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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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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