THE USE OF *GLOMUS INTRARADICES* AND VESICULAR-ARBUSCULAR MYCORRHIZAE FOR ASSESSING THE ENVIRONMENTAL IMPACT OF AZADIRACHTIN

by

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in the Department
of
Biological Sciences

SIMON FRASER UNIVERSITY
June, 1998

ABSTRACT

This research evaluates the eco-toxicological potential of azadirachtin (AZA) and reference pesticides on non-target, subterranean, terrestrial micro-organisms. *Glomus intraradices*, a vesicular-arbuscular (VA) mycorrhizal fungus, and its symbiotic association with roots of Ri T-DNA transformed *Daucus carota* (carrot) in minimal medium (M-medium) and of *Allium porrum* (leek) in vermiculite, were used as non-target biological indicators. Using the M-medium technology, a novel, reliable, reproducible, cost-effective yet simple pesticide toxicity bioassay technique on *G. intraradices* was successfully developed in which the extraradical mycelial growth and extraradical mycelial sporulation of this VA mycorrhizal fungus were used as specific response indicators. After validating that AZA and its formulated products were slightly to moderately persistent in the M-medium and vermiculite, their IC50 (concentration inhibiting 50% growth and development) values, including those of reference pesticides, were estimated by either non-linear regression or linear interpolation statistical analyses. Based on the IC50 values, it was possible, for the first time, to predict that the eco-toxicological potential of AZA, neem extracts and products (Eneem 3G, Neemix 4.5E) to *G. intraradices* and VA mycorrhizal symbiosis in Ri T-DNA transformed *D. carota* roots was negligible when compared with reference pesticides benomyl, chlorothalonil, copper sulphate, dimethoate, and glyphosate and its transformation product, aminomethylphosphonic acid.

To address concerns that the IC50 method was an estimate of effects from an artificial test system, *A. porrum* grown under field-like conditions was used as a VA mycorrhizal-responsive plant in which its growth, biomass, leaf phosphorus (P) status over time, and root colonization by *G. intraradices* were monitored to get an

indication of VA mycorrhizal symbiosis. By comparing these parameters of *A. porrum* grown in AZA, Eneem 3G and reference pesticide treated with those from untreated vermiculites, it was found that there was a positive correlation between the IC50 multiples (field concentration ÷ IC50 values) of these test materials versus their corresponding adverse effects on VA mycorrhizae in the growth substrate. Field concentrations of prescribed application rates of AZA, Eneem 3G, including benomyl and chlorothalonil, had no adverse impact on VA mycorrhizal symbiosis, except when they were introduced at rates used for sterilizing soils or equivalent to accidental spills. Both VA mycorrhizal fungus inoculated and VA mycorrhizal fungus free vermiculite appeared to have a varying capacity to "mitigate" the adverse effects of AZA and reference pesticides on VA mycorrhizal symbiosis. Many factors likely contributed to this "mitigating effect", e.g., pesticide mode of action as well as chemical and biological activities occurring in the growth substrate. Using the IC50 value to *G. intraradices* and the estimated highest no-effect test field concentration of a pesticide on VA mycorrhizal symbiosis as a gauge, it was possible to calculate a "safety factor" to predict the safe use of a pesticide in the terrestrial environment. The application and future consideration of this approach of predicting the safe use of pesticides to enhance the value of eco-toxicological risk evaluation are discussed.

Footnote: *This thesis contains 5 papers. Two papers were published, two were "in press", and one was being reviewed by a journal at the time of thesis submission. These papers were compiled and reformatted according to the regulations/guidelines prescribed by SFU Library. Contributions by co-authors were advisory/collaborative in nature as well as financial/technical support.*

RÉSUMÉ

Cette recherche étudie le potential éco-toxicologique de l'azadirachtine (AZA) et de pesticides de référence sur des micro-organismes terrestres aouterrains non-cibles. *Glomus intraradices*, un champignon format des mycorhizes vésicularies-arbusculaires (VA), et son association symbiotique avec les racines de *Daucus carota* (carrotte) transformées par insertion de l'ADN-T dugéne Ri dans un milieu minimal (milieu-M) et de *Allium porrum* (poireau) dans la vermiculite, furent utilisés comme indicateurs bilogiques non-cibles. En utilisant la technologie au milieu-M, une technique de bio-essai inédite, fiable, reproductible, rentable et pourtant simple, permettant de tester la toxicité des pesticides sur *G. intraradices*, fut mise au point avec succès. Dans cette technique, la croissance mycélienne extra-radicle et al sporulation mycélienne extra-radicle de ce champignon mycorrhizien VA furent utilisées comme indicateurs de réponse spécifiques. Après avoir confirmé que AZA et les produits synthétisés à partir de lui étaint légèment à modérément persistants dans le milieu-M et dans la vermiculite, leurs valeurs de IC50 (la concentration qui inhibe 50% de la croissance et du développement), ainsi que celles des pesticides de référence, durent estimées soit par régression non-linéaire soit par des analyses statistiques d'interpolation linéaire. Sur la base des valeurs de IC50, il fut possible, pour la premiére fois, de prédire que le potential éco-toxicologique de l'AZA, des extraits de neem et de ses produits (Eneem 3G, Neemix 4.5E) sur *G. intraradices* et sur la symbiose mycorhizienne VA dans les racines de D. carota transformées par insertion del'ADN-T de gène Ri était négligible comparé à celui des pesticides de référence que sont de bénomyl, le chlorothalonil, le sulfate de cuivre, le diméthoate, le

glyphosate et al produit transformation de ce dernier, l'acide aminomethyl-phosphonique.

On peut objecter que la méthode utilisant l'IC50 ne fait qu'estimer les effets d'un systéms d'essai artificiel. Afin d'aborder ce probléme, *A. porrum*, cultivé dans des conditionssemblables aux conditions rencontrées dans les champes, fut utilisé comme plante sensible aux mycorhizes et sa croissance, sa biomasse, le statut du phosphore (P) dans ses feuiles au fil du temps et la colonisation de ses racines par *G. intraradices* furent suivis afin d'observer des signes de symbiose mycorhizienne VA. En comparent ces paramétres pour *A. porrum* cultivé dans AZA, Eneem 3G et traité avec les pesticides de référence, avec ceux de vermiculites non traitées, il fut démontré qu'ily avait une corrélation positive entre les multiples de IC50 (concentration dans le terrain ÷ valeur le l'IC50) de ces substances d'essai et leurs effets négatifs correspondants sur les mycorrhizes VA dans le substrat de croissance. Les concentrations dans le terrain, pour les taux d'application recommandés de AZA, Eneem 3G, ainsi que pour le bénomyl, et le chlorothalonil, n'eurent pas d'impact négatif sue la symbiose mycorhizinne VA, sauf lorsqu'ils étaient introduits à des taux utilisé pour la stérilization de sols ou équivalents à des répandements accicentels. Les vermiculite inculées avec des champignons à mycorhize VA comme les vermiculiyes dépourvues de champignon à mycorhize VA semblémeent avoir une capacité variable à "mitiger" les effets négatifs de AZA et des pesticides de référence sur la symbiose mycorhizienne VA. Il est probale que beaucoup de facteurs aient contribué à cet effet "mitigateur", per exemple le mode d'action du pesticide, ainsi que les activités chimiques et biologiques qui ont lieu dans le substrat de croissance. En utilisant la valeur de l'IC50 sur *G. intraradices* et

la plus haute valeur estimée comme étant sans effet, simulant la concentration sur le terrain d'un pesticide sur la symbiose mycorhizienne VA, il fut possible de calculer un "facteur de sécurite" permettant de prédire l'utilisation sans danger du pesticide dans un environnement terrestre. La mise en application st al prise en considération future de cette approche, dans laquelle on prédit l'utilisation sans danger des pesticides pour mettre en valeur l'evaluation des risque éco-toxicologiques, sont discutées.

Note de bas de page: *Cette thése comporte 5 articles. Deux articles furent publiés, deux furent mis "sous presse", et un était en cours de révision par un journal spécialisé lors de la soumission de la thése. Ces articles furent élaborés et reims en forme conformément aux réglements et directives prescrits par la Bibliothéque de SFU. Les contributions des co-auteurs furent apportées sous forme de conseils et collaboration, ainsi que sous la forme de soutien financier/technique.*

ACKNOWLEDGEMENTS

My most profound appreciation and gratitude go to Prof. James E. Rahe of Simon Fraser University for accepting me as his graduate student, and for his encouragement, direction, patience, and strong support at all stages of this project. I also like to thank members of my supervisory committee, Drs. R.A. Nicholson and J.M. Webster, for their guidance and advice. I am grateful to Dr. V. Niemela, Dr. L. Churchland, Mr. B. Kelso, Mr. G. Derksen, and Mr. A. Fabro of Environment Canada, North Vancouver, and Mme. C. Kriz of Pest Management Regulatory Agency, Health Canada, Ottawa, for their support on this project. As well, this research would not have been completed without the co-operation and technical support of staff members of the Pacific Environmental Science Centre of Environment Canada, notably Mr. P. Kluckner, Mr. R. Watts, Mme. C. Wong, Mr. H. Nelson, Mr. B. Holmes, Mr. H. Quon, Mr. R. Englar, and Mr. R. Strub.

I am also indebted to the following scientists for providing me assistance and advice on their expertise: Dr. J.A. Fortin and Mme. L. Nantais (mycorrhizal technology), de l'Université de Montréal, Montreal, QC; Dr. C. Hamel (mycorrhizal technology), McGill University, Montreal, QC; Dr. Y. Dalpé (mycorrhizal technology), Central Experimental Farm, Agriculture & Agri-Food Canada Research Centre, Ottawa, ON; Dr. S. Szeto (residue analytical chemistry), Agriculture & Agri-Food Canada Research Station, Vancouver, BC; Dr. M.B. Isman (azadirachtin technology), University of British Columbia, BC; Mr. R. Balshaw (Statistical Consultant), Simon Fraser University, Burnaby, BC; Dr. M. Habte (plant phosphorus analytical technology) University of Hawaii, Honolulu, HI, USA; and Mme. J. Stroh (Statistician), Environment Canada, North Vancouver, BC.

I reserve my deepest gratitude for Mary, my wife, for her encouragement, patience, financial support, and general technical assistance. I also appreciate the help of Andrew, my son, in the operation of various computer programmes, and the morale support of Esther and Ethel, my daughters.

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GENERAL INTRODUCTION

The delivery of preventative and remedial measures necessary to protect and manage the environment from the adverse impacts of pesticides requires the effective use of a selected battery of well-defined toxicity tests. There are a number of such tests now available for testing indicator organisms of various trophic levels (Environment Canada, 1996; U.S. EPA, 1996; Table 1). To date, however, no standard method is available for assessing pesticide impacts on non-target symbiotic fungi, e.g., vesicular-arbuscular (VA) mycorrhizal fungi and their associations with plant roots (Trappe et al., 1984). These beneficial micro-organisms are associated with the roots of more than 90% of vascular plants (Allen, 1991; Giovannetti and Gianinazzi-Pearson, 1994). With the formulation of slow release soil insecticides from new active ingredients such as azadirachtin (AZA) and other bio-pesticides, there is now an urgent need to develop effective, sensitive, and standardized methods to examine more closely the impacts such compounds may have on this very important but often neglected group of micro-organisms and their symbiotic association with plants to better manage the environment and to promote eco-sustainability.

Vesicular-arbuscular mycorrhiza

Vesicular-arbuscular mycorrhiza (VAM) is a mutually beneficial symbiotic association between a fungus and the roots of a higher plant (Schenck, 1981; Harley and Smith, 1983; Fig. 1). VA mycorrhizal fungi belonging to the

Table 1. An example eco-risk assessment scheme showing indicator organisms of different trophic levels, associated measurement endpoints, and where VA mycorrhizal symbiotic fungi would likely fit

Indicator organisms	Measurement endpoints
Aquatic plants	7-d IC50[a] (green algae)
Aquatic invertebrates	48-h LC50[b] (water fleas)
Fish	96-h LC50 (Rainbow trout)
Amphibians	48-h LC50 (frogs)
Soil bacteria	28-d EGP[c] (nitrogen-fixing bacteria)
Soil fungi	*14-d IC50 (symbiotic fungi)*
Soil invertebrates	14-d LC50 (earthworms)
Terrestrial plants	21-d growth reduction (green beans)
Birds	8-d oral LD50[d] (Mallard duck)
Mammals	7-d oral LD50 (rats)

[a] - IC50 = inhibition concentration of test material to 50% growth

[b] - LC50 = lethal concentration of test material to 50% population

[c] - EGP = ethylene gas production of control versus treated soils

[d] - LD50 = lethal dose of test material to 50% population

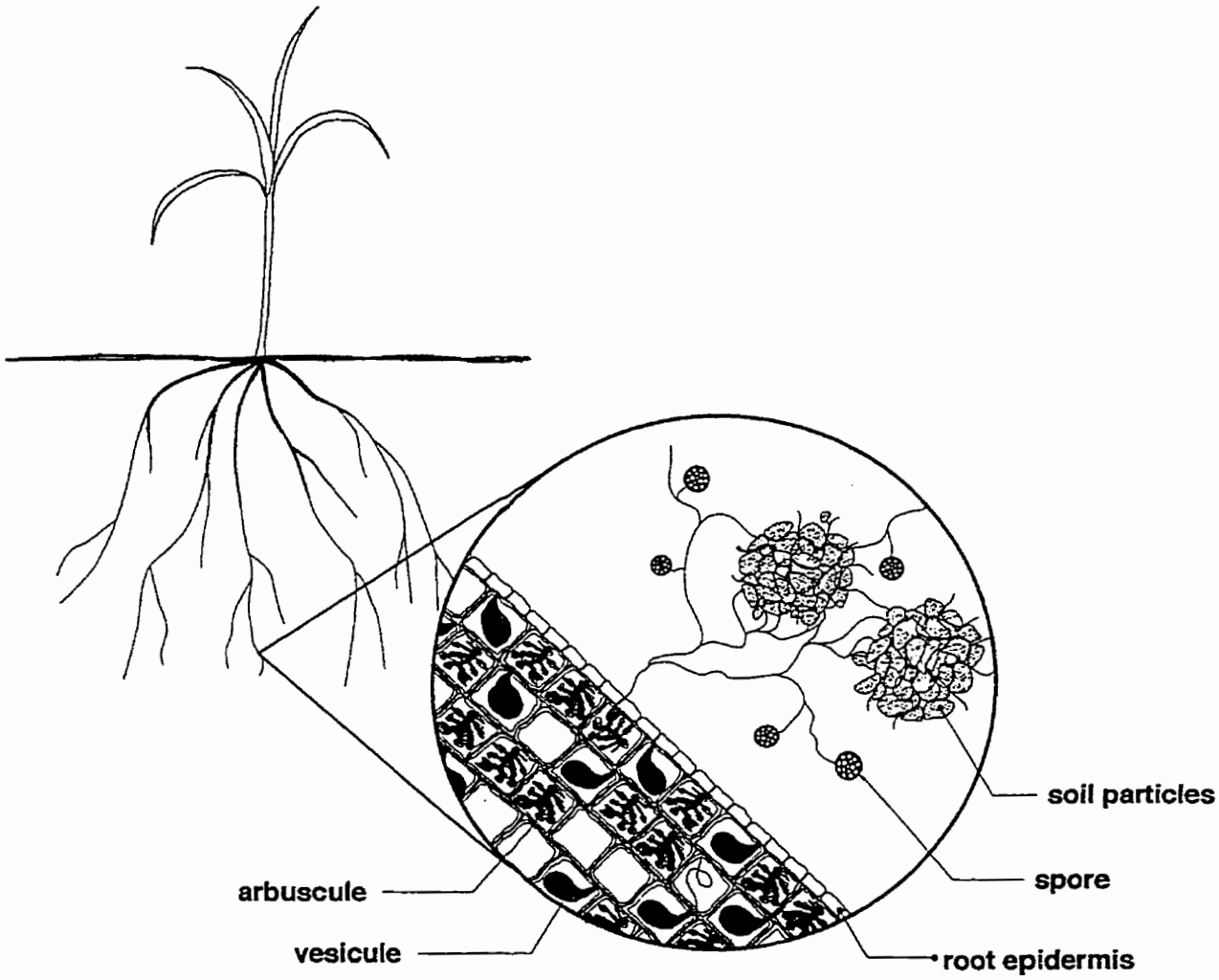


Figure 1. Diagrammatic sketch of vesicular-arbuscular mycorrhizae and host association (Bethlenfalvay and Linderman, 1992).

Class Zygomycetes (Dalpe, 1993). They are obligate biotrophs and do not grow on synthetic media. There are at least 120 species, grouped into six genera and three families: Acaulosporaceae (*Acaulospora, Enthrophospora*), Gigasporaceae (*Gigaspora, Scutellospora*) and Glomaceae (*Glomus, Sclerocystis*) (Schenck and Perez, 1988; Morton and Benny, 1990; Bagyaraj, 1991).

VA mycorrhizal fungi are geographically cosmopolitan and ubiquitous, occurring over a broad ecological range in plants growing in the arctic, temperate, and tropical regions (Mosse et al., 1981). They have the widest host range and distribution of all the mycorrhizal associations (Harley, 1969). Examples of some common temperate/tropical crops associated with VA mycorrhizal fungi are: apples, asparagus, banana, beans, blueberries, cereals, cocoa, coffee, conifers, corn, grapes, legumes, millet, oranges, peanuts, pepper, potatoes, rubber, soybeans, strawberries, sugar cane, tea, tomatoes, tobacco, various grasses (Bagyaraj, 1991).

The presence of VAM in the roots of host plants has been linked to the maintenance and enhancement of plant growth (Abbott and Robson, 1984) attributable to increased nutrient capture (Frey and Schuepp, 1993), uptake (Marschner and Dell, 1994) and transport (Smith et al., 1994); improved drought (Nelson, 1987), disease (Dehne, 1982; Jalali and Jalali, 1991) and cold temperature (Charest et al., 1993) tolerance; and positive synergistic interactions with other soil micro-organisms (Powell and Bagyaraj, 1984; Jawson et al., 1993; Fitter and Garbaye, 1994). As well, VA mycorrhizal fungi play a role in promoting

sustainable agriculture (Linderman, 1992; Miller and Jastrow, 1992; Hamel, 1996) and eco-sustainability (Allen, 1991). Accordingly, it is vital that the impact of soil pesticides, particularly slow release formulations, on these micro-organisms and their mycorrhizal association be evaluated in the overall conservation, protection, and management of the environment.

A more in depth review is provided in Chapters 2 (page 39) on VA mycorrhizal fungi and the rationale of using a representative of this group as an indicator organism for pesticide bioassay. *Glomus intraradices* was selected over the other species (e.g., *G. moseae, G. aggregatum, Gigaspora margarita*, etc) for this research project at the recommendation of Dr. A. J. Fortin (Director, Mycological Research, University of Montreal, Montreal, Quebec) and Dr. Y. Dalpé (Research Mycologist, Central Experimental Farm, Agriculture Canada, Ottawa, Ontario). Both scientists indicated the availability of this VA mycorrhizal fungus in Ri T-DNA transformed carrot root cultures and its propagules (fungal hyphae, spores, mycorrhized roots) for pot cultural system at their respective institutions. More importantly, they agreed to assist and provide the technological know how relating to the culturing of *G. intraradices* if it were selected for this research project. In addition, high quality (quality control certified by Dr. Y. Dalpé) and viable propagules of *G. intraradices* for pot experiments were commercially available from Premer Tech Research Incorporate, Riviére-du-Loop, Quebec.

The taxonomic position (Morton and Benny, 1990; Dalpé, 1993) of *G. intraradices* is as follows:

Kingdom: *Zygomycetes*

Order: *Glomales*

Family: *Glomaceae*

Genus: Glomus

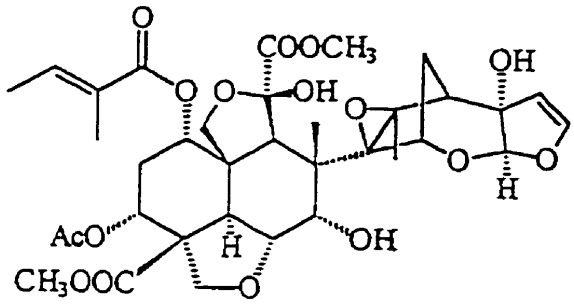
Species: *intraradices* (Schenck & Smith, 1982)

Strain: DAOM *181602* (Department Agriculture Ottawa Mycology)

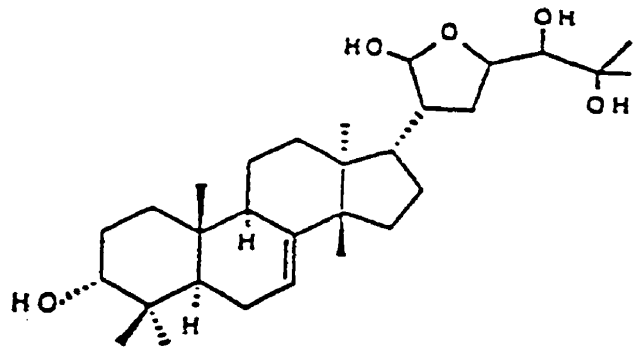
Azadirachtin

There is now considerable interest in the search for and development of new classes of pesticides that are less damaging to the environment and non-target organisms (Rembold, 1990; Schmutterer 1990; Ascher, 1993). One candidate ingredient having the potential to replace many synthetic chemicals for the control of subterranean insect pests is azadirachtin (AZA), a naturally occurring phyto-chemical that is obtained from the neem tree *Azadirachta indica* A. Juss (Koul et al., 1989; Saxena, 1990; Lee et al., 1991).

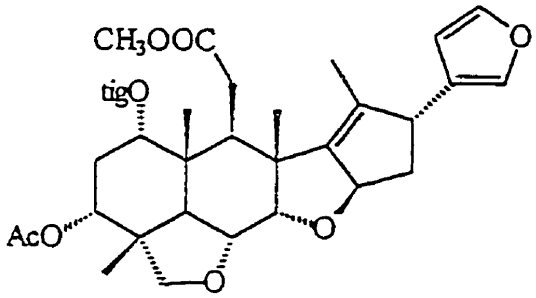
AZA (Fig. 2) is derived from neem kernels, which contain about 45% by weight of neem oil (Jones et al., 1988; Isman, 1997). Solvent extraction of neem seed cake (the residues remaining after oil expulsion by cold pressing of seeds) produces neem extract (NEX). NEX contains AZA and a mixture of more than 30 other neem limonoid compounds, e.g., meliantriol, nimbin, nimbidin, salannin and others (Fig. 2). These compounds occur in varying quantities but generally < 0.05%, except for AZA (Jones et al., 1988). AZA content in NEX can range from <



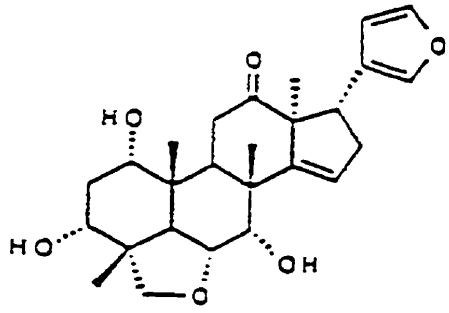
Azadirachtin



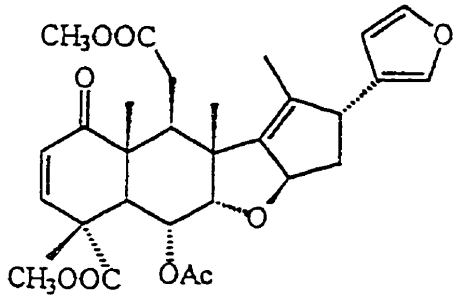
Meliantriol



Salannin



Nimbidin



Nimbin

Figure 2. Neem seed compounds with bioactivity (National Research Council, 1992; Schmutterer, 1995)

0.05% to > 4% (Isman et al., 1990a; Tewari, 1992), depending on the genome of the neem tree from which the seeds are collected. Of the AZA fraction, AZA-A (Fig. 2) is the dominant compound, averaging about 79% in most samples (Hansen et al., 1994), while the remaining fraction consists of about 20% of AZA-B, and 1% of the so-called "positional isomers" (ie., AZA-C, -D, -E, -F, -G, -H, -I, and the 22, 23-dihydro-23-a-, -b-, and -methoxy-AZA). AZA-A, AZA-B and isomers are collectively referred to as AZA, as they have the same basic molecular structure and similar bioactivity.

Presently, synthetic AZA is not available (Ley et al., 1993; Grossman and Ley, 1994). The formulation of commercial preparations of neem-based products for sale as insecticides now depends exclusively on the use of natural NEX. Proprietary solvent extraction methods can enrich AZA to a purity of > 90-95%, but at great expense. AZA (purity 92%) will be used for this research study. AZA may be applied to the soil as a water miscible formulation by drenching, or a granular slow release formulation by soil incorporation.

Unlike synthetic insecticides, which are mostly general contact neuro-toxins, AZA is very selective. It affects the endocrine system (feeding, development, reproduction and the general metabolism) of insects (Mordue and Blackwell, 1993). When compared with some of the common synthetic carbamate and organo-phosphorus insecticides, AZA is not very toxic to aquatic invertebrates (Scott, 1993) and fish (Wan et al., 1996). It is claimed that since it is unstable in UV light, it degrades rapidly when exposed to sunlight.

Although most of the research work on AZA is focused on its effects to insects (Isman et al, 1990b; Ascher, 1993; Mordue and Blackwell, 1993), there is evidence indicating that it also has amoebicidal, bactericidal, fungicidal, nematicidal, and piscicidal properties (Shetty et al., 1989; Tewari, 1992; and NRC, 1992). This research focussed on the neglected fungicidal characteristic of AZA.

Research objectives

To date, no studies have been conducted to evaluate the impact of AZA on non-target organisms such as VA mycorrhizal fungi or VAM in plants (AGRICOLA, 1998; BIOSIS, 1998; CAB, 1998; CA SEARCH, 1998; CRIS/USDA, 1998).

Accordingly, the primary aim of this research program is to investigate the direct and indirect effects of AZA on a representative VA mycorrhizal fungus, *Glomus intraradices,* and its VA mycorrhizal activity in two biological systems. These systems are: (1) the minimal medium (M-medium) system (Fig. 3) using Ri T-DNA transformed *Daucus carota* roots as the symbiotic partner cultured in a gellan gum-based substrate (also see review on page 14), and (2) the vermiculite system (Fig. 4) using leek *Allium porrum* as the host plant symbiont grown under conditions simulating those occurring in nature (also see review on page 67).

The first objective was to observe and validate growth characteristics of *G. intraradices* and Ri T-DNA transformed *D. carota* roots in the M-medium system. There was a need to identify suitable response indicators of the VA mycorrhizal fungus and to characterize their attributes in the biological system used to culture it.

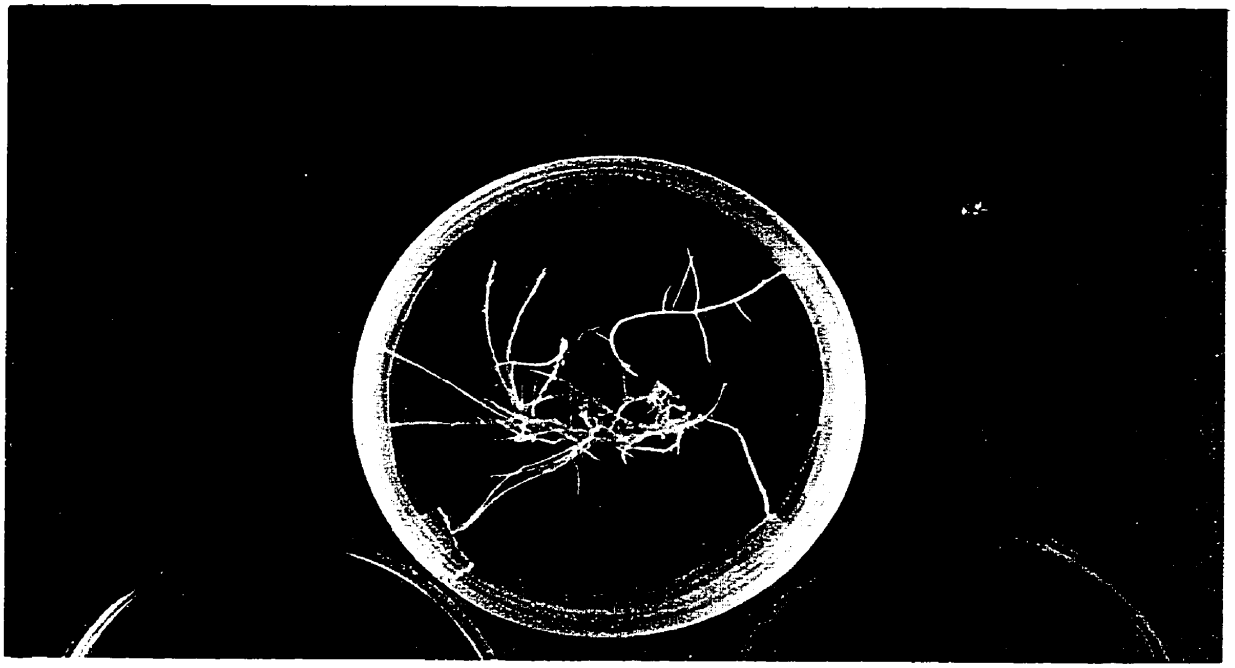


Figure 3. A minimal medium (M-medium) biological system consisting of Ri T-DNA transformed roots of *Daucus carota* and vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus intraradices*. Photograph shows a piece of VAM inoculum at three weeks after inoculation in a Parafilm® taped 100 x 15 mm Petri dish (scale = 0.5 x actual size; carrot roots appear as radiating, thick and yellowish filaments; extraradical mycelial growth of *G. intraradices* appears as faint and threadlike structures at bottom right of dish).

Figure 4. A vermiculite biological system consisting of leek *Allium porrum* growing in a 125 cc plastic Cone-tainer®, which contains 120 cc vermiculite inoculated with about 100 propagules of *Glomus intraradices*. Photograph shows an experiment in progress with Cone-tainer® units and support stands in a growth chamber having a 16-h d cycle; day:night T°C = 25°C:17°C; mean RH = 75%; and average light intensity = 4000 lx/cm^2. (Cone-tainer® scale = 0.1 x actual size).

The information generated would facilitate the design and development of a suitable pesticide toxicity bioassay technique, and would also verify the technical feasibility of using the M-medium system for this purpose.

To date, there is no method available to effectively determine the toxicity of pesticides on VA mycorrhizal fungi for comparison. As a result, their toxicological data are not available in the literature. For example, information such as NOEC (no observed effect concentration), LOEC (lowest observed effect concentration), and IC50 (concentration causing 50% fungal inhibition) are non-existent for this group of non-target organisms. Accordingly, the second objective was to develop a bioassay technique to determine the toxicity of reference pesticides and solvents to *G. intraradices* for comparison. To confirm its presence during the period of test observations, the third objective was to determine the distribution, fate, and persistence of AZA in both the M-medium and the vermiculite biological systems.

Using the novel toxicity bioassay technique developed, the fourth objective was to determine the toxicity of AZA and neem formulated products on *G. intraradices* and VA mycorrhizal infection in the M-medium system. The data generated was used to evaluate and predict the potential eco-toxicological impact of this new pesticide active ingredient on the terrestrial environment, particularly on non-target subterranean micro-organisms.

Bioassay information from the M-medium system represented data obtained from an unnatural system, and they may not be applicable to biologically complex systems occurring in nature. The fifth objective, therefore, was to examine the

relevancy of IC50 values of AZA and reference pesticides and their relation to impacts on the VA mycorrhizal symbiosis of *G. intraradices* in leek, *A. porrum*, host plants grown under environmental conditions simulating those occurring in nature.

The symbiotic relationship of VA mycorrhizal fungi plays a vital role in the growth, physiology, and survival of higher plants, many of which are important food crops. In addition to this and other known beneficial attributes, they may also play an important role as a sensitive biological indicator for assessing the environmental impact of pesticides such as "slow release" granular pesticides, e.g., AZA and other pesticide products. Accordingly, this research program will offer the possibility of an innovative approach of producing toxicity data of VA mycorrhizal fungi for comparison and prediction of eco-toxicological impact potential of pesticides. It will also contribute new knowledge to a better understanding on the potential eco-system impact of AZA on VA mycorrhizal fungi and VAM in host plants for which, hitherto, there are no scientific data available in the published literature.

CHAPTER 1

Growth Characteristics of *Glomus intraradices* and Ri T-DNA Transformed *Daucus carota* Roots Cultured in the Minimal Medium System for Pesticide Toxicity Bioassays[1]

INTRODUCTION

Despite considerable research on mycorrhizal fungi over more than 3 decades (Schenck, 1982; Smith, 1995), a biological system of potential use for short term sub-lethal bioassays involving vesicular-arbuscular mycorrhizal (VAM) fungi was developed only recently (Becard and Fortin, 1988). Although several laboratory techniques were reported for the culturing and maintenance of this system (Chabot et al., 1992; Diop et al., 1992; Becard and Piche, 1992; Declerck et al., 1996; St. Arnaud et al., 1996), none was, however, specifically developed for toxicological tests.

To date, information on a bioassay protocol using *Glomus intraradices* Schenck & Smith, growing on *Daucus carota* L. in a suitable biological system is not available in published literature (AGRICOLA, 1997; BIOSIS, 1997; CAB, 1997; CA SEARCH, 1997; and CRIS-USDA, 1997). Specifically, there is a lack of quantitative data on the growth characteristics of *G. intraradices* as indicated by

[1] - Reprinted/reformatted from Wan & Rahe (1997) *Regional Program Report 97-31* by courtesy of co-author and Environment Canada.

extraradical mycelial growth (EMG) and extraradical mycelial sporulation (EMS), and the relationship between these two structures. The suitability of these morphological structures as criteria to measure the effects of toxicants such as pesticides depends on the degree of reproducibility (Rand et al., 1995) associated with their development in a biological system.

The objective of this study is to examine the suitability of EMG and EMS, and to validate the various culturing techniques relevant to the use of the M-medium biological system as standard test protocols for pesticide bioassays of the VAM fungus *G. intraradices*. For convenience and ease of making observations, these structures were selected rather than the intraradical spores of the fungus.

MATERIALS AND METHODS

The M-medium system

This biological system (Fig. 3) of culturing *G. intraradices* for this research was supplied by the Institut de Recherche en Biologie Végétale, de l'Université de Montréal, Montreal, Quebec. It consisted of the VAM fungus, *G. intraradices,* growing on self-propagating Ri T-DNA transformed roots of *D. carota* in sterilized M-medium (containing various salts, sucrose, vitamins, and water; Appendix 1) solidified with 0.4% (w/v) gellan gum (ICN Biomedical, Cleveland, Ohio) in a Petri dish, as previously described by Becard and Fortin (1988) and Wan et al., (1997). Cultures were initiated by aseptically placing single pieces of M-medium (~ 2.5 cm x 2.5 cm x 0.7 cm or equivalent size) containing VAM inoculum obtained from a 4- to

5-week old mother culture on the centre of virgin M-medium (40 ml plated) in 100 x 15 mm Petri dishes. The pieces of VAM inoculum usually contained two or more pieces of self-propagating Ri T-DNA transformed carrot root pieces with apices (1 - 2 cm in length) and active propagules of *G. intraradices* in the form of EMG network and EMS. Each plate was defined as an experimental unit. The plates were incubated in an inverted position in darkness at 27°C.

Growth of *G. intraradices, D. carota* roots, and VAM infection

Growth of *G. intraradices* was quantified by using EMG and EMS as indicators. As well, the relationship between EMG and EMS in the M-medium system was determined. Cultures were established from five different 5-week old mother culture plates received in January 1995 from the Institut Recherche en Biologie Végétale, de l'Université de Montréal, Quebec, Canada. Estimates of EMG (cm/cm^2)and EMS ($spores/cm^2$) in the VAM inoculum and the plated M-medium were determined by using the grid method (Appendix 2) described by Newman (1966) and modified by Tennant (1975) and Sylvia (1992). Observations of both structures in the plated M-medium were made for 8 consecutive weeks.

Grid observations in each plate were made weekly along 3, 4, 5, 6 and 8 radial transects set apart at about 120°, 90°, 70°, 60°, and 45°, respectively, and the numbers of plate replicates observed were 3, 4, 5, 10, 15, and 25. The first grid count for each transect at week 1 was taken adjacent to the VAM inoculum. Counts for week 2, and so on, were taken from the same transect with the field of

observation progressively moved about 0.5 cm toward the edge of the plate from the location of previous readings. Counts of EMG and EMS were focussed on the top 1-2 mm and bottom ~3-6 mm of the M-medium. A Meiji EMT-2 TR dissecting binocular microscope with a 10 x 10 grid micrometer fitted to the right eyepiece at 15 x magnification (calibrated as equivalent to 0.95 cm x 0.95 cm = 0.90 cm^2) was used for all counts of EMG and EMS.

Growth of *D. carota* roots (cm/cm^2), root biomass (mg/30 cm^2 dry weight), and VAM colonization (%) in the M-medium system established from five different mother cultures were recorded weekly. After new root growth was measured for each observation period, roots from one half (visually determined by placing plate on top of a plate imprint having equal halves drawn) of each plate were carefully removed with forceps and dried to constant weight at 25°C for 48 hours. Mycorrhizal colonization of carrot roots growing on the other half of the plate was determined by the grid-line intersect and slide method of Giovannetti and Mosse (1980) after clearing and staining root materials with trypan blue (Phillips and Hayman, 1970; Koske and Gemma, 1989; and Dalpe, 1993). Weekly estimates of EMG and EMS from 3 to 8 visual transects per plate and 3 to 25 plate replicates were subjected to a regression analysis, a one-way ANOVA, Student's *t* and 5% probability test (SAS Institute, 1996).

Effect of different types of Petri dishes on VAM cultures

D. carota root growth *and G. intraradices* EMG were compared in regular

and two-section Petri dishes. In the two section dishes, each section received 20 mL M-medium, and the growth and development of roots and VAM fungus were initiated in one section of the dish. A piece of VAM inoculum was aseptically placed at the centre of the regular Petri dishes, each of which contained 40 ml M-medium per plate. In the two-section dishes, a similar piece of VAM inoculum was placed on one section of the plate mid-way between the edge and the central divider. Visual observations were made to determine the consistency and configuration of root and mycelium growth in both types of dishes 4 weeks after inoculation.

Effect of age of VAM inocula on establishment of cultures

The establishment of VAM inocula from mother cultures of different ages was determined when used for sub-culturing. Vesicular-arbuscular mycorrhizal inocula were obtained for sub-culturing from 2, 4, 6, 8, 10, 12, and 16-week old (as determined from the time of inoculation) mother cultures of the same batch. The percentage of development (in terms of the number of plates having successful establishment of cultures) of new carrot root branches, EMG, and EMS for each plate (n = 25) was visually observed and recorded at 1, 2, 3, 4, and 5 weeks after inoculation. Observation continued for 12 weeks on the changes in colouration of carrot roots developed from 4-week old VAM inocula.

Effect of aging pre-sterilized M-medium on VAM cultures

The growth of VAM cultures initiated from 4- to 5-week old mother

mycorrhizae was compared on sterilized M-medium, which had been stored in darkness at 20°C for 0, 1, 2, 4, 8, and 12 weeks prior to inoculation. Visual observations of VAM growth were made on each batch of 10 replicate plates 5 weeks after inoculation. They were in turn used as inocula for subculturing onto M-medium that had been stored for the same duration. This procedure was continued for six cycles.

Root harvesting by non-chemical and chemical dissolution of the M-medium

Non-chemical and chemical methods of root harvesting were compared in terms of time efficiency and yield of root biomass. Non-chemical method of harvesting roots was carried out by using a pair of steel forceps to physically remove roots from the M-medium. Chemical dissolution of M-medium was conducted by using two buffers. Roots from 4-week old cultures selected for uniform growth were removed from equal halves (visually determined by placing each plate on top of a plate imprint having equal halves drawn). The following methods were used: (a) a pair of #6 stainless steel forceps in 5 mL water, (b) 6:1 buffer (10 mM sodium citrate buffer, pH = 6.5):M-medium (Doner and Becard, 1991), or (c) 3:1 buffer (50 mM Tris-HCl-TRIZMA base + 10mM EDTA, pH = 7.5): M-medium (Nagahashi et al. 1993). Five samples of 100 mg fresh roots from each method were dried in the fume hood at 25°C and RH = 50% for 24, 48, 72, and 96 h.

RESULTS

Growth characteristics of *G. intraradices*

The development of *G. intraradices* EMG was biphasic (Fig. 5A) with slow growth occurring during the first week (0.5 $\pm$ 0.04 cm/cm^2/wk; n = 25), and a more rapid growth occurring in the second week (4.1 $\pm$ 0.7 cm/cm^2/wk; n = 25) and the following 6 weeks (7.7 $\pm$ 0.4 cm/cm^2/wk; n = 25). EMS was also biphasic (Fig. 5A). Sporulation occurred at the ends of hyphal branches shortly after EMG colonized the plated virgin M-medium. EMS occurred at an estimated rate of 1.6 $\pm$ 0.2 spores/cm^2/wk (n = 25) and 9.3 $\pm$ 0.4 spores/cm^2/wk (n = 25) for the first and second week, respectively, and more than 18.9 $\pm$ 0.9 spores/cm^2/wk (n = 25) during the next 6 weeks. A positive correlation (R^2 = 0.9901**) was obtained between weekly estimates of EMG and EMS (Fig 5B), indicating that the number of spores formed was directly related to hyphal growth.

Most of the new EMG colonizing the plated virgin M-medium originated from the growth and elongation of hyphae of infected root pieces in the VAM inocula. Once extended to the plated M-medium, the hyphae branched profusely in the top 1-2 mm part of the medium during the first 2 weeks of growth, and subsequently in the bottom ~3-6 mm. Some hyphae produced spores, some infected new carrot roots, while others continued to grow and expand in the medium, producing more branches and spores. EMG from the network of hyphae in the VAM inocula did not appear to directly colonize the plated virgin M-medium. Although germination of spores in the VAM inocula occurred, it was sporadic and

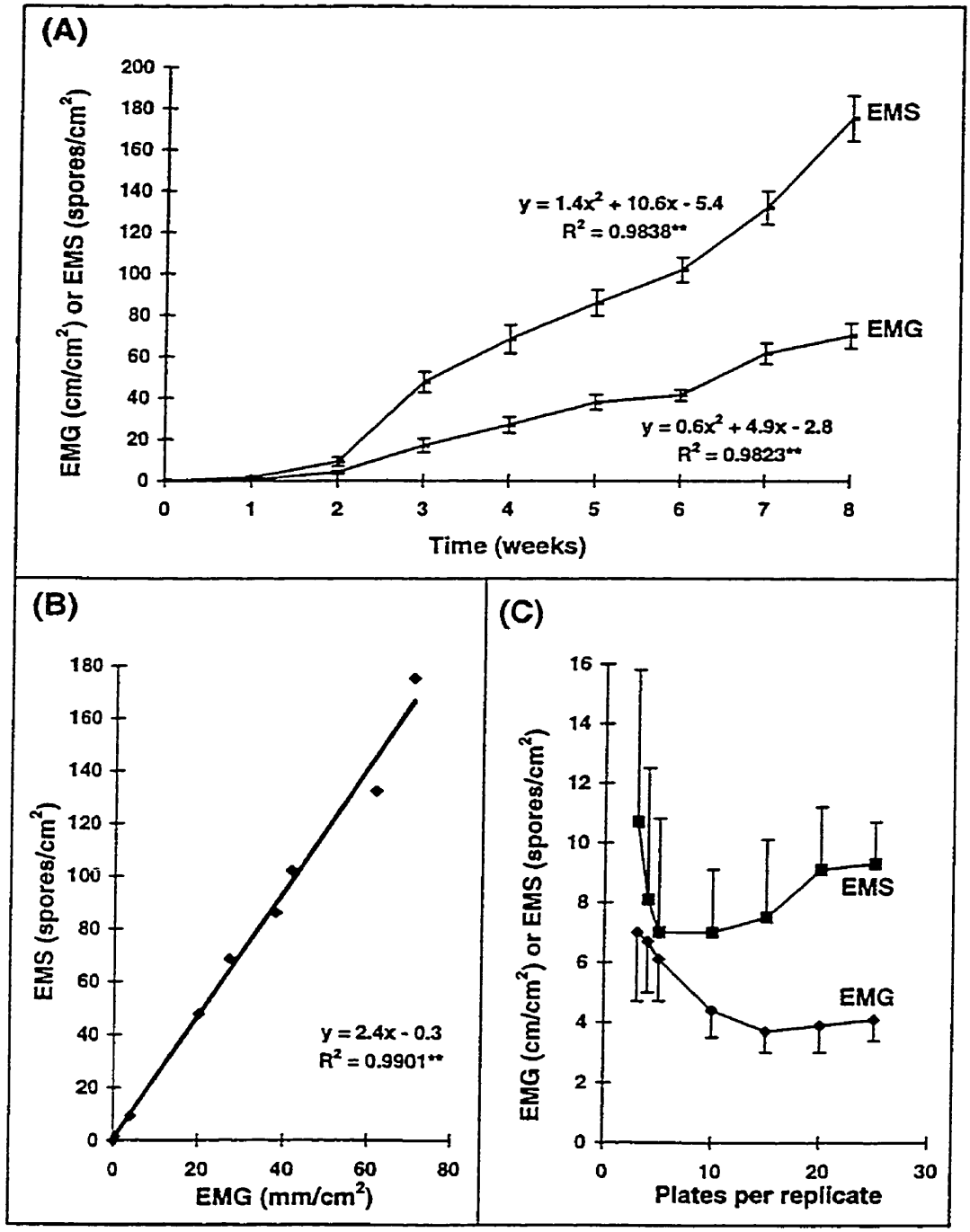


Figure 5. Growth characteristics of *Glomus intraradices* in the M-medium system. **A** Growth phases as indicated by extraradical mycelial growth (EMG) and extraradical mycelial sporulation (EMS). Data points are means ± 95% C.L. (n = 25). **B** Relationship between EMG and EMS. **C** Variation in estimates of EMG and EMS in relation to n replicate plates per data point at two weeks after inoculation. Data points are means and SEM (n = 3 to 25)

a minor contribution to the development of EMG in the plated virgin M-medium.

There was no significant difference ($P < 0.05$) between EMG estimates (five visual fields/plate) of 3- to 25-plate replicates (Fig. 5C) at 2 weeks after inoculation. This indicates that the EMG measurement of a 3-plate replicate was as good as values obtained from 4- to 25-plate replicates. Likewise, the same conclusion can be drawn for EMS estimates (Fig. 5C) at 2 weeks after inoculation. The standard error of mean (SEM) values of both EMG and EMS were generally quite large for data points of 3-, 4-, and 5-plate replicates, suggesting large data variation. However, the trend of EMG and EMS data point SEM values of 10- to 25-plate replicates (Fig. 5C) indicates that these values decreased as the number of plate per replicate increased. This suggests that more than 25 plates/replicate were required to further reduce SEM values and data variation of EMG and EMS.

The trend of the 8-week development for EMG (Fig. 6A) and EMS (Fig. 6B) was basically similar to that of observations made of 3- to 25-plate replicates (five visual grid fields per plate). Estimates for EMG obtained from 3, 4, 5, 10, 15, and 25 plates during the eight weeks of growth did not differ significantly ($P < 0.05$) (Fig. 6A), except on the sixth week, between the 3-plate replicate and the rest of the plate replicates. A positive correlation was obtained from a regression analysis of EMG estimates of different plate replicates with time. The R^2 for 3-, 4-, 5-, 10-, 15-, and 25-plate replicates was 0.9910**, 0.9878**, 0.9943**, 0.9962**, 0.9868**, and 0.9823**, respectively, suggesting that relatively accurate EMG estimates can be obtained at different times from three or more plates replicates.

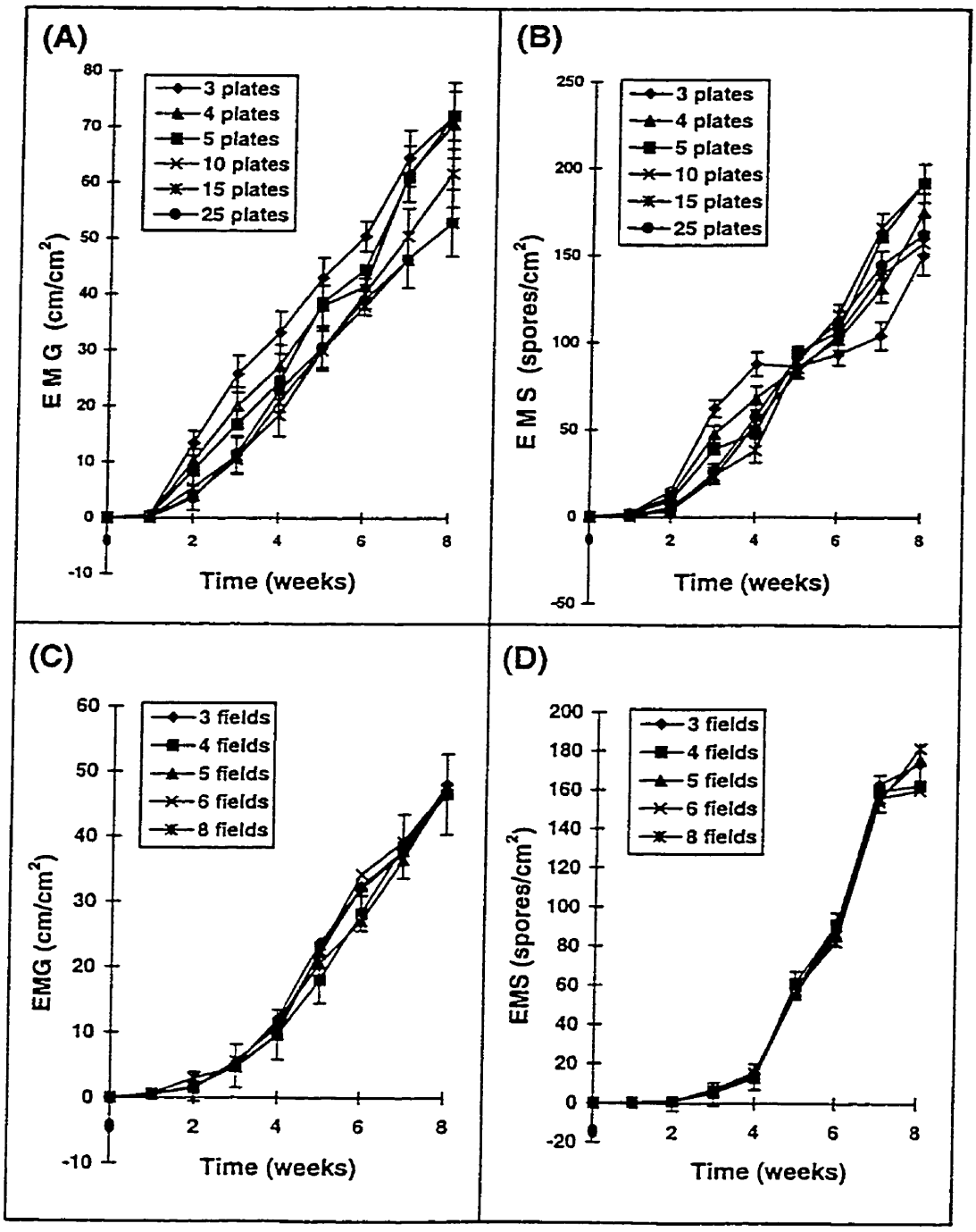


Figure 6. Variation in estimates of rates of extraradical mycelial growth (EMG) and extraradical mycelial sporulation (EMS) for *Glomus intraradices* growing in a M-medium system in relation to the number of plates (5 observation fields/plate) and observation fields (3 to 8/plate). **A** and **B** Plates observed. Data points are means ± 95% C.L. for n = 3, 4, 5, 10, 15 and 25 plates. **C** and **D** Fields observed. Data points are means ± one 95% C.L. (5 fields only; n = 4 plates)

No significant differences ($P < 0.05$) of EMS estimates were obtained during the first 2 weeks among all plate replicates (Fig. 6B). Significant differences were noted from estimates between the 3-plate replicate and the rest of the plate replicates on the 3rd , 4th, and 7th week. As well, a positive correlation of EMS estimates from different plate replicates with time was obtained. The R^2 for 3-, 4-, 5-, 10-, 15-, and 25-plate replicates was 0.9290**, 0.9854**, 0.9895**, 0.9877**, 0.9843**, and 0.9838**, respectively, indicating that relatively accurate EMS estimates can also be obtained at different times from three or more plate replicates.

Variation of EMG and EMS estimates was not significantly different ($P < 0.5$) when observations were taken from three to eight visual grid fields/plate (for a four plate replicate) (Figs. 6C & 6D). This suggests that EMG and EMS estimate variation caused by observing three or more observation fields/plate was likely a minor factor.

Growth characteristics of *D. carota* roots and VAM infection

The growth and development of *D. carota* roots during the 8-week observation period was triphasic with a slow growth rate in week one (0.5 ± 0.1 cm/cm^2/wk; n = 5), a more rapid rate of growth from week two to four (1.8 ± 0.1 cm/cm^2/wk; n = 5), and a stationary or declining phase from the fifth to eighth week (1.0 ± 0.1 cm/cm^2/wk; n = 5) after inoculation (Fig. 7A). A one-way ANOVA on the amount of root growth during the 4th , 5th, 6th, 7th and 8th weeks indicated that

growth (n = 5) from each period was not significantly ($P < 0.05$) different. This indicated that root growth probably peaked at week four. Subsequently, a stationary rather than a declining phase in root elongation, but not in growth of biomass (Fig. 7B), began and continued to the eighth week. It is speculated that possibly limitation due to space or nutrients or a combination of both factors may have initiated the stationary phase of root elongation. The network of carrot roots grew and expanded in all directions in the plated M-medium. The biomass (dry weight) of roots during the 8-week period was biphasic, with a lag phase of 1 week, and a rapid rate of increase from the second to the eighth week following inoculation (Fig. 7B). The weekly VAM infection of carrot roots was also biphasic (Fig. 7C). Root infection by *G. intraradices* increased gradually and averaged about 5%, 7%, 17%, 23%, 28% 35%, and 50% in 2-, 3-, 4-, 5-, 6-, 7-, and 8-week old cultures, respectively. Mycorrhizal infection was positively correlated (R^2 = 0.9854**) with the age (as determined from day of inoculation) of root growth (Fig 7D).

Effect of different types of Petri dishes on VAM cultures

A 100% development of VAM inocula was obtained from both regular and two-section Petri dishes plated with virgin M-medium. Carrot roots and EMG originating from the VAM inocula in the regular Petri dishes and the inoculated section of two-section dishes developed and grew rapidly, expanding in all directions during the first 4 weeks. During this period, carrot root and EMG growth

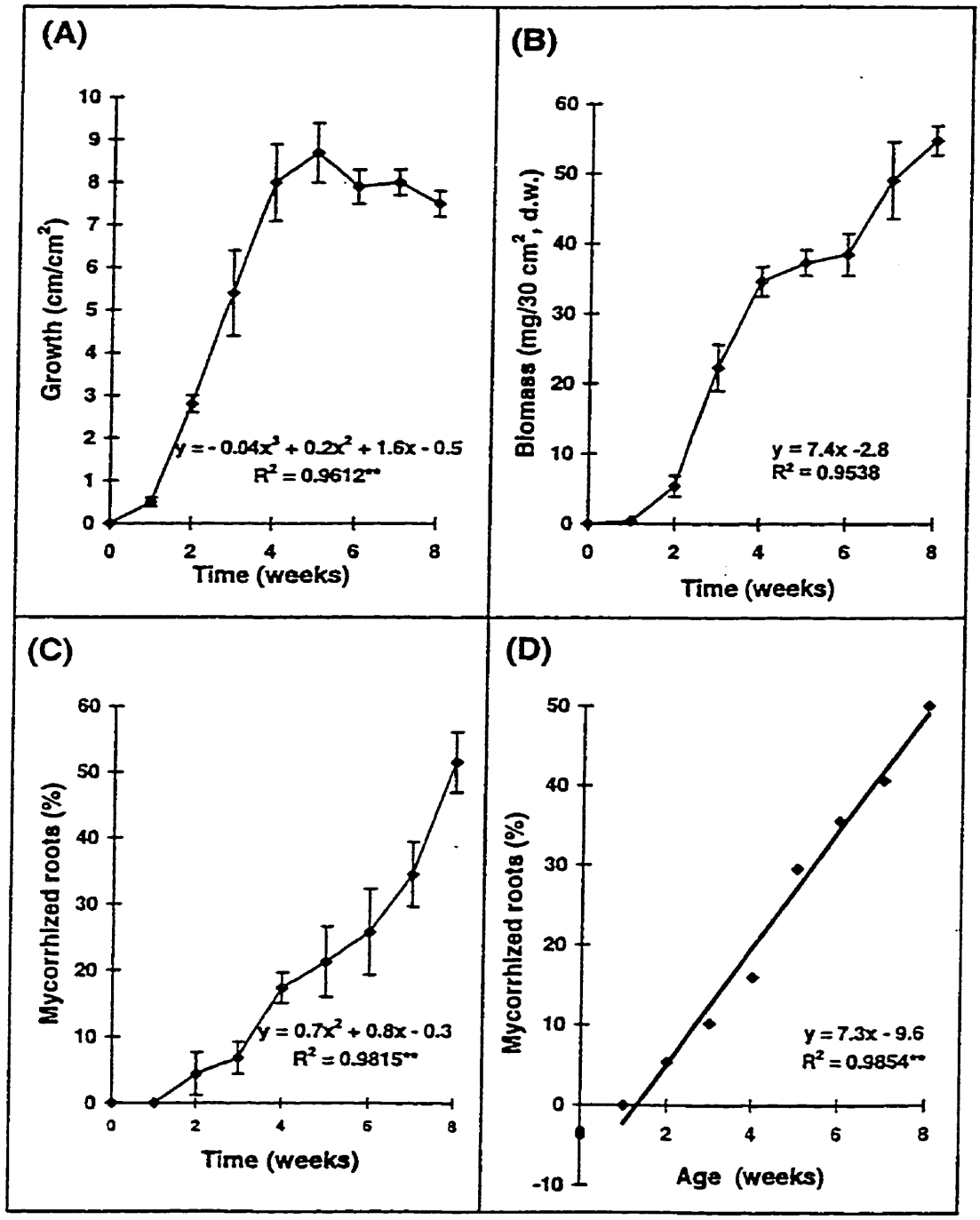


Figure 7. Growth characteristics of Ri T-DNA transformed roots of *Daucus carota* and colonization by the vesicular-arbuscular mycorrhizal fungus *Glomus intraradices* in a M-medium system. **A.** Growth. **B.** Biomass. **C.** Mycorrhized roots. **D.** Relationship between mycorrhizal infection and age of roots. Data points are means and SEM (n = 4)

and expansion in the two-section dishes was initially restricted to the inoculated section. Both the carrot roots and EMG of *G. intraradices* eventually grew across the plastic physical barrier separating the two-sections in about 40% of the two-section Petri dishes. However, the timing of growth into the non-inoculated sections of the dishes occurred at different times for different dishes, i.e., some at 3, 4, 5, and 6 weeks after inoculation. It was observed also that the configuration of the growth network was more variable in the two-section dishes than in regular dishes.

Effect of age of VAM inocula on establishment of new cultures

The effect of using VAM cultures of different ages (as determined from the time of inoculation) as inocula for sub-culturing is illustrated in Figure 8. When observed at 2 weeks after inoculation, 100% of the cultures initiated with 2-, 4-, and 6- week old VAM inocula had new carrot root growth, while 90%, 50%, 30%, and 0% of cultures had new carrot root growth when initiated from 8-, 10-, 12-, and 16-week old VAM inocula, respectively (Fig. 8A). This success rate of establishing carrot roots remained basically the same during the next 4 weeks of observation (Figs. 8B, 8C, and 8D). For *G. intraradices,* root mycorrhizal establishment from 2-week old VAM inocula remained poor (about 15%) during the 5 week observation period (Figs. 8A, 8B, 8C, and 8D). Successful establishment of cultures was initiated with 4, 6, 8-week old VAM inocula gradually increased to 100%, 90% and 85%, respectively, during the 5 week observation period, while development of

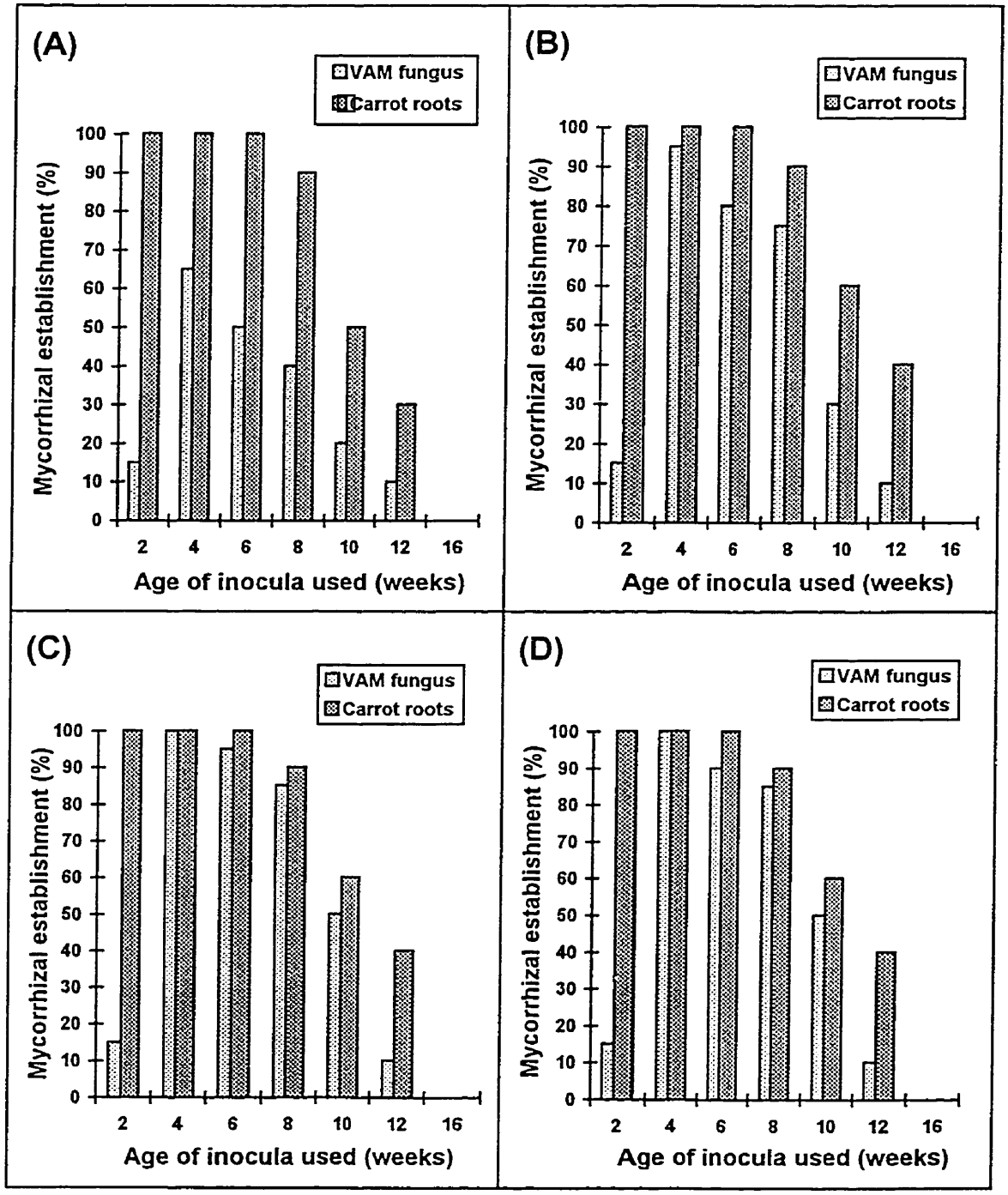


Figure 8. The effect of age of vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus intraradices* and Ri T-DNA transformed roots of *Daucus carota* inocula on their establishment in a M-medium system (n = 25) observed at **A** 2, **B** 3, **C** 4 and **D** 5 weeks after inoculation

VAM fungus remained poor at 50%, 10%, and 0% for 10, 12, and 16-week old VAM inocula, respectively. The colour of carrot roots sub-cultured from 4-week old VAM mother cultures changed progressively from pale white to dark brown during the 12-week visual observation period. Newly established carrot roots and branches in the M-medium were generally pale white. They remained whitish in color during the first 3 weeks of growth and development, then became yellowish white at 4 weeks, light yellow at 5-6 weeks, light brown at 7-8 weeks, and brown at 9 weeks; progressively darker shades of brown developed in roots 10 weeks old and beyond, indicating a change in the physiology of the roots, probably the onset of death due to depletion of nutrients.

Effect of aging pre-sterilized M-medium on VAM cultures

The effect of aging pre-sterilized M-medium by storing in darkness at 20°C for 0, 2, 4, 8, and 12 weeks on the establishment of VAM cultures, quality, and vigour of the growth and development of carrot roots and EMG from 4- to 5-week mother VAM inocula is presented in Figure 9. Establish-ment of 100% in all five treatments was obtained for six consecutive 5-week growth cycles. Moreover, the success of VAM establishment, quality, and the vigour of both carrot root and fungal EMG appeared to be unaffected by storage of the pre-sterilized M-medium for up to 12 weeks.

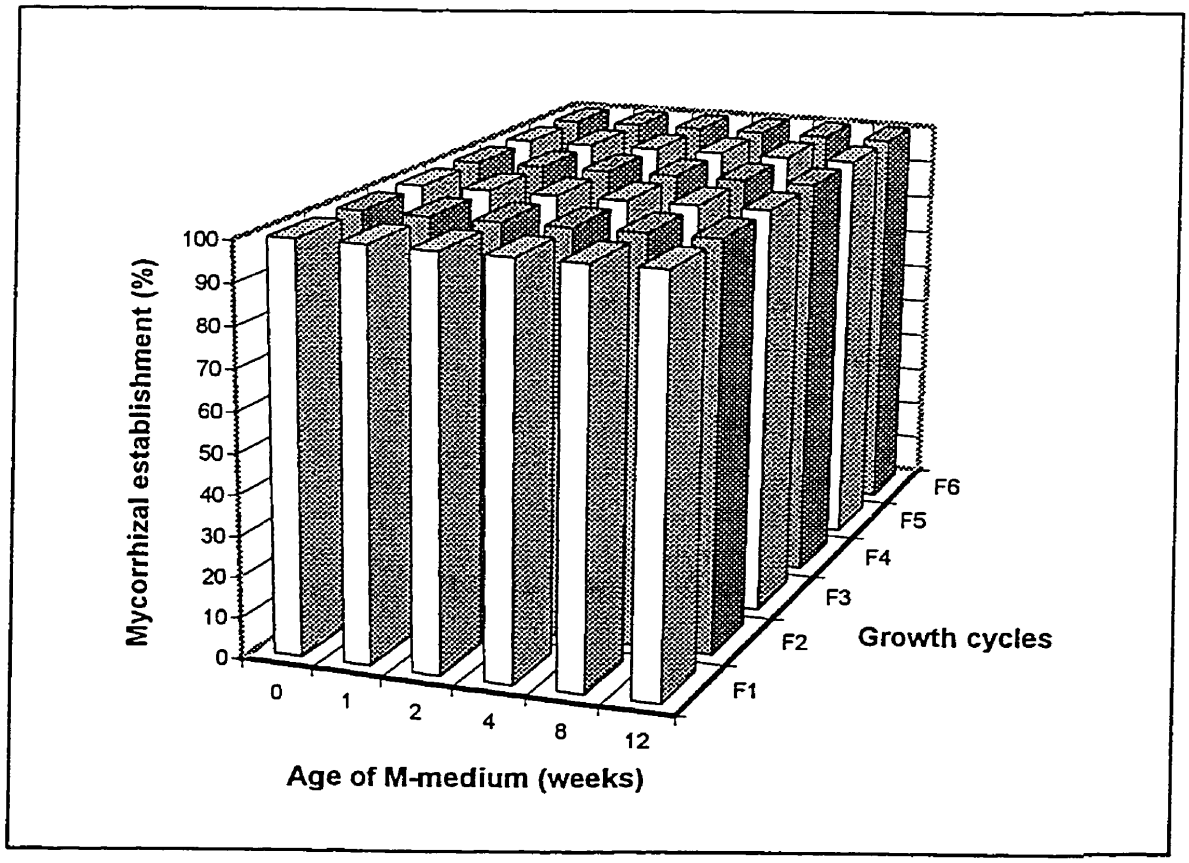


Figure 9. The effect of pre-sterilized M-medium stored at different times on the establishment of vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus intraradices* and Ri T-DNA transformed roots of *Daucus carota* inocula in the M-medium system (n = 10)

Root harvesting by non-chemical and chemical dissolution of the M-medium

The time required for harvesting carrot roots using a pair of forceps to physically remove roots from the M-medium averaged about 2.0 $\pm$ 0.1 min/20 cm^3 plate (n = 10) with no preparation time needed. Roots harvested using citrate and tris-HCl-TRIZMA buffers were both approximately 4.5 $\pm$ 0.5 min/20 cm^3 plate. However, both buffers required about 1.5 h preparation time per sample, e.g., buffer preparation, dissolution time, etc. As well, complete dissolution of the M-medium was not achieved by both buffers. After 1.5 h buffer treatment, about 90% and 10% of the M-medium dissolved in the citrate and TRIZMA buffers, respectively. Furthermore, roots (d.w.) obtained from the buffering method (Fig. 10) averaged about 35% heavier when compared with those from the physical removal method. This experiment suggests that non-chemical method of harvesting carrot roots from the M-medium was more time efficient and reliable than the chemical dissolution method.

DISCUSSION

This study confirms that *G. intraradices* EMG follows closely the growth of transformed carrot roots. Within a batch of M-medium system units, EMG appeared to be more consistent during the first 2 weeks of the growth phase. At this stage of growth, EMG occurred mainly on the top 1-2 mm of the plated M-medium. Grid counts of EMG could be estimated easily and accurately at this time. Beyond 3 weeks after inoculation, the mycelial network began to thicken, making

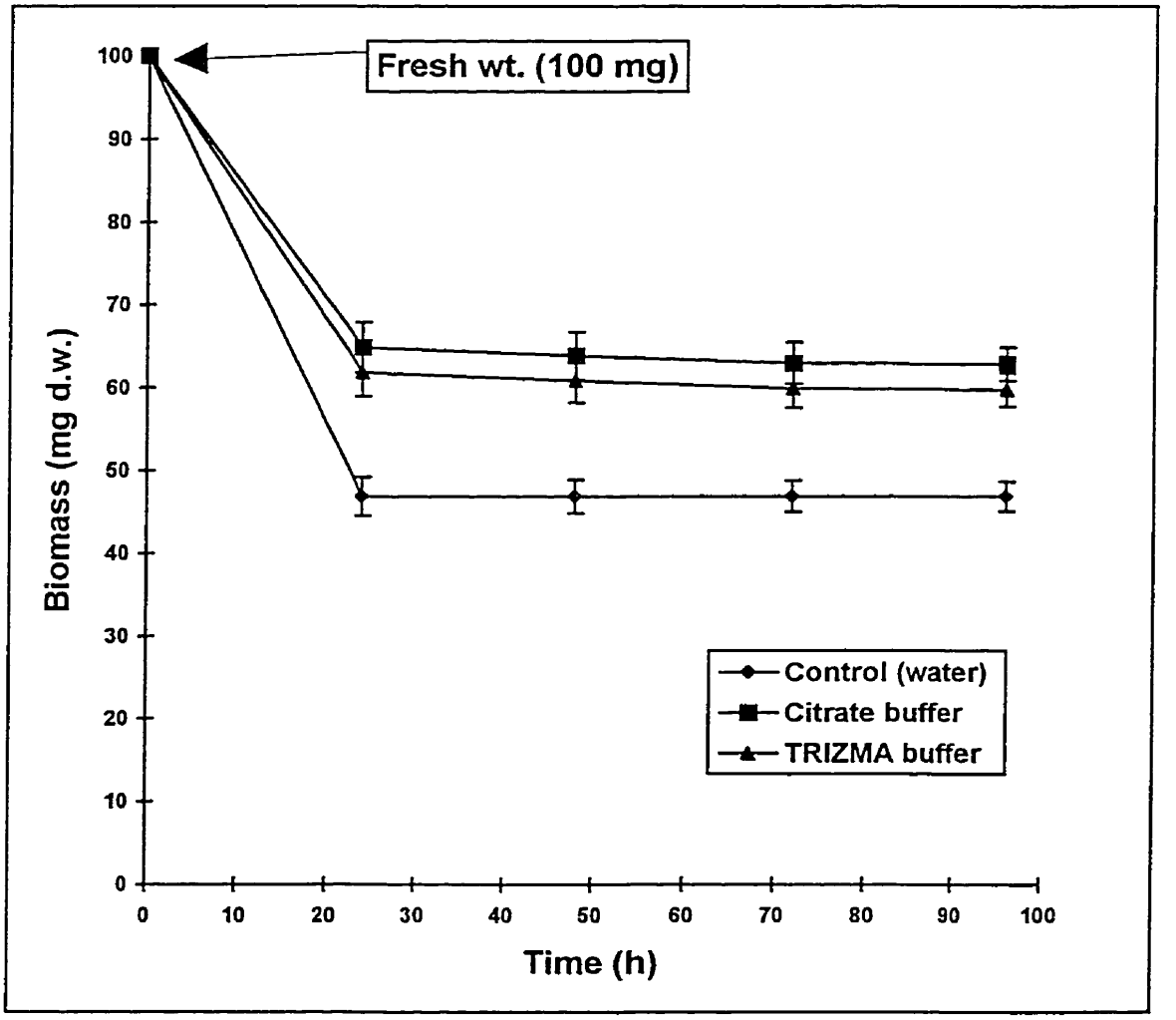


Figure 10. Biomass (dry weight) of Ri T-DNA transformed *Daucus carota* roots obtained by non-chemical and chemical dissolution of the M-medium. Data points are means and SEM (n = 5)

visual counts a challenging and more difficult task. This study also confirmed that sporulation of *G. intraradices* occurs shortly after EMG begins, and it continued in this case during the 8 week period of observation on the M-medium system (Becard and Fortin, 1988; Chabot et al., 1992). EMS could also be estimated accurately during the first 2 weeks following inoculation, and this structure is a potential response indicator of the inhibitory effects of pesticides and other possibly other toxicants.

The use of EMG or EMS as criteria to measure the inhibitory effects of toxicants would be the natural and logical choice. If a toxicant had any adverse effects on these structures, it would most likely affect, and be reflected in, their early growth and development. If EMG and EMS proceeded normally in treated M-medium system units when compared with the control units beyond 3 weeks post inoculation, it would be likely that the toxicant had little or no inhibitory effect on the VAM or VAM fungus. Accordingly, the critical time for observing the inhibitory effect of toxicants on VAM and VAM fungus is likely 2 weeks after inoculation. Beyond 2 weeks, estimating EMG and EMS is more difficult and becomes relatively less accurate due to the thickness of the mycelium, which impairs visual observation, and the large numbers of spores, which makes spore counts a challenging task.

There are several factors that affect the viability of VAM inocula and their suitability for establishing consistent new cultures in virgin M-medium. Age of the mother VAM inocula has a major effect. Vesicular-arbuscular mycorrhizal inocula obtained from 4- and 6-week old mother cultures appeared to have a greater and

generally more consistent success of root establishment than those obtained from 2-, 8-, 10-, 12- and 16-week old mother cultures. It was unclear why such differences occurred. Becard and Fortin (1988) reported that 20-day old roots were much more efficient as host than 10-d old roots. This study confirmed Becard and Fortin's observations that young carrot roots were less host efficient than older roots. As well, it indicated that optimum root host efficiency seemed to extend from 20 to 56 days. Beyond this time and as roots aged, the root host efficiency declined. Perhaps the aging of roots and the subsequent changes in cellular physiology affects the colonization of the VAM fungus. This finding suggests that to ensure 100% VAM development and establishment for toxicant bioassay, 4-week old VAM mother cultures should be selected for sub-culturing M-medium system units. Changes in carrot root colouration with time may give a rough visual indication of the age, and hence the viability, of VAM mother cultures for sub-culturing. Four-week old mycorrhizal carrot roots are generally yellowish in colour.

Both the growth of active hyphae originating from infected carrot root pieces and spore germination of the VAM inocula contributed to the overall colonization of EMG and EMS in plated virgin M-medium. The degree of contribution of each type of structure is presently not determined. To ensure consistent success of VAM establishment, it is essential that each piece of VAM inoculum should contain several root pieces containing apices. Regular Petri dishes, inoculated in the centre, provided conditions that produced relatively consistent establishment and patterns of VAM development when compared with two-section Petri dishes of the

same dimension. As well, to facilitate the maintenance of VAM mother cultures, pre-sterilized M-medium could be stored for at least 12 weeks and used for sub-culturing M-medium system units with no apparent impact on the establishment and quality of EMG and EMS development.

A minimum number of observations/plate and plates/replicate is required in order to obtain a desired level of accuracy (e.g., 5% or 10% error) and reliability of statistical test results (McClave and Dietrich, 1991; Ellersieck and La Point, 1995). However, the number of plate replicates per test concentration used for toxicant bioassays of micro-organisms varies considerably in practice, e.g., from a 1-, 3-, to 10-plate replicates/test concentration for *Bacillus larvae,* a causative agent of American foulbrood (Calderone et al., 1994), *Selenastrum capricornutum* (Environment Canada, 1992a), and *Ascosphaera aggregata* (Goettel et al., 1992), respectively. In fish toxicity tests, two replicates/test concentration are the norm (Wan et al., 1988, 1991; Environment Canada, 1992b).

This study shows that, with three or more observation fields/plate, a 3- to 25-plate replicate/test concentration could be used. Although there is evidence to suggest that plate replicates greater than 25 per test concentration would likely reduce data variation, this consideration of plate replicates for VAM fungus bioassays would be impractical and unmanageable because of experimental and logistic problems. A typical test run involves the use of a control, a solvent control, and five test concentrations. As well, each toxicant is usually tested three times, and the mean of these tests is then taken as the final point estimate. Based on our

experience, it is recommended that a combination of a five-visual field/plate and four-plate/replicate/test concentration be considered for the standard sub-lethal toxicity bioassay of VAM fungus, bearing in mind that large variation of data may occur.

In the eco-risk evaluation of pesticides, indicator organisms from different trophic levels are represented (Environment Canada, 1996; U.S. EPA, 1996). Presently, beneficial soil micro-organisms such as the mycorrhizal fungi are not represented. This absence is possibly and largely due to the lack of a standardized method to measure the toxicity of these indicator organisms for meaningful toxicity comparison (Trappe et al., 1984). In our opinion, there is a need to include mycorrhizal fungi to the existing battery of toxicity tests for the following reasons. Mycorrhizal fungi, e.g., *Glomus* and many other groups, are geographically cosmopolitan and ubiquitous (Mosse et al., 1981). They are commonly and widely found in roots of plants growing over a broad ecological range in the arctic, temperate, and tropical regions. Besides their symbiotic role in promoting plant growth and survival, they are also implicated in enhancing eco-sustainability (Allen, 1991). The addition of symbiotic fungi to the existing battery of toxicity tests has the potential to further enhance (Lyne et al., 1992) the over-all value of eco-risk assessment of pesticides.

In summary, this study suggested that *G. intraradices* is a potentially suitable response indicator organism of pesticide toxicity. As well, it validated the technical feasibility of using the M-medium system to culture this VAM fungus for pesticide

toxicity bioassays.

CONCLUSIONS

This study confirmed selected aspects of growth and related characteristics of *G. intraradices* and Ri T-DNA transformed *D. carota* roots in the M-medium system. Some information relevant to toxicity tests on the growth of these organisms could be inferred from the limited publications available to date, but they lack specificity and are usually incomplete. Accordingly, this study validates growth attributes such as consistency, phases, quantity, timing, etc., of *G. intraradices* in association with its host symbiont in the M-medium system to facilitate the design and development of a suitable pesticide toxicity bioassay technique.

The EMG and EMS of the VAM fungus *G. intraradices* and the root growth and biomass of Ri T-DNA transformed *D. carota* had two or more growth phases during the 8-week observation period when cultured in a 40 mL gellan gum-based M-medium system in darkness at 27°C. Estimates of EMG and EMS could be determined, relatively accurately, from combinations of 3- to 8-visual fields/plate and 3 to 25 plate replicates, using the method (Appendix 2) of length estimation described by Tennant (1975). A positive and good correlation was obtained between weekly EMG and EMS values for *G. intraradices*. Growth consistency and phases of EMG and EMS, and the timing of establishment of *G. intraradices* were more predictable in regular than in two- section Petri dishes. Accordingly, EMG and EMS are potentially suitable response indicators of the inhibitory or toxic

effects of pesticides (and other toxicants) on *G. intraradices.* For practical purposes, observation of EMG and EMS in VAM fungus pesticide toxicity tests should be focussed on the top 1-2 mm layer of the M-medium, and at 2 weeks after inoculation with a combination of five-visual grid fields/plate and a four-plate replicate/test concentration. A 100% establishment of VAM inocula was consistently obtained from materials of 4-week old mother VAM cultures. Infection of carrot roots by *G. intraradices* increased steadily from 5% in 2-week to about 50% in 8-week old cultures. Non-chemical method was more time efficient than the chemical dissolution of M-medium method of harvesting carrot roots. As well, biomass of roots (d.w.) harvested by the chemical dissolution method appeared to be about 35% heavier than those from non-chemical method. Pre-sterilized M-medium stored at 20°C and in darkness for up to three months neither deteriorates in quality nor affect the establishment of VAM inocula. Based on the above considerations, it is technically feasible to use the M-medium system to produce consistent establishment and growth of *G. intraradices* for pesticide toxicity bioassays.

CHAPTER 2

A New Technique for Determining the Sub-lethal Toxicity of Pesticides to Vesicular-arbuscular Myocrrhizal Fungus *Glomus intraradices*[1]

INTRODUCTION

In the eco-risk evaluation of toxic substances and pesticides (Environment Canada, 1996; US EPA, 1996) indicator organisms from different trophic levels are used. Presently, beneficial soil micro-organisms such as VAM symbiotic fungi are not represented (Landis and Yu, 1995; ASTM, 1993). Their absence is probably due to the lack of a standardized method for assessing toxicity to this group of subterranean microorganisms (Trappe et al., 1984) In our opinion, there is a need to include VAM fungi in the existing battery of indicator organisms (Table 1) to further enhance (Lyne et al., 1992) the over-all value of eco-risk assessment of pesticides. VAM fungi, e.g., *Glomus* spp. and many other groups, are geographically cosmopolitan (Mosse et al., 1981). They are commonly found in the rhizosphere and in roots of plants growing in arctic, temperate, and tropical regions. Beside their symbiotic role in promoting plant growth and survival, they are also implicated in enhancing eco-sustainability (Allen, 1991).

[1] - Reprinted/reformatted from Wan, Rahe & Watts (1998) *Environ Toxicol Chem* 17:1421-1428 by courtesy of co-authors and SETAC PRESS.

The conventional method of non-target impact assessment of pesticides on VAM fungi and VAM symbiosis uses inoculated and uninoculated pesticide-treated plants grown in pots (Nemec and Tucker, 1983; Jabaji-Hare and Kendrick, 1987; Perrin and Plenchette, 1993). After a period of growth varying from 6 to 20 weeks (Spokes et al., 1981), the plants are harvested, their shoot biomass determined, and roots are cleared and stained to estimate VAM colonization. Pesticide effects are then evaluated by comparing treatment responses to the controls. Although this is a useful method, the results obtained by different researchers are often not comparable because of the lack of a standardized technique (Trappe et al., 1984; Habte et al., 1992). A method that is simple, standardized, and relatively short term is needed for bioassay of pesticide toxicity to VAM fungi.

The objective of this part of research is to develop a new technique to determine a point estimate of a pesticide concentration that causes 50% inhibition (IC50) in the growth and development of the VAM fungus *Glomus intraradices* Schenck & Smith (DAOM 181602), using the M-medium technology described in Chapter 1. The method of assessment developed in this research is practical, cost effective, and gives reproducible results.

MATERIALS AND METHODS

The M-medium system

This biological system of culturing *G. intraradices* as described by Wan and

Rahe (1997) was used in the development of an appropriate IC50 testing technique for the VAM fungus.

Test Chemicals

The following reference solvents and pesticides were used for the IC50 determination of *G. intraradices*: acetone (100%), and methanol (99.9%) - Baxter Corporation, Mississauga, ON, Canada; benomyl (99%), chlorothalonil (99%), and dimethoate (99%) - Chem Service, West Chester, PA, USA.; copper sulphate (99.9%) - BDH Chemicals, Vancouver, BC, Canada; aminomethylphosphonic acid (97%) and glyphosate (99.9%) - Monsanto Company, St Louis, MO, USA. The rationale for selecting these pesticides was their different biological effects and modes of action. Benomyl is a systemic fungicide. Chlorothalonil and copper sulphate are non-systemic and contact fungicides. Dimethoate is a systemic insecticide, and glyphosate is a systemic herbicide. Aminomethylphosphonic acid (AMPA) is a metabolite of glyphosate.

The test concept

A short term, 14-d sub-lethal test was developed using the growth of *G. intraradices* in the M-medium system to determine whether several reference pesticides and their solvents had more or less inhibitory effects on the VAM fungus relative to controls. The indicators of response to these toxicants were EMG and EMS of *G. intraradices*, and the growth of *D. carota* roots. Their responses to the

inhibitory effects of each toxicant at 2 weeks when compared with the control were used for the IC50 estimation. A nonlinear regression (SAS Institue, 1996) and a linear interpolation method (Norberg-King, 1993) for sub-lethal toxicity were used for the calculation of IC50s. As well, the biomass, and VAM colonization of Ri T-DNA transformed carrot roots were determined for comparison of pesticide impact.

Test procedure

This static, non-renewable test was initiated when a piece of 4-week old VAM inoculum (2.5 cm x 2.5 cm x 0.7 cm or equivalent size) was aseptically placed on the medium containing each toxicant. The M-medium mixture was prepared with distilled water (pH = 5.8). Exactly 40 mL of M-medium mixture was pipetted into each 200 ml Pyrex® bottle and autoclaved for 16 minutes at 121°C and 240 Kpa. After autoclaving, the bottles were allowed to cool to about 58°C $\pm$ 2°C in a water bath prior to toxicant spiking. Toxicant was added with an Eppendorf pipette, and the bottle was then gently shaken for about 10 s to ensure that the pesticide was uniformly and completely incorporated in the molten M-medium before plating (100 cm x 15 cm Petri dishes). The molten M-medium was allowed to set in the plates for about 30 min prior to inoculation with VAM. Following inoculation, the Petri dishes were covered, taped at two places (180° apart) with Ruban® invisible cellophane tape, and incubated in darkness at 27°C in an inverted position.

Each bioassay had a water and/or solvent control and at least five test concentrations. Sterilized water was used for controls of water soluble pesticides

such as $CuSO_4$, dimethoate, glyphosate and AMPA. For acetone and methanol soluble pesticides such as chlorothalonil and benomyl, respectively, the solvent control contained an equivalent amount of solvent that was used to deliver the highest pesticide concentration of the test run. Response to the solvent control was used in the IC50 determination of test materials containing a solvent.

Estimation of EMG, EMS, and carrot root growth were made at days 7, 14, and 21 after inoculation. The measurement of EMG, EMS, and Ri T-DNA transformed carrot roots was conducted according to the method described by Wan and Rahe (1997) and Wan et al. (1997). Observations were made with a dissecting microscope in five grid fields per plate and on four replicate plates for each test concentration. The bioassay of each pesticide was repeated three times. An initial test to determine concentration range was conducted for all materials. Once the inhibitory range of concentrations was established, the individual test concentrations within the range varied serially by two to five fold. The mean IC50 values for the final two tests are presented in the results. The bioassay was declared invalid if more than one plate in each four-plate replicate of each test concentration series was contaminated by other microorganisms.

The biomass (mg dry wt.), and VAM colonization (%) of carrot roots transformed with Ri T-DNA were determined at day 28. For dry weight determination, the original piece of VAM inoculum (2.5 cm x 2.5 cm x 0.74 cm or equivalent size) was first carefully removed from each plate using a surgical blade. Carrot roots from one half (visually determined by placing each plate on top of a

plate imprint having equal halves drawn) of the M-medium were then carefully removed using forceps, placed in a weighing dish, and dried for 48 h in a fume hood at 25°C and a RH of about 45%. The roots from the remaining half of the M-medium were used for estimating VAM colonization. The grid-line intersect and slide method of Giovannetti and Mosse (1980) was used for this determination after the roots were cleared and stained using the trypan blue procedure described by Dalpé (1993).

Two methods of statistical analysis were used to estimate the IC50 - nonlinear regression test of SAS Institute (1996), and linear interpolation calculated by the ICPIN program Version 2.0 of Norberg-King (1993). Root dry weights, VAM fungus colonization of roots, and estimates of root growth were subjected to a one-way ANOVA and Student's *t* test at the 5% level.

The nonlinear regression (NLR)

The NLR estimate is based on this model:

$y = \alpha^{*}\exp(-x/\beta) + \epsilon$

where: ϵ = a normal random variable with mean = 0, and variance σ^2

or $y = \alpha^{*}\exp(-x/\beta)$

α = mean response at 0 concentration of toxicant

β = slope of response function

exp = exponential function

y = response, e.g., hyphal length or spores

x = concentration of toxicant

Standard error (SE) of the estimated mean ICp = ln(p)SE(β), and an estimated 95% confidence limit, if required, for ICp is:

$-\ln(p)\beta^* \pm 1.96[-\log(p)SE(\beta^*)]$; * = estimate from NLR

The IC50 estimate is then calculated directly from the observed parameters as IC50 = -βln(0.5). The NLR method also makes the assumption that the ϵ term is normally distributed with the mean 0 and constant variance.

The linear interpolation (LI)

The LI estimate is based on the following function:

$$ICp = X_j + [M_1(1 - p/100)] (X_{j+1} - X_j)/(M_{j+1} - M_j)$$

where:

X_j = tested concentration whose observed mean response is greater than $M_1(1 - p/100)$

X_{j+1} = tested concentration whose observed mean response is less than $M_1(1 - p/100)$

M_1 = smoothed mean response for the control

M_j = smoothed mean response for concentration J

M_{j+1} = smoothed mean response for concentration J + 1

p = percent reduction in response relative to the control response

ICp = estimated concentration at which there is a percent reduction from the smoothed mean control response.

The basic assumption of this model is that the response to increasing concentrations of any toxicant ranges from no response to a significant decrease relative to controls. Using the IC*p* approach, successive means of the responses at each concentration are considered in order of increasing concentration, starting with the control group. If the observed mean at the next higher concentration(s) is larger than the estimated mean response (with monotonicity taken into account) at the lower concentrations, then it is averaged with the lower groups before the IC*p* is calculated. The "smooth means" M are calculated using isotonic regression (Norberg-King, 1993). The standard error (SE) of IC*p* is calculated from the standard deviation (SD) of the "bootstrap" estimates mean.

For both the NLR and LI methods, parameters that can be used to calculate the ICp are biomass per replicate, mean weights of indicator organisms per replicate, young per original female and additional endpoint [Norberg-King, 1993; R. Balshaw, personal communication]. Accordingly, this approach is suitable for the estimation of pesticide IC50s to *G. intraradices* in which the total hyphal length of EMG or the total number of spores (EMS) are used as inhibitory response indicators. The value assigned to *p* for both methods can be anything from 0.01 and 0.99. For example, an IC50 is the estimated concentration resulting in 50% inhibition relative to the control. Both methods of IC50 determination can be obtained graphically (Fig. 11), although appropriate software was used for the computation of all data. Each plate replicate consisted of a mean value for five observations. Data were not trimmed nor winsorized to adjust for extreme outliers.

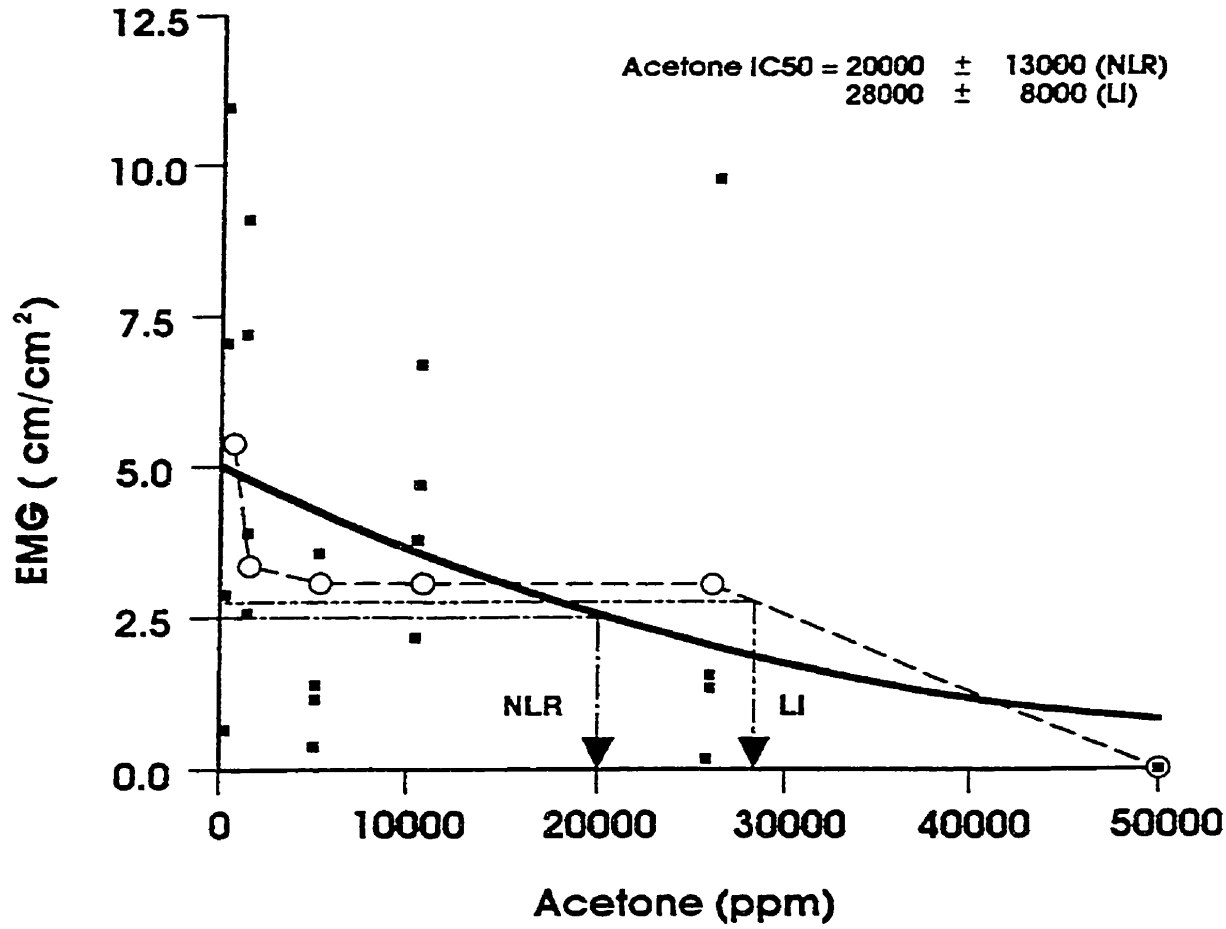


Figure 11. Response to different concentrations of acetone by extraradical mycelial growth (EMG) of *Glomus intraradices* 14 d after inoculation. Estimation of 50% inhibitory concentration (IC50 ± SE ppm) of the solvent by non linear regression (NLR) and linear interpolation (LI) method of determination. (■ - datapoint; o - "smooth" mean of LI)

RESULTS

Toxicity to *G. intraradices*

The estimates for acetone IC50 for *G. intraradices* EMG by the NLR and LI methods of determination were approximately 20,000 and 28,000 ppm, respectively. Both methods of estimation (Table 2) using EMG as a response indicator indicate that solvents such as acetone and methanol were relatively non-toxic to the VAM fungus when compared to reference fungicides such as benomyl, chlorothalonil, and $CuSO_4$. Systemic pesticides such as dimethoate (an insecticide) and glyphosate (a herbicide), including its metabolite AMPA, were also relatively toxic to the fungus when compared to both solvents. Based on the estimates by both methods, the order of increasing toxicity for the compounds tested was acetone, methanol, AMPA, dimethoate, $CuSO_4$, glyphosate, chlorothalonil and benomyl. IC50 estimates obtained using EMS (Table 2) as the response indicator provided a similar toxicity ranking for both reference solvents.

With the exception of the solvents, the IC50 estimates for reference pesticides by NLR and LI methods of determination were comparable, when either EMG or EMS were used as the response indicators. The question of whether EMG or EMS is a more appropriate response indicator to use for IC50 determination is debatable. However, from our previous study (Wan and Rahe, 1997), we showed that EMG is positively correlated to EMS (R^2 ranging from 0.9823** to 0.9962**). In our opinion, both fungal structures could and should be used as a response

Table 2. 50% inhibitory concentration (IC50[a]) of test materials on the extraradical mycelial growth (EMG) and extraradical mycelial sporulation (EMS) of *Glomus intraradices* 14-d after inoculation

Compounds[b]	Nonlinear regression[c] IC50 ± SE (ppm)	Linear interpolation[d] IC50 ± SE (ppm)
EMG response indicator		
Acetone (S)	20000 ± 13000	28000 ± 8000
Methanol (S)	14800 ± 7000	14200 ± 8000
Benomyl (F)	0.004 ± 0.0009	0.003 ± 0.0008
Chlorothalonil (F)	0.05 ± 0.01	0.03 ± 0.002
$CuSO_4$ (F)	0.6 ± 0.2	0.7 ± 0.2
Dimethoate (I)	3.0 ± 1.5	3.1 ± 0.5
Glyphosate (H)	0.5 ± 0.3	0.5 ± 0.2
AMPA	4.2 ± 2.8	5.1 ± 1.6
EMS response indicator		
Acetone (S)	11000 ± 9400	7500 ± 4000
Methanol (S)	1900 ± 170	3400 ± 300
Benomyl (F)	0.003 ± 0.001	0.003 ± 0.0005
Chlorothalonil (F)	0.04 ± 0.009	0.01 ± 0.007
$CuSO_4$ (F)	0.3 ± 0.2	0.4 ± 0.2
Dimethoate (I)	1.4 ± 0.6	0.8 ± 0.4
Glyphosate (H)	0.5 ± 0.3	0.4 ± 0.09
AMPA	3.8 ± 2.8	5.5 ± 1.1

[a] - mean of two estimates
[b] - F = fungicides, H = herbicides, I = insecticides, S = solvents
[c] - SAS Institute Inc.; Department of Statistics, Simon Fraser University
[d] - ICPIN, US Environmental Protection Agency and pesticides.

indicator to calculate point estimates of pesticide effects to obtain an accurate IC50 estimation. There may be an effect of differentiation of EMG and EMS, e.g., an inhibitory effect of a pesticide on EMG versus its stimulatory effect on EMS or vice versa. From the environmental impact view point, an inhibitory effect on EMG of *G. intraradices* by pesticides would likely mean reduced symbiotic activity; a stimulatory effect, on the other hand, would probably increase symbiotic activity. An inhibitory effect on EMS could possibly have a negative impact on the propagation of the VAM fungus.

Effects on *D. carota*

Neither acetone (Fig. 12A) nor methanol (Fig. 12B) affected the growth of Ri T-DNA transformed roots of *D. carota* roots at 25,000 ppm over the 3-week observation period. At 50,000 ppm, both solvents suppressed ($P < 0.05$) root elongation. Benomyl (up to 0.025 ppm in 1,250 ppm methanol) (Fig. 12C) and chlorothalonil (0.625 ppm in 2,500 ppm acetone) (Fig. 12D) did not suppress root growth. Water soluble pesticides such as $CuSO_4$ (Fig. 13A), glyphosate (Fig. 13C) and AMPA (Fig. 13D), however, reduced root elongation significantly ($P < 0.05$) at 6.25 ppm, 0.25 ppm, and 25 ppm, respectively. Dimethoate (Fig. 13B) had no impact on root elongation at 10 ppm. Acetone (Fig. 14A) and methanol (Fig. 14B) did not significantly ($P < 0.05$) reduce root biomass at 25,000 ppm when harvested at 4 weeks. Root dry weight was significantly ($P < 0.05$) reduced by both solvents at 50,000 ppm. As well, neither benomyl (Fig. 14C) nor chlorothalonil (Fig.

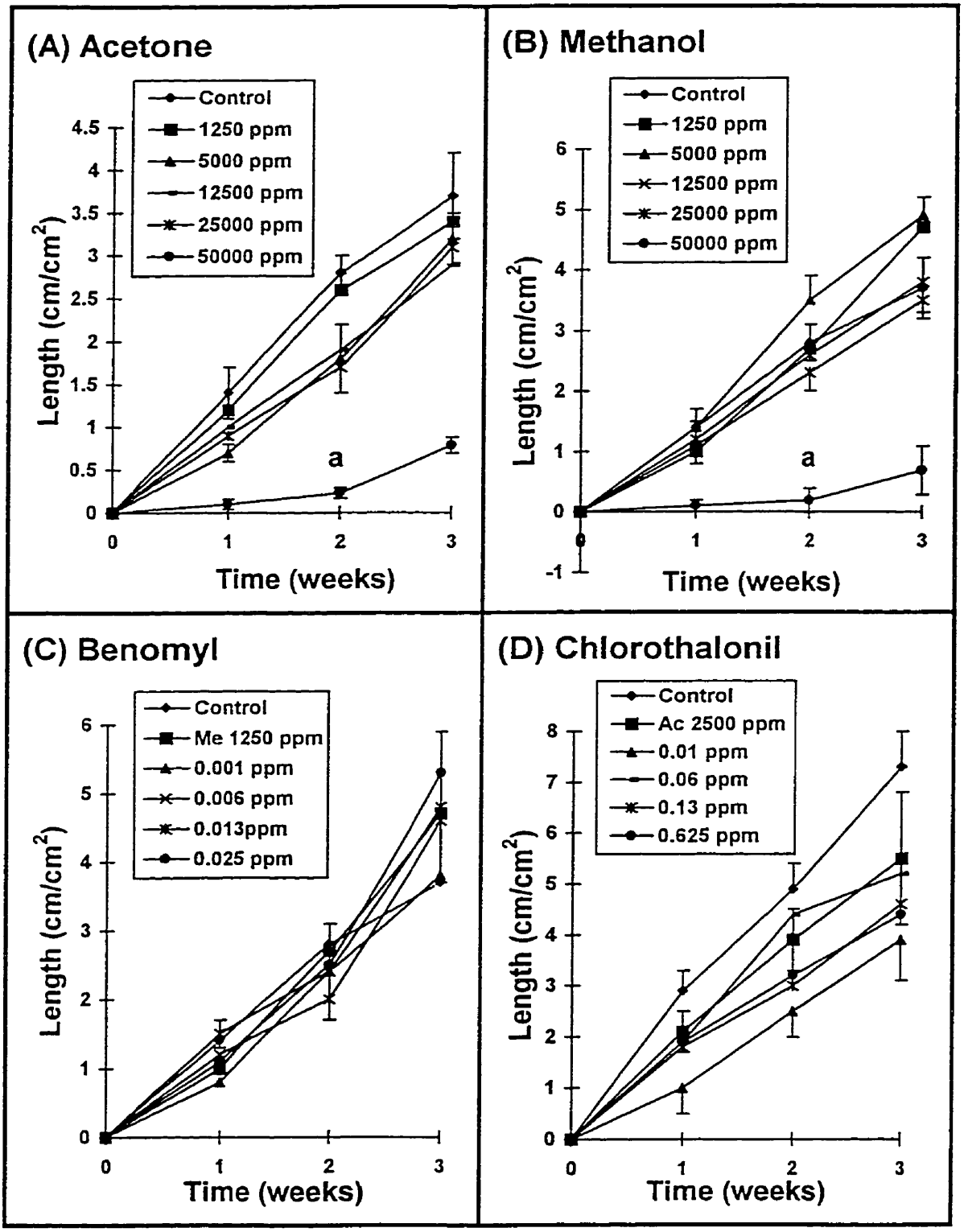


Figure 12. Effect of solvents and pesticides on root growth of Ri T-DNA *Daucus carota*, (Ac = acetone, Me = methanol). Values = means ± SE (n = 4) of selected test concentration series; a - indicates significant differences ($p < 0.05$) between control or solvent control and test concentration series.

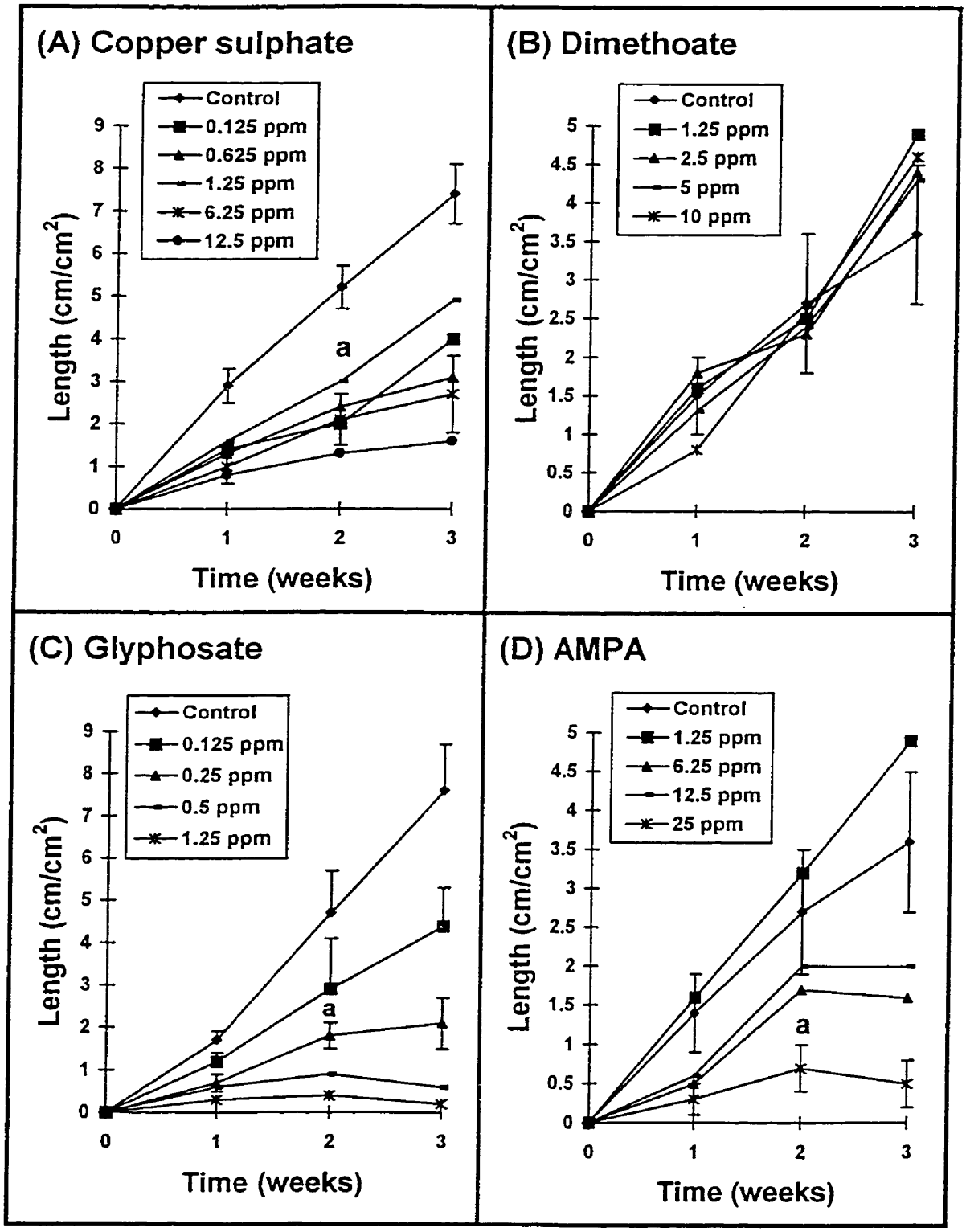


Figure 13. Effect pesticides on root growth of Ri T-DNA *Daucus carota*, (AMPA = aminomethylphosphonic acid). Values = means ± SE (n = 4) of selected test concentration series; a - indicates significant differences ($p < 0.05$) between control or solvent control and test concentration series.

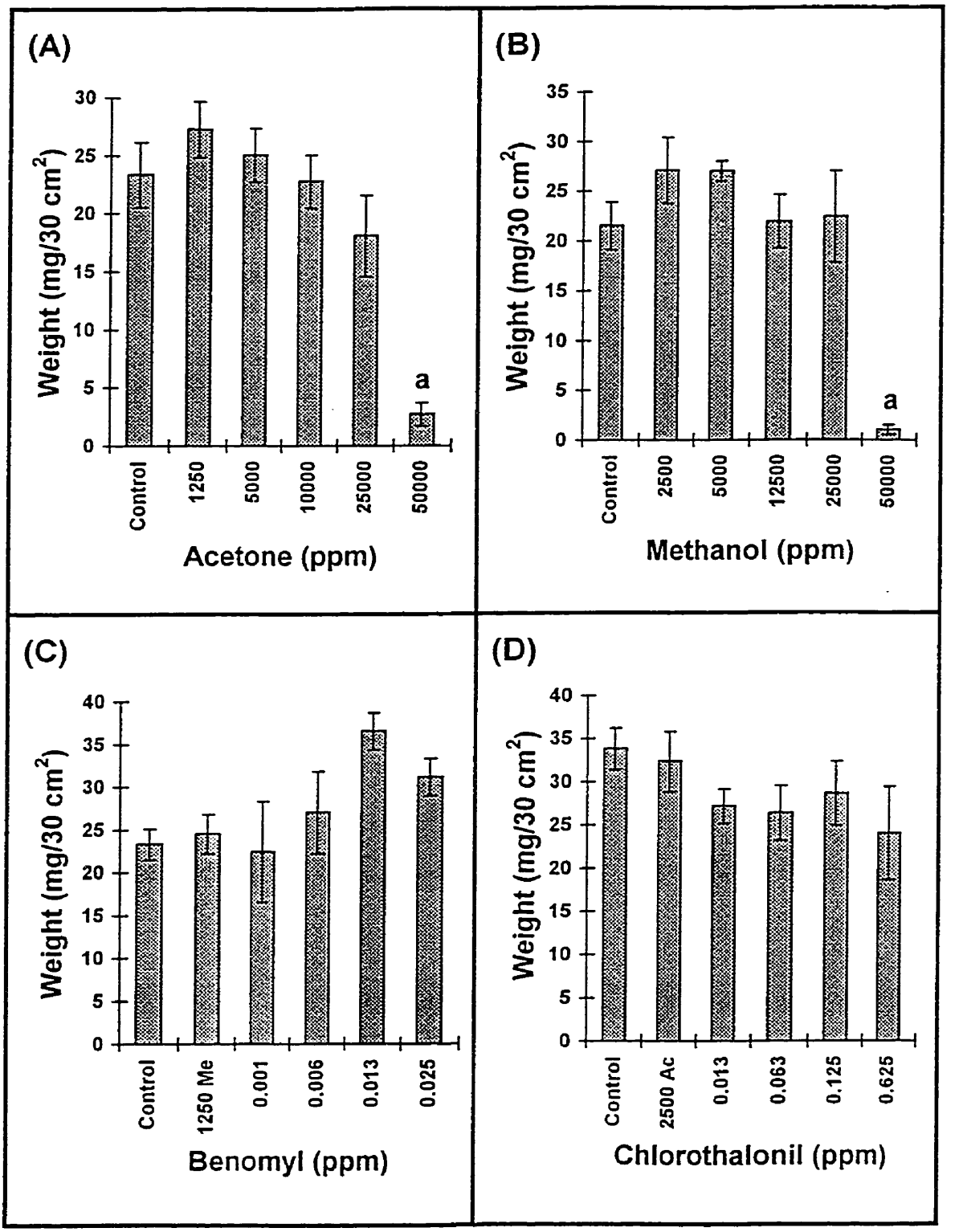


Figure 14. Effect solvents and pesticides on root dry weight of Ri T-DNA *Daucus carota*, (Ac = acetone, Me = methanol). Values = means $\pm$ SE (n = 4); a - indicates significant differences ($p < 0.05$) between control or solvent control and test concentration series.

14D) affected root biomass even at the highest concentration tested. While $CuSO_4$ (Fig. 15A) dimethoate (Fig. 15B) and AMPA (Fig. 15D) reduced root biomass significantly ($P < 0.05$) at fairly high concentrations of 6.25 ppm, 25 ppm and 12.5 ppm, respectively, glyphosate (Fig. 15C) had a significant ($P < 0.05$) impact on the biomass at the lowest test concentration of 0.125 ppm.

Effects on VAM infection

Neither of the two solvents (Figs. 16A, 16B) affected VAM formation when roots were treated at 25,000 ppm. At 50,000 ppm, however, both solvents severely ($P < 0.05$) reduced VAM development. Benomyl (Fig. 16C) reduced ($P < 0.05$) VAM formation at test concentrations as low as 0.003 ppm when compared with the methanol control, while chlorothalonil (Fig. 16D) had a similar effect at 0.13 ppm when compared with the acetone control. $CuSO_4$ (Fig. 17A), dimethoate (Fig. 17B), glyphosate (Fig. 17C),and AMPA (Fig. 17D) reduced VAM fungus colonization significantly ($P < 0.05$) in carrot roots at 6.25 ppm, 2.5 ppm, 1.25 ppm and 50 ppm, respectively.

Distinction of effects

By nature of their symbiotic association, it may be difficult to distinguish the direct and indirect effects of some solvents and test pesticides on *G. intraradices* and Ri T-DNA transformed roots of *D. carota* in the M-medium system. For most pesticides, however, it is possible to obtain an indication of the nature of effects on

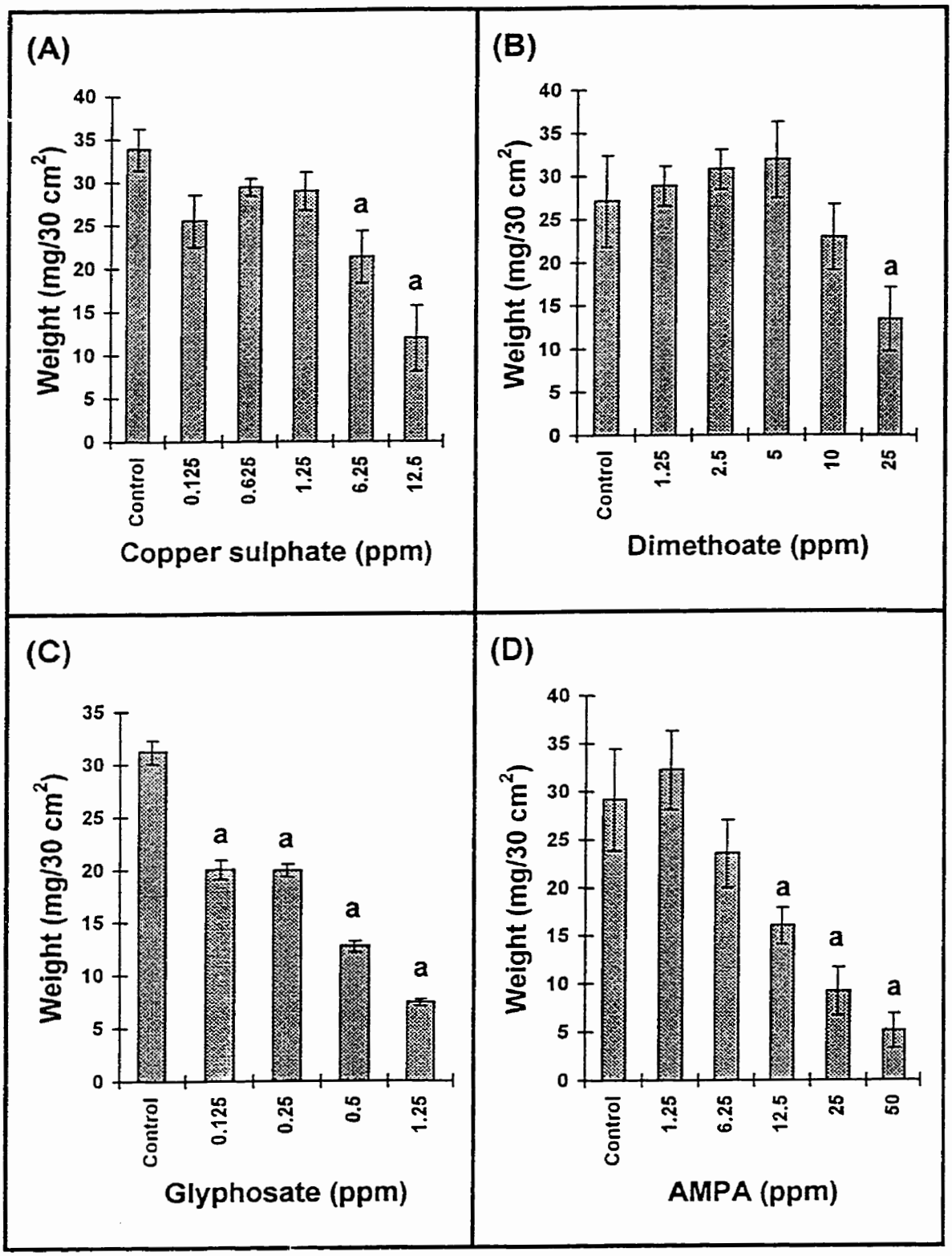


Figure 15. Effect pesticides on root dry weight of Ri T-DNA *Daucus carota* (AMPA = aminomethylphosphonic acid). Values = means ± SE (n = 4); a - indicates significant differences ($p < 0.05$) between control or solvent control and test concentration series.

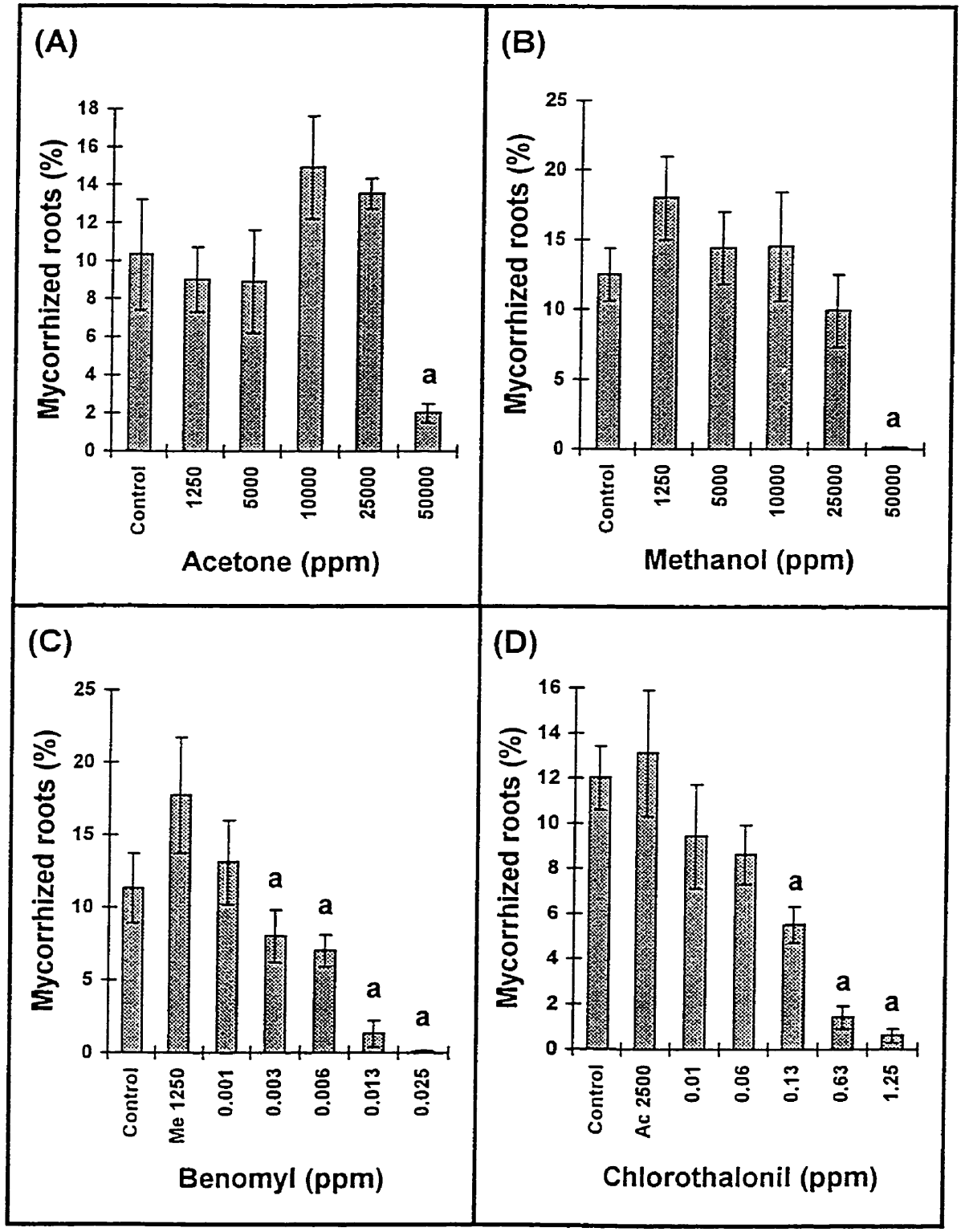


Figure 16. Effect of solvents and pesticides on colonization of Ri T-DNA *Daucus carota* roots by *Glomus intraradices*, (Ac = acetone, Me = methanol). Values = means ± SE (n = 4); a - indicates significant differences ($p < 0.05$) between control or solvent control and test concentration series.

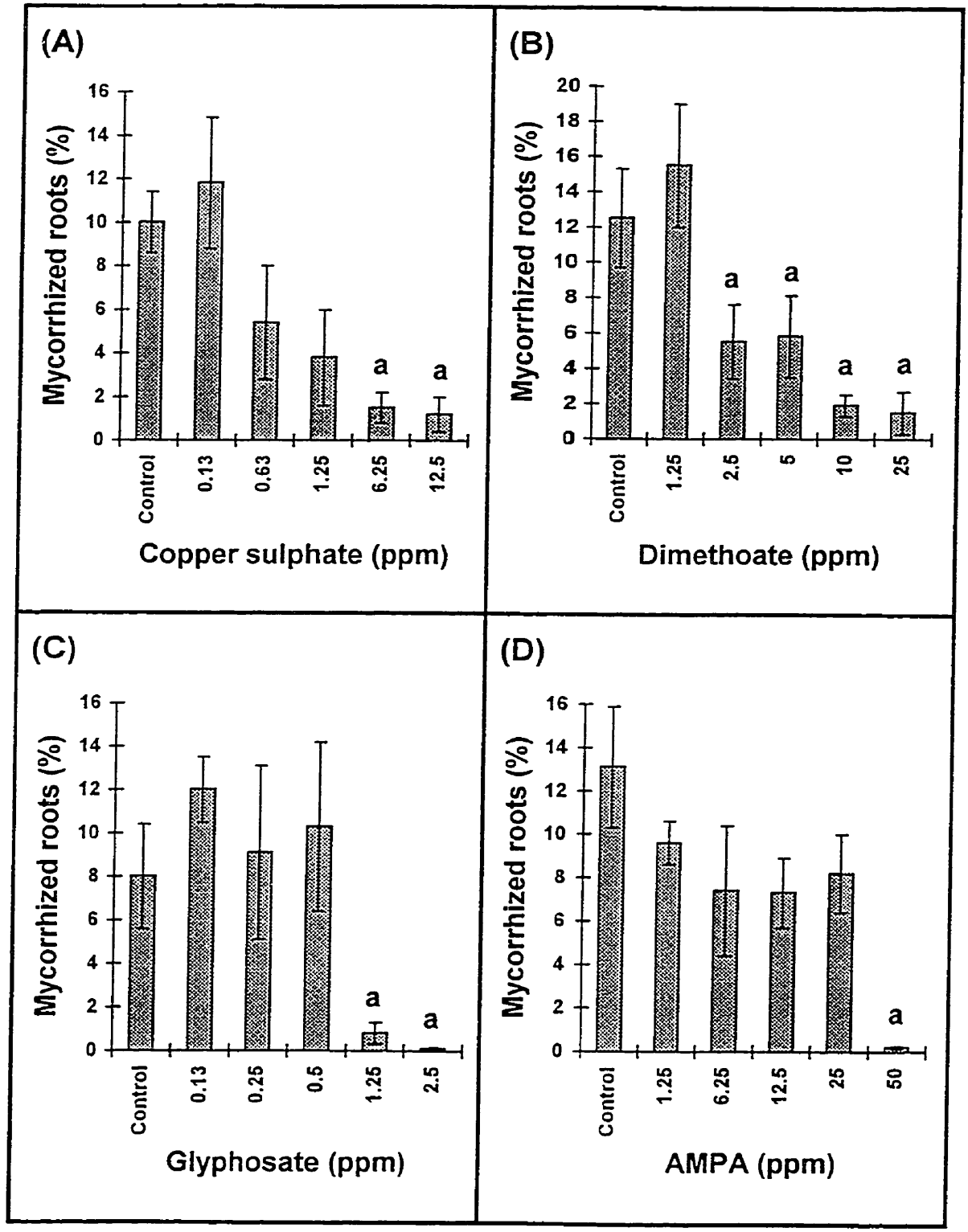


Figure 17. Effect of pesticides on colonization of Ri T-DNA *Daucus carota* roots by *Glomus intraradices*, (AMPA = aminomethylphosphonic acid). Values = means ± SE (n = 4); a - indicates significant differences ($p < 0.05$) between control or solvent control and test concentration series.

the VAM fungus and its host by comparing the impacts of solvents and test concentrations on carrot root growth, biomass, and VAM colonization with controls. For example, benomyl (systemic fungicide), chlorothalonil (contact fungicide), and dimethoate (systemic insecticide) had no significant ($P < 0.05$) impact on root growth (Figs. 12C, 12D, 13B) and biomass (Figs. 14C, 14D, 17B) at test concentrations lower than the IC50 values, but significantly ($P < 0.05$) reduced VAM fungal growth and infection (Figs. 16C, 16D, 16B) at or near the IC50 concentrations. Hence, it is reasonable to infer that these pesticides directly inhibited *G. intraradices*. In contrast, glyphosate (systemic herbicide) significantly ($P < 0.05$) inhibited root growth (Fig. 13C) and reduced its biomass (Fig. 15C), but had no significant ($P < 0.05$) effect on VAM colonization (Fig. 17C) at the IC50 concentration. This suggests that the toxicity of glyphosate to *G. intraradices* was likely indirect, and was the consequence of its direct inhibitory action on the growth of carrot roots. However, pesticides such as $CuSO_4$ (contact fungicide) and AMPA (a major degradation product of glyphosate) did not appear to have any distinct effects. Both compounds either had indistinct or little effects on root growth (Figs. 13A, 13D), its biomass (Figs. 15A, 15D) and VAM fungal colonization (Figs. 17A, 17D) at the IC50 values. This indicates that both compounds probably had some direct as well as indirect effects on the two organisms. Accordingly, in any IC50 determination, it is important to include a determination of root growth, biomass, and VAM infection to help distinguish the effects of test materials on roots and the mycorrhizal fungus.

Effect of temperature

Spiking of solvents and pesticides occurred in 200 ml Pyrex® bottles when 40 ml of the molten M-medium was cooled to 58°C ± 2°C in a water bath. It was critical and desirable, however, to spike the solvent-based reference test pesticides to the molten M-medium at a temperature that would ensure the disappearance of as much solvent via evaporation as possible without at the same time affecting the integrity of each test compound. A temperature of 58°C ± 2°C in a water bath was chosen after laboratory cooling gradient tests of molten M-medium showed that, at this temperature, the medium remained in a liquid state, which facilitated the incorporation and uniform mixing of reference pesticides during the agitation process. Below 55°C, the M-medium began to solidify rapidly, rendering uniform mixing of solvents and test pesticides a challenging task, particularly when large amounts of solvents were introduced to produce some of the required high test pesticide concentrations. The reference pesticides used in this study had b.p. ranging from 117°C to 251°C (Tomlin, 1996). Their chemical integrity was not expected to be affected when they were introduced to the molten M-medium at 58°C ± 2°C. The process of pesticide spiking, mixing, and M-medium plating of each experimental plate was completed within 20 s. After plating, the molten M-medium solidified within 10 min, cooling rapidly to a room temperature of about 25°C.

DISCUSSION

Two statistical approaches are commonly used today for estimating effects of toxicants (Stephan, 1977; Newman, 1995; Slob, 1996). One approach uses the ANOVA and null hypothesis to determine the no-observed-effect-levels (NOELs) and lowest-observed-effect-levels (LOELs) of test concentrations when compared with a control response. The other option uses regression-based models to estimate lethal or inhibitory concentrations (LCp or ICp; p = 0.01 to 0.99). Although both methods have merit, a recent analysis of 198 toxicity data found that 76.9% of the NOELs and 100% of the LOELs exceeded their corresponding 10% effect point estimates from best-fit model equations (Moore and Caux, 1997), suggesting that NOELs and LOELs are poor indicators of low toxic effects (Hoeckstra and Van Ewijik, 1993; Chapman et al., 1996; Suter, 1996). In this study, only regression-based models were considered for the determination of toxicity point estimates.

Estimates of LC50 or IC50 are used to get an idea of the toxic effect of a test compound to the normal population of a test organism when compared with a control. Choice of model matters little when estimating median effects (e.g., IC50, LC50, LD50) as long as the model fits the data (Moore and Caux, 1997). Although several regression-based statistical models were tested, the NLR and LI methods of estimating IC50 were selected because they appear to better fit the variable and sometimes wide dose-response data of test concentrations of reference compounds. Both NLR and LI methods, however, have limitations and require certain assumptions. NLR assumes normally distributed errors with constant

variance. On the other hand, LI assumes that the response to increasing concentrations of any pesticide ranges from no increase to significant decrease when compared to controls. As well, it is assumed that the SD of the "bootstrap" estimates mean could be used to obtain an estimate of the SE for the LI mean, if the difference between the two mean values did not exceed 10%. Despite these limitations, either model of statistical analysis could, in our opinion, be used to obtain an estimate of IC50, where p could range from 0.01 to 0.99. Whether the NLR or LI method is the more appropriate method to estimate IC50 is debatable.

Both the NLR and LI methods used in this study produced comparable IC50 estimates for the reference test compounds, with the exception of acetone. The IC50 estimate of acetone as determined by the NLR method appeared to be more conservative than the estimate obtained by the LI method. However, the large SE of both methods of determination negated any statistical difference of the IC50 estimate for this solvent. On the other hand, the IC50 estimates for methanol obtained by both methods of determination were almost identical. As in acetone, the IC50 estimates for methanol had large SE for both methods of determination.

Large SE indicate data variation. This is likely and collectively caused by a number of contributing factors. For the solvents, the large SE was in part caused by the relatively low b.p. of acetone (56.5°C) and methanol (64.7°C). It is conceivable that varying amounts of solvents evaporated during the 10 s agitation (to ensure uniform mixing) after spiking in each bottle prior to and during plating. Another contributing factor to data variation could be the use of a 4-plate

replication. Our previous study identified that the use of a 3-, 4- or 5-plate replicate per test concentration produced large data point variation (Wan and Rahe, 1997), due mainly to the biological variation in hyphal growth of *G. intraradices* in plated M-medium. Unlike the uniform mycelial radial growth of non-symbiotic fungi in other artificial media, the pattern of mycelial growth of the VAM fungus from Ri T-DNA transformed carrot roots into M-medium was somewhat less regular and consistent. Moreover, data variation could also be caused by the different modes of action of pesticides and their biological effects on *G. intraradices* and *D. carota*. As stated in "Results", pesticides could either effect the VAM fungus directly such as benomyl, chlorothalonil, and dimethoate, or indirectly, e.g., glyphosate, and those that appeared to cause partial effects on both *G. intraradices* and carrot roots. In addition, it was generally known that test results of any new experimental techniques tend to produce relatively large data variation and SE due to uncertainties (Hattis and Burmaster, 1994; Hoffman and Hammond, 1994).

To date, there are no published data available (AGRICOLA, 1997; BIOSIS Preview, 1997; CAB Abstracts, 1997; CA Search, 1997; and CRIS/USDA, 1997) on the toxicity of pesticides to VAM fungi for comparison with our data. However, efficacy tests of benomyl (50% WP) using ED50 (effective dosage) as a point estimate in malt-agar or potato-dextrose-agar growth systems indicate that this fungicide is highly toxic to test organisms such as *Penicillium* spp. (ED50 = 0.2 ppm), *Fusarium* spp. (ED50 = 1 ppm), and *Verticillium* spp (ED50 = 2 ppm) (Bollen and Fuch, 1970). No comparison could be made with other reference pesticides

since data on similar test organisms are not available. In our study, it is evident that the VAM fungus *G. intraradices* is even more highly sensitive to benomyl (99%), with an estimated IC50 of 0.004 ppm when compared to non-VAM fungi stated earlier.

The IC50 of pesticides to *G. intraradices* was determined by exposing the mycorrhizal fungus to test materials under conditions that would represent a "worst case" contamination scenario. These data, however, should not be extrapolated to represent the toxic effect of pesticides occurring in the field. Under field conditions, soil microbial and chemical activities would influence the effects of pesticides (Lyr, 1995). However, IC50 could and should be used for the initial screening of new candidate pesticides before registration, similar to the use of data such as LC50 (lethal concentration to 50% population of an aquatic organism) and LD50 (lethal dose to 50% population of a terrestrial animal). The potential is high for pesticides to have an adverse impact on beneficial subterranean microorganisms such as the VAM fungi, since soil is the ultimate "sink" for pesticide residues in the terrestrial environment. To enhance the usefulness of IC50 value in the eco-risk assessment of pesticides, research is presently underway to evaluate the relationship of this toxicity value with mycorrhizal symbiosis.

CONCLUSIONS

The VAM fungus *G. intraradices* is a highly sensitive indicator organism to pesticides. The research described in this study shows the potential of the Ri T-

DNA transformed carrot root-*G. intraradices* system as a simple, reproducible, practical and efficient technique for obtaining point estimates of pesticide toxicity to mycorrhizal fungi. The technique can be standardized and would make possible meaningful and useful comparisons of the effects of different pesticides on this important component of the soil eco-system. Both the NLR and LI method of ICp determination produce comparable results when EMG and EMS of the VAM fungus are used as response indicators. The order of increasing toxicity of reference test compounds to *G. intraradices* was acetone, methanol, AMPA, dimethoate, $CuSO_4$, glyphosate, chlorothalonil, and benomyl. Accordingly, ICp presents an option for comparing the short term impact of pesticides to VAM fungi, and a possible tool to further enhance the value of eco-risk assessment of pesticides and toxic substances.

CHAPTER 3

Persistence of Azadirachtin-A in Two Biological Systems Used for Culturing Mycorrhizal Fungus *Glomus intraradices*[1]

INTRODUCTION

There is now considerable interest in the search for and development of new classes of pesticides that are less damaging to the environment and non-target organisms (Rembold, 1990; Ascher, 1993). One candidate ingredient having the potential to replace synthetic chemicals for the control of subterranean insect pests is azadirachtin (AZA), a naturally occurring phytochemical with bioactivity from the neem tree *Azadirachta indica* A. Juss (Koul et al. 1989, Saxena, 1990; Lee et al., 1991). AZA is derived from neem seed kernels, which also contain many other liminoid compounds (Isman et al., 1990b; Tewari, 1992; Ley et al., 1993). Of the AZA fraction, AZA-A (Fig. 18) is the dominant compound, averaging about 79% in most samples (Hansen et al., 1994), while the remaining fraction consists of about 20% of AZA-B, and 1% of the so-called "positional isomers" (i.e., AZA-C, -D, -E, -F, -G, -H, -I, and the 22, 23-dihydro-23-a-, -b-, and methoxy-AZA) (Rembold, 1989).

To date, no studies have been conducted to evaluate the impact of AZA-A on non-target soil micro-organisms such as vesicular-arbuscular mycorrhizal fungi

[1] - Reprinted/reformatted from Wan, Rahe & Wong (1997) *J Environ Sci Health B32(6):929-953* by courtesy of co-authors and Marcel Dekker, Inc.

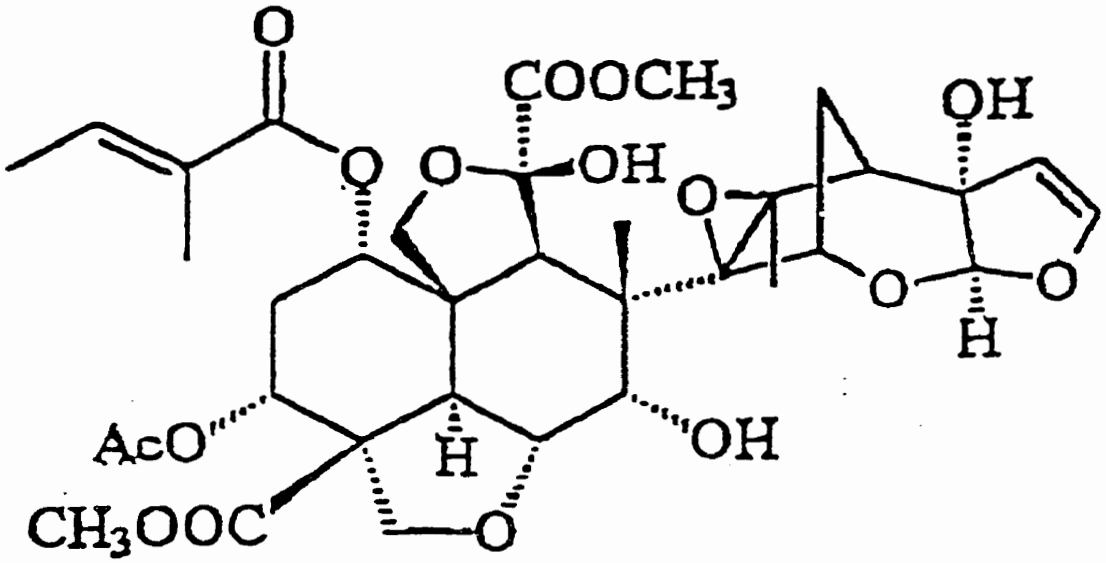


Figure 18. Structure of Azadirachtin-A (Rembold, 1989; Ley et al., 1993)

and mycorrhizal symbiosis (AGRICOLA, 1997; BIOSIS, 1997; CAB, 1997; CA SEARCH, 1997; CRIS-USDA, 1997). The objective of this research was to ascertain the presence of AZA-A in M-medium and vermiculite following its application during the period of impact assessment. Both substrates were used to culture or grow the vesicular-arbuscular mycorrhizal fungus *G. intraradices* for non-target impact evaluation under controlled environmental conditions. This chapter highlights findings of AZA-A persistence in these biological systems as described by Wan et al. (1997).

MATERIALS AND METHODS

Biological Systems Used for Culturing *G. intraradices*

Two types of biological systems were used for this study: (1) the M-medium system as described in Chapter 1, and (2) the vermiculite system. The vermiculite system (Fig. 4) consisted of a leek (*Allium porrum* L., cv. Giant Musselburg) plant growing in a 3 x 20 cm plastic Cone-tainer® Stuewe plastic vessel (Stuewe & Sons, Inc., Corvallis, Oregon) containing 120 mL of vermiculite or a soil-vermiculite mixture. Each leek plant was supported upright with a piece of twist tie attached to a wooden skewer. When required, the substrate was inoculated with about 100 propagules (hyphal and mycorrhized root pieces, viable spores, etc.) of *G. intraradices.* Inoculum of *G. intraradices* was obtained from Premier Tech Research Centre, Riviere-du-Loop, Quebec, which guaranteed a minimum of 2100

active propagules per litre. The vermiculite system was then placed in a growth chamber having a 16-h day cycle; day:night temperature of 25°C:17°C; mean RH = 68% (range, 63 -75%); and average light intensity = 4000 lx/cm^2 (range, 3850 - 4100 lx/cm^2). The plant received 40 ml Hoagland solution by drenching, and about 5 ml distilled water via spraying (simulating rain) weekly. A 100 ml plastic cup was placed under each Cone-tainer® to collect excess Hoagland solution.

Test Materials

AZA-A analytical test standard (95% pure as determined by TLC) was obtained from Sigma Chemicals, Milwaukee, WI, U.S.A. The AZA-A (~92% pure as determined by HPLC) used in this research program other than the analytical standard was obtained from Dr. M.B. Isman of the University of British Columbia (UBC), Vancouver, British Columbia, Canada. This latter material was used for the preparation of stock solutions to fortify the M-medium, vermiculite, and water.

Eneem 3G containing the equivalent of about 3% AZA-A, is an experimental "slow release" granular form of neem extracts. Neem extracts contain AZA-A (31.2%), AZA-B (5.2%), nimbin (3.0%), salannin (5.1%), and other neem compounds (55.5%) (*pers com* Isman, 1996). Eneem 3G was formulated by Phero Tech Inc., Delta, British Columbia, Canada, for trials against soil pests such as onion root maggots (*Delia antiqua* Meigen) and potato wireworms (*Agriotes obscurus* L).

Neemix® 4.5E is a water emulsifiable formulation of neem extracts formulated by Thermo Trilogy Corp, Waltham, MA, USA; it contains the equivalent of 4.5% AZA-A. This product was registered in USA. in 1996 for the control of various agricultural insect pests. Test samples were supplied by W.R. Grace & Co (the former producer), Cambridge, MA, USA.

Distribution of AZA-A in the M-medium

To find out how uniformly AZA-A was dispersed in solidified M-medium, aliquots of 0.1 ml of a solution containing 250 mg AZA/ml in 95% ethanol were added with an Eppendorf pipette to duplicate 200 ml Pyrex® bottles (~3 cm x 3 cm x 14 cm, narrow mouth), each containing 40 ml of autoclaved, molten M-medium (in a waterbath, ~58° ± 2°C) to produce a nominal concentration of 625 ppm. The bottles were then agitated by hand in a circular motion for about 10-15 s before their contents were poured into two Petri dishes. Five pieces (2 cm x 2 cm x 0.7 cm, ~1.5 g each) of M-medium were sampled from different parts of each Petri dish about 30 minutes after the medium had solidified to determine AZA-A concentrations. A plate containing M-medium without AZA-A served as the control. The M-medium pieces were extracted and purified as described under the section "Extraction and Cleanup Procedure".

AZA-A in the M-medium

AZA-A was incorporated into the M-medium of three Petri dishes at a nominal concentration of 12.5 mg AZA-A/L/plate, using the method described above. A plated M-medium without AZA-A was used as a control. The molten M-medium was allowed to solidify for 30 minutes before being inoculated with a piece of M-medium (~2.5 cm x 2.5 cm x 0.7 cm) containing mycorrhized Ri T-DNA transformed carrot roots. After inoculation, a piece (~ 2.5 cm x 2.5 cm x 0.7 cm) of M-medium from each Petri dish was aseptically sampled for the determination of AZA-A (representing day zero concentration). The remaining inoculated M-medium in each plate following sampling was covered, Parafilm® taped, and stored in darkness in an incubator at 27°C. M-medium from each plate was subsequently sampled as described earlier at 1, 3, 7, 14, 20, 42, 56, and 90 d. The M-medium samples were extracted and purified for AZA-A residue analysis as outlined under the section "Extraction and Cleanup Procedure".

AZA-A in Vermiculite

The persistence of AZA-A was monitored in (a) vermiculite (medium grains, Terra-lite®, WR Grace, Ajax, Ontario) alone (control), (b) vermiculite + AZA-A, (c) vermiculite + mycorrhizal inoculum carrier (perlite + sphagnum peat) + AZA-A, (d) vermiculite + mycorrhizal inoculum (*G. intraradices* + perlite + sphagnum peat) + AZA-A, (e) vermiculite + mycorrhizal inoculum + sandy loam soil (40 ml moist soil/L

of vermiculite). AZA-A (92% pure), Neemix® 4.5E, and Eneem 3G were used as sources of AZA-A at nominal concentration of 12 mg AZA-A/kg of vermiculite. Appropriate amounts of AZA-A (92%) or Neemix® 4.5E in 250 ml water were added to aliquots of 125 g of vermiculite (approximately 1 L in volume) in a stainless steel bucket and mixed thoroughly. For Eneem 3G, an appropriate amount of granules was added to 125 g of vermiculite in a 1.5 L wide mouth plastic bottle and mixed. The bottle was then capped and tumbled for 1 h.

Sixteen replicate 120 cc quantities of each vermiculite treatment were placed in individual containers (Cone-tainers®). A week-old leek seedling was then transplanted to each container. The containers were kept in a growth chamber having ambient conditions described earlier, and sampled at 1 h, 1, 3, 7, 14, 28, 56, and 112 d after treatment. At each sampling occasion, the leek seedling was removed, and the vermiculite was emptied into a steel vessel. Twenty five 1-g sub-samples were taken at random and pooled in a 100-mL Pyrex® bottle to make a 25-g composite sample. Duplicate composite samples were taken from each treatment time for determination of AZA-A residues. The vermiculite samples were extracted and purified for determination of AZA-A.

Extraction and Cleanup Procedure

The M-medium sample (~2 g) was homogenized with 2.5 ml of acetonitrile:water (1:3) in a tissue grinder for 1 minute. Carrot roots were removed

from the M-medium system samples taken beyond 14 d. The homogenate was then filtered under vacuum through Whatman #1 filter paper. The tissue grinder was rinsed once with 1 ml acetonitrile:water (1:3) and combined with the sample filtrate. Sample cleanup prior to analysis for AZA-A residues was done by solid phase extraction, using 6 ml tubes containing 1 g of Supelclean LC-18 reversed phase packing (Supelco, Bellefonte, PA). The tubes were prewashed with 10 ml of acetonitrile, followed by 10 ml of distilled water. Using a Pasteur pipette, samples were then quantitatively transferred to the tubes, and the eluates collected. An additional 1.5 ml of acetonitrile was then passed through each tube and combined with the sample eluate. The samples were adjusted to 5 ml for analysis by HPLC.

A mixture of 50 ml methanol:water (1:1) was added to 25 g of vermiculite or soil/vermiculite mixture in a 100-ml screw-topped centrifuge tube, and the sample was agitated for 2 h in a wrist-action shaker. The sample was then filtered under vaccum through Whatman #1 filter paper into a 500-mL round-bottom flask. The screw-topped centrifuge tube and the filter cake were washed three times with 3 x 50 ml of the solvent mix. The combined extract was quantitatively transferred into a 500-ml separatory funnel. After the addition of 10 ml 2% aqueous sodium chloride solution, the aqueous phase was partitioned three times with 50 ml aliquots of hexane and the hexane phase was discarded. The aqueous phase was then extracted three times with 60 ml aliquots dichloromethane.

The combined dichloromethane extracts were dried by adding about 5 g of

anhydrous sodium sulfate and allowed to stand for 30 minutes, then transferred to a 500-ml round bottom flask and flash evaporated to dryness. The AZA-A residues were dissolved in 3 ml of acetonitrile:water (1:3) and cleaned up by solid phase extraction as previously described. The tube was then eluted with an additional 2 ml of acetonitrile. The combined eluates were adjusted to 5 ml for HPLC analysis.

HPLC Determination of AZA-A

The method of Sundaram and Curry (1993) was modified for the determination of AZA-A by HPLC. A Hewlett-Packard HP 1090 high-pressure liquid chromatograph equipped with a Diode-Array UV-visible detector and a temperature-controlled column oven was used. The operating parameters were as follows: column, HP LiChrospher RP-Select B (5 µm, 4.6 i.d. X 250 mm); mobile solvent system, 35% acetonitrile and 65% water, isocratic at 1 ml/min; UV detector wavelength, 217 ± 2 nm; injection volume 25 µL. Quantification of AZA-A was based on peak areas of the external standards injected before and after the samples. The detector response was calibrated for each analysis with the analytical standard ranging from 1 to 50 µg/mL of AZA-A in acetonitrile:water (35:65) mix. Under the described chromatographic conditions, the absolute retention time of AZA-A was 8.448 minutes, and the detection limits were 20 µg/L for water and 200 µg/kg for vermiculite.

RESULTS AND DISCUSSION

Analysis of M-medium Extracts

During the analysis of M-medium extracts, the back pressure of the HPLC columns increased steadily from 74 to 325 millibars, indicating the gradual buildup of micro particles at the inlet end of the column. Although the HPLC column remained in working order during the analysis of all M-medium extracts, complete blockage eventually occurred. As a result, both the guard column and the analytical column had to be replaced. Accordingly, an alternative clean up procedure should be sought to improve the purification of the M-medium extracts before HPLC analysis.

Distribution of AZA-A in M-medium

There was no chromatographic response detected in the M-medium blank that interfered with the determination of AZA-A. The concentrations of AZA-A in the five duplicate pieces of gellan gum-based M-medium taken from different parts of the Petri dish varied from 563 to 625 ppm (Figure 19A). The ANOVA (One Group Variance Test) results show no significant difference ($P < 0.05$) in AZA-A concentrations in the M-medium samples from different plate locations. This suggests that AZA-A was uniformly distributed in the plated M-medium when the compound was applied to the molten medium at 58° ± 2°C by injection using an Eppendorf pipette, and followed by 10-15 seconds of agitation by shaking the

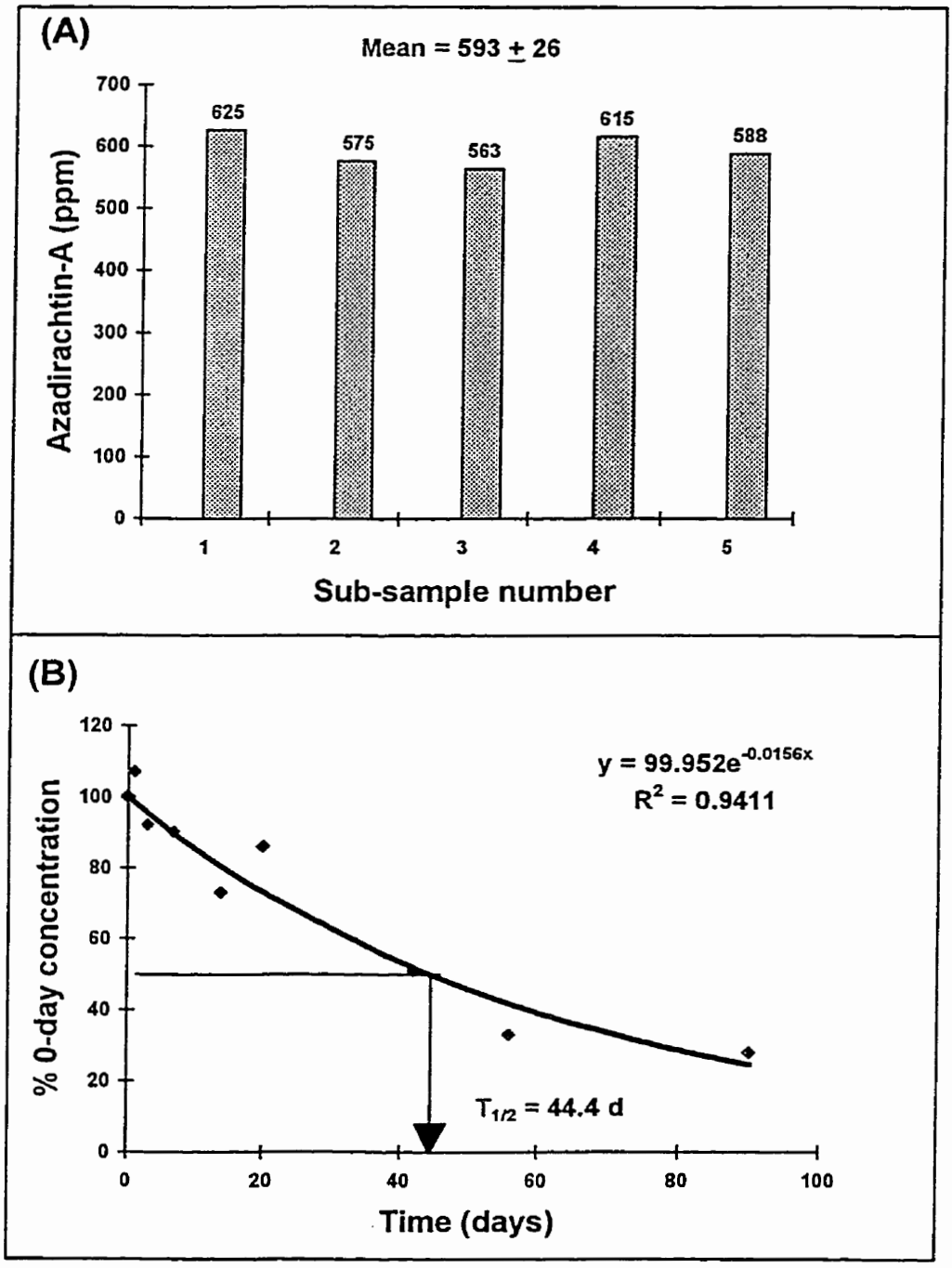


Figure 19. **A** Distribution of Azadirachtin-A in the M-medium (nominal concentration = 625 ppm) in duplicate Petri dishes (mean ± S.D., n = 5). **B** Persistence of azadirachtin-A in the M-medium system (mean values, n = 3)

bottle before pouring.

Persistence of AZA-A in M-medium

Taking the concentration at day zero as the initial concentration, the degradation of AZA-A in M-medium followed simple pseudo-first-order kinetics. The rate constants for the disappearance of AZA-A was $\log_n C_o/C = 0.0156t + 0.0011$ ($n = 9$, $r = 0.9719^*$ significant at $P = 0.05$) (Fig. 19B). The calculated half-life ($T_{1/2}$) of AZA-A in M-medium subjected to a constant temperature regime of 27°C in darkness was 44.4 d. The HPLC elution profiles of extracts of M-medium that was incubated for 7 d or longer contained two additional peaks that could be AZA-A conversion products. However, the identities of these substances were not confirmed.

Persistence of AZA-A in Vermiculite

There was no chromatographic response detected in the vermiculite blank that interfered with the determination of AZA-A. Taking the concentration at day zero as the initial concentration, the disappearance of AZA-A in vermiculite followed simple pseudo-first-order kinetics. The rate constants for the disappearance of AZA-A in vermiculite and the various amended treatments were:

(a) vermiculite + AZA-A (Fig. 20A):

$\log_n C_o/C = 0.0270t + 0.1075$ ($n = 8$, $r = 0.9785^*$, $P < 0.05$);

(b) vermiculite + mycorrhizal inoculum carrier + AZA-A (20B):

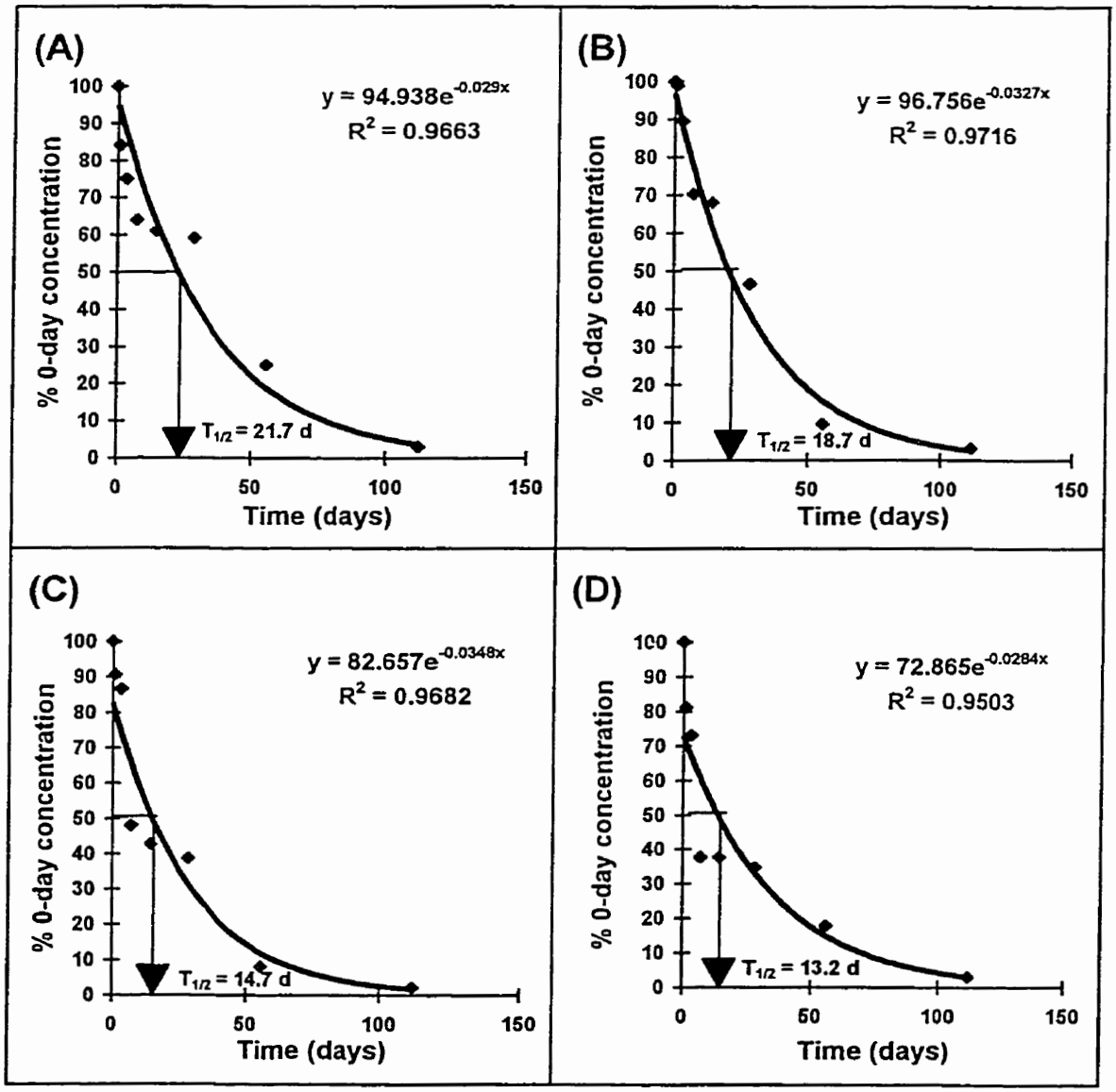


Figure 20. Persistence of Azadirachtin-A in the vermiculite system (mean values; n = 2 composite samples, each containing 25 sub-samples): **A** vermiculite; **B** vermiculite + mycorrhizal inoculum carrier; **C** vermiculite + mycorrhizal inoculum; **D** vermiculite + mycorrhizal inoculum + sandy loam soil

$\log_n C_o/C = 0.0401t - 0.0552$ ($n = 7$, $r = 0.9814^*$, $P < 0.05$);

(c) vermiculite + mycorrhizal inoculum + AZA-A (Fig. 20C):

$\log_n C_o/C = 0.0416t + 0.1098$ ($n = 7$, $r = 0.9697^*$, $P < 0.05$);

(d) vermiculite + mycorrhizal inoculum + sandy loam soil (40g wet weight) (Fig. 20D):

$\log_n C_o/C = 0.0282t + 0.3221$ ($n = 8$, $r = 0.9737^*$, $P < 0.05$).

The calculated $T_{1/2}$ lives of AZA-A were 21.7 d in vermiculite alone, 18.7 d in vermiculite + mycorrhizal inoculum carrier, 14.7 d in vermiculite + mycorrhizal inoculum, and 13.2 d in vermiculite + mycorrhizal inoculum + sandy loam soil (40g wet wt). Based on the chromatograms of the vermiculite extracts from the various treatments, there was evidence of the formation of AZA-A conversion products in vermiculite incubated for 7 d and longer. However their identities were not confirmed. An example of these chromatograms is illustrated in Figure 21.

Persistence of AZA-A from Eneem 3G and Neemix® 4.5E in Vermiculite

There was no chromatographic response detected in the vermiculite blank that interfered with the determination of AZA-A. Taking the concentration at day zero as the initial concentration, the disappearance of AZA-A from Eneem 3G and Neemix® 4.5E in vermiculite containing vesicular arbuscular mycorrhizal fungus followed simple pseudo-first-order kinetics. The rate constants for the disappearance of AZA-A from these two formulated products in vermiculite were:

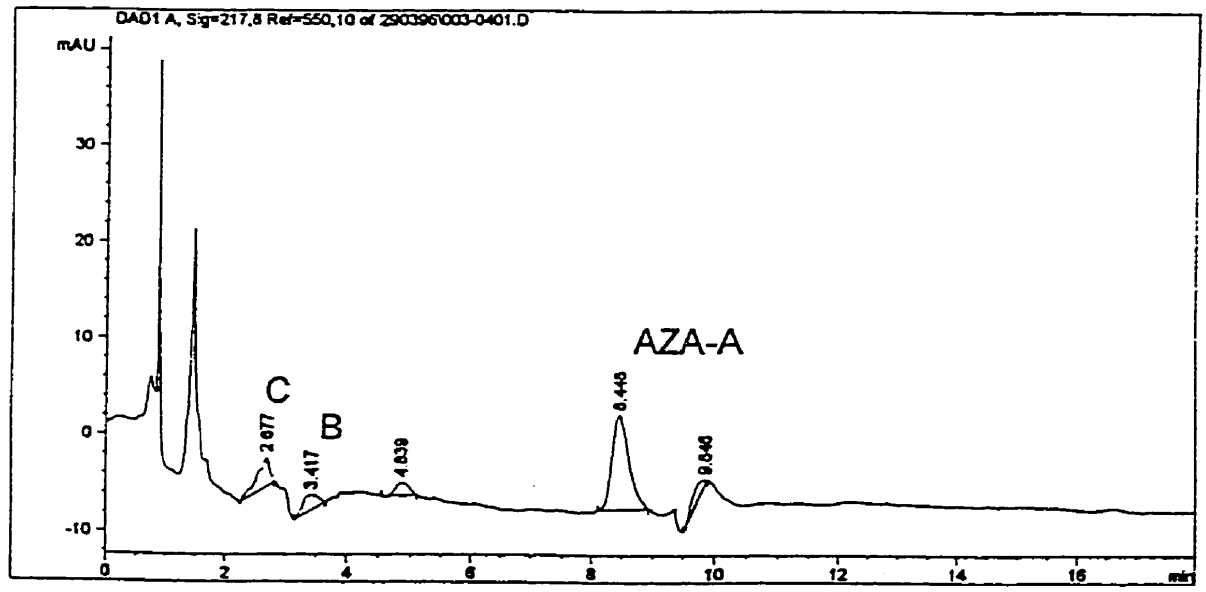


Figure 21. Elution profile of UV-absorbing substances in extract from vermiculite treated with 12 ppm AZA-A (~92% pure), 56 days post treatment. (B, C = unidentified peaks not present in samples from treatments incubated for 7 days or less)

(a) vermiculite + mycorrhizal inoculum + AZA-A from Eneem 3G (Fig. 22A):

$\log_n C_o/C = 0.0150t + 0.0006$ ($n = 9$, $r = 9668^*$, $P < 0.05$);

(b) vermiculite + mycorrhizal inoculum + AZA-A from Neemix® 4.5E (Fig. 22B):

$\log_n C_o/C = 0.0334t + 0.0369$ ($n = 9$, $r = 9022^*$, $P < 0.05$).

The calculated $T_{1/2}$ lives of AZA-A in vermiculite were 46.2 and 20.5 d for Eneem 3G and Neemix® 4.5E, respectively. The chromatograms of the vermiculite extracts from these two products showed at all times of analyses a second peak appearing immediately after AZA-A, which was possibly AZA-B residues. As well, HPLC elution profiles of the extracts of amended vermiculite that were incubated for 7 d or longer contained additional peaks that could possibly be AZA-A and AZA-B conversion products. However, the identity of none of these peaks was confirmed.

Persistence of AZA-A in M-medium and Vermiculite Systems

Although it is known that AZA-A degrades rapidly when exposed to heat (> 165°C), moisture, air (Mordue and Blackwell, 1993) and sunlight (Barnby et al., 1989), there is no information on its persistence in matrices such as M-medium, vermiculite, and vermiculite/soil mixture. Recently, Sundaram and Curry (1993) and Szeto and Wan (1996) determined the persistence of AZA-A in water. In sterilized aqueous buffers of pH 4.1-8.1 and in four natural waters of pH 6.2-8.1 at

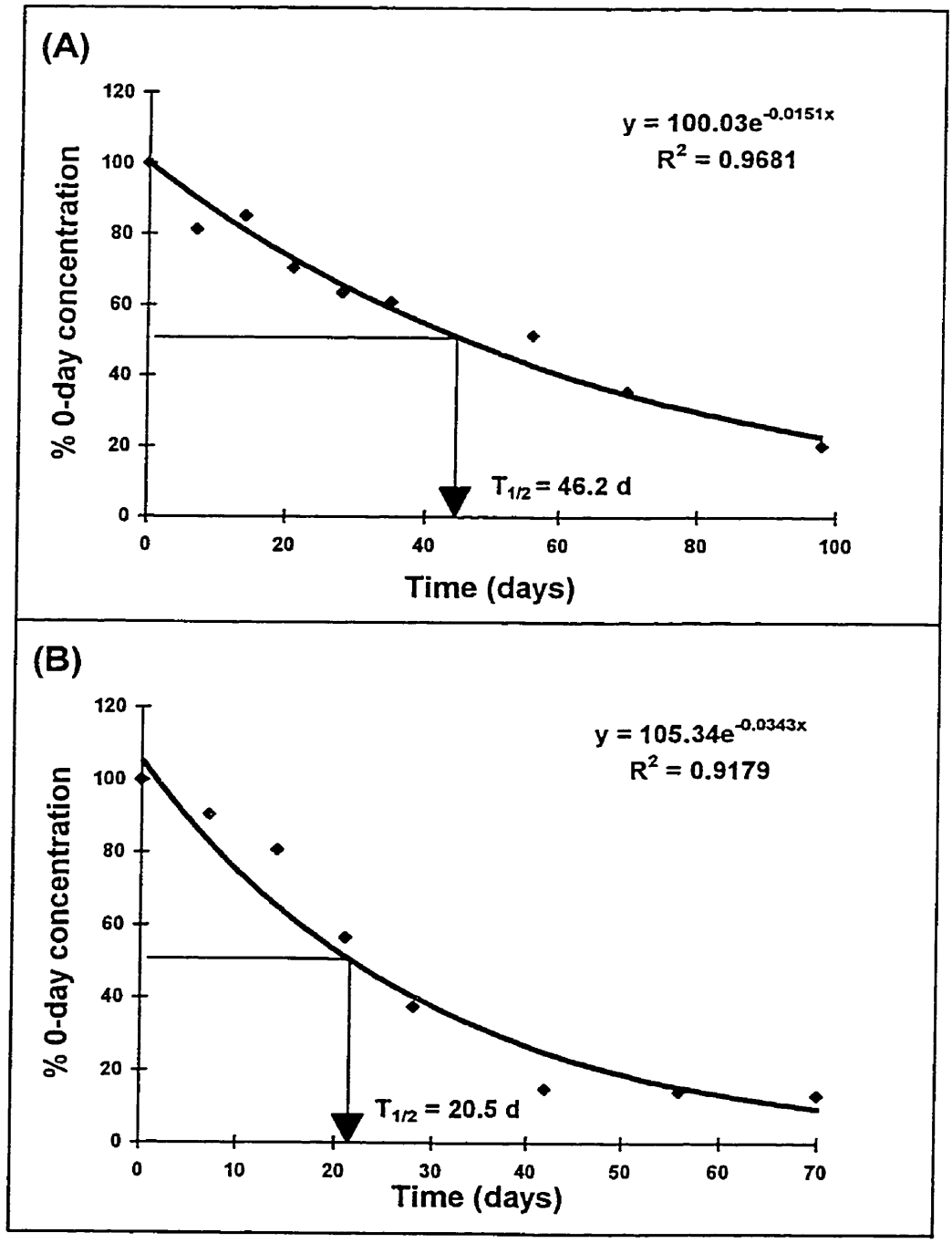


Figure 22. Persistence of Azadirachtin-A obtained from **A** Eneem 3G or **B** Neemix® 4.5E in the vermiculite system (mean values; n = 2 composite samples, each contained 25 sub-samples)

20-45°C, AZA-A hydrolyzed readily (Szeto and Wan, 1996). Its disappearance in water incubated in darkness followed simple pseudo-first-order kinetics, and was more rapid at basic than acidic pH. Sundaram et al. (1993) reported that AZA-A degrades much faster in sterilized water (DT50 = 6.91 d; pH = 8.08; 20° C) when compared to unsterilized water (DT50 = 11.94 d; pH = 7.36; 20° C), and attributed this phenonmenon to the faster rate of hydrolysis at pH 8.08 than pH 7.36 and reduction of microbial degradation due to the probable bactericidal effect of AZA-A on the unsterilized water.

All of the above studies were conducted in matrices that are simple relative to those required for microbial culture, such as the M-medium and vermiculite systems needed for culturing of vesicular arbuscular mycorrhizae. AZA-A had a $T_{1/2}$ of 9.5 d in pH 5.5 water at 35°C (Szeto and Wan, 1996). This pH was quite close to the water used for the preparation of M-medium (pH = 5.8), but the temperature was 8°C higher than the incubation temperature of 27°C for the medium. The calculated $T_{1/2}$ for AZA-A in sterile M-medium incubated in darkness at 27°C was 44.4 d. The calculated $T_{1/2}$ of AZA-A incoporated in M-medium by injection was 34.9 d longer than its half-life in distilled water possibly because of the lower temperature as well as other factors. AZA-A persisted longer in sterilized M-medium than in sterile water. It is speculated that the water molecules in the M-medium may not be as freely available for AZA-A hydrolysis as in pure water. AZA-A persisted in the M-medium for more than 6 weeks. This duration of AZA-A

persistence is crucial because a minimum of 4- to 5-week long observation period may be required for the vesicular-arbuscular mycorrhizal bioassay when using the M-medium system.

To date, little information is available on the behaviour of AZA-A in terrestrial environments. A study by Stark and Walter (1995) indicates that the half-lives for AZA-A in sterilized and unsterilized sandy loam soil at 25° C in a day:night cycle of 16h:8h were 32 and 20 d, respectively. In this study, the calculated half-lives for AZA-A in unsterilized vermiculite, vermiculite + mycorrhizal inoculum carrier, vermiculite + mycorrhizal inoculum, and vermiculite + mycorrhizal inoculum + sandy loam soil were 21.7, 18.7, 14.0 and 13.2 d, respectively. The conclusion that microbial activity contributes substantially to the disappearance of AZA-A from soil and vermiculite based matrices is supported by both studies. The inclusion of mycorrhizal inoculum (consisting of a mixture of peat, perlite, soil particles and about 2100 active *G. intraradices* propagules/L) in the vermiculite system appears to have accelerated the degradation of AZA-A. This could be attributed to the presence of a rich population of microorganisms in the mycorrhizal inoculum (actinomycetes, 2.3×10^8; bacteria, 8.4×10^8; fungi, 9.5×10^5 CFU/g dry weight, as determined in the laboratory by using Starch-Casein agar, Thornton's Standard agar, and Neopeptone-Glucose-Rose Bengal-Aureomycin agar, respectively) and sandy loam soil (actinomycetes, 1.3×10^6; bacteria 4.3×10^7; fungi, 1.4×10^6 CFU/g dry weight) when compared to vermiculite alone (actinomycetes, $< 2.5 \times 10^3$;

bacteria, < 2.5 x 10^3; fungi, < 2.5 x 10^3 CFU/g dry weight). These microorganisms may have contributed to the increased rate of biodegradation of AZA-A.

The half-lives of pure AZA-A in the vermiculite system having the various amendments ranged from 13.2 to 21.7 d, while the half-lives of AZA-A from the formulated products Eneem 3G and Neemix® 4.5E in unsterilized vermiculite were 46.2 and 20.5 d, respectively. AZA-A from Eneem 3G, the granular product of neem extracts, had a half-life that was 2.1 and 3.5 times longer in vermiculite than those AZA-A obtained from Neemix® 4.5E and technical AZA-A, respectively.

Evidence for the presence of unidentified AZA-A conversion products in M-medium, vermiculite, and vermiculite/soil mixture was obtained. Studies by Stark and Walter (1995) and Szeto and Wan (1996) also reported the occurrence of unidentified peaks that may have been AZA-A conversion products. As expected, there was evidence of the occurrence of AZA-B in the HPLC chromatograms of Eneem 3G and Neemix® 4.5E. Both materials are formulated products of natural Neem extracts, which contain other isomers of AZA besides AZA-A (*pers com* Isman, 1997). Quantification of AZA-B was not undertaken, as a standard of this compound was not available at the time of the study.

Both the M-medium and vermiculite systems will be used for the non-target impact evaluation of AZA-A on vesicular arbuscular mycorrhizae. The M-medium system will be used for sub-lethal bioassays of the compound on the mycorrhizal fungus *G. intraradices*. A 2- to 4-week observation period will be needed to

determine the impact, if any, of AZA-A on the growth response of this organism. Accordingly, this compound must be present in sufficient concentration after its incorporation into the M-medium during that period of time. The vermiculite system is intended to simulate growing conditions occurring in nature, and it will be used for the impact evaluation of AZA-A on the mycorrhizal symbiosis. It is also essential that AZA-A persists for at least 2 to 4 weeks after the time of inoculation of mycorrhizal fungus and transplantation of host plants, to allow sufficient time for the fungus to come in contact with, and respond to, the toxicant in the growth substrate. The results of this study confirm that the persistence of AZA-A in both the M-medium and vermiculite systems meet this criterion.

CONCLUSIONS

Refinements to the clean up procedures for gellan gum-based M-medium extracts for HPLC analysis are needed. AZA-A was completely incorporated and uniformly distributed in the M-medium when it was introduced by injection to the molten solution at 56-60°C. The disappearance of AZA-A in M-medium and in vermiculite systems followed pseudo-first order kinetics. The half-life of AZA-A was 44.4 d in M-mediumcontaining *G. intraradices* and *D. carota*. In the systems containing vermiculite, vermiculite and soil mixture, mycorrhizal fungus and leeks, the half-lives for technical and formulated AZA-A ranged from 13.2 to 46.2 days. Evidence for possible AZA-A conversion products in AZA-A treated and aged M-

medium, vermiculite, and soil-vermiculite mixture, as well as the presence of AZA-B residues in products formulated with natural neem extracts was observed. AZA-A persisted long enough in both systems to allow non-target impact assessment of the chemical to be conducted on vesicular arbuscular mycorrhizal symbiosis.

CHAPTER 4

Impact of Azadirachtin on *Glomus intraradices* and Vesicular-arbuscular Mycorrhiza in Ri T-DNA Transformed Roots of *Daucus carota*[1]

INTRODUCTION

The adverse impacts of many synthetic pesticides on the environment and non-target organisms have motivated the search for and development of new classes of compounds that are less damaging (Rembold, 1990; Isman et al., 1990a; Wan et al., 1997). One candidate having the potential to replace some synthetic chemicals for the control of insect pests is azadirachtin (AZA), a naturally occurring phytochemical from the neem tree *Azadirachta indica* A. Juss (Saxena, 1990; Schmutterer, 1995). AZA is derived from neem seed kernels, which also contain many other limonoid compounds (Jones et al., 1988; Tewari, 1992; Ley et al., 1993). Of the total AZA (Fig. 17) fraction, AZA-A is the major component, averaging about 75% - 90% in most samples (Hansen et al., 1994), while the remainder consists of about 10% - 25 % of AZA-B (3-tigloylazadirachtol), and 1% of the so-called "positional isomers" (i.e., AZA-C, -D, -E, -F, -G, -H, -I; 22, 23-dihydro-23-a-, -b-, and methoxy-AZA) (Rembold, 1989). AZA-A, AZA-B and isomers are

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collectively referred to as AZA, as they have the same basic molecular structure and similar bioactivity. AZA is photosensitive and has a MW of 721 and a m.p. of 165°C. This botanical compound is an insecticide acting mainly as an antifeedant and/or insect growth regulator at low concentrations (0.05 - 5 ppm), and a contact poison at concentrations > 50 ppm (Isman et al., 1996; Mordue and Blackwell, 1993). There are also claims that it has fungicidal properties at all these concentrations (Nagarajan and Reddy, 1980; Shetty et al., 1989). Recently, an experimental slow release granular AZA product was developed for use against subterranean insect pests. Several AZA products have been registered since 1996 in the USA for the control of agricultural insect pests (Wan et al., 1997), but none is yet registered in Canada.

In the eco-toxicological risk evaluation of pesticides, indicator organisms from different trophic levels (Environment Canada, 1996; US EPA, 1996) are represented but these do not include beneficial soil microorganisms such as vesicular-arbuscular mycorrhizal (VAM) fungi (Wan et al., 1998). In our opinion, there is a need to include VAM fungi to further enhance the overall value of eco-toxicological risk assessment of soil applied pesticides. VAM fungi, e.g., *Glomus* spp., are geographically cosmopolitan (Mosse et al., 1981). They are commonly found in the rhizosphere and in roots of more than 90% of vascular plants growing in the arctic, temperate, and tropical regions. Beside their symbiotic role in promoting plant growth and survival, they are implicated in enhancing eco-sustainability (Allen, 1991). To date, no studies have been conducted to evaluate the impact of AZA on

VAM fungi and VAM symbiosis (AGRICOLA, 1997; BIOSIS Previews, 1997; CA Search, 1997; CAB Abstracts, 1997; CRIS/USDA, 1997). The objective of this study was to determine the effect of AZA, NEX and its formulated products, Eneem 3G and Neemix 4.5E, on *Glomus intraradices* Schenck & Smith (DAOM #181602) and on its symbiotic association in Ri T-DNA transformed roots of *Daucus carota* L., using the technique developed and described by Wan et al. (1998). The data generated were used to evaluate and predict the eco-toxicological impact potential of azadirachtin and neem compounds.

MATERIALS AND METHODS

The M-medium system

This system of culturing *G. intraradices* for research purposes was supplied by the Institut Recherche en Biologie Végétale, de l'Université de Montréal, Montreal, Quebec. The system was selected for this study for convenience, reliability, results reproducibility, and also for its novelty. It consists of self-propagating mycorrhized Ri T-DNA transformed roots of *D. carota* growing in sterilized minimal medium (M-medium), which contains various salts, vitamins, sucrose and water solidified with 0.4% (w/v) gellan gum (ICN Biomedical, Cleveland, Ohio) in a Petri dish as described by Becard and Fortin (1988) and Wan and Rahe (1997). The roots serve as the host symbiont for the VAM fungus *G. intraradices*, while the plated M-medium serves as a source of nutrients for the roots and a medium for colonization by the fungus. The closed Petri dishes (100 x 15

mm) containing the M-medium system were taped with a piece of Ruban® invisible cellophane tape at two places (~ 180° apart) to facilitate laboratory handling, and then incubated for 28 d in an inverted position in darkness at 27°C.

Test Materials

The AZA (~92% pure as determined by HPLC) and neem extracts (NEX) used in this research program were obtained from Dr. M.B. Isman of the University of British Columbia (UBC), Vancouver, British Columbia, Canada. NEX contained AZA (31.2%), AZA-B (5.2%), nimbin (3.0%), salannin (5.1%), and other neem compounds (55.5%). These materials were used for the preparation of stock solutions to spike the M-medium. Formulated products of AZA, i.e., Eneem 3G and Neemix 4.5E, also were studied. Eneem 3G containing the equivalent of about 3% AZA, is an experimental "slow release" granular formulation of NEX. Eneem 3G (27.8% NEX, 70% ammonium sulphate, 2% silica, and 0.2% magnesium stearate) and its solid carrier was formulated by Phero Tech Inc., Delta, British Columbia, Canada, for trials against soil pests such as onion root maggot (*Delia antiqua* Meigen) and potato wireworm (*Agriotes obscurus* L). Neemix® 4.5E is a water emulsifiable formulation of NEX formulated by Thermo Trilogy Corp, Waltham, MA, U.S.A. This product contains the equivalent of 4.5% AZA and 95% proprietary alcohol-based solvent/carrier. Neemix® 4.5E was registered in the U.S.A. in 1996 for the control of various agricultural insect pests. Test samples were supplied by W.R. Grace & Co (the former producer), Cambridge, MA, U.S.A. Acetone (100%),

ethanol (99.9%), methanol (99.8%), and propanol (99.5%) were used as solvents for AZA, and they were obtained from Commercial Alcohols Ltd., Brampton, Ontario, Canada.

Test procedure

A short term, 14-d sub-lethal test developed by Wan et al. (1998) was used to determine the IC50 to *G. intraradices* of AZA, NEX, NEX formulated products and their carriers/solvents for comparison with reference pesticides. The indicators of response to the test compounds used for the IC50 estimation were the extraradical mycelial growth (EMG) and extraradical mycelial sporulation (EMS) of *G. intraradices*. Their responses to the inhibitory effects of each test compound at 2 weeks when compared with the water or solvent/carrier controls were used for the IC50 estimation. The measurement of EMG, EMS, and Ri T-DNA transformed carrot roots was conducted by using the grid method (Tennant, 1975; Wan and Rahe, 1997). The bioassay of each test material was repeated three times. An initial concentration range-finding test was conducted for all materials. Once the inhibitory range concentrations were established, the individual test concentrations within the range varied serially by two to five fold. For each bioassay, five test concentrations were used. The mean IC50 values of the final two tests are presented in the results. The bioassay was declared invalid and repeated if more than one plate in each four-plate replicate of each test concentration series was contaminated by micro-organisms. The IC50 values of all test materials, including reference

pesticides and solvents, were also determined after each compound was aged for a 30-d period in plated M-medium at 27°C in darkness before inoculation, in order to determine the residual bioactivity, if any, of the original test materials.

Growth of Ri T-DNA transformed carrot roots was observed for three weeks in water, solvent, or pesticide spiked M-medium for comparison. The biomass (mg dry wt), and VAM colonization (%) of roots were then determined at week four for the same reason. For dry weight determination, the original piece of VAM inoculum (2.5 cm x 2.5 cm x 0.74 cm or equivalent size) that was placed on each plate was first carefully removed using a surgical blade. Carrot roots from one half (visually determined by placing the bioassay plate on top of a plate imprint having equal halves drawn) of the M-medium of each plate were then carefully removed using forceps, placed in a 60 mm x 15 mm Al weighing dish, and gently dried for 48 h in a laboratory hood at 25°C and a RH of about 50%. The roots from the remaining half of the M-medium were used for the determination of VAM colonization. The grid-line intersect and slide method of Giovannetti and Mosse (1980) were used for this determination after the roots were cleared and stained using the trypan blue procedure described by Dalpe (1993).

Two methods of statistical analysis could be used to estimate and produce comparable IC50 values (Wan et al., 1998): the nonlinear regression test (SAS, 1996) and the linear interpolation calculated by the ICPIN program Version 2.0 (Norberg-King, 1993). For simplicity and convenience, however, only the nonlinear regression test was used in this study. Root dry weight and VAM mycorrhizal

colonization were subjected to ANOVA and Student's *t*-test at the 5% level (SAS, 1996).

Unless indicated otherwise, methanol was the solvent used for AZA and neem products. The rationale for selecting this solvent will be elaborated on in the "Discussion".

RESULTS

Effect on *Glomus intraradices*

Solvents and carriers were toxic to *G. intraradices* to varying degrees (Table 3) when EMG was used as the response indicator. Acetone and methanol were of relatively low toxicity when compared with ethanol and propanol. Eneem 3G and Neemix 4.5E carriers in methanol were as toxic to this VAM fungus as propanol. The data indicate that solvents influence the toxicity of AZA to *G. intraradices*, as AZA in methanol (IC50 = 230 ppm) was about 2, 70, and 210 times less toxic than in acetone, ethanol, and propanol, respectively. The toxicity of NEX to this VAM fungus, however, was more difficult to determine. Despite several attempts, the IC50 values obtained were less accurate due to what appeared to be the development of and interference by methanol tolerant and thermophilic micro-organisms present in the NEX. From a toxicological perspective, neither AZA nor NEX and its formulated products, Eneem 3G and Neemix 4.5E in methanol solvent (Table 3) were less toxic to *G. intraradices*, when compared with reference systemic and contact fungicides such as benomyl, chlorothalonil, and $CuSO_4$. IC50

Table 3. 50% inhibitory concentration (IC50[a]) of test materials on the extraradical mycelial growth (EMG) of *Glomus intraradices* 14-d after inoculation on pesticide treated M-medium aged for 0 d and 30 d

Compounds[b]	IC50 ± SE (ppm)	
	0-d	30-d
Solvents/carriers		
Acetone (S)	20000 ± 13000[c]	18000 ± 1000
Ethanol (S)	170 ± 30	2000 ± 1100
Methanol (S)	14800 ± 7000	10000 ± 6000
Propanol (S)	70 ± 35	100 ± 45
Eneem carrier (C)	60 ± 30	1200 ± 900
Neemix carrier (C)	80 ± 40	110 ± 50
Neem compounds		
Azadirachtin (F, I) in acetone	105 ± 45	115 ± 70
Azadirachtin (F, I) in ethanol	3.3 ± 1.7	11.7 ± 9.5
Azadirachtin (F, I) in methanol	230 ± 70	70 ± 40
Azadirachtin (F, I) in propanol	1.1 ± 0.6	7.3 ± 6.5
Neem extract (F, I)	~60 ± 25	> 300
Eneem 3G (I)	210 ± 120	70 ± 35
Neemix 4.5E(I)	130 ± 55	90 ± 25
Reference pesticides		
Benomyl (F)	0.004 ± 0.001[c]	0.003 ± 0.001
Chlorothalonil (F) in acetone	0.05 ± 0.02[c]	0.05 ± 0.03
$CuSO_4$ (F)[d]	0.6 ± 0.2[c]	0.10 ± 0.03
Dimethoate (I)[d]	3 ± 1.5[c]	1.5 ± 0.9
Glyphosate (H)[d]	0.5 ± 0.3[c]	0.5 ± 0.3
AMPA (H)[d]	4.2 ± 2.8[c]	1.1 ± 0.5

[a] - mean of two nonlinear regression estimates, nearest whole or decimal; solvent = methanol unless indicated

[b] - C = carriers, F = fungicides, H = herbicides, I = insecticides, S = solvents

[c] - data from Wan et al. (1998)

[d] - water

estimates obtained using EMS as the response indicator (Table 4) provided a similar toxicity ranking for AZA, neem pesticides, reference pesticides, carriers, and solvents. Generally, EMS appeared to be a slightly more sensitive response indicator than EMG.

Using both EMG and EMS as response indicators, the toxicity of solvents and carriers to *G. intraradices* either remained unchanged or decreased to varying degrees after the treated M-medium (under sterilized conditions) was aged for 30-d in darkness at 27 °C before inoculation (Tables 3 and 4). This was to be expected as varying amounts of solvents/carriers would have probably disappeared via evaporation. For AZA (except in methanol), the 30-d aging process of treated M-medium tended to generally decrease its toxicity to the VAM fungus to varying degrees. This decrease in toxicity was probably due to the degradation of AZA. In contrast, both Eneem 3G and Neemix 4.5E appear to increase in toxicity to the fungus after the 30-d aging process. This was probably due to the presence of AZA and other metabolites. The toxicity of reference pesticides, as a whole, remained unchanged after the 30-d aging process.

Effect on *Daucus carota*

Growth in length of Ri T-DNA transformed carrot roots in the M-medium is triphasic (Wan and Rahe , 1997). Typically, roots grow slowly one week after inoculation, then elongate rapidly from 2 to 4 weeks, and reach a stationary phase after 5 weeks. At 2 weeks, acetone (Fig. 23A) and methanol (Fig. 23B) inhibited (P

Table 4. 50% inhibitory concentration (IC50[a]) of test materials on the extraradical mycelial sporulation (EMS) of *Glomus intraradices* 14-d after inoculation on pesticide treated M-medium aged for 0 d and 30 d

Compounds[b]	IC50 ± SE (ppm)	
	0-d	30-d
Solvents/carriers		
Acetone (S)	11000 ± 9000[c]	22000 ± 18000
Ethanol (S)	120 ± 50	1900 ± 1500
Methanol (S)	20000 ± 2000[c]	16000 ± 11000
Propanol (S)	14.2 ± 6.9	40 ± 20
Eneem carrier (C)	75 ± 30	2200 ± 1200
Neemix carrier (C)	30 ± 25	40 ± 30
Neem compounds		
Azadirachtin (F, I) in acetone	110 ± 60	26 ± 9
Azadirachtin (F, I) in ethanol	1.9 ± 1.4	16.1 ± 16
Azadirachtin (F, I) in methanol	80 ± 45	70 ± 40
Azadirachtin (F, I) in propanol	1.3 ± 1.0	7 ± 7
Neem extract (F, I)	~35 ± 20	> 300
Eneem 3G (I)	50 ± 17	100 ± 45
Neemix 4.5E (I)	20 ± 13	50 ± 30
Reference pesticides		
Benomyl (F)	0.003 ± 0.001[c]	0.003 ± 0.001
Chlorothalonil (F) in acetone	0.04 ± 0.01[c]	0.05 ± 0.02
$CuSO_4$ (F)[d]	0.3 ± 0.2[c]	0.09 ± 0.04
Dimethoate (I)[d]	1.4 ± 0.6[c]	0.7 ± 0.5
Glyphosate (H)[d]	0.5 ± 0.3[c]	0.05 ± 0.03
AMPA (H)[d]	3.8 ± 2.7[c]	2.1 ± 1.5

[a] - mean of two nonlinear regression estimates, nearest whole or decimal; solvent = methanol unless indicated

[b] - C = carriers, F = fungicides, H = herbicides, I = insecticides, S = solvents

[c] - data from Wan et al. (1998)

[d] - water

< 0.05) the growth of *D. carota* roots relative to water controls only at 50,000 ppm. Ethanol (Fig. 23C) and propanol (Fig. 23D) had a similar effect ($P < 0.05$) at 2,500 ppm and 1500 ppm, respectively. AZA in acetone (Fig. 24A) inhibited ($P < 0.05$) root growth only at 625 ppm when compared with acetone (12,500 ppm) control, while AZA in methanol (Fig. 24B) produced the same effect ($P < 0.05$) at 312.5 ppm and 625 ppm, respectively, when compared with the methanol (12,500 ppm) control. AZA in ethanol (Fig. 24C) did not suppress ($P < 0.05$) root growth at the highest test concentration of 5 ppm when compared with the ethanol (2,500 ppm) control. However, both ethanol control and AZA (5 ppm) in ethanol, inhibited ($P < 0.05$) root growth when compared with the water control. The highest test concentrations of AZA (5 ppm) in propanol (Fig. 24D) did not inhibit ($P < 0.05$) root growth when compared with the water control and propanol (2,500 ppm) control.

At the highest test concentration of 250 ppm, Eneem carrier (in methanol) (Fig.25A) did not inhibit ($P < 0.05$) root growth when compared with methanol (2,500 ppm) control. However, Eneem carrier at 50 ppm, 100 ppm, and 250 ppm, inhibited ($P < 0.05$) root growth when compared with the water control. None of the test concentrations of Neemix carrier (Fig. 25B) inhibited root growth when compared with methanol control and water control. Eneem 3G (Fig. 25C) and Neemix 4.5E (Fig. 25D), had no ($P < 0.05$) impact on the growth of carrot roots when compared with the corresponding methanol (2,500 ppm) or carrier (500 ppm) controls.

Acetone (Fig. 26A) and methanol reduced ($P < 0.05$) *D. carota* root biomass when harvested at 4r weeks only at 50,000 ppm. Neither ethanol (Fig. 26B) nor

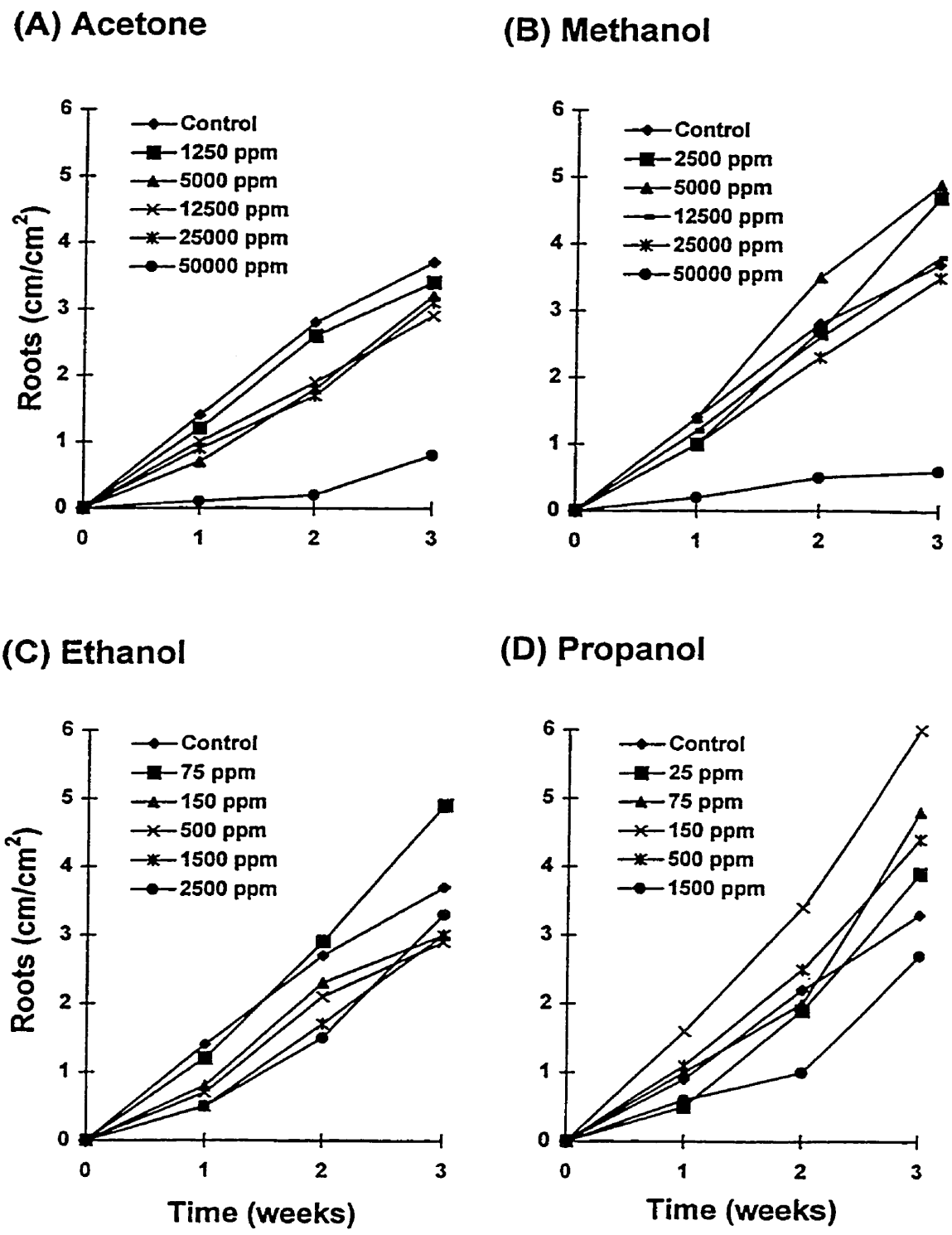

Figure 23. Effect of four solvents on root growth of Ri T-DNA *Daucus carota* grown in solvent spiked M-medium.

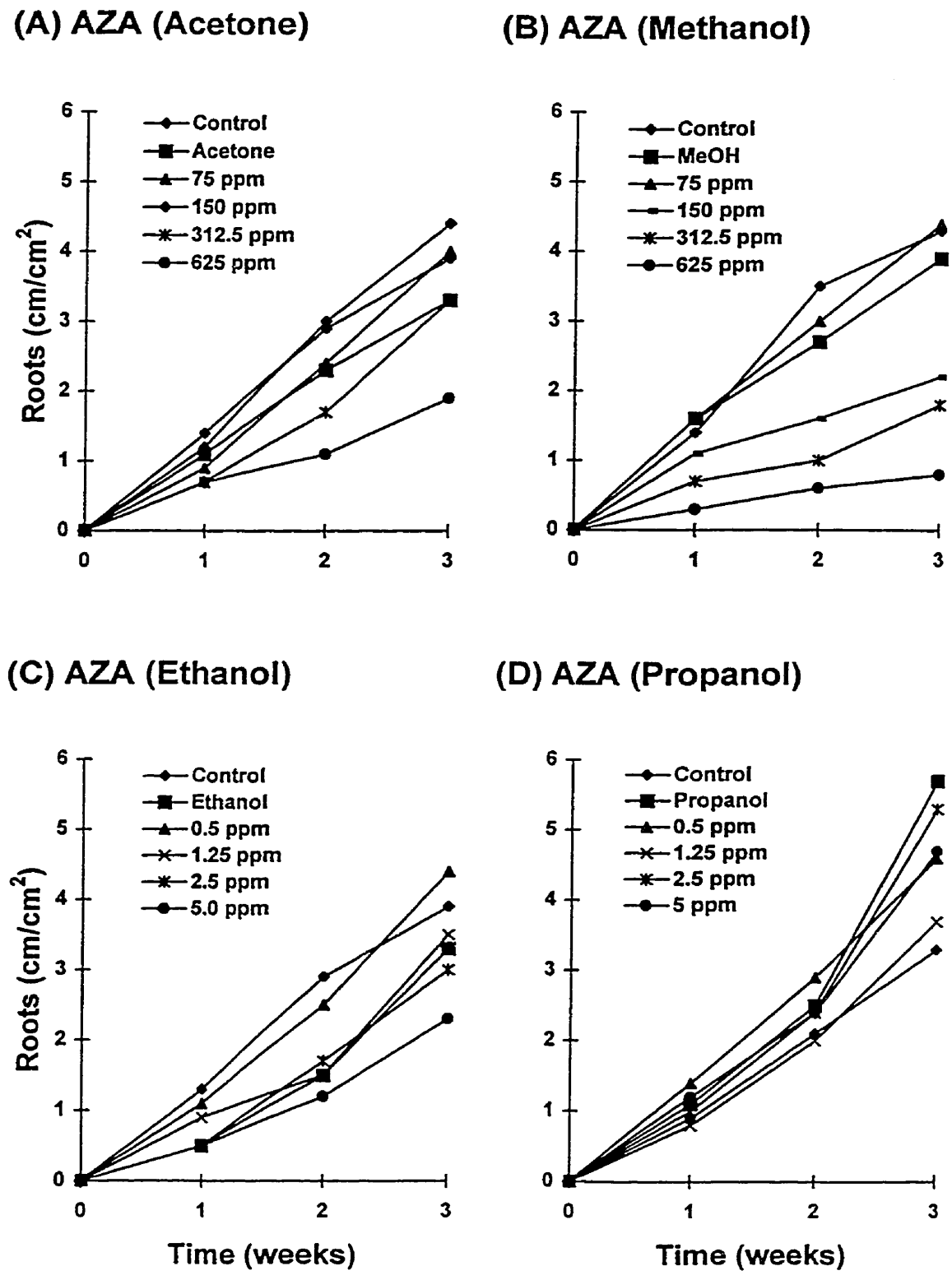

Figure 24. Effect of azadirachtin (AZA) in different solvents on root growth of Ri T-DNA *Daucus carota* grown in AZA spiked M-medium.

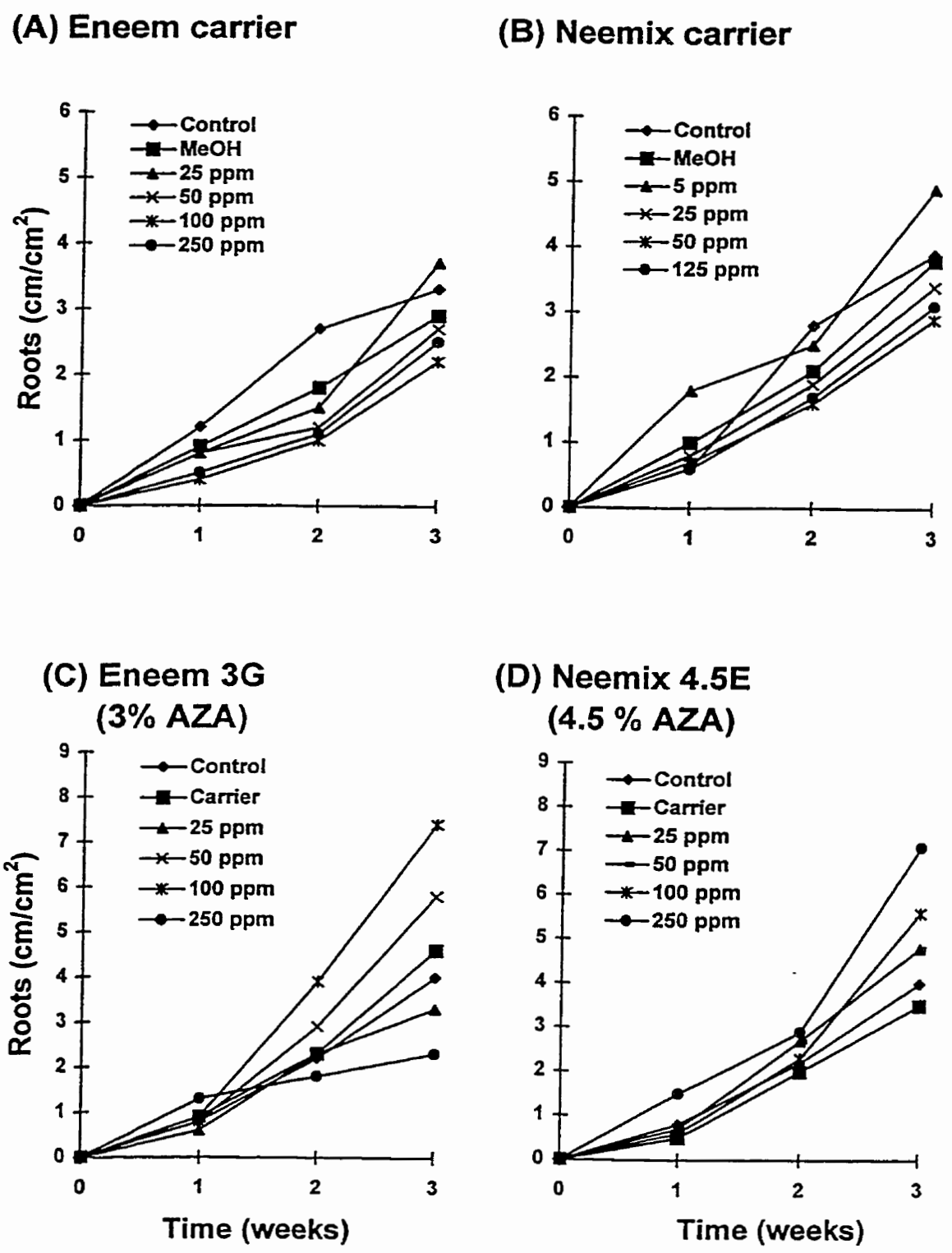

Figure 25. Effect of neem product carriers and neem products on root growth of Ri T-DNA *Daucus carota* grown in spiked M-medium.

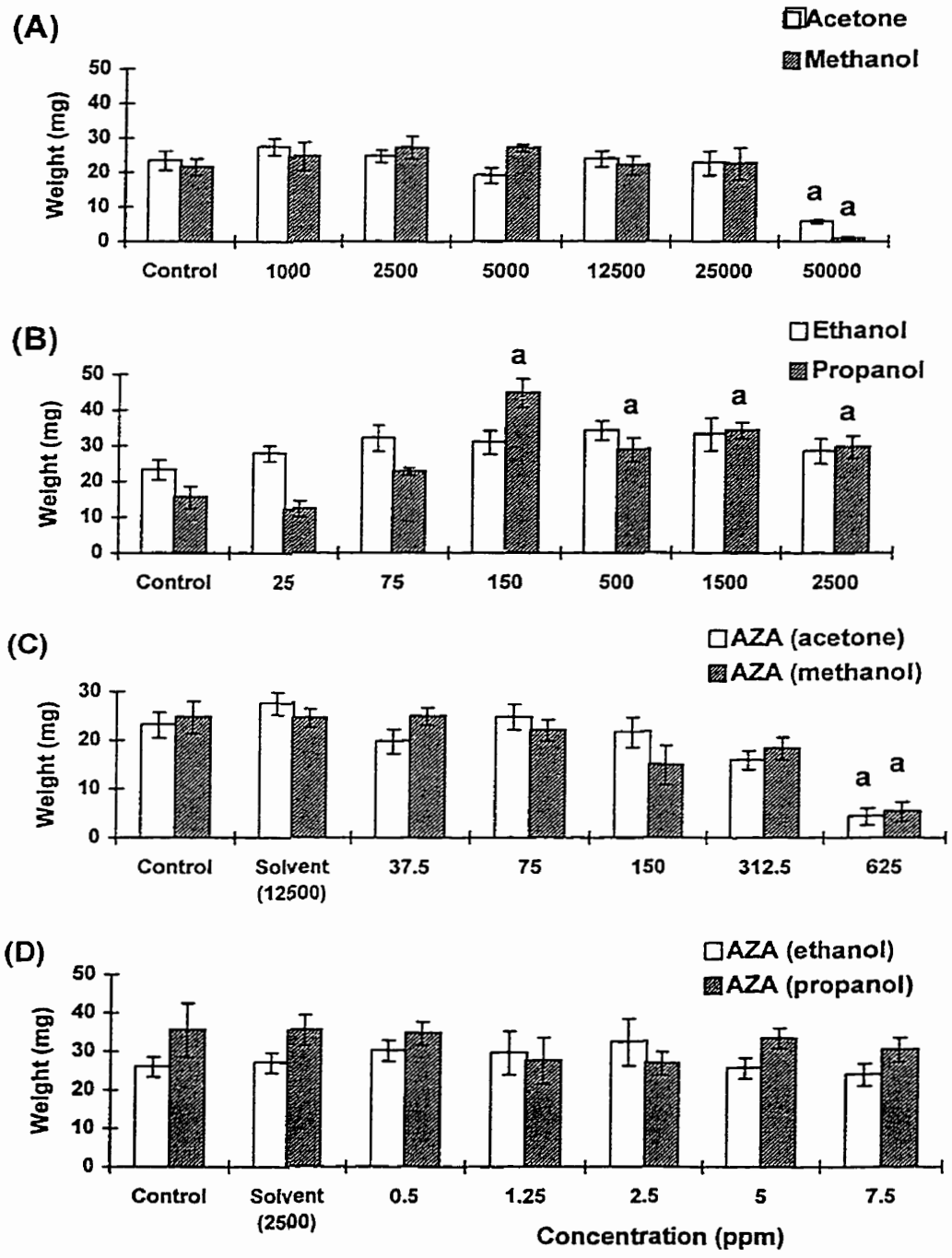

Figure 26. Effect of solvents and azadirachtin (AZA) in various solvents on root dry weight (mg/30 cm^2) of Ri T-DNA *Daucus carota* grown in solvent and AZA spiked M-medium. Values = means ± SE (n = 4); a - indicates significant difference ($p < 0.05$) between means of control and test solvent or AZA concentration series.

propanol suppressed ($P > 0.05$) root dry weight at the highest concentration (2,500 ppm) tested. Propanol, however, appeared to stimulate ($P > 0.05$) root growth at concentrations > 150 ppm, as indicated by the increase in biomass. AZA in both acetone (Fig. 26C) and methanol reduced ($P < 0.05$) root biomass only at the highest concentration (625 ppm). Neither AZA in ethanol (Fig. 26D) nor in propanol, at the highest concentration (7.5 ppm) tested had any impact ($P > 0.05$) on root dry weights. Likewise, Eneem carrier (Fig. 27A) and Neemix carriers and neem products (Fig. 27B) had no effect ($P > 0.05$) on carrot root biomass at the highest concentration (250 ppm) tested.

Effect on vesicular-arbuscular mycorrhiza

VAM formation in Ri T-NA transformed carrot roots by *G. intraradices* averaged about 20% in 4-week old cultures (Wan and Rahe, 1997). Acetone (Fig. 27C) and methanol reduced ($P < 0.05$) VAM colonization of carrot roots at 50,000 ppm when compared with controls. In contrast, ethanol (Fig. 27D) and propanol suppressed ($P < 0.05$) VAM colonization at much lower concentrations, i.e., at >1,500 ppm and >150 ppm, respectively, when compared with the control and the two previous solvents. AZA in acetone (Fig. 28A) and in methanol impacted ($P < 0.05$) VAM colonization of carrot roots only at the highest test concentration (625 ppm) relative to solvent controls. Both AZA in ethanol (Fig. 28B) and propanol, however, reduced ($P < 0.05$) VAM colonization of carrot roots at much lower concentrations of 7.5 ppm and >1.25 ppm, respectively. As well, Eneem carrier

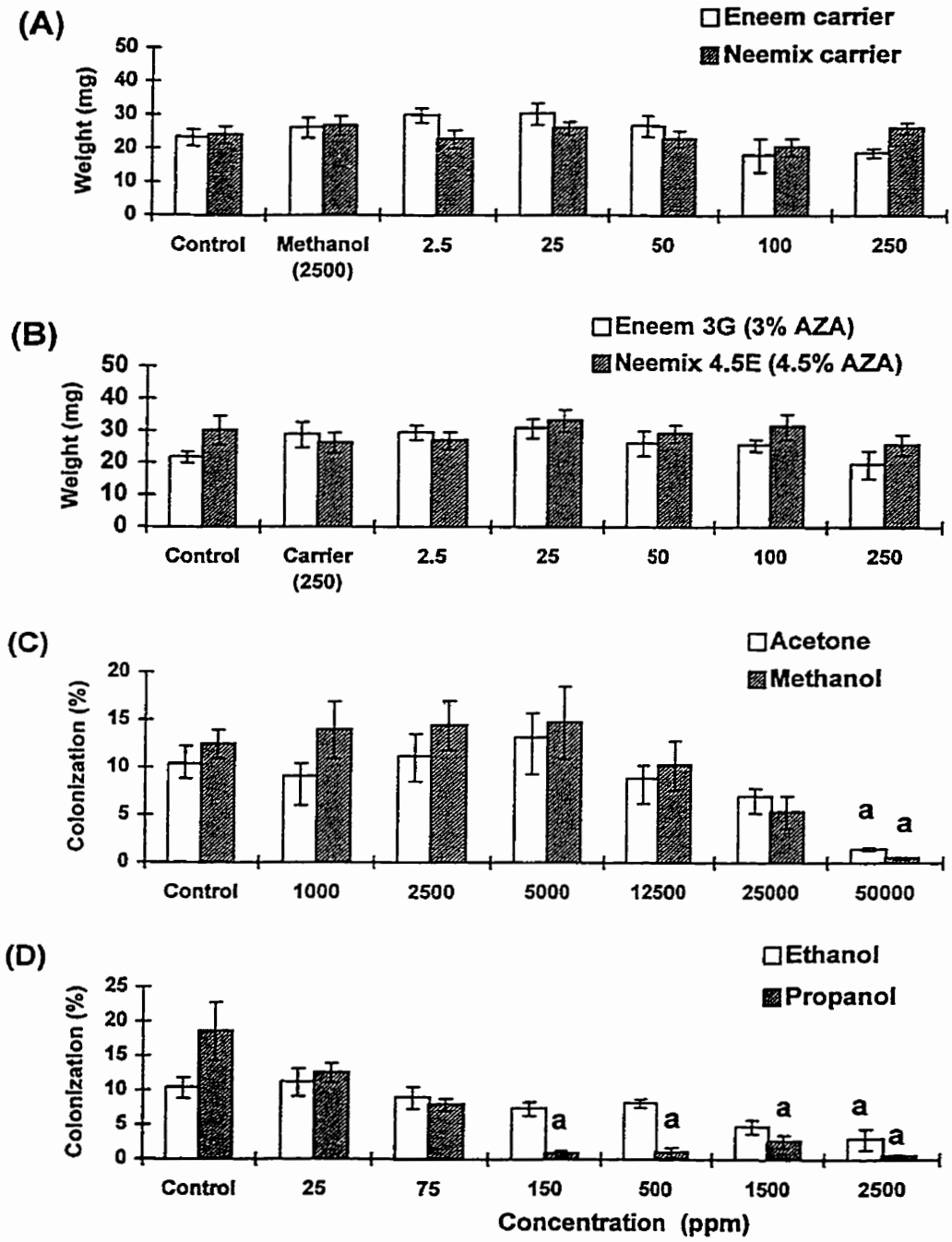

Figure 27. Effect of carriers and neem products on root dry weight (mg/30 cm^2) of Ri T-DNA *Daucus carota* grown in carrier or neem product spiked M-medium: **A** Eneem and Neemix carrier, **B** Eneem 3G and Neemix 4.5E. Effect of solvents on colonization by *Glomus intraradices* of Ri T-DNA *Daucus carota* roots grown in solvent spiked M-medium: **C** Acetone and methanol, **D** Ethanol and Propanol. Values = means $\pm$ SE (n = 4); a - indicates significant difference ($p < 0.05$) between means of control, solvent control, and test concentration series.

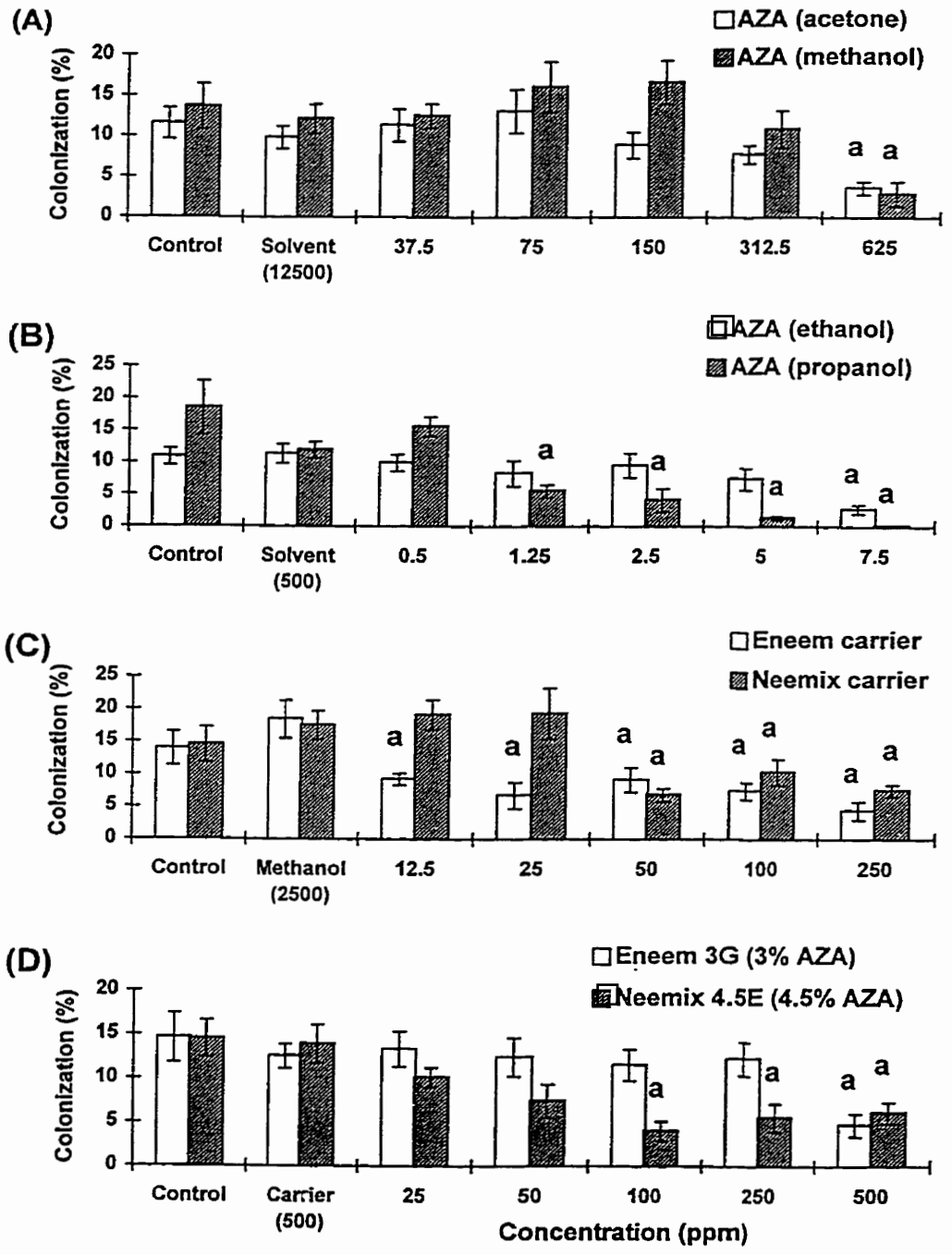

Figure 28. Effect of azadirachtin (AZA) in different solvents, carriers, and neem products on colonization by *Glomus intraradices* of Ri T-DNA *Daucus carota* roots grown in spiked M-medium. Values = means ± SE (n = 4); a - indicates significant differences ($p < 0.05$) between means of solvent control and test concentration series.

(Fig. 28C) and Neemix carrier reduced ($P < 0.05$) VAM colonization of carrot roots at concentrations >12.5 ppm and >50 ppm, respectively, relative to methanol control. Eneem 3G (Fig. 28D) and Neemix 4.5E suppressed ($P < 0.5$) VAM colonization at 500 ppm and >100 ppm, respectively.

DISCUSSION

In the toxicity determination of a test compound, it is important to select a solvent that is neither an antagonist nor a synergist (Casarett and Doull, 1986; Rand et al., 1995). An aqueous solution would be ideal but this was not feasible for AZA due to its low water solubility (Wink, 1993) of about 100 ppm at 25°C (Isman, personnal communication). However, this neem compound, is soluble in acetone and alcohols (Tewari, 1992; National Research Council, 1992). Of the four solvents (acetone, ethanol, methanol, and propanol) tested, methanol was selected for optimal use. Methanol has the lowest b.p. (65°C) when compared with ethanol (79°C) and propanol (99°C). It is both critical and desirable to spike AZA to the molten M-medium at the selected temperature of 58°C $\pm$ 2°C (Wan et al., 1998) so that as much of the solvent as possible evaporates without affecting the integrity of test materials. Methanol was the logical choice, and at 58°C $\pm$ 2°C, a large proportion of the solvent would have evaporated. Nevertheless, it was conceivable that small amounts of methanol may still remain in the plated M-medium due to the short exposure time of 10 - 15 s, especially at concentrations that were >1,000 ppm. As well, methanol (IC50 = 14,800 ppm) was the least toxic solvent to *G.*

intraradices when compared with ethanol (IC50 = 170 ppm) and propanol (IC50 = 70 ppm). Accordingly, the antagonistic or synergistic effect of AZA in methanol would probably be minimal, and this combination would most likely reflect its inhibitory or toxic effect, if any, to the VAM fungus. Acetone (IC50 = 20,000 ppm) could be an alternate solvent, since its b.p. of 56°C is lower than methanol. It would have evaporated completely at the spiking temperature of 58°C ± 2°C. However, its use is not recommended because it deactivates AZA rapidly (Tewari, 1992) and would probably cause deterioration of AZA stock solutions even under refrigeration. Moreover, acetone, especially at concentrations > 25,000 ppm, corrodes plastic plates and can interfere with the visual observation of fungal hyphae and spores.

Data variation is indicated by the large standard errors (SE) for the IC50 values of AZA, NEX, Eneem 3G, Neemix 4.5E and their carriers and solvents. This may be attributed to several factors, viz., incomplete evaporation of solvent/carriers, non-uniform growth pattern of *G. intraradices*, effect of test materials on the growth of *D. carota*, or the inherent characteristic of using a four-plate replicate per test concentration (Wan and Rahe, 1997). Another possible factor contributing to the variation was the effect of heat on the chemical integrity of AZA, thereby affecting its bioactivity. However, with a m.p. of 165°C, a 10 -15 s exposure of the compound to 58°C ± 2°C molten M-medium would be unlikely to cause any chemical degradation. The degradation of AZA via hydrolysis could be another factor affecting its bioactivity. The $t_{1/2}$ of AZA at 35°C in non-sterilized natural water (pH = 6.1) was 10.7 d (Szeto and Wan, 1996), and in sterilized M-medium (pH = 5.8) incubated in

darkness at 27°C was 44.4 d (Wan et al., 1997). However, despite its brief exposure to 58°C ± 2°C M-medium, it was estimated that more than 85% of the initial amount of AZA introduced to the M-medium persisted 14 d after inoculation, when the inhibitory effects of the compound were evaluated. But whether or not heat had any affect on the biological potency of AZA is presently not determined. In spite of the generally large SE, IC50 values provide a meaningful and useful measure of the effects of solvents, AZA, neem compounds, and reference pesticides on *G. intraradices*.

To date, there are no published data available on the toxicity of AZA and neem products to VAM fungi for comparison. Our previous study (Wan et al., 1998) provided a basis for toxicity comparison to *G. intraradices* of reference solvents and pesticides. On the same basis, it appears that AZA in methanol has a very low inhibitory effect on this VAM fungus. With an estimated IC50 value of 230 ppm (using EMG as the response indicator) in methanol, it suggests that AZA has minimal fungistatic/fungicidal effects on *G. intraradices.* This is apparent when AZA toxicity was compared with reference fungicides such as benomyl, chlorothalonil, and copper sulphate, whose IC50 values were 0.004 ppm, 0.05 ppm and 0.6 ppm, respectively. It also had less inhibitory action than the reference herbicide glyphosate, and its metabolite AMPA, and the insecticide dimethoate with the following IC50 values of 3 ppm, 0.5 ppm, and 4.2 ppm, respectively. Likewise, Eneem 3G (IC50 = 210 ppm), Neemix 4.5 G (IC50 = 130 ppm), and NEX (IC50 = ~60 ppm), were rather weak fungicides when tested against VAM fungus.

The fungistatic/fungicidal property of a chemical depends on its ability to penetrate the fungal cell wall and membrane - the principal barriers to toxicants (Hartley and Graham-Bryce, 1980). Benzimidazole and substituted aromatic fungicides such as benomyl (MW = 290) and chlorothalonil (MW = 266), respectively, are not only smaller molecules than AZA (MW = 721) but are also highly lipophilic, enabling them to enter fungal cells in toxic amounts (Delp, 1995). The penetration of AZA in methanol through the cell wall membrane of fungal hyphae and spores may have been impeded. However, AZA in less volatile solvents such as ethanol and propanol appears to have greater cell penetration. When compared with AZA in methanol, the synergistic effect of this compound in ethanol and propanol increased toxicity to *G. intraradices* by about 70 times and 200 times, respectively. With an IC50 of 1.1 $\pm$ 0.6 ppm to *G. intraradices* in propanol, the short term toxic effect of AZA to the VAM fungus is comparable with the fungicide $CuSO_4$ (IC50 = 0.6 $\pm$ 0.2 ppm). However, Cu^{++} ions from $CuSO_4$ are indestructible. Their presence in appropriate amounts, e.g., > 1 ppm, in the environment produces toxic effects to microorganisms indefinitely. Whether or not the toxic effect of AZA in propanol could be sustained beyond 30-d is not determined. Fungicides such as benomyl and chlorothalonil inhibit DNA synthesis (Ware, 1983) and cell enzymes (Bohmont, 1997), respectively. The mode of fungicidal action of AZA in propanol on VAM fungus, however, is not known. Although crude extract of neem fruits was reported to have fungicidal activity (Nagarajan and Reddy, 1980; Shetty et al., 1989), AZA was unlikely to be the main

active ingredient responsible for the bioactivity. The fungicidal activity of the crude extract was probably due to the synergistic effects of AZA in the presence of an array of other triterpenoid and limonoid compounds (Schmutterer, 1995; Isman et al., 1996). This study suggests that without the synergism of an appropriate solvent, AZA would be a weak fungicide.

It is interesting to note that the growth and development of Ri T-DNA transformed carrot roots in terms of root length and biomass were unaffected relative to controls when roots were exposed to fairly high concentrations (150 - 250 ppm) of AZA, NEX, Eneem 3G, Neemix 4.5E, including their carriers and solvents. This indicates that these toxicants, unlike glyphosate which suppresses root elongation and reduces root biomass at 0.25 ppm (Wan et al., 1998), had little or no impact on the host. The effect of glyphosate on *G. intraradices* is probably due to the inhibitory action of the herbicide on the roots rather than on the growth of the VAM fungus. Fungicides such as benomyl and chlorothalonil, both of which were highly toxic to *G. intraradices,* had no effect on root growth and biomass relative to controls even at the highest concentration tested. On the other hand, $CuSO_4$ and AMPA, a metabolite of the herbicide glyphosate, were phytotoxic to carrot roots at 6.25 ppm and 12.5 ppm, respectively.

The VAM colonization of carrot roots was unaffected relative to controls at fairly high concentrations (100 - 625 ppm) of AZA, NEX, Eneem 3G, and Neemix 4.5E in methanol. Although AZA in ethanol and propanol, and Eneem carrier reduced VAM colonization of roots at 7.5 ppm, 2.5 ppm, and 12.5 ppm,

respectively, it was only within the range of concentrations required to produce a similar effect (Wan et al., 1998) by the insecticide dimethoate (10 ppm) and fungicide $CuSO_4$ (6.25 ppm). When compared with benomyl, however, AZA in ethanol and propanol, and Eneem carrier, were much less effective in suppressing VAM colonization, as about 2500, 830, and 4170 times higher concentrations, respectively, of AZA in these solvents were required to produce the same effect. AZA in ethanol and propanol, and Eneem carrier required approximately 60, 20, and 100 times higher concentrations, respectively, to produce the same effect of chlorothalonil on the VAM colonization of carrot roots.

From the view point of eco-toxicological significance, it should be emphasized here that the IC50 values of AZA, NEX, and formulated neem compounds to *G. intraradices* were determined by exposing the VAM fungus to test materials under artificial conditions that would represent a "worst case" contamination scenario, e.g., accidental applications and spills. These data should not be extrapolated to represent the toxic effect of AZA and formulated neem compounds occurring in the field or eco-systems when they are used under prescribed circumstances. Under field conditions, soil microbial and chemical activities would influence the effect of these compounds (Munnecke, 1967; Woodcock, 1967). However, in the absence to date of an alternate testing method that is rapid, reliable, and cost effective in determining pesticide toxicity to VAM fungus (Trappe et al., 1984), IC50 values obtained from the technique described in this report can and should be used for the initial screening of new candidate AZA

and neem products, as well as other pesticides before registration. IC50 values of VAM fungus should be included in a manner similar to the use of data such as LC50 (lethal concentration to 50% population of an aquatic organism) and LD50 (lethal dose to 50% population of a terrestrial animal) not only to get an indication of, but also to enhance, the overall value of the eco-toxicological risk potential assessment of AZA and its formulated products.

An example of how IC50 data from *G. intraradices* could be used to evaluate the eco-toxicological potential of AZA and other pesticides is shown in Table 5. The IC50 multiple (estimated environmental concentration ÷ IC50 value) of each test material is determined by assuming that normal recommended rates (Tomlin, 1996; Meister, 1997a; Meister, 1997b; Meister, 1997c) of plant or soil applied pesticides would generate an average of 1.5 ppm residues in the top 7.5 cm (3 inches) of field soils during the growing season. The small IC50 multiples of AZA, NEX, and neem formulated products suggest that their potential for adverse eco-toxicological impact on non-target soil micro-organisms is much lower than reference fungicides benomyl and chlorothalonil (Table 5). With small IC50 multiples (0.006 - 0.03) and a short residual life ($t_{1/2}$ = 20.5 - 46.2 d) (Wan et al., 1997), we predict that AZA in methanol and in formulated products are unlikely to have any acute or sub-acute toxic impact on non-target fungi. Accordingly, the high *G. intraradices* IC50 values (resulting in small IC50 multiples when expected concentrations in the environment are considered) for AZA and neem compounds suggest their low potential for adverse impacts on beneficial subterranean

Table 5. Eco-toxicological potential of neem compounds, reference pesticides, carriers and solvents in the terrestrial environment as indicated by using *Glomus intraradices* IC50 multiples[a] as an index

Pesticides	IC50 values (ppm)[b] of *G. intraradices*	IC50 multiples of *G. intraradices*
Benomyl	0.004	375
Chlorothalonil	0.05	30
Glyphosate	0.5	3
$CuSO_4$	0.6	2.5
Dimethoate	3	0.5
AMPA	4.2	0.4
Eneem carrier	60	0.03
Neem extracts	~60	~0.03
Neemix carrier	80	0.02
Neemix 4.5E[c]	130	0.01
Eneem 3G[c]	210	0.007
AZA (in methanol)	230	0.006
Methanol	14800	0.0001
Acetone	20000	0.00008

[a] - IC50 multiples = expected pesticide concentration in the environment ÷ IC50 value; calculation here assumes ~1.5 ppm carrier, solvent or pesticide residues in top 7.5 cm (3 inches) soils; large multiples = high eco-risk potential

[b] - IC50 estimate, using EMG as response indicator; data from this study, and Wan et al. (1998)

[c] - Neem formulated products

microorganisms, thereby posing little or no eco-toxicological risk to the terrestrial environment. The relevancy of IC50 values as an index in the eco-risk assessment of AZA and neem compounds, and its relationship with VAM symbiosis and associations are presently being evaluated in host plant symbionts growing under natural conditions.

CONCLUSION

Toxicity of AZA and neem compounds to *G. intraradices* appeared to be solvent dependent. Using EMG as the response indicator, the order of increasing toxicity to this VAM fungus is AZA (methanol), Eneem 3G (methanol), Neemix 4.5E (alcohol-based carrier), AZA (acetone), NEX (methanol), AZA (ethanol), and AZA (propanol). In methanol, neem compounds such as AZA, NEX, Eneem 3G and Neemix 4.5E, including their carriers, have low, short term toxicity to *G. intraradices.* Neither its host *D. carota* nor the VAM infection was affected by these AZA compounds when compared with reference pesticides such as benomyl, chlorothalonil, $CuSO_4$, dimethoate, glyphosate, and AMPA. The relatively high *G. intraradices* IC50 values, and the corresponding low IC50 multiples when the estimated residues in the environment are taken into consideration, suggest that AZA in methanol and neem insecticides are not expected to have an adverse impact on non-target terrestrial organisms or pose an eco-toxicological risk to the environment. Further research is required to study the relevancy of IC50 values as an index in the eco-risk assessment of AZA.

CHAPTER 5

Impact of Azadirachtin on Vesicular-arbuscular Mycorrhizae of *Glomus intraradices* in Leek, *Allium porrum,* and Eco-toxicological Implications[1]

INTRODUCTION

Pesticides have a high potential to cause harmful effects on beneficial subterranean microorganisms because soil is the major repository for pesticides and their residues in the terrestrial environment (Pimentel, 1971; Krogh, 1991; Wan and Rahe, 1998). Of particular concern is the effect of persistent synthetic pesticides on beneficial and non-pathogenic soil populations, and hence on bio-chemical processes involved in nutrient acquisition and cycling, e.g., the formation and function of VAM and symbiotic activity of vesicular-arbuscular (VA) mycorrhizal fungi (Hicks et al., 1990; Habte and Manjunath, 1992). One candidate ingredient having the potential to replace some synthetic pesticides for the control of insect pests is azadirachtin (AZA), a phytochemical that is obtained from the neem tree *Azadirachta indica* A. Juss (Koul et al., 1989; Saxena, 1990; Schmutterer, 1995).

AZA is derived from neem seed kernels, which also contain many other limonoid compounds (Jones et al., 1988; Ley and Denholm, 1993; Hansen et al., 1994). AZA acts mainly as an antifeedant and/or insect growth regulator at low

[1] - Submitted by Wan & Rahe (1998) to *Environ Toxicol Chem* for publication consideration, and by courtesy of co-author

concentrations (0.05 - 5 ppm), and as a contact poison at concentrations > 50 ppm (Isman et al., 1996; Mordue and Blackwell, 1993). There are also claims that it has fungicidal properties at these concentration ranges (Nagarajan and Reddy, 1980; Shetty et al., 1989). Recently, an experimental slow release granular AZA product was developed to test its potency on subterranean insect pests, e.g., onion root maggots (*Delia antiqua* Meigen) and potato wireworms (*Agriotes obscurus* L). Although not yet registered in Canada, several AZA products were registered in 1996 for the control of agricultural insect pests in the USA.

To date, no study has been conducted to evaluate the impact of AZA on VAM and VA mycorrhizal fungi, particularly using the concept of IC50 (concentration inhibiting 50% VAM hyphal growth and development) as a measure of toxicity (AGRICOLA, 1998; BIOSIS, 1998; CAB, 1998; CA SEARCH, 1998; CRIS-USDA, 1998). We recently introduced this concept to indicate the toxicity of pesticides to the VA mycorrhizal fungus *Glomus intraradices* Schenck & Smith (Wan and Rahe, 1998). The IC50 values were determined by adding different concentrations of AZA (and reference pesticides) to a sterilized minimal medium (M-medium) system, and comparing the resulting effects on hyphal growth and spore formation. The M-medium (containing various salts, sucrose, vitamins, and water solidified in 0.4% w/v gellan gum) served as a medium for *G. intraradices* hyphal growth and sporulation, while at the same time providing nutrients for the self propagating host Ri T-DNA transformed roots of *Daucus carota* (Wan and Rahe, 1997). We found that AZA in methanol (IC50 = 230 ppm) and Eneem 3G (IC50 =

210 ppm), a granular slow release formulation designed for the control of soil insect pests, had low toxicity to the VA mycorrhizal fungus *G. intraradices* (Wan and Rahe, 1998). As well, they did not affect VAM and its symbiotic activity in Ri T-DNA transformed roots of *Daucus carota* L, when compared with the reference fungicides benomyl (IC50 = 0.004 ppm) and chlorothalonil (IC50 = 0.05 ppm). There are concerns, however, that this method utilizes an artificial test system, and that the IC50 values produced reflect effects under unnatural conditions. Moreover, this value may not have any bearing on VAM at all in plants grown under natural settings.

The objectives of this study were (a) to determine the effect of AZA, Eneem 3G, and the reference pesticides benomyl and chlorothalonil on VAM of *G. intraradices* in leek, using the vermiculite biological system, and (b) to complete the evaluation of using pesticide IC50 values as an index of eco-toxicity to non-target subterranean micro-organisms, as proposed by Wan and Rahe (1998).

MATERIALS AND METHODS

The vermiculite biological system

This system consisted of a VA mycorrhizal-responsive plant, leek (*A. porrum* cv. Giant Musselburg), growing in non-sterilized vermiculite in a 125 cc Cone-tainer® (Stuewe & Sons, Corvalis, OR, USA) plastic vessel. The vermiculite was amended with a plant nutrient (Hoagland) solution, and was either inoculated or not inoculated with *G. intraradices*. The container was supported by a plastic stand in a

growth chamber having a 16-h day cycle; day:night temperature of 25°C:17°C; mean RH = 75% (range, 65% - 85%); and average full spectrum light (Duro-Test Vita Lite) intensity = 4000 lx/cm^2 (range, 3850 - 4100 lx/cm^2). Each plant received weekly 40 ml of modified Hoagland solution (to produce either 0% P or about 18% P of regular Hoagland solution, i.e., equivalent to 0 mg P/l or about 0.01 mg P/l, respectively (Habte, 1992; Hoagland and Arnon, 1938) by drenching, and about 5 ml distilled water via spraying (simulating rain). A 100 ml plastic cup was placed under each Cone-tainer® to collect and retain excess Hoagland solution or water. Each plant was supported upright by a piece of twist tie attached to a wooden skewer.

Test Materials

The following materials were used for this research: AZA (~92% by HPLC) - Dr. M.B. Isman, University of British Columbia, Vancouver, BC, Canada; Eneem 3G (experimental "slow release" granules containing 3% AZA from neem extract); blank granular carrier (ammonium sulphate + silica + magnesium stearate) - Phero Tech, Delta, BC, Canada; methanol (99.8%) - Commercial Alcohols, Brampton, ON, Canada; benomyl (99%) and chlorothalonil (99%) - Chem Service, West Chester, PA, USA; VAM fungal inoculum (perlite + sphagnum peat + soil + ~2,100 active *Glomus intraradices* propagules/L), blank inoculum carrier (perlite + sphagnum peat + soil) - Premier Tech Research Centre, Riviere-du-Loop, QC, Canada; vermiculite (medium grains, Terra-lite®) - WR Grace Ajax, ON, Canada.

Test concept

This method of assessing pesticide impact on VA mycorrhizal fungi and VAM utilized inoculated and uninoculated pesticide-treated vermiculite to grow the leek host plant. Responses (height, biomass, root colonization) of plants to pesticide treatments were determined at 14 weeks and compared with those of controls. In addition, leaf samples were taken at regular intervals to estimate their phosphorus (P) content during that period. P uptake by VA mycorrhizal-responsive plants has been shown to be related to VA mycorrhizal symbiosis, and the level of P uptake by plant could be used as an indicator of VA mycorrhizal activity (Habte and Turk, 1991; Habte, 1992). These researchers suggested that the optimum level of P for host plants dependent on symbiotic activity of VAM was between 0.01 ppm and 0.02 ppm P.

Test procedure

Observations were made on growth (plant height and frequency of leaf production) of leek grown in vermiculite to determine an appropriate sampling regime and technique. Baseline observations were made on plant height, biomass, root colonization, and leaf P status of leek grown in vermiculite that was either inoculated or not inoculated with *G. intraradices* and amended with modified Hoagland solutions that contained P at 0.00 and 0.01 ppm . Spot checks were made on the P content for the same blade and different leaf tips of leek grown only in P and VA mycorrhizal inoculum amended vermiculite fortnightly (4 weeks after

transplanting to 14 weeks) to characterize P level over time. Triplicate samples of vermiculite and VA mycorrhizal fungus inoculum were also analysed to determine and quantify their chemical elements and pH for comparison with other candidate plant growth substrates, e.g., sterilized sand and a sandy loam agricultural soil from Aldergrove, BC, Canada.

Two series of AZA, Eneem 3G and benomyl test concentrations were set up at different times. The 1996 test series reflected estimated environmental concentration ranges generated by normal field applications rates, while the 1997 test series represented higher field application rates or accidental spill situations. Chlorothalonil was added to the 1997 test series. The tests consisted of uninoculated and VA mycorrhizal inoculated leeks grown in P amended (a) vermiculite + blank inoculum carrier (containing perlite + sphagnum peat + soil particles) (control), (b) vermiculite + blank inoculum carrier + methanol or product blank carrier (control), and (c) vermiculite + VA mycorrhizal fungus inoculum (~100 active *G. intraradices* propagules/treatment of 120 cc vermiculite) + different nominal concentrations of test materials.

The vermiculite was treated at the time of transplanting and VA mycorrhizal fungus inoculation and with the two nominal test concentration series (w/w, d. wt.) of AZA (1996: 5 ppm - 250 ppm; 1997: 250 ppm - 1250 ppm), Eneem 3G (1996: 5 ppm - 250 ppm; 1997: 250 ppm - 12500 ppm), benomyl (1996: 0.005 ppm - 1.25 ppm; 1997: 1 ppm - 125 ppm), and chlorothalonil (1997: 1 ppm - 125 ppm), including water and appropriate solvent controls, using the method described by

Wan et al. (1998). Five replicates of 120 cc vermiculite of each treatment were placed in individual Cone-tainers® plastic vessels. A week-old leek seedling germinated from seed sterilized in 1% sodium hypochlorite for 3 minutes (followed by five distilled water rinses) was then transplanted to each container. The containers were arranged in a completely randomized block design in the growth chamber.

The P content of leek leaves was monitored, using a modification of the approach described by Habte and Turk (1991), starting at week four after transplanting. A 1 cm section of the oldest leaf tip was sampled from each plant; subsequent samples were taken from the next oldest leaf blade, and so on. The leaves were air dried at room temperature (25°C , RH = ~ 50%) to constant weight for about 24 h before weighing. They were then ashed in a muffle furnace at 500°C for 4 h. The P content was determined colorimetrically after the ash was dissolved in 2.5 mL of a 0.5N sulfuric acid mixture (containing 1:37:41 = antimony potassium tartrate:ascorbic acid: ammonium molybdate, wt/vol) and diluted with 10 mL de-ionized water. The colour that developed was quantified spectro-photometrically at 882 nm. At harvest (14 weeks), the height of plants was determined. A sub-sample of 50% fresh weight of the entire fibrous root system was used for dry weight determination, while the other 50% was used for the estimation of colonization by *G. intraradices*. Shoot biomass (dry matter) was also determined after the samples were dried for 48 h at 70°C. The grid-line intersect and slide method of Giovannetti and Mosse (1980) were used for the determination of root

colonization by *G. intraradices*, after the roots were cleared and stained by using the trypan blue procedure described by Dalpe (1993).

Data on leaf tip P also were analyzed by the procedure of ANOVA (SAS Institute, 1996), and when F values were significant, $LSD_{0.05}$ were used to separate treatment means. Student's *t* tests (5% level) were carried out for data on plant growth (height), root and shoot biomass, and colonization by *G. intraradices* at harvest 14 weeks after transplanting. The relationship between root colonization by *G. intraradices* of leeks versus vermiculite amended with different concentrations of reference fungicides benomyl and chlorothalonil at harvest (14 weeks) was determined by regression analysis. Likewise the relationship between leaf P at harvest (14 weeks) versus root colonization by *G. intraradices* of leeks was determined.

RESULTS

Leaf sampling technique

Under controlled environmental conditions, a newly transplanted week-old leek seedling in P amended vermiculite produced a new leaf blade every 2 - 3 weeks. About 6 to 8 leaf blades were produced during the 14-week study period. The seedlings required 4 weeks to grow to a three-leaf stage (about 15 cm height). To ensure that regular excising of a small portion (1 cm) of leaf tip for P determination had minimum impact on plant growth and development, it was undesirable to begin leaf sampling sooner than the three-leaf blade stage.

Accordingly, it was necessary to limit the timing and frequency of sampling to one leaf tip every 2 weeks, starting with the first leaf tip at four weeks after transplanting, followed by the leaf tip of the next oldest leaf 2 weeks later, and so on. By following this procedure, the effect of removing young leaf tips on the overall plant growth and development was minimized. As well, the average age of each successive leaf tip sampled for P determination was consistently about 4 weeks during the 14 week growing period.

Response of leek to untreated, P fertilized, VA mycorrhizal fungus inoculated vermiculite

Leeks grown in I+P (inoculated, P amended) vermiculite were tallest (Fig. 29A) and heaviest (Fig. 29C) when compared with those grown in vermiculite that was U+P (uninoculated, P amended), I-P (inoculated, not P amended), and U-P (uninoculated, not P amended).

P levels over time in leaf tips was higher in leeks grown in I+P vermiculite when compared with plants from U-P vermiculite (Fig. 29B). However, P content was significantly ($p < 0.05$) greater in plants grown in I-P vermiculite than in leeks from U-P vermiculite at 10 weeks after transplanting. It appeared that uptake of P occurred in plants grown in both U-P and I-P vermiculites, indicating the availability of limited sources of P in the vermiculite. However, the uptake of P by plants in I-P vermiculite seemed to be greater than by those grown in U-P vermiculite, suggesting that the symbiotic activity of VAM promoted absorption of P from the

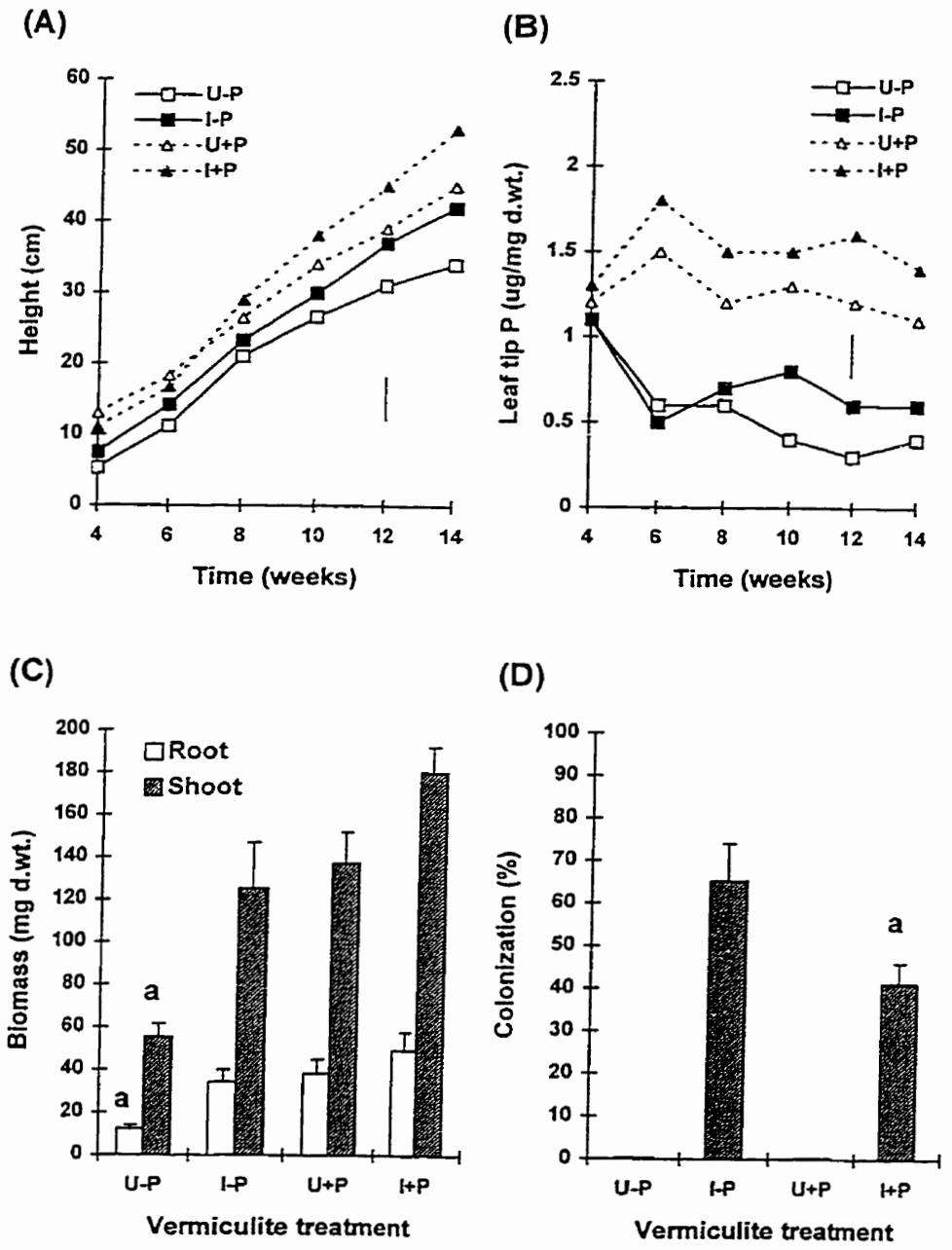

Figure 29. **A** Height, **B** phosphorus (P) content in leaf tips, **C** biomass, and **D** colonization of leek (*Allium porrum)* grown in vermiculite inoculated and uninoculated with *Glomus intraradices* and amended with weekly modified Hoagland solutions containing 0 ppm P (U-P = uninoculated, no P; I-P = inoculated, no P) or 0.01 ppm P (U+P = uninoculated, P amended; I+P = inoculated, P amended). Vertical bar represents LSD at the 5% level or mean ± SEM (n = 5); a - indicates significant differences ($p < 0.05$).

TABLE 6. Chemical elements of a sandy loam soil, sand, vermiculite, vesicular-arbuscular mycorrhizal (VAM) inoculum, and Hoagland solution

Elements (ppm)	Soil[1]	sand[2]	Vermiculite[3]	VAM inoculum[4]	Hoagland
Al	27000	17200	66900	1020	-
Ba	130	100	2210	15	-
Ca	12100	8300	3570	3200	200
Cu*	24	29	< 1	1.2	0.02
Fe	28100	23700	39100	1840	5
K	2060	2000	51900	1500	500
Mg	3925	9400	159000	495	50
Mn*	793	400	227	36	0.5
Mo*	4	< 2	4	< 2	0.01
Na	230	850	10000	5200	-
Ni	31	32	360	4	-
P	2400	570	40	100	30
Pb	25	9	< 10	< 10	-
Si	1000	1900	2100	1000	-
Zn*	85	45	47	5	0.7
pH (rel. unit)	7.2	6.7	9.2	6.6	5.0

[1] - Aldergrove sandy loam, BC, Canada

[2] - Target® sterilized sand, Revy's Home Centres, Vancouver, BC, Canada

[3] - Terra-lite®, medium grain, WR Grace, Ajax, ON, Canada

[4] - Sphagnum peat + perlite + soil particles + *Glomus intraradices* propagules, Premier Tech Research Centre, Riviere-du-Loop, QC, Canada

* - essential trace elements

vermiculite. Spot checks found that P content in leeks of I+P vermiculite generally increased during the first 8 weeks and then decreased gradually, regardless whether leaf tip samples were taken for P determination from the same leaf blade or different blades. Colonization of leek roots by *G. intraradices,* however, was significantly ($p < 0.05$) greater in leeks grown in I-P vermiculite (Fig. 29D) than I+P vermiculite.

Based on these observations, leeks grown in U-P and U+P vermiculite were unsuitable for pesticide bioassays because of the absence of VAM. Leeks grown in I+P vermiculite were selected for bioassays because they produced less variation in height, biomass, leaf P, and root colonization by *G. intraradices* than plants grown in I-P vermiculite.

Although various chemical elements were found in vermiculite (Table 6), it was necessary to amend it with Hoagland solution containing 0.01 ppm P to enhance plant growth (Fig. 29A and 29C). Plant growth in vermiculite was not adversely affected in spite of the presence of high concentrations of Al, Fe, K, Mg, Na, and Ni, when compared with a sterilized sand and a typical local sandy-loam agricultural soil (data not shown). Most of these chemical elements probably occurred in forms that were not readily absorbed by the leek plant. Vermiculite was selected as the growth substrate for this research, as both sterilized sand and the sandy-loam soil contained very high P concentrations, which may have interfered with symbiotic activity of VAM.

Effect of AZA, Eneem 3G and reference pesticides on leaf P, biomass, and VA mycorrhizal colonization:1996-low test concentrations

None of the test concentrations of AZA and Eneem 3G (both up to 250 ppm), benomyl (0 to 1.25 ppm) MeOH, (250 ppm) or Eneem carrier (250 ppm) had any significant ($p < 0.05$) effect on leaf tip P. For simplicity, only test results of the highest test concentration of these series are presented (Fig. 30). As expected, P content in leeks grown in P amended and *G. intraradices* inoculated controls Ctr.(I), MeOH(I), and Car.(I) appeared to be greater than those grown in unamended and uninoculated controls for water Ctr.(U), methanol MeOH(U) and Eneem product carrier Car.(U) treated vermiculite (Fig. 30A).

No significant difference ($p < 0.05$) in leaf P was observed within and among the uninoculated versus inoculated controls. AZA (Fig. 30B), Eneem 3G (Fig. 30C), and benomyl (Fig. 30D) did not have any significant ($p < 0.05$) impact on the VAM activity in leeks grown in vermiculite when compared with their respective controls. This test concentration series also had no significant ($p < 0.05$) impact on the root colonization by *G. intraradices* (Fig. 31A), root dry weight (Fig. 31B), shoot biomass (Fig. 31C), and plant height (Fig. 31D) when compared with their respective MeOH and carrier controls. These results indicate that the test materials of this concentration series had negligible impact on *G. intraradices* and VAM in leeks. To verify and extend these findings, another test series consisting higher test concentrations was conducted 1997.

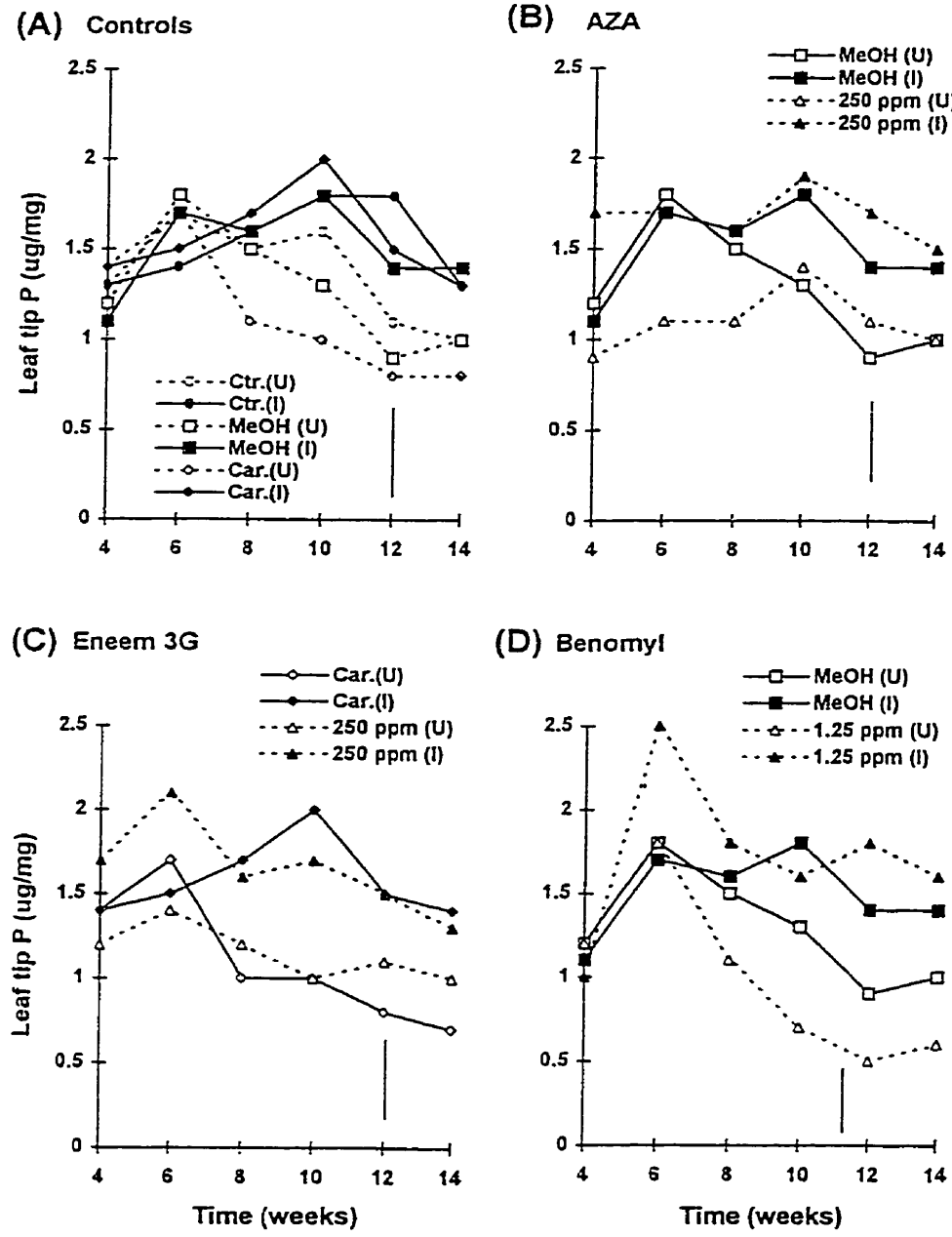

Figure 30. Phosphorus (P) content (µg/mg leaf d. wt.) In leaf tips of leek (*Allium porrum*) grown in vermiculite inoculated and uninoculated with *Glomus intraradices* and amended and not amended weekly with modified Hoagland solution containing 0.01 ppm P **A** controls: Ctr. (U) = water control, uninoculated; Ctr.(I) = water control, inoculated; MeOH (U) = methanol (250 ppm) control, uninoculated; MeOH (I) = methanol (250 ppm) control, inoculated; Car. (U) = Eneem carrier (250 ppm) control, uninoculated; Car. (I) = Eneem carrier (250 ppm) control, inoculated, **B** AZA (250 ppm), **C** Eneem 3G (250 ppm), **D** Benomyl (1.25 ppm). Vertical bar represents LSD value at the 5% level.

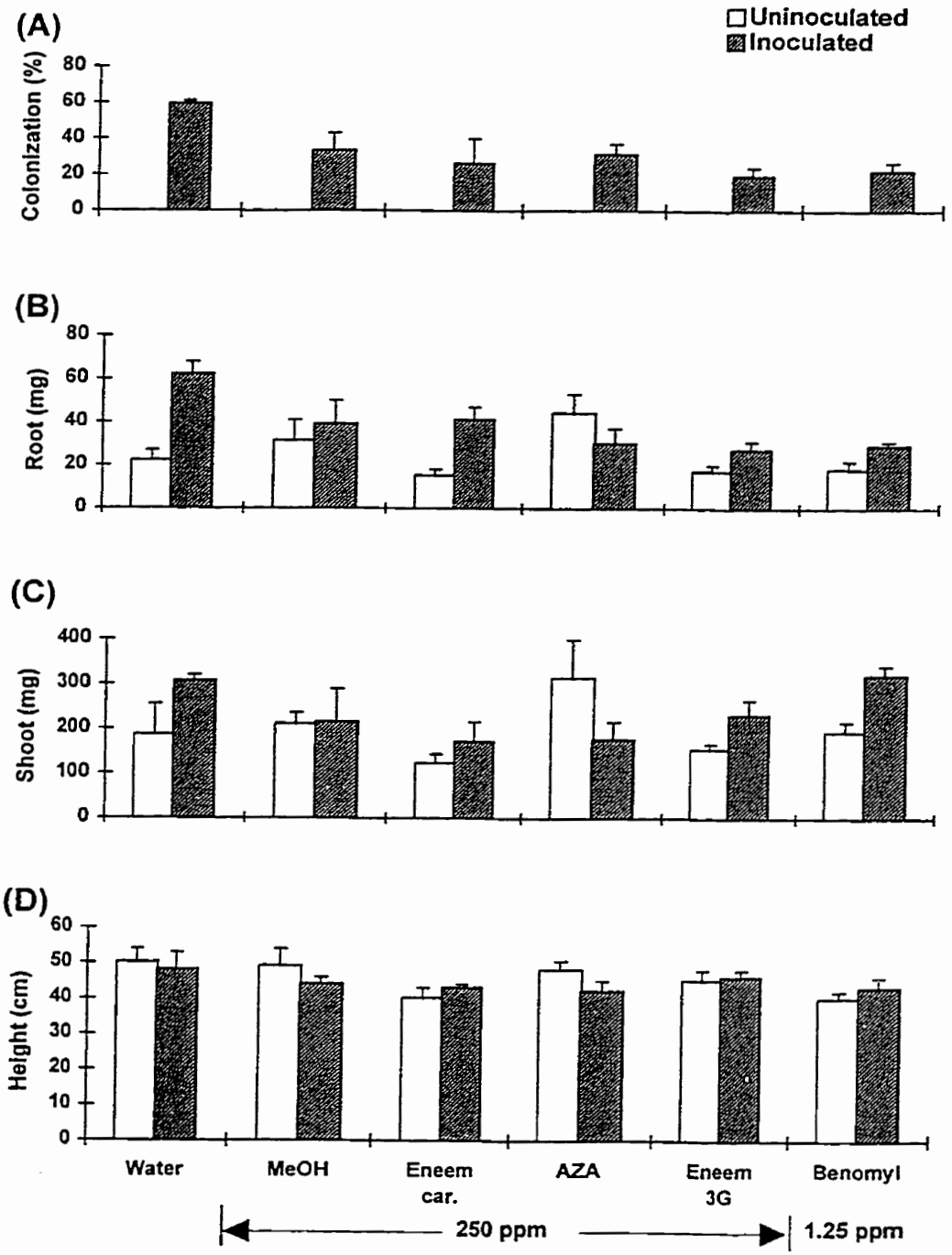

Figure 31. Effect of vermiculite amended with azadirachtin, Eneem 3G, benomyl, methanol (MeOH) solvent, and Eneem carier on *Allium porrum* grown in a 16-h day cycle of day:night temperature = 25°C:17°C, RH = 75%, light intensity = 4000 lx/cm^2, and amended weekly with modified Hoagland solution containing 0.01 ppm P. **A** colonization (%) of roots by *Glomus intraradices*, **B** root dry weight (mg), **C** shoot dry weight (mg), and **D** plant height (cm) at 14 weeks. Vertical bars = mean ± SEM (n= 5).

Relationship of leek leaf tip P to VA mycorrhizal colonization

A dose-response relationship was obtained for reference fungicides benomyl (R^2 = 0.77) and chlorothalonil (R^2 = 0.67) in the 1997 test concentration series (Fig. 32A). As well, there was a positive relationship between leaf tip P and colonization of leek roots by *G. intraradices* when grown in vermiculite amended with benomyl (R^2 = 0.65; Fig. 32B) and chlorothalonil (R^2 = 0.52; Fig. 32C). These data suggest that leaf tip P content was positively related to development of VAM in leek, and that it could be used as an indicator of VA mycorrhizal symbiosis and activity in leeks.

Effect of AZA, Eneem 3G and reference pesticides on leaf P, biomass, and VA mycorrhizal colonization:1997-high test concentrations

The results of this test series confirmed the findings that the highest test concentration of the 1996 series, i.e., AZA and Eneem 3G (both at 250 ppm) and benomyl (1.25 ppm) did not ($p < 0.05$) have any impact on leaf tip P content. Accordingly, only results of the three higher concentrations of the 1997 test series are shown. MeOH (25000 ppm) (Figs. 33A, 35A), Eneem carrier (12500 ppm) (Fig. 34A), and Acetone (12500 ppm) (Fig. 36A) had no significant ($p < 0.05$) impact on leaf P when compared with their respective inoculated and uninoculated water controls. Likewise, AZA at 250 ppm (Fig. 33B), 500 ppm (Fig. 33C), and 1250 ppm (Fig. 33D) had no significant ($p < 0.05$) impact on leaf P. Although Eneem 3G at 500 ppm (Fig. 34B) and 2500 ppm (Fig. 34C) had no effect on leaf P, the highest

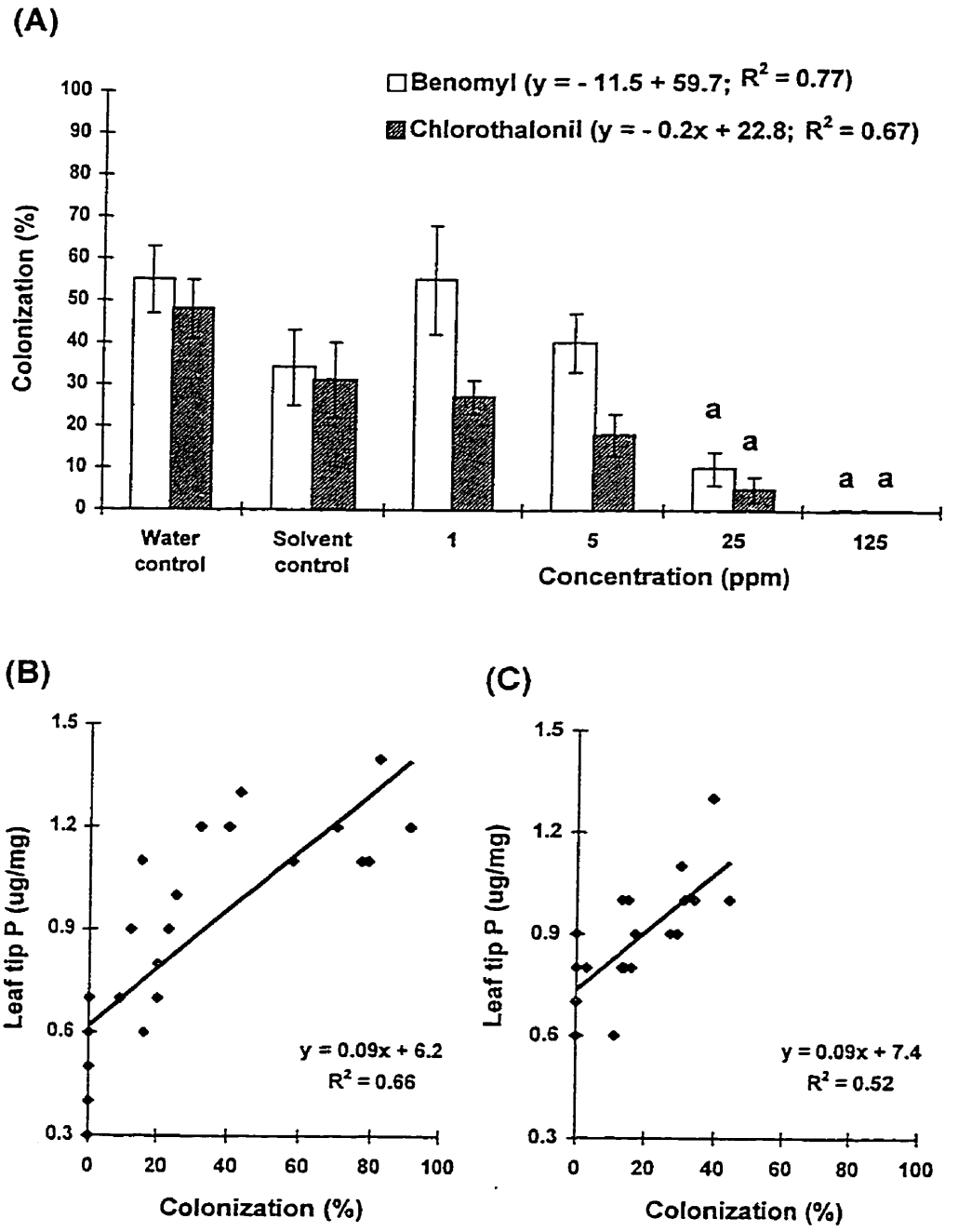

Figure 32. **A** Effect of benomyl and chlorothalonil amended vermiculite on the colonization by *Glomus intraradices* of roots of *Allium porrum* grown in a 16-h day cycle of day:night temperature = 25°C:17°C, RH = 75%, light intensity = 4000 lx/cm^2, and amended weekly with modified Hoagland solution containing 0.01 ppm P; a - indicates significant difference ($p < 0.05$) between means of solvent control and test concentration series. Relationship of leek leaf tip P and VAM fungus colonization of leek roots in benomyl **(B)** and chlorothalonil **(C)** treated vermiculite.

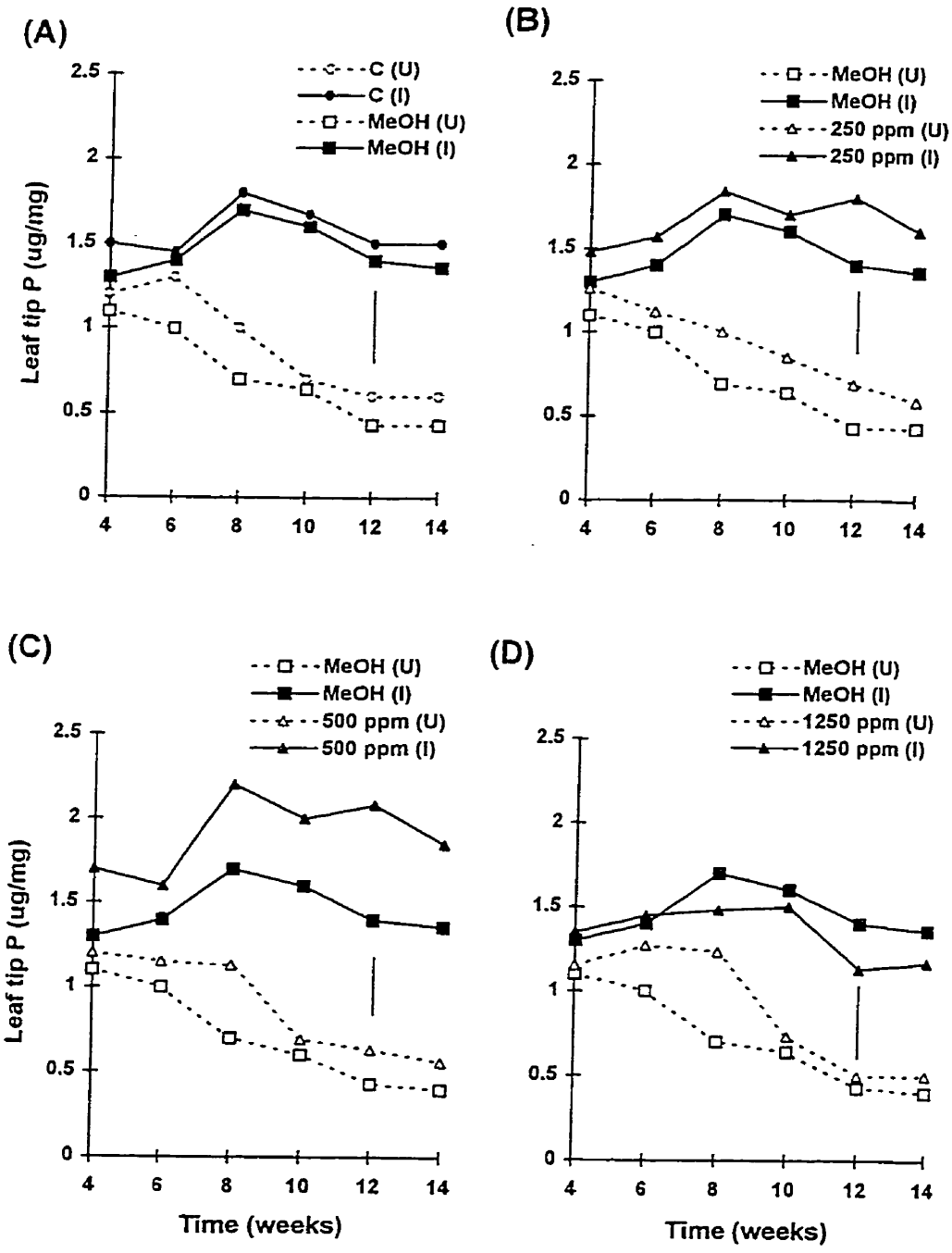

Figure 33. Phosphorus (P) content (µg/mg leaf d. wt.) In leaf tips of leek (*Allium porrum*) grown in vermiculite inoculated (I) and uninoculated (U) with *Glomus intraradices* and amended weekly with modified Hoagland solution containing 0.01 ppm P. **A** water and MeOH (12500 ppm) controls, **B** AZA (250 ppm), **C** AZA (500 ppm), **D** AZA (1250 ppm). Vertical bar represents LSD value at the 5% level.

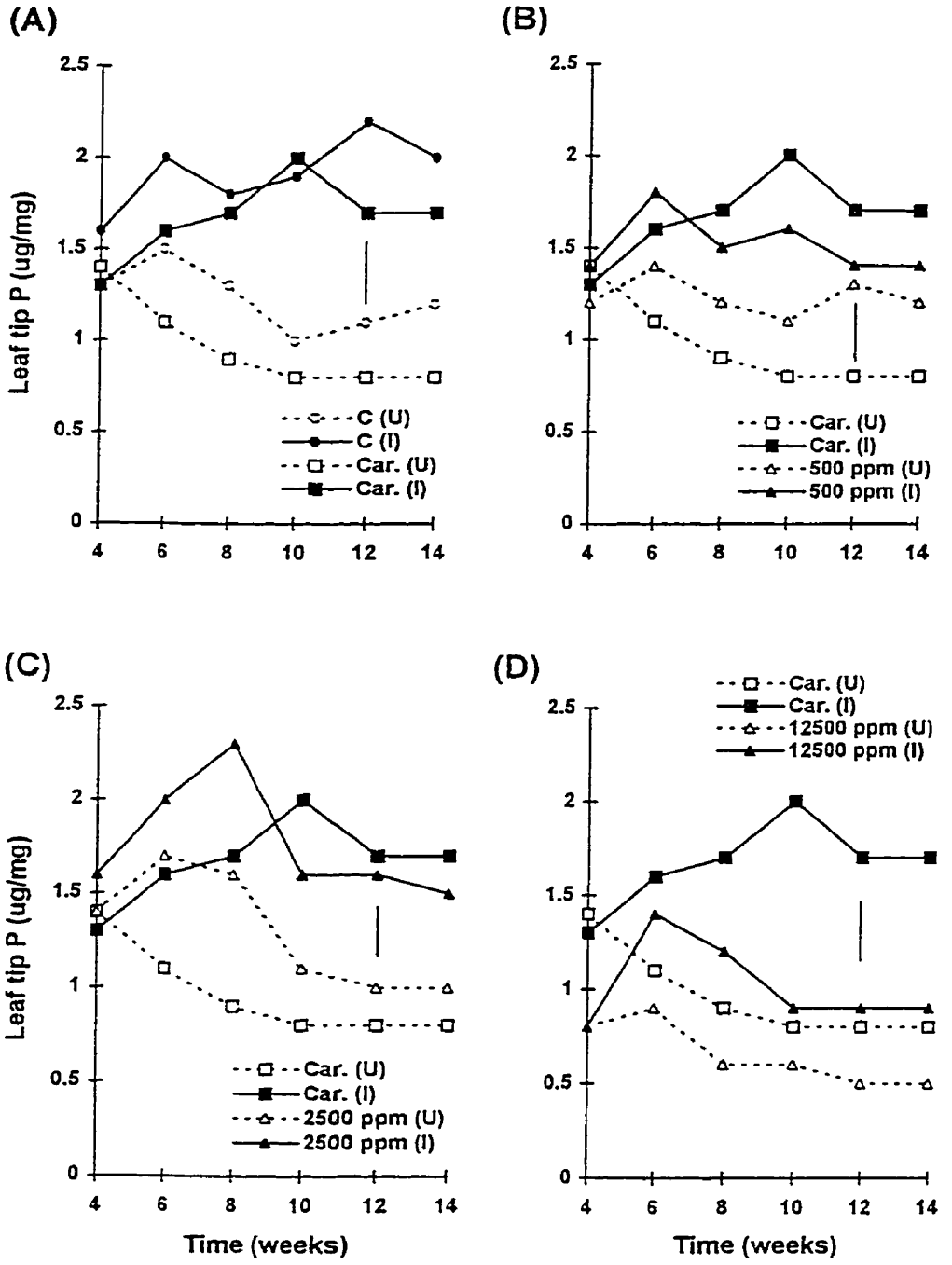

Figure 34. Phosphorus (P) content (µg/mg leaf d. wt.) In leaf tips of leek (*Allium porrum*) grown in vermiculite inoculated (I) and uninoculated (U) with *Glomus intraradices* and amended weekly with modified Hoagland solution containing 0.01 ppm P. **A** water and carrier (12500 ppm) controls, **B** Eneem 3G (500 ppm), **C** Eneem 3G (2500 ppm), **D** Eneem 3G (12500 ppm). Vertical bar represents LSD value at the 5% level.

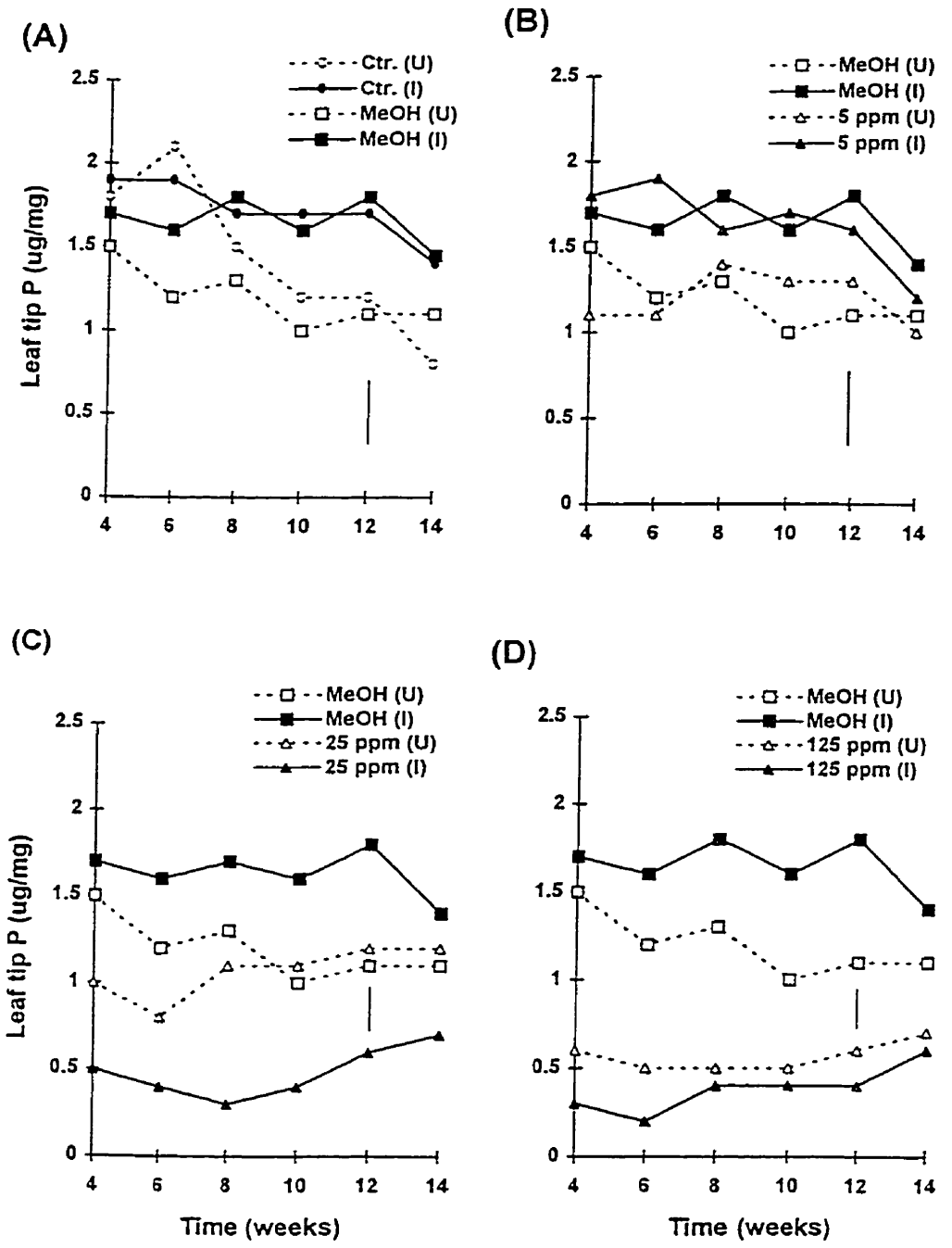

Figure 35. Phosphorus (P) content (µg/mg leaf d. wt.) In leaf tips of leek (*Allium porrum*) grown in vermiculite inoculated (I) and uninoculated (U) with *Glomus intraradices* and amended weekly with modified Hoagland solution containing 0.01 ppm P. **A** water and methanol (MeOH) (25000 ppm) controls, **B** benomyl (5 ppm), **C** benomyl (25 ppm), **D** benomyl (125 ppm). Vertical bar represents LSD value at the 5% level.

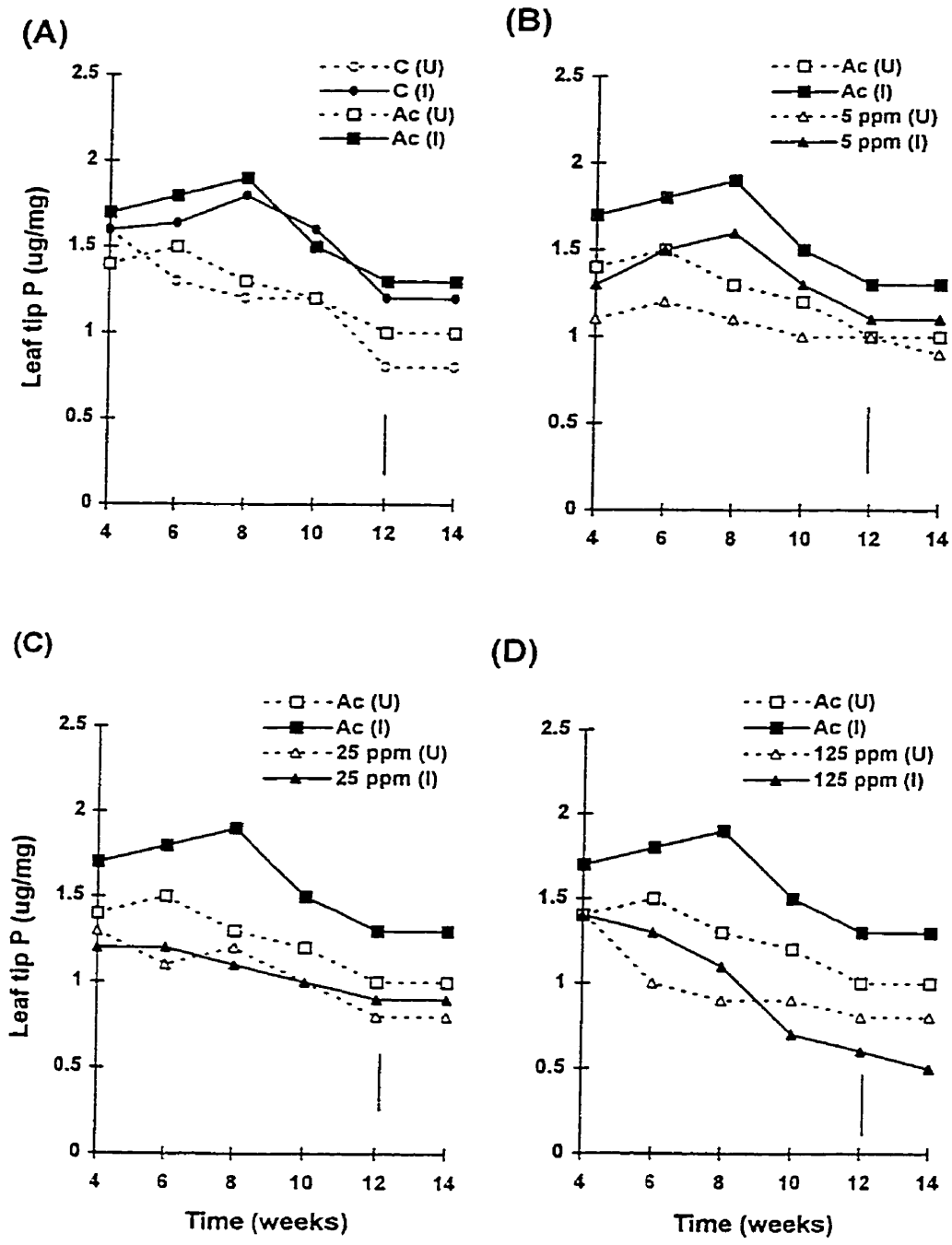

Figure 36. Phosphorus (P) content (µg/mg leaf d. wt.) In leaf tips of leek (*Allium porrum*) grown in vermiculite inoculated (I) and uninoculated (U) with *Glomus intraradices* and amended weekly with modified Hoagland solution containing 0.01 ppm P. **A** water and acetone (Ace) (25000 ppm) controls, **B** chlorthalonil (5 ppm), **C** chlorothalonil (25 ppm), **D** chlorothalonil (125 ppm). Vertical bar represents LSD value at the 5% level.

test concentration (12500 ppm) (Fig. 34D) suppressed ($p < 0.05$) P level of inoculated plants. Both reference fungicides benomyl (Fig. 35C, 35D) and chlorothalonil (Fig. 36C, 36D) reduced leaf P significantly ($P < 0.05$) at 25 ppm and 125 ppm.

Growth (height) of leeks was not significantly ($p < 0.05$) affected by the highest test concentrations of AZA, benomyl and chlorothalonil in either uninoculated or inoculated plants (Table 7). The highest test concentration of Eneem 3G, however, suppressed ($p < 0.05$) the growth of uninoculated leeks by 50% when compared with carrier control. Neither AZA nor Eneem 3G affected the shoot biomass (mg d. wt.) of inoculated leeks. However, Eneem 3G (12500 ppm) reduced shoot biomass significantly ($p < 0.05$) of uninoculated plants. While benomyl at 25 ppm significantly ($p < 0.05$) reduced shoot biomass of only uninoculated leeks (Table 7), at 125 ppm it reduced shoot dry weight of both plants. Chlorothalonil (Table 7) reduced shoot biomass of inoculated plants at 25 ppm and above. AZA and Eneem 3G reduced root biomass at the highest test concentration only in uninoculated plants, while benomyl retarded root growth of both plants at 25 ppm and 125 ppm. Likewise, chlorothalonil reduced ($p < 0.05$) root biomass of only inoculated leeks. AZA (Fig. 37A) did not reduce formation of VAM in leeks, while Eneem 3G (Fig. 37B) suppressed the development of VAM at 12500 ppm. Both benomyl (Fig. 37C) and chlorothalonil (Fig. 37D) reduced the development of VAM significantly ($p < 0.05$) at 25 ppm and 125 ppm when compared with their respective solvent controls. A positive correlation was obtained between IC50 multiples

Table 7. Effect of vermiculite amended with test materials on the growth and biomass of leek *Allium porrum*

Test materials	Height (cm)	Shoot (mg d.wt.)	Root (mg d.wt.)
AZA (in Me[1])			
Me[1] (12500 ppm)	nsr[2]	nsr	nsr
125 ppm	nsr	nsr	nsr
250 ppm	nsr	nsr	nsr
500 ppm	nsr	nsr	nsr
1250 ppm	nsr	nsr	U[3]
Benomyl (in Me[1])			
Me[1] (1250 ppm)	nsr	nsr	nsr
1 ppm	nsr	nsr	nsr
5 ppm	nsr	nsr	nsr
25 ppm	nsr	U	U, I[4]
125 ppm	nsr	U, I	U, I
Chlorothalonil (in Ac[5])			
Ac[5] (2500 ppm)	nsr	nsr	nsr
1 ppm	nsr	nsr	nsr
5 ppm	nsr	nsr	nsr
25 ppm	nsr	I	I
125 ppm	nsr	I	I
Eneem 3G (in solid carrier[6])			
Eneem carrier (12500 ppm)	nsr	nsr	nsr
250 ppm	nsr	nsr	nsr
500 ppm	nsr	nsr	nsr
2500 ppm	nsr	nsr	nsr
12500 ppm	U	U	U

[1] - Me = methanol control
[2] - nsr = no significant ($p < 0.05$) reduction when compared with the water, carrier, or appropriate solvent controls
[3] - U = uninoculated plants significantly ($p < 0.05$) reduced
[4] - I = inoculated plants significantly ($p < 0.05$) reduced
[5] - Ac = acetone control
[6] - solid carrier = mixture of ammonium sulphate + magnesium stearate

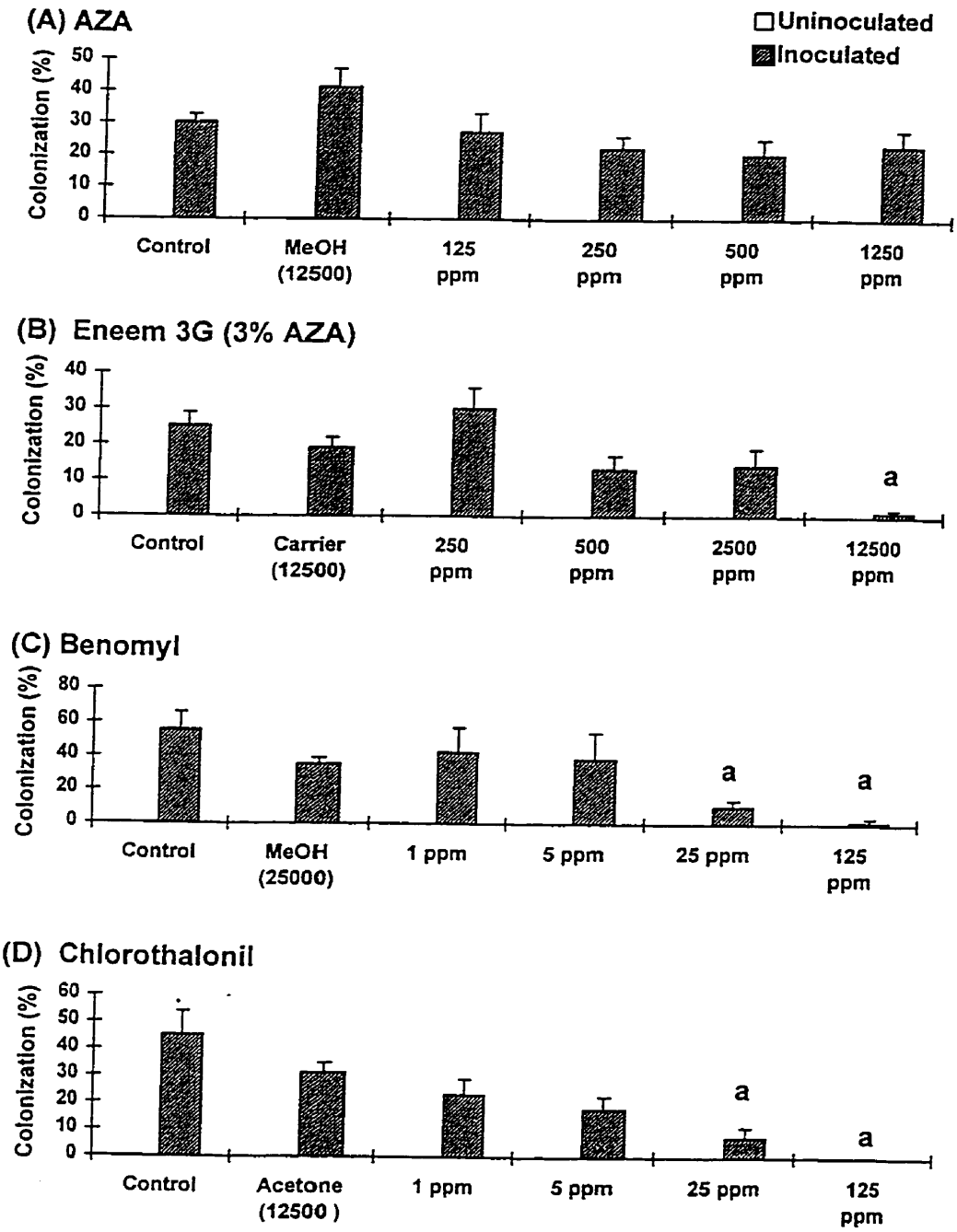

Figure 37. Effect of vermiculite amended with azadirachtin, Eneem 3G, benomyl, and chlorothalonil on the colonization by *Glomus intraradices* of *Allium porrum* grown in a 16-h day cycle of day:night temperature = 25°C:17°C, RH = 75%, light intensity = 4000 lx/cm^2, and amended weekly with modified Hoagland solution containing 0.01 ppm; a - indicates a significant difference ($p < 0.05$) between means of solvent control and test concentrations.

(concentration in substrate ÷ IC50 value) and reduction in root colonization (%) by *G. intraradices* for AZA (R^2 = 0.57), Eneem 3G (R^2 = 0.72), benomyl (R^2 = 0.66), and chlorothalonil (R^2 = 0.50). This suggests that the toxicity to VAM of pesticides was related to their field concentrations expressed as IC50 multiples.

DISCUSSION

It has long been established that VAM enhance P uptake in vascular plants (Hayman, 1982; Marschner and Dell, 1994). Because of the mobility of P within the plant, the youngest leaf of a plant is the most sensitive indicator of the activity of VAM (Habte and Turk, 1991; Habte 1992). The amount of P uptake is related to the development of VAM in roots (Habte et al., 1987). Although *A. porrum* was a suitable VA mycorrhizal indicator plant (Berta et al., 1990; Smith, 1995), the frequency of sampling young tissues for P determination was limited to leaf tips and the number of leaf blades produced. Unlike *Leucaena leucocephala*, a legume tree seedling from which sub-leaflets of the youngest compound leaflet could be sampled regularly 10 d after planting (Habte, 1992; Habte et al., 1987), it was not possible to sample leek leaf tips sooner than 4 weeks after transplanting. This was to allow time for the transplanted leek seedling to fully establish itself in vermiculite, so that the subsequent regular removal of a small section of leaf tip would have a minimum impact on the overall plant growth and development.

P concentration values were variable. Several factors, either singly or in combination, could have contributed to this variation: (a) the need to sample the

same leaf blade twice, particularly in plants responding to higher concentrations of pesticide treatments, (b) the possibility of varying amounts of P from vermiculite and VA mycorrhizal inoculum carrier being absorbed by VAM, (c) variation in colonization by *G. intraradices* in leek roots, and (d) different rates of growth by individual leek associated with inherent genetic variation. Despite the large variation, the basic trends in leaf tip P content of leeks grown in optimum P-amended and *G. intraradices*-inoculated vermiculite were generally higher than those of unamended and uninoculated plants. These trends were generally similar to the findings of Aziz and Habte (1987) and Habte et al. (1992). Since a positive relationship between leaf tip P and VAM in roots of the leek plants grown in fungicide treated vermiculite was shown earlier, P variation over time was used to indicate VA mycorrhizal activity, and to detect the effect of pesticides, if any, on VAM in *A. porrum.*

Vermiculite treated with AZA, Eneem 3G and the reference fungicides benomyl and chlorothalonil had negligible impact on the VA mycorrhizal activity and symbiosis of *G. intraradices* at the 1996 lower test concentration series. This test series was designed to reflect estimated field concentrations when these pesticides were used under prescribed conditions. Impact on VA mycorrhizal activity, however, was detected in the 1997 higher test concentration series, e.g., Eneem 3G (12500 ppm), and both benomyl and chlorothalonil at 25 ppm and 125 ppm. The higher test concentrations represented levels that might occur in the field during repeated oversprays, an accidental spill situation, or when test materials are

used at the maximum recommended rates as soil sterilants.

The lack of impact of test materials on VAM until high levels of residues occurred in the growth substrate as indicated in the higher test concentration series could be attributed to several factors: (a) timing of application, (b) mode of pesticide action, (c) persistence of pesticides, and (d) transformation by chemical actions and microbial activities in the vermiculite. The timing of application could not have been the cause of pesticide ineffectiveness, as test materials and VA mycorrhizal inoculum were mixed with the vermiculite just before transplantation of leeks occurred. Nor was pesticide persistence responsible for the lack of bioactivity since these test materials were slightly to moderately persistent. The $t_{1/2}$ of AZA, Eneem 3G, benomyl and chlorothalonil in soils or vermiculite was 14.7 d, 46.2 d, ~135 d, and ~80 d, respectively (Wan et al., 1997; Szeto and Wan, 1996; Davies, 1988). The lower concentration series was obviously ineffective on the VA mycorrhizal fungus, since an adverse effect on the activity of VAM was only detected at higher concentrations.

The mode of action of individual test materials could be one of the factors contributing to the ineffectiveness of test materials on VA mycorrhizal activity and symbiosis in vermiculite. Our previous study indicated that AZA, a contact pesticide, had no fungicidal activity, and this study confirms that finding (Wan and Rahe, 1998). Likewise, the formulated product of AZA, Eneem 3G, had no effects on VA mycorrhizal activity, until an excessively high test concentration was used, e.g., 12500 ppm Eneem 3G (3% AZA, 97% carrier consisting of a mixture of

ammonium sulphate and magnesium stearate). At this concentration, it was unclear whether the impact on VA mycorrhizal activity was caused by AZA, the carrier, or the synergistic action of both. In contrast, benomyl, a systemic fungicide, may be reduced to ineffective concentrations in soil by absorption into plants. Rouchaud et al. (1974) found that in soils, up to 90% of the material was rapidly absorbed, translocated, and concentrated in the leaves of seedlings. Although a contact fungicide, some of chlorothalonil's six toxic transformation products were similarly removed from the soil (Nelson et al., 1983). Absorption by roots and subsequent translocation to shoots, therefore, probably removed substantial amounts of soil incorporated residues of these compounds, thereby reducing the remaining residues to concentrations harmless to VA mycorrhizal fungus propagules.

Another factor that may have contributed to decreased bioactivity of residues in the vermiculite was the transformation of the original pesticide molecules to inactive metabolites by chemical action and microbial activities (Munnecke, 1967; Woodcock, 1967). Our previous study found that the inclusion of VA mycorrhizal inoculum (containing a mixture of peat, perlite, soil particles and about 2100 active *G. intraradices* propagules/L) in the vermiculite appeared to accelerate AZA degradation (Wan et al., 1997). This was most likely attributed to the abundance of microorganisms found in the VA mycorrhizal inoculum (actinomycetes, 2.3×10^8; bacteria, $8,4 \times 10^8$; fungi, 9.5×10^5 CFU/g dry weight, as determined in the laboratory by using starch-casein agar, Thornton's agar, and

neopeptone-glucose-rose bengal-aureomycin agar, respectively). These microorganisms would likely contribute to the biodegradation of not only AZA but also benomyl and chlorothalonil.

There is one other consideration that may play a role in the low bioactivity (Wan and Rahe, 1998) of AZA, including neem compounds, on the VA mycorrhizal fungi. VA mycorrhizae occur in the roots of the neem tree, *Azadirachta indica* (Diem et al., 1981; Kiran et al., 1989). It is speculated that this association may have allowed VA mycorrhizal fungi to develop tolerances for these compounds.

From the eco-toxicological viewpoint, this study shows that the IC50 test for VAM fungus was a very sensitive test. The IC50 values could be used as an indication of the eco-toxicity potential of pesticides to subterranean microorganisms. Pesticides having large IC50 values are likely to have less eco-toxicity potential than those having small values. For example, the IC50 to *G. intraradices* of benomyl, chlorothalonil, Eneem 3G, and AZA was 0.004 ppm, 0.05 ppm, 210 ppm, and 230 ppm, respectively (Wan and Rahe, 1998). This indicated that benomyl has a greater potential than AZA to have an adverse impact on VA mycorrhizal symbiosis at low concentrations, especially when the actual field concentration was taken into consideration in the estimation of a "Safety Factor" (Table 8).

A Safety Factor is a value equivalent to the IC50 multiples obtained by dividing the actual field concentration of a pesticide by its IC50 value to *G. intraradices*. This factor serves as a gauge to give an indication of the potential of

Table 8. Estimation of a Safety Factor for soil application of pesticides

Pesticides	IC50 (ppm)	HONEC[1] (ppm)		Safety Factor[2]
		Nominal	Actual[3]	
Azadirachtin	230	1250	860 (-31%)	3.7
Eneem 3G	210	1500	1542 (-38%)	7.3
Benomyl	0.004	5	4.6 (-10%)	1150
Chlorothalonil	0.05	5	5.2 (+4%)	104

[1] - HONEC = highest observed no effect concentration at day 0 on *Allium porrum* (biomass, leaf P, and colonization of roots by *Glomus intraradices*) in pesticide amended growth media

[2] - Safety Factor = a value obtained by dividing the actual field concentration of pesticide by its IC50 to *Glomus intraradices*

[3] - Actual HONEC = concentration of a pesticide in amended growth media as determined by chemical analysis; percentage in brackets indicates loss or gain when compared with the nominal concentration (Baude et al., 1974; Rouchaud & Roucourt 1988; Wan et al., 1997).

a pesticide to cause harm to soil microorganisms. Accordingly, the likelihood of a pesticide to have an adverse impact on the terrestrial environment increases when its use resulted in IC50 multiples that exceeded the Safety Factor. Indeed, our vermiculite trials confirmed the low toxic potential of AZA and Eneem 3G, when they were used at the estimated minimum, moderate, and maximum rates of soil application in the top 15 cm (6 inches) soil (Table 9). The use of benomyl and chlorothalonil at the minimum recommended rate (i.e., 3.5 ppm top 15 cm soil for both) of field application was not expected to cause harm to soil microorganisms. However, at moderate and maximum recommended rates, the estimated IC50 multiples of both reference pesticides exceeded the Safety Factor (Table 9), an indication that their use would likely cause harm to the terrestrial environment.

It was evident that a growth substrate such as vermiculite has the capacity to "mitigate" the toxic effects of both benomyl and chlorothalonil at concentrations much greater than their IC50 values. It is not known what causes this phenomenon. It is speculated, however, that this "mitigating factor" was likely the result of a combination of factors described earlier, notably the behaviour of both fungicides, and the chemical and microbial actions occurring in the vermiculite. At 5 ppm, neither fungicides appeared to have any harmful effects on VA mycorrhizal fungus and its symbiotic activity. At 25 ppm, however, both benomyl and chlorothalonil suppressed VA mycorrhizal activity. Test concentrations greater than 25 ppm were equivalent to amounts used for sterilizing soils. Our study confirms that there was a positive relationship between IC50 multiples of pesticides versus

Table 9. Eco-toxicity potential of azadirachtin and reference pesticides in the terrestrial environment (using IC50 value to *Glomus intraradices* and an estimated field concentration to calculate the IC50 multiples for comparison with an estimated Safety Factor[1])

Pesticides	Field rates[2] Kg a.i./ha (ppm)		IC50 (ppm)	IC50 multiples	Safety Factor
Azadirachtin	5	(3.5)	230	0.015	3.7
	50	(35)	230	0.15	3.7
	150	(105)	230	0.45	3.7
Eneem 3G	5	(3.5)	210	0.017	7.3
	50	(35)	210	0.17	7.3
	150	(105)	210	0.51	7.3
Benomyl	5	(3.5)	0.004	875	1150
	50	(35)	0.004	***8750***	1150
	150	(105)	0.004	***26250***	1150
Chlorothalonil	7	(5)	0.05	100	104
	21	(15)	0.05	***300***	104
	210	(150)	0.05	***3000***	104

[1] - Safety Factor = a value obtained by dividing the actual field concentration (as determined by chemical analysis of pesticide amended growth media at day 0) of a pesticide by its IC50 to *Glomus intraradices*

[2] - estimated minimum, moderate, and maximum soil application rates (Trappe et al., 1984); numbers in brackets indicate an approximate concentration on the top 15 cm (6 inches) soil

their corresponding reduction in root colonization by *G. intraradices*. Accordingly, the IC50 value to *G. intraradices* could and should be used for the initial screening of new candidate pesticides before registration to determine their potential to cause harm to non-target soil microorganisms. The "Safety Factor" could be used as a gauge to predict the safe use of the pesticides in the terrestrial environment (Rand et al., 1995).

CONCLUSIONS

Leaf tip P status over time and root colonization by *G. intraradices* were more reliable indicators of VAM and its symbiotic activity in *A. porrum* than plant height and biomass. AZA and Eneem 3G had negligible effects on the VA mycorrhizal symbiosis when used at prescribed application rates under conditions similar to the field. Neither plant growth nor root development nor colonization by *G. intraradices* of *A. porrum* was affected by benomyl, chlorothalonil, and Eneem 3G, except when subjected to concentrations equivalent to very high application rates. There was a positive relationship between the IC50 multiples to *G. intraradices* of these pesticides in the M-medium and their corresponding toxicity to VAM in *A. porrum* grown in vermiculite that was used as an experimental field substrate. For the protection of non-target soil micro-organisms and the terrestrial environment from prescribed use, it is feasible to consider the use of the IC50 value to *G. intraradices* of a pesticide and its estimated highest no effect simulated field test concentration to VAM to determine a "safety factor". This "safety factor" could

be used to predict acceptable field concentrations to minimize the potential adverse eco-toxicological impact of the pesticide.

CONCLUDING DISCUSSION

Although there are a number of well-defined toxicity tests now in place for testing indicator organisms of various trophic levels (Environment Canada, 1996; US EPA, 1996), no standard method is available to date for assessing pesticide impacts on VA mycorrhizal fungi and their symbiotic association (Trappe et al., 1984). This research confirmed that the M-medium technology consisting of both *G. intraradices* and VA mycorrhizal symbiosis from Ri T-DNA transformed *D. carota* roots cultured in the medium could offer a possible instrument for standardizing pesticide bioassays of VA mycorrhizal fungi. It specifically identified the suitability of using the extraradical mycelial growth (EMG), extraradical mycelial sporulation (EMS), and the VA mycorrhizal activity (expressed in terms of % root colonization) of *G. intraradices* on carrot roots as response indicators.

To date, many technical details relating to the use of the M-medium technology for pesticide bioassays are unavailable in the open literature (AGRICOLA, 1998; BIOSIS, 1998: CA ABSTRACTS, 1998; CAB, 1998). This research identified specific techniques required to culture the VA mycorrhizal fungus, make observations of response indicators, and produce data in a consistent, efficient, and standardized manner. EMG and EMS of the VA mycorrhizal fungus could be determined, relatively accurately, from combinations of 3- to 8-visual fields/plate and 3 to 25 plate replicates. It was also found that growth consistency and phases of EMG and EMS, and the timing of establishment of *G. intraradices* were more predictable in regular than in two-section Petri dishes.

For practical purposes, it was suggested that observation of EMG and EMS in VA mycorrhizal fungus pesticide toxicity tests should be focussed on the top 1-2 mm layer of the M-medium, and at 2 weeks after inoculation with a combination of five-visual radial fields/plate and a four-plate replicate/test concentration. VA mycorrhizal association could be measured in terms of percent colonization of carrot roots. A 100% establishment of VA mycorrhizal inocula was consistently obtained from materials of 4-week old mother VA mycorrhizal cultures. Colonization of carrot roots by *G. intraradices* increased steadily from 5% in 2-week to about 50% in 8-week old cultures. As well, this research found that non-chemical method was more time efficient than the chemical dissolution of M-medium method of harvesting carrot roots. Moreover, biomass of roots (d.wt.) harvested by the chemical dissolution method appeared to be about 35% heavier than those from non-chemical method. Pre-sterilized M-medium stored at 20°C and in darkness for up to 3 months does not deteriorate, and allows the establishment of VA mycorrhizal inocula.

Becard and Fortin (1988) indicated the possibility of using the M-medium technology for research applications other than VA mycorrhizal work related to enhancing agricultural and horticultural crop production. This study confirmed their conviction, as it succeeded in developing a simple, reproducible, practical and efficient technique for obtaining point estimates of pesticide toxicity to VA mycorrhizal fungi, using the concept of IC50 (concentration inhibiting 50% EMG or EMS). The IC50 value to *G. intraradices* of reference pesticides, e.g., benomyl,

chlorthalonil, etc., was determined by spiking different concentrations of the test materials to the M-medium and comparing the resulting effects on EMG and EMS with those from controls. Both the nonlinear regression (NLR) and linear interpolation (LI) method of estimating the IC50 value produced comparable results. It was found that the order of increasing toxicity of reference test compounds to *G. intraradices* was acetone, methanol, AMPA, dimethoate, $CuSO_4$, glyphosate, chlorothalonil, and benomyl. Accordingly, IC50 value presented an option for comparing the short term impact of pesticides to VA mycorrhizal fungi, and a possible tool to further enhance the value of eco-risk assessment of pesticides and toxic substances.

There is some information on the persistence of AZA in soils (Stark and Walter, 1995) but to date, there are no data available on the behaviour of AZA in M-medium and vermiculite (Wan et al., 1997). This research validated that AZA was slightly to moderately persistent in both the M-medium and vermiculite biological systems. As well, it found that AZA was completely incorporated and uniformly distributed in the M-medium when it was introduced by injection to the molten solution at 56°-60°C. However, the disappearance of AZA in M-medium and in vermiculite systems followed pseudo-first order kinetics. The half-life of AZA was 44.4 days in M-medium containing *G. intraradices* and *D. carota*. In the systems containing vermiculite, vermiculite and soil mixture, VA mycorrhizal fungus and leek, the half-lives for technical and formulated AZA ranged from 13.2 to 46.2 days. Evidence was observed of the possible occurrence of AZA conversion products in

AZA treated and aged M-medium, vermiculite, and soil-vermiculite mixture, as well as the presence of AZA-B residues in products formulated with natural neem extracts. This study also indicated that refinements to the clean up procedures for gellan gum-based M-medium extracts for HPLC analysis are needed to avoid the blockage of glass columns by micro particles.

Using the IC50 technique developed in this study, it was found that the toxicity of AZA and neem compounds to *G. intraradices* appeared to be solvent dependent. The order of increasing toxicity to this VA mycorrhizal fungus was AZA (methanol), Eneem 3G (methanol), Neemix 4.5E (alcohol-based carrier), AZA (acetone), NEX (methanol), AZA (ethanol), and AZA (propanol) when EMG was used as the response indicator. In methanol, neem compounds such as AZA, NEX, Eneem 3G and Neemix 4.5E, including their carriers, had low, short term toxicity to *G. intraradices.* Neither its host *D. carota* nor the VAM infection was affected by these AZA compounds when compared with reference pesticides such as benomyl, chlorothalonil, $CuSO_4$, dimethoate, glyphosate, and AMPA. The relatively high IC50 values suggested that AZA in methanol and neem insecticides were not expected to have an adverse impact on non-target subterranean micro-organisms nor pose an eco-toxicological risk to the terrestrial environment. There were concerns, however, that the IC50 test results were derived from an artificial system and that the values obtained might not be relevant to field situations.

It has been established that the P status of young VA mycorrhizal-responsive plant leaves monitored as a function of time would provide an indication of VAM

activity and symbiosis (Habte at al., 1987; Habte and Turk, 1991; Habte, 1992). To address the concerns stated earlier, *Allium porrum* grown in non-sterilized and optimum P-amended vermiculite was used, and found suitable, as a VA mycorrhizal-response indicator plant for pesticide bioassays. However, adjustment to the sampling technique and regime of leaf tips for P analysis was necessary to minimize impact on normal plant growth and development. As well, nutrient amendments for vermiculite were needed to promote normal plant growth in the presence of the VA mycorrhizal fungus. When used at prescribed application rates under conditions similar to the field, AZA, Eneem 3G, benomyl and chlorothalonil had negligible effects on the symbiosis of VAM. Neither plant growth nor root development and colonization by *G. intraradices* of *A. porrum* was affected by these pesticides, except when subjected to concentrations equivalent to very high application rates. There was a positive relationship between IC50 multiples of test pesticides in the M-medium and the corresponding concentrations adversely affecting VAM in vermiculite used as an experimental field substrate.

This study confirms that it is feasible to use *G. intraradices* and VAM as indicators for assessing the environmental impact of AZA and pesticides. An IC50 value of a candidate pesticide can be determined using the Ri T-DNA transformed carrot root system. It is suggested that IC50 can be used as an indication of the potential toxicity and adverse field impact of the pesticide, as the smaller its value, the greater is the likelihood of the pesticide causing harmful effects on soil micro-organisms (Wan and Rahe, 1998). Although large differences

in the "sensitivities" of the Ri T-DNA transformed carrot system and the "in vivo" (leek seedlings in vermiculite) system were observed, the relative toxicities of the tested materials in the two systems were similar. There is a need, however, to conduct more research to show the practical value of "in vivo" systems for assessment.

FUTURE CONSIDERATIONS AND PERSPECTIVES

This research demonstrated the use of the IC50 value to *G. intraradices* of a pesticide and its highest no-effect field test concentration on VAM to estimate a safety factor to predict the eco-toxicological potential of the toxicant. As a follow up, there is a need to validate, and perhaps refine, this approach in another laboratory, using this VA mycorrhizal fungus and other indicator species and symbiont host plants. In order to provide a more complete picture, *in situ* field studies are necessary to verify the safety factors obtained from experiments conducted under field-like conditions in a growth chamber. It is preferable to carry out these studies using several VA mycorrhizal fungus species, symbiont host plants, and soil types under defined environmental conditions. Once this task has been accomplished, a standard testing protocol could then be developed so that results on IC50 values and safety factors produced elsewhere are comparable. In the meantime, proponents of pesticides and evaluators from pesticide industries and government agencies should be informed of the potential usefulness of this new approach of evaluation. They should be encouraged to consider the inclusion

of this approach in their evaluation protocol to enhance the value of eco-risk assessment of pesticides and toxicants.

APPENDICES

Appendix 1. Preparation of minimal medium (M-medium) solution (Institut Recherche en Biologie Végétale, de l'Université Montréal, Quebec, Canada)

I. Stock solutions

		grams per litre
1.	Macronutrients	
	$MgSO_4.7H_2O$	7.31
	KNO_3	0.8
	KCL	0.65
	KH_2PO_4	0.048
2.	Calcium nitrate	
	$Ca(NO_3)_2.4H_2O$	2.88
3.	Potassium iodide	
	KI	0.75
4.	Vitamins	
	Glycine	0.6
	Thiamine	0.02
	Pyridoxine	0.02
	Nicotinic acid	0.10
	Myo-inositol	10.00
5.	NaFe EDTA	1.6
6.	Micronutrients	

A. Dissolve 3g $MnCl_2.4H_2O$ (or 2.33g $MnSO_4.H_2O$) in 100 ml pure distilled water.

B. Dissolve 1.325g of $ZnSO_4.7H_2O$ in 100 ml of pure distilled water.

C. Dissolve 0.75g of H_3BO_3 in 100 ml of pure distilled water.

D. Mix A, B and C.

E. Weigh 0.65 of $CuSO_4.5H_2O$ and dissolve in 50 ml of pure distilled water. When totally dissolved, pipette 5 ml of this solution into A, B, C mixture.

F. Dissolve 0.12 of $Na_2MoO.2H_2O$ (or 0.11g $NH_4(Mo_7O_2)_4$ in 100 ml of distilled water. When totally dissolved, pipette 1 ml of this solution into the A, B, C mixture.

G. Adjust the volume of the A, B, C mixture to 500 ml.

**Store all stock solutions in clean bottles in the fridge, except for the vitamin solution which should be kept frozen until required.

II. M-Medium preparation

1. Agitate NaFe EDTA solution
2. Add 700 ml H_2O in 1000 ml graduated cylinder
3. Add 100 ml macronutrient solution
4. Add 100 ml Calcium nitrate solution
5. Add 5 ml NaFe EDTA
6. Add 1 mL micronutrients
7. Add 1 ml KI
8. Add 5 ml thawed vitamin solution
9. Add 10 g sucrose (Sigma cell culture quality)
10. Dissolve completely and bring up to volume
11. Adjust pH to 5.5 or 6.0 (if required)
12. Add 4 g Gelgro or 10 g purified agar
13. Autoclave for 16 min (500 mL per 1000 ml bottle)
14. Wait 5 min after autoclave (Gel sets at about 55°C)
15. Use auto pipetter to transfer the required volume (25 or 40 ml, etc.) to each Petri dish when bottle feels just hot to handle

15. Sterilize fume hood area with 70 % alcohol before laying out Petri dishes

16. Sterilize instruments (e.g., forceps, surgical blade, etc.) with 70% alcohol + 10 % propanol + 20% water mixture, and heat them in flame of spirit lamp for about 5-10 seconds before use each time.

Appendix 2. Estimation of hyphal Length (EMG) and spores (EMS)

A. Length of extraradical mycelial growth (EMG)

Length Conversion Factor (LCF) used in Tennant's method (1975):
EMG = no. Intercepts x LCF (Tennant's simplified Formula)

Grid square (containing 10 x 10 sub-grid square units)

Grid size	½	1	2	3 cm
or	5	10	20	30 mm
LCF	0.3928	0.7856	1.5714	2.3571 (Tennant, 1975)

Meiji EMT-2-TR dissecting scope

At 15 x mag. (setting #1), size of one sub-grid square unit = 0.95 mm
∴ applying LCF (for 1 cm grid size) = 0.95 x 0.7856 (Tennant, 1975)
= 0.7463 mm/crossing/sub-grid square unit

If total counts of EMG crossings = 500 per grid square (of 10 x 10 sub-grid units),
then total length of EMG = 500 x 0.7463 mm/cm^2
= 373.2 mm/cm^2
or = 37.32 cm/cm^2

B. Extraradical mycelial sporulation (EMS)

Meiji EMT-2-TR dissecting scope

At 15 x mag., size of one sub-grid square = 0.95 mm
one grid square (containing 10 x 10 sub-grid sq. units) = 9.5 mm x 9.5 mm
= 90.25 mm^2
= 0.9025 cm^2

If one grid square (of 10 x 10 sub-grid square units) contains 100 spores
therefore, 1 cm^2 = 1 ÷ 0.9025 x 100 = 111 spores/cm^2.

Appendix 3. Preparation of Hoagland's solution (Hoagland and Arnon, 1938)

A. Molar stock solution of each of the following four salts are prepared separately. Each stock solution is used as indicated.

	ml, in a litre of nutrient solution
1 *M* KH_2PO_4, potassium dihydrogen phosphate (MW = 136)	1
1 *M* KNO_3, potassium nitrate (MW = 101)	5
1 *M* $Ca(NO_3)_2.4H_2O$, calcium nitrate (MW = 236)	5
1 *M* $MgSO_4.7H_2O$, magnesium sulfate (MW = 246)	2

B. To each litre of nutrient solution prepared in A is added 1 ml of a solution of the following five salts.

	gm dissolved in 1 litre of water
H_3BO_3, boric acid	2.86
$MnCl_2.4H_2O$, manganese chloride	1.81
$ZnSO_4.7H_2O$, zinc sulfate	0.22
$CuSO_4.5H_2O$, copper sulfate	0.08
$H_2MoO_4.H_2O$, molybdic acid	0.02

C. To each litre of nutrient solution prepared in A, 1 ml of an iron solution is added.

Note: Hoagland recommended the addition of a solution of iron tartrate (0.5%), at the rate of 1 ml/litre of nutrient solution about once or twice a week. Since about 1950, however, it has become common practice to supply iron to nutrient solutions as ferric salts of ethylenediaminetetraacetic acid (EDTA). The addition of 1 ml of a

solution of FeEDTA containing 5 mg Fe per ml to 1 litre of nutrient solution provides 5 ppm iron by weight. This level of iron is adequate for the growth of most higher plants. For a report on the best method of preparation of FeEDTA, consult Steiner and va Windoen, 1970.

Appendix 4. Root staining and estimation of VAM colonization (Dalpe, 1993)

A. Preparation of materials

1. Aqueous solution of 2.5 to 19% KOH (w/v) for root clearing.

2. Aqueous solution of 3% H_2O_2 solution or aqueous solution of 20% NH_4OH for root bleaching.

3. Trypan blue solution (Phillips and Hayman 1970; Koske and Gemma 1989): trypan blue (0.5 g) in glycerol (500 ml), H_2O (450 ml), and Hcl 1% (50 ml) for root coloration.

4. Destaining solutions prepared as in previous step, but without the stain.

5. PVLG mounting media (Omar et al. 1979): polyvinylalcohol, high viscosity (24-32 cP), 1.66 g; H_2O, 10 ml; lactic acid, 10 ml; glycerol, 1.0 ml. Dissolve PVA in water; stir vigorously while adding lactic acid and glycerol; gentle heating may be necessary.

B. Root preparation

1. Use fresh or fixed roots; gently wash them with water to remove attached soil particles and soil debris. Cut them in short pieces to facilitate their staining and mounting on microscope slides.

2. Clear roots in KOH solution (according to the root sensitivity) by autoclaving for 3 minute 121°C or heat at 90°C for 10 to 45 min (use fume hood to avoid breathing harmful vapor of KOH).

3 Bleach roots (use only for highly pigment materials) in H_2O_2 or NH_4OH for 10 to 45 min at room temperature.

4. Rinse roots in several changes of water.

5. Acidify roots in 1% HCl prior to the staining (trypan blue staining is effective under low pH).

6. Stain roots in trypan blue reagent by either autoclaving roots for 3 min at 121°C or in a 90°C water bath for 15 to 60 min. Root material stains

completely blue.

7. Destain roots in destaining solution at room temperature or at 50 to 65°C Destained roots can be stored for a long time (several months) at room temperature.

C. Scoring root colonization

1. With the grid line intersect method (Giovannetti and Mosse, 1980), a grid made of 1.27 cm (0.5 in) squares is etched on a plastic Petri dish lid. Stained roots are deposited in the lid, flattened with the Petri dish bottom, and examined under the microscope at 50 to 100 x. Colonized root sections that intersect the lines of the grid are counted and compared with the total number of roots intersecting the grid lines. Results are expressed in percent of observed that are colonized.

2. With the slide method (Giovannetti and Mosse, 1980): large numbers (50-100) of 1 cm root sections are mounted on slides. The length of colonized root tissue is measured and compared to the total length of root observed. Results are expressed as a percent.

D. Comments

1. Intramatrical vesicles and hyphae are indicators of the presence of VAM fungi in the root system, but only the presence of intracellular arbuscules which are the site of metabolite exchanges between plant and fungi confirms the symbiotic behaviour of the fungal partner. Depending upon the physiological state of the observed mycorrhizae, arbuscules may appear as highly ramified structures originating from a single hypha or as an irregular mass of stained material without recognizable ramification.

2. As different plant roots react differently to clearing, bleaching, and staining procedures, preliminary tests followed by adaptations of techniques are often necessary to optimize staining results. Before staining, extramatrical hyphae can be detected by examining the surface of gently washed roots under a dissecting microscope; their presence will not be necessarily indicate root infection. The removal of phenol from the lactophenol/trypan blue solution (Phillips and Hayman, 1970; Kormanik and McCraw 1982), as used by Koske and Gemma (1989), can provide adequate contrast with several hosts and VAM partners.

Appendix 5. Phosphorus (P) monitoring of subleaflets/leaf tips (Habte et al., 1987)

Standard P solutions

1. Stock P standard solution: 1000 mg/l phosphate as P. Oven dry approximately 5 g KH_2PO_4 (potassium dihydrogen phosphate) for 1 h at 105°C. Cool in a dessicator for 1 h. Dissolve 4.394 g of KH_2PO_4 in deionized water and dilute to 1 litre.

2. Immediate P standard solution: 10 mg/l. Dilute 1.0 ml of stock solution to 100 ml with dionized water.

3. Working standard P solution: prepare daily serial dilutions of 0.002, 0.005, 0.01, 0.03, 0.05, 0.1, 0.15 0.2 mg/l for calibration purposes.

Reagent A

1. To 2.7 l distilled H_2O, add o.35 g antimony potassium tartrate; stir solution to material dissolve well.

2. Add 168 ml concentrated sulfuric acid.

3. Add 14.43 g ammonium molybdate.

4. Add 120 ml H_2O.

5. Store solution in dark bottle in a refridgerator.

Reagent B

1 Add 100 ml reagent A to 0.428 g ascorbic acid.

2. Always use freshly prepared reagent B solution.

Preparation of leaf materials

1. Remove 1-2 cm leaf tip of leak; dry at room temperature for 1 h.

2. Weigh leaf tip.

3. Ash leaf tip at ~500°C for 4 h in a muffle furnace.
4. Cool to room temperature in a dessicator.

5. Add 2.5 ml freshly prepared reagent B (ascorbic acid, 0.428 g/100 ml reagent A).

6. Add 10 ml de-ionized (DI) water; mix well.

7. Stand mixture for 20 min.

8. Read intensity of color at 822 nm optical density (OD) or absorbance (8700 Series UV/VIS Spectrophotometer).

9. Add 1 ml P standard (20 mg/l) to 2.5 ml reagent B + 6.5 ml DI H_2O in a test tube (this gives a concentration of 2 mg P/l).

10. Read OD of P Standard, and ash mixture as prepared.

Calculation

12.5 x OD samples ÷ OD standard x conc. standard = µg P

or µg P/weight of leaf x 100 = P%.

Comments

The method described above is an estimation of P content in leaf tissues. A standard curve is required for more accurate determination. Several P concentrations and their respective absorbances (absorbance on Y axis and P concentration on X axis) are determined to produce a calibration (standard) graph. The reciprocal value of the slope of the straight line so obtained will serve as the curve factor. Each absorbance value will be multiplied by it and then by 12.5 to obtain µg P. The % P is then calculated as described above.

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IMAGE EVALUATION
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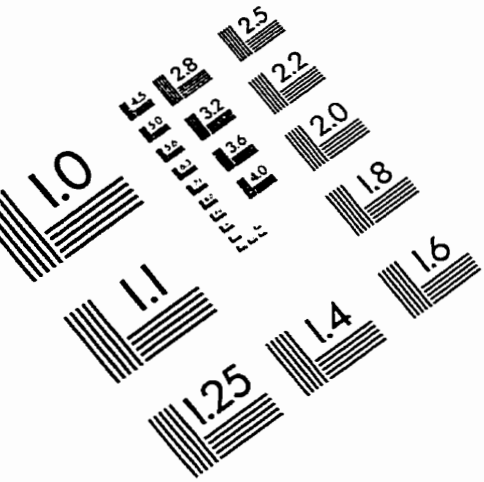

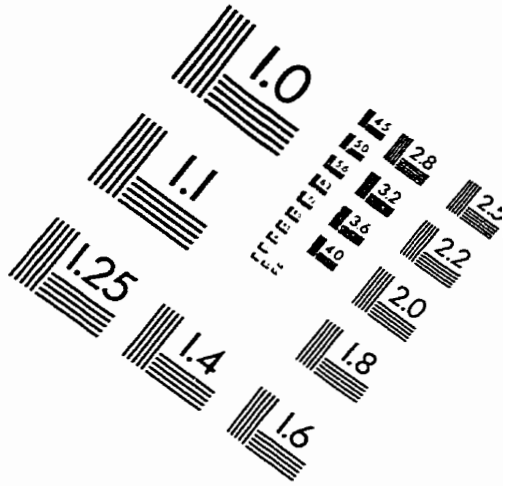

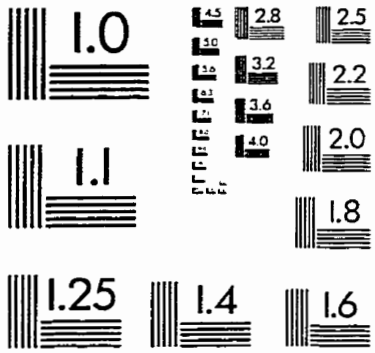

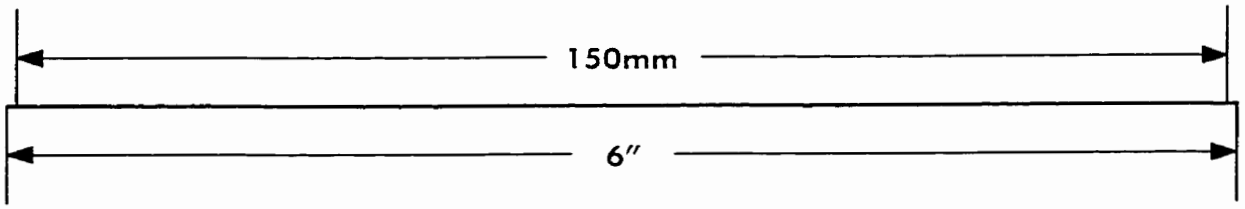

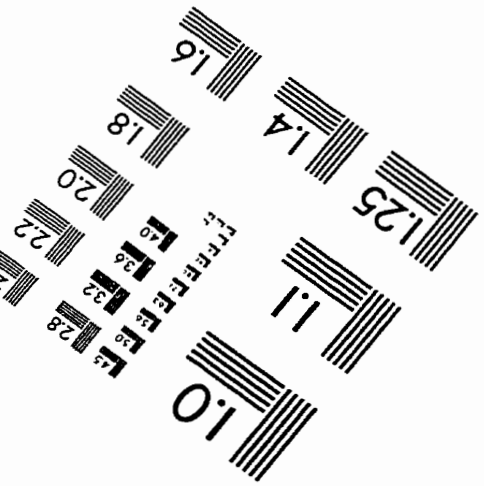

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