

OPTIMIZING BIOCONTROL OF PURPLE NUTSEDGE
(*Cyperus rotundus*)

by
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ABSTRACT

Cyperus rotundus L. CYPRO (purple nutsedge) and *Cyperus esculentus* L. CYPES (yellow nutsedge) are problematic weeds on every continent. At present there is no comprehensive means of controlling these weeds. The primary means of control is herbicides, although the weeds are becoming more resistant. Bioherbicide control of purple and yellow nutsedge is an important avenue of research, with much of the focus being to increase the virulence of current fungal pathogens of *C. rotundus* and *C. esculentus*.

The primary aim of this study was to increase the virulence of a fungal pathogen of *C. rotundus* and *C. esculentus*, with the objective of creating a viable bioherbicide.

A possible means of increasing the virulence of a pathogen would be to increase the amount of amino acid produced by the fungus. This was proposed as a means of increasing the virulence of *Dactylaria higginsii* (Luttrell) M. B. Ellis. Overproduction of amino acids such as valine and leucine result in the feedback-inhibition of acetolactate synthase (ALS), an enzyme which is a target for many herbicides currently on the market. By applying various amino acids to tubers of purple nutsedge and comparing the results with a reputable herbicide, glyphosate, it was possible to determine the success of the amino acid applications. Only glutamine treatment at 600 mg.l⁻¹ resulted in significantly less ($P < 0.001$) germination compared with the water control, while the glyphosate application resulted in no germination. Four treatments were significantly different ($P < 0.001$) from the water control in terms of shoot length, but no pattern or conclusion could be drawn from the results. Injecting amino acids and glyphosate into the leaves of the plants gave similar results to those obtained with the tubers, with no visible damage on those plants injected with the amino acids and complete plant death of those injected with glyphosate. Amino acids had little effect on the growth of the *C. rotundus* plant or tuber. It was later determined by a colleague (Mchunu¹, unpublished) working on the same project, that *D. higginsii* does not infect the local ecotypes of *C. rotundus* in Pietermaritzburg, South Africa.

A second fungus, *Cercospora caricis* Oud., was isolated from *C. rotundus* growing in the region, and confirmed as a *Cercospora* species by conidial identification. Like many *Cercospora* species, *C. caricis* produces a phytotoxin, cercosporin. An increase in production of cercosporin would theoretically lead to an increase in virulence of *C. caricis*. Mutation of hyphae by

ultraviolet-C light was perfected on *C. penzigii* Sacc., where 5 min exposure to UV-C light resulted in approximately 99% cell death. Surviving colonies were analysed by spectrophoresis, and the surviving mutant gave an absorbance value of approximately 5% more than the median. Samples were analysed by high-performance liquid chromatography (HPLC) to determine the presence of cercosporin. No definitive result was obtained. Exposure of *C. caricis* to UV-C for 5 min. resulted in approximately 65% hyphal cell death, with 20 min. resulting in approximately 95% death. A spontaneous mutant was observed in a colony that had been exposed to UV-C. This mutant showed sectorial growth with red and grey growth patterns. The red section of the mutant was subcultured and analysed by spectrophoresis and HPLC. The red *C. caricis* gave an absorbance reading of approximately 140 on HPLC compared with about 22 from the grey colony. HPLC analysis of the wild-type *C. caricis* did not produce a peak corresponding to that of the cercosporin standard, although no conclusion could be obtained on the presence or absence of the toxin.

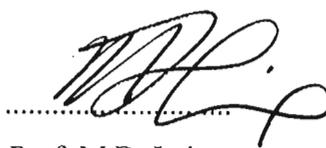
The virulence of the mutant *C. caricis* could not be determined as inoculation experiments were unsuccessful, and had to be discontinued due to time constraints.

DECLARATION

I, Edward J. Brooks, hereby declare that the content of this report is my own research, except where indicated. This thesis has not been submitted for any degree or examination at any other university.



.....
Edward J. Brooks.



.....
Prof. M.D. Laing

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RESEARCH OBJECTIVES AND OVERVIEW

The aim of this study was to increase the virulence of a fungal pathogen of *Cyperus rotundus* L. CYPRO and *Cyperus esculentus* L. CYPES, by means of mutation, with the objective of creating a viable bioherbicide.

The means used to achieve the objectives follow:

Chapter 1 is a review of the literature describing herbicides and pathogens of *C. rotundus* and *C. esculentus*, the necessity of this research, the impact these weeds have on crops, and the strategies used to mutate the fungal pathogens.

Chapter 2 assesses the possibility of using *Dactylaria higginsii* (Luttrell) M. B. Ellis as a biological control agent, looking primarily at the possible use of amino acids to kill the weeds.

Chapter 3 involves the mutation of *Cercospora penzigii* Sacc. The methods tested were used as a model for mutation of *Cercospora caricis* Oud.

Chapter 4 describes the mutation of *C. caricis* with the aim of increasing cercosporin production.

Chapter 5 provides a conclusion to the research conducted in this study and presents recommendations for future research.

CHAPTER 1

LITERATURE REVIEW

1.1 NUTSEdge

Sedges are placed in the order Graminales, class Angiospermae, subclass Monocotyledoneae, and family Cyperaceae (Gleason, 1963). It has been suggested that there are approximately 75 genera and over 4000 species in this family, with an estimated 600 species in the genus *Cyperus* (Gleason, 1963). Of these 600 species, two are most notable for the negative impact they have on farming. These two weeds are *Cyperus rotundus* L. CYPRO (purple nutsedge) and *Cyperus esculentus* L. CYPES (yellow nutsedge). These sedges are especially prolific in disturbed soils such as ploughed fields, where they benefit from irrigation, fertilization and tillage (Barreto and Evans, 1995). Reasons for their success can be attributed to their perennial nature, prolific tuber production, and the length of time that tubers can survive while remaining dormant (Bariuan *et al.*, 1999). Many methods have been employed to control the dominance of these weeds with varying degrees of success. A comprehensive removal technique, where the weed is eradicated from an area, has yet to be discovered. The reason for the success of these two weeds lies in their botanical characteristics, described below, and ability to survive unfavourable environmental conditions.

1.1.1 Botany

The Cyperaceae resemble the Gramineae (grass family), both of which are Monocotyledoneae, but have a number of distinct differences. Wills (1987) noted that the Cyperaceae possess “three-ranked leaves with one-third phyllotaxy and leaves that have closed leaf sheaths”, they usually have solid stems, but have no ligule, and each flower is “subtended by a single glume or scale”.

1.1.1.1 *Cyperus rotundus*

The following structural characteristics of *C. rotundus* were reported by Wills (1987) (Fig. 1.1). The leaves of the purple nutsedge, which average 6 – 10 mm in width and 100 – 350 mm in length, are dark green, shiny, three-ranked and corrugated in cross-section. Wills (1987)

observed that the rachis, which grows through the centre of the leaf bundle and supports the terminal inflorescence, is simple, smooth, erect, and triangular in cross-section and 100 – 600 mm in length. Each inflorescence, which is either simple or slightly compound, is subtended by two or more involucreal leaves or leaf-like bracts, each of which is the same length or longer than the flower-bearing rays. The rays are formed from three to nine three-sided peduncles of unequal length. Narrow spikelets are in clusters near the end. They are 8 – 25 mm long and 2 mm wide, possess between 10 - 40 flowers, and are acute and compressed with a variation in colours ranging from red and reddish-brown to purplish-brown. The glumes, which have three to seven nerves, are ovate and nearly blunt, and are 2 - 3.5 mm long. Individual seeds are achenes. An achene is a dry fruit with one seed and thin outer layer that remains intact even after maturity. Achenes are ovate or oblong-ovate, three angled, black or brown to olive grey in colour with a number of grey lines covering the surface. Each achene, 1.5 mm in length, is sessile on a spikelet and is subtended and covered by a single glume or scale 2 - 3.5 mm long, which has three to seven nerves, is ovate and nearly blunt (Holm *et al.*, 1977).

Although achenes are produced by *C. rotundus*, a perennial, it rarely reproduces by seed (Thullen and Keeley, 1979). The most common method of increasing numbers and quantity is by the production of rhizomes and tubers. Rhizomes, when first produced, are white and fleshy with scale leaves which, as they mature, become ligneous or wiry (Wills, 1987). Rhizomes that grow upwards and reach the surface swell to form a basal bulb, or corm, 3 – 10 mm in diameter that grows into a new plant producing shoots, roots and rhizomes (Wills, 1987). Rhizomes that do not grow towards the surface may produce chains of tubers, each of which may separate from the parent plant and lie dormant for years before growing into a new plant (Ranade and Burns, 1925; Hauser, 1962; Holm *et al.*, 1977). The tubers grow to various sizes in the field, from 7.5 – 35 mm in length and 3 – 10 mm in width (Wills, 1987).

Purple nutsedge can be distinguished from yellow nutsedge by the colour of its inflorescence (from which the name is derived), being red, reddish-brown, or purplish-brown, its dark green leaves, which have boat-shaped leaf tips and grow low on the ground, and the rhizomes, which are initially scaly becoming wiry and difficult to break, and produce bulbs in chains (Wills, 1987).

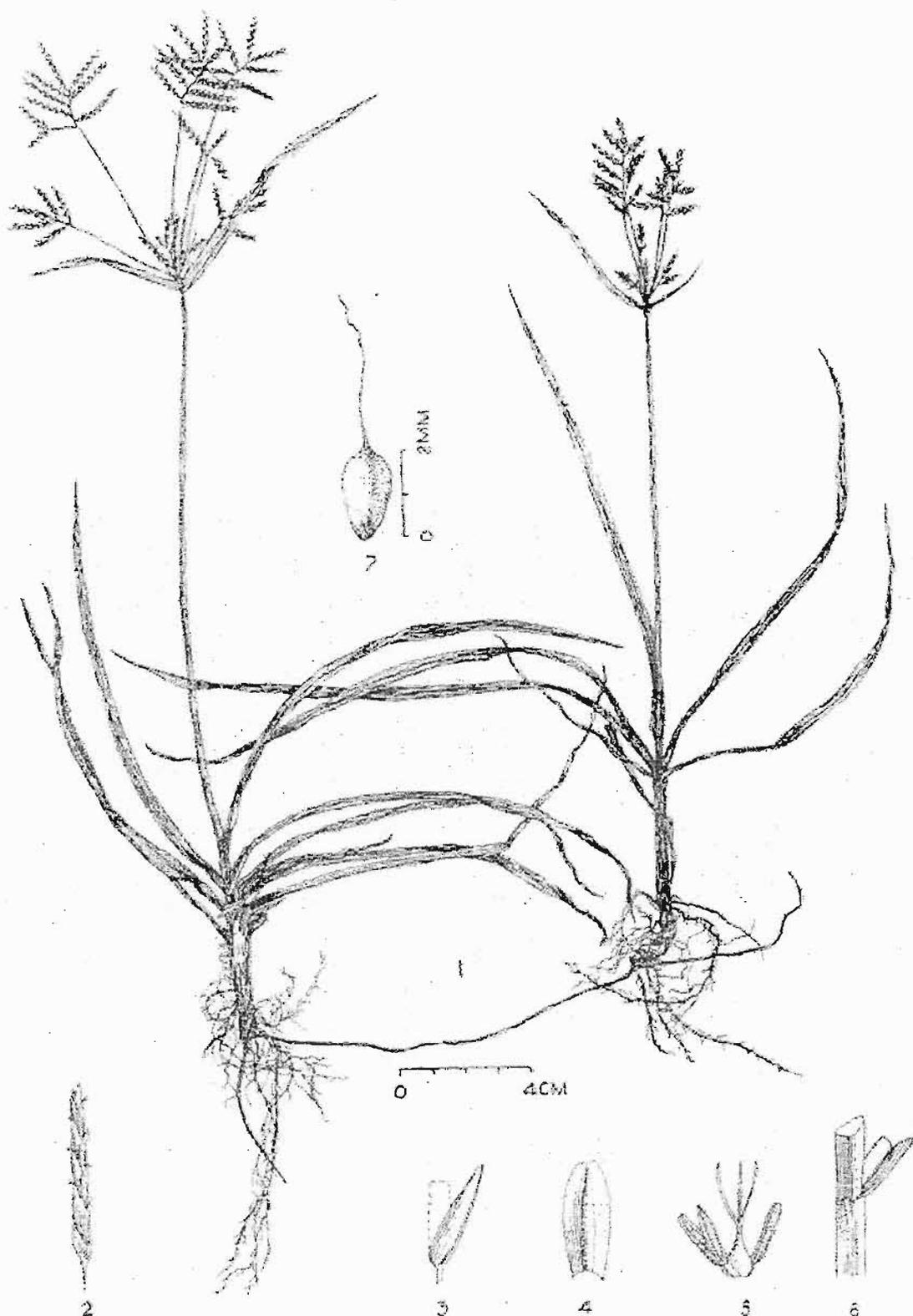


Fig. 1.1. Botany of "*Cyperus rotundus*: 1, habit; 2, portion of inflorescence; 3, flower with glume; 4, glume; 5, flower, glume removed; 6, portion of leaf sheath and blade; 7, achene" (taken directly from Holm *et al.* (1977))

1.1.1.2 *Cyperus esculentus*

The following structural characteristics of *C. esculentus* were reported by Wills (1987) (Fig. 1.2). The leaves of the yellow nutsedge are 4 – 9 mm wide and 200 – 900 mm in length, pale green, three-ranked and corrugated in cross-section. The rachis is 20 – 90 mm in length and is terminated by an umbel. Each umbel, an inflorescence of a cluster of flower-bearing rays all emanating from the same level, possess a number of short rays with two to nine longer rays. At the same level as the umbel are between three and nine involucral leaves, which are all longer than the longest ray. Each inflorescence has golden-brown, simple to compound spikelets arranged along an elongated axis. The spikelets are generally four-ranked but may be two-ranked; they are flattened seed-bearing structures and are between 1.5 – 3 mm wide and 5 – 30 mm long. The achenes are 1.2 - 1.5 mm in length, yellowish-brown and three-angled. They are sessile on a spikelet and are covered by a thin oblong glume or scale.

Unlike purple nutsedge, *C. esculentus* reproduces annually by seeds and perennially by either corm-like bulbs from the base of a leaf fascicle, thread-like rhizomes that extend outward from the bulbs, or by tubers 10 - 20 mm long that are often produced at the end of rhizomes (Wills, 1987). During the growing season the rhizomes produced by the plant may either develop into a new bulb, from which a new plant will grow, or into a dormant tuber which may lie underground for a number of years (Thumleson and Kommedahl, 1961; Garg *et al.*, 1967; Jansen, 1971; Stoller *et al.*, 1972; Holm *et al.*, 1977).

Yellow nutsedge is distinguished from the purple by its yellowish-brown inflorescence which is pinnately arranged along an axis, its pale green leaves which have long needle-like tips and grow upright, and its weak rhizomes which often terminate in bulbs and rarely produce chains of tubers (Wills, 1975).

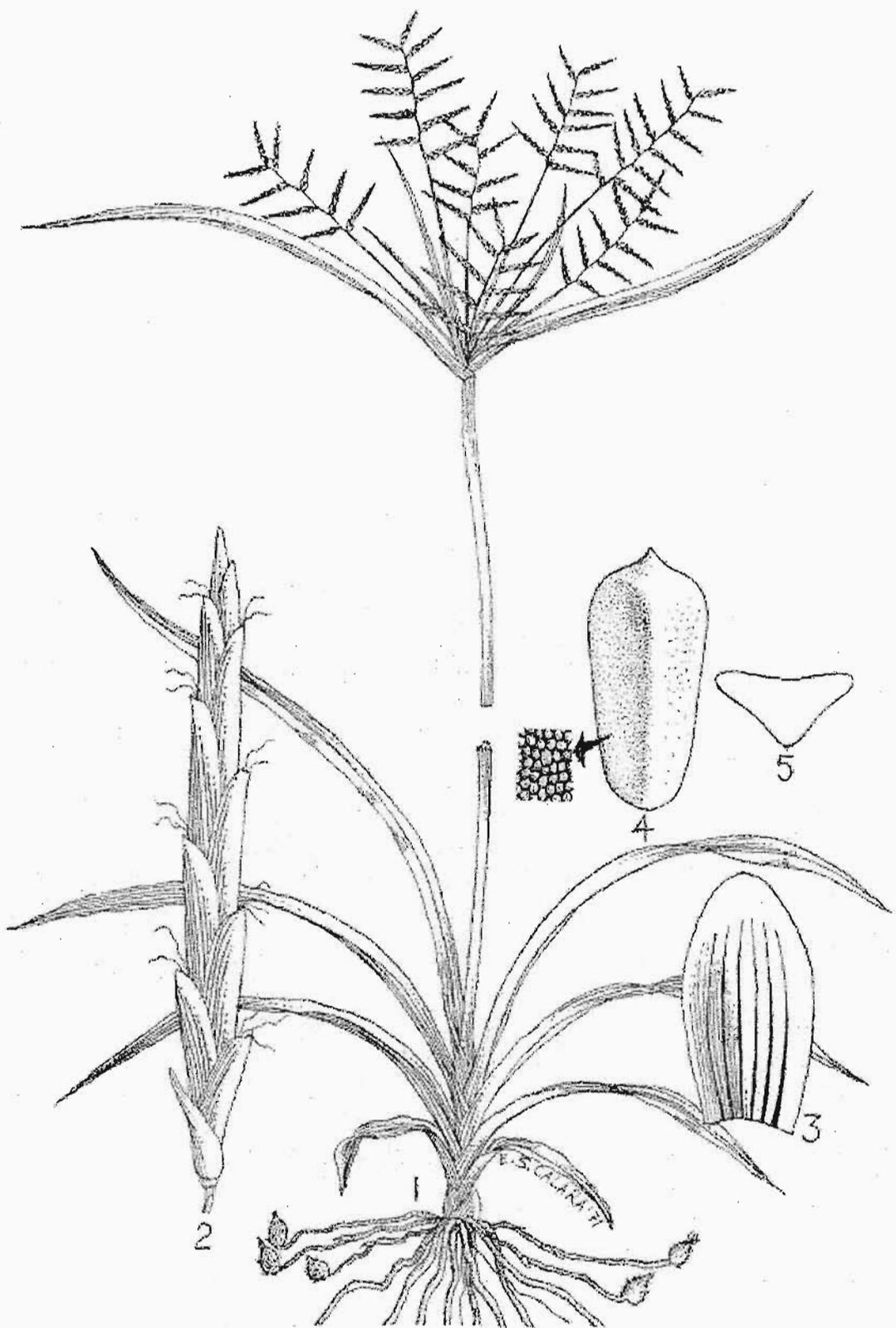


Fig. 1.2. Botany of "*Cyperus esculentus*: 1, habit; 2, spikelet; 3, bract; 4, seed, with close-up detail of surface reticulation; 5, seed, cross section" (taken directly from Holm *et al.* (1977))

1.1.1.3 Common characteristics

Both nutsedges possess two photosynthetic pathways, the conventional C₃ Calvin cycle as well as the efficient C₄ dicarboxylic acid photosynthetic pathway (Downton and Tregunna, 1968; Black *et al.*, 1969; Chen *et al.*, 1970). Plants with the C₄ pathway are characterised by the Kranz-type leaf structure where sheaths around the vascular bundles compartmentalize the photosynthetic events (Wills, 1987). This pathway allows for efficient uptake of CO₂ from the adjacent air as well as from CO₂ produced by cellular respiration (Wills, 1987). The benefits of this system are that the plants can incorporate CO₂ from warmer air and at greater light intensities than conventional C₃ plants (Downton and Tregunna, 1968; Black *et al.*, 1969). Consequently, C₄ plants grow best between day/night temperatures of 30/25°C and 36/31°C, which is 10 - 20°C warmer than those observed for C₃ plants (Downton and Tregunna, 1968). This pathway also influences the optimum amount of light a plant needs, with C₃ plants being saturated between 20 - 30% of full sunlight compared to C₄ plants that saturate between 50% and more of full sunlight (Wills, 1987). Furthermore, Black *et al.* (1969) reported that plants that fix CO₂ at higher temperatures and light conditions, as well as having rhizomes that spread quickly and give rise to new plants, have the potential to become serious weeds.

1.1.1.4 Tubers

Tubers and basal bulbs resemble each other and are often difficult to distinguish. It is only the activity of the structure that separates the two: the basal bulb differentiates into shoots while the tuber remains temporarily dormant (Holm *et al.*, 1977). Rochecouste (1956) reported that *C. rotundus* produces 6 and 14 times more tubers in humid conditions (1250 to 2500 mm of annual rainfall) than in sub-humid (less than 1250 mm of rain) and super-humid (greater than 2500 mm of rain) conditions, respectively. Rochecouste (1956) conducted experiments in Mauritius and reported that no tubers were present below 450 mm of rainfall. This discrepancy may be attributed to soil and growing conditions. Rochecouste (1956) reported that on plants under good tuber-producing conditions (humid conditions), between 15 - 20% more tubers were found below 150 mm than on those grown in sub-optimal conditions. Further studies conducted in 1968 by Rao (1968) indicate that in 90 days one *C. rotundus* tuber had the potential to produce 99 tubers, which equates to 4.8 million per hectare in uncultivated land and eight million in

cultivated land. Holm *et al.* (1977) reported that as much as 40 000 kg of tubers from *C. rotundus* may be produced per hectare.

Tuber germination and dormancy are two areas that farmers have tried to target in order to remove *C. rotundus*. For germination of the tuber, Ueki (1969) found that temperatures between 30°C and 35°C were optimum with no germination at temperatures above or below 45°C and 10°C, respectively. Andrews (1940), performing experiments on moisture content for germination, observed that, after eight days, optimum soil moisture content was between 30% and 40%, with no germination occurring below 10% or above 50%. Further studies have shown that tubers lose viability when desiccated. Smith and Fick (1937) reported that tubers lost viability and died when left for four days in the sun, 16 days in an open lab or desiccator, and 32 days in a storeroom. In each case the moisture content of the tuber was 15% at death except for the tubers left in the sun, which were 24% at death. Ploughing the land in order to desiccate the tubers is not a practical method of weed removal as this process may take a few years during which time the land must lie fallow. The reason for this is that not all tubers are exposed after a single ploughing event, therefore repeated ploughing is required.

There is currently no simple method for the removal of tubers, which may, if not removed, lie dormant for a number of seasons before germinating. These tubers are resilient and can survive extreme conditions of flooding, drought, heat, and lack of aeration (Holm *et al.*, 1977). Most herbicides target the aerial part of the plant which brings limited success. The plant dies back for a short period before it sprouts again with no sign of damage.

1.1.2 Distribution and habitat preferences

Purple and yellow nutsedges are located in a number of countries around the world, with the purple variety being found in more countries and localities than any other weed species; colder climates are the only restriction on its dominance in the remaining countries (Holm *et al.*, 1977). By the late 1970's it was considered a weed in 90 tropical and subtropical countries where it had a negative impact on approximately 52 crop species, and has consequently been given the notorious title of being the world's worst weed (Holm *et al.*, 1977). This is a highly capable plant that is able to survive in almost every conceivable habitat with little regard for soil type, pH, humidity, soil moisture and elevation (Holm *et al.*, 1977). Ranade and Burns (1925)

reported that it does not grow well in soils with high concentrations of salt. Furthermore, due in part to its C₄ photosynthetic pathway, the plant cannot tolerate shade. Holm *et al.* (1977) reported that when other plants, such as cane or plantation trees, grow to a sufficient height to block out the sun, nutsedge withers and dies, leaving only dormant tubers behind. Yellow nutsedge is more tolerant of colder conditions and wet soils. It is found primarily along river banks, roadsides and ditches, and in moist fields, heavily irrigated crops and on low ground. It can grow at a pH between five and seven without any observable problems and is relatively tolerant of shading. In some wet areas it has been known to replace *Cynodon dactylon* (L.) Pers. and *C. rotundus*.

Both nutsedges are prime examples of pioneer species. They are found primarily on cultivated land where the soil is often tilled and, when larger plants become established, they are unable to compete. These weeds colonize other areas by the movement of the tubers, which may be washed away by irrigation or river water, or picked up unintentionally in the mud by man, animals or vehicles. They are able to colonize areas that other plants cannot due to their ability to produce vast numbers of tubers that may lie dormant for a few years. Consequently, flood, fire or disease that might destroy other plants have less impact on nutsedges.

1.1.3 Uses

Although there is much literature regarding the negative impact of this weed, these plants are known to benefit certain communities. In India the plants are sometimes used as soil binders (Holm *et al.*, 1977). *Cyperus esculentus* has been cultivated for their edible tubers in Africa and Europe (Wills, 1987). They are also sometimes used as fodder for animals when regular plants are not available (Holm *et al.*, 1977). The oil of yellow nutsedge has been extracted and used for food and soap in both Egypt and Italy (Wills, 1987). In Europe the tubers, which possess no caffeine, have been ground and roasted and used as a replacement for coffee and cocoa (Wills, 1987). It has been reported that the tubers of yellow nutsedge contain 12 - 30% sugars, 25 - 30% starch and approximately 30% oil, although these figures fluctuate depending on the ecotype (Holm *et al.*, 1977).

El-Moghazy-Shoaib (1967) reported that purple nutsedge was used for medicine in the Stone Age. In Africa, China, southern Europe and India the tubers have been used as an insect

repellent, an aphrodisiac, a remedy for malaria and a cure for headaches (Watt and Breyer-Brandwijk, 1962). Further studies have revealed that the tubers possess triterpenoid compounds that have antipyretic (fever-reducing), analgesic, and anti-inflammatory effects (Gupta *et al.*, 1971). An alcoholic extract has also been found that has muscle-relaxing, antihistaminic, antiemetic (nausea-reducing) and tranquillising activities (Singh *et al.*, 1970). Although purple nutsedge may produce up to six tonnes per hectare of underground dry weight, primarily as tubers, it is not used much as food due to its bitter taste (Wills, 1987).

1.1.4 Impact

Cyperus rotundus has been recognised worldwide as one of the most problematic weeds. Holm *et al.* (1977) undertook a survey to determine the seriousness of the weed and reported that, of the major crop species, it is regarded as one of the three most troublesome weeds of: maize in Ghana and the Philippines; cotton in Turkey, Sudan, Swaziland, and Uganda; rice in Ghana, Indonesia, Iran, Peru, South Africa and Taiwan; and sugarcane in Argentina, India, Indonesia, Peru, Taiwan and Venezuela (Figs 1.3 and 1.4). *Cyperus rotundus* is considered a serious or principle weed in many more countries. One reason for the emergence of nutsedge as a serious weed is the removal and control of many other weeds (Anonymous, 1976; Bendixen and Stoube, 1977; Anonymous, 1979). A decrease in competitive pressure from other weeds has resulted in nutsedge dominance; it has been reported that it is not a problem where quackgrass (*Agropyron repens* (L.) Beauv.), johnsongrass (*Sorghum halepense* (L.) Pers.), bermudagrass (*C. dactylon*), and broadleaf weeds are established (Bendixen and Stoube, 1977).

Studies on *C. rotundus* revealed that crop losses could be as high as 35% in cabbage, 39 - 50% in carrot, 41% in bean, 43% in cucumber, 53% in tomato, 54% in lettuce, 62% in okra, 70% in radish, 73% in bell pepper and 89% in onion (William and Warren, 1975; Keeley, 1987; Morales-Payan *et al.*, 1996; Santos *et al.*, 1996; Morales-Payan *et al.*, 1998). It has been determined that in extreme cases in Argentina the yield of cane might be reduced by 75% with the sugar yield being reduced by 65% (Cerrizuela, 1965).

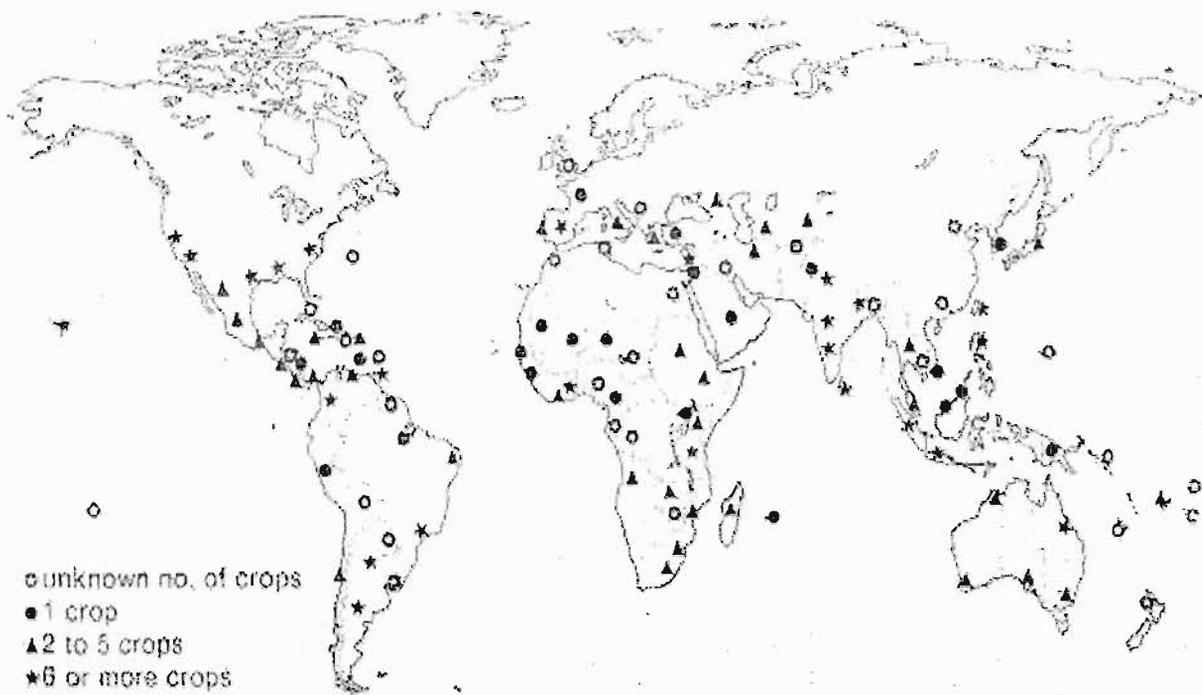


Fig. 1.3. Worldwide distribution of *Cyperus rotundus* indicating the areas in which it is considered a weed (Holm *et al.*, 1977)

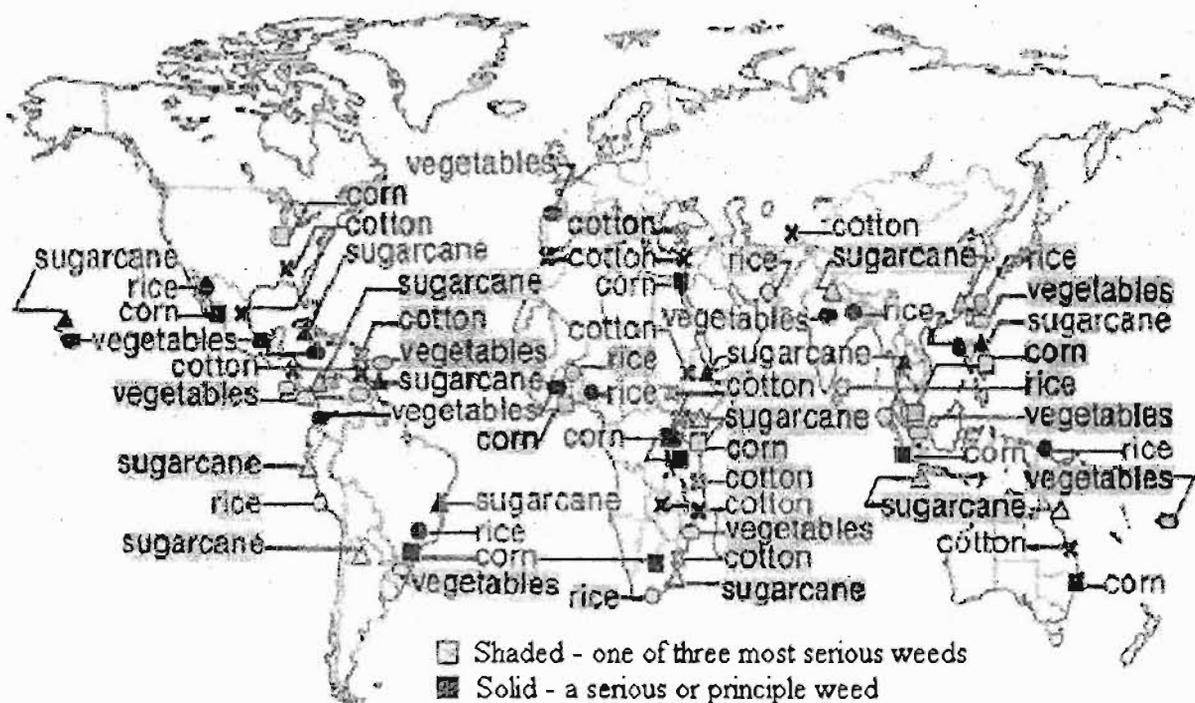


Fig. 1.4. Worldwide distribution of *Cyperus rotundus* indicating the crops for which it is considered a weed (Holm *et al.*, 1977)

Cyperus esculentus is not as serious a weed as purple nutsedge with densities of 40 shoots per square meter having no effect on cotton yield, while 70 shoots per square metre, resulted in yield losses of only 12 - 36% (Keeley and Thullen, 1983). Elsewhere it was reported that infestations of 300 and 1200 tubers per square metre in maize reduced yield by 17 and 41%, respectively, with an average of 8% yield reduction for every 100 shoots per square metre of yellow nutsedge present (Stoller *et al.*, 1979). In 1977 it was present on all continents, but most problematic in southern and eastern Africa, and North and Central America (Figs 1.5 and 1.6) (Holm *et al.*, 1977).

Reduction in crop yields due to purple and yellow nutsedge, and many other weeds, can be largely attributed to two factors, namely competition and allelopathy. The success of a weed is therefore proportional to its ability to out-compete the crop or produce toxic substances that inhibit the crop in some manner.

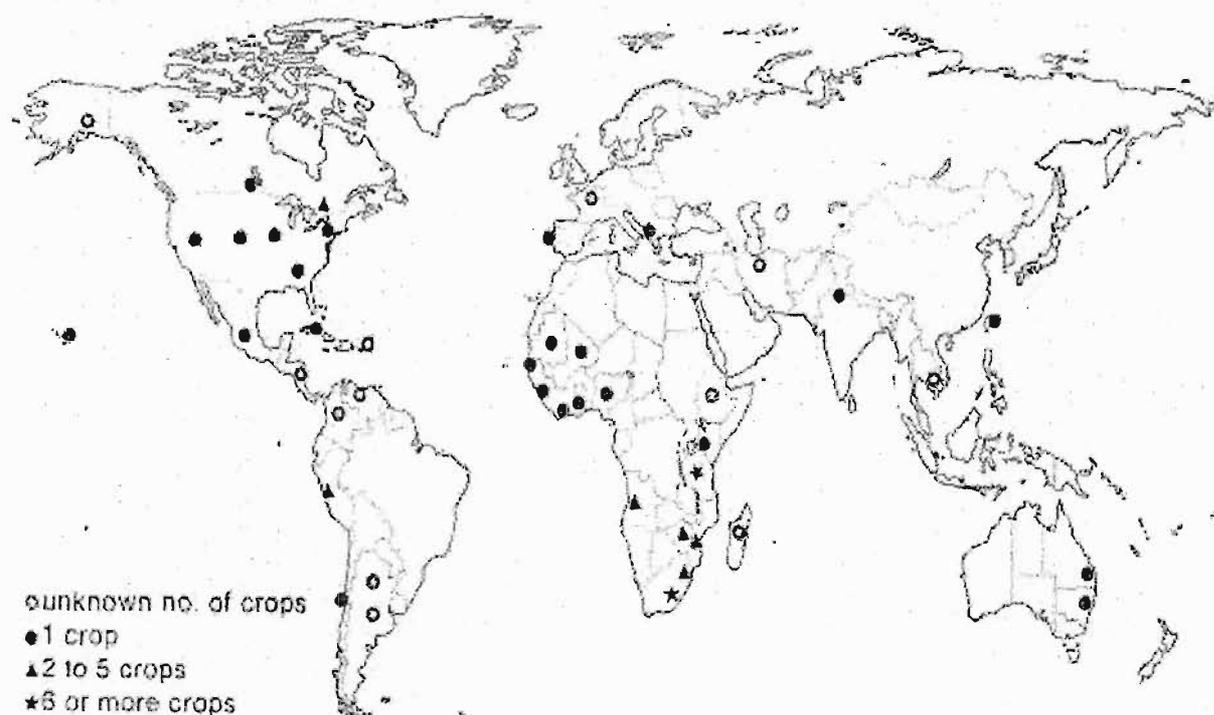


Fig. 1.5. Worldwide distribution of *Cyperus esculentus* indicating the areas in which it is considered a weed (Holm *et al.*, 1977)

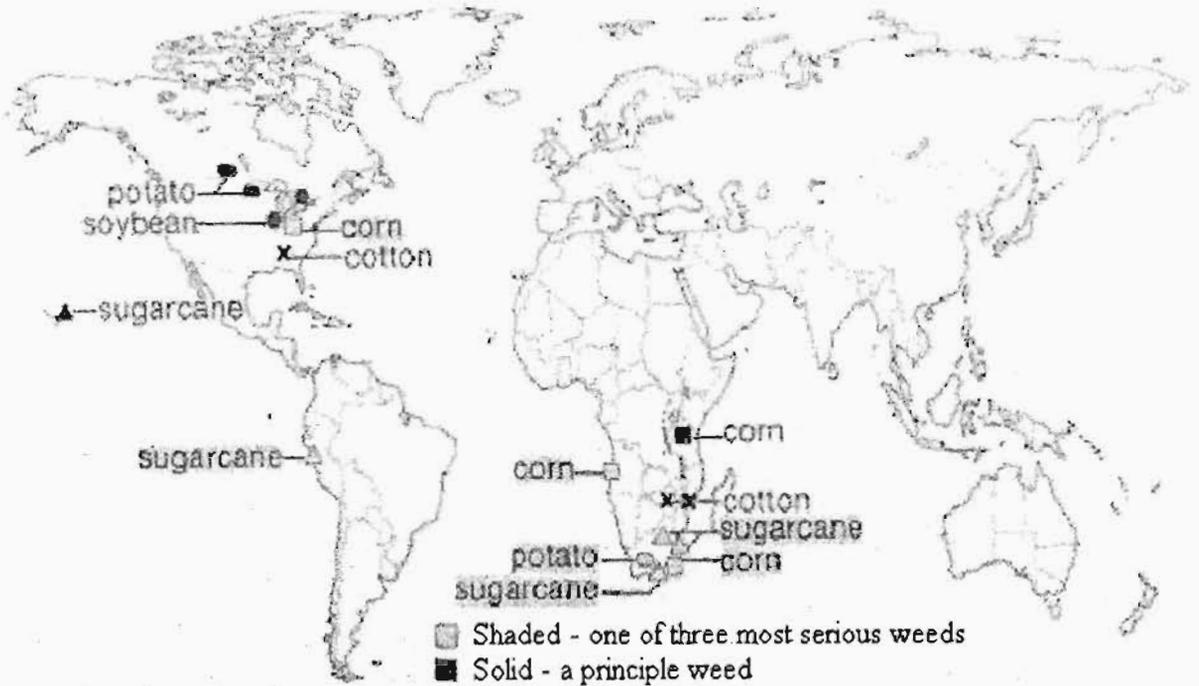


Fig. 1.6. Worldwide distribution of *Cyperus esculentus* indicating the crops for which it is considered a weed (Holm *et al.*, 1977)

1.1.4.1 Competition

Plants are in constant competition with each other for light, nutrients and water. It is this competition that allows for the development and evolution of a species, and it is this competition that makes *C. rotundus* so troublesome. Rochecouste (1956), when studying the weed's impact on sugarcane in Mauritius, reported that under humid conditions (1250 mm of annual rainfall) purple nutsedge produced 30 000 kg per hectare of tubers and shoots. This would substantially increase competition for water and nutrients.

These weeds are not tall plants and are unable to compete effectively for light when most crops mature. They often do most damage to crops early in the plant's life while nutsedges are bigger than the surrounding plants. Keeley and Thullen (1975) reported that yellow nutsedge which was 110 mm tall shortly after emergence of cotton, was 440 mm tall after 49 days of growth and was tall enough to compete for light for most of the growing season. These weeds are far more successful in competing against crops that do not have a dense canopy, or grow more slowly (Cherry, 1973). Examples of these crops include vegetables and irrigated cotton (Keeley, 1987).

Shading of 30% and 80% of yellow nutsedge can result in 32% and 80% reductions in dry matter and tuber production, respectively (Keeley and Thullen, 1978). Tubers are, however, still produced by the plant when grown in 94% shade (Patterson, 1982). This reduction in growth due to shading is substantial and indicates a potential for further research into the control of the weed.

As water is essential to the survival of all plants, competition for water, especially in drier regions, can have detrimental effects. Keeley and Thullen (1975) reported that a concentration of 100 shoots per square metre of yellow nutsedge depleted the soil moisture sufficiently to reduce cotton stands. Fewer canes and poor yields of sugar cane can be attributed to loss of soil moisture at stooling time by purple nutsedge (Chapman, 1966).

Many nutrients are drained from agricultural land due to irrigation and run-off. It is therefore important that the crop has full access to the fertilizers applied to a field. Rochecouste (1956) determined that large quantities of fertilizer meant for the crop could be used or stored by nutsedge. The quantities reported were: 815 kg per hectare for ammonium sulfate, 320 kg for potash, and 200 kg for superphosphate. Purple nutsedge is most sensitive to decreases in nitrogen (N), potassium (K) and phosphorus (P) concentrations in the soil (Nel *et al.*, 1976; Shamsi and Al-Ali, 1983). Reduction in soil nutrients may reduce the dry matter of purple nutsedge but it results in an increase in the root to shoot ratio, which is an automatic response by the plant to increase nutrient uptake (Shamsi and Al-Ali, 1983). Another response to lack of nutrients is the increased production of tubers that, if the nutrient level remains low, can lie dormant until more favourable conditions arise (Shamsi and Al-Ali, 1983). In contrast to this, an increase in nutrient concentration in the soil results in a decrease in tuber formation and an increase in shoot and rhizome production (Nyahoza, 1973). This competition for nutrients has a direct effect on crop species. Reduction in N and P concentrations in wheat by purple nutsedge was reported throughout its life cycle, while yellow nutsedge has been reported to decrease N in silage maize, tomato, fruit and soybean (Soni and Ambasht, 1977; Volz, 1977).

Many of these results have been attributed solely to competition between the weed and the crop without allelopathy being taken into consideration.

1.1.4.2 Allelopathy

Allelopathy can be described as the inhibition of the growth of one plant by the release of substances, liquid or airborne, of another plant. Friedman and Horowitz (1970, 1971) observed that the radicle growth of crop plants was inhibited when grown in an extract of soil containing purple nutsedge tubers and rhizomes. In subsequent experiments tubers and rhizomes of nutsedge were placed in the soil and began to decay, releasing allelopathic chemicals. After 30 – 60 days, barley was planted in the soil and it was observed that growth was inhibited by 15 – 25%. The chemicals responsible were reported to be phenolic acids. It has been shown that *C. rotundus*, when alive, produces a substance that is toxic to radish (*Raphanus sativa* L.) (Elmore, 1985).

Cyperus esculentus also possesses allelopathic properties, with phenolic compounds again implicated (Thumblason and Kommedahl, 1962; Jangaard *et al.*, 1971; Tames *et al.*, 1973). The weed inhibits the growth of maize, soybean and sweet potato (Peterson and Harrison, 1995). It is, however, interesting to note that sweet potato produces a substance that is toxic to both nutsedges, and, although the potato itself is inhibited, yellow nutsedge is inhibited to a far greater degree (Peterson and Harrison, 1995).

The production of allelopathic chemicals by purple and yellow nutsedge aids weeds by inhibiting the growth of other plants that would compete for water, light and nutrients, while not inhibiting the growth of surrounding nutsedge plants.

1.2 CONTROL OF PURPLE AND YELLOW NUTSEGE

Although some herbicides are able to kill the shoots of purple and yellow nutsedge, their inability to destroy the tubers is an important factor in control of nutsedge. It is unclear what impact many herbicides have on the resultant production of tubers. Tubers merely lie dormant for a short period before sprouting again. Therefore, any control measure needs to reduce both the shoot mass and the number of viable tubers if any degree of long-term success is to be achieved (McElroy *et al.*, 2003).

1.2.1 Cultural control measures

A number of methods for curbing the dominance of nutsedge as a weed have been introduced over the years with varying degrees of success. One method that has proved relatively successful is the date of planting so that the crop grows outside the growing seasons for nutsedge. This can be achieved by either planting before the nutsedge sprouts or near the end of its growing season. In North America where yellow nutsedge infestations are a problem, squash planted in early June produced approximately 45% more yield than those planted in late June (Stilwell and Sweet, 1974). Another experiment revealed that okra required less weeding when planted in late summer rather than early spring (William and Warren, 1975). In spring-planted okra, the critical amount of weeding for purple nutsedge was 10 weeks as opposed to five weeks in summer-planted okra. Although this has been met with a degree of success, one of the disadvantages is that by missing the optimum growing period for nutsedge, the optimum growing period for the crop may be missed as well.

Due to the requirement of nutsedge for light, a simple method for the removal or control of the weed is to reduce the amount of light received by the plant. One such method is that of reducing the row spacing between the crops to increase shading. Row spacing of no more than 600 mm for maize and 380 mm for soybean proved more successful in controlling yellow nutsedge than 900 and 760 mm, respectively (Chappel and Leasure, 1980; Choudhary, 1981). Tomatoes grown with row spacings of one metre as opposed to two metres only required weeding for 24 days rather than 36 days (Anonymous, 1981). Again, although narrowing the row spacing is a form of control of nutsedge, the reason the rows are wide apart is because that is the optimum width for maximum yield of the crop. Narrowing the row may control nutsedge by a small degree but the yield potential of the crop is sacrificed.

Another method for reducing light is alternating competitive crops with the primary crop overtime, rotational cropping. Keeley *et al.* (1983) reported that growing a competitive crop such as maize or soybean, rotating with cotton, the primary crop, reduced the number of yellow nutsedge tubers by 97 - 99% in three years. Although this method may never fully remove a nutsedge infestation, it may be sufficient to control it so that crop yield is not severely reduced. This method reduces the chance of weeds, insects and fungi becoming established in an area due

to alternating the primary crop and the introduction of a competitive secondary crop, or a plant that is able to out-compete the weed.

Other sound farming practices such as appropriate soil pH, row and drill spacing, fertilization and recommended cultivars all improve the competitive nature of the crop and decrease the impact of nutsedges (Nieto, 1970; Hammerton, 1972; Miller, 1973; Osgood *et al.*, 1977; Doll, 1981). If certain strategies can be employed that benefit the crop more than the weed, then the negative effect of the weed will be reduced.

1.2.2 Herbicides

Presently, herbicides are the primary tool in the control of purple and yellow nutsedge. Bioherbicides are still in their infancy with regards to these weeds, and cultural methods of control can only reduce the effects of these weeds, not eradicate them. For a foliar-applied herbicide to be successful it needs to be absorbed and transported to the meristematic regions of the tubers, rhizomes and basal bulbs (Sprankle *et al.*, 1975). Several herbicides being used include dazomet, metam sodium, halosulfuron, sulfentrazone, with the soil fumigant methyl bromide proving to be the most effective (Stall, 1994). A post-emergence herbicide, CGA-362622, has recently been tested for weed control in transgenic and non-transgenic cotton and sugarcane crops (Holloway *et al.*, 2000; Hudetz *et al.*, 2000; Wells *et al.*, 2000; Porterfield *et al.*, 2002a, 2002b). CGA-362622 is a sulfonylurea herbicide, which, like imidazolinones and triazolopyrimidines, inhibits the growth of plants by inhibiting acetolactate synthase (ALS) (Ray, 1984; Shaner *et al.*, 1984; Yadav *et al.*, 1986; Hawkes *et al.*, 1989). ALS is the first enzyme in the pathway to the branched-chain amino acids valine, leucine and isoleucine (Hervieu and Vaucheret, 1996). CGA-362622 showed promise when compared with other sulfonylurea herbicides. After only four days, purple and yellow nutsedge absorbed about 55% of the herbicide, compared with eight days required for similar results obtained for imazaquin, chlorimuron, and foliar applied pyriithiobac (Nandihalli and Bendixen, 1988; Reddy and Bendixen, 1988; Vencill, 1998; Troxler *et al.*, 2003). Furthermore, only 6% of the absorbed CGA-362622 remained in the leaf compared with over 70% for both chlorimuron and imazaquin (Nandihalli and Bendixen, 1988; Reddy and Bendixen, 1988; Troxler *et al.*, 2003). The significance of the former is that the herbicide was being translocated throughout the entire plant thereby resulting in the death of tubers. In separate experiments it was observed that a

combination of imazaquin and MSMA provided similar results to CGA-362622 in the reduction of growth of both nutsedges (McElroy *et al.*, 2003). In field studies performed in cotton and turf, more than 80% control of purple and yellow nutsedge was reported when using CGA-362622 (Porterfield *et al.*, 2002a; Troxler *et al.*, 2002). Troxler *et al.* (2003) indicated there is good potential for CGA-362622 to be used as an effective herbicide for purple and yellow nutsedge.

In a study by Ferrel *et al.* (2004), five herbicides were tested on yellow nutsedge to determine their efficacy. Halosulfuron and imazapic, both ALS-inhibiting herbicides, reduced nutsedge competition with other plants more rapidly than the other herbicides. Bentazon was considered the least effective of the herbicides tested as photosynthetic recovery occurred after just five days with 44% total regrowth, compared with the untreated control. MSMA was least effective of the remaining three, as competition for water was not significantly reduced. Halosulfuron and imazapic were considered the most effective herbicides and glyphosate third. As effective as halosulfuron may be, Grichar *et al.* (2003) reported that post-emergence herbicide applications resulted in chlorosis, stunting and leaf margin necrosis of potato plants. It is possible that similar side effects may be observed in other crops. Kadir and Charudattan (2000) reported that many herbicides do not only affect the weed but have a residual effect that can be very damaging to sensitive crops such as cotton, maize and many vegetable crops.

1.2.3 Bioherbicides

To date the primary focus of research into the biocontrol of purple nutsedge has been the use of insects as natural enemies (Habib, 1976a, 1976b; Phatak *et al.*, 1987). According to Evans (1990) these methods have been unsuccessful due to low specificity of the biocontrol organisms and the relatively poor establishment on the host.

The difficulty in developing a successful fungal bioherbicide is understanding, and then obtaining, the correct environmental and growth conditions for the fungus and host, in both lab and field conditions. The temperature required for most fungal bioherbicides to function at their optimum, or at all, is generally between 10 and 30°C, although this will vary for each pathogen (Daigle and Connick, 1990). The dew period is also crucial; if the dew duration, dew temperature or incubation temperatures are not within the correct range there may be no infection (Kadir *et al.*, 2000a). Spore germination, disease severity, infection and possible control of the

weed are affected by the interaction between moisture and temperature (TeBeest *et al.*, 1978; Capo and TeBeest, 1981). A third critical factor is the knowledge of when the host is most susceptible to infection (Watson and Wymore, 1990).

A number of fungi have been identified as possible bioherbicides of purple and yellow nutsedge. Of these, two have shown the most promise, *Dactylaria higginsii* (Luttrell) M. B. Ellis and *Cercospora caricis* Oud.

1.2.3.1 *Dactylaria higginsii*

Dactylaria higginsii is a fungal pathogen that is highly host-specific to *Cyperus* spp. and *Kyllinga brevifolia* Rottb. (both of which are part of the Family Cyperaceae) (Kadir, 1997; Kadir and Charudattan, 2000). This fungus is highly pathogenic to both purple and yellow nutsedge with symptoms first appearing on the plant four to five days after inoculation (Kadir and Charudattan, 2000). The following description of the infection of *D. higginsii* on purple nutsedge was reported by Barreto and Evans (1995). The lesions on the leaf are elliptical, grey, with a reddish brown to black margin. These lesions are 1 - 6 mm long and 0.5 - 2 mm wide. These may occur as small individual spots or coalesce to form large lesions that may result in the death of an entire leaf. Leaf death may occur 15 - 20 days after inoculation (Kadir and Charudattan, 2000). The internal mycelium is intracellular with a diameter of 1 - 3 μm . Hyphae are branched, septate, and hyaline to pale brown. No stromata or external mycelia are present. Conidiophores moving through the cuticle are amphigenous, solitary, and subulate. They are straight, but inflated at the base, tapering toward the apices, unbranched, pale-brown and smooth. Conidia are solitary, holoblastic and obclavate. They are subacute, eguttulate, pale-brown and smooth. The pathogen causes foliar lesions, reduces the growth components of the plant, and, under certain conditions, kills the weed (Kadir *et al.*, 2000a).

Under greenhouse conditions, Kadir and Charudattan (2000) obtained reductions in shoot and tuber number of purple nutsedge of 73% and 80%, respectively with their respective dry weight reductions being 71% and 67%. In separate experiments, Kadir *et al.* (2000a) managed to obtain reductions in shoot and tuber dry weights in purple nutsedge of 50% and 66%, respectively. With an increase in dew temperatures from 20 - 30°C, they observed an increase in disease severity, with 98% obtained between 25° and 30°C on four- and six-leaf-stage plants, but only

75% obtained on eight-leaf-stage plants. A post-inoculation dew period of 24 hours was required to achieve the maximum disease severity of 95% on the four- and six-leaf-stage plants and 75% on eight-leaf-stage plants. It is therefore important to spray purple nutsedge early in its life cycle as waiting a few days results in less disease severity which would have detrimental effects on the yield of the crop. Although it is unlikely that these figures will be replicated in field trials, it is important to know the optimum growth and infection conditions.

Kadir *et al.* (2000b) observed that two inoculations of *D. higginsii* on purple nutsedge greatly enhanced disease severity compared with one inoculation, while there was no significant increase in severity with three inoculations. In the field this is even more apparent as the first inoculation may kill the existing shoots but is unable to do the same to the new shoots sprouting from the tubers and bulbs. A second and possibly a third inoculation is required to infect subsequent growth (Kadir *et al.*, 2000b).

According to Barreto and Evans (1995) and Kadir *et al.* (2000a), the pathogen has good potential as a bioherbicide for *C. rotundus*. Kadir *et al.* (2000a) cites its virulence and infection at a broad range of temperatures and dew durations as reasons for this conclusion. They do, however, note that the requirement for a long dew period, which might be overcome by adding appropriate formulations, may limit the success of the pathogen as a biocontrol agent.

1.2.3.2 *Cercospora* spp.

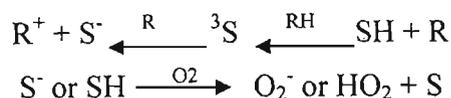
Cercospora caricis is a known phytopathogen of *C. rotundus* and *C. esculentus* and, according to a survey conducted in Israel between 1986 and 1989, has good potential as a biocontrol agent (Quimby *et al.*, 1991). *Cercospora caricis* is a highly host-specific pathogen and only infects plants of the genus *Cyperus*. There is therefore relatively little known about this pathogen as it does not damage any economically important crops. There has been significantly more research directed towards closely related species such as *C. beticola* Sacc. (causing a leaf spot on sugar beet and beetroot), *C. kikuchii* (Matsumoto & Tomoyasu) Gardner (purple seed stain on soybean pathogen), *C. nicotianae* Ellis & Everhart (frog-eye leaf spot on tobacco), *C. zea-maydis* Tehon & Daniels (grey leaf spot on maize), and *C. coffeicola* Berkeley & Cooke (brown eye spot on coffee) (Daub, 1982a; Milat and Blein, 1995; Daub and Ehrenshaft, 2000). According to Farr *et al.* (1989), there are over 500 species of *Cercospora* that cause plant diseases.

Cercospora spp. grow throughout the intercellular spaces of the host plant, without actually penetrating the cell walls (Upchurch *et al.*, 1991). The following description of the infection of *C. caricis* on purple nutsedge was reported by Barreto and Evans (1995). The lesions on the leaves and bracts are originally brown and circular within a chlorotic halo. Later they become pale reddish-brown and elongate with dark margins. Lesions coalesce to cover the width of the leaf, leading to the death of the leaf. Internal mycelia are inconspicuous, external mycelia are absent, and the stomata are either absent or very poorly developed. The conidiophores, which arise through the stomata, are hypophyllous, fasciculate, cylindrical and tapering towards the apices. They are straight or sinuose, unbranched, pale brown and smooth. Conidia are generally solitary, dry, holoblastic and acicular to subcylindrical. They are straight or curved, guttulate, hyaline and smooth, and the apex is rounded to subacute.

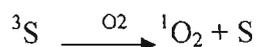
Cercospora spp. produce a number of toxins, with cercosporin and the beticolins the most well known. Beticolins are yellow compounds produced by *C. beticola*, the only documented fungus to produce these toxins. They are photodynamically active compounds that can begin the peroxidation of membrane lipids (Ducrot, 2001). Gomes *et al.* (1996), analysing beticolin-1, one of 15 such toxins isolated, reported that it inhibited the ATP-dependent H^+ -transport across membranes. This was achieved by the inhibition of H^+ -ATPase.

The most important toxin produced, in terms of pathogenicity, is cercosporin, which degrades the host membrane to allow the fungus to receive its nutrients (Daub and Ehrenshaft, 2000). The first isolation of this toxin was by Kuyama and Tamura (1957) from *C. kikuchii*. Cercosporin has subsequently been found in many species of *Cercospora* including *C. caricis* (Blaney *et al.*, 1988). This toxin is one of the reasons that this group of fungal pathogens is successful. When this toxin was isolated and applied to various host species the symptoms were similar to that of the disease caused by *Cercospora* (Daub, 1982a). Mutant *C. kikuchii* that produced approximately 2% of the cercosporin found in wild type cultures showed little or no evidence of pathogenicity on soybean leaves in relation to the wild type (Upchurch *et al.*, 1991). This gives further evidence that cercosporin is an integral part in the virulence of this fungus. It has subsequently been reported to be toxic to plants, mice and bacteria (Yamazaki *et al.*, 1975; Daub, 1982a). Daub (1982b) reported that tobacco tissue leaked ions, while protoplasts burst one to two minutes after being treated with cercosporin.

Daub (1982a) confirmed that cercosporin acts as a toxic photosensitizing agent in plants, which means that it is only capable of killing cells in the presence of light. Photosensitisers are common in all plants in the form of chlorophyll, coumarins, thiophenes and acetylenes, all of which are strong photosensitisers (Heitz and Downum, 1995). Photosensitisers work by absorbing light to create a long-lived electronically excited state (triplet state, 3S) that reacts with oxygen to form a compound that is toxic to the plant (Daub and Hangarter, 1983). This 3S may react with a reducing substrate (R or RH) by the transfer of a hydrogen atom or electron, and then react with O_2 to produce superoxide ions O_2^- (Foote, 1976). The following three formulae are taken directly from Daub and Hangarter (1983):



A second option is that 3S reacts directly with O_2 “yielding the electronically excited singlet state of O_2 (1O_2)” (Daub and Hangarter, 1983):



Daub and Hangarter (1983) reported that, although O_2^- is produced by cercosporin, it is the 1O_2 that is responsible for the toxicity of cercosporin in plants. There are a large number of plants resistant to O_2^- -generating agents - one example is certain cultivars of rye grass which have significantly higher levels of superoxide-scavenging enzymes, peroxidase and catalase, and are resistant to paraquat (Harper and Harvey, 1978). Resistance to 1O_2 , however, is rarer. Carotenoids in plants are good quenchers of photo-oxidation processes sensitized in the chloroplasts, as well as quenching 1O_2 successfully in experiments, but no plant is known to be resistant to 1O_2 (Foote, 1976; Krinsky, 1979). With this knowledge, a few herbicides have been produced that act exclusively by inhibiting the synthesis of carotenoids, thus resulting in photobleaching and cell death by 1O_2 (Sandmann and Boger, 1989; Sollod *et al.*, 1992). In contrast, all species of *Cercospora* and many other fungi appear to be resistant to the toxin (Daub and Hangarter, 1983). Not only are *Cercospora* spp. resistant to cercosporin, but they are resistant to a number of structurally diverse 1O_2 -generating photosensitisers such as porphyrin, thiophene and xanthene dye (Jenns *et al.*, 1995; Chung *et al.*, 1999). This suggests that the fungus is not resistant to cercosporin, or any other compound, but rather to 1O_2 .

Cercosporin plays an important role in disease severity of *Cercospora* spp. A number of papers have reported that disease severity of the host plant was reduced when grown in low light conditions, or where plants were close together and shading was a factor (Calpouzos and Stalknecht, 1967).

Cercospora caricis is a relatively damaging fungus to purple nutsedge, and methods have been examined to alter the pathogenicity of the fungus. One such method, although indirect, was reported by Aly *et al.* (2001). They observed that, when inoculating the plants, a combination of plant hormones and *C. caricis* increased the damage to purple nutsedge by 30%. They subsequently surmised that increased expression of the plant's genes encoding the hormones, such as indole acetic acid (IAA) and cytokinin, might enhance the virulence of the fungus. Another method is to increase the production of cercosporin.

Barreto and Evans (1995), when studying a strain of *C. caricis* found in Rio de Janeiro, observed that the fungus is not restricted to specific seasons and has potential to cause great damage to purple nutsedge. Its distribution suggests that the fungus has a large tolerance for temperature variations, but it is restricted to regions where the mean annual precipitation is below 1400 mm. Barreto and Evans (1995), however, suggested that the use of *C. caricis* as a mycoherbicide is a possibility, but that its slow growth and the difficulty researchers have in obtaining spores mean that it is very difficult to work with this fungus.

1.2.3.3 Other potential bioherbicides

There are a number of other pathogens that infect *C. rotundus* and *C. esculentus*, each one having the potential to be used as a biocontrol agent. Barreto and Evans (1995) reported a few possibilities from a study of *C. rotundus* in Brazil.

Cintractia limitata G. B. Clinton is a rare smut in Rio de Janeiro, although the reason for its scarcity is not understood. This pathogen only causes damage to the inflorescence of the weed resulting in fewer seeds than would ordinarily be available. As this weed does not propagate readily by seed, but rather by tubers and rhizomes, this smut does not show great potential as a biocontrol agent.

Duosporium cyperi K. S. Thind & Rawla is more commonly found on *Cyperus iria* L., but it has been found on *C. rotundus*. It causes dark-brown lesions on the leaves with a linear necrosis that may be present on either half or the entire leaf breadth. This may lead to leaf death. It is better adapted to the warmer climates, as it was only present at locations with a mean annual temperature above 22°C. It was reported that this Hyphomycete caused serious foliage disease of *C. rotundus*. This fungus grows slowly in culture but sporulates readily; a good attribute for bioherbicides. Evans (1987) indicated that this fungus has potential as a biocontrol agent.

Puccinia canaliculata (Schwein.) Lagerh. results in lesions on the leaves that are often overgrown by a hyperparasitic Coelomycete. Lesions are initially uredinial, irregular and chlorotic, and become linear and reddish-brown. They finally become telial. These lesions often coalesce, leading to the death of the leaf. The fungus is relatively common, but only under very specific conditions. Requirements are mean annual temperatures above 22.5°C and mean annual rainfall below 1400 mm. It is not restricted to seasons and occurs throughout the year. Although this fungus has been reported to infect *C. esculentus*, it also causes serious disease on *C. rotundus* (Phatak *et al.*, 1983). This rust shows potential as a bioherbicide, and the fact that it causes disease on both *Cyperus* spp. is advantageous as both are recognized weeds. *Puccinia romagnoliana* Maire & Sacc. is closely related to, if not in fact being, *P. canaliculata*. The biocontrol potential of this fungus on *C. rotundus* was investigated by Bedi and Sokhi (1994). A number of greenhouse trials proved successful as the number and weight of tubers were reduced. Problems in inoculum production, however, resulted in the further development of this fungus as a bioherbicide being abandoned.

1.3 MUTATION OF FUNGI

When mutating an organism, whether plant, fungus or animal, it is important to have certain targets and objectives. In the mutation of a bioherbicidal fungus the objective is to increase the virulence of that fungus and therefore the target is any substance or mechanism that might aid in this.

Certain herbicides such as sulfonylureas, imidazolinones and triazolopyrimidines inhibit the growth of plants by inhibiting acetolactate synthase (ALS) (Ray, 1984; Shaner *et al.*, 1984; Yadav *et al.*, 1986; Hawkes *et al.*, 1989). As mentioned earlier, ALS is the first enzyme in the

pathway to the branched-chain amino acids valine, leucine, and isoleucine (Hervieu and Vaucheret, 1996). Valine is known to inhibit the growth of the seedlings and protoplasts of higher plants (Mifflin, 1969; Bourgin, 1978). It has also been reported that valine inhibits the activity of the ALS enzyme; this indicates that valine is involved in end-product inhibition of ALS, and subsequently inhibits the growth of plants by inhibiting ALS production (Mifflin, 1971; Relton *et al.*, 1986). There is, therefore, the prospect that increasing the amount of valine produced by a pathogen would increase the virulence of that pathogen. Another possible target in the mutation of a bioherbicidal fungus would be the natural toxins produced by that fungus. An example of this would be to increase levels of either cercosporin or one of the beticolins produced by *Cercospora* spp. by mutation. Aly *et al.* (2001) indicated that increasing the expression of hormones such as IAA or cytokinin might increase the virulence of *C. caricis* on purple nutsedge.

A less direct target for mutation may include a number of factors that aid the fungus in survival or in the infection process. These include thicker cell walls of the spores, greater sporulation, higher infection levels, greater tolerance of the fungus to desiccation and temperature variations, etc. The objective of most mutations should be to include a combination of these factors with the primary mutation target.

1.3.1 Mutation

Biolistics, or microprojectile bombardment, is a method by which DNA is shot into the cells of the organism. This DNA is then incorporated into the host genome and transformation is complete. This method has been used successfully to transform *C. caricis* and, according to Aly *et al.* (2001) has great potential in improving the efficacy of the fungus as a biocontrol agent. Aly *et al.* (2001) initially attempted to transform *C. caricis* using protoplasts and electroporation. These methods proved unsuccessful so biolistics was attempted. They were able to achieve expression by two introduced marker genes, the β -glucuronidase (GUS) and the hygromycin B resistance genes. They reported greatest transformation of two transformants per μg of DNA under the following conditions: target cells inoculated onto PDA five days previously, helium pressure of 1100 psi, M17 tungsten particles, three bombardments per Petri dish, and 60 mm between the macrocarrier launcher and the target cells.

Upchurch *et al.* (1991) reported a simple method for obtaining mutants. In their experiments *C. kikuchii* was being selected for a reduction in cercosporin production. A concentration of 2×10^6 spores per ml was prepared. From this, 300 μ l was irradiated with ultraviolet (UV) light at $500 \mu\text{W}\cdot\text{cm}^{-2}$. This solution was then plated out onto a mutant selection minimal medium. Potential mutants, colonies either white or tan as opposed to red, were selected for further analysis. They observed that only eight minutes were required to kill 95 - 99% of the spores. Of the 4820 survivors, only 0.37% were white-tan, of which only three (0.06% of the total number of survivors) were identified as stable mutants producing less cercosporin.

Cercospora spp. are notorious for producing very few spores, and for being very difficult to induce sporulation in the lab (Gwinn and Daub, 1989). The use of protoplasts has been reported as an alternative when mutating *C. nicotianae* as the majority of mycelial cells are uninucleate (Gwinn and Daub, 1989). It is possible to mutate these protoplasts with UV light or a mutagen. Gwinn and Daub (1989) reported successful mutation of protoplasts of *C. nicotianae* using MNNG (N-methyl-N'-nitro-N-nitrosoguanidine). Protoplasts were exposed to $20 \mu\text{g}\cdot\text{ml}^{-1}$ of MNNG for 30 min. After two weeks benomyl resistant mutants were obtained at a frequency of 10^{-9} spores per ml.

1.3.2 Screening

Screening for transformants of the microprojectile bombardment is a relatively simple method as all fungi that have incorporated the GUS gene have blue spots within the hyphae, while the wild-type, where the GUS gene is not being expressed, has no blue spots (Aly *et al.*, 2001). Southern blot analysis is then used to determine whether the second gene has been incorporated into the genome of the transformed fungus (Aly *et al.*, 2001).

Milat and Blein (1995) described a technique for determining the presence of cercosporin and six beticolin toxins from *C. beticola*. After growing the colony and extracting the toxins they purified the samples using flash chromatography. The basis of flash chromatography is that different-sized particles will travel through silica gel at different speeds. In this way, substances produced by the fungus would be separated into layers based on size. These layers can then be collected and analysed. The flash chromatography was monitored by thin-layer chromatography (TLC).

Before purification by flash chromatography, a drop of the extracted toxins was placed on a TLC plate and, similar to flash chromatography, the smaller units move faster than the larger ones resulting in a number of small spots on the plate. This gives an indication as to the number of layers that should be present in the flash chromatography unit. When the layers have been separated, another TLC can be performed to determine the purity of the extracted sample. Each sample should produce only one spot.

High-performance liquid chromatography (HPLC) was used to determine the presence and/or identity of the various toxins. Each purified sample was passed, separately, through a column and analysed using UV detection. The different peaks obtained in the chromatogram can then be compared with other chromatograms produced or with pure samples of each specific toxin.

With flash chromatography Milat and Blein (1995) were able to obtain large amounts (a few milligrams) of cercosporin and the beticolin toxins, with good reproducibility, by pre-treating the silica gel with phosphoric acid and calcium bis(dihydrogenphosphate). Pre-treating the TLC plates with the same two chemicals and then heating to 120°C to reactivate the plates gave better results than using untreated plates. Milat and Blein (1995) reported that the HPLC method described was a convenient method for analysing secondary metabolites produced by *C. beticola*, and that there is possible correlation between pathogenicity and toxin production.

The above method is a very thorough and precise method for determining the amount of cercosporin, or other toxins, produced, but it is a rather tedious method with each step being time consuming. A far simpler method, although less accurate, was used by Velicheti and Sinclair (1994), where they used spectrophotometry to determine the amount of cercosporin produced. This technique is based on the fact that cercosporin has a slight red colour to it and, when *Cercospora* spp. are grown on agar, the cercosporin produced moves into the agar and the difference in colouration can be determined.

The fungus is grown on a potato dextrose agar medium for no less than 21 days, after which a sample is placed in 5 ml of 5.0 N KOH and the absorption recorded at 480 nm. The amount of cercosporin in that sample can then be determined using the formula put forward by Yamazaki and Ogawa (1972): [(absorbance at 480 nm/23300) 534] µg, where 23300 is the molar extinction

coefficient (ϵ) at 480 nm and 534 is the molecular weight of cercosporin. Subtle variations of this technique have been reported (Upchurch *et al.*, 1991).

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CHAPTER 2

Effects of differing concentrations of amino acids on *Cyperus rotundus*

ABSTRACT

Dactylaria higginsii (Luttrell) M. B. Ellis is, in many parts of the world, a fungal pathogen of *Cyperus rotundus* L. CYPRO and *Cyperus esculentus* L. CYPES, two major weeds worldwide. The increase in virulence of *D. higginsii* would have a major financial impact. A potential target for increasing virulence is by increasing the production of certain amino acids, a number of which are involved in feedback-inhibition systems. Two amino acids, valine and leucine, regulate the production of ALS (acetolactate synthase), an enzyme already targeted by many herbicides. When testing these amino acids and several others on *C. rotundus* tuber germination, only glutamine at 600 mg.l⁻¹ was significantly ($P < 0.001$) different from the percentage germination of the water control. Four treatments were significantly ($P < 0.001$) different from the control when shoot length of southern African *C. rotundus* was tested. Furthermore, after injecting the leaves with the amino acids, no effect was observed. These contrasted with the glyphosate control, which resulted in total death of the plants in both experiments.

2.1 INTRODUCTION

Evans (1987) reported that *Dactylaria higginsii* (Luttrell) M. B. Ellis is a fungal pathogen of *Cyperus rotundus* L. CYPRO (a nutsedge that has been referred to as the world's worst weed, Holm *et al.*, 1977) in a number of countries including Australia, Cuba, Sarawak, Sudan, USA and Venezuela. Damage to crops by weeds results in massive financial losses every year. *Cyperus rotundus* alone can result in the yield reduction of sugar cane by up to 75% (Cerrizuela, 1965). Herbicides remain the primary means of weed control, but the efficacy of most herbicides is limited by lack of specificity and increasing tolerance of many weeds to previously effective chemicals. Furthermore, many herbicides damage the weed as well as the crop and the natural ecosystem surrounding the plots. This has led to many effective herbicides being taken off the market. For this reason there has been an increase in the research of biological control agents, such as fungi, that have the ability to target the weed and leave the crop undamaged.

A major concern for farmers is the presence and proliferation of tubers by *C. rotundus* and *Cyperus esculentus* L. CYPES. Rao (1968) observed that in 90 days, one tuber could produce 99 tubers. This equates to approximately eight million tubers per hectare of cultivated land. For this reason it is important for any herbicide or bioherbicide to target the roots and tubers as well as the aerial portions of the plant. Even the most successful current herbicides are unable to eliminate all the tubers. For this reason acetolactate synthase (ALS)-inhibiting herbicides were developed. This is also the aim of many researchers developing bioherbicides. A number of herbicide families are currently used that target this ALS pathway, they include: sulfonylureas, imidazolinones, triazolopyrimidines, glyphosate and pyrimidinyl oxybenzoates (Eberlein *et al.*, 1997). ALS is the first enzyme in the production of branched-chain amino acids and catalyses two parallel reactions; these are the condensation of 1 mole of pyruvate with 1 mole of 2-oxobutyrate to form acetohydroxybutyrate; and the condensation of 2 moles of pyruvate to form acetolactate (Eberlein *et al.*, 1997). The amino acids synthesised, i.e., valine, leucine and isoleucine, are part of a feedback-inhibition system that regulates the production of ALS. Mifflin (1969) reported that seedlings of higher plants are inhibited by the addition of valine.

Barreto and Evans (1995) reported on the potential of a number of fungal pathogens as biocontrol agents for this weed. There are a number of favourable characteristics of *D. higginsii* that make it a good prospect for use as a bioherbicide. Firstly, it grows well in culture. This is important in the production of the bioherbicide because slow-growing pathogens take time for the inoculum to be created and, if not looked after, often lose viability after a number of sub-cultures. Secondly, it sporulates readily in culture. Fungi can increase in number by the breaking off of mycelial fragments which land on a leaf, or favourable substrate, and continue to grow; or more commonly, by spores. In the field *D. higginsii* produces a prolific number of spores which are distributed by wind. Spores that rest on a host plant of *C. rotundus* produce germ tubes which penetrate the leaf and subsequently start a new colony. The same concept is used when developing a biocontrol agent. The fungus produces a number of spores which are then collected and sprayed onto the leaves. It is therefore important that the fungus can produce a large number of spores in culture. The third reason *D. higginsii* is a good prospect is due to the damage it causes on the infected leaf. In an experiment where purple nutsedge shoots were spray-inoculated with *D. higginsii*, a decrease in shoot and tuber numbers by 73% and 80%, respectively, was observed (Kadir and Charudattan, 2000).

There are, however, a number of concerns about the potential of this fungus in controlling *C. rotundus*. When Barreto and Evans (1995) reported on *D. higginsii*, it was observed that the fungus was only present in 10% of the locations where *C. rotundus* was present. Two possible explanations were given: first, that temperature probably affects the distribution, as no fungus was present where the average temperature was less than 22.5°C; and second, that the poor distribution may be attributed to its possible recent introduction into the state of Rio de Janeiro, where the samples were collected. These are two hypotheses which await confirmation. It is possible that some ecotypes of *C. rotundus* have developed resistance to the fungus, or, have physical or chemical attributes which render them less suitable as hosts of *D. higginsii*. Ranade and Burns (1925) described four ecotypes in India based on glume colour ranging from a yellow-white, light red, coppery red to dark red. The presence of these different ecotypes suggests that ecotypes probably exist that exhibit different resistances to pathogens. Although no trials were carried out with *D. higginsii*, this would have a detrimental impact on its use as a bioherbicide.

The primary aim of the experimental work in this chapter was to determine the effect that an increase in amino acid production, particularly valine, by the fungus would have on the growth and well-being of the leaves as well as the germination and shoot growth of the tubers of *C. rotundus*. A secondary aim was, provided *C. rotundus* was susceptible to an increase in valine or another amino acid, to increase the virulence of *D. higginsii* by creating a mutation that resulted in increased production of the harmful amino acid.

2.2 MATERIALS AND METHODS

2.2.1 Tuber germination and shoot length

Tubers of *C. rotundus* were collected from Ukulinga (30°24'0" E, 29°39'0" N), a research farm in Pietermaritzburg (30°25'0" E, 29°35'0" N), South Africa. Petri dishes (70 mm) were prepared by placing a thin layer of cotton wool at the base. Four tubers were placed in each dish and the cotton wool dampened with either L-lysine monohydrochloride, glycine, DL-tryptophan, L-asparagine, L-glutamine, L-valine, in concentrations of 100, 200, 300 and 400 mg.l⁻¹. Water was used as the control. Two Petri dishes were set up for each amino acid concentration; eight tubers were tested per concentration. Twenty tubers were used in the water control with f. The cotton wool was monitored daily to ensure it remained damp. The Petri dishes were stored in the

dark in a 25°C incubator where germination was monitored. After 8 days (d), shoot lengths were measured. Shoot lengths for glyphosate (round-up), an effective herbicide, were taken from the second experiment and incorporated into the results. Glyphosate was used as a control indicating the expected results of high concentrations of amino acids.

A second, separate, experiment was performed with three controls: a negative control, water; a positive control, glyphosate; and a dry control, no liquid added, to ensure the tubers were not germinating with the aid of the liquid present inside the tubers. Five tubers were placed in each Petri dish, with four Petri dishes used for each concentration; 20 tubers per concentration, 100 tubers per amino acid. The tubers were collected from the same field. In addition to the six amino acids used in the first experiment, leucine was also tested. The experiment ran for 12 d. The cotton wool was dampened with the amino acids in concentrations of 200, 400, 600, 800, and 1000 mg.l⁻¹. Glyphosate was applied in three concentrations: 1, 2 (full strength), and 4 ml.l⁻¹. Dishes were then stored in the dark in a 25°C incubator and monitored as in the previous experiment. Tubers that lost viability in the course of the experiment were regarded as failing to germinate, even though they may have shown initial signs of germination.

2.2.2 Leaf inoculation of *Cyperus rotundus*

The response to a foliar application of water, valine, leucine and glyphosate on *C. rotundus* was tested. Approximately 0.5 ml of the solution was injected, using a syringe and needle, into separate leaves of the weed. The flattened tip of the needle was pressed firmly on the underside of the leaf and a finger applying pressure on the top. The liquid was then slowly forced out of the syringe and into the veins of the leaf. Once the solution had moved approximately 2 mm up the leaf, no more was added. Three solutions were tested, i.e., valine (600 mg.l⁻¹), leucine (600 mg.l⁻¹), glyphosate (2 ml.l⁻¹), and water. Five leaves, of varying age and size, per plant were injected with one of the four solutions. Fifteen plants were tested per solution. After the plants injected with glyphosate had died the experiment ran for a further two weeks.

2.2.3 Statistical analysis

Effects of amino acids on germination and shoot length of tubers was analysed statistically. Data were subjected to analysis of variance (ANOVA) using GenStat[®] Executable Release 6

Statistical Analysis Software to determine any difference between treatment means. Arc sine (angular) transformation was used for germination data due to percentage data lying from 0 – 100%. All least significant differences were determined at $P < 0.05$. Interpretation of analysis of variance is only valid when specific assumptions are made, which are: additive effects, independence of errors, homogeneity of variance and normal distribution. If one or more of these assumptions are not met, the sensitivity of the F test and the level of significance in the analysis of variance are affected.

2.3 RESULTS

2.3.1 Tuber germination

Only results from the second trial are presented. Figs 2.1 and 2.2 show the results of the germination of *C. rotundus* tubers at the mean concentration for amino acids and glyphosate tested (2 ml.l^{-1} is full strength concentration for glyphosate). There was much variation in germination at Day 2, but by Day 12 only glutamine, at 600 mg.l^{-1} , was significantly different (at $P < 0.001$) from the water and glyphosate controls, and the majority of other amino acids tested. It had the lowest germination at Day 12 of 80% (Tables 2.1 and 2.2). Valine and leucine, the two amino acids anticipated to have some effect on the germination of the tubers, had no significant effect, with 90% and 100% being the lowest germinating percentages at Day 12 respectively.

Initially the tubers soaked in glyphosate germinated, but by Day 12 the shoots and tubers had died and germination had ceased. Tubers that had initially germinated but were dead were regarded as not germinating (Fig. 2.1). Effects of glyphosate are significantly ($P < 0.001$) different from all treatments tested for Day 7 and Day 12. The dry control is not shown here as there was no germination from any tubers.

Similar results were obtained from the first experiment where only 8 tubers were tested per concentration (data not shown).

Table 2.1. Percent germination of *Cyperus rotundus* tubers grown in Petri dishes with amino acids lysine, tryptophan and valine at a range of concentrations. Water and glyphosate were used as controls. (n = 20 tubers per treatment)

Treatments	Germination (%)	Germination (%)	Germination (%)
	Day 2	Day 7	Day 12
Water	70 (57.1) ^{de}	100 (88.7) ^c	100 (88.72) ^c
Glyphosate 1 ml.l ⁻¹	10 (13.9) ^a	20 (25.8) ^a	0 (1.28) ^a
Glyphosate 2	20 (25.8) ^{abc}	20 (25.8) ^a	0 (1.28) ^a
Glyphosate 4	15 (22.5) ^{ab}	15 (22.5) ^a	0 (1.28) ^a
Lysine 200 mg.l ⁻¹	90 (76.1) ^{ef}	100 (88.7) ^c	100 (88.72) ^c
Lys 400	80 (63.4) ^{def}	100 (88.7) ^c	100 (88.72) ^c
Lys 600	90 (76.1) ^{ef}	90 (76.1) ^{bc}	90 (76.08) ^{bc}
Lys 800	90 (76.1) ^{ef}	100 (88.7) ^c	100 (88.72) ^c
Lys 1000	90 (76.1) ^{ef}	100 (88.7) ^c	100 (88.72) ^c
Tryptophan 200 mg.l ⁻¹	90 (76.1) ^{ef}	100 (88.7) ^c	100 (88.72) ^c
Try 400	70 (57.1) ^{de}	90 (76.1) ^{bc}	100 (88.72) ^c
Try 600	50 (45) ^{bcd}	100 (88.7) ^c	100 (88.72) ^c
Try 800	80 (63.4) ^{def}	100(88.7) ^c	100 (88.72) ^c
Try 1000	90 (76.1) ^{ef}	100 (88.7) ^c	100 (88.72) ^c
Valine 200 mg.l ⁻¹	80 (63.4) ^{def}	90 (76.1) ^{bc}	90 (76.08) ^{bc}
Val 400	90 (76.1) ^{ef}	100 (88.7) ^c	100 (88.72) ^c
Val 600	70 (57.1) ^{de}	90 (76.1) ^{bc}	90 (76.08) ^{bc}
Val 800	20 (25.8) ^{abc}	90 (76.1) ^{bc}	90 (76.08) ^{bc}
Val 1000	80 (63.4) ^{def}	90 (76.1) ^{bc}	100 (88.72) ^c
F test	(<0.001)	(<0.001)	(<0.001)
l.s.d.	(26.91)	(18.11)	(15.534)
s.e.d.	(13.30)	(8.96)	(7.673)
cv%	(21.50)	(11.30)	(9.7)

- 1-way ANOVA with transformed means.

- Values in parenthesis represent transformed means using angular transformation.

- Means with the same letter in each column are not significantly different at P<0.05.

Table 2.2. Percent germination of *Cyperus rotundus* tubers grown in Petri dishes with amino acids asparagine, glycine, glutamine and leucine at a range of concentrations. Water and glyphosate were used as controls. (n = 20 tubers per treatment)

Treatments	Germination (%)	Germination (%)	Germination (%)
	Day 2	Day 7	Day 12
Water	70 (57.1) ^{de}	100 (88.7) ^c	100 (88.72) ^c
Glyphosate 1 ml.l ⁻¹	10 (13.9) ^a	20 (25.8) ^a	0 (1.28) ^a
Glyphosate 2	20 (25.8) ^{abc}	20 (25.8) ^a	0 (1.28) ^a
Glyphosate 4	15 (22.5) ^{ab}	15 (22.5) ^a	0 (1.28) ^a
Asparagine 200 mg.l ⁻¹	80 (63.4) ^{def}	100 (88.7) ^c	100 (88.72) ^c
Asp 400	70 (57.1) ^{de}	80 (63.4) ^b	90 (76.08) ^{bc}
Asp 600	50 (45) ^{bcd}	90 (76.1) ^{bc}	90 (76.08) ^{bc}
Asp 800	90 (76.1) ^{ef}	100 (88.7) ^c	100 (88.72) ^c
Asp 1000	80 (63.4) ^{def}	100 (88.7) ^c	100(88.72) ^c
Glycine 200 mg.l ⁻¹	80 (69.7) ^{def}	100 (88.7) ^c	100 (88.72) ^c
Gly 400	90 (76.1) ^{ef}	100 (88.7) ^c	100 (88.72) ^c
Gly 600	50 (45) ^{bcd}	90 (76.1) ^{bc}	90 (76.08) ^{bc}
Gly 800	90 (76.1) ^{ef}	100 (88.7) ^c	100 (88.72) ^c
Gly 1000	90 (76.1) ^{ef}	100 (88.7) ^c	100 (88.72) ^c
Glutamine 200 mg.l ⁻¹	90 (76.1) ^{ef}	100(88.7) ^c	100 (88.72) ^c
Glu 400	70 (57.1) ^{de}	100 (88.7) ^c	100 (88.72) ^c
Glu 600	70 (57.1) ^{de}	80 (63.4) ^b	80 (63.4) ^b
Glu 800	90 (50.8) ^{cde}	100 (88.7) ^c	100 (88.72) ^c
Glu 1000	70 (57.1) ^{de}	100 (88.7) ^c	100 (88.72) ^c
Leucine 200 mg.l ⁻¹	70 (57.1) ^{abcde}	100 (88.7) ^c	100 (88.72) ^c
Leu 400	90 (76.1) ^{ef}	90 (76.1) ^{bc}	100 (88.72) ^c
Leu 600	80 (63.4) ^{def}	100 (88.7) ^c	100 (88.72) ^c
Leu 800	100 (88.7) ^f	100 (88.7) ^c	100 (88.72) ^c
Leu 1000	100 (88.7) ^f	100 (88.7) ^c	100 (88.72) ^c
F test	(<0.001)	(<0.001)	(<0.001)
l.s.d.	(26.91)	(18.11)	(15.534)
s.e.d.	(13.30)	(8.96)	(7.673)
cv%	(21.50)	(11.30)	(9.7)

- 1-way ANOVA with transformed means.

- Values in parenthesis represent transformed means using angular transformation.

- Means with the same letter in each column are not significantly different at P<0.05.

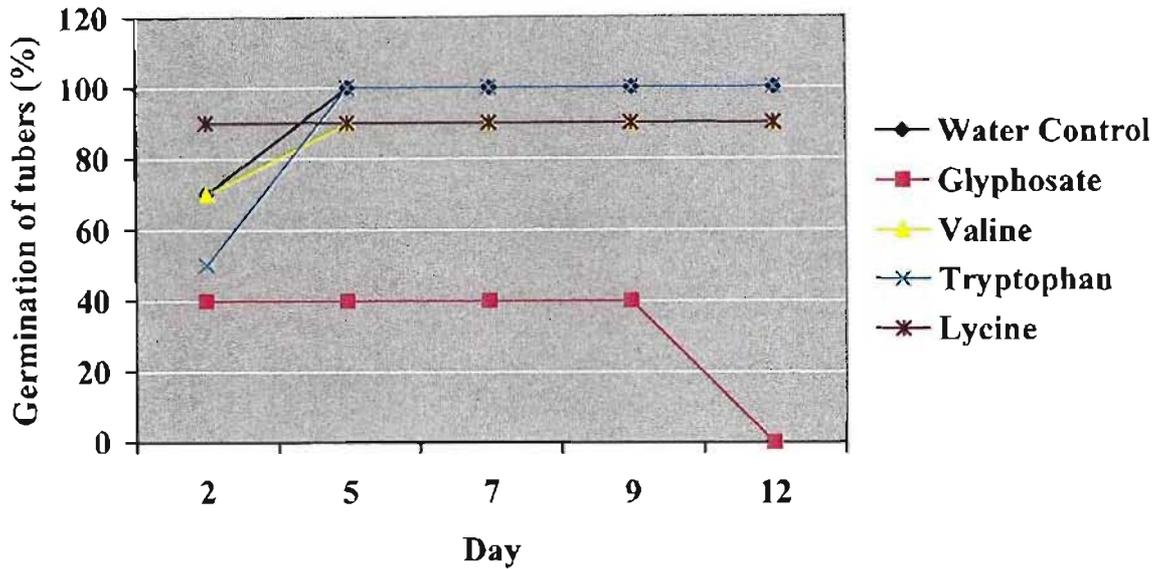


Fig. 2.1. Percentage germination of tubers of *Cyperus rotundus* with valine, tryptophan and lysine added at a concentration of 600 mg.l⁻¹, and glyphosate added at a concentration of 2 ml.l⁻¹. (n = 20 tubers per treatment)

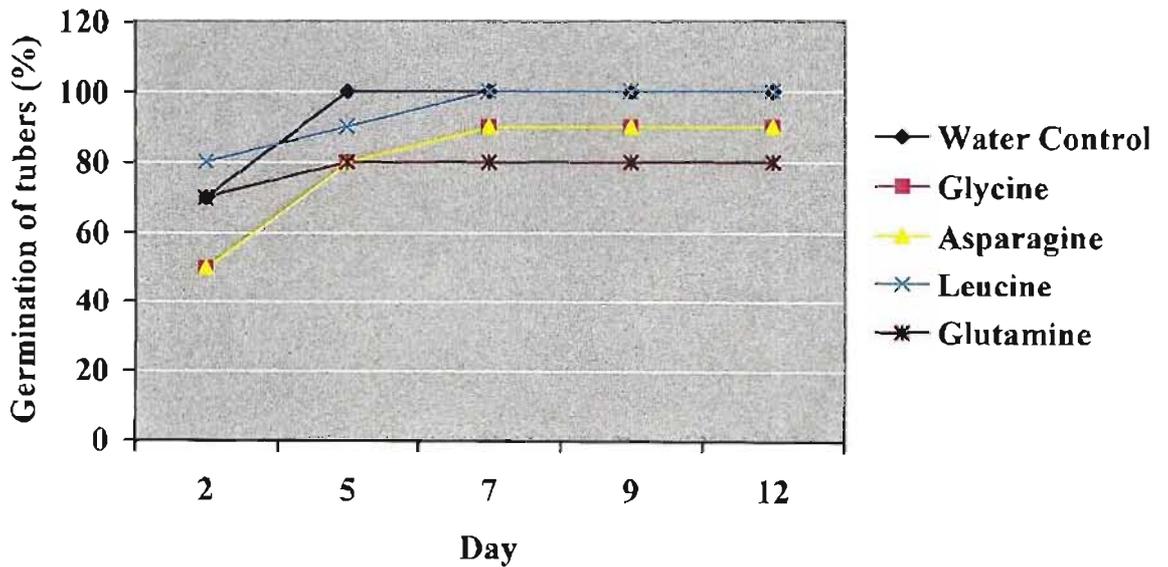


Fig. 2.2. Percentage germination of tubers of *Cyperus rotundus* with glycine, asparagine, leucine and glutamine added at a concentration of 600 mg.l⁻¹, with the water control. (n = 20 tubers per treatment)

2.3.2 Shoot length

There was significant variation in shoot lengths ($P < 0.001$) for *C. rotundus* tubers treated with separate amino acids at a range of concentrations (Table 2.3; Fig. 2.3). Asparagine at 100 mg.l^{-1} was the only treatment which gave a significantly ($P < 0.001$) shorter average shoot length to that of the water control. Glycine at 200 mg.l^{-1} , and lysine at 200 and 400 mg.l^{-1} were the only other treatments significantly ($P < 0.001$) different to that of the water control, all of which had greater average shoot lengths.

Table 2.3. Average shoot lengths of *Cyperus rotundus* tubers grown in Petri dishes with amino acids asparagine, glycine, glutamine, lysine, tryptophan and valine at concentrations indicated. Water and glyphosate were used as controls. (n = 20 tubers per treatment)

Treatment	Average Shoot Length after 12 d (mm)
Glyphosate 1 ml.l ⁻¹	0 ^a
Glyphosate 2	0 ^a
Glyphosate 4	0 ^a
Water	42.51 ^{cdefgh}
Asparagine 100 mg.l ⁻¹	22.85 ^b
Asp 200	53 ^{hijk}
Asp 300	51.4 ^{ghijk}
Asp 400	36.25 ^{cde}
Glycine 100 mg.l ⁻¹	43.25 ^{defgh}
Gly 200	60.29 ^k
Gly 300	35.13 ^{cde}
Gly 400	35 ^{cde}
Glutamine 100 mg.l ⁻¹	35.25 ^{cde}
Glu 200	44.75 ^{efghi}
Glu 300	38 ^{cdef}
Glu 400	32.92 ^{bcd}
Lysine 100 mg.l ⁻¹	48 ^{ghij}
Lys 200	54.5 ^{ijk}
Lys 300	32.15 ^{bc}
Lys 400	55.55 ^{jk}
Tryptophan 100 mg.l ⁻¹	41.3 ^{cdefg}
Try 200	52.25 ^{hijk}
Try 300	38.45 ^{cdef}
Try 400	33.25 ^{bcd}
Valine 100 mg.l ⁻¹	50.45 ^{ghijk}
Val 200	33.11 ^{bcd}
Val 300	36 ^{cde}
Val 400	38.19 ^{cdef}
F test	<0.001
l.s.d.	10.610
s.e.d.	5.179
cv%	13.9

- Means with the same letter in each column are not significantly different at P<0.05.

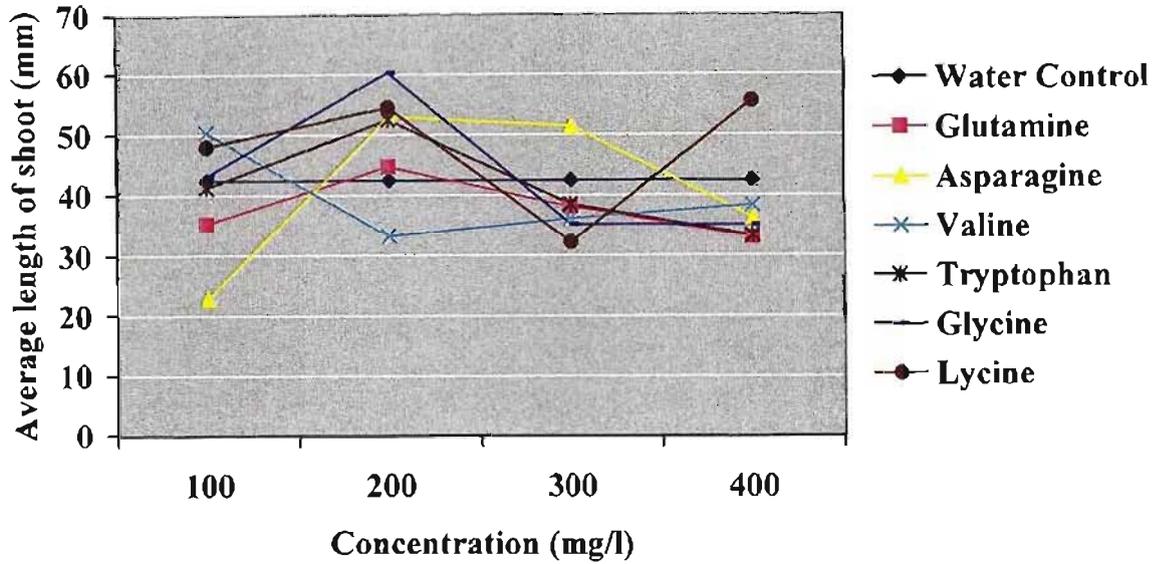


Fig. 2.3. Average length of *Cyperus rotundus* shoots after tubers were incubated for 8 d in amino acids glutamine, asparagine, valine, tryptophan, glycine, and lysine at different concentrations. Tubers were grown in water as a control.

2.3.3 Leaf inoculation

Fig. 2.4 shows two pots, each with a few *C. rotundus* plants. The photographs were taken 11 d after the commencement of the experiment. The experiment ran for a further two weeks with no change in the results obtained at this point. All plants injected with glyphosate were dead after 11 d, while the plants injected with valine, leucine and water showed no damage or lesions.

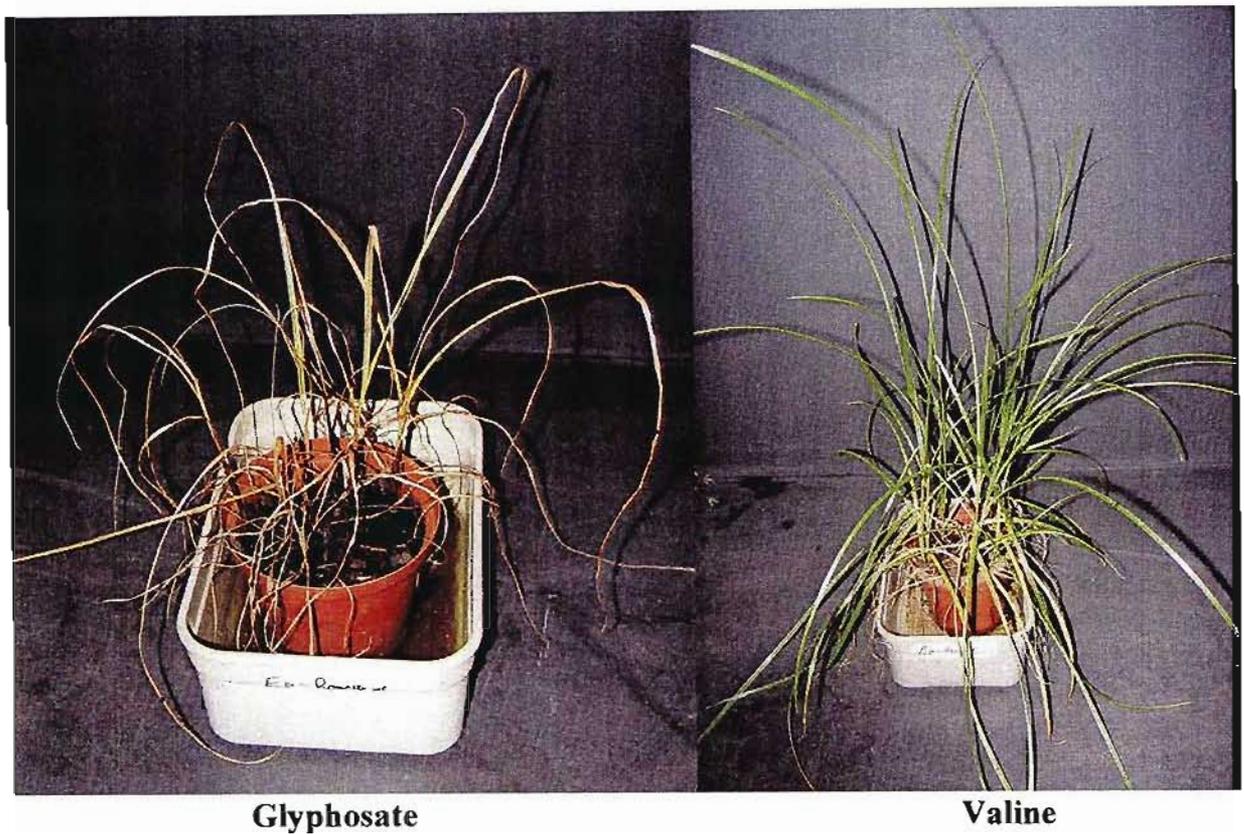


Fig. 2.4. Photographs taken of *Cyperus rotundus* injected with glyphosate at a concentration of 2 ml.l⁻¹ and valine at a concentration of 600 mg.l⁻¹.

2.4 DISCUSSION

Results from tuber germination of *C. rotundus* with the addition of varying concentrations of amino acids (Figs 2.1 and 2.2; Tables 2.1 and 2.2), showed that the amino acids tested had little effect on germination, with only glutamine at 600 mg.l⁻¹ being significantly different (at $P < 0.001$) from the water control at Day 12. This was not an expected result as Mifflin (1969) reported that valine inhibits growth of seedlings of higher plants. Using glyphosate as a control, a relatively successful herbicide used in clearing *C. rotundus*, it is possible to observe the expected results of a harmful amino acid. All treatments on Day 12 were significantly different (at $P < 0.001$) from the glyphosate treatment.

The length of the shoots measured from the germinating tubers was erratic, with no obvious pattern between amino acid concentration and shoot length. Further experiments, with a larger

sample size and, perhaps, stronger concentrations would be required to further confirm the negative results obtained in this study and establish correlations between the effects of amino acids at different concentrations. A larger sample size would reveal a pattern more substantially than may be present in this study.

It would be expected that the leaves of a plant, where the solution was inserted directly into the veins of the leaf, would be more sensitive than the hardy tubers where the test solution was not forced into the plants. As this experiment showed, valine and leucine had no observable effect on the health of the plant. The control, glyphosate (Fig. 2.4), resulted in the death of all plants in the experiment, while the plants with water, valine and leucine (not shown in the figure) showed no signs of damage. It was expected that there would be damage around the point of entry by the solutions, where the concentration of amino acid would be greatest, but no lesions were observed.

According to the ALS pathway it was expected that the addition of valine or leucine would inhibit the ALS enzyme, thereby blocking any further products in the chain, leading to damage and the possible death of the leaves and/or tuber (Eberlein *et al.*, 1997). As many ALS-inhibiting herbicides such as: imazaquin, imazapic, imazethapyr, chlorimuron, and pyriithiobac that target *C. rotundus* already exist, it was expected that the addition of valine would have a detrimental effect on the plant (Troxler *et al.*, 2003).

The results obtained in the various experiments indicate that amino acids, with particular reference to valine and leucine, have little effect, even at relatively high concentrations, on the growth and well-being of southern African *C. rotundus*. It is important to note that there is a difference between a once-off application of an amino acid to the external tuber to what would be experienced by a plant infected with a fungal pathogen continuously producing an amino acid. Indications are, however, that there is no market for the use of a bioherbicide that has a mutation for increased valine production in this region. Furthermore, in recent trials performed by a colleague (Mchunu¹, unpublished), it was observed that *D. higginsii* is not a pathogen of the locally occurring *C. rotundus* or *C. esculentus*, and, when inoculated on the plants, did not cause lesions or even establish itself on the leaves.

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CHAPTER 3

Increasing metabolite production of *Cercospora penzigii* by ultraviolet mutagenesis

ABSTRACT

The wavelength of ultraviolet-C (UV) light is between 240 – 280 nm and is harmful to many organisms by causing damage to DNA molecules. This damage may result in a mutation of the DNA in that cell. *Cercospora penzigii* Sacc. is a fungal pathogen of citrus. When grown on an artificial medium, it releases yellow metabolites into the medium, changing the colour of that medium. An increase in this metabolite production was desired, as cercosporin, a red fungal toxin, may appear yellow in small concentrations. Mutation of *C. penzigii* was achieved using UV-C light. Exposure to UV light for 5 min or more resulted in approximately 99% death of hyphal cells. The result in the survivors was an increase of approximately 5% in the production of the yellow metabolite, compared with the median, when analysed by spectrophoresis. Experiments to determine the structure of the metabolites produced were unsuccessful. The phytotoxin cercosporin, produced by many *Cercospora* species, may be present in *C. penzigii*.

3.1 INTRODUCTION

Mutation is a constant and ongoing process, with simple point mutations in the DNA occurring at every cell division. These are spontaneous mutations that come about due to reading errors in the process of multiplication. The vast majority of these go unnoticed due to the fact that the mutations occur in non-coding regions of the DNA, namely introns. This means that the mutation is not expressed and has no effect on the phenotype of the organism. Mutations that occur in regions that code for genes, namely exons, often do not affect the functioning of the organism for two reasons. Firstly, the mutation might affect only one or two base-pairs and have little effect on the product; and secondly, the product, although a malformed enzyme and ineffective, does not have a large enough effect on the organism to be observed. Therefore, only a very few mutations will be observable, and of these, only a fraction would be of the desired trait. With this in mind, any mutation experiment using physical or chemical mutagens requires an easy and effective means of mutation, large numbers of cells to mutate, and a quick and easy method for determining whether or not the individual is a mutant. In a study by Upchurch *et al.* (1991), more than 4800 *Cercospora kikuchii* (Matsumoto & Tomoyasu) Gardner survivors from

ultraviolet (UV) mutagenesis were collected. Only 18 of these were tested (due to physical changes of the fungi compared with the wild-type), and of these, only three were stable mutants.

UV light is a very effective mutagen. Light can be separated into three wavelength groups. UV-A is visible light and has a wavelength of 320 – 400 nm, UV-B is 280 – 320 nm, while UV-C is 240 – 280 nm (Jiang and Taylor, 1993). UV-C causes the most damage to DNA. In the DNA molecule, adenine (A) binds with thymine (T), and cytosine (C) with guanine (G). According to Jiang and Taylor (1993), UV light results in C – T transitions at dipyrimidines, which come about from the incorporation of A opposite C in dipyrimidine photoproducts instead of G opposite C. Stomato *et al.* (1995), working with hamster cells, observed that mutagenesis with UV light produces mutations for 11 generations following initial exposure.

Very little has been documented about *C. penzigii* Sacc. although it was recognised over 50 years ago by Chupp (1954). According to Pretorius *et al.* (2003), it is morphologically very similar to the many cercosporoid species that form part of the *C. apii* Fresen complex. *Cercospora penzigii* is a citrus pathogen and has been reported in a number of countries including Italy, Mexico, Papua New Guinea, South Africa, Swaziland, and USA (Pretorius *et al.*, 2003). It is not recorded whether this species produces the phytotoxin, cercosporin, which is produced by a large number of the fungi belonging to the genus *Cercospora*. The fungus releases a yellow metabolite which hints at the presence of a toxin, possibly one of the beticolins, a yellow toxin that has been recorded from *C. beticola* Sacc., or possibly cercosporin a very low concentrations (Milat and Blein, 1995).

The primary aim of this experiment was to conduct mutation breeding with the objective of increased production of the yellow metabolites (possibly the phytotoxin cercosporin) of *C. penzigii*. This would provide a model for other *Cercospora* species. A secondary aim was to confirm or refute the presence of cercosporin in the wild-type cultures of *C. penzigii*.

3.2 MATERIALS AND METHODS

3.2.1 Culture used

A pure culture of *C. penzigii* was obtained from the Plant Protection Research Institute (PPRI) of the Agricultural Research Council in Pretoria, South Africa. The sample of *C. penzigii* had been collected from citrus leaves in Malelane, Mpumalanga, South Africa.

3.2.2 Ultraviolet light mutation of cultures

Cultures of *C. penzigii* were grown in potato dextrose broth (PDB) and kept at 25°C in a water bath. After a period of growth a few drops of the liquid, without any colony fragments larger than 1 mm in diameter, were transferred to potato dextrose agar (PDA), and spread over the plate. This was done in such a way as to ensure there were approximately 50 colonies per plate. Each plate was then immediately placed in an enclosed structure, 500 mm below two UV-C lamps, with the top of the Petri dishes removed. The plates were exposed to UV-C light for a period ranging from 5 - 30 min.

Each plate was then incubated at 25°C in the dark for 10 days (d). A 5 mm x 5 mm block from each surviving colony was then transferred to the centre of an agar plate containing 20 ml of PDA and incubated at 25°C in the dark for a further 21 d. Colour change and colony size was noted.

3.2.3 Spectrophoretic analysis of samples

Five 4 mm x 4 mm blocks from just inside the growing margin of the fungus were collected 21 d post-inoculation and immersed in 5 ml of 5.0 N KOH in a glass bottle. Tubes were stored in the dark at room temperature for 24 hr. Filtered samples were analysed at 480 nm in a Milton Roy Spectronic 301 spectrophotometer. Absorbance values were plotted on a calibration curve of cercosporin standard, obtained from SIGMA, to obtain a concentration in mg.l⁻¹. Colonies with the greatest concentrations were then subcultured and used for the next generation mutation trials, with the mutations being cultured for four generations. This ensured that there was not

only mutation driving the colonies towards an increase in the yellow metabolite production, but also a selective breeding pressure.

3.2.4 High-performance liquid chromatography (HPLC) of samples

The extraction method of the yellow component produced was adapted from research by Balis and Payne (1971). *Cercospora penzigii* was grown at 25°C in PDB, with a light cycle of 10 hr light and 14 hr dark. Mycelium was removed from the medium by filtration and ground in a blender with ethyl acetate (20 ml.g⁻¹ of fresh mycelium). The resulting suspension was filtered and the liquid blended once again. This process was repeated until the organic extract was colourless. The extract was then washed once with water, filtered, and the two layers separated using a separation funnel. The ethyl acetate layer was then dried by pouring sodium sulfate powder into the solution, followed by filtration. The remaining ethyl acetate was then evaporated off in a Heidolph vacuum rotary evaporator where the water is heated to the boiling point of the solvent, i.e., 77.1°C. The resulting solid component was then resuspended in methanol and run through high-performance liquid chromatography (HPLC).

Another method for the extraction of the yellow component produced was also examined. Five 4 mm x 4 mm blocks of agar were removed from just inside the growing margin of the culture. These blocks were suspended in 5 ml of methanol and stored for 24 hr in the dark. The solution was then run through the HPLC.

A cercosporin standard was obtained from SIGMA for comparison with the samples.

A Phenomenex[®] Luna 5 µm C18 (255 x 4.60 mm) column was used in the HPLC. Analysis of the samples was performed with a Perkin Elmer Series 200 with autosampler, pump and diode array detector.

The running conditions for the HPLC were as follows: mobile phase was 50% acetonitrile (MeCN) and 50% water containing 5% acetic acid; injection volume was 20 µl and flow rate was set at 1 ml.min⁻¹. Detection was set at 255 and 340 nm.

3.3 RESULTS

3.3.1 Ultraviolet light mutation of cultures

Cercospora penzigii cells exposed to UV-C light for different lengths of time, from 5, 10, 20 and 30 min (Fig. 3.1) showed a very high mortality rate varying between 98.6% at 10 min and 99.6% at 30 min. Survival rate was only marginally higher (0.06%) between the 5 and 10 min exposures.

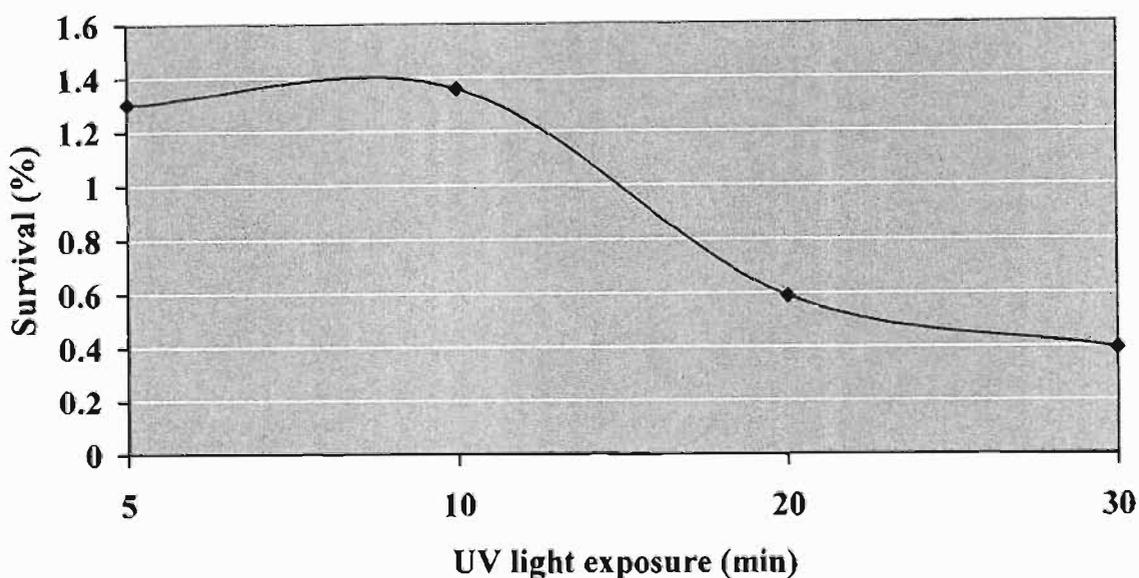


Fig. 3.1. Percentage survival of *Cercospora penzigii* cells exposed to varying amounts of UV-C light.

3.3.2 Spectrophoretic analysis of samples

A yellow metabolite was excreted from *C. penzigii* into the agar, with a darker shade of yellow present closer to the culture (Fig. 3.2). Spectrophoretic analysis gives an absorbance value that can be used to determine concentration. An increase in this value describes a darker shade of yellow, i.e., a higher concentration of compound present. An increase in absorbance readings of one of the samples from Generation 2 was obtained (Fig. 3.3). This absorbance value was 0.286, which, if the compound is cercosporin, corresponds to a concentration of 18.57 mg.l⁻¹.

Absorbance readings were plotted on a calibration curve of cercosporin standard to give concentrations of cercosporin, if present (Fig 3.4). For Generation 2 the mean absorbance was 0.063 and the median 0.057, corresponding to 6.85 and 6.20 mg.l⁻¹ of cercosporin, respectively. As it was unclear whether the yellow compound secreted by *C. penzigii* was cercosporin, the absorbance values have been shown as the primary result, with the possible concentrations added.

After each analysis, the culture with the highest absorbance value was selected for the next generation of mutation. Results show a general increase in the median absorbance with each generation (except for that of Generation 2), from an absorbance value of 0.015 (1.67 mg.l⁻¹ cercosporin) from the first analysis, 0.111 (12.06 mg.l⁻¹) from the second, 0.051 (5.54 mg.l⁻¹) from the third, and 0.084 (9.13 mg.l⁻¹ cercosporin) in the fourth and final generation.

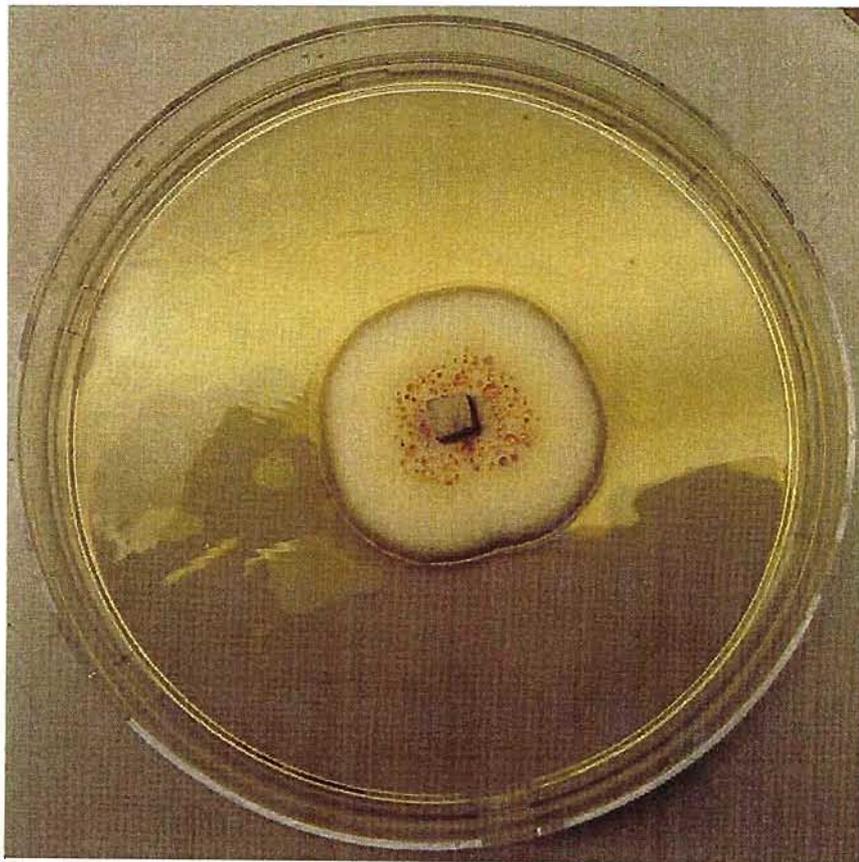


Fig. 3.2. Photograph of *Cercospora penzigii* growing in a Petri dish on potato dextrose agar.

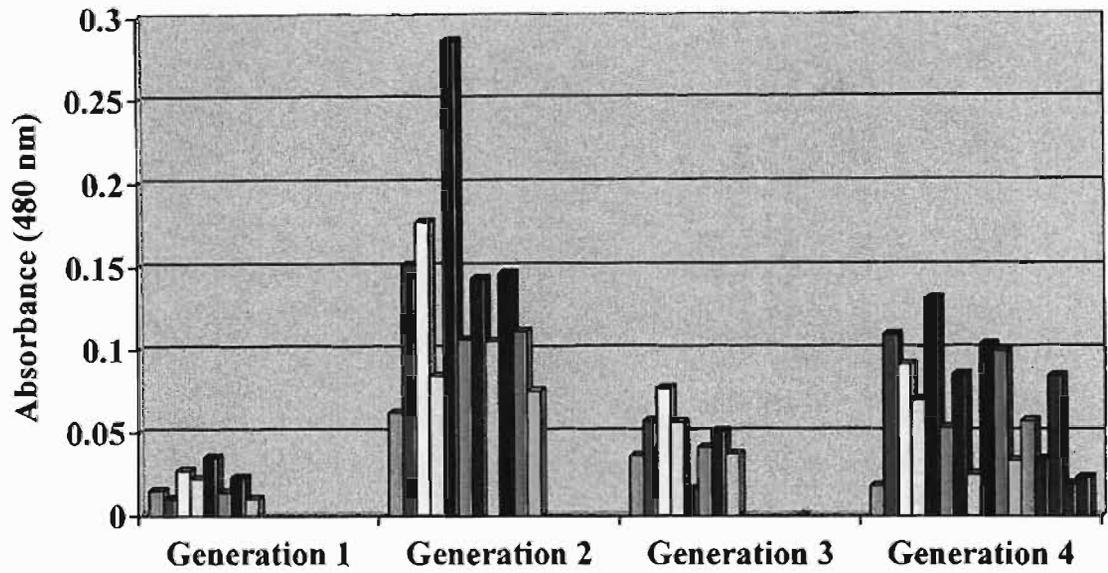


Fig. 3.3. Absorbance values (at 480 nm) obtained by spectrophoresis of separate generations of *Cercospora penzigii*. Each bar represents a separate colony analysed within a generation. Similar colours are for observation purposes only and do not indicate any correlation between samples.

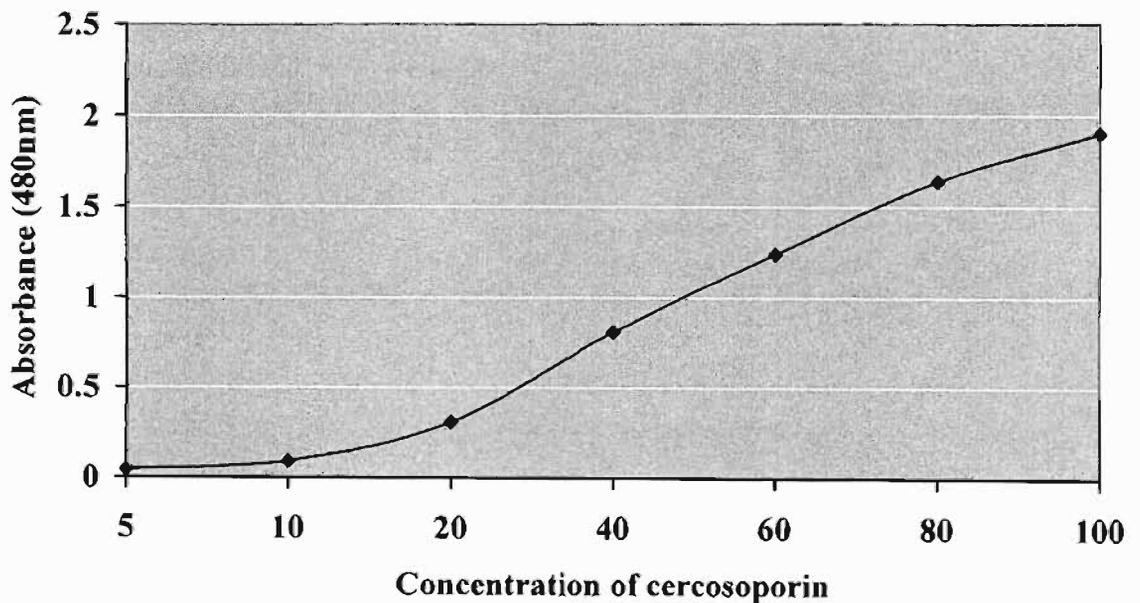


Fig. 3.4. Calibration curve of absorbance (at 480nm) vs known concentrations of a cercosporin standard (from SIGMA).

3.3.3 High-performance liquid chromatography (HPLC) of samples

Good clear chromatograms of the samples were obtained through HPLC. The cercosporin standard (Figs 3.5 and 3.6) shows the peak at approximately 11 min 30 sec. A peak of this magnitude was not observed in any of the *C. penzigii* chromatograms. In Fig. 3.7, a concentrated sample from *C. penzigii* collected by placing blocks of agar in methanol shows a fluctuation at approximately the same time as that of the standard, although the peak is very small. An overlay, not to scale, of the standard and a sample collected by the same method as Fig. 3.7 shows that the two peaks align at about 11 min 30 sec (Fig. 3.8). Samples collected by blending in ethyl acetate followed by vacuum evaporation (Fig. 3.9), did not have this peak but instead had a peak at 17 min. At 340 nm this peak is still visible, but much reduced in size (Fig. 3.10). There is, however, a single strong peak at 3 min, which is observed in samples collected by placing blocks of agar in methanol (Fig. 3.11). Fig. 3.12 is a scaled overlay of three samples of *C. penzigii* collected by placing blocks of agar in methanol. The three samples each gave different absorbance readings when analysed by spectrophoresis. The size of the peak in each case corresponds to the absorbance reading obtained, i.e., the higher the absorbance reading, the larger the peak.

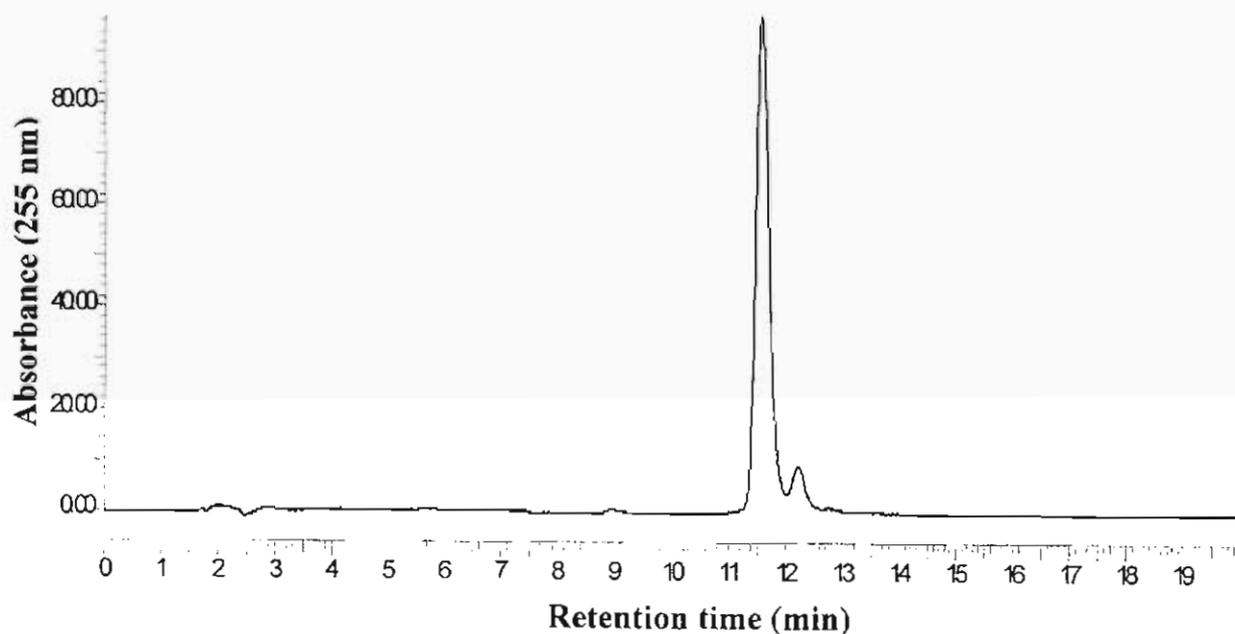


Fig. 3.5. Chromatogram of cercosporin standard from SIGMA. Absorbance at 255 nm.

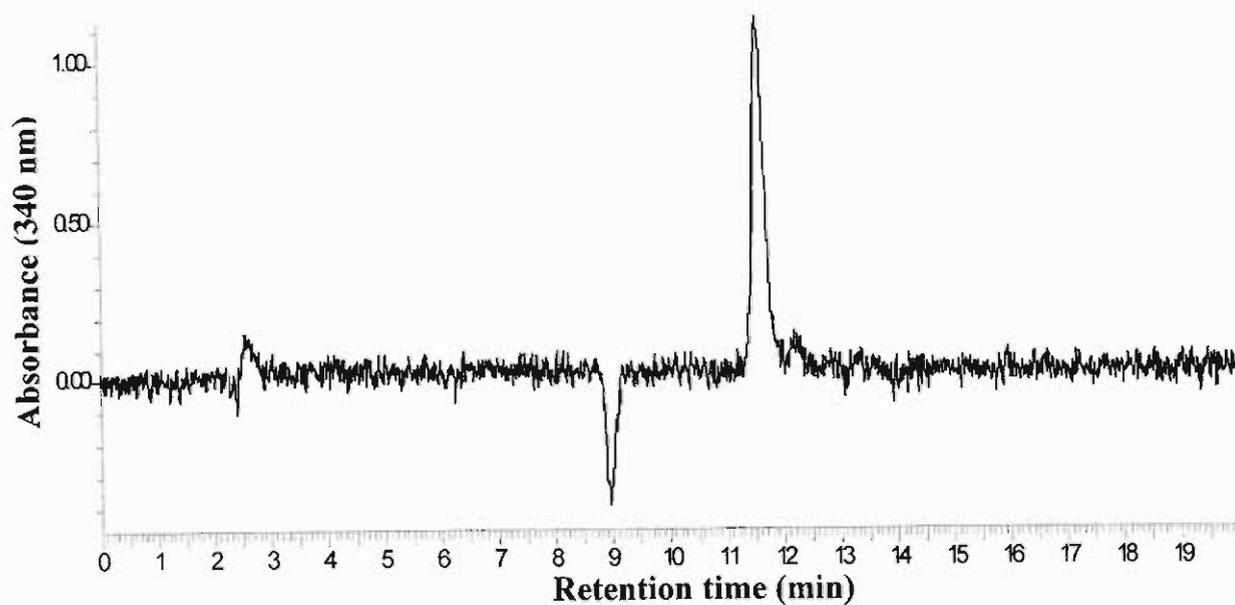


Fig. 3.6. Chromatogram of cercosporin standard from SIGMA. Absorbance at 340 nm.

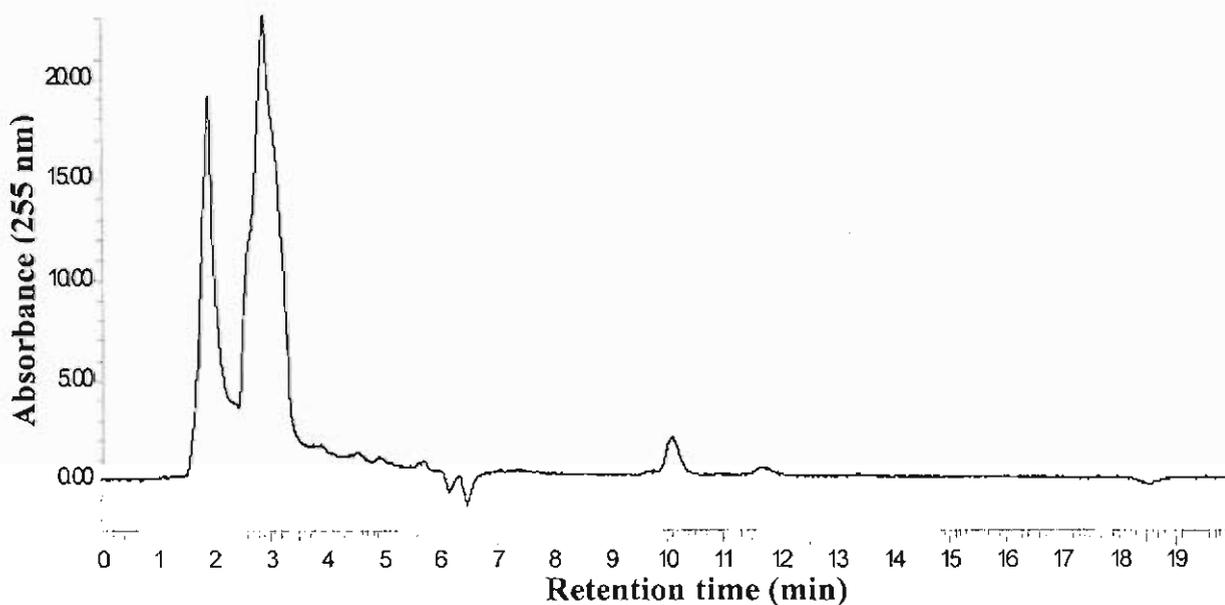


Fig. 3.7. Chromatogram of *Cercospora penzigii* collected by placing blocks of culture in methanol. Absorbance at 255 nm.

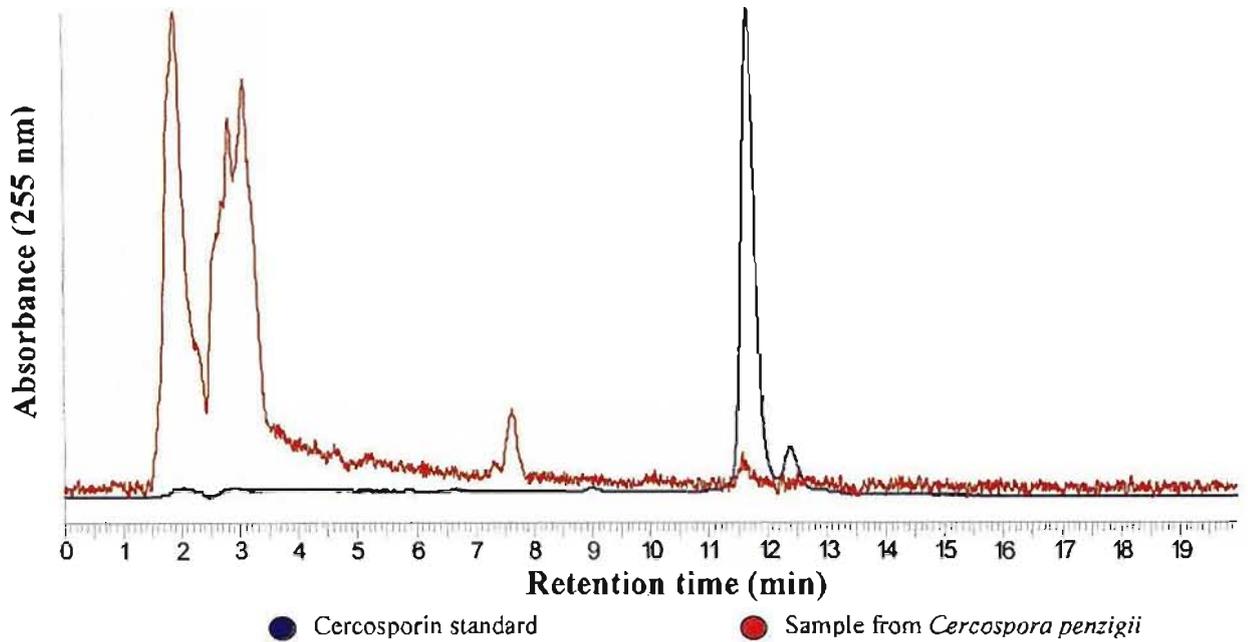


Fig. 3.8. Unscaled overlay of cercosporin standard and a sample of *Cercospora penzigii* collected by placing blocks of agar into methanol. Absorbance at 255 nm.

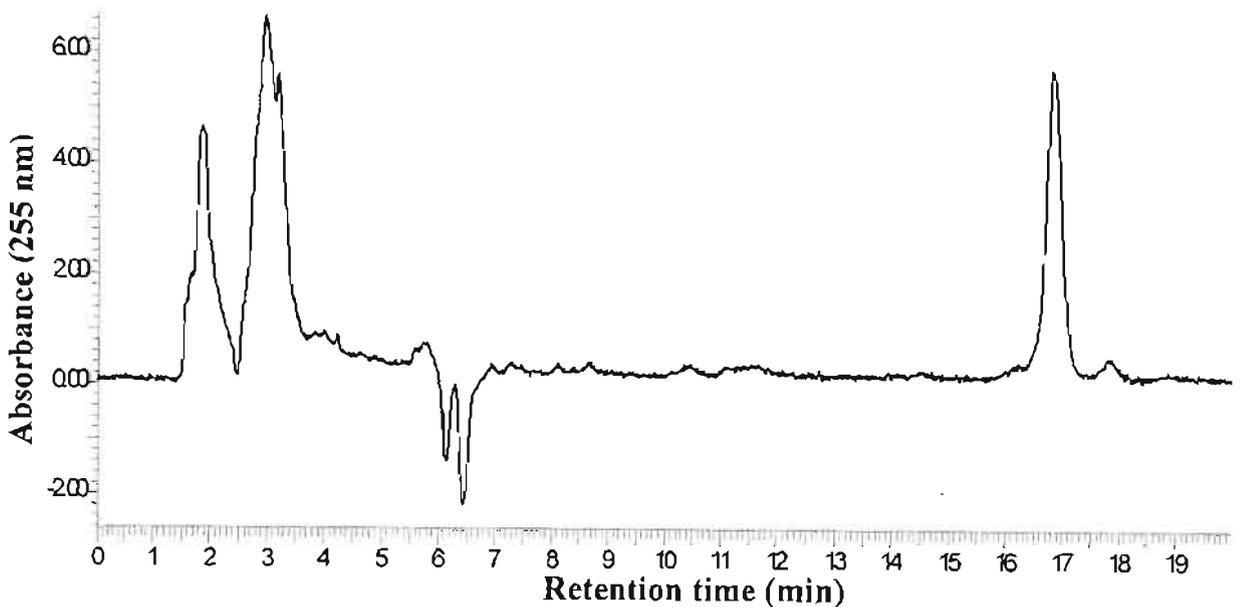


Fig. 3.9. Chromatogram of *Cercospora penzigii* collected by blending in ethyl acetate followed by vacuum evaporation. Absorbance at 255 nm.

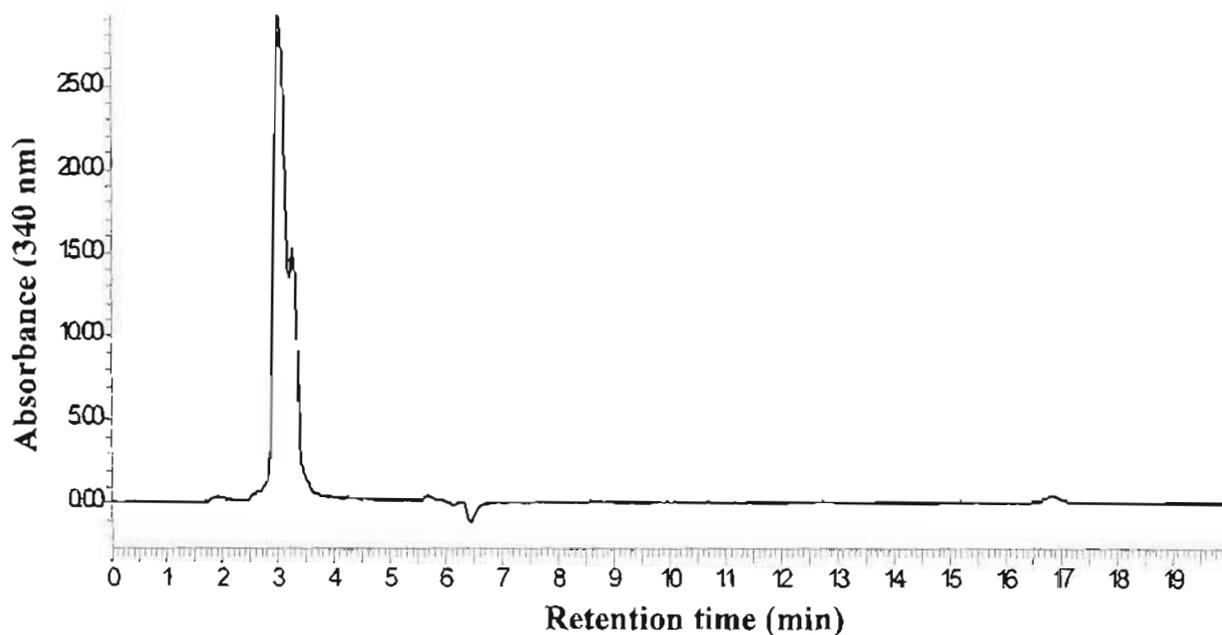


Fig. 3.10. Chromatogram of *Cercospora penzigii* collected by blending in ethyl acetate followed by vacuum evaporation. Absorbance at 340 nm.

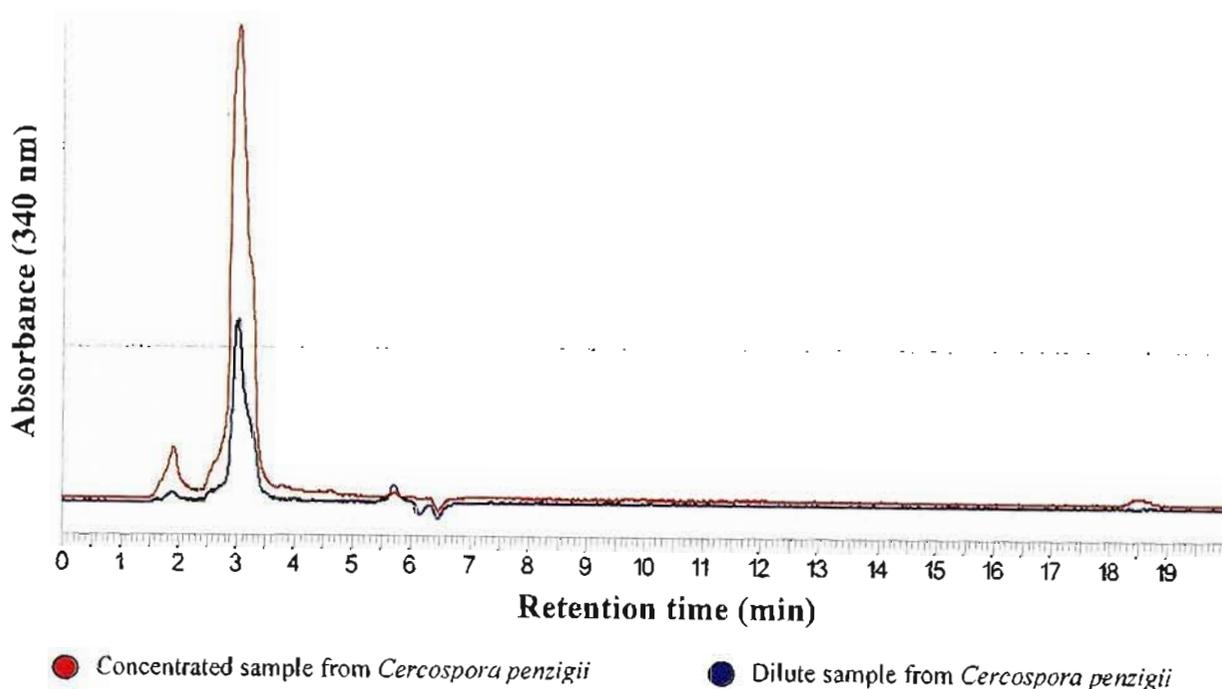


Fig. 3.11. Scaled overlay of two chromatograms of *C. penzigii* collected by placing blocks of culture in methanol. Absorbance at 340 nm.

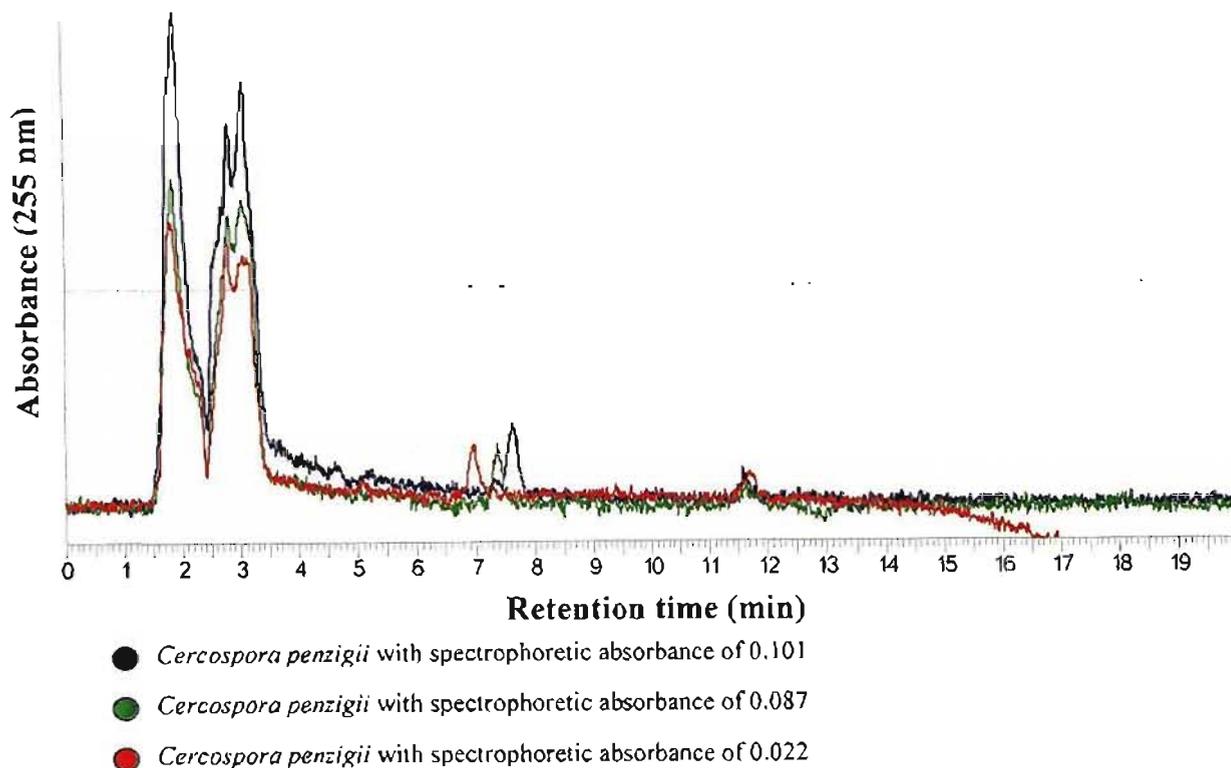


Fig. 3.12. Scaled overlay of three chromatograms of *C. penzigii* collected by placing blocks of culture in methanol. Absorbance at 255 nm.

3.4 DISCUSSION

Upchurch *et al.* (1991) observed that 95 – 99% of conidia of *C. kikuchii* were killed when exposed to approximately 8 min of UV light ($500 \mu\text{W}\cdot\text{cm}^{-1}$). This is in line with the results obtained in this study, where approximately 99% of cells were killed on plates exposed between 5 and 10 min to UV-C light. As expected, an increase in exposure time resulted in a decrease in survival rate. The fewer cells surviving UV light exposure increases the chance that one of these will be a mutant.

A favourable mutation was obtained by means of UV light exposure. One colony showed an increase of approximately 250% in the production of the yellow compound when compared with the median. It is important to note that the mutant obtained was not stable and this result could not be replicated and confirmed as the subcultures of this colony did not give similar absorbance readings.

This study not only investigated the mutation of wild-type *C. penzigii* cultures, but also looked at the possibility of increasing the production of the compound by selective breeding. This was done by selecting the colony with the greatest absorbance reading for the next generation of mutation. This proved successful, with a shift in absorbance of 0.069 (7.5 mg.l⁻¹ cercosporin) over four generations. There is a limit to the increase in yellow compound production by this means, but it is a viable way of increasing production. The second generation of recorded mutations (Fig. 3.3) showed the biggest increase in absorbance value from the overall median, with 0.111 (12.07 mg.l⁻¹ cercosporin) compared with 0.057 (6.20 mg.l⁻¹ cercosporin). When these colonies were subcultured, the absorbance readings dropped back to a level more similar to the overall average.

Although a number of colonies had a yellow, instead of the usual white, fungal growth, all absorbance readings were similar to that of the average, with none giving the highest or lowest readings (data not shown). It is possible that these cultures had a particular mutation that resulted in colour change of the mycelia, but did not affect the production of the yellow compound being released into the agar (Fig. 3.2). A similar observation was made for absorbance reading and colony size (data not shown). There did not appear to be any pattern between colony size and metabolite production. As cercosporin is toxic to many plants, bacteria, animals and fungi, it was initially thought that an increase in toxin production by the fungus, i.e., large concentrations of cercosporin in the agar, may result in decreased fungal viability and growth, but, if this occurred, it was not an observable difference (Daub and Ehrenshaft, 2000). Provided this compound is cercosporin, it confirms what is understood about *Cercospora* species, that they show complete resistance to cercosporin (Daub and Ehrenshaft, 2000).

When run on HPLC, the cercosporin standard gave a peak at approximately 11 min 30s (Fig. 3.5). None of the *C. penzigii* samples gave a strong peak at this time. The *C. penzigii* sample collected by soaking blocks of agar in methanol (Figs 3.7 and 3.8) did give a small peak at this point, approximately 1% of the standard. This indicates the possible presence of cercosporin, but in minute amounts. This peak is too small to be able to confirm the presence of the toxin, but the fact that it is present means that it is possible that *C. penzigii* does produce cercosporin. The fact that cercosporin was not produced at a higher level could be due to a number of factors including light, temperature and growth medium. However, the cultures were all grown on PDA, a growth medium that is generally favourable for cercosporin production (Jenns *et al.*, 1989).

In the chromatogram of the sample of *C. penzigii* collected by blending the mycelium in ethyl acetate followed by vacuum evaporation, a solid peak was present at approximately 17 min (Fig. 3.9). The method of extraction used by Milat and Blein (1995) was followed. They used this method to extract toxins produced by *C. beticola*, which contains cercosporin as well as a number of beticolin toxins. These toxins are bright yellow in colour, the same colour noted in the PDA in the present study. This suggests the possibility that *C. penzigii* does produce one or more of the beticolin toxins. In the study by Milat and Blein (1995), it was shown that all the beticolin toxins present in *C. beticola* had a longer retention time than that of cercosporin. No standard of the beticolin toxins could be obtained for this study so the production of these toxins by *C. penzigii* could not be confirmed.

The peak at 17 min (Fig. 3.9) is the only difference observed between the two methods of toxin extraction from *C. penzigii*. If further research indicates this peak corresponds to a phytotoxin, then the simpler method of extraction by placing blocks of agar in methanol is not sufficient for testing a variety of metabolites. However, if it is simply a result of contamination, then this simple method is a step forward in the analysis of samples. This is due to its ease and time-saving nature. The method of blending the mycelium followed by vacuum evaporation is very tedious and it can take two days to complete one sample. Whereas, by using the simpler method, as many samples as required can be processed in one day.

Furthermore, at 340 nm a large peak was observed at about 3 min (Figs 3.10 and 3.11). Fig. 3.11 shows two different concentrations for the same method of collection. It is likely that this is an injection peak, but a *C. penzigii* metabolite may be present. Both Figs 3.5 and 3.6 show small irregularities at about 2 min 30 sec. Again, it is unclear as to what this compound is and whether, or not, it is a compound produced by *C. penzigii*.

The fact that absorbance values obtained by spectrophoresis correspond to similar-sized peaks when analysed by HPLC is an expected result (Fig. 3.12), and confirmed that both methods of analysing the samples worked. If there had been no correlation between the peaks and the absorbance values then it would have been assumed that one of the systems was faulty.

This study was not able to confirm the presence of cercosporin in *C. penzigii*, but there was enough evidence to suggest its presence. Further analysis using different media, temperatures

and light, and perhaps the use of mass spectrophotometry, would be required to confirm or disprove the presence of this toxin. It is unclear as to what the other peaks represent, but it is possible that the compound with the retention time of approximately 17 min (Fig. 3.9) is a beticolin toxin. Further studies are required for confirmation. The peaks at 3 min (Figs 3.11 and 3.12) are most likely injection peaks and do not correspond to any significant metabolite produced by *C. penzigii*.

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CHAPTER 4

Optimization of pathogenicity of *Cercospora caricis*, a fungal pathogen of *Cyperus rotundus* and *Cyperus esculentus*, by ultraviolet mutagenesis

ABSTRACT

Cercospora caricis Oud., isolated for this experiment from *Cyperus rotundus* L. CYPRO, is also a fungal pathogen of *Cyperus esculentus* L. CYPES, two problematic weeds present on every continent. *Cercospora caricis*, like many *Cercospora* species, produces the phytotoxin cercosporin. Cercosporin is the primary means of pathogenicity of *C. caricis*. An increase in cercosporin production would likely result in an increase in virulence of the fungus. This increase was achieved by a spontaneous mutation, following ultraviolet-C mutation, of *C. caricis*. Five minutes exposure to ultraviolet (UV) light resulted in approximately 65% cell death, while 20 min exposure resulted in approximately 95% cell death. Wild-type *C. caricis* produced no cercosporin, or in amounts that were undetectable, while the red mutant gave an absorbance value of approximately 140 compared to grey mutants which gave readings of approximately 22 when analysed by high-performance liquid chromatography.

4.1 INTRODUCTION

Cercospora caricis Oud. is a known pathogen of *Cyperus esculentus* L. CYPES (yellow nutsedge) and *Cyperus rotundus* L. CYPRO (purple nutsedge). It is known to infect yellow and purple nutsedge in a number of countries and regions including Australia, Brazil, Libya, Nigeria, southern Africa, and Uganda (Evans, 1987; Barreto and Evans, 1995). *Cyperus rotundus* is a substantial problem around the world, and has been reported in more countries and areas than any other weed (Holm *et al.*, 1977). It flourishes in high temperatures, surviving at the highest known temperatures for agriculture, and in almost every soil type, humidity, soil moisture, elevation and pH; the only limiting factor to the spread of the weed appears to be cold temperatures (Holm *et al.*, 1977). Zandstra and Nishimoto (1977) suggested that the success of *C. rotundus* is due to its ability to grow rapidly from tubers and rhizomes and the inability of foliar-applied herbicides to be translocated to the tubers.

In Brazil, *C. caricis* was only found in locations with a mean annual rainfall below 1400 mm, but where present, it was reported to be a very damaging pathogen of *C. rotundus* (Barreto and Evans, 1995). Barreto and Evans (1995) further suggest that *C. caricis* is an option for the biological control of nutsedge, but that it has a few limitations: it grows slowly and does not sporulate readily in culture. The following description of the infection of leaves by *C. caricis* is taken from Blaney *et al.* (1988) and Barreto and Evans (1995):

Leaf spots range from oval to elongate with the colour changing from an initial mottled yellow-orange to brown at maturity. The leaf spot is often surrounded by a yellow halo. The lesions usually coalesce, resulting in leaf death. External mycelium is absent while internal mycelium is inconspicuous. Conidiophores, which rise through stomata, are hypophyllous, fasciculate, cylindrical and tapering towards the apices. *Cercospora caricis* produces chlamydospores in the leaves but no spores have been found in culture. A black liquid is sometimes observed oozing from the colony when grown in culture, but not observed on infected leaves.

Cercosporin is a compound produced by many known *Cercospora* spp. Blaney *et al.* (1988) confirmed that cercosporin was produced by a *C. caricis* isolate from yellow nutsedge. Cercosporin has been referred to as the epitome of non-host-specific toxins due to the fact that it is lethal to a number of organisms other than plants, e.g., other fungi, bacteria and animals (Daub and Ehrenshaft, 2000). Cercosporin is a compound that is photodynamically activated and damages membranes causing lipid peroxidation (Daub and Ehrenshaft, 1993). The structure of this red compound is: 1,12-bis(2-hydroxy-propyl)-2,11-dimethoxy-6,7-methylenedioxy-4,9-dihydroxy-perylene-3,10-quinone, with a molecular weight of 534 (Fig. 4.1) (Upchurch *et al.*, 1991). According to Daub and Ehrenshaft (2000) environmental cues, especially light, trigger the production of cercosporin. Light then activates the cercosporin molecule to generate activated oxygen species. This oxygen results in the peroxidation of the host's membrane lipids, which leads to membrane damage and finally cell death. This membrane damage results in the leakage of nutrients from the cell into the intercellular spaces where, it is hypothesised, they are taken up by the fungus.

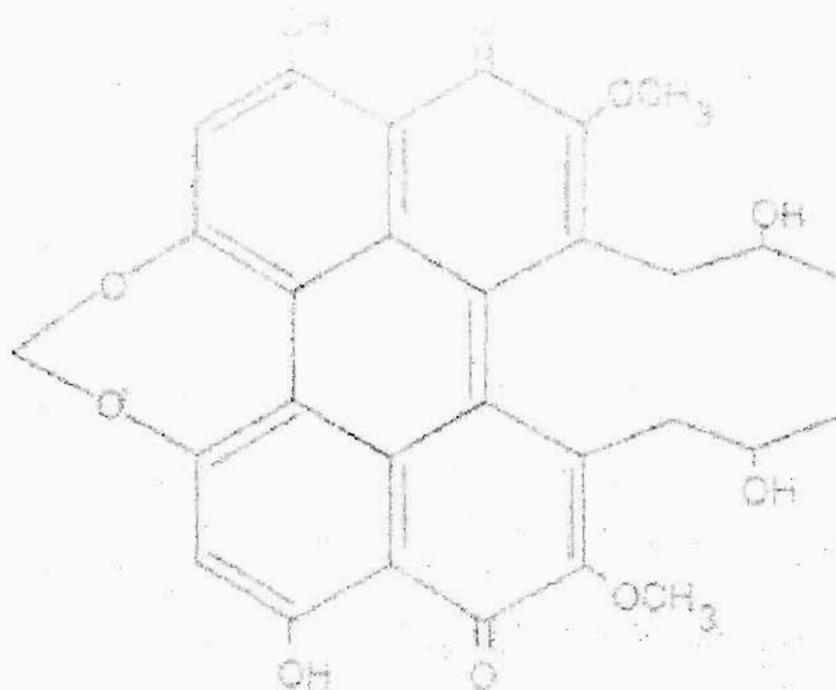


Fig. 4.1. Structural formula of cercosporin (Upchurch *et al.*, 1991).

Mutants showing a decrease in cercosporin production were obtained from *C. kikuchii* (Matsumoto & Tomoyasu) Gardner, a pathogen of soybean (Upchurch *et al.*, 1991). Conidia from *C. kikuchii* were exposed to 500 $\mu\text{W}\cdot\text{cm}^{-2}$ ultraviolet (UV) light for varying amounts of time. Colonies differing in colour from the red-coloured wild-type were selected for analysis. Analysis was performed by treating samples with 5.0 N KOH and then determining the absorbance by means of spectrophoresis. The UV-induced mutants produced no more than 2% of the level of cercosporin produced by the wild-type. Inoculation of soybean leaves confirmed the results obtained by spectrophoresis that a smaller concentration of cercosporin production resulted in decreased disease severity. This study confirmed that cercosporin production by *C. kikuchii* was essential for lesion production on soybean under greenhouse conditions.

Aly *et al.* (2001) successfully expressed two genes in *C. caricis* by biolistic transformation. The genes were the β -glucuronidase gene (GUS) and the hygromycin B resistance gene. The aim of the experiment was to improve the virulence of *C. caricis* for its possible use as a biocontrol agent. Aly *et al.* (2001) reported that the transformation efficiency was not high, but stable, 2 transformants per μg of DNA under optimal conditions. This shows potential in the production of a bioherbicide for *C. rotundus*.

The aims of the present study were three-fold. Firstly, to isolate *C. caricis* from infected leaves of *C. rotundus*. Secondly, to confirm the presence of the toxin, cercosporin. Thirdly, to achieve a significant increase in the production of this toxin.

4.2 MATERIALS AND METHODS

4.2.1 Culture isolation

A pure culture of *C. caricis* was obtained from infected leaves of *C. rotundus*, which were obtained from a local research farm, Ukulinga (30°24'0" E, 29°39'0" N), in Pietermaritzburg, South Africa. Two methods of isolation were tested.

In the first method, the living area of the leaf directly adjacent to the dead lesion was excised. This was sterilized in 70% ethanol or 1% JIK for 1 - 2 min. Leaf sections were then washed twice in sterile water and transferred to potato dextrose agar (PDA). After 7 days' (d) incubation in the dark at 25°C, any *Cercospora* growth was removed, subcultured and monitored for contamination.

In the second method, the entire lesion was removed from the leaf, surface-sterilised with 70% ethanol, and either rinsed or ground in a droplet of sterile water. This droplet was then spread over a plate of PDA and incubated in the dark at 25°C for 24 hr. Each plate was then examined under a dissecting microscope and all *Cercospora*-like conidia were removed and transferred to a clean PDA plate. These colonies were monitored for contamination.

4.2.2 Ultraviolet light mutation of cultures

Cultures of *C. caricis* were grown in potato dextrose broth (PDB). Cultures were kept at 25°C in a water bath. After a period of growth, a few drops of the liquid culture, without any mycelial fragments larger than 1 mm in diameter, were transferred to PDA and spread over the plate. This was done in such a way to ensure there were approximately 50 colonies per plate. Each plate was then immediately placed in an enclosed structure, approximately 500 mm below two UV-C lamps, with the tops of the Petri dishes removed. Plates were exposed to the UV-C light for 2 - 20 min.

Each plate was then incubated at 25°C in the dark for 10 d. A 5 mm x 5 mm block from each surviving colony was then transferred to the centre of an agar plate containing 20 ml of PDA and incubated at 25°C in the dark for a further 21 d. Colour changes and size of the colonies were noted.

4.2.3 Spectrophoretic analysis of samples

Five 4 mm x 4 mm blocks from just inside the growing margin of the fungus were collected 21 d post-inoculation and soaked in 5 ml of 5.0 N KOH in a glass tube. This tube was stored in the dark at room temperature for 24 hr. Samples were analysed at 480 nm in a Milton Roy Spectronic 301 spectrophotometer. All absorbance values were plotted on a calibration curve of cercosporin standard, obtained from SIGMA, to obtain a concentration in mg.l⁻¹.

4.2.4 Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) of samples

Five 4 mm x 4 mm blocks of agar were removed from just inside the growing margin of the culture. These blocks were suspended in 5 ml of methanol and stored for 24 hr in the dark. This solution was filtered and used for thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analysis.

A cercosporin standard was obtained from SIGMA for analysis of the samples.

Merck 60 F-254 plates were used for TLC analysis of the samples. The mobile phase consisted of chloroform-methanol-water (80:20:2), as used by Milat and Blein (1995).

A Phenomenex[®] Luna 5 µm C18 (255 x 4.60 mm) column was used for HPLC analysis of the samples. Analysis was performed with a Perkin Elmer Series 200 with autosampler, pump and diode array detector.

The running conditions for the HPLC were as follows: mobile phase was 50% acetonitrile (MeCN) and 50% water containing 5% acetic acid; injection volume was 20 µl and flow rate was set at 1 ml.min⁻¹. Detection was set at 255 and 340 nm.

4.2.5 Inoculation of *Cyperus rotundus* leaves

A number of methods were attempted for the inoculation of *C. rotundus* leaves. Techniques were adapted from Upchurch *et al.* (1991) where pathogenicity was observed on the leaves. All methods required 3 mm x 3 mm blocks of fungus being excised from the growing margin of *C. caricis*. All three isolates (wild-type and mutated red and grey isolates) were used in each experiment. Observations were made every 24 hr.

Method 1: Agar blocks were placed on the upper surface (fungus side down) of *C. caricis* leaves and infection observed. Plants were kept at a temperature of 25°C and relative humidity of 50% with regular sprinkler action.

Method 2: Agar blocks were placed on the upper surface of *C. caricis* and kept moist and in position by a section of cotton wool wrapped around the leaf and agar block. Plants were kept at 25°C and watered daily from the ground.

Method 3: Leaves were removed from the plants and placed in a Petri dish with moist cotton wool at the base of the leaf to prevent desiccation. Agar blocks were placed on the upper and lower surfaces of the separate leaves. Half of the agar blocks were moistened with a water droplet at the start of the experiment.

Method 4: The same as Method 3 but the outer cell layer of the leaf, upper or lower depending on the point of inoculation, was scraped away to aid infection. Agar blocks were placed on these sections. Half of the agar blocks were moistened with a water droplet at the start of the experiment.

Method 5: The same as Method 3 but pin pricks were made to penetrate the epidermal cells of the entire leaf. Agar blocks were placed on these pin pricks, both upper and lower surfaces of separate leaves. Half of the agar blocks were moistened with a water droplet at the start of the experiment.

4.3 RESULTS

4.3.1 Culture isolation

Both methods of fungal isolation described in Chapter 4.2 worked with a high degree of success. The second method proved more tedious and time-consuming than the first method as it required the use of a light dissecting microscope and a steady hand to remove individual conidia. The isolates were confirmed as a *Cercospora* species by electron microscopy of conidia by a colleague (Mchunu¹, unpublished).

4.3.2 Ultraviolet light mutation of cultures

Hyphal cells of *C. caricis* were exposed to UV-C light for lengths of time varying from 5 - 20 min (Fig. 4.2). With each increase in exposure time percentage of surviving cells decreased. At 5 min exposure over 35% of the cells survived to form viable colonies, while after 20 min only about 5% of the cells survived.

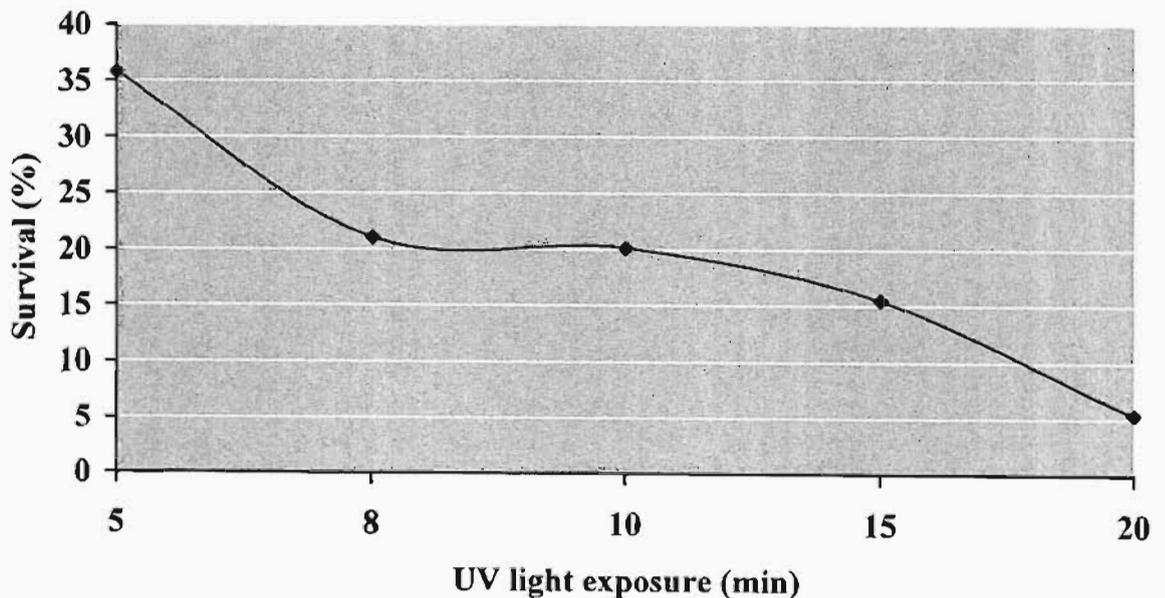


Fig. 4.2. Percentage survival of *Cercospora caricis* cells exposed to varying amounts of UV-C light.

After the final set of mutations involving the wild-type *C. caricis* colony (Fig. 4.3), surviving colonies were subcultured for a few generations without exposure to UV-C light. During this period a spontaneous mutation was observed in one of the cultures (Fig. 4.4). Sectoring was observed in one culture as red sections grew outward from the point of inoculation. These were excised and subcultured to form pure cultures of the red *C. caricis* (Fig. 4.5). The growth medium of these cultures was stained red in much the same way that *C. penzigii* stained the agar yellow (Chapter 3). Two mutants were observed, those that were red in colour and stained the agar red, and gave the highest absorbance values (referred to as red *C. caricis*), and those that were grey in colour and did not alter the colour of the agar, but gave positive absorbance readings (referred to as grey *C. caricis*).



Fig 4.3. Photograph of wild-type *Cercospora caricis*.

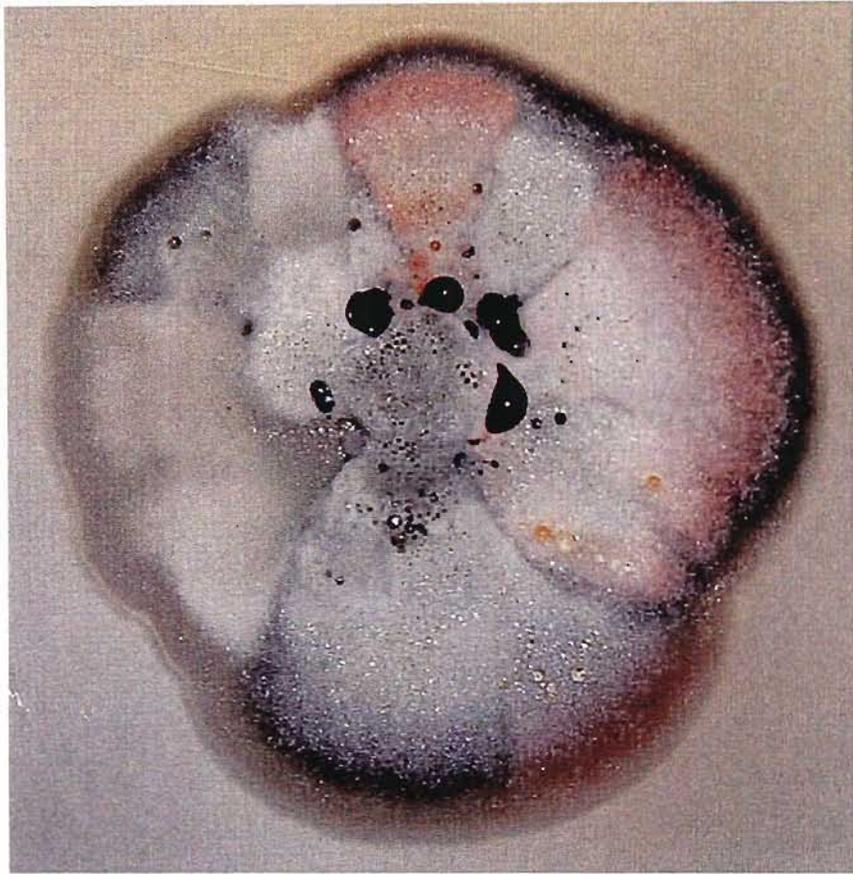


Fig 4.4. Photograph showing sectoring of *Cercospora caricis* with the mutant strain and a red liquid present.

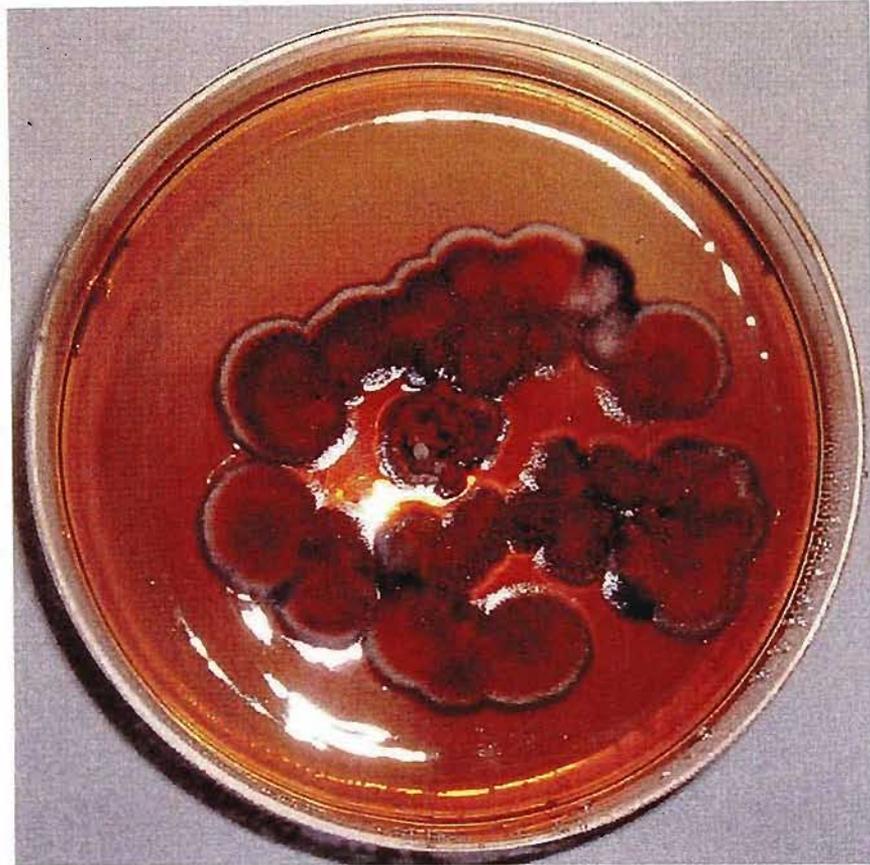


Fig. 4.5. Photograph of mutated red *Cercospora caricis* colony.

4.3.3 Spectrophoretic analysis of samples

All absorbance readings for *C. caricis* were negligible until mutation occurred. Prior to mutation, all readings were less than 0.010 (1.11 mg.l⁻¹ cercosporin). After mutation the median absorbance value was 0.043 (9.55 mg.l⁻¹ cercosporin), observed from a grey *C. caricis* colony, with a maximum absorbance reading of 0.12 (13.04 mg.l⁻¹ cercosporin) observed from the red *C. caricis* colony. Cercosporin concentrations were calculated by plotting absorbance values on a calibration curve (Fig. 4.6).

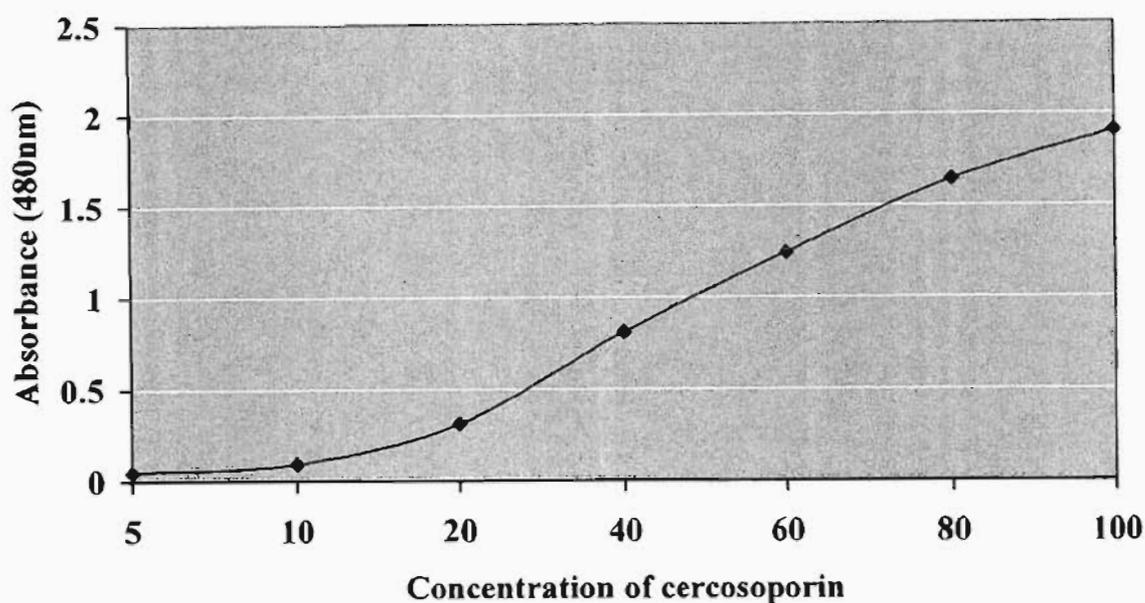
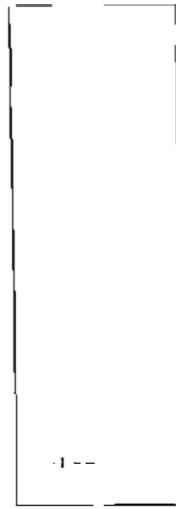


Fig. 4.6. Calibration curve of absorbance (at 480nm) vs known concentrations of a cercosporin standard (from SIGMA).

4.3.4 Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) of samples

TLC analysis of red and grey *C. caricis* gave positive results for the presence of cercosporin when analysed by TLC (Fig. 4.7). Wild-type *C. caricis* did not yield any results using this technique.

The cercosporin standard (Fig. 4.8) produced a peak at approximately 11 min 30 sec. No such peak was visible on the chromatogram of wild-type *C. caricis* (Fig. 4.9), but a fluctuation was observed at about the equivalent time. Fig. 4.10 shows that the retention time of the peaks for cercosporin and the sample from red *C. caricis* correspond. The different amounts of cercosporin produced by the three cultures, red, grey and wild-type, is shown in Fig. 4.11. Red *C. caricis* gave an absorbance value of approximately 140, grey *C. caricis* of about 22, and the wild-type absorbance reading, if present, was negligible.



A Std

Fig. 4.7. Thin-layer chromatography (TLC) of two samples: A – Sample collected from *Cercospora caricis*; Std Cercosporin standard.

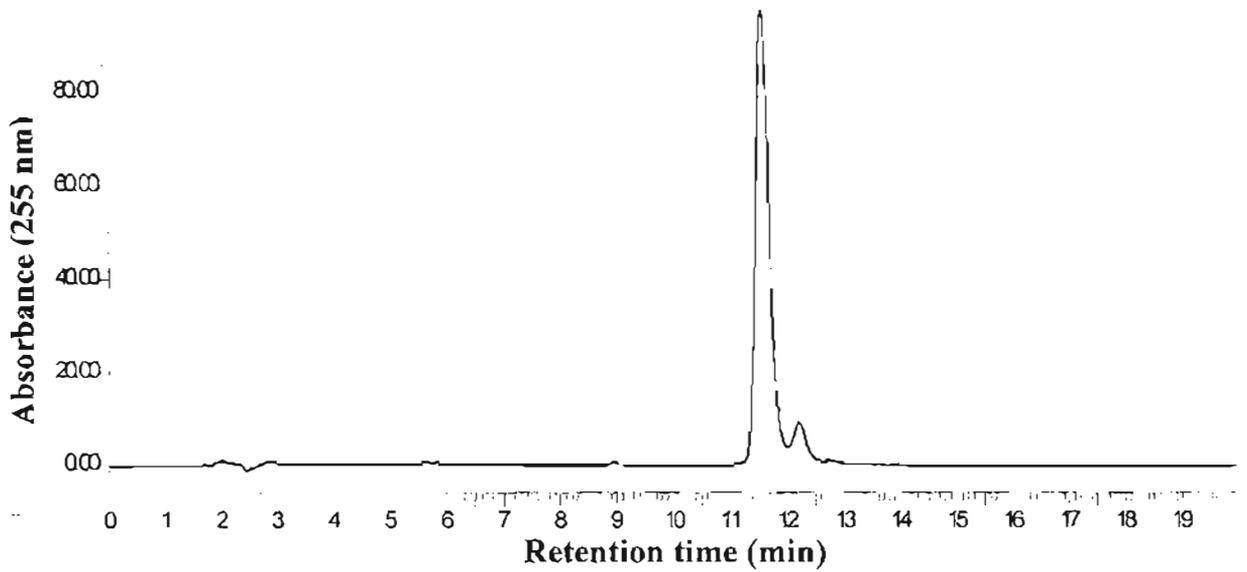


Fig. 4.8. Chromatogram of cercosporin standard from SIGMA. Absorbance at 255 nm.

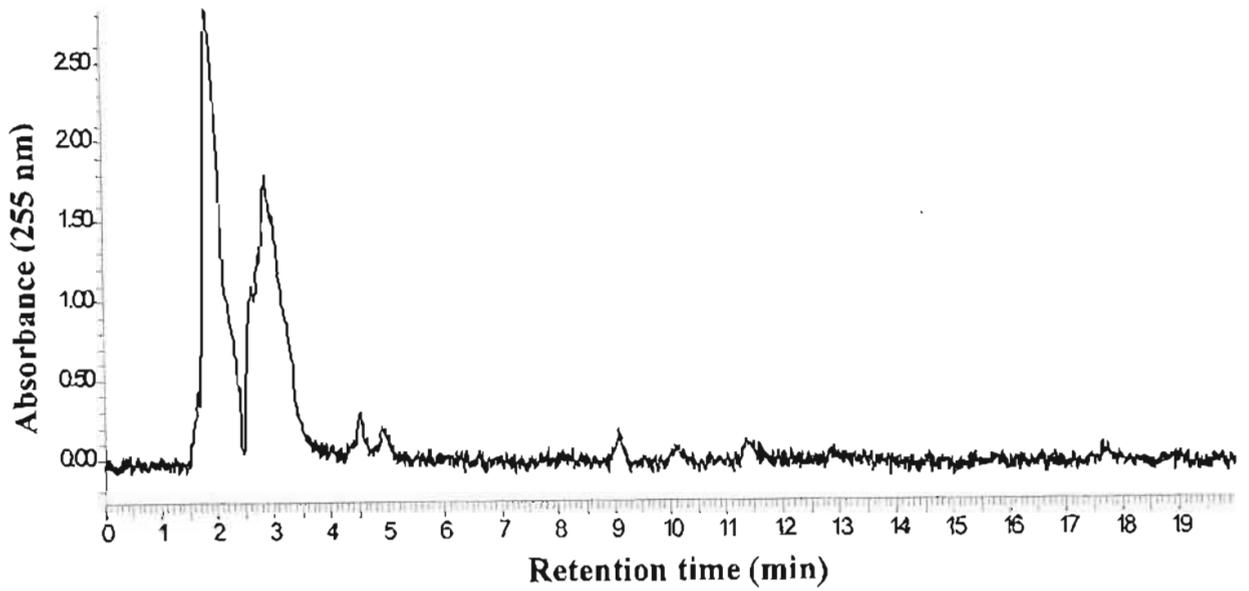


Fig. 4.9. Chromatogram of a sample collected from wild-type *Cercospora caricis*. Absorbance at 255 nm.

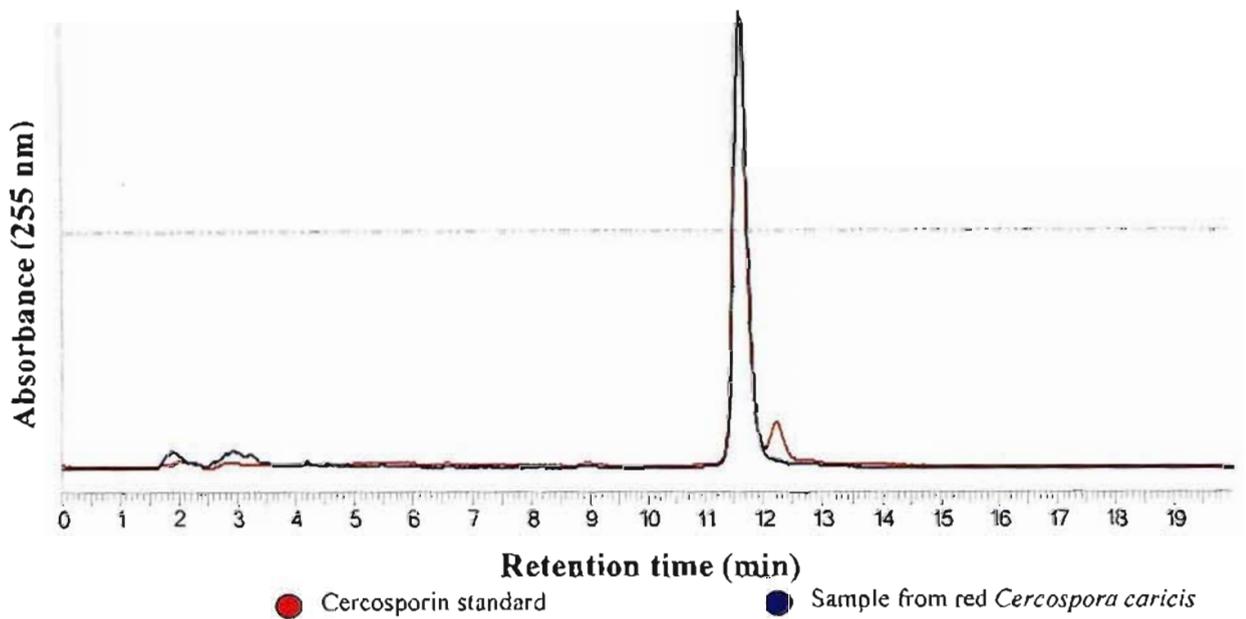


Fig. 4.10. Scaled overlay of a cercosporin standard, from SIGMA, chromatogram and a chromatogram of a sample from red *Cercospora caricis*. Absorbance at 255 nm.

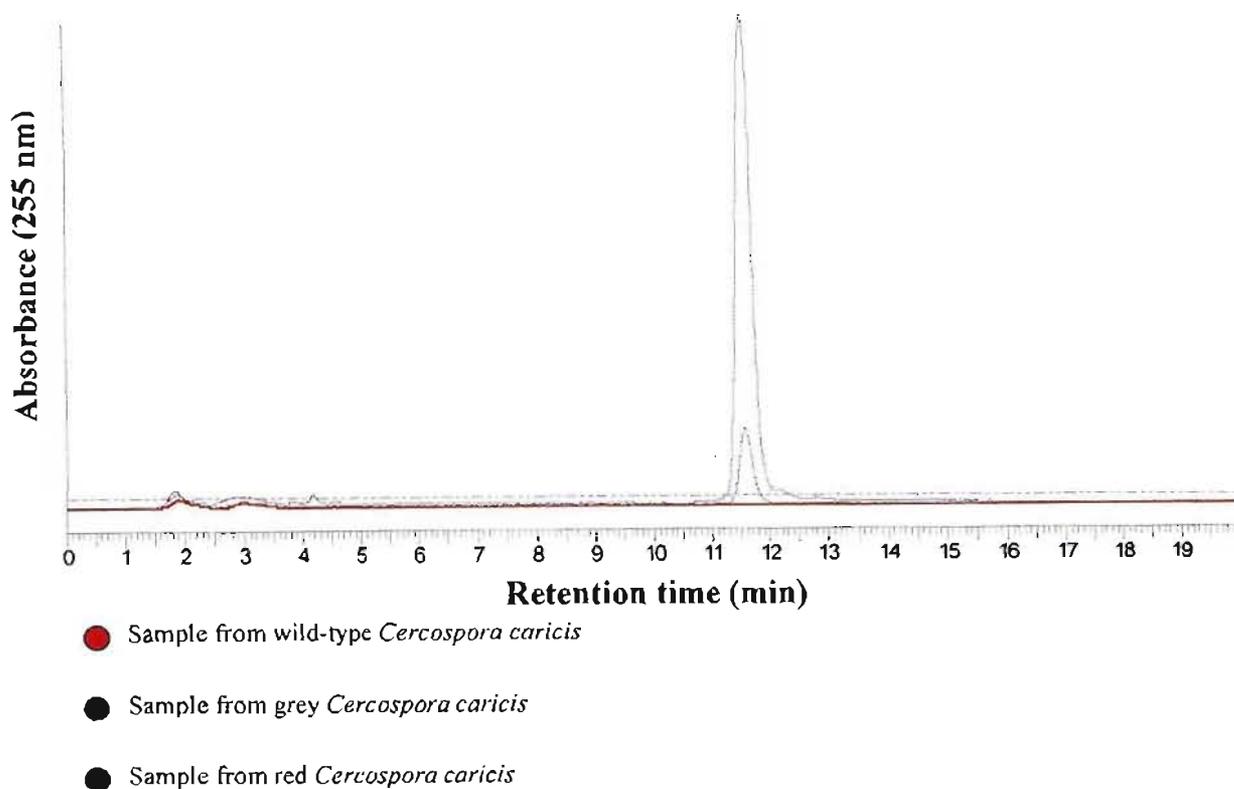


Fig. 4.11. Scaled overlay of equal concentrations of samples collected from red, grey and wild-type *Cercospora caricis*. Absorbance at 255 nm.

4.3.5 Inoculation of *Cyperus rotundus* leaves

No infection was observed in any of the experiments. No visible difference in appearance of the inoculated leaves and those of the control was observed.

4.4 DISCUSSION

Both methods of obtaining a fungal isolate of *C. caricis* proved successful. From the study, the first method was the preferred means of isolation as it was simpler, while the number of clean fungal isolates obtained was not affected by the simplicity. This method requires a large number of fungal lesions as only one, or a maximum of two, isolates can be obtained from each leaf, and only approximately 10% of leaf cuttings used resulted in a positive result.

The second method tested was more tedious and time-consuming, but more precise. Fewer lesions were required for this method as a large number of isolates could be obtained from two or three infected leaves. Removing the conidia from the agar after one day's incubation required dexterity and a steady hand as there were a number of contaminants growing on the agar. As a result, not every conidium subcultured resulted in a contaminant-free culture, but many of the cultures obtained were clean.

Two cultures of *C. caricis* lost viability during the course of this study. This was due to the fact that the fungus does not grow well on artificial media. To increase the longevity of an isolate it is recommended that the culture be subcultured on a regular basis (every two months) from a nutrient rich medium to a nutrient poor medium, and vice versa. The fungus did not survive being stored by placing blocks of agar in sterile distilled water. The method recommended is to take liquid media, with small amounts of *C. caricis* present, mixed with an equal part of sterilized benzene, and store at -80°C.

The results obtained for mutation of *C. caricis* are very different to those obtained for *C. penzigii* (Chapter 3). Exposure to UV-C light for 5 min resulted in approximately 65% death in *C. caricis* cells, while almost 99% of the *C. penzigii* cells were killed. A possible explanation of the discrepancy may be that the hyphal cells of *C. caricis* are hardier than those of *C. penzigii*, but the reason for this is not certain. It may be that the growing conditions resulted in the growth of hardier *C. caricis* cells, possibly with thicker cell walls. Upchurch *et al.* (1991) observed that only 8 min of UV light was sufficient to kill 95 – 99% of *C. kikuchii* conidia. Cultures were grown in PDB before being transferred to PDA for mutation purposes. From observations throughout this study it was noted that *C. caricis* does not grow readily in liquid culture. For *C. caricis* to obtain the same amount of biomass in PDB, it took three times the period of that of *C. penzigii*. This may have had an influence on the reaction of the cultures to UV light.

The wild-type *C. caricis* did not change the colour of the growing medium (Fig. 4.3). None of the isolates collected from *C. rotundus* secreted any coloured compound, red or yellow, into the growth medium. Furthermore, all isolates were grey-white in colour. After mutation a spontaneous mutant was observed that had a red mycelial growth and changed the colour of the agar to a rust-red colour (Fig. 4.5). It is unclear whether this is a completely spontaneous mutation or whether the previous mutation of the culture triggered an response that was only

observed in a later generation. Stomato *et al.* (1995) noted that, in hamster cells, exposure to UV light irradiation resulted in mutations up to 11 generations after initial mutagenesis. Spectrophoretic analysis revealed an increase in toxin production from the wild-type to the mutated *C. caricis*. As observed by Upchurch *et al.* (1991), analysis by spectrophoresis is a valid means of detecting any changes in cercosporin production. The single mutant red *C. caricis* culture gave the highest absorbance value, while the rest of the absorbance readings were obtained from mutated *C. caricis* cultures that had remained grey.

Analysis of wild-type *C. caricis*, by means of TLC, gave positive results for a lack of cercosporin production, with no mark present on the plate. HPLC analysis seemed to confirm this, with no peak present (Fig. 4.9) at the retention time of the standard (Fig. 4.8). However, it was not possible to confirm that no cercosporin was being produced by the wild-type due to the fact that a fluctuation was present at the approximate retention time of the standard. Further analysis of the wild-type would need to be performed to confirm the absence of cercosporin from this culture.

TLC analysis of the red *C. caricis* sample was the first indication that this mutant was producing cercosporin (Fig. 4.7). The R_f values for the standard and the sample were the same. This result indicated that cercosporin was present and being produced by the mutant red *C. caricis*. HPLC analysis of the samples confirmed the results obtained by spectrophoresis and TLC.

In Fig. 4.10, the HPLC chromatogram confirmed that the one red *C. caricis* colony produced the toxin cercosporin as the peak aligns with the cercosporin standard. The amount of cercosporin produced by the three different *C. caricis* cultures is shown in Fig. 4.11. Red *C. caricis* produces more than 100 times that of the grey, while the wild-type produces a minimal amount, if any. This is a significant increase in the production of cercosporin.

A number of methods for testing the pathogenicity of the wild-type compared with that of the mutant *C. caricis* were examined. None of the methods resulted in infection of the leaves. Further methods could not be tested due to the time constraints of this study, but infection of *C. rotundus* with conidia would need to be examined in order to test the virulence of the red mutant *C. caricis*.

This study has successfully produced a mutant strain of *C. caricis*. Furthermore, the red colony produces large amounts of cercosporin, while the grey colonies produce considerably less toxin. Although the increase in cercosporin production would enhance the performance of a bioherbicide, the fungal strain must be suitably infectious. As yet, this has not been successfully tested. Jenns *et al.* (1989) does not believe a reliable correlation is possible between toxin production *in vitro* and virulence. Further studies therefore need to be undertaken to test the pathogenicity of the new mutant on host plants under field, or even lab, conditions.

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CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The aim of this study was to increase the virulence of a fungal pathogen of *Cyperus rotundus* L. CYPRO and *Cyperus esculentus* L. CYPES, by means of mutation, with the objective of creating a viable bioherbicide.

5.1 EFFECTS OF AMINO ACIDS ON *Cyperus rotundus*

Research performed by a colleague, Mchunu¹ (unpublished), showed that *Dactylaria higginsii* (Luttrell) M. B. Ellis is not a fungal pathogen of the South African ecotypes of purple nutsedge, *C. rotundus*, while in the present study, experiments on the use of amino acids to enhance the virulence of a fungus proved that this is not a viable option. Concentrations of amino acids as high as 1000 mg.l⁻¹ had no significant (P<0.001) effect on the germination of tubers, except for glutamine, at 600 mg.l⁻¹, which resulted in significantly less germination than that of the water control. Four treatments showed significant differences (P<0.001) in shoot length to that of the water control, however, there was no underlying pattern that would allow suitable conclusions to be drawn. Injection of amino acids into the leaves of *C. rotundus* resulted in no damage, while the glyphosate control killed the plants. According to Mifflin (1969), the growth of higher plant seedlings is inhibited by valine. This statement was not confirmed in this study and, from the results obtained, increasing the production of a number of amino acids including L-lysine monohydrochloride, glycine, DL-tryptophan, L-asparagine, L-glutamine and L-valine by a fungus would have little effect on the viability and health of *C. rotundus*. This may be a viable avenue of research for other plants, and perhaps even different ecotypes of *C. rotundus*, but not for *C. rotundus* ecotypes found in Pietermaritzburg, South Africa.

5.2 INCREASING METABOLITE PRODUCTION OF *Cercospora penzigii* BY ULTRAVIOLET MUTAGENESIS

Mutagenesis of *Cercospora penzigii* Sacc. by ultraviolet (UV)-C light exposure was successful. Five or more minute's exposure gave the desired percentage of survivors, approximately 1%.

Spectrophoretic analysis of the samples indicated that one mutant produced approximately 250% more yellow metabolite than the median. This mutant, however, was not stable, and when subcultured did not yield the same absorbance values as was observed with the parent mutant. A stable mutant is required if the virulence of a fungus is to be increased, so although this was a favourable mutation, it was of no use.

Analysis of the samples by high-performance liquid chromatography (HPLC) could not prove or refute the presence of cercosporin. It does seem likely though, that another metabolite was present, possibly a yellow toxin such as one of the beticolins that has been observed in *C. beticola* Sacc. (Milat and Blein, 1995). The reason for this hypothesis was the colour of the metabolite, a bright yellow, and the fact that a peak was observed approximately five minutes after that of the cercosporin standard. It is possible that this peak was contamination and not a metabolite. Milat and Blein (1995), however, reported a number of beticolin toxins with retention times considerably greater than that of cercosporin.

Further HPLC analysis, and perhaps mass spectrophotometry, need to be performed in order to confirm or refute the presence of cercosporin, and to determine the structure of the metabolite with the peak at 17 min.

5.3 OPTIMIZATION OF PATHOGENICITY OF *Cercospora caricis*, A FUNGAL PATHOGEN OF *Cyperus rotundus* AND *Cyperus esculentus*, BY ULTRAVIOLET MUTAGENESIS.

The isolation techniques used for the collection of *Cercospora caricis* Oud. from *C. rotundus* were simple and effective. A large number of pure cultures were obtained using both methods of isolation. The method of excising the tissue surrounding the lesion, sterilizing it and then placing on potato dextrose agar (PDA) was the most simple of the two methods, and the number of clean cultures obtained was sufficient for this method to be favoured over the other, more precise method.

Although cercosporin production has previously been confirmed in *C. caricis* (Blaney *et al.*, 1988), the wild-type did not provide a peak corresponding with that of the cercosporin standard. The presence of a fluctuation in the line of the chromatogram means that cercosporin may be present in a small amount. *Cercospora penzigii*, when grown on PDA, changed the colour of the

medium yellow. Red *C. caricis*, the mutant, changed the colour red. None of the *C. caricis* isolates collected from infected leaves changed the colour of the agar, a factor not necessarily indicative of a lack of cercosporin production, but a sign that large amounts of cercosporin were not being produced.

The mutant red *C. caricis*, as noted, turned the PDA rust red in colour, while the mycelium itself was red in colour, in contrast to the usual grey white colour. HPLC analysis confirmed that this colour change was a result of cercosporin production. According to Daub and Ehrenshaft (2000), cercosporin is the archetypal non-host-specific toxin in that it is harmful to plants, bacteria, fungi and animals. An increase in the production of cercosporin by a fungus would theoretically result in an increase in pathogenicity of that fungus. Jenns *et al.* (1989) suggested, however, that drawing any correlations between increased cercosporin production *in vitro* and virulence of the fungus is unreliable.

It is therefore important that the virulence of this mutant *C. caricis* be determined by inoculation of the fungus onto *C. rotundus* under field conditions. Furthermore, Barreto and Evans (1995) queried the ability of this fungus to be a successful biocontrol agent due to its slow growth and inability to sporulate readily in culture. For the success of this line of research, these two aspects need to be addressed. Most importantly, an isolate that sporulates readily needs to be obtained, or another means of obtaining spores needs to be determined. Or, conversely, a different means of inoculation needs to be devised.

The aims of this study were met. By increasing the production of the phytotoxin cercosporin, the potential virulence of a fungal pathogen, of *C. rotundus* and *C. esculentus*, *C. caricis*, was increased. As mentioned above, further research into the growth and sporulation of this fungus and the enhancement of its pathogenicity is required before any conclusions can be drawn as to its use as a bioherbicide for the control of *C. rotundus* and *C. esculentus*.

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