ±0.1**a**
580534	3.0 ±0.2**b**	2.8 ±0.1**b**
580520	1.6 ±0.1**c**	1.7 ±0.2**c**
580148	2.9 ±0.05**b**	3.0 ±0.1**b**

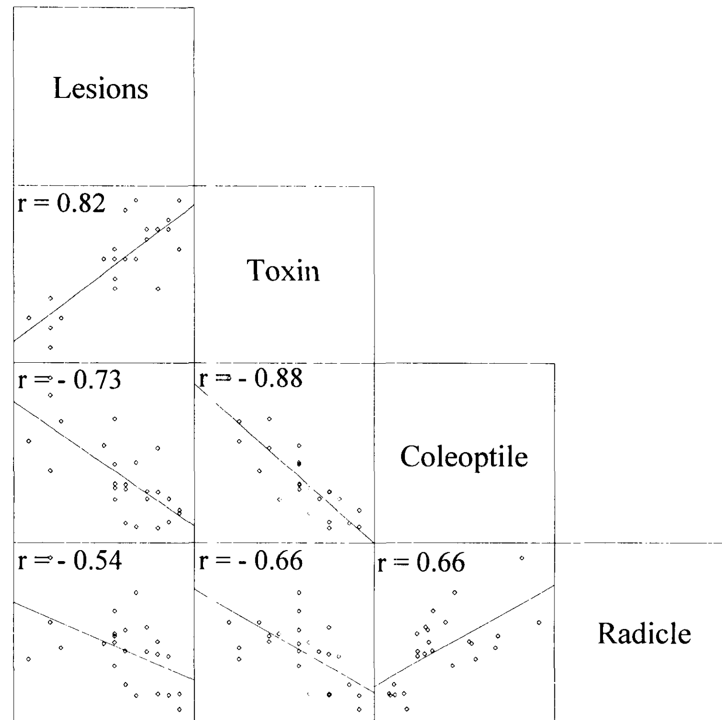

Fig. 5.3. Scatterplot matrix of correla ions between disease severity (lesions) in the glasshouse, reaction to filtrates (toxin), coleoptile length and radicle length of seedling wheat plants at 7 days after inoculation with either conidia or filtrates of *P. semeniperda*. All correlations (r-values) were significant at P<0.01, except for the correlation between number of lesions and radicle length(P<0.05), according to Pearson's correlation with Bonferroni adjusted probabilities. The experiment was repeated with similar results.

5.3.4 Toxin production in relation to filtrate age.

The pH of filtrates dropped markedly over the duration of the experiments. The pH of inoculated filtrates fell from pH 6.5 at day 6 to pH 4.0 at day 30. The pH of uninoculated filtrates dropped from pH 6.5 at day 6 to pH 6.0 at day 30. Mycelial dry weight increased from 0.055 g at day 6 to 0.515 g at day 30. After 6 days incubation, coleoptile lengths of wheat and *B. diandrus* were reduced by 26 and 17 % respectively compared to controls. The inhibitory effects of filtrates on coleoptile lengths of wheat and *B. diandrus* were maximal when filtrates were obtained from cultures that had been incubated for 12 days (Fig. 5.4).

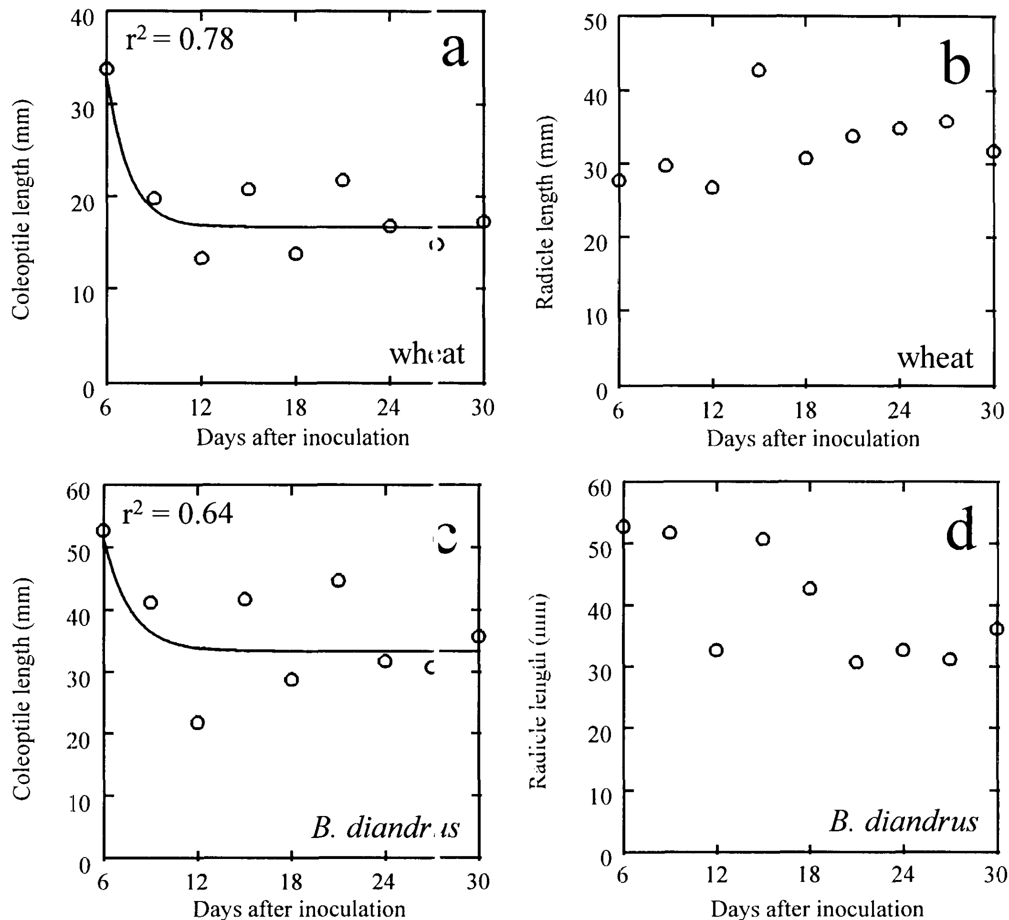

Fig. 5.4. The effect of culture filtrates of *P. semeniperda* (isolate 580170) harvested at different days after culture inoculation on the length of coleoptiles and radicles of wheat and *B. diandrus* after 7 days growth in a bioassay chamber. Data are means of 5 replicates each consisting of 20 observations.

a) The regression equation is $Y = (1 + 69.7 \pm 19.7^* (e^{-0.71 \pm 0.2^* X}))/0.06 \pm 0.002$

c) the regression equation is $Y = (1 + 18.0 \pm 6.2^* (e^{-0.58 \pm 0.15^* X}))/0.03 \pm 0.002$

where Y = coleoptile length of seedlings at 7 days after application of filtrate and X = harvest time (days) of filtrates after inoculation.

The effects of filtrates on radicle growth of both wheat and *B. diandrus* did not change markedly with the length of culture filtrate incubation, although a downward trend was observed for the effect on *B. diandrus* radicles. The linear regressions calculated for data in Fig. 5.4. (b and d) were not significantly different than a horizontal line ($P>0.05$).

A significant negative correlation was observed between filtrate pH and mycelial dry weight (P<0.01) (Fig. 5.5). A positive correlation was observed between coleoptile

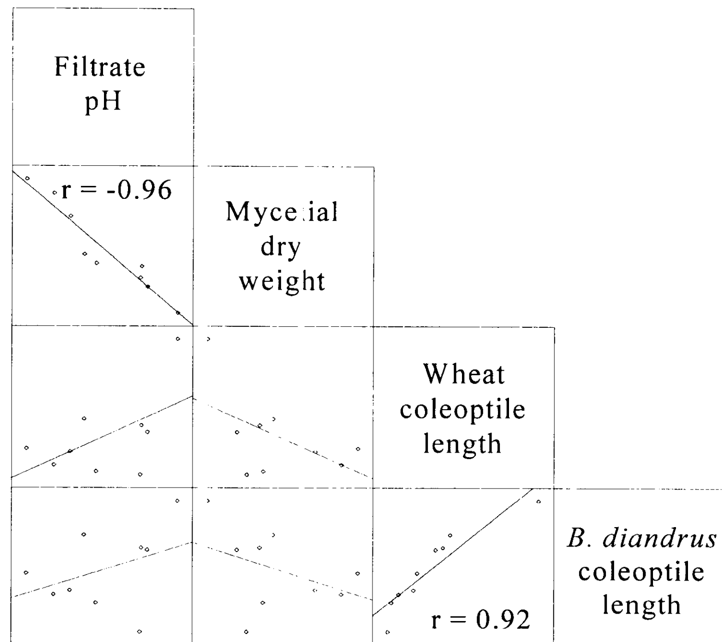

Fig. 5.5. Scatterplot matrix of correlations between filtrate pH, mycelial dry weight and coleoptile length of seedling wheat and *B. diandrus* plants at 7 days after filtrate application. Correlations with r-values were significant at P<0.01.

lengths of wheat and *B. diandrus* (P<0.01). No significant correlations were observed between filtrate pH and mycelial dry weight and coleoptile length of wheat or *B. diandrus*.

5.3.5 Relationship between filtrate toxicity and pH.

Amending the pH of culture filtrates did not affect the toxicity of filtrates (Table 5.5). The effect of inoculated filtrates on coleoptile and radicle length of wheat plants remained similar irrespective of filtrate pH (P>0.01). Similarly, the effect of uninoculated filtrates on coleoptile and radicle length of wheat plants was statistically similar irrespective of filtrate pH (P>0.01).

Table 5.5. The effect of pH amended culture filtrates of *P. semeniperda* (isolate 580170) on coleoptile and radicle lengths of wheat after 7 days growth in a bioassay chamber. Data are means of 4 replicates each with 20 observations. Data within the same column followed by a common letter are not significantly different according to Tukey's HSD test (P>0.01).

Treatment	Coleoptile length (mm)	Radicle length (mm)
Water	57 ±1.6**b**	100 ±4**c**
Uninoculated pH 5	60 ±3.2**b**	53 ±5.8**b**
Uninoculated pH 6.5	63 ±3.3**b**	70 ±4.8**bc**
Inoculated pH 5	9 ±0.6**a**	18 ±2.6**a**
Inoculated pH 6.5	9 ±0.2**a**	18 ±2.5**a**

5.3.6 Effect of filtrate concentration on reaction type.

The most severe reaction types occurred on both wheat and *B. diandrus* after application of undiluted filtrates of *P. semeniperda* (isolate 580170) (Table 5.6). The latent period for symptom expression varied from <12 h to 7 days. The latent period was shorter in infiltrated leaves of wheat than those of *B. diandrus*.

Table 5.6. The latent period and reaction type on seedling leaves of wheat and *B. diandrus* to different concentrations of filtrates of *P semeniperda* (isolate 580170) 7 days after infiltration. Data are means of duplicate experiments consisting of 5 replicates each of 5 observations. Data within the same column followed by a common letter are not significantly different according to Tukey's HSD test (P>0.05).

	Wheat		***B. diandrus***	
Filtrate concentration	**Latent period**	**Reaction type**	**Latent period**	**Reaction type**
1	<12 h	4.5 ±0.2**a**	>72 h	3 ±0.2**a**
1/5	>72 h	3.8 ±0.5**ab**	7 days	1.5 ±0.4**b**
1/10	5 days	3.0 ±0.05**b**	NR	1
1/100	7 days	2.2 ±0.4**c**	NR	1
1/1000	NR	1	NR	1

NR = No symptoms observed at 7 days after filtrate application.

Similarly, reaction type in leaves of *B. diandrus* was lower than that for the corresponding filtrate concentration in wheat leaves. Symptoms were observed in wheat leaves infiltrated with filtrates of 1/100 concentration. Symptoms were not observed in either wheat or *B. diandrus* leaves infiltrated with filtrates at 1/1000 concentration. Slight chlorosis was observed in some wheat seedlings infiltrated with 1X uninoculated filtrates. Otherwise, controls of different concentrations of uninoculated culture filtrates and sterile water resulted in no reactions when infiltrated into seedling leaves of wheat and *B. diandrus*.

5.3.7 Effect of filtrates at different plant growth stages.

Reaction types of wheat plants infiltrated with culture filtrates were higher when applied to plants at the 3-leaf seedling stage (GS13) or when the first node was detectable (GS31) than when plants were infiltrated when the first awns were visible (GS49) or at anthesis (GS60) ($P<0.05$) (Table 5.7) The period for disease expression was also shorter at earlier growth stages. Lesions were generally bigger when plants were infiltrated at earlier growth stages.

Table 5.7. The reaction of wheat leaves infiltrated with filtrates of *P. semeniperda* (isolate 580170) at 4 different growth stages (according to the scale of Zadoks, *et al.,* 1974). Data are means of 10 replicates, each consisting of 9 observations. Data followed by a common letter are not significantly different according to Tukey's HSD test ($P>0.05$). The experiment was repeated with similar results.

Growth stage	Latent period	Reaction type
13	<12 h	4.2 ±0.3**a**
31	<12 h	4.0 ±0.1**a**
49	48 h	3.2 ±0.6**b**
60	36 h	3.3 ±0.5**b**

5.3.8 Host specificity of culture filtrates.

Symptoms on leaves of wheat and *B. diandrus* were observed when leaves were infiltrated with culture filtrates. No symptoms were observed on leaves of *L. purpureus*, *G. hirsutum*, *H. annuus*, or *X. occidentale* after infiltration with culture filtrates. Occasionally, slight chlorosis was observed in plants which were infiltrated with uninoculated filtrates.

5.3.9 A comparison of filtrates produced by P. semeniperda and P. teres.

Culture filtrates of both isolates of *P. semeniperda* (580170 and 580681) significantly reduced the length of wheat coleoptiles and radicles after 7 days growth in a CR bioassay chamber ($P<0.05$) (Table 5.8). Isolates of *P teres* (WI 1535 and WI 8712) did not reduce the length of wheat coleoptiles or radicles when compared to uninoculated filtrates ($P>0.05$). Filtrates from both *P. semeniperda* and *P. teres* significantly reduced the length of coleoptiles and radicles of *B. diandrus* when compared to uninoculated filtrates and water ($P<0.05$). *P. teres* (isolate WI 8712) reduced coleoptile length of *B. diandrus* by 33 %.

Table 5.8. The effect of culture filtrates of *P. semeniperda* (isolates 580170 and 580681) and *P. teres* (isolates WI 1535 and WI 8712) on coleoptile and radicle lengths of wheat and *B. diandrus* after 7 days growth in a bioassay chamber. Data are means of duplicate experiments each consisting of 4 replicates with 20 observations. Data within the same column followed by a common letter are not significantly different according to Tukey's HSD test ($P>0.05$).

	Wheat		***B. diandrus***	
Treatment	**Coleoptile length (mm)**	**Radicle length (mm)**	**Coleoptile length (mm)**	**Radicle length (mm)**
580170	14 ±0.8**a**	29 ±2.8**a**	17 ±2.4**a**	32 ±2.9**a**
580681	39 ±3.6**b**	46 ±7**b**	51 ±2.7**b**	57 ±2.4**b**
WI 1535	60 ±4.1**c**	74 ±8.5**c**	57 ±2.8**b**	53 ±2.4**b**
WI 8712	64 ±3.3**c**	79 ±5.2**c**	49 ±0.7**b**	51 ±4.7**b**
Uninoculated	60 ±3.7**c**	61 ±11**bc**	74 ±0.9**c**	69 ±1.4**bc**
Water	60 ±2**c**	107 ±3.2**d**	73 ±2.1**c**	84 ±2.6**c**

5.4 Discussion

The investigations reported in this chapter provide further evidence that a toxin is produced by *P. semeniperda*. Unconcentrated culture filtrates reduced the length of seedling coleoptiles of wheat and *B. diandrus* by up to 75 % relative to controls consisting of uninoculated culture media and sterile water. Radicle lengths were also reduced by culture filtrates, however, the uninoculated media also decreased radicle length in the CR bioassay when compared to seedlings grown after only sterile water application. A possible explanation for this

retardation of radicle length is the high sugar content of the filtrates which makes carbohydrates more available to the growing coleoptile, since coleoptile length was not reduced by uninoculated media. Toxic metabolites were only produced by *P. semeniperda* if cultures were grown on a rotary shaker. The reason for this is uncertain. However, it may be due to an increase in oxygenation and the more rapid diffusion of nutrients required for toxin production (Shaw, 1981). For routine production of toxins by *P. tritici-repentis* and *P. teres*, the cultures are often grown without agitation (Tomás & Bockus, 1987; Tomás *et al.*, 1990; Friis *et al.*, 1991).

The toxic metabolite produced in agitated culture filtrates was not affected by autoclaving for 15 min. This suggests that the toxic metabolite produced by *P. semeniperda* is not a protein, as proteins would have been denatured by this treatment. Wallace (1951) reported that boiling filtrates of *P. semeniperda* for 5 min did not reduce their toxicity to seed. Lamari & Bernier (1989) reported that culture filtrates produced by *P. tritici-repentis* contained a compound that was denatured by autoclaving. Lamari *et al.* (1993) later found that the toxin produced by *P. tritici-repentis* was a high molecular weight protein. Tomás *et al.*(1990) also reported the production of a high molecular weight protein by *P. tritici-repentis*. In contrast, the toxins produced by *P. teres* are low molecular weight compounds and are not de-activated by autoclaving (Smedegård-Peterson, 1976; Friis *et al.*, 1991). It is possible that the toxic metabolite produced after *in vitro* growth of *P. semeniperda* may be more closely related to the compounds produced by *P. teres* than those produced by *P. tritici-repentis*.

Significant differences were observed between the toxic effects of culture filtrates produced by different isolates of *P. semeniperda*. Differences in the toxicity of culture filtrates produced by different isolates of *P. semeniperda* has also been recorded by Wallace (1951). He found that all isolates reduced the length of wheat shoots by at least 50 %. In the present study, some isolates did not reduce the coleoptile lengths of wheat and *B. diandrus*. Similar findings have been demonstrated for isolates of *P. tritici-repentis* (Tomás & Bockus, 1987), *P. teres* (Friis *et al.*, 1991) and *P. graminea* (Hammouda, 1988).

The symptoms expressed by plants infiltrated with filtrates derived from *P. semeniperda* were very similar to those produced after conidial infection. The use of visible symptoms resulting from the application of toxins is often used as evidence of their role in pathogenesis (eg Tomás & Bockus, 1987). However, as pointed out by Yoder (1980),

symptoms should be used in conjunction with other assays in the evaluation of toxins since the production of visible symptoms may not be reliable in evaluating factors of pathogenesis.

Disease symptoms on leaves of wheat seedlings resulting from inoculation with conidia correlated closely with the rating of seedlings following toxin application (correlation coefficient 0.82). This correlation indicates that the toxin assays can be used to rank isolates for their virulence as pathogens. Furthermore, the correlation between the toxin infiltration assay and the coleoptile length assay (-0.88) shows that the bioassay technique used gave a reliable indication of the degree of toxicity of filtrates derived from different isolates of *P. semeniperda*. By corollary, the bioassay also gave a good idea of the relative virulence of the isolates. Although the radicle lengths of wheat seedlings, as measured using the CR assay technique, provided significant correlations with disease severity and toxin reaction, the effect of uninoculated culture media on radicle length negated the use of this parameter. The correlations between the various assay techniques provides further evidence of the role of a toxin in the disease caused by *P. semeniperda* because the virulence of isolates appeared to be related to toxin production *in vitro*. Tomás & Bockus (1987) described a strong correlation between field disease severity and toxic infiltrations of various isolates of *P. tritici-repentis*. They concluded that toxic infiltrations could be a useful method of screening wheat cultivars for disease resistance to *P. tritici-repentis*. It appears that the *in vitro* toxic metabolites produced by *P. semeniperda* could be used in a similar fashion to determine the most virulent isolates for further research on the mycoherbicidal potential of the pathogen. However, this assumption needs to be validated.

The pH of culture filtrates of *P. semeniperda* decreased with incubation time. However, filtrate pH did not correlate significantly with toxicity of filtrates as measured by coleoptile lengths of wheat and *B. diandrus* seedlings. Adjustment of the pH of culture filtrates after incubation did not affect the toxicity of filtrates. It seems unlikely therefore, that the toxic compound involved in the *P. semeniperda* system involves the lysis of cells by acidity. The results also showed that increasing mycelial dry weight did not correlate with toxicity of culture filtrates. In contrast, maximum toxicity of *P. teres* isolates coincided with the peak for mycelial weight (Smedegård-Peterson, 1976). The production of toxic metabolites in culture filtrates as measured by the CR bioassay occurred by 6 days after media inoculation. This also provides further evidence for the production of a toxin by *P. semeniperda* since staling compounds are unlikely to have formed in culture at this early stage

of the growth. The production of toxic metabolites increased from day 6 to day 12 of the incubation period. After 12 days no further increase in toxin production was observed using the CR assay technique. Strobel *et al.* (1991) suggested that in general, biosynthesis of toxins usually declines after long periods of culture. It appears that this may also be the case for *P. semeniperda*. Isolates of *P. teres* also showed a marked decrease in the pH of the growth medium, with the minimum value reached between 2.8 and 4.5 (Friis *et al.*, 1991). However, unlike the isolate of *P. semeniperda* tested in the present study, the isolates of *P. teres* which produced low pH values were found to be those which produced the highest amounts of toxins (Smedegård-Peterson, 1976; Friis *et al.*, 1991).

Culture filtrates infiltrated into seedling leaves of wheat produced symptoms with concentrations as low as 1/100 of the stock solutions. Concentrations below 1/5 did not produce any symptoms on *B. diandrus*. This observation is in agreement with the effects of conidial inoculation of *P. semeniperda* on *B. diandrus* (Chapter 7), which showed that conidial infection was not readily expressed as leaf spot symptoms. Furthermore, the latent period of the same concentration of filtrates was shorter in wheat leaves than in those of *B. diandrus*. The reason for this is uncertain although it may be linked to the relative virulence of *P. semeniperda* to wheat and *B. diandrus*. Sugawara *et al* (1985) found that the latent period in plants to toxins could be related to light intensity. The possibility exists that *B. diandrus* may be less sensitive because of an environmental parameter. Slight chlorosis was observed in some leaves infiltrated with uninoculated culture filtrates. This can be attributed to the higher concentrations of sugar and salts in the uninoculated medium because no fungal utilisation of the nutrients had occurred (Tomás & Bockus, 1987).

Wheat plants were more susceptible to the toxic metabolites in culture filtrates when plants were inoculated at the seedling or 1st node stage of growth than at either, booting or anthesis. This contrasts with the findings of Tomás & Bockus (1987) for *P. tritici-repentis*. They found that reaction to fungal culture filtrate remained constant regardless of plant age, although reaction to uninoculated medium was less pronounced in older plants. The differences between the growth stages could be attributed to the more waxy nature of older leaves and the thickened cuticles. These factors could have reduced the extent of infiltration of filtrates into the leaves. Furthermore, the results suggest that the physiology of older wheat leaves may be more resistant to the effects of the toxic metabolites. This might result from the deposition of lignin in the cell walls of older leaves.

Culture filtrates that were infiltrated into several different plant species only produced symptoms on wheat and *B. diandrus*. This suggests that the toxic metabolite produced *in* vitro by *P. semeniperda* has some degree of host-specificity. However, a wider range of plant species would need to be tested to further elucidate the specificity of the toxin/s. Other toxins produced by *Pyrenophora* species are reported to be host-specific. *P. tritici-repentis* toxin is reputed to be specific to wheat cultivars that are susceptible to the disease (Tomás & Bockus, 1987; Lamari & Bernier, 1989). Lamari & Bernier (1989) also reported that the toxin produced by *P. tritici-repentis* did not affect any of 11 other plant species tested, including several gramineous hosts. The toxins produced by *P. teres* have been demonstrated to be host-selective when tested against 11 plant species (Smedegård-Peterson, 1976). Interestingly, in the host specificity studies performed by Smedegård-Peterson (1976) tomato was found to be highly sensitive to the toxin but not to conidial infection. He postulated that tomato possessed a resistance mechanism which operated independently of its sensitivity to the toxins. This finding may have important implications for the use of toxins as herbicides. Clearly, it is important to test not only those plant species which may be closely related to the original host when exploring herbicidal potential of phytotoxins. Some degree of host-specificity has also been demonstrated for culture filtrates produced by *P. avenae* (Graniti & Porta-Puglia, 1984).

P. teres has been reported to infect *B. diandrus* but only rarely to infect wheat (Sivanesan, 1987). In the study reported in this chapter, culture filtrates derived from *P. teres* did not affect the length of wheat coleoptiles. However, a decrease in the length of *B. diandrus* coleoptiles was observed. Although culture filtrates from *P. semeniperda* decreased the length of *B. diandrus* coleoptiles significantly more than *P. teres,* the possibility exists for the use of *P. teres* toxins as herbicidal compounds because of its greater host-specificity.

5.5 Summary

P. semeniperda was shown to produce toxic metabolites under *in vitro* conditions. The toxin/s are heat stable and were only produced when cultures are agitated. Using a bioassay which used coleoptile and radicle length as an assay after application of toxic filtrates, it was shown that filtrates of *P. semeniperda* cultures had a severe impact on seedling growth of both wheat and *B. diandrus*. Furthermore, infiltration of wheat and *B. diandrus*

leaves with filtrates produced symptoms similar to those produced by conidial inoculation. The relative toxicity of filtrates derived from several isolates of *P. semeniperda* and infiltrated into wheat leaves was highly correlated with the relative infectivity of conidia produced by these isolates. Toxicity of filtrates was not related to culture pH or mycelial growth. Culture filtrates were toxic to wheat and *B. diandrus* as soon as 6 days after culture inoculation and was maximal in filtrates derived from cultures that were 12 days old. Incubation for a further 18 days did not result in any increase in toxicity of culture filtrates. Culture filtrates which had been diluted to 1/100 were capable of producing symptoms in wheat seedlings. However, only filtrates with greater than 1/5 concentration were able to produce symptoms on *B. diandrus* seedling leaves. Toxicity of filtrates was related to plant age. Plants older than GS31 were significantly less susceptible to the filtrates than plants at younger growth stages. A degree of host specificity was observed. Filtrates infiltrated into leaves of wheat *B. diandrus*, *L. purpureus*, *G. hirsutum* . *H. annuus*, and *X. occidentale* produced characteristic symptoms only on wheat and *B. diandrus*. A comparison of the toxicity of *P. semeniperda* and *P. teres* grown under the same cultural conditions was made. *P. teres* did not affect wheat coleoptile growth, but affected the coleoptile growth of *B. diandrus*. In all cases, filtrates produced by *P. semeniperda* had a greater effect.