TOXIN PRODUCTION BY ~~Development of a transformation protocol for~~ *Ascochyta rabiei*, the causal agent of Ascochyta blight of chickpea (*Cicer arietinum* L.), and ~~toxin production by the wild type and transformants *in vitro*~~ DEVELOPMENT OF A TRANSFORMATION PROTOCOL FOR THE FUNGUS.

By Estelle Cécile Gewiss

Thesis submitted for the degree of Ph.D. in Plant Pathology

At University College London, Department of Biology, London WC1E 6BT

2004

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To my dear father and grandfather

Acknowledgments

I would like to thank Dr Richard Strange for his guidance during this research work.

I acknowledge the financial support conceded to the project by the European Union (contract number ICA4-CT-2000-30003).

I am endebted to Dr Yannick Gachet for his helpful advice, and Dr Brigitte Meunier and Mr Ian Buchanan for their technical help. I would like also to thank Dr Mike Challen, Christopher Rogers and Valérie Odon from Horticulture Research International for welcoming me in their laboratory and giving me the opportunity to learn the *Agrobacterium tumefaciens* mediated transformation technique.

I am thankful to Subramanian Gopalakrishnan for his great help throughout my work, as well as to Nathalie Pourtau and Elise Pelzer for their advice and contribution to this work. I greatly appreciated their friendship.

Finally, I would like to express my deepest gratitude to my mother for her love and Martin for his precious daily support and encouragements.

Abstract

Ascochyta rabiei is the causal agent of blight of chickpea, an important food legume crop for human populations in Developing Countries. All reliably identified isolates of the fungus produce toxins in culture, known as the solanapyrones, of which solanapyrone A is the most frequently found and also the most toxic. The principal aim of the project was to determine the role of this toxin in the disease syndrome by producing toxin-minus mutants and testing them for virulence. Four transformation techniques were attempted: Restriction Enzyme Mediated Integration (REMI), electroporation, particle bombardment and *Agrobacterium tumefaciens*-mediated transformation. With the last, employing a T-DNA containing a hygromycin resistance gene, 908 transformants were obtained from germinated pycnidiospores on a selective medium containing hygromycin. Genuine transformants were tested for the production of solanapyrone A using an assay in microtitre plates. Loss of toxin production by transformants was confirmed by reversed phase High Performance Liquid Chromatography. Sixteen transformants produced significantly less solanapyrone A than the wild-type strain. Transformants were also screened for integration events by PCR, using primers specific to the hygromycin resistance gene and homologous hybridisation to a probe consisting of this gene. Among the four transformants tested, three have integrated two copies of T-DNA and one had a single insertion.

In order to optimise the production of solanapyrone A so as to provide a source of the compound for screening chickpea genotypes, three types of cultures of *A. rabiei* were tested: still culture, shake culture and fermenter culture. The toxin was purified from culture filtrates by solvent partitioning followed by flash chromatography.

The effect of two safeners on the sensitivity of chickpea shoots to solanapyrone A was tested using bioassays. Dichlormid (300 or 800 μg per shoot) and fenclorim (18 μg per shoot) decreased the sensitivity of chickpea shoots to solanapyrone A 1.6 and 2.5-fold, respectively.

Table of Contents

List of Figures

List of Tables

Abbreviations

Biological molecules

Anti-DIG-AP Fab fragments	Fab fragments from an anti-digoxygenin antibody conjugated with alkaline phosphatase (AP).
ATP	Adenosine triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
GSH	glutathione
GST	Glutathione S-transferase
GUS	β-glucoronidase
T-DNA	Transferred DNA

Chemicals

DAPI	4', 6-diaminido-2-phenylindole
DIG	Digoxygenin
EDTA	Ethylenediaminetetraacetic acid
FDA	fluorescein diacetate
MBTH	3-methyl-2-benzothiazolone hydrazone hydrochloride

SDS	Sodium dodecyl sulphate
TAE	Tris, acetic acid, EDTA buffer
Tris	Tris-hydroxymethyl-aminomethane

Techniques

ATMT	*Agrobacterium tumefaciens*-mediated transformation
HPLC	High Performance Liquid Chromatography
PCR	Polymerase Chain Reaction
REMI	Restriction Enzyme-Mediated Integration
SPE	Solid Phase Extraction
TAIL-PCR	Thermal Asymmetric Interlaced-PCR

Units

A	Ampere
Å	Angstrom
bp	base pairs
cm	centimetre
d	day
F	Farad

g	gravitational acceleration
g	gram
h	hour
Hg mm	millimetre of mercury
kV	Kilovolt
l	litre
LD_{50}	Lethal dilution that leads to 50% cell death
M	molar
m	milli
min	minute
mm	millimetre
µ	micron
n	nano
OD	optic density
p	pico
psi	pounds per squared inches
sec	second
SD	standard deviation
U	Units
v	volume

V	Volt
°C	degree Celcius
Ω	Ohm

Media and buffers

CDCLM	Czapek-Dox Liquid Cations Medium
CD-V8	Czapek-Dox V8 broth

Miscellaneous

B.C.	Before Christ
hph	hygromycin B phosphotransferase
ITS	ribosomal DNA internal transcribed spacers
ODS	Octo Decyl Silica
UV	Ultra-violet

Chapter 1

Introduction

1.1. Chickpea

Chickpea (*Cicer arietinum* L.), also named Bengal gram or gram, is a self-pollinated annual plant belonging to the Fabaceae family. It originated from South-Eastern Anatolia (Turkey) where the wild subspecies *Cicer arietinum reticulatum* arises (Sauer, 1993). Archeological evidence shows that in 7500 B.C. people were already harvesting seed from wild chickpea plants (Sauer, 1993). The earliest domestication dates from 6500 B.C. (Zohary and Hopf, 1993). Chickpea is the fourth most important grain legume crop worldwide after soya beans, dry beans and groundnuts, with a total cultivated area exceeding 8.7 million hectares and an annual production of about 6.4 million tonnes (FAO, 2001). This pulse crop ranks as the third non-oilseed grain legume in the world, after beans and peas (FAO, 2001). Chickpea is grown in tropical, sub-tropical and temperate regions but is mainly cultivated in India (Pandey *et al.*, 2001), which contributes 60% of world production (FAO 2001). It is also grown in the Mediterranean Basin, especially Spain, which is the principal producer in this area (Cachinero *et al.*, 2002), Turkey and Tunisia. Canada (Chongo *et al.*, 2004) and Australia (Siddique and Khan, 1993) are important producers of chickpea as well. Chickpea crops are planted in moist soil after the rainy season and are able to grow in marginal, low fertility areas without irrigation. The two main types of chickpea are called desi, which is thought to have originated first and produces small brown seeds, and kabuli, which produces larger pale yellow seeds with lower fibre content (Malhotra *et al.*, 1987).

Chickpea seeds represent a rich source of high quality proteins (17.42 to 21.25%; Agrawal and Singh, 2003; Akem, 1999) and carbohydrates (56.08 to 60.46%; Agrawal and Singh, 2003) and also contain fibres (3.86 to 4.40%; Agrawal and Singh, 2003), which make them a nutritious food. For that reason the sobriquet "poor Man's meat" has been attributed to chickpea in Pakistan.

As a legume, chickpea forms a symbiotic relation with *Rhizobium* which allows the fixation of nitrogen (Laranjo *et al.*, 2002). As a result, soil fertility is improved which is beneficial to crops grown in rotation with chickpea.

1.2. Chickpea diseases

Chickpea is susceptible to various abiotic factors, such as drought (Johansen *et al.*, 1994), cold (Singh *et al.*, 1998), heat and salinity (Singh *et al.*, 1994), which reduce yields. The plant is also affected by pathogens including fungi, viruses, nematodes and other pests such as insects (Makkouk *et al.*, 2003a; Makkouk *et al.*, 2003b; Mouhouche and Fleurat-Lessard, 2003; Cherry *et al.*, 2000; Nene *et al.*, 1996). Amongst fungi, the main pathogens are *Fusarium oxysporum* f. sp. *ciceri*, which causes chickpea wilt, and *Ascochyta rabiei* (Pass.) Labrousse causing Ascochyta blight, the most widespread and economically destructive disease of chickpea (Singh *et al.*, 1994). Ascochyta blight was first reported from Pakistan in 1911 (Butler, 1918), and later from 35 countries worldwide (Nene, 1996). It causes severe losses in seed yield (10 to 100%) and quality (Chongo and Gossen, 2001; Singh and Reddy, 1990; Nene, 1982).

1.3. Ascochyta blight

1.3.1. Symptoms

Ascochyta blight affects all aerial parts of the chickpea plant. The description of the disease is very similar from one country to the other: *A rabiei* causes circular necrotic lesions surrounded by dark margins on leaflets and pods, and brown elongated lesions on stems and petioles. Often the infected seed carry lesions too. Infected stems break, killing all of the tissue above the lesion. When lesions girdle the stem at the collar region, the whole plant dies (Nene, 1982). *A. rabiei* also causes epinasty and chlorosis, which are lethal to the plant (Latif *et al.*, 1993). In infected cells, membrane permeability and turgor drop rapidly and dramatically, respiratory

activity increases, and phenolic compounds and phytoalexins accumulate, leading to the death of some of these cells that turn brown (Dolar and Gurcan, 1993). The development of chickpea blight is affected by cultivar and plant age, young plants being more resistant than older ones. Such differences are not so distinct in susceptible cultivars (Trapero-Casas and Kaiser, 1992).

1.3.2. Ascochyta rabiei

The pathogen has two spore stages. The asexual stage is characterised by the production of pycnidiospores from fruiting bodies called pycnidia. Pycnidiospores are oval to oblong (about 9.0 x 4.3 μm), hyaline and occasionally bicelled (Nene, 1982). The sexual stage of *A. rabiei* occurs when the temperature is cool (Navas-Cortés *et al.*, 1998). It was originally observed on overwintering chickpea debris in Bulgaria by Kovachevski (1936) and named *Mycosphaerella rabiei* Kovachevski (syn. *Didymella rabiei*). Its presence has since been documented many times, especially from Russia (Gorlenko and Bushkova, 1958), Greece (Zachos *et al.*, 1963), Hungary (Kövics *et al.*, 1986), the USA (Kaiser and Hannan, 1987), Spain (Jiménez-Díaz *et al.*, 1987), Syria (Haware, 1987) and Australia (Galloway and MacLeod, 2003). Ascospores, formed in asci contained in pseudothecia, are ovoid and divided into two unequal cells. The teleomorph is heterothallic and results from the mating of individual colonies with two compatible mating-types MATI-1 and MATI-2, two alleles present at a single locus (Wilson and Kaiser, 1995). The occurrence of the sexual stage of *A. rabiei* may explain its genetic diversity that entails the development of new pathotypes by genetic recombination, and its potential for adaptation to new cultivars of its host and to fungicides (Peever *et al.*, 2004).

1.3.3. Epidemiology and dissemination

Although Khan *et al.* (1999) showed that *D. rabiei* caused blight symptoms on four cultivars of common bean (*Phaseolus vulgaris* L.) when inoculated artificially, its

natural hosts seem to be restricted to the genus *Cicer* (Akem, 1999).

Dispersal of spores is essential for the survival and the spread of *A. rabiei.* Both pycnidiospores and ascospores are involved in the disease cycle of the fungus. Pycnidia develop within lesions, often forming concentric rings around the infection site (Akem, 1999; Nene and Reddy 1987; Nene, 1982). The pycnidiospores they contain are spread by wind or rain splash over short distances within fields. On susceptible hosts multiplication is rapid, producing further generations of spores and resulting in epidemics (Nene, 1982). Low temperatures (5-10°C) and high relative humidity (at least 95%) are required for the production and maturation of pseudothecia (Jhorar *et al.*, 1998; Trapero-Casas and Kaiser, 1992). Under such climatic conditions, *D. rabiei* sporulates prolifically. Ascospores are discharged from pseudothecia within 2 h of wetting (Kaiser, 1992). The teleomorph plays an important role in spreading quickly the disease among fields and initiating epidemics due to the long-distance dissemination of ascospores (Armstrong *et al.* 2001; Shtienberg *et al.*, 2000; Trapero-Casas *et al.*, 1996). Isolated chickpea fields have been reported to be infected by blight at 10 to 15 km from the nearest chickpea planting (Trapero-Casas and Kaiser, 1992). Even "resistant" cultivars can become heavily infected (Nene and Reddy, 1987).

The primary sources of inoculum for Ascochyta blight are infected seed and diseased debris that have overwintered on the soil surface (Armstrong *et al.* 2001; Shtienberg *et al.*, 2000; Akem, 1999; Khan *et al.*, 1999; Kaiser, 1997; Kaiser and Okhovat, 1996; Trapero-Casas *et al.*, 1996). Infected seed results in local and international (by introduction of seed into different countries) spread of the disease (Kaiser, 1997). They have been shown to be responsible for the introduction of the pathogen into Iran in 1968 (Kaiser, 1972), Canada in 1974 (Morrall and McKenzie, 1974), and the United States from 1983 (Guzman *et al.*, 1995). *A. rabiei* has been reported to survive in infected chickpea seed in storage for at least five years (Kaiser, 1987). Infected tissues from diseased plants may be dispersed for several hundred meters and reach other crops of healthy chickpea plants, where they also act as a source of infection in wet weather. The teleomorph *D. rabiei* develops on chickpea debris (Nene, 1987). Many farmers use chickpea conservation tillage to prevent soil erosion and to preserve soil moisture. This practice leads to a large quantity of chickpea

debris on the soil after harvest, where *D. rabiei* survives for at least two years, allowing pseudothecia to develop. Infected debris can also be transported by wind to surrounding fields (Kaiser, 1997; Trapero-Casas *et al.*, 1996; Navas-Cortés *et al.*, 1995; Nene, 1982).

1.3.4. *C. arietinum* L. / *A. rabiei* interaction

1.3.4.1. Invasion of the pathogen

Histological and ultrastructural alterations of resistant and susceptible chickpea plants by *A. rabiei* have been studied by Ilarsan and Dolar (2002). Pycnidiospores germinate on leaflets and stems of both resistant and susceptible chickpea plants already within 12 to 24 h post-inoculation. Germ tubes expand and form appressoria that penetrate the cuticle and stomata. *A. rabiei* develops inter- or intracellular hyphae that invade predominantly the space between the middle lamellae and the parenchyma cells. Ilarsan and Dolar (2002) proposed the hypothesis that hyphae of *A. rabiei* present surface proteins able to recognising resistant and susceptible plant cell wall and to initiate their degradation. They spread subepidermally inside the leaves and stems, creating necrotic lesions. The host wall becomes swollen. Hyphae aggregate to form pycnidia in stems and leaflets of susceptible cultivars.

1.3.4.2. Response of *C. arietinum* to *A. rabiei*

In response to attack by *A. rabiei*, chickpea plants build up structural defences, and synthesise antimicrobial secondary metabolites and antimicrobial proteins.

Lignification of the cell wall of the epidermal cells is a pre-infectional defence mechanism of resistant cultivars of chickpea. Pandey *et al.* (1987) observed that only the lignified tissue was not attacked by *A. rabiei.* Epidermal cells are the site of storage of the isoflavones biochanin A and formononetin, the two precursors of phytoalexins in chickpea (Barz and Mackenbrock, 1994).

Post-infectional defence mechanisms include:

(i) the synthesis of two pterocarpan phytoalexins (medicarpin and maackiain) (Kolattukudy *et al.*, 1994; Jaques *et al.*, 1987). These phytoalexins may be induced by glutathione (Armero *et al.*, 2001). However, *A. rabiei* is able to degrade these compounds (Tenhaken *et al.*, 1991);

(ii) the increase of chitinase activity in leaves and pods of plants inoculated with *A. rabiei* (Nehra *et al.*, 1994);

(iii) the activation of the two enzymes peroxidase and diamine oxidase which are involved in the process of lignification and suberization (Angelini *et al.*, 1993);

(iv) the accumulation of PR-2a, a vacuolar class I β-1,3-glucanase that was purified from chickpea cells of *A. rabiei*-infected leaves and cell cultures of chickpea (Vogelsang and Barz, 1993) and PR-2b, another pathogenesis-related protein accumulating in the apoplast of chickpea leaves 1 d before *A. rabiei* enters the apoplastic space (Hanselle and Barz, 2001);

(v) the increase of the activity of copper amine oxidase under infection conditions, which catalyses the production of hydrogen peroxide (H_2O_2) that is essential in response to the attack by *A. rabiei* (Rea *et al.*, 2002). The production of H_2O_2 may lead to an enhanced resistance of plant cell walls, restricting fungal growth and diffusion of solanapyrone toxins (Rea *et al.*, 2002).

The degree of resistance of chickpea to *A. rabiei* depends upon the cultivar, the age of the leaflets, the race of the pathogen and the concentration of inoculum (Ilarsan and Dolar, 2002).

1.3.5. Control of Ascochyta blight

Three main methods are used for the control of Ascochyta blight of chickpea:

cultural practices, chemical control and breeding for resistance. Given that a very low infection level is sufficient to introduce the pathogen in new unaffected areas, an efficient control of the disease is required. The best way to provide long-term solutions to microbial diseases is to apply in synergy different types of control.

1.3.5.1. Cultural practices

Phytosanitary methods are used to reduce the amount of inoculum of *A. rabiei* which can be transmitted by seed or crop debris. They include the use of clean seed to establish new planting areas and destruction of crop residues or deep ploughing of chickpea fields to bury infected debris (Navas-Cortés *et al.*, 1995; Wilson and Kaiser, 1995; Nene and Reddy, 1987).

The incidence of blight can be reduced by adjusting sowing dates. However, late planting of chickpea reduces seed yields because crops are subject to moisture and heat stress (Singh *et al.*, 1997). Crop rotation with non host plants interrupts the normal life cycle of the pathogen by changing its environment, hence reducing the spread of the disease (Singh and Rathi, 2003).

1.3.5.2. Chemical control

Fungicides can be used to treat seed and spray plants (Demirci *et al.*, 2003; Akem, 1999). However, as stated in Section 1.3.2, *A. rabiei*, similarly to other pests, may develop resistance to the chemicals employed by genetic recombination during its sexual stage. Many pesticides are phytotoxic, expensive and ecologically damaging, as well as hazardous to consumers' health.

1.3.5.3. Breeding for resistance

The most effective strategy to control Ascochyta blight is host resistance, achieved

by breeding resistant cultivars (Porta-Puglia *et al.*, 1997). However, only a limited number of accessions with partial resistance to Ascochyta blight have been reported so far (Collard *et al.*, 2001; Stamigna *et al.*, 2000). Variation in disease depends upon the virulence of local isolates of *A. rabiei* and the prevailing climatic conditions. Resistance in chickpea is quantitatively inherited (Flandez-Galvez *et al.*, 2003; Santra *et al.*, 2000). Thus, the quantitative trait loci mapping approach is appropriate for identifying the number and position of genes conferring resistance to Ascochyta blight.

1.4. Phytotoxins

Phytotoxins are a group of mainly low molecular weight fungal secondary metabolites. They are classified as host-selective (or host-specific), defined as toxins which damage only plants that are hosts of the pathogen-producing toxin, or non-host-selective, which can cause symptoms on both hosts and other plants (Strange, 2003). More importantly, host-selective toxins are essential for pathogenicity, whereas non-host-selective toxins are not mandatory for pathogenicity but may enhance virulence (Strange, 2003).

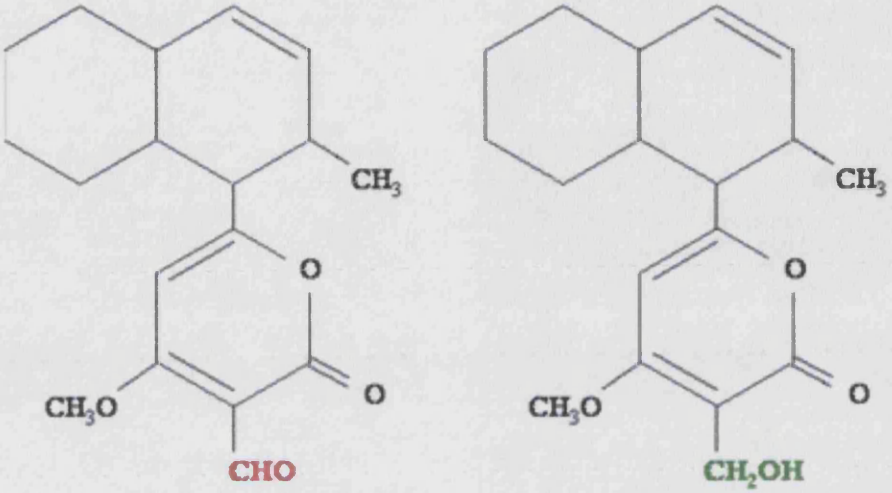
A. rabiei produces three phytotoxins: the solanapyrone A, B and C. Although no evidence has been reported regarding their classification, solanapyrones are considered to be non-host-selective toxins.

The chemical structures of the solanapyrone toxins have been identified (Ichihara *et al.*, 1983). These toxins are decalins with a pyrone moiety carrying an ethanolamine or a methoxy group as well as an aldehydic or alcohol group (Figure 1.1). All solanapyrone toxins are biosynthesised from the same precursor prosolanapyrone III, which has a polyketide structure (Katayama *et al.*, 1998). The formation of the decalin ring structure occurs via a pathway involving a Diels-Alder reaction (Oikawa *et al.*, 1998; Oikawa *et al.*, 1995).

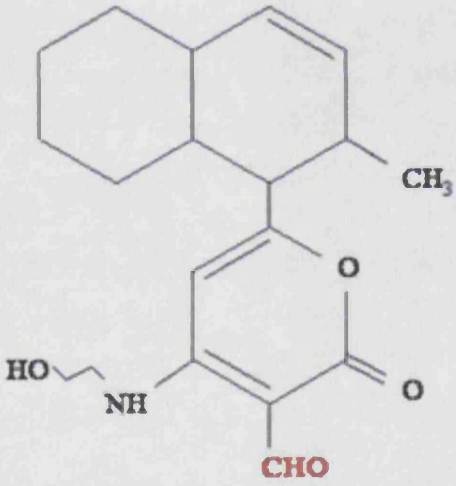
Although *A. rabiei* grows in Czapek-Dox liquid medium, the production of solanapyrones A, B and C by *A. rabiei* requires a supplement of chickpea extract

or five metal cations (calcium, cobalt, copper, manganese and zinc; CDCLM, Appendix 1); Chen and Strange, 1991). The quantity of solanapyrone A produced varies depending on the isolates of *A. rabiei* (Porta-Puglia *et al.*, 1997).

The mechanism of action of solanapyrone toxins is still unknown but they have been shown to affect the integrity of the plasma membranes of chickpea plants. Three lines of evidence suggest that solanapyrone A, the most toxic compound, could play a role in pathogenicity or virulence of *A. rabiei:* (i) all reliably identified isolates of the fungus produce solanapyrone A, (ii) stem breakage, a typical symptom of Ascochyta blight which may be caused by loss of turgor of parenchyma cells surrounding the stele, was reproduced by allowing healthy chickpea shoots to take up a pure preparation of solanapyrone A, and (iii) there is a weak positive correlation between the relative sensitivities of chickpea cells to solanapyrone A and their susceptibility to the disease (Hamid and Strange, 2000).



Solanapyrone A **Solanapyrone B**



Solanapyrone C

Figure 1.1. Structures of the solanapyrone phytotoxins A, B and C

1.5. Plant defence against xenobiotics

1.5.1. The glutathione/glutathione-S-transferase system

Glutathione (GSH) is the tripeptide L-γ-glutamyl-L-cysteinylglycine. It is found in many plant, bacterial and mammalian cells.

Glutathione-S-transferases (GSTs) are a family of dimeric multifunctional enzymes (Dixon *et al.*, 1998). In plants, they are involved in endogenous functions such as cellular protection against pathogen attack (Marrs, 1996) and oxidative damage (Marrs, 1996), and have a role as a carrier for auxins (Chen and Singh, 1999). GSTs catalyse the conjugation of the thiol group of glutathione to various endogenous or exogenous substrates to form polar and nontoxic peptide conjugates (Hatzios, 2000; Cole *et al.*, 1997; Marrs, 1996; Wilce and Parker, 1994). Such conjugates are more hydrophilic than the original compounds, which decreases their ability to integrate into biological membranes and restricts their accumulation within cells and tissues (Coleman *et al.*, 1997). The conjugates are then sequestrated to the vacuole (Cole and Edwards, 2000; Davies and Caseley, 1999; Rea *et al.*, 1998; Coleman *et al.*, 1997; Neuefeind *et al.*, 1997; Marrs, 1996) and may be further metabolised, eventually appearing as bound residues in the extracellular matrix (Lamoureux *et al.*, 1991). Plants GSTs play a role in the detoxification of a diverse range of xenobiotics such as herbicides and toxins (Cole and Edwards, 2000). In chickpea, GSTs were shown to covalently link solanapyrone A to GSH and to form less reactive and more polar glutathione-S-conjugates in the cytoplasm (Hamid and Strange, 2000).

1.5.2. Safeners

Herbicide safeners, also known as herbicide antidotes or crop protectants, are employed to protect crops from herbicide injury. They are divided in seven chemical groups: the dichloroacetamide derivatives, the naphthopyranones, the dichloromethyl acetals and ketals, the oxime ether derivatives, the 2,4-disubstituted thiazolecarboxylates, the phenylpyrimidines, the phenylpyrazoles, the

quinolinoxycarboxylic acid esters, the thiocarbamates and the methylbenzyl-tolylureas.

Safeners enhance crop tolerance through increased herbicide detoxification (Farago *et al.*, 1994). They have mostly been used to protect graminaceous crops such as maize and sorghum from injury by chloroacetanilide and thiocarbamate herbicides, but safeners for wheat, barley and rice have also been marketed (Hatzios and Wu, 1996; Hatzios, 1989; Devlin *et al.*, 1983).

In plants, herbicides undergo GSH conjugation as a primary route of detoxification (Cole *et al.*, 1997). Safeners conjugate herbicides either by elevating the level of GSH or by inducing the activity of glutathione-dependant enzymes that degrade herbicides. Chloroacetamide herbicides have been shown to be detoxified by conjugation with GSH in corn (Gronwald, 1989; O'Connell *et al.*, 1988), sorghum (Dean *et al.*, 1990; Gronwald *et al.*, 1987; Fuerst and Gronwald, 1986; Zama and Hatzios, 1986), wheat (Edwards and Cole, 1996) and maize (Ekler and Stephenson, 1991). Pretreatment of maize shoots with the safener benoxacor or a formulated combination of the herbicide metolachlor with benoxacor induced their GST activities (Cottingham *et al.*, 1998). A correlation between GST activities and chloroacetanilide herbicide susceptibility in maize and competing weeds has been established, suggesting the importance of GSTs in herbicide selectivity (Hatton *et al.*, 1996). The GSTs involved in herbicide detoxification in maize have been characterized (Marrs, 1996). All GST subunits in maize are encoded by distinct genes which are differentially regulated in response to treatment with herbicide safeners (Dixon *et al.*, 1998; Jepson *et al.*, 1994). Gronwald (1989) suggested that safeners may elevate GSH levels indirectly by inducing the activity of glutathione reductase which catalyses the reduction of oxidized glutathione (GSSG) to GSH, and that they maintain a high GSH/GSSG ratio in cells of protected plants to compensate the use of GSH as a reductant in glutathione conjugates. Safeners may also increase the synthesis of GSH by interfering with the normal feedback inhibition of glutamyl-cysteine synthase (Tal *et al.*, 1995). Hatzios (2000) showed that safeners can stimulate transport of glutathione conjugates of specific herbicides to the vacuole.

1.6. Insertional mutagenesis

Mutagenesis can be performed using UV radiation (Skowronek and Fiedurek, 2003) or chemicals such as ethylmethanesulfonate (Hsiao and Ko, 2001). However, these techniques do not allow recognition of the locus of the mutation. This can be achieved by integrating exogenous DNA into fungal cells by means of a vector, usually a plasmid, which contains a selectable marker and permits the selection of cells that have been successfully transformed. When uncharacterised genes have to be mutagenised, the integrative plasmid used must contain DNA that presents little or no homology with the host genome in order to prevent homologous recombination and to allow random insertion. Insertional mutagenesis requires that the target cells are made competent to take up the incoming vector DNA by rendering the fungal cell walls and membranes permeable to the DNA.

1.6.1. Restriction-Enzyme-Mediated Integration (REMI)

The REMI technique requires the presence of restriction endonucleases during the transformation process. This technique was first described by Schiestl and Petes (1991) who studied the mechanism of illegitimate recombination in the yeast *Saccharomyces cerevisiae*. They reported that the transformation frequency increased by a factor of seven compared with a conventional mutagenesis technique and that most of the integrations occurred at the site of the restriction enzyme used during transformation. The addition of restriction enzyme during genetic transformation in the presence of linearised integrative vector increased transformation frequency in the Ascomycetes *Cochliobolus heterostrophus* race T (Lu *et al.*, 1994), *M. grisea* (Shi and Leung, 1995) and *Alternaria alternata* (Akamatsu *et al.*, 1997). Restriction enzymes favour the integration of the transforming vector into the genomic DNA by cutting the host's DNA. Transformation efficiency may be increased by up to hundred-fold in various Ascomycetes including *Colletotrichum graminicola* (Thon *et al.*, 2000), *Gibberella fujikuroi* (Linnemannstöns *et al.*, 1999), *Aspergillus nidulans* (Sánchez *et al.*, 1998), *Mycosphaerella zeae-maydis* (Yun *et al.*, 1998), *A. alternata* (Akamatsu *et al.*,

1997), *Penicillium paxilli* (Itoh and Scott, 1997), *M. grisea* (Shi *et al.*, 1995) and *C. heterostrophus* (Lu *et al.*, 1994). Kuspa and Loomis (1992) reported that all restriction sites of the enzyme used are equally competent to integrate transforming DNA. However, cytotoxic effects have been observed when titres of restriction enzyme were too high as this leads to irreversible digestion of genomic DNA, hence to decrease in transformation efficiency (Sato *et al.*, 1998; Akamatsu *et al.*, 1997; Granado *et al.*, 1997; Shi *et al.*, 1995). Moreover, the restriction enzymes themselves can cause genomic mutations owing to repair errors. Mutations unrelated to the site of integration of the plasmid have been observed in *A. nidulans* and *M. zeae-maydis* (Sánchez *et al.*, 1998 and Yun *et al.*, 1998, respectively).

The mechanism by which REMI occurs is still not fully understood but it is thought to follow three main steps: (i) the linearised plasmid and the restriction enzyme pass through both the cytoplasmic and the nuclear membranes of the cell during transformation; (ii) the restriction enzyme cleaves the host genome at specific sites, creating chromosomal breaks; and (iii) the plasmid integrates randomly into these breaks by *in vivo* ligation (Riggle and Kumamoto, 1998). Most of the time, the restriction recognition sites at both ends of the plasmid are restored. However, different patterns of plasmid integration can occur when restriction sites are not correctly restored after plasmid integration (Bölker *et al.*, 1995; Shi and Leung, 1995). Sweigard (1996) reported, after sequencing the plasmid/genomic DNA junction in rescued plasmids in *M. grisea*, that the original vector ends do not remain intact.

The presence of restriction enzyme in REMI limits the number of plasmid integrations (Granado *et al.*, 1997; Itoh and Scott, 1997; Lu *et al.*, 1994; Kuspa and Loomis, 1992; Schiestl and Petes, 1991), the formation of concatemers (Bölker *et al.*, 1995) and increases the frequency of single-copy integration events (Hirano *et al.*, 2000; Redman *et al.*, 1999; Granado *et al.*, 1997). The use of restriction enzyme also enhances stability and efficiency of integrations by linearising the plasmid (Yakoby *et al.*, 2001; Thon *et al.*, 2000; Linnemannstöns *et al.*, 1999; Lu *et al.*, 1994; Redman and Rodriguez, 1994; Rodriguez, 1994; Kuspa and Loomis, 1992).

In order to facilitate the study of the mutations created by REMI, the production of unicellular and mononuclear structures, namely protoplasts, is required. Protoplasts are obtained by digesting the cell walls of young mycelia, spores or germlings with lytic enzymes. They must be produced in the presence of an osmotic stabiliser, consisting of high salt or sugar concentration, to prevent cell lysis. Protoplasts are exposed to DNA in the presence calcium ions ($CaCl_2$), which induce permeability to DNA and polyethylene glycol, which promotes DNA uptake (Fincham, 1989).

The REMI technique has been applied successfully to pathogenic fungi in order to identify pathogenicity-related genes or genes required for toxin biosynthesis (Idnurm and Howlett, 2001), particularly in *M. grisea* (Balhadère *et al.*, 1999; Sweigard *et al.*, 1998; Shi and Leung, 1995; Shi *et al.*, 1995), *C. albicans* (Brown *et al.*, 1996) and *C. graminicola* (Epstein *et al.*, 1998). This technique has allowed the efficient isolation of genes important for pathogenicity from several fungal pathogens (Balhadère *et al.*, 1999; Linnemannstöns *et al.*, 1999; Redman *et al.*, 1999; Sweigard *et al.*, 1998; Yun *et al.*, 1998; Akamatsu *et al.*, 1997; Granado *et al.*, 1997; Bölker *et al.*, 1995). Kim *et al.* (2002) showed that a sterol glycosyl transferase produced by *Colletotricum gloeosporioides* is a virulence factor by creating mutants of the fungus in which the gene encoding this factor was disrupted. These mutants had decreased virulence for avocado fruits. Similarly, *Fusarium graminearum* mutants with a disrupted *tri5* gene encoding a trichodiene synthase that catalyzes the first reaction in the trichothecene pathway were shown to be drastically reduced in virulence (Bai *et al.*, 2001; Desjardins *et al.*, 1996; Proctor *et al.*, 1995).

After REMI mutagenesis, the recovery of genomic sequences flanking the inserted plasmid is facilitated by the presence of known restriction sites flanking the tagged sequence. However, although REMI represents an efficient tool for tagging and cloning pathogenicity genes from fungal pathogens, its efficiency seems to be significantly limited due to a substantial portion (20 to 100%) of generated mutants that appear to be untagged by the transforming DNA (Balhadère *et al.*, 1999; Linnemannstöns *et al.*, 1999; Epstein *et al.*, 1998; Sánchez *et al.*, 1998; Sweigard *et al.*, 1998). Moreover, Redman *et al.* (1999) and Sweigard *et al.* (1998) reported the production of mutants with complex integration events (tandem or multiple REMI events, or illegitimate integration when the integration did not restore either one or

both restriction sites) in *Colletotrichum magna* and *M. grisea*, respectively, instead of REMI events (single integrations in the sites of the restriction enzyme used).

1.6.2. Electroporation

In this technique a transient electric pulse is applied to create temporarily some pores in the cell membrane of the host in order to allow the uptake of DNA (Lurquin, 1997). Three electrical parameters that can be separately adjusted influence the value of the current: the voltage, the capacitance and the resistance. The cell membrane is perforated at a given voltage, enhancing permeability to macromolecules, but membrane integrity is restored after termination of the pulse (Lurquin, 1997). The electrical parameters vary according to the type of cells to be electroporated.

Electroporation has been recently employed with success to transform the Ascomycetes *Beauvaria bassiana* (Sandhu *et al.*, 2001), *Thermomyces lanuginosus* (Chadha *et al.*, 2000), *C. gloeosporioides f. sp aeschynomene* (Robinson and Sharon, 1999), *Aspergillus fumigatus* (Brown *et al.*, 1998), *Curvularia lunata* (Długoński and Wilmánska, 1998) and *Humicola grisea* var. *thermoidea* (Dantas-Barbosa *et al.*, 1998). It was performed with three types of cells: ungerminated conidia (Brown *et al.*, 1998), germinated conidia (Chadha *et al.*, 2000; Robinson and Sharon, 1999; Dantas-Barbosa *et al.*, 1998; Sánchez and Aguirre, 1996; Ozeki *et al.*, 1994; Shi and Leung, 1994) and protoplasts (Sandhu *et al.*, 2001; Długoński and Wilmánska, 1998; Tsai *et al.*, 1992).

1.6.3. Particle bombardment

This technique is more commonly called biolistics (for biological ballistics). Microprojectiles, generally of tungsten or gold, are coated with plasmid DNA and accelerated at high velocity directly into fungal spores or hyphae. This technique was originally developed to transform plant cells (Datta *et al.*, 2001; Kemp *et al.*, 2001; Schaffrath *et al.*, 2000). It has also been successfully applied to a number of

Ascomycetes such as *A. nidulans* (Barcelos *et al.*, 1998; Fungaro *et al.*, 1997; Herzog *et al.*, 1996), *Trichoderma reesei* (Hazell *et al.*, 2000), *Cercospora caricis* (Aly *et al.*, 2001) and the three phytopathogenic fungi *Cryphonectria parasitica*, *Valsa ceratosperma* and *Phomopsis* G-type (Sasaki *et al.*, 2002).

1.6.4. *Agrobacterium tumefaciens*-mediated transformation

Agrobacterium tumefaciens is a gram-negative soil bacterium that causes crown gall tumors at wound sites of infected dicotyledonous plants (Hooykaas and Beijersbergen, 1994; Zambryski, 1992). The *Agrobacterium*-plant cell interaction is the only known natural example of DNA transfer between kingdoms (Sheng and Citovsky, 1996). During tumor induction, *Agrobacterium* transfers parts of its 200 kb tumour-inducing (Ti) plasmid, the T-DNA (transferred DNA), to plant cells (Zhu *et al.*, 2000; Hooykaas and Beijersbergen, 1994). The oncogenic T-DNA, flanked by 25 bp direct repeats called the left and right borders, integrates subsequently into the plant nuclear genome as a single-strand DNA at random sites in the plant genome. The transfer of T-DNA to the plant nucleus requires the induction of an operon of virulence (*vir*) genes, which are also located on the Ti plasmid and composed of seven genes (*virA, virB, virC, virD, virE, virG* and *virH*). Under induction by compounds secreted from wounded plant cells, such as acetosyringone, the *vir* genes synthesise virulence proteins (Mullins *et al.*, 2001; Pardo *et al.*, 2002; Rho *et al.*, 2001). The expression of the integrated oncogenes present in the T-DNA results in loss of cell division control in the plant and, ultimately, in the formation of crown gall tumors (Christie, 1997).

Phenolic compounds are sensed by the kinase VirA associated with the bacterial inner membrane. This chemical stimulus induces VirA auto phosphorylation, resulting in the phosphorylation of the response regulator VirG (Winans, 1992). Phosphorylated VirG activates *vir* promoters which autoregulate positively the *vir* regulon (Sheng and Citovsky, 1996; Winans *et al.*, 1994; Stachel and Zambryski, 1986). The virulence proteins VirE2 and VirD2 mediate the nuclear import of the T-complex into the cell's nucleus (Rossi *et al.*, 1996). VirD2 is an endonuclease that

cleaves the bottom strand of the T-DNA at four nucleotides in each border sequence in the presence of VirD1 and produces a single strand copy of the T-DNA, called the T-strand (Filichkin and Gelvin, 1993; Stachel *et al.*, 1986). VirD2, which is essential for the precise ligation of the right border into the host genome (Tinland, 1996), covalently links to the 5' end of the released T-DNA, forming the T-complex. This complex is coated with the single-stranded DNA-binding protein VirE2 (Zupan *et al.*, 2000) which provides protection from nucleolytic degradation of the T-strand that can occur in the plant cytoplasm (Binns, 2002; Tzfira and Citovski, 2002; Gelvin, 2000; Zupan *et al.*, 2000). Genetic evidence showed that VirE2 is transferred separately from the T-DNA to the plant cell cytoplasm where they form a complex (Sundberg *et al.*, 1996). The export of VirE2 requires VirE1, which acts as a chaperone (Deng *et al.*, 1999; Sundberg *et al.*, 1996), and the transfer of T-DNA requires VirC1, VirC2 and the ATPase activity of VirB4 and VirB11 providing the energy required (Shirasu *et al.*, 1994; Christie *et al.*, 1988). The intercellular transport of the T-complex from the bacterium to the cytoplasm of the host plant cell occurs through a channel composed of the proteins VirB and VirD4 (Christie and Vogel, 2000; Gelvin, 2000; Tzfira *et al.*, 2000; Zupan *et al.*, 2000). The T-strand integrates into the host genome at random sites by illegitimate recombination (Mayerhofer *et al*, 1991). Genetic studies using deletion mutants showed that the transfer of the T-DNA is polar from the right to the left border repeats (Sheng and Citovsky, 1996). The right border is essential for the *cis*-activation of the transfer of the T-DNA, whereas the left border only delimits the extremity of the T-DNA (Jen and Chilton, 1986; Caplan *et al.*, 1985).

The natural virulence system of *A. tumefaciens* has been used to transform eukaryotes. *A. tumefaciens* cells containing a binary vector system composed of a T-DNA donor plasmid (Ti plasmid without any *vir* genes) and a helper plasmid harboring the *vir* operon are used for this purpose (Sheng and Citovsky, 1996). In these conditions, a *trans*-activation of the T-DNA by the *vir* operon takes place. Given that the T-DNA is defined by its borders, the coding region of the T-DNA can be replaced by genes of interest, for instance the gene for resistance to hygromycin that will tag the mutated gene, without affecting its transfer to the host.

The *Agrobacterium* transformation system has been used for more than 25 years in plants. It has been developed for gene transfer to a wide variety of plants, especially *Arabidopsis thaliana* (Clough and Bent, 1998; Koncz *et al.*, 1992; Feldman, 1991) as well as important crops such as rice (Hiei *et al.*, 1997) and maize (Ishida *et al.*, 1996). Besides plants, transformable hosts include the yeasts *S. cerevisiae* (Bundock *et al.*, 2002; Piers *et al.*, 1996; Bundock *et al.*, 1995) and *Kluyveromyces lactis* (Bundock *et al.*, 1999), and more recently human cells (Kunik *et al.*, 2001) and filamentous fungi, particularly the Ascomycetes *Venturia inaequalis* (Fitzgerald *et al.*, 2003), *Calonectria malonii* (Malonek and Meinhardt, 2001), *F. oxysporum* (Mullins *et al.*, 2001), *Fusarium circinatum* (Covert *et al.*, 2001), *M. grisea* (Rho *et al.*, 2001), *Coccidioides immitis* (Abuodeh *et al.*, 2000) and *Aspergillus awamori* (Gouka *et al.*, 1999; de Groot *et al.*, 1998). In these various hosts, DNA transfer to fungal nuclei seems to happen via the same mechanisms as to plant nuclei. Whereas the whole machinery for the transfer of the T-DNA from *A. tumefaciens* to the fungus seems to be provided by the bacterium, the mode of T-DNA integration into the genome is determined by host factors (Pardo *et al.*, 2002; Bundock *et al.*, 1999). The molecular mechanism of integration of T-DNA by illegitimate recombination seems conserved in higher and lower eukaryotes (Bundock and Hooykaas, 1996).

A. tumefaciens is able to transform different physiological structures of Ascomycetes, such as protoplasts (de Groot *et al.*, 1998), hyphae, conidia (Degefu and Hanif, 2003; Covert *et al.*, 2001; Malonek and Meinhardt, 2001; Mullins *et al.*, 2001; Rho *et al.*, 2001; Abuodeh *et al.*, 2000; de Groot *et al.*, 1998) and mycelium (Fitzgerald *et al.*, 2003).

1.7. Aims of the work

The work presented in this thesis was part of a European Union project that aimed to develop integrated pest management techniques for the control of Ascochyta blight of chickpea. This thesis focuses specifically on the resistance of chickpea plants and the amelioration of disease symptoms. The selection of cultivars of chickpea that are most resistant to Ascochyta blight could be facilitated by the use of the phytotoxin

solanapyrone A. A prerequisite for such selection was to ascertain the potential involvement of the solanapyrone A phytotoxin in the pathogenicity or the virulence of *A. rabiei,* hence in the severity of Ascochyta blight symptoms. This can be achieved by producing toxin-minus mutants and by confirming the reduction or loss of their virulence compared to the parent strain. The reduction of the disease symptoms in an economic and environmentally friendly manner was sought to be accomplished by the use of safeners. Therefore, the two main objectives of this work were:

(i) to develop an efficient transformation system in order to create a library of mutants of *A. rabiei* by random insertional gene inactivation and to select for toxin-minus mutants. This objective required the optimisation of the germination of pycnidiospores of the fungus, the production of protoplasts and the implementation of a reliable screening technique for the production of solanapyrone A;

(ii) to test the effect of different safeners on the sensitivity of chickpea to solanapyrone A using bioassays. The production of a large amount of solanapyrone A was essential to carry out the bioassays.

Chapter 2

Culture of *Ascochyta rabiei* and production of protoplasts from the fungus

2.1. Introduction

Since a major aim of the project described in this thesis was to produce toxin-minus mutants it was necessary to establish techniques for maintaining the fungus in a genetically stable form and for routine culture. In addition, since two of the mutagenesis techniques required protoplasts as fungal material, it was necessary to define conditions under which viable protoplasts of *A. rabiei* could be produced and regenerated into mycelium.

One way of maintaining the genetic stability of fungi is to store them as spore suspensions in liquid nitrogen (Costa and Ferreira, 1991). Isolates of *A. rabiei* have been maintained as pycnidiospore suspensions in 10% glycerol as cryoprotectant at the temperature of liquid nitrogen for many years and when such pycnidiospores are plated on agar media the fungus resumes growth (Strange, personal communication). The mutagenesis techniques described in Chapter 5 required germinated or ungerminated pycnidiospores, young mycelium or protoplasts. Therefore, in this Chapter, techniques for the production, storage and germination of pycnidiospores are described as well as protocols for obtaining protoplasts.

Germination of pycnidiospores of *A. rabiei* has been previously reported by Weltring *et al.* (1995) but not its efficiency.

Fungal cell walls are mainly composed of the polysaccharides glucan and chitin, which are responsible for maintaining mycelial integrity (Farkaš, 2003; Roncero, 2002; Hon, 1996; Wessels, 1993; Ruiz-Herrera *et al.*, 1992). Protoplasts are obtained by digesting the cell walls of the fungi with lytic enzymes that degrade these polysaccharides. Most protoplasts are mononucleate, which facilitates genetic analyses and particularly insertional mutagenesis studies. Although the literature

describes various protocols for the production and regeneration of fungal protoplasts, each fungal group, species or even strain requires specific conditions for protoplasting because of the diversity of the composition of their cell walls (Jung *et al.*, 2000; Lalithakumari, 1996). Three main variables affect the production of fungal protoplasts: the development stage of the fungi, the lytic enzymes and the osmoticum. Weltring *et al.* (1995) and Köhler *et al.* (1995) used protoplasts for the genetic transformation of *A. rabiei* with the pisatin demethylase or the β-glucoronidase (GUS) gene, respectively. However, they did not give any information about the efficiency of protoplast production. Consequently, a step-by-step approach for protoplasting *A. rabiei* was taken in order to develop empirically an efficient method.

2.2. Materials and Methods

2.2.1. Fungal strain

As the Tk-21 isolate (obtained from the University of Ankara, Turkey) of *A. rabiei* produces high toxin titres compared with other strains of *A. rabiei*, it was used in all the experiments described in this thesis (Dolar and Strange, unpublished).

2.2.2. Pycnidiospore production and storage

Chickpea seeds (Cypressa, Katsouris Brothers Ltd, U.K., *ca.* 60) were softened by boiling in 250 ml conical flasks containing water for 30 min and, after draining, sterilised by autoclaving for 20 min at 121°C. They were inoculated with 300 µl of a pycnidial suspension of *A. rabiei* (10^7 spores.ml^{-1}), gently shaken to ensure a good distribution of the inoculum and incubated at room temperature until pycnidia appeared (10 to 12 d). Addition of sterile distilled water to the flasks after incubation resulted in a spore suspension free of mycelium which was decanted. Pycnidiospores were pelleted by centrifugation at 1,000 *g* for 10 min and washed 3 times with sterile distilled water. They were finally resuspended in sterile 10% glycerol at 10^7

spores.ml^{-1} and stored in liquid nitrogen.

2.2.3. Pycnidiospore germination

Stored pycnidiospores. Pycnidiospores of *A. rabiei* (10^6.ml^{-1} medium) that had been stored in liquid nitrogen for 1 year were inoculated to 50 ml of the following media contained in 250 ml conical flasks: Czapek-Dox Cation Liquid Medium (CDCLM) with or without glucose (1 or 3%), chickpea extract broth with or without glucose (1 or 3%), and yeast extract peptone (YEP) broth containing 3% glucose (for formulae, see Appendix 1). They were incubated at 20°C on an orbital shaker rotating at 160 rpm (orbit diameter 5 cm) or in an incubator without shaking for up to 24 h.

Fresh pycnidiospores. Pycnidiospores (10-fold dilutions from 10^9 to 10^6.ml^{-1} medium) were inoculated to 50 ml of the same media as for stored pycnidiospores and to 50 ml of Richards' medium (Appendix 1) with or without glucose (1 or 3%). They were incubated in the same conditions as before for up to 35 h. The effects on germination of a heat shock for 30 min at 40°C or 45°C, the addition of 5 mM $CaCl_2$ to YEP containing 3% glucose , and washing the pycnidiospores (10^6.ml^{-1}) once in ice-cold ultra-pure water and twice in the same medium as the one used for germination were also tested.

Germination of both stored and fresh pycnidiospores was monitored at 2 h intervals by transferring aliquots in duplicate from the culture flasks (four batches) into a haematocymeter (Hausser Scientific, Horsham, P.A., U.S.A.) and observing the numbers germinated under the microscope. Pycnidiospores were considered germinated if the length of the emerging germ tube was at least that of the pycnidiospore (about 12.5 µm).

2.2.4. Protoplast production

Protoplast production was attempted with mycelium, ungerminated pycnidiospores

and germinated pycnidiospores as follows:

Mycelium. Czapek-Dox-V8 broth (CD-V8 broth, Appendix 1; 100 ml in 250 ml flasks) was inoculated with pycnidiospores (100 µl per flask at 10^7 spores.ml^{-1}). Cultures were incubated at 20°C for 72 h on an orbital shaker at 120 rpm (orbit diameter 5 cm) to ensure good aeration of the medium. The culture was filtered through four thicknesses of muslin and the collected mycelium was homogenised in a small volume of fresh CD-V8 broth in a blender (Sorvall Omni-mixer, Newton, USA; 3 pulses; 5 seconds each). The homogenate was added to CD-V8 broth in a 250 ml flask to give a culture volume of 100 ml and incubated for 48 h on the shaker. Filtration, homogenisation of the mycelium and re-inoculation of CD-V8 medium was repeated but the incubation periods was reduced to 24 h in order to obtain young mycelium suitable for protoplasting. Approximately 1 g of fresh mycelium was filtered as previously and rinsed with sterile distilled water followed by MN buffer (1.2 M $MgSO_4$; 10 mM Na_2HPO_4/NaH_2PO_4; pH 5.8). The mycelium was suspended in the combinations of osmoticum, buffer and enzyme specified in Table 2.1 and incubated at 30°C for up to 8 h on an oscillating shaker (70 oscillations per min; 61 cm throw).

Ungerminated pycnidiospores. Freshly produced pycnidiospores (5 X 10^6 pycnidiospores per ml) were suspended in the solutions specified in Table 2.1 and incubated as for mycelium.

Germinated pycnidiospores. Freshly produced pycnidiospores (5 X 10^7 in 50 ml of YEP broth (Appendix 1) containing 3% glucose) were germinated for 18-20 h. They were recovered by centrifugation at 1,000 *g* for 10 min, suspended in the solutions specified in Table 2.1 at 5 X 10^6 per ml and incubated as for mycelium.

For each experiment the time course of protoplast release was monitored by microscopy. Protoplast viability was assessed by plating serial dilutions on agarised medium containing 0.6 M sucrose, 1% yeast extract and 2% peptone (pH 5.0), immediately or after incubation in complete medium (10 g.l^{-1} glucose; 2 g.l^{-1} peptone; 1 g.l^{-1} yeast extract; pH 6.5) at 20°C overnight.

2.3. Results

2.3.1. Pycnidiospore germination

Stored pycnidiospores. Pycnidiospores that were stored in liquid nitrogen for 1 year and inoculated at 10^6 pycnidiospores.ml^{-1} medium did not germinate at 20°C over a period of 24 h whether they were incubated in either CDCLM or chickpea extract broth, supplemented or not with glucose (1 or 3%) and submitted or not to agitation. In contrast, when incubated at 10^6 pycnidiospores.ml^{-1} and 20°C, such spores reached a maximum germination rate of 29.8% in YEP broth (1% yeast extract; 2% peptone; pH 5.0) containing 3% glucose for 24 h.

Fresh pycnidiospores. No germ tubes were formed when freshly produced pycnidiospores (10^6 pycnidiospores.ml^{-1}) were incubated in CDCLM, Richards' medium or Richards' medium supplemented with glucose (1% or 3%) at 20°C over a period of 24 h.

A rich medium such as YEP broth containing 3% glucose was able to stimulate the production of germ tubes (Figure 2.1). In these conditions, pycnidiospore germination (10^6 per ml medium) exceeded 80% between 14 and 20 h incubation, whether the pycnidiospores were washed or unwashed (Figure 2.2). The addition of 5 mM $CaCl_2$ or application of a heat shock did not enhance the germination rate of washed and unwashed pycnidiospores.

However, pycnidiospores did not germinate in YEP broth containing 3% glucose when their concentrations were 10^7, 10^8 or 10^9 per ml.

Starting material	Osmoticum	Enzyme	Result
Mycelium (1)	MN buffer (1.2 M $MgSO_4$; 10 mM Na_2HPO_4/NaH_2PO_4; pH 5.8)	Novozym 234	No protoplasts, fragmented cell walls
Mycelium (2)	MN buffer [a]	Novozym 234 + Driselase	No protoplasts, fragmented cell walls
Mycelium (3)	MN buffer [a]	Novozym 234 + Chitinase	No protoplasts, fragmented cell walls
Mycelium (4)	MN buffer [a]	Novozym 234 +Driselase+Chitinase	No protoplasts, fragmented cell walls
Mycelium (5)	MN buffer with 0.5 M $MgSO_4$	Novozym 234	No protoplasts, fragmented cell walls
Mycelium (6)	MN buffer with 1.0 M Mg SO_4	Novozym 234	No protoplasts, fragmented cell walls
Mycelium (7)	MN buffer with 1.5 M Mg SO_4	Novozym 234	No protoplasts, fragmented cell walls

Mycelium (8)	0.6 M mannitol	Novozym 234	No protoplasts, fragmented cell walls
Mycelium (9)	0.6 M sucrose	Novozym 234	No protoplasts, fragmented cell walls
Mycelium (10)	0.6 M sorbitol	Novozym 234	No protoplasts, fragmented cell walls
Ungerminated pycnidiospores (1)	MN buffer [a]	Novozym 234	No protoplasts
Ungerminated pycnidiospores (2)	0.6 M mannitol	Novozym 234	No protoplasts
Ungerminated pycnidiospores (3)	0.6 M sucrose	Novozym 234	No protoplasts
Ungerminated pycnidiospores (4)	0.6 M sorbitol	Novozym 234	No protoplasts

Germinated pycnidiospores (1)	MN buffer [a]	Novozym 234	No protoplasts
Germinated pycnidiospores (2)	0.6 M mannitol	Novozym 234	No protoplasts
Germinated pycnidiospores (3)	0.6 M sucrose	Novozym 234	No protoplasts
Germinated pycnidiospores (4)	0.6 M sorbitol	Novozym 234	No protoplasts
Germinated pycnidiospores (5)	KC buffer (0.7 M KCl; 0.2 M $CaCl_2$; pH 6.0)	Driselase (10 $mg.ml^{-1}$)+ Cellulase Onozuka R-10 (5 $mg.ml^{-1}$)	2.72×10^7 protoplasts.ml^{-1}

Table 2.1. Composition of lytic solutions and osmotica used to produce protoplasts from mycelium, ungerminated and germinated pycnidiospores of *A. rabiei*. Various lytic enzymes were tested alone or in combinations in different osmotica. Novozym 234 was used at a concentration of 5 or 10 $mg.ml^{-1}$ with mycelium and 5, 10 or 20 $mg.ml^{-1}$ with ungerminated or germinated pycnidiospores. Novozym 234, Driselase and chitinase were manufactured by Sigma and Cellulase Onozuka R-10 by Yakult Pharmaceutical Ind., Co, Ltd, Japan. Experiment numbers are mentioned into parentheses.

[a] In these experiments MN buffer contained 1.2 M $MgSO_4$.

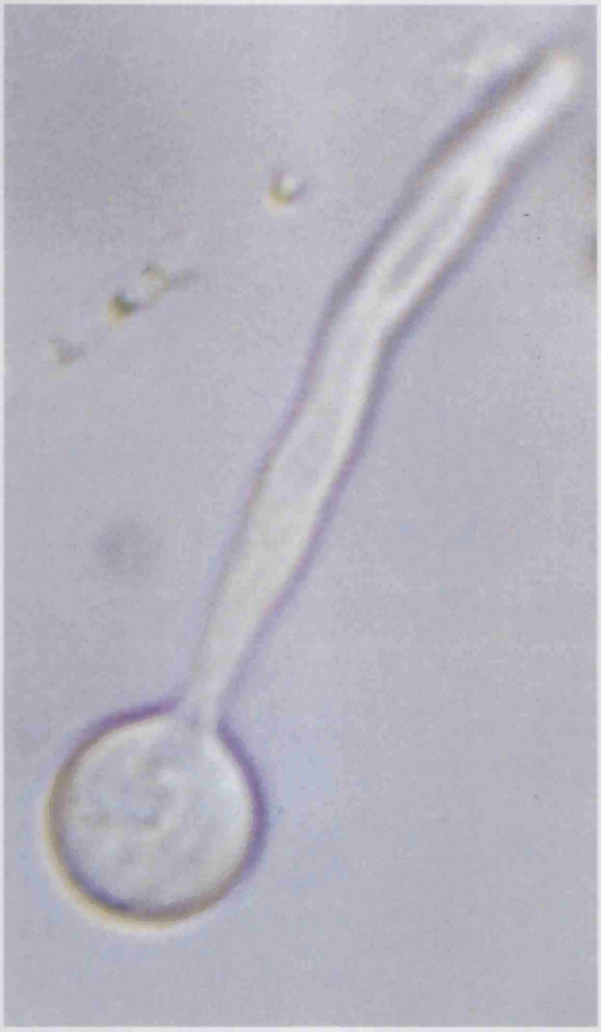


Figure 2.1. A pycnidiospore of *A. rabiei* germinating after inoculation in YEP broth (Appendix 1) containing 3% glucose and incubation on an orbital shaker at 20°C for 14 h.

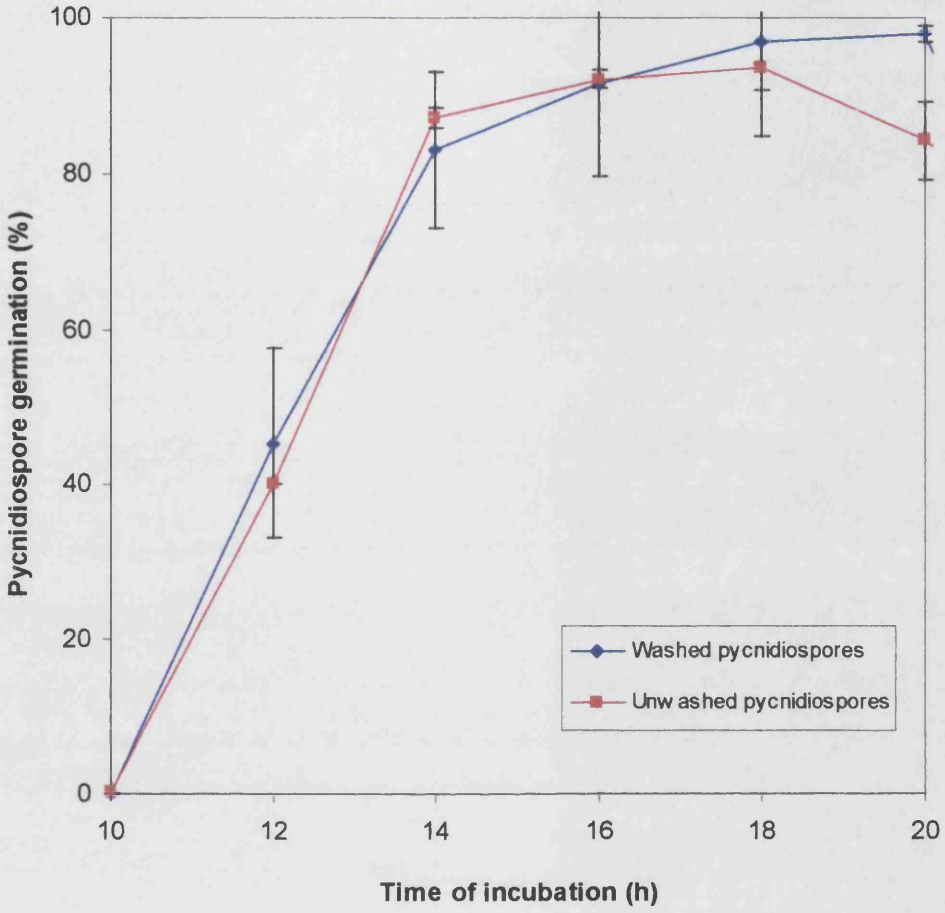


Figure 2.2. Time course of germination of pycnidiospores of *A. rabiei*. YEP broth containing 3% glucose was inoculated with freshly produced pycnidiospores (10^6 per ml in 50 ml medium final concentration) and incubated at 20°C on an orbital shaker. Each value represents the mean of eight readings (four batches for which each measure was taken in duplicate) and is expressed as a percentage.

2.3.2. Protoplast production

Mycelium. Young mycelium of *A. rabiei*, incubated in MN buffer containing Novozym 234 alone or combined with Driselase and / or chitinase, did not form any protoplasts (Table 2.1). The lytic enzymes, used alone or in combination, degraded the walls of 24- to 72-hour-old mycelium, leading to the release of the contents of the hyphae rather than releasing viable protoplasts. Protoplasting was also unsuccessful with mycelium grown and homogenised as for the production of young mycelium, except that the last growing step for 24 h was omitted, and submitted to the action of the same lytic solutions.

No protoplasts were produced with homogenised mycelium incubated with Novozym 234 (10 $mg.ml^{-1}$) dissolved in MN buffer containing from 0.5, 1.0 or 1.5 M $MgSO_4$. Identical results were obtained using 0.6 M mannitol or 0.6 M sucrose as osmoticum (Table 2.1).

Ungerminated pycnidiospores. No protoplasts were obtained when freshly produced pycnidiospores were incubated with Novozym 234 (5; 10 or 20 $mg.ml^{-1}$) dissolved in MN buffer as osmoticum. Other osmotica tried without success were 0.6 M sucrose, 0.6 M mannitol and 1 M sorbitol.

Germinated pycnidiospores. Freshly germinated pycnidiospores (18-20 h) did not produce any protoplasts when incubated with Novozym 234 (5; 10 or 20 $mg.ml^{-1}$) dissolved in MN buffer or the three other osmotica mentioned above. In contrast, digestion of germinated pycnidiospores with Driselase and Cellulase Onozuka R-10 suspended in KC osmoticum generated up to 2.72 X 10^7 protoplasts per ml of lytic solution, representing 5.44 protoplasts released per pycnidiospore (Figure 2.3).

Direct plating of 5 X 10^4 protoplasts allowed the regeneration of only 650 protoplasts (1.3% regeneration). Incubating protoplasts in complete medium at 20°C overnight enhanced their viability to 5.9%. Regenerants were morphologically identical with the parent strain.

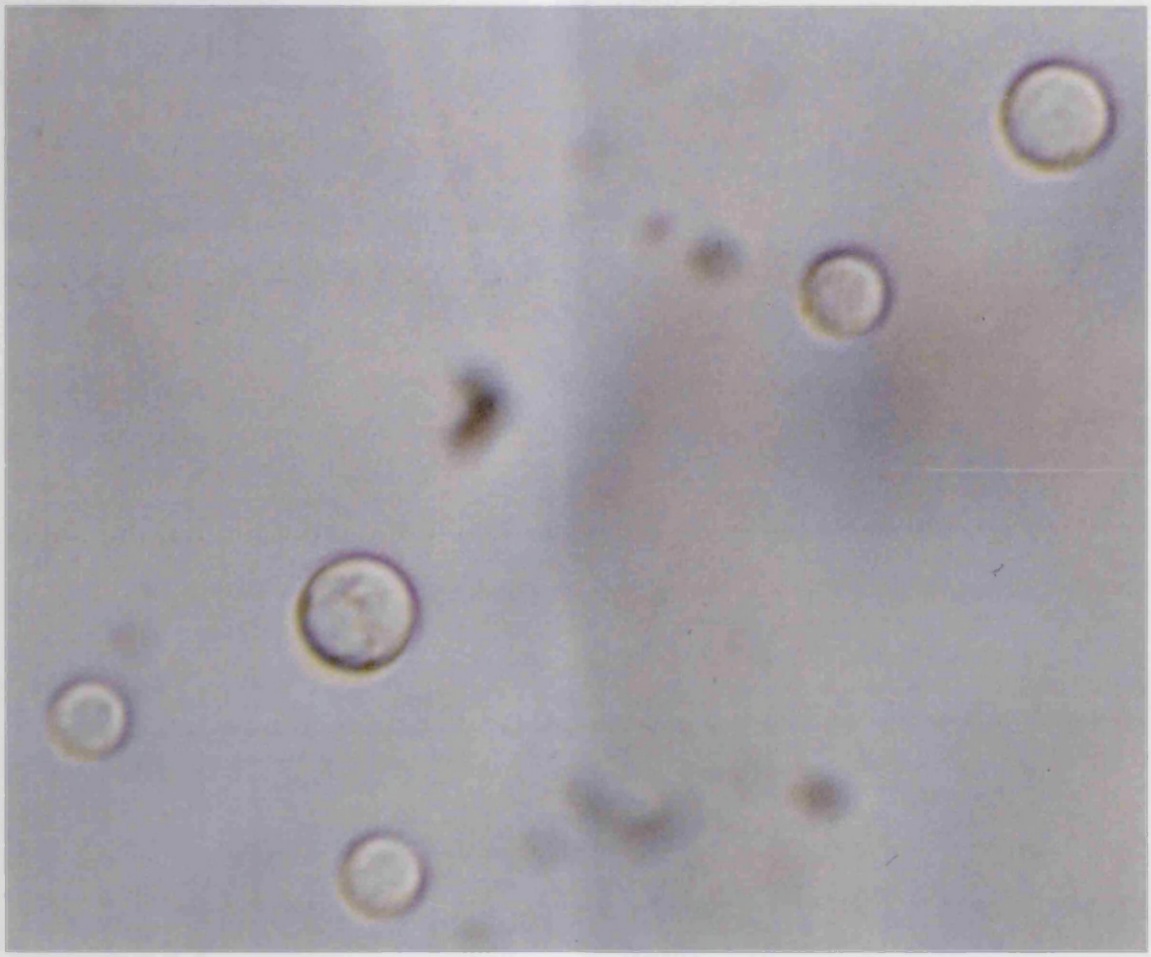


Figure 2.3. Protoplast production with *A. rabiei.* Germinated pycnidiospores (5 X 10^6 per ml germination medium) were incubated in a mixture of Driselase (10 $mg.ml^{-1}$) and Cellulase Onozuka R-10 (5 $mg.ml^{-1}$) dissolved in an osmotic stabiliser (0.7 M KCl, 0.2 M $CaCl_2$, pH 6.0) and incubated at 30°C on an oscillating shaker.

2.4. Discussion

2.4.1. Pycnidiospore germination

Germination of *A. rabiei* required a relatively long time (18-20 h) in YEP broth containing 3% glucose due to the slow growth of the fungus.

Dormancy in spores may be exogenous or endogenous, depending if it is influenced by environmental factors such as temperature, nutrients or application of a heat shock, or by the intrinsic physiology of the spores.

Exogenous dormancy. Storage of spores at low temperature affects their germination. James (2001) and Alves *et al.* (1996) reported that germination of fresh conidia of *Beauveria bassiana* was significantly higher (22-25%) than that of conidia stored at 5-7°C for up to 22 months (15%), after incubation for 12 to 14 h. Pycnidiospores of *A. rabiei* previously stored in liquid nitrogen did not germinate in CDCLM or chickpea extract broth, containing glucose or not, over a period of 24 h. Growth of mycelium was observed after 5-7 d after inoculation of stored pycnidiospores in 30 ml CDCLM, demonstrating that germination and vegetative development of stored pycnidiospores requires more than 24 h owing to their low metabolic activity during storage and that cryopreservation resulted in dormancy of the pycnidiospores.

Nutrients also affect exogenous dormancy of spores, as reported for pycnidiospores of *Coniothyrium minitans,* which germinated best on potato-dextrose agar and malt extract agar medium compared to other nutritive media (McQuilken *et al.*, 1997). Similarly, increased germination of conidia of *Beauveria bassiana* and *Paecilomyces fumosoroseus* increased in the presence of yeast extract and peptone (James, 2001). A nitrogen source was reported to be required for emergence of germ tubes of *Aspergillus niger* (Morozova *et al.*, 2001) and *Rhizopus oligosporus* (Breeuwer *et al.*, 1997). Spores of *A. rabiei* were reported to germinate in Richards' medium but after a minimum incubation time of 24 h (Weltring *et al.*, 1995). In the present study, pycnidiospores of *A. rabiei* did not germinate in CDCLM, chickpea extract broth or Richards' medium over a period of 24 h, whether stored or freshly produced. This

result suggests that the development of germ tubes may require a longer time in these media and that this process may occur within 24 h if the germination medium was enriched with other sources of carbon and nitrogen such as yeast extract and peptone. This was confirmed when the germination rate of stored or fresh pycnidiospores reached about 30% or exceeded 80%, respectively, within 24 h in YEP broth containing 3% glucose.

Shaw and Hoch (2000) demonstrated that germination of pycnidiospores of *Phyllosticta ampelicida* was initiated earlier and was maximal at high concentration of calcium (millimolar level). However, addition of calcium ions (5mM) in YEP broth containing 3% glucose did not improve the germination rate of *A. rabiei.* Similarly, pycnidiospores of most fungi do not require the presence of calcium ions to germinate.

Martínez-Cadena *et al.* (1995) reported that germination of sporangiospores of *Phycomyces blakesleeanus* was activated by heat shock at 48°C for 20 min. Similarly, basidiospores of *Agaricus bisporus* germinated after heat activation at 45°C for 20 min. This result contrasts with that of pycnidiospores of *A. rabiei* as a heat shock did not improve their germination rate. The temperature used may not have been high enough.

Endogenous dormancy. The spores of numerous fungal species do not germinate *en masse.* Self-inhibitors, produced within the fruiting body or the spore, cause endogenous dormancy of spores. They may prevent spore germination prior to their dispersal to ensure their survival in fluctuating environmental conditions. Self-inhibition of germination was reported to be spore density dependent (Ayliffe *et al.*, 1997). For instance, germination of conidia of *C. graminicola* was inhibited when they were inoculated at a titre of 10^7 per ml medium but reached 40% and 80% when the titre was 10^6 and 10^5 per ml, respectively (Leite and Nicholson, 1992). Self-inhibition also occurs in *Colletotrichum* spp. (Robinson and Sharon, 1999; Tsurushima *et al.*, 1995). Even though the mechanism of action of self-inhibitors is still not well understood, a number of self-inhibitors have been identified, in particular mycosporine-alanine in the mucilage of *C. gloeosporioides f. sp. jussiaea* and *C. graminicola* (Ayliffe *et al.*, 1997; Tsurushima *et al.*, 1995; Leite and

Nicholson, 1992), two compounds known as CG-SI1, 2 and 3 in *Colletotrichum gloeosporioides f. sp. Jussiaea* (Tsurushima *et al.*, 1995) and nonanoic acid in *Rhizopus oligosporus* which was also shown to prevent the increase of intracellular pH (Breeuwer *et al.*, 1997). The activity of inhibitors can be reversed by dilution (Robinson and Sharon, 1999; Ayliffe *et al.*, 1997; Leite and Nicholson, 1992). Pycnidiospores of *A. rabiei* germinated when their final concentration was 10^6 spores.ml^{-1}. A higher density of pycnidiospore suspension had a negative effect on germination, possibly owing to the production of inhibitors in the external matrix. Washing pycnidiospores of *A. rabiei* inoculated at a concentration higher than 10^6 per ml would confirm if they produce self-inhibitors, as germination rate in YEP medium containing glucose was not significantly improved with washed pycnidiospores when their final concentration was 10^6 per ml (Figure 2.2). The lack of germination at high pycnidiospore density may as well be due to decreased levels of oxygen or nutrient competition.

Different strains of *Melampsora lini* differed in their ability to germinate and produce a germination self-inhibitor (Ayliffe *et al.*, 1997). Similarly, even though spores of *A. rabiei* were reported to germinate in Richards' medium after incubation for more than 24 h (Weltring *et al.*, 1995), the strain Tk-21 of *A. rabiei* used in this work may possess a different capability for germinating than the strains used by these authors, possibly owing to differences in permeability of their membranes to nutrients.

2.4.2. Protoplast production

The physiological state of the fungus, the lytic solution and the osmotic stabiliser were limiting factors for the production and the regeneration of protoplasts of *A. rabiei*, as reported with various fungi (Campoy *et al.*, 2003; Robinson and Deacon, 2001; Cheng and Bélanger, 2000; Cheng *et al.*, 2000; Gallmetzer *et al.*, 1999; Redman *et al.*, 1999; Reyes *et al.*, 1998).

2.4.2.1. Physiological state

In the present investigation, mycelium, ungerminated pycnidiospores and germinated pycnidiospores were used in attempts to obtain viable protoplasts of *A. rabiei* that could be regenerated. However, only experiments with germinated pycnidiospores were successful.

Protoplasts from various filamentous fungi were produced from mycelium, especially the Ascomycetes, *Penicillium griseofulvum* (Muralidhar *et al.*, 2003), *Aspergillus nidulans* (Koukaki *et al.*, 2003), *Alternaria alternata* (Akamatsu *et al.*, 1997), *Aspergillus niger* (Shuster and Connelley, 1999). Mycelial homogenate was used as starting materials for protoplasting *Fusarium culmorum* (Doohan *et al.*, 1998) and *Venturia inaequalis* (Fitzgerald *et al.*, 2003). However, protoplasting of *A. rabiei* was not successful with mycelia, even though young cultures of the pathogen were used in order to prevent the mycelium becoming too dense and resistant to the action of lytic enzymes. One hypothesis that can explain this result is that the physiological state of the mycelium or the pycnidiospores, used as starting materials for protoplasting, may be inappropriate to enzymatic digestion. Generation of protoplasts was successful with germinated pycnidiospores of *A. rabiei,* suggesting that the cell wall of pycnidiospores of *A. rabiei* during the germination stage are thinner than that of mycelium. Similarly, among Ascomycetes, protoplasts of *Aspergillus nidulans* (Koukaki *et al.*, 2003) were produced from germinated spores, and spores of *Neonectria galligena* were shown to be more appropriate than mycelium for the generation of protoplasts (Tanguay *et al.*, 2003). With the latter, the number of protoplasts obtained was twice the number of conidiospores (Tanguay *et al.*, 2003), whereas, on average, more than 5 protoplasts per pycnidiospore were generated with *A. rabiei.* This value was due to the emergence of one or two germ tubes from each pycnidiospore.

2.4.2.2. Lytic enzymes

A large variety of lytic enzymes, used alone or in combination, have been reported in previous studies to allow the release of viable fungal protoplasts (Wiebe *et al.*, 1997). Among them, Novozym 234, a commercial preparation of enzymes from *Trichoderma spp.* containing principally glucanases and chitinases, has been widely used with Ascomycetes. Novozyme 234 was used with the Ascomycetes *Neonectria gallinea* (Tanguay *et al.*, 2003), *Colletotrichum gloeosporioides* (Yakoby *et al.*, 2001), *Aspergillus niger* (Shuster and Connelley, 1999), *Glomerella graminicola* (Epstein *et al*, 1998) and *Penicillium paxilli* (Young *et al.*, 1998). However, the composition of cell wall degrading enzymes that Novozym 234 contains varies significantly from one batch to another (Farina *et al.*, 2004; Jung *et al.*, 2000). Consequently, their lytic activity and their toxicity differ. Experiments with two batches of Novozym 234 did not result in the production of protoplasts from *A. rabiei.* Likewise, protoplasting of *Verticillium fungicola* with Novozym 234 was not very efficient and generated only 15-20 X 10^3 protoplasts.ml^{-1} from about 5 X 10^4 germinated spores (Amey *et al.*, 2002). The combination of Novozym 234 with Driselase and chitinase also did not yield any protoplasts with germinated pycnidiospores of *A. rabiei*, although Novozym 234 is rich in chitinases. Moreover, during the course of this work, Novozym 234 became unavailable. Consequently, other combinations of lytic enzymes had to be used. When Driselase (10 mg.ml^{-1}) was combined with Cellulase Onozuka R-10 (5 mg.ml^{-1}), many protoplasts were produced. Similarly, protoplasts of *Mycosphaerella spp.* have been produced with these two enzymes (Balint-Kurti *et al.*, 2001).

2.4.2.3. Osmoticum

Protoplasts are osmotically sensitive cells owing to the absence of cell walls. Thus, protoplast preparations have to be protected during enzymatic digestion by the presence of an osmotic stabiliser in the medium. The osmoticum employed protects protoplasts from rupture, hence playing an essential role in protoplast formation and regeneration. Various compounds, used in a wide range of concentrations, have been

proposed as osmotic stabilizers for fungal protoplast isolation and regeneration (Lalithakumari, 1996). Osmotic stabilisers used for fungal protoplasts include inorganic salts, sugars and sugar alcohols (Davis, 1985). It has been observed that inorganic salts are more effective with filamentous fungi, and sugar and sugar alcohols with yeasts and higher plants (Lalithakumari, 1996). Based on the published literature magnesium sulphate (Koukaki *et al*, 2003; Akamatsu *et al.*, 1999; Jones *et al.*, 1999; Young *et al.*, 1998), mannitol (Reyes *et al.*, 1998; Kim and Kim, 1997), sorbitol (Shuster and Connelley, 1999) and sucrose (Kim *et al.*, 2000) were first selected as osmotic stabilisers in order to determine the most suitable osmoticum for the isolation and the regeneration of protoplasts from *A. rabiei.* However, none of these stabilisers were effective for protoplasting *A. rabiei.* No difference in terms of protoplast production was observed when using a wide range of concentrations of $MgSO_4$ (from 0.5 M to 1.5 M). These osmotica may not be suitable for protoplasting *A. rabiei.* However, as they were all tested with Novozym 234 only, and as this enzyme did not produce any protoplasts, their protective role might have been appropriate with Driselase and Cellulase Onozuka R-10. Potassium chloride, used with these two enzymes, successfully protected protoplasts from *A. rabiei.* This osmoticum was also used for protoplasting other Ascomycetes such as *Trichothecium roseum* (Balasubramanian *et al.*, 2003), *Aspergillus nidulans* (Jung *et al*, 2000), *Trichoderma reesei* (Deane *et al.*, 1999), *Mycosphaerella zeae-maydis* (Yun *et al.*, 1998), *Paecilomyces fumosoroseus* (Barreto *et al.*, 1997), *Mycospaerella spp.* (Balint-Kurti *et al.*, 2001) and *Rhynchosporium secalis* (Rohe *et al.*, 1996).

2.4.2.4. Regeneration

Protoplast regeneration was increased 4.5 times when they were incubated in complete medium (Section 2.2.4) at 20°C overnight before plating compared with plating directly on regeneration medium. One explanation of this result may be the greater availability of nutrients in broth, and another the protection of the regenerating protoplasts from the oxidative properties of air. Similarly, regeneration of protoplasts from the plant pathogen *Rhynchosporium secalis* was enhanced when

protoplast suspensions were incubated for 3 d in Fries medium supplemented with sucrose and yeast extract before plating on selective medium (Rohe *et al.*, 1996).

Protoplast regeneration reached a maximum of 5.9% which is within the wide range for filamentous fungi of 0.1 – 50% reported by Peberdy (1991). For instance, low viability was obtained with *Lentinus lepideus* (3.28%, Kim *et al.*, 2000) *Lentinula edodes* (0.08%-0.18%, Kim and Kim, 1997) and *Hebeloma cylindrosporum* (2%, Debaud *et al.*, 1997), and high viability was achieved with *Metarhizium anisopliae* (43.6%, AbuBakar and Chua, 1996) and *Fusarium* spp. (53%, Solis *et al.*, 1996).

Osmoticum plays an important role in regeneration of protoplasts. Furlaneto *et al.* (1999) reported that 31% of protoplasts of *Metarhizium flavoviride* regenerated in the presence of sucrose compared, while only 15% regenerated in KCl, and none in the presence of 0.6 M mannitol or 1.0 M sorbitol. Sucrose has been reported to be commonly used with protoplasts of other filamentous fungi (Farina *et al.*, 2004; Cheng and Bélanger, 2000; Kim *et al.*, 2000; Rohe *et al.*, 1996). It was an efficient stabiliser that allowed the regeneration of protoplasts of *A. rabiei*.

Chapter 3

Production, purification and analysis of solanapyrone toxins

3.1. Introduction

Solanapyrone toxins have previously been produced in still culture (Chen and Strange, 1994), in a liquid medium (CDCLM) developed by Chen and Strange (1991). Production of a large amount of solanapyrone A, the most toxic of the three solanapyrones, was necessary in order to obtain enough material to test the sensitivity of cultivars of chickpea (this aspect of the work is developed in Chapter 6). Solanapyrone production was monitored in two types of still culture (in Roux bottles and conical flasks) as well as in shake culture and a fermenter.

Although the three solanapyrone toxins A, B and C have a related polyketide structure (Chapter 1, Figure 1.1), they can be separated, identified and quantified by reverse-phase High Performance Liquid Chromatography (HPLC) owing to their specific retention times and absorption spectra.

3.2. Materials and Methods

3.2.1. Growth of *A. rabiei*

3.2.1.1. Still culture in Roux bottles

Roux bottles (1 l) containing 100 ml CDCLM were inoculated with pycnidiospores of *A. rabiei* at a final dilution of 10^4 per ml medium and incubated at 20°C. Culture filtrates (100 ml from 3 bottles) were collected and pooled every 48 h, from 8 to 26 d post-inoculation. Solanapyrone toxins were purified by ethyl acetate partitioning (Section 3.2.2.2) and analysed by HPLC (Section 3.2.3).

3.2.1.2. Still culture in conical flasks

Conical flasks (250 ml) containing 30 ml CDCLM were inoculated with pycnidiospores (10^4.ml^{-1} final concentration) and incubated at 20°C without shaking. Samples of culture filtrates were collected in triplicates every 48 h from 8 to 24 d post-inoculation. Solanapyrone toxins were purified by SPE (Section 3.2.2.1), concentrated 50 times in acetonitrile and analysed by HPLC (Section 3.2.3).

3.2.1.3. Shake culture

Flasks (1 l) containg 250 ml CDCLM were inoculated with pycnidiospores (250 µl at 10^7 spores.ml^{-1}) or with mycelial homogenate (10 ml; OD_{620} = 1; produced as described in Chapter 2, Section 2.2.4) and incubated at 20°C on an orbital shaker rotating at 120 rpm (orbit diameter18.5 cm). Cultures were sampled (5 ml samples) at various time intervals according to the growth of the fungus. Samples were filtered through four thicknesses of muslin and centrifuged at 10,000 *g* for 10 min. The supernatants were purified by SPE (Section 3.2.2.1) before analysis by HPLC (Section 3.2.3).

3.2.1.4. Culture in a fermenter

Flasks (250 ml) containing 100 ml CD-V8 broth (Appendix 1) were inoculated with pycnidiospores (100 µl at 10^7 conidiospores.ml^{-1}) and incubated on an orbital shaker (120 rpm; orbit diameter 5 cm) at 20°C for 60 hours. The mycelium was harvested by centrifugation at 12,500 *g* for 10 min, washed and homogenised in CDCLM (Appendix 1). The mycelial homogenate was inoculated in CDCLM (giving a final volume of 4.5 l) previously sterilised in the fermenter vessel (Applikon 7 litres, type Bio Bench 7, Schiedam, Holland) and incubated at 20°C for 5 to 8 d. In all fermentations, pH, temperature, agitation rate and dissolved oxygen (initially set up at 20 psi) were constantly monitored. Samples (20 ml) were collected every 3 h during the day (four times a day). They were filtered through four thicknesses of muslin and centrifuged at 10,000 *g* for 10 min. The supernatants were purified by

SPE (Section 3.2.2.1), the toxins being eluted in 2 ml and analysed by HPLC (Section 3.2.3).

3.2.2. Solanapyrone purification

3.2.2.1. Solid phase extraction (SPE)

An ODS reverse phase end-capped Isolute cartridge (1g; C18; Jones Chromatography) was conditioned by passing through 5 ml methanol followed by 5 ml ultra-pure water. Culture filtrates of *A. rabiei* were passed through the cartridge and non-adsorbed materials were eluted with 5 ml ultra-pure water. Solanapyrone toxins were desorbed with acetonitrile (1 ml or 2 ml).

3.2.2.2. Solvent partitioning

After growth of the fungus, the culture was filtered through four thicknesses of muslin to remove the mycelium. The culture filtrate was centrifuged at 10,000 *g* (10°C) for 20 min to sediment pycnidiospores. After adjusting the pH of the supernatant to 3.0 with 1M H_2SO_4, it was partitioned three times against 1/3 volume of ethyl acetate. The ethyl acetate phases were combined, dried over anhydrous sodium sulphate and the solvent removed on a rotary evaporator. The residue containing the toxins was redissolved in methanol.

3.2.2.3. Flash chromatography

The solvent (methanol or acetonitrile) contained in the solanapyrone samples obtained after solvent partitioning or SPE was evaporated on a rotary evaporator (Type R110; Büchi) at less than 35°C and the toxins were dissolved in dichloromethane. Samples were injected onto a silica based cartridge (40g; particle size 32-63 μm; pore size 60 Å; surface area 573 m^2.g; Biotage Ltd, Hertford, UK) and the cartridge was washed with cyclohexane (110 ml). The toxins were eluted

under low air pressure (15-17.5 psi) with a solvent consisting of a mixture of dichloromethane: cyclohexane: ethyl acetate (3:3:1; 625 ml) followed by dichloromethane: cyclohexane: ethyl acetate (1:1:1; 400 ml) and pure ethyl acetate (150 ml) as mobile phases. Fractions (20 ml) were collected in individual universal bottles and monitored by spectrophotometry to determine the presence of the solanapyrone toxins. Each toxin was quantified with the following formula:

$C = (A \times MW) / \varepsilon l$

Where:

- C is the concentration of solanapyrone toxin ($mg.ml^{-1}$)
- A is the optical density at the λ_{max} specific for each solanapyrone toxin (λ_{max} = 327 nm; 303 nm and 320 nm for solanapyrones A, B and C respectively).
- MW is the molecular weight of the solanapyrone toxins (= 302 $g.mol^{-1}$; 304 $g.mol^{-1}$ and 331 $g.mol^{-1}$ for solanapyrones A, B and C respectively).
- ε is the extinction coefficient specific for each toxin (= 9400; 8500; 7300 for solanapyrones A, B and C respectively).
- l is the width of the spectrophotometer cuvette (= 1 cm).

The purity of solanapyrone toxins was determined by HPLC and the flash chromatography fractions containing either solanapyrone A, B or C were pooled resulting in three major fractions.

3.2.3. Toxin analysis by HPLC

Solanapyrone toxins were separated and quantified on a Philips HPLC equipped with a PU4021 diode array detector and a PU4100 quaternary pump. The system was fitted with a reverse phase ODS column as stationary phase (Apex 2; 5 micron; 4.6 x 150 mm; Jones Chromatography, Hengoed, U.K.) connected to an octodecyl silica (ODS) guard column (Apex 2; 5 µ; 4.6 x 20 mm; Jones Chromatography). The

mobile phase consisted of 60% ultra pure water; 20.1% methanol; 1.8% acetonitrile and 18.1% tetrahydrofuran (all HPLC grade; BDH laboratory supplies, U.K.). A high pressure of 100-200 bar was applied to the solvent and the flow rate was established at 1,000 $\mu l.min^{-1}$. The solanapyrone toxins A, B and C were recognized by their specific retention times and their characteristic UV spectra (λ_{max} = 327; 303 and 320 nm, respectively) which were compared to spectra of reference samples of the compounds. Solanapyrone A was quantified by integration of the area under the peaks of chromatograms cut at the λ_{max} for this compound (327 nm) using the Philips PU6000 integration software.

3.3. Results

3.3.1. Production in Roux bottles

The production of solanapyrone A reached a maximum of 4.05 mg of solanapyrone A per litre of culture filtrate after incubation for 16 d (Figure 3.1).

3.3.2. Production in conical flasks

Solanapyrone A produced in 4 different batches peaked respectively at 16; 14; 16 and 18 d post-incubation reaching 23.0; 32.7; 20.7 and 42.7 mg of toxin per litre of fungal culture filtrate (Figure 3.2).

3.3.3. Production in shake culture

Shake cultures from pycnidiospores or mycelial homogenate, as opposed to still culture, produced the highest titres of solanapyrone A (up to 80 and 150 mg per l of culture filtrate, respectively; Figures 3.3 and 3.4). The toxin peaked after 10 d in cultures from pycnidiosores and after only 5-6 d in cultures derived from mycelial homogenates.

3.3.4. Production in a fermenter

When 500 ml of mycelial homogenate (OD_{620} = 1) were inoculated in a total volume of 4.5 l, the growth of the fungus was fast, leading to the production of a large amount of mycelium. In the two following batches, even though the inoculum (OD_{620} = 1) was reduced to 160 ml and 100 ml, respectively, the amount of mycelium produced was still abundant after 5 d post-inoculation. However, no solanapyrone toxins were detected in any of these three fermentations, with an agitation rate of 400 rpm. In order to slow down fungal growth to increase the chance of detecting the production of solanapyrone toxins, the amount of inoculum was reduced to 100 ml at OD_{620} = 0.2 and the agitation rate decreased to 300 rpm. Again, no solanapyrone toxins were detected.

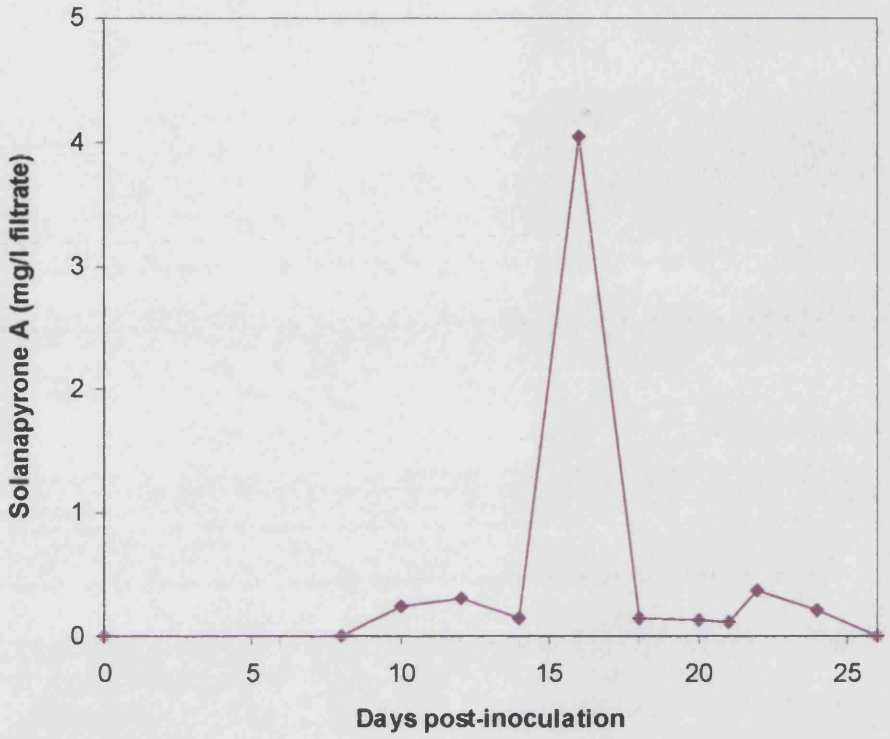


Figure 3.1. Production of solanapyrone A in Roux bottles. CDCLM (100 ml) was inoculated with pycnidiospores of *A. rabiei* (10^4.ml^{-1}) and incubated at 20°C. The time course of solanapyrone toxin production was followed from 8 to 26 d post-inoculation. Each sample corresponds to culture filtrates of the fungus collected from three bottles and pooled before solid phase extraction of the solanapyrone toxins.

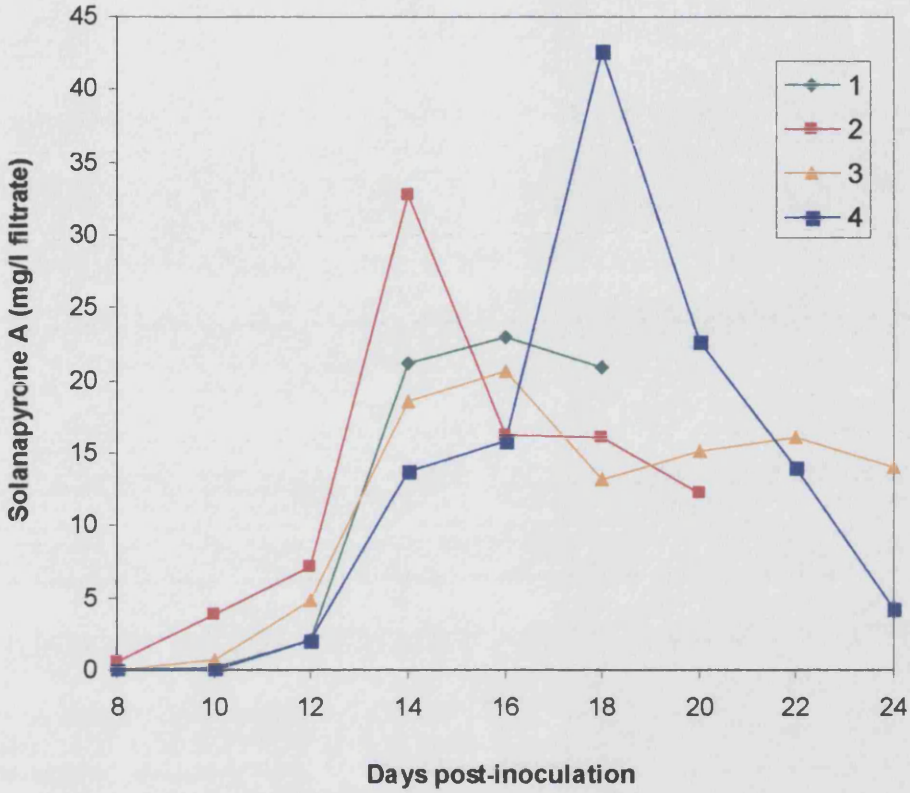


Figure 3.2. Solanapyrone A production in conical flasks. Flasks (250 ml) containing 30 ml CDCLM were inoculated with pycnidiospores (10^4.ml^{-1}) and incubated at 20°C for up to 24 h. Four independent time courses were run. For each time course data represent the mean value of triplicates.

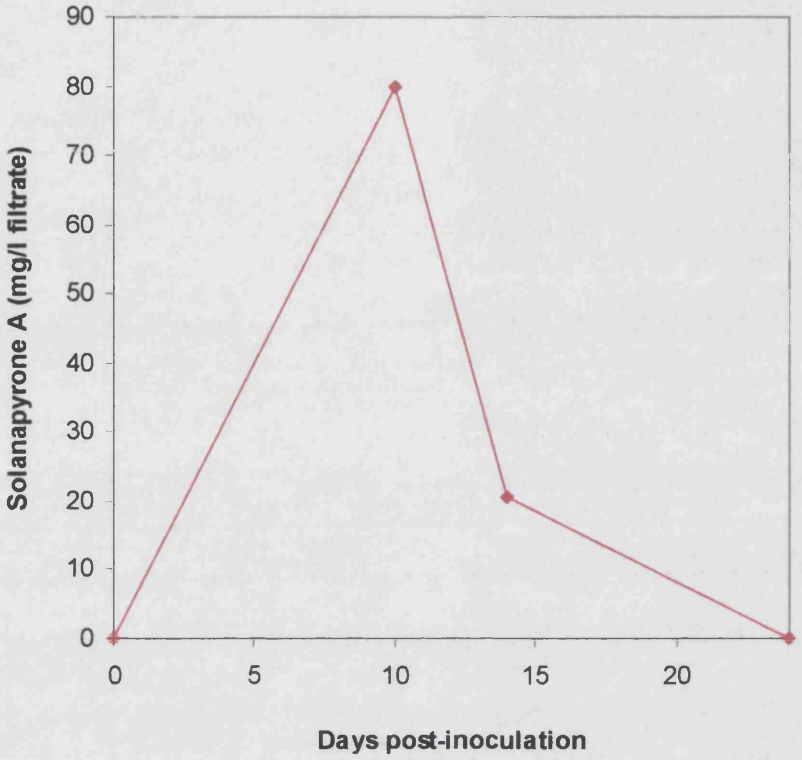


Figure 3.3. Production of solanapyrone A in shake culture from pycnidiospores. Flasks (1 l) containing 250 ml CDCLM were inoculated with pycnidiospores (10^4.ml^{-1}) and incubated at 20°C on an orbital shaker.

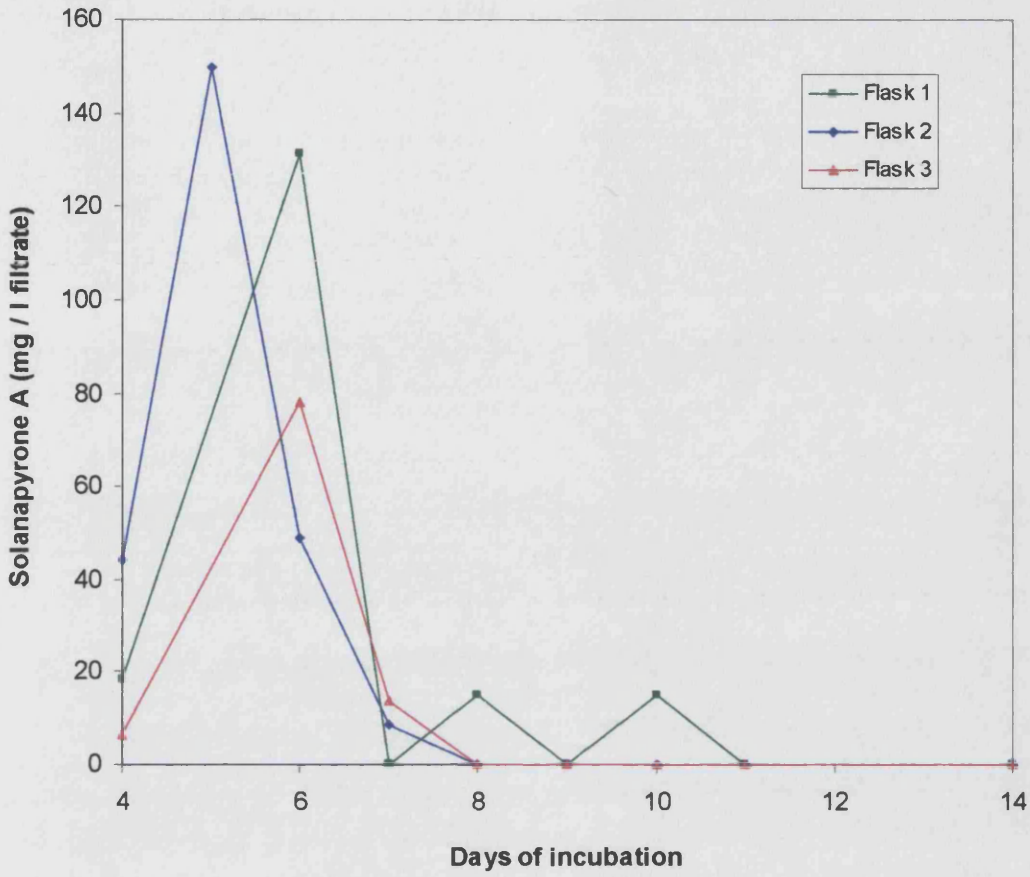


Figure 3.4. Solanapyrone A production in shake culture from mycelial homogenate. Flasks (1 l) containing 250 ml CDCLM were inoculated with mycelial homogenate and incubated at 20°C, under shaking conditions.

3.3.5. Flash chromatography

The presence of each solanapyrone toxin was determined for each fraction by scanning their respective UV spectra on a spectrophotometer (Table 3.1). The three solanapyrone toxins were well separated in different fractions, which facilitated their purification.

3.3.6. HPLC

The production of solanapyrone toxins in culture filtrates of *A. rabiei* was determined by HPLC. The elution of the toxins can be followed according to the time on the chromascan that displays a three-dimensional graph (absorbance versus wavelength versus time, Figure 3.5). The presence of solanapyrone toxins was confirmed by comparing the spectra (absorbance versus the wavelength) of the toxin fractions analysed with the spectra of reference compounds (pure solanapyrone A, B or C) as represented in Figure 3.6. A match better than 95% between the spectra of the samples and references was considered sufficiently close for identification purposes.

Solanapyrone A was eluted at about 800 sec.

Chromatogram of the same toxin fractions were extracted from their respective chromascan by matching the absorbance versus the elution time at its specific wavelength. The area under the peak of the chromatogram was used to quantify the compound that was responsible for it (Figure 3.7).

	Solanapyrone A	Solanapyrone B	Solanapyrone C
Batch 1	Fractions 9 to 23	-	-
Batch 2	Fractions 8 to 23	Fraction 24 to 33	-
Batch 3	Fractions 11 to 18	-	-
Batch 4	Fractions 10 to 21	Fractions 28 to 31	Fractions 35 to 37

Table 3.1. Solanapyrone A collected after flash chromatography of *A. rabiei* filtrates from four different batches of cultures in conical flask. Dash (-) indicates no production of toxin. Fraction 1 corresponds to the first fraction collected with the solvent dichloromethane: cyclohexane: ethyl acetate (3:3:1).

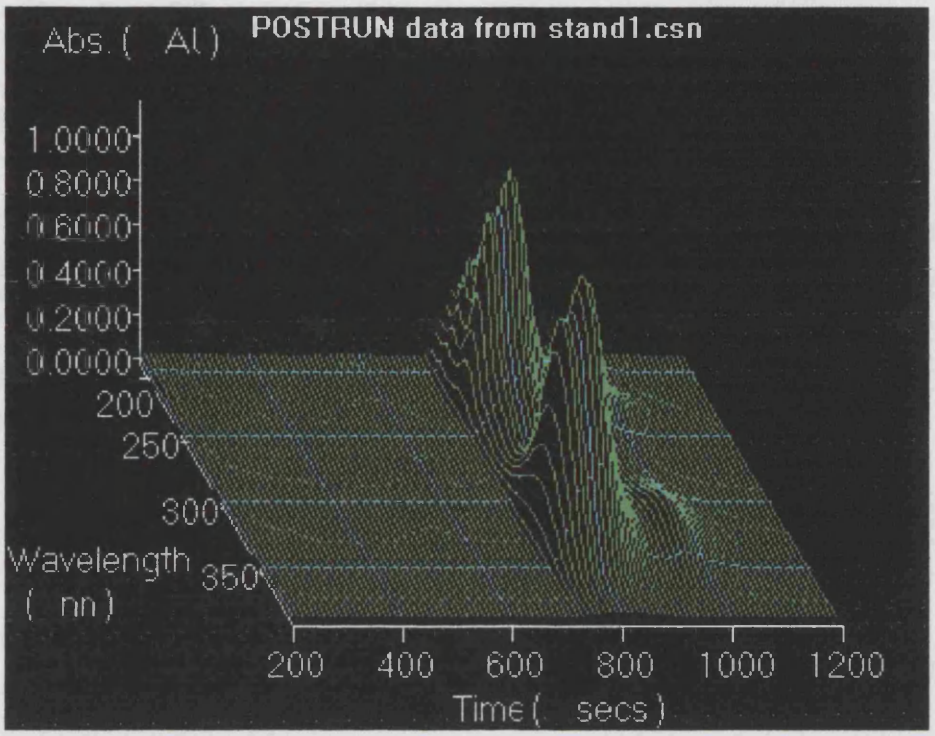


Figure 3.5. Chromascan of solanapyrones A and C. The toxins were produced from a 14 day-old flask culture of *A. rabiei* (Section 3.2.1.2) and purified by solvent partitioning (Section 3.2.2.2). This three-dimensional graph represents the absorbance of the toxins plotted versus the time and the wavelength. The spectra and the chromatograms of the solanapyrone toxins can be extracted from the chromascan.

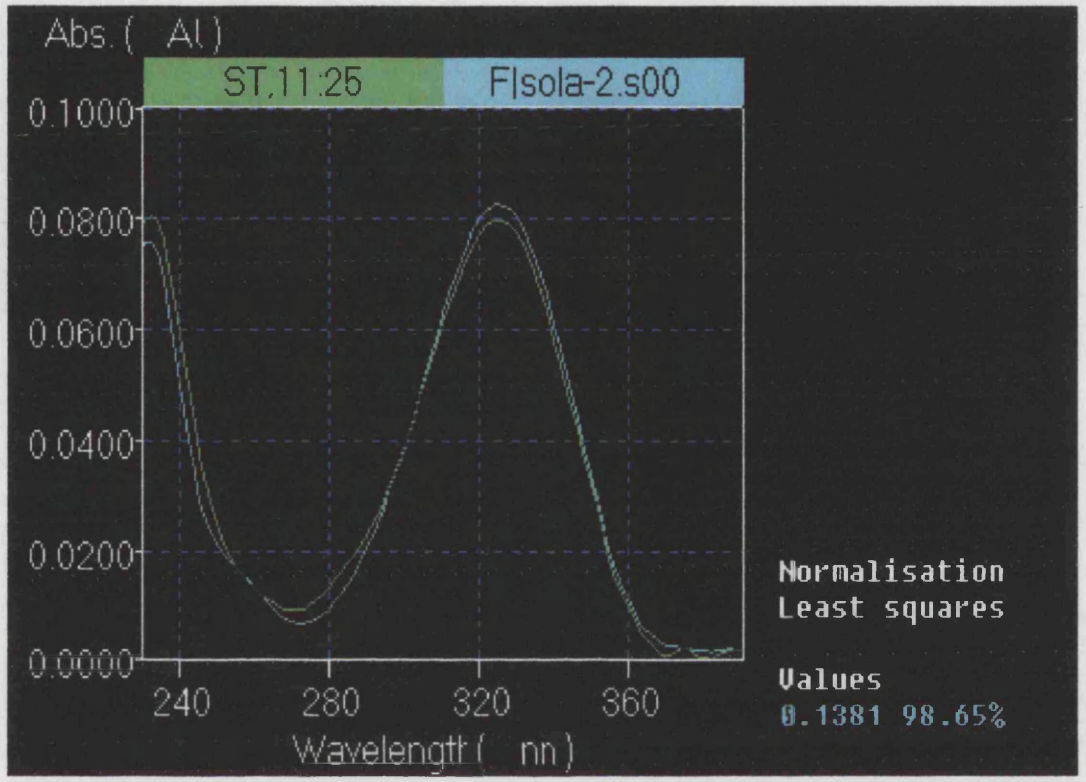


Figure 3.6. Spectra of solanapyrone A. The spectrum of the sample (ST, 11:25) was produced from a 10 day-old shake culture of *A. rabiei* grown on a shaker before purification by SPE (Section 3.2.2.1). It was overlaid with that of authentic solanapyrone A (F| sola-2.s00). A match of 98.65% was obtained for the two spectra over a range from 230 to 390 nm.

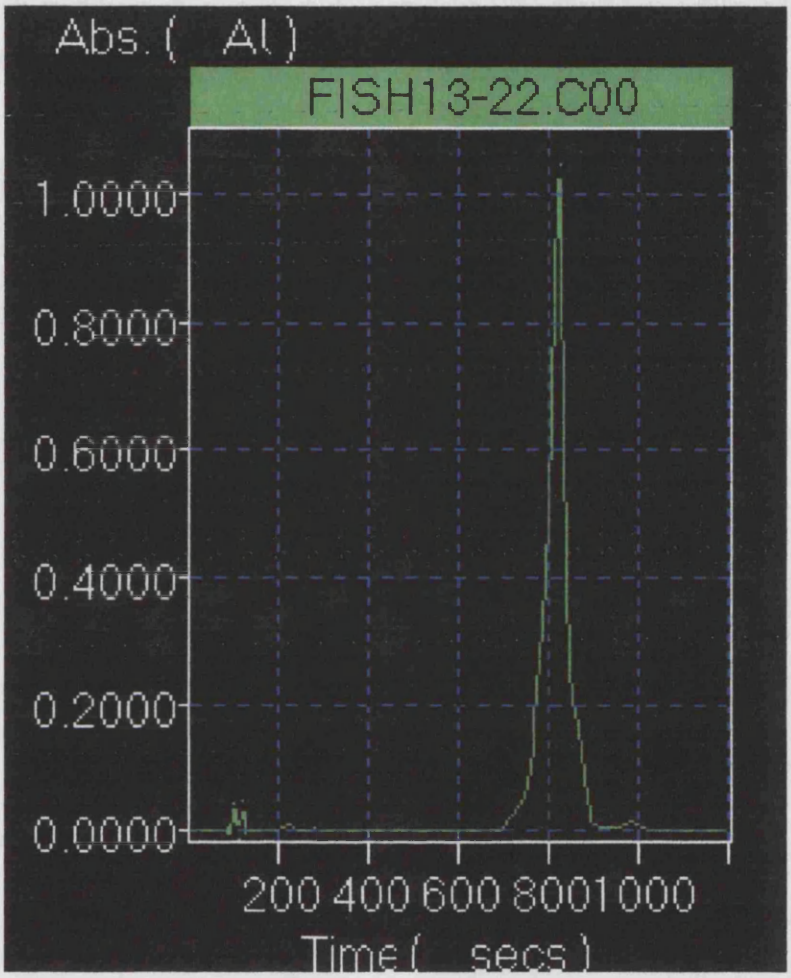


Figure 3.7. Chromatogram of solanapyrone A. The chromatogram of a sample obtained as mentioned in Figure 3.5 was extracted at 327 nm showing a retention time of 800 sec, and used to quantify the toxin.

3.4. Discussion

3.4.1. Still culture in Roux bottles

This technique allowed the production of only a low amount of solanapyrone A (4.05 mg per l of culture filtrate). For this reason, production in conical flasks was tested.

3.4.2. Still culture in conical flasks

The production of solanapyrone A in conical flasks was 5.11 to 10.54 times higher than the production in Roux bottles. However, the differences in production of solanapyrone A between the four time courses demonstrates the difficulty of recovering optimal amounts of toxin. For example, at day 18 post-inoculation the amount of solanapyrone A had already decreased to 16.07 mg.l filtrate^{-1} from a maximum of 32.7 mg.l^{-1} for the second time course, while for the fourth time course, it had reached its maximum value of 42.7 mg.l filtrate^{-1} (Figure 3.2). These results showed that this technique was not totally reproducible from one batch to the other. Consequently, production of solanapyrone A was attempted in shake culture.

3.4.3. Shake culture

The shake culture technique has been used to produce various mycotoxins. Spores of *Metarhizium anisopliae*, inoculated in 250 ml flasks containing 100 ml Czapek Dox broth, produced destruxin (Hsiao and Ko, 2001). Ochratoxins were obtained by *Aspergillus ochraceus* in submerged shake cultures (100 ml in 500 ml flasks) inoculated with 2 to 4 X 10^6 spores per ml medium (Harris and Mantle, 2001). Similarly, solanapyrone A was produced from pycnidiospores of *A. rabiei* in shake culture and peaked at 10 d post-inoculation. Homogenisation of mycelium of *A. rabiei* seemed judicious in order to increase and accelerate the production of solanapyrone A. Culture medium was inoculated with homogenised mycelium grown from pycnidiospores in order to speed up fungal growth. Solanapyrone A production

appeared earlier than in still culture, as it peaked at 5-6 d post-inoculation instead of 14-18 d when mycelial homogenate was used as inoculum. Cultures from homogenate produced solanapyrone A 4-5 d earlier compared to cultures from pycnidiospores. However, the sharp peak of solanapyrone A production increased the difficulty of recovering the toxin since the amount of toxin decreased dramatically and very quickly (Figure 3.4). The shift in timing of the maximum yield of solanapyrone A between experiments may be due to the inoculation step as the quantification of mycelial homogenate by spectrophotometry may not be accurate enough. In other fungi such as *Cunninhamella echinulata*, inoculum density was reported to affect mycelial growth and secondary metabolite production (Chen and Liu, 1997).

3.4.4. Culture in a fermenter

It was attempted to produce solanapyrone A on a larger scale by growing *A. rabiei* in a fermenter. However, this toxin was not detected. The pathogen may have synthesised the toxin but its production may have been evanescent. Solanapyrone A may have peaked sharply as in shake culture and its production may have been missed. Likewise, the production of chitinase by *Verticillium lecanii* peaked sharply after incubation for 6 d (Liu *et al.*, 2003). Alternatively, solanapyrone A may not have been synthesised because the fermentation conditions may not have been favourable to its biosynthesis.

A large number of variables are encountered in fermenter culture, in particular agitation rate and pH.

During fermentation, agitation rate seems to play an important role in mycelial morphology (formation of dispersed mycelial filaments or dense pellets) which affects the concentration of dissolved oxygen and the yield of fungal secondary metabolites (Liu *et al.*, 2003; Papagianni, 2004; Piccoli-Valle *et al.*, 2003; Park *et al.*, 2002; Sinha *et al.*, 2001; Riscaldati *et al.*, 2000; Lejeune and Baron, 1995). Higher yield of biopolymers was obtained with the Ascomycete *Cordyceps militaris* using a gentle agitation (150 rpm). Similarly, the production of polysaccharide pullulan,

citric acid or chitosan with *Aureobasidium pullulans* (Gibbs and Seviour, 1996), *Aspergillus niger* or *Gongronella butleri* (Nwe *et al.*, 2001), respectively, were optimised with low agitation rate. Possibly, the agitation rate of 400 rpm may have been inappropriate for solanapyrone A production.

Controlling the pH of the culture medium was also shown to affect metabolite yield (Liu *et al.*, 2003; Lee *et al.*, 2002; Riscaldati *et al.*, 2000; Papagianni *et al.*, 1999; Carlsen *et al.*, 1996; Wang and McNeil, 1995; Papagianni *et al.*, 1994). Maintaining the pH constant with *A. rabiei* may have affected the production of solanapyrone A.

Chapter 4

Screening techniques for potential toxin-minus mutants

4.1. Introduction

In order to screen putative mutants for loss or reduction in the production of solanapyrone A, it was necessary to implement a specific, sensitive, reproducible and facile technique.

Various techniques have been used to screen fungi for the production of toxin. Among them, detached leaves assays (Mahoney et al., 2003; Johnson *et al.*, 2000; Tanaka *et al.*, 1999; Akamatsu *et al.*, 1997) and microbial assays (Rose *et al.*, 2002; Yun *et al.*, 1998; Yang *et al.*, 1996; Lu *et al.*, 1994; Ciufetti *et al.*, 1992) were the two most common techniques. Another type of bioassay was used to study the biodegradation of the polyketide toxin cercosporin produced by various species of plant pathogens belonging to the genus *Cercospora*. It consisted in testing different bacterial isolates for their capability to degrade the toxin by detecting the presence of cleared zones surrounding bacterial colonies on culture medium containing cercosporin. The amount of toxin remaining in the medium was determined spectrophotometrically after extraction and purification (Mitchell *et al.*, 2002). A similar bioassay was used with penicillin V (Keszenman-Pereyra *et al.*, 2003). These three types of assay require the manipulation of the pathogen as well as host plant tissues or bacteria. Immunoassays have also been used to study the production of fungal toxins, using specific antibodies against the toxins linked to a hapten (Lutz *et al.*, 2003; Maimala *et al.*, 2002; Maragos and Plattner, 2002; Gathumbi *et al.*, 2001; Harris *et al.*, 1999; Akamatsu *et al.*, 1997; Woloshuk *et al.*, 1994). However, immunodetection involves the production and the availability of specific antibodies. Other techniques for the detection of toxins, such as gas chromatography (Apoga *et al.*, 2002; Nielsen and Thrane, 2001; Proctor *et al.*, 1995) or mass spectrometry (Churchill *et al.*, 2001) include processes that are costly or that require specific equipment.

Solanapyrone A has an aldehyde group (Chapter 1, Figure 1.1). This chemical property was used to set up reactions that involve aldehydes, such as the production of a chromophore in the presence of 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) and ferric ions. Silver nitrate leads to the formation of a precipitate of silver metal by reacting with aldehydes in the presence of 0.4 M NaOH and 1 M ammonia (silver mirror reaction). This reagent was tested for its potential to give a water-insoluble product by reacting specifically with solanapyrone A. However, in order to avoid any interference between the silver mirror reaction and the chloride anions (Cl^-) present in CDCLM (Appendix 1), solanapyrone A must be produced in a medium containing no Cl^- anions. The specific light absorption of solanapyrone A at 327 nm was investigated too as a means of detecting the presence of the compound in culture filtrates of *A. rabiei*. In order to determine the specificity of this assay the absorbance was measured on both plate reader and spectrophotometer.

4.2. Materials and Methods

4.2.1. Colorimetric assay with MBTH

A colorimetric assay was developed, using MBTH, which reacts with aldehydes such as solanapyrone A to give a blue formazan dye.

A solution of 0.5% MBTH (Sigma; 500 µl aqueous solution) and 0.5% $FeCl_3$ (500 µl aqueous solution) was added to a range of concentrations (0-100 $\mu g.ml^{-1}$) of solanapyrone A dissolved in CDCLM (1 ml) in test tubes. After incubation at room temperature for 30 min, the production of the blue formazan compound was determined spectrophotometrically at 629 nm.

Another assay with MBTH was carried out in agarised CDCLM. Pycnidiospores of *A. rabiei* (10 µl at 10^7 $spores.ml^{-1}$) were inoculated on agarised CDCLM containing MBTH and $FeCl_3$, both at a concentration ranging from 0.005 to 0.05%. The plates were incubated at 20°C for 13 d.

4.2.2. Assay with silver nitrate ($AgNO_3$)

In order to use the silver nitrate test, chloride anions had to be eliminated from the fungal growth medium. Three different media consisting of CDCLM in which the Cl^- anions associated with the four cations Mn, Ca, Co and Cu were replaced with a sulphate or nitrate group (Table 4.1). Mycelium grown in CD-V8 (100 ml) for 60 h was harvested, homogenised and resuspended in 100 ml alternative medium. Homogenate (10 ml; absorbance at 620 nm = 1) was inoculated in 500 ml of each of the four different alternative media and incubated at 20°C on an orbital shaker (orbit diameter 5 cm; 120 rpm). In order to test for the production of solanapyrone A in the three media and in CDCLM, samples (5 ml) from the four cultures were purified by SPE (Chapter 3, Section 3.2.2.1), eluted with 500 µl acetonitrile and analysed by HPLC (Chapter 3, Section 3.2.3).

Two assays were performed:

(i) Test tube assay: a range of concentration of $AgNO_3$ (500 µl of aqueous solution; 0.01%; 0.05%; 0.1%; 0.5%; 1%; 2% and 5%) was added to a range of concentration of solanapyrone A (500 µl; 1; 5; 10 and 15 µg per ml of solution) and incubated at room temperature or at 50°C. A similar test was carried out by adding Tollen's reagent ($AgNO_3$; 0.4 M NaOH; 1 M ammonia) to the same range of concentration of solanapyrone A. A positive control reaction consisted in adding Tollen's reagent to formaldehyde.

(ii) Petri dish assay: *A. rabiei* was grown on agarised CDCLM containing $AgNO_3$ (concentrations ranging from 0.01% to 5%) and incubated at 20°C.

Cations	Anions			
	CDCLM	Medium 1	Medium 2	Medium 3
Zn	SO_4	SO_4	SO_4	SO_4
Mn	Cl_2	SO_4	Cl_2	SO_4
Ca	Cl_2	NO_3	NO_3	Cl_2
Co	Cl_2	NO_3	Cl_2	NO_3
Cu	Cl_2	SO_4	Cl_2	SO_4

Table 4.1. Composition of three media, derived from CDCLM, used for the production of solanapyrone A by *A. rabiei*. In the three alternative media the Cl^- anions (present in CDCLM) associated with four cations were substituted with a sulphate or nitrate group. Medium 2 contained $Ca(NO_3)_2$ instead of $CaCl_2$ present in CDCLM and, conversely, $Ca(NO_3)_2$ present in medium 1 was replaced by $CaCl_2$ in medium 3.

4.2.3. Assay in microtitre plates

Colonies of *A. rabiei* were grown in CDCLM (2 ml) in the wells of culture plates (Nunc, Denmark) at 20°C for 5 to 7 d. Culture filtrates were purified by SPE (Chapter 3, Section 3.2.2.1) on a C18 cartridge, the successive eluates collected separately and the absorbance at 327 nm (λ_{max} on HPLC), 330 nm and 340 nm measured on a plate reader (Spectra MAX 250; Molecular Devices, Wokingham, U.K.).

In order to confirm that the wavelengths specific to the toxin had been chosen, the absorbance of samples containing solanapyrone A dissolved in CDCLM was scanned between 230 and 390 nm in a spectrophotometer (CDCLM was used as reference). The absorbance of the CDCLM was also scanned (against distilled water) following the same procedure.

4.3. Results

4.3.1. Colorimetric assay with MBTH

In liquid assay, the correlation between the concentration of solanapyrone A in the range 1-100 $\mu g.ml^{-1}$ and the absorbance at 629 nm (wavelength that gives the maximum absorbance) was linear (Figure 4.1).

When *A. rabiei* was grown on solid medium, the substrate turned blue in the vicinity of the fungal colonies with concentrations of MBTH and $FeCl_3$ ranging from 0.01 to 0.025% (Figure 4.2). With concentrations of MBTH and $FeCl_3$ below 0.01% or above 0.025%, the formazan dye was not detectable. No colouration appeared on plates where *A. rabiei* was inoculated on CDCLM containing no MBTH and no $FeCl_3$. At any concentration tested the reagent MBTH was not inhibitory to the growth of the fungus. Concentration of MBTH and $FeCl_3$ was optimal at 0.017% for the detection of the blue dye (Figure 4.2). However, the blue coloration of the formazan dye diffused through the medium.

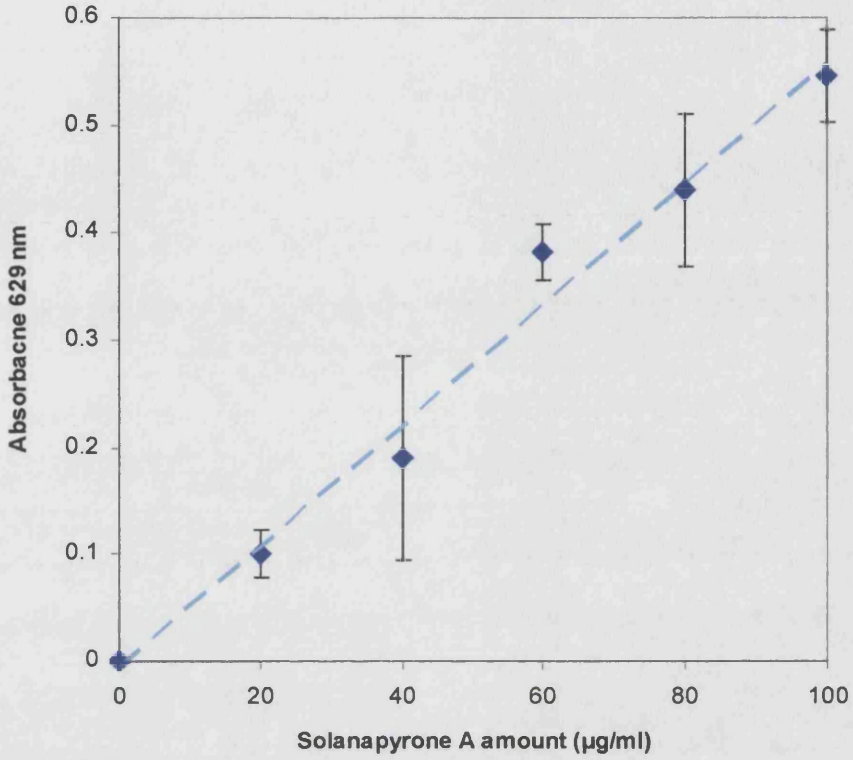


Figure 4.1. Correlation between the amount of solanapyrone A and the absorbance at 629 nm in the presence of 0.5% MBTH and 0.5% $FeCl_3$. Solutions of 0.5% MBTH (500 µl) and 0.5% $FeCl_3$ (500 µl) were added to solanapyrone A (ranging from 0 to 100 $\mu g.ml^{-1}$ in 1ml). After a 30 min-incubation the absorbance at 629 nm was measured on a plate reader. Points represent are means of triplicates and error bars represent the standard deviation.

Figure 4.2. Colorimetric assay with MBTH to detect the production of solanapyrone A by *A. rabiei*. *A. rabiei* was inoculated onto agarised CDCLM supplemented with different amounts of MBTH and $FeCl_3$ (from 0.01 to 0.025%). Optimal concentrations of MBTH and $FeCl_3$ for the detection of the blue formazan dye were 0.017%.

4.3.2. Assay with silver nitrate

The production of solanapyrone A in CDCLM was slightly lower than in the three alternative media. The same amount (8.5 $mg.l^{-1}$ on average) of solanapyrone A was produced in shake culture in all three alternative media, reaching a maximum at 6 to 7 d post-inoculation (Figure 4.3). This result showed that *A. rabiei* is able to synthesise as much solanapyrone A when the chloride anion, associated with four of the five essential elements present in CDCLM, was replaced by other anions such as nitrate or sulphate, as when it is present in the culture medium.

No silver mirror reaction occurred in the test tube assay with silver nitrate, even when the solution containing $AgNO_3$ and solanapyrone A was warmed to 50°C. A silver mirror was formed when Tollen's reagent was added to formaldehyde but did not appear when added to solanapyrone A in solution. The formation of silver mirror did not occur in the absence of NaOH and ammonia in the reaction with solanapyrone A or formaldehyde.

Silver nitrate inhibited the growth of *A. rabiei* on agarised CDCLM, when added in the medium at a concentration equal to or higher than 0.5%.

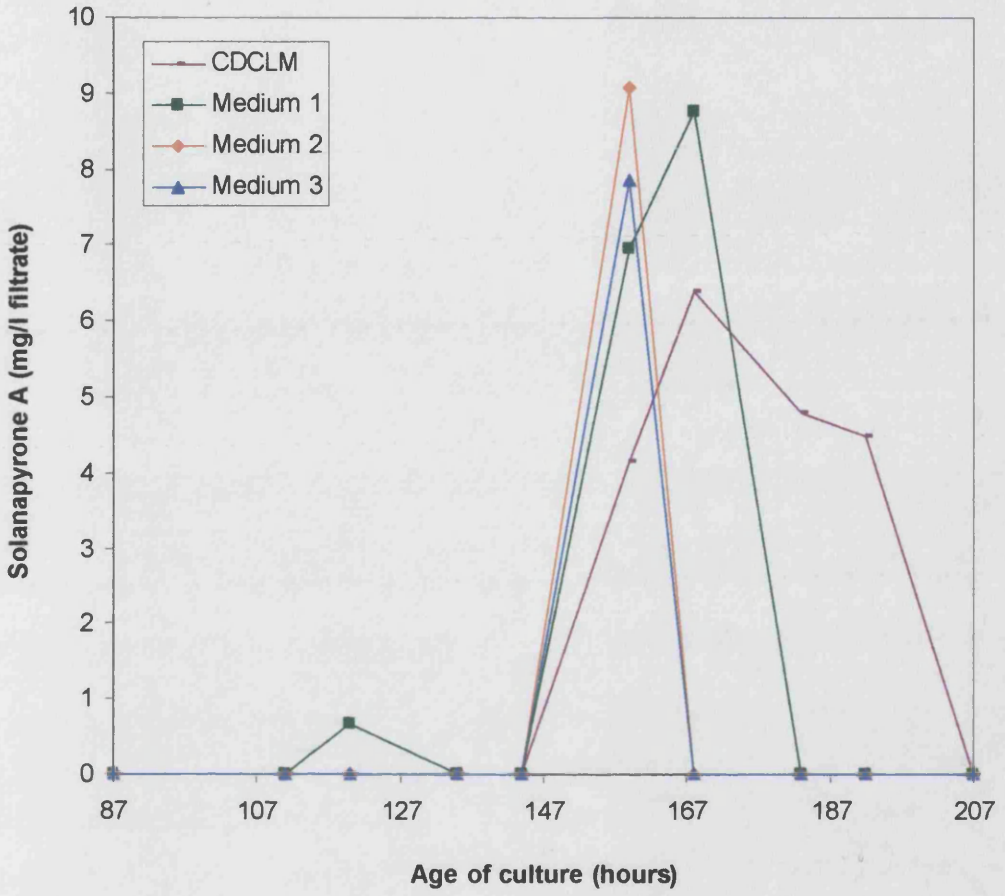


Figure 4.3. Production of solanapyrone A in CDCLM and three alternative media in shake culture of *A. rabiei*. Mycelial homogenate of the fungus (10 ml; OD_{620} = 1) was inoculated in each of the three alternative media (Table 4.1) and incubated at 20°C on an orbital shaker. Samples of culture filtrates were purified by SPE (Chapter 3, Section 3.2.2.1) and the solanapyrone A production determined by HPLC (Chapter 3, Section 3.2.3).

4.3.3. Assay in microtitre plates

Measurement of the absorbance at 327 nm of the different eluates collected after SPE of culture filtrates of *A. rabiei* showed that only the absorbance of the eluate containing the solanapyrone A toxin was significantly different from the absorbance of the control (acetonitrile) (Figure 4.4).

The absorbance of solanapyrone A dissolved in acetonitrile peaked at 325 nm when measured in a spectrophotometer (Figure 4.5).

The fungus produces enough solanapyrone A in microtitre plates to be quantified (5 to 50 $\mu g.ml^{-1}$ culture filtrate). For each sample containing solanapyrone A dissolved in CDCLM, a peak of absorbance was revealed at 320 nm (Figure 4.6). The absorbance of the CDCLM scanned against distilled water revealed no peak (Figure 4.7).

The absorbance measured with a spectrophotometer at 327, 330 and 340 nm of culture filtrates of *A. rabiei* grown in microtitre plates represent respectively 90%, 86% and 56% of the absorbance maximum at 320 nm (Figure 4.6).

The correlation between the absorbance at 340 nm or 320 nm measured on plate reader and the concentration of solanapyrone A is linear up to 40 µg toxin per ml (Figure 4.8). Absorbance at 340 nm was about 56% of the absorbance at 320 nm (Figure 4.8).

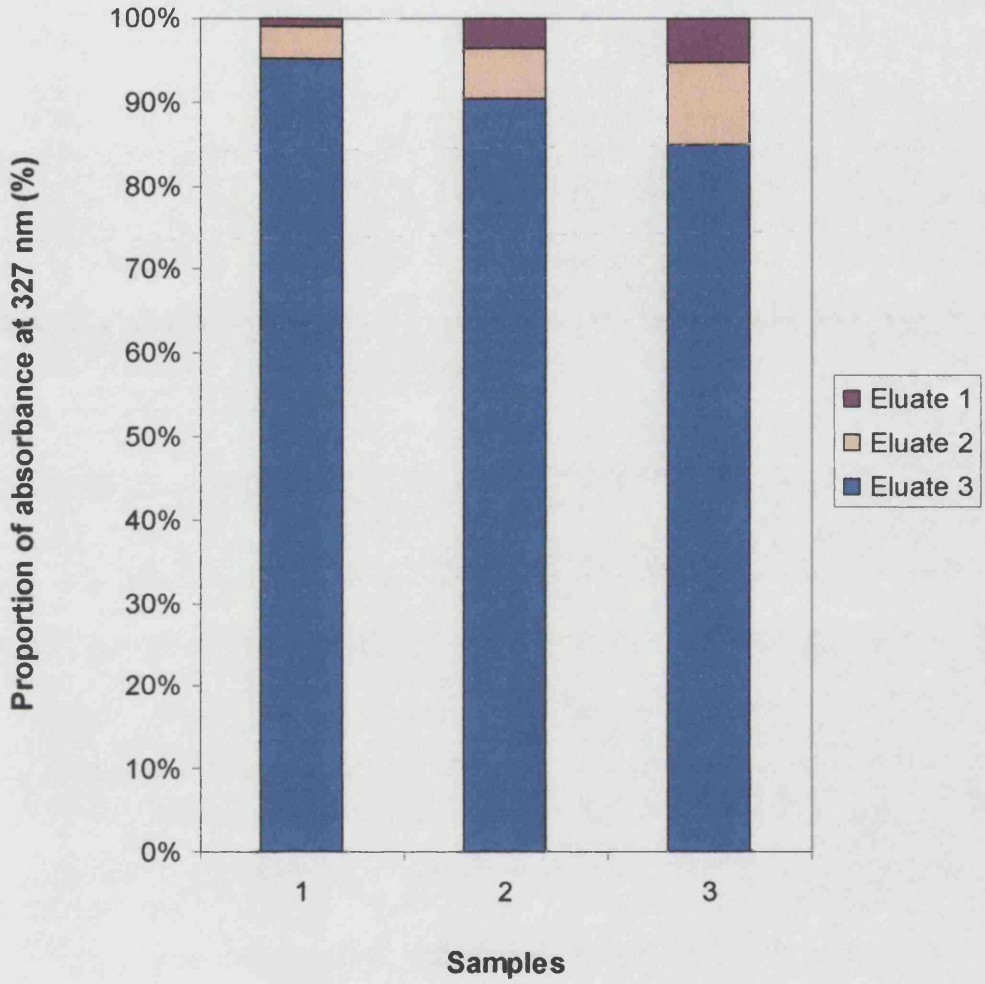


Figure 4.4. Proportion of the absorbance at 327 nm of the different eluates collected after purification by solid phase extraction of three culture filtrates of *A. rabiei*. Eluate 1: non-adsorbed materials of culture filtrate of *A. rabiei*; Eluate 2: non-adsorbed materials eluted with ultra-pure water; Eluate 3: solanapyrone toxins eluted with acetonitrile. A proportion of 100% corresponds to an absorbance of 1.668, 0.649 and 0.642 for the three samples, respectively.

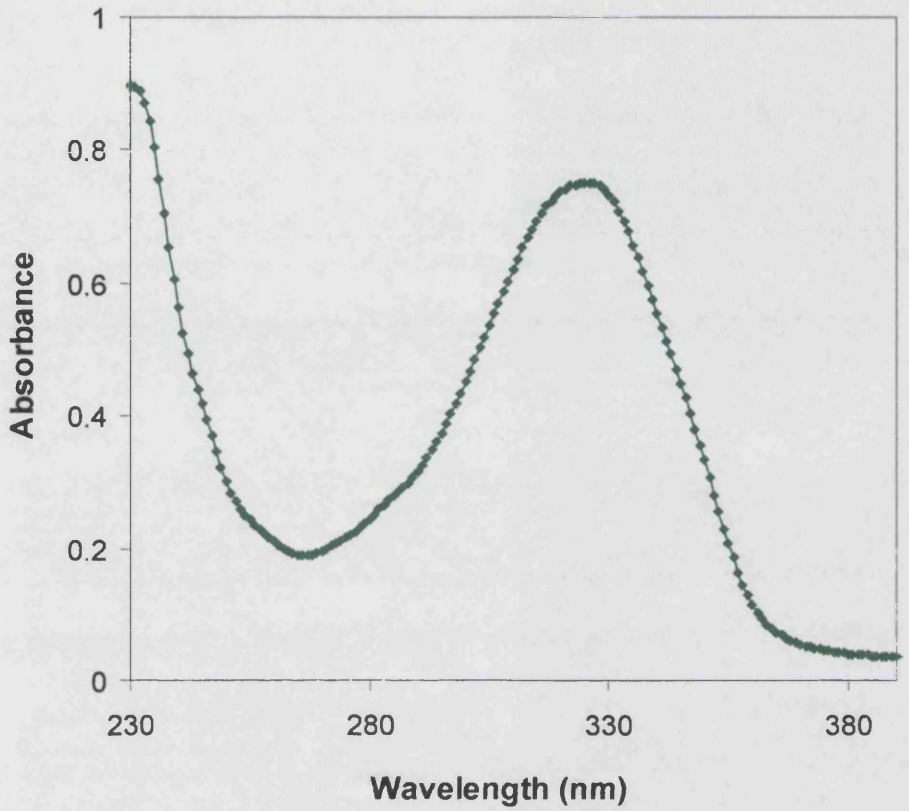


Figure 4.5. Spectrum of solanapyrone A in acetonitrile. The absorbance of solanapyrone A, dissolved in acetonitrile, was determined against the same solvent, for wavelength values ranging from 230 and 390 nm. The λ_{max} was 325 nm.

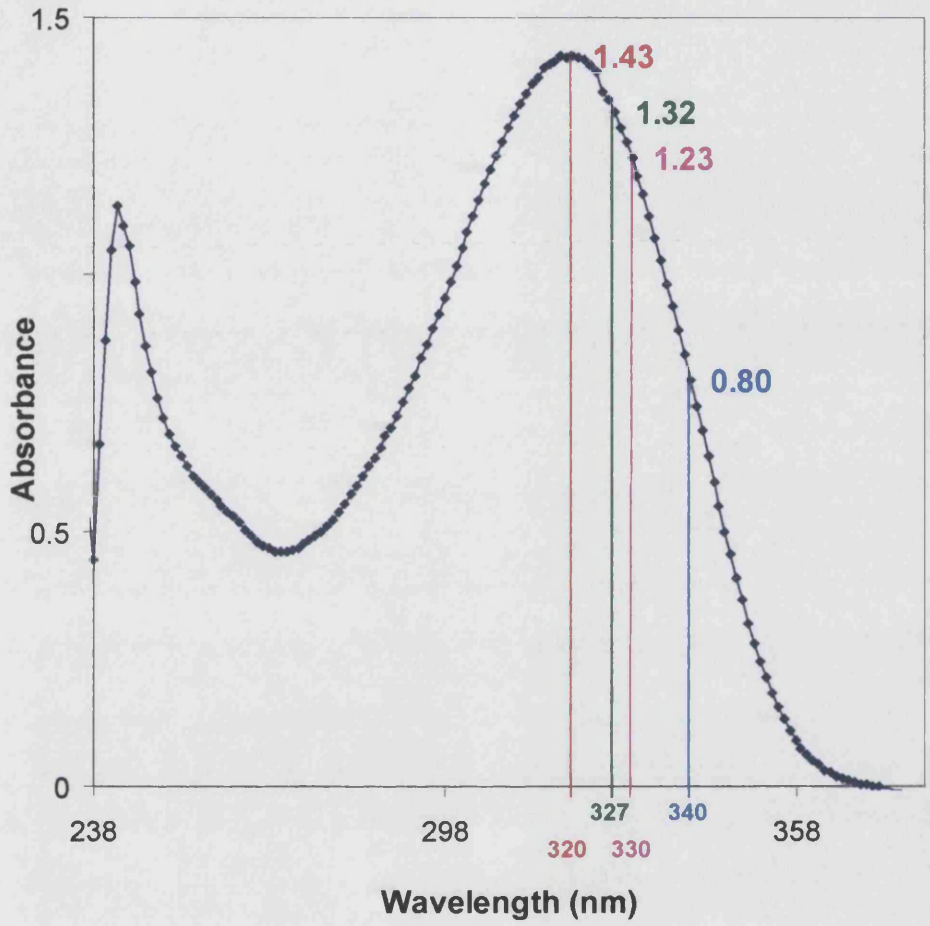


Figure 4.6. Spectrum of solanapyrone A in CDCLM. The toxin was dissolved in CDCLM and the sample read against the same medium. The absorbance was scanned between 238 and 380 nm. Values on the graph show the absorbance at 320, 327, 330 and 327 nm, respectively. The data labelled in colour represent the absorbance of solanapyrone A at the corresponding wavelengths (same colour code).

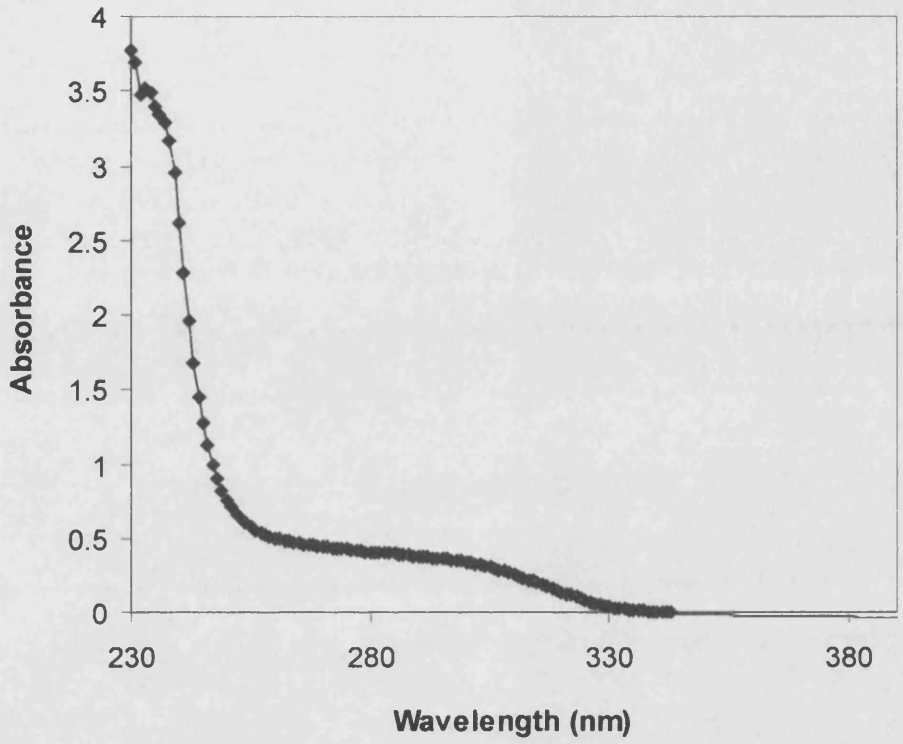


Figure 4.7. Spectrum of CDCLM against distilled water.

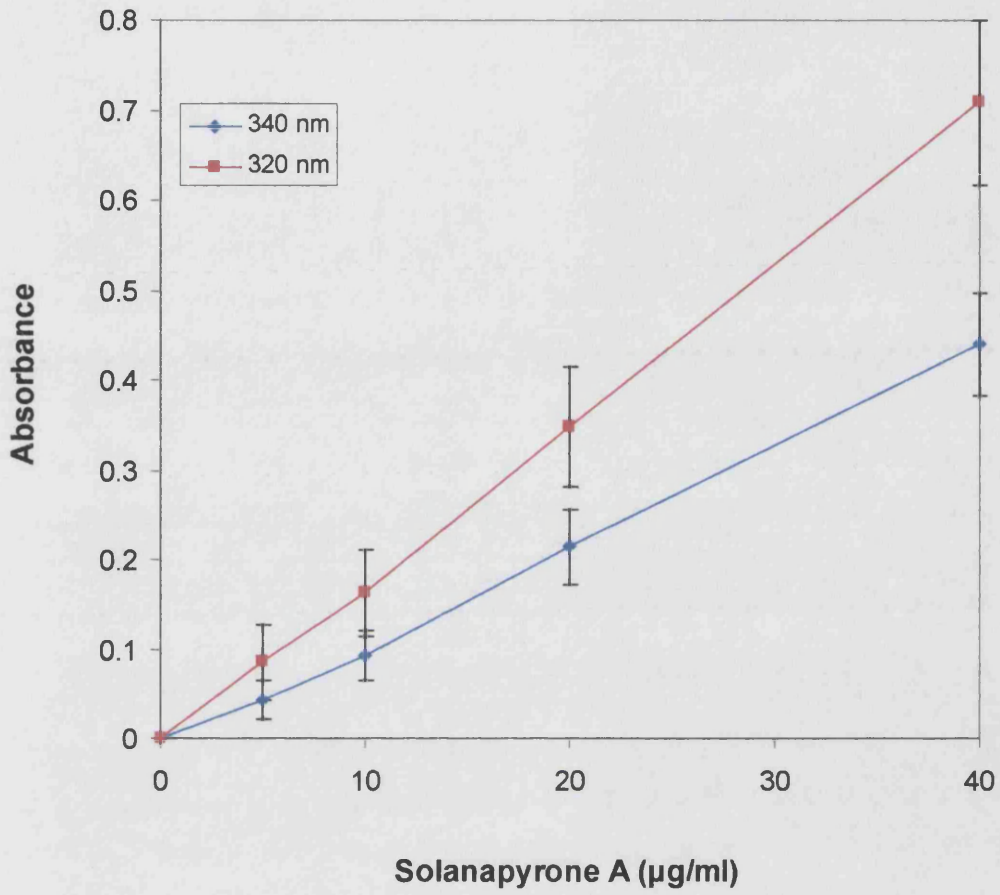


Figure 4.8. Standard curve of solanapyrone A on a plate reader. The absorbance of a solanapyrone A dissolved in CDCLM (ranging from 0 to 40 µg. ml^{-1}) was measured on a plate reader at 320 and 340 nm.

4.4. Discussion

4.4.1. Colorimetric assay with MBTH

Several authors have used MBTH to detect aldehydes in biological models, for instance aliphatic aldehyde groups in animal collagen fibres (Horvath *et al.*, 1983), glycolaldehydes produced from hydroxyl-amino acids by human neutrophils at sites of inflammation (Anderson *et al.*, 1997) or aldehydes produced by nitroalkane-oxidising enzymes in *Streptomyces ansochromogenes* (Zhang *et al.*, 2002). Other applications of the use of the colorimetric reaction include the quantification of aldehydes in fungi. Ride and Drysdale (1972) exploited the reaction to estimate the amount of filamentous fungi in plant tissue, as MBTH reacted with aldehydes which were formed after deamination of N-acetylglucosamine produced from chitin. The amount of aldehyde correlated with the dry weight of the fungi. Similarly, Kaminskyj and Heath (1982) used MBTH to evaluate the growth of the bean rust and cowpea rust fungi by assaying the fungal chitin with this reagent. The same authors also showed that urediospores of *Uromyces phaseoli* var. *vignae* contain aliphatic aldehydes and that the correlation between the absorbance at 650 nm and hexosamine concentration was linear with 0.05% MBTH (Kaminskyj and Heath, 1983).

In the present study, using the model *A. rabiei,* the correlation between the concentration of solanapyrone A produced by *A. rabiei* and the absorbance at 629 nm was linear, showing that the formation of a blue chromophore was the result of the reaction of MBTH with the toxin in the presence of ferric chloride (Figure 4.1). This reaction can therefore be used to quantify the production of toxin by *A. rabiei.*

A minimum amount of MBTH (0.01%) was required for the formation of a blue formazan dye in agarised CDCLM inoculated with *A. rabiei.* However, the dye could not be detected when 0.05% MBTH was used, as the CDCLM turned darker in the presence of such an amount of MBTH before inoculation of *A. rabiei.*

The blue dye diffused in agarised medium where the fungus was grown. Therefore, should a toxin-minus mutant be plated close to a toxin-plus strain, the former may

not be recognised. Consequently, an alternative chemical assay was therefore performed with silver nitrate, a reagent which produces a precipitate with aldehydes.

4.4.2. Assay with silver nitrate

The chloride anions present in CDCLM were not essential for the production of solanapyrone A, as the toxin was produced in comparable amounts in three alternative CDCL media. Hence, the reaction of silver nitrate with solanapyrone A produced in a CDCL medium containing no Cl^- could be tested without interfering with the formation of a silver mirror. However, the toxicity of silver nitrate on *A. rabiei* growth limited its use for the detection of solanapyrone A by the pathogen. In addition, both NaOH and ammonia were necessary for the formation of a silver mirror but would also inhibit the fungal growth. Therefore, the silver nitrate assay could not be used to screen *A. rabiei* for the production of solanapyrone A and an assay in microtitre plates was developed.

4.4.3. Assay in microtitre plates

The absorbance at 327 nm was proved to detect specifically solanapyrone A after SPE (Chapter 3, Section 3.2.2.1) of culture filtrates of *A. rabiei*. The absorbance at 340 nm, which represents 61% of the absorbance at 327 nm on spectrophotometer (Figure 4.6), can be measured on a plate reader directly from culture filtrates to quantify solanapyrone A. The absorbance maximum of solanapyrone A read on spectrophotometer shifted from 325 nm when the toxin was dissolved in acetonitrile (Figure 4.5) to 320 nm when dissolved in CDCLM (Figure 4.6). This hypsochromic shift of 5 nm may be due to the pH of the CDCLM as protonation of the compound may lead to such a shift (El-Sayed *et al.*, 2003; Fiallo *et al.*, 1998).

The CDCLM presented a low absorbance against distilled water, which confirmed that the absorbance of the medium does not interfere with that of the toxin (Figure 4.7).

These results validate the efficiency and the specificity of the microtitre plate technique to quantify solanapyrone A toxin production. Compared to the MBTH technique, the assay in microtitre plates was easy to perform as the direct measurement of the absorbance at 340 nm circumvents the use of any chemical or biological reaction. More importantly, it is very sensitive and specific to the toxin. Consequently, this screen can be used to follow the time course of biosynthesis of solanapyrone A by mutants of *A. rabiei* and is suitable for analysing large numbers of samples.

Chapter 5

Insertional mutagenesis of *Ascochyta rabiei*

5.1. Introduction

5.1.1. Insertional mutagenesis of fungi

Random insertional mutagenesis has been used to inactivate, tag and identify fungal genes. It has been applied to Ascomycetes in order to characterise genes of interest, in particular genes encoding the production of toxins. Toxin-minus mutants were created by REMI with *Alternaria alternata* (Tanaka *et al.*, 1999; Kodama *et al.*, 1998; Akamatsu *et al*, 1997), *Cercospora nicotianae* (Chung *et al.*, 2003), and two Ascomycetes which produce polyketide toxins: *Mycosphaerella zeae-maydis* (Yun *et al.*, 1998) and *Cochliobolus heterostrophus* (Lu *et al.*, 1994). The creation of cercosporin-deficient mutants of *C. nicotianae* enabled Chung *et al.* (2003) to identify a polyketide synthase gene (*CTB1*) encoding for this toxin.

5.1.2. Insertional mutagenesis of *A. rabiei*

As stated in Chapter 1 (Section 1.4), solanapyrone A is thought to play a role in the pathogenicity or the virulence of *A. rabiei.* One or other of these roles can be proved by creating toxin-minus mutants and demonstrating their non-pathogenicity or reduced virulence, respectively, compared with that of the parent strain. Such a strategy requires the development of a high frequency genetic transformation system for *A. rabiei* in order to construct a library of plasmid-tagged mutants.

Knowledge of the genetics of *A. rabiei* is very limited and the genes encoding solanapyrone toxins have not been identified. Consequently, the aim was to create mutants with random and single insertions of a plasmid in the pathogen's genome. In order to select insertional mutants and to locate gene disruptions, hygromycin

resistance, controlled by the *hph* gene, was used as a dominant selective marker.

A. rabiei transformants have been produced by the polyethylene glycol technique using the GUS-(β-glucoronidase) reporter gene to study the infection process of the pathogen in chickpea plants (Köhler *et al.*, 1995), or using a plasmid carrying a pisatin demethylase gene in order to study the metabolism of the phytoalexin pisatin from pea by the pathogen (Weltring *et al.*, 1995). However, a very low transformation frequency was achieved (maximum of two transformants per μg of DNA, when specified). To date, no other report has been published on genetic transformation of *A. rabiei.* Therefore, owing to the lack of an efficient transformation system for *A. rabiei* and to the variability in transformation efficiency among different techniques, the objective of this work was to develop and optimise empirically an appropriate insertional mutagenesis technique for *A. rabiei.* As described in Chapter 1, four techniques of insertional mutagenesis were used: REMI, electroporation, particle bombardment and *Agrobacterium tumefaciens* mediated integration (ATMT).

5.2. Materials and Methods

5.2.1. Plasmid materials

Integrative plasmids pCB 1004 (Carroll *et al.*, 1994) and pHA 1.3 (Powell & Kistler, 1990), both obtained from Prof. Sweigard, Dupont Company, U.S.A., carry a chloramphenicol or ampicillin resistance gene, respectively (Figures 5.1 and 5.2). Plasmids pBin7-1 and pGhph1 were kindly provided by Dr. Mike Challen, Horticulture Research International, Wellesbourne, U.K (Figures 5.3 and 5.4). They all carry a region from *Escherichia coli* encoding a hygromycin resistance gene (hygromycin B phosphotransferase, *hph*) under the control of Ascomycete promoter and terminator sequences. Plasmid pAN7-1 (Punt *et al.*, 1987), provided by Dr. Mike Challen, also contains the *hph* gene.

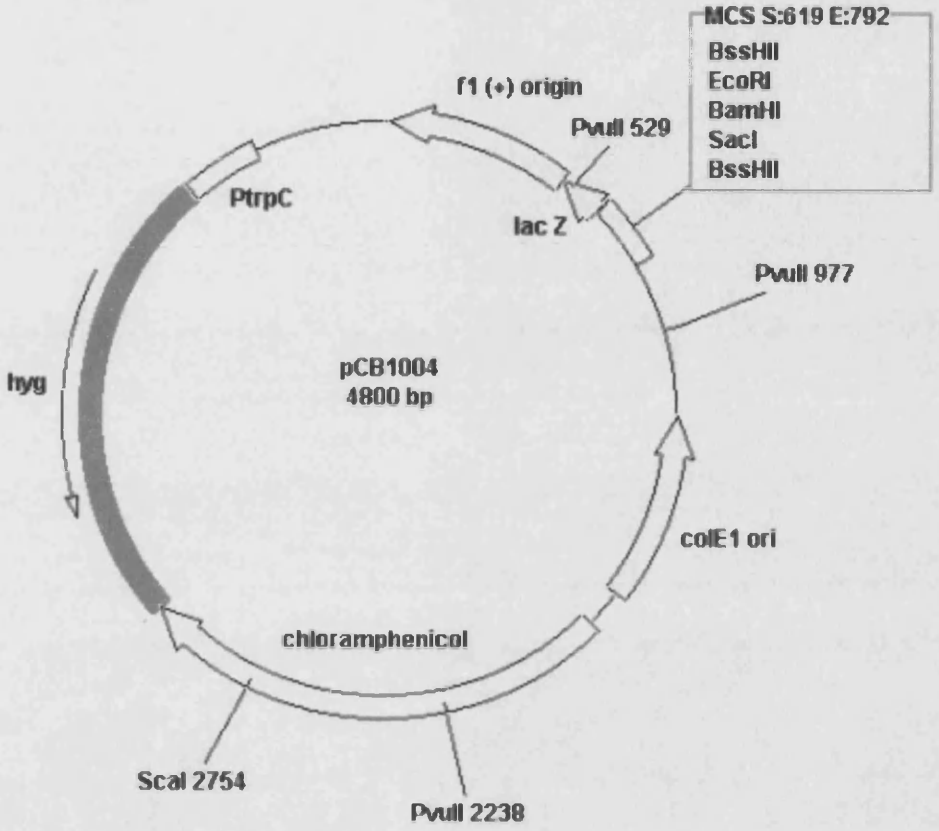


Figure 5.1. Restriction map of the plasmid pCB 1004. The plasmid pCB1004 (Carroll *et al.*, 1994) contains a 1.4 kb gene for hygromycin resistance (*hyg* = *hph*) encoding hygromycin phosphotransferase under the control of the *Aspergillus nidulans* promoter P*trp*C and a chloramphenicol resistance gene. MCS: Multiple Cloning Site.

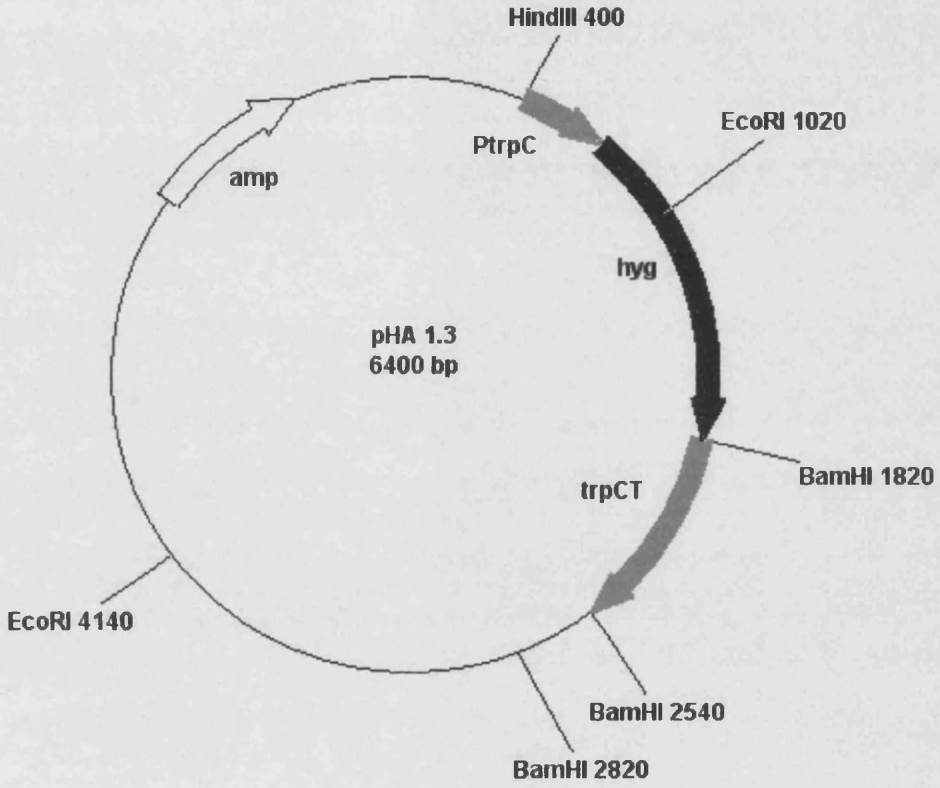


Figure 5.2. Restriction map of the plasmid pHA 1.3. The plasmid pHA 1.3 (Powell and Kistler, 1990) carries the *hyg* (= *hph*) gene under control of the *A. nidulans trpC* promoter as well as an ampicillin resistance gene (*amp*).

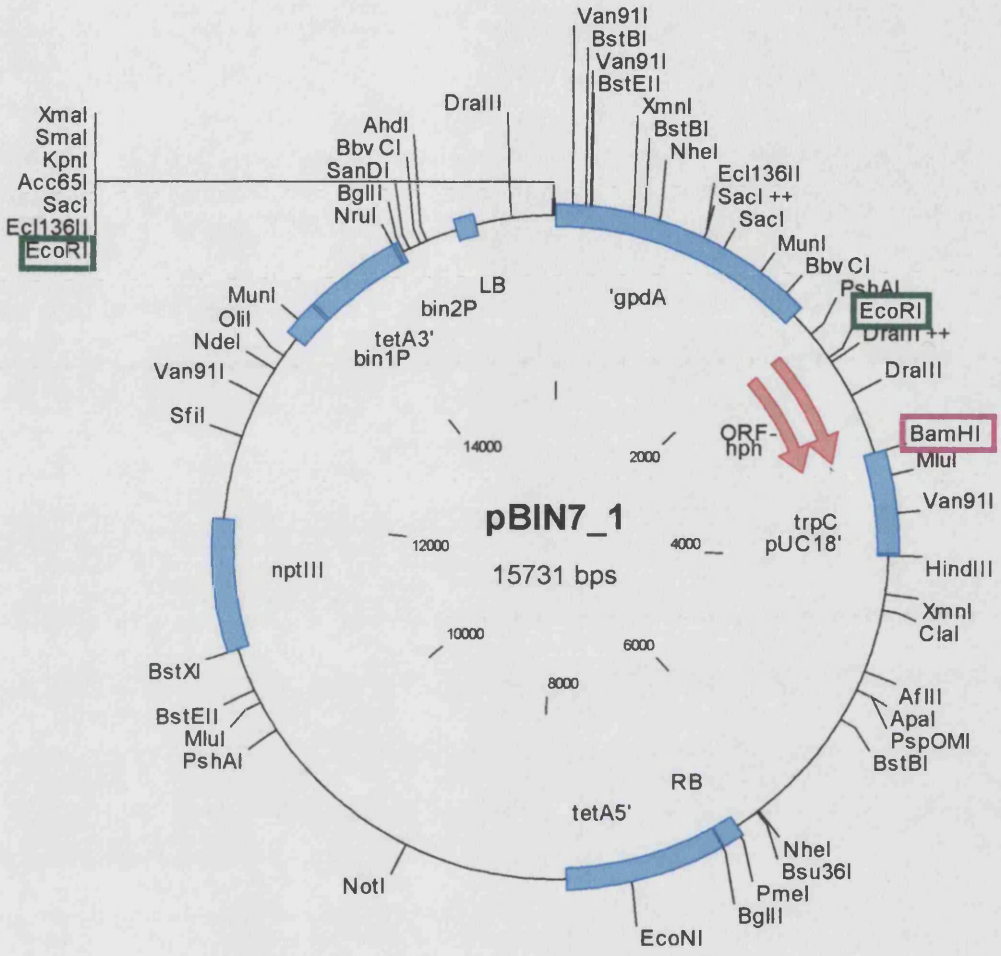


Figure 5.3. Restriction map of the plasmid pBin7-1. The plasmid contains the encoding region from an *Escherichia coli* hygromycin-resistance gene (*hph*), the *gpd* (glyceraldehyde-3-phosphate dehydrogenase) promoter and *trpC* terminator sequences between the left border (LB) and right border (RB) repeats. It contains two *Eco*RI restriction sites (indicated in the green boxes) and a single *Bam*HI restriction site (indicated in the pink box).

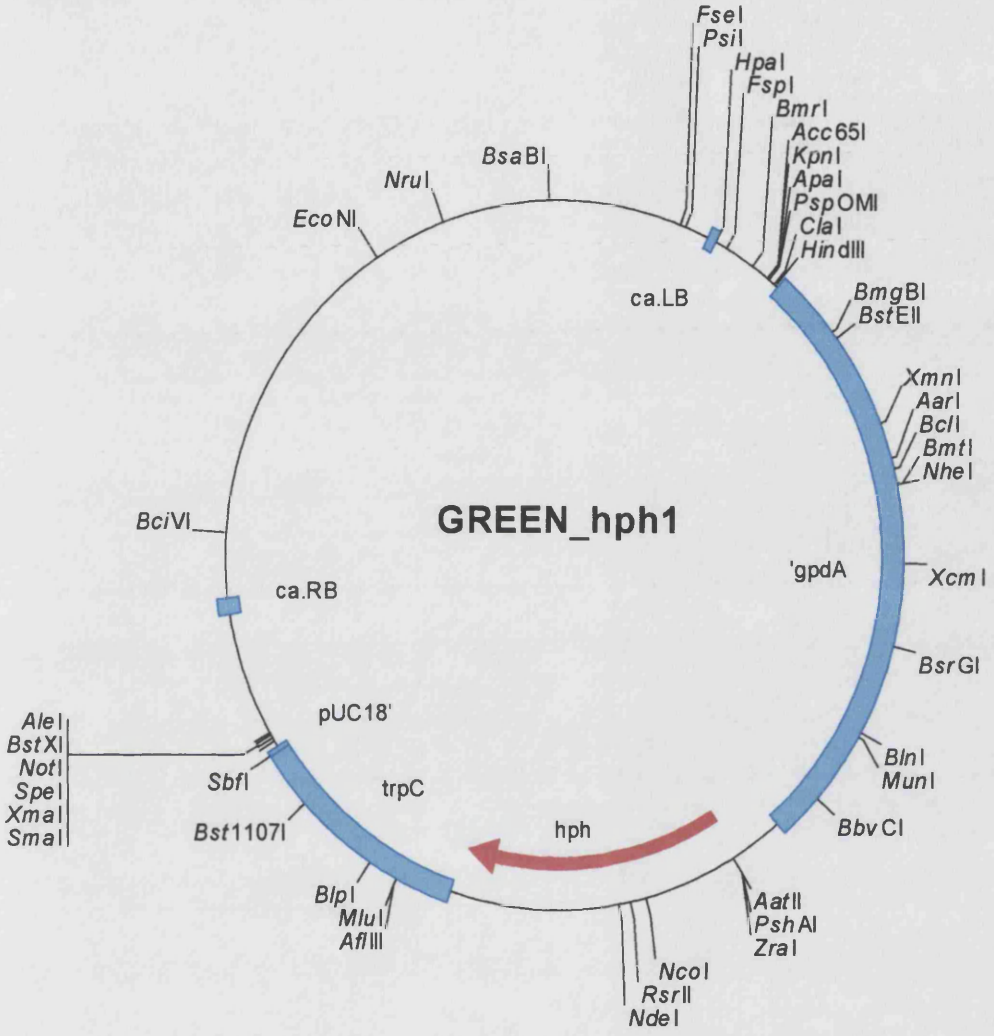


Figure 5.4. Restriction map of the plasmid pGhph1. The plasmid (7292 bps) contains the same *hph* gene, promoter and terminator sequences as well as the *Eco*RI and *Bam*HI restriction sites as plasmid pBin7-1 (Figure 5.3).

5.2.2. Plasmid amplification and purification

Plasmids pCB 1004 and pHA 1.3 were amplified in competent *E. coli* cells (Amersham, Buckinghamshire, U.K.) grown in sterile LB broth (10 $g.l^{-1}$ tryptone; 5 $g.l^{-1}$ yeast extract; 10 $g.l^{-1}$ NaCl) containing 50 $\mu g.ml^{-1}$ chloramphenicol or 30 $\mu g.ml^{-1}$ ampicillin, respectively. Plasmids pCB 1004 or pHA 1.3 (1 µl of each) were separately added to 20 µl of competent cells in a microcentrifuge tube. Cell preparations were first incubated for 30 min on ice, then for exactly 40 sec at 42°C (heat shock) and 2 min on ice. They were transferred to a microcentrifuge tube containing SOC medium (20 $g.l^{-1}$ tryptone; 5 $g.l^{-1}$ yeast extract; 10 ml of 1 M NaCl; 2.5 ml of 1 M KCl; 20 mM Mg^{2+}; 20 mM glucose; pH 7.0) and incubated at 37°C with shaking for 1 hour. The mixtures were spread with a glass rod on the surface of Petri dishes containing agarised LB medium supplemented with the appropriate antibiotic and incubated overnight at 37°C. Single colonies of bacteria were transferred to LB broth and grown at 37°C overnight. Plasmids were subsequently purified either by using the Qiaprep® Spin Miniprep Kit Protocol (Qiagen, Hilden, Germany) or the HiSpeed™ Plasmid Midi Kit Protocol (Qiagen), according to the manufacturer's instructions.

Plasmid preparations were precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. Precipitation was achieved at -20°C for 1 to 2 h. After centrifugation at 4°C for 30 min (13,000 *g*), salts were removed by washing the pellets three times with 70% ethanol and recentrifuging at 4°C for 5 min (13,000 *g*). The pellets were dried for 10 min in a desiccator and resuspended in ultra pure water to a titre of 1 $\mu g.\mu l^{-1}$.

5.2.3. Plasmid linearisation

Plasmids were linearised with the appropriate restriction endonucleases and purified by phenol-chloroform extraction and ethanol precipitation (Sambrook *et al.*, 2002).

5.2.4. Pycnidiospore germination

Conical flasks (250 ml) containing 50 ml YEP broth (Appendix 1) supplemented with 3% glucose were inoculated with freshly produced pycnidiospores of *A. rabiei* (final concentration of 10^6 per ml). They were incubated at 20°C on an orbital shaker rotating at 160 rpm (orbit diameter 5 cm) for 18-20 h.

5.2.5. Selection of transformants

The concentration of hygromycin that inhibited the growth of *A. rabiei* was determined by inoculating the pathogen on potato dextrose agar without hygromycin (control) or with two-fold dilutions of the antibiotics ranging from 100 to 12.5 $\mu g.ml^{-1}$.

5.2.6. REMI technique

When the number of protoplasts (produced as detailed in Chapter 2, Section 2.2.4, Protocol 3) reached a plateau, the lytic reaction was stopped in order to obtain the greatest number of protoplasts and to avoid the formation of impaired cells. Protoplasts were recovered by filtration through glass wool. They were washed by centrifugation at 4°C for 15 min (3,000 *g*) once in 10 ml KC osmoticum (Chapter 2, Table 2.1) and twice in 10 ml ice-cold STC buffer (1.2 M sorbitol; 10 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$) and finally resuspended in ice-cold STC.

Genetic transformation was a modification of the third protocol described by Balhadère *et al.* (1999). Two series of experiments were carried out, using a 100 µl aliquot of protoplasts suspension in each experiment:

Series 1: Protoplasts (5 X 10^6) were incubated for 20 min on ice in the presence of the plasmid pCB1004 linearised with *Eco*RI (two-fold dilution series from 40 to 5 µg) and 60 U of *Eco*RI. After incubation on ice for 20 min and addition of 2 ml polyethylene glycol solution (Appendix 2; 2 ml) the mixture was further incubated

on ice for 20 min and mixed with ice-cold STC buffer (Appendix 2; 30 ml). Protoplasts were collected in the pellet formed after centrifugation at 4°C for 12 min (1,000*g*). Three different conditions of regeneration were tested with suspensions of protoplasts: they were plated immediately after transformation on agarised YEPS regeneration medium (Appendix 2) containing 50 $\mu g.ml^{-1}$ hygromycin, or incubated in complete liquid medium (Appendix 2) at room temperature or at 20°C overnight before plating on selective regeneration medium. Plates were incubated at 20°C overnight before being overlaid with selective regeneration medium.

Series 2: Protoplasts (1.3×10^5) were treated as in Series 1 except that they were incubated in the presence of pCB1004 plasmid linearised with *Bam*HI (10 µg) and a dilution series of *Bam*HI (80 to 5 U), and that protoplast suspensions were incubated in complete liquid medium at 20°C overnight before plating on selective regeneration medium. Plates were incubated and overlaid as in Series 1.

For each experiment of both series, untransformed protoplasts were used as a control in order to determine their viability.

5.2.7. Electroporation

For this technique the plasmids used were washed three successive times in 70% ethanol in order to remove any residue of salt which could interfere with the electric current delivered by the electroporator. Electroporation was carried out with pycnidiospores, protoplasts or mycelium.

Fresh pycnidiospores. Freshly produced pycnidiospores (1.6×10^8 per experiment) were harvested by centrifugation at 1,000 *g* for 5 min. The pellet was washed once in ice-cold ultra-pure water and twice in 1 M ice-cold sorbitol by spinning at 1,000 *g* for 5 min. Pycnidiospores were finally resuspended in 1 M ice-cold sorbitol. The final pycnidiospore suspension (40-100 µl) was added to a chilled microcentrifuge tube containing plasmid pCB1004 (two-fold dilution series from 80 to 2.5 µg). After a 30-minute incubation on ice the mixture was transferred to a 0.4 cm electroporation cuvette (BioRad, Hempstead, UK) and submitted to electrotransformation in a

gene pulser (Gene Pulser II, BioRad) set to 1.6 kV; 25 μF; 200 Ω. Ice-cold 1 M sorbitol (900 μl) was immediately added to the cuvette and the contents returned to the microcentrifuge tube and placed on ice while other electroporations were carried out. Transformed pycnidiospores were plated as soon as possible onto selective sorbitol medium (1 M sorbitol; 0.3% yeast extract; 0.3% casamino acids; 2% agar or 1 M sorbitol; 1% yeast extract; 2% peptone; pH 5.0) containing 50 $\mu g.ml^{-1}$ hygromycin and incubated at 20°C.

Germinated pycnidiospores. Freshly produced pycnidiospores were germinated (Section 5.2.4). They were harvested, washed and resuspended as for ungerminated pycnidiospores. The final pycnidiospore suspension (7-8 X 10^6 per experiment) was submitted to electrotransformation in the same conditions used with ungerminated pycnidiospores, except that the plasmid DNA used was circular pCB1004, pCB1004 linearised with *Eco*RI or pHA1.3 linearised with *Hin*dIII (two-fold dilution series from 40 to 2.5 μg of each), and four combinations of electrical parameters were used: 1.6 kV, 25 μF, 200 Ω; 1.6 kV, 25 μF, 400 Ω; 1.6 kV, 25 μF, 800 Ω or 2.5 kV, 25 μF, 200 Ω. Treated pycnidiospores were plated and incubated as for ungerminated pycnidiospores.

Protoplasts. Protoplasts were produced from germinated pycnidiospores as mentioned in Chapter 2 (Section 2.2.4). They were electroporated (1.3 X 10^6 protoplasts per experiment) in the presence of circular pCB1004 (two-fold dilution series from 20 to 2.5 μg), in the same conditions as ungerminated pycnidiospores.

Mycelium. Mycelium (48 h old), derived from pycnidiospores by shake culture in CD-V8 broth, was harvested by filtration through 4 thicknesses of sterile muslin and washed once in sterile distilled water, then twice with 1 M ice-cold sorbitol and finally resuspended in 1 M ice-cold sorbitol. The mycelial suspension (40 μl at OD_{620} = 0.04) was electroporated under the same conditions as for ungerminated pycnidiospores in the presence of pCB1004 (75, 100, 150, 1,000 ng or two-fold dilution series from 80 to 2.5 μg) or pHA1.3 (100 ng).

Pycnidiospores, protoplasts and mycelium submitted to electroporation without plasmid DNA were plated on non-selective regeneration medium and used as

controls.

5.2.8. Particle bombardment

Microcarriers (Bio-Rad, Hemel Hempstead, U.K.), prepared in 50% glycerol (60 $mg.ml^{-1}$), were vortexed for 5 min in order to resuspend the particles. This stock solution (50 µl containing 3 mg particles) was transferred to a 1.5 ml microcentrifuge tube. Plasmid DNA (10 µg at 1 $\mu g.ml^{-1}$) was added, followed by 50 µl $CaCl_2$ (2.5 M) and 20 µl spermidine (0.1 M), and the mixture was vortexed for 2 to 3 min. The particles were allowed to settle by gravity for 1 min and pelleted by spinning for a short time at high speed in a microcentrifuge. The supernatant was discarded and 140 µl of 70% ethanol was gently added to the microcarriers. After centrifugation and discarding the supernatant as before, 140 µl of 100% ethanol was added. The particles were washed a third time by centrifugation with 48 µl of 100% ethanol. Aliquots of particles (6 µl) were transferred to the centre of each microcarrier in aseptic conditions and desiccated. Two types of target pycnidiospores were used:

Ungerminated pycnidiospores. Pycnidiospores (5×10^3; 10^4 and 2×10^4) were plated on agarised SYC medium (0.6 M sorbitol; 0.3% yeast extract; 0.3% casamino acids) containing 50 $\mu g.ml^{-1}$ hygromycin. They were submitted to particle bombardment, within 1-2 h, with microparticles of tungsten (M10 of about 0.7 µm diameter or M17 type of about 1.1 µm diameter) or gold, coated with 10 µg circular pCB 1004 plasmid. Shooting was performed with a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad) using a vacuum of 711 mm Hg and helium pressure of 1,300 psi. The distance between the stopping screen and the targets cells was set at 6 cm. Pycnidiospores were then incubated at 20°C.

Germinated pycnidiospores. Pycnidiospores were germinated as mentioned in Section 5.2.4. They were recovered by centrifugation at 1,000 *g* for 10 min and plated as before at a final concentration of 10^5; 3×10^5 and 1.7×10^6. Particle bombardment and incubation of these pycnidiospores was performed as for ungerminated pycnidiospores, except that both the circular and the linear (using

*Eco*RI) form of the plasmid pCB 1004 were used.

Control plates consisted of pycnidiospores submitted to bombardment with microparticles but in the absence of plasmid and plated on non-selective SYC medium.

5.2.9. *A. tumefaciens*-mediated transformation

5.2.9.1. Culture and induction of *A. tumefaciens*

Agrobacterium strains AGL1 or LBA1126 (provided by Dr. Mike Challen, Horticulture Research International, Wellesbourne, U.K.) were grown on agarised LB (10 $g.l^{-1}$ tryptone; 5 $g.l^{-1}$ yeast extract; 10 $g.l^{-1}$ NaCl) supplemented with kanamycin (100 $\mu g.ml^{-1}$), rifampicin (20 $\mu g.ml^{-1}$) and carbenicillin (75 $\mu g.ml^{-1}$). A single colony was resuspended in 5 ml of LB broth supplemented with kanamycin (100 $\mu g.ml^{-1}$) and incubated overnight at 28°C (150 rpm). The culture was diluted 26 fold in a final volume of 50 ml of minimal medium (Appendix 2) in a 250 ml conical flask and further incubated at 28°C on an orbital shaker (orbit diameter 5cm; 150 rpm) overnight. The volume of the bacterial culture required to provide an OD_{600} of 0.5 when diluted to 50 ml was transferred to a sterile 50 ml Falcon tube and centrifuged at 2,500 *g* for 10 min. The resulting bacterial pellet was resuspended in 50 ml of induction medium (Appendix 2) containing freshly prepared acetosyringone (2% in ethanol; 100 µl) and kanamycin (100 $\mu g.ml^{-1}$) and further incubated at 28°C for 6 h (150 rpm).

5.2.9.2. Fungus culture

Freshly produced pycnidiospores of *A. rabiei* (10^5 per plate) were spread on overlay discs of cellophane placed on agarised medium (1% yeast extract; 2% peptone; 3% glucose) in Petri plates and incubated at 20°C for 1 or 2 overnights to allow germination.

5.2.9.3. Genetic transformation

Each plate, containing germinated pycnidiospores, was flooded with 2 ml of induced *Agrobacterium* culture induced with acetosyringone (Section 5.2.9.1) and gently agitated manually in order to cover the entire surface with bacteria. After incubation for 2 min, the excess of *Agrobacterium* suspension was removed with a micropipette and the cellophane disc was transferred to agarised induction medium (Appendix 2) that had been freshly prepared and contained 0.2% acetosyringone. The co-cultivation plates were incubated at 20°C for 48 h. Control plates consisted of germinated pycnidiospores treated in the same way but in the absence of *Agrobacterium.*

5.2.9.4. Selection of putative mutants

Each cellophane disc was cut into eight sectors using a scalpel and four transferred to each of two plates containing freshly prepared potato dextrose agar medium or agarised CD-V8 (Appendix 1) medium supplemented with cefotaxime (200 μM) to counter-select *Agrobacterium* cells and hygromycin (50 $\mu g.ml^{-1}$) to select for transformants. Co-cultures were incubated at 20°C and regenerants were further isolated on agarised chickpea extract medium (Appendix 1) or CD-V8 (Appendix 1) supplemented with the same two antibiotics.

5.2.10. Screening putative mutants for the production of solanapyrone A

5.2.10.1. In microtitre plates

One disc of mycelium cut with a cork borer number 3 from the margin of actively growing colonies of putative mutants was transferred to each well of a 24-well culture plate (Nunc, Denmark) containing 2 ml of CDCLM and incubated at 20°C. Each putative mutant was tested individually in triplicate. In order to test for any cross-contamination, the wild-type strain was inoculated in wells both next to

CDCLM (reference) and next to a transformant. Samples of the culture medium (200 µl) were transferred to wells of a 96-well microtitre plate (Nunc) over a time course. The absorbance at 340 nm of each sample was determined on a plate reader (EL_x808_{IU} Ultra Microplate Reader; Bio-Tek Instruments, Inc.). The wild-type was used as control for the production of solanapyrone A.

Simultaneously, the mycelial growth of each transformant was evaluated using a range from 0 (no growth) to 10 (maximum growth) (Figure 5.5).

5.2.10.2. In conical flasks

In order to confirm the low production of solanapyrone A by the transformants, their respective mycelia were transferred to 250 ml conical flasks containing 30 ml of CDCLM and further incubated at 20°C for 14 days. Growth of each transformant was determined both visually on a 1-10 scale (Figure 5.6) and by drying the mycelium to constant weight. The solanapyrone toxins produced by cultures that presented a growth score ≥ 6 were purified by SPE (Chapter 3, Section 3.2.2.1) and eluted in 1 ml acetonitrile. Solanapyrone A was quantified spectrophotometrically by measuring the absorbance at 327 nm and the presence and amount of solanapyrone A confirmed by HPLC (Chapter 3, Section 3.2.3). The other transformants (that presented a growth rate < 6) were incubated at 20°C for a further 7 d prior to purification and analysis of the solanapyrone toxins they produced. Only the transformants with a growth rate ≥ 4 after the second incubation were used for SPE purification and quantification.

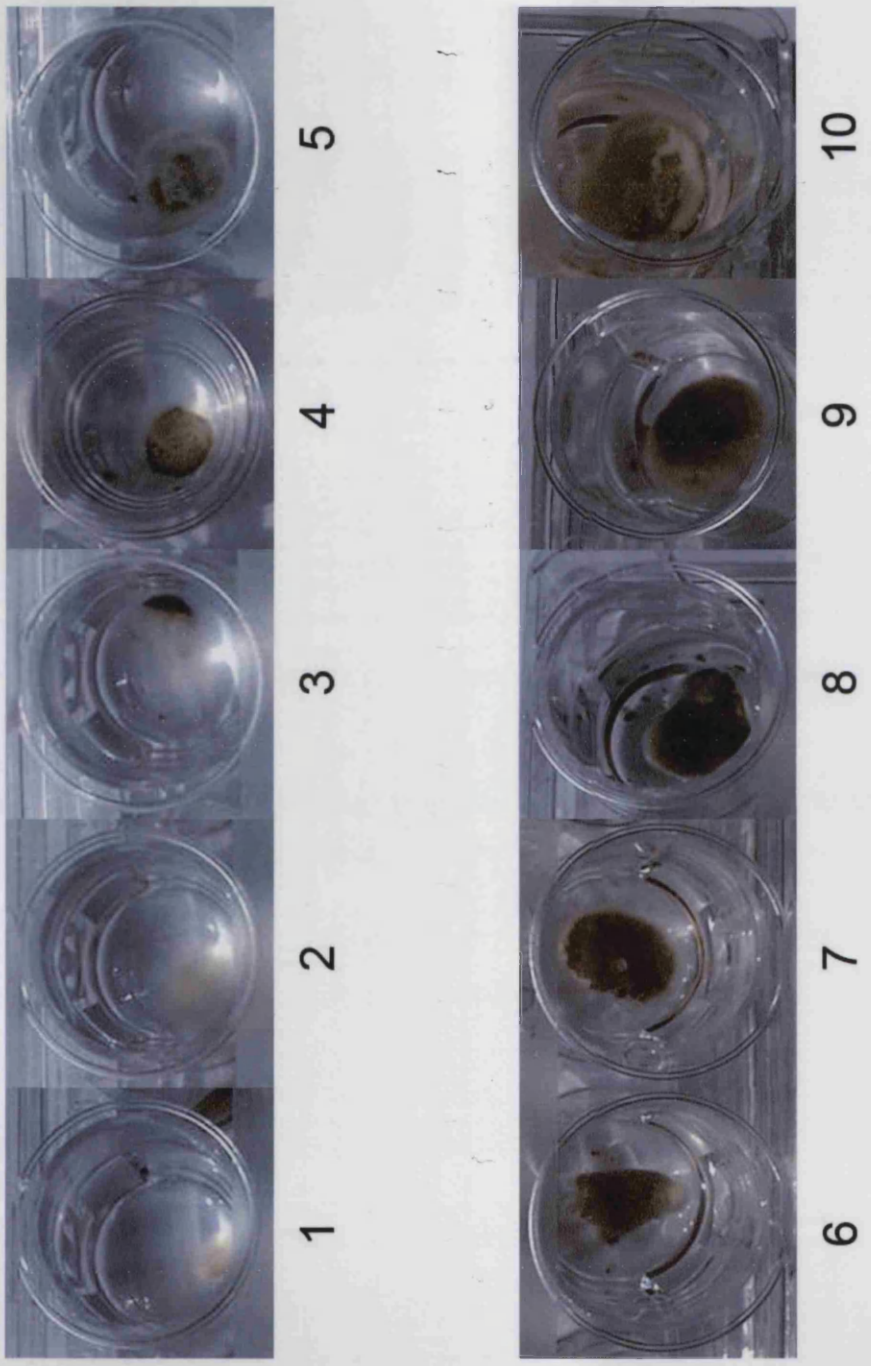


Figure 5.5. Scoring scale for mycelial growth in microtitre plate.

Figure 5.6. Scoring scale for mycelial growth in 250 ml conical flasks.

5.2.11. Sporulation of the transformants

Chickpea seed (Cypressa, Katsouris Brothers Ltd, U.K., *ca.* 60) was softened by boiling in water for 30 min in 250 conical flasks and, after draining, sterilised by autoclaving for 20 min at 121°C. They were inoculated with three discs of cultures of each transformant grown on agarised chickpea extract medium (Appendix 1) or CD-V8 medium (Appendix 1), and incubated at room temperature for 10 to 12 days.

5.2.12. Molecular analyses of transformants

5.2.12.1. Isolation of genomic DNA of *A. rabiei*

Two techniques were used to extract and purify genomic DNA:

(i) DNA suitable for PCR was isolated by the Chelex technique. Five agar plugs of 4 mm diameter were cut with a cork borer from each putative transformant growing on agarised chickpea extract medium (Appendix 1) containing 50 $\mu g.ml^{-1}$ hygromycin. The plugs were transferred to a 1.5 ml microcentrifuge tube and 1 ml of Chelex-Tris suspension (3% Chelex 100 resin (BioRad); 1mM Tris; pH 8.0) added along with 100 mg of glass beads (106 microns; Sigma). The samples were vortexed for 1 min and subjected to three alternate freeze/thaw cycles of 1 min each in liquid nitrogen and boiling water with the final boiling stage performed for 5 min. The samples were vortexed for 1 min, incubated at 55°C for 30-40 min and centrifuged at 13,000 *g* for 5 min. The top 100 µl of supernatant containing the genomic fungal DNA was transferred to a fresh micro-centrifuge tube and stored at -20°C.

(ii) Purer DNA was extracted using the Nucleon Phytopure Plant DNA Extraction Kit (Tepnel, Manchester, U.K.). Three plugs of each transformant were inoculated to 250 ml conical flasks containing 100 ml CD-V8 broth (Appendix 1) and incubated at 20°C for 72 h. Mycelium was harvested by filtering through four thicknesses of muslin and ground in

liquid nitrogen. DNA was purified with the kit according to the manufacturer's recommendations and quantified by spectrophotometry.

5.2.12.2. Polymerase Chain Reaction (PCR)

PCR was carried out using PCR "puReTaq Ready-To-Go PCR Beads" (Amersham) containing, for 25 µl reactions, 200 µM of each dNTP in 10 mM Tris-HCl (pH 9.0), Taq DNA polymerase (about 2.5 U), 50 mM KCl and 1.5 mM $MgCl_2$. In order, 5 or 10 µl of genomic fungal DNA extracted by the Chelex technique (Section 5.2.12.1) was added to each tube, followed by 4 µl (at 2.5 pmol.µl^{-1}) of each of the primers specific to ribosomal DNA internal transcribed spacers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), various amounts of $MgCl_2$ at 10 mM (1.5 mM; 2.0 mM; 2.5 mM and 3.5 mM final concentration) and 12 µl to 2 µl of sterile ultra-pure water to adjust the final volume of each reaction to 25 µl. A positive control consisted of 2 µl of Lambda DNA, 2 µl of control primer 1 (at 2.5 pmol.µl^{-1}), 2 µl of control primer 2 (at 2.5 pmol.µl^{-1}) and 19 µl of sterile ultra-pure water added to PCR beads. A negative control consisted of 2 µl of control primer 1 (at 2.5 pmol.µl^{-1}), 2 µl of control primer 2 (at 2.5 pmol.µl^{-1}) and 21 µl of sterile ultra-pure water added to PCR beads. Amplifications were carried out in a thermocycler (Progene; Techne) and consisted of 1 cycle of 2 min at 95°C (initial denaturation), followed by 30 cycles of 30 s at 95°C (denaturation), 30 s at 55°C (annealing) and 1 min 30 s at 72°C (elongation), and 1 cycle of 5 min at 72°C (final elongation). Amplification products were separated on a 1.2% agarose gel in 1X TAE buffer (Appendix 2) at 74 V for 90 min in an horizontal tank and detected by UV transillumination.

PCR was also performed with 5 µl fungal genomic DNA as in the previous paragraph but extracted either by the Chelex method or using the Nucleon Phytopure Kit (Section 5.2.12.1) with primers specific to the *hph* gene (*hph 1*: 5' -ATG CCT GAA CTC ACC GCG- 3' and *hph 2*: 5' -TCG GTT TCC ACT ATC GGC- 3'). Amplification, separation and detection were performed as before.

5.2.12.3. Southern blotting and homologous hybridisation using digoxigenin

Digoxigenin (DIG)-labelled probe was synthesised with the PCR DIG labelling kit (Roche Diagnostics Ltd., Lewes, U.K.) as recommended by the manufacturer using *hph1* and *hph2* primers, with amplification cycles similar to those used for previous PCR amplification (Section 5.2.12.2). Various concentrations of DNA template (plasmid pAN7-1, which contains the *hph* gene, in the range of 10 to 50 pg) and of *hph* primers (from 0.05 to 1 mM) were used in order to optimise the labelling of the probe (Table 5.1). Synthesis and labelling of the probe was confirmed by differences in electrophoretic mobility between labelled and unlabelled probe on a 1.2% agarose gel (run at 76 V for 1h).

The probe (120 ng) was added to 20 ml of pre-warmed hybridisation solution (5X SSC (from 20 X SSC: 0.3 M trisodium citrate; 3 M NaCl; pH 7.0); 0.1% sarkosyl; 0.02% sodium dodecyl sulphate (SDS)) containing 2% casein-blocking agent (Roche) and 200 µl of Salmon Testes DNA (Sigma) at 10 $mg.ml^{-1}$. It was stored at -20°C until needed.

Genomic DNA (4-15 µg) from putative transformants of *A. rabiei* and from the wild-type was digested for 15 h at 37°C with *Bam* HI (40 U) which cuts only once in the T-DNA or with both *Bam*HI and *Eco*RI (40 U each) which cuts twice in the T-DNA region (Figure 5.3). Restricted DNA was separated by electrophoresis in a 0.7% agarose gel, transferred to Hybond-N^+ nylon membranes (Amersham) and cross-linked by exposure to UV light for 2 min.

Dried membranes were rinsed in hybridisation solution, rolled in a cloth (rinsed with the same solution) in a glass bottle (Hybaid Ltd., U.K.) and pre-hybridised twice at 65°C for 1 h in 15 ml of hybridisation solution containing 2% casein-blocking agent. The probe (120 ng) was denaturated by boiling for 10 min and added to the membranes and further incubated overnight at 65°C. Following hybridisation, the membranes were placed in a tray and washed in solutions of increasing stringency at 65°C with constant agitation as follows:

(i) twice in washing solution I (3X SSC; 0.1% SDS), once for 5 min and once for 15 min;

(ii) twice in washing solution II (1X SSC; 0.1% SDS) for 15 min each and

(iii) twice in washing solution III (0.2X SSC; 0.1% SDS) for 15 min each.

The membranes were washed under constant agitation for 5 min at room temperature in TN1 solution (100 mM Tris, pH 7.5; 150 mM NaCl). They were further washed in the same conditions for 30 min in TN2 solution (TN1 containing 2% casein-blocking agent), followed by a 30 min-wash in TN2 solution containing 150 $mU.ml^{-1}$ anti-DIG-AP Fab fragments (Roche) and two successive 15 min washes in TN1 to remove any unbound antibody. The membranes were finally washed for 5 min in TN3 (100 mM Tris, pH 9.5; 100 mM NaCl and 50 mM $MgCl_2$). Chemiluminescent detection of hybridisations was performed by adding a 1:100 dilution in TN3 of the substrate, CDP-star (Roche), to the membranes. The membranes were covered with Saran wrap and incubated for 5 min. After removing excess substrate the membranes were exposed to X-ray films (Biomax MS-I films, Kodak, Sigma) which were developed using a processor (Compact X2, Xograph Ltd.).

The sensitivity of the DIG technique was tested by hybridising a series of dilutions of the plasmid pAN7-1 (two-fold series dilution from 100 ng to 1 ng) with the *hph* gene as a probe as for genomic DNA.

	Experiment number							
	Labelled probe				Unlabelled probe			
Reagent	1	2	3	4	5	6	7	8
Template DNA (pg)	10	10	10	50	10	10	10	50
Buffer (µl)	5	5	5	5	5	5	5	5
dNTPs with DIG-dUTP (µl)	5	5	5	5	-	-	-	-
hph1 forward primer (µM)	0.05	0.4	1.0	1.0	0.05	0.4	1.0	1.0
hph2 reverse primer (µM)	0.05	0.4	1.0	1.0	0.05	0.4	1.0	1.0
Enzymes (U)	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6
Ultra-pure water (µl)	27.25	13.25	28.25	19.25	27.25	13.25	28.25	19.25
dNTPs (µM)	-	-	-	-	200	200	200	200

Table 5.1. Combinations of reagents used for the synthesis and labelling of the probe specific for the *hph* gene with DIG by PCR. DNA template: plasmid pAN7-1 containing the *hph* gene. Dash (-): no reagent added.

5.2.12.4. Southern blotting and homologous hybridisation using radioactivity

The probe was synthesised by PCR amplification of the *hph* gene as mentioned in Section 5.2.12.2 and purified using the QIAquick PCR Purification kit (Qiagen). The probe (5 µl at 15 ng.µl^{-1}) was labelled with [α-^{32}P]dCTP by random primed labelling using the Megaprime DNA labelling systems kit (Amersham). Unincorporated nucleotides were removed using the Micro Bio-Spin S2000-HR columns (Amersham).

Hybridisations were first performed with the *hph* gene amplified by PCR as in Section 5.2.12.2 using 5-20 µg genomic DNA. The products of the PCR were separated by electrophoresis in a 1% agarose gel, transferred to Hybond-N$^+$ nylon membranes (Amersham) and cross-linked by exposure to UV light for 2 min. Southern blotting of the DNA was performed as mentioned in Section 5.2.12.3. Prehybridisation was carried out at 65°C in 50 ml of 6 X SSPE (0.9 M NaCl; 150 mM NaH_2PO_4; 6 mM EDTA; pH 7.4), 20 mM EDTA, 5 X Denhardt solution (Appendix 2), 0.5% SDS for 4 h in the presence of Salmon Testes DNA (10 mg.ml^{-1}) previously denatured by boiling for 10 min. The membrane was hybridised in the same solution with the probe (75 ng) at 65°C overnight. It was washed twice at 65°C in 2 X SSPE, 0.1% SDS for 20 min, once at 65°C in 1 X SSPE, 0.5% SDS for 10 min and once at 65°C in 0.1 X SSPE, 0.1% SDS for 5 min. The membrane was exposed to X-Ray film for 2 min 20 sec at room temperature and the film was developed in a processor (Xograph Ltd.).

Hybridisations were also performed with genomic DNA (5 µg) digested for 15 h at 37°C with *Bam* HI alone (40 U) or *Bam* HI and *Eco*RI (40 U of each). Restricted DNA was separated by electrophoresis in a 0.7% agarose gel, transferred to Hybond-N$^+$ nylon membranes (Amersham). Cross-linking, Southern blotting of the DNA and hybridisation were performed as mentioned above. The membrane was exposed to a film overnight at -80°C and developed as before.

5.2.13. Nucleation of pycnidiospores

Pycnidiospores were stained with 4', 6-diaminido-2-phenylindole (DAPI) and dried for 5 min before microscopic observation.

5.3. Results

5.3.1. Insertional mutagenesis

Hygromycin resistance was used as the selectable marker for all experiments. The minimum concentration that completely inhibited growth of *A. rabiei* was 25 $\mu g.ml^{-1}$. Therefore, a concentration of 50 $\mu g.ml^{-1}$ was used to select transformants.

5.3.1.1. REMI

Two series of experiments were performed, Series 1 with the restriction enzyme *Eco*RI and Series 2 with *Bam*HI.

Series 1. When protoplasts were treated with various amounts of linearised pCB1004 in the presence of 60 U *Eco*RI and immediately plated on selective YEPS medium, no transformants were obtained. In such conditions, 0.77% of untreated protoplasts regenerated. When protoplasts were incubated in complete liquid medium at room temperature overnight, no transformants were produced and the regeneration of control protoplasts reached 1.83%. However, 16 colonies regenerated after transformation and incubation of protoplasts in complete liquid medium at 20°C overnight but presented a low growth rate compared to the wild-type strain. The viability of untreated protoplasts was 6.65% after such incubation. Transformation efficiency ranged from 0.005 (with 20 µg plasmid) to a maximum of 0.040 (with 10 µg plasmid) transformants per U of *Eco*RI and per 10^5 viable protoplasts (Table 5.2).

Series 2. Putative mutants (21) were produced from protoplasts transformed with linearised pCB1004 and a variable amount of *BamHI*. The regeneration of control protoplasts was 5.20%. Transformation efficiency peaked at 23.67 transformants

per microgram of plasmid and per 10^5 viable protoplasts with 40 U *Bam*HI (Table 5.3).

5.3.1.2. Electroporation

Pycnidiospores. Pycnidiospores (1.6 X 10^8 per experiment) were electroporated with a two-fold dilution series from 80 to 2.5 µg of the plasmid pCB1004. However, no transformants were created. Therefore, electroporation was attempted with germinated pycnidiospores and protoplasts in the presence of variable amounts of plasmids and different settings of field strength (expressed in kV) and resistance (in Ω).

Initially, germinated pycnidiospores (7-8 X 10^6) were electroporated in the presence of 2.5 to 40 µg of circular pCB1004, pCB1004 linearised with *Eco*RI or pHA1.3 linearised with *Hin*dIII. The electroporator was set up at 1.6 kV; 25 µF; 200 Ω, giving a time constant of 5.32 ms and an amperage of 8 A. No transformants were produced with these conditions. Five to eight percent of the treated pycnidiospores used as control were able to regenerate.

A second experiment was designed to increase the viability of the pycnidiospores in order to improve the probability of obtaining transformants. The resistance was doubled to 400 Ω and the other two parameters kept constant, giving a decrease in current to 4 A. Moreover, germinated pycnidiospore suspensions (7-8 X 10^6 per experiment) were incubated at 20°C for 4 h and plated on selective regeneration medium in order to optimise the regeneration of putative mutants. No transformants were obtained and 0.1% to 0.45% of pycnidiospores treated without plasmid regenerated. Therefore, the resistance was doubled again to 800 Ω or the voltage was set to the maximum value (2.5 kV) during electroporation of 10^7 germinated pycnidiospores, while keeping the other parameters constant, giving a current of 2 A and 12.5 A, respectively. No transformants were produced. The regeneration of control pycnidiospores was 0.22% and 0.35%, respectively.

Protoplasts. Electroporation was then attempted on protoplasts (1.3 X 10^6) in the presence of circular pCB1004 (2.5 to 20 µg). Three protoplasts regenerated after transformation with 5 µg plasmid, giving a transformation efficiency of 0.046 transformants per µg of plasmid and per 10^5 viable protoplasts. No transformants were obtained with 2.5, 10 or 20 µg plasmid pCB1004. The viability of control protoplasts was only 0.9%.

Mycelium. Mycelium of *A. rabiei* (40 µl containing mycelial suspension at OD_{620} = 0.04) was also subjected to electroporation in the presence of pCB1004 (75, 100, 150 or 1,000 ng) or pHA1.3 (100 ng) but no transformants regenerated. As the amount of integrative vector can affect transformation efficiency, electroporation of mycelium was then carried out with higher amounts of pCB 1004 plasmid (two-fold dilutions series from 80 to 2.5 µg). Although some colonies were obtained they did not survive subculture.

5.3.1.3. Particle bombardment

Ungerminated pycnidiospores. Pycnidiospores (5 X 10^3; 10^4 and 2 X 10^4) submitted to particle bombardment with microparticles of tungsten (M10 or M17 type) or gold coated with circular pCB 1004 plasmid, were not transformed. The regeneration rate of pycnidiospores bombarded with uncoated particles reached 3.16% on non-selective medium.

Germinated pycnidiospores. Therefore, particle bombardment was attempted with germinated pycnidiospores (10^5; 3 X 10^5 and 1.7 X 10^6) using tungsten microparticles coated with 10 µg of circular or linear pCB 1004 plasmid. However, no colonies regenerated on selective medium. Regeneration of control pycnidiospores was 6.22 %.

Plasmid pCB1004 (µg)	5	10	20	40
Number of transformants	0.6	2.4	0.3	1.5

Table 5.2. Transformation efficiency of protoplasts of *A. rabiei* by REMI according to plasmid amount. Protoplasts (5 X 10^6 per experiment), giving 3.32 X 10^5 viable protoplasts, were treated with 60 U *Eco*RI and a variable amount of the plasmid pCB1004. Results are expressed as the number of transformants per 10^5 viable protoplasts.

*Bam*HI (U)	5	20	35	50	65	80
Number of transformants	4.44	2.96	0	23.67	0	0

Table 5.3. Transformation efficiency of protoplasts of *A. rabiei* by REMI according to the amount of restriction enzyme. Protoplasts (1.3 X 10^5 per experiment, among which 6.76 X 10^3 were viable) were treated with 10 µg of plasmid pCB1004 and a variable amount of *Bam*HI. Results are expressed as the number of transformants per µg of plasmid pCB1004 and per 10^5 viable protoplasts.

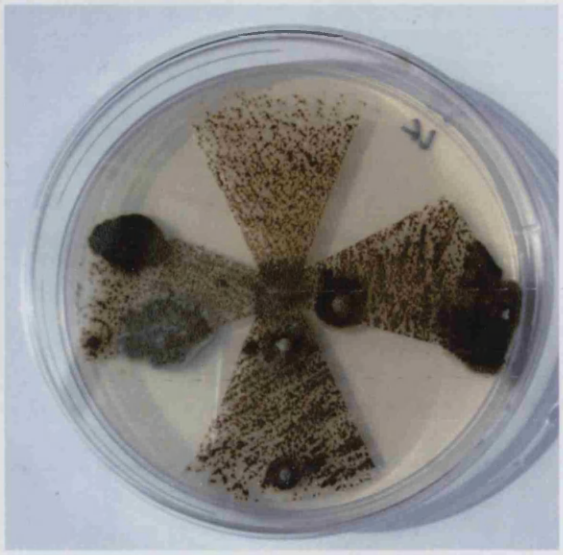
5.3.1.4. ATMT

Transformants were produced from all four experiments performed and arose after incubation for 10 days at 20°C on selective medium. In the first experiment four combinations of *A. tumefaciens* strains and binary vectors were used to transform germinated pycnidiospores of *A. rabiei* (Figure 5.7). The highest transformation efficiency was achieved with the two combinations AGL1/pBin 7-1 and LBA1126/pGhph1 (8.0 and 10.7 transformants per 10^5 pycnidiospores, respectively) and was 5 to 27 times higher than the efficiency reached with the two other combinations AGL1/pGhph1 and LBA1126/pBin7-1 (Figure 5.8).

Consequently, the two most efficient combinations were chosen for a second experiment (Figure 5.8.). A transformation efficiency similar to the one obtained in the first experiment was obtained with the *A. tumefaciens* strain LBA1126 used with the plasmid pGhph1. The combination AGL1/pBin7-1 produced twice as many transformants as in the first experiment.

A total of 908 putative mutants regenerated from the six transformation series (22 to 36 plates per series) and 668 were isolated on selective medium.

Pycnidiospores used as controls were not able to regenerate, demonstrating that transformants did not spontaneously acquire hygromycin resistance (Figure 5.7).



Transformation plate

Control plate (absence of *A. tumefaciens*)

Figure 5.7. Regeneration of *A. rabiei* on selective medium after transformation with *A. tumefaciens*. Fungal colonies regenerated on transformation plate after co-culture of pycnidiospores of *A. rabiei* with *A. tumefaciens* for 2 overnights and transfer to a selective medium. No transformants appeared on control plates when pycnidiospores were treated in the absence of *A. tumefaciens*.

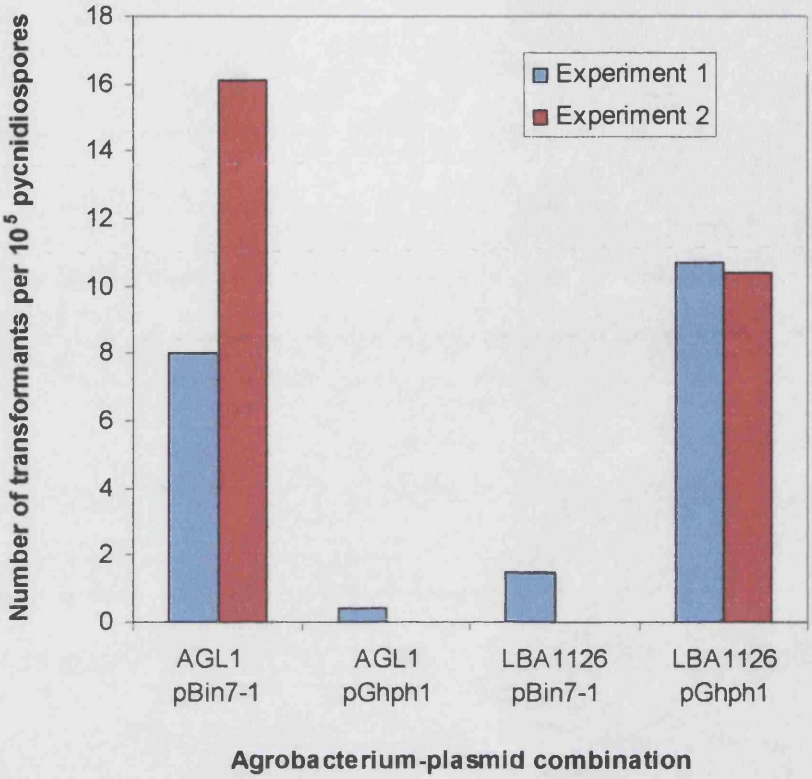


Figure 5.8. Transformation efficiency of *A. rabiei* using four combinations of two strains of *A. tumefaciens* and two plasmids. Two experiments were carried out independently, the first one using the four combinations of *A. tumefaciens* and plasmid, the second one using the two more efficient combinations AGL1/pBin 7-1 and LBA1126/pGhph1.

5.3.2. Screening putative mutants for the production of solanapyrone A

5.3.2.1. In microtitre plates

One putative mutant (called 1A1), isolated after REMI mutagenesis of protoplasts with 10 µg of linearised pCB1004 plasmid and 6 U *Bam*HI, was subcultured in microtitre plate wells containing 2 ml of CDCLM (four replicates). After incubation at 20°C for 9 days, the culture filtrate of the transformant was purified by SPE. HPLC analysis of the purified toxins showed that the fungus produced solanapyrone A (the profile of its spectrum matched at 95.92% with the profile of a reference spectrum; Figure 5.9) but no solanapyrone B nor C. However, the transformant presented a significant reduction of the toxin production (8.5 $\mu g.ml^{-1}$ culture filtrate on average; Table 5.4) compared with the production of 40-45 µg per ml^{-1} of culture filtrate by the parent strain (t-test, $p < 0.001$). This transformant was not able to sporulate.

Putative mutants created by the *A. tumefaciens*-mediated technique were screened for the production of solanapyrone A in microtitre plates as mentioned in Section 5.2.10.1. Transformants that were inoculated in the wells of microtitre plates next to wells containing the wild-type strain (with ratios of absorbance at 340 nm to mycelial growth reaching up to 0.35) presented a ratio varying between 0.01 and 0.31. These values demonstrated that there was no cross-contamination between samples in the microtitre plates and that there is variation in the production of solanapyrone A. The varying levels of production of solanapyrone A is also represented in Figure 5.10 and detailed in Appendix 3.

5.3.2.2. In conical flasks

Out of the 226 transformants obtained from the first transformation experiment, the fifteen presenting a ratio of absorbance at 340 nm to mycelial growth equal to or below 0.05 (arbitrary value) were grown in 30 ml CDCLM (Section 5.2.10.2). Among the cultures of these transformants, nine produced significantly less

solanapyrone A (9.8 to 56.5% of the wild-type), with a majority (five transformants) producing 20.1% of the solanapyrone A of the wild-type or less (Figure 5.11). Four of these fifteen transformants produced a minute amount of solanapyrone C (0.018 $\mu g.ml^{-1}$ on average). None of the nine transformants were capable of sporulating on chickpea seed (they were tested as mentioned in Section 5.2.11).

Among the 682 transformants produced from the second experiment, seven presented a significantly reduced amount of solanapyrone A compared to the wild-type strain. All of them produced less than 30% of the solanapyrone A of the wild-type, four of them producing less than 10% (Figure 5.12; the screening of these transformants was performed by Elise Pelzer). Five of these transformants were able to sporulate (2AB39, 2AB49, 2AB148, 2LG150 and 2LG236).

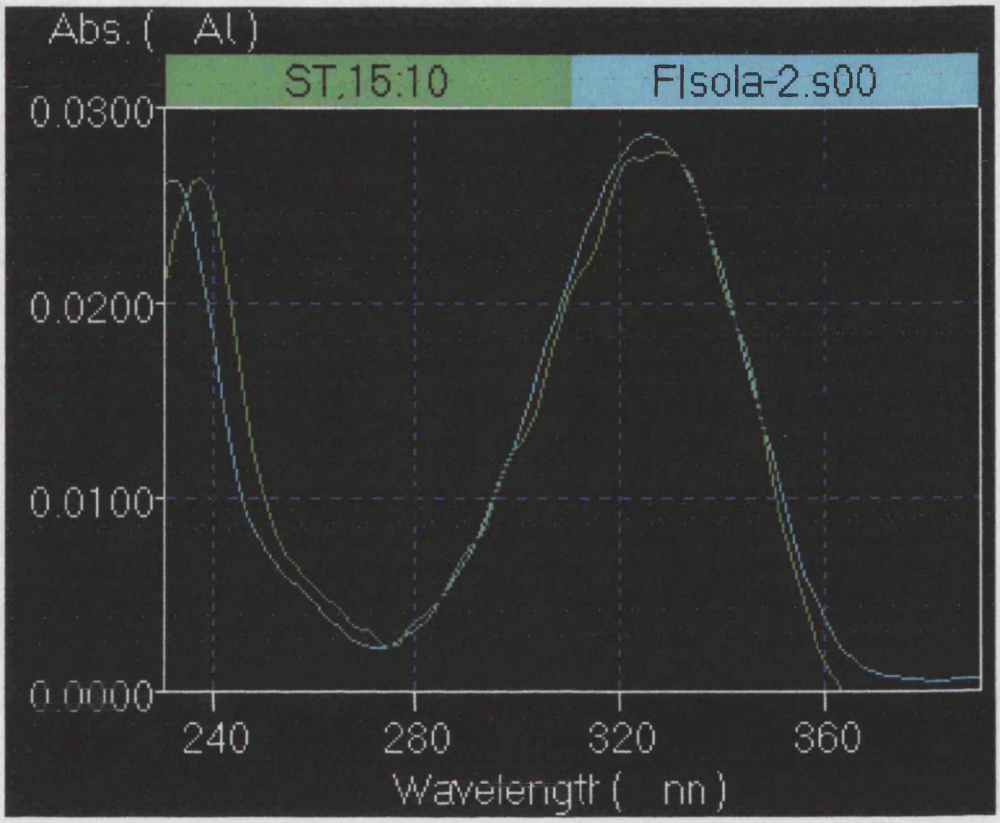


Figure 5.9. Comparison of solanapyrone A spectrum from the regenerant 1A1 with a spectrum of reference (pure solanapyrone A). The spectrum of toxin produced by the regenerant 1A1 (ST, 15:10) was overlaid with that of solanapyrone A (F|sola-2.s00). The spectra matched at 95.92%, confirming that the regenerant 1A1 produced solanapyrone A. However, the absorbance was low, showing that this regenerant was affected in its capability of producing solanapyrone A.

Transformant 1A1 or wild-type	Solanapyrone A ($\mu g.ml^{-1}$)
1A1 (1)	7.2
1A1 (2)	8.0
1A1 (3)	9.8
1A1 (4)	8.9
Wild-type (1)	42.49
Wild-type (2)	28.55
Wild-type (3)	26.88
Wild-type (4)	26.67

Table 5.4. Production of solanapyrone A by the wild-type and by the transformant 1A1 created by REMI. The transformant and the wild-type were inoculated in four replicates in microtitre plates, the absorbance at 340 nm measured on a plate reader and the amount of solanapyrone A produced determined by means of a standard curve. The number of the replicates is indicated in brackets.

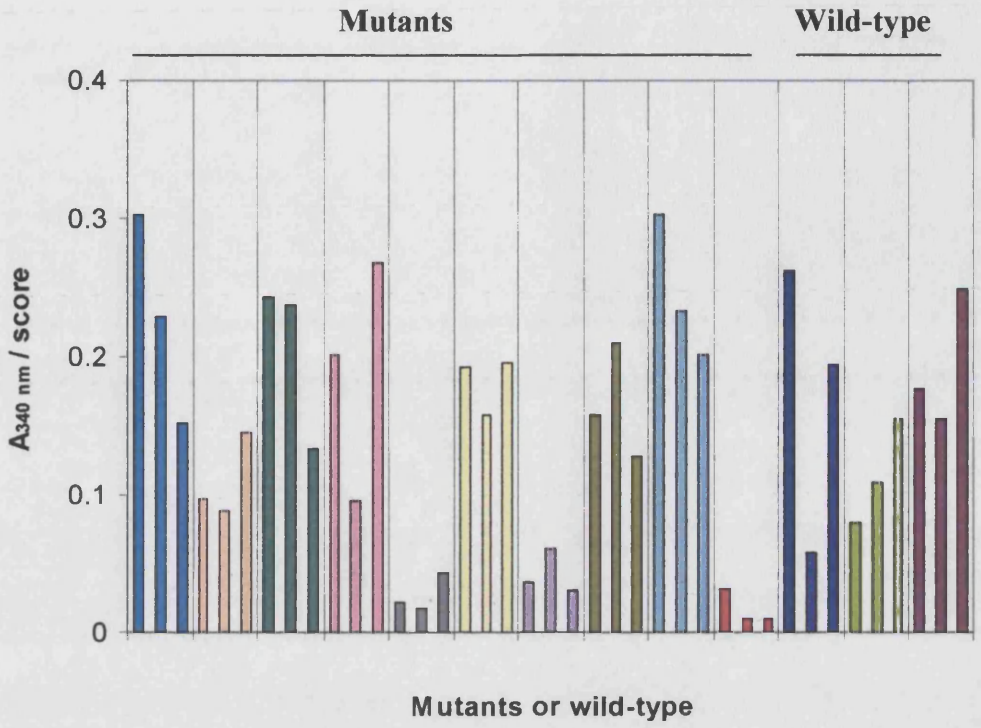


Figure 5.10. Ratio of the absorbance at 340 nm of culture filtrates of ten transformants and three wild-types (all arbitrarily selected from the first experiment) to their growth evaluated on a 1-10 scale. Transformants and wild-types were assayed in triplicate. Wild-types averaged between 0.06 (only one replicate) to 0.19 (11 replicates presented a ratio above 0.10). Ratios of transformants varied from 0.01 to 0.59 but nine had ratios below 0.05 (Appendix 3).

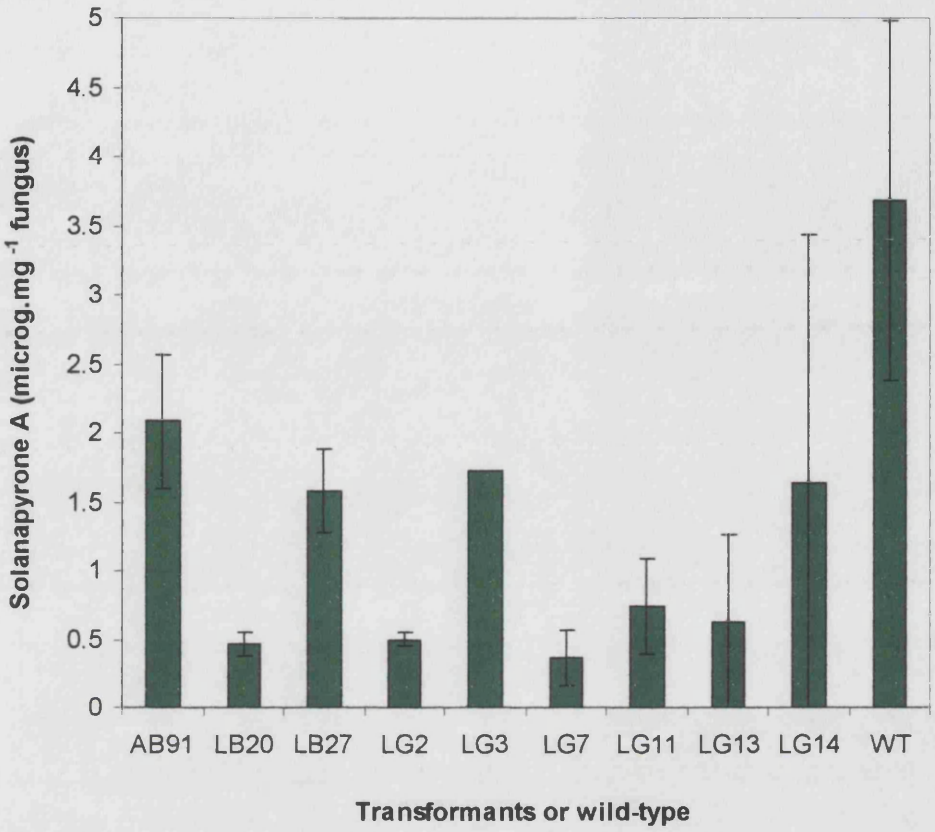


Figure 5.11. Solanapyrone A production by nine transformants of *A. rabiei* produced from the first experiment of the ATMT technique and selected after screening in a microtitre plate. The transformants were grown in triplicate in 30 ml CDCLM in conical flasks for 14 days. Solanapyrone A produced by each transformant was purified by SPE, quantified spectrophotometrically and expressed per mg of fungus (dry weight). Bars represent the standard deviation. No standard deviation was reported for the LG3 as the amount of solanapyrone A was obtained from only one replicate of this transformant.

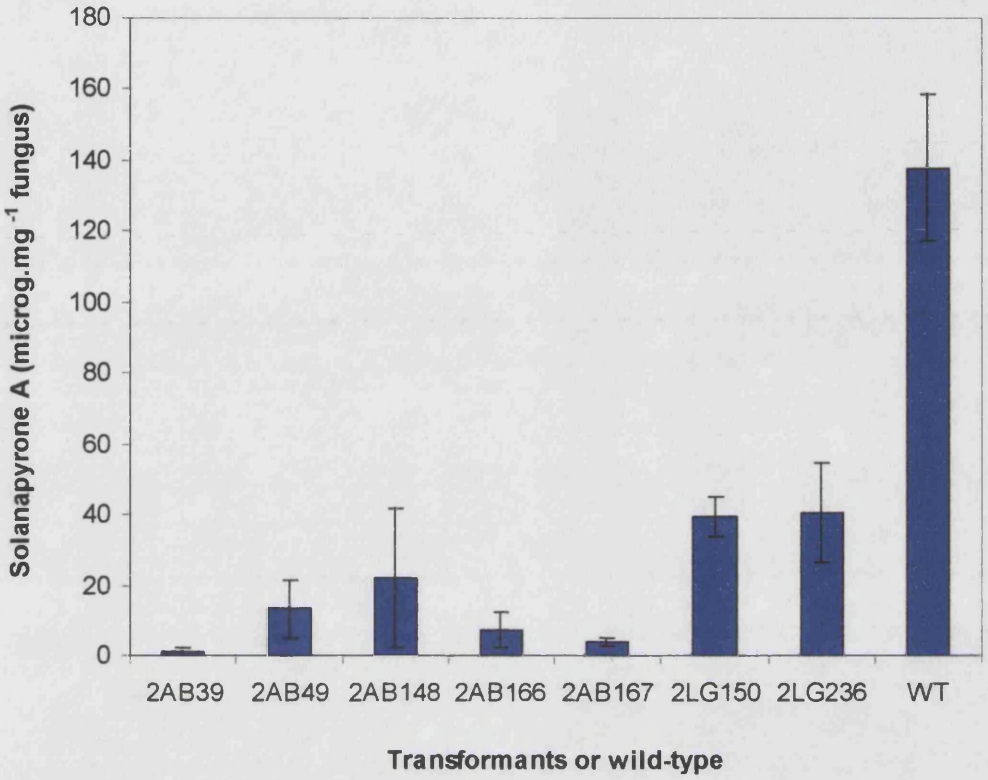


Figure 5.12. Production of solanapyrone A by seven transformants produced by ATMT (second experiment) and selected after screening in microtitre plate. Conical flasks containing 30 ml CDCLM were inoculated with the mycelium (triplicates) of the transformants grown in microtitre plate, and incubated at 20°C for 14 days. The solanapyrone A toxin produced by each of these transformants was purified by SPE, quantified spectrophotometrically and expressed per mg of fungus (dry weight). Bars represent the standard deviation.

5.3.3. Recognition of the *hph* gene in transformants by PCR

An initial screening of transformants for the presence of the T-DNA in the genomic DNA of the regenerant colonies was performed by PCR using primers designed to amplify the *hph* gene.

In order to ensure that the quality of the extracted DNA was suitable for PCR amplification and to determine the optimal conditions for DNA amplification, reactions were firstly set up with genomic DNA of the wild-type strain and primers ITS1 and ITS 4 which recognise the ribosomal repeat units. As shown in Figure 5.13, PCR amplicons of about 600 bp were obtained in all conditions tested which demonstrates that 5 µl of DNA solution was sufficient for amplification of the ribosomal DNA internal transcribed spacers and that additional magnesium chloride was not required for the reaction. As the quality of the DNA extracted by the Chelex technique was satisfactory for PCR amplification, the same protocol was used to amplify the *hph* gene using specific primers.

Thirteen putative mutants were randomly chosen and tested for the presence of the *hph* gene. They all presented a fragment at 987 pb which corresponds to the *hph* gene (Figure 5.14). Five of these transformants (AB91, LB20, LG3, LG7 and LG11) had a significant reduction in the production of solanapyrone A compared to the wild-type (Figure 5.11). The wild-type strain used as a control did not present any band.

However, PCR reactions do not allow the differentiation between autonomous and integrative copies of the T-DNA in the transformants. Consequently, in order to confirm if the T-DNA carrying the *hph* gene has been integrated into the fungal genome and to determine the copy numbers of T-DNA integrations, Southern blot analyses and homologous hybridisations using the *hph* gene as a probe were carried out.

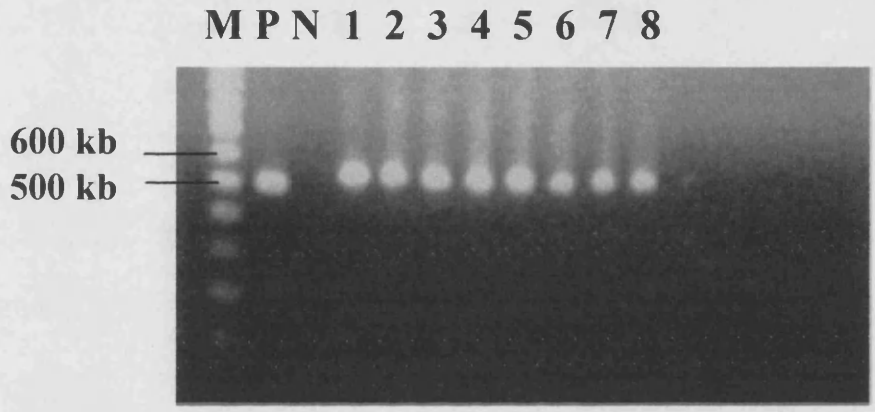


Figure 5.13. PCR amplification of DNA with primers ITS1 and ITS4 from the wild-type strain of *A. rabiei*. Marker (M): 100 bp ladder; P: positive control (λ DNA); N: negative control (no template); Lanes 1, 3, 5 and 7: 5 µl DNA; Lanes 2,4,6 and 8: 10 µl DNA; Concentration of $MgCl_2$ in the PCR reactions: Lanes 1 and 2: 1.5 mM; Lanes 3 and 4: 2.0 mM; Lanes 5 and 6: 2.5 mM; Lanes 7 and 8: 3.5 mM.

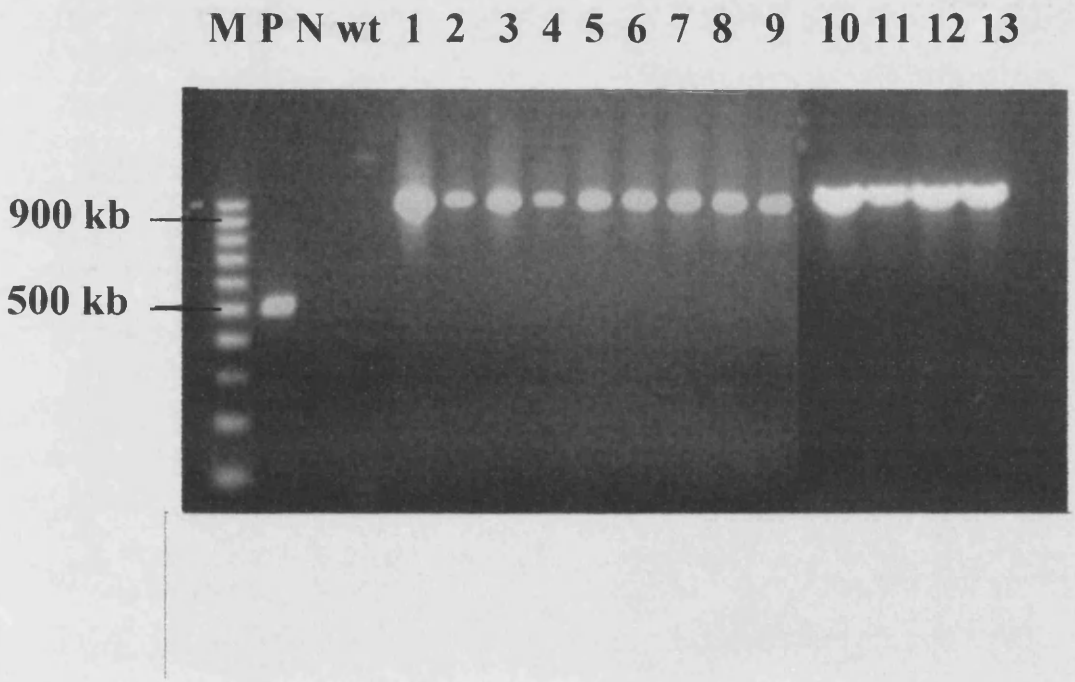


Figure 5.14. Amplification of the *hph* gene from the transformants of *A. rabiei* by PCR. Genomic DNA was extracted using the Chelex technique. M: Marker 100 bp ladder; P: positive control (λ DNA); N: negative control (no template); wt: wild-type; samples 1-13: transformants tested AB26, AB27, AB55, AB60, AB61, AB66, AB67, AB71, AB72, AB91, AB113, AB145 and AG34.

5.3.4. Southern blotting and homologous hybridisation using digoxigenin

A. rabiei transformants created by the ATMT technique which gave the expected PCR product were further analysed by Southern blotting and homologous hybridisation using the *hph* gene as a probe to determine the presence, location and copy number of the inserted T-DNA.

The unlabelled probe appeared as a fragment of 987 bp as expected. Labelling the probe increased its size to about 1.8 kb, due to the incorporation of digoxygenin (Figure 5.15). A minute amount of probe was synthesised using 10 pg of template DNA with primers at 0.05 µM whereas about 6 $ng.\mu l^{-1}$ of probe was obtained with 10 pg of template DNA and 0.4 µM primers, showing that 0.05 µM of primers was not sufficient to produce the probe. The optimal conditions for the synthesis of DIG-labelled *hph* gene was 10 pg of template DNA and primers at 0.4 µM or 50 pg of template DNA and primers at 1.0 µM, giving about 7 $ng.\mu l^{-1}$ probe (Figure 5.15).

Hybridisation of the digested genomic DNA of transformants as well as of the *hph* gene amplified by PCR from transformants created from the first and second experiments was not successfully achieved using the DIG technique.

Hybridisation of the plasmid pAN7-1 which contains the *hph* gene with the DIG-labelled *hph* gene revealed two fragments: the first one at 6.5 kb corresponding to the open circular conformation of the plasmid and the second one at about 4 kb corresponding to its supercoiled conformation. The minimum amount of the plasmid pAN7-1 that gave a distinguishable signal of hybridisation was 1.56 ng (Figure 5.16).

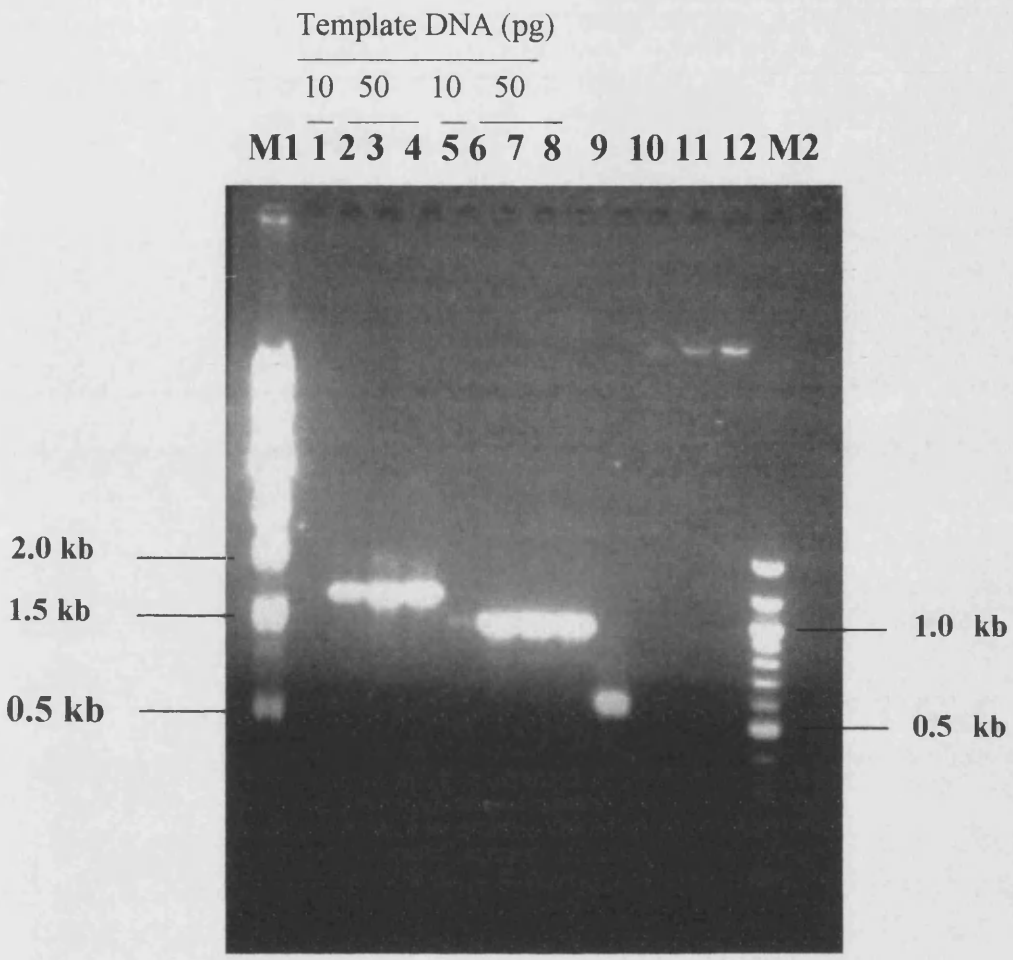


Figure 5.15. Synthesis and DIG-labelling of the *hph* gene used as a probe for Southern blot analyses of genomic DNA of putative mutants of *A. rabiei*. M1: marker 1kb; lanes 1-4: DIG-labelled probe (*hph* gene amplified from pAN7-1 plasmid); lanes 5-8: unlabelled probe; lane 9: unlabelled positive control (tissue plasminogen activator); lane 10-12: λ DNA (1; 5 and 10 ng); M2: marker 100 bp. Lane numbers correspond to the experiment numbers mentioned in Table 5.1. Concentrations of primers: Lanes 1 and 5: 0.05 µM; lanes 2 and 6: 0.4 µM; lanes 3, 4 and 7, 8: 1.0 µM.

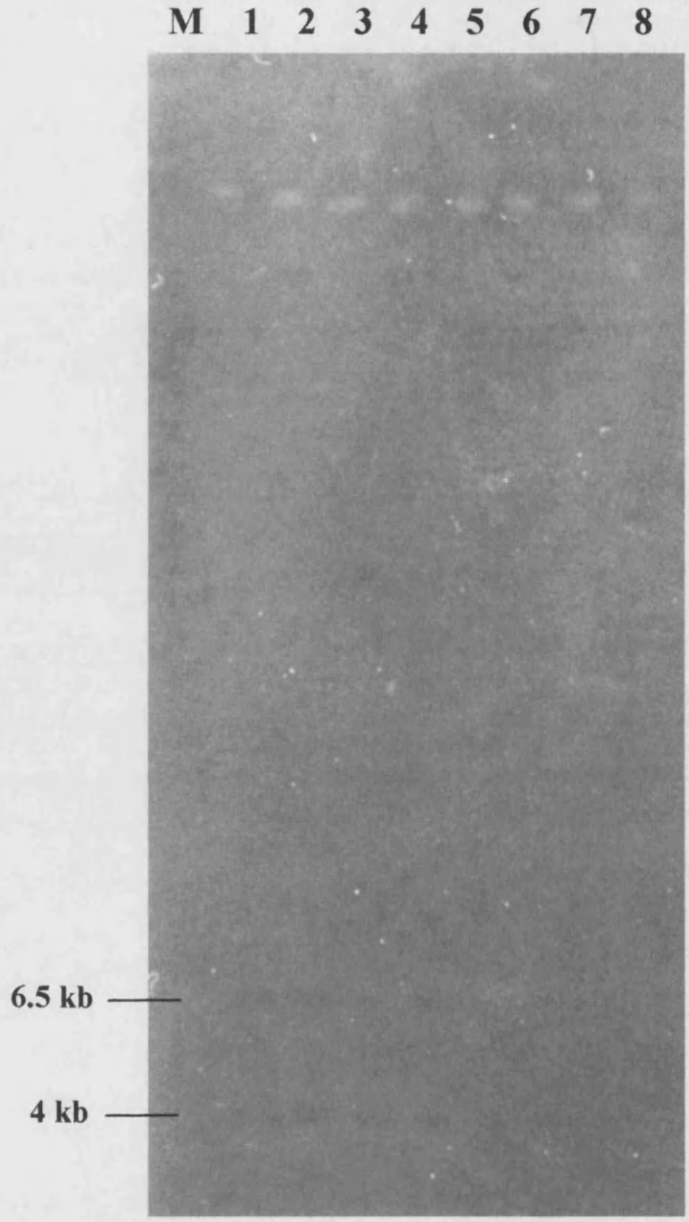


Figure 5.16. Hybridisation of a series of dilution of the the plasmid pAN7-1 with the *hph* gene labelled with digoxygenin. M: marker (1kb); lanes 1 to 8: 100 ng, 50 ng, 25 ng, 12.5 ng, 6.25 ng, 3.125 ng, 1.56 ng and 1 ng of plasmid pAN7-1.

5.3.5. Southern blotting and homologous hybridisation using radioactivity

First, this technique was tested by hybridising the PCR amplicons obtained after amplification of the *hph* gene with six transformants, using the radiolabelled *hph* gene as a probe. An expected fragment at 987 bp corresponding to the *hph* gene was detected (Figure 5.17). This result confirms that the band detected by PCR is the *hph* gene and shows that this hybridisation technique was more appropriate than the DIG system.

Hybridisation was then performed with genomic DNA of four transformants that were created from the second experiment and that presented a reduction in their production of solanapyrone A compared with the wild-type strain. Their respective genomic DNA was digested with *Bam*HI alone or *Bam*HI and *Eco*RI (Figures 5.18 and 5.19). Some fragments that were detected on one of the two hybridisation films did not appear clearly on the other one. Consequently, the results of both hybridisations were combined. The number and size of the different fragments produced are summarised in Table 5.5. The hybridisation profile of the different transformants confirmed that the T-DNA has been integrated into the genome of the transformants studied as no single fragments of the size of the T-DNA (about 7 kb) were detected. The single digest with *Bam*HI generated hybridised fragments of different sizes for each transformant, showing that the T-DNA has been integrated at different loci in their genome. Only the double digestion with *Bam*HI and *Eco*RI generated fragments of identical size (2.5-3 kb and 0.8 kb) for all four transformants, as the probe hybridised to one *Eco*RI-*Eco*RI fragment and one *Eco*RI-*Bam*HI fragment (Figures 5.3 and 5.4).

Two fragments were obtained after hybridisation of the plasmid pAN7-1 containing the *hph* gene with the probe (Figure 5.19). They correspond to the supercoiled (lower band) and open circular conformations (upper band) of the plasmid.

The hybridisation patterns of the four transformants studied demonstrated that the transformants 2AB39 had integrated two copies of the T-DNA, in tandem in a head-to-head form or independently (Figure 5.20). Two copies of the T-DNA have been integrated in tandem in the genome of the transformants 2AB49 and 2LG236.

Three fragments hybridised to the probe when their genomic DNA was digested with *Bam*HI and *Eco*RI, demonstrating that a recombination between two restriction sites (*Bam*HI and/or *Eco*RI sites) of the T-DNA occurred before or during integration. The hybridisation of one fragment of 4-5 kb with the single digest of the transformant 2AB148 and two fragments of about 3 kb and 0.8 kb with the double digest showed that it had integrated only one copy of the T-DNA.

5.3.6. Nucleation of pycnidiospores

Staining the nuclei of pycnidiospores of the strain Tk-21 of *A. rabiei* with DAPI showed that most of them are binucleate (as illustrated in Figure 5.21) and bicellular.

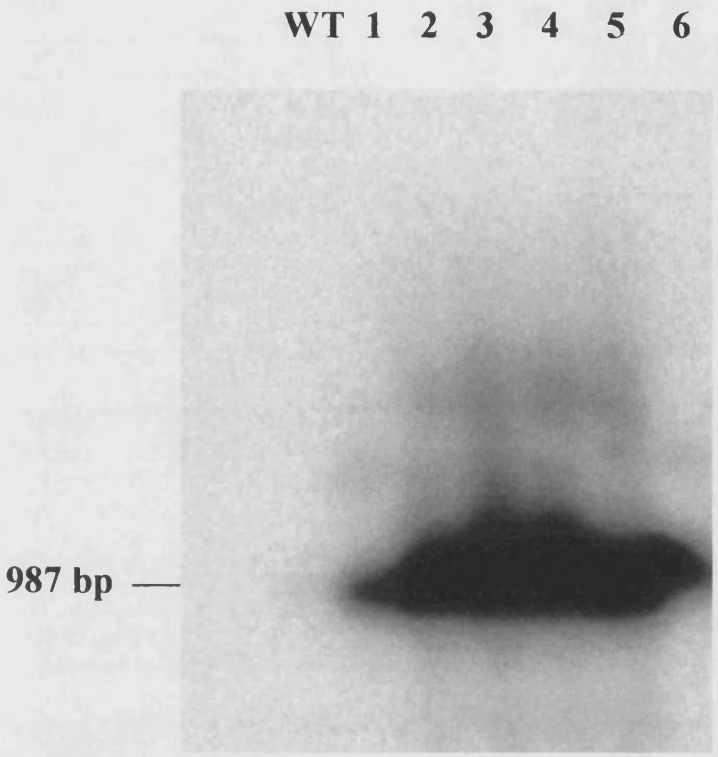


Figure 5.17. Hybridisation of the *hph* gene amplified by PCR from the genomic DNA of transformants produced by the *A. tumefaciens-mediated* technique. Hybridisation was performed using [α-^{32}P]dCTP (as described in Section 5.2.12.4) with the wild-type strain (WT) and the transformants 2AB148, 2AB49, 2AB39, LG7, LG3 and LB20 (lanes 1 to 8, respectively).

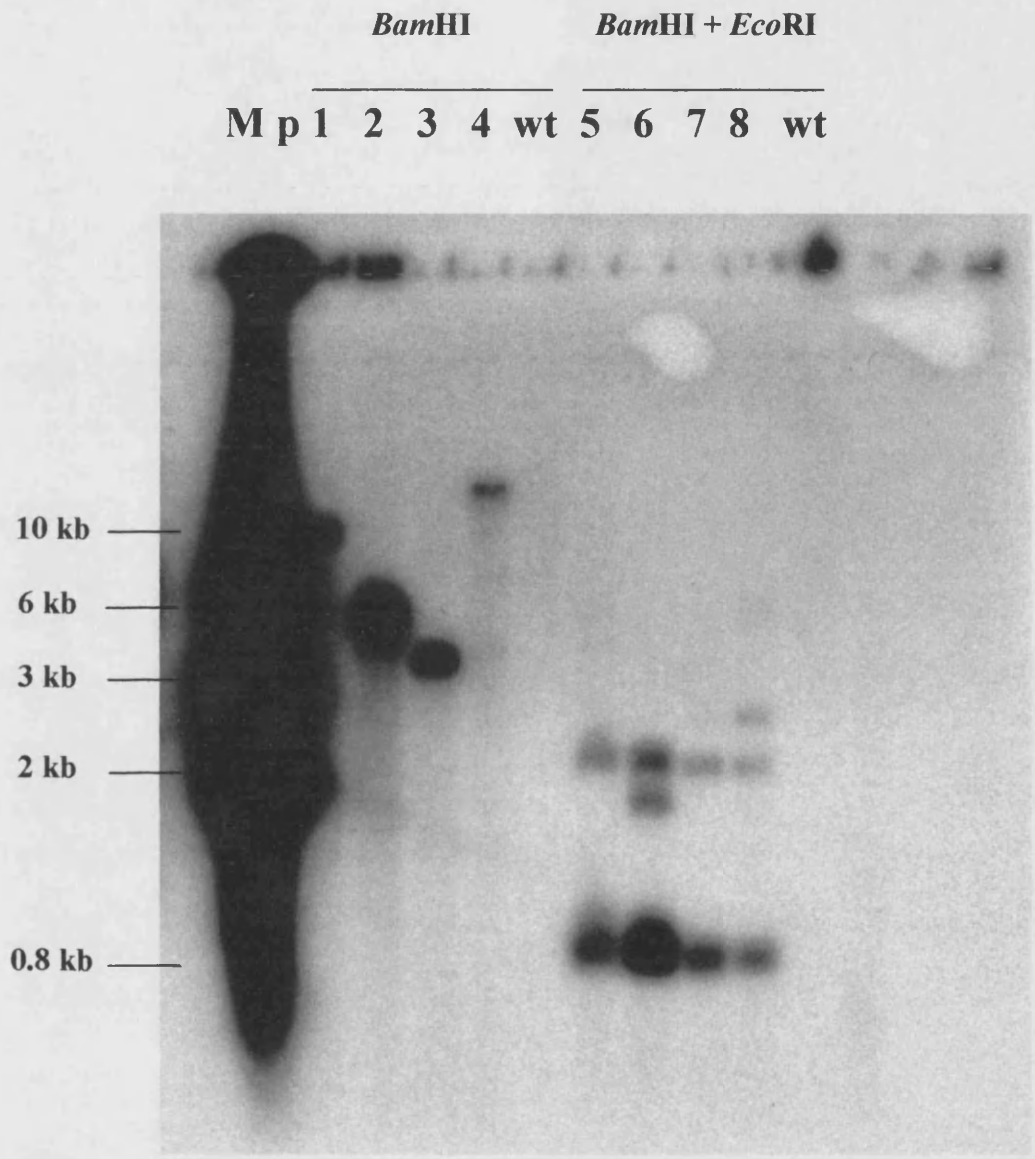


Figure 5.18. Hybridisation of genomic DNA (5 µg per lane) of the transformants 2AB39 (lanes 1 and 5), 2AB49 (lanes 2 and 6), 2AB148 (lanes 3 and 7) and 2LG236 (lanes 4 and 8) digested by *Bam*HI (lanes 1-4) or *Bam*HI and *Eco*RI (lanes 5-8). The *hph* gene synthesised by PCR was labelled with [α-^{32}P]dCTP (as described in Section 5.2.12.4). M: marker (1kb); p: plasmid pAN7-1 (15 ng); wt: wild-type strain.

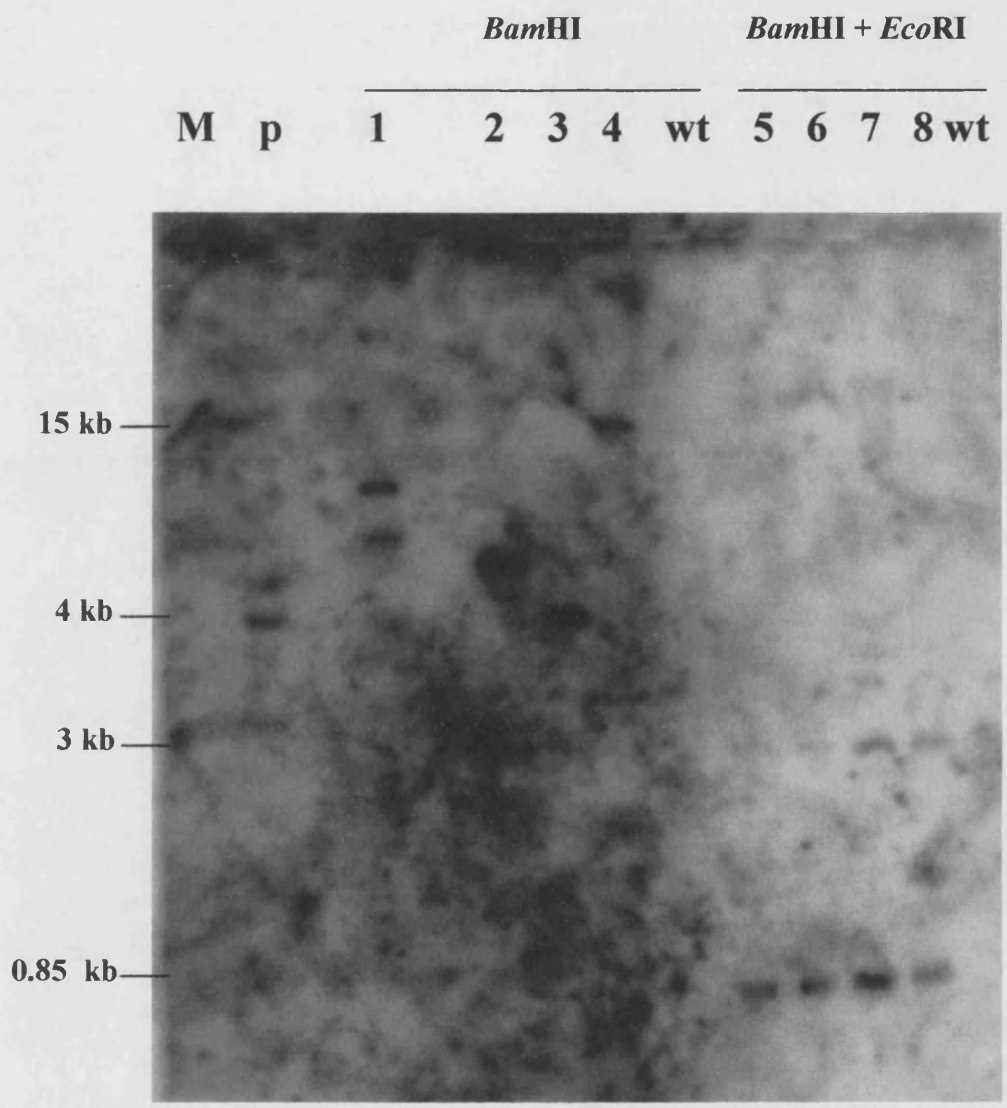


Figure 5.19. Homologous hybridisation of genomic DNA (5 µg per lane) of the transformants 2AB39 (lanes 1 and 5), 2AB49 (lanes 2 and 6), 2AB148 (lanes 3 and 7) and 2LG236 (lanes 4 and 8). Genomic DNAs were digested with *Bam*HI (lanes 1-4) or *Bam*HI and *Eco*RI (lanes 5-8). M: marker (1 kb); p: plasmid pAN7-1 (25 pg); wt: wild-type strain.

Transformant	Single digest with *Bam*HI	Double digest with *Bam*HI and *Eco*RI
2AB39	**1:** 11.0 **2:** 8.0	**1:** 2.5 - 3.0 **2:** 0.8
2AB49	**1:** 7.5 **2:** 6.0	**1:** 2.5 **2:** 2.0 **3:** 0.8
2AB148	**1:** 4.0-5.0	**1:** 2.5-3.0 **2:** 0.8
2LG236	**1:** 14.0-15.0	**1:** 3.0 **2:** 2.5 **3:** 0.8

Table 5.5. Number and size of fragments obtained after digestion of genomic DNA of four transformants with *Bam*HI alone or with both *Bam*HI and *Eco*RI and hybridisation with the radiolabelled *hph* gene. These results combine the results of the two hybridisation performed (Figures 5.18 and 5.19). The number of fragments for each transformant is indicated in bold and their size expressed in kb.

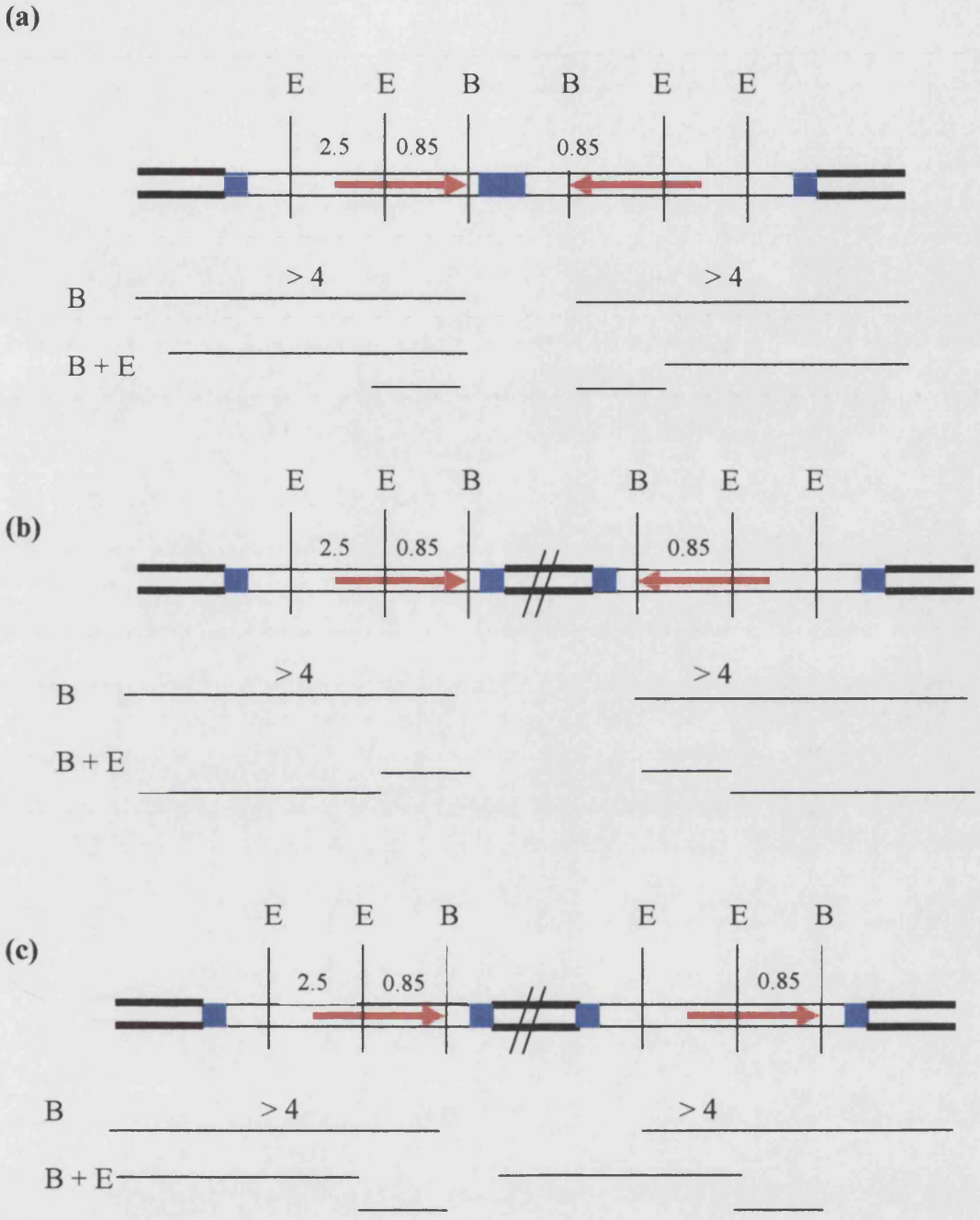


Figure 5.20. Interpretation of the copy number of integrations for the transformant 2AB39. (a) two head-to-head integrations in tandem. (b) and (c) two independant integrations (head-to-head or head-to-tail). In the three interpretations (a), (b) and (c), lines in bold represent the genomic DNA of the transformant and the thinner lines the T-DNA (see map of plasmids pBin7-1 and pGhph1 Figures 5.3 and 5.4), with the left and right borders (blue squares). The red arrow symbolises the *hph* gene. The sizes of the fragments are expressed in kb. B: *Bam*HI; E: *Eco*RI.

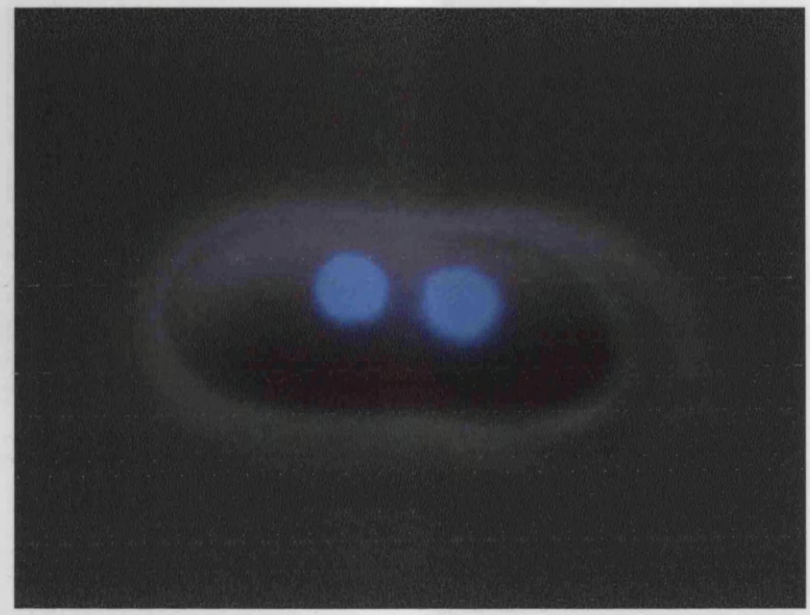


Figure 5.21. Pycnidiospore of *A. rabiei* stained with DAPI. Some pycnidiospores were binucleate.

5.4. Discussion

5.4.1. Insertional mutagenesis

In filamentous fungi, non-homologous integrations occur at variable frequencies, depending on the gene knocked-out and the fungal species (Fincham, 1989). However, if all genes are considered to have the same chance of mutation, the number of mutants required to be created in order to obtain a mutant of interest (toxin-minus mutant) can be evaluated. Considering a genome containing G base pairs and a gene composed of g base pairs, the probability that the mutation is not in this gene is: $1 - (g/G) = (G-g)/G$

For n mutants, the probability that no mutations occur in this gene is: $((G-g)/G)^n$

Hence, the probability that at least one mutation is in this gene is: $1 - ((G-g)/G)^n$

If $1 - ((G-g)/G)^n = 0.9$, then $((G-g)/G)^n = 0.1$

So: $(G-g)/G = \sqrt[n]{0.1}$

Then: $n = \ln(0.1) / \ln((G-g)/G)$

According to the literature, the genome size of filamentous fungi varies between 35 and 50 Mbp and a gene size varies between 3 and 5 kb (Xu and Xue, 2002; Tzeng *et al.*, 1992). Therefore, on the basis of the extremes, and assuming that a single gene is responsible for the production of solanapyrone A, the number of mutants required to be created in order to be able to select at least one toxin-minus mutant affected in one gene that encodes for the production of the toxin, with a probability of 90%, is between 16,117 and 38,375. Consequently, the creation of random genomic mutations requires the production and the screening of a large number of transformants in order to isolate a single mutant defective in the production of solanapyrone A.

5.4.1.1. REMI

The plasmid pCB1004, used to transform *A. rabiei* by REMI, was constructed from the plasmid pCB1003 used to transform *Magnaporthe grisea* by REMI, giving between 108 and 166 transformants per µg plasmid (Balhadère *et al.*, 1999). Moreover, transformation efficiencies of *Ophiostoma piceae* and *O. quercus* with pCB1004 ranged between 1.2 X 10^3 and 7.7 X 10^5 transformants per µg plasmid (Wang *et al.*, 1999) from 1.0-1.5 X 10^7 protoplasts. However, the authors did not record the number of viable protoplasts. Consequently, it was not possible to compare this result with the transformation efficiency of *A. rabiei*, which gave a maximum efficiency of 23.67 transformants per µg plasmid and per 10^5 viable protoplasts with 40 U *Bam*HI and 10 µg plasmid pCB1004 (Table 5.3). This value can be explained by the low number of transformants obtained, thus a difference of a few transformants can lead to a big increase in the efficiency. In this study, transformation efficiency was low with *Eco*RI, suggesting that the amount of enzyme used was not optimal for transformation (Table 5.2). However, transformation efficiency peaked when 10 µg of plasmid was used (Table 5.2). The efficiency of transformation increased with 40 µg plasmid pCB1004 compare with 20 µg but was lower compared with 10 µg plasmid. Transformation efficiency might be enhanced by testing 10 µg plasmid pCB1004 and a variable amount of *Eco*RI. Consequently, the optimum amount of plasmid (10 µg) was used in the second series of transformation with the enzyme *Bam*HI. Transformation frequency presented a sharp peak at 40 U *Bam*HI. The optimum reached 0.6 transformants per 10^5 viable protoplasts per µg plasmid and per U restriction enzyme, which is 150 times the optimum obtained with *Eco*RI, suggesting that *Bam*HI was more efficient for the transformation of *A. rabiei* by REMI. However, the optimal amount of enzyme could be determined more precisely by testing more dilutions between 20 and 60 U.

No transformants were created when protoplasts treated with linearised plasmid and *Eco*RI were incubated at room temperature before plating or immediately plated after transformation. Incubation of protoplasts at 20°C treated in the same conditions improved protoplast regeneration (3.6 to 8.6 times) and transformation efficiency. Transformants were also produced when protoplasts treated in the presence of

linearised plasmids and 40 U *Bam* HI were incubated at 20°C before plating. These results showed that transformation efficacy of *A. rabiei* depends upon incubation conditions and that the capability of protoplasts to regenerate may be enhanced at 20°C. Their regeneration could be further studied by testing different incubation times and incubation conditions such as shaking.

Among the conditions tested, the maximum transformation efficiency (18.46 transformants per µg plasmid and per 10^5 viable protoplasts) was achieved using 10 µg pCB1004 plasmid and 40 U *BamHI*. This result may be enhanced by testing a greater range of concentration of restriction enzyme. Optimisation of the nature and the titre of restriction enzymes raised the transformation efficiency of the Ascomycetes *Colletotrichum magna* (Redman *et al.*, 1999), *Aspergillus niger* (Shuster and Connelley, 1999) and *M. grisea* (Shi *et al.*, 1995). Transformation efficiency of *Glomerella graminicola* by REMI increased when a restriction enzyme other than the one used to linearise the plasmid was used (Epstein *et al.*, 1998). Sweigard (1996) showed that the optimum enzyme concentration must be determined empirically and the optimum is usually represented by a sharp peak. Moreover, high concentration of enzyme decreases transformation frequency because of its cytotoxic effect on fungal genome, preventing the repair mechanisms of the DNA and causing cell death (Sato *et al.*, 1998; Akamatsu *et al.*, 1997; Granado *et al.*, 1997; Shi *et al.*, 1995).

Transformation rate is also affected by the nature and the amount of plasmid used. Redman and Rodriguez (1994) demonstrated that REMI-mediated transformation of *Colletotrichum lindemuthianum* was increased 675-fold using the plasmid pHA1.3 compared to the plasmid pFOL1.

The REMI technique presents two other important limitations. Firstly, some genes may not carry any restriction site and may consequently be inaccessible by this technique. Secondly, treatment with polyethylene glycol promotes protoplast fusion in addition to DNA uptake (Smith, 1994). Therefore, transformation of fungal protoplasts may yield heterokaryotic transformants (Lorito *et al.*, 1993).

Reducing the concentration of hygromycin in the regeneration medium to 25 $\mu g.ml^{-1}$

(the minimum inhibitory concentration for *A. rabiei*) might enhance protoplast regeneration and growth of transformants.

Furthermore, Akamatsu *et al.* (1997) showed in *Alternaria alternata* that with the REMI technique, transformation frequency depends on the pathotype of the fungus. The strain of *A. rabiei* used (Tk-21) might be more resistant to REMI than other strains.

In view of the low number of transformants produced by REMI, the probability of obtaining a toxin-minus mutant was low. Moreover, the REMI method is still empirical. As the transformation rate varies mainly according to the fungal model, the plasmid and the restriction enzymes used (Kahmann and Basse, 1999; Maier and Schäfer, 1999; Riggle and Kumamoto, 1998), the optimisation of all the parameters that affect the REMI procedure would require a considerable amount of time-consuming work. Therefore, it was more judicious to carry out other insertional mutagenesis methods with *A. rabiei:* electroporation, particle bombardment and *A. tumefaciens*-mediated transformation.

5.4.1.2. Electroporation

Some colonies regenerated on selective medium after electroporation of mycelium of *A. rabiei*. However, they did not grow after a first subculture, which indicates that they may be not true transformants, i.e. they have not integrated the plasmid or they lost the plasmid after the first mitotic division, and that mycelial cells of *A. rabiei* were not competent for electroporation in the conditions used, even in the presence of a large range of plasmid (from 75 ng to 80 µg).

No transformants either were produced from pycnidiospores, showing that they were recalcitrant to electroporation. Conversely, electroporation of spore suspensions of most of the *Colletotricum* isolates tested resulted in higher transformation efficiencies compared with hyphal tips (Redman and Rodriguez, 1994).

The cell wall of pycnidiospores may be ruptured where the germ tube emerges, as

observed with spores of *Rhizopus oligosporus* (Breeuwer *et al.*, 1997). Therefore, pycnidiospores may be more competent in DNA uptake and to transformation when they are at an early stage of germination. For these reasons, two other types of fungal cells were used for electroporation: germinated pycnidiospores which present weak spots in their cell walls, or protoplasts which lack cell-walls. Electroporation of germinated pycnidiospores, using the same electrical parameters as those used with mycelia or ungerminated pycnidiospores, did not generate any transformants. This result indicates that the cell wall of the spores, even after germination, was resistant to the applied electric field and that higher electric current may be more suitable for electrotransformation. However, 5 to 8% of the electroporated control pycnidiospores regenerated. This relatively low value suggests that the applied current affected their viability. Consequently, a good balance must be found between regeneration rate and transformation efficiency. When the resistance was doubled twice to 400 and 800 Ω no transformants were generated. This result indicates that these values may be too high for electroporating pycnidiospores, giving too low a field strength (4 A and 2 A, respectively). Surprisingly, germinated pycnidiospores were recalcitrant to electroporation when submitted to higher voltage as well (2.5 kV), even though this value was expected to increase the transient formation of pores in the fungal cell-wall. Similarly to these results, electroporation of germinating spores of *Thermomyces lanuginosus* using a current of 0.6 kV, 25 µF, 200 Ω with 2.5-10 µg plasmid (Chadha *et al.*, 2000) and *A. niger* using 2.4 kV, 8 ms and 1-10 µg plasmid (Ozeki *et al.*, 1994) presented a low efficiency of 5 and 0.6 transformants per µg plasmid, respectively. Conversely, transformants were obtained with germinated conidia of *Colletotrichum gloeosporioides f. sp. aeschynomene* using 1.4 kV, 25 µF, 800 Ω (Robinson and Sharon, 1999), and of *Neurospora crassa* using 2.5 kV, 25 µF, 200 Ω (Chakraborty *et al.*, 1991), electrical parameters that were also tested with germinated pycnidiospores of *A. rabiei*. Sánchez and Aguirre (1996) reported that the time of germination of conidia of *A. nidulans* significantly affected transformation efficiency. Germinating pycnidiospores of *A. rabiei* for a shorter or longer time may improve their transformation by electroporation.

Application of a current of 12.5 A to germinated pycnidiospores decreased the regeneration of control pycnidiospores treated in the absence of plasmid from 5-8%

to 0.35%, which shows that a higher electric current affected the viability of the pycnidiospores and compromised a good transformation efficiency. Unexpectedly, the regeneration of germinated pycnidiospores also decreased to 0.1-0.45% or 0.22% when these were submitted to a current of 4 A or 2 A, respectively.

Apart from electric settings, several other parameters affect the success of fungal uptake of DNA by electroporation. These include pre-treatment of fungal cells with various concentrations of lithium acetate as lithium ions induce cell wall permeability, enhancing their competency (Thompson *et al.*, 1998), dithiothreitol which has the property of increasing cell-wall porosity, hence weakening the fungal cell-walls (De Backer *et al.*, 1999; Thompson *et al.*, 1998) or β-glucoronidase, a cell-wall degrading enzyme (Chakraborty *et al.*, 1991). Mechanical damage with glass beads, creating breaks in cell-walls and increasing potential sites of DNA uptake, increased transformation efficiency of *Colletotrichum spp.* (Redman and Rodriguez, 1994).

Electroporation of protoplasts was only partially successful as the transformation efficiency was only 0.046 transformants per microgram of plasmid and per 10^5 viable protoplasts. The low regeneration rate of control protoplasts (0.9%) compared to germinated pycnidiospores (5-8%) can be explained by their fragility due to the absence of cell-wall. Likewise, Weiland (2003) reported a low transformation efficiency (0.1-0.4 transformants from 10^5 viable protoplasts per microgram of plasmid) of protoplasts of *Pythium aphanidermatum.* However, this author showed that 10-15% of control protoplasts were viable using 500 V, 50 μF and 13 Ω as electric parameters and that an extensive rupture of protoplasts occurred with a value of field strength exceeding 1 kV, suggesting that a lower electric pulse may increase the regeneration of protoplasts of *A. rabiei* and therefore that of transformants. Consequently, as the electric parameters differ for each fungal model, they must be adjusted empirically in order to obtain a high transformation rate for *A. rabiei.*

The nature and conformation of the plasmid significantly influenced transformation efficiency of *A. nidulans.* Sánchez and Aguirre (1996) showed that about 60 times more transformants were produced using the plasmid DHG25 compared to the plasmid pREN2, and that for each plasmid this number was increased twice using

their linear form. Using the linear conformation of a plasmid increased by 70% the transformation rate of *C. gloeosporioides f. sp. aeschynomene* compared to the circular configuration (Robinson and Sharon, 1999). However, in the present study, the use of two different plasmids (pCB1004 and pHA1.3), circular or linear, during electroporation of germinated pycnidiospores, did not improve transformation efficiency. Moreover, few transformants were created with protoplasts in the presence of the plasmid pCB1004. These results suggest that the plasmids do not seem to be the limiting factor for electroporation of *A. rabiei.* Redman and Rodriguez (1994) reported high electroporation efficiency (up to 1,000 transformants per µg plasmid) with *Colletotrichum spp* using the pHA1.3 plasmid.

The major parameters that influence transformation frequency of *A. rabiei* appeared to be the physiological state of the fungus and the electric values (voltage, capacitance and internal resistance). Electroporation rate of *A. rabiei* might also depend on the isolate used, as reported for *Colletotricum spp.* (Redman and Rodriguez, 1994). Cell concentration also affects electroporation efficacy. Above a specific number of fungal cells, saturation may occur due to heterogeneity of the electric field caused by cell-to-cell contact (Suga and Hatakeyama, 2001) but a minimal number of cells is required to allow the propagation of the electric current between them during the electroporation process (Hogan and Klein, 1997).

Owing to the limited success of electroporation-mediated transformation of protoplasts of *A. rabiei,* and owing to the requirement to produce protoplasts, particle bombardment was used to attempt the transformation of the fungus.

5.4.1.3. Particle bombardment

Despite the use of particle bombardment with tungsten or gold microparticles to transform intact or germinated conidia of the Ascomycetes *Trichoderma harzianum Rifai* TM (Viterbo *et al.*, 2001), *Trichoderma reesei* (Hazell *et al.*, 2000), *Metarhizium anisopliae* (Inglis *et al.*, 2000; Bogo *et al.*, 1996; St Leger *et al.*, 1995), *Coccidioides immitis* (Yu and Cole, 1998), *Paecilomyces fumosoroseus* (Barreto *et al.*, 1997) and *A. nidulans* (Fungaro *et al.*, 1997 and 1995), and the yeast

Saccharomyces cerevisiae (Heiser, 2000), this technique did not transform pycnidiospores of *A. rabiei*, although they were subjected to particle bombardment in a wide range of concentration (5 X 10^3 to 1.7 X 10^6), with different types of microparticles and with circular or linear plasmid. This result demonstrates that pycnidiospores of *A. rabiei*, previously germinated or not, are recalcitrant to transformation by particle bombardment under the conditions of the experiments performed.

Particle bombardment of *A. rabiei* might be achievable by varying some physical parameters that have been proved to affect transformation efficiency of conidia of *A. nidulans*, such as the helium pressure or the size of microparticles (Herzog *et al.*, 1996). For instance, transformation of *A. nidulans* by bombardment was optimal using 10^8 conidia per plate, a helium pressure of 1,200 psi, a target distance of 6 cm, a chamber vacuum of 711 mm Hg and microparticles of tungsten (M5, M10 or M17 type). Identical conditions were used with *A. rabiei*, except that a slightly higher helium pressure was used and that a lower concentration of pycnidiospores was plated. Consequently, increasing the number of pycnidiospores per experiment might enhance the probability of obtaining transformants. Transformation frequency may be affected by biological parameters as well. Conidia of the Ascomycete *P. fumosoroseus* were successfully transformed by particle bombardment when plated at a titre of 3 X 10^7 per plate, giving rise to 140 to 153 transformants per µg of DNA (Barreto *et al.*, 1997). This technique produced a relatively high, although very variable, number of transformants (32 to 201 per µg of DNA) with conidia of *M. anisopliae* (5 X 10^8 per plate; Bogo *et al.*, 1996). Therefore, increasing the pycnidiospore titre may improve transformation efficiency of *A. rabiei*.

In view of the results of the genetic transformation of *A. rabiei* by REMI, electroporation and particle bombardment, all these three techniques required to be empirically optimised in order to obtain a large number of transformants. However, optimisation is time consuming and may not be successful. Consequently, a fourth technique was attempted, the *A. tumefaciens*-mediated transformation, which was thought to be more successful as it is a natural transformation process.

5.4.1.4. ATMT

The *A. tumefaciens* technique was successfully used to transform germinated pycnidiospores of *A. rabiei*. Hygromycin-resistant colonies of *A. rabiei* regenerated after transformation, suggesting that *A. tumefaciens* is able to use its virulence system to transform this pathogen.

Covert *et al.* (2001) successfully transformed three isolates of *Fusarium circinatum* with the *A. tumefaciens* strain AGL1, obtaining a transformation efficiency of 2 to 150 transformants per 10^5 conidia. The same bacterium strain was also used by Combier *et al.* (2003) for the transformation of *Hebeloma cylindrosporum*. Conidia of *Botrytis cinerea* and *Verticillium fungicola* (Amey *et al.*, 2002 and Rolland *et al.*, 2003, respectively) were transformed with the *A. tumefaciens* strain LBA1126. The two strains AGL1 and LBA1126 were also successfully used to transform *A. rabiei*. Two combinations of *A. tumefaciens* strain/plasmid (AGL1/pBin7-1 and LBA1126/pGhph1) out of the four tested gave a high transformation efficiency (up to 16.1 transformants per 10^5 pycnidiospores). This significant difference in efficiency suggests that the *A. tumefaciens* strains AGL1 and LBA1126 exhibit a difference in their capability of transferring the T-DNA present in two different binary vectors (pBin7-1 and pGhph1) that are activated by different helper plasmids (the hypervirulent strain AGL1 contains pTiBO542 and LBA1126 contains pTiB6 containing mutations in the *vir A* and *vir G* genes).

Several Ascomycetes submitted to the ATMT technique produced variable numbers of transformants. For instance, 2 to 150 transformants per 10^5 pycnidiospores from *F. circinatum* (Covert *et al.*, 2001) and 10 to 90 transformants per 10^5 pycnidiospores from *Aspergillus awamori* (De Groot *et al.*, 1998). Transformation efficiency was reproducible in two independent experiments with *A. rabiei* using the combination LBA1126/pGhph1 (10.7 and 10.4 transformants per 10^5 pycnidiospores) and was enhanced with the combination AGL1/pBin7-1 but varied in a range of only two-fold (8.0 and 16.1 transformants per 10^5 pycnidiospores). These efficiency values are high compared to the efficiency observed with *Fusarium venenatum* (0.25 transformants per 10^5 pycnidiospores, De Groot *et al.*, 1998) and *B. cinerea* (1.5 transformants per

10^5 pycnidiospores, Rolland *et al.*, 2003).

A library of 908 putative mutants was generated by the ATMT procedure in two independent experiments. However, only 668 of them could be isolated as the mycelium of the remaining grew too close to each other and could be cross-contaminated. This may be improved by inoculating fewer pycnidiospores per plate.

The ATMT technique was used as a means to circumvent the production of protoplasts. Protoplasts can be enucleate, leading to a low regeneration rate, or multinucleate, with only one nucleus integrating the transforming DNA, therefore generating a low number of stable transformants (Smith, 1994; Herzog *et al.*, 1996). Lima *et al.* (2003) reported that only 37.5% of protoplasts from the plant pathogen pathogen *Crinipellis perniciosa* were uninucleate. Moreover, protoplasting *A. rabiei* is time consuming as a maximum number of protoplasts were released only after incubation for 8 h in lytic solution. The difficulty of producing regenerating protoplasts was overcome by direct transformation of intact pycnidiospores.

Conidia of *Botrytis cinerea* were transformed with the LBA1126 strain of *A. tumefaciens* after co-cultivation for 48 h (Rolland *et al.*, 2003). Rho *et al.* (2001) reported a transformation efficiency greater than 1,000 transformants per 10^6 conidia when the time of co-cultivation of conidia with *Agrobacterium* cells was 36 or 48 h. Transformants of *A. rabiei* were obtained by ATMT after 48 h of co-cultivation. This period of time might be increased in order to optimize the transformation frequency of *A. rabiei*. However, it should not be too long to avoid the overgrowth of the bacterium, leading to the potential death of the fungus.

If an efficiency of 16.1 transformants per 10^5 pycnidiospores can be reproducibly obtained with the *A. tumefaciens* strain AGL1 and the plasmid pBin7-1 (Figure 5.8) and if 30 plates on average are used per series of transformation, a total of 34 to 80 series of transformation are required in order to obtain a sufficient number of transformants to isolate a single mutant defective in the production of solanapyrone A, as discussed at the beginning of Section 5.4.1.

5.4.2. Characterisation of the transformants

5.4.2.1. Screening for the production of solanapyrone A

Among the sixteen transformants (produced from the two experiments performed) which present a reduced production of solanapyrone A, six were created with the combination AGL1/pBin7-1 (AB91, 2AB39, 2AB49, 2AB148, 2AB166 and 2AB167), two with LBA1126/pBin7-1 (LB20 and LB27) and eight with LBA1126/pGhph1 (LG2, LG3, LG7, LG11, LG13, LG14, 2LG150 and 2LG236). It was then interesting to determine if these particular transformants had integrated the T-DNA and the number of copies.

5.4.2.2. PCR

Five of the nine transformants altered in the production of solanapyrone A obtained from the first experiment were tested by PCR. All five presented a fragment corresponding to the *hph* gene (AB91, LB20, LG3, LG7 and LG11 (Figure 5.14), which indicated that the T-DNA carrying the *hph* gene was present.

5.4.2.3. Southern blot and homologous hybridisation using digoxygenin

Detection of integration of vector in genomic DNA of various Ascomycetes has been performed using DIG-labelled probes, in particular *A. niger* (Shuster and Connelley, 1999) and *A. alternata* (Johnson *et al.*, 2000) after transformation by REMI. The *hph* gene was also detected using the DIG system after transformation of *T. reesei* by particle bombardment (Hazell *et al.*, 2000). However, no hybridisation signal was obtained with *A. rabiei* using this technique, whether the digested genomic DNA of the transformants or the amplified *hph* gene from their genomic DNA was used. A relatively high amount of plasmid pAN7-1 (at least 1.56 ng) was required to obtain a hybridisation signal with the DIG-labelled *hph* gene. These results showed that the DIG system was not sensitive enough to detect integrations of the T-DNA in the

genomic DNA of *A. rabiei.*

5.4.2.4. Southern blotting and hybridisation using radioactivity

The number of integrations of ectopic DNA into the genome of transformants has been determined with various Ascomycetes after genetic transformation by ATMT. Single copy integration events were detected for all the transformants analysed with *V. fungicola* (Amey *et al.*, 2002) and *Calonectria morganii* (Malonek and Meinhardt, 2001), whereas some transformants of *F. circinatum* and *A. awamori* had integrated more than one copy of T-DNA (Covert *et al.*, 2001 and De Groot *et al.*, 1998, respectively). Both single and multiple integrations occurred with *A. awamori* (Gouka *et al.*, 1999) and *Fusarium oxysporum* (Mullins *et al.*, 2001). T-DNA insertions in *A. rabiei* occurred randomly and mostly at one site in tandem or at two sites. Only one transformant presented a single insertion. However, the copy number of T-DNA integrations into the fungal genome must be limited in order to facilitate the identification and the subsequent isolation of the mutated genes. Sullivan *et al.* (2002) reported that all the transformants of *Blastomyces dermatitidis* presented insertions at single sites, although these carried multiple DNA copies when high densities of *Agrobacterium* were used. At low densities of bacteria no concatemers were detected but insertions were identified at multiple sites. Transformation efficiency of *M. grisea* and *F. oxysporum* was significantly enhanced by pretreating *Agrobacterium* cells with acetosyringone prior to co-cultivation with the fungi and by prolonging the co-cultivation time. However, the absence of acetosyringone in the culture medium of *A. tumefaciens* prior to transformation produced a higher percentage of single copy integrations of T-DNA compared to the presence of acetosyringone (Rho *et al.*, 2001 and Mullins *et al.*, 2001, respectively). Consequently, varying the density of *Agrobacterium* cells and the molarity of acetosyringone for the transformation of *A. rabiei* might enhance the number of single insertions of the T-DNA.

The combination of bacteria and plasmid AGL1/pBin7-1 produced two transformants with two copies of the T-DNA and one transformant with only one copy. The

analysis of more transformants is required to determine if the combination used affects the number of integrations of T-DNA.

5.4.3. Nucleation of pycnidiospores

Transformants were selected by their resistance to hygromycin. However, most pycnidiospores of the strain Tk-21 of *A. rabiei* are binucleate, suggesting that transformed pycnidiopores may contain one nucleus with T-DNA insertion(s) and another one without. Consequently, these two nuclei must be segregated by single-spore isolating the transformants several times consecutively in order to isolate the nuclei that contain the insertion(s) and facilitate the subsequent genetic analyses for the identification of the mutated gene(s).

Chapter 6

The effect of safeners on the sensitivity of chickpea to solanapyrone A

6.1. Introduction

As stated in Chapter 1, solanapyrone A was shown to be toxic to chickpea cells (Section 1.4). The differential susceptibilities of various chickpea cultivars to solanapyrone A might be explained by their differential ability to detoxify this compound. Safeners protect crops from herbicide injury by enhancing their detoxification (Chapter 1, Section 1.5.2). Among the most widely used safeners are the dichloroacetamides (Walton and Casida, 1995), including dichlormid (N, N-diallyl-2,2-dichloroacetamide). This safener has been shown to decrease the sensitivity of chickpea plants to solanapyrone A and to boost the detoxification of this toxin in bioassays by raising both GST activity and the level of GSH (Hamid and Strange, 2000). Dichlormid is able to protect crops from chloroacetanilide herbicide injury by inducing GST isoenzymes specific to the herbicides (Dixon et al., 1998; Gronwald and Plaisance, 1998). Hirase and Molin (2002) showed that dichlormid protected sorghum plants from growth inhibition by alachlor. In corn, it increased activities of ATP-sulfurylase and adenosine-5'-phosphosulfate sulfotransferase, two enzymes that are sensitive to environmental stimuli and that enhance sulphate assimilation, leading to the increase of cysteine required for glutathione (GSH) synthesis (Farago and Brunold, 1994). This safener also increased indirectly the specific activity of cysteine synthase in sorghum shoots (Hirase and Molin, 2001). Edwars and Cole (1996) reported that dichlormid enhanced the activities of glutathione-S-transferases (GST) in wheat, specifically toward the herbicide fenoxaprop. This safener increased levels of GSH in maize (Ekler *et al.*, 1993), sorghum (Gronwald *et al.*, 1987) and tobacco (Rennenberg *et al.*, 1982) as well as the GST content in sorghum (Gronwald *et al.*, 1987). It enhanced the conjugation of GSH with the herbicides metolachlor, metazachlor and acetochlor in maize (Ekler *et*

al., 1993).

Another safener, fenclorim (4,6-dichloro-2-phenylpyrimidine), belongs to the phenylpyrimidine group. It protects rice, maize and sorghum against injury caused by pretilachlor (Wu *et al.*, 2000) by promoting the conjugation of glutathione with the herbicide, reducing its phytotoxic activity (Deng *et al.* 1997; Wu *et al.*, 1996). This safener induced the expression of the GST genes in rice (Usui *et al.*, 2001; Wu *et al.*, 1999). Deng *et al.* (2002) reported that a gene coding the GST II subunit of rice plays an important role in the detoxification of pretilachlor. Apart from rice, fenclorim protects also maize and sorghum from injury caused by the herbicides pretilachlor and metolachlor (Wu *et al.*, 2000).

One objective of this work was to assay the effect of the safeners dichlormid and fenclorim on the sensitivity of chickpea plants to solanapyrone A.

6.2. Materials and Methods

6.2.1. Plant growth

Seeds of chickpea cultivar Amdoun1 (susceptible to Ascochyta blight; provided by INRAT, Tunisia) were incubated in 2.5% sodium hypochloride (in sterile distilled water) for 1 min and washed three times with sterile distilled water. They were incubated on sterile water agar (20 $g.l^{-1}$ agar) for about 5 d until they germinated and were transferred to pots (18 cm diameter; 8 seeds per pot) containing compost (John Innes number 2) and silver sand (1:1 v/v). Plants were grown in a greenhouse at 25°C ± 2°C with a 14 hour-photoperiod.

6.2.2. Treatment of chickpea shoots with safeners

Safeners, supplied by Greyhound Chromatography and Allied Chemicals Company (Merseyside, UK), were dissolved in acetone and diluted with water to various standard concentrations. Chickpea shoots (0.61-2.91 g; 5 petioles each) were

collected from 21 to 28 day-old healthy plants. They were transferred to 15 ml Falcon tubes and allowed to take up an aqueous solution of dichlormid (112.5, 300.0 and 800.0 μg per shoot) or of fenclorim (9, 18 and 36 μg per shoot). The treated shoots were transferred to fresh tubes containing distilled water in which they were maintained in a greenhouse for 72 h. Shoots that were allowed to take up distilled water containing the same amount of acetone as in the safener solution were used as controls. All experiments were performed in triplicate.

6.2.3. Cell isolation

The leaflets of control and treated chickpea shoots were bisected transversely and placed in 10 ml of digestion solution (Appendix 4). A vacuum was gently applied twice to the leaflet suspension to allow the enzyme solution to infiltrate the intracellular spaces. The suspension was stirred for 20 min at about 100 rpm to avoid damaging the chickpea cells, filtered through four thicknesses of muslin into a 15 ml Falcon tube and centrifuged at 85 *g* for 5 min. Pelleted cells were resuspended in 5 ml of ice-cold holding buffer (Appendix 4) and centrifuged. After repeating this step twice, the pellet was finally resuspended in a sufficient volume of ice-cold holding buffer to allow efficient counting of cell viability under the microscope. The cell suspension was kept on ice prior to the bioassay.

6.2.4. Cell bioassay

Cell viability was assessed by adding in three wells of a 96 well-microtitre plate 50 μl of vital stain fluorescein diacetate (FDA, Sigma) solution (5 mg FDA per ml acetone, diluted 50 times in holding buffer) to 50 μl of cell suspension. After incubation for 5 min, 50 cells per well were scored by observation under an inverted microscope equipped with epifluorescence optics (Olympus; IM model). Viable cells emitted a yellow-green fluorescence, whereas dead cells appeared red (Figure 6.1). The bioassay was carried out when the cell viability of the suspension was sufficiently high. Holding buffer (50 μl) was transferred into all the wells of a 96

well-microtitre plate, except the wells of the columns numbered 1 and 11. Pure preparation of solanapyrone A (100 µl at 750 $\mu g.ml^{-1}$; toxin purified by flash chromatography as in Chapter 2, Section 3.2.2.3) was added to the wells of the first column. A serial series of 2-fold dilution was obtained by transferring 50 µl of the contents of these wells to the following wells, up to the eleventh. After adding chickpea cell suspension to all the wells (50 µl per well), the mixture was incubated at room temperature for 3 h. Every sample was assayed in duplicate (2 rows per sample).

6.2.5. Bioassay scoring

To score the bioassay, 50 µl of freshly prepared FDA solution was added to each well of the microtitre plate. After incubation for 5 min, the number of living cells was assessed by observation of the fluorescence. The control (column 12) was scored before the other samples. The results were expressed in percentage of death according to the following formula:

$$\frac{C-T}{C} \times 100$$

With: C = sum of viable cells in duplicate controls

T = sum of viable cells in duplicate samples

These values of percentage of cell death were converted to probits (Appendix 5) and plotted versus the $\log_2$ of the dilution factor of solanapyrone A in order to determine the dilution factor of toxin that led to the death of 50% of the cells, which was arbitrarily defined as 1 U of toxin activity and abbreviated LD_{50}.

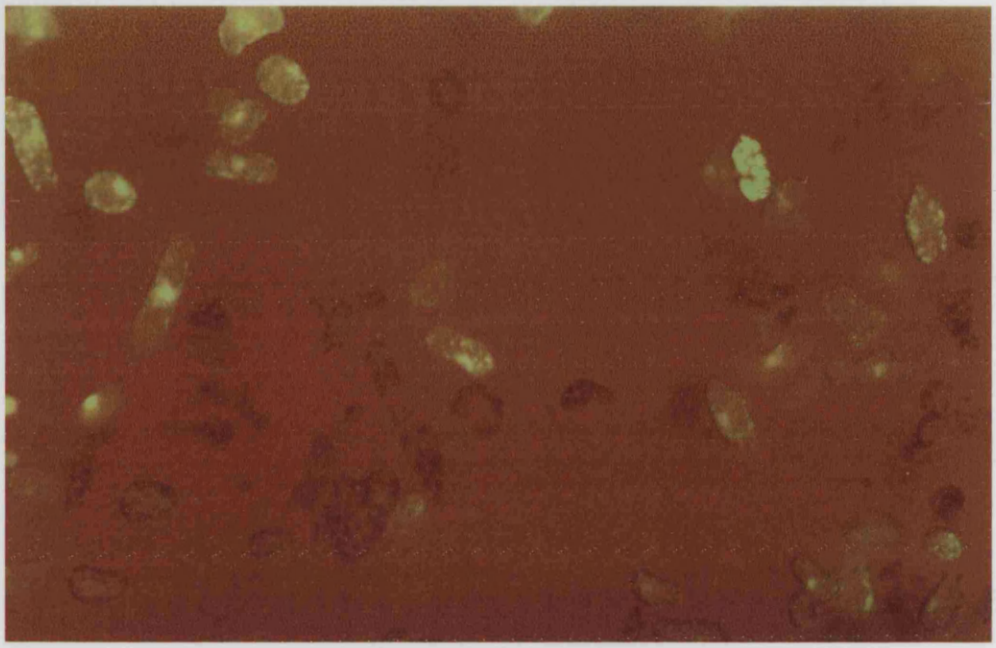


Figure 6.1. Assessment of the viability of chickpea cells after enzymatic digestion and incubation with fluorescein diacetate. The fluorescein diacetate gave a yellow-green fluorescence in viable cells and dead cells appeared red.

6.3. Results

6.3.1. Treatment of chickpea shoots with dichlormid

Cells from shoots that were allowed to take up 300 and 800 µg dichlormid had significantly increased LD_{50} values (t-test, $p = 0.043$ and $p = 0.012$, respectively): these cells were 1.58 and 1.64 times less sensitive to solanapyrone A, respectively, than those isolated from controls incubated in water when treated with solanapyrone A compared to controls (Figure 6.2). These results demonstrated that dichlormid (300 and 800 µg per shoot) decreased the sensitivity of chickpea shoots of the cultivar Amdoun 1 to solanapyrone A and that 112.5 µg per shoot was not sufficient.

6.3.2. Treatment of chickpea shoots with fenclorim

The LD_{50} value of chickpea cells from shoots that took up 18 µg fenclorim and that were treated with solanapyrone A was significantly higher compared to controls (t-test, $p = 0.017$). The sensitivity of chickpea cells isolated from shoots treated with fenclorim was 2.47 times lower than that of control shoots, showing that that this safener enhanced the ability of Amdoun 1 plants to protect themselves against the toxic effect of solanapyrone A (Figure 6.3).

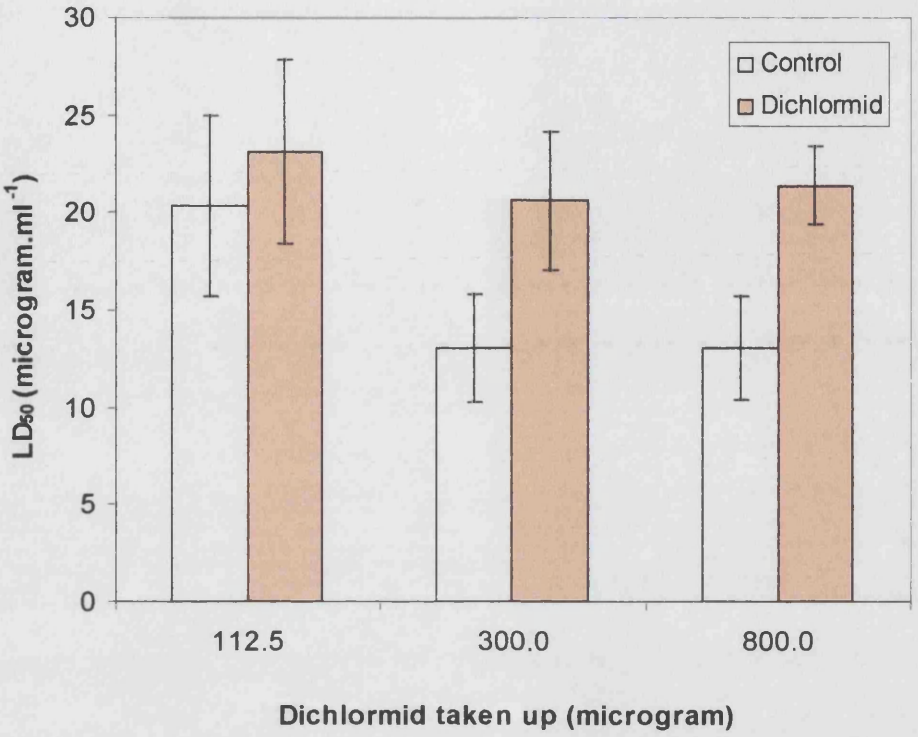


Figure 6.2. LD_{50} values of solanapyrone A after treatment of chickpea shoots of the cultivar Amdoun 1 with dichlormid. Chickpea shoots were allowed to take up an aqueous solution of dichlormid (112.5, 300.0 and 800.0 µg per shoot), transferred to fresh tubes containing distilled water and incubated for 72 h. Controls were allowed to take up distilled water and acetone instead of a solution of safener. Controls and treated shoots were assayed for their insensitivity to solanapyrone A. All experiments were performed in triplicate. Error bars correspond to standard deviation.

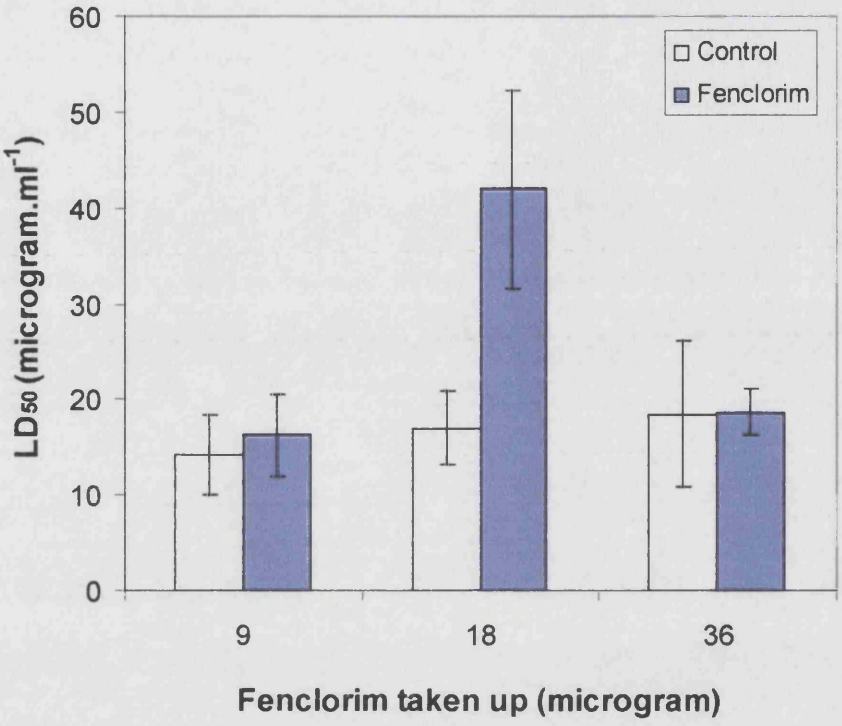


Figure 6.3. LD_{50} values of solanapyrone A after allowing chickpea shoots of the cultivar Amdoun 1 to take up 9, 18 and 36 µg of the safener fenclorim. After treatment with fenclorim (or water for the controls), chickpea shoots were transferred to tubes containing distilled water and incubated for 72 h. Controls and treated shoots were assayed for their insensitivity to solanapyrone A. All experiments were performed in triplicate. Error bars correspond to standard deviation.

6.4. Discussion

6.4.1. Treatment of chickpea shoots with dichlormid

The decrease in sensitivity (1.58-fold) of chickpea cells from shoots of the cultivar Amdoun 1 treated with 300 µg of dichlormid is in agreement with the results of Hamid and Strange (2000) who showed, with the cultivar ILC 3279, a loss of sensitivity of chickpea shoots treated in the same conditions (2.66-fold) compared to controls. The decrease of the sensitivity of the cultivar ILC 3279 was greater compared to the cultivar Amdoun 1, reflecting the fact that ILC 3279 is resistant to Ascochyta blight and that Amdoun 1 is susceptible to this disease. The same authors reported a safening activity of dichlormid when chickpea shoots took up 150 µg of this safener. Therefore, it would be interesting to test intermediate concentrations of dichlormid (both between 112.5 and 150 µg per shoot, and between 150 and 300 µg per shoot) in order to determine more precisely the optimal amount of safener that affects the sensitivity of chickpea plants to solanapyrone A.

In wheat, the GST activity toward fenoxaprop was increased by both safeners dichlormid and fenchlorazoleethyl (Edwards and Cole, 1996). The safening activity of dichlormid in sorghum was reported to be lower than those of other safeners such as flurazole and naphtalic anhydride (Gronwald *et al.*, 1987). Hirase and Molin (2001) showed that not only dichlormid but also fluxofenim, naphtalic anhydride and benoxacor protected sorghum shoots from growth inhibition by the herbicide alachlor. Consequently, it would be of interest to test the effect of other safeners than dichlormid on the sensitivity of chickpea to solanapyrone A, and to compare the effect of those that belong to the same or to a different chemical family.

6.4.2. Treatment of chickpea shoots with fenclorim

Fenclorim was more effective in reducing the sensitivity of chickpea cells of the cultivar Amdoun 1 than dichlormid, as it increased the LD_{50} 2.47-fold compared to

the control. Miyauchi *et al.* (2002) reported that both fenclorim and dymron protected rice against injury caused by pretilachlor. Moreover, Wu *et al.* (2000) showed that the safeners fenclorim, benoxacor and fluxofenim reduced the phytotoxic effect of pretilachlor and metalochlor on rice, maize and sorghum. Dymron, benoxacor, and fluxofenim might present a safening activity on chickpea plants.

6.4.3. Sensitivity of different cultivars of chickpea

Values of the LD_{50} obtained for the controls (shoots that were allowed to take up water) were between 13.03 and 20.37 $\mu g.ml^{-1}$, which demonstrates a day-to-day variation for the bioassays, as shown previously by Hamid (1999).

It would be interesting to test the sensitivity of different cultivars of chickpea that have different degree of resistance or susceptibility to *Ascochyta* or *Fusarium*, another pathogen of chickpea plants causing chickpea wilt, to solanapyrone A. Cultivars ILC-3279 (moderately resistant to Ascochyta blight) and Amdoun 1 (very susceptible to Ascochyta blight) could be used as an internal control in order to circumvent the influence of day-to-day variations in the assays. If solanapyrone A is an important pathogenicity or virulence factor, assaying the sensitivity of chickpea plants to this toxin would be a useful tool to select plants that are resistant to Ascochyta blight.

Chapter 7

General discussion and future prospects

As part of a wider project on the integrated management of Ascochyta blight of chickpea, this study focused on the production of toxin-minus mutants of *A. rabiei* and the discovery of safeners that reduce the sensitivity of chickpea plants to the toxin, solanapyrone A. The production of toxin minus mutants was required in order to help ascertain the role played by the toxins in the disease, since if these were non-pathogenic or reduced in virulence this would be strong evidence for their importance in the disease syndrome. Moreover, such evidence would strengthen the validity of an approach to breeding for resistance by selecting genotypes of the plant that were toxin insensitive. The interest in safeners was sparked by the fact that they are able to protect plants against xenobiotics such as herbicides and these could include the solanapyrones. Thus safeners could be part of an integrated package for the control of Ascochyta blight.

The production of toxin minus mutants required the optimisation of several techniques. These include germination of the pycnidiospores of the fungus, production of fungal protoplasts and development of methods for insertional mutagenesis and screening of the mutants produced for the toxin-minus phenotype. Consequently, these techniques are discussed in the paragraphs below.

In order to test whether safeners protected plants treated with solanapyrone A, it was necessary to have reasonable amounts of the compound at hand. Therefore, attempts were made to optimise the production of solanapyrone A and these are also discussed.

Finally, some comments are made about the contribution of this study to the integrated control of Ascochyta blight of chickpea and suggestions are made as to the future course of research.

7.1. Germination of pycnidiospores of *A. rabiei*

Germination of freshly produced pycnidiospores in YEP broth (Appendix 1), containing 3% glucose, exceeded 80% after incubation for 14 to 20 h, whereas germination of pycnidiospores stored in liquid nitrogen was below 30% in the same medium after incubation for 24 h. However, fresh pycnidiospores incubated in CDCLM (Appendix 1) or Richards' medium (Appendix 1) supplemented or not with glucose (1% or 3%) did not germinate over a period of 24 h. Moreover, germination did not occur when pycnidiospores were incubated at 10^7, 10^8 or 10^9 per ml in YEP broth containing 3% glucose. These results showed, as described in Chapter 2 (Section 2.3.1), that germination of pycnidiospores of *A. rabiei* was affected by three factors: storage in liquid nitrogen, nutrient supply and pycnidiospore density. The dormancy of *A. rabiei* seems to be mainly exogenous as spore density may cause a competition for nutrients among pycnidiospores. This type of dormancy was also observed with conidia of *Aspergillus niger* (Morozova *et al.*, 2001). Nutritive elements rich in nitrogen activated their germination. Optimal germination of pycnidiospores of *A. rabiei* was obtained when freshly produced pycnidiospores were inoculated in YEP medium (Appendix 1) supplemented with 3% glucose incubated at 20°C on a rotary shaker for 18 to 20 h.

7.2. Production of protoplasts of *A. rabiei*

The developmental state of *A. rabiei* appeared critical for the production of protoplasts. No protoplasts were produced from mycelium or ungerminated pycnidiospores treated with various lytic enzymes and osmotica (Chapter 2, Table 2.1). Protoplasts (2.72×10^7 per ml of lytic solution) were only generated when germinated pycnidiospores were treated with Driselase and Cellulase Onozuka suspended in KC buffer (Chapter 2, Table 2.1). Similarly, protoplasts were produced with germinated spores of *Neonectria galligena* (Tanguay *et al.*, 2003). The choice of the lytic enzyme is essential to the success of protoplasting fungi, in particular Ascomycetes. Different lytic enzymes have different activities on different Ascomycetes. For instance, Lysing Enzyme (5

mg/ml) is capable of protoplasting *Coniothyrium minitans* (Jones *et al.*, 1999), whereas protoplasts from spores of *Mycosphaerella graminicola* were obtained with Novozym 234 (Payne *et al.*, 1998). Lytic enzymes have previously been shown to be more effective when used as mixtures rather than individually (Robinson and Deacon, 2001; Cheng *et al.*, 2000; Lalithakumari, 2000; Redman *et al.*, 1999). For example, Akamatsu *et al.* (1999) produced protoplasts from *Alternaria alternata* with Novozym 234 and Kitalase. Protoplasts from *Trichoderma reesei* were obtained with Novozym 234 and cellulase. Jung *et al.* (2000) produced protoplasts of *Aspergillus nidulans* using a combination of β-D-glucanase, Driselase and lyticase but showed that this combination led to significant protoplast death. They also used Driselase alone, which was less toxic but decreased the protoplast yield.

Both lytic enzymes and osmoticum must be selected carefully as they may affect protoplast regeneration. Barreto *et al.* (1997) reported that lysing enzymes from *Trichoderma harzianum* and KCl optimised protoplast production of *Paecilomyces fumosoroseus* but that a mixture of Novozym 234 and cellulose and sorbitol were more appropriate for protoplast regeneration. Regeneration of protoplasts of *A. rabiei* submitted to the action of Driselase and Cellulase Onozuka suspended in an osmoticum containing potassium chloride reached 5.9%, a satisfactory value for a filamentous fungus. However, this value may be enhanced by submitting the protoplasts to agitation during incubation at 20°C in liquid complete medium (Chapter 2, Section 2.2.4) overnight and/or by plating them onto an agarised medium containing an osmoticum different from sucrose.

7.3. Insertional mutagenesis

The production of toxin-minus mutants of *A. rabiei* required the creation of a large number of transformants, hence the implementation of an efficient insertional mutagenesis technique was required.

Different mutagenesis methods have different efficiencies with fungi. For instance, electroporation of conidia of *Aspergillus fumigatus* produced up to 1,300 transformants per μg plasmid, whereas 141 transformants were created with the same fungus by REMI (Brown et al., 1998). Electroporation and particle bombardment were inappropriate in transforming *Aspergillus giganteus*, whereas ATMT increased 240 fold the efficiency of the conventional transformation of protoplasts using polyethylene glycol (Meyer *et al.*, 2003). Similarly, with *A. rabiei*, no transformants were produced by particle bombardment, few were obtained by REMI and electroporation but nearly 1,000 were generated by ATMT.

This work presents the first report on the successful genetic transformation of a fungus from the genus *Ascochyta* using *Agrobacterium*. The ATMT technique is an effective approach to transform *A. rabiei* with heterologous DNA since 908 transformants were created that grew on a medium supplemented with hygromycin. Among these transformants, 16 had a significant reduction in the production of solanapyrone A compared to the wild-type.

Direct transformation of pycnidiospores of *A. rabiei* makes *A. tumefaciens* a powerful and facile tool for genetic transformation of *A. rabiei*. ATMT circumvents some of the limitations of the REMI technique, such as the production of protoplasts and the use of appropriate restriction enzymes, and of electroporation and particle bombardment, two techniques which require the empirical adjustment of several variables (as discussed in Chapter 5). It is an effective tool to tag, identify and subsequently clone the genes of *A. rabiei* that encode the production of solanapyrone toxins, in particular solanapyrone A. This technique was used to generate a library of transformants, which can subsequently be screened for the phenotype of interest (loss or reduction of virulence). It also offers the potential to transform other *Ascochyta* species which are pathogens of different crops.

7.4. Screening putative mutants for the production of solanapyrone A

Assessment of the production of solanapyrone A by putative mutants required the implementation of a reliable assay. Two assays were developed: a colorimetric test using MBTH and an assay in microtitre plates.

A blue dye was formed when *A. rabiei* was grown on agarised CDCLM containing MBTH (0.01 to 0.025%) and $FeCl_3$ (0.01 to 0.025%). This dye indicated the presence of an aldehyde, probably solanapyrone A. However, this assay was not appropriate for the screening of a large number of transformants owing to the diffusion of solanapyrone A in the culture medium. An assay in microtitre plates was then developed. *A. rabiei* was grown in 24-well culture plates containing 2 ml CDCLM per well. Culture filtrates of each transformant were transferred to a 96-well plate and solanapyrone A was quantified by reading directly the absorbance at 340nm (close to the λ_{max} of solanapyrone A). The accuracy of this assay was confirmed by growing some selected transformants in conical flasks and by analysing the toxins synthesised by HPLC, after solid phase extraction. This assay had the advantage of circumventing bioassays on host plants, as performed with *Alternaria alternata* (Ito *et al.*, 2004).

7.5. Perspective with regard to the created mutants

Although colonies of *A. rabiei* were obtained by ATMT that grew on nutrient agar supplemented with hygromycin several other criteria would have to be met before such a mutant could be considered to be genuinely toxin-minus. First, it would be necessary to ascertain that the fungus was homokaryotic with regard to a single insertion in the gene of interest. Secondly, the mutant would have to be stable and thirdly it would be desirable to put the fungus through meiosis and demonstrate that hygromycin resistance and the toxin-minus phenotype cosegregated.

7.5.1. Single spore purification of the mutants

The majority of the pycnidiospores of *A. rabiei* produced in this study were binucleate. Consequently, the initial transformants are likely to be heterokaryons with a mixed population of transformed and untransformed nuclei. Similarly, 99.5% of the conidia of *Botrytis cinerea* are multinucleate. Transformants of this fungus, obtained by ATMT, were single-spore purified and one round of single-conidium isolation appeared sufficient to obtain monokaryons (Rolland *et al.*, 2003). A single isolation may be sufficient with transformants of *A. rabiei* if the multiple nuclei of each pycnidiospore derive from the mitosis of a single inherited nucleus. However, if the nuclei originate from different nuclei of the parent mycelium, several isolations must be performed.

In the second ATMT experiment, five selected mutants had reduced production of solanapyrone A compared to the wild-type but were unimpaired with regard to sporulation. Single spore isolation of these mutants is in progress in order to select for the nuclei that have been transformed. The colonies that are able to regenerate on selective medium will be sent to partners involved in the project who will test them for virulence on various chickpea cultivars. A reduction in the virulence of these mutants compared to the parent strain would be a strong evidence of the involvement of solanapyrone A in the development of Ascochyta blight. The best proof for the role of toxin would be the loss of virulence of a mutant that does not produce any solanapyrone A.

7.5.2. Stability of the transformants

It would be important to determine if the integrations of the T-DNA are mitotically stable by confirming the expression of hygromycin resistance gene after serial transfer of the transformants to non-selective and selective medium, alternately.

To ensure that the integrations of T-DNA are responsible for the mutations and that these mutations are meiotically stable, the mutant phenotype (reduction or loss in the

production of solanapyrone toxin) and the integrations must co-segregate in the progeny after back-crossing with a compatible wild-type isolate.

7.5.3. Determination of the mutated gene(s)

In order to facilitate the isolation of the mutated gene(s), mutants with single integration of T-DNA must be used. The mutated gene(s) can be identified by isolating the fungal genomic DNA flanking the T-DNA, sequencing it and looking for similarities with sequences present in gene databases. Isolation of the flanking DNA can be performed by plasmid rescue or inverse PCR. Plasmid rescue was used by Rose *et al.* (2002) to identify a polyketide synthase required for the biosynthesis of T-toxin in *C. heterostrophus*. The sequences flanking the integrated T-DNA can be amplified by inverse PCR (Clergeot *et al.*, 2000; Shuster and Connelley, 1999). The integrity of the sequences of the left and right borders is of importance if the identification of the mutated genes is performed by inverse PCR, as primers specific to these sequences must be used. However parts of the sequences of the borders may be lost. Covert *et al.* (2001) reported that half of the tested transformants of *F. circinatum* contained non-T-DNA sequences. An alternative to inverse PCR is the thermal asymmetric interlaced (TAIL)-PCR using a set of nested primers specific to the T-DNA border sequences and a short arbitrary degenerate primer (Combier *et al.*, 2003; Mullins *et al.*, 2001).

Solanapyrone A is synthesised from a polyketide precursor. Yang *et al.* (1996) have isolated a gene (*PKS1*) that encodes a polyketide synthase necessary for both biosynthesis of T-toxin and high virulence of *Cochliobolus heterostophus* race T to maize. However, polyketides constitute a group of secondary metabolites with a broad range of biological activities (O'Hagan, 1995). Polyketide synthases are responsible for the biosynthesis of several mycotoxins and other secondary metabolites, in particular for the biosynthesis of solanapyrone toxins, aflatoxin and fumonisin. Consequently, cloning the gene(s) which encode(s) polyketide synthase is not specific enough. The reduction in the production of solanapyrone A by sixteen transformants indicates that these have

been mutagenised in genes that are not specific to the pathway of synthesis of solanapyrone A or in genes that are at the beginning of the pathway. A mutation in a gene encoding an enzyme that is involved in the Diels-Alder reaction leading to the formation of solanapyrone A would be useful to clone a gene that encode the production of this toxin. To date, only three natural Diels-Alderases are known, namely solanapyrone synthase which catalyses the formation of solanapyrone A, lovastatin nonaketide synthase and fungal macrophomatesynthase (Ose *et al.*, 2003).

Osbourn (2001) reported that the genes required for the synthesis of toxins are often closely clustered in fungal genomes, *e.g.* the *TOX2* locus of *Cochliobolus carbonum*. The genes involved in aflatoxin biosynthesis are clustered in *Aspergillus* spp. (Yu *et al.*, 2004). Similarly, clustered genes encode trichothecenes, toxic compounds produced by *Fusarium* spp. (Brown *et al.*, 2004). It can be expected that the expression of several genes are necessary for the production of solanapyrone A as its biosynthetic pathway involves several successive steps (Oikawa *et al.*, 1998). Therefore, the mutated gene(s) in *A. rabiei* might be part of a cluster.

7.6. Production of solanapyrone A

Solanapyrone A production varied in the four techniques tested: still cultures in Roux bottles or in conical flasks, shake cultures and cultures in a fermenter. Among these four techniques, 23.0 to 42.7 mg of toxin was routinely obtained. The highest amounts of solanapyrone A (up to 150 $mg.l^{-1}$ of culture filtrate) were obtained in shake culture of *A. rabiei* after 5-6 days of incubation only. Similarly, a higher amount of toxin ascochitine was produced in shake culture of *Ascochyta fabae* compared to still cultures, unless the surface to volume ratio was large (Beed *et al.*, 1994). In contrast, *Penicillium roqueforti* produced more eremofortin C and PR toxin in still cultures than shake cultures (Chang *et al.*, 1991). The difficulty encountered with the shake culture technique was the risk of missing the peak of production of the toxin. No solanapyrone toxins were detected from cultures of the fungus in a fermenter. However, they may have been been

synthesised as observed in shake culture, but they may have had a sharp peak owing to the fast growth of *A. rabiei.* Nevertheless, it would be of interest to develop this technique if a large amount of solanapyrone A is required for screening chickpea genotypes, as it presents the advantage to work with a large volume of fungal culture.

Three main parameters affect fungal growth and the synthesis of secondary metabolites: the level of dissolved oxygen, the agitation rate which also affects the amount of dissolved oxygen (Feng *et al.*, 2004; Kim *et al.*, 2003) and the pH (Papagianni, 2004; O'Donnell *et al.*, 2001). Better control of these parameters may allow the production of solanapyrone A by *A. rabiei.* The growth of *A. rabiei* may also be better controlled by using pycnidiospores as inoculum instead of mycelial homogenate as they are easier to quantify. Carlsen *et al.* (1996) produced α-amylase from fermenter cultures of *Aspergillus oryzae* by inoculating spores (3 to 6 X 10^8 per litre culture medium).

7.7. Safeners

The high cost of fungicides, the rising concern about the widespread use of large amounts of chemicals to control plant pathogens and the development of populations of plant pathogens more resistant to chemicals has emphasised the need to use resistant cultivars. In the expectation that solanapyrone A may be involved in the development of Ascochyta blight, the role of safeners toward the insensitivity of chickpea to solanapyrone A was investigated.

Safeners enhance herbicide resistance in a crop and therefore the selectivity of herbicides for competing weeds (Davies and Caseley, 1999). In this study, dichlormid (300 and 800 µg per shoot) and fenclorim (18 µg per shoot) decreased the sensitivity of chickpea shoots to solanapyrone A. The role of these two safeners in the reduction of disease symptoms caused by Ascochyta blight must be confirmed by assays on chickpea plants in natural conditions. This piece of work can be performed in the field by partners of the project. The role of other safeners towards the sensitivity of various chickpea

cultivars to solanapyrone A must be assessed as well.

The use of safeners which decrease the susceptibility of chickpea to blight would allow the reduction of the amount of fungicides which are at present applied to the field. Applying herbicides in combination with safeners may simultaneously protect chickpea plants and control weeds which compete with crops for resources such as nutrients, water and light, reducing crop yields.

7.8. Role of solanapyrone A

The mechanism of action of solanapyrone A has not yet been established. Mizushina *et al.* (2002) showed that solanapyrone A selectively inhibited the activities of mammalian DNA polymerase β and λ *in vitro*. However, the reproduction of the symptom of stem breakage on chickpea plants by solanapyrone A suggests that the toxin may disrupt the cell membranes owing to its detergent-like structure composed of a hydrophobic decalin moiety and a hydrophilic pyrone moiety (Strange, 1997). Death of chickpea cells observed in bioassays involving fluorescein diacetate as a vital dye (Chapter 6) support the evidence that cell membranes are the site of action of solanapyrone A.

7.9. Integration of pest management techniques

Regarding the important impact of Ascochyta blight on chickpea cultivation worldwide, sustainable pest management techniques that are socially and economically acceptable as well as environmentally safe must be implemented. No true resistance to Ascochyta blight has been reported, rather Chongo and Gossen (2001) found that susceptibility increased with plant age. The best long-term prospect for Ascochyta blight is thought to be through the use of the partially resistant germplasm that is available integrated with other control measures which respect the farmers' needs.

Determining the role of solanapyrone A in the development of Ascochyta blight of chickpea would be a major contribution towards disease control via breeding of chickpea. If solanapyrone A is a pathogenicity or virulence factor for Ascochyta blight, the cultivars of chickpea that are more resistant to the disease can be selected for their reduced sensitivity to the toxin. Breeding resistant cultivars is used as a means to reduce the use of chemicals in agriculture. However, plant breeding is expensive, time and space consuming. More importantly, a suitable scale for scoring accurately blight symptoms and quantifying the disease severity is still lacking.

As part of a project that aims to control Ascochyta blight of chickpea, the breeding and the safener approaches are associated with three other control strategies performed by four partners:

(i) the escape and the forecasting of the disease by defining the best planting times to reduce the risk of epidemics;

(ii) the reduction of inoculum via field sanitation and

(iii) the determination of the most effective fungicides for seed and plant treatment.

The complementarity of these approaches is contributing to knowledge about both *C. arietinum* L.and *A. rabiei* and it is hoped that this knowledge will allow better control of Ascochyta blight.

References

AbuBakar FD and Chua HP, 1996. Formation and regeneration of protoplasts in *Metarhizium anisopliae*. *Asia-Pacific Journal of Molecular Biology and Biotechnology* **4:** 20-29.

Abuodeh RO, Orbach MJ, Mandel MA, Das A and Galgiani JN, 2000. Genetic transformation of *Coccidioides immitis* facilitated by *Agrobacterium tumefaciens*. *Journal of Infectious Diseases* **181:** 2106-2110.

Agrawal K and Singh G, 2003. Physico-chemical and milling quality of some improved varieties of chickpea (*Cicer arietinum*). *Journal of Food Science and Technology-Mysore* **40:** 439-442.

Akamatsu H, Itoh Y, Kodama M, Otani H and Kohmoto K, 1997. AAL-toxin-deficient mutants of *Alternaria alternata* tomato pathotype by restriction enzyme-mediated integration. *Phytopathology* **87:** 967-972.

Akamatsu H, Taga M, Kodama M, Johnson R, Otani H and Kohmoto K, 1999. Molecular karyotypes for Alternaria plant pathogens known to produce host-specific toxins. *Current Genetics* **35:** 647-656.

Akem C, 1999. Ascochyta blight of chickpea: present status and future priorities. *International journal of Pest Management* **45**: 131-137.

Alves SB, Pereira RM, Stimac JL and Vieira SA, 1996. Delayed germination of *Beauveria bassiana* conidia after prolonged storage at low, above-freezing temperatures. *Biocontrol Science and Technology* **6:** 575-581.

Aly R, Halpern N, Rubin B, Dor E, Golan S and Hershenhorn J, 2001. Biolistic transformation of *Cercospora caricis*, a specific pathogenic fungus of *Cyperus rotundus*. *Mycological Research* **105:** 150-152.

Amey RC, Athey-Pollard A, Burns C, Mills PR, Bailey A and Foster GD, 2002.

PEG-mediated and *Agrobacterium*-mediated transformation in the mycopathogen *Verticillium fungicola*. *Mycological Research* **106:** 4-11.

Anderson MM, Hazen SL, Hsu FF and Heinecke JW, 1997. Human neutrophils employ the myeloperoxidase-hydrogen peroxide- chloride system to convert hydroxy-amino acids into glycolaldehyde, 2-hydroxypropanal, and acrolein - A mechanism for the generation of highly reactive alpha-hydroxy and alpha, beta-unsaturated aldehydes by phagocytes at sites of inflammation. *Journal of Clinical Investigation* **99:** 424-432.

Angelini R, Bragaloni M, Federico R, Infantino A and Porta-Puglia A, 1993. Peroxidase in resistance of chickpea to *Ascochyta rabiei*. *Journal of Plant Physiology* **142**: 704-709.

Apoga D, Akesson H, Jansson HB and Odham G, 2002. Relationship between production of the phytotoxin prehelminthosporol and virulence in isolates of the plant pathogenic fungus *Bipolaris sorokiniana*. *European Journal of Plant Pathology* **108:** 519-526.

Armero J, Requejo R, Jorrin J, Lopez-Valbuena R and Tena M, 2001. Release of phytoalexins and related isoflavonoids from intact chickpea seedlings elicited with reduced glutathione at root level. *Plant Physiology and Biochemistry* **39**: 785-795.

Armstrong CL, Chongo G, Gossen BD and Duczek LJ, 2001. Mating type distribution and incidence of the teleomorph of *Ascochyta rabiei* (*Didymella rabiei*) in Canada. *Canadian Journal of Plant Pathology* **23**: 110–113.

Ayliffe MA, Lawrence GJ, Ellis JG and Pryor AJ, 1997. Production of a self-inhibitor of urediospore germination in *Melampsora lini* (flax rust) segregates as a recessive, single gene trait. *Canadian Journal of Botany-Revue Canadienne de Botanique* **75:** 74-76.

Bai GH, Plattner R, Desjardins A and Kolb, 2001. Resistance to *Fusarium* head blight and deoxynivalenol accumulation in wheat. *Plant Breeding* **120**: 1-6.

de Backer MD, Maes D, Vandoninck S, Logghe M, Contreras R and Luyten WHML, 1999. Transformation of *Candida albicans* by electroporation. *Yeast* **15:** 1609-1618.

Balasubramanian N, Juliet GA, Srikalaivani P and Lalithakumari D, 2003. Release and regeneration of protoplasts from the fungus *Trichothecium roseum. Canadian Journal of Microbiology* **49:** 263-268.

Balhadère PV, Foster AJ and Talbot NJ, 1999. Identification of pathogenicity mutants of the rice blast fungus *Magnaporthe grisea* by insertional mutagenesis. *Molecular Plant-Microbe Interactions* **12:** 129-142.

Balint-Kurti PJ, May GD and Churchill ACL, 2001. Development of a transformation system for Mycosphaerella pathogens of banana: a tool for the study of host/pathogen interactions. *FEMS Microbiology Letters* **195:** 9-15.

Barcellos FG, Fungaro MHP, Furlaneto MC, Lejeune B, Pizzirani-Kleiner AA and de Azevedo JL, 1998. Genetic analysis of *Aspergillus nidulans* unstable transformants obtained by the biolistic process. *Canadian Journal of Microbiology* **44:** 1137-1141.

Barreto CC, Alves LC, Aragao FJL, Rech E, Schrank A and Vainstein MH, 1997. High frequency gene transfer by microprojectile bombardment of intact conidia from the entomopathogenic fungus *Paecilomyces fumosoroseus. FEMS Microbiology Letters* **156:** 95-99.

Barz W and Mackenbrock U, 1994. Constitutive and elicitation induced metabolism of isoflavones and pterocarpans in chickpea (*Cicer arietinum*) cell-suspension cultures. *Plant Cell Tissue and Organ Culture* **38**: 199-211.

Beed FD, Sue RE and Strange RN, 1994. Variation in the Production of Ascochitine by Ascochyta-Fabae. *Mycological Research* **98:** 1069-1076.

Binns AN, 2002. T-DNA of *Agrobacterium tumefaciens*: 25 years and counting. *Trends in Plant Science* **7**: 231-233.

Bliffeld M, Mundy J, Potrykus I and Futterer J, 1999. Genetic engineering of wheat for increased resistance to powdery mildew disease. *Theoretical and Applied Genetics* **98:** 1079-1086.

Bogo MR, Vainstein MH, Aragao FJL, Rech E and Schrank A, 1996. High frequency gene conversion among benomyl resistant transformants in the entomopathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiology Letters* **142:** 123-127.

Bölker M, Bohnert HU, Braun KH, Gorl J and Kahmann R, 1995. Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). *Molecular and General Genetics* **248:** 547-552.

Breeuwer P, deReu JC, Drocourt JL, Rombouts FM and Abee T, 1997. Nonanoic acid, a fungal self-inhibitor, prevents germination of *Rhizopus oligosporus* sporangiospores by dissipation of the pH gradient. *Applied and Environmental Microbiology* **63:** 178-185.

Brown DH, Slobodkin IV and Kumamoto CA, 1996. Stable transformation and regulated expression of an inducible reporter construct in *Candida albicans* using restriction enzyme mediated integration. *Molecular and General Genetics* **251**: 75-80.

Brown DW, Dyer RB, McCormick SP, Kendra DF and Plattner RD, 2004. Functional demarcation of the *Fusarium* core trichothcene gene cluster. *Fungal Genetics and Biology* **41**: 454-462.

Brown JS, Aufauvre-Brown A and Holden DW, 1998. Insertional mutagenesis of Aspergillus fumigatus. *Molecular and General Genetics* **259:** 327-335.

Bundock P and Hooykaas PJJ, 1996. Integration of *Agrobacterium tumefaciens* T-DNA in the *Saccharomyces cerevisiae* genome by illegitimate recombination. *Proceedings of the National Academy of Science of the United States of America* **93**: 15272-15275.

Bundock P, Dendulkras A, Beijersbergen A and Hooykaas PJJ, 1995. Transkingdom

T-DNA transfer from *Agrobacterium-tumefaciens* to *Saccharomyces cerevisiae. EMBO Journal* **14**: 3206-3214.

Bundock P, Mroczek K, Winkler AA, Steensma HY and Hooykaas PJJ, 1999. T-DNA from *Agrobacterium tumefaciens* as an efficient tool for gene targeting in *Kluyveromyces lactis. Molecular and General Genetics* **261**: 115-121.

Bundock P, van Attikum H, den Dulk-Ras A, and Hooykaas PJJ, 2002. Insertional mutagenesis in yeasts using T-DNA from *Agrobacterium tumefaciens. Yeast* **19**: 529-536.

Butler EJ, 1918. *Fungi and disease in plants.*M. Bishen Singh Mahendra Pal Singh, Dehradun and M/s. *Periodical Experts*, Dehli, 547 pp. (reprinted 1973).

Cachinero JM, Hervas A, Jiménez-Díaz RM and Tena M, 2002. Plant defence reactions against Fusarium wilt in chickpea induced by incompatible race 0 of *Fusarium oxysporum f.sp ciceris* and nonhost isolates of *F. oxysporum. Plant Pathology* **51**: 765-776.

Campoy S, Perez F, Martin JF, Gutierrez S and Liras P, 2003. Stable transformants of the azaphilone pigment-producing *Monascus purpureus* obtained by protoplast transformation and *Agrobacterium*-mediated DNA transfer. *Current Genetics* **43:** 447-452.

Caplan AB, Vanmontagu M and Schell J, 1985. Genetic analysis of integration mediated by single T-DNA borders. *Journal of Bacteriology* **161**: 655-664.

Carlsen M, Spohr AB, Nielsen J and Villadsen J, 1996. Morphology and physiology of an alpha amylase producing strain of *Aspergillus oryzae* during batch cultivations. *Biotechnology and Bioengineering* **49:** 266-276.

Carroll AM, Sweigard JA and Valent-Central B, 1994. Improved vectors for selecting resistance to hygromycin. *Fungal Genetics Newsletter* **41**: 22.

Chadha BS, Kaur R, Saini HS and Singh S, 2000. Characterization of hygromycin-resistant transformants of thermophilic fungus *Thermomyces lanuginosus. World*

Journal of Microbiology & Biotechnology **16:** 303-306.

Chakraborty BN, Patterson NA and Kapoor M, 1991. An electroporation-based system for high-efficiency transformation of germinated conidia of filamentous fungi. *Canadian Journal of Microbiology* **37:** 858-863.

Chang SC, Wei YH, Wei DL, Chen YY and Jong SC, 1991. Factors Affecting the Production of Eremofortin-C and Pr Toxin in Penicillium-Roqueforti. *Applied and Environmental Microbiology* **57:** 2581-2585.

Chen HC and Liu TM, 1997. Inoculum effects on the production of gama-linolenic acid by the sahke culture of *Cunninghamella echinulata* CCRC 31840. *Enzyme and Microbial Technology* **21**:137-142.

Chen WQ and Singh KB, 1999. The auxin, hydrogen peroxide and salicylic acid induced expression of the *Arabidopsis* GST6 promoter is mediated in part by an OCS element. *Plant Journal* **19**: 667-677.

Chen YM and Strange RN, 1991. Synthesis of the solanapyrone phytotoxins by *Ascochyta rabiei* in response to metal cations and development of a defined medium for toxin production. *Plant Pathology* **40**: 401-407.

Chen YM and Strange RN, 1994. Production of a proteinaceous phytotoxin by *Ascochyta rabiei* grown in expressed chickpea Sap. *Plant Pathology* **43:** 321-327.

Cheng YL and Bélanger RR, 2000. Protoplast preparation and regeneration from spores of the biocontrol fungus *Pseudozyma flocculosa. FEMS Microbiology Letters* **190:** 287-291.

Cherry AJ, Rabindra RJ, Parnell MA, Geetha N, Kennedy JS and Grzywacz D, 2000. Field evaluation of *Helicoverpa armigera* nucleopolyhedrovirus formulations for control of the chickpea pod-borer, *H. armigera* (Hubn.), on chickpea (*Cicer arietinum* var. Shoba) in southern India. *Crop Protection* **19**: 51-60.

Chongo G and Gossen BD, 2001. Effect of plant age on resistance to *Ascochyta*

rabiei in chickpea. *Canadian Journal of Plant Pathology* **23**: 358-363.

Chongo G, Gossen BD, Buchwaldt L, Adhikari T and Rimmer SR, 2004. Genetic diversity of *Ascochyta rabiei* in Canada. *Plant Disease* **88:** 4-10.

Christie PJ and Vogel JP, 2000. Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends in Microbiology* **8**: 354-360.

Christie PJ, 1997. *Agrobacterium tumefaciens* T-complex transport apparatus: A paradigm for a new family of multifunctional transporters in Eubacteria. *Journal of Bacteriology* **179**: 3085-3094.

Christie PJ, Ward JE, Winans SC and Nester EW, 1988. The *Agrobacterium tumefaciens* VirE2 gene product is a single-stranded DNA-binding protein that associates with T-DNA. *Journal of Bacteriology* **170**: 2659-2667.

Chung KR, Ehrenshaft M, Wetzel DK and Daub ME, 2003. Cercosporin-deficient mutants by plasmid tagging in the asexual fungus *Cercospora nicotianae*. *Molecular Genetics and Genomics* **270:** 103-113.

Churchill ACL, Dunkle LD, Silbert W, Kennedy KJ and Macko V, 2001. Differential synthesis of peritoxins and precursors by pathogenic strains of the fungus *Periconia circinata*. *Applied and Environmental Microbiology* **67:** 5721-5728.

Ciufetti LM, Yoder OC and Turgeon BG, 1992. A microbial assay for host-specific fungal polyketide toxins. *Fungal Genetics Newsletter* **39**: 18-19.

Clergeot PH, Gourgues M, Cots J, Laurans F, Latorse MP and Pepin R, 2001. PLS1, a gene encoding a tetraspanin-like protein, is required for penetration of rice leaf by the fungal pathogen *Magnaporthe grisea*. *Proceedings of the National Academy of Science of the United States of America* **98**: 6963-6968.

Clough SJ and Bent AF, 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16**: 735-43.

Cojean N, Entz-Werle N, Eyer D, Becmeur F, Kehrli P, Marcellin L, Christmann D, Babin A and Lutz P, 2003. Dumbbell nephroblastoma: an uncommon cause of spinal cord compression. *Archives de Pediatrie* **10:** 1075-1078.

Cole DJ and Edwards, 2000. Secondary metabolism of agrochemicals in plants. In: *Metabolism of agrochemicals in plants* (eds. T. Roberts).

Cole DJ, Cummins I, Hatton PJ, Dixon D and Edwards R, 1997. Glutathione transferases in crops and major weeds. In: Hatzios KK (ed.) Regulation of enzymatic systems detoxifying xenobiotics in plants, Kluwer Academic Publishers, Amsterdam, pp.139-154.

Coleman JOD, BlakeKalff MMA and Davies TGE, 1997. Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. *Trends in Plant Science* **2**: 144-151.

Collard BCY, Ades PK, Pang ECK, Brouwer JB and Taylor PWJ, 2001. Prospecting for sources of resistance to Ascochyta blight in wild *Cicer* species. *Australian Plant Pathology* **30**: 271-276.

Combier JP, Melayah D, Raffier C, Gay G and Marmeisse R, 2003. *Agrobacterium tumefaciens*-mediated transformation as a tool for insertional mutagenesis in the symbiotic ectomycorrhizal fungus *Hebeloma cylindrosporum*. *FEMS Microbiology Letters* **220:** 141-148.

Costa CP and Ferreira MC, 1991. Preservation of microorganisms – a review. *Revista de Microbiologia* **22**: 263-268.

Cottingham CK, Hatzios KK and Meredith S, 1998. Influence of chemical treatments on glutathione S-transferases of maize with activity towards metolachlor and cinnamic acid. *Journal of Biosciences* **53:** 973-979.

Cournoyer JJ, Kshirsagar T, Fantauzzi PP, Figliozzi GM, Makdessian T and Yan B, 2002. Color test for the detection of resin-bound aldehyde in solid- phase combinatorial synthesis. *Journal of Combinatorial Chemistry* **4:** 120-124.

Covert SF, Kapoor P, Lee MH, Briley A and Nairn CJ, 2001. *Agrobacterium tumefaciens*-mediated transformation of *Fusarium circinatum*. *Mycological Research* **105:** 259-264.

Dantas-Barbosa C, Araujo EF, Moraes LMP, Vainstein MH and Azevedo MO, 1998. Genetic transformation of germinated conidia of the thermophilic fungus *Humicola grisea* var. *thermoidea* to hygromycin B resistance. *FEMS Microbiology Letters* **169:** 185-190.

Datta K, Tu JM, Oliva N, Ona I, Velazhahan R, Mew TW, Muthukrishnan S and Datta SK, 2001. Enhanced resistance to sheath blight by constitutive expression of infection-related rice chitinase in transgenic elite indica rice cultivars. *Plant Science* **160**: 405-414.

Davies J and Caseley JC, 1999. Herbicide safeners: a review. *Pesticide Science* **55:** 1043-1058.

Davis BD, 1985. Factors affecting protoplasts isolation. In: Fungal protoplasts: Application in biochemistry and genetics, Peberdy JF and Ferenczy L eds., pp. 45-72. Marcel Dekker, New York.

Dean JV, Gronwald JW and Eberlein CV, 1990. Induction of glutathione S-transferase isozymes in sorghum by herbicide antidotes. *Plant Physiology* **92:** 467-473.

Deane EE, Whipps JM, Lynch JM and Peberdy JF, 1999. Transformation of *Trichoderma reesei* with a constitutively expressed heterologous fungal chitinase gene. *Enzyme and Microbial Technology* **24:** 419-424.

Debaud JC, Marmeisse R and Gay G, 1997. Genetics and molecular biology of the fungal partner in the ectomycorrhizal symbiosis *Hebeloma cylindrosporum* x *Pinus pinaster*. In: The Mycota V Part B, Plant relationships, Carroll/Tudzynski eds., pp.95-112.

Degefu Y and Hanif M, 2003. *Agrobacterium tumefaciens*-mediated integration of *Helminthosporium turcicum*, the maize leaf-blight fungus. *Archives of*

Microbiology **180**: 279-284.

Demirci F, Bayraktar H, Babaliogullu I, Dolar FS and Maden S, 2003. In vitro and in vivo effects of some fungicides against the chickpea blight pathogen, *Ascochyta rabiei*. *Journal of Phytopathology* **151**: 519-524.

Deng F, Jelesko J, Cramer C, Wu Jingrui and Krinton K, 2002. Characterisation of glutathione S-transferase on the physiological function in rice and *Arabidopsis* by antisense genes. Abstract of Poster, American Society of Plant Biologists, ref. 153.

Deng WY, Chen LS, Peng WT, Liang XY, Sekiguchi S, Gordon MP, Comai L and Nester EW, 1999. VirE1 is a specific molecular chaperone for the exported single-stranded-DNA-binding protein VirE2 in *Agrobacterium*. *Molecular Microbiology* **31**: 1795-1807.

Deng F, Nagao A, Shim IS and Usui K, 1997. Induction of glutathione S-transferase isozymes in rice shoots treated with a combination of pretilachlor and fenclorim. *Journal of Weed Science and Technology* **42**: 277–283.

Desjardins AE, Proctor RH, Bai GH, McCormick SP, Shaner G, Buechley G and Hohn TM, 1996. Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. *Molecular Plant Microbe Interactions* **9**: 775-781.

Devlin DL, Moshier LJ, Russ OG, and Stahlman PW, 1983. Antidotes reduce injury to grain sorghum (*Sorghum bicolor*) from acetanilide herbicides. *Weed Science* **31**: 790-795.

Dixon DP, Cummins I, Cole DJ and Edwards R, 1998. Glutathione-mediated detoxification systems in plants. *Current Opinion in Plant Biology* **1**: 258-266.

Dlugonski J and Wilmanska D, 1998. Application of protoplasts from *Curvularia lunata* for steroid hydroxylation and genetic transformation. *Microbios* **94**: 113-121.

Dolar S and Gurcan A, 1993. The role of the phytoalexins on the resistance to chickpea blight (*Ascochyta rabiei* (Pass.) Labr. in chickpeas. *Journal of Turkish Phytopathology* 22: 17-26.

Doohan FM, Smith P, Parry DW and Nicholson P, 1998. Transformation of *Fusarium culmorum* with the β-D-glucuronidase (GUS) reporter gene: a system for studying host-pathogen relationships and disease control. *Physiological and Molecular Plant Pathology* **53**: 253-268.

Edwards R and Cole DJ, 1996. Glutathione transferases in wheat (*Triticum*) species with activity toward fenoxaprop-ethyl and other herbicides. *Pesticide Biochemistry and Physiology* **54**: 96-104.

Ekler Z and Stephenson GR, 1991. Comparative effectiveness and mode of action of safeners for chloroacetamide herbicides in maize seedlings. *Journal of Biosciences* **46**: 828-835.

Ekler Z, Dutka F and Stephenson GR, 1993. Safener effects on acetochlor toxicity, uptake, metabolism and glutathione-S-transferase activity in maize. *Weed Research* **33:** 311-318.

El-Sayed M, Müller H, Rheinwald G, Lang H and Spange S, 2003. UV/Vis spectroscopic properties of N-(2′-Hydroxy-4′-N,N-dimethyl-aminobenzylidene)-4-nitroaniline in various solvents and solid environments. *Chemical Monthly* **134**: 361-370.

Epstein L, Lusnak K and Kaur S, 1998. Transformation-mediated developmental mutants of *Glomerella graminicola* (*Colletotrichum graminicola*). *Fungal Genetics and Biology* **23**: 189-203.

Farago S and Brunold C, 1994. Regulation of thiol contents in maize roots by internediates and effectors of glutathione synthesis. *Journal of Plant Physiology* **144**: 433-437.

Farago S, Brunold C and Kreuz K, 1994. Herbicide safeners and glutathione metabolism. *Physiologia Plantarum* **91**: 537-542.

Farina JI, Molina OE and Figueroa LIC, 2004. Formation and regeneration of protoplasts in *Sclerotium rolfsii* ATCC 201126. *Journal of Applied Microbiology* **96:** 254-262.

Farkaš V, 2003. Structure and biosynthesis of fungal cell walls: Methodological approaches. *Folia Microbiologica* **48:** 469-478.

Feldmann KA, 1991. T-DNA insertion mutagenesis in *Arabidopsis* mutational spectrum. *Plant Journal* **1**: 71-82

Feng XL, Guan CJ and Zhao CX, 2004. Ion exchange resin catalyzed condensation of indole and carbonyl compounds - Synthesis of bis-indolylmethanes. *Synthetic Communications* **34:** 487-492.

Fiallo ML, Tayeb H, Suarato A and Garnier-Suillerot A, 1998. Circular dichroism studies on anthracycline antitumor compounds. Relationship between the molecular structure and the spectroscopic data. *Journal of Pharmaceutical Sciences* **87**: 967-975.

Filichkin SA and Gelvin SB, 1993. Formation of a putative relaxation intermediate during T-DNA processing directed by the *Agrobacterium tumefaciens* VirD1, D2 endonuclease. *Molecular Microbiology* **8**: 915-926.

Fincham JRS, 1989. Transformation in fungi. *Microbiological Reviews* **53:** 148-170.

Fitzgerald AM, Mudge AM, Gleave AP and Plummer KM, 2003. *Agrobacterium* and PEG-mediated transformation of the phytopathogen *Venturia inaequalis. Mycological Research* **107**: 803-810.

Flandez-Galvez H, Ades PK, Ford R, Pang ECK and Taylor PWJ, 2003. QTL analysis for Ascochyta blight resistance in an intraspecific population of chickpea (*Cicer arietinum* L.). *Theoretical and Applied Genetics* **107**: 1257-1265.

Food and Agriculture Organisation (FAO), 2001.

Fuerst E.P. and Gronwald JW, 1986. Induction of rapid metabolism of metolachlor in

sorghum shoots by CGA-92194 and other antidotes. *Weed Science* **34**: 354-361.

Fungaro MHP, Poli RC, PizziraniKleiner AA, Besin E, Lejeune B and Azevedo JL, 1997. Genetic and molecular analysis of *Aspergillus nidulans* transformants obtained by the biolistic process. *Canadian Journal of Microbiology* **43**: 718-722.

Fungaro MHP, Rech E, Muhlen GS, Vainstein MH, Pascon RC, Dequeiroz MV, PizziraniKleiner AA and Deazevedo JL, 1995. Transformation of *Aspergillus nidulans* by microprojectile bombardment on intact conidia. *FEMS Microbiology Letters* **125:** 293-297.

Furlaneto MC, Paião FG, Pinto FG and Fungaro MH, 1999. Transformation of the entomopathogenic fungus *Metarhizium flavoviride* to high resistance to benomyl. *Canadian Journal of Microbiology* **45**: 875-878.

Gallmetzer M, Burgstaller W and Schinner F, 1999. An optimized method for the isolation of protoplasts from *Penicillium simplicissimum* to produce sealed plasma membrane vesicles. *Mycologia* **91:** 206-212.

Galloway J and MacLeod WJ, 2003. *Didymella rabiei*, the teleomorph of *Ascochyta rabiei*, found on chickpea stubble in Western Australia. *Australian Plant Pathology* **32**: 127-128.

Gathumbi JK, Usleber E and Martlbauer E, 2001. Production of ultrasensitive antibodies against aflatoxin B-1. *Letters in Applied Microbiology* **32:** 349-351.

Gelvin SB, 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**: 223-256.

Gibbs PA and Seviour RJ, 1996. Does the agitation rate and/or oxygen saturation influence exopolysaccharide production by *Aureobasidium pullulans* in batch culture? *Applied Microbiology and Biotechnology* **46:** 503-510.

Gorlenko MV and Bushkova LN, 1958. Perfect of the causal agent of ascochytosis of

chickpea. Abstract in: *Review of Applied Mycology* **37**: 695.

Gouka RJ, Gerk C, Hooykaas PJJ, Bundock P, Musters W, Verrips CT and de Groot MJA, 1999. Transformation of *Aspergillus awamori* by *Agrobacterium tumefaciens*-mediated homologous recombination. *Nature Biotechnology* **17**: 598-601.

Granado JD, KerteszChaloupkova K, Aebi M and Kues U, 1997. Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. *Molecular & General Genetics* **256:** 28-36.

Gronwald JW and Plaisance KL, 1998. Isolation and characterization of glutathione S-transferase isozymes from sorghum. *Plant Physiology* **117:** 877-892.

Gronwald JW, 1989. Influence of herbicide safeners on herbicide metabolism. In: *Crop safeners for herbicides: development, uses and mechanisms of action* (Hatzios KK and Hoagland RE, Eds.), p. 103, Academic Press, New York.

Gronwald JW, Fuerst EP, Eberlein CV and Egli MA, 1987. Effect of Herbicide Antidotes on Glutathione Content and Glutathione-S-Transferase Activity of Sorghum Shoots. *Pesticide Biochemistry and Physiology* **29:** 66-76.

de Groot MJA, Bundock P, Hooykaas PJJ and Beijersbergen AGM, 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnology* **16:** 839-842.

Guzman P, Davis RM, Gilbertson RL, Smith SN and Temple S, 1995. First report of *Ascochyta rabiei* causing Ascochyta blight of garbanzo in California. *Plant Disease* **79**: 82.

Hamid K and Strange RN, 2000. Phytotoxicity of solanapyrones A and B produced by the chickpea pathogen *Ascochyta rabiei* (Pass.) Labr. and the apparent metabolism of solanapyrone A by chickpea tissues. *Physiological and Molecular Plant Pathology* **56**: 235-244.

Hamid K., 1999. Separation and phytotoxicity of solanapyrone compounds produced

by *Ascochyta rabiei* (Pass.) Labr. and their metabolism by chickpea (*Cicer arietinum*). Ph.D. thesis, University College London, U.K.

Hanselle T and Barz W, 2001. Purification and characterisation of the extracellular PR-2b beta-1,3-glucanase accumulating in different *Ascochyta rabiei*-infected chickpea (*Cicer arietinum* L.) cultivars. *Plant Science* **161**: 773-781.

Harris JP and Mantle PG, 2001. Biosynthesis of ochratoxins by *Aspergillus ochraceus*. *Phytochemistry* **58:** 709-716.

Harris LJ, Desjardins AE, Plattner RD, Nicholson P, Butler G, Young YC, Weston G, Proctor RH and Hohn TM, 1999. Possible role of trichothecene mycotoxins in virulence of *Fusarium graminearum* on maize. *Plant Dis*ease **83:** 954-960.

Hatton PJ, Dixon D, Cole DJ and Edwards R, 1996. Glutathione transferase activities and herbicide selectivity in maize and associated weed species. *Pesticide Science* **46:** 267-275.

Hatzios KK, 1989. Development of herbicide safeners: industrial and university perspectives. In *Crop safeners for herbicides: development, uses and mechanisms of action* (Hatzios KK and Hoagland RE, Eds.), p. 103, Academic Press, New York.

Hatzios KK, 2000. Herbicide safeners and synergists. In: Roberts T, ed. *Metabolism of agrochemicals in plants*. New York: John Wiley, 259–294.

Hatzios KK and Wu JG, 1996. Herbicide safeners: Tools for improving the efficacy and selectivity of herbicides. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes* **31:** 545-553.

Haware MP, 1987. Occurence of the perfect state of *Ascochyta rabiei* in Syria. *International Chickpea Newsletter* **17**: 29-30.

Hazell BW, Te'O VSJ, Bradner JR, Bergquist PL and Nevalainen KMH, 2000. Rapid transformation of high cellulase-producing mutant strains of *Trichoderma reesei* by microprojectile bombardment. *Letters in Applied Microbiology* **30:** 282-286.

Heiser W, 2000. Optimization of biolistic transformation using the helium-driven PDS-1000/He system. *US/EG Bulletin* 1688.

Herzog RW, Daniell H, Singh NK and Lemke PA, 1996. A comparative study on the transformation of *Aspergillus nidulans* by microprojectile bombardment of conidia and a more conventional procedure using protoplasts treated with polyethyleneglycol. *Applied Microbiology and Biotechnology* **45:** 333-337.

Hiei Y, Komari T and Kubo T, 1997. Transformation of rice mediated by *Agrobacterium tumefaciens. Plant Molecular Biology* **35**: 205-218.

Hirano T, Sato T, Yaegashi K and Enei H, 2000. Efficient transformation of the edible basidiomycete *Lentinus edodes* with a vector using a glyceraldehyde-3-phosphate dehydrogenase promoter to hygromycin B resistance. *Molecular and General Genetics* **263**: 1047-1052.

Hirase K and Molin WT, 2002. Differential cysteine synthase activity and alachlor susceptibility in five crops and six weed species. *Pesticide Biochemistry and Physiology* **72**: 169-177.

Hirase K and Molin WT, 2001. Effect of flurazole and other safeners for chloroacetanilide herbicides on cysteine synthase in sorghum shoots. *Pesticide Biochemistry and Physiology* **71:** 116-123.

Hogan LH and Klein BS, 1997. Transforming DNA integrates at multiple sites in the dimorphic fungal pathogen *Blastomyces dermatitidis. Gene* **186:** 219-226.

Hon DNS, 1996. Chitin and chitosan: Medical applications. In: Polysaccharides in Medicinal Application, Dumitri S eds., New York: Marcel Dekker, pp. 631–649.

Hooykaas PJJ and Beijersbergen AGM, 1994. The virulence system of *Agrobacterium tumefaciens. Annual Review of Phytopathology* **32**: 157-179.

Horvath A, Gonzalez E, Farias G, Cabrera R, Parraguez O and Vial E, 1983. Source of the colors produced in tissue-slices with the MBTH reaction color sources. *Acta Anatomica* **115:** 238-243.

Hsiao YM and Ko JL, 2001. Determination of destruxins, cyclic peptide toxins, produced by different strains of *Metarhizium anisopliae* and their mutants induced by ethyl methane sulfonate and ultraviolet using HPLC method. *Toxicon* **39**: 837-841.

Ichihara A, Tazaki H and Sakamura S, 1983. Solanapyrones A, B and C, phytotoxic metabolites from the fungus *Alternaria solani. Tetrahedron Letters* **24**: 5299-5432.

Idnurm A and Howlett BJ, 2001. Characterization of an opsin gene from the ascomycete *Leptosphaeria maculans. Genome* **44**: 167-171.

Ilarslan H and Dolar FS, 2002. Histological and ultrastructural changes in leaves and stems of resistant and susceptible chickpea cultivars to *Ascochyta rabiei. Journal of Phytopathology* **150**: 340-348.

Inglis PW, Aragao H, Frazao H, Magalhaes BP and Valadares-Inglis MC, 2000. Biolistic co-transformation of *Metarhizium anisopliae* var. *acridum* strain CG423 with green fluorescent protein and resistance to glufosinate ammonium. *FEMS Microbiology Letters* **191:** 249-254.

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T and Kumashiro T, 1996. High efficiency transformation of maize (*Zea mays* L) mediated by *Agrobacterium tumefaciens. Nature Biotechnology* **14**: 745-750.

Ito K, Tanaka T, Hatta R, Yamamoto M, Akimitsu K and Tsuge T, 2004. Dissection of the host range of the fungal plant pathogen Alternaria alternata by modification of secondary metabolism. *Molecular Microbiology* **52:** 399-411.

Itoh Y and Scott B, 1997. Effect of de-phosphorylation of linearized pAN7-1 and of addition of restriction enzyme on plasmid integration in *Penicillium paxilli. Current Genetics* **32**: 147-151.

Jain SM, Okerblom C, Pehu E and Newton RJ, 1992. Genetic engineering - an additional tool for plant improvement. *Agricultural Science in Finland* **1:** 323-338.

James RR, 2001. Effects of exogenous nutrients on conidial germination and virulence against the silverleaf whitefly for two hyphomycetes. *Journal of Invertebrate Pathology* **77:** 99-107.

Jaques U, Kessmann H and Barz W, 1987. Accumulation of phenolic compounds and phytoalexins in sliced and elicitor-treated cotyledons of *Cicer arietinum* L. *Journal of Biosciences* **42**: 1171-1178.

Jen GC and Chilton MD, 1986. The right border region of PTIT37 T-DNA is intrinsically more active than the left border region in promoting T-DNA transformation. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 3895-3899.

Jepson I, Lay VJ, Holt DC, Bright SW and Greenland AJ, 1994. Cloning and characterization of maize herbicide safener-induced cDNAs encoding subunits of glutathione S-transferase isoforms I, II and IV. *Plant Molecular Biology* **26:** 1855-1866.

Jhorar OP, Butler DR and Mathauda SS, 1998. Effects of leaf wetness duration, relative humidity, light and dark on infection and sporulation by *Didymella rabiei* on chickpea. *Plant Pathology* **47**: 586-594.

Jiménez-Díaz RM, Navas-Cortés JA and Trapero-Casas, 1987. Ocurrence of *Mycosphaerella rabiei*, the teleomorph of *Ascochyta rabiei* in Andalucia. Proceedings of the 7th Congress of the Mediterranean Phytopathology Union, Granada, Spain, 124-125.

Johansen C, Krishnamurthy L, Saxena NP and Sethi SC, 1994. Genotypic variation in moisture response of chickpea grown under line-source sprinklers in a semiarid tropical environment. *Fields Crops Research* **37**: 103-112.

Johnson RD, Johnson L, Itoh Y, Kodama M, Otani H and Kahmoto K, 2000. Cloning and characterization of a cyclic peptide synthetase gene from *Alternaria alternata* apple pathotype whose product is involved in AM-toxin synthesis and pathogenicity. *Molecular Plant-Microbe Interactions* **13:** 742-753.

Jones E, Carpenter M, Fong D, Goldstein A, Thrush A, Crowhurst R and Stewart A, 1999. Co-transformation of the sclerotical mycoparasite *Conothyrium minitans* with hygromycin B resistance and β-glucuronidase markers. *Mycological Research* **103**: 929-937.

Jung K, Ovechkina Y, Prigozhina N, Oakley CE and Oakley BR, 2000. The use of beta-D-glucanase as a substitute for Novozym 234 in immunofluorescence and protoplasting. *Fungal Genetics Newsletter* **47**: 65-66.

Kahmann R and Basse C, 1999. REMI (Restriction Enzyme Mediated Integration) and its impact on the isolation of pathogenicity genes in fungi attacking plants. *European Journal of Plant Pathology* **105:** 221-229.

Kaiser WJ and Hannan RM 1987. First report of *Mycosphaerella rabiei* on chickpeas in the Western Emisphere. *Plant Disease* **71**: 192.

Kaiser WJ and Okhovat M, 1996. Distribution of *Didymella rabiei*, the teleomorph of *Ascochyta rabiei*, in Iran. *Iranian Journal of Plant Pathology* **32**: 158-163.

Kaiser WJ, 1972. Occurrence of three fungal diseases of chickpea (*Cicer arietinum*) in Iran. *Food and Agriculture Organisation Plant Protection Bulletin* **20**: 73-78.

Kaiser WJ, 1987. *Testing and production of healthy plant germplasm.* Technical Bulltin No. 2 (Copenhagen, Denmark: Danish Government Institute of Seed Pathology for Developing Countries), 30 pp.

Kaiser WJ, 1992. Epidemiology of *Ascochyta rabiei.* In: Singh KB, Saxena MC, eds. *Disease resistance breeding in kabuli chickpea.* Aleppo, Syria: ICARDA, 117-143.

Kaiser WJ, 1997. Inter- and intranational spread of Ascochyta pathogens of chickpea, faba bean and lentil. *Canadian Journal of Plant Pathology* **19**: 215-224.

Khan MSA, Ramsey MD and Scott ES, 1999. Host range studies with an Australian isolate of *Ascochyta rabiei. Australian Plant Pathology* **28**: 170-173.

Kaminskyj SGW and Heath MC, 1982. An evalution of the nitrous-acid-3-methyl-2-bnzothiazolinone hdrazone hdrochloride ferric-chloride assay for chitin in rust fungi and rust-infected tissue. *Canadian Journal of Botany-Revue Canadienne de Botanique* **60:** 2575-2580.

Kaminskyj SGW and Heath MC, 1983. Histological responses of infection structures and intercellular mycelium of *Uromyces Phaseoli* var. *typica* and *Uromyces Phaseoli* var. *vignae* to the Hno2-MBTH-$FeCl_3$ and the Iki-H_2SO_4 tests. *Physiological Plant Pathology* **22:** 173-179.

Katayama K, Kobayashi T, Oikawa H, Honma M and Ichhara A, 1998. Enzymatic activity and partial purification of solanapyrone synthase: first enzyme catalysing Diels-Alder reaction. *Biochimica et Biophysica Acta* **1384**: 387-395.

Kemp A, Parker J and Grierson C, 2001. Biolistic transformation of *Arabidopsis* root hairs: a novel technique to facilitate map-based cloning. *Plant Journal* **27**: 367-371.

Keszenman-Pereyra D, Lawrence S, Twfieg ME, Price J and Turner G, 2003. The *npgA/cfwA* gene encodes a putative 4 '-phosphopantetheinyl transferase which is essential for penicillin biosynthesis in *Aspergillus nidulans*. *Current Genetics* **43:** 186-190.

Kim BK, Kang JH, Jin M, Kim HW, Shim MJ and Choi EC, 2000. Mycelial protoplast isolation and regeneration of *Lentinus lepideus*. *Life Sciences* **66:** 1359-1367.

Kim C and Kim BK, 1997. Liquid culture enhances protoplast formation from the auxotroph (Ser) of *Lentinula edodes*. *Archives of Pharmacal Research* **20:** 206-211.

Kim YK, Wang YH, Liu ZM and Kolattukudy PE, 2002. Identification of a hard surface contact-induced gene in *Colletotrichum gloeosporioides* conidia as a sterol glycosyl transferase, a novel fungal virulence factor. *Plant Journal* **30**: 177-187.

Kodama M, Akamatsu H, Itoh Y, Narusaka Y, Sanekata T, Otani H and Kohmoto K, 1998. Host-specific toxin deficient mutants of the tomato pathotype of *Alternaria alternata* obtained by restriction enzyme-mediated transformation. *Molecular Genetics of Host-Specific Toxins in Plant Disease*, Kohmoto K and Yoder OC (eds): 35-42.

Köhler G, Linkert C and Barz W, 1995. Infection studies of *Cicer arietinum* (L) with GUS-(*E-coli* beta- glucuronidase) transformed *Ascochyta rabiei* strains. *Journal of Phytopathology-Phytopathologische Zeitschrift* **143:** 589-595.

Kolattukudy PE, Kamper JU and Kamper W, 1994. Fungus-induced degradation and reinforcement of defensive barriers of plants. In: Petrini O, Quellette (eds.), Host wall alterations by parasitic fungi, pp. 67-80.APS Press, The American Phytopathological Society, St. Paul, Minnesota, USA.

Koncz C, Nemeth K, Redei GP and Schell J, 1992. T-DNA insertional mutagenesis in *Arabidopsis*. *Plant Molecular Biology* **20**: 963-976.

Koukaki M, Giannoutsou E, Karagouni A and Diallinas G, 2003. A novel improved method for *Aspergillus nidulans* transformation. *Journal of Microbiological Methods* **55:** 687-695.

Kovachevski IC, 1936. The blight of chickpea (*Cicer arietinum*), *Mycosphaerella rabiei* sp. Ministry of Agriculture and National Domains, Plant Protection Institute, Sofia, Bulgaria.

Kövics G, Holly L and Simay EI, 1986. An ascochytosis of the chickpea (*Cicer arietinum* L.) caused by *Didymella rabiei* (Kov.) v. Arx: Imperfect: *Ascochyta rabiei* (Pass.) in Hungary. *Acta Phytopathologica et Entomologica Hungarica* **21**: 147-150.

Kunik T, Tzfira T, Kapulnik Y, Gafni Y, Dingwall C and Citovsky V, 2001. Genetic transformation of HeLa cells by *Agrobacterium*. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 1871-1876.

Kuspa A and Loomis WF, 1992. Tagging developmental genes in *Dictyostelium* by

restriction enzyme-mediated integration of plasmid DNA. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 8803-8807.

Lalithakumari D, 2000. Fungal protoplast – a biotechnological tool. Science Publishers, Inc.

Lalithakumari D, 1996. Protoplasts - A biotechnological tool for plant pathological studies, 1996. *Indian Phytopathology* **49**: 199-212.

Lamoureux GL, Rusness DG, Schroder P and Rennenberg H, 1991. Diphenylether herbicide metabolism in spruce cell suspension culture - the identification of 2 novel metabolites. *Abstracts of Papers of the American Chemical Society* **201:** 53-AGRO.

Lapidot M, Raveh D, Sivan A, Arad S and Shapira M, 2002. Stable chloroplast transformation of the unicellular red alga *Porphyridium* species. *Plant Physiology* **129:** 7-12.

Laranjo M, Branco C, Soares R, Alho L, Carvalho MDE and Oliveira S, 2002. Comparison of chickpea R*hizobia* isolates from diverse Portuguese natural populations based on symbiotic effectiveness and DNA fingerprint. *Journal of Applied Microbiology* **92**: 1043-1050.

Latif Z, Strange RN, Bilton J and Riazuddin S, 1993. Production of the phytotoxins, solanapyrones A and C and cytochalasin D among nine isolates of *Ascochyta rabiei. Plant Pathology* **42**: 172-180.

Lee SD, Park SW, Oh KK, Hong SI and Kim SW, 2002. Improvement for the production of clavulanic acid by mutant *Streptomyces clavuligerus. Letters in Applied Microbiology* **34**: 370-375.

Leite B and Nicholson RL, 1992. Mycosporine alanine – a selfinhibitor of germination from the conidial mucilage of *Colletotrichum graminicola. Experimental Mycology* **16:** 76-86.

Lejeune R and Baron GV, 1995. Effect of agitation on growth and enzyme

production of *Trichoderma reesei* in batch fermentation. *Applied Microbiology and Biotechnology* **43:** 249-258.

Lima JO, dos Santos JK, Pereira JF, de Resende MLV, de Araújo EF and de Queiroz MV, 2003. Development of a transformation system for *Crinipellis pernisiosa*, the causal agent of witches' broom in cocoa plants. *Current Genetics* 42:236-240.

Linnemannstöns P, Voss T, Hedden P, Gaskin P and Tudzynski B, 1999. Deletions in the gibberellin biosynthesis gene cluster of *Gibberella fujikuroi* by restriction enzyme-mediated integration and conventional transformation-mediated mutagenesis. *Applied and Environmental Microbiology* **65**: 2558-2564.

Liu BL, Kao PM, Tzeng YM and Feng KC, 2003. Production of chitinase from *Verticillium lecanii* F091 using submerged fermentation. *Enzyme and Microbial Technology* **33**: 410-415.

Lorito M, Hayes CK, Dipietro A and Harman GE, 1993. Biolistic transformation of *Trichoderma harzianum* and *Gliocladium virens* using plasmid and genomic DNA. *Current Genetics* **24:** 349-356.

Lu SW, Lyngholm L, Yang G, Bronson C, Yoder OC and Turgeon BG, 1994. Tagged mutations at the Tox1 locus of *Cochliobolus heterostrophus* by Restriction Enzyme-Mediated Integration. *Proceedings of the National Academy of Sciences of the United States of America* **91:** 12649-12653.

Lurquin PF, 1997. Gene transfer by electroporation. *Molecular Biotechnology* **7:** 5-35.

Lutz MP, Feichtinger G, Défago G and Duffy B, 2003. Mycotoxigenic *Fusarium* and deoxynivalenol production repress chitinase gene expression in the biocontrol agent *Trichoderma atroviride* P1. *Applied and Environmental Microbiology* **69**: 3077-3084.

Mahoney N, Lardnen R, Molyneux RJ, Scott ES, Smith LR and Schoch TK, 2003. Phenolic and heterocyclic metabolite profiles of the grapevine pathogen *Eutypa lata*. *Phytochemistry* **64**: 475-484.

Maier FJ and Schafer W, 1999. Mutagenesis via insertional or restriction enzyme-mediated integration (REMI) as a tool to tag pathogenicity related genes in plant pathogenic fungi. *Biological Chemistry* **380:** 855-864.

Maimala S, Tartar A, Boucias D and Chandrapatya A, 2002. Detection of the toxin hirsutellin A from *Hirsutella thompsonii. Journal of Invertebrate Pathology* **80:** 112-126.

Makkouk KM, Hamed AA, Hussein M and Kumari SG, 2003a. First report of faba bean necrotic yellows virus (FBNYV) infecting chickpea (*Cicer arietinum*) and faba bean (*Vicia faba*) crops in Sudan. *Plant Pathology* **52**: 412.

Makkouk KM, Rizkallah L, Kumari SG, Zaki M and Abul Enein R, 2003b. First record of chickpea chlorotic dwarf virus (CpCDV) affecting faba bean (*Vicia faba*) crops in Egypt. *Plant Pathology* **52**: 413.

Malhotra, R.S., R.P.S. Pundir and A.E. Slinkard. 1987. Genetic resources of chickpea. In: Saxena MC and Singh KB (ed.), *The Chickpea.* C.A.B. International Cambrian News Ltd, Aberystwyth, UK, p.67-81.

Malonek S and Meinhardt F, 2001. *Agrobacterium tumefaciens*-mediated genetic transformation of the phytopathogenic ascomycete *Calonectria morganii. Current Genetics* **40:** 152-155.

Maragos CM and Plattner RD, 2002. Rapid fluorescence polarization immunoassay for the mycotoxin deoxynivalenol in wheat. *Journal of Agricultural and Food Chemistry* **50:** 1827-1832.

Marrs KA, 1996. The functions and regulation of glutathione S-transferases in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**: 127-158.

Martínez-Cadena G, Saavedra-Calixto J, Messina-Valencia G, Domínguez-Gutiérrez G and Novoa- Martínez G, 1995. Effect of carbon source and pH of the growth medium on spore germination in Phycomyces blakesleeanus, 1995. *Archives of Microbiology* **164**:231-234.

Mayerhofer R, Koncz-Kalman Z, Nawrath C, Bakkeren G, Crameri A, Angelis K, Redei GP, Schell J, Hohn B, and Koncz C, 1991. T-DNA integration: a mode of illegitimate recombination in plants. *EMBO Journal* **10**: 697–704.

McQuilken MP, Budge SP and Whipps JM, 1997. Effects of culture media and environmental factors on conidial germination, pycnidial production and hyphal extension of *Coniothyrium minitans*. *Mycological Research* **101:** 11-17.

Meyer V, Mueller D, Strowig T and Stahl U, 2003. Comparison of different transformation methods for *Aspergillus giganteus*. *Current Genetics* **43:** 371-377.

Mitchell TK, Chilton WS and Daub ME, 2002. Biodegradation of the polyketide toxin cercosporin. *Applied and Environmental Microbiology* **68:** 4173-4181.

Miyauchi N, Kobayashi K and Usui K, 2002. Differential safening activity of dymron and fenclorim on pretilachlor injury in rice seedlings in soil. *Weed Biology and Management* **2**: 46-51.

Mizushina Y, Kamisuki S, Kasai N, Shimazaki N, Takemura M, Asahara H, Linn S, Yoshida S, Matsukage A, Koiwai O, Sugawara F, Yoshida H and Sakaguchi K, 2002. A plant phytotoxin, solanapyrone A, is an inhibitor of DNA polymerase beta and lambda. *Journal of Biological Chemistry* **277:** 630-638.

Morozova EV, Baranova MV, Kozlov VP, Tereshina VM, Memorskaya AS and Feofilova EP, 2001. Peculiarities of exogenous dormancy of *Aspergillus niger* conidia. *Microbiology* **70:** 527-534.

Morrall RAA and Mckenzie DL, 1974. A note on the inadvertent introduction to North America of *Ascochyta rabiei*, a destructive pathogen of chickpea. *Plant Disease Reporter* **58**: 342-345.

Mouhouche F and Fleurat-Lessard F, 2003. Susceptibility of several cultivars of cowpea seeds (*Cicer arietinum*) to the infestation either by the specialised pest *Callosobruchus maculatus* (F.) (Coleoptera; Bruchidae) or an unspecialised pest *Sitophilus oryzae* (Coleopterae; Curculionidae). *Sciences des Aliments* **23**: 633-653.

Mullins ED, Chen X, Romaine P, Raina R, Geiser DM and Kang S, 2001. *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. *Phytopathology* **91:** 173-180.

Muralidhar R, Gummadi SN, Dasu VV and Panda T, 2003. Statistical analysis on some critical parameters affecting the formation of protoplasts from the mycelium of *Penicillium griseofulvum*. *Biochemical Engineering Journal* **16:** 229-235.

Navas-Cortés JA, Trapero-Casas A and Jiménez-Díaz RM, 1995. Survival of *Didymella rabiei* in chickpea straw debris in Spain. *Plant Pathology* **44**: 332-339.

Navas-Cortés JA, Trapero-Casas A, Jiménez-Díaz RM, 1998. Influence of relative humidity and temperature on development of *Didymella rabiei* on chickpea debris. *Plant Pathology* **47**: 57-66.

Nehra KS, Chugh LK, Dhillon S and Singh R, 1994. Induction, purification and characterization of chitinases from chickpea (*Cicer arietinum* L.) leaves and pods infected with *Ascochyta rabiei*. *Journal of Plant Physiology* **144**: 7-11.

Nene YL, 1982. A review of Ascochyta blight of chickpea. *Tropical Pest Management* **28**: 61-70.

Nene YL, 1996. Sustainable agriculture: future hope for developing countries. *Canadian Journal of Plant Pathology* **18**: 133-140.

Nene YL and Reddy MV, 1987. Chickpea diseases and their control. In: Saxena MC, Singh KB eds. *The chickpea*. Oxford: CAB International.

Nene YL, Sheila VK and Sharma SB 1996. A world list of chickpea and pigeonpea pathogens. ICRISAT Pulse Pathology Progress Report.

Neuefeind T, Reinemer P and Bieseler B, 1997. Plant glutathione S-transferases and herbicide detoxification. *Biological Chemistry* **378**: 199-205.

Nielsen KF and Thrane U, 2001. Fast methods for screening of trichothecenes in fungal cultures using gas chromatography-tandem mass spectrometry. *Journal of*

Chromatography A **929:** 75-87.

Nwe N, Chandrkrachang S, Stevens WF, Maw T, Tan TK, Khor E and Wong SM, 2001. Production of fungal chitosan by solid state and submerged fermentation. *Carbohydrate Polymers*, Press.

O'Connell KM, Breaux EJ and Fraley RT, 1988. Different rates of metabolism of two chloroacetanilide herbicides in pioneer 3320 corn. *Plant Physiology* **86**: 359.

O'Donnell D, Wang LP, Xu JF, Ridgway D, Gu TY and Moo-Young M, 2001. Enhanced heterologous protein production in *Aspergillus niger* through pH control of extracellular protease activity. *Biochemical Engineering Journal* **8:** 187-193.

O'Hagan D, 1995. Biosynthesis of fatty acids and polyketide metabolites. *Natural Product Reports* **12**: 1-32.

Oikawa H, Katayama K, Suzuki Y and Ichihara A, 1995. Enzymatic activity catalyzing exo selective Diels-Alder reaction in solanapyrone biosynthesis. *Journal of the Chemical Society* **13**: 1321-1322.

Oikawa H, Kobayashi T, Katayama K, Suzuki Y and Ichihara A, 1998. Total synthesis of (-) solanapyrone A via enzymatic Diels-Alder reaction of prosolanapyrone. *Journal of Organic Chemistry* **63**: 8748-8756.

Osbourn AE, 2001. Tox-boxes, fungal secondary metabolites, and plant disease. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 14187-14188.

Ose T, Watanabe K, Mie T, Honma M, Watanabe H, Yao M, Oikawa H and Tanaka I, 2003. Insight into a natural Diels-Alder reaction from the structure of macrophomate synthase. *Nature* **422:** 185-189.

Ozeki K, Kyoya F, Hizume K, Kanda A, Hamachi M and Nunokawa Y, 1994. Transformation of intact *Aspergillus niger* by electroporation. *Bioscience Biotechnology and Biochemistry* **58:** 2224-2227.

Pandey B K, Singh US and Chaube HS, 1987. Mode of infection of Ascochyta blight as caused by *Ascochyta rabiei*. *Journal of Phytopathology* **119:** 88–93.

Pandey R, Sharma-Natu P and Ghildiyal MC, 2001. Irrigation decreases loss of leaf nitrogen and Rubisco during pod development in chickpea. *Journal of Agronomy and Crop Science* **187:** 177-183.

Papagianni M, 2004. Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances* **22:** 189-259.

Pardo AG, Hanif M, Raudaskoski M and Gorfer M, 2002. Genetic transformation of ectomycorrhizal fungi mediated by *Agrobacterium tumefaciens*. *Mycological Research* **106:** 132-137.

Park JP, Kim YM, Kim SW, Hwang HJ, Cho YJ, Lee YS, Song CH and Yun JW, 2002. Effect of agitation intensity on the exo-biopolymer production and mycelial morphology in *Cordyceps militaris*. *Letters in Applied Microbiology* **34**: 433-438.

Payne AC, Grosjean-Cournoyer MC and Hollomon DW, 1998. Transformation of the phytopathogen Mycosphaerella graminicola to carbendazim and hygromycin B resistance. *Current Genetics* **34:** 100-104.

Peberdy, J.F., 1991. Fungal protoplasts. In: More gene manipulations in fungi (Bennett J.W. and Lasure L.L., eds.), pp. 307-318. Academic Press, California.

Peever TL, Salimath SS, Su G, Kaiser WJ and Muehlbauer FJ, 2004. Historical and contemporary multilocus population structure of *Ascochyta rabiei* (teleomorph : *Didymella rabiei*) in the Pacific Northwest of the United States. *Molecular Ecology* **13**: 291-309.

Piccoli-Valle RH, Passos RJV, Brandi IV, Peternelli LA and Silva DO, 2003. Influence of different mixing and aeration regimens on pectin lyase production by *Penicillium griseoroseum*. *Process Biochemistry* **38:** 849-854.

Piers KL, Heath JD, Liang XY, Stephens KM and Nester EW, 1996. *Agrobacterium*

tumefaciens-mediated transformation of yeast. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 1613-1618.

Porta-Puglia A, Infantino A, Crino P, Angelini R and Venora G, 1997. Ascochyta blight of chickpea: present status and prospects. *Pakistani Journal of Phytopathology* **9**: 8-15.

Powell WA and Kistler HC, 1990. *In vivo* rearrangement of foreign DNA by *Fusarium oxysporum* produces linear self-replicating plasmids. *Journal of Bacteriology* **172**: 3163-3171.

Proctor RH, Hohn TM and McCormick SP, 1995. Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. *Molecular Plant-Microbe Interactions* **8**: 593-601.

Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Hondel CAMJJ, 1987. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli. Gene* **56**: 117-124.

Rea G, Metoui O, Infantino A, Federico R, and Angelini R, 2002. Copper amine oxidase expression in defense responses to wounding and *Ascochyta rabiei* invasion. *Plant Physiology* **128:** 865–875.

Rea PA, Li ZS, Lu YP, Drozdowicz YM and Martinoia E, 1998. From vacuolar GS-X pumps to multispecific ABC transporters. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**:727-760.

Redman RS and Rodriguez RJ, 1994. Factors affecting the efficient transformation of *Colletotrichum* species. *Experimental Mycology* **18:** 230-246.

Redman RS, Ranson JC and Rodriguez RJ, 1999. Conversion of the pathogenic fungus *Colletotrichum magna* to a nonpathogenic, endophytic mutualist by gene disruption. *Molecular Plant-Microbe Interactions* **12:** 969-975.

Rennenberg H, Birk C and Schaer B, 1982. Effect of N,N-Diallyl-2,2-Dichloroacetamide (R-25788) on Efflux and Synthesis of Glutathione in Tobacco

Suspension-Cultures. *Phytochemistry* **21:** 5-8.

Reyes RG, Eguchi F, Iijima T and Higaki M, 1998. Regeneration of protoplasts from hyphal strands of *Volvariella volvacea*. *Journal of Wood Science* **44:** 401-407.

Rho HS, Kang S and Lee YH, 2001. *Agrobacterium tumefaciens*-mediated transformation of the plant pathogenic fungus, *Magnaporthe grisea*. *Molecules and Cells* **12:** 407-411.

Ride JP and Drysdale RB, 1972. A rapid method for the chemical estimation of filamentous fungi in plant tissue. *Physiological Plant Pathology* **2**: 7-15.

Riggle PJ and Kumamoto CA, 1998. Genetic analysis in fungi using restriction-enzyme-mediated integration. *Current Opinion in Microbiology* **1:** 395-399.

Riscaldati E, Moresi M, Federici F and Petruccioli M, 2000. Effect of pH and stirring rate on itaconate production by *Aspergillus terreus*. *Journal of Biotechnology* **83:** 219-230.

Robinson HL and Deacon JW, 2001. Protoplast preparation and transient transformation of *Rhizoctonia solani*. *Mycological Research* **105:** 1295-1303.

Robinson M and Sharon A, 1999. Transformation of the bioherbicide *Colletotrichum gloeosporioides f. sp aeschynomene* by electroporation of germinated conidia. *Current Genetics* **36:** 98-104.

Rohe M, Searle J, Newton AC and Knogge W, 1996. Transformation of the plant pathogenic fungus, *Rhynchosporium secalis*. *Current Genetics* **29:** 587-590.

Rose MS, Yun SH, Asvarak T, Lu SW, Yoder OC and Turgeon BG, 2002. A decarboxylase encoded at the *Cochliobolus heterostrophus* translocation-associated Tox1B locus is required for polyketide (T-toxin) biosynthesis and high virulence on T-cytoplasm maize. *Molecular Plant Microbe Interactions* **15**: 883-893.

Rolland S, Jobic C, Fevre M and Bruel C, 2003. *Agrobacterium*-mediated transformation of *Botrytis cinerea*, simple purification of monokaryotic

transformants and rapid conidia-based identification of the transfer-DNA host genomic DNA flanking sequences. *Current Genetics* **44:** 164-171.

Roncero C, 2002. The genetic complexity of chitin synthesis in fungi. *Current Genetics* **41:** 367-378.

Rossi L, Hohn B and Tinland B, 1996. Integration of complete T-DNA units is dependant on the activity of VirE2 protein of *Agrobacterium tumefaciens. Proceedings of the National Academy of Sciences of the United States of America* **93**: 126-130.

Ruiz-Herrera J, Sentandreu RR and Martínez JP, 1992. Chitin biosynthesis in fungi. In: Handbook of Applied Mycology, Vol. 4., Fungal Biotechnology, Dilip KA, Richard PE and Mukerji KG eds., pp. 281–312, New York: Marcel Dekker.

Sambrook J, and Russell DW, 2001. *Molecular cloning- A laboratory manual,* 3rd ed. Cold Spring Harbour Laboratory Press, New York.

Sánchez O and Aguirre J, 1996. Efficient transformation of *Aspergillus nidulans* by electroporation of germinated conidia. *Fungal Genetics Newsletter* **43:** 48-51.

Sánchez O, Navarro RE and Aguirre J, 1998. Increased transformation frequency and tagging of developmental genes in *Aspergillus nidulans* by restriction enzyme-mediated integration (REMI). *Molecular and General Genetics* **258:** 89-94.

Sandhu SS, Unkles SE, Rajak RC and Kinghorn JR, 2001. Generation of benomyl resistant *Beauveria bassiana* strains and their infectivity against *Helicoverpa armigera. Biocontrol Science and Technology* **11:** 245-250.

Santra DK, Tekeoglu M, Ratnaparkhe M, Kaiser WJ and Muehlbauer FJ, 2000. Identification and mapping of QTLs conferring resistance to Ascochyta blight in chickpea . *Crop Science* **40**: 1606-1612.

Sasaki A, Onoue M, Kanematsu S, Suzaki K, Miyanishi M, Suzuki N, Nuss DL and Yoshida K, 2002. Extending chestnut blight hypovirus host range within diaporthales by biolistic delivery of viral cDNA. *Molecular Plant-Microbe*

Interactions **15:** 780-789.

Sato T, Yaegashi K, Ishii S, Hirano T, Kajiwara S, Shishido K and Enei H, 1998. Transformation of the edible basidiomycete *Lentinus edodes* by restriction enzyme-mediated integration of plasmid DNA. *Biosciences Biotechnology and Biochemistry* **62**: 2346-2350.

Sauer JD, 1993. Historical geography of crop plants - a select roster. CRC Press, Boca Raton, Florida.

Schaffrath U, Mauch F, Freydl E, Schweizer P and Dudler R, 2000. Constitutive expression of the defense-related *Rir1b* gene in transgenic rice plants confers enhanced resistance to the rice blast fungus *Magnaporthe grisea. Plant Molecular Biology* **43:** 59-66.

Schiestl RH and Petes TD, 1991. Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America* **88**: 7585-7589.

Shaw BD and Hoch HC, 2000. Ca^{2+} regulation of *Phyllosticta ampelicida* pycnidiospore germination and appressorium formation. *Fungal Genetics and Biology* **31:** 43-53.

Sheng JS and Citovsky V, 1996. *Agrobacterium* plant cell DNA transport: have virulence proteins, will travel. *Plant Cell* **8**: 1699-1710.

Shi Z and Leung H, 1994. Genetic analysis and rapid mapping of a sporulation mutation in *Magnaporthe grisea. Molecular Plant-Microbe Interactions* **7**: 113-120.

Shi Z, Christian D and Leung H, 1995. Enhanced transformation in *Magnaporthe grisea* by restriction enzyme-mediated integration of plasmid DNA. *Phytopathology* **85**: 329-333.

Shi ZX and Leung H, 1995. Genetic analysis of sporulation in *Magnaporthe grisea* by chemical and insertional mutagenesis. *Molecular Plant-Microbe Interactions*

8: 949-959.

Shirasu K, Koukolikovanicola Z, Hohn B and Kado CI, 1994. An innner membrane associated virulence protein essential for T-DNA transfer from *Agrobacterium tumefaciens* to plants exhibits ATPase activity and similarities to conjugative transfer genes. *Molecular Microbiology* **11**: 581-588.

Shirasu K, Nielsen K, Piffanelli P, Oliver R and Schulze-Lefert P, 1999. Cell-autonomous complementation of mlo resistance using a biolistic transient expression system. *Plant Journal* **17:** 293-299.

Shtienberg D, Vintal H, Brener S and Retig B, 2000. Rational management of *Didymella rabiei* in chickpea by integration of genotype resistance and postinfection application of fungicides. *Phytopathology* **90**: 834-842.

Shuster JR and Connelley MB, 1999. Promoter-tagged restriction enzyme-mediated insertion (PT-REMI) mutagenesis in *Aspergillus niger. Molecular and General Genetics* **262:** 27-34.

Siddique KHM and Khan TN, 1993. Adaptation and yield improvement of chickpea of Western Australia. In: *Proceedings of chickpea workshop.* Tamworth, New South Wales, Australia, 22-30.

Singh KB and Reddy MV, 1990. Patterns of resistance and susceptibility to races of *Ascochyta rabiei* among germplasm accessions and breeding lines of chickpea. *Plant Disease* **74**: 127-129.

Singh KB, Malhotra RS, Halila MH, Knights EJ and Verma MM, 1994. Current status and future strategy in breeding chickpea for resistance to biotic and abiotic stresses. *Euphytica* **73**: 137-149.

Singh KB, Malhotra RS, Saxena MC and Bejiga G, 1997. Superiority of winter sowing over traditional spring sowing of chickpea in the Mediterranean region. *Agronomy Journal* **89**: 112-118.

Singh KB, Ocampo B and Robertson LD, 1998. Diversity for abiotic and biotic stress

resistance in the wild annual Cicer species. *Genetic Resources and Crop Evolution* **45**: 9-17.

Singh KK and Rathi KS, 2003. Dry matter production and productivity as influenced by staggered sowing of mustard intercropped at different row ratios with chickpea. *Journal of Agronomy and Crop Science* **189**: 169-175.

Sinha J, Bae JT, Park JP, Kim KH, Song CH and Yun JW, 2001. Changes in morphology of *Paecilomyces japonica* and their effect on broth rheology during production of exo-biopolymers. *Applied Microbiology and Biotechnology* **56:** 88-92.

Skowronek M and Fiedurek J, 2003. Selection of biochemical mutants of *Aspergillus niger* resistant to some abiotic stresses with increased inulinase production. *Journal of Applied Microbiology* **95**: 686-692.

Smith JE, 1994. In: *Aspergillus*, Plenum, New York and London, Biotechnology Handbooks vol. 7, pp. 41-76.

Solis S, Flores ME and Huitron C, 1996. Protoplasts from pectinolytic fungi: Isolation, regeneration and pectinolytic enzyme production. *Letters in Applied Microbiology* **23:** 36-42.

Stachel SE and Zambryski PC, 1986. *Agrobacterium tumefaciens* and the susceptible plant cell – a novel adaptation of extracellular recognitionand DNA conjugation. *Cell* **47**: 155-157.

Stachel SE, Timmerman B and Zambryski PC, 1986. Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. *Nature* **322**: 706-712.

Stamigna C, Crino P and Saccarodo F, 2000. Wild relatives of chickpea: multiple disease resistance and problems to introgression in the cultigen. *Journal of Genetics and Breeding* **54**: 213-219.

St Leger RJ, Shimizu S, Joshi L, Bidochka MJ and Roberts DW, 1995. Cotransformation of *Metarhizium anisopliae* by electroporation or using the

gene gun to produce stable GUS transformants. *FEMS Microbiology Letters* **131:** 289-294.

Strange RN, 2003. Introduction to plant pathology, 464 pp, Wiley, Chichester.

Strange RN, 1997. Phytotoxins associated with *Ascochyta* species. In: *Toxins in plant disease development and evolving biotechnology,* Upadhyay RK and Mukerji KG eds., pp. 167-181.

Suga M and Hatakeyama T, 2001. High efficiency transformation of *Schizosaccharomyces pombe* pretreated with thiol compounds by electroporation. *Yeast* **18:** 1015-1021.

Sullivan TD, Rooney PJ and Klein BS, 2002. *Agrobacterium tumefaciens* integrates transfer DNA into single chromosomal sites of dimorphic fungi and yields homokaryotic progeny from multinucleate yeast. *Eukaryotic Cell* **1:** 895-905.

Sundberg C, Meek L, Carroll K, Das A and Ream W, 1996. VirE1 protein mediates export of the single-stranded DNA-binding protein VirE2 from *Agrobacterium tumefaciens* into plant. *Journal of Bacteriology* **178**: 1207-1212.

Sweigard J, 1996. A REMI primer for filamentous fungi. *IS-Molecular Plant-Microbe Interactions Reporter*, pp. 3-5.

Sweigard JA, Carroll AM, Farrall L, Chumley FG and Valent B, 1998. *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. *Molecular Plant-Microbe Interactions* **11**: 404-412.

Tal JA, Hall JC and Stephenson GR, 1995. Nonenzymatic conjugation of fenoxaprop-ethyl with glutathione and cystein in several grass species. *Wed Research* **35**: 133-139.

Tanaka A, Shiotani H, Yamamoto M and Tsuge T, 1999. Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-specific AK-toxin in the japanese pear pathotype of *Alternaria alternata. Molecular Plant-Microbe Interactions* **12:** 691-702.

Tanguay P, Coupal J and Bernier L, 2003. Genetic transformation, electrophoretic karyotyping and isolation of insertional mutants in the tree pathogenic fungus *Neonectria galligena*. *Forest Pathology* **33:** 413-428.

Tenhaken R, Salmen HC and Barz W, 1991. Purification and characterization of pterocarpan hydroxylase, a flavoprotein monooxygenase from the fungus *Ascochyta rabiei* involved in pterocarpan phytoalexin metabolism. *Archives of Microbiology* **155**: 353-359.

Thompson JR, Register E, Curotto J, Kurtz M and Kelly R, 1998. An improved protocol for the preparation of yeast cells for transformation by electroporation. *Yeast* **14:** 565-571.

Thon MR, Nuckles EM and Vaillancourt LJ, 2000. Restriction enzyme-mediated integration used to produce pathogenicity mutants of *Colletotrichum graminicola*. *Molecular Plant-Microbe Interactions* **13**: 1356-1365.

Tinland B, 1996. The integration of T-DNA into plant genomes. *Trends in Plant Science* **1**: 178-184.

Trapero-Casas A and Kaiser WJ, 1992. Development of *Didymella rabiei*, the teleomorph of *Ascochyta rabiei*, on chickpea straw. *Phytopathology* **82**: 1261-1266.

Trapero-Casas A, Navas-Cortés JA and Jiménez-Díaz RM, 1996. Airborne ascospores of *Didymella rabiei* as a major primary inoculum for Ascochyta blight epidemics in chickpea crops in southern Spain. *European Journal of Plant Pathology* **102**: 237-245.

Tsai HF, Siegel MR and Schardl CL, 1992. Transformation of *Acremonium coenophialum*, a protective fungal symbiont of the grass *Festuca arundinacea*. *Current Genetics* **22**: 399-406.

Tsurushima T, Ueno T, Fukami H, Irie H and Inoue M, 1995. Germination self-inhibitors from *Colletotrichum-Gloeosporioides F sp jussiaea*. *Molecular Plant-Microbe Interactions* **8:** 652-657.

Tzeng TH, Lyngholm LK, Ford CF and Bronson CR, 1992. A Restriction-Fragment-Length-Polymorphism map and electrophoretic karyotype of the fungal maize pathogen *Cochliobolus heterostrophus*. *Genetics* **130:** 81-96.

Tzfira T and Citovsky V, 2002. Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. *Trends in Cell Biology* **12**: 121-129.

Tzfira T, Rhee Y, Chen MH, Kunik T and Citovsky V, 2000. Nucleic acid transport in plant-microbe interactions: the molecules that walk through the walls. *Annual Review of Microbiology* **54**: 187-219.

Viterbo A, Haran S, Friesem D, Ramot O and Chet I, 2001. Antifungal activity of a novel endochitinase gene (chit36) from *Trichoderma harzianum Rifai* TM. *FEMS Microbiology Letters* **200:** 169-174.

Vogelsang R and Barz W, 1993. Purification, characterization and differential hormonal regulation of a beta-1,3-glucanase and two chitinases from chickpea (*Cicer arietinum*). *Planta* **189**: 60-69.

Walton JD and Casida JE, 1995. Specific Binding of A Dichloroacetamide Herbicide Safener in Maize at A Site That Also Binds Thiocarbamate and Chloroacetanilide Herbicides. *Plant Physiology* **109:** 213-219.

Wang HL, Kim SH, Siu H and Breuil C, 1999. Transformation of sapstaining fungi with hygromycin B resistance plasmids pAN7-1 and pCB1004. *Mycological Research* **103:** 77-80.

Wang YC and Mcneil B, 1995. pH effects on exopolysaccharide and oxalic acid production in cultures of *Sclerotium glucanicum*. *Enzyme and Microbial Technology* **17:** 124-130.

Weiland JJ, 2003. Transformation of *Pythium aphanidermatumto* to geneticin resistance. *Current Genetics* **42**: 344-352.

Weltring KM, Schaub HP and Barz W, 1995. Metabolism of pisatin stereoisomers by *Ascochyta rabiei* strains transformed with the pisatin demethylase gene of

Nectria haematococca Mp-Vi. Molecular Plant-Microbe Interactions **8:** 499-505.

Wessels JGH, 1993. Wall Growth, Protein Excretion and Morphogenesis in fungi. *New Phytologist* **123:** 397-413.

Wiebe MG, Novakova M, Miller L, Blakebrough ML, Robson GD, Punt PJ and Trinci APJ, 1997. Protoplast production and transformation of morphological mutants of the Quorn(R) myco-protein fungus, *Fusarium graminearum* A3/5, using the hygromycin B resistance plasmid pAN7-1. *Mycological Research* **101:** 871-877.

Wilce MCJ and Parker MW, 1994. Structure and function of glutathione S-transferases. *Biochimica et Biophysica Acta – Protein Structure and Molecular Enzymology* **1205**: 1-18.

Wilson AD and Kaiser WJ, 1995. Cytology and genetics of sexual incompatibility in *Didymella rabiei*. *Mycologia* **87**: 795-804.

Winans SC, 1992. Two-way chemical signalling in *Agrobacterium*-plant interactions. *Microbiological Reviews* **56**: 12-31.

Winans SC, Mantis NJ, Chen CY, Chang CH and Han DC, 1994. Host recognition by the VirA, VirG, two component regulatory proteins of *Agrobacterium tumefaciens*. *Research in Microbiology* **145**: 461-473.

Woloshuk CP, Foutz KR, Brewer JF, Bhatnagar D, Cleveland TE and Payne GA, 1994. Molecular characterization of Aflr, a regulatory locus for aflatoxin biosynthesis. *Applied and Environmental Microbiology* **60:** 2408-2414.

Wu JR, Hwang IT and Hatzios KK, 2000. Effects of chloroacetanilide herbicides on membrane fatty acid desaturation and lipid composition in rice, maize, and sorghum. *Pesticide Biochemistry and Physiology* **66:** 161-169.

Xu JR and Xue CY, 2002. Time for a blast: genomics of *Magnaporthe grisea*. *Molecular Plant Pathology* **3:** 173-176.

Yakoby N, Zhou R, Kobiler I, Dinoor A and Prusky D, 2001. Development of

Colletotrichum gloeosporioides restriction enzyme-mediated integration mutants as biocontrol agents against anthracnose disease in avocado fruits. *Phytopahtology* **91**: 143-148.

Yang G, Rase MS, Turgeon BG and Yode OC, 1996. A polyketide synthase is required for fungal virulence and production of the polyketide T-toxin. *The Plant Cell* **8**: 2139-2150.

Young C, Itoh Y, Johnson R, Garthwaite I, Miles CO, Munday-Finch SC and Scott B, 1998. Paxilline-negative mutants of *Penicillium paxilli* generated by heterologous and homologous plasmid integration. *Current Genetics* **33:** 368-377.

Young LY, Lorenz MC and Heitman J, 2000. A STE12 homolog is required for mating but dispensable for filamentation in *Candida lusitaniae*. *Genetics* **155:** 17-29.

Yu JJ and Cole GT, 1998. Biolistic transformation of the human pathogenic fungus *Coccidioides immitis*. *Journal of Microbiological Methods* **33:** 129-141.

Yu JJ, Chang PK, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP and Bennett JW, 2004. Clustered pathway genes in aflatoxin biosynthesis. *Applied and Environmental Microbiology* **70**: 1253-1262.

Yun SH, Turgeon BG and Yoder OC, 1998. REMI-induced mutants of *Mycosphaerella zeae-maydis* lacking the polyketide PM-toxin are deficient in pathogenesis to corn. *Physiological and Molecular Plant Pathology* **52**: 53-66.

Zachos DG, Panagopoulos CG and Makris SA, 1963. Recherches sur la biologie et la lutte contre l'anthracnose du pois-chiche. *Annales de l'Institut Phytopathologique Benaki* **5**: 167-192.

Zama P and Hatzios KK, 1986. Effects of CGA-92194 on the chemical reactivity of metolachlor with glutathione and metabolism of metolachlor in grain sorghum (*Sorghum bicolor*). *Weed Science* **34**: 834-841.

Zambryski PC, 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**: 465-490.

Zhang J, Ma WB and Tan HR, 2002. Cloning, expression and characterization of a gene encoding nitroalkane-oxidizing enzyme from *Streptomyces ansochromogenes*. *European Journal of Biochemistry* **269:** 6302-6307.

Zhu J, Oger PM, Schrammeijer B, Hooykaas PJJ, Farrand SK and Winans SC, 2000. The bases of crown gall tumorigenesis. *Journal of Bacteriology* **182**: 3885-3895.

Zohary D and Hopf M, 1993. Domestication of plants in the old World - The origin and spread of cultivated plants in West Asia, Europe, and the Nile Valley. Clarendon Press, Oxford.

Zupan J, Muth TR, Draper O, and Zambryski P, 2000. The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *Plant Journal* **23**:11–28.

Appendix 1 – Media, buffers and solutions for the culture of *A. rabiei*

Unless otherwise stated, the media, buffers and solutions were sterilised by autoclaving at 121°C for 20 min.

Czapek-Dox Liquid Cations Medium (CDLCM)

Czapek-Dox nutrients	33.4 $g.l^{-1}$
$ZnSO_4.7H_2O$	50 $mg.l^{-1}$
$MnCl_2.4H_2O$	20 $mg.l^{-1}$
$CaCl_2.2H_2O$	100 $mg.l^{-1}$
$CoCl_2.6H_2O$	20 $mg.l^{-1}$
$CuCl_2.2H_2O$	20 $mg.l^{-1}$

Chickpea extract broth

Extract of chickpea boiled for 30 min in 500 ml distilled water	60 $g.l^{-1}$
Sucrose	20 $g.l^{-1}$

Yeast extract peptone (YEP) broth

Yeast extract	1%
Peptone	2%

Richards' medium

KNO_3	10 $g.l^{-1}$
KH_2PO_4	5 $g.l^{-1}$
$MgSO_4.7H_2O$	2.5 $g.l^{-1}$
$FeCl_3$	0.02 $g.l^{-1}$
glucose	50 $g.l^{-1}$

Czapek-Dox-V8 (CD-V8) broth

Czapek-Dox nutrients	33.4 $g.l^{-1}$
Peptone	1 $g.l^{-1}$
Yeast extract	1 $g.l^{-1}$
Casein hydrolysate	1 $g.l^{-1}$

The nutrients were dissolved in 1l distilled water, supplemented with 200 ml of V8-juice filtered through four thickness of muslin and centrifuged for 5 min at 2,000 *g*.

Appendix 2 – Solutions and media required for insertional mutagenesis of *A. rabiei*

Polyethylene glycol solution

Polyethylene glycol 4,000	60%
Tris-HCl, pH 7.5	25 mM
$CaCl_2$	25 mM

The solution was filter-sterilised.

STC buffer

Sucrose	0.6 M
Tris-HCl, pH 7.5	10 mM
$CaCl_2$	10 mM

The solution was filter-sterilised.

YEPS medium

Yeast extract	1%
Peptone	2%
Sucrose	20%

Complete medium

Glucose	10 $g.l^{-1}$
Peptone	2 $g.l^{-1}$
Yeast extract	1 $g.l^{-1}$
Glucose	20%

The pH was adjusted at 6.5 and the solution sterilised.

TAE 50X buffer, pH 7.2

Tris base	242 $g.l^{-1}$
Glacial acetic acid	57.1 $ml.l^{-1}$
0.5M EDTA, pH 8.0	100 $ml.l^{-1}$

K-buffer, pH 7.0

K_2HPO_4	200 $g.l^{-1}$
KH_2PO_4	145 $g.l^{-1}$

M-N solution

$MgSO_4.7H_2O$	30 $g.l^{-1}$
NaCl	15 $g.l^{-1}$

Minimal medium

K-buffer, pH 7.0	10 $ml.l^{-1}$
M-N solution	20 $ml.l^{-1}$
1% $CaCl_2.2H_2O$	1 $ml.l^{-1}$
20% glucose	10 $ml.l^{-1}$
0.01% $FeSO_4$	10 $ml.l^{-1}$
20% $(NH_4)_2SO_4$	2.5 $ml.l^{-1}$

Induction medium

Minimal medium supplemented with:

2-morpholino-ethanesulfonic acid	40 mM
glucose	10 mM
glycerol	0.5%

50 X Denhardt's solution

Ficoll	10 $g.l^{-1}$
Polyvinylpyrrolidone	10 $g.l^{-1}$
Bovine Serum Albumine Fraction 5	10 $g.l^{-1}$

The solution was filter-sterilised and stored at -20°C in small aliquots.

Appendix 3 - Solanapyrone A production by 226 putative mutants created by *A. tumefaciens*-mediated transformation (first experiment)

For each putative mutant the mean value (ratio of the absorbance at 340 nm to the growth scoring) from three replicates ± the standard deviation is represented in the following table.

M: value missing.

Mutant	Solanapyrone A			Mutant	Solanapyrone A		
AB1	0.17	±	0.13	AB34	0.24	±	0.09
AB2	0.13	±	0.05	AB35	0.19	±	0.10
AB3	0.16	±	0.05	AB36	0.15	±	0.08
AB4	0.23	±	0.08	AB37	0.27	±	0.08
AB5	0.03	±	0.02	AB38	0.28	±	M
AB6	0.18	±	0.06	AB39	0.18	±	0.06
AB7	0.15	±	0.04	AB40	0.06	±	0.02
AB8	0.27	±	0.11	AB41	0.17	±	0.15
AB9	0.04	±	0.03	AB42	0.22	±	0.10
AB10	0.11	±	0.05	AB43	0.15	±	0.06
AB11	0.26	±	0.10	AB44	0.27	±	M
AB12	0.16	±	0.13	AB45	0.10	±	0.07
AB13	0.15	±	0.02	AB46	0.20	±	0.06
AB14	0.09	±	0.03	AB47	0.12	±	0.09
AB15	0.20	±	0.04	AB48	0.11	±	0.01
AB16	0.22	±	0.05	AB50	0.21	±	0.05
AB17	0.17	±	0.02	AB51	0.19	±	0.04
AB18	0.06	±	0.05	AB52	0.23	±	0.08
AB19	0.21	±	0.14	AB53	0.22	±	0.05
AB20	0.23	±	0.09	AB54	0.25	±	0.07
AB21	0.15	±	0.15	AB55	0.10	±	0.03
AB22	0.32	±	0.06	AB56	0.15	±	0.06
AB23	0.15	±	0.04	AB57	0.12	±	0.09
AB24	0.05	±	0.02	AB58	0.23	±	0.03
AB25	0.10	±	0.02	AB59	0.18	±	0.04
AB26	0.07	±	0.02	AB60	0.29	±	0.08
AB27	0.21	±	0.07	AB61	0.04	±	0.02
AB28	0.21	±	0.08	AB62	0.18	±	0.08
AB29	0.25	±	0.12	AB63	0.10	±	0.08
AB30	0.21	±	0.07	AB64	0.09	±	0.11
AB31	0.19	±	0.11	AB65	0.19	±	0.04
AB32	0.18	±	M	AB66	0.19	±	0.14
AB33	0.13	±	0.08	AB67	0.16	±	0.16

Mutant	Solanapyrone A			Mutant	Solanapyrone A		
AB68	0.24	±	0.05	AB114	0.15	±	0.06
AB69	0.17	±	0.05	AB115	0.16	±	0.03
AB70	0.27	±	0.08	AB116	0.16	±	0.09
AB71	0.13	±	0.02	AB117	0.19	±	0.07
AB72	0.13	±	0.03	AB118	0.26	±	0.03
AB73	0.22	±	0.11	AB119	0.16	±	0.06
AB74	0.17	±	0.10	AB120	0.21	±	0.07
AB76	0.23	±	0.14	AB121	0.02	±	0.00
AB77	0.15	±	0.03	AB122	0.21	±	0.03
AB78	0.23	±	0.05	AB123	0.11	±	0.02
AB80	0.20	±	0.04	AB124	0.16	±	0.05
AB81	0.03	±	0.02	AB125	0.06	±	0.05
AB82	0.10	±	0.04	AB126	0.16	±	0.06
AB83	0.09	±	0.04	AB127	0.11	±	0.06
AB84	0.21	±	0.07	AB128	0.23	±	0.02
AB85	0.10	±	0.03	AB129	0.23	±	0.09
AB86	0.24	±	0.11	AB130	0.16	±	0.07
AB87	0.22	±	0.05	AB131	0.12	±	0.04
AB88	0.07	±	0.09	AB132	0.59	±	0.32
AB89	0.12	±	0.05	AB133	0.27	±	0.08
AB90	0.16	±	0.09	AB134	0.14	±	0.05
AB91	0.05	±	0.01	AB135	0.20	±	0.08
AB92	0.20	±	0.06	AB136	0.10	±	0.05
AB93	0.23	±	0.06	AB137	0.13	±	0.10
AB94	0.21	±	0.04	AB138	0.12	±	0.03
AB95	0.11	±	0.03	AB139	0.07	±	0.04
AB96	0.20	±	0.07	AB140	0.22	±	0.09
AB97	0.27	±	0.09	AB141	0.02	±	0.01
AB98	0.10	±	0.04	AB142	0.21	±	0.06
AB99	0.26	±	0.05	AB143	0.15	±	0.04
AB100	0.12	±	0.06	AB144	0.07	±	0.06
AB101	0.27	±	0.03	1B145	0.24	±	0.05
AB102	0.10	±	0.06	AB146	0.09	±	0.12
AB103	0.27	±	0.12	AB147	0.22	±	0.03
AB104	0.10	±	0.01	AB148	0.15	±	0.06
AB105	0.03	±	0.01	AB149	0.15	±	0.05
AB106	0.13	±	0.11	AB150	0.19	±	0.08
AB107	0.14	±	0.09	AB151	0.18	±	0.01
AB108	0.21	±	0.17	AB152	0.09	±	0.06
AB109	0.25	±	0.10	AB153	0.06	±	0.03
AB110	0.28	±	0.14	AB154	0.20	±	0.03
AB111	0.12	±	0.07	AB155	0.12	±	0.13
AB112	0.25	±	0.04	AB156	0.11	±	0.09
AB113	0.02	±	0.01	AB157	0.15	±	0.08

Mutant	Solanapyrone A			Mutant	Solanapyrone A		
AB158	0.18	±	0.09	AG1	0.03	±	0.01
AB159	0.04	±	0.02	AG3	0.09	±	0.01
AB160	0.10	±	0.09	AG4	0.28	±	0.01
AB161	0.17	±	0.07	AG5	0.17	±	0.11
AB162	0.12	±	0.06	AG6	0.04	±	0.01
AB163	0.17	±	0.03	AG7	0.08	±	0.01
AB164	0.23	±	0.08	AG8	0.24	±	0.05
AB165	0.23	±	0.12	AG9	0.15	±	0.09
AB166	0.14	±	0.11	AG10	0.11	±	0.03
AB167	0.19	±	0.09				
AB168	0.20	±	0.05				
AB169	0.28	±	0.04				
AB170	0.17	±	0.14				
AB171	0.23	±	0.15				
AB172	0.19	±	0.15				
AB173	0.22	±	0.06				
AB174	0.17	±	0.04				
AB175	0.25	±	0.05				

Mutant	Solanapyrone A			Mutant	Solanapyrone A	
LB1	0.10	±	0.07	LG2	±	0.02
LB2	0.12	±	0.07	LG3	±	0.03
LB3	0.21	±	0.11	LG4	±	0.02
LB4	0.07	±	0.07	LG7	±	0.05
LB5	0.08	±	0.09	LG8	±	0.03
LB6	0.03	±	0.01	LG9	±	0.05
LB7	0.08	±	0.05	LG10	±	0.11
LB8	0.25	±	0.06	LG11	±	0.02
LB9	0.12	±	0.08	LG13	±	0.01
LB10	0.18	±	0.02	LG14	±	0.03
LB11	0.08	±	0.08	LG15	±	0.03
LB12	0.05	±	0.04	LG16	±	0.03
LB13	0.14	±	0.07	LG17	±	0.08
LB14	0.10	±	0.04	LG20	±	0.03
LB15	0.05	±	0.05			
LB16	0.14	±	0.03			
LB17	0.04	±	0.00			
LB18	0.16	±	0.18			
LB19	0.02	±	0.01			
LB20	0.01	±	0.00			
LB21	0.18	±	0.08			
LB24	0.11	±	0.06			
LB25	0.11	±	0.03			
LB26	0.25	±	0.07			
LB27	0.02	±	0.00			
LB28	0.17	±	0.12			
LB29	0.15	±	0.15			
LB30	0.16	±	0.05			
LB32	0.18	±	0.04			
LB33	0.18	±	0.16			
LB34	0.08	±	0.02			

Appendix 4 – Buffers and solutions used for bioassays

Digestion solution

Pectolyase Y-23 (ICN Biochemicals, Ohio, USA)	50 $\mu g.ml^{-1}$
Macerozyme R-10 (Yakult Pharmaceutical Ind. Co, Ltd, Tokyo, Japan)	15 $mg.ml^{-1}$
Bovine serum albumin (Sigma)	500 $\mu g.ml^{-1}$

The enzymes and the protein were dissolved in holding buffer and kept at -20°C in 10 ml aliquots until use.

Holding buffer

Citric acid	50 mM
Magnesium sulphate	1 mM
KH_2PO_4	1 mM
NaOH	5.8 $g.l^{-1}$
Glucose	100 $g.l^{-1}$

The pH of the buffer was adjusted to 5.8.

Appendix 5 - Probit Table

Conversion of percentage of death to probit

%	0	1	2	3	4	5	6	7	8	9
0	2.42	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
	0.00	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80	0.90
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Contributions to the Integrated Pest Management project for the control of Ascochyta blight of chickpea

Posters

- 5th European Conference on grain legumes, Dijon, France (7-11 June 2004). "Development of a transformation protocol for *Ascochya rabiei*, the causal agent of Ascochyta blight of chickpea". Abstract published (p. 200, Ref. 543).

- 8th International Congress of Plant Pathology, Christchurch, New Zealand (2-7 February 2003). "Development of Integrated Pest Management Techniques for the control of Ascochyta blight of chickpea". Abstract published (p. 277, Ref. 20.26).

- UCL Biology postgraduate symposia, London (2003, 2002, 2001). "Development of Integrated Pest Management Techniques for the control of Ascochyta blight of chickpea".

- British Society for Plant Pathology Presidential meeting: "Plant pathology and global food security", Imperial College, London (8-10 July 2002). "Development of Integrated Pest Management Techniques for the control of Ascochyta blight of chickpea". Abstract published (p. 31, Ref. PP2). Poster recommended by the jury.

Oral presentations

- Molecular Biology of Fungal Pathogens XIV Conference, Ambleside, U.K. (15-17 September 2003). "Development of a transformation protocol for *Ascochyta rabiei*".

- Biology postgraduate symposium, University College London (2 May 2003).

"Development of Integrated Pest Management Techniques for the control of Ascochyta blight of chickpea".

- Project meeting, Ankara, Turkey (21-24 October 2002). "Production of toxin-minus mutants by Restriction Enzyme Mediated Integration" and "Use of solanapyrone toxins to select for resistance"
- Project meeting, London, UK (2-4 June 2004). "Transformation of *A. rabiei* by *Agrobacterium tumefaciens*: a route to the production of toxin-minus mutants" and "Use of solanapyrone toxins to select for resistance".

Creation of a webpage presenting the project and the partners involved. Address: http://www.ucl.ac.uk/biology/strange/chindex.htm