Virulence and dissemination enhancement of a mycoherbicide
by Kanat Slyambekovich Tiourebaev

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
The pathogen Fusarium oxysporum was originally isolated from diseased Cannabis sativa plants in the Chu River Valley, Djambul region, Kazakhstan. This fungus, which causes severe wilting, eventually resulting in plant death, was identified as Fusarium oxysporum f. sp. cannabis. A host range experiment confirmed that the virulence of this pathogen is limited to C. sativa. Under greenhouse conditions, 40-80% of the C. sativa plants inoculated with conidial formulations of the pathogen succumbed to this disease. In field tests conducted in Kazakhstan during the summers of 1996 and 1997 about 35% of Cannabis plants in experimental plots treated with the sawdust formulation of Fusarium oxysporum f. sp. cannabis died or exhibited severe wilting symptoms.

This study compared the vertical and lateral movement of Fusarium oxysporum spores through soil columns. In columns infested with the liquid spore suspension and the granular formulations, the pathogen moved down the soil profile 3- 5 cm. When a surface sterilized Cannabis, tomato, or grass seed was placed on the surface of the infested soil with these formulations, the fungal spores were recovered from the same depth, 3-5 cm. In the treatment using live seed, {Cannabis, tomato and Bluebunch Wheatgrass) coated with a CMC/spore suspension, the pathogen could be detected 9 cm below the soil surface, which was the limit of root growth in the tubes. These results suggest that the downward movement of the pathogen in the soil is facilitated by the seedling root growth of the host plant, and similarly by some non-host seedlings. . This method of inoculation may allow target weed species to be controlled using biologicals and, in addition, allow for selection of the successional plant species.

Valine analogs were used to select for valine-excreting mutants of Fusarium oxysporum f. sp. cannabis, casual agent of Fusarium wilt of Cannabis sativa. The comparative evaluation of pathogenicity of valine excreting mutants and their wild type parent showed increased virulence of the mutant strains to C. sativa. Host range studies on selected nonhost plants, did not reveal pathogenicity beyond C. sativa. Valine excreting mutants of Fusarium oxysporum f. sp. papaver were also generated. Valine excretion by these mutants was 10-50 times higher than by wild type strains.

•Keywords: Biocontrol, mycoherbicide, Cannabis sativa, Fusarium oxysporum f. sp. cannabis, live seed formulation, virulence enhancement.

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ABSTRACT

The pathogen *Fusarium oxysporum* was originally isolated from diseased *Cannabis sativa* plants in the Chu River Valley, Djambul region, Kazakhstan. This fungus, which causes severe wilting, eventually resulting in plant death, was identified as *Fusarium oxysporum* f. sp. *cannabis*. A host range experiment confirmed that the virulence of this pathogen is limited to *C. sativa*. Under greenhouse conditions, 40-80% of the *C. sativa* plants inoculated with conidial formulations of the pathogen succumbed to this disease. In field tests conducted in Kazakhstan during the summers of 1996 and 1997 about 35% of *Cannabis* plants in experimental plots treated with the sawdust formulation of *Fusarium oxysporum* f. sp. *cannabis* died or exhibited severe wilting symptoms.

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Valine analogs were used to select for valine-excreting mutants of *Fusarium oxysporum* f. sp. *cannabis,* casual agent of Fusarium wilt of *Cannabis sativa*. The comparative evaluation of pathogenicity of valine excreting mutants and their wild type parent showed increased virulence of the mutant strains to *C. sativa*. Host range studies on selected nonhost plants, did not reveal pathogenicity beyond *C. sativa*. Valine excreting mutants of *Fusarium oxysporum* f. sp. *papaver* were also generated. Valine excretion by these mutants was 10-50 times higher than by wild type strains.

Keywords: Biocontrol, mycoherbicide, *Cannabis sativa*, *Fusarium oxysporum* f. sp. *cannabis*, live seed formulation, virulence enhancement.

CHAPTER 1

LITERATURE REVIEW

Biological Control of Weeds

The biological control of weeds can be defined as the field application of selective plant pathogens or insects to control unwanted plant species. Biological control of weeds has many positive attributes including specificity, persistence, and the possibility of secondary spread. In nonagricultural areas chemical herbicides will never be a long-term, economic, or environmental solution to weed control (Evans, 1994).

Biological Control Strategies

Biological control strategies may or may not include plant death. The choice of a certain strategy will depend on the target weed, on the biocontrol agent used, or on the area (agricultural or conservation area) where the weed is considered a problem. Several distinct types of biological control of weeds have been described in the literature (Wapshere, 1989; Huffaker, 1964; Batra, 1982; Frick, 1974). According to Wapshere (1989) there are four main types of approaches to control of unwanted plants: a) - Classical/inoculative, b) - Inundative/augmentative, c) - Broad-spectrum, d) - Conservative.

Classical/inoculative biological weed control involves importation and release of natural enemies of a weed from the areas where the weed originated. Examples of such an

approach include control of Skeleton weed (*Chondrilla juncea*) in south-eastern Australia by the rust fungus *Puccinia chondrilla* to the extent that the plant is no longer of economic importance as a weed. This fungus was introduced from the Mediterranean region (Hassan, 1981), where the plant was also believed to have originated.

The "inundative/augmentative" biological control of weeds involves mass-production and release of naturally occurring enemies against native weeds. Collego®, a formulation of *Colletotrichium gloeosporioides* f. sp. *aeschynomene* is an example of this approach (Wapshere, 1989). A strain of this fungus, endemic to the USA, causes anthracnose disease of northern joint vetch, and was used to control this weed in rice fields in Arkansas (Daniel, 1973).

The "broad-spectrum" biocontrol of weeds involves manipulation of agents with broad-spectrum range (polyphagous insects, polyphagous herbivores, polyphagous fish, non specific phytopathogens). The prime requirement in this method seems to be containment of the broad-spectrum biocontrol agent, delimination of a broad-spectrum biocontrol agent in time and space. Development of auxotrophic - nutrient requiring mutants of *Sclerotinia sclerotiorum* (Miller and Sands, 1989 a) and mutants of *S. sclerotiorum* incapable of forming sclerotia (Miller and Sands, 1989 b) are examples of such a strategy.

The "conservative" method is based on reduction of antagonists of the weed's natural enemies and is more applicable to phytophagous biocontrol agents. This method is less often used and sometimes is considered as a part of Integrated Pest Management (IPM), although all the approaches with pathogens could be considered as components of

IPM. Use of phytopathogens (exotic or/and native) in combination with chemical herbicides is considered as a part of integrated weed management. This may allow decreased application rates of both the chemical herbicide and the mycoherbicide. The efficacy of a mycoherbicide can be enhanced when applied in combination with the chemical herbicide (Hougland, 1996).

In all types of biological control there is opportunity for selection of the most effective strains of biocontrol agent. Such selection involves directly screening for the most efficacious strain or selection of individual traits that might result in strain improvement.

The current investigation is a part of the ongoing development of an inundative /augmentative biological control method for non-cultivated *Cannabis sativa* in Kazakhstan.

Narcotic Plants as Unwanted Plants

The illegal use of narcotics derived from the plant species *Cannabis sativa* (marijuana), *Papaver somniferum* (opium), *Erythroxylum coca* (cocaine) is pervasive around the world. The sources for drug supply originate from illegal plantations as well as from naturally occurring stands (mainly *Cannabis*). The prohibition and control of illegal growth of these plant species is the concern of government institutions and law enforcement agencies. This usually involves manual or chemical eradication of plants. Since naturally occurring stands that usually are not continuous may cover large areas,

methods of manual and chemical eradication are infeasible to be annually applied for both economic and ecological reasons. At least in these uses the development of control strategies for narcotic crops should include biological control methods (Bayley, 1997). Unlike other weed plants, narcotic plants are high valued crop plants, and once eradicated might be illegally planted again. This could be stemmed by introducing a plant pathogen that is specific enough to allow other crop species to be grown while it provides long-term control of the target plant.

In South Kazakhstan non-cultivated *Cannabis* plants can be found in continuous and spatial stands, with one stand in the Chu river Valley covering approximately one hundred twenty five thousand hectares.

Cannabis

Origins. *Cannabis* is generally believed to be of Asiatic origin. According to R. E. Schultes (1970), Alphonse de Candole, the first authority on the origin of cultivated plants, specifies that "the species has been found wild, beyond a doubt, to the south of the Caspian Sea (border sea in west Kazakhstan), near the Irtish (river in north-eastern Kazakhstan) , in the desert of Kirgiz (southern Kazakhstan), beyond Lake Baikal in Dahuria.... and further to the east". This general area as origin was also supported by other authors (Vavilov, 1924; Zhukovskii, 1964). Archeological findings date early cultivation of cannabis in China back to Neolithic times, about 6,000 years ago (Hui-Lin Li, 1974). The first post-Linnean binomial distinction of two species of *Cannabis* was made by Lammarck in 1783, when he gave a detailed description of *C. indica*, "grown in,

found in Oriental Indies" (Emboden, 1974). At the present time, the possible origins of *Cannabis* spp., include China, India, and Central Asia.

Classification. The taxonomic characterization of *Cannabis* species has been a long and controversial process, only somewhat resolved in the last 25 years. It was first described by Linneaeus in 1735, and named as *Cannabis sativa* L., (Schultes, 1970). Lammarck and Janishewsky later described two more species (Emboden, 1974) *Cannabis indica* Lam., and *Cannabis ruderalis* Janish. However, the concepts of a monotypic genus were supported by Small, et al. (1972) in their findings that interspecies hybrids could be obtained by cross-pollination. According to Schultes (1970), W. Postma recognized only one species, to be split into two types of *C. sativa*: the northern, Russian hemps; and the southern, Indian hemps. Further, an alternative scheme of classification was proposed based on chemical phenotypes of the psychotropic compounds, that include six cannabinol classes: Δ^8-tetrahydrocannabinol (Δ^8-THC), Δ^9- tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), cannabinol (CBN), cannabichromene (CBC), and cannabigerol monemethyl ether (CBGM). The first two are believed to be psychotomimetic (Small, 1975). In this way, Fetterman, et al. (1971) recognized at least two classes of cannabis. Small, et al. (1975) proposed recognition of four cannabinoid phenotypes based on differences in the THC : CBD ratio (tetrahydrocannabinol : cannabidiol). Phenotype I consisted of populations with the highest THC : CBD ratio both in female and male plants, whereas the lowest ratio was in phenotype III with females plants being more potent than males. Evaluation of geographical distribution of the phenotypes showed that phenotype I

usually originates in countries south of latitude 30^0 N (Small, et al. 1973). There was disagreement among taxonomists on whether *Cannabis* is a monotypic or polytypic genus until Schultes provided keys to three distinct species of Cannabis: *Cannabis sativa* L., *Cannabis indica* Lam., and *Cannabis ruderalis* Janish., (Schultes, 1974).

Botanical Characteristics. Cannabis is an annual plant, growing each season from seed. When seed germinate, the radicle grows downward and ultimately develops into the primary root of the plant. The extent of this development depends, to a considerable extent, upon the nature of the soil in which the seed has been planted. If the soil is well "worked" and not overly compact, the primary root penetrates readily and lateral roots develop. However, if there is only a shallow layer of top soil over a hardened layer of subsoil, the resulting root system will be spreading and shallow. *Cannabis* is typically dioecious, but monoecious plants do appear in some populations.

In *C. sativa*, the leaves are most often opposite one another, except near the inflorescence, where they become alternate. The seed of *C. sativa* is large, often exceeding five mm, usually without marbling, and is compressed. The stem may grow to a considerable height of 4 - 8 m in river deltas.

In *C. indica*, the leaves are constantly alternative, stems are more cylindrical. *C. indica* plants are shorter (rarely exceeds 1.5 m) than *C. sativa* , seeds are smaller, darker, spherical. *C. indica* usually has a strong odor.

C. ruderalis is a small plant with marbled seeds, about one-half the size of *C. sativa* seed and over twice the size of the achenes of *C. indica*. This species is not found

in the USA and is restricted to central Asia, the Volga region and western Siberia. This species was considered to be a truly wild plant by Vavilov, and was called weedy hemp by Zhukovskii.

Diseases of *Cannabis*

Quite a few organisms attack or associate with Cannabis, including insects (Batra, 1976), bacteria and fungi (McPartland, 1991, 1984; Lentz, 1974; Noviello, 1962). Among these are some capable of causing severe disease, such as grey mold caused by *Botrytis cinerea*; hemp canker by *Sclerotinia sclerotiorum*; damping-off by *Pythium* spp., *Rhizoctonia solani*, and *Fusarium oxysporum*. Leaf spot diseases caused by *Septoria* spp., and *Phoma* spp.; wilts caused by *Verticillium* spp., and *Fusarium oxysporum* have also been documented.

Fusarium Wilt of *Cannabis*

Fusarium wilt of *Cannabis sativa* L. was reported to cause serious losses in cultivated hemp in Italy in 1959-1960 (Noviello, 1962). As a result by 1975 the area devoted to the cultivation of hemp in Italy declined from 20,000 hectares to 80 hectares. Crop cultivation was totally discontinued in areas where fusarium wilt was most severe. In this disease caused by *Fusarium oxysporum* f. sp. *cannabis* Noviello and Snyder, the first symptoms consist of yellowing of the foliage of infected plants, followed by wilting, leaves dry up and hang on the plant. A dark brown discoloration of the vascular system

also occurs. As a rule, infected plants are killed (Noviello, 1962; McCain, 1984). Host specificity studies showed that the host range of the pathogen was restricted to *Cannabis* (McCain and Noviello, 1984).

Fusarium oxysporum

The asexual fungus *Fusarium oxysporum* Schlect. emend. Snyd.& Hans., is a soilborne fungus with worldwide distribution. It is most often a host-specific pathogen, but is also successful as a saprophyte in that all strains are able to grow on organic matter in the soil, and are able to survive for long periods of time even when host tissue is unavailable. The main survival and dissemination forms of *Fusarium oxysporum* are chlamydospores, macroconidia and microconidia. Historically, strains of *Fusarium oxysporum* have been divided into formae specialis on the basis of their pathogenicity to various host plants. These formae specialis are further divided into races based on differences in virulence to various host cultivars. There are over 120 described forma specialis and races of pathogenic *Fusarium oxysporum* (Armstrong, et al. 1981). Some of them show a high level of host specificity, causing wilt of only a single host species (Snyder and Hansen, 1940). However, *Fusarium oxysporum* ff. sp. *apii, cassiae,* and *vasinfectum* have wide host ranges in more than one family of plants (Armstrong, et al. 1975). It has been demonstrated that wilt fusaria may invade plants with no apparent external or internal symptoms of disease, e.g. *F. oxysporum* f. sp. *battatas,* the sweet-potato wilt fungus, was reisolated from stems of inoculated cotton and the weed, Mexican clover (Armstrong, et al. 1948). Price uses the term "symptomless host" for such plants.

He also defines another term "symptomless carrier" as being restricted to those plants which support the active growth and actually contribute to an increase in number of propagules without any fungal invasion of tissue (Price, 1977). Nonpathogenic isolates of *F. oxysporum*, like pathogenic *F. oxysporum*, are highly competitive saprophytes. It has been shown that nonpathogenic and weakly virulent isolates of *F. oxysporum* can effectively reduce Fusarium wilt of carnation, or cucumber due to competition for nutrients and space in soil (Cugudda, et al. 1987; Mandeel, et al. 1991). Within formae specialis of *F. oxysporum* there are well known pathogens that cause wilt diseases on many agricultural crops, including tomato, cotton, peas and beans.

Fusarium wilt has been reported to cause serious epidemics in cultivated *Erythroxylum coca* caused by *Fusarium oxysporum* f. sp. *erythroxylum* (Darlington, 1996; Sands, 1997), and *Cannabis sativa* caused by *Fusarium oxysporum* f. sp. *cannabis* (Noviello and Snyder, 1962). *Fusarium oxysporum* f. sp. *papaverum* has been shown to cause severe wilt of opium poppy *Papaver somniferum* under greenhouse conditions (McCarthy, 1995; Anderson, 1996). Due to restricted host range, pathogens of this species were proposed as potential biocontrol agents of the above listed major narcotic plant species (McCain, 1984; McCarthy, 1995; Anderson, 1996; Sands, 1997).

Bioherbicides

Bioherbicides are biological control agents applied to control weeds in ways similar to chemical herbicides. The active ingredient in a bioherbicide is a living organism,

applied in inundative doses of propagules. Most commonly the microorganism used is a fungus and its propagules are spores or fragments of mycelium; in this case the bioherbicide is also referred to as a mycoherbicide (Auld, 1995). Development of a bioherbicide requires studies in identification, fermentation, formulation, efficacy, host range, and testing for nontarget safety.

Over the past 25 years more than one hundred microorganisms have been identified as candidates for development as commercial bioherbicidal agents (Zorner, 1993). However only four bioherbicides have been registered in the United States or Canada (Cook, et al. 1996; TeBeest, et al. 1992). In 1981, DeVine® (*Phytophtora palmivora*), was registered for control of strangler vine (*Morrenia odorata*) in Florida citrus groves (Kenney, 1986). Collego®, (*Colletotrichium gloeosporioides* f. sp. *aeschynomene*), was registered shortly thereafter to control northern jointvetch (*Aeschynomene virginica*) in Arkansas (Bowers, 1986). BioMal® (*Colletotrichium gloeosporioides* f. sp. *aeschynomene*) was registered for control of round-leaved mallow (*Malva pusilla* L.) in Canada and the United States (Grant, et al. 1990a, 1990b). Dr. Biosedge® (*Puccinia canalicularta*) was registered for control of yellow nutsedge (*Cyperus esculentus* L.) (Phatak, et al. 1983). In Japan, *Xanthomonas campestris* pv. *poae* was registered for biological control of annual bluegrass (Imaizumi, et al. 1997). Most of these agents are not commercially available in part due to small non-profitable markets, or for several connected reasons. High and long-term effectiveness may result in reduced sales of the bioherbicides. Often the results of using biological control are not as dramatic or quick as the results of using chemicals, and the results are not guaranteed.

Slow development of potential bioherbicides mostly results from difficulties in producing and stabilizing these agents and from lack of consistently effective weed control in field situations.

As has been pointed out (Zorner, et al. 1993; Jackson, et al. 1996), research efforts must be shifted from discovery of bioherbicides to solving the production, storage, and efficacy problems that plague all bioherbicides.

Strategies for Improvement of Bioherbicides

Improvement of Formulation and Inoculation

Formulation involves the blending of active ingredients, such as fungal spores, with inert carriers, such as diluents, surfactants, and/or solid substrates. It has been shown that addition of nutritional factors such as carbon sources, nitrogen sources, vitamins, trace metals, and manipulation of carbon-to-nitrogen ratio in liquid (Walker, 1981; Jackson, 1996) or granular preparations of a potential bioherbicide (Hildebrand, 1978) may enhance effectiveness of pathogens or increase sporulation on the surface of granules. Improved effectiveness has also been achieved by enhanced stability and/or biological activity of the phytopathogens (Connick, 1998). These improvements reduce the need to apply high dosages of inoculum.

Generally, choice of inoculation method depends on the method of production of the mycoherbicide formulation. The most practical ways of inoculation are spray or soil surface inoculation for foliar and soilborne root pathogens, respectively. However, the

amount of inoculum to produce and formulate may be expensive and not environmentally sound. One reason for rare use of plant pathogens for weed control is that they are usually not lethal enough at low concentrations. Typically, >10,000 spores/square cm of weed foliage are sprayed to inundate and control weeds (Gressel, 1996). The most critical event for maximum efficacy of an applied soilborne mycoherbicide is the successful and relatively fast penetration into the rhizosphere of the target weed and in numbers high enough to cause the disease, and to survive in the soil environment.

In studies of dispersal of *F. oxysporum* in soil by growth outward from a food base, D. Park (1959) showed that it was limited to small distances (3-4 mm in 4 weeks). He also showed that dispersal of the fungus in the soil by continuous growth could occur in the presence of continuous organic matter. Also when bulbs of *Narcissus*, which is a biennial crop in the United Kingdom, were planted 7 cm distant from bulbs infected with *F. oxysporum* f. sp. *narcissi*, no infection took place after one season (Price, 1977). On the other hand dispersal of the fungus in soil by migration of spores under influence of water may cover long distances (Burke, 1965), both in lateral and vertical directions. This passive dispersal follows a cone shape with the greatest lateral spread near the source of the propagules and in the direction of the water current (Park, 1959). Dispersal facilitated by water depends not only on the extent and rate of water input (Hepple, 1960), but also on characteristics of the microorganism (cell size, type of inoculum, spore concentration) and of the soil (texture, pH, clay mineral concentration). The greatest vertical movement of antagonistic *Fusarium* spp. by water transportation was found in lighter, sandy soils (Gulino, 1995; Gracia-Garza and Fravel, 1998). The lowest was found in clay soils. As

was shown by Connick, et al (1998) when using a PESTA food based formulation, the concentration of the mycoherbicide *Fusarium oxysporum* f. sp. *papaver* was logarithmically reduced in the first cm of clay soil.

There are several examples indicating that inoculum density can have an effect on disease development with species of *Fusarium*. A positive correlation was demonstrated between the depth of a pathogen and it's corresponding virulence (Ben-Yephet, 1994; Sippell and Hall, 1982). It was shown that the number of *F. oxysporum* f. sp. *dianthi*, (which is specific to carnation) propagules decreased linearly as soil depth increased (Ben-Yephet, 1994). Therefore one way to increase disease severity is to achieve high levels of the pathogen in the rhizosphere of the target weed species.

Several reports indicate that the pathogenesis and survival of many soilborne pathogens is largely dependent on association with underground plant parts. Outside of the rhizosphere, *Fusarium oxysporum* exists as resting spores. As the plant grows through the habitat of the fungus, it creates through exudation a micro-environment favorable for spore germination and subsequent vegetative growth (Griffin, 1969). The response of pathogens to plant root exudates may be host specific (Buxton, 1957) or non-host specific (Oritsejafor and Adeniji, 1990). In either case, subsequent growth of the fungus can lead to an increased inoculum or a decline in the population. Similarly, some plant species may have a selective effect on soilborne nonpathogenic populations of *Fusarium oxysporum* and this effect seems to be plant specific (Edel, et al. 1997).

Therefore, one approach to enhance efficacy of the mycoherbicide can be improvement of movement of the pathogen to the root zone of the weed, and to increase

mycoherbicide propagule density in the rhizosphere. Due to the ability of pathogenic *Fusarium oxysporum* to grow saprophytically on organic matter, the use of a non-host plant species as a carrier plant for the mycoherbicide could facilitate its relatively fast soil penetration into the rhizosphere of the target weed. Also association of the mycoherbicide with underground parts of non-host carrier plant could provide a microenvironment favorable for vegetative growth along the root surface subsequently increasing propagule numbers through the soil profile.

Enhancement of Virulence

Several approaches to virulence enhancement may include: genetic transformation of the fungus with a gene for phytotoxin production (Greaves, et al. 1989); enhanced production of degradative enzymes (Dickman, 1989); enhanced detoxification of plant defense compounds (Schafer, et al. 1989). These methods of increasing virulence are feasible, but food safety or environmental or technical considerations have limited their commercial use. Another approach would be to enhance the virulence of biocontrol agents without producing potentially dangerous metabolites. On the contrary, the increased production of one or more essential amino acids by the pathogen seems to be a more environmentally benign approach, and possibly requires less time and testing involved with biosafety considerations. The basic background behind this study involves aspects of intermediary metabolism in plants and microbes.

The strategy in the development of a chemical herbicide is basically focused on developing a compound that will target a specific step of a biochemical process in plants,

and possibly the one which is absent in humans and animals. One such process is biosynthesis of a branched-chain amino acid. The first enzymatic step common to the biosynthesis of the branched-chain amino acids is catalyzed by acetohydroxy acid synthase (AHAS; E.C.4.1.3.18, also referred to as acetolactate synthase ALS). Genetics and biochemistry of this enzyme have been extensively studied in microorganisms (for review see De Felice, et al. 1982) and in higher plants (Bryan, 1980; Haughn, 1986). A number of the sulfonylurea compounds are toxic to both plants and microorganisms. Two classes of agriculturally important herbicides, the sulfonylureas and the imidazolinones, have been shown to act by specifically inhibiting ALS (La Rossa and Falco, 1984; Shaner, et al. 1984). Inhibition of ALS by sulfunylureas is competitive with respect to pyruvate (or 2-ketobutyrate) binding site. Inhibition of ALS by imidazolinones (activity against monocots, dicots) is uncompetitive with respect to pyruvate. The growth of both shoot and root apical meristems of wild type *Arabidopsis thaliana* on agar-solidified mineral media was completely inhibited by chlorsulfuron concentrations of 28 nM or higher. However, growth was not inhibited by 280 nM chlorsulfuron if 1mM valine and 1mM isoleucine were included in the medium (Haughn and Somerville, 1986). This is consistent with previous studies in tobacco and pea indicating that growth inhibition by chlorsulfuron was due to inhibition of branched-chain amino acid biosynthesis (Chaleff and Ray, 1984; Ray, 1984).

Mutation in ALS confers resistance to sulfunylureas both at enzyme and organismal levels in microorganisms and plants. Dominant mutations that confer resistance to sulfonylurea herbicide sulfometuron methyl in *Salmonella typhimurium* (La

Rossa and Schloss, 1984) and Saccharomyces cerevisiae (Falco and Dumas, 1985) have been shown to map to the structural gene for ALS. In each case the mutations result in the synthesis of a herbicide-resistant ALS. Analogous mutants have also been isolated in higher plants by selecting for sulfonylurea-resistant mutants in tobacco (Chaleff and Ray, 1984), and in *Arabidopsis thaliana* (Haughn and Somerville, 1986). These mutations occur in the large subunit and result in single amino acid substitution at the amino terminal conserved region. The role of the small subunit of ALS is unclear, most of active site appears to reside in large subunit (Chaleff and Ray, 1984). The analysis of the effects of sulfonylureas on ALS activity in extracts from resistant mutants of *Arabidopsis thaliana* indicated that resistance is due to a sulfonylurea-resistant enzyme activity. The mutant enzymes retain sensitivity for feedback inhibition by valine (Haughn, 1986).

Essential Amino Acids

Microorganisms and plants can generally synthesize all the essential amino acids. Many of oxo-acids produced by transamination of the amino acids are common metabolic intermediates. As well as providing a pathway for degradation of surplus amino acids which is integrated with other metabolic sequences, transamination allows synthesis of many of the amino acids from intermediates of carbohydrate metabolism.

Other amino acids are referred to as essential or indispensable: they are dietary essentials for man and most mammals. These amino acids have oxo-acids which cannot be synthesized by the animal from any source other than the amino acid itself. For man, leucine, isoleucine, valine, threonine, lysine, methionine, phenylalanine and tryptophan are

essential. Tyrosine formed from phenylalanine, and cysteine, formed from methionine, are not essential amino acids, but since they are synthesized from essential amino acids their synthesis places strain on the available precursor. In children histidine and arginine are also essential, since, although they can be synthesized, the requirement for growth is greater than the synthetic capacity. Thus, an essential amino acid is one which cannot be synthesized, or not in sufficient quantity, by the animal or organism concerned (Bender, 1975).

Biosynthesis of Isoleucine and Valine. These two amino acids are synthesized by parallel pathways, and there is a considerable amount of evidence that in all systems examined the same enzymes are responsible for synthesis of both: in microorganisms (Umbarger, 1962; De Felice, et al. 1982); in the higher plants (Bryan, 1980). Wagner, et al. (1965) showed that mitochondrial fractions from *Neurospora crassa* will catalyze simultaneous synthesis of isoleucine and valine, and that pyruvate and L-oxobutyrate, as well as L-acetolactate and L-aceto-L-hydroxybutyrate, are mutually competitive. Furthermore, both pyruvate metabolism and the synthesis of isoleucine and valine are intra-mitochondrial. The precursor of isoleucine, L-oxo-butyrate, is formed from threonine, either by catabolic threonine deaminase (induced by growth on threonine rich media) or by biosynthetic threonine deaminase (which is repressed by growth on media rich in isoleucine). Thus while pyruvate will be available for entry into the pathway of branched-chain amino acid biosynthesis at a relatively constant rate, the proportion of isoleucine produced can be controlled by the activity of the biosynthetic threonine

deaminase. In fungi, the enzymes of isoleucine and valine biosynthesis are mitochondrial; in bacteria, which do not have mitochondria, the enzymes have been shown to be tightly membrane-associated, forming membrane bound multi-enzyme complexes. Preparations of mitochondrial membrane from *N. crassa* having acetolactate synthase activity are sensitive to inhibition by both valine, and to lesser extent, isoleucine.

Two distinct branched-chain amino acid aminotransferases have been isolated from *N. crassa*. Both are active toward valine, leucine and isoleucine. One enzyme is mitochondrial, and will utilize only glutamate as an amino donor, while the other is cytoplasmic, and will utilize phenylalanine, tyrosine or methionine to aminate the branched-chain oxo-acid. Growth of the organism on media rich in the branched-chain amino acids leads to induction of the cytoplasmic aminotransferase, but has no effect on the activity of the mitochondrial enzyme. Since biosynthesis of the branched-chain amino acids is wholly mitochondrial in Neurospora, it is probable that the cytoplasmic aminotransferase is mainly concerned with catabolism rather than biosynthesis (Bender, 1975). In plants the biosynthesis of branched-chain amino acids is located in chloroplasts (Hagelstein, et al. 1993).

Nonprotein Amino Acids

Nonprotein amino acids are those which are not found in protein main chains. As a group nonprotein amino acids are extremely diversified. In addition to the 20 or so universally distributed protein amino acids, over 400 others have been obtained from natural sources. About 240 nonprotein amino acids are found in various plants.

Prokaryotic organisms are the source for an additional 50, while fungi provide 75 others. Animals produce about 50. In most of the source organisms the nonprotein amino acids are most frequently present in the free state (Hunt, 1985). Some nonprotein amino acids are potent toxicants. Furthermore many amino acid analogs have been synthesized, often in the hope of obtaining an antimicrobial or antitumor agent. A structural change in a protein amino acid may yield a product which no longer functions normally in metabolism, and which inhibits the metabolism of the natural analog, and sometimes produces effects comparable to a deficiency of the natural metabolite (Meister, 1965). Formation of aberrant, analog-containing protein represents the most frequently cited basis for the antimetabolic properties of certain toxic amino acid antagonists (Hunt, 1985). Other common modes of action may include: inhibition of enzyme function competitively by virtue of the structural analogy to the natural substrate molecule; noncompetitive inhibition also occurres. Among other common modes of action are disruption of amino acid uptake and translocation, generation of erroneous repression signal, false end-product inhibition, and alternation of cellular structural components, etc. (Lea and Norris, 1976; Fowden, et al. 1979; Rosenthal, 1982).

Isoleucine, Valine and Leucine Antagonists. Among the effective antagonists of branched-chain amino acids are: methylglycine, 2-amino-4-methylhexanoic acid - the most effective competitive antagonists of leucine utilization; O-methylthreonine and cyclopentaneglycine - competitive antagonists of isoleucine incorporation into proteins; α-amino-β-chlorbutyric acid is a potent antagonist for valine incorporation into protein, and

inhibition can be prevented by valine (Rabinovitz and McGrath, 1959). The similarity in structure of valine and isoleucine not only results in mutual antagonism, but analogs frequently are antagonists of both amino acids. Among valine antagonists studied for inhibitory effect on growth are: L-isoleucine, L-leucine, D-valine, α-aminoisobutanesulfonic acid , aminochlorbutiric acid, α-aminobutyric acid, methylglycine, L-norvaline, L-penicillamine, L-valinol, β-hydroxyvaline, valine hydroxamate, ω-dehydroalloisoleucine (Shive and Skinner, 1963). The α-aminobutyric acid toxicity for E. coli is prevented by valine, isoleucine and very effectively by leucine (Dittmer, 1950). The norvaline, or 2-aminopentanoic acid, [$CH_3CH_2CH_2CH(NH_2)COOH$] - structural analog of valine, causes growth inhibition of *E. coli,* but is reversed in a competitive manner only by a mixture of the amino acids, suggesting that more than one antagonism is involved (Kisum, et al. 1976; Dittmer, 1950). The norvaline was less toxic to the duckweed than aminochlorbutiric acid (also a valine analog, unavailable) at concentration of 0.2 millimoles (Shive and Skinner, 1963). The L-penicillamine [$(CH_3)_2C(SH)CH(NH_2)COOH$] toxicity for *E. coli* is reversed by branched-chain amino acids; isoleucine is the most competitive reversing agent (Gleinstein and Winitz, 1961). β-hydroxyvaline [$(CH_3)_2CHC(OH)(NH_2)COOH$] is inhibitory to the growth of *Lactobacillus arabinosus* and is reported to be an antagonist of valine (Meister, 1965; Rosenthal, 1982).

Feedback Inhibition

The regulation of any branched metabolic pathway is a complex process. Common

patterns of regulation appear to be simple feedback inhibition. The first demonstration of the end product inhibition of the formation of an intermediate in the biosynthetic pathway was the inhibitory effect of purines upon the synthesis of 5-amino-4-imidazolecarboxamide in *E. coli* (Gots and Chu, 1952).

The pathways of amino acid biosynthesis are to a great extent under precise control by feed-back mechanisms, which regulate the rate of synthesis of each end product to correspond to its rate of utilization by the growing cell. Each amino acid synthesized by branched pathway inhibits the first step unique to its own biosynthesis. Thus, lysine inhibits the conversion of aspartic semialdehyde to dehydro-dipicolinic acid; methionine inhibits the O-succinylation of homoserine isoleucine inhibits the biosynthetic threonine diaminase; valine competitively inhibits the conversion of pyruvate to acetolactate in *E. coli* and in *Aerobacter aerogenes* (Umbarger, et al. 1957); isoleucine competitively inhibits the deamination of threonine to α-ketobutyric acid and prevents overproduction of threonine deaminase (Umbarger and Brown, 1958).

Valine inhibits the growth of higher plants, both as seedlings (Miflin, 1965 a) and protoplasts (Bourgin, 1976). This inhibition is relieved by isoleucine. Studies on the ALS enzyme isolated from barley have demonstrated concerted feedback inhibition by valine and leucine (Miflin, 1969 b, 1971), and synthesis of branched-chain amino acids in isolated chloroplasts has been reported to be regulated by exogenous valine and isoleucine (Schulze-Siebert, et al. 1984). The ALS enzyme isolated from valine-resistant mutants of tobacco was appreciably less sensitive to inhibition to valine and leucine than wild type (Relton, 1986).

Inhibition of the ALS - the first enzyme in the biosynthetic pathway of the branched-chain amino acids by excessive amounts of either one of the three amino acids added exogenously leads to starvation of the organism for other two branched amino acids, causing inhibition of growth and death.

False Feedback Inhibition

It was recognized that not only the end product of a biosynthetic pathway, but also its analogs, can exert feedback control over the action of the initial enzyme of the pathway. Moyed (1960) observed that inhibition by certain tryptophan analogs could be noncompetitively reversed by tryptophan and also by intermediates in its formation. Also there was direct evidence that several histidine analogs block the histidine biosynthetic pathway in the same way as histidine itself does. Such mimicry of the end product in inhibiting the action of the initial enzyme has been called "false feedback" (Umbarger and Davis, 1962).

End Product Overproduction

Studies on end product control revealed that mechanisms include repression of the synthesis of enzymes involved in the biosynthesis, and also metabolic inhibition of enzymic activity. There are some instances of escape of such control, which have resulted in the overproduction and substantial excretion of certain amino acids (Moyed, 1960; Umbarger, 1962; Davis, 1952; Adelberg, 1958).

One method of overcoming the controls is the cultivation of auxotrophic mutants

on a growth limiting amount of the required end product. This procedure frequently results in the heavy accumulation of metabolic intermediates. Such an accumulation of diaminopimelate by a lysine auxotroph (Davis, 1952) has made possible a commercial process (Casida, 1956) in which a second organism is used to convert the diaminopimelate to lysine (accumulation of diaminopimelate 0.5 g/l).

Another approach, not based on auxotrophic mutations, is simple random screening of soil samples for microorganisms that excrete amino acids. One such organism *Micrococcus glutamicus* was found to excrete L-glutamate in an amount equal to about one-fifth of the glucose consumed (Konoshita, 1957). Such overproduction of an amino acid could be due to sequential loss of several control mechanisms in a normal pathway.

Organisms that excrete amino acids can be isolated by a more rationally directed method: selection of mutants resistant to growth inhibition by amino acid analogs. Resistant mutants of *E. coli* were obtained for a variety of amino acid analogs, and each was shown to excrete the corresponding amino acid (Adelberg, 1958). It was initially thought that the overproduction of the amino acid, due to a loss of feedback control, was itself responsible for reversing the inhibition (Adelberg, 1958). However, it was shown, at least in some cases, that the excretion is not responsible for resistance (Moyed, 1960). In these cases the analog mimics the inhibitory effect of the end product, in the wild type organism, on the initial enzyme of the pathway. The mutant has an altered enzyme, selected for resistance to the analog, and since this enzyme is also resistant to feedback by the normal end product, this product is excreted.

Altering the amount of the end product of the pathway in the media will often influence the level of the bisynthetic enzymes of that pathway. Thus growth in the presence of an excess of the end product will usually yield a majority of the cells with decreased (or even none) of the enzymes of that pathway. Conversely, the intracellular levels of an amino acid can be lowered to less than the normal steady-state value by supplying the wild type with an extensive enrichment that lacks the end product under investigation. Under these conditions the cell becomes "derepressed", and has been observed to synthesize as much as 50 times the normal amount of the enzyme of the derepressed pathway, relative to the rest of the protein of the cell (Vogel, 1956).

Amino Acid Imbalance

Antagonism between naturally occurring amino acids has often been observed in nutritional experiments on bacteria, and much effort has been expended in designing "balanced" media for the optimal growth of microorganisms. Several examples of this type of antagonism may be cited. Glycine, serine, threonine and β-alanine inhibit the growth of *Streptococcus faecalis*, and this effect is reversed by increasing alanine in the medium (Snell, 1943). A lysine auxotroph of *Neurospora crassa* was competitively inhibited by L-arginine, such that 50% inhibition was observed with an arginine:lysine ratio of one. Mutual antagonism have been reported between branched-chain amino acids (Brickson, 1948).

Departure from the optimal amino acid ratio leads to an amino acid imbalance whose effects are somewhat similar to those observed in amino acid deficiency. Thus, in

both amino acid imbalance and amino acid deficiency the organisms exhibit markedly reduced growth. There are associated increases in degradative metabolism and in excretion of amino acids (Meister, 1965)

Concluding Remarks and Objectives

In this study we report the efficacy of a Kazakhstan strain of *Fusarium oxysporum* as a potential bioherbicide candidate for biological control of *Cannabis sativa* in the Chu river Valley, Kazakhstan. In addition we study the possibility of improvement of soil dispersal of a mycoherbicide by coating beneficial seeds with the weed-specific pathogen to enhance spread in the soil and mycoherbicidal efficacy. Also we describe the process of generating specific mutants of plant pathogens, that by virtue of overproducing one or more inhibitory metabolites, they are more virulent and more efficacious as weed biocontrol agents.

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CHAPTER 2

FUSARIUM OXYSPORUM F. SP. *CANNABIS*, A PROMISING CANDIDATE FOR BIOCONTROL OF *CANNABIS* IN KAZAKHSTAN

Introduction

The wide-spread distribution of *Cannabis* species (*C. sativa* L., *C. indica* Lam., *C. ruderalis* Janisch.) in the southern part of Kazakhstan has led to considerable degradation of pasture and agricultural areas. In the Chu River Valley, *Cannabis* plants grow on an estimated one hundred twenty thousand hectares (Intl. Confer. Alma-Ata 1991). The Chu River Valley is located in a semi-arid region in the southern Kazakhstan with low annual precipitation and average summer temperatures of 30-35 C. Noncultivated *Cannabis* plants in this area can be as tall as 2.5 meters. It is the largest known natural stand of *Cannabis* in Central Asia. This area is capable of producing about five hundred metric tons of marijuana per year (Intl. Confer. Alma-Ata 1991). The occurrence of such a vast, non-cultivated stand of wild *Cannabis* plants causes an enormous impact on the economical and sociological character of the region.

Two methods of controlling this problem plant species involve annual applications of chemicals and manual removal. Both control measures have limited effectiveness, for one reason or another. Chemical controls are effective at reducing the existing vegetation, but do nothing to the residual seed base, which quickly germinates to cover available

space. Manual removal of plants from 120,000 hectares is physically prohibitive. However, there is potential to control this weed species using phytopathogenic microorganisms.

The probable origin of *Cannabis* could very well be somewhere within the wide span of temperate Asia from the Caucasus Mountains and the Caspian Sea through western and central Asia into eastern Asia (Schultes, 1970; Zhukovskii, 1964; Vavilov, 1924). There is no concerted agreement among botanists as to where the plant originally grew wild and where its cultivation first began. *Cannabis sativa* is a well known source of oil, fiber and marijuana and is one of man's oldest cultivated plants. Due to centuries of cross-pollination, there is no easy way to distinguish between wild, adventitious, semi-cultivated, or cultivated plants. However, Schultes in 1974 presented a key to three species of *Cannabis*: *C. sativa*, *C. indica*, and *C. ruderalis*, leaving the total number of species as "still open to question" (Emboden, 1974). In addition, the properties of the plant change depending on the climate in which it grows. Plants imported to Egypt and Tunisia from Europe for fiber supply lost fiber qualities and began to produce more intoxicating resin. Plants grown in England and in France from seed imported from India after several generations were morphologically indistinguishable from the race long acclimatized to European conditions (Schultes, 1970). This all makes determining the taxonomical characterization and exact origin of Cannabis species very difficult.

The most commonly recognized species of *Cannabis* (*C. sativa* L., *C. indica* Lam., *C. ruderalis* Janisch.) are found in Southern Kazakhstan. This region is also known as a part of what used to be called the Great Silk Road that connected trade between

Europe and Eastern Asia. It likely that introduction or/and emigration of *Cannabis* through this region was a continuous process for centuries, resulting in continuous *Cannabis* crossbreeding and adaptation; thus, Southern Kazakhstan is a natural choice to collect *Cannabis* pathogens. Early Chinese literature recognized the *Cannabis* plant as dioecious. The male plant was called *I* or *hsi* and the female plant was *tsu* or *chu* (Hui-Lin Li). It may be no coincidence that the river valley known for the largest *Cannabis* stand in Central Asia is named the Chu River Valley.

Classical biocontrol philosophy advocates the collection of natural plant pathogens and pathogenic insects from the area of a plant's origin (Wapshere, 1989). Or in case of the inundative approach to collect local natural pathogens causing disease in the existing local population of the weed. The first step in this study was to observe local *Cannabis* populations and any related disease incidence. Several hundred samples of diseased *Cannabis* tissue were collected from the Chu River Valley. The majority of samples collected were from *Cannabis* plants that were exhibiting symptoms of vascular wilt: stunted growth, yellowed leaves and vascular tissue discoloration. These disease symptoms were the most commonly observed in the region. The isolated plant pathogens were then subjected to Koch's postulates (i.e., isolated, cultured, inoculated, reisolated, identified). Most of the collected plant material yielded morphologically similar isolates of *Fusarium*, identified as *F. oxysporum* (Nelson, et al. 1983; Brayford, 1993) based on microscopic analysis and colony morphology (Sands, et al. 1997). *Fusarium oxysporum* is a soilborne fungus, which has many formae specialis that are known to cause vascular wilts on many crops world wide. There are over 120 described formae specialis and races

of pathogenic *Fusarium oxysporum* that have been separated on the basis of specificity to various host plants, and are characterized by a high level of host specificity (Armstrong and Armstrong, 1981). However there can be exceptions.

Fusarium wilt was reported to cause serious loss in the cultivated hemp crop in Italy (Noviello and Snyder, 1962) and *Fusarium oxysporum* was proposed as a potential biocontrol agent for unwanted *C. sativa* (McCain and Noviello, 1984). In the interest of developing a host specific biological control agent for *Cannabis sativa*, our evaluations focused on these *F. oxysporum* isolates. In this study we describe the use of pathogenic strains of *Fusarium oxysporum* for biological control of wild *Cannabis sativa.*

The objectives of this investigation were: 1) to acquire pathogens of *Cannabis sativa* and evaluate the pathogenicity of selected fungal isolates to *Cannabis sativa* and selected crop species under greenhouse and field conditions; 2) to determine the level of pathogenicity of selected strains of *Fusarium oxysporum* at different temperature regimes to *Cannabis sativa*; 3) to compare virulence of generated nitrate nonutilizing mutants to *C. sativa* with their wild type parents. Paramount considerations in the development of this weed control program were host specificity, environmental safety and human safety.

Materials and Methods

Fungal Isolation

Stem, leaf, and root tissue samples were collected from plants exhibiting the following symptoms of vascular wilt: stunted growth, yellowed leaves, and vascular tissue

discoloration. Plant material was surface sterilized by soaking tissue samples in a 0.5% sodium hypochlorite solution for one minute prior to plating sections onto 2 % water agar amended with alcohol sterilized 50 mg/L streptomycin sulfate and 15 mg/L tetracycline (Sigma Chemicals, St. Louis, MO). Plates were incubated at ambient temperature for 48 hours and hyphal tips were transferred to Potato Dextrose Agar (PDA) (Difco Laboratories, Detroit, MI) for purification and identification (Nelson, et al. 1983).

Culture Preservation

The method used for fungal culture preservation was described by Sands, et al. (1997). Single spore cultures were plated onto PDA and incubated at ambient temperature. After 48-72 hours, sterile toothpicks were placed on the actively growing colonies and the fungal cultures were further incubated until the toothpicks were completely colonized by mycelia. Toothpicks were harvested, placed in sterile No. 2 dram vials with the lids askew (Wheaton Scientific, Millville, NJ) and dried overnight under a sterile air stream in a laminar flow hood. The lids of the vials were tightened and the cultures were stored at 5 C. Viability of cultures stored on toothpicks exceeded three years.

Fungal Cultures and Inoculum Production

Fusarium oxysporum f. sp. *lycopersici* 0-1080 (ATCC) and *Fusarium oxysporum* cultures isolated from collected plant material were grown in 250 ml flasks containing 100 ml Potato Dextrose Broth (PDB) (Difco Laboratories, Detroit, MI) for 5-7 days at

ambient temperature with agitation (200 rpm). The concentration of conidia in the culture (primarily microconidia) was determined with a hemacytometer and adjusted to the desired concentration with sterile water (10^7sp/ml). A granular food based formulation involving Birch sawdust (BSF), is readily available, inexpensive and can be easily prepared as follows: 50 milliliters of a 0.1 % yeast extract solution (Difco Laboratories, Detroit, MI) and 0.1 M citric acid solution (pH 5.5) (Sigma) were added to 100 g of Birch sawdust. The spore suspension was added to the formulation at a rate of 10 ml of 10^7 sp/ml spore suspension per 100 grams of sterilized BSF. The control consisted of noninoculated PDB poured over sterile BSF. This mixture was stirred well and then air dried under a laminar flow hood for 4-6 hours. In growth-chamber inoculations the formulation was applied to the soil surface at a rate of 0.5 g /16.5 cm diameter pot, which contained one *C. sativa* plant. Larger field plots, 1 square meter, containing 20 *C. sativa* plants were inoculated with 10 g of formulation per plot spread over the soil surface.

Vegetative Compatibility

In order to determine whether the selected isolates of *Fusarium oxysporum* belong to the same vegetative compatibility group, nitrate non-utilizing auxotrophs (*nit*1, *nit*M, *nit*3) from wild type strains of Cs72, Cs95 and Cs109-2 were generated on PDA media containing 3% potassium chlorate (Correll, et al. 1987). The nitrate non-utilizing auxotrophs were paired in all combinations on nitrate minimal media to determine vegetative compatibility among *Cannabis* isolates. Also it has been shown that nitrate non-utilizing mutants can be reisolated from the soil or plant material using selective media

(PDA media containing 3% potassium chlorate) and therefore easily discriminated from other *Fusarium* (Hadar, 1989).

Evaluation of *C. sativa* Plants Affected by Fusarium Wilt

Individual plants were rated for disease severity on a scale of 1 to 5, where 1 = no disease, 2 = minor wilting of lower leaves, 3 = wilting symptoms observed on 25-50% of leaves, 4 = >75% leaves show severe wilting symptoms, 5 = dead plant.

Disease Average Index (DAI) for each treatment was calculated as = (1 n + 2 n + 3 n + 4 n + 5 n) divided by Total n, where n = number of plants with symptoms from 1 through 5.

In addition Disease Incidence Percent (% disease) was estimated for each treatment as = a/b x 100%, where a = number of *C. sativa* plants with severe disease symptoms (from 3 through 5) and b = total number of *C. sativa* plants per treatment (from 1 through 5). Disease incidence percent was used to monitor the progression of Fusarium wilt in treated and control field plots of *Cannabis sativa*.

Greenhouse Evaluation of *F. oxysporum* Isolates on *C. sativa*

Preliminary Screening. All one hundred fifteen *Fusarium oxysporum* isolates obtained from the Chu River Valley plant samples collected during 1995-1996 were screened for pathogenicity in preliminary studies under greenhouse conditions 28 C (19 hours day, 5 hours night) (data not shown). In these preliminary screening tests each isolate was applied to five *Cannabis sativa* plants with two - three pairs of true leaves by

placing 0.5 g of BSF formulation at the base of each plant. Control plants were inoculated with autoclaved inoculum. *Lycopersicon esculentum* plants (cv. Bonnie Best) inoculated with *Fusarium oxysporum* f. sp. *lycopersici* strain 0-1080 (ATCC) were included as a positive control. Plants were watered daily for 2 minutes by an automatic sprinkler system. Plants were evaluated daily for symptoms of vascular wilt. Tissue from plants exhibiting disease symptoms was surface sterilized in 0.5% NaOCl and plated onto 2 % water agar amended with antibiotics. Hyphal tips were transferred to PDA for purification and identification.

Out of 115 isolates tested, three (Cs72, Cs95, Cs109-2) caused characteristic symptoms observed on the majority of the diseased *Cannabis* plants collected in the Chu River Valley. These consisted of stunted growth, yellowed leaves and vascular tissue discoloration Figure B.1. (Appendix B). These three isolates were selected for more detailed evaluation. The level of pathogenicity of these selected strains of *Fusarium oxysporum* were examined at different temperature regimes in environmental growth chambers.

Disease Severity at Different Temperatures. Plants inoculated with the selected *Fusarium oxysporum* isolates (Cs72, Cs95, Cs109-2) and their nitrate nonutilizing auxotrophs (*nit* mutants) Cs95nit1, Cs109-2nit3 were maintained in Conviron environmental growth chambers with computer controlled conditions set at the following parameters: Test A = 22 C (19 hours day, 5 hours night); Test B = 28 C (19 hours day, 5 hours night); and Test C = 35 C day and 28 C night time (19 hours day, 5 hours night).

The other four treatments included were *C. sativa* and tomato plants (cv. Bonnie Best) inoculated with autoclaved BSF formulation and *Fusarium oxysporum* f. sp. *lycopersici* strain 0-1080 (ATCC). Plants were watered daily for 2 minutes by an automatic sprinkler system. Inoculations of the test plants were performed in a complete randomized design. Plants were inoculated at the same leaf stage, and at the same rate of inoculum as stated earlier. Each experiment included nine treatments. An experimental unit consisted of ten plants replicated twice. The disease average index was determined and data were analyzed as a factorial with temperature and inoculum as two factors in a completely randomized design with two replications per treatment. Reisolation of the wild type strains was as described earlier. Reisolation of *nit* mutants from treated plants exhibiting wilting symptoms was performed using PDA media containing 3% potassium chlorate.

Greenhouse Host Range Study. The host range of strains Cs72, Cs95 and Cs109-2, Cs95 nit1 exhibiting pathogenicity to *C. sativa* in greenhouse virulence tests was evaluated on various crop plant species in environmental growth chambers. The choice of non target plant species for host range studies was mainly dictated by the number of non target crop plant species grown in the region of the Chu River Valley. Isolates were evaluated for virulence against *Lycopersicon esculentum* (cv. Bonnie Best), *Zea mays* (cv. Sakota), *Triticum aestivum* (cv. Penewawa), *Hordeum vulgare* (cv. Clark), *Phaseolus vulgaris* (cv. Blue Lake Bush), *Festuca arundinaceae* (cv. Apache), and Blue Bunch wheatgrass (*Agropyron spicatum*). The strain Cs95 was also evaluated against *Gossypium hirsutum* (cv. Stoneville), *Pisum sativum* (cv. Alaska), *Raphanus sativus* (cv. Cherry

Bomb), *Melilotus indica* (cv. Toudo), *Cucumus sativus* (cv. Straight eight), *Daucus carota* (cv. Sweet sunshine). Control plants were inoculated with autoclaved inoculum. *Fusarium oxysporum* f. sp. *lycopersici* strain 0-1080 (ATCC) was included as a positive control. Plants were maintained in environmental growth chambers with computer controlled conditions: 28 C (19 hours day, 5 hours night). Preliminary data showed that the temperature regime of 35 C was too stressful for some non target plants tested, resulting in death of noninoculated control tomato plants. Plant spp. were randomized within treatments. An experimental unit consisted of ten plants of each species replicated twice. One week old plants were soil surface inoculated by placing 0.5 g of BSF inoculum at the base of each plant. Plants were watered daily. The plants were evaluated daily for symptoms of vascular wilt. The plants exhibiting symptoms of fungal infection were plated onto Komada's medium for reisolation of the pathogen. At the end of the experiment, one arbitrary plant from each treatment was assessed for the presence of *Fusarium* by plating plant tissue on Komada's medium (Komada, H. 1975).

Field Pathogenicity Studies 1996-97

Field evaluation of the *F. oxysporum* isolates Cs72, Cs95, Cs109-2 was carried out at the experimental station of the Kazakh Institute of Agriculture, Academy of Agriculture, Kazakhstan. The field experimental plots were located about 300 kilometers east of the Chu River Valley. This location was dictated mostly by security reasons. The climatic conditions of the experimental station do not differ significantly from those in the Chu River Valley. The climatic data including temperature fluctuations at the

experimental station during summers of 1996 and 1997 are presented in Figure B.2. (Appendix B).

Fusarium oxysporum isolates Cs95, Cs72, Cs109-2 that did not show virulence activity in greenhouse studies on tested non target plants but caused vascular wilt of *C. sativa* in greenhouse conditions, were selected for field evaluation. Each field experiment included four treatments: Cs95, Cs72, Cs109-2, and control treatment. Inoculations were performed in late May on *C. sativa* plants with two- three pairs of true leaves. Plants were soil surface inoculated with BSF of the selected *F. oxysporum* isolates or with the control formulation by placing 0.5 g of inoculum at the base of each plant. Control plots were treated with autoclaved inoculum. Fungal strains were reisolated from diseased plants by plating vascular tissue onto Komada's medium. Reisolates were compared with the original culture based on colony morphology and microscopic analysis.

Each treatment was tested on four experimental plots, which were assigned in a complete randomized fashion. Individual one square meter research plots contained twenty *C. sativa* plants. Plots were separated from each other by a 20 cm deep trench 1 meter wide. Plants were watered daily. Plants were evaluated for disease symptoms every third day to monitor disease progression. End of season readings of experimental units were statistically analyzed.

The field tests of the selected *Fusarium* isolates were repeated in two consecutive years (1996 and 1997). In both field experiments an experimental unit consisted of twenty *C. sativa* plants in one square meter experimental plot. The disease average index was obtained and data from each year were analyzed separately in complete randomized design

with four replications per treatment.

Statistical Analysis

Data were studied by analyses of variance in the SAS program. To compare treatment mean differences between more than two treatments, least significant differences were calculated at $\alpha = 5\%$.

Results

Greenhouse Evaluation of *F. oxysporum* Isolates on *C. sativa*

One hundred fifteen cultures of *Fusarium oxysporum* were isolated from the plant material collected from eleven locations within the Chu River Valley. All isolates of *Fusarium oxysporum* were screened for pathogenicity to *C. sativa* under greenhouse conditions (data not shown). Three isolates Cs72, Cs95 and Cs109-2 were selected due to their ability to cause wilting symptoms on treated *Cannabis* plants similar to those observed in most of the locations where the collections of the diseased plant material were done.

The first symptoms of disease on *Cannabis* plants, wilting and death of a few lower leaves, were observed one-two weeks after inoculation. Early symptoms further developed into severe wilt and death of the plant. The period of time between initial symptoms and plant death was variable, ranging from weeks to months.

The development of wilt disease in treated plants in terms of time and severity depended on conditions in the environmental growth chambers (Table 2.1.).

Table 2.1. Disease Severity in *C. sativa* Plants Grown in Greenhouse Soil Infested with Nitrate Nonutilizing Mutants of *F. oxysporum* and Their Wild Type Parents at Different Temperatures

Strain number	Plant spp. tested	Temperature 28C-day, 28C-night		35C-day, 28C-night	
		DAI [1]	%disease [2]	DAI	%disease
Cs95	*C. sativa*	2.58 c [3]	42.2%	3.5 e	80%
Cs95 nit	*C. sativa*	2.52 c	42.2%	3.2 de	60%
Cs109-2	*C. sativa*	2.57 c	42.2%	2.4 c	40%
Cs109-2 nit	*C. sativa*	2.63 c	42.2%	2.8 cd	55%
Cs72	*C. sativa*	2.37 c	36.7%	3.4 e	80%
0-1080[4]	*C. sativa*	1.21 ab	0%	1.6 b	5%
Control[5]	*C. sativa*	1.10 a	0%	1.4 ab	5%
Control	Tomato	1.16 a	0%	-[6]	-
0-1080	Tomato	2.81 cd	53.4%	-	-
LSD(0.05)	0.445				

[1] Mean disease average index. Disease average index was rated using 1 (no disease) to 5 (dead plant) rating scale.

[2] Mean percent disease was calculated using = a/b x 100, where a - number of plants with disease symptoms (3-5), and b- total amount of *C. sativa* plants per treatment (1-5).

[3] Means with the same letter are not significantly different ($p \leq 0.05$).

[4] *Fusarium oxysporum* f. sp. *lycopersici* (ATCC 0-1080).

[5] Control plants inoculated with autoclaved food based formulation.

[6] Data not included.

At constant 28 C 35-40% of treated plants were dead or severely wilted in a period of 6-8 weeks, while 40-80% of treated *C. sativa* plants grown at 28 C night and 35 C day showed severe wilt and death in period of 3-5 weeks. Virulence at 22 C was inconsistent and is therefore not presented.

The average disease ratings in treatments of all *F. oxysporum* isolates were significantly higher than disease ratings of the control plants in both growth chamber experiments (Table 2.1). The average disease ratings of plants inoculated with *F. oxysporum* isolates Cs72, Cs95, Cs95 *nit*-1 were significantly higher at 28 C/night, 35 C/day temperature than disease ratings of plants inoculated with the same isolates when grown at constant 28 C day and night . The level of virulence of the nitrate nonutilizing mutants of strains Cs95, Cs109-2 did not differ significantly from their wild type parents.

Vegetative Compatibility

The ability of the nitrate nonutilizing mutants of *F. oxysporum* isolates from the Chu River Valley to form heterokaryons between each other with wild type mycelial growth on nitrate containing media suggest that they are closely related and belong to the same vegetative compatibility group.

Greenhouse Host Range Study

The data involving host range evaluation at 28 C is presented in Table A.1. (Appendix A). The temperature regimes of 35 C day and 28 C night was too stressful for some non-host plants tested, resulting in death of noninoculated control tomato plants.

The host range tests included mostly non target crop plant species grown in the region. Symptoms of *Fusarium* wilt were not observed on any of the tested plant species inoculated with *F. oxysporum* isolates Cs72, Cs95, Cs109-2, Cs95 nit1 with exception of *C. sativa*. Wilt symptoms were observed in tomato plants inoculated with *F. oxysporum* f. sp. *lycopersici*, but were not observed in *C. sativa* plants inoculated with *F. oxysporum* f. sp. *lycopersici*.

Field Pathogenicity Studies 1996-97

First symptoms of disease on *Cannabis* plants in the field were characteristic wilting symptoms of the lower leaves followed by progression of wilting of upper leaves. The symptoms observed in diseased *Cannabis* plants in experimental plots were identical to the wilt symptoms observed in the growth chamber inoculations and in diseased plants in natural stands in the Chu River Valley. Severe wilting symptoms resulting in stunted growth and death of *C. sativa* plants were observed on most of the experimental plots treated with the pathogen. The average disease ratings of the *C. sativa* plants in the field plots inoculated with *F. oxysporum* were significantly higher ($p \leq 0.05$) than disease ratings of the control plants in field experiments in two consecutive years (Table 2.2).

Discoloration of the vascular tissue was also observed in the pathogen treated plants that did not show external wilting symptoms. *F. oxysporum* was reisolated from all surface sterilized diseased tissue and was morphologically indistinguishable from original isolates.

Table 2.2. Disease Severity in *C. sativa* Plants Grown in Field Soil Infested with *F. oxysporum* f. sp. *cannabis* Isolates During the Summers of 1996 and 1997

Strain number	1996		1997	
	DAI [1]	%disease[2]	DAI	%disease
Cs95	2.51 c [3]	36.7%	2.49 c	41.3%
Cs109-2	3.05 d	53.8%	2.61 c	40.0%
Cs72	2.28 b	31.3%	2.11 b	25.0%
Control	1.15 a	3.8%	1.05 a	1.3%
LSD(0.05)	0.207		0.248	

[1] Means of disease average indexes. DAI was rated using a 1- (no disease) to 5 - (dead plant) rating scale for each of four experimental plots within a treatment.
[2] Means of percent disease. Percent disease was estimated for each of four experimental plots within a treatment. Percent disease = a/b x 100, where a - amount of *C. sativa* plants with disease symptoms per experimental plot (3-5), and b - total amount of *C. sativa* plants per experimental plot (1-5).
[3] Means with the same letter are not significantly different ($p \leq 0.05$).

Death in the control treatments 3.8% (3 out of 80 control plants) in 1996 and 1.3% (1 out of 80 control plants) in 1997 was probably due to other factors, because vascular tissue did not show discoloration and surface sterilized tissue did not yield *F. oxysporum*.

The progression of disease on *Cannabis sativa* inoculated with the *F. oxysporum* isolates during summer 1997 field experiments was similar to that from the previous year field experiment and is presented in Figure B.3. (Appendix B). Plant death was recorded 4 weeks after inoculation in all *F. oxysporum* treatments. In about 8-9 weeks after inoculation, the progression of Fusarium wilt leveled out in most of the pathogen treated

experimental plots. At the end of the 1996 field experiment about 40.6 % of all *C. sativa* plants treated with *F. oxysporum* isolates were dead or showed severe wilting symptoms. At the end of the 1997 field experiment about 35.4 % of all *C. sativa* plants treated with *F. oxysporum* isolates were dead or showed severe wilting symptoms.

Discussion

Several hundred samples of diseased *Cannabis* tissue were collected from eleven locations within the Chu River Valley. The majority of samples collected from diseased *Cannabis* plants exhibiting symptoms of vascular wilt, including stunted growth, yellowed leaves and vascular tissue discoloration yielded the fungus identified as *Fusarium oxysporum*. Koch's postulates were confirmed in growth-chamber studies. The pathogenicity of the isolates of *F. oxysporum* to *C. sativa* was verified in field and growth-chamber studies. In growth-chamber studies the observed level of vascular wilt in *F. oxysporum* treated plants was significantly different from the control treatments ($p \leq 0.05$) both at 28 C constant (day and night) and at 35 C day temperature sets (Table 2.1).

Limited host specificity studies did not reveal any pathogenicity outside the host genus. The pathogenicity and host range indicate that the pathogen is a distinct formae specialis of *F. oxysporum*, and therefore referred as *F. oxysporum* f. sp. *cannabis* (Noviello and Snyder, 1962). This suggested that *Fusarium oxysporum* f. sp. *cannabis* could be used as a mycoherbicide for control of *Cannabis* population in the Chu River Valley. In addition to this study, Fusarium wilt was reported to cause serious loss in the

cultivated hemp crop in Italy (Noviello and Snyder, 1962) and was proposed as a potential biocontrol agent for unwanted *C. sativa* (McCain and Noviello, 1984).

Positive isolation of the selected *Fusarium oxysporum* strains from infected plant material was made possible by generating nitrate non-utilizing mutants. Vegetative compatibility studies indicated that tested *Fusarium oxysporum* isolates Cs72, Cs95 and Cs109-2 are closely related. These isolates originated from the diseased plant material collected from three different locations within the Chu River Valley. The pathogenicity evaluation of all three isolates, despite their relatedness, was conducted to see if they had similar level of virulence to *C. sativa*. The level of virulence of the isolates was not significantly different ($p \leq 0.05$) at constant 28 C (Table 2.1). We found that nitrate non-utilizing mutants of *F. oxysporum* f. sp. *cannabis* retained their pathogenicity. This has been shown to be true for *F. oxysporum* f. sp. *vasinfectum* by Katan, T., and Katan, J. (1988). Hadar, et al. (1989) showed that the pattern and rate of *nit* mutant survival in the soil were similar to their wild type parents.

Disease incidence was higher under growth-chamber conditions than observed in the field experimental plots. Environmental factors, such as temperature fluctuations, water stress, soil type, and interactions with other soil organisms, may influence the activity of the pathogen or disease progress and severity. For example the results showed that under greenhouse conditions, a higher incidence of wilting disease was observed at higher temperatures. This is not surprising because temperatures of 35- 45 C during summer time are not unusual in the Chu River Valley. In at least three other cases, high temperatures are known to favor Fusarium wilt. Constant high temperature (35 C) is

known to favor development of Fusarium wilt of chrysanthemum caused by *Fusarium oxysporum* f. sp. *chrysanthemi* (Gardiner, 1987). Clayton (1923) found that the most favorable conditions for tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* was 28 C with short periods where temperature rise to 33 or 35 C. For Fusarium wilt of chrysanthemum, 29 C with increases to 35 C during the day time were the best for symptom development (Gardiner, 1989).

Significant differences in the field pathogenicity of tested strains to *C. sativa* were observed as compared to control treatments ($p \leq 0.05$). In field tests conducted in Kazakhstan during the summers of 1996 and 1997 about 35% of *Cannabis* plants in experimental plots treated with the sawdust formulation of *F. oxysporum* f. sp. *cannabis* died or exhibited wilting symptoms. The impact of this disease in native stands suggests that higher disease levels could possibly be achieved. Also eradication of every last weed is not necessary if the population is reduced enough to prevent the weed from competing with desirable plants (Wapshere, et al. 1989; Strobel, 1991). Therefore for the long term control of a *Cannabis* population, this pathogen could be a very promising biocontrol agent in the Chu River region. Development of more effective inoculum formulations is a possible way to enhance the performance of the mycoherbicide, as well as an improvement of the biocontrol agent itself may be possible.

The search for more effective biocontrol agents against *Cannabis* is an ongoing process in the Chu Valley region and in other locations throughout Kazakhstan. The search for mycoherbicidal pathogens is not restricted to the pathogens of *Cannabis*, but also other weed species such as leafy spurge and spotted knapweed.

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CHAPTER 3

A NOVEL INOCULATION METHOD: SOIL PENETRATION OF A MYCOHERBICIDE FACILITATED BY CARRIER SEEDLINGS

Introduction

Fusarium wilt was reported to cause serious loss in the cultivated hemp crop *Cannabis sativa* in Italy (Noviello and Snyder, 1962). Due to its restricted host range this pathogen was proposed as a potential biocontrol agent for unwanted *Cannabis sativa* (McCain and Noviello, 1984).

Screening of *F. oxysporum* isolates for their potential as biocontrol agents relies mostly on pathogenicity tests, which determine the ability of an organism to kill a plant species. These tests are useful, but bear some inherent problems, one of which is an effective method of inoculation (Kraft and Haglund, 1978; Armstrong and Armstrong, 1981). In addition, these pathogens can be sensitive to numerous environmental factors, such as temperature, relative humidity, rainfall, etc. They are also dependent on the age and susceptibility of the host cultivar (Hart and Endo, 1981).

The pathogenic strains of *Fusarium oxysporum* f. sp. *cannabis* used in the experiments described herein were originally isolated from diseased *Cannabis sativa* plants in the Chu River Valley, Kazakhstan. Greenhouse tests in quarantine have proven the virulence of these *Fusarium oxysporum* f. sp. *cannabis* isolates on *C. sativa*. In these trials mortality varied between 40 and 80% at 28 C and 35 C, respectively (Tiourebaev, et

al. 1998). The host range tests that have been conducted previously included mostly non target crop plant species grown in the region, and the pathogenicity of these isolates was limited to *C. sativa*. Field trails conducted in Kazakhstan during the summers of 1996 and 1997, using a Birch Sawdust Formulation (BSF), resulted in 35% of *Cannabis* plants either dying or exhibiting severe wilt symptoms (Tiourebaev, et al. 1998).

However, the observed level of naturally occurring *Fusarium* wilt in some locations in the Chu River Valley plus the fact that the disease incidence was higher under growth-chamber conditions at higher temperatures indicates many environmental factors such as temperature fluctuations (Gardiner, 1989), water stress, soil type (Grazia-Garza, 1998), and interactions with other soil organisms may influence the activity of the pathogen or disease progress and severity. Some of these factors i.e. soil type, soil water potential, and soil pH (Oritzejafor, 1986) may interfere with successful penetration from the soil surface into rhizosphere of the target plant (Connick, et al. 1998). Several reports showed that there can be significant decrease in the inoculum density from the site of application (soil surface) to the site of infection (roots of target weed) (Ben-Yephet, 1994), as well as significant decline in the population density of the mycoherbicide with time (Couteaudier, 1990; Toyota, 1992).

I theorize that the mycoherbicide kill rate could be increased by improving the delivery of the pathogen to the target plant. Downward movement from the soil surface into the rhizosphere and propagule density of a mycoherbicide are two factors limiting the success of possible soilborne biocontrol agents. To overcome these limitations, I evaluated the effectiveness of using live seed of non target plant spp., coated with fungal

spores as a delivery system for the mycoherbicide. If *Fusarium oxysporum* f. sp. *cannabis* is nonpathogenic on plant species other than *Cannabis*, then one might expect that as a saprophyte it would successfully grow on the root surface of a non-host plant. As the live seed germinates and sends roots into the soil, the *Fusarium* multiplies, continues to colonize emerging tissue, and is carried further into the soil. This effectively carries the fungus through the soil environment, where it can contact host tissue and cause disease in the target species. An added benefit of live seed formulations, besides their ability to effectively deliver pathogens, is the ability to introduce a reclamation plant species as part of the treatment.

The objective of this study was to investigate the movement of *Fusarium oxysporum* strain Cs95, which is pathogenic to *Cannabis sativa*, through soil. The following were steps in this process:

a) Compare the efficacy of various pathogen delivery systems, including traditional soil surface inoculation methods and coated live seed formulations.

b) Establish experimental field plots to demonstrate the efficiency of this novel inoculation system in the field.

Materials and Methods

Fungal Cultures and Preservation

Fusarium oxysporum f. sp. *cannabis* strains were isolated from diseased *Cannabis sativa* plants in the Chu River Valley, Djambil Region, Kazakhstan from 1995 to 1996.

Stem, leaf, and root samples were collected from plants exhibiting symptoms of vascular wilt, stunted growth, yellowed leaves, and vascular tissue discoloration. Plant material was surface sterilized in a 0.5% sodium hypochlorite solution and plated onto 2 % water agar amended with 50 mg/L streptomycin sulfate and 15 mg/L tetracycline (Sigma Chemicals, St. Louis, MO). Plates were incubated at ambient temperature for 48 hours and hyphae were transferred to Potato Dextrose Agar (PDA) (Difco, Detroit, MI) for purification and identification (Nelson, 1983; Barnett, 1987; Brayford, 1993). Fungal isolates used during this experiment were preserved by placing sterile toothpicks onto actively growing cultures, that previously had been single spored. Once toothpicks were completely colonized by mycelia (4-5 days), they were harvested, placed into sterile No. 2 dram vials (Wheaton Scientific, Milleville, NJ) and dried overnight with loose lids in a laminar flow hood. The lids of the vials were then tightened and the cultures stored at 5 C. Viability of cultures stored on toothpicks has been observed to exceed three years.

Marking of Fungal Cultures. Chlorate mutants were selected to positively identify the fungi that were reisolated during the experiment (Hadar, 1989). Nitrate non-utilizing mutants were generated by plating a liquid suspension of *Fusarium oxysporum* f. sp. *cannabis* Cs95 onto media containing 3% potassium chlorate (Correll, et al. 1987). Colonies showing wild type growth were isolated and characterized on minimal media containing sodium nitrate, sodium nitrite or hypoxanthine as the sole nitrogen source. An auxotroph of strain Cs95 lacking nitrate dehydrogenase (*nit* 1) was selected for further analysis.

Fungal Spore Suspension. Fungal cultures were grown in 0.5 L flasks containing 200 ml PDB (Difco) for 7-10 days on a shaker (200 rpm) at ambient temperature, until a concentration of 10^8 microconidia/ml was achieved. The liquid cultures were filtered through 4 layers of cheese cloth and the final spore concentration was adjusted to 10^7 sp/ml with additional sterile water.

Test Plants and Seeds

The seeds used during this experiment were *Cannabis sativa* (var. Afghan), *Festuca arundinaceae* (var. Apache), Blue Bunch wheatgrass (*Agropyron spicatum*), *Lycopersicon esculentum* (cv. Bonnie Best). The seeds of selected plant species were surface sterilized in a 0.5% sodium hypochlorite solution for 3 minutes, rinsed with sterile distilled H_2O and dried in a laminar flow hood.

Inoculum Production

Food Based Granular Formulation. Canola Flour Mix (CFM) employed in greenhouse studies was prepared by combining equal parts ground canola flour and wheat flour with 1% by volume activated charcoal and enough water amended with 0.01% Silwet L-77 surfactant to make the mixture stick together. This formulation is very effective in greenhouse studies, but is fairly labor intensive and expensive to produce.

In contrast, another granular food based formulation used both in greenhouse and field inoculations - Birch sawdust formulation (BSF) involves a readily available and inexpensive carrier and it was effectively formulated by coating 100 g of birch sawdust

with 50 milliliters of a 0.1 % yeast extract and 1 M citric acid solution (pH 5.5). This formulation is well mixed and then autoclaved. The spore suspension (10^7 spores/ml) is added to both of these formulations at a rate of 10 ml of spore suspension/ 100 grams of carrier. This mixture is air dried under a laminar flow hood for 4-6 hours. The formulations were stored in sealed paper bags.

Live Seed Formulation. Live seed formulations (LSF) of *C. sativa* and non host seeds *Festuca arundinaceae* (var. Apache), Blue Bunch wheatgrass (*Agropyron spicatum*), *Lycopersicon esculentum* (cv. Bonnie Best) were prepared following the protocol described by (Grey and Mathre, 1988). One hundred seeds of each plant spp. were surface sterilized in a 0.5% NaOCl solution for 3-5 min, then immersed into different available coating solutions, stirred, and allowed to air dry in a laminar flow hood for 1-2 hours. Available coating agents used were 5% carboxymethyl cellulose (CMC), 2% methyl cellulose (MC), Mycotech oil (Oil) and liquid Potato Dextrose Broth spore suspension (SS). After drying the coated seeds were allowed to soak in 10 ml of a conidia suspension (10^7 spores/ml) of *Fusarium oxysporum* f. sp. *cannabis* for 10 minutes. The coated seeds were then air dried for 24 hours and stored at 4 C.

To determine the fungal spore concentration per seed, 10 seeds from each treatment were placed into 1 ml of sterile H_2O, shaken for 5 min and the resulting spore suspension was counted with a hemacytometer (4 DF, Neubauer 0.1 mm). The test included five treatments with replication (n = 5). The mean fungal spore concentrations per seed were obtained and data were analyzed in completely randomized design with five

replications per treatment.

Colonization of *C. sativa* Seedlings by *F. oxysporum* f. sp. *cannabis*

The ability of *Fusarium oxysporum* f. sp. *cannabis* to colonize emerging root tissue of the host seedlings was examined. Live seed formulation of *C. sativa* seeds was prepared as described above using CMC and a spore suspension of *Fusarium oxysporum* f. sp. *cannabis* isolates Cs72, Cs109-2, Cs95. Coated pregerminated *Cannabis* seeds were then placed on water agar plates, three plates per treatment, each containing ten seeds. One week after incubation at ambient temperature ten seedlings from each treatment selected for root homogeneity (~ 5 cm) were washed with 50 ml of dH_2O. The number of cfu per root was determined after dilution plating of the resulting suspension on Komada's medium (Komada, 1975). Three replicates per dilution level were spiral plated for the enumeration of fungal colonies. Each colonization experiment was performed at least three times. The concentration of conidia in CFU washed from the seedlings' roots was compared to that initially applied.

Analysis of the Depth and Concentration of *Fusarium* Spores

The effect of inoculum formulations on the downward movement of *Fusarium oxysporum* f. sp. *cannabis* Cs95 through a soil profile was examined using the following system shown in Figure B.4. (Appendix B). Five milliliter plastic pipetor tips (15 cm - length, 1 cm - diameter) were filled with Montana State University Plant Growth Center (PGC) soil mix amended with the wetting agent Aqua-Gro 2000. The soil columns were

placed into 25 ml glass test tubes, covered with plastic caps and sterilized with two autoclaving cycles (60 min, 120 C). Four ml of sterile water was added aseptically to each soil column and allowed to equilibrate, and excess water was allowed to drain off. No more water was added during the experiment. This system allows plant seedlings to grow for more than two weeks in sterile conditions without watering. This allowed us to exclude movement of the fungal spores facilitated by added water. The soil columns were inoculated with a nitrate nonutilizing mutant (*nit*-1) of *Fusarium oxysporum* f. sp. *cannabis* strain Cs95 in different inoculum formulations in a complete randomized fashion. The inoculum formulations used in this experiment were: Live seed formulation (*Cannabis sativa* seeds), food based formulation (CFM), and liquid spore suspension. In treatments using live seed formulation, each individual soil column was inoculated with a single coated seed. The concentration of *Fusarium oxysporum* microconidia on each seed was approximately 10^4 spores. The spore concentration on the food based formulation, CFM, was adjusted to the same spore concentration per tube. In the treatment with liquid spore suspension the top of the soil column was infested with 20 microliters of 10^6 spores/ml suspension. Sterile seeds of *C. sativa* coated in CMC were used in the control treatment. The soil columns were then placed inside glass test tubes, covered with plastic caps and incubated at 28 C (19 hours light / 5 hours dark). The space between the plastic cap and soil surface provided enough room for seedlings to grow for 10 days after germination. Ten days after germination, the soil columns were cut into 1 cm segments, which contained 1 g of wet soil. Soil segments were serially diluted in a 1% gelatin solution. Soil dilutions were spiral-plated (Spiral plater Model C, Spiral System Inc) onto PDA

plates containing 3% $KClO_3$ (Sigma). The roots in all soil columns tested reached a depth of 9 cm at the end of the experiment. The experiment was conducted in autoclaved and non autoclaved PGC soil and repeated at least once. Each experiment contained four treatments with replication (n=5). Treatments included were: Live seed formulation - *Cannabis sativa* (LSFcs), Food based formulation (CFM), Liquid spore suspension (SS), non inoculated soil columns - control. The results from the tests are presented (Table 3.1; 3.2). Soil dilution data (0's) from control treatment were not included in the data analysis. The mean fungal concentration was estimated and data was analyzed as a factorial with soil depth and inoculum as two factors in completely randomized design with five replications per treatment.

A similar experiment examined the effect of different Live seed formulations. The nitrate non utilizing mutant of *Fusarium oxysporum* f. sp. *cannabis* Cs95 (*nit*-1) was formulated onto the live host seeds of *Cannabis sativa,* and also on seeds of the non host species Bluebunch wheatgrass and tomato. The experiment involved the following treatments: *Cannabis* seeds, Bluebunch wheatgrass and tomato seeds coated with CMC without fungal spores = sterile control (Ctrl); liquid spore suspension (ss); Food based formulation (CFM); autoclaved CFM (aCFM); Live seed formulation - *Cannabis* seeds (LSFcs); Live seed formulation - Bluebunch wheatgrass seeds (LSFbwg); Live seed formulation - Tomato seeds (LSFt). Each treatment was applied to 5 soil columns assigned in complete randomized fashion. This experiment contained 9 treatments with replication (n=5). The experiment was repeated at least once. Soil dilution data (0's) from control treatment were not included into data analysis. The mean fungal

concentration was estimated and data was analyzed as a factorial with soil depth and inoculum as two factors in a completely randomized design with five replications per treatment.

Evaluation *C. sativa* Plants Affected by Fusarium Wilt

Individual plants were rated for disease severity on a scale of 1 to 5, where 1 = no disease, 2 = minor wilting of lower leaves, 3 = wilting symptoms observed on 25-50% of leaves, 4 = >75% leaves show severe wilting symptoms, 5 = dead plant.

Disease incidence percent (%) was estimated for each treatment as = a/b x 100%, where a = number of *C. sativa* plants with severe disease symptoms (from 3 through 5) and b = total number of *C. sativa* plants per treatment (from 1 through 5).

Field Studies of Potential of Live Seed Formulation

Field plots to determine the virulence of live seed formulations of *Fusarium oxysporum* f. sp. *cannabis* strain Cs95 were established at the Kazakh Institute of Agriculture Experiment Station, Academy of Agriculture, Republic of Kazakhstan. Viable *Festuca arundinaceae* seeds were chosen for LSF (LSFf), due to their availability, their high germination rate and mainly because this plant species is already present in the region. Also an important feature of *Festuca arundinaceae* is its wide adaptation, growing on a wide variety of soils, from highly acid to highly alkaline, which is probably the widest of any grass species (Wheeler and Hill, 1957).

Field testing of the performance of the live seed formulations involved two

experiments. In one experiment inoculations of experimental plots (one meter square) were performed prior to the planting of *Cannabis*. The following treatments were included: Live seed formulation (*Festuca arundinaceae)* with the mycoherbicide = LSFf; sterile LSFf (without mycoherbicide); Food based formulation (birch sawdust formulation) = BSF; autoclaved BSF; non inoculated plots. Each treatment was applied to three experimental plots. After *Festuca arundinaceae* seeds germinated (about one week after inoculation) the experimental plots were seeded with 100 *C. sativa* seeds. Upon germination *C. sativa* seedlings were thinned down to 20 plants per plot.

In the second experiment inoculations were performed on established *Cannabis* plots. The experiment included the following treatments: BSF, autoclaved BSF, LSFf, sterile LSFf, non inoculated *C. sativa*. Each treatment was applied in completely randomized fashion to three, one meter square plots containing 20 *C. sativa* seedlings. Plants were inoculated at 2-3 true pair leaf stage.

In both experiments all plots were inoculated with 10 g of inoculum per plot. All plots were watered by hand on a daily basis. Formulations were prepared according to the protocol established in the previous sections.

Each experiment included five treatments. An experimental unit consisted of 20 *C.sativa* plants replicated three times. The average disease ratings of the *Cannabis* plants in the field experimental plots were obtained and data from each experiment were analyzed separately in complete randomized design with three replicates per treatment. Analysis of variance was performed using the SAS program. To compare differences between more than two treatment means, least significant differences were calculated at $\alpha = 5\%$.

Results

Adhesive Ability of Tested Seed Coating Substances to Hold Fungal Spores

The choice of the coating agent depended on its ability to hold fungal spores on the seed surface. The CMC coating was chosen over methyl cellulose (MC), Mycotech oil (Oil) and liquid Potato dextrose broth spore suspension (SS) (Table 3.1).

Table 3.1. Mean Fungal Spore Concentration Washed off from Seeds Coated with Available Coating Agents

Coating Agent	*Cannabis* seed	tomato seed	grass seed
	log sp/ml [1]	log sp/ml	log sp/ml
Control	0 a [3]	0 a	0 a
Spore suspension	4.39 bc	4.27 b	4.78 d
Mycotech oil	4.60 cd	5.25 e	5.30 e
Methyl cellulose	4.81 d	4.78 d	5.49 e
Carboxymethyl cellulose	5.38 e	5.52 e	5.34 e
LSD (0.05) 0.309			

[1] Data were log transformed prior to analysis.
[2] Values are means of 5 replicates, and values followed by the same letter are not significantly different at $p \leq 0.05$. Ten seeds from each treatment were placed into 1 ml of sterile H_2O, shaken for 5 min and the resulting spore suspension was counted with a hemacytometer (4 DF, Neubauer 0.1 mm). The test included five treatments with replication (n = 5).

All of the treatments resulted in successful conidia germination and mycelium growth was observed after 2-3 days in all treatments.

Colonization of *C. sativa* Seedlings by *F. oxysporum* f. sp. *cannabis*

Due to its ability to grow saprophytically, *Fusarium oxysporum* f. sp. *cannabis* could colonize emerging root tissue initially without killing plant seedlings Figure B.5. (Appendix B). The regulation mechanism responsible for the balance between the saprophytic and the pathogenic state of the pathogen is not well understood. The propagule concentration washed from the seedling roots was compared to that initially applied (Table 3.2). The increase in propagule numbers on emerging root tissues was up to four fold when compared to that initially applied in LSF. Further studies were carried out using mainly *F. oxysporum* f. sp. *cannabis* strain Cs95.

Table 3.2. Increase in *F. oxysporum* f. sp. *cannabis* Propagules During Colonization of Emerging Root Tissue of *C. sativa* Seedlings

Strain	Initial cfu/ml	After colonization cfu/ml	Increase cfu/ml
Control	0	0	0x
Cs95	10^4	10^8	10^4x
Cs109-2	10^4	10^8	10^4x
Cs72	10^4	10^6	10^2x

Live seed formulation of *F. oxysporum* f. sp. *cannabis* isolates Cs72, Cs109-2, Cs95 on *C. sativa* seeds was prepared using Carboxymethyl cellulose.

Analysis of the Depth and Concentration of *Fusarium* Spores

The data suggests that the propagule concentration decreases with increasing soil depth (Table 3.3 and Table 3.4). When a pathogen is applied as a liquid spore suspension, downward movement of the pathogen in a soil column without any additional water was

limited to 3 cm. When the pathogen was applied as a granular food based formulation (CFM), fungal spores could be detected in the soil to a depth of 5 cm, but the spore concentration decreased about three logs.

Table 3.3. Movement of *F. oxysporum* f. sp. *cannabis* Isolate Cs95 (*nit*-1) in Soil Columns Facilitated by *C. sativa* Roots in Autoclaved Soil

Treatment[1]	Concentration of *F. o. c.* in a soil column cfu/g (log)				
	1 cm	3cm	5cm	7cm	9cm
Control	0 a[2]	0 a	0 a	0 a	0 a
ss	5.7 de	4.42 c	0 a	0 a	0 a
CFM	5.91 e	4.52 c	2.47 b	0 a	0 a
LSFcs	5.64 de	4.82 cd	4.71 c	4.37 c	3.91 b
LSD(0.05) 0.88					

[1] Control = *C. sativa* seeds coated with Carboxymethyl cellulose (sterile control).
ss = liquid spore suspension.
CFM = Canola Flour Mix inoculum.
LSFcs = *C. sativa* Live seed formulation.

[2] Values are means of 5 replicates; data were log transformed prior to analysis and values followed by the same letter are not significantly different at $p \leq 0.05$.

However when the pathogen was applied in the form of Live seed formulation, it was present on the entire length of the root as it grew into the soil. The propagule concentration did not increase as it descended, but rather maintained a relatively constant spore concentration along the entire root length. These results suggest that movement of

the pathogen in the soil was facilitated by the root growth of the host plant which also allowed multiplication of the pathogen on the root surface. Live seed formulation resulted in significantly higher propagule concentration than Food based formulation at increasing depth of the soil columns filled either with autoclaved (Table 3.3) or non-autoclaved soil (Table 3.4). Further investigation was carried out in non-autoclaved soil.

Table 3.4. Movement of *F. oxysporum* f. sp. *cannabis* Isolate Cs95 (*nit*-1) in Soil Columns Facilitated by *C. sativa* Roots in Nonautoclaved Soil

Treatment[1]	Concentration of *F. o. c.* in a soil column cfu/g (log)				
	1 cm	3cm	5cm	7cm	9cm
Control	0 a[2]	0 a	0 a	0 a	0 a
ss	5.56 e	4.06 c	0 a	0 a	0 a
CFM	5.59 e	4.15 c	1.91 b	0 a	0 a
LSFcs	5.68 e	4.69 d	4.22 cd	4.04 c	3.76 c
LSD(0.05) 0.539					

[1] Control = *C. sativa* seeds coated with Carboxymethyl cellulose (sterile control).
ss = liquid spore suspension.
CFM = Canola Flour Mix inoculum.
LSFcs = *C. sativa* Live seed formulation.

[2] Values are means of 5 replicates; data were log transformed prior to analysis and values followed by the same letter are not significantly different at $p \leq 0.05$.

Effect of Live Seed Formulations on Movement of the Mycoherbicide

In the treatments when *F. oxysporum* Cs95 was applied in the form of Live seed formulation using Bluebunch wheat grass, the pathogen was recovered from soil at the

same depth as when applied to the host plant (*C. sativa*) as a carrier (Table 3.5).

Table 3.5. Movement of *F. oxysporum* f. sp. *cannabis* Isolate Cs95 (*nit*-1) in Soil Columns Facilitated by Roots of Host and Non-host Plants in Nonautoclaved Soil

Treatment[1]	Concentration of *F. o. c.* cfu/g (log)				
	1 cm	3cm	5cm	7cm	9cm
Control	0 a[2]	0 a	0 a	0 a	0 a
ss	5.65 i	3.7 fe	0 a	0 a	0 a
CFM	5.75 i	4.36 fg	0.10 a	0 a	0 a
LSFcs	5.63 i	4.77 hg	4.45 g	4.27 feg	3.59 e
LSFbwg	5.18 hi	2.59 d	2.49 cd	2.53 dc	1.06 b
LSFt	4.69 hg	1.86 c	1.06 b	0.49 a	0 a
LSD(.05) 0.71					

[1] Control = *C. sativa* seeds, as well as Bluebunch wheatgrass and tomato seeds were coated with Carboxymethyl cellulose (sterile control).
ss = liquid spore suspension.
CFM = Canola Flour Mix inoculum.
LSFcs = *C. sativa* Live seed formulation.
LSFbwg = Bluebunch wheatgrass Live seed formulation.
LSFt = Tomato Live seed formulation.

[2] Values are means of 5 replicates; data were log transformed prior to analysis. Values followed by the same letter are not significantly different at p = 0.05 according to LSD test.

However the concentration of the recovered mycoherbicide was significantly less than in the case of the host seed formulation ($p \leq 0.05$).

Field Studies

The presence of the pathogen within the treated *C. sativa* plants exhibiting wilt disease symptoms was detected by reisolation of the pathogen on Komada's medium. Higher level of disease incidence was achieved on *C. sativa* plants with Live seed formulation than with Food based formulation either on experimental plots inoculated before *Cannabis* was planted or on experimental plots with grown *Cannabis* plants (Table 3.6).

Table 3.6. Disease Severity in *C. sativa* Plants Grown in Field Soil Infested with Different Inoculum Formulations of *F. oxysporum* f. sp. *cannabis* isolate Cs95

Treatment	A Inoculation after planting		B Inoculation before planting	
	DAI[4]	% disease[6]	DAI	% disease
LSF sterile[1]	1.30 b[5]	1.8%	1.23 a	0%
LSF	2.70 d	48.3%	2.52 c	41.7%
BSFsterile[2]	1.08 a	0%	1.13 a	0%
BSF	2.12 c	30%	1.98 b	28.3%
Control[3]	1.05 a	0%	1.10 a	0%
LSD(0.05)	0.264		0.275	

[1] Live seed formulation without pathogen.
[2] Food based formulation autoclaved.
[3] Uninoculated *C. sativa* plants.
[4] Means of disease average indexes. DAI was rated using a 1 (no disease) to 5 (dead plant) rating scale for each of three experimental plots within each treatment.
[5] Means with the same letter are not significantly different ($p \leq 0.05$).
[6] Means of percent disease. Percent disease was estimated for each of three experimental plots within each treatment. Percent disease = a/b x 100, where a - amount of *C. sativa* plants with disease symptoms per experimental plot (3-5), and b - total amount of *C. sativa* plants per experimental plot (1-5).

Experiment included two parts: A - Experimental plots were infested after *C. sativa* reached the stage of 2-3 true leaves, and B - Experimental plots were infested before *C. sativa* was planted (after germination of *Festuca arundinacea* Live Seed formulation). Each test included five treatments, each treatment applied to three individual experimental plots, each plot contained 20 *C. sativa* plants.

Discussion

In field tests in Kazakhstan during the summers of 1996 and 1997, about 35% of *Cannabis* plants in experimental plots treated with a sawdust formulation of *F. oxysporum f. sp. cannabis* died or exhibited wilting symptoms (Tiourebaev, et al. 1998). We believe that the performance of the biocontrol agent can be improved by using other inoculation methods. In the case of most root pathogens, their effectiveness depends on successful soil penetration toward the root zone.

We compared downward movement of the mycoherbicide in the soil columns when applied as different formulations. Recently Grazia-Garza (1998) studied effect of water percolation on movement of *Fusarium oxysporum* propagules in soil columns. He showed that, in general, 10 fold fewer cfu were recovered at 8- to 10-cm depth compared to a 0- to 2-cm depth. The effect of water on downward movement of fungal propagules in most of the soils depended on the amount of water added (Hepple, 1960). However in semi-arid regions with low annual precipitation such as the Chu River Valley it would be hard to rely on passive transport of mycoherbicide by water. In our study we were able to compare downward movement of fungal propagules in soil columns without any water added during the experiment after inoculation. Our study indicated that *Fusarium*

oxysporum f. sp. *cannabis* propagule density declined in the top 3-5 cm of the soil column when applied in the form of food based formulation, both in autoclaved and nonautoclaved soils. In both CFM and SS treatments most of the cfu were recovered from the top 1-3 cm of the column ($p \leq 0.05$) (table 3.3, 3.4, 3.5). Live seed formulation proved to be superior to other tested formulations (liquid spore suspension, Canola flour mix). The propagules of the mycoherbicide could be recovered from significantly deeper soil depth and at significantly higher numbers when applied as Live seed formulation, as compared to the tested food based formulations. Due to the ability of *Fusarium oxysporum* to grow saprophytically on non-host tissues, the mycoherbicide can be applied at lower rates when applied in a form of Live seed formulation. Initial pathogen concentration can be reduced 10^3- 10^4 times. This could be important for large scale application of mycoherbicides, both in terms of the cost and the reduction of the impact of introduced microorganisms on soil microflora.

The significant change in fungal spore concentration from the soil surface downward varied depending on which carrier seed was used for the Live seed formulation. This suggests that more efficacious carrier seed/seedling systems may yet be discovered.

The average disease ratings of *Cannabis sativa* plants in the field experimental plots inoculated with the Live seed formulation of *Fusarium oxysporum* were significantly higher than in plots inoculated with Food based formulation. Higher disease incidence caused by Live seed formulation was observed both in established *Cannabis sativa* plots and in plots inoculated prior to *Cannabis sativa* planting than caused by Food based formulation.

In conclusion, this novel method of mycoherbicide application improves penetration of the biocontrol agent into the rhizosphere zone of the target weed. In addition to providing a possible spore delivery mechanism, a beneficial species can also be introduced by serving as the seed carrier, thus providing a hand picked successor species. Live seed delivery systems would offer the added benefit of providing a means to eradicate weeds and re-seed treated areas with a single treatment.

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CHAPTER 4

A NOVEL APPROACH TO VIRULENCE ENHANCEMENT OF MYCOHERBICIDES

Introduction

Most mycoherbicides are host-specific pathogens incapable of effectively destroying the target plant. Can pathogenic fungi be safely enhanced for virulence without increasing their host range? The approach we describe is a novel way to enhance the virulence of biocontrol agents without producing potentially dangerous metabolites. In addition, this approach does not use recombinant genetics, thereby decreasing the time and testing involved with biosafety considerations. The basic background behind this study involves aspects of intermediary metabolism in plants and microbes.

Most modern chemical herbicides inhibit a single biosynthetic enzyme in a plant, rendering the plant incapable of producing a metabolite essential for plant growth. The lack of this metabolite eventually leads to plant death. Some examples of chemical herbicides that interfere with amino acid biosynthesis are Glyphosate, sulfonylureas, imidazolinones, 1, 2, 4-triasol pyrimidines, etc. glyphosate inhibits 5'enolpyruvylshikimate 3-phosphate synthase (EPSP), which is the key enzyme in the shikimic acid pathway (Amrhein, 1986). Another common target enzyme is acetolactate synthase (ALS), which is an early enzyme in branched amino acid biosynthesis. ALS is a unique herbicide target, because several structurally different compounds inhibit the enzyme (sulfonylureas,

imidazolinones, 1, 2, 4-triasol pyrimidines). This enzyme can also be inhibited by its own end product amino acids. Feedback inhibition, is a regulatory mechanism by which organisms, including plants, efficiently regulate the synthesis of cellular metabolites. Within a plant cell, the biosynthetic activity of essential enzymes is feedback regulated by the concentration of the end-product of the pathway. Accumulation of end-product shuts down the enzyme, preventing overproduction. In the case of branched biosynthetic pathways of amino acids, there are multiple end-products. However, the regulating enzyme in the branched pathway is often inhibited by a single end product. This may lead to shutdown of biosynthesis of the entire branched pathway leading to inhibition of growth. For example, isoleucine inhibits ALS which is important for biosynthesis of not only isoleucine, but also two other essential amino acids, valine, and leucine, and the essential vitamin pantothenic acid.

The other biosynthetic families of amino acids and corresponding essential enzymes under feedback inhibition and repression are well documented. In higher plants (as in bacteria) lysine, threonine, and methionine are synthesized in a branched pathway from aspartate (Bryan, 1980; Umbarger and Davis, 1962). The first and the third enzyme in this pathway, aspartate kinase and homoserine dehydrogenase, respectively, are also known to be regulated by end product feedback inhibition (Green and Phillips, 1974). Hence the exogenous application of one end product to the plant can lead to mis-regulation of the pathway, resulting in starvation of the plant for one of the other end products of the pathway. Reversal of this amino acid inhibition usually involves providing all of the end-products (usually just amino acids) in the family simultaneously, to

overcome the requirement for all of the products of the pathway.

Examples of such mis-regulation by exogenous end products are common throughout the plant kingdom. Examples include the "frenching disease" of tobacco where unusual strains of *Pseudomonas flourescens*, a bacterium in the root zone, produce a significant amount of the essential amino acid isoleucine. By overproducing isoleucine in the rhizosphere of the plant, this bacterium inhibits the plant's growth (Steinberg, 1950). Steinberg showed that isoleucine can be used to duplicate frenching symptoms of tobacco in aseptic culture (Steinberg, 1946). The earliest symptom of frenching is chlorosis along the margins of young leaves, which spreads gradually over the entire leaf surface. As the leaf develops, only the midrib elongates thereby producing a distorted narrow leaf. Terminal growth is greatly retarded and apical dominance is lost, resulting in a stunted plant with small distorted leaves. In severe cases, the axillary buds are stimulated into growth (Steinberg, 1950; Lucas, 1965). According to G. Lucas, Steinberg in 1960 reported *Bacillus cereus* is also responsible for the appearance of severe "frenching" symptoms in tobacco seedlings (Lucas, 1965). Higher populations of this wide spread non-pathogenic microorganism were found in the rhizosphere of frenched tobacco than in the rhizosphere of normal tobacco. Roots of frenched tobacco showed unusually large populations of *B. cereus*. Accumulation of large amounts of free isoleucine and other amino acids were also found in the leaf lamina of frenched plants.

Mutants - Overproducers

Regulatory mutants, resistant to feedback inhibition or enzyme repression, have been well defined in prokaryotes (Adelberg, 1958; Umbarger and Davis, 1962), in lower eukaryotes (Maiti, 1988), and in plants (Wu, 1994; Relton, 1986; Carlson, 1973). Feedback inhibition resistant mutants result in overproduction of the pathway end product(s) due to altered regulatory sites, which do not allow normal inhibition of the enzyme. A number of feedback derepressed mutants were obtained by selecting organisms able to grow in the presence of toxic concentrations of an end product amino acid.

An alternative approach would be to consider that the common mechanism of antimetabolite resistance in mutants of microorganisms is the over production of the corresponding metabolite. Lists of candidate amino acid analogs are available, and the choice of several for testing is advisable, as not all amino acid analogs will be inhibitory. Amino acid analogs are known to act as false end product inhibitors or as false repressors for enzymes involved in biosynthesis of amino acids (Rosenthal, 1982; Umbarger and Davis, 1962). A number of amino acid analogues undergo enzymatic transformations the same as the corresponding natural amino acid, while others inhibit various enzymatic activities. An analogue which acts as a competitive substrate and is incorporated into protein prevents the incorporation of only the corresponding natural amino acid; whereas, an inhibitory analogue which is not incorporated prevents protein synthesis, and this prevents incorporation of amino acids other than the corresponding natural amino acid (Rosenthal, 1982; Lea, 1976; Rabinovitz, 1959). An important step in the process of

obtaining mutants is to select an appropriate amino acid analog that inhibits the pathogen by virtue of its structure being similar to the amino acid that we want the pathogen to produce.

The intent of this study is to describe the process of generating specific mutants of plant pathogens, that may be more virulent and more useful as weed biocontrol agents, by the virtue of overproducing one or more inhibitory metabolites.

Screening Amino Acid Excreting Mutants

A commercial bioassay system is available for screening putative amino acid excreters (Difco Products). *Pediococcus cerevisiae* (ATCC 8042) is the assay organism because of its strict requirement for amino acids. In an agar medium with all amino acids except one, the microbe will not grow. The mutants that are being screened are patched onto this agar seeded with *Pediococcus.* After incubation the size of zone of the *Pediococcus* around the patched mutant is an indication of its relative excretion. More quantitative excretion results can be obtained by assay of a fungal or bacterial culture filtrate (Sands and Hankin, 1974).

Materials and Methods

Fungal Cultures

Fusarium oxysporum f. sp. *cannabis* Cs95 - the cause of fusarium wilt of *Cannabis sativa, Fusarium oxysporum* f. sp. *papaver* CP3A, 4P-23 - the cause of

fusarium wilt of *Papaver somniferum*, and *Fusarium oxysporum* f. sp. *lycopercici* 0-1080 (ATCC) - the cause of fusarium wilt of tomato were used for mutant selection. Cultures were maintained as described in previous chapters. For inoculum preparation fungal cultures were grown in 250 ml flasks containing 100 ml Potato Dextrose Broth (PDB) (Difco Laboratories, Detroit, MI) for 5-7 days at ambient temperature with agitation (200 rpm). The concentration of conidia in the culture (primarily microconidia) was estimated with a hemacytometer (4 DF, Neubauer 0.1 mm) and adjusted to the appropriate concentration with sterile water (10^7 sp/ml).

Bacteria

Pediococcus cerevisiae (ATCC 8042) - an auxotrophic strain with strict requirement for valine and other amino acids was used in the standard assay. In an agar medium containing all of the amino acids except one, the microbe will not grow. A bacterial suspension was maintained at - 4 C in 3% glycerol. A single cell colony grown overnight was used in each assay, and for assay media preparation.

Media and Culture Conditions

The following media were used: Minimal agar medium [(3% Sucrose (w/v), 1.7 g/l Yeast extract base without amino acids (Difco Laboratories), and 16 g/l Agar Ultrapure, USB, Cleveland, Ohio)], Valine assay medium (Difco), Potato Dextrose Broth (PDB), Potato Dextrose Agar (PDA), (Difco Laboratories, Detroit, MI), Minimal media + NO_3 , MRS broth (Sigma), MRS agar (Sigma), and L-amino acids (Sigma).

Amino Acid Toxicity Tests on Plants

Germinated seeds of *C. sativa* - marijuana, *Papaver somniferum*- opium poppy, and *Nicotiana tobacum* - tobacco were transferred to minimal media agar plates supplemented with increasing concentrations of amino acids added singly and in combination. Each amino acid or combination of amino acids tested was supplemented at eight concentrations (0 mM, 0.01 mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM, 3 mM, 4 mM), and each treatment was replicated six times. Five pregerminated seeds were placed on each plate, per replication. Plates were incubated at ambient temperature with 12 hour diurnal period for two weeks. The fresh weight per seedling was calculated at the end of the experiment.

Mutant Selection

The inhibitory effect of valine and valine analogs was tested against the selected *Fusarium* spp., using a well zone–diffusion assay on a minimal medium. Seven available valine analogs were tested. The gradient plates were prepared as follows: Minimal media was poured into Petri dishes, then a disk (diameter = 10 mm) of the solidified medium was cut out (with number 5 cutter) from the center of the plate and filled with the sterile solution of an analog (0.5 ml of a 1000 ppm of analog in sterile distilled H_2O). The solution was gradually applied to the well upon its drying out in the hood. The plates were allowed to dry and form a gradient under the hood for 24 hours. A *Fusarium oxysporum* microconidia suspension of 10^7 sp/ml was obtained by washing a 10-14 day fungal culture grown on minimal medium (with nitrate) with 10 ml of sterile H_2O. Five

hundred microliters of the spore suspension was thoroughly spread across the plate with a glass rod. These plates were then incubated at 28 C. Resistant colonies observed within inhibition zones (Figure B.6.) were transferred to Minimal agar medium containing 500 ppm of a corresponding analog, those that retained the ability to grow, were further evaluated.

Screening Amino Acid Excreting Mutants

A bioassay system was used for screening possible amino acid excreters (Difco Products). *Pediococcus cerevisiae* ATCC 8042 was used as the assay organism (1 ml of 10^8 cells/ml suspension per one liter of Valine assay agar media) because of its strict requirement for amino acids. The mutant colonies that were being screened were patched onto this agar seeded with *Pediococcus*. After incubation for 2-3 days at 28 C, the size of the zone of the *Pediococcus* around the patched mutant is an indication of relative excretion Figure B.7. (Appendix B).

Semiquantitative Assay of Excreted Valine

The same assay system described above was used to estimate the amount of valine excreted by the mutants, i.e., Valine assay medium (Difco Products) and *Pediococcus cerevisiae* ATCC 8042 as the assay organism (20 µl of 10^4 cells/ml suspension per 5 ml of valine assay broth). Single celled colonies of valine analog resistant mutants and the wild type strain were grown in minimal medium+NO_3 broth for two weeks at ambient temperature with agitation (200 rpm). Fungal liquid cultures were

centrifuged (30 min at 15,000 rpm), the supernatant was filter sterilized (22 µm pore size). One ml aliquotes of filter sterilized supernatant of each culture were added aseptically to 3 separate 5 ml glass tubes containing 4 ml of valine assay broth inoculated with *Pediococcus cerevisiae*. The tubes were incubated at 37 C until bacterial growth reached its stationary phase between 24- 48 hours. The maximum growth response of *Pediococcus cerevisiae* ATCC 8042 in the presence of valine in fungal filtrates was measured by a Klett apparatus (Klett units). The range of valine concentrations (5 µM, 10 µM, 20 µM) where the linear growth response of *Pediococcus cerevisiae* was observed was used as a standard reference.

Greenhouse Studies

Virulence Evaluation of the Mutants on the Target Host

Cannabis sativa. The virulence of wild type and mutant strains of *Fusarium oxysporum* f. *sp. cannabis* were compared in environmental growth chambers (28 C, 19 hours day, 5 hours night). Both the rate of kill and final mortality were considered important criteria. *Cannabis sativa* plants with two - three pairs of true leaves were soil surface inoculated by placing 0.5 g of food based formulation (CFM) on the soil surface at the base of each plant. Control plants were inoculated with autoclaved inoculum. The experiment included five treatments: wild type (Cs95), mutant isolates 4nv (Cs95), 6pa (Cs95), 8pa (Cs95), and a control treatment. The experimental unit consisted of ten individual *C. sativa* plants grown in 16 cm pots, replicated twice. Plants were watered daily for 2 minutes by an automatic sprinkler system. Plants were evaluated daily for symptoms of

vascular wilt. Individual plants were rated for disease severity as described in Chapter 2. The average disease index was obtained and data was analyzed in randomized block design with two replications per treatment. Tissue from plants exhibiting disease symptoms was surface sterilized in 0.5% NaOCl and plated onto 2 % water agar amended with antibiotics (hyphal tips were transferred to PDA for purification and identification) and onto a minimal medium supplemented with the corresponding amino acid analog (500 ppm).

Papaver somniferum. A similar experiment was performed with the wild type and mutant strains of *Fusarium oxysporum* f. *sp. papaver* (CP3A and 4p-23-2) in environmental growth chambers (28 C, 19 hours day, 5 hours night). *P. somniferum* plants with three-four pairs of true leaves were soil surface inoculated by placing 0.5 g of food based formulation (CFM) at the base of each plant. Control plants were inoculated with autoclaved inoculum. The experiment included six treatments: wild type (CP3A), mutant - 1nv (CP3A), wild type 4p-23-2, mutant isolates 43pa (4p-23-2), 44nv (4p-23-2), and control treatment. An experimental unit consisted of ten individual *P. somniferum* plants, replicated twice. Plants were watered daily for 2 minutes by an automatic sprinkler. Plants were evaluated daily for symptoms of vascular wilt. Individual plants were rated for disease severity as: 1 - healthy, 2 - slight wilt symptoms (leaf yellowing/discoloring, leaf tips curling), 3 - severe wilt symptoms (several leaves wilting, crown discoloring/rot), 4 - dead. The disease average index was obtained and data were analyzed in randomized block design with two replications per treatment. Tissue from plants exhibiting disease

symptoms was surface sterilized in 0.5% NaOCl and plated onto 2 % water agar amended with antibiotics (hyphal tips were transferred to PDA for purification and identification) and onto minimal medium supplemented with the corresponding amino acid analog (500 ppm).

Host Range Evaluation. The host range of selected mutants and wild type isolates was evaluated for virulence in environmental growth chambers on the following crop plant species: *Lycopersicon esculentum* (cv. Bonnie Best), *Zea mays* (cv. Sakota), *Triticum aestivum* (cv. Penewawa), *Hordeum vulgare* (cv. Clark), *Phaseolus vulgaris* (cv. Blue Lake Bush), *Festuca arundinaceae* (cv. Apache), and Blue Bunch wheatgrass (*Agropyron spicatum*), *Cucumus sativus* (cv. Straight eight), *Daucus carota* (cv. Sweet sunshine), *Gossypium hirsutum* (cv. Stoneville), *Pisum sativum* (cv. Alaska), *Raphanus sativus* (cv. Cherry Bomb), *Melilotus indica* (cv. Toudo). Control plants were inoculated with autoclaved inoculum. Plants were maintained in environmental growth chambers with computer controlled conditions: 28 C (19 hours day, 5 hours night). One week old plants were inoculated by placing 0.5 g of CFM inoculum at the base of each plant. An experimental unit consisted of five individual plants, replicated twice. Plants were watered daily for 2 minutes by an automatic sprinkler. The plants were evaluated daily for symptoms of vascular wilt. Tissue from plants exhibiting disease symptoms was plated as described earlier in the section. At the end of the experiment, one plant from the wild type isolate treatment was arbitrarily chosen and assessed for the presence of *Fusarium* by plating plant tissue on Komada's medium (Komada, 1975). Similarly, one arbitrary plant

from each treatment with mutant strains was assessed for the presence of *Fusarium* by plating plant tissue on minimal medium supplemented with the corresponding amino acid analog.

Results

Amino Acid Toxicity Tests on Plants

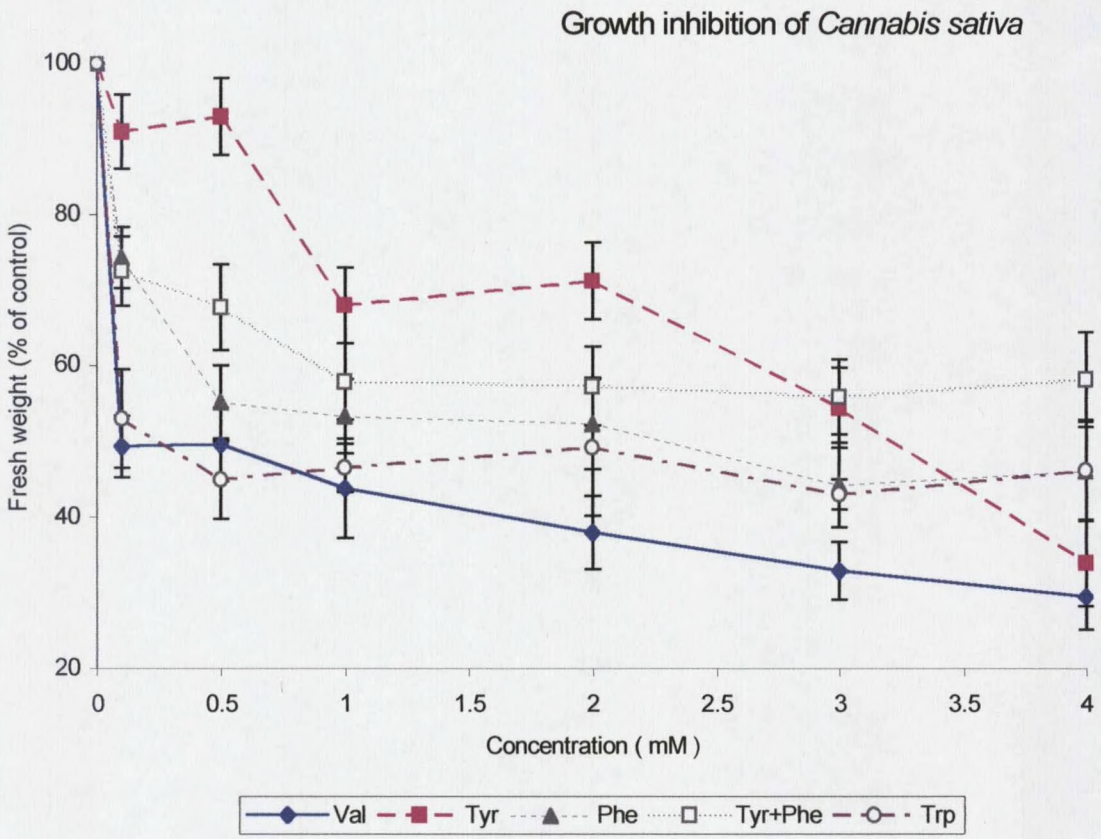
Inhibition of plant growth of three plant species (*Cannabis sativa, Papaver somniferum, Nicotiana tobacum*) with various exogenous L-amino acids (valine, tyrosine, phenylalanine, tryptophan and combination of tyrosine and phenylalanine) was studied. The toxicity of increasing concentrations of some exogenously added amino acids resulted in decreased fresh weight per plant/ seedling.

Cannabis sativa. Increasing concentrations of exogenous amino acids tested in media with nitrate as a sole source of nitrogen were inhibitory to normal growth of *Cannabis sativa* seedlings. The inhibitory effect resulted in reduced root system and overall biomass of *C. sativa* seedlings. Inhibitory effect was observed starting at a concentration of 0.1 mM for all amino acids tested (Figure 4.1.). The amino acids tested are known as effective feedback inhibitors. The levels of growth inhibition by each of the amino acids is presented in Figure 4.1. The fresh weight of *C. sativa* seedlings grown on the media supplemented with 0.1 mM valine was half of the control *C. sativa* seedlings grown on the media without the amino acid. Phenylalanine at 0.1 mM reduced fresh

weight of the seedlings by about 40 - 45% that of the control. A similar growth inhibition effect was observed on media supplemented with 0.5 mM tryptophan. Tyrosine was less inhibitory than other amino acids tested on *C. sativa* seedlings. The fresh weight was reduced by 25-30% with 1 mM tyrosine. The combination of tyrosine and phenylalanine was more inhibitory than tyrosine alone, but less inhibitory than phenylalanine alone. Complete inhibition of root apical growth of all seedlings was observed at 1 mM - 2 mM of any amino acid tested.

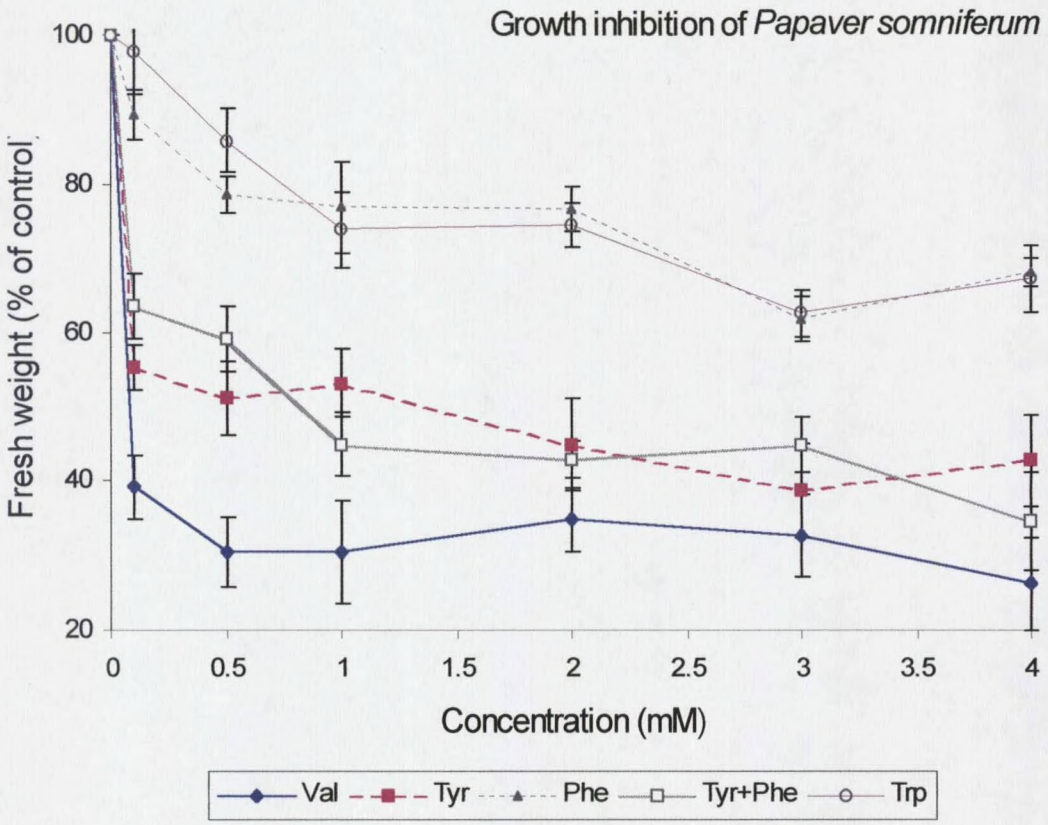
Papaver somniferum. The inhibition effect on *Papaver somniferum* seedlings by each of the amino acids is presented in Figure 4.2. *Papaver somniferum* seedling grown on media supplemented with 0.1 mM valine were reduced in fresh weight by about 60% as compared to the control seedlings. Tyrosine 0.1 mM reduced fresh weight of the seedlings by about 40% compared to the control. Phenylalanine and tryptophan were less inhibitory to *Papaver somniferum* seedling growth than other amino acids tested. Fresh weight was reduced by 20% at 1 mM. Maximal reduction of the fresh weight, 30-40%, was observed starting at 3 mM of phenylalanine and tryptophan. The combination of tyrosine and phenylalanine was more inhibitory than phenylalanine, but less than tyrosine alone.

Figure 4.1. Growth Inhibition of *Cannabis sativa* Seedlings by Increasing Concentrations of Exogenous Amino Acids



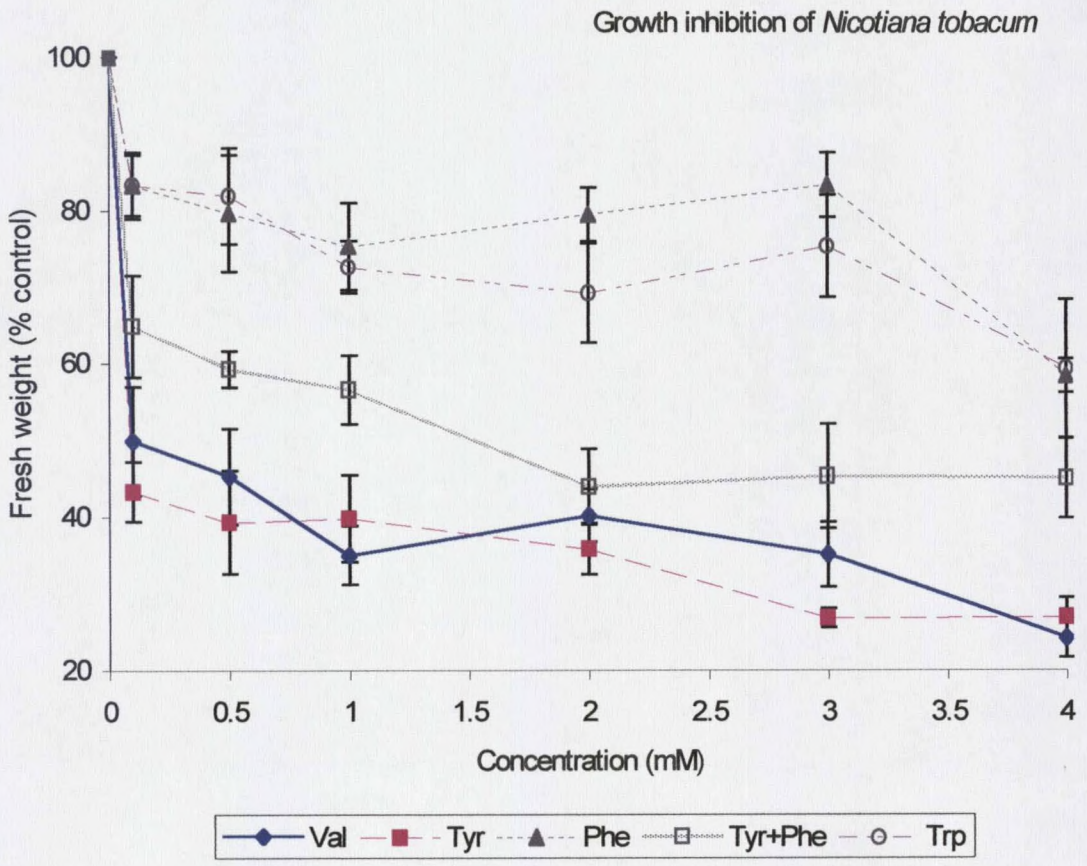
Two-week old seedlings grown in the presence of an amino acid were weighted in groups of ten and recorded as percent of weight of control seedlings. Values presented are the means +/- Standard error.

Figure 4.2. Growth Inhibition of *Papaver somniferum* Seedlings by Increasing Concentrations of Exogenous Amino Acids



Two-week old seedlings grown in the presence of an amino acid were weighted in groups of ten and recorded as percent of weight of control seedlings. Values presented are the means +/- Standard error.

Figure 4.3. Growth Inhibition of *Nicotiana tobacum* Seedlings by Increasing Concentrations of Exogenous Amino Acids



Two-week old seedlings grown in the presence of an amino acid were weighted in groups of ten and recorded as percent of weight of control seedlings. Values presented are the means +/- Standard error.

Nicotiana tobacum. Growth inhibition of tobacco seedlings on agar by increasing concentrations of the amino acids was also observed starting at 0.1 mM (Figure 4.3.). Tyrosine reduced fresh weight of the seedlings by 50-60% at a concentration of 0.1 mM. A similar level of inhibition was observed on agar media supplemented with 0.1 mM valine. At the same concentration phenylalanine and tryptophan separately reduced fresh weight by 20% of the fresh weight of control seedlings.

Valine Analogs Inhibitory to *Fusarium oxysporum*

Analogs of valine were tested against the pathogen using a well zone – diffusion assay on minimal medium, Table A.2. (Appendix A). Available valine analogs used were: VH = L-valine hydroxamate, VA = L-valine amide, PA = L-penicillamine, KMVA = DL-α-keto-β- methyl valeric acid, NV = L-norvaline, VL = L-valinol and ABA = L-amino-n- butyric acid. Of all analogs tested, only norvaline and penicillamine were inhibitory to *Fusarium oxysporum*. After several days of incubation, a clear zone of inhibition was formed, Figure B.6. (Appendix B). Several days later, single mutant colonies appeared within the zone. Single mutant colonies observed within inhibition zones were transferred to plates containing minimal media supplemented with 500 ppm of the corresponding analog. Single celled mutant colonies capable of wild type growth in the presence of the inhibitory analog were transferred to toothpicks and stored for future analysis.

Valine Excreting Mutants

Valine excreting mutants of *Fusarium oxysporum* f. sp. *cannabis* (Cs95), *Fusarium oxysporum* f. sp. *papaver* (CP3A, 4p-23-2) were generated on gradient plates with minimal media containing either norvaline or penicillamine. Analog resistant mutants were generated at a rate of approximately 1: 1,000,000.

Semiquantitative Assay of Excreted Valine

The resultant mutant colonies were screened for their ability to secrete valine into the medium. A bioassay system media was used (Valine assay medium, *Pediococcus cerevisiae* auxotroph bacteria) both in agar medium and in broth medium. Preliminary data showed that mutants excrete 10-55 times as much valine in liquid nitrate-containing minimal medium as the wild type parent after an incubation period of 14 days on the shaker (200 rpm, 28 C) (Table 4.1).

The semiquantitative analysis showed that the concentration of valine excreted by mutants could be between 20 and 93 μM in the fungal filtrates, in contrast to the wild type between 0 and 3 μM of valine.

Virulence Evaluation on the Target Host

Mutants excreting substantially higher quantities of valine into the medium were selected for virulence evaluation on target plants.

Table 4.1. Semiquantitative Estimation of Valine Excreted by Wild Type and Valine Analog Resistant Isolates of Various formae specialis of *F. oxysporum*

		Resistant mutants		
F. oxy. f. sp.	Isolate	PA	NV	mg/l
cannabina	C95	-	-	0-0.18
cannabina	C95	-	4nv	2.84
cannabina	C95	6pa	-	2.48
cannabina	C95	8pa	-	9.93
papaver	CP3A	-	-	0.35
papaver	CP3A	-	1nv	3.19
papaver	4p-23-2	-	-	0-0.18
papaver	4p-23-2	-	44nv	10.83
papaver	4p-23-2	-	45nv	4.26
papaver	4p-23-2	43pa	-	4.61
papaver	4p-23-2	25pa	-	2.31

PA - Penicillamine resistant mutants
NV - Norvaline resistant mutants

Cannabis sativa. Virulence of wild type *Fusarium oxysporum* f. sp. *cannabis* Cs95 and its valine excreting mutants (4nvCs95, 6paCs95, 8paCs95) was evaluated on *Cannabis sativa*. The average disease ratings of the *C. sativa* plants in treatments of all *F. oxysporum* isolates were significantly higher than disease ratings of the control plants (Table 4.2). The average disease ratings of plants inoculated with *F. oxysporum* mutant isolates 4nv (Cs95), 6pa (Cs95), 8pa (Cs95) were significantly higher than disease ratings

of plants inoculated with the parent wild type (Cs95) isolate ($p \leq 0.05$). The studies conducted in growth chambers showed an increased level of virulence. The wild type strain resulted in 25% control of the target plant, while the mutants showed increased control to 70-90% (Table 4.2). Significant differences were noticed in the virulence of mutant strains to *C. sativa* when compared to wild type and control treatments ($p \leq 0.05$).

Table 4.2. Disease Severity in *C. sativa* Plants Grown in Greenhouse Caused by Valine Excreting Mutants of *F. oxysporum* f. sp. *cannabis* Cs95

Isolate	DAI[1]	%disease[2]	duration[5]
Control[3]	1.35 a [4]	0%	8 weeks
wt (Cs95)	2.40 b	25%	6-8 weeks
4nv (Cs95)	3.75 c	70%	2-3 weeks
6pa (Cs95)	4.45 cd	90%	2-3 weeks
8pa (Cs95)	4.60 d	90%	2 weeks
LSD (0.05) 0.775			

[1] Mean disease average index. Disease average index was rated using a 1 (no disease) to 5 (dead plant) rating scale.
[2] Mean percent disease. Percent disease was calculated using = a/b x 100, where a- amount of plants with disease symptoms (3-5), and b- total amount of *C. sativa* plants per treatment (1-5).
[3] Control plants inoculated with autoclaved food based formulation.
[4] Means with the same letter are not significantly different ($p \leq 0.05$).
[5] Duration means time after inoculation till the last readings of the presented disease average index.

The development of wilt disease symptoms in treated plants was faster in the case of mutant isolates, resulting in plant death two weeks after inoculation. Most of the plants killed reached the 5-6 true leaf stage. *Cannabis* plants inoculated with wild type parent strain were dead or severely wilted in a period of 6-8 weeks, while mutant treated *C.*

sativa plants showed severe wilt and death in period of 2 - 3 weeks (Table 4.2).

Interestingly, in addition to wilt symptoms we observed leaf distortion, loss of stem apical dominance, and stunted growth in some *C. sativa* plants treated with valine excreting mutants, but not often. The symptoms of leaf distortion can be characterized by chlorosis of leaflet margins of some of the younger leaves, loss of the characteristic cannabis coarse saw-toothed edge and pointed tip. Reisolation of the pathogen from infected plant tissues on the media supplemented with corresponding valine analogs confirmed that survival of the plants was not due to plant escape from the pathogen. The systemic distribution of the wild type and mutant isolates within the plants was recorded (Table 4.3.). The nodes of the plants were plated on minimal media supplemented with 500 mg/l of norvaline. In plants treated with the valine excreting mutant 4nv(Cs95) about 84 % of nodes with wilted leaves and 39 % of nodes with leaves not showing wilting yielded the pathogen. In plants treated with wild type parent strain, 87 % of nodes with wilted leaves and 7.5 % of nodes with nonwilted leaves yielded the pathogen. Symptoms of leaf distortion were associated with the presence of the pathogen in vascular tissues of the symptomatic leaf in all plants tested (Table 4.3.).

Papaver somniferum. Pathogenicity evaluation of valine excreting mutant 1nv (CP3A) of *Fusarium oxysporum* f. sp. *papaver* on *Papaver somniferum* showed increased virulence compared to the wild type parent isolate CP3A (Table 4.4.).

Table 4.3. Systemic Distribution of the Wild Type and Valine Excreting Mutant 4nv(Cs95) within *C. sativa* Plants 8 Weeks after Inoculation

Nodes with	Mutant[1]		wild type[2]		Control	
	%nodes[3]	%infect[4]	%nodes	%infect	% nodes	%infect
wilted leaves	38.9	83.9	72	97	9.1	0
not wilted leaves	54.5	38.5	27.9	7.5	90.9	0
distorted leaves	4.6	100	0	0	0	0

1 Plants inoculated with mutant 4nv(Cs95).
2 Plants inoculated with wild type (Cs95).
3 Mean percent of nodes with each symptom of leaves was calculated as Number of nodes with leaves with symptoms / Total number of nodes.
4 Mean percent of nodes yielded the pathogen for each symptom of leaves was calculated as number of nodes yielded pathogen / total number of nodes with leaves with the symptom.

Table 4.4. Disease Severity in *P. somniferum* Plants Grown in Greenhouse Caused by Valine Excreting Mutants of *F. oxysporum* f. sp. *papaver*

Isolate	DAI[1]	%disease[2]	duration[5]
Control[3]	1.1 a [4]	0 %	2 weeks
wt (CP3A)	1.9 b	30 %	2 weeks
1nv (CP3A)	2.8 c	60 %	2 weeks
LSD (0.05) 0.71			

1 Mean disease average index. Disease average index was rated using a 1 (no disease) to 4 (dead plant) rating scale.
2 Mean percent disease. Percent disease was calculated using = a/b x 100, where a- amount of plants with disease symptoms, and b- total amount of *P. somniferum* plants per treatment.
3 Control plants inoculated with autoclaved food based formulation.
4 Means with the same letter are not significantly different ($p \leq 0.05$).
5 Duration means time after inoculation till the last readings of the presented disease average index.

Host Range Evaluation

Symptoms of Fusarium wilt were not observed on any of the test plant species inoculated with *Fusarium oxysporum* isolates, either the wild type or valine excreters, with exception of symptoms on *Cannabis sativa*, Table A.1. (Appendix A). One sweet clover plant died during the experiment in the treatment with the 4nv (Cs95) mutant. No kill was recorded in 80 individually inoculated sweet clover plants with wild type strain and valine excreting mutant 4nv (Cs95).

Discussion

The development of the pathogenic formae specialis of *Fusarium oxysporum* as a mycoherbicide is favored, because of its restricted host range (McCain, 1984; Sands, et al. 1997), and its relatively high ability to cause wilt of the target weed, at least in greenhouse studies (McCarthy, 1995; Anderson, 1996). However, in some instances, performance of *Fusarium oxysporum* mycoherbicide formulations under field conditions was less effective and inconsistent as compared to greenhouse evaluations. Therefore considerable research efforts were concentrated on improvement studies. These included: a) optimization of conditions for large-scale production of the biomass, preferably chlamydospores, either using solid substrate fermentation, or liquid culture fermentation (Boyette, 1993; Hebbar, 1997); b) improvement of suitable and inexpensive agricultural byproducts as formulation substrates (Hebbar, 1996; Hildebrand, 1978); c) stabilization of granular formulations of pathogenic *Fusarium oxysporum* (Connick, et al. 1998); d)

selection of oils and adhesives for liquid formulations (Connick and Quimby, 1991; Womack, 1996).

The objective of this investigation was to improve, via mutagenesis and selection, *Fusarium oxysporum* as a potential candidate for biological weed control. Described here are mutants that are resistant to toxic analogs of the amino acid valine. Due to loss of sensitivity (false feedback inhibition) to valine analogs - norvaline, or penicillamine, and consequently loss of feedback control mechanism by valine, these mutants over excrete valine. Bioassay using growth response in valine assay media (Difco Products) of auxotrophic bacterium *Pediococcus cerevisiae* ATCC 8042 to culture filtrates of mutant strains of *Fusarium oxysporum* showed that these mutants excrete 10-55 times more valine than the wild type parent strain. Semiquantitative analysis showed that the concentration of secreted valine by mutants could be between 20 and 93 μM in the fungal filtrates, in contrast to wild type between 0 and 3 μM of valine (Table 4.1).

Increasing concentrations of exogenous valine in minimal media starting at 0.1 mM were inhibitory to normal growth of *Cannabis sativa* seedlings. The inhibitory effect resulted in reduced root system and overall biomass of *C. sativa* seedlings. Fresh weight of *C. sativa* seedlings grown on the media supplemented with 0.1 mM valine was reduced by 50 % as compared to that of the control *C. sativa* seedlings.

The pathogenicity studies conducted in growth chambers showed significant differences in the virulence of tested mutant strains of *Fusarium oxysporum* f. sp. *cannabis* to *C. sativa* as compared to wild type and control treatments ($p \leq 0.05$). Twenty five - thirty percent of plants inoculated with wild type strain Cs95 were killed or

showed severe wilt symptoms 6- to 8-weeks after inoculation. In comparison mutant strains 4nv(Cs95), 6pa (Cs95) and 8pa(Cs95) killed or caused severe wilt in 70 - 90% of inoculated plants 2-3weeks after inoculation (Table 4.2.). Moreover, some of the *C. sativa* plants inoculated with either norvaline- or penicillamine-resistant mutants, besides characteristic wilting, showed symptoms of leaf distortion, chlorosis of the leaflet margins, and loss of apical dominance resulting in stimulation of branching (Figure A.9). These additional symptoms were somewhat similar to the symptoms of "frenching" disease in tobacco described by Steinberg (1950, 1956) and by Lucas (1965). "Frenching" symptoms included chlorosis along the margins of young leaves, loss of apical dominance resulting in a stunted plant with leaf distortion. Steinberg (1950) showed that *Pseudomonas flourescens* overproducing isoleucine in the rhizosphere of the plant was the casual agent of "frenching" disease of tobacco. He also demonstrated that isoleucine can be used to duplicate frenching symptoms of tobacco in aseptic culture (Steinberg, 1946). Reisolation of the pathogen on the medium supplemented with the corresponding valine analog showed that the leaf distortion was accompanied with the presence of the mutant strains in the distorted leaf.

The limited host range studies on fourteen plant species did not reveal increased pathogenicity of valine excreting mutants toward non-host plant species Table A.1. (Appendix A).

A similar approach was used to obtain valine analog-resistant mutants of *Fusarium oxysporum* f. sp. *papaver*. Semiquantitative analysis using *Pediococcus cerevisiae* ATCC 8042 showed that the concentration of secreted valine by *Fusarium*

oxysporum f. sp. *papaver* mutants could be between 10.83 mg/l - 44nv(4p-23-2) and 4.62 mg/l - 43pa(4p-23-2) compared to 0-0.18 mg/l - wild type 4p-23-2 (Table 4.1).

The evaluation of resulting valine excreting mutants of *Fusarium oxysporum* f. sp. *papaver* also showed increased virulence of the mutant 1nv (CP3A) then of the wild type parent isolate (Table 4.4).

Feedback control inhibition by valine both on organism and enzymatic level was shown for *Nicotiana tobacum* and *Arabidopsis thaliana, Hordeum vulgare* (Relton, 1986; Wu, 1994; Miflin, 1969, 1971). In this study we showed that valine was also growth inhibitory to *Cannabis sativa* and *Papaver somniferum* seedlings starting at concentrations as low as 0.1 mM in minimal media with nitrate as the sole source of nitrogen. The inhibitory effects of aromatic amino acids (tyrosine, phenylalanine and tryptophan) on the growth of *Cannabis sativa, Papaver somniferum* and *Nicotiana tobacum* (Figure 4.1; 4.2; 4.3) suggest that overproduction of some of these amino acids by phytopathogens could also be used as pathogen improvements. We found that 0.1 mM tyrosine is more toxic for growth of *Papaver somniferum* and *Nicotiana tobacum* than are the same concentrations of phenylalanine or tryptophan. In contrast, tyrosine is less inhibitory to growth of *Cannabis sativa* than are the same concentrations of phenylalanine or tryptophan. Therefore the choice of the amino acid analog for generation "false feedback" control insensitive mutants of the mycoherbicide would depend on the which amino acid is more toxic to the target weed. For example, to improve the *Cannabis* pathogen, one should chose valine analogs, or tryptophan analogs, such as 5-methyltryptophan - a successful false feedback inhibitor of tryptophan biosynthesis

(Moyed, 1960), and similarly, valine or tyrosine analogs for *Papaver* pathogen.

In conclusion, we propose the method of selecting valine excreting mutants of *Fusarium oxysporum* with enhanced pathogenicity as a method of improving virulence of existing *Fusarium oxysporum* mycoherbicides. Also incremental increases in virulence can be made by selection for additional excretion of the same or different amino acid. In addition the resistance of the mutant strains to various amino acid analogs enables the strains thus to be marked for their field release.

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CHAPTER 5

SUMMARY

Biocontrol of narcotic crops remains an untried alternative to standard control methods (Bailey, 1997). Whether used in a classical release strategy or with innundative methods, biocontrol offers a highly selective and environmentally friendly means of removing noxious and narcotic production potential of a field or region with low environmental impact.

At the present time several biocontrol strategies involving fusarium wilt are being developed for major narcotic plant species, such as: *Fusarium oxysporum* f. sp. *erythroxyli* for control of *Erythroxylum coca* - coca (Darlington, 1996; Sands, 1997); *Fusarium oxysporum* f. sp. *papaver* for control of *Papaver somniferum* - opium poppy (McCarthy, 1995; Anderson, 1996); *Fusarium oxysporum* f. sp. *cannabis* for control of *Cannabis sativa* - marijuana (McCain, 1984; Tiourebaev, et al. 1998).

In this investigation we describe evaluation of pathogenic *Fusarium oxysporum* as a potential mycoherbicide for control of marijuana - *Cannabis sativa,* both in environmentally controlled growth chambers and in field conditions. Koch's postulates were confirmed for the isolate Cs95 identified as *Fusarium oxysporum* originally isolated from diseased *Cannabis sativa* plants in the Chu River Valley, Kazakhstan. The limited host range studies including plant species present in the Chu River Valley showed that the pathogenicity of the pathogen was restricted to *Cannabis sativa.* The wilt disease was

observed in 40-80% of the inoculated plants in the greenhouse conditions at 28 C and 35 C respectively and in 35% of inoculated plants in the field experimental plots.

For *Fusarium oxysporum* f. sp. *cannabis* to be useful as a mycoherbicide, it must be able to proliferate from a formulated preparation into soil. Because *Fusarium oxysporum* infects plant roots, it must disperse from the formulation into the root zone to be effective.

The movement of *Fusarium oxysporum* propagules through soil is passive and greatly affected by water percolation. The passive dispersal of propagules from soil surface downward facilitated by water movement, depending on soil characteristics (sandy soils vs soil with high content of clay), can reach significant distances or be restricted to few centimeters from the soil surface, respectively. Hepple (1960) concluded that the rate of the infiltration of water through soil is seldom high enough to move fungal spore. Similarly, Burke (1965) concluded that "dissemination of the pathogen is largely limited to washing over soil surface" and that "propagules of the *Fusarium* apparently are rapidly filtered out as water percolates through soil". Recently Gracia-Garza (1998) measured the effect of percolating water on *Fusarium oxysporum* propagules through different types of soil columns following simulated rain. No significant differences were observed among total number of colony forming units (cfu) recovered from different soils used. In all soils, most of cfu were recovered from the top 2 centimeters of the column ($p<0.01$).

In this investigation we evaluate efficacy of live seed formulation as a delivery system for mycoherbicide. In soil columns inoculated with live seed formulations the total number of cfu recovered was significantly greater than with granular food based or liquid

formulations. In a soil column inoculated with CFM and liquid spore suspension most of cfu were recovered from the top 1-3 centimeters of the column. In contrast the propagules of the mycoherbicide could be recovered from significantly deeper soil depth and at significantly higher numbers when applied as Live seed formulation, as compared to the tested Food based formulations. Moreover no water was added to the soil columns inoculated with different formulations during the experiment to eliminate the effect of water percolation on fungal movement. Therefore the significant increase in cfu numbers along the soil column inoculated with LSF was mainly due the root elongation of the carrier plant. This method of mycoherbicide application using non-target plant species as a carrier agent can improve downward dispersal of the mycoherbicide propagules in soil profile especially in semiarid regions with low annual precipitation. It is also noteworthy that the application of the mycoherbicide in the form of live seed formulation allows simultaneous introduction of successive beneficial plant species. Introduced beneficial plant species in addition to the mycoherbicide can also add the pressure on the fitness of the weed plant in the region.

In the last chapter we described another approach of overall improvement of the mycoherbicide performance. The induced excretion of one of the essential amino acids by the phytopathogenic *Fusarium oxysporum*, directly or indirectly enhanced the virulence of the pathogen. The application of this method toward enhancement of the virulence of the mycoherbicides has not been demonstrated before. The excretion of one of the essential amino acids may well not be hazardous to the environment or man. The hypothesis behind enhanced virulence due excretion of the amino acid valine (known as an agent of feedback

inhibition in plant species investigated), can also include disturbance of amino acid imbalance, or co-excretion of some toxic intermediates of valine synthesis, or transformation of the exogenous amino acids into toxic products by the plant itself.

In conclusion, we discovered and evaluated a Kazakhstan phytopathogenic isolate of *Fusarium oxysporum* as a potential candidate for innundative control of *Cannabis sativa* in the Chu River Valley, Kazakhstan. Secondly, we propose the use of Live seed formulation as more efficacious mycoherbicide delivery system. Thirdly, we propose an alternative method of mycoherbicide virulence enhancement by generating false feedback inhibition insensitive mutants over secreting one of the essential amino acids.

APPENDICES

APPENDIX A

Table A.1. Incidence of Vascular Wilt on Crop Plant Species Grown in Greenhouse Soil Infested with *F. oxysporum* Isolates

Plant spp.	Control[1]	Strains of *Fusarium oxysporum*						
		0-1080[2]	Cs72	Cs109-2	Cs95	nv95	pa95	nit95
Lycopersicon esculentum	-	+[3]	-[4]	-	-	-	-	-
Zea mays	-	-	-	-	-	-	-	-
Triticum aestivum	-	-	-	-	-	-	-	-
Phaseolus vulgaris	-	-	-	-	-	-	-	-
Hordeum vulgare	-	-	-	-	-	-	-	-
Festuca arundinaceae	-	-	-	-	-	-	-	-
Agropyron spicatum	-	-	-	-	-	-	-	-
Cannabis sativa	-	-	+	+	+	+	+	+
Cucumus sativus	-	-	nt[5]	nt	-	-	-	-
Gossypium hirsutum	-	-	nt	nt	-	-	-	-
Pisum sativum	-	-	nt	nt	-	-	-	-
Daucus carota.	-	-	nt	nt	-	-	-	-
Melilotus indica	-	-	nt	nt	-	-	-	-
Raphanus sativus	-	-	nt	nt	-	-	-	-

[1] Control plants were inoculated with autoclave-treated inoculum.
[2] *F. oxysporum* f. sp. *lycopersici* strain 0-1080 (ATCC) was included as a positive control.
[3] No wilt symptoms observed in all plants treated.
[4] Severe wilt symptoms resulting in plant death.
[5] Not tested.

Plants were maintained in environmental growth chambers with computer controlled conditions: temperature 28 C, diurnal period (19 hours light, 5 hours dark).

Table A.2. Growth Inhibitory Effect of Valine Analogs Tested

	VH[1]	VA	PA	KMVA	NV	NL	ABA	Val	Ctrl
Cz[2]	+[3]	+	+	+	+	+	+	+	+
MM	+	+	zone[4]	+	zone	-[5]	+	+	+
MM/NO_3	+	+	+	+	+/zone	zone	+	+	+
PDA	+	+	+	+	+	+	+	+	+

1 VH - valine hydroxamate; VA - valine amide; PA - penicillamine; KMVA - keto methyl valeric acid; NV - norvaline; NL - norleucine; AKBA - amino ketobutyric acid; Val - valine; Control = sterile ddH_2O.

2 Inhibitory effect was tested using well zone diffusion assay on four types of media: CZ - Czapek media, PDA - Potato Dextrose Agar, NO_3 - minimal NO_3 media, MM - minimal media.

3 + = growth, no zone.

4 zone = inhibition zone.

5 - = no growth.

APPENDIX B

Figure B.1. Symptoms of Wilt Disease on *Cannabis sativa* Plants Caused by *Fusarium oxysporum*

Figure B.2. Temperature Fluctuation During Summers 1996 and 1997 at the Experimental Station of the Kazakh Institute of Agriculture, Academy of Agriculture of Kazakhstan

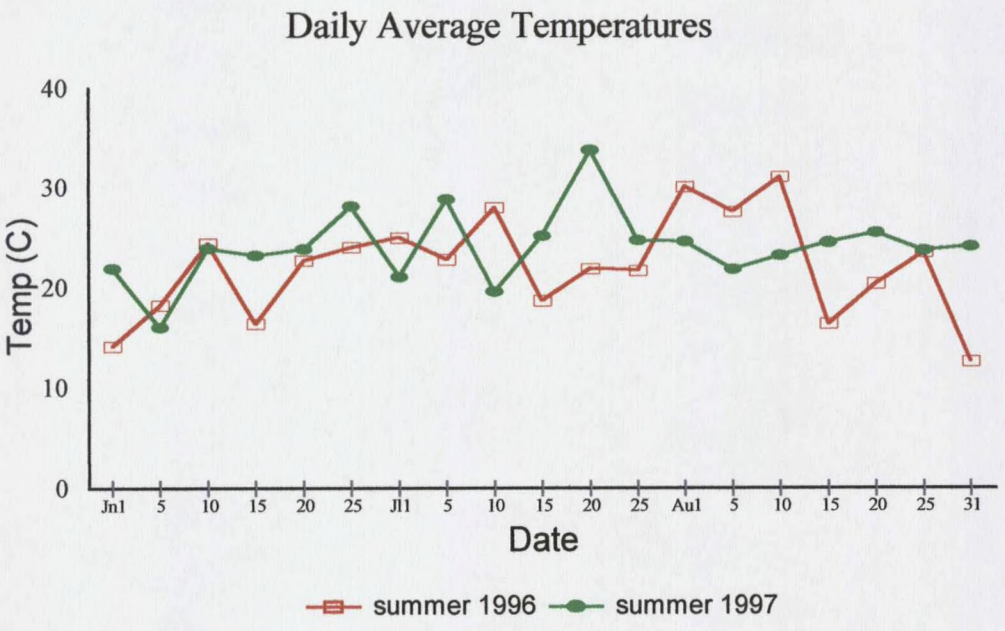


Figure B.3. Progression of Fusarium Wilt in Treated and Control Plots of *C. sativa* During Field Experiment in Summer 1997

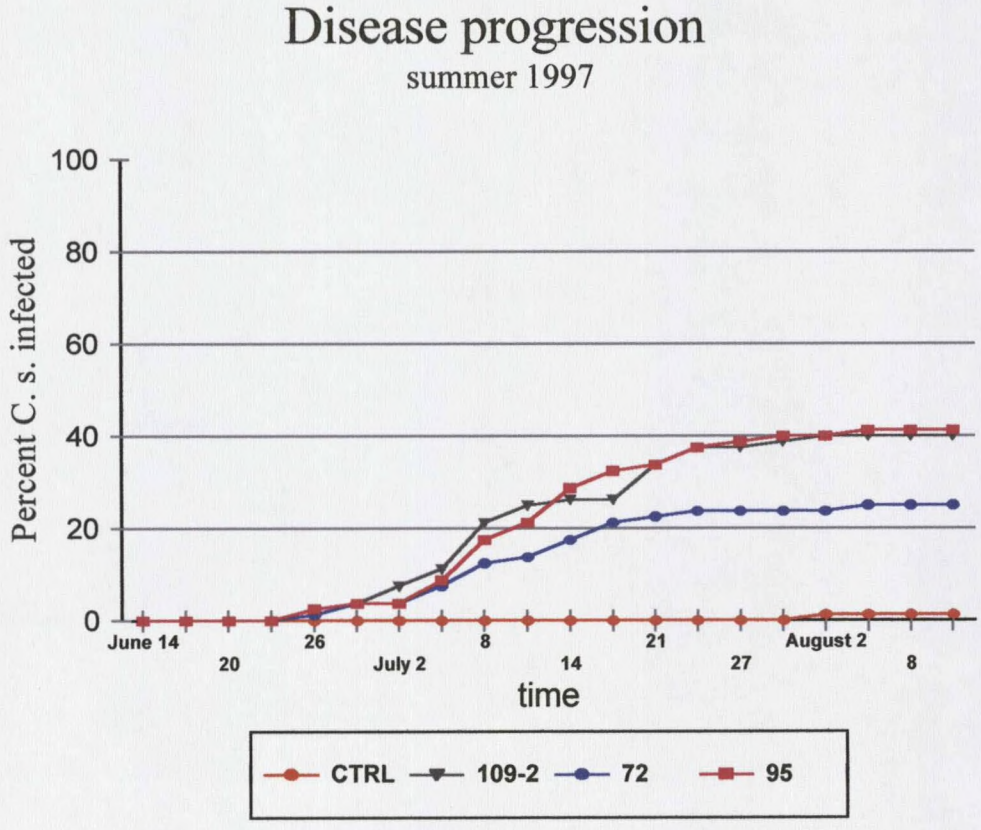


Figure B.4. The Soil Columns were Used to Evaluate Downward Movement of the Mycoherbicide

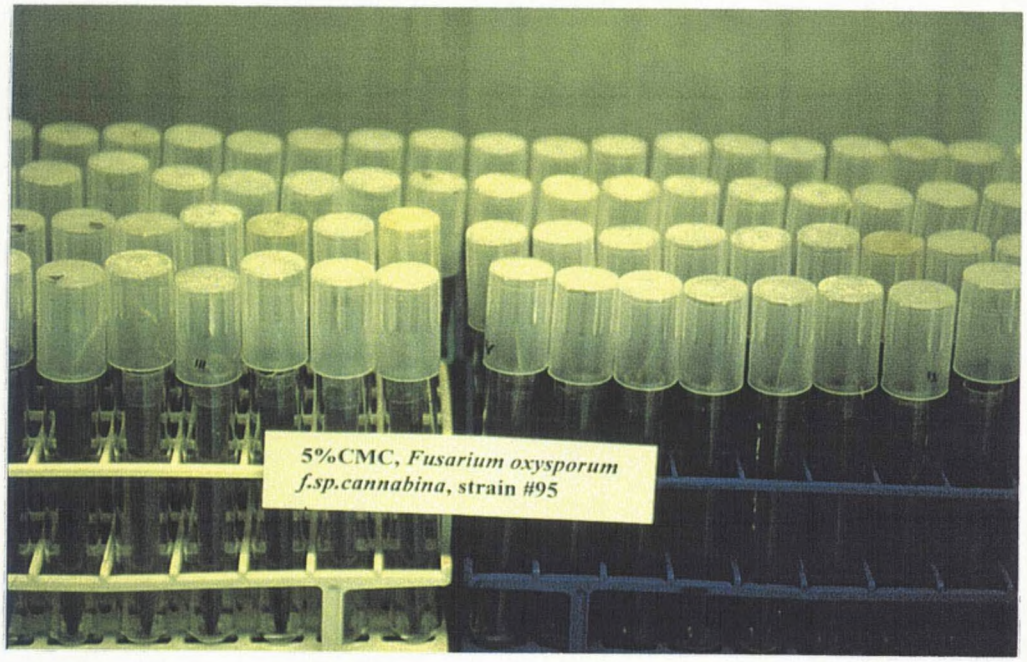


Figure B.5. Colonization of *C. sativa* Seedlings by *F. oxysporum* f. sp. *cannabis*

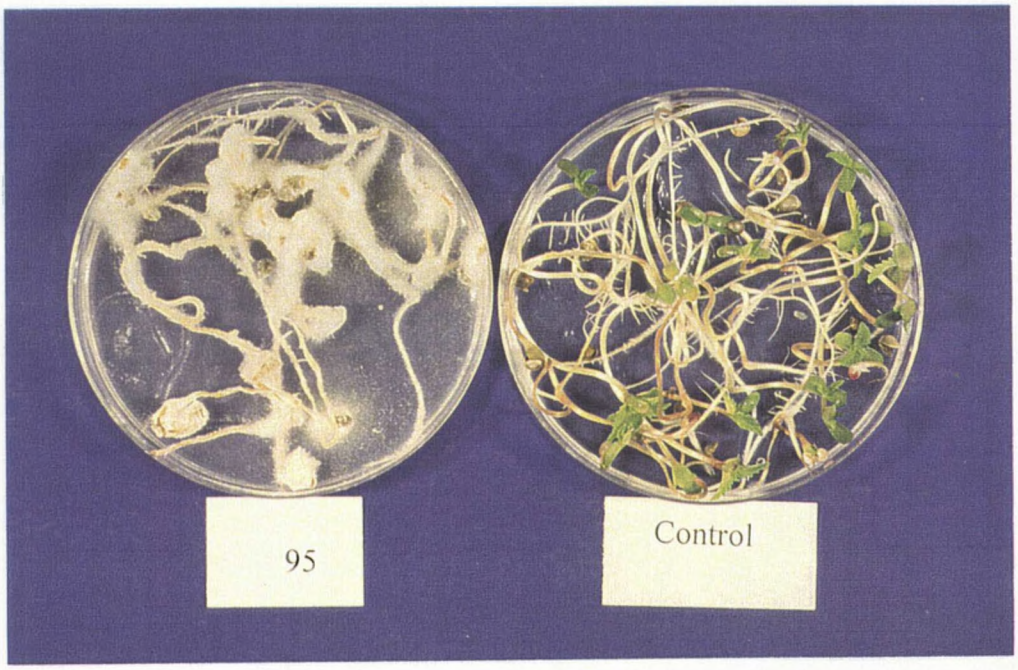
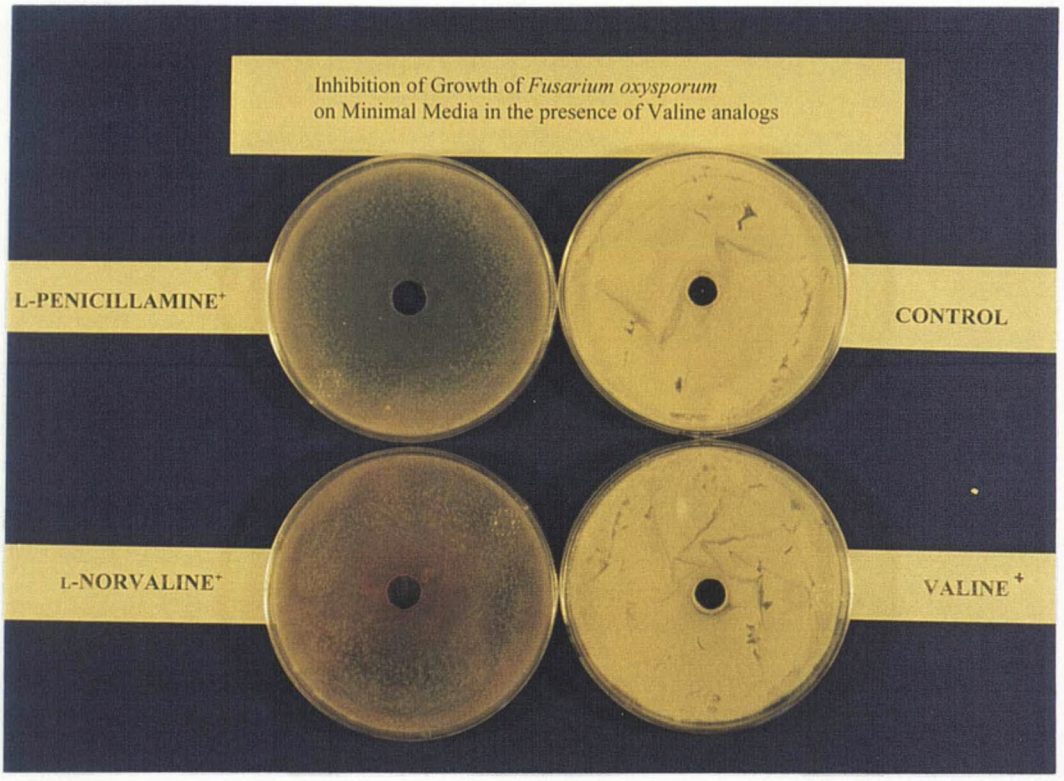


Figure B.6. Growth Inhibition of *F. oxysporum* Cs95 by Toxic Valine Analogs



From all available valine analogs tested only L- norvaline and L-penicillamine were inhibitory to tested isolates of *Fusarium oxysporum*. Single colonies appeared within the inhibition zones were transfered to plates with minimal media supplemented with 500 ppm of the corresponding analog. Colonies capable of wild type growth in the presence of an analog were used for further analysis.

Figure B.7. Formation of a Halo of Bacterial Colonies of Auxotrophic Bacterium *Pedicoccus cerevisae* around Valine Excreting Mutant of *F. oxysposrum* on the Valine Assay Medium

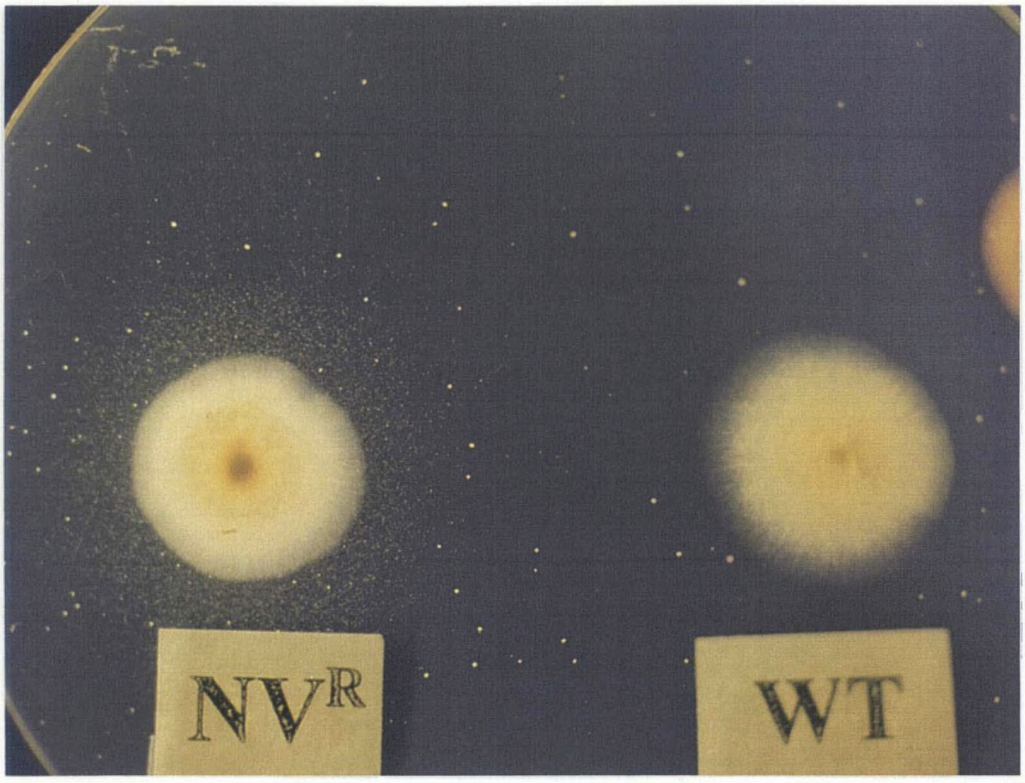


Figure B.8. Symptoms of Leaf Distortion on *C. sativa* Inoculated with Valine Excreting Mutant