EVALUATION OF *SEPTORIA GALEOPSIDIS* WESTD. AS A BIOHERBICIDE FOR HEMP-NETTLE (*GALEOPSIS TETRAHIT* L.).

by

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ABSTRACT

M. Sc. 1988 **Plant Science**

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Evaluation of Septoria galeopsidis Westd. as a bioherbicide for hemp-nettle (Galeopsis tetrahit L.).

Hemp-nettle is an important weed in field crops in Canada and is difficult to control in broadleaf crops such as canola (Brassica napus L.). A fungal pathogen, Septoria galeopsidis Westd., was isolated from diseased hemp-nettle plants and its potential as a bioherbicide was evaluated. The fungus did not sporulate in liquid media, but inoculum was produced under near ultra-violet light on agar containing hemp-nettle extract. Over 60 % of the pycnidiospores germinated on water agar after 24 hours, at temperatures ranging from 15 to 30 C. Optimum temperature for mycelial growth was 24-26 C. The pathogen required over 24 hours of dew in order to infect its host. Maximum infection was obtained with 60 hours of dew at 24 C. The pathogen reduced plant fresh and dry weight but did not kill the plants. Plant weight decreased as inoculum density increased within the range 1.0×10^6 to 1.0×10^9 spores/m^2. Maximum aboveground biomass reductions were obtained at an inoculum density of 1.0×10^9 spores/m^2. The pathogen infected hemp-nettle plants at the cotyledon, 2-, 4-, 6-, and 8-true-leaf stages, but reductions in dry weight were observed only on seedlings at the cotyledon stage and on 8-leaf plants. New leaves were produced by all diseased plants in all experiments.

RESUME

M. Sc. 1988 **Phytotechnie**

Hélène Gadoury

Evaluation du champignon Septoria galeopsidis Westd. en tant que bioherbicide contre l'ortie royale (Galeopsis tetrahit L.)

L'ortie royale est une mauvaise herbe répandue dans les grandes cultures au Canada, et la répression de cette mauvaise herbe dans les cultures à feuilles larges telles que le colza (Brassica napus L.) est présentement difficile. Un champignon pathogène, Septoria galeopsidis Westd., fut isolé de plants d'ortie royale malades, et le potentiel de ce pathogène en tant qu'agent de lutte biologique fut évalué. Le champignon n'a pas produit de spores dans les milieux de culture liquides, mais suffisamment d'inoculum a été produit sur des plats de Pétri contenant de l'agar a base d'extrait d'ortie royale irradiés avec une lumière de longueur d'onde voisine de l'ultra-violet. Plus de 60 % des spores ont germé sur des plats d'agar après 24 heures d'incubation à des températures de 15 à 30 C. La température optimale pour la croissance des hyphes était de 21-26 C. Le pathogène a requis plus de 24 heures en atmosphère saturée d'eau pour infecter son hôte. Un maximum d'infection a été obtenu après 60 heures en atmosphère saturée d'eau, à 24 C. Le pathogène a réduit le poids frais et le poids sec des plants mais ne les a pas tués. Une réduction maximale de la biomasse a été obtenue avec une densité d'inoculum de 1.0 X 10^9 spores/m^2.

Le poids sec des plants a diminué a mesuré que la densité d'inoculum a augmenté entre 1.0 X 10^6 et 1.0 X 10^9 spores/m^2. Le pathogène a infecté des plants d'ortie aux stades de cotylédons et de deux, quatre, six et huit feuilles, mais une réduction du poids sec n'a été observée que chez les plants inoculés au stade de cotyledons et au stade de huit feuilles. De nouvelles feuilles ont été produites par tous les plants dans toutes les expériences.

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CHAPTER 1.

General Introduction.

Hemp-nettle (*Galeopsis tetrahit* L.) is a widespread weed in Canada, Europe, and Asia (Hanf, 1983; O'Donovan and Sharma, 1987). Although chemical herbicides provide adequate control in cereals, the repression of this broadleaf weed in broadleaf crops such as canola (rapeseed, *Brassica napus* L.) is difficult. This incites the search for new control methods. One of the ways to decrease weed competition is to use mycoherbicides, or bioherbicides made with fungi that are pathogenic to the target weed. Potential biological control agents have been evaluated for a large number of weeds, but no pathogen has yet been studied for the repression of hemp-nettle.

Today's growing concern for the degradation of the environment has set new challenges to agriculture. Besides the fact that bioherbicides may provide an efficient alternative control method for aggressive weeds, they also exhibit other desirable characteristics. They do not leave any toxic residues in the soil, they are specific to the target weed and thus do not cause any damage to other plants in the habitat, and they are harmless to man (Watson and Colette, 1986).

The pathogens that cause disease on plants are not all of equal potential as bioherbicides (Templeton, 1982a). Their potential can be assessed by understanding the disease cycle and the constraints to development of epiphytotics, and by deduction from our knowledge of similar pathogens of economic crops (Templeton, 1982a; Walker and Riley, 1982).

After a review of the literature concerning important aspects of the subject, this thesis presents the results of investigations performed in order to 1) find an endogenous pathogen attacking hemp-nettle, 2) determine the optimum conditions for disease development, and 3) assess the potential of this pathogen as a bioherbicide for hemp-nettle. The fungal pathogen that was found and investigated is *Septoria galeopsidis* Westd. The investigations included microscopic and macroscopic observations of the fungus, and evaluation of the effect of different factors (dew period temperature and duration, inoculum density and spray adjuvants, plant age) on infection and disease severity on hemp-nettle.

Chapter 2.

Literature review.

2.1 Hemp-nettle biology and control.

2.1.1 Description of hemp-nettle.

Hemp-nettle (bee nettle, dog nettle, flowering nettle, ortie royale) was introduced from Eurasia (Frankton and Mulligan, 1970). It occurs throughout North America and is widely distributed in Canada in every province to the northern limit of agriculture. It belongs to the family Labiatae and is an annual, reproducing only by seed (Alex and Switzer, 1976). Cotyledons are round to oval, each with a distinct notch and auricles at the point of attachment with the petiole. First true leaves differ little from the following ones (Bouchard et al., 1979). Leaves are opposite, light green, ovate to elliptic with 5 to 10 large, obtuse teeth on each side (Alex and Switzer, 1976). Veins mostly end in notches between the teeth. Both leaf surfaces are pubescent. The stem is erect, 30 to 80 cm high, branched, square, usually swollen at the nodes, and covered with rather harsh, straight, long, somewhat downward-pointing hair. The root system consists of a tap root with branched laterals (O'Donovan and Sharma, 1987). Hemp-nettle is a very variable species with respect to flower colour and size and leaf shape (Frankton and Mulligan, 1970). Flowers are in dense clusters in the axils of leaves near the ends of stems and branches (Alex and Switzer, 1976). The calyx is short, tubular, 10-ribbed, ending in 5 sharp, narrow teeth which are very spiny when mature. The corolla is pinkish to light purplish or whitish, often variegated, 12-23 mm long, and usually with two yellow spots. There are five united petals which are irregular, tubular, two-lipped at the end, the upper lip two-lobed, lower lip three-lobed. Each flower produces four egg-shaped, somewhat triangular seeds. The species flowers from mid-July to mid-August.

Other Galeopsis species found in Canada include G. ladanum L., G. ladanum var. litifolia (Hoffm.) Wallr., G. speciosa Mill., G. tetrahit var. arvensis Schlecht., G. tetrahit var. bifida (Boenn.) Lej. & Court., and G. tetrahit var. bifida f. albiflora House (Scoggan, 1978). G. versicolor Curt. can also be found in Quèbec city area (Quèbec) (Marie-Victorin, 1964).

Despite its abundance and the stability of its characters, hemp-nettle seems to be a tetraploid hybrid, since it has successfully been produced by crossing two diploid species : G. pubescens Besser (n=8) and G. speciosa (n=8) (Marie-Victorin, 1964).

Cultivated plants that belong to the family Labiatae include oregano (Oreganum spp.), mint (Mentha spp.), basil (Satureja spp.), thyme (Thymus spp.), sage (Salvia spp.), lavander (Lavandula spp.), germander (Teucrium spp.) catnip (Nepeta cataria L.), coleus (Coleus spp.), bergamot (Monarda spp.), false dragonhead (<u>Physostegia</u> spp.), woundwort (<u>Stachys</u> spp.), and many others. No cultivated species belongs to the genus <u>Galeopsis</u> (Scoggan, 1978; Polunin, 1969).

2.1.2 Biology, ecology and reproduction of hemp-nettle.

Hemp-nettle occurs in grainfields, seeded pastures, gardens, waste areas, along roadsides, and around buildings (Alex and Switzer, 1976; Marie-Victorin, 1964). The first peak of emergence occurs in early spring, the earliest recorded date being April 8, in Alberta (O'Donovan and Sharma, 1987). Several climatic factors, however, determine the time of emergence (Ervio, 1981).

Temperature and photoperiod have limited effect on most aspects of the vegetative growth of hemp-nettle (O'Donovan and Sharma, 1987). Low moisture levels, however, reduce it. It can thrive on most agricultural soils in the temperate regions of the world but prefers well-aerated, well-watered, humus soils, and cool, damp climate (Hanf, 1983; O'Donovan and Sharma, 1987). Optimum soil pH range for growth lies between 5 and 6. In the Prairie provinces, it is found mostly in fertile soils.

Ecological studies were performed on natural populations in Quebec (Lègere and Deschênes, 1982). These studies showed that mortality is low for all but very high densities (720 and 1040 plants/m^2) and individual dry weight production is higher for early emerging specimens. Dry weight and number of seeds per plant varied inversely with density. More seeds per dry weight unit were produced by late emerging individuals, but they reached maturity at the same time as early emerging ones and dry weight production per unit area increased constantly with time until flowering stage, at all density levels. Hemp-nettle is frequently found in association with *Polygonum* species, stinkweed (*Thlaspi arvense* L.), chickweed (*Stellaria media* (L.) Vill.), shepherd's-purse (*Capsella bursa-pastoris* (L.) Medic.), Tartarian buckwheat (*Fagopyron tataricum* (L.), Gaertn.), lamb's-quarters (*Chenopodium album* L.), dandelion (*Taraxacum officinale* Weber), Canada thistle (*Cirsium arvense* (L.) Scop.), and wild oats (*Avena fatua* L.) (O'Donovan and Sharma, 1987).

Hemp-nettle is self-fertile, mainly autogamous, and does not require insect pollination (O'Donovan and Sharma, 1987). At day/night temperatures of 23/15 C and a 16 hour photoperiod, it produces an average of 387 seeds per plant. The seeds become loose when they ripen and they fall off easily from the persistent calyx. They are mostly scattered and dispersed by wind, water, and farm machinery.

Hemp-nettle is a quantitative long-day species with respect to its flowering response. Low light intensities delay the onset and development of flowers and seeds. Reproductive growth is also delayed and reduced by low temperature regimes and low soil moisture. (O'Donovan and Sharma, 1987).

2.1.3 Seed dormancy and germination requirements.

Hemp-nettle seeds have strong dormancy characteristics which play a role in the difficulty and expense of controlling it (O'Donovan and Sharma, 1987). Seeds of *Galeopsis tetrahit* germinate poorly due to dormancy and low fertility (Andersen, 1968; Pawlowski et al., 1968). The optimum temperature for germination is 13 C, no germination occur at 25 C or above, and alternating temperatures of 5 to 15 C are more favorable than the optimum constant temperature (Andersen, 1968). Removal of the seed coat and endosperm, as well as treatment with aqueous solutions of the potassium salt of gibberellic acid stimulated germination of dormant lines (O'Donovan and Sharma, 1987; Corns, 1960). Increasing depth of burial increases the longevity of seeds of *G. tetrahit* and they retain viability better in soil than when they are stored in dry conditions (Hopp, 1957).

2.1.4 Economic importance.

Hemp-nettle is listed under "Other weeds" in the Canada Seeds Act and has been designated as a "nuisance weed" in Alberta (O'Donovan and Sharma, 1987). Populations of hemp-nettle have increased in Alberta and Saskatchewan from 1948 to 1977 (Anonymous, 1979). They reduce yields of several crops, including wheat (*Triticum* spp.), canola (*Brassica napus* L.), oats (*Avena sativa* L.), and alfalfa (*Medicago sativa* L.) (O'Donovan and Sharma, 1987). Hemp-nettle competes with these crops for moisture, nutrients, and light, with the highest recorded yield reduction of 85% in alfalfa. In oats and alfalfa, the time of onset of hemp-nettle competition occurs between the second and fourth week after seeding. Hemp-nettle is more competitive than quackgrass (Agropyron repens L.) but less than wild oats (Avena fatua L.).

The seeds of this weed are a serious contaminant of small grains and are difficult to clean from the crop seed (O'Donovan and Sharma, 1987; Kohout and Zimova, 1970). Hemp-nettle plant extracts also contain poisonous compounds (O'Donovan and Sharma, 1987).

Galeopsis tetrahit is a symptomless host for some crop pathogens, including Phoma exigua Desm., the tobacco rattle virus, and several nematodes including Ditylenchus dipsaci (Kühn 1837) Filipjev1936, and Heterodera species (O'Donovan and Sharma, 1987; Kaczmarek, 1985).

2.1.5 Conventional control of hemp-nettle.

Cultural practices that have efficiently reduced hemp-nettle populations include delaying seeding and well-timed tillage prior to seeding, summer fallowing, the use of competitive cereal crops in the rotation, zero-tillage, and improved fertilization (Nakoneshny and Friesen, 1961; O'Donovan and Sharma, 1987).

Chemical control is widely used. The herbicide 2-methyl-4-chlorophenoxyacetic acid (MCPA) and a number of newly registered herbicides are available for the control of this weed in cereal crops (table 2.1)(O'Donovan and Sharma, 1987). However, chemical control of this broadleaf weed in broadleaf crops such as canola and soybean is presently not available.

The hormone herbicide (2,4-dichlorophenoxy) acetic acid (2,4-D) does not control hemp-nettle but there is no clear evidence that hemp-nettle resistance has developed over time (Hay, 1968; O'Donovan and Sharma, 1987). Selection for resistance between species rather than within species is more likely to have occurred as a result of the use of herbicides, which could have contributed to the general observation that hemp-nettle is more abundant now than 40 years ago, when 2,4-D first became commercially available. Hemp-nettle is probably favored by selective removal of susceptible species. In addition, natural selection and other changes in farm practices that could have made the environment more conducive to the success of this species have probably played a role in this situation.

2.2 Biological control.

2.2.1 Biological control of weeds - theory.

Extensive reviews of theory and practices of biological weed control have been written (Shroeder, 1983; Templeton,

Table 2.1 Postemergence herbicides registered for control of hemp-nettle in cereals (partly reproduced from O'Donovan and Sharma, 1987).

Herbicide	kg/ha
Bromoxynil/MCPA (1:1)[1]	0.60
Chlorsulfuron	0.01
Cyanazine/MCPA-K (1:2)[1]	0.85
Dicamba/MCPA-K (1:4)	0.50
Fluroxypyr	0.15-0.20
Linuron + MCPA amine (1:2)[1]	0.85
MCPA	0.55-0.85
MCPA/Mecoprop/Dicamba (6:15:1.5)	0.45-0.60
MCPB/MCPA (15:1)	0.45-0.68
Metribuzin	0.20-0.30
Metribuzin + MCPA	0.20+0.40
Metribuzin + Dicamba	0.20+0.01
Metsulfuron methyl	0.004

[1]Herbicides registered for flax (*Glycine max* L.) as well.

1982b; Wilson, 1969; Wilson, 1964; Huffaker, 1959; Huffaker, 1957). Biological control of weeds is defined as "the deliberate use of natural enemies to suppress or control weeds" (Watson and Colette, 1986). Several types of organisms can be used as biological control agents : bacteria, fungi, nematodes, viruses, insects, and herbivores of all kinds.

Biological weed control with insects is well documented, and numerous examples of successful attempts exist, such as the famous introduction of the larvae of the moth *Cactoblastis cactorum* Berg. in Australia, in 1925, to repress species of *Opuntia* cactus (Templeton, 1982b). Substantially fewer attempts to control weeds with agents other than insects have been made, but in the past 10 to 15 years, numerous plant pathogens, especially fungi, have been evaluated as agents for weed control. Certain biological attributes and technological advantages of fungi over other pathogens, as well as the fact that most plant diseases are caused by fungi, are responsible for the dominance of fungi in biological control programs.

When fungal pathogens are used as biocontrol agents, either the classical or the inundative strategy can be identified (Templeton, 1982b). In the classical (or inoculative) strategy, the fungus is simply introduced or released into a weed population where it did not exist before to establish an epiphytotic and no further manipulations are made. The introduction of *Puccinia chondrillina* Bubak. & Syd. into Australia from Europe to control skeleton weed

(*Chondrilla juncea* L.) is an example of this method. Prior to release, pathogens introduced in regions where they do not naturally occur must be studied in a specialized containment or quarantine facility in order to eliminate any danger of unplanned escapes (Watson and Sackston, 1985). Two quarantines in the United States (at Frederick, Maryland, and at Gainesville, Florida) and one in Canada (at Macdonald College, Ste-Anne-de-Bellevue, Québec) are approved for the containment of foreign plant pathogens for study as biological control agents for weeds.

In the inundative (or mycoherbicide) strategy, large quantities of fungal spores are applied similar to chemical herbicide application, to infect and kill susceptible weeds present in fields and groves (Templeton, 1982b). Two examples of this type of use are the commercialized mycoherbicides Devine ® and Collego ® (TeBeest and Templeton, 1985). Devine ® is a formulation of *Phytophtora palmivora* (Butler) Butler, registered in 1981 for the control of strangler (milkweed) vine (*Morrenia odorata* Lindl.) in Florida citrus groves. Collego ® is a formulation of *Colletotrichum gloeosporioides* (Penz.) Sacc. f.sp. *aeschynomene*, registered in 1982 for the control of northern jointvetch (*Aeschynomene virginica* (L.) B.S.P.) in rice and soybean fields.

When the inundative strategy is used, several steps must be followed, including production of spores in large quantities, formulation of the mycoherbicide, assessment of the fungus resistance to environmental factors (Watson and Colette, 1986). Also, the host weed as well as the pathogen must possess certain characteristics. The weed must be an important weed, and must be found in an important crop. It must be difficult to control by any other method, and must be attacked by some pathogens. The pathogen must be easy to culture, host specific, efficient, genetically stable, tolerant to a wide range of environmental conditions, and it must produce large quantities of spores.

2.2.2 Biological control of hemp-nettle.

Although hemp-nettle is a good candidate for biological control, no programs have been developed to evaluate potential biological control agents for this plant. *Galeopsis tetrahit* is the only host of the beetle *Dlochrysa fastuosa* Motsch. (Coleoptera: Chrysomelidae). Larvae of the small rivulet moth (*Perizoma alchemillata* L., Lepidoptera: Geometridae), which is widely distributed in Newfoundland, feed on the leaves of the weed and may provide limited natural control (Morris and Bolte, 1977). The nematodes *Ditylenchus dipsaci* and *Heterodera galeopsidis* Goffart 1936 have been found growing in association with hemp-nettle, the latter pathogen causing severe stunting of the plant (O'Donovan and Sharma, 1987).

Fungi found in association with hemp-nettle in North America include *Erysiphe galeopsidis* DC., *Septoria galeopsidis* Westd., *Phoma exigua* var. *foveata* Desm., and *Phyllosticta* decidua Ell. & Kellerm. (O'Donovan and Sharma, 1987; Seymour, 1929).

2.3 Taxonomy and description of Septoria galeopsidis Westd.

2.3.1 Introduction.

One of the fungi found in association with hemp-nettle, Septoria galeopsidis West., and the disease that it causes on hemp-nettle are the objects of the present study. The potential of this pathogen as a biological control agent has never been investigated before. This section is devoted to the taxonomy and description of the genus Septoria and the species Septoria galeopsidis Westd.

2.3.2 Classification and characteristics of the genus Septoria.

The genus Septoria contains more than 2000 described taxa (Sutton, 1980). The genus is heterogeneous, however, and still requires considerable revision. The species in that genus are classified by Sutton in the group Coelomycetes, which are the Fungi Imperfecti that produce pycnidia, acervuli, and stromata.

Septoria species produce immersed, branched, septate, and pale brown mycelium, and immersed, separate or aggregated, globose, brown, thin-walled pycnidia, with a single, circular ostiole (pore by which spores are freed). Conidiophores are absent. Conidia (pycnidiospores) are filiform (thread-shaped), with many septa or guttules, hyaline, smooth, continuous or restricted at the septa (Sutton, 1980; Jørstad, 1967; Saccardo, 1882-1931).

2.3.3 Species of Septoria as causal agents of diseases on plants of economic importance.

The genus Septoria occurs throughout the world. It incites diseases on numerous crops, causing mainly leaf spots and blights (Agrios, 1978). Septoria glume blotch of wheat (caused by Septoria nodorum (Berk.) Berk. - perfect state: Leptosphaeria nodorum Müller) and Septoria leaf blotch of wheat (caused by Septoria tritici Rob. ex. Desm. - perfect state : Mycosphaerella graminicola [Fuckel] Schroeter) are major wheat diseases in several areas of the world, causing serious yield reductions (Eyal,1981). In the United States, the percent loss may reach 30-50% in a severe epiphytotic, for susceptible wheat cultivars with the resulting grain shrivelled and unfit for milling (Danon et al., 1982). Other common and serious diseases caused by Septoria species include leaf blotch and glume blotch of other cereals and grasses, leaf spots of celery (Septoria apii-graveolentis Dorogin), beet, carrot, cucurbits, lettuce, tomato (Septoria lycopersici (Speg.) Sacc.), soybean, bramble, aster, azalea, carnation (on which they cause a corm rot), chrysanthemum, marigold, and many others (Agrios, 1978).

2.3.4 Species of Septoria associated with plants in the genus Galeopsis and the family Labiatae.

Only two Galeopsis species, G. speciosa and G. tetrahit are reported as hosts of Septoria galeopsidis. No other Galeopsis species is reported to be found in association with a Septoria pathogen. Septoria species have been found on several other members of the family Labiatae, however, including mint (S. menthicola Sacc. & Let.), catnip (S. nepetae Ell. & Ev.), woundwort (S. stachydis Rob. ex Desm.), and many others (Seymour, 1929; Jørstad, 1967).

2.3.5 Species of Septoria as potential control agents for other weeds.

A few studies have been conducted on the potential of Septoria spp. as biocontrol agents for weeds. A leaf spot disease was found on the widespread weed Chromolaena odorata (L.) Kr. (Russo, 1985). Necrotic spots with red margins were observed on leaves of flowering plants. The spots coalesced over time and covered the entire leaf surface, and Septoria sp. was isolated from the spots. Healthy plants were inoculated with spores (about 10^4/ml) and mycelial fragments from cultures of the isolated Septoria sp. and incubated in plastic bags. Five to eight days after inoculation, necrotic areas similar to those originally observed developed on the oldest leaves. The fungus seemed to attack only senescing tissue. Naturally infected plants usually were not killed, and resprouting occurred.

Smith and Self (1984) assessed the pathogenicity of Septoria polygonorum Desm. on Pennsylvania smartweed (Polygonum pennsylvanicum L.). Inoculum was produced in shake cultures of modified V-8 medium within 5 days. Conidia and mycelial fragments caused leaf spots on older, but not younger, leaves. No stem lesions developed. Wetting periods exceeding 36 hours were necessary for infection to occur. Field infections and greenhouse infections were established but local epiphytotic did not develop.

Both of the above studies were not exhaustive and conclusions concerning the suitability of these Septoria species for development of a bioherbicide cannot be drawn.

2.3.6 Taxonomy of Septoria galeopsidis Westd.

Septoria galeopsidis seems to accompany its widespread host Galeopsis tetrahit everywhere (Jørstad, 1967). The leaf spots produced by the pathogen are hypophyllous, greenish or brown, usually with a paler center and a dark border zone, irregular and angular, bounded by the veins. Pycnidia are scattered, brown, punctiform, thin-walled, epiphyllous on the lesions and up to 80 µm in diameter. Pycnidia produce cylindrical spores which are straight, flexuous, or curved, with up to three mostly indistinct septa (Jørstad, 1967; Grove, 1935). Conidial size for G. tetrahit has been reported as 28-60 X 1-2 µm (Jørstad, 1967) and 30-40 X 1-1.5 µm (Grove, 1935; Saccardo, 1882-1931). On G. speciosa conidia have been reported to range in size from 29-46 X 1-1.5 μm (Jørstad, 1967).

Septoria cotylea Pat. & Har., a form of *S. galeopsidis* is said to occur on the cotyledons, but does not seem to differ from the typical form. *Rhabdospora galeopsidis* seems to represent the winter form of *S. galeopsidis* (Jørstad, 1967). *Ascochyta galeopsidis* can be found in association with *Septoria* species, on the same spots (Grove, 1935).

Chapter 3.

Studies of the pathogen.

3.1 Introduction.

On June 1st, 1986, diseased hemp-nettle plants were found next to a barn located near Montebello (Quèbec). The leaves were covered with dark, more or less circular, coalescing spots. Diseased plant material was brought to the laboratory for pathogen isolation, observation, identification, and studies of the ecology of the pathogen.

3.2 Isolation, observation and identification of the pathogen.

3.2.1 Isolation.

Diseased plant leaves were surface disinfested by placing them in 70% (v/v) ethanol for 30 seconds followed by 2% (v/v) sodium hypochlorite for 15 seconds, and finally rinsing them in sterile distilled water. Surface disinfested leaves were then cut into smaller pieces (0.5 X 0.5 cm), plated on potato dextrose agar (PDA) in Petri dishes, and incubated at 24 C for one week. Black, dense fungal mycelium grew out from these tissue pieces, and subcultures were made by transferring hyphal tips to PDA plates. A small quantity of spores exuded from pycnidia produced on these subcultures after three weeks. Single spore isolates were obtained from these cultures by streaking a needle bearing spores on a PDA plate. Stock cultures on PDA slants were covered with mineral oil and stored in a refrigerator.

3.2.2 Koch's postulates.

The following steps, referred to as Koch's Postulates (Agrios, 1978), were taken to verify the hypothesis that the isolated organism was the causal agent of the disease: 1) the fungus was found associated with the disease in all the diseased plants examined; 2) the fungus was isolated and grown in pure culture on PDA (see section 3.2.1 above); 3) healthy hemp-nettle plants were inoculated with spores of the fungus grown in pure culture, and the same symptoms appeared on the plants; and 4) the fungus was reisolated in pure culture and colonies had the same visual characteristics as the original colonies. These observations demonstrated that the isolated fungus was the causal agent of the disease.

3.2.3 Microscopic observations.

Fruiting bodies and spores produced by the fungus were observed under the microscope (100X and 400X) for identification. Conidia were produced in pycnidia and emerged from the ostiole in a gel-like substance, or matrix. They were filiform, several-celled, and hyaline. Seventy-five pycnidiospores mounted in distilled water were measured using a microscope at 400X and a micrometer.

3.2.4 Identification.

Characteristics of the fungus (shape, appearance, and size of conidia, and type of structure in which conidia are produced) corresponded to the description of the genus Septoria (Sutton, 1980). Septoria galeopsidis Westd. was reported as a pathogen of Galeopsis tetrahit (Seymour 1929; Jørstad, 1967). Descriptions and dimensions of the fungus as reported by several authors (Jørstad, 1967; Grove, 1935; Saccardo, 1882-1931) were similar to those observed here (table 3.2.1). Cultures of the fungus were sent to the Commonwealth Mycological Institute (Identification Services, Kew, Richmond, Surrey, TW9 3AF U.K.), and the identification of the fungus as S. galeopsidis Westd. was confirmed by Dr. E. Punithalingam.

A hemp-nettle specimen bearing spots caused by a fungus identified as Septoria galeopsidis Westd. from the McGill University Herbarium was examined. It had been collected in Nova Scotia in 1909. The lesions had the same appearance as the ones that were observed on the diseased specimen found in Montebello area. Small pieces of diseased plant material from the specimen were immersed in water, and rehydrated spores were measured (table 3.2.1). These measurements were similar to those for the pathogen under investigation.

Table 3.2.1 Measurements of *Septoria galeopsidis* pycnidiospores.

Origin or reference	No of spores measured	range (μm)	mean (μm)
Saccardo, 1882-1931	-	1.0-1.5 X 30.0-40.0	-
Jørstad, 1967	-	1.0-2.0 X 28.0-60.0	-
Montebello	75	1.0-2.5 X 27.5-55.0	1.8 X 38.0
McGill University Herbarium	25	1.0-2.0 X 27.5-55.0	1.3 X 40.2

3.3 Ecology of the pathogen.

3.3.1 Introduction.

Optimum conditions for growth, sporulation, and spore germination of a fungus must be determined before studies on development and severity of the disease caused by the fungus on a plant are conducted. Information gathered through these investigations on the pathogen separate from its host give valuable indications about optimum conditions for disease development. It also allows the assessment of the limitations of the pathogen, which is critical in the evaluation of a pathogen as a potential bioherbicide for a weed.

Septoria galeopsidis was first studied separately from its host. Different growth media and incubation conditions were tested for maximum spore production. The effects of incubation temperature and medium composition on radial growth, and the effect of temperature on percent spore germination were also investigated.

3.3.2. Optimum conditions for spore production.

One of the essential criteria for successful use of plant pathogens as biological herbicides is the development of methods to produce abundant and viable inoculum (Walker and Riley, 1982). The production of spores often requires more specific conditions than does vegetative growth (Deacon, 1980).

A fungus may grow under certain conditions and yet be unable to complete its life cycle. Several factors were evaluated for their effect on sporulation of S. galeopsidis. These factors included medium composition and texture, type of inoculum, light quality, and temperature.

3.3.2.1 Materials and methods.

Five liquid and 15 agar media were prepared according to the recipes given in appendix A. One hundred ml of each liquid medium in 250-ml Erlenmeyer flasks were autoclaved 17 minutes. Agar media were autoclaved and poured into sterile plastic 9-cm Petri plates. A third type of medium consisted of boiled soybean (Glycine max L.) seeds, barley (Hordeum vulgare L.) seeds, or stems of approximately two month-old hemp-nettle plantsthat were put into 250- or 125-ml Erlenmeyer flasks and autoclaved 30 minutes. Liquid, agar, seed, and stem media were seeded with either spores (approximately 1.0×10^4 to 5.0×10^5 spores/ml) produced on PDA, PDA plugs (6 mm in diameter) bearing mycelium, or small pieces of mycelium (1 X 1mm) from colonies grown on PDA. Flasks containing inoculated liquid media were placed on a rotary shaker at 250 rpm for three weeks and observed at weekly intervals.

The effect of light quality on spore production was assessed by providing four-day-old pieces of mycelium growing on water agar containing hemp-nettle plant parts (HPA) with either artificial light from fluorescent and incandescent bulbs (14-hour photoperiod, 250 uE/m^2/s) in a growth cabinet, continuous near ultra-violet (NUV) light (366 nm) in a cubbard, or no light, in an incubator. Temperature was approximately 24 C, and plates were incubated for three weeks and observed at weekly intervals.

The effect of temperature was tested by placing water agar (WA), Czapek's dox agar (CDA), hemp-nettle plant parts agar (HPA), lima bean agar (LBA), nutrient agar (NA), and violet red bile agar (VRBA) plates inoculated with pieces of mycelium in different incubators at 5, 15, 25, and 30 C in the dark to evaluate the effect of temperature on spore production.

These investigations were not conducted with the intent of performing statistical analyses afterwards, but were rather a series of preliminary manipulations made in order to discover a way of producing sufficient quantities of inoculum for subsequent investigations. Treatments were therefore not replicated, and sporulation was visually assessed.

3.3.2.2 Results and discussion.

Results from these investigations are summarized in table 3.3.1. *Septoria galeopsidis* did not sporulate in any liquid medium tested. *Septoria* spp. generally sporulate well on agar media (Cooke and Jones, 1970), but only a few species are reported to sporulate in liquid media. *S. polygonorum*, for example, was reported to sporulate in shake cultures of V-8

Table 3.3.1 Visual evaluation of pycnidiospore production by *Septoria galeopsidis* as influenced by medium and incubation conditions.

Medium	Inoculum[1]	Incubation conditions	Sporulation[2]
Liquid media			
Beef peptone broth	m	room temp., daylight	-
Hemp-nettle extract	m	room temp., daylight	-
		room temp., NUV light	-
Potato dextrose broth	m	room temp., daylight	-
		room temp., NUV light	-
	p	room temp., daylight	-
Richard's modified solution	m	room temp., daylight	-
		room temp., NUV light	-
	p	room temp., daylight	-
Richard's modified solution with leaves	m	room temp., daylight	-
Agar media			
Bacto-agar (water agar)	m	15 C, darkness,	-
		25 C, darkness,	-
		31 C, darkness,	-
Bacto-mycological	m	room temp., NUV light	-
Bacto-yeast agar	m	room temp., NUV light	-
Czapek's dox	m	15 C, darkness	-
		25 C, darkness	-
		31 C, darkness	-
Czapek's dox-V-8	m	room temp., NUV light	-
	p	room temp., NUV light	*
Hemp-nettle extract agar	m	room temp., NUV light	*
	p	room temp., NUV light	****
		16 C, NUV light	-
Hemp-nettle plant parts agar	m	5 C, darkness	-
		15 C, darkness	-
		25 C, darkness	-
		31 C, darkness	-
		room temp., NUV light	**

Table 3.3.1 Continued.

Medium	Inoculum[1]	Incubation conditions	Sporulation[2]
		24/18 C, artificial light	*
		15/10 C, artificial light	*
	p	room temp., NUV light	-
Hemp-nettle extract with PDA	p	room temp., NUV light	****
Lima bean agar	m	15 C, darkness	-
		25 C, darkness	-
		31 C, darkness	-
	p	room temp., NUV light	-
Malt extract	p	room temp., NUV light	-
Malt extract and yeast	m	room temp., NUV light	-
Nutrient	m	15 C, darkness	-
		25 C, darkness	-
		31 C, darkness	-
Potato dextrose	m	room temp., NUV light	-
	m	room temp., darkness	*
	p	room temp., NUV light	-
<u>S. nodorum</u> sporulation	p	room temp., NUV light	-
Violet red bile	m	15 C, darkness	-
		25 C, darkness	-
		31 C, darkness	-
<u>Other media</u>			
Barley seeds	m	room temp., daylight	-
	p	room temp., daylight	***
	p	room temp., NUV light	-
Hemp-nettle stems	p	room temp., daylight	-
Soybean seeds	m	room temp., daylight	-
	p	room temp., daylight	***

[1] m mycelium plugs
p pycnidiospores

[2] - no sporulation
* trace sporulation
** good sporulation
*** very good sporulation
**** excellent sporulation

media (Smith and Self, 1984). Although biologically feasible, mass production of organisms that require solid substrates is not favored by the equipment and technology that are presently available in the industry (Bowers, 1982; Kenney and Couch, 1981). Spore production would therefore be more complex and costly for a fungus sporulating only on solid substrates. For these reasons, sporulation in submerged liquid culture might be an essential requirement for a fungus to be commercialized as a mycoherbicide.

A small number of solid media were suitable for spore production. The fungus sporulated well on water agar containing plant parts (HPA) when pieces of mycelium were used as inoculum. Transferring the hyphae directly to pieces of plant material emerging from the agar favored sporulation. Sutton (1980) mentioned the beneficial effect of incorporating sterilized natural materials such as wheat straw or macerated leaves and lupin stems into the basic agar medium. Some fungal species sporulated better on the plant material than on the agar itself.

Agar media containing concentrated hemp-nettle extract (HEA) with or without potato extract and dextrose were the best media for sporulation when a spore suspension was used as inoculum. Inoculum densities of approximately 1.0×10^5 spores/ml produced high quantities of spores per plate. Although the two wheat pathogens S. tritici and S. nodorum were reported as producing large numbers of spores on Czapek's dox-V-8 agar (CDVA), only a very small quantity of inoculum was produced on that medium by S. galeopsidis (Cooke and Jones, 1970). The only other medium on which spores were produced, although in a very small quantity, was PDA. On HEA, spores of S. galeopsidis were extruded from pycnidia as droplets or tendrils (cirrhi), similar to S. nodorum spores on wheat leaves (Griffiths and Peverett, 1980). These droplets and cirrhi are observed in figure 3.2.1 A and B, respectively. Figure 3.2.1 C shows an enlarged pycnidium releasing spores in water.

Abundant sporulation was obtained on boiled soybean and barley seed, when the inoculum consisted of a suspension of 5.0 X 10^5 spores/ml, but no sporulation was obtained when the seeds were inoculated with mycelium plugs. These results, however, were observed only once, and failure to obtain sporulation in subsequent trials was attributed to contamination, changes in temperature, and/or not viable inoculum.

Very little sporulation occurred in darkness. As in the case of S. tritici and S. nodorum, NUV light (366 nm) was most effective to induce sporulation from plates inoculated with either mycelium or spores (Cooke and Jones, 1970). Smaller, but substantial quantities of pycnidiospores were produced in artificial light, but the accompanying condensation promoted contamination. The success of artificial light tubes in inducing sporulation probably largely depended upon the small but significant amount of NUV radiation emitted from them

Figure 3.2.1 Spore production by *Septoria galeopsidis*.

A) Spores extruded in droplets from pycnidia on hemp-nettle extract agar.

B) Spores extruded in tendrils (cirrhi) from pycnidia on hemp-nettle extract agar.

C) Pycnidium releasing spores in water.


A
100 µm

(Cooke and Jones, 1970). The stimulatory effect of NUV light on sporulation is recognized, and Sutton (1980) stated that fungal cultures submitted to 12 h NUV light irradiation/12 h dark in plastic Petri dishes were more likely to sporulate than when incubated in other conditions. Visible light has two major effects on reproduction (Deacon, 1980), it often induces the formation of sexual and asexual reproductive structures, and it orients these structures during spore dispersal. NUV light is most effective for these functions, and a sporogenic substanc (called P310) occurs in hyphae soon after their irradiation (Deacon, 1980). This substance might have been produced by Septoria galeopsidis cultures that were irradiated with either artificial light and NUV light, since greater quantities of spores were produced than in darkness.

Temperature had no effect on sporulation in darkness. Cool temperatures did not induce sporulation by S. galeopsidis.

These preliminary investigations led to the development of a standard procedure for spore production. The most efficient way to produce spores for further experimentation was to transfer pieces of mycelium on or near hemp-nettle plant material in water agar, incubate these cultures for four days in darkness to allow the fungus to grow, and then place the cultures under NUV light at room temperature (21-24 C). The small quantities of conidia thus produced were then collected with a sterile syringe and expelled dropwise on HEA plates. These cultures were incubated at room temperature under continuous NUV light for production of inoculum used in experiments.

Septoria galeopsidis exhibited cultural variability as differences in colony appearance were often observed throughout these studies. In a study on S. avenae Frank. cultural variability was observed and was believed to be due to a process of continuous mutations and subsequent vegetative association of different nuclear types within a heterocaryotic system (Hooker, 1957). Spore production on most suitable media by Septoria galeopsidis was also variable even when all other factors were unchanged.

3.3.3 Experiment 1. Effect of incubation temperature and medium composition on radial growth.

When a fungus is grown on a solid medium the hyphae grow in a radial fashion from the inoculum and the resultant colony usually has a circular outline (Robinson, 1978). Hyphae extend at a rate which is normally constant for a given set of conditions, and radial growth is thus one of the parameters that are used in assessing the suitability of a certain environment to sustain the growth of a fungus. This experiment was performed to determine the effect of temperature and medium composition on radial growth of Septoria galeopsidis

3.3.3.1 Materials and methods.

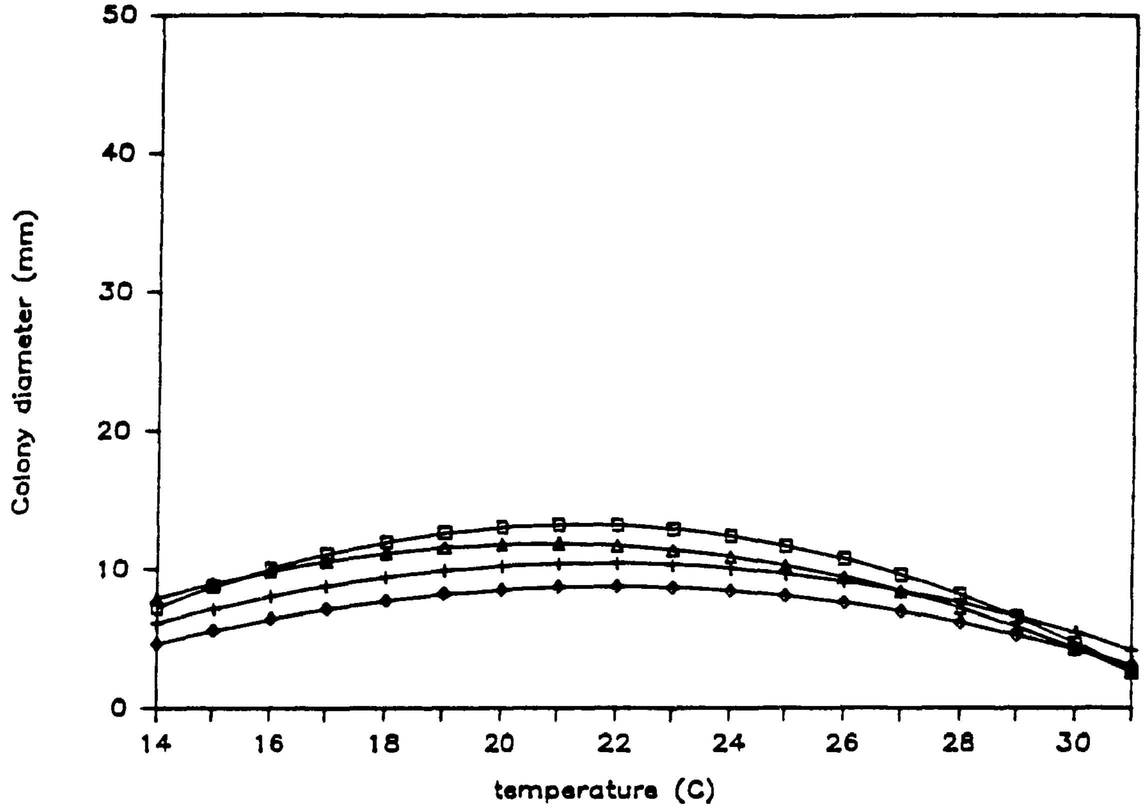
Twelve agar media (beef peptone (BPA), Czapek's dox

(CDA), Czapek's dox-V-8 (CDVA), hemp-nettle plant parts (HPA), lima bean (LBA), malt extract (MEA), malt extract and yeast (MEYA), nutrient (NA), potato dextrose (PDA), violet red bile (VRBA), water (WA), yeast extract (YEA)) and four temperatures (14, 21, 26 and 31 C) were assessed for their effects on radial growth. The media were poured into sterile plastic Petri plates using an automatic pipeter 17 ml/plate. Plates of each medium were seeded with a 4-mm-diameter PDA plug from a single spore culture that had grown for three days in the dark at 24 C. Plates were placed in incubators at the different temperatures and colony diameter was measured after 28 days. Each treatment was replicated three times. Data were analyzed using the general linear models procedure.

3.3.3.2 Results and discussion.

Septoria galeopsidis is a slow-growing fungus and the margins of the colonies did not reach the edges of the Petri plates in any instance. The relationship between colony diameter and incubation temperature on each medium is presented in figures 3.3.1, 3.3.2, and 3.3.3. Means of colony diameter at each temperature for each medium are given in table E.1, in appendix E. The analysis of variance demonstrated that both temperature and medium composition had an effect on colony growth, and there was an interaction between the two factors (table E.2).

Regression analyses were performed on colony diameter

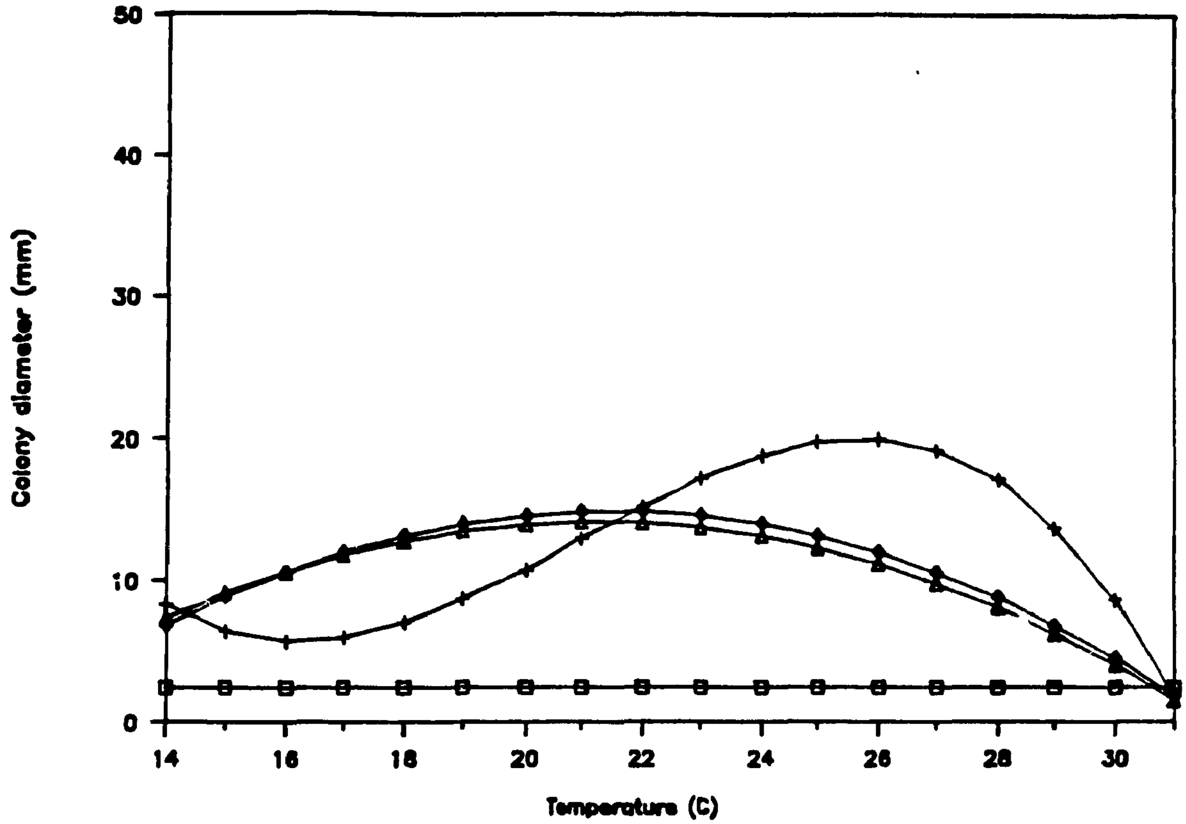


Legend: Agar media

□	Beef peptone	$Y = -37.66 + 4.78*T - 0.11*T^2$
+	Czapeck Dox	$Y = -23.55 + 3.14*T - 0.07*T^2$
◇	Malt Extract	$Y = -23.36 + 2.95*T - 0.07*T^2$
△	Yeast Extract	$Y = -25.56 + 3.62*T - 0.09*T^2$

Y = Estimate of colony diameter (mm)
T = Temperature (C)

Figure 3.3.1 The effect of incubation temperature on colony diameter of Septoria galeopsidis on different agar media.

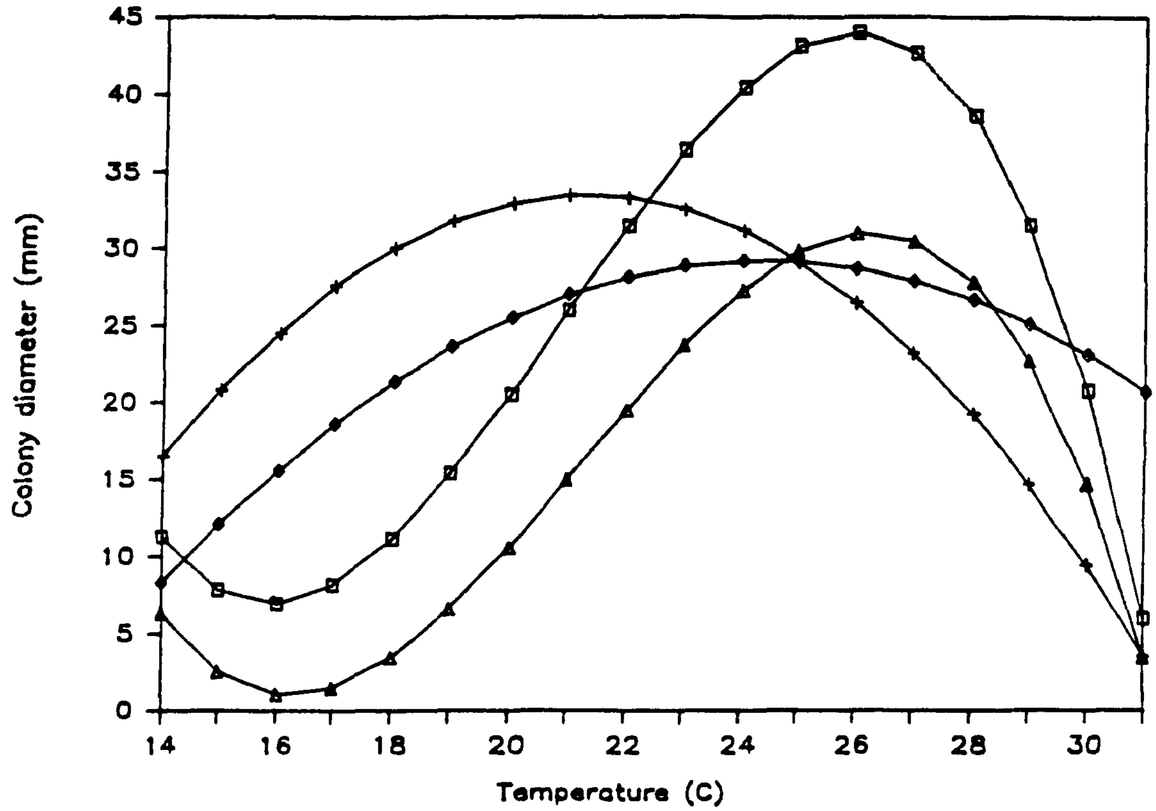


Legend: Agar media

□ Violet Red Bile
+ Nutrient
$Y = 271.07 - 41.50*T + 2.09*T^2 - 0.03*T^3$
◇ MaltExtract and Yeast
$Y = -51.48 + 6.18*T - 0.14*T^2$
△ Water
$Y = -44.75 + 5.56*T - 0.13*T^2$

Y = Estimate of colony diameter (mm)
T = Temperature (C)

Figure 3.3.2 The effect of incubation temperature on colony diameter of <u>Septoria galeopsidis</u> on different agar media.



Legend: Agar media

□ Czapeck Dox-V-8
$Y = 578.86 - 90.50*T + 4.59*T^2 - 0.07*T^3$

\+ Hemp-nettle Plant parts
$Y = -110.73 + 13.54*T + 0.32*T^2$

◇ Lima Bean
$Y = - 86.17 + 9.47*T - 0.19*T^2$

△ Potato Dextrose
$Y = 501.77 - 77.68*T + 3.87*T^2 - 0.06*T^3$

Y = Estimate of colony diameter (mm)
T = Temperature (C)

Figure 3.3.3 The effect of incubation temperature on colony diameter of *Septoria galeopsidis* on different agar media.

data for each medium separately (table E.3). On most growth media, colony diameter had a curvilinear relationship with incubation temperature, with largest diameters occurring at temperatures of 21 or 26 C. A cubic relationship between radial growth and incubation temperature was observed on CDVA, PDA and NA. A quadratic relationship was observed on BPA, CDA, MEA, YEA, LBA, HPA, MEYA, and WA. Mycelium growth on VRBA was not affected by temperature.

Colony growth was greater on CDV, HPA, LBA, and PDA. Colony morphology differed greatly from one medium to the other. A thin, loose, immersed mycelium was produced on HPA and WA, while mycelium growing on PDA and CDV was very dense, and completely covered with pycnidia. On the other media, mycelium had an intermediate texture.

The morphology of fungal colonies is affected by environmental and genetic factors (Robinson, 1978). It can vary considerably with the medium on which it grows. For many fungi, the maximum radial growth rate of the hyphae is achieved at very low glucose (or some other growth-limiting carbon source) concentrations, while increasing the glucose concentration will result in an increase in the hyphal density of the colony. At the lowest glucose concentrations, the apical cells of the marginal hyphae rarely branch and there are relatively large areas of the medium uncolonized within the colony perimeter, as was observed in this experiment on HPA and WA. On richer media, the extensive branching results in good coverage of the medium.

Temperature is an important factor in the growth of fungi (Cooke, 1979). During different stages of their life cycle, however, the optimal temperature may be different. Most fungi are mesophillic, they grow at moderate temperatures of 10-40 C, with an optimum between 25 and 35 C (Deacon, 1980). Pathogens of temperate plants may not grow at all above 30 C. S. galeopsidis grew at all temperatures between 14 and 31 C, but the optimum temperature was 21-26 C, as indicated by the regression curves, and a temperature of 31 C greatly slowed the rate of hyphal elongation. The results obtained for a fungus in pure culture, however, may not apply to the natural environment, where the species range may be restricted by other organisms (Deacon, 1980). The optimum temperature for growth of a species may also be the optimum for another organism that will compete with it.

Fungi may tolerate one unfavorable factor provided that all others are optimal (Deacon, 1980). This could explain the interaction between temperature and medium composition. The fungus may have better chances to grow under less than optimal temperatures (14 and 31 C) when its nutrient requirements are met in the agar, and inversely, the lack of essential nutrients in the medium can impede fungal growth, even under a favorable temperature.

These results gave indications on the requirements for growth of *S. galeopsidis* and about the most efficient way to produce the mycelium used in inoculum production for subsequent experiments. The fungus is a relatively slow-growing one. According to these results, dense and abundant mycelium would be produced on CDVA, LBA, and PDA at a temperature of 26 C.

3.3.4 Experiment 2. Percent spore germination over time as influenced by incubation temperature.

Germination is the initial stage in the development of a fungus mycelium from its spore (Gottlieb, 1950). It involves a large number of metabolic and ultrastructural changes (Deacon, 1980; Gottlieb, 1950). Spores can generally germinate in water or very simple media, due to their ability to utilize their stored reserves, thus differing from hyphal growth. Environmental factors such as temperature, moisture, and light can influence both percent germination and the time required by spores to germinate. This experiment was conducted to determine the optimum temperature and the time required for germination of *S. galeopsidis* pycnidiospores on water agar.

3.3.4.1 Materials and methods.

Pycnidiospores produced on HEA were collected and suspended in water, and inoculum density was adjusted to 3.0 X 10^5 spores/ml. Aliquots of 0.1 ml of the spore suspension were spread on water agar plates with a glass rod and plates were distributed to four incubators at 15 $\pm$ 1, 20 $\pm$ 1, 25 $\pm$ 1, and

30 ± 1 C. After approximately 6, 12, 18, and 24 hours, one hundred spores were counted per plate using the microscope at 100X. Percent germination was calculated and recorded. No single event could be associated with the onset of germination of S. galeopsidis pycnidiospores because the spores are naturally elongated and the time at which the germ tube emerged was not well defined. A conservative approach was therefore used in declaring that a spore had germinated or not. Spores were considered germinated when changes in the shape, increases in width and length as compared to ungerminated spores, and/or branching were obvious. The experiment was performed twice, with four blocks the first time and five blocks the second time.

The experiment was stopped after 24 hours because extensive mycelium growth under some of the temperature regimes made the counting difficult. Due to the distribution of percentage values, the data were arcsine transformed prior to analysis (Gomez and Gomez, 1984). The data of the two experiments had homogeneous variances and were pooled for analysis with the general linear models procedure.

3.3.4.2 Results and discussion.

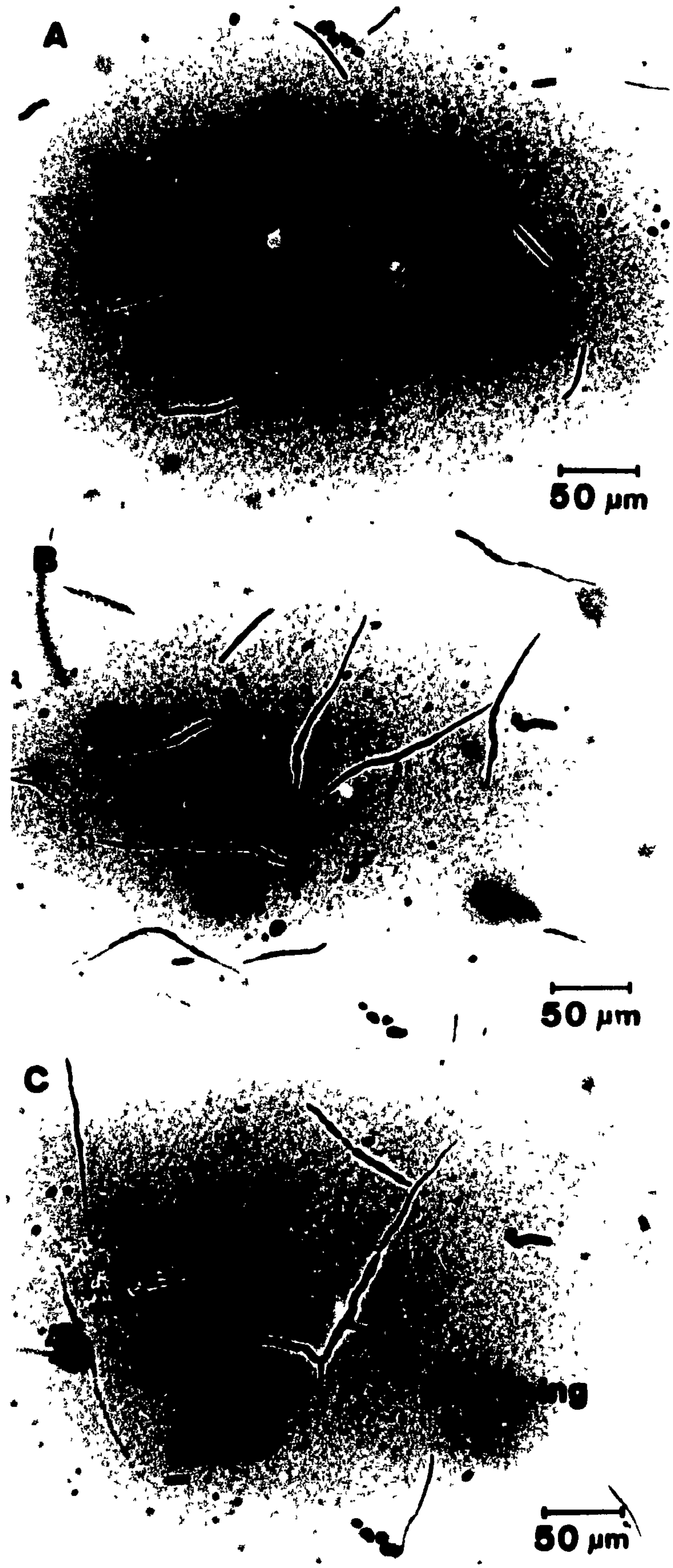
The process of S. galeopsidis pycnidiospore germination is illustrated on figure 3.4.1. Ungerminated pycnidiospores, elongation of germ tubes at 15 C, and beginning of branching at 24 C after 24 hours are shown.

Figure 3.4.1 _Septoria galeopsidis_ spore germination on water agar.

A) Ungerminated spores at 0 hour.

B) Germinated spores after 24 hours at 15 C.

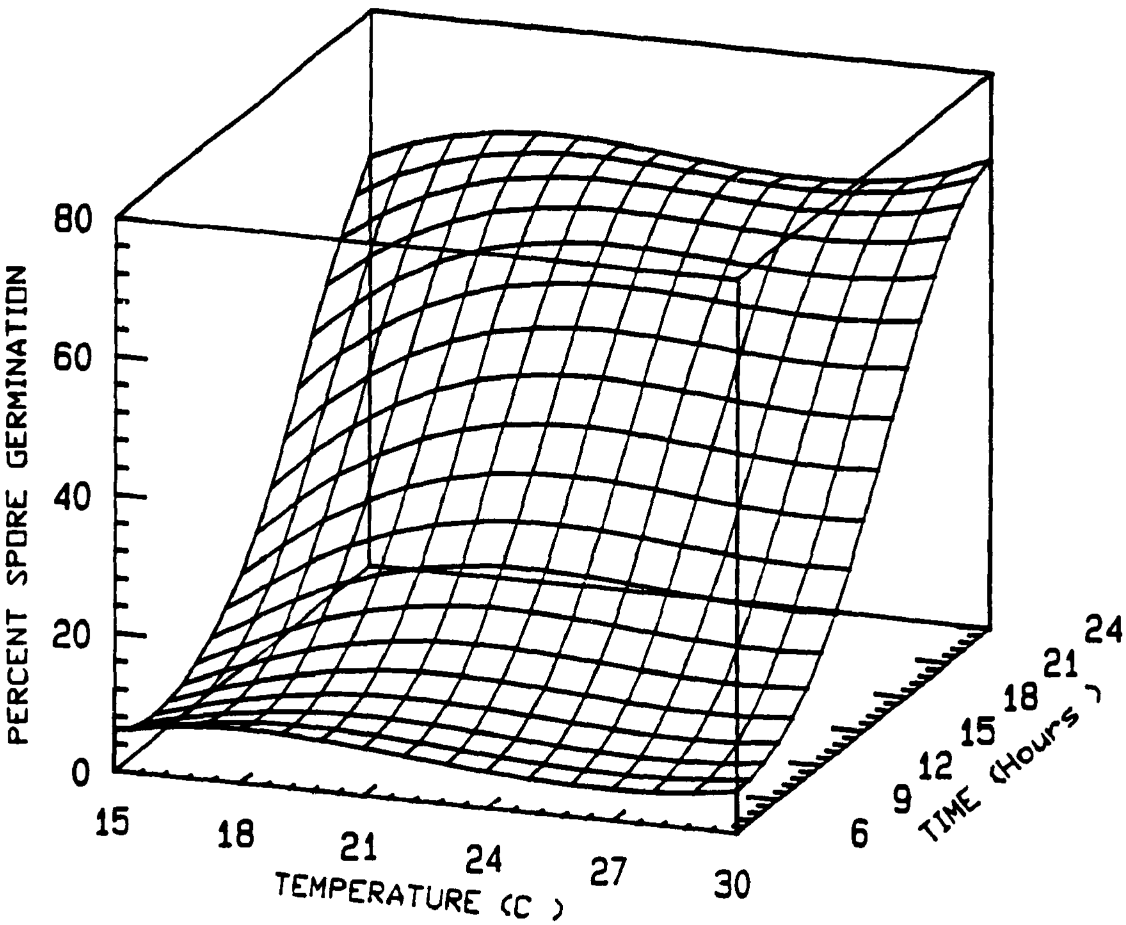
C) Spore starting to branch after 24 hours at 24 C.


A
50 µm
B
50 µm
C
50 µm

The means of percent spores germination and transformed data at each temperature for each 6-hour interval are presented in table E.4. The analysis of variance indicated a highly significant effect ($p<0.01$) of temperature on percent germination (table E.5). After 24 hours, however, there was no significant difference in percent germination between the temperature treatments (table E.6). Although percent germination was similar at all temperatures after 24 hours, mycelium growth was much less extensive at 15 C than 20, 25, or 30 C.

A regression equation taking time and temperature factors into account was fitted to the transformed data (table E.7). Percent germination had a curvilinear relationship with time and temperature, and the two factors interacted in a complex fashion. Figure 3.4.2 shows the relationship between these three variables, as predicted by the equation. The values obtained from the regression equation were retransformed into percent values prior to plotting ($[\text{arcsine}(x)]^2$).

According to these results, when moisture is available, over 60% spore germination would be expected after 24 hours at any temperature. Germination of S. galeopsidis spores on the surface of hemp-nettle leaves, however, might be different from that on water agar. A variety of factors, including nutrients, phytochemicals, microorganisms, free moisture, oxygen and carbon dioxide, pH, enzymes, dormancy, radiation, inoculum density, and senescence may affect spore germination in a



$$Y = 0.81 - 0.61*T + 0.03*T^2 - 0.0003*T^3 + 0.12*C - 0.01*C^2 + 0.0002*C^3 + 0.04*C*T - 0.001*C*T^2 - 0.0008*C^2*T + 0.00003*C^2*T^2$$

Y = Estimate of arcsine value of percent spore germination
C = Incubation temperature (C)
T = Incubation period (hours)

Figure 3.4.2 *Septoria galeopsidis* pycnidiospore germination over time as influenced by incubation temperature (pooled data for two experiments).

positive or negative way (Gottlieb, 1950). Even before it initiates infection, a spore that lands on the surface of a plant must be able to overcome competition from the resident saprophytic microflora in order to germinate and grow (Deacon, 1980). Infection of a host may thus require a considerable inoculum potential, which is the "energy of growth of a parasite available for infection of the host at the surface of the host organ to be infected" (Deacon, 1980). Moreover, spores can interact in several ways and a spore may behave differently when isolated from neighbouring spores (Robinson, 1978). Fungal spores may produce one or several metabolites that stimulate or inhibit germ-tube emergence.

Germination starts with the swelling of the spore, followed by a conversion of food reserves, predominantly lipids, into pools of metabolically active compounds, accompanied by a rapid increase in respiration (Deacon, 1980). A new wall layer may then be formed, through which soon emerges a germ tube. It is well known that temperature affects the percentage of germination, the time required for germination, and the rate of elongation of germ tubes (Gottlieb, 1950). Low temperatures slow biochemical reactions down whereas increased temperatures cause them to occur at a more rapid rate. Two of the possible ways in which germination is affected are the different temperature requirements for enzymes and the effect of temperature on the viscosity of water, and thus on imbibition of water by the spore. For most fungal species, the minimum temperature for spore germination is between 0 and 10

C, and the optimum temperature is between 24 and 28 C (Gottlieb, 1950).

Stages of germination of S. apii-graveolentis Dorogin, the cause of late blight of celery (Apium graveolens L.), were described by MacMillan (1942). Freshly exuded, three-celled, elongated spores were suspended in celery juice and observed. After four hours, the spores had swollen and elongated. After 12 hours, growth was resumed and branching was beginning, and it was evident at that stage that the spores had germinated.

S. galeopsidis spores followed the same steps in their germination. The percentage of spores that produce germ tubes is the most commonly used criterion to determine the effect of different physiological agents on germination (Gottlieb, 1950). The time required for germination, length of the tube, and its rate of elongation are also used. In this experiment, as was mentioned in section 3.3.4.1, it was difficult to determine precisely the time of emergence of the germ tube because S. galeopsidis pycnidiospores are naturally elongated. A relatively high percentage of germination occurred within a broad range of temperatures. The species did not have very restrictive temperature requirements. Subsequent growth of mycelium, however, was less extensive at 15 C than at any other temperature. Time required by spores to germinate was relatively long, this indicates that the period of high moisture necessary for substantial germination might be a limiting factor to the production of the disease.

CHAPTER 4.

Etiology of the disease.

4.1 Introduction.

Investigations were performed to determine how *Septoria galeopsidis* affects hemp-nettle and the conditions required for maximum disease production. These experiments evaluated the effects of 1) dew period duration and temperature, 2) inoculum density and spray adjuvants, and 3) plant age on disease development and severity. Information gathered through these experiments will be used to assess the potential of *S. galeopsidis* as a biological control agent for hemp-nettle.

4.2 General materials and methods.

4.2.1 Seed germination and plant growth.

Seeds collected in August, 1985 from naturally occurring hemp-nettle plants were used in the initial experiments. Subsequently, seeds were periodically obtained from plants grown in the greenhouse in order to maintain a fresh supply of seeds.

Seeds were washed in running water for approximately two hours and then placed in glass Petri plates on filter papers moistened with 3 ml of a gibberellic acid solution (1000 ppm). Seeds were then incubated at 15 C in the dark for 9 days. Seed germination was poor (30-35%), not uniform, and at planting time radicle lengths were between 2 and 20 mm. Six germinated seeds were planted 3 cm deep in pasteurized ProMix® (ProMix BX, Premier Brands, Inc., New York, NY) in each 10-cm plastic pot. Pots were placed in a controlled environment chamber and supplied with a 14-hour photoperiod with both incandescent and fluorescent light at an average intensity of 255 uE/m2/s. Day/night temperature regime was 24/18 C. Plants were watered daily with tap water and fertilized weekly beginning two weeks after planting with 5-11-26 fertilizer (1.25 g/L, 50 ml/pot) unless otherwise specified. Plants were thinned to three per pot after eight days, and since the stems usually did not stand upright, the plants were provided with a training system (composed of three wooden stakes connected with a piece of galvanized wire for each pot) 24 hours before spraying.

4.2.2 Inoculum production.

Cultures were started on PDA from small pieces of mycelium (1 X 1 mm) taken from the stock culture. After approximately one month of incubation at 24 C in darkness, small pieces of mycelium from these subcultures were transferred to HPA plates. Cultures were incubated in the dark at 24 C for four days, and then moved to a cabinet at room temperature under NUV light for four to seven days. After that period of time, pycnidia exuded matrix containing filiform spores which were collected with the tip of a sterile syringe and discharged dropwise on HEA plates. Five to seven days after inoculation, HEA plates were covered with pycnidia releasing pycnidiospores. Pycnidiospores were harvested by adding distilled water and gently scraping the agar surface with a glass rod. These spores were used as inoculum for plants in experiments.

After collection from HEA plates, the spore suspension was filtered through a 250-um mesh sieve to remove pieces of mycelium and then the filtrate was centrifuged for 10 minutes at 6500 G. The supernatant was discarded, and the pellet was resuspended in distilled water. Inoculum density was determined with the aid of a haemacytometer and further adjusted to the desired concentration(s) by diluting with distilled water.

4.2.3. Inoculation method.

Plants were inoculated with fungal spores using one of two different methods. The first method used two tandem DeVilbiss atomizers (Model 163, DeVilbiss Inc., Somerset, PA) powered with compressed air and placed 10 cm apart on a rod at a height of 46 cm above the soil surface. Plants were moved by hand under the spray, using a 0.09 m^2 tray. The second method used a single full cone nozzle (TG 0.7, Spraying Systems Co.) in a spray chamber (Research Instrument Manufacturing Co. Ltd., Guelph, Ont.). In both cases, the spray volume was adjusted to 500 L/ha, according to the height of the plant and the area covered by the spray.

4.2.4 Data collection.

Disease ratings were performed at 2-day intervals. The disease period started on the 10th to 14th day after inoculation and ended on the 18th or 20th day depending on the experiment. The percent leaf area covered by necrotic lesions was visually assessed for each leaf present on plants at the time of inoculation, using Horsfall and Barratt's system (Horsfall and Barratt, 1945; appendix B) and a corresponding assessment key made with the aid of a dot grid (appendix C). This grading system is based on the recognized fact that a person's ability to distinguish disease improves in accuracy toward the end of the percentage scale, i.e. when a plant has little disease or when it is almost completely diseased. The average grade was calculated for each plant and for each pot. Pot averages were used to draw disease progress curves, and the area under the disease progress curve (AUDPC) was calculated for each experimental unit and used for non-parametric statistical analysis. The formula that was used for AUDPC calculation is presented in appendix D (Shaner and Finney, 1977).

At the end of the disease rating period plants were harvested and their heights and aboveground biomass (fresh weight) were measured. Plants were then dried at 60 C for 8 days and weighed.

4.2.5 Statistical analyses.

Analysis of variance was performed on height, fresh weight, and dry weight data using the general linear models procedure. Regression analysis was performed on variables having a significant effect on these dependent variables.

A Friedman two-way analysis of variance was performed on the AUDPC data, and a non-parametric simple linear regression analysis was performed on the significant effects where relevant. Non-parametric statistical tests were used with AUDPC values because of the weak scale of measurement (visual disease ratings) (Daniel, 1978).

4.3 Experiment 1. The effect of dew period duration and temperature on disease development and severity.

4.3.1 Introduction.

For a disease to occur and develop, a susceptible plant, a virulent pathogen, and a favorable environment must be available to interact at the same time (Agrios, 1978; Holcomb, 1982). Environmental factors can predispose a plant to disease by altering the plant's susceptibility or by directly affecting the interactions of host and pathogen. Presence, absence, amount, and duration of environmental factors may act as constraints to disease development.

Moisture and temperature are among the environmental factors that most affect the initiation and development of plant diseases (Holcomb, 1982). The moisture requirements and optimum temperatures vary widely from one plant pathogen to another, and must be known in order to assess the potential of a specific microorganism as a biological herbicide. This experiment was conducted to determine the optimum dew period duration and temperature for infection of hemp-nettle plants by *S. galeopsidis* and to evaluate the subsequent severity of the disease.

4.3.2 Materials and methods.

The experiment, a 3 X 4 X 2 factorial, was performed as three separate runs, since only one temperature could be tested at one time in the dew chamber. The independent variables were dew period temperature (18 ± 2, 24 ± 1, and 30 ± 2 C), dew period duration (24, 36, 48, and 60 hours), and level of inoculation (uninoculated vs inoculated). Hemp-nettle plants at the 8-leaf stage were used since the disease was not produced on younger seedlings in preliminary experiments. Plants were sprayed with either corn syrup (2% v/v) or inoculum of *Septoria galeopsidis* at a rate of 1.0×10^8 spores/m^2, using the two laboratory atomizers. The spore suspension also contained 2% (v/v) corn syrup.

Plants were put in the dew chamber immediately after spraying. There were three plants per pot and three pots per treatment. After their respective dew period duration, plants were placed on a bench in the greenhouse to allow disease development, and were supplied with natural daylight. Day/night temperature regime during that period was 25/15 C on the average. Plants were arranged in a randomized complete block design because it was believed that a gradient in the amount of dew forming on the plants and gradients in temperature and light existed in the dew chamber and the greenhouse, respectively. The experiment was replicated once.

Disease ratings were performed 10, 12, 14, 16, and 18 days after inoculation for the first eight leaves. Plants were harvested on day 18.

4.3.3 Results.

Over 50 % of both control and inoculated plants that received the 30 C-60 hour-dew period treatment combination were killed by the high temperature-long dew period stress. This treatment combination could therefore not be included in the statistical analyses. Consequently, two separate analyses of variance were conducted for each dependent variables. The first one tested the effect of all dew period durations, at the temperatures of 18 and 24 C only (data set A), and the second one tested the effect of all temperatures for dew periods of 24, 36, and 48 hours only (data set B). Further analyses were performed on uninoculated and inoculated plants separately. Non-parametric tests on AUDPC data were performed on the data for inoculated plants only.

Disease development

Symptoms appeared eight to ten days after inoculation, first as chlorotic, roughly circular areas on inoculated leaves. These lesions soon became necrotic and pycnidia were visible within a few days. Some lesions were found on control plants due to their proximity to inoculated plants in the dew chamber, but the level of infection was negligible. Non-parametric statistical analyses were performed on inoculated plant data only. Figure 4.3.1 shows lesions with pycnidia on a leaf, a severely infected leaf about to absciss, and inoculated plants that received 48 hours of dew at 24 C compared to uninoculated plants receiving the same treatment. A large number of leaves have abscissed from infected plants.

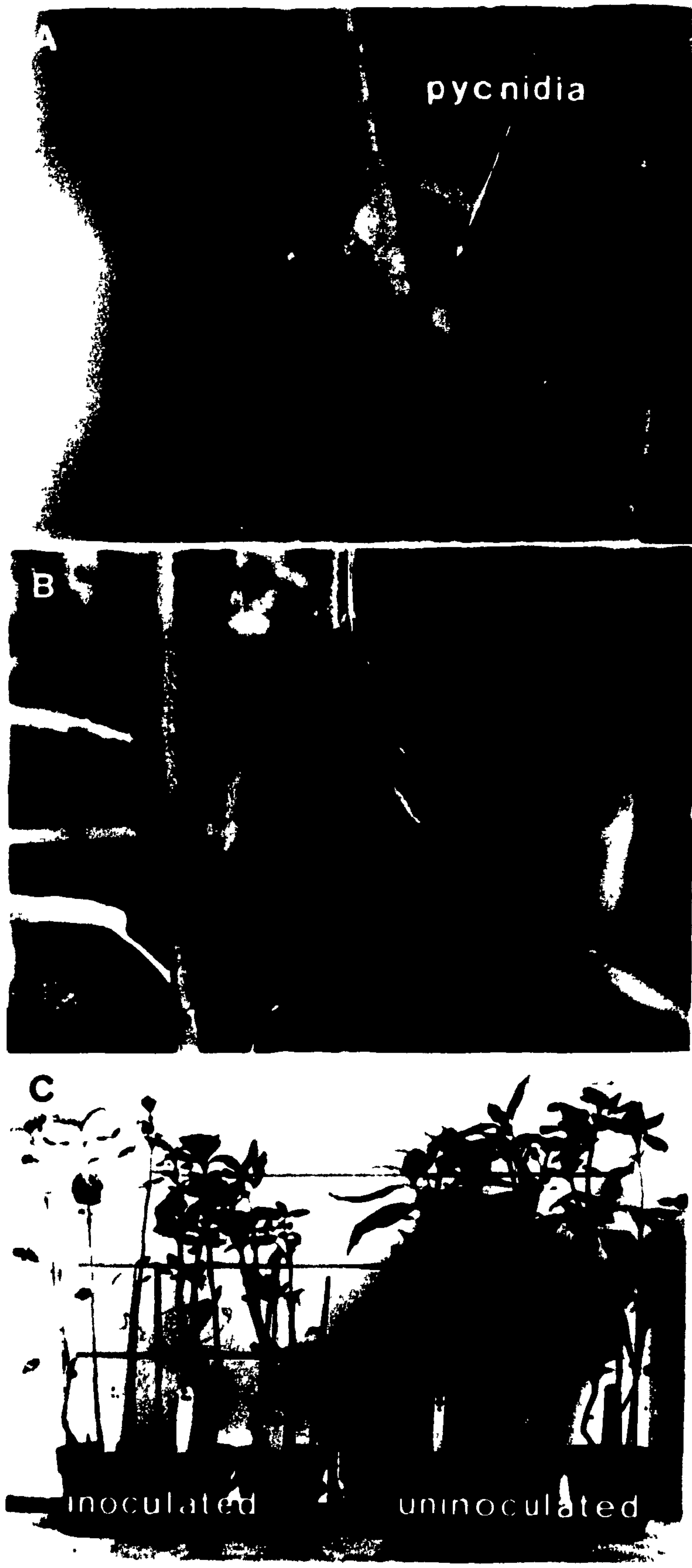
Mean AUDPC values are given for each dew period duration and each temperature in tables E.8 and E.9. Both dew period duration and temperature significantly affected disease development ($p<0.01$, table E.10). Maximum disease production was obtained after 60 hours of dew, and AUDPC values for that duration was significantly higher than after 24 and 36, but not 48 hours. The relationship between AUDPC and dew period duration was described by a non-parametric simple linear regresssion equation, for each temperature (table E.11). The area under the disease progress curve was significantly larger at 24 C than 18 C and 30 C, and the difference between 18 and 30 C was not significant. Disease progress curves for each dew period duration are shown in figures 4.3.2, 4.3.3, and 4.3.4,

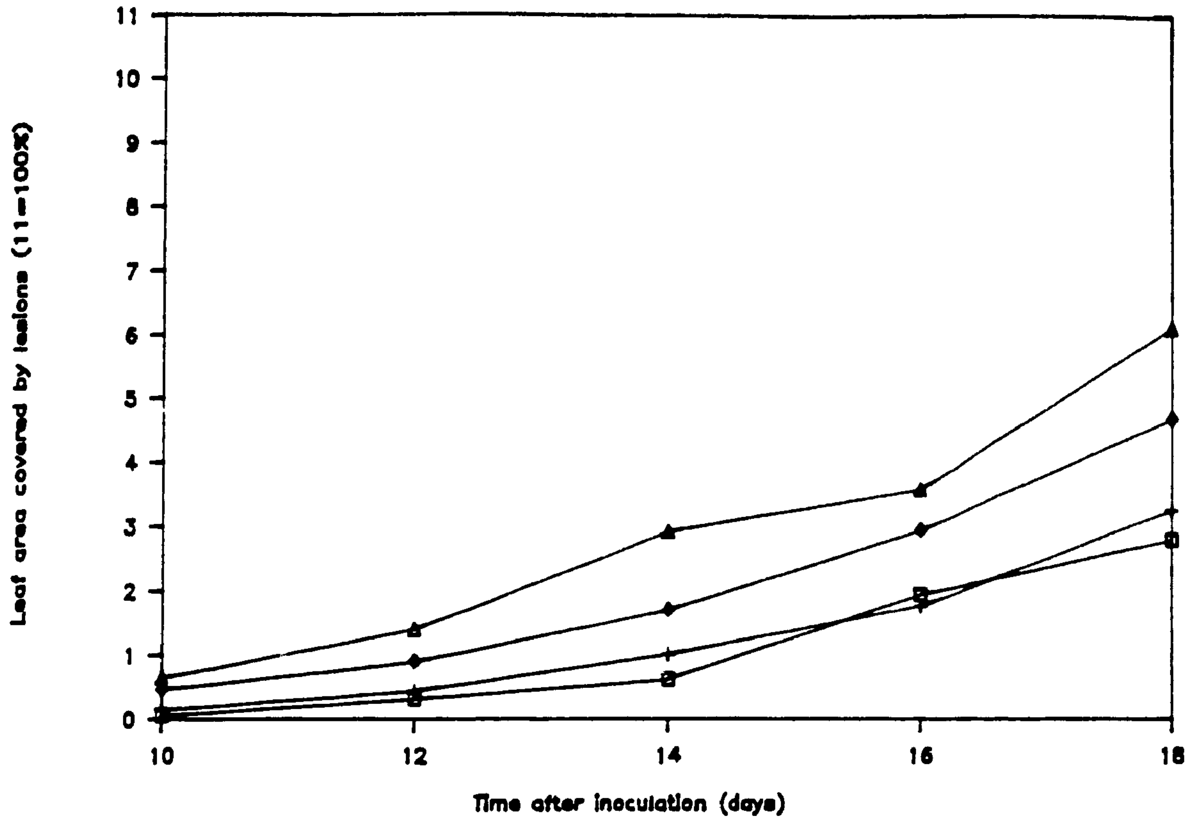
Figure 4.3.1 Lesions caused by Septoria galeopsidis and their effect on hemp-nettle plants.

A) Enlarged pycnidia-bearing lesions on a leaf.

B) Leaf covered with lesions and about to absciss.

C) Inoculated and uninoculated plants that have received a 48-hour dew period at 24 C.

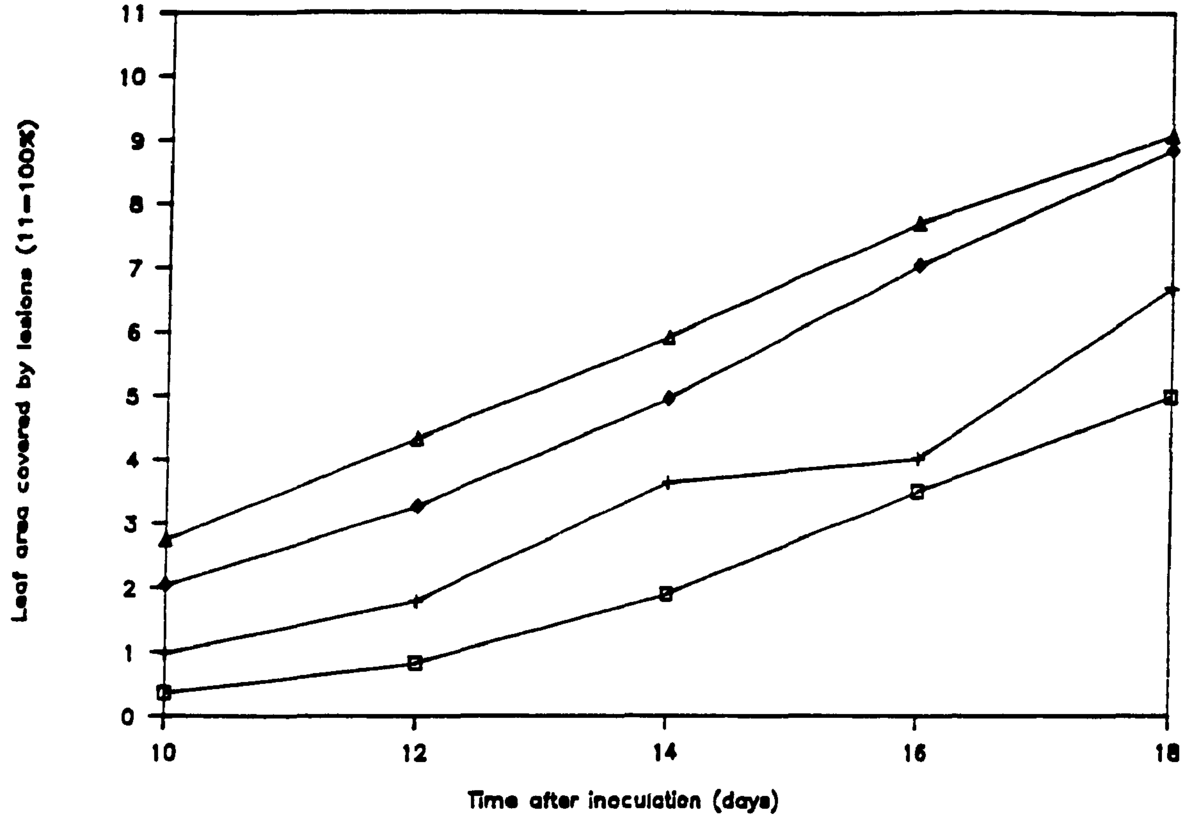

A
pycnidia
B
C
inoculated
uninoculated



Legend: Dew period duration

□ 24 hours
+ 36 hours
◇ 48 hours
△ 60 hours

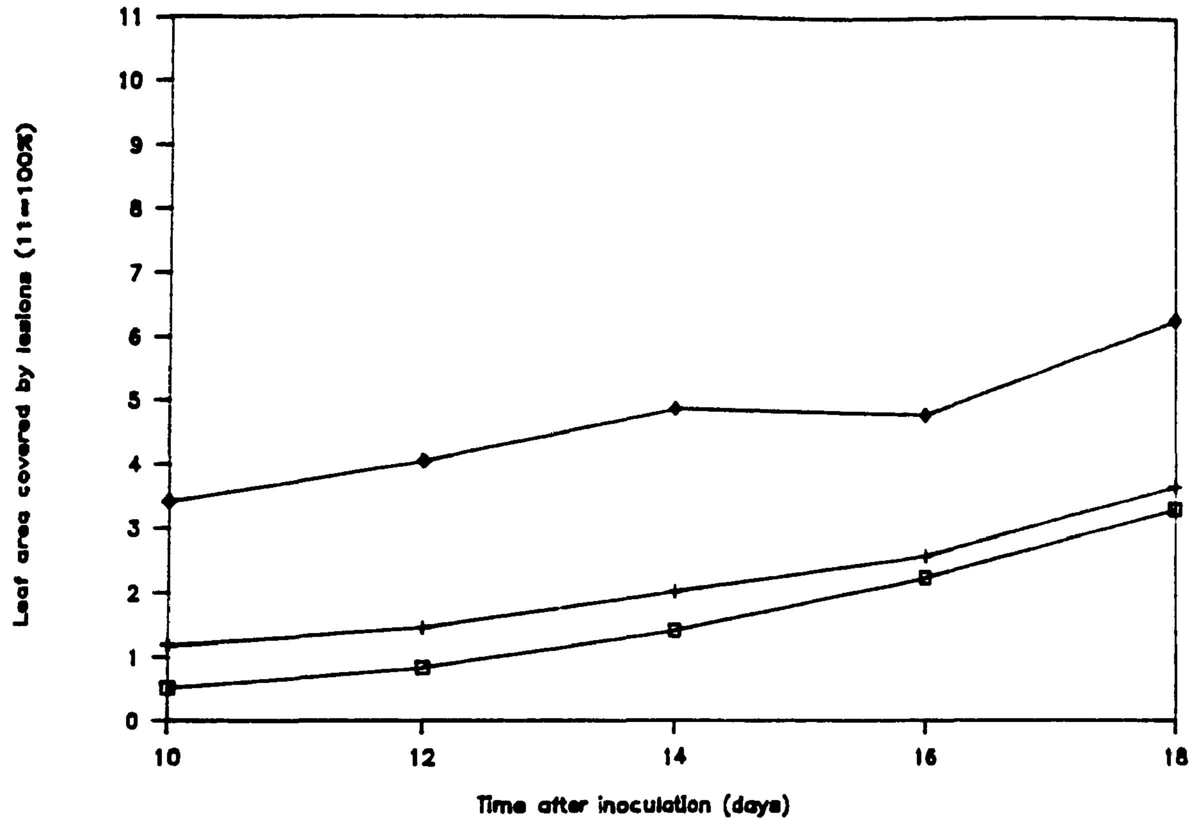
Figure 4.3.2 Disease progress curves for each dew period duration at a temperature of 18 C (pooled data for two experiments).



Legend: Dew period duration

- □ 24 hours
- + 36 hours
- ◇ 48 hours
- △ 60 hours

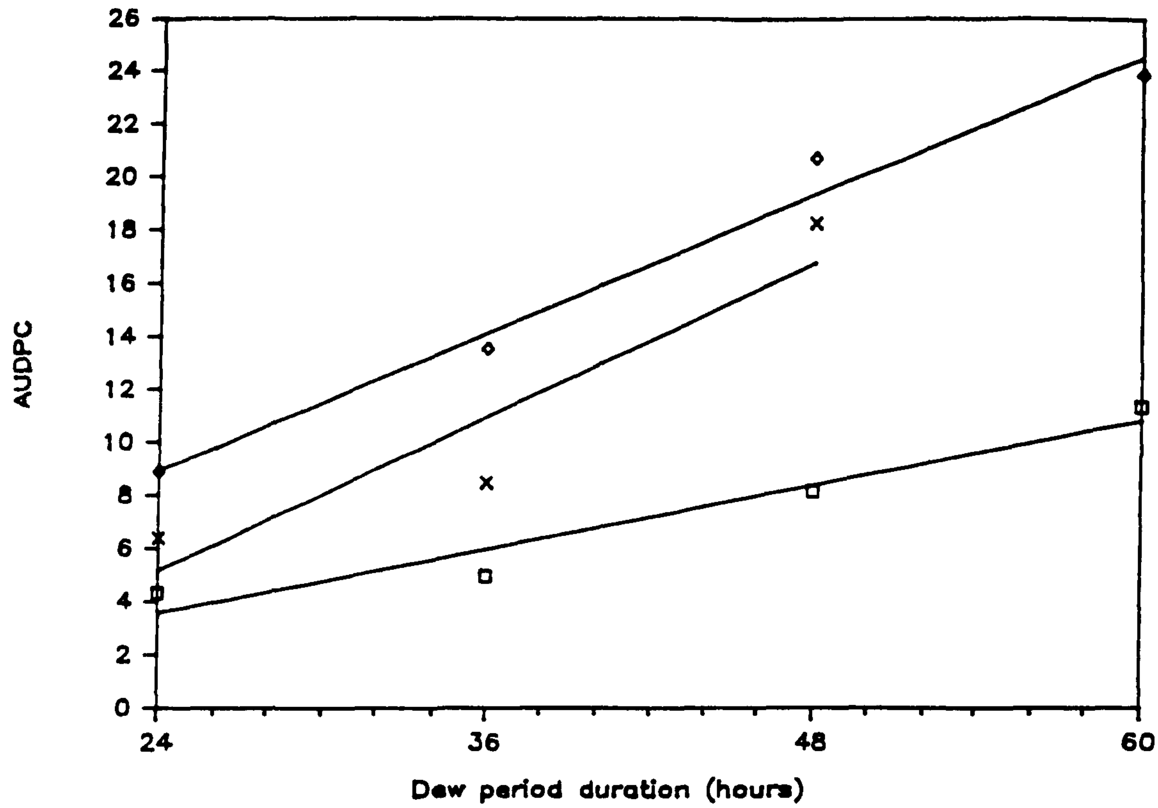
Figure 4.3.3 Disease progress curves for each dew period duration at a temperature of 24 C (pooled data for two experiments).



Legend: Dew period duration

□ 24 hours
+ 36 hours
◇ 48 hours

Figure 4.3.4 Disease progress curves for each dew period duration at a temperature of 30 C (pooled data for two experiments).



Legend: Temperature

□	18 C	Y = -1.24 + 0.20*D
◇	24 C	Y = -1.46 + 0.43*D
×	30 C	Y = -6.42 + 0.48*D

Y = Estimate of the area under the disease progress curve
D = Dew period duration (hours)

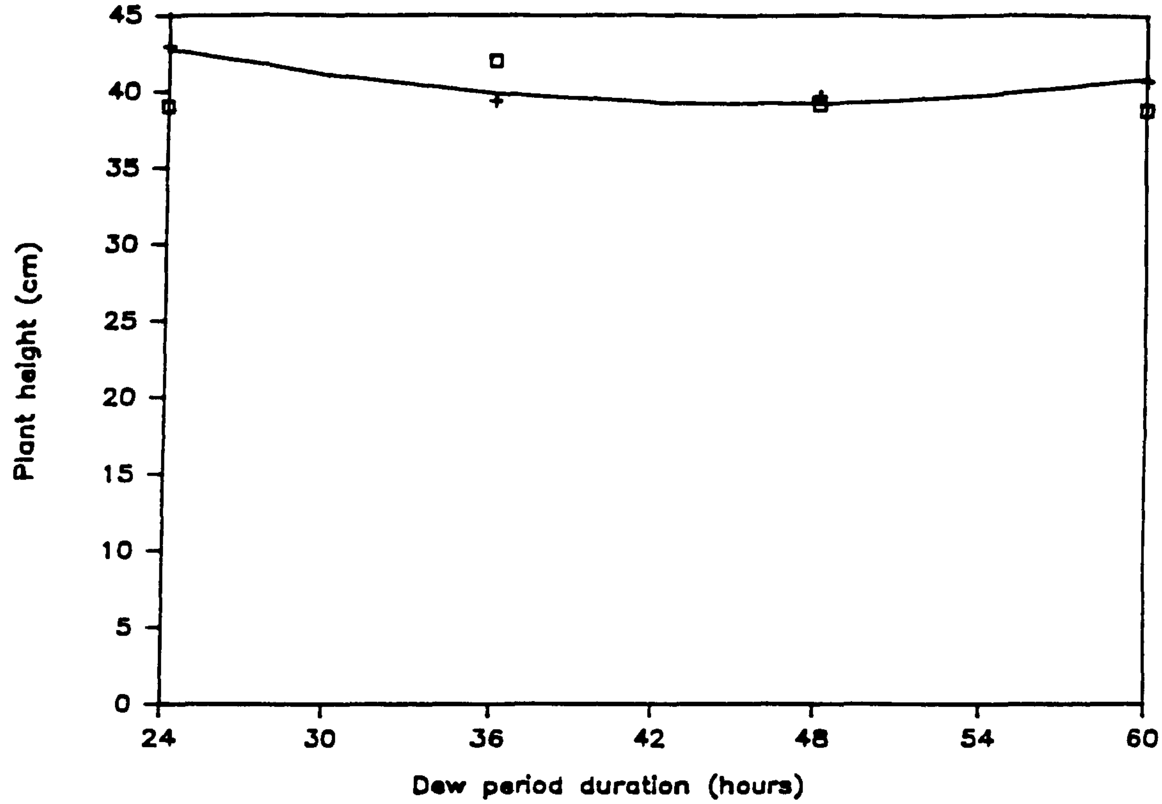
Figure 4.3.5 The effect of dew period duration on Area Under the Disease Progress Curve at 18, 24, and 30 C (pooled data for two experiments).

for temperatures of 18, 24, and 30 C. respectively. Points representing mean disease ratings on the graphs were connected to show the area under the curves. Regression lines at the different temperatures are presented on figure 4.3.5.

Height

Height values for uninoculated and inoculated plants that received the different dew period duration treatments were averaged over the temperature treatments of 18 and 24 C, and height values for the different temperature treatments were averaged over dew period duration treatments of 24, 36, and 48 hours (tables E.12 and E.13).

Inoculation and temperature had no significant effect on plant height and the interaction between the two factors was not significant (tables E.14 and E.15). There was an interaction between dew period duration and inoculation, however, which indicated a difference in the response of control and inoculated plant height to the length of dew period. The analysis of the data set A sorted by inoculation level showed that dew period duration had a significant effect on height of inoculated plants but not control plants (table E.16). A regression curve was fitted to the height data of inoculated plants, and is presented on figure 4.3.6 along with the means for inoculated and control plants (table E.17).



Legend:

□ Uninoculated plants
+ Inoculated plants $Y = 55.86 - 0.74*D + 0.01*D^2$

Y = Estimate of plant height (cm)
D = Dew period durarion (hours)

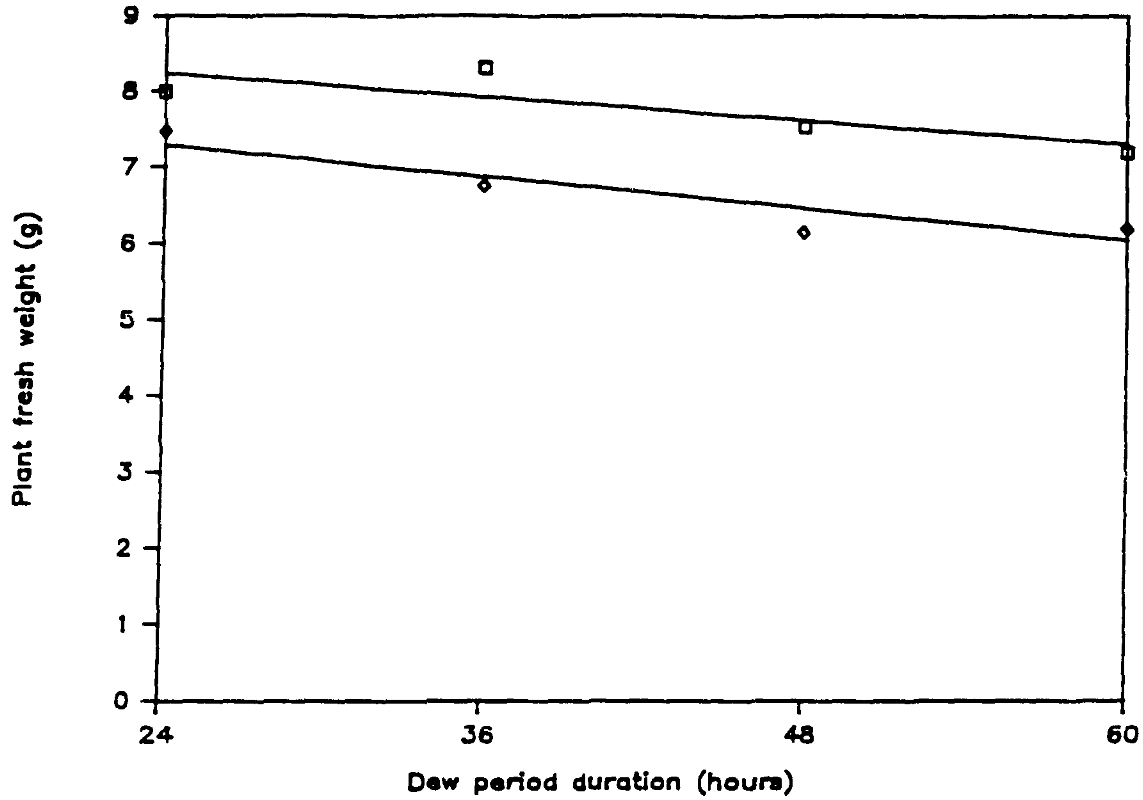
Figure 4.3.6 The effect of dew period duration on height of uninoculated and inoculated hemp-nettle plants (pooled data for two experiments - 30 C treatment deleted).

Plant fresh and dry weight

The means of plant fresh and dry weight for each dew period duration averaged over the temperatures of 18 and 24 C are presented in tables E.18 and E.19, respectively. Tables E.20 and E.21 present the fresh and dry weight values for each temperature averaged over the dew period durations of 24, 36, and 48 hours.

Inoculation of hemp-nettle plants with S. galeopsidis had a highly significant effect ($p < 0.01$) on both plant fresh and dry weights (tables E.22 to E.25). The fresh weight mean of inoculated plants was 14.2 - 14.6 % (depending on the data set) lower than that of uninoculated plants, and the mean of inoculated plant dry weight was 20.0 - 21.4 % lower than that of controls. Again, the two variables were not affected by temperature in the 18 - 30 C range, and there was no significant interaction between inoculation and temperature. Both inoculated and control plant fresh and dry weights were significantly affected by dew period duration (tables E.26 and E.27), but the absence of interaction between this factor and inoculation indicated that the length of dew period had the same effect on both inoculated and control plant biomass.

Regression equations were fitted to the fresh and dry weight data of control and inoculated plants (tables E.28 and E.29). Figures 4.3.7 and 4.3.8 illustrate the relationship between the two dependent variables and dew period duration,

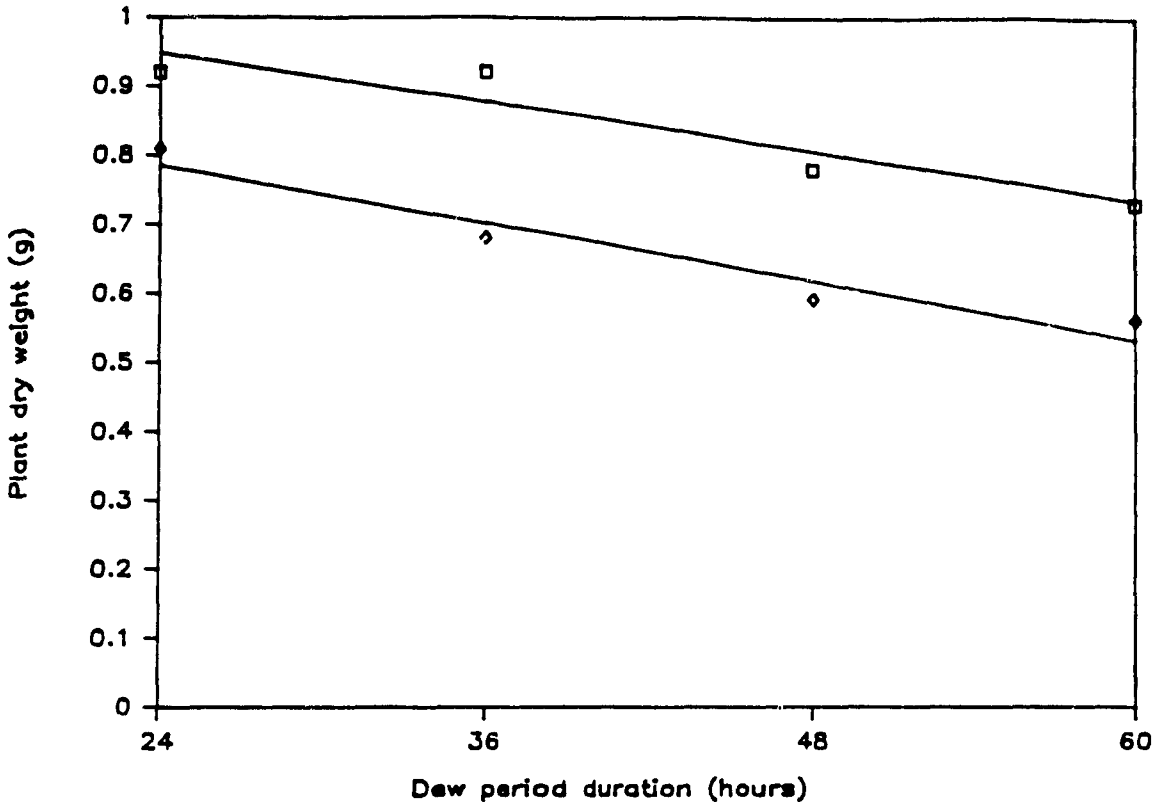


Legend:

□ Uninoculated plants Y = 8.88 - 0.03*D
◇ Inoculated plants Y = 8.14 - 0.04*D

Y = Estimate of plant fresh weight (g)
D = Dew period duration (hours)

Figure 4.3.7 The effect of dew period duration on fresh weight of uninoculated and inoculated hemp-nettle plants (pooled data for two experiments- 30 Ctreatment deleted).



Legend:

□ Uninoculated plants $Y = 1.09 - 0.01*D$
◇ Inoculated plants $Y = 0.96 - 0.01*D$

Y = Estimate of plant dry weight (g)
D = Dew period duration (hours)

Figure 4.3.8 The effect of dew period duration on dry weight of uninoculated and inoculated hemp-nettle plants (pooled data for two replications - 30 C treatment deleted).

for control and inoculated plants. Fresh and dry weight were linearly and inversely related to the length of dew period.

4.3.4 Discussion.

Disease development

As expected, temperature and duration of dew period affected the degree of infection. In preliminary experiments, dew periods less than or equal to 24 hours were not long enough for infection to occur. Disease was produced at all temperatures, but was more favored at 24 C, and the level of infection increased with the time spent in the dew chamber. Such a positive correlation between increase in postinoculation dew period and disease severity was also reported for S. tritici and S. nodorum (Eyal et al., 1977; Hess and Shaner, 1987).

Optimum conditions for infection are known for many plant pathogens (Jeger et al., 1985). Although all Septoria species require high moisture for infection and severe disease development, dew period duration and temperature requirements vary within the genus (Agrios, 1978; Holmes and Colhoun, 1974). At 12 C, S. nodorum needed only three hours of high humidity to attack mature wheat plants, whereas a minimum of 20 hours was required by S. tritici. S. galeopsidis falls in the category of long dew period-requiring species. This can be a severe limitation regarding its potential as a biological control agent. The pathogen was not restricted in its dew period temperature requirements, however, since substantial disease development occurred within the range 18-30 C. It is known that Septoria species can cause disease over a wide range of temperatures between 10 and 27 C (Agrios, 1978). The optimum temperature for disease development, 24 C, was similar to the optimum temperature for radial growth of the pathogen in pure culture (section 3.3). Usually, the most rapid disease development occurs when the temperature is optimum for the development of the pathogen but is not optimal for development of the host (Agrios, 1978).

Studies on other potential biological control agents showed that in general, pathogens under evaluation do not require very long dew periods in order to severely infect or even kill their host weeds. Colletotrichum gloeosporioides (Penz.) Sacc. f.sp. aeschynomene required only 12 hours of dew period, at 24, 28, or 32 C in order to infect northern jointvetch (Aeschynomene virginica (L.) B.S.P.) (TeBeest et al., 1978). Those conditions were sufficient for anthracnose to develop rapidly, and the pathogen is a good candidate mycoherbicide. Another fungus, Colletotrichum malvarum (A. Braun & Casp.) Southworth, severely infected prickly sida (Sida spinosa L.) when plants were exposed to only 16 hours of dew period at 24 C (Kirkpatrick and Templeton, 1982). Only 8 hours of dew were necessary for Alternaria cassiae Jurair and Khan in order to produce reductions of 95 to 100 % in plant number and dry weight, within the range of 20-30 C (Walker and Riley, 1982).

S. galeopsidis did not kill hemp-nettle plants, and although the plants were severely diseased after 60 hours of dew, they were still able to sustain vigorous growth of new leaves and thus compensate for the weight and vigor loss. Percent decrease in aboveground biomass of inoculated plants as compared to controls did not reflect the high levels of infection after longer dew periods.

Moisture primarily affects germination of the spores (which require a film of moisture on the tissues) and penetration of the host by the germ tube (Agrios, 1978). The fungal pathogen becomes independent of the presence of free moisture once it can obtain nutrients and water from the host. *S. galeopsidis* pycnidiospores probably required a long time to germinate (see section 3.4) and infect their host. In order to obtain maximum disease production in a field situation, plants would have to be inoculated when long periods of high moisture are forecasted, and this considerably limits the flexibility of use of the pathogen, unless ways are found to overcome the need for free moisture. For example, a technique was developed and evaluated with *A. cassiae* which may increase leaf wetness duration (Quimby Jr. and Fulgham, 1986). Spores of the pathogen were applied on their host in an aqueous carrier and this was followed with the application of an invert emulsion. Under these conditions, the pathogen was able to cause 88 % mortality of sicklepod (*Cassiae obtusifolia* L.)

without any dew. Although promising, this method is still experimental.

Height

Overall, plant height was not affected by the pathogen, and even under most severe infection, there was no significant height difference between control and inoculated plants. Height did not reflect the health status of hemp-nettle plants. Although a regression curve was fitted to height data of inoculated plants as influenced by the duration of the dew period, there does not seem to be any biological reason for the behaviour of the curve.

Plant fresh and dry weight

The lesions and stress caused by *S. galeopsidis* on inoculated plants were sufficient to decrease the weight of the plants, although not to a very large extent. Dew period duration did not have a more pronounced effect on plant fresh and dry weights of inoculated plants than control plants. Although more disease was produced with increasing durations, the difference in biomass between control and inoculated plants receiving 60 hours of dew was the same as the difference in biomass between inoculated and control plants receiving 24 hours of dew. The pathogen made the fresh and dry weight of every inoculated plant approximately 14 and 21%, respectively, lower than controls receiving the same dew period and temperature. The rate of decrease in fresh and dry weight for inoculated plants as dew period increased (i.e. as infection level increased) was expected to be more pronounced than for uninoculated plants, which would have been demonstrated by a steeper slope of the regression line. Hemp-nettle plants were able to overcome the loss of weight by vigorous growth of newly emerged leaves.

4.4. Experiment 2. The effect of inoculum density andspray adjuvants on disease development and severity.

4.4.1 Introduction.

The determination of the optimum inoculum density for maximum disease development is important in the development of a biological herbicide, since the presence of a large population of an aggressive and virulent pathogen is one of the essential factors for the occurrence of epiphytotics (Quimby Jr., 1982). Sometimes, the addition of adjuvants to the spore suspension can also enhance infection. This experiment was performed to determine the impact of inoculum density and different adjuvants on subsequent development and severity of the disease caused by <u>Septoria</u> <u>galeopsidis</u> on hemp-nettle.

4.4.2 Materials and methods.

The experiment was a 5 X 4 factorial, with four replicates. The independent variables were inoculum density (0, 1.0 X 10^6, 1.0 X 10^7, 1.0 X 10^8, and 1.0 X 10^9 spores/m^2) and spray adjuvants (corn syrup (2% v/v in water), sorbo (sorbitol, Atkemix Inc., Brantford, Ontario; 20% v/v), gelatin (2% w/v), and control (water alone). Pycnidiospores were applied using the two laboratory atomizers. There were two plants per pot, at the 10-leaf stage. After spraying, plants were placed in the dew chamber at 25 $\pm$ 2 C, in a randomized complete block design for 48 hours. Plants were then taken out and placed on a greenhouse bench in a randomized complete block design and provided with natural daylight.

The experiment was repeated once, with some modifications. In the second experiment, the adjuvants were not assessed. Plants were sprayed at the early 8-leaf stage and there were six pots per treatment. The first experiment was peformed in July (average day/night greenhouse temperature approximately 35/25 C) and the second experiment was performed in September (average day/night temperature approximately 25/15 C).

Disease assessments were performed 12, 14, 16, 18, and 20 days after inoculation. Plants were harvested on day 20. A species of Pythium was responsible for the death of a few plants (four out of 60) in the second experiment.

4.4.3 Results.

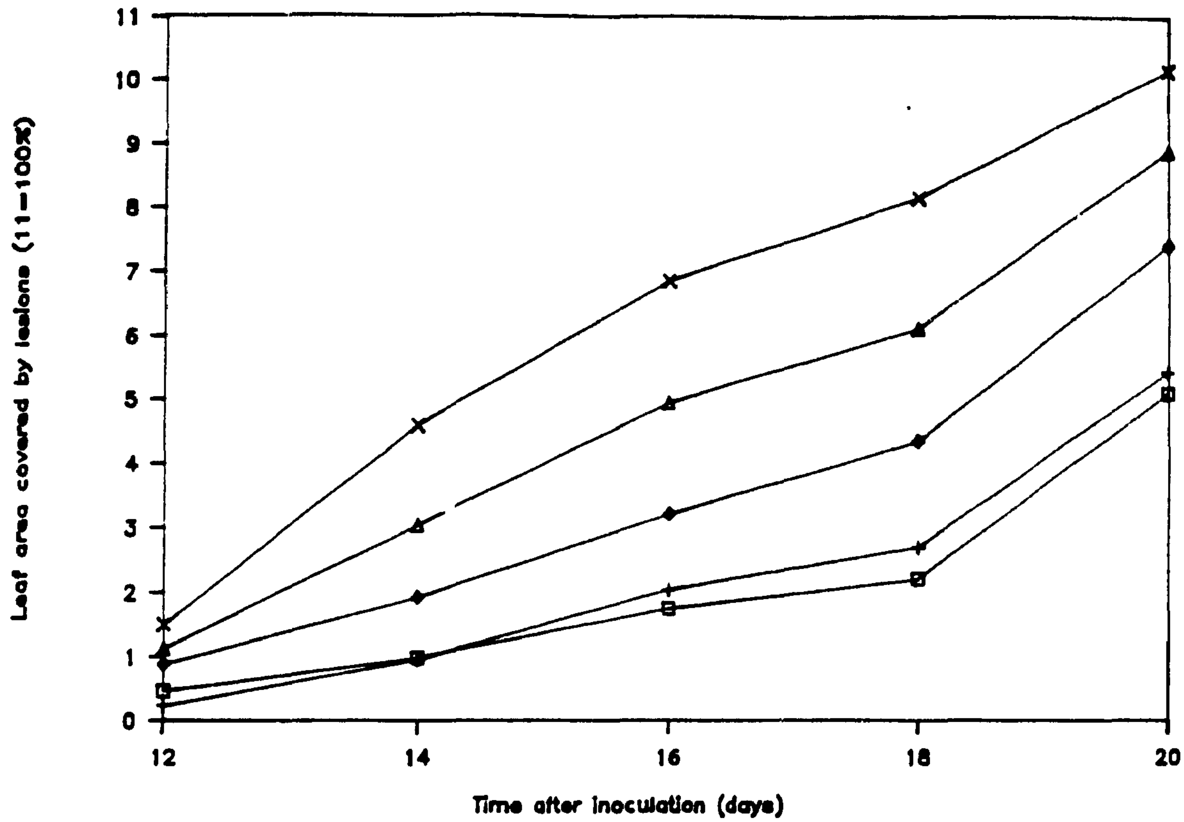
Disease development

Some symptoms of infection appeared on control plants. This was due to the proximity of inoculated plants and uninoculated controls in the dew chamber during the dew period. The infection level was very low, however, and controls were still suitable for comparisons.

Inoculum density had a significant effect on disease development but adjuvants had no effect (Table E.30). Leaf area covered by lesions increased over time as the inoculum density increased. Figures 4.4.1 and 4.4.2 illustrate the disease progress on control and inoculated plants of experiments 1 and 2, respectively. The differences between each disease progress curve were best shown by AUDPC values, and a regression line was obtained from performing a non-parametric simple linear regression analysis on the AUDPC values for concentrations between 1.0 X 10^6 and 1.0 X 10^9 spores/m^2 (Table E.31). Higher values for AUDPC were obtained in experiment 2 than 1. The mean values for each inoculum rate as well as the regression lines are shown on figures 4.4.3 and 4.4.4.

Plant height

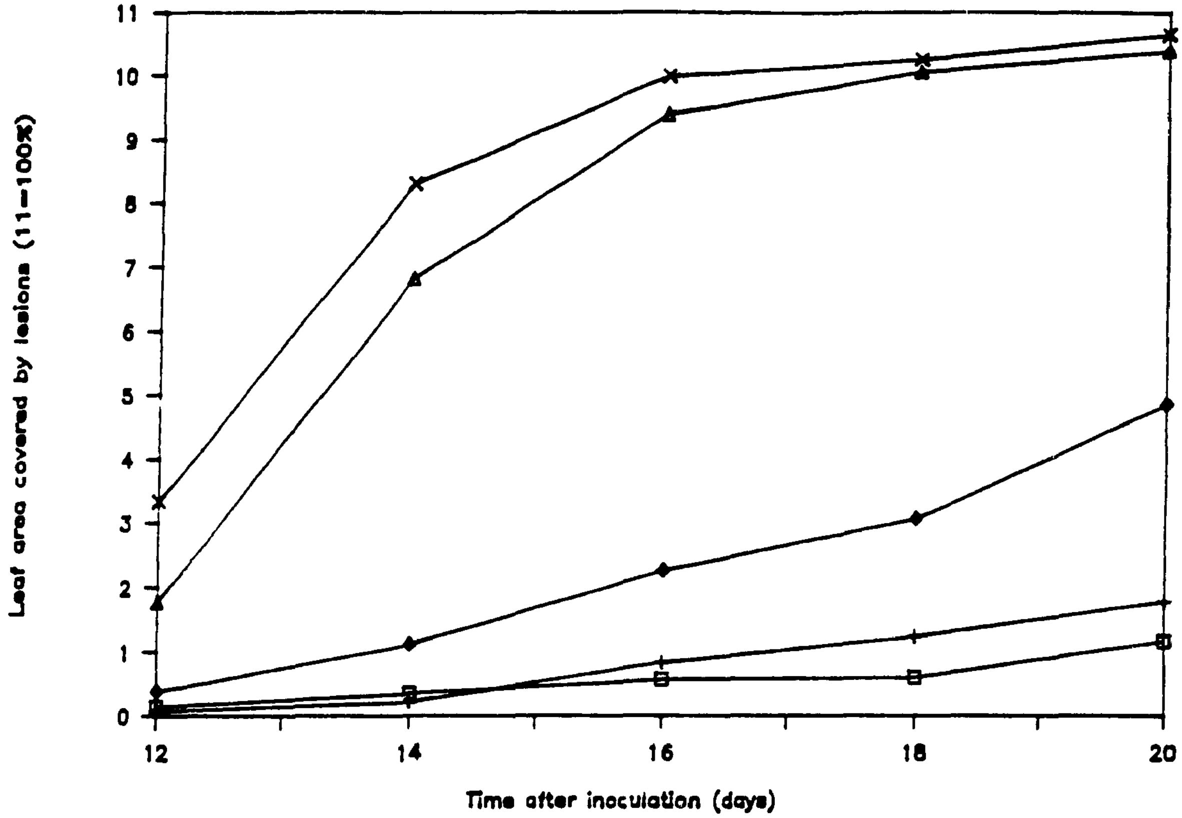
The height of hemp-nettle plants remained constant over all treatments, although plants in the first experiment were



Legend: Inoculum density

□ Control
+ 1.0 X 10^6 spores/m^2
◇ 1.0 X 10^7 spores/m^2
△ 1.0 X 10^8 spores/m^2
× 1.0 X 10^9 spores/m^2

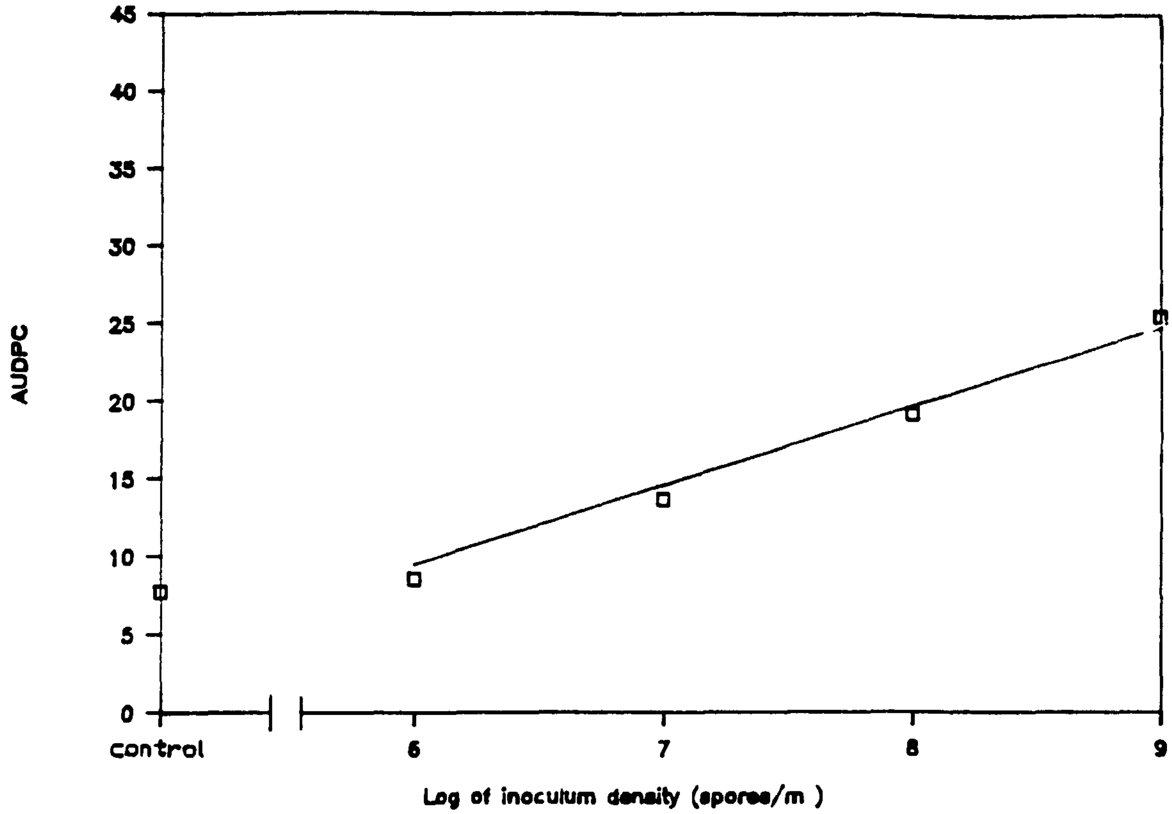
Figure 4.4.1 Disease progress curves for each inoculum density (experiment 1 - averaged over all adjuvants).



Legend: Inoculum density

□ Control
\+ 1.0 X 10^6 spores/m^2
◇ 1.0 X 10^7 spores/m^2
△ 1.0 X 10^8 spores/m^2
× 1.0 X 10^9 spores/m^2

Figure 4.4.2 Disease progress curves for each inoculum density (experiment 2).

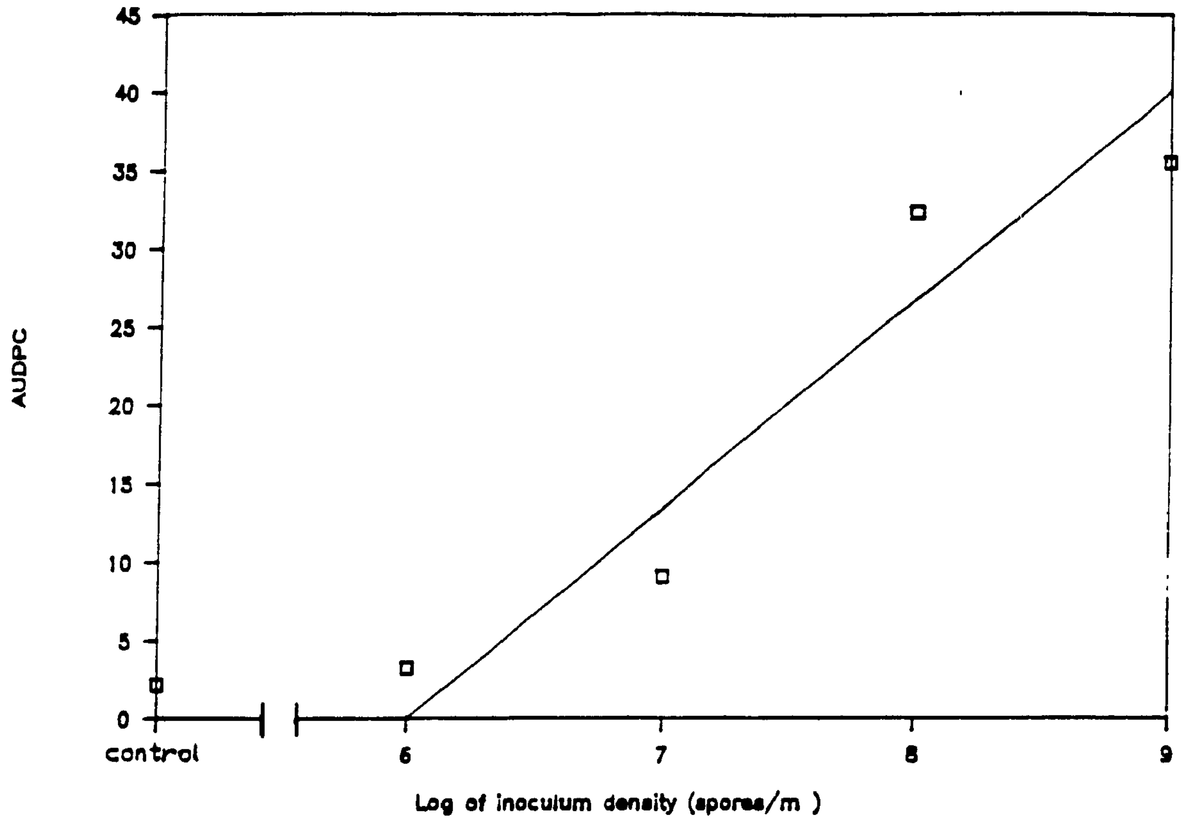


Y = -21.12 + 5.11*I

Y = Estimate of Area Under the Disease Progress Curve

I = Log of inoculum density

Figure 4.4.3 The effect of inoculum density on area under the disease progress curve (experiment 1 -- averaged over all adjuvants).



Y = -80.06 + 13.35*I

Y = Estimate of the Area Under the Disease Progress Curve
I = Log of inoculum density

Figure 4.4.4 The effect of inoculum density on area under the disease progress curve (experiment 2).

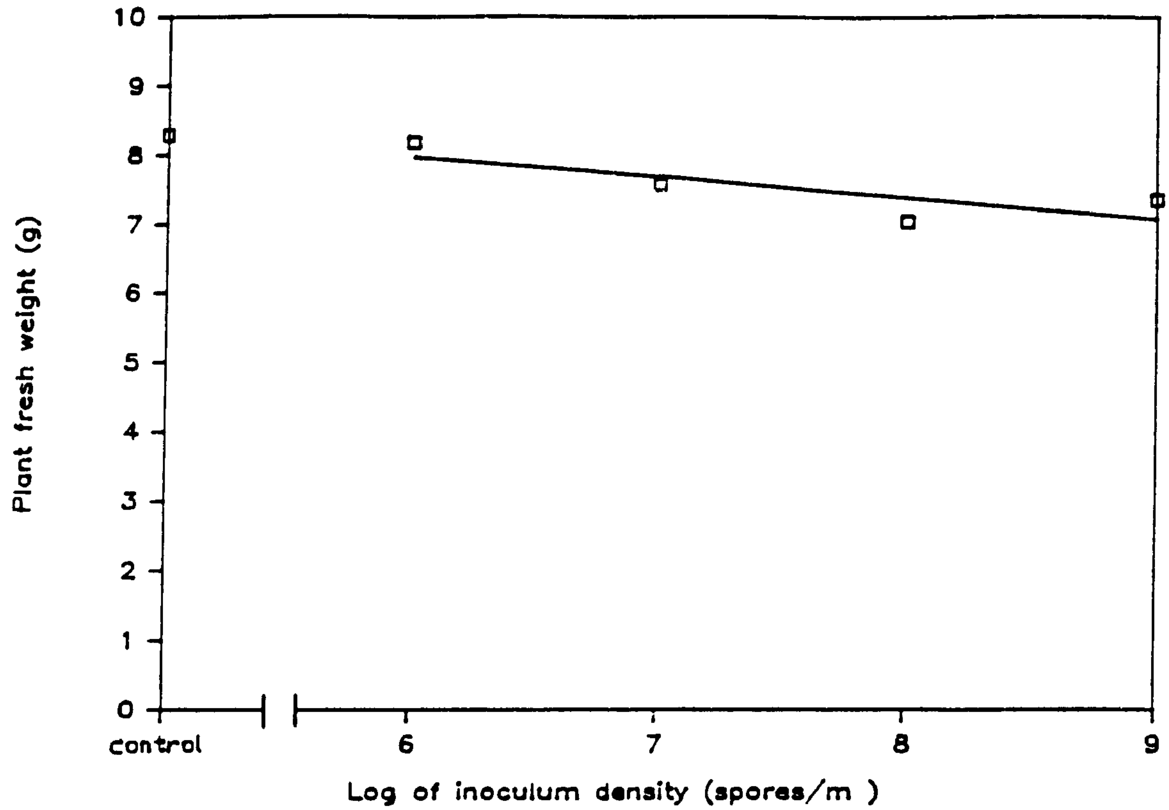
twice as tall as plants in the second experiment (table E.32). The inoculum density of *Septoria galeopsidis* spores and the different spray adjuvants used had no significant effect on plant height in either experiment (table E.33).

Plant fresh and dry weight

Means of plant fresh weight and dry weight are presented in table E.32. Inoculum density had a highly significant effect ($P<0.01$) on plant fresh and dry weight in both experiments (Tables E.34 and E.35).

Fresh weight means of the plants sprayed with 1.0×10^6, 1.0×10^7, 1.0×10^8, and 1.0×10^9 spores/m^2 were 1.3, 8.9, 15.2, and 11.5% lower than that of uninoculated controls, respectively, in the first experiment. In the second experiment, the differences in weight between controls and inoculated plants were greater. Mean fresh weights of inoculated plants were 2.3, 7.4, 32.7, and 39.5% lower than that of controls, respectively. Figures 4.4.5 and 4.4.6 show the effect of increasing inoculum density on fresh weight for experiments 1 and 2, respectively. In both experiments, fresh weight had an inverse, linear relationship with inoculum density (table E.36).

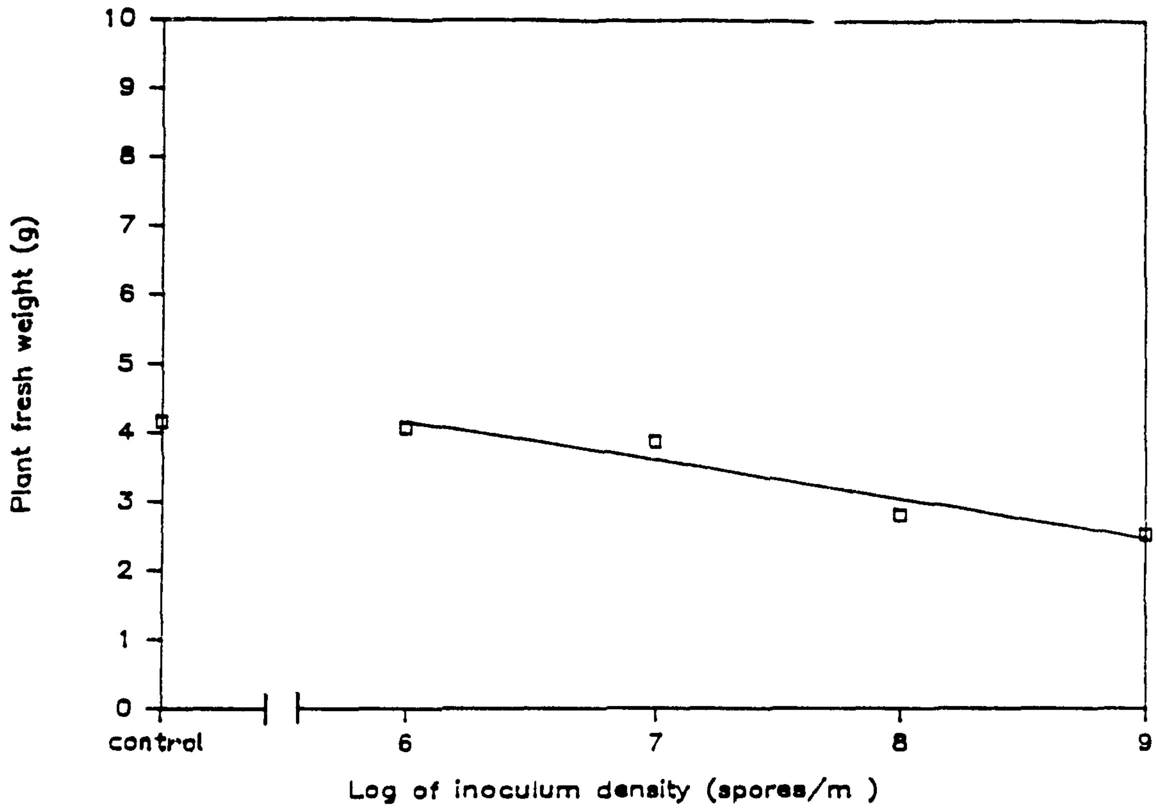
Adjuvants had no significant effect on plant fresh weight and there was no interaction between additives and inoculation rate. The addition of gelatin, sorbo, or corn syrup to the



$$Y = 9.81 - 0.30*I$$

Y = Estimate of plant fresh weight (g)
I = Log of inoculum density

Figure 4.4.5 The effect of inoculum density on fresh weight of hemp-nettle plants (experiment 1 - averaged over all adjuvants).



Y = 7.60 - 0.57*I

Y = Estimate of plant fresh weight (g)
I = Log of inoculum density

Figure 4.4.6 The effect of inoculum density on fresh weight of hemp-nettle plants (experiment 2).

spore suspension did not increase the effect of the pathogen.

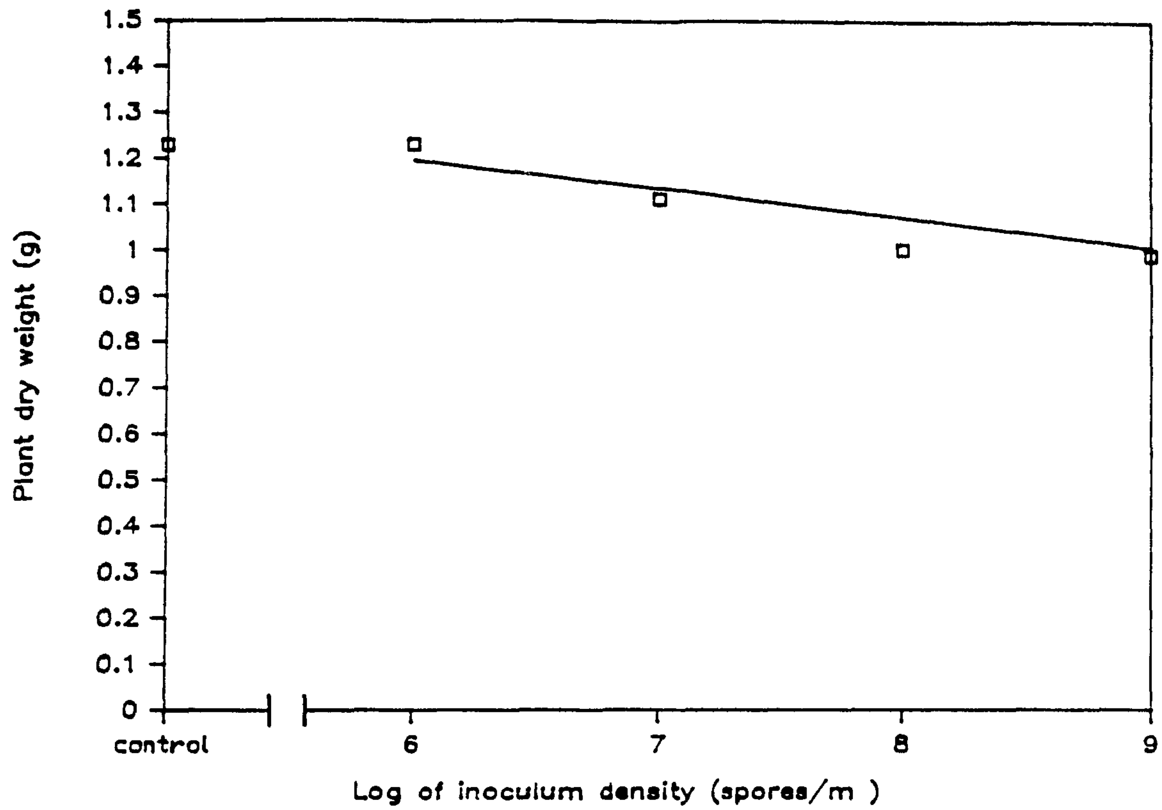
The decrease in plant weight was more apparent for the dry weight values. The decrease in plant dry weight due to application rates of 1.0×10^6, 1.0×10^7, 1.0×10^8 and 1.0×10^9 spores/m^2 was 0.00, 9.76, 18.70, and 19.51%, respectively, in experiment 1, and 4.74, 16.07, 46.90, and 53.50% in experiment 2, respectively, compared to controls. Figures 4.4.7 and 4.4.8 show the plot of dry weight means, including those of the controls, along with the regression curves, for experiments 1 and 2, respectively. In experiment 1, dry weight had an inverse, linear relationship with the log of inoculation rate, while the inverse relationship between these two variables in experiment 2, was best described by a third degree polynomial (Table E.37).

No significant effect of adjuvants on plant dry weight was detected. There was no significant interaction between adjuvants and inoculum density.

4.4.4 Discussion.

Disease development

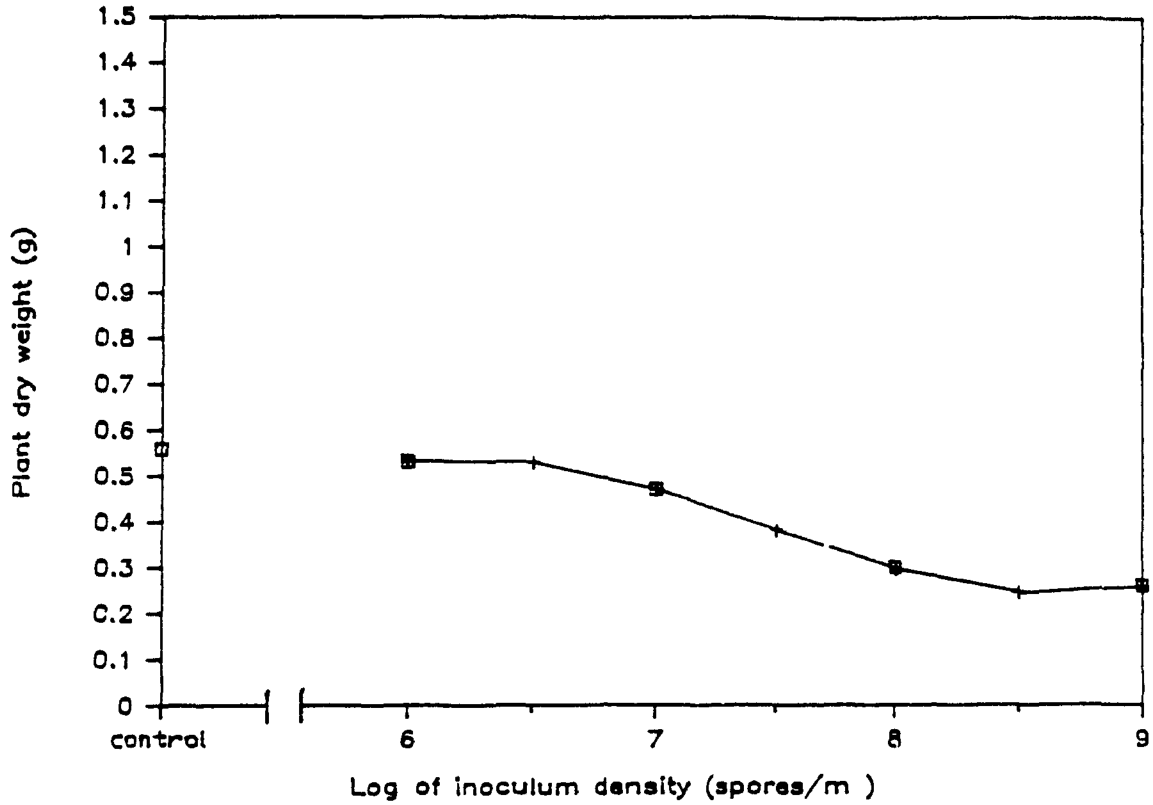
There was a marked increase in leaf area covered by lesions, from low to high inoculum densities. The difference in infection level between plants receiving 0 and 1.0×10^6 spores/m^2 was very low. Probably no substantial disease



Y = 1.58 - 0.06*I

Y = Extimate of plant dry weight (g)
I = Log of inoculum density

Figure 4.4.7 The effect of inoculum density on dry weight of hemp-nettle plants (experiment 1 - averaged over all adjuvants).



$$Y = -15.07 + 6.59*I - 0.91*I^2 + 0.04*I^3$$

Y = Estimate of plant dry weight (g)
I = Log of inoculum density

Figure 4.4.8 The effect of inoculum density on dry weight of hemp-nettle plants (experiment 2).

development would have occurred at inoculum rates under 1.0 X 10^6 spores/m^2. Experiment 2 showed a very marked difference between AUDPC at low concentrations (1.0 X 10^6 and 1.0 X 10^7 spores/m^2) and the ones at high concentrations (1.0 X 10^8 and 1.0 X 10^9 spores /m^2). Although a regression line was fitted to the AUDPC data of experiment 2, the relationship between AUDPC and log of inoculation rate might have been best described by a third degree polynomial, as the increments in AUDPC were moderate between the values of 1.0 X 10^6 and 1.0 X 10^7, very high from 1.0 X 10^7 and 1.0 X 0^8, and again, small, from 1.0 X 10^8 to 1.0 X 10^9 spores/m^2. Unfortunately, no non-parametric test is available to detect curvilinear relationships. In experiment 1, however, the AUDPC values increased more progressively between the inoculation rates of 1.0 X 10^6 and 1.0 X 10^7 spores/m^2 and the relationship between the two variables clearly was a linear one.

It is recognized that a minimal level of inoculum (numerical threshold) is necessary to obtain satisfactory infection and disease development (Tuite, 1969). High levels of inoculum usually decrease the time for symptoms to appear. In rare instances, however, too much inoculum may decrease infection. An inhibitor which prevents germination can be produced by the spores when their population is dense (Robinson, 1978). Conidial matrix can also play an important role in the decrease in germinability under high population densities (Louis and Cooke, 1985). This decrease in germinability, in the case of the species S. nodorum, is in fact only a delay in germination of the spores (Rapilly and Skajennikoff, 1974). High concentrations of cirrhus extract appeared to inhibit germination through a physical phenomenon.

Overall, the AUDPC values were higher in experiment 2 than 1, probably because of lower, more optimal temperatures for disease development prevailing in the greenhouse in September. Disease progress was less rapid under the very high July temperatures. As was observed in an earlier section (3.3.3), growth of the pathogen in pure culture is substantially slowed down by high temperatures. The relationship between the number of propagules of a pathogen and the host response is a function of the virulence of the pathogen and the host resistance, but both may however be affected by environmental factors (Lapwood and McKee, 1966).

Experiments performed on crop species with other pathogens have shown similar results. In a research conducted on the effect of the density of zoospores of *Phytophthora infestans* (Mont.) de Bary on the incidence of infection on potato leaves, plotting of the percentage response against dose (1 to 256 zoospores per drop) gave a characteristic sigmoid curve for each variety under investigation (Lapwood and McKee, 1966). On spring wheat cultivars, the percentage of leaf area covered by *Septoria tritici*. lesions, recorded 22 days after inoculation, was significantly linearly correlated with the logarithm of the inoculum density applied to the wheat plants (Shearer, 1978). A gradual decrease in latency period (defined by the author as the period, in days, from inoculation to the observation of the first black mature pycnidia) was recorded as the inoculum density increased, from 2.0×10^4 to 1.2×10^6 conidia/ml.

Latency period was not recorded in the present experiments. By extrapolating the disease progress curves, however, it is possible to see that at high concentrations (1.0×10^8 and 1.0×10^9 spores/m^2), lesions probably appeared earlier than at low concentrations. This corresponded to the incubation period rather than the latency period. Incubation period is defined as " the time between inoculation and the development of symptoms " (Tuite, 1969).

Area under the disease progress curve was found a more suitable parameter to evaluate the stress caused to the weed than the final percentage of leaf area covered by lesions. AUDPC takes into account the period of time during which the stress was sustained by the plant; in other words, the speed at which the disease developed. Therefore, AUDPC data gave more information than single disease rating data. It also decreased the variance of the data.

A higher percentage of leaf area covered with lesions was expected on plants sprayed with spores in combination with sorbo, corn syrup, or gelatin, than with spores and water alone. The incorporation of gelatin into the spore suspension is recognized to minimize the effect of plant surface characteristics or inconsistent performance of the spraying apparatus (Shearer, 1978). In experiments on infection of French beans (*Phaseolus vulgaris* L.) by *Botrytis cinerea* Pers., glucose added to the spore suspension was apparently important in conidial germination and further superficial development, but not to penetration (Van den Heuvel and Waterreus, 1983). The addition of infection stimulants (KH_2PO_4 or Na-ATP) to the inoculum was necessary to bring about germination. In the same study, the type of infection structures depended on inoculum density. Highest inoculum concentrations (2×10^6 conidia/ml) lead to 80% of infection hyphae originating from germ tube apices, while at low concentrations (2×10^5 conidia/ml and lower), penetration was derived from appressoria or infection cushions. At high inoculum concentrations, the high proportion of germ tube apices was associated with a rapid penetration (within 12-24 hours after inoculation).

Plant height

The results demonstrated the lack of correlation between inoculation rate and plant height. Adjuvants did not increase the effectiveness of inoculum on plant height. The height differential between the two experiments wa)robably due to the higher greenhouse temperatures in July than September (Quisenberry, 1975). Differences in other environmental factors such as photoperiod and light intensity may also be in part responsible for this situation.

It is known that damage to the terminal growing point, either mechanical or caused by insects or diseases, can affect the height of plants (Quisenberry, 1975). The lack of response of plant height to disease stress might have been due to the fact that the growing point of hemp-nettle plants was resistant to or protected against the attack by S.galeopsidis. Only the fully-expanded, senescent (advanced in age) leaves that were present at inoculation time were affected.

Plant height is an important factor to consider for weed/crop interactions. The projection of a weed canopy over a crop canopy results in a considerable advantage to the weed with respect to light capture (Radosevich and Holt, 1984). If stem elongation occurs early in the life cycle or at a critical development stage, reductions in crop yield may result. In these experiments, healthy, upper hemp-nettle leaves continued to grow, no matter how diseased the lower leaves were. Although smaller, these upper leaves might still intercept substantial sunlight and have a negative effect on crop growth.

Plant fresh and dry weight

In the case of annual plants, aboveground biomass is a good indicator of vigor. Results showed that hemp-nettle biomass could be significantly decreased by S. galeopsidis, especially with high inoculation rates (1.0 X 10^8 and 1.0 X 10^9 spores/m^2). Dry weight measurements were more sensitive than fresh weight measurements in detecting this inverse relationship.

The decrease in hemp-nettle biomass as the inoculum density increased was due to 1) an increasing number of abscissing leaves, 2) a general decrease in plant vigor due to disease stress, and 3) a drastic decrease in photosyntetic leaf area due to increasing necrotic areas. Although the upper, uninoculated leaves of the plants continued to grow, even on most heavily infected plants, the overall health and probably the competitiveness of those plants were negatively affected.

It is known that injury can cause leaf abscission (Carns, 1966). Depletion of metabolites in the abscission zone may be responsible for that. A small number of leaves were shed by uninoculated plants, probably due to organ maturity or senescence, but a considerably larger number of leaves were shed by inoculated plants. On severely diseased plants, almost all the leaves that were inoculated had abscissed three weeks after inoculation.

The weight of a seedling is proportionnal to the space it occupies, and the preemption of resources that constitute space is critical in weed-crop competition (Radosevich and Holt, 1984). In experiment 2, hemp-nettle plants treated with <u>S. galeopsidis</u> at a rate of 1.0 X 10^9 spores/m^2 had less than half the dry weight of uninoculated plants. In the case where hemp-nettle would be growing in association with a crop, the space that diseased weeds would fail to occupy would be available to the crop.

In order to get a substantial reduction in hemp-nettle aboveground biomass, inoculum rates higher than 1.0×10^8 spores/m^2 had to be used. This might be considered as a critical inoculum density in order to get substantial disease production. An inoculum rate of 1.0×10^{10} spores/m^2 might have further decreased plant vigor, but such large quantities of inoculum were difficult to obtain.

In most dose-response experiments with potential biological herbicides for weeds, the inoculum rates required to substantially reduce dry weight or kill plants were relatively low. For example, 90 to 100% of the spurred anoda (*Anoda cristata*) plants in the cotyledon to the first-leaf stage were killed within four to five days with an inoculum concentration of only 3.0×10^4 conidia of *Alternaria macrospora* per ml, and an 8-hour dew period (Walker, 1981). In another study, all leafy spurge (*Euphorbia esula* L.) shoot systems were killed with conidia of *Alternaria* sp. at inoculum densities above 1.0×10^6 spores/ml (Kuprinsky and Lorenz, 1983). Yellowing and death of individual leaves occurred with concentrations above 6.0×10^4 spores/ml. A study on control of velvetleaf (*Abutilon theophrasti* Medic.) and prickly sida (*Sida spinosa*)with *Fusarium lateritium*, showed that inoculum concentrations of 7.5×10^5 and 1.5×10^6 conidia/ml killed 64 and 91% of the two weeds, respectively, at the 10th leaf stage (Boyette and Walker, 1985). Ninety to 100% of sicklepod (*Cassia obtusifolia*) seedlings were killed within 4 or 5 days following inoculation with as little as 3.0×10^4 conidia of *Alternaria cassiae* per ml (Walker and Riley, 1982). The pathogen Phomopsis convolvulus Sp. Nov. produced complete necrosis of field bindweed (Convolvulus arvensis L.) leaves when sprayed at inoculum densities equal to or higher than 5.0 X 10^6 spores/ml (Ormeno-Nunez, 1986). No subsequent regrowth was observed from cotyledonary axillary buds. In all these studies, plants were sprayed until runoff, and at different growth stages. It is difficult to make comparisons in these conditions. However, if hemp-nettle plants had been sprayed until runoff with the same spore suspensions that were used in the experiments, the decrease in plant weight might have been higher.

Adjuvants had no significant effect on disease severity compared to water alone. It was believed at first that corn syrup, sorbo and gelatin would favor disease development by nourishing spores during prepenetration stages, favoring their adherence to the surface of the leaves during the dew period, and/or improving the coverage of the leaf surface by the production of smaller droplets in the spray. It is known that the size of the inoculation droplets may be important for infection potential (Lapwood and McKee, 1966). No such effect was observed.

In summary, the highest disease production occurred under an inoculation rate of 1.0 X 10^9 spores/m^2, with no effect of additives. This high level of infection was accompanied with a significant decrease in plant fresh and dry weights, although no effect on plant height was observed.

4.5 Experiment 3. The effect of plant age on disease development and severity.

4.5.1 Introduction.

The susceptibility of plants to fungal parasites changes throughout their lifetime (Tuite, 1969). In general, young plants are more susceptible to disease than older plants. In certain host-pathogen combinations, however, immature plants or plant parts are resistant and mature plants or plant parts are susceptible to the pathogen (Holcomb, 1982). Septoria species have various requirements with respect to plant age for maximum disease development. For example, S. tritici can infect wheat at all growth stages and produce extensive infection of seedlings often resulting in death, while S. nodorum is increasingly prevalent as the crop matures (Holmes and Colhoun, 1974).

In order to achieve maximum infection and disease development on hemp-nettle plants, inoculation with Septoria galeopsidis pycnidiospores should be made when the plants are at the most susceptible age. An experiment was performed to determine the growth stage(s) at which maximum disease development will occur.

4.5.2 Materials and methods.

Hemp-nettle plants at five different growth stages (cotyledon, 2- 4-, 6-, and 8-leaf stages) were either sprayed with a suspension of 1.0 X 10^8 spores/m^2 or distilled water using the spray chamber. After spraying, plants were put in the dew chamber at 24 C for 48 hours. They were then placed on a greenhouse bench in a randomized complete block design and provided with natural daylight. Avergage day and night temperatures were 25 and 15 C, respectively, during the period that plants remained in the greenhouse. Disease was assessed 14, 16, 18, and 20 days after inoculation, and plants were harvested on day 20. The experiment was performed twice, with six pots per treatment, and two plants per pot.

4.5.3 Results.

Height, fresh weight and dry weight data were analyzed on a per-age basis, since comparisons between ages were of no relevance. Variances of the two experiments were homogeneous for each growth stage and each dependent variable, but experiments were analyzed separately for height and fresh weight data due to a significant interaction between the experiment and the inoculation treatment. The two experiments were pooled, however, for the analysis of variance of dry weight data, since the interaction between the two factors was not significant. A contaminating Pythium species killed most of the plants at the 4-leaf stage in the second experiment. Consequently, the analysis of variance for 4-leaf stage plants is based on the data of the first experiment only.

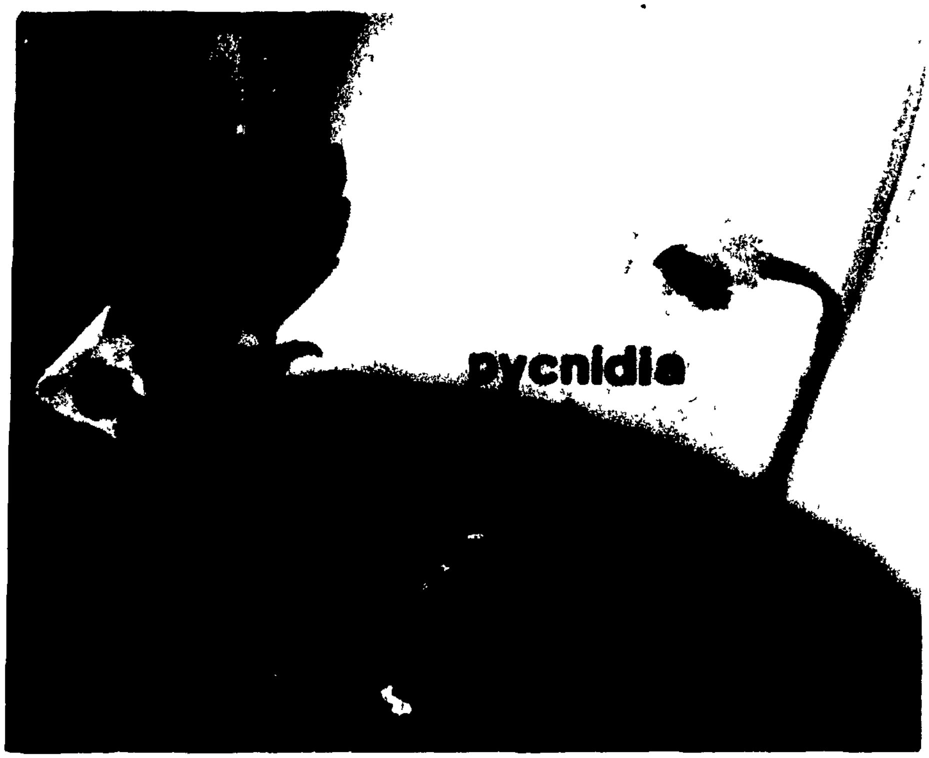
AUDPC data of all ages could be compared because the data consisted of proportions. The two experiments were analyzed separately, however. Percent leaf area covered by lesions was averaged for only those leaves or cotyledons that were present at inoculation time. Cotyledons were taken into account for plants inoculated at the cotyledon stage only. Since Friedman two-way analysis of variance requires an equal number of replicates of each treatment, blocks 4 and 6 had to be deleted from the data for analysis of experiment 1, due to missing data, and blocks 2 and 4 were deleted from the data of experiment 2. Only four blocks were therefore used in each test.

Disease development

Symptoms appeared on plants at all growth stages. Figure 4.5.1 shows a young seedling inoculated at the cotyledon stage. Although cotyledons were severely diseased, and almost completely covered with pycnidia, new emerging leaves were healthy and vigorous.

AUDPC averages are presented in table E.38. The non-parametric analysis of variance indicated that plant age at inoculation time had a significant effect on disease development in the first experiment but not in the second experiment (table E.39). Multiple comparisons indicated that the difference in AUDPC between plants at the cotyledon stage and plants at the 2-leaf stage was significant at the 0.20

Figure 4.5.1 Hemp-nettle seedling inoculated with *Septoria galeopsidis* spores at the cotyledon stage. Pycnidia are visible on cotyledons.


pycnidia

experimentwise error rate in experiment 1. Figures 4.5.2 and 4.5.3 show the disease progress curves at each age for experiments 1 and 2, respectively, and the variation in AUDPC values as influenced by plant age is shown in figures 4.5.4 and 4.5.5, respectively.

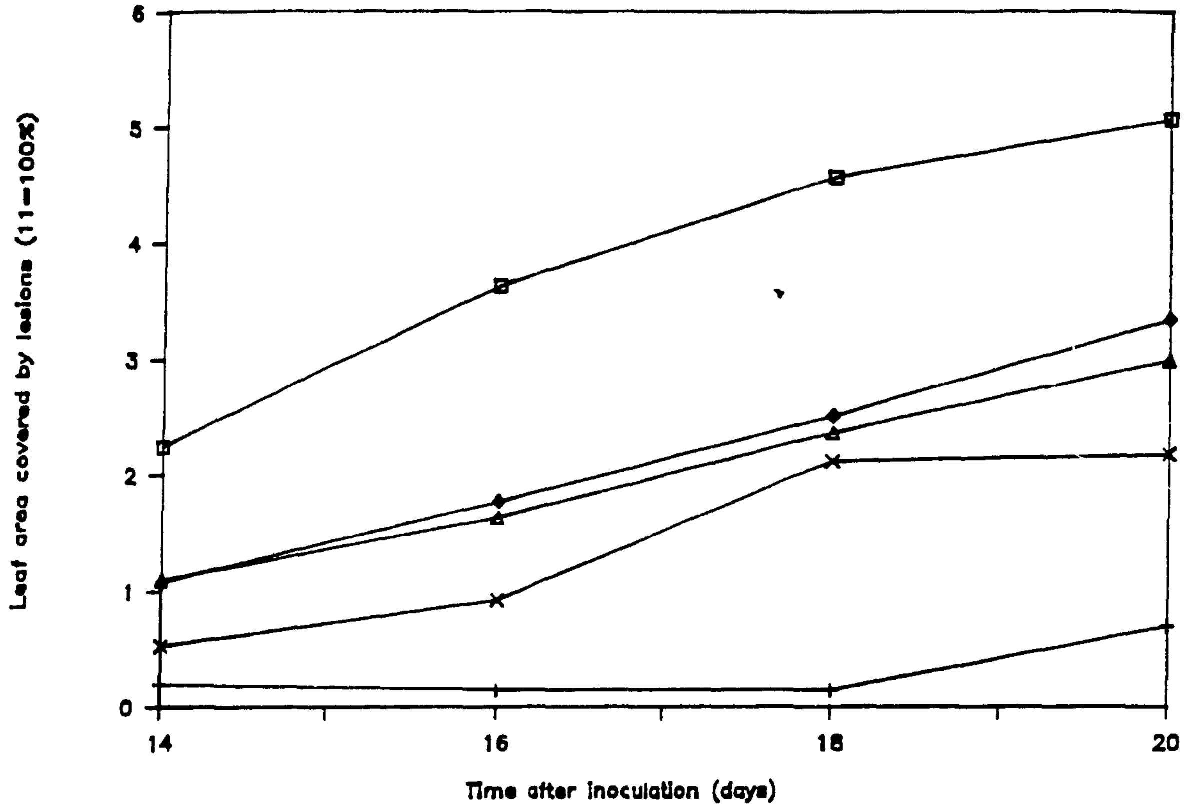
All plants continued to grow and produce new leaves after inoculation. These leaves were healthy and no lesions were found on any of them.

Height

Means of uninoculated and inoculated plant heights and percent difference between them for each growth stage were calculated separately for the two experiments (table E.40). Plant height was not significanlty reduced by the pathogen at any growth stage. The height of plants inoculated at the 2-leaf stage was significantly higher, however, than that of controls. Plants that were inoculated at other stages of growth were either slightly taller or slightly shorter than controls, but not significantly. The analyses of variance for each experiment are presented in tables E.41 and E.42.

Fresh and dry weight

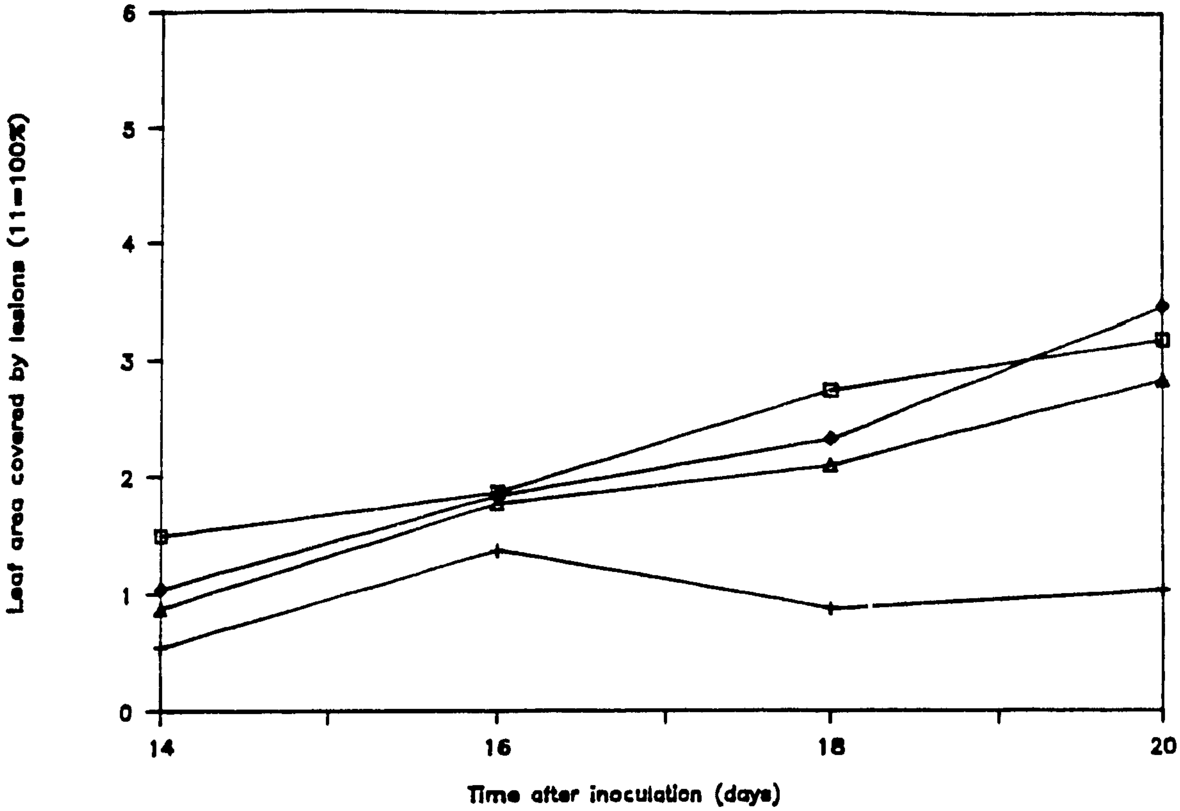
Despite relatively high differences between inoculated and uninoculated plants at cotyledon and 2-leaf stages (table E.43), analyses of variance indicated that plant fresh weight



Legend: Plant growth stage

□ cotyledon
+ two-leaf
◇ four-leaf
△ six-leaf
× eight-leaf

Figure 4.5.2 Disease progress curves at each growth stage of hemp-nettle plants (experiment 1).



Legend: Plant growth stage

□ cotyledon
+ two-leaf
◇ six-leaf
△ eight-leaf

Figure 4.5.3 Disease progress curves at each growth stage of hemp-nettle plants (experiment 2).

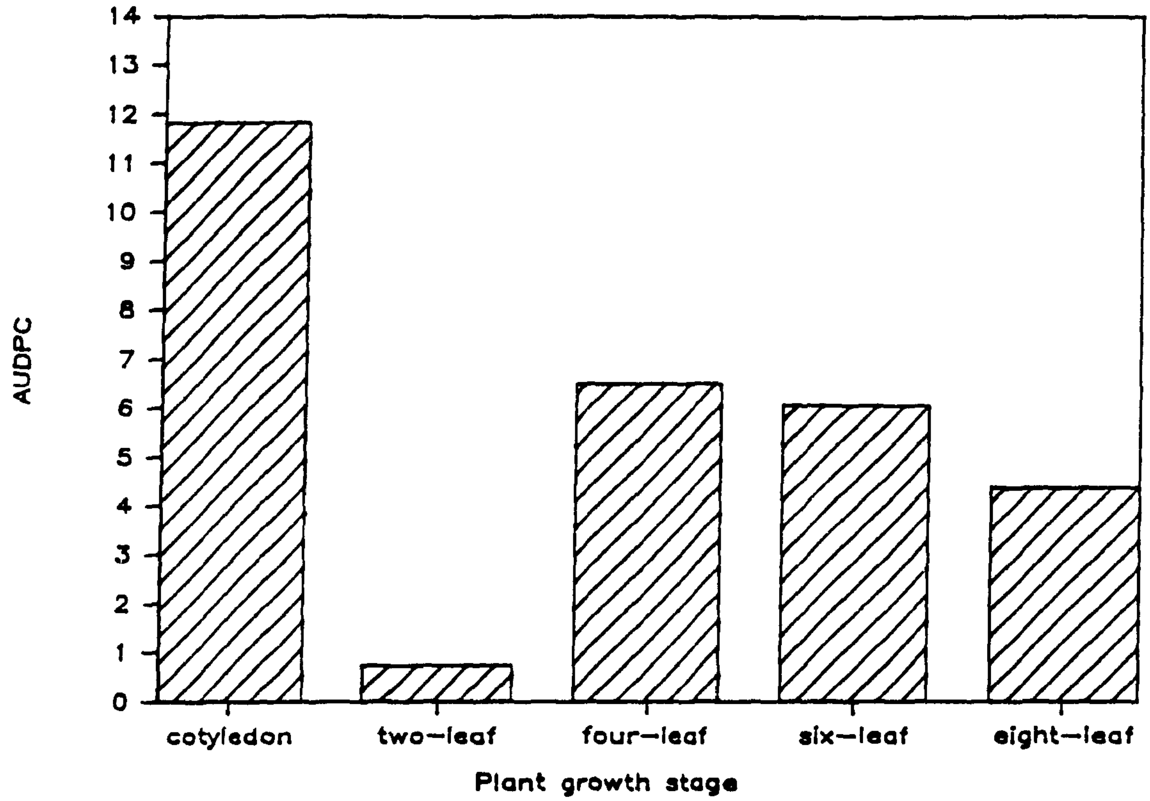


Figure 4.5.4 The effect of hemp-nettle growth stage on Area Under the Disease Progress Curve (experiment 1).

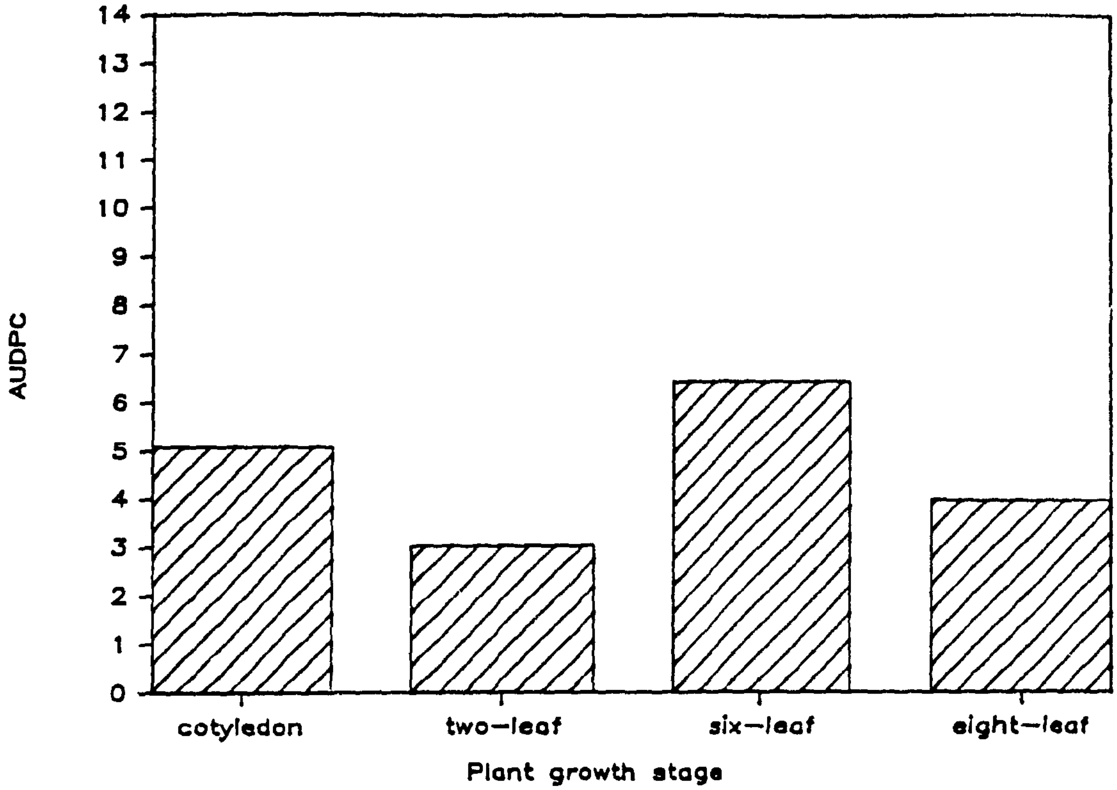


Figure 4.5.5 The effect of hemp-nettle growth stage on Area Under the Disease Progress Curve (experiment 2).

was not significantly affected by incculation, at any growth stage (tables E.44 and E.45). The pooled dry weight data also indicated high differences between control and inoculated plants (table E.46), and this time, the dry weight of plants inoculated at the cotyledon stage was significantly lower than that of the controls (table E.47). Dry weights of plants inoculated at other growth stages were not significantly different from uninoculated controls.

4.5.4 Discussion.

Disease development

The percentage of leaf area covered by lesions was higher on plants at cotyledon stage than on any other plants, but not significantly, except for 2-leaf-stage plants in experiment 1. The comparatively lower leaf area of plants at the cotyledon stage might have been a determinant factor. The vertical overlapping of hemp-nettle leaves at older stages of growth may have prevented inoculum from reaching the whole leaf area of the plant. Tolerance due to structural and/or biochemical defenses may have built up in plants as they aged. Some plants may escape disease because of rapid growth and of some inherent qualities which make them resistant for a period of their life, and others show tolerance to a disease because of exceptional vigor (Agrios, 1978). Although differences were not significant in experiment 2, by examining figure 4.5.3 it appears that plants at the 2-leaf stage were resistant. Almost no symptoms appeared on the first two true leaves. However, results might have been different if cotyledons had been included in pot averages for two-leaf-stage plants. Lesions did appear on the cotyledons of these plants. Therefore, plants were not resistant, but the young, newly emerged leaves exhibited resistance to the pathogen. This resistance was probably only temporary, since the two first leaves of plants inoculated at other growth stages exhibited substantial numbers of lesions.

Plants can generally withstand a certain level of damage due to infection without having their growth or yield affected in any significant way (Agrios, 1978). This level was probably reached for hemp-nettle at the cotyledon stage. The decrease in dry weight reflected the extensive degree of infection of the seedlings. The level of infection was high on some plants at other growth stages, but the threshold value for decrease in aboveground biomass was not reached. These plants had a high level of tolerance towards attack by *S. galeopsidis*.

Height

Hemp-nettle plant height was not decreased by the fungus, at any growth stage. All plants continued to grow despite infection.

The significant increase in height of plants inoculated at 2-leaf stage was an unexpected result. It is recognized that some plant pathogens have the ability to cause imbalances in plant growth hormones (Agrios, 1978). The fungus *Gibberella fujikuroi* causing the foolish seedling disease of rice, for example, apparently secretes gibberellin in its host plant which then grows rapidly and becomes much taller than healthy plants. It is doubtful that *Septoria galeopsidis* produced a similar effect on hemp-nettle plants, since it has not been observed on any other plants, and no *Septoria* spp. are reported as producing that kind of effect on their host.

Fresh weight and dry weight

Although lesions appeared on inoculated plants of all ages, only plants inoculated at the cotyledon stage had aboveground biomass significantly reduced by *S. galeopsidis* inoculation. This reduction was only detected by analysis of variance of dry weight in experiment 1, however.

Cotyledons seemed to be more vulnerable than other leaves to the disease. The surface area of cotyledons was small compared to true leaves. Assuming that the lesions expanded at the same rate on cotyledons as they did on true leaves, it took a shorter time for the cotyledons to be covered with necotic tissue, thus reducing photosynthetic leaf area. The lesions only appeared 8 to 10 days after inoculation, after the first true leaves had emerged. Still the plants did not completely compensate for the loss of green cotyledon tissue, and their vigor was more affected by the pathogen at that stage than at any other stage.

Plants that were inoculated at other growth stages did not exhibit significant differences in fresh or dry weights as compared to controls. In previous experiments (sections 4.3 and 4.4), however, the fresh and dry weights of plants inoculated at the 8-leaf stage were significantly reduced compared to uninoculated plants. This discrepancy between results may indicate an unnoticed difference in growing conditions of either the fungus or the plants, differences in post-inoculation environmental conditions, and/or genetic variability of the fungus or the plants.

The effect of plant age on weed control has been studied for other potential biological control agents. Fusarium lateritium killed almost 100% of velvetleaf and prickly sida seedlings when inoculated at the cotyledon to 2-leaf stages. When inoculated at the 7- to 10-leaf stage, however, only 64% of velvetleaf plants and 91% of prickly sida plants were killed (Boyette and Walker, 1985). In another study, sicklepod (Cassia obtusifolia) plants at all stages of growth were infected by Alternaria cassiae Jurair and Khan, but seedlings in the cotyledon to first-leaf stage were most sensitive (96% were killed within 14 days). There was an increase in tolerance to the pathogen as the plants increased in age (Walker and Riley, 1982). Bracken fern (Pteridium aquilinum (L.) Kuhn) also exhibited an increase in tolerance to infection by Ascochyta pteridis with increasing age (Webb and Lindow, 1987).

In all these studies, the tolerance of the plants to the pathogens increased as they were advancing in age. The results of the experiment with *S. galeopsidis* supported these findings. The detrimental effect of the pathogen was restricted to very young seedlings and the period of high susceptibility was thus very short. It could be concluded from this experiment that plants should be inoculated at the cotyledon stage only. This is impractical in a field situation, however, where many factors can prevent a proper timing of spray applications, including nonuniform germination of weed seeds and unfavorable weather conditions. Weeds do not need to be killed for adequate control to be achieved, but control should be sufficient during critical periods (Daniel and Templeton, 1973). If the application of the pathogen is delayed until after all hemp-nettle plants have reached the 8-leaf stage, when the plants are also susceptible, then the weed has had adequate time to compete with the crop and substantially decrease yields (O'Donovan and Sharma, 1987).

Chapter 5

Conclusions

5.1 Summary and conclusions.

Although perfect pathogens for biological control probably do not exist in nature, if a certain pathogen presents too many constraints, it should not be retained as a potential mycoherbicide. This study demonstrated that the use of Septoria galeopsidis Westd. as a biological herbicide for hemp-nettle had severe limitations. Results of investigations are summarized hereafter.

1. The pathogen did not sporulate in common liquid media. Although pycnidiospores were produced on hemp-nettle extract agar under near ultra-violet light, production was not reliable due to the variable nature of both the medium and the pathogen itself.

2. Over 60% of the pycnidiospores had germinated on water agar after 24 hours within the temperature range 15-30 C. Although the number of viable spores was relatively high, the long period of moisture required to achieve that germination percentage could impede the infection of hemp-nettle plants when periods of moisture are limited.

3. Dense hyphal growth was favored by a temperature of

21-26 C, and by rich agar media such as Czapeck Dox-V-8 agar and Potato Dextrose Agar.

4. The incubation period of the pathogen was relatively long. New, healthy hemp-nettle leaves emerged before lesions were visible. The first symptoms usually appeared eight to ten days after inoculation, first as chlorotic spots on the leaves, which soon became necrotic and coalescent. Abscission of infected leaves was frequently observed.

5. The pathogen required long dew periods to infect its host. At least 24 hours of high relative humidity were necessary to obtain substantial infection, and maximum level of infection was obtained with a dew period duration of 60 hours. Significant decreases in fresh and dry weight of plants were obtained as dew period increased, but differences between control and inoculated plants did not increase with increasing dew periods. Disease developed at all temperatures between 18 and 30 C, but was more favored by a temperature of 24 C. There was no effect of temperature on fresh and dry weight, however.

6. Infection level increased and fresh and dry weight decreased with increasing inoculum density, from 1.0 X 10^6 to 1.0 X 10^9 spores/m^2. Maximum infection and aboveground biomass reduction were obtained with 1.0 X 10^9 spores/m^2.

7. The pathogen produced lesions on plants at all growth stages. Only the fresh and dry weight of seedlings inoculated

at the cotyledon stage, however, were significantly decreased bv infection. A high degree of tolerance was observed at older ages, and the level of infection was not reflected by plant aboveground biomass.

8. Septoria galeopsidis did not kill hemp-nettle plants in any instance. Height was not affected by infection, and new leaves were always produced even when lower leaves were severely infected and had abscissed.

5.2 Suggestions for future research.

Considering the limitations of Septoria galeopsidis as a biological herbicide, another pathogen should be sought for the control of hemp-nettle. A number of factors, however, might improve development and severity of the disease caused by S. galeopsidis. More virulent isolates of the pathogen might be discovered in nature or through genetic manipulations. Double inoculation or higher inoculum density might substantially increase the stress on the plant. Also, a synergistic effect may result from simultaneous applications of chemical herbicides and spore suspension (Wymore et al., 1987; Scheepens, 1987). Finally, the application of the pathogen in an aqueous carrier followed by an invert emulsion might considerably improve the tolerance of the fungus to adverse environmental conditions (Quimby Jr.and Fulgham, 1986). If further studies on this pathogen are undertaken, an extensive host range should be performed.

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APPENDICES

APPENDIX A

Receipes of media tested for spore production and hyphal growth of *Septoria galeopsidis*.

Medium	Ingredients	quantity/L[1]
Liquid media		
Beef Peptone Broth (BPB)(Kendrick and Walker, 1948)	Dextrose	10 g
	Beef extract[2]	3 g
	Peptone	10 g
Hemp-nettle Extract	Infusion from hemp-nettle[3]	1 L
Potato Dextrose broth	Potato Dextrose Broth[2]	24 g
Richard's modified solution (Walker 1980)	Sucrose	10 g
	KNO_3	10 g
	$MgSO_4 \cdot H_2O$	2.5 g
	KH_2PO_4	5 g
	$FeCl_3$	0.02 g
	V-8 juice[4]	150 ml
Richard's modified solution with hemp-nettle leaves	Richard's modified solution	1 L
	hemp-nettle leaf pieces	25 g
Agar media		
Beef peptone	Beef Extract[2]	3 g
	Dextrose	10 g
	Peptone	10 g
Czapek's Dox	Czapek's dox broth[5]	35 g
	Bacto-agar[2]	15 g
CzapeckDox-V-8 (modified from Cooke and Jones, 1970)	Czapek's dox broth[5]	35 g
	V-8 juice[4]	200 ml
	Bacto-agar[2]	15 g
Hemp-nettle Extract	Infusion from hemp-nettle[3]	500 ml
	Bacto-agar	15 ml

Appendix A (Continued)

Medium	Ingredients	quantity/L
Hemp-nettle Extract with infusion from potato and dextrose	Infusion from hemp-nettle[3]	500 ml
	Bacto-agar[2]	15 g
	Potato Dextrose Agar[2]	7.5 g
Hemp-nettle plant part	Hemp-nettle leaf, petiole and stem pieces	20 g
	Bacto-agar[2]	15 g
Hemp-nettle seed (modified from Tuite, 1969)	Crushed hemp-nettle seeds	100 g
	Bacto-agar[2]	20 g
Lima Bean	Bacto Lima Bean Agar[2]	23 g
Malt extract (Tuite, 1969)	Malt extract[6]	25 g
	Bacto-agar[2]	20 g
Malt extract and yeast	Yeast extract[2]	4 g
	Malt extract[6]	10 g
	Dextrose	4 g
	Bacto-Agar[2]	15 g
Mycological	Bacto-Mycological agar[2]	35 g
Nutrient	Bacto-nutrient agar[2]	3 g
Potato Dextrose	Potato Dextrose agar[2]	39 g
Septoria _nodorum_ sporulating agar (Dhingra and Sinclair, 1985)	Sucrose	20 g
	$MgSO_4.7H_2O$	0.5 g
	Zinc sulfate	1.92 mg
	Bacto-agar	20 g
	Glycine	2.43 g
	Fe^{++}	0.2 mg
	Mn^{++}	0.1 mg
Violet Red Bile	Bacto-Violet Red Bile Agar[2]	41.5 g
Yeast extract	Yeast extract[2]	3 g
	Bacto-agar[2]	15 g
Water agar	Bacto-agar[2]	15 g

Appendix A (Continued).

Medium	Ingredients
<u>Seed and stem media</u>	
Soybean seeds (Sarwar Alam and Strange, 1987)	25 g of dry soybeans boiled in distilled water/250-ml flask
Barley seeds	10 g dry barley boiled in distilled water/125-ml flask
Hemp-nettle stems	10 g of autoclaved hemp-nettle stem pieces/125 flask

[1] All media were prepared with distilled water.
[2] Difco Laboratories, Detroit, Michigan.
[3] 100 g of plant material boiled for 30 minutes in 1L of distilled water and strained through a 250-um sieve.
[4] Campbell Soup Company Ltd., Toronto, Ontario.
[5] BBL, Cockeysville, Maryland.
[6] Sigma Chemical Company, St-Louis, Missouri.

APPENDIX B

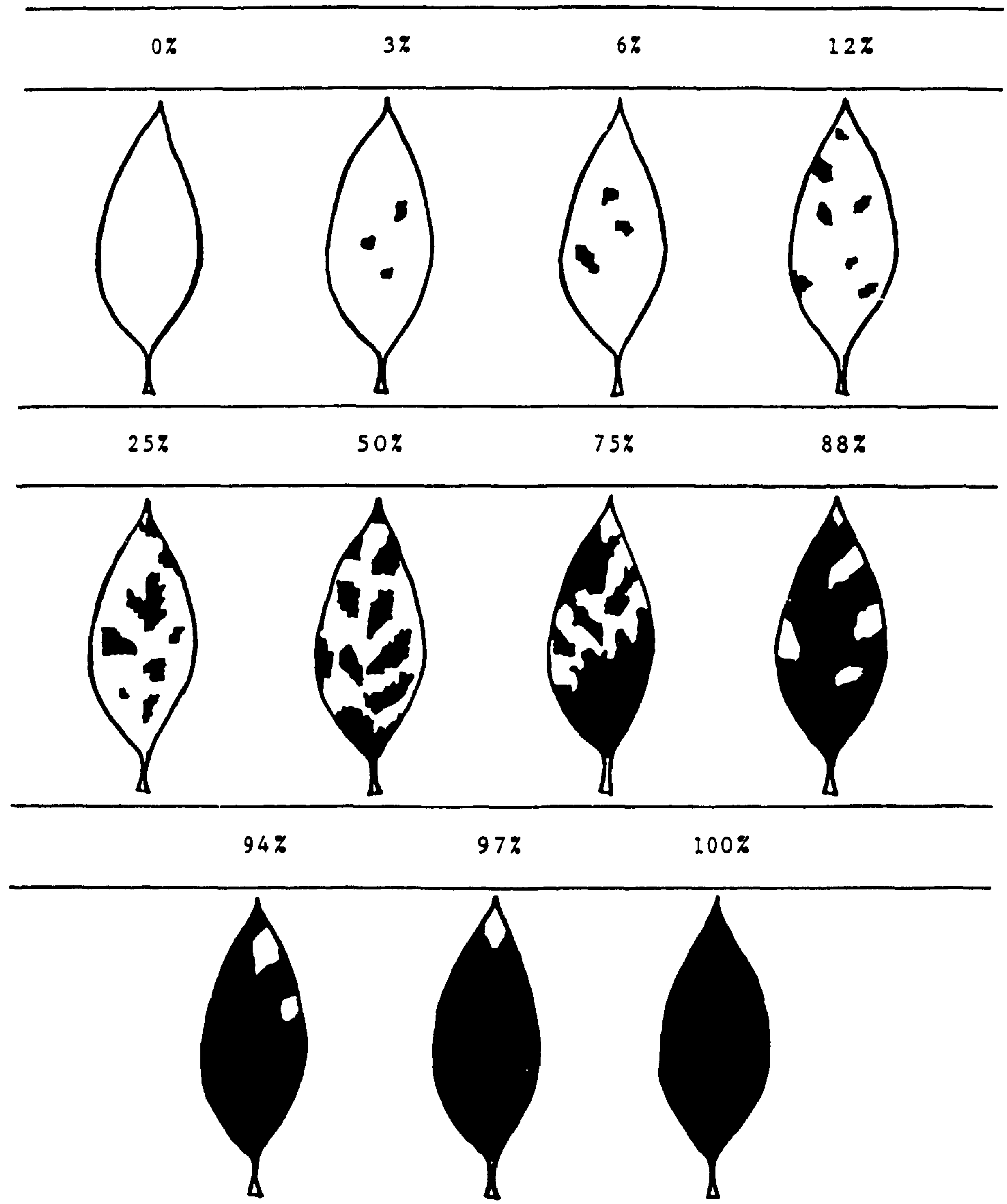
Horsfall and Barratt's grading system

Grade	Percent infected	Percent healthy
0	0	100
1	0 to 3	97 to 100
2	3 to 6	94 to 97
3	6 to 12	88 to 94
4	12 to 25	75 to 88
5	25 to 50	50 to 75
6	50 to 75	25 to 50
7	75 to 88	12 to 25
8	88 to 94	6 to 12
9	94 to 97	3 to 6
10	97 to 100	0 to 3
11	100	0

(Horsfall and Barratt, 1945)

APPENDIX C

Assessment key for hemp-nettle leaves infected by <u>Septoria</u> <u>galeopsidis</u> Westd. using Horsfall and Barratt's grading system.



APPENDIX D

Formula for the calculation of the area under the disease progress curve (AUDPC) (modified from Shaner and Finney, 1977).

$$AUDPC = \sum_{i=1}^{n} [(Y_i + Y_{i+1})/2][X_{i+1} - X_i]$$

Y_i = Average grade for leaf area covered by lesions on the ith assessment day

Y_{i+1} = Average grade for leaf area covered by lesions on the (i+1)th assessment day

n = Number of assessment days (n=4 or n=5 depending on the experiment)

$[X_{i+1} - X_i]$ was considered equal to 1 since the time interval between two consecutive disease assessments was constant (=2 days) throughout the studies.

APPENDIX E

TABLES OF MEANS AND ANALYSIS OF VARIANCE

Table E.1 Means of colony diameter (mm) for each medium-temperature treatment combination (3 reps per treatment).

Agar medium	Incubation temperature (C) 14	21	26	31
Violet Red Bile	1.7	3.7	3.7	0.8
Malt Extract	4.7	8.7	7.7	3.0
Czapeck Dox	6.3	10.0	9.7	4.0
Yeast Extract	8.0	11.7	9.7	2.5
Beef Peptone	7.7	11.7	13.5	2.0
Malt Extract and Yeast	7.0	14.0	14.0	1.5
Water	7.0	15.7	8.5	2.5
Nutrient	8.3	13.0	20.0	1.8
Lima Bean	8.3	27.0	28.7	- [1]
Potato Dextrose	6.3	15.0	31.0	3.6
Hemp-nettle Plant parts	16.7	33.0	27.0	3.0
Czapeck Dox-V-8	11.3	26.0	44.0	6.0

[1] Missing data due to contamination or failure of mycelium of growing from the germinated spores.

Table E.2 Analysis of variance for colony diameter.

Source of variation	df	SS	F value
Total	125	12133.00	
Temperature (T)	3	3526.70	204.14**
Block in T (error a)	8	46.07	
Medium (M)	11	3941.64	51.79**
M*T	32	477.68	11.19**
Error	71	491.20	

** Significant at the 0.01 level

Table E.3 Analysis of variance of the fit of a regression curve to colony diameter data for each medium.

Source of variation	df	SS	F value	Fitted equation	R^2
Beef Peptone					
T	1	32.69	4.81*		
T*T	1	151.44	22.27**	Y = -37.6606	
T*T*T	1	24.22	3.56	+ 4.7830*T	0.96
Pooled error[1]	79	6.80		- 0.1123*T^2	
Czapek's dox					
T	1	4.86	0.82		
T*T	1	67.19	9.88**	Y = -23.5535	
T*T*T	1	1.62	0.27	+ 3.1413*T	0.61
Pooled error[1]	79	6.80		- 0.0725*T^2	
Czapek's Dox-V-8					
T	1	12.84	0.82	Y = +578.8557	
T*T	1	1834.87	269.80**	- 90.4970*T	0.95
T*T*T	1	761.29	111.94**	+ 4.5942*T^2	
Pooled error[1]	79	6.80		- 0.0733*T^3	
Hemp-nettle Plant parts					
T	1	0.21	0.03		
T*T	1	865.36	127.24**	Y = -110.7250	
T*T*T	1	1.87	0.27	+ 13.5420*T	0.91
Pooled error[1]	79	6.80		- 0.3179*T^2	
Lima Bean					
T	1	672.89	98.94**		
T*T	1	91.78	13.50**	Y = -86.1667	
T*T*T	-	-	-	+ 9.4722*T	0.93
Pooled error[1]	79	66.80		- 0.1944*T^2	
Malt Extract					
T	1	1.32	0.19		
T*T	1	39.04	5.74*	Y = -23.3593	
T*T*T	1	0.04	0.01	+ 2.9461*T	0.46
Pooled error[1]	79	6.80		- 0.0675*T^2	

Table E.3 (Continued).

Source of variation	df	SS	F value	Fitted equation	R^2
---------- MaltExtractandYeast ----------					
T	1	17.30	2.54		
T*T	1	171.71	25.25**	Y = -51.4813	
T*T*T	1	5.99	0.88	+ 6.1817*T	0.88
Pooled error[1]	79	6.80		$-0.1439*T^2$	
---------- Nutrient ----------					
T	1	13.43	1.98	Y = +271.0672	
T*T	1	359.51	52.86**	- 41.4954*T	0.93
T*T*T	1	156.75	23.05**	$+ 2.0889*T^2$	
Pooled error[1]	79	6.80		$- 0.0332*T^3$	
---------- Potato Dextrose ----------					
T	1	5.88	0.86	Y = +501.7733	
T*T	1	367.32	54.01**	- 77.6797*T	0.91
T*T*T	1	251.63	37.00**	$+ 3.8719*T^2$	
Pooled error[1]	79	6.80		$- 0.0608*T^3$	
---------- Violet Red Bile ----------					
T	1	0.41	0.06		
T*T	1	17.62	2.59	-	-
T*T*T	1	0.53	0.08		
Pooled error[1]	79	6.80			
---------- Water ----------					
T	1	22.24	3.27		
T*T	1	185.17	22.23**	Y = -44.7495	0.96
T*T*T	1	22.92	3.37	+ 5.5582*T	
Pooled error[1]	79	6.80		$- 0.1311*T^2$	
---------- Yeast Extract ----------					
T	1	20.65	3.04		
T*T	1	85.91	12.63**	Y = -25.5575	
T*T*T	1	0.33	0.05	+ 3.6158*T	0.75
Pooled error[1]	79	6.80		$- 0.0873*T^2$	

[1]Pooled error SS = R(T)SS + Error SS (from table E.   )
T = Incubation temperature
* = Significant at the 0.05 level
** = Significant at the 0.01 level
Y = Estimate of colony diameter

Table E.4 Means of percent spore germination at each incubation temperature after 6, 12, 18, and 24 hours (pooled for two experiments).

Time after Plating (hours)	Incubation temperature (C) 15 %	15 Asine[1] (rad.)	20 %	20 Asine (rad.)	25 %	25 Asine (rad.)	30 %	30 Asine (rad.)
6	6	0.23	9	0.30	4	0.21	7	0.26
12	8	0.29	14	0.39	18	0.43	15	0.40
18	25	0.53	48	0.76	50	0.79	48	0.76
24	60	0.89	64	0.93	61	0.90	67	0.97

[1] Arcsine transformation = arcsine $\sqrt{x}$

x = percent spore germination/100

Table E.5 Analysis of variance for transformed percent spore germination data (pooled for two experiments).

Source of variation	df	SS	F value
Total	143	11.5243	
Replication (N)	1	0.0013	0.29
Block in N (error a)	7	0.0325	
Time (T)	3	9.8905	521.11**
Temperature (C)	3	0.3308	17.27**
H*C	9	0.2603	4.53**
N*T	3	0.0314	1.64
N*C	3	0.0166	0.87
N*T*C	9	0.0070	0.12
Error	105	0.6703	

** Significant at the 0.01 level

Table E.6 Analysis of variance for transformed data of percent spore germination at each counting period (pooled for two experiments).

Source of variation	df	SS	F value
6 hours			
Total	35	0.1839	
Replication (N)	1	0.0020	0.27
Block in N (error a)	7	0.0522	
Temperature (C)	3	0.0466	4.06*
N*C	3	0.0052	0.45
Error	21	0.0802	
12 hours			
Total	35	0.2132	
Replication (N)	1	0.0022	0.66
Block in N (error a)	7	0.0234	
Temperature (C)	3	0.1003	9.66**
N*C	3	0.0110	1.06
Error	21	0.0727	
18 hours			
Total	35	0.6734	
Replication (N)	1	0.0121	1.43
Block in N (error a)	7	0.0593	
Temperature (C)	3	0.4080	14.87**
N*C	3	0.0027	0.10
Error	21	0.1921	
24 hours			
Total	35	0.2780	
Replication (N)	1	0.0165	2.47
Block in N (error a)	7	0.0466	
Temperature (C)	3	0.0362	1.44
N*C	3	0.0047	0.19
Error	21	0.1763	

* Significant at the 0.05 level
** Significant at the 0.01 level

Table E.7 Analysis of variance of the fit of a regression curve to transformed percent spore germination data (pooled for two experiments).

Source of variation	df	SS	F value	Fitted equation	R^2
Total	143	11.5243			
N	1	0.0013	0.29		
R(N)	7	0.0325			
T	1	9.9147	1640.30**		
T*T	1	0.0694	11.48**		
T*T*T	1	0.1917	31.72**	Y = 0.8107	0.93
C	1	0.1926	31.86**	− 0.6150*T	
C*C	1	0.0838	13.87**	+ 0.0278*T^2	
C*C*C	1	0.0479	7.93**	− 0.0003*T^3	
T*C	1	0.0211	3.49	+ 0.1183*C	
T*T*C	1	0.0978	16.18**	− 0.0107*C^2	
T*T*T*C	1	0.0152	2.52	+ 0.0002*C^3	
T*C*C	1	0.0000	0.01	+ 0.0411*T*C	
T*T*C*C	1	0.0866	14.33**	− 0.0014*T^2*C	
T*T*T*C*C	1	0.0227	3.76	− 0.0008*T*C^2	
T*C*C*C	1	0.0012	0.20	+ 0.00003*T^2*I^2	
T*T*C*C*C	1	0.0128	2.12		
T*T*T*C*C*C	1	0.0074	1.23		
Error	120	0.7253			

N = Replication
R = Blocks
T = Time
C = Temperature
Y = Estimate of arcsine value of percent spore germination
** = Significant at the 0.01 level

TableE.8 Means of AUDPC values for inoculated plants at each dew period duration (averaged over temperatures of 18 and 24 C).

Dew period duration (hours)	AUDPC [1]
24	6.63 a
36	9.24 ab
48	14.42 bc
60	17.59 c

[1] Means followed by the same letter are not significantly different at the 0.20 experimentwise error rate according to multiple comparison procedure of the Friedman test.

Table E.9 Means of AUDPC values for inoculated plants at eachtemperature (averaged over dew period durations of 24, 36, and 48 hours.)

Dew period temperature (C)	AUDPC [1]
18	5.56 a
24	13.94 b
30	10.62 a

[1] Means followed by the same letter are not significantly different at the 0.15 experimentwise error rate according to multiple comparison procedure of the Friedman test.

Table E.10 Friedman two-way analysis of variance for AUDPC values.

Source of variation	df	X^2 value
Dewperiod duration	3	22.50**
Temperature	2	17.29**

** Significant at the 0.01 level

Table E. 11 Non-parametric simple linear regression equations for AUDPC at each temperature.

Temperature (C)	Regression equation [1]
18	Y = -1.241 + 0.201*D [2]
24	Y = -1.462 + 0.433*D [2]
30	Y = -6.420 + 0.483*D [3]

[1] D = dew period duration (hours)
[2] for D values between 24 and 60 hours
[3] for D values between 24 and 48 hours

Table E.12 Means of plant height for uninoculated and inoculated plants at each dew period duration (averaged over temperatures of 18 and 24).

Dew period duration (hours)	Mean height (cm) Control plants	Inoculated plants
24	39.08	43.00
36	42.00	39.36
48	39.17	39.69
60	38.81	40.75
Column means	39.76	40.70

Table E.13 Means of plant height for uninoculated and inoculated plants at each dew period temperature (averaged over dew period durations of 24, 36 and 48 hours).

Dew period temperature (C)	Plant height (cm) Control plants	Inoculatedplants
18	40.67	41.80
24	39.50	39.57
30	39.48	39.13
Column means	39.88	40.19

Table E.14 Analysis of variance for plant height(data set A).

Sourceof variation	df	SS	F value
Total	95	2137.81	
Replication (N)	1	19.26	0.02
Temperature (T)	1	129.89	0.17
T*N (error a)	1	772.56	
Block	2	4.39	0.18
Inoculation (I)	1	21.09	1.77
Duration (D)	3	40.93	1.15
D*I	3	137.09	3.84*
T*I	1	13.25	1.11
T*D	3	53.90	1.51
T*D*I	3	41.37	1.16
Error	76	904.07	

* Significant at the 0.05 level

Table E. 15 Analysis of variance for plant height (data set B).

Source of variation	df	SS	F value
Total	106	2817.25	
Replication (N)	1	7.97	0.02
Temperature (T)	2	89.09	0.16
T*N (error a)	2	572.42	
Block	2	21.11	0.66
Inoculation (I)	1	0.69	0.04
Duration(D)	2	320.21	10.04**
D*I	2	199.31	6.25**
T*I	2	15.27	0.48
T*D	4	253.46	3.97**
T*D*I	4	38.39	0.60
Error	84	1339.42	

** Significant at the 0.01 level

Table E.16 Analysis of variance for uninoculated and inoculated plant height (data set A).

Source of variation	df	SS	F value
Control plants			
Total	46	969.22	
Replication (N)	1	0.75	0.00
Temperature (T)	1	38.19	0.10
T*N (error a)	1	364.38	
Block	2	6.14	0.23
Duration (D)	3	88.07	2.16
T*D	3	6.98	0.17
Error	36	377.86	
Inoculated plants			
Total	47	1147.17	
Replication	1	39.72	0.09
Temperature	1	113.06	0.26
T*N (error a)	1	430.00	
Block	2	7.39	0.35
Duration	3	97.16	3.09*
T*D	3	81.97	2.60
Error	36	377.86	

* Significant at the 0.05 level

Table E17 Analysis of variance of the fit of a regression curve to the height of inoculated plants (data set A).

Source of variation	df	SS	F value	Fitted equation	R^2
Total	47	1147.17			
N	1	458.79	1.07		
T	1	234.08	0.54		
N*T (error a)	1	430.00		Y = +55.8570	0.61
D	1	24.70	2.25	- 0.7381*D	
D*D	1	66.11	6.02*	+ $0.0082*D^2$	
D*D*D	1	6.34	0.58		
D*T	1	27.79	2.53		
Error	40	439.44			

N = replication
T = temperature
D = dew period duration
Y = estimate of plant height
* = significant at the 0.05 level

Table E. 18 Means of fresh weight for uninoculated and inoculatedplantsat each dew period duration (averaged over the temperatures of 18 and 24 C.)

Dew period duration (hours)	Fresh weight (g)	
	Control plants	Inoculated plants
24	8.00	7.48
36	8.30	6.75
48	7.53	6.14
60	7.19	6.28
Column means	7.76	6.66

Table E .19 Means of dry weight for uninoculated and inoculated plants at each dew period duration (averaged over temperatures of 18 and 24 C.)

Dew period duration (hours)	Dry weight (g)	
	Control plants	Inoculated plants
24	0.92	0.81
36	0.92	0.68
48	0.78	0.59
60	0.73	0.56
Column means	0.84	0.66

Table E.20 Meaus of fresh weight for uninoculated and inoculated plants at each dew period temperature (averaged over dew period durations of 24, 36, and 48 hours).

Dew periodtemperature (C)	freshweight (g) Control plants	Inoculated plants
18	8.72	7.70
24	7.17	5.88
30	7.69	6.53
Column means	7.86	6.71

Table E.21 Means of dry weight for uninoculated and inoculated plantsat each temperature (averaged over dew period durations of 24, 36, and 48 hours).

Dew period temperature (C)	Dry weight (g) Control plants	Inoculated plants
18	0.94	0.76
24	0.81	0.61
30	0.78	0.66
Column means	0.85	0.68

Table E.22 Analysis of variance for plant fresh weight (data set A).

Source of variation	df	SS	F value
Total	95	221.98	
Replication (N)	1	11.11	1.05
Temperature (T)	1	70.19	6.66
T*N (error a)	1	10.54	
Block	2	1.62	0.92
Inoculation (I)	1	28.65	32.56**
Duration (D)	3	17.96	6.80**
D*I	3	3.94	1.49
D*T	3	0.16	0.06
T*I	1	1.62	1.84
T*I*D	3	9.36	3.55*
Error	76	66.85	

* Significant at the 0.05 level
** Significant at the 0.01 level

Table E.23 Analysis of variance for plant fresh weight (data set B).

Source of variation	df	SS	F value
Total	106	267.30	
Replication (N)	1	12.79	2.76
Temperature (T)	2	53.67	5.09
T*N (error a)	2	10.54	
Block	2	0.95	0.45
Inoculation	1	38.05	35.63**
Duration (D)	2	40.56	18.99**
D*I	2	4.99	2.29
T*I	2	0.41	0.19
D*T	4	11.75	2.75*
D*T*I	4	8.30	1.94
Error	84	89.72	

* Significant at the 0.05 level
** Significant at the 0.01 level

Table E.24 Analysis of variance for plant dry weight (data set A).

Source of variation	df	SS	F value
Total	95	3.85	
Replication (N)	1	0.30	2.49
Temperature (T)	1	0.50	4.13
N*T (error a)	1	0.12	
Block	2	0.03	1.07
Inoculation (I)	1	0.78	49.29**
Duration (D)	3	0.77	16.29**
D*I	3	0.05	1.13
T*I	1	0.01	0.78
D*T	3	0.00	0.09
D*T*I	3	0.07	1.48
Error	76	1.20	

** Significant at the 0.01 level

Table E.25 Analysis of variance for plant dry weight (data set B).

Source of variation	df	SS	F value
Total	106	4.50	
Replication (N)	1	0.20	1.79
Temperature (T)	2	0.45	2.05
N*T	2	0.22	
Block	2	0.04	1.14
Inoculation (I)	1	0.80	44.06**
Duration (D)	2	1.03	28.47**
D*I	2	0.08	2.27
T*I	2	0.02	0.42
T*D	4	0.14	1.86
T*D*I	4	0.06	0.78
Error	84	1.52	

** Significant at the 0.01 level

Table E.26 Analysis of variance for fresh weight of control and inoculated plants (data set A).

Source of variation	df	SS	F value
------------------- Control plants -------------------			
Total	46	90.41	
Replication (N)	1	5.22	0.60
Temperature (T)	1	26.31	3.01
N*T (error a)	1	8.75	
Block	2	0.26	0.12
Duration (D)	3	9.34	2.89*
D*T	3	4.84	1.50
Error	35	37.71	
------------------- Inoculated plants -------------------			
Total	47	102.56	
Replication	1	6.63	2.12
Temperature	1	46.55	14.91
N*T	1	3.12	
Block	2	4.33	3.16
Duration	3	13.08	6.38**
T*D	3	4.26	2.08
Error	36	24.60	

* Significant at the 0.05 level
** Significant at the 0.01 level

Table E.27 Analysis of variance for dry weight of control and inoculated plants (data set A).

Source of variation	df	SS	F value
----------------------- Control plants -----------------------			
Total	46	1.47	
Replication (N)	1	0.23	4.15
Temperature (T)	1	0.18	3.43
N*T	1	0.05	
Block	2	0.01	0.14
Duration (D)	3	0.37	6.83**
D*T	3	0.05	0.99
Error	35	0.62	
---------------------- Inoculated plants ----------------------			
Total	47	1.58	
Replication	1	0.10	1.32
Temperature	1	0.34	4.56
N*T	1	0.07	
Block	2	0.10	3.65*
Duration	3	0.47	11.45**
T*D	3	0.02	0.52
Error	36	0.49	

* Significant at the 0.05 level
** Significant at the 0.01 level

Table E.28 Analysis of variance of the fit of a regression line to fresh weight of uninoculated and inoculated plants (data set A).

Source of variation	df	SS	F value	Fitted equation	R^2
---------- Uninoculated plants ----------					
Total	46	90.41			
N	1	5.09	0.59		
T	1	9.73	1.13		
N*T (error a)	1	8.54		Y = 8.8771	0.53
D	1	6.53	6.32*	-(0.0265*D)	
D*D	1	0.97	0.94		
D*D*D	1	1.88	1.82		
D*T	1	2.60	2.52		
Error	39	40.24			
---------- Inoculated plants ----------					
Total	47	102.56			
N	1	6.63	2.12		
T	1	0.33	0.11	Y = 8.1364	0.68
N*T	1	3.12		-(0.0351*D)	
D	1	10.63	13.84**		
D*D	1	2.21	2.88		
D*D*D	1	0.24	0.32		
D*T	1	2.47	3.22		
Error	40	30.71			

N = Replication
T = Temperature
D = Dew period duration
Y = Estimate of plant fresh weight
* = Significant at the 0.05 level
** = Significant at the 0.01 level

Table E.29 Analysis of variance of the fit of a regression line todry weight of control and inoculated plants (data set A).

Source of variation	df	SS	F value	Fitted equation	R2
---------- Uninoculated plants ----------					
Total	45	1.44			
N	1	0.22	4.30		
T	1	0.07	1.29		
N*T	1	0.06		Y = 1.0924 -(0.0060*D)	0.53
D	1	0.31	17.97**		
D*D	1	0.01	0.80		
D*D*D	1	0.02	1.45		
D*T	1	0.02	1.17		
Error	38	0.64			
---------- Inoculated plants ----------					
Total	47	1.58			
N	1	0.10	1.32		
T	1	0.01	0.09	Y = 0.9582 -(0.0071*D)	0.60
N*T	1	0.07			
D	1	0.44	29.28**		
D*D	1	0.03	1.86		
D*D*D	1	0.00	0.01		
D*T	1	0.01	0.68		
Error	40	0.60			

N = Replication
T = Temperature
D = Dew period duration
Y = Estimate of plant dry weight
* = Signiicant at the 0.05 level
** = Significant at the 0.01 level

TableE.30 Friedman two-way analysis of variance for AUDPC.

Source of variation	df	X^2 calculated
---------- Expriment 1 ----------		
Additives	3	1.20
Inoculation rates	4	13.00*
---------- Experiment 2 ----------		
Inoculation rate	4	22.27 **

* Significant at the 0.05 level
** Significant at the 0.01 level

Table E.31 Non-parametric simple linear regression equations for AUDPC values.

Experiment 2A	Experiment 2B
Y = -80.055 + (13.35*R)	Y = -21.121113 + (5.1062525*R)

Y = Estimate of AUDPC
R = Log of inoculation rate
Table 4.4.10 AUDPC mean values.

Table E.32 Means of height, fresh weight and dry weight for each inoculum density.

Inoculation rate (spores/m^2)	Height (cm)	Fresh weight (g)	% reduction	Dry weight (g)	% reduction
Experiment 1					
0.0	62.53	8.3	-	1.23	-
1.0×10^6	61.81	8.19	1.33	1.23	0.00
1.0×10^7	61.74	7.56	8.92	1.11	9.76
1.0×10^8	61.38	7.04	15.18	1.00	18.70
1.0×10^9	63.09	7.35	11.45	0.99	19.51
Experiment 2					
0.0	30.17	4.17	-	0.56	-
1.0×10^6	30.92	4.08	2.34	0.53	4.74
1.0×10^7	31.08	3.87	7.39	0.47	16.07
1.0×10^8	27.58	2.81	32.67	0.30	46.90
1.0×10^9	28.75	2.53	39.50	0.26	53.50

Table E.33 Analysis of variance for plant height.

Source of variation	df	SS	F value
Experiment 1			
Total	79	1567.37	
Block	3	568.98	13.15**
Additives	3	54.51	1.26
Inoculum density	4	30.54	0.53
Additives*inoculum density	12	100.51	0.58
Error	57	821.83	
Experiment 2			
Total	29	287.30	
Block	5	89.70	2.50
Inoculum density	4	13.49	1.88
Error	20	143.63	

** Significant at the 0.01 level

Table E.34 Analysis of variance for plant fresh weight.

Source of variation	df	SS	F value
Experiment 1			
Total	79	119.16	
Block	3	27.52	9.17**
Additives (A)	3	6.36	2.13
Inoculum density (I)	4	19.23	4.84**
A*I	12	9.40	0.79
Error	57	56.65	
Experiment 2			
Total	29	18.69	
Block	5	0.61	0.61
Inoculum density	4	14.06	17.49**
Error	20	4.02	

** Significant at the 0.01 level

Table E.35 Analysis of variance for plant dry weight.

Source of variation	df	SS	F value
---------- Experiment 1 ----------			
Total	79	4.22	
Block	3	0.89	8.16**
Additives	3	0.14	1.28
Inoculum density	4	0.85	5.83**
Additives*inoculum density	12	0.26	0.59
Error	57	2.08	
---------- Experiment 2 ----------			
Total	29	0.53	
Block	5	0.01	0.65
Inoculum density	4	0.45	33.36**
Error	20	0.07	

** Significant at the 0.01 level

Table E.36 Analysis of variance of the fit of a regression line for plant fresh weight.

Source of variation	df	SS	F value	Fitted equation	R^2
------------------------			Experiment 1	------------------------	
Total	79	125.15			
R	3	30.38	10.07**		
A	3	5.70	1.89	Y = + 9.8111	0.46
D	1	15.42	15.33**	- 0.3047*D	
D*D	1	0.83	0.82		
D*D*D	1	3.31	3.29		
D*A	3	2.14	0.71		
Error	67	67.37			
------------------------			Experiment 2	------------------------	
Total	29	18.69			
R	5	0.61	0.57		
D	1	12.48	58.37**	Y =+ 7.5981	0.76
D*D	1	0.63	2.96	- 0.5705*D	
D*D*D	1	0.47	2.19		
Error	21	4.49			

R = Blocks
A = Adjuvants
D = Log of inoculum density
Y = Estimate of plant fresh weight
* = Significant at the 0.05 level
** = Significant at the 0.01 level

Table E.37 Analysis of variance of the fit of regression line for plant dry weight.

Source of variation	df	SS	F value	Fitted equation	R^2
Experiment 1					
Total	78	4.40			
R	3	1.02	9.77**		
A	3	0.11	1.09		
D	1	0.77	22.07**	Y = + 1.5795	0.46
D*D	1	0.01	0.16	- 0.0638*D	
D*D*D	1	0.09	2.53		
A*D	3	0.11	1.10		
Error	66	2.29			
Experiment 2					
Total	29	0.53			
R	5	0.01	0.60		
D	1	0.42	115.02**	Y = - 15.0663	0.86
D*D	1	0.01	1.99	+ 6.5933*D	
D*D*D	1	0.02	4.89*	- 0.91*D^2	
Error	21	0.08		+ 0.0407*D^3	

R = Blocks
D = Log of inoculum density
Y = Estimate of plant dry weight
* Significant at the 0.05 level
** Significant at the 0.01 level

TableE.38 Means of Area Under the Disease Progress Curve (AUDPC) for inoculated plants at each growth stage.

Age	Plant growth stage	AUDPC [1] Experiment 1	Experiment 2
1	cotyledons	11.844 a	4.625 a
2	2 leaves	0.750 b	3.042 a
3	4 leaves	6.479 ab	-
4	6 leaves	6.042 ab	6.437 a
5	8 leaves	4.397 ab	5.749 a

[1] Means in the same column followed by the same letter are not significantly different at the 0.20 experimentwise error rate.

Table E.39 Friedman two-way analysis of variance for AUDPC.

Source of variation	df	Chi-square value
Experiment 1		
Growth stage	4	11.5*
Experiment2		
Growth stage	3	3.3

* Significant at the 0.05 level

Table E.40 Means of plant height for uninoculated and inoculated treatments at each growth stage.

	------ Height (cm) ------		
Plant growth stage	Uninoculated plants	Inoculated plants	% Difference
---------- experiment 1 ----------			
cotyledons	12.25	10.25	-16.3
2-leaf	14.42	17.80	+23.4*
4-leaf	28.17	27.25	-3.3
6-leaf	35.08	32.75	-6.6
8-leaf	40.50	42.33	+4.5
---------- experiment 2 ----------			
cotyledons	10.83	9.75	+10.0
2-leaf	14.25	13.75	-3.5
6-leaf	30.50	29.42	-3.5
8-leaf	25.67	26.42	+2.9

* Significant at the 0.05 level

Table E.41 Analysis ofvariance for plant height at each growth stage (experiment 1).

Source of variation	df	SS	F value
---------- Cotyledons ----------			
Total	9	49.23	
Blocks	5	36.25	6.44
Inoculation	1	6.13	5.44
Error	3	3.38	
---------- 2-leaf ----------			
Total	22	90.65	
Blocks	5	9.91	0.71
Inoculation	1	32.40	11.68*
Error	4	11.10	
---------- 4-leaf ----------			
Total	11	275.73	
Blocks	5	161.35	1.44
Inoculation	1	2.52	0.11
Error	5	111.85	
---------- 6-leaf ----------			
Total	11	285.42	
Blocks	5	157.92	1.42
Inoculation	1	16.33	0.73
Error	5	111.17	
---------- 8-leaf ----------			
Total	11	223.92	
Blocks	5	202.17	17.33**
Inoculation	1	10.08	4.32
Error	5	11.67	

* Significant at the 0.05 level
** Significant at the 0.01 level

Table E.42 Analysisof variance for plant height at each growth stage (experiment 2).

Source of variation	df	SS	F value
Cotyledons			
Total	9	28.40	
Blocks	5	22.96	5.25
Inoculation	1	3.13	3.57
Error	3	2.63	
2-leaf			
Total	11	16.50	
Blocks	5	13.25	5.30*
Inoculation	1	0.75	1.50
Error	5	2.50	
6-leaf			
Total	11	175.73	
Blocks	5	145.35	5.41*
Inoculation	1	3.52	0.66
Error	5	26.85	
8-leaf			
Total	11	52.23	
Blocks	5	28.10	1.25
Inoculation	1	1.69	0.38
Error	5	22.44	

* Significant at the 0.05 level

Table E.43 Mean plant fresh weight for uninoculated and inoculated treatments at each growth stage.

	---- Fresh weight (g)----		
Plantgrowth stage	Uninoculated plants	Inoculated plants	% difference
Experiment 1			
cotyledons	0.16	0.10	-37.5
2-leaf	0.40	0.61	+52.5
4-leaf	2.57	2.77	+7.8
6-leaf	4.89	4.00	-18.2
8-leaf	5.85	5.75	-1.7
Experiment 2			
cotyledons	0.11	0.08	-2.7
2-leaf	0.39	0.36	-7.7
6-leaf	3.38	3.31	-2.1
8-leaf	5.36	5.87	+9.5

Table E.44 Analysis of variance for plant fresh weight at each growth stage (experiment 1).

Source of variation	df	SS	F value
Cotyledons			
Total	9	0.0430	
Blocks	5	0.0330	8.09
Inoculation	1	0.0046	5.62
Error	3	0.0024	
2-leaf			
Total	10	0.3001	
Blocks	5	0.0939	0.90
Inoculation	1	0.1262	6.07
Error	4	0.0832	
4-leaf			
Total	11	2.8441	
Blocks	5	2.3705	6.69*
Inoculation	1	0.1194	1.69
Error	5	0.3542	
6-leaf			
Total	11	10.2423	
Blocks	5	5.1005	1.83
Inoculation	1	2.3506	4.21
Error	5	2.7912	
8-leaf			
Total	11	11.8184	
Blocks	5	7.9924	2.10
Inoculation	1	0.0276	0.04
Error	10	18.4312	

* Significant at the 0.05 level

Table E.45 Analysis of variance for plant fresh weight at each growth stage (experiment 2).

Source of variation	df	SS	F value
------------------------- Cotyledons -------------------------			
Total	9	0.0080	
Blocks	5	0.0059	4.90
Inoculation	1	0.0017	6.99
Error	3	0.0007	
------------------------- 2-leaf -------------------------			
Total	11	0.1486	
Blocks	5	0.1134	3.59
Inoculation	1	0.0037	0.58
Error	5	0.0316	
------------------------- 6-leaf -------------------------			
Total	11	3.8563	
Blocks	5	2.3719	1.62
Inoculation	1	0.0182	0.06
Error	5	1.4662	
------------------------- 8-leaf -------------------------			
Total	11	0.2460	
Blocks	5	19.0724	1.30
Inoculation	1	0.7916	0.27
Error	5	14.6328	

Table E.46 Mean plant dry weight for uninoculated and inoculated treatments at each growth stage (pooled data for two experiments).

		----- Dry weight (g) ------		
Age	Plant growth stage	Uninoculated plants	Inoculated plants	% Difference
1	Cotyledons	0.010	0.007	-26.0**
2	2 leaves	0.034	0.042	+23.5
3	4 leaves	0.270	0.280	+3.7
4	6 leaves	0.500	0.440	-12.0
5	8 leaves	0.780	0.750	-3.8

** Significant at the 0.01 level

Table E.47 Analysis of variance for plant dry weight at each growth stage (pooled data for two experiments).

Source of variation	df	SS	F value
Cotyledons			
Total	19	0.0002928	
Experiment (E)	1	0.0000688	3.55
Block in experiment	10	0.0001939	7.40*
Inoculation (I)	1	0.0000238	9.07*
E*I	1	0.0000004	0.15
Error	6	0.0000157	
2-leaf			
Total	22	0.0059	
Experiment (E)	1	0.0012	5.60*
Block in experiment	10	0.0022	1.26
Inoculation (I)	1	0.0005	2.67
E*I	1	0.0007	3.84
Error	9	0.0016	
4-leaf			
Total	11	0.0373	
Block	5	0.0303	4.99
Inoculation	1	0.0008	0.68
Error	5	0.0061	
6-leaf			
Total	23	0.3963	
Experiment (E)	1	0.1570	14.48**
Block in experiment	10	0.1084	1.19
Inoculation (I)	1	0.0286	3.15
E*I	1	0.0113	1.24
Error	10	0.0910	
8-leaf			
Total	23	0.7037	
Experiment	1	0.1408	3.40
Block in experiment	10	0.4136	2.94
Inoculation	1	0.0079	0.56
E*I	1	0.0007	0.05
Error	10	0.1407	

* Significant at the 0.05 level
** Significant at the 0.01 level