SEPARATION AND PHYTOTOXICITY OF SOLANAPYRONE COMPOUNDS PRODUCED BY *ASCOCHYTA RABIEI* (PASS.) LABR. AND THEIR METABOLISM BY CHICKPEA (*CICER ARIETINUM* L.)

BY

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ABSTRACT

An isolate of *Ascochyta rabiei* secreted the phytotoxins, solanapyrones A, B and C when grown on Czapek Dox nutrients supplemented with five cations. The toxins were identified and quantified by high performance liquid chromatography with diode array detection and isolated from culture filtrates by partitioning into ethyl acetate and flash chromatography on silica gel.

Cells isolated from leaflets of 12 chickpea cultivars differed by up to five fold in their sensitivity to solanapyrone A and this compound was 2.6-12.6 times more toxic than solanapyrone B, depending on cultivar.

When chickpea shoots were placed in solanapyrone A, the compound could not be recovered from the plant and symptoms developed consisting of turgor loss of stems and flame-shaped, chlorotic zones in the leaflets. In similar experiments with solanapyrone B, only 9.4% of the compound taken up was recovered and stems remained turgid but their leaflets became twisted and chlorotic.

Glutathione reacted with solanapyrone A, rapidly reducing the amount of free toxin and forming a Sol.A-glutathione conjugate as well as reducing its activity when incorporated in the cell assay. Measurement of reduced glutathione concentration and GST activity among cultivars showed that the differences of their means were highly significant and both were negatively correlated with their relative sensitivity to solanapyrone A. Treatment of shoots with solanapyrone A enhanced total, reduced and oxidized glutathione content as well as GST activity 1.26, 1.23, 1.50 and 1.94 fold, respectively.

Similarly, treatment of shoots with the safener, dichlormid, also raised total, oxidized and reduced glutathione levels and GST activity. Cells isolated from shoots treated with dichlormid at 150 µg/shoot and 300 µg/shoot were 2.45 times and 2.66 times less sensitive to solanapyrone A with LD_{50} values of 71.5 µg/ml and 77.8 µg/ml, respectively as compared to 29.2 µg/ml for controls.

In preliminary experiments designed to identify microbial genes capable of detoxifying the solanapyrones a basal mineral salts medium caused demethylation of solanapyrone A. Demethylated solanapyrone A was 16.4 fold less toxic than solanapyrone A in the cell assay, requiring 514.0 µg/ml to kill 50% of the cells compared with 31.3 µg/ml for Sol.A.

Dedicated to my parents

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ABBREVIATIONS

BM	basal medium
BMM	basal mineral medium
CDLM	Czapek Dox liquid medium
CDLMC	Czapek Dox liquid medium amended with five cations
cm	centimetre
cv	cultivar
g	gram
h	hours
HB	holding buffer
HPLC	high performance liquid chromatography
ICARDA	International Centre for Agricutural Research in the Dry Areas
L	litres
LD_{50}	Dose of the toxin required to kill 50% of the cells
LII	linear infection index
M	molar
mM	millimolar
mm	millimetre
min	minutes
ng	nanograms
nm	nanometre
r_s	Spearman's correlation coefficient
Rf	retention factor
TLC	thin layer chromatography

CHAPTER 1

INTRODUCTION

1.1. THE CHICKPEA

Chickpea (*Cicer arietinum* L.) is a self-pollinated annual plant, generally growing from 20 to 100 cm in height. Glandular hairs are present on all aerial parts of the plant except most of the corolla. The leaves are pseudo-imperipinnate (the apical leaflet is not in a truely terminal position) and leaflets are typically toothed, 8-17 mm in length and 5-14 mm in width. The shape of the leaflets varies from obovate to elliptical.

Flowers are typically papilionaceous and emerge from pedicel or peduncle racemes 6-13 mm long. Sepals consist of five deeply lanceolate teeth with prominent midribs, ranging from 5-6 mm. Corollas are purple, red, pink, blue or white in colour. The vexillum is obovate, 8-11 mm long and 7-10 mm wide. Wings are also obovate, 6-9 mm long and 4 mm wide with short pedicels. The keel is 6-8 mm long, rhomboid, with a pedicel 2-3 mm in length.

The androecium is diadelphous consisting of nine stamens with fused filaments and the tenth completely free. The ovary contains 1-3 ovules, rarely 4 and the style is 3-4 mm long, glabrous except at the bottom, linear and upturned, ending in a globose stigma which is either slightly broader or the same size as the style.

Pods are rhomboid, oblong or ovate in shape, typically inflated and ending in a mucro that sometimes looks like a thorn. The number of pods per plant varies from 30 to 150. Each pod consists of exocarp, mesocarp and endocarp containing one or two, rarely up to four, spherical or angular seeds. There are two main commercial types of chickpea, the Kabuli type with large, smooth, and light coloured seeds and the Desi type with smaller and darker coloured seeds which may vary from yellow to black (Cubero, 1987; Singh, 1985).

1.1.1. The agricultural importance of chickpea

Chickpea is the third most important pulse crop in the world, but it ranks first in the Indian subcontinent and Mediterranean basin (Anonymous, 1994). It is a member of the family Leguminosae which is second in size only to the Gramineae (Aykroyd and Doughty, 1964), providing the second most important source of food after cereals for humans and animals (Anonymous, 1979). Among the world's grain food legumes, chickpea is second to dry beans (*Phaseolus vulgaris* L.) in area grown and third to dry beans and dry peas (*Pisum sativum* L.) in production (Singh, 1985).

The annual area sown to chickpea worldwide is 9.94 million hectares and the annual production is 7.038 million tonnes (Anonymous, 1993). India is the largest chickpea producer, growing 4 million tons annually on 6.5 million ha. and Pakistan is second, producing 0.5 million tons annually on 1 million ha. Mexico, Turkey, Ethiopia and Burma (in descending order) are other countries where chickpea is important and jointly comprise around 14 percent of both total area grown and production (Malik and Tufail, 1981; Jodha and Rao, 1987; Ladizinsky, 1995; Horn and Reddy, 1996).

Chickpea fixes atmospheric nitrogen when inoculated with appropriate strains of *Rhizobium* and improves soil fertility for any crop grown subsequently as, on decay, nitrogen is released to the soil (Anonymous, 1981; Papastylianou, 1987).

1.1.2. Nutritional value

Chickpea seed is mainly used as food because of its high protein (12.4-31.5%) and carbohydrate (52.4-70.9%) content (Singh, 1985; Williams and Singh, 1987; Dutta *et al.*, 1988; Khan, 1990; Awasthi *et al.*, 1991; Dhawan *et al.*, 1991). On the Indian subcontinent it is known as the poor man's meat (Strange *et al.*, 1992). It is eaten raw, boiled or as dhal, which consists of the cotyledons separated from their seed coat. The leaves are used as green vegetables. When eaten with cereals chickpea gives a balanced diet. It is also one of the major constituents of various sweets. The dry stalks and husks containing small broken pieces of grain obtained during milling are fed to animals (Malik and Tufail, 1981).

Medicinal properties have been claimed for the exudates from glandular hairs of the plant which contain 94% malic acid and 6% oxalic acid and they are also used in vinegar (Alam, 1989). The proteins of chickpea are helpful in reducing cholesterol levels in blood serum owing to their beneficial effect on lipid metabolism (Zulet and Martinez, 1995).

1.1.3. Constraints to chickpea production

The world's average chickpea yield is 586 Kg ha^{-1} and is very low when compared with the yields of important cereals such as wheat (1927 Kg ha^{-1}), rice (2823 Kg ha^{-1}), maize (3318 Kg ha^{-1}) and even sorghum (1397 Kg ha^{-1}) and millets (668 Kg ha^{-1}). The yields of some pulses

such as faba beans (1153 Kg ha^{-1}) and lentils (634 Kg ha^{-1}) are also higher than those of chickpea (Jodha and Rao, 1987).

A recent report by Ladizinsky (1995) revealed that the area under chickpea has been almost static for the last 30 years at 9 - 10 million ha worldwide and, over the same time period, the average yield, which the author puts at 700 Kg ha^{-1} rather than the earlier figure of 586 Kg ha^{-1} quoted by Jodha and Rao (1987), has risen by only 10%. Jodha and Rao (1987) give the average yield in the USA, where agriculture is more advanced, as 993 Kg ha^{-1}. Factors limiting yield are discussed in the following paragraphs.

Chickpea has received less attention than cereals such as wheat, rice and other cash crops and has remained a crop for poor people in poor environments (Malik and Tufail, 1981; Jodha and Rao, 1987). It is mainly grown in countries where yields are low whereas in countries such as the USA, where agriculture is more advanced, chickpea represents only 5% of the world's production (Jodha and Rao, 1987). Moreover, in developing countries, the crop is mostly confined to marginal and rainfed lands without inputs. A further factor is that, with the advent of the green revolution based on high yielding cultivars of wheat, chickpea has been relegated to even more marginal lands (Ali *et al.*, 1991; Malik and Tufail, 1981; Jodha and Rao, 1987).

Lack of mechanized farming also contributes to low yields of the crop. In Pakistan manual harvesting is practised and threshing is mostly done by bullock treading, although hand flailing is also common. Both produce unsatisfactorily cleaned seed (Malik and Tufail, 1981).

The crop is mostly raised without fertilizer application. As it is a legume it does not require nitrogen but phosphorous application is very important for grain development, one recommended rate being 50 Kg P_2O_5/ha (Malik and Tufail, 1981).

Weeds are also a major limiting factor as chickpea yields can be increased by about 25% by weeding twice, 30-40 and 80-90 days after emergence (Ali *et al.*, 1991).

Other important limiting factors are the lack of resistance to diseases and insect pests and the non-availability of improved seeds to the grower (Ali *et al.*, 1991; Malik and Tufail, 1981). Yield increases of 70% have been achieved in Pakistan by replacing old cultivars with new improved ones that are blight tolerant (Ali *et al.*, 1991). Among insects, the most damaging ones are pod borer (*Heliothis armigera, Heliothis viriplaca, Helicoverpa armigera*), cut worm (*Agrotis* spp.), leaf miner (*Liriomyza cicerina*) and seed weevils (*Bruchus* spp.; Anonymous, 1990; Nene and Reddy, 1987). Among diseases, blight caused by *Ascochyta rabiei* (Pass.) Labr. is the most damaging. Diseases are discussed further in the next section.

1.2. CHICKPEA DISEASES

More than 50 pathogens have been reported so far on chickpeas from different parts of the world and a few of them have the potential to devastate the crop (Nene and Reddy, 1987).

The most serious fungal diseases in descending order of importance are Ascochyta blight caused by *Ascochyta rabiei* (Pass) Labr., Fusarium wilt caused by *Fusarium oxysporum* Schlecht. emnd. Snyd. & Hans. f. sp. *ciceri* (Padwick) Snyder. & Hans., dry root rot caused by *Rhizoctonia bataticola* (Taub). Butler, charcoal rot caused by *Macrophomina phaseolina* (Maubi) Ashby, Botrytis grey mould caused by *Botrytis cinerea* Pers. ex. Fr., black root rot caused by *Fusarium solani* (Mart.) Appel & Wr., Phytophthora root rot caused by *Phytophthora megasperma* Drechs. and Pythium root rot caused by *Pythium ultimum* Traw.

Other less important fungal diseases are Alternaria blight caused by *Alternaria alternata* (Fr.) Kiessler, Colletotrichum blight caused by *Colletotrichum dematium* Pers. ex Fr.,

Phoma blight caused by *Phoma medicaginis* Malbre & Roum, Stemphylium blight caused by *Stemphylium sarciniforme* (Cav.) Wilts., rust caused by *Uromyces ciceris - arietini* (Grogn.) Jacz & Beyer, powdery mildew caused by *Leveillula taurica* (Lev.) Arnaud, Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, Verticillium wilt caused by *Verticillium albo-atrum* Reinke & Berth, wet root rot caused by *Rhizoctonia solani* Kuhn, collar rot caused by *Sclerotium rolfsii* Sacc. and foot rot caused by *Operculella padwickii* Kheswalla.

Important viral diseases include stunt caused by Pea Leaf Roll Virus, Chickpea Chlorotic Dwarf Virus and a Gemini Virus, mosaic caused by Alfalfa Mosaic Virus, proliferation caused by Cucumber Mosaic Virus, narrow leaf caused by Bean Mosaic Virus, Necrosis caused by Lettuce Necrotic Yellow Virus, while other viral diseases such as Pea Enation Mosaic Virus and Pea Streak Virus have also been reported in the USA but their importance is not known (Nene and Reddy, 1987; Horn and Reddy, 1996).

Among the nematodes infesting chickpea, *Meloidogyne* spp., *Heterodera* spp. and *Pratylenchulus* spp. cause heavy losses to the crop in several countries. *M. incognita* and *M. javanica* are of economic importance in India and *M. artiellia* is important in the Mediterranean Region. In Syria, a species of cyst nematode of the *H. trifolii* group and *P. thorei* have caused marked yield losses. *Rotylenchulus reniformis, Helicotylenchulus sharafati, Hoplolaimus dimorphicus* are other nematodes associated with chickpea but they are of less importance (Greco, 1987; Di-Vito *et al.*, 1996).

Bacterial blight caused by *Xanthomonas cassiae* Kulkarni et al. was also found to be damaging to chickpea in India (Rangaswamy and Prasad, 1960; Nene, 1980).

1.2.1. Chickpea blight

Ascochyta blight is the most destructive disease of chickpea as it can devastate the crop over large areas if weather conditions favour infection and spread (Nene, 1982; Vishunavat *et al.*, 1985; Kaiser, 1987; Haware *et al.,* 1995; Singh and Reddy, 1996).

The disease has been reported from 28 countries, Algeria, Australia, Bangladesh, Bulgaria, Canada, Cyprus, Ethiopia, France, Greece, India, Iran, Iraq, Israel, Italy, Jordan, Lebanon, Mexico, Morocco, Portugal, Pakistan, Romania, Spain, Syria, Tanzania, Tunisia, Turkey, USA and USSR (Nene and Reddy, 1987). Losses in some of these countries have been reported. For example, in Greece Demetriades *et al.* (1959) reported 10 and 20% loss in 1957 and 1958, respectively. In 1959, it appeared in severe epiphytotic form in the Punjab province of India (Bedi, 1961). In Spain it caused severe losses wherever chickpeas were grown in 1964 (Puerta Romero, 1964). Losses in Russia in 1968 were 15 - 83% depending on the area affected (Askerov, 1968) and a severe epiphytotic was reported in 1968 and 1969 in Rumania by Radulescu *et al.*, 1971. Malaiki and Hamdi (1984) reported a 40% losses in Tunisia in 1981.

In Pakistan it appeared in 1920-1930, 1936 and 1978-79 causing losses of 50%, 20-50% and 17%, respectively and in 1979-82 it attacked in epidemic form resulting in approximately 50% loss each year (Sattar, 1933; Luthra *et al.*, 1935; Malik and Tufail, 1981; Malik and Bashir, 1984).

1.2.2. Nomenclature of causal organism

The causal agent of chickpea blight is the fungus *Ascochyta rabiei* (Pass.) Labr. but it was first named as *Zythia rabiei* by Passerini in 1867 because of its unicellular and hyaline pycnidiospores. Later, overlooking or not accepting Passerini's diagnosis, Comes (1891)

identified the fungus as *Ascochyta pisi* Lib. but in 1893 Prillieux and Delacroix named it *Phyllosticta cicerina.* In 1918, Trotter suggested that the fungus resembled *Phyllosticta* and hence proposed the combination of *Phyllosticta rabiei* (Pass.) Trotter (Khune and Kapoor, 1980). Owing to the absence of bicellular spores on the host, although a few were observed in culture, Labrousse (1930) also described the fungus as *Phyllosticta rabiei* but a year later he suggested it should be called *Ascochyta rabiei* as it produced 2-4% single septate spores on artificially inoculated plants. Now the International Mycological Institute and the majority of workers have accepted *Ascochyta rabiei* (Pass.) Labr. as the correct name (Nene, 1982).

1.2.2.1. Imperfect stage

Pycnidia containing pycnidiospores on live and dead chickpea material are the main characteristic of the asexual stage of the fungus and they can be seen as minute dots, embedded in host tissue. They are tan brown or grey, spherical or pear-shaped, ostiolate and generally vary in size from 80-240 x 60-215 µm. They contain numerous hyaline spores on short conidiophores embedded in a mucilaginous mass. When the pycnidia are wet, the mucilage absorbs moisture and swells causing the spores to ooze out. Pycnidiospores are oval to oblong, straight or slightly bent at both ends, hyaline and occasionally bicelled, 8.2 - 10.4 x 3.9-4.0 µm (Sattar, 1934; Luthra *et al.*, 1935). However, according to Kovacheski (1936) the size of pycnidiospores from the host is 6.0 - 16.0 x 3.4 - 5.5 µm and on artificial media 4.8 - 14.0 x 3.2 - 5.2 µm. Colonies of the fungus on liquid artificial media are flat and submerged with sparse mycelium, white when young and later turning dark.

1.2.2.2. Perfect stage

The sexual, teleomorph or perfect stage of *Ascochyta rabiei* was reported by Kovachevski in Bulgaria in 1936 and named *Mycosphaerella rabiei* Kovachevski. Its occurrence on overwintering chickpea residues was later reported from the former USSR (Gorlenko and Bushkova, 1958), Greece (Zachos *et al.*, 1963), Hungary (Kovics *et al.*, 1986), USA (Kaiser and Hannan, 1987), Spain (Jimenez-Diaz *et al.*, 1987) and Syria (Haware, 1987).

Pseudothecia are dark brown to black, subglobose, 120-270 µm in diameter with an inconspicuous ostiole. Asci are cylindrical to subclavate and eight spored. Ascospores are 9.5-16.0 x 4.5-7.0 µm and hyaline. They are strongly constricted at the septum which divides the spore into two unequal cells.

On the basis of the pattern of development of pseudothecia, the lack of fasciculate asci arising from preformed pseudoparaphyses and constriction of ascospores at the septum, Trapero-Casas and Kaiser, (1992a) named the perfect stage *Didymella rabiei* (Kovachevski) v. Arx rather than *Mycosphaerella rabiei* Kovachevski.

1.2.2.3. Races of *A. rabiei*

Luthra *et al.* (1939) and Arif and Jabbar (1965) found no evidence of races of the fungus. However, Reddy and Kabbabeh (1985), on the basis of 50 isolates collected from farmers' fields and experimental plots in Syria and Lebanon, proposed the existence of six races. Fischer *et al.* (1995) also suggested that races of the fungus existed owing to the variability of host-pathogen interaction and the sudden loss of resistance of some chickpea cultivars.

Contrary to these popular claims, Weigand (1989) showed that although the six 'races' differed in virulence, this was not specific, the more virulent 'races' being more virulent on all test cultivars and the less virulent also less virulent on all cultivars. The resolution of this point will have to await more stringent experimentation under controlled conditions with genetically pure cultures of the fungus and true breeding lines of the plant.

1.2.2.4. Symptoms

The fungus attacks all aerial parts of the plant throughout the growing season (Fig. 1.1). During the early stages of infection, petioles, leaflets and young branches lose their turgidity and develop epinasty. Small water-soaked spots appear on stems, leaves and pods which expand and become necrotic and covered with concentric rings of pycnidia. Lesions on stems and petioles often girdle the affected portion, causing breakage. Infected pods produce shrivelled seeds or are sometimes empty (Nene, 1982; Nene and Reddy, 1987; Alam, 1989).

1.3. EPIDEMIOLOGY

The occurrence of epidemics of Ascochyta blight of chickpea clearly indicates the successful survival of the fungus from one season to another either in crop residues or infected seed. Polycyclic pathogens such as *A. rabiei* have more than one and often many generations per season and may cause explosive damage under favourable environment. For example pycnidiospores of *Ascochyta rabiei* alighting on a susceptible chickpea plant produces a new generation of spores within 7-10 days in a cool and damp environment (Strange, 1993), while under laboratory conditions, a single inoculated seed may give as many as 10^8 pycnidiospores (Alam *et al.*, 1987).

Fig. 1.1. Disease symptoms consisting of necrotic lesions on stem, leaflets, pods and seeds of chickpea (cv. ILC 482). Note the dark concentric rings of pycnidia.

1.3.1. Survival of the fungus in crop residues

Survival of *A. rabiei* on diseased crop residues was first reported by Luthra *et al.* (1935) who also observed that when diseased material was buried in moist soil at a depth of 2 inches or more the fungus was killed. In contrast, Zachos *et al.* (1963) found that infected debris left on the surface of the soil for 2 years became covered with pycnidia and pseudothecia containing live spores. Similarly, Kaiser (1973) reported that the fungus survived for more than two years in naturally infected tissue at 10-35 °C and 0-30% relative humidity on the soil surface. Trapero-Casas and Kaiser (1992a) were able to induce the teleomorph of *A. rabiei* on artificially infested chickpea straw and found that, under field conditions in the Palouse region of the Pacific Northwest of the USA, the teleomorph developed extensively on overwintered chickpea crop residues remaining on the soil surface. Pseudothecia discharged ascospores in the spring (from the beginning of March to the end of May) and these probably served as primary inoculum for epidemics. In Spain, Navas-Cortes *et al.* (1995) found that the fungus grew saprophytically on infested chickpea stems and pods and formed pycnidia and pseudothecia. Under natural conditions the fungus grew rapidly on the tissue, formed abundant pseudothecia and remained alive throughout the two years of study. Trapero-Casas *et al.* (1996) exposed potted trap plants for one week periods near infected chickpea debris or grew trap plants 100 m from such debris. They found that the incidence and severity of Ascochyta blight developing on the plants correlated with pseudothecial maturity and ascospore production of the fungus.

1.3.2. Survival of the fungus in infected seed

Many workers have reported the survival of *A. rabiei* through seed. Butler (1918) was probably first to demonstrate the infection of chickpea seeds by *A. rabiei* and described the growth of the pathogen from infected seed to seedling during germination.

Many workers such as Zachos *et al.* (1963), Maden *et al.* (1975), Kumar *et al.* (1983), Qureshi, (1984), Vishunavat *et al.* (1985), Haware *et al.* (1986), Vishunavat and Chaube (1986), Kaiser (1987), Tripathi *et al.* (1987d), Porta-Puglia (1990) and Dey and Singh (1994) have suggested that infected seeds are an efficient means of transmission of the pathogen from one season to another. Infected seeds are also thought to be the means by which the pathogen has been introduced into countries which were previously free of the fungus. Such countries include Canada (Morrall and Mckenzie, 1974; Tu and Hall, 1984), Egypt (Abdel Monem *et al.*, 1984) and the USA (Kaiser and Muehlbauer, 1984).

The fungus is carried mainly superficially and predominantly as spores on seed surfaces but sometimes internally, both as mycelium and spores in the seedcoat and rarely in the embryo (Luthra and Bedi, 1932; Sattar, 1933; Maden *et al.*, 1975; Vishunavat *et al.*, 1985 and Dey and Singh, 1994).

The fungus survived for longer times when seeds were stored at low temperatures rather than high temperatures. Maden *et al.* (1975) found that spores of the fungus obtained from infected seeds stored at 3 ± 2°C for 14 months showed 33% germination. Periodic isolations revealed that the fungus survived for 14 to 15 months in infected seeds stored at 5°C and 10°C but only for 12 and 10 months in seeds stored at 20°C and 30°C, respectively (Tripathi *et al.*, 1987d). Sattar (1933) found that 50% spores obtained from seed survived for five months at 25-30 °C and only 5% for the same period at 35°C. Another report revealed that

although the storing of the infected seeds at 55°C, 60°C and 65°C for 6 to 12 h caused complete eradication of the fungus, it also reduced seed germination by 50% (Tripathi *et al.*, 1987b). The exposure of seed to direct sunlight for 8h per day for 15 days in May in India (approx. temperature 40°C) resulted in 50% reduction in the recovery of *A. rabiei*, while in seeds exposed to sunlight for the same period but covered with black polythene sheet, the recovery of the fungus was reduced by approximately 68% without reducing seed germination (Tripathi *et al.*, 1987c).

1.3.3. Dispersal of the pathogen

The spread of the disease has been attributed mainly to pycnidiospores produced at primary foci of infection which may originate from infected crop debris or infected seed (Nene, 1982). However, Trapero-Casas and Kaiser (1992a) found that airborne ascospores may serve as primary inoculum for epidemics in the USA. Sattar (1933) observed that infected plant material containing pycnidia may be blown for hundreds of metres under wet and windy weather conditions and Luthra *et al.* (1935) found that infected tissue breaks off from the brittle diseased plant and is transported hundreds of metres by winds, explaining the rapid spread of disease when rain is accompanied by strong winds.

Outbreaks of the disease in fields are associated with temperatures of 20-25°C, cloudy days and intermittent rains accompanied by winds (Haware *et al.*, 1986). Under moist conditions, conidia of *A. rabiei* ooze from the pycnidia in a gelatinous mucilage which dissolves in the water and allows dispersal by rain splash from plant to plant. Splash dispersal is essentially short range but the combination of splash with strong winds may spread spores over long distances. Pseudothecia protrude through the ostiole of the pseudothecium and forcibly

discharge their ascospores into the air where they can be carried for up to 3 km (Ingold, 1978; Kaiser, 1987).

1.3.4. Mode of infection

Spore germination of *A. rabiei* and the development of disease symptoms in chickpea cultivars is strongly influenced by humidity. Relative humidity of 98-100% is considered the optimum for infection, development of disease symptoms and sporulation (Chauhan and Sinha, 1973; Hohl *et al.* 1990; Trapero-Casas and Kaiser, 1992b; Jhorar *et al.*, 1998). The optimum temperature for infection and disease development is 20°C (Trapero-Casas and Kaiser, 1992b, Chauhan and Sinha, 1973). Temperatures below 18°C and above 28°C inhibited disease development owing to retardation of fungal growth (Hohl *et al.* 1990) and no symptoms were noted at 10°C and 30°C owing to lack of growth of the fungus at these temperatures (Chauhan and Sinha, 1973).

Spore germination, comprising swelling of the spore and development of a germ tube, occurs in 12-48 hours depending upon humidity and exposure of the leaflets to light (Hohl *et al.*, 1990; Pandey *et al.*, 1987). Hohl *et al.* (1990) observed the development of appressoria at the site of penetration, while Pandey *et al.* (1987) did not notice these. The young germ tubes secrete a mucilaginous exudate which helps attachment to the plant and provides protection against desiccation, while inside the leaf the mucilage may be a reservoir for fungal toxins and enzymes (Hohl *et al.*, 1990). Pandey *et al.* (1987) and Hohl *et al.* (1990) reported that the fungus penetrated through the cuticle between two epidermal cells but none of these workers saw any intracellular hyphae.

Hohl *et al.* (1990) also compared the histology of disease development in resistant and susceptible chickpea cultivars. They found that time of spore germination, development of a polar tube, fungal colonisation, secretion of mucilaginous exudate and appressoria formation were identical in both resistant and susceptible cultivars but, in resistant cultivars, cells died around the site of penetration during the early stages of infection, forming a small necrotic spot. They suggested that autoflourescence of cells in resistant cultivars is an early response indicative of a hypersensitive reaction and suggested that resistance may involve both hypersensitivity and detoxification of fungal toxins.

1.3.5. Host range

Kaiser (1973) reported that the fungus can infect cowpea (*Vigna sinensis*) and bean (*Phaseolus vulgaris*) when inoculated artificially. The fungus produced small reddish brown spots on the stems, petioles and leaves of these plants but the lesions did not increase in size. Later, Kaiser (1990) was able to isolate the fungus from several plant species growing in blight infected chickpea plantings or from fields where debris from blighted chickpeas remained on the soil surface over winter. These included *Amaranthus albus, Convolvolus arvense, Descurainia sophia, Galium aparine, Lanium amplexicaule, Lens culinaris, Medicago sativa, Pisum sativum, Solanum nigrum, Thlapsi arvense* and *Triticum aestivum.* Montorsi *et al.* (1992) isolated *A. rabiei* from seeds of berseem clover (*Trifolium alexandrinum*) and conducted successful pathogenicity trials on this plant.

1.4. CONTROL

Three methods have been used for the control of the disease, cultural practices, chemical control and breeding for resistance (Ahmad *et al.*, 1949; Nene, 1982; Mitsueda *et al.*, 1997). However, the most economical method would be through developing varieties with durable resistance while retaining other agronomically desirable characters.

1.4.1. Cultural practices

Sattar (1933) recommended the use of healthy seed for sowing, deep sowing to prevent emergence of infected seed, crop rotation and destruction of diseased crop residues as control measures. Mitsueda *et al.* (1997) suggested the destruction of infected plants by burning or deep ploughing in order to eliminate inoculum but the survival of the fungus on alternate hosts may reduce the efficacy of this measure (Kaiser, 1990; Montorsi *et al.*, 1992). In addition to field sanitation, Luthra *et al.* (1935) recommended intercropping with wheat, barley, taramira (*Eruca sativa*) and sarson (*Brassica campestris*) in order to reduce the spread of inoculum.

1.4.2. Chemical control

Infected seeds have been responsible for the introduction of the pathogen into new areas so seed treatment with effective fungicide is a useful method for reducing initial inoculum and preventing spread of the disease (Morrall and Mckenzie, 1974; Cother, 1977; Abdel Monem *et al.*, 1984; Kaiser and Muehlbauer, 1984; Nene, 1982).

Sattar (1933) recommended immersion of seed for 10 min in 0.5% copper sulphate, or pre-soaking in water at 20°C for 6 hours followed by immersion in hot water (53°C) for 15 minutes, but germination was adversely affected by the latter treatment. Seed borne inoculum

was controlled by immersing seed for 2 hours in malachite green (0.005%) or pimaricin (150 μg/ml) for 12h (Zachos 1951; Zachos *et al.*, 1963). Maden (1983) controlled the transmission of *A. rabiei* from seed to the aerial parts of the plant by treating seed with thiram (80% wettable powder) plus benomyl (50% wettable powder; 1:1 mix) at 6g product/Kg seed. Bhatti *et al.* (1984) reported that when calixin M and captan were used to dress infected seed at a dosage of 0.1%, control was 60% and germination and seedling vigour was enhanced.

Sugha *et al.* (1992) evaluated 12 fungicides *in vitro* for inhibition of spore germination of *A. rabiei*. They found that topsin-M (19.96 μg ml^{-1}) was the most effective followed by benomyl, galben R-4-33 and tecto.

Foliar applications of various fungicides have been reported to reduce disease spread significantly. Guar and Singh (1985) found that out of 21 chemicals sprayed four times at 10 day interval on artificially inoculated blight susceptible plants dithianon, chlorothalonil, captafol and captan controlled the disease best. Reddy and Singh (1990) reported that two sprays of chlorothalonil, one each during the seedling and early podding stages gave the highest cost benefit ratio (1:5) for controlling Ascochyta blight in the field.

Tripathi *et al.* (1987a) were able to completely eradicate seed-borne inoculum by treatment with calixin M, calixin M + thiram, thiram + brassicol and bavistin + thiram, when tested by the blotter method. They recommended treatment with bavistin + thiram (1:3) at 2.5 g/Kg seed with 3 sprays of bavistin at 0.5 Kg/ha at 10 days intervals to reduce disease severity and enhance grain yield. The crop was sown in November 7 and foliar application was started in the last week of January.

1.4.3. Difficulties in the development of resistant varieties

Singh and Reddy (1996) claimed that Ascochyta blight of chickpea can be managed by the use of resistant cultivars while Mitsueda *et al.* (1997) stressed that other methods of control are either impractical or uneconomical. In order to develop resistant cultivars there is a need to standardize inoculation and screening techniques.

1.4.3.1. Inoculation techniques

Labrousse (1931) was perhaps the first to make an effort to identify resistance through artificial inoculations. He scattered infected tissues on test plants and carried out repeated sprinklings with an aqueous spore suspension. In other experiments the test plants were inoculated with infected plant debris containing viable pycnidiospores (Luthar *et al.* 1938; Sattar and Hafiz, 1951). Singh *et al.* (1981) developed a field method for large scale screening which involved interplanting a susceptible spreader line, scattering infected debris between rows, spraying with spores from infected plants and providing high humidity by sprinkler irrigation. The material was scored on a 1-9 scale which will be discussed in section 1.4.3.2.

Timing of inoculum application is also important. Sattar (1933) found that plants were most susceptible at flowering and fruiting and therefore suggested these stages for inoculation, otherwise even plants of a susceptible variety may show a deceptive appearance of resistance. Spreading diseased crop residues over soil both in the autumn and spring in India has also been recommended (Vedysheva, 1966).

Environmental factors such as relative humidity and temperature influence the occurrence and severity of the disease which can complicate field screening. Consequently,

plant growth rooms with controlled environments have been used for screening germplasm against the pathogen (Reddy and Nene, 1979; Haware *et al.*, 1995).

1.4.3.2. Disease rating scales

The use of different disease rating scales by different research workers for scoring blight symptoms is a major problem in evaluating resistance of germplasm. Vir and Grewal (1974) used a 0-4 scale in pot and field screening where 0 = no infection, 1 = a few minute localized lesions on stems and/or up to 5% foliage infection, 2 = stem lesions 2-6 mm long which may girdle the stem and/or 5-25% foliage infection, 3 = stem lesion larger than 6 mm and girdling of the stem and/or 25-75% foliage infection, 4 = all young leaves and shoots killed.

Morrall and Mckenzie (1974) used a 0-5 scale for field screening as follows: 0 = no visible lesion on any plant in the plot; 1 = a few scattered lesions on the plant, usually found when observed carefully; 2 = lesions common and readily observed on plants but defoliation and damage not great, or in only one or two patches in the plot; 3 = lesions very common and damaging; 4 = all plots with extensive lesions, defoliation, and dying branches; 5 = all plants, or all but parts of a few plants, killed. Reddy and Nene (1979) extended the scale of Morrall and Mckenzie (1974) from 5 points (0-4) to 9 points (1-9). In their scale 1 = resistant, (no lesion visible); 2 = resistant to moderately resistant (lesions on a few plants, usually not visible); 3 = moderately resistant (a few scattered lesions usually seen after careful searching); 4 = moderately resistant to tolerant (lesions and defoliation on some plants, not damaging); 5 = tolerant (lesions common and easily observed on all plants, but defoliation and/or damage not great); 6 = tolerant to moderately susceptible (lesions very common, killing a few plants); 7 = moderately susceptible (lesions very common, damaging and killing 25% of plants); 8 =

moderately susceptible to susceptible (extensive lesions on all plants, causing defoliation and drying of branches and killing 50% of the plants); 9 = susceptible (lesions extensive on all plants, causing defoliation and drying of branches and killing of 75% of the plants). In this scale there are two points which are not clear. First, in point 2 of the scale if the lesions are not visible then how it can be known that they are there? Second, definite percentages (25, 50 and 75) of plants killed are given rather than a range. Later Singh *et al.* (1981) developed a 1-9 scale but with only five points. They scored their material as: 1 = no visible lesion on any plants (highly resistant); 3 = lesions visible on less than 10% of the plants, no stem girdling (resistant); 5 = lesions visible on up to 25% of the plants, stem girdling on less than 10% of the plants but little damage (tolerant); 7 = lesions on most plants, stem girdling on less than 50% of the plants resulting in death of a few plants (susceptible); 9 = lesion profuse on all plants, stem girdling on more than 50% of the plants and death of most plants (highly susceptible). A 9 point scale which is currently being used at the International Centre for Agricultural Research in the Dry Areas (ICARDA) is as follows: 1 = Highly resistant, no infection; 2 = Highly resistant, some leaf infection observed; 3 = Very resistant, some stem infection; 2-6 mm lesions; 4 = Resistant; stem starting to be girdled, larger lesions, no breakage; 5 = Moderately resistant; stem girdling and some breakage but < 50%; 6 = Susceptible; about 50% stems showing breakage; 7 = Very susceptible, most branches broken, 75% of plants killed; 8 = Highly susceptible, nearly all stems affected, tops broken, but green and 9 = Completely killed, no green material (Akem, pers. com.).

A quantitative scale, the linear infection index (LII), based on number and lesion size has also been introduced by (Riahi *et al.*, 1990). The index is calculated as follows:

$$\frac{NL \times ALL}{SL} \times 100$$

where NL = number of lesions on stems, ALL = average lesion length on stems and SL = stem length. The linear infection index was used to establish a new scale in which plants with LII% values 0 to 4 were considered resistant, and those with LII% values 5 or more were considered as susceptible. However, this scale does not include plants in which symptoms are confined to leaves.

1.4.3.3. Sources of resistance

Many workers have reported sources of resistance in chickpea against *A. rabiei.* Singh *et al.* (1981) tested 9,385 genotypes involving germplasm and segregating populations. They identified 21 lines and 36 progeny as resistant i.e. ≤ 3 on their 1-9 scale in the F_4 to F_7 generation. Reddy *et al.* (1983) found that out of 2000 accessions in Pakistan only two lines, ICC-7389 and 8536, were rated 4 or less, and fifteen others showed a rating of 5 on the 1-9 scale of Singh *et al.* (1981). Six lines, ICC-7067, -7192, -8476, -8540, -8565 and -8566, showed moderate resistance (3 on the 1-9 scale of Singh *et al.* [1981]) in both Syria and Pakistan but no cultivar was recorded as fully resistant in both countries. Reddy and Singh (1984) evaluated 9,574 desi and 3,836 kabuli germplasm accessions on their own 1-9 scale and identified 11 kabuli and 6 desi accessions as resistant in the vegetative and podding stages. These were as follows: the resistant kabuli accessions (rating 4) were: ILC 72, ILC 196, ILC 201, ILC 202, ILC 2506, ILC 2956, ILC 3274, ILC 3279, ILC 3346, ILC 3956 and ILC 4421.

The resistant desi accessions (rating 3) were: ICC 3634, ICC 4200, ICC 4248, ICC4368, ICC 5124 and ICC 6981.

More than 10,000 germplasm accessions of chickpea were tested against the pathogen at the National Agricultural Research Centre (NARC), Islamabad, Pakistan. The most promising lines of the desi type reported were ICC-76, ICC-607, ICC-641, CGP-1468, CGP-8519, NEC-138-2, NEC-1526, E-100Y, CM-72, CM68, RC-32, C-44, AUG 480. The kabuli lines showing resistance were ILC-72, ILC-183, ILC-194, ILC 195, ILC 484, ILC-201, ILC-202 and PUC-128. The resistant/tolerant lines have been used in hybridization programmes for the development of resistant varieties and CM-72, CM-88, C-44, Punjab 91, Paidar-91 and Noor-91 have been released for commercial cultivation in Pakistan (Mitsueda *et al.*, 1997).

Singh and Reddy (1993) evaluated 201 accessions of eight annual wild species of *Cicer* in field and greenhouse experiments over a 3 year period in Syria. They found that one accession each of *C. judaicum* Bois (ILWC 165) and *C. pinnatifidum* Jaub. & Spach (ILWC 159) were consistently rated resistant with scores of 2-4 on the 1-9 scale of Reddy and Singh (1984) in both field and greenhouse evaluations.

Singh and Reddy (1996) claimed to have developed 1584 chickpea lines which were resistant to Ascochyta blight with a range of maturity, plant height, and seed size not previously available to growers in blight endemic areas in the Mediterranean region. These include 92 lines which were claimed to be resistant on the 1-9 scale of Singh *et al.* (1981) to 6 'races' of *Ascochyta*, and 15 large seeded and 28 early maturity lines. However, as previously discussed (section 1.2.2.3) the existence of races of *A. rabiei* is not established. Furthermore, the ratings of most of the resistant lines is not given and also some described as resistant had ratings as high as 8 on the 1-9 scale of Singh *et al.* (1981)!

Strange (1997) suggested four problems confronting breeders in search of useful resistance in chickpea to Ascochyta blight:

1. Variation in estimates of disease severity owing to lack of a suitable scale for scoring blight symptoms and operator error.

2. Heterogeneous inocula.

3. The habit of regarding all reactions less than 5 on the 1-9 scale as resistant and those that are 5 or above as susceptible (Reddy *et al.*, 1981).

4. Lack of knowledge about the fundamentals of the interaction of host and pathogen regulating disease severity.

1.5. TOXINS

The involvement of toxins produced by pathogenic micro-organisms was first suggested by de Bary in the latter half of the 19th century. Toxins were defined by Scheffer and Briggs (1981) as microbial products other than enzymes, which cause obvious damage to plant tissues and which are known with reasonable confidence to be involved in disease development. Most are produced both *in vitro* and *in planta*. These compounds may play important roles in virulence or pathogenesis, especially when produced in the early stages of disease development. In some cases they are capable of producing many or all of the disease symptoms (Upadhyay and Mukerji, 1997; Durbin, 1983). Most toxins are low molecular weight compounds and diffuse from the site of infection to the surrounding tissue or are translocated within the plant via the apoplast (Upadhyay and Mukerji, 1997).

Toxins produce different symptoms in plants according to their chemical nature. At the macroscopic level they cause chlorosis, wilting, growth abnormalities and the production of

water-soaked lesions, while at the microscopic and biochemical levels toxins adversely affect the structural components of the plants including the plasma membrane, chloroplasts and mitochondria (Wheeler, 1981; Otani and Kohmoto, 1992; Strange, 1993). Otani *et al.* (1995) classified toxins in terms of their primary site of action as follows:

1. Plasma membrane. Examples are: ACT- toxin produced by the *Alternaria alternata* tangerine pathotype also denoted as *A. citri*, AF- toxin produced by *Alternaria alternata* strawberry pathotype and AK- toxin produced by *Alternaria alternata* Japanese pear pathotype. These toxins appear to have early effects on the plasma membranes of susceptible cells (Otani and Kohmoto 1992). As reviewed by Otani *et al* (1995), an increase in electrolyte loss from tissues and invagination of plasma membranes are common characteristics of toxin action. In addition, AK-toxin and AF-toxin induce a depolarization of membrane electropotential in 5-10 min.

2. Mitochondria: Toxins such as ACR (L)- toxin produced by *Alternaria alternata* rough melon pathotype comes into this category. The toxin induces a swelling of mitochondria, reduction in numbers and vesiculation of cristae, and a decrease in the electron density of the matrix (Akimitsu *et al.*, 1989). After affecting mitochondria, the toxin induces a rapid increase in electrolyte loss from leaf tissue and eventually causes a veinal necrosis on the leaves. Continuous irradiation of leaves immediately after toxin exposure inhibits toxin-induced electrolyte loss and necrosis but not the action of the toxin on mitochondria (Kohmoto *et al.*, 1989).

3. Plasma membrane and chloroplast: AM-toxin produced by *Alternaria alternata* apple pathotype is an example of this class. Physiological and ultrastructural studies suggested that susceptible apple cultivars have two primary sites for AM-toxin action. One site is in the

chloroplasts, where the toxin induces dissociation and vesiculation of grana lamellae and inhibits photosythetic CO_2 fixation. The other site is in the plasma membrane where the toxin causes modification of plasma membranes, increasing electrolyte loss (Kohmoto *et al.*, 1982).

Generally toxins are classified into two groups, host selective and non host selective (Strange, 1993, Scheffer and Briggs, 1981).

1.5.1. Host selective toxins (host specific toxins)

Host selective toxins (HSTs) are those which affect only plants that are hosts of the toxin-producing organism (Strange, 1993). For pathogens producing host selective toxins, the virulence of the organism is correlated with the amount of toxin produced (Oku, 1994).

At present 14 pathogenic fungi are known to produce more than 20 host selective toxins and most of them belong to the genera *Alternaria* and *Helminthosporium* (Oku, 1994; Strange, 1993; Otani *et al.*, 1995; Upadhyay and Mukerji 1997). Tanaka (1933) probably was the first to report an HST when he was working with leaf spot disease of pear caused by *Alternaria alternata* (previously called *A. kikuchiana*). He observed that fungal-free culture filtrates were toxic to leaves of a variety susceptible to the pathogen while resistant varieties remained unaffected. Toxin preparations were found to contain three closely related compounds, two major and a minor one (Nishimura *et al.*, 1979). Later, in 1982 Nakashima and co-workers were able to isolate two toxins called AK-toxins I and II in crystalline form. The chemical structure of AK-toxin I was determined by mass spectrometry, nuclear magnetic resonance, infra red and ultra violet spectroscopy. AK-toxin II was found to be a derivative of AK-toxin I. AK-toxin I was highly toxic and induced veinal necrosis and rapid K^+ loss on leaves

of the pear cultivar, Nijisseiki, at concentrations of 10^{-8} to 5 x 10^{-9} M but no visible symptoms on leaves of a number of resistant cultivars at 1.2 x 10^{-4} M (Nishimura and Kohmoto, 1983).

Maekawa *et al.* (1984) isolated three compounds, AF-toxins, I, II, III from a strawberry pathotype of *A. alternata* which caused black spot disease on the cultivar, Morioka-16. A pathotype *A. alternata* which infected apples (formerly, *A. mali*) produced three toxins AM-toxins I, II, and III. On analysis, AM I toxin was found to be a cyclic depsipeptide and AM II and III were derivatives (Otani *et al.*, 1995).

Gilchrist *et al.* (1995) reported the production of AAL-toxin by *A. alternata* f. sp. *lycopersici*, causing stem canker on tomatoes both in the host plant and in culture medium.

Victoria blight of oats caused by *Cochliobolus victoriae* (= *Helminthosporium victoriae*) was first described by Meehan and Murphy (1946). Only oat lines containing Victoria-type resistance to crown rust were susceptible. Cell free culture filtrates of *C. victoriae* contained a toxic compound that not only produced the disease symptoms but also exhibited the same specificity as the fungus. The most abundant toxic compound purified from culture filtrates was victorin C (HV-toxin). Victorin A, B, D, E and victoricine were minor components (Macko *et al.*, 1985; Wolpert *et al.*, 1985; Wolpert *et al.*, 1988). The susceptibility to the fungus and sensitivity to its toxin was controlled by a single dominant gene with the homozygous recessive genotype conditioning resistance to the pathogen and insensitivity to the toxin. Thus victorin was a specific toxin affecting only those genotypes of host that carry the dominant gene for susceptibility to *C. victoriae.* The gene also conferred resistance to crown rust of oats (Scheffer, 1976).

The fungal pathogen *Cochliobolus carbonum* (= *Helminthosporium carbonum*), causal organism of leaf spot and ear mold of maize, produces a host selective toxin named HC-toxin.

Maize containing the *Hm1* gene is resistant to the pathogen and tolerant of the toxin since the gene encodes an enzyme which inactivates HC-toxin by reducing an essential carbonyl group of the toxin (Meeley and Walton 1991; Meeley *et al.*, 1992). Yoder (1973) reported that *Pyllosticta maydis* causing yellow leaf blight of corn, produced a host-selective toxin which may be similar to HM-T toxin which is produced by *Helminthosporium maydis*, because both toxins are host-selective for corn with T cytoplasm and also both cause damage to mitochondria from corn with T but not N cytoplasm. Macko *et al.* (1992) found that *Periconia circinata* causing root and crown rot disease of sorghum produced peritoxins A and B which had molecular weights of 574 and 558, respectively and were selectively toxic to genotypes of *Sorghum bicolor*.

1.5.2. Non host selective toxins (non host specific toxins)

Non host selective toxins are those which produce symptoms not only on host of the pathogen but on other plants as well (Strange, 1993).

A cyclic tetrapeptide compound, tentoxin, is produced by *Alternaria tenuis* and was found to be toxic to all members of the Compositae and Umbelliferae tested while Cruciferous species were insensitive (Durbin and Uchytil, 1977). Later Klotz (1988) reported that tentoxin disturbed coupling factor 1 which is involved in photosynthetic phosphorylation. *Alternaria tagetica* which causes water-soaked lesions on marigold produces zinniol toxins (Cotty and Misaghi, 1985).

Manulis *et al.* (1986) investigated structure-activity relationships of the phytotoxins stemphyloxin I and II from *Stemphylium botryosum* f. sp. *lycopersici* by quantitative comparison of their biological activity with chemically related phytotoxins from *Phoma betae*,

betaenones A, B and C. They found that the β-ketoaldehyde moiety was essential for biological activity and its toxicity was influenced by its spatial orientation.

Protein-lipopolysaccharide complexes (PLPC) were isolated from culture filtrates of *Verticillium albo-atrum* (WCS 800) and *V. dahliae* (WCS 070) virulent to tomato and potato and were also obtained from an isolate of *V. albo-atrum* (V22W) which was non-virulent to these hosts. PLPC from the virulent isolates were toxic to tomato in a leaf bioassay at 4 $\mu g/ml^{-1}$ (WCS 800) and 20 $\mu g/ml^{-1}$ (WCS 070) but the two PLPCs from the non-virulent isolate required concentration of 100 and 1000 $\mu g/ml^{-1}$ for toxicity. Production of less PLPC in V22W was suggested as the reason for its non-virulence (Harling *et al.*, 1986).

Sirodesmin PL was isolated from the culture filtrate of *Phoma lingam*, causal organism of black leg disease of several cruciferous crops, especially cabbage and rape. This toxin reduced the viability of freshly isolated protoplasts of *Brassica napus*, *Nicotiana tabacum* and *Solanum tuberosum* while cell cultures of the host plant, *B. napus* were more sensitive than those of the two non hosts plants (Sjodin *et al.*, 1988). Badawy and Hoppe (1989) were able to isolate sirodesmin PL from culture filtrates of *Leptosphaeria maculans* (perfect stage of *Phoma lingam*).

Non-host selective toxins are therefore chemically heterogeneous and their modes of action have seldom been established.

1.5.3. Toxins produced by the genus *Ascochyta*

Strange (1997) divided the toxins produced by the genus *Ascochyta* into five groups on the basis of their chemical structures which are as follows:

Group 1: Comprising of ascochitine, ascosalitoxin, pyrenolide A, hyalopyrone and the pinolidoxins (Fig. 1.2).

Group 2 : Chrysanthones A, B and C, chrysophanic acid, pachybasin, cyperine, epoxydon, epoxydone acetate and decumbin (brefeldin A) (Fig. 1.3).

Group 3 : Ascochlorin, ascofuranone and ascofuranol (Fig. 1.4).

Group 4 : The cytochalasins (Figs. 1.5 and 1.6).

Group 5 : The solanapyrones (Fig. 1.7).

Ascochyta pisi and *A. fabae* produced ascochitine in culture (Beed *et al.*, 1994; Lepoivre, 1982; Oku and Nakanishi, 1963). The phytotoxins pinolidoxin and ascosalitoxin were produced by *Ascochyta pinodes* and *Ascochyta pisi* var. *pisi*, respectively, the causal agents of pea anthracnose, when grown on wheat kernels. Three pinolidoxins called epi-, dihydro- and epoxy-pinolidoxin were also isolated from *Ascochyta pinodes* grown on wheat (Evidente *et al.*, 1993a; 1993b; 1993c). Venkatasubbaiah and Chilton (1992) reported that *A. hyalospora* produced ascochitine along with two other phytotoxins, pyrenolide A and hyalopyrone. A strain (L.1.1) of *A. chrysanthemi* causal agent of ray blight of *Chrysanthemum* produced chrysanthone A, B and C. When the fungus was grown on potato dextrose broth it gave chrysanthone A and C but when grown on wort agar medium it produced chrysanthone B in the mycelium extracts (Albinati *et al.*, 1989; Arnone *et al.*, 1990).

Assante and co-workers (1981) isolated epoxydone and epoxydone acetate from a culture of *A. chrysanthemi.*

Ascotoxin was extracted from *A. imperfecta* causal organism of foot rot of lucerne and clover (Suzuki *et al.*, 1970). Later, ascotoxin was found to be identical with decumbin which

was isolated from *Penicillium decumbens* (Singleton *et al.*, 1958) and brefeldin A isolated from *P. brefeldiana* (Härri *et al.*, 1963).

Tamura *et al.* (1968) isolated ascochlorin from *A. viciae*. Later, ascofuranone and ascofuranol were isolated by Sasaki and co-workers (1972, 1973) from the same fungus.

Seven cytochalasins were identified by Bottalico *et al.* (1990) from *A. heteromorpha*, causal agent of a foliar disease of oleander. Later, Vurro *et al.* (1992) tested 13 species of *Ascochyta* for the production of the cytochalasins by growing them on wheat and found that only *A. lathyri* produced cytochalasins A and B. Recently Latif *et al.* (1993) reported that out of nine isolates of *Ascochyta rabiei* tested, one strain produced cytochalasin D in still culture containing Czapek Dox liquid medium supplemented with aqueous extract of chickpea seed.

The solanapyrones A, B and C were first isolated from culture filtrates of *Alternaria solani,* a pathogen causing early blight of potato (Ichihara *et al.*, 1983). These three compounds contain a pyrone moiety in which alternative aldehyde, hydroxyl and ethanolamine substitution groups describe their different structures (Ichihara *et al.*, 1983; Strange, 1997: Fig. 1.7).

Benning and Barz (1995) found by incorporation experiments that the solanapyrone carbon skeleton was synthesized from eight acetate units. The head to tail arrangement of acetate units in the main chain of the polyketide results from a series of condensation-reduction steps similar to fatty acid formation. Further reduction to a triene moiety in an intramolecular Diels-Alder reaction leads to the cis-decalin moiety of the solanapyrones (Oikawa *et al.*, 1994). Recently Ichihara and Oikawa (1997) showed that this reaction is catalyzed by the enzyme Diels-Alderase. Previously, Oikawa *et al.* (1989) showed by experiments with [S-$^{13}CH_3$] labelled methionine and cultures of *A. solani* that the methoxy group of the pyrone ring in

Sol.A and Sol.B was introduced via the C_1-pathway. *A. rabiei* also produced Sol.A and C when grown on Czapek Dox liquid medium supplemented with chickpea seed extract (Alam *et al.*, 1989; Latif *et al.*, 1993). Chen and Strange (1991) found that *A. rabiei* produced not only Sol.A and Sol.C but also produced Sol.B when the fungus was grown on Czapek Dox medium supplemented with the metal cations Zn, Mn, Ca, Cu, Co and Li and that Zn was essential for production of the toxins.

1.6. DETOXIFICATION OF TOXINS

Plants are constantly exposed to a variety of toxic compounds. These foreign compounds may be of natural origin, e.g. microbial toxins, or man made e.g. herbicides and chemical waste pollutants (Coleman *et al.*, 1997b).

As discussed earlier, *A. rabiei* produces the solanapyrone toxins and the possibility now exists that they play an important role in host parasite relations as they are produced by many pathogenic isolates of the fungus (Strange, 1997; Latif *et al.* 1993; Benning and Barz, 1995).

Strange (1997) suggested three methods for exploiting toxins in disease control: screening whole plants for resistance, selection for resistance in tissue culture with regeneration of resistant plants and genetically engineering plants to destroy the toxic compounds.

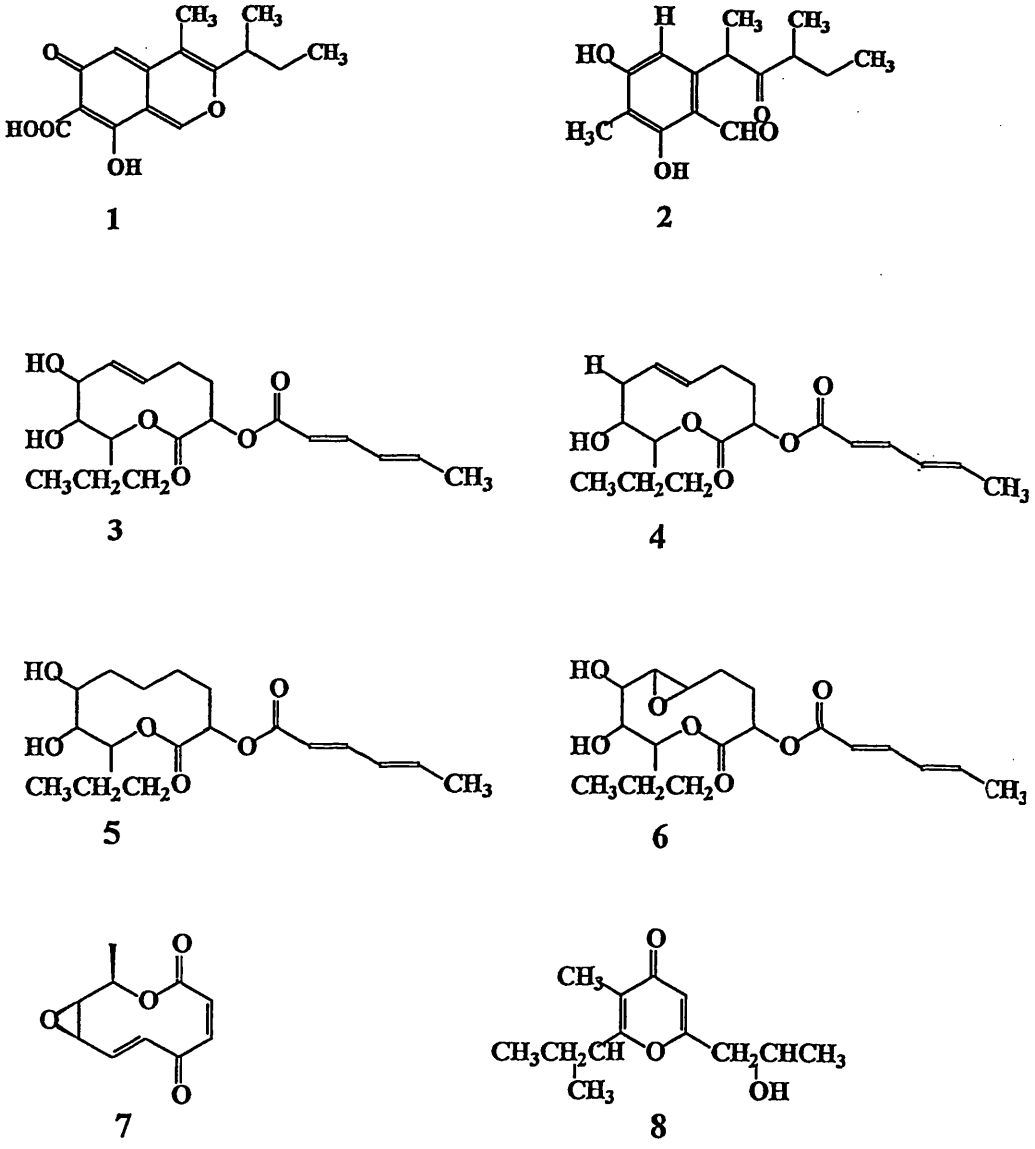


Fig. 1.2. Structures of toxins produced by the genus *Ascochyta*, I. 1 = ascochitire; 2 = ascosalitoxin; 3 = pinolidoxin; 4 = epipinolidoxin; 5 = dihydropinolidoxin; 6 = epoxypinolidoxin; 7 = pyrenolide A; 8 = hyalopyrone.

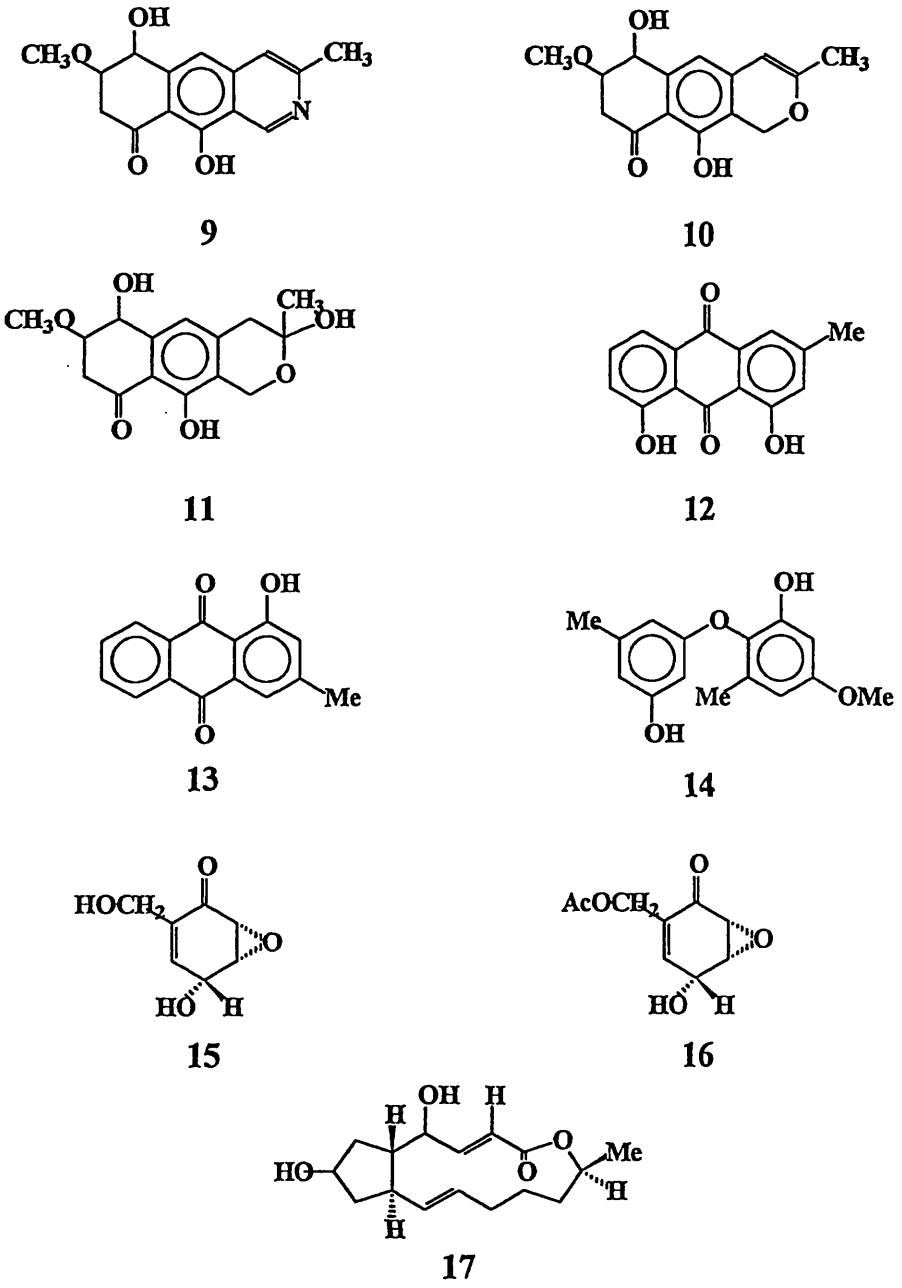


Fig. 1.3. Structures of toxins produced by the genus *Ascochyta*, II. 9 = chrysanthone A; 10 = chrysanthone B; 11 = chrysanthone C; 12 = chrysophanic acid; 13 = pachybasin; 14 = cyperine; 15 = epoxydon; 16 = epoxydon acetate; 17 = decumbin (= brefeldin A or ascotoxin).

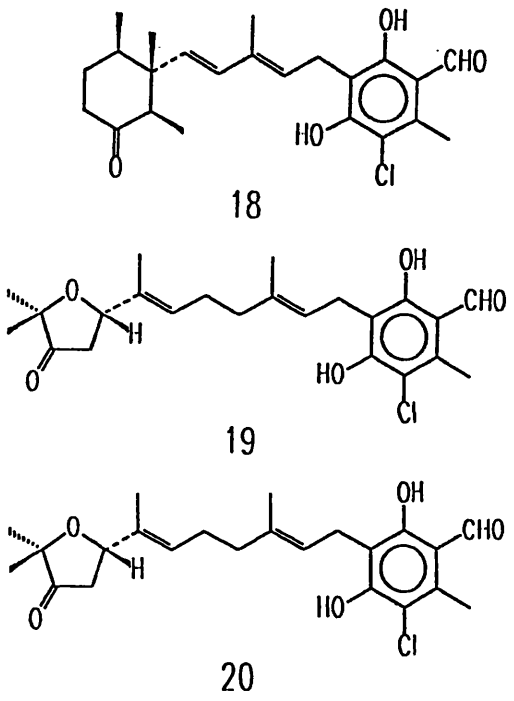


Fig. 1.4. Structures of toxins produced by the genus *Ascochyta*, III. 18 = ascochlorin; 19 = ascofuranone; 20 = ascofuranol.

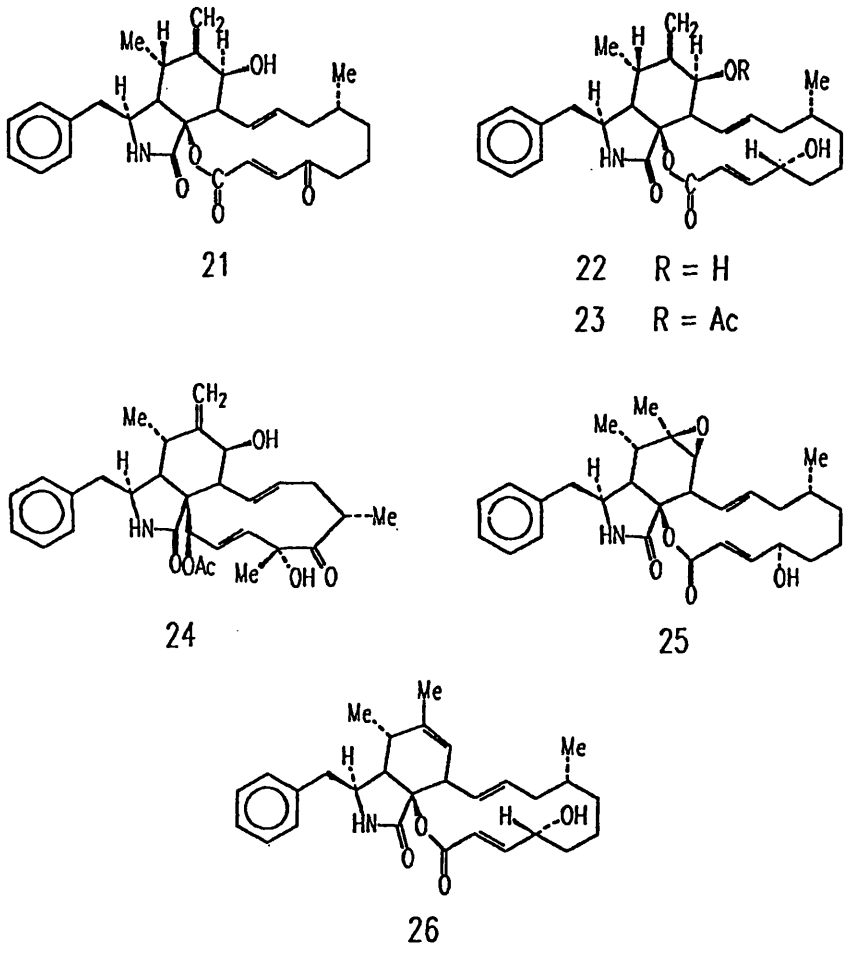


Fig. 1.5. Structures of toxins produced by the genus *Ascochyta*, IV. 21 = cytochalasin A; 22 = cytochalasin B; 23 = 7-O-acetylcytochalasin B; 24 = cytochalasin D; 25 = cytochalasin F; 26 = cytochalasin T.

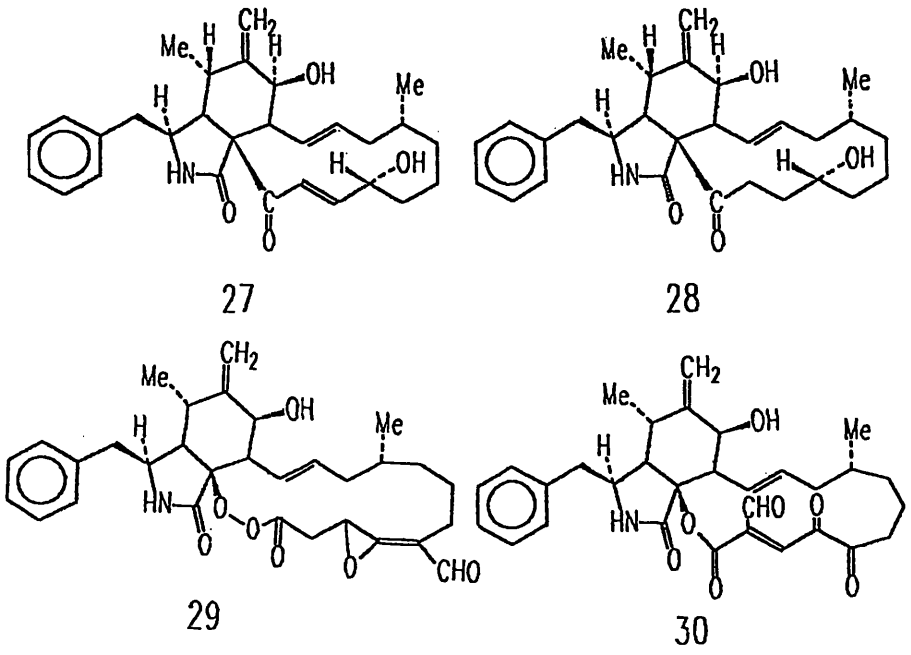


Fig. 1.6. Structures of the toxins produced by the genus *Ascochyta*, IV. 27 = deoxyaphomin; 28 = ascochalasin; 29 = cytochalasin U; 30 = cytochalasin V.

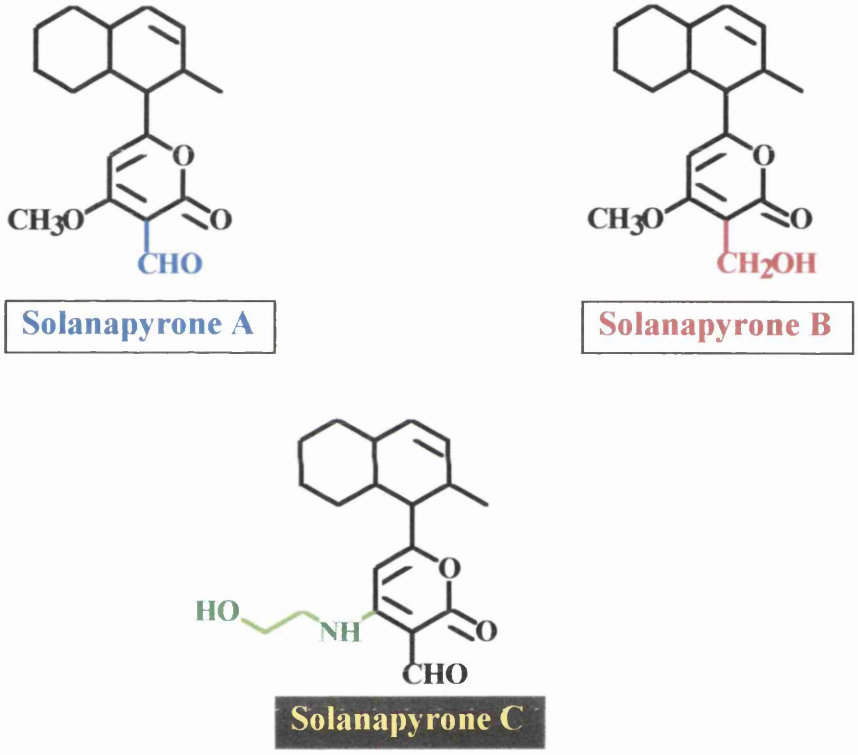


Fig. 1.7. Structures of the solanapyrone toxins produced by *A. rabiei.*

1.6.1. Detoxification by plants

Plants have developed a number of biochemical processes to counter the toxic effects of xenobiotics. One of the most important is chemical modification of the foreign compound. For example, HC-toxin (as previously discussed in section 1.5.1) is detoxified by an enzyme produced by resistant maize plants containing the *Hm1* gene (Meeley and Walton, 1991; Meeley *et al.*, 1992). Similarly, Zweimuller *et al.* (1997) detected detoxification of fomannoxin by investigating the interaction of the phytotoxin with *Pinus sylvestris* cells. Fomannoxin is a phytotoxic secondary metabolite produced by the pathogenic Basidiomycete, *Heterobasidion annosum,* during the infection process. They found that the aromatic aldehyde group is reduced by the plant cells producing the non toxic fomannoxin alcohol, while after longer incubation times, fomannoxin acid -β-glucoside could be isolated as another detoxification metabolite.

Another way in which xenobiotics are detoxified is by covalent linkage to endogenous molecules such as glutathione (Coleman *et al.*, 1997a, 1997b). By using the xenobiotic, monochlorobimane, which reacts with glutathione (GSH) to yield a strong blue fluorescent conjugate, bimane-glutathione, these workers demonstrated that this xenobiotic was detoxified by conjugation to GSH in the cytoplasm, followed by the transport of the conjugate into the vacuole. They also reported that the detoxification pathway shared many features with the pathway used by plants for the synthesis and vacuolar deposition of secondary metabolites such as anthocyanins.

GSH (γ-glutamyl-cysteinyl-glycine) acts as a cellular nucleophile through the thiol group of its cysteinyl residue and can undergo spontaneous or glutathione S-transferase (GST) catalyzed conjugation to a wide range of xenobiotic electrophiles resulting in their

detoxification. For example several herbicides and their derivatives which contain electrophilic sites are metabolized by conjugation with GSH (Lamoureux *et al.*, 1991).

Mozer *et al.* (1983) was able to purify two glutathione transferase enzymes GST I and GST II and concluded that both enzymes catalyzed the formation of a glutathione-alachlor conjugate *in vitro* when alachlor was used as a substrate. Later, Breaux *et al.* (1987) suggested that the basis for selective phytotoxicity is often the lack of metabolic deactivation in susceptible plants. For example, the selective chloroacetanilide herbicides alachlor, acetochlor and metachlor are metabolized less readily by susceptible weeds such as barnyardgrass than by tolerant corn seedlings. They reported that conjugation with glutathione was the cause of tolerance to chloroacetanilide. They also observed corn seedlings had more GSH compared with barnyardgrass which contained less GSH i.e. 182.4 ± 10.2 μg/g fresh wt. and 57.3 ± 3.0 μg/g fresh wt., respectively.

In plants, potentially toxic compounds are ultimately deposited in the large central vacuole. Wolf *et al.* (1996) showed that alachlor rapidly accumulates as GS-conjugates in the plant vacuole and that the first step of its degradation, formation of the γ-glutamylcysteinyl-S-conjugate, is catalyzed by a vacuolar carboxypeptidase. They suggested that the glutathione conjugate is a transport form but not a storage form of xenobiotic molecules.

Herbicide safeners are chemicals which protect crop plants from injury by certain herbicides, without affecting their weed control efficacy. They act by causing increase in the activities of GST, glucosyl transferases and cytochrome P-450 dependent monooxygenases, as well as increase in GSH content (Farago *et al.*, 1994). Gaillard *et al.* (1994) found that treatment of barley with the safener cloquintocet-mexyl increased the activity of GST by 50% and also promoted transport of both glutathione and glucoside conjugates of the compound

into the vacuole. Hunaiti and Ali (1991) observed 2.69 and 2.7 fold increase in GST activity in chickpea shoots when treated with 10 ppm and 20 ppm oxidiazon, respectively for 24 hours. Generally, the corn herbicide antidotes such as 5-(2,4-dichlorophenyl)-4-isoxazolecarboxylic acid, ethyl ester; 2-[(cyanomethoxy)imino]-benzeneacetonitrile; naphthalic anhydride; 2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylic acid, benzyl ester and N,N-diallyl-2-,2-dichloro-acetamide, raised enzyme levels between 1.5- and 2.5- fold in both roots and shoots (Mozer *et al.*, 1983). Recently, modest enhancement of GST activity towards 1-chloro 2,4-dinitrobenzene was reported in soybean when treated with the herbicide safeners dichlormid, naphthalic anhydride and BAS 145-148 (Andrews *et al.*, 1997). These safeners could also be helpful in increasing GSH content and GST activity in chickpea, another dicotyledonous plant.

1.7. AIMS OF THE PROJECT

The overall aim of the project was to contribute to an understanding of the role of the solanapyrone toxins in Ascochyta blight of chickpea. In order to do this it was necessary to do the following:

1. To isolate the solanapyrone toxins.
2. To determine the relative toxicity of the solanapyrone toxins in a range of chickpea cultivars.
3. To seek an explanation for differences among cultivars in sensitivity to the toxins.
4. To attempt to use these data to ameliorate the effects of the disease.

CHAPTER 2

PRODUCTION, SEPARATION AND SOME CHEMICAL REACTIONS OF THE SOLANAPYRONE TOXINS

2.1. INTRODUCTION

Alam *et al.* (1989) reported that isolates of *A. rabiei* produced the solanapyrone toxins A and C when grown in Czapek Dox liquid medium (CDLM) supplemented with chickpea seed extract. Later, Chen and Strange (1991) found that *A. rabiei* not only produced solanapyrones A and C but also solanapyrone B when it was grown on CDLM supplemented with the metal cations Zn, Ca, Cu, Co and Mn rather than chickpea seed extract and that zinc was essential for toxin production. In this chapter, production of the solanapyrone toxins A, B and C by *A. rabiei* (isolate PUT 7) when grown on this medium is described as well as experiments in which the addition of zinc was delayed or was omitted altogether.

In order to study the toxicity of the individual solanapyrones, a technique was needed to separate them in sufficient quantities for experimentation. Therefore solid and liquid phase extraction and radial and flash chromatography were evaluated for the purification and isolation of the toxins from culture filtrates. Purity and yield of the toxins was assessed by reversed phase analytical HPLC.

Some chemical reactions of the compounds were also tested by spraying thin layer chromatograms of the compounds with several reagents.

2.2. MATERIALS AND METHODS

2.2.1. Toxin production

A. rabiei was grown on Czapek Dox liquid medium to which was added the following salts:-

Salts	Concentration mg/l
1. $ZnSO_4.7H_2O$	50
2. $CaCl_2.2H_2O$	100
3. $CoCl_2.6H_2O$	20
4. $CuCl_2.6H_2O$	20
5. $MnCl_2.4H_2O$	20

The medium (CDLMC) was dispensed in conical flasks (30 ml medium/250 ml conical flask) and inoculated with 0.03 ml/flask spore suspension of *A. rabiei* (isolate PUT 7) containing 10^7 spores/ml (Alam *et al.*, 1987). Incubation was at 20 ± 1 °C without shaking for 12 days. In some experiments the addition of zinc was delayed until the cultures were 8 days old or this cation was omitted altogether.

2.2.2. Partial purification of the solanapyrone toxins by solid phase extraction (SPE)

For bulk preparation of the solanapyrones, mycelium of the fungus was removed from a total of about 1 litre of culture medium (33 flasks containing 30 ml each) by filtration through four layers of muslin cloth. Spores were removed from the filtrate by

centrifugation at *c.* 10,000 *g* for 20 minutes. After centrifugation, the supernatant was passed through three reversed phase end-capped Isolute cartridges (5g: C18: International Sorbent Technology, Duffryn Industrial Estate, Ystrad Mynach, Hengoed, Glamorgan, UK) which had been conditioned with 25 ml methanol, followed by 25 ml distilled water. Culture filtrates were run slowly through the conditioned cartridges, followed by distilled water (25 ml). The toxins were eluted with 25 ml acetonitrile (HPLC grade). Anhydrous sodium sulphate (1g) was added to the acetonitrile solution and, after filtration through Whatman No. 1 filter paper, the acetonitrile was removed by film evaporation at <40°C. Residues were dissolved in acetonitrile (5 ml: HPLC grade) and the solanapyrones were quantified by running 20 µl samples on high performance liquid chromatography (section 2.2.6).

For smaller scale experiments, individual flasks (250 ml containing 30 ml medium) were processed in essentially the same way except that 1g Isolute cartridges rather than 5g cartridges were used.

2.2.3. Partial purification of the solanapyrone toxins in bulk by liquid phase extraction (LPE)

The pH of culture filtrates (1 litre) was adjusted to 3.00 with 1M H_2SO_4 before partitioning three times against 1/3rd volume of ethyl acetate. The ethyl acetate phases were combined, dried over 20 g of anhydrous sodium sulphate and taken to dryness on a rotary evaporator at < 35°C. The residue containing the toxins was dissolved in 5 ml ethyl acetate and the solanapyrones were quantified by running samples (20 µl) on HPLC (section 2.2.6).

2.2.4. Separation of the solanapyrone toxins

2.2.4.1. By chromatotron

Silica gel 60 PF_{254} (TA-460649, Merck, D-6100 Darmstadt, F.R. Germany) 45 g was mixed thoroughly with distilled water (90 ml) in a glass bottle. The suspension was cooled to 0-5°C and poured on a circular glass plate (Fig. 2.1a). The plate was tapped to spread the Silica gel evenly and kept at room temperature for 1 h followed by incubation overnight at 55 ± 5 °C. Preparation of the plate was finished by a special blade which was rotated from the centre to give a thickness of 1 mm and to remove Silica from the centre and the edges (Fig. 2.1b).

The solvent for separation of the toxins (dichloromethane, cyclohexane and ethyl acetate 4:4:1 v/v/v) was placed in the mariotte flask and used to equilibrate the plate for 30 min (Fig. 2.2). The concentrated samples (1.5 ml) of toxins in ethyl acetate, representing 500 ml culture filtrate, were injected onto the plate through the solvent inlet with the help of a syringe and 80 fractions (3 ml/fraction) were collected. Each fraction was observed on a spectrophotometer and pooled according to their $\lambda_{max.}$ (section 2.2.5). Solvent was removed from the pooled fractions by a rotary evaporator operating at <40°C. The residues were dissolved in methanol (5 ml) and checked on HPLC for their purity (section 2.2.6).

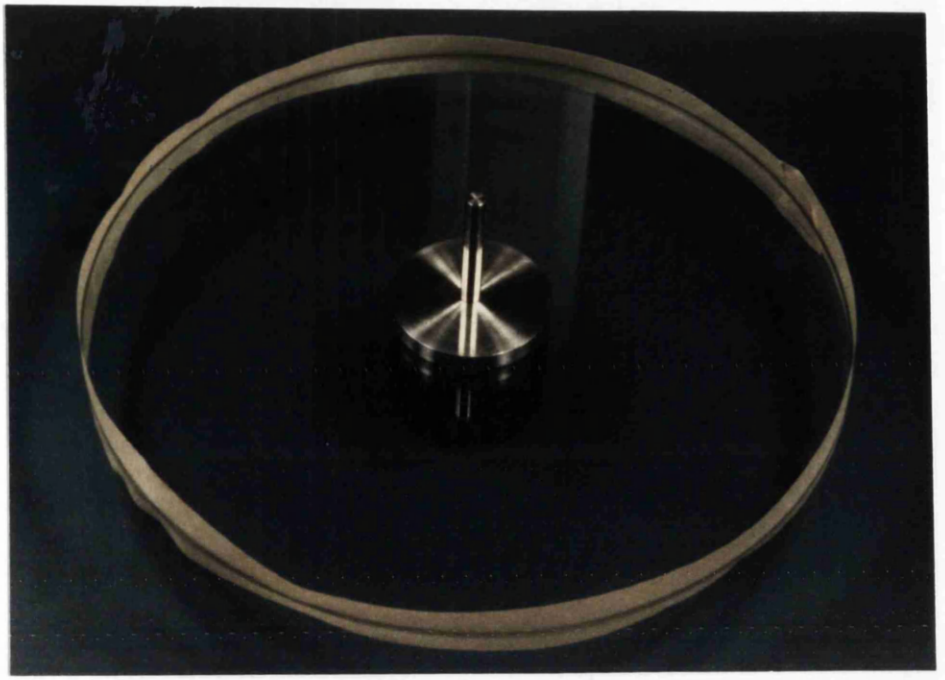


Fig 2.1a. A chromatotron glass plate. Note the tape around the edge of the plate to retain the silica gel.

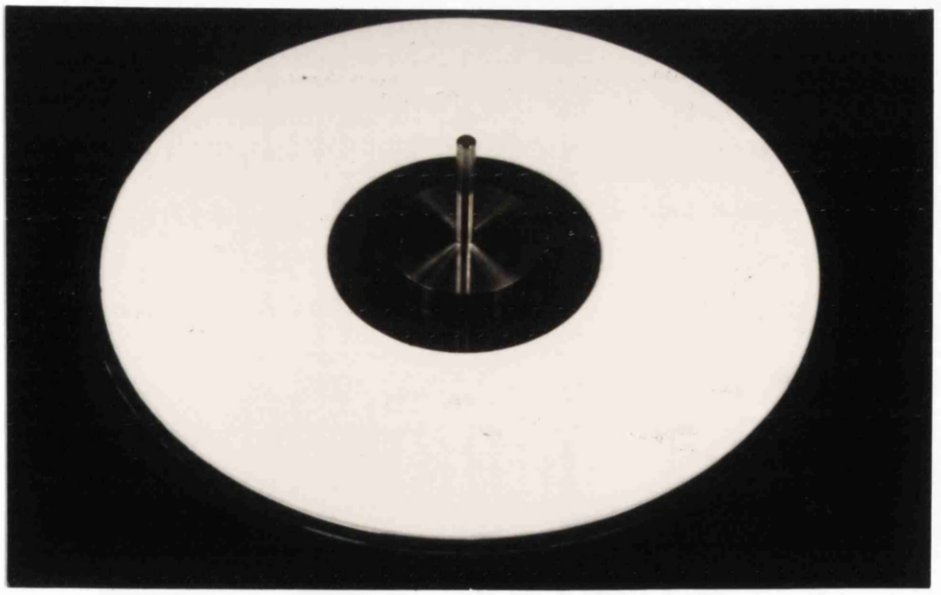


Fig. 2.1b. A chromatotron glass plate after pouring silica gel and incubating overnight at 55 ± 5 °C. The tape shown in Fig. 2.1a was removed once the silica gel had set.

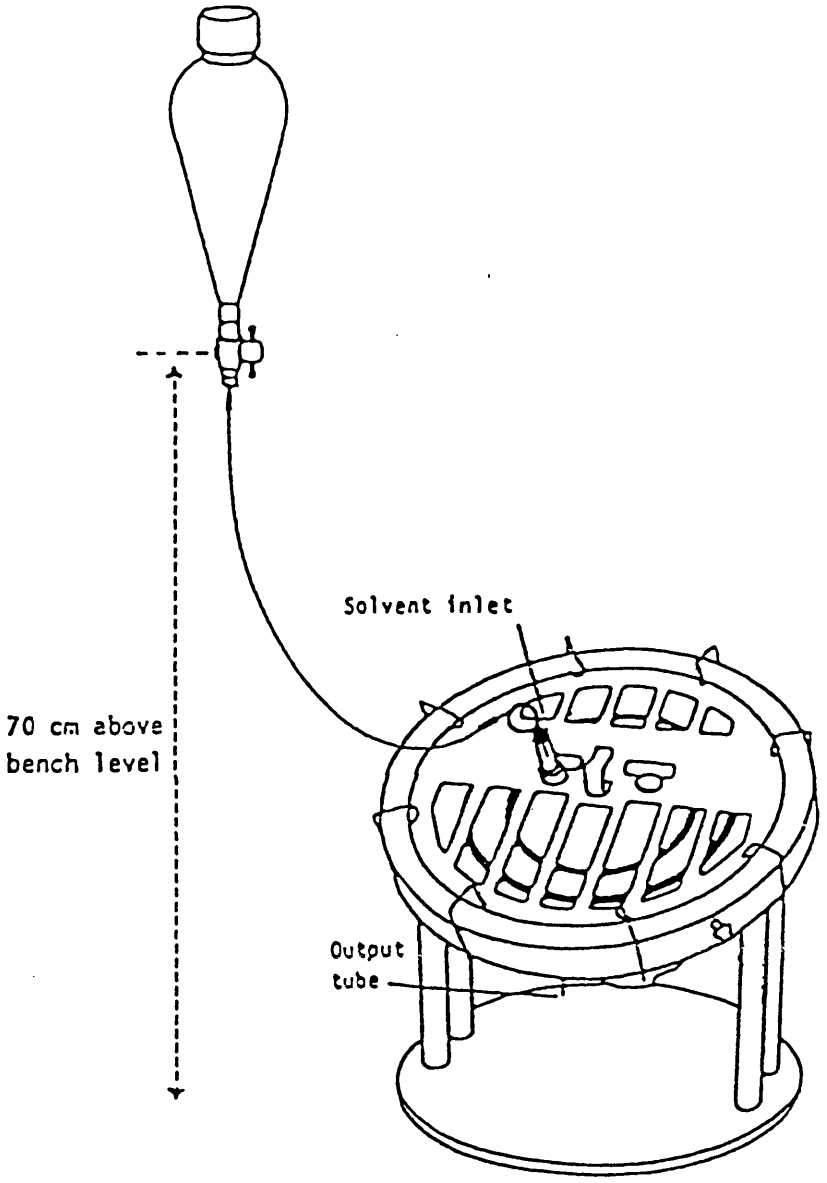


Fig. 2.2. The chromatotron showing the set up of the apparatus with the mariotte flask containing the solvent. The silica gel plate is located in the angled chamber.

2.2.4.2. By flash chromatography using a glass column

A glass column (45 x 2.5 cm) fitted with a stop cock was used for flash chromatography (Fig. 2.3). The bottom of the column was plugged with glass wool and 1 cm acid washed sand was placed on top of this. Silica gel 60 (250-400 mesh, Merck) was suspended in a solvent solution consisting of dichloromethane, cyclohexane and ethyl acetate (3:3:1) and poured into the column to give a packed height of about 24 cm. The top of the silica column was covered with filter paper and a flow controller was fitted tightly to the column with strong rubber bands. The main air line valve leading to the flow controller was opened slightly to increase pressure and the column was washed with 10 column volumes (117 ml = 1 column volume) of methanol and equilibrated with 10 column volumes of dichloromethane, cyclohexane and ethyl acetate (3:3:1). After equilibration, concentrated sample of toxins in ethyl acetate (1.5 ml preparation from a total of 2 litre culture filtrate) was placed gently on the column with a Pasteur pipette without disturbing the adsorbent bed. Fractions (50 ml) were collected using solvents of the following composition:

Fractions	**Solvent**	**Composition (v/v/v)**
1-30	dichloromethane + cyclohexane + ethyl acetate	3:3:1
31-60	"	2:2:1
61-90	"	1:1:1
91-100	ethyl acetate	100%
101-130	methanol	100%

These fractions were observed on a spectrophotometer individually, pooled according to their spectra and $\lambda_{max.}$ (section 2.2.5) and checked on HPLC to confirm their purity (section 2.2.6).

2.2.4.3. By flash chromatography using a commercial apparatus

An ethyl acetate sample (5 ml) of the toxins representing 1 litre of culture filtrate (section 2.2.3) was evaporated. The residue was dissolved in dichloromethane (2.5 ml) and injected onto a dry cartridge of silica gel with particle size 32-63 μm, pore size 60 Å and surface area 573 m^2/gm (40 g: Biotage UK. Ltd., 15 Harforde Court, Foxholes Business Park, John Tate Road, Hertford, UK: Fig. 2.4). The cartridge was washed with cyclohexane (110 ml) and toxins were eluted with dichloromethane, cyclohexane, ethyl acetate (3:3:1; 625 ml), dichloromethane, cyclohexane, ethyl acetate (1:1:1; 400 ml) and finally ethyl acetate (150 ml) under pressure from an airline. The eluates were collected as 25 ml fractions in universal glass bottles. Each fraction was observed on a spectrophotometer (section 2.2.5) and the recovery of the solanapyrones was ascertained by comparison with the amounts of toxins measured in the crude preparation by HPLC (section 2.2.6).

2.2.5. Observation of fractions on a spectrophotometer

The UV spectrum of each fraction was recorded on a spectrophotometer (Philips; Model PU 8720). Fractions with the same spectra and having absorption > 0.5 at their $\lambda_{max.}$ were pooled and concentrated by rotary evaporation. The solanapyrones dissolved in methanol were quantified according to their extinction coefficients (Solanapyrone A UV

$\lambda_{max.}$ nm [$\in$] 327 [9,400], Solanapyrone B UV $\lambda_{max.}$ nm [$\in$] 303 [8,500] and Solanapyrone C UV $\lambda_{max.}$ nm [$\in$] 320 [7,300]; Ichihara *et al.*, 1983). Solvent was removed from the pooled fractions by a rotary evaporator operating at <40°C. The residues were dissolved in methanol (5 ml) and checked on HPLC for purity (section 2.2.6.).

2.2.6. Analytical HPLC

Toxins samples were separated on a Philips HPLC equipped with a diode array detector essentially according to Chen and Strange (1991) except that the solvent system consisted of methanol 23.1%, water 56.3% and tetrahydrofuran 20.6% (v/v/v) which was pumped at flow rate 1 ml/min. The stationary phase was an ODS column (Spherisorb ODS 2; 150 x 4.5 mm diam.; Jones Chromatography, Glamorgan, UK) and was protected by a guard column (20 x 4.6 mm diam.).

The solanapyrones were recognised by their UV spectra which were compared by superimposition on those of authentic samples already available in the laboratory.

For quantification of the solanapyrones, chromatograms were abstracted from the three dimensional chromascans (absorption x wavelength x time) at $\lambda_{max.}$ = 327, $\lambda_{max.}$ = 303 and $\lambda_{max.}$ = 320 for solanapyrones A, B and C, respectively and the areas of the peaks were compared with those of standard solutions of the authentic compounds.

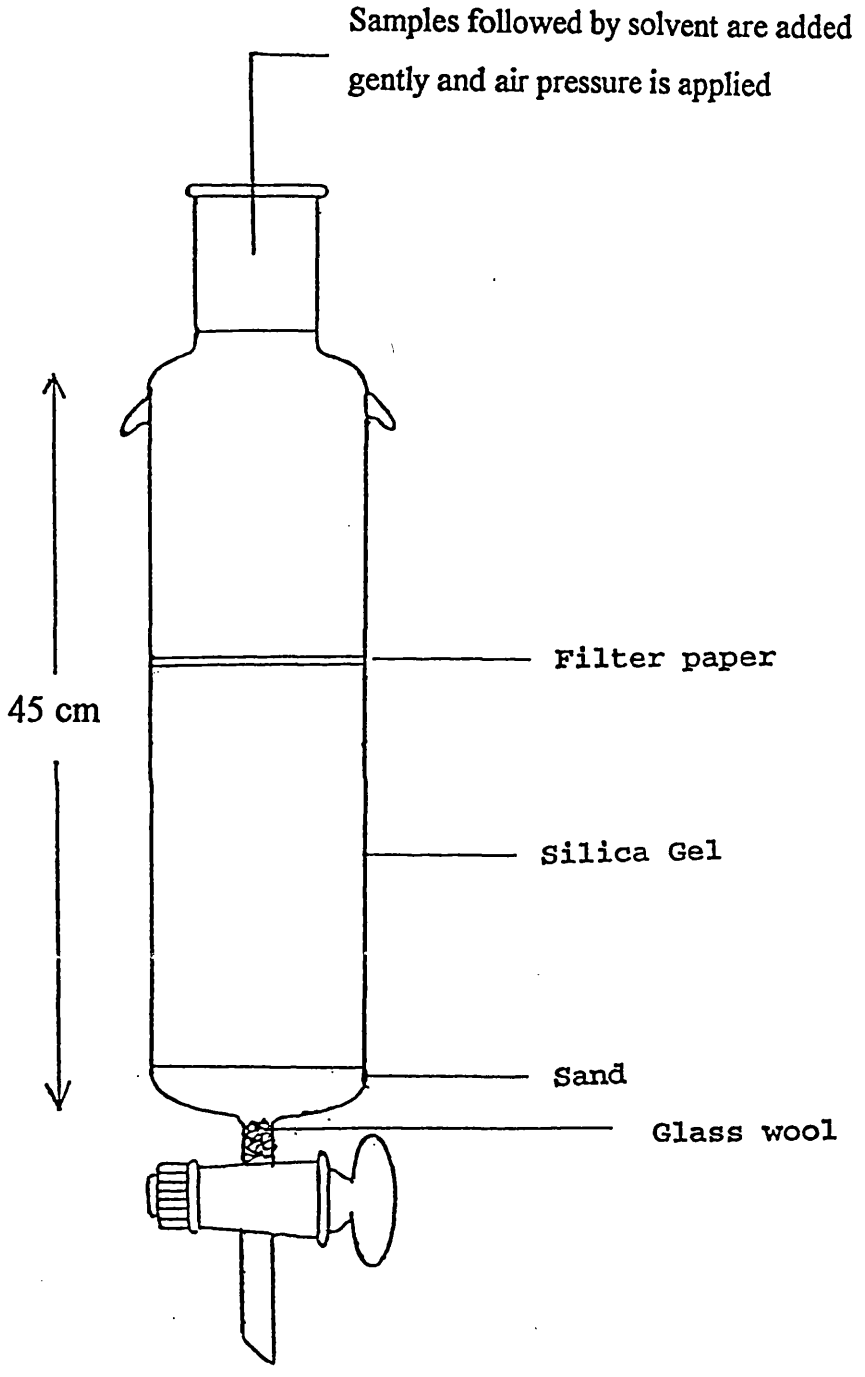


Fig. 2.3. Diagram to show the construction of a flash chromatography column.

Fig.2.4. A commercial flash chromatography apparatus (Biotage UK. Ltd., 15 Harforde Court, Foxholes Business Park, John Tate Road, Hertford, UK).

2.2.7. Detection of solanapyrones on TLC plates

Samples of solanapyrones A, B and C were chromatographed on TLC plates (Silica gel 60 F_{254}, Merck) with dichloromethane, cyclohexane and ethyl acetate 3:3:1, 2:2:1 or 1:1:1 as mobile phases. Compounds were observed under long and short wave using a chromatovue UV light box (Ultra Violet Products, INC, USA. Model, CC-20).

TLC plates were also sprayed with the following reagents with some modifications of the methods given by Stahl (1969) :-

1. Anisaldehyde. The reagent was prepared from glacial acetic acid (5 ml), anisaldehyde (0.5 ml) and concentrated sulphuric acid (0.1 ml). After spraying, the plates were heated at 100-105 °C in an oven for 1 minute.

2. Saturated solutions of o-dianisidine in glacial acetic acid.

3. o-dianisidine 0.5 g/10 ml of glacial acetic acid.

4. Saturated solution of 2,7 diaminofluorene in glacial acetic acid.

5. 2,4 dinitrophenylhydrazine 0.05 g/10ml of ethanol.

6. TLC plates were also placed in a glass tank for 15-20 minutes containing 15 g of iodine crystals.

2.3. RESULTS

2.3.1. Production of the solanapyrone toxins

The isolate of *Ascochyta rabiei* (PUT 7) grew well on CDLMC (Fig. 2.5). Solanapyrones A, B and C were recovered from culture filtrates of the fungus by solid phase extraction and liquid phase extraction (Fig. 2.6 and 2.7, respectively).

In crude preparations, obtained by extracting culture filtrates in ethyl acetate, the fungus produced Sol.A (45.51 ± 8.46 mg/L), Sol. B (42.33 ± 23.15 mg/L) and Sol.C (2.18 ± 0.33 mg/L: Fig. 2.8).

2.3.2. Separation and purification of the solanapyrone toxins

2.3.2.1. By the chromatotron

Out of 160 fractions separated by the chromatotron, only seven fractions showed the presence of Sol.A when observed on a spectrophotometer. These fractions when pooled gave 14.45 mg/L of the pure compound compared with 45 mg, as determined by HPLC, in the crude ethyl acetate preparation. Neither Sol.B nor Sol.C could be distinguished in any of the other fractions.

2.3.2.2. By flash chromatography using a glass column

Sol.A was obtained in fractions 20 to 31. Fractions from 32 to 46 gave a mixture of solanapyrones A and B. Fractions 47 to 60 yielded Sol.B while 61 to 67 gave a mixture of Sol.A and B. Fractions 68-94 also had Sol.B. The 100% methanol fractions 101-107

also had Sol.B. Sol.C appeared in fractions 108-113 but was contaminated with Sol.B (Fig. 2.9).

On pooling the relevant fractions 50.20 mg and 19.80 mg of pure Sol.A and Sol.B, respectively, from 2 L of culture filtrate were obtained.

2.3.2.3. By flash chromatography using a commercial apparatus

Solanapyrones were separated by flash chromatography using the Biotage apparatus. Sol.A generally appeared in fractions 12 to 25, Sol.B in 29 to 37 and Sol.C in 42-47. The last was synthesized in low concentrations by the isolate of the fungus used (PUT 7: Fig. 2.10). On pooling these fractions according to their UV spectra, samples of Sol.A (34.65 ± 7.15 mg/L), Sol.B (17.94 ± 9.1 mg/L) and Sol.C (0.66 ± 0.39 mg/L) were obtained with % recoveries of 75.92 ± 3.33, 42.95 ± 1.61 and 32.5 ± 23.19, respectively (Fig. 2.8).

2.3.2.4. Confirmation of purity of solanapyrones A, B and C

The samples of the toxins prepared by flash chromatography using the Biotage apparatus gave single peaks on HPLC, Sol.B eluting at 525 ± 30 seconds, Sol.A at 640 ± 96 and Sol.C at 871 ± 32 seconds (Figs. 2.11, 2.13 and 2.15, respectively). Spectra of the compounds gave a better than 99% match with those of authentic samples (Figs. 2.12, 2.14 and 2.16).

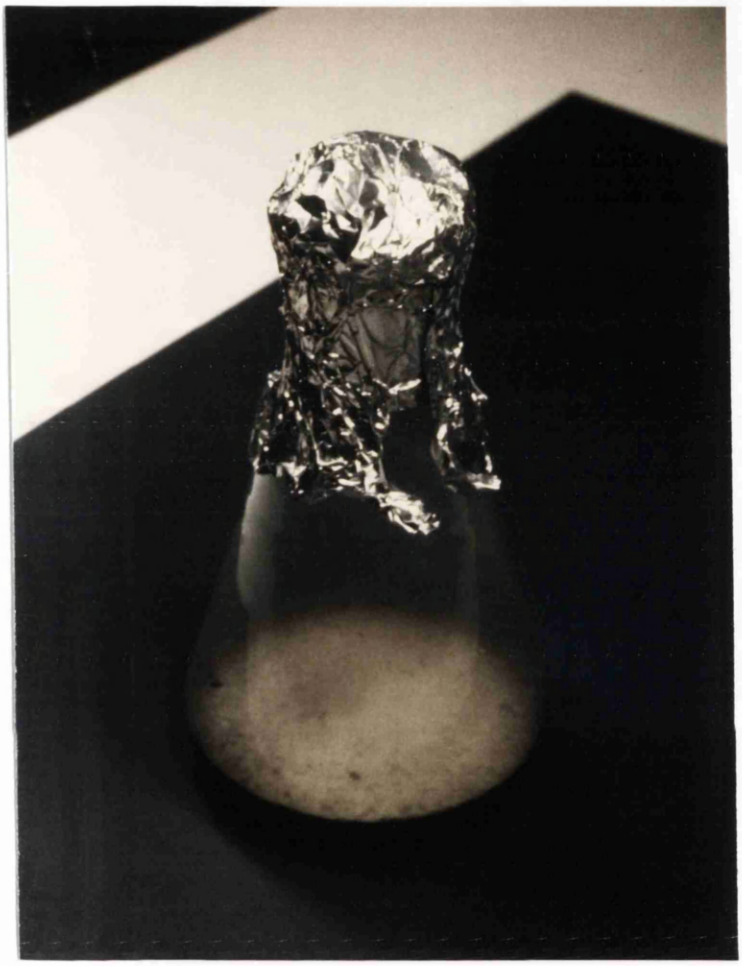
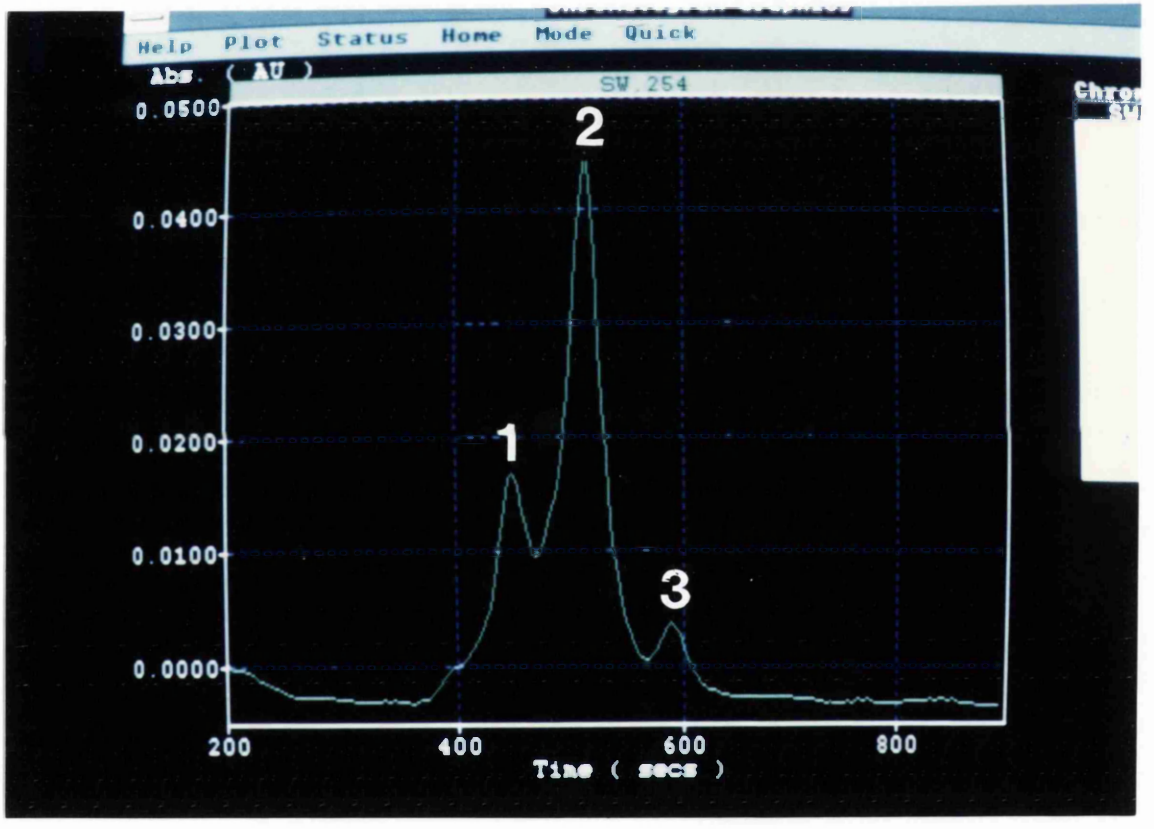


Fig. 2.5. An isolate (PUT 7) of *Ascochyta rabiei* growing on CDLMC medium.

Fig. 2.6. A chromatogram at 254 nm of solanapyrones toxins prepared by solid phase extraction (for details see text). A sample of the acetonitrile fraction equivalent to 80 µl of the original culture filtrate was run on HPLC using an ODS column (Spherisorb ODS 2: 150 x 4.5 mm) with a solvent system consisted of methanol 23.1%, water 56.3% and tetrahydrofuran 20.6% (v/v/v), pumped at flow rate of 1 ml/min. Peaks 1, 2 and 3 eluting at 432 sec., 501.6 sec. and 570.6 sec. were identified as Sol.B, Sol.A and Sol.C, respectively by superimposing their UV spectra on those of authentic samples.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0.0200
0.0100
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2
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200
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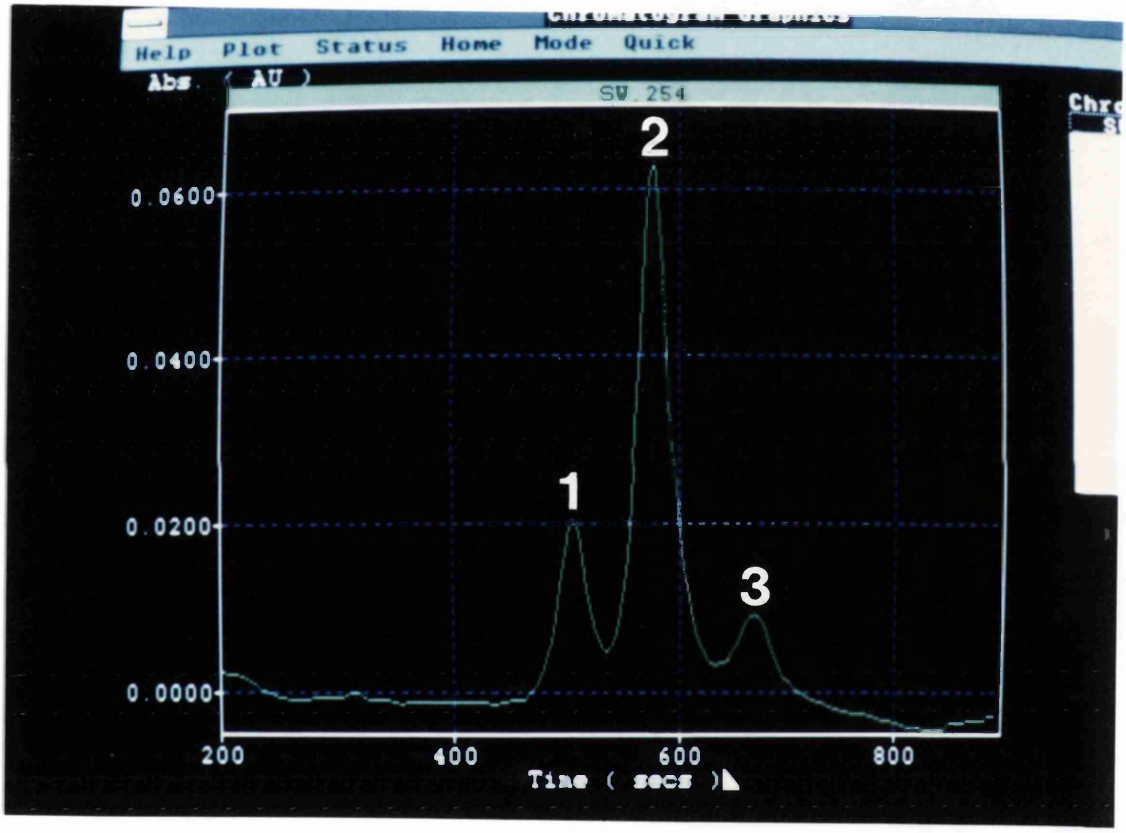
600
800
Time (secs)

Fig. 2.7. A chromatogram at 254 nm of solanapyrones toxins prepared by liquid phase extraction (for details see text). A sample of the ethyl acetate fraction equivalent to 80 µl of the original culture filtrate was run on HPLC using an ODS column (Spherisorb ODS 2: 150 x 4.5 mm) with a solvent system consisted of methanol 23.1%, water 56.3% and tetrahydrofuran 20.6% (v/v/v), pumped at flow rate of 1 ml/min. Peaks 1, 2 and 3 eluting at 495.6 sec., 558.6 sec. and 666.6 sec. were identified as Sol.B, Sol.A and Sol.C, respectively by superimposing their UV spectra on those of authentic samples.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Time (secs)

Fig. 2.8. Recovery of the solanapyrones toxins extracted by liquid phase extraction from 1l culture filtrate and separated by flash chromatography using a Biotage commercial apparatus. Recovery of the solanapyrones was ascertained by quantitative HPLC. For details see text. Error bars are Standard Deviations.

Fig. 2.8. Recovery of the solanapyrone toxins from 1L culture filtrate of *A. rabiei*

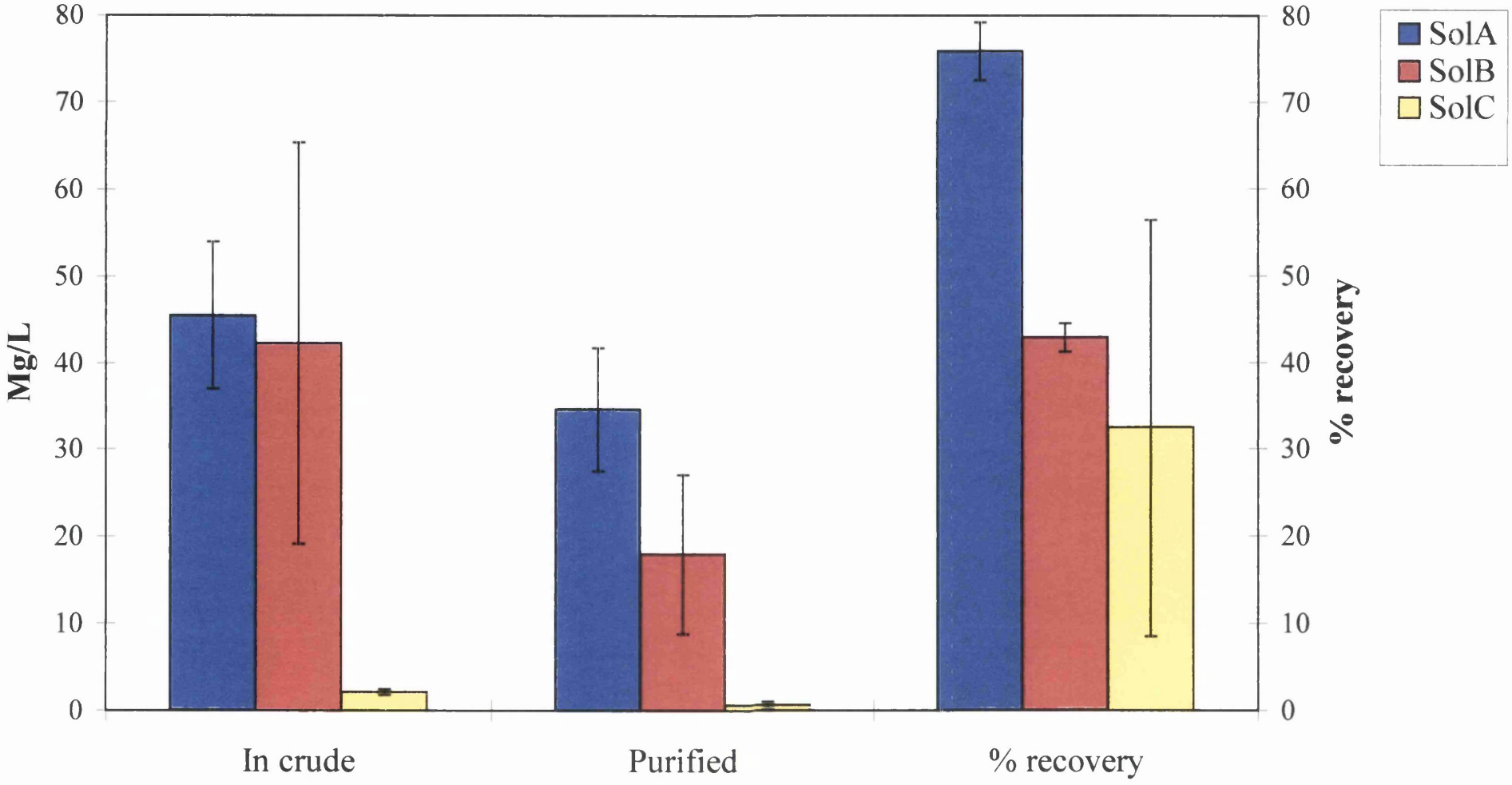


Fig. 2.9. Separation of the solanapyrone toxins by flash chromatography using a glass column. Samples of solanapyrones eluted in the following fractions; Sol.A (blue coloured) 20-31, Sol.B (red coloured) 47-60, 68-94 and then 101 to 107. Intermediate fractions gave mixtures of the solanapyrones.

Fig. 2.9. Separation of the solanapyrone toxins from 2L culture filtrate of *A. rabiei* by flash chromatography using a glass column

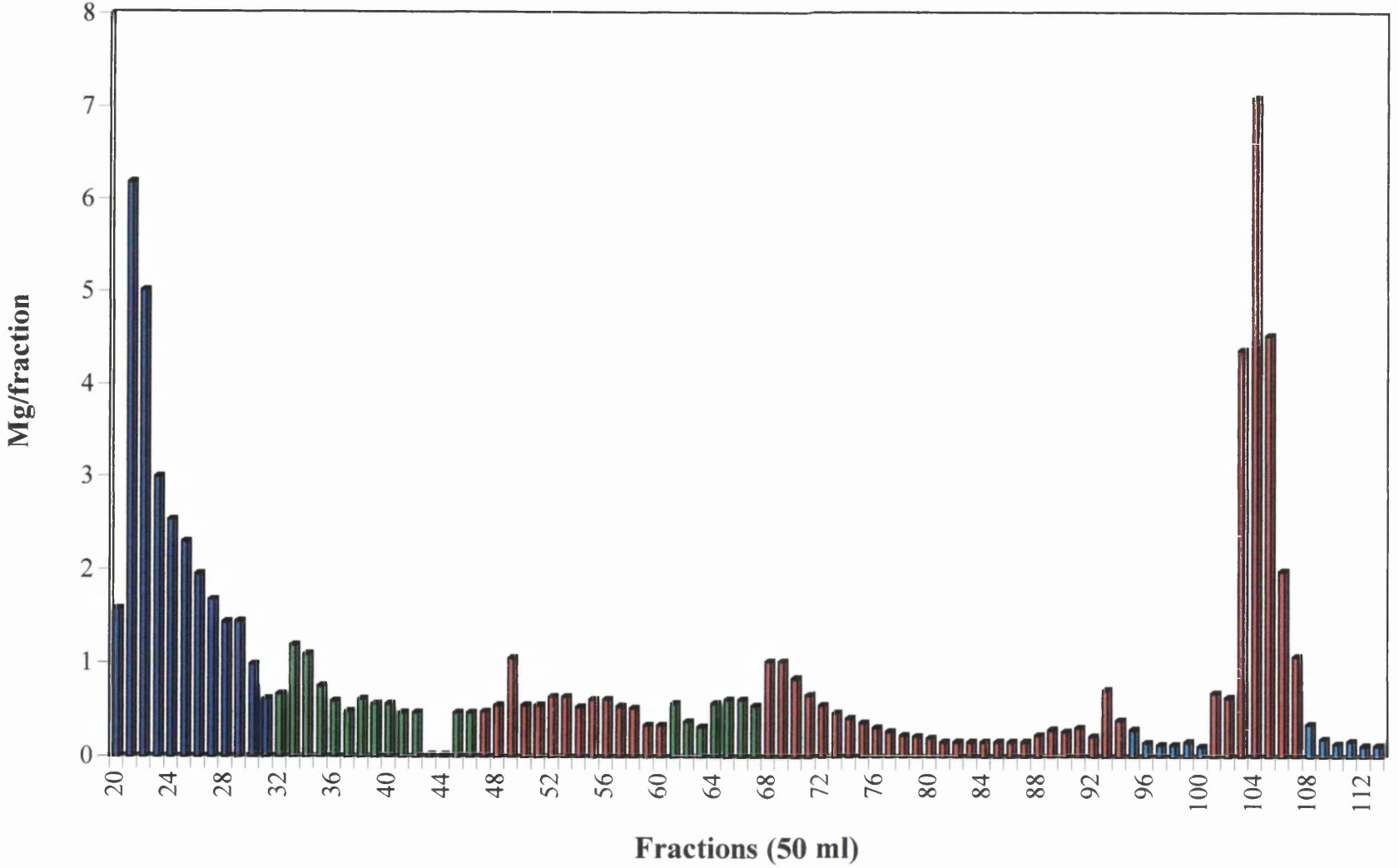
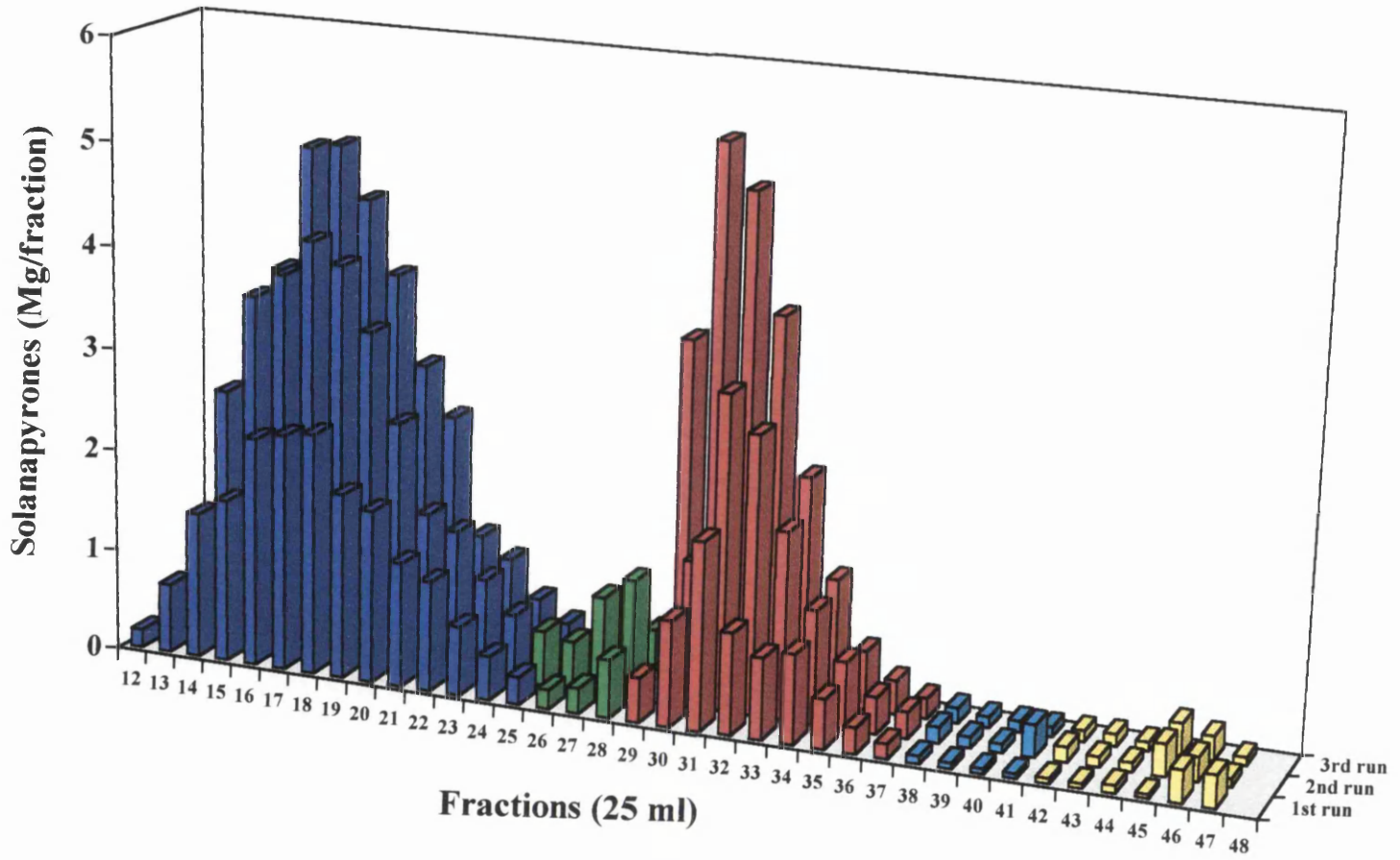


Fig. 2.10. Histogram showing the separation of the solanapyrone toxins from 3 samples of culture filtrate (1 L each) of culture filtrate. Individual toxins were separated by flash chromatography using a commercial apparatus (Biotage) with a succession of solvents (for details see text). Samples of the solanapyrones generally eluted in the following fractions; Sol.A (blue coloured) 12-25, Sol.B (red coloured) 29-37 and Sol.C (yellow coloured) 42-47. Intermediate fractions gave small quantities of mixtures.

Fig. 2.10. Histogram showing the separation of the solanapyrone toxins from 1L culture filtrate of *A. rabiei*



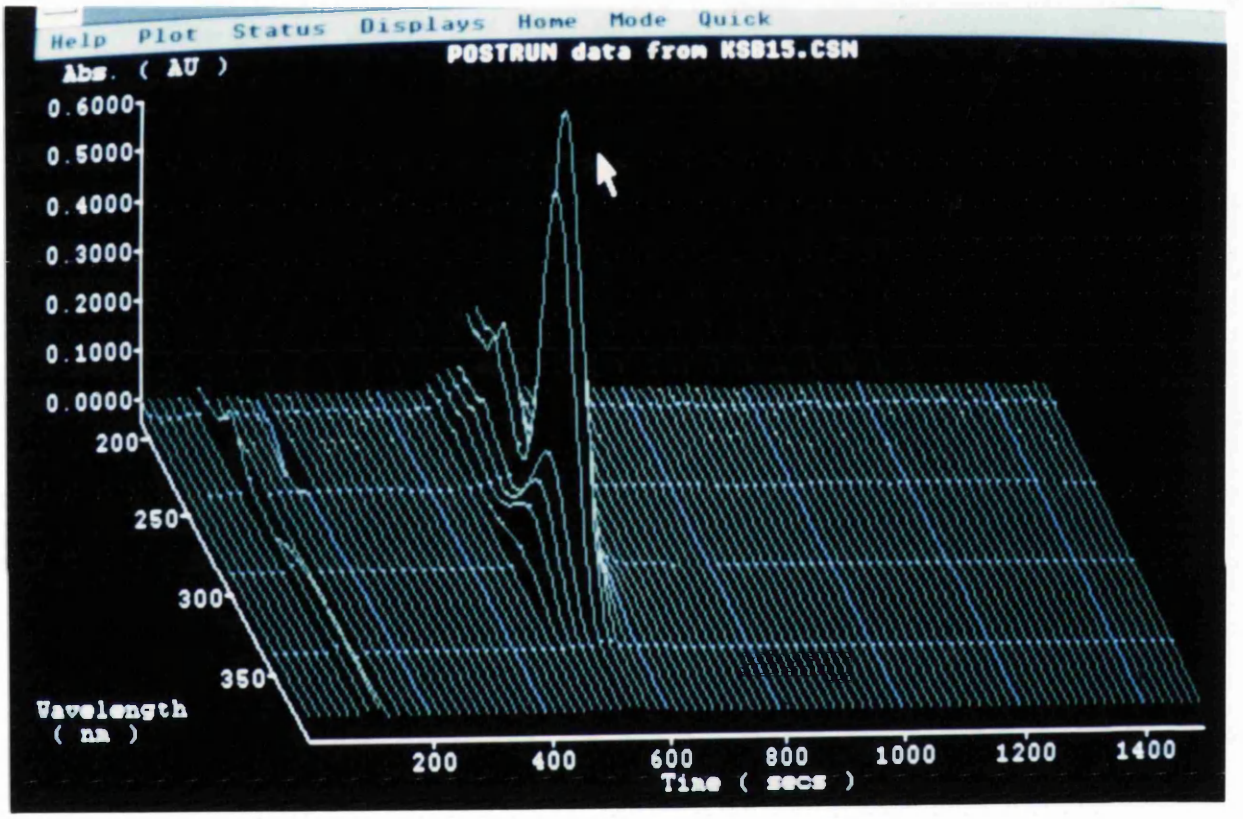
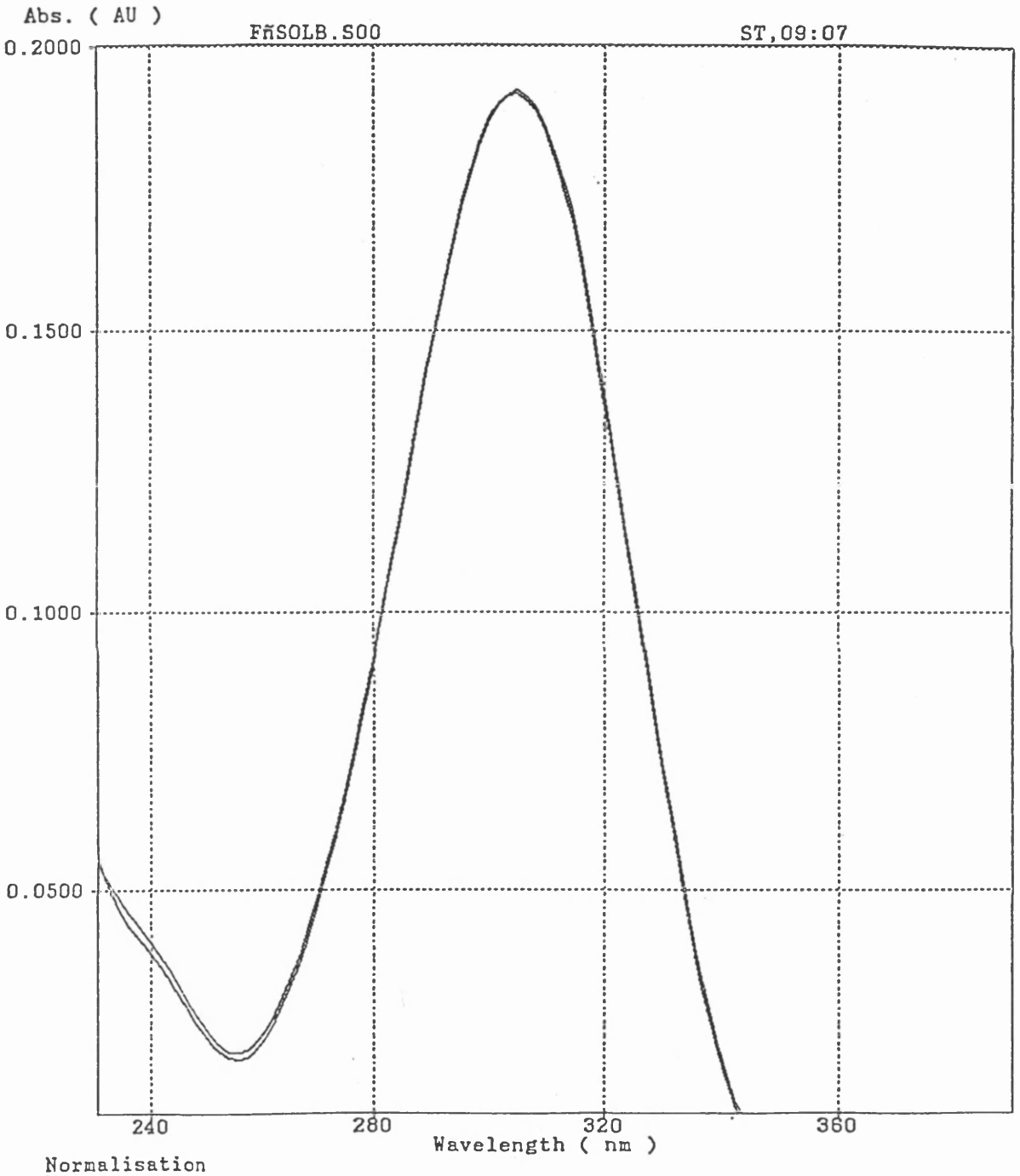


Fig. 2.11. A chromascan of a sample of Sol.B on HPLC, separated by flash chromatography using a commercial apparatus (Biotage).

Fig. 2.12. Spectrum of a sample of Sol.B, separated by flash chromatography using a commercial apparatus (Biotage), superimposed on the spectrum of an authentic sample of the compound showing a 99.95% match by least squares between 230 and 345 nm with the authentic compound.



Normalisation
Least squares

Values
0.6360 99.95%

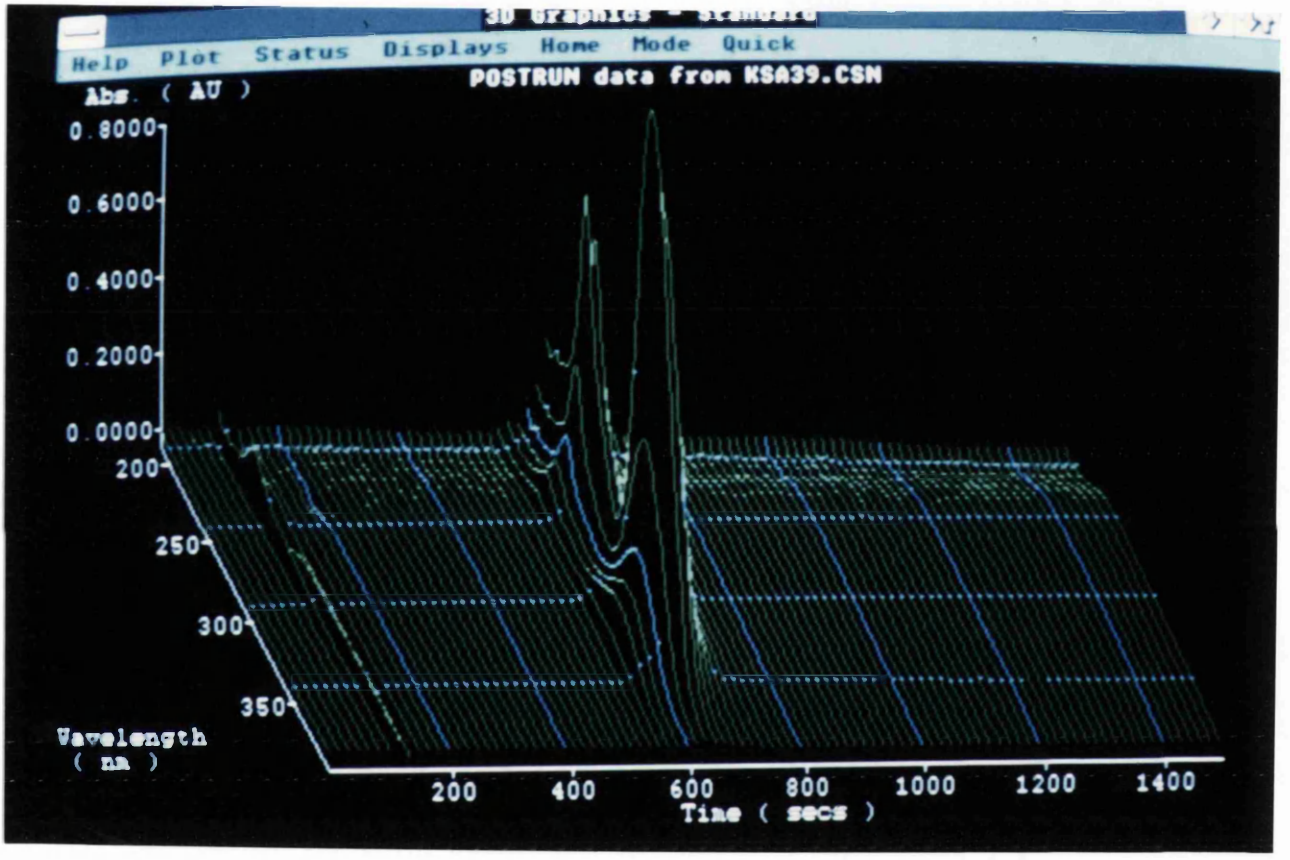
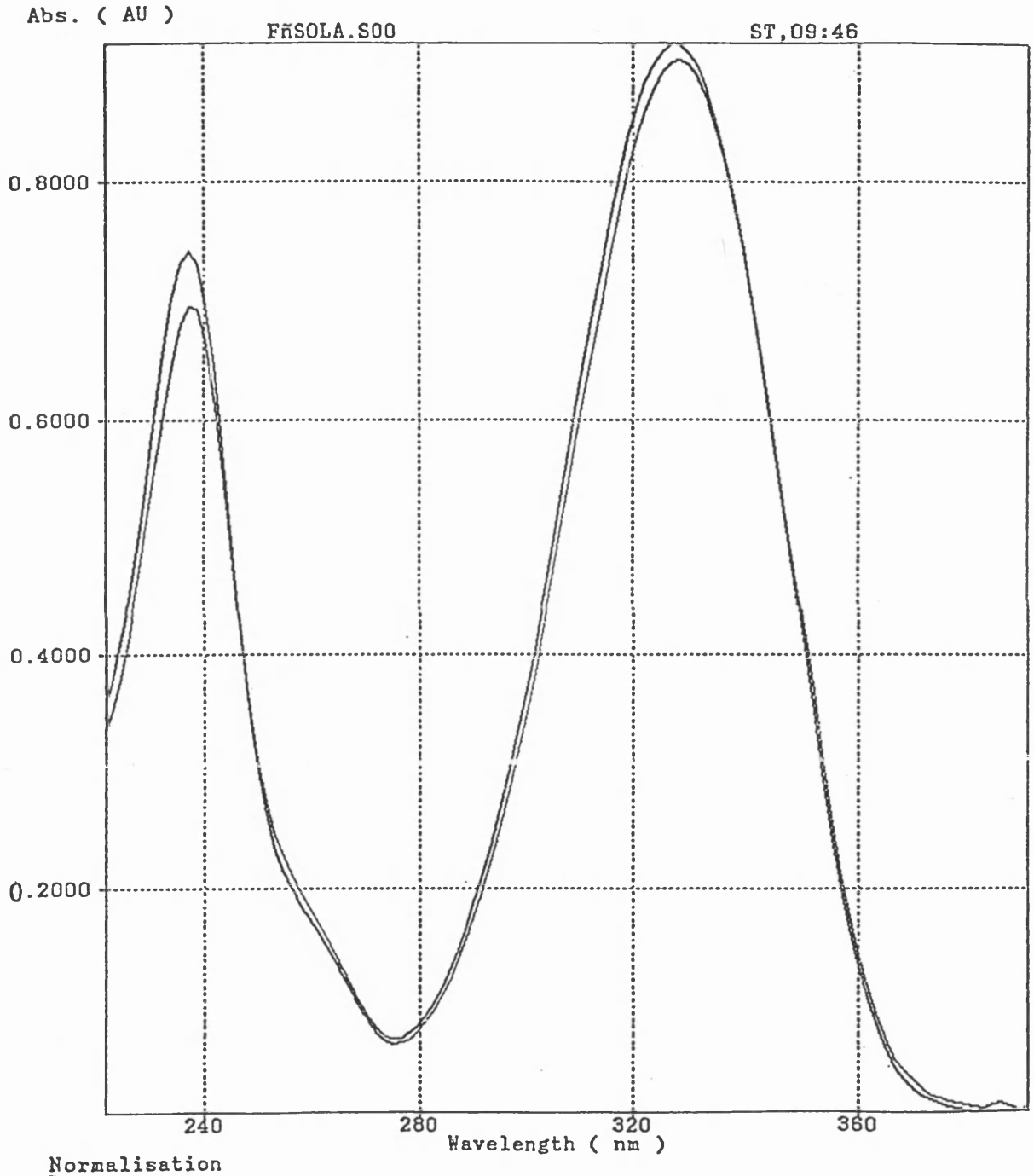


Fig. 2.13. A chromascan of a sample of Sol.A on HPLC, separated by flash chromatography using a commercial apparatus (Biotage).

Fig. 2.14. Spectrum of a sample of Sol.A, separated by flash chromatography using a commercial apparatus (Biotage), superimposed on the spectrum of an authentic sample of the compound showing a 99.57% match by least squares between 230 and 380 nm with the authentic compound.



Normalisation
Least squares

Values
6.5630 99.57%

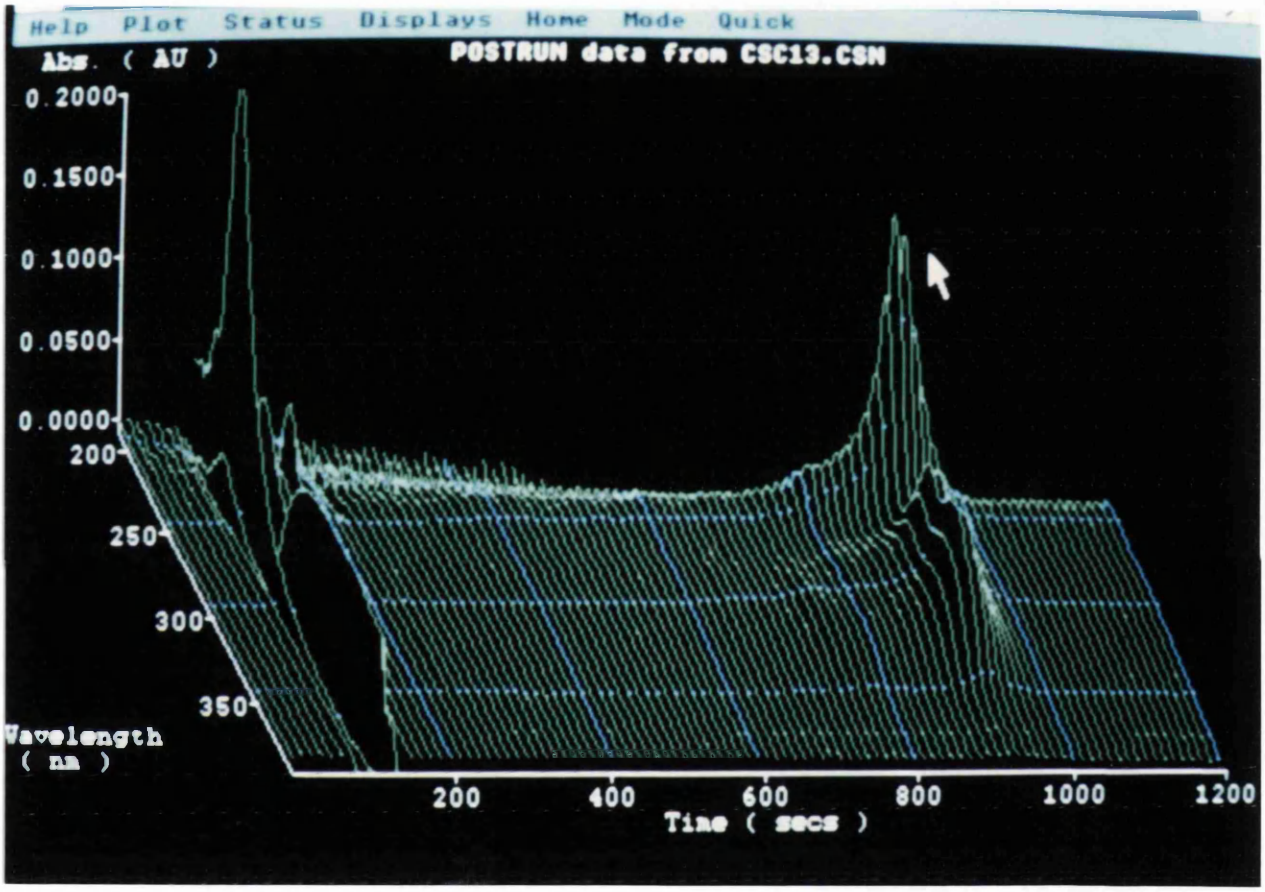
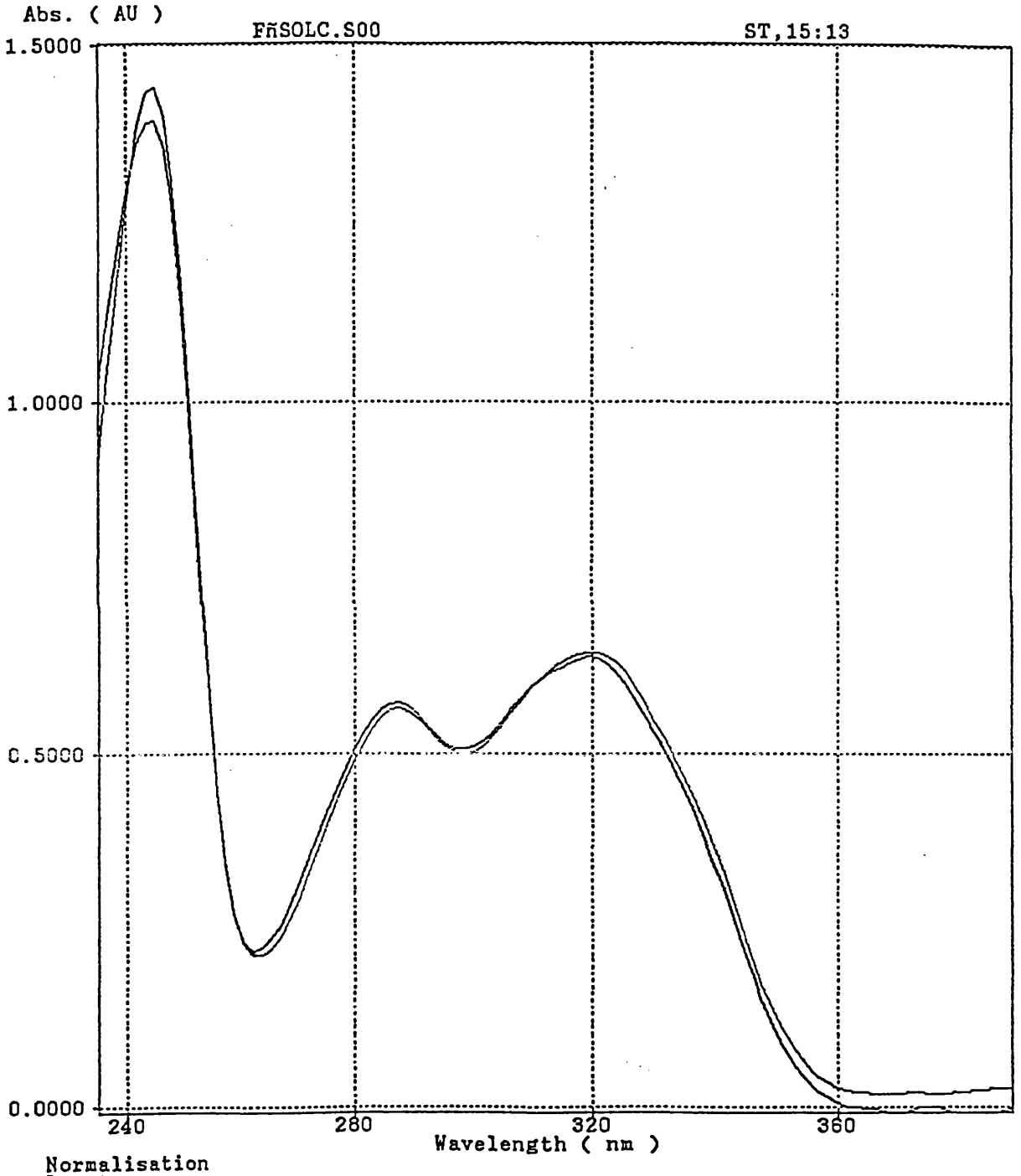


Fig. 2.15. A chromascan of a sample of Sol.C on HPLC, separated by flash chromatography using a commercial apparatus (Biotage).

Fig. 2.16. Spectrum of a sample of Sol.C, separated by flash chromatography using a commercial apparatus (Biotage), superimposed on the spectrum of an authentic sample of the compound showing a 99.52% match by least squares between 235 and 380 nm with the authentic compound.



Normalisation
Least squares

Values
7.9786 99.52%

2.3.3. The effect of delaying or omitting the addition of zinc ions to cultures of *A. rabiei* growing on CDLMC

2.3.3.1. Toxin production

When the addition of zinc was delayed until cultures were 8 days old, more Sol.A (65.0 ± 12.1 mg/L: Fig. 2.17) was produced compared with cultures when zinc was present at the time of inoculation (45.5 ± 8.46 mg/L: Fig. 2.8). In contrast, the concentrations of Sol.B and Sol.C were reduced by the delayed addition of zinc and this was particularly marked for Sol.B (compare Figs. 2.8 and 2.17). Omission of zinc altogether resulted in greatly reduced solanapyrone production (Fig. 2.18)

2.3.3.2. Dry weight of mycelium

The dry weights of the mycelium from day 9 to day 13 ranged from 264 ± 48 mg to 408 ± 29 mg but weights for individual days did not differ significantly whether the fungus was grown on CDLMC with zinc added at day 8 or without zinc.

2.3.3.3. pH of culture filtrates

The pH of culture filtrates measured at daily intervals from day 9 to day 13 inclusive varied between 8.4 and 8.9, those cultures receiving zinc at day 8 generally being slightly lower than those receiving no zinc.

Fig. 2.17. Histogram showing the recovery of the solanapyrone toxins extracted by liquid phase extraction from 1L culture filtrate of *A. rabiei* growing on CDLMC. Zn was added 8 days after inoculation of the medium. Individual solanapyrones were separated by flash chromatography using a commercial apparatus (Biotage). Recovery of the compounds was ascertained by HPLC. Error bars are Standard Deviations.

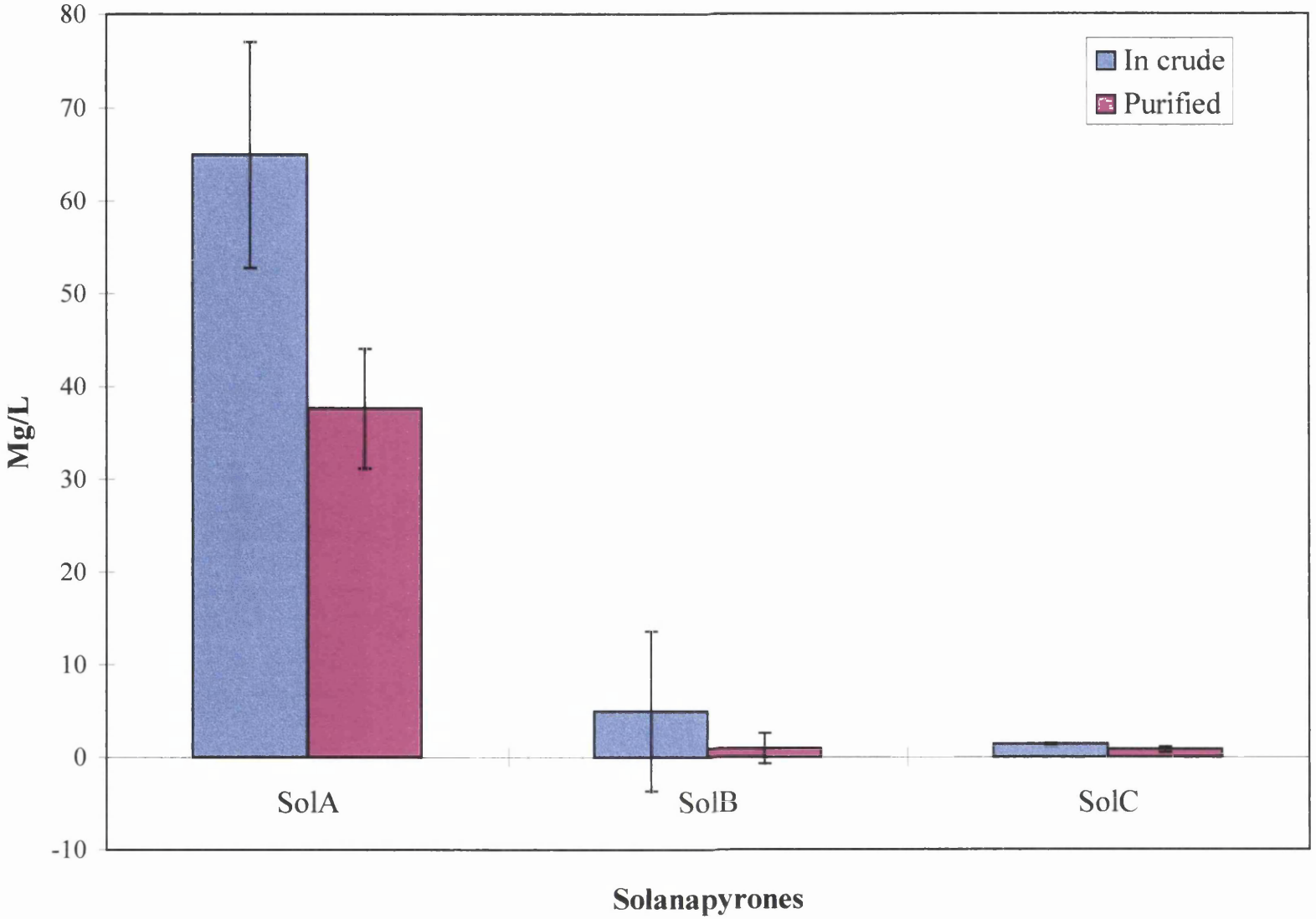
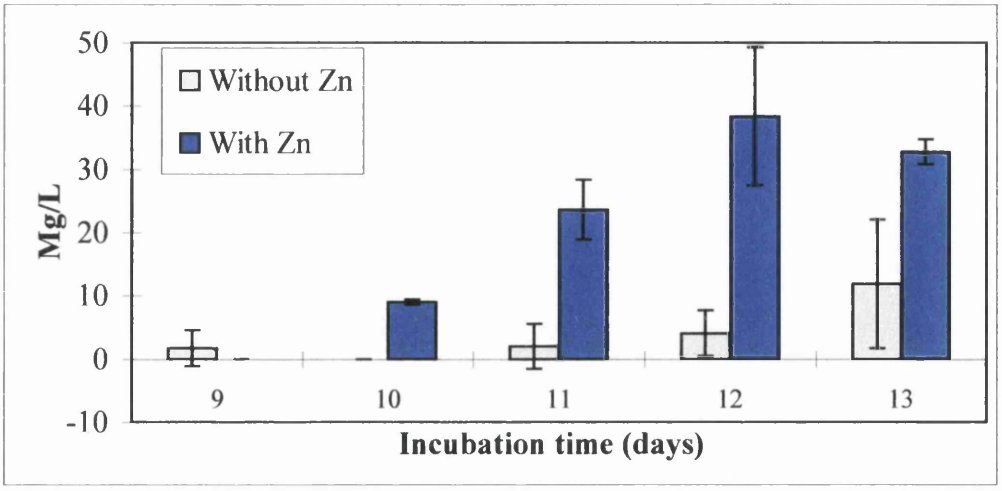
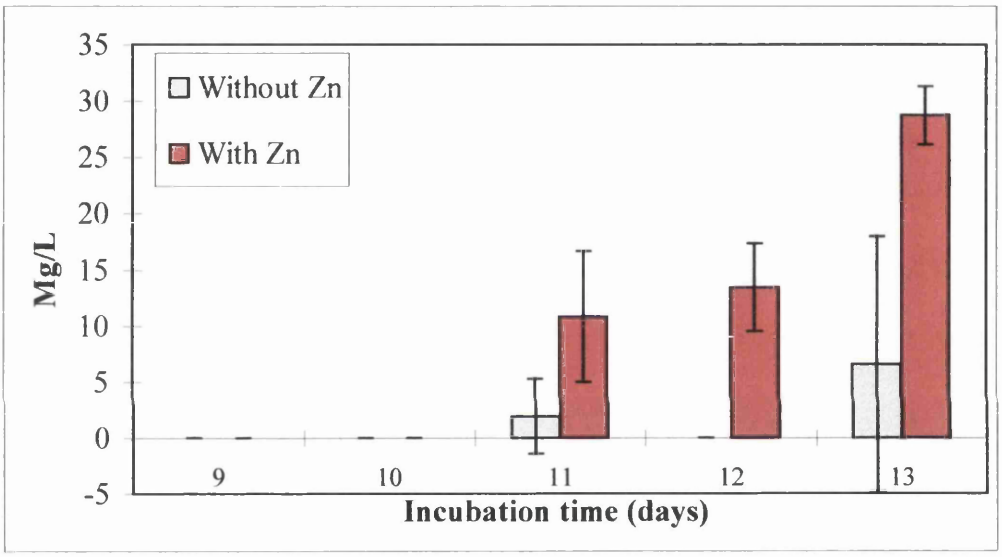

80
70
60
50
40
30
20
10
0
-10
Mg/L
In crude
Purified
SolA
SolB
SolC
Solanapyrones

Fig. 2.18. Histogram showing production of solanapyrones A, B and C by *A. rabiei* growing on CDLMC with Zn ions either added to 8 day old cultures or omitted. The solanapyrones were extracted from culture filtrates by solid phase extraction using 1g C18 Isolute cartridge and quantified by HPLC. Error bars are Standard Deviations.

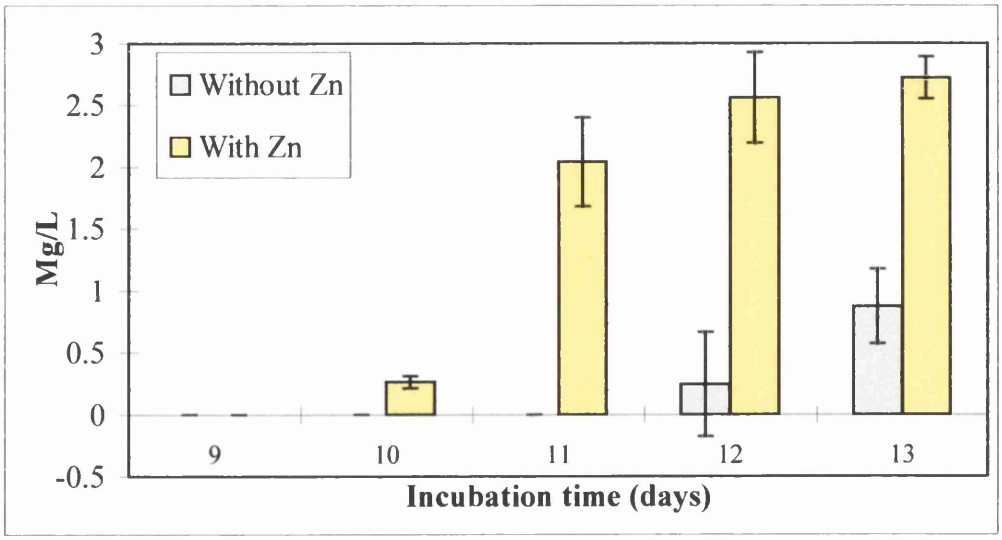
Sol.A)



Sol.B)



Sol.C)



2.3.4. The detection of the solanapyrone toxins on TLC plates

Solanapyrones were also identified by their Rf values on TLC plates developed in mixtures of the solvents cyclohexane, dichloromethane and ethyl acetate. Sol.A had the highest Rf values of three solanapyrones i.e. 0.89, 0.60 and 0.25 in solvents with the compositions 1:1:1, 2:2:1 and 3:3:1 (v/v/v), respectively, whereas the Rf values for Sol.C were 0.20, 0.09 and 0.06. Sol.B was intermediate (Table 2.1: Fig. 2.19).

o-Dianisidine (saturated solution in glacial acetic acid or 0.5 g/10 ml), diaminofluorene and 2,4 dinitrophenylhydrazine reacted with both Sol.A and Sol.C but not Sol.B. Only iodine vapour reacted with all three solanapyrones and anisaldehyde did not react any of them (Figs. 2.20a, 2.20b, 2.20c, and 2.20d). The limits of detection of the toxins by these reagents on TLC is given in Table 2.2.

Table 2.1. Rf values of the solanapyrone toxins on TLC plates (Silica gel 60 F_{254}) developed with mixtures of dichloromethane, cyclohexane and ethyl acetate in different proportions

Solvent composition	Rf values		
dichloromethane : cyclohexane : ethyl acetate	Sol.A	Sol.B	Sol.C
1:1:1	0.89 ± 0.008	0.52 ± 0.01	0.20 ± 0.007
2:2:1	0.60 ± 0.01	0.26 ± 0.008	0.09 ± 0.007
3:3:1	0.25 ± 0.01	0.12 ± 0.007	0.06 ± 0.0008

Table 2.2. The limits of detection of the solanapyrone toxins by various reagents on Si gel TLC plates

	Reagents	Limits of detection (µg/spot)		
		Sol.A	**Sol.B**	**Sol.C**
1.	**o-Dianisidine**	0.05	NR	0.05
2.	**Diaminofluorene**	0.05	NR	0.25
3.	**2,4 dinitrophenylhydrazine**	0.50	NR	0.25
4.	**Iodine vapours**	4.0	0.05	4.0

NR = no reaction

Solanapyrones were spotted on TLC plates, developed in dichloromethane, cyclohexane and ethyl acetate 3:3:1 (v/v/v) and, after drying, the plates were sprayed with reagents 1-3 or exposed to iodine vapours (reagent 4).

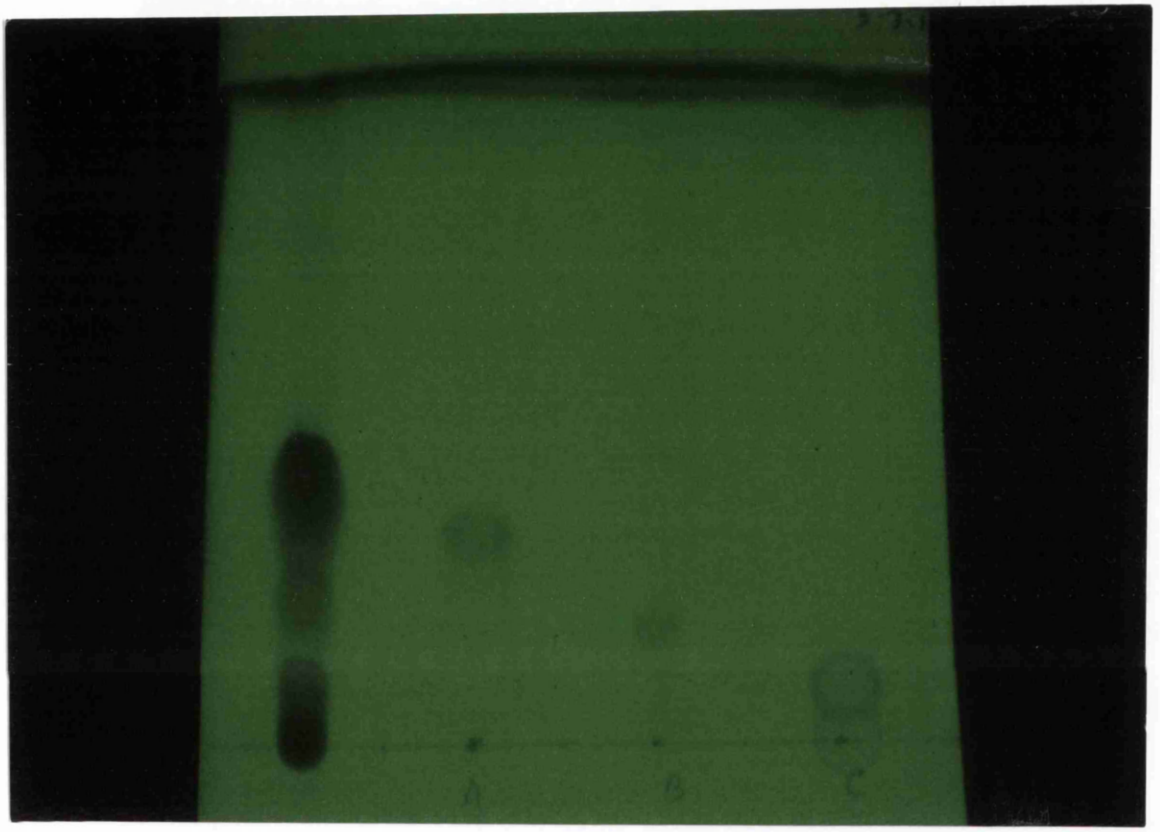


Fig. 2.19. Thin Layer Chromatograhy of the solanapyrone toxins on silica before and after purification. **1**) Ethyl acetate extract from culture filtrates. **2**) Sol.A separated by flash chromatography using a commercial apparatus (Biotage). **3**) Sol.B separated as above. **4**) Sol.C separated as above. Plates were developed in a solvent mixture consisting of dichloromethane: cyclohexane: ethyl acetate (3:3:1 v/v/v), dried in a fumecupboard and observed under short wave UV.

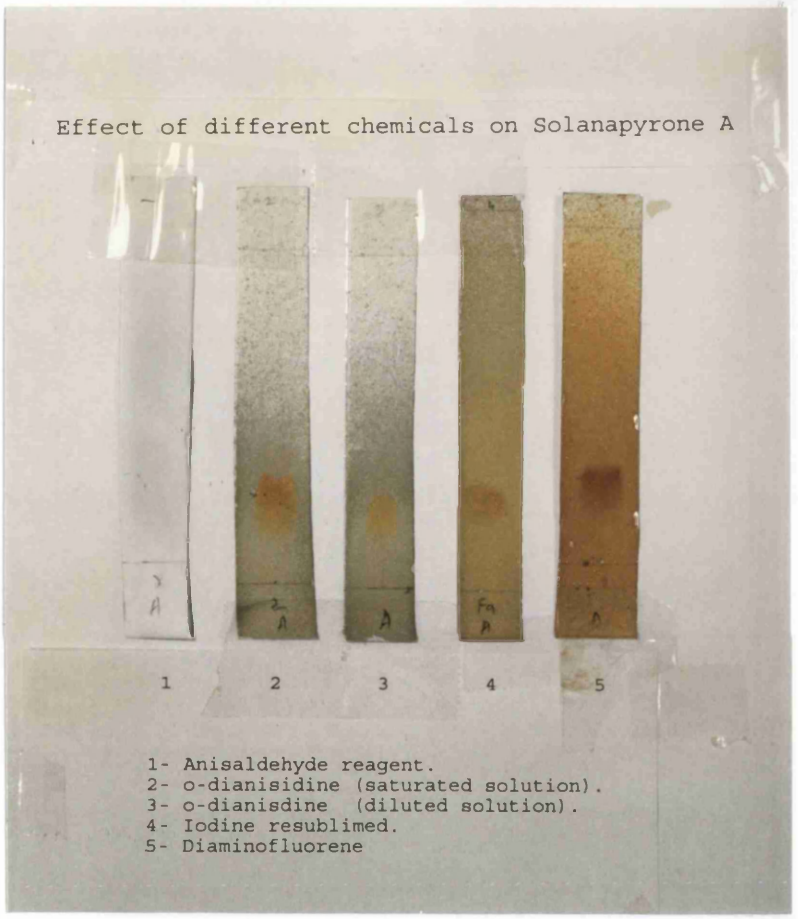


Fig. 2.20a. Reaction of Sol.A with various spray reagents showing that it reacted with all reagents except anisaldehyde. Compounds were developed on Si gel TLC plates with dichloromethane: cyclohexane: ethyl acetate (3:3:1 v/v/v).

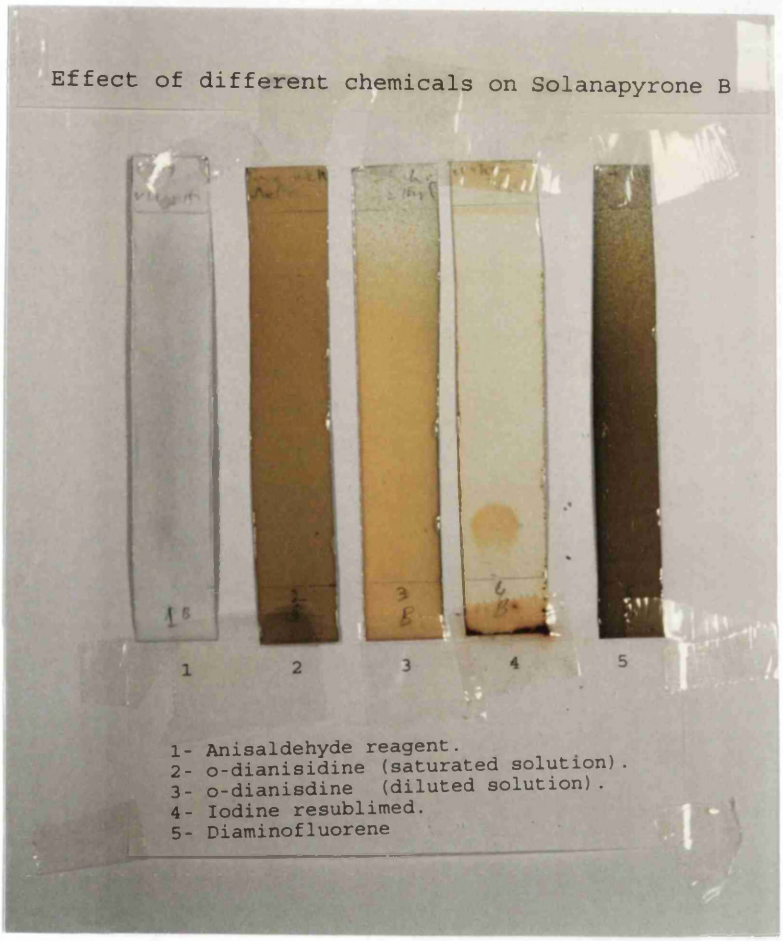


Fig. 2.20b. Reaction of Sol.B with various spray reagents showing that this compound reacted with only iodine. Compounds were developed on Si gel TLC plates with dichloromethane: cyclohexane: ethyl acetate (3:3:1 v/v/v).

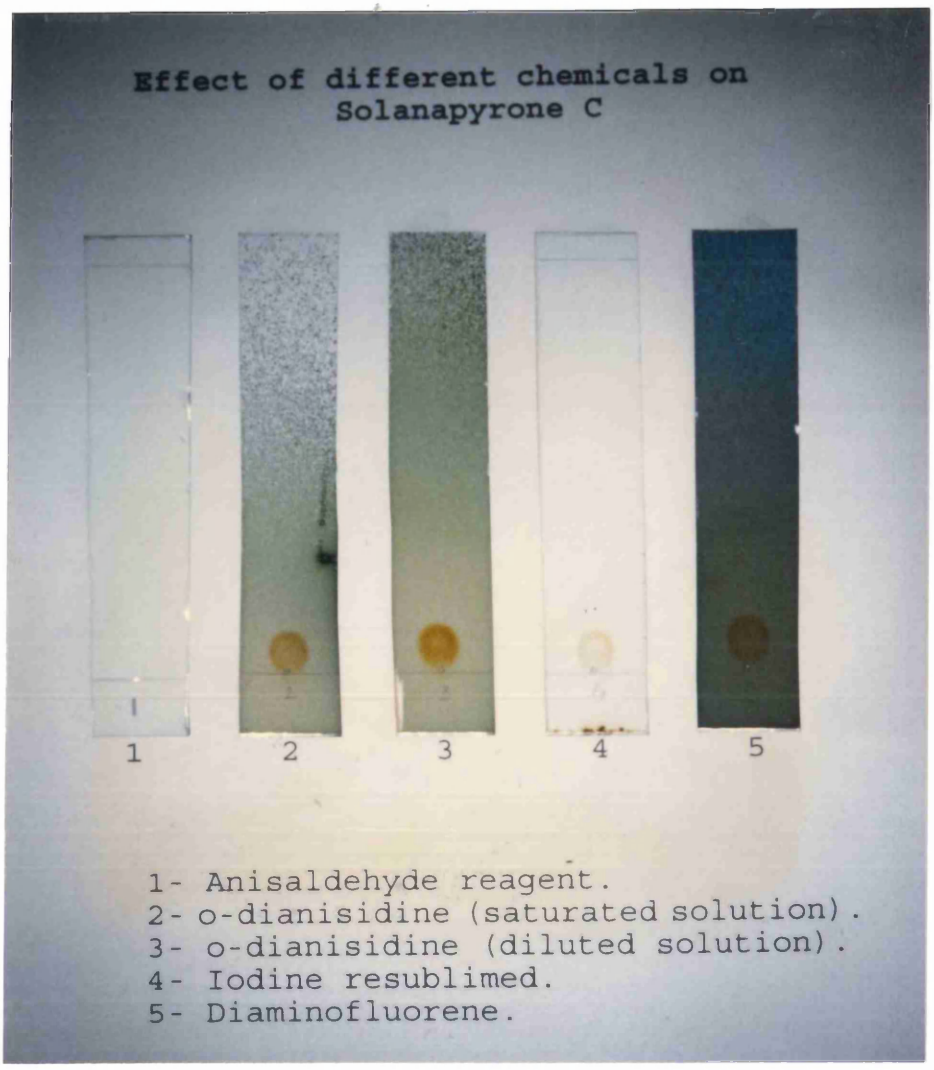
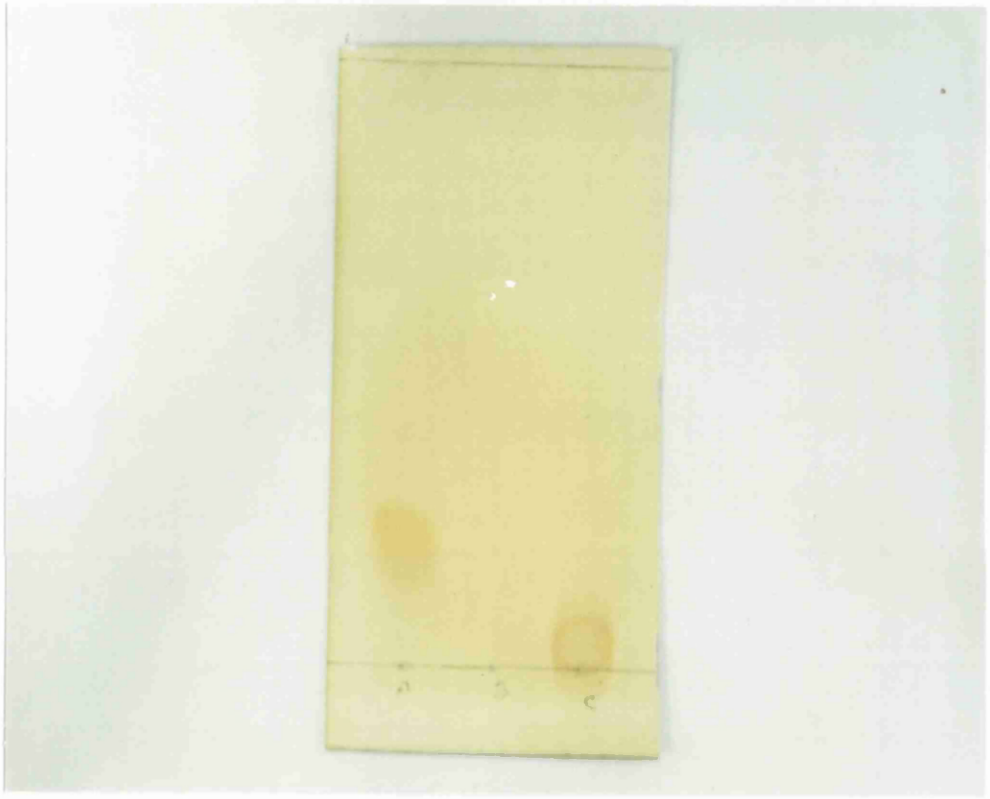


Fig. 2.20c. Reaction of Sol.C with various spray reagents showing that it reacted with all reagents except anisaldehyde. Compounds were developed on Si gel TLC plates with dichloromethane: cyclohexane: ethyl acetate (3:3:1 v/v/v).



A B C

Fig. 2.20d. TLC of solanapyrones A, B and C sprayed with 2,4 dinitrophenylhydrazine showing that the reagent reacted with Sol.A and Sol.C but not with Sol.B. Compounds were developed on Si gel TLC plates with dichloromethane: cyclohexane: ethyl acetate (3:3:1 v/v/v).

2.4. CONCLUSIONS

Alam *et al.* (1989) showed that *A. rabiei* only produced toxins when Czapek Dox nutrients were supplemented with chickpea seed extract. Later, Chen and Strange (1991) showed that divalent cations were the components of chickpea extract required for the production of the toxins by the fungus and that their removal by a cation exchange resin prevented the production of the solanapyrones without affecting growth. The fungus grew well on CDLMC whether zinc was added or not but large differences in solanapyrone productions were observed. When the addition of zinc was delayed until 8 days after inoculation, the fungus produced 1.4 times more Sol.A (65.0 mg/L of culture filtrate compared with 45.5 mg/L) while the production of Sol.B and Sol.C was reduced (5.0 mg/L and 1.4 mg/L compared with 42.3 mg/L and 2.18 mg/L, respectively). Very little of the three compounds was obtained when zinc was omitted from the medium.

Metal ions are important in primary metabolism. For example, zinc has long been known to be essential for the growth of microorganism including fungi and is important in the structure and function of many enzymes, such as those involved in nucleic acid metabolism and cell division (Failla, 1977). Zinc has also been implicated in the synthesis of several secondary metabolites of fungi. For example, the production of an unidentified phytotoxin by *Fusarium vasinfectum* Atk. was only detectable in sucrose-nitrate basal medium supplemented with ≥ 2 µg zinc /25ml and was optimum when zinc was supplied at 6 µg/25 ml. Higher concentrations of zinc markedly reduced toxin production and none occurred at 100 µg/25 ml (Kalyanasundaram and Saraswathi-Devi, 1955). Similarly, zinc

was found essential for versicolorin synthesis by *Aspergillus parasiticus* and it was suggested that it acted at the transcriptional level (Niehaus, 1989). According to Smith and Moss (1985), zinc ions stimulate glycolysis during the stationary phase of growth of *Aspergillus flavus* and *Aspergillus parasiticus,* ensuring the availability of sufficient acetyl coenzyme A for the production of the intermediary polyketides required for aflatoxin biosynthesis. Since the carbon skeleton of the solanapyrones is synthesized via the polyketide pathway and these toxins are also produced in the stationary phase (Benning and Barz, 1995) zinc may be playing a similar role in the synthesis of these compounds. The large reduction in toxin production by *A. rabiei* with no concomitant reduction in biomass of the fungus when zinc is witheld is further evidence for this view (Fig. 2.18).

In order to investigate the toxicity and metabolism of the solanapyrone toxins, the subjects of Chapters 3, 4 and 5, it was necessary to separate them from culture filtrates and from each other. An initial separation from culture filtrates by partitioning into ethyl acetate was found to be most appropriate for bulk preparations since it was inexpensive relative to solid phase extraction and there was no risk of blocking the pores of the cartridges. Solid phase extraction was useful for analytical work since the product could be put straight onto the HPLC without running the risk of fouling the column as the packing material of both cartridge and HPLC column was C18 silica.

Once the solanapyrones were separated from culture filtrates, it was necessary to obtain pure preparations of the individual compounds. Three methods were attempted, radial chromatography using a chromatotron, flash chromatography using a home-made glass column or flash chromatography using a commercial apparatus (Biotage). The last of these was the most successful giving pure preparations of the compounds in good yield.

In order to detect the solanapyrones conveniently, a number of reagents were tested for reaction with the compounds on silica gel TLC plates. Sol.A and Sol.C, both of which have an aldehyde group, reacted with all but one of the reagents tried but Sol.B which has an alcohol rather than an aldehyde group only reacted with iodine vapour.

CHAPTER 3

THE SENSITIVITY OF CHICKPEA CULTIVARS TO SOLANAPYRONES A AND B

3.1. INTRODUCTION

During the early stages of infection of chickpea by *Ascochyta rabiei*, petioles and young branches develop epinasty and leaflets become flaccid owing to the loss of turgor of their cells. Small, water-soaked spots appear on stems, leaves and pods which become necrotic and when the necrosis girdles the stems and petioles, they usually break. These symptoms are consistent with toxin production and can be explained by plasma membrane dysfunction. The plasma membrane affected by toxins loses its selective permeability and this allows leakage of cell sap into intercellular spaces giving rise to water soaking as well as destroying the turgor necessary for support of plant organs since plant cells act as mini-hydroskeletons when they are turgid.

A variety of techniques has been used to assay toxins depending on the data required and the knowledge of the toxin(s) already available. Where a toxin is only suspected, a non-specific assay is appropriate. Wilting is one of the most common symptoms caused by toxins but it is difficult to quantify since cuttings are usually incubated with toxin preparations which may be taken up at variable rates. Another disadvantage of the technique is that comparatively

large amounts of toxin solution are required. Other non-specific assays are more quantitative such as the inhibition of root growth (Rasmussen and Scheffer, 1988b; Kaur, 1995) and the inhibition of incorporation of labelled ^{14}C-leucine into proteins of cell suspension cultures (Manulis *et al.*, 1986). Chlorosis may also be a non-specific symptom of toxin action and this has been used as an assay for phaseolotoxin and HC-toxin, produced by *Pseudomonas phaseolicola* and *Helminthosporium carbonum*, respectively (Hoitink *et al.*, 1966; Rasmussen and Scheffer, 1988a).

Since toxins often cause the dysfunction of membranes, assays to measure this, such as electrolyte leakage (Damann *et al.*, 1974) and failure of cells to retain fluorescein when treated with fluorescein diacetate (FDA) may also form the bases of assays. In the FDA assay, isolated cells or protoplasts are incubated with toxins followed by the addition of the dye. Cells or protoplasts with intact plasma membranes take up FDA and cleave the acetate groups from the molecule by means of non-specific esterases. The resulting fluorescein is not able to permeate intact membranes and is therefore retained in cells which fluoresce under UV light. In contrast, cells with ruptured membranes do not retain fluorescein and remain dark (Strange *et al.*, 1982; Shohet and Strange, 1989; Alam *et al.*, 1989; Strange and Alam, 1992; Latif *et al.*, 1993; Strange, 1993; Widholm, 1972).

Once the biochemical properties of the toxin are known, other assay techniques may be used. For example, the accumulation of ornithine in bean leaves treated with phaseolotoxin suggested that the inhibition of ornithine carbamoyltransferase could be used as an assay for the toxin. Further studies supported the hypothesis that the inhibition of this enzyme was the cause of chlorosis (Mitchell, 1979; Turner and Mitchell, 1985). Similarly, if physico-chemical data are available it may be possible to exploit these in techniques such as high performance liquid

chromatography (HPLC). For example, Hayashi *et al.* (1990) were able to quantify as little as 10 ng of AK and AF toxins produced by *Alternaria alternata* using HPLC.

In the last chapter HPLC was used to quantify the solanapyrone toxin of *A. rabiei.* In this chapter the FDA technique was adopted in order to determine the sensitivity of 12 gentoypes of chickpea to the two solanapyrones A and B which were isolated in sufficient quantity as described in Chapter 2.

3.2. MATERIALS AND METHODS

3.2.1. Plants

Seed of five kabuli cultivars ILC 3279, ILC 482, ILC 249, INRAT 88, Kasseb and seven desi cultivars AUG 424, CM 88, CM 68, 6153, C 44, C 235 and CM 72 was soaked for 12 h in water and planted in John Innes No.2 compost in plastic pots (13 cm diam: 8 seeds per pot). Plants were raised in a greenhouse at 25 ± 2 °C.

3.2.2. Preparation of holding buffer and digestion solution

Holding buffer (HB) consisted of glucose, 0.55 M; citric acid monohydrate, 50 mM; $MgSO_4.7H_2O$, 1 mM; K_2HPO_4, 1 mM; $CaCl_2.2H_2O$, 5 mM and NaOH, 0.15 M. The pH was adjusted to 5.8 with 1 M H_2SO_4 and the buffer was stored in a refrigerator at 4 °C.

The digestion solution was prepared by dissolving macerozyme R-10 (Yakult Honsha Co. Ltd., Japan: 15 g/l), pectolyase Y-23 (ICN Biomedicals, Inc.: 0.05 g/l) and bovine serum albumin (Sigma: 0.5 g/l) in holding buffer.

3.2.3. Isolation of cells

Leaflets from newly opened leaves of 33-55 day old plants were cut into small pieces (approximately 2 cm square), placed in digestion solution (8 ml) and vacuum infiltrated twice for 40 to 50 seconds. They were then agitated gently by a magnetic stirrer at room temperature until they began to disintegrate (20-25 min). The resultant cell suspension was passed through four thicknesses of muslin cloth and centrifuged for 5 minutes at 100 *g* to pellet the cells. Cells were freed from enzymes by resuspending in ice-cold HB (10 ml) and centrifuging twice before suspending in sufficient holding buffer to give an absorbance of 0.2 A units at λ = 620 nm ($\approx$ 2.25 x 10^5 cells/ml).

3.2.4. Bioassay

Two fold dilution series of toxin preparations in duplicate were made across a microtest plate (96 wells: Greiner, Labortechnik, UK) with a Digital Multichannel pipette (50 µl/well: Chen and Strange, 1994). Cell suspension (50 µl: 2.25 x 10^5 cells/ml) with $\geq$ 70% viability was added to each well. Plates were wrapped in aluminium foil and incubated for 3 hours at 25 °C. After incubation, fluorescein diacetate (FDA: 50 µl) was added to each well and incubated for 5 minutes. FDA was prepared weekly as a stock solution (5 mg/ml) in acetone, stored at -20 °C and diluted (1:49) in HB immediately before use. After incubation for 5 minutes the cells were observed under an Olympus inverted microscope (Model IMT) equipped with epifluorescene optics (Strange *et al.*, 1982: Chen and Strange 1994). Fifty cells were scored in each well. The

cells fluorescing green were counted as live and those not fluorescing as dead. Dead cells were easily visualized when plates were illuminated by low intensity red light from above the plate.

The percentage cell death was ascertained by using the following formula:

$$\frac{C - T}{C} \times 100 = \%\ \textbf{Cell death}$$

Where C = Number of live cells in control wells

and T = Number of live cells in test wells

The percentage cell death was converted to probits using the table in Appendix 3.1 and plotted against the $\log_2$ dilution factor. This allowed the factor by which the toxin preparation had to be diluted to kill 50% of the cells, the LD_{50}, to be obtained. The LD_{50} was arbitrarily defined as 1 unit of activity. Units of activity per ml of a toxin preparation of unknown concentration were calculated by multiplying the dilution factor giving 50% cell death by 20 since only 50µl of preparation or its dilutions were used per well. Where the concentration of the toxin preparation was known, this was divided by the dilution factor giving the LD_{50} value in order to express the toxicity in terms of µg/ml.

Since sensitivity varied among assays done on different days, sensitivity of cultivars to the toxins was expressed relative to that of Sol.A acting on cells of ILC 3279 which was included in each assay. Thus the dose required to kill 50% of the cells of ILC 3279 was divided by the dose required to kill 50% of the cells of the cultivar being tested.

3.3. RESULTS

Live cells fluoresced green under the microscope owing to fluorescein while the dead cells remained brown (Fig. 3.1). Probit % cell death was linearly related to the $\log_2$ of toxin dose (Fig. 3.2).

The relative sensitivity of ILC 3279 to Sol.A, which was used as internal control, varied among the assays done on different days as the dose of the compound to kill 50% of the cells of this cultivar ranged from 10.1 ± 1 to 93.3 ± 13.8 µg/ml (Appendix 3.2 and 3.3).

The differences of means of relative sensitivity to Sol.A and Sol.B among the chickpea cultivars were highly significant for both toxins ($P<<0.001$: Table 3.1 and 3.2). Cultivars ILC 249, AUG 424 and 6153 were the most sensitive to Sol.A and cultivars Kasseb and CM 72 the least. The range of sensitivity was 0.65 to 3.3 on a scale in which ILC 3279 was rated as 1. Cultivars were less sensitive to Sol.B, the range being 0.14 to 0.46 on the same scale (i.e. sensitivity of ILC 3279 to Sol.A =1: Fig. 3.3). Sol.A was 2.62 to 12.64 times more toxic than Sol.B depending upon the cultivar (Fig. 3.4).

Comparison of the relative sensitivity of cultivars to Sol.A with their disease ratings to Ascochyta blight showed that those which were most sensitive to Sol.A such as 6153 and AUG 424, were also the most susceptible to the disease scoring 9 on the 1-9 scale of Singh *et al.* (1981) while less sensitive cultivars such as Kasseb, CM 72 and INRAT 88 scored 4.5, 6 and 4, respectively (Fig. 3.5). Spearman's correlation coefficient values between the susceptibility of the cultivars to *A. rabiei* and their relative sensitivity to Sol.A (+0.5166) and to Sol.B (+0.5229) were positive but non-significant (Fig. 3.5 and Fig. 3.6, respectively).

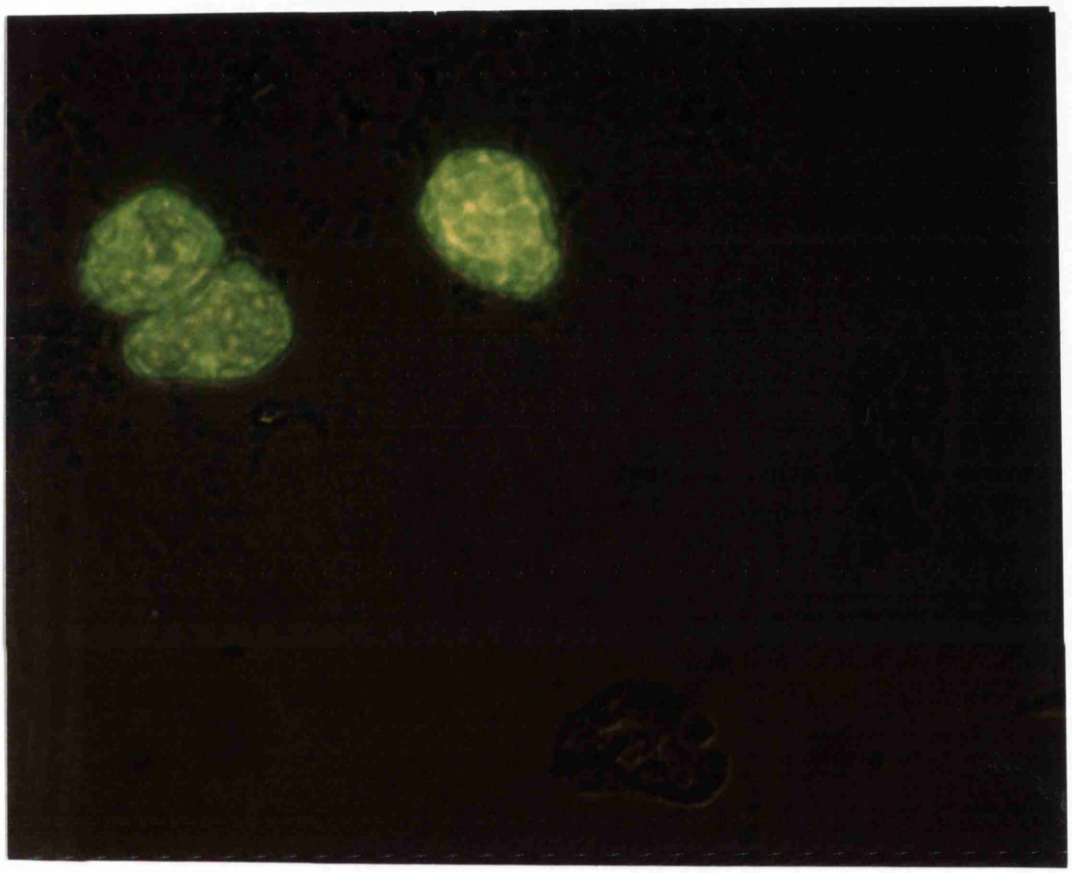


Fig. 3.1 Cells isolated from chickpea leaflets and treated with fluorescein diacetate. Those fluorescing green owing to the accumulation of fluorescein were scored as live. Non-fluorescent cells were scored as dead.

Fig. 3.2. Graph of probit % cell death versus $\log_2$ dilution factor of toxin dose. From the graph the dilution corresponding to a probit value of 5 (= 50 % cell death) was extracted (dotted line).

Fig. 3.2. Plot of probit % cell death versus log_2 dilution factor

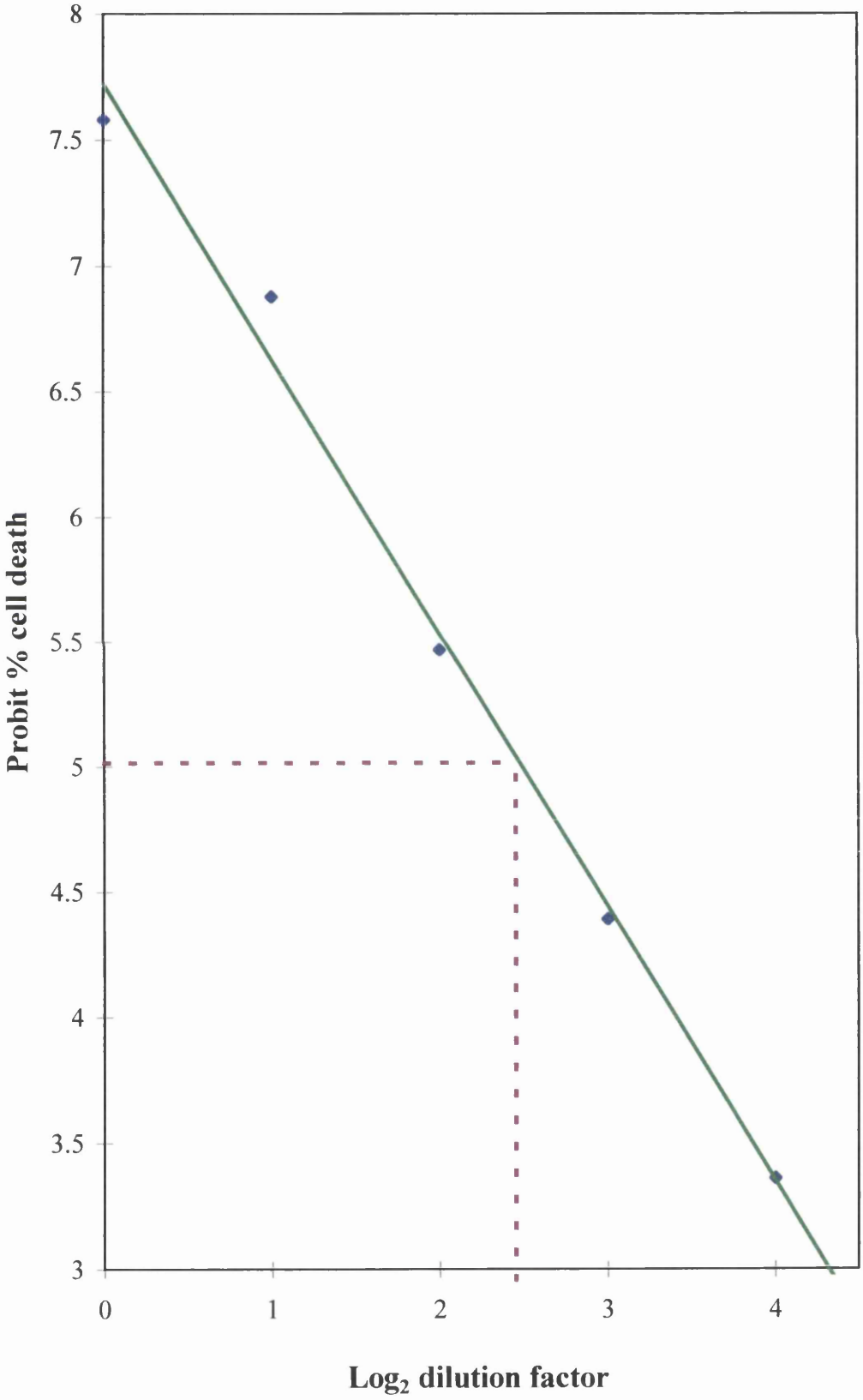


Table 3.1. Sensitivity of chickpea cultivars to solanapyrone A relative to the sensitivity of ILC 3279 to Sol.A (= 1)

Cultivars	Relative sensitivity to Sol.A	
ILC 249	3.40 ± 1.03	A
AUG 424	3.28 ± 0.48	A
6153	2.97 ± 0.20	A
C 44	1.45 ± 0.21	B
ILC 482	1.45 ± 0.56	B
CM 88	1.28 ± 0.16	BC
CM 68	1.22 ± 0.20	BC
C 235	1.03 ± 0.14	BC
ILC 3279	1.00 ± 0.00	BC
INRAT 88	0.95 ± 0.22	BC
CM 72	0.69 ± 0.09	C
Kasseb	0.65 ± 0.14	C

ANOVA showed $P<<0.001$. Having shown a highly significant difference using ANOVA, the least significant difference (LSD) test with alpha set at 0.05 was used to examine differences within the data set. Means followed by the same letters are not significantly different at the 95 % confidence level where LSD value = 0.67. Relative sensitivity of the test cultivars was calculated by dividing the dose of Sol.A to kill 50% cell of ILC 3279 (=1) by the dose required to kill 50% of the cells of the cultivar being tested.

Table 3.2. Sensitivity of chickpea cultivars to solanapyrone B relative to the sensitivity of ILC 3279 to Sol.A (= 1)

Cultivars	Relative sensitivity to Sol.B	
6153	0.46 ± 0.10	A
ILC 249	0.40 ± 0.02	A
INRAT 88	0.39 ± 0.11	A
ILC 482	0.28 ± 0.07	B
AUG 424	0.27 ± 0.03	BC
CM 68	0.24 ± 0.03	BCD
CM 88	0.23 ± 0.005	BCDE
C 235	0.19 ± 0.01	BCDE
CM 72	0.19 ± 0.01	BCDE
ILC 3279	0.18 ± 0.01	CDE
C 44	0.16 ± 0.01	DE
Kasseb	0.14 ± 0.01	E

ANOVA showed $P<<0.001$. Having shown a highly significant difference using ANOVA, the least significant difference (LSD) test with alpha set at 0.05 was used to examine differences within the data set. Means followed by the same letters are not significantly different at the 95 % confidence level where LSD value = 0.09. Relative sensitivity of the test cultivars was calculated by dividing the dose of Sol.A to kill 50% cell of ILC 3279 (=1) by the dose required to kill 50% of the cells of the cultivar being tested.

Fig. 3.3. Sensitivity of chickpea cultivars to Sol.A and Sol.B relative to the sensitivity of ILC 3279 to Sol.A (=1). Absolute values for the LD_{50} for this cultivar varied from 10.1 to 93.3 μg/ml according to assay conditions. Error bars are Standard Deviations.

Fig. 3.3. Sensitivity of chickpea cultivars to Sol. A and Sol. B relative to the sensitivity of ILC 3279 to Sol.A (=1)

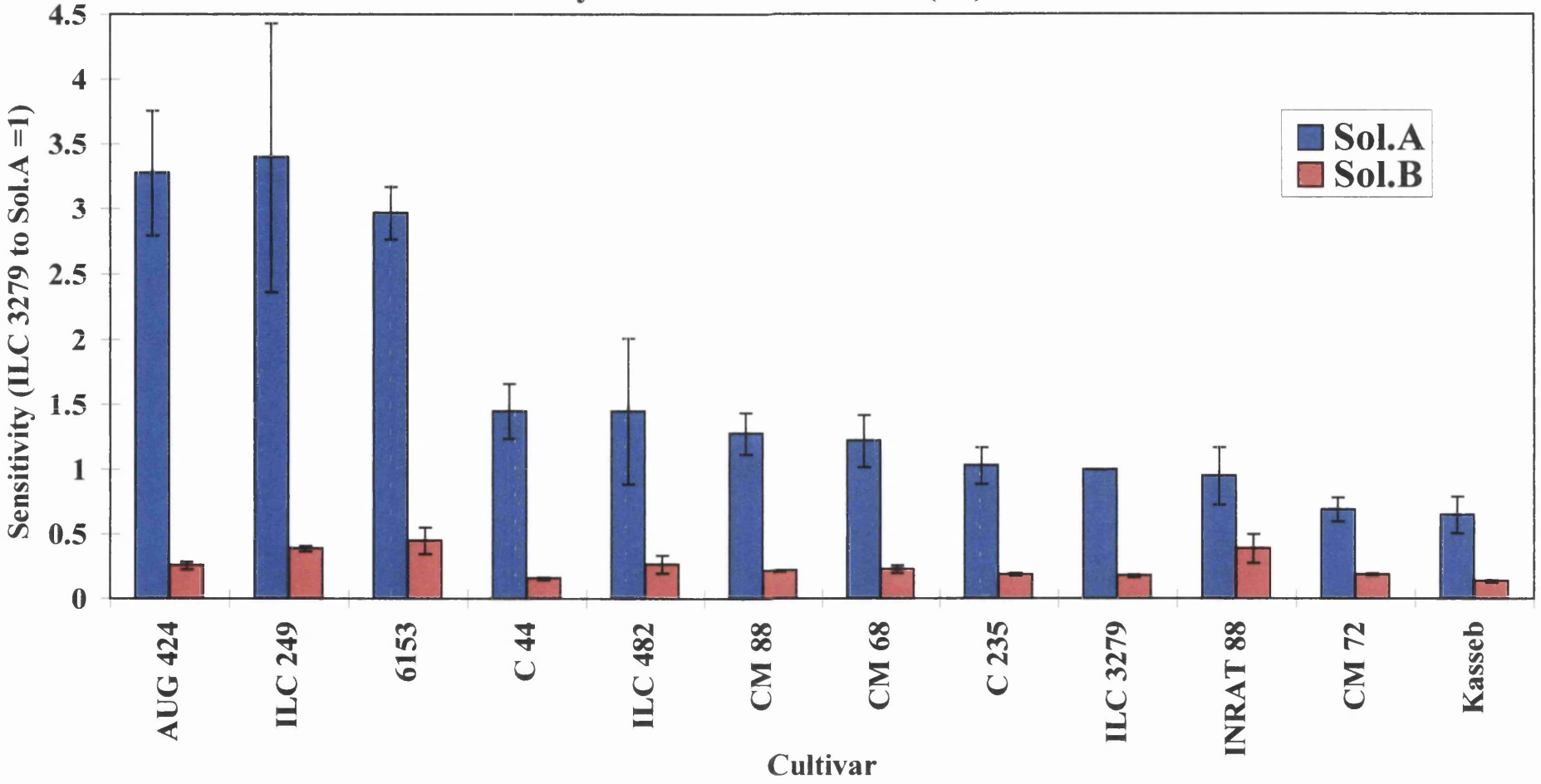


Fig 3.4. The relative toxicity of Sol.A and Sol.B for 12 cultivars of chickpea. Values were obtained for toxicity relative to Sol.A acting on ILC 3279 and dividing that for Sol.A by that for Sol.B for each cultivar. Error bars are Standard Deviations.

Fig. 3.4. Relative toxicity of solanapyrone toxins (Sol. A/Sol. B) to chickpea cultivar

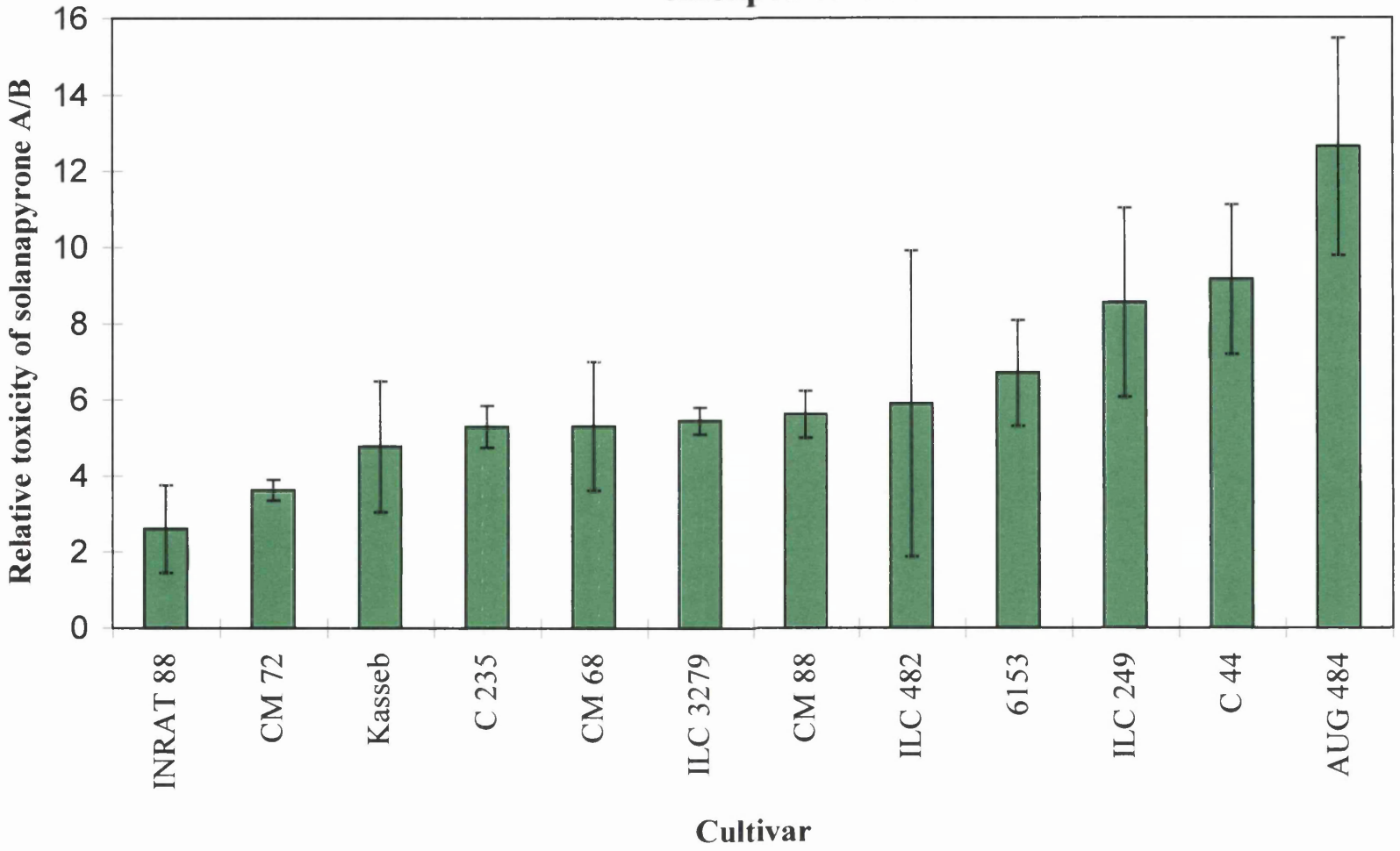


Fig. 3.5. The relationship between relative sensitivity of the cultivars to Sol.A and their susceptibility to *A. rabiei*. Sensitivity of the cultivars to Sol.A was expressed relative to sensitivity of ILC 3279 to Sol.A (=1). Cultivars ILC 3279, CM 72, ILC 482, C 44, C 235, 6153 and AUG 424 were rated on the 1-9 scale of Singh *et al.* (1981) whereas the cultivars Kasseb and INRAT 88 were scored on the 1-9 scale of ICARDA (Akem, pers. Com.). Spearman's correlation coefficient value showed a positive correlation (+ 0.5166) between the relative sensitivity of the cells to Sol.A and their susceptibility to the fungus but this was non-significant.

Fig. 3.5. Relationship between the relative sensitivity of the cultivars to Sol.A and their susceptibility to *A. rabiei*

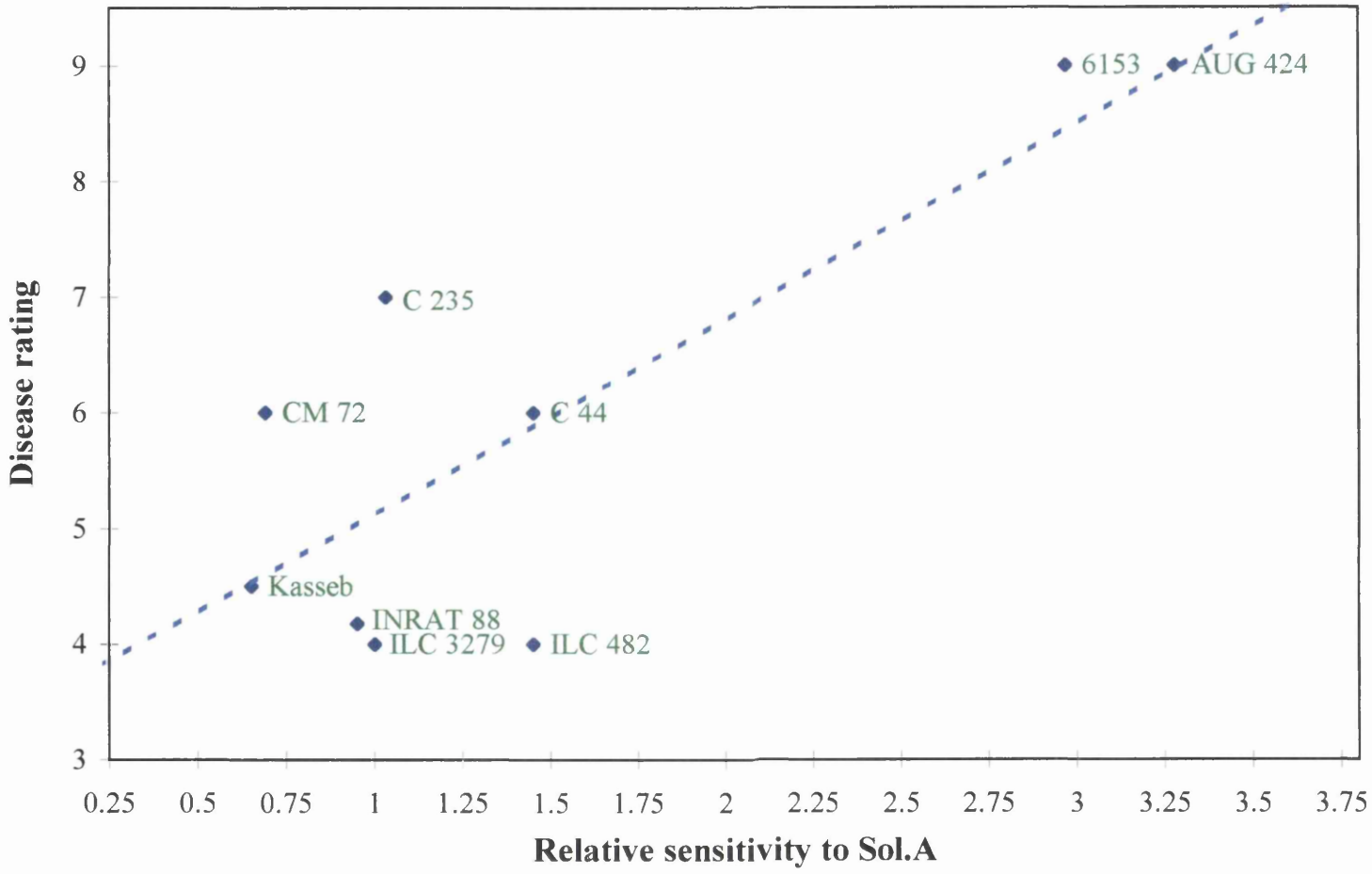
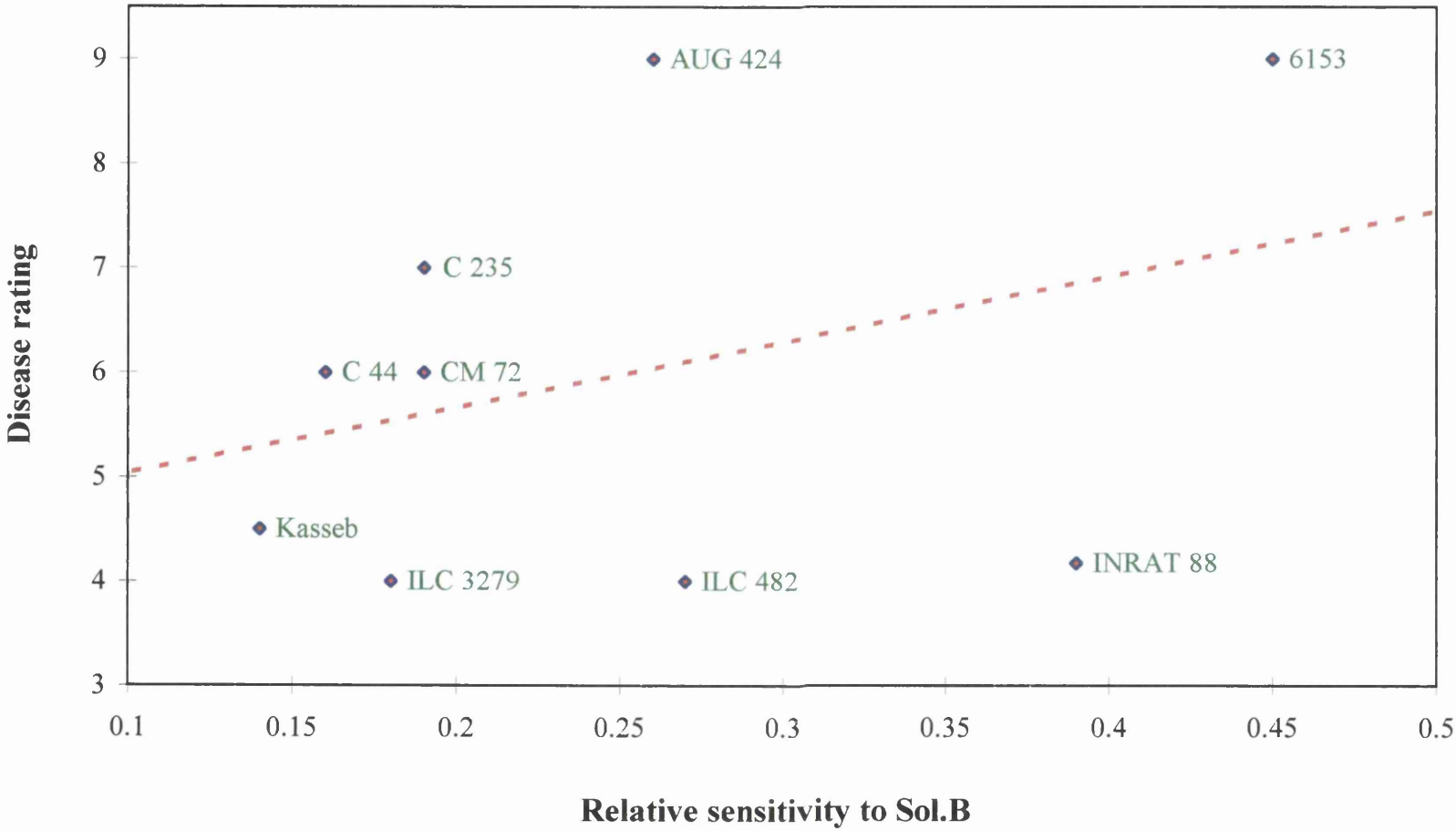


Fig. 3.6. The relationship between relative sensitivity of the cultivars to Sol.B and their susceptibility to *A. rabiei*. Relative sensitivity of the cultivars to Sol.B was expressed relative to sensitivity of ILC 3279 to Sol.A (=1). Cultivars ILC 3279, CM 72, ILC 482, C 44, C 235, 6153 and AUG 424 were rated on the 1-9 scale of Singh *et al.* (1981) whereas the cultivars Kasseb and INRAT 88 were scored on the 1-9 scale of ICARDA (Akem, pers. Com.). Spearman's correlation coefficient value showed a positive correlation (+ 0.5229) between the relative sensitivity of the cells to Sol.B and their susceptibility to the fungus but this was non-significant.

Fig. 3.6. Relationship between relative sensitivity of the cultivars to Sol.B and their their susceptibility to *A. rabiei*



3.4. CONCLUSIONS

In these studies day to day variation of almost 9 fold in the sensitivity to Sol.A of chickpea cultivar ILC 3279 which was used as internal control was observed. As reviewed by Yoder (1981), bioassays are variable because biological systems are complex and there may be several factors involved in the variation of sensitivity of the plants to the toxins, such as temperature, nutritional status and age of the plant. For example, sorghum leaves incubated at temperatures ranging from 35 to 50 °C were found less sensitive to PC-toxin of *Periconia circinata* than those incubated at lower temperatures (Bronson and Scheffer, 1977); oat seedlings grown in the presence of nutrients rather than on water appeared more sensitive to HV-toxin when toxicity was measured by electrolyte leakage (Damann *et al.*, 1974); seedlings also appeared to become increasingly sensitive up to 18 days in this assay (Damann *et al.*, 1974). Preparation of tissue for assay is also a potential source of variation. For example Damann *et al.* (1974) found that toxin-induced leakage from leaves decreased with increase in size of leaf pieces from 0.5 cm to 2.0 cm. Light was also found to be an important factor in some toxin assays. Light was required for tabtoxin to cause chlorosis in tobacco (Durbin and Sinden, 1967) whereas chlorosis caused by tentoxin was more dramatic when cucumber seedlings were incubated in darkness during the period of toxin treatment, complete chlorosis occurring when they were further held in the dark for 24 h after treatment (Templeton 1972). In these studies phenotypic variation in shape and size of leaves among plants grown from seed labelled as ILC 3279 suggesting genetic variability which might account for some of the variation in toxin sensitivity.

Since biological variation is unavoidable and all bioassays could not be done on one day the cultivar ILC 3279 was used as internal control and the results of the sensitivity of the other cultivars included in the trial were expressed relative to sensitivity of this standard. Similarly, Mitchell (1978) quantified phaseolotoxin production using a group of 13 *Pseudomonas* isolates, but this could only be done in three different experiments. In order to make comparisons among the isolates he included one, number 4612, as a reference in each experiment. Also, Scheffer and Livingston (1980) included standard sensitive and resistant clones when evaluating the relative sensitivity of 17 sugarcane clones to HS-toxin at various seasons of the year. Results were expressed as a percent of the sensitive standard and showed that the ranking order was the same from season to season despite variation in absolute sensitivities.

Sol.A was 2.62 to 12.64 times more toxic than Sol.B according to cultivar (Fig. 3.4) possibly because Sol.A has an aldehyde group while in Sol.B this group is reduced to the corresponding alcohol (Fig. 1.7). Other workers have found that Sol.A was about four times as toxic to chickpea cells as Sol.C (Strange and Alam 1992: Alam *et al.*, 1989) and Sol. A has also been reported as being 2.4 and 1.8 times more toxic than Sol.B and Sol.C, respectively, in an inhibition of root growth assay (Kaur, 1995).

The comparison of the relative sensitivity of the cultivars to Sol.A with their susceptibility to the fungus showed that the cultivars which are the most sensitive such as 6153 and AUG 424 are also highly susceptible to the blight disease caused by the fungus as these scored 9 on the 1-9 scale of Singh *et al.* (1981). On the other hand, cultivars which were less sensitive such as Kasseb, CM 72, INRAT 88 and ILC 3279 were tolerant to resistant in the field, scoring 4-6 on the scales of Singh *et al.* (1981) or ICARDA (Fig. 3.5). Spearman's

correlation showed that the relative sensitivity of 9 cultivars to Sol.A was positively correlated with their susceptibility to the fungus but the value of the coefficient (+ 0.5166) was non-significant. It will be interesting to know if such a correlation becomes significant when more chickpea genotypes are included in the test and when their reaction to disease is scored on a less subjective basis.

A more stringent test of the role of the solanapyrones in blight of chickpea caused by *A. rabiei* would be mutants in which a gene necessary for their production is disrupted. A marked decrease in the virulence of such tox-minus mutants would be a clear indication of their role in the disease syndrome. Should this situation obtain, the solanapyrones could be used as surrogates for the pathogen in screening plants for resistance to the disease. This approach has been adopted by other workers with diseases in which toxins are involved and will be further discussed in Chapter 6 (Kohmoto *et al.*, 1991: Wheeler and Luke, 1955: Hartman *et al.*, 1984: Nadel and Spiegel-Roy, 1988: Vidhyasekaran *et al.*, 1990: Jin *et al.*, 1996).

The variability of the relative sensitivity to the toxins among the cultivars raised the interesting question as to why some cultivars are less sensitive than others. For example, do the less sensitive cultivars have an ability to detoxify them by metabolic activity as in the case of HC-toxin which is reduced to a non-toxic form by maize containing the *Hm1* gene (Meeley and Walton, 1991: Meeley *et al.*, 1992)? Alternatively, since plants often detoxify compounds by forming adducts with glutathione, are levels of this compound and the activity of the enzyme that catalyses the formation of the adduct, glutathione-S-transferase, higher in the less sensitive genotypes (Coleman *et al.*, 1997a and 1997b)?

Should insufficient ability to detoxify the solanapyrones be found in chickpea germplasm, another possibility for producing resistant plants would be to find a gene from

another organisms which encodes an enzyme with this property. The gene could then be engineered into chickpea, preferably under the control of a wound promoter so that it is activated on penetration of the plant by the fungus. Accordingly, the next chapter is concerned with the chemical reactions of SolA.

CHAPTER 4

CHEMICAL REACTIONS OF SOLANAPYRONE A

4.1. INTRODUCTION

Solanapyrones A, B and C were separated successfully and the toxicity of Sol.A and B to cells isolated from 12 chickpea cultivars was tested as discussed in chapter 2 and 3, respectively. Strange (1997) suggested three methods of exploiting toxins in disease control: in screening for resistance, selection for resistance in tissue culture from which resistant plants may be regenerated and genetically engineering plants to destroy the toxic compounds. The genes capable of detoxifying the toxins could be found in plants or in micro-organisms. For example, maize containing the *Hm1* gene confers resistance to strains of *Helminthosporium carbonum* producing HC-toxin as it is responsible for the synthesis of an enzyme which reduces the carbonyl group of the toxin giving rise to the corresponding non-toxic alcohol (Meeley and Walton, 1991; Meeley *et al.*, 1992). Kneusel *et al.*, (1990) described the detoxification of brefeldin A, a toxin that plays an important role in the development of disease symptoms in safflower infected by *Alternaria carthami*, by a strain of soil-borne bacteria *Bacillus subtilis,* designated BG3 to a more hydrophilic compound in which the lactone ring was hydrolysed. The authors suggested that the detoxification of the toxin by the lactonase activity of the enzyme may be exploited in the future to introduce resistance to Alternaria leaf blight of safflower.

In order to find a micro-organism capable of degrading the solanapyrones there was a need for a medium suitable for the growth of the micro-organisms which did not affect the stability of the compounds during incubation. For this purpose the sensitivity of Sol.A to heat, incubation in basal medium (section 4.2.1), basal mineral medium (4.2.2.2), basal medium including some carbon sources or in individual components of basal mineral medium were investigated and are described in this chapter.

The solanapyrones A, B and C are decalins with a pyrone moiety in which the alternatives of an ethanolamine or a methoxy group and an aldehydic or alcohol group give rise to the variation in structure found in the three compounds (Fig. 1.7).

Since Sol.A contains an aldehyde group, some reactions of this group are reviewed. Aldehydes contain three regions of reactivity; the electrophilic carbonyl carbon is attacked by nucleophilic compounds, the nucleophilic oxygen by electrophilic compounds and hydrogen by acidic compounds (Vollhardt and Schore, 1994). In many reactions, however, it is the electrophilic carbonyl carbon that plays the dominant role (Geissman, 1959). For example, the addition of hydrogen cyanide gives cyanohydrin in which the attacking cyanide acts as nucleophile. Ammonia reacts with formaldehyde to give hexamethylene tetramine (urotropine; Fig. 4.1. a, b) and amines react with aldehydes to form imines. Addition of water to aldehydes give aldehyde hydrates which are usually not stable and are seldom isolatable. Aldehydes react with sodium bisulphite ($Na^+HSO_3^-$) in a reversible reaction to form bisulphite addition compounds (Geissman, 1959; Fig. 4.2. c, d, e). Primary alcohols are formed when organometallic compounds are added to formaldehyde (Vollhardt and Schore, 1994; Fig. 4.2. f). The addition of H_2S to an aldehyde or ketone can result in a variety of products such as α-hydroxythiols, thioketones, gem-dithiols, but most usual product is trithiane. Thiols add to aldehydes and ketones to give hemimercaptal or dithioacetal (March, 1992; Fig. 4.2. g, h).

Plants are constantly exposed to natural and man made xenobiotics. To reduce the toxicity of these compounds, plants have developed a number of biochemical processes. One of these is the chemical modification of the toxic compound by covalent linkage to an endogenous molecule. Glutathione (GSH) is one of the commonest of these (Coleman *et al.*, 1997b). So the reactions of Sol.A with glutathione, and toxicity of Sol.A to plant cells in the presence of glutathione were studied and are described in this chapter.

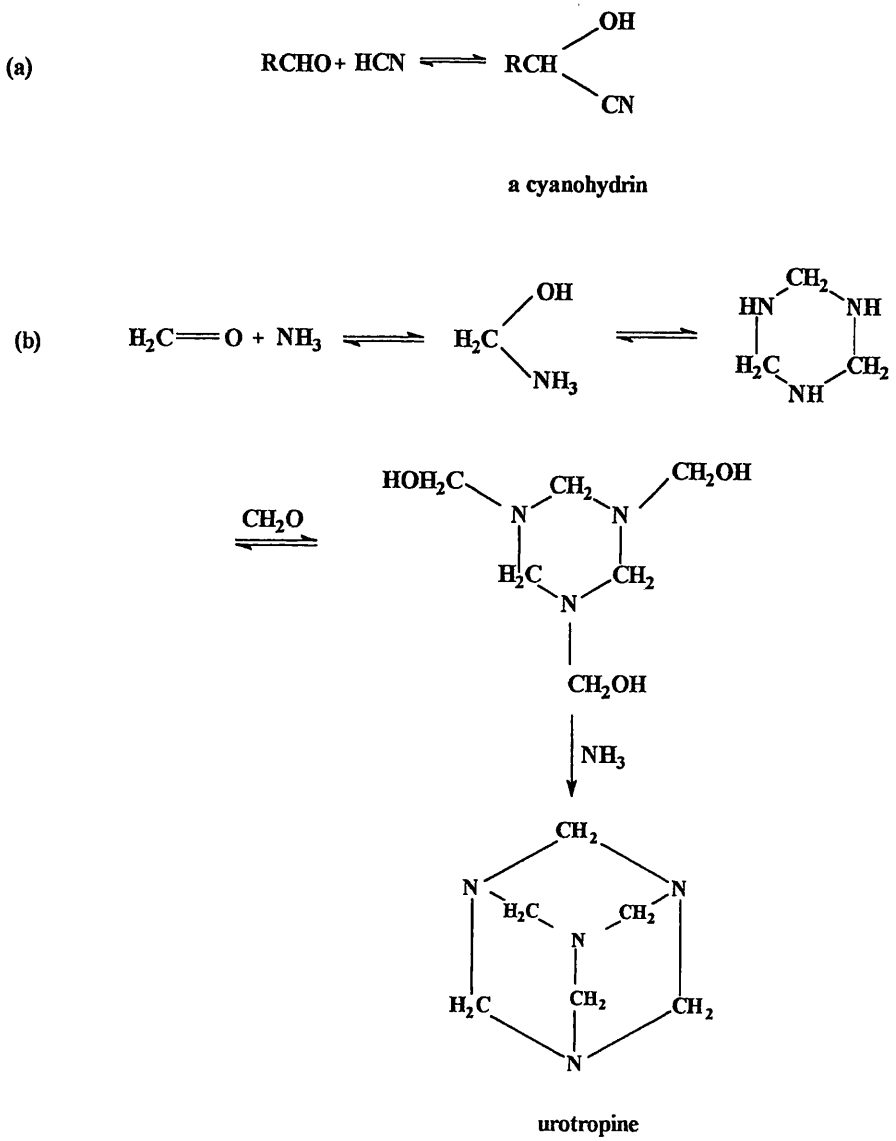


Fig. 4.1. Reactions of aldehydes and ketones, I.

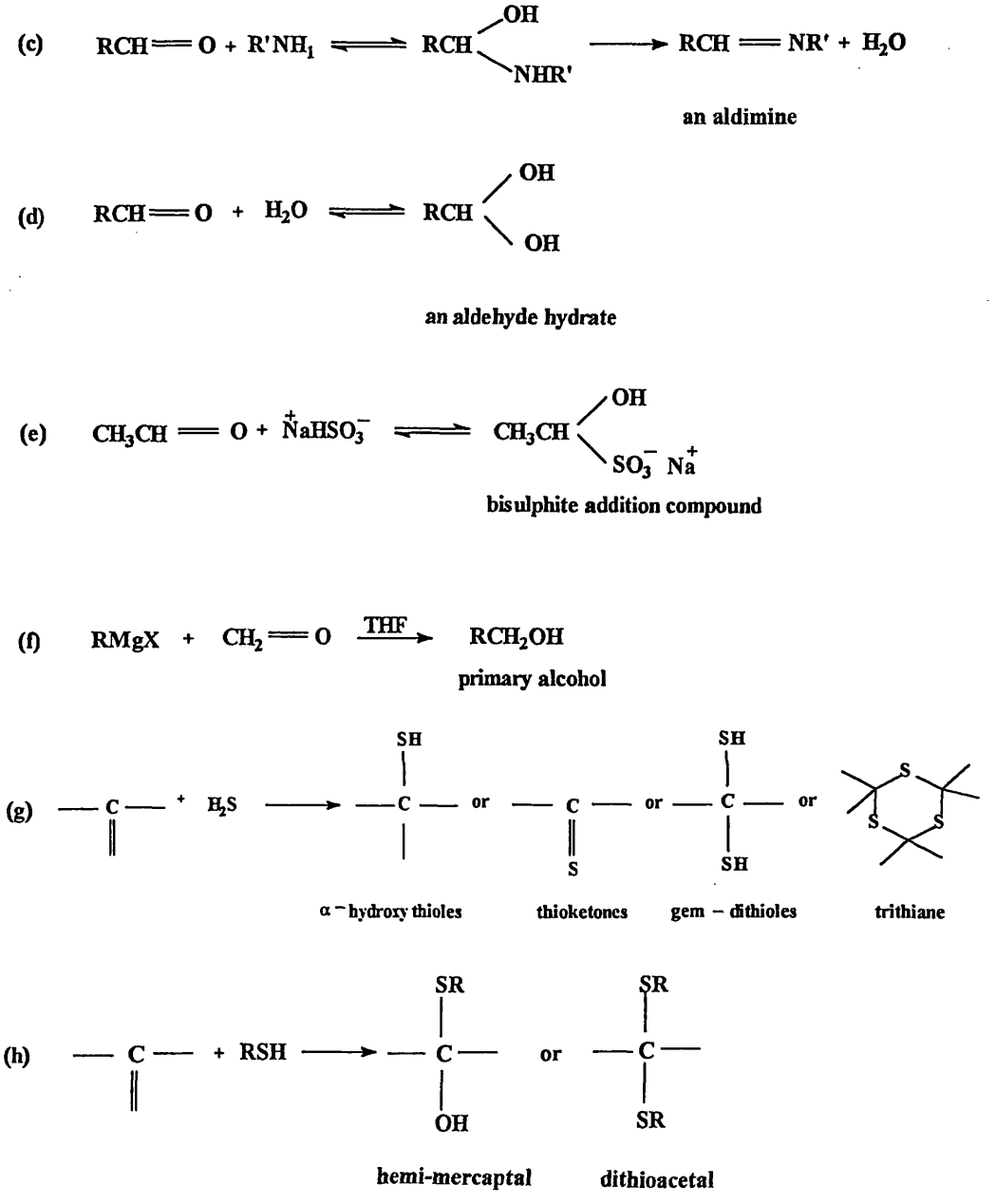


Fig. 4.2. Reactions of aldehydes and ketones, II.

4.2. MATERIALS AND METHODS

4.2.1. Testing the stability of Sol.A to autoclaving in basal medium

Sol.A (2 mg/ml) was autoclaved at 121 °C for 20 minutes in a basal medium containing (NaCl, 0.50 %; $MgSO_4$, 0.02 %; $NH_4H_2PO_4$, 0.10 %; K_2HPO_4, 0.10 %; pH = 6.8) in sample glass vials (58 x 17 mm [diam.]). Controls were not autoclaved. Compounds were extracted by running samples through 1g C18 Isolute cartridges and eluted in acetonitrile (2 ml: section 2.2.2). A sample (20 µl) was run on HPLC for analysis and quantification of the compounds (section 2.2.6).

4.2.2. Chemical stability of Sol.A

4.2.2.1. Effect of different carbon sources in basal medium (BM) on Sol.A

Basal medium (section 4.2.1) was supplemented with 0.2 % of different carbon sources such as sodium benzoate, glucose, lactic acid, arginine, sodium acetate and sodium succinate. After adjusting the pH to 6.8 with NaOH, the media were autoclaved (section 4.2.1). The medium (2 ml/vial: 58 x 17 mm [diam.]) was dispensed separately under sterile conditions. Sol.A (2 mg in 111.0 µl of ethanol/vial) was added to each vial and incubated for 96 hours at 30 °C in a shaker bath. Sol.A incubated in basal medium served as controls.

4.2.2.1.1. Extraction of hydrophobic compounds from basal medium and supplemented basal medium

After incubation, the contents of each vial were centrifuged at 3000 *g* for 3 minutes and the supernatants were transferred to universal bottles (25 ml). Residues in the tubes were dissolved in methanol (3 x 0.5 ml) and added to the supernatants. The preparations were diluted to 25 ml with distilled water before solid phase extraction. The compounds were extracted on a 1g C18 Isolute cartridge and eluted in 2 ml of acetonitrile (section 2.2.2) before HPLC analysis (2.2.6).

4.2.2.2. Growing of bacteria in basal mineral medium containing 360 μM Sol.A

Escherichia coli (JM 109) was grown in basal mineral medium (BMM) containing 360 μM Sol.A as a carbon source using the method of Kneusel *et al.*, (1990) in which brefeldin A was used as a carbon source for *Bacillus subtilis* spp.

The BMM consisted of the following constituents:

Salt	Concentration (mg/100 ml)
1. Na_2HPO_4	700
2. KH_2PO_4	300
3. NaCl	50
4. NH_4Cl	100
5. $CaCl_2$	1.1
6. $MgSO_4$	20

pH = 7.25

Sterilized BMM (5 ml) was dispensed in conical flasks (25 ml). Sol.A (360 µM: 544 µg/flask) was added and incubated for 96 hours at 37 °C. After incubation, the hydrophobic compounds were extracted by solid phase extraction using 1g C18 Isolute cartridges and eluted in acetonitrile (2 ml) as in section 4.2.2.1.1 and analysed by HPLC (section 2.2.6).

4.2.2.3. Effect of individual constituents of basal mineral medium on Sol.A

Sol.A (360 µM) was incubated in distilled water (5 ml) containing individual constituents of basal mineral medium (section 4.2.2.2) for 72 hours at 37 °C. In controls Sol.A was incubated in distilled water only. After incubation compounds from each flask were treated as in section 4.2.2.1.1 and analysed by HPLC (section 2.2.6).

4.2.2.4. Effect of 0.5 M NH_4OH and 0.5 M NaOH on Sol.A

NH_4OH and NaOH 0.5M solutions (5 ml/flask) containing 360 µM Sol.A were incubated in a water bath at 50°C for an hour. Distilled water with the same concentration of Sol.A served as a control. After incubation the hydrophobic compounds were extracted by solid phase extraction, eluted in acetonitrile (2 ml) as in section 4.2.2.1.1 and analysed by HPLC (section 2.2.6).

4.2.2.5. Effect of basal medium and basal mineral medium without Na_2HPO_4

Sol.A (360 µM) was incubated in basal medium (section 4.2.1: 5 ml) and basal mineral medium (BMM: section 4.2.2.2) without Na_2HPO_4. In controls, Sol.A was incubated with water only. After incubation for 96 h, the contents of the flasks were treated as in section 4.2.2.1.1. Compounds in the acetonitrile eluates were analysed by HPLC (section 2.2.6).

4.2.2.6. Effect of glutathione on Sol.A

Tris HCl buffer 10 mM (pH 8: 2 ml) containing 50 mM glutathione was dispensed in sample glass vials (58 x 17 mm [diam.]). Sol.A (272 µg/vial) was added and vortexed for 30 seconds. The contents of each vial were filter sterilized through 0.22 µm filters (MSi, Microseparations, INC, USA). In controls Sol.A was incubated in Tris HCl buffer (10 mM: pH 8). The mixture was allowed to react in the dark at 25 °C for 0, 1, 2, 3, 4, 5, 6 and 16 h. After incubation overnight, the contents of the vials were treated as in section 4.2.2.1.1 while the samples incubated for shorter time periods were injected (without solid phase extraction) into the HPLC and analysed (section 2.2.6).

4.2.3. Isolation of reaction products of solanapyrone A

4.2.3.1. Isolation of SLC-4

Sol.A (360 µM) was incubated in conical flasks in BMM (24 x 5 ml) at 37 °C for 96 h as in section 4.2.2.2. Contents of the flasks were treated as in 4.2.2.1.1. Compounds from each flask were eluted from the solid phase extraction cartridges in acetonitrile (2 ml) and amalgamated (= 48 ml), dried on a rotary evaporator at < 30 °C and dissolved in acetonitrile (1 ml). The product was spotted on TLC plates (20 µl/spot; Silica gel 60 F_{254}, Merck). TLC plates were developed in cyclohexane: dichloromethane: ethyl acetate (1:1:1 v/v/v) and dried in a fume cupboard. Spots were observed under short wave length UV light, and scraped from the plates. They were eluted from the silica in methanol (3 x 0.5 ml) and, after centrifuging, the supernatants were combined and dried on a rotary evaporator. The residue was dissolved in methanol (500 µl) and a sample (20 µl) was run on HPLC (section 2.2.6) to check purity and

another sample (150 µl) was sent for mass spectrometry. Samples of SLC-4 were also spotted on a TLC plate (20 µl/spot). The plate was developed in the same solvent as before and sprayed with 2,4 dinitrophenylhydrazine (section 2.2.7).

4.2.3.2. Isolation of Sol.A-glutathione conjugate

Sol.A (272 µg) was added to 10 mM Tris HCl buffer (pH 8: 2 ml) in glass sample vials (58 x 17 mm [diam.]) containing 500 µM glutathione. In controls, Sol.A was incubated in Tris HCl buffer only. A second control contained only Tris HCl buffer (10 mM: pH 8) and glutathione (500 µM). Nine vials were included for each treatment. The vials were vortexed for 15 seconds, filter sterilized through 0.22 µm filters (Msi, Micron Separations, INC. USA) and incubated at 25 °C overnight. The contents of the vials for each treatments were amalgamated and freeze dried. The residues were dissolved in methanol (2 ml) and spotted on cellulose TLC plates (Cellulose F, Merck, Germany). Plates were developed in butan-1-ol/acetic acid/pyridine/water (15:3:10:12 v/v/v/), dried in a fume cupboard and observed under long and short wave length UV light. Spots differing in Rf from those in controls were scraped off and eluted from the cellulose in methanol (3 x 1 ml). After concentration to 600 µl, compounds were further purified by HPLC using the same conditions as in section 2.2.6 except that the solvent system consisted of tetrahydrofuran, methanol and water (10:10:80 v/v/v/). After injecting samples (50 µl/run), fractions (6 ml) were collected, amalgamated and freeze dried overnight. Residues were dissolved in methanol (150 µl) and tested for purity on HPLC.

The samples purified by HPLC (20 µl) was spotted again on cellulose TLC plates. Plates were developed in the same solvents as above, observed under long and short wavelength UV light and sprayed with 2,4, dinitrophenylhydrazine (section 2.2.7).

4.2.3.3. Mass spectrometry of Sol.A-glutathione conjugate

Sol.A was incubated in Tris HCl buffer (pH 8) containing 500 µM glutathione (4.2.3.2). After incubation the contents of the vials for each treatments were freeze dried, dissolved in methanol (2 ml), dried on rotary evaporator and dissolved in the same solvent (600 µl) and sent for mass spectrometry.

4.2.4. Phytotoxicity

4.2.4.1. Testing the phytotoxicity of SLC-4

Toxicity of the compound was tested as previously described (sections 3.2.3 and 3.2.4).

4.2.4.2. Effect of different concentrations of glutathione on the phytotoxicity of Sol.A to cells

Glutathione (50 mM) was placed in the first wells of microtest plates (96 wells) and two fold dilutions were made from wells 2-10. Sol.A (125 µg/ml [0.414 mM]) in holding buffer was added to each well. Control wells contained only holding buffer or glutathione (50 mM). Plates were incubated for 16 hours at 25°C before adding cells isolated from chickpea leaflets (cv. ILC 3279: 3 weeks old; section 3.2.3) and viability of cells was scored after incubation for 3 h (section 3.2.4).

4.2.4.3. Toxicity of Sol.A with or without glutathione

The effect of a standard concentration of glutathione (50 mM) on the toxicity of a dilution series of Sol.A was tested as follows. An ethanolic solution of Sol A was added to well 1 of a microtest plate so that on evaporation of the ethanol the well contained 15.1 μg of the compound. Holding buffer (100 μl) containing 50 mM glutathione was added to well one and a two fold dilution series made across the plate. Controls contained no Sol.A. Another plate with the same dilution series of Sol.A but without glutathione was also set up for comparison. Plates were incubated for 16 hours at 25 °C before adding cells isolated from chickpea leaflets (cv. ILC 3279: 3 weeks old; section 3.2.3) and viability of cells was scored after incubation for 3 h (section 3.2.4).

4.3. RESULTS

4.3.1. Effect of autoclaving Sol.A in basal medium

Only 2170.2 µg of Sol.A (54.3 % of the starting material) was recovered when incubated in basal medium but autoclaving caused a further reduction of 1859.1 µg leaving 7.8% of the starting material. Possible reasons for the low recovery of Sol.A in controls and tests were that the residues were not recovered properly from the incubation vials and samples were not diluted before solid phase extraction on C18 Isolute cartridge (Table 4.1). A new compound was formed which had a UV spectrum with λ_{max} 313 nm and a retention time of 1201 ± 58 seconds as compared to that of Sol.A which had λ_{max} 327 nm and retention time of 660.6 ± 23 seconds (Fig. 4.3).

4.3.2. Effect of basal medium supplemented with different carbon sources on Sol.A

The means of the recovery of solanapyrone A differed significantly among the treatments when incubated for 96 h in basal medium supplemented with various carbon sources ($P<<0.001$). In controls where Sol.A was incubated in basal medium (without carbon source), 1518.3 µg of the compound was recovered (75.9 % of the starting material [2000 µg]). Out of five carbon sources used to supplement basal medium, lactic acid was the least interfering as recovery of Sol.A was 1460.1 µg (73.0 % of the starting material). In contrast, only 758.8 µg of the compound (37.9 %) of the starting material was recovered when basal medium was supplemented with arginine (Table 4.2).

Table 4.1. Recovery of Sol.A (μg) after autoclaving in basal medium

Treatment	R1	R2	R3	Mean	% recovery of starting material
Control (4000 μg of Sol.A + basal medium [2 ml]: not autoclaved)	2230.4	2232.8	2047.4	2170.2 ± 106.3	54.3
Test (4000 μg of Sol.A + basal medium [2 ml]: autoclaved)	214.2	343.1	376.1	311.1 ± 85.6	7.8

Sol.A 4000 μg/2 ml of basal medium was autoclaved at 121 °C for 20 minutes. After autoclaving, the hydrophobic compounds were extracted by solid phase extraction using a 1g C18 Isolute cartridge, eluted in acetonitrile (2 ml) and analysed by HPLC.

Fig. 4.3. Chromatogram of Sol.A at λ = 327 nm after autoclaving in basal medium. Peak 1 is for Sol.A left after autoclaving and peak 2 is a compound which appeared as a result of autoclaving.

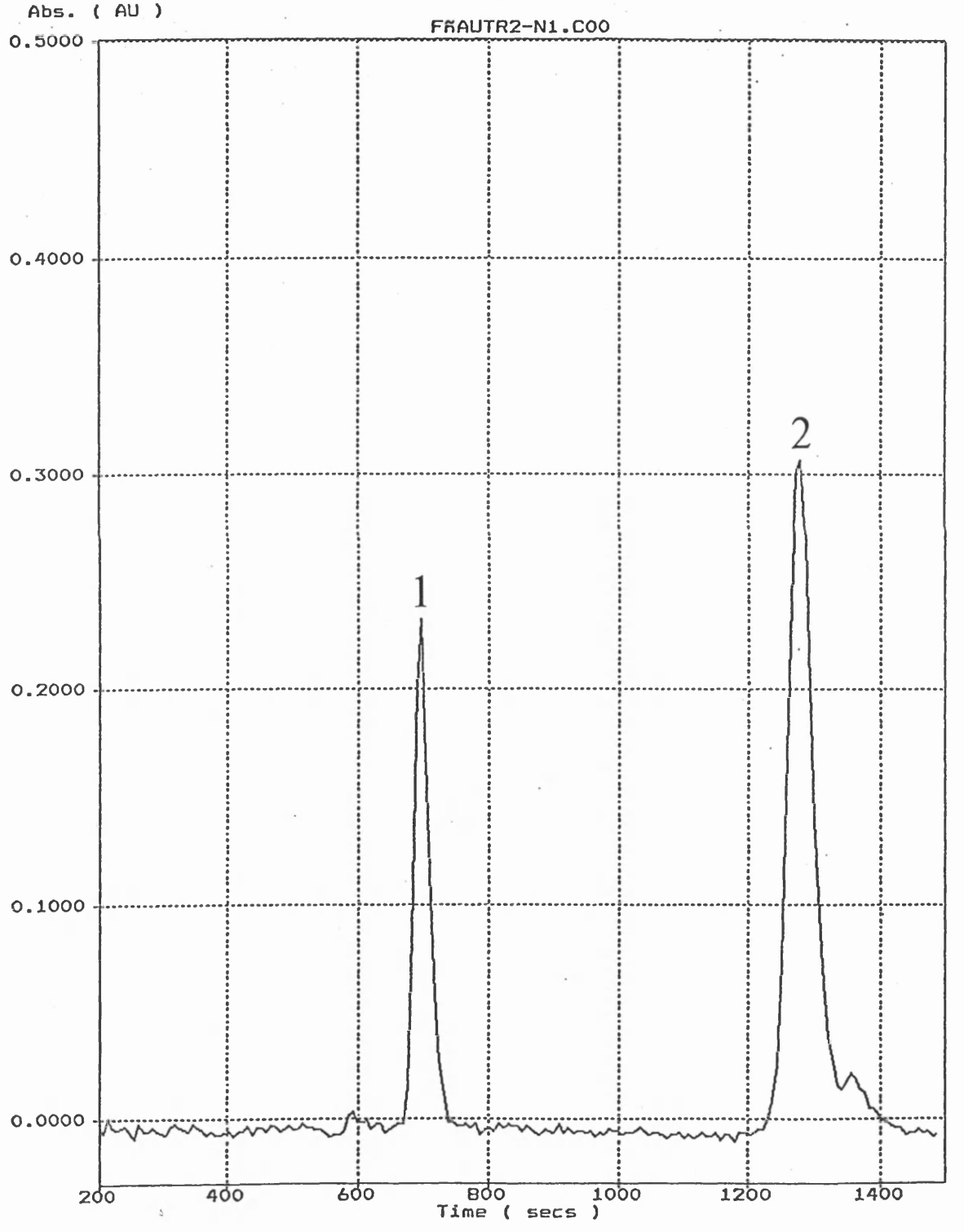

Abs. (AU)
FRAUTR2-N1.C00
0.5000
0.4000
0.3000
0.2000
0.1000
0.0000
1
2
200
400
600
800
1000
1200
1400
Time (secs)

Table 4.2. Recovery of Sol.A (μg) after incubation in basal medium (2 ml) amended with different carbon sources for 96 h at 37°C

Treatments	Sol.A (μg)		% recovery of starting material
Control (Basal medium only: B.M)	1518.3 ± 161.4	A	75.9
B.M + Lactic acid	1460.1 ± 65.5	A	73.0
B.M + Sodium benzoate	1403.4 ± 47.9	AB	70.1
B.M + Glucose	1332.5 ± 125.7	ABC	66.6
B.M + Na-acetate	1223.7 ± 36.5	BC	61.2
B.M + Na-succinate	1205.2 ± 170.7	C	60.3
B.M + Arginine	758.8 ± 32.2	D	37.9

Sol.A (2000 μg) was incubated in basal medium (2 ml) supplemented with the carbon sources supplied at 2 %. After incubation, the hydrophobic compounds were extracted by solid phase extraction using 1g C18 Isolute cartridge, eluted in acetonitrile and analysed by HPLC. ANOVA showed that $P<<0.001$. The Least Significant Difference (LSD) test with alpha set at 0.05 was used to examine differences within the data set (LSD value: 187.2). Means followed by the same letters are not significantly different at the 95 % confidence level.

4.3.3. Degradation of Sol.A by basal mineral medium (BMM)

When attempts were made to find a bacterial species able to degrade Sol.A, using the compound as a carbon source, the basal mineral medium itself was found to cause degradation of the phytotoxin, producing a new compound SLC-4. The recovery of Sol.A decreased from 434.21 ± 25.5 µg (79 % of the starting material) after incubation at 37 °C for 24 h to 180.9 ± 5.1 µg (33 % of the starting material) after incubation for 96 h. Concomitantly, SLC-4 increased from 49.7 ± 7.4 µg to 134.2 ± 7.0 µg over the same period assuming an extinction coefficient of 7,300 at 320 nm based on Sol.C which the spectrum resembles (compare Figs 4.6 and 2.16: Fig. 4.4).

4.3.3.1. Purification of SLC-4 by TLC

The Rf of SLC-4 was (0.77) than that of Sol.A (0.55: Fig. 4.5). On HPLC SLC-4 had retention time of 1123.9 ± 21.2 seconds and a UV spectrum with λ_{max} = 310, 278 and 241 nm (Fig. 4.6). Mass spectrometry showed that SLC-4 was a demethylated product of Sol.A since major ions at 287, 259 and 138 had mass values that were 15 less than those of Sol.A 302, 274 and 153, respectively: compare Fig. 4.7 with Fig. 4.8.

When chromatographed on thin layer plates and sprayed with 2,4 dinitrophenylhydrazine SLC-4 gave a brown colour (Fig 4.9).

4.3.3.2. Phytotoxicity of SLC-4

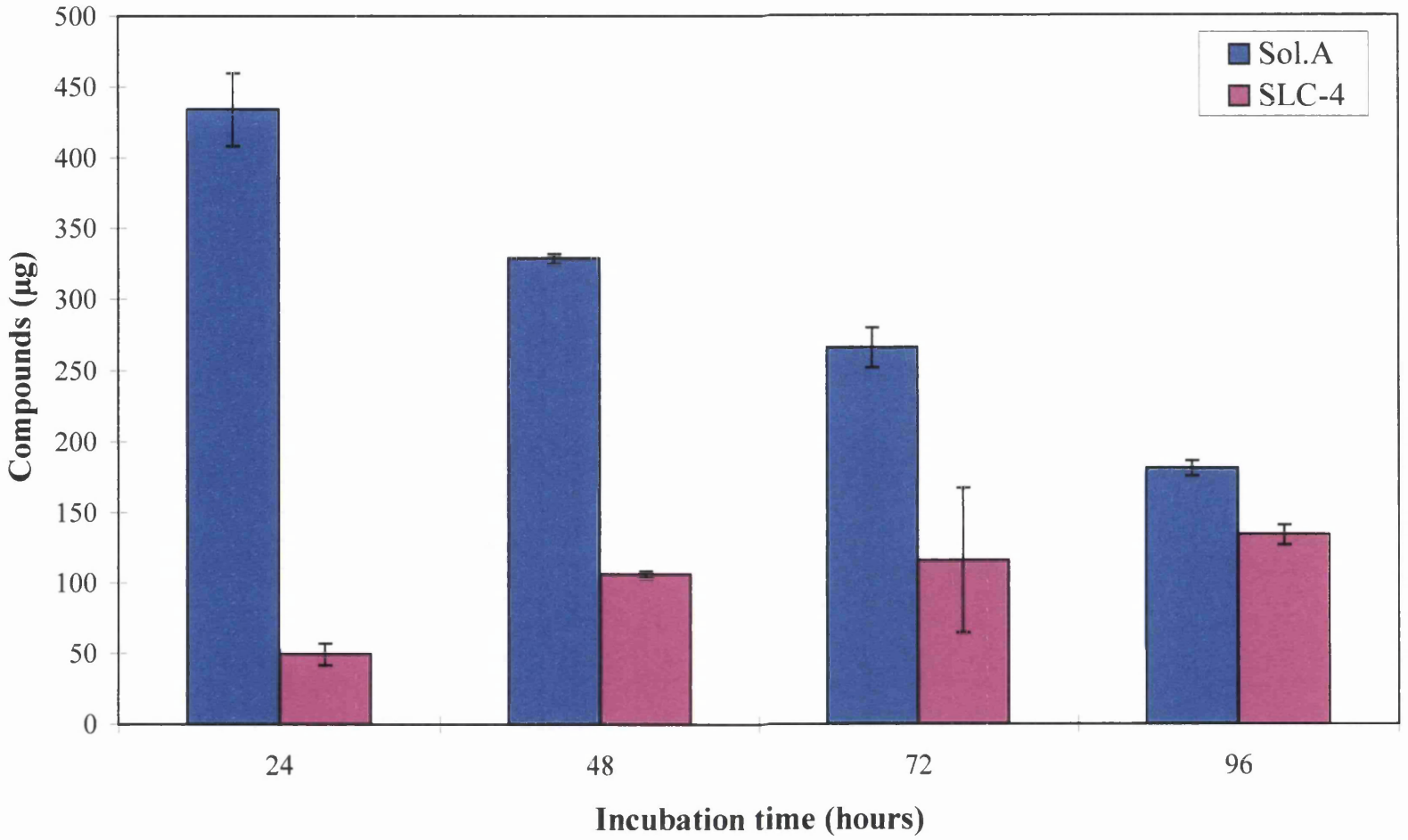
The LD_{50} for Sol.A and SLC-4 were 31.3 ± 2.1 and 514.0 ± 55.0 µg/ml, respectively (Table 4.3).

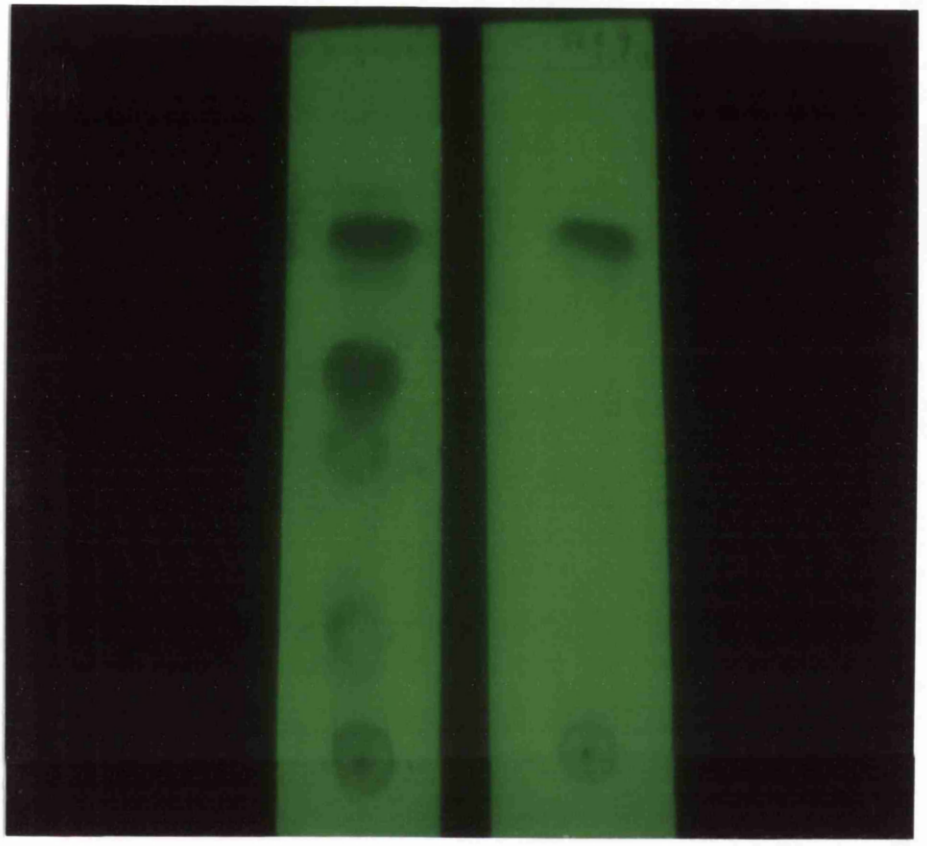
Table 4.3. Toxicity of Sol.A and SLC-4 to cells isolated from chickpea leaflets (cv. ILC 3279)

Compound	LD_{50} values (doses of the compounds [µg/ml] required to kill 50 % cells)				
	R1	R2	R3	Mean	SD
SLC-4	467.3	574.7	500.0	514.0	55.0
Sol.A	33.5	31.2	29.2	31.3	2.1

Fig. 4.4. Histogram showing the recovery of Sol.A and formation of a new compound SLC-4 during incubation in basal mineral medium from 24 to 96 h at 37 °C. Sol.A (544 μg/vial) was incubated in basal mineral medium (5 ml) and after incubation the hydrophobic compounds were extracted by solid phase extraction using 1g C18 Isolute cartridge, eluted in acetonitrile (2 ml) and quantified by HPLC using an extinction coefficient of 7,300 at 320 nm for SLC-4. Error bars are Standard Deviations.

Fig. 4.4. Recovery of Sol.A and formation of SLC-4 during incubation in basal mineral medium





1 **2**

Fig. 4.5. Thin Layer Chromatography of SLC-4 before and after purification. **1**) Sol.A (544 µg) incubated in basal mineral medium (5 ml/flask) at 37 °C for 96 h. After incubation compounds were extracted by solid phase extraction, eluted in acetonitrile (48 ml), concentrated and spotted on silica gel TLC plates. The plates were developed in cyclohexane, dichloromethane and ethyl acetate (1:1:1 v/v/v) and observed under short wave length UV light. Note the spot with highest Rf value of 0.77 of SLC-4 and lower one with Rf value of 0.55 of remaining Sol.A. **2**) Note the single spot of SLC-4 with Rf value of 0.77 after isolation.

Fig. 4.6. The top half of the figure shows the UV spectrum of SLC-4 and the bottom half of the figure shows the chromatogram of the compound at 310 nm. .

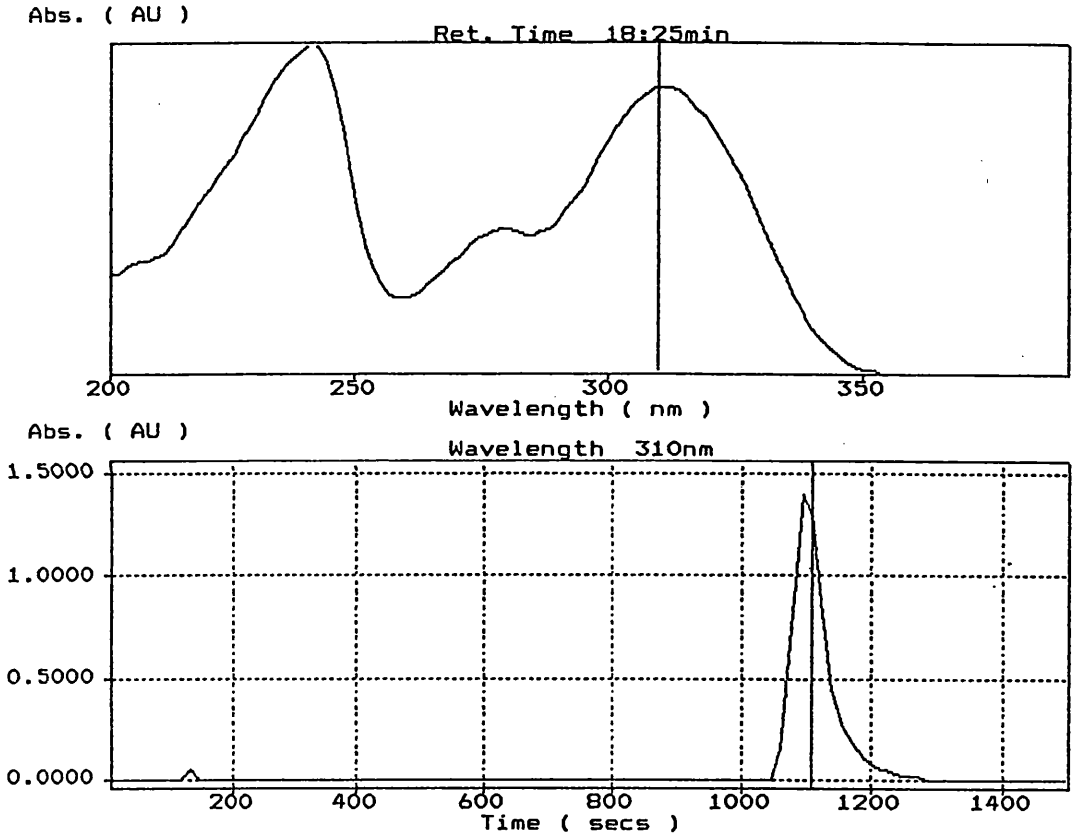

Abs. (AU)
Ret. Time 18:25min
200
250
300
350
Wavelength (nm)
Abs. (AU)
Wavelength 310nm
1.5000
1.0000
0.5000
0.0000
200
400
600
800
1000
1200
1400
Time (secs)

Fig. 4.7. Mass spectrum of SLC-4.

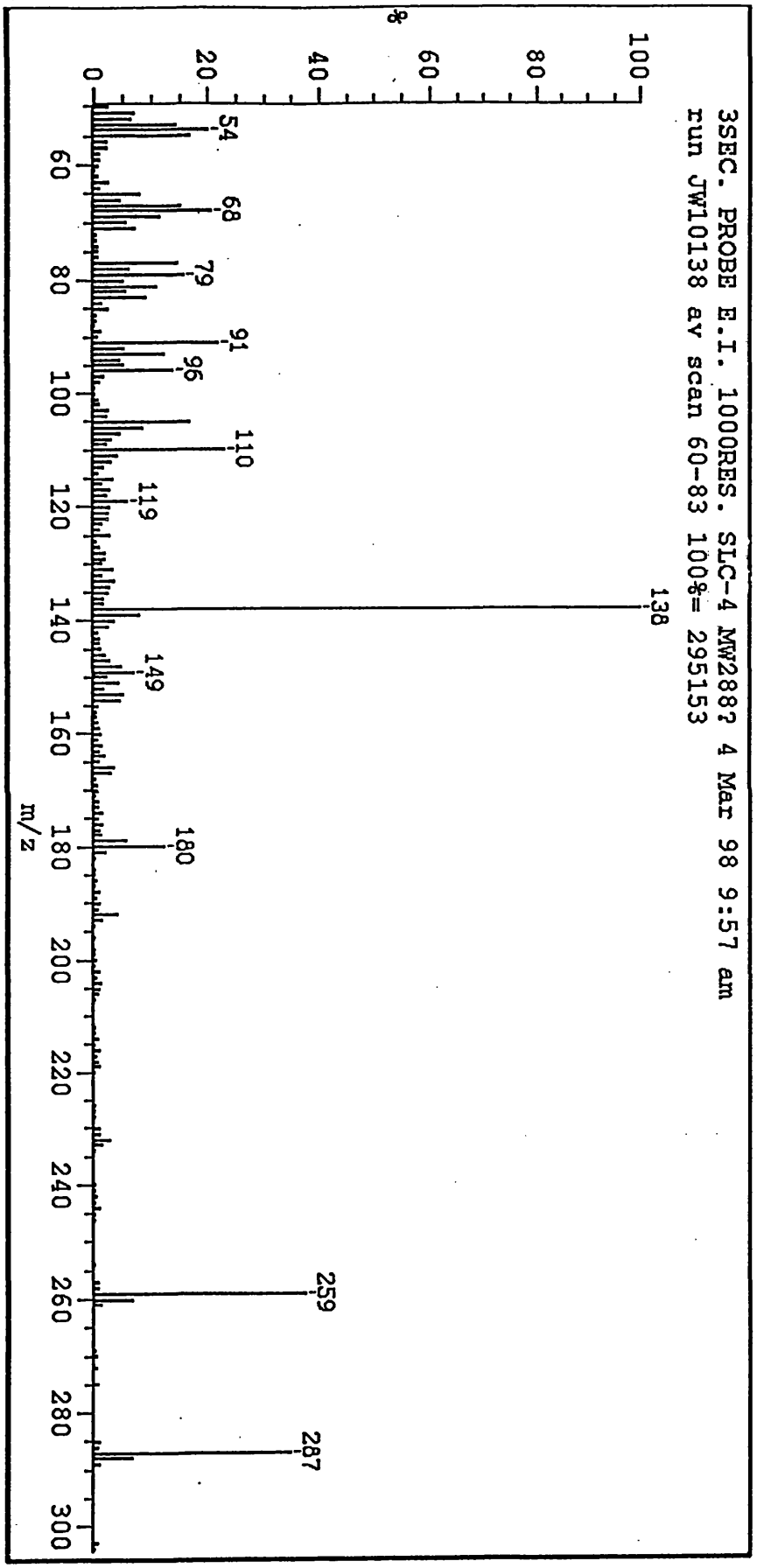
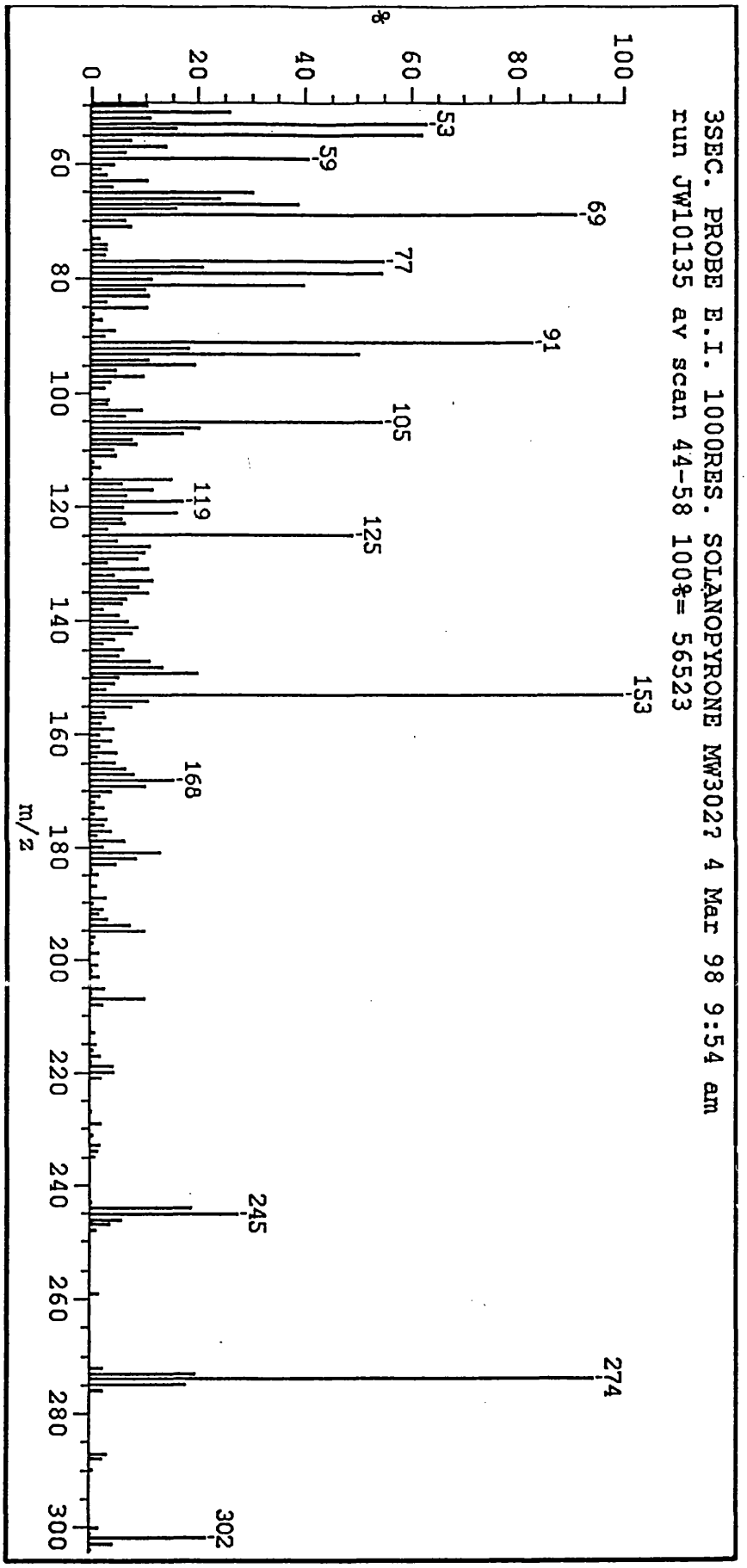

3SEC. PROBE E.I. 1000RES. SLC-4 MW288? 4 Mar 98 9:57 am
run JW10138 av scan 60-83 100%= 295153
%
m/z
54
68
79
91
96
110
119
138
149
180
259
287

Fig. 4.8. Mass spectrum of Sol.A.


3SEC. PROBE E.I. 1000RES. SOLANOPYRONE MW302? 4 Mar 98 9:54 am
run JW10135 av scan 44-58 100%= 56523
%
m/z
53
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77
91
105
119
125
153
168
245
274
302

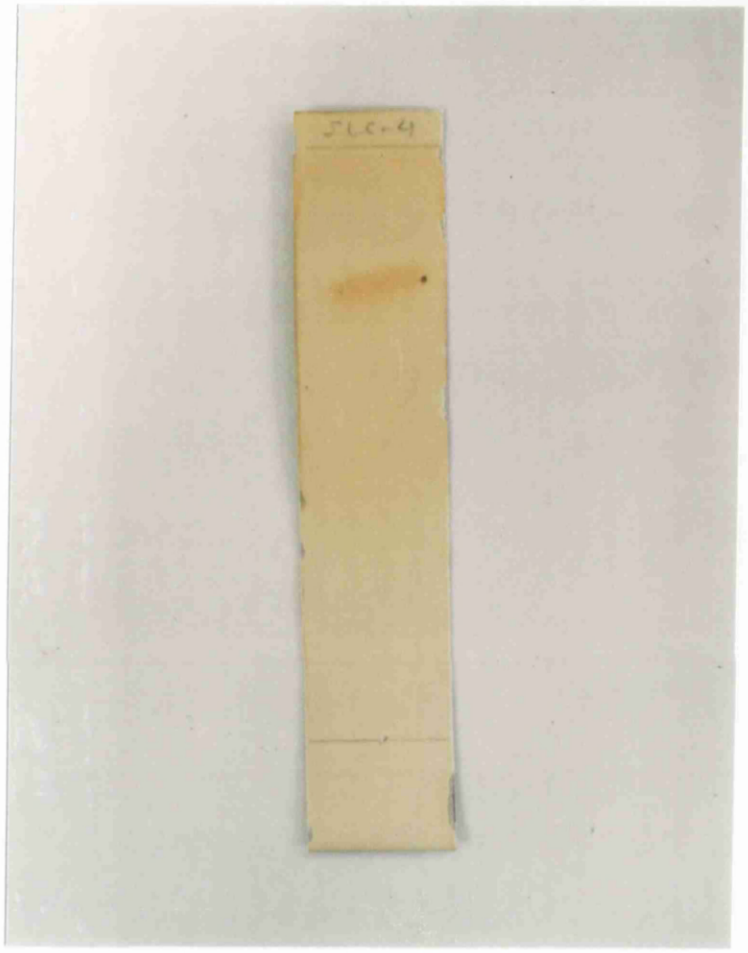


Fig. 4.9. The reaction of SLC-4 with 2,4-dinitrophenylhydrazine. SLC-4 was spotted on silica gel TLC plates which were developed in cyclohexane, dichloromethane and ethyl acetate (1:1:1 v/v/v) and sprayed with the reagent.

4.3.4. Effects of individual constituents of BMM on Sol.A

Recovery of Sol.A after incubation for 72 h at 37 °C in the individual constituents of basal mineral medium differed significantly ($P << 0.001$). Out of the six constituents, Na_2HPO_4 was the most reactive compound since only 90.5 ± 6.0 µg Sol.A was recovered from 544 µg of starting material (16.6 %). The second most reactive compound was NH_4Cl where recovery of the Sol.A was 319.4 ± 43.7 µg (58.7 % of the starting material: Table 4.4).

4.3.5. Effect of NaOH and NH_4OH

In controls when Sol.A 544 µg was incubated for an hour at 50 °C in water (5 ml), the recovery of the compound was 96.2 % and 84.7 % in two experiments. Incubation of the compound in 0.5 M solution of NaOH or NH_4OH at 50 °C allowed recoveries of only 18.1 and 38.4 %, respectively (Table 4.5 and 4.6).

Incubation with NH_4OH resulted in the production of a compound similar to SLC-4 since it had retention time of 1047.6 ± 7.6 seconds (SLC-4: 1123.9 ± 21.2 seconds) and a UV spectrum (λ_{max} = 239, 281 and 310 nm) which matched 95.4 % with that of SLC-4 when compared over the range of 230-360 nm by the method of least squares (Fig. 4.10).

4.3.6. Effect of basal medium and basal mineral medium without Na_2HPO_4 on Sol.A

When Sol.A (544 µg) was incubated for 72 h at 37 °C in water (5 ml), only 297.1 µg of the compound was recovered (54 % of the starting material). Incubation of Sol.A in basal medium and basal mineral medium without Na_2HPO_4 caused a further reduction of 66.9 µg and 166.4 µg, respectively of the compound, showing the greater stability of the compound in basal

medium than in basal mineral medium. Statistically the means of recovery of the Sol.A by incubating Sol.A in basal medium did not differ from that of controls (water) at 95 % significance (Table 4.7).

Table 4.4. Recovery of solanapyrone A (μg) after incubation for 72 h at 37 °C with aqueous solutions of individual constituents of basal mineral medium

Constituents	Recovery of Sol.A (μg)	
	Mean	% recovery of the starting material
Aqueous sol. of $CaCl_2$ (5 ml) + Sol.A (544 μg)	369.7 ± 12.31 A	67.9
Control (water: 5 ml) + Sol.A (544 μg)	369.1 ± 29.3 A	67.8
Aqueous sol. of KH_2PO_4 (5 ml) + Sol.A (544 μg)	368.0 ± 19.6 A	67.6
Aqueous sol. of NaCl (5 ml) + Sol.A (544 μg)	354.5 ± 23.4 AB	65.2
Aqueous sol. of $MgSO_4$ (5 ml) + Sol.A (544 μg)	343.2 ± 12.6 AB	63.1
Aqueous sol. of NH_4Cl (5 ml) + Sol.A (544 μg)	319.4 ± 43.7 B	58.7
Aqueous sol. of Na_2HPO_4 (5 ml) + Sol.A (544 μg)	90.5 ± 6.0 C	16.6

Sol.A (544 μg) was incubated for 72 h at 37 °C in salts solutions. After incubation the hydrophobic compounds were extracted by solid phase extraction and quantified by HPLC. ANOVA showed $P<<0.001$. The Least Significant Difference (LSD) test with alpha set at 0.05 which gave LSD value (42.15) was used to examine differences within the data set. Means followed by the same letters are not significantly different at 95 % confidence level.

Table 4.5. Recovery of Sol.A (μg) after incubation in 0.5 M NaOH solution for an hour at 50°C

Treatments	R1	R2	R3	Mean	% recovery of starting material
Control (544 μg of Sol.A) + water (5 ml) H_2O)	548.6	487.6	533.8	523.3 ± 31.8	96.2
Sol.A (544 μg) + 0.5 M NaOH (5 ml)	79.3	104.2	111.8	98.4 ± 17.0	18.1

Sol.A (544 μg) was incubated in 5 ml of 0.5 M NaOH solution. after incubation for an hour at 50°C, the hydrophobic compounds were extracted by solid phase extraction, eluted in acetonitrile (2 ml) and quantified by HPLC.

Table 4.6. Recovery of Sol.A and formation of a new compound (μg) after incubation in 0.5 M NH_4OH solution for an hour at 50°C

Treatments	R1	R2	R3	Mean	% recovery of the starting material
Control (544 μg of Sol.A + H_2O (5 ml)	445.0	430.0	507.8	460.9 ± 41.3	84.7
Sol.A (544 Mg) + 0.5 M NH_4OH (5 ml)	228.2	191.4	207.0	208.9 ± 18.5	38.4
New compound resembling SLC-4	126.6	115.8	113.0	118.5 ± 7.2	---

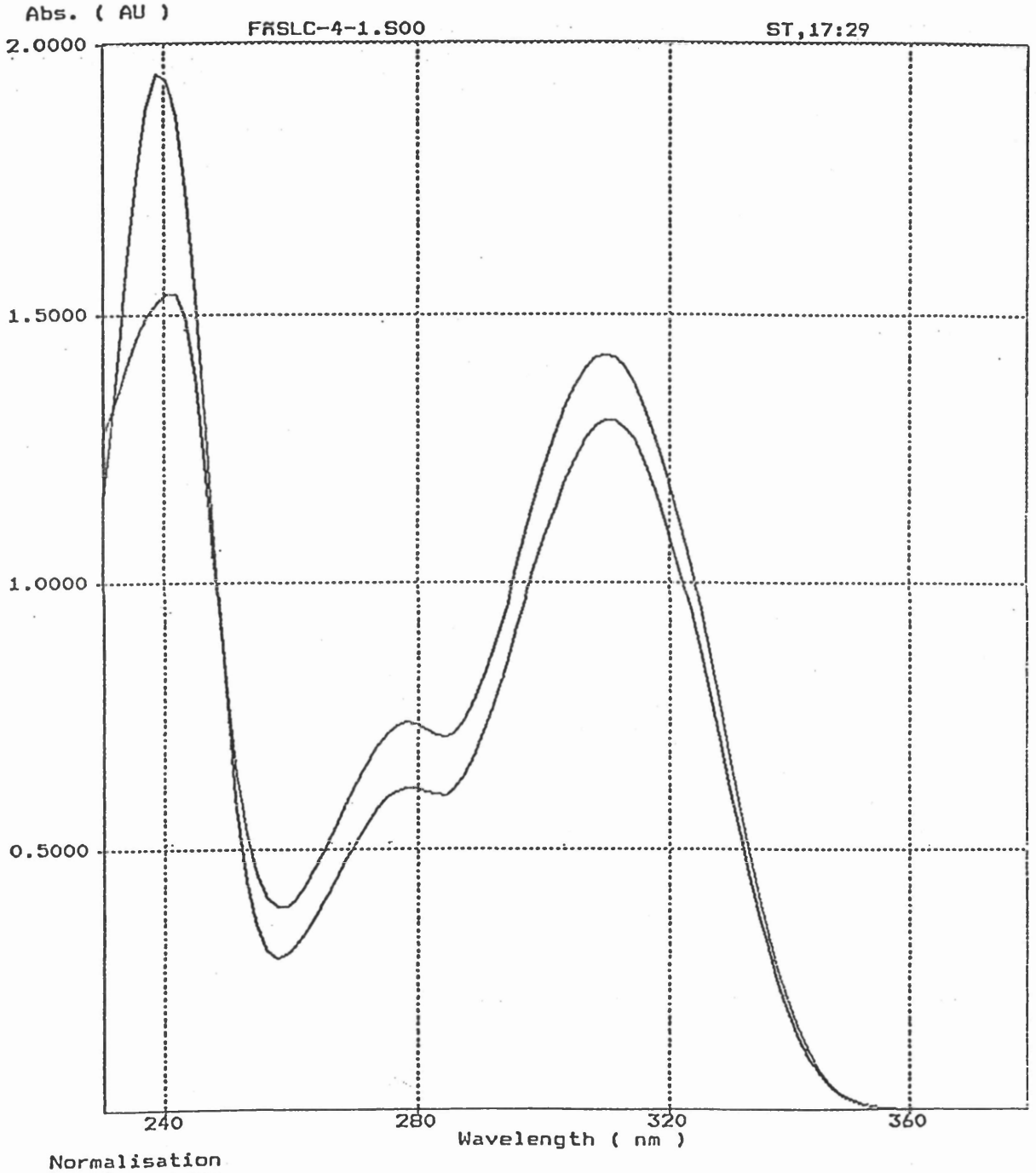
Sol.A (544 μg) was incubated in 5 ml of 0.5 M NH_4OH solution. after incubation for an hour at 50 °C, the hydrophobic compounds were extracted by solid phase extraction, eluted in acetonitrile (2 ml) and analysed by HPLC. The quantity of new compound was measured by using external standards of known concentrations of Sol.C during integration on HPLC and assuming an extinction coefficient of 7,300 at 320 nm.

Table 4.7. Recovery of Sol.A (μg) after incubation at 37 °C for 72 hours in basal medium and basal mineral medium without Na_2HPO_4

Treatments	Mean		% recovery of starting material
Control (544 μg of Sol.A + water [5 ml])	297.1 ± 56.5	A	54.6
Sol.A (544 μg) + Basal medium (5 ml)	230.2 ± 25.7	A	42.3
Sol.A (544 μg) + Basal mineral medium without Na_2HPO_4: 5 ml	130.7 ± 11.7	B	24.0

Sol.A (544 μg) was incubated in 5 ml of basal medium and basal mineral medium. After incubation for an hour at 50 °C, the hydrophobic compounds were extracted by solid phase extraction, eluted in acetonitrile (2 ml) and quantified by HPLC. ANOVA showed that $P<<0.001$. The Least Significant Difference (LSD) test with alpha set at 0.05, which gave a LSD value of 72.95 was used to examine differences within the data set. Means followed by the same letter are not significantly different at the 95 % confidence level.

Fig. 4.10. The UV spectrum of a new compound similar to SLC-4 formed when Sol.A was incubated in 0.5M NH_4OH solution, compared to SLC-4 (demethylated Sol.A). Note that comparison of the spectra by the method of least squares gave a match of 95.44%.



Normalisation
Least squares

Values
22.023 95.44%

4.3.7. Effect of glutathione (GSH) on Sol.A

4.3.7.1. Effect of glutathione (50 mM) in Tris HCl buffer (10 mM: pH 8) on Sol.A incubated for 16 hours at 25 °C in dark

When Sol.A was incubated in 10 mM Tris HCl buffer for 16 h recovery of the compound was 82.5% but 8.9% when the buffer contained 50 mM glutathione (Table 4.8). Most of the loss (69.5%) occurred within the first hour.

Table 4.8. Effect of glutathione (50 mM) in Tris HCl buffer (10 mM) on Sol.A incubated for 16 hours at 25 °C

Treatments	Recovery of Sol.A (µg)				% recovery of starting material
	R1	R2	R3	Mean	
Control (10 mM Tris HCl buffer [2 ml]) + Sol.A (272 µg)	237.4	210.9	224.5	224.3 ± 13.2	82.5
Glutathione 50 mM in 10 mM Tris HCl buffer (2 ml) + Sol.A (272 µg)	22.80	28.9	20.8	24.2 ± 4.2	8.9

Sol.A (272 µg) was incubated in 2 ml of 10 mM Tris HCl buffer containing 50 mM glutathione at 25 °C in the dark for 16 hours. After incubation the hydrophobic compounds were extracted by solid phase extraction, eluted in acetonitrile (2 ml) and analysed by HPLC.

4.3.7.2. Isolation of a Sol.A-glutathione conjugate

Two spots appeared on TLC plates with lower Rf values of (0.74 ± 0.01) and (0.64 ± 0.02) than those of Sol.A (0.92 ± 0.01) when the compound was incubated with glutathione (Fig. 4.11). These spots were absent in controls when Sol.A was incubated in Tris HCl buffer only. After purification the new compound SCL-5 which had Rf value of 0.74 ± 0.01 appeared on HPLC with a spectrum similar to that of Sol.C having peaks at λ_{max} 321, 289 and 248 nm. However, its retention time was much less (192.6 seconds: Fig. 4.12). On superimposition, the spectrum of SCL-5 over the range 230-390 nm gave a match of 83.0 % with that of Sol.C. The spectrum of compound of the second spot SCL-6 with Rf value 0.64 could not be observed on HPLC owing to its very low concentration.

2,4 dinitrophenylhydrazine reacted with SCL-5 and SCL-6 and also with Sol.A but did not react with glutathione.

4.3.7.3. Mass spectrometry of Sol.A-glutathione conjugate

Mass spectrometry of Sol.A and glutathione showed molecular ions of 302 and 308, respectively (Fig. 4.8 and 4.13). When Sol.A was incubated with glutathione in Tris-HCl buffer at pH 8.0 overnight (section 4.2.3.3) a product was obtained with a molecular ion of 606 (Fig. 4.14) which when accurately measured gave a mass of 606.214300 corresponding to a formula of $C_{28}H_{36}N_3O_{10}S$ (calculated 606.212142) and suggesting the conjugate as shown in Fig. 4.15. This, however, is 1 dalton greater than expected from the mass spectrum. One possible interpretation of this result is that the protons of the two terminal carboxyl groups on the glutathione moiety of the molecule are lost and the molecular ion is for M + 1 of this species as usually obtained for fast atom bombardment mass spectrometry.

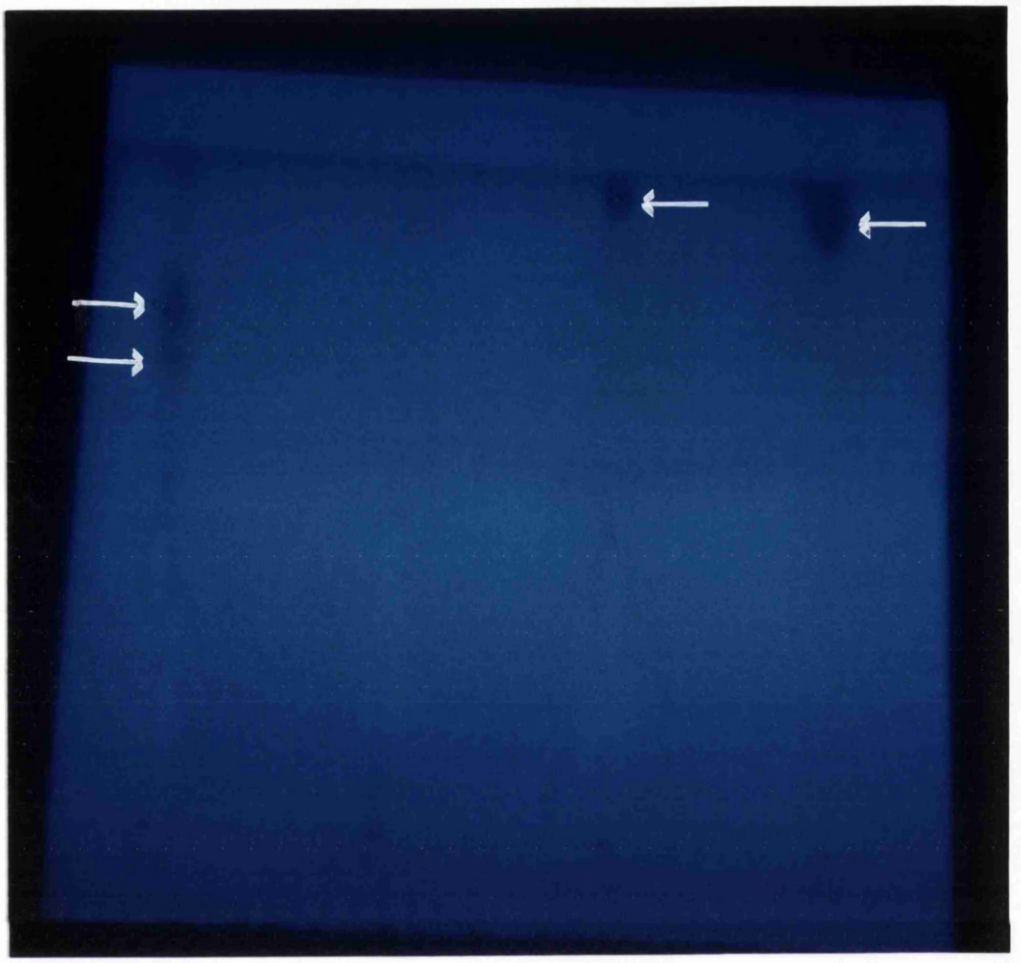
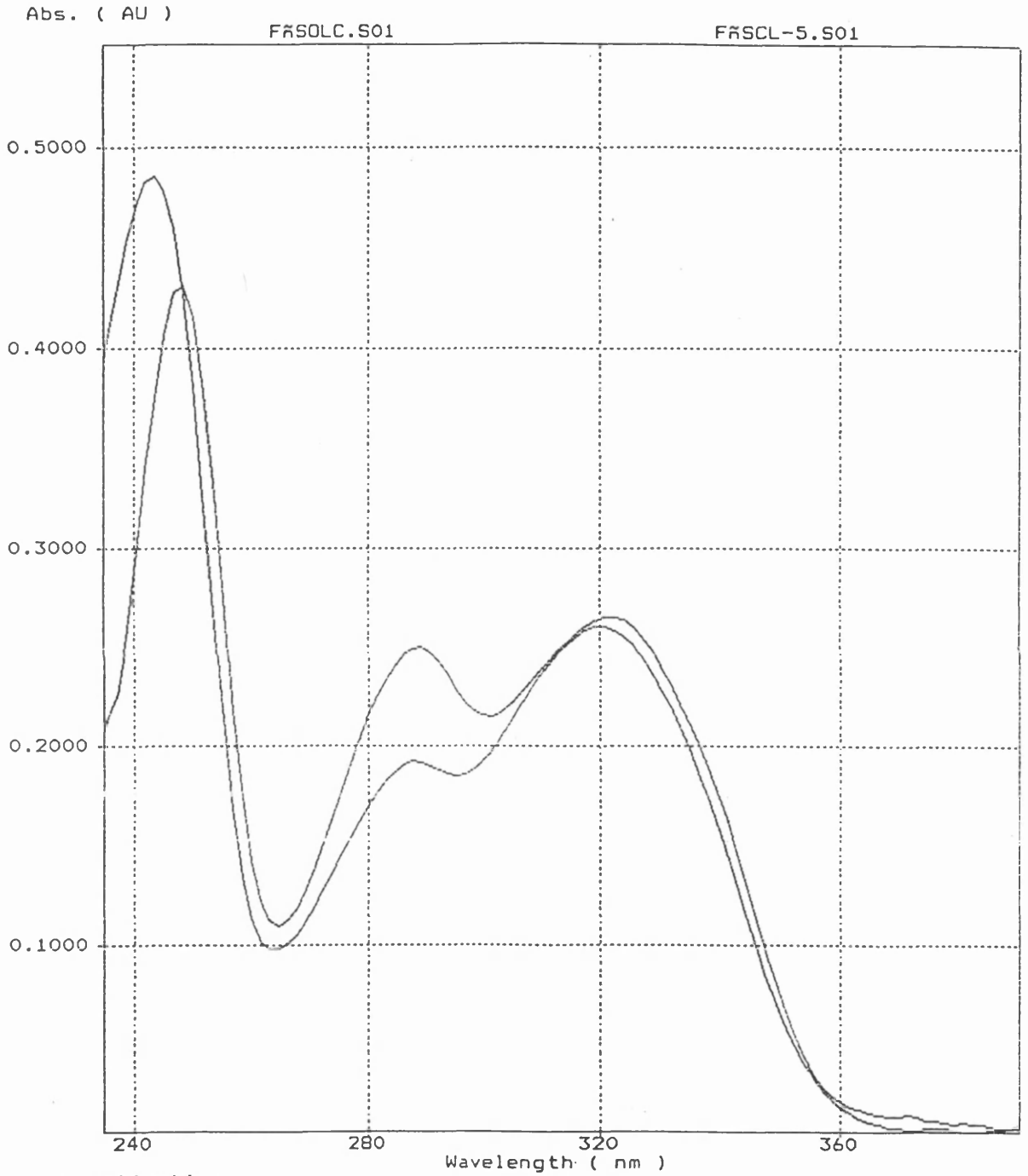


Fig. 4.11. Thin Layer Chromatography of Sol.A-glutathione conjugate. **Lane 1)** After incubation Sol.A (272 µg) in 2 ml Tris HCl buffer (10 mM) containing 500 µM GSH. Note the spots of SCL-5 and SCL-6 with Rf values 0.74 and 0.64, respectively (arrowed) may be caused by Sol.A-glutathione conjugates. Plates were developed in buta-1-ol/acetic acid/pyridine/water (15:3:10:12 v/v/v).). **Lane 2**), Control, where Tris HCl buffer (10 mM) containing 500 µM GSH was spotted. **Lane 3**), Control, where Sol.A was incubated in Tris HCl buffer without GSH (note spot with Rf value 0.94 [arrowed] is for Sol.A. **Lane 4**) Note spot with Rf value 0.92 (arrowed) is for standard Sol.A.

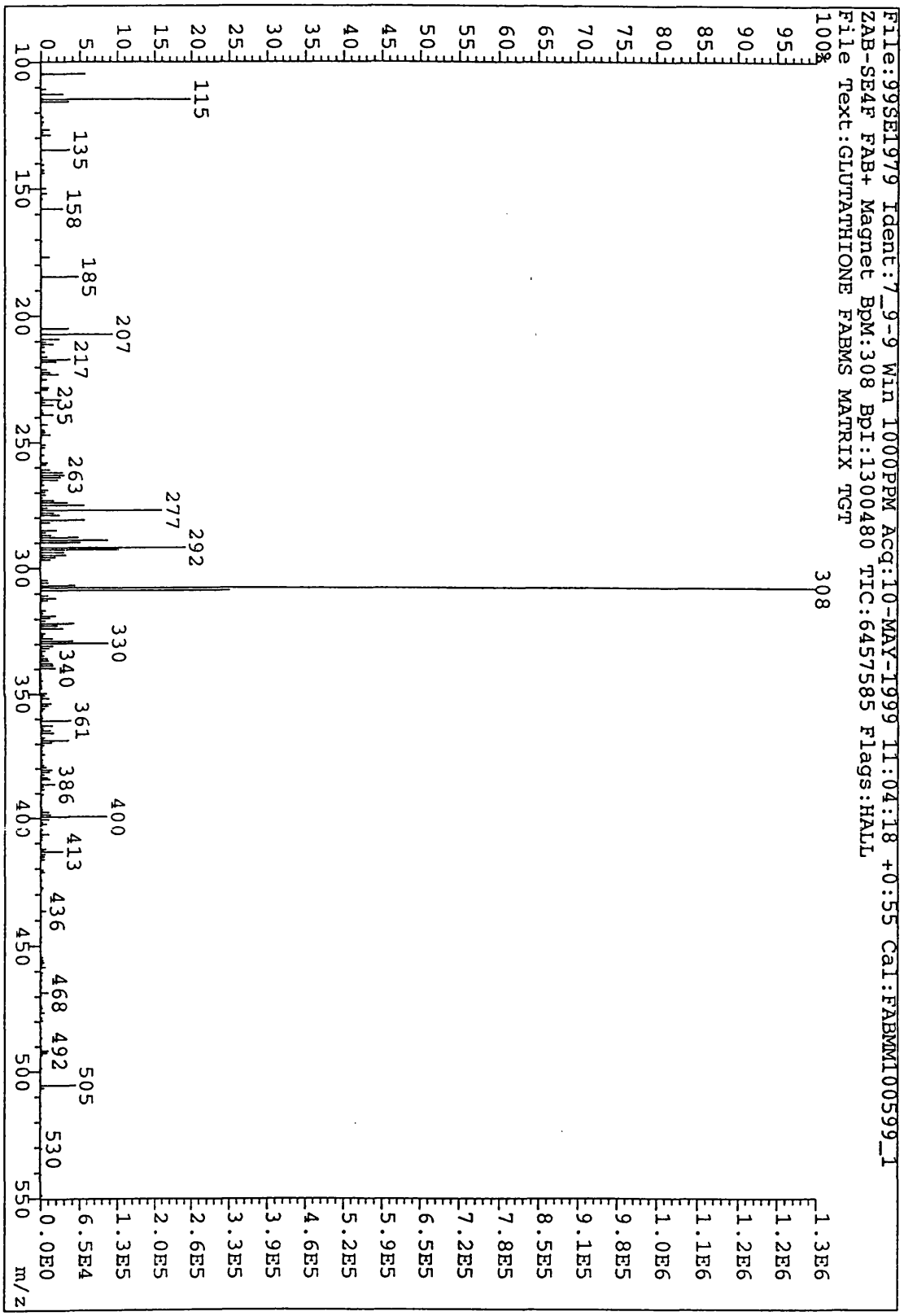
Fig. 4.12. The UV spectrum of SCL-5 when superimposed on that of Sol.C had a match of (83.0 %).



Normalisation
Least squares

Values
2.9764 83.04%

Fig. 4.13. Mass spectrum of glutathione.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ZAB-SE4F FAB+ Magnet BpM:308 BpI:1300480 TIC:6457585 Flags:HALL
File Text:GLUTATHIONE FABMS MATRIX TGT
100%
308
115
135
158
185
207
217
235
263
277
292
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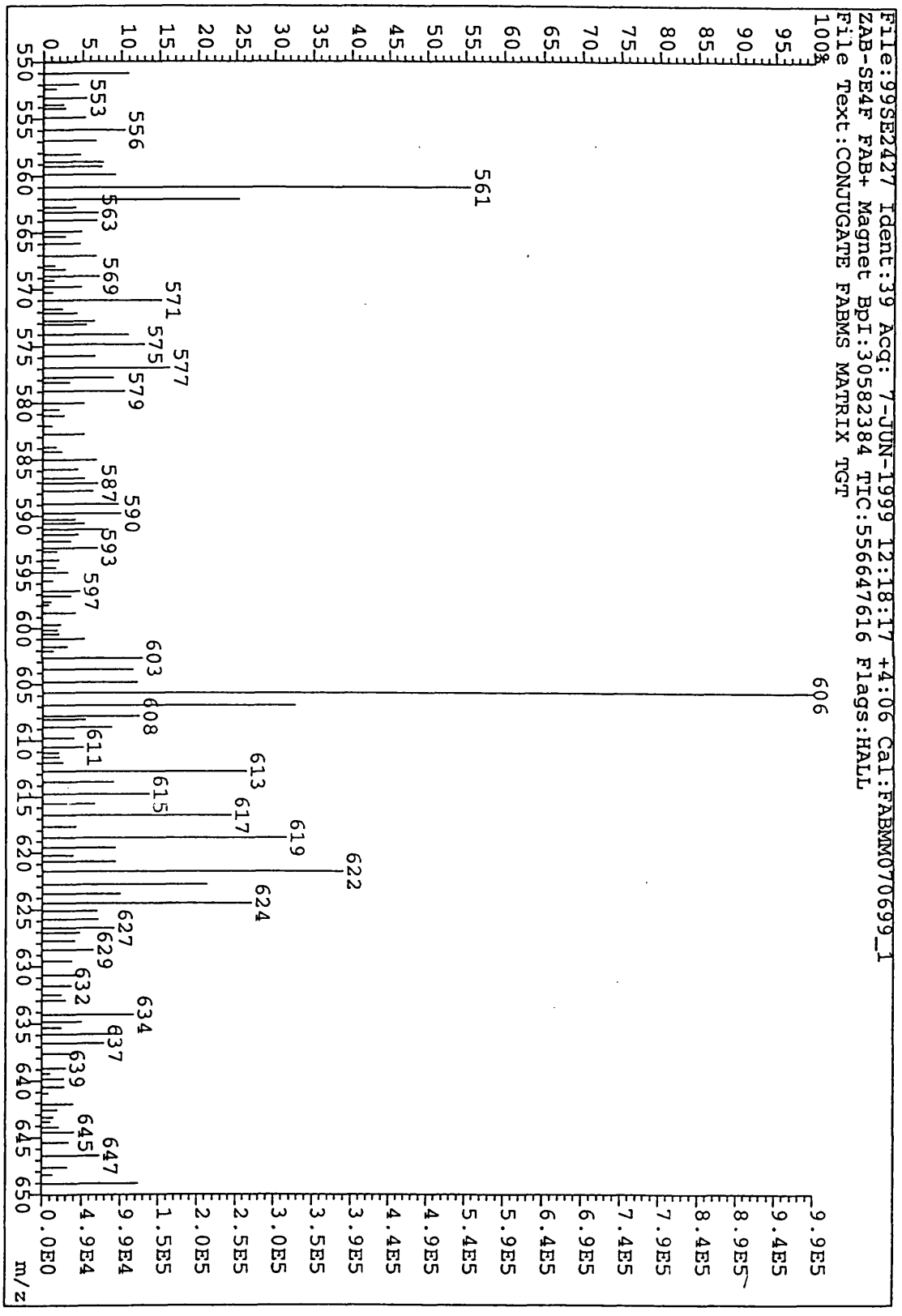
340
361
386
400
413
436
468
492
505
530
m/z

Fig. 4.14. Mass spectrum of Sol.A-glutathione conjugate.


File:99SE2427 Ident:39 Acq: 7-JUN-1999 12:18:17 +4:06 Cal:FABMM070699_1
ZAB-SE4F FAB+ Magnet BpI:30582384 TIC:556647616 Flags:HALL
File Text:CONJUGATE FABMS MATRIX TGT
100%
606
561
556
553
563
569
571
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603
608
611
613
615
617
619
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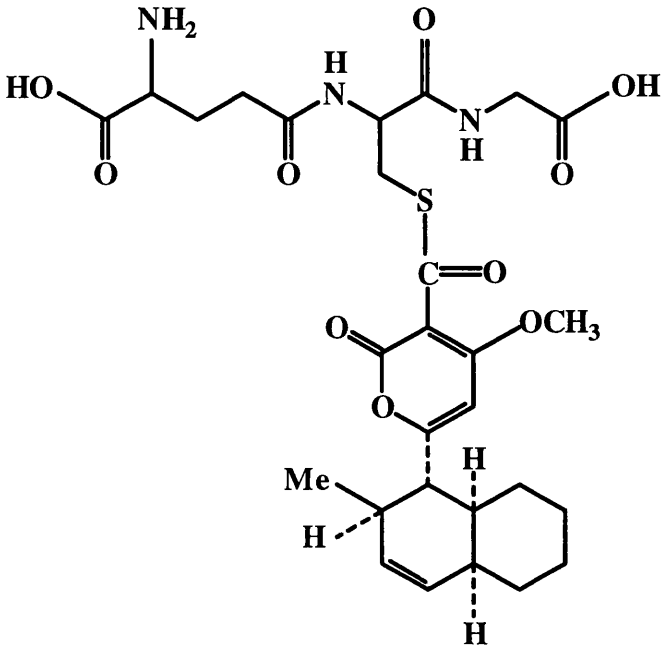


Fig. 4.15. Structure of Sol.A-glutathione. Sol.A was incubated with glutathione in Tris-HCl buffer (pH 8) overnight (section 4.2.3.3), a product was obtained with a molecular ion of 606 which when accurately measured gave a mass of 606.214300 corresponding to a formula of $C_{28}H_{36}N_3O_{10}S$ (calculated 606.212142) and suggesting the above structure of the conjugate.

4.3.7.4. Effect of different concentrations of glutathione on phytotoxicity of Sol.A to plant cells

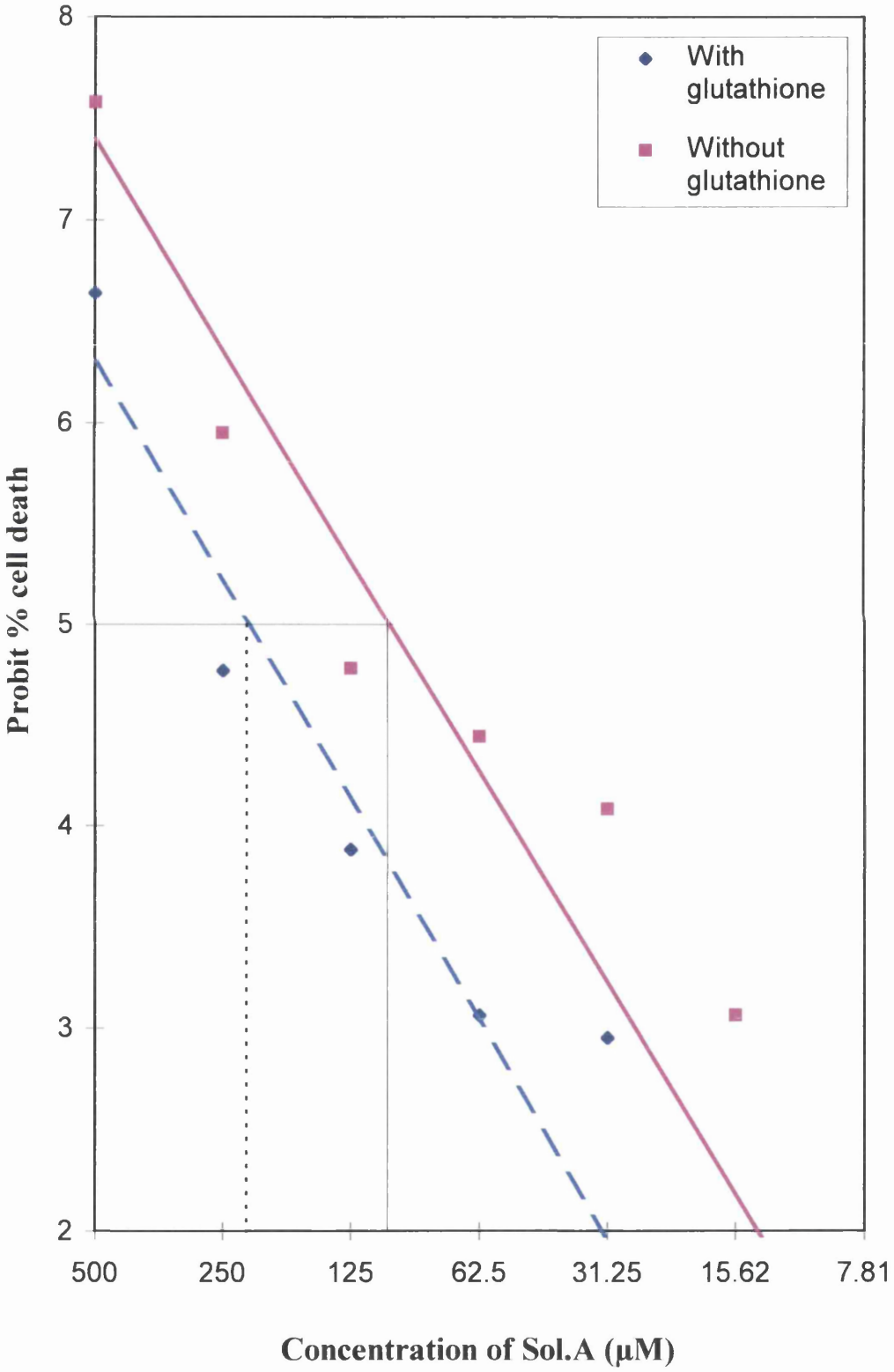
When different concentrations of glutathione were included in an assay with 0.414 mM Sol.A which killed all the cells (a concentration about 2.4 times greater than the average LD_{50} value) only the highest concentration of glutathione tested (50 mM) reduced cell death to less than 50%. Holding buffer containing glutathione (50 mM) did not affect the viability of cells.

4.3.7.5. Toxicity of different concentrations of Sol.A to plant cells incubated in holding buffer with or without glutathione

When the toxicity of Sol.A was determined, more than twice as much of the compound (215.3 μM) was required to reach the LD_{50} value in the presence of glutathione (50 mM) compared with its absence 100.7 μM (Fig. 4.16).

Fig. 4.16. Cell assay showing toxicity of Sol.A to cells isolated from chickpea leaflets (cv. ILC 3279) with and without glutathione. - - - - toxicity in the presence of glutathione, —— without glutathione. LD_{50} values arrowed on the x-axis.

Fig. 4.16. Cell assay showing toxicity of Sol.A with and without glutathione



4.4. CONCLUSIONS

Sterilizing solutions of Sol.A in basal medium was impractical since over 85% of the compound was lost. Sol.A incubated at low concentrations (544 μg/5 ml of media: 360 μM) for 72 h at 37 °C was found more stable in basal medium than in basal mineral medium without Na_2HPO_4 as the recoveries of the compound were 42.3 % and 24.0 %, respectively (Table 4.7). At higher concentrations (2000 μg of Sol.A/2 ml of basal media) recovery was better almost 76 % remaining after incubation at 37 °C for 96 h (Table 4.2). Sol.A was found also unstable in water when incubated (544 μg/5 ml of water) as the recovery of the compound was only 54.6 % after 72 h of incubation at 37°C (Table 4.7). When basal medium was supplemented with different carbon sources, lactic acid was found the least interfering with Sol.A (Table 4.2). The high reactivity of Sol.A can be explained by its possession of an aldehyde group (Ichihara *et al.*, 1983). These experiments were done to find a suitable medium for the growth of the micro-organism capable of degrading the solanapyrones without affecting the stability of the compound itself.

Basal mineral medium caused demethylation of Sol.A and yielded a new compound (SLC-4) which was isolated by thin layer chromatography. This compound was found to be 16.4 times less toxic than Sol.A in the cell assay. Further experiments showed that out of the six constituents of the basal mineral medium, Na_2HPO_4 and NH_4Cl were the most reactive causing 76.5% and 13% losses, respectively. Incubation of Sol.A in NH_4OH yielded a compound that was very similar to SLC-4 as its UV spectrum had a match of 95.4% when superimposed on that of SLC-4 (Fig. 4.10).

A rapid reduction in the recovery of Sol.A (69.5 %) was observed when the toxin was incubated with glutathione and two new compounds, SCL-5 and SCL-6 with Rf values of 0.74 and 0.64, respectively on TLC were observed. These compounds may be caused by the conjugation of the compound with glutathione. The mass spectrometry of the reaction mixtures of Sol.A and glutathione showed that Sol.A-glutathione conjugate had a molecular ion of 606 (Fig. 4.14) which when accurately measured gave a mass of 606.214300 corresponding to a formula of $C_{28}H_{36}N_3O_{10}S$ (calculated 606.212142) and suggesting the conjugate as shown in Fig. 4.15. An explanation of the mass found in the spectrometery is given in section 4.3.7.3 but its validity requires confirmation by further studies of the reaction.

Xenobiotics containing electrophilic sites (i.e. compounds that have centres of low electron density and can accept electrons to form a covalent bond) such as aldehydes are particularly hazardous. GSH acts as a cellular nucleophile through the thiol group of its cysteinyl residue and can undergo spontaneous or GST catalyzed conjugation with wide range of electrophiles, resulting in their detoxification. The products of GSH conjugation are usually more water-soluble than the compound and are either non toxic or less toxic (Coleman *et al.*, 1997a). Coleman *et al.* (1997b) were able to show conjugation of monochlorobimane (BmCl) with glutathione since the non fluorescent compound reacted with glutathione to yield a strong blue fluorescent conjugate, bimane-glutathione (Bm-SG). Similarly, in corn seedlings chloroacetanilide (a herbicide) tolerance was found to be due to its conjugation with glutathione (Breaux *et al.*, 1987). In these studies, only a concentration of 50 mM glutathione (the highest tested) reduced the death of chickpea cells to <50% when treated with a concentration of Sol.A that was 100% lethal in the absence of glutathione (section 4.3.7.4.). In another assay in which a concentration range of Sol.A was tested in the presence of 50 mM glutathione, toxicity of the compound was reduced by a factor of 2.13 (section 4.3.7.5.).

Since glutathione was found to react with Sol.A, forming a conjugate and reducing its toxicity to cells isolated from chickpea leaflets, it became of interest to know whether glutathione levels and GST activity vary among various chickpea cultivars and whether such variation might explain the variation in sensitivity among cultivars to the compound demonstrated in Chapter 3, a subject to be investigated in the next chapter.

CHAPTER 5

BIOCHEMICAL REACTIONS OF THE SOLANAPYRONE TOXINS *IN PLANTA*

5.1. INTRODUCTION

Although *A. rabiei* produces solanapyrones A, B and C in culture, only Shahid and Riazuddin (1998) claimed to have found Sol.C in infected plants. Hohl *et al.* (1991), for example, did not find any of the three compounds. Similarly, the tree pathogen, *Heterobasidion annosum*, produces a series of compounds with phytotoxic activity in culture, in particular, fomajorin, fomajorin S and D, fomannosin, dihydrobenzofuran fomannoxin but up to now only fomannoxin has been detected *in vivo* and isolated from naturally infected wood (Heslin *et al.*, 1983). Recently, fomannoxin was found to be metabolised by cell cultures of *Pinus sylvestris* to fomannoxin alcohol and subsequently to fomannoxin acid β-glucoside both of which are less toxic than fomannoxin (Zweimuller *et al.*, 1997).

Since there are precedents for the metabolism of toxins by plants which explain the inability to extract them from plants infected by toxigenic organisms, experiments were conducted in order to determine how chickpea might metabolise solanapyrone A, the most toxic of the solanapyrone toxins, and Sol.B. The biochemical reactions of Sol.A and Sol.B were determined by incubating the compounds with cell suspensions and Sol.A was also

incubated with a protein preparation of chickpea shoots. Attempts were also made to reisolate Sol.A and Sol.B from plant shoots after they had been allowed to take up these compounds.

As discussed in the last chapter, the reduced glutathione (GSH)-glutathione S-transferase enzyme (GST) system is a mechanism by which plants detoxify xenobiotics (Coleman *et al.*, 1997a; Lamoureux *et al.*, 1991). Furthermore, evidence was presented for this mechanism in the detoxification of Sol.A by chickpeas. Accordingly, the concentration of GSH and activity of GST in a range of chickpea cultivars were determined in order to ascertain if there were any correlation between them and the sensitivity of the cultivars to Sol.A.

Herbicides safeners prevent herbicide damage in cereals such as maize and wheat and some dicotyledonous species such as soybean by enhancing the levels of GST and GSH. It was therefore of interest to determine whether chickpea was similarly affected and whether safener treated plants were less sensitive to Sol.A.

5.2. MATERIALS AND METHODS

5.2.1. Incubation of Sol.A and Sol.B with cell suspensions

Cells were isolated from chickpea (cv. ILC 3279: 3 weeks old) as previously described and adjusted to $\approx 2.25 \times 10^5$ cells/ml (section 3.2.3). Ethanolic solutions of Sol.A and Sol.B were placed in conical flasks (25 ml) and, after evaporation of the ethanol, cell suspension (5 ml: viability 68%) was added to each flask. The final concentration of solanapyrones was 360 μM. Flasks were shaken gently for 30 seconds and incubated at

25°C in the dark for 24 h without agitation. Controls consisted of solanapyrones in holding buffer without cells or cells in holding buffer without solanapyrones. After incubation, the contents of the flasks were centrifuged at 10,000 *g* for 2 min. and the supernatants were transferred to universal bottles. The incubation flasks were washed out with methanol (3 x 0.5 ml) in order to remove all traces of the solanapyrones and the washings were added to the universal bottles. After dilution with distilled water to 25 ml, the solutions were fractionated on end-capped Isolute cartridge (1g: C18), eluted in acetonitrile (2 ml) and quantified by HPLC as described in sections 2.2.2 and 2.2.6.

5.2.2. Incubation of Sol.A and Sol.B with plant shoots

Sol.A and Sol.B were placed in separate glass vials (58 x 17 mm [diam]). After evaporation of the ethanol, distilled water (4 ml) was added to each vial and vortexed for 3 min. to give a 450 µM solution. Weighed shoots of chickpea (ILC 3279: 3 weeks old) were placed in each vial and incubated in a greenhouse at 25 ± 2 °C for 24 h. To see the effect of toxins on symptom development, shoots were incubated in toxin solutions for 72 hours. Transpiration was aided by an electric fan. Shoots incubated in water served as controls. After incubation the shoots were weighed again and the contents left in each vial were transferred to universal bottles. Incubation vials were washed out with methanol (3 x 0.5 ml) and washings were added to the universal bottles. After dilution with distilled water to 25 ml, hydrophobic compounds were extracted by end-capped Isolute cartridges (1g: C18), eluted in acetonitrile (2 ml) and quantified by HPLC (section 2.2.2 and 2.2.6).

5.2.2.1. Recovery of the solanapyrones from shoots

Shoots were cut into small pieces with scissors, homogenized in a Sorval Omni-mixer with 80% ethanol (3 x 10 ml) for 5 min. The homogenate (30 ml) was centrifuged at 3000 *g* for 5 minutes and the supernatant evaporated to dryness on a rotary evaporator at 30°C. Residues were dissolved in methanol (1.5 ml) and transferred to universal bottles. After dilution with distilled water to 25 ml, hydrophobic compounds were extracted in acetonitrile (2 ml) by solid phase extraction (section 2.2.2) and quantified by HPLC (section 2.2.6).

5.2.2.2. Purification of a new compound (TLA-1) isolated from plant shoots incubated in Sol.A

The acetonitrile preparations (section 5.2.2.1) were concentrated on a rotary evaporator to 500 µl and samples were spotted on silica TLC plates (Silica gel 60 F_{254}, Merck, Germany). The plates were developed in cyclohexane/dichloromethane/ethyl acetate (1:1:1 v/v/v) and, after evaporation of the solvent in a fume cupboard, were observed under short wavelength UV light. Spots at Rf 0.81 were scraped from the TLC plates, placed in Eppendorf tubes (1.5 ml) and vortexed in acetonitrile (3 x 1 ml). Supernatants were evaporated to dryness on a rotary evaporator and the residues dissolved in the same solvent (50 µl). Samples (20 µl) were spotted on TLC plates and plates were developed in cyclohexane, dichloromethane and ehtyl acetate (1:1:1 v/v/v/). TLC plates were also sprayed with 2,4 dinitrophenylhydrazine (section 2.2.7). In order to check for purity samples (20 µl) were run on HPLC (section 2.2.6).

5.2.3. Protein extraction from chickpea

Proteins were extracted using a modification of the method described by Mayer *et al.* (1987). Shoots of chickpea (cv. ILC 3279: 1g) from three week old plants were ground with liquid N_2 in a pestle and mortar. The resulting powder was transferred to a centrifuge tube (15 ml) and suspended in ice cold extraction buffer (14 ml) consisting of 10 mM phosphate buffer (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% mercaptoethanol and 1% sodium dodecyl sulphate. After mixing thoroughly by shaking the tubes for 15 min., they were centrifuged at 13000 *g* at 4°C for 6 min. Supernatants were stored on ice until use.

5.2.4. Incubation of protein extract with solanapyrone A

An ethanolic solution of Sol.A was placed in glass vials (58 x 17 mm [diam.]) and, after evaporation of ethanol, protein preparation (2 ml: section 5.2.3) was dispensed in each glass vial; the final concentration of Sol.A was 180 µM. Contents were vortexed for 2 minutes, passed through filters (0.22 µm) and incubated at 25°C for 24 hours in the dark. Sol.A incubated in extraction buffer served as control. After incubation, contents of the vials were transferred to universal bottles. The incubation vials were washed out with methanol (3 x 0.5 ml) in order to remove all traces of the solanapyrone and the washings were added to universal bottles. After dilution with distilled water to 25 ml the hydrophobic compounds were subjected to solid phase extraction (section 2.2.2) and quantified by HPLC (section 2.2.6).

5.2.5. Measurements of glutathione in chickpea

5.2.5.1. Extraction of glutathione from leaflets

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured using modifications of the methods of Anderson (1985) and Coleman *et al.* (1997b). Leaflets (0.5 g) of chickpea cultivars (21-25 days old) were pulverized with liquid nitrogen in a pestle and mortar. The resulting powder was suspended in extraction buffer (2 ml) consisting of 5% (w/v) sulphosalicylic acid (SSA: Sigma) and 6.3 mM diethyltriaminepentaacetic acid (DETAPAC: Sigma), vortexed for 45 seconds, kept on ice for 10 minutes, centrifuged for 12 minutes at 10,000 *g* and further centrifuged at 10,000 *g* for 3 minutes at 4°C to remove floating particles. The supernatant was used for the determination of glutathione.

5.2.5.2. Measurement of total glutathione (GSH+GSSG)

Supernatant (50 µl: section 5.2.5.1) was added to 750 µl of 0.143 M potassium phosphate buffer containing 6.3 mM (DETAPAC: pH 7.5), 100 µl of 6 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and 100 µl of 2.1 mM β-nicotinamide adenine dinucleotide phosphate in the reduced form (β-NADPH). The reaction was initiated by the addition of 25 µl of glutathione reductase (20 U/ml: Sigma) and the change in absorption was measured at 412 nm for 6 minutes on a spectrophotometer.

A standard curve with a concentration range of 0-45 µM GSH was drawn and used to determine the concentration of total glutathione (GSH + GSSG) which was expressed as n moles/g fresh wt. of leaflets (Coleman *et al.*, 1997b).

5.2.5.3. Measurement of oxidized glutathione (GSSG)

Supernatant (400 µl: section 5.2.5.1) was mixed with 8 µl of 2-vinylpyridine (2VP) and 40 µl of triethanolamine (TEA: Sigma). The mixture was vortexed for 15 seconds and incubated at 25 °C for 1 h.

GSSG was determined as described for total glutathione (section 5.2.5.2) from a standard curve with a concentration range from 0-40 µM of GSSG. The amount of GSSG was calculated and expressed as n mole/g fresh wt. of leaflets.

5.2.5.4. Measurement of reduced glutathione (GSH)

The amount of GSH was calculated by subtracting the amount of GSSG from the total amount of glutathione GSSG + GSH and also expressed as n mole/g fresh wt of leaflets.

5.2.6. Measurement of glutathione S-transferase (GST) activity in chickpea cultivars

5.2.6.1. Extraction of GST from leaflets

GST was extracted using a modification of the method of Hunaiti and Ali, (1990). Leaflets (0.5 g) from each cultivar were ground in liquid nitrogen in a pestle and mortar until a fine powder was obtained. All further steps were carried out at 0-4°C. The resulting powder was suspended in 0.1 M potassium phosphate buffer (pH 7.0: 0.5 ml), containing polyvinylpyrrolidone (PVP-40: 5%: Sigma) vortexed for 1 minute and centrifuged at 15000 *g* for 15 min. After diluting the supernatant to 1 g of tissue/ml with extraction

buffer, it was passed through a 0.45 μm filter (Gelman Sciences, USA) to remove floating particles and the filtrate was tested for GST activity.

5.2.6.2. Enzyme assay

GST activity was measured by the formation of the conjugate of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) determined spectrophotometrically at 340 nm essentially according to the procedures of Simons and Jagt (1977) and Habig *et al.* (1974). The assay mixtures (3 ml) consisted of 0.1 M phosphate buffer (pH 6.5: 2,865 μl), 1-chloro-2,4-dinitrobenzene (CDNB: Sigma: 30 μl in ethanol, final concentration 0.1 mM), glutathione (GSH) 75 μl (final concentration 2.5 mM) and the reaction was started by adding the enzyme solution (30 μl). A complete assay mixture without enzyme served as a control. The reaction was monitored spectrophotometrically by the increase in absorbance at 340 nm for 6 min. (ϵ_{340} of the conjugate = 10 mM^{-1} cm^{-1}) for 6 minutes (Mannervik and Guthenberg 1981). Units of activity were calculated where one unit of activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of S-2,4-dinitrophenylglutathione per minute at room temperature. Total units of enzyme activity were calculated by subtracting the units of activity obtained in controls without enzyme.

5.2.7. Glutathione and GST activity in shoots treated with Sol.A

Solanapyrone A (60.4 µg in 2.13 µl ethanol) was vortexed with 2 ml water in polypropylene conical tubes (115 mm x 30 mm [diam.]) to give a 100 µM solution. Shoots (0.75 g) of chickpea (cv. ILC 3279: 3 weeks old) were placed in the tubes and were allowed to take up 1.5 ml of the solution (= 45.3 µg Sol.A) while being incubated in a green house at 23 ± 2 °C (5 to 6h). Shoots were transferred to tubes containing only distilled water (25 ml) and incubated for a further 96 h under the same conditions. The water level was maintained throughout the incubation period. Shoots incubated in distilled water without Sol.A served as controls. After the incubation period the parts of shoots covered by Sol.A solution or water were discarded and the remaining 0.5 g was ground in liquid nitrogen and used for the estimation of glutathione and GST activity. Glutathione content and GST activity were measured as described in sections 5.2.5 and 5.2.6 except that the plant material consisted of complete shoots rather than leaflets.

5.2.8. Induction of GST activity (units) in shoots treated with dichlormid

Induction of GST activity was determined as in section (5.2.7) except that shoots were placed in aqueous solutions of dichlormid (Fig. 5.1: 100 and 200 µg/ml prepared from a stock solution of 10 mg/ml in acetone). Shoots were allowed to take up 1.5 ml solution (=150 µg or 300 µg/shoot). Shoots incubated in water served as controls. Glutathione levels and GST activity were determined as described in sections 5.2.5 and 5.2.6.

5.2.8.1. Sensitivity of cells to Sol.A treated with dichlormid

Cells were isolated from leaflets of shoots treated with dichlormid as described in section 5.2.8 and tested for their sensitivity to Sol.A as in section 3.2.3 and 3.2.4. Cells isolated from leaflets obtained from shoots incubated in water served as controls.

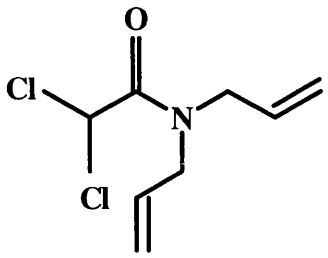


Fig. 5.1. Structure of dichlormid

5.3. RESULTS

5.3.1. Recovery of Sol.A and Sol.B incubated with cell suspensions of chickpea

When Sol.A and Sol.B were incubated in holding buffer at 25°C for 24 h, recovery was 46.9 % and 70.3 %, respectively showing that incubation in holding buffer was itself causing loss of the compounds but that this was less for Sol.B. Incubation of the compounds with cell suspensions caused further losses of 12.8% and 16.4% of Sol.A and Sol.B, respectively, and these losses were significant ($P < 0.05$: Tables 5.1 and 5.2).

5.3.2. Incubation of Sol.A and Sol.B with plant shoots

When chickpea shoots were incubated in Sol.A for 24 h, a highly significant loss of 16.3% in weight occurred. In contrast, in controls where shoots were incubated in water a significant increase in weight of 17.5% was observed (Table 5.3).

Only 391.7µg out of 544 µg (72.0%) of Sol.A remained after incubation in water for 24 h but a further significant ($P<0.01$) 175.6 µg of the compound (32.3%) was removed over the same period when incubated with chickpea shoots. No Sol.A was recovered from extracts of the shoots (Table 5.4).

When chickpea shoots were incubated in Sol.B, a non-significant loss of 7 % in weight occurred. In contrast, in controls where shoots were incubated in water a significant increase in weight of 16.4 % was observed (Table 5.5).

Out of 547 µg of Sol.B, 453.5 (82.9%) remained after incubation in water for 24 h but a further significant ($P<0.01$) loss of 234.0 µg of the compound (42.8%) occurred over the same period when the compound was incubated with chickpea shoots of which

22.0 μg (4% of the original starting material but 9.4% of the amount taken up by the shoots) was recovered from extracts of the shoots (Table 5.6).

To see the effect of toxins on disease symptoms, two types of experiments were conducted. In the first experiment, shoots were placed in Sol.A or Sol.B solutions (450 μM) and incubated for 72 h (section 5.2.2) while in the second one, shoots were allowed to take up 45.3 μg/shoot of Sol.A and further incubated in water for 96 h (section 5.2.7).

When shoots were incubated in Sol.A (544 μg/4ml H_2O: 450 μM) for 72 hours (section 5.2.2) the stems became shrivelled, brown and corky and the leaflets developed flame-shaped chlorotic zones. Controls incubated in water remained turgid and green (Fig. 5.2).

When shoots were allowed to take up 45.3 μg/shoot of Sol.A and incubated further for 96 h in water, breaking just below the first leaflet and bleaching at the base of the stems occurred. In controls, where shoots were placed in water, stems remained intact and green at the base (Figs. 5.3 and 5.4).

Incubation of shoots in Sol.B (547 μg/4 ml H_2O: 450 μM) for 72 h (section 5.2.2) produced different symptoms: leaflets became wilted, chlorotic, brown and appeared scorched whereas those of controls remained intact, turgid and green (Fig. 5.5).

5.3.2.1. Recovery of the solanapyrones from shoots incubated in the compounds

No Sol.A could be recovered from shoots incubated in the compound but a compound (TLA-1), not found in controls, was extracted. On HPLC TLA-1 had a retention time of 778.8 seconds and a UV spectrum with peaks at λ_{max} = 238 and 288 nm (Fig.5.6). When extracts were chromatographed on TLC plates an extra spot (Rf 0.81)

was also noticed under short wave-length UV light in those from shoots incubated in Sol.A (Fig. 5.9) which gave a brown colour when sprayed with 2,4 dinitrophenylhydrazine. When the spot was scraped from unsprayed plates and the compound eluted from the silica it was resolved into two peaks on HPLC. Compound 1 (TLA-1A) had a retention time of 725.4 seconds and the spectrum was a better than 95% match with that of TLA-1 (Fig. 5.7). In contrast, compound 2 (TLA-1B) had a retention time 1047 seconds and a UV spectrum with peaks at λ_{max} = 238, 278 and 310 nm which was a 97% match with that of SLC-4 (demethylated Sol.A, previously identified from samples of Sol.A incubated in mineral salts medium: Fig. 5.8: see Chapter 4).

5.3.3. Incubation of protein extract from chickpea with solanapyrone A

When Sol.A was incubated in extraction buffer for 24 h recovery of the compound was >80% but only 35% when the buffer included the proteinaceous extract from chickpea shoots ($P<0.01$: Table 5.7).

Table 5.1. Recovery of Sol.A (μg/flask) after incubation at 25°C for 24 hours in holding buffer or holding buffer containing cell suspension prepared from chickpea leaflets

Treatments	R1	R2	R3	Mean	% recovery of starting material
Control (544 μg of Sol.A + Holding buffer [5 ml])	283.4	246.2	235.8	255.1 ± 25.0	46.9
544 μg of Sol.A + Cell suspension in holding buffer (5 ml)	195.2	189.2	172.2	185.5 + 11.9	34.1

Sol.A (544 μg) was incubated in holding buffer containing cell suspension (5 ml: 2.25 x 10^5 cells/ml) for 24 h at 25°C. Sol.A was incubated in holding buffer in controls. Means of recovery of Sol.A from cell suspension compared with buffer alone differed significantly ($P<0.05$).

Table 5.2. Recovery of Sol.B (μg/flask) after incubation at 25°C for 24 hours in holding buffer or holding buffer containing cell suspension prepared from chickpea leaflets

Treatments	R1	R2	R3	Mean	% recovery of starting material
Control (547 μg of Sol.B + Holding buffer: 5 ml)	330.4	415.2	408.0	384.5 ± 47.0	70.3
547 μg of Sol.B + Cell suspension in holding buffer (5 ml)	296.2	264.8	321.2	294.3 ± 28.3	53.8

Sol.B (547 μg) was incubated in holding buffer containing cell suspension (5 ml: 2.25 x 10^5 cells/ml) for 24 h at 25°C. Sol.B was incubated in holding buffer alone in controls. Means of recovery of Sol.B in cell suspension compared with buffer alone differed significantly ($P < 0.05$).

Table 5.3. Weight of shoots of chickpea (ILC 3279) before and after incubation for 24 hours at 25 °C in H_2O (4 ml) containing Sol.A (544 µg)

Treatments	Wt. of shoots (mg)		Difference in weight after incubation
Shoots incubated in Sol.A (544 µg) in water (4 ml)	Before incubation	After incubation	(mg)
R1	560	470	- 90
R2	570	480	- 90
R3	590	490	- 100
Mean	573.3	480	- 93.3
SD	15.3	10.0	5.8
Control (shoots incubated in H_2O)			
R1	660	800	+140
R2	660	760	+ 100
R3	570	670	+ 100
Mean	630	743.3	+113.3
SD	52	66.6	23.1

+ = Increase in weight

- = Loss in weight

Statistical analysis was done using Student's t-Test of the means of two sets of paired observations. Mean of losses in weight owing to incubation of shoots in Sol.A differed significantly ($P<0.005$). In controls the mean of gain in weight of shoots also differed significantly ($P<0.05$).

Table 5.4. Recovery of Sol.A (μg) after incubation for 24 hours at 25 °C with chickpea shoots

Treatments	R1	R2	R3	Mean	% recovery of starting material
Control vials containing Sol.A 544 μg/4 ml of H_2O without plant shoots	418.4	374.2	382.6	391.7 ± 23.4	72.0
Sol.A remaining in vials after removal of plant shoots	246.8	180.1	221.0	216.1 ± 33.6	39.7
Sol.A extracted from plant shoots incubated in Sol.A	Nil	Nil	Nil	Nil	Nil

Sol.A 544 μg/4ml of water was incubated with chickpea shoots for 24h. In controls the compound was incubated in water without plant shoots. Sol.A was recovered from vials after removing the shoots but not from shoots placed in Sol.A solution. Means of the recovery of Sol.A differed significantly between controls and tests ($P<0.01$).

Table 5.5. Weight of shoots of chickpea (ILC 3279) before and after incubation for 24 hours at 25 °C in Sol.B (547 µg/4 ml of H_2O)

Treatments	Wt. of shoots (mg)		Difference in weight after incubation
Shoots incubated in Sol.B (547 µg) in water (4 ml)	Before incubation	After incubation	(mg)
R1	360	380	+ 20
R2	500	410	- 90
R3	420	410	- 10
Mean	426.7	400	- 26.7
SD	70.2	17.3	43.6
Control (shoots incubated in H_2O)			
R1	400	470	+ 70
R2	390	470	+ 80
R3	430	480	+ 50
Mean	406.7	473.3	+ 66.7
SD	20.8	5.8	15.3

+ = Increase in weight

- = Loss in weight

Statistical analysis was done using Student's t-Test of the means of two sets of paired observations. Difference in means of losses in weight of shoots owing to incubation in Sol.B did not differ significantly. In controls the mean of gain in weight of shoots differed significantly ($P<0.05$).

Table 5.6. Recovery of Sol.B (μg) after incubation for 24 hours at 25 °C with chickpea shoots

Treatments	R1	R2	R3	Mean	% recovery of starting material
Control vials containing Sol.B (547 μg/4 ml of H_2O) without plant shoots	433.7	442.2	484.6	453.5 ± 27.2	82.9
Sol.B remaining in vials after removal of plant shoots	268.4	199.6	190.6	219.5 ± 42.5	40.1
Sol.B extracted from plant shoots incubated in Sol.B	27.3	31.1	7.6	22.0 ± 12.6	9.4 (of Sol.B taken up)

Sol.B 547 μg/4ml of water was incubated with chickpea shoots for 24h. In controls the compound was incubated in water without plant shoots. Sol.B was recovered from vials after removing the shoots and also from shoots placed in Sol.B solution.

Means of the recovery of Sol.B between treatments differed significantly ($P<0.01$).

Table 5.7. Metabolism of Sol.A by a protein preparation from chickpea

Treatments	R1	R2	R3	Mean	% recovery of starting material
Control (Sol.A 272 µg in extraction buffer [2 ml] without plant proteins)	210.2	202.5	243.9	218.9 ± 22.0	80.5
Test (Sol.A [272 µg] incubated with plant proteins in extraction buffer [2 ml])	94.8	90.1	101.3	95.4 ± 5.6	35.1

Means of recovery of Sol.A in protein preparation compared with buffer only differed significantly ($P<0.01$). Sol.A (272 µg) was incubated with protein preparation from chickpea shoots (2 ml) in extraction buffer for 24 h at 25°C. In controls Sol.A was incubated in extraction buffer alone.

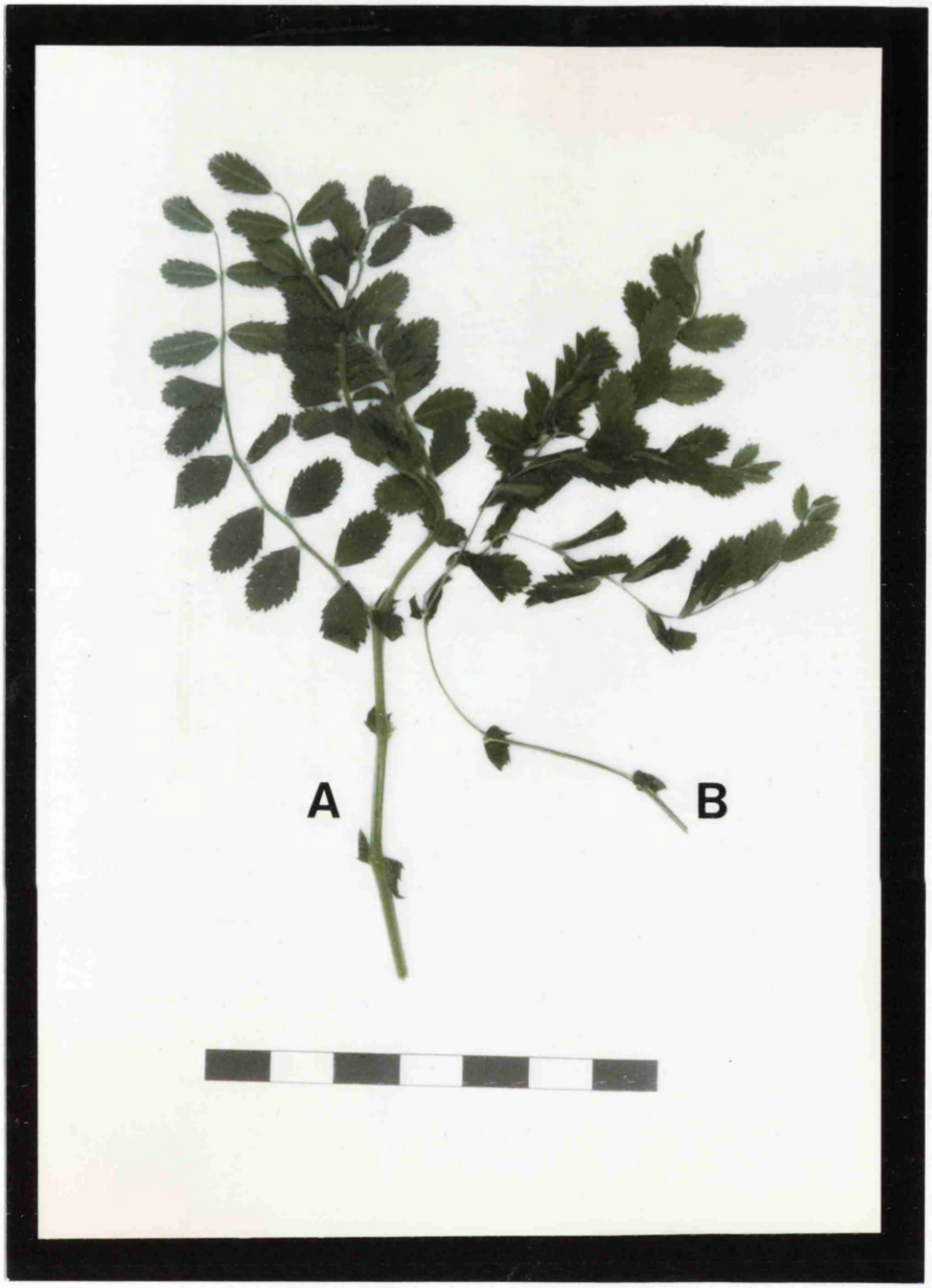


Fig. 5.2. Effect of incubating shoots in a solution of Sol.A. **A**) Shoots of chickpea cv. ILC 3279 incubated in water remained turgid. **B**) Stems of shoots incubated in Sol.A (544 µg/4 ml of H_2O) for 72 hours at 25 °C became shrivelled and flame-shaped discolouration appeared on the leaflets. Scale (1 small unit = 1 cm).

Fig. 5.3. Effect of incubating shoots in a solution of Sol.A and then in water. **A**) Shoots of chickpea cv. ILC 3279 incubated in water remained turgid and the bases of the stems remained green. **B**) Shoots of chickpea (ILC 3279) after taking up 45.3 µg of Sol.A and incubated further for 96 hours in water. Note breaking of stem just below the first leaflet and bleaching at the base.

Fig. 5.4. Shoots of chickpea (ILC 3279) after taking up 45.3 µg of Sol.A and incubated further for a 96 hours in water. Note breakage of stem just below the uppermost leaf.

Fig. 5.5. Effect of incubating shoots in a solution of Sol.B. **A**) Shoots incubated in Sol.B. (547 µg/4 ml of H_2O) for 72 hours at 25°C. Leaflets became wilted, chlorotic and appeared scorched. Note abscission of two leaflets. **B**) Shoot incubated in water, the leaflets remained intact, green and turgid. (Scale 1 small unit = 1 cm)

Fig. 5.6. The UV spectrum of compound TLA-1 extracted from shoots incubated in Sol.A.

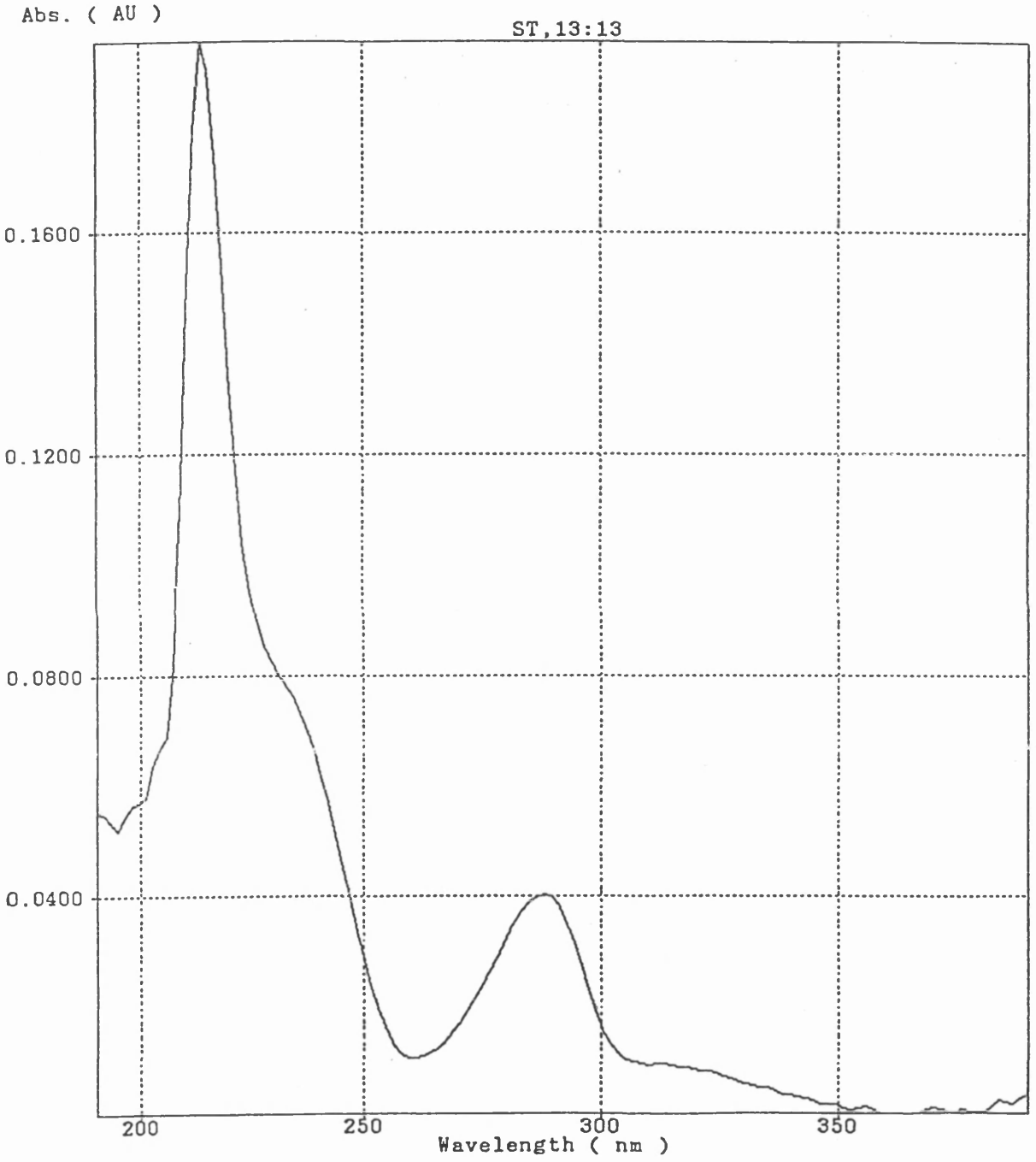
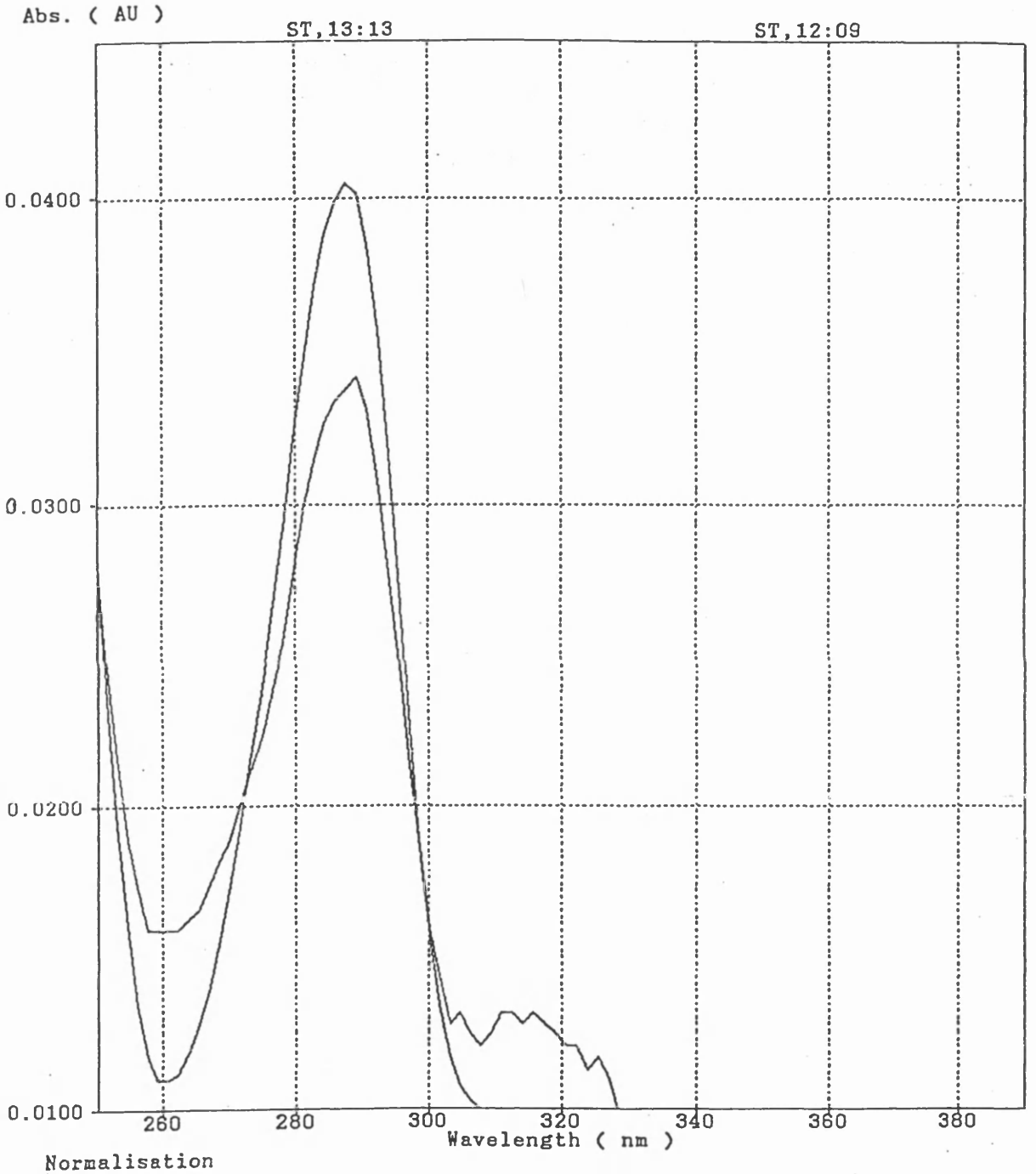

Abs. (AU)
ST,13:13
0.1600
0.1200
0.0800
0.0400
200
250
300
350
Wavelength (nm)

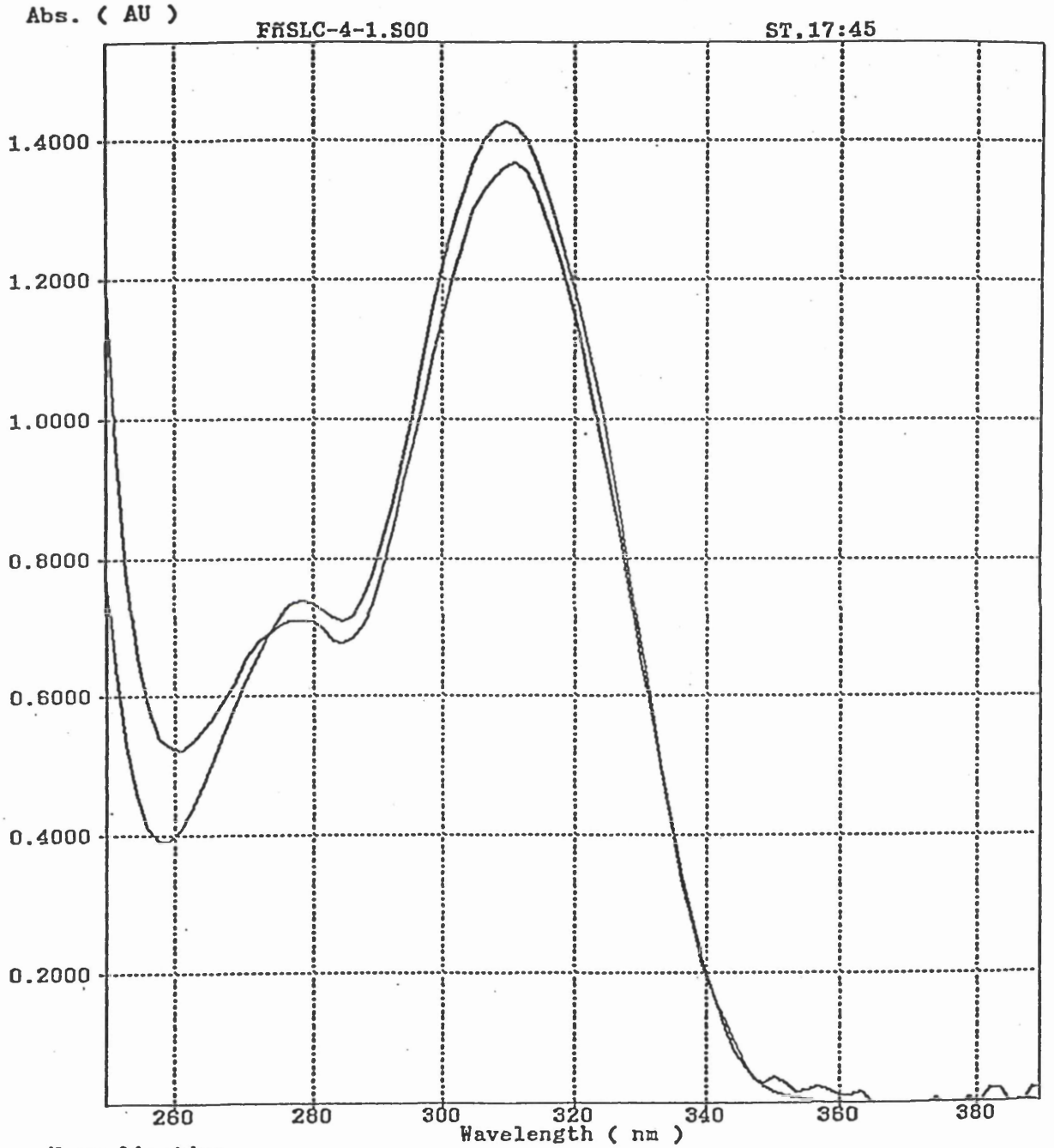
Fig. 5.7. The UV spectrum of TLA-1A superimposed on that of TLA-1 showing a match of 95.9 %.



Normalisation
Least squares

Values
3.7933 95.90%

Fig. 5.8. The UV spectrum of TLA-1B superimposed on that of SLC-4 and showing a 97.15 % match.



Normalisation
Least squares

Values
46.861 97.15%

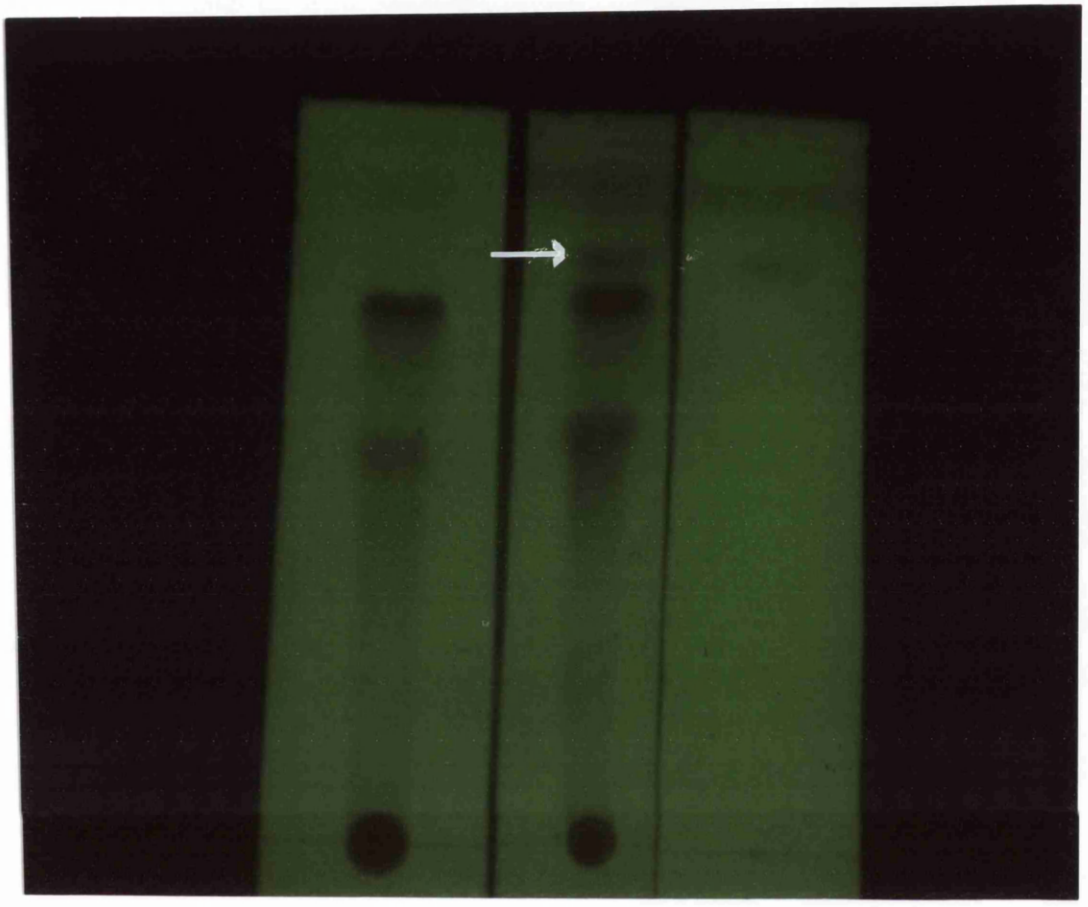


Fig. 5.9. Thin layer chromatograms of plant extracts viewed under short wavelength UV. **1**) extract from shoots incubated in water. **2**) extract from shoot incubated in Sol.A 544 µg/4 ml of water. Note the new spot with an Rf value of 0.81 (arrowed). **3**) purified preparation of the spot running at Rf 0.81(TLA-1). The TLC plate was developed in cyclohexane, dichloromethane and ethyl acetate (1:1:1 by v/v/v).

5.3.4. Levels of total, oxidized (GSSG) and reduced glutathione (GSH) in chickpea cultivars

Standard curves for reduced glutathione and oxidized glutathione were linear from 0 to 45 μM and 0 to 40 μM, respectively (Figs. 5.10 and 5.11). When higher concentrations of GSH were used, the reaction was initially fast but soon slowed giving a curve.

Measurement of reduced glutathione in chickpea cultivars showed that the difference of their means was highly significant ($P << 0.001$: Table 5.8). Cultivar AUG 424 had the least and Kasseb the most (200.7 ± 27.2 and 561.0 ± 112.9 n moles/g fresh wt. of leaflets, respectively: Fig. 5.12). Comparison of the means of reduced glutathione levels of the cultivars at 0.05% confidence separated the cultivars into six overlapping groups in which cultivars within a group did not differ significantly from each other (Table 5.8). Sensitivity to Sol.A was inversely related to GSH levels, cultivars that were least sensitive such as Kasseb and CM 72 having GSH concentrations that were 1.7 - 2.8 times greater than those of the most sensitive cultivars such as 6153 and AUG 424. When all cultivars were analysed a Spearman's correlation coefficient value (r_s) of -0.7323: $P<0.01$ was obtained (Table 5.8: Fig. 5.13).

Cultivars had low levels of oxidized glutathione which ranged from 11.9 ± 5.2 to 67.2 ± 19.3 n moles/g fresh wt. of leaflets (Fig. 5.12).

Fig. 5.10. Standard curve of reduced glutathione (GSH)

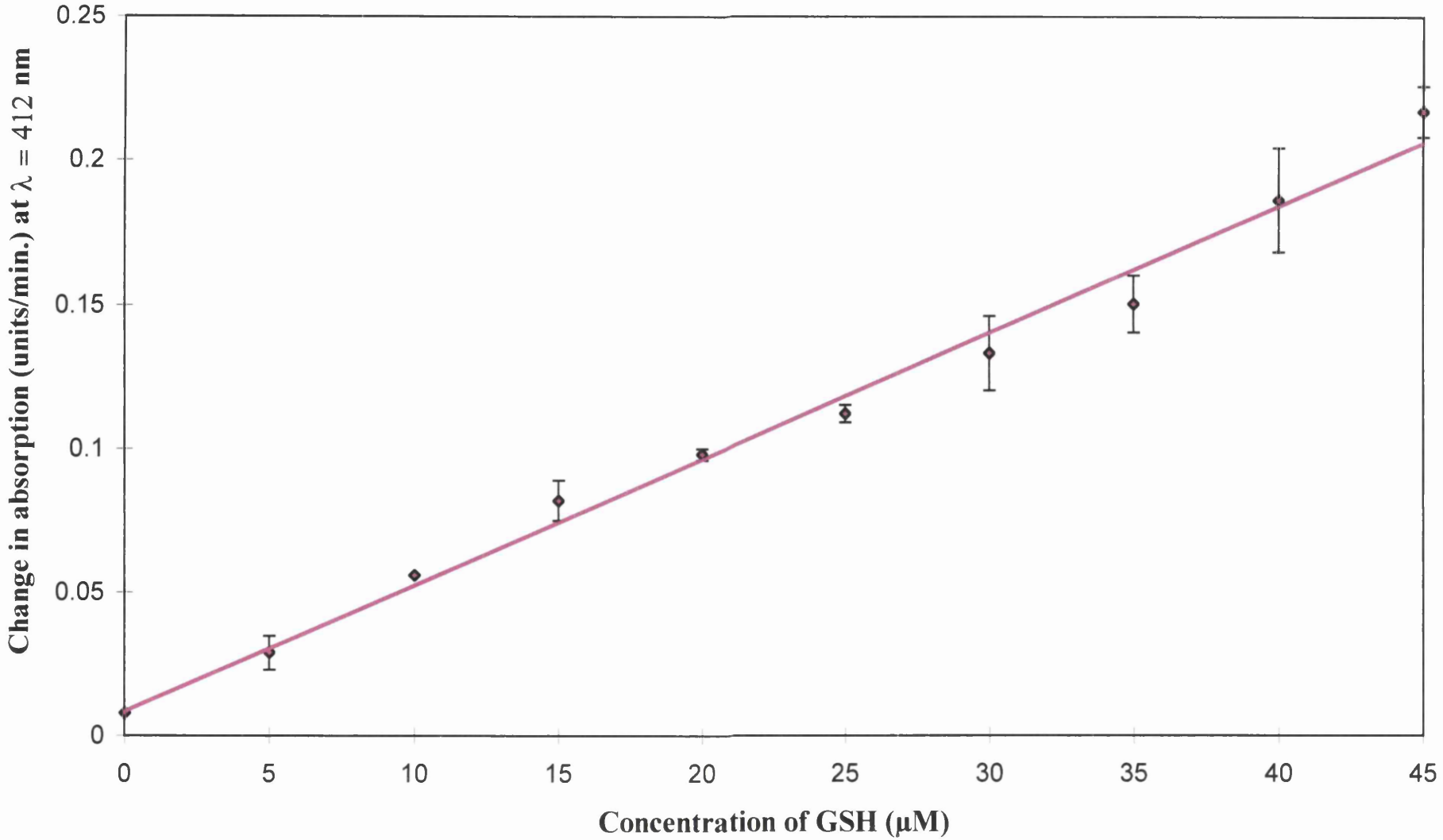


Fig. 5.11. Standard curve of oxidized glutathione (GSSG)

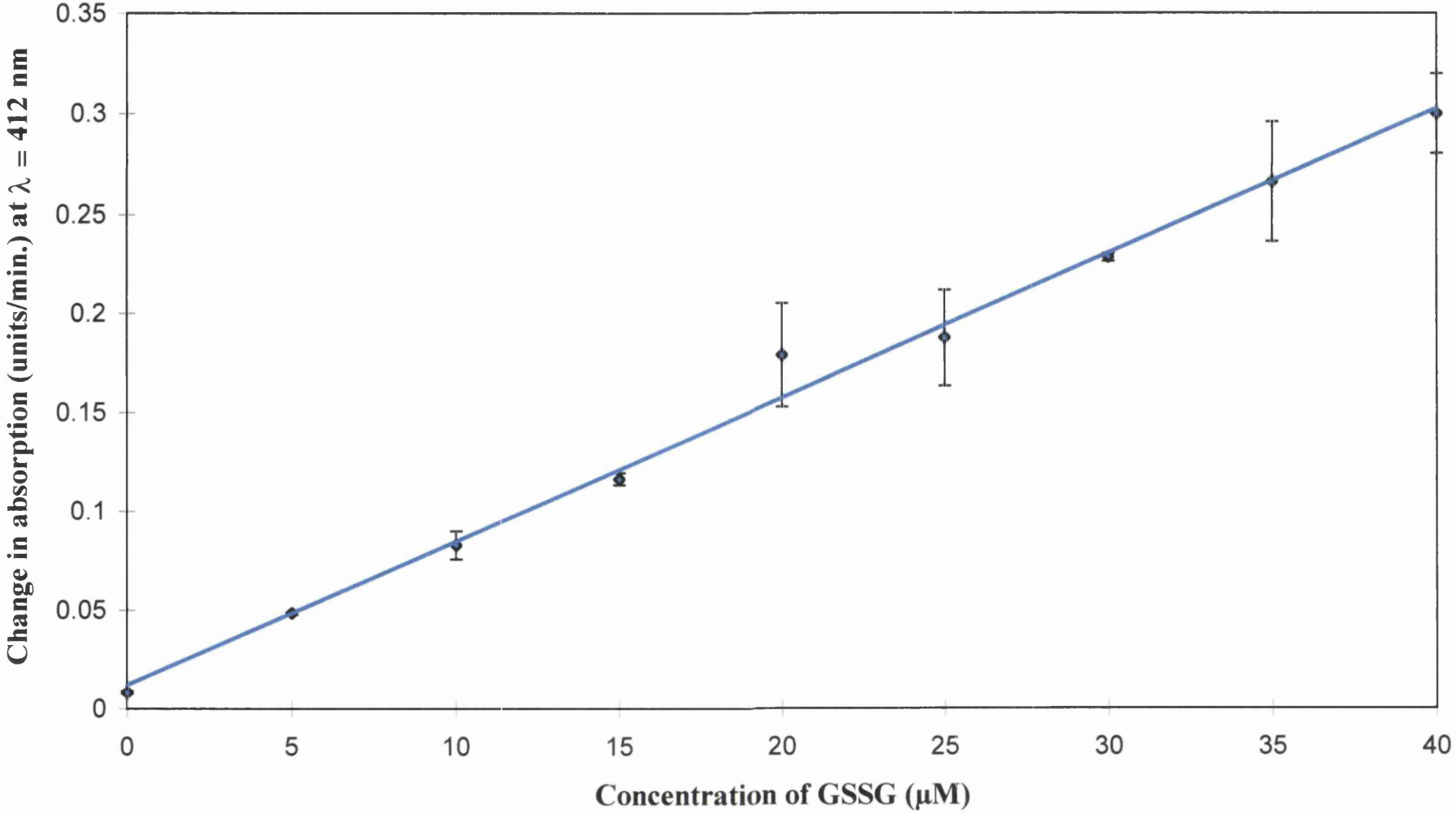


Table 5.8. Reduced glutathione (n moles/g fresh wt. of leaflets) of 12 chickpea cultivars

Cultivars	GSH content	
Kasseb	561.0 ± 112.9	A
CM 72	512.7 ± 83.0	AB
C-44	489.9 ± 28.0	AB
CM-68	482.1 ± 103.2	AB
ILC 3279	462.5 ± 18.1	AB
INRAT 88	440.3 ± 88.7	BC
ILC 482	422.3 ± 35.9	BCD
C-235	411.6 ± 42.7	BCD
ILC 249	354.7 ± 31.6	CDE
CM 88	331.4 ± 37.2	DE
6153	298.9 ± 7.8	EF
AUG 424	200.7 ± 27.2	F

ANOVA showed $P<<0.001$. Having shown a highly significant difference using ANOVA, the least significant difference (LSD) test with alpha set at 0.05 was used to examine differences within the data set. Means followed by the same letters are not significantly different at the 95 % confidence level where LSD value = 103.9.

Fig. 5.12. Measurements of total, reduced and oxidized glutathione (n moles/g fresh wt. of leaflets) of 12 chickpea cultivars

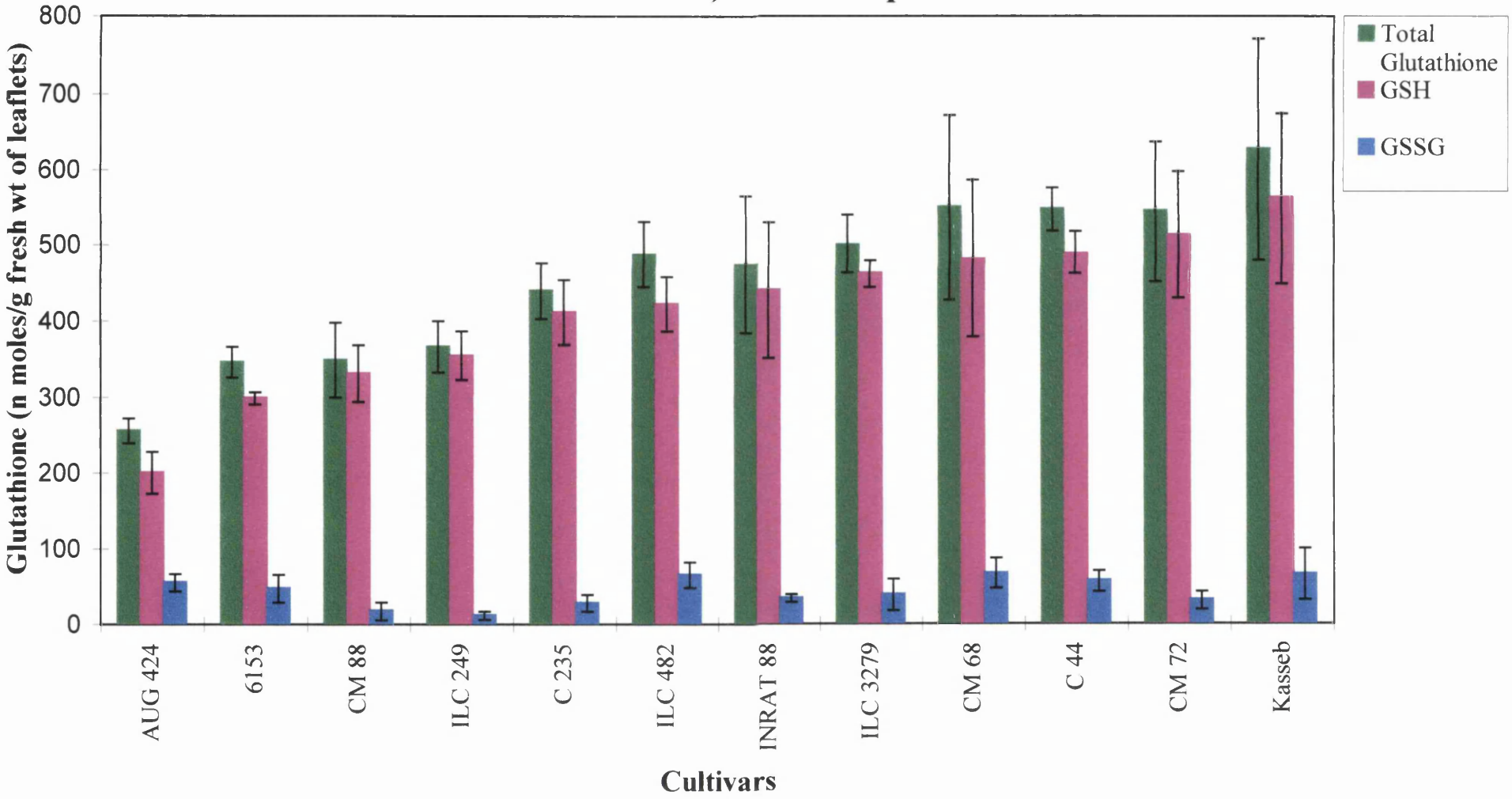
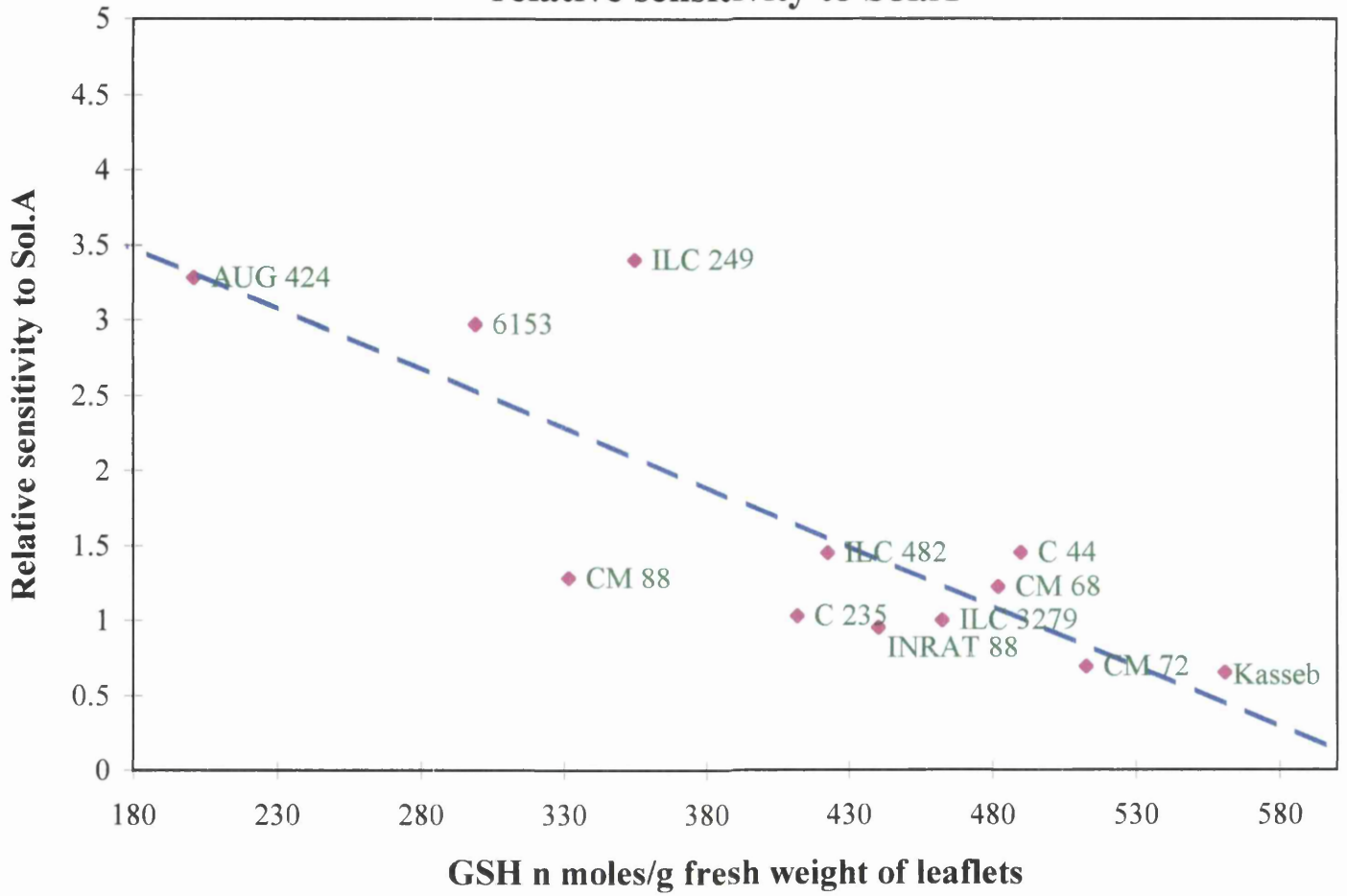


Fig. 5.13. Relationship between reduced glutathione (GSH) content (n moles/g fresh wt. of leaflets) of chickpea cultivars and the sensitivity of their cells to Sol.A. Sensitivity was inversely correlated with GSH levels with a Spearman's correlation coefficient value (r_s) of -0.7323 ($P<0.01$).

Fig. 5.13. Relationship between GSH levels of 12 chickpea cultivars and their relative sensitivity to Sol.A



5.3.5. Glutathione S-transferase (GST) activity in chickpea cultivars

Measurement of GST activity as units showed that the difference of their means among the cultivars was highly significant ($P<<0.001$). Cultivar AUG 424 had the least and INRAT-88 the most, 40.1 ± 3.8 and 66.8 ± 3.9 units of activity/g fresh wt. of leaflets, respectively. Comparison of the means of activity at 0.05% confidence separated the cultivars into five overlapping groups in which cultivars within a group did not differ significantly from each other (Table 5.9). Sensitivity to Sol.A was inversely related to GST activity, cultivars that were least sensitive such as Kasseb and INRAT-88 having 1.40 - 1.66 times greater activity than the most sensitive cultivars such as 6153 and AUG 424. When all 12 cultivars were analysed a Spearman's correlation coefficient value (r_s) of -0.8094: $P<0.01$ was obtained (Fig. 5.14).

5.3.6. The effect of Sol.A on glutathione concentration and glutathione S-transferase activity in shoots treated with the toxin

When shoots of cv. ILC 3279 were allowed to take up Sol.A (45.3 µg/shoot) there was a significant increase in glutathione content ($P<0.05$). The levels of total, oxidized and reduced glutathione increased from 672 ± 55.4, 59.7 ± 6.8 and 612.3 n moles/g fresh weight of shoots, respectively to 848 ± 92.2, 89.6 ± 15.5 and 758.4 ± 82.6 n moles/g fresh weight, respectively (Fig. 5.15).

Treatment of shoots with Sol.A also caused a significant 1.9 fold increase in GST activity from 57.9 ± 10.0 units /g fresh wt. in controls to 112.3 ± 18.6 units/g fresh wt. in tests ($P<0.05$: Table 5.10).

5.3.7. The effect of dichlormid on glutathione concentration and glutathione S-transferase activity in shoots treated with the compound

When shoots were allowed to take up dichlormid (150 and 300 µg/shoot), glutathione levels of shoots treated with the higher concentration rose significantly ($P<0.001$: Fig. 5.16). GST activity was also enhanced 1.38 and 1.42 fold by the treatments but the enhancement was not significant (Table 5.11).

5.3.8. Decrease in sensitivity to Sol.A of cells isolated from chickpea shoots treated with dichlormid

When shoots of chickpea cv. ILC 3279 were treated with dichlormid 150 µg/shoot and 300 µg/shoot, the cells isolated from leaflets of the treated shoots were 2.45 times and 2.66 times less sensitive to Sol.A, respectively, than those isolated from controls incubated in water only (Table 5.12).

Table 5.9. GST activity (units) of chickpea cultivars/g fresh weight of leaflets

Cultivars	Units of activity/g fresh wt.of tissue
INRAT-88	66.77 ± 3.9 A
Kasseb	57.87 ± 5.1 B
ILC 3279	54.57 ± 5.1 BC
CM 72	54.53 ± 3.9 BC
CM 88	52.33 ± 6.6 BC
C-235	51.23 ± 6.9 BC
ILC 249	50.10 ± 3.8 BC
CM-68	49.03 ± 5.8 CD
C-44	49.00 ± 3.3 CD
6153	41.23 ± 5.1 DE
ILC 482	41.20 ± 1.9 DE
AUG 424	40.10 ± 3.8 E

ANOVA showed $P<<0.001$. Having shown a highly significant difference using ANOVA, the least significant difference (LSD) test with alpha set at 0.05 was used to examine differences within the data set. Means followed by the same letters are not significantly different at the 95 % confidence level where LSD value = 8.093

Fig. 5.14. Relationship between GST activity of chickpea cultivars and the sensitivity of their cells to Sol.A. Sensitivity was inversely correlated with GST activity with a Spearman's correlation coefficient value (r_s) of -0.8094 ($P<0.01$).

Fig. 5.14. Relationship between GST activity of 12 chickpea cultivars and their relative sensitivity to Sol.A

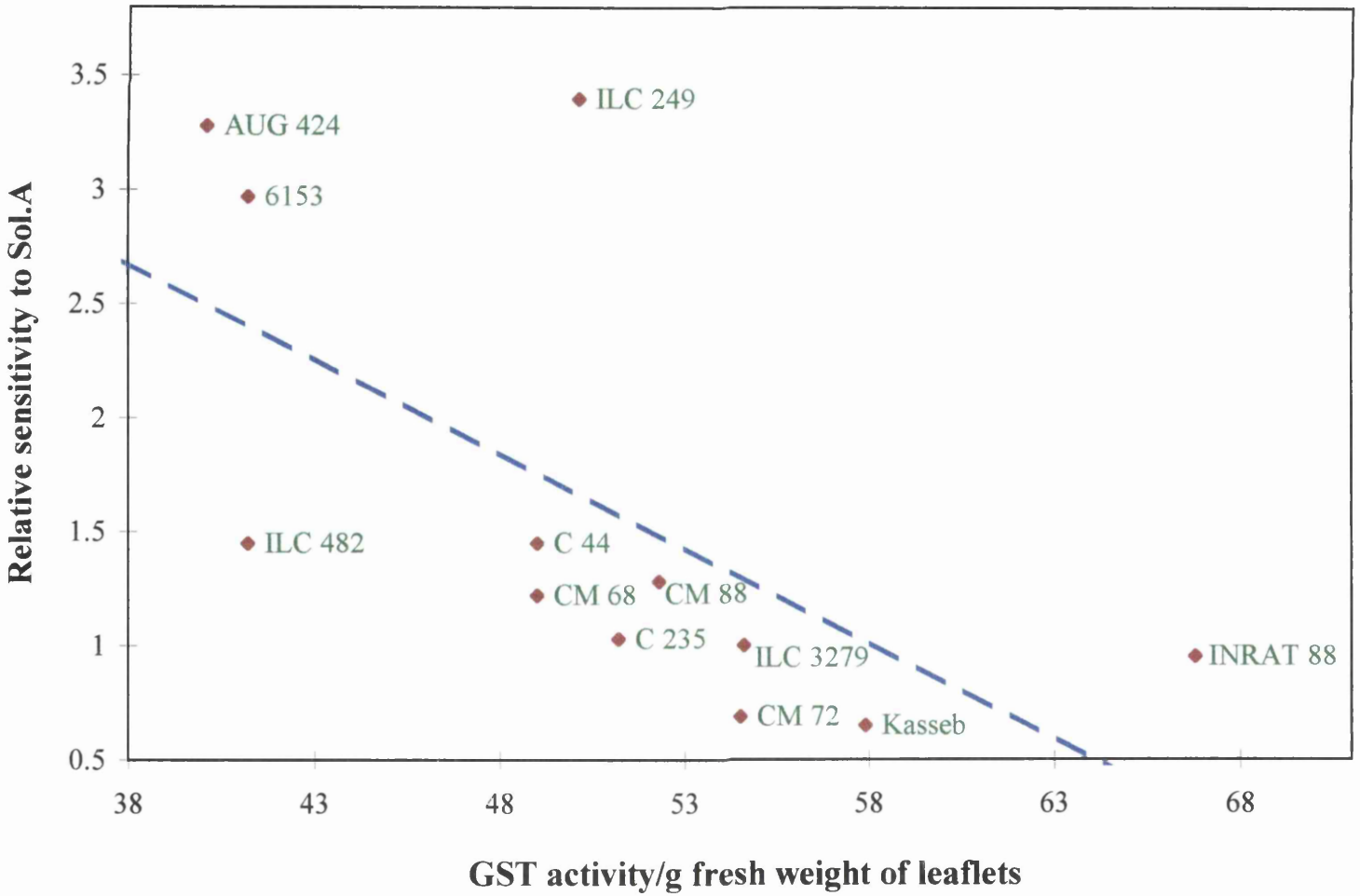


Fig. 5.15. Total glutathione (GSSG+GSH), GSSG and GSH/ g fresh weight of shoots of chickpea (ILC 3279) treated with Sol.A (45.3 µg/shoot) or water and incubated at 23 ± 2 °C for 96 hours

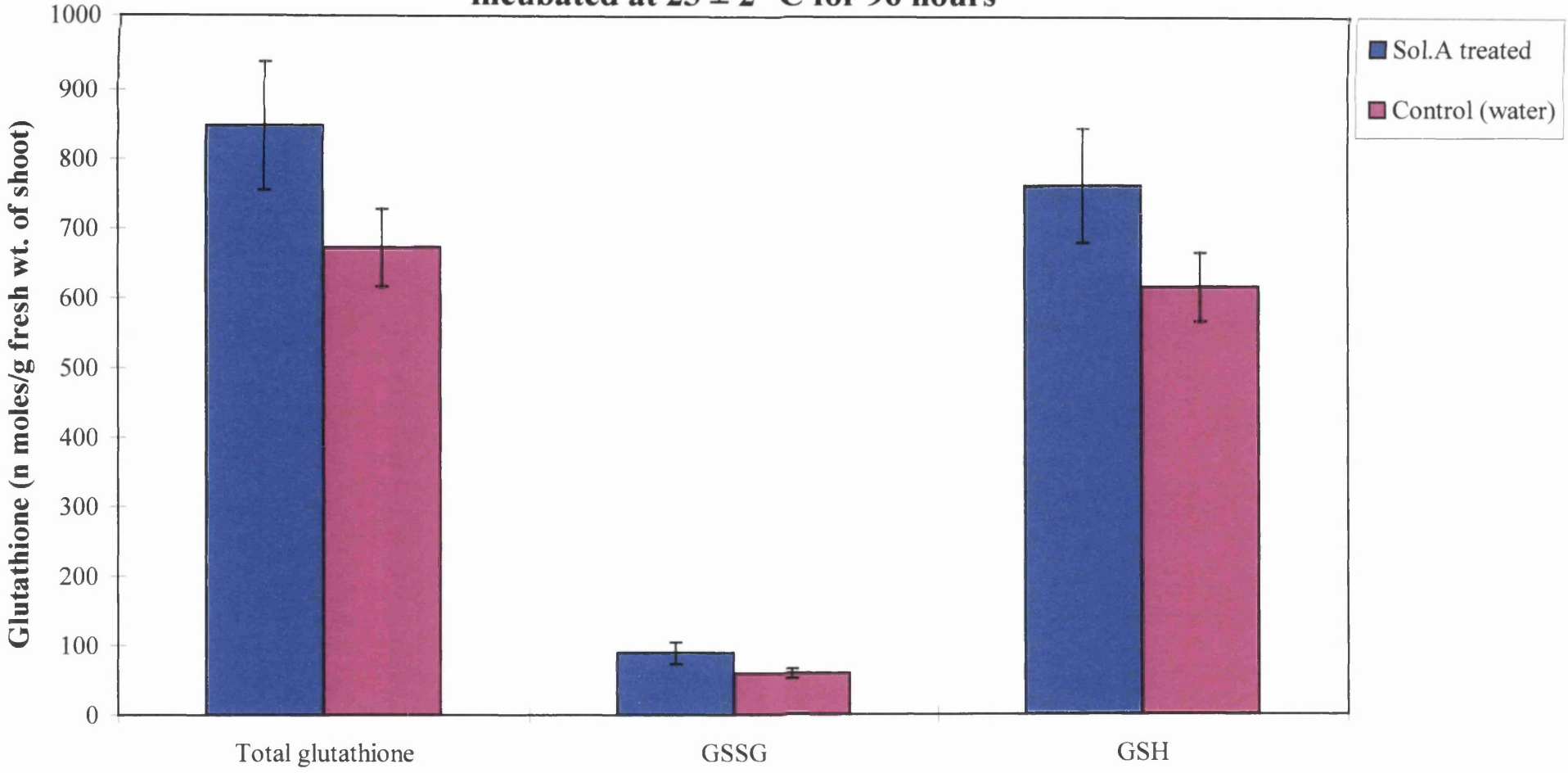


Table 5.10. Glutathione S-transferase activity (units/g fresh wt. of chickpea shoots cv. ILC 3279) 96 h after treating with Sol.A (45.3 µg/shoot)

Treatments	R1	R2	R3	Mean	SD
Shoots incubated in Sol.A	95.6	132.3	109.0	112.3	18.6
Control (shoots incubated in H_2O without Sol.A)	65.7	62.3	45.7	57.9	10.7

Means of treatments differed significantly ($P<0.05$). Plant shoots were incubated in Sol.A (100 µM: 30.2 µg/ml). Each shoot was allowed to transpire 1.5 ml of Sol.A solution (≈ 45.3 µg/shoot) for 5 to 6 h and further incubated in water for 96 h. Control shoots were kept in water without Sol.A throughout the incubation period.

Fig.5.16. Total glutahtione (GSSG+GSH), GSSG and GSH/g fresh weight of chickpea shoots (cv. ILC 3279) treated with dichlormid 150 µg/shoot, 300 µg/shoot or water and incubated at 23±2 °C for 96 h

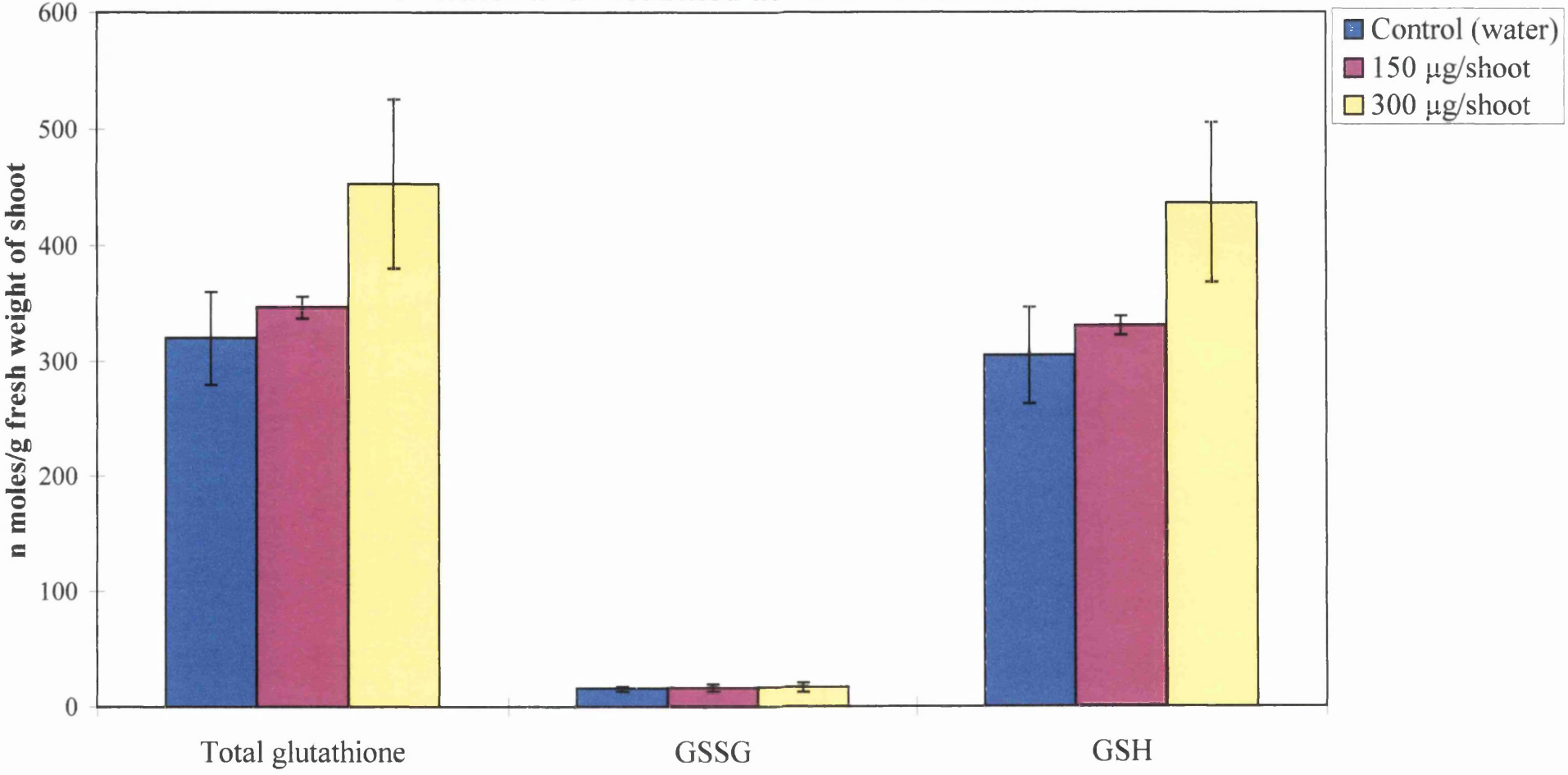


Table 5.11. Glutathione S-transferase activity (units/g fresh wt. of chickpea shoots cv. ILC 3279) 96 h after treating with dichlormid (150 µg/shoot and 300 µg/shoot)

Treatments	R1	R2	R3	Mean	SD
control (shoots incubated in water)	79.0	62.3	69.0	70.1	8.4
Shoots treated with dichlormid (150 µg/shoot)	115.7	72.3	102.3	96.8	22.2
Shoots treated with dichlormid (300 µg/shoot)	129	89.0	82.3	100.1	25.2

Shoots were incubated in dichlormid solutions of (200 µg/ml and 100 µg/ml), allowed to transpire 1.5 ml from each incubation tube for 5 to 7 hours (≈ 150 µg and 300 µg, respectively) and further incubated in water for 96 h. Control shoots were kept in water without Sol.A throughout the incubation period.

Table 5.12. Sensitivity of cells isolated from leaflets of chickpea shoot after treatment with dilchlormid

Treatments	Sol.A (µg/ml) required to cause 50% cell death				
	R1	R2	R3	Mean	SD
Control (cells isolated from shoots incubated in water)	26.0	39.6	22.1	29.2	9.2
Cells isolated from shoots allowed to take (150 µg/shoot) of dichlormid	74.74	67.95	72.5	71.7	3.4
Cells isolated from shoots allowed to take (300 µg/shoot) of dichlormid	74.74	72.48	86.1	77.8	7.3

Shoots were incubated in dichlormid solutions of (200 µg/ml and 100 µg/ml), allowed to take 1.5 ml from each incubation tube for 5 to 7 hours (≈ 150 µg/shoot and 300 µg/shoot, respectively) and further incubated in water for 96 h. Control shoots were kept in water throughout the incubation period. Cells from leaflets were isolated and tested for their sensitivity to Sol.A.

5.4. CONCLUSIONS

Sol.A and Sol.B caused a highly significant loss of 15.8 % and a non-significant loss of 7%, respectively in shoots weighed 24 h after incubation in these compounds (Tables 5.3 and 5.5). These losses may have resulted from the dysfunction of the plasma membrane leading to the leakage of cell contents. Stems of shoots placed in Sol.A (544 µg/4 ml water) lost turgor and became shrivelled and their leaflets developed flame-shaped chlorotic zones. Treatment of shoots with lower concentrations of Sol.A, of which 45.3 µg/shoot was taken up and subsequent incubation for 96 h in water led to the breaking of stems just below the uppermost leaflet and bleaching of stems at the base. In contrast, when shoots were placed in Sol.B (547 µg/4 ml of water), their stems remained turgid but their leaflets become twisted, appeared scorched and chlorotic and some abscised. All these symptoms engendered by purified toxin preparations are also seen in plants infected by *A. rabiei.*

Although *A. rabiei* produces the phytotoxins solanapyrones A, B and C in culture (Alam *et al.*, 1989), only Sol.C has been claimed to have been recovered from plants infected with the fungus (Shahid and Riazuddin, 1998). One possibility is that the pathogen does not produce the compounds in the plant. Alternatively, the plant may metabolise them. The studies reported in this chapter showed that Sol.A and Sol.B were metabolised by cells and shoot cuttings of chickpea (cv. ILC 3279). Sol.A was also metabolised by a protein preparation from shoots of chickpea (cv. ILC 3279). No Sol.A was recovered from shoots incubated in Sol.A but a new compound (TLA-1) was extracted. When TLA-1 was isolated from TLC plates and run on HPLC it gave two compounds TLA-1A and

TLA-1B. TLA-1B and SLC-4 (a product of Sol.A in which the methoxyl group is demethylated to give the corresponding alcohol: see Chapter 4) had similar retention times on HPLC and their spectra, taken between 250 and 360 nm, gave a 95% match. SLC-4 is far less toxic than Sol.A recalling similar detoxification mechanisms in other systems. For example, fomannoxin, a toxin produced by *Heterobasidion annosum* was metabolised by conifer cells to give fomannoxin alcohol (Zweimuller *et al.*, 1997). Similarly, the reduction of a carbonyl group of HC-toxin, produced by *Helminthosporium carbonum,* again giving an alcohol, by an enzyme of resistant maize encoded by the *Hm1* gene detoxified the compound and the gene conferred resistance on the plant (Meeley and Walton, 1991; Meeley *et al.*, 1992). This left the question of the identity of TLA-1B although from the retention time on HPLC and its UV spectrum it seems likely that it is closely related to SLC-4.

As shown in the last chapter, the presence of glutathione markedly reduced the toxicity of Sol.A for cells isolated from chickpea leaflets. Therefore the GSH-GST system was investigated as a means by which the plant detoxified Sol.A and raised the question as to whether TLA-1 was a glutathione conjugate of Sol.A. The GSH-GST system is a well known mechanisms for detoxifying xenobiotics, GSH forming a conjugate with the xenobiotic either spontaneously or catalysed by GST. For example, both alachlor and chloracetanilide form conjugates with glutathione and their conjugation is catalysed by GST. Subsequently the conjugate accumulates in the vacuole where it may be degraded (Breaux *et al.* 1987; Sandermann., 1992 and 1994; Marrs 1996; Wolf *et al.* 1996; Coleman *et al.*, 1997a).

Measurement of GSH content and GST activity among cultivars showed that differences of their means were highly significant and both were found to be negatively

correlated to their relative sensitivity to Sol.A. Among 12 chickpea cultivars GSH content ranged from 200.7 ± 27.2 n moles/g fresh wt. of leaflets for cv. AUG 424, which was the most sensitive to Sol.A to 561.0 ± 112.9 n moles/g fresh wt. of leaflets for cv. Kasseb, which was the least sensitive. Generally the least sensitive cultivars to Sol.A (Kasseb and CM 72) had 1.9 - 2.8 and 1.7 - 2.5 times greater GSH content than those of the most sensitive cultivars (6153 and AUG 424). These figures are similar to those reported by Breaux *et al.* (1987) who reported that shoots of corn and sorghum tolerant to the herbicide chloroacetanilide had a 2.6 fold higher concentration of GSH than that of the susceptible species, giant foxtail and barnyard grass.

GST activity ranged from 40.1 ± 3.8 to 66.8 ± 3.9 units/g fresh wt. of leaflets among 12 cultivars of chickpea. The cultivars least sensitive to Sol.A (INRAT 88 and Kasseb) had 1.62 - 1.66 times and 1.40 - 1.44 times more GST activity than those of the most sensitive cultivars (6153, ILC 482 and AUG 424). It had been reported that crude extracts made from various atrazine-resistant corn lines possess elevated levels of GST whereas an atrazine-sensitive corn line (GT112) had less than 1% of the GST activity found in the resistant lines (Shimabukuro *et al.*, 1971). The involvement of GST in catalyzing the conjugation of glutathione with xenobiotics to form non toxic compounds is well known. For example, two GST enzymes GSTI and GSTII which catalyzed the formation of a glutathione conjugate with the herbicide alachlor were purified from etiolated corn tissue. This conjugation resulted in elimination of the biological activity of the herbicide (Mozer *et al.*, 1983).

Xenobiotics may enhance the GSH content and GST activity of plants (Mozer *et al.*, 1983; Breaux *et al.*, 1987; Hunaiti and Ali, 1991; Andrews *et al.*, 1997). In this study, increased total, reduced and oxidized glutathione as well as increased GST activity were

found in shoots allowed to take up Sol.A (Fig. 5.15; Table 5.10). Similarly, treatment of shoots with the safener, dichlormid, also raised total, oxidized and reduced glutathione levels and GST activity (Fig. 5.16; Table 5.11). Cells isolated from shoots treated with dichlormid at 150 μg/shoot and 300 μg/shoot were found to be 2.45 times and 2.65 times, respectively, less sensitive to Sol.A than cells from control shoots that had not been treated with the compound, giving circumstantial evidence that the decrease in sensitivity was caused by the increased GSH levels and GST activity (Table 5.12).

Since GSH and GST are involved in the metabolism and detoxification of several xenobiotics as reviewed earlier and GSH forms a conjugate with Sol.A (Chapter 4) it may be that this system is also responsible for the detoxification of the solanapyrone toxins. Unfortunately, time did not permit completion of the investigation of TLA-1 as the glutathione conjugate of Sol.A. However, the enhancement of GSH levels and GST activity by the safener, dichlormid, and the reduced sensitivity of cells from plants treated with the compound hold out the possibility that safeners could be used to mitigate the symptoms of Ascochyta blight of chickpea.

CHAPTER 6

DISCUSSION

Ascochyta blight of chickpea is still a major disease of the crop and difficult to control for a number of reasons. Firstly, pycnidiospores of the fungus alighting on a susceptible chickpea plant produce a new generation of spores within 7-10 days in a cool and damp environment. Thus a polycyclic pathogen can cause explosive epidemics under favourable environmental conditions (Strange, 1993). Secondly, genetic variability of the cultivars may give considerable variation in the reaction of individual lines to the fungus even though chickpea is self-pollinated crop (Reddy *et al.*, 1981). Thirdly, lack of a suitable standard scale for the screening of disease reaction has further confounded the issue of variation in disease reaction although recently a quantitative scale, the linear infection index scale (LII) based on number and lesion size has been introduced (Riahi *et al.*, 1990). Fourthly, inocula may be heterogeneous. Finally, the existence or non-existence of races of the pathogen is still debated. Weigand (1989) showed that although six races differed in virulence this was not specific, the more virulent 'races' being virulent on all test cultivars and vice-versa.

The solanapyrones toxins may be important pathogenicity or virulence factors of the fungus since they are produced by all reliably identified isolates and reproduce symptoms of the disease. In the studies reported in this thesis, an isolate of the fungus (PUT 7) produced Sol.A, B and C when grown on Czapek Dox liquid medium amended

with cations. Stems of shoots placed in Sol.A (544 µg/4 ml water) lost turgor and became shrivelled and their leaflets developed flame-shaped chlorotic zones. Treatment of shoots with lower concentrations of Sol.A, of which 45.3 µg/shoot was taken up, and subsequent incubation for 96 h in water led to the breaking of stems just below the uppermost leaflet and bleaching of stems at the base. In contrast, when shoots were placed in Sol.B (547 µg/4 ml of water), their stems remained turgid but their leaflets become twisted, appeared scorched, chlorotic and some abscised. These symptoms are also seen in plants infected by *A. rabiei.* Alam *et al.* (1989) also found that chlorosis and epinasty, symptoms characteristics of early infection of the blight, occurred when shoot cuttings were placed in solutions of the solanapyrones. Chen and Strange (1994) found that the fungus produced not only Sol.A and C but also Sol.B when grown on Czapek Dox nutrients supplemented with cations. Later, Latif *et al.* (1993) reported that eight isolates of the fungus produced Sol.A and Sol.C but a nineth did not produce these compounds. However, this isolate produced cytochalasin D, raising the possibility that the fungus was an isolate of *Phoma* which produced most of the cytochalasins and has also been isolated from chickpea (Strange, 1997).

To study the individual roles of the solanapyrone phytotoxins in disease development, their relative toxicity and the relative sensitivity of chickpea cultivars to them, there was a need for a facile technique to separate them. This was achieved by flash chromatography using a commercial apparatus (Biotage) along with a silica cartridge and a specific solvent system (for details see section 2.3.2.3 chapter 2).

Strange (1997) suggested that toxins can be exploited in three ways to develop resistance against the toxigenic pathogen: (i) screening for toxin insensitivity at the whole plant level, (ii) use of the toxin at the tissue culture level and regeneration of toxin

insensitive plants from surviving cells and (iii) genetically engineering plants to destroy the toxins. Probably the most common approach for selecting for disease resistance has been to use pathogen toxins as the selecting agent in cell cultures as the cells are exposed uniformly to the toxins (Daub, 1986). Selection of resistant germplasm using toxins has been reported. For example, the toxin produced by *Helminthosporium victoria* causal agent of victoria blight on oats was used as screening agent. Genotypes resistant to the toxin were also resistant to the fungus (Wheeler and Luke, 1955). Cell lines of alfalfa (*Medicago sativa*) insensitive to the toxins produced by *Fusarium oxysporum* f. sp. *medicaginis* were selected and plants regenerated from them were resistant; the resistance was both stable and inheritable (Hartman *et al.*, 1984). Nadel and Spiegel-Roy (1988) selected lemon cell cultures resistant to toxin produced by the fungus *Phoma tracheiphila*, the causal agent of a Mal secco, a serious disease of lemon (*Citrus limon* Burm. f.) and citron (*C. medica* L). The resistance was found to be stable. In another success, Vidhyasekaran *et al.* (1990) used a partially purified toxin preparation of *Helminthosporium oryzae* to select for resistance to brown spot of rice. Four toxin resistant calli were selected and plants regenerated from two of these were resistant to the pathogen; the resistance was heritable and stable. Using non-selective toxins produced by *Alternaria solani,* toxin sensitive and toxin insensitive clones of Russet Burbank were regenerated from protoplasts; the toxin insensitive clones were resistant to the pathogen (Matern *et al.*, 1978). Now purified preparations of the solanapyrones are available these techniques could be used to screen chickpea. Data reported in this thesis suggest that the less sensitive plants would also be more resistant to the disease.

During the early stages of infection of chickpea by *Ascochyta rabiei*, petioles and young branches develop epinasty and leaflets become flaccid owing to the loss of turgor of their

cells. Small, water-soaked spots appear on stems, leaves and pods which become necrotic and when the necrosis girdles the stems and petioles, they usually break. These symptoms are consistent with toxin production and can be explained by plasma membrane dysfunction. The plasma membrane affected by toxins loses its selective permeability and allows leakage of cell sap into intercellular spaces giving rise to water soaking as well as destroying the turgor necessary for the support of plant organs since plant cells act as mini-hydroskeletons when they are turgid. On the basis of these observations the relative toxicity of the toxins to cells isolated from 12 cultivars and the relative sensitivity of these cultivars to Sol.A and B was investigated using fluorescein diacetate (FDA) as a test of viability.

The cell assay results revealed that the relative sensitivity of the cultivars to Sol.A or B was highly significant among the cultivars and that the most sensitive cultivars to Sol.A such as 6153 and AUG 424 were also highly susceptible to the disease since these scored 9 on the 1-9 scale of Singh *et al.* (1981). On the other hand, cultivars which were less sensitive such as Kasseb, CM 72, INRAT 88 and ILC 3279 were also found tolerant to resistant in the field, scoring 4-6 on the scales of Singh *et al.* (1981) or ICARDA (Chapter 3: Fig. 3.5). Spearman's correlation showed that the relative sensitivity of 9 cultivars to Sol.A was positively correlated with their susceptibility to the fungus but the value of the coefficient (+ 0.5166) was non-significant. It will be interesting to know if such a correlation becomes significant when more chickpea genotypes are included in the test and when their reaction to disease is scored on a less subjective basis. These preliminary data encourage the view that toxin insensitivity may be an important component of resistance of chickpea to the fungus. There is a need to check the sensitivity of more cultivars of chickpea preferably true breeding lines including wild species to establish the range of sensitivity to the toxins among species of *Cicer*.

Sol.A was 2.62 to 12.64 folds more toxic than Sol.B depending upon cultivar. The greater toxicity of Sol.A may be caused by its aldehyde group and has been previously reported. For example, Sol.A was found to be about four times as toxic to chickpea cells as Sol.C (Strange and Alam 1992: Alam *et al.*, 1989). In another report Sol.A was 2.4 and 1.8 times more toxic than Sol.B and Sol.C, respectively, in a root growth inhibition assay (Kaur, 1995).

Another attractive prospect for the use of the solanapyrones is in the search for genes encoding enzymes that degrade them which could be incorporated into the plant (Strange, 1998). The gene responsible for degradation could be found in plants or micro-organisms. For example, in plants, the reason for the resistance of maize containing the *Hm1* gene to strains of *Helminthosporium carbonum* producing HC-toxin is that the gene is responsible for the synthesis of an enzyme which inactivates HC-toxin by reducing a carbonyl group essential for toxicity (Meeley and Walton 1991: Meeley *et al.*, 1992). In the case of micro-organisms, Kneusel *et al.* (1990) found a strain of *Bacillus subtilis* (BG3) capable of detoxifying brefeldin A, a toxin of *Alternaria carthami*, that plays an important role in disease development in safflower. As mentioned by the authors, transformation of safflower with the gene that encodes the enzyme could be a valuable alternative to traditional breeding methods for resistance.

As a prelude finding a gene in a micro-organism capable of detoxifying the solananpyrones there was a need to develop a medium. The medium would allow growth of the micro-organism when supplemented with the solanapyrones as a carbon source. In these studies stability of Sol.A proved to be a problem owing to its reactive aldehyde group. Different media were tried and Sol.A was found to be most stable in basal medium. Incubation of Sol.A in basal mineral medium resulted in demethylation of the methoxyl group giving the corresponding alcohol (see Chapter 4). The demethylated Sol.A (SLC-4) was found to be

16.4 folds less toxic than Sol.A. In a similar vein fomannoxin, a toxin produced by *Heterobasidion annosum* was metabolised by conifer cells to give non-toxic fomannoxin alcohol (Zweimuller *et al.*, 1997). In this thesis, rather than pursuing the possibility of degradation of Sol.A by a micro-organism because of its instability in media suitable for microbial growth, attention was turned to the possibility of its metabolism by the chickpea plant itself.

Detoxification and elimination of potentially phytotoxic compounds such as microbial toxins and agrochemicals (xenobiotics) present in the environment is essential for the survival of plants (Gaillard *et al.*, 1994; Wolf *et al.*, 1996) and an important detoxification mechanism is the chemical modification of the xenobiotics by covalent linkage to the endogenous tripeptide glutathione (Coleman *et al.* 1997a).

In these studies it was found that incubation of Sol.A with glutathione resulted in the rapid loss of the free compound, possibly as a result of conjugation. The mass spectrometry of the reaction mixtures of Sol.A and glutathione showed that Sol.A-glutathione conjugate had a molecular ion of 606 (Fig. 4.14) which when accurately measured gave a mass of 606.214300 corresponding to a formula of $C_{28}H_{36}N_3O_{10}S$ (calculated 606.212142) and suggesting the conjugate as shown in Fig. 4.15. An explanation of the mass found in the spectrometry is given in section 4.3.7.3 but its validity requires confirmation by further studies of the reaction. The sulphur group in glutathione may be acting as nucleophilic site (i.e. an electron rich centre) and the aldehydes group of Sol.A as an electrophile (centre of low electron density). The conjugation between glutathione (soft nucleophile) and aldehydes (soft electrophile) takes place spontaneously and can be enhanced by GSTs (Coleman *et al.* 1997a).

As reviewed by Coleman *et al.* (1997a) in plants the metabolism and detoxification of xenobiotics can be divided into three phases: in the first step which is generally mediated by cytochrome P-450 dependent monooxygenases, a foreign compound may be oxidized, reduced or hydrolyzed to reveal or introduce a functional group. At this stage the phytoxicity of the compound may not necessarily be decreased. In a second step, the xenobiotic is deactivated by covalent linkage to an endogenous hydrophilic substance such as glutathione, malonate or glucose to form a water-soluble conjugate, reactions which are catalyzed by -malonyl, -gluconyl and -glutathione transferases, respectively. In the third step, the inactive water-soluble conjugates secreted into the vacuole. In the bioassays reported in this thesis the toxicity of Sol.A was reduced 2.13 fold by the presence of glutathione. This observation together with the fact that Sol.A could be not recovered from shoots incubated in the compound led to the investigation of the GSH / GST system as one possible mechanism by which the solanapyrones could be metabolised.

Measurements of GSH content and GST activity among 12 chickpea cultivars differed significantly and both were found to be negatively correlated with their relative sensitivities to Sol.A. Generally the least sensitive cultivars to Sol.A such as Kasseb and CM 72 had 1.9 - 2.8 and 1.7 - 2.5 times more GSH content than those of the most sensitive cultivars such as 6153 and AUG 424. These figures are similar to those reported in a rather different contest by Breaux *et al.* (1987). They found that shoots of corn and sorghum tolerant to the herbicide chloroacetanilide had a 2.6 fold higher concentration of GSH than that of the susceptible species giant foxtail and barnyard grass.

The cultivars least sensitive to Sol.A such as INRAT 88 and Kasseb had 1.62 - 1.66 times and 1.40 - 1.44 times more GST activity than those of the most sensitive cultivars 6153, ILC 482 and AUG 424. The involvement of GST in catalyzing the

conjugation of glutathione with xenobiotics to form non toxic compounds is well known. For example, two GST enzymes GSTI and GSTII which catalysed the conjugation of alachlor to glutathione were purified from etiolated corn tissue. Conjugation resulted in elimination of the biological activity of the herbicide (Mozer *et al.*, 1983). The variation in GSH content and GST activity among 12 chickpea cultivars suggested that the GSH/GST system through its ability to form conjugates with Sol.A might be a factor of resistance to *A. rabiei.*

The level of GST has been shown to be chemically inducible in plants and animals. Such increases have been proposed to play important role for increasing resistance to the toxic chemicals (Mozer *et al.*, 1983). Treatment of shoots with Sol.A enhanced total, reduced and oxidized glutathione content as well as GST activity 1.26, 1.23, 1.50 and 1.94 fold, respectively. These experiments encouraged the investigation of safeners since these compounds are reported to protect crops by increasing the GSH/GST system (Farago *et al.*, 1994). There are many reports showing increases in GSH and GST caused by treatment with safeners. For example, GST activity was found to be increased by 1.51 fold in barley plants treated with cloquintocet-mexyl (Gaillard *et al.*, 1994). In another report Mozer *et al.* (1983) found that in general, corn antidotes (5-(2,4-dichlorophenyl)-4-isoxazolecarboxylic acid, ethyl ester; α-[(cyanomethoxy)imino]-benzeneacetonitrile naphthalic anhydride; 2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylic acid, benzyl ester and N,N-diallyl-2,2-dichloroacetamide) raised enzyme levels between 1.5- and 2.5- fold in both roots and shoots and Hunaiti and Ali (1991) found an increase of almost 2.7 fold in GST activity of chickpea shoots when treated with 10 ppm or 20 ppm oxadiazon. Safeners are not themselves phytotoxic and do not cause any phenotypic alterations or dramatic alterations in protein synthesis.

The study reported in chapter 5 showed that treatment of shoots with dichlormid 300 µg/shoot raised total, oxidized and reduced glutathione levels by 1.42, 1.07 and 1.43 fold, respectively as compared to control shoots. Breaux *et al.* (1987) also found an increase in GSH content by 1.26 fold and 1.58 fold in corn and sorghum seedlings, respectively by seed treatment with the safener flurazole. Treatment of chickpea shoots with dichlormid increased GST activity but the standard deviation was very high. One reason for this may have been that the lines were not genetically pure, a possibility borne out by variation in the phenotype of leaflets. Another reason may have been the rate of uptake of the dichlormid solutions varied from 5 to 8 h.

Cell isolated from shoots treated with dichlormid 150 µg/shoot and 300 µg/shoot were 2.45 times and 2.66 times, respectively less sensitive to Sol.A than controls (Table 5.12) encouraging the view that the safeners could be used to decrease the sensitivity of chickpea to Sol.A and thus increase its resistance to *A. rabiei.* This hypothesis now requires testing, not only with dichlormid, but also with other safeners as well.

Appendix 3.1

Probit table

Transformation of percentages to probits

%	0	1	2	3	4	5	6	7	8	9
0	2.42	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
	0.00	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80	0.90
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Appendix 3.2

Dose of solanapyrone A (µg/ml) required to kill 50% cells of chickpea cultivars. Cultivar ILC 3279 was used as internal control for comparison of each cultivar to standardise the assay conditions

Cultivars	R1	R2	R3	Mean	S.D
ILC 3279	10.31	8.97	11.05	10.11	1.05
ILC 482	9.62	8.37	4.81	7.6	2.49
6153	3.40	3.64	3.17	3.40	0.23
ILC 3279	10.31	9.62	6.80	8.91	1.85
ILC 249	1.95	2.96	3.40	2.77	0.74
ILC 3279	13.60	14.58	12.69	13.62	0.94
INRAT 88	19.23	13.60	11.84	14.89	3.86
Kasseb	22.62	25.39	16.75	21.58	4.41
ILC 3279	27.30	31.25	29.17	29.24	1.97
CM 88	23.68	25.39	19.23	22.76	3.17
CM 68	27.21	25.39	20.16	24.25	3.65
ILC 3279	16.75	25.39	22.62	21.58	4.41
CM 72	27.21	35.91	31.25	31.45	4.35
C 44	12.69	16.75	15.62	15.02	2.09
ILC 3279	44.20	38.49	35.91	39.53	4.24
C 235	38.49	44.20	33.51	38.73	5.34
AUG 424	13.60	12.69	10.30	12.19	1.70

Appendix 3.3

Dose of solanapyrone A (µg/ml: on ILC 3279) and solanapyrone B (µg/ml: required to kill 50% cells of chickpea cultivars) relative to dose required to 50% cells of ILC 3279

Cultivars	R1	R2	R3	Mean	S.D
ILC3279 (Sol.A)	50.81	47.39	67.02	55.05	10.48
ILC 3279 (Sol.B)	287.35	308.64	287.35	294.44	12.29
ILC 3279 (Sol.A)	38.49	41.25	29.17	36.30	6.32
ILC 482 (Sol.B)	104.16	125.0	176.99	135.38	37.50
6153 (Sol.B)	62.5	94.78	88.40	81.89	17.09
ILC 3279 (Sol.A)	82.50	88.49	108.93	93.30	13.85
INRAT 88 (Sol.B)	176.99	268.09	287.35	244.14	58.94
ILC 249 (Sol.B)	233.64	217.86	250.0	233.83	16.07
ILC 3279 (Sol.A)	77.04	62.50	82.5	74.01	10.33
Kasseb (Sol.B)	467.28	467.28	588.23	507.59	69.83
CM 88 (Sol.B)	308.64	330.03	308.64	315.77	12.34
ILC 3279 (Sol.A)	82.50	77.04	88.49	82.67	5.72
CM 72 (Sol.B)	406.50	467.28	406.50	426.76	35.09
CM 68 (Sol.B)	308.64	330.03	406.50	348.39	51.44
ILC 3279 (Sol.A)	82.5	62.50	67.02	70.67	10.48
C 44 (Sol.B)	467.28	406.50	436.68	436.82	30.39
C 235 (Sol.B)	380.22	354.60	330.03	354.95	25.09
AUG 424 (Sol.B)	233.64	287.35	303.03	283.67	48.30

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Table 1. Resistant chickpea lines showing <10% wilt incidence, 1990/91 – 1993/94, Yezin, Myanmar.

Year	Chickpea lines resistant to wilt
1990/91	Karachi
1991/92	ICC 4918, ICC 4936, ICC 11320, ICC 11329, ICCV 3, ICCV 5, ICCV 89224, ICCV 89305, ICCV 89342, ICCL 84204, ICCL 85311
1992/93	ICC 13025, ICC 14196, ICC 14309, ICC 14528, ICC 15167, ICCV 88101, ICCV 90010, ICCV 90039, ICCV 90041, ICCX 830155, -BH - 14H - BH, ICCX 830240 - BH - BH - 5H - BH, ICCX 830256 -BH - BH - 4H - BH, ICCX 850627 - BH - 93H - BH
1993/94	E 100 YM, ICCC 32, ICCC 37, ICCV 3, ICCV 5, ICC 11320, ICC 11323, ICCX 580627 BH - 92H - BH - ICCX 830235 - BH - BH - BH - 5H

diameter earthen pots. The pots were watered and allowed to stand for 4 days for the establishment of the fungus in the soil mixture.

The seeds of susceptible variety (JG 62) were sown in each pot to test the pathogenicity of the fungus. Almost all the seedlings (>90%) wilted within 20 to 30 days after sowing, and they were incorporated into the soil. Then the pots were used to screen the test genotypes. Seven seeds of the test lines, and three seeds of the susceptible control (JG 62) were sown in each pot in four replications. The observations were taken after 20 and 40 days of sowing.

One hundred twenty-four lines and three local varieties were tested from 1990/91 to 1993/94. Lines that showed <10% wilt incidence were considered resistant (Table 1).

These resistant lines should be evaluated further for tolerance to fusarium wilt in multilocational research station trials, and on-farm trials to select high-yielding chickpea lines that are well adapted to Myanmar.

Reference

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An Easy Method for Isolating the Solanapyrone Toxins from Culture Filtrates of *Ascochyta rabiei*

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Blight, caused by *Ascochyta rabiei*, is one of the most serious diseases of chickpea (Nene 1982). The fungus attacks all aerial parts of the plant causing epinasty of petioles and young branches followed by water soaking and necrosis. These symptoms are consistent with the breakdown of the semipermeable properties of plasmamembranes of the plant. Using fluorescein diacetate, a dye which causes live cells with intact membranes to fluoresce under long wavelength UV light, culture filtrates of the pathogen were found to kill cells isolated enzymically from leaflets of the plant. The assay was used to monitor purification of the toxins. Three compounds were obtained and identified as solanapyrones A, B, and C (Alam et al. 1989, Latif et al. 1993, Chen and Strange 1991). The purpose of this report is to make available and speedy method for obtaining pure preparations of the toxins.

Ascochyta rabiei was grown on Czapek Dox medium supplemented with $ZnSO_4 \cdot 7H_2O$, 0.05 g L^{-1}; $CuCl_2$ $2H_2O$, 0.02 g L^{-1}; $MnCl_2 \cdot 4H_2O$, 0.02 g L^{-1}; $CaCl_2 \cdot 2H_2O$, 0.10 g L^{-1}, and $CoCl_2 \cdot 6H_2O$, 0.02 g L^{-1} (Chen and Strange 1991). The medium was dispensed in 250 mL flasks (30 ml per medium flask) and inoculated with 0.03 ml per flask spore suspension of *A. rabiei* (isolate PUT $7:10^7$ spores mL^{-1}). Flasks were incubated at 20±1°C without shaking for 12 days.

Routinely, 33 flasks (≈1 L) were harvested at a time. Mycelium was removed by filtration through four layers of muslin cloth, and the filtrate centrifuged at 10 000 G for 20 minutes at 10°C to sediment spores. The pH of the supernatant was adjusted to 3.0 with 1 M H_2SO_4, and partitioned three times against $^1/_3$ volume of ethyl acetate. The ethyl acetate phases were combined, dried over 20 g of anhydrous sodium sulfate, and taken to dryness on a rotary evaporator at less than 35°C. The residue containing the toxins was dissolved in 5 mL ethyl acetate and the toxins quantified in a small sample (20 µL) by high performance liquid chromatography (HPLC), essentially according to Chen and Strange (1991) except that the solvent system consisted of methanol 23.1%, water 56.3%, and tetrahydrofuran 20.6%.

In order to separate the toxins in the remainder of the sample, the ethyl acetate was removed by film evaporation, dissolved in dichloromethane, and injected on the top of a dry cartridge of silica gel (40 g; Biotage UK Ltd., 15 Haforde Court, Foxholes Business Park, John Tate Road, Hertford, UK). The cartridge was washed with cyclohexane (110 mL) and eluted with dichloromethane, cyclohexane, ethyl acetate (3:3:1; 625 mL), dichloromethane, cyclohexane, ethyl acetate (1:1:1; 400 mL) and finally ethyl acetate (150 mL) under pressure from an airline. The eluates were collected as 25-mL fractions.

Fractions were pooled and quantified according to their UV spectra and extinction coefficients (Solanapyrone A UV λ_{max} nm [ϵ] 327 [9,400], Solanapyrone B UV λ_{max} nm [ϵ] 303 [8,500], and Solanapyrone C UV λ_{max} nm [ϵ] 320 [7,300]) and their recovery ascertained by comparison with the amounts measured in the crude preparation by HPLC (Table 1).

Solanapyrone A normally appeared in fractions 12–25, Solanapyrone B in fractions 29–37, and Solanapyrone C which was synthesized in low concentrations by this isolate in fractions 42–47 (Fig. 1).

The technique was speedy (10–13 seconds for each fraction) and yielded pure preparations of the compounds as determined by HPLC and their UV spectra.

Table 1. Recovery of the solanapyrone toxins from 1 L culture filtrate of *Ascochyta rabiei*. The toxins were extracted in ethyl acetate and separated by flash chromatography.

Compound	In crude ethyl acetate extract (mg)	Pure (mg)	Recovery (%)
Solanapyrone A	45.51 ± 08.46	34.65 ± 7.15	75.92 ± 03.33
Solanapyrone B	42.33 ± 23.15	17.94 ± 9.10	42.95 ± 01.61
Solanapyrone C	2.18 ± 00.33	0.66 ± 0.39	32.50 ± 23.91

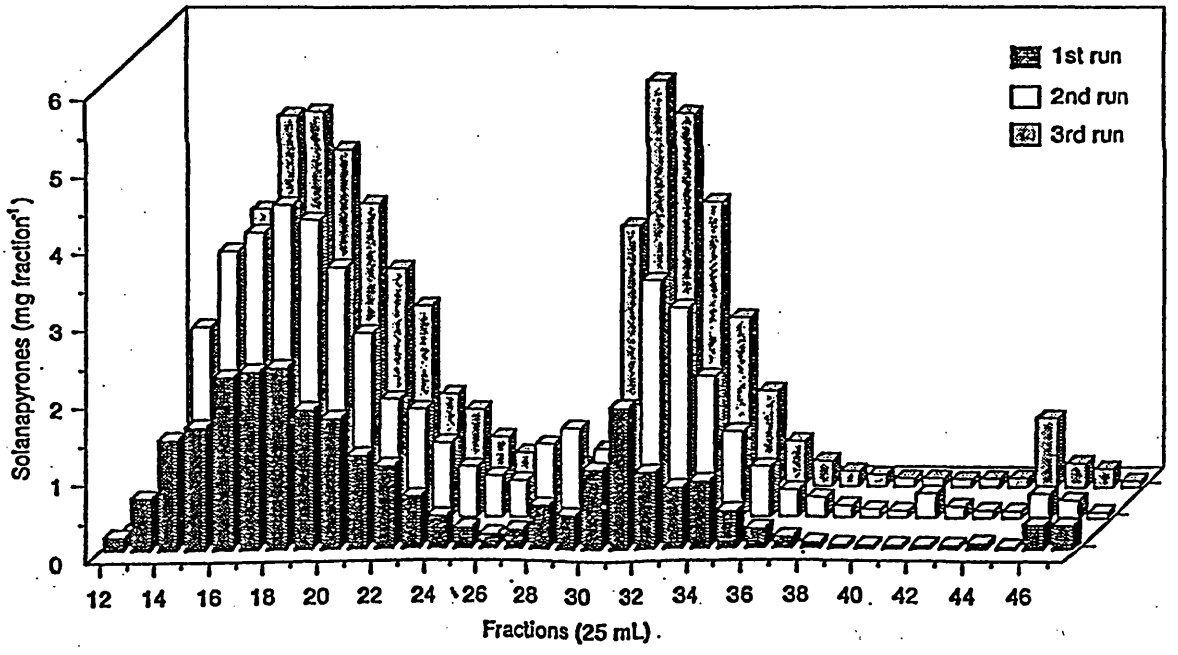

Figure 1. Histogram showing the separation of the solanapyrone toxins from three samples of 1 L of culture filtrate. Pure samples of the solanapyrones eluted in the following fractions; solanapyrone A 12–25, solanapyrone B 29–37, and solanapyrone C 42–47. Intermediate fractions gave small quantities of mixtures.

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Entomology

Evaluation of Some Insecticides Against *Helicoverpa armigera* on Chickpea at Badaun, Uttar Pradesh, India

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Chickpea is one of the most widely cultivated pulses in India. It suffers from damage by the pod borer, *Helicoverpa armigera* (Hübner)—a major yield reducing factor. Pod-borer damage varies considerably in different agroclimatic regions in India (Sehgal 1990). Pest damage surveys conducted in Uttar Pradesh (UP) by Lal et al. (1985) revealed that only 4.4% chickpea farmers sprayed/dusted with common and cheap insecticides. The few farmers who apply insecticide complain that insecticides are not effective because plants develop insecticide resistance (Armes et al. 1992). Given this situation, new insecticides need to be evaluated to manage the pest effectively.

Field experiments were conducted in Ujhani Regional Research Station of Govind Ballabh Pant University of Agriculture and Technology in the postrainy seasons of 1993/94 and 1994/95. There were nine treatments including chemical insecticides with some new formulations, microbial insecticides, and neem-based formulation (Table 1). Chickpea variety, Pant G 114, was sown on 7 Dec 1993 and 30 Nov 1994 in 5 × 3-m plots at an interrow spacing of 30 cm in a randomized block design with four replications. The soil of the experiment was light sandy in texture. The crop was irrigated twice at 25 and 50 days after germination. Two applications of each insecticide were made at pod initiation and 15 days later. Pod-borer damage was recorded on 16 randomly selected plants from each plot at crop maturity. Yield of sampled plants was also added to the plot yield to calculate total yield.

The data from 2 years are given in Table 1. The data indicate that pod-borer damage was significantly lower in all the treatments except Nimbecidine® (azadirachtin) and Dipel® (*Bacillus thuringiensis* ssp *kurstaki*)