The significance of volatile antifungal metabolites produced by *Trichoderma harzianum* biotype Th4, in green-mould disease of commercial mushroom crops

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ABSTRACT

The aggressive mushroom competitor, *Trichoderma harzianum* biotype Th4, produces volatile antifungal secondary metabolites both in culture and during the disease cycle in compost. Th4 cultures produced one such compound only when cultured in the presence of *Agaricus bisporus* mycelium or liquid medium made from compost colonised with *A. bisporus*. This compound has TLC and UV-absorption and characteristics indicating that it belongs to a class of pyrone antibiotics characterised from other *T. harzianum* biotypes. UV absorption spectra indicated this compound was not 6-pentyl-2H-pyran-one (6PAP), the volatile antifungal metabolite widely described in Th1. Furthermore, this compound was not produced by Th1 under any culture conditions. Mycelial growth of *A. bisporus, Botrytis cinerea and Sclerotium cepivorum* was inhibited in the presence of this compound through volatility , diffusion and direct application. This indicates that Th4 produces novel, volatile, antifungal metabolites in the presence of *A. bisporus* that are likely involved in green mould disease of mushroom crops.

ACKNOWLEDGMENTS

This MSc thesis was begun in January 1998 and completed in early 2001. The time effort that has gone into this project would have been many times greater were it not for the help and co-operation of people from Brock University's Department of Biological Sciences and University of Guelph's Department of Plant Agriculture.

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O.K.

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LITERATURE REVIEW

I. Commercial mushroom production

i. Composting

Successful cultivation of the button mushroom *Agaricus bisporus* (Lange) Imbach, requires a growth medium with nutritional content that will allow luxuriant growth of *A. bisporus* mycelium, while resisting colonisation by competitor bacteria and fungi. Production of this material, called compost, is dependent upon the metabolic activity of thermophilic microorganisms that occur naturally in its primary ingredient; straw-bedded horse manure. These bacteria and fungi (Fermor and Grant, 1985) facilitate the aerobic degradation of simple carbohydrates found in the starting materials into water, carbon dioxide and heat. Since this degradative process requires oxygen, aeration throughout the compost is critical during this process. If anaerobic conditions are widespread throughout the materials during this process, poor quality compost, favouring the proliferation of "weed" moulds (*Penicillium* sp., *Chaetomium* sp., *Trichoderma* sp.) and other competitor microorganisms, results. Raw materials usually undergo two separate composting phases prior to inoculation or 'spawning' with *A. bisporus* mycelium.

Phase I composting begins by mixing starting materials with nitrogen-rich supplements, which raise nitrogen levels to those optimal for mushroom production. Initially, raw materials are turned and watered daily for a period of one to two weeks, providing uniform moisture and aeration. After this period, nitrogen and gypsum are added and the material is formed into windrows. The windrows are turned and watered every other day for ten days. It is during this period that simple carbohydrates are eliminated by thermophiles, leaving complex constituents such as lignin. This

complex polymer, composed of phenylpropane units bonded to one another in various ways (figure 1), can be degraded by fungi expressing oxidising enzymes, such as laccase that initiate cleavage of the aromatic ring (Deacon, 1997). The action of laccase (Matcham and Wood, 1992) and other enzymes (Bonnen *et al.*, 1994; Reddy and D'Souza, 1994; Kersten *et al.*, 1995) involved in generating or transferring oxidants, begins the uncontrolled enzymatic combustion responsible for lignin degradation (Deacon, 1997). *A. bisporus* is one of relatively few fungi that exhibit such enzymatic activity (figure 2). Thus, the mushroom mycelium can degrade lignin and effectively colonise the compost medium.

After phase I is complete, compost is filled onto shelves in growing rooms or into bulk chambers. Thus begins Phase II, which eliminates pests that may compete or interfere with mushroom production while completing the metabolic processes begun in Phase I. Four stages, defined by environmental conditions, can be identified during Phase II. The first stage, pre-pasteurisation, provides generous ventilation to alleviate temperature differences in the compost, usually lasting less than one day. After pre-pasteurisation, the crop air temperature is permitted to rise to 60 °C. Steam may be injected into the room to augment this process. The compost temperature is maintained at this level for four to twelve hours depending on the system. Pasteurisation is followed by a conditioning process, which allows thermophilic microorganisms to convert ammonia and nitrates into protein that can be utilised by the mushroom mycelium. This is achieved by lowering the compost temperature to 47 °C, and is usually maintained for three to six days, or until ammonia levels have been lowered to an acceptable level. Following this a cool-down period of two to four

hours brings the compost temperature to 25 °C for inoculation with *A. bisporus* mycelium.

ii. Spawning

Compost is inoculated with mushroom 'spawn'. Spawn is produced by inoculating sterile cereal grain (barley, rye, wheat, etc.) with a specific strain of *A. bisporus*. This axenic culture is incubated, allowing the mycelium to fully colonise the grains. Once colonised, the grains are ready to be used in mushroom production. Spawn is spread over the surface of phase II-compost-filled shelves at a rate of 0.5 %, on a wet weight basis, and mixed throughout the entire depth. During 'spawn run' optimal environmental conditions for *A. bisporus* mycelial growth are maintained as follows: air temperature 24-26 °C, CO_2 concentration 5000 parts per million (ppm) and relative humidity 90-95 %. After approximately fourteen days, the compost should be 80-90 % colonised with mushroom mycelium. At this time the fully colonised compost is top dressed with a material called the casing layer.

iii. Casing

The casing layer is usually composed of sphagnum peat moss, which is mixed with lime ($CaCO_3$) to increase its pH to 7.5. This mixture is then added on top of colonised compost to a depth of 5-8 cm. Environmental conditions established during the spawn run are maintained during the first week of the case run, allowing the mycelium to colonise the casing layer.

iv. Pinning and Cropping

Induction of mushroom primordia, often referred to as 'pinning', is initiated when the mushroom mycelium reaches the surface of the casing layer. This is achieved by lowering compost temperature to 20 °C, reducing relative humidity to 85

% and CO_2 levels to 1000 ppm. Mushrooms are produced by the mycelium in a series of 'breaks' or 'flushes', each approximately one week apart. The first two breaks yield approximately 80 % of the total mushrooms from a cropping cycle. For this reason, it is often more economical for growers to terminate a crop before the fourth break and begin anew.

v. Crop termination

Crop termination, also called 'steaming-off', is accomplished by maintaining a compost temperature of 70 °C for at least twelve hours, thus eliminating any insects or diseases that could interfere with a new crop. The spent compost is then cooled, removed and transported away from the farm to reduce contamination risk to subsequent crops.

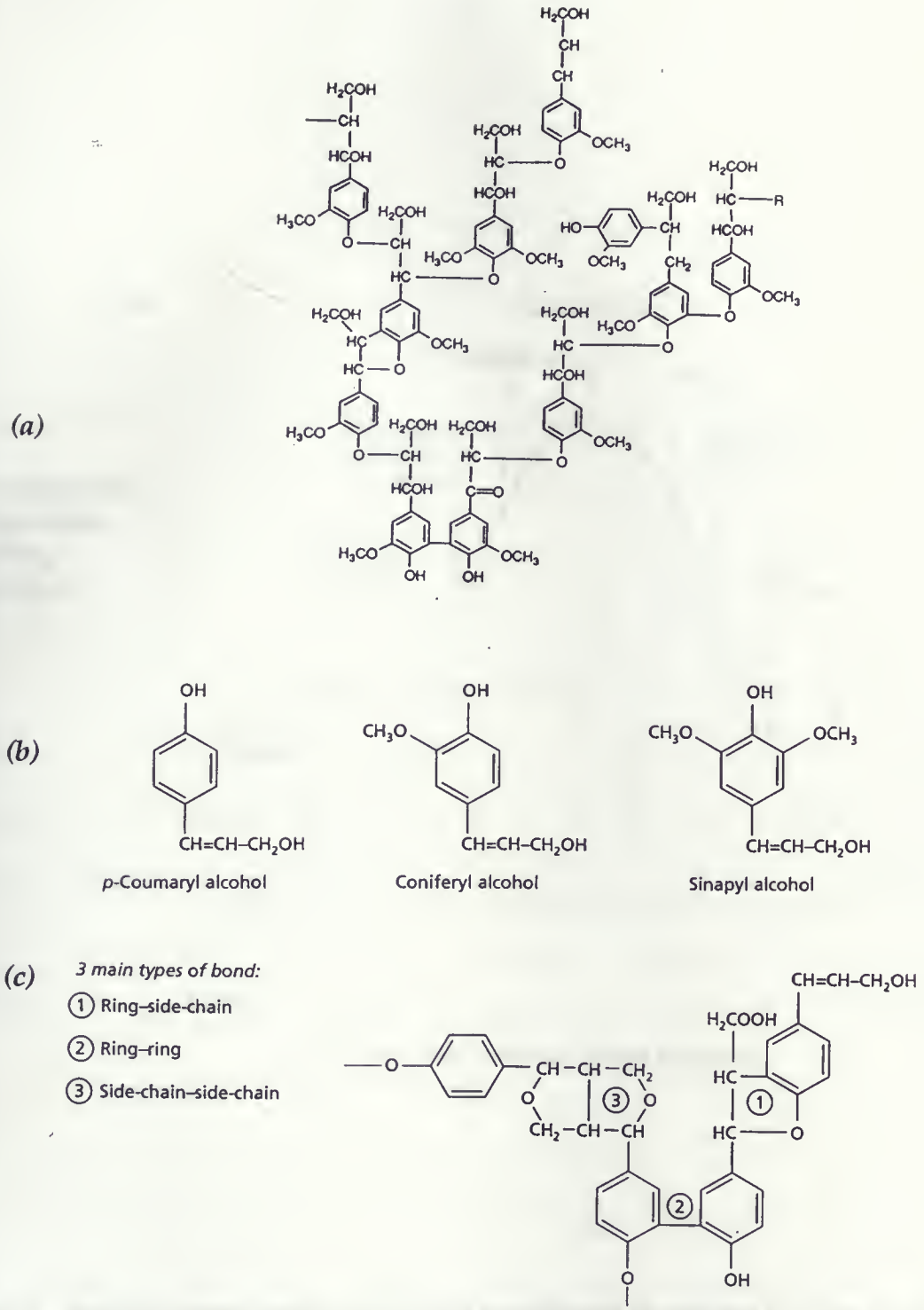


Figure 1. ***(a) Diagrammatic representation of the structure for a part of the lignin polymer. R indicates that the structure of the polymer extends beyond that shown in the figure. (b) Three types of phenylpropane units that constitute lignin. (c) Three main types of bonds linking these into a three-dimensional polymer. (Modified from Barr and Aust, 1994 and Deacon, 1997)***

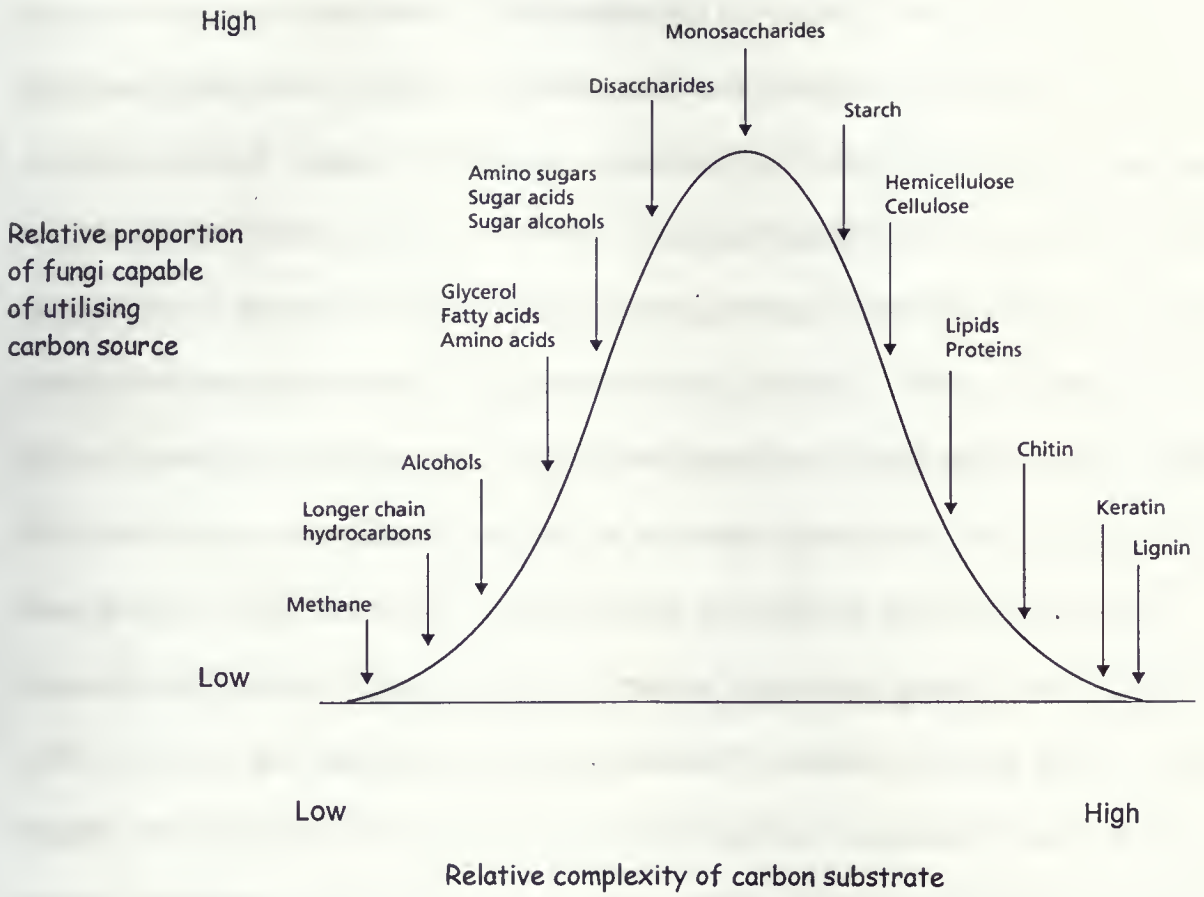


Figure 2. ***Several representative carbon substrates degraded by fungi. These are arranged such that the chemical structure or complexity of the substrate increases from left to right and the proportion of fungi capable of utilising them increases vertically. (Modified from Deacon, 1997)***

II. Green-mould disease of mushrooms

i. History

Trichoderma infections have been associated with production of the commercial mushroom since its domestication in France three centuries ago (Ospina-Giraldo *et al.,* 1998). Appearing as green patches of sporulation on the compost surface, these infections are considered an indicator of poor compost quality or preparation (Rinker, 1993). In 1985, mushroom facilities in Ireland described a devastating 'green-mould' that infected entire growing rooms the loss of mushroom production was proportional to the area infected (Staunton, 1987). Disease symptoms (green sporulation covering the casing of infected areas) appeared two to five weeks after spawning into, what appeared to be, a normal spawn run. Unlike green-mould seen prior to 1985, this organism proliferated aggressively through compost, a characteristic used in naming this new disease, aggressive green-mould (Staunton, 1987). During this epidemic, red pepper mites (*Pygmephorus* spp.), which spread rapidly through mushroom houses, were often found in association with this disease, carrying infectious spores with them (Seaby, 1987). These arachnids are extremely difficult to control and, in addition to exacerbating the spread of green-mould, cause mushrooms that do grow to be unmarketable (figure 3). This infection spread throughout farms in the British Isles, resulting in economic losses of an estimated ten million English pounds by the end of 1986 (Seaby, 1987). This prompted a thorough analysis of the problem by the scientific community.

Strains of *Trichoderma* were isolated from various farms, and an isolate of what appeared to be *T. harzianum* Rifai, was identified as the causal agent (Seaby, 1987). When inoculated into *A. bisporus* spawned compost, this isolate yielded

symptoms identical to those seen on mushroom farms in all trials (Seaby, 1987). This new strain was dubbed Th2. *Trichoderma harzianum* strains that do not cause green mould disease in mushroom crops are referred to as the Th1, or non-aggressive biotype (Seaby, 1987), and are common soil inhabitants. A third *T. harziaum* isolate, morphologically distinct from Th1 and Th2, was found in association with this disease on mushroom farms, and was dubbed Th3 (Seaby, 1987).

In 1990, a disease similar to that seen in the British Isles, began to appear on North American mushroom farms (Rinker, 1993). Disease symptoms identical to those observed in the British Isles spread rapidly throughout major mushroom farming regions, causing massive production losses in Pennsylvania, Ontario, British Columbia and California. Samples taken from infected mushroom farms identified the causal agent morphologically as another biotype of *T. harzianum*. This biotype was named Th4.

ii. Taxonomy and identification

Many infections that are green in colour occur on mushroom farms (Rinker *et al.*, 1997b; Rinker, 1993). These include *Gliocladium* spp., *Cladosporium* spp., *Aspergillus* spp., *Penicillium* spp. and *Chaetomium* spp. as well as many *Trichoderma* species other than those in the *harzianum* species group. Although these fungi exhibit a green colouration at some point during their infection cycle, only aggressive biotypes (Th2 and Th4) of *T. harzianum* cause devastating crop loss. Thus, such infections are referred to as 'green-mould' by the mushroom community (Rinker, 1993).

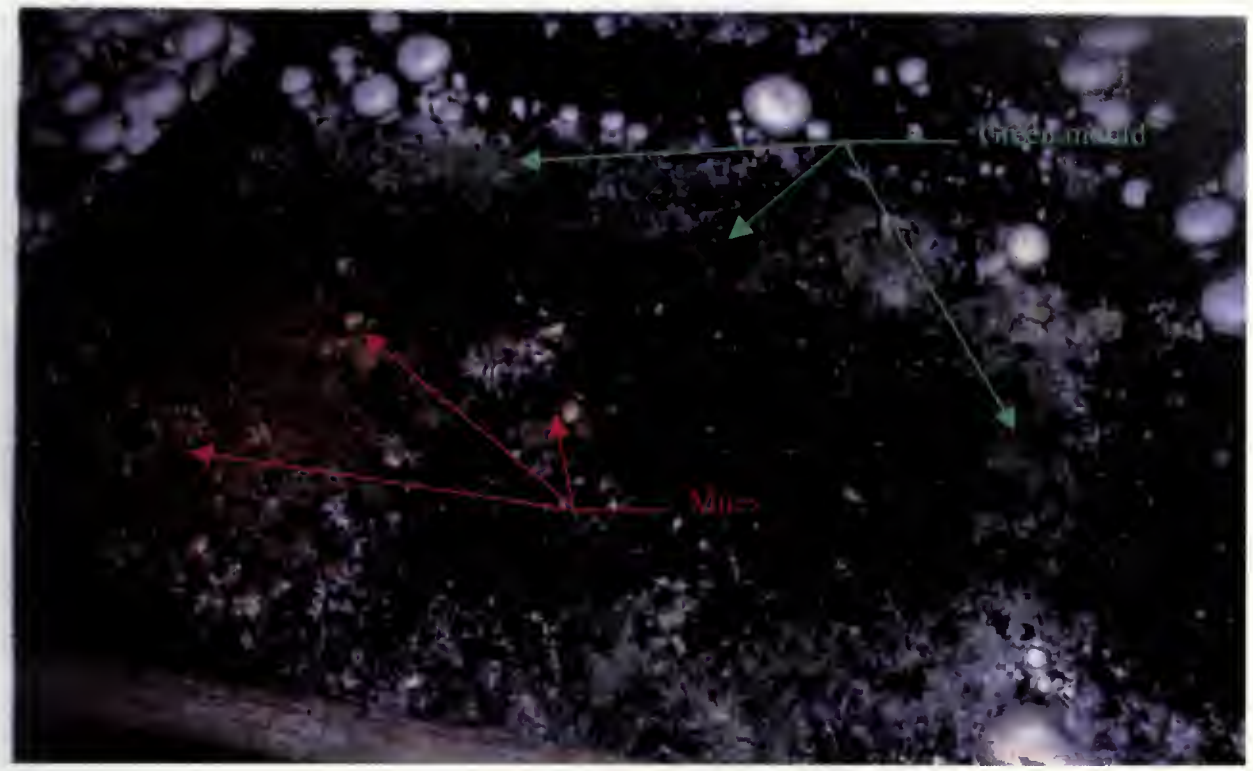


Figure 3. *Compost infected with aggressive green mould disease at a commercial mushroom farm. Note the green sporulation and the red pepper mites on the mushroom caps.*

Three distinct biotypes (Th1, Th2, and Th4), classified as *T. harzianum* by Rifai's (1969) morphological criteria, have been identified in mushroom crops and verified as distinct from one another using molecular markers (Muthumeenakshi and Mills, 1995; Castle *et al.*, 1998; Ospina-Giraldo, *et al.*, 1998). Aggressive green-mould biotypes exhibit slight microscopic differences in culture when compared to each other and to non-aggressive Th1 isolates (A. Castle, personal communication) but cannot be separated from Th1 or each other using Rifai's (1969) morphological criteria (Seaby, 1996b). From this point on, unless explicitly described as Th2 or Th4, the name *T. harzianum* will be in reference to the type-culture or non-aggressive 'Th1' types of this species group, defined by Rifai (1969).

Specific molecular markers for identification of Th4 green-mould in North American mushroom crops have recently been developed (Castle *et al.*, 1998). Applying the randomly amplified polymorphic DNA (RAPD) method using the polymerase chain reaction (PCR), samples can be assayed for presence of Th4 and the information returned to the grower quickly (A. J. Castle, personal communication). This allows growers to determine whether initially small patches of 'green-mould' found on their farms are the aggressive Th4 biotype, or another less problematic fungus. It is also a useful technique for locating inoculum sources of involved in a persistent green-mould problem.

A fungus similar in appearance to these three biotypes has also been found to cause problems on mushroom farms in the United Kingdom when spore loads are very high. Conidia of this strain are extremely similar to those of Th4 (Ospina-Giraldo *et al.*, 1998). Due to these similarities, this fungus was initially described as Th3. However, subsequent molecular analyses using RAPD primers, ribosomal and

mitochondrial restriction fragment length polymorphisms (RFLPs) (Muthumeenakshi and Mills, 1995; Castle *et al.,* 1998), nucleotide sequence of internal transcribed spacers (ITS-1 and ITS-2) and 5.8 S gene of the ribosomal DNA (Ospina-Giraldo *et al.*, 1998), show that this biotype is more closely related to the species group *T. atroviride* (Castle *et al.*, 1998; Ospina-Giraldo *et al.*, 1998).

iii. Epidemiology and origin

The systematic analysis of Th2, undertaken following the green-mould outbreak in the British Isles, provided information that helped European growers develop stringent sanitation procedures that effectively managed this disease in absence of a registered chemical application (Seaby, 1989). This research also aided North American growers in managing Th4 once it appeared on mushroom farms. These initial reports, in conjunction with subsequent research on Th4, helped dispel misconceptions common in the mushroom industry regarding spread and epidemiology of this disease (Rinker, 1993).

The massive crop losses mushroom growers incurred due to aggressive green-mould in both North America and the United Kingdom prompted analyses to determine inoculum sources responsible for these outbreaks. During the British Isles epidemic of 1985 and 1986, it was a common practice for growers to dump compost bags, often contaminated with aggressive green-mould, onto fields near farms or compost production facilities (Seaby, 1996b). This, combined with the finding that green-mould was transmitted in supplied compost and not spawn (Staunton, 1987), generated concern as to whether this dumping practice was providing a source of inoculum, perpetuating the epidemic (Seaby, 1996b). Another concern was the possibility that a mutant strain of 'heat resistant' *T. harzianum* had

developed. If this strain was able to survive the peak heat cycle during compost production in compost yards, this would serve as a source of inoculum (Seaby, 1996b).

Using both molecular and morphological characteristics for identification of aggressive green-mould on various farm areas involved in mushroom and compost production, researchers found active aggressive green-mould spores in rooms that had been steamed, 'cleaned' and prepared for clean phase II compost material (Rinker *et al.*, 1997a, Seaby, 1996b). Green-mould spores were often isolated from areas involved in pre-production, production run and post-production. Transmission of these spores into 'clean' pre-production areas from post-production and dumping areas was also high due to poor sanitation techniques. Lack of a thorough post-crop clean-up, improper removal of spent compost and debris from interior and exterior areas of farms, and movement of equipment and personnel contaminated with spores between pre- and post-production areas were identified as key factors in the spread of green-mould on infected farms (Rinker *et al.*, 1997a). Animal vectors also spread green-mould on infected farms. Red-pepper mites, mice and sciarid flies, translocate spores and are not mutually exclusive of one another; mice were often found to be carrying both spores and mites with spores attached to them. Due to their abundance and frequent association with green-mould on infected mushroom farms, mites are the most troublesome and difficult to control vector of spores (Seaby, 1987).

Researchers have proposed Th2 derived from a mutational event of Th1, giving rise to this aggressive phenotype (Seaby, 1987). With the outbreak of Th4 in North American farms however, this seems unlikely. *Trichoderma* species reproduce clonally via conidia and the occurrence of multiple independent mutational events

giving rise to two genetically distinct strains (Th2 and Th4) exhibiting the same aggressive phenotype is unlikely (Castle *et al.*, 1998). A more plausible explanation is the derivation of these aggressive biotypes from natural populations that were, by chance, adapted to the environment present in mushroom farms (Castle *et al.*, 1998). Consequently, they have proliferated in this environment, producing the devastating effects we now observe.

iv. Disease management

Data gathered regarding the sources of perpetuating inoculum present on a mushroom farm infected with Th2 (Seaby, 1987) or Th4 (Rinker *et al.*, 1997a), demonstrated that a high degree of control may be achieved simply through improved sanitation procedures. Informing growers of potential infection risks on their farms, lead to implementation of stringent hygiene and sanitation programmes on infected farms. Though costly, this level of cultural control is effective in preventing disease outbreak in epidemic proportions (Castle *et al.*, 1998).

Although chemical control means had been developed for management of green-mould in mushroom crops, the registered product, Benlate 50WP (DuPont Canada Inc., Box 2200, Streetsville, Mississauga, ON), is no longer produced by DuPont. Application of Benlate 50WP for mushroom growing involves coating the spawn grains with dry powder product prior to spawning the phase II compost (Rinker and Alm, 1998b). Experimental trials show no significant yield loss when compost was inoculated with Th4 and treated with half a gram of Benlate 50WP mixed with twenty-two grams of gypsum per kilogram of spawn. In the absence of this treatment, 100% yield loss occurred (Rinker and Alm, 1998a). When compared to the cost required to maintain the sanitation programmes previously outlined, the cost of

applying such a fungicide is minimal. To the uninformed grower the choice is clear; apply fungicide to the spawn grains during each spawn-run. If many growers choose this method of control for green-mould, fungicide resistance may develop quickly.

Fungi rapidly develop resistance to benzimidazole-based fungicides; a single point mutation in the β-tubulin gene is enough to confer resistance (Koenraadt *et al.*, 1992). Repeated fungicide applications in a situation such as that described above, provide strong selective pressure for resistant strains in a population. Such selection has occurred in *Verticillium fungicola* (Preuss.) Hassebr., the etiological agent of dry bubble disease in mushroom crops; benomyl treatments on this pathogen are now less effective in some mushroom-producing regions (D. L. Rinker, personal communication). UV-induced mutation of Th4 in Dr. Peter Romaine's laboratory (Penn State University) has produced an isolate of Th4 that is resistant to benomyl, illustrating that Th4 is capable of such resistance. Thus, fungicide dependence for control of green-mould will result in only a brief respite from its potentially devastating effects.

Although control techniques currently employed are effective in keeping the incidence of green-mould disease at relatively low levels, periodic crop losses are still a major concern, as is the threat of benomyl resistance in Th4. Cases of green-mould infection on previously unaffected farms are being reported each year (Rinker and Alm, 1997a). Economic losses attributed to Th4 outbreaks in Pennsylvania alone were estimated at over twenty million dollars at the end of 1997 (Ospina-Giraldo *et al.*, 1998). Seventy percent of farms in Ontario have experienced crop losses due to this disease and in 1997 four farms reported crop losses attributed to Th4 for the first time (Rinker and Alm, 1997c). Although research has aided management of this disease,

the mechanism by which Th4 causes crop devastation remains unknown. UV-mutagenesis has shown benomyl resistant Th4 strains can exist. Persistent green-mould problems in many areas require fungicide application on a regular basis, thus selecting for resistant phenotypes. When this occurs, alternative control methods must be available to prevent crop loss in epidemic proportions.

III. Fungal antagonism by *Trichoderma* species.

i. Focus of present research

Microscopic examination of green-mould infected compost has shown that there is no evidence of physical interaction between Th4 and *A. bisporus* hyphae that is indicative of active parasitism. Similar results have been obtained when examining hyphal interactions on a variety of semi-solid media (figure 4); coiling or other evidence of hyphal interaction were not observed (Droganes, 1998). Although hyphal interactions may occur when *T. harzianum* is confronted with certain host fungi (figure 4) (Elad *et al.*, 1983, Benhamou and Chet, 1993), such events are not prerequisite for growth inhibition of host fungi in the presence of *T. harzianum* (Dennis and Webster, 1971c; Calistru *et al.*, 1997).

Possible factors contributing to the antagonistic effect of *T. harzianum* on host fungi include the following: hydrolytic enzymes that degrade the host cell wall (Elad *et al.*, 1982; de la Cruz *et al.*, 1995; Noronha and Ulhoa, 1996), volatile (Claydon *et al.*, 1987; Ghisalberti and Rowland, 1993; Faull *et al.*, 1994), and non-volatile antibiotics (Brewer *et al.*, 1987; Corley *et al.*, 1994; Correa *et al.*, 1996). These all inhibit growth

(a)

(b)

Figure 4. *(a) Absence of hyphal interaction between* Agaricus bisporus *and* Trichoderma harzianum *Th4 in vitro (Droganes, 1998). (b) Coiling of* T. harzianum *Th1 biotype hyphae around hyphae of* Rhizoctonia solani *in vitro (Benhamou and Chet, 1993).*

of host hyphae, albeit via different mechanisms. Recent observations indicating differences between volatile metabolites produced by Th1 and Th2 (Mumpuni *et al*, 1998) have inspired the following research, which focuses on determining whether Th4 produces volatile antifungal metabolites involved in the establishment of green-mould disease.

ii. Antifungal secondary metabolites

The potential role of antibiotics in biocontrol of plant pathogens has received much attention recently (Deacon, 1997). *Trichoderma* spp. have dominated this field and were among the first fungi known to produce antibiotics (Weindling, 1934). Several biocontrol formulations containing *T. harzianum* are now marketed to be used in management strategies for a variety of fungal pathogens (Papavizas, 1985; Schoeman *et al.*, 1996), most notably *Botrytis cinerea* Pers. *ex* Fr. (Deacon, 1997). Antibiotic production plays an important role in fungal antagonism by *T. harzianum*. Scarselletti and Faull (1994) illustrated a strong relationship between the production of antibiotics by *T. harzianum* and the antagonistic ability of this fungus *in vitro*. *Trichoderma* species produce antibiotics through a variety of metabolic pathways, many of which are silent unless induced by mutational events and/or specific environmental or culture conditions (Faull *et al.*, 1994; Bonnarme *et al.*, 1997). This includes enhanced antibiotic production in the presence of myelium from another fungus (Cooney and Lauren, 1998).

Many of the antibiotics produced by *Trichoderma* spp. are defined as secondary metabolites, owing to their production during idiophase or stationary 'growth' phase. Such metabolic products are not essential to growth of the organism but often confer some advantage to an established colony. The biosynthetic pathways involved in their

production are diverse. However, many of the described antibiotics are produced through the polyketide pathway (figure 5). This pathway appears to have no role other than secondary metabolism and its products include pigments, toxins and antibiotics (Moore-Landecker, 1996). This pathway has been described almost exclusively in fungi, most notably in the genera *Aspergillus, Trichoderma* and *Penicillium* (Deacon, 1997). Other pathways, responsible for the production of isonitrile, peptide and other antibiotics (figure 5), have also been described in *T. harzianum*.

iii. Volatile antifungal compounds

6-*n*-pentyl-2*H*-pyran-2-one (6PAP) is perhaps the most widely described volatile antibiotic produced by certain *T. harzianum* isolates (Deacon, 1997). Its structure (figure 5) indicates that it is a product of the polyketide pathway. Mumpuni *et al.* (1998) studied the effects of volatile compounds produced by aggressive and non-aggressive biotypes of *T. harzianum*, namely Th2 and Th1, on *A. bisporus* cultures *in vitro*. Although compounds responsible for the observed effects were not identified, these findings did illustrate differences in the effect of volatile metabolites from Th1 and Th2 on *A. bisporus* mycelial growth. Production of 6PAP and related compounds, varies between *Trichoderma* isolates classified as the same species (Dennis and Webster, 1971b), between single spore isolates from the same progenitor (Worasatit *et al.*, 1994), under different nutritional conditions (Bonnarme *et al.*, 1997) and in the presence of other fungi (Cooney *et al.*, 1997). These facts raise a number of interesting questions in terms of the effects that aggressive *T. harzianum* biotypes have towards mushroom crops.

In 1997, Rinker and Alm (1997b) performed experiments analysing the effect of supplementation on green-mould expression in mushroom crops. Supplements are

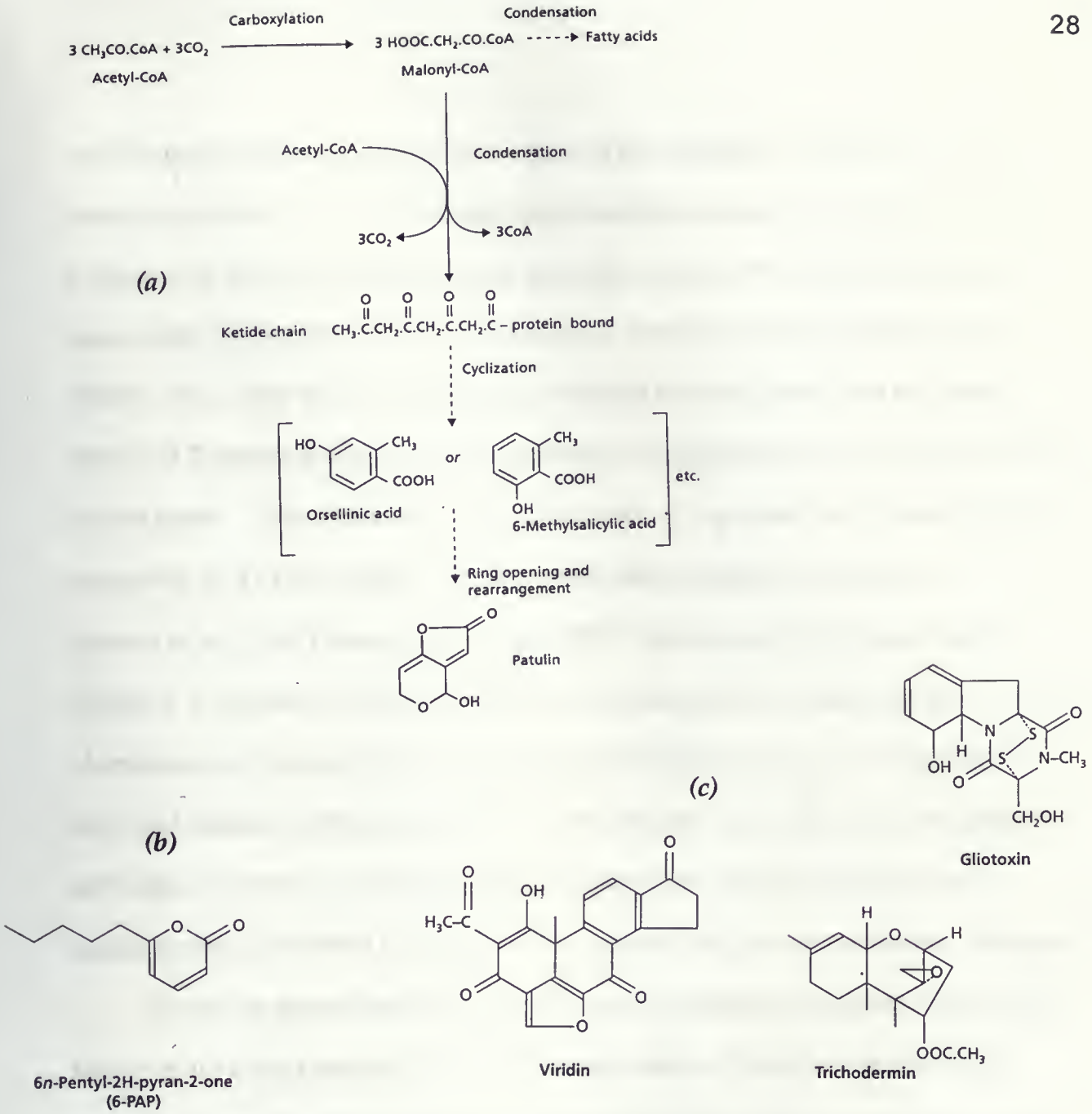


Figure 5. ***(a) One of the main pathways through which fungal secondary metabolites are produced; the polyketide pathway (b) A volatile antifungal secondary metabolite of*** **Trichoderma harzianum, *6-n-pentyl-2H-pyran-2-one (6PAP) (c) Other antibiotic compounds produced by* Trichoderma *spp.***

commercially available vegetable by-products that increase mushroom yield when added to compost. It was found that aggressive green-mould is more devastating to a mushroom crop when supplements, such as soya or corn meal-based products, were added to the compost. This correlates to research done by Bonnarme *et al.* (1997). Their work showed a significant increase in 6PAP production by various strains of *Trichoderma in vitro* when commercial vegetable by-products were added to culture media. This correlation may be indicative of increased antifungal compound production by Th4 in compost supplemented with vegetable by-products. Cooney *et al.*, (1997) illustrated increased 6PAP production by *Trichoderma* spp. including *T. harzianum*, when cultured with various plant-pathogenic fungi. This level of response, as indicated by 6PAP titre, varied significantly with the test organism used and between different isolates of *T. harzianum*. Such differential expression of antifungal compounds in the presence of a given host, may explain the varied aggressiveness exhibited by different *T. harzianum* biotypes in mushroom compost.

Based on reports from mushroom growers claiming that brown strains of *A. bisporus* are more tolerant to green-mould epidemics, Rinker and Alm (1997a) carried out experiments attempting to determine whether such a relationship existed. They found that trays of brown mushrooms inoculated with Th4 produced significantly more mushrooms than trays containing either white or off-white hybrid strains. If aggressive *T. harzianum* produces antifungal compounds that are involved in the establishment of green-mould disease, then tolerance to such an antibiotic in brown mushroom strains may account for these differences.

INTRODUCTION

Trichoderma harzianum Rifai is an imperfect ubiquitous soil fungus renowned in the scientific community for its biological control potential. Widely studied as the classical example for chitinase and glucanase production, this facultative mycoparasite is also well described in terms of antifungal antibiotic production. In the mid-1980's a biotype of *T. harzianum* emerged in commercial mushroom (*Agaricus bisporus* [Lange] Imbach) crops causing a disease known as green mould. This biotype, called Th2, caused epidemic crop losses in the U.K. throughout the late 1980's and continues to be a major pest in mushroom farms. In the early 1990's a similar disease appeared in North American mushroom crops also resulting in epidemic crop loss in many mushroom growing regions. The etiological agent isolated was similar to Th2 but distinct differences were described, hence this North American biotype was called Th4. These aggressive biotypes are both distinct from that described by Rifai (1969) and other *T. harzianum* type cultures, now referred to as Th1 biotypes.

Characteristics of Th1 and Th4 biotypes that could account for their differential aggressiveness towards mushrooms have yet to be identified. In 1998 Mumpuni *et al.* described differences in the volatile metabolites produced by Th1 and Th2, and postulated that these may be related to aggressiveness of the biotypes towards a mushroom crop. Isolation and identification of volatile pyrones produced by *T. harzianum* Rifai (Th1) in liquid culture has been described by Claydon *et al.* (1987). Antifungal and antibiotic activity of such pyrones and other secondary metabolites produced by *Trichoderma* species has been described (Weindling, 1941; Dennis and Webster, 1971b; Scarselletti *et al.*, 1994; Worasatit *et al.*, 1994; Abe *et al.*, 2000).

This work describes the isolation of a putative secondary metabolite from *T. harzianum* Th4 liquid culture that has significant antifungal activity against a number of fungi including *A. bisporus*. In addition, data presented indicates that production of this metabolite is increased in the presence of *A. bisporus* mycelia and is limited to aggressive biotypes of the *T. harzianum* form-species.

MATERIALS AND METHODS

I. Fungal cultures

Agaricus bisporus commercial spawn strains Somycel 608 large off-white hybrid, Sylvan SB65 large brown, Sylvan 130 mid-range hybrid (Sylvan America Inc., Kittanning, PA); Horst U1 hybrid off-white, Amycel 2400 large brown (Amycel Inc., Avondale, PA), were maintained on a modified *Schizophyllum* complete medium (SCM) [2% dextrose (Caledon Laboratory Products Inc., Georgetown, ON), 0.2% yeast extract (Difco, Detroit, MI), 0.2% peptone (Difco), 0.1% K_2HPO_4, 0.05% $MgSO_4*7H_20$, 0.046% KH_2PO_4, 2% agar (Bioshop Canada Inc., Burlington, ON)]. *Trichoderma harzianum* isolates (table 1) were transferred periodically from liquid nitrogen stock cultures to malt extract agar medium (2% malt extract, 2% agar). *Botrytis cinerea* Pers. isolate Bc2 (provided by Dr. M. S. Manocha, Brock University) and *Sclerotium cepivorum* Berk. MCG3 (provided by Dr. M. R. MacDonald, University of Guelph, Guelph, ON) were transferred from liquid nitrogen periodically and maintained on potato dextrose agar (PDA) (Bioshop). All stock and experimental cultures were incubated at 25°C with 1 hour of fluorescent lighting every 24 hours, unless otherwise stated.

Table 1. Trichoderma harzianum *isolates (courtesy of A. J. Castle and D. L. Rinker) used based on molecular and morphological characteristics.*

Isolate	T number*	RAPD group**	Biotype
Shig1	-	2	Th1
Shig3	T40	2	Th1
Skui2	T128	2	Th1
Slea12	-	2	Th1
Ssyl5	T123	2	Th1
Ssyl6	T110	2	Th1
Swel4	T52	2	Th1
H89atr1	-	2	Th1
H89vir3	-	2	Th1
H89vrd1	-	3	Th1
T22 ®	-	-	Th1
Root shield ®	-	-	Th1
Sbro2	T80	1	Th4
Sgre12	T50	1	Th4
Shol1	T98	1	Th4
Shol2	T99	1	Th4
Slea25	-	1	Th4
Slea26	T20	1	Th4
Smon1	T85	1	Th4
H89har3	-	1	Th4
Ss7c	T586	1	Th4

* University of Guelph, Department of Plant Agriculture, Vineland Station, ON.

** Castle *et al.*, 1998

II. Opposition experiments

Figure 6 illustrates the experimental set-up outlined in the following two sections.

i. *Botrytis cinerea*

To determine whether *T. harzianum* cultures produced volatile antifungal compounds, a test fungus (*A. bisporus* or *B. cinerea* Bc2) was exposed to headspace gasses produced by *T. harzianum* cultures. Due to its rapid growth, *B. cinerea* Bc2 was used in initial experimentation. This allowed data to be gathered more quickly since its growth rate is at least 5 times (by radial assessment) that of *A. bisporus*. Inoculum plugs (5 mm diameter) were excised with a #3 cork borer from the edge of an actively growing Bc2 culture and placed in the centre of petri dishes (90 mm) filled with PDA. These plates were then uncovered, inverted and placed over uncovered, MEA-filled petri dishes (90 mm) that were either uninoculated, or freshly inoculated with 5 mm diameter plugs of Th1, Th4 or Bc2, excised from the growing margin of active cultures. The two plates were then sealed together with Parafilm "M" laboratory film (American National Can, Chicago, IL). Area covered by Bc2 mycelium was quantified by using a grid and colony diameter was determined by taking the average of ten measurements through the point of inoculation in millimeters. These measurements were performed every 24 hours from the time when the plates were sealed and incubated. A mm^2 grid was used to determine the area of mycelial coverage. The results of these experiments (tables 2 and 3) prompted similar experimentation using *A. bisporus* as the test fungus.

ii. *Agaricus bisporus*

Inoculum plugs (5 mm diameter) were excised with a #3 cork borer from the edge of an actively growing *A. bisporus* culture and placed in the centre of petri dishes (90 mm) filled with SCM. Unless otherwise stated, fungal inoculum plugs for all subsequent experimentation were excised in this manner. Initially *A. bisporus* strain Somycel 608 was selected for these experiments. However, growth of this strain was not as uniform as that of Sylvan 130. Hence, S130 was used in later experimentation.

In initial experiments (table 4), these plates were inverted and placed over (figure 6) uncovered, MEA-filled petri dishes (90 mm) that were uninoculated, or freshly inoculated with 5 mm diameter plugs of either Th1, Th4 or Bc2, excised from the growing margin of active cultures. The diameter of *A. bisporus* mycelium was then measured every 10 days, by taking the average of ten measurements through the point of inoculation using a standard ruler. This method did not control for differences in *A. bisporus* colony diameter that may have appeared between replicates as a result of inoculation methods used. Thus, in subsequent experiments (appendix 3), *A. bisporus* cultures were allowed to grow for 10 days prior to exposing them to headspace gasses from Th1, Th4 and Bc2 cultures. The colony diameter was measured at this time, as described above.

Fungal contamination was a persistent problem on the SCM dishes due to the duration of these experiments. To alleviate this, benomyl was added at a rate of 10 ppm to the SCM after sterilisation, when the molten agar had cooled to a temperature of 50 °C. This prevented *T. harzianum* biotypes or *B. cinerea* Bc2 from colonising the upper plate throughout the duration this experiment. *A. bisporus* Sylvan 130 was

inoculated onto SCM plates and allowed to incubate as described above, and the colony diameter was measured. After this, the plates were uncovered, inverted and placed over uncovered, MEA-filled petri dishes (90 mm), that were uninoculated, or freshly inoculated with 5 mm diameter plugs of either Th1, Th4 or Bc2. The two plates were then sealed together with Parafilm and incubated. Colony diameter was measured every 10 days thereafter (table 5).

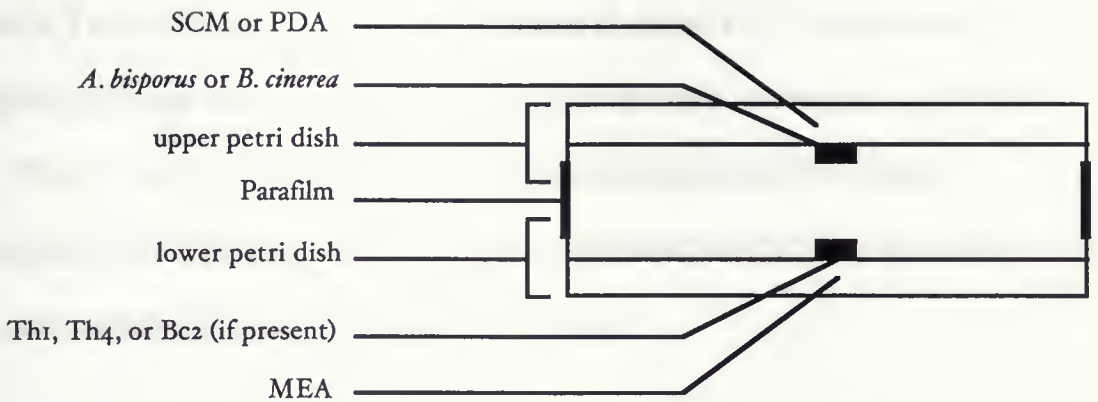


Figure 6. ***Diagrammatic representation of petri dish arrangement used in opposition experiments assessing antifungal activity of volatile compounds produced by*** **Botrytis cinerea** ***isolate Bc2 and*** **Trichoderma harzianum** ***biotypes Th1 and Th4, on mycelial growth of the test fungus.***

III. Isolation of metabolites from *Trichoderma harzianum* liquid culture

i. General

Liquid, shake cultures of *T. harzianum* biotypes were created by inoculating 2% malt extract broth (MEB) with a final concentration of 10^5-10^6 conidia per mL. Conidia were harvested from 14-day-old *T. harzianum* colonies on MEA by adding 10 mL of sterile 5% Tween 20 (which reduced clumping of conidia) in distilled water. A sterile, bent glass rod was then used to stroke the agar surface, releasing conidia into the liquid. This created a suspension of conidia that was quantified using a haemocytometer. For Th1 and Th4 cultures, isolates Ssyl5 (T123) and Ss7c (T586) were used respectively, unless stated otherwise.

After shaking at 20-22 °C and 150 rpm for thirty days (unless stated otherwise) with 12 h of light and 12 h of darkness, the culture broth containing *T. harzianum* mycelium was vacuum-filtered through pre-labelled, pre-weighed Whatman #4 filter paper, and washed twice with 50 mL distilled water. This pre-weighed filter paper was then dried for 24 hours at 50-55 °C; and the dry weight of the mycelium determined on an analytical balance.

ii. Extraction from mycelium

For experiments determining the antifungal activity of metabolites extracted from organic material in *T. harzianum* cultures, filtered mycelium was not dried. Instead, mycelium was macerated with distilled water using a mortar and pestle. The resulting slurry was extracted three times with 50 mL of dichloromethane (DCM) in a separatory funnel. All solvents used throughout the duration of this research were obtained from Caledon Laboratory Products Inc., Georgetown, ON. These extracts

were combined, dried over sodium sulfate (Na_2SO_4) and passed through an hydrophobic filter with pore size of 0.5 μm (Millipore Corporation, Bedford, MA). The solvent was then removed under vacuum, yielding a yellow-coloured oil.

iii. Extraction from culture medium

The filtrate obtained from *T. harzianum* cultures was placed in a continuous extraction apparatus for three days, using dichloromethane (DCM) as the solvent. This facilitated the extraction of all non-polar compounds from the culture filtrate, concentrating them in a round-bottom collecting flask. The DCM in the collecting flask was dried over anhydrous sodium sulfate, decanted into another flask and passed through a Millipore filter. The solvent was then removed under vacuum, yielding a yellow-coloured oil with an odour reminiscent of buttered popcorn. This and other residues obtained throughout this research were weighed on an analytical balance and stored under argon at –20 °C.

Extracts from uninoculated MEB were obtained in a manner similar to that described above. These were used to determine whether the extraction technique from MEB introduced any compounds inhibitory to fungal growth.

Due to the viscous nature of these crude products, further experimentation assessing antifungal activity required that they be dissolved in methanol (MeOH) at a ratio of 1:10 (volume MeOH: volume of medium extracted) for application.

Unless explicitly stated, all references to 'Th4 extract(s)', 'Th1 extract(s)' or 'MEB extract(s)' in the following documentation imply metabolites extracted from the filtrate derived from 30-day cultures and suspended in methanol as outlined above.

IV. Antifungal activity of crude extracted compounds

i. Inhibition of *Botrytis cinerea* mycelial growth

Inoculum plugs, 5 mm in diameter, were excised from the growing margin of a *B. cinerea* Bc2 culture and placed in the centre of 90 mm petri dishes containing PDA. To determine the antifungal activity of extracted compounds, 20 µL of methanol, Th4 extract or MEB extract were added directly to the top surface of inoculum plugs. These cultures and control cultures were sealed with Parafilm and incubated at 22°C. The area of mycelial coverage was quantified every 24 hours.

ii. Inhhibition of *Agaricus bisporus* mycelial growth

In attempts to determine the minimum quantity of Th4 extract required to significantly inhibit *A. bisporus* mycelial growth, a dilution series of the 100% crude extract in methanol (10%, 1% and 0.1%) was created. One mL of 100% crude extract was added to 9 mL methanol. One mL of this dilution (10%) was added to 9 mL methanol and so on. These dilutions, the methanol control, the organic Th4 extract and the MEB extract were then added, in volumes of 20 µL each, to *A. bisporus* Horst U1 plugs that had been freshly inoculated onto SCM agar. These cultures were then sealed with Parafilm and incubated.

Experiments were also performed to determine if aqueous Th4 extracts have different effects on growth of white or brown *A. bisporus* strains. This involved adding 40 µL of extract suspended in methanol to inoculum plugs of strains Somycel 608 or Amycel 2400 (table 8). Appropriate controls were also included.

Experiments indicating an inhibitory effect of methanol when added to inoculum plugs (table 9), prompted further experimentation to determine whether this was in

fact occurring. A volume of 20 µL of either methanol alone or Th4 extract in methanol were added to Horst U1 inoculum plugs.

For all the experiments in this section, the area of *A. bisporus* growth was quantified using a mm^2 grid every 5 days for 15 days (Horst U1) or every 10 days for 30 days (all other *A. bisporus* strains). This shorter interval was chosen for Horst U1 due to its relatively rapid growth compared to other *A. bisporus* strains used.

iii. ***Sclerotium cepivorum***

The antifungal activity of metabolites isolated from Th4 liquid cultures was also assessed by direct application of Th4 extract to the actively growing margin of established *S. cepivorum* cultures. Inoculum plugs of *S. cepivorum* were excised from 15-day-old cultures with a #3 cork borer and placed in the centre of PDA-filled petri dishes. After 3 days, when the colony had grown to a diameter of approximately 5 cm, 10 µL of Th4 extract were added directly to the advancing margin of mycelium.

Ten µL of Th4 extracts were also applied directly to fresh *S. cepivorum* inoculum plugs and growth was assessed after 4 days.

V. Volatile antifungal activity of extract

Volatile antifungal activity of extracted metabolites was determined using a procedure similar to that depicted in figure 6. However, the bottom plate was empty and contained different crude culture extracts dissolved in MeOH. In light of results indicating the toxic effects of MeOH on *A. bisporus* Horst U1 mycelium (table 9), it was necessary to evaporate MeOH in the bottom plate prior to sealing it in opposition to such cultures. Leaving the dish open at room temperature achieved this. Immediately after inoculation the upper plate, prepared as in section II. ii., was uncovered, inverted and sealed over uncovered petri dishes containing 1 mL of

extracts from one of the following sources: Th1 in MEB, Th4 in MEB, or uninoculated MEB. As indicated in section III. iii. above, this volume contains 10 mg of crude extracted residue for both Th1 and Th4 extracts. The area of *A. bisporus* mycelial growth was quantified after ten and thirty days using a mm^2 grid.

VI. Diffusable antifungal activity of extract

Fifty µL of aqueous extracts (MEB control extract, Th4 extract, Th1 extract and methanol control) were added to 10 mm diameter, paper disks cut from bibulous paper in 10 µL applications, allowing 5 minutes between each for evaporation of the MeOH. This volume corresponds to 0.5 mg of crude extract for each experimental addition. These disks were then placed in the centre of a petri dish containing SCM agar that was previously inoculated with a uniform lawn of *A. bisporus* Horst U1 mycelium. This mycelial lawn was prepared by adding sterile distilled water and 10 mm diameter *A. bisporus* plugs (taken from the leading edge of a 20 to 30 day old colony with a number 5 cork borer), at a ratio of one plug per mL of water, to a sterile Sorvall homogenising tube. This mixture was homogenised in a Sorvall omni-mixer for 5 minutes at speed #6. One hundred and fifty microlitres of the resulting homogenate was spread evenly onto each SCM plate. The inhibitory zone around the filter paper disk, if present, was quantified after ten days by averaging ten measurements of its diameter made using a standard ruler.

VII. Antifungal activity against brown and white strains of *Agaricus bisporus*

To determine whether brown strains of *A. bisporus* were more tolerant to the antibiotic effects of compounds extracted from Th4 culture filtrate, different quantities of the crude compounds were added to bibulous paper disks (10mm) as described

above. To determine the effect of metabolite concentration on diameter of the inhibitory zone, disks with 10, 20, 30, 40 and 50 μL of crude Th4 metabolite extracts were placed on SCM agar with macerated mycelia of *A. bisporus* strain Sylvan SB65 or Sylvan 130 and incubated. Diameter of the inhibitory zone was quantified after ten days using the mean of ten measurements made with a standard ruler.

VIII. Extraction of metabolites from compost

Phase II compost from the Vineland campus of the University of Guelph was either unspawned, spawned with *A. bisporus* Sylvan 130 or spawned with S130 and inoculated with *T. harzianum* biotype Th4 (T586) and placed in trays or bags. After three weeks,1 kg of material was removed from each compost sample. Extensive green-mould sporulation was present in spawned compost inoculated with Th4, *A. bisporus* mycelium had fully colonised the spawned compost and the unspawned compost remained unchanged. These samples were mixed with two litres of distilled water and stirred occasionally for one hour. The resulting broth was gravity filtered through cheesecloth and the resulting filtrate was placed in a continuous extraction apparatus with DCM. After three days, the DCM containing extracted compounds was dried over anhydrous sodium sulfate and the solvent was removed under vacuum.

IX. Co-culture of *Agaricus bisporus* and *Trichoderma harzianum*

Liquid medium (SCM) was inoculated with *A. bisporus* Sylvan 130 (S130) homogenate at a rate of 1 μL homogenate per mL of broth. Homogenate was prepared as described in section III vi. Liquid cultures (150 rpm, 22°C) were maintained for 30 days, and then inoculated with 10^5-10^6 conidia of Th4 isolate Ss7c (T586) or Th1 isolate Ssyl5 (T123), per mL of culture. In some cases, these established *A. bisporus* cultures were autoclaved prior to the addition of *T. harzianum*

inoculum. This was done in order to determine whether a heat labile component of the culture was responsible for eliciting production of the different metabolites observed when Th4 is in the presence of *A. bisporus* mycelium (results section VII.).

After inoculation with *T. harzianum* and another thirty days shaking at room temperature, cultures were vacuum-filtered and filtrate was extracted with DCM in a continuous extraction apparatus. After three days, extracted metabolites in DCM were dried over anhydrous Na_2SO_4, Millipore filtered and the solvent was removed under vacuum. This yielded a sweet-smelling yellow oil. The odour of Th1 extracts was notably different than that of Th4 extracts. These extracts were subsequently used in experimentation as described below.

X. Compost medium

i. General

Experiments were performed to determine whether *A. bisporus* mycelium facilitated the degradation of complex compost constituents, such as lignin, into less complex breakdown products that can be utilised by *T. harzianum*. One kilogram samples of fresh phase II compost that was either spawned (*A. bisporus* Sylvan 130) or unspawned, were filled into plastic bags and incubated at 25 °C. Three bags of each spawned and unspawned compost, were removed each week, including week zero, for a total of eight weeks. These bags were individually emptied into buckets containing 2 L of distilled water and allowed to sit for two hours, stirring occasionally. The broth of each was then passed through cheesecloth and a number of filters, ending with Whatman #4 filter paper, to obtain a broth that was representative of the water soluble nutritional constituents contained in the compost sample at that time. These broth samples were kept at –20 °C for future use.

ii. Semi-solid culture

Compost agar medium (CAM) was made by adding 2% agar to each broth collected above, and sterilising the mixture. These plates were inoculated with either Th1 isolate Ssyl 5 (T123) or Th4 isolate Ss7c (T586) (n=3). Mycelial growth was assessed qualitatively after 5 days (figure 11).

iii. Liquid culture

Three 125 mL cultures of each of the aforementioned broths (X.i.) were prepared by sterilising and inoculating with 10^5-10^6 conidia of either Th1 isolate Ssyl 5 (T123) or Th4 isolate Ss7c (T586). These were allowed to shake at 150 rpm at 20 – 22 °C for 10 days, at which point stationary phase had been reached (figure 13). Cultures were then filtered through pre-weighed Whatman #4 filter paper, which was subsequently dried. Dry weight of mycelium was then determined using an analytical balance. This provided quantitative data indicative of the maximum (stationary phase) *T. harzianum* biomass supported by each broth.

iv. Isolation of metabolites from Th4 cultures in compost broth

Thirty-day-old cultures of Th4 in compost broth derived from 4 week old spawned compost, unspawned compost and Th4 infected compost, as described in the previous section, were filtered and the culture filtrate was placed in a continuous extraction apparatus with DCM. Residues were obtained as described above in section III. ii. and resuspended in methanol.

XI. Visualisation of chromophores characteristic of Th1 or Th4 using TLC

i. General

Thin-layer chromatography plates (Silica gel 60 F_{254}, EM Science, Gibbstown, NJ) were used with a solvent system described by Claydon *et al.*, (1987) 97% DCM

and 3% MeOH, to observe spots characteristic of green-mould-infected compost extracts and Th1 or Th4 cultures in MEB, compost media, and co-culture with *A. bisporus*. Using this solvent system, Claydon *et al.* (1987) found major antifungal metabolites produced by *T. harzianum in vitro* (2% MEB) to have R_F values around 0.7. In attempts to observe similar compounds, R_F values of spots from the aforementioned extracts were determined by visualising TLC plates under UV light (254 nm) and by spraying with 5% H_2SO_4 in methanol and charring at 80 °C. The diameter of the spots visualised on TLC that corresponded to the R_F 0.79 and 0.56 chromphores was used as a measure of chromophore intensity which likely corresponds to their concentration in the extracts.

ii. Assay for *T. harzianum* chromophores in semi-solid media cultures

A variety of *T. harzianum* isolates (table 1) were assayed for the chromophores they produced on semi-solid media. MEA plates were inoculated with *T. harzianum* isolates and incubated for 15 days. Plugs were then removed from these cultures with a 3-mm diameter cork borer. These plugs were placed on TLC plates and a drop of DCM was added to each plug with a Pasteur pipette. These plugs were then allowed to dry on the TLC plates. Thus, the metabolites present in the media plugs were translocated onto the TLC plate. These plates were then eluted and visualised as described above.

XII. Purification of major chromophores in Th4 extracts

i. General

Silica (70-230 mesh 60Å, Aldrich Chemical Company Ltd., Milwaukee, WI) column chromatography was used to separate compounds from Th4 infected compost, Th4 in MEB and *A. bisporus*/Th4 co-culture. Initially the same solvent

system used for TLC was used in attempts to separate compounds found in the crude extracts. This system did not give adequate separation and therefore was replaced with the following solvent: 199 parts toluene (methylbenzene, phenylmethane. C_7H_8) to 1 part *tert*-butyl alcohol (2-methyl-2-propanol, $(CH_3)_3COH$). This solvent was mixed with silica gel, creating a slurry to be loaded into a glass column. The glass column contained a piece of cotton at the tapered end just before the stopcock to prevent silica from passing through. Upon loading the glass column with a sufficient quantity of silica, roughly 100mg per mg of crude extract to be purified, a layer of fine glass beads was loaded on top of the column to a thickness of about 2 mm. To begin separation, the stopcock was opened, allowing the solvent to run through. When the solvent level on top of the column approached the glass bead interface, the crude extract, suspended in a minimal volume of solvent, was added to the column. The glass bead layer aided in absorbing this extract, translocating it to the silica below and preventing its resuspension into the solvent which was subsequently added. Fractions of 15 mL each were collected from the column until the desired compounds had passed through, as assessed by TLC. Fractions containing the same compound were then combined, dried over anhydrous Na_2SO_4. The solvent was removed under vacuum, yielding purified residues of specific R_F values.

ii. Antifungal activity assays

Ten millilitres of crude extract from 30-day-old Th4 cultures were divided into two 5 mL portions. One half was run through a silica column to obtain fractions containing the major chromophore identified in such extracts (results section VII. i.). All other fractions were combined to obtain an extract containing all compounds found

in 30-day-old Th4 extracts except the major chromophore. The solvent was removed from these two extracts and each residue was resuspended in 5 mL of methanol. This roughly standardised the concentrations of compounds in all extracts. These extracts and the other half of the crude extract were then added to bibulous paper disks as described above, in section VI. A total volume of 50 µL was added to each disk for each extract tested and these disks were placed in the centre of a mycelial lawn of *A. bisporus* Sylvan 130, the preparation of which is also in section VI. After 10 days incubation, diameter of the inhibitory zone was quantified by taking the mean of ten measurements made with a standard ruler.

An identical procedure was employed when testing purified extracts derived from mushroom compost infected with Th4. However, the purification process yielded an extract containing only the major chromophore associated with green-mould disease (results section VII. i.) and an extract containing all other compounds present in compost extracts. The purified compost chromophore, crude Th4 extract, purified Th4 *in vitro* chromophore and an extract containing all other compounds from *in vitro* Th4 culture extracts other than the major chromophore, were added individually to bibulous paper disks as outlined in the previous paragraph. These were placed on a uniform lawn of *A. bisporus* Horst U1 mycelium and the diameter of the inhibitory zone was quantified after 10 days.

XIII. Gas chromatograms of chromophores purified from Th4 extracts

A 5890A Hewlett-Packard gas chromatograph with ChemStation software was used to analyse purified extracts containing chromophores isolated green-mould-infected compost, Th4 cultures in MEB and co-culture of Th4 with *A. bisporus*. Optimised GC conditions were as follows: initial temperature 70 °C, initial time 3 min.,

injector temperature 150 °C, rate 5 °C per min., final temperature 100 °C. Samples obtained as described in section XI. and 1 µL samples in methanol were loaded into the injector using a Hamilton syringe. A detailed explanation of the experiments that helped determine these conditions is found in results section XII. Using this procedure, retention times for the major chromophores purified from these extracts were identified.

XIV. Spectroscopic analyses of Th4 chromophores

i. UV spectroscopy

UV absorbance spectra (225 nm – 375 nm) for the two major chromophores were determined in MeOH, using a Beckmann DU 7400 spectrophotometer.

ii. Mass spectroscopy and nuclear magnetic resonance spectroscopy

Electron ionisation or fast atom bombardment mass spectroscopy and GC/MS were performed by T. Jones, Department of Chemistry, Brock University, on a Carlo Erba/Kratos HRGC/MS, using DCM as the solvent.

^{1}H nuclear magnetic resonance (NMR) spectra of purified compounds were determined at 300 MHz in deuterochloroform ($CDCl_3$. Cambridge Isotope Laboratories, Inc., Andover, MA) and performed by J. Li, Brock University, Department of Chemistry on a Bruker Avance DPX_{300} digital NMR spectrometer.

RESULTS

I. Opposition experiments

i. *Botrytis cinerea* Bc2

After 72 hours of growth in the presence of headspace gasses produced by *T. harzianum* isolate Ss7c (T586), size of *B. cinerea* Bc2 mycelial colony was significantly reduced compared to control treatments (table 2). Mycelial growth of Bc2 was also reduced in the presence of Th1 cultures, significantly more than the reduction observed in the presence of Th4 (table 3). No significant differences between Bc2 cultures grown in the absence of another fungus and those grown in the presence of other Bc2 cultures, were observed (table 3).

ii. Agaricus bisporus

Compared to control cultures, *A. bisporus* Sylvan 130 mycelial growth was significantly reduced in the presence of headspace gasses produced by Th1, Th4 and Bc2 fungal cultures (table 4 and 5, and appendix III). This growth reduction occurred after one week of incubation in the presence of these headspace gasses and continued for the duration of the experiment. Significant *A. bisporus* growth reduction occurred in the presence of Th1 or Th4 headspace gasses compared to cultures grown in the presence of Bc2 headspace gasses (table 5). The experimental data in table 4 and appendix III represent experiments performed without adding benomyl to the SCM prior to inoculation with *A. bisporus*. This resulted in contamination of some plates by the opposing fungus, (Th1, Th4 or Bc2). Notwithstanding this contamination, trends in these data indicate an increased reduction in *A. bisporus* growth in the presence of *B. cinerea* Bc2 and *T. harzianum* biotypes that is similar to that observed when benomyl was added to the *A. bisporus* culture medium (table 5).

Table 2. ***Reduction of* Botrytis cinerea *Bc2 mycelial growth in the presence of headspace gasses produced by* Trichoderma harzianum *isolate Ss7c T586, biotype Th4***

	Bc2 mean colony size (mm^2)*		
Opposing petri dish	24 hours	48 hours	72 hours
MEA control (n=10)	**129 a**	**1130 a**	**3361 a**
Th4 culture - T586 (n=5)	**103 a**	**917 a**	**2802 b**

* Means followed by a different letter in each column are significantly different from each other at P<0.05 according to One-way ANOVA (Fisher's LSD). See Appendix I for raw data and statistical analyses.

Table 3. ***Reduction of*** **Botrytis cinerea** ***Bc2 mycelial growth in the presence of headspace gasses produced by*** **Trichoderma harzianum** ***cultures***

	Bc2 mean colony diameter (mm)*			
Opposing petri dish	24 hours	48 hours	96 hours	168 hours
MEA control (n=5)	**14.5 a**	**37.6 a**	**56.2 a**	**73.5 a**
Bc2 (n=5)	**14.1 a**	**38.0 a**	**59.1 a**	**76.1 a**
Th4 - T586 (n=5)	**13.9 a**	**35.1 a**	**48.8 b**	**55.8 b****
Th1 - T123 (n=5)	**12.5 b**	**29.7 b**	**37.5 c**	**53.8 b**

* Means followed by a different letter in each column are significantly different from each other at P<0.05 according to One-way ANOVA (Fisher's LSD). See Appendix II for raw data and statistical analyses.

** n=3 due to contamination of two samples.

Table 4. ***Reduction of* Agaricus bisporus *Somycel 608 mycelial growth in the presence of headspace gasses produced by* Trichoderma harzianum *cultures***

	A. bisporus* mean colony diameter (mm)		
Opposing petri dish	10 days	20 days	30 days
MEA control (n=4)	**23.5 a**	**44.3 a**	**59.8 a**
Bc2 (n=5)	**20.9 b**	**39.6 b**	**54.7 b**
Th4 - T586 (n=4)	**20.4 b**	**37.2 b**	**49.2 c****
Th1 - T123 (n=5)	**19.0 b**	**36.8 b**	**47.8 c*****

* Means followed by a different letter in each column are significantly different from each other at P<0.05 according to One-way ANOVA (Fisher's LSD). See Appendix IV for raw data and statistical analyses.

** n=2 due to contamination of two samples.

*** n=4 due to contamination of one sample.

Table 5. ***Reduction of* Agaricus bisporus *Sylvan 130 mycelial growth on SCMB media in the presence of headspace gasses produced by* Botrytis cinerea *or* Trichoderma harzianum *cultures***

	A. bisporus* mean colony size (mm^2)		
Opposing petri dish	10 days**	20 days	30 days
MEA control (n=10)	**78 a**	**487 a**	**1212 a**
Bc2 (n=10)	**79 a**	**381 b**	**730 b**
Th4 - T586 (n=10)	**79 a**	**271 c**	**594 c**
Th1 - T123 (n=10)	**77 a**	**290 c**	**528 c**

* Means followed by a different letter in each column are significantly different from each other at P<0.05 according to One-way ANOVA (Fisher's LSD). See Appendix V for raw data and statistical analyses.

** *A. bisporus* cultures sealed with freshly inoculated test fungi on MEA at t=10 days.

II. Antifungal activity of crude extracted compounds

i. *Botrytis cinerea*

Growth of *B. cinerea* Bc2 was significantly reduced when metabolites extracted from thirty day old Th4 culture filtrate and suspended in methanol were added directly to inoculum plugs (table 6). Bc2 inoculum plugs treated with Th4 extracts produced colonies that were significantly smaller in area than colonies arising from untreated plugs, those treated with methanol or with MEB extract in methanol. This reduction of mycelial growth, first measured at 24 hours, was observed in cultures up to 72 hours after inoculation and treatment. No difference in mycelial growth was observed between untreated inoculum plugs, plugs treated with methanol and those treated with MEB extract.

ii. *Agaricus bisporus*

Table 7 displays results of an experiment performed to determine the effects of different extracts and dilutions thereof, on radial growth of *A. bisporus* mycelium. Compared to untreated control cultures, radial growth of *A. bisporus* Horst U1 was inhibited by adding 20 μL of Th4 extract directly to each inoculum plug immediately following inoculation. This volume is equivalent to 200 μg of crude extract (see materials and methods III. iii.). Dilutions of this volume (in methanol) also reduced *A. bisporus* growth compared to untreated controls. Quantities of crude extract as small as 0.2 μg (1000X dilution or 0.1% on table 7) resulted in significantly smaller *A. bisporus* colonies compared to untreated cultures (table 7). This effect was observed for up to ten days following application. One replicate that was treated with 200 μg of crude extract did not show any growth and was transferred to fresh SCM agar after 20 days. After this transfer, still no growth occurred (figure 7). Metabolites extracted from

the organic portion of 30 day Th4 cultures had no noticeable effect on growth of *A. bisporus* mycelium. The MEB extract used in this experiment had a significant inhibitory effect on *A. bisporus* growth, equivalent to that of the Th4 extract. This extract was clear and green in colour, in contrast to the clear yellow characteristic of all other MEB extracts, indicating that an unknown contaminating factor was present.

Table 6. ***Reduction of* Botrytis cinerea *Bc2 mycelial growth by metabolites extracted from* Trichoderma harzianum *Th4 culture filtrate suspended in methanol and added directly to inoculum plugs***

	B. cinerea* mean colony size (mm^2)		
Treatment	24 hours	48 hours	72 hours
Untreated control (n=3)	**228 a**	**1391 a**	**4004 a**
20 µL methanol (n=3)	**260 a**	**1519 a**	**4191 a**
20 µL MEB extract (n=3)	**284 a**	**1544 a**	**4078 a**
20 µL Th4 extract (n=3)	**110 b**	**835 b**	**2804 b**

* Means followed by a different letter in each column are significantly different from each other at $P<0.05$ according to One-way ANOVA (Fisher's LSD). See Appendix VI for raw data and statistical analyses.

Table 7. ***Reduction of* Agaricus bisporus *Horst U1 mycelial growth by metabolites extracted from* Trichoderma harzianum *biotype Th4 culture filtrate and suspended in methanol added directly to inoculum plugs***

	A. bisporus* mean colony size (mm^2)			
Treatment	5 days	10 days	15 days	20 days
Untreated control (n=4)	**334 a**	**1013 b**	**2097 a**	**3690 a**
20 µl methanol (n=3)	**342 a**	**1061 a**	**2105 a**	**3794 a**
20 µl MEB extract ** (n=3)	**28 c**	**58 d**	**439 b**	**1235 b**
20 µl Th4 extract 0.1% (n=4)	**228 b**	**872 c**	**1946 a**	**3468 a**
20 µl Th4 extract 1% (n=4)	**237 b**	**856 c**	**1956 a**	**3489 a**
20 µl Th4 extract 10% (n=3)	**223 b**	**837 c**	**1905 a**	**3383 a**
20 µl Th4 extract 100% (n=5)	**32 c**	**109 d**	**385 b**	**1145 b**
Th4 mycelial extract (n=3)	**342 a**	**1017 b**	**1989 a**	**3626 a**

* Means followed by a different letter in each column are significantly different from each other at P<0.05 according to One-way ANOVA (Fisher's LSD). See Appendix VII for raw data and statistical analyses.

** Extract from uncharacteristic MEB

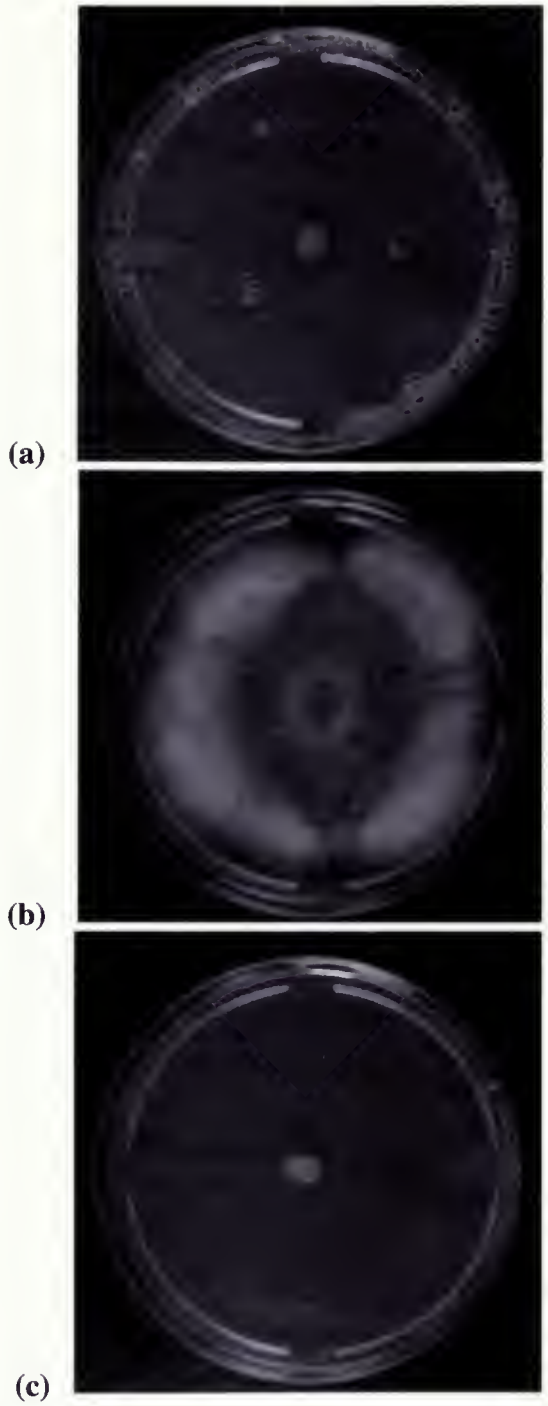


Figure 7. *(a) Inhibition of* Agaricus bisporus *Horst U1 mycelial growth by metabolites isolated from 30-day-old liquid (2% MEB) culture of* Trichoderma harzianum *biotype Th4, 20 days after application. (b) Untreated control culture of Horst U1 20 days after inoculation. (c) Horst U1 plug shown in (a), 30 days after being transferred to fresh SCM agar.*

Table 8 illustrates an attempt at determining differential inhibitory activity of the Th4 extract towards growth of mycelia from brown and off-white hybrid strains of *A. bisporus*. Growth of *A. bisporus* mycelium did not differ significantly between plugs treated with Th4 extract and those treated with methanol only, indicating a toxic effect of methanol on the fungal hyphae.

The inhibitory effect of methanol was also observed when 20 μL of methanol were applied directly to inoculum plugs (table 9) or when SCM agar was amended with 3% methanol (data not shown). Based on these observations, direct application of metabolites suspended in methanol to inoculum plugs was no longer performed.

iii. *Sclerotium cepivorum*

When added to the growing margin of established *S. cepivorum* cultures, visible inhibition of fungal mycelium in the area of the addition was observed after 24 hours (figure 8). When extracts were applied directly to inoculum plugs, no growth was seen after 4 days (figure 9).

Table 8. ***Reduction of* Agaricus bisporus *Somycel 608 (off-white hybrid) and Amycel 2400 (large brown) mycelial growth by application of either methanol or Th4 extract in methanol directly to inoculum plugs***

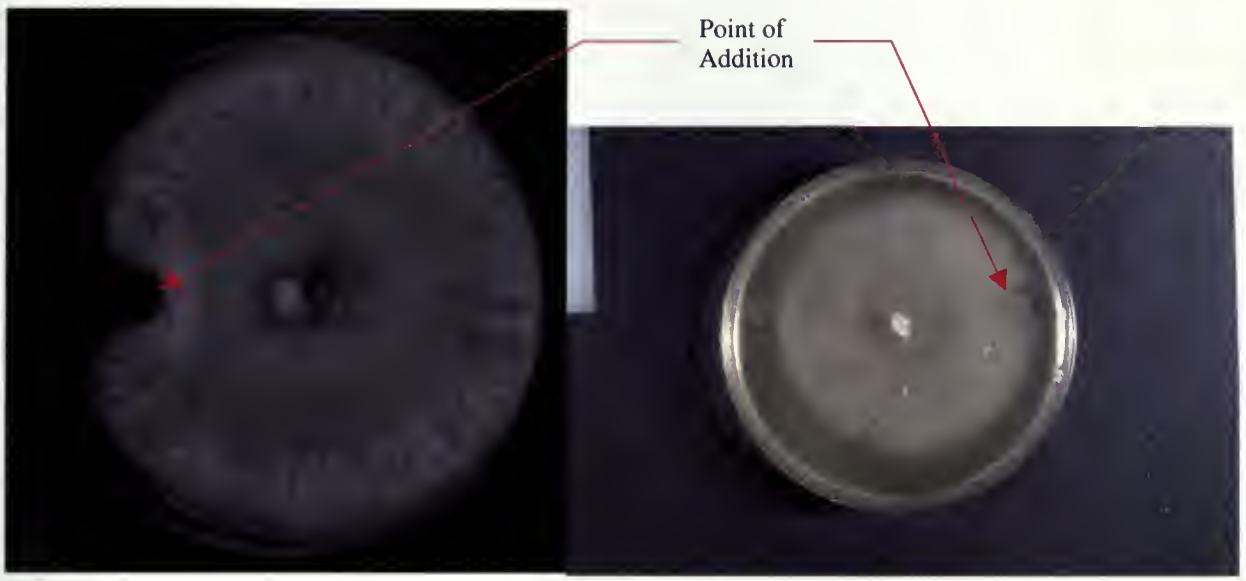
	A. bisporus* mean colony size (mm^2)		
Treatment (40 µl)	10 days	20 days	30 days
Somycel 608 + methanol (n=4)	**124 a**	**294 a**	**496 a**
Somycel 608 + Th4 extract (n=5)	**133 a**	**441 a**	**830 a**
2400 + methanol (n=5)	**76 A**	**258 A**	**499 A**
2400 + Th4 extract (n=5)	**76 A**	**276 A**	**536 A**

* Means followed by a different letter in each column are significantly different from each other at $P<0.05$ according to Mann-Whitney U-test. See Appendix VIII for raw data and statistical analyses.

Table 9. ***Reduction of* Agaricus bisporus *Horst U1 mycelial growth by application of either methanol or Th4 extract in methanol directly to inoculum plugs***

	A. bisporus* mean colony size (mm^2)		
Treatment	5 days	10 days	15 days
Untreated control	**410 c**	**1435 b**	**3183 b**
20 µl methanol	**296 b**	**1130 a**	**2769 a**
20 µl Th4 extract	**226 a**	**970 a**	**2576 a**

* Means followed by a different letter in each column are significantly different from each other at P<0.05 according to One-way ANOVA (Fisher's LSD). See Appendix IX for raw data and statistical analyses.



(a) (b)

Figure 8. Sclerotium cepivorum *mycelial growth 24 hours after 10 uL of the following extracts, suspended in methanol, were added to the advancing colony margin: (a) compounds extracted from filtrate of a 30 day* Trichoderma harzianum *Th4 culture in 2% malt extract broth, (b) 10 µL of compounds extracted from uninoculated 2% MEB. Note the inhibition of mycelial growth at the point where the* T. harzianum *extract was added.*

(a)

(b)

Figure 9. *(a) Inhibition of* Sclerotium cepivorum *growth 4 days following the addition of 10 µL of metabolites extracted from* Trichoderma harzianum *Th4 culture broth. (b) Mycelial growth of* S. cepivorum *4 days following the addition of 10 µL of compounds extracted from 2% MEB.*

III. Volatile antifungal activity of crude extracted compounds

Section V. of materials and methods explains the experimental design used to determine antifungal effects of *T. harzianum* culture extracts that can be attributed to volatile compounds. Table 10 illustrates the inhibitory effects of volatile compounds isolated from both Th1 and Th4 cultures on *A. bisporus* mycelial growth.

A. bisporus cultures shared only airspace with the various treatments listed in table 10 for thirty days. After this time, only Th1 and Th4 extracts had a significant effect on *A. bisporus* mycelial growth compared to untreated control cultures. In the presence of either of these extracts, radial growth of *A. bisporus* mycelia was reduced. Data illustrating this effect were first gathered after only ten days of exposure to these volatile compounds.

IV. Diffusable antifungal activity of crude extracted compounds

The ability of the extracted metabolites to diffuse through aqueous media, exhibiting antifungal activity at a distance from the point of application, was assessed using the techniques outlined in materials and methods section VI. When placed on a lawn of freshly inoculated *A. bisporus* Horst U1 mycelium, a zone of growth inhibition was noted around bibulous paper disks impregnated with compounds extracted from either Th1 or Th4 cultures (figure 10); control treatments of methanol and MEB extract did not induce an inhibitory zone (table 11). Furthermore, in the presence of disks containing Th1 extracts, the zone of *A. bisporus* inhibition was significantly larger in diameter than in the presence of disks containing Th4 extracts. Figure 10 also illustrates that after several weeks, growth of *A. bisporus* occurred in areas previously devoid of mycelium. This growth typically continued until *A. bisporus* mycelium was actively growing in the entire 'inhibitory zone', as marked after 10 days.

Table 10. ***Reduction of* Agaricus bisporus *Somycel 608 mycelial growth in the presence of volatile metabolites extracted from* Trichoderma harzianum *culture broth***

	A. bisporus* mean colony size (mm^2)	
Treatment	10 days	30 days
Untreated control (n=5)	**116 a**	**586 a**
1 mL methanol (n=4)	**97 b**	**559 a****
1 mL MEB extract (n=5)	**119 a**	**526 a****
1 mL Th1 extract (n=4)	**42 c**	**264 b**
1 mL Th4 extract (n=4)	**26 c**	**296 b**

* Means followed by a different letter in each column are significantly different from each other at P<0.05 according to LSD-based One-way ANOVA. See Appendix X for raw data and statistical analyses.

** n=x-1 due to contamination of one sample in each replicate set.

Table 11. ***Zone of fungal growth inhibition caused by bibulous paper disks impregnated with diffusable metabolites extracted from*** **Trichoderma harzianum** ***culture broth and placed on a fresh, uniform lawn of*** **Agaricus bisporus** ***Horst U1 inoculum***

Compound added to disk (50 μl)	**Diameter of inhibitory zone (mm) after 10 days***
Methanol (n=4)	**0 a**
MEB extract (n=10)	**0 a**
Th1 extract (n=10)	**54 c**
Th4 extract (n=10)	**19 b**

* Means followed by a different letter in each column are significantly different from each other at P<0.05 according to LSD-based One-way ANOVA. See Appendix XI for raw data and statistical analyses.

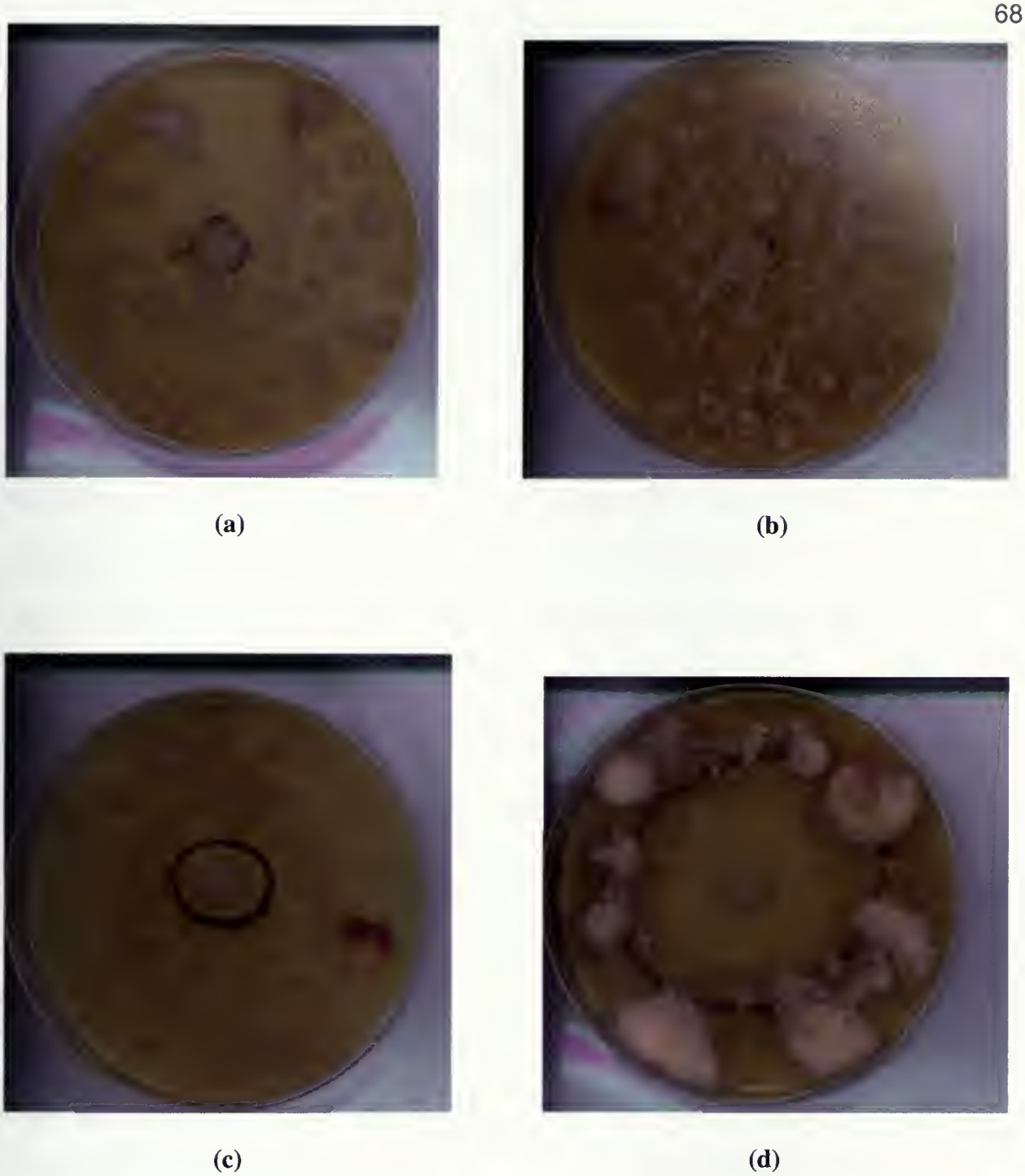


Figure 10. *Ninety-day-old lawn of* Agaricus bisporus *Horst U1 mycelium on SCM agar with paper disks containing 50 µL of (a) methanol (b) MEB extract (c) Th4 extract and (d) Th1 extract. The circles on the reverse side of the plates indicate zone of mycelial growth inhibition marked after ten days of incubation (table 11).*

V. Activity of extracted compounds on brown and white strains of *A. bisporus*

Differential antifungal activity of the Th4 extract towards brown and white strains of *A. bisporus* was determined using bibulous paper discs impregnated with extracted metabolites (materials and methods section VII.). The data in table 12 illustrate a significant difference in the antifungal effect of metabolites from Th4 on brown (Sylvan SB65) and white (Sylvan 130) strains of *A. bisporus*. The different quantities of metabolites added also had an effect on the size of the inhibitory zone. As the total quantity of crude metabolites added was increased, the size of the inhibitory zone increased accordingly. In all cases, for all the different quantities added, the size of the inhibitory zone was smaller when disks were placed on a mycelial lawn of SB65 compared to when placed on a lawn of Sylvan 130. Thus, to produce inhibitory zones on a SB65 mycelial lawn of equal size to those on a S130 mycelial lawn, significantly higher titres of antifungal compounds isolated from Th4 are required.

VI. Growth of *T. harzianum* in compost media

In order to determine whether *A. bisporus* colonisation of compost contributed to the proliferation of green-mould in compost, Th1 and Th4 biotypes were grown in semi-solid and liquid media prepared from both spawned and unspawned compost (materials and methods section X). Figure 11 clearly illustrates how *A. bisporus* colonisation of compost effects growth luxuriance of *T. harzianum* mycelium on semi-solid compost medium. Media derived from unspawned compost supports minimal *T. harzianum* growth compared to media derived from spawned compost. This effect was also observed in liquid compost media (figure 12). This figure illustrates that after 3 weeks, media prepared from spawned compost reaches a maximum with

respect to the quantity of Th4 mycelium it will support. This was assessed by dry weight of Th4 mycelium present in each type of medium after stationary phase was reached (materials and methods section X. iii.). This trend is also illustrated by visual assessment of *T. harzianum* grown on semi-solid compost media (figure 11).

Table 12. ***Zone of fungal growth inhibition caused by bibulous paper disks impregnated with diffusable metabolites extracted from Th4 culture broth and placed on a fresh, uniform lawn of*** **Agaricus bisporus** ***Sylvan 130 or Sylvan SB65 inoculum***

	Diameter of inhibitory zone (mm) after 10 days*	
Quantity of metabolites added to disk (mg)	130 (off-white)	SB65 (brown)
	n=5	n=5
0.1	**23 a**	**17 b**
0.2	**26 a**	**20 b**
0.3	**33 a**	**26 b**
0.4	**45 a**	**32 b**
0.5	**48 a**	**35 b**

* Means followed by a different letter in each row are significantly different from each other at P<0.05 according to Mann-Whitney U-test. See Appendix XII for raw data and statistical analyses.

(a) Th4 on 0 wk. SCAM **(b) Th4 on 2 wk. SCAM** **(c) Th4 on 3wk. SCAM**

(d) Th4 on 8wk. SCAM **(e) Th4 on 0wk. UCAM** **(f) Th4 on 3wk. UCAM**

(g) Th4 on 8wk. UCAM **(h) Th1 on 8wk. SCAM** **(i) Th1 on 8wk. UCAM**

Figure 11. *Growth of* Trichoderma harzianum *biotypes after 5 days on semi-solid media derived from spawned (SCAM) and unspawned (UCAM) compost. Th4 on media from 0- (a), 2- (b) , 3- (c) and 8-week-old (d) spawned compost and on media derived from 0- (e), 3- (f) and 8-week-old (g) unspawned compost. Th1 on media from 8-week-old spawned (h) and unspawned (i) compost.*

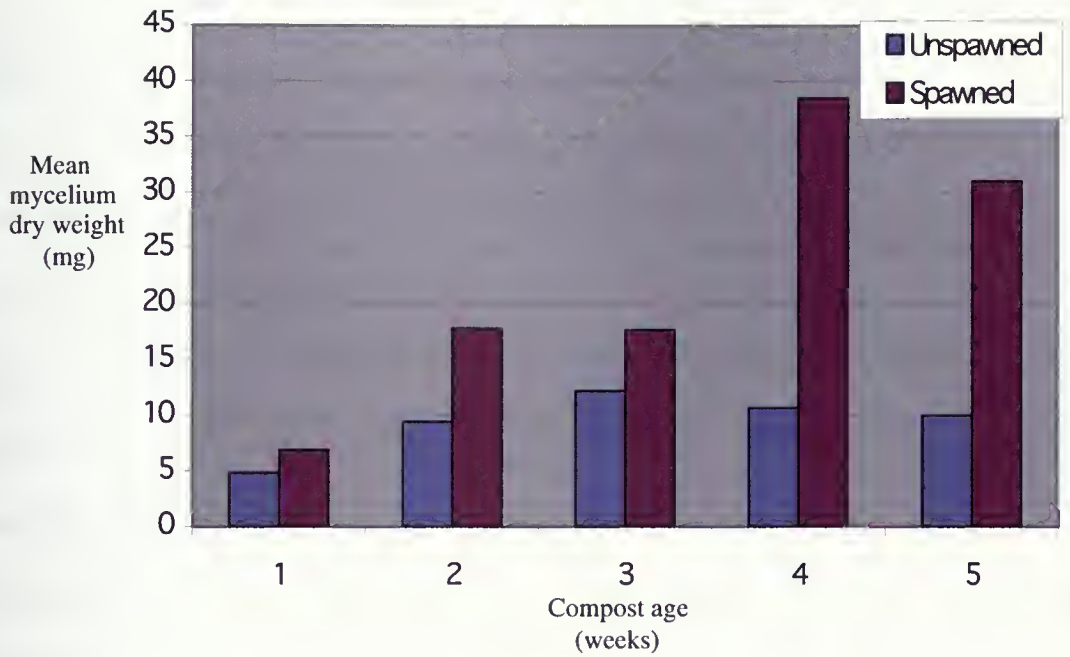


Figure 12. *Mean dry weight of* Trichoderma harzianum *Th4 mycelia after reaching stationary growth phase in one hundred millilitres of liquid media derived from spawned and unspawned compost of different ages.*

VII. Purification of major chromophores produced by Th4

i. General

Metabolites extracted from MEB and compost broth cultures of Th1 and Th4, from co-cultures of *A. bisporus* and Th4 or Th1 and from compost infected with Th4 were analysed for major compounds that may be responsible for antifungal activity according to the protocol described in materials and methods section XI.

Two major chromophores, with R_F 0.56 and 0.79, were identified with TLC in extracts from 30-day-old Th4 cultures in 2% MEB. Visualisation of these spots under UV light indicated that the 0.79 spot was present in this extract at a higher concentration than the 0.56 spot. Via TLC, the R_F 0.79 compound was first detectable in extracts from Th4 cultures five days old. The increase in chromophore intensity, quantified by spot size, was proportional to the culture duration, the most intense spot being present in extracts from cultures sixty days old. This relationship was not linear, as much of the increase in chromophore titre occurred during stationary phase of Th4 cultures (figure 13). The R_F 0.56 compound in Th4 extracts could only be detected on TLC plates after 30 days, after mycelial growth had stopped and culture was in stationary phase (figure 13). Extracts from Th1 cultures did not contain either of these compounds, regardless of culture duration or medium. A chromophore with R_F 0.72 was observed in Th1 extracts, corresponding to the major antifungal compound identified by Claydon *et al.* (1987) in *T. harzianum* cultures.

The R_F 0.79 compound was purified from the 30-day-old Th4 culture extracts as described in materials and methods section XII. i. Purified fractions containing only this compound were combined while the remaining fractions were combined, yielding

an extract that contained all other compounds including the R_F 0.56 chromophore. These extracts were used in subsequent antifungal activity assays.

Thin layer chromatography revealed a chromophore with R_F 0.56 in metabolite extracts from green-mould infected compost. This compound had identical chromatographic properties to the R_F 0.56 compound found in culture extracts, indicating they are the same compound. The R_F 0.79 chromophore was not present. The R_F 0.56 compound was found only in compost infected with green-mould.

Compared to extracts of Th4 grown in MEB, intensity of this chromophore was increased *in vitro* by co-culturing Th4 and *A. bisporus* or by growing Th4 in medium prepared from the broth of compost fully colonised with *A. bisporus* (three weeks after spawning). The intensity of these chromophores was not altered by autoclaving *A. bisporus* cultures prior inoculation with Th4. *Agaricus bisporus* and Th1 co-culture extracts did not contain either of these compounds nor did extracts of Th4 cultures grown in a medium derived from unspawned compost.

Compost extract was purified as described in materials and methods section XII. i. to obtain compound with chromophore having and R_F value of 0.56 on TLC as described. Antifungal activity of this extract was assessed in subsequent experiments. Extracts from co-cultures of *A. bisporus* and *T. harzianum* biotypes were also purified and the purified compounds analysed for antifungal activity.

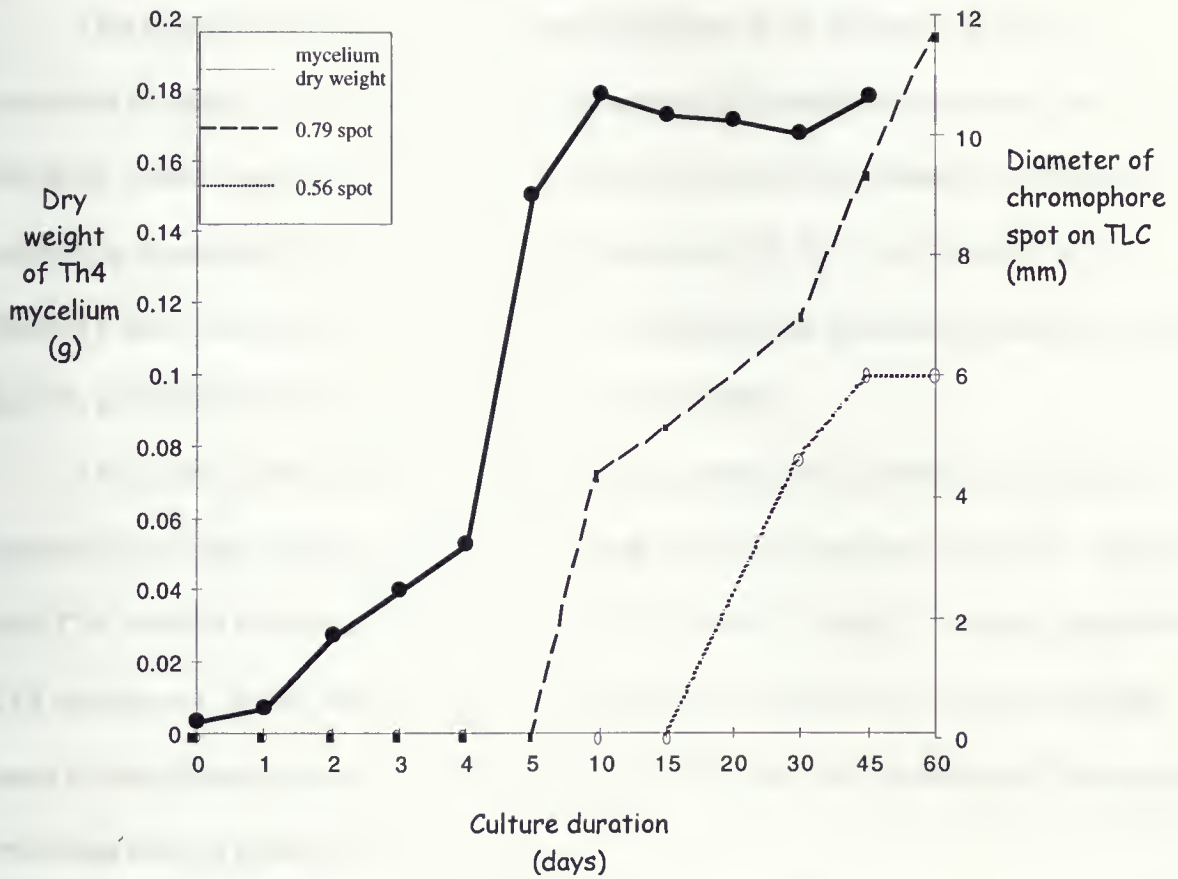


Figure 13. *Dry weight of mycelium and relative quantities of two major chromophores in* Trichoderma harzianum *Th4 cultures (250 mL MEB) over sixty-day culture duration.*

ii. Antifungal activity assays

In order to determine whether the major chromophores identified in Th4 culture extracts were responsible for the antifungal activity exhibited by these extracts, bibulous paper disks were impregnated with each of these purified compounds and placed on a lawn of *A. bisporus* mycelium (materials and methods section XII. ii.).

The compound with R_F 0.79 caused inhibition of *A. bisporus* mycelium as illustrated in table 13. This inhibitory zone was significantly smaller in diameter than inhibitory zones caused by disks impregnated with crude Th4 extract or the extract containing all metabolites in the Th4 extract except R_F 0.79. The diameter of the inhibitory zone caused by crude Th4 extracts and extracts containing metabolites other than R_F 0.79 did not differ significantly from one another.

The compound found in association with green-mould-infected mushroom compost (R_F 0.56) caused inhibition of *A. bisporus* as illustrated in table 14. Extracts from Th4 in MEB also caused mycelial growth inhibition. Additions of the purified R_F 0.79 compound, crude extract and metabolites other than R_F 0.79, show a similar trend to that shown in table 13; the purified 0.79 compound showed significantly less antifungal activity than the crude extract.

VIII. Assay for *T. harzianum* chromophores in semi-solid media cultures

Media plugs taken from cultures of *T. harzianum* isolates (materials and methods section XI. ii.) previously identified as Th4 (table 1) all contained the R_F 0.79 chromophore while Th1 cultures did not contain this chromophore (results not shown).

Table 13. ***Zone of* Agaricus bisporus *Sylvan 130 growth inhibition caused by bibulous paper disks impregnated with metabolites extracted from* Trichoderma harzianum *culture broth.***

Compound added to disk (50 µl)	**Diameter of inhibitory zone (mm) after 10 days***
Crude Th4 extract (n=10)	**21.0 b**
Pure R_F 0.79 compound from Th4 extract (n=10)	**15.9 a**
Crude Th4 extract minus R_F 0.79 (n=10)	**22.2 b**

* Means followed by a different letter in each column are significantly different from each other at P<0.05 according to LSD-based One-way ANOVA. See Appendix XIII for raw data and statistical analyses.

Table 14. ***Zone of* Agaricus bisporus *Horst U1 growth inhibition caused by bibulous paper disks impregnated with the major metabolite associated with green-mould infection (R_F 0.56), crude metabolites extracted from* Trichoderma harzianum *Th4 MEB culture, the major chromophore found in MEB cultures (R_F 0.79), or all metabolites present in crude Th4 extracts other than R_F 0.79.***

Compound added to disk (50 μL)	Diameter of inhibitory zone (mm) after 10 days*
Crude Th4 extract (n=3)	**24.0 a**
Pure R_F 0.56 compound from compost (n=3)	**16.6 b**
Pure R_F 0.79 compound from Th4 extract (n=3)	**14.6 b**
Other metabolites from Th4 extract (n=3)	**21.9 ab**

* Means followed by a different letter in each column are significantly different from each other at P<0.05 according to LSD-based One-way ANOVA. See Appendix XIV for raw data and statistical analyses.

IX. Gas chromatography of purified, major chromophores produced by Th4

In order to determine whether the aforementioned chromophores had been separated from other constituents of the compost or culture extracts from which they were obtained, purified samples were analysed with a gas chromatograph. Initial experiments indicated that no compounds other than the solvent (MeOH) were present in the samples (appendix XV). Visualisation of chromophores on TLC plates illustrated the presence of compounds in these purified extracts, thus samples were run through GC under a variety of conditions. It was determined that the initial conditions did not give adequate separation of compounds due to the volatile nature of the compounds being analysed. This caused the peaks corresponding to the solvent and the purified compound to move though the GC column at the same rate and hence produce overlapping signals observed as a single peak, as seen in appendix XV. By reducing the temperature, flow rate and adding initial time (materials and methods section XIII.), the methanol peak was separated from the peak corresponding to the compounds of interest.

Gas chromatograms of R_F 0.56 and 0.79 spots purified from extracts of infected compost and co-cultures of *A. bisporus* and Th4 revealed two major peaks other than the solvent peak (figure 14). As illustrated, these two peaks had similar retention times (2.94 and 3.84 minutes) in both the samples. The quantitative data shown in these chromatograms indicates that the concentration of the compound with retention time of 2.94 minutes is greater than ten-fold that of the second compound. TLC data (figures 15 and 16) combined with this indicate that the compounds R_F 0.56 and R_F 0.79 likely correspond to the compounds shown on the GC with retention times of 2.94 and 3.84 minutes respectively.

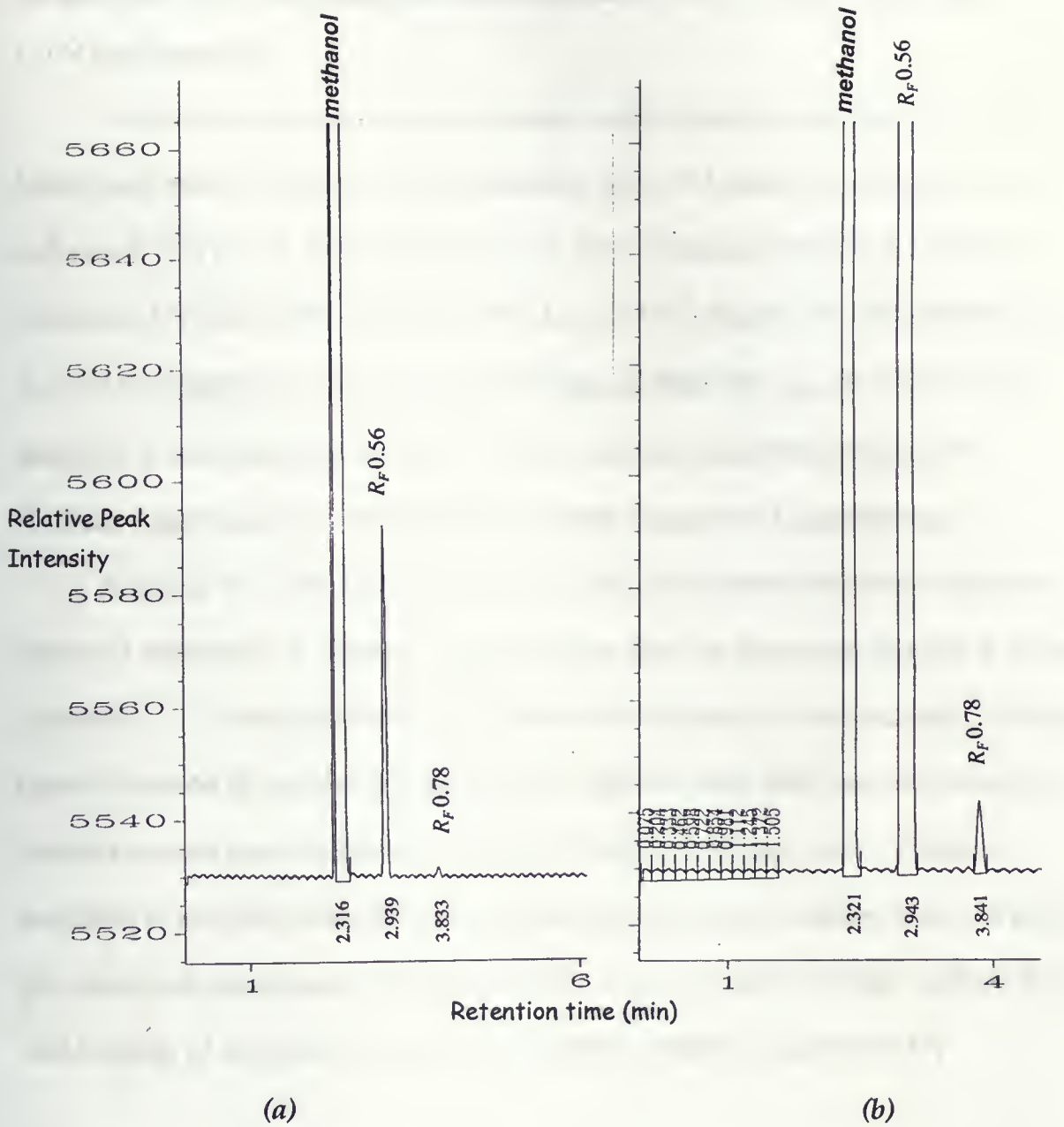


Figure 14. ***Gas chromatograms of purified extracts containing chromophores possessing antifungal activity. Analytes were purified from (a) extracts derived from green-mould-infected compost and (b) co-cultures of*** **Trichoderma harzianum** ***Th4 and*** **Agaricus bisporus,** ***and suspended in methanol for injection into GC.***

X. Spectroscopic analyses of major chromophores found in Th4 cultures

i. UV spectroscopy

UV absorbance spectra were determined for major chromophores isolated from green-mould-infected compost extracts, from Th4 liquid cultures and from co-cultures of Th4 and *A. bisporus* (figure 15). The compound with R_F 0.79 had a distinctive UV absorption spectrum, with λ_{max} 313 nm (figure 15). UV spectra for the R_F 0.56 chromophore were not clear, although at least one λ_{max} at 275 nm was apparent in samples from compost and from co-culture extracts (figure 15).

ii. Mass spectroscopy and nuclear magnetic resonance spectroscopy

Analysis of a pure sample of the R_F 0.79 spot revealed the mass spectrum shown in appendix 15. Proton resonance spectrum for this same sample is shown in appendix 16. These data indicate a pyrone structure may be present, although future experimentation is required to validate this. Unfortunately NMR and MS data from further samples were inconclusive due to the solvent system used. Toluene persisted in samples despite removal attempts and many samples were not purified with ultra-pure spectroscopy grade solvents. Due to these impurities, spectra for the vast majority of samples could not be accurately analysed (appendix 17)

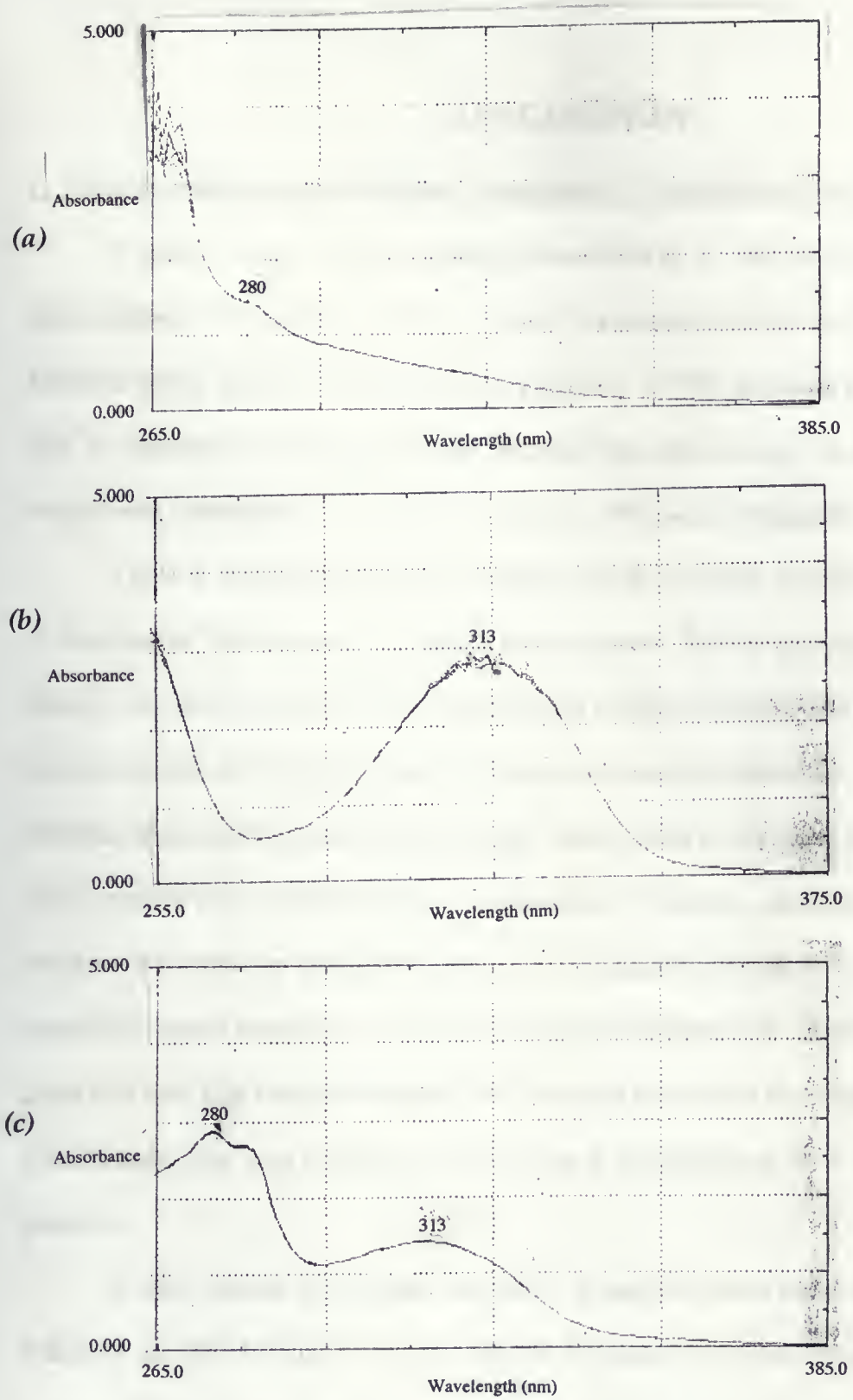


Figure 15. Ultraviolet absorbance spectra of chromophores isolated from extracts of (a) green-mould infected compost (b) Trichoderma harzianum Th4 in MEB culture and (c) Th4 in co-culture with Agaricus bisporus.

DISCUSSION

I. Volatile antifungal metabolites produced by *T. harzianum* cultures

Production of volatile antibiotic compounds by *T. harzianum* is widely documented and plays a significant role in the antagonistic effect this fungus has towards many other fungi (Dennis and Webster, 1971b; Bruce *et al.*, 1984). In light of this, an analysis of volatile antibiotic metabolites produced by the aggressive mushroom competitor, *T. harzianum* biotype Th4, was conducted.

Table 2 and 3 illustrate the inhibitory effect of volatile metabolites produced by *T. harzianum* Th4 on mycelial growth of *B. cinerea*. Similar experimentation by Hutchinson and Cowan (1972) indicated that inhibitory effects can be attributed to carbon dioxide and ethanol; waste products of fungal metabolism. To determine whether this was the case the test fungus was grown in the presence of a fungus that had a growth rate to similar that of *T. harzianum*. *Botrytis cinerea* strain Bc2 was chosen to provide an adequate control for the possible inhibitory effects of such metabolic waste products. The data in table 3 illustrate that volatile metabolites from both Th1 and Th4 biotypes significantly reduced *B. cinerea* mycelial growth and no growth reduction was observed when grown in the presence of *B. cinerea* headspace gasses.

Similar results were observed when *A. bisporus* was used as the test fungus (tables 4 and 5). In table 4 an inhibition of *A. bisporus* mycelial growth by volatile metabolic waste products from *B. cinerea* cultures is observed. This supports the aforementioned claims by Hutchinson and Cowan (1972). *B. cinerea* does not produce volatile compounds other than those described as metabolic waste products such as CO_2 and alcohol (Deacon, 1997). Although *T. harzianum* cultures produce

these volatile metabolic waste products, the degree of *A. bisporus* inhibition observed in the presence of *T. harzianum* headspace gasses is significantly higher than in the presence of *B. cinerea* headspace gasses. This indicates the presence of a volatile antifungal compound, as is widely described in *T. harzianum* as a secondary metabolite (Claydon *et al.*, 1987; Ghisalberti and Rowland, 1993; Scarselletti and Faull, 1994).

II. Isolation of volatile antifungal compounds from *T. harzianum* cultures

Crude *T. harzianum* culture extracts exhibited antifungal activity against *B. cinerea* (table 6), *S. cepivorum* (figure 8) and *A. bisporus* (table 7 and figure 7) when applied directly to these fungi. Although fungal toxicity associated with the methanol in which these metabolites were dissolved was shown, this artefact was later eliminated, and the antifungal activity of extracted metabolites towards *A. bisporus* mycelia was illustrated (table 11 and figure 7).

Crude extracted metabolites from both Th1 and Th4 culture broth exhibited volatile antifungal activity (table 10), indicating that the metabolites responsible for the effects seen in tables 2 through 5, had been isolated. These extracts also exhibited diffusible antifungal activity when disks impregnated with these compounds were placed on a uniform lawn of *A. bisporus* mycelia (table 11). This method proved to be an efficient way to assay the antifungal effects of crude or pure *T.harzianum* metabolites isolated from various sources.

Metabolite extracts from both Th1 and Th4 biotypes exhibited inhibitory activity towards *A. bisporus* mycelia (tables 7, 10 and 11). These extracts contained different major chromophores as shown by TLC. Th1 extracts contained a compound with R_F 0.72, which corresponds to that reported for 6PAP. a volatile antifungal compound

produced by type-cultures (Th1 biotypes) of *T. harzianum* (Claydon *et al.*,1987). The presence of this compound in Th1 metabolite extracts is therefore not surprising, and as one would expect, these extracts exhibit antifungal activity towards *A. bisporus*. Although differential activity of Th1 and Th4 extracts towards *A. bisporus* is shown in tables 10 and 11, these differences are of little significance due to the lack of quantitative information regarding specific compounds in either extract.

III. Production of a major *T. harzianum* antifungal metabolite elicited by *A. bisporus*

Table 14 illustrates the antifungal activity of this purified compound towards *A. bisporus* mycelia. This compound (R_F 0.56) was produced by Th4 in compost, in co-culture with *A. bisporus* and in media derived from compost colonised with *A. bisporus* mycelia. Production of this compound was not altered by autoclaving *A. bisporus* mycelia prior to inoculation with Th4 in co-culture experiments, indicating that a heat-labile *A. bisporus* factor is not involved in inducing production of this compound (data not shown).

These observations suggest that a non-protein product of *A. bisporus* metabolism is required for the production of the antifungal metabolite with R_F 0.56 by *T. harziánum* biotype Th4. This non-protein may be produced extracellularly through the enzymatic processes of *A. bisporus* or may be a component of *A. bisporus* hyphae. Since this compound is found in infected compost, is inhibitory to *A. bisporus* mycelium and is not produced by non-aggressive (Th1) biotypes of *T. harzianum* under any culture conditions studied, it is likely that this compound plays a role in green-mould disease of mushroom crops.

Since a heat-labile factor is not involved in the production of this antifungal compound associated with green mould infection, a highly specific protein-based

host-parasite recognition system is not involved in eliciting production of this compound.

IV. Differential activity of extracted compounds from Th4 towards brown and white strains of *A. bisporus*

Rinker and Alm (1997a) illustrated that aggressive *T. harzianum* Th4 caused less extensive crop loss and did not proliferate as extensively in compost spawned with a brown strain of *A. bisporus* (Sylvan SB65) compared to compost spawned with white strains of *A. bisporus* (including Sylvan 130). This finding supported claims of mushroom growers indicating that brown mushroom strains were more tolerant to green-mould infection. Initial experiments indicated a differential effect of metabolites extracted from Th4 cultures on mycelia of brown and white strains of *A. bisporus in vitro* (table 8). This experiment was repeated using the same mushroom strains studied by Rinker and Alm (1997a); antifungal metabolites extracted from Th4 cultures showed an increased level of inhibition towards mycelia from the white *A. bisporus* strain, Sylvan 130 (table 12). This exciting discovery further indicated the role of volatile antifungal metabolites produced by *T. harzianum* Th4 in the establishment of green-mould disease iin mushroom crops.

V. Requirement of *A. bisporus* in green-mould infestation of compost

It is widely accepted that the presence of *A. bisporus* mycelium is required in order for green-mould to proliferate and sporulate in compost (Seaby, 1987). Although observations indicate that *A. bisporus* metabolism or mycelium is required in order for Th4 to produce certain antifungal metabolites, they do not explain why Th4 cannot proliferate through compost that is not spawned with *A. bisporus*.

The data in figures 11 and 12 clearly show that unspawned mushroom compost and compost not fully colonised by *A. bisporus* mycelium do not support significant growth of *T. harzianum* mycelium. This is true for both Th1 and Th4 biotypes. Since the composting process gives rise to a substrate that is highly selective for *A. bisporus* growth, these data are not surprising. Once *A. bisporus* has colonised the compost, its digestive enzymes metabolise the complex structures present (such as lignin) into simpler carbohydrates. These can be utilised by *T. harzianum* biotypes, which explains why media derived from spawned compost can support luxuriant growth of both *T. harzianum* biotypes.

VI. Significance of antifungal compounds produced by Th4 in green-mould disease

Why can Th4 proliferate through mushroom compost, causing the crop devastation characteristic of green-mould disease, while Th1 cannot? The answer to this question, wh has puzzled mushroom researchers since green-mould epidemics began, may be answered by further study of the antifungal compounds produced by Th4.

Although non-aggressive *T. harzianum* biotypes exist in a very limited sense throughout mushroom compost, they do not sporulate or colonise compost as do aggressive biotypes. The antifungal compound specific to Th4 may be involved in this colonisation. Production of this compound by Th4 during certain mushroom cropping stages would inhibit mushroom growth, thereby allowing the aggressive biotype to flourish in the mushroom compost, utilising the breakdown products of *A. bisporus* metabolism and causing green-mould disease.

Active *A. bisporus* mycelium can be isolated from green-mould-infected compost (Rinker, personal communication), and from *A. bisporus* petri dishes inoculated with Th4 (Droganes, 1998), indicating that Th4 does not cause complete cell-death in the mushroom mycelium. Further evidence indicating that Th4 does not

kill *A. bisporus* mycelium is occasionally seen in green-mould-infected mushroom crops several weeks after infection has been established. In this phenomenon a flush or break of mushrooms occurs at the focal point of green-mould-infection, at a time corresponding to what would be a third or fourth break in a healthy mushroom crop (Rinker, personal communication). The results shown in figure 10 support the notion that the antibiotic compounds isolated are inhibitory but not toxic. As illustrated, after several weeks *A. bisporus* begins to grow in areas that previously showed complete inhibition of mycelial growth. Whether this is due to dissipation of the volatile antifungal compounds, slow growth of *A. bisporus* in the presence of these compounds or a combination of both, is unknown and a topic of future research.

Studies by Bonnarme *et al.* (1997) indicate that certain vegetable oils significantly enhance the production of 6PAP *in vitro*. With this in mind, observations indicating that green-mould infection can intensify in the presence of vegetable-based supplements (Rinker and Alm, 1997a) may also support the involvement of an antibiotic compound in the establishment of green-mould disease.

CONCLUSIONS

The data shown indicate that *T. haarzianum* biotype Th4 produces volatile antifungal compounds that are likely a product of secondary metabolism. The production of one such compound was found to be increased in the presence of *A. bisporus* culture contents, whether viable or autoclaved. This same compound was also found in green-mould-infected compost and is likely involved in disease establishment. Although the identity of this antifungal compound was not determined, data presented suggests that it is related to the pyrone class of antifungal compounds widely described in *Trichoderma* species, and that it has yet to be described.

With this knowledge, further research will hopefully lead to the development of *A. bisporus* strains resistant to this compound or Th4 strains deficient in its production. Such developments will aid in more accurately determining the role this compound has in green-mould disease of mushrooms.

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APPENDICES

APPENDIX I

Reduction of *Botrytis cinerea* Bc2 mycelial growth in the presence of headspace gasses produced by *Trichoderma harzianum* isolate Ss7c T586, biotype Th4 (Table 2)

Mac Daddy:Desktop Folder:Documents:MSc:stats:BotvsTh4andMEA

	lower	area24h	area48h	area72h
1	MEA	110	935	2413
2	MEA	71	684	2161
3	MEA	71	755	2548
4	MEA	52	465	2090
5	MEA	135	1065	2923
6	MEA	155	1187	3348
7	MEA	84	748	2710
8	MEA	103	1071	3219
9	MEA	161	1329	3548
10	MEA	90	935	3058
11	Th4 on M	90	1226	3671
12	Th4 on M	148	1168	3110
13	Th4 on M	187	1445	3368
14	Th4 on M	77	1039	3181
15	Th4 on M	142	774	3477

- - - - - O N E W A Y - - - - -

Variable AREA24H
By Variable LOWER lower

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	1	2219.9009	2219.9009	1.4092	.2564
Within Groups	13	20478.5861	1575.2759		
Total	14	22698.4871			

Variable AREA48H
By Variable LOWER lower

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	1	151092.0075	151092.0075	2.3175	.1519
Within Groups	13	847547.0781	65195.9291		
Total	14	998639.0856			

Variable AREA72H
By Variable LOWER lower

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	1	1042921.947	1042921.947	5.5192	.0353
Within Groups	13	2456518.790	188962.9838		
Total	14	3499440.737			

APPENDIX II

Reduction of *Botrytis cinerea* Bc2 mycelial growth in the presence of headspace gasses produced by *Trichoderma harzianum* cultures (Table 3)

Mac Daddy:Desktop Folder:Documents:MSc:stats:BotvsTh1Th4Bc2

	lower	diam24h	diam48h	diam96h	diam168h
1	MEA	14.4	37.8	58.2	78.4
2	MEA	14.0	37.3	56.4	79.8
3	MEA	14.4	38.7	54.4	72.5
4	MEA	14.5	37.0	59.6	73.4
5	MEA	15.0	37.1	52.6	63.3
6	BC2 on M	14.4	37.0	57.0	71.4
7	BC2 on M	14.8	39.2	59.4	85.0
8	BC2 on M	13.5	37.8	61.8	77.0
9	BC2 on M	13.8	37.6	61.2	74.4
10	BC2 on M	14.0	38.2	56.3	72.8
11	Th1 on M	10.8	27.2	33.3	.
12	Th1 on M	13.4	29.6	35.2	.
13	Th1 on M	13.2	28.1	35.6	58.8
14	Th1 on M	11.5	28.6	36.8	50.2
15	Th1 on M	13.6	34.8	46.5	52.4
16	Th4 on M	14.0	35.6	48.0	51.1
17	Th4 on M	14.8	38.5	52.8	52.8
18	Th4 on M	13.7	33.4	46.2	49.0
19	Th4 on M	13.9	37.0	56.3	67.0
20	Th4 on M	13.0	31.0	40.5	52.2

- - - - - O N E W A Y - - - - -

Variable DIAM24H Diameter (mm) of Bc2 mycelium at 24h
By Variable LOWER

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	11.0255	3.6752	6.1125	.0057
Within Groups	16	9.6200	.6012		
Total	19	20.6455			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= .5483 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.00

(*) Indicates significant differences which are shown in the lower triangle

		G r p 3	G r p 4	G r p 2	G r p 1
Mean	LOWER				
12.5000	Grp 3				
13.8800	Grp 4	*			
14.1000	Grp 2	*			
14.4600	Grp 1	*			

Homogeneous Subsets (highest and lowest means are not significantly different)

Subset 1

Group Grp 3

Mean 12.5000

- - - - - - - - - - -

Subset 2

Group	Grp 4	Grp 2	Grp 1
Mean	13.8800	14.1000	14.4600

- - - - - O N E W A Y - - - - -

Variable DIAM48H Diameter (mm) of Bc2 mycelium at 48h
By Variable LOWER

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	219.6055	73.2018	15.4573	.0001
Within Groups	16	75.7720	4.7357		
Total	19	295.3775			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 1.5388 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.00

(*) Indicates significant differences which are shown in the lower triangle

		G r p 3	G r p 4	G r p 1	G r p 2
Mean	LOWER				
29.6600	Grp 3				
35.1000	Grp 4	*			
37.5800	Grp 1	*			
37.9600	Grp 2	*			

Homogeneous Subsets (highest and lowest means are not significantly different)

Subset 1

Group Grp 3

Mean 29.6600

- - - - - - - - - - -

Subset 2

Group	Grp 4	Grp 1	Grp 2
Mean	35.1000	37.5800	37.9600

- - - - - O N E W A Y - - - - -

Variable DIAM96H Diameter (mm) of Bc2 mycelium at 96h
By Variable LOWER

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	1400.5455	466.8485	23.9115	.0000
Within Groups	16	312.3840	19.5240		
Total	19	1712.9295			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 3.1244 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.00

(*) Indicates significant differences which are shown in the lower triangle

Mean	LOWER	G r p 3	G r p 4	G r p 1	G r p 2
37.4800	Grp 3				
48.7600	Grp 4	*			
56.2400	Grp 1	*	*		
59.1400	Grp 2	*	*		

Homogeneous Subsets (highest and lowest means are not significantly different)

Subset 1

Group Grp 3

Mean 37.4800

- - - - - - - - - - -

Subset 2

Group	Grp 4
Mean	48.7600

Subset 3

Group	Grp 1	Grp 2
Mean	56.2400	59.1400

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Variable DIAM168H Diameter (mm) of Bc2 mycelium at 168h
By Variable LOWER

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	1906.4788	635.4929	16.7624	.0001
Within Groups	14	530.7640	37.9117		
Total	17	2437.2428			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 4.3538 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.03

(*) Indicates significant differences which are shown in the lower triangle

		G r p 3	G r p 4	G r p 1	G r p 2
Mean	LOWER				
53.8000	Grp 3				
54.4200	Grp 4				
73.4800	Grp 1	*	*		
76.1200	Grp 2	*	*		

APPENDIX III

Agaricus bisporus **Sylvan 130 mycelial growth was significantly reduced in the presence of headspace gasses produced by Th1, Th4 and Bc2 fungal cultures (Referred to in Results I. i.)**

Mac Daddy:Desktop Folder:Documents:MSc:stats:130FacewoBenomyl

	treatmnt	tenday	twntyday	thrtyday
1	MEA	17.0	35.1	52.4
2	MEA	20.4	38.6	55.6
3	MEA	18.7	37.4	55.0
4	MEA	15.2	31.2	44.4
5	MEA	18.0	35.6	52.6
6	MEA	19.3	40.0	58.4
7	Bc2 on M	18.2	35.3	.
8	Bc2 on M	14.3	28.6	43.3
9	Bc2 on M	16.3	33.3	48.7
10	Bc2 on M	18.0	36.9	.
11	Bc2 on M	18.0	37.0	52.7
12	Bc2 on M	18.2	35.6	.
13	Th1 on M	19.3	31.8	44.8
14	Th1 on M	15.7	.	.
15	Th1 on M	17.0	.	.
16	Th1 on M	16.3	.	.
17	Th1 on M	17.1	.	.
18	Th1 on M	15.3	.	.
19	Th1 on M	17.4	.	.
20	Th4 on M	19.6	33.3	48.3
21	Th4 on M	15.3	31.4	47.4
22	Th4 on M	16.2	31.8	49.7
23	Th4 on M	17.4	.	.
24	Th4 on M	16.0	.	.
25	Th4 on M	17.2	33.3	45.7

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Variable TENDAY S130 mycelial diam (mm) at 10 days
By Variable TREATMNT

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	5.9190	1.9730	.8108	.5021
Within Groups	21	51.1026	2.4335		
Total	24	57.0216			

Levene Test for Homogeneity of Variances

Statistic	df1	df2	2-tail Sig.
.2610	3	21	.853

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 1.1031 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 2.94

- No two groups are significantly different at the .050 level

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Variable TWNTYDAY S130 mycelial diam (mm) at 20 days
By Variable TREATMNT

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	43.9114	14.6371	1.8804	.1827
Within Groups	13	101.1933	7.7841		
Total	16	145.1047			

Levene Test for Homogeneity of Variances

Statistic	df1	df2	2-tail Sig.
1.3765	3	13	.294

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 1.9728 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.06

- No two groups are significantly different at the .050 level

- - - - - O N E W A Y - - - - -

Variable THRTYDAY S130 mycelial diam (mm) at 30 days
By Variable TREATMNT

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	112.5611	37.5204	2.2426	.1459
Within Groups	10	167.3075	16.7308		
Total	13	279.8686			

Levene Test for Homogeneity of Variances

Statistic	df1	df2	2-tail Sig.
.9316	3	10	.461

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 2.8923 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.15

- No two groups are significantly different at the .050 level

APPENDIX IV

Reduction of *Agaricus bisporus* Somycel 608 mycelial growth in the presence of headspace gasses produced by *Trichoderma harzianum* cultures (Table 4)

Mac Daddy:Desktop Folder:Documents:MSc:stats:608vsTh1Th4Bc2

	lower	diam10d	diam20d	diam30d	diam40d
1	nothing	22.1	42.3	59.7	72.0
2	nothing	25.0	45.9	59.3	74.1
3	nothing	26.5	47.1	60.6	70.2
4	nothing	20.4	41.9	59.5	75.7
5	Bc2	19.1	42.7	55.5	64.2
6	Bc2	21.1	38.2	55.8	73.1
7	Bc2	20.0	37.9	49.8	59.7
8	Bc2	23.4	40.8	58.9	78.4
9	Bc2	21.1	38.3	53.3	76.5
10	Th1	18.3	35.0	46.1	64.8
11	Th1	18.2	35.8	49.6	72.2
12	Th1	18.3	38.9	.	.
13	Th1	21.1	37.6	.	.
14	Th4	21.6	39.6	.	.
15	Th4	19.9	35.6	52.5	.
16	Th4	19.9	37.2	44.2	.
17	Th4	20.4	36.3	49.0	.
18	Th4	20.4	37.4	51.0	70.3

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Variable DIAM10D Diameter (mm) of 608 mycelium after 10 d
By Variable LOWER Fungus on lower MEA plate

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	42.9085	14.3028	4.8659	.0160
Within Groups	14	41.1515	2.9394		
Total	17	84.0600			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 1.2123 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.03

(*) Indicates significant differences which are shown in the lower triangle

		G r p 3	G r p 4	G r p 2	G r p 1
Mean	LOWER				
18.9750	Grp 3				
20.4400	Grp 4				
20.9400	Grp 2				
23.5000	Grp 1	*	*	*	

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Variable DIAM20D Diameter (mm) of 608 mycelium after 20 d
By Variable LOWER Fungus on lower MEA plate

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	146.4593	48.8198	12.1607	.0003
Within Groups	14	56.2035	4.0145		
Total	17	202.6628			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 1.4168 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.03

(*) Indicates significant differences which are shown in the lower triangle

		G r p 3	G r p 4	G r p 2	G r p 1
Mean	LOWER				
36.8250	Grp 3				
37.2200	Grp 4				
39.5800	Grp 2				
44.3000	Grp 1	*	*	*	

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Variable DIAM30D Diameter (mm) of 608 mycelium after 30 d
By Variable LOWER Fungus on lower MEA plate

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	302.5453	100.8484	12.0932	.0008
Within Groups	11	91.7320	8.3393		
Total	14	394.2773			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 2.0420 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.11

(*) Indicates significant differences which are shown in the lower triangle

		G r p 3	G r p 4	G r p 2	G r p 1
Mean	LOWER				
47.8500	Grp 3				
49.1750	Grp 4				
54.6600	Grp 2	*	*		
59.7750	Grp 1	*	*	*	

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Variable DIAM40D Diameter (mm) of 608 mycelium after 40 d
By Variable LOWER Fungus on lower MEA plate

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	30.8587	10.2862	.2688	.8462
Within Groups	8	306.1480	38.2685		
Total	11	337.0067			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 4.3743 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.26

- No two groups are significantly different at the .050 level

APPENDIX V

Reduction of *Agaricus bisporus* Sylvan 130 mycelial growth on SCMB media in the presence of headspace gasses produced by *Botrytis cinerea* or *Trichoderma harzianum* cultures (Table 5)

Mac Daddy:Desktop Folder:Documents:MSc:stats:130FacingWBenomyl

	treatmnt	area10d	area20d	area30d
1	nothing	90	516	1252
2	nothing	84	419	877
3	nothing	84	400	1123
4	nothing	84	503	1265
5	nothing	77	574	1452
6	nothing	58	406	1245
7	nothing	90	652	1606
8	nothing	77	516	1213
9	nothing	71	458	1181
10	nothing	65	426	910
11	Bc2	71	394	806
12	Bc2	71	465	806
13	Bc2	103	445	748
14	Bc2	77	361	768
15	Bc2	71	297	568
16	Bc2	84	400	684
17	Bc2	77	452	884
18	Bc2	71	323	703
19	Bc2	84	374	684
20	Bc2	77	297	652
21	Th1	71	213	348
22	Th1	58	258	574
23	Th1	97	258	542
24	Th1	71	239	613
25	Th1	84	284	626
26	Th1	90	368	742
27	Th1	77	265	484
28	Th1	84	361	677
29	Th1	84	394	729
30	Th1	77	329	606
31	Th4	65	239	452
32	Th4	77	245	426
33	Th4	84	213	387
34	Th4	77	277	523
35	Th4	90	335	548
36	Th4	65	323	587
37	Th4	97	400	710
38	Th4	71	245	400
39	Th4	71	258	581
40	Th4	71	361	671
41	.	.	.	.

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Variable AREA20D Area (mm2) of 130 mycelium after 20 days
By Variable TREATMNT facing fungus on MEA added at t=10 days

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	254721.1455	84907.0485	18.7750	.0000
Within Groups	36	162804.7598	4522.3544		
Total	39	417525.9053			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 47.5518 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 2.87

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	G r p 4	G r p 3	G r p 2	G r p 1
		4	3	2	1
289.6768	Grp 4				
296.7736	Grp 3				
380.6444	Grp 2	*	*		
487.0958	Grp 1	*	*	*	

Homogeneous Subsets (highest and lowest means are not significantly different)

Subset 1

Group	Grp 4	Grp 3
Mean	289.6768	296.7736

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Subset 2

Group Grp 2

Mean 380.6444

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Subset 3

Group Grp 1

Mean 487.0958

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Variable AREA30D Area (mm2) of 130 mycelium after 30 days
By Variable TREATMNT facing fungus on MEA added at t=10 days

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	2863950.043	954650.0142	46.3077	.0000
Within Groups	36	742153.1188	20615.3644		
Total	39	3606103.161			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 101.5268 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 2.87

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	G r p 4	G r p 3	G r p 2	G r p 1
		4	3	2	1
528.3860	Grp 4				
594.1924	Grp 3				
730.3211	Grp 2	*	*		
1212.2556	Grp 1	*	*	*	

Homogeneous Subsets (highest and lowest means are not significantly different)

Subset 1

Group	Grp 4	Grp 3
Mean	528.3860	594.1924

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Subset 2

Group Grp 2

Mean 730.3211

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Subset 3

Group Grp 1

Mean 1212.2556

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APPENDIX VI

Reduction of *Botrytis cinerea* Bc2 mycelial growth by metabolites extracted from *Trichoderma harzianum* Th4 culture filtrate suspended in methanol and added directly to inoculum plugs (Table 6)

Mac Daddy:Desktop Folder:Documents:MSc:stats:BC2vsMeOHMEBandTh4

	treatmnt	area24h	area48h	area72h
1	nothing	219	1374	4077
2	nothing	187	1265	3774
3	nothing	277	1535	4161
4	MeOH	258	1452	4342
5	MeOH	284	1606	4161
6	MeOH	239	1490	4071
7	MEB	277	1465	4000
8	MEB	277	1535	3994
9	MEB	297	1632	4239
10	Th4	142	923	2858
11	Th4	103	716	2652
12	Th4	84	865	2903

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Variable AREA24H Area of BC2 mycelium after 24 hours
By Variable TREATMNT 10 ul direct addition

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	53790.9746	17930.3249	19.8821	.0005
Within Groups	8	7214.6780	901.8348		
Total	11	61005.6526			

Levene Test for Homogeneity of Variances

Statistic	df1	df2	2-tail Sig.
1.5933	3	8	.266

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 21.2348 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.26

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	G r p 4	G r p 1	G r p 2	G r p 3
		4	1	2	3
109.6772	Grp 4				
227.9565	Grp 1	*			
260.2145	Grp 2	*			
283.8704	Grp 3	*			

Homogeneous Subsets (highest and lowest means are not significantly different)

Subset 1

Group Grp 4

Mean	109.6772

Subset 2

Group	Grp 1	Grp 2	Grp 3
Mean	227.9565	260.2145	283.8704

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Variable AREA48H Area of BC2 mycelium after 48 hours
By Variable TREATMNT 10 ul direct addition

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	988702.2540	329567.4180	30.3077	.0001
Within Groups	8	86992.3680	10874.0460		
Total	11	1075694.622			

Levene Test for Homogeneity of Variances

Statistic	df1	df2	2-tail Sig.
.3835	3	8	.768

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 73.7362 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.26

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	Grp 4	Grp 1	Grp 2	Grp 3
		4	1	2	3
834.4069	Grp 4				
1391.3951	Grp 1	*			
1516.1260	Grp 2	*			
1544.0829	Grp 3	*			

Homogeneous Subsets (highest and lowest means are not significantly different)

Subset 1

Group	Grp 4
Mean	834.4069

Subset 2

Group	Grp 1	Grp 2	Grp 3
Mean	1391.3951	1516.1260	1544.0829

- - - - - O N E W A Y - - - - -

Variable AREA72H Area of BC2 mycelium after 72 hours
By Variable TREATMNT 10 ul direct addition

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	3778642.534	1259547.511	51.3983	.0000
Within Groups	8	196045.0015	24505.6252		
Total	11	3974687.535			

Levene Test for Homogeneity of Variances

Statistic	df1	df2	2-tail Sig.
.5206	3	8	.680

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 110.6924 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.26

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	G r p 4	G r p 1	G r p 3	G r p 2
		4	1	3	2
2804.2955	Grp 4				
4004.2931	Grp 1	*			
4077.4112	Grp 3	*			
4191.3895	Grp 2	*			

Homogeneous Subsets (highest and lowest means are not significantly different)

Subset 1

Group	Grp 4
Mean	2804.2955

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Subset 2

Group	Grp 1	Grp 3	Grp 2
Mean	4004.2931	4077.4112	4191.3895

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APPENDIX VII

Reduction of *Agaricus bisporus* Horst U1 mycelial growth by metabolites extracted from *Trichoderma harzianum* biotype Th4 culture filtrate and suspended in methanol added directly to inoculum plugs (Table 7)

Mac Daddy:Desktop Folder:Documents:MSc:stats:E1

	treatmnt	area5d	area10d	area15d	area20d
1	nothing	361	1065	2148	3742
2	nothing	323	1032	2168	4006
3	nothing	323	1006	2039	3626
4	nothing	329	948	2032	3387
5	MeOH	381	1123	2077	3865
6	MeOH	348	1065	2265	3742
7	MeOH	297	994	1974	3774
8	green ME	32	71	355	1168
9	green ME	26	52	658	1471
10	green ME	26	52	303	1065
11	100% Th4	39	168	523	1742
12	100% Th4	32	252	897	2097
13	100% Th4	32	32	181	723
14	100% Th4	32	65	297	1135
15	100% Th4	26	26	26	26
16	10% Th4	232	897	1839	3503
17	10% Th4	232	845	2142	3394
18	10% Th4	206	768	1735	3252
19	1% Th4	239	910	1871	3200
20	1% Th4	232	916	2058	3665
21	1% Th4	258	877	2129	3710
22	1% Th4	219	723	1768	3381
23	0.1% Th4	310	1058	2135	3671
24	0.1% Th4	245	903	2161	3761
25	0.1% Th4	187	781	1819	3323
26	0.1% Th4	168	748	1671	3116
27	organic	323	955	1871	3181
28	organic	400	1200	2110	4032
29	organic	303	897	1987	3665

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Variable AREA5D Area of U1 mycelium (mm2) after 5 days
By Variable TREATMNT 20 ul direct addition at t=0

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
veen Groups	7	427731.0148	61104.4307	54.7015	.0000
in Groups	21	23458.1094	1117.0528		
l	28	451189.1242			

iple Range Tests: LSD test with significance level .05

difference between two means is significant if
AN(J)-MEAN(I) >= 23.6332 * RANGE * SQRT(1/N(I) + 1/N(J))
th the following value(s) for RANGE: 2.94
*) Indicates significant differences which are shown in the lower triangle

		G r p 3	G r p 4	G r p 5	G r p 7	G r p 6	G r p 1	G r p 2	G r p 8
Mean	TREATMNT								
27.9569	Grp 3								
32.2580	Grp 4								
23.6555	Grp 5	*	*						
27.4189	Grp 7	*	*						
37.0963	Grp 6	*	*						
33.8703	Grp 1	*	*	*	*	*			
41.9348	Grp 2	*	*	*	*	*			
41.9348	Grp 8	*	*	*	*	*			

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Variable AREA15D Area of U1 mycelium (mm2) after 15 days
By Variable TREATMNT 20 ul direct addition at t=0

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	7	14863127.17	2123303.881	46.1499	.0000
Within Groups	21	966185.5213	46008.8343		
Total	28	15829312.69			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 151.6721 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 2.94

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	G r p 4	G r p 3	G r p 5	G r p 7	G r p 6	G r p 8	G r p 1	G r p 2
384.5154	Grp 4								
438.7088	Grp 3								
1905.3725	Grp 5	*	*						
1946.7703	Grp 7	*	*						
1956.4477	Grp 6	*	*						
1989.2433	Grp 8	*	*						
2096.7700	Grp 1	*	*						
2105.3721	Grp 2	*	*						

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Variable AREA20D Area of U1 mycelium (mm2) after 20 days
By Variable TREATMNT 20 ul direct addition at t=0

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	7	33576576.42	4796653.775	26.2796	.0000
Within Groups	21	3833003.745	182523.9879		
Total	28	37409580.17			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 302.0960 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 2.94

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	G r p 4	G r p 3	G r p 5	G r p 7	G r p 6	G r p 8	G r p 1	G r p 2
1144.5138	Grp 4								
1234.4061	Grp 3								
3382.7889	Grp 5	*	*						
3467.7350	Grp 7	*	*						
3488.7027	Grp 6	*	*						
3625.7992	Grp 8	*	*						
3690.3152	Grp 1	*	*						
3793.5408	Grp 2	*	*						

APPENDIX VIII

Reduction of *Agaricus bisporus* Somycel 608 (off-white hybrid) and Amycel 2400 (large brown) mycelial growth by application of either methanol or Th4 extract in methanol directly to inoculum plugs (Table 8)

Mac Daddy:Desktop Folder:Documents:MSc:stats:2400and608vsMeOHandTh4

	treatmnt	area10d	area20d	area30d
1	608 and	123	200	361
2	608 and	181	465	716
3	608 and	84	187	323
4	608 and	110	323	587
5	608 and	103	277	497
6	608 and	97	258	497
7	608 and	123	465	1239
8	608 and	213	832	1271
9	608 and	129	374	645
10	2400 and	52	194	426
11	2400 and	90	303	561
12	2400 and	65	239	465
13	2400 and	65	194	432
14	2400 and	110	361	613
15	2400 and	77	290	529
16	2400 and	65	258	497
17	2400 and	71	226	503
18	2400 and	58	226	445
19	2400 and	110	381	710

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

AREA10D area of agaricus mycelium (mm2) at 10d
by TREATMNT agaricus strain and treatment (40 ul)

Mean Rank	Cases	
4.63	4	TREATMNT = 1 608 and MeOH
5.30	5	TREATMNT = 2 608 and Th4
	9	Total

		Exact	Corrected for ties	
U	W	2-Tailed P	Z	2-Tailed P
8.5	18.5	.7302	-.3690	.7122

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

AREA20D area of agaricus mycelium (mm2) at 20d
by TREATMNT agaricus strain and treatment (40 ul)

Mean Rank	Cases	
3.88	4	TREATMNT = 1 608 and MeOH
5.90	5	TREATMNT = 2 608 and Th4
	9	Total

		Exact	Corrected for ties	
U	W	2-Tailed P	Z	2-Tailed P
5.5	15.5	.2857	-1.1069	.2683

AREA30D area of agaricus mycelium (mm2) at 30d
by TREATMNT agaricus strain and treatment (40 ul)

Mean Rank	Cases	
3.75	4	TREATMNT = 1 608 and MeOH
6.00	5	TREATMNT = 2 608 and Th4
	9	Total

		Exact		Corrected for ties
U	W	2-Tailed P	Z	2-Tailed P
5.0	15.0	.2857	-1.2299	.2187

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

AREA10D area of agaricus mycelium (mm2) at 10d
by TREATMNT agaricus strain and treatment (40 ul)

Mean Rank	Cases	
5.30	5	TREATMNT = 3 2400 and MeOH
5.70	5	TREATMNT = 4 2400 and Th4
	10	Total

		Exact	Corrected for ties	
U	W	2-Tailed P	Z	2-Tailed P
11.5	26.5	.8413	-.2121	.8320

AREA20D area of agaricus mycelium (mm2) at 20d
by TREATMNT agaricus strain and treatment (40 ul)

Mean Rank	Cases	
5.00	5	TREATMNT = 3 2400 and MeOH
6.00	5	TREATMNT = 4 2400 and Th4
	10	Total

		Exact	Corrected for ties	
U	W	2-Tailed P	Z	2-Tailed P
10.0	25.0	.6905	-.5254	.5993

AREA30D area of agaricus mycelium (mm2) at 30d
by TREATMNT agaricus strain and treatment (40 ul)

Mean Rank	Cases	
4.80	5	TREATMNT = 3 2400 and MeOH
6.20	5	TREATMNT = 4 2400 and Th4
	10	Total

Exact Corrected for ties

U	W	2-Tailed P	Z	2-Tailed P
9.0	24.0	.5476	-.7311	.4647

APPENDIX IX

Reduction of *Agaricus bisporus* Horst U1 mycelial growth by application of either methanol or Th4 extract in methanol directly to inoculum plugs (Table 9)

Mac Daddy:Desktop Folder:Documents:MSc:stats:U1vsMeOHandTh4

	treatmnt	area5d	area10d	area15d
1	nothing	458	1465	3219
2	nothing	394	1497	3258
3	nothing	355	1245	2832
4	nothing	387	1406	3316
5	nothing	458	1561	3290
6	MeOH	303	1077	2768
7	MeOH	348	1161	2619
8	MeOH	265	1077	2748
9	MeOH	303	1148	2826
10	MeOH	265	1187	2884
11	Th4	200	916	2529
12	Th4	206	839	2310
13	Th4	271	1155	2890

- - - - - O N E W A Y - - - - -

Variable AREA5D Area (mm2) of U1 mycelium after 5d
By Variable TREATMNT 20 ul direct addition at t=0

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	2	69889.7389	34944.8694	21.3518	.0002
Within Groups	10	16366.2197	1636.6220		
Total	12	86255.9585			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 28.6061 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.15

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	Grp 3	Grp 2	Grp 1
225.8060	Grp 3			
296.7736	Grp 2	*		
410.3218	Grp 1	*	*	

- - - - - O N E W A Y - - - - -

Variable AREA10D Area (mm2) of U1 mycelium after 10d
By Variable TREATMNT 20 ul direct addition at t=0

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	2	457455.4129	228727.7064	18.7635	.0004
Within Groups	10	121900.3102	12190.0310		
Total	12	579355.7231			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 78.0706 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.15

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	G r p 3	G r p 2	G r p 1
969.8905	Grp 3			
1130.3203	Grp 2			
1434.8358	Grp 1	*	*	

APPENDICES

APPENDIX X

Reduction of *Agaricus bisporus* Horst U1 mycelial growth in the presence of volatile metabolites extracted from *Trichoderma harzianum* culture broth (Table 10)

Mac Daddy:Desktop Folder:Documents:MSc:stats:U1vs1mlFacingMeOHMEBTh1Th4

	treatmnt	area10d	area30d
1	control	116	581
2	control	90	703
3	control	123	574
4	control	142	510
5	control	110	561
6	1ml MeOH	103	600
7	1ml MeOH	90	.
8	1ml MeOH	110	555
9	1ml MeOH	84	523
10	1ml MEB	116	497
11	1ml MEB	116	510
12	1ml MEB	129	568
13	1ml MEB	110	.
14	1ml MEB	123	529
15	1ml Th1	45	258
16	1ml Th1	26	232
17	1ml Th1	45	290
18	1ml Th1	52	277
19	1ml Th4	26	361
20	1ml Th4	26	206
21	1ml Th4	26	361
22	1ml Th4	26	258

- - - - - O N E W A Y - - - - -

Variable AREA10D area (mm2) of U1 mycelium after 10 days
By Variable TREATMNT

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	16298.6977	5432.8992	31.3980	.0000
Within Groups	14	2422.4669	173.0333		
Total	17	18721.1646			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 9.3014 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.03

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	Grp 4	Grp 2	Grp 1	Grp 3
		4	2	1	3
41.9354	Grp 4				
96.7740	Grp 2	*			
116.1288	Grp 1	*	*		
118.7094	Grp 3	*	*		

- - - - - O N E W A Y - - - - -

Variable AREA30D area (mm2) of U1 mycelium after 30 days
By Variable TREATMNT

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	268408.2222	89469.4074	38.1307	.0000
Within Groups	12	28156.6685	2346.3890		
Total	15	296564.8907			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 34.2519 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.08

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	Grp 4	Grp 3	Grp 2	Grp 1
264.5156	Grp 4				
525.8054	Grp 3	*			
559.1387	Grp 2	*			
585.8053	Grp 1	*			

APPENDIX XI

Zone of fungal growth inhibition caused by bibulous paper disks impregnated with diffusable metabolites extracted from *Trichoderma harzianum* culture broth and placed on a fresh, uniform lawn of *Agaricus bisporus* Horst U1 inoculum (Table 11)

StatsMSc:U1vs50ulTh1Th4ondisc

	treatmnt	zone10d
1	1	.00
2	1	.00
3	1	.00
4	1	.00
5	2	.00
6	2	.00
7	2	.00
8	2	.00
9	2	.00
10	2	.00
11	2	.00
12	2	.00
13	2	.00
14	2	.00
15	3	54.40
16	3	57.60
17	3	44.80
18	3	52.40
19	3	57.20
20	3	49.80
21	3	56.30
22	3	55.70
23	3	52.90
24	3	57.40
25	4	20.70
26	4	16.80
27	4	17.20
28	4	17.80
29	4	23.40
30	4	20.20
31	4	17.90
32	4	19.60
33	4	19.00
34	4	21.80

1 Mon
2 MEP
3 30d Th1
4 30d Th4

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

ZONE10D Diam of inhib Zone (mm) of U1 mycelium i
by TREATMNT 50 ul on disc

Mean Rank	Cases	
7.50	4	TREATMNT = 1 MeOH
7.50	10	TREATMNT = 2 MEB extract
	--	
	14	Total

		Exact		Corrected for ties
U	W	2-Tailed P	Z	2-Tailed P
20.0	30.0	1.0000	.0000	1.0000

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

ZONE10D Diam of inhib Zone (mm) of U1 mycelium i
by TREATMNT 50 ul on disc

Mean Rank	Cases	
2.50	4	TREATMNT = 1 MeOH
9.50	10	TREATMNT = 3 30d Th1 extract
	--	
	14	Total

		Exact	Corrected for ties	
U	W	2-Tailed P	Z	2-Tailed P
.0	10.0	.0020	-2.8600	.0042

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

ZONE10D Diam of inhib Zone (mm) of U1 mycelium i
by TREATMNT 50 ul on disc

Mean Rank	Cases	
2.50	4	TREATMNT = 1 MeOH
9.50	10	TREATMNT = 4 30d Th4 extract
	--	
	14	Total

		Exact		Corrected for ties
U	W	2-Tailed P	Z	2-Tailed P
.0	10.0	.0020	-2.8600	.0042

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

ZONE10D Diam of inhib Zone (mm) of U1 mycelium i
by TREATMNT 50 ul on disc

Mean Rank	Cases	
5.50	10	TREATMNT = 2 MEB extract
15.50	10	TREATMNT = 3 30d Th1 extract
	--	
	20	Total

		Exact		Corrected for ties
U	W	2-Tailed P	Z	2-Tailed P
.0	55.0	.0000	-4.0384	.0001

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

ZONE10D Diam of inhib Zone (mm) of U1 mycelium i
by TREATMNT 50 ul on disc

Mean Rank	Cases	
5.50	10	TREATMNT = 2 MEB extract
15.50	10	TREATMNT = 4 30d Th4 extract
	--	
	20	Total

		Exact		Corrected for ties
U	W	2-Tailed P	Z	2-Tailed P
.0	55.0	.0000	-4.0384	.0001

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

ZONE10D Diam of inhib Zone (mm) of U1 mycelium i
by TREATMNT 50 ul on disc

Mean Rank	Cases	
15.50	10	TREATMNT = 3 30d Th1 extract
5.50	10	TREATMNT = 4 30d Th4 extract
	--	
	20	Total

		Exact		Corrected for ties
U	W	2-Tailed P	Z	2-Tailed P
.0	155.0	.0000	-3.7796	.0002

APPENDIX XII

Zone of fungal growth inhibition caused by bibulous paper disks impregnated with diffusable metabolites extracted from Th4 culture broth and placed on a fresh, uniform lawn of *Agaricus bisporus* Sylvan 130 or Sylvan SB65 inoculum (Table 12)

StatsMSc:sb65and130vsTh4extract

	abstrain	ul10	ul20	ul30	ul40	ul50
1	1	20.00	30.00	28.00	44.00	47.00
2	1	21.00	26.00	30.00	44.00	49.00
3	1	23.00	24.00	33.00	48.00	50.00
4	1	25.00	22.00	36.00	45.00	48.00
5	1	27.00	26.00	36.00	43.00	46.00
6	2	19.00	24.00	30.00	37.00	34.00
7	2	16.00	20.00	28.00	35.00	35.00
8	2	15.00	18.00	23.00	31.00	40.00
9	2	16.00	18.00	24.00	29.00	37.00
10	2	18.00	21.00	26.00	29.00	30.00
11	.	.	.	.	.	.
12	.	.	.	.	.	.
13	.	.	.	.	.	.
14	.	.	.	.	.	.
15	.	.	.	.	.	.
16	.	.	.	.	.	.
17	.	.	.	.	.	.
18	.	.	.	.	.	.
19	.	.	.	.	.	.
20	.	.	.	.	.	.
21	.	.	.	.	.	.
22	.	.	.	.	.	.
23	.	.	.	.	.	.
24	.	.	.	.	.	.
25	.	.	.	.	.	.
26	.	.	.	.	.	.
27	.	.	.	.	.	.
28	.	.	.	.	.	.
29	.	.	.	.	.	.
30	.	.	.	.	.	.
31	.	.	.	.	.	.
32	.	.	.	.	.	.
33	.	.	.	.	.	.

23.2 25.6 32.6 44.8 48

16.8 20.2 1-1 26.2 32.2 35.2

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

UL10 ul of Th4 crude
by ABSTRAIN Agaricus strain

Mean Rank	Cases	
8.00	5	ABSTRAIN = 1 130
3.00	5	ABSTRAIN = 2 sb65
	--	
	10	Total

		Exact		Corrected for ties
U	W	2-Tailed P	Z	2-Tailed P
.0	40.0	.0079	-2.6191	.0088

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

UL20 ul of Th4 extract
by ABSTRAIN Agaricus strain

Mean Rank	Cases	
7.70	5	ABSTRAIN = 1 130
3.30	5	ABSTRAIN = 2 sb65
	--	
	10	Total

		Exact		Corrected for ties
U	W	2-Tailed P	Z	2-Tailed P
1.5	38.5	.0159	-2.3190	.0204

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

UL30 ul of Th4 extract
by ABSTRAIN Agaricus strain

Mean Rank	Cases	
7.60	5	ABSTRAIN = 1 130
3.40	5	ABSTRAIN = 2 sb65
	10	Total

		Exact	Corrected for ties	
U	W	2-Tailed P	Z	2-Tailed P
2.0	38.0	.0317	-2.2136	.0269

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

UL40 ul of th4 extract
by ABSTRAIN Agaricus strain

Mean Rank	Cases	
8.00	5	ABSTRAIN = 1 130
3.00	5	ABSTRAIN = 2 sb65
	10	Total

		Exact	Corrected for ties	
U	W	2-Tailed P	Z	2-Tailed P
.0	40.0	.0079	-2.6271	.0086

- - - - - Mann-Whitney U - Wilcoxon Rank Sum W Test

UL50 ul of Th4 extract
by ABSTRAIN Agaricus strain

Mean Rank	Cases	
8.00	5	ABSTRAIN = 1 130
3.00	5	ABSTRAIN = 2 sb65
	10	Total

		Exact	Corrected for ties	
U	W	2-Tailed P	Z	2-Tailed P
.0	40.0	.0079	-2.6112	.0090

APPENDIX XIII

Zone of *Agaricus bisporus* Sylvan 130 growth inhibition caused by bibulous paper disks impregnated with metabolites extracted from *Trichoderma harzianum* culture broth (Table 13)

Mac Daddy:Desktop Folder:Documents:MSc:stats:130CrudePureOther

	treatmnt	zone10d
1	crude	18.00
2	crude	16.70
3	crude	23.70
4	crude	22.40
5	crude	22.80
6	crude	22.20
7	crude	22.30
8	crude	22.00
9	crude	17.80
10	crude	21.70
11	pure	14.10
12	pure	19.80
13	pure	17.50
14	pure	15.00
15	pure	15.30
16	pure	13.00
17	pure	16.50
18	pure	13.60
19	pure	13.70
20	pure	20.80
21	other	20.20
22	other	21.70
23	other	23.40
24	other	27.10
25	other	25.00
26	other	22.70
27	other	22.30
28	other	15.40
29	other	20.00
30	other	24.70
31	.	.
32	.	.
33	.	.
34	.	.
35	.	.
36	.	.
37	.	.
38	.	.
39	.	.
40	.	.
41	.	.

Mean	20.9600	22.2500

APPENDIX XIV

Zone of *Agaricus bisporus* Horst U1 growth inhibition caused by bibulous paper disks impregnated with the major metabolite associated with green-mould infection (R_F 0.56), crude metabolites extracted from *Trichoderma harzianum* Th4 MEB culture, the major chromophore found in MEB cultures (R_F 0.79), or all metabolites present in crude Th4 extracts other than R_F 0.79 (Table 14)

Mac Daddy:Desktop Folder:Documents:MSc:stats:U1discCrudePureCompostOther

	treatmnt	zone10d
1	crude Th	25.50
2	crude Th	20.60
3	crude Th	26.00
4	0.79 i.v	12.70
5	0.79 i.v	12.50
6	0.79 i.v	18.70
7	0.56 com	19.60
8	0.56 com	13.00
9	0.56 com	17.10
10	non-spot	22.40
11	non-spot	16.80
12	non-spot	26.40
13	.	.
14	.	.
15	.	.
16	.	.
17	.	.
18	.	.
19	.	.
20	.	.
21	.	.
22	.	.
23	.	.
24	.	.
25	.	.
26	.	.
27	.	.
28	.	.
29	.	.
30	.	.
31	.	.
32	.	.
33	.	.
34	.	.
35	.	.
36	.	.
37	.	.
38	.	.
39	.	.
40	.	.
41	.	.

- - - - - O N E W A Y - - - - -

Variable ZONE10D diam of inhib zone in U1 lawn at 10d
By Variable TREATMNT 50 ul addition to disk

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	3	174.7158	58.2386	4.1843	.0468
Within Groups	8	111.3467	13.9183		
Total	11	286.0625			

Multiple Range Tests: LSD test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 2.6380 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 3.26

(*) Indicates significant differences which are shown in the lower triangle

Mean	TREATMNT	Grp 2	Grp 3	Grp 4	Grp 1
14.6333	Grp 2				
16.5667	Grp 3				
21.8667	Grp 4	*			
24.0333	Grp 1	*	*		

Homogeneous Subsets (highest and lowest means are not significantly different)

Subset 1

Group	Grp 2	Grp 3
Mean	14.6333	16.5667

- - - - - - - - - - - - - - - - - -

Subset 2

Group	Grp 3	Grp 4
Mean	16.5667	21.8667

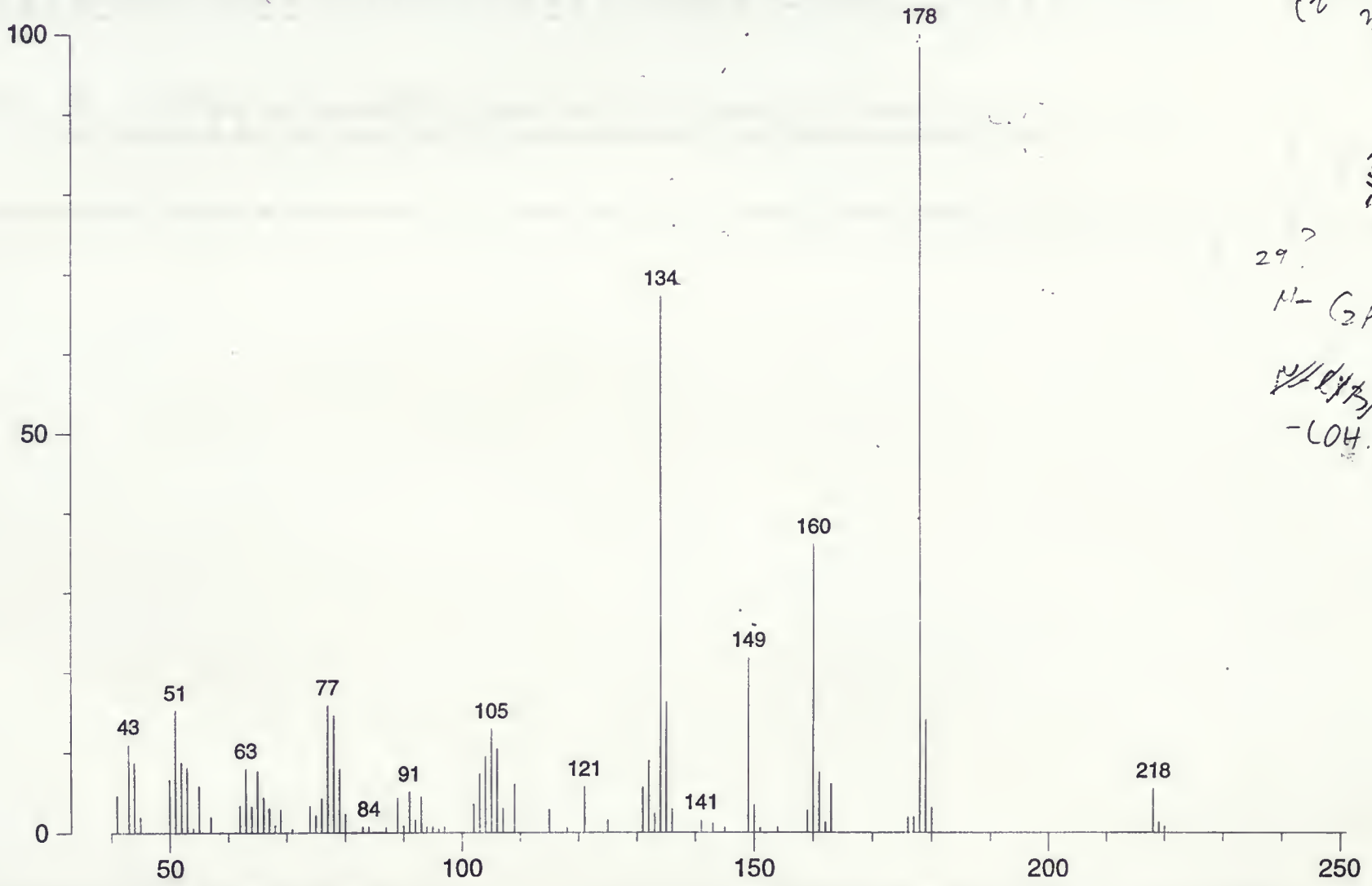
Subset 3

Group	Grp 4	Grp 1
Mean	21.8667	24.0333

APPENDIX XV

EI-Mass Spectra – Crude 30 d Th4 culture in MEA

0819ms0009 Scan 1 (Av 3-5 Acq) 100%=4739 mv 19-Aug-1999 15:49
LRP +EI Holland/Unknown



19ms0009 Scan 1 (Av 3-5 Acq) +EI LRP

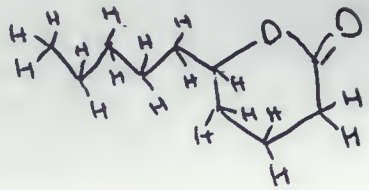
rmalised to largest peak in current list
eraged Nominal data

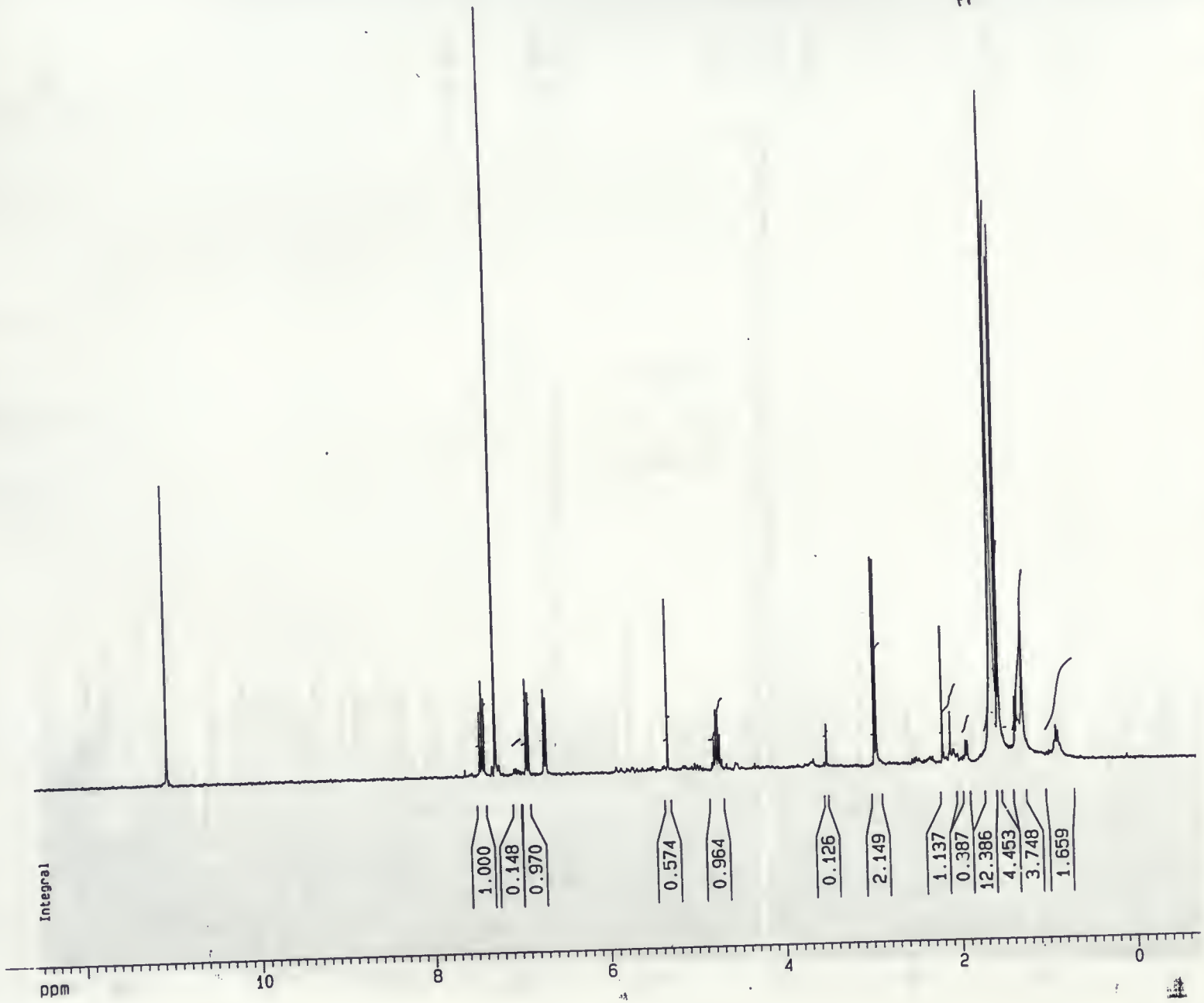
ak	Mass	Intensity	%	Flags
4	41	23006	4.6	N
6	43	54742	11.0	N
7	44	43423	8.7	N
8	45	10110	2.0	N
13	50	32851	6.6	N
14	51	75718	15.2	N
15	52	43804	8.8	N
16	53	40297	8.1	N
18	55	28998	5.8	N
20	57	9883	2.0	N
25	62	16795	3.4	N
26	63	39732	8.0	N
27	64	16386	3.3	N
28	65	38049	7.7	N
29	66	21804	4.4	N
30	67	15200	3.1	N
32	69	14749	3.0	N
37	74	16377	3.3	N
38	75	10862	2.2	N
39	76	21119	4.3	N
40	77	78839	15.9	N
41	78	72697	14.6	N
42	79	39453	7.9	N
43	80	11619	2.3	N
52	89	21643	4.4	N
54	91	25462	5.1	N
55	92	8034	1.6	N
56	93	22690	4.6	N
65	102	18034	3.6	N
66	103	36875	7.4	N
67	104	47170	9.5	N
68	105	64399	13.0	N
69	106	52490	10.6	N
70	107	15225	3.1	N
72	109	30022	6.0	N
78	115	14687	3.0	N
84	121	28723	5.8	N
88	125	8418	1.7	N
94	131	28322	5.7	N
95	132	44678	9.0	N
96	133	11974	2.4	N
97	134	334354	67.3	N
98	135	81432	16.4	N
99	136	15019	3.0	N
.04	141	7828	1.6	N
.06	143	6123	1.2	N
.12	149	108587	21.9	N
.13	150	17479	3.5	N
.22	159	13831	2.8	N
.23	160	179390	36.1	N
.24	161	37520	7.6	N
.25	162	6580	1.3	N

APPENDIX XVI

^{1}H-NMR – Crude 30 d Th4 culture in MEA and Ultraviolet absorbance spectra of chromophores isolated from extracts of green-mould infected compost *Trichoderma harzianum* Th4 in MEB culture and Th4 in co-culture with *Agaricus bisporus*

Brock DPX300
QSIM





Current Data Parameters

NAME	oliver
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	990813
Time	13.31
INSTRUM	dpx300
PROBHD	5 mm QNP 1H
PULPROG	zg30
TD	16384
SOLVENT	CDCl3
NS	16
DS	2
SWH	6172.839 Hz
FIDRES	0.376760 Hz
AQ	1.3271540 sec
RG	1448.2
DW	81.000 usec
DE	4.50 usec
TE	300.0 K
D1	1.00000000 sec

========== CHANNEL f1 =========

NUC1	1H
P1	5.30 usec
PL1	-4.00 dB
SFO1	300.1318534 MHz

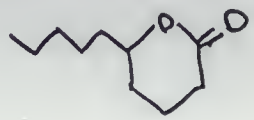
F2 - Processing parameters

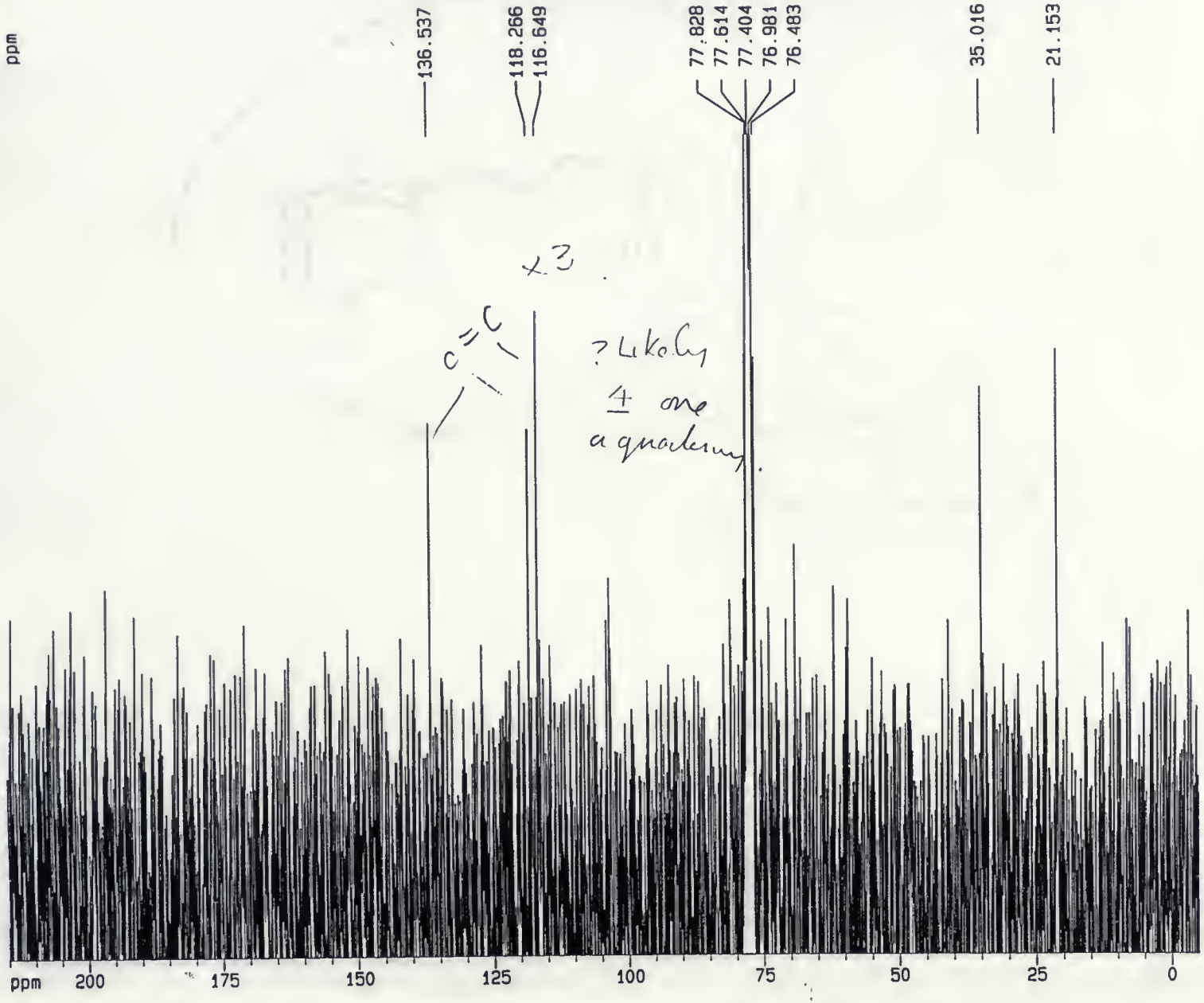
SI	8192
SF	300.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	20.00 cm
F1P	12.567 ppm
F1	3771.62 Hz
F2P	-0.696 ppm
F2	-208.99 Hz
PPMCM	0.66315 ppm/cm
HZCM	199.03082 Hz/cm

Brock DPX300
QSIM





Current Data Parameters

NAME	oliver
EXPNO	2
PROCNO	1

F2 - Acquisition Parameters

Date_	990813
Time	14.13
INSTRUM	dpx300
PROBHD	5 mm QNP 1H
PULPROG	zgdc30
TD	32768
SOLVENT	CDCl3
NS	1337
DS	2
SWH	23809.523 Hz
FIDRES	0.726609 Hz
AQ	0.6881780 sec
RG	8192
DW	21.000 usec
DE	4.50 usec
TE	300.0 K
D1	1.00000000 sec
D11	0.03000000 sec

---------- CHANNEL f1 ----------

NUC1	13C
P1	4.70 usec
PL1	-4.00 dB
SFO1	75.4772501 MHz

---------- CHANNEL f2 ----------

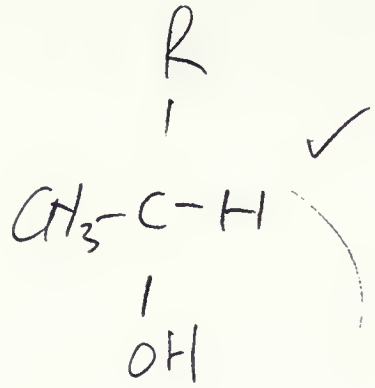
CPDPRG2	waltz16
NUC2	1H
PCPD2	81.00 usec
PL2	-4.00 dB
PL12	22.00 dB
SFO2	300.1312005 MHz

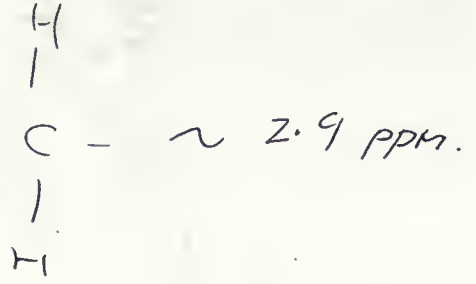
F2 - Processing parameters

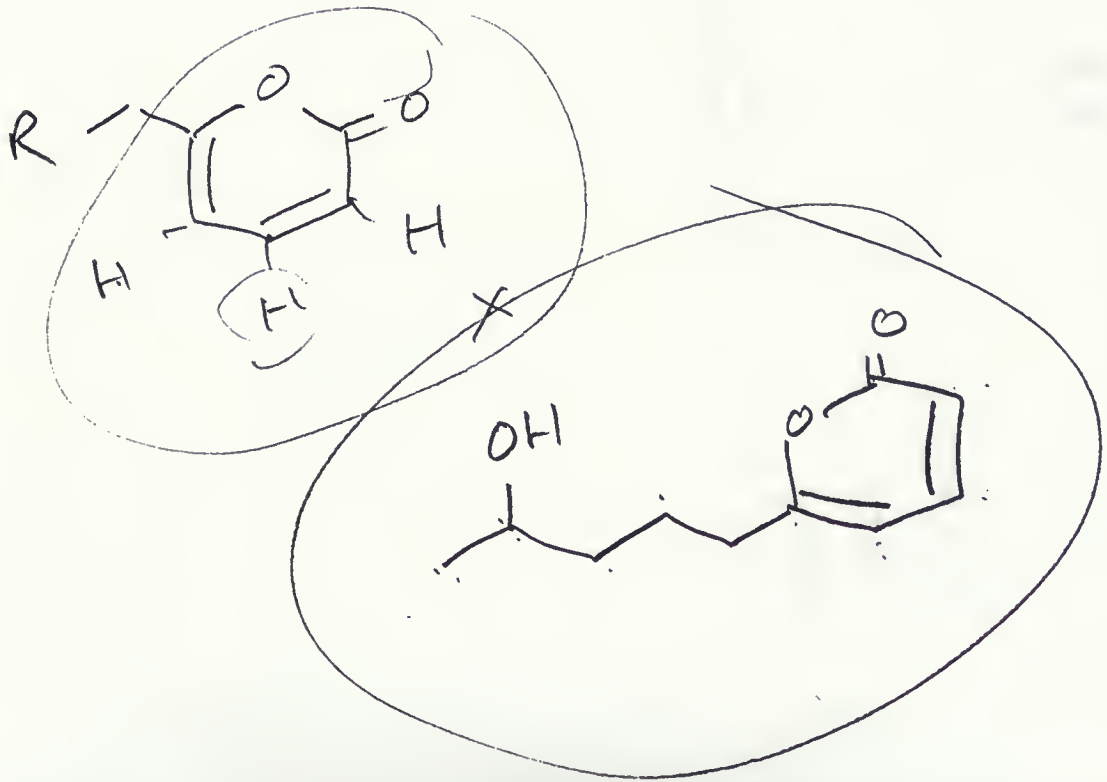
SI	16384
SF	75.4677190 MHz
WDW	EM
SSB	0
LB	2.00 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	215.000 ppm
F1	16225.56 Hz
F2P	-5.000 ppm
F2	-377.34 Hz
PPMCM	11.00000 ppm/cm
HZCM	830.14490 Hz/cm




~ 2.9 ppm.

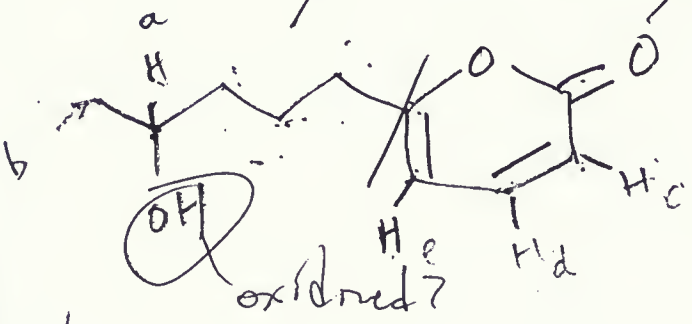


$C_5H_{10}O$ = 87

60 + 11 + 16

$C_5H_3O_2$

60 + 3 + 32 = 95



6

$C_{10}H_{14}O_3$

120 + 14 + 48.

182 ~ 178

166

purify + isolate + structure

correlate peak to spot

GC/MS + NMR

take weights + on column

250mL SCM → purify

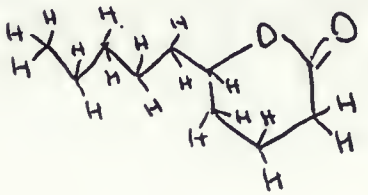
125mL SCM →

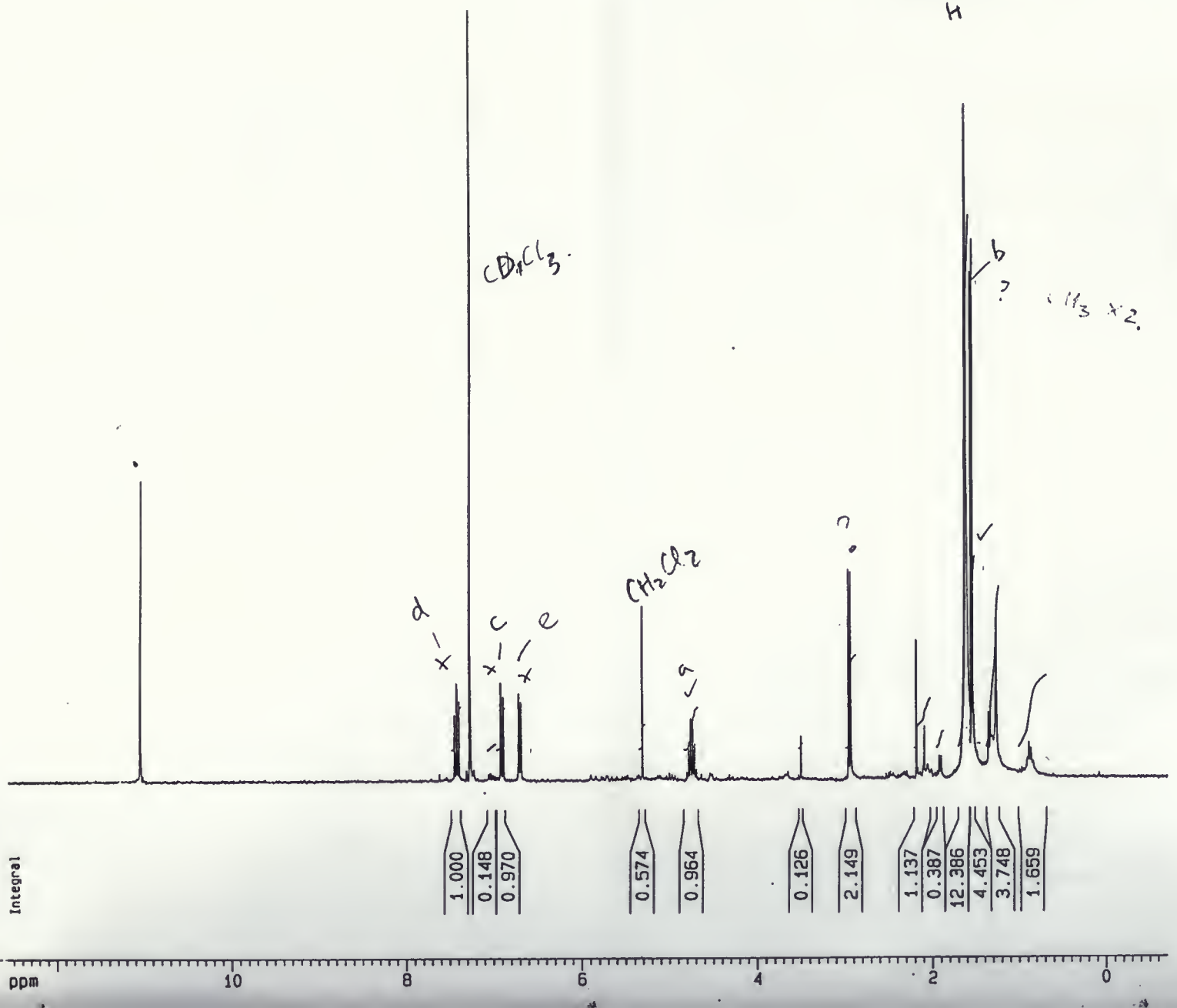
125mL SCM →

166

C_3H_3 $5 \times 12 = 60$

Brock DPX300
QSIM





Current Data Parameters

NAME	oliver
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	990813
Time	13.31
INSTRUM	dpx300
PROBHD	5 mm QNP 1H
PULPROG	zg30
TD	16384
SOLVENT	CDCl3
NS	16
DS	2
SWH	6172.839 Hz
FIDRES	0.376760 Hz
AQ	1.3271540 sec
RG	1448.2
DW	81.000 usec
DE	4.50 usec
TE	300.0 K
D1	1.00000000 sec

---------- CHANNEL f1 ---------

NUC1	1H
P1	5.30 usec
PL1	-4.00 dB
SFO1	300.1318534 MHz

F2 - Processing parameters

SI	8192
SF	300.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	20.00 cm
F1P	12.567 ppm
F1	3771.62 Hz
F2P	-0.696 ppm
F2	-208.99 Hz
PPMCM	0.66315 ppm/cm
HZCM	199.03082 Hz/cm

MEA

n=5

add 255.0

Date: 02/22/C
Time: 12:11

Method name: A:\DEFAULT
Autosave name: [A:\]fraft
Sampling device: None
Read average time: 0.50 sec

A:\WORK_001 A:\WORK_002 A:\WORK_003 A:\WORK_004 A:\WORK_005

Functions: Scan

5.0000

[Abs]

225.0 375.

λc240
↓
solvent
effects

old THV + A.L ...
... than 99.1
0.56 . 0.79

full 2peaks.
N280 -> comp.
713.

BioTek ... 7000

Date: 02/23/0
Time: 10:2

Header Files Templates Variables Control Tests Methods Save/Clear Print Quit

User Λ: Custom: VIEW
Scan: λ: 235 nm
End λ: 385 nm
Overlay scans: [Yes]

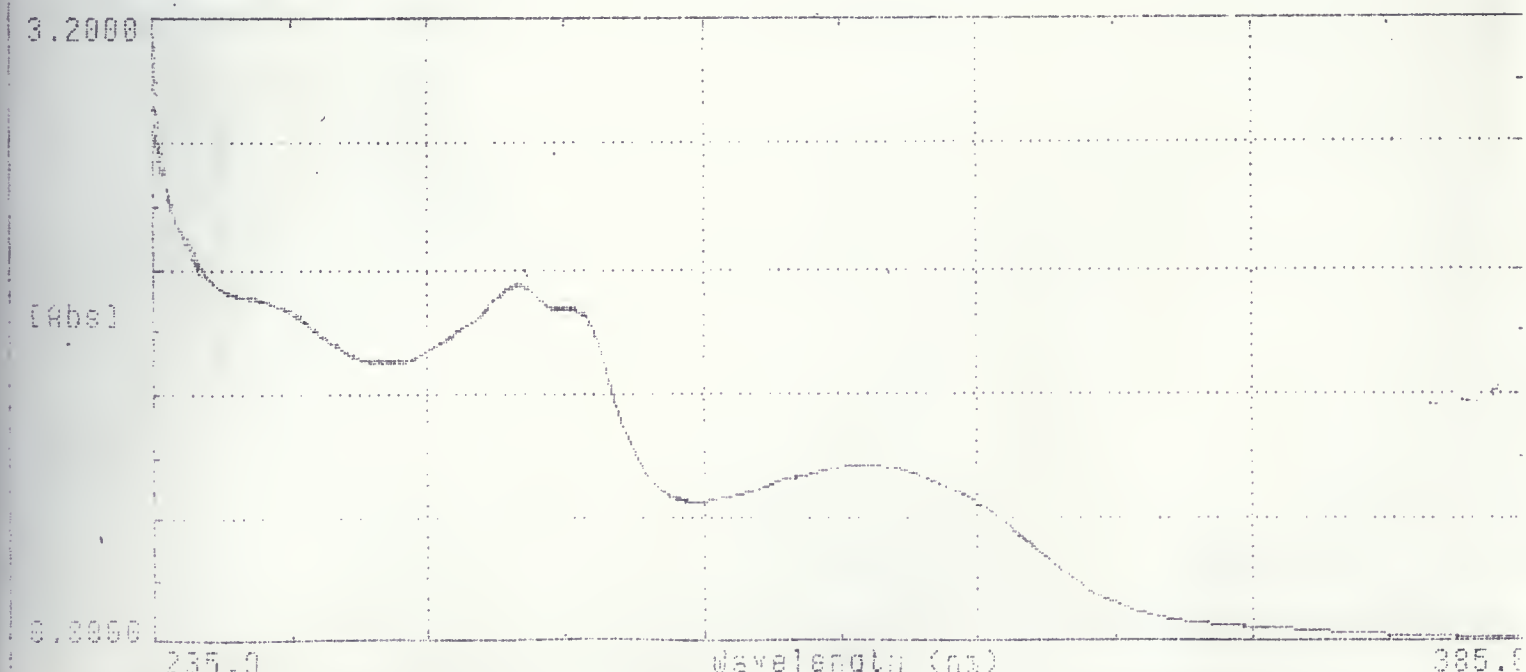
Autoprint: [No]
Autosave: [No]
Scans per sample: 5
Interval: 10.00 [sec]

Method name: A:\DEFAULT
Autosave name: A:\Default
Sampling device: None
Read average time: 0.50 sec

A:\WORK_001 A:\WORK_002 A:\WORK_003 A:\WORK_004 A:\WORK_005

Zoom ZoomOut Trace Function Autoscale Annotate Prin

Functions: Scan



very dilute - compact spot

n = 5

BECKMAN DU-7000

Date: 02/25/(
Time: 12:26

odd 255.0

Read Base Tabulate +-*Scans Scatter NetA Method SaveClear Print Qu

Scan Sequence: VIEW
Start λ: 235 nm
End λ: 385 nm
Overlay scans: [Yes]

Autoprint: [No]
Autosave: [No]
Scans per sample: 5
Interval: 10.00 [sec]

Method name: A:\DEFAULT
Autosave name: [A:\ifraft
Sampling device: None
Read average time: 0.50 se

A:\WORK_001 A:\WORK_002 A:\WORK_003 A:\WORK_004 A:\WORK_005

Zoom ZoomOut Trace Function Autoscale Annotate Pri

Functions: Scan

5.0000

0.0000

235

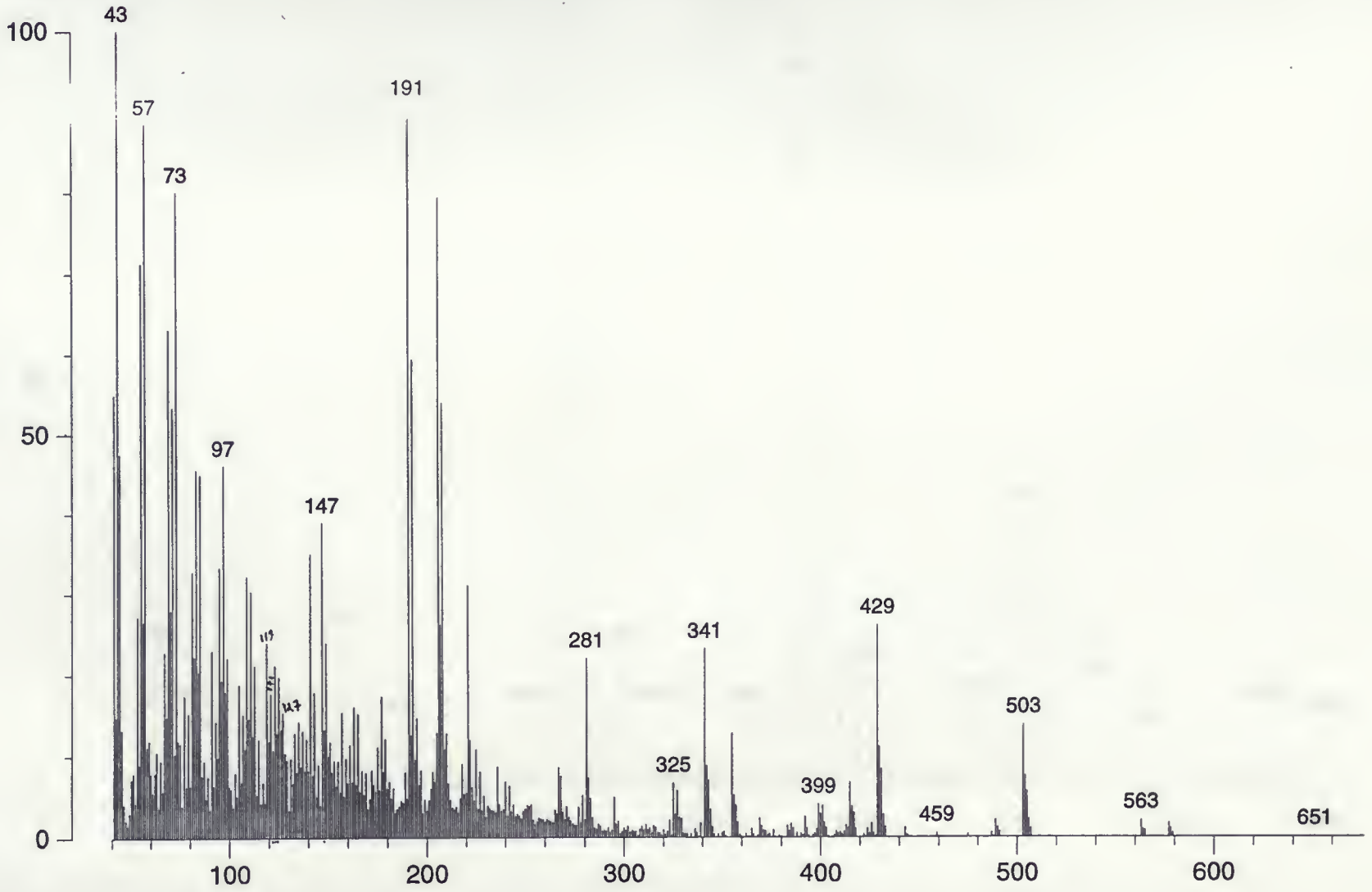
Wavelength (nm)

385.

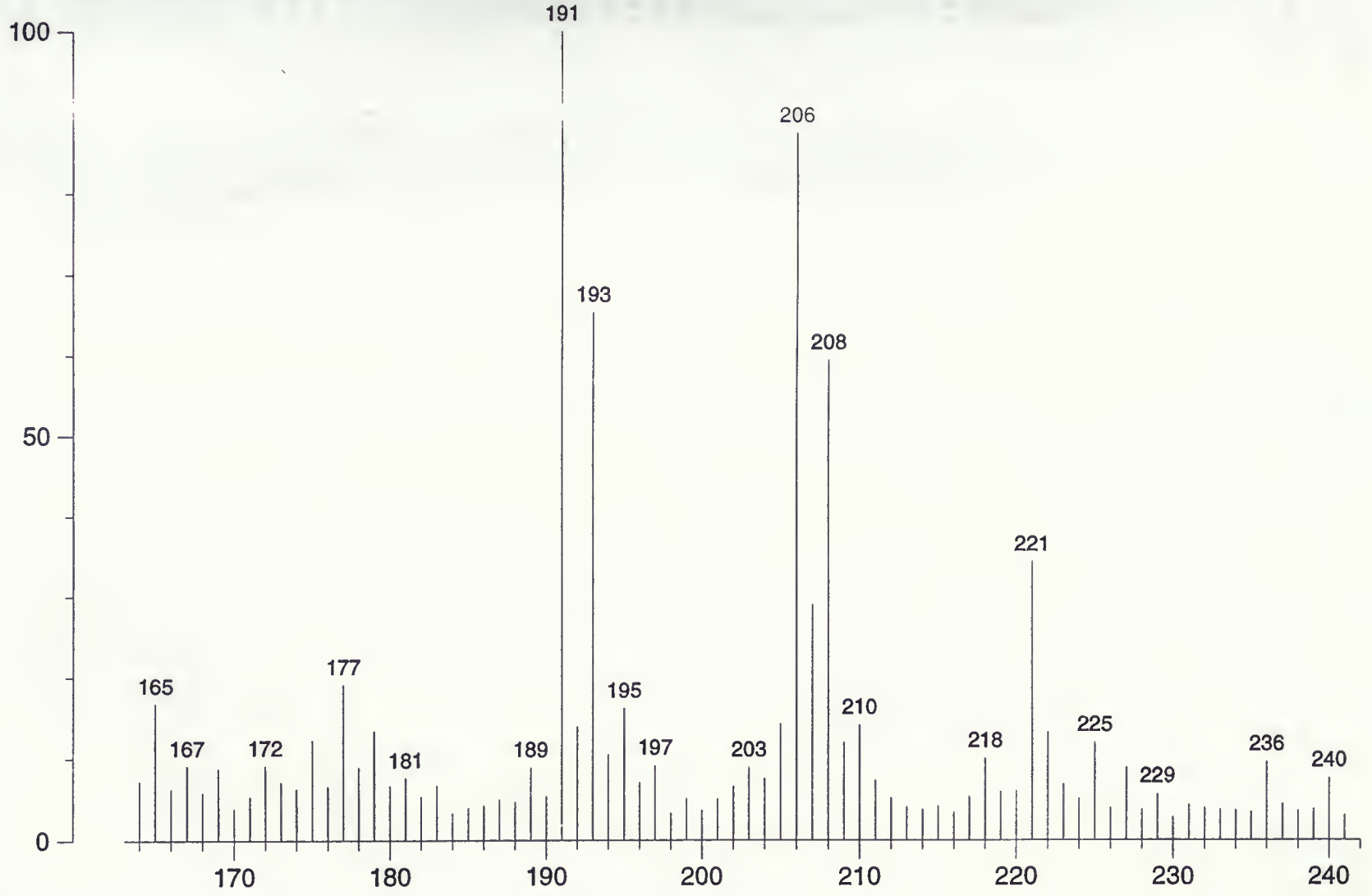
APPENDIX XVII

EI-Mass Spectra – 'Purified' R_F 0.56 (poor spectra due to impurities in solvents used and/or masking of analyte peak by solvent peak)

1217ms0017 Scan 1 (Av 11-17 Acq) 100%=12718 mv 17-Dec-1999 13:09
LRP +EI Oliver Krupke/PUHex unknown/184?



1217ms0017 Scan 1 (Av 11-17 Acq) 100%=11555 mv 17-Dec-1999 13:09
LRP +EI Oliver Krupke/PUHex unknown/184?



-1217ms0017 Scan 1 (Av 11-17 Acq) +EI LRP

Normalised to largest peak in current list
Averaged Nominal data

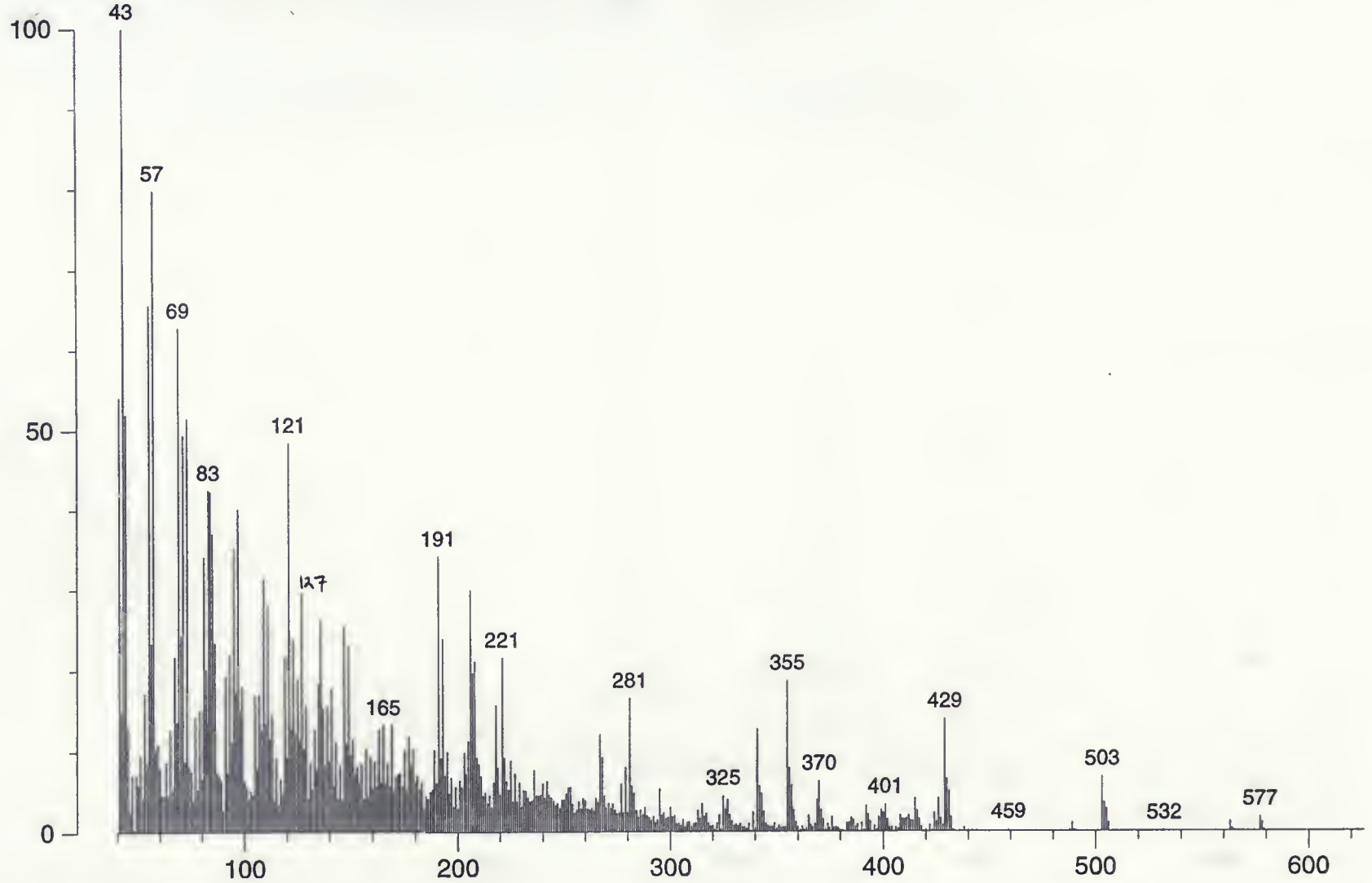
Peak	Mass	Intensity	%	Flags
122	159	129988	9.7	N
123	160	90502	6.8	N
124	161	151883	11.4	N
125	162	91470	6.9	N
126	163	214447	16.1	N
127	164	87502	6.6	N
128	165	202854	15.2	N
129	166	75886	5.7	N
130	167	109975	8.2	N
131	168	70851	5.3	N
132	169	106011	8.0	N
135	172	110393	8.3	N
136	173	86652	6.5	N
137	174	76640	5.7	N
138	175	148378	11.1	N
139	176	79405	6.0	N
140	177	231567	17.4	N
141	178	108180	8.1	N
142	179	162347	12.2	N
143	180	81190	6.1	N
144	181	92026	6.9	N
146	183	81613	6.1	N
152	189	107932	8.1	N
154	191	1211375	90.9	N
155	192	169060	12.7	N
156	193	791728	59.4	N
157	194	128057	9.6	N
158	195	196025	14.7	N
159	196	86659	6.5	N
160	197	110938	8.3	N
165	202	80629	6.0	N
166	203	107896	8.1	N
167	204	91708	6.9	N
168	205	172525	12.9	N
169	206	1059701	79.5	N
170	207	350648	26.3	N
171	208	719603	54.0	N
172	209	145812	10.9	N
173	210	171133	12.8	N
174	211	89392	6.7	N
181	218	121519	9.1	N
182	219	72055	5.4	N
183	220	73818	5.5	N
184	221	415398	31.2	N
185	222	160789	12.1	N
186	223	83173	6.2	N
188	225	144866	10.9	N
190	227	108309	8.1	N
192	229	68697	5.2	N
199	236	116516	8.7	N
203	240	92297	6.9	N
205	242	85416	6.4	N

1217ms0017 Scan 1 (Av 11-17 Acq) +EI LRP

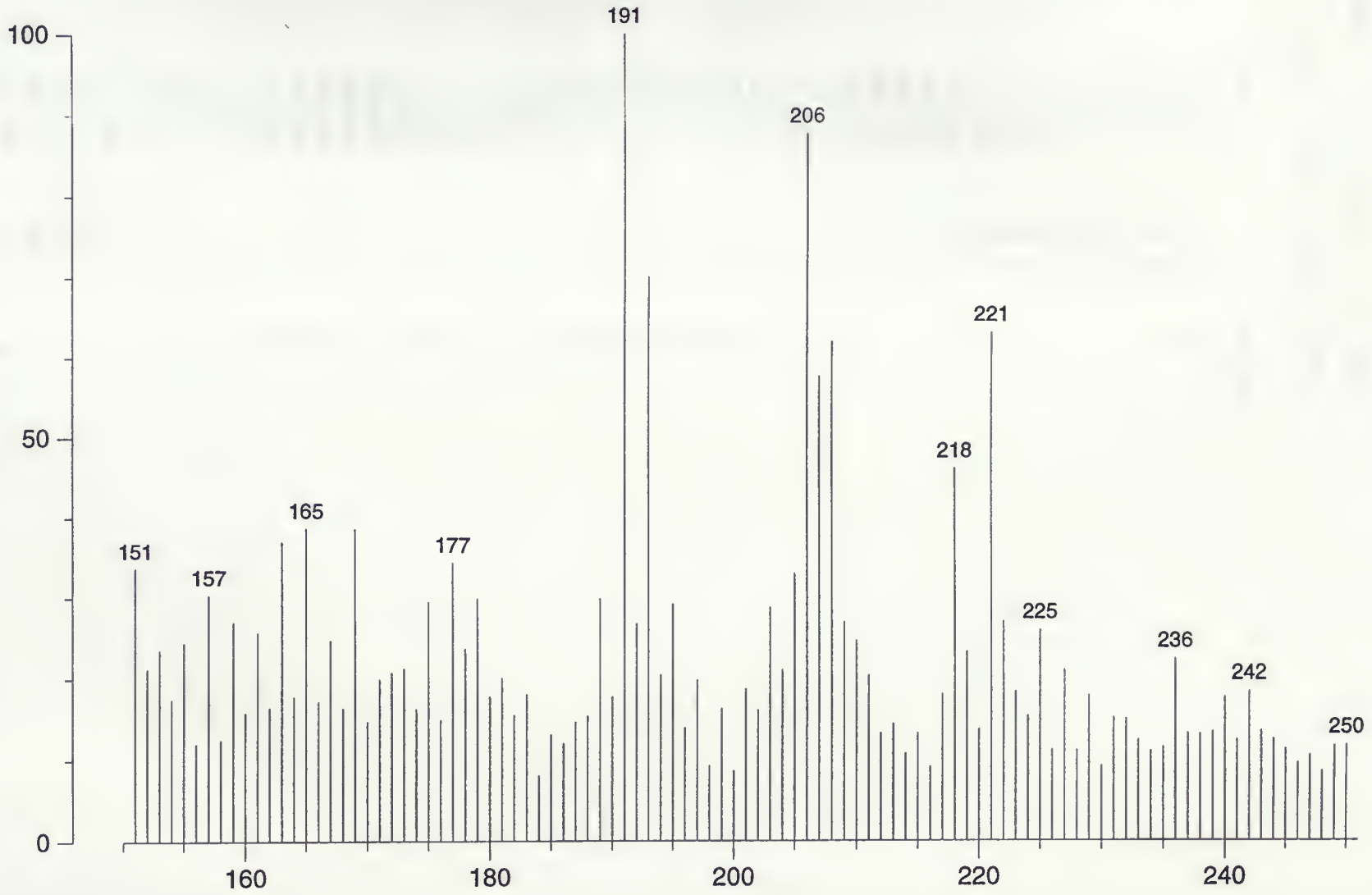
Normalised to largest peak in current list
Averaged Nominal data

Peak	Mass	Intensity	%	Flags
230	267	115634	8.7	N
231	268	101189	7.6	N
242	279	70137	5.3	N
244	281	294721	22.1	N
245	282	98731	7.4	N
258	295	66694	5.0	N
288	325	89518	6.7	N
290	327	77385	5.8	N
304	341	310406	23.3	N
305	342	118286	8.9	N
306	343	95414	7.2	N
318	355	170514	12.8	N
319	356	69634	5.2	N
378	415	90450	6.8	N
392	429	349365	26.2	N
393	430	149117	11.2	N
394	431	112960	8.5	N
466	503	185616	13.9	N
467	504	101936	7.6	N
468	505	77176	5.8	N

1217ms0019 Scan 1 (Av 8-16 Acq) 100%=9889 mv 17-Dec-1999 13:15
LRP +EI Oliver Krupke/sample#3/ unknown/184?



1217ms0019 Scan 1 (Av 8-16 Acq) 100%=3383 mv 17-Dec-1999 13:15
LRP +EI Oliver Krupke/sample#3/ unknown/184?



1217ms0019 Scan 1 (Av 8-16 Acq) +EI LRP

Normalised to largest peak in current list
Averaged Nominal data

Peak	Mass	Intensity	%	Flags
4	41	560696	54.1	N
5	42	153783	14.8	N
6	43	1036671	100.0	N
7	44	538701	52.0	N
8	45	133311	12.9	N
10	47	72940	7.0	N
12	49	73484	7.1	N
13	50	60387	5.8	N
14	51	97521	9.4	N
16	53	177384	17.1	N
17	54	86350	8.3	N
18	55	680111	65.6	N
19	56	242358	23.4	N
20	57	827785	79.9	N
21	58	105626	10.2	N
22	59	112739	10.9	N
23	60	81247	7.8	N
26	63	88537	8.5	N
28	65	130954	12.6	N
29	66	53951	5.2	N
30	67	225364	21.7	N
31	68	141942	13.7	N
32	69	651080	62.8	N
33	70	253006	24.4	N
34	71	512217	49.4	N
35	72	90336	8.7	N
36	73	534265	51.5	N
37	74	85188	8.2	N
38	75	77448	7.5	N
40	77	147387	14.2	N
41	78	54349	5.2	N
42	79	157215	15.2	N
43	80	68640	6.6	N
44	81	353586	34.1	N
45	82	209681	20.2	N
46	83	440909	42.5	N
47	84	439142	42.4	N
48	85	384546	37.1	N
49	86	243589	23.5	N
50	87	76449	7.4	N
51	88	72183	7.0	N
52	89	66610	6.4	N
54	91	199791	19.3	N
55	92	76200	7.4	N
56	93	227317	21.9	N
57	94	115164	11.1	N
58	95	366227	35.3	N
59	96	180285	17.4	N
60	97	416555	40.2	N
61	98	156008	15.0	N
62	99	186318	18.0	N
63	100	65751	6.3	N

1217ms0019 Scan 1 (Av 8-16 Acq) +EI LRP

Normalised to largest peak in current list
Averaged Nominal data

Peak	Mass	Intensity	%	Flags
64	101	57718	5.6	N
66	103	52298	5.0	N
68	105	175205	16.9	N
69	106	81013	7.8	N
70	107	176200	17.0	N
71	108	131447	12.7	N
72	109	325643	31.4	N
73	110	138999	13.4	N
74	111	292125	28.2	N
75	112	119149	11.5	N
76	113	150530	14.5	N
78	115	95026	9.2	N
80	117	68620	6.6	N
82	119	224577	21.7	N
83	120	95244	9.2	N
84	121	502821	48.5	N
85	122	132093	12.7	N
86	123	247683	23.9	N
87	124	125822	12.1	N
88	125	195740	18.9	N
89	126	116951	11.3	N
90	127	307134	29.6	N
91	128	106476	10.3	N
92	129	161429	15.6	N
94	131	88940	8.6	N
95	132	54524	5.3	N
96	133	131950	12.7	N
97	134	79534	7.7	N
98	135	189592	18.3	N
99	136	272900	26.3	N
100	137	157890	15.2	N
101	138	85637	8.3	N
102	139	161066	15.5	N
103	140	90019	8.7	N
104	141	183782	17.7	N
105	142	60067	5.8	N
106	143	115930	11.2	N
108	145	85308	8.2	N
110	147	263870	25.5	N
111	148	113400	10.9	N
112	149	238206	23.0	N
113	150	98521	9.5	N
114	151	119545	11.5	N
115	152	75361	7.3	N
116	153	83619	8.1	N
117	154	62180	6.0	N
118	155	86834	8.4	N
120	157	107664	10.4	N
122	159	95850	9.2	N
123	160	56358	5.4	N
124	161	91184	8.8	N
125	162	58417	5.6	N

1217ms0019 Scan 1 (Av 8-16 Acq) +EI LRP

Normalised to largest peak in current list
Averaged Nominal data

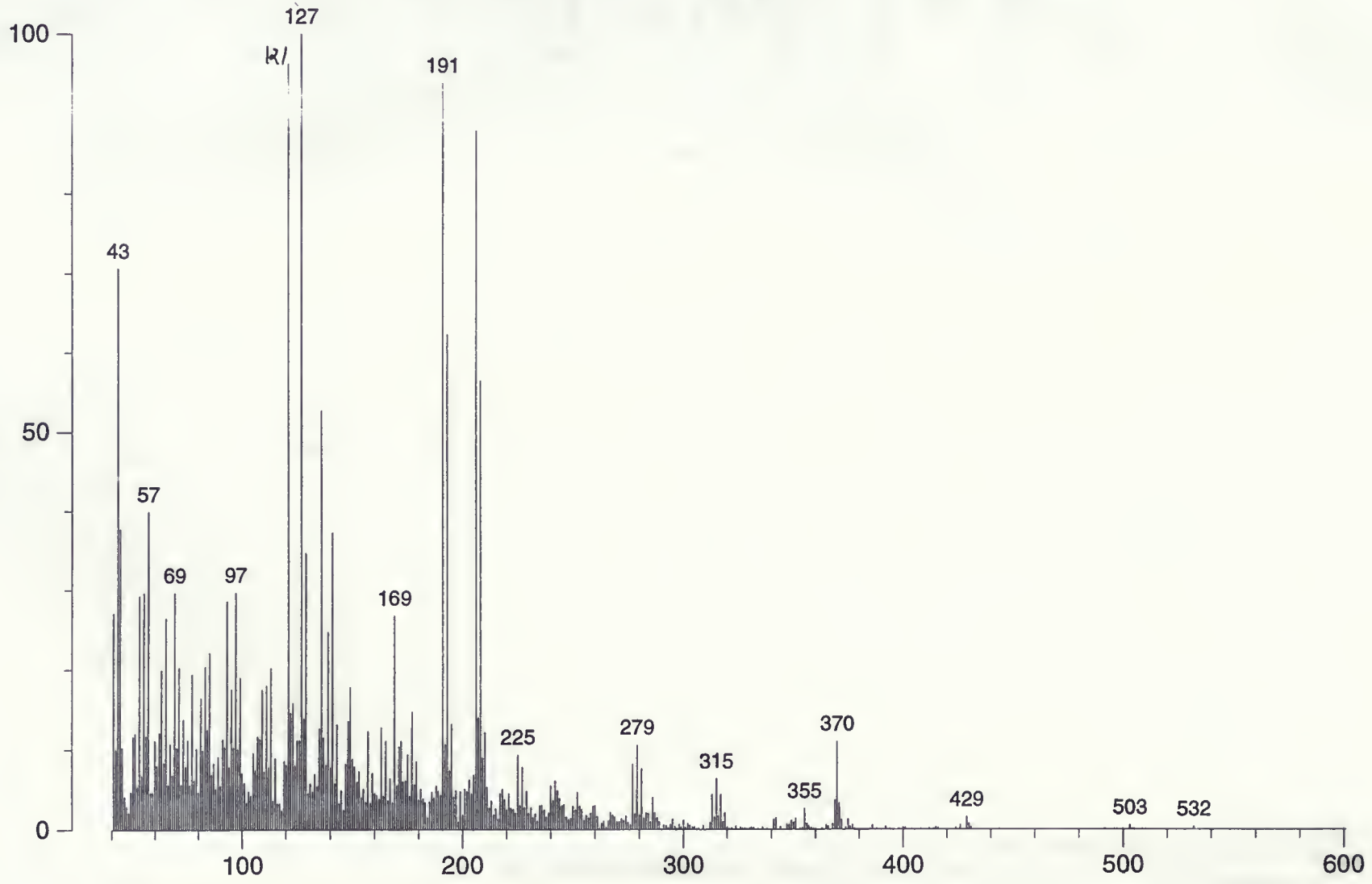
Peak	Mass	Intensity	%	Flags
126	163	131408	12.7	N
127	164	63250	6.1	N
128	165	137281	13.2	N
129	166	61295	5.9	N
130	167	87892	8.5	N
131	168	58182	5.6	N
132	169	137107	13.2	N
133	170	52331	5.0	N
134	171	70749	6.8	N
135	172	73866	7.1	N
136	173	75290	7.3	N
137	174	57829	5.6	N
138	175	104772	10.1	N
139	176	53066	5.1	N
140	177	121990	11.8	N
141	178	84146	8.1	N
142	179	106063	10.2	N
143	180	63214	6.1	N
144	181	71409	6.9	N
145	182	55170	5.3	N
146	183	64287	6.2	N
150	187	52158	5.0	N
151	188	54844	5.3	N
152	189	106045	10.2	N
153	190	63355	6.1	N
154	191	354696	34.2	N
155	192	95135	9.2	N
156	193	248677	24.0	N
157	194	72791	7.0	N
158	195	103462	10.0	N
160	197	70302	6.8	N
162	199	58002	5.6	N
164	201	66479	6.4	N
165	202	57368	5.5	N
166	203	102146	9.9	N
167	204	74677	7.2	N
168	205	116998	11.3	N
169	206	311058	30.0	N
170	207	204360	19.7	N
171	208	219577	21.2	N
172	209	95851	9.2	N
173	210	87551	8.4	N
174	211	72359	7.0	N
180	217	64201	6.2	N
181	218	163257	15.7	N
182	219	82656	8.0	N
184	221	223889	21.6	N
185	222	95755	9.2	N
186	223	65427	6.3	N
187	224	54631	5.3	N
188	225	92153	8.9	N
190	227	74880	7.2	N

1217ms0019 Scan 1 (Av 8-16 Acq) +EI LRP

Normalised to largest peak in current list
Averaged Nominal data

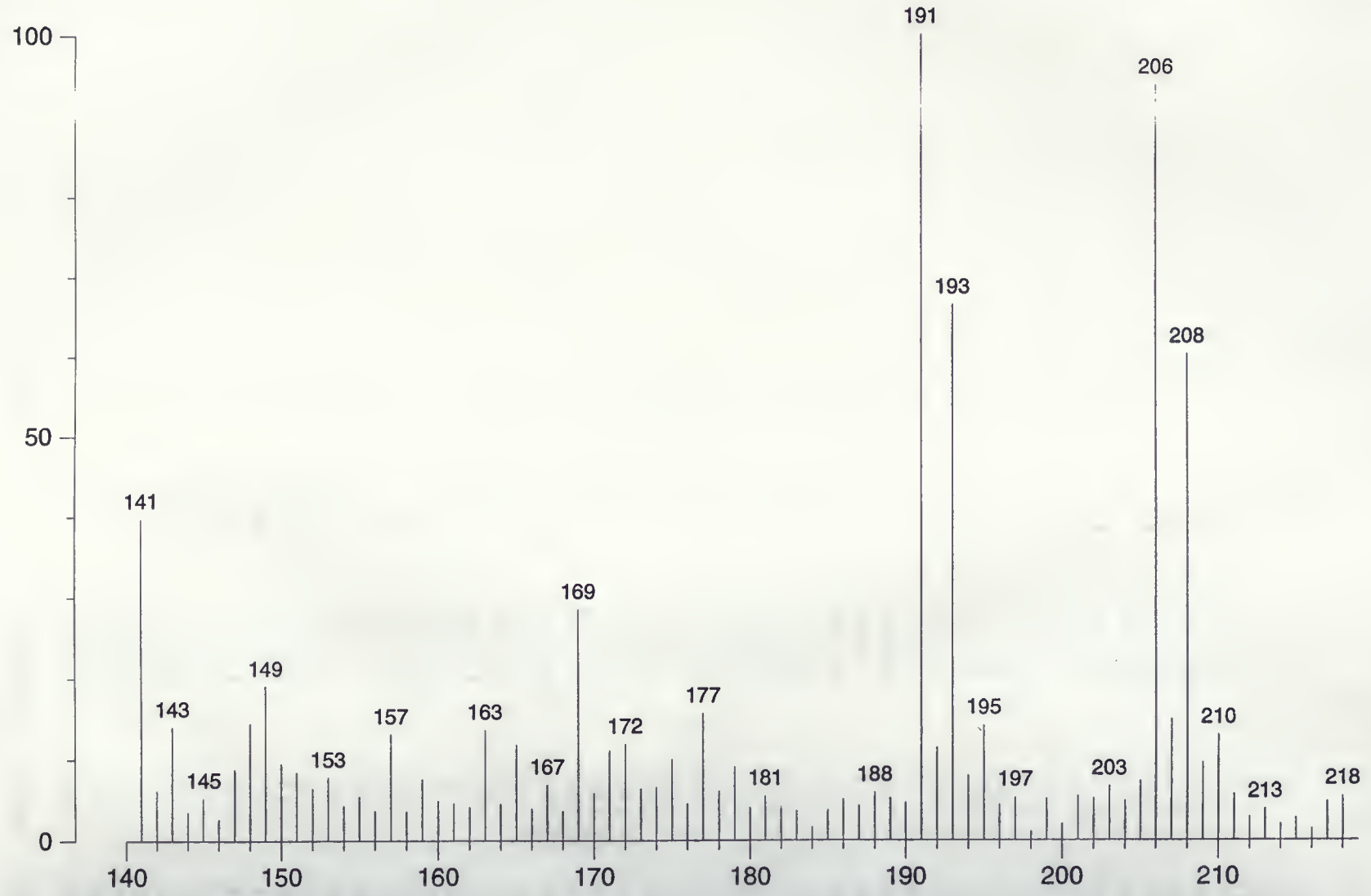
Peak	Mass	Intensity	%	Flags
192	229	63681	6.1	N
194	231	54026	5.2	N
195	232	53489	5.2	N
199	236	79543	7.7	N
203	240	62839	6.1	N
205	242	65413	6.3	N
215	252	57429	5.5	N
216	253	57823	5.6	N
230	267	125403	12.1	N
231	268	96085	9.3	N
240	277	61821	6.0	N
242	279	83026	8.0	N
244	281	171569	16.5	N
245	282	59107	5.7	N
258	295	55685	5.4	N
304	341	131821	12.7	N
305	342	58573	5.7	N
318	355	193864	18.7	N
319	356	81893	7.9	N
320	357	60405	5.8	N
333	370	65044	6.3	N
392	429	144647	14.0	N
393	430	68461	6.6	N
394	431	52258	5.0	N
466	503	70833	6.8	N

1217ms0018 Scan 1 (Av 10-16 Acq) 100%=14802 mv 17-Dec-1999 13:12
LRP +EI Oliver Krupke/sample#2/ unknown/184?



1217ms0018 Scan 1 (Av 10-16 Acq) 100%=13897 mv 17-Dec-1999 13:12
LRP +EI Oliver Krupke/sample#2/ unknown/184?

\just By pulex.



1217ms0018 Scan 1 (Av 10-16 Acq) +EI LRP

Normalised to largest peak in current list
Averaged Nominal data

Peak	Mass	Intensity	%	Flags
63	100	109972	7.1	N
64	101	92037	5.9	N
68	105	149227	9.6	N
69	106	115934	7.5	N
70	107	180939	11.7	N
71	108	176564	11.4	N
72	109	271436	17.5	N
73	110	113799	7.3	N
74	111	280612	18.1	N
75	112	121787	7.8	N
76	113	314086	20.2	N
78	115	139207	9.0	N
82	119	132984	8.6	N
83	120	126398	8.1	N
84	121	1494274	96.3	N
85	122	227607	14.7	N
86	123	246233	15.9	N
87	124	126202	8.1	N
88	125	173919	11.2	N
89	126	174070	11.2	N
90	127	1551765	100.0	N
91	128	216192	13.9	N
92	129	539587	34.8	N
94	131	89614	5.8	N
96	133	108279	7.0	N
97	134	85016	5.5	N
98	135	196760	12.7	N
99	136	818260	52.7	N
100	137	178454	11.5	N
101	138	126331	8.1	N
102	139	384123	24.8	N
103	140	121087	7.8	N
104	141	579244	37.3	N
105	142	90684	5.8	N
106	143	204230	13.2	N
110	147	127663	8.2	N
111	148	210520	13.6	N
112	149	277201	17.9	N
113	150	138097	8.9	N
114	151	123053	7.9	N
115	152	93976	6.1	N
116	153	113367	7.3	N
118	155	79673	5.1	N
120	157	190868	12.3	N
122	159	110441	7.1	N
126	163	198817	12.8	N
128	165	172855	11.1	N
130	167	100329	6.5	N
132	169	416314	26.8	N
133	170	85876	5.5	N
134	171	161722	10.4	N
135	172	173305	11.2	N

1217ms0018 Scan 1 (Av 10-16 Acq) +EI LRP

Normalised to largest peak in current list
Averaged Nominal data

Peak	Mass	Intensity	%	Flags
136	173	93898	6.1	N
137	174	95473	6.2	N
138	175	145919	9.4	N
140	177	228694	14.7	N
141	178	88223	5.7	N
142	179	132508	8.5	N
144	181	79571	5.1	N
151	188	85708	5.5	N
154	191	1456810	93.9	N
155	192	166065	10.7	N
156	193	967126	62.3	N
157	194	115481	7.4	N
158	195	205759	13.3	N
164	201	80171	5.2	N
166	203	97142	6.3	N
168	205	106840	6.9	N
169	206	1364570	87.9	N
170	207	217532	14.0	N
171	208	876266	56.5	N
172	209	140064	9.0	N
173	210	189283	12.2	N
174	211	83028	5.4	N
181	218	79243	5.1	N
188	225	145070	9.3	N
190	227	121958	7.9	N
203	240	85724	5.5	N
205	242	95478	6.2	N
240	277	127185	8.2	N
242	279	164541	10.6	N
244	281	118627	7.6	N
278	315	98984	6.4	N
333	370	171446	11.0	N