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Alternaria alternata f. sp. *sphenocleae*, a Potential Mycoherbicide of Gooseweed (*Sphenoclea zeylanica* Gaertner)

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Short title:

An *Alternaria alternata* to control gooseweed (*Sphenoclea zeylanica)*

Rhomela Favila Masangkay

Abstract

A foliar pathogen identified as a member of the genus *Alternaria* was isolated from blighted *Sphenoclea zeylanica* (gooseweed) collected in 1991 from a rice field near Los Baños, Laguna, Philippines. Inoculum density, dew period, and plant height are factors influencing biocontrol of *S. zeylanica* with this indigenous pathogen. Significantly higher percent reductions in plant height and dry weight were obtained and all plants were killed at higher inoculum concentrations with 8 h of dew. The number, germination, and virulence of conidia were significantly affected by production techniques, temperature, light condition, and incubation period. Exposure to continuous near-ultraviolet (NUV) light at 28°C stimulated sporulation on agar media and on solid substrates. Overall, the best production technique was the use of sorghum seeds using an equal quantity of sorghum seeds and water (w/v) incubated for four weeks. Another conidia production method using the sporulation medium (S-medium) technique was evaluated with the addition of 20 g L^{-1} of calcium carbonate ($CaCO_3$) and 2 ml of sterile distilled water. Primary 1/2 PDA at 18°C in the dark produced the most virulent conidia. This technique produced conidia relatively rapid, but was labour intensive. Host range studies using 49 plant species in 40 genera representing 20 families, selected by using a modified centrifugal phylogenetic and variety strategy indicated that only *S. zeylanica* was susceptible in the absence and presence of supplemental dew. On the basis of morphological and cultural characteristics, pathogenicity on the host, host specificity, and the absence of a previous record of this fungal pathogen on *S. zeylanica*, the binomial *A. alternata* f. sp. *sphenocleae* is proposed.

Résumé

Un agent pathogène foliaire appartenant au genre *Alternaria* a été isolé de feuilles nécrosées par *Sphenoclea zeylanica* dans un champs de riz près de Los Baños aux Philippines en 1991. Des facteurs, tels la densité de l'inoculum, la période de rosée et la taille des plants ont influencé la performance de ce champignon pathogène comme agent de biocontrôle de *S. zeylanica*. Une période de rosée de huit heures et un taux d'inoculum élevé ont permis d'obtenir une réduction significative de la taille et du poids des plants ainsi qu'un taux de mortalité de 100%. Le substrat, les conditions de lumière, la température et la période d'incubation ont eu un effet significatif sur le nombre, le taux de germination et la virulence des conidies. L'exposition continue à une lumière de type ultraviolet-proche à une température de 28°C a stimulé la sporulation sur les milieux gélosés et sur les substrats solides. La meilleure technique de production a été d'utiliser le sorgho comme substrat de croissance avec une proportion égale d'eau (p/v). Une autre technique de production, le milieu S, consistait à incorporer du carbonate de calcium (20 g L^{-1}) à un milieu gélosé et de l'inoculer avec une culture obtenue sur 1/2 PDA, à 18°C et dans le noir. Malgré la performance obtenue, cette technique n'est pas retenue puisqu'elle nécessite beaucoup de manipulations. Les travaux pour déterminer la gamme d'hôtes potentiels ont porté sur 49 espèces de plantes appartenant à 40 genres et regroupées dans 20 familles. La sélection a été effectuée grâce au système phylogénétique centrifuge modifié et a permis de démontrer que seule *S. zeylanica* était susceptible au champignon et ce, avec ou sans période de rosée. Considérant les critères morphologiques de l'isolat, la gamme d'hôte potentiel et l'absence de record précédent, nous proposons l'appellation *A. alternata* f. sp. *sphenocleae*.

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This humble piece of work is not the author's sole effort but with the inspiration and assistance of those who, in one way or another, extended their helping hands, who all deserve to be given recognition and thanks.

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("Maraming-maraming salamat sa inyong lahat").

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Description of Thesis Format

This thesis is comprised of original papers that will be submitted to appropriate scientific journals for publication. In accordance with Part B, Section 2 of the "Guidelines Concerning Thesis Preparation" from the Faculty of Graduate Studies and Research, McGill University, I quote the entire text that applies to this format:

> *2/ Manuscripts and Authorship: "Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of the published paper(s). These texts must be bound as an integral part of the thesis. If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated. The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include a table of contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list. Additional material must be provided (e.g. in appendices) and in sufficient detail 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 as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements 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 interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as*

an examiner for that thesis." In order for this thesis to be consistent with the above statements, it is structured in the following manner:

The thesis begins with abstracts in English and French, followed by a table of contents. Chapter 1 comprises a general introduction in which background knowledge and current status of the thesis research subject is presented. This section concludes with an outline of the specific objectives of the thesis. The next five chapters constitute the body of the thesis, each chapter being one complete manuscript.

Chapter 2 manuscript is to be submitted for publication to *Mycologia.*

Chapter 3 manuscript is to be submitted for publication to *Plant Disease.*

Chapter 4 manuscript is to be submitted for publication to *Canadian Journal of Microbiology.*

Chapter 5 manuscript is to be submitted to *Biocontrol Science and Technology.*

Chapter 6 manuscript is to be submitted to *Crop Protection.*

The various manuscript chapters are linked via connecting texts so as to establish logical bridges between the different papers.

A general discussion and synthesis of the major conclusions of the thesis are presented in Chapter 7. The main contributions to knowledge of this research are outlined in Chapter 8. An Appendix section contains all the analysis of variance tables from the experiments conducted in this thesis.

Manuscripts from Chapters 2, 3, 4, and 5 are co-authored by Dr. T. C. Paulitz and Dr. S. G. Hallett. The candidate (Rhomela F. Masangkay) performed all the experimental research, statistical analyses, and is the primary author of all four manuscripts. Dr. T. C. Paulitz and Dr. S. G. Hallett provided supervisory guidance and assisted in manuscript preparation. The manuscript from Chapter 6 is co-authored by Ms. M. A. Galon, Dr. T. C. Paulitz, and Dr. A. K. Watson. Ms. M. A. Galon, a research assistant at the International Rice Research Institute (IRRI), completed the experiment at IRRI in Los Baños, Laguna, Philippines under the supervision of Dr. A. K. Watson. The candidate (Rhomela F. Masangkay) developed and wrote the protocol of this study and initiated the research during her six months in 1993 at IRRI

conducting research for her Ph.D. thesis. The candidate also performed all the experimental analyses and is the primary author of this manuscript. Dr. T. C. Paulitz provided supervisory guidance and assisted in manuscript preparation.

Chapter 1. General Introduction

1.1. General remarks on the biology of *Sphenoclea zeylanica*

1.1.1. Importance, distribution, and nomenclature

Sphenoclea zeylanica Gaertner, commonly known as gooseweed (English) or silisilihan (Pilipino), is a member of the family Sphenocleaceae (Lindl.) Mart. ex DC. (Cardenas *et al.*, 1972; Holm *et al.*, 1977). It is an annual herbaceous broadleaf weed species native to tropical Africa (Holm *et al.*, 1977; Waterhouse, 1993) that is distributed in all tropical and subtropical regions of the world. It is considered a serious weed of rice (*Oryza sativa* L.) throughout the Caribbean area and in Guyana, India, Pakistan, Southeast Asia, and West Africa and a principal weed of rice in Surinam, Trinidad, and the United States (Holm *et al.*, 1977). *S. zeylanica* is among the 32 worst agricultural weeds in Southeast Asia (Waterhouse, 1993; 1994) and is important locally in Myanmar, Laos, Vietnam, Brunei, and Indonesia, widespread and important in the Philippines and Kampuchea, and very widespread and very important in Malaysia. It is the dominant broadleaf weed species in irrigated rice in Central Luzon, Philippines (Medrano *et al.*, 1993), in major rice granaries in Peninsular Malaysia (Azmi *et al.*, 1994), and in lowland rice in northwestern Nigeria (Imeokparia, 1989). It is among the top four noxious weed species in rice in Chiang Mai, Thailand (Kittipong *et al.*, 1995); a troublesome weed in paddy fields, especially in submerged areas in Central Thailand (Premasthira and Zungsonthiporn, 1995) and in India (Mukhopadhyay, 1995); and a common broadleaf paddy weed in Indonesia (Tjitrosemito, 1994) and in Pakistan (Shad, 1986).

S. zeylanica thrives in almost any kind of wet habitat at altitudes below 350 m (Holm *et al.*, 1977). It grows in seasonal swamps or depressions, which are periodically inundated (Pancho *et al.*, 1969; Holm *et al.*, 1977); in irrigation and drainage canals (Smith and Shaw, 1966); in open wet places, in mudholes, and in poorly drained soils (Pancho *et al.*, 1969); on the mud of tidal creeks in Africa (Holm

et al., 1977); and in submerged soils during the dry season (Premasthira and Zungsonthiporn, 1995).

It has been reported that *S. zeylanica* strongly competes for light, water, and nutrients in rice systems, reducing yields by as much as 45% (Laganao, 1981; Ampong-Nyarko and De Datta, 1991; Biswas and Sattar, 1991). Significant yield reductions in transplanted rice can, however, occur at weed densities as low as 20 plants m^{-2} (IRRI, 1989). It can also pose problems when it remains green and succulent by interfering with harvesting procedures (Migo *et al.*, 1986).

However, research has also demonstrated that this weed can be beneficial. Young plants and tips of older plants are steamed and eaten with rice in Java, Indonesia (Holm *et al.*, 1977) and in Thailand (Jackquat and Bertussa, 1990). In India, the production of toxic exudates by *S. zeylanica* has been shown to provide 99% control of rice root nematodes (*Hirschmanniella* spp.) (Mohandas *et al.*, 1981). In Thailand, decomposed *S. zeylanica* in submerged soil can inhibit growth and can delay flowering of such weeds as white head (*Eclipta prostrata* Linn.), red sprangletop (*Leptochloa chinensis* (L.) Ness), and barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) (Premasthira and Zungsonthiporn, 1995). Interestingly, *S. zeylanica* residues have some manurial value in rice (Dubey, 1982).

Early reports indicated that *S. zeylanica* was only a weed in rice (Holm *et al.*, 1977). However, recent findings have revealed that this weed is also associated with other crops. It is an important weed in cotton (*Gossypium hirsutum* L.) in Louisiana, U.S.A. (Sanders, 1990). It is also a serious weed in wheat (*Triticum aestivum* L.) in wheat-rice rotations in India (Khan *et al.*, 1988) and in soybean [(*Glycine max* (L.) Merr.)] in rice-soybean rotations in Thailand (Vongsaroj, 1994). Moreover, Harada (1994) claimed that *S. zeylanica* is a piscicidal weed when grown in aquatic environments.

1.1.2. Description of Sphenoclea zeylanica

Holm *et al.* (1977) described *S. zeylanica* as *"a fleshy, erect, annual marsh herb; **stems** erect, often much-branched, 7 to 150 cm; **leaves** alternate, oblong to lanceolate-oblong, gradually tapering at both ends, apex sometimes acute, smooth, 2.5 to 16 cm long, 0.5 to 1.5 rarely 5 cm wide; **petiole** 0.3 to 30 mm long; **inflorescence** in dense spikes, cylindrical, 0.75 to 7.5 cm long, 5 mm in diameter; **peduncle** slender, 1 to 8 cm long; **flowers** sessile, wedge-shaped below, attached longitudinally to the rachis by a linear base; **calyx** five-lobed, triangular (deltoid), semi-circular; **corolla** whitish or greenish white, occasionally mauve-tinged, 2.5 to 4 mm long, united slightly more than half way; **stamens** five, alternating with the corolla lobes and free or attached at the corolla base, filaments slightly dilated at base; **ovary** two-celled, ovules numerous; **style** short; **fruit** a flattened globose capsule, 4 to 5 mm in diameter opening transversely; **seed** yellowish brown, 0.5 mm long."*

S. zeylanica can easily be recognized by its cord-like roots, hollow stems, bracts that subtend the inflorescence, and white, terminal densely spike-like inflorescence. The flowers develop first at the base, then toward the apex and stamens are inserted halfway between the corolla lobes (Pancho *et al.*, 1969; Holm *et al.*, 1977). Moreover, it has 15 to 100 fruits which are non-fleshy and dehiscent and the capsules are circumscissile.

Vegetative growth in *S. zeylanica* is rapid, with flowering typically taking place within 35 to 70 days (Laganao, 1981). In the Philippines, flowering occurs throughout the year. Almost every flower on every inflorescence sets fruit and only one or two flowers are open at once on any one head (Holm *et al.*, 1977). *S. zeylanica* is hermaphroditic (2n = 24) and its mode of reproduction is solely via seed (Pancho *et al.*, 1969; Cardenas *et al.*, 1972; Holm *et al.*, 1977). A single *S. zeylanica* plant can produce 60,000 to 100,000 seeds without nitrogen fertilization and this total can double or triple if an additional 60 kg N ha^{-1} are provided (Migo *et al.*, 1986).

1.1.3. Classification and related species of Sphenoclea zeylanica

S. zeylanica is one of two species belonging to the family Sphenocleaceae, a monogeneric family (Rosatti, 1986). The other species is *S. dalzielli* N.E. Brown which has only been found in northern Nigeria (Anonymous, 1912). The family Sphenocleaceae was placed in the Campanulales order of the Asteridae subclass based on floral characteristics. This family has a densely spicate inflorescence and each sessile flower is subtended by a bract and two bracteoles (Rosatti, 1986). The flowers have imbricate lobes, tetracytic stomates, and a circumscissile capsule (Lammers, 1992). However, it was recently reported that the Sphenocleaceae family is more closely allied to the order Solanales which also includes the genera *Nicotiana*, *Lycopersicon*, *Petunia*, *Convolvulus*, *Ipomoea*, *Montinia*, and *Hydrolea* (Cosner *et al.*, 1994).

1.2. Strategies for the control of *Sphenoclea zeylanica*

1.2.1. Chemical methods of weed control

Various chemical herbicides belonging to the chlorophenoxy, chloroacetamide, and sulfonylurea groups have been widely evaluated for the control of broadleaf weed species including *S. zeylanica* in Asia. These herbicides can be applied pre-emergence, post-emergence, or both. They can also be applied in combination with other herbicides.

In the tropics, the chlorophenoxy herbicide group was routinely used for weed control of broadleaf weeds, including *S. zeylanica*. This group includes 2,4-D ((2,4-dichlorophenoxy) acetic acid) applied at 0.5 to 0.8 kg ai/ha from three to five days after transplanting (DAT) or 15 to 25 DAT or 20 days after sowing (DAS) (Madrid *et al.*, 1972; Migo *at al.*, 1986; Raju and Reddy, 1987; Mercado *et al.*, 1990; Smith and Hill, 1990; Ho, 1995) and MCPA ((4-chloro-2-methylphenoxy) acetic acid) applied at 0.6 to 1.7 kg ai/ha (Smith and Hill, 1990; Moody, 1994; Ho, 1995). Other promising and commonly used herbicides include butachlor (N-(buthoxymethyl)-2-chloro-N-(2,6-diethylphenyl) acetamide) applied at 1.2 to 2.0 kg/ha from three to seven DAT or six

to eight DAS (Zafar, 1989; Ho, 1995), oxadiazon (3-[2,4-dichloro-5-1(1-methylethoxy) phenyl]-5-(1,1-dimethylethyl)-1,3,4-oxadiazol-2-(3H)-one) applied at 0.54 to 1.5 kg/ha from 10 to 14 DAS (Zafar, 1989; Imeokparia, 1994; Ho, 1995), and thiobencarb (S-[(4-chlorophenyl) methyl] diethylcarbamothiate) applied at 2 to 3 kg/ha from six DAS (Zafar, 1989; Ho, 1995). However, the effectiveness of these herbicides is very much dependent on the maintenance of a suitable water level in transplanted rice (Ho, 1995).

The application of two herbicides in mixture have provided effective control against *S. zeylanica*. These include bensulfuron-methyl (2-[[[[[(4,6-dimethoxy-2-pyrimidinyl) amino] carbonyl] amino] sulfonyl] methyl] benzoic acid) (20 g/ha) + quinclorac (3,7-dichloro-8-quinolinecarboxylic acid) (250 g/ha) applied at four DAS (Peudpaichit *et al.*, 1987), ethoxysulfuron (10 to 15 g/ha) + thiobencarb (1.5 kg/ha) applied at five to 10 DAS (Sitchawat and Khattiyakarun, 1995), ethoxysulfuron (15 g/ha) + propanil (N-(3,4-dichlorophenyl)propanamide) (1.5 kg/ha) applied at 15 DAS (Sitchawat and Khattiyakarun, 1995), oxadiazon (0.5 kg/ha) + propanil (1.5 kg/ha) applied at 10 to 14 DAS (Imeokparia, 1990; 1994; Ho, 1995), and pretilachlor + safener at 0.3 to 0.75 kg/ha applied at zero to four DAS or four DAT (Llorente and Evangelista, 1990; Ho, 1995).

In the late 1980's and early 1990's, the sulfonylurea herbicide group became popular because of its excellent control of broadleaf weeds including *S. zeylanica* at relatively low application rates (Ho, 1994). This group of herbicides includes Setoff (cinosulfuron) applied at 20 to 40 g/ha from three to 15 DAS or DAT (Calderon *et al.*, 1987; Ho, 1995), NC-311 (pyrazolsulfuron-ethyl) applied at 14 to 30 g/ha from three to 14 DAT or seven to 14 DAS (Ooi, 1988; Ho, 1995), ACC 322,140 (1-{[O-(cyclopropylcarbonyl) phenyl] sulfamoyl} -3 (4,6-dimethoxy-2-pyrimidinyl)-urea) applied at 20 to 30 g/ha from zero to 15 DAS or DAT (Braddock *et al.*, 1995), HOE 95404 (ethoxysulfuron) applied at 20 g/ha from 21 to 35 DAS, and metsulfuron methyl (2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) amino] carbonyl] amino] sulfonyl] benzoic acid) applied at 6 g/ha from 21 to 35 DAS (Sitchawat and Khattiyakarun, 1995). Another promising new herbicide is KIH-2023 (sodium 2,6-

bis[4,6-dimethoxy-pyrimidin-2-yl)oxy]benzoate) applied at the 15 to 30 g/ha at 1- to 7-leaf stage of the weed (Kobayashi *et al.*, 1995).

Although these herbicides provide fairly effective control, subsistence farmers cannot afford to purchase these chemical herbicides and many of these herbicides are not readily available, especially in remote areas. *S. zeylanica* was reported to be initially susceptible to 2,4-D (Madrid *et al.*, 1972), the least expensive and most readily available herbicide, but subsequently, it was found that some populations of *S. zeylanica* became tolerant to this phenoxy herbicide in both the Philippines and Malaysia (Sy and Mercado, 1983; Baki and Azmi, 1994).

1.2.2. Other weed control strategies

In the Philippines and many other countries in Southeast Asia, the usual practice of farmers in preparing their land consists of plowing twice and harrowing thrice before planting and relying for the most part on hand weeding twice for weed control. Hand weeding, however, is expensive (if labour must be hired), time consuming, and sometimes cannot be finished before the critical period of crop-weed competition (tillering stage) if labour is unavailable. For example, hand weeding one hectare of flooded, transplanted rice in Vietnam requires as much as 300 to 1200 person-hrs and costs at least five times more than chemical herbicides in wet-seeded rice (Moody, 1994).

Efficient water management was also reported to control *S. zeylanica* by early flooding of rice fields with 5 to 7.5 cm of water (Mercado, 1979) as well as by maintaining a depth of 5 to 8 cm of water in the paddies (Medrano *et al.*, 1993). However, this method of control is only applicable where irrigation systems are available.

Mechanical weeding is also available and can lower labour costs, but yields are sometimes reduced especially in direct-seeded rice. Other weed control strategies to control *S. zeylanica* include crop rotation, cultivation, and proper drainage systems (Smith and Shaw, 1966).

1.2.3. Biological methods

1.2.3.1. Biological control of weeds with plant pathogens

Biological control of weeds is the deliberate use of natural enemies to suppress the growth or reduce the population of a problem weed species (Watson, 1991). The most biologically effective alternatives to chemicals for weed control in cultivated crops that have been extensively evaluated are plant pathogens, more specifically, plant pathogenic fungi (Boyette *et al.*, 1991). There is great interest in using plant pathogenic fungi for weed control because many fungi do not require wounds or insect vectors to penetrate and infect plant tissues, are often host-specific, and multiply rapidly by producing stable, infective propagules that can be easily stored (Hasan, 1980; Scheepens and van Zon, 1982; Templeton, 1982b). The use of plant pathogenic fungi also offers an exploitable biotechnology that is an effective supplement to conventional weed control which is mainly based on chemical and mechanical methods (Charudattan, 1990). Moreover, specificity in control, environmental safety, and lower development costs are all plausible reasons for implementing phytopathogenic fungi to control problem weeds (Bowers, 1982; Templeton, 1986; Hasan, 1988; Charudattan, 1990).

Biological weed control using plant pathogens entails two broad strategies; 1) the classical approach and 2) the mycoherbicide (or bioherbicide) approach. In the classical approach, the pathogen (generally an exotic organism) is introduced and allowed to spread unchecked within the susceptible weed population usually because of a lack of any natural enemies (Boyette *et al.*, 1991). The ideal target for this approach is an introduced, aggressive, widespreading perennial weed species that infests large areas such as rangelands or aquatic habitats. However, there has been a successful implementation of the classical biological control strategy in cultivated crops using a rust fungus. *Puccinia chondrillina* Bubak and Syd. from Europe has been successfully used to control skeleton weed (*Chondrilla juncea* L.) in Australia in wheat-fallow systems and in the United States mainly because of the rust's ability to readily disperse (Hasan, 1974; Emge and Kingsolver, 1977; Cullen, 1978; Batra, 1981;

Emge and Templeton, 1981). Similarly, *Puccinia lagenophorae* (Cooke), an indigenous rust has been shown to significantly suppress the competitive ability of common groundsel (*Senecio vulgaris* L.) in lettuce (*Lactuca sativa* L.) (Paul and Ayres, 1987).

In the mycoherbicide (or bioherbicide) approach, the pathogen (generally an endemic organism) is mass-produced and applied to target weeds using techniques and methodologies similar to those used with chemical herbicides (Templeton, 1982b; Mortensen, 1988; Boyette *et al.*, 1991). This strategy is best suited for annual weeds in annual crops, where rapid weed control is generally desired (Templeton, 1986). The use of this approach is based on the fundamental epidemiological principles of plant pathology (Templeton *et al.*, 1979; Shrum, 1982; Watson, 1992; 1994a; 1994b). Plant disease is often suppressed by pathogen, plant, as well as environmental factors. Pathogen factors reducing efficacy include low initial inoculum levels, weak virulence, and low fecundity. Plant factors affecting disease expression include low susceptibility to the disease, defense mechanisms, and spatial plant distribution. Environmental conditions such as low and high temperatures, and the absence of an adequate dew period often limit diseases in plants (Watson, 1994a; 1994b). In the bioherbicide approach, these constraints on disease development can be bypassed by periodically dispersing an abundant supply of virulent inoculum uniformly onto a susceptible weed population (Watson, 1994a; 1994b), proper timing of inoculum application to take advantage of favourable environmental conditions and/or the most susceptible weed growth stage (Charudattan, 1991), and inoculum formulation to avoid unfavourable environmental conditions and facilitate application (Daniel *et al.*, 1973).

A fungal pathogen is, therefore, considered a potential bioherbicide candidate if it is virulent on the target weed, sporulates readily in culture systems, and does not infect desirable plant species. Thus, a prospective bioherbicide should be safe to the user, safe for the environment, inexpensive to use, easy to produce and store, and provide reliable control against the target weed population.

The development of potential bioherbicides to marketable products is hindered by biological, technological, economical, environmental, and governmental constraints (TeBeest and Templeton, 1985; Watson, 1989; Charudattan, 1991). Pathogen virulence and fastidious environmental requirements are the two most critical constraints in the development of a bioherbicide (Templeton, 1982a; Upadhyay and Rai, 1988; Watson and Wymore, 1990; Charudattan, 1991; Watson, 1991). Presently, technological improvements for potential bioherbicide candidates focuses on such factors as: increased pathogen virulence, improved toxin production, altered host range, resistance to crop production chemicals, altered survival or persistence in the environment, broader environmental tolerance, increased propagule production in fermentation systems, enhanced tolerance to formulation process, and innovative formulation approaches (Templeton and Heiny, 1989). Other limiting factors include formulation, specificity, efficacy, and commitment by the industrial sector (Watson, 1989). Advancements have been made in the formulation and application of bioherbicide products including the use of alginate gel technology, microencapsulation, invert emulsions, and various additives to enhance germination, virulence, and efficacy (Connick, 1988; Boyette *et al.*, 1991; Connick *et al.*, 1991; Stowell, 1991). The lack of major industrial involvement has been due to inconsistent field performance, uncertainty in regulatory requirements, development costs, and market potential (Watson, 1989).

The bioherbicide approach has been implemented principally in cultivated crops where intensive efforts to use fungal plant pathogens began more than two decades ago. Most of the research is presently concentrated in North America and Europe, with limited research being conducted in developing countries.

In North America, there have been three registered bioherbicide products from fungal plant pathogens namely; 1) DeVine®, a short-lived liquid formulation of chlamydospores of the soil-borne fungus *Phytophthora palmivora* (Butler) Butler, was registered in 1981 for the control of milkweed vine [*Morrenia odorata* (H.&A.) Lindl.] in Florida citrus groves (Ridings *et al.*, 1976; Kenney, 1986); 2) COLLEGO®, a dry

powder formulation of *Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *aeschynomene*, was registered in 1982 for the control of northern jointvetch [*Aeschynomene virginica* (L.) B.S.P.] in rice and soybeans in Arkansas, Louisiana, and Mississippi (TeBeest and Templeton, 1985; Smith, 1986; Templeton, 1986); and 3) Biomal®, a dry formulation of *C. gloeosporioides* f. sp. *malvae*, was registered in 1992 for the control of round-leaved mallow (*Malva pusilla* Sm.) in flax (*Linum usitatissimum* L.) and lentils (*Lens esculenta* Moench.) (Mortensen, 1988; Makowski and Mortensen, 1992). Outside of North America, there is only one other registered product, LUBOA 1 S_{22} which is composed of *C. gloeosporioides* f. sp. *cuscutae* for the control of parasitic dodder (*Cuscuta* sp.) in soybeans in China (Templeton, 1992; Wan *et al.*, 1994). Other potential bioherbicide products were not marketed because of limited market size, low economic returns, production problems, and the high cost of registration (Templeton, 1992; Charudattan, 1994).

In the tropics, although bioherbicide research is in its infancy stage, prospects are very encouraging (Evans, 1987; Watson, 1991; Bayot *et al.*, 1994). The present status of research is focused on initial surveys, collection of diseased weeds, and isolation of causal organisms with limited laboratory and field studies (Watson, 1994a; 1994b).

A bioherbicide research program initiated in 1991 at the International Rice Research Institute (IRRI) and the University of the Philippines at Los Baños (UPLB), College, Laguna, Philippines, in collaboration with McGill University, Montréal, Canada focuses on nine major weeds of rice including smallflower umbrella sedge (*Cyperus difformis* L.), rice flatsedge (*C. iria* L.), purple nutsedge (*C. rotundus* L.), jungle rice [*Echinochloa colona* (L.) Link.], barnyardgrass [*E. crusgalli* (L.) Beauv.], globe fingerush [*Fimbristylis miliacea* (L.) Vahl], mimosa (*Mimosa invisa* Mart.), monochoria [*Monochoria vaginalis* (Burm.f.) Kunth] and gooseweed (*S. zeylanica* Gaertner) (Watson, 1991; Bayot *et al.*, 1994; Watson, 1994a; 1994b). Virulent pathogens have been isolated from all these target weeds and are potential bioherbicide candidates (Watson, 1994a; 1994b).

Other weed species of interest in the tropics include waterhyacinth [*Eichhornia crassipes* (Mart.) Solms] (Kasno *et al.*, 1980; Caunter, 1982; Napompeth, 1990), water fern (*Salvinia molesta* D. S.) (Watson, 1991; Julien, 1992), goosegrass [*Eleusine indica* (L.) Gaertn.] (Figliola *et al.*, 1988); wild poinsettia (*Euphorbia heterophylla* L.) (Yorinori and Gazziero, 1990); and itchgrass [*Rottboellia cochinchinensis* (Lour.) W.D. Clayton] (Ellison and Evans, 1990). In Japan, as well as in a number of neighbouring countries, biological control candidates are being sought for such important weed genera as *Eleocharis* and *Echinochloa* (Imaizumi *et al.*, 1991; Suzuki, 1991; Yoo, 1991; Gohbara and Yamaguchi, 1994; Tanaka *et al.*, 1994).

1.2.3.2. Status of the biological control of Sphenoclea zeylanica

No insects have been recorded to attack *S. zeylanica* and only two pathogens have been reported to occur on *S. zeylanica*; a leaf mold pathogen and a leaf blight pathogen. *Cercosporidium helleri* Earle, the leaf mold pathogen, was recorded on *S. zeylanica* in paddy fields at Ernakulam, Kerala, India (Ponnappa, 1967). The same pathogen was observed in Prey Phadu, Cambodia in June 1986 (Moody *et al.*, 1994) and at the International Rice Research Institute (IRRI) Experimental Farm, Los Baños, Laguna, Philippines and in Victoria, Laguna, Philippines (Bayot *et al.*, 1994; Moody *et al.*, 1994; Mabbayad and Watson, 1995). Disease symptoms typically appear 12 to 14 days after the spraying of *S. zeylanica* seedlings with spores gathered from heavily infected leaves (Bayot *et al.*, 1994). Unfortunately, this pathogen does not produce spores in culture and disease progress in young plants is rather slow. Hence, this leaf mold pathogen is not a potential bioherbicide candidate.

The second pathogen, *Alternaria* sp. Nees ex Fr., which causes leaf blight in *S. zeylanica*, was collected near a rice field at Los Baños, Laguna, Philippines. This pathogen is more aggressive than the leaf mold pathogen and can be readily produced. Laboratory and field studies with this pathogen suggest that it is a promising bioherbicide candidate (Bayot *et al.*, 1994). Mabbayad and Watson (1995) reported that this fungal pathogen killed *S. zeylanica* seedlings as well as mature plants at the

flowering stage using a concentration of 1 x 10^4 spores ml^{-1} over a wide range of dew periods under greenhouse conditions. This *Alternaria* isolate has also been shown to be effective in controlling *S. zeylanica* under variable rainfall conditions in the field. Substantial reductions in weed biomass have been obtained in the field by using inoculum concentrations of 1 x 10^5 to 1 x 10^6 spores ml^{-1} (Mabbayad and Watson, 1995).

1.3. The biological control agent

1.3.1. Characteristics of the genus Alternaria Nees ex Fr.

Alternaria is a dictyosporic (many-celled spores with transverse and oblique or longitudinal septa) genus based on the conidium-ontogeny system of classification of Vuillemin (1911). According to the classification of Saccardo (1886), *Alternaria* belongs to the family Dematiaceae (conidiophores free, not united in fascicles; hyphae, conidia, or both, dark brown to black) of the order Hyphomycetes, Fungi Imperfecti. The order Hyphomycetes comprises mycelial forms which are either sterile or bear conidia on separate hyphae as synnematous or sporodochial conidiomata but not with discrete conidiomata (Hawksworth *et al.*, 1983).

The genus *Alternaria* was established by Christian Gootfried Nees von Esenbeck (C. G. Nees), with *A. alternata* (Fries) Keissler (= *A. tenuis* Nees ex Pers.) as the type species (Elliot, 1917; Wiltshire, 1933; Groves and Skolko, 1944; Joly, 1967; Simmons, 1967). The dispute over the taxonomic position of *Alternaria* began in the early 1800's, when Elias Magnus Fries described the genus *Macrosporium* and differentiated it from *Cladosporium*, *Helminthosporium*, and *Sporodesmium* and included the *Alternaria*-like specimens in the genus *Torula*. After several researchers questioned this assignment, Fries acknowledged the existence of the genus *Alternaria* and also found that it differed from *Macrosporium* (Tweedy and Powell, 1963).

The generic criterion for *Alternaria* was the form of conidia (obclavate, pointed, and often having beaks) as analyzed and stressed by Elliot (1917) thus, treating *Alternaria* and *Macrosporium* as distinct genera. However, Angell (1929)

found them similar and used the epithet *Macrosporium* to designate both groups. Wiltshire (1933) resolved the confusion when he agreed that *Alternaria* and *Macrosporium* were congeneric and considered *Alternaria* to be a more appropriate epithet for designating the genus. The epithet *Macrosporium* then became the nomen ambiguum. *Pleospora herbarum* (Pers.) Rabenh. was assumed to be the ascogenous stage of *Alternaria* (Ellis, 1971), however, pure cultures of this isolate produced no *Alternaria*-type conidia and pure *Alternaria* isolates produced no ascosporic stages (Tweedy and Powell, 1963).

Bearing in mind all the taxonomic difficulties indicated previously, Ellis (1971) characterized the genus as follows: "***Colonies*** *effuse, usually grey, dark blackish brown or black.* ***Mycelium*** *all immersed or partially superficial.* ***Hyphae*** *colourless, olivaceous brown or brown;* ***stroma*** *rarely formed.* ***Setae*** *and* ***hyphopodia*** *absent.* ***Conidiophores*** *macronematous, mononematous, simple or irregularly and loosely branched, pale brown or brown, solitary or in fascicles.* ***Conidiogenous cells*** *integrated, terminal becoming intercalary, polytretic, sympodial, or sometimes monotretic, cicatrized.* ***Conidia*** *catenate or solitary, dry, typically ovoid or obclavate, often rostrate, pale or mid olivaceous brown, or brown, smooth or verrucose, with transverse and frequently also oblique or longitudinal septa.*"

1.3.2. Species of Alternaria as causal agents of disease on plants of economic importance

Alternaria species are either parasites on living plants or saprophytes on organic substrata. Several studies have also demonstrated that this fungal genus produces toxins which could be involved in any or all stages of infection, from initial penetration of the tissue to establishment, colonization, and death of the tissue (Nishimura and Kohmoto, 1983; Scheffer, 1983; Rotem, 1994).

Alternaria species cause disease on many important cultivated crops (Table 1.1). Many of these species are recorded in most countries of the world including *A. solani* Sorauer on potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon*

esculentum Mill.) (Ellis and Gibson, 1975; Rossman *et al.*, 1987; Rotem, 1994), *A. brassicae* (Berk.) Sacc. and *A. brassicicola* (Schw.) Wiltshire on brassicas (Ellis, 1968; Rossman *et al.*, 1987; Rotem, 1994), *A. dauci* (Kühn) Groves and Skolko on carrots (*Daucus carota* L.) (David, 1988; Rotem, 1994), and *A. porri* (Ell.) Cif. on onions (*Allium cepa* L.) (Ellis and Holliday, 1970; Rossman *et al.*, 1987; Rotem, 1994). To date, there are 50 formally described species in the genus *Alternaria* (Rossman *et al.*, 1987) and most of these species are plant pathogens with restricted host ranges.

1.3.3. Species of Alternaria implicated in biological weed control

In addition to the *Alternaria* isolate from *S. zeylanica*, there are other *Alternaria* species that have been examined as possible biological weed control agents as shown in Table 1.2. To date, there have been no registered bioherbicide products using *Alternaria* pathogens. However, important advancements have been made to increase their efficacy, particularly with respect to their formulation and application. Such modifications include the use of alginate gel technology, microencapsulation, invert emulsions, and various additives to enhance germination, virulence, and efficacy (Walker, 1982; Connick, 1988; Boyette *et al.*, 1991; Connick *et al.*, 1991; Daigle and Cotty, 1992). Moreover, a host-specific toxin, maculosin, was isolated from *A. alternata*, a potential biological control agent for *Centaurea maculosa* (Stierle *et al.*, 1988; Stierle and Cardellina, 1989).

Table 1.1. Important crop diseases caused by *Alternaria* species.

Alternaria species	Disease and Crop	Reference(s)
A. bataticola Ikata ex Yamamoto	leaf spot of sweet potato (*Ipomoea batatas* L.)	David, 1991
A. brassicae (Berk.) Sacc.	leaf spots of Brassicaceae	Ellis, 1968; Rossman *et al.*, 1987
A. brassicicola (Schw.) Wiltshire	leaf spots of Brassicaceae	Ellis, 1968; Rossman *et al.*, 1987
A. burnsii Uppal, Patal and Kamat	blight of cumin (*Cuminum cyminum* L.)	Rossman *et al.*, 1987; Anahosur, 1978
A. carthami Chowdury	leaf spot of safflower (*Carthamus tinctorius* L.)	Ellis and Holliday, 1970; Rossman *et al.*, 1987
A. chrysanthemi Simmons & Crosier	leaf spot of *Chrysanthemum*	Ellis, 1968; Rossman *et al.*, 1987
A. citri Ellis and Pierce	rots of *Citrus* fruits	Ellis and Holliday, 1970; Rossman *et al.*, 1987
A. cucumerina (Ellis & Everh.) Elliot	leaf spot of cucumber (*Cucumis sativus* L.) and melon (*C. melo* L.)	Ellis and Holliday, 1970; Rossman *et al.*, 1987
A. dauci (Kühn) Groves and Skolko	leaf blight of carrot (*Daucus carota* L.)	David, 1988
A. dianthi Stevens and Hall	blight of *Dianthus* spp.	David, 1988
A. dianthicola	bud rot of *Dianthus* spp.	David, 1991

Table 1.1. Continued...

Alternaria species	Disease and Crop	Reference(s)
A. gossypina (Thümen) Hopkins	leaf spot and cotton (*Gossypium hirsutum* L.) boll rot	David, 1988
A. helianthi (Hansf.) Tubaki & Nishihara	leaf spot, head blight, and other diseases of *Helianthus* species	Anahosur, 1978; Rossman *et al.*, 1987
A. kikuchiana Tanaka	black spot of pear (*Pyrus pyrifolia* (N. L. Burm.) Nakai cv. Nijisseiki	David, 1988
A. linicola Groves and Skolko	damping off of flax (*Linum usitatissimum* L.) seedlings	David, 1991
A. longipes (Ellis & Everh.) Mason	brown spot of tobacco (*Nicotiana tabacum* L.)	Ellis and Holliday, 1970; Rossman *et al.*, 1987
A. macrospora Zimmermann	leaf spot of cotton	Ellis and Holliday, 1970; Rossman *et al.*, 1987
A. padwickii (Ganguly) Ellis	stackburn, seedling blight, and leaf spot of rice (*Oryza sativa* L.)	Ellis and Holliday, 1972; Rossman *et al.*, 1987
A. passiflorae Simmonds	brown spot of passion fruit (*Passiflora edulis* Sims.)	Ellis and Holliday, 1970; Rossman *et al.*, 1987

Table 1.1. Continued...

***Alternaria* species**	**Disease and Crop**	**Reference(s)**
A. peponicola (Rabenhorst) Simmons	rind rot of Cucurbitaceae	David, 1991
A. porri (Ell.) Cif.	purple blotch of onion	Ellis and Holliday, 1970;
A. radicina Meier, Drechsler & Eddy	black spot of carrot and other Apiaceae	Ellis and Holliday, 1972; Rossman *et al.*, 1987
A. ricini (Yoshii) Hansf.	diseases of castor (*Ricinus communis* L.)	Ellis and Holliday, 1970; Rossman *et al.*, 1987
A. sesami (Kawamura) Mohanty and Behera	diseases of sesame (*Sesamum indicum* L.)	Ellis and Holliday, 1970; Rossman et al., 1987
A. solani Sorauer	early blight of potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.)	Ellis and Gibson, 1975; Rossman *et al.*, 1987

Table 1.2. *Alternaria* species as potential biological weed control agents.

***Alternaria* species**	**Weed host(s)**	**Reference(s)**
A. alternantherae Holcomb and Antonopoulos	*Alternanthera philoxeroides* (Mart.) Griseb. (alligatorweed)	Holcomb and Antonopoulos, 1976
A. alternata (Fr.) Keissler	*Centaurea maculosa* Lam. (spotted knapweed)	Stierle et al., 1988; Stierle and Cardellina, 1989
A. alternata (Fr.) Keissler	*Eichhornia crassipes* (Mart.) Solms (waterhyacinth)	Aneja and Singh, 1989
A. angustiovoidea Simmons (= *A. tenuissima* f. sp. *euphorbiae*)	*Euphorbia esula* L. (leafy spurge)	Krupinsky and Lorenz, 1983; Yang *et al.*, 1990
A. cassiae Jurair and Khan	*Cassia obtusifolia* L. (sicklepod)	Walker, 1982; Walker and Riley, 1982
A. crassa (Sacc.) Rands	*Datura stramonium* L. (jimsonweed)	Boyette, 1986; Boyette and Turfitt, 1988
A. eichhorniae Nag Raj and Ponnappa	waterhyacinth	David, 1991
A. helianthi (Hansf.) Tubaki and Nishihara	*Xanthium strumarium* L. (ccmmon cocklebur)	Tubaki and Nishihara, 1969; Quimby, 1989
A. macrospora Zimm.	*Anoda cristata* (L.) Schlecht. (spurred anoda)	Walker and Sciumbato, 1979; 1981

Table 1.2. Continued...

Alternaria species	Weed host(s)	Reference(s)
Alternaria sp.	*Carduus pycnocephalus* L. (Italian thistle)	Anderson and Lindow, 1985
Alternaria sp.	*Cuscuta gronovii* Willd. (dodder swamp)	Bewick *et al.*, 1986; 1987
Alternaria sp.	*Scirpus planiculmis* F. Schm.	Park *et al.*, 1992

1.4 Thesis Objectives

The research reported in this thesis focuses on the use of an indigenous fungus, *Alternaria alternata* f. sp. *sphenocleae*, which was collected from blighted *S. zeylanica* plants collected near a rice field at Los Baños, Laguna, Philippines in 1991. The specific objectives of this research were to:

- describe the principal morphological and cultural characteristics of *A. alternata* f. sp. *sphenocleae*,
- establish the etiology of the disease caused by this fungal pathogen,
- find a suitable medium and develop a methodology to produce abundant, viable, and virulent inoculum,
- evaluate the efficacy of a sporulation medium (S-medium) on *A. alternata* f. sp. *sphenocleae*, and
- investigate, under controlled conditions, the host-range of this indigenous fungus.

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Connecting Text

Prior to the development or application of a biological control agent, an adequate biosystematic foundation should first be established. Thus, in this study, the morphological and cultural characteristics of the *Alternaria* species isolated from blighted leaves of *Sphenoclea zeylanica* plants collected in a rice field near Los Baños, Laguna, Philippines are determined, characterized, and compared with other established *Alternaria* species.

Chapter 2 - Morphological and Cultural Characteristics of an *Alternaria* Isolate

2.1. Abstract

On the basis of growth characteristics, conidial measurements, pathogenicity on the host, and the absence of a previous record of *Alternaria alternata* on *Sphenoclea zeylanica*, the binomial *A. alternata* f. sp. *sphenocleae* is proposed. This isolate produces chlamydospores and phytotoxin(s). *S. zeylanica* was immune or highly resistant to other *Alternaria* isolates tested.

2.2. Introduction

Sphenoclea zeylanica Gaertner (gooseweed) is a common, annual herbaceous, broadleaf weed species of wetland rice (*Oryza sativa* L.) in Southeast and South Asia, the United States, the West Indies, and West Africa (Holm *et al.*, 1977). It is also associated with cotton (*Gossypium hirsutum* L.) in Louisiana, U.S.A. (Sanders, 1990), wheat (*Triticum aestivum* L.) in wheat-rice rotations in India (Khan *et al.*, 1988) and in soybean [(*Glycine max* (L.) Merr.)] in rice-soybean rotations in Thailand (Vongsaroj, 1994). *S. zeylanica* can reduce yields of rice by as much as 45% (Laganao, 1981; Ampong-Nyarko and De Datta, 1991; Biswas and Sattar, 1991) and significant yield reductions in transplanted rice occur when weed densities are as low as 20 plants m^{-2} (IRRI, 1989).

Several herbicides including 2,4-D [(2,4-dichlorophenoxy)acetic acid] provide fairly effective control of *S. zeylanica* but subsistence farmers cannot afford to purchase this chemical herbicide and is not readily available, especially in remote areas. Some populations of *S. zeylanica* have become tolerant to the continuous post-emergent application of 2,4-D both in the Philippines and in Malaysia (Sy and Mercado, 1983; Baki and Azmi, 1994). There is, therefore, a need to discover and develop new weed control strategies and to improve existing weed control technologies that are economically and environmentally sustainable. Biological weed

control has great potential to reduce chemical inputs and to provide viable, economic, and effective weed control components within integrated pest management (IPM) programs in rice (Watson, 1991).

In 1991, a biological weed control research program was initiated at the International Rice Research Institute (IRRI) and the University of the Philippines at Los Baños (UPLB), College, Los Baños, Laguna, Philippines, in collaboration with McGill University, Montréal, Québec, Canada. *S. zeylanica* is among the nine original target weed species selected. In 1991, an *Alternaria* species was isolated from blighted *S. zeylanica* collected in a rice field near Los Baños, Laguna, Philippines. This *Alternaria* isolate recovered from *S. zeylanica* has not been previously reported and therefore has never been included in the lists of *Alternaria* species in the literature. However, laboratory, greenhouse, and field studies as well as farmer's field trials in the Philippines have demonstrated that this fungal pathogen is an excellent bioherbicide candidate for *S. zeylanica* (Bayot *et al.*, 1994; Mabbayad and Watson, 1995; Masangkay *et al.*, 1996a; 1996b). In 1993, this fungal pathogen was sent to the International Mycological Institute (IMI), Surrey, UK and was identified as *A. alternata*. However, before a biological control agent could be developed and applied efficiently, an adequate biosystematic foundation should first be established (Huber, 1993). Thus, the objectives of this study were to determine and characterize the cultural and conidial morphology of this *Alternaria* species, and compare its pathogenicity to other established ATCC *Alternaria* isolates.

2.3. Materials and Methods

2.3.1. Isolation and culture maintenance of the *Alternaria alternata* isolate from *S. zeylanica*. Diseased *S. zeylanica* leaves were collected from blighted plants in rice fields at the International Rice Research Institute (IRRI) Experimental Farm in 1991. Leaf pieces were surface sterilized with 0.5% sodium hypochlorite solution and incubated on fresh potato dextrose agar (PDA; Difco, Detroit, MI). Monoconidial isolates were prepared, stored, and maintained on half-strength potato dextrose (1/2

PDA) in culture tubes at IRRI and imported into the quarantine facility of McGill University (Macdonald Campus, Ste-Anne-de-Bellevue, Québec, Canada). These monoconidial isolates were then used to inoculate test plant material, re-isolated, stored, and maintained on 1/2 PDA in small vials under mineral oil at 4°C as stock cultures (Tuite, 1969). Small pieces of mycelium from the stock cultures were aseptically transferred to cooled PDA (20 ml) in plastic petri dishes (90-mm-diameter). Cultures were sealed with Parafilm® (American National Can Co., Greenwich, CT) and incubated at 28°C under continuous near-ultraviolet light (NUV; J-05, UVP, Inc., Circleville, OH) for three days. Agar plugs (4-mm-diameter) from the margins of young actively growing colonies were used as seed inoculum (Tuite, 1969).

2.3.2. Nutrient requirement of the *Alternaria* isolate. Several agar media for culturing species of *Alternaria* were tested including corn meal agar (CMA; Difco, Detroit, MI), malt extract agar (MEA; Difco, Detroit, MI), oatmeal agar (OMA; Difco, Detroit, MI), PDA, 1/2 PDA, and V-8 juice agar (VJA). The different media tested were prepared following recommendations from the label except for VJA which was prepared according to the method described by Simmons (1992; 1993). Twenty ml of media were dispensed into plastic petri dishes (90-mm-diameter). After cooling, inoculation was carried out by inverting and placing in the center of each petri dish an agar plug (4-mm-diameter) from 3-day-old PDA cultures of the *A. alternata* isolate from *S. zeylanica* under aseptic conditions. Subsequently, plates were sealed with Parafilm® and incubated under continuous light condition (400 $\mu Em^{-2}s^{-1}$ PAR) at 28°C.

Growth rates in culture were calculated on the basis of colony measurements (mm) starting after 24 h of incubation and continuing until the plate was completely covered with mycelium. Measurements were made on two radial axes on the reverse of the plate every 24 h. The experiment was laid out in a randomized complete block design with ten replications and was conducted three times.

2.3.3. Light requirement of the *Alternaria* isolate. The different agar media evaluated earlier were incubated under four light conditions including; 1) continuous darkness (D), 2) continuous light (400 $\mu Em^{-2}s^{-1}$ PAR) (L), 3) 12 h of alternating light (400 $\mu Em^{-2}s^{-1}$ PAR) and dark (L/D), and 4) exposure to continuous NUV. The incubation temperature was 28°C. Growth rates were assessed as previously mentioned. The experiment was laid out in a randomized complete block design with six replications and was conducted three times.

2.3.4. Temperature requirement of the *Alternaria* isolate. The most promising agar media were selected and incubated at temperatures between 20 and 36°C at 4°C intervals. Plates were subsequently incubated under continuous light condition (400 $\mu Em^{-2}s^{-1}$ PAR). Growth rates were assessed as previously mentioned. The experiment was laid out in a randomized complete block design with three replications and was conducted three times.

2.3.5. Comparative assessment of the morphological characteristics and pathogenicity of several *Alternaria* isolates. Cultures of several *Alternaria* isolates selected on availability were obtained from the American Type Culture Collection (ATCC) (ATCC 11854, ATCC 24127, ATCC 26669, ATCC 28329, ATCC 36381, and ATCC 60376), Rockville, MD and reproduced by following the specific recommended requirements for growth of each isolate; ATCC 11854 on rabbit food agar (RFA) at 24°C, ATCC 24127 on RFA at 24°C under a 12 h fluorescent-light cycle, ATCC 26669 on PDA at 24°C, ATCC 28329 on CMA at 24°C, ATCC 36381 on VJA at 26°C, and ATCC 60376 on PDA at 26°C. Media were prepared following recommendations from the label except for VJA and RFA which were prepared according to the method described by Stevens (1981). The *Alternaria* isolate from *S. zeylanica* was produced either on VJA or 1/2 PDA at 28°C under continuous NUV.

For morphological characteristics, only ATCC 24127 and ATCC 28329 were compared with the *Alternaria* isolate from *S. zeylanica*. The isolates were grown on

VJA and 1/2 PDA under continuous NUV at 28°C for ten days. Two samples of conidial droplets were selected at random from each of five replicates. Droplets were mounted on glass slides in a drop of distilled water and 10 conidia in two randomly chosen microscopic fields were measured. Conidial measurements were assessed using the image analyzer (Leco 2001®, Leco Instruments Ltd., Mississauga, ON).

For pathogenicity studies, *S. zeylanica* seeds collected from IRRI Experimental Farm in 1994 were soaked with 95% hydrochloric acid (HCl) for 10 min and washed with continuous running distilled water for 30 min. Subsequently, the seeds were soaked in distilled water at room temperature (24 ± 2°C) for 12 h. Seeds were sown in black plastic trays Kord (K 10; Plant Products, Laval, QC) 2/3 filled with a sterilized soil mixture consisting of 3 parts garden soil, 3 parts Pro-mix^{BX} (commercial potting mix, Premier Horticulture Inc., Red Hill, PA), 2 parts vermiculite Holiday® (VIL Vermiculite Inc., Montréal, QC) and 1 part sphagnum peat moss Tourbe® (Les Tourbières Premier Ltée, Rivière-du-Loup, QC). After sowing, the soil was lightly pressed with a 0.5 x 25 x 15 cm wooden block and watered until saturated. Seeded trays were placed in a controlled environment chamber [Conviron® E15 (Winnipeg, Man.); 32/24°C day/night, 400 $\mu Em^{-2}s^{-1}$ photosynthetically active radiation (PAR), 12 h day^{-1}, and 70 to 80% relative humidity (RH)] until the plants were ready for transplanting. Relative humidity was maintained with a humidifier, soil was kept saturated, and the soil surface was misted every 12 h using a hand-held atomizer.

Healthy seedlings were selected 21 days after sowing and transplanted in transparent plastic pots (7 x 10.5 cm, Better Plastics, Inc., Kissimmee, FL) filled with sterilized moistened soil mixture. Three plants were transplanted in each pot and fertilized with 10 ml of 1.25 g L^{-1} of 20-20-20 (N-P-K) fertilizer. Pots were maintained in the controlled environment chamber with conditions as previously described. Water level was maintained at a depth of 2 to 3 cm throughout the experimental period.

Conidia for inoculation were harvested by flooding the plates with 10 ml of sterile deionized water containing 0.01% Triton® X-100 (polyethylene glycol tert-octylphenyl ether, Sigma Chemical Co., St. Louis, MO) as a wetting agent and gently

scraping the surface of the colonies with a sterile glass slide. Resulting suspensions were poured through a 250 mm plastic sieve lined with two layers of sterile cheesecloth. Inoculum concentration was determined with the aid of a haemocytometer and adjusted to 1 x 10^5 conidia ml^{-1} with sterile deionized water containing 0.01% Triton X-100®.

S. zeylanica plants (5 to 6 and 19 to 20 cm tall) were inoculated with 10 ml of the conidial suspension using a hand-held atomizer. Control treatments were sprayed with deionized water containing the wetting agent. Immediately after spraying, pots were placed in a dark dew chamber (Percival®, Boone, IA) with 100% relative humidity at 24°C for 8 h. Subsequently, pots were returned to the controlled environment chamber having the same conditions as previously mentioned.

S. zeylanica-Alternaria interactions were evaluated using disease severity, sporulation, mortality, and dry weight reduction. Disease severity was visually assessed daily for 14 days following inoculation by using a disease rating scale of I (immune) - no visible symptoms; HR (highly resistant) - very few, minute, necrotic flecks present; MR (moderately resistant) - a few small, necrotic lesions present; MS (moderately susceptible) - many necrotic lesions present but no expansion of lesions to cause desiccation of leaves; and S (susceptible) - many necrotic lesions present which expand causing desiccation and leaf abscission. At 14 days after inoculation and after disease severity rating was completed, one or two inoculated leaves were excised from both the treated and untreated plants from each replicate. Excised leaves were surface-sterilized with 0.5% sodium hypochlorite solution for 30 seconds, rinsed, and then incubated on moistened filter paper (Whatman® #1, Whatman International Ltd., Maidstone, England) in glass petri plates at 28°C in the dark. After 24, 48, and 72 h of incubation (HAI), sporulation on leaves was examined with a stereomicroscope. Sporulation was rated at four levels: - = no sporulation, + = light sporulation, ++ = moderate sporulation, and +++ = heavy sporulation. Mortality and dry weight were assessed 14 DAI. Mortality was evaluated for each plant, results pooled, and averaged for each pot. Collapsed plants were considered dead, although the succulent stem base of some blighted plants remained green. Dry weight was obtained by cutting live

aboveground tissue parts at soil level drying in paper bags for seven days at 45°C, and weighing. Dead leaves and dead portions of the stem were not included in the dry weight measurements. The dry weight data were expressed as percent reduction in biomass compared with biomass of the non-inoculated controls.

The study was laid out in a randomized complete block factorial design with three replications and was conducted twice.

2.3.6. Bioassay for phytotoxin production. Various media were evaluated including potato dextrose broth (Difco, Detroit, MI) (Gilchrist and Grogan, 1975; Hayashi *et al.*, 1990), Czapek Dox solution (Difco, Detroit, MI) (Stierle *et al.*, 1988; Hradil *et al.*, 1989), Czapek Dox solution with 1.5% agar (BDH, Darmstadt, Germany) (Häggblom, 1981), Czapek Dox solution supplemented with 5 mg ml^{-1} of $ZnSO_4$ (Gardner et al., 1985), V-8 juice medium [(250 ml of V-8 juice (Campbell Soup Co., Ltd., Toronto, ON), 1 g of $CaCO_3$ (Fisher Scientific Co., Fairlawn, NJ), and distilled water to 1 L] (Bains and Tewari, 1987), modified Richard's solution (25 g of sucrose, 10 g of KNO_3, 5 g of KH_2PO_4, 2.5 g of $MgSO_4$, 0.02 g of $FeCl_3$, 0.005 g of $ZnSO_4$, and 1 L of distilled water) (Kohmoto *et al.*, 1991), and Fries liquid medium (30 g of sucrose, 5 g of ammonium tartrate, 1 g of NH_4NO_3, 1 g of KH_2PO_4, 0.5 g of $MgSO_4$, 0.1 g of NaCl, 0.1 g of $CaCl_2$, 0.5 g of casein hydrolysate, and 1 g of yeast extract in 1 L of distilled water) (Gilchrist and Grogan, 1975). Flasks containing only the liquid media evaluated served as controls. The flasks were placed on rotary shakers at 200 rpm at 28°C under continuous light conditions (400 $\mu Em^{-2}s^{-1}$ PAR). After two to three weeks of incubation, cultures in each flask were centrifuged separately at 3,000 rpm for 20 min to pellet the mycelium. The supernatant in centrifuge tubes was filtered thrice; 1) through three layers of sterile cheesecloth, 2) through a filter paper (Whatman® #4, Whatman International Ltd., Maidstone, England), and 3) through a millipore filter membrane (0.22 µm) (Costar®, Cambridge, MA) and collected in 50 ml sterile polypropylene centrifuge tubes. Test plants were produced similarly as mentioned previously. The stem end of a freshly cut healthy shoot of *S. zeylanica* (5 to 6 cm tall) was immediately immersed in 30 ml of collected culture filtrate and held at 24°C

in the dark. Cuttings placed in the different liquid media from the control flasks and sterile distilled water served as control treatments. Cuttings were examined for 72 h. Chlorosis and wilting of leaves were assumed to indicate the presence of phytotoxin(s) in the culture filtrate. This experiment was laid out in a randomized complete block design (RCBD) with four replications and was conducted three times.

2.3.7. Data Analyses

All percentage data were arc sine transformed prior to analysis (Gomez and Gomez, 1984). Factorial experiments were analyzed with a factorial analysis of variance considering the effect of each factor individually and their interaction. Results were pooled if homogeneity of variances was confirmed by Bartlett's test (Gomez and Gomez, 1984). All analyses were conducted using STATGRAPHICS software for IBM-PC (STSC Inc., 1991). Treatment means were separated using Duncan's multiple range test at the 1% or 5% level of significance.

2.4. Results

2.4.1. Nutrient requirement of the *Alternaria* isolate. There was a significant effect of the different agar media evaluated on radial mycelial growth of the *A. alternata* isolate from *S. zeylanica* (Appendix 2.1). Mycelial growth rates (8.1 to 8.6 mm day^{-1}) on VJA, PDA, 1/2 PDA, and CMA were significantly faster than on MEA and OMA (6.7 and 7.3 mm day^{-1}, respectively) (Table 2.1).

2.4.2. Light requirement of the *Alternaria* isolate. There was a significant interaction between agar medium and light condition on radial mycelial growth of the *A. alternata* isolate from *S. zeylanica* (Appendix 2.2). Rapid radial mycelial growth generally occurred on all agar media except MEA when exposed to continuous light, dark, or NUV (Table 2.2). Under exposure to NUV, growth rate on OMA was also reduced. Exposures to 12 h of alternating light and dark conditions significantly reduced mycelial growth, with growth fastest on VJA and OMA under these conditions.

2.4.3. Temperature requirement of the *Alternaria* isolate. There was a significant interaction between agar medium and temperature on radial mycelial growth of the *A. alternata* isolate from *S. zeylanica* (Appendix 2.3). On all agar media, 36°C did not support the growth of the *A. alternata* isolate from *S. zeylanica*. Different agar media responded differently to temperature. The optimum predicted temperature varied between the different agar media (Figure 2.1). The predicted optimum temperature for maximum radial mycelial growth on 1/2 PDA and VJA was 24.4°C whereas, CMA and PDA required a lower predicted optimum temperature (20.4°C and 21.3°C, respectively).

2.4.4. Comparative assessment of the morphological characteristics of several *Alternaria* isolates. The most distinct conidial features of the *A. alternata* isolate from *S. zeylanica* were size of body and beak, total conidial length, and the percentage of spores with beaks (Tables 2.3a and 2.3b). On VJA, the *A. alternata* isolate from *S. zeylanica* generally gave longer conidial length than the other *A. alternata* isolates (Table 2.3.a). Longer body size, beak size, and total conidial length were also observed for the *A. alternata* isolate from *S. zeylanica* grown on 1/2 PDA compared with the two other *A. alternata* isolates (Table 2.3b). The *A. alternata* isolate from *S. zeylanica* has a lower number of beaked conidia than the other *A. alternata* isolates evaluated.

Conidia of the *A. alternata* isolate from *S. zeylanica* were smoky or golden brown, commonly in chains of three or more; polymorphous, ovoid, ellipsoidal, obpyriform or obclavate, but commonly ovoid or obpyriform and surface walls were usually smooth. Conidia were either beaked or beakless (Figure 2.2a to c) and simple but sometimes branched (Figure 2.2c) with up to three transverse and eight longitudinal or oblique septa. Beaks were conical and narrowly tapering, usually shorter than the body and pale brown. Conidia were frequently slightly constricted at the septa. Earliest formed transepta and major longisepta were slightly thicker and darker than younger septa. Conidiophores were solitary or in fascicles of two to six

unbranched or branched, straight or flexuous, occasionally geniculate with one or several conidial scars, pale to mid-brown, of variable length, up to 269 µm x 4 - 10 µm (Figure 2.2d). First formed conidia were predominantly long-ellipsoid, although some short-ovoid primary conidia were also present (Figure 2.2e). Colonies were olivaceous black or black, cottony or velvety, with a mycelial growth rate of 8.5 to 8.6 mm day^{-1} at 28°C under continuous NUV. Another interesting characteristic of this isolate was the production of chlamydospores (Figure 2.2f).

2.4.5. Comparative assessment of the pathogenicity of several *Alternaria* isolates. *S. zeylanica* responded differently to inoculation of different *Alternaria* isolates (Table 2.4). *S. zeylanica* was susceptible only to the *Alternaria* isolate from *S. zeylanica*. *S. zeylanica* was immune to *A. tenuissima* (ATCC 11854) and *A. alternata* isolates ATCC 24127, ATCC 26669, and ATCC 60376 and highly resistant to *A. alternata* isolates ATCC 28329 and ATCC 36381. These two *A. alternata* isolates only produced very few, minute, necrotic flecks on *S. zeylanica*. Symptoms of wilting and leaf cupping were first observed 12 h after removal from the dew chamber only on plants inoculated with the isolate from *S. zeylanica*. Sporulation was observed on excised leaves when inoculated with the *S. zeylanica* isolate as early as 12 HAI and sporulation did not occur on excised leaves inoculated with any of the other ATCC isolates. Most plants inoculated with the *S. zeylanica* isolate were killed whereas inoculation did not cause mortality in any of the ATCC isolates (data not shown). Dry weight of *S. zeylanica* was reduced by 97 to 99% when inoculated with the *S. zeylanica* isolate (Table 2.4). The two isolates (ATCC 28329 and ATCC 36381) which caused some flecking reduced plant weight by only 3 to 4%.

2.4.6. Bioassay for phytotoxin production. *A. alternata* appeared to produce phytotoxin(s) in all liquid media tested except for V-8 juice medium 24 h after immersion (Figure 2.3a). In all treatments, chlorosis and wilting of leaves were observed 24 h after exposing the *S. zeylanica* cutting to the culture filtrate except for V-8 juice medium in which symptoms were delayed for 24 h. After 72 h, leaves were completely abscised when immersed in modified Richard's and Fries liquid media. In all cases, the sterile media control did not show any phytotoxic activity. As illustrated (Figure 2.3b), the *S. zeylanica* cutting immersed in sterile Richard's medium was as healthy as the cutting immersed in sterile distilled water.

2.5. Discussion

Nutrients, light, and temperature significantly affected mycelial growth rates of the *A. alternata* isolate from *S. zeylanica*. Mycelial growth rates on VJA, PDA, 1/2 PDA, and CMA were significantly faster than on MEA and OMA. Rapid radial mycelial growth generally occurred under exposures to continuous light, dark, or NUV and exposures to 12 h of alternating light and dark conditions significantly reduced mycelial growth. Growth of the *A. alternata* isolate from *S. zeylanica* was completely inhibited at 36°C. The predicted optimum temperature for maximum radial mycelial growth varied with agar media. The predicted optimum temperature for 1/2 PDA and VJA was 24.4°C whereas, CMA and PDA required a lower predicted optimum temperature (20.4°C and 21.3°C, respectively). These growth requirements conform with those used with other *Alternaria* species for taxonomic studies (Simmons, 1967; Nag Raj and Ponnappa, 1970; Grogan *et al.*, 1975; Vakalounakis and Malathrakis, 1988; Simmons, 1992; Shabana *et al.*, 1995).

S. zeylanica responded differently to inoculation of different *Alternaria* isolates. *S. zeylanica* was susceptible only to the *Alternaria* isolate from *S. zeylanica* and was immune or highly resistant to other *Alternaria* isolates. Symptoms of wilting and leaf cupping were first observed 12 h after removal from the dew chamber only on plants inoculated with the *S. zeylanica* isolate. Most plants inoculated with the *S. zeylanica*

isolate were killed and dry weight reductions of over 97% were recorded. Sporulation time and level were used to characterize the compatibility of the *A. alternata* isolates as pathogens of *S. zeylanica*. Sporulation occurred on *S. zeylanica* as early as 12 h when inoculated with the *A. alternata* isolate from *S. zeylanica*. In contrast, no sporulation occurred within 72 h on excised *S. zeylanica* leaves inoculated with any of the other *Alternaria* isolates. This finding indicates that these isolates were not pathogens of *S. zeylanica*.

Host-selectivity, pathogenicity, and virulence of some *A. alternata* are due to the production and release of host-specific toxins (Scheffer and Livingston, 1984). Rapid disease development in *S. zeylanica* when inoculated with the *A. alternata* isolate from *S. zeylanica* and the phytotoxicity of cell free extracts indicated that phytotoxic compounds are being produced which may be selective to the specific host plant. Common metabolites from *A. alternata* include tenuazonic acid, alternaric acid, tentoxin, and zinniol (Stoessl, 1981), radicinin and related compounds (Robeson *et al.*, 1982), alternariol, altenuene and related compounds, certain isocoumarins, brefeldin, altenins, altersolanols, and macrosporin (Stoessl, 1981), and maculosin (Stierle *et al.*, 1988). Studies to isolate, purify, and characterize the phytotoxin(s) produced by this fungal pathogen should be investigated.

The diagnosis of the characteristics of the *A. alternata* isolate from *S. zeylanica* are:

> *Conidiophores* were solitary or in fascicles of two to six unbranched or branched, straight or flexuous, occasionally geniculate with one or several conidial scars, pale to mid-brown, of variable length, up to 269 µm x 4 - 10 µm. *Conidia* from VJA and 1/2 PDA were commonly in branch chains of three or more; polymorphous, ovoid, ellipsoidal, obpyriform or obclavate, but commonly ovoid or obpyriform; majority (57 to 64%) with a beak (rostrate) which is simple or branched, conical and narrowly tapering, usually shorter than the body, pale brown; smoky brown or gold brown, surface walls usually smooth; with up to three transverse and eight longitudinal or oblique septa,

frequently slightly constricted at the septa; measuring 10 - 60 µm (32 µm) x 8 - 49 µm (14 µm) for VJA and 17 - 62 µm (38 µm) x 8 - 49 µm (14 µm) for 1/2 PDA excluding the rostrum which was 5 - 33 µm (18 µm) x 4 - 10 µm (7 µm) for VJA and 5 - 50 µm (24 µm) x 6 - 9 µm (7 µm) for 1/2 PDA, and a total length of 10 - 89 µm (46µm) for VJA and 18 - 93 µm (53 µm) for 1/2 PDA. First formed conidia were predominantly long-ellipsoid, although some short-ovoid primary conidia were also present. Earliest formed transepta and major longisepta were slightly thicker and darker than younger septa. *Colonies* were olivaceous black or black, cottony or velvety, with a mycelial growth rate of 8.5 to 8.6 mm day^{-1} at 28°C under continuous NUV. Moreover, this isolate produces chlamydospores.

The absence of any previous record of *A. alternata* associated with *S. zeylanica*, the unique morphological, cultural, and pathological characteristics of this *A. alternata* isolate from *S. zeylanica* in comparison with other *A. alternata* isolates, and host specificity (Chapter 6 of this study) indicate that there are sufficient grounds to state that this *A. alternata* causing leaf blight of *S. zeylanica* corresponds to a subspecific forma speciales thus, the binomial, *A. alternata* f. sp. *sphenocleae* is proposed.

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Table 2.1. Effect of agar medium on radial mycelial growth of the *Alternaria alternata* isolate from *Sphenoclea zeylanica*.[y]

Agar medium	Radial mycelial growth (mm day^{-1})
VJA (V-8 juice agar)	8.6 a[z]
PDA (potato dextrose agar)	8.5 a
1/2 PDA (half-strength PDA)	8.5 a
CMA (corn meal agar)	8.1 a
OMA (oatmeal agar)	7.3 b
MEA (malt extract agar)	6.7 b

[y] Plates were incubated at 28°C under continuous light condition (400 $\mu Em^{-2}s^{-1}$ PAR). Results are from pooled experiments.

[z] Means having common letters are not significantly different according to Duncan's multiple range test at the 5% level of significance.

Table 2.2. Effect of agar medium and light condition on radial mycelial growth of the *Alternaria alternata* isolate from *Sphenoclea zeylanica.*[w]

Agar medium	Radial mycelial growth (mm day^{-1})			
	Light condition			
	L[x]	L/D	D	NUV
1/2 PDA[y]	8.2 ab[z]	6.3 e	8.6 a	8.5 ab
PDA	8.0 abc	6.3 e	8.6 a	8.5 ab
VJA	8.6 a	7.9 bc	8.6 a	8.6 a
CMA	8.6 a	5.3 f	8.6 a	8.1 ab
MEA	5.7 ef	5.6 f	6.7 de	6.7 de
OMA	8.6 a	7.4 cd	8.6 a	7.2 d

[w] Plates were incubated at 28°C. Results are from pooled experiments.

[x] L = continuous light (400 $\mu Em^{-2}s^{-1}$), L/D = 12 h of alternating light (400 $\mu Em^{-2}s^{-1}$) and dark, D = continuous dark, NUV = continuous near ultra-violet light (J-205, UVP, Inc.).

[y] 1/2 PDA = half-strength potato dextrose agar, PDA = potato dextrose agar, VJA = V-8 juice agar, CMA = corn meal agar, MEA = malt extract agar, OMA = oatmeal agar.

[z] Means having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

Table 2.3a. Morphological characteristics of the *Alternaria alternata* isolate from *Sphenoclea zeylanica* in comparison with other *A. alternata* isolates grown on V-8 juice agar at 28°C under continuous near-ultraviolet light for ten days.

***Alternaria* isolate**	**Body (µm)**		**Beak (µm)**		**Total length (µm)**	**Number of septa**	**Conidia with beaks (%)**
	Length	**Width**	**Length**	**Width**			
A. alternata from *Sphenoclea zeylanica*	32[y] (10 - 60)[z]	14 (8 - 49)	18 (5 - 33)	7 (4 - 10)	46 (10 - 89)	1 - 8	64
A. alternata (ATCC 24127)	25 (10 - 46)	12 (6 - 18)	4 (1 - 15)	5 (3 - 8)	29 (13 - 52)	1 - 7	87
A. alternata (ATCC 28329)	34 (13 - 48)	9 (5 - 18)	12 (2 - 28)	5 (2 - 8)	40 (12 - 72)	1 - 7	78

[y] Indicates the mean.

[z] Indicates extreme values.

Table 2.3b. Morphological characteristics of the *Alternaria alternata* isolate from *Sphenoclea zeylanica* in comparison with other *A. alternata* isolates grown on half-strength potato dextrose agar at 28°C under continuous near-ultraviolet light for ten days.

***Alternaria* isolate**	**Body (µm)**		**Beak (µm)**		**Total length (µm)**	**Number of septa**	**Conidia with beaks (%)**
	Length	**Width**	**Length**	**Width**			
A. alternata from *Sphenoclea zeylanica*	38[y] (17 - 62)[z]	17 (13 - 29)	24 (5 - 50)	7 (6 - 9)	53 (18 - 93)	1 - 7	57
A. alternata (ATCC 24127)	28 (17 - 41)	11 (8 - 14)	5 (1 - 14)	4 (2 - 6)	33 (14 - 56)	1 - 12	94
A. alternata (ATCC 28329)	32 (12 - 36)	12 (5 - 18)	7 (2 - 20)	4 (2 - 6)	43 (15 - 60)	1 - 5	72

[y] Indicates the mean.

[z] Indicates extreme values.

Table 2.4. Effects of different *Alternaria* isolates on *Sphenoclea zeylanica*.[w]

Alternaria isolate	Original host plant	Disease reaction(s)[x]	Dry weight reduction (%)[y]	
			5 to 6 cm tall	19 to 20 cm tall
A. tenuissima (ATCC 11854)	*Petroselinum crispum* (Mill.) Mansfeld (parsley)	I	0 c[z]	0 c
A. alternata (ATCC 24127)	*Plantago aristida* Michx.	I	0 c	0 c
A. alternata (ATCC 26669)	*Capsicum annuum* L. (sweet pepper)	I	0 c	0 c
A. alternata (ATCC 28329)	*Lycopersicon esculentum* Mill. (tomato)	I-HR	3.6 b	2.9 b
A. alternata (ATCC 36381)	*Gossypium hirsutum* L. (cotton)	I-HR	3.8 b	2.9 b

Table 2.4. Continued...

Alternaria isolate	Original host plant	Disease reaction(s)	Dry weight reduction (%)	
			5 to 6 cm tall	19 to 20 cm tall
A. alternata (ATCC 60376)	*Brassica oleracea* L. (Chinese cabbage)	I	0 c	0 c
Alternaria species	*Sphenoclea zeylanica* Gaertner (gooseweed)	S	99.0 a	97.1 a

[w] Plants (5 to 6 and 19 to 20 cm tall) were inoculated with 1 x 10^5 conidia ml^{-1}.

[x] Disease severity rating until 14 days after inoculation (DAI) based on a disease rating scale of I (immune) = no visible symptoms, HR (highly resistant) = very few, minute, necrotic flecks present, S (susceptible) = many necrotic lesions present which expand causing desiccation and leaf abscission.

[y] Results are from pooled experiments.

[z] Means having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

Figure 2.1. Effect of agar medium and temperature on radial mycelial growth of the *Alternaria alternata* isolate from *Sphenoclea zeylanica*. Agar media evaluated were 1/2 PDA (half-strength dextrose agar) = ——●——, PDA (potato dextrose agar) = ——■——, VJA (V-8 juice agar) = ——▲——, CMA (corn meal agar) = ——▼——. Regression equations for: 1/2 PDA ($Y = -29.61 + 3.12X - 0.06X^2$; $r^2 = 0.98$), PDA ($Y = -11.70 + 1.85X - 0.04X^2$; $r^2 = 0.79$), VJA ($Y = -30.42 + 3.22X - 0.07X^2$; $r^2 = 0.97$), CMA ($Y = -8.49 + 1.68X - 0.04X^2$; $r^2 = 0.80$). Results are from pooled experiments.

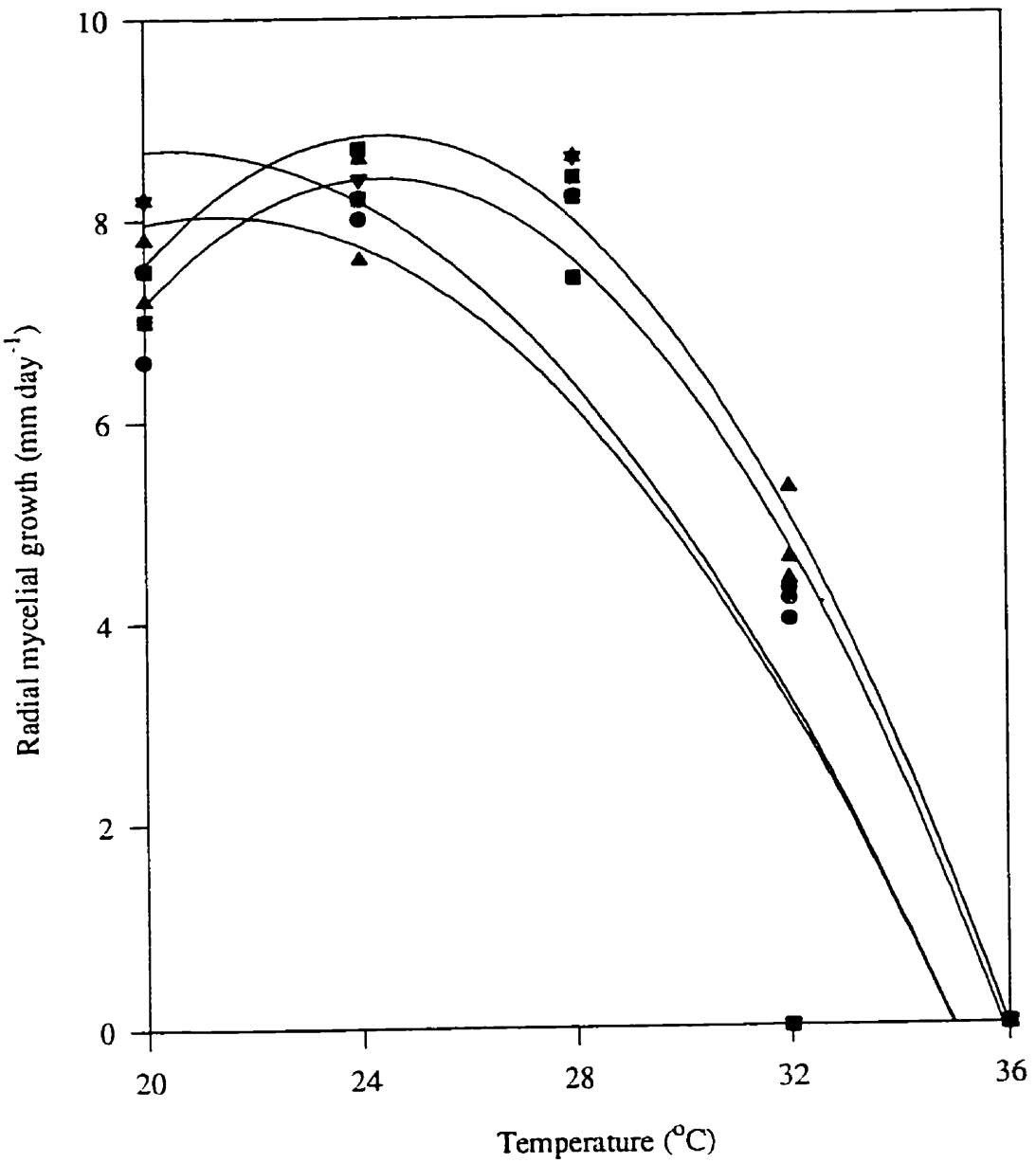
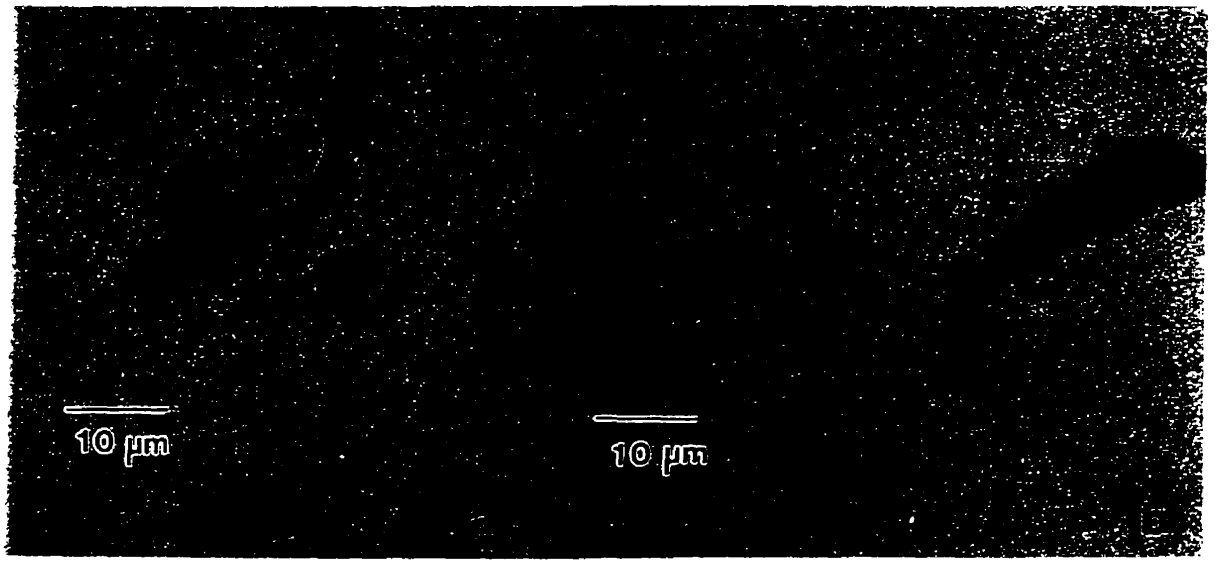
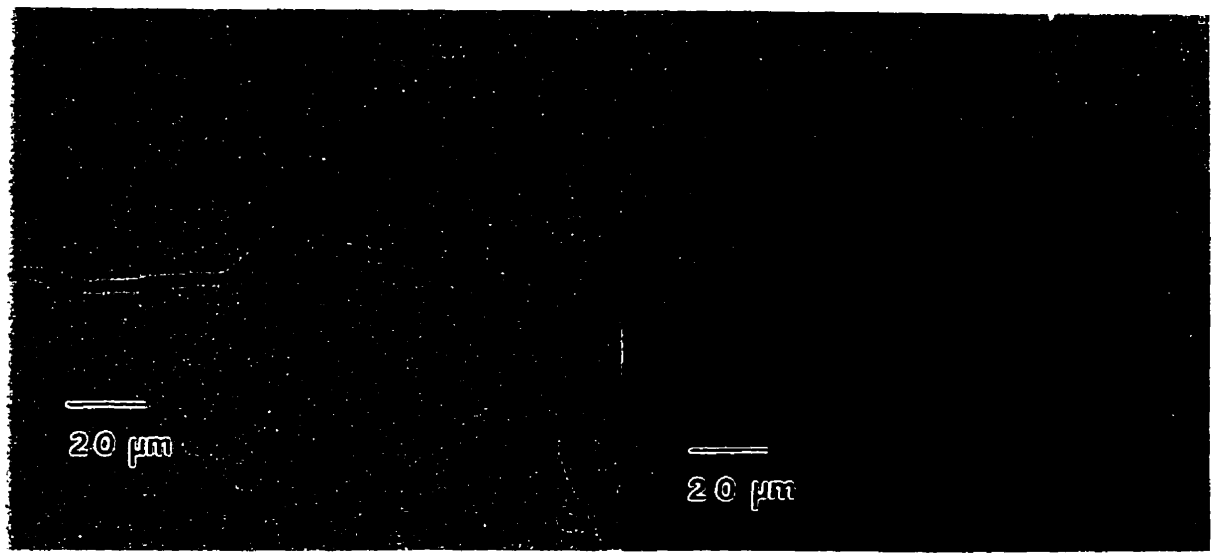

10
8
6
4
2
0
Radial mycelial growth (mm day-1)
20
24
28
32
36
Temperature (°C)

Figure 2.2. Conidial characteristics of the *Alternaria alternata* isolate from *Sphenoclea zeylanica* grown on V-8 juice agar at 28°C under continuous near-ultraviolet light for ten days. a) and b) conidia showing beaked and beakless sporulating types, c) conidium with branched beak, d) conidiophore, e) conidium on conidiophore showing secondary sporulation, f) chlamydospore formation.


10 µm
10 µm


20 µm
20 µm

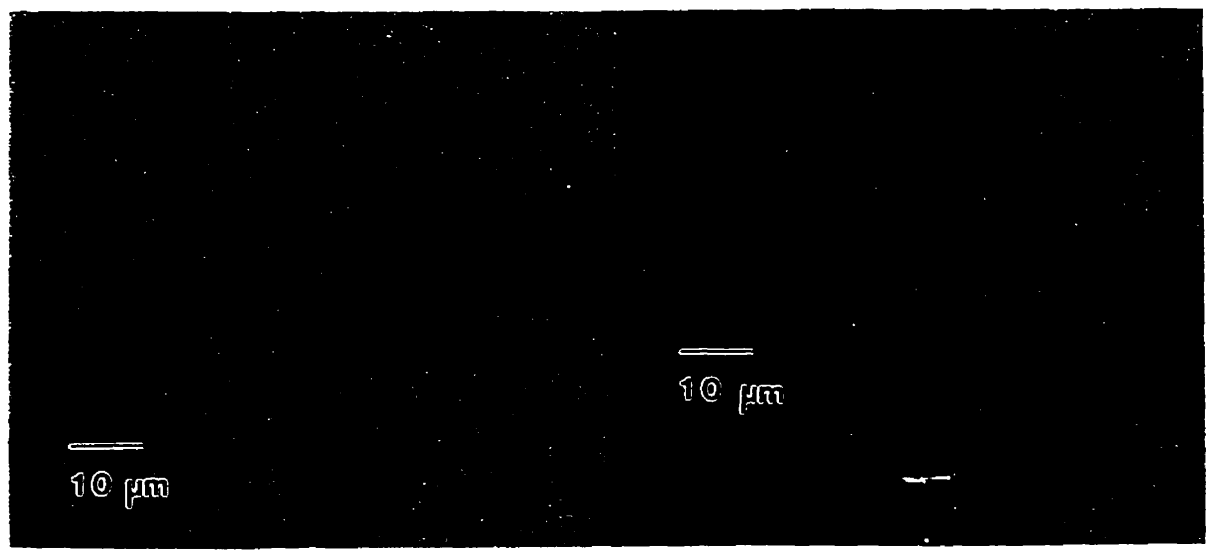
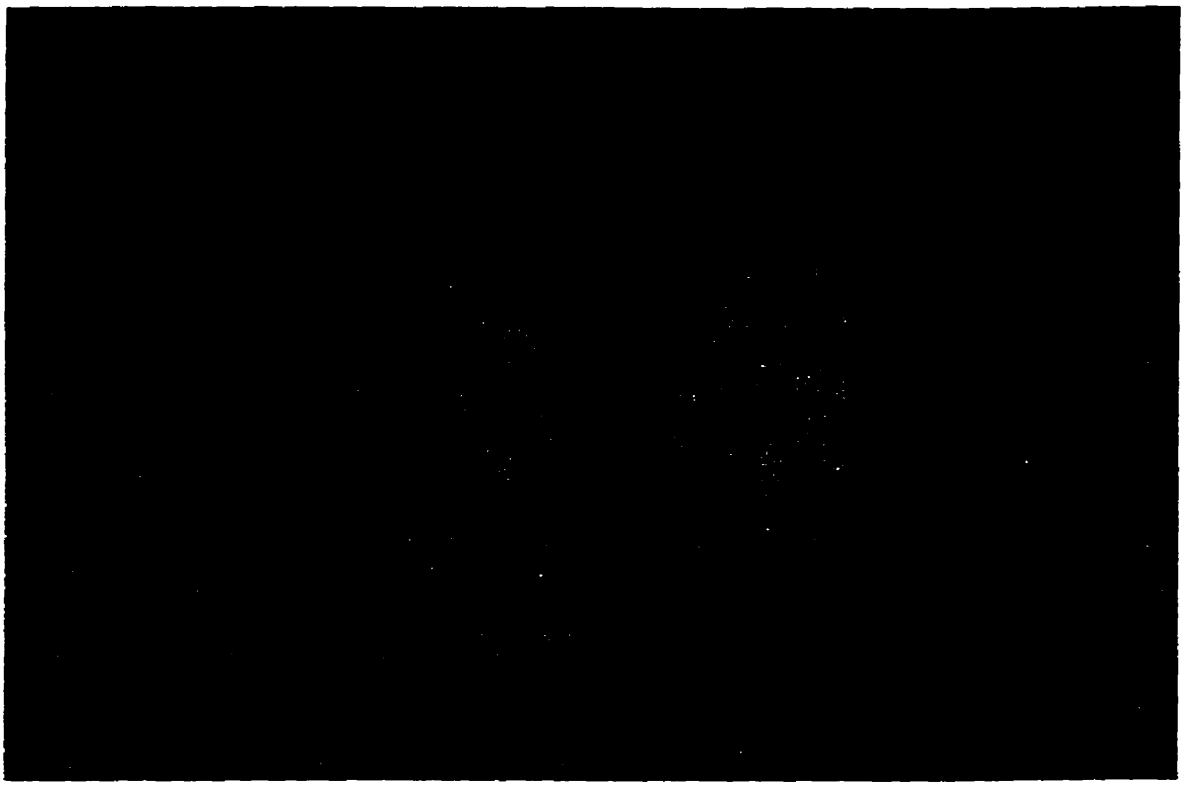

10 µm
10 µm

Figure 2.3a. Effect of culture filtrate of *Alternaria alternata-Sphenoclea zeylanica* on shoot cuttings of *S. zeylanica*. C_1 = sterile distilled water; T_1 = V-8 juice medium; T_2 = potato dextrose broth; T_3 = Czapek Dox broth medium; T_4 = Czapek Dox broth medium supplemented with 5 mg ml^{-1} of $ZnSO_4$; T_5 = Czapek Dox broth medium with 1.5% agar; T_6 = modified Richard's medium; and T_7 = Fries liquid medium. Liquid media were incubated until 14 days at 28°C under continuous light (400 $\mu Em^{-2}s^{-1}$ PAR). Photo was taken 24 h after immersion.

Figure 2.3b. Wilting of leaves on shoot cuttings of *Sphenoclea zeylanica* when immersed in the culture filtrate of the *Alternaria alternata* isolate from *S. zeylanica*. C_1 (Control 1) = cutting in sterile distilled water; T_6 = cutting in culture filtrate from modified Richard's medium; and C_2 (Control 2) = cutting in sterile modified Richard's medium. Photo was taken 24 h after immersion.



Connecting Text

During the development phase of a biological weed control research program, it is necessary to determine various aspects of the potential bioherbicide candidate including optimal conditions and limitation for disease development and host damage. In this chapter, the effect of inoculum density, dew period, and plant height on disease development of *Alternaria alternata* (Fr.) Keissler f. sp. *sphenocleae* and control of *Sphenoclea zeylanica* Gaertner are examined.

Chapter 3 - Factors Influencing Biocontrol of Gooseweed (*Sphenoclea zeylanica*) with *Alternaria alternata* f. sp. *sphenocleae*

3.1. Abstract

Inoculum density, dew period, and plant height are factors influencing biocontrol of *Sphenoclea zeylanica* with *Alternaria alternata* f. sp. *sphenocleae*. When higher inoculum concentrations were applied to plants of different heights with a long dew period, 100% leaf area damage (LAD) occurred earlier than when lower inoculum concentrations were used. At higher inoculum densities, significantly greater plant height reductions were obtained and all plants were killed. A dew period of 8 h was sufficient to cause 100% mortality of plants inoculated with 1 x 10^6 conidia ml^{-1} whereas, 16 h of dew was required with 1 x 10^4 conidia ml^{-1} to attain the same effect. For all dew periods, significantly greater dry weight reductions were obtained at higher inoculum densities.

3.2. Introduction

Gooseweed, *Sphenoclea zeylanica* Gaertner, is a common, annual herbaceous, broadleaf weed species of wetland rice (*Oryza sativa* L.) in Southeast Asia, the United States, the Caribbean area, India, Pakistan, and West Africa (Holm *et al.*, 1977). It has been reported that it was only a weed in rice (Holm *et al.*, 1977), however, recent findings have indicated that it is also associated with other crops including cotton (*Gossypium hirsutum* L.) in Louisiana, U.S.A. (Sanders, 1990), wheat (*Triticum aestivum* L.) in wheat-rice rotations in India (Khan *et al.*, 1988) and in soybean [(*Glycine max* (L.) Merr.)] in rice-soybean rotations in Thailand (Vongsaroj, 1994). *S. zeylanica* can reduce rice yields by as much as 45% (Laganao, 1981; Ampong-Nyarko and De Datta, 1991; Biswas and Sattar, 1991) and significant yield reductions in transplanted rice can occur at weed densities as low as 20 plants m^{-2} (IRRI, 1989).

There are several management strategies available to control *S. zeylanica* including handweeding, cultural techniques, mechanical methods, and chemical

herbicides. Also, the possibility of utilizing an indigenous fungal pathogen as a biological control agent is being investigated (Watson, 1994). In 1991, *Alternaria alternata* (Fr.) Keissler f. sp. *sphenocleae* was isolated from blighted *S. zeylanica* collected in a rice field near Los Baños, Laguna, Philippines. Laboratory and field studies suggest that this fungal pathogen is a promising bioherbicide candidate (Bayot *et al.*, 1994; Mabbayad and Watson, 1995; Masangkay *et al.*, 1996).

Infection, disease development, and subsequently weed control efficacy caused by a fungal pathogen are usually suppressed under natural conditions (Holcomb, 1982; Watson and Wymore, 1990; TeBeest, 1991). It is, therefore, necessary to examine the biology of the pathogen-host interaction and determine optimal environmental conditions for disease development and effective control of *S. zeylanica* by *A. alternata* f. sp. *sphenocleae*. Thus, the objective of this study was to evaluate the interaction of inoculum density with plant height and dew period on infection, disease development, and control efficacy of *A. alternata* f. sp. *sphenocleae* on *S. zeylanica*.

3.3. Materials and Methods

3.3.1. Inoculum production

Diseased *S. zeylanica* leaves were collected from blighted plants in rice fields at the International Rice Research Institute (IRRI) Experimental Farm in 1991. Leaf pieces were surface sterilized with 0.5% sodium hypochlorite solution and incubated on fresh potato dextrose agar (PDA; Difco, Detroit, MI). Monoconidial isolates of *A. alternata* f. sp. *sphenocleae* were prepared, stored, and maintained on half-strength potato dextrose (1/2 PDA) in culture tubes at IRRI and imported into the quarantine facility of McGill University (Macdonald Campus, Ste-Anne-de-Bellevue, Québec, Canada). These monoconidial isolates were then used to inoculate test plant material, re-isolated, stored, and maintained on 1/2 PDA in small vials under mineral oil at 4°C as stock cultures (Tuite, 1969). Small pieces of mycelium from the stock cultures were aseptically transferred to cooled PDA (20 ml) in plastic petri dishes (90-mm-diameter). Cultures were sealed with Parafilm® (American National Can Co.,

Greenwich, CT) and incubated at 28°C under continuous near-ultraviolet (NUV; J-05, UVP, Inc., Circleville, OH) light for three days. Agar plugs (4-mm-diameter) from the margins of young actively growing colonies were used as seed inoculum (Tuite, 1969). Plastic petri dishes (90-mm-diameter) with cooled 1/2 PDA (20 ml) were inoculated under aseptic conditions by inverting and placing an agar plug in the center of each dish. Plates were sealed with Parafilm® and incubated either under NUV or dark conditions at 28°C for three to four weeks. Conidia were harvested by flooding the plates with 10 ml of sterile deionized water containing 0.01% Triton® X-100 (polyethylene glycol tert-octylphenyl ether, Sigma Chemical Co., St. Louis, MO) as a wetting agent and gently scraping the surface of the colonies with a sterile glass slide. Resulting suspensions were poured through a 250 mm plastic sieve lined with two layers of sterile cheesecloth. Inoculum concentration was determined with the aid of a haemocytometer and adjusted to the desired concentration with sterile deionized water containing 0.01% Triton X-100®.

3.3.2. Plant production

S. zeylanica seeds collected from IRRI Experimental Farm in 1994 were soaked with 95% hydrochloric acid (HCl) for 10 min and washed with continuous running distilled water for 30 min. Subsequently, the seeds were soaked in distilled water at room temperature (24 ± 2°C) for 12 h. Seeds were sown in black plastic trays Kord (K 10; Plant Products, Laval, QC) 2/3 filled with sterilized soil mixture consisting of 3 parts garden soil, 3 parts Pro-mix^{BX} (commercial potting mix, Premier Horticulture Inc., Red Hill, PA), 2 parts vermiculite Holiday® (VIL Vermiculite Inc., Montréal, QC) and 1 part sphagnum peat moss Tourbe® (Les Tourbières Premier Ltée, Rivière-du-Loup, QC). After sowing, the soil was lightly pressed with a 0.5 x 25 x 15 cm wooden block and watered until saturated. Seeded trays were placed in a controlled environment chamber [Conviron® E15 (Winnipeg, Man.); 32/24°C day/night, 400 $\mu Em^{-2}s^{-1}$ photosynthetically active radiation (PAR), 12 h day^{-1}, and 70 to 80% relative humidity (RH)] until plants were ready for transplanting. Relative humidity was

maintained with a humidifier, soil was kept saturated and the soil surface was misted every 12 h using a hand-held atomizer.

Healthy seedlings were selected 21 days after sowing and transplanted in transparent plastic pots (7 x 10.5 cm, Better Plastics, Inc., Kissimmee, FL) filled with sterilized moistened soil mixture. Three plants were transplanted in each pot and fertilized with 10 ml of 1.25 g L^{-1} of 20-20-20 (N-P-K) fertilizer. Pots were transferred back to the controlled environment chamber with conditions as previously described. Water level was maintained at a depth of 2 to 3 cm throughout the experimental period.

Different aged plants, as characterized by plant height, were used in this study. In controlled environment conditions, the minute seedlings remained in a small rosette-like form for two weeks, after which the uniculm succulent shoots elongated. Stem branching occurred when plants were 15 to 16 cm tall, flower bud initiation started when plants were 20 to 21 cm tall, and flowering occurred when plants were 25 to 26 cm tall. During the flowering stage, branches also produced flowers.

3.3.3. General inoculation procedure

Each treatment consisted of inoculating three pots of *S. zeylanica* (each pot containing three plants) with 10 ml of conidial suspension containing 0.01% Triton® X-100 as a wetting agent, using a hand-held atomizer. Control treatments were sprayed with deionized water containing the wetting agent. Immediately after spraying, pots were placed in a dark dew chamber (Percival®, Boone, IA) with 100% relative humidity at 24°C depending on the treatments indicated. Pots were then transferred to the controlled environment chamber having the same conditions as previously mentioned.

3.3.4. Assessment of efficacy

Disease intensity was assessed as percentage leaf area damage (% LAD) and recorded visually every two days for 14 days after inoculation (DAI). Plant height, mortality of plants, and dry weight of living above-ground biomass were assessed 14

DAI. Plant height was determined for each plant by measuring from soil level to the base of the blighted portion of infected stems or to the base of the uppermost leaf of unaffected plants, results were pooled, and averaged for each pot. Plant height data were expressed as percent reduction in plant height compared with plant height of non-inoculated controls. Mortality was evaluated for each plant, results pooled, and averaged for each pot. Collapsed plants were considered dead, although the succulent stem base of some blighted plants remained green. Dry weights were obtained by cutting live aboveground tissue at soil level, drying in paper bags for seven days at 45°C, and weighing. Dead leaves and dead portions of the stem were not included in the dry weight measurements. Dry weight data were expressed as percent reduction in biomass compared with biomass of the non-inoculated controls.

3.3.5. Effect of inoculum density and plant height

S. zeylanica seedlings at different plant heights (5 to 6, 10 to 11, 15 to 16, 20 to 21, and 25 to 26 cm tall) were inoculated at inoculum densities of 0, 1 x 10^4, 1 x 10^5, and 1 x 10^6 conidia ml^{-1}, placed in a dark dew chamber with 100% relative humidity at 24°C for 8 h. Pots were then transferred to the controlled environment chamber having the same conditions as previously mentioned.

3.3.6. Effect of dew period and inoculum density

Plants (16 to 17 cm tall) were inoculated at inoculum densities of 0, 1 x 10^4, 1 x 10^5, and 1 x 10^6 conidia ml^{-1}. After spraying, pots with no supplemental dew were immediately placed in the controlled environment chamber and the rest of the pots placed in a dark dew chamber with 100% relative humidity at 24°C and exposed to dew periods of 8, 16, and 24 h. The controlled environment chamber was programmed to simulate the dark period in the dew chamber. Following the dew period treatment, pots were transferred to the controlled environment chamber having the same conditions as previously mentioned.

3.3.7. Data Analyses

All experiments were performed twice and laid out in a randomized complete block design (RCBD) with three replicates. All percentage data were arc sine transformed prior to analysis (Gomez and Gomez, 1984). Factorial experiments were analyzed using a factorial analysis of variance considering the effect of each factor individually and their interaction. Results from the two trials were pooled if homogeneity of variances was confirmed by Bartlett's test (Gomez and Gomez, 1984). All analyses were conducted using STATGRAPHICS software for IBM-PC (STSC Inc., 1991). Treatment means were separated using Duncan's multiple range test at the 1% level of significance.

3.4. Results

3.4.1. Effect of inoculum density and plant height. There was a significant interaction between inoculum density and plant height on percent reduction in plant height (Appendix 3.1), mortality (Appendix 3.2), and dry weight (Appendix 3.3) of *S. zeylanica* inoculated with *A. alternata* f. sp. *sphenocleae*. Inoculum densities of 1 x 10^5 to 1 x 10^6 conidia ml^{-1} on 5- to 6-cm-tall plants gave 100% LAD at 8 DAI. Using an inoculum density of 1 x 10^4 conidia ml^{-1} on 5- to 6- cm tall plants, resulted in a 99% LAD 14 DAI. This slight reduction in disease severity was also observed on 20- to 21- and 25- to 26-cm-tall plants inoculated with 1 x 10^5 conidia ml^{-1} (Figure 3.1). With the highest inoculum density (1 x 10^6 conidia ml^{-1}), 100% LAD was observed on 20- to 21- and 25- to 26-cm-tall plants 14 DAI.

With all plant heights, a conidial suspension of 1 x 10^6 conidia ml^{-1} provided 100% reduction in plant height however, this was not significantly different when 5- to 6-, 10- to 11-, and 15- to 16-cm-tall plants were inoculated with 1 x 10^5 conidia ml^{-1} (Figure 3.2). The lowest percent reduction in plant height (52%) was observed with 25- to 26-cm-tall plants inoculated with the lowest inoculum density (1 x 10^4 conidia ml^{-1}).

When 1 x 10^6 conidia ml^{-1} were applied to all the different plant heights, 100% mortality was obtained. One hundred percent mortality was also obtained with 5- to 6-cm-tall plants when inoculated with 1 x 10^5 conidia ml^{-1} (Figure 3.3). Lower plant mortality (44 to 61%) occurred when 1 x 10^4 conidia ml^{-1} were applied to 15- to 16-, 20- to 21-, and 25- to 26-cm-tall plants. Dry weight reduction data (Figure 3.4) mirrored mortality data for 5- to 6-cm-tall plants. With older plants, blight did not extend down the entire stem and at the lower inoculum density, limited regrowth occurred in some older plants.

3.4.2. Effect of inoculum density and dew period. There was a significant interaction between inoculum density and dew period on percent mortality (Appendix 3.4) and dry weight reduction (Appendix 3.5.) of *S. zeylanica* inoculated with *A. alternata* f. sp. *sphenocleae*. Plants inoculated with 1 x 10^4 conidia ml^{-1} supplemented with 16 and 24 h of dew gave 100% LAD at 14 DAI and only 6% LAD occurred at 14 DAI with no supplemental dew (Figure 3.5). Inoculated plants with 1 x 10^5 conidia ml^{-1} with a dew period of 24 h gave 100% LAD at 10 DAI whereas, a dew period of 16 h required 12 DAI to produce the same effect. With no supplemental dew, 90% LAD was still achieved. With the highest inoculum density (1 x 10^6 conidia ml^{-1}), 100% LAD was obtained at 12 DAI with 8 h of dew, at 10 DAI with 16 h of dew, and at 8 DAI with 24 h of dew and with no supplemental dew, 98% LAD was observed.

A dew period of 8 h was sufficient to cause 100% mortality when plants were inoculated with 1 x 10^6 conidia ml^{-1} whereas, a lower inoculum concentration (1 x 10^5 conidia ml^{-1}) required 16 h of dew to attain the same effect (Figure 3.6). Plants were not killed when inoculated with 1 x 10^4 conidia ml^{-1} having no supplemental dew.

Plants inoculated with 1 x 10^6 conidia ml^{-1} supplemented with 16 to 24 h of dew gave 100% dry weight reduction, but this was not significantly different from 1 x 10^5 conidia ml^{-1} having 24 h of dew (Figure 3.7). The lowest dry weight reduction (18%) occurred when plants were inoculated with 1 x 10^4 conidia ml^{-1} having no supplemental dew.

3.5. Discussion

The level of infection and damage caused by *A. alternata* f. sp. *sphenocleae* on *S. zeylanica* was influenced by inoculum density, dew period, and plant height. Low initial inoculum level contributes to the failure of disease epidemics to develop and persist in weed populations (Holcomb, 1982; Watson and Wymore, 1990). Greater disease expression was obtained on plants inoculated with higher inoculum densities. The minimum inoculum density required for effective control of *S. zeylanica* was 1 x 10^4 conidia ml^{-1}. In the bioherbicide strategy, low application rates capable of causing severe or lethal damage to the host weed are desired to minimize production costs (Baker and Henis, 1990).

The minimum dew period to achieve 100% mortality was 8 h. This does not conform with the findings of Mabbayad and Watson (1995) wherein they reported 100% mortality even when no supplemental dew was provided. Differences in results can be attributed to the varying environmental conditions that inoculated plants were subjected once they were transferred from dew chambers. Mabbayad and Watson (1995) transferred plants to a mist room set at 26 ± 2°C with 85 to 95% relative humidity while in this experiment, plants were transferred to a controlled environment chamber with a 32/24°C day/night temperature and a relative humidity of 70 to 80%. However, the findings presented in this study are similar to those reported for commercial as well as potential bioherbicides in which supplementary dew was required to obtain high levels of efficacy. Examples include *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (Penz.) Sacc. for the control of northern jointvetch [*Aeschynomene virginica* (L.) B.S.P.] (TeBeest and Templeton, 1978), *C. coccodes* (Wallr.) Hughes for the control of eastern black nightshade (*Solanum ptycanthum* Dun.) (Anderson and Walker, 1985), *Fusarium lateritium* Nees ex Fr. for the control of velvetleaf (*Abutilon theophrasti* Medik.) and prickly sida (*Sida spinosa* L.) (Boyette and Walker, 1985), *Alternaria cassiae* Jurair and Khan for the control of sicklepod (*Cassia obtusifolia* L.) (Walker and Boyette, 1986), and *C. gloeosporioides*

(Penz.) Sacc. f. sp. *malvae* for the control of round-leaved mallow (*Malva pusilla* Sm.) (Makowski, 1993).

The most important environmental factors affecting plant disease are moisture and to a lesser extent, temperature. Free moisture on the leaf surface is needed for the germination and penetration by infective propagules of most foliar plant pathogens (Rotem, 1978; Zadoks and Schein, 1979). In the field, free moisture may be provided by several natural and man-made processes such as rain, dew, fog, and irrigation (Wallin, 1963). The duration of leaf surface moisture is more important than the amount that is present (Rotem, 1978; Van der Wal, 1978).

An important prerequisite for any potential bioherbicide candidate to be developed is the determination of the plant stage in which the host is most susceptible to disease development (Holcomb, 1982; Watson and Wymore, 1990). The susceptibility of *S. zeylanica* to *A. alternata* f. sp. *sphenocleae* decreased with increasing plant height. This finding is similar to reports of other potential bioherbicides in which younger seedlings were shown to have the greatest susceptibility to disease (TeBeest and Templeton, 1978; Boyette and Walker, 1985; Charudattan, 1990).

Interestingly, these laboratory results tend to support the results reported from field trials conducted in Talavera, Nueva Ecija, Philippines (IRRI, 1995) and Baybay, Leyte, Philippines (IRRI, 1997).

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Figure 3.1. Effect of inoculum density and plant height on disease development caused by *Alternaria alternata* f. sp. *sphenocleae* on *Sphenoclea zeylanica* expressed as percent leaf area damage (% LAD). Data recorded every two days until 14 days after inoculation. Inoculum densities of A = 1 x 10^4 ml^{-1}, B = 1 x 10^5 ml^{-1}, C = 1 x 10^6 ml^{-1} and plant height (5 to 6 cm = ——●——, 10 to 11 cm = ——■——, 15 to 16 cm = ——▲——, 20 to 21 cm = ——▼——, 25 to 26 cm = ——◆——). Each pot was inoculated with 10 ml of conidial suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. Sprayed potted plants were placed in a dark dew chamber with 100% relative humidity at 24°C for 8 h. Regression equations **for (A):** for 5- to 6-cm-tall plants ($Y = 18.62 + 14.94X - 0.67X^2$; $r^2 = 0.95$); for 10- to 11-cm-tall plants ($Y = 21.78 + 12.53X - 0.51X^2$; $r^2 = 98$); for 15- to 16-cm-tall plants ($Y = -8.52 + 12.67X - 0.35X^2$; $r^2 = 0.98$); for 20- to 21-cm-tall plants ($Y = -2.13 + 10.74X - 0.25X^2$; $r^2 = 0.98$); for 25- to 26-cm-tall plants ($Y = -8.60 + 10.30X - 0.18X^2$; $r^2 = 0.97$), **for (B):** for 5- to 6-cm-tall plants ($Y = 31.34 + 13.86X - 0.66X^2$; $r^2 = 0.94$; for 10- to 11-cm-tall plants ($Y = 9.14 + 15.59X - 0.66X^2$; $r^2 = 98$); for 15- to 16-cm-tall plants ($Y = -6.57 + 14.55X - 0.49X^2$; $r^2 = 0.98$); for 20- to 21-cm-tall plants ($Y = -5.42 + 11.79X - 0.30X^2$; $r^2 = 0.98$); for 25- to 26-cm-tall plants ($Y = -14.60 + 11.74X - 0.25X^2$; $r^2 = 0.98$), **for (C):** for 5- to 6-cm-tall plants ($Y = 37.28 + 12.98X - 0.63X^2$; $r^2 = 0.93$; for 10- to 11-cm-tall plants ($Y = 31.83 + 12.96X - 0.59X^2$; $r^2 = 98$); for 15- to 16-cm-tall plants ($Y = -4.84 + 17.89X - 0.75X^2$; $r^2 = 0.98$); for 20- to 21-cm-tall plants ($Y = -8.15 + 13.15X - 0.38X^2$; $r^2 = 0.99$); for 25- to 26-cm-tall plants ($Y = -9.77 + 13.54X - 0.40X^2$; $r^2 = 0.98$). Results are from pooled experiments.

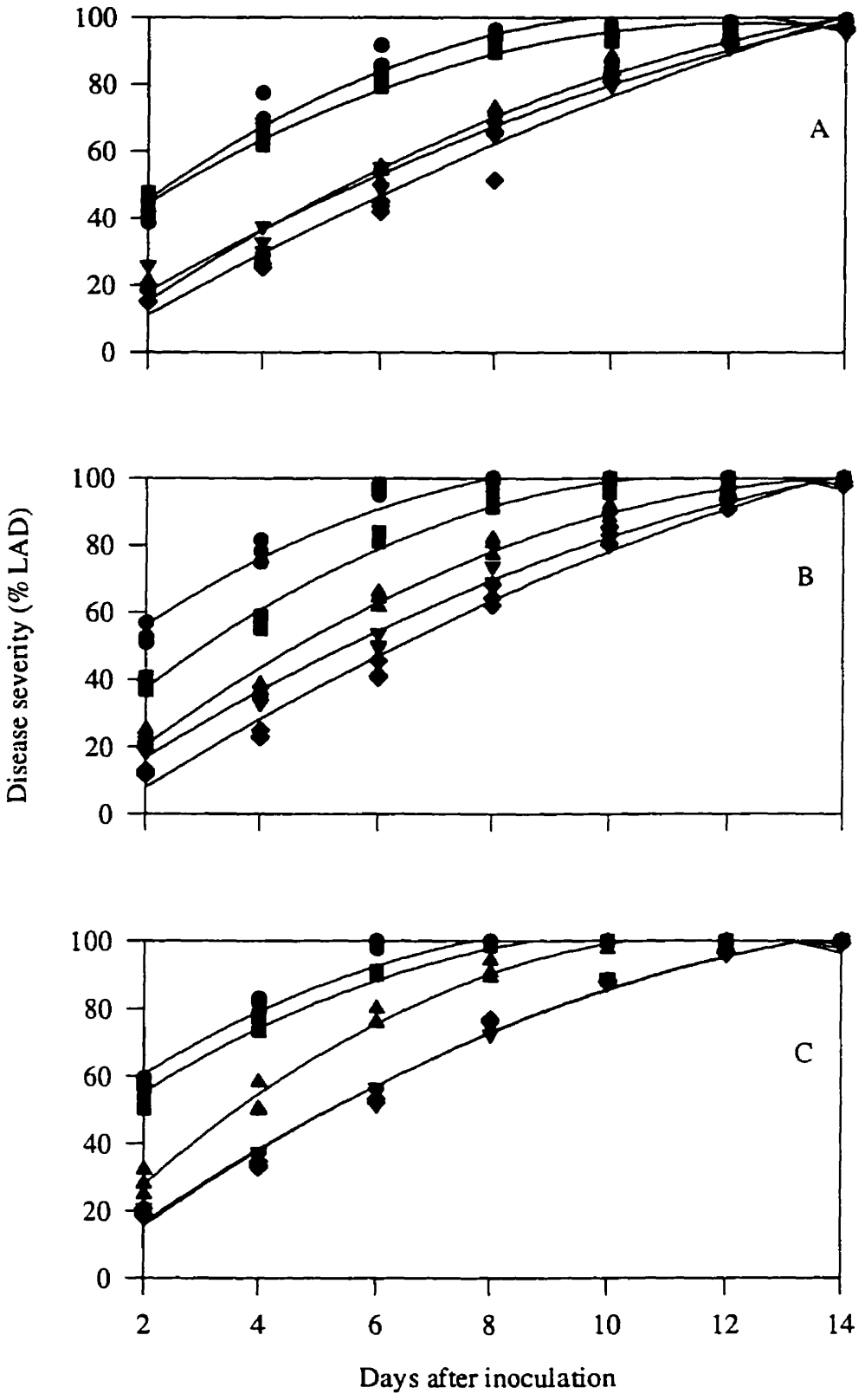

100
80
60
40
20
0
A
B
C
Disease severity (% LAD)
2
4
6
8
10
12
14
Days after inoculation

Figure 3.2. Effect of inoculum density and plant height on disease development caused by *Alternaria alternata* f. sp. *sphenocleae* on *Sphenoclea zeylanica*, expressed as percent reduction in plant height 14 days after inoculation. Each pot was inoculated with 10 ml of conidial suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. Sprayed potted plants were placed in a dark dew chamber with 100% relative humidity at 24°C for 8 h. Results are from pooled experiments. Bars having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

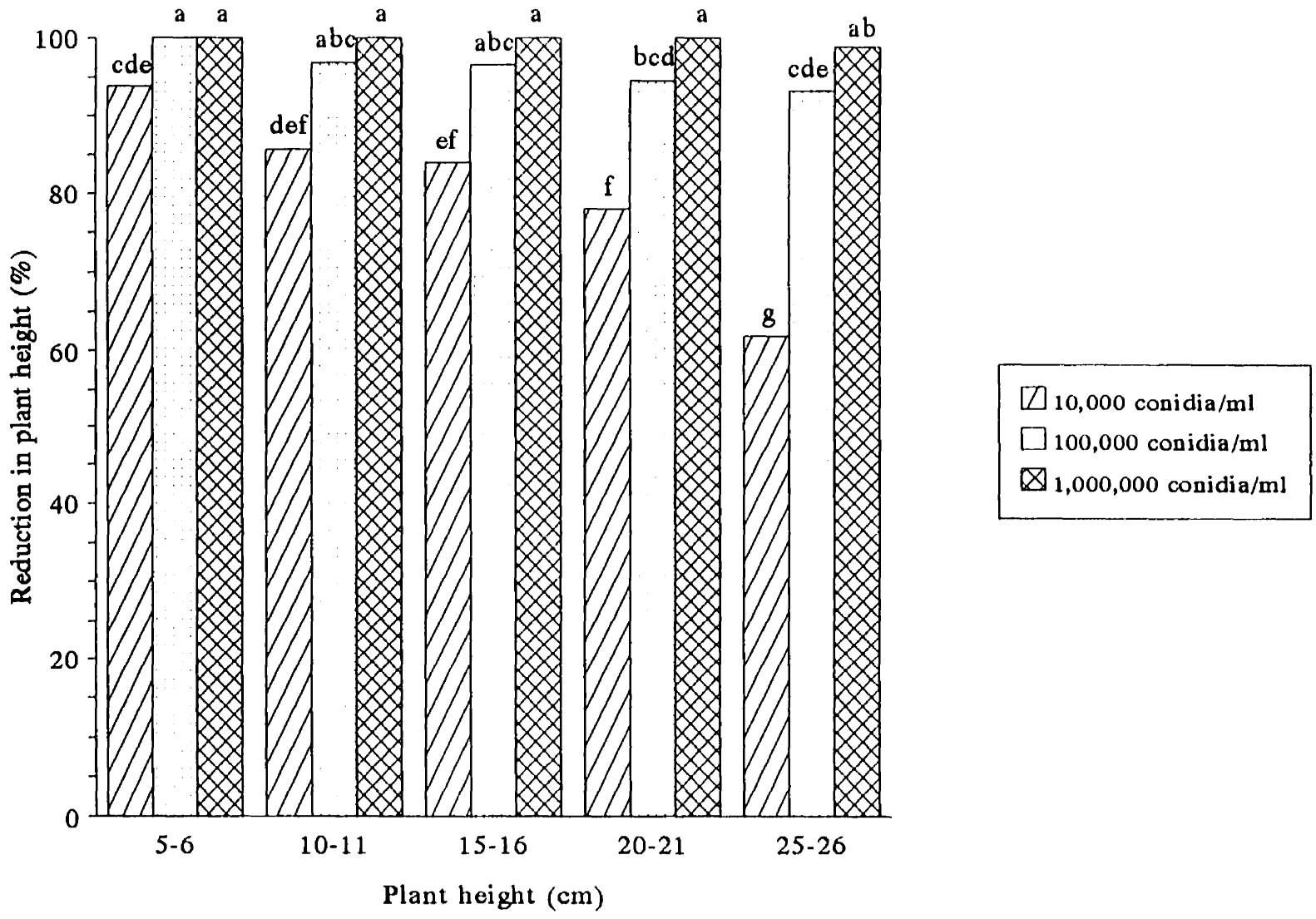

100
80
60
40
20
0
Reduction in plant height (%)
cde
a
a
def
abc
a
ef
abc
a
f
bcd
a
g
cde
ab
5-6
10-11
15-16
20-21
25-26
Plant height (cm)
10,000 conidia/ml
100,000 conidia/ml
1,000,000 conidia/ml

Figure 3.3. Effect of inoculum density and plant height on disease development caused by *Alternaria alternata* f. sp. *sphenocleae* on *Sphenoclea zeylanica*, expressed as percent mortality 14 days after inoculation. Each pot was inoculated with 10 ml of conidial suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. Sprayed potted plants were placed in a dark dew chamber with 100% relative humidity at 24°C for 8 h. Results are from pooled experiments. Bars having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

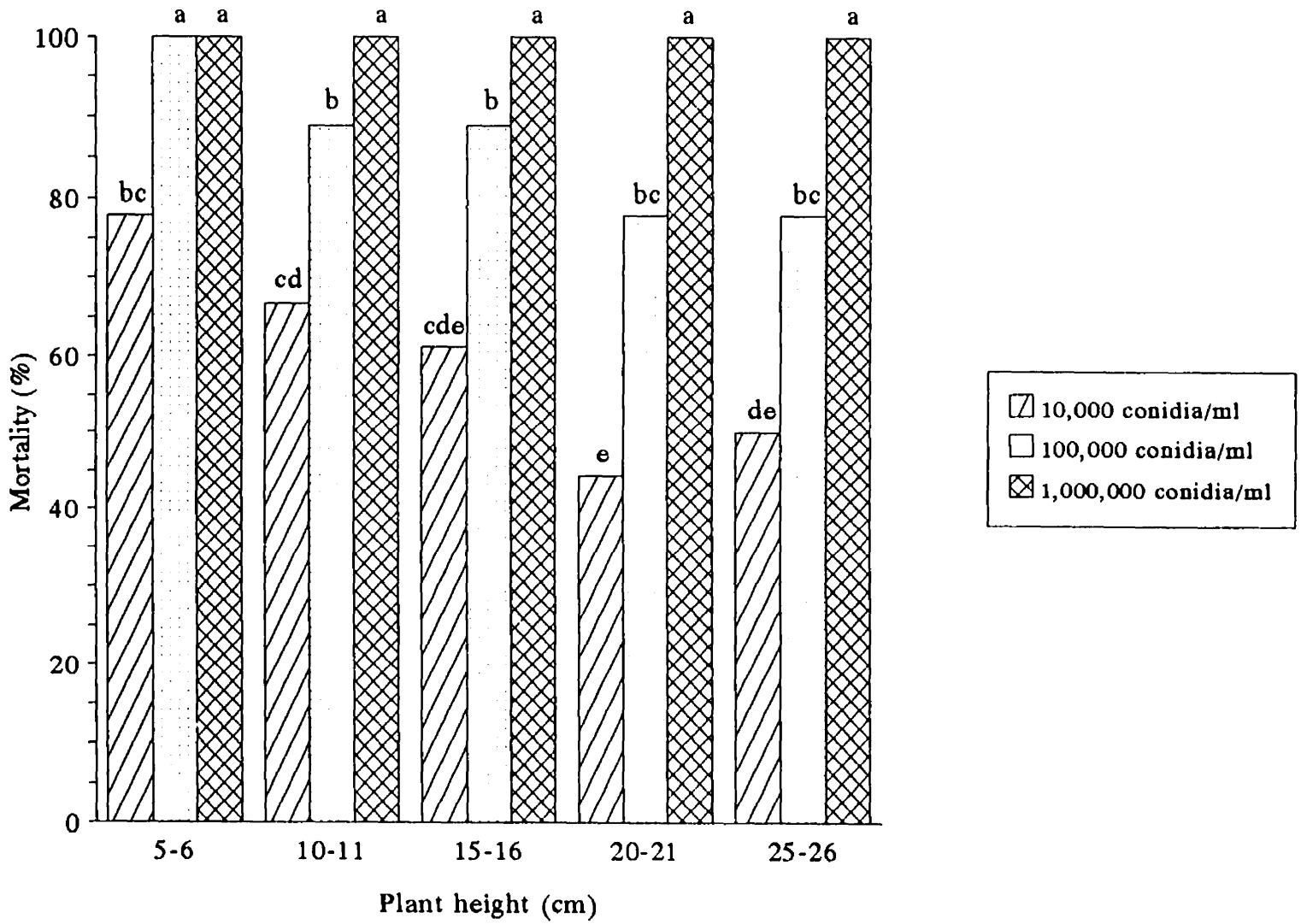

100
80
60
40
20
0
Mortality (%)
a a
bc
a
b
cd
a
b
cde
a
bc
e
a
bc
de
a
5-6
10-11
15-16
20-21
25-26
Plant height (cm)
10,000 conidia/ml
100,000 conidia/ml
1,000,000 conidia/ml

Figure 3.4. Effect of inoculum density and plant height on disease development caused by *Alternaria alternata* f. sp. *sphenocleae* on *Sphenoclea zeylanica*, expressed as percent dry weight reduction 14 days after inoculation. Each pot was inoculated with 10 ml of conidial suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. Sprayed potted plants were placed in a dark dew chamber with 100% relative humidity at 24°C for 8 h. Results are from pooled experiments. Bars having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

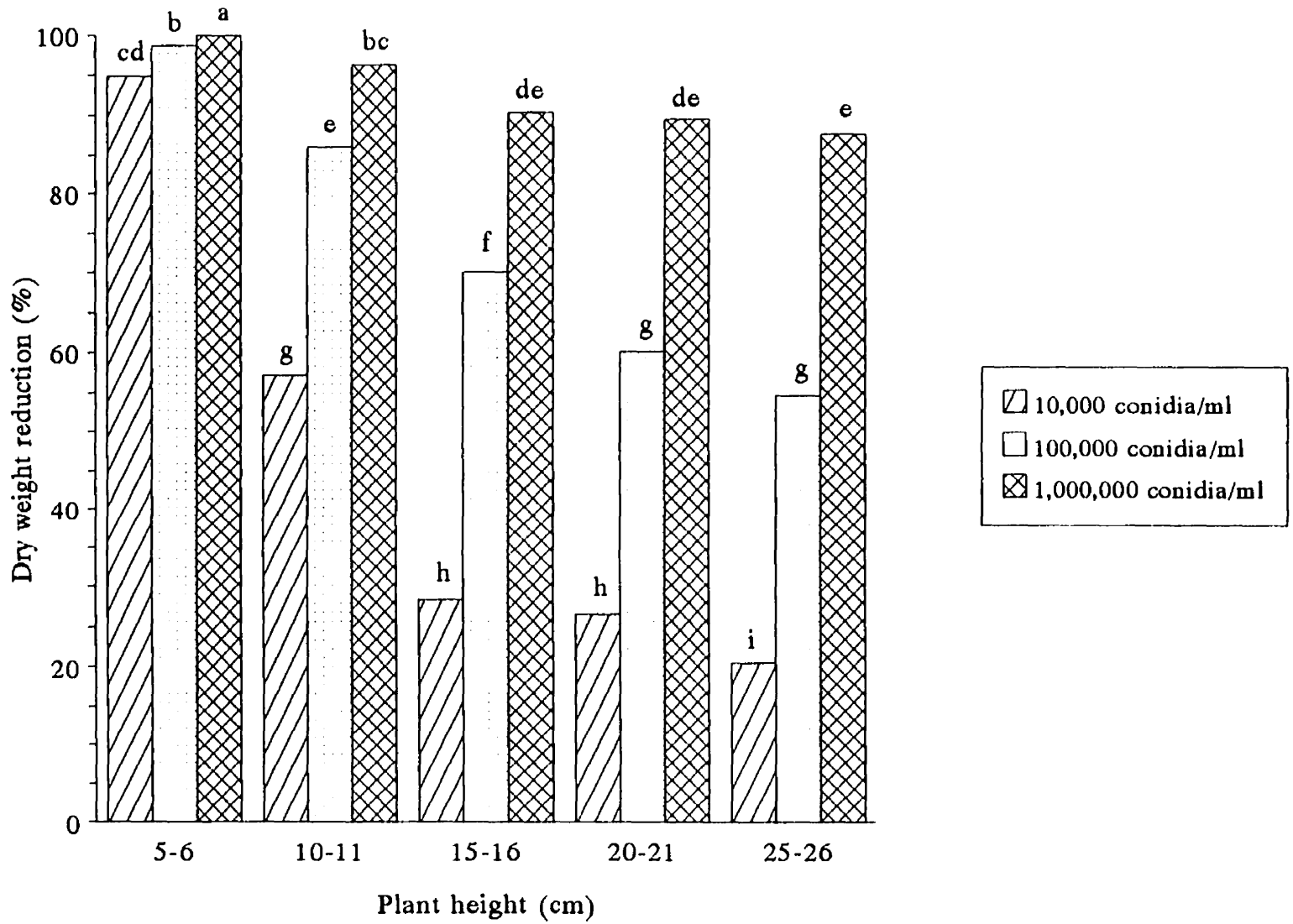

Dry weight reduction (%)
100
80
60
40
20
0
cd
b
a
g
e
bc
h
f
de
h
g
de
i
g
e
5-6
10-11
15-16
20-21
25-26
Plant height (cm)
10,000 conidia/ml
100,000 conidia/ml
1,000,000 conidia/ml

Figure 3.5. Effect of inoculum density and dew period on disease development caused by *Alternaria alternata* f. sp. *sphenocleae* on *Sphenoclea zeylanica*, expressed as percent leaf area damage (% LAD). Data were collected every two days until 14 days after inoculation. Plants (16 to 17 cm tall) were inoculated with 10 ml of conidial suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. Inoculum densities were A = 1 x 10^4 ml^{-1}, B = 1 x 10^5 ml^{-1}, and C = 1 x 10^6 ml^{-1} and dew periods were 0 h = ——●——, 8 h = ——■——, 16 h = ——▲——, and 24 h = ——▼—— . Sprayed potted plants were placed in a dark dew chamber with 100% relative humidity at 24°C. Regression equations **for (A):** for 0 h dew period ($Y = 0.94 - 0.57X + 0.07X^2$; $r^2 = 0.96$); for 8 h dew period ($Y = -15.50 + 12.10X - 0.27X^2$; $r^2 = 98$); for 16 h dew period ($Y = 3.68 + 16.42X - 0.70X^2$; $r^2 = 0.97$); for 24 h dew period ($Y = 24.47 + 14.01X - 0.63X^2$; $r^2 = 0.96$), **for (B):** for 0 h dew period ($Y = -7.02 + 3.63X - 0.26X^2$; $r^2 = 0.97$; for 8 h dew period ($Y = -16.88 + 14.64X - 0.45X^2$; $r^2 = 98$); for 16 h dew period ($Y = 15.22 + 15.68X - 0.70X^2$; $r^2 = 0.97$); for 2h h dew period ($Y = 32.79 + 13.40X - 0.63X^2$; $r^2 = 0.93$), **for (C):** for 0 dew period ($Y = -13.97 + 10.50X - 0.17X^2$; $r^2 = 0.98$; for 8 h dew period ($Y = -12.24 + 19.40X - 0.82X^2$; $r^2 = 98$); for 16 h dew period ($Y = 33.23 + 13.93X - 0.68X^2$; $r^2 = 0.89$); for 24 h dew period ($Y = 44.48 + 11.74X - 0.57X^2$; $r^2 = 0.88$). Results are from pooled experiments.

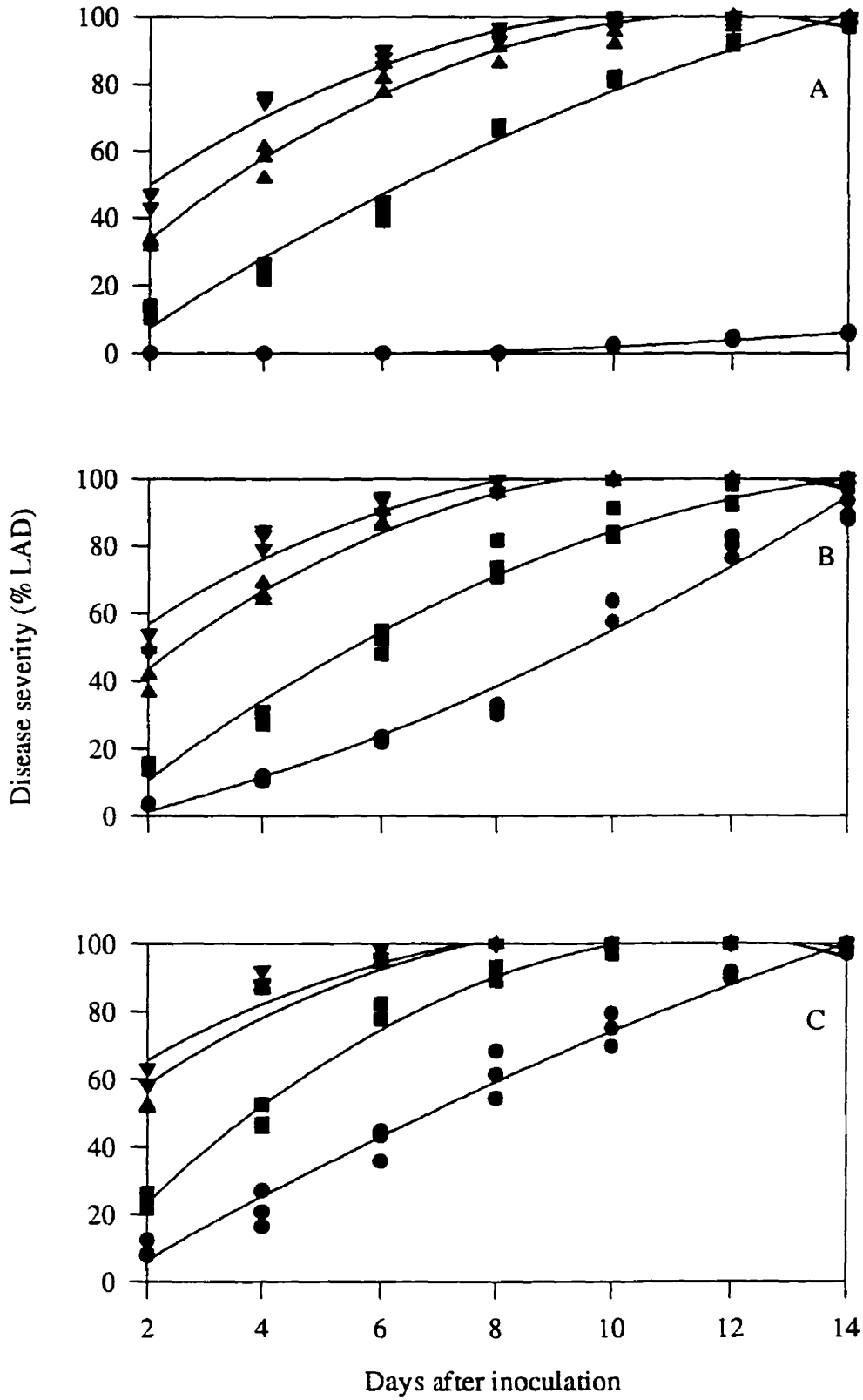

100
80
60
40
20
0
A
B
C
Disease severity (% LAD)
2
4
6
8
10
12
14
Days after inoculation

Figure 3.6. Effect of inoculum density and dew period on disease development caused by *Alternaria alternata* f. sp. *sphenocleae* on *Sphenoclea zeylanica*, expressed as percent mortality 14 days after inoculation. Plants (16 to 17 cm tall) were inoculated with 10 ml of conidial suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. Sprayed potted plants were placed in a dark dew chamber with 100% relative humidity at 24°C. Results are from pooled experiments. Bars having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

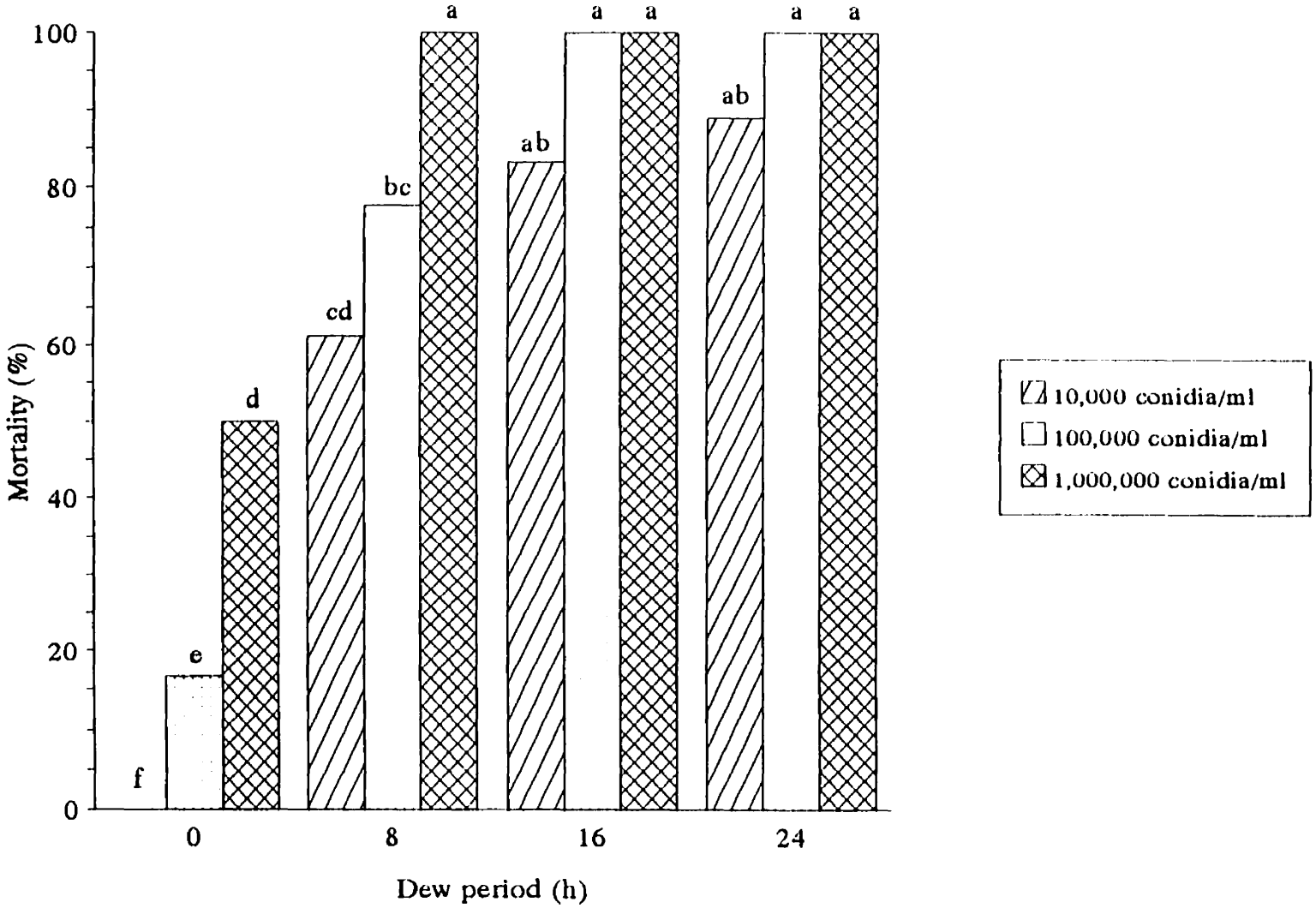
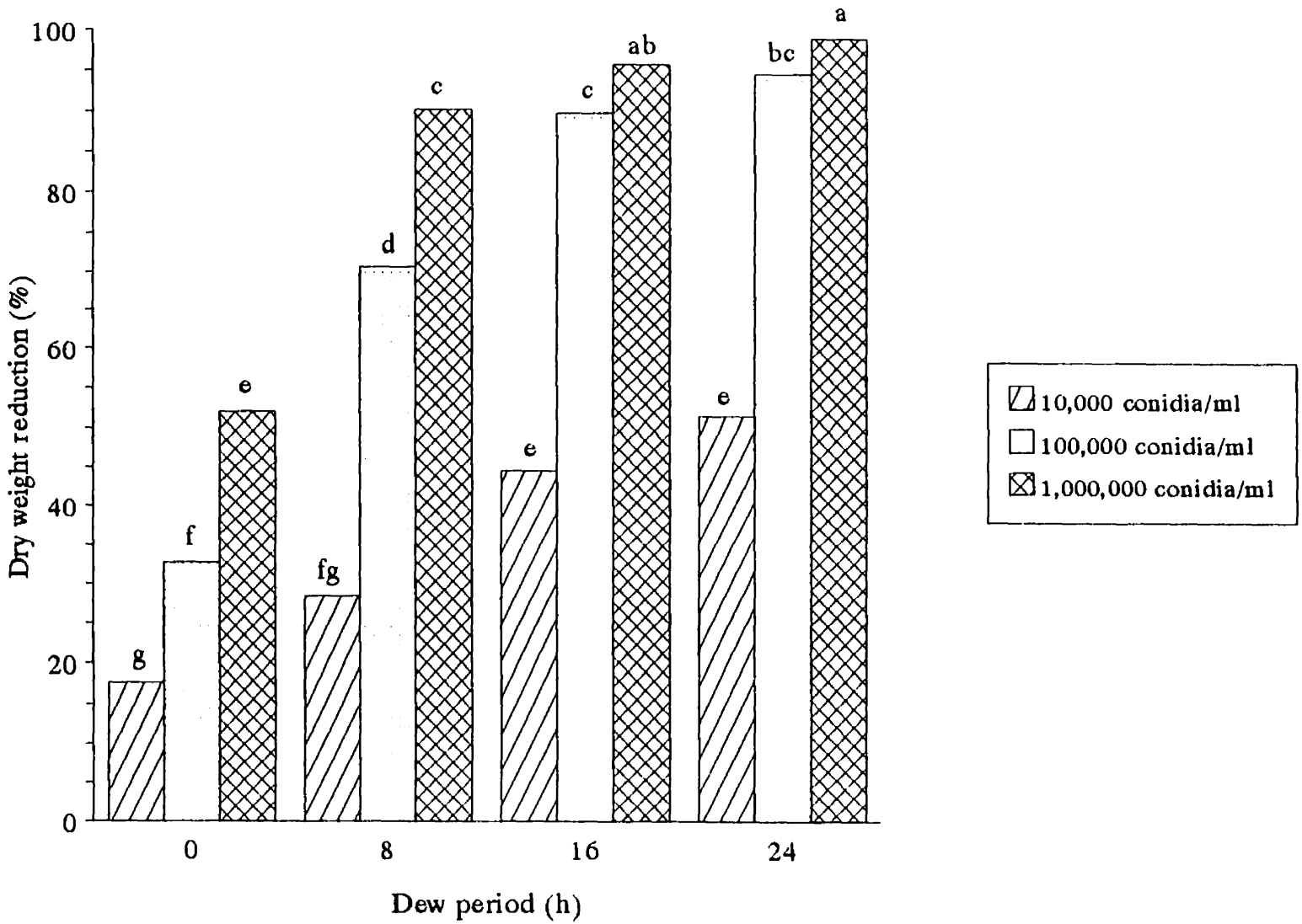

100
80
60
40
20
0
Mortality (%)
f
e
d
cd
bc
a
ab
a
a
ab
a
a
0
8
16
24
Dew period (h)
10,000 conidia/ml
100,000 conidia/ml
1,000,000 conidia/ml

Figure 3.7. Effect of inoculum density and dew period on disease development caused by *Alternaria alternata* f. sp. *sphenocleae* on *Sphenoclea zeylanica*, expressed as percent dry weight reduction 14 days after inoculation. Plants (16 to 17 cm tall) were inoculated with 10 ml of conidial suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. Sprayed potted plants were placed in a dark dew chamber with 100% relative humidity at 24°C. Results are from pooled experiments. Bars having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.


100
80
60
40
20
0
Dry weight reduction (%)
g
f
e
fg
d
c
e
c
ab
e
bc
a
0
8
16
24
Dew period (h)
10,000 conidia/ml
100,000 conidia/ml
1,000,000 conidia/ml

Connecting Text

One of the critical aspects in determining the success or failure of a potential bioherbicide candidate is the optimization of conidia production. At present, little is known about the specific culture and conidia production requirements of *Alternaria alternata* f. sp. *sphenocleae*. As with other potential bioherbicides, large amounts of infective propagules of this fungal pathogen are required for field application. In this chapter, the effects of media (i.e. standard agar media and various agricultural-based solid substrates), temperature, and light conditions on production, viability, and virulence of *A. alternata* f. sp. *sphenocleae* conidia, are presented.

Chapter 4 - Mass Production Techniques of *Alternaria alternata* f. sp. *sphenocleae*, a Biocontrol Agent for *Sphenoclea zeylanica*

4.1. Abstract

On all agar media at 28°C, sporulation under continuous near-ultraviolet (NUV) light was similar to continuous dark exposure except that under continuous NUV, sporulation increased greatly on V-8 juice agar (VJA) and decreased substantially on half-strength potato dextrose agar (1/2 PDA). The most virulent conidia were produced on 1/2 PDA four weeks after incubation (WAI) at 28°C under continuous NUV. On solid substrates, sorghum seeds produced the greatest number of conidia at 28°C under NUV. Further evaluation of sorghum seeds, however, indicated that maximum number and most virulent conidia were produced four WAI at 28°C either under continuous light or dark conditions. An equal quantity of sorghum seeds and water (w/v) maximized conidia production. Overall, the best production technique was the use of sorghum seeds.

4.2. Introduction

Gooseweed (*Sphenoclea zeylanica* Gaertner) is an annual herbaceous broadleaf weed species native to tropical Africa (Holm *et al.*, 1977; Waterhouse, 1993) that is distributed in all subtropical and tropical regions of the world. It is considered a serious weed of rice (*Oryza sativa* L.) (Holm *et al.*, 1977) and is among the 32 worst agricultural weeds in Southeast Asia (Waterhouse, 1993; 1994). This weed species is one of the nine major weeds of rice included within the biological weed control research program initiated in 1991 at the International Rice Research Institute (IRRI) and the University of the Philippines at Los Baños, Laguna, Philippines in collaboration with McGill University, Montréal, Québec, Canada (Watson, 1991; Bayot *et al.*, 1994). In 1991, a fungal pathogen, *Alternaria alternata* (Fr.) Keissler f. sp. *sphenocleae*, was isolated from blighted *S. zeylanica* collected from a rice field

near Los Baños, Laguna, Philippines. Laboratory and field studies with this pathogen suggest that it is a promising bioherbicide candidate (Bayot *et al.*, 1994; Mabbayad and Watson, 1995; Masangkay *et al.*, 1996).

As with other potential bioherbicides, large amounts of infective propagules of this fungus are required for field application. Several media and methods have been used to produce sufficient amounts of inoculum of various fungi used in biological weed control programs (Daniel *et al.*, 1973; Walker, 1982; Boyette *et al.*, 1991; Wymore *et al.*, 1988; Winder and van Dyke, 1990). Although several studies recommended some general growth media (Miller, 1955; Tuite, 1969; Stevens, 1981), temperature (Ellis, 1968; Ellis and Holliday, 1970; Ellis and Gibson, 1975), light conditions (Ellis, 1968; Ellis and Holliday, 1970; Vakalounakis, 1991), addition of supplements (Srinivasan *et al.*, 1971; Anahosur, 1978; Park *et al.*, 1992), and various combinations of these factors (Rands, 1917; Groves and Skolko, 1944; Neergaard, 1945; Aragaki, 1964) to stimulate sporulation in the genus *Alternaria*, little is known about the specific culture and conidia production requirements of *A. alternata* f. sp. *sphenocleae*. Thus, the objective of this study was to determine the effects of media, temperature, and light conditions on *A. alternata* f. sp. *sphenocleae* conidia production, viability, and virulence.

4.3. Materials and Methods

4.3.1. Pathogen isolation and culture maintenance

Diseased *S. zeylanica* leaves were collected from blighted plants in rice fields at the International Rice Research Institute (IRRI) Experimental Farm in 1991. Leaf pieces were surface sterilized with 0.5% sodium hypochlorite solution and incubated on fresh potato dextrose agar (PDA; Difco, Detroit, MI). Monoconidial isolates of *A. alternata* f. sp. *sphenocleae* were prepared, stored, and maintained on half-strength potato dextrose (1/2 PDA) in culture tubes at IRRI and imported into the quarantine facility of McGill University (Macdonald Campus, Ste-Anne-de-Bellevue, Québec, Canada). These monoconidial isolates were then used to inoculate test plant material,

re-isolated, stored, and maintained on 1/2 PDA in small vials under mineral oil at 4°C as stock cultures (Tuite, 1969). Small pieces of mycelium from the stock cultures were aseptically transferred to cooled PDA (20 ml) in plastic petri dishes (90-mm-diameter). Cultures were sealed with Parafilm® (American National Can Co., Greenwich, CT) and incubated at 28°C under continuous near-ultraviolet light (NUV; J-05, UVP, Inc., Circleville, OH) for five to seven days. Agar plugs (4-mm-diameter) from the margins of young actively growing colonies were used as seed inoculum (Tuite, 1969).

4.3.2. Plant production

S. zeylanica seeds collected at IRRI Experimental Farm in 1994 were soaked with 95% hydrochloric acid (HCl) for 10 min and washed with continuous running distilled water for 30 min. Subsequently, the seeds were soaked in distilled water at room temperature (24 ± 2°C) for 12 h. Seeds were sown in black plastic trays Kord (K 10; Plant Products, Laval, QC) 2/3 filled with sterilized soil mixture consisting of 3 parts garden soil, 3 parts Pro-mix^{BX} (commercial potting mix, Premier Horticulture Inc., Red Hill, PA), 2 parts vermiculite Holiday® (VIL Vermiculite Inc., Montréal, QC) and 1 part sphagnum peat moss Tourbe® (Les Tourbières Premier Ltée, Rivière-du-Loup, QC). After sowing, the soil was lightly pressed with a 0.5 x 25 x 15 cm wooden block and watered until saturated. Seeded trays were placed in a controlled environment chamber [Conviron® E15 (Winnipeg, Man.); 32/24°C day/night, 400 $\mu Em^{-2}s^{-1}$ photosynthetically active radiation (PAR), 12 h day^{-1}, and 70 to 80% relative humidity (RH)] until plants were ready for transplanting. Relative humidity was maintained with a humidifier, soil was kept saturated and the soil surface was misted every 12 h using a hand-held atomizer.

Healthy seedlings were selected 21 days after sowing and transplanted in transparent plastic pots (7 x 10.5 cm, Better Plastics, Inc., Kissimmee, FL) filled with sterilized moistened soil mixture. Three plants were transplanted in each pot and fertilized with 10 ml of 1.25 g L^{-1} of 20-20-20 (N-P-K) fertilizer. Pots were transferred to the controlled environment chamber with conditions as previously

described. Water level was maintained at a depth of 2 to 3 cm throughout the experimental period.

4.3.3. General inoculation procedure

Only treatments that produced more than 1 x 10^5 conidia $plate^{-1}$ or g $substrate^{-1}$ were assessed. Plants (19 to 20 cm tall) were inoculated with 10 ml of 1 x 10^5 conidia ml^{-1} suspension with 0.01% Triton® X-100 (polyethylene glycol tert-octylphenyl ether, Sigma Chemical Co., St. Louis, MO) as a wetting agent using a hand-held atomizer. Control treatments were sprayed with deionized water containing the wetting agent. After spraying, pots were placed in a dark dew chamber (Percival®, Boone, IA) with 100% relative humidity at 24°C for 8 h. Pots were then transferred to the controlled environment chamber having the same conditions as previously mentioned.

4.3.4. Assessment of germination (viability)

Droplets (50µl) of conidial suspension (1 x 10^5 conidia ml^{-1}) in sterile deionized water were placed on 1.5% water agar disks (20-mm-diameter), allowed to air dry for 5 min, covered with a cover slip, and incubated in petri dishes at 24°C under continuous darkness for 8 h. Each treatment replicate had two sample units (agar disks) for each conidia suspension. Prior to observation with a microscope (500X), germinating conidia were killed and stained with lactophenol-cotton blue (Tuite, 1969). Conidia were considered to have germinated when the length of the germ tube was greater than the width of the conidium. Several randomly selected fields of view were observed per sample until a total of 100 conidia per agar disk had been assessed for germination.

4.3.5. Pathogenicity test (virulence)

Virulence of conidia was assessed as percent reduction in dry weight 14 days after inoculation (DAI) where % reduction in dry weight = (dry weight of check plant - dry weight of treated plant)/dry weight of check plant x 100. Dry weight was

obtained by cutting live aboveground tissue at soil level, drying in paper bags for six to seven days at 45°C, and weighing. Dead leaves and dead portions of stems were not included in the dry weight measurements.

4.3.6. Conidia production on standard agar media

4.3.6.1. Effect of agar media, temperature, and light condition on conidia production. Conidia production of *A. alternata* f. sp. *sphenocleae* was observed on several agar media under different light conditions. Agar media tested were corn meal agar (CMA; Difco, Detroit, MI), malt extract agar (MEA; Difco, Detroit, MI), oatmeal agar (OMA; Difco, Detroit, MI), PDA, 1/2 PDA, and V-8 juice agar (VJA). The different media tested were prepared following recommendations from the label except for VJA which was prepared according to the method described by Tuite (1969). Twenty ml of media were dispensed into plastic petri dishes (90-mm-diameter). After cooling, inoculation was carried out by inverting and placing in the center of each petri dish an agar plug (4-mm-diameter) from 5-day-old PDA cultures of the *A. alternata* f. sp. *sphenocleae* isolate under aseptic conditions. Subsequently, plates were sealed with Parafilm®. Five incubation temperatures between 20 and 36°C at 4°C intervals and three light conditions; 1) continuous darkness (D), 2) continuous light (400 $\mu Em^{-2}s^{-1}$ PAR) (L), and 3) 12 h of alternating light (400 $\mu Em^{-2}s^{-1}$ PAR) and dark (L/D) periods were evaluated. For 28°C, exposure to continuous NUV was also included as a treatment. Conidia were harvested four weeks after inoculation (WAI) by flooding the plates with 10 ml of sterile deionized water and scraping the surface of the colonies with a sterile rubber policeman. Resulting suspensions were poured through a 250 mm plastic sieve lined with two layers of sterile cheesecloth. Conidia of the collected suspension were counted with the aid of a haemocytometer under a light microscope (Tuite, 1969). Eight readings for each colony were recorded and averaged.

4.3.6.2. Effect of agar media, light condition, and incubation period on production and virulence of conidia. Production and virulence of *A. alternata* f. sp. *sphenocleae* conidia were further tested on 1/2 PDA and VJA using 16 light conditions and conidia were harvested at three and four WAI. The different light conditions included; 1) D, 2) D + L, 3) D + NUV, 4) D + L/D, 5) L, 6) L + D, 7) L + NUV, 8) L + L/D, 9) L/D, 10) L/D + D, 11) L/D + L, 12) L/D + NUV, 13) NUV, 14) NUV + L/D, 15) NUV + L, and 16) NUV + D. In the combined light treatments (e.g. D + L, D + NUV, D + L/D, etc), after three days in the initial condition, cultures were transferred to the other light treatment and maintained for the duration of the experiment. Production and virulence of conidia were evaluated as described above.

4.3.7. Conidia production on agricultural-based solid substrates

4.3.7.1. Effect of agricultural-based solid substrates, temperature, and light condition on conidia production. The following agricultural-based products were evaluated for conidia production of *A. alternata* f. sp. *sphenocleae*: barley [*Hordeum vulgare* L.] seed; black-eyed bean [*Vigna unguiculata* (L.) Walp.] seed; chickpea [*Cicer arietinum* L.] seed; corn [*Zea mays* L.] seed; corn meal; cracked corn; cowpea [*Vigna sinensis* (L.) Engl.] seed; millet [*Setaria italica* (L.) Beauv.] seed; mungbean [*Vigna radiata* (L.) R. Wilcz.] seed; cracked mungbean seed; oat [*Avena sativa* L.] seed; peanut [*Arachis hypogea* (L.) R. Wilcz.] seed; rice [*Oryza sativa* L.] polished grain; sorghum [*Sorghum bicolor* (L.) Moench] seed; sorghum leaves; sorghum stalks; soybean [*Glycine max* (L.) Merr.] seed; common sunflower [*Helianthus annuus* L.] seed; durum wheat (*Triticum durum* Desf.) seed; and hard spring red wheat (*T. aestivum* L.) seed. Mature sorghum leaves and stalks were collected from the Horticulture Research Centre of McGill University. Leaves were cut into approximately 1 x 1 cm pieces and dried at 60°C for seven days. One g of dried leaf material was placed into each 250 ml Erlenmeyer flask and soaked with 10 ml of deionized water overnight before autoclaving. Sorghum stalks were cut into 1 cm lengths and dried at 80°C for seven days. Two g of dried stalk material were placed

into each 250 ml Erlenmeyer flask and soaked in 10 ml of deionized water overnight before autoclaving. For the other substrates, 20 g of each substrate was combined with 20 ml of deionized water for one hr before autoclaving in a 250 ml Erlenmyer flask. Flasks were covered with cotton plugs and autoclaved for 17 min at 120°C and 100 kPa; and subsequently shaken by hand to disperse the particles. After cooling, an agar plug (4-mm-diameter) from 7-day-old PDA cultures was placed in each flask under aseptic conditions. Incubation temperatures were 24, 28, and 32°C under continuous light (400 $\mu Em^{-2}s^{-1}$ PAR). For 28°C, exposure to continuous NUV was included as an additional treatment. Inoculated flasks were shaken by hand every other day until 14 DAI to prevent aggregation of solid particles and to improve aeration (Mudgett, 1986). Conidia were harvested at four WAI by adding 50 ml of sterile deionized water to each flask, shaking the flasks on a rotary shaker at 200 rpm for 10 min at room temperature (24 ± 2°C), and then pouring the contents through a 250 mm plastic sieve lined with two layers of sterile cheesecloth. Conidia production was determined with the aid of the haemocytometer as described above.

4.3.7.2. Effect of temperature, light condition, and incubation period on production and virulence of conidia grown on sorghum seeds. Production and virulence of *A. alternata* f. sp. *sphenocleae* conidia were further tested using sorghum seeds under different temperature, light conditions, and incubation periods. Inoculation materials were prepared as described earlier. Incubation temperatures included 24, 28, and 32°C and light conditions were continuous dark (D), continuous light (400 $\mu Em^{-2}s^{-1}$ PAR) (L), and 12 h of alternating light and dark (L/D) conditions. Conidia were harvested at one, two, three, and four WAI and the number and virulence of conidia produced were determined.

4.3.7.3. Effect of moisture content and quantity of sorghum seeds on conidia production. Inoculation materials were prepared as described earlier, except that moisture content (10, 20, and 30 ml) and quantity of sorghum seeds (10, 20, and 30 g) were compared. Percent moisture content of seeds after autoclaving was evaluated on

a wet basis using the formula: (g H_2O/g wet sorghum seed) x 100. Inoculated flasks were incubated at 28°C under continuous light. Conidia were harvested at four WAI and conidia production was evaluated.

4.3.7.4. Effect of storage period on the number, germination, and virulence of conidia grown on sorghum seeds. Inoculation materials were prepared as described earlier. Inoculated flasks were incubated at 28°C under continuous light for seven days and were subsequently stored under room conditions (24 ± 2°C) until harvest. Conidia were harvested after one, two, three, four, nine, and 12 months of storage. Production, germination, and virulence of conidia were determined.

4.3.8. Comparison of production methods on conidia production, germination, and virulence

Two agar media (1/2 PDA and VJA) and one agricultural-based solid substrate (sorghum seed) were selected from the production methods evaluated earlier. Plates or flasks were inoculated following the procedures outlined above and were incubated at 28°C in the dark. Conidia were harvested at four WAI. Production, germination, and virulence of conidia were determined.

4.3.9. Data Analyses

All experiments were performed twice and laid out in either a randomized complete block design (RCBD) or a completely randomized design (CRD) with three replicates. Data from conidial counts were subjected to log (x+1) transformation for zero values and log (x) for non-zero values and percentage data were arc sine transformed before analysis (Gomez and Gomez, 1984). Factorial analysis of variance considered the effect of each factor individually and their interaction. Results from the two trials were pooled if homogeneity of variances was confirmed by Bartlett's test (Gomez and Gomez, 1984). All analyses were conducted using STATGRAPHICS software for IBM-PC (STSC, Inc., 1991). Treatment means were separated with the

least significant difference test or Duncan's multiple range test at the 1% or 5% level of significance.

4.4. Results

4.4.1. Effect of agar media, temperature, and light condition on conidia production. There was a significant interaction among agar media, temperature, and light condition on conidia production of *A. alternata* f. sp. *sphenocleae* (Appendix 4.1). The highest number of conidia were produced on 1/2 PDA at 28°C in the dark (Table 4.1). No conidia were produced at 36°C and very few at 32°C on any of the agar media in all the temperatures and light conditions. Poor conidia production was observed on CMA and OMA in all the temperature and light conditions evaluated. With the rest of the agar media, the best temperature and light combination was at 28°C under continuous dark. Zonation of sporulating and non-sporulating sectors was only observed in cultures exposed to 12 h of alternating light and dark periods especially on 1/2 PDA and PDA. Cultures grown on 1/2 PDA, PDA, and VJA under continuous dark were darker olivaceous black than under continuous light.

There was a significant interaction between agar media and light condition on conidia production of *A. alternata* f. sp. *sphenocleae* incubated at 28°C (Appendix 4.2). The greatest number of conidia occurred on VJA under continuous NUV (2.0 x 10^6 conidia plate^{-1}) (Table 4.2). Sporulation under continuous NUV was similar to continuous dark exposure for all agar media except that under continuous NUV, sporulation increased greatly on VJA and decreased substantially on 1/2 PDA.

4.4.2. Effect of light condition and incubation period on production and virulence of conidia grown on 1/2 PDA and VJA at 28°C. There was a significant interaction between the various combinations of light conditions and incubation period on conidia production of *A. alternata* f. sp. *sphenocleae* grown on 1/2 PDA and VJA (Appendix 4.3). Maximum number of conidia were produced on VJA under continuous NUV (Table 4.3). Light generally inhibited conidia production, but the adverse effect of exposure to continuous light or 12 h of alternating light and dark periods was

overcome when cultures were subsequently exposed to continuous NUV or continuous dark conditions. However, the positive effect of continuous dark exposure was negated when cultures were subsequently exposed to continuous light or 12 h of alternating light and dark periods. No conidia were produced on VJA under D + L, L, and L/D + L exposures. On 1/2 PDA, significantly higher number of conidia was produced under D, L + D, L + NUV, L/D + D, NUV + D exposures than VJA. Significantly more conidia were obtained from VJA when exposed to NUV and NUV + L/D. Conidia production was not significantly different on both agar media with the rest of the treatments. Harvesting conidia four WAI significantly gave more conidia (3.75×10^5 conidia plate^{-1}) than at three WAI (3.35×10^5 conidia plate^{-1}) (data not presented).

There was a significant interaction between agar media and light condition on virulence of *A. alternata* f. sp. *sphenocleae* conidia (Appendix 4.4). The highest dry weight reduction (94%) occurred with conidia produced on 1/2 PDA exposed to continuous NUV and the lowest reductions (45%) for conidia produced on VJA exposed to L/D + NUV (Figures 4.1 and 4.2). Under the different light treatments, virulence of *A. alternata* f. sp. *sphenocleae* conidia grown on 1/2 PDA was always significantly higher than those grown on VJA. Significantly more virulent conidia (expressed as dry weight reduction) were produced at four WAI than at three WAI (data not presented).

4.4.3. Effect of various agricultural-based solid substrates and temperature under continuous light on conidia production. Twenty agricultural-based products were evaluated as solid substrates for mass production of *A. alternata* f. sp. *sphenocleae* at different temperatures under continuous light. There was a significant interaction between the different agricultural-based substrates and temperature on conidia production (Appendix 4.5). The highest number of conidia were produced on sorghum and hard red spring wheat seeds at 28°C (2.1×10^5 conidia g substrate^{-1}) (Table 4.4). No conidia were produced at 32°C among the different substrates under continuous light. At 28°C, significantly more conidia were produced on barley, millet, mungbean,

sorghum, durum wheat, and hard red spring wheat seeds, corn meal, cracked corn, and sorghum stalks than at 24°C. At 24 and 28°C, oat seed and polished rice grain produced approximately the same number of conidia whereas, poor conidia production was observed on black-eyed bean, chickpea, corn, cowpea, peanut, soybean, and common sunflower seeds, cracked mungbean, and sorghum leaves.

4.4.4. Effect of various agricultural-based solid substrates incubated under continuous NUV light and continuous light periods at 28°C on conidia production. There was a significant interaction between the various agricultural-based substrates when exposed to continuous NUV or continuous light at 28°C (Appendix 4.6). The most abundant conidia production occurred on sorghum seeds under NUV (1.3×10^6 g substrate^{-1}) (Table 4.5). Exposure to NUV produced a significantly higher number of conidia on barley, oat, sorghum, and hard red spring wheat seeds, corn meal, polished rice grain, and sorghum stalks than continuous light. Conidia production with the rest of the treatments was not significantly affected by either exposure to NUV or continuous light. Of the various solid substrates tested, significantly higher number of conidia were produced under continuous NUV than continuous light.

4.4.5. Effect of temperature, light condition, and incubation period on conidia production and conidia virulence grown on sorghum seeds. There was a significant interaction among temperature, light condition, and incubation period on conidia production of *A. alternata* f. sp. *sphenocleae* grown on sorghum seeds (Appendix 4.7). Maximum number of conidia were produced at 28°C under continuous light at four WAI but this was not significantly different at 28°C in the dark at four WAI and 28°C under continuous light at three WAI (Table 4.6). At 32°C, exposure to 12 h of alternating light and dark periods was detrimental for conidia production in all the incubation periods. No conidia were produced at 32°C four WAI under continuous light. At all temperature and light conditions tested, there was poor conidia production at one to two WAI. Higher number of conidia were produced at three WAI under continuous dark or light conditions at 28°C than at 24 and 32°C

whereas, exposure to 12 h of alternating light and dark periods at 24°C gave more conidia than at 28 and 32°C. At four WAI in all the different light conditions, 28°C gave the greatest number of conidia.

There was also a significant interaction among temperature, light condition, and incubation period on the virulence of *A. alternata* f. sp. *sphenocleae* conidia when grown on sorghum seeds (Appendix 4.8). The most virulent conidia were produced four WAI under either continuous dark or light at 28°C (Table 4.7). In all light conditions and temperatures evaluated, a greater number of virulent conidia were produced at four WAI than at three WAI. In all light conditions and incubation periods, a greater number of virulent conidia were produced at 28°C than at 24°C.

4.4.6. Effect of moisture content and quantity of sorghum seeds on conidia production. There was a significant interaction between the amount of sorghum seeds and volume of water added on conidia production of *A. alternata* f. sp. *sphenocleae* (Appendix 4.9). An equal proportion of the quantity of sorghum seeds and volume of water added to these seeds before autoclaving maximized conidia production (Table 4.8). Conidia production was closely related to the volume of the flask occupied by the seeds and the seed moisture content. Low moisture content (26.6%) restricted growth and sporulation, and high moisture content (93.0%) induced the seeds to become sticky and to agglomerate, resulting in extensive mycelial growth and limited conidia production.

4.4.7. Effect of storage period on the number, germination, and virulence of conidia grown on sorghum seeds. The number, germination, and virulence of conidia were significantly affected by storage period (Appendices 4.10A, 4.10B, 4.10C, respectively). The number, germination, and virulence of *A. alternata* f. sp. *sphenocleae* conidia decreased when stored for up to 12 months (Figures 4.3A, 4.3B, and 4.3C, respectively). However, even after 12 months of storage, there were still more than 1.0×10^5 conidia g substrate^{-1} with conidia germination and virulence (expressed as dry weight reduction) of more than 80% and 75%, respectively.

4.4.8. Comparison of production methods on production, germination, and virulence of conidia. Production methods employed did not have any significant effect on conidia germination (Appendix 4.11A) but did significantly affected virulence of *A. alternata* f. sp. *sphenocleae* conidia (Appendix 4.11B). Conidia produced from the different production methods were morphologically similar, however, sorghum seeds produced more chlamydospores than 1/2 PDA and VJA. Sorghum seeds produced the highest number of conidia (1.85×10^7 conidia flask^{-1}) whereas, VJA gave the least number of conidia (7.0×10^5 conidia plate^{-1}) (Table 4.9). The most virulent conidia (expressed as dry weight reduction) were produced from sorghum seeds (98%) whereas conidia from VJA gave the lowest dry weight reduction (66%).

4.5. Discussion

Alternaria species are well-adapted to natural conditions, with daily fluctuations in temperature and light. Their mechanism of photosporogenesis consists of two distinctive phases; an inductive phase which leads to the formation of conidiophores and a terminal phase which leads to the formation of conidia.

Under laboratory conditions, conidia production of *A. alternata* f. sp. *sphenocleae* was affected by agar media, temperature, light condition, and incubation period. The most suitable agar media, temperature, light condition, and incubation period were 1/2 PDA at 28°C under continuous NUV grown four WAI as reflected by the greatest number of conidia produced and the highest virulence of conidia, expressed as a percentage dry weight reduction within the host plant. Sporulation was stimulated under NUV exposure which supports the findings on *A. brassicicola* (Leach, 1964; Ellis, 1968), *A. chrysanthemi* (Ellis, 1968), *A. kikuchiana* (Ohmori and Nakajima, 1970), *A. cichorii* (Vakalounakis and Christias, 1981), and *A. alternata* strain RL 671-2 and ATCC 36068 (Wei *et al.*, 1985) whereas, continuous light completely inhibited sporulation. However, the detrimental effect of exposing cultures of *A. alternata* f. sp. *sphenocleae* to continuous light or 12 h of alternating light and

darkness was overcome when cultures were subsequently exposed to continuous NUV or dark conditions. This positive effect, however, was negated when cultures were subsequently exposed to continuous light conditions.

Light requirements are very distinct for *Alternaria* species (Leach, 1967). The main element in most formulae for sporulation is irradiation with NUV light irregardless of light. The inductive phase is stimulated by NUV wavelengths in the range of 310-400 nm and the terminal phase proceeds best in darkness and is often inhibited by light. Exposures to NUV followed by dark induced sporulation of *A. solani* (Lukens, 1963), *A. chrysanthemi* (Leach, 1964), *A. dauci* (Leach and Trione, 1966), and *A. cichorii* (Vakalounakis and Christias, 1981). Exposing conidiophores to light stimulates regrowth to vegetative hyphae whereas NUV prevents vegetative growth (Aragaki *et al.*, 1973). Exposure to dark conditions also improved sporulation of *A. alternata* f. sp. *sphenocleae* and this was also truf on *A. brassicae* (Senior *et al.*, 1987). Zonation of sporulating and non-sporulating sectors was only observed in cultures when exposed to 12 h of alternating light and dark periods at 28°C especially on 1/2 PDA and PDA and supports the findings of Kaiser *et al.* (1994). Light inhibits sporulation when the temperature is relatively high but not when the temperature is lower (Aragaki, 1961). Inhibition of the terminal phase by light is initiated at a critical temperature or it might be that the light-inhibited mechanism is bypassed at low temperatures (Lukens, 1966). Generally, on all agar media, sporulation under continuous light gradually decreased with an increase in temperature and this supports the findings of Bashi and Rotem (1975). Exposures to continuous NUV was only evaluated at 28°C due to the limited number of incubators within the quarantine facility.

Some *Alternaria* species need specific light requirements for sporulation. For example, *A. tomato* (Aragaki, 1964) and *A. euphorbiae* (Yoshimura *et al.*, 1986) required cool-white fluorescent light (2,700 lux) for four to six days followed by darkness for 24 h, *A. porri* should be exposed to sunlight for a 2 h period (Ellis and Holliday, 1970), *A. carthami* needed a light requirement of 3,200 lux (Mortensen and

Bergman, 1983), *A. solani* required wavelengths less than 360 nm (Honda and Yunoki, 1981; Vakalounakis, 1991), and *A. macrospora* needed 5,000 lux from a cool white fluorescent light on a 12 h diurnal cycle (Cotty, 1987).

Other methods employed to stimulate sporulation of *Alternaria* species included cutting up 10- to 12-day-old potato agar cultures and exposing them to sunshine and slight drying for *A. porri* f. sp. *solani* (Rands, 1917), placing three- to four-day-old cultures in sunlight for 30 min and exposing them to 12 h of alternating NUV and dark thereafter for *A. cucumerina* and *A. tenuissima* (Stevens, 1981), placing small pieces of agar cultures on the interior surface of the lid of a petri dish and a moist filter paper on the bottom of the dish maintaining a 100% RH at room temperature for *A. porri* (Neergaard, 1945), extensively cutting the mycelium of a 10-day-old culture and exposing the wounded mycelium to a 12 h alternating cycle of light (6.5 W m^{-2}) and dark for two days for *A. carthami* (McRae *et al.*, 1984), and by exposing seven-day-old cultures of *A. cassiae* and *A. crassa* grown in VJA at 25°C to 300 nm UV for 40 min followed by one additional 30 min UV exposure 24 h after the first exposure (Amsellem *et al.*, 1990).

Sporulation of some *Alternaria* species was also stimulated with the addition of some supplements such as biotin and pyridoxine for *A. burnsii* (Anahosur, 1978) and autoclaved leaves of leafy spurge (*Euphorbia esula*) on moistened filter paper on petri dishes for *A. angustiovoidea* (Yang *et al.*, 1990).

Alternaria species grow across a wide range of temperatures. Long-chained species (Longicatenatae) are best cultured at temperatures ranging from 25 to 27°C (Neergaard, 1945), short-chained species (Brevicatenatae) from 19 to 35°C (Neergaard, 1945; Ellis and Holliday, 1970), and non-chained species (Noncatenatae) from 23 to 30°C (Neergaard, 1945; Ellis, 1968; Ellis and Holliday, 1970; Ellis and Gibson, 1975; David, 1991). *A. alternata* f. sp. *sphenocleae* belongs to Brevicatenatae classification.

The use of agar media is time consuming to prepare, expensive, and not feasible for large-scale production of conidia for field studies. It is, therefore, necessary to develop an efficient and economic production system if a fungal pathogen

is to be considered for augmentation in a bioherbicide strategy (Boyette *et al.*, 1991). The viable inocula must be produced in an inexpensive medium and the cost of production for spraying large areas must be competitive with that of chemical herbicides (Churchill, 1982; Upadhyay and Rai, 1988). This can be achieved by developing a medium that is simple in composition such as utilizing agricultural-based products, and a production procedure that is easy to operate with minimal labour requirement (Boyette *et al.*, 1991; Feng *et al.*, 1994). Solid substrate fermentation utilizing agricultural-based products has been used for inoculum production of bioherbicide fungi and is potentially feasible (Morin *et al.*, 1989, Connick *et al.*, 1990). This method is very common for mass production of spore-producing organisms used to transform organic compounds (Mudgett, 1986) and for microbial insecticides (Soper and Ward, 1981). *Alternaria* species do not sporulate in liquid culture (Walker, 1982) however, they are generally very adaptable to solid substrate fermentation (Templeton and Heiny, 1989) and in this study, solid substrate fermentation was shown to be feasible with *A. alternata* f. sp. *sphenocleae*.

Inoculum production was good on some of the solid substrates evaluated. Solid substrates with a lower protein content appear to encourage sporulation as indicated by the high number of conidia produced on barley, millet, oat, sorghum, durum wheat and hard red spring wheat seeds, cracked corn, polished rice grain with sorghum, and hard red spring wheat seeds. The superiority of sorghum and hard red spring wheat seeds to support large numbers of conidia was likely due to their large surface area and structure retention, lack of particle agglomeration, nutrient composition, and appropriate moisture content which restricted vegetative mycelial growth thereby stimulating conidia production. However, sorghum seeds were chosen for further evaluation since they are locally and more readily available at a minimal cost in the Philippines as compared with hard red spring wheat seeds. Large quantities of viable and virulent conidia have been produced on sorghum seeds and used to spray greenhouse and field studies in the Philippines (Mabbayad and Watson, 1995).

Exposure to continuous NUV also stimulated sporulation on solid substrates however, exposure to continuous light or dark produced more conidia than exposure to 12 h of alternating light and dark period. There might have been minimal light penetration at the bottom of the flasks under continuous light exposure thus, some of the particles were protected from the inhibitory effect of light on sporulation unlike on those agar cultures on plastic petri dishes which were uniformly exposed to continuous light. Shaking the flasks up to 14 DAI might also have had an impact on conidia production creating a new physical environment that enhanced conidiophore and/or conidia formation in light. Exposure to 12 h of alternating light and dark period produced extensive mycelial growth, compared with continuous light or dark. The most virulent conidia were produced four WAI under continuous dark or light at 28°C. The use of sorghum seeds is, therefore, a potential method in developing countries to produce conidia at the village level or as a small "cottage" industry. Conidia production could still be improved by optimizing such factors as nutrient balance, pH, temperature, and aeration.

Two production methods at various temperature and light conditions produced sufficiently large quantities of *A. alternata* f. sp. *sphenocleae* conidia. The conidia production from one flask containing 20 g of sorghum seeds at 54% moisture content (at time of inoculation) was equal to the conidia production of approximately 14 and 26 plates for 1/2 PDA and VJA, respectively. The different methods did not affect conidia germinability, however, conidia produced from sorghum seeds were more virulent than those produced on 1/2 PDA and VJA. Solid substrate fermentation offers significant advantages over agar media techniques for future industrial scale-up of *A. alternata* f. sp. *sphenocleae*, but the use of solid substrates also has limitations. This procedure can be time consuming, labour intensive, harvesting conidia from the substrate can be difficult, and contamination of substrates can be problematic (Connick *et al.*, 1990; Boyette *et al.*, 1991).

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Table 4.1. Interaction of agar medium, light condition, and temperature on conidia production of *Alternaria alternata* f. sp. *sphenocleae*.[w]

Agar medium	Light condition	Number of conidia per plate (x 10^4)				
		Temperature (°C)				
		20	24	28	32	36
CMA[x]	D[y]	0.4 e[z]	1.2 e	3.6 de	0.9 e	0 f
CMA	L	0.1 e	3.5 de	0.3 e	0 f	0 f
CMA	L/D	0.3 e	0.7 e	0.4 e	0.1 e	0 f
MEA	D	0.4 e	1.2 e	6.2 cd	1.0 e	0 f
MEA	L	0.2 e	2.0 de	0.1 e	0 f	0 f
MEA	L/D	0.3 e	0.3 e	3.0 de	0.1 e	0 f
OMA	D	0.5 e	1.4 de	4.9 c-e	0.3 e	0 f
OMA	L	0.9 e	2.0 de	0.1 e	0 f	0 f

Table 4.1. Continued...

Agar medium	Light condition	Number of conidia per plate (x 10^4)				
		Temperature (°C)				
		20	**24**	**28**	**32**	**36**
OMA	L/D	0.4 e	0.5 e	1.3 e	0.1 e	0 f
PDA	D	0.5 e	1.1 e	9.6 c	1.0 e	0 f
PDA	L	0.1 e	2.9 de	0.1 e	0 f	0 f
PDA	L/D	0.3 e	0.5 e	3.3 de	0.1 e	0 f
1/2 PDA	D	1.6 de	1.6 de	191.7 a	1.1 e	0 f
1/2 PDA	L	2.3 de	1.2 e	0.9 e	0.1 e	0 f
1/2 PDA	L/D	0.2 e	0.5 e	3.9 de	1.2 e	0 f
VJA	D	0.5 e	1.2 e	48.2 b	1.1 e	0 f

Table 4.1. Continued...

Agar medium	Light condition	Number of conidia per plate (x 10^4)				
		Temperature (°C)				
		20	24	28	32	36
VJA	L	1.3 e	3.1 de	0.1 e	0.1 e	0 f
VJA	L/D	0.3 e	0.6 e	1.6 de	0.3 e	0 f

[w] Number of conidia per plate was counted after four wk of incubation. Results are from pooled experiments.

[x] CMA = corn meal agar, MEA = malt extract agar, OMA = oatmeal agar, PDA = potato dextrose agar, 1/2 PDA = half-strength potato dextrose agar, VJA = V-8 juice agar.

[y] D = continuous dark, L = continuous light (400 $\mu Em^{-2}s^{-1}$), L/D = 12 h of alternating light (400 $\mu Em^{-2}s^{-1}$) and dark.

[z] Means having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

Table 4.2. Influence of agar medium and light condition on conidia production of *Alternaria alternata* f. sp. *sphenocleae* incubated at 28°C.[w]

Agar medium	Number of conidia per plate (x 10^4)			
	Light condition			
	D[x]	L	L/D	NUV
CMA[y]	3.6 e-g[z]	0.3 g	0.4 g	7.4 e-g
MEA	6.2 e-g	0.1 g	3.0 e-g	5.5 e-g
OMA	4.9 e-g	0.1 g	1.3 fg	8.1 ef
PDA	9.6 e	0.1 g	3.3 e-g	10.3 e
1/2 PDA	191.7 b	0.9 fg	3.9 e-g	22.2 d
VJA	48.2 c	0.1 g	1.6 fg	200.0 a

[w] Number of conidia per plate was counted after four wk of incubation. Results are from pooled experiments.

[x] D = continuous dark, L = continuous light (400 $\mu Em^{-2}s^{-1}$), L/D = 12 h of alternating light (400 $\mu Em^{-2}s^{-1}$) and dark, NUV = continuous near ultra-violet light (J-205, UVP, Inc.).

[y] CMA = corn meal agar, MEA = malt extract agar, OMA = oatmeal agar, PDA = potato dextrose agar, 1/2 PDA = half-strength potato dextrose agar, VJA = V-8 juice agar.

[z] Means having common letters are not significantly different according to Duncan's multiple range test at the 5% level of significance.

Table 4.3. Interaction of various combinations of light conditions on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on 1/2 PDA and VJA at 28°C.[v]

Light condition	Number of conidia per plate (x 10^4)		
	1/2 PDA[w]	VJA	Difference
D[x]	110.0 b[y]	42.3 fg	67.7 ***[z]
D + L	1.6 h	0 i	1.6 ns
D + NUV	40.5 g	45.3 e-g	- 4.8 ns
D + L/D	7.4 h	8.3 h	- 0.9 ns
L	0.2 h	0 i	0.2 ns
L + D	41.9 g	16.1 h	25.7 ***
L + NUV	71.0 d	35.9 g	35.1 ***
L + L/D	12.8 h	6.4 h	6.4 ns
L/D	3.9 h	1.2 h	2.7 ns
L/D + D	43.8 fg	6.6 h	37.2 ***
L/D + L	0.1 h	0 i	0.1 ns
L/D + NUV	43.4 fg	35.6 g	7.6 ns
NUV	77.0 cd	155.3 a	-78.3 ***
NUV + L/D	63.3 de	95.9 bc	-32.5 ***
NUV + L	13.5 h	1.8 h	11.7 ns
NUV + D	110.6 b	61.4 d-f	49.3 ***

[v] Results are from pooled experiments.

[w] 1/2 PDA = half-strength potato dextrose agar, VJA = V-8 juice agar.

[x] D = continuous dark, D + L = D + continuous light (400 $\mu Em^{-2}s^{-1}$ PAR), D + NUV = D + continuous near ultra-violet light (J-205, UVP, Inc.), D + L/D = D + 12 h of alternating light (400 $\mu Em^{-2}s^{-1}$ PAR) and dark, L = continuous light, L + D = L + continuous dark, L + NUV = L + continuous near ultra-violet light, L + L/D = L + 12 h of alternating light and dark, L/D = 12 h of alternating light and dark, L/D + D = L/D + continuous dark, L/D+ L = L/D + continuous light, L/D + NUV = L/D + continuous near ultra-violet light, NUV = continuous near ultra-violet light, NUV + L/D = NUV + 12 h of alternating light and dark, NUV + L = NUV + continuous light, NUV + D = NUV + continuous dark.

[y] Means having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

[z] *** = significant at 0.1% level and ns = not significant according to the t-test.

Table 4.4. Influence of various agricultural-based solid substrates and temperature on conidia production of *Alternaria alternata* f. sp. *sphenocleae* incubated under continuous light.[y]

Solid substrate	Number of conidia per g substrate (x 10^4)		
	Temperature (°C)		
	24	28	32
Barley seed	9.6 c[z]	12.9 b	0 l
Black-eyed bean seed	0.1 k	0.4 k	0 l
Chickpea seed	0.1 k	0.4 k	0 l
Corn seed	0.6 jk	0.8 jk	0 l
Corn meal	1.2 jk	2.4 g-i	0 l
Cracked corn	3.4 f-h	5.5 e	0 l
Cowpea seed	0.1 k	0.4 k	0 l
Millet seed	1.1 jk	3.8 e-g	0 l
Mungbean seed	0.1 k	3.3 f-h	0 l
Cracked mungbean	0.5 k	0.8 jk	0 l
Oat seed	11.2 bc	10.6 c	0 l
Peanut seed	0.1 k	0.1 k	0 l
Polished rice grain	1.1 jk	1.9 h-j	0 l
Sorghum seed	7.5 d	21.0 a	0 l
Sorghum leaves	0.1 k	0.6 jk	0 l

Table 4.4. Continued...

Solid substrate	Number of conidia per g substrate (x 10^4)		
	Temperature (°C)		
	24	28	32
Sorghum stalks	0.9 jk	3.2 f-h	0 l
Soybean seed	0.1 k	0.4 k	0 l
Common sunflower seed	0.1 k	0.3 k	0 l
Durum wheat seed	0.9 jk	4.2 ef	0 l
Hard red spring wheat seed	1.6 h-j	21.0 a	0 l

[y] Number of conidia per flask was counted after four wk of incubation. Results are from pooled experiments.

[z] Means having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

Table 4.5. Influence of various agricultural-based solid substrates and light condition on conidia production of *Alternaria alternata* f. sp. *sphenocleae* incubated at 28°C.[w]

Solid substrate	Number of conidia per g substrate (x 10^4)		
	Light condition		
	L[x]	NUV	Difference
Barley seed	12.9 f-h[y]	58.5 b	- 45.6 ***[z]
Black-eyed bean seed	0.4 k	0.8 k	- 0.4 ns
Chickpea seed	0.4 k	0.8 k	- 0.4 ns
Corn seed	0.8 k	1.1 k	- 0.3 ns
Corn meal	2.4 jk	23.7 d	- 21.3 ***
Cracked corn	5.5 h-k	9.1 g-j	- 3.6 ns
Cowpea seed	0.4 k	0.6 k	- 0.2 ns
Millet seed	3.8 i-k	6.1 g-k	- 2.3 ns
Mungbean seed	3.3 i-k	4.4 i-k	- 1.1 ns
Cracked mungbean	0.8 k	1.5 jk	- 0.7 ns
Oat seed	10.6 g-i	60.5 b	- 49.9 ***
Peanut seed	0.1 k	0.2 k	- 0.1 ns
Polished rice grain	1.9 jk	18.5 d-f	- 16.6 ***
Sorghum seed	21.0 de	127.8 a	-106.8 ***
Sorghum leaves	0.6 k	1.2 k	- 0.6 ns

Table 4.5. Continued...

Solid substrate	Number of conidia per g substrate (x 10^4)		
	Light condition		
	L	NUV	Difference
Sorghum stalks	3.2 i-k	13.5 e-g	- 10.3 ***
Soybean seed	0.4 k	0.8 k	- 0.4 ns
Common sunflower seed	0.3 k	0.5 k	- 0.2 ns
Durum wheat seed	4.2 i-k	6.8 g-k	- 0.6 ns
Hard red spring wheat seed	21.0 de	45.3 c	- 24.3 ***

[w] Number of conidia per flask was counted after four wk of incubation. Results are from pooled experiments.

[x] L = continuous light (400 $\mu Em^{-2}s^{-1}$), NUV = near ultra-violet light (J-05, UVP, Inc.).

[y] Means having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

[z] *** = significant at 0.1% level and ns = not significant according to the t-test.

Table 4.6. Interaction of incubation period, light condition, and temperature on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on sorghum seeds.[x]

Incubation period (weeks)	Light condition	Number of conidia per g substrate (x 10^4)		
		Temperature (°C)		
		24	28	32
1	D[y]	0.1 g[z]	0.4 fg	0.1 g
1	L	0.1 g	0.9 e-g	0.1 g
1	L/D	0.1 g	0 h	0 h
2	D	0.1 g	1.4 e-g	0.2 g
2	L	0.9 e-g	1.5 e-g	0.1 g
2	L/D	0.3 fg	0.1 g	0 h
3	D	0.7 e-g	21.0 b	0.3 fg
3	L	5.4 d	22.7 ab	0.1 g

Table 4.6. Continued...

Incubation period (weeks)	Light condition	Number of conidia per g substrate (x 10^4)		
		Temperature (°C)		
		24	28	32
3	L/D	2.3 ef	0.6 g	0 h
4	D	2.0 e-g	23.7 a	0.6 fg
4	L	6.2 d	23.8 a	0 h
4	L/D	2.7 e	8.4 c	0.1 g

[x] Results are from pooled experiments.

[y] D = continuous dark, L = continuous light (400 $\mu Em^{-2}s^{-1}$), L/D = 12 h of alternating light (400 $\mu Em^{-2}s^{-1}$) and dark, NUV = continuous near ultra-violet light (J-205, UVP, Inc.).

[z] Means having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

Table 4.7. Interaction of light condition, incubation period, and temperature on virulence of *Alternaria alternata* f. sp. *sphenocleae* conidia grown on sorghum seeds.[w]

		Dry weight reduction (%)[x]	
Light condition	**Incubation period (weeks)**	**Temperature (°C)**	
		24	**28**
D[y]	3	70.1 e[z]	91.0 c
D	4	73.3 e	100.0 a
L	3	65.4 f	93.1 bc
L	4	70.4 e	100.0 a
L/D	3	40.2 h	86.9 d
L/D	4	62.2 g	94.3 b

[w] Results are from pooled experiments.

[x] Dry weight reduction (%) as an indication of virulence of conidia.

[y] D = continuous dark, L = continuous light (400 $\mu Em^{-2}s^{-1}$), L/D = 12 h of alternating light (400 $\mu Em^{-2}s^{-1}$) and dark.

[z] Means having common letters are not significantly different according to Duncan's multiple range test at the 1% level of significance.

Table 4.8. Influence of quantity of sorghum seeds and moisture content on conidia production of *Alternaria alternata* f. sp. *sphenocleae* in 250 ml Erlenmeyer flask.[w]

Weight of sorghum seed (g)	Number of conidia per g substrate (x 10^5)		
	Amount of water added (ml)		
	10	20	30
10	1.46 a[y] (55.42)[z]	1.12 b (76.13)	0.98 b (92.96)
20	0.09 c (34.75)	1.60 a (54.49)	1.18 b (66.76)
30	0.07 c (26.59)	1.05 b (42.98)	1.48 a (54.07)

[w] Flasks were incubated at 28°C under continuous light.

[x]Number of conidia per flask was counted after four wk of incubation. Results are from pooled experiments.

[y] Means having common letters are not significantly different according to Duncan's multiple range test at the 5% level of significance.

[z] Values in parentheses are % moisture content of sorghum seeds after autoclaving.

Table 4.9. Influence of production methods on the number, germination, and virulence of conidia of *Alternaria alternata* f. sp. *sphenocleae*.[u]

Production method[v]	Number of conidia per plate or flask (x 10^6)[w]	Germination (%)[x]	Dry weight reduction(%)[y]
1/2 PDA	1.3	98.3 a[z]	86 b
VJA	0.7	96.5 a	66 c
Sorghum seed	18.5	97.2 a	98 a

[u] Cultures were incubated at 28°C under continuous dark. Results are from pooled experiments.

[v] 1/2 PDA = half-strength potato dextrose agar, VJA = V-8 juice agar.

[w] Conidia were harvested after four wk of incubation.

[x] Germination of conidia was assessed 8 h after incubation at 24°C under continuous dark.

[y] Dry weight reduction (%) as an indication of virulence of conidia.

[z] In a column, means having common letters are not significantly different according to Duncan's multiple range test at the 5% level of significance.

Figure 4.1. Influence of various combinations of light conditions on virulence of *Alternaria alternata* f. sp. *sphenocleae* conidia, expressed as reduction in dry weight of *Sphenoclea zeylanica* plants 14 days after inoculation, grown on 1/2 PDA and VJA. Cultures were incubated at 28°C. Conidia were harvested after three and four wk of incubation. D = continuous dark, D + NUV = D + continuous near ultra-violet light (J-205, UVP, Inc.), L + D = continuous light (400 $\mu Em^{-2}s^{-1}$ PAR) + continuous dark, L + NUV = L + continuous near ultra-violet light, L/D + D = 12 h of alternating light (400 $\mu Em^{-2}s^{-1}$ PAR) and dark + continuous dark, L/D + NUV = L/D + continuous near ultra-violet light, NUV = continuous near ultra-violet light, NUV + L/D = NUV + 12 h of alternating light and dark, NUV + D = NUV + continuous dark. 1/2 PDA = half-strength potato dextrose agar, VJA = V-8 juice agar. Plants (19 to 20 cm tall) were inoculated with 10 ml of 1 x 10^5 conidia ml^{-1} suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. After spraying, pots were placed in a dark dew chamber with 100% relative humidity at 24°C for 8 h. Results are from pooled experiments. Bars having common letters are not significantly different according to Duncan's multiple range test at the 5% level of significance.

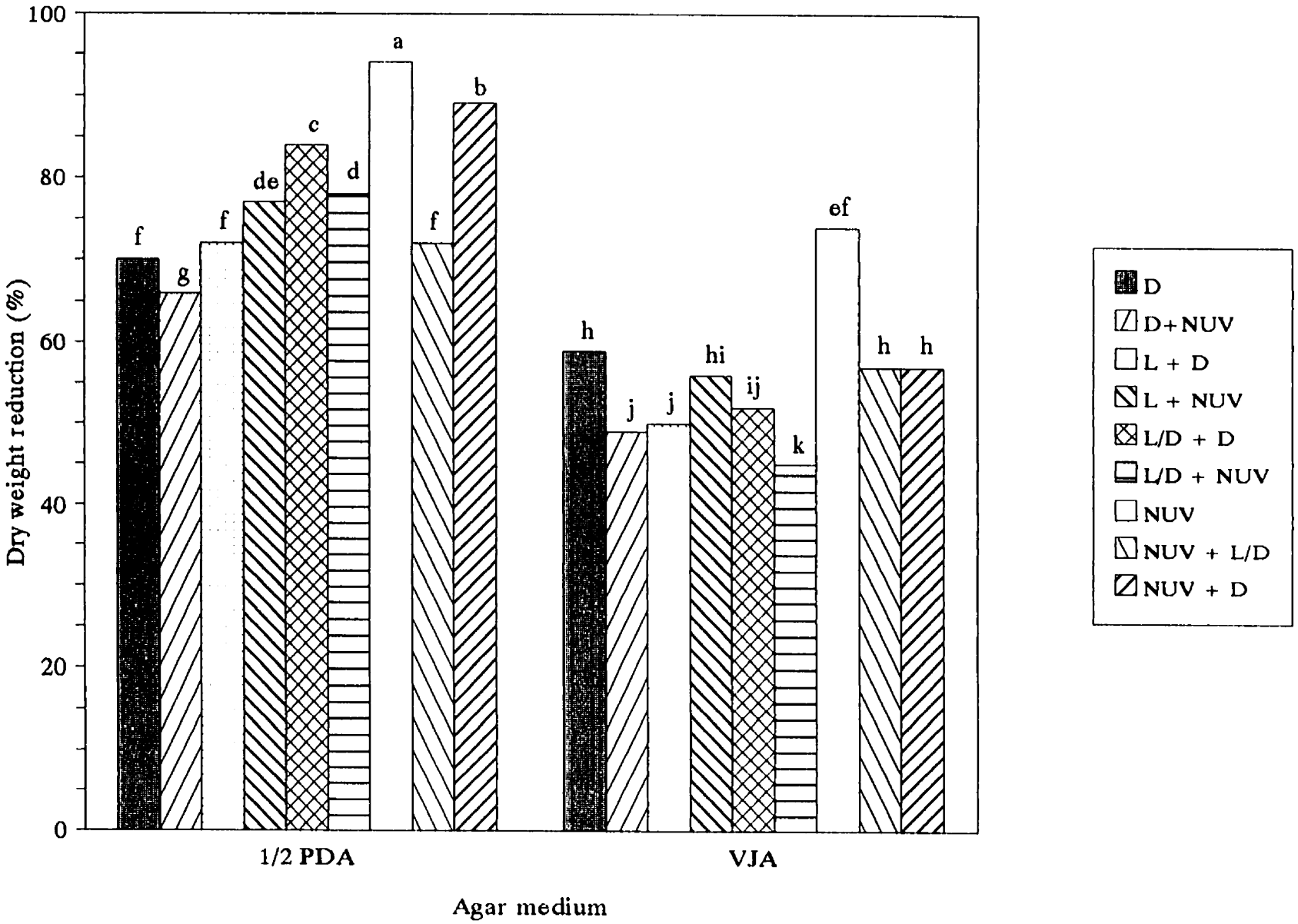

100
80
60
40
20
0
Dry weight reduction (%)
f
g
f
de
c
d
a
f
b
h
j
j
hi
ij
k
ef
h
h
1/2 PDA
VJA
Agar medium
D
D+NUV
L + D
L + NUV
L/D + D
L/D + NUV
NUV
NUV + L/D
NUV + D

Figure 4.2. The effect of *Alternaria alternata* f. sp. *sphenocleae* on *Sphenoclea zeylanica* plants 14 days after treatment with conidia grown on either half-strength potato dextrose agar (1/2 PDA) and V-8 juice agar (VJA) at 28°C under near ultra-violet light. a) control plants, b) plants treated with conidia from VJA, c) plants treated with conidia from 1/2 PDA. Plants (19 to 20 cm tall) were inoculated with 10 ml of 1 x 10^5 conidia ml^{-1} suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. After spraying, pots were placed in a dark dew chamber with 100% relative humidity at 24°C for 8 h.

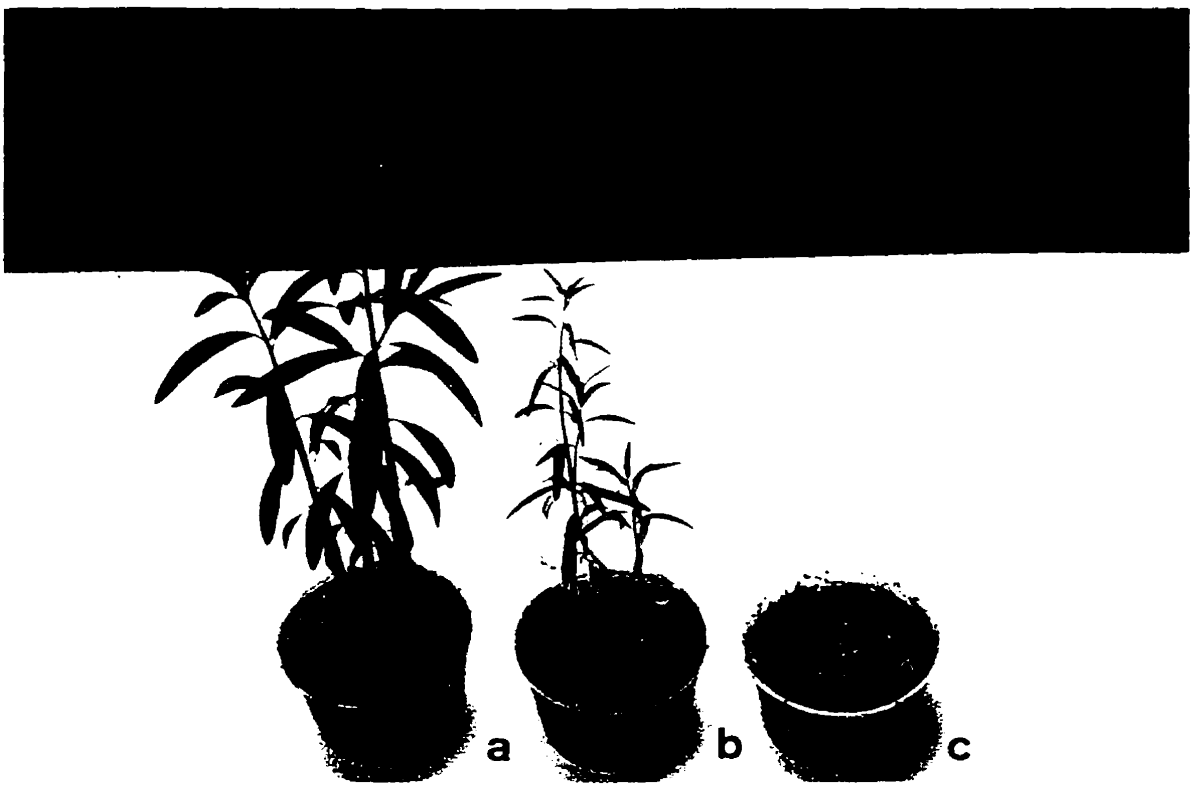
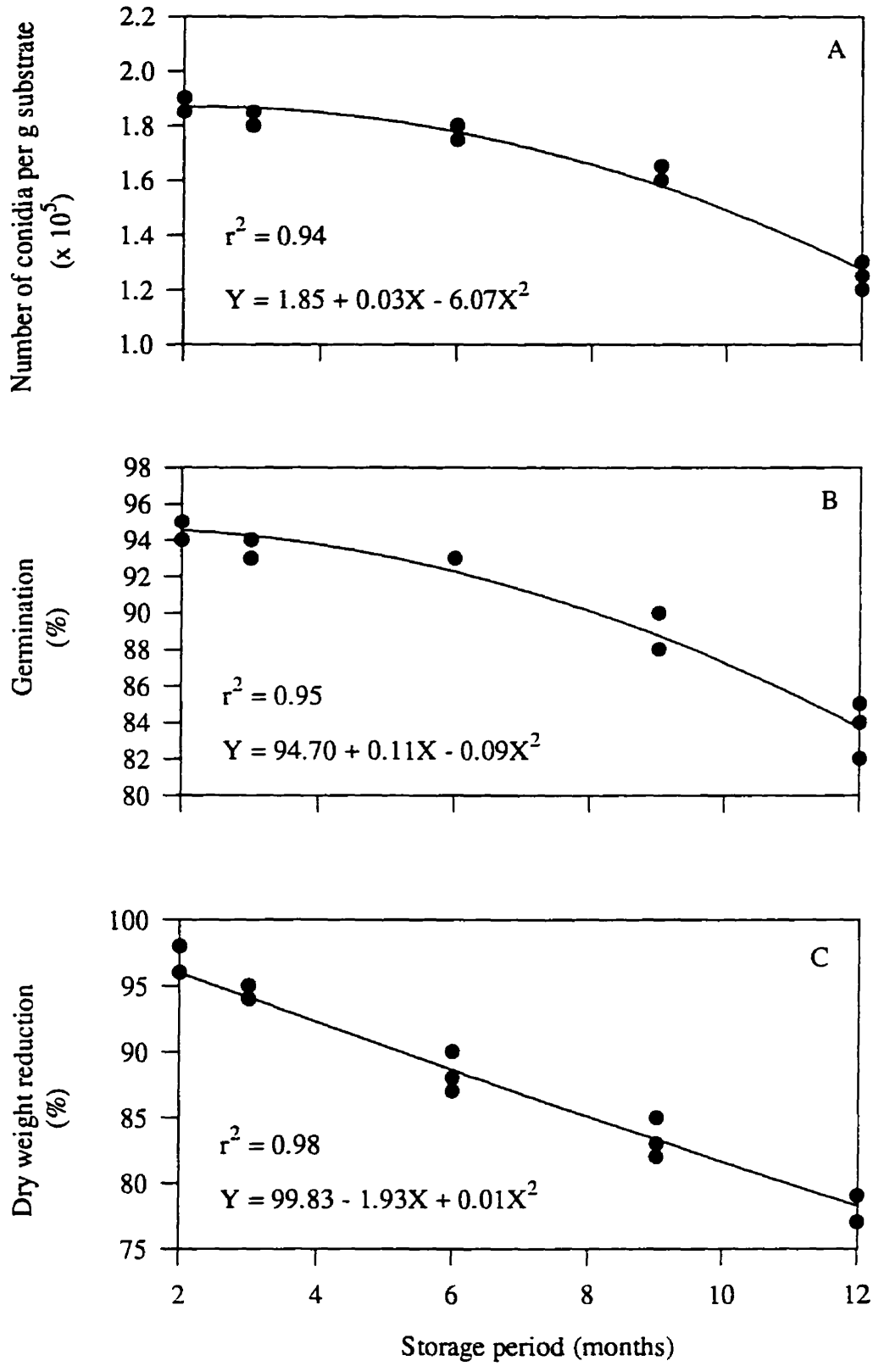

a
b
c

Figure 4.3. Relationship between storage period and the number (A), germination (B), and virulence (C) of *Alternaria alternata* f. sp. *sphenocleae* conidia grown on sorghum seeds. Virulence of conidia was expressed as reduction in dry weight of *Sphenoclea zeylanica* plants 14 days after inoculation. Inoculated flasks were incubated at 28°C under continuous light for seven days and were subsequently transferred under room conditions until harvest time. For virulence testing, plants (19 to 20 cm tall) were inoculated with 10 ml of 1 x 10^5 conidia ml^{-1} suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. After spraying, pots were placed in a dark dew chamber with 100% relative humidity at 24°C for 8 h. Results are from pooled experiments.


A
Number of conidia per g substrate (x 10^5)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
$r^2 = 0.94$
$Y = 1.85 + 0.03X - 6.07X^2$
B
Germination (%)
98
96
94
92
90
88
86
84
82
80
$r^2 = 0.95$
$Y = 94.70 + 0.11X - 0.09X^2$
C
Dry weight reduction (%)
100
95
90
85
80
75
$r^2 = 0.98$
$Y = 99.83 - 1.93X + 0.01X^2$
2
4
6
8
10
12
Storage period (months)

Connecting Text

Different production techniques have been evaluated for the production of *Alternaria alternata* f. sp. *sphenocleae* conidia however, they require at least three to four weeks of incubation to produce large quantities of viable and virulent propagules for field application. Thus, a technique to induce profuse sporulation within 24 h of incubation developed by Shahin and Shepard (1979)[1] was evaluated. In this chapter, the efficacy of the sporulation medium (S-medium) technique for conidia production of *A. alternata* f. sp. *sphenocleae* and factors affecting production and virulence of *A. alternata* f. sp. *sphenocleae* conidia using this technique, are reported.

[1]**Shahin, E. A. and Shepard, J. F. 1979.** An efficient technique for inducing profuse sporulation of *Alternaria* species. Phytopathology 69:618-620.

Chapter 5 - Evaluation of the S-medium Technique for the Sporulation of *Alternaria alternata* f. sp. *sphenocleae*, a Biocontrol Agent for *Sphenoclea zeylanica*

5.1. Abstract

Sporulation of *Alternaria alternata* f. sp. *sphenocleae* using the sporulation medium (S-medium) technique was relatively rapid. Potato dextrose agar (PDA) and half-strength PDA (1/2 PDA) were the best primary agar media for conidia production, while water agar (WA) was the worst. The addition of 20 g L^{-1} of calcium carbonate ($CaCO_3$) and 2 ml of sterile distilled water optimized conidia production. Primary 1/2 PDA at 18°C in the dark produced the most virulent conidia whereas, the predicted optimum $CaCO_3$ concentration to produce the most virulent conidia was 22.7 g L^{-1}.

5.2. Introduction

Alternaria alternata (Fr.) Keissler f. sp. *sphenocleae* causes leaf blight in *Sphenoclea zeylanica* Gaertner (gooseweed), an annual herbaceous broadleaf weed species and a serious weed of rice (*Oryza sativa* L.) (Holm *et al.*, 1977). *S. zeylanica* is one of the nine major rice weeds included for the biological weed control research program initiated in 1991 at the International Rice Research Institute (IRRI) and the University of the Philippines at Los Baños, Laguna, Philippines in collaboration with McGill University, Montréal, Québec, Canada (Watson, 1991; Bayot *et al.*, 1994; Watson, 1994). Laboratory and field studies with *A. alternata* (Fr.) Keissler f. sp. *sphenocleae* suggest that it is a promising bioherbicide candidate (Bayot *et al.*, 1994; Mabbayad and Watson, 1995; Masangkay *et al.*, 1996a). Two production techniques have been evaluated for the production of *A. alternata* f. sp. *sphenocleae* conidia however, they require at least four weeks of incubation to produce 7 x 10^5 to 1.3 x 10^6 conidia $plate^{-1}$ for the agar media technique and 9.5 x 10^5 conidia g $substrate^{-1}$ for solid substrate fermentation technique (Masangkay *et al.*, 1996b).

A sporulation medium (S-medium) for inducing profuse sporulation of *Alternaria* species was developed by Shahin and Shepard (1979) and may be a suitable method to produce abundant, viable, and virulent inocula of *A. alternata* f. sp. *sphenocleae* within 24 h of incubation. The S-medium production system of Shahin and Shepard (1979) involved culturing *Alternaria* isolates on various primary agar media [including potato dextrose agar (PDA), malt extract agar (MEA) and others] in glass petri plates for 48 to 72 h. Before the appearance of aerial mycelium, cultures with developing mycelium were cut with a sterile scalpel into 4 mm^2 blocks. These agar blocks were transferred individually and placed on the surface of the S-medium. The S-medium was composed of 20 g sucrose, 30 g calcium carbonate ($CaCO_3$), and 20 g of agar per liter of distilled water (pH 7.4). Two ml of sterile distilled water were added to the plates and incubated at 18°C in the dark. Conidia were collected 18 to 24 h after seeding. Conidia production ranged from 1.8×10^5 to 2.8×10^6 spores $plate^{-1}$ from the different primary agar media evaluated.

Thus, the objectives of this study were to: 1) evaluate the efficacy of the S-medium technique for conidia production of *A. alternata* f. sp. *sphenocleae*; and, 2) determine factors affecting production and virulence of *A. alternata* f. sp. *sphenocleae* conidia using this technique.

5.3. Materials and Methods

5.3.1. Pathogen isolation and culture maintenance

A. alternata f. sp. *sphenocleae* isolated and maintained by following the same procedure as described in section **4.3.1. Pathogen isolation and culture maintenance** of Chapter 4 (pp. 87-88).

5.3.2. Plant production

S. zeylanica plant production followed the same procedure as indicated in section **4.3.2. Plant production** of Chapter 4 (pp. 88-89).

5.3.3. General inoculation procedure

The same procedure as described in section **4.3.3. General inoculation procedure** of Chapter 4 (p. 89) was used.

5.3.4. Conidia production assessment

Conidia were harvested after 24 h of incubation unless otherwise indicated by flooding the plates with 10 ml of sterile deionized water with 0.01% Triton® X-100 (polyethylene glycol tert-octylphenyl ether, Sigma Chemical Co., St. Louis, MO) and scraping the surface of the colonies with a sterile rubber spatula. Resultant conidia suspensions were poured through a 250 mm plastic sieve lined with two layers of sterile cheesecloth. Conidia of the collected suspension were counted with the aid of a haemocytometer under a light microscope (Tuite, 1969). Six readings for each colony were recorded and averaged.

5.3.5. Pathogenicity test (virulence)

Virulence of the conidia produced was assessed by following the same procedure described in section **4.3.5. Pathogenicity testing** of Chapter 4 (pp. 89-90).

5.3.6. Effect of various primary agar media and sequential harvesting on total conidia production

Czapek-Dox agar (CZA; Difco, Detroit, MI), corn meal agar (CMA; Difco, Detroit, MI), MEA (Difco, Detroit, MI), oatmeal agar (OMA; Difco, Detroit, MI), PDA, 1/2 PDA, V-8 juice agar (VJA), and water agar (WA; BDH, Darmstadt, Germany) were evaluated as the primary agar media. The media were prepared following recommendations from the label except for VJA which was prepared according to the method described by Tuite (1969). Twenty ml of media were dispensed into sterile plastic petri dishes (90-mm-diameter). After cooling, each plate was seeded with a centrally positioned 4-mm-diameter plug taken from the edge of actively growing seven day old PDA cultures of the *A. alternata* f. sp. *sphenocleae* isolate under aseptic conditions. Cultures were sealed with Parafilm® and maintained

on the various primary agar media at 28°C under continuous NUV. Forty-eight to 72 h after seeding, but before the appearance of aerial mycelium, the cultures were cut with a sterile scalpel into 4 mm^2 blocks. Under aseptic conditions, 20 agar blocks were individually positioned on the surface of the S-medium. The S-medium of Shahin and Shepard (1979) was modified by reducing the $CaCO_3$ (BDH Inc., Toronto, ON) from 30 to 20 g L^{-1}. Two ml of sterile distilled water was added to each plate. Plates were then sealed with Parafilm® and incubated at 18°C in the dark. Conidia were harvested every 24 h for five days. Harvested conidia were counted with the aid of a haemocytometer as described above. At each harvest time, the plates were re-sealed with Parafilm® and maintained in conditions described above.

5.3.7. Effect of temperature on conidia production

Cultures were grown on the various primary agar media, transferred to S-medium as described above, and then incubated at 18, 24, or 28°C in the dark. Conidia were harvested after 24 h and conidia production was determined with the aid of a haemocytometer as mentioned previously.

5.3.8. Effect of light condition on production and virulence of conidia

Cultures were grown on the various primary agar media, transferred to S-medium as described above, and incubated under continuous dark (D), continuous light (400 $\mu Em^{-2}s^{-1}$ PAR) (L), or 12 h of alternating light (400 $\mu Em^{-2}s^{-1}$ PAR) and dark (L/D) conditions at 18°C. Conidia were harvested after 24 h and counted. Virulence of conidia was determined as described above.

5.3.9. Effect of $CaCO_3$ added to the S-medium on production and virulence of conidia

The addition of different amounts of $CaCO_3$ (10, 20, 30, 40, and 50 g L^{-1}) to the S-medium were evaluated for production and virulence of *A. alternata* f. sp. *sphenocleae* conidia. Cultures were grown on primary PDA then transferred to S-medium as described above. S-medium plates were incubated at 18°C in the dark.

Conidia were harvested after 24 h, counted, and the virulence of conidia evaluated as described above.

5.3.10. Effect of volume of water added to the S-medium on conidia production

The effect of the addition of different volumes of sterile distilled water (1, 2, 3 and 4 ml) after seeding the S-medium on conidia production was evaluated. Cultures were grown on primary PDA and transferred to S-medium as described above. S-medium plates were incubated at 18°C in the dark. Conidia were harvested after 24 h and conidia production was determined with the aid of a haemocytometer as mentioned previously.

5.3.11. Data Analyses

All experiments were performed twice and laid out in either a randomized complete block design (RCBD) or a completely randomized design (CRD) with three replicates. Data from conidial counts were subjected to log (x+1) transformation for zero values and log (x) for non-zero values and percentage data were arc sine transformed before analysis (Gomez and Gomez, 1984). Factorial analysis of variance considered the effect of each factor individually and their interaction. Results from the two trials were pooled if homogeneity of variances was confirmed by Bartlett's test (Gomez and Gomez, 1984). All analyses were conducted using STATGRAPHICS software for IBM-PC (STSC Inc., 1991). Treatment means were separated using Duncan's multiple range test at the 5% level of significance.

5.4. Results

5.4.1. Effect of various primary agar media and sequential harvesting on total conidia production. There was a significant interaction between the different primary agar media and sequential harvesting of conidia every 24 h until 120 h on conidia production (Appendix 5.1). The best primary agar media for conidia production were PDA and 1/2 PDA whereas WA produced the fewest number of conidia (Figure 5.1).

5.4.2. Effect of incubation temperature of S-medium on conidia production. There was a significant interaction between primary agar media and S-medium incubation temperature on conidia production of *A. alternata* f. sp. *sphenocleae* in the dark on S-medium (Appendix 5.2). The best primary agar medium and incubation temperature combination for conidia production were PDA and S-medium incubation at 18°C (3.0×10^6 conidia plate^{-1}) (Table 5.1). For all primary agar media tested, more conidia were produced when the S-medium incubation was 18°C rather than 24 or 28°C. Conidia production was very poor when WA was the primary media and no conidia were produced at 28°C.

5.4.3. Effect of light condition on production and virulence. There was a significant interaction between the primary agar media and incubation light condition on conidia production of *A. alternata* f. sp. *sphenocleae* at 18°C on S-medium (Appendix 5.3). The most suitable primary agar medium and light condition for conidia production was PDA under continuous dark (Table 5.2). Exposure to continuous dark gave the highest conidia production for all primary agar media while continuous light completely inhibited conidia production. No conidia were produced from CMA, MEA, OMA, and WA when S-medium was exposed to 12 h of alternating light and dark periods.

Only conidia produced at 18°C under continuous dark were evaluated for virulence since exposure to continuous light did not produce conidia and 12 h of alternating light and dark produced insufficient number of conidia or no conidia for inoculation of host plants. Virulence of *A. alternata* f. sp. *sphenocleae* conidia at 18°C on S-medium was significantly affected by the various primary agar media evaluated (Appendix 5.4). The most virulent conidia were produced from primary 1/2 PDA whereas, primary CMA gave the least virulent conidia, but this was not significantly different from primary MEA, CDA, and OMA (Figure 5.2).

5.4.4. Effect of $CaCO_3$ added to the S-medium on production and virulence of conidia. The addition of different amounts of $CaCO_3$ to the S-medium significantly affected production and virulence of conidia (Appendix 5.5A and 5.5B, respectively). Maximum number of conidia were produced with the addition of 20 g L^{-1} of $CaCO_3$ to the S-medium (6.05 x 10^6 conidia $plate^{-1}$) whereas, greater or lesser $CaCO_3$ concentrations decreased conidia production (Figure 5.3). There was a quadratic relationship between the addition of $CaCO_3$ to the S-medium and virulence of *A. alternata* f. sp. *sphenocleae* conidia (Figure 5.4). The predicted optimum amount of $CaCO_3$ for maximum dry weight reduction was 22.7 g L^{-1}.

5.4.5. Effect of volume of water added on S-medium for conidia production. The volume of water added to the S-medium greatly affected conidia production (Appendix 5.6). The greatest number of conidia was produced with the addition of 2 ml of sterile distilled water (6.97 x 10^6 conidia $plate^{-1}$) whereas, greater or lesser amounts of water reduced conidia production (Table 5.3).

5.5. Discussion

Primary agar media, temperature, light condition, amount of $CaCO_3$ added to the S-medium, and volume of sterile distilled water added after seeding the S-medium greatly affected conidia production of *A. alternata* f. sp. *sphenocleae*. All the primary agar media investigated gave abundant number of conidia except for primary WA. Primary agar media rich in nutrients were, therefore, required to stimulate growth of the fungus before transferring mycelial blocks to the S-medium. The preferred primary agar media and S-medium incubation temperature were PDA and 18°C, respectively. Cultures incubated at 24 to 28°C did not produce enough conidia to conduct virulence testing. S-medium cultures exposed to continuous dark produced the highest number of conidia. Light completely inhibited conidia production and exposure to 12 h of alternating light and dark period likewise prevented conidia production in most of the primary agar media tested. Conidia production could be optimized with the addition of 20 g L^{-1} of $CaCO_3$ to the S-medium and this contradicts

findings of Shahin and Shepard (1979). They indicated that different amounts of $CaCO_3$ added gave comparable conidia production. The addition of 2 ml of water to the S-medium was optimum for maximum conidia production, and these results are similar to those of Shahin and Shepard (1979).

Conidia of *A. alternata* f. sp. *sphenocleae* could be produced in a relatively short time and subsequent harvesting could be carried out up to 120 h in all the primary agar media evaluated. This could be attributed to the addition of 20 g of sucrose L^{-1} to the S-medium (Shahin and Shepard, 1979).

Light conditions and amount of $CaCO_3$ added to the S-medium greatly affected virulence of *A. alternata* f. sp. *sphenocleae* conidia. Since insufficient numbers of conidia were produced with S-medium cultures exposed to continuous light and 12 h of alternating light and dark period, only those conidia produced at 18°C in the dark were evaluated for virulence. Primary 1/2 PDA produced the most virulent conidia when expressed as percent dry weight reduction in host plants. The predicted optimal amount of $CaCO_3$ to be added to the S-medium to produce the most virulent conidia was found to be 22.7 g L^{-1} of $CaCO_3$.

In subsequent studies, primary PDA was utilized because cutting and positioning mycelial blocks on the S-medium was easier and faster. When 1/2 PDA was used, agar blocks were softer and needed considerably more time and care in cutting and positioning the mycelial blocks on the S-medium.

Primary 1/2 PDA produced a low number of conidia during the first harvest time but abruptly increased after 48 h of incubation, and then gradually decreased up to 120 h of incubation. This might be attributed to the excessive amount of moisture in culture plates during the first harvest period. However, during the second harvest time, excessive moisture might have evaporated while conidia were being harvested during the first harvest period thereby increasing conidia production in the second harvest. There were difficulties encountered during conidia harvesting on 1/2 PDA because the surface of this medium was soft thereby requiring extra care and time to collect all the conidia produced.

The S-medium technique is basically a simple water agar medium containing $CaCO_3$ that promoted extensive sporulation of *A. alternata* f. sp. *sphenocleae* when the fungus was first established on any of several primary agar media. Water was added to the S-medium after mycelial blocks were seeded to aid in spreading the mycelia all throughout the petri plates and increase sporulation efficiency. Added sucrose was sufficient to maintain continued production of conidia up to 120 h of incubation. Several physical and environmental parameters, however, were involved in attaining high production and virulent conidia. These included cool temperature (18°C), addition of 2 ml of sterile distilled water to the surface of the S-medium, addition of supplements (22.7 g L^{-1} of $CaCO_3$ and 20 g of sucrose L^{-1}), primary agar media (except WA), wounding of the mycelium, and absence of light. This production technique could produce high numbers of conidia within 24 h of incubation. Moreover, harvesting could still be carried out sequentially unlike with agar media and solid substrate fermentation which require four weeks after incubation to produce large quantities of conidia (Masangkay *et al.*, 1996b). However, this technique is time-consuming, expensive, labour-intensive, and presents a number of difficulties with respect to the maintainance of sterile conditions and in the harvest of conidia. Therefore, the use of this technique in developing countries is likely to be limited, in part, because of the 18°C temperature requirement of this fungus for effective and abundant sporulation.

5.6. Literature Cited

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Table 5.1. Influence of various primary agar media and temperature on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on S-medium.[v,w]

Primary agar medium[x]	Number of conidia per plate ($\times 10^4$)[y]		
	Temperature (°C)		
	18	**24**	**28**
CDA	113.3 c[z]	6.2 h	0.1 k
CMA	34.7 f	1.8 i	0.1 k
MEA	60.8 d	1.8 i	0.1 k
OMA	49.3 e	2.3 i	0.1 k
PDA	300.0 a	9.0 g	0.1 k
1/2 PDA	198.3 bc	7.9 gh	0.2 j
VJA	250.0 b	8.3 gh	0.1 k
WA	7.0 gh	0.1 k	0 l

[v] S-medium consisted of 2% water agar containing 20 g L^{-1} sucrose and 30 g L^{-1} of $CaCO_3$, pH 7.4.

[w] The fungus was first established on several primary agar media and maintained at 28°C under continuous NUV light (J-05, UVP, Inc.) for 48 to 72 h then transferred to S-medium. The S-medium cultures were incubated at three temperatures in the dark.

[x] CDA = Czapek-Dox agar, CMA = corn meal agar, MEA = malt extract agar, OMA = oatmeal agar, PDA = potato dextrose agar, 1/2 PDA = half-strength potato dextrose agar, VJA = V-8 juice agar, WA = water agar.

[y] Number of conidia per plate was counted after 24 h of incubation. Results are from pooled experiments.

[z] Means having common letters are not significantly different according to Duncan's multiple range test at the 5% level of significance.

Table 5.2. Influence of various primary agar media and light condition on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on S-medium.[u,v]

Primary agar medium[x]	Number of conidia per plate (x 10^4)[w]		
	Light condition[y]		
	D	L	L/D
CDA	112.2 d[z]	0 j	0.1 i
CMA	34.7 g	0 j	0 j
MEA	61.7 e	0 j	0 j
OMA	47.9 f	0 j	0 j
PDA	295.1 a	0 j	0.1 i
1/2 PDA	199.5 c	0 j	0.1 i
VJA	251.2 b	0 j	0.1 i
WA	6.9 h	0 j	0 j

[u] S-medium consisted of 2% water agar containing 20 g L^{-1} sucrose and 30 g L^{-1} of $CaCO_3$, pH 7.4.

[v] The fungus was first established on several primary agar media and maintained at 28°C under continuous NUV light (J-05, UVP, Inc.) for 48 to 72 h then transferred to S-medium. The S-medium cultures were incubated at 18°C under three light conditions.

[w] CDA = Czapek-Dox agar, CMA = corn meal agar, MEA = malt extract agar, OMA = oatmeal agar, PDA = potato dextrose agar, 1/2 PDA = half-strength potato dextrose agar, VJA = V-8 juice agar, WA = water agar.

[x] Number of conidia per plate was counted after 24 h of incubation. Results are from pooled experiments.

[y] D = continuous dark, L = continuous light (400 $\mu Em^{-2}s^{-1}$), L/D = 12 h of alternating light (400 $\mu Em^{-2}s^{-1}$) and dark.

[z] Means having common letters are not significantly different according to Duncan's multiple range test at 5% level of significance.

Table 5.3. Influence of water volume on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on S-medium.[w,x]

Volume of water added (ml)	Number of conidia per plate[y] ($\times 10^5$)
1	0.21 d[z]
2	69.72 a
3	1.25 b
4	0.69 c

[w] S-medium consisted of 2% water agar containing 20 g L^{-1} sucrose and 30 g L^{-1} of $CaCO_3$, pH 7.4.

[x] The fungus was first established on PDA and maintained at 28°C under continuous NUV light (J-05, UVP, Inc.) for 48 to 72 h then transferred to S-medium. The S-medium cultures were incubated at 18°C in the dark.

[y] Number of conidia per plate was counted after 24 h of incubation. Results are from pooled experiments.

[z] Means having common letters are not significantly different according to Duncan's multiple range test at the 5% level of significance.

Figure 5.1. Effect of various primary agar media and sequential harvesting on total conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on S-medium. S-medium consisted of 2% water agar containing 20 g L^{-1} sucrose and 30 g L^{-1} of $CaCO_3$, pH 7.4. Seeded primary agar media such as PDA = potato dextrose agar, 1/2 PDA = half-strength potato dextrose agar, VJA = V-8 juice agar, CDA = Czapek-Dox agar, MEA = malt extract agar, CMA = corn meal agar, OMA = oatmeal agar, and WA = water agar were maintained at 28°C under continuous NUV light (J-05, UVP, Inc.) for 48 to 72 h then transferred to S-medium. The S-medium cultures were incubated at 18°C in the dark. Number of conidia per plate was counted 24 h after incubation (HAI) until 120 HAI. Results are from pooled experiments.

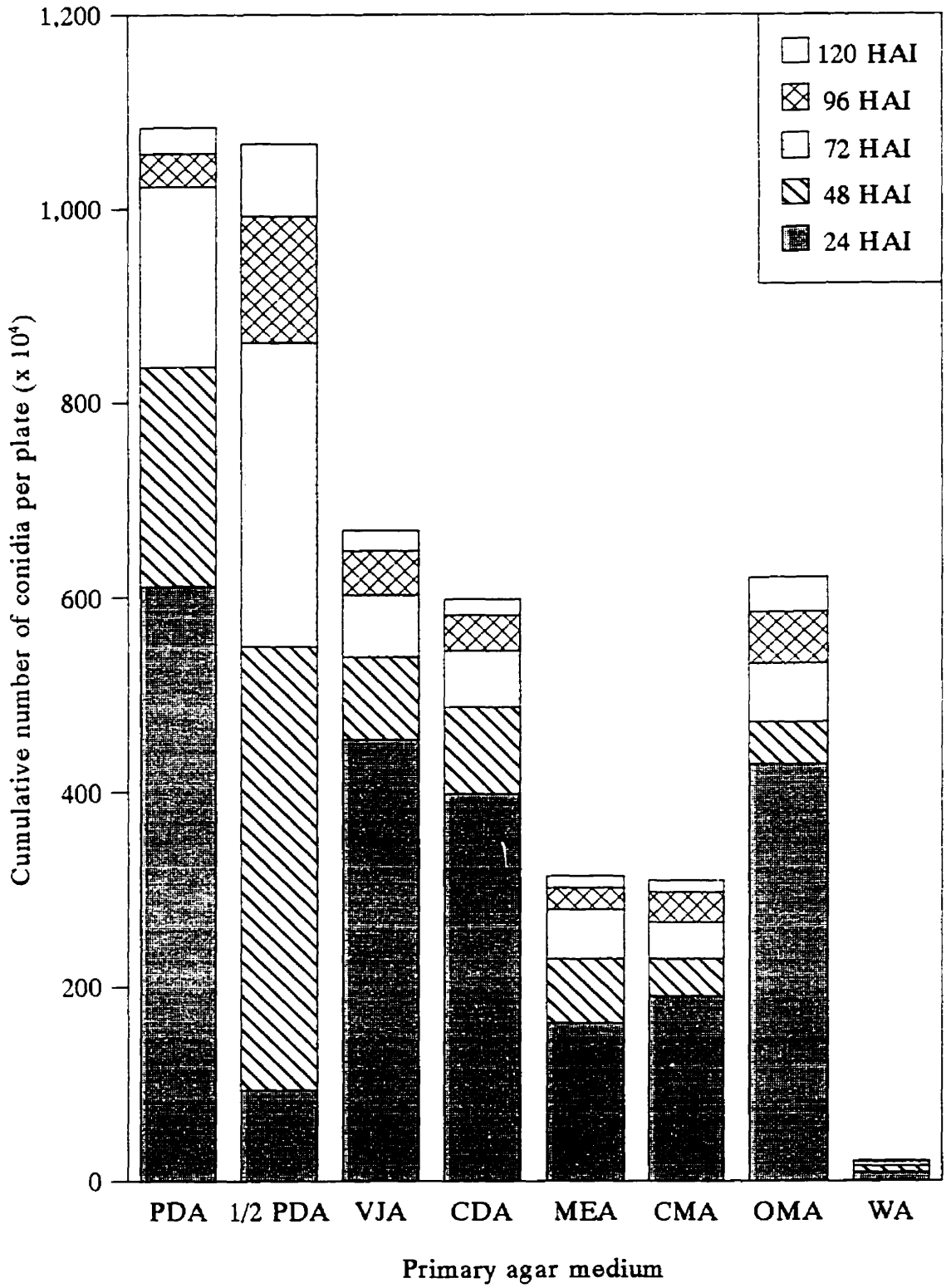

1,200
1,000
800
600
400
200
0
Cumulative number of conidia per plate (x 10⁴)
120 HAI
96 HAI
72 HAI
48 HAI
24 HAI
PDA
1/2 PDA
VJA
CDA
MEA
CMA
OMA
WA
Primary agar medium

Figure 5.2. Virulence of *Alternaria alternata* f. sp. *sphenocleae* conidia when colony was established on various primary agar media and then transferred to the S-medium. Results are expressed as percent reduction in dry weight of *Sphenoclea zeylanica* plants 14 days after inoculation. Plants (19 to 20 cm tall) were inoculated with 10 ml of 1 x 10^5 conidia ml^{-1} suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. Sprayed potted plants were placed in a dark dew chamber with 100% relative humidity at 24°C for 8 h. S-medium consisted of 2% water agar containing 20 g L^{-1} sucrose and 30 g L^{-1} of $CaCO_3$, pH 7.4. Seeded primary agar media such as CDA = Czapek-Dox agar, CMA = corn meal agar, MEA = malt extract agar, OMA = oatmeal agar, PDA = potato dextrose agar, 1/2 PDA = half-strength potato dextrose agar, and VJA = V-8 juice agar were maintained at 28°C under continuous NUV light (J-05, UVP, Inc.) for 48 to 72 h then transferred to the S-medium. The S-medium cultures were incubated at 18°C in the dark. Results are from pooled experiments. Bars having common letters are not significantly different according to Duncan's multiple range test at the 5% level of significance.

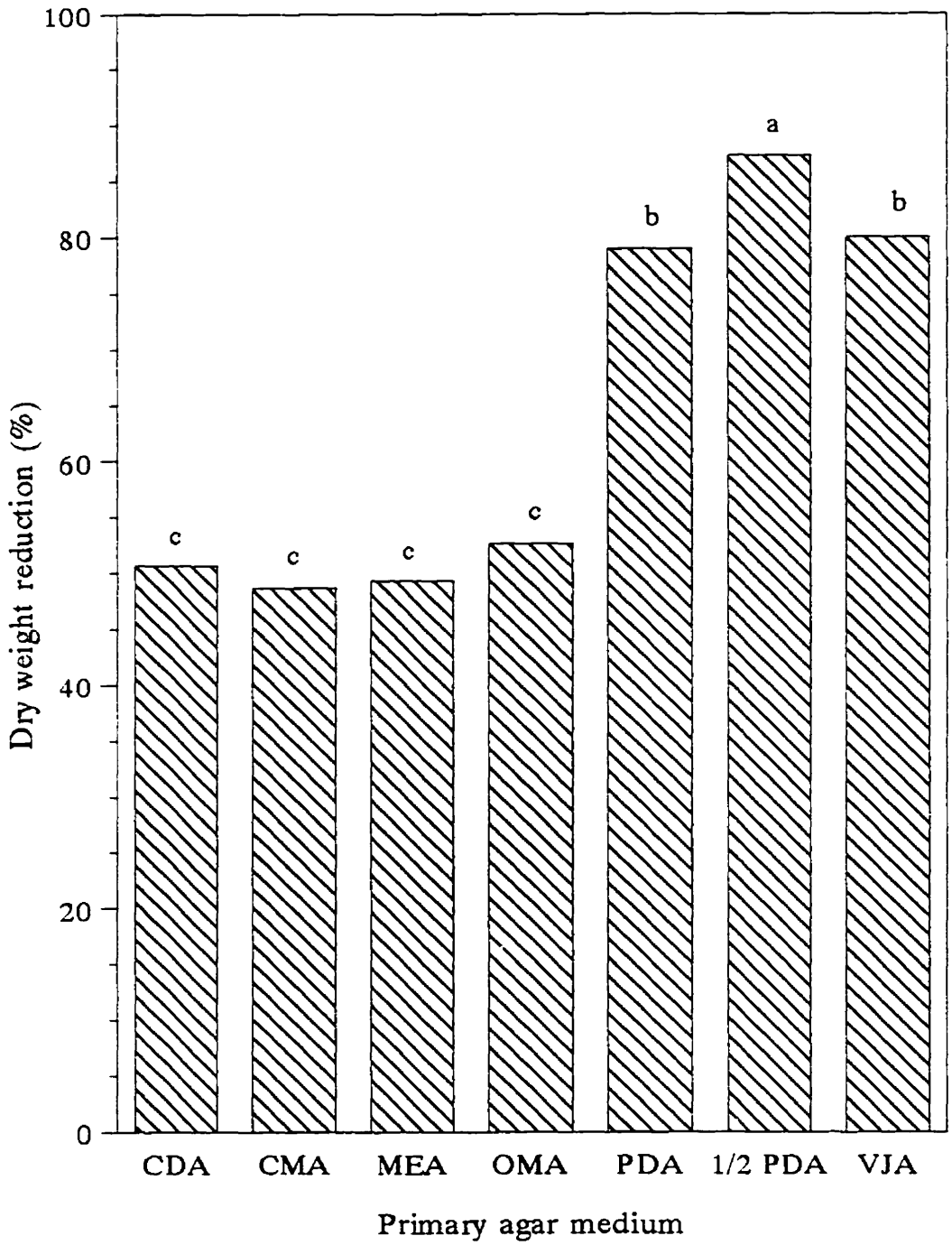

100
80
60
40
20
0
Dry weight reduction (%)
c
c
c
c
b
a
b
CDA
CMA
MEA
OMA
PDA
1/2 PDA
VJA
Primary agar medium

Figure 5.3. Influence of $CaCO_3$ concentration on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on S-medium. S-medium consisted of 2% water agar containing 20 g L^{-1} sucrose and 30 g L^{-1} of $CaCO_3$, pH 7.4. Seeded primary PDA plates were maintained at 28°C under continuous NUV light (J-05, UVP, Inc.) for 48 to 72 h then transferred to S-medium. The S-medium cultures were incubated at 18°C in the dark. Number of conidia per plate was counted after 24 h of incubation and results are from pooled experiments. Bars having common letters are not significantly different according to Duncan's multiple range test at the 5% level of significance.

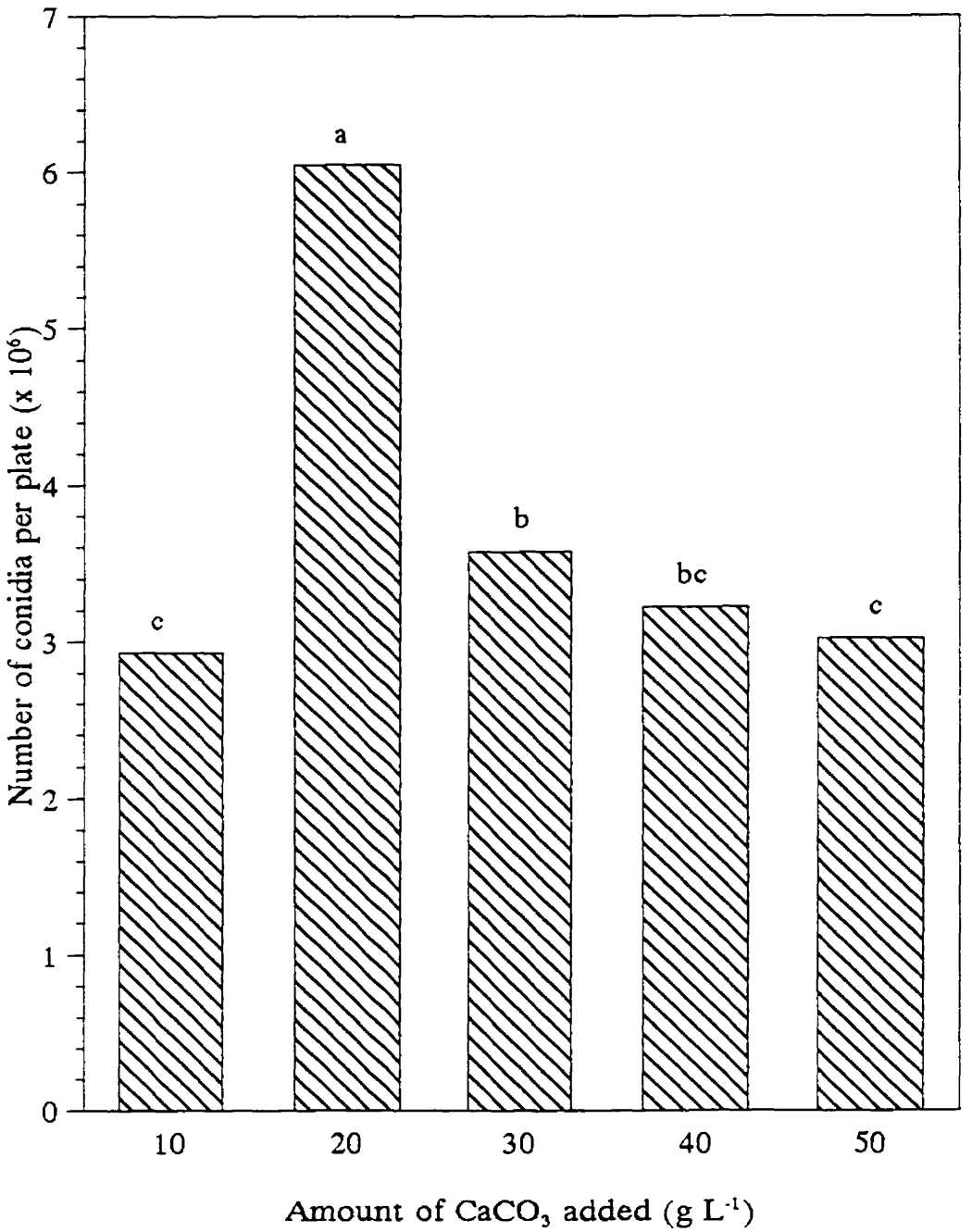
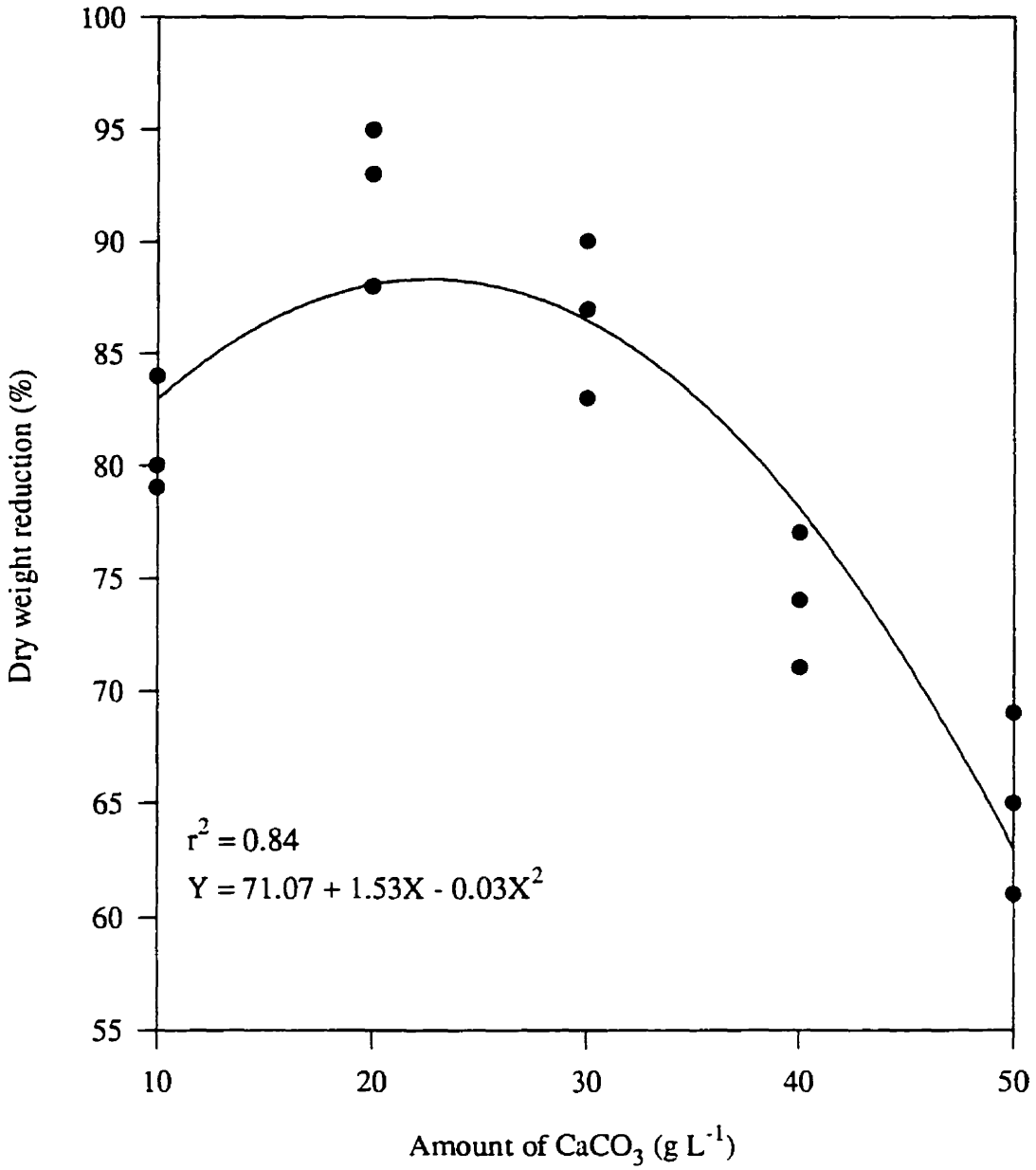

7
6
5
4
3
2
1
0
Number of conidia per plate (x 10⁶)
a
b
bc
c
c
10
20
30
40
50
Amount of CaCO₃ added (g L⁻¹)

Figure 5.4. Effect of $CaCO_3$ concentration on virulence of *Alternaria alternata* f. sp. *sphenocleae* conidia grown on S-medium. Expressed as percent reduction in dry weight of *Sphenoclea zeylanica* plants 14 days after inoculation. Plants (19 to 20 cm tall) were inoculated with 10 ml of 1 x 10^5 conidia ml^{-1} suspension with 0.01% Triton X-100 as a wetting agent using a hand-held atomizer. Sprayed potted plants were placed in a dark dew chamber with 100% relative humidity at 24°C for 8 h. S-medium consisted of 2% water agar containing 20 g L^{-1} sucrose and 30 g L^{-1} of $CaCO_3$, pH 7.4. Seeded primary PDA plates were maintained at 28°C under continuous NUV light (J-05, UVP, Inc.) for 48 to 72 h then transferred to S-medium. The S-medium cultures were incubated at 18°C in the dark. Results are from pooled experiments.


100
95
90
85
80
75
70
65
60
55
Dry weight reduction (%)
$r^2 = 0.84$
$Y = 71.07 + 1.53X - 0.03X^2$
10
20
30
40
50
Amount of $CaCO_3$ (g L^{-1})

Connecting Text

One of the most important factors to consider in the development of a biological weed control strategy is the delimitation of the host range of the biological control agent. Host specificity of the fungal pathogen must assure the safety of desirable plants if the biological control agent is to be mass reared and applied as inundative inoculum. To date, the host range testing of *Alternaria alternata* f. sp. *sphenocleae* is very limited. In this chapter, the findings of host range experiments with this indigenous fungal pathogen from blighted *Sphenoclea zeylanica* (gooseweed) leaves, are reported.

Chapter 6. Host range of *Alternaria alternata* f. sp. *sphenocleae* Causing Leaf Blight of Gooseweed

6.1. Abstract

Forty-nine plant species in 40 genera representing 20 families, selected by using a modified centrifugal phylogenetic and variety strategy, were screened against *Alternaria alternata* f. sp. *sphenocleae*. Only *Sphenoclea zeylanica* (gooseweed), the original host, was susceptible to *A. alternata* f. sp. *sphenocleae* in the presence or absence of supplemental dew. All *S. zeylanica* plants were killed when subjected to a 14 to 15 h dew. Lettuce, soybean, common bean, winged bean, mungbean, stringbean, banana, and some rice cultivars showed highly resistant reactions. Cabbage, radish, and okra showed highly resistant to moderately resistant reactions while cotton showed moderately resistant to moderately susceptible reactions. However, sporulation was only observed on detached excised *S. zeylanica* leaves and no sporulation occurred on other plant species evaluated. Moreover, *S. zeylanica* showed 100% leaf area damage (LAD) and exhibited a rapid reaction to the application of *A. alternata* f. sp. *sphenocleae*. Initial field trials also indicated that only *S. zeylanica* was susceptible to this fungal pathogen. These results demonstrate that *A. alternata* f. sp. *sphenocleae* can be safely used to control *S. zeylanica* in irrigated rice fields.

6.2. Introduction

Sphenoclea zeylanica Gaertner (gooseweed) is a common, annual herbaceous, broadleaf weed species of wetland rice (*Oryza sativa* L.) in Southeast and South Asia, the United States, the West Indies, and West Africa (Holm *et al.*, 1977). Holm *et al.* (1977) reported that *S. zeylanica* was only a weed in rice, however, recent findings have revealed that this weed is also associated with other crops including cotton (*Gossypium hirsutum* L.) in Louisiana, U.S.A. (Sanders, 1990), wheat (*Triticum aestivum* L.) in wheat-rice rotations in India (Khan *et al.*, 1988) and in soybean [(*Glycine max* (L.) Merr.)] in rice-soybean rotations in Thailand (Vongsaroj, 1994). *S.*

zeylanica strongly competes for light, water, and nutrients in rice systems, reducing yields by as much as 45% (Laganao, 1981; Ampong-Nyarko and De Datta, 1991; Biswas and Sattar, 1991). Significant yield reductions in transplanted rice occurred at weed densities as low as 20 plants m^{-2} (IRRI, 1989). It can also pose problems when it remains green and succulent by interfering with harvesting procedures (Migo *et al.*, 1986). There are various management strategies to control this weed species such as handweeding, cultural and mechanical methods as well as chemical herbicides however, each method has its limitations.

The possibility of utilizing a biological control agent against this weed species was initiated in 1991 when a fungal pathogen, *Alternaria alternata* (Fr.) Keissler f. sp. *sphenocleae*, was isolated from blighted *S. zeylanica* collected in a rice field near Los Baños, Laguna, Philippines. Laboratory, greenhouse, and field studies with this pathogen suggest that it is a promising bioherbicide candidate (Bayot *et al.*, 1994; Mabbayad and Watson, 1995; Masangkay *et al.*, 1996a; 1996b).

One of the most important factors to consider in a biological weed control program is host specificity. It is of critical importance that the safety of desirable plants are not threatened by the release of a potential biological weed control agent (Hasan, 1983; Schroeder, 1983; Watson, 1985). To date, host range testing of *A. alternata* f. sp. *sphenocleae* is limited. Thus, the objective of this study was to determine the response of selected plant species inoculated with *A. alternata* f. sp. *sphenocleae*.

6.3. Materials and Methods

This study was conducted at the International Rice Research Institute, Los Baños, Laguna, Philippines.

6.3.1. Inoculum production

A single-conidium isolate of *A. alternata* f. sp. *sphenocleae* growing on half-strength potato dextrose agar (1/2 PDA) (Difco, Detroit, MI) slants in small vials was

maintained at 4°C as a stock culture. Small pieces of mycelium from the stock cultures were aseptically transferred to cooled 1/2 PDA (20 ml) in glass petri plates (100-mm-diameter). Cultures were sealed with Parafilm® (American National Can Co., Greenwich, CT) and incubated at 28°C under continuous light [80 $\mu Em^{-2}s^{-1}$ photosynthetically active radiation (PAR)] for two to three days and served as the seed inoculum. Four agar plugs (6-mm-diameter) from the margins of young colonies were inoculated by inverting and placing them equidistantly into each glass petri plate with cooled 1/2 PDA (20 ml) under aseptic conditions. Subsequently, plates were sealed with Parafilm® and incubated in conditions as previously described for three to seven days. Conidia were harvested by flooding each plate with distilled water containing 0.05% Tween 20 (polyoxyethylene-sorbitan-monolaureate, Sigma Chemical Co., St. Louis, MO) and brushing the surface with a sterile camel's hair brush. The resulting suspension was filtered through a layer of cheesecloth. Inoculum concentration was determined with the aid of a haemocytometer and adjusted to the desired concentration with distilled water containing 0.05% Tween 20.

6.3.2. Plant production

Forty genera and 49 plant species belonging to 20 families were selected for the host range trial (Table 6.1) using the modified centrifugal phylogenetic and variety strategy proposed by Wapshere (1974). Plants tested came from Los Baños, Laguna and nearby areas. These plants belonged to either one or more of three groups: (1) plant species commonly reported to be infected by species of *Alternaria*; (2) plants commonly found in close proximity to irrigated rice fields; and (3) taxonomically related plants. Group 1 included plant species reported to be attacked by the fungal pathogen, *Alternaria* species, and designations for this group follow Farr *et al.* (1989) and Tangonan and Quebral (1992). Group 2 plants were assigned according to Aycardo (1977), Carandang *et al.* (1977), Harwood (1977), and Gomez and Gomez (1983). The phylogenetically related plants of group 3 were based on the systematic classification of Cosner *et al.* (1994) including commonly grown economically

important crops of the genera *Ipomoea*, *Lycopersicon*, and *Capsicum*. Plants were either grown from seed or propagated vegetatively depending on the plant species.

6.3.3. General inoculation procedure

Seedlings at the 2- to 3-leaf stage were inoculated with a conidial suspension of 3.5×10^5 ml^{-1} using a hand-held atomizer until run-off. Control plants were sprayed with distilled water containing 0.05% Tween 20. One inoculated set and one uninoculated set of test plants were placed in a dark dew chamber with 100% relative humidity (RH) at 25°C for 14 to 15 h. Subsequently, pots were transferred to a corner of the greenhouse having a temperature of 24 to 28°C and 85 to 95% RH. The other inoculated and uninoculated set of test plants were placed outdoors immediately after inoculation and exposed to prevailing natural conditions (in the absence of a dew supplement). The experiment was conducted as a factorial experiment with species tested, inoculation level (0 and 3.5×10^5 ml^{-1}), and dew supplements (0 and 14 to 15 h) as factors. Each treatment was replicated four times having two to four plants per pot depending on the plant species. Inoculated pots were randomly placed in the greenhouse and outdoors until the termination of the experiment. The experiment was repeated twice.

Plant-pathogen interactions were evaluated using two parameters: disease severity and sporulation. Disease severity was visually assessed daily for 14 days following inoculation by using the disease rating scale of I (immune) - no visible symptoms; HR (highly resistant) - very few, minute, necrotic flecks present; MR (moderately resistant) - a few small, necrotic lesions present; MS (moderately susceptible) - many necrotic lesions present but no expansion of lesions to cause desiccation of leaves; and S (susceptible) - many necrotic lesions present which expand causing desiccation and leaf abscission. Percent leaf area damage (LAD) was estimated and the number of days after inoculation when the disease symptoms first appeared were also recorded. At 14 days after inoculation and after disease severity rating was completed, one or two inoculated leaves were excised from both the treated and untreated plants from each replicate. Excised leaves were surface-sterilized with

0.5% sodium hypochlorite solution for 30 seconds, rinsed, and then incubated on moistened filter paper (Whatman® #1, Whatman International Ltd., Maidstone, England) in glass petri plates at 28°C in the dark. After 24, 48, and 72 h of incubation (HAI), sporulation on leaves was examined with a stereomicroscope. Sporulation was rated at four levels: - = no sporulation, + = light sporulation, ++ = moderate sporulation, and +++ = heavy sporulation. Sporulation time and level were used to characterize the compatibility of host-pathogen interactions. If no sporulation occurred within 72 h on excised leaves, then the fungus was designated as being incompatible with the host plant.

6.4. Results

Different plant species responded differently to inoculation of *A. alternata* f. sp. *sphenocleae*. Among the 40 genera and 49 plant species representing 20 families evaluated, only *S. zeylanica* was susceptible to *A. alternata* f. sp. *sphenocleae* in the presence of supplemental dew (Table 6.1) or with the absence of dew (data not shown). *Lactuca sativa* L., *Glycine max* (L.) Merr., *Phaseolus vulgaris* L., *Psophocarpus tetragonolobus* (L.) DC., *Vigna radiata* (L.) R. Wilcz., *V. unguiculata* (L.) Walp., *Musa sapientum* L., and some rice cultivars (breeding lines) showed highly resistant reactions having very few, minute, necrotic flecks. *Brassica oleracea* L. var. *capitata* L., *Raphanus sativus* L., and *Abelmoshus esculentus* (L.) Moench. showed moderately resistant to highly resistant reactions having few small, necrotic lesions. *Gossypium hirsutum* L. was the only plant species that showed moderately resistant to moderately susceptible reactions having many necrotic lesions, which did not expand and were not severe enough to cause defoliation or desiccation of leaves. The rest of the test plant species were immune to *A. alternata* f. sp. *sphenocleae*.

Only *S. zeylanica* exhibited 100% LAD while the rest of the test plant species had very low LAD values ranging from 2 to 14 (Table 6.1). *S. zeylanica* showed symptoms of wilting and leaf cupping 12 h after removal from the dew chamber (Table 6.1). The fungal pathogen causes necrosis of the host tissue, initially killing

the leaves, then the stems and eventually the entire plant. All *S. zeylanica* plants were killed when subjected to a 14 to 15 h dew. For other test plant species that showed the minor symptoms, the latent period for disease development was 24 to 144 h after removal from the dew chamber.

Sporulation only occurred on excised *S. zeylanica* leaves. Sporulation was light at 24 HAI and heavy at 48 HAI and occurred in the presence and absence of supplemental dew (data not presented). Sporulation did not occur on other test plant species.

6.5. Discussion

An important component of all biological weed control programs is host specificity testing. Before a pathogen can be widely used in the field as a biocontrol agent, the safety of non-target economic and wild plants must first be assured. However, host specificity is a less rigorous requirement when employing the bioherbicide strategy since the biological control agent is already present in the environment and non-target plants should have been already exposed to the pathogen (Watson, 1985). Mabbayad and Watson (1995) reported that no symptoms were observed on associated plant species (*Echinochloa* spp., *Fimbristylis miliacea* (L.) Vahl., *Cyperus difformis* L., *Cyperus iria* L., *Eclipta prostrata* L., *Leptochloa chinensis* (L.) Nees, *Ludwigia octovalvis* (Jacq.) Raven, *Monochoria vaginalis* (Burm. f.) Presl., *Ammannia* sp., *Sesbania* sp., and volunteer rice) during field trials. Initial findings also indicated that rice, corn, wheat, sorghum, okra, tomato, potato, soybean and mungbean were not infected when inoculated with 1 x 10^5 conidia ml^{-1} (Bayot *et al.*, 1994).

To assure the specificity and safety of using *A. alternata* f. sp. *sphenocleae*, the modified centrifugal phylogenetic and variety strategy (Wapshere, 1974), which has received wide acceptance in biological weed control research, was used to select the 49 plant species in 40 genera in 20 families for host range testing. In the absence or presence of dew for 14 to 15 h, only *S. zeylanica* was susceptible to *A. alternata* f. sp.

sphenocleae among the 49 plant species and crop varieties evaluated. Among the test plant species that showed highly resistant to moderately susceptible reactions with the application of *A. alternata* f. sp. *sphenocleae* were actually reported host plants for other *Alternaria* species except for *P. tetragonolobus* and *M. sapientum*. This is, therefore, the first report that winged bean and banana are infected with an *Alternaria* species. Rice is a known host of an *Alternaria* species and most of the rice cultivars that showed visible symptoms were not the modern varieties that are being currently cultivated. Moreover, initial field trials indicated that only *S. zeylanica* was susceptible to *A. alternata* f. sp. *sphenocleae* and the rest of the plant species evaluated were not infected (data not presented). *S. zeylanica* is the only test plant species that gave 100% LAD and an immediate reaction (12 h after removal from the dew chamber) to *A. alternata* f. sp. *sphenocleae*. LAD values from infected test plant species were very low (2 to 14%) and the appearance of symptoms ranged from one to six days after removal from the dew chamber. Sporulation was observed on excised leaves of *S. zeylanica* as early as 24 HAI and no sporulation occurred on any of the other test plant species demonstrating that these infected plant species were incompatible hosts for this fungal pathogen even under optimal conditions for disease development. The appearance of symptoms in several of the test species might have been due to other factors (e.g. wounds) which facilitated the penetration of the pathogen, especially given that many *Alternaria* species are considered secondary invaders or saprophytes.

From these experiments, it can be assumed, it can be assumed that *S. zeylanica* is the only host plant of *A. alternata* f. sp. *sphenocleae*. However, further host-range testing particularly of different *G. hirsutum* varieties should be conducted under field conditions using inoculum produced from sorghum seeds to confirm the restricted host range of *A. alternata* f. sp. *sphenocleae*.

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Table 6.1. Results of host-specificity screening for *Alternaria alternata* f. sp. *sphenocleae.*

Plant family	Test plant species	Common name	Cultivar[a]	Host reaction[b]	LAD (%)[c]	Latent period (days)[d]
A. Amaranthaceae	*Amaranthus spinosus* L. [(1) e]	spiny amaranth	——	I	0	N.A.[f]
B. Apiaceae	*Apium graveolens* L. [(1,2)]	celery	Tall Utah	I	0	N.A.
	Daucus carota L. [(1,2)]	carrot	Kuroda	I	0	N.A.
C. Araceae	*Colocasia esculenta* (L.) Schott. [(2)]	taro	——	I	0	N.A.
D. Asteraceae	*Lactuca sativa* L. [(1,2)]	lettuce	Denise Red	HR	2	3-5
E. Brassicaceae	*Brassica oleracea* L. var. *capitata* L. [(1,2)]	cabbage	Rareball	HR-MR	7	1-2

Table 6.1. Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
	B. pekinensis (Lour.) Rupr. [1,2]	pechay	Black Behi	I	0	N.A.
	Raphanus sativus L. [1,2]	radish	Ra-1	HR-MR	13	1-2
F. Bromeliaceae	*Ananas comosus* (L.) Merr. [1]	pineapple	(Not known)	I	0	N.A.
G. Caricaceae	*Carica papaya* L. [1]	papaya	——	I	0	N.A.
H. Convolvulaceae	*Ipomoea aquatica* Forssk. [1,2,3]	kangkong	——	I	0	N.A.
	I. batatas (L.) Lam. [1,2,3]	sweet potato	——	I	0	N.A.

Table 6.1. Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
I. Cucurbitaceae	*Cucurbita pepo* L. [1,2]	squash	A-193	I	0	N.A.
	Cucumis melo L. [1,2]	cantaloupe	Gulf Coast	I	0	N.A.
	C. sativus L. [1,2]	cucumber	UPLCu 6	I	0	N.A.
	Citrullus lanatus (Thunb.) Matsum. & Nakai [1,2]	watermelon	Sugar baby	I	0	N.A.
	Lagenaria leucantha (Lam.) Rusby. [2]	white-flowered gourd	1042-1-1 Long	I	0	N.A.
	Momordica charantia L. [2]	bitter gourd	Sta. Rita	I	0	N.A.

Table 6.1. Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
J. Cyperaceae	*Cyperus difformis* L. [2]	umbrella sedge	——	I	0	N.A.
	C. iria L. [2]	rice flatsedge	——	I	0	N.A.
	Fimbristylis miliacea (Lour.) Vahl. [2]	globe fingerush	——	I	0	N.A.
K. Dioscobeaceae	*Dioscorea alata* L. [2]	purple yam	(Not known)	I	0	N.A.
L. Euphorbiaceae	*Manihot esculenta* Crantz. [1]	cassava	(Not known)	I	0	N.A.
M. Fabaceaea	*Arachis hypogea* L. [1]	peanut	(Not known)	I	0	N.A.

Table 6.1. Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
	Glycine max (L.) Merr. [1,2]	soybean	PSBSy-2	HR	4	3-6
	Phaseolus vulgaris L. [1,2]	common bean	CES 18-6	HR	3	3-7
	Psophocarpus tetragonolobus (L.) DC. [2]	winged bean	Batangas medium	HR	5	3-5
	Sesbania sp. [1,2]	sesbania	——	I	0	N.A.
	Vigna radiata (L.) R. Wilcz. [1,2]	mungbean	Pag-asa 3	HR	6	3-6
	V. unguiculata (L.) Walp. [1,2]	stringbean	UPLPS 2	HR	3	3-6

Table 6.1. Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
N. Liliaceae	*Allium cepa* L. [1,2]	onion	(Not known)	I	0	N.A.
	A. sativum L. [1,2]	garlic	(Not known)	I	0	N.A.
O. Malvaceae	*Abelmoschus esculentus* (L.) Moench. [1,2]	okra	Smooth Green	HR-MR	5	2-3
	Gossypium hirsutum L. [1]	cotton	(Not known)	MR-MS	14	1-2
P. Musaceae	*Musa sapientum* L. [2]	banana	(Not known)	HR	3	4

Table 6.1. Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
			IR 26	HR	6	3-5
			IR 28	I	0	N.A.
			IR 29	I	0	N.A.
			IR 30	I	0	N.A.
			IR 32	HR	2	4-6
			IR 34	HR	6	3-5
			IR 36	HR	2	4-6
			IR 38	HR	2	4-6
			IR 40	HR	2	4-6
			IR 42	HR	3	4-6
			IR 43	HR	6	3-5

Table 6.1 Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
Q. Poaceae	*Echinochloa colona* (L.) Link. [1,2]	junglerice	——	I	0	N.A.
	E. crus-galli (L.) Beauv. [1,2]	barnyardgrass	——	I	0	N.A.
	E. glabrescens Munro ex Hook. f. [1,2]	————	——	I	0	N. A.
	Oryza sativa L. [1]	rice	IR 5	HR	3	4-6
			IR 8	I	0	N.A.
			IR 20	HR	6	3-5
			IR 22	HR	2	4-6
			IR 24	HR	2	4-6

Table 6.1. Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
			IR 44	HR	6	3-5
			IR 45	I	0	N.A.
			IR 46	HR	4	4-6
			IR 48	I	0	N.A.
			IR 50	HR	6	3-5
			IR 52	I	0	N.A.
			IR 54	I	0	N.A.
			IR 56	HR	2	4-6
			IR 58	I	0	N.A.
			IR 60	I	0	N.A.
			IR 64	I	0	N.A.

Table 6.1. Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
			IR 65	I	0	N.A.
			IR 66	I	0	N.A.
			IR 68	I	0	N.A.
			IR 70	I	0	N.A.
			IR 72	I	0	N.A.
			IR 74	I	0	N.A.
			PSBRC 1	I	0	N.A.
			PSBRC 2	I	0	N.A.
			PSBRC 4	HR	2	4-6

Table 6.1 Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
			PSBRC 10	HR	2	4-6
			PSBRC 20	HR	2	4-6
			Indica 7532	I	0	N.A.
			Japonica 7674	I	0	N.A.
			Javanica 8034	I	0	N.A.

Table 6.1. Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
	Saccharum officinarum L. [1]	sugarcane	(Not known)	I	0	N.A.
	Sorghum bicolor (L.) Moench [1]	sorghum	(Not known)	I	0	N.A.
	Triticum aestivum L. [1]	wheat	(Not known)	I	0	N.A.
	Zea mays L. [1]	corn	Super sweet	I	0	N.A.
R. Solanaceae	*Capsicum annuum* L. [1,2,3]	sweet pepper	California wonder	I	0	N.A.
	C. frutescens L. [1,2,3]	chili	(Not known)	I	0	N.A.

Table 6.1. Continued...

Plant family	Test plant species	Common name	Cultivar	Host reaction	LAD (%)	Latent period (days)
	Lycopersicon esculentum Miller [1,2,3]	tomato	KS Apollo	I	0	N.A.
	Solanum melongena L. [1,2,3]	eggplant	Claveria	I	0	N.A.
S. Sphenocleaceae	*Sphenoclea zeylanica* Gaertner [1]	gooseweed	——	S	100	0.5
T. Zingiberaceae	*Zingiber officinale* Roscoe [2]	ginger	——	I	0	N.A.

[a] Includes varieties and breeding lines.

[b] Disease severity rating until 14 days after inoculation (DAI) using 3.5×10^5 conidia ml^{-1} and based on a disease rating scale of I (immune) - no visible symptoms; HR (highly resistant) - very few, minute, necrotic flecks present; MR (moderately resistant) - a few small, necrotic lesions present; MS (moderately susceptible) - many necrotic lesions present

but no expansion of lesions to cause desiccation of leaves; and S (susceptible) - many necrotic lesions present which expand causing desiccation and leaf abscission.

[c] LAD = leaf area damage 14 DAI as an indication of disease severity.

[d] Number of days after inoculation when first disease symptoms appeared.

[e] (1) Plant species selected for host-range testing because it was reported to be infected by species of *Alternaria*.

(2) Plant species selected for host-range testing because it is commonly found in close proximity to irrigated rice fields in the Philippines.

(3) Plant species selected for host-range testing because it is taxonomically related to *Sphenoclea zeylanica*.

[f] Not applicable.

Chapter 7. General Conclusions

The research reported in this thesis investigated the possibility of utilizing an indigenous fungal pathogen, *Altenaria alternata* f. sp. *sphenocleae*, to control *Sphenoclea zeylanica* (gooseweed) in irrigated lowland rice systems so as to reduce the use of chemical inputs and provide a viable, economic, and effective weed control component for incorporation within integrated pest management programs, especially in major rice growing areas in Southeast Asia.

Inoculum density, dew period, and plant height are factors influencing biocontrol of *S. zeylanica* with this indigenous pathogen. Significantly higher percent reduction in plant height and dry weight were obtained and all plants were killed at higher inoculum concentrations (1×10^6 conidia ml^{-1}) with 8 h of dew.

The number, germination, and virulence of conidia were significantly affected by production techniques, temperature, light condition, and incubation period. Exposure to continuous near-ultraviolet (NUV) light at 28°C stimulated sporulation on agar media and on agricultural-based solid substrates. The most virulent conidia were produced on half-strength potato dextrose agar (1/2 PDA) and sorghum seeds four weeks after incubation. Overall, the best production technique was the use of sorghum seeds using an equal quantity of sorghum seeds and water (w/v). More than 1×10^5 conidia g $substrate^{-1}$ could still be extracted from colonized sorghum seeds stored up to 12 months at room conditions. Germination and virulence (expressed as dry weight reduction) of these conidia were more than 80% and 75%, respectively.

Conidia production using the sporulation medium (S-medium) technique was relatively rapid, but labour intensive. The addition of 20 g L^{-1} of calcium carbonate ($CaCO_3$) and 2 ml of sterile distilled water optimized conidia production on the S-medium. Primary 1/2 PDA at 18°C in the dark produced the most virulent conidia. Its use in developing countries may be limited however, because of the 18°C temperature requirement of this fungus for abundant sporulation.

Host range studies using 49 plant species in 40 genera representing 20 families, selected by using a modified centrifugal phylogenetic and variety strategy were

conducted in the absence or presence of supplemental dew. Most of the test plant species were immune while some test plant species displayed highly resistant to moderately susceptible reactions to *A. alternata* f.sp. *sphenocleae*, but these plants were incompatible hosts as no sporulation of the fungal pathogen occurred. *S. zeylanica* was the only plant susceptible to *A. alternata* f.sp. *sphenocleae*.

In light of the lack of previous reports of *A. alternata* on *S. zeylanica*, and in view of its pathogenicity, conidial morphology and cultural characteristics, and host specificity, the binomial *A. alternata* f. sp. *sphenocleae* is proposed for this fungal pathogen that causes leaf blight in *S. zeylanica*.

Chapter 8. Contributions to Knowledge

The following are considered to be key contributions to original knowledge arising from the research described in this thesis:

- The *Alternaria* species isolated from blighted *Sphenoclea zeylanica* has been named *A. alternata* f. sp. *sphenocleae* based upon the pathogenicity on the host, morphological characteristics, host specificity, and absence of records of an *Alternaria* species on *S. zeylanica*.

- The etiology of the disease caused by *A. alternata* f. sp. *sphenocleae* on *S. zeylanica* has been further characterized.

- The specific culture and conidia production requirements of *A. alternata* f. sp. *sphenocleae* on agar media, agricultural-based solid substrates, and a specific sporulation medium (S-medium) have been determined.

- The host range study with *A. alternata* f. sp. *sphenocleae* demonstrated that this fungal pathogen is virulent only on *S. zeylanica*.

Appendix 2.1. Analysis of variance for the influence of different agar media on radial mycelial growth of *Alternaria alternata* isolate from *Sphenoclea zeylanica.*

Source	d.f.	Mean square	F value	Significance level
Agar medium	5	1.78	30.48	0.0000
Error	10	0.06		

Appendix 2.2. Analysis of variance for the influence of agar media and light conditions on radial mycelial growth of *Alternaria alternata* isolate from *Sphenoclea zeylanica.*

Source	d.f.	Mean square	F value	Significance level
Agar medium (M)	5	7.07	126.71	0.0000
Light condition (L)	3	11.93	213.75	0.0000
M x L	15	1.16	20.84	0.0000
Error	30	0.06		

Appendix 2.3. Analysis of variance for the influence of agar media and temperature on radial mycelial growth of *Alternaria alternata* from *Sphenoclea zeylanica*.

Source	d.f.	Mean square	F value	Significance level
Agar medium (M)	3	12.50	267.41	0.0000
Temperature (T)	4	267.91	5732.67	0.0000
M x T	12	4.09	87.43	0.0000
Error	24	0.05		

Appendix 2.4a. Analysis of variance on percent dry weight reduction of *Sphenoclea zeylanica* (5 to 6-cm-tall plants) inoculated with different *Alternaria* isolates.

Source	d.f.	Mean square	F value	Significance level
Alternaria isolates	6	4123.78	10730.67	0.0000
Error	12	0.38		

Appendix 2.4b. Analysis of variance on percent dry weight reduction of *Sphenoclea zeylanica* (19 to 20-cm-tall plants) inoculated with different *Alternaria* isolates.

Source	d.f.	Mean square	F value	Significance level
Agar medium	5	3967.36	13730.76	0.0000
Error	10	0.29		

Appendix 3.1. Analysis of variance for the influence of inoculum density and plant height on percent plant height reduction of *Sphenoclea zeylanica* inoculated with *Alternaria alternata* f. sp. *sphenocleae*.

Source	d.f.	Mean square	F value	Significance level
Inoculum density (I)	4	218.28	21.50	0.0001
Plant height (P)	2	1546.36	152.29	0.0000
I x P	8	114.06	11.23	0.0010
Error	16	10.15		

Appendix 3.2. Analysis of variance for the influence of inoculum density and plant height on percent mortality of *Sphenoclea zeylanica* inoculated with *Alternaria alternata* f. sp. *sphenocleae*.

Source	d.f.	Mean square	F value	Significance level
Inoculum density (I)	3	29499.66	707.96	0.0001
Plant height (P)	4	377.33	9.06	0.0000
I x P	12	136.58	3.28	0.0100
Error	24	41.67		

Appendix 3.3. Analysis of variance for the influence of inoculum density and plant height on percent dry weight reduction of *Sphenoclea zeylanica* inoculated with *Alternaria alternata* f. sp. *sphenocleae.*

Source	d.f.	Mean square	F value	Significance level
Inoculum density (I)	2	8500.51	1049.10	0.0000
Plant height (P)	4	2913.85	359.62	0.0000
I x P	8	531.41	65.58	0.0001
Error	16	8.10		

Appendix 3.4. Analysis of variance for the influence of inoculum density and dew period on percent mortality of *Sphenoclea zeylanica* inoculated with *Alternaria alternata* f. sp. *sphenocleae.*

Source	d.f.	Mean square	F value	Significance level
Dew period (D)	3	8155.58	149.23	0.0000
Inoculum density (I)	3	17754.84	324.87	0.0000
D x I	9	1113.62	20.38	0.0001

Error	18	54.65		

Appendix 3.5. Analysis of variance for the influence of inoculum density and dew period on percent dry weight reduction of *Sphenoclea zeylanica* inoculated with *Alternaria alternata* f. sp. *sphenocleae.*

Source	d.f.	Mean square	F value	Significance level
Dew period (D)	3	3906.60	153.99	0.0000
Inoculum density (I)	2	7212.30	284.30	0.0000
D x I	6	233.52	9.21	0.0010
Error	12	25.37		

Appendix 4.1. Analysis of variance for the influence of agar medium, light condition, and temperature on conidia production of *Alternaria alternata* f. sp. *sphenocleae.*

Source	d.f.	Mean square	F value	Significance level
Medium (M)	5	1.18×10^{11}	348.91	0.0000
Light condition (L)	2	2.24×10^{11}	665.25	0.0000
Temperature (T)	4	2.42×10^{11}	714.86	0.0000
M x L	10	1.10×10^{11}	325.39	0.0000
M x T	20	1.13×10^{11}	333.28	0.0000
L x T	8	2.19×10^{11}	647.33	0.0000
M x L x T	40	1.09×10^{11}	321.38	0.0000
Error	80	3.38×10^{8}		

Appendix 4.2. Analysis of variance for the influence of agar medium and light condition on conidia production of *Alternaria alternata* f. sp. *sphenocleae*.

Source	d.f.	Mean square	F value	Significance level
Medium (M)	5	9.61×10^{11}	694.89	0.0000
Light condition (L)	3	1.05×10^{12}	761.67	0.0000
M x L	15	8.33×10^{11}	602.20	0.0000
Error	30	1.38×10^{9}		

Appendix 4.3. Analysis of variance for the influence of various combinations of light conditions and incubation period on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on 1/2 PDA and VJA at 28°C.

Source	d.f.	Mean square	F value	Significance level
Medium (M)	1	3.12×10^{11}	59.42	0.0000
Light condition (L)	15	1.62×10^{12}	307.39	0.0000
Incubation period (I)	1	4.20×10^{10}	8.00	0.0000
M x L	15	3.34×10^{11}	63.53	0.0000
M x I	1	1.09×10^{10}	2.08	0.1599
L x I	15	8.10×10^{9}	1.54	0.1522
M x L x I	15	3.66×10^{9}	0.70	0.7684
Error	30	5.26×10^{9}		

Appendix 4.4. Analysis of variance for the influence of various combinations of light conditions and incubation period on virulence of *Alternaria alternata* f. sp. *sphenocleae* conidia grown on 1/2 PDA and VJA at 28°C.

Source	d.f.	Mean square	F value	Significance level
Medium (M)	1	5631.89	7308.15	0.0000
Light condition (L)	8	351.17	455.70	0.0000
Incubation period (I)	1	69.99	90.82	0.0000
M x L	8	90.38	117.28	0.0000
M x I	1	0.0009	0.001	0.9728
L x I	8	1.24	1.61	0.1975
M x L x I	8	1.75	2.27	0.0777
Error	16	0.77		

Appendix 4.5. Analysis of variance for the influence of various agricultural-based solid substrates and temperature on conidia production of *Alternaria alternata* f. sp. *sphenocleae* incubated under continuous light.

Source	d.f.	Mean square	F value	Significance level
Solid substrate (S)	19	8.31×10^{9}	170.05	0.0000
Temperature (T)	2	3.33×10^{10}	680.83	0.0000
S x T	38	3.93×10^{9}	80.38	0.0000
Error	76	4.89×10^{7}		

Appendix 4.6. Analysis of variance for the influence of various agricultural-based solid substrates and light condition on conidia production of *Alternaria alternata* f. sp. *sphenocleae* incubated at 28°C.

Source	d.f.	Mean square	F value	Significance level
Solid substrate (S)	19	2.15×10^{11}	267.16	0.0000
Light condition (L)	1	6.21×10^{11}	771.12	0.0000
S x L	19	1.05×10^{11}	130.27	0.0000
Error	38	8.06×10^{8}		

Appendix 4.7. Analysis of variance for the influence of incubation period, light condition, and temperature on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on sorghum seeds.

Source	d.f.	Mean square	F value	Significance level
Incubation period (I)	3	3.74×10^{10}	660.05	0.0000
Light condition (L)	2	1.52×10^{10}	268.88	0.0000
Temperature (T)	2	7.47×10^{10}	1320.08	0.0000
I x L	6	4.08×10^{9}	72.11	0.0000
I x T	6	2.22×10^{10}	391.42	0.0000
L x T	4	1.19×10^{10}	211.00	0.0000
I x L x T	12	3.49×10^{9}	61.66	0.0000
Error	24	5.66×10^{7}		

Appendix 4.8. Analysis of variance for the influence of light condition, incubation period, and temperature on virulence of *Alternaria alternata* f. sp. *sphenocleae* conidia grown on sorghum seeds.

Source	d.f.	Mean square	F value	Significance level
Light condition (L)	2	609.26	884.70	0.0000
Incubation period (I)	1	689.85	1001.72	0.0000
Temperature (T)	1	8541.15	12402.44	0.0000
L x I	2	66.78	96.97	0.0004
L x T	2	203.03	294.81	0.0000
I x T	1	8.79	12.77	0.0233
L x I x T	2	78.70	114.27	0.0003
Error	4	0.69		

Appendix 4.9. Analysis of variance for the influence of moisture content and quantity of sorghum seeds on conidia production of *Alternaria alternata* f. sp. *sphenocleae* incubated under continuous light at 28°C.

Source	d.f.	Mean square	F value	Significance level
Weight of sorghum (W)	2	2.40×10^{9}	42.48	0.0001
Volume of water (V)	2	1.50×10^{10}	264.78	0.0000
W x V	4	1.06×10^{7}	188.41	0.0000
Error	8	5.65×10^{7}		

Appendix 4.10A. Analysis of variance for the influence of storage period on the number of *Alternaria alternata* f. sp. *sphenocleae* conidia grown on sorghum seeds incubated under continuous light at 28°C.

Source	d.f.	Mean square	F value	Significance level
Storage period	5	1.76	64.49	0.0000
Error	10	2.72		

Appendix 4.10B. Analysis of variance for the influence of storage period on germination of *Alternaria alternata* f. sp. *sphenocleae* conidia grown on sorghum seeds incubated under continuous light at 28°C.

Source	d.f.	Mean square	F value	Significance level
Storage period	5	58.59	77.54	0.0000
Error	10	0.76		

Appendix 4.10C. Analysis of variance for the influence of storage period on virulence of *Alternaria alternata* f. sp. *sphenocleae* conidia grown on sorghum seeds incubated under continuous light at 28°C.

Source	d.f.	Mean square	F value	Significance level
Storage period	5	179.92	108.66	0.0000
Error	10	1.66		

Appendix 4.11A. Analysis of variance for the influence of production methods on germination of *Alternaria alternata* f. sp. *sphenocleae* conidia.

Source	d.f.	Mean square	F value	Significance level
Production method	2	2.58	3.26	0.1444
Error	4	0.79		

Appendix 4.11B. Analysis of variance for the influence of production methods on virulence of *Alternaria alternata* f. sp. *sphenocleae* conidia.

Source	d.f.	Mean square	F value	Significance level
Production method	2	774.08	1161.13	0.0000
Error	4	0.67		

Appendix 5.1. Analysis of variance for the influence of various primary agar media and sequential harvesting on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on S-medium.

Source	d.f.	Mean square	F value	Significance level
Medium (M)	7	8.22×10^{12}	982.12	0.0000
Harvest time (H)	4	3.87×10^{13}	4625.92	0.0000
M x H	28	2.15×10^{12}	256.59	0.0000
Error	56	8.37×10^{9}		

Appendix 5.2. Analysis of variance for the influence of various primary agar media and temperature on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on S-medium.

Source	d.f.	Mean square	F value	Significance level
Medium (M)	7	1.27×10^{12}	259.86	0.0000
Temperature (T)	2	1.24×10^{13}	2529.25	0.0000
M x T	14	1.16×10^{12}	237.00	0.0000
Error	28	4.89×10^{9}		

Appendix 5.3. Analysis of variance for the influence of various primary agar media and light condition on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on S-medium.

Source	d.f.	Mean square	F value	Significance level
Medium (M)	7	1.20×10^{12}	243.57	0.0000
Light condition (L)	2	1.28×10^{13}	2612.58	0.0000
M x L	14	1.19×10^{12}	243.19	0.0000
Error	28	4.91×10^{9}		

Appendix 5.4. Analysis of variance for the influence of various primary agar media on virulence of *Alternaria alternata* f. sp. *sphenocleae* conidia grown on S-medium incubated at 18°C in the dark.

Source	d.f.	Mean square	F value	Significance level
Medium	6	890.94	203.00	0.0000
Error	12	4.39		

Appendix 5.5A. Analysis of variance for the influence of $CaCO_3$ concentration on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on S-medium incubated at 18°C in the dark .

Source	d.f.	Mean square	F value	Significance level
Amount of $CaCO_3$	4	5.11×10^{12}	127.60	0.0000
Error	8	4.00×10^{10}		

Appendix 5.5B. Analysis of variance for the influence of $CaCO_3$ concentration on virulence of *Alternaria alternata* f. sp. *sphenocleae* conidia grown on S-medium .

Source	d.f.	Mean square	F value	Significance level
Amount of $CaCO_3$	4	337.57	24.79	0.0001
Error	8	13.62		

Appendix 5.6. Analysis of variance for the influence of water volume on conidia production of *Alternaria alternata* f. sp. *sphenocleae* grown on S-medium incubated at 18°C in the dark.

Source	d.f.	Mean square	F value	Significance level
Water volume	3	3.55×10^{13}	16863.30	0.0000
Error	6	2.10×10^{9}		

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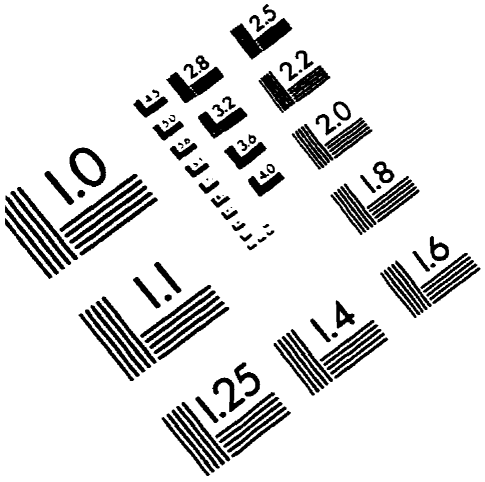

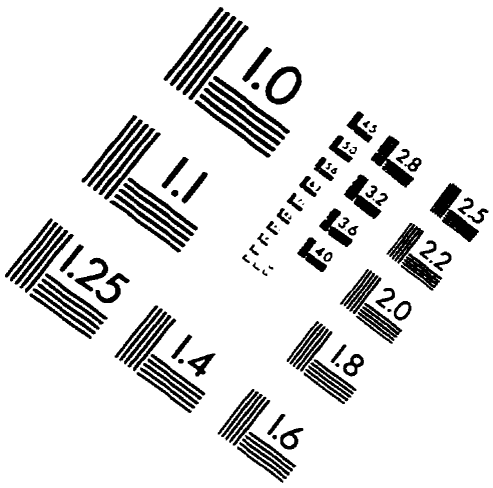

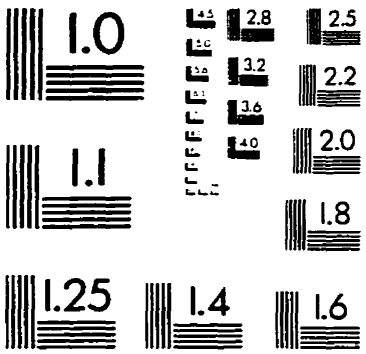

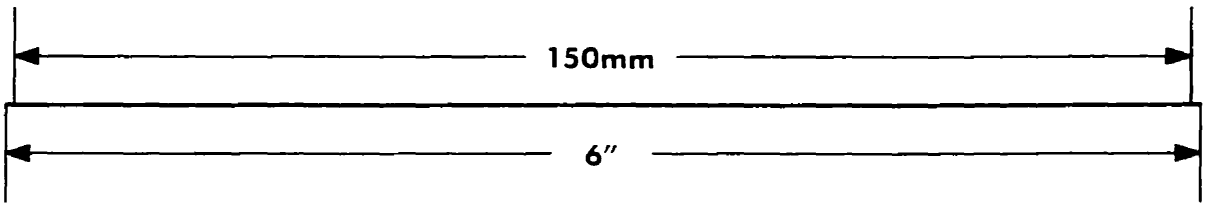

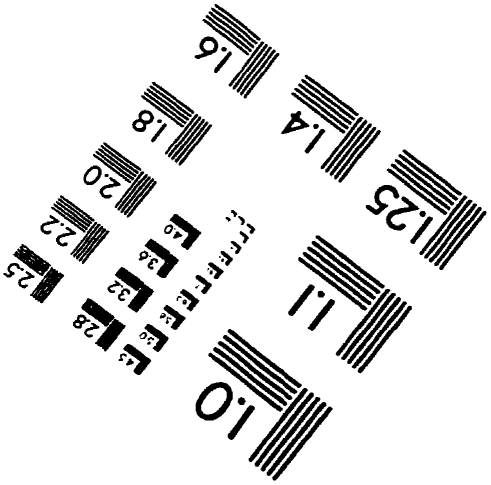

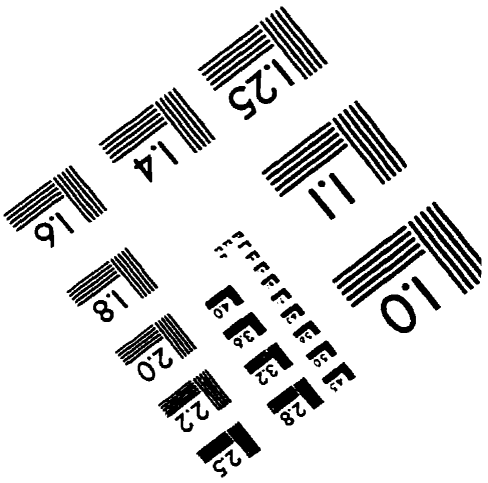

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