IDENTIFICATION OF *Fusarium* spp. AND MYCOTOXINS ASSOCIATED WITH HEAD BLIGHT OF WHEAT IN ETHIOPIA

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a candidate for the degree of DOCTOR OF PHILOSOPHY

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ABSTRACT

Wheat seeds produced in 1987 and stored under various conditions at research stations, seed farms, state farms and farmer fields in different regions of Ethiopia were checked for *Fusarium* infection. *Fusarium* was detected in 35 to 65% of the samples. *Fusarium* Head Blight (FHB) survey in wheat fields in 1988 indicated as many as 85% of the plants in some state farms showed head scab. Lower levels of FHB were recorded at farmer fields. The results indicated that FHB can be a major problem to wheat production in Ethiopia and a breeding program against the disease should be initiated.

Seventeen and thirteen *Fusarium* species were identified from stored seed and scabby wheat heads in the field, respectively. *F. nivale* and *F. avenaceum* were dominant species in samples collected from cool, moist, high altitude areas. *F. graminearum* was more frequent at lower altitude and in northwestern regions. In both state farms and farmer fields, *F. sporotrichioides* and *F. poae* occurred less

frequently and had more limited distribution than F. graminearum. F. equiseti was more commonly isolated from stored seed samples.

The majority of the Fusarium species did not produce detectable amounts of either trichothecenes or zearalenone. Two isolates of F. graminearum produced low levels of 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol and zearalenone. An isolate of F. poae produced a low level of neosolaniol.

A single strain, F. sporotrichioides #48d produced a variety of trichothecenes. They were tentatively identified by thin layer chromatography and gas chromatography-mass spectrometry as neosolaniol, 8-acetylneosolaniol, 8-propionylneosolaniol, 8-n-valerylneosolaniol, 8-isovalerylneosolaniol (T-2 toxin), 8-n-butyrylneosolaniol, 8-isobutyrylneosolaniol, 8-hexynylneosolaniol and 4,8-diacetyl T-2 tetraol (NT-1). The 8-n-valerylneosolaniol and 8-hexynylneosolaniol are reported for the first time.

Sensitivity of wheat cultivars to Fusarium toxins in coleoptile elongation assay did not, generally, correlate with their reaction to FHB in the field. The cultivars did not respond similarly to all the mycotoxins or to different concentrations of a single mycotoxin. Line HAR424, however, exhibited low sensitivity to all mycotoxins as well as low FHB in the field.

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CHAPTER 1

INTRODUCTION

Wheat is one of the major cereal crops grown in the highlands of Ethiopia at altitudes between 1700 and 2800 m. In 1986/87, 639,000 hectares of land was devoted to wheat production with an average yield of 12 q/ha [1]. Private peasant holdings, producer cooperatives, and state farms, comprised 82, 8, and 10 percent of the total land area devoted to wheat, respectively.

Durum wheat (Triticum turgidum L) and bread wheat (Triticum aestivum L. em. Thell) are the two most widely grown species, although other wheat species are also cultivated [8]. Ethiopia is recognized as a center of evolution of tetraploid wheat and a number of species have been identified by workers who made scientific expeditions to the country [5]. Presently, it is estimated that durum wheat is grown on 60-70% of the total land devoted to wheat production [7], all of which is produced by peasant farmers under rain fed conditions. Although a few high-yielding durum wheat cultivars have been recently distributed to farmers, nearly all durum cultivars used are landrace cultivars consisting of a mixture of several tetraploid types [8]. In contrast, bread wheat has only recently been introduced to Ethiopia. It has wider adaptation

and is higher yielding than durum wheat. It is presently grown on 30 to 40 percent of the total wheat acreage devoted to wheat production [8]. The majority of bread wheat cultivars grown by peasant farmers and state farms are improved cultivars and have higher yields than the national average [1]. Irrigated wheat production is only in the experimental stage. However, unpredictable amounts of rainfall in the country and a national need for self-sufficiency in food production will require utilization of an estimated 3.3 million hectares of irrigable land the country possesses in its numerous fertile river valleys [3]. The potential of introducing wheat as a double-crop with cotton in the Awash Valley has been investigated and reported to be feasible [4].

Several factors have been attributed to the low yield of wheat in Ethiopia. These include use of landrace cultivars that have low genetic potential for yield and disease resistance, primitive traditional agronomic practices, and wheat diseases [6]. Wheat suffers from a number of diseases in all wheat-producing regions in the country. Three rust diseases, stem rust (Puccinia graminis f. sp tritici Eriks.), leaf rust (P. recondita Rob. ex Desum), and stripe rust (P. striiformis west) and leaf and glume blotches (Septoria tritici Rob. ex Desm. and S. nodorum Berk, respectively) are the major disease problems in most regions [2]. Both pathological and genetic (breeding for resistance) studies have been undertaken on these diseases in the past

resulting in considerable progress. For some other diseases, however, few or no studies have been initiated.

Fusarium Head Blight (FHB), caused by a number of Fusarium species, has been reported as one of the major diseases in the high altitude areas having cool and wet climate [2]. Previous work on FHB in Ethiopia has been limited to preliminary surveys on large state farms and research plots. The threat of FHB to the Ethiopian wheat crop in terms of its importance, distribution, fungal species involved, reduction in quality of wheat and the levels and types of mycotoxins produced have not been investigated. This study was undertaken to: 1. determine the identity and distribution of Fusarium species causing FHB on wheat in Ethiopia, 2. identify mycotoxin producing strains and the mycotoxins they produce, and 3. assess reactions of some of the wheat cultivars currently growing or being released for use in the country to FHB and Fusarium toxins.

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CHAPTER 2

LITERATURE REVIEW

Fusarium Head Blight (FHB):

Fusarium Head Blight (FHB), a disease that occurs on all small grain cereal crops, is also known as scab, pink mold, whiteheads and tombstone scab [166]. The disease is best recognized on emerged immature heads of wheat where one or more spikelets or the entire head appears prematurely bleached. Perithecia and superficial pink or orange mycelium and spore masses may be seen on, and especially at the base of diseased spikelets. Bleached spikelets are often sterile or contain poor quality seed that give rise to low vigor seedlings [13,101,166].

FHB occurs on wheat world-wide. Wheat scab was reported as a major disease in the USA as early as 1891 [33]. Despite the many years of research on the nature and control of the disease, FHB remains one of the major threats to wheat production. Recent reports indicate that the incidence of affected spikes within a field may range from a trace to 100 percent each year in major wheat-producing areas such as the Red River Valley of Northwest Minnesota [167]. Severe head blight epidemics have occurred at intervals of several years between 1927 and 1980 in many wheat growing areas of Canada [33,146]. In 1985, abnormally high summer rainfall in southern

Manitoba resulted in a high incidence of FHB in wheat [2]. FHB is reported to be more important than any other disease of wheat and barley in the People's Republic of China [34]. FHB is also one of the major diseases of wheat in western Europe [28], eastern Europe, and the Soviet Union [100]. In Ethiopia where most of the wheat is grown in cool and wet highland regions, FHB periodically becomes severe, especially during extended rainy seasons [5,6,7].

The fungi that cause FHB are facultative parasites that may also infect other plant parts [166]. Seedling blight results from seed-borne inoculum of blighted and scabby wheat [33,166]. Root, crown and foot rots of wheat, barley and oats are due to infection from soil-borne inoculum of these fungi [21,32,33]. Up to 50% loss due to Fusarium root and foot rot has been reported in winter wheat in the Pacific Northwest [32]. Some of the fungi that cause FHB can also infect leaves and leaf sheaths causing leaf blotch [28,33].

The Pathogens:

All fungi that cause head blight or scab in wheat are in the genus Fusarium. The pathogens have simple nutritional requirements and are omnipresent as saprophytes [166]. Cook (1981) and Wiese (1987) listed the principal Fusarium species that cause head blight or scab as Fusarium graminearum Schwabe (syn. F. roseum Lk. emend. Snyd & Hans. f.sp cerealis (Cke.) Snyd. & Hans 'Graminearum') and its

teleomorph Gibberella zeae (Schw) Petch (Syns. G. roseum f. sp cerealis 'Graminearum', G. saubinettii (Mont) Sacc), F. avenaceum (Corda ex Fr.) Sacc. (syn. F.roseum Lk. emend. Snyd. & Hans. f. sp cerealis (Cke.) Snyd. & Hans. 'Avenaceum') and its teleomorph G. avenacea Cook, F. culmorum (Smith.) Sacc. (syn. F. roseum LK emend. Snyd. & Hans f.sp cerealis (cke.) Snyd. & Hans. 'culmorum'), Microdochium nivale (Ces. ex Berl. & Vogl.) Sammuels & Hallett (Syns. Gerlachia nivales (Ces. ex Berl. & Vogl.) Gams & Muller, F. nivale (Fr.) Ces. ex Berl & Vogl.) and its teleomorph Monographella nivalis (Schaff.) Muller (syn. Calonectaria nivalis Schaff).

Several of these species have been reported to cause ear and stalk rot in corn. Purss (1971) demonstrated that isolates of F. graminearum from crown rot of wheat, stalk rot of maize, and head blight of wheat are all capable of causing FHB of wheat [123]. F. culmorum isolated from foot rot of wheat and barley is reported to cause stalk rot of maize. Crown rot of wheat, however, is caused only by F. graminearum isolated from the crown of wheat, indicating that a specialized form of F. graminearum is responsible for crown rot in wheat.

Gordon (1952) in his taxonomic study of Fusarium species identified a total of 16 species and varieties of Fusarium from cereal seeds grown in Canada during the period 1939 to 1943 [49]. These species and varieties are F. poae Pk, Wr., F. sporotrichioides Sherb., F. avenaceum (Fr.) Sacc., F.

arthrosporioides Sherb., F. semitectum Berk. and Rav., F. equiseti (Cda.) Sacc., F. acuminatum Ell. & Ev., F. culmorum (W.G.Sm) Sacc., F. graminearum Schwabe, F. sambucinum Fuckel, F. sambucinum var. coeruleum Wr., F. lateritium Nees emend Snyder & Hans, F. moniliforme Sheld. emend. Snyder & Hans., F. oxysporum var. redoens (Wr.) n. Comb., F. solani (App. & Wr.) Wr. emend. Snyder & Hans.. Abramson et al (1985) reported that F. graminearum, F. sporotrichioides, F. poae, F. acuminatum, F. avenaceum, and F. equiseti were the most frequent species identified from wheats grown in 1985 in southern Manitoba [2]. The same Fusarium species were identified from scabby spring wheat grown in 1984,1985 and 1986 on farm fields and agricultural experiment stations in Minnesota [167]. F. poae, F. avenaceum and F. acuminatum were the most frequently isolated species in Canada while in Minnesota, F. graminearum and F. poae comprised 75% and 17% of the isolates, respectively. Sturz (1983) reported that F. poae colonized the ears and leaf sheaths of wheat and barley plants prior to emergence of the ear from the boot [143]. However, Stack (1985) studied the head blighting potential of seven Fusarium species isolated from spring wheat heads and reported that only F. graminearum and F. culmorum caused severe blighting of wheat heads following inoculation [140]. F. tricinctum, F. sporotrichioides, F. equiseti, F. acuminatum and F. poae did not blight heads but caused wheat heads to become moldy under extremely favorable (eg. constant moisture saturation and

suitable temperature) conditions. F. sporotrichioides was routinely isolated from all parts of spring wheat plants grown in 1979 in Minnesota [159]. It caused leaf and glume necrosis but kernels on affected spikelets appeared to fill and ripen normally.

Cook (1981) discussed the respective temperature preferences of F. graminearum, F. culmorum and F. avenaceum [33]. F. graminearum is the predominate species found on cereals in warmer regions such as the corn belt states of the USA [8], eastern Australia [22], and southern Europe [28]. F. culmorum is intermediate in temperature preference being predominant in the north western states of the USA, prairie and maritime provinces of Canada, Europe, and the Soviet Union. F. avenaceum occurs in areas characterized by cool climate during the growing season. F. nivale is the principal causal agent of leaf and sheath blotche, head blight and snowmold in cooler regions of northern Europe, the Soviet Union, and the People's Republic of China [28,33,34,100]. In South Africa, F. moniliforme predominates in subtropical areas, F. moniliforme var. subglutinans in temperate areas and F. graminearum in areas with an intermediate climate [95].

F. nivale was known to cause only pink snowmold of cereals and turf in North America until the report of Inglis and Cook (1981) that Calonectaria nivalis also caused scab of wheat in north western Washington in 1980 [66]. Gordon (1960) identified F. graminearum and F. oxysporum on bread wheats

from Kenya and Uganda in Eastern Africa [52]. In Ethiopia, F. culmorum, F. graminearum, F. longipes, F. semitectum and F. dimerum, causing root rot and/or head blight, were identified in the 1970's on wheats grown at various locations [5,80,142]. These reports were, however, based on preliminary surveys, mainly from research plots.

Taxonomy of Fusaria:

In discussing species of the genus Fusarium, one has to consider the problems and various views expressed in the taxonomy and classification of the genus. The taxonomy of Fusarium is complex and often difficult because many species exhibit extreme variability in their morphological characteristics, even when grown from monospore cultures. By the early 1930s, more than 100 species of Fusarium had been described [30,138]. It was the work of two German mycologists (H.W. Wollenweber and O.A. Reinking, 1935) that established the basis of a systematic approach to Fusarium taxonomy [172]. They reduced the number to 65 species and 78 varieties. Snyder and Hansen (1940, 1941, 1945) further reduced the number of the species to nine, all of them identifiable by morphological characteristics observed in culture on a standard agar medium [134,135,136]. The nine species described are F. solani, F. episphaeria (perfect stage: Nectria), F. rigidiuscula (perfect stage: Calonectaria), F. nivale (perfect stage: Calonectaria), F. roseum (perfect stage: Gibberella), F.

lateritium (perfect stage: Gibberella), F. moniliforme (perfect stage: Gibberella), F. oxysporum, and F. tricinctum. There were no varieties described for these species, but a number of sub-specific names at the formae speciales levels were listed to denote important behavioral characteristics such as pathogenesis on a particular host or hosts. Species differentiation was based on differences in the shape of the macroconidium together with such characteristics as the presence or absence of microconidia, their position and kind, and the formation of chlamydospores. Later, Snyder et al (1957), introduced the cultivar concept [137]. What they referred to as morphologically different strains of F. roseum were given cultivar names such as F. roseum 'Graminearum' or if pathogenic, F. roseum f. sp. cerealis cultivar 'Graminearum', 'Culmorum' or 'Avenaceum'. This has introduced a rather complex three or four-stage nomenclature for the species without any simplification of identification [17].

Environmental factors have been shown to cause great variability in morphological (as well as physiological) characteristics of isolates of many species [178]. This has made the Snyder and Hansen's approach less usable regardless of its simplicity. Joffe (1977) suggested that the Snyder and Hansen system is oversimplified for use in identification of species of Fusarium for toxicological purposes [73].

Gordon (1952) working in Canada, Booth (1971) in England, Joffe (1974) in Israel and many others have developed

taxonomic systems based on Snyder and Hansen and Wollenweber and Reinking [17,49,72]. Still there has been no satisfactory system developed that is accepted by all involved in Fusarium taxonomy. However, manuals such as those published by Booth (1977) and Nelson et al (1983) by selecting and combining the best features of several systems remain useful tools in Fusarium identification [18,118].

Epidemiology of FHB:

Fungi that cause FHB overwinter on host residues which are the principal reservoirs for the pathogens, although soils, seeds, and various other hosts are also inoculum sources [146,166]. The pathogens survive in old stalks and ears of maize, and on stubble and debris of wheat, barley and other cereals [49,62,131,163]. They are primarily located in the soil organic debris fractions and are more abundant in continuously cropped cereal fields [144]. Warren and Kommedahl (1973) noted that F. graminearum overwintered on wheat straw with the proportion of straw that was colonized declining during the winter [163]. The pathogens have been isolated from soils collected from cereal fields, insects and grains; infected grains being one of the major sources of inoculum when used as seed [8,50,51,146,168].

The inoculum may be ascospores, macroconidia, chlamydospores, and hyphal fragments [17,132,146]. The formation and role of chylamydospores as a survival mechanism

for Fusarium have been reviewed by Schippers et al [127]. Since inoculum for head blight requires aerial dispersal to infection sites, ascospores and macroconidia probably are the most important inocula causing primary and secondary infection, respectively. Ayers et al (1975) reported that mature perithecia of G. zeae were found in Pennsylvania on corn debris and wheat stubble from April through October [9]. Thus, abundant ascospore inoculum may be available for an extended period of time. Optimal temperature for perithecial production and ascospore discharge in G. zeae differ significantly [153]. Perithecial production increases with increasing temperature to 29 C and decreases sharply at higher temperatures. This optimum is very close to the optimum for macroconidial production and mycelial growth [4,153]. Ascospore discharge, on the other hand, is favored by low temperatures with no discharge occurring above 26 C.

Andersen (1948) reported that wheat susceptibility to G. zeae was highest during the flowering to post-flowering stages, with susceptibility decreasing in the late stages of seed development [4]. The optimum temperature for infection and disease development was 25 C and continued wetness was necessary for infection. The critical exposure period of wetness for infection was the shortest at 25 C. Studies on optimal temperature and moisture requirements for the other head blight causing Fusarium species are very limited. Generally, anthesis and prevailing weather conditions at

anthesis seem to be the major factors influencing scab development.

Mycotoxins:

Mycotoxins are secondary metabolites produced by fungi that are toxic to animals. They are considered chemical pollutants of biological origin, presenting the most serious hazard to man and animals through contamination of food and feed stuffs [46]. Mycotoxins have been implicated in a number of human and animal disease syndromes known as mycotoxicoses. Tanaka et al (1988) demonstrated the world- wide occurrence of mycotoxins in agricultural products [150]. The United Nation's Food and Agriculture Organization (FAO) has estimated that 25 percent of the world's food crops are ruined by mycotoxins [94] and has suggested that 10 to 50 percent of the crops in Africa and the Far East are contaminated. Many countries, recognizing the dangers associated with mycotoxin contamination of food and feeds, have instituted regulatory controls on foods and feeds moving in domestic and international commerce. The global impact of mycotoxins on the health of man and animals as well as on trade and economics was reviewed in 1977 by a joint conference of the Food and Agriculture Organization, the World Health Organization and the United Nations Environmental Program [46]. It was recognized that information was needed to establish where hazards exist and their extent. To facilitate

this, FAO has prepared a general guideline for developing effective mycotoxin surveillance programs to be used in many countries [47]. Certain regions of the world are more prone to the hazard than others. The hot and humid tropics provide conditions most favorable for the rapid proliferation of most mycotoxin-producing fungi [61].

A large number of isolates of fungi, including Aspergillus, Penicillium, and Fusarium species are known to produce a variety of potent mycotoxins [26,111,130,177]. Fusarium is one of the principal genera of the field fungi that invade wheat and barley kernels while they are developing in the field [29]. Aspergillus and Penicillium, on the other hand, are storage fungi that do not commonly invade seeds of cereal plants to any serious degree before harvest. More than a dozen Fusarium species are known to produce toxins [73,96]. Marasas et al (1984) listed 203 toxigenic strains in twenty species of Fusarium from the International Toxic Fusarium Reference Collection (Table 1) [96].

Mycotoxins produced by Fusarium spp.:

Two groups of mycotoxins are known to be produced by many toxigenic strains of Fusarium species.

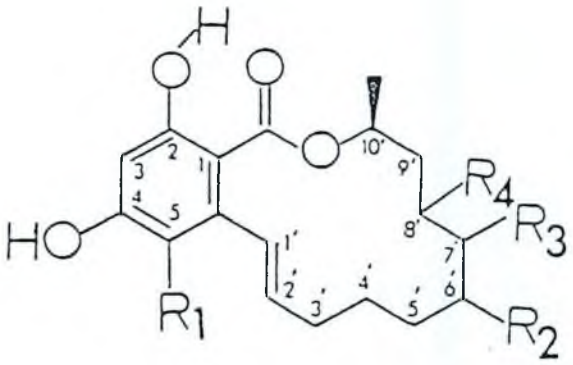
Zearalenone: Zearalenone or F-2 and its derivatives are produced by F. graminearum, F. roseum, F. nivale, F. tricinctum, F. sporotrichioides, F. oxysporum, F. moniliforme, and Gibberella zeae [16,99,111,112].

Table 2-1 Toxigenic *Fusarium* strains in the International Toxic *Fusarium* Reference Collection (ITFRC).

Section	Species	No. of Toxigenic strains
Eupionnotes	*F. merismoides*	1
Archnites	*F. nivale*	1
	F. larvarum	1
Sporotrichiella	*F. sporotrichioides*	43
	F. chlamydosporum	3
	F. poae	8
	F. tricinctum	8
Roseum	*F. avenaceum*	5
Arthrosporiella	*F. semitectum*	8
Gibbosum	*F. equiseti*	15
	F. acuminatum	4
Discolor	*F. sambucinum*	6
	F. culmorum	12
	F. graminearum	34
Liseola	*F. moniliforme*	32
	F. proliferatum	1
	F. subglutinans	4
	F. anthophilum	1
Elegans	*F. oxysporum*	9
Martiella	*F. solani*	7
	Total	203

The chemical structure of zearalenone is 6-(10-hydroxy-6-oxo-trans-1-undecenyl)β-resorcyclic acid lactone [158]. The structure of zearalenone and some naturally occurring derivatives are shown in Table 2-2 [114].

Table 2-2 Zearalenone and its naturally occurring derivatives.



Name	R_1	R_2	R_3	R_4
Zearalenone	H	=O	H_2	H_2
8'-Hydroxyzearalenone	H	=O	H_2	OH
7'-Dehydrozearalenone	H	=O	H	H
5'-Formylzearalenone	HC=O	=O	H_2	H_2
6'-8'-Dihydroxyzearalenone	H	OH	H_2	OH

Zearalenone is a white crystalline compound with a molecular weight of 318, and a melting point of 164-165 C. It fluoresces under UV irradiation and has UV maxima at 236, 274 and 316 nm [16]. The toxin is insoluble in water , carbon disulphide and carbon tetrachloride. It is soluble in aqueous alkali, diethyl ether, benzene, chloroform, methylene chloride, ethyl acetate, acetonitrile, and alcohols [16,158]. Zearalenone is biosynthesized by head-to-tail condensation of acetate units via the acetate-malonyl-coenzyme A enzyme system [141]. The enzymes for zearalenone synthesis are either induced or activated at low temperatures (12-14 C), conditions not optimum for fungal growth but produce physiological stress [114]. Zearalenone functions as a sex-regulating hormone in F. roseum [170,171]. Perithecial formation is enhanced by as much as 100 percent when zearalenone is added to a medium on which the fungus is growing. Conversely, inhibition of biosynthesis of zearalenone by dichlorovos inhibits the production of perithecia by F. roseum [169]. This suggests zearalenone may be a true fungal hormone which governs development of the sexual stage.

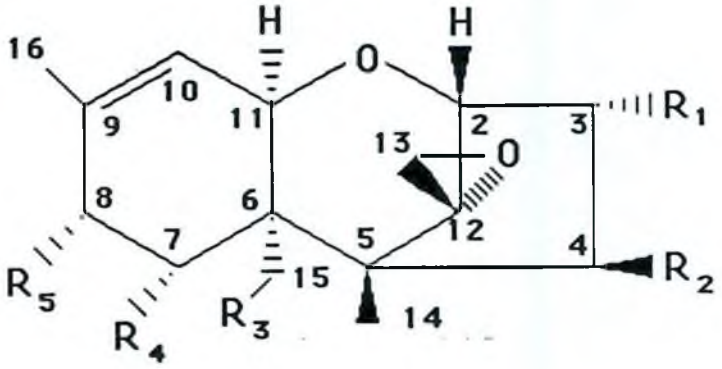
Trichothecenes: The trichothecenes are a group of naturally occurring sesquiterpenoids produced by various genera of fungi such as Trichothecium, Cephalosporium, Myrothecium, Trichoderma, Stachybotrys, Fusarium, Verticimonosporium, and Cylindrocarpon [2,67,114]. This group of compounds has also

been identified from a higher plant, Baccharis megapotamica [84,85]. Grove (1988) reported several Fusarium species which produced a variety of trichothecenes [59]. These include F. acuminatum, F. avenaceum, F. chlamydosporum, F. culmorum, F. equiseti, F. graminearum, F. moniliforme, F. nivale, F. oxysporum, F. poae, F. sambucinum, F. semitectum, F. solani, and F. sporotrichioides.

Most naturally-occurring trichothecenes contain a 12,13-epoxide and its elimination results in loss of toxicity to animals [12,111,149]. The trichothecenes are divided into two groups - macrocyclic and non-macrocyclic - depending on the presence/absence of a macrocyclic ring linking C-4 and C-15 with diesters. The macrocyclic trichothecenes are produced by the genera Myrothecium, Cylindrocarpon, Stachybotrys and Verticimonosporium, but have not been reported from Fusarium [71]. The non-macrocyclic trichothecenes are divided into three classes based upon the ring oxidation patterns [67]. Many Fusarium strains produce Class I (Table 2-3) and Class II (Table 2-4) trichothecenes [59,67]. The Class III trichothecenes are 7,8-epoxides produced by Cephalosporium and Trichothecium species [67].

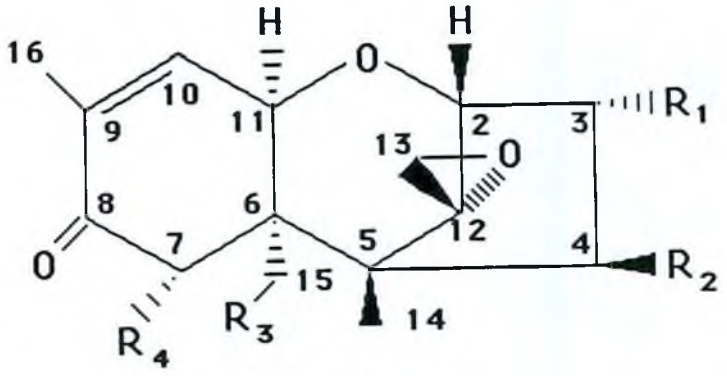
The number of naturally-occurring trichothecenes isolated from natural sources has steadily increased. In 1974, Mirocha et al, reported the occurrence of 27 trichothecene derivatives [111]. This number increased to 37 in 1977 [114] and by 1981 the number had grown to 58 [149]. Grove (1988), in

Table 2-3 Class I Fusarium trichothecenes.



Compound	R_1	R_2	R_3	R_4	R_5	ref.
T-2 toxin	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$	10,11
HT-2	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$	11
DAS	OH	OAc	OAc	H	H	156
Scirpentriol	OH	OH	OH	H	H	119
Neosolaniol	OH	OAc	OAc	H	OH	69
8-Acetyl-neosolaniol	OH	OAc	OAc	H	OAc	68
T-2 tetraol	OH	OH	OH	H	OH	113

Table 2-4 Class II Fusarium trichothecenes.



Compound	R_1	R_2	R_3	R_4	ref.
Nivalenol	OH	OH	OH	OH	156
Diacetylnivalenol	OH	OAc	OAc	OH	156
Fusarenon-x	OH	H	OH	OH	156
Deoxynivalenol	OH	H	OH	OH	176
3-Acetyldeoxy-nivalenol	OAc	H	OH	OH	176

his review of the literature published prior to December 1986, reported a total of 148 trichothecenes that had been isolated from natural sources [59]. These included 83 non-macrocyclic and 65 macrocyclic compounds. Twenty five of the macrocyclic compounds, the baccharinoids, have been isolated only from plants of the genus Baccharis. Grove suggested these compounds are probably products of a plant-fungus interaction in which fungal macrocyclic trichothecenes have been taken up by the plant and modified further. Recently, a number of new trichothecene mycotoxins produced by F. sporotrichioides have been reported [35,36,37]. Chemical and physical properties, structural formulas and biological activities of many of these trichothecenes have been reviewed by Mirocha et al [114], Tamm et al [149] and Ishii [67].

The various steps, precursors and intermediates in the biosynthesis of trichothecenes have been thoroughly reviewed by Tamm [148]. Jones and Lowe (1960) were the first to observe the incorporation of three molecules of [2-^{14}C]-mevalonate into the trichothecane nucleus of trichothecene [76]. The positions of the labeled carbon atoms were determined after extensive chemical degradation. Later, Godtfredsen and Vangedal (1965) demonstrated the radioactivity from [2-^{14}C]-mevalonate was distributed between C(4), C(10) and C(14) of trichothecalone, indicating that carbons 4, 10, and 14 originated from carbon 2 of the mevalonate [48].

Conditions for mycotoxin production by Fusarium spp.:

Mycotoxin production is affected by a number of factors. Not only the strain of the fungus, but nutrition, climate, host resistance, substrate availability, storage conditions and interaction with other fungi influence the production of mycotoxins [89]. Probably the most important factor is the interaction between water availability and temperature [44].

FHB can be caused by a variety of Fusarium species and strains. Each strain of a species may make a different amount and mixture of toxins [54,55,56,57,90]. Most strains of F. graminearum from North America, for example, produce 15-acetyldeoxynivalenol whereas most strains from Europe, Japan, and China produce 3-acetyldeoxynivalenol [106,110].

Miller et al (1983,1985) reported the concentration of ergosterol (as a measure of fungal biomass) and deoxynivalenol in infected corn ears and wheat spikes peaked six weeks after inoculation and declined thereafter [107,109]. The concentration of 15-acetyldeoxynivalenol increased rapidly in the first two weeks and then declined. No appreciable amount of zearalenone was observed until 9 weeks after inoculation. These results suggest that grains from a field or different fields infected by a single strain of a given species, or harvested at different times after infection may have different toxins or different levels of the same toxins [45].

Culturing Fusarium for mycotoxin production:

Mycotoxin production by Fusarium species is greatly influenced by the medium on which the fungus is grown. Miller et al (1986) demonstrated that nutrient limitation induces the synthesis of trichothecenes [104]. Major factors that stimulate the biosynthesis of deoxynivalenol and related compounds by F. graminearum include reduced oxygen level, depletion of carbohydrate in the medium, a low concentration of an organic nitrogen source and pH of about 6.5 [106].

Fusarium isolates are usually grown on solid substrate for high production of zearalenone and trichothecenes [1,23,44,58,67]. Greenhalgh et al (1983) reported a number of F. graminearum isolates produced deoxynivalenol and zearalenone on corn and rice media [58]. The highest level of both mycotoxins was obtained on these media with 35 to 40% initial moisture (before autoclaving) and 24 days of incubation. At 19 C incubation, the main product was zearalenone whereas at 25 C both zearalenone and deoxynivalenol were formed. A higher incubation temperature (28 C) favored deoxynivalenol formation.

These mycotoxins can also be produced, often in much lower quantities, by Fusarium strains cultured in liquid media such as glucose yeast extract peptone or GYEP [106], Czapek Dox medium supplemented with peptone [15] and modified Gregory or Vogel synthetic medium [38]. The culture can be grown as stationary surface culture or as a shaken (on a rotary shaker)

submerged culture [157]. Prolonged culturing on liquid media may result in the metabolism of some Fusarium toxins into other products. Bergers et al (1985) reported that T-2 toxin produced by F. tricinctum in liquid Czapek Dox medium (supplemented with 1% casein peptone) was metabolized and converted into HT-2 toxin and T-2 tetraol [15]. The conversion proceeded faster when the culture was incubated at a higher temperature (27 C verse 15 C). Greenhalgh et al also reported that 3-acetyldeoxynivalenol produced by F. culmorum in liquid culture was chemically converted to deoxynivalenol [55].

El-Kady (1982) developed a suitable liquid medium for the production of zearalenone by many Fusarium species [40]. Fungal growth and zearalenone production were the highest when starch was the carbon source, ammonium or nitrate was the nitrogen source and the medium was supplemented with glutamine and riboflavin. The maximum amount of zearalenone appeared at pH 7, after 12 days of incubation at 20 C. Miller et al (1983,1986) described a liquid culture method in a stirred jar fermentor for the production of deoxynivlenol and 3-acetyldeoxynivalenol [104,106]. The advantages of liquid media over solid media are the composition can easily be varied and the analysis for mycotoxins requires fewer purification steps.

Analysis of Fusarium mycotoxins:

A versatile method for the isolation, detection, and

quantitation of zearalenone in maize and barley was devised by Mirocha et al [116]. This method employs either thin-layer chromatography (TLC), gas-liquid chromatography (GLC), GC-mass spectrometry (GC-MS), or a combination of these procedures. The sample is extracted with ethyl acetate using a Soxhlet or Batch extraction procedure. The extract is purified using one molar sodium hydroxide (base clean-up) or acetonitrile - light petroleum. A number of other extraction and fractionation procedures have been reported. Eppley's extraction procedure with chloroform:water (10:1) was reported to be very efficient for zearalenone [42]. Simultaneous extraction, separation and qualitative analysis procedure for zearalenone and other mycotoxins has been reported [165]. In this procedure, the sample is extracted with acetonitrile, defatted with isooctane and dried over sodium sulphate. The extract is dissolved in benzene:methanol (99:1) and added to a dry mini-silica gel-column and washed with benzene, benzene:acetone (95:5), diethyl ether, chloroform:methanol (95:5), and acetone to elute the various fractions.

Zearalenone can be detected and quantified by TLC, GLC, or GC-MS [116]. A variety of solvent systems have been described to develop silica gel TLC plates for zearalenone detection [16,53,152]. Zearalenone appears as a greenish-blue fluorescent spot under short wave (254 nm) UV light [42]. For further confirmation of zearalenone, a freshly developed and dried plate is sprayed with 0.7% aqueous Fast Violet B

followed by a buffer solution spray, and zearalenone appears as a pink spot upon heating [126]. The pink spot turns purple when sprayed with 30% sulfuric acid in methanol. Other procedures for quantitative analysis of zearalenone have also been described [70].

Isolation, purification, and chromatographic procedures for a number of trichothecenes have been reviewed by Tamm and Tori [149]. Generally, trichothecenes are extracted with ethyl acetate or chloroform. Lipids are removed by suspending the concentrated ethyl acetate extract in acetonitrile which retains the trichothecenes when partitioned against petroleum ether (liquid-liquid partition). Trichothecenes can also be purified from crude extracts by precipitation of impurities with ammonium sulfate [14,78] or ferric chloride [31], and column chromatography with silica gel [14,78,115] or florisil [175].

Various solvent systems (eg 3:1 benzene:acetone or 1:1 ethyl acetate:ethyl ether) are used to develop TLC plates spotted with trichothecenes [53]. Detection and quantitation by TLC becomes difficult since trichothecenes, unlike zearalenone, exhibit no fluorescent emission when irradiated with UV. The compounds are made visible, however, by spraying with reagents such as p-anisaldehyde, aluminum chloride or the blue spray [147].

GC-MS procedures have also been developed for both zearalenone and the trichothecenes [14,116,161]. The advantage

of these procedures lies in relatively precise identification of minute amounts of toxins. The use of high-performance liquid chromatography (HPLC) for the determination of zearalenone and trichothecenes has been described [63,77,83]. The application of this technique is rapidly increasing due to its many advantages over the other chromatographic techniques. The analysis is very fast, the need for sample fractionation is reduced and compounds which are thermally labile or too polar for GC can be analyzed easily without derivatization [53].

The new technique of mass spectrometry-mass spectrometry (MS-MS) has been applied to crude extracts of wheat , corn, oats, and rice to detect DON and zearalenone in levels below 1 ppm [14,121]. MS-MS is an elegant means of rapidly screening and identifying mycotoxins.

A simple and rapid method of confirmation of DON and 3-ADON residues in cereal crops was developed by King et al [81]. It involves a one-step oxidative treatment with aqueous periodate or freshly precipitated lead oxide in which DON or 3-ADON is quantitatively converted (oxidative transformation) into a unique seven-membered ring lactone analogue. This analogue can be quantified by electron capture gas-liquid chromatography at levels equivalent to 0.05 ppm.

Fusarium mycotoxicoses:

A comprehensive review of human and animal

mycotoxicoses caused by toxins produced by different genera of fungi has been published [173,174]. The most studied and well documented case of Fusarium mycotoxicoses in humans involved alimentary toxic aleukia (ATA), a disease wide-spread among the population of the Soviet Union during the post World War II years [74]. The disease was associated with the ingestion of over-wintered grains which became infected with F. poae and F. sporotrichioides. The clinical course of ATA follows a number of stages, progressing from mild inflammation in the digestive system to a leukopenic stage in which leukocytes decrease in number due to disturbances in the bone marrow. This loss of leucocytes leads to impaired functioning of the immune system. Petechial hemorrhages appear on the skin and necrotic angina in the mouth and in the lungs, stomach, and intestine. The last stage is the stage of convalescence in which either death or a period of recovery occur. It was later established that strains of F. sporotrichioides and F. poae associated with ATA produce the trichothecenes neosolaniol, HT-2 toxin, T-2 tetraol, and a large quantities of T-2 toxin [113,155].

Literature on Fusarium mycotoxicoses in domestic animals and poultry is abundant. The various Fusarium mycotoxicoses reported to occur in cattle and sheep [86,124], horses [20], swine [87,88], and poultry [75] have been thoroughly reviewed. Moldy corn toxicoses in cattle, reported by Hsu et al [65], and Marasas et al [98] was associated with

many different fungi, but predominantly F. tricinctum. T-2 toxin produced by these fungi was the cause of death of many lactating cows after prolonged ingestion of a diet containing 60% ground moldy corn [65]. Infected corn fed to swine caused emesis [41,159] and estrogenic syndrome [112].

Biological activities of Fusarium toxins:

The trichothecenes, when applied to shaved skin of rats or rabbits, produce severe local irritation, inflammation, desquamation, subepidermal hemorrhaging and general necrosis [164]. When trichothecenes are administered orally, necrotic lesions may develop in the mouth, stomach and intestine. This is usually accompanied by a hemorrhage and degeneration of cells of the bone marrow, and lymph nodes [133].

These biological activities have been used as bioassay methods to detect the presence of the trichothecenes [43,154,156]. The bioassay techniques include the dermal (rabbit or mice) toxicity test, cytotoxicity, and inhibition of protein synthesis in rabbit reticulocytes. These tests are more sensitive than the earlier TLC and GLC techniques, but do not permit differentiation of the different trichothecenes.

The LD50 values of the trichothecenes vary somewhat with the particular toxin, the animal species studied and the route of exposure of the animals [117]. The more toxic members of the group, ie. with lower LD50s, include T-2 toxin,

Fusarenon-x, nivalenol and DAS. One of the least toxic compounds in the group is DON [117].

The trichothecenes are metabolized in animals. DON was converted by rumen microorganisms into 3,7,15-trihydroxy trichothec-9,12-dien-8-one within 24h [82]. The major metabolites in urine and feces of rats following intravenous, oral and dermal administrations of tritium-labeled T-2 toxin were 3'-OH-HT-2 and T-2 tetraol [120]. DON was found in the eggs following oral administration to laying hens [122].

Present evidence indicates that trichothecene toxins are not tumorigenic or mutagenic [117]. However, a few trichothecenes (T-2 toxin and DON) showed teratogenic effects on embryonic and fetal development in mice [64,79].

Zearalenone is an estrogenic mycotoxin that affects reproduction. Estrogenic syndrome in swine includes swollen and edematous vulva, enlarged and edematous uterus, general atrophy of the ovaries, and abortion [112]. Zearalenone also inhibits fetal development and litter size [91].

Phytotoxicity of Fusarium toxins:

Most of the trichothecenes tested have been found to be phytotoxic. For example, T-2 toxin is able to produce significant growth inhibition of tobacco callus tissue at very low concentrations [60]. The phytotoxicity of T-2 toxin has been confirmed by Marasas, et al [98], Burmeister and Hasseltine [25] and Burmister et al [24]. Moniliformin

metabolites produced by F. moniliforme cause disruption of mitotic cell division in maize [145]. Diacetoxyscirpenol, a trichothecene produced by F. equiseti, has been reported to be toxic to pea seedlings [19]. DON and T-2 toxin are able to inhibit the incorporation of ^{3}H-leucine in maize and wheat tissue [27]. At the whole plant level, germinating seeds of higher plants are extremely sensitve to the presence of trichothecenes [25,39,98]. Because of the sensitivity of plants to the trichothecene mycotoxins, Marasas et al [98] and Burmeister and Hesseltine [25] have suggested that plant systems such as pea seedlings be used for biological assay of low-order trichothecene contamination in food and feeds.

Manka et al (1985) reported the more virulent species such as F. culmorum and F. graminearum, that caused severe FHB, produced considerably higher quantities of DON, 3-ADON, and zearalenone than the less virulent ones such as F. avenaceum, F. equiseti and F. solani [93]. In contrast, a recent report by Adams et al (1989) indicated that DON and 15-ADON were not pathogenicity or virulence factors for G. zeae [3]. They demonstrated that some strains of the species were nontoxigenic but highly virulent ear mold pathogens of maize.

Resistance of wheat cultivars to FHB and Fusarium toxins:

A number of factors may play a role in the resistance of wheat to FHB. Previously, two types of resistance - Type I: resistance to initial infection and Type

II: resistance to hyphal invasion - were reported [128]. Type I resistance involves a number of characteristics many of which have to do with the morphology of the spike of a cultivar and are essentially escape mechanisms rather than resistance *per se*. Type II resistance is not adequately understood. It is in part related to the chemical and physical structure of the plant and may include the concentration of such compounds as phenols and triticenes [139] and the lignification process that takes place after infection [125]. Miller *et al* (1985,1986) recently demonstrated the presence of another type (Type III) of resistance resulting from the ability of FHB-resistant cultivars to degrade DON [103,107,108]. A fourth type of resistance (Type IV) was also recently reported by Wang and Miller (1987). They found that coleoptile tissue segments from FHB resistant wheat cultivars tolerated significantly high concentrations of DON and 3-ADON when compared to the susceptible cultivars [161].

Sources of resistance and field screening techniques for use in wheat breeding for FHB resistance are now available [92,162]. The level of mycotoxin contamination in grain and the amount of fungal biomass on infected heads also can be used to evaluate the relative resistance of cultivars in the field [108,151]. Greenhouse and laboratory inoculation methods for *Fusarium* on wheat seedlings have been demonstrated [102]. Coleoptile bioassay techniques using purified *Fusarium* toxins have been suggested for rapid *in vitro* germplasm screening for

FHB resistance [105]. Such early testing showed good correlation to the field tests.

Cultural practices such as field sanitation, crop rotation, the use of high quality seed, proper fertilization and seed and foliage fungicide applications can substantially reduce Fusarium diseases including FHB in cereals [101,163]. The use of resistant cultivars, however, remains the most reliable means of controlling the disease.

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CHAPTER 3

Fusarium INFECTION OF STORED SEEDS AND THE INCIDENCE OF *Fusarium* HEAD BLIGHT (FHB) IN WHEAT FIELDS IN ETHIOPIA

ABSTRACT

Fusarium infection of wheat was surveyed on 301 seed samples collected from basic (breeder) seeds, seed production, demonstration fields, state farms and farmers in Ethiopia. Samples were from wheat produced in 1987 which was a relatively dry season in most growing areas. *Fusarium* was detected in 35 to 63% of the samples. The levels of seed infection per sample ranged from 1 to 17%.

Conditions during the 1988 growing season were conducive to the development of FHB in most wheat growing areas. On some state farms, as many as 85% of the plants exhibited head scab symptoms with the percentage of infected spikelets per head ranging from 5 to 80%. Low levels of FHB were recorded at farmer fields which were planted late with mostly landrace varieties. The newly released high yielding cultivar Dashen was very susceptible to FHB in the field. The old cultivar Enkoy exhibited some field resistance.

While harvesting by machine significantly reduced the proportion of bad seeds as compared to manually harvested samples, machine harvested grain still retained a high level

of shriveled and Fusarium infected seeds.

The results of both seed infection and FHB field surveys indicated that Fusarium diseases in general and FHB in particular can be major problems to wheat production. It is suggested that breeding cultivars for FHB resistance should be one of the objectives of the wheat improvement program.

INTRODUCTION

Cereal diseases incited by Fusarium species are distributed world wide [19]. Fusarium diseases such as head blight, seedling blight, root, crown and foot rots of wheat, barley and oats have frequently been reported [2,8,10,11,12,13,14,17]. The importance of Fusarium head blight has significantly increased with the knowledge of mycotoxins produced by the pathogens and the health hazards they present to human and animals by ingesting contaminated grain and grain products [9,16,21].

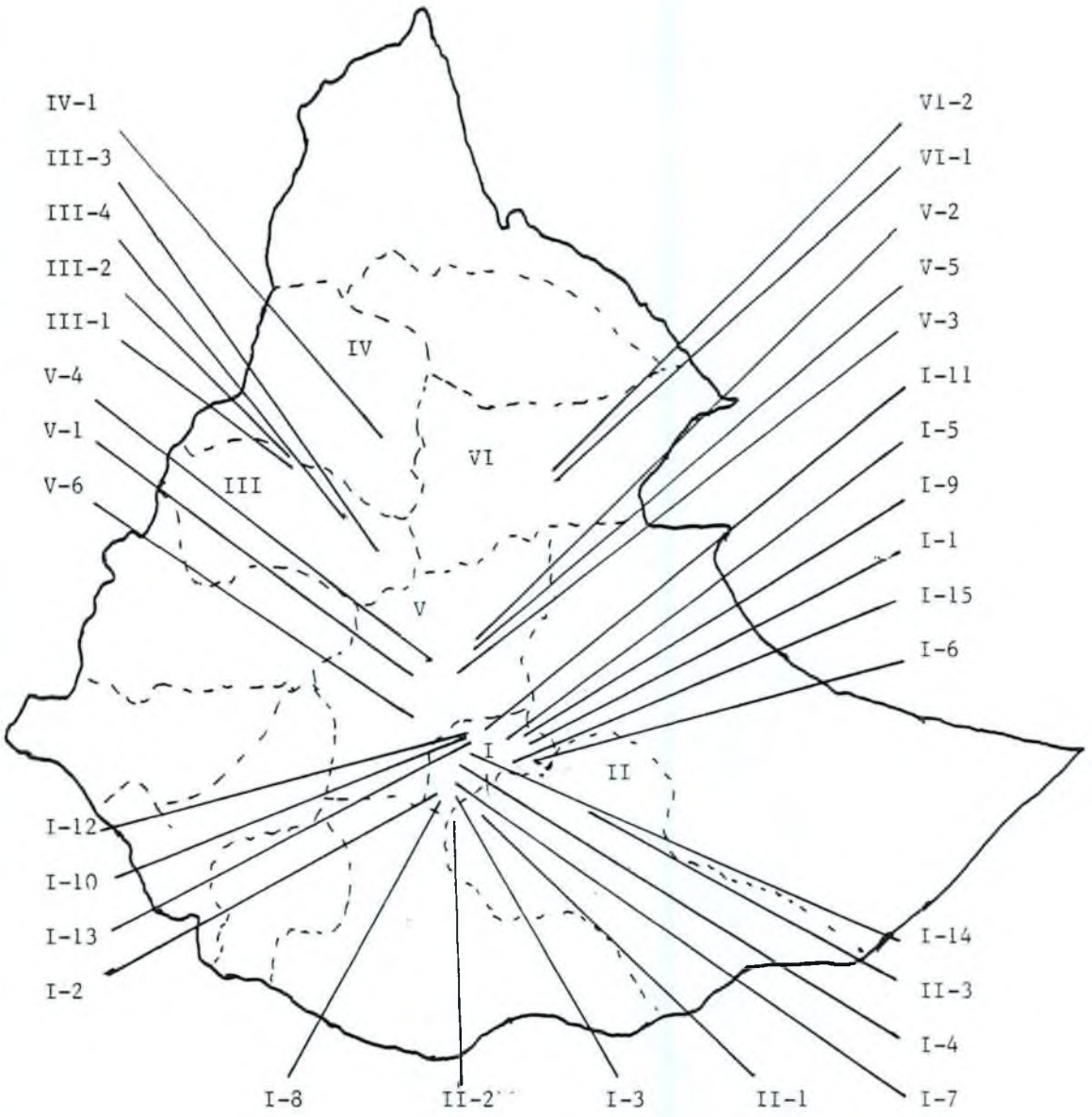
Fusarium head blight is best recognized on emerged immature heads of wheat where one or more spikelets or the entire head appears prematurely bleached [19]. Bleached spikelets are often sterile or contain poor quality seed that produce seedlings of low vigor [5,15]. Severe epidemics of head blight have frequently been reported in North America [1,18,20]. In Ethiopia, Fusarium has occasionally been reported to cause foot rot and head blight [3,6]. The extent of the problem, however, has not been investigated. This study

was undertaken to determine the level of Fusarium infection or contamination on stored wheat seeds and to survey the occurrence and distribution of Fusarium head blight (FHB) in wheat fields in the country.

MATERIALS AND METHODS

Fusarium contamination on stored wheat seeds:

Seed samples: Fifty to 100 g of wheat seeds were collected during field trips and/or by research staff in Arsi, Bale, Gojam, Gonder, Shoa and Wollo administrative regions during May through September, 1988. Approximate locations of the sampling areas are shown in Fig 3-1. Thirty four samples were collected from basic (breeder) seed production, 16 from demonstration fields, 16 from seed farms, 19 from state farms and 216 from peasant farmers. A total of 301 seed samples were collected. Thirty of the farmers' samples that were collected from Gojam, Gonder and Shoa were kindly provided by the Plant Genetics Resource Center (PGRC). The majority of seeds were harvested during the 1987 growing season and stored under various storage conditions that ranged from primitive farmer methods to improved seed farms and state farms methods.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V
I
II
I-12
I-10
I-13
I-2
I-14
II-3
I-4
I-7
I-8
II-2
I-3
II-1

Detection of Fusarium on wheat seeds: Seeds from each sample were rolled in cheesecloth and surface disinfected by immersing first in 70% ethanol for 15 sec. and then in 1% sodium hypochlorite (Clorox, 5.25% sodium hypochlorite) for 30 sec. Seeds were allowed to dry in the inoculation hood for a few minutes and plated on potato sucrose agar (PSA) plates as described elsewhere [7]. Potato sucrose agar was prepared as follows:

500 ml potato extract.

20 g sucrose.

7.5 g agar

500 ml deionized water

These were mixed and autoclaved at 15 psi for 20 min. (The potato extract was prepared by boiling 200 g of peeled and diced potato in 500 ml water for 10 min. The suspension was filtered through cheesecloth and the volume was adjusted to 500 ml with water). Two milliliters of 50% lactic acid was added to a liter of autoclaved PSA to reduce bacterial contamination. Approximately 20 ml PSA was poured into 100 x 15 mm sterilized disposable plastic petri dishes. One to two hundred seeds per sample, twenty seeds per petri dish, were plated. The plates were incubated at 20 to 24 C with 12 h fluorescent light for 7 to 10 days.

Fusarium colonies were identified by the presence of typical conidia when viewed under a microscope and then

counted. Representative colonies were transferred to new plates to be used for the identification of the species (Chap. 4). Non-sporulating colonies suspected to be Fusarium were also subcultured on new PSA plates and incubated at a lower temperature (when F. nivale was suspected). Slow growing and late sporulating colonies were re-examined after 15 and 20 days of incubation. The percentage of seed infection was determined from the colony count.

Survey of FHB in wheat fields:

Fields surveyed: FHB surveys were made from September to late November 1988 when the wheat was in flowering to late dough stages depending upon location. Research plots, seed farms, state farms and farmer fields in Arsi, Bale, Gojam, Gonder and Shoa regions (Fig 3-1) were surveyed. FHB was more severe in earlier planted sections (blocks) on some state farms. Collections were made from these sections. Most farmer fields were planted late in the season and only early planted fields were surveyed.

Evaluation method: Plants with blighted and non-blighted heads were counted in 0.5 x 0.5 m quadrants to determine the incidence of FHB. Depending on the size of the field, four to six diagonal counts were made for each field. The severity of the disease was determined by counting the number of infected spikelets per head.

Detection of Fusarium: A few heads with typical FHB symptoms were collected, threshed and plated on PSA plates as described

earlier. The number of Fusarium colonies were counted and representative colonies were sub-cultured on new PSA plates for species identification (Chapt. 4).

Evaluation of seed quality: Seeds from severely FHB infected fields in four state farms were collected and the percentage of shriveled, germinating and Fusarium infected seeds were determined. Infection on seeds was determined by plating 100 seeds per sample on PSA as described before. Fusarium colonies were counted 7 to 10 days later. The effect of machine (combine) harvesting of such fields on the percentage of shriveled and infected seeds was determined. Machine harvested seed samples were compared with manually harvested seed samples from the same fields in terms of the percentage of shriveled, infected and germinating seeds they contained. Manually harvested samples were assumed to contain all of the possible shriveled, infected and healthy seeds.

All laboratory work was carried out from June to December 1988 at Holetta Research Center, Institute of Agricultural Research, Ethiopia.

RESULTS

Fusarium seed infection:

The level of Fusarium infection or contamination of stored seed samples collected from basic seeds, seed production, demonstration fields, state farms and farmer's fields are shown in Tables 3-1 to 3-5. Fifty percent of the

Table 3-1. Percent *Fusarium* infection on breeder (basic) seed sample harvested in 1987 from four locations.

Location[a]	Wheat species	Cultivars	% *Fusarium* seed infection
Bichena (III-3)	Tetraploid	Boohai	0
	"	Cocorit71-CandealII	0
	"	Bichena local	0
Debre Zeit (V-3)	Tetraploid	Boohai	3
	"	Cocorit 71	0
	"	Cocorit71-CandealII	1
	"	Dz04-118	2
	"	Gerardo	1
Holetta (V-4)	Hexaploid	Bonde	0
	"	Dereselign	13
	"	Gara	9
	"	Israel	8
	"	Kanga	17
	"	Laketch	15
	"	Mamba	1
	"	Romany BC	0
	"	Salmayo	2
	"	6290-Bulk	4
Kulumsa (I-13)	Hexaploid	Bonde	0
	"	Dashen	0
	"	Dereselign	0
	"	Enkoy	1
	"	ET-13	0
	"	Gara	0
	"	HAR-407	1
	"	HAR-416	0
	"	Kanga	2
	"	Laketch	5
	"	Mamba	0
	"	Pavon-76	2
	"	Romany BC	0
	"	Salmayo	0
	"	6290-Bulk	0

a. Numerals in parenthesis are location codes, see Fig. 3-1 for description.

Table 3-2. *Fusarium* infection of wheat seeds grown for seed production by Arsi Rural Development Unit (ARDU) in 1987.

Farms	Location[a] code	Cultivars	% *Fusarium* infection
Ardayta	I-2	K6295-4A	0
Asasa	I-1	Enkoy	1
		Romany BC	0
Dinkity	I-6	Enkoy	0
Gonde	I-10	Enkoy	0
		Dashen	0
		Gara	0
Herero	II-1	Enkoy	1
Iteya	I-12	Dashen	3
		Enkoy	0
		ET-13	0
		K6295-4A	0
Kulumsa	I-13	Boohai (Tetraploid)	3
		K6290-Bulk	1
		Romany BC	2
Serufta	II-2	Enkoy	1

a. See Fig. 3-1 for description of locations.

Table 3-3. Fusarium infection of wheat seeds produced at IAR/ADD demonstration field at Bichena (location=III-3) in 1987.

Wheat species	Cultivars	% Fusarium infection
Hexaploid	Dashen	2
	ET-13	0
	Enkoy	0
	HAR-407	1
	HAR-416	0
	Bulga (Local)	0
Tetraploid	Boohai	0
	Boohai "s'	1
	Cocorit 71	2
	Cocorit 71-Candeal II	0
	Cr"s'mexi"s'L-92	2
	DZ-04-118	0
	Hora	1
	Illumilo/Cit71-Bahun	0
	Yemen Cit"s"	0
	Wassma	0

Table 3-4. *Fusarium* infection of wheat seeds produced by the state farms in 1987.

State farms	Location code[a]	Cultivares	% *Fusarium* Infection
Adele	I-1	Dashen	2
		Enkoy	0
Diksis	I-5	Dashen	13
		Enkoy	0
Dinkiti	I-6	Dashen	8
		Enkoy	0
Garadella	I-7	Dereselign	3
		Enkoy	0
		K6290-Bulk	0
		K6295-4A	0
Gofer	I-8	Dashen	4
		Enkoy	2
Gololcha	I-9	Dashen	5
		Enkoy	0
Lole	I-14	Dashen	9
Robe	I-15	Enkoy	3
Serufta	II-2	Dashen	6
		Enkoy	1
Sinana	II-3	Enkoy	1

a. See Fig. 3-1 for location description.

Table 3-5. *Fusarium* infection on wheat seeds (mostly landraces of tetraploids) produced by peasant farmers in 1987 in different wheat producing regions of Ethiopia.

Regions & locations[a]	Location code[b]	No. of samples[c]	% infect. samples	% *Fusarium* infection
Arsi	I			
Asasa	I-3	33	64	1-7
Bekoji	I-4	8	0	0
Huruta	I-11	9	44	1-13
Robe	I-15	19	21	1-6
Bale	II			
Sinana	II-1	11	0	0
Gojam	III			
Adet	III-1	2	100	1-4
Bahir-dar	III-2	11	18	1-3
PGRC/E[d]	III	10	50	1-6
Gonder	IV			
Debre-tabor	IV-1	2	0	0
PGRC/E[d]	IV	10	20	2
Shoa	V			
Debre-brhan	V-2	13	54	1-3
Debre-zeit	V-3	21	29	1-5
Holetta	V-4	34	24	1-3
Wolisso	V-6	7	43	1-2
PGRC/E[d]	V	10	20	1-2
Wello	VI			
Sirinka	VI-1	10	60	1-4
Woldia	VI-2	6	50	1-2
	Total	216		

a. Samples collected from farmers approximately 20 to 30 km radius from the locations shown.
b. See Fig. 3-1 for description of locations.
c. seed samples constitute of mixtures of land races of mostly tetraploid (durum) wheats.
d. collected and provided by the Plant Genetic Resource Center of Ethiopia.

tetraploid and 56% of the hexaploid wheat in the samples from basic seed production showed 1-2% and 1-17% Fusarium infection, respectively (Table 3-1). Forty-four percent of the samples from seed production fields of the Arsi Rural Development Unit (ARDU) showed 1-3% seed infection (Table 3-2). Low level (1-2%) seed infection was observed on 37% of the samples collected from the demonstration field at Bichena (Table 3-3). About 63% of the samples showed 1 to 13% seed infection on different cultivars grown on the state farms (Table 3-4). These results also indicate that seeds from the cultivar 'Dashen' were more frequently infected than seeds from the cultivar 'Enkoy'. Fusarium was detected on about 35% of the seed samples collected from farmers in diverse wheat growing regions of the country (Table 3-5). The percent of seed infected per sample ranged from 1 to 13%.

Incidence of FHB in wheat fields:

Conditions in the 1988 growing season were unusually favorable for FHB development in many wheat growing areas. Temperature and rain fall in some of the surveyed areas for August, September and October, 1988, during which most wheat was flowering and FHB development peaked, are shown in Table 3-6. The temperature ranged from 10 to 25 C for most areas during this period. There were 22 to 30, 17 to 26, and 4 to 18 rainy days in August, September and October, respectively. The amount of rainfall shown represents a 9 to 135% increase

Table 3-6. Altitude, mean daily temperature ranges and monthly rainfall for the three months in 1988 during which FHB was severe in some selected locations[a].

Locations codes[b]	Altitude (m)	Temperature(C)[c] Aug.	Sept.	Oct.	Rainfall (mm)[d] Aug.	Sept.	Oct.
Exept. centers							
Adet (III-1)	2080	11-22	10-23	9-23	270(26)	169(23)	130(18)
Debrezeit(V-3)	1900	13-24	12-25	9-25	25(22)	190(23)	17(4)
Holetta(V-4)	2400	10-19	9-19	5-21	284(30)	240(26)	32(5)
Kulumsa(I-13)	2200	11-19	11-21	11-22	123(27)	149(25)	30(7)
Mota	2440	18-21	19-22	17-22	323(24)	148(17)	159(12)
State farms							
Dixis(I-5)	2680	-	-	-	218(28)	183(25)	36(15)
Garadela(I-7)	2420	4-25	4-24	5-25	166(24)	91(17)	48(7)
Gofer(I-8)	2550	4-20	3-22	2-22	234(26)	121(19)	113(14)
Hunte(II-1)	2380	9-22	8-22	6-22	200(28)	11(19)	48(10)
Lole(I-14)	2430	5-20	5-21	5-23	146(22)	100(19)	24(9)
Serufta(II-2)	2300	9-19	8-20	7-21	158(21)	117(17)	50(11)

a. The major growing season is from June to end of November
b. See Fig. 3-1 for location descriptions.
c. Temperature range is minimum-maximum.
d. Numbers in parenthesis are number of rainy days in the month.

over the monthly mean rainfall for the three months in these locations.

Percentage of wheat plants with scabby heads (incidence), percentage of infected spikelets per head (severity) and crop stage taken on experimental plots, state farms, seed farms and farmer fields are presented in Tables 3-7,3-8,3-9 and 3-10, respectively. The values given for the experimental plots at each location are means of a large number of evaluations on different breeding lines and cultivars. FHB incidence and severity at the research centers ranged from 0 at Bekoji where the crop was just flowering to 56 and 44%, respectively, at Kulumsa where the crop was maturing (Table 3-7). At some state farms, as high as 85% incidence and 80% severity were recorded in early sown fields (Table 3-8). The cultivar Dashen was the most severely affected. The wheat cultivars planted at the relatively drier Garadel state farm matured early and no FHB was observed. Two cultivars, HAR407 and HAR416, were affected more (57 and 34% incidence and 40 and 30% severity, respectively) than 'Dashen' in seed production fields (Table 3-9). These are newly released cultivars at the seed multiplication stage. Low level FHB was recorded on wheat at farmer fields (Table 3-10). Most wheat grown by farmers were landrace cultivars and were planted late in the season. They were tillering or just

Table 3-7. FHB field survey, mean values for different wheat cultivars and lines at experimental plots during the 1988 season.

Experiment stations	Location code[a]	Incidence[b] (%)	Severity[c] (%)	Crop stage
Asasa	I-3	0	0	matured
Bekoji	I-4	0	0	flowering
Kulumsa	I-13	56 (5-80)	44 (30-80)	dough
Robe	I-15	0	0	flowering
Sinana	II-1	0	0	heading
Adet	III-1	10 (1-20)	15 (0-20)	dough
Mota	III-4	10	10	tillering
Debre-tabor	IV-1	8 (5-10)	10	dough
Ambo	V-1	trace	trace	flowering
Debre-zeit	V-3	5 (0-10)	10	maturing
Holetta	V-4	28 (1-70)	35 (10-80)	dough
Sheno	V-5	0	0	matured

a. See Fig. 1 for location code
b. Percentage of FHB infected plants in 0.5 x 0.5 m., mean of 4 to 6 counts. Values in parenthesis are ranges.
c. Percentage of FHB infected spikelets per head. Values in parenthesis are ranges.

Table 3-8. FHB evaluation on different cultivars planted at the state farms during the 1988 season.

State farms[a]	Cultivars	Incidence (%)	Severity (%)	Crop stage
Diksis (I-5)	Enkoy	<1	0	dough
	Dashen	60	30	dough
Garadela (I-7)	6290-Bulk	-	-	matured
	Dereselign	-	-	matured
	Enkoy	-	-	matured
	K6295-4A	0	0	maturing
	Pavon-76	0	0	maturing
Gofer (I-8)	Dashen	85	65	dough
Herero (II-1)	6290-Bulk	<1	5	dough
	Dereselign	0	0	dough
	Enkoy	0	0	dough
	ET-13	0	0	dough
Hunte (II-1)	Dashen	19	20	dough
Lole (I-14)	Batu	65	70	dough
	DAshen	84	80	dough
Serufta (II-2)	Batu	70	80	dough
	Dashen	80	45	dough

a. Each state farm is several thousands of hectares, divided in a number of blocks with different sowing dates. Some blocks have up to 30 days difference in sowing dates. FHB was more severe on earlier planted blocks and these evaluations were made on those blocks. See location codes (parenthesis) in Fig. 3-1.

Table 3-9. FHB evaluation of different cultivars in seed production fields during the 1988 season.

	Locations[a]							
	V-4		I-13		I-12		I-10	
Cultivar	Inc.[b]	Sev.[c]	Inc.		Sev.		Inc.	Sev.
Inc.	Sev.							
Dashen	21	60	5	20	6	10	4	4
Enkoy	<1	<1	-[d]	-	-	-	0	0
ET-13	5	<1	-	-	<1	<1	-	-
HAR407	57	40	18	35	-	-	-	-
HAR416	34	30	-	-	-	-	-	-
Pavon76	-	-	23	30	-	-	-	-

a. See Fig. 3-1 for locations
b. Inc. = Incidence (Percentage of plants with FHB symptom)
c. Sev. = Severity (percentage of infected spikelets per head)
d. - = The cultivar was not planted at that location

Table 3-10. FHB evaluation on wheats grown on peasant farmer fields and cooperative farms during the 1988 season.

Regions & locatins[a]	Location code	Incidence (%)[b]	Severity (%)[b]	Crop stage[c]
Arsi	I			
Asasa	I-3	0	0	maturing
Bekoji	I-4	0	0	flowering
Huruta	I-12	<1	<1	flowering
Robe	I-16	0	0	flowering
Bale	II			
Herero		<1	<1	dough
Hunte		<1	<1	dough
Gojam	III			
Adet	III-1	5	<1	dough
Mota	III-4	0	0	tillering
Gonder	IV			
Debre-tabore	IV-1	7	5	milk
Shoa	V			
Debre-brhan	V-2	0	0	milk
Debre-zeit	V-3	5	1	dough
Holetta[d]	V-4	0-35	0-50	dough

a. Location include a 10 to 20 km. radius .
b. Values are means of several fields at each location .
c. The wheats grown were mostly landrace cultivars and were planted late in season. FHB evaluation was only on a few heading fields.
d. Almost all evaluations were on fields planted with improved cultivars.

flowering at the time of the survey. The highest incidence and severity of FHB was recorded on farmer fields near the Holetta Research Center where improved cultivars and early planting (as recommended) have been used.

Seed quality of FHB infected Crop:

The percentage of shriveled, germinating and Fusarium infected seeds were determined from severely infected blocks in four state farms (Table 3-11). Some of the samples contained as much as 78% shriveled seed with a germination rate as low as 44%. Fusarium infection of the seed ranged from 17 to 56%. The small differences in sowing date of some fields seemed to have no effect on FHB severity. Machine harvesting significantly reduced the amount of shriveled seed at Dixis, Lole and Serufta state farms when compared to manually harvested (which included all bad seeds) seed samples from the same blocks (Fig. 3-2). The harvesting machines at Serufta state farm were more efficient in reducing both shriveled and Fusarium infected seeds and increasing the proportion of germinating seeds than the machinery used at Gofer farm.

DISCUSSION

The high level of seed infection by Fusarium in 35 to 63% of the samples collected from various sources and regions indicated the potential importance of FHB to wheat

CHAPTER 4

Fusarium SPECIES ON STORED SEEDS AND SEEDS FROM SCABBY HEADS OF WHEAT IN ETHIOPIA

ABSTRACT

Seventeen *Fusarium* species were identified from stored wheat seed samples obtained from basic (breeder) seed, seed production, farmers' demonstration fields, state farms and farmers. The most frequent species that constituted 10% or more of the isolates were *F. avenaceum*, *F. lateritium*, and *F. equiseti* in basic seed samples; *F. avenaceum* and *F. nivale* in seed production samples; *F. poae* and *F. nivale* in samples from demonstration fields; *F. nivale* and *F. semitectum* in the state farm samples; and *F. equiseti*, *F. graminearum* and *F. nivale* in farmers' samples. A larger number of species were isolated from samples collected in Arsi and Shoa regions where wheat is grown more extensively.

Thirteen *Fusarium* species were identified from scabby wheat heads collected during the field surveys in 1988. The most frequent species that make up 10% or more of the isolates were *F. nivale*, *F. graminearum*, and *F. avenaceum* at research centers in four regions; *F. nivale* and *F. avenaceum* at the state farms; and *F. nivale*, *F. avenaceum* and *F. graminearum* in farmer fields. *F. nivale* and *F. avenaceum* were most prevalent at the cool and moist high altitude areas such

as Holetta Research Center and Arsi region where the Kulumsa Experiment Station and most of the state farms are located. F. graminearum was more frequent in the northwestern regions (Gojam and Gonder) and also at the relatively lower and drier areas such as Debrezeit. Species like F. sporotrichioides and F. poae which are important because of their ability to produce mycotoxins also occurred but less frequently and with limited distribution.

INTRODUCTION

Problems in identifying species of Fusarium and the existence of several systems of nomenclature frequently make literature comparisons difficult at the species level. There are, however, a large number of Fusarium species reported to incite diseases such as scab, snow mold, and foot, root and crown rots in cereals. Gordon (1952) identified 16 species and varieties of Fusarium from a large number of wheat, barley and oat seed samples collected from all over Canada [7]. F. poae, F. avenaceum and F. acuminatum were the most frequently identified species. Abnormally high summer rainfall in 1985 in southern Manitoba resulted in a high incidence of Fusarium head blight symptoms in wheat [1]. The major species identified in samples of wheat delivered to primary elevators were F. graminearum, F. sporotrichioides, F. poae, F. acuminatum, F. avenaceum, F. equiseti and F. culmorum. Wilcoxson, et al (1988), identified 15 Fusarium species from

scabby wheat collected in 1984, 1985 and 1986 from farm fields and agricultural experiment stations in Minnesota [18]. F. graminearum, and F. poae were prevalent. In Central Washington State, F. graminearum, F. culmorum, F. nivale and F. avenaceum have been associated with scab in irrigated wheat fields [15]. F. nivale was frequently reported as a major species causing both leaf disease and head scab in Europe [6]. This species predominantly occurs in relatively colder areas of Northern Europe and the Alpine region. It was also reported to cause both snow mold and scab of wheat in Northwestern Washington in 1980 [9].

A very limited survey was made in Ethiopia to determine the level of Fusarium on cereal seeds and its role as a causal agent of head blight. F. graminearum was identified in 1973 on wheat seed samples during routine seed health testing [2]. A year later, in 1974, this species was reported for the first time in the country causing head blight in wheat and triticale at Holetta Research Center [3]. F. longipes and F. semitectum were identified later from blighted heads of wheat [10]. Stewart and Yirgu (1967) identified F. culmorum from roots of rotting wheat [14]. Wheat seeds produced in 1979 at Holetta Research Center were found to be infected with F. avenaceum, F. dimerum, F. graminearum and F. moniliforme [4]. These identifications were based on a few samples, usually collected from research plots. The purpose of the study described here was to identify the Fusarium species

isolated from stored seeds and scabby heads of wheat (Chapter 3) and report their distribution in major wheat growing areas of Ethiopia.

MATERIALS AND METHODS

Isolation and culturing of *Fusarium*:

Fusarium species were isolated from stored seed samples and from kernels of scabbed spikes collected during field surveys as described before (Chapter 3). Representative colonies were transferred to new potato sucrose agar (PSA) plates and incubated at 20 to 24 C with 12 h fluorescent light for 7 to 10 days. Petri dishes were rechecked after 15 and 20 days for slow growing isolates.

Attempts were made to stimulate sporulation in nonsporulating isolates. Isolates suspected of being *F. nivale* were re-incubated at a lower (14-15 C) temperature. In some nonsporulating isolates, aerial mycelium was scraped off the surface of the agar and washed with several changes of sterile distilled water and re-incubated [5]. Other isolates were transferred to carnation Leaf Agar (CLA) to promote sporulation rather than mycelial growth as suggested by Nelson *et al* [11].

CLA was prepared as follows: Young carnation leaves were harvested , cut into approximately 10 mm long pieces, placed in a fruit jar and dried in an oven at 50 C for 2 h. The leaf pieces were then sterilized by propylene oxide

fumigation as described by Hansen et al [8]. The leaf pieces were moistened slightly by lightly atomizing them with water. Propylene oxide at a rate of one ml per liter capacity of the container was introduced in the jar and the jar was tightly closed. The jar was shaken, kept in a desiccator and placed in a hood overnight. The lid was then loosened to permit the fumigant to escape. CLA was prepared by placing several sterile leaf pieces in a petri dish and floating them on 2% water agar cooled to 45 C. The dishes were left at room temperature for 4 days before use to allow the growth of possible contaminants from leaf pieces.

Single spore isolation:

Single spore isolation was made from each of the the Fusarium isolates as described by Booth [5]. A drop of sterile water was placed on a sterile slide under a dissecting microscope. Spores were picked up from a sporodochium by touching it with the wet tip of a needle. The tip of the needle was then introduced into the drop of water on the slide. The needle was withdrawn when the individual spores in the suspension were distinguishable (not obscured by overlapping). The spore suspension was then picked up by a sterile loop and streaked across a water agar plate along the lines made on the bottom of the petri dish. The plates were incubated for 15-20 h at 20-22 C. Clearly positioned individual germinating spores were located using a low power compound microscope, picked up with some agar on a sterile

needle and transferred to slant of PSA. The tubes were incubated at 20-24 C and 12 h fluorescent light (40 watt) for 7 to 15 days.

Identification of Fusarium species:

Fusarium species were identified according to the keys outlined by Booth [5]. Such characters as growth rate, presence or absence of microconidia, formation of micro-and macroconidia (from simple phialides or polyphialides), shape, septation and size of both types of conidia, and culture pigmentations were considered. Additional information was obtained from the manual by Nelson et al [11]. Most identifications were made on 7 to 10 day cultures, but also on 15 day or older cultures when dealing with slow growing isolates. All isolates used for identification were subcultured from either the single spore isolates on PSA slants or from cultures stored on silica gel.

Storage of Fusarium on silica gel:

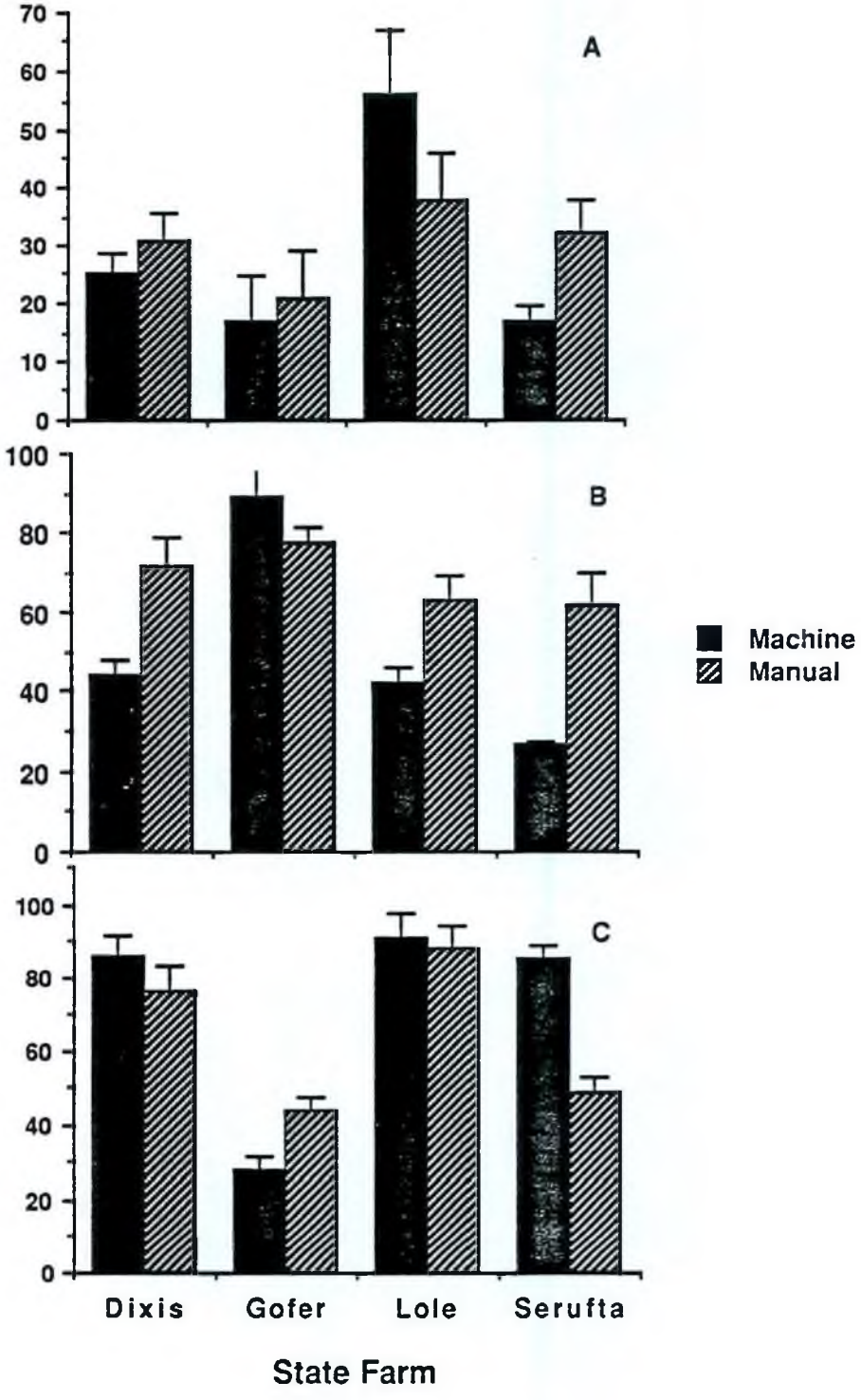
Representative isolates were transferred to silica gel for long term storage according to the procedure described by Perkins [12] and Windels et al [19] with some modifications. Pyrex screw-cap tubes (16x150 mm) were filled with approximately 8g silica gel (specification Mil-D-3716, grade H type 2, Mesh size 6-12, code 400808237, Davidson Chem. Co., Baltimore, MD) and eight tubes were placed in wide mouth Mason jars with about 1 inch Drierite crystal (with indicator dye and No. 8 mesh, Hammond Drierite Co. Xenia, Ohio). The

Table 3-11. Characterization of wheat seeds harvested from severely FHB infected blocks of four state farms during the 1988 season.

State farms[a]	Cultivar	Field no. (Pl.date)	Inc. (%)	Shr. (%)	Ger. (%)	Inf. (%)
Dixis	Dashen	C-19(29/5)	24	59	83	24
(I-5)		C-21(29/5)	50	59	82	34
		C-13(2/6)	90	44	86	25
		B-12(3/6)	97	29	89	37
Gofer	Batu	C-53(4/6)	15	32	65	18
(I-8)	Dashen	C-47(4/7)	71	40	67	39
		C-44(5/6)	77	69	43	35
		B-28(10/6)	95	78	44	21
Lole	Batu	B-9(11/5)	65	23	86	34
(I-14)	Dashen	B-A1(1/7)	84	42	91	56
Serufta	Batu	1-34(26/5)	82	50	70	27
(II-2)		1-15(26/5)	91	26	89	31
	Dashen	2-73(13/6)	62	23	78	19
		1-23(27/5)	90	27	85	17

a. Location codes are in parenthesis, see Fig. 1 for location.

Note: Pl. date = planting date.
Inc. = incidence
Shr. = shriveled
Ger. = germinating
Inf. = infection


70
60
50
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A
100
80
60
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20
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B
100
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40
20
0
C
Machine
Manual
Dixis
Gofer
Lole
Serufta
State Farm

production in Ethiopia. Most samples with high seed infection were from basic (breeder) seeds produced at research centers and wheat grown at state farms. The variation in the level of seed infection among the different wheat species and cultivars seemed to be mainly due to differences in location. The tetraploid cultivars produced as basic seeds had zero infection at Bichena, a testing site in the middle of farmer fields (Table 3-1). Seeds from the same cultivars grown at Debre-Zeit Experiment Center showed 1 to 3% infection. The cultivar Dereselign also showed no seed infection when produced as basic seed at Kulumsa while seeds of this cultivar produced at Holetta Research Center showed as high as 13% infection. It should be noted, however, that not only environment but conditions such as cultural practices, cultivars planted, harvesting methods and storage conditions of these seed samples differed greatly and these differences may have affected the survival and, hence, the level of Fusarium infection and/or contamination of the seeds in the samples.

The cool and humid conditions on the high-lands of Ethiopia where most wheats are grown favor FHB development. Environment, temperature and moisture in particular, play a significant role in FHB development. The occurrence of continued wetness, as happened during the 1988 season in most growing areas, favors FHB which may become epidemic in proportion [2]. The results from both seed infection and FHB

field surveys indicate that the problem is more severe at the state farms and research centers. This may confirm some previous observations that diseases in general cause more problems in the research centers than in farmer fields [6]. Monocropping of wheat by the state farms provides a readily available source of inoculum for the disease. Wheat stubble and infected seeds were reported to be the major means of survival of the pathogen and sources of inoculum for the disease [4,18]. It should be noted that most basic (breeder) seeds are produced at research centers and most commercial seed farms are located on the state farms.

The very high proportion of shriveled seeds (23-78%) in early planted fields of some state farms could indicate the extent of yield losses. However, other diseases such as yellow rust (Puccinia striiformis West.) were observed in most fields and may have contributed to the shriveling. The content of shriveled and infected seeds can be reduced to some extent by machine harvesting. But such grains, especially those harvested from severely affected fields, were still not clean enough.

The old cultivar 'Enkoy' seemed to resist FHB in most state farms. It exhibited a low level of seed infection (Table 3-4) and a low level of incidence and severity in the field (Table 3-8). The cultivar 'Dashen', on the other hand, is quite susceptible to FHB. It exhibited the highest seed infection, incidence and severity of FHB. Dashen is a newly

tested for resistance to FHB since the disease has never been considered a major threat to the crop. These survey results indicate that FHB can be a major problem to wheat production in Ethiopia during wet seasons like occurred in 1988. A breeding program needs to be initiated against the disease. Other means of controlling the disease (eg. crop rotation practices at the state farms) should also be investigated.

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tubes were then dry sterilized in an oven at 180 C for 1.5 h. Seven grams of instant nonfat dry milk (Carnation brand) was dissolved in 100 ml of distilled water. About 5 ml was poured into each culture tube and autoclaved at 110 C for 15 min. The milk was used on the same day. Spores from 7 to 10 day cultures were suspended in 3 ml sterile milk. About a milliliter of the milk-spore suspension was pipetted into each silica gel tube. The tube was held nearly horizontal to distribute the suspension uniformly over the silica gel surface. The screw caps were replaced tightly, shaken immediately on a vortex mixer and plunged in crushed ice for at least 5 min. to cool the tubes. The tubes were put back into the Mason jars and stored for a week at room temperature. The cultures were checked for growth by sprinkling a few silica gel crystals on PSA plates. The silica gel cultures were stored in a refrigerator at 4 C.

The silica gel culture tubes in the Mason jars were packed and brought to the Department of Plant Pathology, University of Missouri (with USDA permit IPPQ573) for further identification and confirmation of the species and testing for their ability to produce mycotoxins.

RESULTS

Fusarium species on stored seeds:

Fusarium species and their frequencies, identified from stored seed samples collected from basic (breeder) seed,

Table 4-1. Percentage of Fusarium isolates in different species and percentage and range of seed infection on wheat seeds grown as breeder (basic) seeds in 1987

Fusarium species[a]	%	Locations[b] I-13	V-3	V-4
F. avenaceum	38	0	0	49 (0-29)[c]
F. lateritium	31	12 (0-2)	14 (0-1)	37 (0-17)
F. equiseti	10	53 (0-6)	29 (0-1)	0
F. nivale	8	0	0	11 (0-8)
F. graminearum	5	18 (0-2)	29 (0-2)	0
F. heterosporum	3	17 (0-3)	0	0
F. oxysporum	1	0	0	1 (0-1)
Fusarium spp.[d]	4	0	28 (0-2)	2 (0-2)

a. Based on 3300 isolates.
b. See Fig. 3-1 for location description.
c. Values in parenthesis are ranges of percent infection per sample.
d. Species that could not be identified due to contamination and/or insufficient structural evidence.

Table 4-2. Percentage of *Fusarium* isolates in different species and percentage and ranges for each species from wheat seeds grown for seed production at Arsi Rural Development Unit (ARDU) in 1987.

Fusarium species[b]	%	Location[a] I-2	I-3	I-6	I-10	I-12	I-13	II-1	II-2
F. avenaceum	50	0	0	0	0	100	67	0	0
F. nivale	22	0	0	0	0	0	33	0	100
F. lateritium	7	0	0	0	0	0	0	100	0
F. oxysporum	7	0	100	0	0	0	0	0	0
Fusarium spp.[c]	14	0	0	100	0	0	0	0	0

a. See Fig. 3-I for location.
b. Based on 1600 isolates.
c. Species that could not be identified due to contamination and or insufficient structural evidence.

Table 4-3. Percentage of *Fusarium* isolates in different species and percentage and range of percent seed infection from wheat seeds grown at demonstration field at Bichena (region III-3)[a] in 1987

Fusarium species[b]	%	% seed infection range
F. poae	38	0-2
F. nivale[c]	25	0-2
Fusarium species[d]	37	0-2

a. See Fig. 3-1 for the location of Bichena.
b. Based on 1600 isolates.
c. Identification based on very few late sporulating cultures.
d. Species that could not be identified due to contamination and/or insufficient structural evidence.

Table 4-4. Percentage of Fusarium isolates in different species and percentage for each species in wheat seeds grown at different state farms in 1987..

Fusarium species[a]	%	State farms[b] I-1	I-5	I-6	I-7	I-8	I-9	I-14	1-15	II-1	II-2
F. nivale	66	0	100	100	100	50	0	0	100	100	0
F. semitectum	17	0	0	0	0	33	0	100	0	0	100
F. graminearum	4	100	0	0	0	0	0	0	0	0	0
F. poae	3	0	0	0	0	17	14	0	0	0	0
Fusarium spp.[c]	10	0	0	0	0	0	86	0	0	0	0

a. Based on 1900 isolates.
b. See Fig. 3-1 for state farm locations.
c. Species could not be identified due to contamination and/or insufficient structural evidences.

Table 4-5. Mean percentage of Fusarium species isolated from wheat kernels grown by farmers in 1987 in six major wheat growing regions of Ethiopia.

Fusarium species[b]	%	Regions[a] I	II	III	IV	V	VI
F. equiseti	21	28	-	26	-	10	18
F. graminearum	15	15	-	9	-	23	6
F. nivale	11	6	-	13	-	21	-
F. poae	9	10	-	9	-	6	12
F. semitectum	7	9	-	9	-	6	-
F. sambucinum	6	12	-	-	-	2	-
F. oxysporum	4	2	-	-	-	4	24
F. heterosporum	4	3	-	13	-	2	-
F. solani	3	2	-	-	-	2	12
F. moniliforme	2	2	-	-	-	2	6
F. lateritium	2	-	-	-	-	6	-
F. avenaceum	1	2	-	-	-	2	-
F. stilboides	1	3	-	-	-	-	-
F. tricinctum	<1	-	-	-	-	2	-
F. decemcellulare	<1	2	-	-	-	-	-
F. merismoides	<1	-	-	-	33	-	-
F. udum	<1	-	-	-	-	-	6
Fusarium spp.[c]	13	4	100	21	67	12	18

a. See Fig. 3-1 for regions.
b. Based on 21500 isolates.
c. Species could not be identified due to contamination and/or insufficient structural evidence.

seed production, demonstration fields, state farms and farmers in different regions are presented in Tables 4-1,4-2,4-3,4-4 and 4-5, respectively. Three species (F. avenaceum, F. lateritium, and F. equiseti) dominated the species identified from the basic seed samples. All of the F. avenaceum and most of the F. lateritium were identified from the basic seed grown at Holetta Research Center (Table 4-1). Most F. equiseti were identified from samples grown at Kulumsa. Major species identified from seed production samples were F. avenaceum and F. nivale, all of which were identified from two locations (I-13 and I-14, Table 4-2). F. poae and F. nivale were the only species identified from demonstration field samples at Bichena (III-3, Table 4-3). F. nivale and F. semitectum were the major species identified from the state farm samples (Table 4-4). F. equiseti and F. graminearum which were less frequent on basic seed and state farm samples were more prevalent and widely distributed in farmer samples (Table 4-5). Seventeen species were identified from the farmers samples. More species were identified from samples collected in Arsi and Shoa regions; the largest wheat growing regions in Ethiopia.

Fusarium species from scabbed wheat heads:

Fusarium species identified from seeds of scabby wheat heads collected during field surveys during the 1988 growing season are summarized in Tables 4-6 to 4-8. More Fusarium species were identified from samples collected from

research centers (Table 4-6) than from state farms (Table 4-7) and farmer fields (Table 4-8). F. nivale was the most frequently identified species from research centers but it was restricted to the Holetta (V-4) and Kulumsa (I-I3) stations (Table 4-6). F. graminearum was the most widely distributed species. It was identified in five of the six research centers surveyed and was more frequent at the northern experiment stations (III-1, III-4, and IV-1) and at the Debrezeit (V-4) station in the central region. F. avenaceum was identified from samples collected from four of the six stations sampled.

F. nivale was the most frequent and widely distributed species in the state farms (Table 4-7). It constituted about 52% of the species identified and was found in all farms where FHB was recorded. F. avenaceum was the second most frequent species (37%) but was identified in samples from only 3 out of 5 state farms.

F. nivale was again the most frequently identified species (35%) from farmer field samples and distributed in three of the five administrative regions considered (Table 4-8). F. avenaceum and F. graminearum were also frequently found with the later being identified in samples from all regions. F. poae (2%) and F. lateritium (1%) were identified only from

Table 4-6. Percentage of Fusarium isolates in different species from scabby wheats and percentage of each species in six research stations in 1988

Fusarium species[b]	%	Research stations[a] I-13	III-1	III-4	IV-1	V-3	V-4
F. nivale	48	53(0-67)[c]	-	-	-	-	67(5-78)
F. graminearum	22	-	97(0-47)	39(0-22)	78(0-60)	78(0-63)	5(0-28)
F. avenaceum	15	22(0-41)	-	25(0-14)	22(0-66)	-	16(0-23)
F. stilboides	<1	4(0-7)	-	-	-	-	-
F. heterosporum	<1	-	-	-	-	-	-
F. tricinctum	<1	2(0-5)	-	-	-	-	-
F. semitectum	<1	-	-	-	-	-	4(0-5)
F. equiseti	<1	1(0-5)	-	-	-	-	-
F. sporotrichioides	<1	-	-	-	-	9(0-15)	-
F. poae	<1	-	3(0-2)	-	-	-	-
Fusarium spp.[d]	13	17(0-34)	-	36(0-20)	-	13(0-20)	6(0-13)

a. See Fig 3-1 for locations.
b. Based on 4500 isolates.
c. Numbers in parenthesis are ranges of each species per sample.
d. Species that could not be identified.

Table 4-7. Fusarium species and their frequencies (%) isolated and identified from scabbed wheat heads collected from state farms in 1988.

Fusarium species[a]	%	State farms[b] I-5	I-7	I-8	I-14	II-1	II-2
F. nivale	52	63(5-50)[c]	-	26(5-14)	100(5-73)	70(5-73)	23(22-25)
F. avenaceum	37	19(0-16)	-	63(11-32)	-	-	64(57-72)
F. graminearum		2	-	-	11(0-8)	-	-1(0-3)
F. sambucinum	2	11(1-8)	-	-	-	-	-
F. moniliforme	<1	-	-	-	-	-	1(0-2)
Fusarium Spp.[d]	8	7(0-6)	6	-	-	30(0-13)	11(0-22)

a. Based on 1800 isolates.
b. See Fig. 3-1 for locations.
c. Numbers in parenthesis are ranges per sample.
d. Species could not be identified due to contamination and/or insufficient structural evidence.

CHAPTER 5

SCREENING OF *Fusarium* SPECIES AND ISOLATES FROM ETHIOPIAN WHEATS FOR MYCOTOXIN PRODUCTION

ABSTRACT

The majority of *Fusarium* species isolated from stored wheats and scabby wheats in the field, did not produce detectable amounts of either trichothecenes or zearalenone. The production of low levels of 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and neosolaniol by two isolates of *F. graminearum*, and an isolate of *F. poae*, respectively, was confirmed by GC-MS. One of the isolates of *F. graminearum* also produced low levels of zearalenone.

A single strain, *F. sporotrichioides* #48d produced a variety of trichothecenes. The trichothecenes were tentatively identified by thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS) as neosolaniol, 8-acetylneosolaniol, 8-propionylneosolaniol, 8-n-butyrylneosolaniol, 8-isobutyrylneosolaniol, 8-n-valerylneosolaniol, 8-isovalerylneosolaniol (T-2 toxin), 8-hexynylneosolaniol and 4,8-diacetyl T-2 tetraol (NT-1). T-2 toxin and neosolaniol have been reported to be produced as major metabolites by some strains of *F. sporotrichioides* while the 8-acetylneosolaniol, 8-propionylneosolaniol,

8-butyrylneosolaniol, 8-isobutyrylneosolaniol and NT-1 have been reported as minor metabolites. The 8-n-valerylneosolaniol and 8-hexynylneosolaniol are new trichothecenes that are being reported for the first time.

INTRODUCTION

A number of Fusarium species are toxigenic and produce secondary metabolites deleterious to animal and human health [1,3,16]. The production of these mycotoxins often depends on environmental conditions such as substrate availability, storage conditions (moisture and temperature) and competition with other organisms [15]. These mycotoxins can also be produced in the laboratory by culturing Fusarium species in liquid media [2,17,24] or on solid substrates [4,11]. Many toxigenic strains of Fusarium species produce zearalenone (F-2) and trichothecenes. The structure of zearalenone is 6-(10-hydroxy-6-oxo-trans-1-undecenyl)β-resorcyclic acid lactone [25] and it is known to cause an estrogenic syndrome in swine [18]. The trichothecenes are generally characterized by a tetracyclic 12,13-epoxytrichothec-9-ene skeleton [22]. The 12,13-epoxide and the -9-ene functionalities are required for their biological activity [12]. Trichothecenes produced by Fusarium species are divided into several classes based upon ring oxidation patterns. The majority of the known trichothecenes belong to the first class (Type A) and have α-hydroxy substituents at

the 8 position (eg. T-2 toxin has an isovaleryl ester at the eight position) [12]. The most studied trichothecene in this class is T-2 toxin due to its link with Alimentary Toxic Aleukia (ATA) in humans [19]. The 8-keto trichothecenes constitute the second class (Type B) and cause feed refusal in swine (eg. deoxynivalenol or vomitoxin) [5,9].

The objectives of this study were to screen: 1. wheat seed samples collected during the 1988 season in Ethiopia for mycotoxin contamination, and 2. Fusarium species isolated from Ethiopian wheat for mycotoxin production.

MATERIALS AND METHODS

Solvents and mycotoxin standards:

All solvents used for extraction and column chromatography were A.C.S. grade purchased from Fisher Sci. Co. All mycotoxin standards (T-2 toxin, T-2 tetraol, 4-propanoyl HT-2, HT-2, diacetoxyscirpenol (DAS), neosolaniol, nivalenol, deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), and zearalenone) were obtained from Sigma Chemical or were isolated and purified in our laboratory.

Mycotoxin analysis of wheat seed samples:

Fifty samples of seed were collected from Ethiopian wheat fields with 0 to 97% Fusarium head blight (FHB) infection during the 1988 season. The samples constituted different breeding lines and cultivars grown at experiment

stations, state farms, seed farms and farmer's fields in different regions of Ethiopia. Some of the samples contained as high as 78% shriveled and 39% Fusarium infected seeds (Chapters 3&4). Because of the high Fusarium infection, the seeds were examined for mycotoxin contamination.

Samples were examined by a modification of the method of Rottinghaus et al [20]. The ground wheat samples (25 g) were extracted with 100 ml acetonitrile:4% aqueous KCl (9:1) in a 250 ml screw-capped wide mouth polyethylene bottle. The samples were shaken for 1 hour on a wrist-action shaker and the extracts filtered through Whatman #4 filter paper.

For detection of trichothecenes, a disposable charcoal column was prepared by adding a Schlicher and Schuell (S&S) 1/2 inch diameter biological disc to a 6 ml Monoject (Sherwood Medical) disposable syringe barrel. One gram Darco G-60 charcoal:diatomaceous earth (3:1) was added to the syringe barrel. A thin layer of neutral alumina was added and then two S&S 1/2 inch diameter biological discs were placed on top the alumina. A 20 ml fraction of the filtrate (equivalent to 5 g sample) was passed through the charcoal column under vacuum and the column was washed twice with 10 ml acetonitrile:water (9:1). The column eluants were concentrated to dryness.

For detection of zearalenone, a C_{18} disposable column was prepared by adding an S&S 1/2 inch diameter biological disc to a 6 ml Monoject disposable syringe barrel and 1 g C_{18}

reversed phase silica gel (Baker Chemical, 40 μm size) was added to the syringe. Two S&S 1/2 inch biological discs were placed on top the C_{18}. The column was activated by washing with 5 ml methanol followed by 5 ml water. Distilled water (8.5 ml) was added to 10 ml of the filtrate, mixed and applied to the activated C_{18} column. The column eluant was collected in a 50 ml screw capped test tube under vacuum. The column was washed with an additional 5 ml wash solution (9:1:8.5 acetonitrile:4% aqueous KCl:water). The combined eluant from the C_{18} column was extracted twice with 5 ml chloroform. The chloroform layers were filtered through Whatman #1PS filter paper and concentrated to dryness.

TLC analysis:

Both normal phase (Analtech, 10x20 cm scored silica gel HL) and reverse phase (Whatman 10x10 cm KC18) TLC plates were used. Extracts from the charcoal and C_{18} columns were redissolved in 100-300 μl acetone: methanol (2:1) and 10 to 20 μl were spotted on TLC plates along with appropriate mycotoxin standards.

The TLC plates were developed using the following mobile phases:

Silica gel TLC:

Solvent system 1 = 97:3 chloroform:methanol.

Solvent system 2 = 1:1 ethyl acetate:ethyl ether.

Solvent system 3 = 3:1 benzene:acetone.

C_{18} TLC:

Solvent system 4 = 35:20:1 methanol:water:acetic acid.

The mycotoxins were detected on the developed TLC plates using the following spray reagents:

Spray reagent #1 (Fast violet B):

For zearalenone detection, silica gel plates developed in solvent system 1 were sprayed with 0.7% aqueous Fast Violet B (Eastman Kodak) followed by a buffer solution (50 ml 0.025 M sodium borate and 4.6 ml 0.1 M HCl, pH 9) and upon heating at 100 C for 5 to 10 min, zearalenone appeared as a pink spot. The pink zearalenone spot turned purple when sprayed with 30% sulfuric acid in methanol [20].

Spray reagent #2 (Aluminum chloride):

For deoxynivalenol and 3-acetyldeoxynivalenol detection silica gel TLC plates were developed in solvent system 2, dried and sprayed heavily with 20% aluminum chloride in methanol [23]. The plates were heated at 110 C for 15 min and observed under long wavelength UV light; the trichothecenes appeared as bright blue fluorescent spots.

Spray reagent #3 (Blue spray):

T-2 toxin and the rest of the trichothecenes were detected on silica gel plates developed in solvent system 3. The plates were first sprayed with 1% 4(p-nitrobenzyl)pyridin (Sigma) in 2:3 chloroform:carbon tetrachloride and heated at 150 C for 30 min. The plates were cooled and sprayed with 10% tetraethylenepentamine (Eastman Kodak) in 2:3 chloroform:carbon tetrachloride. The epoxide-containing

trichothecenes appeared as blue spots on a white background [23].

Spray reagent #4 (p-anisaldehyde):

Trichothecenes on C_{18} TLC plates were detected when spotted plates were developed in solvent system 4, dried and sprayed with para-anisaldehyde (85:10:5:0.5 methanol:glacial acetic acid:concentrated sulfuric acid:p-anisaldehyde). The trichothecenes appeared as pink to reddish-brown spots.

Screening Fusarium isolates for mycotoxins:

Fusarium isolates: Seventy eight isolates of 17 Fusarium species (Table 5-1), isolated and identified from Ethiopian wheat seed samples (Chapters 3&4), were screened for mycotoxin production. The isolates were maintained on silica gel at 4 C as described earlier. Inoculum was prepared by placing several silica gel particles onto potato sucrose agar (PSA) plates and incubating at 24 C with a 12 h fluorescent light for 7 days as described earlier (Chapter 3).

Culturing the fungus, extraction and detection of mycotoxins:

First, all 78 isolates were grown in potato dextrose broth (PDB) liquid medium. PDB was prepared by adding 15 g dextrose to a liter of potato extract (made by boiling 200 g of scrubbed and diced potatoes in a liter of water) and autoclaving at 15 psi for 20 min. Two 8 mm diameter discs from each of the 7 day old PSA plate cultures were placed in a 250

Table 5-1. *Fusarium* species and isolates screened for mycotoxin production.

Fusarium species	No. of isolates	% of isolates field[a]	% of isolates stored[b]
F. equiseti	13	8	92
F. avenaceum	13	100	0
F. semitectum	9	56	44
F. graminearum	8	88	12
F. nivale	8	100	0
F. lateritium	7	43	57
F. poae	5	40	60
F. oxysporum	4	0	100
F. heterosporum	3	33	67
F. tricinctum	1	0	100
F. solani	1	0	100
F. udum	1	0	100
F. decemcellulare	1	0	100
F. stilboides	1	100	0
F. sambucinum	1	100	0
F. moniliforme	1	100	0
F. sporotrichioides	1	100	0

a. Isolated from scabbed heads collected from the field in 1988
b. Isolated from stored seed samples of the 1987 crop

ml Erlenmeyer flask containing 50 ml PDB. These were incubated on a rotary shaker (125 rpm) at room temperature (25 to 27 C) for 21 days with 12 h day light and 12 h darkness.

At the end of the incubation period, 50 ml ethyl acetate was added to each flask of the PDB cultures. The flasks were shaken and left overnight. The ethyl acetate extracts were decanted into beakers containing 5 g sodium sulfate to remove water, decanted again into other beakers and left overnight in the hood to dry. The extraction was repeated with an additional 50 ml ethyl acetate. The culture residue was transferred with 2 ml chloroform into small vials and taken to dryness. The extracts, dissolved in 9:1 acetonitrile:water, were passed through disposable charcoal columns as described earlier. The column eluant was taken to dryness and spotted on silica gel TLC plates.

In the second trial, 3 isolates of F. nivale (#23, #43a, #43b), F. avenaceum (#3, #18, #43), and F. graminearum (DZ1a, ADT1c, HR24); two isolates of F. poae (#5, #17SF,), F. semitectum (#34d, #36d) and F. lateritum (#38b, #41c); and one isolate of F. sporotrichioides (#48d) and F. equiseti (#15FFb) were cultured on three different media - glucose yeast extract peptone (GYEP), rice and corn. GYEP was prepared as described by Miller et al [17]. The medium contained 10 g glucose (Sigma), 1 g yeast extract (Difco), and 1 g peptone (Difco) in a liter of distilled water. Fifty milliliters of the medium was transferred to 250 ml Erlenmeyer flasks and autoclaved at

15 psi for 20 min. The rice and corn media were prepared as described by Greenhalgh et al [11]. About 50 g Uncle Ben's converted long-grain rice or white corn grits (Quaker Oats) were placed in 250 ml Erlenmeyer flasks. Distilled water (25 ml) was added to each flask and capped with aluminum foil before autoclaving at 15 psi for 30 min. The moisture content of the media after autoclaving was approximately 35%. Two 8 mm diameter discs of 7 day old PSA plate cultures were transferred to one flask of each medium and incubated for 10 days at 24 C followed by an additional 11 days at 15 C.

GYEP liquid culture was extracted with 50 ml ethyl acetate as described before. The cultures grown on rice and corn were extracted with 9:1 acetonitrile:4% aqueous potassium chloride. One hundred milliliters of extraction solvent was added to each flask, the clumps were broken-up and left overnight to soak. The cultures were placed in a blender (low speed) for 3 min and then filtered through Whatman #1 filter paper. A portion (20 ml) of the filtrate was passed through a small charcoal column as described above, concentrated and examined by silica gel TLC.

Large scale mycotoxin production:

Five isolates (F. nivale #43b, F. avenaceum #18, F. graminearum HR24, F. poae #5 and F. sporotrichioides #48d), that had been shown to produce trichothecenes, were selected for large scale mycotoxin production. Canning jars (1 qt) containing 100 g white corn grits (Quaker Oats) and 50 ml

Table 4-8. Percentage of Fusarium isolates in different species from scabby wheats collected from farmer fields in 1988 and percentage of each species in four administrative regions[a]

Fusarium species[c]	%	Regions[b] I	II	IV	V
F. nivale	35	43(0-18)[d]	33(0-3)	-	54(0-44)
F. avenaceum	31	50(0-21)	-	25(0-13)	28(0-23)
F. graminearum	29	7(0-3)	44(0-4)	75(0-39)	10(0-8)
F. poae	2	-	-	-	5(0-4)
F. lateritium	1	-	-	-	2(0-2)
F. sporotrichioides	1	-	22(0-2)	-	-

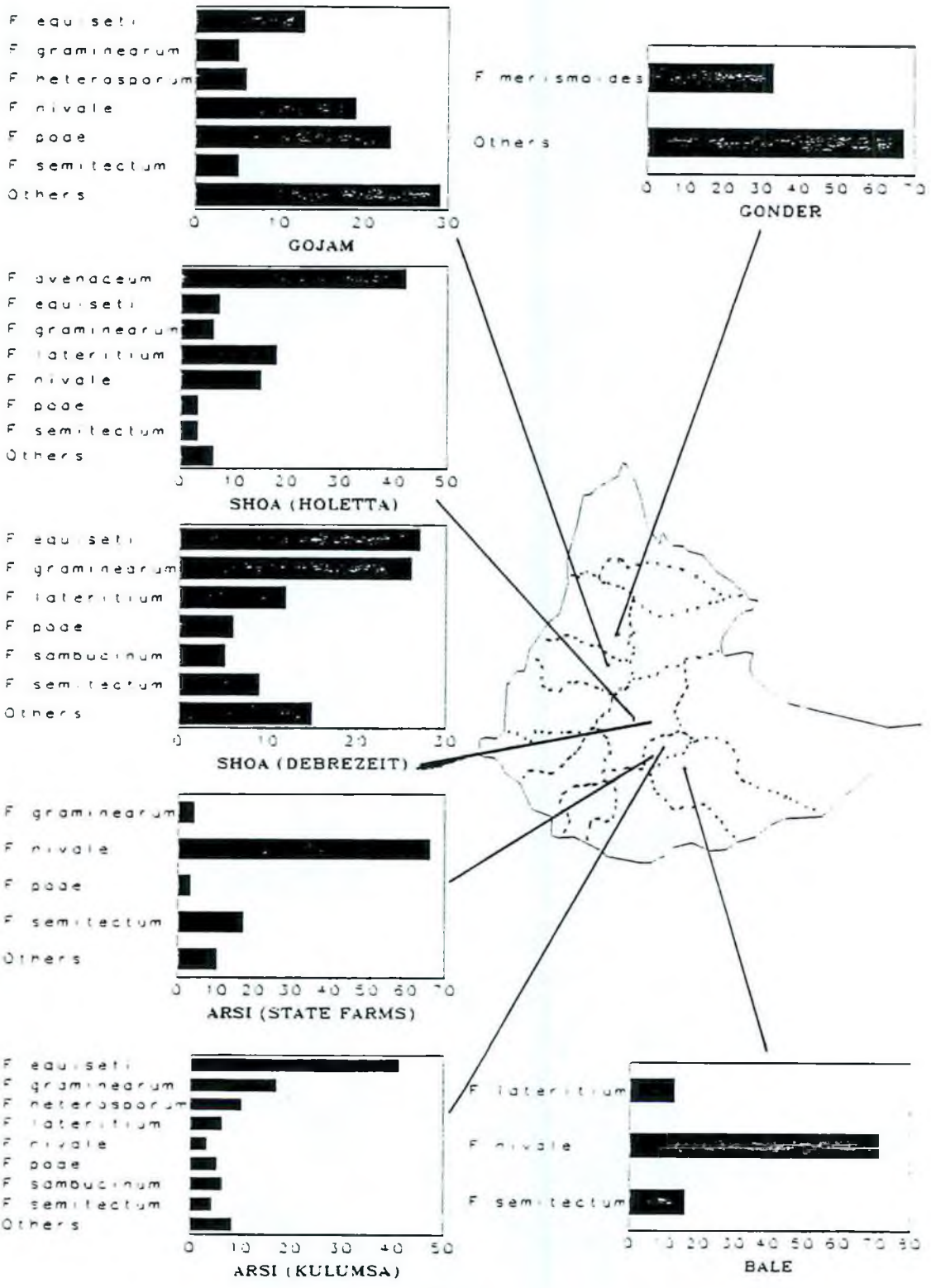
a. Most farmer fields were planted late and were just flowering during the survey. These evaluations are on a few early planted fields.
b. See Fig. 3-1 for regions.
c. Based on 1200 isolates.
d. Values in parenthesis are ranges of percent infection per sample.

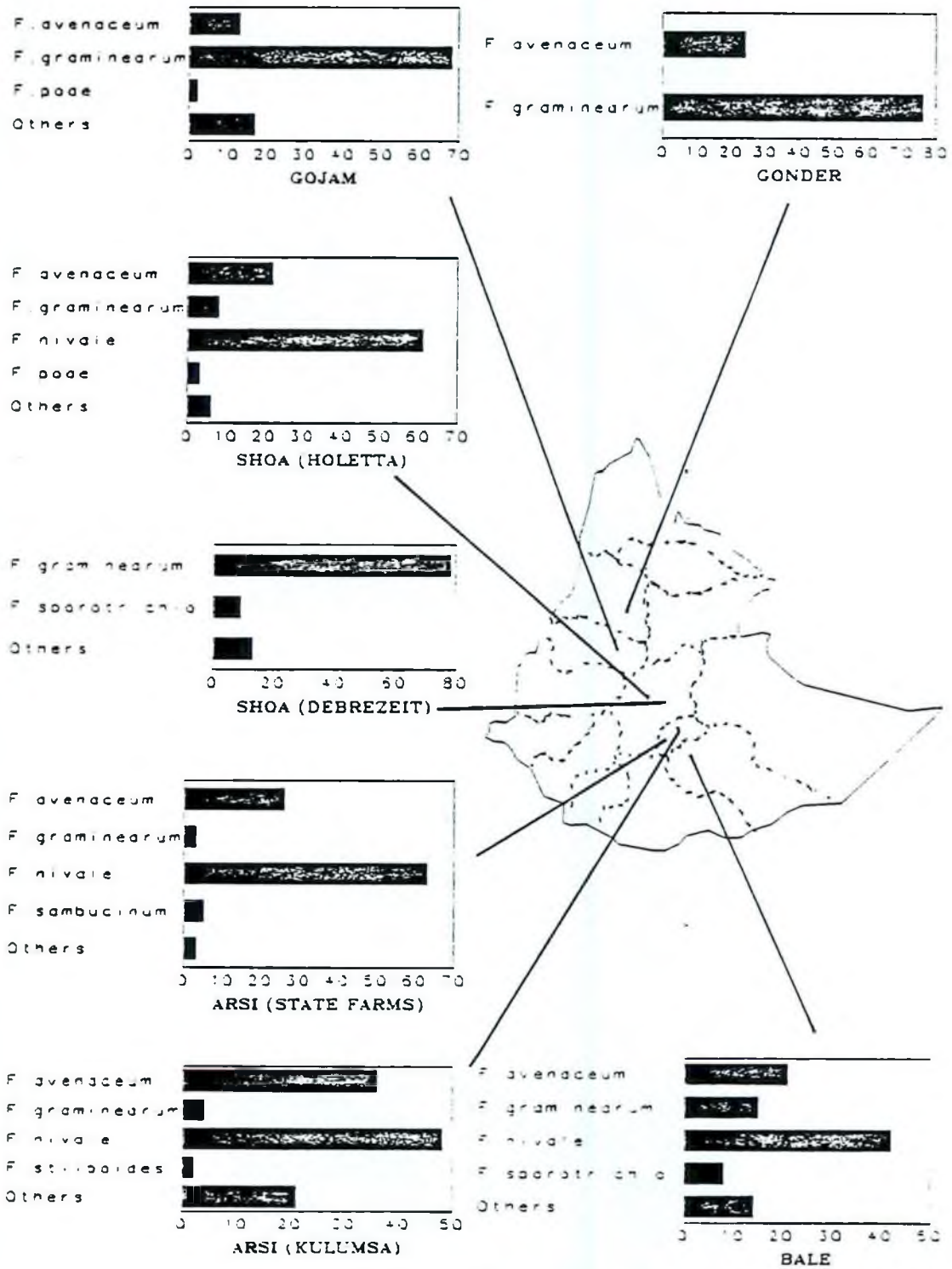
samples collected from region V (Shoa). F. sporotrichioides in farmer samples was identified only from Region II (Bale).

DISCUSSION

A total of seventeen Fusarium species were identified from stored seed samples collected from various sources. Frequencies and distributions of the major species are shown in Fig. 4-1. The most frequently isolated and widely distributed species were F. equiseti, F. nivale, F. graminearum, F. poae and F. semitectum. It seems that F. avenaceum is restricted to seed samples from Holetta and Kulumsa research centers. More species were identified from the farmer samples which may reflect the large number of isolates made from these samples as compared to others.

Thirteen Fusarium species were identified from scabby head samples collected during the field survey. Frequencies and distributions of the major species are shown in Fig. 4-2. F. nivale and F. avenaceum dominated the isolates made from high and cool areas such as Holetta (V-4), Kulumsa (I-13) and most state farms in region I. F. graminearum was prevalent in lower and dryer regions like Debrezeit (V-3) and north western regions of Gojam and Gonder (III-1 and IV-1). A smaller number of species were identified from the farmers field than from the farmers stored seed samples. This may be due to the small number of farmer fields surveyed (since most


F equiseti
F graminearum
F heterosporum
F nivale
F poae
F semitectum
Others
0 10 20 30
GOJAM
F merismoides
Others
0 10 20 30 40 50 60 70
GONDER
F avenaceum
F equiseti
F graminearum
F lateritium
F nivale
F poae
F semitectum
Others
0 10 20 30 40 50
SHOA (HOLETTA)
F equiseti
F graminearum
F lateritium
F poae
F sambucinum
F semitectum
Others
0 10 20 30
SHOA (DEBREZEIT)
F graminearum
F nivale
F poae
F semitectum
Others
0 10 20 30 40 50 60 70
ARSI (STATE FARMS)
F equiseti
F graminearum
F heterosporum
F lateritium
F nivale
F poae
F sambucinum
F semitectum
Others
0 10 20 30 40 50
ARSI (KULUMSA)
F lateritium
F nivale
F semitectum
0 10 20 30 40 50 60 70 80
BALE


F. avenaceum
F. graminearum
F. poae
Others
0 10 20 30 40 50 60 70
GOJAM
F. avenaceum
F. graminearum
0 10 20 30 40 50 60 70 80
GONDER
F. avenaceum
F. graminearum
F. nivale
F. poae
Others
0 10 20 30 40 50 60 70
SHOA (HOLETTA)
F. graminearum
F. sporotrichioides
Others
0 20 40 60 80
SHOA (DEBREZEIT)
F. avenaceum
F. graminearum
F. nivale
F. sambucinum
Others
0 10 20 30 40 50 60 70
ARSI (STATE FARMS)
F. avenaceum
F. graminearum
F. nivale
F. sporotrichioides
Others
0 10 20 30 40 50
ARSI (KULUMSA)
F. avenaceum
F. graminearum
F. nivale
F. sporotrichioides
Others
0 10 20 30 40 50
BALE

were at an early growth stage) as compared to the large number of stored seed samples analyzed.

Most species, except F. sporotrichioides, that were identified from scabby heads in the field were also identified from the stored seed samples. On the other hand, some species (F. oxysporum, F. solani, F. decemcellulare, F. merismoides and F. udum) which were identified from stored seeds were not identified from the field samples. The frequency of these species was, however, very low, ranging only from <1 to 4%. F. equiseti was less than one percent in the field sample, but was the most frequent and widely distributed species in stored seed sample. Most of these species were identified previously in the USA from scabbed wheat heads [18]. The involvement of the minor species identified from the farmers' seed samples in causing FHB is not clear.

The relative head blighting potential of some of these species have been studied previously [13]. F. tricinctum, F. sporotrichioides, F. equiseti, F. acuminatum and F. poae did not blight but cause wheat heads to become moldy under extremely favorable (eg. constant moisture saturation) conditions while F. graminearum and F. culmorum caused severe blighting. F. nivale, F. poae and F. sporotrichioides were shown to colonize leaves and leaf sheaths prior to emergence of the ear [9,16,17]. It was suggested that such early colonization by certain Fusaria may predispose the tissues to infection by late season species

such as F. graminearum and F. culmorum [16].

F. culmorum, F. dimerum and F. longipes which were reported to occur on wheat in Ethiopia previously [4,10,14] were not identified from either the stored or field samples during this study. The population of these species could have been too low to be detected or may have been named differently using another system of nomenclature. This can often happens in Fusarium taxonomy. It should also be noted that a large proportion of the isolates from both stored and field samples could not be identified for various reasons. Most did not sporulate even after some stimulating treatments described earlier. Others were severely contaminated by other fungi and/or bacteria.

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The GC/MS total ion chromatogram of fraction 16-II-1&2 (Fig. 5-2) showed a similar retention time (19:55 min) as fraction 16-III (Fig.5-8). The MS of this fraction (Fig. 5-11) also showed an identical pattern of fragmentation as that of fraction 16-III (Fig. 5-9) with all the diagnostic ions. This fraction was, therefore, identified as 8-acetoxyneosolaniol (Fig. 5-10) and was probably a carry-over from fraction 16-III during the fractionation process.

3. Neosolaniol (fraction 16-V):

The GC/MS total ion chromatogram of the TMS derivative of fraction 16-V (Fig. 5-12) had a major peak at a retention time of 23:20 min (scan # 1461) which was similar to the retention time of the neosolaniol standard. The protonated molecular ion (MH^+) in the PCI mode was 526 which was same as the MH^+ of the TMS-neosolaniol standard. The EI fragmentation pattern of fraction 16-V scan# 1461 (Fig. 5-13) was identical to that of the 3-TMS-neosolaniol standard (Fig. 5-14). The difference between the molecular ion (MH^+ = 526) and the 3-TMS-neosolaniol fragment (m/z 436) was m/z 90 which was the loss of TMS ($(CH_3)_3SiOH$) from the 8 position. Based on the same retention time, PCI and EI mass spectra as the standard, the compound was identified as neosolaniol (Fig. 5-15).

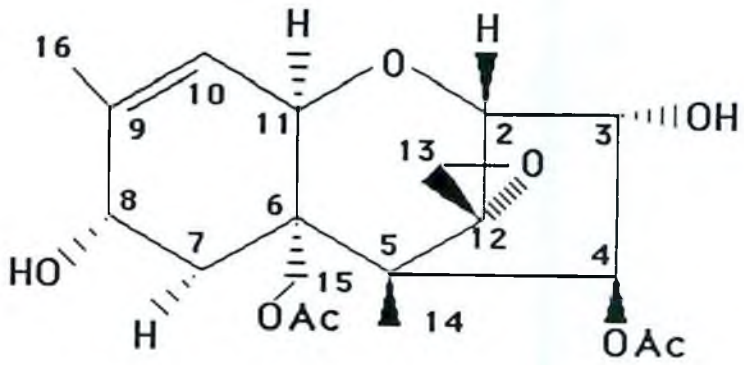

Figure 5-15. Chemical structure of neosolaniol.

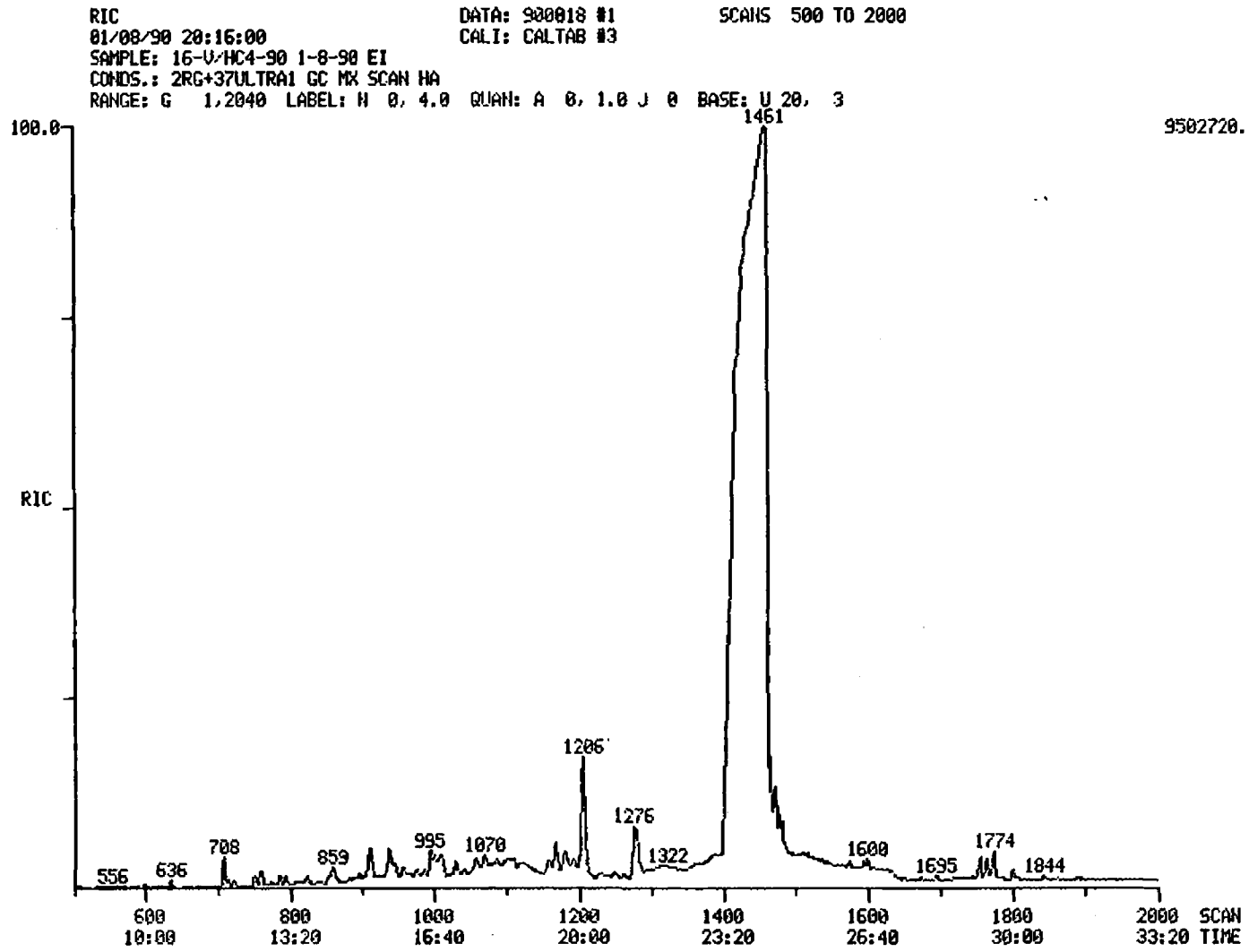

Figure 5-12. GC/MS total ion chromatogram (EI mode) of TMS derivative of fraction 16-V.

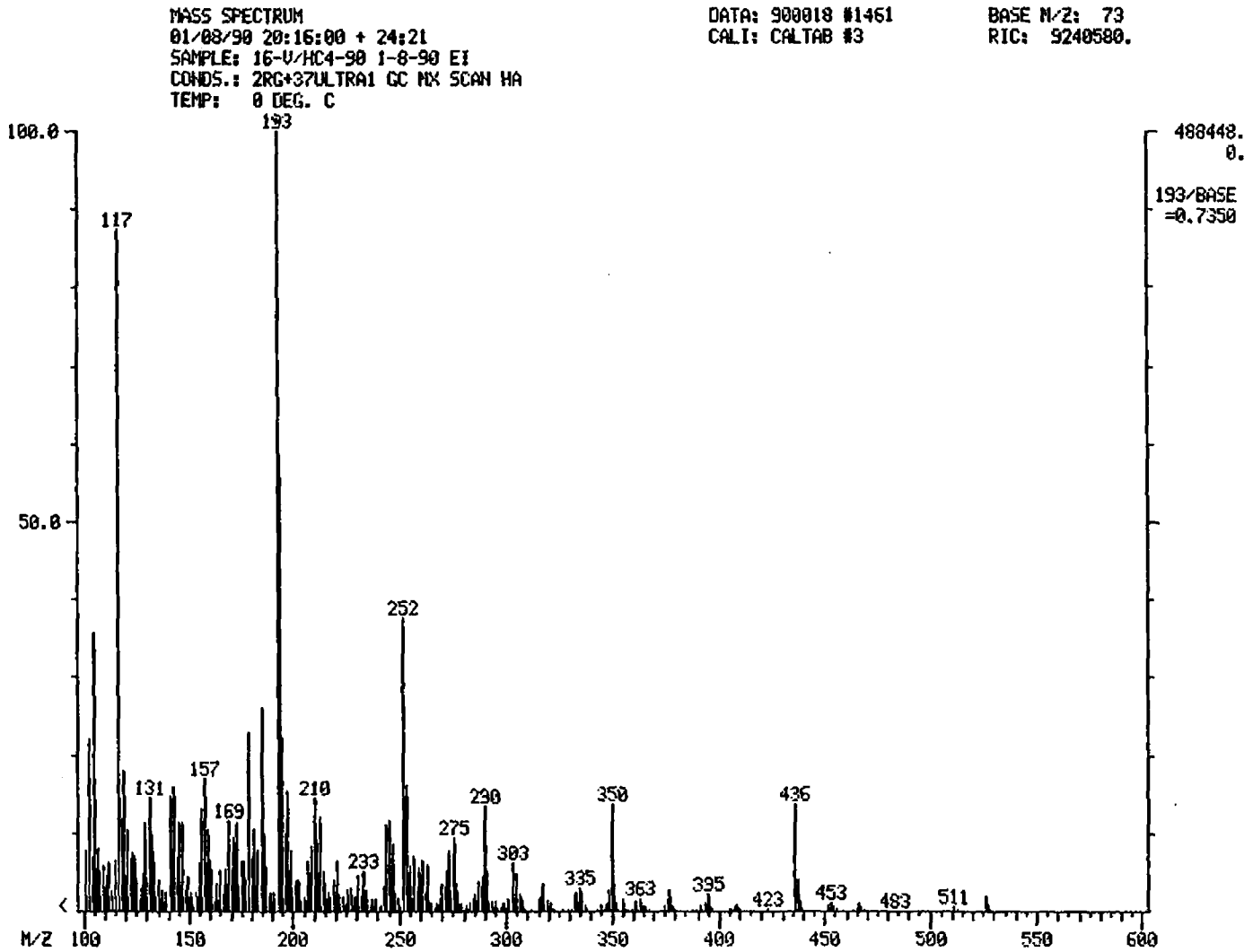

Figure 5-13. EI mass spectrum of TMS derivative of fraction 16-V, scan# 1461.

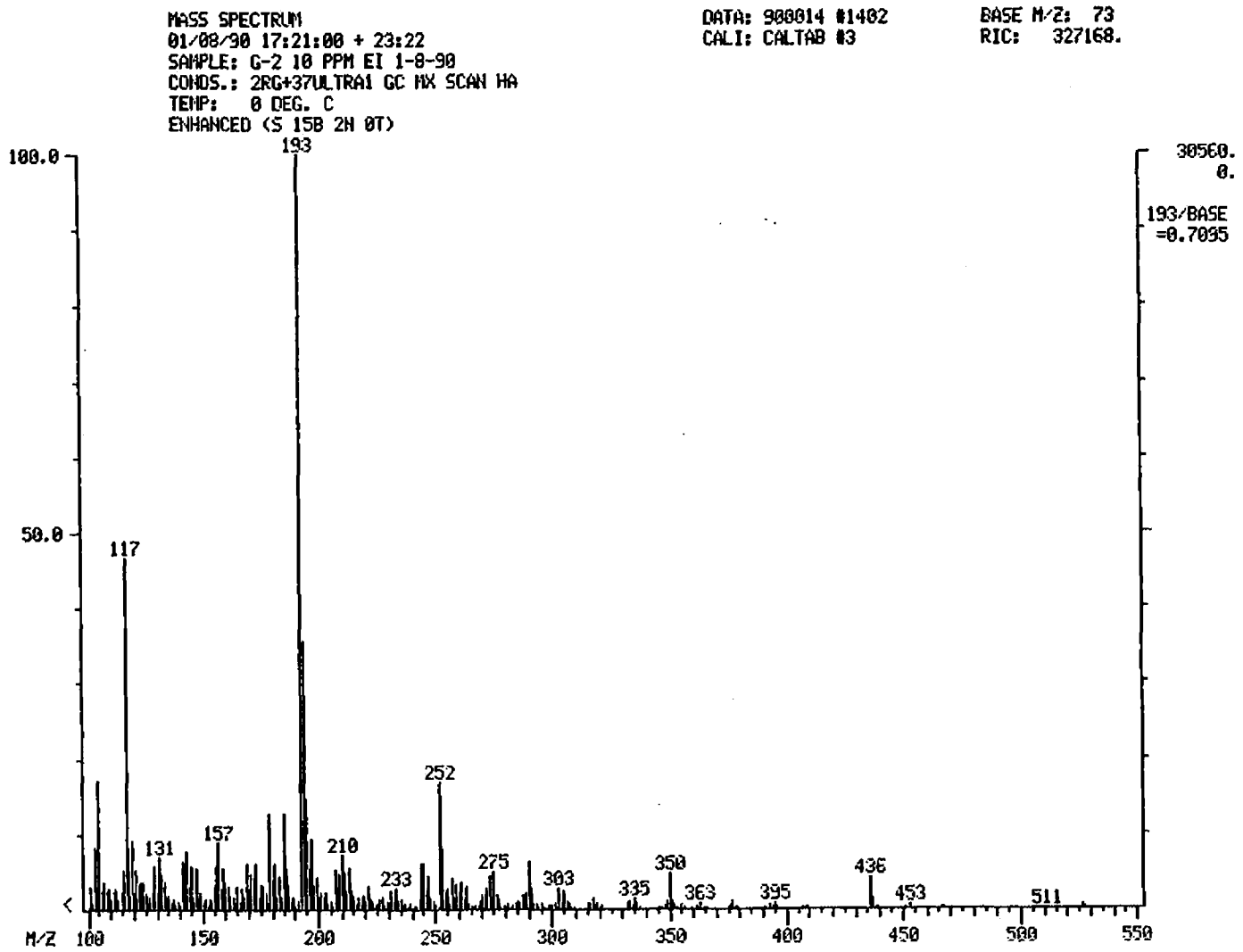

Figure 5-14. EI mass spectrum of TMS derivative of neosolaniol standard.

4. 8-isovaleryIneosolaniol or T-2 toxin (16-II-5, scan# 1603):

The GC/MS total ion chromatogram of the TMS derivative of fraction 16-II-5 showed two distinct peaks (Fig. 5-16). The smaller peak had a retention time of 24:20 min (scan # 1603) which corresponded to the retention time of T-2 toxin standard. The PCI MH^+ of the TMS derivative of this fraction was 538 which was the same as the MH^+ of TMS-T-2 toxin standard. The EI mass spectrum of this fraction (Fig. 5-17) was identical to the EI mass spectrum of the T-2 toxin standard (Fig 5-18). The EI spectrum was similar to the MS of neosolaniol except for the addition of the mass fragment at m/z 85. The difference between the MH^+ = 538 and the 3-TMS-neosolaniol fragment (m/z 436) was the loss of an isovaleric acid (m/z 102) from the 8 position. The m/z 85 was the fragment C_5H_9O resulting from the isovaleric ester. The compound was, therefore, identified as 8-isovalerylneosolaniol or T-2 toxin (Fig. 5-19).

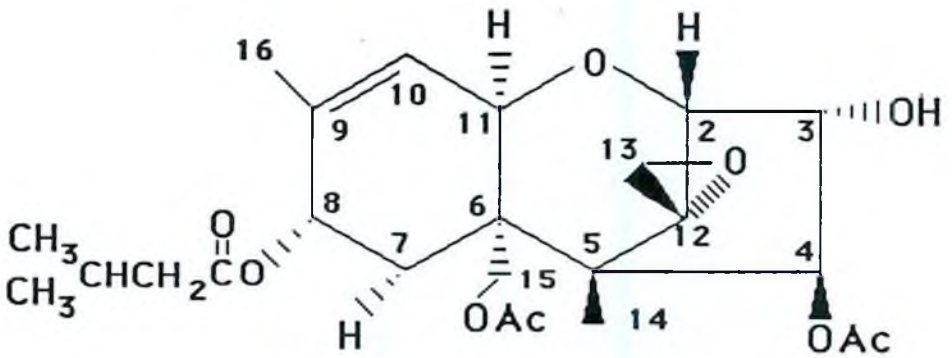

Figure 5-19. Chemical structure of 8-isovalerylnecsolaniol.

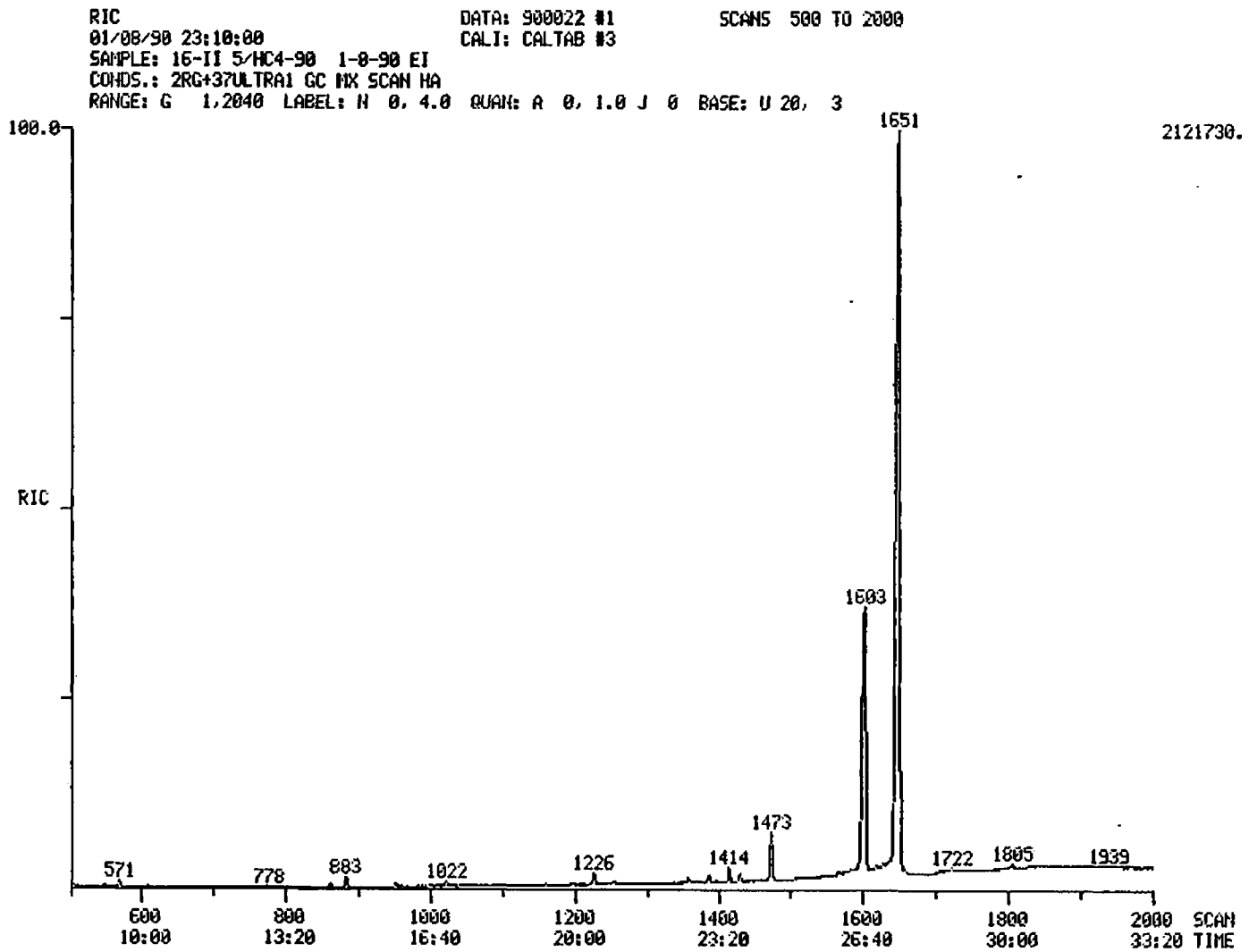

Figure 5-16. GC/MS total ion chromatogram (EI mode) of TMS derivative of fraction 16-II-5.

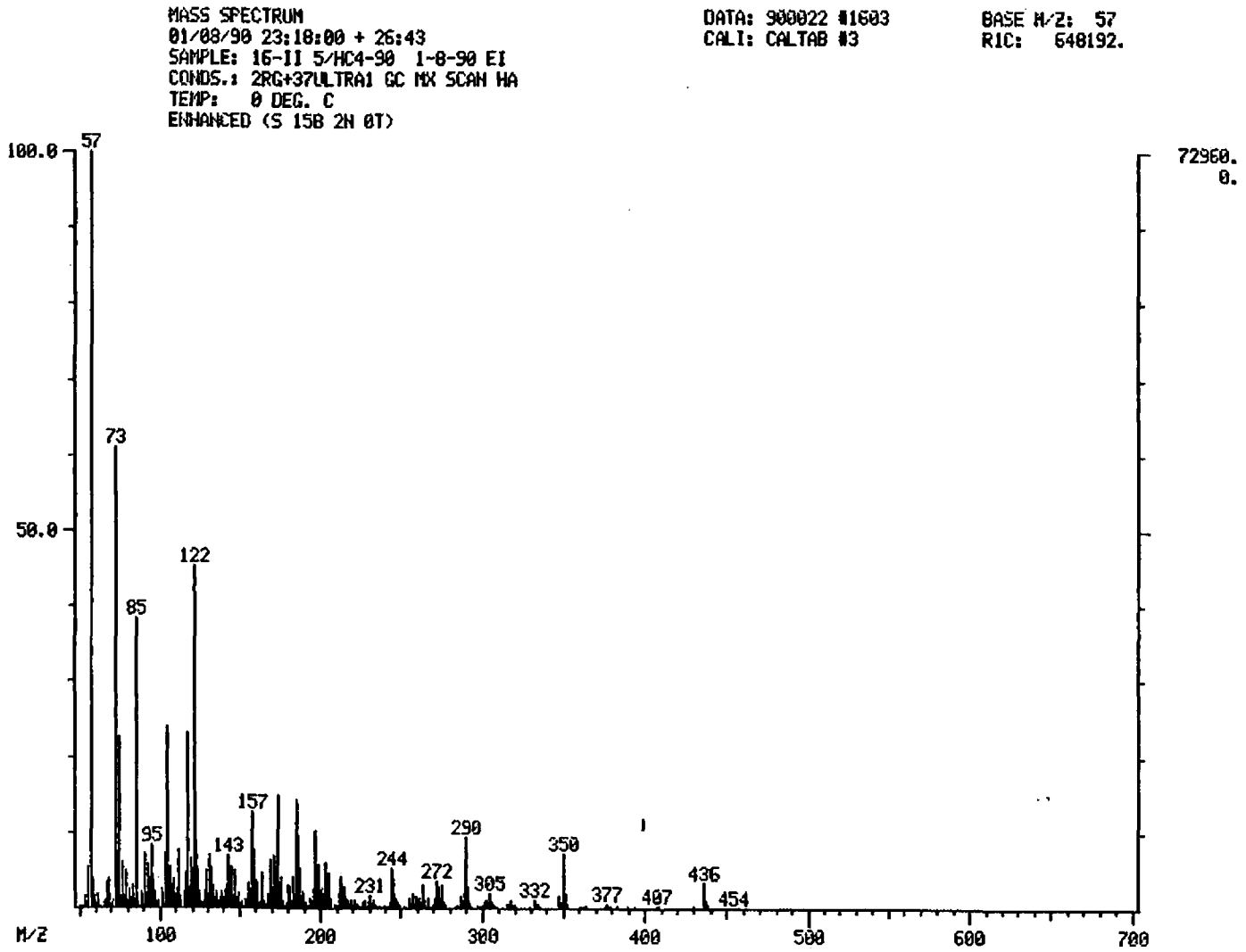

Figure 5-17. EI amss spectrum of TMS derivative of fraction 16-II-5, scan# 1603.

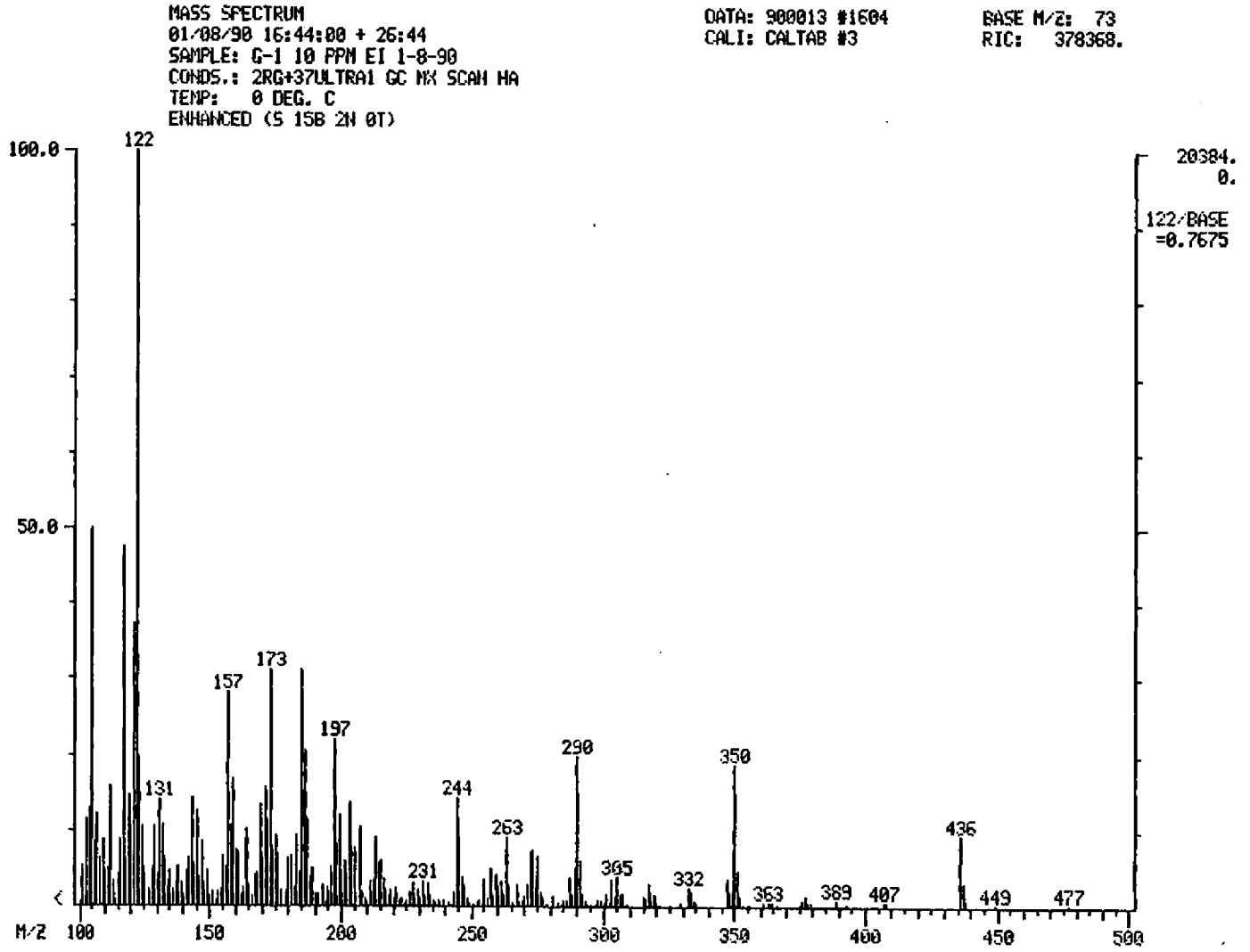

Figure 5-18. EI mass spectrum of TMS derivative of T-2 toxin standard.

For the purpose of easy literature comparisons, the PCI MODE GC/MS data of the TFA derivatives of fractions 16-II 1&2, 16-III and 16-IV are discussed. The electron impact (EI) mass spectra of the TMS derivatives of the other fractions indicated structural similarities. between the fractions. The MS of these fractions also exhibited EI fragmentation patterns similar to the MS of 3-TMS-neosolaniol (Fig. 5-14). Major masses common to the MS of these fractions included m/z 117, 122 (193 for TMS derivatized fragments of neosolaniol), 157, 290, 350 and 436. The m/z 436 was 3-TMS neosolaniol fragment with a loss of the functional group (RCOOH) at the 8 position (Fig. 5-3). The ester gruops have a tendency to split at the 8 position first followed by loss at the 15 followed by the 4 position (Howard Casper, personal communication). Considering the consistent appearance of the m/z 436 (3-TMS-neosolaniol fragment, the pattern of loss of the functional

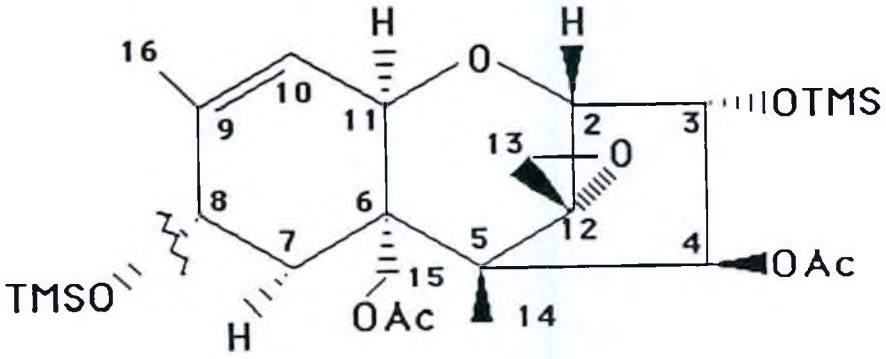

Figure 5-3. 3-TMS-neosolaniol fragment.

groups and the appearance of a distinct additional mass in the lower EI mass spectra of each fraction, it was believed that all the functional groups were at the 8 position.

The molecular (parent) ion of the TMS derivatized fractions were weak or absent in the EI mode, but were determined in the positive chemical ionization or PCI mode. The PCI mass spectra of the fractions were not included because there was very little additional information. Based on the GC/MS analysis, the following trichothecenes were identified from the culture extract of the isolate (F. sporotrichioides #48d).

1. 4,8-diacetoxy T-2 tetraol or NT-1 (Fraction 16-IV):

The GC/MS total ion chromatogram of the TFA derivative of fraction 16-IV has a major peak (scan # 470) at a retention time of 15:27 min (Fig. 5-4) which had the same retention time as the TFA-NT-1 standard. The mass spectrum (PCI mode) of this fraction (Fig. 5-5) was identical to that of the NT-1 standard (Fig. 5-6). A molecular ion of (MH^+) 575, a base peak at m/z 515 (loss of acetic acid at the C-8 position), m/z 401 (loss of trifluoroacetic acid at the C-15 position) and m/z 341 (loss of two acetic acids at the C-4 and C-8 positions) were diagnostic ions in the PCI mode. The mass spectrum was identical to the published mass spectrum of TFA-NT-1 [27]. Based on the retention time, MS comparison with a standard and literature reports, the compound was identified as 4,8-diacetoxy T-2 tetraol or NT-1 (Fig. 5-7).

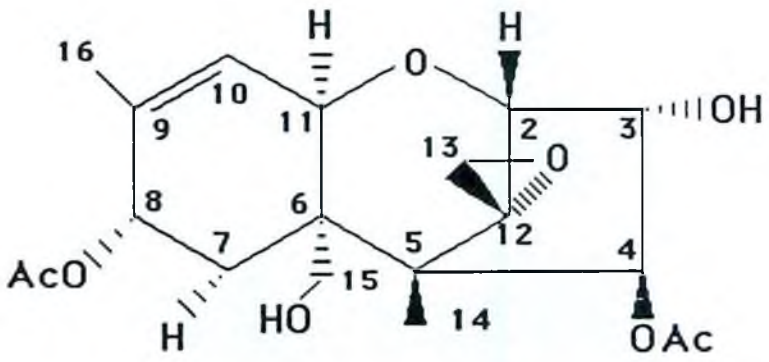

Figure 5-7. Chemical structure of 4,8-diacetoxy T-2 tetraol.

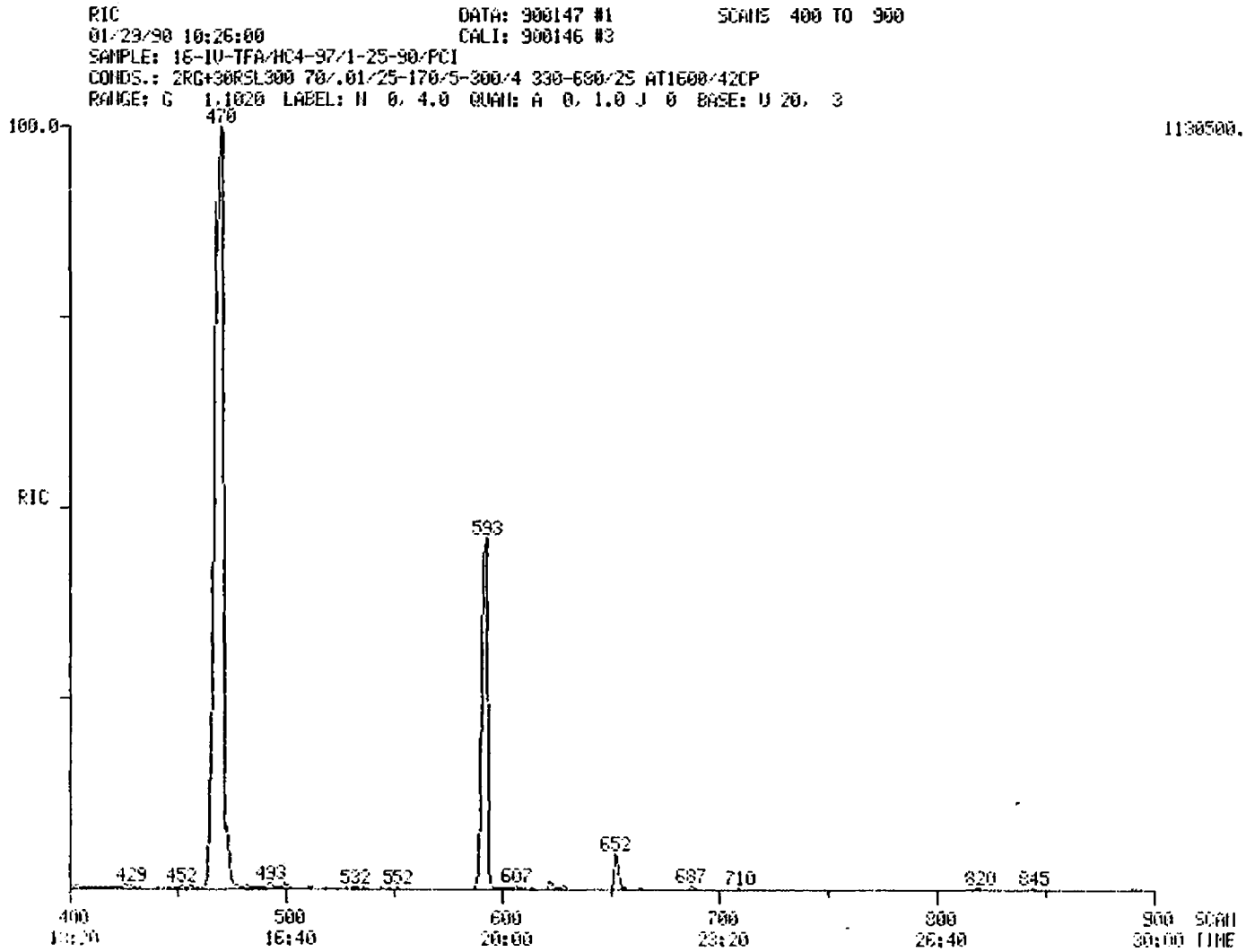

Figure 5-4. GC/MS total ion chromatogram (PCI mode) of TFA derivative of fraction 16-IV.

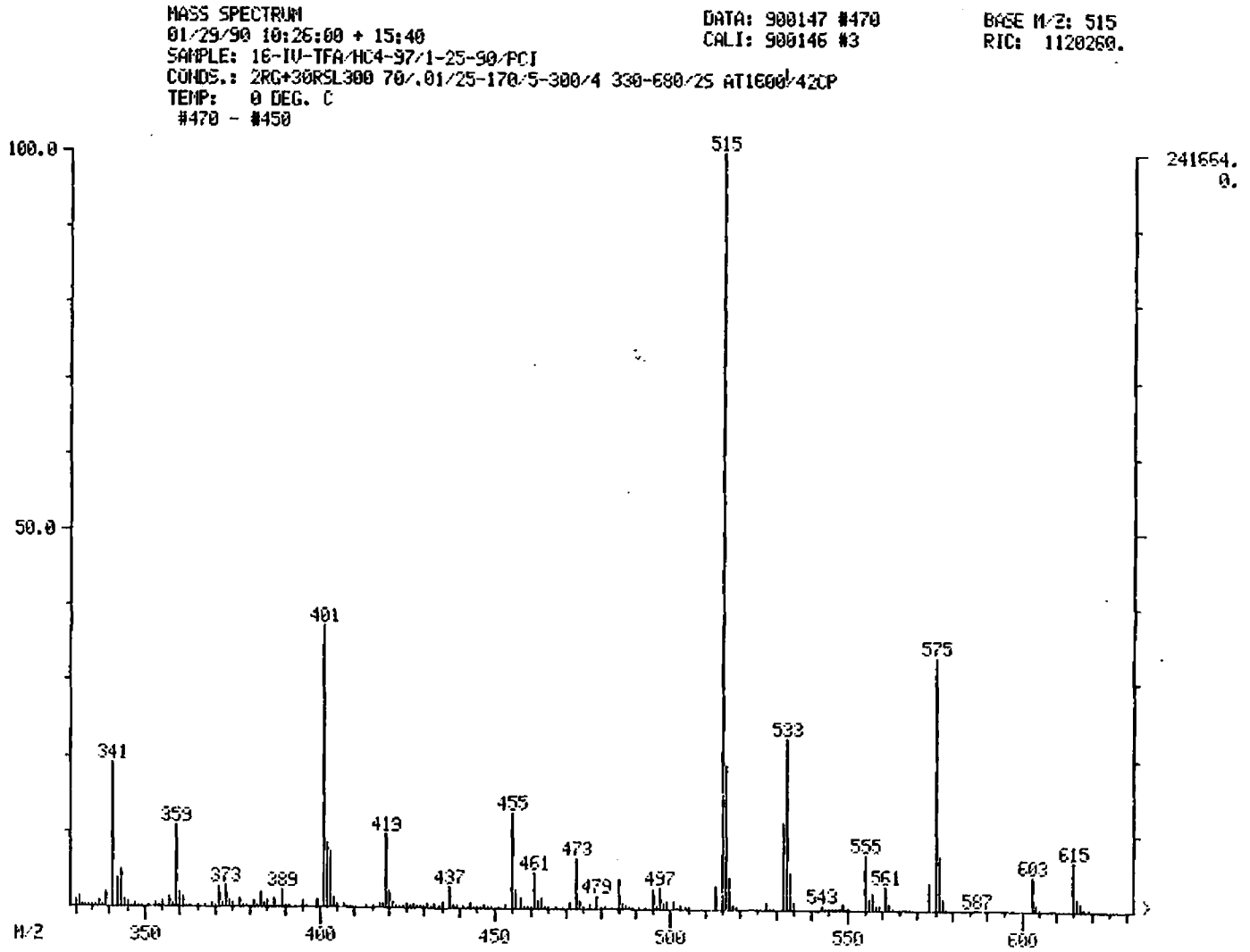

Figure 5-5. MS (PCI mode) of TFA derivative of fraction 16-IV, scan# 470.

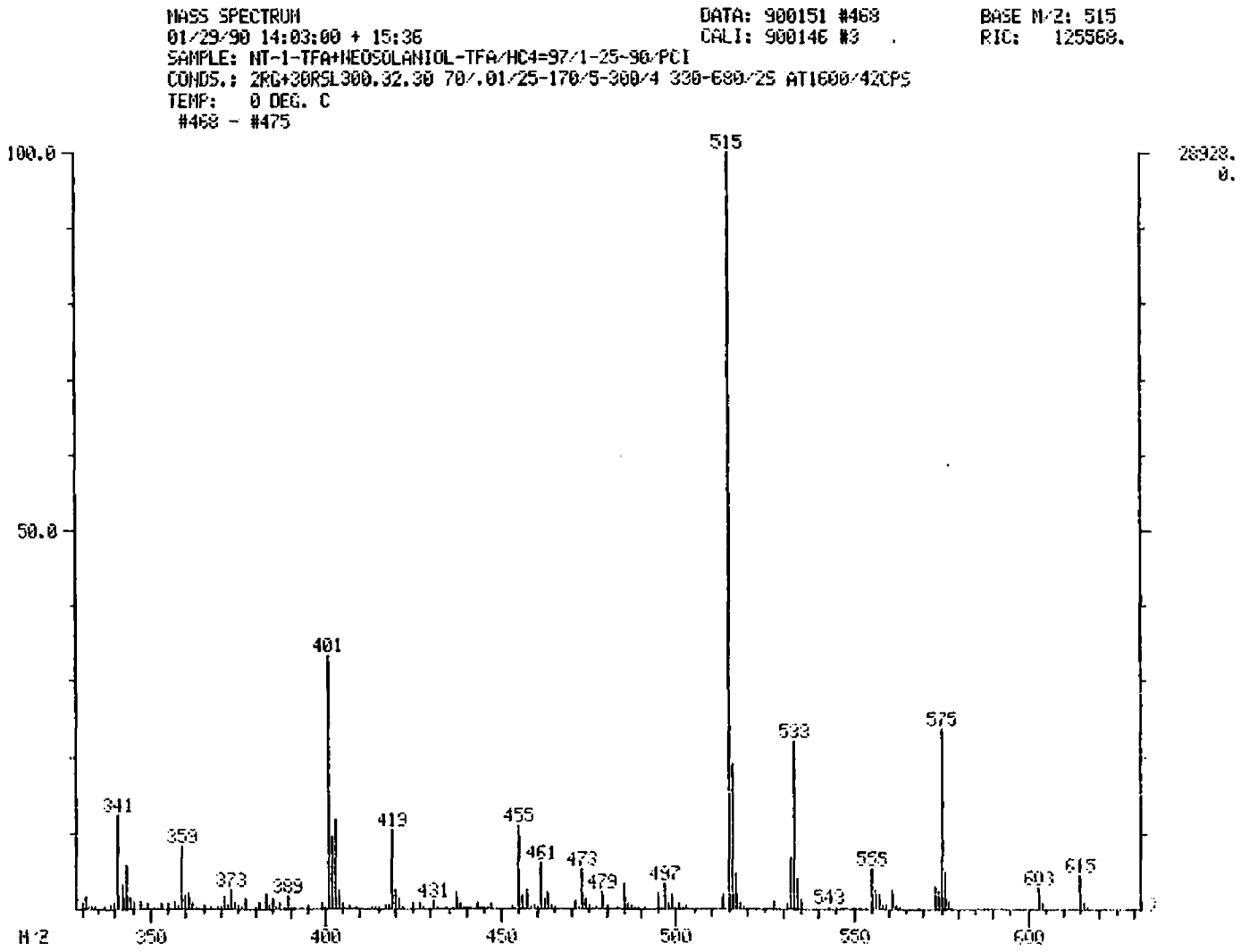

Figure 5-6. MS (PCI mode) of TFA-NT-1 standard.

2. 8-acetoxyneosolaniol (Fractions 16-III, scan # 610 and 16-II-1&2, scan # 592):

The GC/MS total ion chromatogram of the TFA derivative of fraction 16-III had a major peak (scan # 610) at a retention time of 19:52 min (Fig. 5-8). The PCI mass spectrum of this fraction (Fig. 5-9) was identical to the mass spectrum of 8-acetoxyneosolaniol reported previously [27]. A molecular ion (MH^+) of 521, and main fragment ions at m/z 461 (loss of acetic acid from the C-8 position), 401 (additional loss of acetic acid from the C-15 position) and m/z 341 (loss of a third acetic acid from the C-4 position) were diagnostic ions in the spectrum. Based on comparison of the mass spectra of the fraction with the literature reports, the compound was identified as 8-acetoxyneosolaniol (Fig. 5-10).

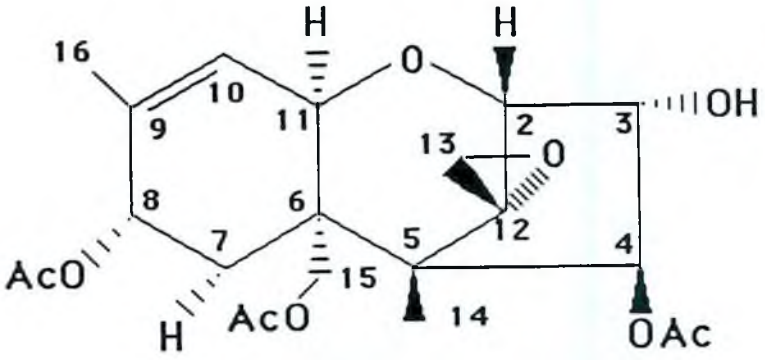

Figure 5-10. Chemical structure of 8-acetoxyneosolaniol.

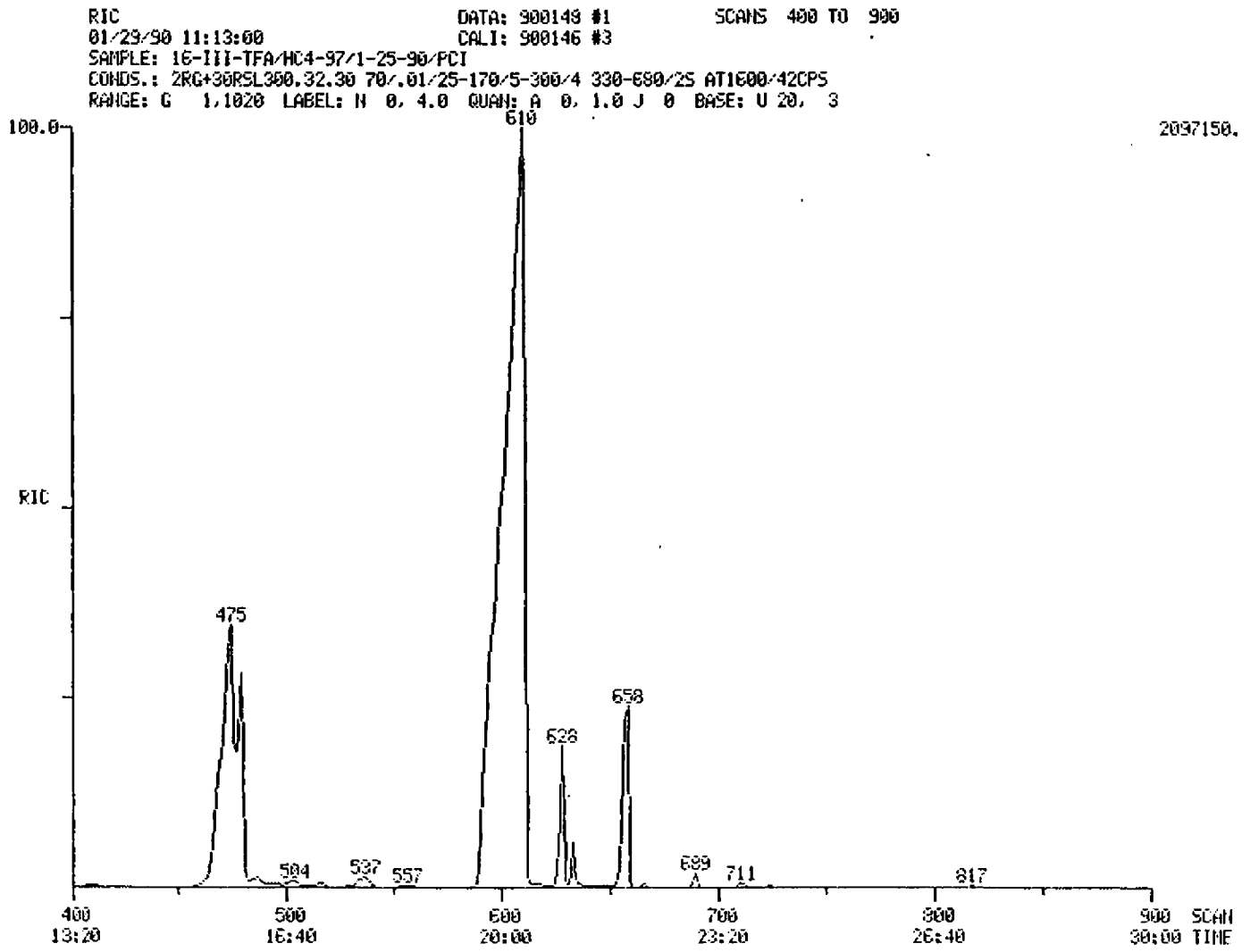

Figure 5-8. GC/MS total ion chromatogram (PCI mode) of TFA derivative of fraction 16-III.

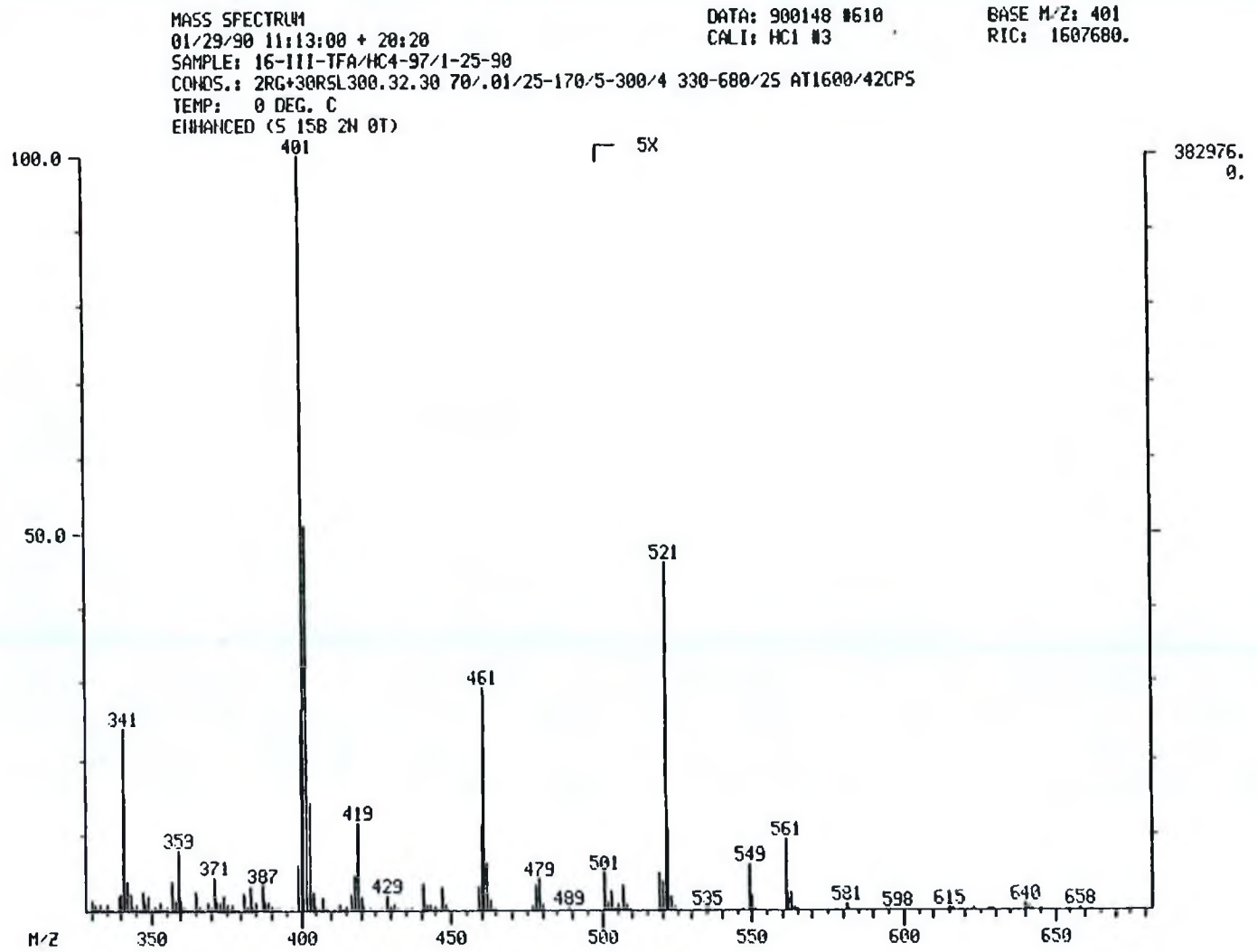

Figure 5-9. MS (PCI mode) of TFA derivative of fraction 16-III, scan# 610.

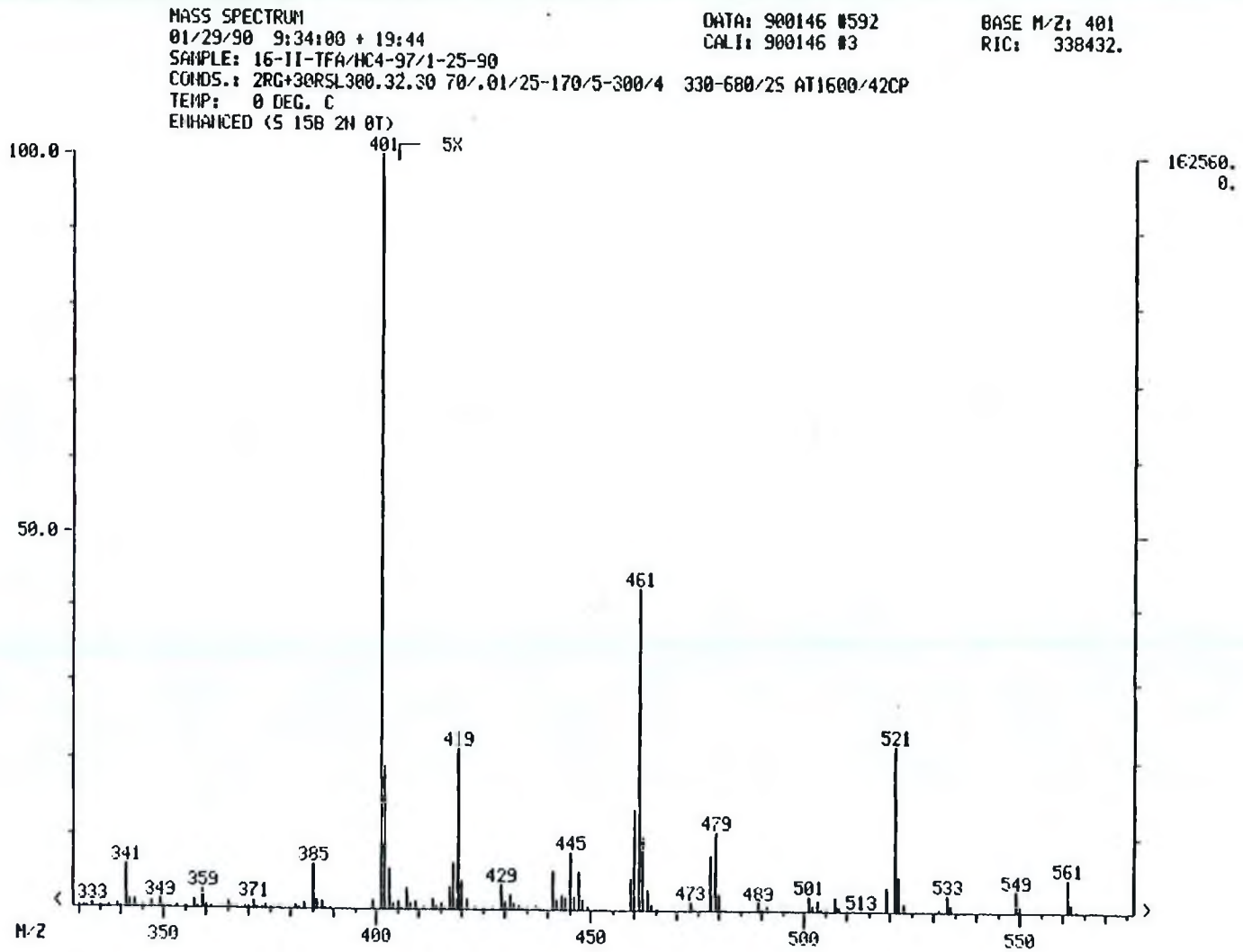

Figure 5-11. MS (PCI mode) of TFA derivative of fraction 16-II-1&2, scan# 592.

distilled water were loosely capped and autoclaved for 20 min at 15 psi. The moisture content after autoclaving was approximately 35%. A spore suspension was prepared by adding sterilized water to 7 day old PSA plates of each isolate (10 ml/plate) and scraping the surface of the culture with an inoculating loop. A 2 ml aliquot of the mycelium-spore suspension was pipetted into each jar (10 jars/isolate). The jars were shaken, the lids loosened to allow respiration and the cultures were incubated for 28 days at 10 to 12 C in darkness. F. graminearum DZ1a was also cultured in ten 250 ml Erlenmeyer flasks containing 50 ml GYEP liquid medium.

At the end of the incubation period, 400 ml 85:15 chloroform:acetone was added to each jar, clumps were broken up and left overnight to soak. The cultures were placed in a blender for 3 min and filtered through Whatman #1 filter paper under vacuum. The culture medium was re-extracted in the blender with acetone and filtered. The GYEP liquid culture was extracted as described earlier. The extracts were concentrated to an oil-like residue on a rotary evaporator.

The oily-residue was passed through a florisil column and the different fractions separated by silica gel flash chromatography as described below.

Florisil column: Florisil (Fisher, 60-100 mesh) was packed into 5 x 20 cm gravity column (containing 2:1 benzene:hexane) to a depth of about 10 cm. A 5 cm layer of anhydrous sodium sulfate (Fisher) was added to the top of the florisil. The

oily-residue was dissolved in benzene:hexane (2:1) and approximately 30 ml was added to the column. The column was washed successively with 500 ml benzene:hexane (2:1); methylene chloride and chloroform: acetone (9:1). The column was finally stripped with acetone. The chloroform:acetone and acetone fractions were concentrated in a rotary evaporator and examined by silica gel TLC.

Silica gel flash chromatography: The trichothecenes present in the florisil extracts of each isolate were separated by flash chromatography as described by Still [21]. A standard 4 x 45 cm flash column, equipped with a regulator head, was slurry packed with 40 to 60 μm silica gel (Baker) in benzene:acetone (4:1) to a level of about 15 cm. A 1 cm layer of Ottawa sand was placed on top of the silica gel. The florisil eluant was dissolved in 0.5 ml benzene:acetone (4:1) and layered on the column. The column was eluted successively with 4:1, 3:1 and 2:1 benzene:acetone with a solvent head flow rate of 5 ml/min. Eighty tubes of 2 ml fractions were collected. Every third tube was concentrated and examined by silica gel TLC. Similar fractions were combined.

C_{18} flash chromatography: Compounds that could not be separated by silica gel chromatography were separated on a C_{18} flash chromatography column. Twenty grams of 40 μm C_{18} (Baker) was mixed with 50 ml solvent (35:20:1 methanol:water:acetic acid) and slurry packed in a 2 x 45 cm flash column to a height of about 10 cm. Half a milliliter of culture extract dissolved in

the same solvent was layered on the column. The column was eluted with 100 ml solvent under pressure. Fifty fractions (2 ml) were collected in tubes, and every third tube was extracted twice with chloroform. The chloroform layer was taken to dryness, redissolved in acetone:methanol (2:1) and spotted on C_{18} TLC plates. The plates were developed in solvent system 4, dried, and sprayed with spray reagent #4. Based on the spots on the C_{18} plate, similar fractions were combined, extracted with chloroform and taken to dryness.

Preparatory TLC on silica gel and C_{18}: Normal phase pre-adsorbent silica gel HL (Analetch) and reverse phase C_{18} (Whatman, KC18F) TLC plates were used to isolate trichothecenes that co-eluted on flash chromatography. Each extract from the flash column was dissolved in 2:1 acetone:methanol and approximately 100 μl was applied to the pre-adsorbent strip on the silica gel plates or carefully spotted on the C_{18} plates. The silica gel and C_{18} plates were developed in solvent systems 3 and 4, respectively. Strips (2 cm wide) were cut from each side of the developed silica gel and C_{18} plates and sprayed with spray reagents # 3 and #4, respectively. The strips were realigned and bands were scraped from the unsprayed portions of the plates. The trichothecenes were eluted (3x) from the silica gel or C_{18} with 10 ml acetone. The acetone extracts were filtered through Rainin 0.45 micron nylon-66 membrane filters, concentrated, and re-checked for purity by TLC. The purified compounds were

submitted for GC-MS analysis.

Gas Chromatography-mass spectroscopy (GC-MS): The GC-MS analysis of the purified toxins and the interpretation of the mass spectra were made by Dr. Howard Casper, North Dakota State University, Dept. of Vet. Sci., Fargo, ND. A Finnigan INCOS 50 system quadrapole mass spectrophotometer interfaced with a Hewlitt Packard 5840 gas chromatograph was used. Chromatographic separations were made on a 37 mm x 0.32 mm (i.d.) fused silica capillary column Ultra I (0.50 μm). Helium was used as the carrier gas at 25 psi. The column oven was programmed from 70 C to 170 C at 25 C/min, then ramped from 170 C to 300 C at 5 C/min and held for 4 min.

Mass spectrometer conditions: ion source temperature, 175 C; transfer line, 300 C; scan rate, 50-700 aMU/s; ionizing voltage, 70 eV. EI (electron impact) and PCI (positive chemical ionization) used methane gas with ion source at 120 C.

Sample derivatization: Each toxin was taken up in acetonitrile and a 10 to 50 μg aliquot of each toxin was evaporated to dryness with nitrogen. The aliquot was then treated with 200 μl TMSI (N-trimethylsilylimidazole, Pierce Chemical Co.) in a reaction vial for one hour at 65 C. One ml water and 1 ml isooctane were added, vortexed and centrifuged. The isooctane layer was analyzed by GC-MS. The sample residue was also reacted with 50 μl TFAA (trifluoroaceticanhydride, Pierce Chemical Co.) in a reaction vial at 65 C for 30 min.

After excess reagent was evaporated with a stream of nitrogen, the residue was reconstituted in 100 μl toluene and 1 μl was injected in the GC/MS.

RESULTS

Detection of Fusarium toxins on infected seeds:

Wheat seeds collected from FHB infected fields were screened for the presence of trichothecenes and zearalenone on silica gel TLC plates. Only one sample was found to be contaminated with mycotoxins. Low level (less than 1 ppm) T-2 toxin and DAS were detected by silica gel TLC. This was confirmed by GC-MS analysis at the National Veterinary Services Lab., Ames, Iowa. The sample (cultivar Dashen) was collected from Dixis state farm.

Screening Fusarium isolates for mycotoxin production:

Extracts from 78 isolates of Fusarium, cultured in potato dextrose broth (PDB) were examined by silica gel TLC. No detectable amount of zearalenone, DON, 3-ADON, T-2 toxin, DAS, HT-2, neosolaniol, nivalenol or T-2 tetraol was produced by the majority of the isolates in this medium under the culturing conditions described. The detection limit was 1 ppm for each of these mycotoxins. However, some spots from extracts of F. sporotrichioides #48d and F. poae #5 did correspond to T-2 toxin and neosolaniol standards. There were additional spots that did not correspond to any of the

standards. The quantity of these compounds was too small for further analysis. The culture conditions were not conducive to produce large amounts of the mycotoxins.

These two isolates as well as 15 other isolates of F. nivale, F. avenaceum, F. graminearum, F. lateritium and F. equiseti were selected and cultured on GYEP, rice and corn media. Upon examination by silica gel TLC, only three isolates of F. graminearum (DZ1a, ADT1c and HR24) grown on rice medium produced spots that corresponded to an estimated 500 to 1000 ppm zearalenone. F. graminearum (HR24) cultured on rice and corn also produced spots that corresponded to an estimated 10 and 100 ppm DON and 3-ADON, respectively. The extract from F. graminearum DZ1a culture grown on GYEP liquid medium produced a large spot that occurred between the spots of the standards DON and 3-ADON. F. avenaceum #18, F. graminearum HR24, F. poae #5 and F. sporotrichioides #48d grown on corn medium produced spots that did not match any of the standards. Some of the spots were, however, very close to the T-2 toxin or DAS and neosolaniol standards. The remaining extracts were too small to be used for further purification and identification.

Based on these preliminary results, five isolates (F. graminearum HR24, F. poae #5, F. sporotrichioides #48d, F. avenaceum #18 and F. nivale #43b) were cultured on corn grits for large scale mycotoxin production. The last two species were included only because they were the most frequently identified species from scabby Ethiopian wheats in 1988. F.

graminearum DZ1a was also cultured in GYEP liquid medium. The extracts from these cultures were cleaned up by florisil column chromatography and the trichothecenes were separated by silica gel flash chromatography.

Silica gel TLC analysis of these fractions showed that only F. graminearum HR24 produced spots that corresponded to an estimated 100 ppm DON and 3-ADON. All Fusarium species produced various trichothecenes or trichothecene-like compounds which were detected by silica gel TLC. Fractions with spots that appeared blue with the blue spray (spray reagent #3) and had relative R_f values between T-2 acetate (Rel. R_f 1.60) and T-2 tetraol (Rel. R_f 0.05) were collected from each isolate (Table 5-2). Several spots had relative R_f values similar to T-2 toxin (Rel. R_f 1), DAS (Rel. R_f 0.93) and neosolaniol (Rel. R_f 0.45). Most spots, however, did not correspond to any of the standards.

The result of the gas chromatography-mass spectra (GC-MS) analysis of these fractions showed that only 3-ADON in fractions II and III of F. graminearum HR24, 15-ADON in fraction II of F. graminearum DZ1a and neosolaniol in fraction VI of F. poae #5 extracts were detected. The presence of any known trichothecene in all the fractions of F. nivale #43b, F. avenaceum #18, and the rest of the fractions of F. graminearum HR24, F. graminearum DZ1a and F. poae #5 was not confirmed by GC-MS. The levels of these compounds were very low, therefore, restricting further study.

Table 5-2 Relative R_f values of fractions from culture extracts of *Fusarium* isolates and standards on silica gel TLC[a].

Fusarium isolates	Fraction	Rel.R_f.[b]
F. nivale #43b	I	1.50
	II	1.38
	III	1.10
	IV	0.91
	V	0.63
F. avenaceum #18	I	1.28
	II	1.00
	III	0.78
	IV	0.50
F. graminearum HR24	I	1.54
	II	1.00
	III	0.93
	IV	0.68
	V	0.55
F. graminearum DZ1a[c]	I	0.97
	II	0.67
	III	0.56
F. poae #5	I	1.46
	II	1.36
	III	1.18
	IV	0.93
	V	0.64
	VI	0.50
F. sporotrichioides #48d	16-I	1.36
	16-II	0.97
	16-III	0.86
	16-IV	0.69
	16-V	0.47
Standards		
T-2 acetate		1.60
4-propanoyl HT-2		1.16
T-2 toxin		1.00
DAS		0.93
Neosolaniol		0.45
HT-2		0.32
Nivalenol		0.07
T-2 tetraol		0.05

a. 3:1 benzene:acetone solvent system; HL silica gel plate
b. Relative to T-2 toxin.
c. Cultured on GYEP liquid medium.

F. sporotrichioides #48d, on the other hand, produced high levels of trichothecene mycotoxins. Further study was, therefore, concentrated on separation and identification of the trichothecenes produced by this isolate. Five fractions were separated by silica gel flash chromatography. The relative R_f of these fractions are presented in Table 5-2. Based on the relative R_fs of the spots compared with the standards on TLC, two of the fractions, fraction 16-II (Rel. R_f = 0.97) and fraction 16-V (Rel. R_f = 0.47) were tentatively identified as either T-2 toxin or DAS and neosolaniol, respectively. The other fractions were not identified by silica gel TLC.

The GC/MS analysis of these fractions revealed several important points. Fraction 16-I was found not to be a trichothecene. The GC/MS total ion chromatogram of fraction 16-II (which appeared as one spot on silica gel TLC and was suspected to be either T-2 toxin or DAS) actually contained a mixture of trichothecenes (Figs. 5-1 & 5-2). These compounds could not be separated by silica gel TLC. The compounds were, however, easily separated by C_{18} flash chromatography or C_{18} TLC into five fractions (16-II-1&2, 16-II-3, 16-II-4, 16-II-5 and 16-II-6). The relative R_fs (relative to T-2 toxin) of these fractions on C_{18} TLC are shown in Table 5-3. Fraction 16-II-5 had a similar R_f to that of T-2 toxin.

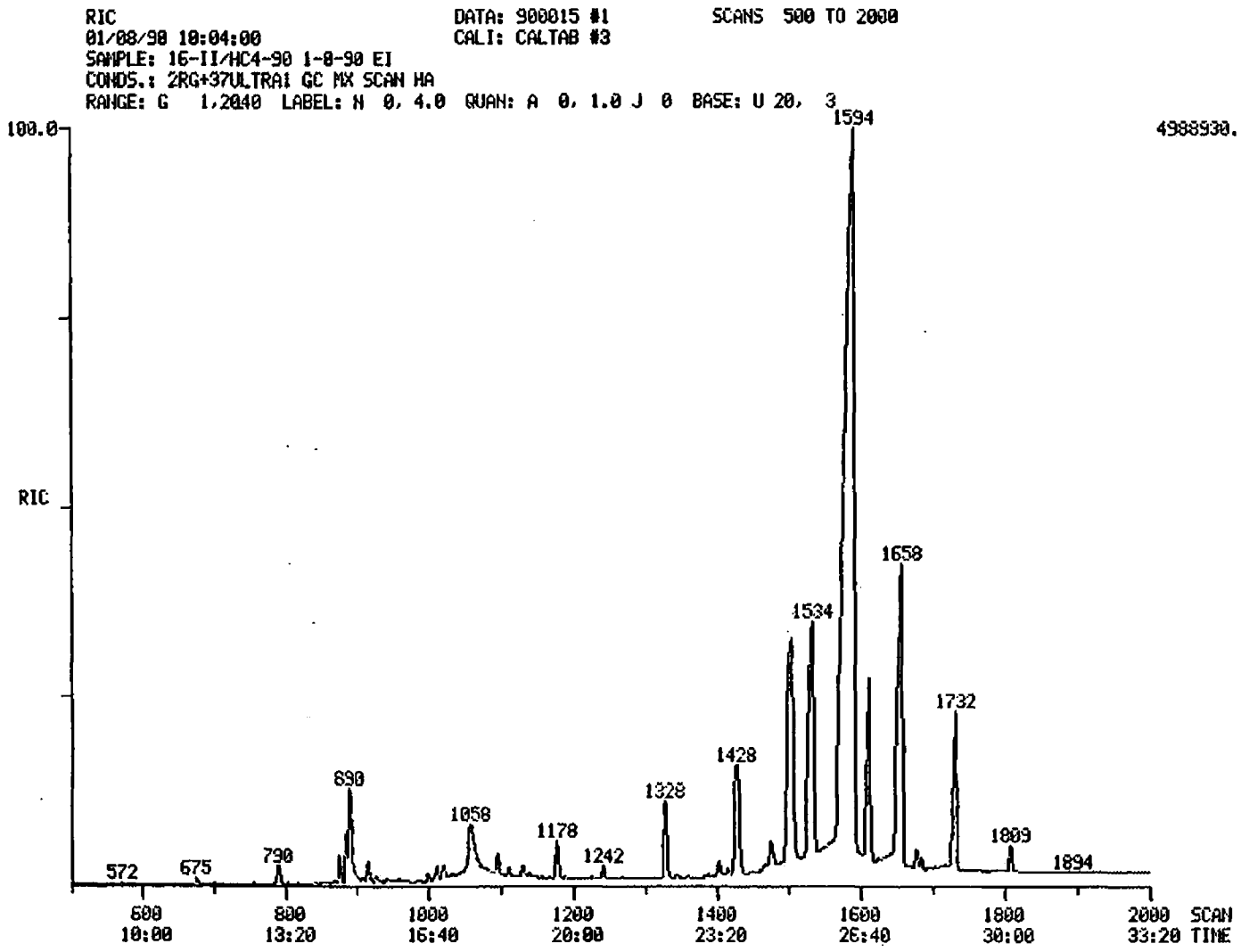

Figure 5-1. GC/MS total ion chromatogram (EI mode) of TMS derivative of fraction 16-II.

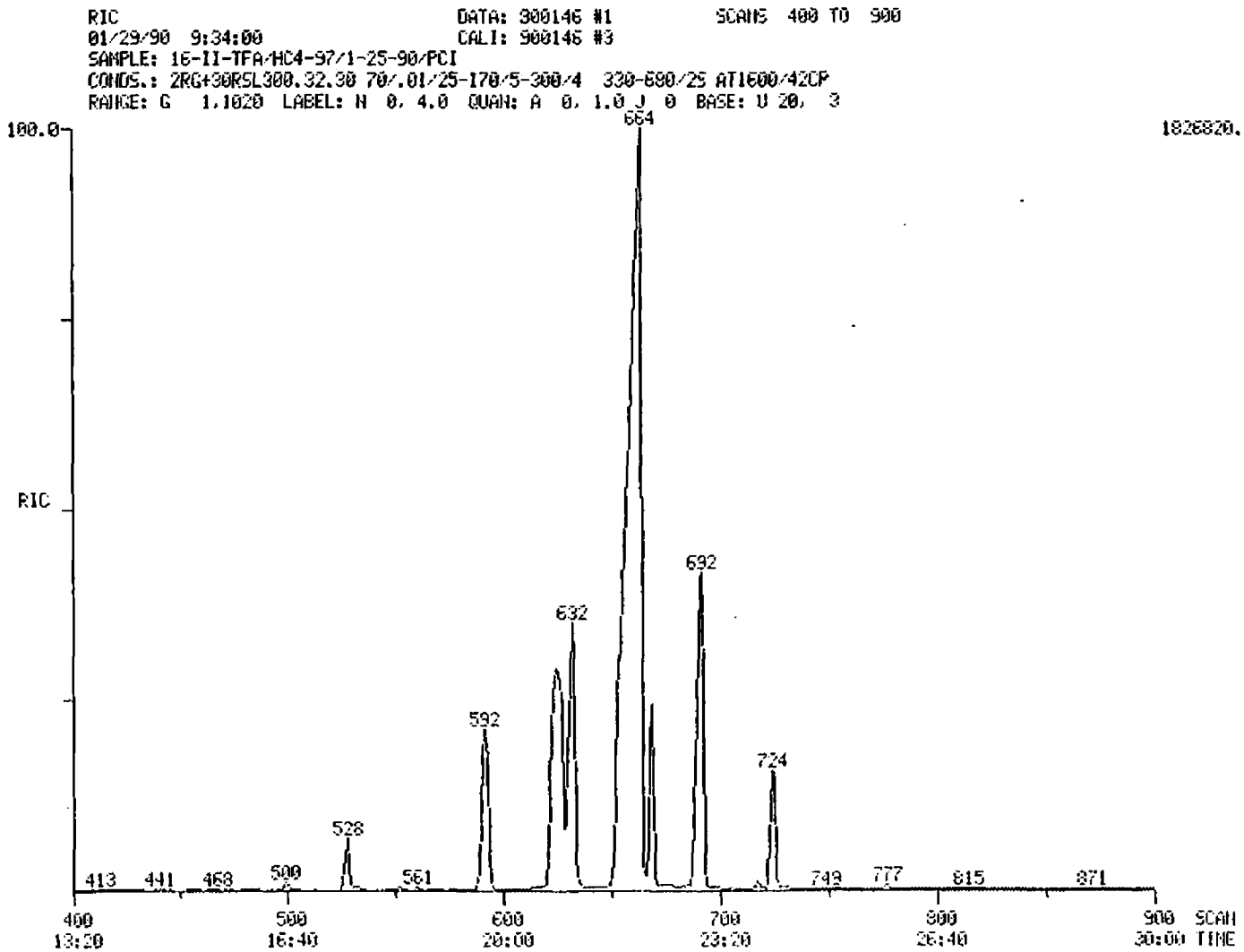

Figure 5-2. GC/MS total ion chromatogram (PCI mode) of TFA derivative of fraction 16-II.

Table 5-3. Relative R_f values (Rel. R_f) on C_{18} TLC of fraction 16-1I isolated from culture extract of F. sporotrichioides #48d.

Fraction	C-18 TLC[a] Rel. R_f[b]
16-II-1&2	2.00
16-II-3	1.73
16-II-4	1.41
16-II-5	1.03
16-II-6	0.65

a. 35:20:1 methanol:water:acetic acid.
b. Relative to T-2 toxin.

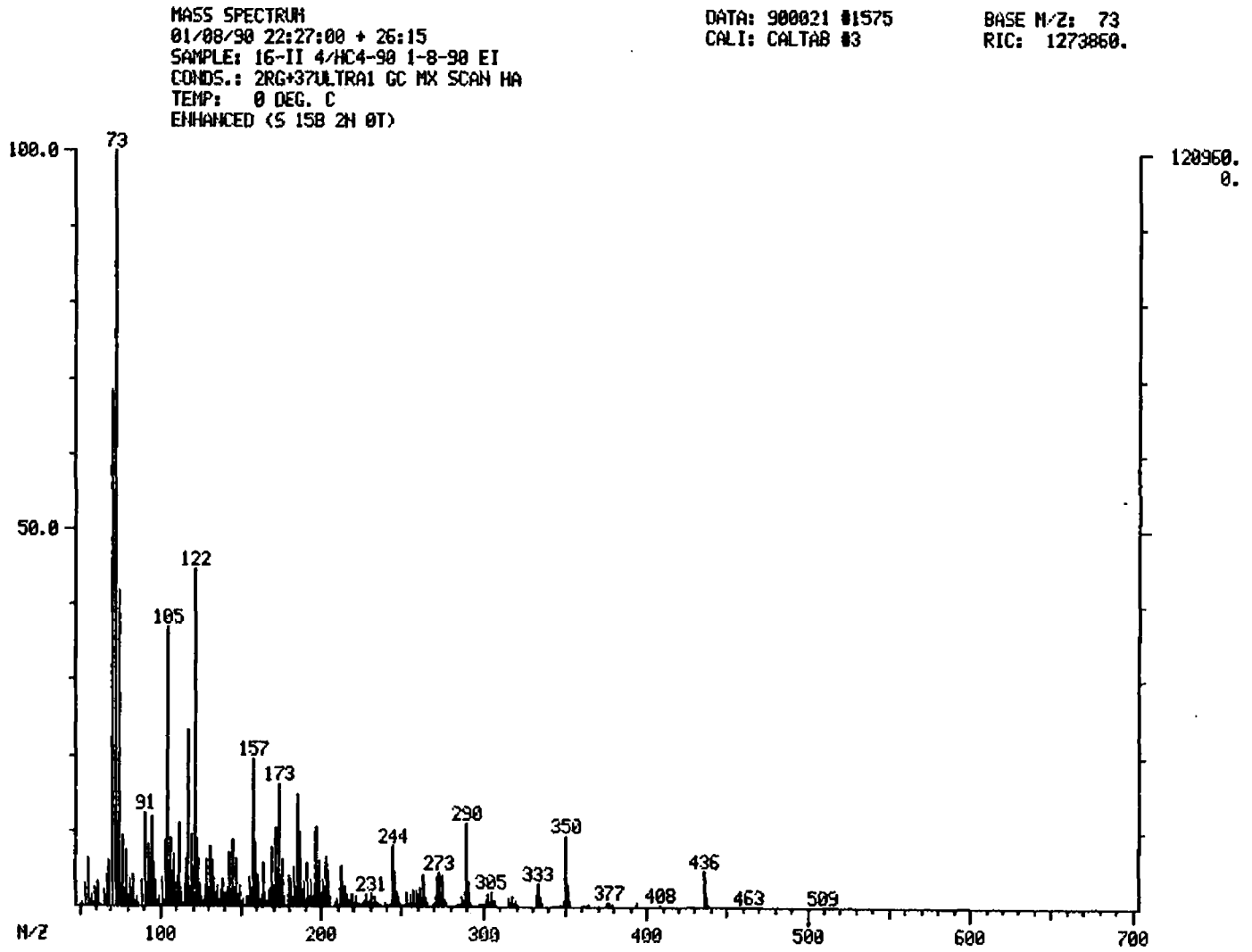

Figure 5-25. EI mass spectrum of TMS derivative of fraction 16-II-4, scan# 1575.

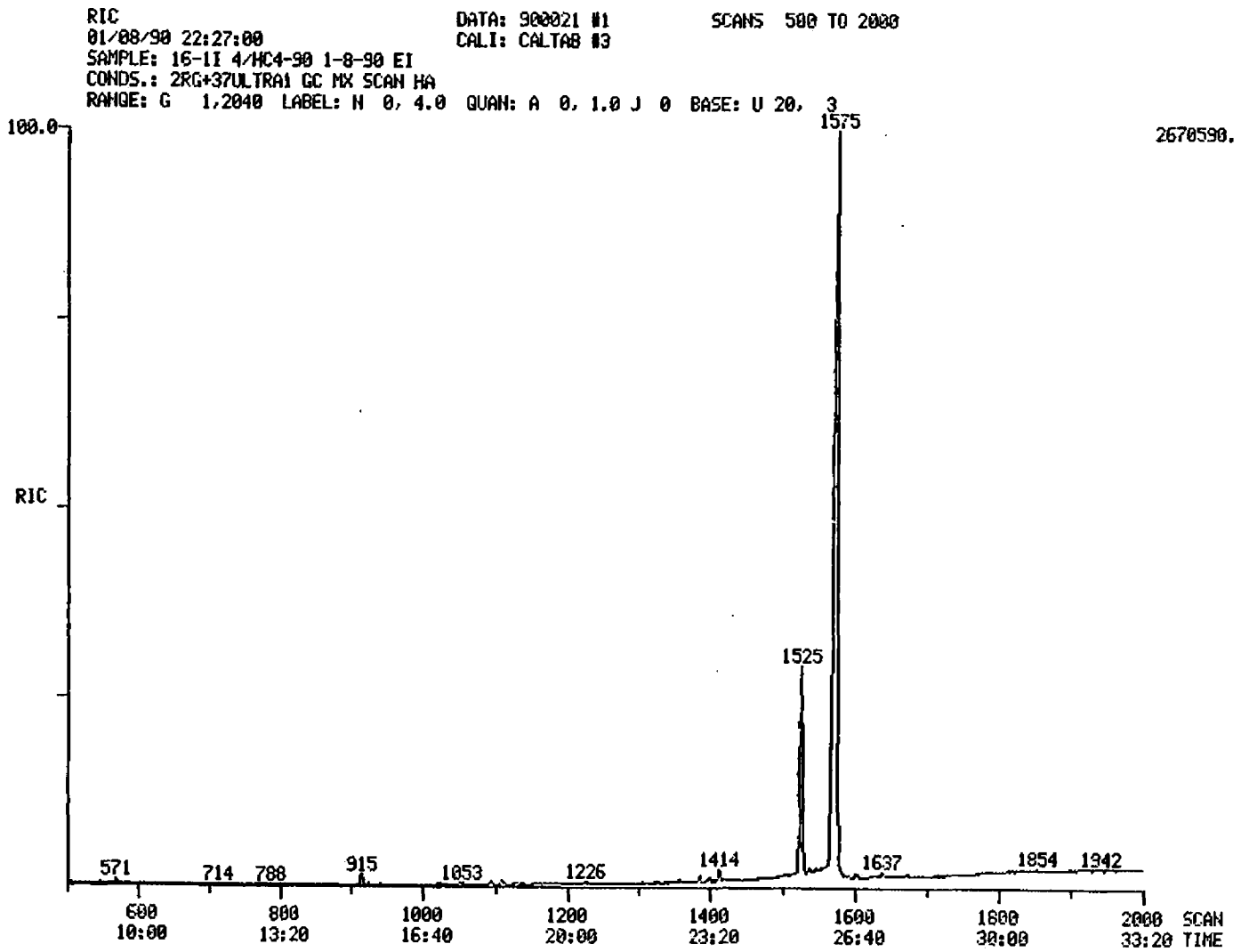

Figure 5-22. GC/MS total ion chromatogram (EI mode) of TMS derivative of fraction 16-II-4.

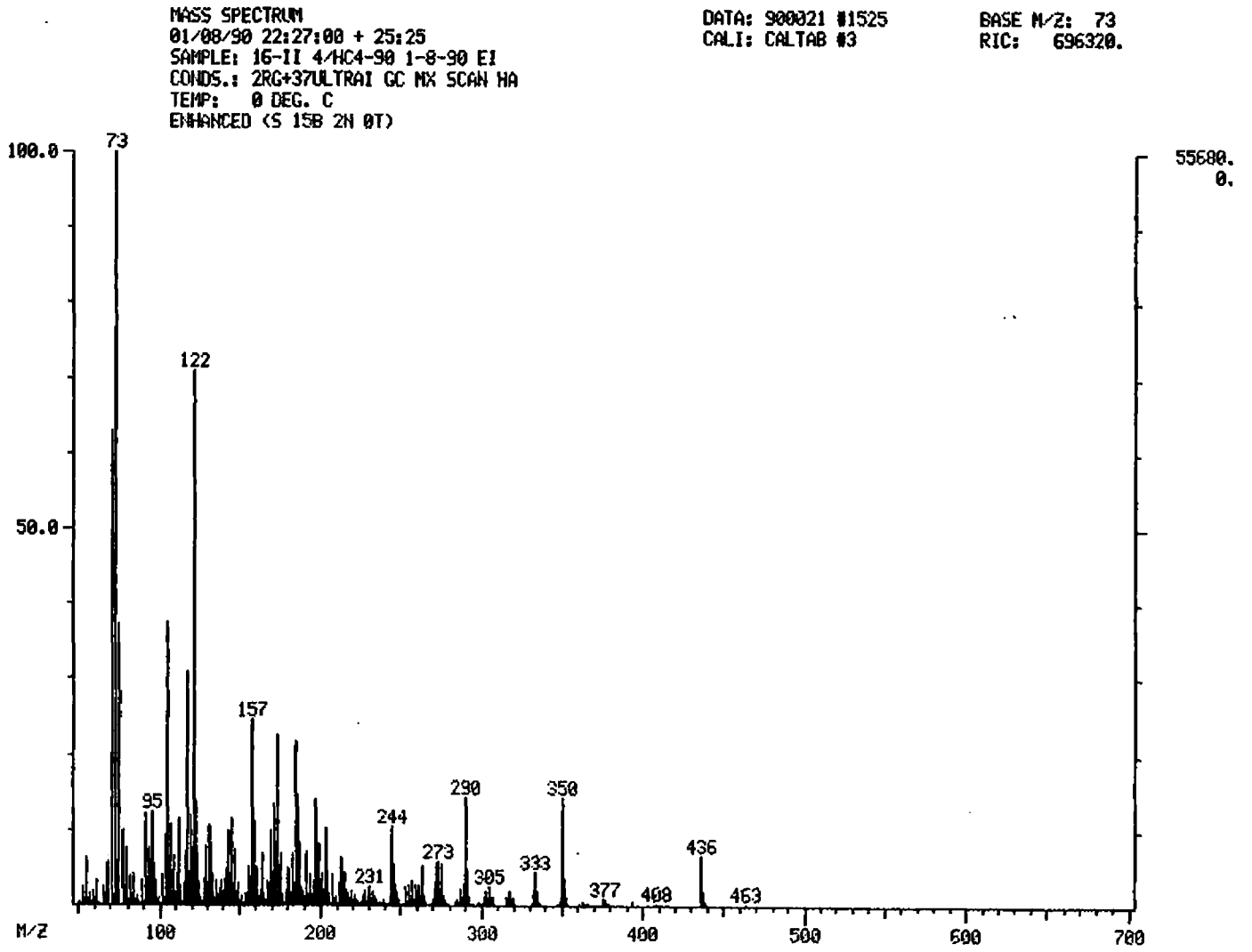

Figure 5-23. EI mass spectrum of TMS derivative of fraction 16-II-4, scan# 1525.

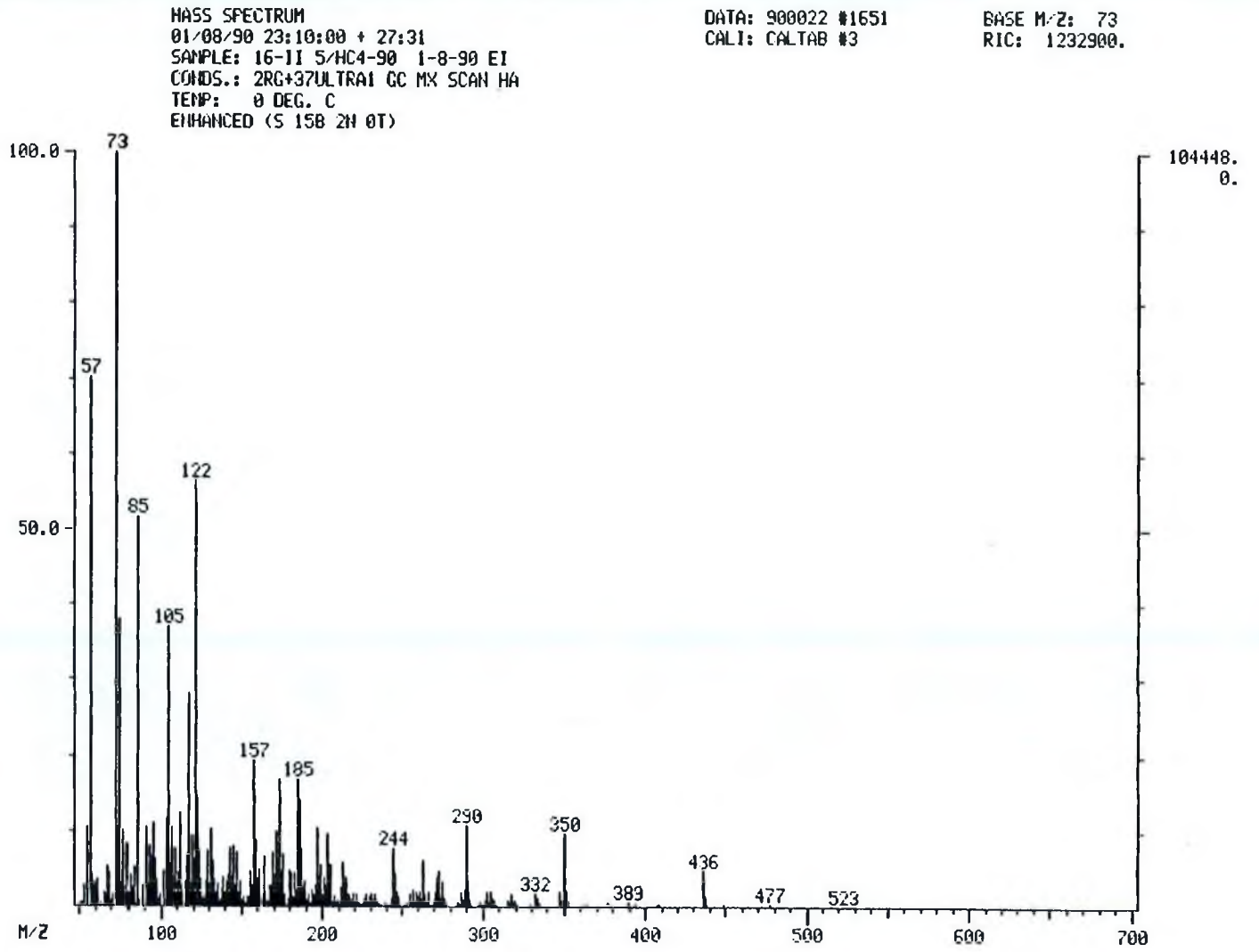

Figure 5-20. EI mass spectrum of TMS derivative of fraction 16-5, scan# 1651.

6. 8-isobutyrylneosolaniol (fraction 16-II-4, scan 1525):

The GC/MS total ion chromatogram of fraction 16-II-4 (Fig. 5-22) showed two distinct peaks. The retention time of the smaller peak (scan# 1525) was 25:24 min and the larger peak (scan# 1575) was 26:07 min. The MH^+ (PCI mode) of both compounds was 524. The EI mass spectra of both scan#1525 and 1575 (Fig. 5-23 and Fig. 5-25) were similar to the EI MS of the 3-TMS-neosolaniol standard, except for the presence of a large fragment at m/z 71. The difference between the molecular ion (MH^+ =524) and the 3-TMS-neosolaniol fragment (m/z 436) was the loss of a butyric acid (m/z 88) from the 8 position. The m/z 71 fragment corresponds to C_4H_7O group which is either n-butyric or isobutyric acid. From the GC/MS total ion chromatogram of isovaleryl and n-valeryl neosolaniol (Fig. 5-16), it was shown that the compound with the iso group had a shorter retention time. Therefore, the compound with the smaller peak and shorter retention time was tentatively identified as 8-isobutyrylneosolaniol (Fig. 5-24).

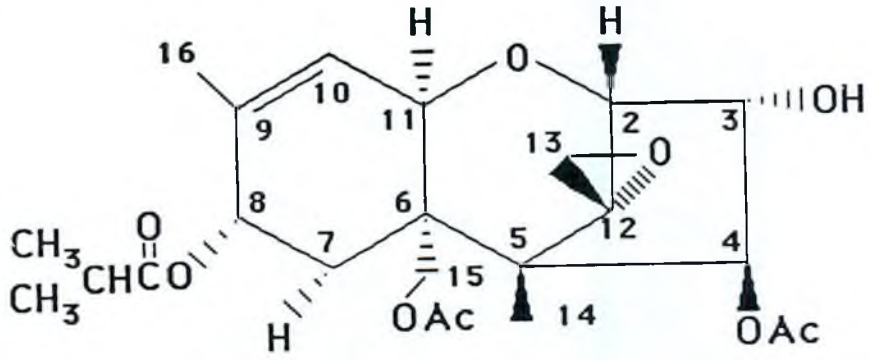

Figure 5-24. Chemical structure of 8-isobutyrylneosolaniol.

5. 8-n-valerylneosolaniol (Fraction 16-II-5, scan 1651):

The major peak in the GC/MS total ion chromatogram of fraction 16-II-5 (Fig. 5-16) had a retention time of 27:23 min (scan # 1651). Both the smaller and the major peak had the same molecular ion (MH^+ = 538) by PCI mode and identical EI mass spectra (Fig. 5-20). The fragment at m/z 85 appeared in the EI MS of this fraction indicating that it also contained the C_5H_9O fragment. The loss of this fragment suggested that the difference in structure was the n-valeryl vs isovaleryl of T-2 toxin. The difference in retention time also showed that the compounds were different. The iso- configuration had a shorter retention time than the n-valeryl derivative. Based on the identical EI MS and molecular ion (PCI mode) and difference in retention time, the compound in the major peak (scan # 1651) was identified tentatively as 8-n-valerylneosolaniol (Fig. 5-21).

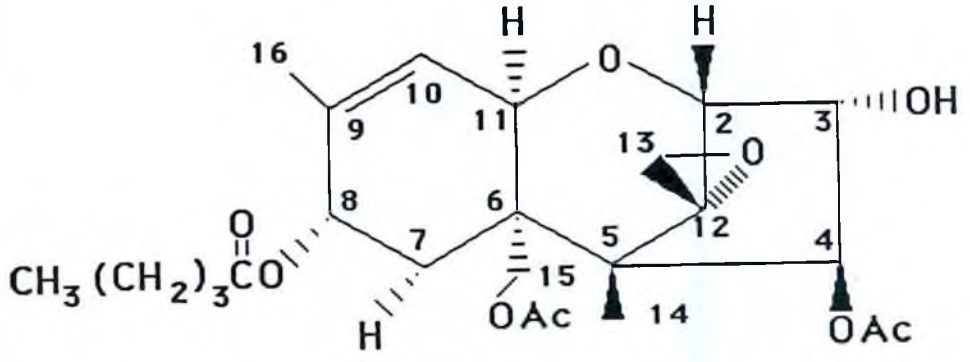

Figure 5-21. Chemical structure of 8-n-valerylneosolaniol.

DISCUSSION

The majority of the Fusarium isolates identified from both stored seed and from scabby wheat in the field did not produce detectable amounts of known trichothecenes or zearalenone. The productions of 3-ADON and 15-ADON by a few isolates of F. graminearum and neosolaniol by an isolate of F. poae were very low under the different culturing conditions used. Isolates of F. nivale and F. avenaceum, which were the most common species in the field samples, did not produce mycotoxins in culture.

Only one of the fifty seed samples collected from FHB infected wheat fields in 1988 was found to be contaminated with low levels of mycotoxins, eventhough some of the samples contained over 50% infected seeds (primarily by F. nivale and F. avenaceum) and were severely shriveled. The results indicated that the importance of the Fusarium species was primarily as pathogens to the crop, causing loss in yield and quality, rather than from being toxigenic.

F. sporotrichioides #48d, which occurred at a low frequency, however, produced a variety of known and new trichothecene mycotoxins. Other strains of the species were previously known to produce over a dozen trichothecenes [13,26]. These include T-2 toxin, 3'-hydroxy T-2, acetyl T-2, T-2 tetraol, T-2 triol, DAS, HT-2, 3'-hydroxy HT-2, neosolaniol and 4-deacetylneosolaniol. Several minor

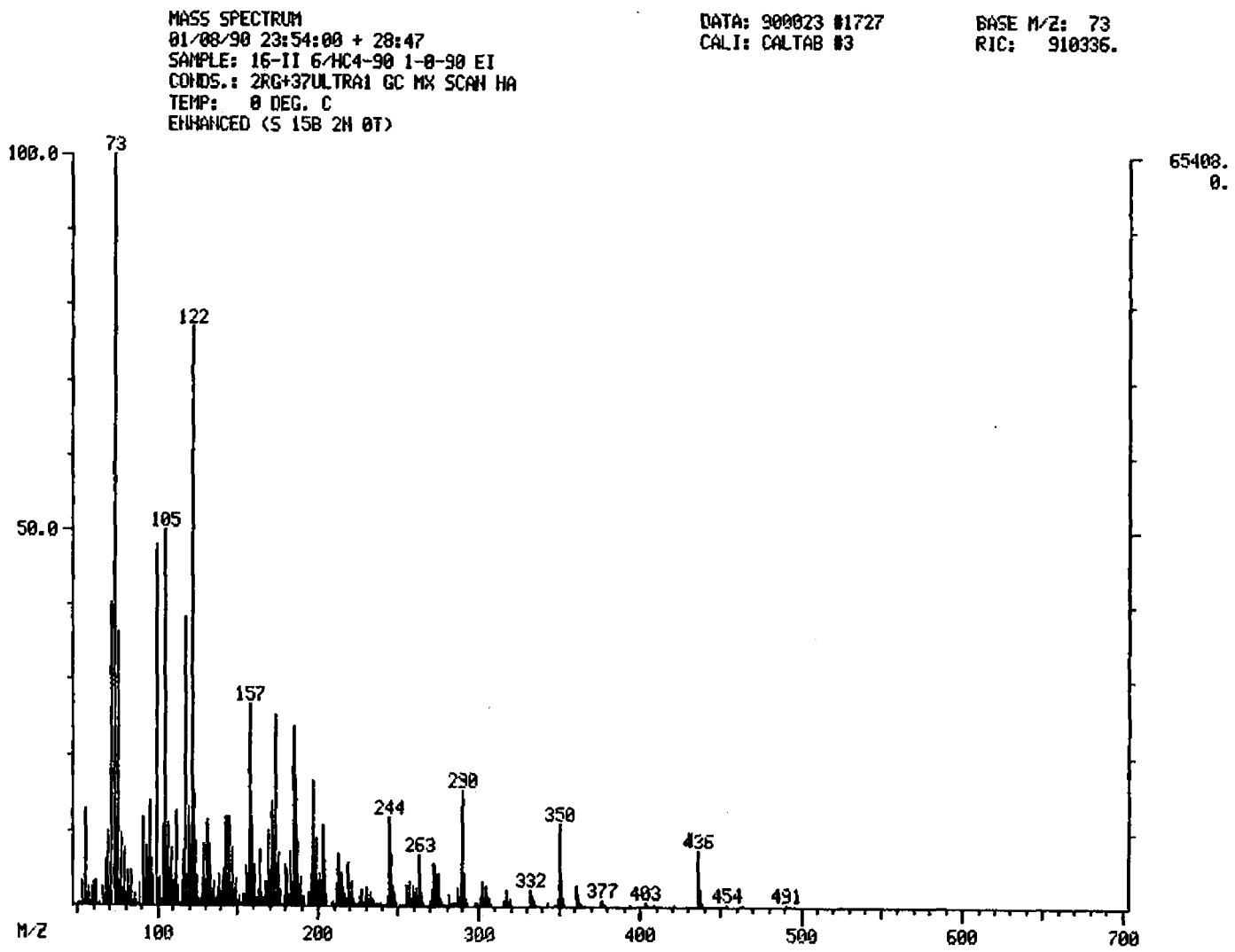

Figure 5-31. EI mass spectrum of TMS derivative of fraction 16-II-6, Scan# 1727.

8. 8-propionylneosolaniol (Fraction 16-II-3, scan 1499):

The GC/MS total ion chromatogram of fraction 16-II-3 (Fig. 5-27) had a major peak at a retention time of 24:50 min (scan# 1499). The MH^+ = 510 was obtained from the MS of the fraction in PCI mode. The EI mass spectrum of the fraction (Fig. 5-28) was similar to the spectrum of the 3-TMS-neosolaniol except for the major fragment at m/z 57. The difference between the molecular ion (MH^+=510) and the 3-TMS-neosolaniol fragment (m/z 436) was a loss of propionic acid (m/z 74) from the 8 position. The fragment m/z 57 was CH_3CH_2CO- of the propionic acid. This fraction was, therefore, tentatively identified as 8-propionylneosolaniol (Fig. 5-29).

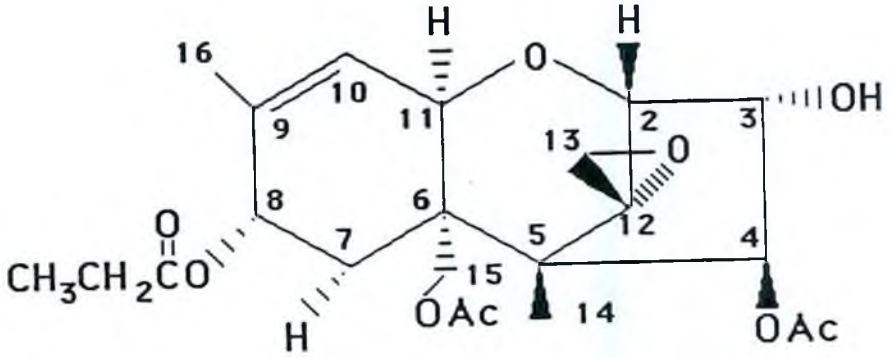

Figure 5-29. Chemical structure of 8-propionylneosolaniol.

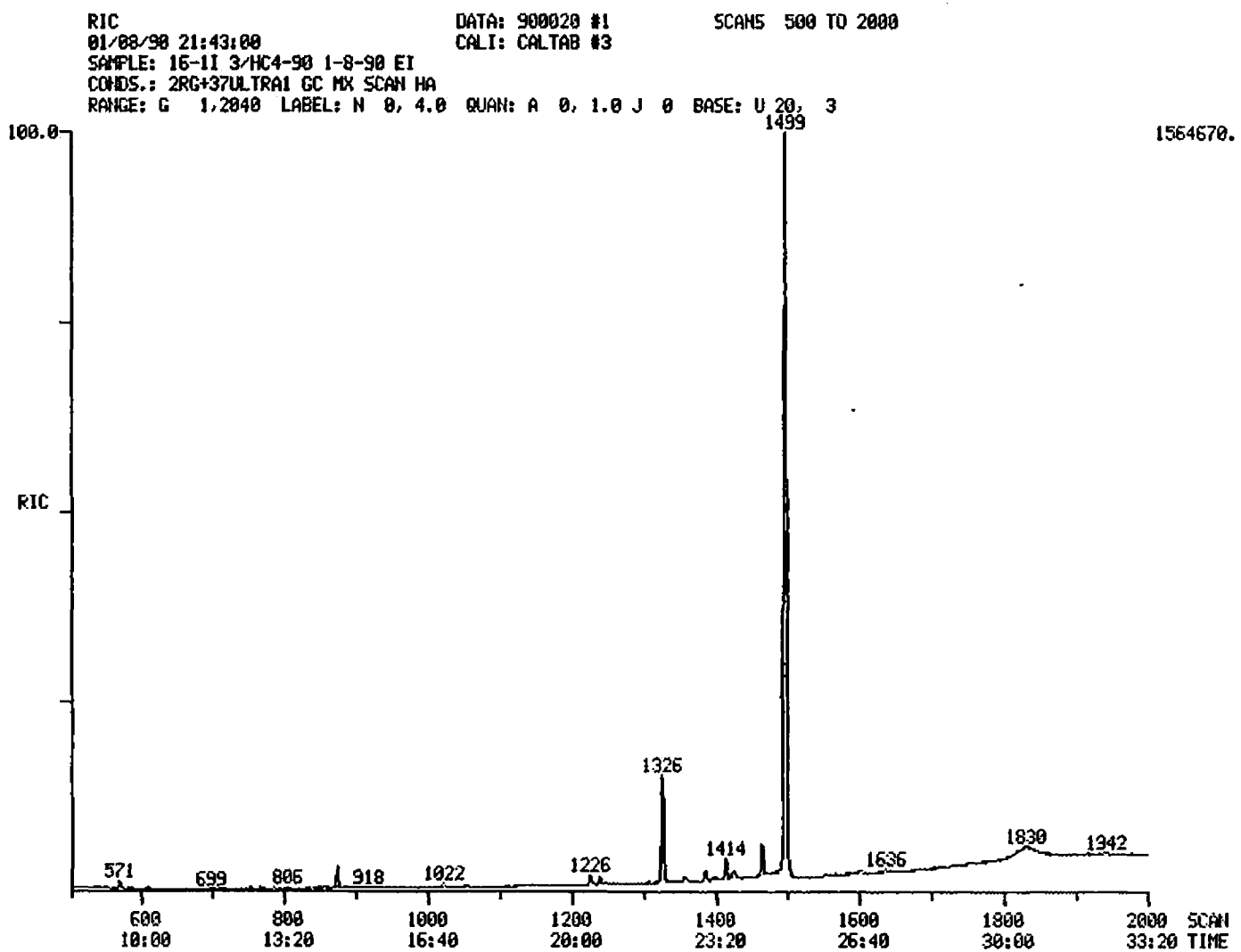

Figure 5-27. GC/MS total ion chromatogram (EI mode) of TMS derivative of fraction 16-II-3.

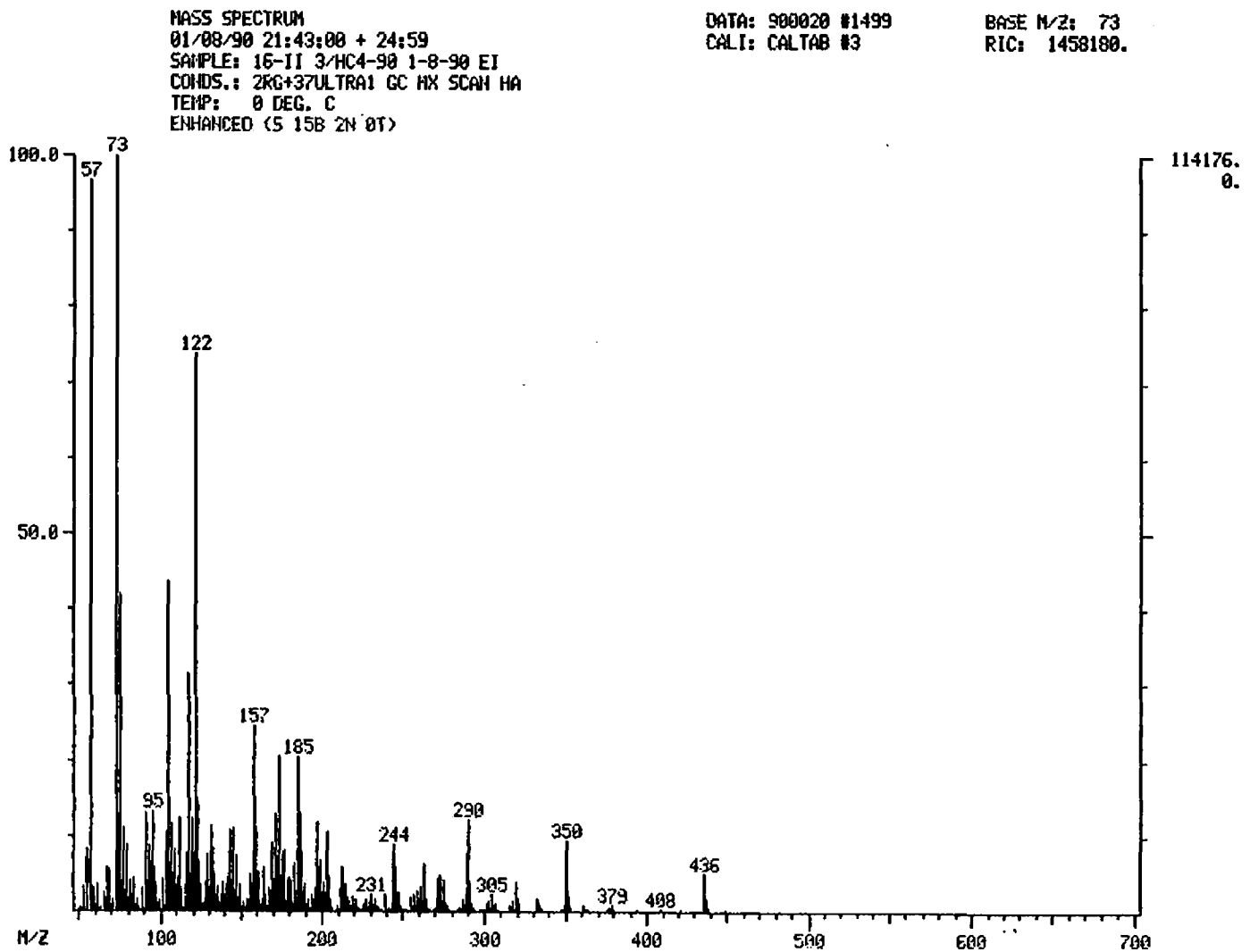

Figure 5-28. EI mass spectrum of TMS derivative of fraction 16-II-3, scan# 1499.

9. 8-hexynylneosolaniol (Fraction 16-II-6 scan 1727):

The GC/MS total ion chromatogram of fraction 16-II-6 (Fig. 5-30) had one major peak with a retention time of 28:40 min (scan# 1727). The molecular ion (MH^+ = 552) was obtained from the PCI mode. The EI mass spectra of the fraction (Fig. 5-31) was similar to the EI mass of 3-TMS-neosolaniol with addition of the mass m/z 99. The difference between the molecular ion (MH^+ = 552) and the 3-TMS-neosolaniol fragment (m/z 436) was a loss of hexonic acid (m/z 116) from the 8 position. The m/z 99 fragment was $C_6H_{11}O$ of the hexonic acid. From the molecular weight and the mass at m/z 99 in the MS of the fraction, the compound was tentatively identified as 8-hexynylneosolaniol (Fig. 5-32).

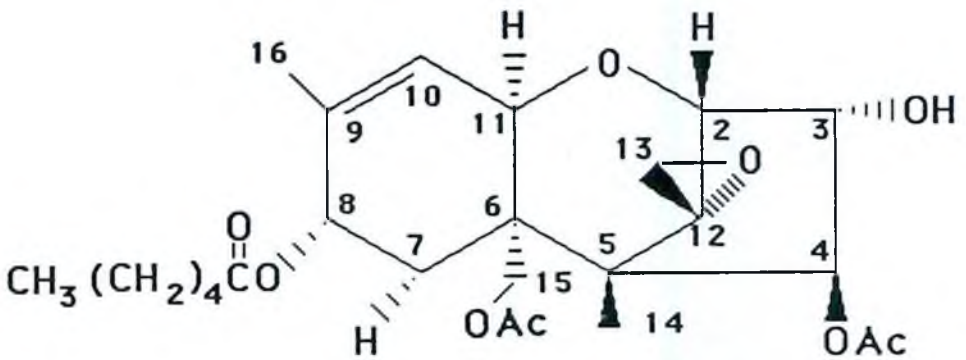

Figure 5-32. Chemical structure of 8-hexynylneosolaniol.

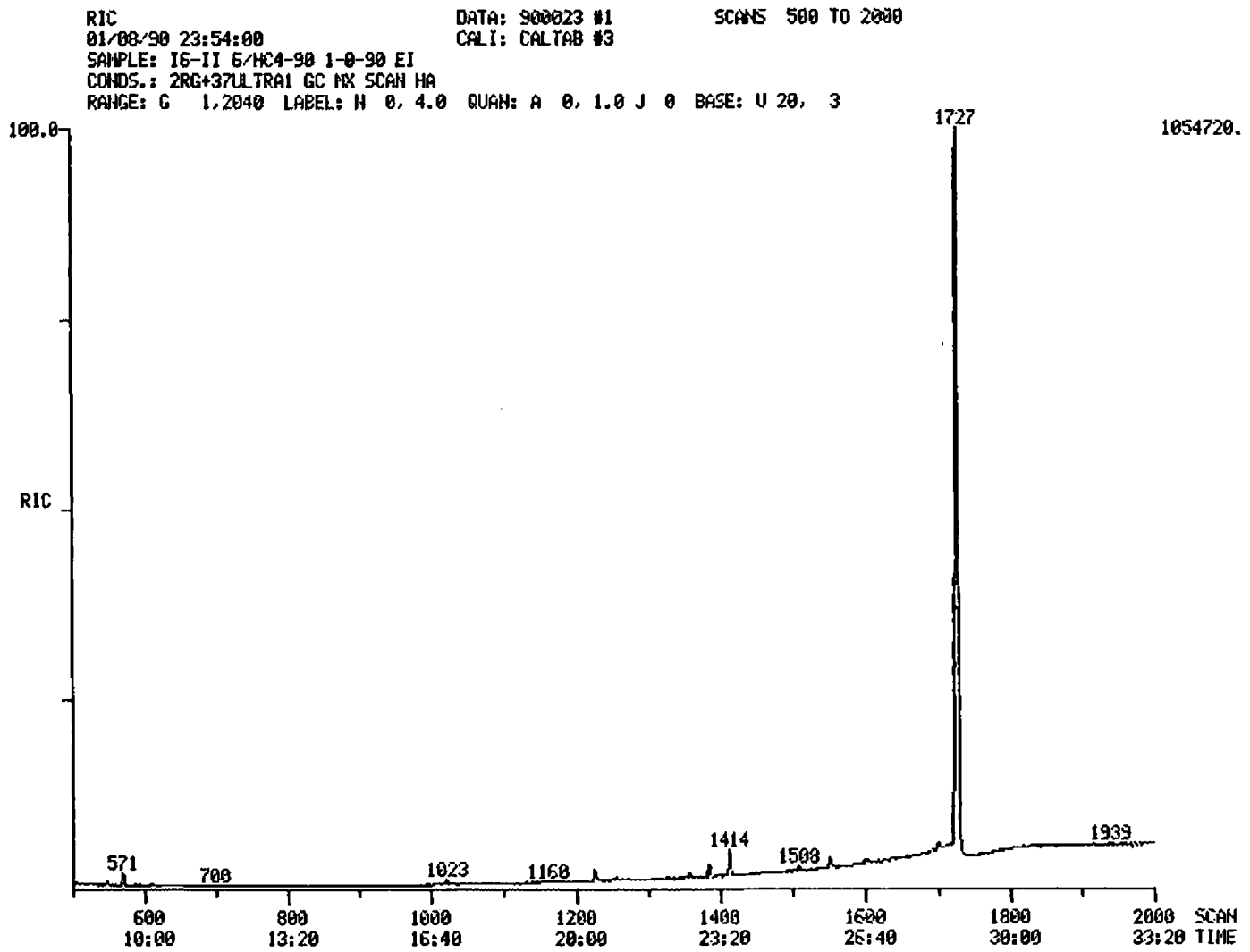

Figure 5-30. GC/MS total ion chromatogram (EI mode) of TMS derivative of fraction 16-II-6.

Table 6-1.- Evaluation of wheat breeding lines in advanced test(Pre-Verification Variety Test or PVVT) for FHB field resistance at Holetta and Kulumsa Experiment centers, Ethiopia, during the 1988 season.

	Holetta		Kulumsa	
Cultivars	Incidence (%)	Severity (%)	Incidence (%)	Severity (%)
HAR727	3c	10d	10f	14e
HAR424	8c	25bcd	15f	30de
HAR719	12c	20cd	25ef	35cd
Dashen	25b	30bc	16f	40cbd
HAR604	25b	30bc	34dc	30de
HAR609	27b	30bc	13f	25ed
HAR715	30b	20bcd	40cd	50b
HAR627	31b	30bc	42cbd	40cbd
HAR720	32c	35b	47cbd	40cbd
HAR605	36b	25bcd	54b	30cbd
HAR716	38b	35b	39d	40cbd
HAR600	65a	80a	54b	65a
HAR712	70a	75a	69a	45bc

Percentage values with the same letters are not significantly different according to Duncan's multiple range test (P=0.05).

Table 6-2. The highest FHB incidence recorded for cultivars and lines (selected for coleoptile bioassay test) in the field during the 1988 season.

Cultivars	Incidence[a] (%)
Dashen	95
Batu	91
Pavon 76	23
HAR-712	70
HAR-407	57
HAR-416	34
HAR-609	27
HAR-424	8
ET-13	1
Enkoy	1

a. The highest percentage of incidence recorded at any one location; locations and sowing dates vary for most cultivars and lines.

Table 6-3. Percent growth inhibition of wheat coleoptile tissue by T-2 toxin

Cultivar	Concentration (M) 10^{-3}	10^{-4}	10^{-5}	10^{-6}	Cultivar mean
HAR-416	100a	98a	74a	57a	83a
HAR-407	97a	98a	61ab	51ab	77ab
HAR-609	100a	88abc	70a	52ab	77ab
ET-13	97a	92ab	61ab	36bc	73abc
Batu	100a	95ab	57abc	32cd	71abc
Enkoy	96a	88abc	56bc	35bc	69bc
HAR-712	97a	88abc	63ab	30cd	67bc
Dashen	94a	84bc	54bcd	41abc	66bc
Pavon 76	98a	82c	42d	37bc	63c
HAR-424	100a	87bc	46cd	18d	62c
T-2 mean	98a	90b	59c	39d	

Least square means (LSM) followed by same letters are not significantly different (P=0.05)

Table 6-4. Percent growth inhibition of wheat coleoptile tissue by diacetoxyscirpenol (DAS)

	Concentration (M)				Cultivar
Cultivar	10^{-3}	10^{-4}	10^{-5}	10^{-6}	mean
HAR-712	84ab	73a	69a	56a	71a
HAR-416	93a	86a	67a	30a	69a
Pavon 76	85ab	81a	69a	39a	68a
HAR-609	70bc	69a	61a	30a	57a
Enkoy	77abc	74a	63a	8a	57a
HAR-407	85ab	66a	45ab	24a	55a
Dashen	87ab	63a	48ab	5ab	55a
HAR-424	65c	65a	35ab	13a	53ab
Batu	79abc	73a	18b	(56)b	45b
ET-13	73bc	(9)b	(82)c	(9)b	(6)c
DAS mean	80a	64b	39c	14d	

Least square mean (LSM) followed by same letters are not significantly different (P=0.05)

Note: Values in brackets refer to % increase in growth.

Table 6-5. Percent growth inhibition of wheat coleoptile tissue by deoxynivalenol (DON)

	Concentration (M)				Cultivar
Cultivar	10^{-3}	10^{-4}	10^{-5}	10^{-6}	mean
HAR-407	94ab	93abc	91a	46a	81a
HAR-416	93ab	100a	90a	31ab	79a
Dashen	95a	96ab	85a	0bc	78ab
Pavon 76	92ab	89bc	68b	33ab	71ab
HAR-609	88abc	92bc	79a	8bc	70ab
Batu	96a	85bcd	62b	(5)bc	64ab
HAR-712	89ab	93abc	89a	(16)c	64ab
ET-13	89ab	80d	62b	19abc	62b
Enkoy	78c	82cd	65b	11abc	60b
HAR-424	84bc	75d	64b	11abc	59b
DON mean	90a	89a	77b		15c

Least square mean (LSM) followed by same letters are not significantly different (P=0.05)

Note: Values in brackets refer to % increase in growth.

Table 6-6. Percent growth inhibition of wheat coleoptile tissue by zearalenone

Cultivar	Concentration (M) 10^{-3}	10^{-4}	10^{-5}	10^{-6}	Cultivar mean
HAR-407	48a	44a	19ab	4a	28a
Batu	41a	42ab	31a	2a	28a
HAR-609	43a	40ab	9b	21a	26a
HAR-712	53a	28b	19ab	0.6a	25a
Enkoy	40a	48a	8b	6a	25a
ET-13	43a	40ab	8b	5a	24a
Pavon 76	43a	34ab	17ab	2a	23a
HAR-416	43a	27b	11ab	6a	23a
Dashen	55a	6c	21ab	4a	22a
HAR-424	13b	35ab	25ab	3a	19a
Zear. mean	42a	35b	17c	6d	

Least square mean (LSM) followed by same letters are not significantly different (P=0.05)

Table 6-7. Percent inhibition in growth of coleoptile tissue of the cultivar Fortuna by Fusarium culture extracts.

Extracts	Dilutions 1	2	3
F. nivale #43b	100**	46* (CV=80)	42* (CV=82)
F. avenaceum #18	100**	64** (CV=35)	14 (CV=132)
F. graminearum HR24	100**	87** (CV=14)	31** (CV=54)
F. poae #5	100**	71** (CV=37)	37* (CV=79)
F. sporotrichioides #48d	100**	97** (CV=5)	94** (CV=7)
Corn extract	100**	65** (CV=50)	22 (CV=112)
Buffer + methanol	100**	23 (CV=140)	26 (CV=91)

** significantly different from the control at p=0.01
* significantly different from the control at p=0.05
Note: 1 = 1:9 culture or corn extract or methanol : buffer
2 = 10 fold dilution of 1
3 = 10 fold dilution of 2

mycotoxins, (eg. DAS), the inhibition of the coleoptile elongation of the cultivars/lines was significant at all concentrations (Table 6-4).

The mean percentage inhibition of the coleoptile elongation of the cultivars/lines (across all the concentrations of the mycotoxins) was considered to evaluate the cultivars. Significantly low inhibition in coleoptile elongation were obtained with Pavon-76 and HAR424 to T-2 toxin (Table 6-3); with Batu and ET-13 to DAS (Table 6-4) and with ET-13, Enkoy and HAR424 to DON (Table 6-5). HAR424 was less sensitive to all of the tested mycotoxins with a significantly lower inhibition in coleoptile elongation. This line also had low FHB incidence in the field (Table 6-2). On the other hand, HAR416, with moderate FHB incidence in the field, was the most sensitive to T-2, DAS and DON. The other wheat lines responded differently to the different compounds, being more tolerant to one than to the other.

ET-13 and Enkoy, on which very low FHB incidence was recorded in the field, were moderate in their response to T-2. ET-13 was more tolerant to DAS and both tolerated DON. The two FHB susceptible (in the field) cultivars, Dashen and Batu, were both moderately tolerant to T-2 and DON. Batu was more tolerant to DAS than Dashen. All the cultivars and lines were similar in their response to zearalenone.

Except for HAR424, no correlation was apparent between the coleoptile elongation inhibition by any of the

mycotoxins and the FHB field evaluation of these wheats.

Coleoptile bioassay with culture extract:

The result of the coleoptile bioassay using extracts from five Fusarium species grown on corn medium is given in Table 6-7. In dilution 1 (1 ml methanol extracted culture filtrate or corn extract or methanol was added to 9 ml buffer) no coleoptile section showed any elongation and inhibition was 100% (Table 6-7). In dilution 2 (10 fold dilution of 1) all culture and corn extracts significantly inhibited the elongation. The effect of methanol was reduced significantly at this dilution. The effect of both methanol and corn extract were significantly reduced in the dilution 3 (10 fold dilution of 2). All culture extracts at this dilution, except that of F. avenaceum, significantly inhibited coleoptile elongation. Extracts from the F. sporotrichioides culture was the most inhibitory at all concentrations. A very high coefficient of variation (CV) was calculated for most observations within each extract and concentration.

DISCUSSION

The cultivar Dashen was highly susceptible to FHB at the state farms. However, it was not as susceptible when grown with the breeding lines at both Kulumsa and Holetta research stations. Not only locations, but sowing dates and weather condition for these locations were different. The difference in response of the cultivars to FHB could also be attributed

to the high build-up of inoculum at state farms where monocropping of wheat is practiced. In any case, under the conditions prevailing at the two experiment stations, Dashen was not the most susceptible and there were lines more resistant or susceptible than this cultivar. The incidence of FHB on Dashen was as low as that of the relatively resistant lines such as HAR424, HAR727 or HAR609 at Kulumsa.

The use of the coleoptile assay with mycotoxins to determine resistance of wheat to FHB has been suggested previously [5,16]. In our work, however, the field data for most cultivars/lines did not significantly correlate to any of the coleoptile bioassay data. The lack of correlation between the field evaluation and coleoptile bioassay may be due to the involvement of different mechanism(s) of resistance in these systems or lack of control of the environment. HAR424 was the only line that showed consistent and significantly lower FHB in the field and lower sensitivity to T-2 toxin, DAS and DON. This line may have the resistance mechanism for the disease in the field due to its insensitivity to the mycotoxins, a characteristic that may make the line useful as a source of resistance for a breeding program against the pathogen(s).

Variations among the cultivars/lines in their response to the mycotoxins and mycotoxin concentrations may suggest the complexity of resistance of wheat to FHB. If the fact that mycotoxins as factors of pathogenicity or virulence is established, this result then indicates that cultivars

could have different responses to FHB according to the type and concentrations of the mycotoxins produced by that particular isolate infecting the crop. It also indicates that in determining resistance of wheat to FHB using the coleoptile assay, one has to use all mycotoxins that could be produced by Fusarium species causing the disease. Resistance to different mycotoxins may have entirely different mechanisms.

The coleoptile bioassay test using crude culture extract shows that there were metabolites in the culture filtrate of all the isolates that were inhibitory to coleoptile elongation. The inhibition caused by culture filtrate of F. nivale was of particular importance. This species was the major species isolated from Ethiopian wheats in 1988. Isolates of this species did not produce detectable amounts of known mycotoxins (Chapter 5). Mycotoxin analysis of infected wheat seeds (mainly by F. nivale) were negative for any of the trichothecenes. The inhibition caused by F. nivale culture filtrate to the coleoptile elongation might be either by some known trichothecenes occurring at very low levels (lower than the detection limit used in Chapter 5) or by some other metabolites.

The coleoptile bioassay test using culture filtrate is an inexpensive means of testing a large number of cultivars, in a very short time. But the interference from the residue of the media on which the fungus is growing and the solvent used to extract the cultures has to be minimized.

Improving the extraction and fractionation methods may help in obtaining better results.

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Vita

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trichothecene metabolites were also reported to be produced by a strain of F. sporotrichioides MC-72083 [6,7,8]. These metabolites included sporol, sporotrichiol, 8-hydroxytrichthecene, FS-1, FS-2, 4-propanoyl HT-2 and trichotriol. F. sporotrichioides #48d, however, produced, neosolaniol, 8-acetylneosolaniol, 8-propionylneosolaniol, 8-n-butyrylneosolaniol, 8-n-valerylneosolaniol, 8-hexynyl-neosolaniols and 4,8-diacetyl T-2 tetraol as major products. The 8-isobutyrlneosolaniol, and 8-isovalerylneosolaniol (T-2 toxin) were minor products of the fungus. The 8-propionyl-neosolaniol, 8-isobutyrylneosolaniol and 8-n-butyrylneosolaniol were reported previously to be produced as minor metabolites by a Canadian strain of F. sporotrichioides DAOM 165006 in liquid culture [10]. The 4,8-diacetyl T-2 tetraol was also reported as a minor product of another strain, F. sporotrichioides strain M-1-1 [14]. The n-valeryl-neosolaniol and hexynylneosolaniol, which are pending confirmation, are reported for the first time.

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CHAPTER 6

REACTION OF SOME WHEAT CULTIVARS AND LINES TO *Fusarium* HEAD BLIGHT AND *Fusarium* TOXINS

ABSTRACT

The wheat cultivars and lines showed a significant difference in their reaction to FHB in the field. However, the observed field reaction type did not correlate significantly with the sensitivity of the cultivars and lines to *Fusarium* toxins in the coleoptile elongation assay. This lack of correlation may indicate toxin-independent resistance mechanisms to fungal infection in the field or that toxins are not involved in FHB caused by *Fusarium* strains endemic to Ethiopia.

The wheat cultivars did not respond in a similar fashion to all the mycotoxins tested or to different concentrations of a single mycotoxin. The data indicated that there were interactions between the cultivars/lines and the mycotoxins. However, some lines, such as HAR424, consistently exhibited low sensitivity to all mycotoxins tested. This toxin insensitvity did correlate with the field reaction of HAR424 to FHB.

The coleoptile bioassay test using culture filtrate rather than purified toxins may be an inexpensive method of testing resistance of a large number of wheat germplasm to the

pathogens. The use of the coleoptile bioassy test as a supplement to field and greenhouse tests and the limitations of this test are discussed.

INTRODUCTION

Intervarietal differences in susceptibility to Fusarium head blight (FHB) or scab were recognized at the turn of the century and since then many workers have attempted to find sources of scab resistance [4,6]. Recently, lines with high levels of resistance have been identified, especially among materials from China, Japan, and Brazil [7,8,15]. No source of immunity or near-immunity to scab has been reported.

A number of factors play a role in the resistance of wheat to FHB. Two types of resistance, Type I - resistance to initial infection and Type II - resistance to hyphal invasion of the plant tissue, have been reported [12]. Type III resistance to the pathogen(s) was suggested by Miller et al (1986) who demonstrated that FHB resistant cultivars apparently possessed the ability to degrade deoxynivalenol, whereas susceptible cultivars did not [10,11]. A new type of resistance (Type IV) was proposed by Wang and Miller (1987) who demonstrated that coleoptile tissue from resistant cultivars could tolerate much higher concentrations of trichothecenes than the same tissue from susceptible cultivars [16]. Teich et al (1987) reported wheat cultivars and lines grown in 1986 in Ontario, Canada, differed significantly in

scab incidence and in deoxynivalenol (DON) concentration in the grain [14]. Concentration of DON in the grains of the various cultivars and lines were correlated with scab incidence.

The involvement of mycotoxins in the FHB development remains controversial. On the one hand, many trichothecene mycotoxins produced by Fusarium species have been shown to be phytotoxic [2,5,9]. Some of these metabolites were shown to interfere with cell division [13] or inhibit protein synthesis [3]. Adams and Hart (1989), on the other hand, demonstrated that DON and 15-ADON were not pathogenicity or virulence factors, at least for G. zeae on maize [1].

The main purpose of this study was: 1. to assess the levels of field resistance of some bread wheat lines to FHB, 2. to determine the relationship of the field response of some cultivars/lines to FHB with the response of coleoptile tissue segments to Fusarium toxins, and 3. to evaluate the use of culture filtrates of some Fusarium isolates collected in Ethiopia for the coleoptile elongation assay.

MATERIALS AND METHODS

Field evaluation:

Twelve wheat lines from the advanced test of the wheat breeding program (pre-verification variety test or PVVT) and the cultivar Dashen were evaluated for FHB resistance under natural infection at Kulumsa and Holetta research

stations, where FHB was severe during the 1988 season. Incidence (percentage of plants with FHB symptom in 0.5 x 0.5 m, 4 counts/plot) and severity (percentage of spikelets with FHB symptom per head, 5 random heads/plot) were recorded. Arcsine transformed data were analyzed by Duncan's multiple range test (P = 0.05). Non-transformed data are presented in the Tables.

Coleoptile bioassay with pure mycotoxins:

The coleoptile bioassay was carried out according to the methods described by Cutler and Jarvis [5] and Wang and Miller [16]. Seeds from 10 wheat cultivars and lines (with low to high FHB incidence in the field) were first surface sterilized by immersion in 70% ethanol for 15 sec followed by immersion in 1% sodium hypochlorite (Clorox, 5.25% sodium hypochlorite) for 30 sec. The seeds were planted in a sterile moist sand:vermiculite (1:1) mixture in a tray and grown in the dark (covered with aluminum foil) for 3 days at room temperature (25 C). Using a device consisting of two razor blades mounted 4 mm apart on a handle, two 4 mm coleoptile sections were cut from each seedling after the apical 2 mm had been discarded. Concentrations of 10^{-3} M, 10^{-4} M, 10^{-5} M and 10^{-6} M of purified deoxynivalenol (DON), diacetoxyscirpenol (DAS), T-2 toxin and zearalenone were prepared in buffer (1.794 g KH2PO4, 1.019 g citric acid monohydrate and 20 g sucrose in a liter of distilled water, pH 5.6). All the mycotoxins were obtained from the Veterinary Medical Diagnostic Laboratory,

University of Missouri, Columbia. Ten coleoptile sections were added to a 4 ml plastic vial containing 2 ml of preparation at each concentration of mycotoxin, plus a control containing no mycotoxin. The vials were then incubated in the dark (covered with aluminum foil) at room temperature (25 C) on a rotary shaker at 125 rpm. After 20 h incubation, the segments were measured by projecting their images, magnified 6x, using an overhead projector. Percentage inhibition in coleoptile elongation (compared to the mean elongation in the control) was analyzed by the General Linear Model procedure and least square means (P=0.05) are reported.

Coleoptile bioassay with culture extracts:

Five species of Fusarium (F. nivale #3, F. avenaceum #5, F. graminearum #9, F. poae #10 and F. sporotrichioides #16) were cultured on corn grits and extracted as described for large scale mycotoxin production in Chapter 5. The 50 ml extract saved from the chloroform:acetone and acetone strip (Chapter 5) were concentrated to an oil-like residue in a rotary evaporator. Each of these was resuspended in 2 ml methanol and purified by XAD-2 minicolumn chromatography. The minicolumn was prepared by packing Amberlite XAD-2 in 1 x 10 cm column to about 5 cm height, washing with 10 ml methanol and rinsing three times with distilled water. One half ml of the extract was added to the column and retained materials were eluted with 25 ml of 60% methanol. The eluant was concentrated in a rotary evaporator and redissolved in 1 ml

methanol. Nine ml buffer (described above) was added to this extract. Indole 3-acetic acid (Sigma) at a concentration of 10^{-4} M was added to all extracts and the buffer ccntrol. Two additional 10 fold dilutions were made from the first preparation. The bioassay was carried out as described above using seedlings of the cultivar Fortuna (obtained from Dr. A.L. Scharen, Montana State University). The effect of corn extract and methanol on coleoptile elongation was determined since the culture filtrate also contained some residue from the medium and the solvent. Percentage inhibition in coleoptile elongation (compared to the control) was calculated and data were analyzed for differences in percent inhibition of coleoptile elongation between treatments using the Student's t test.

RESULTS

Field evaluation:

FHB incidence and severity for the wheat lines, assessed at two locations, are summarized in Table 6-1. The lines differ significantly in their reaction to the pathogen and these differences seem to be consistent at both locations. The lines HAR727, HAR719, HAR609 and HAR424 showed good field resistance although some of these were not significantly different from the cultivar Dashen. Some of the lines (eg. HAR712, HAR600) were more susceptible than Dashen. It should be noted that Dashen was the cultivar most highly affected by

the disease at the state farms.

Coleoptile bioassay with pure toxins:

The cultivars/lines used for the bioassay and the highest FHB incidence recorded in a field on these wheats are given in Table 6-2. Since the data were taken from fields in different environments at different sowing dates, the results may only indicate the general trend of responses of the cultivars and lines to FHB in the field.

The results of the coleoptile bioassay using different concentrations of T-2 toxin, DAS, DON and zearalenone are shown in Tables 6-3, 6-4, 6-5 and 6-6, respectively. All mycotoxins, including zearalenone, inhibited elongation of the coleoptiles, on the average, from 19 to 83%. In all cases inhibition of coleoptile elongation decreased significantly with the decrease in mycotoxin concentration. The order of the extent of inhibition , from high to low, was T-2 toxin, DON, DAS and zearalenone. DAS and DON at lower concentrations stimulated elongation of the coleoptile of some cultivars,(eg. ET-13).

Most differences among the cultivar/lines under each concentration of a mycotoxin were, however, significant, but not necessarily in a similar fashion. At higher concentrations of some mycotoxins (eg. T-2 toxin at 10^{-3} M, Table 6-3) the inhibition was not significant among the cultivars/lines, but differed at lower concentrations (eg. 10^{-5} M). For the other

7. 8-n-butyrylneosolaniol (Fraction 16-II-4, scan 1575):

The larger peak (scan# 1575) in the GC/MS total ion chromatogram of fraction 16-II-4 (Fig. 5-22) had a retention time 26:07 min. Similarities in EI mass spectra and molecular ion (in PCI mode) between scan# 1525 and scan# 1575 have been discussed in the preceding section. The compound with scan# 1575, having a longer retention time than scan# 1525, was identified as 8-n-butyrylneosolaniol (Fig. 5-26).

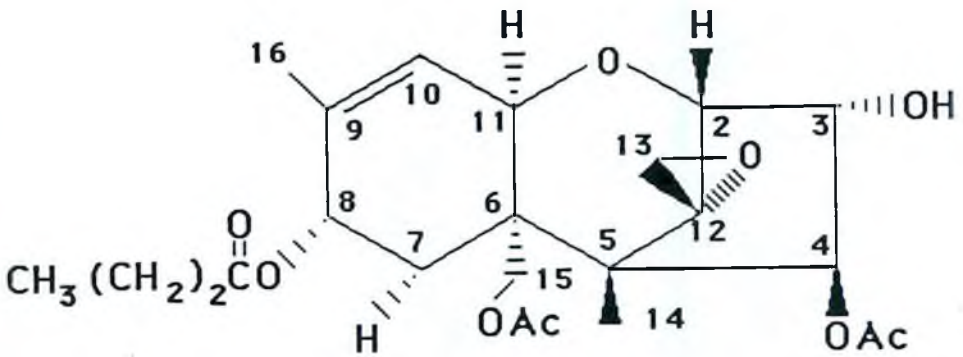

Figure 5-26. Chemical structure of 8-n-butyrylneosolaniol.