The Role Of Hydroxamic Acids In Take-all Resistance

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by
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Statement of Originality

Unless indicated otherwise, the results presented in this thesis are from original works of the author.

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ABSTRACT

The occurrence of hydroxamic acids (Hx) and their affects on take-all have been investigated in this study. An improved HPLC procedure for the separation and quantification of Hx in wheat, rye and triticale roots was established. This method completely separated 2,4-Dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2 , 4-Dihydroxy - 7- methoxy - 1 4- benzoxazin -3-one (DIMBOA), 2(3)-benzoxazolinone (BOA) and 6-methoxybenzoxazolinone (MBOA) within 17 min.

DIMBOA was the only Hx found in wheat roots, whereas both DIMBOA and DIBOA were present in the roots of triticale and rye. The Hx content of whole roots of wheat, rye and triticale reached a maximum 3 to 4 days after germination, depending on species. The DIMBOA content of wheat roots ranged from 0.4 to 1.5 µmoles/g f.wt in the varieties studied. The DIMBOA content of the triticale varieties ranged from 0.9 to 2.0 µmoles/g f.wt, and DIBOA from 0.26 to 1.1 µmoles/g f.wt. DIMBOA concentrations in rye roots ranged from 0.3 to 0.5 µmoles/g f.wt, whereas DIBOA levels ranged from zero to 1.1 µmoles/g.f.wt.

The Hx content of wheat, rye and triticale roots was highest in the youngest parts of the root. The root tip of these cereals always contained significantly higher levels of Hx than the older parts of the root.

When extracts prepared from triticale and rye roots were incorporated into the nutrient media, growth of two isolates of *Gaeumannomyces graminis* var.*tritici* (*Ggt*) (EBI and WP 28) was inhibited. Similar extracts prepared from wheat did not inhibit the growth of *Ggt*. The fungal strain WP 28 actually grew more rapidly on medium containing extracts from wheat (cv. Sunstar) roots. The inhibitory effect of triticale and rye extracts was attributed to the presence of DIBOA. The inhibitory effect of these

particular extracts correlated to the resistance of the respective plant to take-all in the field as reported by Hollins *et al* . (1986).

Hydroxamic acids inhibited the growth of *Ggt* when incorporated into the growth media. DIBOA and BOA significantly inhibited the growth of both strains of the fungus at concentrations as low as 0.5 mM. DIMBOA and 6-methoxybenzoxazolinone (MBOA) did not significantly inhibit the growth of *Ggt* EBI at 0.5 mM. However, at higher concentrations DIMBOA and MBOA were inhibitory. The Hx at the concentrations studied (0.5 to 5.0 mM) were only fungistatic, though, as the fungal colonies resumed growth when removed from the inhibitor. There was no significant difference in the growth of the two fungal isolates on media containing extracts or Hx.

The wheat variety with the lowest DIMBOA content was the most susceptible to infection by the fungus. Wheat contained only DIMBOA, which was undetectable by 21 days. The cereals, rye and triticale, which contined both DIMBOA and DIBOA were more resistant to the take-all fungus. Hydroxamic acid levels in triticale and rye were low or not detectable at 21 and 35 days. Rye was the more resistant species out of the two. Increased synthesis of Hx was not observed in roots of these cereals as a response to infection by the take-all fungus. On the basis of these results, it was concluded that DIBOA was more effective than DIMBOA in conferring resistance to take-all.

Wheat varieties which had an individual rye chromosome inserted were assayed for Hx content. All lines contained DIMBOA but one line (CSB 5R) also contained DIBOA. This preliminary result indicates that the gene(s) responsible for DIBOA synthesis may be on chromosome 5 of rye.

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ABBREVIATIONS

AWB	Australian Wheat Board
BOA	2(3)-Benzoxazolinone
cv(v)	Cultivar(s)
DIBOA	2,4-Dihydroxy-1,4-benzoxazin-3-one
DIBOA-Glc	2,4-Dihydroxy-1,4-benzoxazolinone-β-glucoside
DIMBOA	2,4-Dihydroxy-7-methoxy-1,4 benzoxazin-3-one
DIMBOA-Glc	2,4-Dihydroxy-7-methoxy-1,4 benzoxazolinon-β-glucoside
f.wt	Fresh weight
Gg	*Gaeumannomyces graminis*
Gga	*Gaeumannomyces graminis* var. *aveneae*
Ggg	*Gaeumannomyces graminis* var. *graminis*
Ggt	*Gaeumannomyces graminis* var. *tritici*
HBOA	2-Hydroxy-1,4-benzoxazin-3-one
HMBOA	7-Methoxy-2-hydroxy-1,4-benzoxazin-3-one
HPLC	High pressure liquid chromatography
Hx	Hydroxamic acid
M	Molar
MBOA	6-methoxybenzoxazolinone
mol	Mole
PDA	Potato dextrose agar
SE	Standard error
TAD	Take-all decline
w/v	Weight for volume

CHAPTER ONE

Introduction

1.1 General introduction

Take-all is a crown and root-rotting disease most commonly found in wheat and barley. It is caused by a soil-borne fungal pathogen and was recognized in Australia as early as 1852 (Garrett, 1981). Due to limited means of control it is considered as one of the most serious diseases of wheat in Australia.

In terms of area devoted to production and total value to the economy, wheat is Australia's most important crop (Fig 1.1). In the 1994-95 growing season, nearly 8 000 000 ha were dedicated to the growing of wheat, which contributed $1.993 b to the Australian economy (AWB, 1994-95).

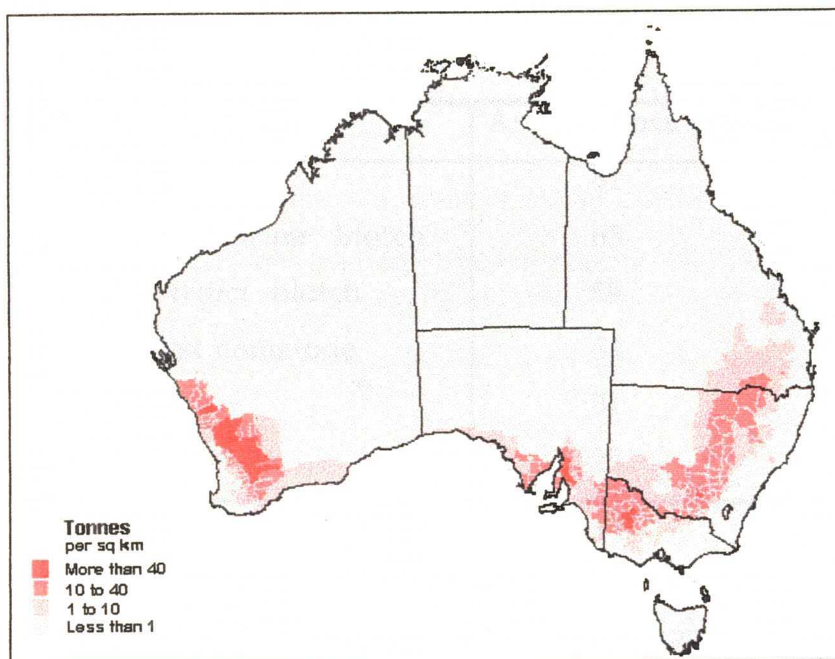


Fig 1.1 : Wheat growing areas of Australia showing production for 1993-94. (Year Book Australia, 1996).

Take-all is more severe in the southern wheat growing areas than in the northern wheat belt. It is also the most serious root disease of wheat occurring throughout the Western Australian grain belt and has led to wheat cropping being discontinued in some of the southern or high rainfall areas of this region (Cotterill and Sivasithamparam, 1989).

The quantity and quality of the grain yield can be reduced by wheat diseases. Recent figures for losses incurred due to take-all and other diseases are not readily available in literature. Brennan and Murray, however, performed a study in 1988 that reported the annual loss due to wheat diseases in Australia was around $400 m, and more recently an article in a publication aimed at wheat growers reported that take-all alone cost the economy as much as $200 m (Mussared, 1996; Murray and Brown, 1987; Brennan and Murray, 1988). In 1992, take-all cost growers in South Australia around $60 m (Mussared, 1996).

Disease	Annual Loss ($mn)
Take-all	81
Septoria nodorum blotch	63
Septoria tritici blotch	59
Cereal cyst nematode	54
Black point	31
Yellow spot	25

Table 1.1: ***Major diseases of wheat in Australia.*** The 6 major diseases of wheat in Australia according to Brennan and Murray (1988) in order of estimated annual losses, taking into account available control practices.

1.2 Geographical distribution of take-all

In addition to its occurrence in Australia, take-all occurs widely around the world in temperate climates causing severe yield losses in many cereal growing regions. Take-all has been reported to affect wheat in the USA (Cook and Reis, 1981; Duffy and Weller, 1994), England (Polley and Thomas, 1991), Sweden (Nilsson, 1977) and high rainfall areas of South Africa (Yarham, 1981). It has also been reported in Canada, Belgium, The Netherlands, Switzerland, Brazil, Japan, (Yarham, 1981), Kenya and China (Garrett, 1981) but is generally a disease of lesser importance in these countries (Fig 1.2).

The fungus responsible for take-all is widespread in soils closely associated with the roots of native grasses and it is likely that the ploughing of native grassland for cultivation of cereals brings about the onset of disease (Garrett, 1981). In the field, root infection is favoured by soil temperatures of 12-20°C (Butler, 1961) and areas where precipitation is greater than the regional average (Smiley *et al.* , 1996).

1.3 Causative organism

When take-all symptoms were first noted, various suggestions as to their cause were proposed, but it was not until the late 1800's that the disease in wheat was correctly attributed to a fungal pathogen. Confusion surrounded the correct naming of the fungus for some time until 1972, when Walker named it *Gaeumannomyces graminis* (Sacc.) von Arx & Olivier var. *tritici* .

In 1875, Saccardo originally named the fungus *Rhaphidophora graminis*, after isolating it from the base of a rotting grass culm. The fungus was later transferred to the genus *Ophiobolus* as the generic name *Rhaphidophora* had already been used (Walker 1972, 1975). In 1890 Prillieux and Delacroix compared isolates from wheat in France with those described by Saccardo and identified the fungus as *Ophiobolus graminis* (Sacc.) (Walker, 1981).

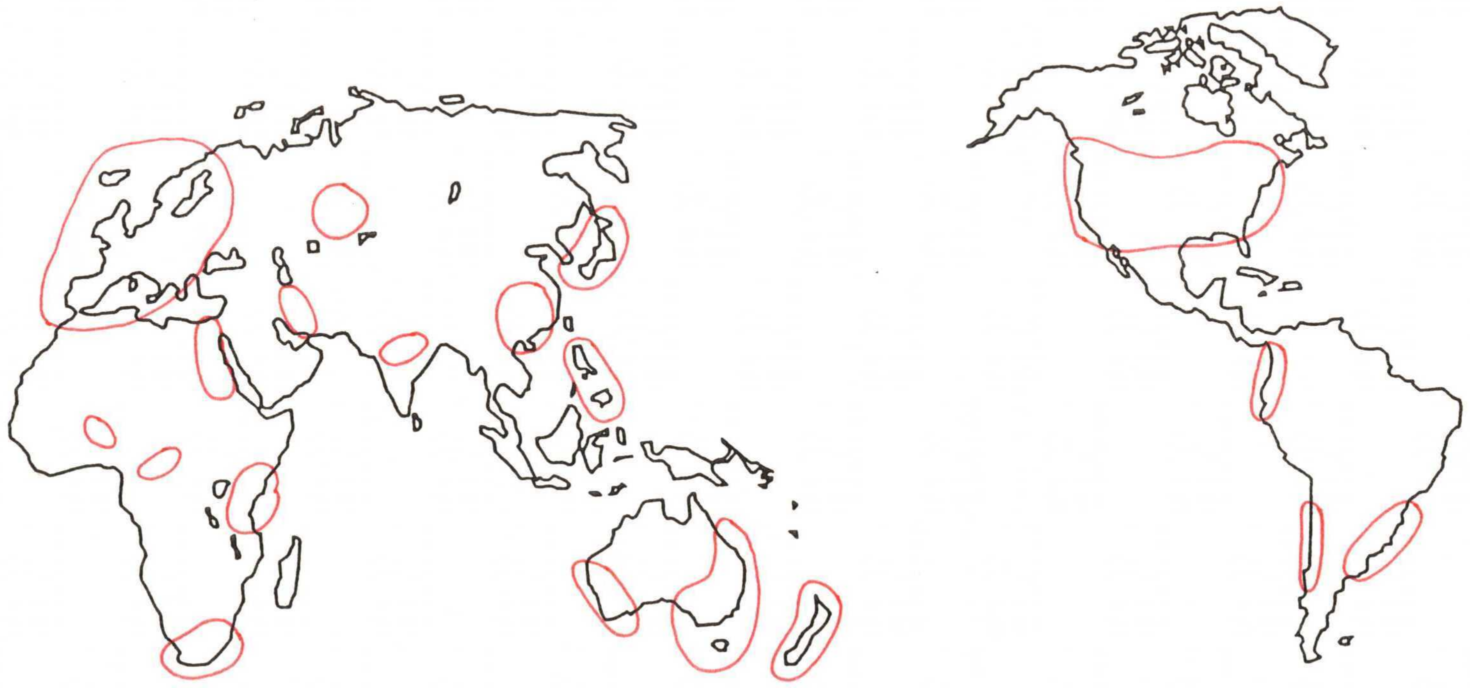


Fig 1.2 : Worldwide distribution of take-all. (C M I).

In 1952 von Arx and Olivier determined that the fungus did not technically belong in the genus *Ophiobolus* and created a new genus *Gaeumannomyces* , with the species name being *G.graminis* (Sacc.) von Arx and Olivier (*Gg*) (Walker, 1981).

In 1972, Walker examined several specimens and came to the conclusion that sheath rot in rice and the take-all disease of oats and wheat were all caused by different varieties of *Gg*. He assigned the cause of take-all in wheat to *Gaeumannomyces graminis* von Arx and Olivier var. *tritici* Walker (*Ggt*).

There are two other species which belong to this genus, *G. graminis* (Sacc.) von Arx and Olivier var. *aveneae* (Turner) Dennis (*Gga*) which causes take-all in oats, and *G.graminis* (Sacc.) von Arx and Olivier var. *graminis* (*Ggg*) which causes take-all of grasses. These three varieties are all closely related and form a compact taxonomic group.

1.4 Host species

Within the *Gramineae*, *G.graminis* (*Gg*) has a wide host range with numerous species reported to be susceptible to some degree. Due to its low saprophytic activity, inoculum levels depend on the presence of host species which harbour *Gg* in their roots (Cotterill and Sivasithamparam, 1987). Common hosts include non-cereal grasses which can act as a source of initial inoculum when cereals are planted (Cotterill and Sivasithamparam, 1989).

1.4.1 Ggg

Ggg is the least pathogenic of the three varities of *Gaeumannomyces* and causes little reduction in yields of cereals. It occurs in tropical and subtropical areas on *Oryza* (rice) and is commonly found in warmer parts of the world, colonizing the roots, stolons and crowns of various grasses (Walker, 1975).

1.4.2 Gga

The variety *Gga* is more virulent than *Ggg* and has a wider host range than *Ggt* (Nilsson and Smith, 1981; Walker, 1972). *Gga* can be found on the cereals *Avena* (oat), *Hordeum* (barley), *Triticum* (wheat) and turf grasses. *Gga* has the enzyme avenacinase which breaks down the glucoside avenacin in oats. Avenacin inhibits attack by *Ggt* and *Ggg* (Walker, 1975).

1.4.3 Ggt

Many cereals and grasses are susceptible to *Ggt*. Wheat is highly susceptible, barley is usually less so and rye can be considered moderately resistant. Triticale, the amphiploid between wheat and rye has been reported to be closer to wheat than rye in susceptibility (Hollins *et al.*, 1986). Oats are highly resistant to most isolates of *Ggt*. Cereals resistant to *Ggt* include *Oryza*, *Zea mays* (maize), *Setaria* (millet) and *Sorghum* (Scott, 1981). Brassicas such as canola and Indian mustard are also highly resistant and are recognized as break crops (Angus *et al.* , 1994). Field peas and other legumes, rape and linseed are also resistant and are effective rotation crops.

1.5 Symptoms of take-all

Although symptoms of take-all vary with soil conditions, seasons, hosts and geographic location, infection is most commonly indicated by blackening of the roots and crown and the premature death of plants before the grain matures (Wong, 1983; Penrose, 1991). Root rot can occur on plants at all growth stages (Walker, 1975). Some avirulent strains show no sign of these symptoms (Dewan and Sivasithamparam, 1990) and sometimes a generalized infection occurs where yield may be reduced without other obvious symptoms (Walker, 1975).

The first indication of disease in a crop is the appearance of patches in the crop where many young seedlings have been killed outright, hence the name take-all (Jones and Clifford, 1978).

1.5.1 Above ground

If seedlings survive, take-all patches may become interspersed with stunted plants and are often overcome with weeds (Jones and Clifford, 1978; Clarkson and Polley, 1981). Tillering in these plants is reduced and the ears ripen prematurely and produce badly pinched grain or no grain at all. The empty bleached ears are called whiteheads and they become visible under hot and dry conditions when crown root development is restricted and there is water stress resulting from earlier root rot (Walker, 1975; Wong, 1983). Wheats may, however, differ in their incidence of whiteheads due to environmental effects or through the presence of additional root pathogens.

The reduction of plant height and number of tillers are good indicators of take-all severity. The more pathogenic the isolate, the greater the reduction in shoot and root weight (Bassett *et al.*, 1986).

1.5.2 Below ground

The root system of infected plants becomes stunted and blackened and the whole plant can be pulled easily from the soil (Jones and Clifford, 1978). Discolouration usually spreads 2-5 cm up the stem (Wong, 1983) and black plate mycelium on the stem base may also be revealed by stripping off the lowest leaf sheaths (Clarkson and Polley, 1981). The degree of blackening varies greatly with seasons and climate (Walker, 1975). In wet weather, perithecia may be readily observed in this region as small black raised spots (Jones and Clifford, 1978).

In dry areas, such as in Western Australia and South Africa, disease development is slower and symptoms may differ. The drier conditions are usually considered unfavorable to the fungus (Clarkson and Polley, 1981; Cotterill and Sivasithamparam, 1989) and blackening of the stem base is rarely observed. Symptoms may include uneven ripening, reduced tillering,

furling of the leaves and dark greenish-brown discolouration of stem-base and roots.

1.5.3 In the laboratory

When examined in the laboratory infected roots show brown to black lesions, vascular discolouration and various degrees of blocking of the xylem vessels. Dark external runner hyphae can be seen. The stelar discolouration and the runner hyphae can be distinguished easily against a white background (Cotterill and Sivasithamparam, 1988).

1.6 Infection process

Take-all in a cereal crop usually originates from infected stubble residues in which *Ggt* has survived in the soil from a previous infected crop or on perennial grass weeds (Garrett, 1981). Although infection from residues is most common, disease may also result from the germination of ascospores present in the soil. Ascospores are not a common source of infection, however, due to microbial competition (Weste, 1972). Macrohyphae grow over the root surface which is then penetrated by microhyphae. The hyphae constrict in diameter while penetrating the cell wall and the plant cell forms lignitubers around the the hyphae as a penetration response. The fungus dissolves the lignituber and makes its way through the plant cells until it finally plugs the xylem and causes death of the plant.

1.6.1 Contact and penetration

The contact between *Ggt* and host roots is established by trophical growth with the hypha growing towards the root rather contacting it by chance (Weste, 1972). The mycelium of *Gg* consists of brown thick-walled macrohyphae or runner hyphae and hyaline, thin-walled microhyphae or infection hyphae (Skou, 1981). The macrohyphae are formed by the fusion of three to five of the fine hyaline hyphae which subsequently thicken and darken to form the runner hyphae (Weste, 1972). The runner hyphae are able

to spread readily over the surface of the host root system to form a mat of mycelium from which the penetration of the root by microhyphae occurs (Fig 1.3)(Walker, 1975; Penrose,1985).

Penetration of the root by the fungus initially occurs through the root hair, then through the intact epidermis in the regions of the elongating zone of the root. Penetration does not occur at the root tip. Infection of the root hairs may occur within seven to eight hours of ascospore germination (Weste, 1972). When the hypha penetrates through the root hair or any cell inside the root a penetration peg is formed. From this penetration peg, a narrow infection hypha invades the cell wall. Once the cell has been penetrated, the hypha regains its normal diameter and grows in all directions, though mainly transversely (Weste, 1972; Skou, 1981).

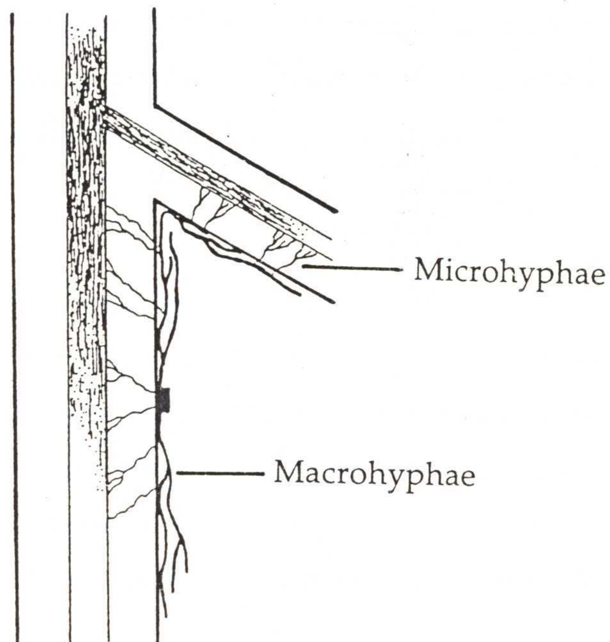


Fig 1. 3 : Diagrammatic representation of infection of roots by *Ggt* showing the two types of hypha and the plugging of the xylem.

1.6.2 Lignituber production

When an infection hypha forms a penetration peg the area around it becomes lignified as a host cell reaction. The thickening of the cell wall grows into tubular structures around the hypha called lignitubers. Lignitubers have been shown to be impregnated with lignin as observed following staining with safranin. Lignitubers may develop within 21 hours after infection (Weste, 1972). There is a correlation between the length of the lignituber and the infectivity of the fungus. A long lignituber results from a strong fungal infection with a prolonged production of lignituber (Holden, 1976). As long as lignitubers are being produced, the plasmalemma remains intact.

The infection hypha grows forward by dissolving the end of the lignituber. The lignituber is dissolved by the production of proteolytic, pectinolytic and cellulolytic enzymes (Weste, 1972). The hyphae within the lignitubers are always constricted in diameter when compared to other hyphae (Holden, 1976). However, once a lignituber has been broken the hypha regains its normal diameter and it continues to grow forward to either attack the next cell or fill the invaded cell with mycelium.

1.6.3 Growth in the root

Once the penetration of the root hair or epidermis has occurred, the macrohyphae are able to grow longitudinally through the cortical tissue to infect other cells. The hypha keeps growing like this until it reaches the endodermis. The endodermis constitutes a barrier to the fungus, such that all of the cortex may be invaded before the stele is penetrated. Penetration into the stele is believed to occur through the plasmodesmata (Skou, 1981).

Lignitubers may form in the stele as they do in the cortex, but only severe attacks reach the stele and from this point further disease development is rapid. Large macrohyphae grow longitudinally through the cortical tissue, and microhyphae grow out from these macrohyphae to attack the

endodermis. Hyphae grow along the xylem elements and the stele becomes blackened (Weste, 1972; Holden, 1976; Gilligan, 1980; Penrose, 1985) (Fig 1.4). The most extensive growth is achieved by hyphae in the xylem vessels (Holden, 1976). The rapid onset of stelar blackening reflects a greater rate of hyphal entry into the host's vascular tissue and therefore a more susceptible host (Penrose, 1985). In comparing infection between *Ggt* and *Ggg*, only *Ggt* penetrates the stele (Holden, 1976) and in oat roots infected with *Ggt* lignitubers and the plugging of the xylem are not observed (Weste, 1972).

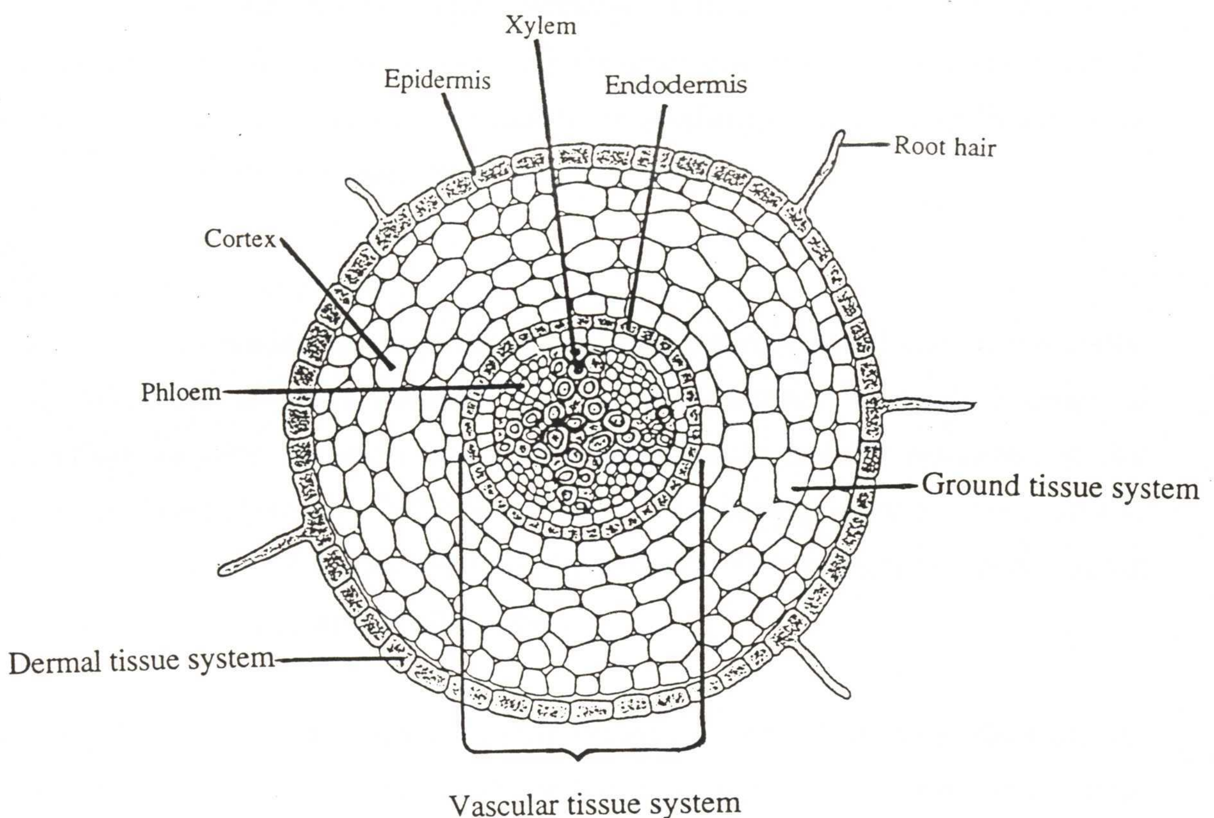


Fig 1.4 : The cross section of a root.

There is a definite preference in the growth of the pathogen up the roots from the initial sites of infection (Gilligan, 1980). The discolouration of the stele also follows this pattern, with the characteristic blackening occurring around the region of infection and up the root (Holden, 1976). Growing upwards facilitates the faster spread to healthy roots via the crown, whereas if the fungus were to grow down the root, the pathogen would be carried down the soil profile into regions of lower root density (Gilligan, 1980).

Only when all of the stele has been invaded does translocation cease. It has been shown that the translocation of isotopically labelled assimilates does not occur into regions below stelar blockage (Gilligan, 1980). The effects of blockage can be compared to severing the root and the induced water stress leads to the reduced vigour or death of seedlings, tillers and heads and shrivelling of grain (Holden, 1976; Penrose, 1991).

1.6.4 Growth in the crown

The crown is invaded through the subcoronal internode and the crown roots. The coleoptile is often covered with runner hyphae or with a crust of mycelium causing the stem base to blacken. This is usually restricted to the outer cell layers (Walker, 1975; Skou, 1981). The fungus may advance up the base of the tillers and may lead to tiller death and premature head death (Penrose, 1985; Duffy and Weller, 1994).

In the final phase of attack the fungus reverts to the saprophytic state on the remains of the dead plant where it produces perithecia on or in the straw base or in the roots.

In resistant hosts, penetration and occupation of the roots does occur but there is less mycelial growth and root destruction. The rate of root decay is equivalent to the rate of root replacement in resistant hosts (Weste, 1972).

When the host tissue is dead or is resistant, lignitubers are not produced (Skou, 1981).

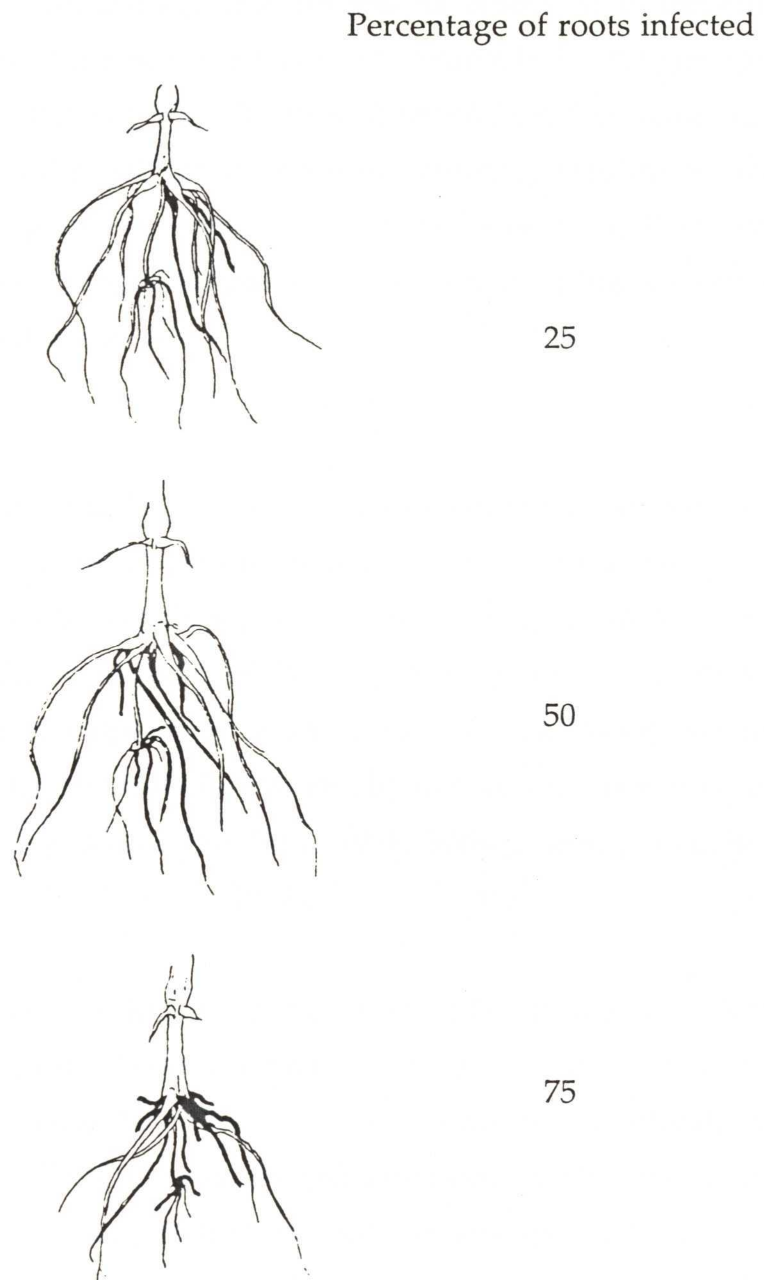


Fig 1.5 : A diagrammatic representation of take-all infection rated as the percentage of roots infected.

1.7 Control of take-all

One of the reasons that take-all is regarded as so important is that there are only limited means of controlling this disease in wheat. It is difficult to control by chemicals and little or no resisitance is available in the germplasm of cultivated wheats. Control of take-all can be effected by either reducing the inoculum potential in the soil or by creating growing conditions which favour the host more than the pathogen (Cotterill and Sivasithamparam, 1989). The only successful control measure is to use resistant break crops such as oats, rye, legumes and brassicas.

1.7.1 Crop rotation

Where it is economically viable, crop rotation still offers the best method to control take-all. Break crops in cereal rotations can increase the yield of subsequent wheat crops by depriving soil borne pathogens of a host and reducing infection of the subsequent wheat crop (Kirkegaard *et al.*, 1996). The use of resistant rotation crops such as rape, lupins, sunflower, sorghum, legumes, oat and rye may be limited, however, by factors such as soil type and length of growing season (Cook and Reis, 1981; Wong, 1983; Cotterill and Sivasithamparam, 1989; Angus *et al* , 1994).

Rotation of crops reduces the inoculum potential of *Ggt* in the soil (Walker, 1975; Jones and Clifford, 1978). Legumes, in particular, enhance the development of suppressive microflora to *Ggt*, which is a relatively weak competitive organism (Cotterill and Sivasithamparam, 1989) and therefore has more difficulty establishing itself on the roots of susceptible hosts.

Angus *et al* (1994) suggested that the differing effectiveness of break crops may involve disease inhibition as well as absence of the host. The effect of brassicas may be associated with the release of antifungal compounds from the roots or decaying tissue which supress soil borne fungal pathogens. Their study found that canola and Indian mustard released isothiocyanates (ITC's)

from their roots which inhibit the growth of *Ggt in vitro,* especially during the early stages of mycelial growth.

Due to some grass species maintaining the take-all fungus at high levels, wheat and barley should not be used as the first crop on new land, or after pasture (Walker, 1975; Wong, 1983). It is also important to maintain a weed free fallow. The take-all fungus may be better able to survive in unaffected rye grass roots, where it can establish an almost symbiotic relationship, than in roots of wheat susceptible to rotting, and subsequent breakdown from invasion by secondary colonizers (Dewan and Sivasithamparam, 1990).

1.7.2 Delayed sowing

Delayed sowing may be effective in reducing take-all but does not necessarily result in increased yields (Cotterill and Sivasithamparam, 1989). Reduced severity of take-all is related to a reduced inoculum level at the later sowing date. Resistance of the roots increases with age, and, as a result infection develops more slowly when the plants are invaded after the seedling stage. The delayed onset of infection gives the plant a better capacity to produce new crown roots and therefore escape the most severe damage (Nilsson, 1973).

1.7.3 Fertilizers

Fertilizers applied to wheat enhance plant growth and yield and they often exert a secondary effect on yield through their effects on the take-all fungus. A close inverse relationship between the nutritional status of the plant and the severity of take-all has been noted (Butler, 1961; Brennan, 1992). Generally, NH_4^+ - N is an effective means of reducing take-all. Smiley and Cook (1973) found that the form of N affects take-all due to a pH effect in the rhizosphere. Disease incidence is reduced by NH_4^+ - N through increasing rhizosphere acidity, whereas NO_3^- - N increases disease severity by making the rhizosphere more alkaline, which favours the growth of the fungus. The NH_4^+ - N effect is negated by liming (Walker, 1975).

The application of fertilizers containing phosphate also helps to reduce losses due to take-all. This has been suggested to be due to more vigorous host root development and, in conditions of adequate soil N, it is a particularly valuable measure for control (Butler, 1961). In field studies, the percentage yield increased, due to declines in the severity of take-all as the level of P applied increased. Where levels of take-all were high (>65% of roots infected), the P was less effective in reducing the levels of *Ggt* (Brennan, 1995).

Chloride ions in fertilizers have also been found to decrease take-all under some conditions, although the mechanism is unknown (Cook and Reis, 1981).

1.7.4 Fungicides

Fungicides and fumigants offer little advantage in control of take-all because of cost, and their effects on the soil and subsequent crops are questionable (Huber, 1981). Baytan, (4-chlorophenoxy-1,1-dimethyl-1,2,4-triazole-1-ethanol) is the only fungicide registered for take-all in the USA and it is not widely used because its performance has been inconsistent (Harrison, 1993). Baytan causes mild stunting and limited tiller formation, although the tillers on plants treated with Baytan were healthier and yields greater.

1.7.5 Tillage practices

The effect of different tillage practices on take-all severity has been variable in the past with conflicting results found for each practice. The incorporation of straw or grass residues into the seedbed is said to increase disease severity by distributing inoculum throughout the soil profile. The soil is also much looser enabling Ggt to grow through the soil more easily to infect wheat roots (Cotterill and Sivasithamparam, 1989). Conservation tillage leaves crop residues on the soil surface for protection against soil erosion and water evaporation, however, this enables the fungus to survive in the soil and

infect subsequent crops. As a result it has been proposed that stubbble or grass should be removed before wheat is planted (Yarham, 1981).

Direct drilling is the extreme tillage practice, whereby all residue of the previous crop is left on the soil surface and the crop is drilled directly through the residue and into the soil (Cook and Haglund, 1991). Direct drilling leaves inoculum in a layer at the surface ideally positioned next to the crown (Cotterill and Sivasithamparam, 1989; Cook and Reis, 1981). As a result seeds need to be sown below this infective layer, but deeply sown wheat is subject to more physical damage (Butler, 1961). Direct drilling also favours the development of grass weeds which can act as a carrier for Ggt (Yarham, 1981). Direct-drilled plots usually show less take-all even though they have similar infectivity and propagule numbers to deep cultivated plots at harvest (Cotterill and Sivasithamparam, 1989).

1.7.6 Biocontrol

Cross-protection or biocontrol is the protection of a plant from infection by a microbial pathogen following the simultaneous or prior exposure to another microorganism (Wong, 1981). Organisms known to protect wheat from take-all include *Phialophora, Trichoderma, Pseudomonas, Streptomyces, Bacillus* and *Ggg*. These organisms protect the plant either by occupying the area that would have been occupied by *Ggt,* or by producing chemicals which inhibit the growth of the fungus.

Competitive species such as *Phialophora, Ggg,* and hypovirulent isolates of *Ggt* have been shown to protect wheat against take-all in glasshouse and field experiments (Jones and Clifford, 1978; Deacon, 1974; Cook and Reis, 1981 and Wong, 1981). It is possible to establish *P. radicicola* and *Ggg* directly by seed inoculation because these fungi grow well over the seed and stem base, depending on inoculum levels (Deacon, 1974, Prew, 1981). Avirulent fungi such as these are successful primarily because they compete for the same

ecological niche as the pathogen and appear to displace it by their prior occupation (Wong, 1981).

The use of cold-tolerant isolates of *Ggg* as biocontrol agents is considered important as the temperature of soil is between 5-15°C during the early growing period when cereals are most susceptible. The cold isolates can compete favourably against the take-all fungus for colonization of wheat roots, thereby protecting the root system. The yields from wheat plants colonized by these competitive fungi were not significantly different from those of healthy wheat not inoculated with take-all. These fungi also protect wheat roots, by inducing host resistance. *Ggg* and *Phialophora* spp. induce greater lignification and suberization of the endodermis and xylem vessels in roots which are more longer term protection mechanisms than the production of labile antibiotics or fungitoxic compounds (Wong *et al.*, 1996)

Dewan and Sivasithamparam (1988) found that the presence of *Trichoderma hamatuum* or *T.koningii* reduced the mortality of wheat and rye-grass plants inoculated with the take-all fungus. They found an increase in the fresh shoot and root weights, presumably as a result of the higher frequency of *T. koningii* observed on the roots at seedling stage. Recovery of *Ggt* from wheat roots was low when populations of *Trichoderma* spp. were high.

Fluorescent pseudomonads that produce antibiotics also belong to the microflora antagonistic towards *Ggt* (Thomashow and Weller, 1988, 1990; Renwick *et al.*, 1991; Harrison *et al*, 1993). These beneficial strains compete aggressively for sites on roots or in the rhizosphere where nutrients are available. They have also been shown to antagonize pathogens by producing a variety of metabolites that include antibiotics and siderophores (Thomashow and Weller, 1990).

Harrison *et al* (1993) showed that strains of *Pseudomonas* produced strong zones of inhibition against *Ggt* in growth trials conducted on sterile media. The antibiotic 2,4-diacetylphloroglucinol is believed to be the primary reason for take-all suppression in these studies. Thomashow and Weller (1988, 1990) have found an antibiotic in *Pseudomonas fluorescens* called phenazine-1-carboxylate (PCA) which is active against *Ggt* at concentrations as low as 1µg/ml. *Pseudomonas* mutants unable to produce phenazine (*phz*$^-$) are no longer inhibitory to *Ggt in vitro*, and even though they are still able to colonize roots, they provide less control of take-all on seedlings. Significant control of take-all occurs in the presence of PCA in concentrations as little as 25-30 ng/g root.

A range of microbial antagonists were isolated from wheat roots after the plants showed a reduction in the levels of take-all *in vivo*. Bacteria including *Bacillus, Streptomyces* and *Pseudomonas* were the most common isolates found. These bacterial isolates were able to grow on agar containing mycelia from *Gg*. The mycelia possessed chitinase or β-glucanase activity, or both, which caused the lysis of the fungal cells. The suppression shown by this group of bacteria may be due to the bacteria competing for space on the root, or these isolates might damage fungal cell walls, thereby protecting plants from fungal attack (Renwick *et al* , 1991).

1.7.7 Resistance

Some cereal varieties show a degree of resistance to take-all and the possibility of increasing resistance in commercial varieties is a worthwhile objective. Increasing resistance may be especially useful when used in conjunction with other methods aimed at minimizing take-all.

The resistance of triticale has great potential benefit to cereal growers. Although less resistant than rye, triticale may provide an improved alternative crop to wheat where yield is limited by take-all. It could also

substitute for barley, which is nearly as susceptible to take-all as wheat (Hollins *et al*, 1986). Cross-breeding with triticale may provide a means for eventually transferring resistance from rye into wheat.

1.8 Take-all Decline

Take-all Decline (TAD) is defined as the spontaneous reduction in take-all and an increase in yield with the continued cropping of wheat or barley (Rovira and Wildermuth, 1981; Andrade *et al.*, 1994). Take-all decline was first reported in England (Walker, 1975) and has also been observed in Canada, USA, Holland, France, Yugoslavia, Switzerland and Denmark (Rovira and Wildermuth, 1981). Take-all decline can occur under continuous cereal cropping in Australia but it is more short-lived than in other countries and its effects are often lost completely (Yarham, 1981). Take-all decline occurs when there is an abundance of old diseased plant material and a susceptible cereal monoculture is maintained (Walker, 1975). If there is a break from the monoculture the TAD is destroyed.

It has been reported that the number of root infections on wheat in TAD soil is the same as that in non-decline soil but there is much less rapid lesion development on plants in the decline soil (Walker, 1975). There have also been suggestions that TAD is due to changes in the soil microflora as antagonistic organisms are found in greater abundance in TAD soils than in other soils. Andrade *et al.* (1994) found populations of *Bacillus* spp., fluorescent pseudomonads and actinomycetes antagonistic to *Ggt* were higher in suppressive soils than in the nonsuppressive soils. Mycoparasitism is also involved in TAD. Two fungi were isolated that suppressed *Ggt* in a field test. One of these strains was then shown to parasitize *Ggt* on potato dextrose agar.

Cook and Reis (1981) found that the factors for TAD could be transferred from field to field and that they became ineffective if the monoculture was

interrupted with a break crop. They found the responsible agents to be very small and sensitive to moist heat (60°C, 30 min) and methyl bromide.

1.9 Summary

From this review of the literature it can be seen that take-all has been a particularly destructive disease due to its widespread occurrence, its reduction in yields and subsequent cost to wheat growers and the few available control practices. As has been shown, some microorganisms can protect wheat from take-all by producing compounds that inhibit the growth of the fungus. This however, requires the microorganisms to be present in the soil and to be able to compete for an ecological niche on the wheat root. It would be more beneficial for the plant to be able to produce inhibitory compounds as an inbuilt defence mechanism. This would overcome the need to sow wheat with seed treatments to ensure the growth of antagonistic organisms. Therefore the development of wheat varieties that have some natural resistance is important for control of take-all. Resistance could be enhanced by the production of compounds such as secondary plant products which are naturally present in the plant, which may also inhibit the growth of the fungus.

1.10 Secondary plant products

Secondary products are "those natural products usually of plant origin which do not function directly in the primary biochemical activities that support the growth, development and reproduction of the organism in which they occur" (Conn, 1980).

Secondary plant products are thought to have evolved as a survival mechanism. As plants lack the ability to move, secondary plant products may have a role in the survival of the organism by deterring predators and competing plant species or attracting pollinators or symbionts. Some of the more well known secondary plant products include alkaloids, isoprenoids, and phenylpropanoids, however, these classifications are quite broad and are not representative of all the secondary plant products that have been identified.

Alkaloids are nitrogenous compounds that occur in higher plants. To date over 10 000 alkaloids have been identified. Alkaloids can be classified into several groups, including indole alkaloids, tropane alkaloids, benzylisoquinoline alkaloids and bisbenzylisoquinoline alkaloids. The indole alkaloids are based on tryptophan and include quinine (antimalarial agent) and strychnine (tetanic poison). Tropane alkaloids are based on putrescine and include atropine (anticholinergic antidote) and cocaine (topical anaesthetic). Benzylisoquinoline alkaloids are based on reticuline and include morphine (narcotic analgesic) and codeine (analgesic). Bisbenzylisoquinoline alkaloids are a smaller group containing less well known alkaloids (Kutchan, 1995).

Isoprenoids are compounds synthesized by a repetitive joining of isoprene (2-methyl-1,3-butadiene) units. Isoprenoids include mono-, sesqui-, di- and tri-terpenes, high molecular weight polymers such as rubber, carotene and other carotenoids and steroids. Terpenes include essential oils characteristic

for plant fragrances for example menthol (peppermint), pinene (turpentine) and limonene (oil of lemon and orange). Rubber is produced by many dicotyledenous plants and may contain between 1500-60 000 isoprene units depending on species (McGarvey and Croteau, 1995).

Phenylpropanoids include flavonoids and isoflavonoids, coumarins, suberin and lignin and other wall bound phenolics. The function of phenylpropanoids is varied with some serving as flower pigments, UV protectants, insect repellants and also as complex polymeric constituents of surface and support structures such as lignin (Hahlbrock and Scheel, 1989). Phenylpropanoids are so named because of the basic structure of a three-carbon side chain on an aromatic ring which is derived from phenylalanine (Whetten and Sederoff, 1995). Phenylalanine is converted to 4-coumarate from which the individual phenylpropanoids are derived (Hahlbrock and Scheel, 1989).

Lignin is a polymer of aromatic subunits however the composition differs between species and cell types within a species. It serves as a matrix around the polysaccharide components of some plant cell walls giving strength and repelling water. Flavonoids are induced to protect plants against UV light and are also a response to wounding. Furanocoumarins and suberin are induced by the attack of fungal pathogens (Hahlbrock and Scheel, 1989; Whetten and Sederoff, 1995).

Hydroxamic acids are secondary metabolites which are found in numerous plant species. Theses compounds are derived from the shikimic acid pathway (Reimann and Byerrum, 1964). The function of Hx in plants is not well defined but they have been shown from previous studies to inhibit the growth of *Ggt in vitro* (Klein and Marshall, 1989). It has since been proposed that hydroxamic acids may provide cereals with resistance to the take-all fungus *in vivo*.

1.11 *Chemistry of hydroxamic acids*

1.11.1 General introduction

Hydroxamic acids (Hx) are secondary metabolites which have a 4-hydroxy-1,4-benzoxazin-3-one structure (Niemeyer, 1988a). The hydroxamic acid moiety occurs in compounds that have activity as antibiotics, growth factors, tumour inhibitors, cell-division factors and pigments and may have a role in iron uptake (Maehr, 1971).

Hydroxamic acids were first discovered in 1955 by Virtanen and Hietala when incorporation of rye extracts containing Hx into a growth medium were found to inhibit the growth of *Fusarium nivale*. The inhibitory compound was found to be 2(3)-benzoxazolinone (BOA) which was originally regarded as the active compound but it was later found to be a decomposition product of an aglucone precursor (Virtanen and Hietala, 1960).

1.11.2 Classification of hydroxamic acids

Hydroxamic acids may be classified according to the number of hydroxamate units per molecule (Fig 1.6). There are three major classes of hydroxamic acids each having one, two or three units per molecule. Primary hydroxamic acids have one hydroxamic acid unit while di- and tri-hydroxamic acids have two and three units respectively per molecule. Di-hydroxamic acids may also be linear or cyclic and to date compounds with more than three hydroxamic acid functions per molecule have not been reported (Maehr, 1971).

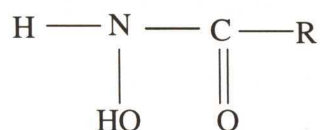


Fig 1.6 : A primary hydroxamic acid unit.

The 4-hydroxy-1,4-benzoxazin-3-ones belong to the secondary cyclic Hx group. 2,4-Dihydroxy-7-methoxy-1,4-benzoxazolinone (DIMBOA) and 2,4-dihydroxy-1,4-benzoxazolinone (DIBOA) can be further classified as containing an oxazine skeleton (Fig 1.7).

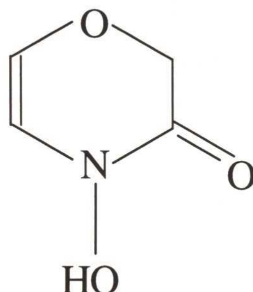


Fig 1.7 : Oxazine skeleton.

1.11.3 DIBOA related compounds

Virtanen and Hietala (1955) first isolated Hx as a substance which inhibited the growth of *Fusarium nivale* when incorporated into agar medium. The physical and chemical properties of the substance and the empirical formula led to the structure and name of 2(3)-benzoxazolinone (BOA). It was later found that BOA is formed from an aglucone precursor which in turn is the product of an enzymic hydrolysis of the primary glucoside compound found in intact rye (Virtanen and Hietala, 1960).

Hydroxamic acid-glucosides (Hx-Glc) are the primary precursors and Hx occur in this form in intact plants. Hydrolysis of the Hx-Glc by β-glucosidase leads to the formation of the corresponding aglucone. The main Hx in rye is 2,4-dihydroxy-1,4-benzoxazolinone-β-glucoside (DIBOA-Glc) which undergoes hydrolysis to form the aglucone 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA).

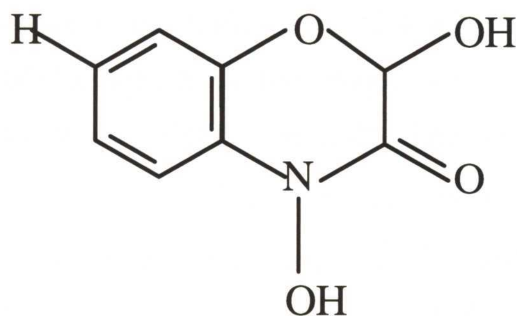


Fig 1.8 : 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA).

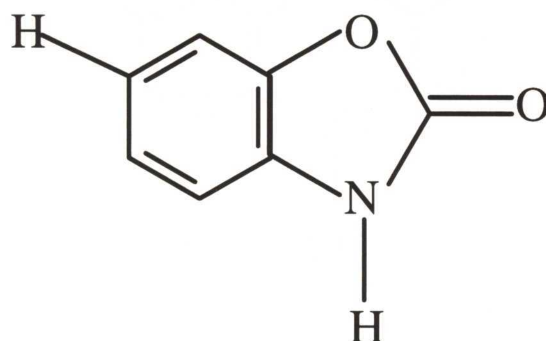


Fig 1.9 : 2(3)-benzoxazolinone (BOA).

By comparing the UV spectra of the glucoside and aglucone, and characterizing the sugar formed on hydrolysis, it was confirmed that only the release of glucose takes place in the enzymic decomposition of the glucoside. The enzymatic hydrolysis of the glucoside occurs rapidly at room temperature in aqueous extracts.

1.11.4 DIMBOA related compounds

The methoxylated analogue of DIBOA is 2,4-dihydroxy-7-methoxy-1,4-benzoxazolinone (DIMBOA), which is the major Hx found in wheat and maize (Wahlroos and Virtanen, 1959) (Fig 1.10). The primary precursor of DIMBOA is 2 , 4 -dihydroxy- 7- methoxy - benzoxazolinone - β - glucoside (DIMBOA-Glc) which also undergoes enzymic hydrolysis to form the aglucone DIMBOA. DIMBOA forms the benzoxazolinone MBOA (Fig 1.11) and formic acid upon heating aqueous solutions (Wahlroos and Virtanen, 1959; Virtanen and Hietala, 1960).

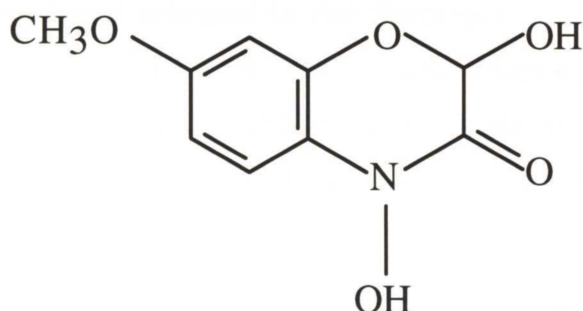


Fig 1.10 : 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA).

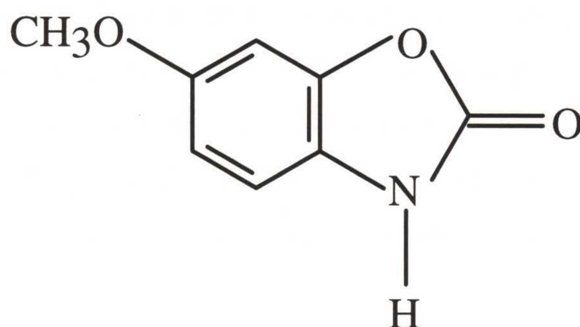


Fig 1.11 : 6-methoxybenzoxazolinone (MBOA).

1.11.5 Decomposition of hydroxamic acids

At pH values above the pK_a of the Hx group (*ie.*, above a pH of about 6.95) the 2,4-dihydroxy compounds are unstable (Ioannou *et al.*, 1980). Upon heating in aqueous solution, the six membered ring of the aglucone decomposes to the benzoxazolinone and formic acid (Bredenberg *et al.*, 1962; Virtanen and Hietala, 1960).

Benzoxazolinones account for 60-70% of the decomposition products of the Hx. The other major product is formic acid. Other products are not well characterized but include a red-brown pigment which may come from the oxidation of *o*-aminophenol released in the decomposition process (Kumar *et al.*, 1994). In conditions of low pH Bravo and Niemeyer (1986) detected another decomposition product of DIMBOA in yields of around 10%. Their data shows that this compound is most likely an isomer of DIMBOA.

The amount of benzoxazolinone formed from the aglucone is quantitative in the pH range 4 to 8, with the slow formation of a small amount of side products noted at pH 9.7 (Bredenberg *et al*, 1962). The amount of benzoxazolinone formed decreases when the aglucone concentration rises (Hietala and Virtanen, 1960). The benzoxazolinones are relatively stable and do not undergo any further degradation (Tipton *et.al.*, 1973).

For the decomposition of aglucone to benzoxazolinone, the presence of an N-hydroxyl group and an easily ruptured C1-C2 bond is a prerequisite. The first step is pH dependent and involves the formation of a hemiacetal-aldehyde equilibrium (Fig 1.12). The rate determining step is the breaking of N-O, C-C bonds in the hemiacetal-aldehyde function, with the subsequent formation of the formate ion.

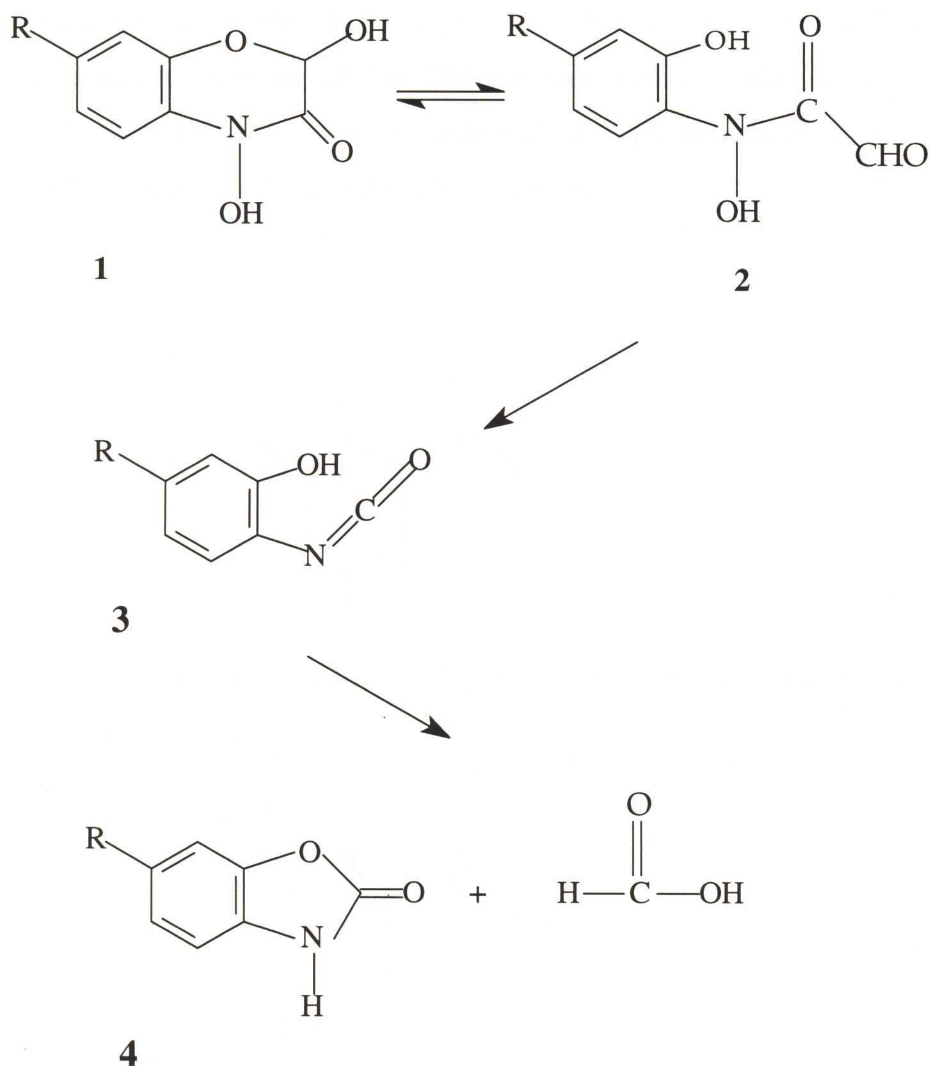


Fig 1.12 : The chemical decomposition of the aglucone to the benzoxazolinone. 1 - Hydroxamic acid, 2 - aldol form, 3 - isocyanate, 4 - benzoxazolinone and formic acid. The formation of isocyanate is the rate limiting step (Niemeyer, 1988a).

1.11.6 Reaction centres in the Hx molecule

The hydroxamic acid function (Fig 1.13) and the hemi-acetal at C-2 (Fig 1.14) are the main reactive centres of Hx (Bravo and Lazo, 1993). As discussed previously, the formation of the hemi-acetal is also the first step in the breakdown of the aglucone to the benzoxazolinone.

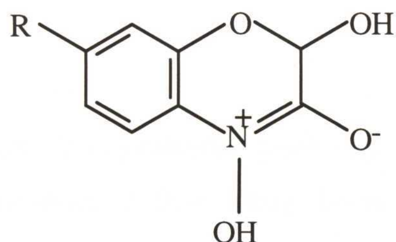


Fig 1.13 : Resonance form of 2,4-dihydroxy-1,4-benzoxazin-3-ones.

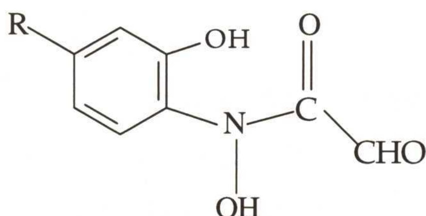


Fig 1.14 : Opening of the hemi-acetal at C-2 to form the acyclic structure.

The aldehyde function of the acyclic form is a highly reactive electrophile as the adjacent carbonyl group drains electron density from it. As a result, in the presence of nucleophiles, DIMBOA should form addition products readily. The N atom is prone to attack by reducing agents due to electron withdrawal from the adjacent carbonyl groups. Reducing agents cause the hydroxamic N to be slowly reduced to an amide N. It is therefore possible that DIMBOA may react with sulphydryl groups in enzymes causing their inactivation.

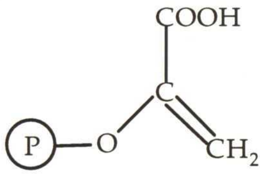
Hydroxamic acids are able to form coordination compounds. As a bidentate group, the anionic hydroxamate function resembles the acetylacetonato ligand in its behaviour towards various chelate ions. Hydroxamic acids have a distinct preference for ferric iron [Fe(III)] (Maehr,1971), with stability constants for ferric ion complexes with DIMBOA and DIBOA being higher than those for citric acid which is known to participate in microbial iron metabolism and absorption (Barnes and Putnam, 1987).

1.12 Biosynthesis of Hx

To date the complete biosynthetic pathway for Hx has not been fully elucidated. In the few studies that have been carried out, the biosynthetic pathway has been shown to be related to the shikimic acid pathway (Fig 1.15). Label from the shikimate precursors quinic acid , methionine, ribose, glycine and glycerate are specifically and extensively incorporated into DIMBOA.

1.12.1 Biogenesis of the aromatic ring

In one of the first studies performed on the biosynthesis of Hx, Reimann and Byerrum (1964) found that quinic acid, glycerate and ribose were incorporated in DIMBOA. All three metabolites can be converted to shikimic acid which is then utilized in the biosynthesis of the aromatic ring. Glycerate enters the shikimic acid pathway as phosphoenolpyruvate. Degradation studies showed that essentially all the ^{14}C incorporated from quinic acid was located in the benzene ring carbons with ^{14}C-glycerate and ^{14}C-ribose being incorporated to a lesser extent. Tryptophan was incorporated with a very large dilution when compared to other precursors, and therefore seems unlikely to play an active role in the biosynthesis of the aromatic ring (Reimann and Byerrum, 1964).



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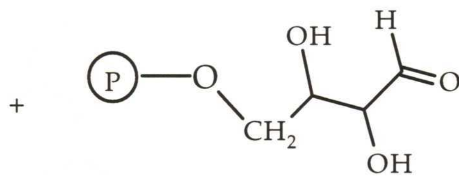
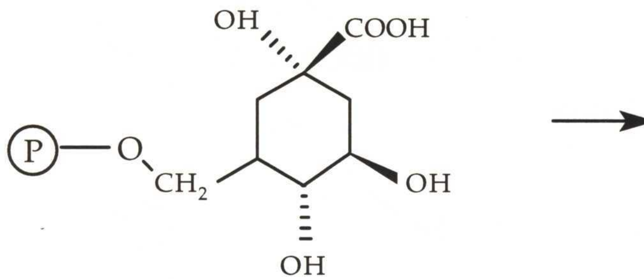
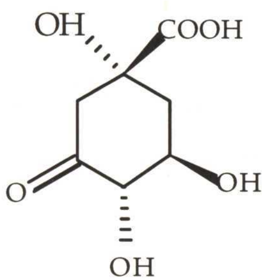


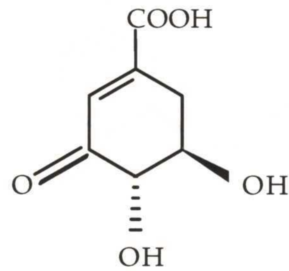
Phosphophenolpyruvate

Erythrose 4-phosphate



3-Deoxy-arabino-heptulosonate-7-phosphate





3-Dehydroquinate

3-Dehydroshikimate

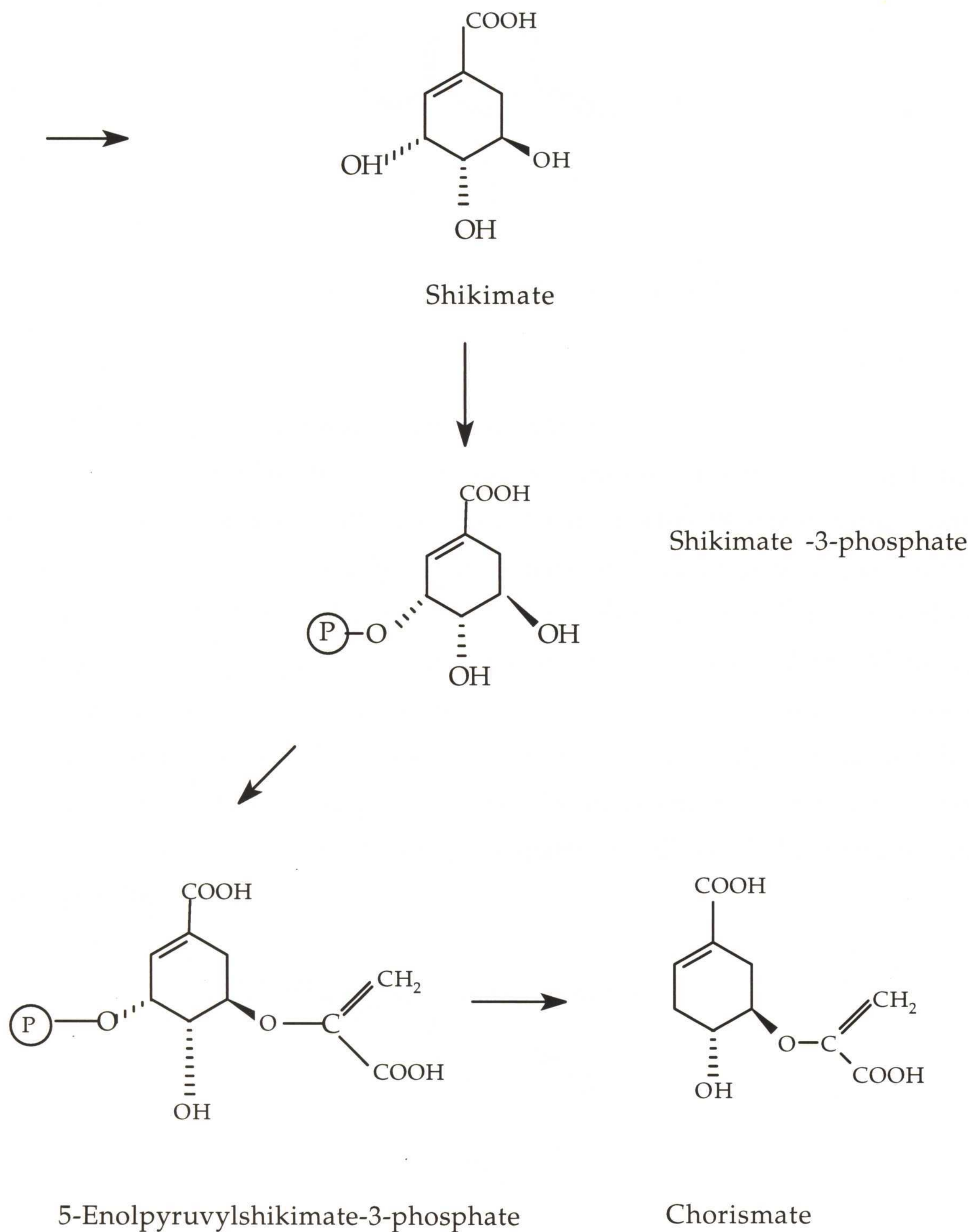


Fig 1.15 : The shikimic acid pathway.

Fig 1.16 : The aromatic ring of DIMBOA.

1.12.2 Origin of the methoxyl group of DIMBOA

The 7-methoxyl of DIMBOA is formed by a transmethylation process from methionine. Essentially all the label from methyl-^{14}C-methionine was recovered from the methoxyl position. Glycine and glycerate were also found to be incorporated into the methoxyl group. Glycine is a known methyl precursor and the isotope was present in the methoxyl C of DIMBOA although the activity was 4-times less than that from methionine. Glycerate also provides C with 37.2% of the incorporated radioactivity associated with the methoxyl carbon. It is possible that the glycerate may have been first converted to serine which in turn participated in 1-C metabolism (Reimann and Byerrum, 1964).

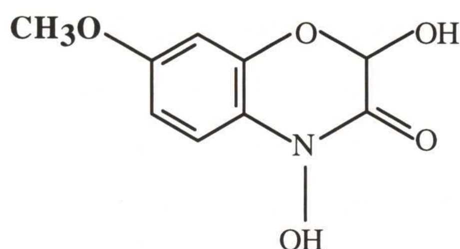


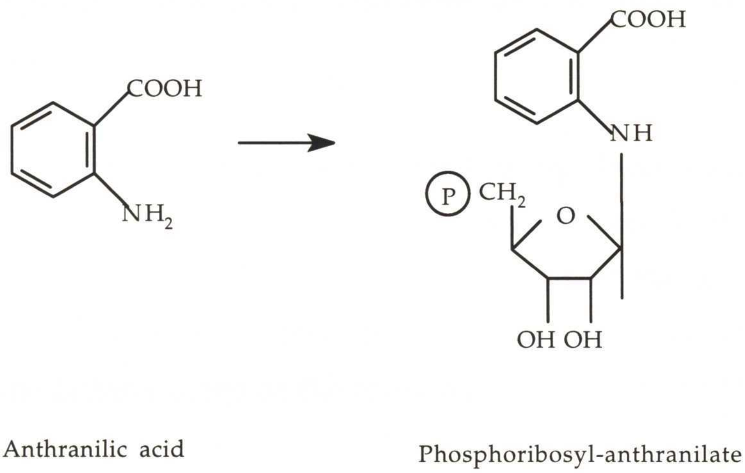
Fig 1.17 : The methoxyl group of DIMBOA.

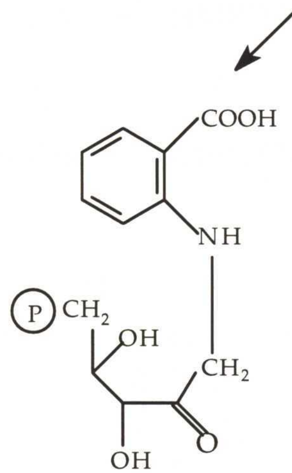
1.12.3 Biosynthesis of the oxazine ring

Degradation studies show that the two C atoms completing the oxazine ring are derived from carbons 1 and 2 of ribose. Reimann and Byerrum (1964) and Tipton *et al.* (1973) found 62% and 40% respectively of 1-^{14}C-ribose in the C-3 of DIMBOA.

Fig 1.18 : The oxazine ring of DIMBOA.

Reimann and Byerrum (1964) proposed that the ring was formed by a reaction of ribose or a ribose phosphate with an aromatic amine followed by cyclization and modification of the heterocyclic ring. Tipton *et al.* (1973) identified the aromatic amine as anthranilic acid. Anthranilic acid labelled with ^{14}C in the ring or with ^{15}N in the amino group is incorporated into the benzoxazinones with low dilution of the isotope suggesting a direct route of incorporation. Nakagawa *et al.* (1995) also found that label from anthranilic acid was incorporated into DIBOA-Glc and DIMBOA-Glc. Neither hydroxyanthranilic acid or *o*-aminophenyl is incorporated into the benzoxazinones. It is therefore unlikely that anthranilic acid is converted to another aromatic amine before reacting with a ribose phosphate (Tipton *et al.*, 1973). (Fig 1.19).





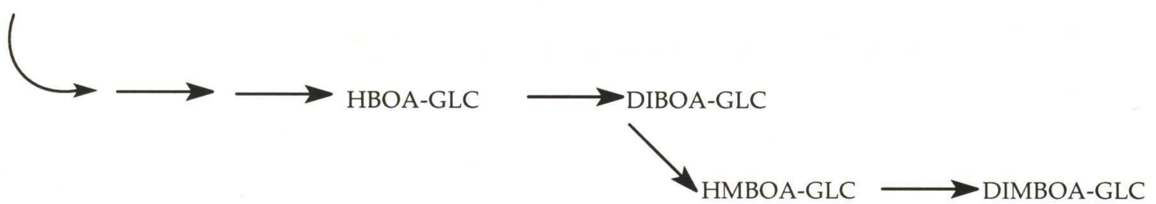
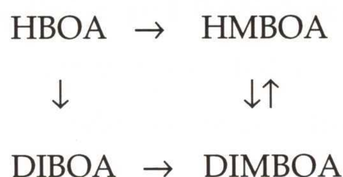


Fig 1.19 : The conversion of anthranilic acid to DIMBOA and DIBOA with the steps that have been identified.

The steps between the conversion of anthranilic acid to 1-(*o*-carboxyphenylamino)-1-deoxyribulose have been identified but the steps from 1-(*o*-carboxyphenylamino)-1-deoxyribulose to the final Hx have not been fully elucidated.

Hydroxamic acids which possess an N-hydroxyl group, have corresponding lactams which lack the N-hydroxyl group (Bailey and Larson, 1991). Tipton *et al.* (1973) were able to demonstrate the interconversion of the lactam HBOA and DIBOA *in vivo* but were unable to do so *in vitro*. A scheme was proposed where the lactams acted as the immediate precursors of Hx.



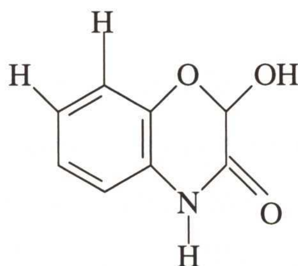


Fig 1.20 : 2-Hydroxy-1,4-benzoxazin-3-one (HBOA).

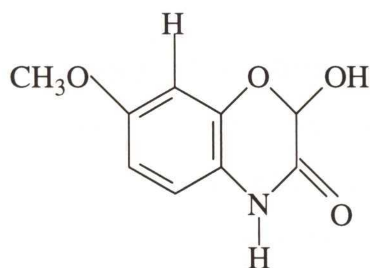


Fig 1.21 : 7-Methoxy-2-hydroxy-1,4-benzoxazin-3-one (HMBOA).

Bailey and Larson (1991) isolated a cytochrome P-450 dependent N-monooxygenase from corn that converts the lactam aglucone HBOA to DIBOA. Monooxygenase catalyzes the hydroxylation at the N-4 position of HBOA and the reaction can only occur after the formation of the bi-ring structure. Monooxygenase activity reached a maximum two days after germination and was not observed in tissues such as seeds, which lack Hx.

Experiments performed by Leighton *et al.* (1994) have shown that the lactam aglucones and not the lactam glucosides are the intermediates in the pathway. Lactam glucosylating activity could not be detected when HBOA or HMBOA were the substrate, providing further evidence that the lactam aglucones are the intermediates and that glucosylation of Hx would be the last step in the pathway. Furthermore, Bailey and Larson (1989) isolated a glucosyltransferase from corn which catalyzes the addition of glucose at the 2-position of DIMBOA, consistent with the specific glucosyltransferase found by Leighton *et al.* (1994).

1.13 Distribution of hydroxamic acids

Hydroxamic acids occur in many plant species and may account for up to 5% dry weight of many *Gramineae* (Argandoña *et al.* , 1981) but it is generally accepted that barley (Argandoña *et al*, 1981) and sorghum (Zúñiga *et al*, 1983) do not contain Hx. Wheat and corn contain mainly DIMBOA, rye contains DIBOA and triticale contains both DIMBOA and DIBOA (Niemeyer, 1988a). (Table 1.2). Wild and cultivated cereals contain similar concentrations of Hx.

The concentration of Hx varies with different cultivars and with the stage of growth (Venis and Watson, 1978). Xie *et al.* (1992) found that the concentrations of Hx were significantly different in different corn lines, whereas Niemeyer *et al.* (1992) found that within the common wheats, durum wheats showed substantially higher concentrations of Hx than did bread wheats. Niemeyer (1988 b) found that Hx contents ranged from 0.21-0.46

to 12.4-16.0 μmol/g.f.wt which compares with a range of 0.99 to 8.07 μmol/g.f.wt found by Nicol *et al.* (1992).

Tribe	**Species**	**DIMBOA**	**DIBOA**
Bambuseae	*Chusquea cumigii*	+	-
Arundinea	*Arunda donax*	-	+
Festuceae	*Bromus*	-	-
	Poa	-	-
	Dactylis	-	-
Phalarideae	*Phalaris*	-	-
Hordeae	*Lolium*	-	-
	Elymus	+	-
	Secale cereale	-	+
	Triticum durum cv. SNA-3	+`	+
	*cv.*Huenfuen	+	-
	Hordeum chilensis	-	-
	H.distichum	-	-
Chlorideae	*Cynodon*	-	-
Andropogoneae	*Sorghum*	-	-
Paniceae	*Setaria*	-	-
Maydeae	*Zea mays cv.* T129s	+	-
	cv. BxBx	+	+

Table 1.2 : The occurrence of hydroxamic acids in various *Gramineae* . Plus signs indicate that Hx were detected while dashes indicate that Hx were not detected (Zúñiga *et al.*, 1983).

1.13.1 Geographical distribution

In a study on the geographical distribution of Hx, Xie *et al.* (1992) found no significant difference between regions. Although there was no significant difference in Hx concentration, they did find a trend showing that plants in intermediate temperate regions had higher levels of all individual compounds assayed. Large variations in Hx concentrations within each geographical group indicate that germ-plasm with high DIMBOA content may be found in accessions from a wide range of the geographic areas.

1.13.2 Distribution of Hx in intact plants

Hx are absent from the seeds of cereals (Argandoña *et al.*, 1981) and become detectable around the second day after germination. Maximum Hx levels are usually reached by the fourth day after germination and decline rapidly after (Nicol *et al.*, 1992; Morse *et al.*, 1991; Long *et al.*, 1974; Klun and Robinson, 1969; Argandoña *et al.*, 1980 and Baker and Smith, 1977). The decline may be due to a growth dilution of the Hx within the plant. Morse *et al.*, (1991) suggested that Hx accumulate during early growth and that the decline in levels is due to metabolism, transportation or a combination of both.

DIMBOA is found in both the root and shoot of seedlings and coleoptiles (Argandoña *et al.*, 1981; Kumar *et al.*, 1994). Klun and Robinson (1969) found that in corn the concentration of DIMBOA was highest in the root, then in the stem, whorl and leaf tissues, whereas Richardson and Bacon (1993) found that the highest concentration of cyclic Hx in 5-day-old corn was in the shoot followed by the radicle. The lowest concentration of Hx in corn was in the seminal roots. There appears to be general agreement, however, that maximum Hx levels occur in the youngest parts of the plants.

1.13.3 Distribution of Hx in leaves

The concentrations of Hx in both wild and cultivated species are highest in the younger leaves and decline rapidly with age (Zúñiga *et al.*, 1983; Xie *et al.*, 1992). Each leaf shows the highest content when it is newly emerged (Argandoña *et al.*, 1981; Zúñiga *et. al.*, 1983). Hydroxamic acids are not homogeneously distributed among the various parts of leaves. Niemeyer *et al.* (1989) found that the DIMBOA content in the tip of wheat leaves was substantially higher than in the base, the magnitude of these differences depending on the cultivar. Argandoña and Corcuera (1985) found that the concentrations of Hx in the lateral veins of corn was higher than in the rest of the leaf and Massardo *et al.* (1994) found that Hx were present in both the mesophyll parenchyma and vascular bundle tissues of corn. The mesophyll parenchyma protoplasts contained large amounts of DIMBOA, but it was not found within the vacuole, mitochondria or chloroplast of corn leaves.

1.13.4 Distribution of Hx in roots and root exudates

As has been reported for leaves, Hx content within roots varies significantly with root age (Xie *et al.*, 1991). DIMBOA levels in root tissue also decline rapidly with age (Xie *et al.*, 1992).

Xie *et al.*, (1991) found that the concentration of all individual Hx was higher in nodal roots than in other parts of root. The nodal root system is the youngest part in the corn root system.

DIMBOA was the major Hx in all tissues of various parts of corn roots. It accounted for a major proportion of total Hx in the stele of nodal roots, cortex of primary roots and all tissues of adventitious roots. The concentrations of DIMBOA in the cortex of the nodal roots were significantly higher than those in the stele of nodal roots (Xie *et al.*, 1991). As Hx are proposed to be a protection mechanism, it is plausible that the levels in the cortex would be

higher than in the stele, which is surrounded by the endodermis, a protective barrier.

DIBOA was the only Hx detected in root exudates of rye. Hydroxamic acids were not detected in root exudates from wheat. The amount of DIBOA exuded by rye plants is not proportional to its content in the roots. Additionally the wheat variety Alifén contained both DIMBOA and DIBOA in its roots and none of them was exuded (Pérez and Ormeño-Núñez, 1991).

1.13.5 Compartmentation of hydroxamic acids

In 1960 Virtanen and Hietala found that the aglucone was not formed if the enzymes were destroyed by placing intact plants in boiling water. Virtanen and Wahlroos (1963) came to the conclusion that Hx occur as glucosides in intact plants as neither the aglucone nor MBOA was found in young plants.

Hofman and Hofmanová (1971) then found that corn plants stopped growing and died in the presence of a small amount of aglucone. They concluded that the free aglucones are toxic and are absent from uninjured plants.

Massardo *et al.* (1994) subsequently proposed that there was compartmentation between DIMBOA-Glc and its hydrolytic enzyme. The glucoside was present only in the extravacuolar space of the mesophyll parenchyma protoplasts of corn whereas DIMBOA-β-glucosidase was found only in the vacuole. This compartmentation within a parenchyma cell prevents enzymatic breakdown of the glucosides, thus providing protection from the toxic effects of Hx under normal physiological conditions. On pest attack, membranes in the cells are damaged and the enzymes could disperse in the cell and come into contact with the glucosides and release the toxic aglucones. The released aglucone could then damage the pathogen and surrounding cells, impairing the pathogens establishment (Massardo *et al.*, 1994).

1.13.6 Accumulation of Hx in callus tissue

Zúñiga and Massardo (1991) found that undifferentiated tissues of wheat produce only aglucones and differentiated tissues glucosides. Two-day-old apical sections of seedlings contain only glucosides. As tissues age the amount of aglucones decreases and glucosides appear so that in 13 to 18-day-old seedlings only DIMBOA-Glc was detected. Zúñiga *et al.* (1990) also proposed that Hx may be glucosylated and translocated to differentiated tissues or translocated to differentiated tissues and glucosylated before being incorporated into differentiated tissues.

Zúñiga *et al.* (1990) found the main Hx in 5-day-old callus of wheat was DIBOA. As the callus aged the level of DIBOA decreased with a subsequent increase in DIMBOA content up to around 27 days. After 50 days DIMBOA and DIBOA were undetectable and when the callus differentiated into the shoots and roots, only DIMBOA was detected. They concluded that DIMBOA originates from DIBOA.

1.14 Function

As with many secondary plant products, the function of hydroxamic acids is not clearly understood. They have been suggested to be associated with the resistance of plants to insects and microbial pathogens, the detoxification of herbicides and allelopathic interactions between species. The finding that the Hx content of wounded plants is higher than that of undamaged plants lends evidence to the theory that they act as plant protection factors (Morse *et al.*, 1991).

1.14.1 Fungi

The presence of Hx in plants was first noted due to their effects on the growth of fungi and many of the early studies were concentrated in this area. Hydroxamic acids have been shown to inhibit the growth of many fungi including *Fusarium* spp., *Sclerotinia trifolium, Puccinia graminis* and

Septoria nodorum (Virtanen and Hietala, 1955; Whitney and Mortimore, 1959; El Naghy and Linko, 1962; El Naghy and Shaw, 1966 and Baker and Smith, 1977). BOA inhibits the growth of *Fusarium nivale* and *Sclerotinia trifolium* (clover-rot fungus) at concentrations of 0.05% when incorporated into growth medium (Virtanen and Hietala, 1955).

Whitney and Mortimore (1959) found that when an extract from corn was incorporated into agar, the growth of *Fusarium moniliforme* was inhibited by 45% and that of *Giberella zeae* by 22%. The inhibitory substance was identified as 6-methoxybenzoxazolinone (MBOA) which was active at a concentration of 0.12 mg/ml.

El Naghy and Shaw (1966) and El Naghy and Linko (1962) showed that wheat varieties resistant to stem rust caused by *Puccinia graminis tritici* consistently had a higher content of the Hx-Glc than the less resistant varieties. In *in vitro* studies to determine the nature of this resistance El Naghy and Linko (1962) showed that rust development was clearly inhibited by BOA at concentrations above 0.05% with no development occurring at 0.09% or above. The germination of uredospores was also inhibited. MBOA gave similar results to those of BOA. The aglucone and the benzoxazolinones were shown to cause death of the injured plant cells so that the further development of the pathogen was inhibited.

Hydroxamic acids have been shown to be involved in the resistance of corn to stalk rot (BeMiller and Pappelis, 1965 a and b) and of wheat to *Septoria nodorum* (Baker and Smith, 1977) but in these cases Hx are more important as protection agents in younger plants.

1.14.2 Bacteria

Hydroxamic acids have been shown to be active agents in the resistance of crops to bacteria. Hartman *et al.* (1975) tested 30 bacterial pathogens and found that the corn pathogens *Erwinia* spp. were among the least sensitive organisms to Hx. As corn is known to contain high levels of Hx, those organisms able to parasitize corn must have developed a mechanism whereby they are able to withstand high concentrations of Hx. At concentrations that were non-bacteriocidal to the isolates, the inhibitory effect was mainly an increase in the lag phase of growth. Corcuera *et al* (1978) also found that DIMBOA prolonged the lag phase of several *Erwinia* spp. At equivalent concentrations, DIMBOA was more inhibitory than MBOA, and there was only a slight increase over the effect of DIMBOA when DIMBOA and MBOA were used synergistically. DIMBOA was effective at concentrations as low as 0.3 mM. They also found that when extracts prepared from the genotype *bxbx,* which does not contain Hx, the growth of *Erwinia* was not inhibited but when DIMBOA was added to these extracts the inhibitory effect was observed.

Bravo and Lazo (1993) tested 3 bacteria, *Staphylococcus aureus, Escherichia coli,* and *Candida albicans* and found that all the tested hydroxamic acids gave moderate inhibition of the growth of the bacteria.

These studies show a correlation between the content of Hx in plants and their resistance to various fungal and bacterial pathogens.

1.14.3 Aphids

Another area where Hx may act is in the resistance of cereals to insects such as aphids and borers. As the concentration of Hx in cereals increases, the population growth and degree of infestation by the aphids *Metopolophium dirhodum* and *Schizaphis graminum* decreases (Argandoña *et al.*, 1980; Argandoña *et al.*, 1981; Zúñiga *et al.*, 1983; Niemeyer *et al.* , 1989 and Thackray

et al., 1990). Hydroxamic acids exhibit both antibiotic and antifeedant effects towards aphids (Niemeyer, 1988a).

The aglucones DIMBOA and DIBOA have similar effects on the survival of aphids (Zúñiga *et al.*, 1983), but DIMBOA has a greater effect on aphids than its decomposition product MBOA (Argandoña *et al.*, 1980).

It was originally thought that Hx were not important for resistance in mature plants as older plant parts have lower Hx content and have more aphids. However, evidence now suggests that hydroxamic acids are more concentrated in the site of attack by the aphid than in the leaf as a whole, and that they play a role in plants of all ages (Niemeyer *et al.* , 1989 and Thackray *et al.*, 1990). Niemeyer *et al.* (1989) have also shown that for some cultivars of wheat, DIMBOA levels actually increase following aphid infestation.

1.14.4 Corn borer

Research has shown that hydroxamic acids were active compounds in the resistance of corn to the European corn borer *Ostrinia nubialis* , and that corn lines highly resistant to the borer contained about ten times more Hx than the highly susceptible lines (Klun and Brindley,1966). MBOA only retards the rate of population growth and does not affect survival of the borer whereas DIMBOA inhibited larval development and caused 25% mortality and appeared to function as a repellant and/or feeding deterrant (Klun and Brindley, 1967). The loss of resistance as the plant ages can be attributed to the decrease of DIMBOA, as cultivars which maintain higher concentrations of DIMBOA tend to be more resistant (Klun and Robinson, 1969)

1.14.5 Allelopathy

Allelopathy occurs when one plant produces a chemical or chemicals that influences the germination, growth or development of another plant (Barnes and Putnam, 1987). DIBOA and BOA have been suggested to be involved in

allelopathy (Barnes and Putnam, 1987; Pérez and Ormeño-Núñez, 1991). An important requirement for allelopathy is the exudation of Hx into the rhizosphere.

DIBOA is the only hydroxamic acid detected in root exudates and the amount exuded is not dependent on its content in the roots. Hydroxamic acids have not been detected in exudates from wheat (Pérez and Ormeño-Núñez,1991).

A rye exuding DIBOA was found to significantly reduce total weed biomass in the field (Pérez and Ormeño-Nuñez, 1993). Barnes and Putnam (1987) found that DIBOA significantly reduced the germination of monocot species, whereas BOA inhibited both the germination and seedling growth of dicot species. DIBOA-glucoside, DIBOA and BOA all inhibited radicle and coleoptile growth of wild oats (Pérez and Ormeño-Núñez,1991). Pérez (1990) found in *in vitro* studies that MBOA was more inhibitory on the root growth of *Avena fatua* than its parent aglucone DIMBOA.

The important point to remember from these studies is that Hx do exhibit allelopathic effects *in vitro* and these effects will only be exhibited *in vivo* if they are exuded from the roots.

1.14.6 Pesticides

Ioannou *et al.* (1980) showed *in vivo* degradation of the organophosphate, diazinon by subcellular fractions of corn. Trypsin digestion of extracts did not result in any loss of degradative activity suggesting that the degradation was due to a non-enzymatic system. Purification of the extracts showed that DIMBOA was the active agent in the *in vitro* hydrolysis of diazinon. This hydrolysis is specific for diazinon as other organophosphate insecticides, carbamates and pyrethroids were not degraded. Maximum degradation occurs at pH 5 and declines as pH increases. This is probably due to the fact that DIMBOA decomposes as pH increases. The higher the DIMBOA

concentration the larger the amount of diazinon degraded, however, longer incubation periods do not result in increased degradation. This is also probably due to the breakdown of DIMBOA to MBOA.

Hydroxamic acids have also been associated with the breakdown of atrazine. In higher plants there are three possible pathways for the detoxification of atrazine. These pathways are : glutathione conjugation, hydroxylation and dealkylation (Shimabukuro, 1968). Hydroxylation results in the total detoxification of atrazine to hydroxyatrazine while dealkylation results in the partial detoxification to 2-chloro-4-amino-6-isopropyl-*s*-triazine which is subsequently metabolized to non-toxic forms. Evidence indicates that hydroxylation may be limited to species which contain benzoxazinone. Sorghum does not contain benzoxazinone and when treated with atrazine, no hydroxylated derivatives are detected. All three hydroxylated derivatives appear in atrazine treated corn containing benzoxazinone (Shimabukuro, 1967, 1968).

The participation of Hx in the detoxification of insecticides enables the plant to withstand high doses while the target insects are readily killed.

1.15 Biological activity

The biological activity of Hx has not been extensively studied and from the results found definite conclusions on how they act can not be drawn. Hydroxamic acids have been found to inhibit ATPase activity in spinach, acetylcholine esterase in aphids and respiration in mitochondria.

Queirolo *et al.* (1983) found that 5 mM DIMBOA caused 50% inhibition of the ATPase activity of chloroplast coupling factor 1 (CF_1) in spinach chloroplasts. One mole of Hx per mole of active site caused reversible inhibition of ATPase. The fact that the inhibition was reversible indicates that DIMBOA does not bind at the substrate binding site. Protection of the enzyme by

iodoacetamide (iodoacetamide irreversibly inhibits the catalytic activity of enzymes by modifying cysteine) was not complete suggesting that cysteine residues are not the only groups that react with DIMBOA. Activated CF_1 has 4 sulfhydryl groups and Hx have been shown to react with nucleophiles such as thiols. Therefore the enzyme inactivation caused by DIMBOA reacting with thiols is also possible.

Cuevas and Niemeyer (1993) found that inactivation of acetyl cholinesterase in aphids occurred at concentrations as low as 1 mM DIMBOA and increased with DIMBOA concentration. Acetyl cholinesterases from various sources were all affected to some degree. Cysteine was not the only reaction site as all the cholinesterases, some of which had different structures, were inactivated by DIMBOA. It was proposed that in this case DIMBOA reacted with the serine residues of acetyl cholinesterase.

Bravo and Lazo (1993) proposed that the antimicrobial activity of hydroxamic acids towards *Staphylococcus aureus, Escherichia coli* and *Candida albicans* may be due to the inactivation of microbial urease by two possible mechanisms. Hydroxamic acids complex well with the metal ion at the active site and also react with the sulphydryl groups. No distinction between the two mechanisms could be made.

Massardo *et al.* (1994) found that DIMBOA reduced electron transport in mitochondria and chloroplasts. The addition of succinate reversed this inhibition suggesting that DIMBOA inhibited electron transfer from NADH to O_2 in maize mitochondria. In bovine mitochondria DIMBOA inhibits both the electron transport chain and oxidative phosphorylation reactions suggesting that DIMBOA acts between succinate dehydrogenase and cytochrome c sites.

1.16 Objectives of the study

The broad aims of the study were to further our understanding of

i) the role of Hx in the resistance of cereals to the take-all fungus and,

ii) the relationship between various Hx in wheat, rye and triticale.

To reach these objectives the first aim to be achieved is the establishment of a rapid but sensitive method of detecting Hx in the root tissue of the cereals under investigation. The types of Hx and the levels at which they occur in root tissue can then be assayed. The next aim is to design studies to determine which Hx are the most potent inhibitors of fungal growth and at what concentration they are active at. The next part of the study involves monitoring the concentration of Hx of roots when infected with the take-all fungus. The last part of the study involves assaying the Hx content of wheat genotypes carrying individual rye chromosomes to look at the possible transfer of take-all resistance from rye to wheat.

CHAPTER TWO

Materials and methods

2.1 *Materials*

Seeds of wheat (*Triticum aestivum*) , rye (*Secale cereale*) , triticale (*Triticosecale*) and corn (*Zea mays*) were obtained from the Plant Breeding Institute, The University of Sydney.

Take-all fungal cultures (*Gaeumannomyces graminis* var. *tritici*) were provided by Dr Paul Harvey and Dr Fred Stoddard, Department of Crop Sciences, The University of Sydney.

Authentic samples of DIMBOA and DIBOA were kindly donated by Professor Hermann Niemeyer, Facultad de Ciencias, Universidad de Chile, Santiago, Chile. BOA was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin, USA, and MBOA from Sigma Chemical Company, St Louis, MO, USA. Agar and potato dextrose agar (PDA) were from Difco Laboratories, Detroit, MI, USA.

Cellulose acetate filters (0.45µm) were from Millipore Products Division, Bedford, Ma, USA and a Zorbax (SB C18 4.6mm x 15cm) HPLC column was used for Hx separation. Diethyl ether (Nanograde), hexane (Nanograde) and methanol (HPLC grade) were all from Mallinckrodt Specialty Chemicals Co., Paris, Kentucky, USA. All other chemicals were AR grade and distilled, deionized water was used unless stated otherwise.

2.2 Methods

2.2.1 Growth of seedlings

Seeds were surface sterilized in 0.5% (*w/v*) sodium hypochlorite (NaOCl) for 10 min, rinsed thoroughly in tap water for 15 min and germinated on plastic mesh floated over nutrient solution in photographic developing trays as shown in Figs 2.1 and 2.2. The nutrient solution contained 2 mM $Ca(NO_3)_2.4H_2O$, 2 mM KNO_3, 0.43 mM NH_4Cl, 0.75 mM $MgSO_4.7H_2O$, 20 μM $NaH_2PO_4.2H_2O$ and 27 μM $FeCl.6H_2O$ (Larkin, 1987) and was aerated continuously with an aquarium pump.

Seedlings were grown in a glasshouse under natural light supplemented with artificial light from 400 W Sylvania discharge lamps with a photon flux of 200 $\mu mol/m^{-2}s^{-2}$ to provide a 16 hr photoperiod. The average day and night temperatures were 26°C and 22°C respectively.

Fig 2.1: The hydroponic system used for growing seedlings. Rye seeds are shown immediately after sowing.

Fig 2.2: Rye seedlings 4 days after sowing.

2.2.2 Preparation of DIMBOA and DIBOA

DIMBOA and DIBOA were isolated from the roots and leaves of 7-day old corn seedlings and the leaves of 4-day-old rye seedlings respectively, according to the procedure of Queirolo *et al.* (1983) as follows. The residual seed was removed and the shoots and roots of seedlings were macerated in a Waring blender in 2 volumes of water. The homogenate was allowed to stand at room temperature (20-22°C) for 60 min, filtered through two layers of muslin and the filtrate adjusted to pH 3 with 1 M HCl and centrifuged for 10 min at 10 000 g. The supernatant was partitioned twice against 2 volumes of diethyl ether and the organic phases combined, dried with anhydrous Na_2SO_4, filtered and the ether removed under vacuum. The residue was dissolved in about 1 ml of acetone, and crystallization initiated by the addition of hexane. Hexane was added slowly until a precipitate began to appear and the mixture was left to form crystals. The excess hexane was

removed and the crystals set aside. The crystals were then treated with acetone and hexane as described until white crystals were obtained.

The identity of the isolated Hx was confirmed by a positive (violet colour) reaction to ferric chloride reagent (50g $FeCl_3.6H_2O$, 500ml ethanol and 5ml HCl), melting point, UV spectrum in ethanol and HPLC (comparison of elution time and co-chromatography with an authentic standard).

2.2.3 Extraction and quantification of Hx in seedlings

Hydroxamic acids were extracted from seedlings according to the method of Niemeyer (1988b) as follows. The roots of seedlings (50-100 mg) were ground twice with 0.33 ml of water in a 10ml Pyrex tissue extractor which was then rinsed with 0.33 ml distilled water and allowed to stand for 15 min at room temperature. The extract was then adjusted to pH 3 using 0.1 M HCl (0.5 ml) and centrifuged at 10 000 g for 10 min. The supernatant was made up to 2 ml, filtered through a 0.45 μm cellulose acetate filter and assayed for Hx using HPLC.

Hydroxamic acids were separated using the HPLC method of Cuevas *et al.* (1992) with slight modifications as follows. Samples of 20μl were chromatographed at ambient temperature (20-22°C) in a Zorbax SB C18 column (4.6 mm x 15 cm) using methanol:water:phosphoric acid (40:59:1) as the mobile phase at a flow rate of 0.4 ml/min. Hydroxamic acids were detected by monitoring the absorbance at 263 nm with a Gilson 118 UV-Vis detector and peak areas were measured with a Shimadzu C-R6A Chromatopac integrator and compared with a calibration curve of different known Hx concentrations. The recovery of Hx using the above procedure was measured by adding known amounts of DIMBOA to the extraction medium.

2.2.4 Culturing of Ggt

2.2.4.1 Isolation of Ggt

Wheat seeds were surface sterilized in 5% (*w/v*) NaOCl and rinsed in sterile distilled water and grown hydroponically for five days as described in 2.2.1. Seedlings exhibiting any visible signs of fungal infection were discarded. The seedlings were then used to isolate Ggt according to the method of Harvey (1994) as follows.

Pots (10 cm diameter) were half filled with sterile sand and the fungal inoculum was introduced as pieces of agar culture (approx. 1.0 cm^2). A seedling was placed on top of each piece of inoculum and a layer of sterile sand was placed over the seedling and inoculum. Plants were grown in a glasshouse at 18°C and watered as needed with tap water.

After five weeks, plants were harvested and *Ggt* was isolated from diseased roots as follows. Blackened root segments were surface sterilized in 1% (*w/v*) $AgNO_3$ for 1 min. The roots were then washed in 5% (*w/v*) NaCl and rinsed twice in sterile distilled water and allowed to dry. The root segments were then plated on 0.25 PDA (4.9 g PDA, 5.0 g agar per 500 ml water) supplemented with streptomycin sulphate (100 mg/ml) and incubated at 22°C. Hyphal tips were removed as they emerged from the root and subcultured onto fresh 0.25 PDA. The cultures were sealed with Parafilm and stored at 4°C until required.

2.2.4.2 Subculturing of Ggt

Actively growing colonies used in studies were obtained by subculturing a square from the edge of a colony on to 0.25 (PDA) containing streptomycin sulphate (100 mg/ml). The cultures were incubated at 22°C for five days before use in growth studies. The strains were kept virulent by re-isolating from susceptible seedlings as described in 2.2.4.1 every six months.

Two strains of *Ggt* were used in all experiments. Strain EBI which attacks rye and wheat and strain WP 28 which only attacks wheat.

2.2.5 *Growth of Ggt on media containing root extracts*

The growth rate of *Ggt* (EBI, WP28) was measured on agar containing extracts prepared from roots of rye, triticale and wheat as follows. Root material (2 g) was macerated in 10 ml of water and allowed to stand for 15 min. The macerate was centrifuged at 10,000 g for 10 min and the supernatant filtered through 0.45 µm cellulose acetate filters and added to molten agar at approximately 60°C. Discs (6 mm) from the edge of an actively growing colony of Ggt were transferred to the agar containing the root extracts and the plates were incubated at 22°C. The diameter of the colony was determined by averaging two measurements taken at right angles to each after five days. The diameters of those colonies grown on agar containing extracts were compared to a control which contained no extracts.

2.2.6 *Growth of Ggt on medium containing Hx*

Agar (0.5 g) and PDA (0.49 g) were dissolved in 40 ml of water and autoclaved. DIMBOA and DIBOA were dissolved in 10ml of sterile distilled water and filtered through a 0.45 µm cellulose acetate filter to sterilize. The filtered Hx solution was added to the molten agar at approximately 60°C, mixed and the plates poured. MBOA and BOA could be added directly to the agar and autoclaved as the benzoxazolinones are stable under these conditions.

Discs (6 mm) of Ggt from the edge of an actively growing colony were transferred to the agar containing Hx and the plates were incubated at 22°C. The diameter of the colony was determined by averaging two measurements taken at right angles to each other daily for four days. After four days any colonies that did not show any growth on this medium were transferred to 0.25 PDA which did not contain Hx. Those colonies whose growth was not

inhibited were retained on the agar containing Hx. The diameter of the colonies were measured for another three days for both treatments.

2.2.7 Take-all challenge study

Plants were grown hydroponically to 3 days as described in 2.2.1. Pots (10 cm diameter) were half filled with sterilized sand and a 1.0 cm^2 square of *Ggt* inoculum was placed on the top of the sand in the middle of the pot. A three day old seedling was placed on the inoculum square and the pot filled with sand. The plants were grown in a glasshouse at 18°C and watered as needed.

Root material was extracted and analyzed for Hx content after three, 21 and 35 days as described in 2.2.3, except that after 21 and 35 days the root material weighed between 0.5-1.0g and consequently the extraction procedure was modified as follows. Roots were extracted twice with 3.33ml of water with a mortar and pestle which was rinsed with 3.33 ml of water and the volume of the 10 000 g supernatant was made up to 25 ml.

At 35 days all plants were assayed for take-all severity by measuring the percentage of roots showing characteristic blackening. The presence of stem base blackening was also noted.

2.2.8 Statistical analysis

All experiments were performed at least twice except for 2.2.6 which was performed with an internal control due to a limited supply of materials. All results were analyzed for significant differences according to the Student's t test using JMP Version 3.0. Results compared to a control for significant difference were analyzed according to Dunnett's Method also using JMP Version 3.0. Significant results have a confidence level of 95%.

CHAPTER THREE

Purification and characterization of hydroxamic acids

3.1 Introduction

Hydroxamic acids are secondary plant products present in many of the *Gramineae,* and as discussed in Chapter One, have been reported to protect these plants in a number of ways, including inhibiting bacterial and fungal attack, deterring aphid feeding, detoxifying pesticides and exerting allelopathic effects on other plants. In a study performed by Klein and Marshall (1989), extracts from rye were found to inhibit the growth of the take-all fungus *in vitro.* The active compound was identified as BOA and it was proposed that other Hx which had been found in cereals may act similarly towards the take-all fungus.

As DIBOA and DIMBOA are not commercially available the isolation and characterization of these Hx was undertaken. Once the Hx were isolated and positively identified, their distribution in cereal roots, their change in concentration over time and the variation of Hx between different cereals were studied.

Results and Discussion

3.2 .1 Identification of DIBOA and DIMBOA

The isolated compounds were tentatively identified in a qualitative test as cyclic Hx by their positive reaction to $FeCl_3$. A dilute solution of hydroxamic acid gave a violet-blue colour when a drop was placed on a filter paper impregnated with $FeCl_3$ solution. Further evidence was obtained by comparing the melting points of the isolated crystals. The melting point for DIBOA was 154°C and the melting point for DIMBOA was 164-167°C. These values agree closely to those of Tipton *et al.* (1967).

The UV spectra (Figs 3.1 and 3.2) show that DIMBOA had a peak at 263 nm and a shoulder at 288 nm which agrees with values obtained by Lyons *et al.* (1988). The spectrum of DIBOA showed a peak at 253 nm and a shoulder at 280 nm which also agrees with published values (Lyons *et al.*, 1988). From the spectra, extinction coefficients were calculated to be 11,707 for DIMBOA and 6,595 for DIBOA. These values agree well with values reported by Woodward *et al.* (1979).

The identity of the Hx were also confirmed using HPLC. No extra peaks were detected when samples of isolated DIMBOA and DIBOA were co-chromatographed with known standards. (Fig 3.3).

The identity of DIMBOA was also confirmed by performing degradation studies. An aqueous solution of DIMBOA (1mg/ml) was heated in a sealed tube at 60°C and a sample chromatographed every 60 min to monitor the formation of breakdown products (Fig 3.4). The major decomposition product detected after 60 min was MBOA as determined by HPLC analysis using a known standard as the reference compound.

3.3 Separation of hydroxamic acids using HPLC

A mixture of standard Hx aglucones containing DIBOA and DIMBOA, and the benzoxazolinones BOA and MBOA, was resolved into four separate peaks within 17 min as shown in the chromatograms presented in Fig 3.3.

Hydroxamic Acid	Retention Time (min)
DIBOA	8.18±0.2
DIMBOA	9.60±0.4
BOA	13.06±0.4
MBOA	15.39±0.6

Table 3.1 : Retention times for hydroxamic acids separated by HPLC.

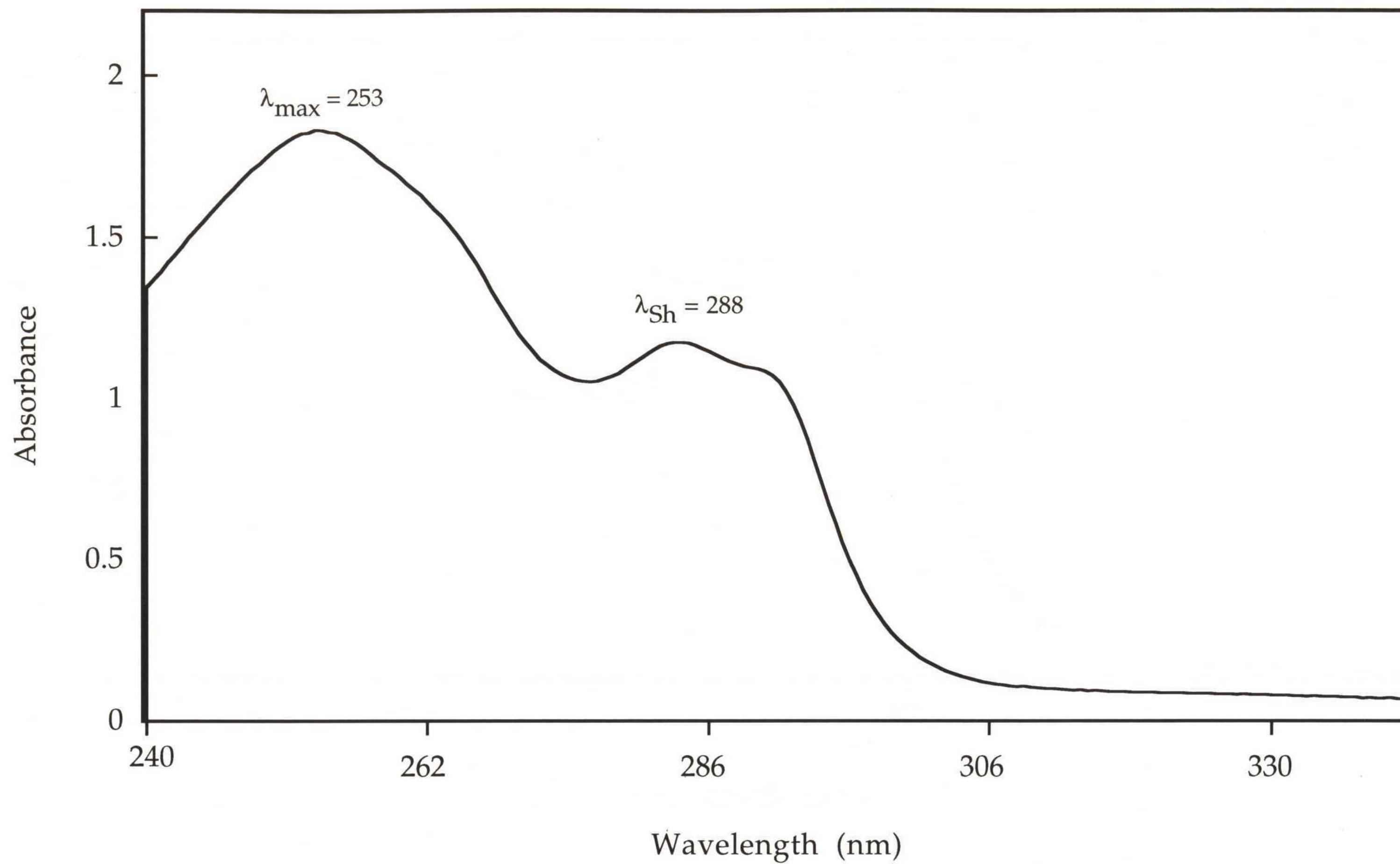


Fig 3.1 UV-Visible spectrum of DIBOA **.** The spectrum was determined with a solution of 0.05 mg/ml DIBOA in ethanol.

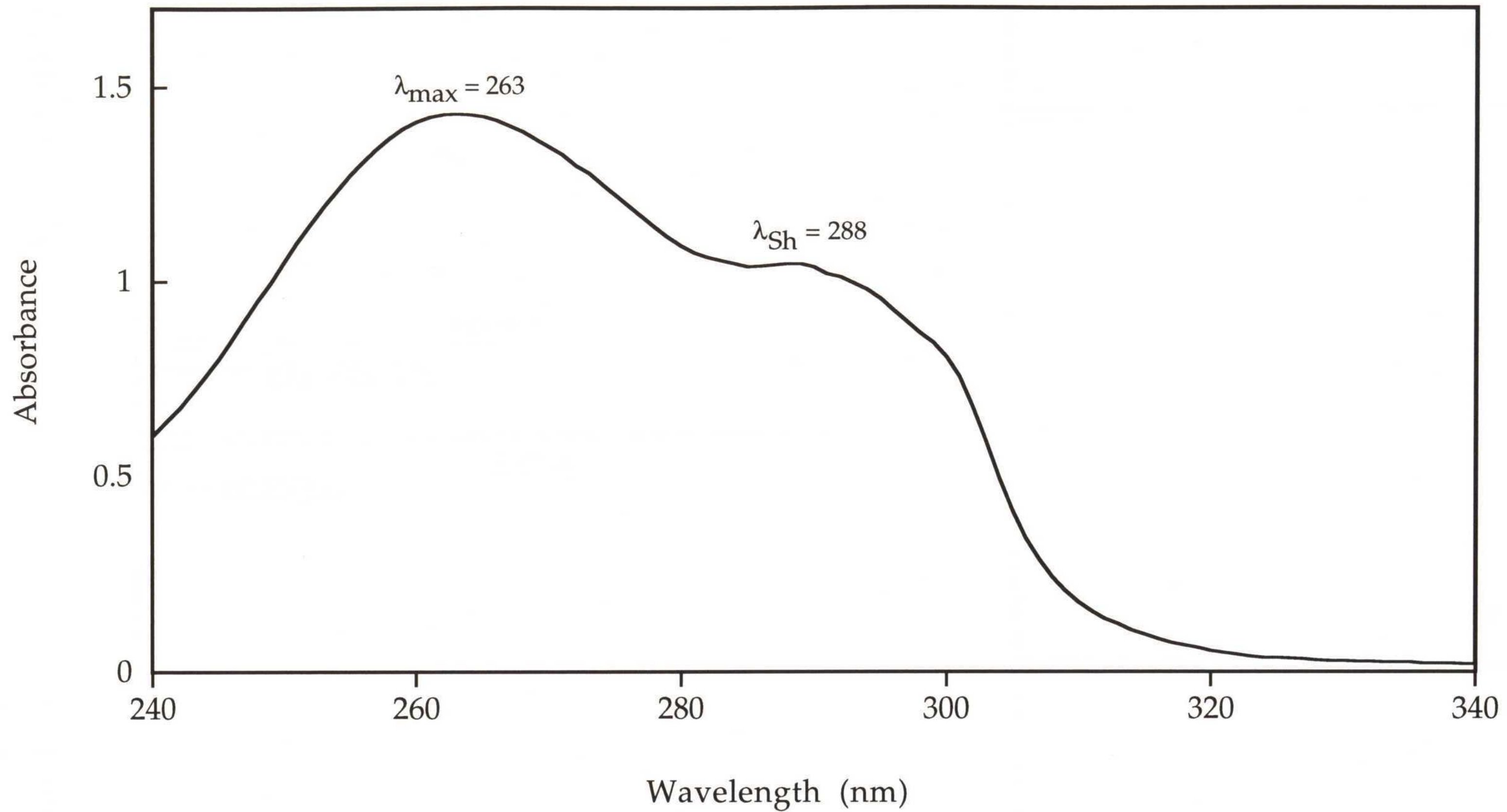


Fig 3.2 : UV-Visible spectrum of DIMBOA . The spectrum was determined with a solution of 0.03 mg/ml DIMBOA in ethanol.

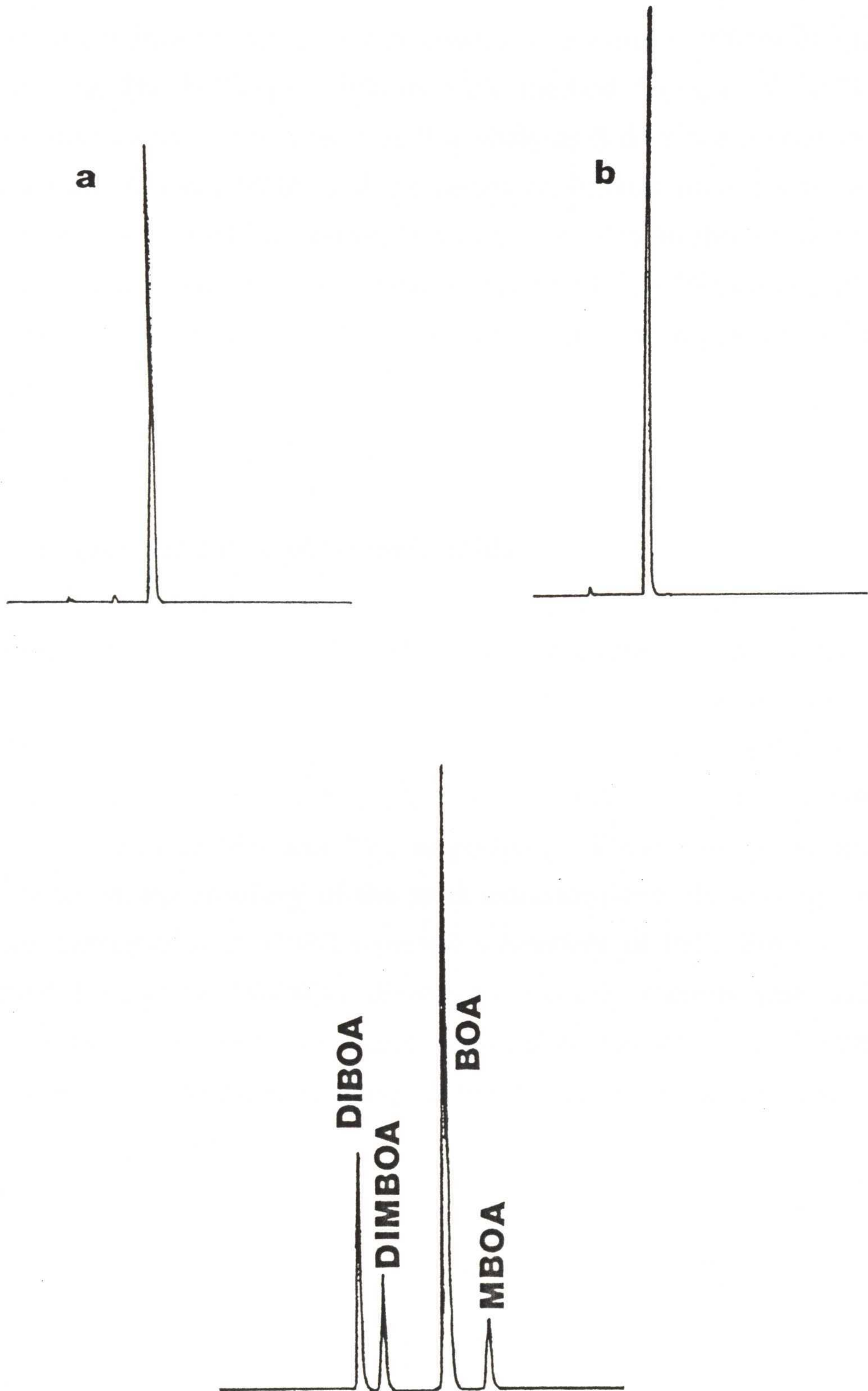


Fig 3.3 : Co-chromatography of DIMBOA (a) and DIBOA (b) and the chromatographic separation of DIBOA (I), DIMBOA (II), BOA (III) and MBOA (IV).

The HPLC procedure chosen gave advantages over other methods in speed and sensitivity. The $FeCl_3$ spectrophotometric method (Long *et al.*, 1974) is not as sensitive as the method used in this study as it does not discriminate between DIMBOA and DIBOA, and the benzoxazolinones do not react with $FeCl_3$ to form the violet-blue colour. It was necessary to be able to detect all compounds separately as rye and triticale contain both DIMBOA and DIBOA and the presence of BOA and MBOA indicates the decomposition of the aglucones.

3.4 Characterization of hydroxamic acids

3.4.1 Recovery of DIMBOA

The recovery of DIMBOA in the extraction procedure was measured by adding known amounts of DIMBOA to the water used to extract the root tissue. The slope of the line in Fig 3.5 shows that the recovery was 83% using this method. Using similar methods, Lyons *et al.* (1988) and Xie *et al.* (1991) obtained recoveries of 78% and 72% respectively. These results are more representative of the recovery of the total extraction and chromatographic procedure. Gutierrez *et al.* (1982) reported a recovery of 104% but this was determined by adding DIMBOA directly to sample extracts just before chromatography. Therefore, the value reported by Gutierrez *et al.* (1982), only represents the recovery in their chromatographic procedure and not the extraction procedure.

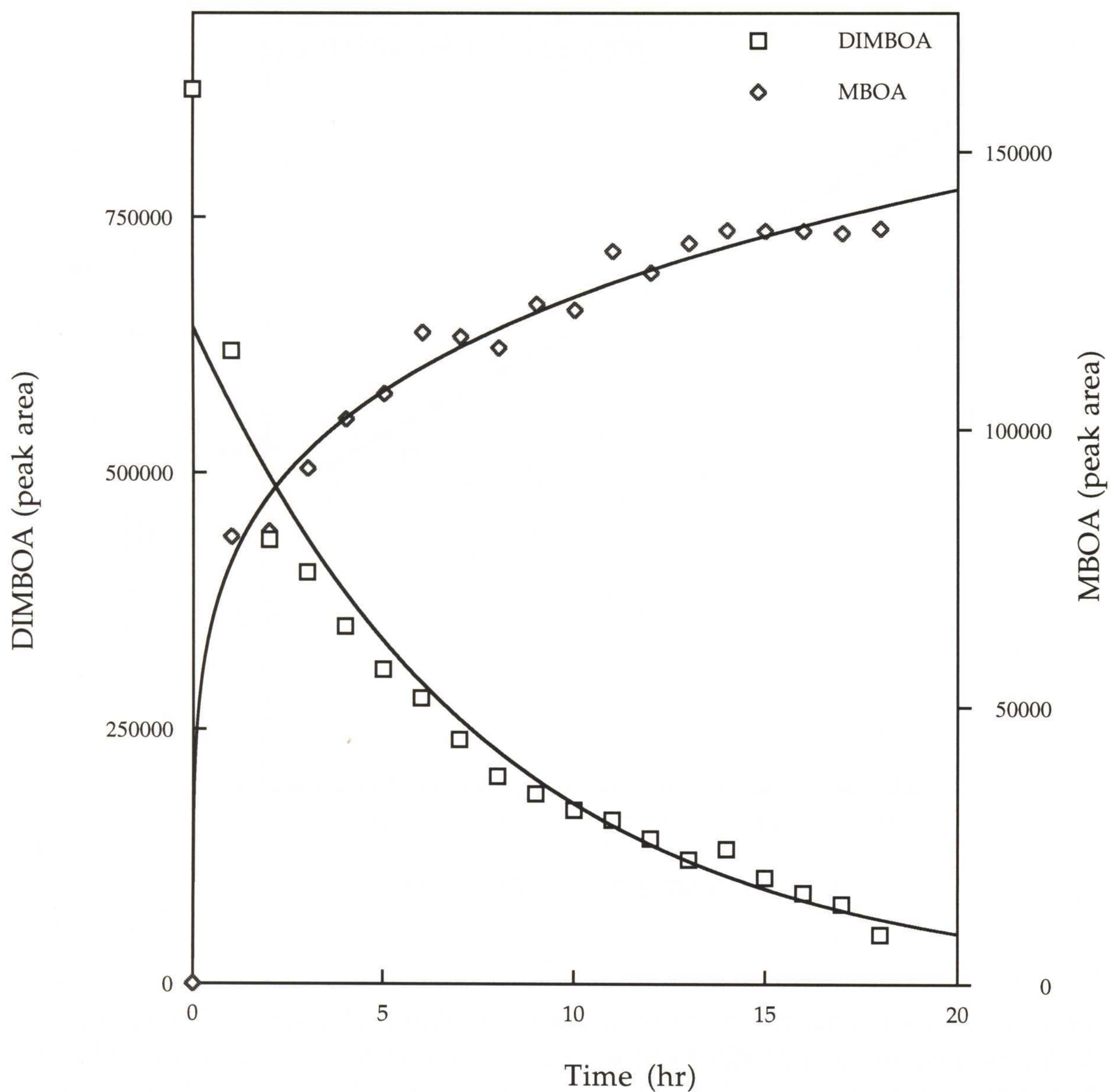


Fig 3.4 : The effect of heating an aqueous solution of DIMBOA. A solution of DIMBOA (1mg/ml) was heated at 60°C and a sample was chromatographed every 60 min. The major decomposition product was MBOA. Peak areas are in arbitary units.

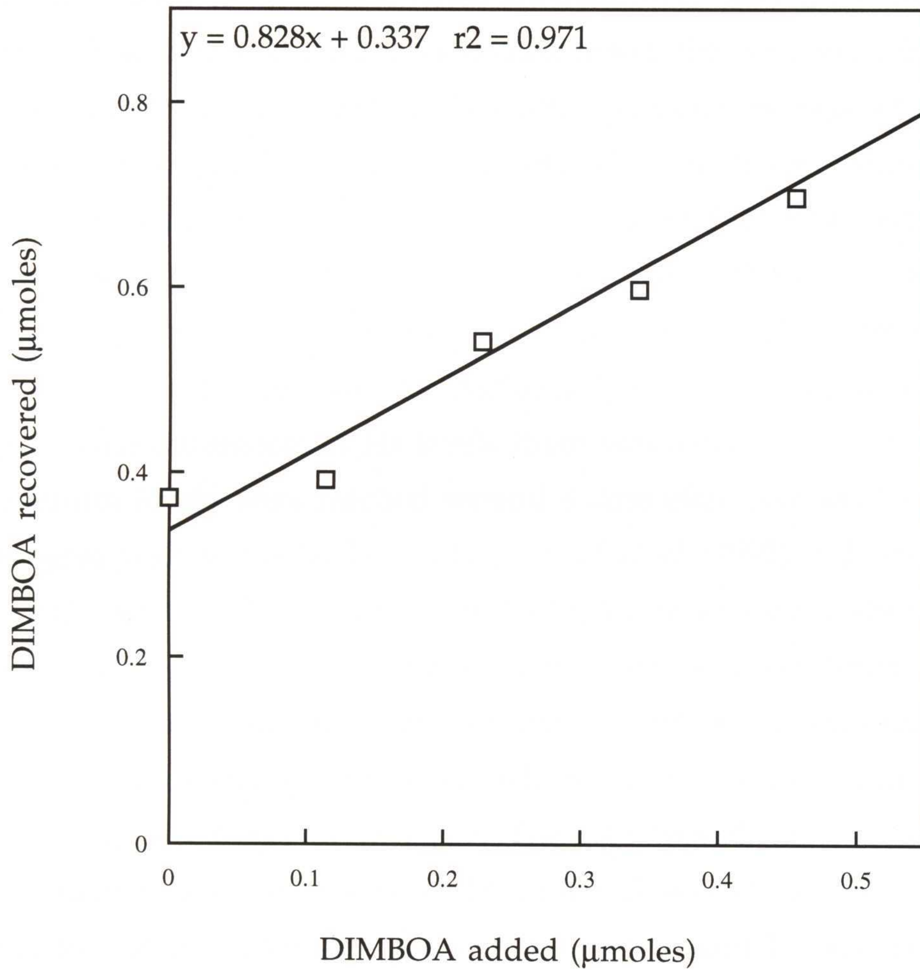


Fig 3.5 : Recovery of DIMBOA from wheat root extracts. Root tissue was extracted with aqueous solutions containing DIMBOA and allowed to stand for 15 min. The pH was taken to 3, the extract centrifuged and the supernatant analyzed for DIMBOA. The slope of the line indicates that recovery of DIMBOA was 83%.

3.4.2 Changes in hydroxamic acid levels in plants over time

Time-course studies to monitor the Hx concentrations in wheat, triticale and rye were performed to determine when maximum levels of the compounds occurred in these plants. For the three cereals tested, the concentration of Hx in roots was maximum three to four days after germination (Figs 3.6, 3.9 and 3.12). The DIMBOA levels in 3 and 4-day-old wheat roots were significantly greater than those in older plants. For triticale, both DIMBOA and DIBOA levels were not significantly different over the course of the assay. In rye the DIMBOA levels were not significantly different but the DIBOA levels were significantly higher in three day old seedlings. For those cereals that did not show significant differences in Hx levels there was a general trend showing that maximum levels were reached around 4 days after germination. These findings agree with the published data of Nicol *et al.* (1992) and Argandoña *et. al.* (1981) who found maximum DIMBOA levels in wheat after 4 days. Argandoña *et al.* (1980) found that Hx levels reach a maximum in rye shortly after germination. Since Hx content content was maximum when plants were approximately four days old, most subsequent studies were performed with seedlings of this age. The fact that Hx reach a maximum after germination is consistent with the proposal that Hx act as protection mechanisms for the youngest parts of the plant which lack structural defences (Zúñiga *et al.*, 1983).

3.4.3 Varietal difference

Studies were performed to compare the Hx content of different cultivars of wheat, rye and triticale. In wheat, DIMBOA levels ranged between 0.35 and 1.48 µmoles/g f.wt whereas DIBOA was not detected. The cultivar Sunstar had significantly higher levels of DIMBOA than the other cultivars assayed, and Vulcan was found to contain significantly lower levels of DIMBOA. These two wheat varieties were chosen for further studies because of these large differences in Hx levels (Fig 3.8).

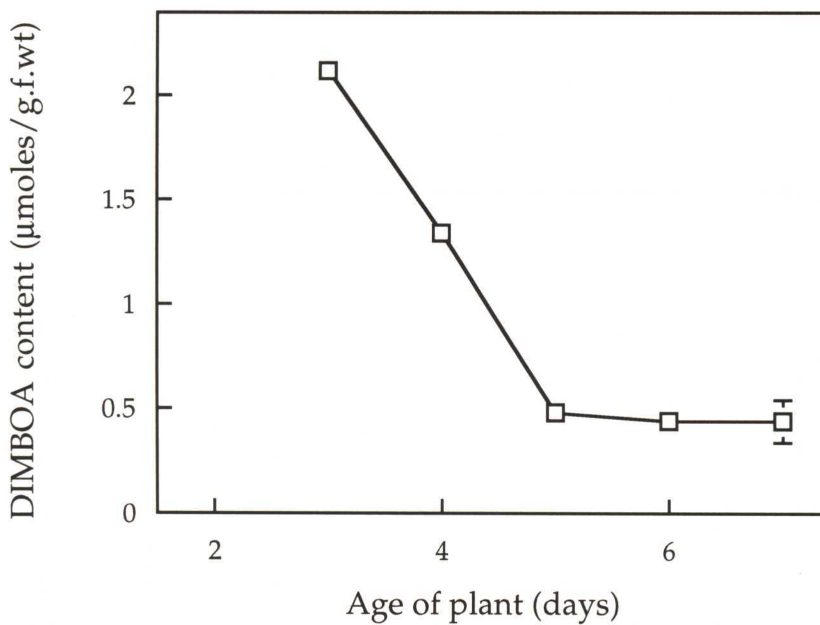


Fig 3.6 : Effect of plant age on DIMBOA content of wheat roots. Wheat (Sunstar) roots were analyzed as described in 2.2.3. The results are the mean of duplicate experiments and the vertical bars are the SE of the mean. Absence of error bars indicates that error is smaller than the data point.

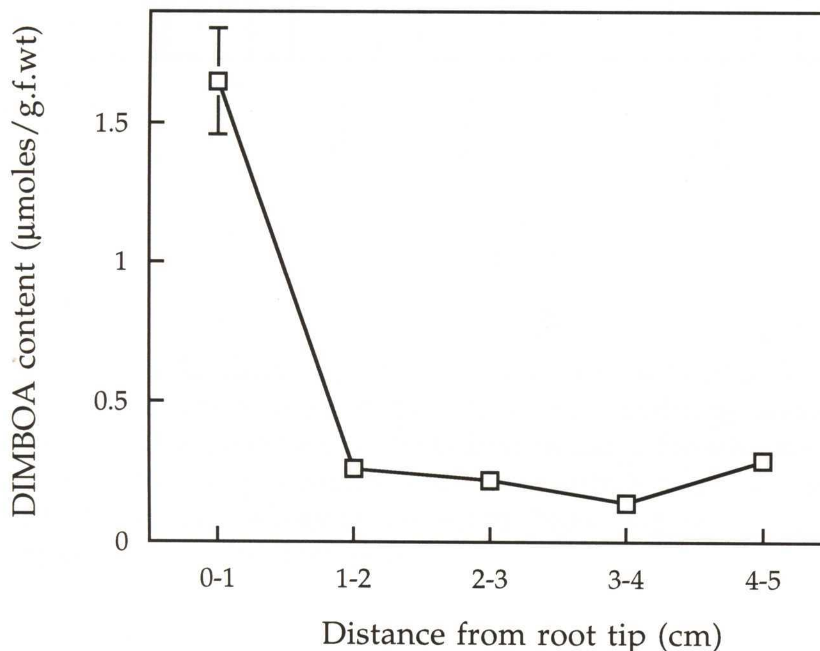


Fig 3.7 : The distribution of DIMBOA in wheat root sections. Roots of 5-day-old wheat (Sunstar) were cut into 1 cm sections which were analyzed for Hx concentration as in 2.2.3. The first section (0-1 cm) represents the root tip. The results are the mean of duplicate experiments and the vertical bars represent then SE of the mean. Absence of error bars indicates that error is smaller than the data point.

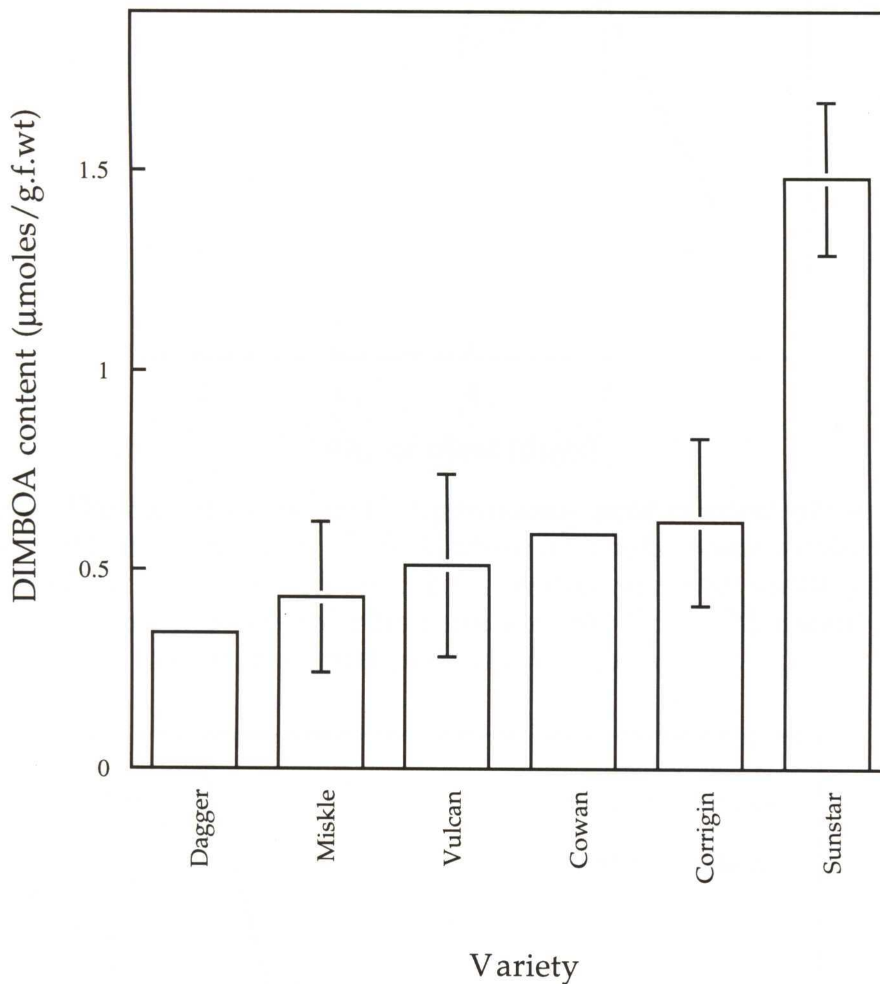


Fig 3.8 : Hxydroxamic acid content of roots of different wheat varieties. Roots from 4-day-old wheat seedlings were analyzed for DIMBOA content as described in 2.2.3. Results are the mean of duplicate experiments and the vertical bars represent the SE of the mean. Absence of error bars indicates that error is smaller than the data point.

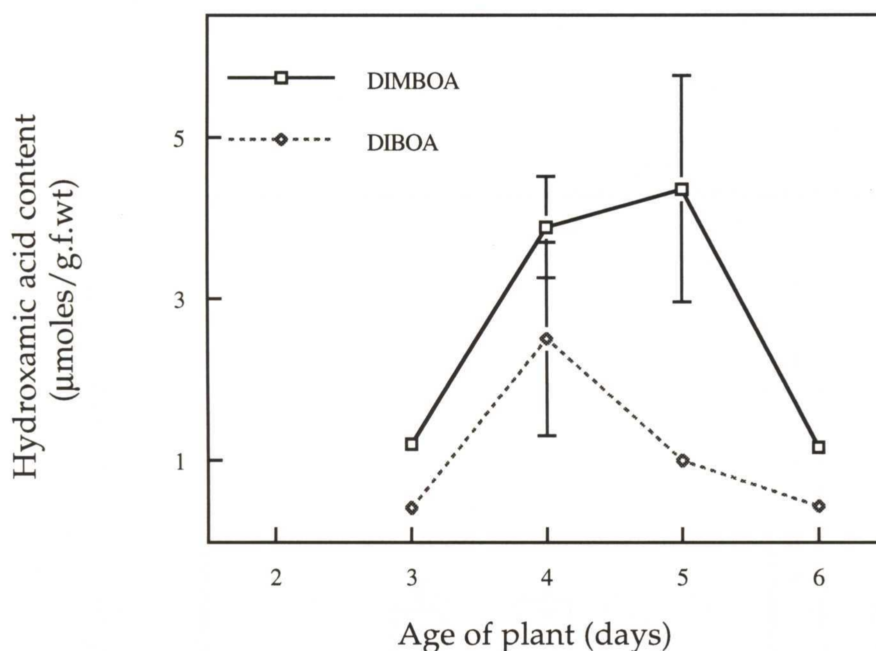


Fig: 3.9 : The variation in cyclic hydroxamic acid concentration with plant age in triticale. Triticale (Tall Madonna) roots were analyzed for Hx concentration as described in 2.2.3. Results are the mean of duplicate experiments and the vertical bars represent the SE of the mean. Absence of error indicates the error is smaller than the data point.

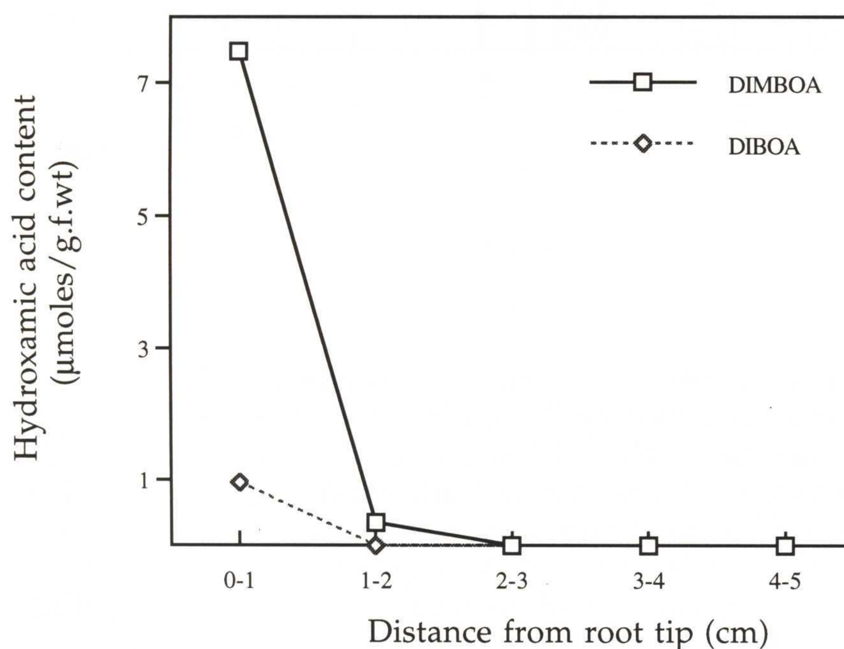


Fig 3.10 : The distribution of cyclic hydroxamic acids in triticale roots. Roots of 5-day-old triticale (Tall Madonna) were cut into 1 cm sections and analyzed for Hx concentration as described in 2.2.3. The first section (0-1 cm) represents the root tip. The results are the mean of duplicate experiments and the vertical bars represent the SE of the mean. Absence of error bars indicates that error is smaller than the data point.

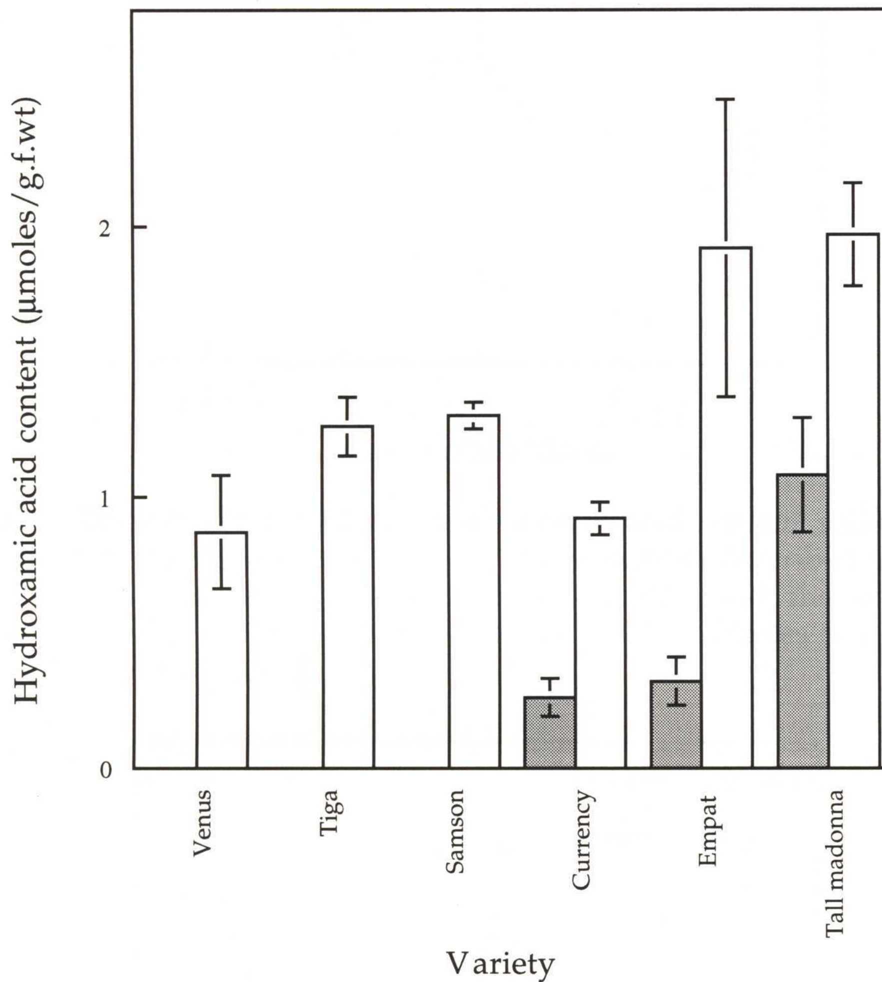


Fig 3.11 : The distribution of cylic hydroxamic acids in selected triticale. Roots from 4-day-old seedlings were analyzed for cyclic Hx concentration as described in 2.2.3. The solid bars represent DIBOA content whereas the unfilled bars represent DIMBOA content. Results are the mean of duplicate experiments and the vertical bars represent the SE of the mean. Absence of error bars indicates the error is smaller than the data point.

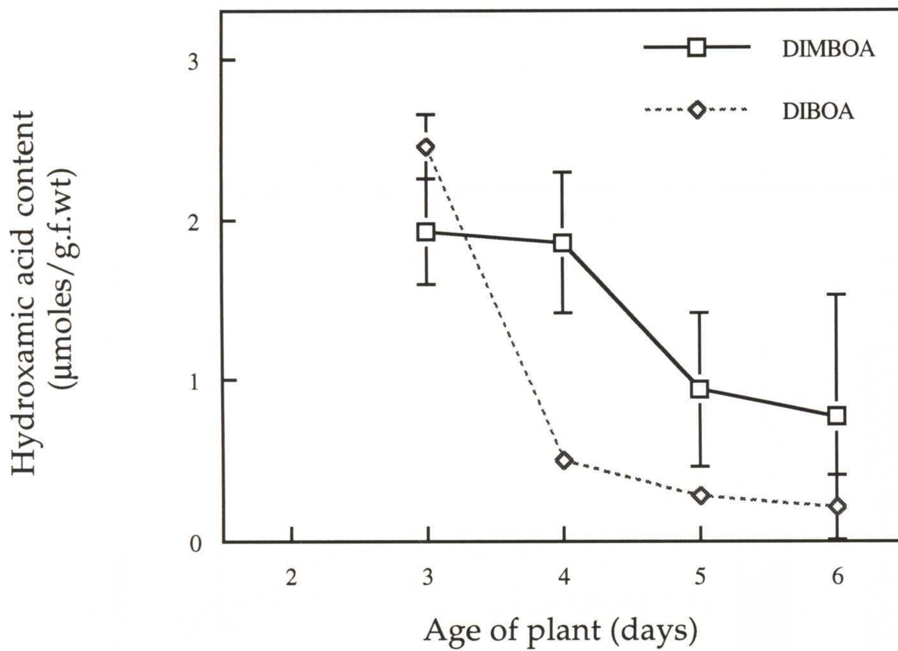


Fig 3.12 : The variation in cyclic hydroxamic acid content with plant age. Rye (Uneveta) roots were analyzed for Hx content as described in 2.2.3. The results are the mean of duplicate experiments and the vertical bars represent the SE of the mean. Absence of error bars indicates that the error is smaller than the data point.

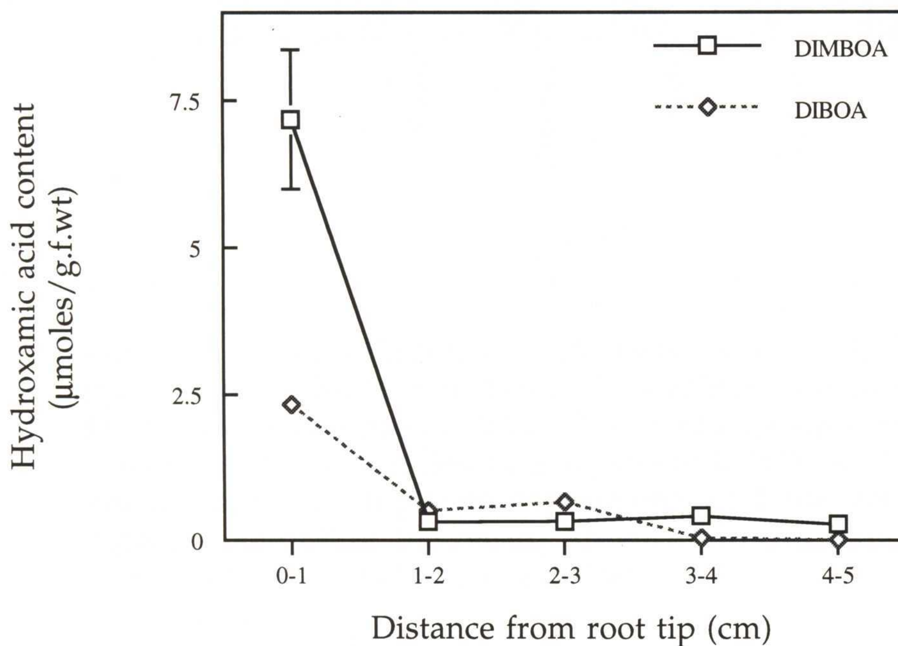


Fig 3.13 : The distribution of cyclic hydroxamic acids in rye root sections. Roots of 5-day-old rye (Uneveta) were cut into 1cm sections which were analyzed for Hx concentration as described in 2.2.3. The first section (0-1 cm) represents the root tip. Results are the mean of duplicate experiments and the vertical bars represent the SE of the mean. Absence of error bars indicates that the error is saller than the data point.

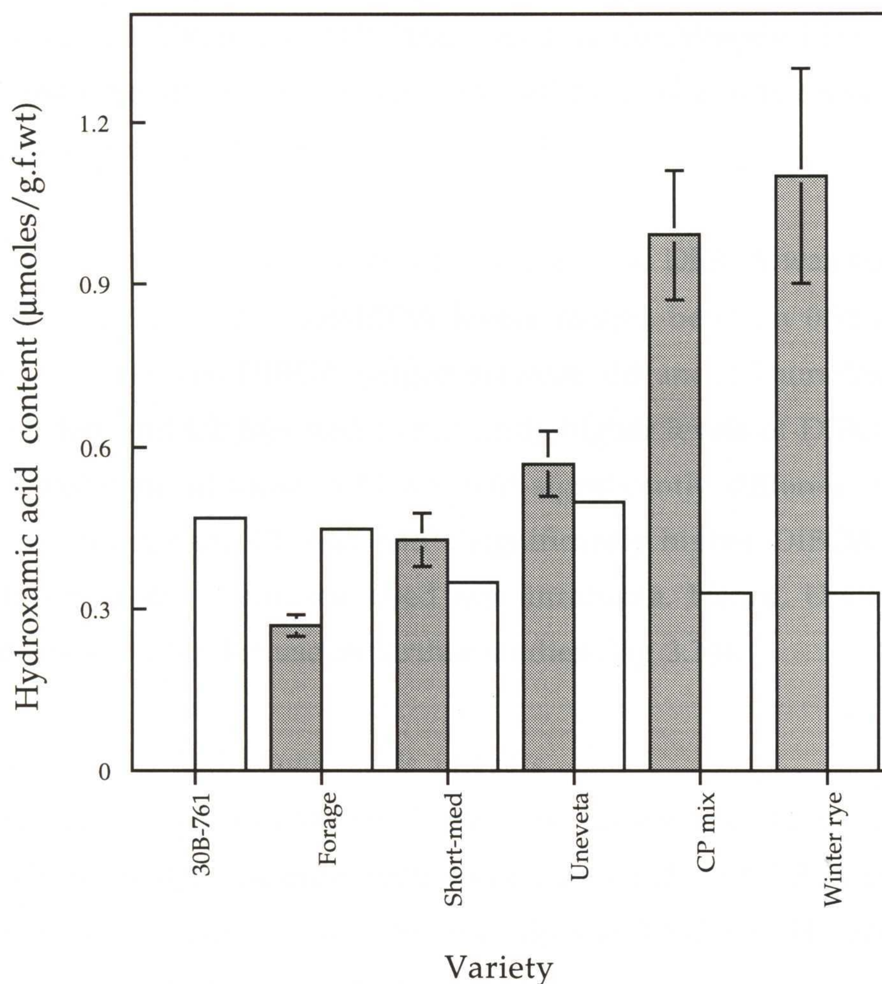


Fig 3.14 : The distribution of cyclic hydroxamic acids in selected rye varieties. Roots from 4-day-old seedlings was analyzed for Hx content as described in 2.2.3. The solid bars represent DIBOA content while the unfilled bars represent DIMBOA. The results are the mean of duplicate experiments and the vertical bars represent the SE of the mean. Absence of error bars indicates that the error is smaller than the data point.

DIMBOA was found in all of the cultivars of triticale, and DIBOA was detected in several of the varieties assayed. DIMBOA levels ranged between 0.87 and 1.97 μmoles/g f.wt., whereas DIBOA, when present, ranged between 0.26 and 1.08 μmoles/g f.wt. Both Empat and Tall Madonna had significantly higher levels of DIMBOA and Tall Madonna had significantly higher levels of DIBOA than the other triticales assayed. Tall Madonna was chosen out of the triticales for use in further studies (Fig 3.11).

DIMBOA was detected in all rye cultivars assayed and DIBOA was present in all but one of the cultivars. DIMBOA levels ranged between 0.33 and 0.50 μmoles/g f.wt., whereas DIBOA ranged between 0.0 and 1.1 μmoles/g f.wt. Both Winter Rye and CP Mix had significantly higher levels of DIBOA of the rye studied but none of these cultivars had significantly different levels of DIMBOA. Winter rye and CP Mix had a significantly higher DIBOA content but growth under the conditions used was unreliable. Hence, Uneveta and Short-med were chosen for use in further studies (Fig 3.14).

3.4.4 Distribution of hydroxamic acids in roots

In the three cereal species examined, the root tip always had significantly higher levels of Hx than the older sections of the root (Figs 3.7, 3.10 and 3.13). The higher concentrations found in the root tip may be due to Hx acting as a protective measure as it has been noted that take-all infection occurs away from the growing root tip (Skou, 1981).

In conclusion, a convenient HPLC method which does not require the use of a solvent gradient was established which completely separates DIBOA, DIMBOA, BOA and MBOA within 17 min. This method was used to determine the Hx content of root tissues. Time-course studies and studies on the distribution of Hx in roots showed that the Hx concentrations are greatest in the youngest tissues and decline rapidly with plant age. The Hx content of different varieties was studied to select varieties for use in subsequent studies as varieties for later studies were chosen on their Hx

content. Varieties which differ in Hx content and the types of Hx will be used to test the hypothesis that Hx act as a defence mechanism for take-all.

CHAPTER FOUR

The effect of hydroxamic acids on the growth of Gaeumannomyces

4.1 Introduction

Hx were first identified after Virtanen and Hietala (1955) found that extracts made from rye seedlings exhibited an anti-Fusarium effect when incorporated into growth medium. From this initial finding more Hx were identified and their effects on other fungi, bacteria and plants were studied.

In studying the root rotting disease take-all caused by *Ggt*, Klein and Marshall (1989) found that root extracts of rye, triticale and wheat incorporated into culture medium exerted different effects on the growth of *Ggt*. They found that hyphal growth of *Ggt* in the presence of rye extracts was neglible, growth was intermediate with triticale extracts, and extensive with wheat extracts. The effect of the extracts was concentration dependent as the effect of the extracts on the growth of *Ggt* was lessened when the extracts were diluted . In 1990, Liu *et al.* suggested that the compound in rye roots which induced resistance to take-all was BOA.

This chapter describes a study in which the effect of DIBOA, BOA, DIMBOA and MBOA on root growth was examined.

4.2 Results

4.2.1 The effect of cereal root extracts on the growth of Ggt

When extracts prepared from wheat roots of cv Sunstar were added to nutrient medium, growth of *Ggt* was not inhibited (Fig 4.1 and 4.2). There was no significant effect on the strain EBI as it grew to a diameter 110% of the control after 5 days, whereas the growth of *Ggt* WP 28 was significantly

greater than the control. The diameter of the WP 28 colony was 150% of the control after 5 days.

The extracts prepared from the wheat cv Vulcan did not significantly affect the growth of *Ggt* (Figs 4.1 and 4.2). After 5 days the diameters of the colonies were 95 and 77% of the control respectively.

The extracts prepared from triticale (cv Tall Madonna) roots significantly inhibited the growth of both strains of *Ggt* studied (Figs 4.1 and 4.2). The strain EBI was inhibited more than WP 28. After 5 days the diameter of *Ggt* EBI was 26% of the control whereas the diameter of WP 28 was 70% of the control.

The greatest inhibition of fungal growth was observed when extracts prepared from rye (cv Uneveta) were incorporated into the nutrient media (Figs 4.1 and 4.2). The growth of both strains of *Ggt* was significantly inhibited with the strain EBI being inhibited by 70% and strain WP 28 being inhibited by 80% after 5 days.

The following figures represent the results of four separate growth experiments for both strains of the fungus presented in the one graph.

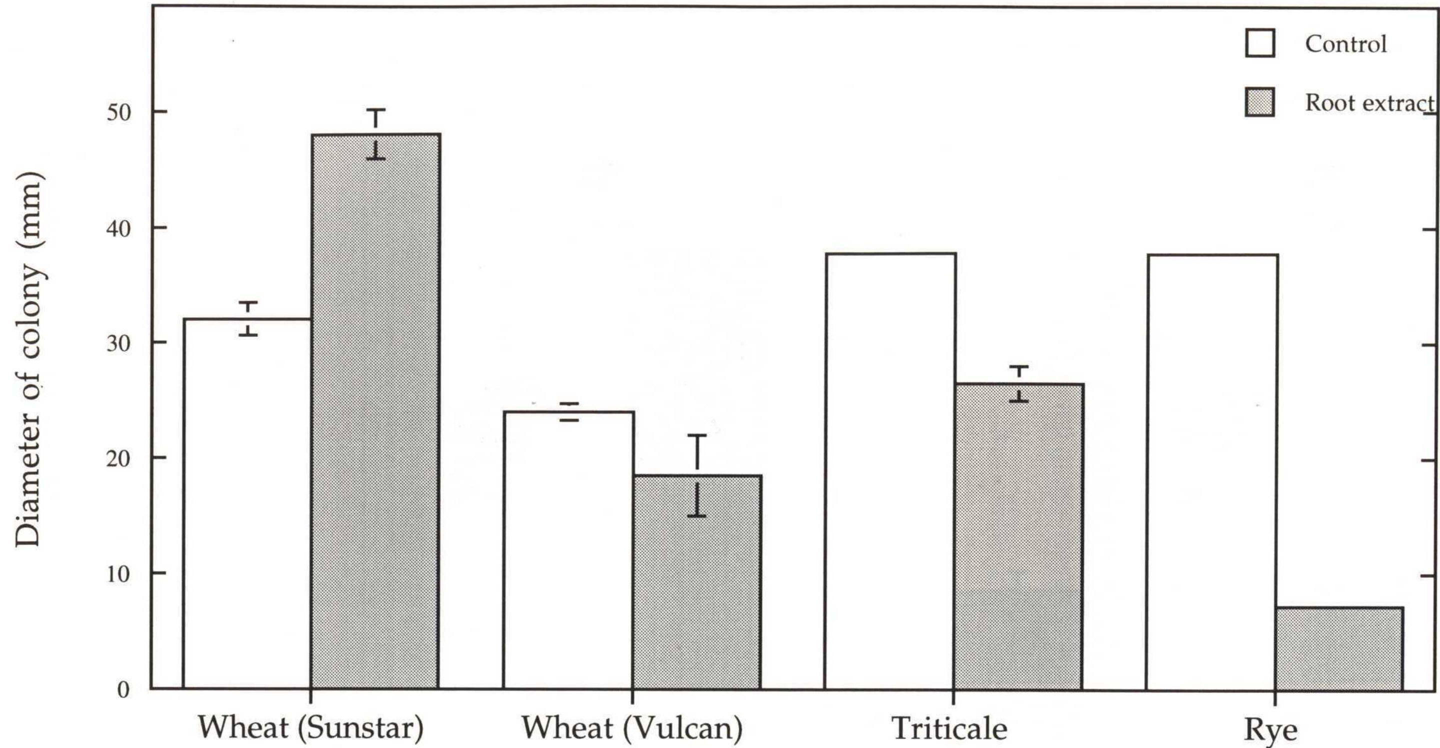


Fig 4.1: The effect of cereal root extracts on the growth of Ggt WP28. Extracts of roots of wheat, triticale and rye were incorporated into growth media and the diameter of the colony of Ggt WP28 was measured after 5 days. The unfilled bars represent the control and the filled bars represent growth media which contained extract from 200 mg f.wt of root tissue/ml. The results are the mean of duplicate experiments and the vertical bars represent the SE of the mean. Absence of error bars indicates the error is smaller than the data point. The differences between the control and the media with root extracts were significant for Sunstar, triticale and rye are significantly different.

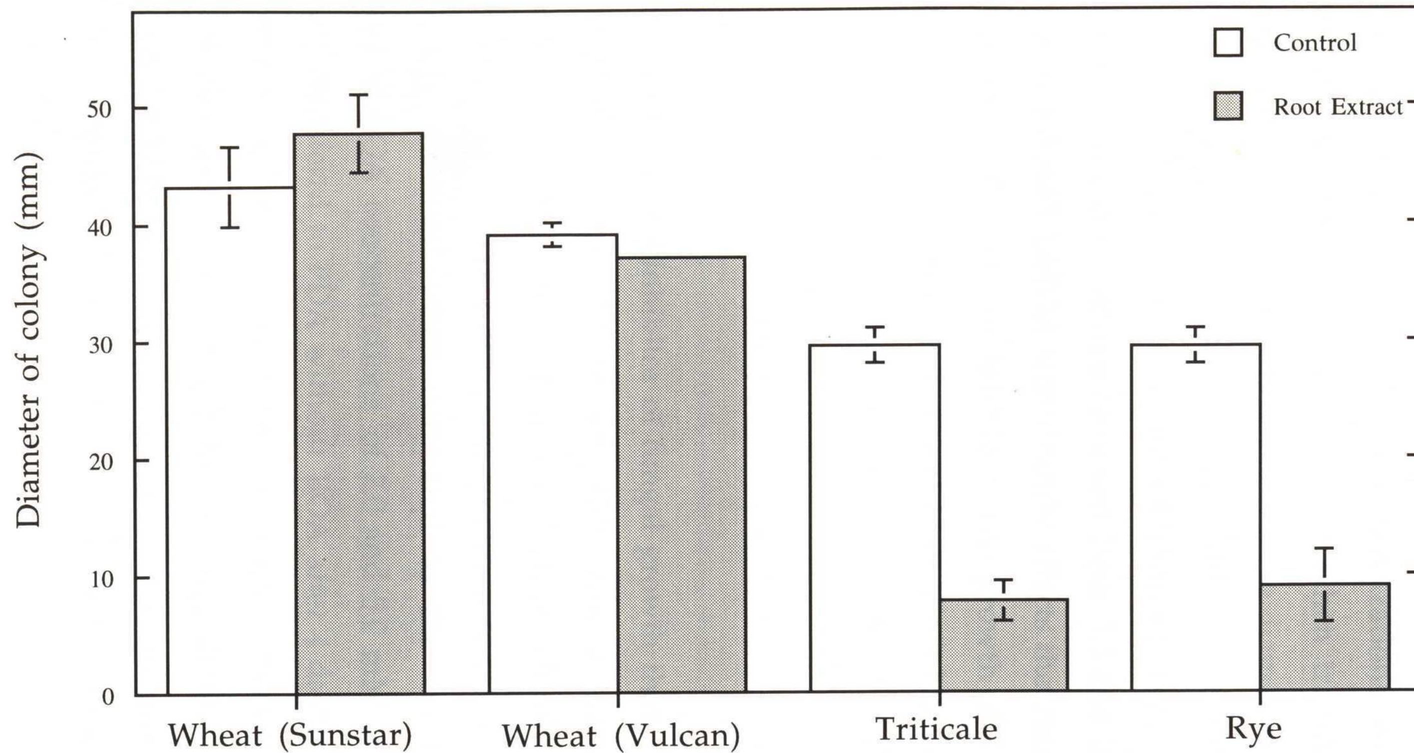


Fig 4.2 : The effect of cereal root extracts on the growth of Ggt EBI. Extracts of roots of wheat, triticale and rye were incorporated into growth media and the diameter of the colony of *Ggt* EBI was measured after 5 days. The unfilled bars represent the control while the filled bars representgrowth media which contain extract from 200 mg f.wt of root tissue/ml. The results are the mean of duplicate experiments and the vertical bars represent the SE of the mean. Absence of error bars indicates that error is smaller than the data point. The differences between the control and the media with root extracts were significant for triticale and rye are significantly different.

4.2.2 The effect of hydroxamic acids on the growth of Ggt.

4.2.2.1 DIBOA

Of the Hx studied, DIBOA was the most effective inhibitor of the growth of *Ggt*. All concentrations of DIBOA studied were significant inhibitors of *Ggt* (Figs 4.3 and 4.4). For both strains of *Ggt*, 1.5 2.0 and 5.0 mM DIBOA totally inhibited the growth of *Ggt*. These concentrations were only fungistatic, however, as the colonies resumed growth when transferred after 4 days to PDA containing no inhibitor. After 7 days, the diameters of the colonies that were transferred were compared to each other. The diameter of the strain EBI, which was removed from 5 mM DIBOA, was significantly smaller than the diameters of those colonies removed from 1.5 and 2.0 mM DIBOA. This indicates that 5 mM DIBOA significantly affects the recovery of the fungus after it is removed from the inhibitor. The growth of the strain WP 28 was not significantly affected after removal from the inhibitor indicating that the fungus able to attack rye is not more able to survive on the HX found in rye.

4.2.2.2 BOA

BOA was a less potent inhibitor of fungal growth than its precursor DIBOA. In both studies, (Figs 4.5 and 4.6), *Ggt* grew to some extent on media containing concentrations up to and including 1.5 mM BOA. After 4 days, the growth of both strains of *Ggt*, at all concentrations of BOA studied was significantly inhibited when compared to the control. Those colonies subjected to BOA concentrations of 2.0 and 5.0 mM were totally inhibited and were transferrd to PDA without BOA after 4 days. When the diameters of the transferred colonies were compared after removal from the inhibitor, those colonies grown on 5 mM BOA were significantly smaller than those grown on 2 mM BOA for both strains of the fungus. This indicates that 5 mM BOA significantly affects the recovery of the fungus after it is removed from the inhibitor.

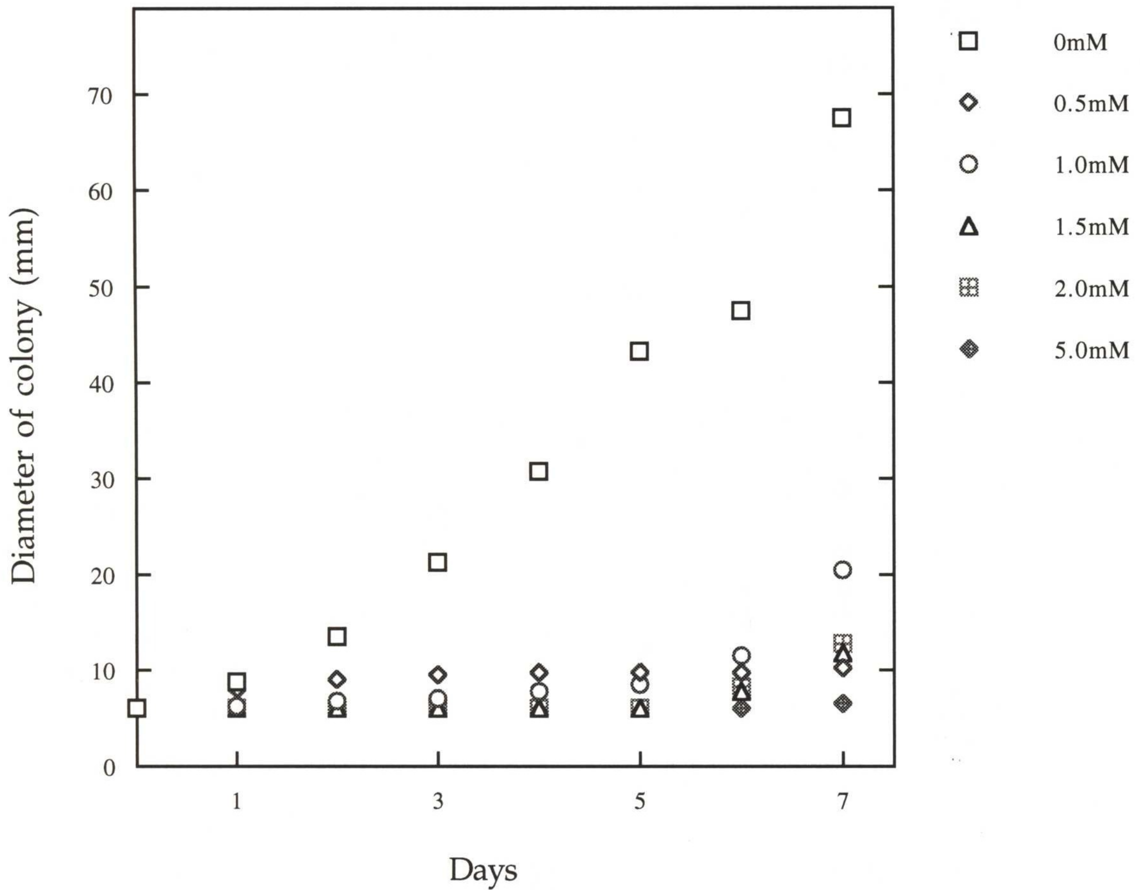


Fig 4.3 : The effect of DIBOA on the growth of Ggt (EBI). DIBOA was incorporated at the concentrations shown into PDA from a filter sterilized solution and the diameter of the colonies measured at the times indicated. After 4 days, colonies that did not exhibit growth (1.5, 2.0 and 5.0 mM) were transferred to PDA without DIBOA. The results are the mean of duplicate experiments. The diameter of the control was significantly greater than the diameter of the colonies grown on media containing DIBOA, at all concentrations tested, after 4 days

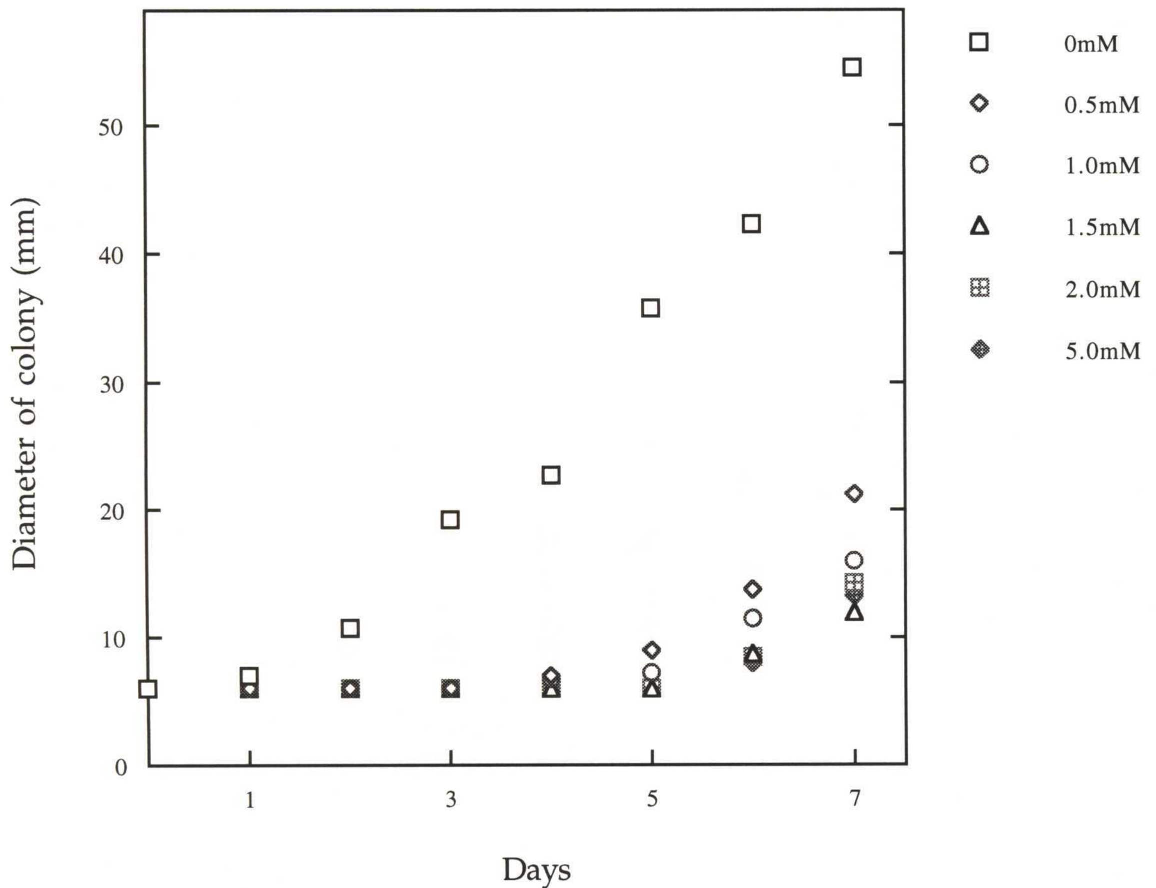


Fig 4.4 : The effect of DIBOA on the growth of Ggt (WP 28). DIBOA was incorporated at the concentrations shown into PDA from a filter sterilized solution and the diameter of the colonies measured at the times indicated. After 4 days, colonies that did not exhibit growth (1.5, 2.0 and 5.0 mM) were transferred to PDA without DIBOA. The results are the mean of duplicate experiments. The diameter of the control was significantly greater than the diameter of the colonies grown on media containing DIBOA, at all concentrations tested, after 4 days.

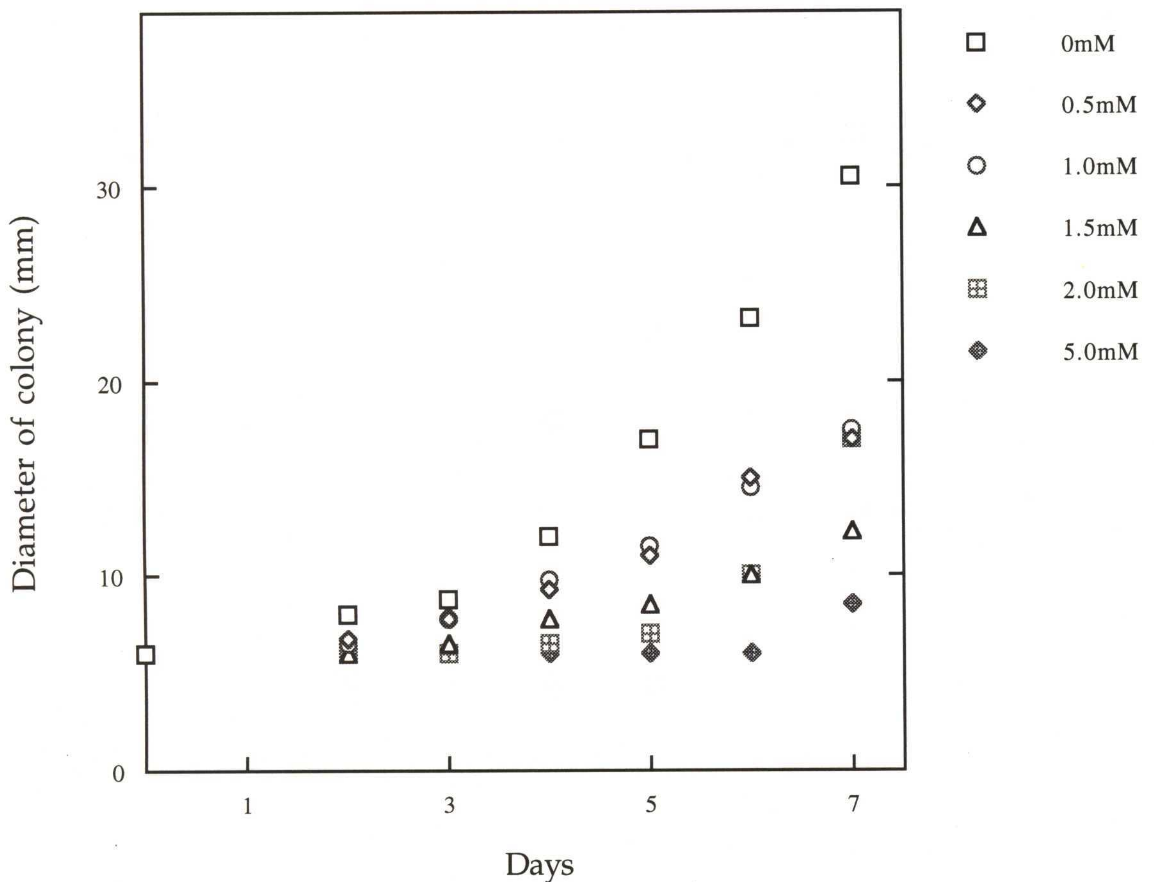


Fig 4.5 : The effect of BOA on the growth of Ggt (EBI). BOA was incorporated at the concentrations shown into PDA from a filter sterilized solution and the diameter of the colonies measured at the times indicated. After 4 days, colonies that did not exhibit any growth (2.0 and 5.0 mM) were transferred to PDA without BOA. The results are the mean of duplicate experiments. The diameter of the control was significantly greater than the diameter of the colonies grown on media containing BOA, at all concentrations tested, after 4 days.

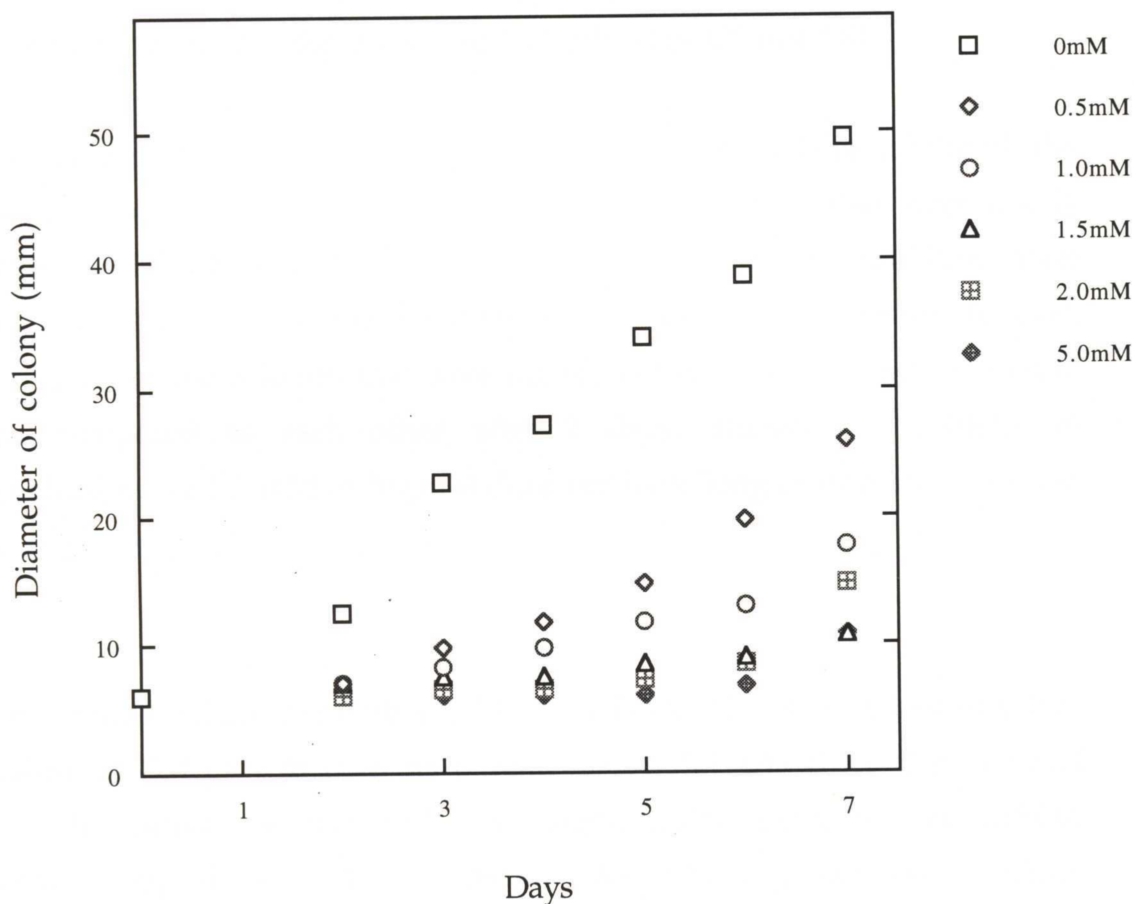


Fig 4. 6 : The effect of BOA on the growth of Ggt (WP 28). BOA was incorporated at the concentrations shown into PDA from a filter sterilized solution and the diameter of the colonies measured at rightat the times indicated. After 4 days, colonies that did not exhinit growth (2.0 and 5.0 mM) were transferred to PDA without BOA. The results are the mean of duplicate experiments. The diameter of the control was significantly greater than the diameter of the colonies grown on media containing BOA, at all concentrations, after 4 days.

4.2.2.3 DIMBOA

DIMBOA was not as effective in inhibiting the growth of *Ggt* as its demethoxylated analogue, DIBOA or BOA. All concentrations of DIMBOA significantly inhibited the growth of *Ggt* except 0.5 mM DIMBOA which did not significantly inhibit the growth of *Ggt* EBI (Figs 4.7 and 4.8).

DIMBOA concentrations of 1.0, 1.5, 2.0 and 5.0 mM totally inhibited the growth of both strains of *Ggt* after 4 days. Those colonies that were totally inhibited by DIMBOA were transferred to PDA without any inhibitor. After 7 days the diameters of the transferred colonies were compared to each other. None of the colonies that were transferred were significantly different when compared to each other after 7 days. Therefore, DIMBOA at concentrations of 1.0 mM to 5.0 mM does not have long lasting effects on the growth of the fungus.

4.2.2.4 MBOA

As was found when comparing DIBOA to BOA, MBOA is a less effective inhibitor of *Ggt* growth than its parent compound DIMBOA (Figs 4.9 and 4.10). The strain of *Ggt* EBI was significantly inhibited at MBOA concentrations of 1.0 mM and above. The colony grown on medium containing 0.5 mM MBOA was not inhibited at all however, as the diameter of the colony was the same as the control after 4 days. All the concentrations of MBOA studied significantly inhibited the growth of *Ggt* WP 28 however.

Only 2.0 and 5.0 mM MBOA totally inhibited the growth of both strains of *Ggt*. After 4 days, these colonies were transferred to PDA without MBOA . When the diameters of the colonies transferred from 2.0 and 5.0 mM MBOA were compared after 7 days there was no significant difference indicating that the recovery was similar for all colonies.

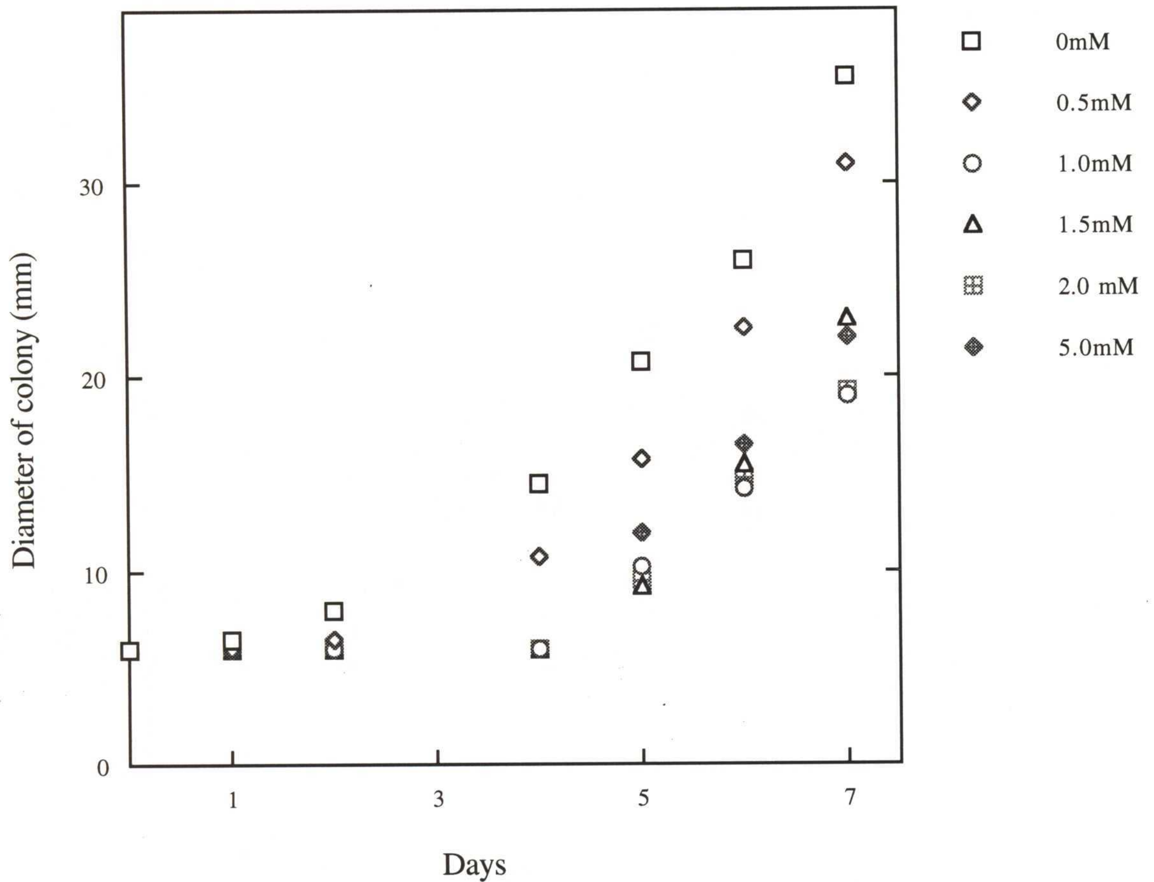


Fig 4.7 : The effect of DIMBOA on the growth of Ggt (EBI). DIMBOA was incorporated at the concentrations shown into PDA from a filter sterilized solution and the diameter of the colonies was measured at the times indicated. After 4 days, colonies that did not exhibit any growth (1.0, 1.5, 2.0 and 5.0 mM) were transferred to PDA without DIMBOA. The results are the mean of duplicate experiments. The diameter of the control was significantly greater than the diameters of the colonies grown on media containing DIMBOA, except for 0.5 mM, after 4 days.

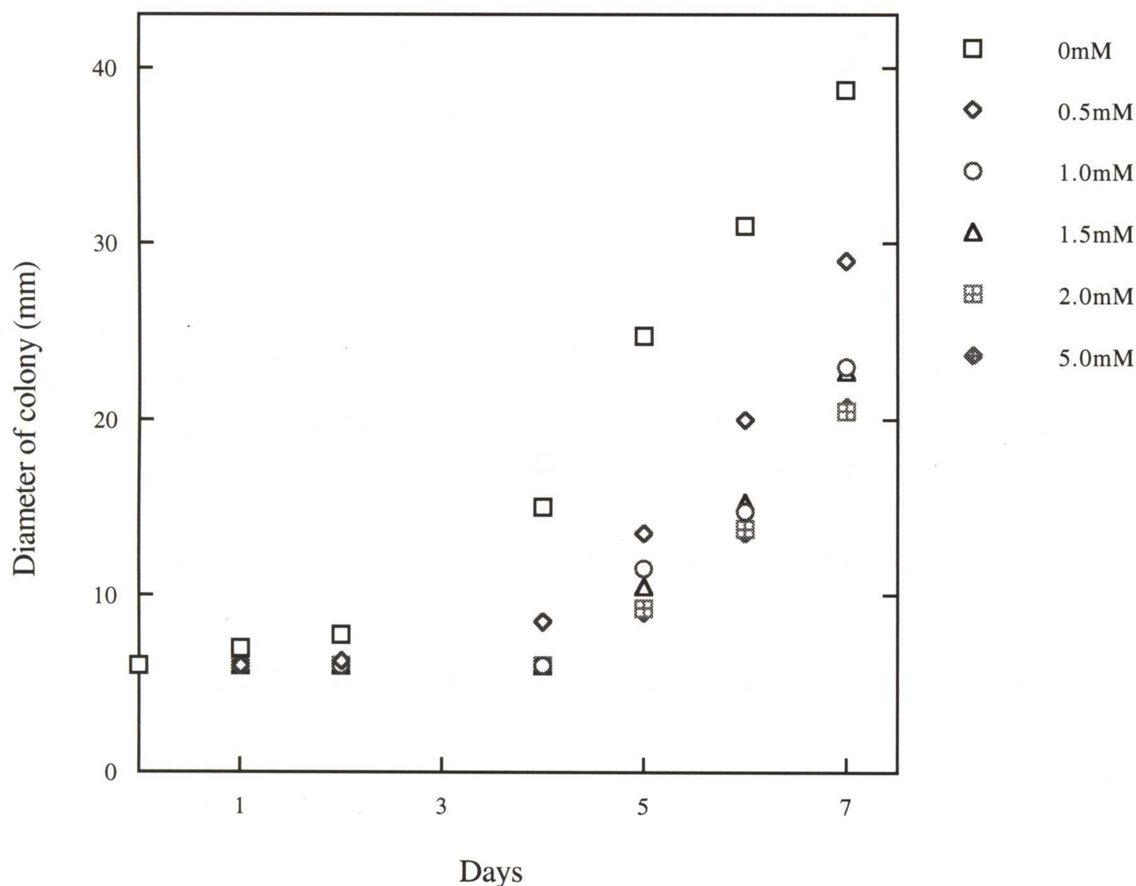


Fig 4.8 : The effect of DIMBOA on the growth of Ggt (WP 28). DIMBOA was incorporated at the concentrations shown into PDA from afilter sterilized solution and the diameter of the colonies measured at the times indicated. After 4 days, colonies that did not exhibit growth (1.0, 1.5, 2.0 and 5.0 mM) were transferred to PDA without DIMBOA. The results are the mean of duplicate experiments. The diameter of the control was significantly greater than the diameter of the colonies grown on media containing DIMBOA, at all concentrations, after 4 days.

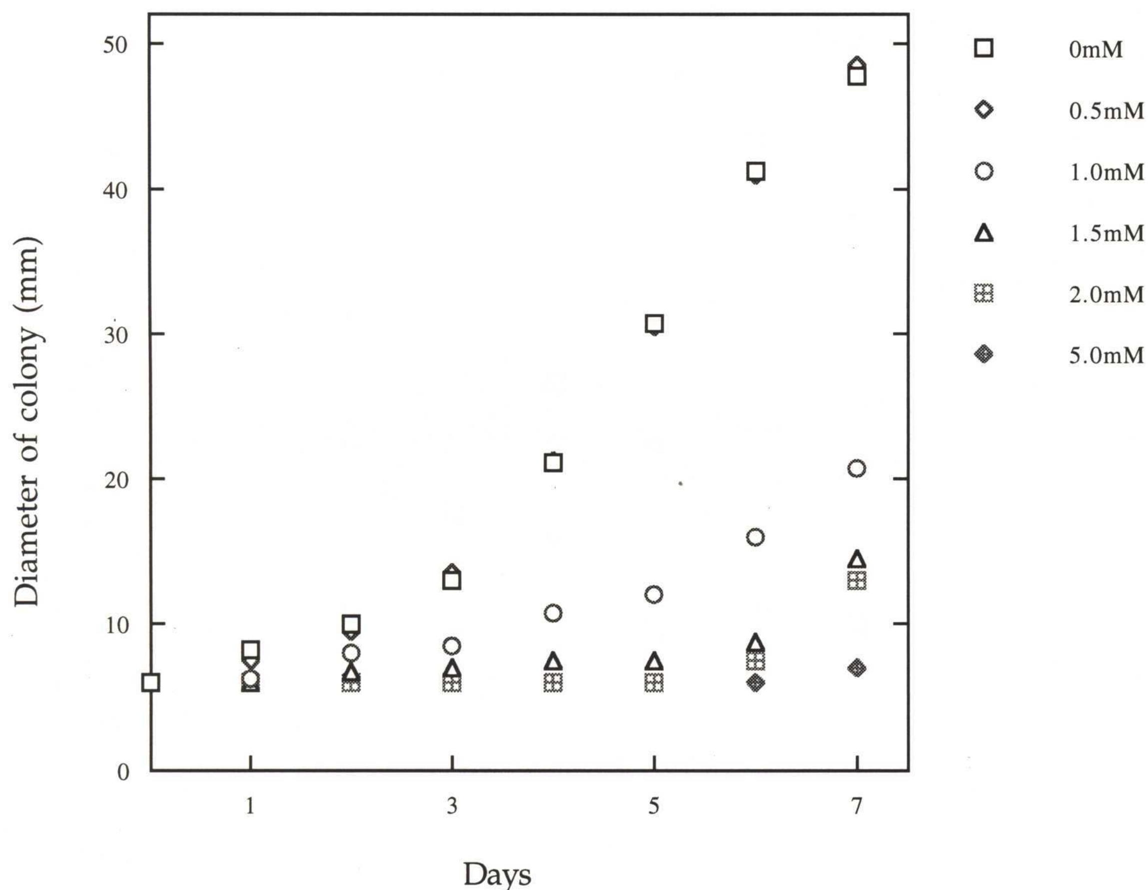


Fig 4.9 : The effect of MBOA on the growth of Ggt (EBI). MBOA was incorporated at the concentrations shown into PDA from a filter sterilized solution and the diameter of the colonies measured at the times indicated. After 4 days, colonies that did not exhibit growth (2.0 and 5.0 mM) were transferred to PDA without MBOA. The results are the mean of duplicate experiments. The diameter of the control was significantly greater than the diameters of the colonies grown on media containing MBOA, except for 0.5 mM, after 4 days.

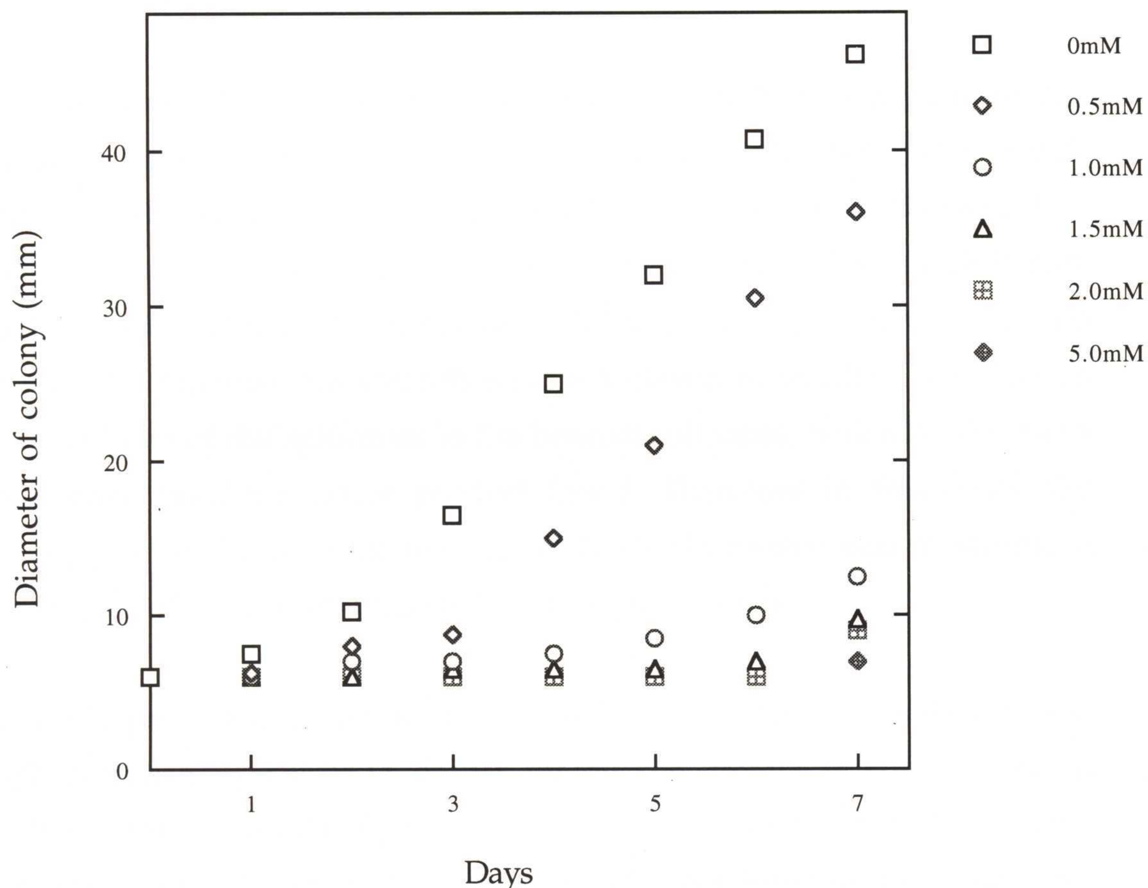


Fig 4.10 : The effect of MBOA on the growth of Ggt (WP 28). MBOA was incorporated at the concentrations shown into PDA from a filter sterilized solution and the diameter of the colonies measured at the times indicated. After 4 days, colonies that did not exhibit growth (2.0 and 5.0 mM) were transferred to PDA without MBOA. The results are the mean of duplicate experiments. The diameter of the control was significantly greater than the diameter of the colonies grown on media containing MBOA, at all concentrations, after 4 days.

4.3 Discussion

The results presented in this study show that the addition of Hx to the media and extracts containing Hx added to media inhibit the growth of *Ggt in vitro*.

The inhibition exhibited by the respective extracts is a reflection of the general resistance of the respective cereals to *Ggt* in the field. The cereals rated on their inhibitory activity towards *Ggt* were rye>triticale>wheat. In a previous study Klein and Marshall (1989) found a similar result and from initial studies attributed the inhibition to BOA. In the experiment conducted by Klein and Marshall the extracts were autoclaved to sterilize resulting in the breakdown of the aglucones to the benzoxazolinones, which is why BOA would have been the major product found. Therefore in this study the extracts were sterilized by filtering and added to the molten agar to maintain the integrity of the compounds contained in the extracts.

The results presented in Figs 4.1 and 4.2 indicate that the extracts from wheat which contain only DIMBOA do not inhibit the growth of either strain of *Ggt*. In fact the strain WP 28 grew to be significantly greater than the control. This result could be explained by these extracts containing nutrients or other compounds which may promote the growth of the fungus and negate the effect of the Hx in the media. Indeed, Klun and Brindley (1966) found that the inclusion of niacin and Brewers yeast in an artificial diet attenuated the inhibitory effects of MBOA. Weste (1972) also found that sugars and amino acids that were present in exudates promoted the growth of the fungus. In previous chapters it was shown that Sunstar had significantly higher levels of DIMBOA to Vulcan, however the concentration of DIMBOA in Sunstar is still not enough to inhibit the growth of *Ggt in vitro*.

The extracts prepared from both triticale and rye had a significant effect on the growth of both strains of *Ggt* when incorporated into the growth

medium. Triticale and rye contained both DIMBOA and DIBOA, and as shown in the latter part of this study, DIBOA is a more inhibitor than DIMBOA. This may be why media containing extracts from rye and triticale are more potent than media containing extracts prepared from wheat. The strain EBI is able to attack rye and this is probably why it grew to a greater extent than WP 28 on media containing rye extracts. There was not, however, a great difference observed between the ability of both strains to grow on the media containing extracts.

To determine which Hx were the most effective, each of the four inhibitors (DIBOA, BOA, DIMBOA and MBOA) were added separately to agar in increasing concentrations. All of the Hx significantly inhibited the growth of the two strains of *Ggt* at 0.05 mM (the lowest concentration tested) except for strain EBI grown on 0.5 mM MBOA and strain EBI grown on 0.5 mM DIMBOA. Even though the concentrations studied effectively inhibited the growth of both strains of *Ggt*, they were only fungistatic as the cultures resumed growing when replated on Hx-free PDA. The rate of growth of the colonies that were transferred was not as great as that of the colonies that were not inhibited. This indicates that the Hx do affect the rate at which the transferred colonies recover. Those colonies removed from 5.0 mM DIBOA and BOA grew significantly slower than the other transferred colonies indicating that these Hx at these concentrations significantly affect the recovery of the fungus. There was, however, no significant difference between the growth of the colonies transferred from DIMBOA and MBOA after 7 days indicating that the higher concentrations do not have a greater effect than lower concentrations on the recovery of the fungus. It may be possible, however, that concentrations above 5.0 mM would have a greater effect on the recovery of the take-all fungus when removed from the inhibitor. Based on the inhibition as a percentage growth of the control the inhibitors can be graded in increasing order of potency as DIBOA>BOA>DIMBOA>MBOA.

From grading of potency where DIBOA and BOA were found to be more potent than the methoxylated analogues could help explain why extracts from rye and triticale (which contain DIBOA) are more inhibitory than extracts from wheat which only contain DIMBOA. It is important to note that these results are from studies performed *in vitro* but this result could help to explain why rye and triticale are more resistant than wheat in the field.

Previous research has found that the growth of fungi may be inhibited by Hx. Hydroxamic acids have been shown to inhibit the growth of fungal pathogens in studies by Virtanen and Hietala (1955), Whitney and Mortimore (1959 a and b), El Naghy and Linko (1962), BeMiller and Pappelis (1965 a and b), ElNaghy and Shaw (1966), Baker and Smith (1977) and Long *et al.* (1975). General trends found that tissues with a high Hx content were more resistant than those tissues that had a low Hx content.

In more recent studies, the growth of *Ggt* has been shown to be inhibited by chemicals released from plant roots. In a study performed by Angus *et al.* (1994) it was shown that the growth of *Ggt* was inhibited by isothiocyanates released from brassicas. Even at the lowest concentration tested, the growth of *Ggt* was inhibited by 60-80% of the control.

In a study which is more relevant to the findings in this chapter, Browdowsky *et al.* (1994) found a Hx that inhibits a dioxygenase in Gg. Gg has a novel fatty acid dioxygenase, linoleic acid 8R-dioxygenase. The Hx N-(3-phenoxycinnamyl)acetohydroxamic acid or BWA4C was found to be a potent inhibitor of 8R-dioxygenase. Hx are strong chelators of the ferric ion of lipoxygenases and this leads to the suggestion that 8R-dioxygenase contains iron in its active site. If Hx do chelate to the ferric ion in enzymes it may help to explain the biological activity of Hx.

An important point to note is that this study and the other studies mentioned were performed *in vitro,* and the findings may not hold *in vivo* due to other factors affecting the growth of *Ggt*.

The main conclusions to draw from this chapter are that extracts of rye had a greater inhibitory effect on the growth of *Ggt* than extracts of triticale which were also found to be inhibitory. Extracts of wheat did not inhibit the growth of the take-all fungus *in vitro*. The next conclusion is that DIBOA is a more potent inhibitor of *Ggt* than DIMBOA. The effect of the aglucones was deemed more important than the benzoxazolinones as the benzoxazolinones are the decomposition product of the aglucones.

CHAPTER FIVE

Hydroxamic acid levels in cereals challenged by Gaeumannomyces

5.1 Introduction

Hx have been proposed to have a protective role in plants when attacked by fungi, bacteria and other pests. Previous chapters have indicated that Hx inhibit the growth of *Ggt in vivo*. In this chapter, the hypothesis that Hx may be produced in increased concentrations when cereal roots are infected by *Ggt* is tested.

5.2 Results

Whole roots of wheat, triticale and rye were assayed for Hx content at 3, 21 and 35 days to monitor Hx concentration over the initial infection period. The assay at day three gave the initial Hx concentration in the plant. At 35 days the plants were scored for take-all infection by the percentage of roots showing characteristic blackening and plants were also scored for stem-base blackening which is another symptom of take-all disease. This experiment was performed twice and both times the same trends were exhibited so the results for one experiment are presented and discussed.

Wheat showed the least resistance to *Ggt* after 35 days. Rated by the percentage of blackened roots, the cultivar Vulcan was the least resistant out of the wheat varieties tested for both strains of *Ggt*. Both Sunstar and Vulcan exhibited the characteristic stem base blackening. Triticale showed intermediate levels of disease severity as rated by the percentage of root blackening. Rye showed the greatest resistance to *Ggt*. The fungal strain WP28 did not infect rye (as is to be expected). The strain EBI infected cv Uneveta but not cv Short-med. There was no stem base blackening evident

in either the triticale or rye cultivars. For all treatments there was no significantly different change in Hx concentration.

5.2.1 Hydroxamic acid levels in wheat challenged with Ggt

The DIMBOA levels in Sunstar followed a natural decline over the 35 days in both the control plants and the plants challenged with fungus. The Hx levels in Sunstar were not detectable by day 21 indicating that there was no increased production of Hx in this cultivar in response to fungal infection. Vulcan showed the greatest percentage infection and that may be due to Hx not being detected (results not shown). The percentage infection for Vulcan was 0, 59 and 72% for the control, strain EBI and strain WP28 respectively. MBOA was not detected in either treatment. (Table 5.1 and 5.2).

Days	**Control**	***Ggt* EBI**	***Ggt* WP28**
3	0.21±0.02	-	-
21	ND	ND	ND
35	ND	ND	ND
% infection	0	43	25

* ND, not detected

Table 5.1: DIMBOA content in wheat (Sunstar) challenged with the take-all fungus. Hx were quantified as in 2.2.3 and 2.2.7. Concentrations are in µmoles/g.f.wt. and results are the mean of three replicates ± SE.

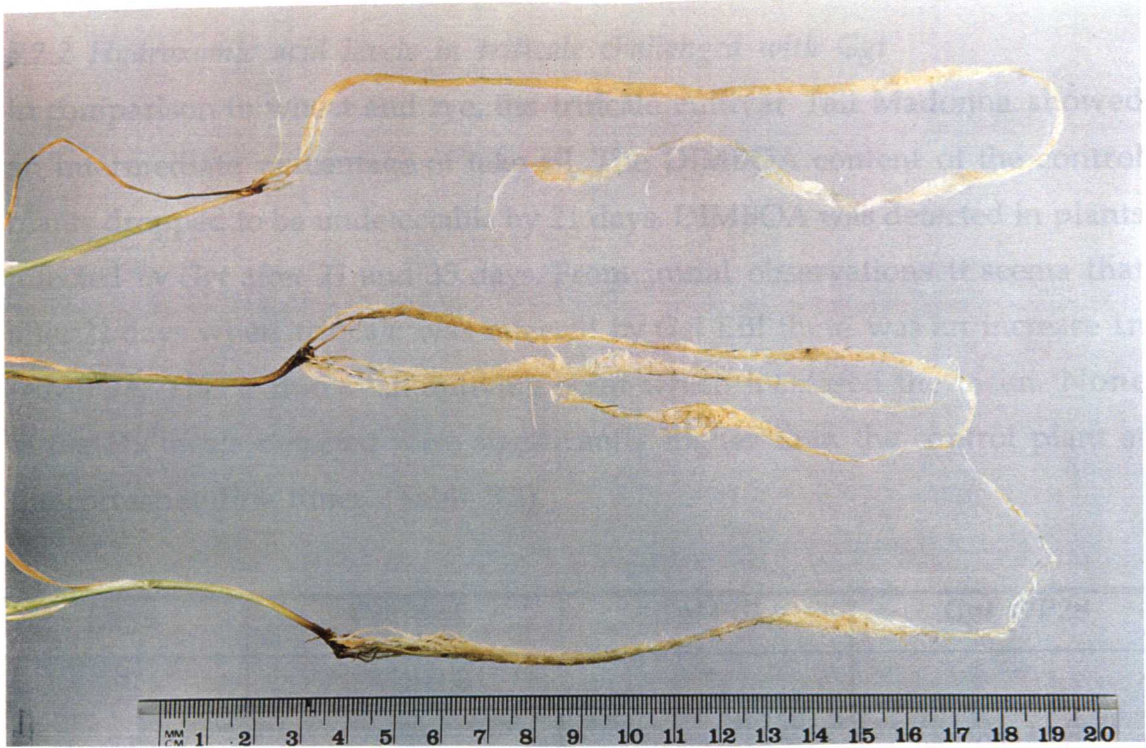


Fig 5.1 Infection of wheat roots with Ggt. The wheat seedlings (cv Sunstar) were harvested five weeks after inoculation with *Ggt*. The top plant is the control, the middle plant is wheat infected with WP28 and the bottom plant is wheat infected with EBI.

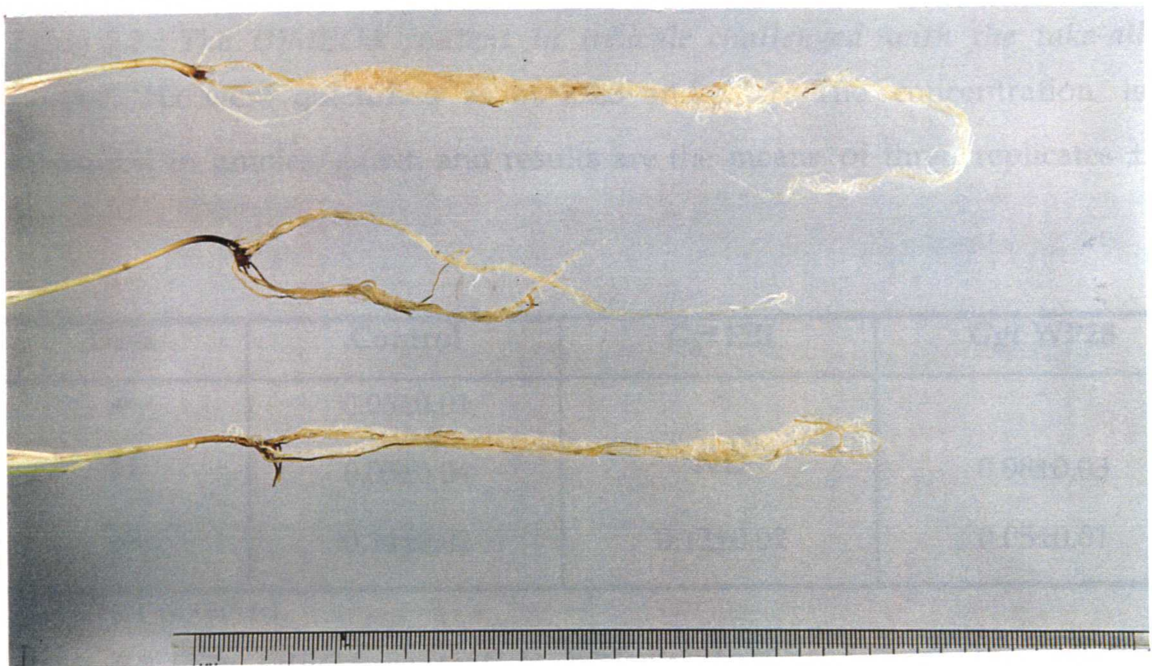


Fig 5.2 Infection of wheat roots with Ggt. The wheat seedlings (cv Vulcan) were harvested five weeks after inoculation with *Ggt*. The top plant is the control, the middle plant is wheat infected with WP28 and the bottom plant is wheat infected with EBI.

5.2.2 Hydroxamic acid levels in triticale challenged with Ggt

In comparison to wheat and rye, the triticale cultivar Tall Madonna showed an intermediate percentage of take-all. The DIMBOA content of the control plants dropped to be undetectable by 21 days. DIMBOA was detected in plants infected by *Ggt* after 21 and 35 days. From initial observations it seems that after 21 days when triticale was infected by *Ggt* EBI there was an increase in DIMBOA. This is due to an outlying point which increased the mean. None of the Hx levels detected were significantly higher than the control plant at the corresponding times. (Table 5.3).

Days	Control	*Ggt* EBI	*Ggt* WP28
3	0.15±0.05	-	-
21	ND	0.27±0.15	0.06±0.03
35	ND	0.06±0.04	0.06±0.04
% infection	0	28	64

*ND, not detected

Table 5.2 : The DIMBOA content in triticale challenged with the take-all fungus. Hx were quantified as in 2.2.3 and 2.2.7. The concentration is measured in µmoles/g.f.wt. and results are the means of three replicates ± SE.

Days	Control	*Ggt* EBI	*Ggt* WP28
3	0.05±0.01	-	-
21	0.05±0.04	ND	0.08±0.03
35	0.14±0.02	0.12±0.02	0.05±0.01

*ND, not detected

Table 5.3: The DIBOA content in triticale challenged with the take-all fungus. Hx were quantified as in 2.2.3 and 2.2.7. The concentrations are measured in µmoles/g.f.wt. and results are the mean of three replicates ± SE.

Fig 5.3 Infection of triticale roots with Ggt. The triticale seedlings (cv Tall Madonna) were harvested five weeks after inoculation with *Ggt*. The top plant is the control, the middle plant is triticale infected with WP28 and the bottom plant is triticale infected with EBI.

5.2.3 Hydroxamic acid levels in rye challenged with Ggt

In the study where the rye Uneveta was used, the DIMBOA levels followed a natural decline to be undetectable by day 21. DIMBOA was not detected in any plant after 21 days. The DIBOA of the control remained stable and DIMBOA was only detected in the plants infected with EBI after 35 days. The levels of DIBOA in the infected plant were not significantly higher than the levels found in the control however (Table 5.4 and 5.5).

In the study using the rye Short-med, DIMBOA was not detected (results not shown). DIBOA was only detected in plants grown in the presence of *Ggt*. None of these exhibited any obvious signs of infection and the DIBOA levels were not significantly higher when compared to the control. (Table 5.6).

Days	**Control**	***Ggt* EBI**	***Ggt* WP28**
3	0.05±0.04	-	-
21	ND	ND	ND
35	ND	ND	ND
% infection	0	40	0

*ND, not detected

Table 5.4 : The DIMBOA content in rye (Uneveta) challenged with the take-all fungus. Hx were quantified as in 2.2.3 and 2.2.7. The concentration is measured in µmoles/g.f.wt. and results are the mean of three replicates ±SE.

Days	Control	*Ggt* EBI	*Ggt* WP28
3	0.06±0.02	-	-
21	0.06±0.04	ND	ND
35	0.14±0.05	0.06±0.04	ND

*ND, not detected

Table 5.5 : The DIBOA concentration in rye (Uneveta) challenged with the take-all fungus. Hx were quantified as in 2.2.3 and 2.2.7. The concentrations are in µmoles/g.f.wt. and results are the mean of three replicates ±SE.

Days	Control	*Ggt* EBI	*Ggt* WP28
3	ND	-	-
21	ND	0.05±0.03	0.03±0.02
35	ND	0.04±0.02	0.01±0
% infection	0	0	0

*ND, not detected

Table 5.6: The DIBOA content in rye (Short-med) challenged with the take-all fungus. Hx were quantified as in 2.2.3 and 2.2.7. The concentrations are in µmoles/g.f.wt. and results are the mean of three replicates ± SE.

Fig 5.4 Infection of rye roots with Ggt. The rye seedlings (cv Short-med) were harvested five weeks after inoculation with *Ggt*. The top plant is the control, the middle plant is rye infected with WP28 and the bottom plant is rye infected with EBI.

Fig 5.5 Infection of rye roots with Ggt. The rye seedlings (cv Uneveta) were harvested five weeks after inoculation with *Ggt*. The top plant is the control, the middle plant is rye infected with WP28 and the bottom plant is rye infected with EBI.

5.3 Discussion

Under the conditions in which these experiments were performed it can be concluded that in wheat, triticale and rye there is no synthesis of Hx induced by infection with *Ggt*. Those plants in which Hx were detected, the levels were not significantly greater than the control indicating that there was no increased synthesis when attacked by *Ggt*.

Cereals are more susceptible to take-all at the seedling stage when the plant lacks structural defence. This study found that the wheat cv Sunstar has significnatly higher DIMBOA levels than the cv Vulcan. It was also found that Sunstar was more resistant to *Ggt* infection than cv Vulcan. This is most likely due to Sunstar having a higher DIMBOA content as in this study Vulcan had undetectable levels of DIMBOA. The results described in this chapter are consistent with those presented in Chapter Four, in that DIBOA seems to be the more important inhibitor of fungal growth. Triticale and rye contained DIBOA and these cereals were found to be less susceptible to *Ggt* infection than wheat. Also, the fact that there is no increased production of Hx induced by the infection of *Ggt* it seems that the low concentration of DIBOA detected was sufficient to impede infection by the fungus.

More evidence that *Ggt* does not induce increased synthesis of Hx is the fact that strain WP 28 is unable to infect rye but DIBOA was still detected in plants (cv Short-Med) grown in the presence of WP 28. As WP 28 is unable to infect rye these levels must be occurring naturally and may act as a prolonged defence mechanism in rye.

There have been varied results reported in the literature on studies performed on Hx in plants when submitted to wounding. Niemeyer *et al.* (1989) showed that of three cultivars of wheat, there were no statistically significant changes in DIMBOA concentration when submitted to infestation by aphids for two of the cultivars but one cultivar did show an

increase. They concluded that the production of Hx as a response to damage varies between cultivars.

Morse *et al.* (1991) showed that for both control plants and plants submitted to artificial wounding the Hx concentration declined with time but found there was a significantly higher concentration of Hx in the wounded plants compared to the intact plants. Even though the wounded plant showed a significant increase of Hx on infection they found the observed increases were not very substantial and they came to the conclusion that if the response was a form of induced resisitance then one would expect a larger increase in Hx than that noted.

Nakagawa *et al.* (1995) also found that the appearance and disappearance of Hx was little affected by infection of pathogens as well as wounding with a razor blade.

In a later study Weibull and Niemeyer (1995) found that the rate of hydrolysis of DIMBOA-Glc in wheat plants depends on the type of fungus involved. Necrotrophs which penetrate by mechanically or enzymically disrupting cell membranes caused a decrease in the glucoside content whereas an obligate parasite did not. Of the two necrotrophs tested the barley pathogen did not invoke the same response as the wheat pathogen. As *Ggt* WP28 is unable to attack rye it may be like the obligate parasite and not cause a response. Weibull and Niemeyer also found that following the decrease of DIMBOA-glc a corresponding increase in DIMBOA was not found. It was first proposed that it was transported away but no DIMBOA was found in the roots. It was then discovered that extracts of the fungus could decompose DIMBOA without the production of MBOA. This was specific to DIMBOA however as it was not able to degrade DIBOA. It may be that *Ggt* is like *Septoria tritici* and causes the decomposition of DIMBOA or DIBOA without the concurrent production of benzoxazolinones as MBOA or BOA were not detected in any of the studies even though the aglucone may have been.

From the results presented in this chapter and the previous chapter it can be concluded that DIBOA is the most potent inhibitor, of the Hx studied, of the growth of *Ggt*. If the resistance of cereals is to be improved the presence of DIBOA at reasonable levels is of paramount importance. As a result of this finding studies were undertaken to understand the inheritance of DIBOA more fully.

CHAPTER SIX

Hydroxamic acid content of wheats carrying single rye chromosomes

6.1 Introduction

Experiments described in previous chapters have indicated that the concentration of Hx in cereals roots did not increase following infection with *Ggt*. This result indicates that the endogenous level of Hx in plants, and the type of Hx present, are major factors in the resistance of cereals to take-all. It was shown that, of the Hx assayed, DIBOA was the most potent inhibitor of the growth of *Ggt*. Therefore, the breeding of wheat which can produce DIBOA may represent a strategy to increase resistance to take-all. This chapter describes the analysis of the Hx content of wheat lines carrying individual chromosomes of 2 different rye varieties to identify lines for potential breeding studies.

6.2 Results

Wheat (cv. Chinese Spring) addition lines carrying individual rye chromosomes were obtained from the University of Sydney's Plant Breeding Institute, Cobbity, NSW. These lines carried chromosomes from two rye cultivars. The two rye cultivars were Blanko and King II, hence the titles CSB and CSK. The wheat cultivar Chinese Spring was chosen because it can be readily crossed to cereal rye. The line designation system indicates which of the rye chromosomes are carried by each genotype. Thus the line CSB 3RS carries only the short arm of chromosome 3, whereas the line CSK 2RL carries the long arm of chromosome 2 of rye. The Hx content of the wheat addition lines are presented in Fig 6.1.

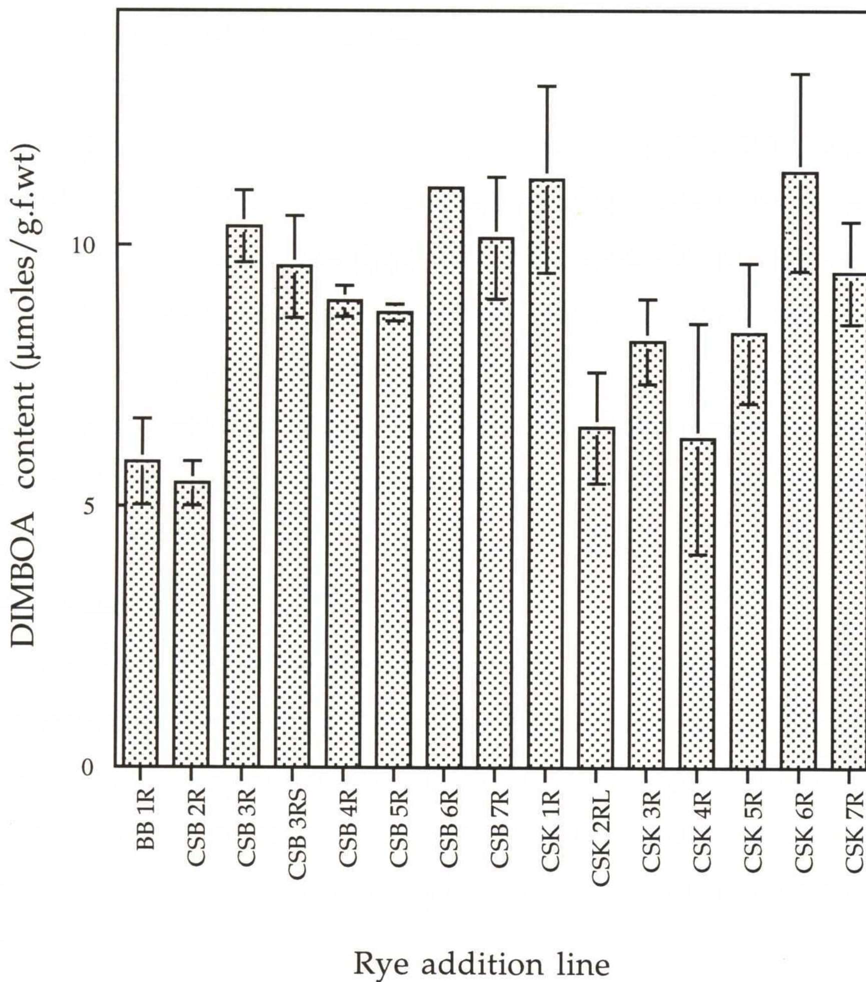


Fig 6.1 DIMBOA content of wheat (Chinese Spring) lines carrying individual or half chromosomes of rye. Root tissue of 4-day-old addition lines was analyzed for DIMBOA content as described in 2.2.3. DIBOA was detected in line CSB 5R. Results are the mean of three replicates and vertical bars represent the SE of the mean. Absence of error bars indicate that error is smaller than the data point.

In the wheat lines with added rye chromosomes, the parent plants should have 2*n*=44 chromosomes, that is, they have 42 chromosomes of wheat plus one pair from rye. In the lines CSB 2R, CSB 3R, CSK 4R and CSK 5R only 2*n*=43 was achieved. This means that the rye chromosome will only be in some of the progeny of these lines.

All of the lines assayed contained high levels of DIMBOA when compared to the DIMBOA content of the varieties used in earlier chapters of this study. The DIMBOA content of the lines CSK 2RL, CSK 4R, BB 1R and CSB 2R was significantly lower than in the other addition lines assayed. The most important result, when considering the aim of this experiment, was that DIBOA was detected in 2 of the 3 plants that were assayed of the line CSB 5R, although at a relatively low concentration of 0.01 μmoles/g.f.wt.

6.3 Discussion

Hydroxamic acids have been shown to inhibit the growth of *Ggt*. Therefore, the breeding of wheat plants with higher levels of DIMBOA, and in particular, plants which contain DIBOA, is a strategy that could improve the resistance of wheat to take-all. This part of the study was aimed at detecting the chromosome(s) which contains genes that control DIBOA formation in rye plants.

DIBOA was detected in the line CSB 5R. This leads to the suggestion that the genetic information for DIBOA synthesis may be located on the 5 chromosome of rye. The fact that DIBOA was not detected in CSK 5R may be due to this line having 2*n*=43 chromosomes which means the rye chromosome may not have been expressed in these plants. The level of DIBOA was very low but its detection in some of the plants is promising for further studies.

There have been few studies conducted on the genetics of Hx synthesis in plants to date. Breeding programmes for the production of cereals with high levels of Hx to promote resistance to pests and disease have contributed more information on the genetics of Hx production.

In corn, the genotype *Bx.Bx* contains DIMBOA whereas corn with the genotype *bx.bx* does not. Dunn *et al.* (1981) showed that the *bx* locus (non Hx producing) is in the short arm of chromosome 4. They also found that in *Bx.Bx* that Hx concentration is conditioned primarily by a major, partially dominant gene. Hydroxamic acid concentration in inbreds appears to be conditioned by genes with smaller effects. In the inbred B49 they found that Hx concentration is conditioned by five loci and in the inbred B37 by two loci. It may be then, that in rye there are a number of genes that have a cumulative effect for the production of DIBOA which were not fully expressed in the wheat addition lines.

Niemeyer (1988b) and Niemeyer *et al.* (1992) looked at different combinations of wheat genomes as a means of breeding wheats more resistant to attack by pathogens. Niemeyer (1988b) proposed that significant increases in Hx levels are unlikely to be obtained by hybridization of wheats sharing the same genome. The B genome of *T. speltoides* has been shown to be associated with the expression of high levels of DIMBOA therefore crossing wheats containing the A and D genome with wild species containing the G and B genome would have greater effect in increasing the production of Hx in wheat. They also suggested that the genome of rye could be useful in transferring resistance against pathogens to cereals. It has already been shown that triticale contains both DIMBOA and DIBOA reflecting a genetic contribution from rye. Therefore, further studies involving wheats with rye shromosomes inserted may enhance our understanding of the genetics of Hx production.

In conclusion the rye insertion lines analyzed in this study contain high levels of DIMBOA and one contained DIBOA. Lines such as these might prove to be useful for breeding more resistant wheats, not only because they may contain higher levels of DIMBOA but because some contain DIBOA. Due to the limited supply of seeds for these lines, more detailed analysis of DIMBOA and DIBOA content and the resistance of these plants to take-all could not be performed.

CHAPTER SEVEN

Conclusions

The results presented in this thesis provide an insight into the role of Hx in the resistance of some cereals to the take-all fungus.

Hydroxamic acids reached maximum concentrations in wheat, rye and triticale roots 4-5 days after germination. Similar results were found in other studies (Nicol *et al.*, 1992; Argandoña *et al.*, 1981, Argandoña *et al.*, 1980). This study showed that the root tip of 5-day-old seedlings of wheat, triticale and rye contained significantly higher levels of Hx than older parts of the root. This is consistent with studies by Argandoña *et al.* (1981) and Zúñiga *et al.* (1983), who showed Hx concentrations to be higher in younger parts of plants. High levels of Hx in the youngest parts of plants may help to explain why take-all infection does not occur at the growing root tip (Skou, 1981) which lacks structural defences. As the root ages it undergoes lignification and becomes more resistant to attack by pathogens due to mechanical strength. The growing root tip may also be more prone to attack by pathogens as it tends to be more metabolically active than the rest of the root and may contain more available nutrients. A higher level of Hx would thus protect this area from infection by pathogens such as *Ggt*.

Varieties of wheat, rye and triticale differing in Hx content were selected to determine if there was a relationship between Hx concentration and take-all resistance. Further, as rye and some triticale varieties contained both DIMBOA and DIBOA, the possibility that a combination of Hx was more effective in inhibiting the growth of *Ggt*

could be tested. The wheat variety Sunstar contained significantly higher levels of DIMBOA than other varieties tested, whereas Vulcan contained relatively low levels of DIMBOA. The triticale Tall Madonna contained significantly higher levels of both DIMBOA and DIBOA than the other triticales assayed, and the rye Short-med and Uneveta contained intermediate levels of both DIMBOA and DIBOA. Two strains of *Ggt* were chosen to observe if there was a difference in the ability of either strain to grow on media containing root extracts or individual Hx. Although strain EBI is able to infect rye in the field, it did not more grow more readily than strain WP 28 on media containing extracts from rye or media containing the Hx present in extracts of rye roots.

A preliminary assay of the effect of Hx on the growth of *Ggt* was performed, in which extracts prepared from the roots of the selected cereals were incorporated into the growth media for *Ggt*. Extracts from wheat did not inhibit the growth of either strain of *Ggt*. On the contrary, extracts prepared from Sunstar roots significantly promoted the growth of *Ggt*. This effect may be due to the presence of other nutrients in the Sunstar root extract promoting the growth of the fungus and thereby masking the effect of DIMBOA. Klun and Brindley (1966) found that when vitamins were addded to an artificial diet containing Hx, the inhibitory effect of MBOA was attenuated. The extracts prepared from triticale and rye significantly inhibited the growth of both strains of *Ggt*. Since DIMBOA contents of the wheat, rye and triticale roots examined in this study were not significantly different, it is possible that the extracts prepared from rye and triticale were more inhibitory than those prepared from wheat because they contained DIBOA. The potency of the extracts *in vivo* correlates to the reported resistance of the cereals in the field (Hollins *et al.*, 1986). To provide further evidence that DIBOA was the most potent inhibitor, studies were undertaken to observe the effect of individual Hx on the

growth of *Ggt* were undertaken. All of the Hx studied significantly inhibited the growth of both strains of *Ggt* when incorporated into growth media at a concentration of 0.5 mM, except for MBOA and DIMBOA. With 0.5 mM MBOA and DIMBOA, the growth of strain EBI was not significantly inhibited. The colonies that were inhibited on growth media containing Hx were transferred to PDA without Hx after 4 days. All colonies resumed growth when removed from the inhibitor, indicating that these concentrations are only fungistatic and not fungicidal. DIBOA was the most potent inhibitor, however, as the colonies that were transferred from media containing DIBOA did not recover as well as colonies removed from the other inhibitors when transferred to media without Hx. The potency of the Hx could be graded as DIBOA>BOA>DIMBOA>MBOA.

This study was followed with a trial designed to examine the correlation between Hx concentration and take-all resistance. After 5 weeks, the wheat varieties showed the greatest amount of infection rated on the percentage of roots exhibiting characteristic blackening. The cv. Sunstar, which had the highest DIMBOA levels was more resistant to infection than cv. Vulcan which had low levels of DIMBOA. The triticale, Tall Madonna, showed intermediate levels of take-all infection when compared to wheat and rye. The rye cvv. Short-med and Uneveta were totally resistant to *Ggt* WP 28 (as is to be expected) and only Uneveta was infected by *Ggt* EBI. Cereals containing DIBOA appeared to be more resistant. There was no increased synthesis of Hx in any of these cereals following infection. Since earlier work indicated that DIBOA was a more potent its absence from wheat may leave this species more susceptible to attack from *Ggt*.

As DIBOA was deemed the most potent inhibitor of fungal growth it would be beneficial to be able to transfer the capacity to synthesize this compound to wheat. The transfer of genes for DIBOA production

could be transferred to wheat using triticale as a bridging species. The Hx content of wheats carrying individual rye chromosomes or chromosome arms were analyzed to determine which chromosome in rye carries the genes for DIBOA synthesis in rye. All of the lines contained DIMBOA and the line CSB 5R also contained low amounts of DIBOA (0.01 μmoles/g.f.wt). These preliminary findings indicate that the gene/s for DIBOA synthesis may be on the 5 chromosome of rye.

DIMBOA and DIBOA are both suggested to be derived from HBOA (Leighton *et al.*, 1994). The lactam aglucones are intermediates in the pathway, and glucosylation of the Hx appears be the last step in biosynthesis (Fig 7.1). Zúñiga *et al.* (1990) showed that callus from wheat accumulates mainly DIBOA, whereas the leaves of seedlings contain DIMBOA. They concluded that DIMBOA originates from DIBOA. Furthermore, Zúñiga and Massardo (1991) found that undifferentiated tissues produce only aglucones, and they proposed that Hx are synthesized by meristematic tissues and are subsequently glucosylated and translocated to differentiated tissues.

Several explanations could be put forward as to why both DIMBOA and DIBOA occur in rye whereas wheat only contains DIMBOA. A possible biosynthetic pathway for DIBOA and DIMBOA is represented in the scheme shown in Fig 7.1. The studies of Leighton *et al.* (1994) indicate that that some of these steps are more likely to occur than others. Their isolation of a Hx glucosyltransferase that did not glucosylate the lactams HBOA or HMBOA led to the conclusion that glucosylation of Hx is the final step in the biosynthetic pathway .

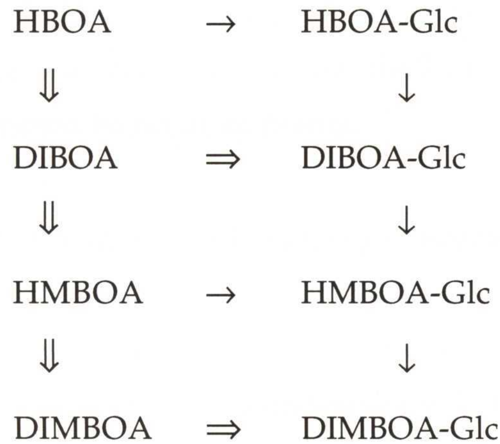


Fig 7.1 : Possible routes for the biosynthetic pathways for DIBOA and DIMBOA. Double arrows (⇒) represent steps that are more likely to occur than steps represented by single arrows (→).

As DIMBOA and DIBOA share the same biosynthetic pathway, it is possible that gene(s) on the 5 chromosome of rye may code for a glucosyltransferase that preferentially glucosylates DIBOA. The glucosylation of DIBOA would prevent it from being converted to DIMBOA. The absence of such an enzyme from wheat could explain the difference in the types of Hx found in wheat and rye. Some possible explanations include;

There may be more than one glucosyltransferase that glucosylates hydroxamic acids : a glucosyltransferase that glucosylates both DIBOA and DIMBOA may be present in rye and one that only glucosylates DIMBOA in wheat.

There may be a difference in the partitioning of DIBOA between the glucosylation and formation of HMBOA based on differences in the affinities of the enzymes concerned : differences in the partitioning may result in only some of the DIBOA being converted to DIMBOA in rye, whereas in wheat all of the DIBOA is converted to DIMBOA.

The formation of DIBOA from DIMBOA is unlikely to take place as this would require the removal of the methoxy group, and such reactions are not known to occur in plants.

In studies on the hydroxamic acid glucosyltransferases Leighton *et al.* (1994) showed that the isolated glucosyltransferase only glucosylates hydroxamic acids. Their results do not, however, conclusively show that there is not a specific glucosyltransferase for DIMBOA and DIBOA respectively. This leads to the conclusion that, the possibility of there being more than one glucosyltransferase that glucosylates hydroxamic acids is most likely.

This study has shown that Hx, in particular DIBOA, do seem to protect triticale and rye from take-all. Only DIMBOA in high concentrations provides some protection to wheat from infection by *Ggt*. If the resistance of these cereals, especially wheat, is to be improved, the breeding of varieties with higher Hx concentrations is important. Preliminary studies have shown that the 5 chromosome of rye may carry some of the genetic information for the synthesis of DIBOA. This information may be a useful tool for determining some of the enzymes involved in the final steps in the biosynthetic pathway of DIMBOA and DIBOA. Once the biosynthetic pathway has been fully elucidated the breeding of wheat which is more resistant to take-all should become an easier task.

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APPENDIX ONE

Statistical results used in Chapter Three

All results that are significant have a confidence level of 95%.

Day	3	4	5	6	7
3	-0.23	0.53	1.40	1.43	1.44
4	0.53	-0.23	0.62	0.66	0.66
5	1.40	0.62	-0.23	-0.20	-0.19
6	1.43	0.66	-0.20	-0.23	-0.23
7	1.44	0.66	-0.19	-0.23	-0.23

Table 1.1: DIMBOA levels in wheat over time. Comparisons for all pairs using the Student's t test. Positive values show pairs of means that are significantly different.

Section	0-1	4-5	1-2	2-3	3-4
0-1	-0.31	1.04	1.07	1.11	1.19
4-5	1.04	-0.31	-0.28	-0.24	-0.16
1-2	1.07	-0.28	-0.31	-0.27	-0.19
2-3	1.11	-0.24	-0.27	-0.31	-0.23
3-4	1.19	-0.16	-0.19	-0.23	-0.31

Table 1.2: Distribution of DIMBOA in wheat root sections. Comparisons for all pairs using the Student's t test. Positive values show pairs of means that are significantly different.

Variety	Sunstar	Corrigin	Cowan	Vulcan	Miskle	Dagger
Sunstar	-0.48	0.33	0.35	0.49	0.57	0.59
Corrigin	0.33	-0.59	-0.56	-0.43	-0.35	-0.33
Cowan	0.35	-0.56	-0.59	-0.45	-0.38	-0.35
Vulcan	0.49	-0.43	-0.45	-0.48	-0.40	-0.38
Miskle	0.57	-0.35	-0.38	-0.40	-0.48	-0.46
Dagger	0.59	-0.33	-0.35	-0.38	-0.46	-0.50

Table 1.3 : DIMBOA levels in different wheat varities. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Day	5	4	3	6
5	-4.30	-3.83	-1.13	-1.11
4	-3.83	-4.30	-1.60	-1.58
3	-1.31	-1.60	-4.30	-4.28
6	-1.11	-1.58	-4.28	-4.30

Table 1.4 : DIMBOA levels in triticale over time. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Day	4	5	6	3
4	-0.51	-3.63	-3.07	-3.04
5	-3.63	-5.12	-4.56	-4.54
6	-3.07	-4.56	-5.12	-5.10
3	-3.04	-4.54	-5.10	-5.12

Table 1.5 : DIBOA levels in triticale over time. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Section	0-1	1-2	3-4	2-3	4-5
0-1	-0.09	7.03	7.38	7.38	7.38
1-2	7.03	-0.09	0.25	0.25	0.25
3-4	7.38	0.25	-0.09	-0.09	-0.09
2-3	7.38	0.25	-0.09	-0.09	-0.09
4-5	7.38	0.25	-0.09	-0.09	-0.09

Table 1.6 : DIMBOA levels in triticale root sections. Comparisons for each pair using Student's t test test. Positive values show pairs of means that are significantly different.

Section	0-1	1-2	3-4	2-3	4-5
0-1	-0.15	6.97	7.32	7.32	7.32
1-2	6.97	-0.15	0.19	0.19	0.19
3-4	7.32	0.19	-0.15	-0.15	-0.15
2-3	7.32	0.19	-0.15	-0.15	-0.15
4-5	7.32	0.19	-0.15	-0.15	-0.15

Table 1.7 : DIBOA levels in triticale root sections. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Variety	Empat	Tall Madonna	Samson	Currency	Venus
Empat	-0.48	-0.07	0.62	1.01	1.02
Tall Madonna	-0.07	-0.48	0.21	0.60	0.61
Samson	0.62	0.21	-0.48	-0.10	-0.09
Currency	1.01	0.60	-0.10	-0.39	-0.38
Venus	1.02	0.61	-0.09	-0.38	-0.48

Table 1.8 : DIMBOA levels in selected triticale. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Variety	Empat	Tall Madonna	Samson	Currency	Venus
Empat	-0.48	-0.07	0.62	1.01	1.02
Tall Madonna	-0.76	-0.48	0.21	0.60	0.61
Samson	0.62	0.21	-0.48	-0.10	-0.09
Currency	1.01	0.60	-0.10	-0.39	-0.38
Venus	1.02	0.61	-0.91	-0.38	-0.48

Table 1.9 : DIBOA levels in selected triticale. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Day	3	4	5	6
3	-1.76	-1.69	-1.06	-0.98
4	-1.69	-1.76	-1.13	-1.05
5	-1.06	-1.13	-1.76	-1.67
6	-0.98	-1.05	-1.67	-1.76

Table 1.10 : DIMBOA levels in rye over time. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Day	3	6	4	5
3	-1.43	-0.09	0.52	0.74
6	-0.09	-1.43	-0.82	-0.60
4	0.52	-0.82	-1.43	-1.21
5	0.74	-0.60	-1.21	-1.43

Table 1.11 : DIBOA levels in rye over time. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Section	0-1	3-4	2-3	1-2	4-5
0-1	-2.74	4.01	4.01	4.11	4.15
3-4	4.01	-2.74	-2.65	-2.64	-2.60
2-3	4.01	-2.65	-2.74	-2.73	-2.69
1-2	4.11	-2.64	-2.73	-2.74	-2.70
4-5	4.15	-2.60	-2.69	-2.70	-2.74

Table 1.12 : DIMBOA levels in rye root sections. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Section	0-1	2-3	1-2	3-4	4-5
0-1	-0.40	1.26	1.42	1.89	1.90
2-3	1.26	-0.40	-0.24	0.22	0.23
1-2	1.42	-0.24	-0.40	0.06	0.07
3-4	1.89	0.22	0.06	-0.40	-0.40
4-5	1.90	0.23	0.07	-0.40	-0.40

Table 1.13 : DIBOA levels in rye root sections. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Variety	Uneveta	30B-761	Forage	Short-med	Winter rye	CP mix
Uneveta	-0.38	-0.34	-0.33	-0.29	-0.25	-0.14
30B-761	-0.34	-0.38	-0.36	-0.33	-0.28	-0.18
Forage	-0.33	-0.36	-0.38	-3.49	-0.30	-0.19
Short-med	-0.29	-0.33	-0.34	-0.46	-0.42	-0.31
Winter rye	-0.25	-0.28	-0.30	-0.42	-0.46	-0.36
CP mix	-0.14	-0.18	-0.19	-0.31	-0.36	-0.46

Table 1.14 : DIMBOA levels in selected rye varieties. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Variety	Winter rye	CP mix	Uneveta	Short-med	Forage	30B-761
Winter rye	-0.35	-0.24	0.20	0.31	0.50	0.77
CP mix	-0.24	-0.35	0.08	0.19	0.38	0.65
Uneveta	0.20	0.09	-0.29	-0.1	0.01	0.28
Short-med	0.31	0.19	-0.18	-0.35	-0.16	0.10
Forage	0.50	0.38	0.01	-0.16	-0.29	-0.02
30b-761	0.77	0.65	0.28	0.10	-0.02	-0.29

Table 1.15 : DIBOA levels in selected rye varieties. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

APPENDIX TWO

Statistical results used in Chapter Four

All results that are significant have a confidence level of 95%.

Cereal Extract	Control
Sunstar	-11.52
Vulcan	-30.32
Triticale	13.07
Rye	6.89

Table 2.1 : The effect of cereal extracts on the growth of Ggt (EBI). Comparisons with a control using Dunnett's method. Positive values show pairs of means that are significantly different.

Cereal Extract	Control
Sunstar	11.18
Vulcan	-15.21
Triticale	4.70
Rye	28.97

Table 2.2 : The effect of cereal extracts on the growth of Ggt (WP 28). Comparisons with a control using Dunnett's method. Positive values show pairs of means that are significantly different.

Concentration	Control	0.5	1.0	1.5	2.0	5.0
Control	-3.25	17.99	20.49	21.49	2149	21.49
0.5	17.99	-3.25	-0.75	0.24	0.24	0.24
1.0	20.49	-0.75	-3.25	-2.25	-2.25	-2.25
1.5	21.49	0.24	-2.25	-3.25	-3.25	-3.25
2.0	21.49	0.24	-2.25	-3.25	-3.25	-3.25
5.0	21.49	0.24	-2.25	-3.25	-3.25	-3.25

Table 2.3 : The effect of DIBOA on the growth of Ggt (EBI) after four days. Comparisons with a control using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	2.0	1.5	5.0
2.0	-4.86	-3.86	1.38
1.5	-3.86	-4.86	0.38
5.0	1.38	0.38	-4.86

Table 2.4 : The growth of DIBOA inhibited Ggt (EBI) 3 days after removal from inhibitor. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	Control	0.5	1.0	1.5	2.0	5.0
Control	-0.49	11.75	12.50	12.75	12.75	12.75
0.5	11.75	-0.49	0.25	0.50	0.50	0.50
1.0	12.50	0.25	-0.49	-0.24	-0.24	-0.24
1.5	12.75	0.50	-0.24	-0.49	-0.49	-0.49
2.0	12.75	0.50	-0.24	-0.49	-0.49	-0.49
5.0	12.75	0.50	-0.24	-0.49	-0.49	-0.49

Table 2.5 : The effect of DIBOA on the growth of Ggt (WP 28) after four days. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	1.5	2.0	5.0
1.5	-4.34	-3.34	-2.09
2.0	-3.34	-4.34	-3.09
5.0	-2.09	-3.09	-4.34

Table 2.6 : The growth of DIBOA inhibited Ggt (WP 28) 3 days after removal from inhibitor. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	Control	1.0	0.5	1.5	2.0	5.0
Control	-1.69	0.55	1.05	2.55	3.80	4.30
1.0	0.55	-1.69	-1.19	0.30	1.55	2.05
0.5	1.05	-1.19	-1.69	-0.19	1.05	1.55
1.5	2.55	0.30	-0.19	-1.69	-0.44	0.05
2.0	3.80	1.55	1.05	-0.44	-1.69	-1.19
5.0	4.30	2.05	1.55	0.05	-1.19	-1.69

Table 2.7 : The effect of BOA on the growth of Ggt (EBI) after four days. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	2.0	5.0
2.0	-1.76	6.23
5.0	6.23	-1.76

Table 2.8 : The growth of BOA inhibited Ggt (EBI) 3 days after removal from inhibitor. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different

Concentration	Control	0.5	1.0	1.5	2.0	5.0
Control	-1.17	14.32	16.32	18.57	19.57	20.07
0.5	14.32	-1.17	0.82	3.07	4.07	4.57
1.0	16.32	0.82	-1.17	1.07	2.07	2.57
1.5	18.57	3.07	1.07	-1.17	-0.17	0.32
2.0	19.57	4.97	2.07	-0.17	-1.17	-0.67
5.0	20.07	4.57	2.57	0.32	-0.67	-1.17

Table 2.9 : The effect of BOA on the growth of Ggt (WP 28) after four days. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	2.0	5.0
2.0	-1.22	2.77
5.0	2.77	-1.22

Table 2.10 : The growth of BOA inhibited Ggt (WP 28) 3 days after removal from inhibitor. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	**Control**	**0.5**	**1.0**	**1.5**	**2.0**	**5.0**
Control	-5.66	-1.91	2.83	2.83	2.83	2.83
0.5	-1.91	-5.66	-0.91	-0.91	-0.91	-0.91
1.0	2.83	-0.91	-5.66	-5.66	-5.66	-5.66
1.5	2.83	-0.91	-5.66	-5.66	-5.66	-5.66
2.0	2.83	-0.91	-5.66	-5.66	-5.66	-5.66
5.0	2.83	-0.91	-5.66	-5.66	-5.66	-5.66

Table 2.11 : The effect of DIMBOA on the growth of Ggt (EBI) after four days. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	**1.5**	**5.0**	**2.0**	**1.0**
1.5	-11.43	-10.43	-7.68	1.56
5.0	-10.43	-11.43	-8.68	0.56
2.0	-7.68	-8.68	-11.43	-2.18
1.0	1.56	0.56	-2.18	-11.43

Table 2.12 : The growth of DIMBOA inhibited Ggt (EBI) 3 days after removal from inhibitor. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	Control	0.5	1.0	1.5	2.0	5.0
Control	-3.60	2.89	5.39	4.58	5.39	5.71
0.5	2.89	-3.60	-1.10	-1.91	-1.10	-0.78
1.0	5.39	-1.10	-3.60	-4.41	-3.60	-3.28
1.5	4.58	-1.91	-4.41	-3.60	-4.41	-4.15
2.0	5.39	-1.10	-3.60	-4.41	-3.60	-3.28
5.0	5.71	-0.78	-3.28	-4.15	-3.28	-2.94

Table 2.13 : The effect of DIMBOA on the growth of Ggt (WP 28) after four days. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	1.0	5.0	1.5	2.0
1.0	-6.41	-4.69	-6.36	-3.91
5.0	-4.69	-5.24	-7.07	-4.52
1.5	-6.36	-7.07	-9.07	-6.86
2.0	-3.91	-4.52	-6.868	-6.41

Table 2.14 : The growth of DIMBOA inhibited Ggt (WP 28) 3 days after removal from inhibitor. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	Control	0.5	1.0	1.5	2.0	5.0
Control	-4.20	-3.95	6.54	9.79	11.29	11.29
0.5	-3.95	-4.20	6.29	9.54	11.04	11.04
1.0	6.54	6.29	-4.20	-0.95	0.54	0.54
1.5	9.79	9.54	-0.95	-4.20	-2.70	-4.20
2.0	11.29	11.04	0.54	-2.70	-4.20	-4.20
5.0	11.29	11.04	0.54	-2.70	-4.20	-4.20

Table 2.15 : The effect of MBOA on the growth of Ggt (EBI) after four days. Comparisons for each pair using Student's test. Positive values show pairs of means that are significantly different.

Concentration	2.0	5.0
2.0	-9.59	-3.59
5.0	-3.59	-9.59

Table 2.16 : The growth of MBOA inhibited Ggt (EBI) 3 days after removal from inhibitor. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	Control	0.5	1.0	1.5	2.0	5.0
Control	-0.70	9.29	16.79	18.29	18.29	18.29
0.5	9.29	-0.70	6.79	8.29	8.29	8.29
1.0	16.79	6.79	-0.70	0.79	0.79	0.79
1.5	18.29	8.29	0.79	-0.70	-0.70	-0.70
2.0	18.29	8.29	0.79	-0.70	-0.70	-0.70
5.0	18.29	8.29	0.79	-0.70	-0.70	-0.70

Table 2.17 : The effect of MBOA on the growth of Ggt (WP 28) after four days. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.

Concentration	2.0	5.0
2.0	-4.20	-2.20
5.0	-2.20	-4.20

Table 2.18 : The growth of MBOA inhibited Ggt (WP 28) 3 days after removal from inhibitor. Comparisons for each pair using Student's t. Positive values show pairs of means that are significantly different.

APPENDIX THREE

Statisticsal results used in Chapter Five

All results that are significant have a confidence level of 95%.

Concentration (after 21 days)	**Control**	**Concentration (after 35 days)**	**Control**
Control	-0.14	Control	-0.16
+EBI	-0.09	+EBI	-0.13

Table 3.1 : DIBOA content of triticale infected with Ggt (EBI) after 21 and 35 days. Comparisons to the control using Dunnett's method. Positive values show pairs of means that are significantly different.

Concentration (after 21 days)	**Control**	**Concentration (after 35 days)**	**Control**
Control	-0.52	Control	-0.16
+EBI	-0.25	+EBI	-0.10

Table 3.2 : DIMBOA content of triticale infected with Ggt (EBI) after 21 and 35 days. Comparisons to the control using Dunnett's method. Positive values show pairs of means that are significantly different.

Concentration (after 21 days)	**Control**	**Concentration (after 35 days)**	**Control**
Control	-0.12	Control	-0.15
+WP 28	-0.07	+WP 28	-0.10

Table 3.3 : DIMBOA content of triticale infected with Ggt (WP 28) after 21 and 35 days. Comparisons to the control using Dunnett's method. Positive values show pairs of means that are significantly different.

Concentration (after 21 days)	**Control**	**Concentration (after 35 days)**	**Control**
Control	-0.18	Control	-0.16
+WP 28	-0.15	+WP 28	-0.11

Table 3.4 : DIBOA content of triticale infected with Ggt (WP 28) after 21 and 35 days. Comparisons to the control using Dunnett's method. Positive values show pairs of means that are significantly different

Concentration (after 35 days)	**Control**
Control	-0.20
+EBI	-0.07

Table 3.5 : DIBOA content of rye (Uneveta) infected with Ggt (EBI) after 35 days. Comparisons to the control using Dunnett's method. Positive values show pairs of means that are significantly different.

Concentration (after 21 days)	Control	Concentration (after 35 days)	Control
Control	-0.12	Control	-0.10
+EBI	-0.08	+EBI	-0.06

Table 3.6 : DIBOA content of rye (Short-med) infected with Ggt (EBI) after 21 and 35 days. Comparisons to the control using Dunnett's method. Positive values show pairs of means that are significantly different.

Concentration (after 21 days)	Control	Concentration (after 35 days)	Control
Control	-0.05	Control	-0.03
+WP 28	-0.09	+WP 28	-0.02

Table 3.7 : DIBOA content of rye (Short-med) infected with Ggt (WP 28) after 21 and 35 days. Comparisons to the control using Dunnett's method. Positive values show pairs of means that are significantly different.

APPENDIX FOUR

Statistical results used in Chapter Six

All results that are significant have a confidence level of 95%.

Line	**CSK6R**	**CSK1R**	**CSB6R**	**CSB3R**	**CSB7R**	**CSK7R**	**CSB3RS**
CSK6R	-3.46	-3.32	-3.58	-2.41	-2.19	-1.52	-1.64
CSK 1R	-3.32	-3.46	-3.72	-2.55	-2.33	-1.66	-1.78
CSB6R	-3.58	-3.72	-4.24	-3.11	-2.89	-2.22	-2.34
CSB3R	-2.41	-2.55	-3.11	-3.46	-3.24	-2.58	-2.69
CSB7R	-2.19	-2.33	-2.89	-3.24	-3.46	-2.80	-2.91
CSB3RS	-1.64	-1.78	-2.34	-2.69	-2.91	-3.35	-3.46
CSK7R	-1.52	-1.66	-2.22	-2.58	-2.80	-3.46	-3.35
CSB4R	-0.98	-1.12	-1.68	-2.03	-2.25	-2.92	-2.80
CSB5R	-0.76	-0.90	-1.46	-1.81	-2.03	-2.70	-2.58
CSK5R	-0.37	-0.51	-1.07	-1.43	-1.65	-2.31	-2.20
CSK3R	-0.21	-0.35	-0.91	-1.26	-1.48	-2.15	-2.03
CSK2RL	1.43	1.29	0.74	0.38	0.16	-0.50	-0.38
CSK4R	1.62	1.48	0.93	0.57	0.35	-0.31	-0.29
BB1R	2.09	1.95	1.39	1.03	0.81	0.15	0.26
CSB2R	2.49	2.35	1.80	1.44	1.22	0.55	0.67

CSB4R	CSB5R	CSK5R	CSK3R	CSK2RL	CSK4R	BB1R	CSB2R
-0.98	-0.76	-0.37	-0.21	1.43	1.62	2.09	2.49
-1.12	-0.90	-0.51	-0.35	1.29	1.48	1.95	2.35
-1.68	-1.46	-1.07	-0.91	0.74	0.93	1.39	1.80
-2.03	-1.81	-1.43	-1.26	0.38	0.57	1.03	1.44
-2.25	-2.03	-1.65	-1.48	0.16	0.35	0.81	1.22
-2.80	-2.58	-2.20	-2.03	-0.38	-0.19	0.26	0.67
-2.92	-2.70	-2.31	-2.15	-0.50	-0.31	0.15	0.55
-3.46	-3.24	-2.86	-2.69	-1.04	-0.85	-0.39	0.01
-3.24	-3.46	-3.08	-2.91	-1.26	-1.07	-0.61	-0.20
-2.86	-3.08	-3.46	-3.30	-1.65	-1.46	-0.99	-0.59
-2.69	-2.91	-3.30	-3.46	-1.81	-1.62	-1.16	-0.75
-1.04	-1.26	-1.65	-1.81	-3.46	-3.27	-2.81	-2.40
-0.85	-1.07	-1.46	-1.62	-3.27	-3.46	-3.00	-2.59
-0.39	-0.61	-0.99	-1.16	-2.81	-3.00	-3.46	-3.06
0.01	-0.20	-0.59	-0.75	-2.40	-2.59	-3.06	-3.46

Table 4.1 : The DIMBOA content of wheat lines carrying a single rye chromosome. Comparisons for each pair using Student's t test. Positive values show pairs of means that are significantly different.