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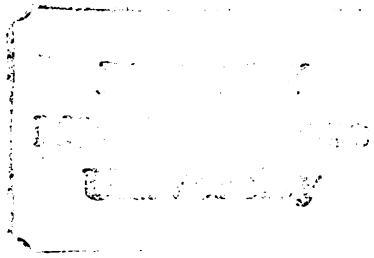

This is to certify that the

thesis entitled

VIRULENCE MUTANTS OF CEPHALOSPORIUM GRAMINEUM

presented by

SALLY LYNN VAN WERT

has been accepted towards fulfillment
of the requirements for

Master of Science degree in Botany and Plant Pathology

Dennis W. Fulbright

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VIRULENCE MUTANTS OF CEPHALOSPORIUM GRAMINEUM

By

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ABSTRACT

VIRULENCE MUTANTS OF CEPHALOSPORIUM GRAMINEUM

By

Sally Lynn Van Wert

To determine if graminin A (GRA) or polysaccharides, metabolites found in culture filtrates of Cephalosporium gramineum, are important disease determinants of Cephalosporium leaf stripe (CLS), mutants were generated by UV light and chemical mutagenesis. Mutants varying in virulence were selected in a wheat seedling bioassay. GRA and polysaccharide porduction were determined by extraction from culture filtrates. No correlation between polysaccharide production and virulence was found. Gas chromatography/mass spectroscopy studies indicated that GRA was not a pathogenicity factor and put its role as a virulence factor in doubt since GRA was not produced by moderately to highly virulent mutants. However, biological activity of GRA was observed in antimicrobial and phytotoxic assays. The seedling assay was also used to screen wheat lines for resistance to CLS. A positive relationship between tolerance and lack of symptom production in the seedling assay was found.

To my mom and dad

ACKNOWLEDGEMENTS

I wish to thank Dr. Dennis Fulbright for his constructive advice, encouragement and financial support throughout my graduate career at MSU. Thanks also goes to my committee members Drs. Bob Scheffer and Ray Hammerschmidt for their interest in my education and the editing of this manuscript. Special thanks goes to Al Ravenscroft for all his technical assistance and emotional support. Particular thanks is extended to Brian Musselman, Facility Manager of the MSU Mass Spectral Facility. Without his assistance and expertise I would not have been able to complete this study.

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Last, but not least, I extend my appreciation to my family for their love and support. Finally, love and appreciation are extended to Maggie McLeod, Jack Dingledine and Dan Shelton for their invaluable friendship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure | Page

LIST OF APPENDICES

LIST OF ABBREVIATIONS

Ac_2O	acetone
CA	Davis' complete agar
CLS	Cephalosporium leaf stripe
EMS	ethyl methanesulfonate
EtOAc	ethyl acetate
EtOH	ethanol
GC	gas chromatography
GC/MS	gas chromatography/mass spectroscopy
GRA	graminin A
MA	minimal agar
MeOH	methanol
NTG	N-methyl-N'-nitro-N-nitrosoguanadine
PDA	potato dextrose agar
PDA + str	potato dextrose agar plus streptomycin
PDB	potato dextrose broth
TLC	thin layer chromatogaphy
UV	ultraviolet

LITERATURE REVIEW

General

Stripe disease of wheat was first observed in Japan where it became an important disease in fields cropped consecutively to wheat. Nisikado and Ikata named the causal fungus Cephalosporium gramineum Nis. and Ika. and the disease Cephalosporium leaf stripe (CLS). Their studies resulted in a comprehensive report, which included discussion of the taxonomic position of the fungus and observations on factors influencing disease development (Nisikado et al, 1934).

Cephalosporium is a member of the Deuteromycetes (Fungi Imperfecti). The genus is characterized by its well developed hyaline mycelium and its slender unbranched, singly arising phialides (conidiophores) (Buchanan, 1911; Barron, 1968; Hawksworth and Waller, 1976). Phialospores (conidia) are abstricted successively in basipetal succession from an open growing point at the apex of the sporogenous cell, which lacks a distinct apical collarette. Phialospores remain in a ball or rarely in fragile chains at the apex. The spore balls on top of solitary, tapering phialides, are the diagnostic feature of the genus (Barron, 1968). In 1971, Gams (in: McGinnis, 1980) concluded the genera Acremonium and Cephalosporium were cogeneric and that

Acremonium should be considered to have priority. However, this suggestion has not been followed; Cephalosporium will be retained as the genus name in this paper.

Grasses are the only known hosts, as is implied by the species ephitet gramineum. Several major cereal crops are susceptible (Wiese, 1977) and CLS has been found on many grasses, including the species of Dactylis, Bromus, Elymus, Agropyron, Poa and Arrhenatherum (Bruehl, 1957; Howell and Burgess, 1967). Spring cultivars of wheat, oats, barley, rye and triticale are susceptible, but they do not suffer serious damage from the disease in the field (Wiese, 1977). The disease is of major importance only on winter wheat (Triticum aestivum L.).

Cephalosporium leaf stripe is found in Japan (Nisikado et al., 1934), Europe (Gray, 1960; Slope, 1965; Hawksworth and Waller, 1976) and North America (Wiese, 1977). It was first reported in the United States by Bruehl (1956a, 1956b). In North America CLS occurs frequently in the Great Lake States, the Pacific Northwest, Kansas and Montana. Disease incidence in these areas has ranged to 80% (Wiese, 1977), and yield has been reduced by 80% under severe disease conditions (Richardson and Rennie, 1970; Johnston and Mathre, 1972). In Michigan, CLS has been observed every year since 1961. Smith et al. (1966) found symptoms in every wheat field observed in 1965. Recently, Kansas estimated the loss of 500,000 bushels of wheat, or 1.5% of

the crop to CLS. This is greater than the losses from powdery mildew and take-all (NCA-Meetings, 1979).

Bruehl (1963) identified a fungus that formed a sporodochial stage on winter wheat stubble and straw as Hymenula cerealis Ellis. and Everth.. Cultural and inoculation experiments showed that H. cerealis is the saprophytic-sporulating stage of C. gramineum. The causal organism of CLS, therefore, has two imperfect names: Cephalosporium gramineum, the parasitic stage, and Hymenula cerealis, the saprophytic stage. Bruehl stated that H. cerealis should take precidence over C. gramineum but recommended that Cephalosporium leaf stripe remain the name of the disease. To date no perfect stage has been described for the fungus.

Saprophytic stage

The saprophytic life cycle of C. gramineum begins after harvest and tillage operations return wheat debris to the soil. Sporodochia appear in cool, wet weather during the autumn months. According to Wiese and Ravenscroft (1978b), growth of the fungus within the straw is non-directional, inter- and intracellular. Stomata and severed ends of straw segments are the avenues to the exterior. Spore production and sporodochial development are initiated only after hyphae emerge from straw. Rapid conidiogenesis along with mucopolysaccharide production results in a mass of tightly adhering phialospores (Bruehl, 1963; Hawksworth and Waller,

1976; Wiese and Ravenscroft, 1978b). Conidiogenesis is highly efficient even without sporodochia formation (Wiese, 1977). Wiese and Ravenscroft (1975) found the half-life of conidia to be 0.5 to 2.5 weeks at 25 C, whether the soil was moist or dry, and 26 weeks at 7 C in moist soils. As long as the integrity of the colonized host tissues was maintained, the population renewed itself. When the host residues remain undisturbed near or at the soil surface, the fungus can survive saprophytically and produce infectious propagules (conida) for at least three growing seasons.

Cephalosporium gramineum is a poor competiter in colonizing refuse, grows slowly (Wiese, 1977). Lai (1967) and Lai and Bruehl (1968) showed that the fungus dominated buried straw for the first six months and then declined at a constant rate. After the C. gramineum titer decreased, Trichoderma spp. and Fusarium spp. became the predominating colonizers, in moist and dry soil, respectively. Thus, C. gramineum, when established in straw, exhibits some ability to dominate the substrate. There is evidence that antimicrobial production may play a role in excluding other fungi from previously infested wheat straw (Lai, 1967; Lai and Bruehl, 1968; Bruehl et al., 1969). An antibiotic produced by H. cerealis (Lai, 1967; Lai and Bruehl, 1968; Bruehl and Lai, 1969) was most active in a medium at pH 5 or lower, and might aid in competition. Bruehl et al. (1972) found antibiotic production to be related to growth rate;

antibiotic production was reduced when the fungus grew rapidly and increased when the fungus was under moderate water stress and growing slowly. Among fungi, effective substrate possession is not unusual, and may be of more importance to pathogens of weak saprophytic ability (Bruehl and Lai, 1966). In summary, until infection occurs, survival during the fall and winter is a function of spore production, longevity and substrate possession.

The development of a green wheat agar as a selective medium (Wiese and Ravenscroft, 1973), allowed the quantitative determination of C. gramineum in soil . Wiese and Ravenscroft (1975) monitored propagule numbers in Michigan wheat fields and showed that propagules began to increase dramatically in September and peaked in midwinter. Little or no detection of propagules occured in June and July. Spores were the primary source of inoculum and declined rapidly in number with the advent of warm temperatures. Once a field became infested with C. gramineum, continued wheat monoculture normally resulted in a build-up of C. gramineum populations. Wiese and Ravenscroft (1978a) demonstrated a longterm decrease in the pathogen population and disease incidence with continued wheat monoculture over an eight-year period. Other than in Michigan, the decline of CLS has not been reported in any major wheat growing area where wheat monoculture is commonly practiced. Bailey (1980), studying the decline phenomenon in the same fields

observed by Wiese and Ravenscroft (1978a), did not demonstrate the transferability of the 'decline factor' from a field in decline to fields which had been in monoculture but not showing decline. Bailey suggested that pathogen populations and disease fluctuations over time may be a characteristic of individual fields regardless of seemingly identical cultural practices.

Parasitic stage

It is generally agreed that the soil-borne pathogen, C. gramineum, invades wheat through the roots at wounds associated with spring heaving of soil (Bruehl, 1957; Pool and Sharp, 1969a; Johnston and Mathre, 1972; Mathre et al., 1977; Wiese, 1977) or through wounds associated with insect injury (Bruehl, 1968; Wiese, 1977). Spring grains escape infection, or avoid injury, as spring infection rarely builds to damaging proportions (Wiese, 1977). In Britain, leaf stripe has been associated with wireworm damage (Slope and Bardner, 1965.). Otiano (1962) presented evidence for the entrance of the pathogen through openings resulting from rupture of the pericarp and coleorhiza by emergence of roots during germination.

Experiments by Mathre and Johnston (1975b) indicated that root exudates increased conidia production by hyphae from infested straw. They suggested that passive entry of conidia into the root system took place in the spring after

heaving had severed the roots. At this time, conidia would be drawn in with transpirational water, enter the vascular system, reproduce and colonize the plant. This type of mechanism would separate C. gramineum from other vascular pathogens such as Fusarium spp. and Verticillum spp., which can actively penetrate and grow into root tissue, as well as entering through wounds.

Bailey (1980; Bailey et al., 1982) showed by electron microscopy, that conidia will germinate and produce runner hyphae on the root surface in response to increased root exudation following freeze stress. Once penetration had occured the epidermis and cortex did not prevent colonization by C. gramineum. Bailey suggested that freeze stress and not wounds per se may be an important factor affecting the predisposition of wheat plants to active penetration and infection by C. gramineum.

When the fungus was introduced directly into the root xylem, at room temperature, visible leaf stripe was seen in about ten days (Wiese and Ravenscroft, 1978a). In cereals, the fungus became established as a vascular pathogen and caused chlorotic and necrotic xylary stripes. Formation of stripes started near the crown and moved acropetally, later accompanied by chlorosis and necrosis of leaf tissue (Nisikado et al., 1934; Bruehl, 1957; Hawskworth and Waller, 1976). One to 4 linear stripes develop on on the culms, leaf blades and leaf sheaths. Acropetal symptom development

leads to early senescence of lower leaves resulting in stunting, the bleaching of spikes prior to normal ripening, poorly filled or unfilled seed heads, smaller seed size and decreased flour quality (Mathre and Johnston, 1975a; Mathre, et al., 1977). Overall quality decrease was measured by kernel weight, flour yield and physical properties of dough.

Microscopic examination of leaf segments showed that the fungus was present within 1-2 vascular bundles per leaf. Only proto- and metaxylem vessels were inhabited (Wiese and Ravenscroft, 1978a). Morton (1980) reported that xylem restriction was associated with xylem maturation gradients between internodes, within nodes, and within leaves. With increased colonization, the disruption of protoplasts in phloem and mesophyll cells bordering vascular bundles was detected. A marked decrease in chloroplast number within affected mesophyll cells was associated with external manifestations of chlorotic stripes. Zones of mesophyll disruption coalesced when vascular bundles were colonized. This evidence inducated that C. gramineum is incapable of penetrating living cells at any time during the disease cycle.

Control

Cephalosporium leaf stripe is favored by cool spring temperatures, soils having high moisture levels, early planting dates and fields planted consecutively to wheat (Nisikado et al., 1934; Pool, 1967; Bruehl, 1968; Bruehl and Lai, 1968a; Pool and Sharp, 1969a; Wiese and Ravenscroft, 1976, 1978a; Bailey, 1980; Bailey et al., 1982). Bruehl and Lai (1968a) indicated CLS was associated with soil types having low pH and poor internal drainage. Pool and Sharp (1967, 1969b) showed that disease was more prevalent in lower and more poorly drained soils. Poor drainage leads to poor aeriation, compact soils and slow decomposition of straw so that survival of the pathogen in refuse is prolonged. Pool and Sharp (1967, 1969b) also indicated that seedlings from early plantings with fertilization were subject to increased root breakage as compared to those from later plantings or early plantings without fertilization. The larger root system in the fertilized seedling could yield more entry points for the pathogen. Latin et al. (1982) recently reported that the amount of applied nitrogen appeared to have no measureable effect on CLS incidence.

The major means of control of CLS are through the cultural practices of crop rotation, sanitation and late sowing. As these three types of control are not always practical, the most desirable control measure would be the use of resistant varieties. Seed treatment is not necessary because

the pathogen is not disseminated through seed (Nisikado et al., 1934). No chemical means of controlling this disease are currently available.

Wiese and Ravenscroft (1975) and Latin et al. (1982) have suggested three year rotations of wheat with nonhost crops. CLS was shown to be self-sustaining in fields with a history of grassy weeds in the 2nd, 3rd or 4th year of wheat production (Wiese and Ravenscroft, 1978a). Late sowing (Wiese and Ravenscroft, 1976; Wiese, 1977) limits autumn root growth, thus minimizing sites for injury and infection. Wiese and Ravenscroft (1976) advocated planting 10 days after the local Hessian flyfree dates in Michigan.

Sanitation is of extreme importance as a cultural means of control, and can be accomplished by burning infested straw and stubble, mechanical removal of residue prior to planting, and disking or deep plowing (Nisikado et al., 1934; Wiese and Ravenscroft, 1975; Latin et al., 1982). Removal of residue eliminates the pathogen. Wiese and Ravenscroft (1975) showed that levels of fungus and disease are related to the amount of residue in and on the uppermost 7.6 cm of soil. Disking was shown to be inferior to deep plowing for removing residue. Mathre and Jphnston (1975b) stated that deep plowing to 30 cm is an excellent means of control. Although Latin et al. (1982) have confirmed Wiese's work in Michigan, they indicated that minimal tillage has promise when combined with longer rotations and

resistant wheat cultivars. This combination was suggested because of significant soil erosion problems in the Pacific Northwest and the need for maintainance of soil conservation practices.

To date there are few winter wheat cultivars showing tolerance to CLS. Severing the roots of wheat seedlings, applying conida in liquid suspension cultures and rating symptoms after a given period, has been used to differentiate between resistant and susceptible varieties (Mathre and Johnston, 1975a; Mathre et al., 1977; Bailey 1980). Mathre (Mathre et al., 1977) contends that a lower inoculum potential of C. gramineum differentiates best and that these levels approximate those encountered in the field. Morton et al. (1980) also discouraged the use of nonvernalized winter wheat plants because it was assumed they provide an atypical environment for the pathogen. Bailey (1980) showed that a freeze screening technique correlated better with field determined resistance than the test utilizing root severing as the predisposition factor.

Mathre et al. (1977) observed that yield components in infected plants (seed size or kernel weight and seed number per head) vary in different wheat lines suggesting that lines may react to the pathogen in different ways. As seed size is usually reduced more than seed number, Morton and Mathre (1980a) suggested that selection on the basis of seed size could be an effective and simple means of identifying

and evaluating resistance in infected plants derived from winter wheat germplasm.

Morton and Mathre (1980b) have identified three types of resistance to CLS: 1) a reduction in the number of diseased plants in a population, 2) a reduction in number of diseased tillers within a plant, and 3) a reduction of the rate and severity of disease development within a plant. The latter two responses restrict the pathogen after successful ingress and have been observed only in one wheat cultivar, Crest LRC 40.

Pathogenesis

There has been much speculation regarding which fungal metabolite, if any, might be a primary agent in symptom production in CLS. Most studies have focused on polysaccharides or toxins. In 1938, Ikata and Kawai (1938), showed that filtrate from nutrient solutions in which the organism had grown contained a toxin which inhibited seedling growth. Bruehl (1963) suggested that symptom development may be the result of toxic metabolites in addition to the plugging of vessels by mycelium, because brown discoloration of vascular elements occured when little mycelium was present. Spalding et al., (1961) found that *C. gramineum* produced polygalacturonase which was isolated only from infected plants. Diseased tissues were also found to have a lower moisture content, which they attributed to increased viscosity of

solutes in the xylem and xylary plugging by a polysaccharide produced by the fungus. As lateral and acropetal dye movement could not take place in stripe tissue, it was felt that hyphae and pectin plugs derived from the degradation of host tissue by pectinolytic enzyme action further inhibited water movement. Such 'vascular distress' was concluded to contribute to cellular dysfunction and death of wheat plants. This work was supported by Pool and Sharp (1969a) who extracted a polysaccharide produced by C. gramineum, only from infected wheat tissue, and showed that it would restrict fluid movement in healthy wheat leaves.

Wiese (1972) suggested that a diffusable fungal by-product(s) and not pectin or hyphal plugging in the xylem was responsible for stripe development. Electron micrographs of infected vascular bundles showed an accumulation of an electron-dense material surrounding conidia. This electron-dense material was found to line the walls of infected vessels after liberation from conidia. In leaves bearing prominent symptoms, xylem vessels were densely packed with conidia but no plugs of polysaccharide or pectin were seen. Wiese believed that occlusion of main vascular bundles as a result of fungal proliferation is of secondary importance in pathogenesis as occlusions were found to follow rather than preceed lateral extension of leaf striping.

In 1977 Kobayashi and Ui (1977b, 1979) isolated and characterized a toxic metabolite from culture filtrates of C. gramineum which reportedly discolored the vascular tissue and induced chlorosis and vascular browning at low concentrations in wheat leaf cuttings. This compound, graminin A, was also found to have antibiotic properties effective against some bacteria and fungi. Gregatin A, isolated earlier by Kobayashi and Ui (1977a) from culture filtrates of C. gregatum, had nearly the same structure and antimicrobial activity as graminin A (Figure 1). Gregatin A is a toxin that apparently mimics the symptoms of brown stem rot of adzuki bean, soybeans and mung beans. Gray and Chamberlain (1975), four years earlier, found that soybeans bred for resistance to brown stem rot did not wilt when placed in a crude culture extract of C. gregatum but susceptible cultivars wilted in 3 days. They suggested that a toxin was involved in pathogenicity and that resistance was partially a result of toxin resistance. More recently, Kobayashi (1980) attempted to extract gregatin A and graminin A from diseased adzuki bean and wheat tissue, respectively, and from plants injected hypodermically in the stem base with their respective toxins. As no toxins were isolated, he postulated that the compounds may have been converted into other compounds in vivo. In both circumstances inoculation with toxin produced symptoms similar to natural infection.

Creatura et al. (1981) found that stomates opened wider and responded slower to water potential changes when leaves were treated with graminin A than leaves not treated with the compound. This response also occured in C. gramineum infeted plants where it preceeded stripe development and was more pronounced under conditions of water stress. They showed that differences in stomatal activity were not a function of differences in the leaf water status. Results indicated that the toxin graminin A, and not blockage of the xylem, was involved in early stages of pathogenesis. Morton and Mathre (1980a) reported that the pattern of stripe formation in C. gramineum infected flag leaves of a susceptible winter wheat cultivar at heading was closely correlated with depression of realitve water content, net photosynthesis, chlorophyll content and stomatal conductance. All four physiological parameters were interrelated as indicated by regression analysis. They concluded that chlorosis around colonized vascular bundles could be attributed to effects of localized restriction of lateral water movement rather than a diffusable toxin, like graminin A. It should be noted that those data were recorded late in pathogenesis, in contrast to the work by Creatura et al., whose results apply to early pathogenesis.

Graminin A (Figure 1) and gregatin A (Figure 1) are

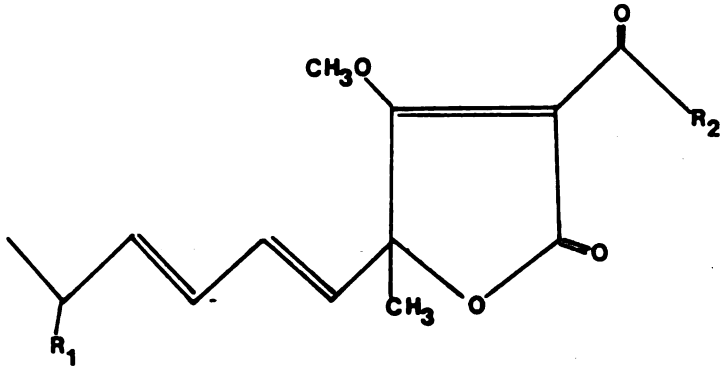
FIGURE 1. Structure of the gregatins and graminin A.

FIGURE 2. Structure of 'butenolide'.

FIGURE 3. Structure of vitamin C.

FIGURE 4. Structure of tetronic acid.

FIGURE 5. Structure of the tautomer of tetronic acid.



		R_1	R_2
GREGATIN	A	H	$CH{=}CH{-}CH_3$
	B	H	CH_3
	C+D	H	$CH_2{-}CH(OH)CH_3$
	E	OH	CH_3
GRAMININ	A	H	$CH{=}CH{-}C_3H_7$

FIGURE 1

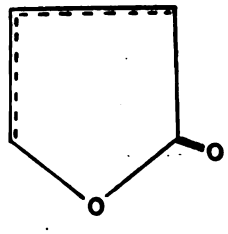


FIGURE 2

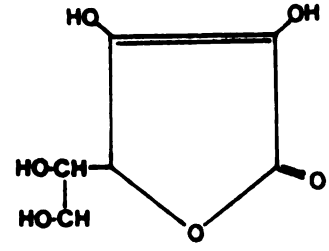


FIGURE 3

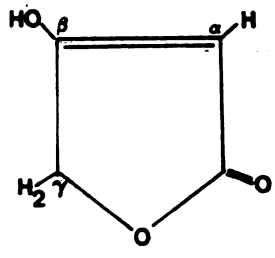
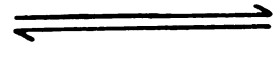


FIGURE 4



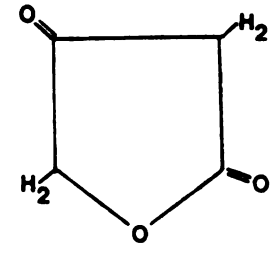


FIGURE 5

secondary metabolites that are derivatives of tetronic acid. The proper name for tetronic acid is 3-hydroxybut-2-enolide (Pattenden, 1978). Tetronic acid and its simple acyl derivatives are polar substances with high melting point (Haynes and Plimmer, 1960). These compounds are found in a group of natural products which includes the butenolides. The term 'butenolide' describes the unsaturated γ-lactone system (Figure 2) (Pattenden, 1978). The nucleus of both butenolides and tetronic acids is the γ-lactone, 5-membered ring; a cyclic ester (Morrison and Boyd, 1975). Tetronic acid derivatives are α and/or γ-substituted derivatives of the parent acid (Figure 4). This form normally predominates in the tautomeric system (Figures 4 and 5) (Haynes and Plimmer, 1960). Those dissubstituted in the γ-position are not regarded as tetronic acids.

The tetronic acid nucleus occurs in the important natural product vitamin C (Figure 3), which is a γ-substituted-α-hydroxytetronic acid (Haynes and Plimmer, 1960). A group of complex tetronic acid derivatives were found responsible for coloration in the lichens (Haynes and Plimmer, 1960; Pattenden, 1978). Tetronic acid derivatives are also found as fungal metabolic products from several Penicillium spp.. The bulk of these have been characterized from P. charlesii, and many other fungi (Haynes and Plimmer, 1960; Pattenden, 1978; Gudgeon et al., 1979).

Apart from vitamin C, most tetronic acid derivatives appear to show little physiological activity. However, penicillic acid is more active against gram-negative bacteria than is penicillin (Haynes and Plimmer , 1960); vulpinic acid, produced by the fungal constituent of lichen, is toxic to animals (Pattenden, 1978); and the tetronic acid derivatives found in marine sponges possess strong antibiotic activity (Pattenden, 1978). Patulin, biosynthesized by P. patulin, has been used as a fungal toxin in food and as a general plant toxin (Ellis and McCalla, 1973). The gregatins and graminin A have been shown to possess both antimicrobial activity and phytotoxicity (Kobayashi and Ui, 1977a, 1979, Kobayashi, 1980; Creatura et al., 1980). Gregatin production has also been reported for Aspergillus panamensis (Anke et al., 1980). Interestingly, aspertonin A and B, produced by A. rugulosus (Ballatine et al., 1969), are enantiomers of gregatin A and D, respectively (Kobayashi and Ui, 1977a; Anke et al., 1980). Many lactone-ring containing compounds are carcinogenic to animals (Ceigler et al., 1971; Uraguchi and Yamazaki, 1978).

Anke et al. (1980) consider the double bond of the α-side chain of gregatin A (Figure 1) as the structural feature most important for biological activity. The reduced activity of gregatin D (Figure 1) is a result of the reduction of this double bond. Gregatin D can theoretically be

derived from gregatin A through addition of various alcohols or water to the double bond of the α-side chain. The longer acyl chain of graminin A leads to a change in selective toxicity, as the data of Kobayashi and Ui (1979) indicated.

Two separate biosynthetic pathways to tetronic acids have been established: a) oxidative cleavages of polyketide derived aromatic intermediates, and b) condensation of an acetate-derived chain(s), usually fatty acid, with tricarboxcylic acid (TCA) cycle intermediates (Turner, 1971; Pattenden, 1978; Gudgeon et al., 1979). The relatively high incorporation of tracer molecules, primarily ^{14}C-labeled precursors, into secondary metabolites in microorganisms has enabled detailed studies to be made of the biosynthesis of several fungal metabolites. Based on these works, Stoessl (1981) suggested that graminin A and gregatins A and D are compounds of mixed fatty acid-TCA cycle origin, with C-4-6 likely from C-1 to C-3 of succinic acid or equivalent and C-7 - C-12 and C-2 - C-16 (or C-18) from fatty acid residues.

Determining the role of a toxin in pathogenesis

In plant pathology, the term toxin is generally defined as a 'non-enzymatic product of a microorganism or a microorganism-host interaction which is harmful to plants in low concentration' (Rudolf, 1976). Toxins in plant pathology have three essential features: a) toxins are products of

microbial pathogens of plants; b) toxins cause obvious damage to plant tissues; and c) toxins are known with confidence to be involved in disease development (Scheffer, 1983). Some authors (Luke and Biggs, 1976; Rudolf, 1976) distinguish between phytotoxin and pathotoxin, the former referring to those which are toxic to plants and the latter defined as a toxin which induces all typical disease symptoms in reasonable concentration and whose production is correlated with pathogenicity. The term 'phytotoxin' can be misunderstood as many mycotoxins may be toxic to plants but may never be involved in the development of plant diseases (Reiss, 1978). The term is also used to indicate a toxic substance from higher plants (Harper and Balke, 1981).

Rather than define toxins in terms of their toxic properties, Yoder (1980, 1983) distinguishes these molecules in accordance with their requirement for successful infection of the host; 'pathogenicity factors' for those toxins required for pathogenicity, and 'virulence factors' for those toxins involved in the degree of disease expression. Phytotoxic metabolites can further be classified as non-specific or host-specific (or host-selective). Non-specific toxins affect plant species other than those hosts of the toxin-producing pathogen. Host-specific toxins affect only hosts of the pathogen. The toxic metabolite graminin A may be a non-specific toxin as it has limited host selectivity (Kobayashi and Ui, 1979; Scheffer, 1983).

There are a number of guidelines proposed for the evaluation of the significance of toxins in disease (Rudolf, 1976, Yoder, 1980). Criteria commonly used include the isolation of toxin from diseased plants, reproduction of typical disease symptoms when toxin is applied to healthy plants, correlation of pathogen and toxin specificities toward plants and correlation of virulence with ability to produce toxin. None of these criteria alone provide adequate evidence for the involvement of a toxin in disease.

Assay systems to evaluate the phytotoxic action of suspected toxins when applied to healthy plants include the use of intact plants, seeds or seedlings, plant parts, tissue culture cells or the use of other target organisms. The placement of cuttings into test solutions has been frequently used to assay for wilt toxins and other toxins (Rudolf, 1976). Van Alfen (Van Alfen and McMillan, 1982) recently discussed the misleading nature of the wilt bioassay method when a suspected wilt toxin is a macromolecule and causes wilt by physically interfering with water movement through cuttings regardless of other activities it might possess. He suggested alternatives to the wilt bioassay which considered the potential of these macromolecules to physically disrupt water transport. An inherent difficulty with the use of cutting assays is that the concentration of the toxin to which living cells are exposed can

never be determined. The toxin dose taken up per gram fresh weight of a cutting must be determined (Rudolf, 1976). The site of primary or secondary site for action of some toxins is the plasmalemma of the host (Rudolf, 1976; Scheffer, 1976; Yoder, 1980). The measurement of electrolyte leakage is used to assess this action when plant parts are exposed to various concentrations of toxin. However, only gross changes in permeability are detected and it is assumed that electolytes leak at the same rate as non-electrolytes (Ayers, 1978). Toxins and allelopathic compounds have been assayed by the inhibition of seedling root growth (Pringle and Braun, 1957; Pringle and Scheffer, 1963; Scheffer and Pringle, 1961; Tang and Young, 1982). The selectivity of the toxin produced by Periconia circinata for susceptible sorghum seedlings but not resistant sorghum seedlings, was demonstrated with this assay (Scheffer and Pringle, 1961). A more rapid and sensitive microbiological assay for toxin, involving inhibition of other organisms by the toxin, may reveal toxic activity at picogram levels (Staskawicz and Panopoulos, 1979).

Screening plant lines for resistance to disease with toxin has been used primarily for host-specific toxins (Wheeler and Luke, 1955; Schertz and Tai, 1969; Steiner and Byther, 1971). Disease-resistant plants or cells can be efficiently selected both in vitro and in vivo. A high level of resistance is expected if a pathogenicity factor is

used; an intermediate level of resistance is expected if a virulence factor is used (Yoder, 1983).

The best test of pathological significance is the specific elimination of the toxin from the biological system and the observation of changes (if any) which may occur in disease development or initiation. The most powerful technique to attempt specific elimination of a molecule from a complex system is genetic manipulation, either by mutational analysis or by the use of naturally occuring variation. An example of elimination of a toxin was afforded by Patil et al. (1974). By mutational analysis, they were able to establish the role of phaseolotoxin, a non-specific toxin, in the bean halo blight disease. A toxin-less mutant of Pseudomonas syringae pv. phaseolicola, though able to multiply as well in vivo as the wild type, could neither cause systemic chlorosis nor invade inoculated host tissue systemically. Phaseolotoxin was therefore determined to be a virulence factor. Naturally occuring variations of toxin production is present in the pathogen Ps. syringae pv. tabaci . Loss of tabtoxin-producing ability results in loss of chlorotic halos, as is characteristic of Ps. syringae pv. angulata (Braun, 1937). These results indicated that the tabtoxin, a non-specific toxin, contributed and was responsible for certain symptoms, but was not required for pathogenicity. In contrast, genetic and other data indicate that the host-specific toxins from Helminthosporium

victoriae and H. carbonum are required for pathogenicity of the producing fungi (Scheffer, 1976).

PART I: ISOLATION AND CHARACTERIZATION OF *CEPHALOSPORIUM GRAMINEUM* MUTANTS THAT VARY IN VIRULENCE

INTRODUCTION

Cephalosporium leaf stripe (CLS), caused by the soil-borne fungus Cephalosporium gramineum, produces vascular chlorosis and necrosis of leaf tissue resulting in yield reduction in winter wheat. Polysaccharides and toxins produced by the fungus have been suggested as the cause of such symptoms. Polysaccharides produced by the fungus were implicated in plugging of the xylem (Spalding et al., 1961; Pool and Sharp, 1969a). However, Wiese (1972) could find no evidence of plugging by polysaccharides. Instead, Wiese found xylary occlusions resulting from fungal proliferation. These developed after leaf striping was evident. Kobayashi and Ui (1977b, 1979) isolated and characterized a toxic substance, graminin A (GRA), from culture filtrates of C. gramineum . Graminin A, which produced vascular browning and chlorosis at low concentrations (25 μg/ml) in excised leaves, is structurally similar to gregatin A, a toxic substance isolated from culture filtrates of C. gregatum . Gregatin A mimics the symptoms of brown stem rot, caused by C. gregatum , on adzuki beans, soybeans and mung beans (Kobayashi and Ui, 1977a). The purpose of my study was to determine whether or not GRA and polysaccharides are important disease determinants of CLS. This was approached by isolating virulence mutants, and screening them for toxin and polysaccharide production in culture.

MATERIALS AND METHODS

Isolates of Cephalosporium gramineum

These are described in Table 1. Cultures of C. gramineum were grown on potato dextrose agar (1.5% agar) plus streptomycin (100 µg/ml) (PDA + str), or grown in potato dextrose broth (PDB) (Tuite, 1969). Cultures to be assayed for GRA and polysaccahride production were grown in a broth described by Kobayashi and Ui (1979).

Toxin

Authentic GRA was kindly supplied by K. Kobayashi (Hokkaido University, Sapporo, Japan). The preparations had a minor contaminant (Kobayashi, personal communication) which could be detected by gas-liquid chromatography (GC), high pressure liquid chromatography, and GC/mass spectroscopy (GC/MS). This toxin preparation was used for all bioassays unless otherwise indicated.

Mutagenesis of Cephalosporium gramineum

Ultraviolet light (UV) induced mutants of C. gramineum were obtained by plating 0.1 ml (10^8 cells/ml) of a 3-day-old shake culture of CG-18 grown on a Lab-line orbit environ shaker (1800 rpm, 27 C) onto PDA + str and exposing the plate to shortwave (200 µW/cm^2) UV light for 2.5 minutes from a height of 3.6 inches with a UVSL-25 Mineralight lamp

TABLE 1. Fungal Isolates Used

Isolates	Morphology on Various Media				Virlulence	Source
	PDA	CA	MA	WA		
CG-82	myc	myc	myc	myc	very high	Michigan field isolate
M-13	myc	myc	myc	myc	very high	Michigan field isolate
CG-18	yst	yst	yst	myc	moderate	UV mutant of M-13 *
N20	yst	yst	yst	myc	high	NTG mutant of CG-18
E53	yst	yst	yst	myc	low	EMS mutant of CG-18
7-54	yst	yst	-	-	very low	UV mutant of CG-18
E67	yst	yst	yst	yst	very low	EMS mutant of CG-18

PDA = potato dextrose agar
CA = Davis' complete agar (Lederberg, 1950)
MA = Davis' minimal agar (Lederberg, 1950)
WA = water agar
myc = mycelial
yst = yeast-like
- = no growth
* Fulbright and Ravenscroft, 1981

(Ultra-Violet Products Inc., San Gabriel, CA 91778). The plates were incubated in the dark at room temperature (24-26 C) for 6 days before selecting colonies for virulence test.

Mutagenesis with N-methyl-N'-nitro-N-nitrosoguanadine (NTG) and ethyl methanesulfonate (EMS) was performed as described by Miller (1972) for NTG mutagenesis. The concentration of NTG was approximately 40 µg/ml. Approximately 150 µl of concentrated EMS was added to 12 ml of a shake culture of mutant CG-18. Aliquots were taken from the shake culture at 0, 1, 2, 4, 10 and 24 hours and plated on PDA + str. After 4 days growth, colonies were selected arbitrarily and tested for virulence using the seedling assay (described below). Auxotrophic requirements were found by supplementing minimal agar (MA) with various combinations of amino acids, vitamins, purines and pyrimidines (Holliday, 1956).

Seedling assay

The soft, winter white wheat cultivar Yorkstar was used for all assays requiring plant material. This variety is very susceptible to CLS. Plants for the seedling assay were grown for 10 days in autoclaved sand in a growth chamber with a 14-hr-day-photoperiod (9 x 10^4ergs/cm^2 · sec) and 21 C. The plants were fertilized at 3 days and 9 days with a solution of Rapid Gro (25, 19, 17; 1 tbsp/gal water). After 10 days growth seedlings were removed, the roots severed to 1 inch in length and washed free of and particles, and plawed im conidial suspensions. Conidial suspensions were

prepared by growing C. gramineum in 50ml PDB for 6 days on a reciprocal shaker (96 strokes/min) at 24-26 C. Six seedlings were placed in each conidial suspension (10^7-10^9 conidia/ml) for at least 15 minutes before transfer to a pot containing sterilized soil. The remaining conidial suspension was poured equally into the holes in which the seedlings were planted.

After inoculation, seedlings were grown in a chamber at 17 C, with a 15-hr-day-photoperiod (9×10^4 ergs/cm$^2 \cdot$ sec). The temperature favored growth of the pathogen. Plants were fertilized 7 days after inoculation and symptoms were rated 14 days after inoculation. If no symptoms appeared the 14 days, the plants were kept in the chamber for another week for observation of possible symptoms.

The initial screening procedure was repeated 3 times, to select mutants with various degrees of virulence. Four mutants, 3 with decreased virulence and 1 with increased virulence, were selected and tested 3 more times. Each seedling assay included the following treatments : uninoculated PDB, isolates CG-18, M-13, CG-82 and autoclaved (20 min, 15 psi) shake cultures of CG-18 and M-13.

A symptom rating system (1-15) was based on observations of symptom development in the growth chamber (Table 2). Each plant in a pot was given a rating and the ratings were totaled per pot. Each pot represented one replication of one treatment. The experiment was set up and analyzed as

a randomized complete block design, blocking over time (Steele and Torrie, 1980). A disease severity rating as related to isolate virulence was obtained by transforming the symptom rating system to a scale of 6-100 (Table 3). This was accomplished by multiplying total pot symptom rating values (symptom rating total for 6 plants) by a factor of 1.11.

A seedling was randomly selected from pots inoculated with each isolates for reisolation of the organism. Sections from the leaf-sheath and the leaves were surface sterilized in a 1.5% NaOCl solution, and placed on PDA + str. Plates were observed for one week for fungal growth from the vascular tissue.

Colony morphology and relative spore production

Growth of mutants was obseved after 4 days on PDA, Davis' complete agar (CA)(Lederberg, 1950), Davis' minimal agar (MA) (Lederberg, 1950) and water agar (WA) for comparison with colonies of mutant CG-18 and isolate M-13. Morphology of conidia and sporogenous cells were examined with the light microscope to confirm identity of the mutants as being members of the genus Cephalosporium.

To determine relative spore production of mutants and isolates, PDB (50 ml in 125 ml Erlenmyer flasks) was inocuated with mutants or wild type isolates grown for 7 days in

TABLE 2

Symptom rating system used in the seedling assay

Rating	Symptom
1	no symptoms
2	faint, genefal chlorosis
3	1st leaf chlorotic
3 *	2nd leaf chlorotic
4	1st and 2nd leaves chlorotic
5	1st leaf striping
5 *	2nd leaf striping
5 **	3rd leaf striping
6	1st leaf chlorotic and striping
6 *	2nd leaf chlorotic and striping
7	1st and 2nd leaves striping
8	1st leaf chlorotic, 2nd leaf striping
8 *	1st leaf striping, 2nd leaf chlorotic
9	1st leaf chlorotic and striping, 2nd leaf chlorotic or striping
9 *	1st leaf chlorotic or striping, 2nd leaf chlorotic and striping
10	1st and 2nd leaf chlorotic and striping
11	1st leaf dead
12	1st leaf dead, 2nd leaf chlorotic
13	1st leaf dead, 2nd leaf striping

TABLE 2 con't.

14	entire plant nearly dead, stem is green
15	entire plant dead, no green tissue

* Astericks indicate the same rating was used for variations of the same type of symptom.

TABLE 3

Disease severity rating as related to isolate virulence

Total Symptom Rating for 6 Seedlings	Disease Severity Rating		Disease Severity	Isolate Virulence
6	6.66	0-25:	no disease or very mild	absent or very low
12	13.32			
18	19.98			
24	26.64	25-45:	mild	low
30	33.30			
36	39.96			
42	46.62	45-60:	moderate	moderate
48	53.28			
54	59.94			
60	66.60	60-85:	severe	high
66	73.26			
72	79.92			
78	86.58			
84	86.58	85-100:	very severe	very high
90	99.90			

PDA + str. Inoculated flasks were incubated on a reciprocal shaker. After 6 days the number of conidia/ml for each culture was determined using a hemacytometer.

Detection of revertants

An auxotrophic, methionine requiring mutant isolated after UV light mutageneis of mutant CG-18 was assayed for reversion by the following method. A culture of approximately 10^8 conidia/ml PDB was centrifuged at 8500 g for 10 minutes. The pellet was resuspended in sterile physiological saline (0.85% NaCl), centrifuged and then resuspended in 10 ml saline. A 0.2 ml sample of the suspension was plated on MA. After one week plates were checked for growth. Any colonies appearing on MA would be considered revertants and tested for virulence in the seedling assay.

Ability of isolates to overwinter and cause disease in the field

Spore suspensions were obtained by growing the wild type isolates CG-82 and M-13 and the mutants CG-18, 7-54 and E-67 in modified Eckert's medium (Johnston and Mathre, 1972) in shake culture (1800 rpm, 27 C). Oat inoculum was prepared by adding 10 ml aliquots of a heavy spore suspension of each isolate to 150 g of oat seeds which had been moistened with 100 ml distilled water and autoclaved for 90 minutes in glass quart jars. The jars were capped with a screw band placed over 3 layers of Whatman #3 filter paper. The bottles were shaken to distribute inoculum and incubated

at room temperature (24-26 C) for 8 weeks. The jars were shaken at 2-3 week intervals and at 4 weeks 50 ml of Eckert's medium was added (Mathre and Johnston, 1975a).

Field plots were inoculated with oat kernel inoculum at Michigan State University in the fall of 1982. Inoculation was accomplished by placing the inoculum in a 7-row, Auger-feed seed drill along with vitavax treated wheat seed. The seed and inoculum were added to the row simultaneously in a field which had been grown in dicots for several years. A completely randomized design was used. Treatments included wild type isolates CG-82 and M-13 and mutant isolates CG-18, 7-54 and E67 grown on oat kernels. Controls included vitavax treated seed and oat seeds treated with PDB.

Isolation of the fungus from infected tillers (leaves showing stripes) was attempted in the spring of 1983. Isolations were attempted from plants at growth stage 5 (pseudo-stem, formed by sheaths and leaves, strongly erect) and 10.1 (first ears just visible, ear escaping through split of sheath) on the Feekes' scale (Large, 1954).

Culture harvest

Isolates (3 replications/isolate) were grown in 4 1 diptheria bottles containing 1 liter of medium (Kobayashi and Ui, 1979) for 28 or 29 days (24-26 C). Three 1 liter replications of each isolate were grown. Cultures were harvested by filtering through 11 cm Whatman #4 filter paper

discs which had been dried to a constant weight in a 45 C drying oven. Filters with mycelium were dried again in the oven and weighed. For all isolates 200 ml of filtrate was centrifuged at 4100 g for 40 minutes. The resuspended conidial pellet placed in a predried, preweighed aluminum pan, and then dried and weighed. The weights of culture medium which collected on filters, along with the mycelium and that which was pelleted by centrifugation, were subtracted from dried mycelial and conidial pellet weights, respectively.

Polysaccharide extraction

Polysaccharide was precipitated by adding an equal volume of 95% EtOH to 200 ml of the culture filtrate. The solution was refigerated (4 C) overnight and then centrifuged at 4100 g for 30 minutes to pellet the precipitate. The pellet was redissolved in a small volume of distilled water and dialyzed in cellulose dialysis tubing (molecular weight cut off 12,000 - 14,000, Spectrapor, VWR Scientific Inc.) against distilled water for 6 hours in the cold (4 C). The polysaccharide content was determined by the Anthrone method (625 nm) (Hodge and Hofreiter, 1962) using galactose was used as the standard. An analysis of variance and the LSD multiple comparison test (Steele and Torrie, 1980) were performed to determine significant differences in polysaccharide production between wild type isolates and mutants in culture.

Toxin extraction and purification

The remaining 800 ml of culture filtrate was evaporated under reduced pressure at 50 C to 100 ml. Each 100 ml sample was then extracted four times with equal volumes of methylenechloride (CH_2Cl_2). The efficiency of extraction was 82% based on recovery of known amounts of GRA. The organic solvent extracts were evaporated to dryness under reduced pressure at 38 C and the residue redissolved in a small volume of chloroform ($CHCl_3$). The $CHCl_3$solutions were transferred to 1 dram vials and dried under a stream of nitrogen gas. Vials were sealed with a teflon lined cap and stored at -20 ^{C} until futher purification.

The contents of each dram vial were redissolved in 200 µl $CHCL_3$ and applied to Supelco silica gel G , preparative thin layer chromatography (TLC) plates containing a phosphor (254 nm). The plates were developed with $CHCl_3$:methanol (MeOH) (98:2, v/v) and the toxin detected as a dark blue band under shortwave (300 $\mu W/cm^2$) UV light, but not longwave (640 $\mu W/cm^2$) UV light. The toxin bands were removed form the TLC plate and eluted with $CHCl_3$. The $CHCl_3$ eluate was filtered through Whatman CG/F, 3.1 cm glass-fiber filters to remove silica gel paricles. The filtrate evaporated to dryness under reduced pressure at 38 C. The residue was redissolved in 50 µl $CHCL_3$ and a small volume spotted on a Fischer silica gel GF, redi-plate to check for homogeneity of the sample. The efficiency of recovery toxin from TLC prepara-

tive plates was 79%. At all times a 1 or 2 µg samples of authentic GRA/µl $CHCl_3$ was used as a standard. Dried culture filtrate preparations were stored at -20 C in 1 dram vials with teflon caps until analysis.

Toxin detection by gas chromatography (GC)

50 1 $CHCl_3$ was added to each vial containing preparations from each isolate culture filtrate. One µl samples were analyzed by injection into a Varian 3700 GC equipped with a Supelco glass column (6 ft, inner diameter 2 mm) packed with 3% OV-17 on 100/120 gas chromosorb Q. Parameters of each run included: 300 C detector, 270 C injector, 190 C (1 min) - 290 C (1 min) column, with a program of 10 C increase/min, range 10^{-10}, attenuation 4 or 8 and flame ionization detection. The carrier gas (N_2) had a flow rate of 30 cc/min. To insure complete delivery, samples were injected by the sandwich technique where the sample being assayed was held in the syringe between two equal volumes of solvent. Along with each set of culture filtrate preparations, a sample of 1 µg authentic GRA/µl $CHCl_3$ was assayed. Samples were also assayed by coinjection with 1 µl autnentic GRA/µl $CHCl_3$ to verify cochromatography of sample and standard.

Toxin detection by gas chromatography/mass spectroscopy (GC/MS)

Culture filtrate preparations and authentic toxin were analyzed by GC/MS using a Hewlett-Packard, 5985 GC/MS

quadropole system equipped with an 18 inch glass column and packed with 3% OV-17. The GC column temperature program for each run was 190 C (1 min) - 285 C, with a 20 C increase per minute. The carrier gas (He) had a flow rate of 30 cc/min. Mass spectra were obtained by electron impact of compounds. In addition to total mass spectra, selective ion intensity and mass spectra were produced by selective monitoring of ions of particular interest.

Toxin detection by ultraviolet spectroscopy

The spectra of culture filtrate preparations were obtained by scanning samples (in MeOH) from 210 nm - 340 nm in a Gilford 2600 spectophotometer. The spectra of preparations from wild type and mutant isolate culture filtrates were compared with the spectrum of the authentic toxin and with published spectral values for GRA (Kobayashi and Ui, 1979).

Disc assay for antimicrobial activity

Toxin in 2% EtOH was added to individual Whatman 540 (2.1 cm) hardened ashless filter discs to final quantities of 0, 30, 50 and 100 µg GRA. The discs were allowed to dry and placed on plates of PDA + str. These plates were then sprayed with a log-phase culture of Escherichia coli, Rhodotorula spp. or Bacillus megaterium ,grown in complete medium, using a DeVilbiss 15 spray atomizer. Plates were incubated at 27 C and observed for zones of inhibition after

24 hours. This assay was repeated using Cladosporium cucumerinum. Plates were incubated at 17 C and observed for zones of inhibition after 30-36 hours.

The disc assay developed by Staskawicz and Ponopoulus (1979) was also used. A 2 ml log-phase E. coli culture grown in minimal broth or Rhodotorula spp. grown in complete broth was mixed with 2 ml of molten WA (2%) kept at 65 C. After overlaying on MA, toxin-impregnated filters were placed on the overlay. Alternatively wells were cut into the overlayed agar plates with a No. 3 cork borer (0.6 cm diameter) and filled with 20-25 µl toxin solution. Plates were incubated as above and observed for zones of inhibition after 5 hours and 24 hours for E. coli and Rhodotorula spp., respectively . This assay was also used with C. cucumerinum. Complete broth (25 ml) was added directly to 10-day-old cultures of C. cucumerinum grown on PDA and the surfaces of the cultures were rubbed lightly with a glass rod. Two ml of the resulting spore suspensions were mixed with 2 ml of molten WA. Toxin-impregnated filters were placed on the overlay or wells were cut in the agar, as above. Plates were incubated at 17 C and observed for zones of inhibition after 30-36 hours. Toxin concentrations from 0 to 100 g GRA in 2% EtOH were used in all assays. the culture filtrate preparations of wild type isolates and mutants were also tested by these methods.

TLC plate assay for antimicrobial activity

A nutrient medium (Allen and Kúc, 1968) was added directly to 10-day-old cultures of C. cucumerinum grown on PDA. The surfaces of the cultures was rubbed lightly with a glass rod and the resulting spore suspension placed in a glass DeVillbiss 15 atomizer. The suspension was sprayed on TLC plates on which authentic toxin samples and preparations from wild type isolate and mutant culture filtrates has been run or spotted. The sprayed plates were placed in humidity chambers (Allen and Kuc, 1968). Plates were supported by 10 ml glass beakers and the chamber was sealed with masking tape. The plates were incubated for 48 hours and inspected for inhibition of C. cucumerinum at locations corresponding to migration of the toxin. Zones of inhibition appeared as white spots on a dark green background of spores and mycelium.

Leaf-sheath assay for phytotoxicity

Plants for the leaf-sheath assays were grown in sterilized soil in a growth chamber with a 12-hr-day-photoperiod ($2 \times 10^5 ergs/cm^2 \cdot sec$) and a 21 C temperature regime for 15 days. These plants were fertilized 7 days after planting. Following the procedure of Kobayashi and Ui (1977b, 1979), 10-day-old wheat seedlings were cut at the soil line, cut again under water and placed through slits in parafilm sealed 1 dram vials containing 1 ml of toxin solution (0, 25, 50 and 100 µg GRA/ml 2% EtOH). Each treatment

was performed in duplicate. Vials were placed under flourescent light banks (6 x 10^3ergs /cm^2 · sec) with a 10-hr-day-photoperiod; the temperature was 28 C. Leaves were evaluated for chlorosis and wilt, as compared to the 2% EtOH control, after 3-5 days. The leaf-sheath assay was also performed by placing cut seedlings in 100 µl of toxin solution (0, 25, 50, and 100 µg GRA/ml 2% EtOH), allowing the toxin solution to be taken up and then filling the vial with distilled water whenever necessary. The leaves were evaluated as above. This procedure allowed the amount of toxin applied per gram fresh weight of wheat tissue to be determined. Fresh weights of five cuttings were averaged to yield a mean fresh weight.

Seedling-flask assay for phytotoxicity

Seeds were surface sterilized by placing in 70% EtOH for 5 minutes, under vacuum, followed by 5 minutes under vacuum in 0.5% NaOCl containing 1 drop of 5% Triton-X 100 per 500 ml 0.5% NaOCl. Seeds were then rinsed twice with sterile distilled water and single seeds were placed in scintillation vials containing 3 ml 0.1% nutrient broth and allowed to germinate (Bailey, 1980). After 4-7 days, noncontaminated, germinated seeds were aseptically transferred to 125 ml Erlenmyer flasks containing 20 ml PDA + str. One week later a 1 ml solution of GRA in 2% EtOH or 1 ml of 10, 20 or 40% EtOH was aseptically pipetted onto the agar surface and the flask swirled so the liquid covered the

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surface. After 2 days roots were inspected for browning.

Inhibition of seed root growth by graminin A

Inhibition of seed root growth was assayed by the methods of both Pringle and Braun (1957) and Tang and Young (1982). Following the procedure of Pringle and Braun, five wheat seeds, with the radicle just beginning to show, were placed in 60 x 15 mm petri dishes with 5 ml of a toxin solution (0, 25, 50, 100 and 200 µg GRA/ml 2% EtOH) or distilled water. After 48 hours the length of roots was measured.

Following the methods of Tang and Young, dormant cress curled seeds (Herpt Brothers Seedmen Inc., Brewster, NY 10509) or pregerminated wheat seeds were placed in petri dishes on filter discs impregnated with toxin and moistened with 200 µl of water. The petri dishes were sealed with parafilm. Cress seed root length was measured after 3 days and wheat seed root length after 8 hours. For both seed types the following toxin solutions and controls were used: 0, 5, 10, 30, 50, and 100 µg GRA in 2% EtOH and distilled water. An analysis of variance was performed on each set of data. If a significant difference was found with the F-test, Dunnett's test (Steele and Torrie, 1980) was performed to determine which toxin treatment varied significantly from the 2% EtOH control.

Leaf-puncture assay for phytotoxicity

Plants for the leaf-puncture assay were grown the same as those grown for the leaf-sheath assay. Cut 15-day-old

wheat leaves were placed in pyrex petri dishes (15 x 100 mm) on glass supports over wet filter paper. A small hole was made near the leaf base and a 5 µl solution of GRA (25, 50, 100, 200 µg GRA/ml 2% EtOH) was placed on the wound (Scheffer and Livingston, 1980). The plates were sealed with parafilm and the wheat leaves observed for symptom production in 24 hours.

Electrolyte leakage assay for phytotoxicity

Ten to twelve day-old greenhouse grown wheat seedlings used for the electrolyte leakage assay. The first and second leaves of seedlings were cut into 1 cm lengths and rinsed. Duplicate 0.2 g samples were tied in 6 x 6 inch pieces of cheesecloth, the cheesecloth bags were placed in distilled water and rinsed 3 times during the following 10 minutes. The bags were then placed in scintillation vials containing 5 ml of toxin solution (0, 25, 50, 100 and 200 µg GRA/ml 2% EtOH) for 1 hour. The leaf pieces were vacuum infiltrated for 10 minutes and then incubated on a shaker (122 strokes/min, 24 C) for the remaining 50 minutes. The bags were rinsed thoroughly with distilled water and 5 ml distilled water were added as a leaching solution. The initial zero reading was taken at this point. Vials were incubated on a shaker and conductivity (µmhos) readings were made at 0.5, 1, 2, up to 10 hours with a pipette-type electrode (k = 1.0) coupled with a conductivity meter (Scheffer and Livingston, 1980). A comparison of electrolyte leakage

between leaves and sheaths was also made using 12-day-old seedlings. Readings were taken hourly up to 12 hours and then sporadically up to 46.5 hours. Duplicate sample readings were averaged and plotted. Data were also analyzed by linear regression (Steele and Torrie, 1980). The slopes (rate of electrolyte leakage) of individual regression lines were compared for homogeneity.

RESULTS

Mutagenesis and isolation of mutants with altered virulence

Propagules of mutant CG-18 were treated with NTG, EMS and UV light. Survival of 10% of the propagules was achieved after 24 hours, 10 hours, and 2.5 minutes, respectively. One auxotrophic mutant requiring methionine was obtained by UV mutagenesis. Revertants of the auxotroph were not obtained after several attempts to isolate prototrophs. An identifying number was given to each mutant. Mutants generated by NTG were given a number preceeded by the letter N. Those generated by EMS were given a number preceeded by the letter E.

Several hundred mutagenized isolates were screened for virulence in The seedling assay. Four mutant isolates, N20, E53, 7-54 and E67, were selected for further study. Seedling plants were inoculated and severity of disease induced by each isolate was determined. Virulence of wild

type isolates and mutants of cultivar Yorkstar are summarized in Table 4. The wild type isolates CG-82 and M-13 were highly virulent, and the mutant CG-18 was moderately virulent. Disease induced by isolate N20 was rated as more severe than that induced by its parent CG-18. Disease severity levels rated for isolates E53, 7-54 and E67 were less severe than CG-18. There was no significant difference among the disease severities produced by the autoclaved cultures, uninoculated PDB, or mutants E67 and 7-54. Disease severity produced by CG-82 and M-13 were not significantly different.

The fungus was routinely isolated from the sheath but rarely from the third leaf of inoculated seedlings, depending on the virulence of the isolate. CG-82 and M-13, highly virulent wild type isolates, were isolated from all parts of seedlings in most cases. E67, a mutant of very low virulence, was sometimes isolated from the sheath and rarely from the first leaf. The same was true for mutant 7-54. E53 was usually isolated from the sheath and first leaf. Mutants CG-18 and N20 were found in the sheath and in the first and second leaf, but rarely in the third leaf.

TABLE 4

Comparative virulence of isolates and disease severity of cultivar Yorkstar

Isolate	Disease Severity Rating[1]	Disease Severity	Virulence[2]
CG-82	93.24 e	very severe	very high
M-13	85.47 e	very severe	very high
CG-18	44.76 c	moderate	moderate
N20	61.43 d	severe	high
E53	27.93 b	low	low
7-54	13.32 a	very low	very low
control (PDB)	6.66 a	very low	very low
M-13 auto	6.66 a	very low	very low
CG-18 auto	6.66 a	very low	very low

1. Isolates with the same letter do not differ signigicantly (p = 0.05) according to the LSD test (Steele and Torrie, 1980).

2. The relationship of disease severity to virulence is summarized in Table 3.

Colony morphology and relative spore production of isolates

Colony morphology of wild type isolates and mutants on four agar types is summarized in Table 1. All fungi produced yellow-beige colored colonies.

Mutant CG-18 was yeast-like when examined with the light microscope. The most common form of CG-18 was as the shape of the letter Y, where each of the three sections making up the letter were elongated, oval sporogenous cells. Sometimes short, randomly branched chains and clumps of sporogenous cells were found. Single, round and elongated, oval conidia were also seen. All sporogenous cells contained two round inclusions, one at either end of each cell. The conidia of M-13 and CG-82 had the same morphology as conidia of CG-18. Examination of spores and sporulating structures of mutants with the light microscope confirmed they were C. gramineum and derived from CG-18.

The relative spore production of wild type isolates and mutant shake cultures used for inoculation of seedlings in the seedling assay was 0.80 - 5.20 x 10^8 conidia/ml PDB. CG-18 produced the most spores and E67 produced the least number of spores in PDB. There was no relationship between virulence and spore production of mutants and isolates.

Ability of isolates to overwinter and cause disease in the field

Reisolation of C. gramineum in the spring of 1983 from the early season (growth stage 5) and the late season

(growth stage 10.1) plants showing striping symptoms was successful for CG-82, M-13 and CG-18. The results were identical for both plant ages. The C. gramineum reisolated from CG-82 and M-13 inoculated plants always had mycelial growth on PDA. The control plants were infected with a C. gramineum which had mycelial growth on PDA. The pathogenic yeast-like CG-18 was always reisolated from CG-18 inoculated plants. A C. gramineum isolate with mycelial growth on PDA was isolated from E67 inoculated plants indicating a natural contaminate from the soil. No C. gramineum was isolated from 7-54 inoculated plants.

Polysaccharide and toxin production by isolates in culture

Polysaccharide production in culture is summarized in Table 5. There were, however, significant differences in the amount of polysaccharide produced in culture between wild type isolates and mutants. However, there was no relationship between virulence in the seedling assay and polysaccharide production in culture.

Characterization of culture filtrate preparations by gas chromatography/mass spectroscopy and ultraviolet spectroscopy

Preparations from culture filtrates coinjected with autnentic GRA resulted in one peak when analyzed by conventional GC. Subsequent analysis of toxin preparations by

TABLE 5

Prodution of polysaccharide in culture by isolates of *Cephalosporium gramineum* which vary in virulence

Isolate	Virulence[1]	mg polysaccharide / mg fungal tissue[2,3]
CG-82	very high	0.33 a
M-13	very high	0.95 c
CG-18	moderate	0.81 bc
N20	high	0.47 ab
E53	low	0.77 bc
E67	very low	0.94 bc

1. Virulence was determined by the seedling assay, using cultivar Yorkstar.

2. Polysaccharide content was determined by the anthrone assay. Fungal tissue was determined by dry weight.

3. Each value is the mean of 3 replications. Values followed by the same letter do not differ significantly ($p = 0.05$) according to the LSD test (Steele and Torrie, 1980).

GC/MS revealed no production of GRA by all mutants (Table 6). CG-82 and M-13 did produce GRA as determined by GC/MS.

The total ion mass spectrum of authentic GRA from Kobayashi is shown in Figure 6. The molecular ion (M) m/e 304 was detected as were (M-1) m/e 303 and (M+1) m/e 305. Two mass spectral fragments of GRA were observed at m/e 207 ($M-C_6H_9O$) and m/e 97 ($-C_6H_9O$) (Kobayashi and Ui, 1977). The base peak was m/e 93. The heights of the ion peaks in the spectra indicate the relative abundance of one peak to that of the base peak.

The total ion mass spectra of culture filtrate preparations from M-13 and CG-18 are shown in Figure 7. The spectrum of culture filtrate preparations from M-13 culture filtrates (Figure 7A) is representative of both CG-82 and M-13. The moleclar ion (M) m/e 304 was observed. The apparent base peak, however, was a spectral fragment at m/e 129. The spectrum of the culture filtrate preparation from CG-18 culture filtrate (Figure 7B) is representative of CG-18, N20, E53 and E67. No molecular ion (M) m/e 304 was detected. The base peak was m/e 129. The spectral fragment, m/e 129, was also seen in the spectrum of authentic GRA (Figure 6).

The authentic GRA sample and preparations from culture filtrates of the tested isolates were examined more closely for GRA by selective ion monitoring. The ions m/e 129, 224, 248 and 304 were chosen. The ion m/e 129 was

TABLE 6

Production of graminin A in culture by isolates which vary in virulence

Isolate	Virulence[1]	Graminin A_2 Production[2]
CG-82	very high	+
M-13	very high	+
CG-18	moderate	-
N20	high	-
E53	low	-
E67	very low	-

1. Virulence was determined by the seedling assay, using the cultivar Yorkstar.

2. The presence of graminin A was determined by gas chromatography/mass spectroscopy.

   + = graminin A produced, - = no graminin A detected.

FIGURE 6. Mass spectrum of authentic graminin A.

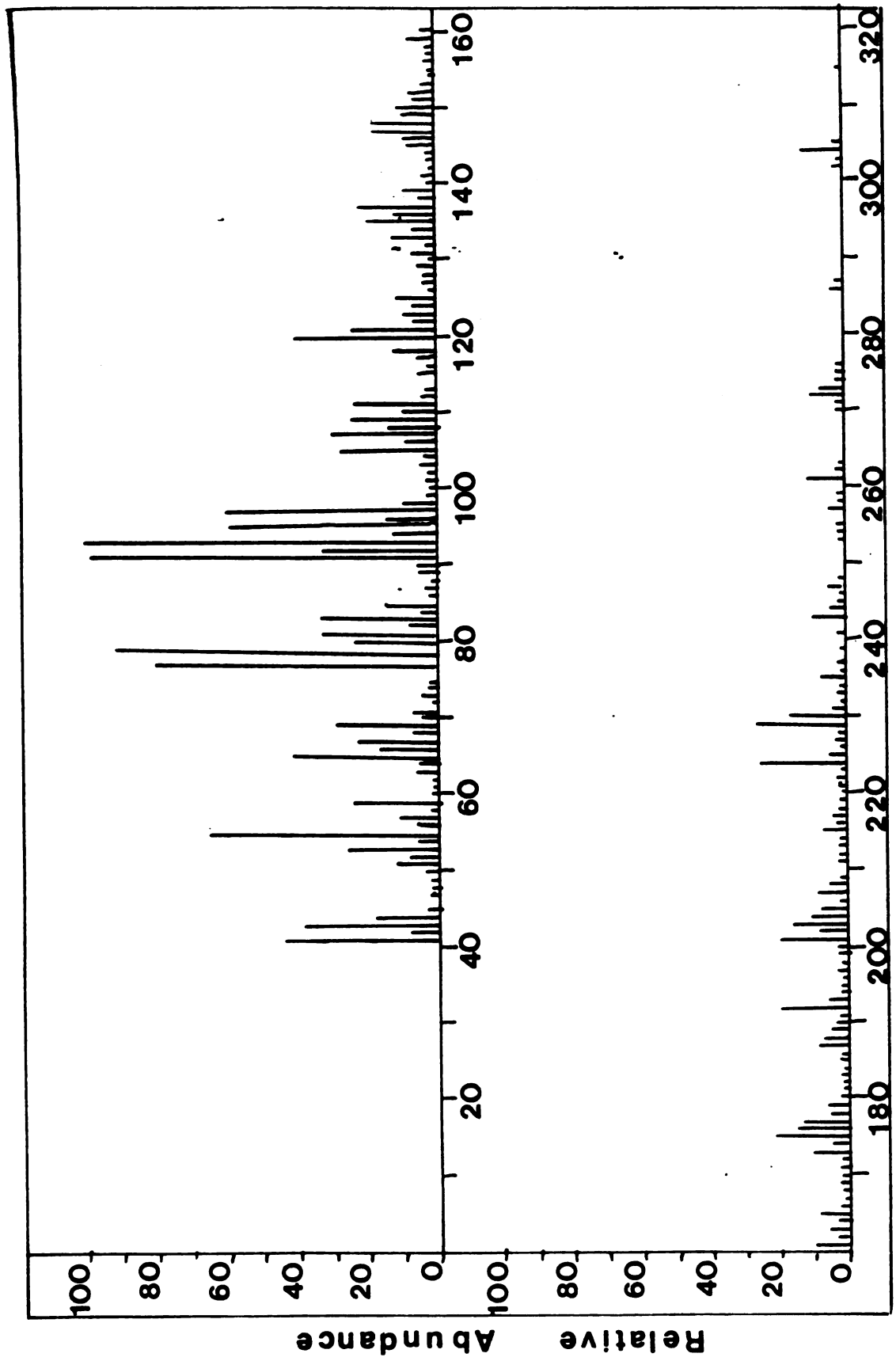
Relative Abundance
100
80
60
40
20
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
m/e

FIGURE 6.

FIGURE 7. Mass spectra of preparations from culture filtrates of M-13 (A) and CG-18 (B).

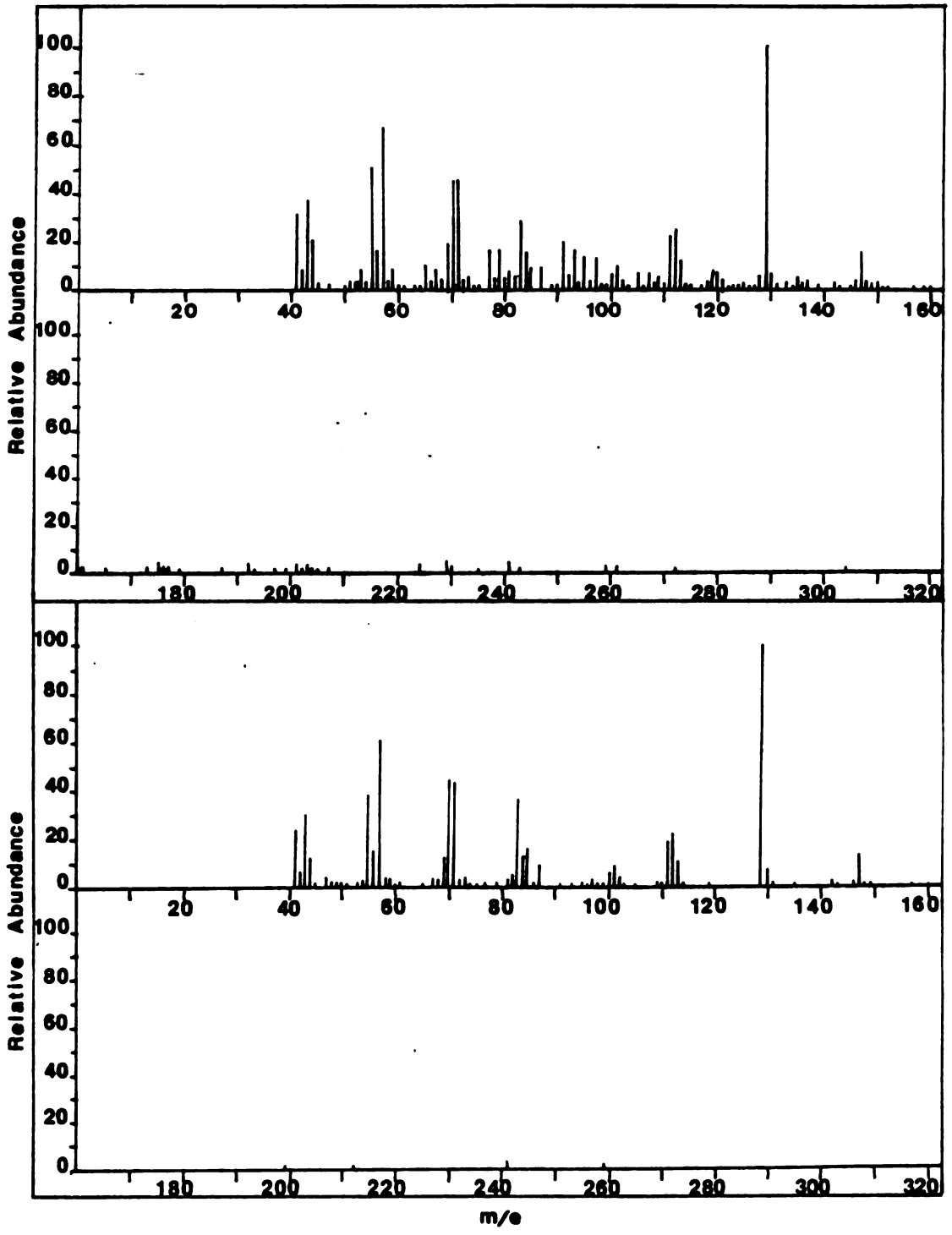
Relative Abundance
100
80
60
40
20
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
m/e

FIGURE 7.

monitored as it was the base peak of the isolate and mutant culture filtrate preparations. The remaining ions were not found in the spectrum of preparations from CG-18 culture filtrates (Figure 7B), but were common to the authentic GRA mass spectrum (Figure 6).

The ion intensities of the selected ions in the authentic GRA sample are shown in Figure 8A. The number of ions detected for a peak reaching 100% on the relative abundance scale is given for each selected ion. The peaks at approximately 180 seconds (3 min) represented GRA. The ratio of the number of ions of m/e 304 detected to the number of ions of m/e 224 detected was 1:2. That the ion peaks for m/e 224, 248 and 304 had the same retention time indicated they were derived from the same molecule. Figure 8B is the mass spectrum of the selected ions in authentic GRA at the retention time 3:02 minutes. The spectral fragment m/e 129 was present in the authentic GRA.

The ion intensities of the selected ions in the preparations from M-13 culture filtrate are shown in Figure 9A. The peaks at approximately 200 seconds (3.5 min) represented GRA. ALthough GC alone did not allow the detection of a contaminating compound, the ion intensity spectrum resolved this compound from GRA. This was seen by the different retention time for m/e 129. The ion m/e 129 had a shorter retention time than the spectral fragments of GRA. There was, however, incomplete resolution of the spectral fragments of

FIGURE 8. Selective ion monitoring spectra of authentic graminin A.

A. Selective ion intensity spectrum.
The number of ions detected is given for each peak reaching 100% on the relative abundance scale.

B. Selective ion mass spectrum.
The height of the ion peaks in the spectra indicate the relative abundance of one peak to that of the base peak.

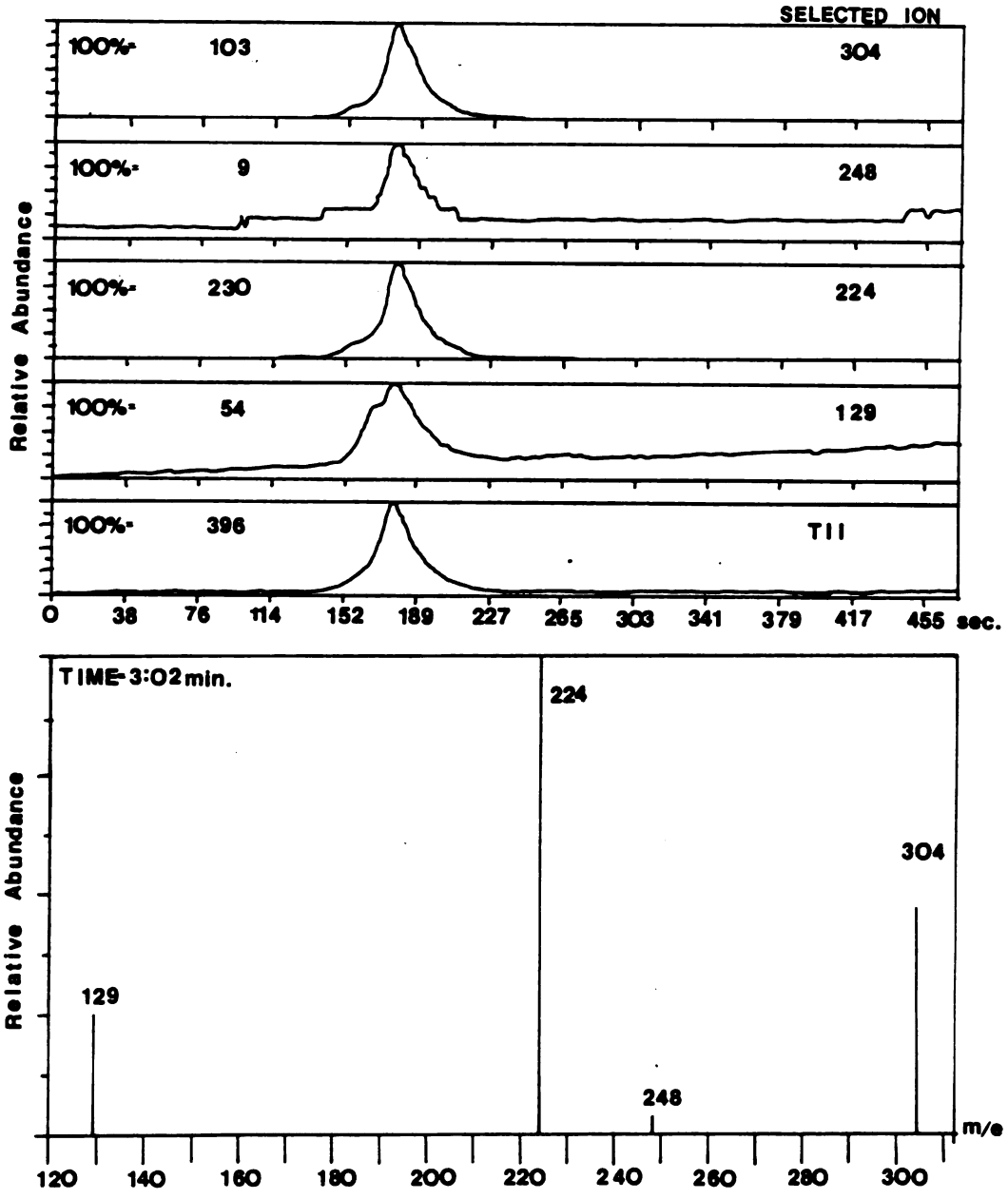
SELECTED ION
100%- 103
304
100%- 9
248
100%- 230
224
100%- 54
129
100%- 396
TII
Relative Abundance
0 38 76 114 152 189 227 265 303 341 379 417 455 sec.
TIME-3:02min.
224
304
129
248
m/e
120 140 160 180 200 220 240 260 280 300

FIGURE 8.

FIGURE 9. Selective ion monitoring spectra of preparations from isolate M-13 culture filtrate.

A. Selective ion intensity spectrum.
The number of ions detected is given for each peak reaching 100% on the relative abundance scale.

B. Selective ion mass spectrum
The height of the ion peaks in the spectra indicate the relative abundance of one peak to that of the base peak.

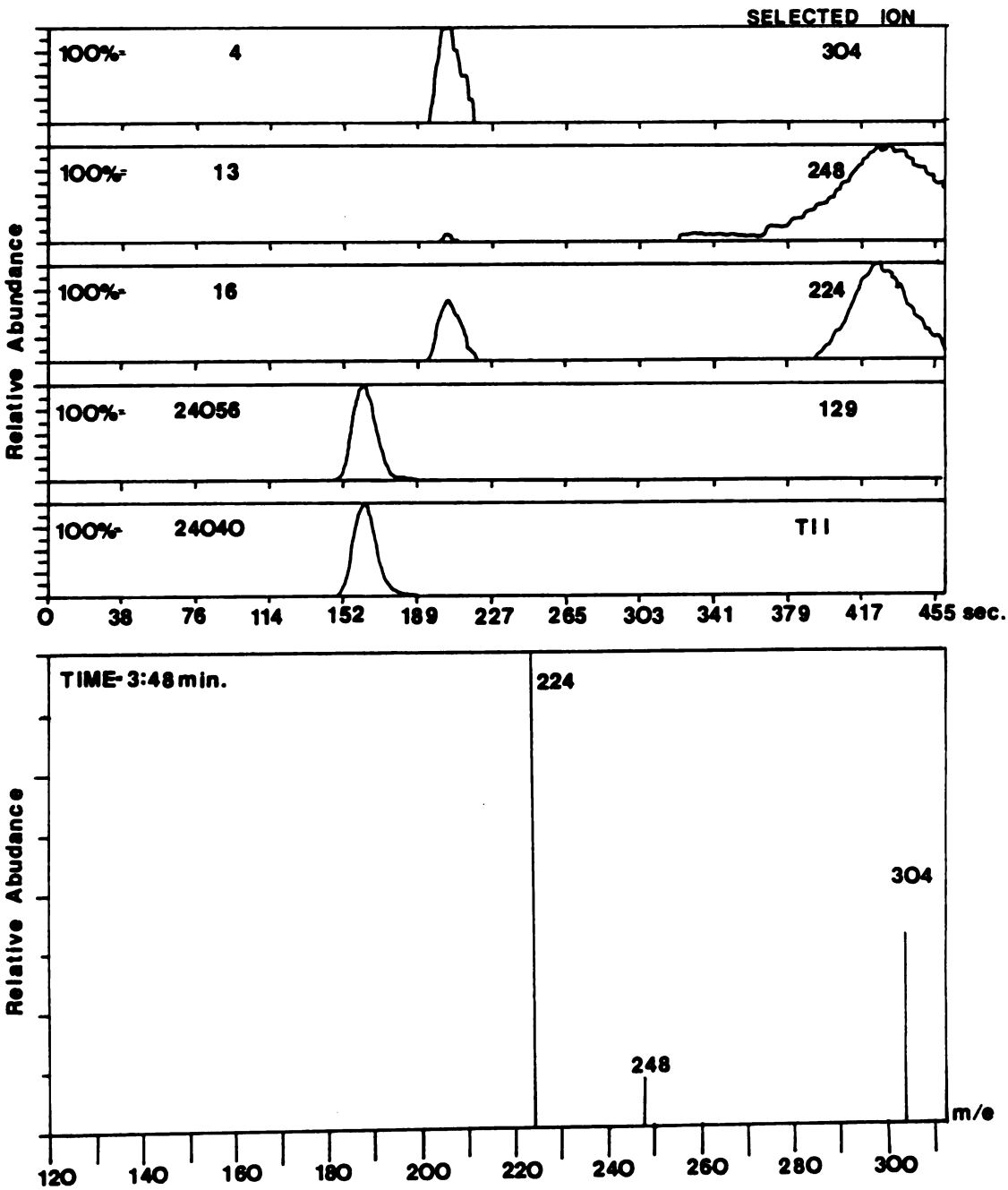
SELECTED ION
100%
4
304
100%
13
248
100%
16
224
100%
24056
129
100%
24040
TII
Relative Abundance
0
38
76
114
152
189
227
265
303
341
379
417
455 sec.
TIME=3:48 min.
224
304
248
m/e
Relative Abudance
120
140
160
180
200
220
240
260
280
300

FIGURE 9.

FIGURE 10. Selective ion monitoring spectra of preparations from mutant CG-18 culture filtrate.

A. Selective ion intensity spectrum.
The number of ions detected is given for each peak reaching 100% on the relative abundance scale.

B. Selective ion mass spectrum.
The height of the ion peaks in the spectra indicate the relative abundance of one peak to that of the base peak.

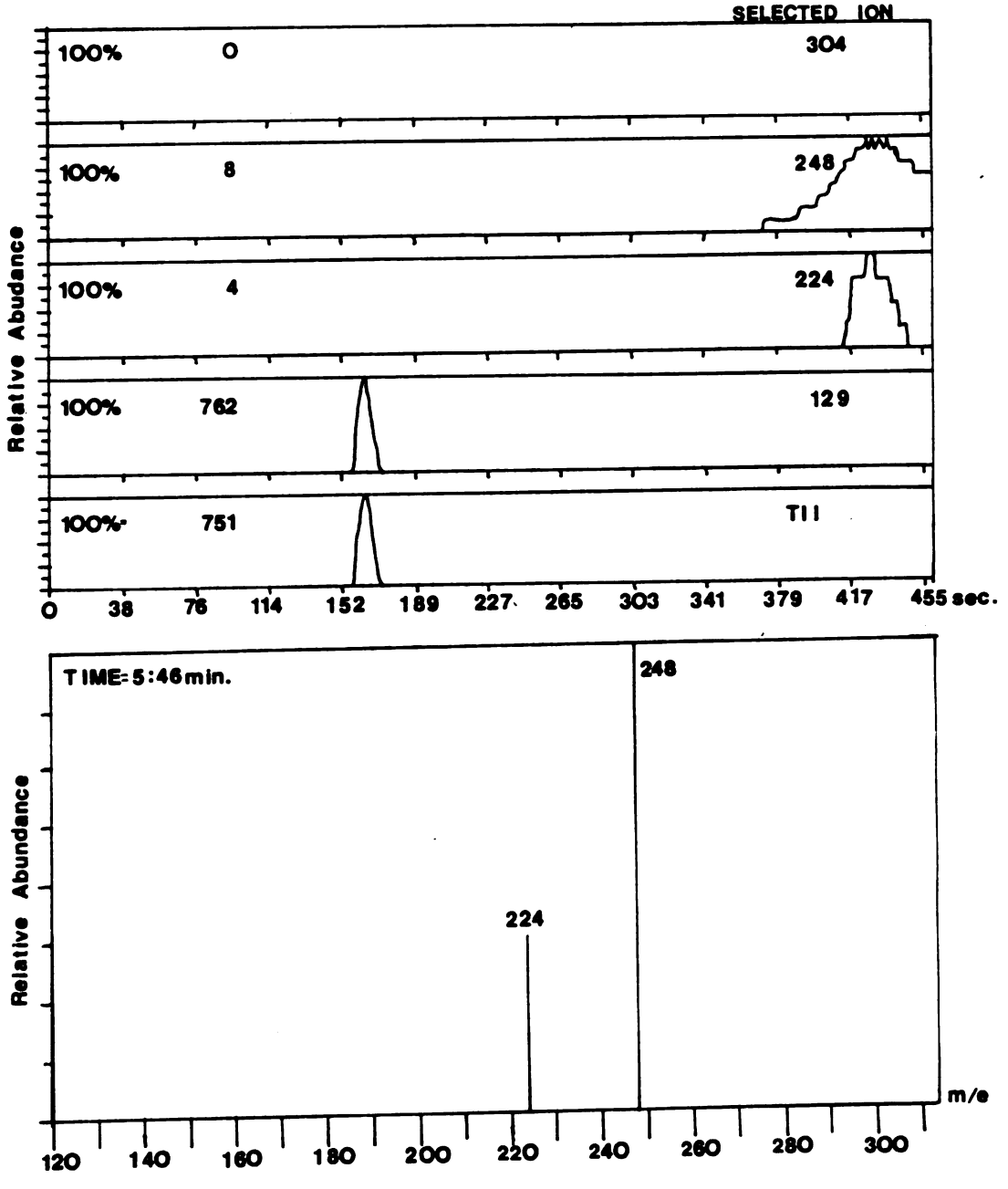
SELECTED ION
100%
0
304
100%
8
248
100%
4
224
100%
762
129
100%
751
TII
Relative Abudance
0
38
76
114
152
189
227
265
303
341
379
417
455 sec.
TIME=5:46min.
248
224
Relative Abundance
m/e
120
140
160
180
200
220
240
260
280
300

FIGURE 10.

GRA from m/e 129. The ratio of the number of ions of m/e 304 detected to the number of ions of m/e 224 detected was 1:2. This ratio was the same as the ratio for these same ions in the authentic GRA selective ion intensity spectrum. This further verified the presence of GRA in the culture filtrate extract of M-13. Figure 9B is the mass spectrum of the selected ions in the preparations from isolate M-13 culture filtrates at retention time of 3:48 minutes. The spectral fragment m/e 129 was not detected at this time. The base peak was m/e 224.

The ion intensities of the selected ions in the preparations from mutant CG-18 culture filtrates are seen in Figure 10A. There were no peaks belonging to GRA. The only spectral fragment present at the approximate retention time for GRA was m/e 129. The retention times for spectral fragments m/e 224 and m/e 248 were too long to belong to GRA. In addition, m/e 304 was not detected. Figure 10B is the spectrum of selected ions m/e 224 and m/e 248 at the retention time 5:46 minutes.

Ultraviolet spectral analysis of authentic GRA and the preparations from M-13 and CG-18 were obtained. Authentic GRA had a spectrum in MeOH of λmax 225 (34,000), 240 shoulder (20,000) and 300 (15,000) nm (Figures 11B and 12A) (Kobayashi and Ui, 1977b). The spectrum of the preparation from M-13 culture filtrate is shown on Figure 11A. The spectrum was similar to authentic GRA, although the 240 nm

FIGURE 11. Ultraviolet spectrum of isolate M-13 culture filtrate preparations (A) and authentic graminin A (B).

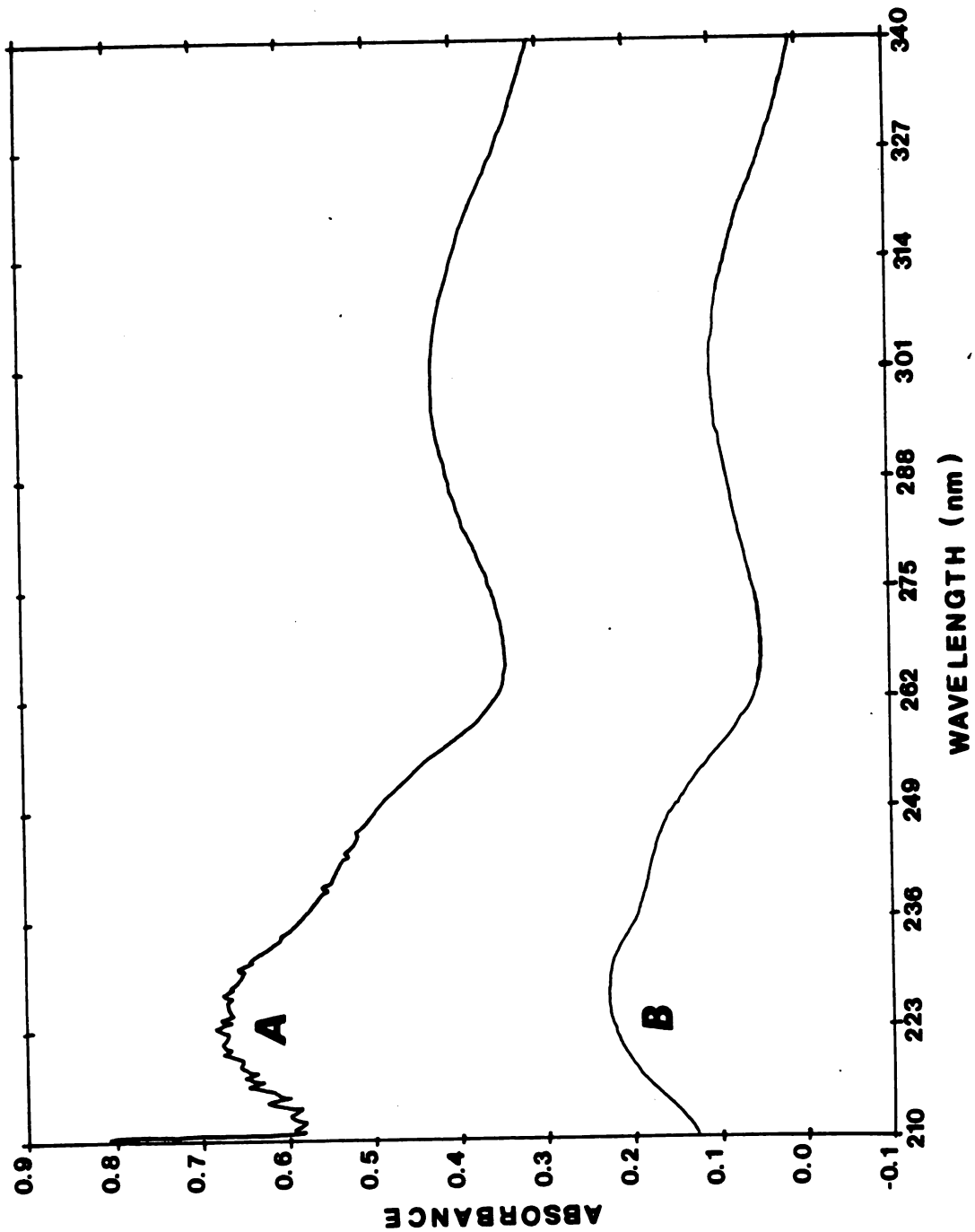
ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
A
B
210
223
236
249
262
275
288
301
314
327
340
WAVELENGTH (nm)

FIGURE 11.

FIGURE 12. Ultraviolet spectrum of authentic graminin A (A) and mutant CG-18 culture filtrate preparations (B).

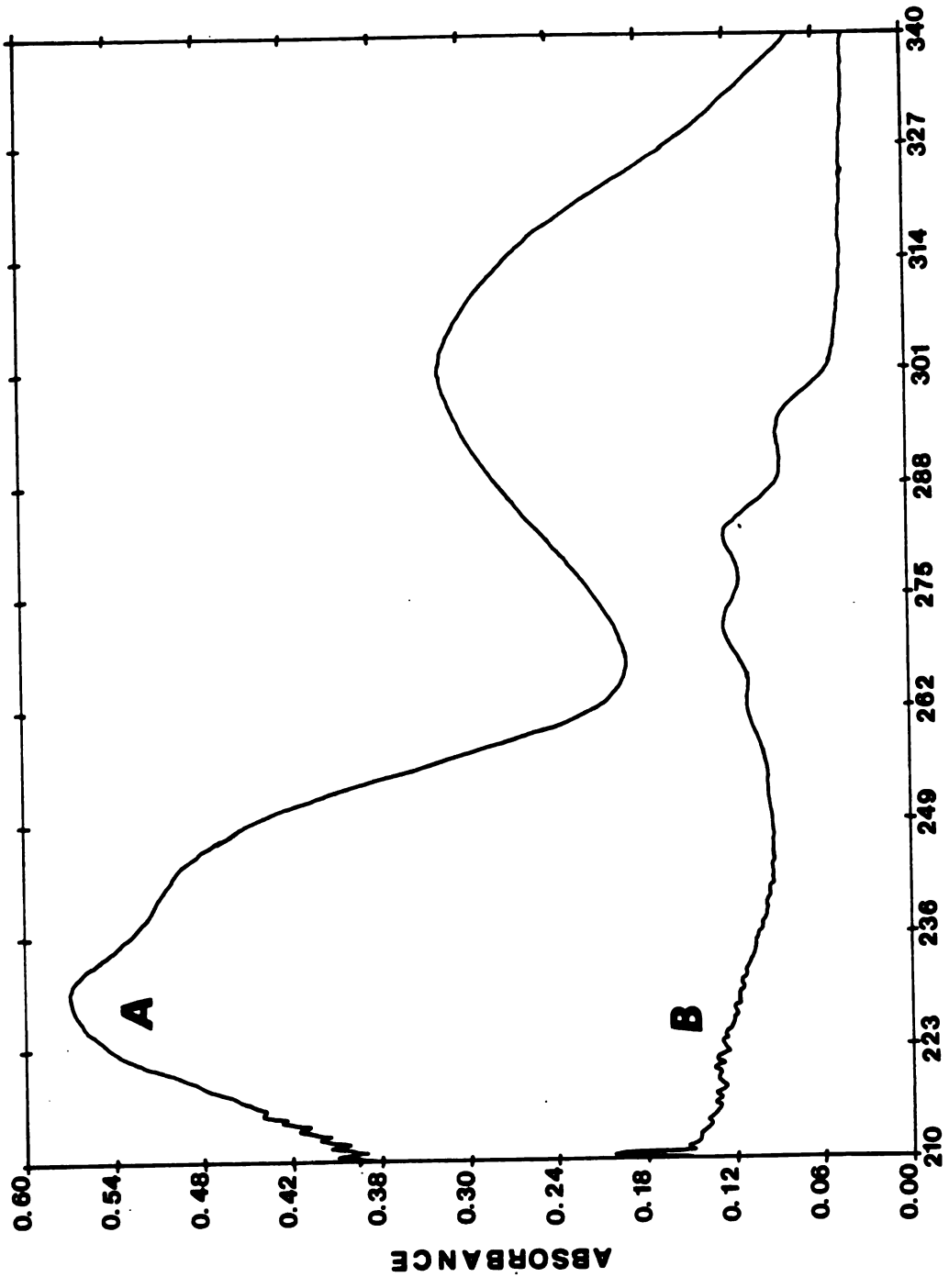
ABSORBANCE
0.60
0.54
0.48
0.42
0.38
0.30
0.24
0.18
0.12
0.06
0.00
A
B
210
223
236
249
262
275
288
301
314
327
340
WAVELENGTH (nm)

FIGURE 12.

shoulder was not as well defined. This spectrum further confirmed GRA production by M-13 in culture. Preparations from CG-82 culture filtrates were not examined by UV spectroscopy. The spectrum of the preparation from CG-18 is shown in Figure 12B and is not similar to that of authentic GRA or preparations from M-13. This spectrum further confirmed the lack of GRA production by CG-18 in culture. Preparations from other mutant culture filtrates were not examined by UV spectroscopy. The contaminating compound which produced the spectrum shown in Figure 12B absorbed UV light primarily between 260 and 300 nm.

Disc assay

The test organisms E. coli , Rhodotorula spp. or B. megaterium appeared to be insensitive to GRA because no zones of inhibition were observed in the filter disc or well assays. However, C. cucumerinum was sensitive to GRA. The zones of inhibition produced by GRA filled wells varied from 2-5 mm for 20 µg/20 µl 2% EtOH to 1-2 mm for 4 µg/20 µl 2% EtOH. Although distinct zones of inhibition were not observed when 2 or 3 µg GRA/20 µl 2% EtOH was placed in wells, growth of C. cucumerinum was faint. Zones of inhibition were also observed in the disc assay. The zones of inhibition were not as large as those produced by GRA filled wells.

The preparation from isolated CG-82 was the only one that inhibited C. cucumerinum in well assays. Zones of inhibition of 4 mm were observed when 20 µl of a 100 µl 2% EtOH-preparation was placed in a well. No zones of inhibition were found in plates overlaid with E. coli and Rhodotorula spp.. This indicated that the organisms are not insensitive to the contaminant found in the isolate and mutant preparations.

TLC Plate Assay

C. cucumerinum was inhibited by GRA spotted of chromatographed on TLC plates. Approximately one third of the inhibition was lost when authentic GRA solutions of known concentrations were chromatographed, versus those spotted after chromatography. This method of assaying for biological activity of GRA allowed the detection of activity when approximately 15 µg of GRA was chromatographed. Inhibition was incomplete when 15 µg GRA was chromatographed and complete when 25 µg GRA was chromatographed.

The growth of C. cucumerinum was almost always inhibited at the R_f value for authentic GRA when preparations from CG-82 and M-13 were chromatographed on TLC plates. Inhibition was also observed when extracts were spotted, but not chromatographed. There was some inhibition by other compounds in semi-purified toxin preparations at R_f values different from that of authentic GRA. The growth of C.

cucumerinum was never inhibited at the R_f value of authentic GRA when preparations from mutant culture filtrates were run on TLC plates. Again this indicated the insensitivity of C. cucumerinum to the contaminant which comigrated with GRA on TLC plates and in GC columns.

Leaf-sheath assay

The results of the leaf-sheath assay (Table 7) are representative of all experiments using known or unknown volume uptake of GRA. Symptoms were rated for each toxin application in each experiment as it compared to the control (2% EtOH). No distinctive differences in symptom production were seen among toxin treatments in both experiments. Symptom production for each toxin treatment was variable. In one experiment the 25 µg toxin treatment produced most severe symptoms, chlorosis and wilt. In the other experiment, this treatment produced no symptoms. In both experiments the control (2% EtOH) exhibited more severe symptoms than some of the toxin treatments.

Seedling-flask assay

Root tip and overall root browning was observed with all concentrations of GRA applied to agar surfaces. The actual concentrations of GRA per ml PDA + str were 1.25, 2.5, 5 and 10 µg GRA. Generally, the higher concentrations of GRA produced more root necrosis and browning. Not all seedling roots treated with a final concentration of 2.5 or 1.25

µg GRA/ml agar became brown. The addition of EtOH solutions (2, 10, 20, 40%) to the agar surface in flasks with Yorkstar seedlings caused no root tip or general root browning.

Inhibition of seed root growth

In seed germination experiments (method of Pringle and Braun, 1957) GRA concentrations of 25, 100 and 200 µg/ml 2% EtOH significantly inhibited root elongation when compared to controls (2% EtOH) (Table 8). In another experiment using this same method (data not shown) only the 25 µg GRA treatment differed significantly from the control. In this case, the mean root length was increased as compared to that of the control. Inhibition of wheat seedling germination and cress seed germination of GRA (method of Tang and Young, 1982) was not observed (Table 8). Although the mean root growth of wheat seedlings for the 10 and 4 µg GRA treatments was less than that of the control, these treatments did not differ significantly.

Leaf-puncture assay

No symptpms appeared 24 hours after placing GA on wounds made in cut wheat leaves. There were still no symptoms present after 3 days incubation. The actual amounts of GRA applied as 5 µl volumes to punctures were 1, 0.05, 0.25 and 0.125 µg of GRA.

TABLE 7

Leaf-sheath assay symptoms for Yorkstar 3 days after treatment with Graminin A[1,2]

	Symptoms Produced[3] Toxin Treatment (µg GRA/100 µl 2% EtOH)				
Experiment	100	50	25	0	water
1	c,w	lc	c,w	c	c
2	lc,w	lc	-	c	lc

1. Data is representative of all experiments using known of unknown volume uptake of GRA. Data from experiment of April, 1983.

2. The mean gram weight of 5 cuttings of Yorkstar at the time of the leaf-sheath assay was 0.1577 g.

3. Symptoms were scored for each toxin treatment in each experiment as compared to the control (2% EtOH):

   c - chlorosis, lc - light chlorosis
   w - wilt, (-) - no symptoms.

TABLE 8

Effect of graminin A on root growth by seeds of wheat and cress

g GA/ml 2% EtOH	Mean Root Length (cm) Wheat (P + B)[1]	Wheat (T + Y)[2]	Cress (T + Y)[2]
200	1.30*[3]	ND[4]	ND
100	1.38*	3.34	1.61
50	1.96	3.56	1.92
30	ND	3.04	1.40
25	1.58*	ND	ND
12.5	3.14	ND	ND
10	ND	1.91	ND
4	ND	1.89	ND
0	2.78	3.26	1.34

1. Method of Pringle and Braun, 1957.

2. Method of Tang and Young, 1982.

3. Mean root lengths with an asterick differ significantly ($p = 0.05$) from the control (2% EtOH) within each experiment as determined by Dunnett's test (Steele and Torrie, 1980).

4. ND - not determined.

FIGURE 13. Electrolyte leakage of leaves (A) and sheaths (B).

Wheat leaf and sheath pieces from 12-day-old wheat seedlings were infiltrated with authentic graminin A for 1 hour. Conductivity (μmhos) readings were made at 0.05 hours and hourly up to 12 hours. Readings were made sporadically up to 46.5 hours.

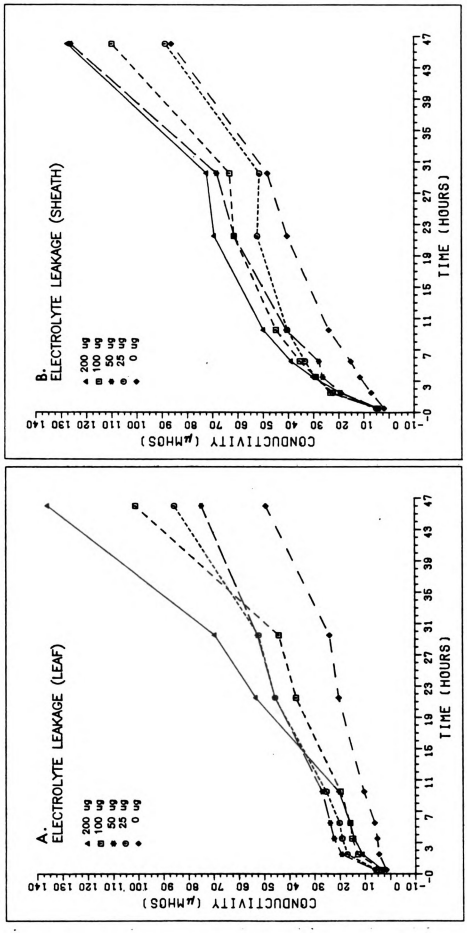
A.
ELECTROLYTE LEAKAGE (LEAF)
200 ug
100 ug
50 ug
25 ug
0 ug
CONDUCTIVITY (μMHOS)
TIME (HOURS)
B.
ELECTROLYTE LEAKAGE (SHEATH)
200 ug
100 ug
50 ug
25 ug
0 ug
CONDUCTIVITY (μMHOS)
TIME (HOURS)

FIGURE 13.

Electrolyte leakage

Results of an electrolyte leakage experiment are shown in Figure 13. The only large difference in rate of electrolyte leakage was observed between the leaf piece toxin treatments of 200 µg GRA and the control (2% EtOH). In other experiments using only leaf tissue (data not shown) there were no differences. No significant differences in electrolyte leakage were observed between the slopes of individual regression lines for each treatment when compared to the control.

DISCUSSION

CLS is a serious vascular disease of winter wheat that produces symptoms suggesting the involvement of xylem plugging compounds, such as polysaccharides, or the action of toxins. This work attempted to elucidate the role of extracellular polysaccharides and a toxic secondary meatabolite, graminin A, in pathogenesis. Graminin A, described by Kobayashi and Ui (1979) was shown to affect stomate function (Creatura et al., 1981). My goal was to isolate avirulent mutants of the previously mutated isolate CG-18. CG-18 was selected because it exhibited a yeast-like growth habit on PDA and was still pathogenic, although somewhat reduced in virulence. The yeast-like morphology enabled the screening of several colonies/plate without the colonies growing into

each other.

The mutants N20, E53, E67 and 7-54 were selected from hundreds of isolates screened in the seedling assay because they consistently exhibited virulence patterns different from their parent, CG-18. E53 and E67 appeared to be stable avirulent strains which can be recovered from inoculated plants. 7-54 is a methionine auxotroph, exhibiting reduced virulence, which can also be recovered from inoculated plant tissue. N20 exhibited increased virulence as compared to the parent isolate, CG-18.

GRA was produced in culture only by the pathogenic isolates CG-82 and M-13, but not by the pathogenic isolates CG-18 and N20. Therefore, it is not a pathogenicity factor. The role of GRA as a virulence factor is questionable since CG-18 and N20 were moderatley to highly virulent but did not produce GRA in culture.

It is possible that GRA was not detected in culture filtrate extracts of CG-18 and CG-18 derived mutants because large enough volumes of filtrate were not extracted. One might expect abundant production of a toxic metabolite possessing the weak biological activity of GRA if the compound were involved in pathogenesis. As is the case with the study of production of any compound <u>in vitro</u> , there is no certainty that production of toxin <u>in vitro</u> will be equivalent to toxin production <u>in vivo</u>.

Elimination of the toxin by mutation was not specific, as the dose of UV light which produced the original yeast-like mutant CG-18 also eliminated toxin production. As GRA is a secondary metabolite, a mutation in primary function could lead to the loss of toxin production. The low virulence of mutants E53 and E67 and the high virulence of mutant N20 may in fact be due to mutations in pathogenicity genes. The possibility exists that mutagenesis could have eliminated the production of another unidentified toxin(s) or factors involved in pathogenesis.

An incompatible interaction between host and parasite may be considered to result from the interaction of the product of the pathogenic Px gene with the product from the corresponding host plant's Rx gene (Ellingboe, 1979). The mutant N20 is of interest because its increased virulence may represent a change in function of a Px gene product. Similar support was obtained in work with constituative mutations to increased virulence in Phyllosticta maydis (Gabriel et al., 1977). The decrease in virulence of mutants E53 and E67 may represent the epistasis of one parasite/host gene pair specifying incompatibility to all parasite/host gene pairs which would give a compatible relationship.

This hypothesis may or may not be extended to the mutant 7-54, a methionine auxotroph. Revertants were not

obtained, therefore it was not determined if the loss of virulence was accounted for by the nutritional requirement. The difficulty in obtaining prototrophic revertants suggests that a deletion was responsible for auxotrophy. Mutant 7-54 was not included in tests for the production of polysaccharide and toxin in culture.

The relative spore production of isolates were similar and could not account for differences in virulence. The high inoculum density used for seedling inoculation reinforces the credibility of the virulence levels obtained for the isolates. Mathre et al. (1977) speculated that inoculum levels in the field were 10^3 conidia/ml.

The extent of vascular colonization may relate to an isolates' virulence. The reduced virulence mutants, E67 and 7-54, were isolated proximal to the point of inoculation; the roots. The most virulent isolates, CG-82 and M-13, could be isolated distal from the point of inoculation, the third leaf. There are no reports which describe the spread of C. gramineum within wheat tissue, although it may be assumed that conidia move with transpirational flow. Microscopic examination of conidia within seedlings inoculated with a wild type isolate showed the fungus to be widely distributed in wheat prior to symptom development (Wiese, 1972). Growth kinetic studies of wild type isolates and mutants may yield some answers. A preliminary study of the growth kinetics of CG-18 and 7-54 indicated the generation

time of CG-18 to be approximately 10 hours and that of 7-54 to be approximately 12 hours in PDB (Appendix G).

The colony morphology expressed by the isolates probably has little to do with virulence. Wiese (1972) observed C. gramineum to be primairly in conidial form in proto- and metaxylem leaf vessels. In addition, the mycelial growth habit of CG-82 and M-13 on WA and MA appeared in contrast to the reported growth habit within the plant.

The pathogenic, yeast-like mutant CG-18 can overwinter in the field; it was reisolated from both early and late season plants with symptoms. The methionine mutant 7-54 was not reisolated and there was no infection by naturally occuring C. gramineum with mycelial growth habit. The inability of 7-54 to overwinter and produce infection in the field may be due to its nutritional requirement. The overwintering and subsequent infection of plants by E67 is open to conjecture since mycelial isolates were found in field plants inoculated with E67. There was a possibility that E67 reverted to mycelial colony morphology while in the field, however, this would be considered a rare event. Low virulence of the mycelial isolate from E67 inoculated plants in the seedling assay would indicate this. It is more probable that the C. gramineum isolate might be a result of natural infestation or contamination from M-13 infested oat inoculum in an adjacent row. The C. gramineum isolated from the control row could also be from natural infection or

contamination by CG-82 infested oat inoculum in an adjacent row.

The lack of correlation between polysaccharide prodcution and virulence did not eliminate the involvement of polysaccharide in pathogenesis. As is the case with toxin production *in vitro*, there is no certainty that production *in vitro* will be equivalent to production *in vivo*. Polysaccharide has been implicated in the development of CLS by plugging xylem and increasing viscosity of xylem sap (Spalding et al., 1966). As polysaccharide was not recovered from noninfected winter wheat tissue, Pool and Sharp (1969a) suggested it may contribute to the disease syndrome by being one of the major factors restricting fluid movement in infected plants. Most polysaccharides which have been shown to be involved in disease development are associated with vascular wilt diseases (Dimond, 1972). Although *C. gramineum* is a vascular pathogen, wilt is not one of the symptoms generally associated with the disease.

GRA may be involved in the more rapid colonization of the plants or of the production of severe symptoms. Both CG-82 and M-13 infection resulted in death of leaves on whole plants more often than infection by other fungal isolates, thereby achieving a very high disease severity rating. Perhaps GRA is involved in stressing the plant and providing the high virulence associated with CG-82 and M-13. GRA can not be consideried the disease determinant

responsible for the diagnostic striping symptoms of CLS as the isolate CG-18 produced the most pronounced striping in the seedling assay but did not produce GRA in culture.

The GC/MS results nullified the results obtained by conventional GC. A compound that cochromatographed with GRA on the GC apparently was a contaminant and was mistakenly assayed as GC in earlier results. The nature of the contaminating compound which cochromatographed with GRA is not known. This contaminant was thought to be GRA when it was first seen on TLC plates, because of its common R_f value and its absorption of shortwave UV light, but not longerwave UV light. Quite often compounds which cochromatograph are isomers and difficult to separate. There were, however, no spectral fragments in the mass spectra of CG-18 and mutants that would indicate the contaminant was an isomer of GRA, ie. the same molecular ion. In addition, the UV spectra of the two compounds were quite different. Since the contaminant inhibited none of the test organisms in the disc assay, the biological activity of the GRA present in culture filtrate preparations of isolates CG-82 and M-13 could be ascertained using C. cucumerinum as the test organism. Biological activity was present in the preparations from isolate CG-82 but not in the preparations from isolate M-13. As the amount of GRA in these extracts was not quantified it was not known if enough of the preparation from M-13 culture filtrates was added to the wells to detect inhibition. The

lowest concentration of authentic GRA producing zones of inhibition was 4 µg GRA/20 µl 2% EtOH.

The disc assay was a more sensitive method of detecting biological activity of GRA than was the TLC plate assay. Using the TLC plate assay inhibition of growth of C. cucumerinum was seen with 10 µg or greater GRA. Since mutant CG-18 or other mutants did not produce GRA, it is now obvious why inhibition was never detected when culture filtrate preparations were developed on TLC plates. In contrast to the disc assay, the inhibition of C. cucumerinum by culture filtrate extracts of M-13 on TLC plates was observed. Kobayashi and Ui (1979) reported the inhibition of growth of B. subtilis (<25 µg GRA/ml) and E. coli (>200 µg GRA/ml). Perhaps the inhibition of growth of E. coli by GRA was not detected in the disc assay as not enough GRA was used.

In contrast to reports by Kobayashi and Ui (1979), GRA did not produce browning of leaves and vascular tissues of wheat cuttings at high or low concentrations. However, chlorosis of leaf-sheath tissue exposed to GRA was seen. Leaf-sheath tissue exposed to 2% EtOH and water also became chlorotic after 3 days. The major distinction between the toxin treated tissues and the control tissues was wilting and leaf drying often associated with toxin treated tissue. Chlorosis was not observed when leaves without intact sheaths were assayed in this manner. Apparently, intact

sheaths were necessary for the observation of the toxic action of GA in the leaf-sheath assay. Toxin application did not reproduce the typical chlorotic striping symptoms of CLS in excised leaves, possibly because the entire vascular system was exposed to GRA.

The remaining bioassays were performed in an attempt to find an assay, more sensitive and less subjective than the leaf-sheath assay, for phytotoxicity. Most of the bioassays were selected because they are commonly used to indicate the phytotoxic action of a suspected toxin, and can be used to screen plant lines for resistance to disease. However, the leaf-sheath assay was the only assay in which GRA consistently exhibited phytotoxicity.

The seedling-flask assay was developed to examine the effect of GRA on root tissue in young seedlings. Although browning of roots and root tips was observed, the assay required too much preparation to be practicle. Contammination of seedling cultures was very high as the large amount of seed pubesence prevented complete surface sterilization. An assay using roots and observing the time required for root hair cell death after toxin treatment might differentiate resistant and susceptible wheat lines. Root hair cell death could be determined by microscopic observation after roots were stained with a vital stain.

Inhibition of seed germination provided an assay for phytotoxicity, but inhibition of root elongation was

inconsistent. Immersing the germinated wheat seeds in toxin solutions (Pringle and Braun, 1937) provided inhibition of root elongation, but results differed in two separate experiments using this method. Nonhomogeneity of wheat seed could be partially responsible for this inconsistency. The lack of phytotoxicity of GRA impregnated filter discs (Tang and Young, 1982) may have been due to the insolubility of GRA in water or binding of GRA to the cellulose ot the paper.

The leaf-puncture assay used to distinguish sugarcane clone sensitivity to Helminthosporium sacchari toxin (Steiner and Byther, 1971) was adapted to wheat. As little as 58 ng of H. sacchari toxin produced runner lesions on susceptible sugarcane leaves (Steiner and Strobel, 1971). The highest amount of GRA applied to wheat leaf punctures was 1 µg GRA. Perhaps greater quantiites were needed to observe toxic action. Additional problems with this assay were the incomplete uptake of the toxin droplet into the puncture and the inability to quantify actual toxin uptake.

The electrolyte-leakage assay would have provided the best assay for quantitative work with authentic GRA preparations. It does not require visual assessment and is the most rapid of the assays discussed. There was no difference in the rate of toxin-induced leakage among all the various concentrations of toxin. Measurement of electrolyte leakage from sheaths was examined because of the necessity for in-

tact leaf-sheaths in the leaf-sheath bioassay. Although the slopes of the lines often appeared different, linear regression analysis did not demonstrate any significant differences. It can be concluded that the plasmalemma of the host is probably not a site of action for GRA.

Phytotoxic activity of GRA was shown by Creatura et al. (1981) . In her study stomates of toxin treated leaves were to open wider and respond more slowly to water potential changes than stomates not treated with the compound. She showed differences in stomatal activity were not a function of the leaf water status. In light of her work, it would be of interest to see if GRA has the same action on guard cells as does fusicoccin (Turner, 1973). Fusicoccin is a toxin produced by Fusicoccum amygdali which opens stomates both in the light and in the dark in a wide range of species (Turner and Graniti, 1969).

In an attempt to determine the role of GRA in pathogenesis, the guidelines proposed for the evaluation of the significance of toxins in disease were followed (Rudolf, 1976, Yoder, 1980). A summary of the results based on commonly used criteria follows. Toxin was not isolated from diseased tissue (Kobayashi, 1980), although Kobayashi (Kobayashi and Ui, 1979, Kobayashi, 1980) reported reproduction of typical disease symptoms when toxin was applied to healthy plants or excised leaves. In contrast, the reproduction of typical disease symptoms in the leaf-sheath assay

was not found in this study. There was no correlation of virulence with the ability of the fungus to produce the toxin in culture. Elimination of the toxin from the biolocical system did not eliminate pathogenicity or greatly affect virulence. Although GRA has antimicrobial and phytotoxic activity, based on this study it does not appear to be a significant determinant of disease.

PART II: SCREENING WHEAT LINES FOR RESISTANCE TO *CEPHALOSPORIUM GRAMINEUM* WITH GRAMININ A AND WITH ISOLATES VARYING IN VIRULENCE

INTRODUCTION

Cephalosporium leaf stripe (CLS), caused by Cephalosporium gramineum , is the only known vascular pathogen of wheat. Presently, resistance in Triticum aestivum L. has not been identified and field tolerance has only been observed in a few lines (Mathre et al., 1977). Using fungal inoculum in field plots Mathre et al. (1977) observed variation in yield components (seed size, kernel weight, and seed number per head) in different wheat lines. Morton and Mathre (1980a) suggested that the intercrossing of wheat lines showing some tolerance in yield components may give rise to lines with resistance to both decreased kernel weight and decreased seed number per head. They suggested this could be an effective means for identifying and evaluating resistance in infected plants of winter wheat germplasm lines. Morton and Mathre (1980b) also identified three types of resistance to CLS: 1) a reduction in the number of diseased plants in a population; 2) a reduction in number of diseased tillers within a plant; and 3) a reduction of the rate and severity of disease symptom development within a plant. With type 1 resistant wheat varieties symptoms appear when the pathogen is inoculated above the root.

Screening plant lines for reistance to disease with toxins has been performed primarily with host-specific toxins. Wheeler and Luke (1955) used HV toxin to screen for

victoria blight resistance in oat lines. Schertz and Tai (1969) used a toxin isolated from Periconia circinata to identify resistance in sorghum. A procedure for identifying resistant clones of sugarcane to eyespot disease was developed using the host-specific toxin isolated from Helminthosporium sacchari (Steiner and Byther, 1971). Graminin A (GRA), a toxic metabolite of C. gramineum , has been shown to possess phytotoxicity and to be specific for wheat (Kobayashi and Ui, 1979), and thus may be useful in screening winter wheat lines for resistance to CLS.

The purpose of this study was to screen wheat lines for resistance to C. gramineum by fungal inoculation and to determine if the use of GRA to identify resistant germplasm is a feasible alternative to inoculation with the pathogen. This was approached by screening, in the seedling stage, wheat lines known to be susceptible or tolerant in the field, for disease severity in a pathogenicity assay. The same wheat lines were also examined for the extent of chlorosis and wilting in a toxin-leaf-sheath assay.

MATERIALS AND METHODS

Isolates of Cephalosporium gramineum

These are described in Table 9. Cultures of C. gramineum were grown and maintained on potato dextrose agar (1.5% agar) plus streptomycin (100 µg/ml) (PDA + str), or

grown in potato dextrose broth (PDB) (Tuite, 1969).

TABLE 9. Isolates of Cephalosporium gramineum

Isolate	Virulence	Source
CG-82	very high	Michigan field isolate
M-13	very high	Michigan field isolate
CG-18	moderate	UV mutant of M-13*
N20	high	NTG mutant of CG-18
E53	low	EMS mutant of CG-18
E67	very low	EMS mutant of CG-18

*Fulbright and Ravenscroft, 1981

Toxin

Refer to Part 1.

Plants

Winter wheat lines, other than Yorkstar, were provided by D. E. Mathre, Montana State University, Bozeman, MT 59717. Wheat lines are described in Table 10.

Table 10

Wheat lines and their disease reaction to *Cepahalosporium gramineum* in the field[1]

Wheat Line	Disease Reaction[2]
Yorkstar	38, 44
Marias	28
Agrotritichum	0
CI07638	4
CI11222	5
PI178383	13
F6-870	14
PI278212	21
Lenore	14
UT89099	10
PI347738	43
MT77077	28
LRC 40	30
Lancer	34
PI094424	42

1. Disease reaction for the 1980-81 growing season, Michigan State University Field Plots. Personal communication, A. Ravenscroft.

2. Percentage of tillers with striping symptoms at growth stage 5 on the Feekes' scale. Stage 5 is characterized by strongly erect pseudo-stems formed by sheaths and leaves.

Seedling assay

Assays were performed as described in Part 1. The screening procedure for the seedlings was repeated 3 times. Data from the 1st replication were eliminated as the symptom rating values varied significantly from the 2nd and 3rd replications. The experiment was set up and analyzed as a factorial design (Steele and Torrie, 1980) with wheat lines and isolates as factors with two levels of the isolate factor: nonautoclaved and autoclaved shake cultures. A total of 15 wheat lines and 7 isolates, including a PDB control, were used.

Another experiment used millipore filtered (0.45 µm filters) and autoclaved shake cultures was performed. Analysis was similar to the above analysis. The symptom rating (1-15) (Part 1, Table 2) for each individual plant was transformed to the disease severity index (6-100) by multiplying by 6.66. Each plant was considered a replicate, making a total of 6 replicates per treatment type.

Leaf-sheath assay

The leaf-sheath assay was performed as described in Part 1. The assay was performed 3 times by placing cut wheat seedlings in 1 ml solutions of toxin of different concentrations. The assay was performed twice by placing cut wheat seedlings in 100 µl solutions of toxin of different concentrations, allowing the solution to be taken up and

then filling the vials with distilled water. Fresh weights prior to toxin treatment of five cuttings (14-day-old) for each wheat line were averaged to yield a mean fresh weight for each wheat line to which known concentrations of toxin were applied.

RESULTS

Disease severity of wheat lines inoculated as seedlings with isolates of Cephalosporium gramineum

Disease severity of wheat lines inoculated with wild type isolates and with mutants is summarized in Table 11 and Figures 14 and 15. For all wheat lines except LRC 40 treatment with mutant E67 did not differ significantly from PDB. This holds true for the mutant E53 in every wheat line except Yorkstar, Marias, PI178383, LRC 40 and PI094424. Mutant N20 often appeared to be more virulent than the isolate CG-18, but the difference may not have been significant. Isolate CG-82 was usually the most virulent, but it may not have differed significantly from isolate M-13 and mutants CG-18 and N20. Isolate M-13 was more severe than siolate CG-82 on the wheat line Yorkstar, F6-870 and PI094424, but not significantly so on wheat line PI094424. Agrotritichum (a cross between Agropyron elongatum and an unknown hard, red winter wheat) is resistant to C. gramineum (personal communication, Mathre, Table 10).

TABLE 11. Disease severity of winter wheat lines UT89099, Lenore, PI347738, MT77077, LRC 40, Lancer and PI09924 inoculated as seedlings with isolates of Cephalsoporium gramineum.

Inoculated seedlings were incubated in the growth chamber for 14 days before assessing disease severity. Isolates with the same letter do not differ significantly ($p = 0.05$) within a wheat line according to the LSD test (Steele and Torrie, 1980).

TABLE 11

Disease Severity of Wheat Lines[1]

Isolate	UT89099	Lenore	PI347738	MT77077	LRC 40	Lancer	PI094424
CG-82	73.26 c	96.00 e	88.25 c	78.79 d	80.49 c	66.03 c	36.86 b
M-13	64.37 c	55.51 d	71.59 c	56.47 cd	42.19 b	35.53 b	79.92 d
CG-18	35.23 b	40.49 bcd	72.71 c	58.81 cd	62.17 bc	43.30 b	36.06 b
N20	36.62 b	42.73 cd	41.09 b	45.52 bc	69.36 c	37.18 b	34.43 b
E53	9.42 a	21.65 abc	26.07 ab	24.97 ab	61.04 bc	14.42 ab	63.27 c
E67	31.07 ab	8.89 a	28.30 ab	17.77 a	63.80 bc	11.12 a	21.11 ab
PDB control	8.89 a	17.78 ab	9.99 a	6.66 a	6.66 a	9.99 a	6.66 a

1. Isolates with the same letter do not differ significantly (p = 0.05) within a wheat line according to the LSD test (Steele and Torrie, 1980).

FIGURE 14. Disease severity of winter wheat lines Yorkstar, Marias, Agrotritichum and CI07638 inoculated as seedlings with isolates of Cephalosporium gramineum.

Inoculated seedlings were incubated in the growth chamber for 14 days before assessing disease severity. Isolates with the same letter do not differ significantly ($p = 0.05$) within a wheat line according to the LSD test (Steele and Torrie, 1980).

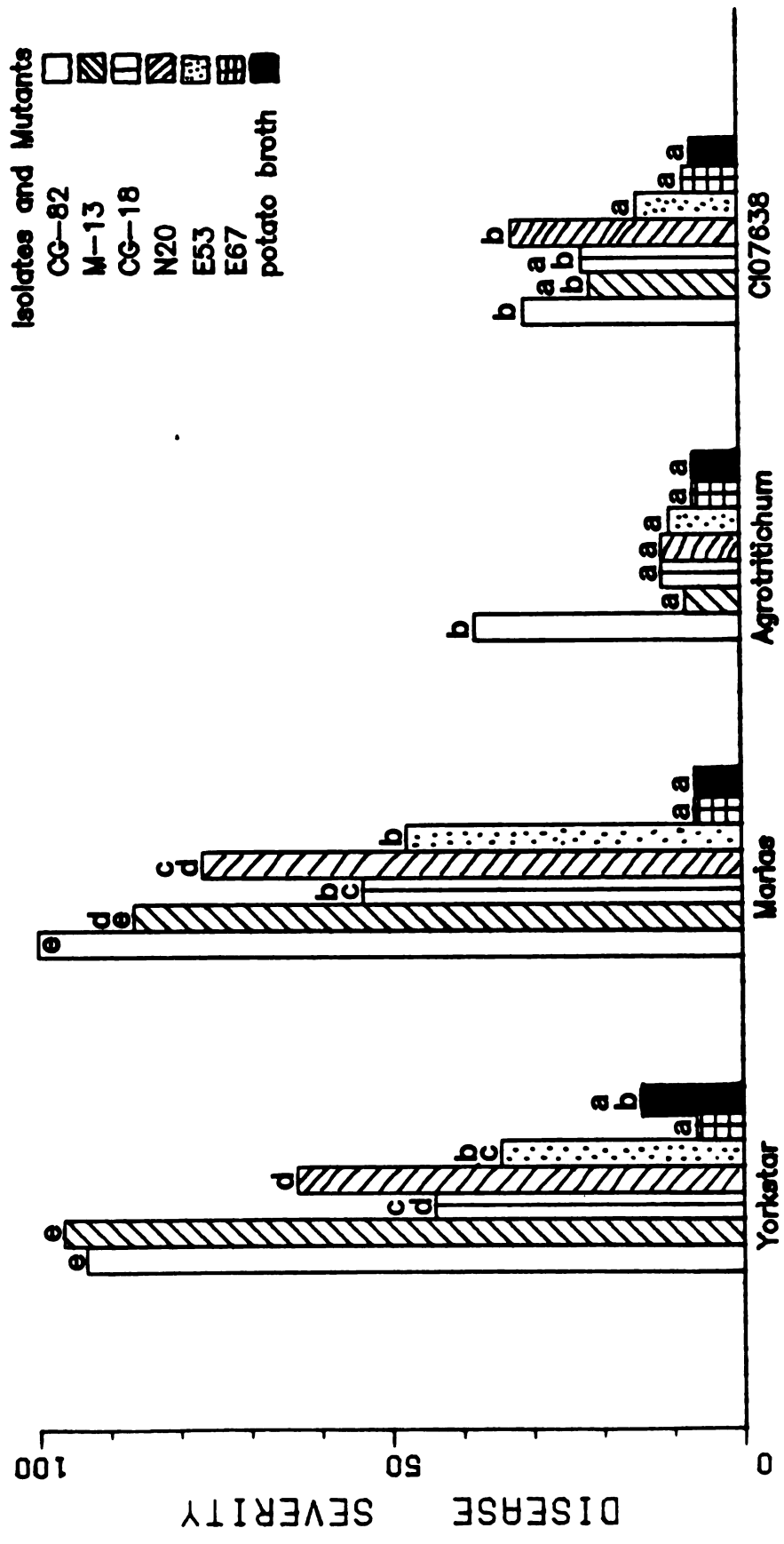
Isolates and Mutants
CG—82
M—13
CG—18
N20
E53
E67
potato broth
DISEASE SEVERITY
100
50
0
WHEAT LINES
Yorkstar
Marfax
Agrotriticum
C107638

FIGURE 14.

FIGURE 15. Disease severity of winter wheat lines CI11222, PI178383, F6-870 and PI278212 inoculated as seedlings with isolates of Cephalosporium gramineum.

Inoculated seedlings were incubated in the growth chamber for 14 days before assessing disease severity. Isolates with the same letter do not differ significantly (p = 0.05) within a wheat line according to the LSD test (Steele and Torrie, 1980).

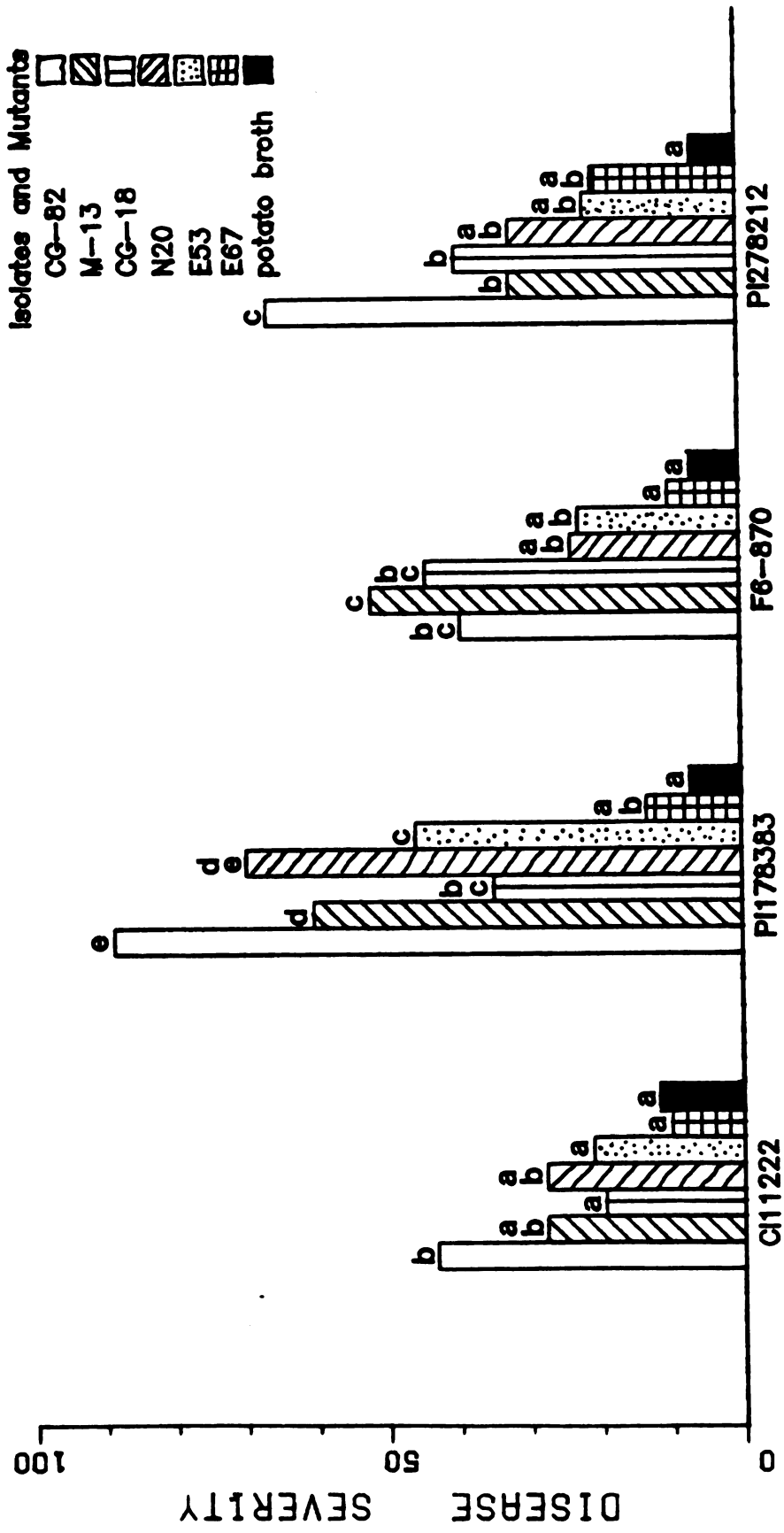
Isolates and Mutants
CG-82
M-13
CG-18
N20
E53
E67
potato broth
DISEASE SEVERITY
100
50
0
WHEAT LINES
CI11222
PI178383
F6-870
PI278212

FIGURE 15.

Table 12

The effect of autoclaving and millipore filtering isolate shake suspensions on disease severity in winter wheat lines Yorkstar and Agrotritichum.

Wheat Line - Yorkstar	Disease Severity[1] Isolate Preparation		
Isolate	Untreated[2]	Millipore Filtered[2,3]	Autoclaved[2]
CG-82	82.14 e	31.08 cd	13.32 ab
M-13	79.92 e	23.31 bc	19.98 abc
CG-18	39.96 d	13.32 ab	13.32 ab
PDB	13.32 ab	13.32 ab	6.66 a

Wheat Line - Agrotritichum	Disease Severity[1] Isolate Preparation		
Isolate	Untreated[2]	Millipore Filtered[2,3]	Autoclaved[2]
CG-82	13.32 a	12.21 a	6.66 a
M-13	19.98 a	13.32 a	6.66 a
CG-18	15.54 a	6.66 a	6.66 a
PDB	6.66 a	6.66 a	6.66 a

1. Values followed by the same letter do not differ significantly ($p = 0.05$) within each wheat line according

Table 12 con't.

to the LSD test (Steele and Torrie, 1980).

2. Each value is the mean of disease severity rating of 6 plants.

3. Shake cultures were centrifuged and millipore filtered (0.45 µm filters).

The data in Table 11 and Figures 14 and 15 indicate that mutants E67 and E53 cause mild disease. Mutants N20 and isolate CG-18 cause moderate disease, and the isolates M-13 and CG-82 cause severe disease. Autoclaving and millipore filtering shake cultures removed compounds possibly active in disease development (Table 12). The data presented are representative of all wheat lines.

The effect of graminin A on wheat lines as determined by the leaf-sheath assay

The effect of GRA on winter wheat lines is presented in Table 13. The data are representative of all experiments using known or unknown volume uptake of GRA. Symptoms were rated for each wheat line toxin application as compared to the contol (2% ethanol (EtOH)) application for that wheat lines. Little difference between symptoms produced by the toxin applications could be seen in each individual wheat line. The lines Agrotritichum, CI07638 and F6-870 appeared to be the least affected by the toxin when very chlorotic and dry leaves were considered the most severe symptoms produced by the toxin. The wheat lines Marias and PI178383 appeared to be the most affected by the toxin. PI178383 expressed wilting and drying of leaves. The gram fresh weight of cut seedling tissue to which toxin was applied did not appear to influence symptom production. A distinctive difference in symptoms produced by the toxin could not be seen

Table 13

Leaf-sheath assay symptoms: The effect of graminin A[1] on cut seedlings of 14-day-old winter wheat lines

	Symptoms Produced[2] Toxin Treatment (µg GRA/100 µl 2% EtOH)				
Wheat Line	100	50	25	0	water
Yorkstar (0.1577)[3]	c,w	lc	c,w	c	c
Marias (0.2197)	c,d	c,d	lc,w	lc,w	w
Agrotritichum (0.1495)	c,w	lc,w	-	lc	-
CI07638 (0.1488)	c,w	lc,w	w	w	-
CI11222 (0.2415)	c,pd,w	c,w	c,w	lc,w	w
PI178383 (0.1691)	c,d	d	lc,w	lc,pd	w
F6-870 (0.1691)	c,w	lc,w	w	w	w
PI278212 (0.2195)	c,pd,w	c,w	c,w	c.w	lc,w

1. Data are representative of all experiments using known or unknown volume uptake of graminin A. Data from experiment of April, 1983.

2. Symptoms were scored for each wheat line toxin treatment as compared to the control (2% EtOH):

Table 13 con't.

vc - very chlorotic, c - chlorotic, lc - light chlorosis, w - wilt, pd - partially dried leaves, d - dried leaves, (-) - no symptoms.

3. Mean gram weight of 5 cut seedlings for each wheat line at the time of the leaf-sheath assay.

among wheat lines, except for the wilting and drying of leaves of PI178383.

DISCUSSION

Differences in resistance among wheat lines were identified by inoculation with several different isolates of the fungus. Although inoculation to determine disease resistance to C. gramineum in winter wheat has been used before (Mathre and Johnston, 1975a; Mathre et al., 1977) its use has been limited to vernalized winter wheat seedlings in the field. The use of nonvernalized winter wheat plants for fungal inoculation has been discouraged because it was assumed that nonvernalized plants provide an atypical environment for the pathogen (Morton et al., 1980). However, spring cultivars of wheat are susceptible to the pathogen (Wiese, 1977), but may not become infected because woulnd caused by spring upheaval, inducing root breakage, may be absent. Nonvernalized winter wheat seedlings are commonly used when screening wheat lines for resistance to rust and powdery mildew. The resistance to rust and powdery mildew demonstrated by these seedlings is also present in the field prior to and following vernalization (personal communication, A. Ravenscroft).

The high virulence of mutants E53 and E67 on LRC 40 was due to high symptom ratings (11-15) for most of the plants

in one of the replications. As these mutants are usually low in virulence the abnormally severe disease in LRC 40 treated with these mutants is suspect. The high virulence of mutant E53 on PI178383 and PI094424 is suspect for the same reasons. When M-13 and CG-82 treatment caused mild disease, the plant symptom ratings were all low (4-7).

Mathre et al. (1977) contends that lower inoculum levels of C. gramineum differentiate best between resistant and susceptible varieties because these levels approximate those encountered in the field (10^3 conidia /ml). The use of high inoculum levels (10^7 - 10^9 conidia/ml), however, did not interfere with evaluation of resistance in this study. The high levels of inoculum used reinforce the credibility of the virulence levels obtained for the isolates and mutants. This is particularly true for the mutants of low virulence, E53 and E67.

Ideally, centrifuging and millipore filtering the cultures would have eliminated only the fungus. However, other fungal byproducts active in disease may have been pelleted during centrifugation or millipore filtering. It is unlikely that GRA would be eliminated by millipore filtration because it is a small molecule. It is possible that a substance, such as a polysaccharide, could have been eliminated by millipore filtration of the culture supernatant. Polysaccharides from C. gramineum have been suggested as the cause of certain symptoms (Spalding et al., 1961; Pool and

Sharp, 1969a). However, polysaccharide production in culture is probably not correlated with virulence in the seedling assay (Part 1).

Differences in the resistance of 8 wheat lines to disease caused by C. gramineum can be seen in Figures 14 and 15. These wheat lines were representative of all the wheat lines and were picked because they best exemplified three broad catagories of disease The wheat lines Yorkstar, Marias and PI178383 had no resistance any of the isolates except mutant E67. Wheat lines F6-870 and PI278212 were intermediate in resistance to all isolates. High resistance was demonstrated by the wheat lines Agrotritichum, CI07638 and CI11222. Agrotritichum was the wheat line showing the highest level of resistance to C. gramineum; however, Figure 14 indicates that it was not immune. The significant difference between disease severity produced by the wild type isolates CG-82 and M-13 on Agrotritichum, PI178383 and PI278212 suggested that races of C. gramineum may exist.

When correlating the resistance of wheat lines screened in the seedling assay to disease reaction in the field (Table 10), it can be concluded that the screening procedure differentiated well between highly resistant (field disease reaction < 5%) and highly susceptible (field disease reaction > 30%) wheat lines, but differentiation was questionable for wheat lines showing intermediate resistance. The screening procedure revealed F6-870 (field disease reaction

14%) as intermediate in resistance and PI278212 (field disease reaction 21%) as low-intermediate in resistance, as might be expected from their field disease reactions. However, PI178383 (field disease reaction 13%) appeared to be susceptible. In this case, if the seedling assay were being used to screen winter wheat lines for resistance, a wheat line with intermediate field resistance may have been eliminated prior to field trials. This illustrates a possible pitfall; the resistance expressed by a wheat line in the field trials may differ from that expressed in the seedling assay.

All the wheat lines showed about the same reaction from toxin in the leaf-sheath assay. regardless of their reaction to the fungus. However, Agrotritichum and CI07638 appeared to be the most tolerant to toxin. This correlated well with resistance determined by the seedling assay and field disease reaction. In contrast, PI178383 (field disease reaction 13%) appeared to be more affected by GRA than was Yorkstar (field disease reaction 38, 44%). The fresh weight of cut seedling to which the GRA was applied did not appear to influence symptom production. The Agrotritichum tissues had a mean fresh weight approximately two-thirds that of Marias tissues, but Agrotritichum was less affected by toxin treatment than was Marias. Tissues of wheat lines PI178383 and F6-870 had the same mean fresh weight but the former was more affected by toxin than was the latter.

Screening wheat lines for resistance to C. gramineum with GRA does not appear to be a feasible alternative to inoculation with the fungus since: 1) the the leaf-sheath assay with toxin was very subjective; and 2) distinctive differences were not evident. This supports the tentative conclusion that GRA is not a significant determinant of disease (Part 1).

The seedling assay might be used as a rapid means of identifying winter wheat germplasm of intermediate and high resistance to C. gramineum. The resistant lines should then be screened in the field to determine field disease reaction. C. gramineum isolates of different, but known virulence should be included in both laboratory and field trials. This practice will aid in identification of germplasm resistant to a potentially greater number of isolates and races of the pathogen in question.

APPENDICES

APPENDIX A

The effect of temperature and pH on graminin A

Samples of graminin A (GRA) in $CHCl_3$ (1 µg/ml) were autoclaved (20 min, 15 psi), evaporated to dryness under reduced pressure at 50 C, and acidified with HCl to pH 1.2. The acidified preparation was then extracted with ethyl acetate and held at room temperature for two days. All treated samples were dissolved in 100 µl $CHCl_3$. Treated samples were quantified by gas chromatography (GC) and tested for biological activity by the thin layer chromatography (TLC) plate assay using Cladosporium cucumerinum as the activity indicator.

Summary

Autoclaving GRA resulted in the quantitative loss of 75% of the compound due to destruction by heat. Evaporation of distilled water-GRA solutions resulted in the quantitative loss of 50% of the compound. GRA may have been lost by heat destruction or volitility or from inefficient recovery of the compund from the inside of the evaporating flask. Treating GRA with acid resulted in the quantitative loss of 60% of the compound. This could be due to destruction of the compound by acid or a poorer efficiency of extaction with ethyl acetate from acidified solutions. GRA did not

break down at room temperature. Inhibition of growth of C. cucumerinum on TLC plates was complete when 10 µg of GRA were spotted and incomplete when 5 µg of GRA wse spotted.

TABLE A.1 Quantification and biological activity of graminin A samples after exposure to heat or pH change.

	Quantification by GC	Biological Activity		
Sample Treatment	µg GRA/µl $CHCl_3$	µl GRA spotted	µg GRA spotted	Inhibition[2]
autoclaved	0.245	15	2.94	-
		20	4.90	+/-
		25	6.12	+/-
evaporated	0.510	10	5.10	-
		15	7.65	-
		20	10.20	+
acidified	0.383			ND
room temp.	0.989	5	4.95	+
		10	9.89	+
		15	14.83	+
		20	19.78	+
standard	1.024[1]	5	5.12	-
		10	10.24	+
		20	20.48	+

1. Weight of GRA present was determined by ultraviolet spectral analysis.

2. Inhibition of growth of *C. cucumerinum* : + complete, +/- incomplete, - absent, ND not determined.

APPENDIX B

Efficiency of extraction of graminin A from water by organic solvents

Samples of authentic graminin A (GRA) (1.63 µg/µl $CHCl_3$) were added to 10 ml distilled water. Solutions were extracted four times, each time with 10 ml of ethyl acetate (EtOAc), $CHCl_3$, or CH_2Cl_2. Organic extracts and remaining distilled water were evaporated to dryness under reduced pressure at 38 C. Compounds were redissolved in 100 µl $CHCl_3$and quantified by gas chromatography (GC). The percent GA recovered from distilled watwe was determined as compared to a non extracted sample. The results indicated that CH_2Cl_2 was the best solvent for extracting GRA from culture filtrate of C. gramineum.

TABLE B.1 Quantification of graminin A in organic solvent extracts.

Sample	µg GRA/µl $CHCl_3$[1]	% Recovery
CH_2Cl_2	1.35	82.82
aqueous residue	0.11	
$CHCl_3$	0.36	21.84
aqueous residue	ND	
EtOAc extracted	0.96	58.65
aqueous residue	ND	
unextracted standard[2]	1.63	

1. ND - not detected by GC.

2. Weight of GRA present was determined by ultraviolet spectral analysis.

APPENDIX C

Migration of graminin A on silica thin layer chromatography using different mobile phases

TABLE C.1 Migration of graminin A[1] on silicic acid thin layer chromatography plates using different mobile phases.

Solvent	Dielectric Constant (25 C)	R_f[2]
acetonitrile	38.80	0.89
methylene chloride (CH_2Cl_2)	8.93	NM
isopropyl alcohol	19.90	0.84
n-propanol	20.30	0.84
ethyl acetate (EtOAc)	6.02	0.81
butanol	17.51	0.93
chloroform ($CHCl_3$)	4.73	NM
ethanol (EtOH)	24.55	0.85
methanol (MeOH)	32.70	0.98
n-hexane	1.88	NM
dioxane	2.21	0.64
distilled water	78.54	NM
acetone (Ac_2O)	20.70	0.90
CH_2Cl_2-EtOAc (75:25)		0.78

TABLE C.1 con't.

Solvent	R_f
$CHCl_3$-EtOAc (75:25)	0.89
$CHCl_3$-AC_2O (75:25)	0.80
CH_2Cl_2-EtOAc (98:2)	0.21
$CHCl_3$-EtOAc (95:5)	0.48
CH_2Cl_2-AC_2O (95:5)	0.65
$CHCl_3$-AC_2O (95:5)	0.68
CH_2Cl_2-MeOH (95:5)	0.70
$CHCl_3$-MeOH (98:2)	0.54
$CHCl_3$-butanol (98:2)	0.33

1. An authentic sample of graminin A from Japan was used.
2. NM - no migration.

APPENDIX D

High pressure liquid chromatography of graminin A

Column: LiChrosorb C_{18}, 10 m, 4.6 x 250mm, Altex Inc.

HPLC: Varian Model 5000 liquid chromatograph with a Hitachi Model 100-40 Spectrophotometer.

Settings: UV 210nm, range 0.05.

Solvent System: distilled water:methanol (50:50), isocratic.

Samples: Graminin A in methanol, 2ppm.

Retention Time: 6 min 7 sec - 6 min 15 sec.

APPENDIX E

Flash chromatography of graminin A

Flash chromatography was tried to see whether some of the yellow contaminating pigments associated with organic extracts could be removed from the extract sample proir to thin layer chromatography (TLC). A slurry of superfine silica (30 g, Chrommedia for column chromatography, LPS-2, Whatman, Inc.) was made with CH_2Cl_2 and poured into glass column (2.5 cm OD, 19 cm length of packing). A dirty culture extract of graminin A (GRA) (0.6 ml) plus 200 µl authentic GRA (200 µg) was loaded onto the column and extracted with four different mobile phases containing CH_2CL_2 and/or ethyl acetate (EtOAc). A total of 80 fractions (10 ml each) were collected. Mobile phases and corresponding fractions eluted from the column are listed in Table E.1. Fractions were combined, evaporated, redissolved in 100 µl $CHCl_3$ and run on TLC plates. TLC plates were illuminated with shortwave (300 nm) ultra-violet light to visualize GRA. Combined fractions were also assayed for GRA by gas chromatography (GC). GRA was quantified by the cut and weigh method. The presence of GRA in samples run on TLC plates and the quantity of GRA present as determined by GC are presented in Table E.2. Biological activity of GRA in

combined fractions was determined by the TLC plate assay (Table E.3).

Summary:

Most of the yellow pigmentation was found in the first 25 fractions and the last 20 fractions. As was the case with all other column chromatography packings tried (silicic acid, LH-20, SX-3; data not reported), GRA was not confined to discrete fractions and could be detected in most fractions. Flash chromatography was the methood of column chromatography which best removed contaminating pigments. GRA was quantified by GC before it was known that a contaminating compound comigrated with GRA (Part 1).

TABLE E.1 Mobile phases used and corresponding fractions eluted from column.

Solvent	Fractions
100% CH_2Cl_2, 100 ml	1-10
CH_2Cl_2:EtOAc (97:3), 250 ml	11-42
CH_2Cl_2:EtOAc (92:8), 200 ml	42-67
100% EtOAc, 100 ml	68-80

TABLE E.2 The presence and quantity of graminin A in samples as determined by thin layer chromatography and gas chromatography, respectively.

Combined Fractions	TLC	GC peak area (cm^2)
11-25	-	0.48
26-40	+	8.29
41-50	?	0.29
51-60	-	0.68
61-67	-	0.07
68-72	-	-
73-80	-	-
authentic GRA 2 µg	+	2.19

TABLE E.3 Biological activity of graminin A in combined fractions as determined by the thin layer chromatography plate assay.

Combined Fractions	µl spotted	Inhibition of *Cladosporium cucumerinum*
26-40	5	-
	10	10

APPENDIX F

The optimun number of days of growth, the optimum temperature for growth and the optimum growth medium for M-13 for maximun production of toxin.

F.1 The optimum number of days of growth of M-13 in culture for the maximum production of toxin

Harvest of 7 liters of fungal cultures took place 13, 21, 28, 32 and 35 days after inoculation of medium (Kobayashi and Ui medium, 1979) in diptheria and roux bottles with a 4-day-old spore suspension of M-13. Only the cultures grown for 35 days were grown in roux flasks. Cultures were grown at 25 C, photoperiod 12 hours. The mycelial dry weight after 3 days of drying at 45 C was measured. Toxin was extracted with $CHCl_3$ after proteins and carbohydrates were precipitated with MeOH. Dried extracts were redissolved in 200 µl $CHCl_3$ and 5 µl of each were run on a thin layer chromatography (TLC) plate (solvent system, 98 $CHCl_3$: 2 MeOH). Darkness of bands visualized with shortwave ultraviolet light (300nm) with R_f similar to authentic GRA was used to determine toxin production. Results are given in Table F1.1.

TABLE F.1.1 Tissue dry weight of M-13 and visualization and migration of graminin A from culture filtrate extracts of M-13, with different harvest times, on thin layer chromatography plates.

Days until harvest	Fungal Tissue dry wt (g)	R_f of toxin band	Darkness of band
13[1]	2.44	0.61	light
21	2.25	0.61	medium
28	3.25	0.60	dark
32	4.69	0.59	dark
35[2]	8.98	0.60	medium
authentic GRA 2 µg		0.63	very dark

1. The 13 day fungal dry weight was measured on a different balance.

2. Growth in the roux flasks was greater than in the diptheria bolltes.

F.2

The optimum temperature for culture growth of M-13 for the maximum production of toxin

Fungal cultures of M-13 were grown in diptheria bottles (Kobayashi and Ui medium, 1979) at 15, 19 and 25 C for 28 days prior to harvest in growth chambers with a 12 hour photoperiod. Toxin was extracted, prepared, run on TLC plates and visualized as in Appendix F.1. Results are given in Table F.2.1.

TABLE F.2.1 Migration and visualization of graminin A from culture filtrate extracts of M-13, grown at different temperatures, on thin layer chromatography plates.

Temperature (C)	R_f	Darkness of Spot
15	0.58	-
19	0.5756	light
25	0.58	dark
authentic GRA 2 µg	0.60	very dark

F.3

The optimum culture medium for growth of M-13 for maximum production of toxin

Cultures of M-13 were grown in diptheria bottles for 32 days at 25 C, photoperiod 12 hours. Three types of culture medium were used: Kobayashi and UI medium (1979), Pool and Sharp medium (1969) and modified Fries No. 3 basal medium (Pringle and Scheffer, 1963). Cultures were extracted, prepared, run on TLC plates and visualized as in Appendix F.1. Results are given in Table F.3.1.

TABLE F.3.1 Migration and visualization of graminin A from culture filtrate extracts of M-13, grown in different media, on thin layer chromatopgraphy plates.

Medium	R_f	Darkness of spot
Kobayashi and Ui	0.46	dark
Pool and Sharp	0.48	light
modified Fries	0.47	very light
authentic GRA 2 µg	0.49	very dark

Conclusion:

The optimum number of days of growth of M-13 for greatest toxin production was from 28-32 days. The optimum temperature for growth of M-13 for greatest toxin production was 25 C. The optimum culture medium for growth of M-13 for greatest toxin production was Kobayashi and Ui medium.

These conclusions were made before it was known that a contaminating compound comigrated with GRA (Part 1). Quantifying the actual amount of GRA present by gas chromatography/mass spectroscopy may alter this conclusion. If the contaminant were removed prior to gas chromatography then this method of quantification would be adequate.

APPENDIX G

Growth kinetics of CG-18 and 7-54

The growth curves obtained for CG-18 and 7-54 in potato dextrose broth (PDB) and PDB plus methionine (20 µl/ml) are in Figure G.1. When grown in PDB, CG-18 remained in lag phase for approximately 30 hours before entering the exponential growth phase. Approximate generation time was 10 hours. The mutant 7-54 remained in lag phase for approximately 75 hours when grown in PDB. The generation time for 7-54 was appoximaely 12 hours. The addition of methionine to the PDB decreased the time CG-18 was in lag phase by approximately 6 hours and increased the generation time by approximately 5 hours. The time in lag phase for 7-54 was decreased by approximatley 11 hours with addition of methionine to PDB. The generation time for 7-54 was reduced to approximately 4 hours. This was one third of the generation time found in PDB.

Figure F.1 Growth kinetics of CG-18 and 7-54 in potato dextrose broth and potato dextrose broth plus methionine (20 µl/ml).

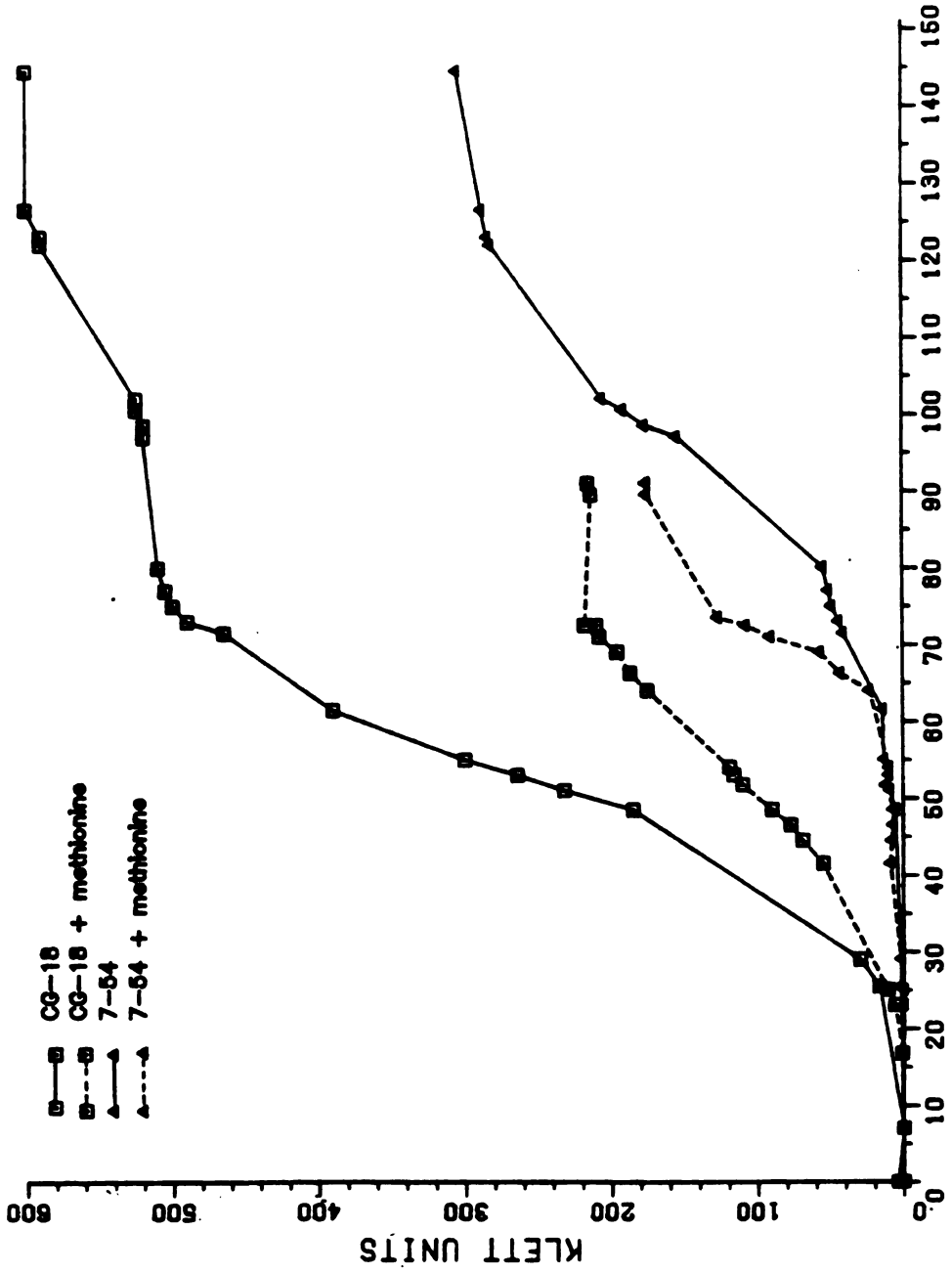
CG-18
CG-18 + methionine
7-54
7-54 + methionine
KLETT UNITS
0
100
200
300
400
500
600
TIME (HRS)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160

FIGURE F.1

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