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Resistance of Common Ragweed *(Ambrosia artemisiifolia* L.) to the Herbicide Linuron and Evaluation of Several Species of Pathogenic Fungi for its Biological Control

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Abstract

Common ragweed *(Ambrosia artemisiifolia* L., Asteraceae) is an annual herbacious weed that is a troublesome pest in carrot fields of Southwestern Québec. Over the past decade, ragweed has shown resistance to linuron, the only herbicide that is registered for post-emergence control of this weed in carrots. In this research, the degree of resistance to linuron was investigated for a ragweed biotype collected from a carrot field in Sherrington, Québec, where a decreased performance of linuron had been noted. This biotype showed a linuron resistance ratio (I_{50}) of 9.09, when compared with ragweed plants collected from a field never sprayed with this herbicide. The fungal pathogen *Phoma* sp., which had been initially isolated from diseased ragweed leaves in 1993, was considered as a potentially effective biological agent for the control of common ragweed. The pathogenicity of *Phoma* sp. was re-evaluated during the current research. This fungus was found not to have any appreciable virulence towards common ragweed; it is likely that virulence was lost during storage. Hence, twenty other fungal species were isolated from diseased common ragweed plants and assayed to determine their potential as biological agents against this noxious weed. Varying dew periods, temperatures, spore concentrations, host growth stages, and different types of carrier were evaluated. Only isolates ATT#9, INNA4a, INNA4b, ATT#10, ISO#65, and ISO#68 were able to induce lesions on ragweed foliage at spore concentrations of 10^6 to 10^7 spores ml^{-1}, but only after an extended dew period of 48 hrs. No interaction effects on the degree of ragweed control were found when combining five fungal isolates and the insect, *Ophraella communa* LeSage. However, a possible interactive effect was detected when the fungal isolate ISO#65 and linuron were used in combination.

Résumé

La petite herbe à poux, ou ambrosia *(Ambrosia artemisiifolia* L., Asteraceae) est une mauvaise herbe annuelle très envahissante dans les champs de carottes du sud-ouest du Québec. Au cours de la dernière decennie, l'ambrosia a développé une résistance au linuron, le seul herbicide qui est homologué pour le contrôle en post-émergence de l'ambrosia dans les carottes. Le degré de résistance au linuron a été étudié pour un biotype d'ambrosia provenant d'un champ de Sherrington, Québec, où une baisse d'efficacité du linuron a été notée depuis quelques années. Ce biotype a démontré un ratio de résistance (I_{50}) au linuron de 9.09, comparé aux plants d'ambrosia provenant d'un champ qui n'a jamais été exposé à cet herbicide. Un champignon pathogène, *Phoma* sp., a été isolé d'un plant d'ambrosia en 1993, et semblait détenir un fort potentiel d'agent de biocontrôle pour l'ambrosia. La pathogenécité de *Phoma* sp. par rapport à l'ambrosia a été ré-évaluée au cours de cette étude, mais n'a pas été démontré. Il est probable que la virulence de *Phoma* sp.ait été perdue au cours de la période d'entreposage. Plusieurs espèces de champignons, isolés d'ambrosia, ont été évalués, en variant la période et la température de rosée, la concentration des solutions de spores, le stade de croissance des plants d'ambrosia, et les formules d'application. Seuls les isolats ATT#9, INNA4a, INNA4b, ATT#10, ISO#65, et ISO#68 ont créé des lésions sur le feuillage d'ambrosia, mais pour ce faire ont requis des périodes de rosée d'au moins 48 hrs. Il n'y a pas eu d'effet d'interaction en ce qui concerne le niveau de contrôle de l'ambrosia lorsque cinq isolats de champignon et l'insecte *Ophraella communa* LeSage ont été utilisés ensemble. Par contre, une interaction possible entre l'isolat ISO#65 et le linuron a été observée.

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Description of thesis format

This manuscript has been submitted in the form of papers suitable for journal publication. The first chapter is a general introduction that reviews the literature concerning all the theory on which this thesis is based. Chapters two, three, and four represent three different aspects of this research, and are complete manuscripts containing an abstract, introduction, material and methods, results, and discussion. Chapter two is a paper that will be submitted to the scientific journal *Weed Technology*. Chapter five is a general conclusion that includes the major conclusions of each chapter. Connecting texts between the chapters provide a comprehensive link from one experiment to the other.

This project was supervised by Dr. Alan K. Watson, and co-supervised by Dr. Antonio DiTommaso. Both supervisors reviewed all manuscripts, and are co-authors of manuscript two. The candidate was responsible for all experimental designs, field, greenhouse, and laboratory work, as well as data analysis and writing the manuscripts. This format follows the conditions outlined in the Guidelines Concerning Thesis Preparation, in part 2 of section B, which states the following:

"The candidate has the option, subject to the approval of their Department, of including as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.

"If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory.

"The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in literary format. It should be more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: 1) a Table of Contents, 2) a general abstract in both English and French, 3) an introduction which clearly states the rationale and objectives of the thesis, 4) a comprehensive review of the background literature to the subject of the thesis, 5) a final overall conclusion and/or summary.

"Additional material (procedural and design data as well as descriptions of equipment) must be provided where appropriate and in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

"In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. oral defense. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's best interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

Chapter 1. General Introduction

1.1. ABSTRACT

Common ragweed *(Ambrosia artemisiifolia* L.) is an annual weed that is widely distributed in Southern Québec and Ontario. It is an aggressive pioneer that can be an important weed in some crops, such as carrots. To date, only the herbicide linuron, a substituted urea, is registered in Québec and Ontario for the control of this weed in carrots. However, ragweed has shown resistance to linuron in many fields of Southwestern Québec. Herbicide resistance is the inherited ability of a species to survive after having been exposed to a dose of herbicide normally lethal to the susceptible type. Biological control is an approach that can be explored in order to control ragweed without the use of chemical herbicides. Herbivore management, phytocenotic approach, and classical and inundative strategies are all different methods of exploiting biological control. The objective of this thesis was to confirm and determine the degree of linuron resistance in a suspected linuron-resistant biotype of ragweed, and to develop a method of biocontrol of ragweed using fungal pathogens and a species of insect.

1.2. INTRODUCTION

For centuries farmers have struggled to overcome undesirable plants that thrive in the spaces where cultivated crops are grown. Although some control has been achieved, efficacious methods of eradication of weeds or other agricultural pests have never been found (Aldrich and Kremer 1997). To this day, weeds continue to plague farmland or any other environment where they are undesired. Weeds can be defined as *plants that originated under a natural environment and, in response to imposed and natural environments, evolved and continue to do so as an interfering associate with our desired plants and activities* (Aldrich and Kremer 1997). In agricultural systems, a substantial portion of expenditures goes towards weed control, by far more costly than the control of other pests such as diseases or insects. For example, in 1993 herbicides comprised approximately 85% of pesticides used on major crops in the United States, and 65% of all pesticides used in that country (Schmuck 1993). Many strategies have been used to control weeds including mechanical, cultural, chemical and biological methods. Mechanical practices include tillage, hand weeding, mowing, mulches, burning and flooding. Cultural practices include crop selection, rotation, variety selection, planting date, plant population, spacing, fertility and irrigation. Chemical control makes use of herbicides that can be broad-spectrum or selective. It is recognized that herbicide selectivity is the single key factor to widespread use of chemical herbicides in agricultural and non-agricultural systems (Ashton and Monaco 1991). Herbicides have proven to be a necessity for large-scale agricultural production, being more efficacious and cost-efficient than mechanical or cultural methods. The biological control strategy has not been widely used in agricultural systems largely because of a relatively narrow spectrum of weed control and fastidious environmental conditions required by the biocontrol agent (Watson

1991). Often however, the most effective and economical approach to controlling weeds involves a combination of several weed management methods (McWhorter and Chandler 1982).

1.3. *AMBROSIA ARTEMISIIFOLIA* L.

1.3.1. Biology and description

Ambrosia artemisiifolia L., common or short ragweed (hereafter referred to as ragweed), is one of the most ubiquitous weeds in Southern Québec and Ontario (Bassett and Crompton 1979; Alex 1997). This plant is thought to be native of Western Provinces of Canada, having quickly colonized Southern Québec and Ontario following deforestation in these regions. In Québec, ragweed has been completely eliminated east of Rivière-du-Loup since the 1940's through a widespread eradication program (Fleurbec 1978). In contrast, it was introduced into Europe in the middle of the nineteenth century and still proliferates in many regions including Russia and France (Marie-Victorin 1964; Igrc *et al.* 1995). Ragweed is an annual species of the Asteraceae (Compositae) family that reproduces exclusively by seeds. It is a pioneer plant that is well adapted to invading highly disturbed and nutrient-impoverished soils, because it is very opportunistic: it grows well in conditions difficult for other plants, and its numerous dormant seeds are among the first to germinate early in the spring (Bassett and Crompton 1979; Bachand and Christin 1996). Thus, like most annuals, it is an R-strategist, but a very aggressive and plastic one (Maryushkina 1990). It is found in neglected cultivated lands, roadsides, rights of ways, gardens, poorly maintained lawns, fence lines, waste places, and in

disturbed areas in pastures and meadows, but is generally absent from well-established plant communities (Bassett and Crompton 1979; Bachand and Christin 1996).

Ragweed stems are erect, 15 to 200 cm high, usually highly branched and hairless or hairy throughout (Britton and Brown 1970; Alex 1997). Its compound leaves are alternate on most of the plant, except for the lower leaves, which are opposite (Marie-Victorin 1964; Britton and Brown 1970). They are thin, 1-2 pinnatified, petioled, 5-10 cm long, and lobes are oblong or lanceolate, obtuse or acute, and the uppermost leaves of the branches are sometimes linear-lanceolate and entire (Britton and Brown 1970). When the plants are young, leaves are bright green to yellowish-green in colour, but become grayish-green with age (Alex 1997). Ragweed is a monoecious species bearing unisexual flower heads that contain male or female flowers, but in some cases only one type of inflorescence can be present on a plant (Alex 1997). Female heads are obovoid or subglobose, and are located at the axils of short, narrow, green bracts. They contain only one female flower that produces a hard, somewhat triangular or diamond-shaped seed with several short, sharp spines around the upper shoulder (Marie-Victorin 1964; Alex 1997). Seeds are pale brown or cream with or without darker stripes or irregular spots, are 3 to 5 mm long, and may remain viable for up to 40 years (Basset and Crompton 1979; Bachand and Christin 1996). The inconspicuous male heads develop on a raceme-like elongated cluster at the extremity on the plant, and are made up of 10 to 20 florets, each with five stamens, borne together in a cupule of bracts (Levetin and McMahon 1996). Approximately 50 to 100 cupules occur on each of the many flowering racemes, each male flower head hanging downward on a short stalk (Levetin and McMahon 1996; Alex 1997). The thousands of staminate flowers on each plant release approximately one billion pollen grains per season (Levetin and McMahon 1996).

1.3.2. Economic importance and control

Common ragweed is mostly known for being the principal cause of hayfever which affects roughly 10% of the population of Québec in the months of August and September (Vincent *et al.* 1992; Christin and Mazur 1994; Goulet *et al.* 1996; Alex 1997). This represents more than 650,000 people, and 37,000 new cases of ragweed pollen allergies are diagnosed each year (Christin and Mazur 1994). All these symptoms are due to the huge quantities of very light pollen that the plants produce yearly. Aerial concentrations of as little as 1 to 3 pollen grains/m^2 is enough to trigger the characteristic hay fever symptoms for most allergic individuals (Comtois and Gagnon 1990). Economic costs of allergies due to ragweed have been estimated to be of at least 49 million dollars in the province of Québec only (Comité de santé environnementale (CSE) et Direction de la santé publique de Montréal-Centre 1994).

Ragweed can also be a highly competitive weed in several crops because of its extensive root system that mobilizes water and nutrients and its luxurious growth habit that can choke out other plants (Marie-Victorin 1964). Indeed, the production of one pound of dry matter of ragweed requires three times more water than is needed by corn (Cox 1985). Furthermore, cows that eat this plant produce milk that taste mildly of ragweed (Fleurbec 1978).

Many approaches have been used to control ragweed both in urban areas and in agricultural systems. Bachand and Christin (1996) reported on several methods that were being assessed in urban environments including manual weeding, use of geotextiles, rubble, or wood chips to physically hinder the emergence of ragweed seedlings, mowing, pressurized hot water applications, and thermal ramps (i.e., fire treatment).

In field crops, ragweed can be controlled by many herbicides including 2,4-D, 2,4-DB, acifluorfen, bentazon, chlorimuron ethyl, chlopyralid, desmedipham, dicamba, diquat, diuron, fomesafen, glyphosate, imazapyr, imazethapyr, linuron, MCPA, metolachlor, metribuzin, naptalam, pyridate, simazine, and triclopyr (Anonymous 1999) (for chemical names refer to Appendix I). However, in vegetable crops, such as carrot *(Daucus carota* L.), onion *(Allium cepa* L.), or cabbage *(Brassica graveolens* L.), these herbicides cannot be used because of unacceptable phytotoxic effects on these crops (Anonymous 1999). In carrots, only linuron can be applied for ragweed control in post-emergence situations. Moreover, in onions and cabbage, there are no herbicides registered to control ragweed once the crops have emerged (Anonymous 1999; Phytodata Inc. personnel, *personal communication).*

1.4. LINURON

Linuron, [*N'*-(3,4-dichlorophenyl)-*N*-methoxy-*N*-methylurea], is a substituted urea herbicide used for the control of broad-leaved and grassy weeds, such as barnyard grass *[Echinochloa crusgalli* (L.) Beauv.], chickweed *(Stellaria media* L.), corn spurry *(Spergulla arvensis* L.), velvetleaf *(Abutilon theophrasti* Medic.), witchgrass *(Panicum capillare* L.), lambsquarters *(Chenopodium album* L.), common purslane *(Portulaca oleracea* L.), and wild buckwheat *(Polygonum convolvulus* L.) (Anonymous 1999). In Québec and Ontario, linuron is registered for use in corn *(Zea mays* L.), soybean *[Glycine max* (L.) Merr.], carrot, celery *(Apium graveolens* L.), potato *(Solanum tuberosum* L.), dill *(Anethum graveolens* L.), parsnip *(Pastinaca sativa* L.), asparagus *(Asparagus officinalis* L.), oat *(Avena sativa* L.), wheat *(Triticum aestivum* L.), barley *(Hordeum vulgare* L.), gladiola *(Gladiolus* x *hybridus* L.), and fruit trees (Anonymous 1999).

The mode of action of linuron is to inhibit the Hill reaction by acting on photosystem II (PSII) in the thylakoid membranes of the chloroplasts, more specifically by binding to the serine 264 of the plastoquinone Q_B niche on the D1 protein (Gronwald 1994). This prevents the attachment of Q_B, thus effectively blocking the normal electron flow and generating a strong oxidative stress that damages the PSII reaction center and photo-oxidizes lipid and chlorophyll molecules (Pallet and Dodge 1980; Barry *et al.* 1990). Linuron is easily absorbed by the roots, less so by the leaves, and is translocated through the xylem (Humburg *et al.* 1989). However, movement in the xylem is so slow that linuron is typically considered a contact herbicide (Anonymous 1999). The residual effects of linuron usually disappear within four months of application, which makes it a safe herbicide to use with respect to crops grown in the same field the following season (Anonymous 1999). Prior to the availability of linuron for use in carrots, Stoddard Solvent (Varsol®), a selective herbicide oil, was the main chemical used in carrot fields to control annual weeds. Stoddard Solvent was a very costly product (US \$12-\$14 ha^{-1}) that did not control ragweed, but provided excellent control of most other troublesome annual weeds such as lambsquarters and redroot pigweed *(Amaranthus retroflexus* L.) (Dickerson and Rahn 1963). As a result of the Solvent's failure to effectively suppress ragweed in carrots, many authors (Dickerson and Rahn 1963; Trevett and Gardner 1963; Kuratle and Rahn 1968) investigated the effects of linuron on this weed, and the excellent performance of this product in controlling ragweed led to its registration, by DuPont in the early 1970's, for pre- and post-emergent use in carrots.

For many years linuron provided excellent control of ragweed in carrots and other vegetables. Since the end of the 1980's, however, an increasing number of carrot growers

in Southwestern Québec have noted a gradual decline in the ability of linuron to control ragweed in their fields. By the beginning of the 1990's, some producers began experimenting with various linuron rates, and other herbicide combinations, in an attempt to delay the appearance of linuron-resistant ragweed. It is now widely recognized among these vegetable producers that linuron has ceased to be an acceptable chemical tool for controlling ragweed. In affected fields, the maximum allowable rate of linuron is no longer effective on established ragweed plants, nevertheless this herbicide is still being used because there are no alternative control strategies available (Phytodata Inc. personnel and carrot producers in Southwestern Québec, *personal communication*).

1.5. HERBICIDE RESISTANCE

The exact definition of the terms "resistance" and "tolerance" are still under debate, such that both terms are often used interchangeably (Moss and Rubin 1993). However, many workers have suggested that the term "herbicide tolerance" be reserved for a plant species' inherent insensibility to a chemical, implying *no genetic modification or selection*, whereas "herbicide resistance" should refer to a species' *inherited* ability to survive and reproduce following exposure to a herbicide dose that is normally lethal to the wild, susceptible type (Moss and Rubin 1993; Anonymous 1998). This resistance may have naturally occurred in the plant species or have been artificially induced by such methods as genetic engineering, or selection of variants produced by tissue culture or mutagenesis techniques (Anonymous 1998).

As early as the 1950's, soon after herbicides had been widely introduced for commercial use, many workers were predicting the eventual appearance of herbicide-resistant weeds (e.g., Abel 1954; Harper 1956). Hence, herbicide rotations were

recommended, but unfortunately they were not systematically applied (Gressel and Segel 1978). Although the first resistance cases concerning other pesticides such as insecticides and rodenticides appeared relatively quickly after introduction of these products in cropping systems, the first case of herbicide resistance was only reported in 1968 (Ryan 1970) and involved common groundsel *(Senecio vulgaris* L.) resistance to the triazine herbicides, simazine, and atrazine. Since then however, the cases of herbicide resistance has been steadily increasing, both in terms of the number of resistant species and in the number of herbicide families to which resistance has evolved (Moss and Rubin 1993; Heap 1999a). Most herbicide resistance cases have been reported for the triazine family (Heap 1999a). Weed species that have most frequently exhibited some form of herbicide resistance are *A. retroflexus, Amaranthus hybridus* L., *Kochia scoparia* L., *C. album, S. vulgaris, Solanum nigrum* L., and *Poa annua* L. (Moss and Rubin 1993).

By 1998, as many as 216 herbicide-resistant weed biotypes (145 weed species) had been recorded in 45 countries by the International Survey of Herbicide-Resistant Weeds (Heap 1999b). The majority of resistant biotypes were reported in the US (71%), and the remainder largely in industrialized countries that rely heavily on chemical herbicides as their main weed control strategy (Heap 1999a). Although the first reports of herbicide resistance involved mostly the triazines, the widespread use of ALS (acetolactate synthase) and ACCase (acetyl-coenzyme A carboxylase) inhibitors over the past 15 years has led to a greater prevalence of resistance to these herbicide groups (Heap 1999a).

Several mechanisms may be responsible for providing resistance to a chemical in a plant. Genetically inherited modifications of the site of action of the herbicide are the principal means by which weeds achieve resistance to triazines. Specifically, resistance is

due to the alteration of a single amino acid on the D1 polypeptide in the PSII complex, where the serine at position 264 on the protein is replaced by a glycine (Moss and Rubin 1993; Gronwald 1994). The D1 protein is encoded by the chloroplast gene pbsA (Trebst 1991), and the substitution of a glycine for serine at the 264 position is the result of a single point-mutation on that gene (Gronwald 1994). Because of this alteration, plants resistant to triazines can withstand herbicide doses much greater than those normally effective, i.e. sometimes greater than 100x (Moss and Rubin 1993). A second method of developing resistance to a herbicide is to rapidly metabolize and transform the product into relatively non-toxic compounds via a number of biochemical pathways including oxidation, reduction, hydrolysis, isomerization, or conjugation (Coupland 1991; Moss and Rubin 1993). Finally, sequestering and compartmentalizing the chemical into various storage areas of the cells, such as vacuoles, or into tissues that are located at some distance from the target site of the herbicide, has also been suggested to play a least a partial role in the development of herbicide resistance (Coupland 1991).

1.6. BIOLOGICAL CONTROL

1.6.1. General

Biological control is *the deliberate use of natural enemies to suppress the growth or reduce the population of a weed species* (Watson 1993). Organisms that have been used as biotic agents include insects, mites, nematodes, plant pathogens and aquatic and terrestrial herbivores (Watson 1993). Insects have been particularly studied and used as biotic agents (Aldrich and Kremer 1997). There are generally three approaches associated with biological control: herbivore management and classical and inundative strategies.

Herbivore management involves grazing animals such as geese used to remove grass weeds in strawberries and cotton, or the white amur *(Ctenopharyngodon idella* Cuvier & Valenciennes) for consuming weeds in aquatic systems (Watson 1993; Aldrich and Kremer 1997). Differential palatability and increased management requirements limit the effectiveness of grazing animals and, although very effective in some cases, this strategy is not applicable to most weed infestation cases (Watson 1993). In the case of the biological control of ragweed, herbivore management has not been an option. However, the classical approach has been used, the inundative approach is being considered, and a third approach, not usually mentioned in current literature, has also been investigated: the phytocenotic approach. These three biological control approaches will be discussed in further details.

1.6.2. Phytocenotic approach

The phytocenotic approach is based on the principle of interspecific plant competition, the essence of which is that *individuals of one species suffer a reduction in fecundity, survivorship or growth as a result of resource exploitation or interference by individuals of another species* (Begon *et al.* 1990). Competition can occur for resources such as water, nutrients, space, and light (Begon *et al.* 1990). The optimization of plant competition is a strategy that has been successfully used for weed control in agricultural systems for a long time. Indeed, vigorous and/or tall-growing crops, such as buckwheat *(Fagopyrum tataricum* Gaerth.), alfalfa *(Medicago sativa* L.), barley *(Hordeum vulgare* L.), sorghum *(Sorghum bicolor* L.), rye *(Secale cereale* L.), and soybean, may effectively compete with weeds (Muzik 1970; Ross and Lembi 1985). Crops that can be maintained

in dense stands and smother the weeds are referred to as smother crops (Ross and Lembi 1985). In agricultural situations, crop competition for light (i.e., shading) and/or space (i.e., smothering) is most often exploited to suppress weeds. Crops can gain a competitive advantage over weeds by possessing a number of physical and physiological features including early emergence from the soil, height advantage over weeds, and a more rapid growth rate (Ross and Lembi 1985).

This approach can also be used to control weeds in non-crop situations such as rights-of-ways. DiTommaso *et al.* (2000) employed such an approach to suppress ragweed growing on roadsides and vacant lots. In northern regions of North America, roadside soils have characteristically high salinity levels because of yearly applications of deicing salt. Common ragweed is particularly suited to tolerate these highly saline conditions and often invades and dominates these unfavourable habitats. In a recent study, DiTommaso *et al.* (2000) investigated the germination capacity of several plant species under variable saline conditions in an effort to select effective competitors for ragweed in roadside areas. Although a number of species showed good potential for use along roadsides, common ragweed showed consistently the greatest germination at the higher salinity concentrations tested (i.e. 200-400mM) compared with the other species evaluated.

1.6.3. Classical approach

The classical or inoculative approach involves the control of weeds that are not native to the habitat which they invade (i.e., that have been imported intentionally or unintentionally from one region to another). Over 70% and 50% of major weed species in

Canada and the United States, respectively, have been introduced (Watson 1991). Furthermore, 13 out of the 15 most noxious weeds in North America are non-native. These troublesome weeds have few if any natural enemies in their new habitat, thus allowing them to proliferate. Biotic agents that are used to control these non-native weeds must therefore be imported from their region of origin. These potential agents must be host-specific, so that their import will not be deleterious to the native flora or to economically important crops (Klingman and Coulson 1982; Ashton and Monaco 1991). The introduced agents should also be free of parasites, predators, and diseases, and should adapt quickly to their new environment so as to establish high population densities fast enough to suppress the weeds (Ashton and Monaco 1991). The classical approach is particularly effective when the target weed is an aggressive, widespread, perennial, introduced weed that spreads over large areas, such as rangelands or aquatic habitats (Watson 1993).

Many success stories are associated with the classical approach to biological control. Perhaps the most famous case of large-scale biological control is that of the prickly-pear cactus *(Opuntia* sp.) in Australia. Introduced as ornamentals in the 1800's from their native North and South Americas, these cacti had, by 1925, infested 24 million hectares of prime grazing lands. Twelve million hectares were heavily infested and rendered useless, and infestations were increasing at the rate of 400,000 hectares per year. After careful screening of some 150 *Opuntia*-feeding insects, 12 were released in Australia. The cactus moth, *Cactoblastis cactorum* Berg. from Argentina, was especially effective by tunneling through the stems, underground bulbs, and roots. The moth was released in Australia and, six years later had controlled *Opuntia* sp. over large areas of grassland. By 1935, 95% and 75% of infested areas in Queensland and New South Wales,

respectively, were free of *Opuntia* sp. (Ashton and Monaco 1991; Aldrich and Kremer 1997). Another example of a successful classical biocontrol program is St. John'swort, or klamath weed *(Hypericum perforatum* L.), in the United States. This plant was introduced from Europe and, by the 1930's, had become a serious problem on rangelands in Western U.S. and Canada. Satisfactory control of St. John'swort was achieved by the beetle *Chrysolina quadrigemina* Rossi three years after its release (Aldrich and Kremer 1997).

The use of fungal pathogens in the classical approach, although not as common as in the inundative strategy, has also been successful. The rust fungus *Puccinia chondrillina* Bubak & Syd. is currently being used in Australia and in the Western United States to control the very problematic skeleton weed *(Chondrilla juncea* L.) (Aldrich and Kremer 1997).

The use of the Crysomelid beetle *Zygogramma suturalis* F. for the control of ragweed within the classical approach has been successful in countries such as the former USSR and Croatia, where ragweed had been accidentally introduced from North America in cereal shipments. *Z. suturalis* was collected in the United States, mass reared, and released in these European countries where ragweed had become an important pest. The insect was able to establish a healthy population in many regions of the affected countries, and acceptable ragweed control has been obtained (Kovalev 1989; Reznik 1991; Igrc *et al.* 1995).

1.6.4. Inundative approach

The inundative approach involves the control of native weeds by naturally occurring enemies. Despite the existence of natural enemies however, weeds can

nonetheless attain high population densities in some regions for several reasons including low phytophageous insect populations, low pathogen inoculum levels, weakly virulent pathogens, poor dispersal mechanisms of the pathogen, unfavourable environmental conditions for the development of disease or insects (Watson 1993). Fortunately, these natural enemies can be identified, collected, mass reared and released in high numbers in areas where weed infestations are high and natural enemy populations are low or nonexistent (Watson 1993). Until recently, insects have been the primary biotic agents employed in the inundative strategy, but more recently weed pathogens have also been used. This "bioherbicide", or "mycoherbicide" approach in the case of fungal pathogens, is analogous to the chemical herbicide approach in that high concentrations of inoculum (i.e., usually fungal spores) are typically used over affected areas (Watson 1993). The inundative approach consists of three major phases namely discovery, development and deployment (Templeton 1982). The discovery phase includes the collection of diseased plants, the isolation of the causal organism, demonstration of Koch's postulates, identification of the pathogen, its culture on artificial media, and the maintenance of pathogen cultures in short- and long-term storage. The development phase involves determination of: (1) optimal conditions for spore production, (2) disease development and host damage, (3) infection process, (4) mode of action of the pathogen, (5) host range, and (6) efficacy of the pathogen to control the target weed. The deployment phase involves the industrial sector with which researchers will collaborate for the production and possible commercialization of the bioherbicide. This step deals with aspects such as formulation, fermentation, regulation, marketing, and implementation (Watson 1993).

To date, three mycoherbicides have been developed for use in cultivated crops in North America. COLLEGO® was registered in 1982 in the US; it is a dry powder

formulation of an anthracnose disease-causing fungus *[Colletotricum gloeosporioides* (Penz.) Penz. & Sacc. f.sp. *aeschynomene]* used to control northern jointvetch *(Aeschynomene virginica* L.) in rice *(Oryza sativa* L.) and soybeans (Bowers 1986). DeVine® was registered in 1981 in the US; it is a liquid formulation of *Phytophthora palmivora* (Butler) Butl. MWV Pathotype that is used to control strangler vine *(Morrenia odorata* (H.&A.) Lindl.) in Florida citrus groves (Ridings 1986). BioMal® was registered in 1992 in Canada, and is a dry formulation of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. f.sp. *malvae* for the control of round-leafed mallow *(Malva pusilla* Sm.) in flax *(Linum usitatissimum* L.) and lentils *(Lens esculenta* Moench) (Makowski and Mortensen 1992). Due to production problems, BioMal® is not currently available.

The prospect of using fungal pathogens for the control of ragweed in vegetable crops within the inundative approach will be examined in Chapters 3 and 4.

1.7. DESCRIPTION OF ORGANISMS INVOLVED IN THE BIOCONTROL PROJECT FOR *A. ARTEMISIIFOLIA*

1.7.1. Fungal pathogens

1.7.1.1. General

Fungi are eukaryotic organisms whose cells are almost always surrounded by a rigid cell wall made of cellulose and chitin, in proportions that vary with each fungal species. Fungal bodies can be of three types: a *plasmodium,* which consists of a single, multinucleate mass of cytoplasm not surrounded by a rigid cell wall, *single cells,* with or without a wall, and a *hypha,* which consists of a series of cells, surrounded by a cell wall,

in the form of a filament. Most species of fungi are found in the form of hyphae, which may be either septate (uninucleate, binucleate, or multinucleate), or not (Moore-Landecker 1996; Agrios 1997).

The classification of fungi is currently under debate, because of the large morphological and reproductive diversity of this group. The "True Fungi" belong to the Kingdom Fungi, which is separated into four phyla: Chytridiomycota (producing zoospores), Zygomycota (producing sporangia), Ascomycota (the sac fungi), and Basidiomycota (the club and mushroom fungi). Some authors also add a fifth phylum, the Deuteromycota (Imperfect Fungi). Some fungi previously considered as "lower" fungi are now placed in the Kingdom Protozoa, a heterogeneous group comprising of microorganisms that may be unicellular, plasmodial, colonial, very simple multicells, or phagotrophic (Agrios 1997). Other "lower" fungi are placed in the relatively new Kingdom Protista, phylum Oomycota, such as the late blight of potato *(Phytophthora infestans* Montagne) (Cavalier-Smith 1997).

Fungi may be saprophytic, heterotrophic, or parasitic, or may use a combination of these modes of nutrition throughout their life cycles. They secrete digestive enzymes into their environment in order to break down complex molecules into simple components that can be absorbed by the hyphae (Moore-Landecker 1996; Agrios 1997). Fungi play an important role as recyclers in ecosystems. Fungi are also a source of food and by-products such as antibiotics, organic acids, and ethanol, and serve as tiny bio-reactants to produce foods and beverages such as cheese, soy sauce, beer, and wine (Levetin and McMahon 1996; Moore-Landecker 1996).

Unfortunately, many animal and plant diseases are caused by parasitic fungi, such as skin and yeast diseases for animals and late blight of potato and corn smut *(Ustilago*

maydis Pers.) (Moore-Landecker 1996; Agrios 1997). But it is this very pathogenic attribute of fungi that provides us with a means to attack and control some insect, plant, and even fungal pests that interfere with our daily lives.

1.7.1.2. Phoma species

Phoma sp. was isolated from several diseased ragweed plants in the fall of 1993, in an urban area on the island of Montréal, Québec (Brière *et al.* 1995). This fungus is morphologically similar to *Phyllostica ambrosinae* Pers., a pathogen of giant ragweed *(Ambrosia trifida* L.). Identification to the species is currently being carried out, but this *Phoma* is most probably a previously undescribed species. The genus or form-genera *Phoma* belongs to the Kingdom Fungi, Form-Phyllum Deuteromycotina (Fungi Imperfecti), and Form-Order Sphaeropsidales. The reason Deuteromycotina is considered as a *form*-subdivision, and that all subsequent taxa are *form*-taxa, is that the Deuteromycotina is an artificial assemblage of fungi. Deuteromycetes are a group of about 15,000 species that have been combined because they lack, or appear to lack, a sexual stage. This feature makes it impossible to place these fungi within the other three sub-divisions of the Fungi, because the sub-divisions are principally based on the mode of sexual reproduction. However, it is generally believed that the majority of the Deuteromycetes are the non-sexual stages, or anamorphs, of sexually reproducing fungi that belong to the Ascomycetes or Basidiomycetes (Moore-Landecker 1996; Agrios 1997). There are reports of perfect stages of *Phoma* in *Pleospora, Leptosphaeria,* and *Mycosphaerella* (Stevens 1981).

The name *Phoma* is probably a corruption of the Greek *phyma,* which means wart or pustule. Indeed, fungi belonging to the genus *Phoma* develop dark brown pycnidia. In

cultures, these are slowly formed, mostly scattered and close to the surface of the mycelium, more or less flask-shaped, sometimes irregular, with short necks, fairly thin-walled and easily crushed. If the fungus is growing on a host, the pycnidia are immersed in the host tissue, and are erumpent or have short beaks piercing the epidermis. The pycnidia produce masses of small, hyaline, ovate to elongate, one-celled spores, called conidia (an asexual spore produced at the ends of mycelia and not enclosed within a specialized cover) (Sussman and Halvorson 1966; Barnett and Hunter 1972; Smith 1981). In culture, most *Phoma* species produce a fair amount of floccose aerial mycelium, white at first and then darkening. In most published keys, three related genera are recognized: *Phoma, Macrophoma* and *Phyllostica,* differentiated mainly by their spore size or location of infection on hosts. However, these distinctions are purely artificial, in the case of *Phoma* and *Macrophoma,* and likely invalid in the case of *Phoma* and *Phyllostica.* The genus *Phoma* and the related genera include a large number of species (i.e., 1700 *Phoma* spp. and 1500 *Phyllostica* spp.) (Smith 1981).

Phoma species are found throughout the world and may be either saprophytic or parasitic. Many agricultural crops are affected by diseases caused by one or more species of *Phoma.* These include: gummy stem blight of curcubits (Keinath *et al.* 1995), gangrene of potatoes (Hide *et al.* 1995), blackleg of canola *(Brassica napus* L.) (Lamey 1995), linseed *(Linum usitatissimum* L.) disease in Ireland (Mercer and Ruddock 1994), early decline of asparagus (Block and Bollen 1994), disease of alfalfa (Soby *et al.* 1996), disease of peas *(Pisum sativum* L.) and green beans *(Phaseolus* sp.) (Biddle and Whaley 1996), root rot of vining pea *(Pisum* sp.) (Persson *et al.* 1997), pink root of onions (Coleman *et al.* 1997), stem and pod rot of lupine *(Lupinus* sp.) (Reddy *et al.* 1996), and black stem of sunflower *(Helianthus annuus* L.) (Carson 1991). *Phoma* species also affect

many horticultural crops, such as gentian *(Gentiana sino-ornata* L.) (Punithalingam and Harling 1993), periwinkle *(Vinca minor* L.) (Koelsch *et al.* 1995) and clematis *(Clematis* spp.) (Smith *et al.* 1994), and fruit crops, such as litchi *(Litchi chinensis* Sonn.) (McMillan 1995) and lemon *(Citrus limon L.)* (Deng *et al.* 1995). Finally, some *Phoma* species have also been found to damage processed food products such as Cheddar cheese (i.e., thread mold) (Hocking and Faedo 1992) and partially processed lettuce *(Lactua sativa* L.) (i.e., rot) (Magnuson *et al.* 1990). However, other *Phoma* species exhibit features that may be beneficial. For example, a *Phoma* sp. has been evaluated for its ability to suppress take-all and common root rot of spring wheat (Shivanna *et al.* 1996). Also, *P. etheridgei* has been investigated to serve as a bioprotectant against aspen *(Populus* spp. L.) decay (Hutchison *et al.* 1994). An interesting yellow pigment has been derived from *P. lingam* and *P. wasabiae* (Pedras *et al.* 1995), and helpful manganese and iron oxidative properties have been discovered in another *Phoma* species growing on building stones in Spain (Torre and Gomez-Alarcon 1994). Several *Phoma* species have been studied as potential mycoherbicides against several troublesome weeds including *P. proboscis* for field bindweed *(Convolvulus arvensis* L.) (Heiny 1994), *P. sorghina* for pokeweed *(Phytolacca americana* L.) (Venkatasubbaiah *et al.* 1992) and purple loosestrife *(Lythrum salicaria* L.) (Nyvall and Hu 1997), and *P. aquilena* for bracken fern *(Pteridium* sp. Gleditsch.) in New Zealand (McElwee *et al.* 1990).

1.7.2. *Ophraella communa* LeSage

O. communa LeSage (Coleoptera, Chrysomelidae, previously referred to as *Galerucella notulata* or *Ophraella notulata*), is native to Québec and occurs throughout

most of the continental United States and parts of Canada (Horn 1893) feeding chiefly, and in eastern North America exclusively, on *Ambrosia* species (Wilcox 1965; Wood 1973; Futuyma *et al.* 1993). Adults overwinter in soil debris and are observed along with eggs on ragweed seedlings in mid-May in Québec. Recently mated females typically cement their fertilized eggs one by one on *Ambrosia* foliage. Newly laid eggs are yellow and become yellow-orange within a few hours; these small eggs (mean length of 0.69 mm) are pyriform when viewed laterally, and circular when viewed dorsally (Welch 1978). Once the larvae hatch, they soon begin skeletonizing the upper or lower epidermal and mesophyll cells. At the end of the 3rd instar, larvae spin loosely woven cocoons of a clear viscous maxillary secretion that hardens and darkens soon after it is extruded. The resulting adults consume all of the leaf. Welch (1978) reports that all stages of *O. communa* occur on common ragweed and total development time for both males and females from egg laying to adult emergence averages 21.8 days. Welch (1978) also reports an observation made in 1972 in New Haven, CT, USA, where two generations of *O. communa* fed, from July 16 to October 30, on 300 ragweed plants. All but 3 plants died as a result of severe skeletonization and defoliation, and only 3 seeds could be found on live plants. Previous research by Teshler *et al.* (1996) evaluated *O. communa* on ragweed under controlled environmental conditions. These workers found that *O. communa* is able to double its population size in 5 to 7 days with an average generation time of 30 to 35 days. *O. communa* is being evaluated as a potential biological control agent for common ragweed in the United States (Futuyma and Floyd 1997) and in Australia (Palmer and Goeden 1991).

1.8. STATUS OF THE RESEARCH (WORK THAT HAS PREVIOUSLY BEEN DONE WITH PHOMA SP. AND O. COMMUNA)

Preliminary research on the biological control of common ragweed using the inundative approach has been carried out (Brière *et al.* 1995). In this research, both a fungal agent, *Phoma* sp., and an insect, *O. communa*, were used. *Phoma* sp. inoculum formulation and pathogenicity were investigated. Plants infected with *Phoma* sp. developed small, dark, necrotic lesions on leaves at first, followed by pycnidia development on the senescing leaves. Frequently, systemic infections in leaf petioles and stems were observed, which often led to their dieback (Brière *et al.* 1995). Due to systemic infection, this fungus caused substantial seedling mortality and reduction in pollen production. However, optimal growth conditions and inoculum formulation for this *Phoma* sp. have not been thoroughly investigated in the laboratory in previous work. Therefore, Fortin (1996) conducted further research on optimizing liquid formulation in *Phoma* sp.. The highest level of disease in common ragweed was obtained for plants sprayed at the 4-leaf stage with a formulation containing 10^8 spores/ml and a 10% concentration of canola and soybean oil mixture. However, long dew periods were required (i.e., 36 hr.) to obtain severe disease symptoms. This requirement for a lengthy dew period represents a significant constraint for the potential use of *Phoma* sp. as a mycoherbicide. To date, no host specificity testing using this fungus has been carried out.

Teshler *et al.* (1996) established the life table for *O. communa* under laboratory conditions and also completed preliminary feeding trials. These workers showed that *O. communa* predisposed ragweed to attack by *Phoma* sp. Indeed, *Phoma* caused systemic infection and the death of inflorescence when applied alone, but rarely killed the whole

plant. Addition of *O. communa* produced a synergistic effect resulting in a high level of plant mortality. Host range studies for *O. communa* have been carried out by Palmer and Goeden (1991) and although sunflower sustained some feeding in no-choice laboratory feeding tests, there is no evidence of *O. communa* ever attacking sunflower in the field. Thus, the result of the laboratory test is most likely an artifact of the no-choice test and does not reflect actual field situations. Current research on *O. communa* focuses on elucidating mechanisms of the diapause cycle, and on developing an artificial diet to facilitate mass rearing (M.P. Teshler, *personal communication).*

1.9. THESIS OBJECTIVES

The general objectives of this research were: (1) to confirm and determine the degree of linuron resistance in field-collected common ragweed plants, and (2) to further investigate the possibility of using the fungal pathogen, *Phoma* sp. to effectively suppress common ragweed alone or in combination with the Chrysomelid beetle, *O. communa.*

The specific objectives of this research were to:

1) Evaluate suspected linuron-resistant common ragweed biotypes against susceptible ragweed biotypes in order to confirm linuron resistance;
2) Assess linuron-resistant common ragweed biotypes for cross-resistance to atrazine;
3) Determine the efficacy of *Phoma* sp. (or other new candidate fungi) to suppress common ragweed;
4) Search for other selective fungal pathogens which may decrease ragweed growth and reproduction;

5) Determine the optimum conditions for mass production of fungal inocula by using different growth media as well as light and temperature conditions;
6) Determine the host range of fungal pathogens showing good potential to suppress common ragweed;
7) Assess the interaction between the different fungal pathogens and *O. communa;*
8) Assess the interaction between the different fungal pathogens and linuron.

This research focuses primarily on the potential of *Phoma* sp. or other possible candidate fungi, to control common ragweed alone or in combination with *O. communa.*

Connecting Text

The information contained in the previous chapter makes a brief review of the wealth of knowledge concerning the weed *Ambrosia artemisiifolia* L., the herbicide linuron, the phenomena of herbicide resistance, biological control of undesirable vegetation, and the fungal and insect organisms that have been proposed for the biological control of common ragweed. The following chapters will present detailed experiments designed to shed more light on common ragweed's particular case of linuron resistance, and on organisms that could eventually serve to control this weed.

Chapter 2. Resistance of Common Ragweed *(Ambrosia artemisiifolia)* to the Herbicide Linuron in Carrot Fields of Southwestern Québec.

2.1. ABSTRACT

Linuron is extensively used in carrot *(Daucus carota* L.) production areas of North America for the control of annual broadleaf weeds, including common ragweed *(Ambrosia artemisiifolia* L.). In Québec, the recommended rates for post-emergence application of linuron in carrots are 1.125 to 2.25 kg ai ha^{-1}. Since the late 1980's, some vegetable producers in Southwestern Québec have observed a gradual decrease in the ability of linuron to control ragweed in their fields. Hence, the goals of this research were to determine the degree and extent of linuron-resistance in ragweed populations within carrot fields of Southwestern Québec. Two consecutive experiments were conducted with suspected linuron-resistant ragweed from a carrot field in Sherrington, Québec. In both experiments, these plants were subjected to increasing rates of linuron under greenhouse conditions. For the first experiment, resistance of ragweed biotypes to linuron was confirmed with a small percentage (3%) of plants surviving to the reproductive phase after being subjected to as much as 10X the maximum recommended linuron rate (22.5 kg ai ha^{-1}). Susceptible plants (controls) collected from fields with no prior history of linuron use were all killed when sprayed with the lowest recommended rate (1.125 kg ai ha^{-1}). The second experiment showed a ragweed herbicide-resistance ratio for linuron (I_{50}) of 9.09. Field observations in the same Sherrington field indicated that the application of reduced rates of linuron when ragweed seedlings are smaller than 5 cm in height is not an option for the control of linuron-resistant common ragweed.

2.2. INTRODUCTION

Ambrosia artemisiifolia, or common ragweed (Asteraceae), is one of the most common summer annual weeds in southern Québec and Ontario (Canada), and in the Central and Northeastern US (Bassett and Crompton 1979). Ragweed is a highly successful pioneer species that is generally absent from well-established plant communities. It is most often found in frequently disturbed habitats such as roadsides, waste places, fence lines, rights-of-ways, and agricultural fields (Marie-Victorin 1964; Bassett and Crompton 1979; Maryushkina 1990; Bachand and Christin 1996).

In Southwestern Québec, ragweed is a major weed in vegetable crops such as carrot, onion, and cabbage; weed surveys by regional agronomists in 1997, estimated the proportion of carrot and cabbage fields heavily infested by ragweed to be at 25% and 50%, respectively (Phytodata Inc. personnel and Southwestern carrot growers, *personal communication*). Ragweed can be controlled successfully by many herbicides including 2,4-D, atrazine, bentazon, dicamba, diuron, linuron, MCPA (see Appendix I for chemical names). However, relatively few herbicides can be safely employed within carrot, onion, or cabbage cropping systems (Anonymous 1999). Those herbicides that are registered for use in these crops have important limitations including a narrow spectrum of crop safety. Moreover, in onions and cabbage, there are no herbicides currently registered for the control of ragweed once the crop has emerged (Anonymous 1999).

Linuron, a substituted urea herbicide, is registered in Québec and Ontario for use in carrots as either a pre-emergence (0.55-1.625 kg ai ha^{-1}) or post-emergence treatment (0.912-2.25 kg ai ha^{-1}), alone or in combination with pre-emergence herbicides such as trifluralin and prometryne (see Appendix I for chemical names) (Ahrens *et al.* 1994; Anonymous 1999). However, linuron is the only post-emergence herbicide registered in

Québec for the control of ragweed in carrots (Anonymous 1999). Hence, linuron has been used extensively within carrot production systems since the 1970's (Dickerson and Rahn 1963; Trevett and Gardner 1963; Kuratle and Rahn 1968).

Since the late 1980's, carrot growers in Southwestern Québec have noted a gradual decline in the ability of linuron to control ragweed in their fields. Presently, Québec carrot producers find that the use of pre- and post-emergence registered rates of linuron do not provide economically acceptable levels of ragweed control, especially since ragweed seeds germinate throughout much of the growing season. Current estimates peg the financial losses from ragweed infestations in this region at as much as $1000 and $500 CDN per hectare for carrot and cabbage production, respectively (Phytodata Inc. personnel, *personal communication)*. Given the approximately 3,500 and 1,500 ha that are currently grown to carrots and cabbage, respectively, in this region of Québec, the total financial loss due to ragweed infestations alone is estimated to be $1 million CDN annually (Phytodata Inc. personnel, *personal communication*). These losses include (1) additional costs for mechanical and manual hoeing, (2) reduced carrot stand density as a result of crop seedlings being mistakenly removed during hand-weeding and manual hoeing operations, (3) lower productivity due to direct competition by weeds for available resources, and (4) losses due to the interference of remaining ragweed plants with mechanical crop harvesting operations. Despite the substantial financial losses, linuron is currently still being used in Québec carrot production systems largely because there are few other acceptable ragweed control strategies available in this crop.

To our knowledge, resistance of common ragweed to linuron in North America has not been reported elsewhere. Thus, the specific objective of this research was to determine the degree of linuron resistance in field-collected common ragweed.

2.3. MATERIAL AND METHODS

2.3.1. First greenhouse experiment

2.3.1.1. Collection of plants

In the spring and summer of 1999, approximately 150 suspected linuron-resistant common ragweed seedlings ('R-group') were collected from a 10.8 hectare field in Sherrington, Québec, Canada, where severe ragweed control problems have been reported. The field is a deep, well-drained and decomposed muck soil (over 1.5m in depth) sitting on a medium loam mineral soil (McKibbin and Stobbe 1936). The typical 4-yr. crop rotation cycle on this field has been corn, onion, and two consecutive years of carrot. Although linuron was applied on this field only when carrots were being grown, the efficacy of linuron in suppressing ragweed has progressively decreased over the past 10 years (Phytodata Inc. agronomists, *personal communication).* Ragweed seedlings were collected at the same time from a field at the Emile A. Lods Agronomy Research Centre, Macdonald Campus of McGill University, Ste-Anne-de-Bellevue, Québec, Canada (approximately 45 km NW of the Sherrington site). This field site had no prior history of linuron use such that the ragweed population present was assumed to be susceptible ('S-group') to linuron. Ragweed plants from both field sites were collected at the two- to four-leaf stage, and transplanted into a potting medium composed of 2/3 commercial potting mix[1] and 1/3 black potting soil. Plants were arranged in groups of three in 13 x 15 cm Styrofoam flats, such that each flat represented one experimental unit. Flats were placed in the Macdonald Campus greenhouse and subjected to a 16-h natural photoperiod,

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[1] Pro-mix BX®, Premier Brands, Inc., New York, NY, USA

33°C/18°C average day/night temperatures, and watered as required. No fertilizer was added throughout the 31 day experimental period. Thirteen days after transplanting and just before treatment application, plants had attained a height of 5-10 cm or the 6- to 12-leaf stage.

2.3.1.2. Herbicide application

Susceptible and resistant ragweed plants were subjected to one of six linuron[2] concentrations: 0X, 0.5X, 1X, 2X, 3X, 4X, and 10X, where X represents the maximum post-emergence recommended rate of 2.25 kg ai ha^{-1} in carrots (Anonymous 1999). Linuron was sprayed without any adjuvants using a spray chamber equipped with an XR-Teejet 8002VS® flat-fan nozzle[3] at a pressure of 200 to 250 kPa, providing a final volume of 260 L ha^{-1}. Control plants (0X) were sprayed with distilled water only. Immediately after spraying, plants were returned to the greenhouse and grown under the same environmental conditions described above.

2.3.1.3. Experimental design

The experiment was set up in a randomized complete block design with six replications. Blocks here represent repetitions of trials in time with the first spray application occurring in May 1999, and the second application taking place in July 1999.

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[2] Lorox 50DF®, E.I. Dupont De Nemours and Company, Wilmington, DE 19898.

[3] Teejet Spray Nozzles. Spraying Systems Co., P.O. Box 7900, Wheaton, IL, 60189.

2.3.1.4. Harvest procedures

In both trials, plants were harvested 31 days after spraying, and mortality and number of staminate inflorescences produced by each live plant were recorded. Plant height was measured from the soil line to the shoot apex before excision. Biomass determination was made by placing living above-ground and below-ground tissues in paper bags, oven-drying at 55°C for three days, and weighing them.

2.3.1.5. Statistical analyses

All data in this thesis were analyzed using the Statistical Analysis Systems® package (SAS 1996) the SigmaPlot® Scientific Graphing software package (SigmaPlot 1998). Mortality and number of male inflorescences were ln-transformed and height data squared in order to obtain homogeneous variances. Mortality data were subjected to an ANOVA, whereas height, above-ground biomass, and number of male inflorescences were subjected to an ANCOVA, where the 0X rate served as the co-variable. Below-ground biomass data were not subjected to an ANOVA because homogeneity of variances could not be achieved. If results for both trials were not significantly different, data were pooled into a CRD with 12 replicates. Variable responses to increasing rates of linuron were further analyzed by regression.

2.3.2. Second greenhouse experiment

Because linuron rates used for the previous experiments were too high to establish a dose-response curve for the S-biotype, a second experiment was conducted using lower linuron rates.

2.3.2.1. Collection of plants

In February 2000, 'R-group' seeds were collected from the same Sherrington field as for the previous experiment. Because the field was still covered with snow, pieces of frozen soil were removed from the field, placed in flats, and left at 4°C for 3 days for slow thawing. The flats were subsequently placed in the greenhouse for germination. 'S-group' seeds were also collected from the same field at the Lods Research Centre. These seeds were collected from dead ragweed plants that were emerging from the snow cover. In the greenhouse, the S-group seeds were immersed in water for approximately one hour before being sown in flats containing the same soil medium as described in the previous experiment. When the seedlings from both biotypes emerged, they were transplanted in groups of three in 13 x 15 cm Styrofoam flats, such that each flat represented one experimental unit. Flats were placed in the Macdonald Campus greenhouse and subjected to a 14-h photoperiod, 21°C/18°C average day/night temperatures, and watered as required. Fertilizer (10-30-10) was added twice during the first week of seedling growth, after which 20-20-20 fertilizer was used once a week until treatment. Fourteen days after transplanting and just before treatment application, plants had attained a height of 5-8 cm or the 6- to 10-leaf stage.

2.3.2.2. Herbicide application

S-group plants were subjected to one of the following linuron concentrations: 0X, 1/32X, 1/16X, 1/8X, 1/4X and 1/2X , whereas R-group plants were subjected 0X, 1/2X, 1X, 2X, and 4X linuron rates. Herbicide application equipment and conditions were as described in the previous experiment (section 2.3.1.2).

2.3.2.3. Experimental design

The experiment was set up in a randomized complete block design with five replications. Blocks here represent repetitions of trials (trial 1 and 2) in time and space, with a one-week interval between sprays, and trials placed on separate greenhouse benches.

2.3.2.4. Data collection procedure

Mortality of the ragweed plants was recorded 15 days after applying the herbicide. No other data was collected for this experiment. Resistance ratios (I_{50}) were determined by dividing the I_{50} of the R-biotype on the I_{50} of the S-biotype.

2.3.2.5. Statistical analyses

Mortality data required no transformation in order to obtain homogeneous variances; the data was subjected to an ANOVA. If the results for both trials were not significantly different, the data was pooled into a CRD with 10 replicates. Variable responses to increasing rates of linuron were further analyzed by regression.

2.3.3. Field observations

In order to observe the effects of reduced rates of linuron have on the dynamics of the ragweed population in field conditions, twenty $0.5m^2$ quadrats were randomly placed, in Spring of 1999, in the Sherrington field site where 'R-group' plants for greenhouse trials were collected. Within each quadrat, the number of ragweed plants was recorded on each of 5 dates (May 24, June 3, 7 11, and July 29), a period during which ragweed seedlings were continuously emerging. In the summer of 1999, this field was treated in

the following manner: 336 g ai ha^{-1} of linuron were applied on May 22, 27, 31, and June 4, and 1.2 kg ai ha^{-1} of linuron were applied on the 1st of July. Prior to these, a pre-emergence application of the non-selective herbicide paraquat[1] was done on May 17 at a rate of 500 g ai ha^{-1}. One application of acifluorfen was done on July 16 at a rate of 600 g ai ha^{-1}.

2.4. RESULTS AND DISCUSSION

2.4.1. Greenhouse experiments

The impact of linuron on susceptible (S) and resistant (R) ragweed biotypes for the first greenhouse experiment is presented in Figures 1 to 5. Mortality increased rapidly with increasing linuron rates, and all other plant variables measured responded in a similar manner to linuron applications (i.e. decreased rapidly with increasing linuron rate). For the first greenhouse experiment, all S-plants were killed at each of the linuron rates used (Figure 1), including the 0.5X rate which is approximately equal to the minimum recommended rate for linuron in carrot (0.912 kg ai ha^{-1}) (Anonymous 1999). However, R-plants were substantially less affected by linuron than S-plants with only 2% control of R-plants achieved at the 0.5X rate and nearly 3% of R-plants surviving the 10X rate (Figure 1). Also, the effect of linuron was very much delayed for R-plants as compared with the S-plants. Indeed, susceptible ragweed plants were killed within 3-4 days following treatment application, whereas R-plants were only killed 6-7 days after treatment, if they were killed at all (data not shown). The maximum recommended linuron rate (1X) resulted in only 25% of mortality, although above-ground biomass was reduced by more than 50% in both trials (Figure 2) and below-ground biomass was

reduced by 89% and 65% for trials 1 and 2, respectively (Figure 3) compared with the control treatment (0X). However, as ragweed is known to recover relatively quickly from many stresses (Bachand and Christin 1996) which may include herbicide injury, reducing the population of this weed is probably more desirable than simply reducing the total biomass, because it is likely that herbicide-injured ragweed plants could resume active growth, thereby eventually intensely competing for available resources.

At the recommended 1X linuron rate, some plants in the second trial had a higher above-ground biomass, height, and staminate inflorescence production, than was predicted by regression analysis (Figures 2, 4 and 5, respectively). This indicates that recommended linuron rates that were formerly fatal to ragweed not only currently offer poor control of this resistant biotype, but may actually provide just enough stress to stimulate plants to produce more biomass and inflorescences, in the same manner as mechanical mowing has been shown to rapidly stimulate ragweed lateral shoot production (Bachand and Christin 1996).

Figures 6 and 7 show the impact of linuron on susceptible (S) and resistant (R) ragweed biotypes, respectively, for the second experiment. Experiment 2 was conducted in order to obtain the precise linuron-resistance ratio, by using lower linuron rates to obtain the S-biotype's herbicide rate-response curve as well, essential to determine the resistance ratios. A linuron rate of 0.5 kg ai ha^{-1} was sufficient to cause 50% mortality in the S-biotype, whereas R-biotypes required 4.5 kg ai ha^{-1} for 50% mortality. Thus, the resistance ratio for linuron was 9.1, which is more than the 3.4 linuron-resistance ratio obtained by Fuerst *et al.* (1986) for smooth pigweed *(Amaranthus hybridus* L.), and the 1.91 ratio obtained by Beuret (1989).

The findings reported here clearly indicate that some ragweed biotypes in Southwestern Québec have developed resistance to linuron. This is the first reported case of common ragweed resistance to linuron in North America. To date, 14 cases of resistance to urea herbicides have been reported, including resistance to linuron (Heap 1999b). Weed species that have shown resistance to linuron are found in a number of plant families and include redroot pigweed *(Amaranthus retroflexus* L.) (Bulgaria, 1984), horseweed *(Conyza canadensis* L.) (France, 1988), wild pointsettia *(Euphorbia heterophylla* L.) (Ecuador, 1994), and common lamb'squarters *(Chenopodium album* L.) (Norway, 1994) (Holt and LeBaron 1990; Heap 1999b). Common purslane *(Portulaca oleracea* L.) and common groundsel biotypes resistant to linuron were found in carrot fields of Michigan (Masabni and Zandstra 1999) and Switzerland (Beuret 1989), respectively. Common ragweed has also been shown to have developed resistance to other groups of herbicides including atrazine, simazine, cyanazine and cloransulam-methyl (Heap 1999b). Kuratle *et al.* (1969) reported that, unlike carrots, common ragweed did not metabolize linuron into non-toxic derivatives as well thus explaining the selectivity of this herbicide. Both Oettmeier *et al.* (1982) and Fuerst *et al.* (1986) have reported that the linuron resistance observed in some weed biotypes are attributable to the alteration of the binding site (in the chloroplast) of the herbicide, but Fuerst *et al.* (1986) conclude that the biotypes used by both research groups differ in the exact mutation that confers resistance. Moreover, Fuerst *et al.* (1986) cautions that the absorption, translocation, and metabolism of herbicides may also play a role in resistance. Studies by Beuret (1988) show that linuron resistance in Canada fleabane *(Erigeron canadensis* L.) is most probably not due to a chloroplastic mutation, and experiments by Beuret (1989) suggest that resistance of common groundsel to linuron might be due to slowed herbicide

penetration or rapid metabolization of the chemical. The exact mechanisms of linuron resistance implicated in the biotype of common ragweed presented in this study is not yet known and remains to be elucidated.

2.4.2. Field observations

The population dynamics of ragweed in the field that was subjected to reduced rate treatments of linuron is shown in Figure 8. The ragweed population continued to increase at the beginning of the season despite linuron applications due to constant recruitment from the seed bank *(personal observation)*. Moreover, the unusually dry spring conditions in this region of Southwestern Québec in 1999 may have also reduced the efficacy of linuron (Anonymous 1999). Indeed, the Sherrington area received only 237 mm of rain from April to mid-August 1999, compared to average normal rainfall for that period of time of 330 mm (data from Ste-Clotilde weather station, Agriculture Canada). In contrast, field trials by Dickerson and Rahn (1963) and Kuratle and Rahn (1968) in Georgetown, Delaware, showed that linuron provided excellent control of ragweed, even with early post-emergence rates as low as 0.31 kg ai ha^{-1}.

The occurrence of linuron-resistant ragweed populations in Southwestern Québec appears variable, with some producers still effectively controlling ragweed with linuron, while others are even contemplating abandoning carrot production altogether because of the lack of ragweed control options currently available to them. At present, it is difficult to find a clear trend between carrot production areas that have seen the appearance of linuron-resistant ragweed biotypes and areas in which resistance has not been detected. It would be expected that in fields in which carrot production has occurred over many years there would be a greater likelihood of finding linuron-resistant ragweed populations than

in fields that have only recently been under carrot production. However, in our field surveys, this trend was not apparent. In fact, for a number field sites having received regular linuron applications for over 10 years, producers stated that they were satisfied with the ragweed control afforded by linuron. In contrast, some producers that have only recently been using linuron in some of their fields have noted a dramatic decrease in the efficacy of this herbicide to control ragweed. Carrot producers establish crop rotation cycles comprising crops such as parsnip, beet *(Beta vulgaris* L.), onion, lettuce, radish *(Raphamus sativus* L.), celery, rutabaga *(Brassica napus* L.), millet *(Milium* sp. L.), corn, wheat, and other cereals. Some ragweed-controlling herbicides, such as pyrazone, clopyralid, and 2,4-D, can be used in beets, rutabaga, corn and most cereals, but for all the other crops included in the rotations, there are no registered herbicides that control ragweed (Anonymous 1999). Some producers in the region have recently resorted to using multiple early-season reduced rate applications of linuron at the more susceptible cotyledon or 1-2 leaf growth stage of ragweed in order to increase control. This strategy, however, does not appear to be equally effective in all fields. In the Maritime Provinces, metribuzin has been registered for use in carrot fields in mineral soils only, with successful control of ragweed. However, there are presently no plans to register this herbicide for use in Québec carrot fields in either mineral or organic soils.

Table I. ANCOVA (mortality) and ANOVA (parameters other than mortality) trial (block) effect results and related coefficient of variation (C.V.) (R-group only for the first greenhouse experiment, and both S- and R-groups for the second greenhouse experiment). Parameters that showed no significant differences between both trials (NS) were pooled.

Parameters mesured	**AN(C)OVA**	**C.V.**
	Pr > F	
First experiment, R-group		
Mortality	0.7621 (NS)	71.9
Above-ground biomass	0.0128 *	70.7
Male inflorescences	0.0001 ***	73.3
Height	0.8090 (NS)	169.1
Second experiment, S- and R-groups		
Mortality (linuron, S-group)	0.8622 (NS)	88.4
Mortality (linuron, R-group)	0.6513 (NS)	78.5

Figure 1: Linuron-rate response of linuron-susceptible ragweed (white circles) and linuron-resistant ragweed (black circles) mortality (%) for the first greenhouse experiment.

Regression equation is:

$Y = -7.14 + 110.72\ (1 - 0.57^{x})$ Adj. $R^2 = 0.96$

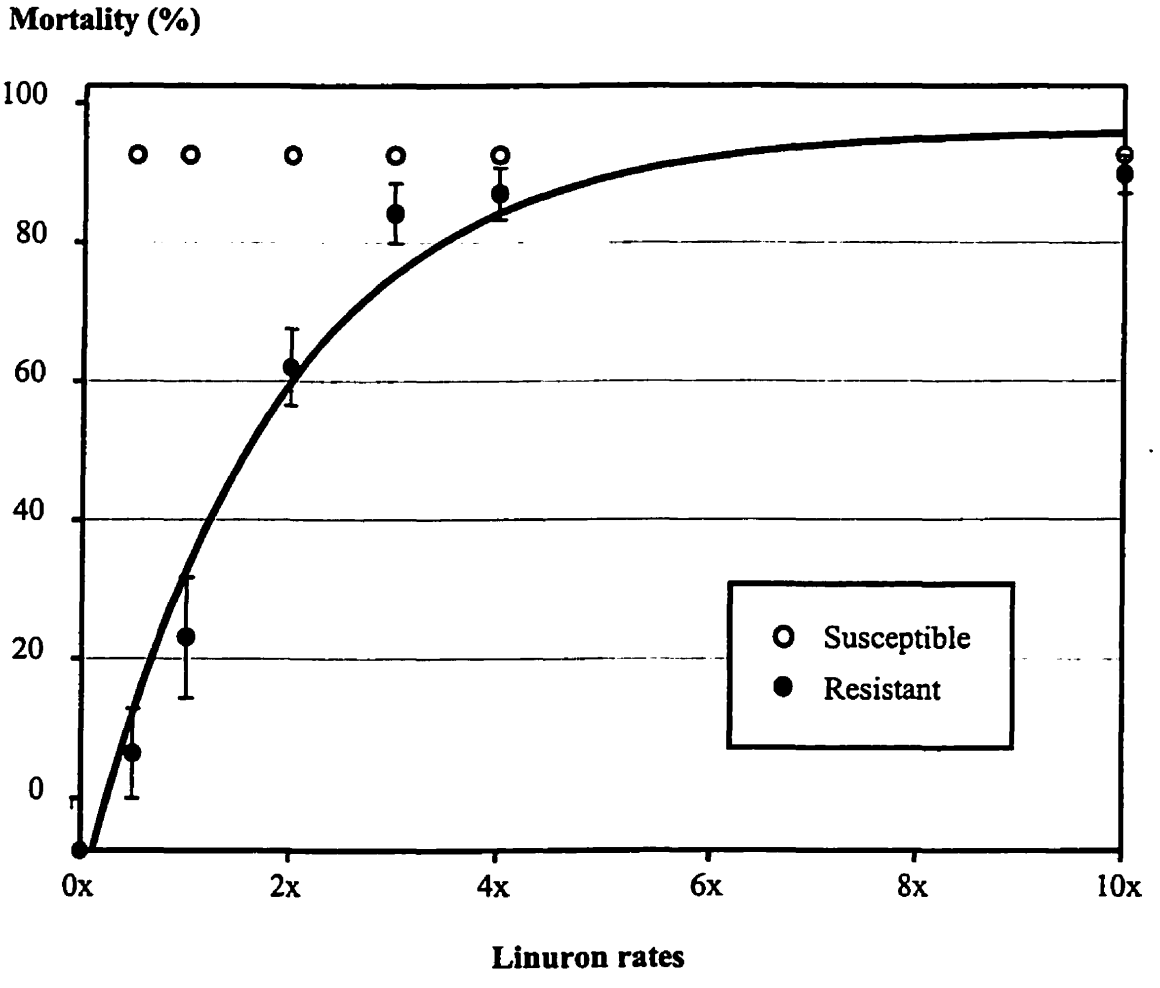

Mortality (%)
100
80
60
40
20
0
0x
2x
4x
6x
8x
10x
Susceptible
Resistant
Linuron rates

Figure 2: Linuron-rate response of linuron-resistant ragweed above-ground biomass (expressed as % of control) for the first greenhouse experiment. Trials were not pooled due to significant differences in the ANCOVA test.

Regression equations are:

$$Y_{(\text{trial 1})} = \frac{99.88 - 23.74x}{1 + 0.09x + 0.56x^2} \qquad \text{Adj. } R^2_{(\text{trial 1})} = 1.00$$

$$Y_{(\text{trial 2})} = 99.79 * \exp(-1.05x) \qquad \text{Adj. } R^2_{(\text{trial 2})} = 0.96$$

Figure 3: Linuron-rate response of linuron-resistant ragweed below-ground biomass (expressed as % of control) for the first greenhouse experiment. Trials were not pooled due to non-homogeneity of variances.

Regression equations are:

$$Y_{(\text{trial 1})} = \frac{1 - 0.22x}{0.01 + 0.06x} \qquad \text{Adj. } R^2_{(\text{trial 1})} = 1.00$$

$$Y_{(\text{trial 2})} = 54.59 * \exp(-3.50x) + 45.49 * \exp(-0.23x) \quad \text{Adj. } R^2_{(\text{trial 2})} = 0.99$$

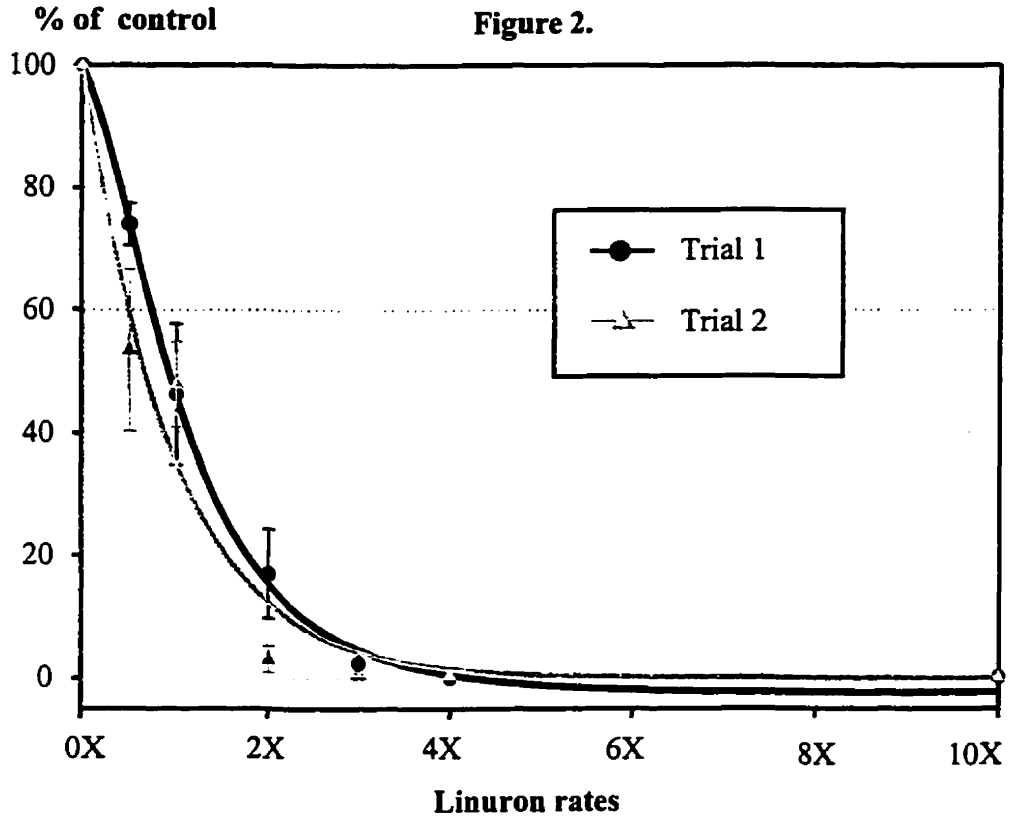


Figure 2.

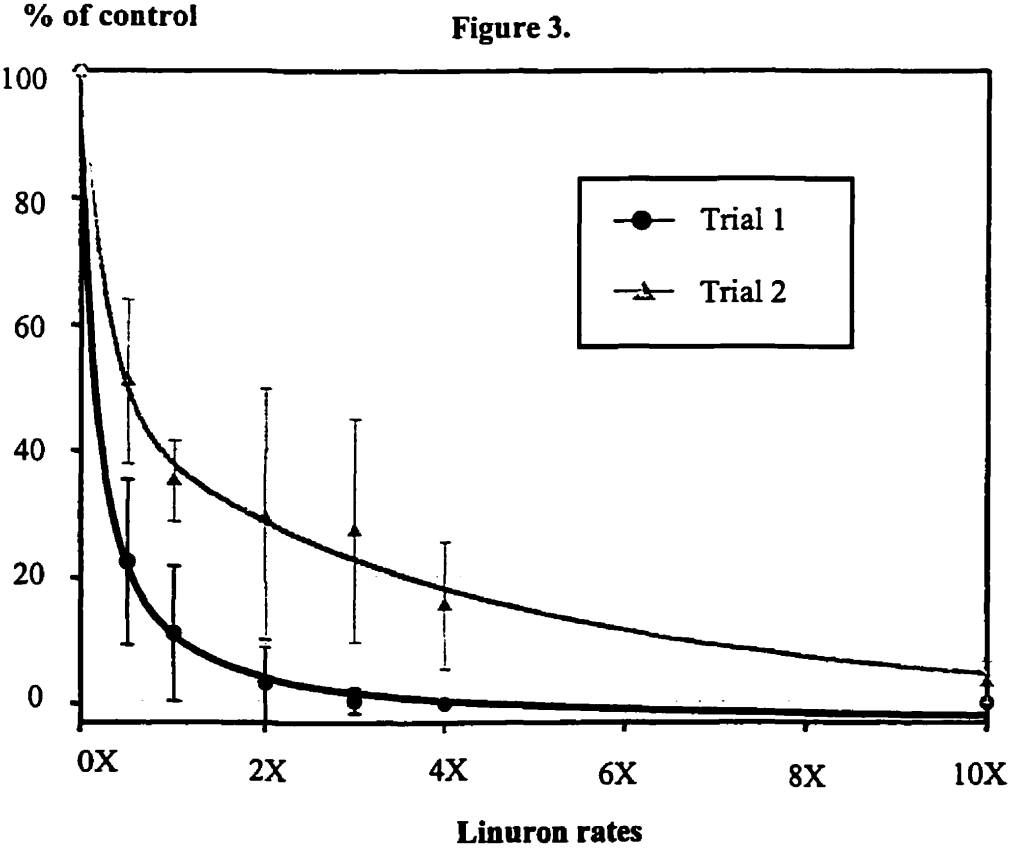


Figure 3.

Figure 4: Linuron-rate response of linuron-resistant ragweed height (expressed as % of control) for the first greenhouse experiment.

Regression equation is:

$Y = -4.75 + 110.67 * \exp(-0.47x)$ Adj. $R^2 = 1.00$

Figure 5: Linuron-rate response of linuron-resistant ragweed in terms of number of male inflorescences produced per plant for the first greenhouse experiment. Trials were not pooled due to significant differences in the ANCOVA test.

Regression equations are:

$Y_{(trial\ 1)} = 1.55 * \exp(-2.72x)$ Adj. $R^2_{(trial\ 1)} = 1.00$

$Y_{(trial\ 2)} = 0.54 + 0.98 * \exp(-0.65x)$ Adj. $R^2_{(trial\ 2)} = 0.83$

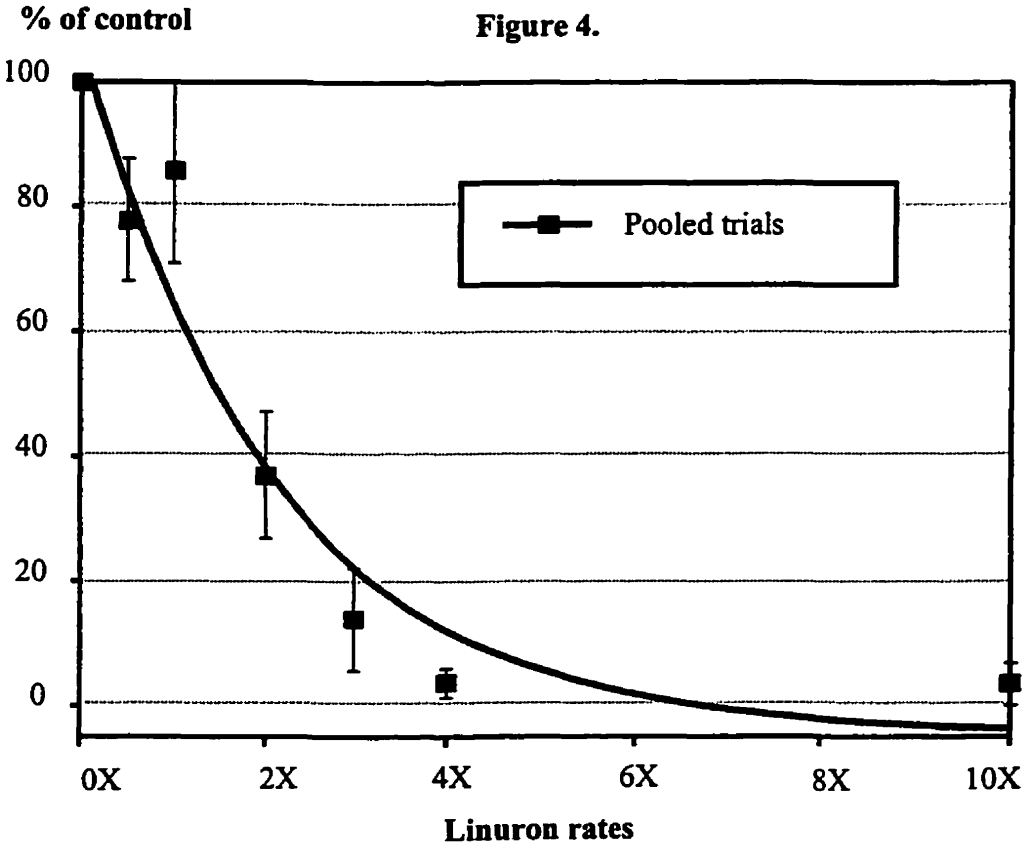


Figure 4.

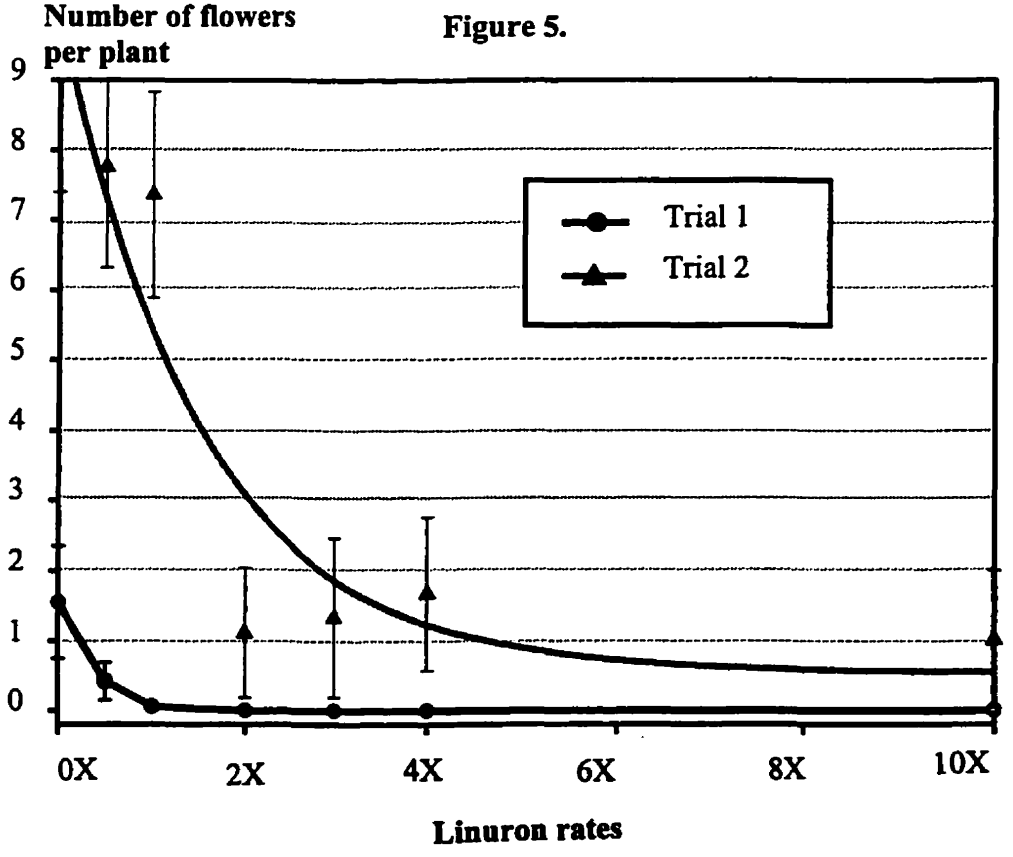


Figure 5.

Figure 6: Linuron-rate response of linuron-susceptible mortality (%) for the second greenhouse experiment. Block 1 and block 2 pooled (10 replicates).

Regression equations are:

$$Y_{(S)} = \frac{69.56}{1 + abs(x/0.15)^{-2.39}} \qquad \text{Adj. } R^2_{(S)} = 0.70$$

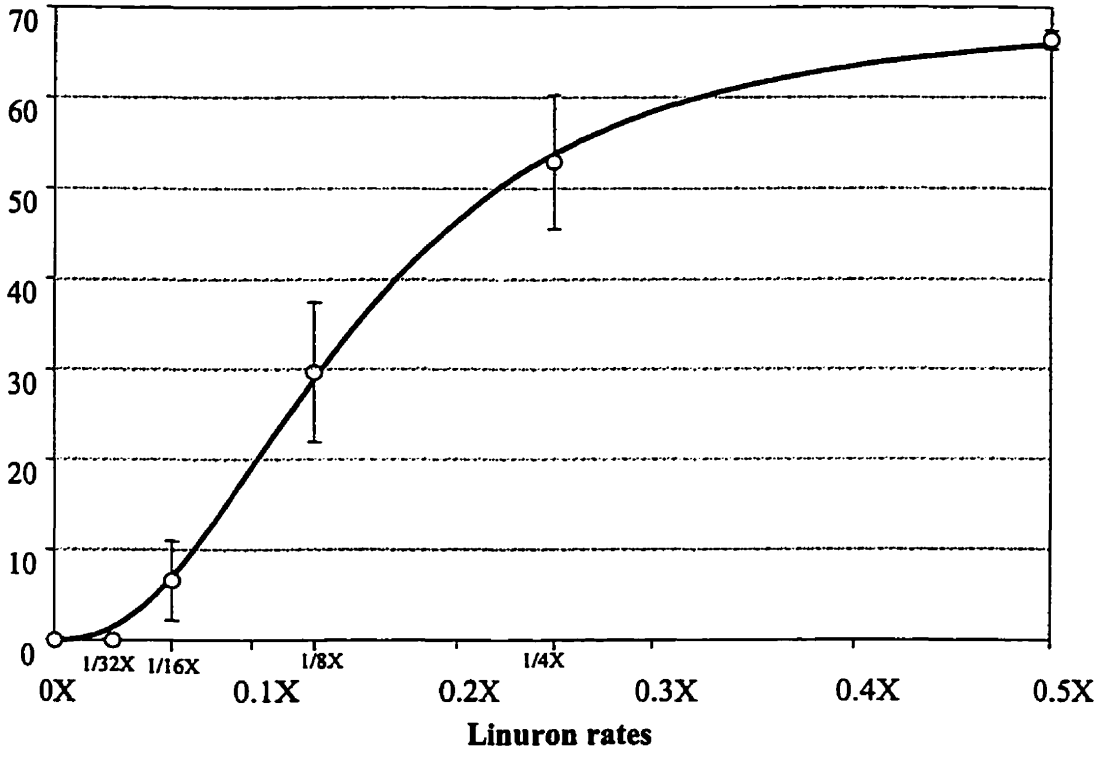
Figure 7: Linuron-rate response of linuron-resistant mortality (%) for the second greenhouse experiment. Block 1 and block 2 pooled (10 replicates).

Regression equations are:

$$Y_{(R)} = \frac{72.72}{1 + abs(x/1.59)^{-3.34}} \qquad \text{Adj. } R^2_{(R)} = 0.76$$

Mortality (%)

Figure 7



Mortality (%)

Figure 6

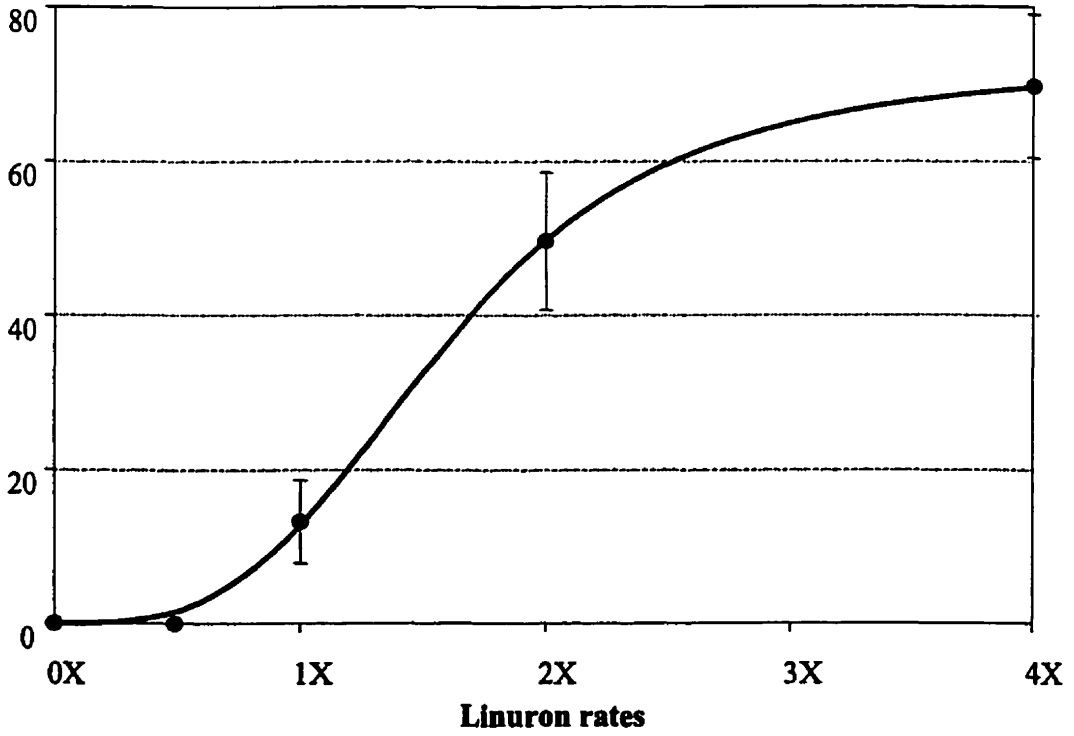
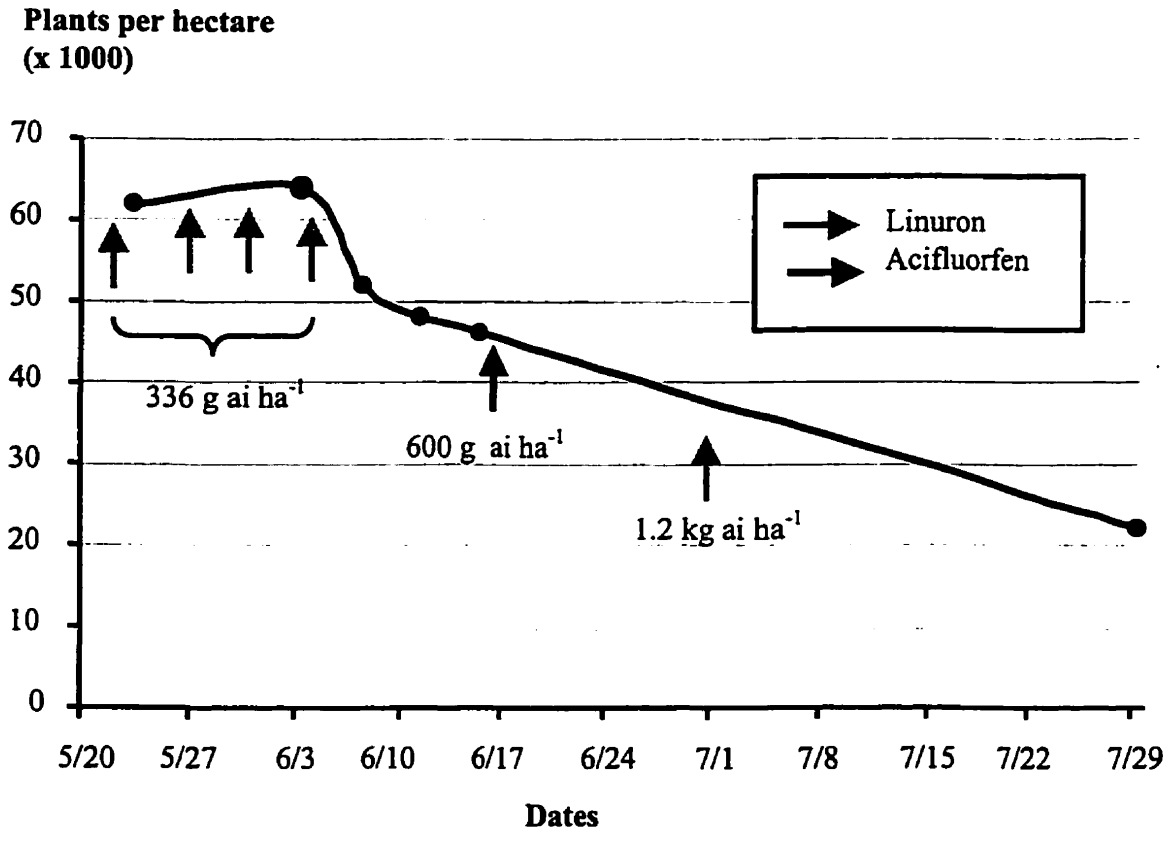


Figure 8: Population dynamics of common ragweed seedlings emerging in a field that was treated with reduced-rates of linuron at five different times in the season (gray arrows), and one application of the herbicide acifluorfen (black arrow).


Plants per hectare
(x 1000)
70
60
50
40
30
20
10
0
5/20
5/27
6/3
6/10
6/17
6/24
7/1
7/8
7/15
7/22
7/29
Dates
Linuron
Acifluorfen
336 g ai ha-1
600 g ai ha-1
1.2 kg ai ha-1

Connecting Text

The presence of linuron -resistant biotypes of *Ambrosia artemisiifolia* L. in carrot fields of Southwestern Québec was demonstrated and discussed in Chapter 2. Given that no other herbicides registered for use in carrots effectively control common ragweed, alternative methods suppressing this troublesome weed in carrots must be sought. In the following chapters, the prospect of controlling *A. artemisiifolia* through biological control strategies and in particular, the mycoherbicide approach, are addressed.

Chapter 3. Spore production of *Phoma* sp. on agar media

3.1. ABSTRACT

Spore production on artificial media is an essential step in laboratory work with fungal organisms. The goal of the following experiments was to determine the optimal environmental conditions to induce sporulation in *Phoma* sp. In a first experiment, five agar media, three light conditions, and two temperature regimes were assessed for their ability to stimulate sporulation of this fungus. *Phoma* sp. grown on potato-dextrose agar (PDA) or V-8 juice extract agar (V8J) under a 12-hr. near-UV light cycle resulted in the greatest sporulation, regardless of temperature tested. In a second experiment, *Phoma* sp. grown on PDA and a 12-hr. cycle under white light and constant temperature was found to produce the maximum number of spores.

3.2. INTRODUCTION

The production of fungal spores on artificial media in the laboratory is an essential step in the mycoherbicide approach to weed control. Numerous environmental factors can affect the ability of a fungus to sporulate, including oxygen, water, pH, nutrients, temperature, and light (Onions *et al.* 1981; Stevens 1981; Moore-Landecker 1996). All fungi require oxygen, water, and an adequate pH (between 4-7) to grow and sporulate, but vary in their requirements for the three other factors.

Klebs (1899) was one of the first researchers to report that sporulation took place when nutrients were depleted in the growth media. Although the precise reason for this process has not been fully elucidated, it has been suggested that cessation of mycelial growth may be the stimulus for initiating reproduction in fungi (Moore-Landecker 1996). Therefore, laboratory media must not be too rich as to stimulate extensive mycelial growth, but instead must be balanced enough to support normal growth and eventual sporulation of the fungus (Onions *et al.* 1981). For example, Czapek agar is an optimum growth medium for most Penicilia and Aspergilli fungi, but may be too rich for most other fungal species. Vegetable extracts such as potato, carrots, and V-8 juice, and other media such as malt, cornmeal, and peptone-yeast may be suitable for culturing most fungi (Onions *et al.* 1981; Stevens 1981).

Although optimum temperature requirements are species-specific, most fungi can grow and reproduce effectively at temperatures between 20 to 25°C (Onions *et al.* 1981; Moore-Landecker 1996). In general, temperature requirements for sexual and non-sexual reproductive phases can vary greatly within a single species, with optimal temperatures

for non-sexual reproduction often similar to temperatures required for mycelial growth (Moore-Landecker 1996).

Light duration and quality can also substantially affect the sporulation of a fungus. Presently, there are five recognized groupings of fungi based on their light requirement for sporulation: Species that (1) are indifferent to light; (2) react unfavorably to light; (3) require alternating light and darkness; (4) produce viable spores under complete darkness but sporulate more readily under light; (5) require light (Moore-Landecker 1996). Not all wavelengths of light are effective in inducing sporulation in fungi. In general, near-ultraviolet, violet, or blue regions of the spectrum (320-490 nm) are most effective (Moore-Landecker 1996).

The objective of the experiments described in this chapter was to determine the optimum environmental conditions in the laboratory for maximum sporulation of *Phoma* sp.

3.3. MATERIALS AND METHODS

3.3.1. Experiment 1: Determination of the most suitable environment for spore production in *Phoma* sp.

This experiment was carried out to determine the most suitable environmental conditions and media for optimum spore production in *Phoma* sp. *Phoma* sp. was isolated from several diseased ragweed plants in the fall of 1993, in an urban area on the island of Montréal, Québec (Brière *et al.* 1995). Five agar growth media were evaluated: potato-

dextrose agar (PDA)[4], malt extract agar (MEA)[5], V-8 juice agar (V8J)[6], oatmeal agar (OAT)[7] and half-PDA agar (H-PDA)[8]. Three light conditions and two temperatures were also tested, creating six different environments: continuous white light (CWL)[9], alternate near UV (AUV)[10], and continuous dark (CD), each at 27 ± 2°C and 20 ± 2°C. The 20°C temperature regime was established in incubators[11], whereas the 27°C temperature regime was established under room conditions. In order to reduce temperature fluctuations under room conditions, Petri dishes were surrounded on all sides by cardboard and heavy fabric. The experiment was set-up in a completely randomized design with four replications for each growth media/environment treatment combination. Spores were collected from all Petri dishes 16 days after inoculation (DAI), and the number of spores within each Petri dish was assessed. Collection of spores was made by flooding the Petri dish with a known volume of distilled water, gently scraping the agar surface with a spatula, and filtering through several layers of cheesecloth. Spore number was determined by using a

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[4] DIFCO Laboratories: Detroit, MI 48232-7058, USA

[5] Tuite 1969; Recipe #128: Malt agar. p.44

[6] Diener 1955 ; Miller 1955 *in* Tuite 1969. Recipe #248: V-8 juice agar. p.73

[7] Kuster 1959 ; Shirling and Gottlieb 1966 *in* Tuite 1969. Recipe #160: Oatmeal agar (Shirling and Gottlieb no.3). p.51

[8] Refer to Appendix II for recipe

[9] TFC: Daylight White fluorescent tubes, FL-15D, 15W, 41cm in length, placed at 25cm over the fungal cultures

[10] 12-hrs cycle, Near-ultraviolet (NUV) fluorescent tubes, F15T8BLB (black light) Sylvania Co., placed at 25cm over the fungal cultures

[11] Sheldon Manufacturing Inc., Cornelius, Oregon

hemocytometer. Colony diameter was also measured at 2, 4, 6, 8, 10 and 12 DAI. Diameter assessments were obtained by substracting the diameter of the initial plug (i.e., 4 mm) from the final diameter of the colony.

Differences in the mean number of spores for the various treatment combinations were evaluated at the P>0.05 level using Tukey's Studentized Range Test. Regression analysis was used to assess differences in mycelial growth for the different treatments.

3.3.2. Experiment 2: Determination of the most suitable environment and harvest time for optimal spore production in *Phoma* sp.

Based on the results obtained in the first experiment, only PDA and V8J agar media were retained for the second experiment. Three light regimes were evaluated: AUV, which appeared to most favour spore production in the first experiment, continuous near-UV (CUV), and alternate white light (AWL)[12]. All light conditions were established at a constant temperature of 24 ± 2°C in an incubator. In this experiment, spores were harvested at five different dates: 7, 9, 11, 13 and 15 DAI. As in the first experiment, the trial was set-up in a completely randomized design with four replications of each harvest date/media/environment treatment combination. Spore collection was as described for experiment 1. Colony diameter was measured at 4, 6, 8, 11, 13 and 15 DAI. Final colony diameter was the mean of two measured diameters (on the same plate) minus the initial plug diameter (i.e., 4 mm).

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[12] 12-hrs cycle

Differences in mean number of spores for the various treatment combinations were evaluated at the P>0.05 level using Tukey's test. Regression analysis was used to assess differences in mycelial diameter growth for the different treatments.

3.4. RESULTS

3.4.1. Experiment 1: Determination of the most suitable environment for spore production in *Phoma* sp.

The general appearance of the *Phoma* sp. colonies varied widely from one growth media to another and from one environmental regime to another. Generally, more aerial mycelium was produced at 27°C than at 20°C, making assessment of pycnidial numbers rather difficult. *Phoma* sp. growth on PDA, H-PDA and V8J media at 27°C resulted in a thick and dark green mat of aerial mycelia. In some instances, this mycelia was white rather than dark green, especially on the top of the mat. Growth on MEA media resulted in nearly no aerial mycelia production, even at 27°C. The mycelia produced on MEA media was initially yellowish or cream, then gradually darkening to a medium brown colour. Moreover, the colonies produced on MEA media were uniform compared with the pronounced sectoring observed for colonies grown on other media, especially at 20°C. However, mycelial growth was very slow on the MEA media, and pycnidial production was low. This media also tended to yield small, malformed 'dirty' spores (i.e., containing many floating drops of an oil-like substance). Large quantities of pycnidia were produced on OAT media, with most of the pycnidia developing and sporulating inside the agar matrix, thus making spore retrieval difficult. Colonies grown on PDA media also showed a large quantity of floating oily drops and small spores, but only when grown in darkness.

Otherwise spores were uniformly shaped and the solution 'clean' as observed in colonies grown on H-PDA and V8J media. When colony sectoring did occur on these media, it appeared that less pycnidia were produced in sectors of dark mycelium than in sectors of white mycelium.

The quantity of spores (mean of four replicates) harvested in each media for the different light/temperature combinations is shown in Table II. Of all treatment combinations, only spore production on PDA under AUV at 20°C was significantly greater than the spore production for the other treatment combinations.

For each of the six light-temperature regimes, only *Phoma* sp. mycelial growth on MEA media was significantly less than mycelial growth for the other four media (Figures 9 to 14). Nonetheless, after 14 DAI, all colonies had attained a diameter of 85 mm, regardless of growth media and light/temperature regime.

3.4.2. Experiment 2: Determination of the most suitable environment and harvest time for optimal spore production in *Phoma* sp.

The general appearance of *Phoma* sp. colonies on each of the two media evaluated was similar to those observed in experiment 1. Similarly, some sectoring of the colonies was detected, with dark sections of colonies generally producing more aerial mycelia, but less pycnidia. As in experiment 1, there was wide variability in the number of spores harvested per plate.

Significant differences in spore production were found for *Phoma* sp. grown on PDA under CUV light at 10 DAI compared with all other sample days, except 12 DAI,

and between the two last sample periods (i.e., 12 and 14 DAI) (Figure 15). Significant differences in spore production were also observed for colonies grown on V8J media under CUV light at 14 DAI compared with all other sample periods, except for the 10 DAI harvest period (Figure 15). Under AUV light, spore production on PDA was significantly lower only at the 6 DAI sample period compared with the 14 DAI harvest date (Figure 16). Spore production on V8J media under AUV light was significantly greater at 14 DAI compared with all other harvest dates, except 12 DAI (Figure 16). Under AWL, *Phoma* sp. spore production on PDA at 14 DAI was significantly greater that at 12 DAI, which was significantly greater that at the other harvest dates (Figure 17). Moreover, significantly more spores were harvested 14 DAI than 12 DAI. In contrast, no significant differences in spore production at each of the harvest dates were found for colonies grown on V8J media under AWL (Figure 17).

Figure 18 shows that significant differences in spore production were obtained for *Phoma* sp. colonies grown PDA media and under AWL light at the 12 and 14 DAI harvest dates compared with all other harvest dates, and that there were no significant differences in spore production between any of the harvest dates for colonies grown under CUV and AUV light. For *Phoma* sp. colonies grown on V8J media, spore production under AWL at 14 DAI was significantly greater than spore production for all other harvest dates, except for 8DAI in AWL, 12 DAI in AWL, and 14 DAI in CUV light (Figure 19).

Under all light regimes, mycelial growth was significantly lower on PDA than on V8J media. However, by the 12 DAI harvest date, all colonies has attained the maximum diameter possible (i.e., 85 mm, or the diameter of the Petri dish) (Figures 20 - 22).

3.5. DISCUSSION

High variability in *Phoma* sp. spore production among replicates was observed in both experiments. In some instances, spore production within a treatment varied from 406 million spores per plate to as much as 6.5 billion spores per plate. This variability may have been due to the strong sectoring that was observed for colonies in several plates. Often, replicate Petri dishes for a single treatment were completely covered with the thick, dark mycelia form of the fungus that produced few pycnidia, whereas other Petri dishes showed little or no aerial mycelia but were instead covered with spore-producing pycnidia. Onions *et al.* (1981) suggested that repeated transferring of fungal cultures may lead to a differentiation, weakening, and eventual death of the cultures. In the first experiment, repeated transfer of *Phoma* sp. colonies may have been responsible for the strong sectoring and reduced pycnidial production observed. Although in the second experiment all new colonies were established from an original source colony, sectoring of colonies in several of the plates was nonetheless observed regardless of growth media used. Another possible explanation for the appearance of sectors in *Phoma* sp. colonies is the fact that prior to these experiments, the colonies were not purified by either hyphal tipping or single sporing, which would most probably have rendered the colonies much more uniform in terms of mycelial growth and pycnidial production.

Mycelial growth of *Phoma* sp. was stimulated under continuous darkness and white light, but not sporulation. Alternating near-UV light resulted in the greatest spore production at both temperature regimes used. MEA was found not to be suitable for *Phoma* sp. sporulation, although mycelial growth was slow but uniform (i.e., no sectoring). Thus, MEA growth media may be an ideal substrate on which to grow *Phoma* sp. mycelia when sporulation is not preferred. The OAT growth media supported

relatively high levels of pycnidia, however the majority of the pycnidia developed inside the agar, thus making the harvesting of spores difficult if not impossible. Hence, OAT is an unsuitable solid media for *Phoma* sp. spore production. The ability of *Phoma* sp. to sporulate in liquid media in not known but warrants further study, as not all fungi will readily sporulate in such media (Auld et Morin 1995). It was hypothesized that H-PDA growth media might enhance spore production in *Phoma* sp. by providing fewer nutrients thereby stimulating this fungus to sporulate more readily (Klebs 1899; Moore-Landecker 1996). Findings in this research did not support this hypothesis as mycelial growth on H-PDA media was low under all light/temperature combinations on this media. The PDA and V8J growth media resulted in the greatest spore production of *Phoma* sp.. Hence, these two media were used for the second experiment.

In experiment 2, the AUV light regime, which proved to be the most suitable light condition for *Phoma* sp. spore production in experiment 1, was compared with the AWL and CUV light regimes for their ability to induce sporulation. Findings demonstrated that on PDA growth media, AWL stimulated *Phoma* sp. to sporulate more readily than all other light regimes tested (Figure 20), but for near-UV light, there were no significant differences in spore production between light regimes, although AWL did appear to favor some spore production (Figure 21). Moreover, *Phoma* sp. subjected to long periods (i.e., 14 DAI) of CUV and AUV light showed low spore production on V8J growth media and no sporulation at all on PDA media. These findings are in contrast to report of Onions *et al.* (1981) where fungi within the Sphaeropsidales Order, such as *Phoma* sp., often sporulate to a greater degree in the presence of near-UV light. The two temperature regimes evaluated (i.e., 20°C and 27°C) had little impact on the sporulation of *Phoma* sp.,

which is in accordance with the theory stating that Deuteromycete fungi have a wide window of optimal temperatures for asexual reproduction (Onions *et al.* 1981; Moore-Landecker 1996)

The findings from this research demonstrate that suitable spore production in *Phoma* sp. can be obtained on PDA, when exposed to a 12-hr. white light cycle, and maintained at a temperature range of 20°C to 27°C.

Table II. Spore yield (and standard errors) of *Phoma* sp. grown on one of five solid media and subjected to different light and temperature regimes for a period of 16 days. Yields having the same letter are not significantly different at the P >0.05 level according to the Tukey's Studentized Range Test.

Treatment	OAT	MEA	V8J
Light, 20°C	3.5 (1.7) **b**	0.1 (0.1) **b**	51.3 (20.3) **b**
Light, 27°C	21.3 (12.3) **b**	0.4 (0.3) **b**	218.6 (123.5) **ab**
Near-UV, 20°C	18.4 (2.8) **b**	68.3 (33.0) **b**	2618.1 (1470.7) **ab**
Near-UV, 27°C	1602.5 (1491.7) **ab**	941.7 (242.8) **ab**	817.0 (622.9) **ab**
Darkness, 20°C	0.0 (0.0) **b**	0.1 (0.0) **b**	2.4 (1.0) **b**
Darkness, 27°C	0.6 (0.5) **b**	0.4 (0.4) **b**	5.0 (2.5) **b**

Treatment	PDA	H-PDA
Light, 20°C	191.3 (11.5) **ab**	1.9 (1.0) **b**
Light, 27°C	14.6 (8.3) **b**	0.4 (0.3) **b**
Near-UV, 20°C	4831.4 (3175.0) **a**	204.2 (96.2) **ab**
Near-UV, 27°C	3971.3 (2768.3) **ab**	116.1 (38.0) **ab**
Darkness, 20°C	2.3 (2.2) **b**	0.2 (0.1) **b**
Darkness, 27°C	4.9 (3.8) **b**	0.3 (0.1) **b**

Figure 9: Relationship between mycelial growth (i.e., colony diameter) and days after inoculation (DAI) for *Phoma* sp. grown on one of five solid media and subjected to white light at 20°C for a period of 14 days.

Regression equations are:

$Y_{(oat)} = -3.3 + 36.6 * \ln |x|$ Adj. $R^2_{(oat)} = 0.94$

$Y_{(malt)} = 32.3 * \ln |x-0.6|$ Adj. $R^2_{(malt)} = 0.96$

$Y_{(V8)} = -1.9 + 36.4 * \ln |x|$ Adj. $R^2_{(V8)} = 0.92$

$Y_{(PDA)} = -4.5 + 36.1 * \ln |x|$ Adj. $R^2_{(PDA)} = 0.96$

$Y_{(H\text{-}PDA)} = -1.2 + 35.9 * \ln |x|$ Adj. $R^2_{(H\text{-}PDA)} = 0.93$

Figure 10: Relationship between mycelial growth (i.e., colony diameter) and days after inoculation (DAI) for *Phoma* sp. grown on one of five solid media and subjected to white light at 27°C for a period of 14 days.

Regression equations are:

$Y_{(oat)} = -28.7 + 80.4 * \ln |x| - 13.7 * (\ln |x|)^2$ Adj. $R^2_{(oat)} = 0.96$

$Y_{(malt)} = -8.7 + 36.7 * \ln |x|$ Adj. $R^2_{(malt)} = 0.98$

$Y_{(V8)} = -31.5 + 85.6 * \ln |x| - 15.5 * (\ln |x|)^2$ Adj. $R^2_{(V8)} = 0.96$

$Y_{(PDA)} = 25.9 + 24.8 * \ln |x-1.1|$ Adj. $R^2_{(PDA)} = 0.93$

$Y_{(H\text{-}PDA)} = 40.1 + 19.9 * \ln |x-1.6|$ Adj. $R^2_{(H\text{-}PDA)} = 0.95$

Figure 9.

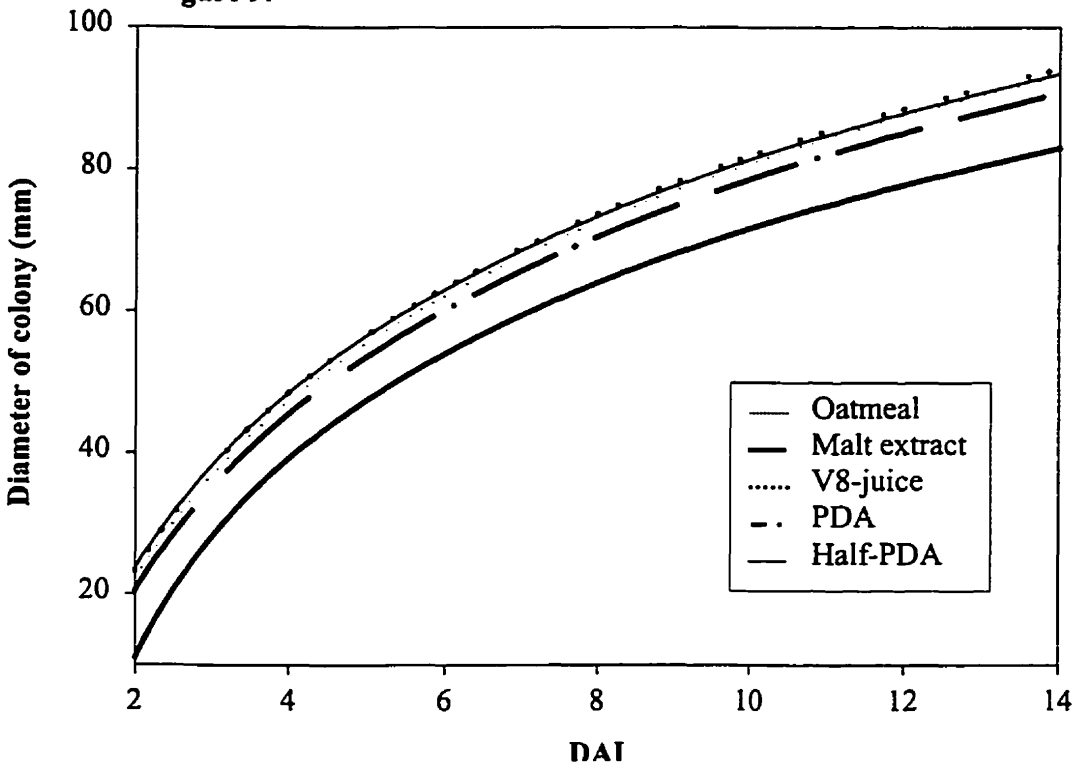


Figure 10.

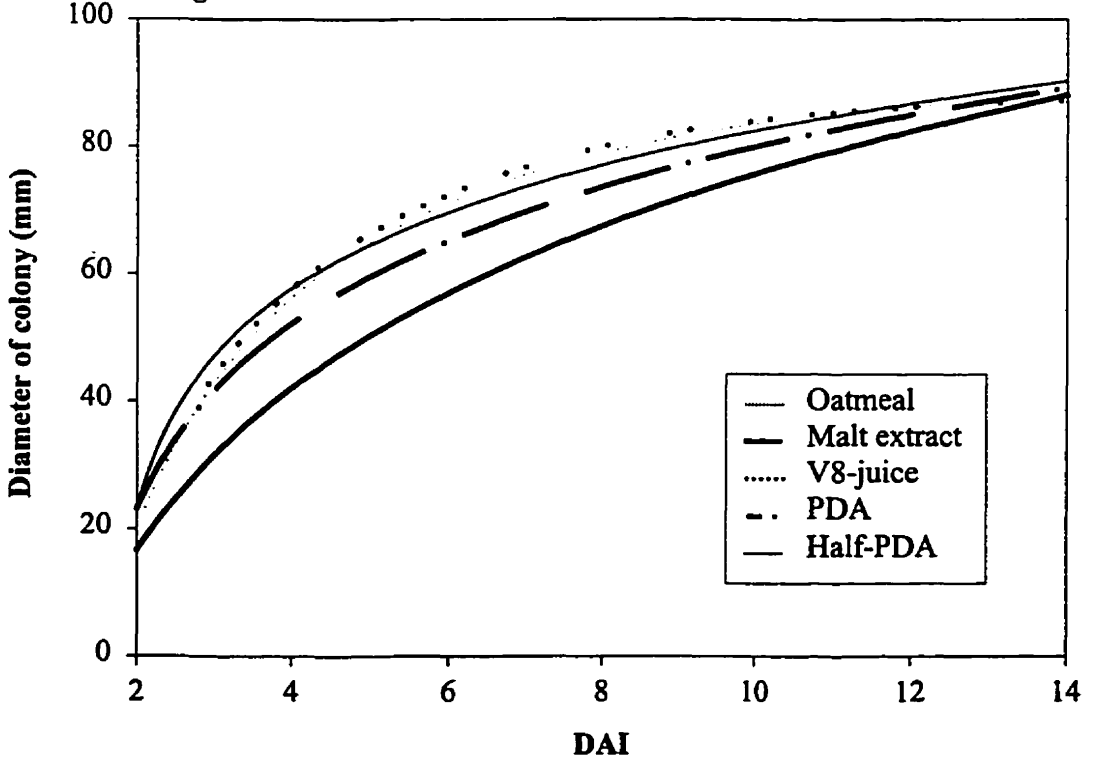


Figure 11: Relationship between mycelial growth (i.e., colony diameter) and days after inoculation (DAI) for *Phoma* sp. grown on one of five solid media and subjected to near-UV light at 20°C for a period of 14 days.

Regression equations are:

$Y_{(oat)} = -0.3 + 35.5 * \ln |x|$ Adj. $R^2_{(oat)} = 0.93$

$Y_{(malt)} = -11.7 + 37.7 * \ln |x|$ Adj. $R^2_{(malt)} = 0.98$

$Y_{(V8)} = 42.8 + 19.0 * \ln |x-1.6|$ Adj. $R^2_{(V8)} = 0.94$

$Y_{(PDA)} = 30.3 + 23.9 * \ln |x-1.3|$ Adj. $R^2_{(PDA)} = 0.94$

$Y_{(H\text{-}PDA)} = 34.1 + 22.4 * \ln |x-2.4|$ Adj. $R^2_{(H\text{-}PDA)} = 0.94$

Figure 12: Relationship between mycelial growth (i.e., colony diameter) and days after inoculation (DAI) for *Phoma* sp. grown on one of five solid media and subjected to near-UV light at 27°C for a period of 14 days.

Regression equations are:

$Y_{(oat)} = -2.5 + 35.2 * \ln |x|$ Adj. $R^2_{(oat)} = 0.98$

$Y_{(malt)} = -10.8 + 36.0 * \ln |x|$ Adj. $R^2_{(malt)} = 0.97$

$Y_{(V8)} = -0.6 + 34.0 * \ln |x|$ Adj. $R^2_{(V8)} = 0.98$

$Y_{(PDA)} = -2.5 + 35.1 * \ln |x|$ Adj. $R^2_{(PDA)} = 0.97$

$Y_{(H\text{-}PDA)} = -2.2 + 35.1 * \ln |x|$ Adj. $R^2_{(H\text{-}PDA)} = 0.97$

Figure 11.

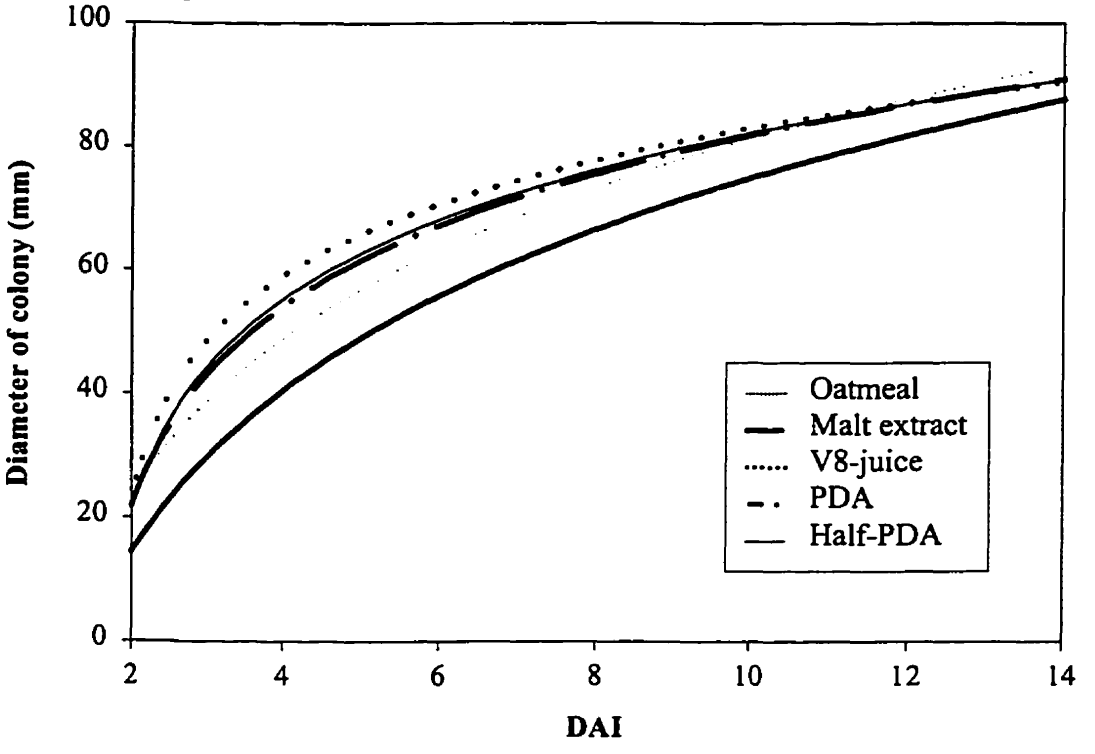


Figure 12.

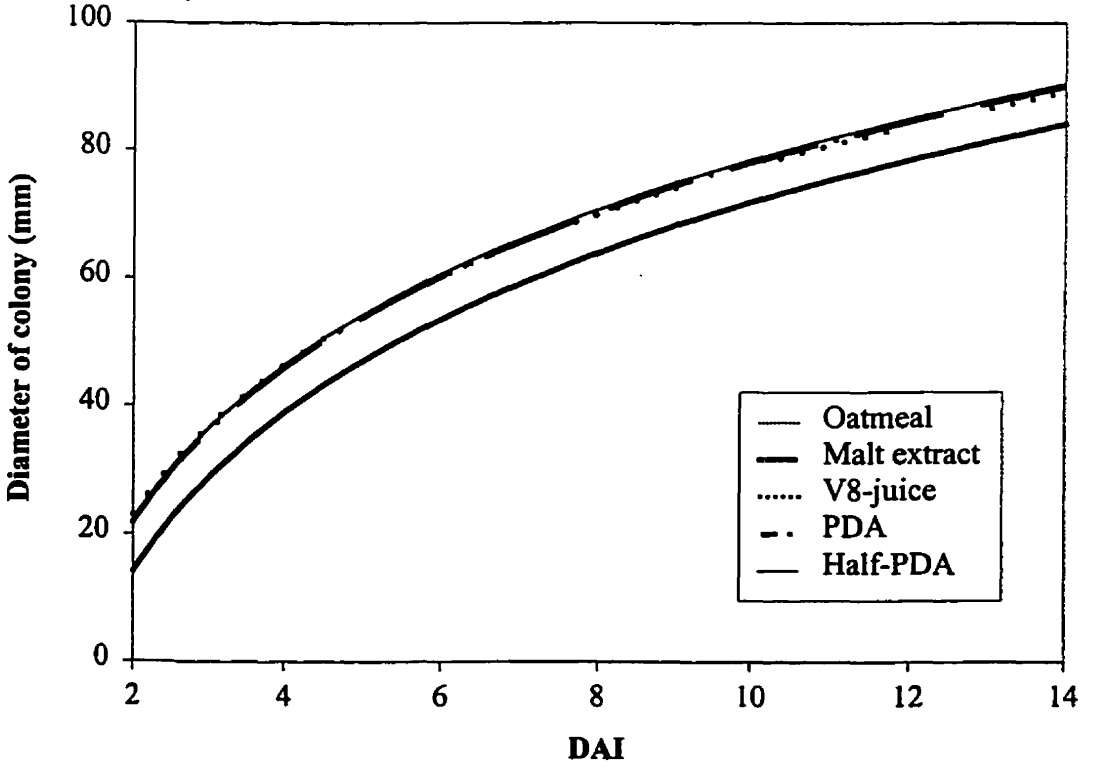


Figure 13: Relationship between mycelial growth (i.e., colony diameter) and days after inoculation (DAI) for *Phoma* sp. grown on one of five solid media and subjected to darkness at 20°C for a period of 14 days.

Regression equations are:

$Y_{(oat)} = -14.0 + 39.6 * \ln|x|$ Adj. $R^2_{(oat)} = 0.97$

$Y_{(malt)} = -2361.8 + 535.6 * \ln|x-82.1|$ Adj. $R^2_{(malt)} = 1.00$

$Y_{(V8)} = -11.7 + 39.0 * \ln|x|$ Adj. $R^2_{(V8)} = 0.97$

$Y_{(PDA)} = -12.9 + 38.2 * \ln|x|$ Adj. $R^2_{(PDA)} = 0.98$

$Y_{(H\text{-}PDA)} = -13.7 + 39.5 * \ln|x|$ Adj. $R^2_{(H\text{-}PDA)} = 0.96$

Figure 14: Relationship between mycelial growth (i.e., colony diameter) and days after inoculation (DAI) for *Phoma* sp. grown on one of five solid media and subjected to darkness at 27°C for a period of 14 days.

Regression equations are:

$Y_{(oat)} = 1.8 + 34.4 * \ln|x|$ Adj. $R^2_{(oat)} = 0.95$

$Y_{(malt)} = -10.2 + 35.5 * \ln|x|$ Adj. $R^2_{(malt)} = 0.98$

$Y_{(V8)} = 37.0 + 21.0 * \ln|x-1.5|$ Adj. $R^2_{(V8)} = 0.96$

$Y_{(PDA)} = -3.1 + 35.0 * \ln|x|$ Adj. $R^2_{(PDA)} = 0.97$

$Y_{(H\text{-}PDA)} = 1.8 + 34.3 * \ln|x|$ Adj. $R^2_{(H\text{-}PDA)} = 0.94$

Figure 13.

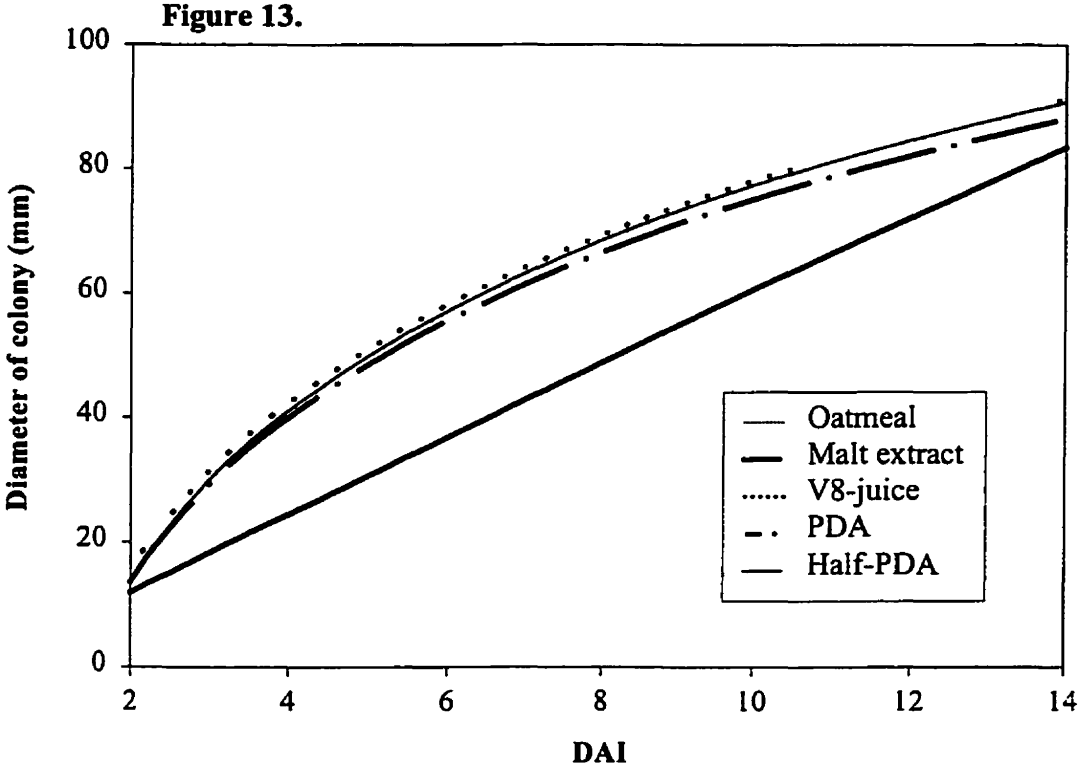


Figure 14.

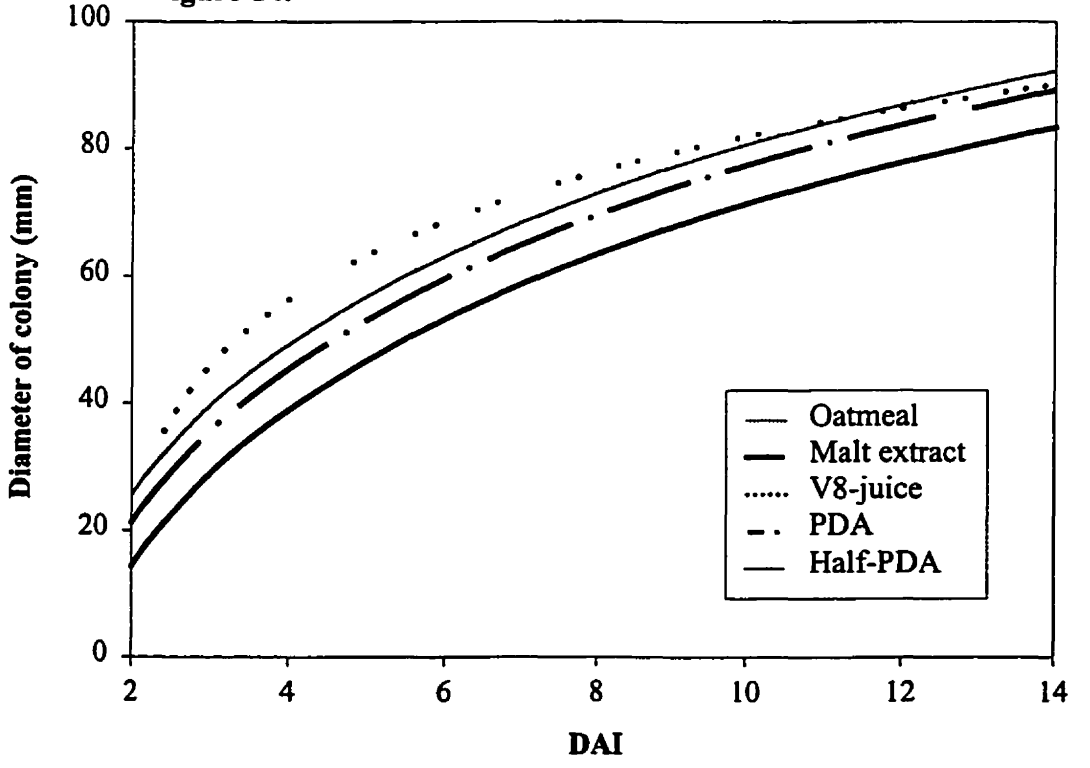


Figure 15: Spore yield (and standard error) of *Phoma* sp. grown on PDA and V8-juice agar media and subjected to continuous near-UV light for a period of 16 days. Yields for bars having the same letter are not significantly different at the P >0.05 level according to the Tukey's Studentized Range Test.

Figure 16: Spore yield (and standard error) of *Phoma* sp. grown on PDA and V8-juice agar media and subjected to alternate near-UV light for a period of 16 days. Yields for bars having the same letter are not significantly different at the P >0.05 level according to the Tukey's Studentized Range Test.

Figure 15.

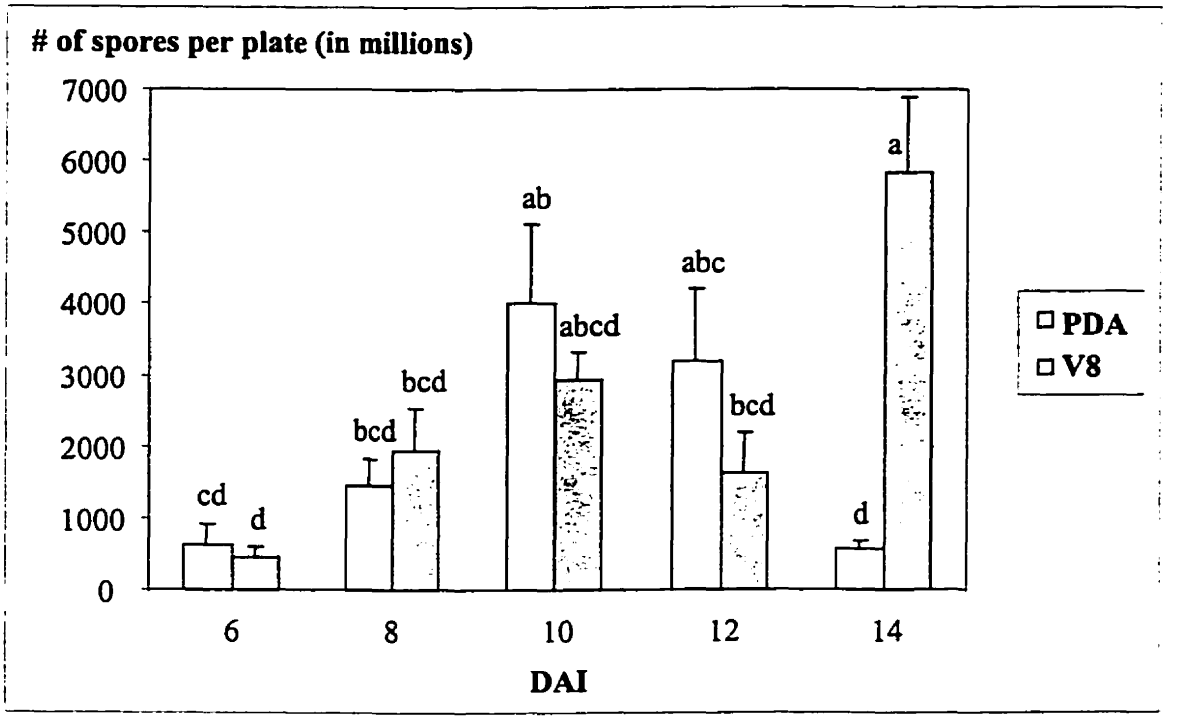


Figure 16.

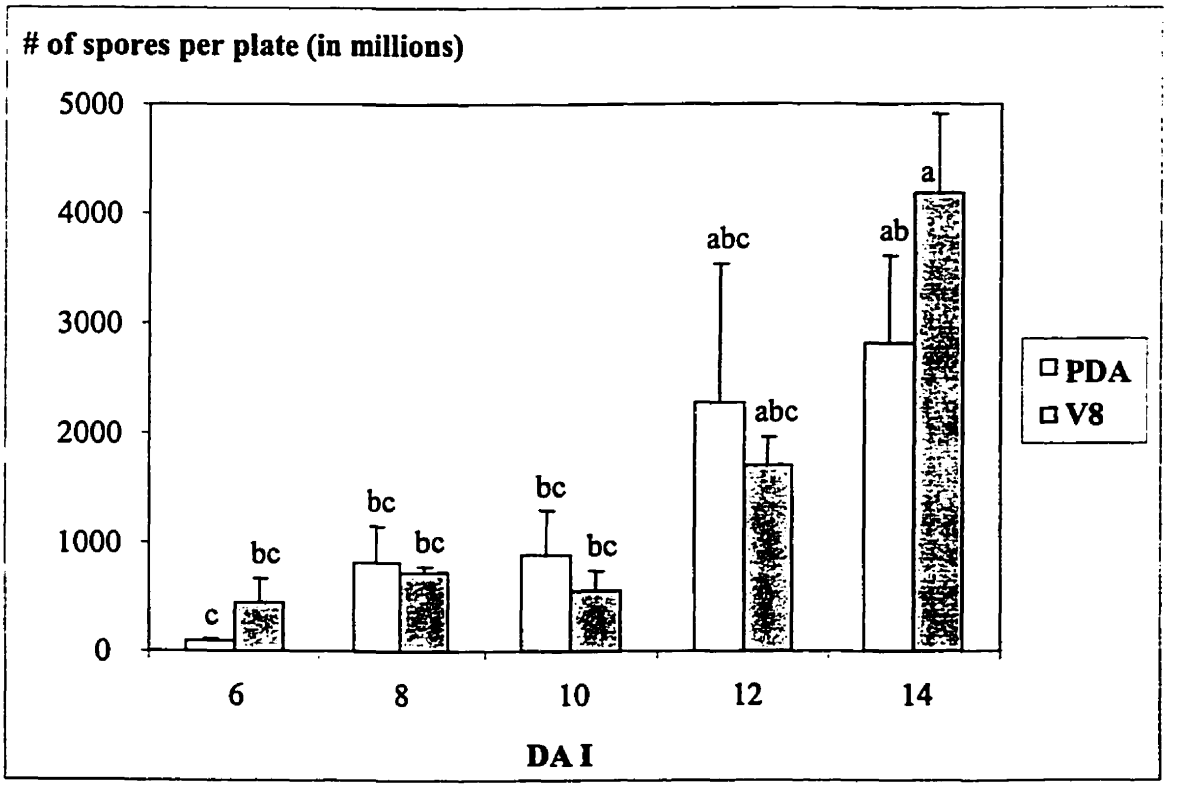


Figure 17: Spore yield (and standard error) of *Phoma* sp. grown on PDA and V8-juice agar media and subjected to alternate white light for a period of 16 days. Yields for bars having the same letter are not significantly different at the P >0.05 level according to the Tukey's Studentized Range Test.

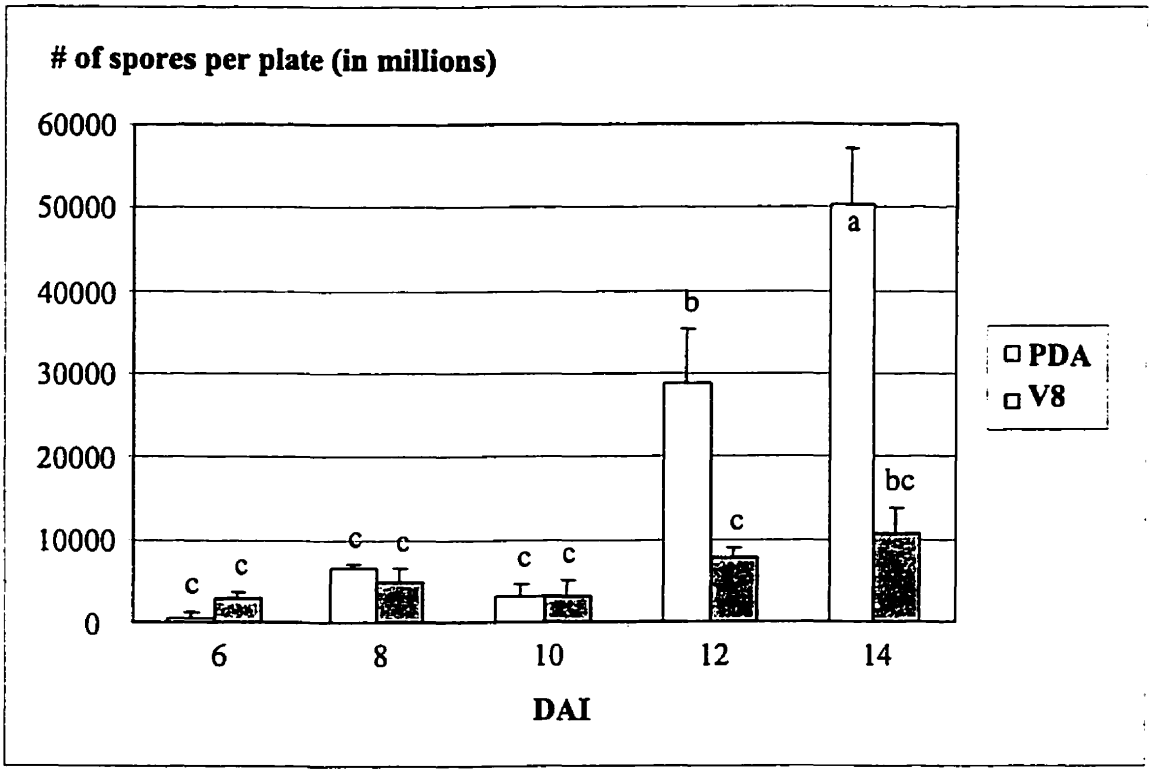

# of spores per plate (in millions)
60000
50000
40000
30000
20000
10000
0
c
c
c
c
c
c
b
c
a
bc
6
8
10
12
14
DAI
PDA
V8

Figure 18: Spore yield (and standard error) of *Phoma* sp. grown on PDA media and subjected to the three different light regimes for a period of 16 days. Yields for bars having the same letter are not significantly different at the P >0.05 level according to the Tukey's Studentized Range Test.

Figure 19: Spore yield (and standard error) of *Phoma* sp. grown on V8-juice media and subjected to the three different light regimes for a period of 16 days. Yields for bars having the same letter are not significantly different at the P >0.05 level according to the Tukey's Studentized Range Test.

Figure 18.

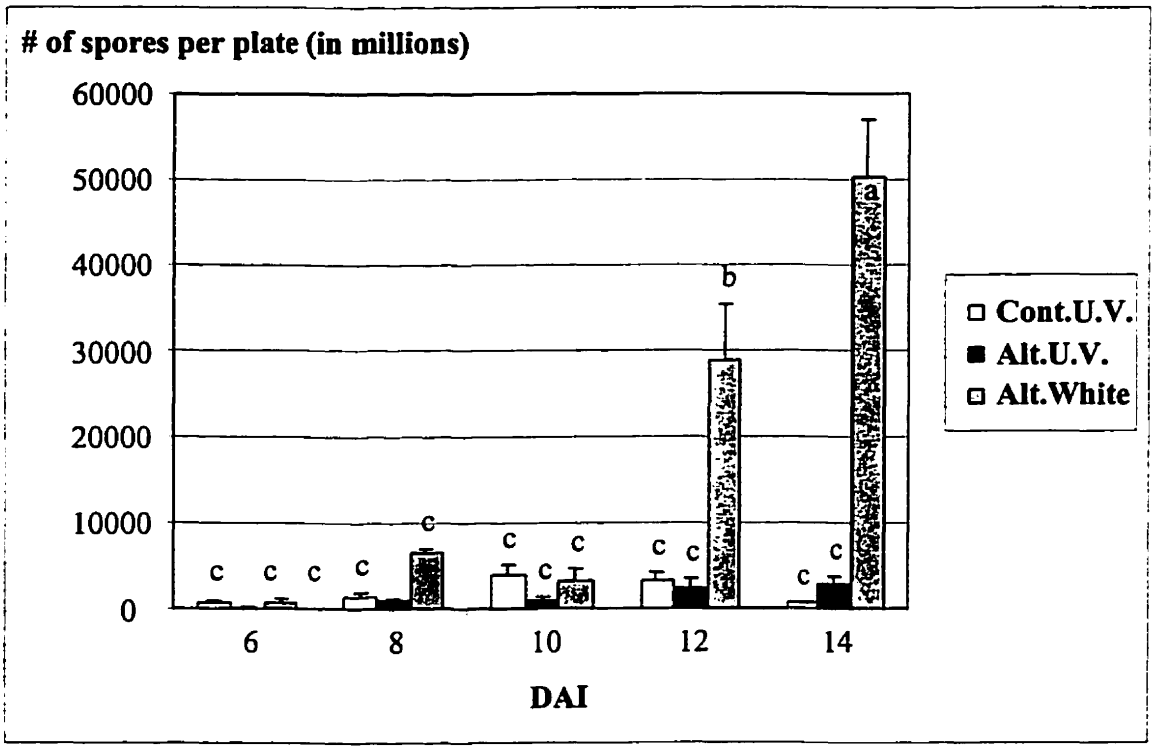


Figure 19.

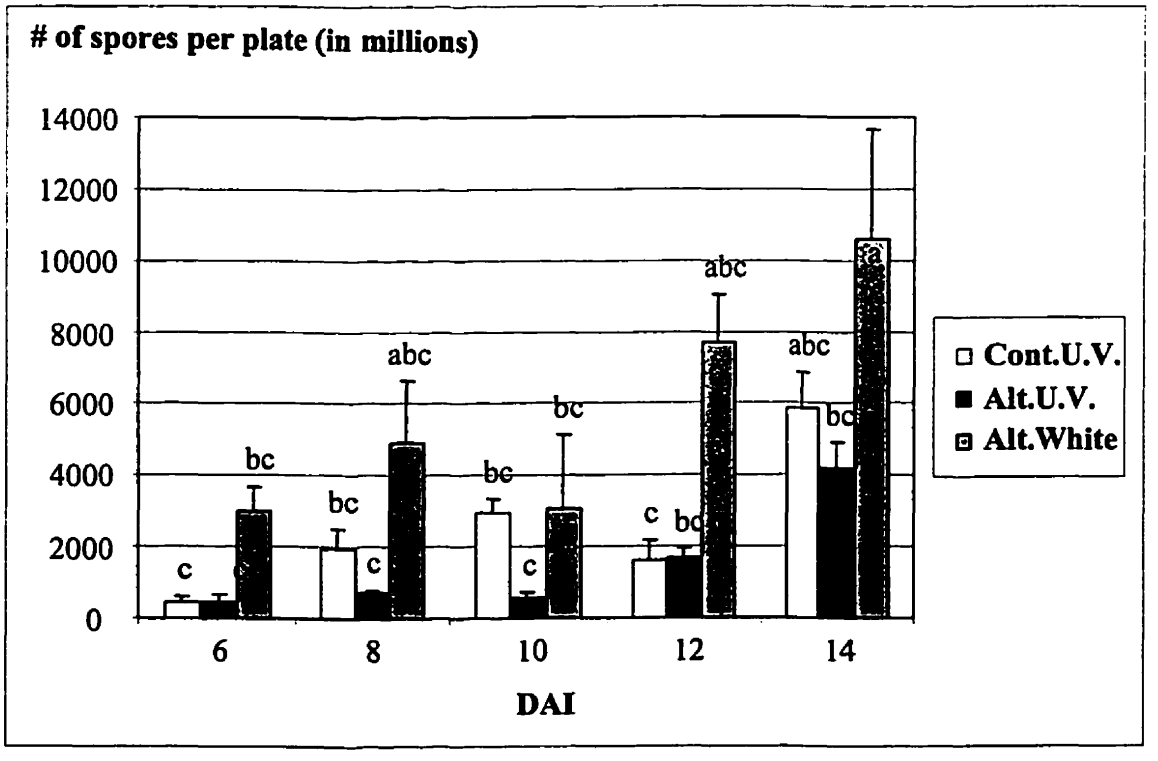


Figure 20: Relationship between mycelial growth (i.e., colony diameter) and days after inoculation (DAI) for *Phoma* sp. grown on PDA and V8-juice media and subjected to continuous near-UV light for a period of 13 days.

Regression equations are:

$Y_{(PDA)} = 35.0 * \ln |x-1.6|$ Adj. $R^2_{(PDA)} = 0.89$

$Y_{(V8)} = -159.4 + 195.6 * \ln |x| - 39.7 * (\ln |x|)^2$ Adj. $R^2_{(V8)} = 0.98$

Figure 21: Relationship between mycelial growth (i.e., colony diameter) and days after inoculation (DAI) for *Phoma* sp. grown on PDA and V8-juice media and subjected to alternate near-UV light for a period of 13 days.

Regression equations are:

$Y_{(PDA)} = 36.0 * \ln |x-1.7|$ Adj. $R^2_{(PDA)} = 0.95$

$Y_{(V8)} = -132.7 + 171.4 * \ln |x| - 34.2 * (\ln |x|)^2$ Adj. $R^2_{(V8)} = 0.85$

Figure 20.

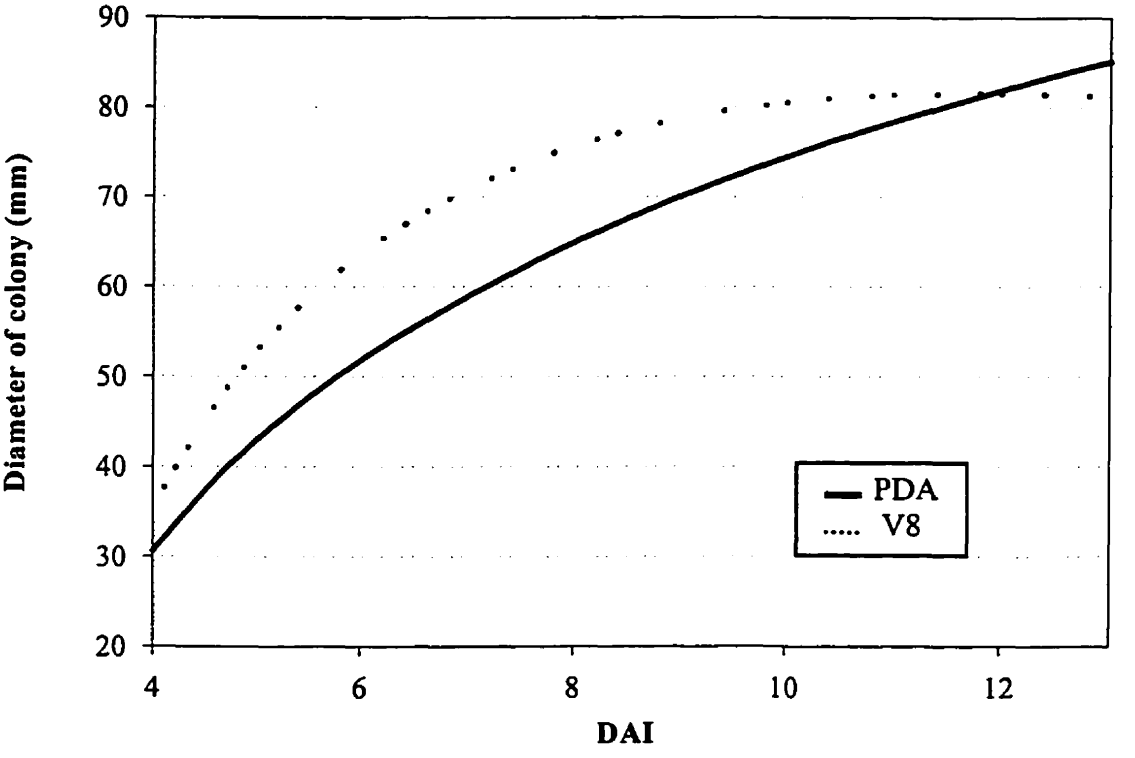


Figure 21.

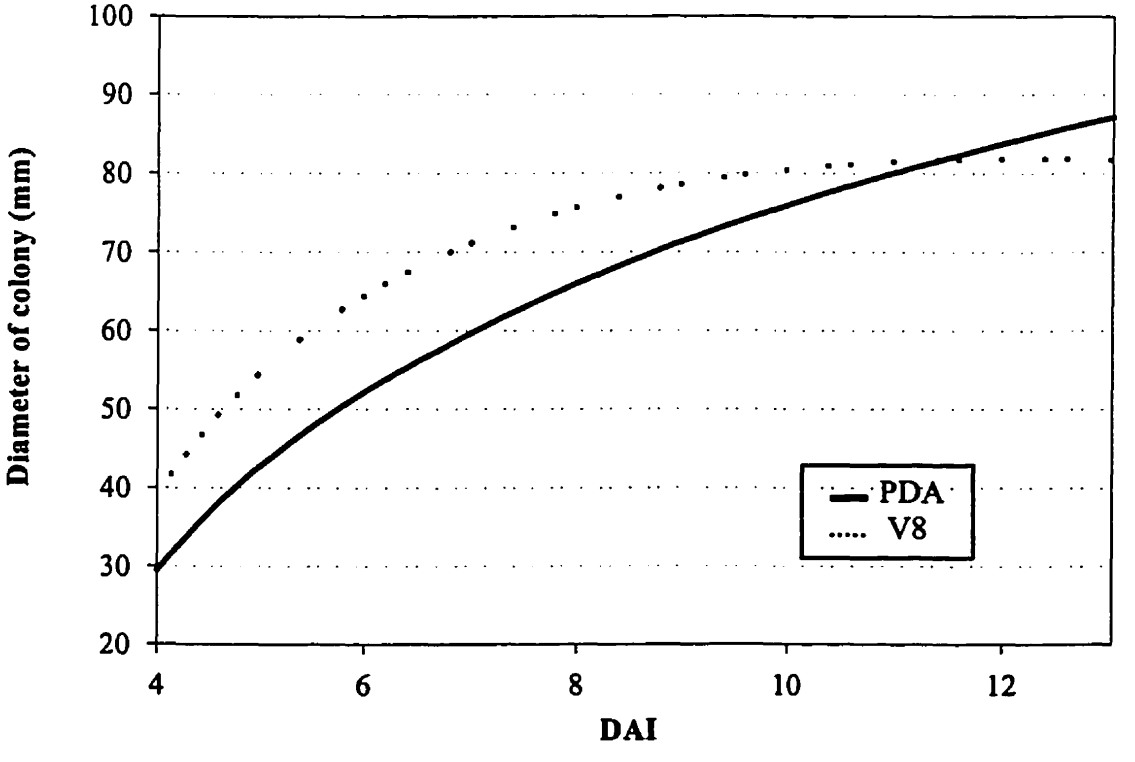
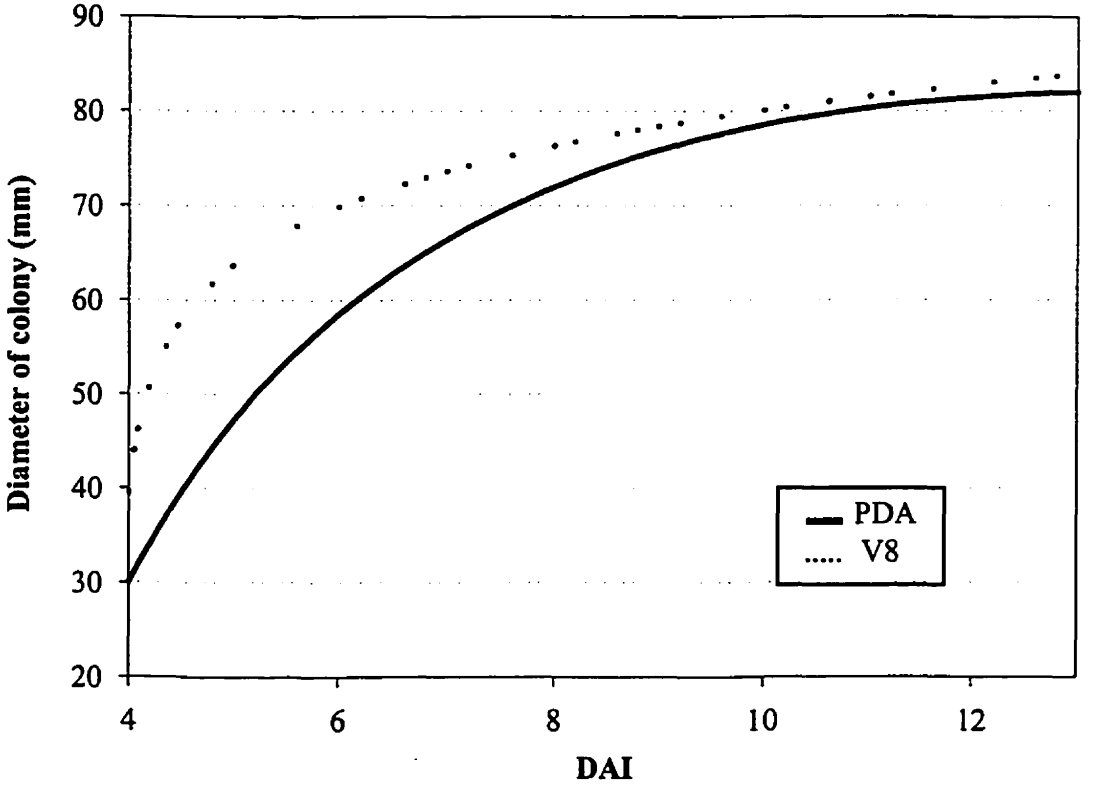


Figure 22: Relationship between mycelial growth (i.e., colony diameter) and days after inoculation (DAI) for *Phoma* sp. grown on PDA and V8-juice media and subjected to alternate white light for a period of 13 days.

Regression equations are:

$Y_{(PDA)} = -151.2 + 177.6 * \ln|x| - 33.8 * (\ln|x|)^2$ Adj. $R^2_{(PDA)} = 0.94$

$Y_{(V8)} = 62.8 + 9.6 * \ln|x-3.9|$ Adj. $R^2_{(V8)} = 0.97$


90
80
70
60
50
40
30
20
Diameter of colony (mm)
4
6
8
10
12
DAI
PDA
V8

Connecting Text

In the previous chapter, the optimum environmental conditions for spore production in *Phoma* sp. were investigated. In the bioherbicide approach, the typical steps that follow the determination of optimum laboratory growth conditions of the fungus include: (1) investigation of the biocontrol candidate's degree of virulence and methods of improving this feature, if necessary; (2) formulation of a carrier that provides efficient spore application, protection, and preferably enhancement of spore virulence as well as a reduction in dew period requirements; (3) determination of the candidate's host range; (4) determination of the optimal methods for long-term storage of the inoculum. However, preliminary treatment of common ragweed plants with *Phoma* sp. revealed that this fungal species had either lost its virulence to ragweed during storage, or is much less virulent than previously believed. As a result of the reduced *Phoma* sp. virulence, the sequence of steps that would usually be implemented in the bioherbicide strategy were set aside, such that the following chapter is dedicated to experiments that were performed in an attempt to restore *Phoma* sp. virulence for common ragweed, as well as to search for new candidate fungi.

Chapter 4. Effects of fungal pathogens and *O. communa* on *Ambrosia artemisiifolia* L.

4.1. ABSTRACT

The bioherbicide approach to the biological control of weeds allows us to take advantage of the weed's natural enemies. Bioherbicides can also be used in conjunction with other weed control methods to enhance levels of suppression. The objectives of the research reported in this chapter were to evaluate (1) the pathogenic potential of several fungi towards *Ambrosia artemisiifolia* L. and (2) the interactive effects of selected fungal pathogens and other ragweed control strategies. All fungal pathogens were isolated from diseased ragweed tissues, cultured, and applied to ragweed seedlings. A number of factors were evaluated including ragweed growth stage, spore carrier used, spore concentration, and dew period duration and temperature. The interaction between several fungi, the insect *Ophraella communa* LeSage, and the herbicide linuron, were also evaluated. Results demonstrated that ragweed performance was little affected by any of the fungi assayed, including *Phoma* sp. No interactions were detected between the five fungal isolates and the insect *O. communa*. However, a possible interaction was found between the fungal isolate ISO#65 and linuron.

4.2. INTRODUCTION

Although parasites and diseases are common in natural plant communities, rarely do pest infestations reach devastating epidemic proportions under typical conditions (Ross and Lambi 1985; Watson and Wymore 1990; Watson 1999). Many factors limit disease development including low pathogen inoculum levels, weakly virulent pathogens, poor dispersal mechanisms of infectious pathogen propagules, unfavourable moisture and/or temperature conditions, and host defense mechanisms (Watson and Wymore 1990; Watson 1993). The bioherbicide approach of biological control attempts to bypass these constraints by applying a sufficient amount of inoculum (i.e., number of pathogenic propagules), at the appropriate time (i.e., host susceptibility), and under the appropriate conditions (i.e., moisture and temperature conditions, carrier formulation) (Watson 1993; Green *et al.* 1997). Regardless of whether bioherbicides are formulated in solid or in liquid form, the goal of a formulation is to protect the pathogenic propagules (i.e., usually spores) against adverse environmental conditions during the critical stages of germination and infection. Formulations can also serve to nourish the propagules during storage and application, and before the pathogen becomes established in the host (Connick *et al.* 1990; Green *et al.* 1997).

Liquid formulations include such carriers as water, invert emulsions, and oil emulsions, with or without the addition of adjuvants (Connick *et al.* 1990; Green *et al.* 1997). In the early stages of a research program, a simple water carrier is used to evaluate the potential and efficacy of the organism in greenhouse or laboratory conditions (Daigle *et al.* 1990). Whereas in field applications, invert emulsions are particularly useful at bypassing or reducing dew period requirements of a pathogen (Daigle and Connick 1990;

Green *et al.* 1990). Several oils may be used for this type of emulsion, such as paraffin wax, mineral oil, soybean oil, and lethicin (Daigle and Connick 1990).

For decades the dangers of relying on a single method of pest control have been apparent and recognized. Moreover, the benefits and ultimate success of using multiple strategies of control have also been acknowledged (Wilson 1969). The need to integrate the biocontrol strategy with other weed management methods may be required for several reasons: (1) the agent may not be providing the desired level of control; (2) there may be more than one weed that requires control and the potential agent most probably is not effective against all of them; (3) some component of the agricultural management system may not be compatible with the biocontrol agent; (4) or for economic reasons, it may not be advantageous to use the same weed control methods over the entire distribution of the weed problem (Watson and Wymore 1989).

Many examples of integration of both fungal pathogens and chemical pesticides can be cited. Smith (1991) and Klerk *et al.* (1985) have experimented with many chemicals in order to determine which could be integrated with the mycoherbicide COLLEGO®, and found several that could be tank mixed with the bioherbicide. Wymore and Watson (1989) found a synergistic interaction between the herbicide thiadiazuron[13] and *Colletotrichum coccoides* (Wallr.) Hughes to increase velvetleaf mortality, and Charudattan (1993) reported that the fungus *Cochliobolus lunatus* (Drechsler) and the herbicide atrazine could be successfully combined to increase control barnyard grass.

Bioherbicides have also been successfully integrated with insect biocontrol agents. Examples include: (1) control of prickly-pear cactus with the insect *Cactoblastis*

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[13] Refer to Appendix I for chemical name

cactorum (Berg) and the fungus *Gloeosporium lunatum* (E & E) (Wilson 1969), (2) control of Crofton weed *(Eupatorium adenophorum* Spreng.) with a gall fly, a native Ceramybycid root borer insect, and a leaf-spot pathogen (Wilson 1969), and (3) control of waterhyacinth *[Eichhornia crassipes* (Mart.) Solms] with two arthropods, *Neochetina eichhorniae* Warner and *Orthogalumna terebrantis* Wallwork (Charudattan *et al.* 1978).

One very interesting interaction between fungal pathogens and insects results from wounding of host plants by the insect, subsequently allowing the fungal organisms to enter host weed tissues more readily (Wilson 1969; Begon *et al.* 1990). However, Hatcher (1995) demonstrated a case in which pathogen entry into a host could actually be inhibited after the plant had been consumed by phytophagous insects. Indeed, proteinase genes in tomato plants can be activated following animal grazing, thus producing proteinase inhibitors (PI) that are deleterious to most fungi. PI production can also be induced in plants after fungal attacks, and are thought to have negative impact on the ability of insects to digest the plant material.

According to Hatcher (1995), there are four principal categories for classifying biotic interactions, namely synergism, addition, equivalence, and inhibition. When integrating different components of weed control, addition and synergism effects are most desirable. A synergistic interaction between two biotic agents is one whose effects are greater than that obtained by adding the effects of each agent alone, whereas an additive interaction is one whose overall effect is equal to the sum of the effects of each of the agents alone (Hatcher 1995). Inhibition occurs when the overall effect of both agents combined is less than the effect of any one of the agents used alone, and equivalence occurs when the effect of both agents combined is equal to that of the most effective of the agents when used alone (Hatcher 1995).

The specific objectives of the experiments described in this chapter were to: (1) evaluate the pathogenic potential of various fungal organisms isolated from *A. artemisiifolia* tissue; (2) evaluate the interactive effects between several fungal organisms and the phytophagous insect, *O. communa,* and (3) evaluate the interactive effects of the various fungal organisms and the herbicide, linuron.

4.3. MATERIAL AND METHODS

4.3.1. Collection of the fungal organisms

4.3.1.1. Isolation

Throughout the summer of 1998 and 1999, diseased ragweed material was collected from areas on and around the island of Montréal, Québec, Canada, including fields and roadsides in the vicinity of the E.A. Lods Agronomy Research Centre and the Macdonald Campus, both of which are located in Ste-Anne-de-Bellevue. Ragweed plant material was also collected from various vegetable fields in Sherrington, Québec as well as the grounds of the Domtar Research Centre, in Senneville, Québec.

When the disease symptoms were observed on ragweed leaves, pieces of leaf of approximately 2-5 mm^2 in size and comprising the tissues immediately adjacent to the lesion, were removed. The leaf pieces were surface-disinfested by immersing them for 15 seconds in a 0.55% sodium hypochlorite solution, followed by 10 seconds in a 75% ethanol solution. The tissues were subsequently rinsed twice in sterile double-distilled water, and were placed on sterile filter paper[14] to absorb excess water. Leaf tissues were

[14] Fisherbrand® #P8, Fisher Scientific Limited, Montréal, Québec

then placed on PDA and water agar (WA)[15] plates amended with chloramphenicol (chl) and streptomycin sulfate (strp)[16], so as to inhibit colonization of the tissues and agar by bacteria. PDA or WA plates were sealed with a strip of Parafilm® [17] and placed in an incubator at 24 ± 2°C with an alternate light/dark regime of 12 hours. The lighting source was a combination of white light[18] and near-UV light[19]. Immediately after fungal cultures began to grow on the PDA or WA surface, hyphal tips were aseptically removed with a syringe needle and placed on fresh PDA-chl-strp plates, sealed, and returned to the incubator. The 12-hr white and near-UV light regime was selected because this light source was most suitable for fungal growth in previous experiments (see preceding Chapter). Although alternating white light resulted in increased spore yield in *Phoma* sp., the recommendation of Onions *et al.* (1981) and Stevens (1981) to use 12-hr cycles of both near-UV and white light was retained because it is generally most suited for Deuteromycete fungi.

When disease symptoms were found on the stem and petioles, pieces of stem or petioles of approximately 1-2 cm in length and comprising both necrotic tissues and surrounding visibly healthy tissues, were surface-disinfested as described previously and placed on a moist, sterile filter paper in a Petri plate and sealed. Plates were placed in an

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[15] Agar-agar: Mikrobiologie, BDH Inc., Toronto, Ontario

[16] Refer to Apprendix II for recipe

[17] American National Can™, Menasha, WI 54952

[18] TFC: Daylight White fluorescent tubes, FL-15D, 15W, 41cm in length, placed at 25cm over the fungal cultures

[19] Near-ultraviolet (NUV) fluorescent tubes, F15T8BLB (black light) Sylvania Co., placed at 25cm over the fungal cultures

incubator under the same conditions described previously. If fungi colonized the tissue, they were allowed to sporulate. Spores were then carefully collected, added to a small quantity of sterile water (the exact volume of water was dependent on the amount and texture of spores harvested), and spread on PDA-chl-strp and WA-chl-strp plates. Six germinated spores (when possible) were collected using a syringe needle, from either the PDA or WA plates, and individually placed on fresh PDA plates. Plates were sealed and returned to the incubator.

4.3.1.2. Culture maintenance

When the fungal cultures sporulated on PDA plates, spores were collected by flooding the plate with water and gently scraping the colony surface with a sterile spatula. The resulting solution was placed in soil-filled test tubes[20] for long-term storage, and kept at room temperature until the soil was entirely colonized by the fungal mycelium. Tubes were then maintained at 4°C until further use. To initiate new colonies, a few grains of test tube soil were sprinkled on PDA plates and then placed in an incubator.

4.3.2. Common ragweed plant source

Common ragweed seeds were collected before the start of these experiments, with additional seeds being collected during the Fall of 1998 and 1999. Seed sources were varied as many people in many areas collected the seeds. The collected seeds were dry-stored at 4°C. In order to break dormancy, the seeds were mixed into moist sand and kept

[20] Refer to Appendix II for recipe

at 4°C for several weeks before use. After rinsing the sand off the seeds with water, ragweed seeds were immersed in a glass beaker containing 95% sulfuric acid for 10 minutes. The seeds were then gently rinsed with flowing lukewarm tap water for 12 hours. An acid-stable, flexible, 1 mm-mesh screen, held in place on the beaker with an elastic band, prevented the seeds from flowing out of the beaker.

Ragweed seeds were sown in trays containing a mixture of 1/2 commercial potting mix[21] and 1/2 black potting soil. The trays were placed in a growth chamber[22] set at 12 hr darkness at 18°C and 12 hr light (250-300 μmol m^{-2} sec^{-1}, provided by both fluorescent and incandescent lights) at 24°C. Individual seedlings having attained the cotyledon stage were transferred to 10 mm-diameter plastic pots, containing the same potting mixture as use for trays. Seedlings were returned to the growth chamber until they reached the appropriate size for treatment applications. Unless otherwise indicated, treated plants were returned to another growth chamber, set to the same conditions as previously described, following fungal treatment applications.

4.3.3. Viability and pathogenicity of fungal isolates

4.3.3.1. Spore germination tests

To assess the effect of the fungal pathogens on ragweed, spore solutions were applied at various rates and to various stages of growth of the plants. However, before applying each spore solution, germination tests were conducted to verify the viability of

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[21] Pro-mix BX®, Premier Brands, Inc., New York, NY, USA

[22] Controlled Environments Ltd, Winnipeg, Manitoba

spores. For each spore solution, approximately 1 ml of diluted solution (1.0×10^2 spores ml^{-1}) was applied to PDA and WA disks of 1.5 cm-diameter in a covered Petri dish, and left at room temperature for approximately 12 hr. Germinated spores were counted with the use of a compound microscope. The percentage of germinated spores out of a total of 50 spores was then determined.

4.3.3.2. Preliminary applications of Phoma sp.

The goal of these two preliminary applications was to observe the type of lesions produced by *Phoma* sp. on ragweed, and to re-isolate these lesions in order to confirm Koch's postulate. For both experiments, fungal spores were applied using a simple carrier consisting of double distilled water with 1% v/v gelatin[23], and sprayed until runoff with a hand-held sprayer on ragweed plants at the 4-leaf stage. For the first application, a spore concentration of 1.9×10^8 spores ml^{-1} was used and plants were placed for 24 hr in a dew chamber[24] (24 ± 2°C), in the dark. For the second application, a spore concentration of 1.0×10^8 spores ml^{-1} was used and plants were placed for 36 hr. in the dew chamber (24 ± 2°C), in the dark. Lesions that developed were isolated using the same procedure as described in section 4.3.1.1. For all experiments involving fungal spore applications, a replicated control treatment was always included, and consisted of ragweed plants being sprayed with the carrier only.

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[23] BDH Inc., Toronto, CAN

[24] Percival Inc., Boone, Iowa 50036

4.3.3.3. Effects of inoculum spore concentration on Phoma sp. efficacy

To determine the optimal spore concentration to be applied, seven different solutions were applied to ragweed plants at the 2-leaf stage. Solution concentrations were 5×10^2, 5×10^3, 5×10^4, 5×10^5, 5×10^6, 5×10^7, and 5×10^8 spores ml^{-1} and were applied until runoff using a hand-held sprayer, with a simple carrier consisting of double distilled water and one drop of surfactant[25]. Plants were placed in the dew chamber for 24 hr (24 ± 2°C). In addition, pycnidial exudate, collected from a sporulating plated *Phoma* sp. colony using a syringe needle, was spot applied to 6-leaf-stage ragweed plants. These exudates consisted of indeterminate spore concentrates. These treated plants were not placed in a dew chamber afterwards.

4.3.3.4. Effects of dew period and host plant growth stage on Phoma sp. efficacy

A 1.1×10^8 spores m^{-1} spore solution was applied to 6- and 12-leaf ragweed plants at a volume of 100 ml m^{-2}, using a standard spray chamber equipped with an XR-Teejet 8002VS® flat-fan nozzle[26]. The distilled water and surfactant carrier was used, and plants were placed for either 24 or 48 hr. in a dark dew chamber set at 24 ± 2°C.

4.3.3.5. Effects of inoculum spore concentration and host plant growth stage on Phoma sp. efficacy

Two ragweed growth stages (3- and 8-leaf stage) and two spore concentrations (9.6×10^6 and 9.6×10^8 spores ml^{-1}) were used. Spore solutions (water and surfactant

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[25] Refer to Appendix II for recipe

[26] Teejet Spray Nozzles. Spraying Systems Co., P.O. Box 7900, Wheaton, IL, 60189

carrier) were applied until runoff with a hand-held sprayer. Dew period consisted of 24 hr at 24 ± 2°C, in the dark.

4.3.3.6. Effects of dew period and temperature on Phoma sp. efficacy

A 2.4 x 10^7 spores ml^{-1} water and surfactant solution was applied until runoff to plants ranging from the 2- to 6-leaf stage with a hand-held sprayer. Combinations of two dew periods (24 and 48 hr) and two dew period temperatures (22 and 26 ± 2°C) were used.

4.3.3.7. Effects of inoculum carrier on Phoma sp. efficacy

A water and oil carrier[27] was tested against the standard water and surfactant carrier (control) to determine the effect of the oil on efficacy of the fungus. The spore concentration was the same for both carriers (i.e., 1.8 x 10^8 spores ml^{-1}), and was applied in a spray chamber at a volume of 50 ml m^{-2}. Dew chamber period was 24 hr at a temperature of 24 ± 2°C.

4.3.3.8. Application of Phoma *sp. re-isolated from a lesion on treated ragweed*

Phoma sp. isolates I3 and I6 were recovered from lesions on a ragweed plant previously treated with a *Phoma* sp. spore solution (refer to section 4.3.3.2.). Spore solutions of both the I3 and I6 isolates were applied with a hand-held sprayer at concentrations of 4.5 x 10^9 spores ml^{-1} until runoff to ragweed plants from the 4- to 8-leaf stage. In contrast to previous trials, the 4- to 6-leaf ragweed plants were placed in a greenhouse where the mean temperature (22 ± 5°C) immediately after a 24 hr dew period

[27] Refer to Appendix II for recipe

at 24 ± 2°C. Ragweed plants at the 8-leaf stage were also placed in the greenhouse, however these plants were covered with a clear plastic bag for 2 weeks. This procedure maintained a high degree of moisture around the plants thereby maximizing fungal infection and sporulation.

A spore solution of isolate I8 that had been recovered from lesions on ragweed plants treated with isolate I6, was applied to 4- and 6-leaf stage ragweed plants at a concentration of 5.4×10^7 spores ml^{-1}. Spore solutions were applied until runoff with a hand-held sprayer. Treated ragweed plants were placed in the dew chamber for 24 hr at a temperature of 24 ± 2°C.

4.3.3.9. Isolate #3, Curvularia inaequalis

Spore solutions of isolate #3 were applied until runoff on two separate occasions using a hand-held sprayer. For the first application, the spore concentration was 2.4×10^6 spores ml^{-1}. Immediately after spraying, ragweed plants at the 4-leaf stage were placed in a dew chamber for 24 hr (24 ± 2°C). For the second application, the spore concentration was 2×10^6 spores ml^{-1} and dew period was simulated by placing clear plastic bags over the plants for 24 hr, while under greenhouse conditions (22 ± 5°C).

4.3.3.10. Isolates ATT#9, ATT#23, ISO#1, ISO#6, ISO#9, and ISO#34

A water-surfactant spore solution of these isolates was applied to 4-leaf-stage plants, until runoff, using a hand sprayer. Dew period was 24 hr at 24 ± 2°C. The solution concentrations were as followed: ATT#23: 6.9×10^6 spores ml^{-1}, ISO#1: 4.0×10^6 spores ml^{-1}, ISO#6: 8.9×10^7 spores ml^{-1}, ISO#9: 2.0×10^5 spores ml^{-1}, and ISO#34:

1.5 x 10^7 spores ml^{-1}. For ATT#9, pycnidial exudate was collected from a plated colony using a syringe needle, and spot applied directly on ragweed leaves.

4.3.3.11. Effects of dew period on isolates INNA4b and ATT#10

For isolates INNA4b and ATT#10, two different dew periods were evaluated, 24 and 48 hr, both at 24 ± 2°C. Spore suspensions were applied until runoff using a hand-held sprayer. Spore concentrations were 1.4 x 10^7 and 1.3 x 10^7 spores ml^{-1} for INNA4b and ATT#10, respectively.

4.3.3.12. Effects of dew period and temperature on various isolates

For isolates ATT#19, ISO#66, ISO#68, and INNA4a, four combinations of two dew periods (24 and 48 hr) and two dew period temperatures (22 and 26 ± 2°C) were evaluated. Spore solution were applied until runoff using a hand-held sprayer. Spore concentrations were 3.0 x 10^7, 1.8 x 10^7, 2.4 x 10^7, and 2.3 x 10^7 spores ml^{-1} for ATT#19, ISO#66, ISO#68 and INNA4a, respectively.

4.3.3.13. Effects of a paraffin formulation and dew period on various isolates

A paraffin formulation was tested against the standard water-surfactant solution as a carrier for the spore suspensions. This formulation had shown promise in previous research involving *Phoma* sp. (S. Brière, *personal communication*). The formulation consisted of a mixture of several oils and surfactants, paraffin, and water[28]. Spore suspensions of isolates INNA4, INNA4aI1, ISO#26, ISO#65, ISO#67, ISO#68I1, and ISO#69 were formulated using this carrier and were assessed for their ability to cause

[28] Refer to Appendix II for recipe

damage to treated ragweed plants. All solutions were applied with a hand-held sprayer until runoff. Dew periods of 24 and 48 hr at 24 ± 2°C were used for ragweed plants treated with the water-based suspensions, whereas plants treated with the paraffin formulation were not placed in the dew chamber, since this formulation was devised in an attempt to bypass dew requirements for fungal infection. Spore concentrations were as followed: INNA4: 5.6 x 10^6 spores ml^{-1}, INNA4aI1: 4.2 x 10^6 spores ml^{-1}, ISO#26: 6.9 x 10^6 spores ml^{-1}, ISO#65: 3.9 x 10^6 spores ml^{-1}, ISO#67: 3.9 x 10^5 spores ml^{-1}, ISO#68I1: 1.3 x 10^6 spores ml^{-1}, and ISO#69: 1.1 x 10^7 spores ml^{-1}.

4.3.4. Interaction effects from using fungi and insects in combination

To test the interaction between *O. communa* insect damage and fungal infection, two of approaches were used:

1) Adult *O. communa* beetles were placed on ragweed plants at the 4-leaf stage and kept until approximately 10% of the leaf surface area was damaged (i.e., 1-2 days). The plants were placed under netting so that the insects could not escape. After the desired level of plant damage was obtained, the insects were removed and the plants were immediately subjected to the various fungal spore applications.

2) Insect damage was simulated by cutting lesions of no more than 2- to 3-cm in length on the ragweed leaves (i.e., mechanical damage) using sterilized (75% ethanol) scissors. Immediately after cutting the foliage, fungal spore applications were carried out. The degree of ragweed damage to ragweed leaf tissue by mechanical cutting was similar to the levels of damage caused by the beetles in the first approach.

The fungal isolates *Phoma* sp., ISO #34, ISO #65, ISO #67, ISO #68, ISO #69 were used for these experiments, with the following spore concentrations: 8.7×10^7, 3.4×10^7, 2.4×10^8, 4.5×10^7, 5.0×10^8, 9.1×10^7 spores ml^{-1}. Four spore application treatments (with four replications) were carried out using the water-surfactant carrier: (1) damaged plants sprayed with a spore solution, (2) intact plants sprayed with the same spore solution, (3) damaged plants sprayed with the carrier only, and (4) intact plants sprayed with the carrier only (controls). Treated plants were subjected to a 24 hr dew period, and returned to the growth chambers.

4.3.5. Interaction effects from using fungi and a herbicide in combination

Spores of two fungal isolates (ISO #34 and ISO #65) were used in combination with the herbicide linuron to assess the possible interaction effects on ragweed growth and development. The experimental design consisted of six treatments: (1) linuron only, (2) ISO#34 only, (3) ISO#65 only, (4) linuron and ISO#34, (5) linuron and ISO#65, and (6) water-surfactant carrier only (controls), with each treatment replicated six times. Spore concentrations were as follows: 3.2×10^6 spores ml^{-1} for ISO #34 and 3.8×10^6 spores ml^{-1} for ISO #65. Linuron (LOROX DF ®) was applied at a rate of 1.13 kg ai ha^{-1}, which is the minimum recommended rate for linuron for post-emergence control of ragweed in carrots (Anonymous 1999). Treatments were applied to suspected linuron-resistant ragweed plants (5-10 cm tall) initially collected from the same field site in Sherrington, Québec as described in Chapter 2.

Biomass of plants was recorded 20 days after spraying. Differences in mean biomass for the various treatment combinations were evaluated at the P>0.05 level using Tukey's Studentized Range test.

4.4. RESULTS AND DISCUSSION

4.4.1. *Phoma* sp.

All parameters measured as well as treatment results for experiments using *Phoma* sp. are summarized in Table III. Although *Phoma* sp. had previously shown much potential as a biological control agent for *A. artemisiifolia* (Brière *et al.* 1995), findings from this research could not duplicate these earlier results. Indeed, *Phoma* sp. was rarely able to infect ragweed plants, regardless of plant growth stage, spore concentration, or form of carrier used. Even when provided with a 24 hr dew period, *Phoma* sp. was unable to infect host plants, often requiring a minimum 36 hr dew period for successful colonization of host plant tissue to occur. Despite initial colonization of ragweed tissues, lesions remained localized and did not increase in size to any extent. Findings from trials evaluating the effects of both dew period duration and temperature showed that only plants subjected to a 48 hr dew period suffered any visible damage from the fungus. In all instances however, infected ragweed plants eventually "grew out" from any deleterious effects caused by the fungus. By this time, foliar lesions generally covered less than 1% of the total leaf surface area of treated ragweed plants.

It is possible that the *Phoma* sp. isolate may have lost its virulence towards ragweed during the 6 years of storage. This fungus had been stored since 1993 on mineral oil-covered PDA slants, and although this method of storage has been shown to preserve

some fungal cultures for over 20 years, other cultures have been shown to lose their original characteristics after only a few months (Onions *et al.* 1981). As a result of this, lesions were isolated from the plants infected after the preliminary *Phoma* sp. treatments (isolates I1 to I7) (section 4.3.3.2.). Isolates I3 and I6 were re-applied to ragweed, and lesions were once again isolated (I8), cultured, and re-applied, all in an attempt to "revive" the fungus and restore its original virulence (Table II). Unfortunately, none of the re-isolates showed higher levels of virulence than the original *Phoma* sp. colonies.

It has been suggested that very high spore concentrations might have a deleterious effect on infection rates because of (1) high intraspecific competition between individual spores or (2) an inhibitory substance present in the spore matrix (the gel-like pycnidial exudate) preventing spores from infecting leaf tissues (Chung and Wilcoxson 1968; Mondal and Parbery 1992; Mahuku and Goodwin 1998). Indeed, the conidial matrix, which may contain many exopolysaccharides, glycoproteins, and enzymes, might play a role in the survival, differenciation, growth, and pathogenicity of some fungi (Mondal and Parbery 1992; Mahuku and Goodwin 1998). Chung and Wilcoxson (1968) found that *Phoma medicaginis* Malbr. & Roum. conidia did not germinate when crowded, presumably because of an inhibitory substance either produced by the conidia themselves, or by the pycnidia and found in the spore matrix. Since fungal spores used in the present study were not centrifuged, high spore solution concentrations necessarily meant that spore matrix concentrations were also relatively high. However, *Phoma* sp. spore solutions used for germination tests before each spore application were always diluted to 1.0×10^2 spores ml^{-1}, so that the inhibitory effect that the spore matrix might have had on spore germination would not have been detected. As is was, spores exhibited high germination levels even on water agar, a medium containing few nutrients.

Results from trials evaluating the effects of both fungal spore concentration and ragweed growth stage showed that host plant development stage had little impact on disease progress. Moreover, the addition of 7% oil to the aqueous *Phoma* sp. spore suspension carrier did not increase fungal infection rates as expected. It had been anticipated that the presence of the oil might have reduced spore dehydration and thus increased the levels of infection caused by *Phoma* sp..

4.4.2. Other fungal isolates

A complete list of all fungal isolates found on ragweed plants including identification to genus or species, when possible, and location where the isolates were collected is shown in Table IV. A description of the various treatments as well as results of treatment effects from fungal infection of treated ragweed plants are shown in Table V. Treatment effects on ragweed following application of other fungal isolates were similar to findings obtained using *Phoma* sp. No isolate was able to infect ragweed plants after only a 24 hr dew period, regardless of dew temperature, with the exception of isolate ISO#68, which showed limited disease development (i.e., lesions covering less than 1% of the leaf surface) for only one of the replicates.

Only the fungal isolates ATT#9, INNA4a, INNA4b, ATT#10, ISO#65, and ISO#68 were able to cause limited infection on ragweed, and only following an extended dew period of 48 hr. However, none of these isolates had a substantial impact on ragweed growth or survival. For all these isolates, Koch's postulate was confirmed by isolating the resulting lesions, and identifying the PDA cultures as being identical to the original colony. A number of other fungal applications (i.e., isolates ISO#3, ISO#34, and

ATT#19) produced lesions on host plants; however, Koch's postulate could not be confirmed as the recovered fungal colonies did not match the originally applied cultures. The recovered cultures were common saprophytic fungi.

As was carried out for *Phoma* sp. trials, spores of some isolates recovered from lesions on ragweed plants following spore application were re-applied in an attempt to increase virulence of these isolates (i.e., INNA4aI1 recovered from INNA4a lesions and ISO#68I1 recovered from ISO#68 lesions). However, as observed for *Phoma* sp. trials, the recovered isolates did not exhibit a greater degree of virulence compared with the original isolates.

At first glance, it appears illogical that fungi recovered from lesions on ragweed leaves would no longer be pathogenic to this same weed. One possible reason that might explain the apparent lack of pathogenicity of these fungi towards ragweed is that most of these isolates were recovered from ragweed plants late in the season (i.e., August and September), when flowering had ceased and natural senescence had begun. At this stage in the plant life cycle, defense mechanisms are typically reduced such that weakly pathogenic and even saprophytic fungal organisms can readily invade host plant tissues (Agrios 1997). In the spring, few fungi were recovered from younger more actively growing ragweed plants, simply because not many young diseased plants were available at this time, with the exception of mildew-infested plants. Unfortunately, the causal agent of mildew *(Erysiphe cichoracearum* var. *latispora* Fl. Fr., anamorph *Oidium* sp.) is an obligate parasite that cannot be cultured under laboratory conditions. It had been hoped that some of the fungi recovered from older senescing ragweed plants might have demonstrated some pathogenicity to younger ragweed plants, but this was not the case. Considering the epidemiology of fungi, a pathogen might not be present on its host early

in the season because there are very few fungal propagules left from the previous season to initiate the infection process, and/or simply because their propagules disperse poorly, such that appreciable infection of the host only occurs late in the season (Agrios 1997). The bioherbicide approach easily by-passes these two constraints. Unfortunately, none of the fungi recovered from diseased ragweed plants demonstrated a high degree of virulence towards host plants, even when exposed to optimal environmental conditions for disease initiation and development.

An oil-paraffin-water mix carrier was also evaluated for some of the fungal isolates. This carrier had been previously used by Brière *et al. (personal communication)* in 1993 trials using the original *Phoma* sp. culture. In all instances where this formulation was used, ragweed plants from both the control group (plants treated with the carrier only) and the fungal spore-treated group exhibited severe phytotoxic effects from the oil formulation. Indeed, characteristic phytotoxic symptoms were observed including leaf deformities such as curling, mottling, 'burning', and generalized stunting of plants (Muzik 1970; Klingman *et al.* 1982). This formulation was thus abandoned as a potential spore carrier because of its noticeable toxicity to ragweed plant tissue, and because its high production cost would be a powerful deterrent to its use on a commercial basis.

4.4.3. Interaction effects from using fungi and insects in combination

No interactions between the various fungi tested and insect- or mechanically-inflicted ragweed damage were observed (Table VI). The fungal isolates used produced relatively low levels of infection, regardless of whether inocula were applied to intact or damaged plants. As a result of the low virulence of the fungal isolates when used alone, it

was not possible to detect any additional adverse effects on ragweed growth and development from applying inocula to damaged foliage.

4.4.4. Interaction effects from using fungi and a herbicide in combination

Above-ground biomass of ragweed plants treated with the various combinations of the fungal isolates ISO #34 and ISO #65, and the minimum post-emergence recommended rate of the herbicide linuron are shown in Figure 23. As expected, there were no significant differences in biomass between control plants and plants treated with fungal spores only. Similarly, there were no significant differences between the biomass of control plants and the biomass of plants treated only with linuron, although ragweed biomass was reduced compared with that of controls and plants treated with only fungal spores. These results were not surprising considering the findings of experiments described in Chapter 2, where the 1/2X rate of linuron (i.e., the rate use in this trial) caused little damage to resistant (R) ragweed biotypes.

Ragweed plants treated with spores from isolate ISO#34 and linuron showed no significant differences in biomass compared with plants treated with linuron only, which indicates that there are no interactive effects between this fungal isolate and linuron.

Although the biomass of ragweed plants treated with fungal spores of ISO#65 and linuron is not significantly different from the biomass of plants treated with linuron only, it is nonetheless significantly lower than the biomass of control plants. This finding indicates a possible interactive effect between the fungal isolate ISO#65 and linuron. The impact of this treatment combination on ragweed growth warrants further research. It is possible that higher use rates of linuron could have increased ragweed mortality.

However, it is also likely that higher herbicide use rates could adversely affect ISO#65 survival and/or virulence. Further research is required to determine possible additive and/or synergistic effects of increasing linuron application rates, use of different carriers, use of different fungal isolates (e.g., ATT#9, INNA4a, INNA4b, ATT#10, and ISO#68) and specific environmental conditions.

Table III: Treatment descriptions and results for all *Phoma* sp. spore application trials

Ragweed growth stage	Reps	Spore concentration (spores ml^{-1})	Volume applied	Spore germination	Carrier	Dew period and temperature	Results
4 leaves	6	1.93×10^8	Runoff	> 95%	Water + 1% gelatin	24 hrs (24 ± 2°C)	No lesions
4 leaves	6	10^8	Runoff	> 95%	Water + 1% gelatin	36 hrs (24 ± 2°C)	Few lesions (< 1% coverage)
2 leaves	4	5×10^2 5×10^3 5×10^4 5×10^5 5×10^6 5×10^7 5×10^8	Runoff	> 95%	Water + surfactant*	24 hrs (24 ± 2°C)	No lesions
6 leaves	6 spots	Very high (pycnidial exudates)	Spot applic-ations	> 95%	---	None	Lesions at the site of application; lesions did not spread
6 leaves 12 leaves	6 4	1.10×10^8	$100ml/m^2$	> 95%	Water + surfactant	24hrs & 48hrs (24 ± 2°C)	Few lesions (48hrs) (< 1% coverage)
8 leaves 8 leaves 3 leaves 3 leaves	4 4 4 4	9.63×10^6 9.63×10^8 9.63×10^6 9.63×10^8	Runoff	> 95%	Water + surfactant	24 hrs (24 ± 2°C)	No lesions
2-6 leaves	3	2.4×10^7	Runoff	> 95%	Water + surfactant	4 combinations: 24hrs & 48hrs 22°C & 26°C	48 hrs (22°C & 26°C): few lesions (< 1% coverage)

* Surfactant: 85% Span 80® and 15% Tween 80®, by volume

Ragweed growth stage	Reps	Spore concentration (spores ml^{-1})	Volume applied	Spore germination	Carrier	Dew Period and Temperature	Results
4 leaves	6	1.78×10^8	$50ml/m^2$	Fresh: 98.5% After 96hrs at 7°C For spores with and without oil	Water and 7% oil** and Water + surfactant only	24 hrs (24 ± 2°C)	No lesions
Phoma I3 and I6						4-6 lves: 24hrs 24 ± 2°C	No lesions on the 4- and 6-leaved plants Very few lesions on the 8-leaved plants, with no pycnidial production
4 leaves	2	4.5×10^9	Runoff	> 95%	Water + surfactant	8 leaves: plastic bag left on them	
6 leaves	2						
8 leaves	2						
Phoma I8						24hrs 24 ± 2°C	No lesions
4 leaves	3	5.4×10^7	Runoff	> 95%	Water + surfactant		
6 leaves	3						

** 2% surfactant (85% Span 80® + 15% Tween 80®) in canola oil, by volume; 7% of this mixture added to distilled water.

Table IV: Origin and identification of each fungal isolate collected from *A. artemisiifolia*, unless otherwise indicated.

Isolates	Origin	Identification
ISO #1	Macdonald Campus of McGill grounds, Ste-Anne-de-Bellevue	N/A
ISO #3	Macdonald Campus of McGill grounds, Ste-Anne-de-Bellevue, on *Taraxacum officinalis*	*Curvularia inaequalis*
ISO #6	Macdonald Campus of McGill grounds, Ste-Anne-de-Bellevue	N/A
ISO #9	Macdonald Campus of McGill grounds, Ste-Anne-de-Bellevue	N/A
ISO #26	Macdonald Campus of McGill grounds, Ste-Anne-de-Bellevue	N/A
ISO #34	Macdonald Campus of McGill grounds, Ste-Anne-de-Bellevue	*Fusarium* sp.
ISO #65	Emile E. Lods Agronomy Research Centre, Ste-Anne-de-Bellevue	*Phoma / Dendrophoma* sp.
ISO #66	Emile E. Lods Agronomy Research Centre, Ste-Anne-de-Bellevue	*Alternaria alternata*
ISO #67	Emile E. Lods Agronomy Research Centre, Ste-Anne-de-Bellevue	*Phoma / Dendrophoma* sp.
ISO #68	Emile E. Lods Agronomy Research Centre, Ste-Anne-de-Bellevue	*Phoma / Dendrophoma* sp.
ISO #68 I1	Isolated from ragweed inoculated with ISO #68 and that developed lesions	*Phoma / Dendrophoma* sp.
ISO #69	Emile E. Lods Agronomy Research Centre, Ste-Anne-de-Bellevue	*Phoma / Dendrophoma* sp.
ATT #9		N/A
ATT #10	Emile E. Lods Agronomy Research Centre, Ste-Anne-de-Bellevue	*Fusarium* sp.
ATT #19	Emile E. Lods Agronomy Research Centre, Ste-Anne-de-Bellevue	N/A
ATT #23	Collected on ragweed seedlings from Californian seeds, USA	*Periconia* sp.
INNA 4	Macdonald Campus of McGill grounds, Ste-Anne-de-Bellevue	*Phoma / Dendrophoma* sp.
INNA 4a	Macdonald Campus of McGill grounds, Ste-Anne-de-Bellevue	*Phoma / Dendrophoma* sp.
INNA 4a I1	Isolated from ragweed inoculated with INNA 4a and that developed lesions	*Phoma / Dendrophoma* sp.
INNA 4b	Macdonald Campus of McGill grounds, Ste-Anne-de-Bellevue	*Phoma / Dendrophoma* sp.

Table V: Treatment descriptions and results for all fungal isolate spore application trials, with the exception of *Phoma* sp.

Isolate	Ragweed growth stage	Reps	Spore concentration (spores ml^{-1})	Volume applied	Spore germination	Carrier	Dew period and temperature	Results
ISO#1	4 leaves	3	4×10^6	Runoff	> 90%	Water + surfactant *	24 hrs (24 ± 2°C)	No lesions
ISO#3	4 leaves	3	2.4×10^6	Runoff	> 90%	Water + surfactant	24 hrs (24 ± 2°C)	No lesions
ISO#3	4 leaves	3	1.98×10^6	Runoff	> 95%	Water + surfactant	24 hrs (plastic bag) 22 ± 5°C	Few lesions (< 1% coverage) Not recovered as *Curvularia* sp.
ISO#6	4 leaves	3	8.9×10^7	Runoff	98%	Water + surfactant	24 hrs (24 ± 2°C)	No lesions
ISO#9	4 leaves	3	2×10^5	Runoff	99%	Water + surfactant	24 hrs (24 ± 2°C)	No lesions
ISO#26	2-4 leaves	3	6.88×10^6	Runoff	Water: >95% P.F.**: 50%	Water + surfactant and P.F.	24 & 48 hrs at 24 ± 2°C None for P.F.	All plants treated with P.F. died, including P.F. controls No lesions for others
ISO#34	4 leaves	2	1.49×10^7	Runoff	> 90%	Water + surfactant	24 hrs (24 ± 2°C)	1 plant completely destroyed Other plant: <1% coverage Not recovered as ISO#34
ISO#65	2-6 leaves	3	3.89×10^6	Runoff	Water: 86% P.F.: 85%	Water + surfactant and P.F.	24 & 48 hrs at 24 ± 2°C None for P.F.	24 hrs: no lesions 48 hrs: 1 plant with many lesions. All P.F. plants dead
ISO#66	2-6 leaves	3	1.75×10^7	Runoff	> 95%	Water + surfactant	4 combinations 24 & 48hrs 22 & 26°C	No lesions

Isolate	Ragweed growth stage	Reps	Spore concentration (spores ml^{-1})	Volume applied	Spore germination	Carrier	Dew period and temperature	Results
ISO#67	2-6 leaves	3	3.88×10^5	Runoff	Water: 84% P.F.: 75%	Water + surfactant and P.F.	24 & 48 hrs at 24 ± 2°C None for P.F.	Water: no lesions P.F.: Oil toxicity damage, but no lesions.
ISO#68	2-6 leaves	3	2.35×10^7	Runoff	> 95%	Water + surfactant	4 combinations 24 & 48hrs 22 & 26°C	24hrs: 0 - < 1% 48hrs, 22°C: 0-26% coverage 48 hrs, 26°C: <1 – 10% coverage Eventually all became < 1%
ISO#68 I1	2-4 leaves	3	1.25×10^6	Runoff	Water: >95% P.F.: 80%	Water + surfactant and P.F.	24 & 48 hrs at 24 ± 2°C None for P.F.	Water: no lesions P.F.: both P.F.+ spores and P.F. controls died
ISO#69	2-6 leaves	3	1.06×10^7	Runoff	Water: 75% P.F.: 70%	Water + surfactant and P.F.	24 & 48 hrs at 24 ± 2°C None for P.F.	Water: no lesions P.F.: both P.F.+ spores and P.F. controls died
ATT#9	4 leaves	4	Very high (pycnidial exudates)	---	> 95%	---	None	Lesions at the site of application that did not spread
ATT #10	2-6 leaves	3	1.28×10^7	Runoff	> 95%	Water + surfactant	24 & 48hrs (24 ± 2°C)	Very few lesions (48 hrs) (< 1% coverage)
ATT #19	2-6 leaves	3	3×10^7	Runoff	> 90%	Water + surfactant	4 combinations 24 & 48hrs 22 & 26°C	24 hrs: no lesions 48 hrs: very few lesions, < 1%, not recovered as att#19
ATT #23	4 leaves	3	6.88×10^6	Runoff	> 95%	Water + surfactant	24 hrs (24 ± 2°C)	No lesions

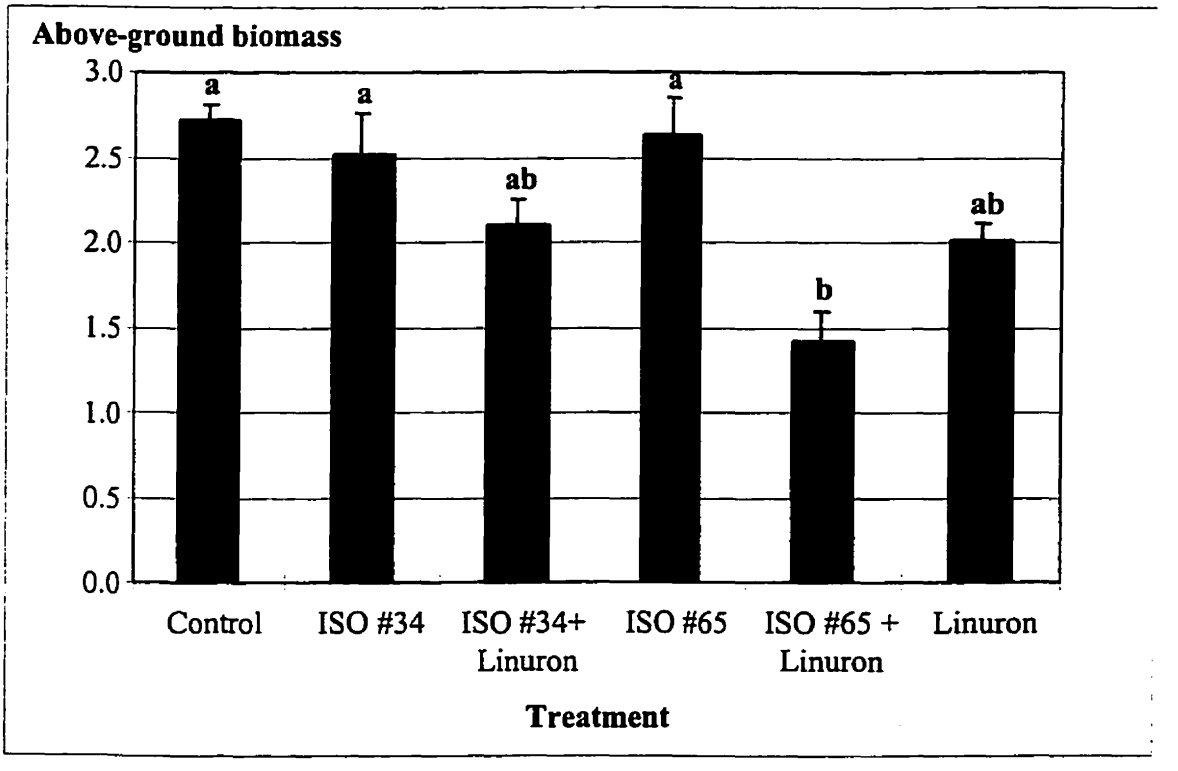
Isolate	Ragweed growth stage	Reps	Spore concentration (spores ml^{-1})	Volume applied	Spore germination	Carrier	Dew period and temperature	Results
INNA4	2-6 leaves	3	5.63×10^6	Runoff	Water: 98% P.F.: > 90%	Water + surfactant and P.F.	24 & 48 hrs at 24 ± 2°C None for P.F.	No lesions. Plants treated with P.F. suffered oil toxicity damage.
INNA 4a	2-6 leaves	3	2.3×10^7	Runoff	> 95%	Water + surfactant	4 combinations 24 & 48hrs 22 & 26°C	24 hrs: no lesions 48 hrs, 22°C: <1% - 45% lesion coverage 48 hrs, 26°C: 0 – 5% coverage See text for details
INNA 4a I1	2-4 leaves	3	4.23×10^6	Runoff	Water: >95% P.F.: > 90%	Water + surfactant and P.F.	24 & 48 hrs at 24 ± 2°C None for P.F.	Same as INNA4a
INNA 4b	2-4 leaves	3	1.28×10^7	Runoff	Water: >95% P.F.: > 90%	Water + surfactant and P.F.	24 & 48 hrs at 24 ± 2°C None for P.F.	Very few lesions (48 hrs) (< 1% coverage)

* Surfactant: 85% Span 80® and 15% Tween 80®, by volume
** Paraffin formulation: Refer to Appendix II for recipe

Table VI. Treatment descriptions and results for all fungal isolate spore applications in combination with either *O. communa* insect feeding damage or mechanical damage

Isolates	Spore concentration (spores ml^{-1})	Type of damage	Results
Phoma sp.	8.7×10^7	Insect	Less than 1% of total foliage surface covered with lesions for damaged plants treated with spores, consisting of small necrotic spots that did not spread.
ISO #34	3.4×10^7	Insect	Same as above
ISO #65	2.4×10^8	Mechanical	No damage for any treatment
ISO #67	4.5×10^7	Mechanical	No damage for any treatment
ISO #68	5.0×10^8	Insect	No damage for any treatment
ISO #69	9.1×10^7	Mechanical	Same as for *Phoma* sp.

Figure 23: Above-ground biomass (g) (and standard errors) of ragweed plants subjected to six different treatments: Controls (water carrier only), fungal isolate ISO#34 spore solution only, ISO #34 spore solution and linuron, fungal isolate ISO#65 spore solution only, ISO#65 and linuron. Bars having the same letter are not significantly different at the $P > 0.05$ level according to the Tukey's Studentized Range Test.


Above-ground biomass
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
ab
a
b
ab
Control
ISO #34
ISO #34+ Linuron
ISO #65
ISO #65 + Linuron
Linuron
Treatment

Chapter 5. General conclusions

The level of linuron-resistance of an *A. artemisiifolia* L. biotype collected from a carrot field in Southwestern Québec was assessed. Optimal environmental conditions for maximum sporulation of *Phoma* sp., a fungus that had shown potential as a bioherbicide for common ragweed, in laboratory conditions, was also determined. Furthermore, the pathogenicity towards common ragweed of *Phoma* sp. and several other fungi species isolated from diseased ragweed plants was assessed.

The resistance of common ragweed to linuron has been suspected by farmers of Southwestern Québec for over a decade, but had never been studied and documented. Results of the experiments described in Chapter 2 confirm this linuron resistance. The ragweed biotype collected in a carrot field in Sherrington showed a resistance ratio of 9.09, which is more than other resistance ratios found for several other weed species. Furthermore, field applications of reduced-rates of linuron when ragweed seedlings are just emerging from the soil do not seem to offer any appreciable control of ragweed that has developed a resistance to linuron.

Many projects concerning biocontrol of ragweed, including this present thesis and an extensive project to develop the insect *O. communa* as a biocontrol agent for this weed, are based on the fact that many carrot growers of Southwestern Québec are observing more and more linuron-resistant ragweed in their fields. Results from experiments described in Chapter 2 will serve to confirm and support this observed herbicide resistance.

The fungus *Phoma* sp. had reportedly shown a good potential as a biological control agent against ragweed in laboratory experiments performed in 1993, shortly after

the fungus' discovery on ragweed plants on the Macdonald Campus grounds. Based on these findings, the experiments in Chapter 3 were conducted, as the original objectives of this thesis were to develop *Phoma* sp. for large-scale production and utilization, and revealed that *Phoma* sp. would yield itself well to laboratory production. However, it became apparent that *Phoma* sp. has perhaps permanently lost its virulence against ragweed during storage, following several applications and re-isolations that were carried out in order to revive *Phoma* sp.'s virulence. A series of experiments using the oil-paraffin formulation showed evidence that ragweed mortality observed during the 1993 trials might have been due to toxicity of the spore carrier itself, and not to *Phoma* sp. infections. Oils have been extensively used as herbicides, but even if this oil-paraffin formulation were the cause of ragweed death, its wide-scale use as a ragweed herbicide would not be possible due to its high viscosity that would hinder its application, and important production costs which would make it uneconomical.

Several other fungal isolates were collected from ragweed because of *Phoma* sp.'s lack of aggressiveness towards this weed. Most of these isolates were collected from senescing ragweed plants, as very few young diseased ragweed individuals were found. Fungi found in senescing plants tissues would most probably be weakly parasitic and even saprophytic species. However virulent species, that could not attack the plants earlier in the season because of poor dispersal mechanisms for example, might have been present on these plants. The results of the experiments described in Chapter 4 show that none of the fungal isolates show much promise as ragweed biocontrol agents, although isolate ISO#65 and linuron used together might achieve interesting ragweed-control results.

Based on the results obtained from the experiments described in the present thesis, further steps to be taken would be to examine ISO#65 and linuron's interaction more closely, by using different spore and herbicide concentrations, carriers, and environmental conditions. Furthermore, continuing the search for fungi on diseased ragweed plants in other parts of the weed's habitat might yield many other fungal candidates, as very virulent common ragweed fungal pathogens might exist but be very limited in their distribution.

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Appendix I: Herbicide Chemical Names

2,4-D: 2,4-(dichlorophenoxy)acetic acid], atrazine [6-chloro-N-ethyl-N'(1-methylethyl)-1,3,5-triazine-2,4-diamine

2,4-DB: 4-(2,4-dichlorophenoxy)butanoic acid

Acifluorfen: 5-[2-chloro-4(trifluoromethyl)phenoxy]-2-nitrobenzoic acid

Atrazine: 6-chloro-*N*-ethyl-*N'*-(1-methylehtyl)-1,3,5-triazine-2,4-diamine

Bentazon: 3-(1-methylethyl)-(1H0-2,1,3-benzothiadiazine-4(3H)-one 2,2-dioxine

Chlopyralide: 3,6-dichloro-2-pyridinecarboxylic acid

Chlorimuron ethyl: ethyl 2-[[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino] carbonyl]amino]sulfonyl]benzoate

Desmedipham: ethyl[3-[[(phenylamino)carboxyl]oxy]phenyl]carbamate

Dicamba: 3,6-dichloro-2-methoxybenzoic acid

Diquat: 6,7-dihydrodipyrido(1,2-α:2',1'-*c*)pyrazinediium ion

Diuron: N'-(3,4-dichlorophenyl)-N,N-dimethylurea

Fomesafen: 5-[2-chloro-4-(trifluoromethyl)phenoxy]-*N*-(methylsulfonyl)-2-nitrobenzamide

Glyphosate: *N*-(phosphonomethyl)glycine

Imazapyr: (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-3-pyridinecarboxylic acid

Imazethapyr: (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid

Linuron: N'-(3,4-dichlorophenyl)-N-methoxy-N-methylurea

MCPA: (4-chloro-2-methylphenoxy) acetic acid

Metolachlore: 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) autamide

Metribuzin: 4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one

Naptalam: 2-[(1-naphthalenylamino)carbonyl]benzoic acid

Paraquat: 1,1'-dimethyl-4-4'bibyridinium ion

Prometryne: *N,N'*-bis(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine

Pyrazone: 5-amino-4-chloro-2-phenyl-3(2*H)*-pyridazinone

Pyridate: *O*-(6-chloro-3-phenyl-4-pyridazinyl) *S*-octyl carbonothioate

Simazine: 6-chloro-*N,N'*-diethyl-1,3,5-triazine-2,4-diamine

Thidiazuron: *N*-phenyl-*N'*-1,2,3-thidiazol-5-yl-urea

Triclopyr: [(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid

Trifluarin: [2,6-dinitro-*N,N*-dipropyl-4-(trifluoromethyl)benzenamine]

Appendix II: Recipes and Formulations

Chloramphenicol and Streptomycin PDA and WA: 0.1g of both antibiotics per litre of DIFCO PDA or Mikrobiologie agar-agar. Chloramphenicol was added to the media prior to autoclaving because of its heat-stability. Streptomycin sulfate must be added after autoclaving under aseptic conditions.

Copper-sulfate glue: This glue is used to maintain the cigarette paper caps on the storage soil tubes, and acts as a mycocide to prevent fungal contamination. Two grams of copper sulfate and 20 g of gelatin are added to 100ml of double-distilled water, and gently heated over a hot plate until all ingredients dissolve.

Half-PDA: 12g DIFCO Potato Dextrose Agar, 20g Mikrobiologie agar-agar, 1L double-distilled water, autoclaved at 121°C for 20 minutes.

Long-term storage in soil-filled test tubes: Oatmeal flour (produced by grinding rolled oat in a domestic coffee grinder and sieving through a 1mm mesh sieve) is uniformly mixed into black soil (sieved in a 2mm mesh sieve) in 1% w/w proportions. Glass test tubes are filled to 3/4 with this soil mix, to which 3-4 ml of distilled water are added. The tubes are plugged with cotton and autoclaved for 45 minutes at 121°C. Forty-eight hours later, the tubes are autoclaved again, and allowed to cool. Once the tubes are inoculated with the desired fungus, the cotton plug is pushed in its entirety inside the tube, making sure that the cotton does not come in contact with the soil surface. Excess cotton is

burned off. Finally, tubes are capped with a layer of cigarette paper held in place by a copper sulfate glue, to keep mites and fungal contaminants out. Excess paper is gently burned off.

Water / oil carrier: The water/oil formulation consisted of 2% surfactant (85% Span 80® + 15% Tween 80®) added, by volume, to vegetable oil (Crisco® canola oil, Procter & Gamble, Toronto, Ontario), and 7% of this mixture added to distilled water.

Paraffin formulation: Melted white paraffin (2.5g) is added to 45ml of Orchex® oil and 2.5ml of BRIJ93 oil (polyoxyethylene, oleyl ether, 0.01% BHA and 0.005% citric acid; ICI Americas Inc., Wilmington, Delaware, 19897), and mixed thoroughly in a blender. A mixture of 50ml distilled water, one drop of Tween 80®, 1g of sucrose, and fungal spores are gently added to the oil mix, blending regularly.

Surfactant: 85% Span 80® and 15% Tween 80®, by volume