1997

Mycorrhizal interactions of selected species of endangered New England flora /

Jeffrey M. Lerner
University of Massachusetts Amherst

MYCORRHIZAL INTERACTIONS OF SELECTED SPECIES OF ENDANGERED NEW ENGLAND FLORA

A Thesis Presented

by

JEFFREY M. LERNER

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

February 1997

Plant and Soil Sciences

MYCORRHIZAL INTERACTIONS OF SELECTED SPECIES OF ENDANGERED NEW ENGLAND FLORA

A Thesis Presented

by

JEFFREY M. LERNER

Approved as to style and content by:

Haim B. Gunner, Chair

Robert L. Wick, Member

Lyle E. Craker, Member

William J. Bramlage, Department Head
Plant and Soil Sciences

DEDICATION

To Christina, whose unceasing love, help, and support enabled me to keep going under sometimes difficult circumstances. I would also like to thank my friends in the Depts. of Plant Pathology and Plant and Soil Sciences, who helped make my graduate experience fun and enjoyable.

ACKNOWLEDGMENTS

I would like to express my appreciation and gratitude to the following individuals for their generous help and support:

- Steve Bentivenga and Dr. Joe Morton, INVAM (The International Culture Collection of Arbuscular and VA Mycorrhizal Fungi), West Virginia University, Morgantown, WV.
- Dr. Paul Somers and Patricia Swain of The Massachusetts Natural Heritage Endangered Species Program (MNHESP), Division of Fisheries and Wildlife, Westboro, MA.
- Pamela Weatherbee, Bob Clark and the Trustees of Reservations, Beverly, MA.
- Heather McCargo, The New England Wildflower Society, Framingham, MA.
- Glenn Griffith and his colleagues at the U.S. Environmental Protection Agency, Environmental Research Lab, Corvallis, OR for the use of their ecozone map.
- Vahram Elaquoz, for his painstaking and elegant translations of several articles.
- Trina Hosmer and Dr. Wes Autio for their help with statistics.
- The members of my thesis committee: Dr. Robert Wick, for his outstanding help and guidance, and for the use of his extraordinary VAM slides; and Dr. Lyle Craker, who as head of the Dept. of Plant and Soil Sciences provided generous financial support.
- Dr. Haim Gunner, a remarkable scientist and gentleman whose warmth, generosity of spirit, wisdom and positive outlook is truly inspiring, and without whom this thesis could not have been completed.

This research was funded in part by grants from the following organizations:

- Massachusetts Agricultural Experiment Station (Hatch Project #738) University of Massachusetts, Amherst, MA.
- Applied Microbial Systems, Inc., Amherst, MA.
- Worcester County Horticultural Society, Tower Hill Botanic Garden, Boylston, MA.

ABSTRACT

MYCORRHIZAL INTERACTIONS OF SELECTED SPECIES

OF ENDANGERED NEW ENGLAND FLORA

FEBRUARY 1997

JEFFREY M. LERNER

B.A., NEW SCHOOL FOR SOCIAL RESEARCH

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Haim B. Gunner

Current strategies geared towards the conservation of endangered plant species call for increased emphasis on research, offsite propagation, and subsequent reintroduction of endangered flora. The success of these efforts can be significantly enhanced through the utilization of vesicular-arbuscular mycorrhizae (VAM). VAM are symbiotic associations formed between the roots of the vast majority of herbaceous plant species and several genera of soil-borne zygomycetous fungi in the order Glomales. Numerous greenhouse and field trials have demonstrated that mycorrhization of a wide range of plant hosts with effective VAM isolates enhances virtually all plant functions, particularly growth, uptake of phosphorus and other nutrients, stress tolerance, pathogen resistance, and transplant survival.

In this study, a mycorrhizal pot-cultured inoculum containing a non-indigenous VAMF isolate (*Glomus etunicatum* UT-316), when incorporated into a compost-based germination and transplant medium, proved effective at enhancing growth and transplant

survival and reducing root and crown disease in three endangered species during greenhouse cultivation - *Penstemon hirsutus* L., *Gentiana clausa* Raf. (Closed gentian), and *Gentianopsis crinita* Froel. (Fringed gentian). This disease-suppressive effect was confirmed using this same inoculum against *Rhizoctonia solani* in radish (*Raphanus sativus*) and *Fusarium oxysporum* f. sp. *basilicum* in basil. A soil dilution and plating series revealed that the mycorrhizal inoculum had a general suppressive effect on soil microflora, particularly soil fungi. While increased survival was the result of suppression of soil-borne fungal pathogens, the mechanism of growth enhancement in Fringed Gentian and the other test plants remained undefined, though it may have been due to the suppression of saprophytic microflora which were competing with the plants for scarce nutrients, as well as the suppression of root-infecting pathogenic fungi. VAM pot culture inoculum significantly enhanced the growth and survival of Fringed gentian and the other test plants by conferring to the growing medium a suppressive influence on soil microflora in general and soil-borne fungi and fungal pathogens in particular. These beneficial effects occurred despite the lack of mycorrhizal root colonization in the test plants during the growing season, suggesting that the underlying basis of lower disease incidence and enhanced growth was the suppressive influence of mycorrhizally-associated microflora from the inoculum rather than the direct influence of mycorrhizal colonization of the plant hosts.

Gentians in nature as well as those grown for this study formed unusual mycotrophic relationships with both mycorrhizal and so-called "pseudomycorrhizal" (dark-septate endophytic, or DSE) fungi. In Fringed gentian, which was the focus of this investigation,

DSE's colonized roots throughout the growing season, but VA mycorrhizae formed after the onset of dormancy in late autumn and remained throughout the overwintering period. The timing and morphology of mycorrhization in *G. crinita* indicates that mycorrhization is a phenologically-based and functionally-defined symbiosis which is largely under the control of the gentian host. The resulting association is not the classic symbiosis seen in most plants, which typically involves nutrient exchange during active growth. Rather, this association is one whose function is relegated to root development in preparation for dormancy, temporary symbiosis, phosphorus embodiment and storage, and finally, the dissolution of the intraradical portion of the mycorrhizal symbiont for its phosphorus reserves, to meet the specific needs of the gentian host at various phenological stages throughout this portion of the gentian's life cycle. This suggests that the gentian is living mycoheterotrophically off of its fungal symbiont, at least for a portion of the symbiosis, obtaining both carbohydrates and phosphorus. Mycorrhizae, and the phosphorus mycorrhizae provide, appear to play an integral role in perennialization, dormancy, overwintering survival, and reemergence of the gentian host. Fringed gentian developed these alternative mycotrophic strategies as a means of survival in the highly-stressed, resource-limited habitats in which this plant species evolved.

This study has demonstrated that VAM inoculum, when incorporated into a compost-based germination and growing medium, has capabilities which can significantly enhance the propagation of greenhouse-grown plants. VAM inoculum shows great potential as a biologically active pathogen-suppressant and plant enhancement agent in sustainable, ecologically-based horticultural production systems.

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS v

ABSTRACT vi

LIST OF TABLES xii

LIST OF FIGURES xiv

Chapter

I. INTRODUCTION 1

A. Rationale. 1
B. Vesicular-arbuscular mycorrhizae 4
C. Fringed gentian (*Gentianopsis crinita* Froel.) 5

II. LITERATURE REVIEW 13

A. The importance of VAM in plant ecology 13
B. Investigations of VAM in Eastern North American flora 15
C. Plant biodiversity conservation: Current strategies 16
D. VAM and conservation horticulture 19
E. Gentians, VAM, and other root-associated fungi 22
F. VAM in commercial horticulture 24
G. VAM and the control of soil-borne root pathogens 27

1. Introduction 27
2. Physiological changes in the root tissues 28
3. VAM-induced changes in the mycorrhizosphere 30
4. Survey and analysis 31
5. Summary 34

H. Other considerations 35
I. Summation of literature review 36

III. OBJECTIVES 38

IV. 1993-94 FIELD RESEARCH AND INOCULUM PRODUCTION 39

A. Field Research 39

1. Materials and Methods. 39
2. Results and Discussion 40

B. Inoculum production 41

1. Materials and Methods 41
2. Results and Discussion 42

V. 1994 PRELIMINARY TRIALS 55

A. Materials and Methods 55
B. Results and Discussion 57

1. *Penstemon hirsutus* 57
2. *Gentiana clausa* 58
3. *Gentianopsis crinita* 58

VI. 1995 GROWTH AND SURVIVAL EXPERIMENTS 73

A. Materials and Methods 73
B. Results 76

1. Germination 76
2. Survival and pathogen contamination 77
3. Growth 82
4. Mycorrhization 85
5. Other root/fungus associations 86

VII. 1996 EXPERIMENTS 131

A. Pathogen suppression experiments 131

1. Materials and Methods 131
2. Results 135

B. Soil dilution and plating experiment 136

1. Materials and Methods 136
2. Results 137

VIII. DISCUSSION 147

A. 1995 growth and survival experiments 147

1. Effect of mycorrhizal inoculum on growth and survival 148
2. Pathogen contamination of growing media 155
3. Mycorrhizal activity in Fringed gentian 159
4. The unusual nature of gentian mycorrhizae 163
5. The role of phosphorus in dormancy and reemergence 167
6. Mycorrhizae and its role in the perennialization of root tissue 169
7. Fringed Gentian and other root-associated fungi 170

B. 1996 Experiments 173

1. Disease suppression experiments 173
2. Soil dilution and plating 179

IX. SUMMARY AND CONCLUSIONS 182

APPENDIX: LAB TECHNIQUES FOR INVESTIGATING VAM 187

BIBLIOGRAPHY 191

LIST OF TABLES

Table — Page

1. Survey of investigations into VAM and disease suppression with various plant hosts. 32

2. *Gentianopsis crinita* (Fringed gentian) field sites in MA 45

3. Field data from soil and plant excavations at five field sites, October-November, 1993. 45

4. VAMF species extracted from field sites or pot cultures and identified. 45

5. Results of the 1994 preliminary experiments: Establishment of cultural guidelines for the propagation of *Gentianopsis crinita*. 62

6. Treatment media used in the 1995 growth and survival experiments 89

7. Incidence of pathogenic fungi in roots or crowns of diseased *G. crinita* from the 1995 growth and survival experiments. 90

8. Incidence of non-pathogenic fungi in roots of healthy and diseased *G. crinita* from the 1995 growth and survival experiments. 90

9. Influence of germination medium on survival of all transplanted *G.crinita* (TM 1-14) from the 1995 experiments 91

10. Influence of germination medium on survival of *G. crinita* in "effective" transplant media (TM 10-14) from the 1995 experiments. 91

11. Influence of germination medium and transplant medium (TM 10-14) on survival of *G. crinita*. 92

12. Influence of transplant medium (TM 10-14) on survival of *G. crinita*. 92

13. Growth of *G. crinita* transplanted into TM 10-14 from mycorrhizal germination medium (GM 1). 93

14. Growth of *G. crinita* transplanted into TM 10-14 from non-mycorrhizal germination medium (GM 2). 93

15. Comparison of growth of *G. crinita* from mycorrhizal and non-mycorrhizal germination media in the "effective" transplant media (TM 10-14) 94

16. Survival of radish (*Raphanus sativus*) seedlings in various media 20 days after inoculation with *Rhizoctonia solani* 139

17. Survival of basil (*Ocimum basilicum*) seedlings in various media four months after inoculation with *Fusarium oxysporum* f. sp. *basilicum*. 139

18. Influence of mycorrhizal inoculum on soil microorganism populations in various transplant media from the 1995 growth and survival experiments. 140

LIST OF FIGURES

Figure — Page

1. Indigenous VAMF spores extracted from soil collected from field site 5. 7

2. Vesicles and intra-radical ("inside root") hyphae formed by VAMF 8

3. Hyphae and arbusucles from VAMF in root cortical cells. 9

4. Vesicular-arbuscular mycorrhizae (VAM) in root tissue. 10

5. Fringed Gentian (*Gentianopsis crinita* Froel). 11

6. Reported extant sites of *G. crinita* in Massachusetts. 12

7. Map of "eco-zones" of each *G. crinita* field site. 46

8. Fringed Gentian and associated vegetation in a calcareous fen, field site 2, Mt. Hope Park, South Williamstown, MA. 47

9. Fringed Gentian in a wet meadow at the edge of a pond, field site 5, North Common Meadow, Petersham, MA. 48

10. Field site 1, Women's Federated State Forest, New Salem, MA. 49

11. Field site 3, wet meadow at Field Farm, Williamstown, MA. 50

12. VAMF spores extracted from field site 5, North Common Meadow, Petersham, MA. 51

13. VAMF spores extracted from field site 4, a calcareous fen in Cheshire, MA. 52

14. *Glomus etunicatum* spores extracted from a pot culture started with soil from field site 3, Field Farm, South Williamstown, MA. 53

15. Vesicles from a Sudex (sorghum/sudangrass hybrid) pot culture, indicating a successful and potentially infective mycorrhizal inoculum. 54

16. Beneficial effect of mycorrhizal inoculum on *Penstemon hirsutus*. 63

17. Influence of mycorrhizal inoculum on leaf surface area of *P. hirsutus* ten weeks after transplant. 64

18. *Penstemon hirsutus* three months after transplant into a mycorrhizally-inoculated transplant medium 65

19. *P. hirsutus* three months after transplant into a non-mycorrhizal transplant medium. 66

20. Post-transplant disease incidence and mortality of *P. hirsutus*. 67

21. Post-transplant survival of *P. hirsutus*. 68

22. The beneficial effect of mycorrhizal inoculum on *G. clausa* and the correlation between the longest leaf span and shoot dry weight. 69

23. Influence of mycorrhizal inoculum on leaf span of *G. clausa* at harvest. 70

24. Mycorrhizal influence on shoot weight of *G. clausa* at harvest. 71

25. Correlation between longest leaf span and shoot weight of *G. clausa* at harvest. 72

26. Influence of germination medium on survival of *G. crinita*. 95

27. Influence of mycorrhizal inoculum on germination of *G. crinita*. 96

28. *G. crinita* seedlings approximately eleven weeks after germination, March, 1995 97

29. *G. crinita* seedlings approximately eleven weeks after germination, March, 1996. 98

30. Timeline of 1995 growth and survival experiments. 99

31. Diseased *G. crinita* root (unstained) sampled June, 1995. 100

32. Fungal root pathogens invading a damaged *G. crinita* root (stained) sampled May, 1995. 101

33. *Pythium* oospores in a diseased *G. crinita* root (unstained), sampled May, 1995. 102

34. *Pythium* oospores in the root of *G. crinita*. 103

35. Conidia of *Fusarium* from diseased root tissue of *G. crinita* (unstained), collected June, 1995. 104

36. Conidia of *Alternaria* in diseased crown tissue of *G. crinita*, collected June, 1995. 105

37. Chlamydospores of an unidentified dematiaceous fungus from the roots of *G. crinita* shortly after it had died in November, 1995. 106

38. Close-up of VAMF spores from site 5, North Common Meadow, Petersham, MA, showing nematode eggs and nematodes (center). 107

39. Influence of germination medium on survival of *G. crinita* through overwintering period. 108

40. Influence of germination medium on survival of *G. crinita* throughout first-year growing season. 109

41. Influence of transplant media (TM 10-14) on survival of *G. crinita* from mycorrhizal germination medium (GM 1) 110

42. Influence of transplant media (TM 10-14) on survival of *G. crinita* from non-mycorrhizal germination medium (GM 2). 111

43. Survival of *G. crinita* from all ten treatments. 112

44. Influence of transplant media on survival of *G. crinita.* 113

45. *Gentianopsis crinita* in early November during onset of dormancy. 114

46. Influence of germination medium on size of *G. crinita,* as measured by leaf span. 115

47. Influence of germination medium on growth of *G. crinita*. 116

48. Influence of transplant media on growth of *G. crinita* from mycorrhizal germination medium (GM 1). 117

49. Influence of transplant media on growth of *G. crinita* from mycorrhizal germination medium (GM 1), with trendlines. 118

50. Influence of transplant media on growth of *G. crinita* from non-mycorrhizal germination medium (GM 2). 119

51. Influence of transplant media on growth of *G. crinita* from non-mycorrhizal germination medium (GM 2), with trendlines. 120

52. *Gentianopsis crinita* plants approximately four months after transplant (August 1995) from each of the germination treatment sets. 121

53. *G. crinita* emerging from dormancy in late February, 1996. 122

54. Mycorrhizal *G. crinita* plants three months after reemergence (May, 1996). 123

55. A nearly full-grown *G. crinita*, June, 1996. 124

56. The beginning of mycorrhizal colonization in the roots of *G. crinita*, early November, 1995. 125

57. The peak of mycorrhization in *G. crinita* root, mid-December, 1995. 126

58. Swollen *G. crinita* root in response to heavy mycorrhizal colonization, mid-December, 1995. 127

59. A dark-septate endophytic (DSE) fungus, probably *Phialocephala fortinii*, inhabiting the roots of a healthy *G. crinita*, August, 1995. 128

60. A dark-septate endophyte (DSE) inhabiting the roots of a healthy *G. crinita*, August, 1995. 129

61. Non-pathogenic chytrids in the roots of a healthy *G. crinita*. 130

62. Survival of radish in various media twenty days after inoculation with *Rhizoctonia*. 141

63. Survival of basil in various media four months after inoculation with *Fusarium*. 142

64. Basil from the 1996 disease suppression experiments, four months after inoculation with the pathogenic fungus *Fusarium oxysporum* f. sp. *basilicum*, April, 1996. 143

65. A close-up of a diseased basil plant inoculated with the *Fusarium* pathogen. 144

66. Influence of mycorrhizal inoculum on total soil microorganism populations of transplant media from 1995 experiments. 145

67. Influence of mycorrhizal inoculum on soil microorganism populations. 146

CHAPTER I

INTRODUCTION

A. Rationale

This research addresses two environmental challenges - the preservation of plant species biodiversity and the need for environmentally-sustainable plant production technologies. Current strategies adopted to address the first issue emphasize habitat preservation in conjunction with conservation horticulture. This is a branch of horticulture concerned with research, offsite propagation, germplasm banking, and reintroduction of endangered plant species into native habitats for the purpose of reestablishing wild populations. The second issue has focused attention on ecologically-based pest and disease management (EBPM) which uses an integrated approach to manage insects and pathogens, incorporating natural processes, naturally-derived compounds, and biologically-based agents wherever possible. Vesicular-arbuscular mycorrhizae (VAM) have been explored as one such agent to enhance growth and protect plants from disease. The ultimate goal is to reduce chemical inputs such as fertilizers and fungicides in plant production.

VAM are ubiquitous and essential symbionts of the vast majority of herbaceous plant species worldwide, integral components of the constellation of ecological interactions in the rhizosphere of plants in nature and, at least in some cases, critical to their survival. Intentional mycorrhization of a wide range of plant hosts with effective VAM isolates has demonstrated enhancement of virtually every plant function and therefore has enormous potential for sustainable plant production. The use of VAM as a growth-promoting, disease-

suppressing biological control and nutritional agent as an alternative to hazardous and increasingly ineffective chemical pesticides and fertilizers currently in widespread use has generated much interest and study.

The preservation of plant biodiversity is widely regarded as a fundamental cornerstone of life on earth. Not only is such diversity vital to the ecological health of natural systems, but diversity can also be considered a form of planetary wealth for humankind which remains largely untapped. Many of these plant species and mycorrhizal symbionts have yet to be explored for their potential applications to horticulture, agriculture, forestry, land reclamation, bioremediation, biotechnology, industry, and medicine. Unfortunately, habitat degradation and environmental contamination are putting tremendous pressures on New England's floral biodiversity, having imperiled over 28% of the region's native plant species (over 560 of 2,000), a situation echoed throughout the U.S. and the rest of the world (Falk and Olwell, 1992). The ecological implications of this situation are not fully known, but there is widespread concern about potentially negative impacts on ecosystems, habitats, and other plant and animal species (Wilson, 1992). Conservation horticulture, which seeks to remedy this situation, demands an increased understanding of the ecology, physiology, reproductive strategies, distribution patterns, and rhizosphere dynamics of native plants. Such efforts will prove critical to the success of endangered species propagation and restoration, and thus to the preservation of floral biodiversity.

One of the principles of conservation horticulture is that native endangered plant species, which are often among the most difficult to grow, should be cultivated under conditions that are as close as possible to their natural environment, including the

incorporation of indigenous mycorrhizal and other fungal endophytes. Unfortunately, information on rare and endangered plant species and their mycorrhizal symbionts is scant. (Falk and Olwell, 1992). One of the goals of this study was to gain knowledge about the role and function of mycorrhizal symbiosis in the selected plant species. An added benefit of this research was the exploration of indigenous VA mycorrhizae from the roots, rhizosphere, and surrounding soils of endangered species and their habitats. This has yielded several new VAM isolates not only adapted to the region's climate, soil, and plants, but perhaps effective at enhancing one or more plant functions. Prior to this study, the International Culture Collection of Arbuscular and VA Mycorrhizal Fungi (INVAM) in West Virginia did not possess VAM isolates from any inland habitats in New England, despite having over 700 isolates from other regions of the U.S. in their collection. INVAM now has four new isolates from this region (Bentivenga, 1995).

The main purpose of this research, however, was to investigate the use of VAM in conservation horticulture. The original hypothesis was that VAM was the missing component which made propagation of these selected endangered species difficult. Due to the unexpected observations and results of this research, however, several new hypotheses could be formulated to explain what had been seen. In the process, it is hoped that some light has been shed on several heretofore unexplored functions and effects of VAM, thereby expanding their potential uses and benefits to conservation horticulture. These benefits may be applicable to commercial horticultural production as well, thereby encouraging the industry to explore the use of VAM and other environmentally-sustainable plant production technologies.

B. Vesicular-arbuscular mycorrhizae

Vesicular-arbuscular mycorrhizae (VAM) are a root-fungus association resulting from the colonization of plant roots by a group of soil-borne fungi in the class Zygomycetes, order Glomales. The fungi which are responsible for this association are known as vesicular-arbuscular mycorrhizal fungi (VAMF). These fungi live in symbiotic association with the roots of the majority of land plants, and are ubiquitous in soils and plants throughout the world. VAMF are obligate symbionts, meaning that they cannot complete their life cycle, or reproduce, in the absence of a living plant host. VAMF reproduce asexually through the formation of chlamydospores and azygospores, which are the largest fungal spores known (30 - 800 μm), and function as reproductive and resting structures (Fig. 1, p. 7). These spores germinate in soil in the presence of plant roots and produce hyphae which penetrate roots, enter cortical cells, and form structures known as vesicles and arbuscules (Figs. 2-4, pp. 8-10). Vesicles are storage organs, and arbuscules function mainly as the site of nutrient transfer and exchange; the fungus provides nutrients, especially phosphorus, to the plant, in exchange for a small amount of photosynthate (carbohydrates) from the plant. The fungal hyphae also grow out from the root into the rhizosphere or "mycorrhizosphere". It is at the end of these extra-radical ("outside root") hyphae that the reproductive spores are usually borne (they are sometimes formed inside the root as well). The benefits of mycorrhizal development to the host plant have been extensively documented, and will be further explored in the literature review section of this paper (pp. 13-37).

C. Fringed gentian (*Gentianopsis crinita* Froel)

Description: *G. crinita* is a native herbaceous annual or biennial, depending on its habitat. Several ecotypes of this species exist; this is also attributed to habitat (Karlsson, 1974). The plant has an upright habit, and can be anywhere from 6" to 3' tall. Bright violet-blue tubular flowers, each with four deeply fringed lobes, are borne singly from September to November (Fig. 5, p. 11).

Habitat: Wet meadows and thickets, seepage banks, brooksides, calcareous fens and hillsides, and other wetland-associated habitats.

Range: From Central Maine south throughout New England.

Endangerment Status: The Center for Plant Conservation and the Nature Conservancy place *G. crinita* in the G3/G4 category, which means that the species is considered to be restricted in range and locally rare (CPC, 1990). The Natural Heritage programs in each of the New England states list the species in either the "watch-listed" or "special concern" categories, with populations in decline throughout its range (MNHESP, 1992). Massachusetts gives Fringed gentian a "special concern" status, which is the third highest level of endangerment after "endangered" and "threatened". There are currently about 25 extant populations reported throughout Massachusetts (Fig. 6, p. 12). Due to the elusive nature of this species, the smaller populations (under 10 individuals) disappear and reappear sporadically, thus making a definitive assessment of population and endangerment status difficult.

Propagation: Fringed gentian seeds are sown in late fall and need to be stratified at 2° C for two months. They do best in a compost-based germination medium. After stratification,

they are placed under mist and germinate in about a month. Approximately three months later (early spring), they are transplanted and moved to a cool greenhouse for two months, until they can be put outside in a partially shaded cold-frame for the summer. They are usually transplanted a second time in early autumn, and then outplanted or overwintered in a cold frame during the first year. Fringed gentian is considered a horticulturally recalcitrant plant, meaning that it is very difficult to grow and maintain in cultivation (Brumback and Curtis, 1986).

Mycorrhizal status: *G. crinita* is a facultative mycotroph, with low to moderate levels of mycorrhizae in nature. Higher levels are possible in cultivation with effective VAMF isolates. Mycorrhizae formation appears to correspond with certain phenological events in the plant's life cycle; namely, dormancy and bloom. This, coupled with the unusual nature of mycorrhizae in gentians (Neumann, 1934; Jacquelinet-Jeanmougin, et al, 1987) suggest that they may function in ways other than those normally attributed to mycorrhizae (e.g., nutrient uptake). In fact, *G. crinita* has been described as an "ecologically-obligate" mycotroph (Janos, 1995), meaning that it may, under certain circumstances, depend on its mycorrhizal symbiont to fulfill just such an unusual role (namely, perennialization, dormancy, reemergence, and in some cases, mycoheterotrophy; this is presented in greater detail in the discussion section, pp. 147-172).

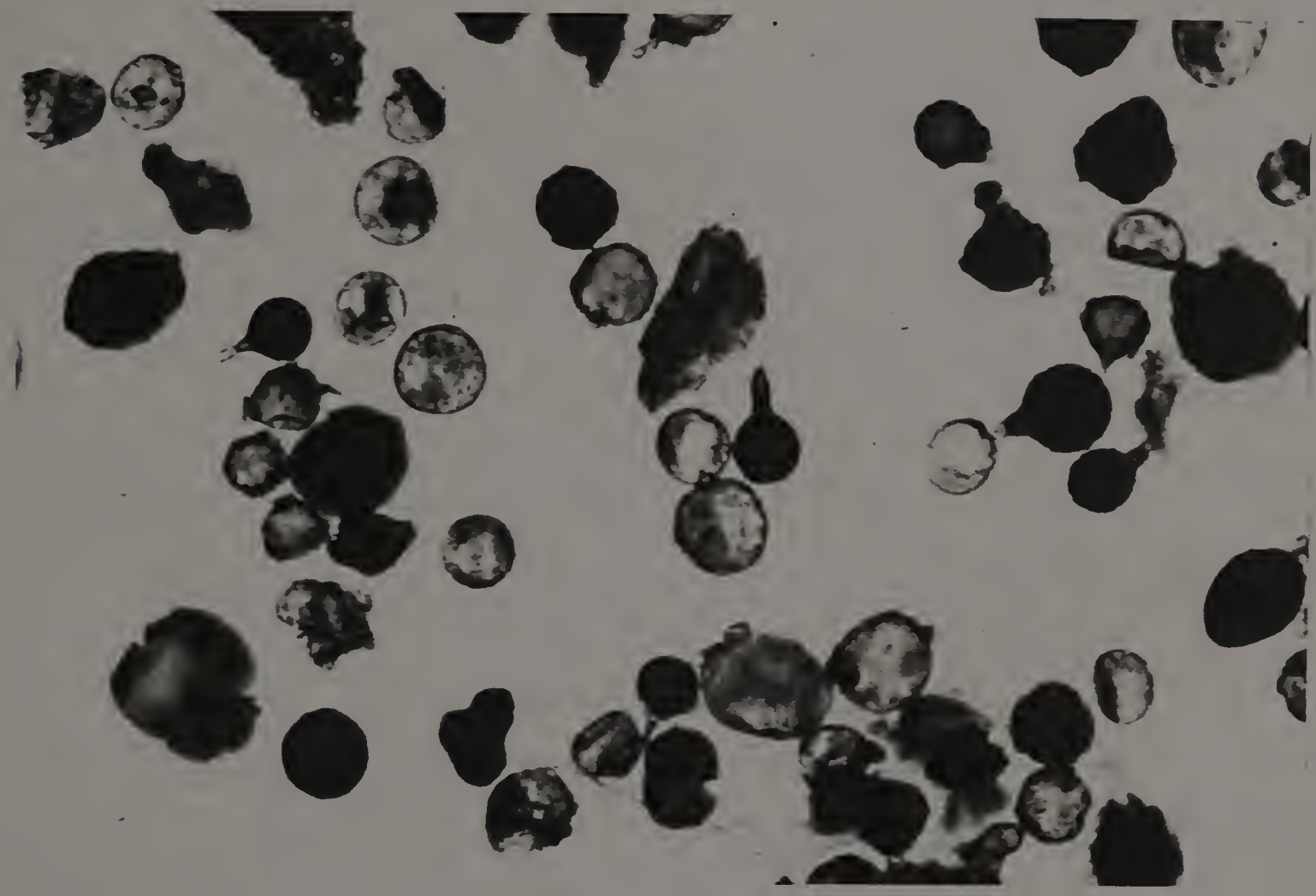


Figure 1. Indigenous VAMF spores extracted from soil collected from field site 5. Note the germinating spore and hyphal extension (center). Diameter of spores pictured here range from 60-150 μm, which falls within the average size range of most VAMF spores.

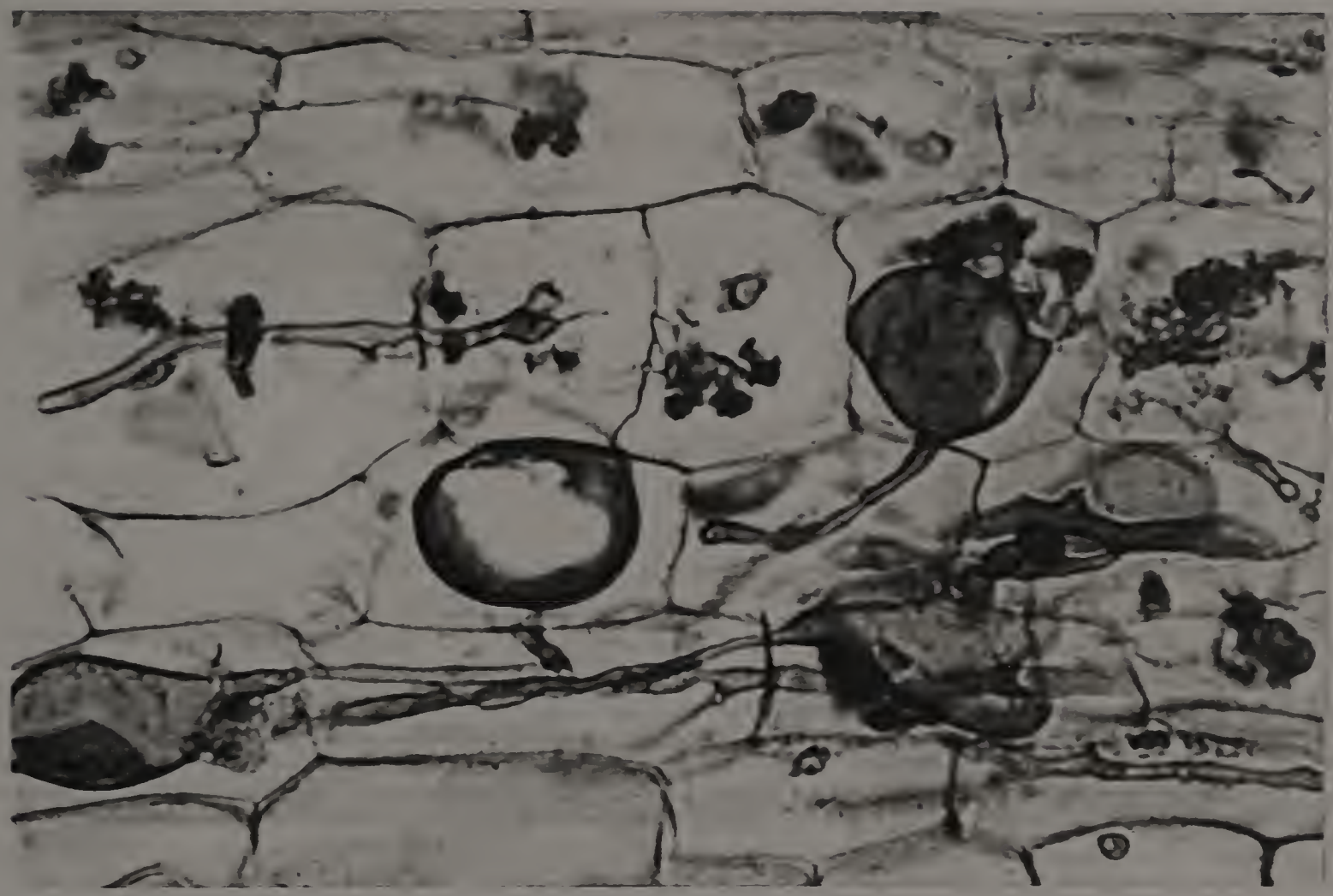


Figure 2. Vesicles and intra-radical ("inside root") hyphae formed by VAMF. (Photo courtesy of Dr. Robert Wick. Used by permission.)

Figure 3. Hyphae and arbuscules from VAMF in root cortical cells. Note the resemblance to a "little tree", which is the literal translation of the word "arbuscule". This morphology represents the more common "Arum" type of mycorrhizae. (Photo courtesy of Dr. Robert Wick. Used by permission.)

Figure 4. Vesicular-arbuscular mycorrhizae (VAM) in root tissue. The distribution of hyphae and arbuscules throughout the root cortex represents a moderately high level of mycorrhizal colonization. (Photo courtesy of Dr. Robert Wick. Used by permission.)

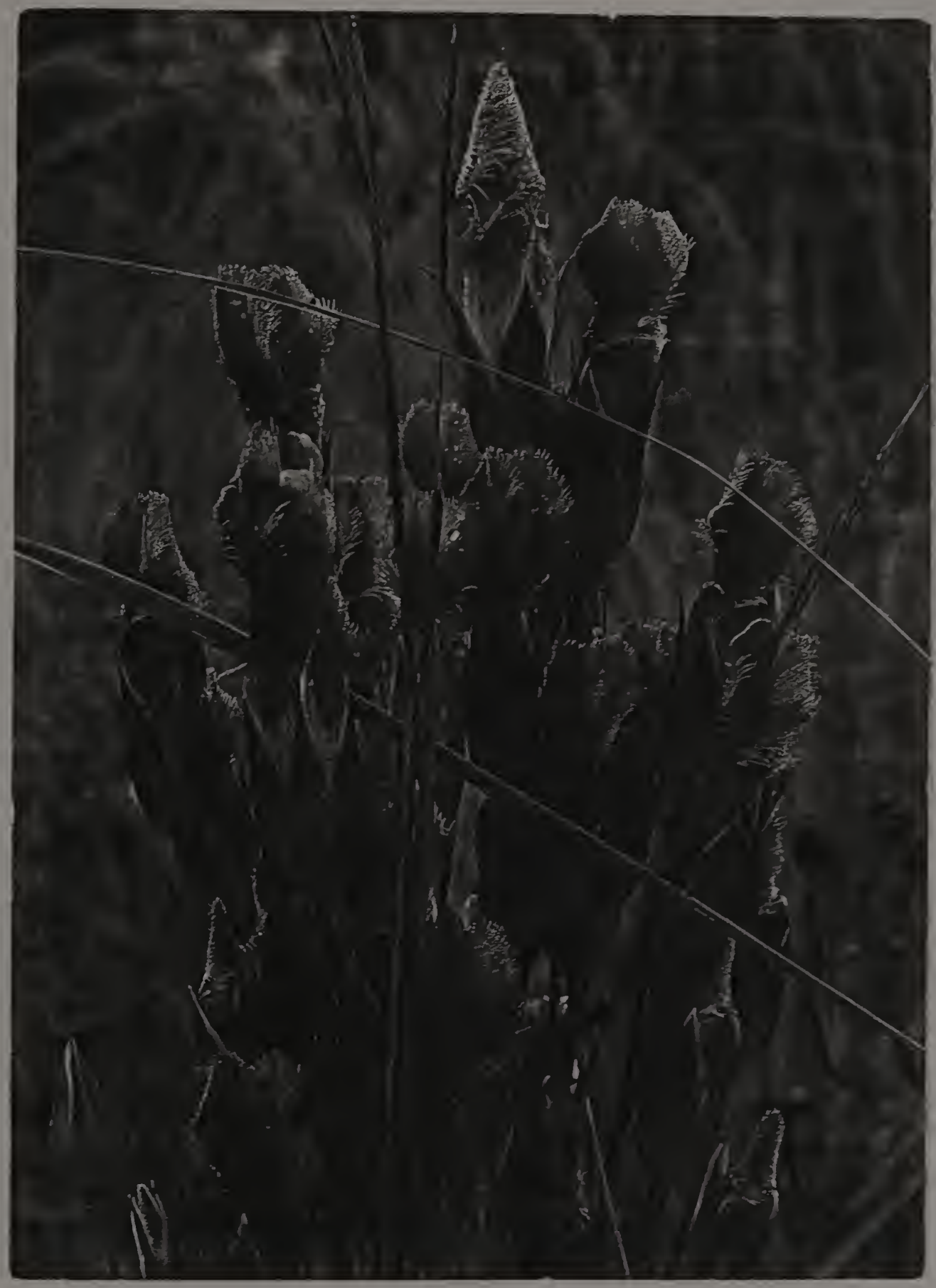


Figure 5. Fringed Gentian (*Gentianopsis crinita* Froel).

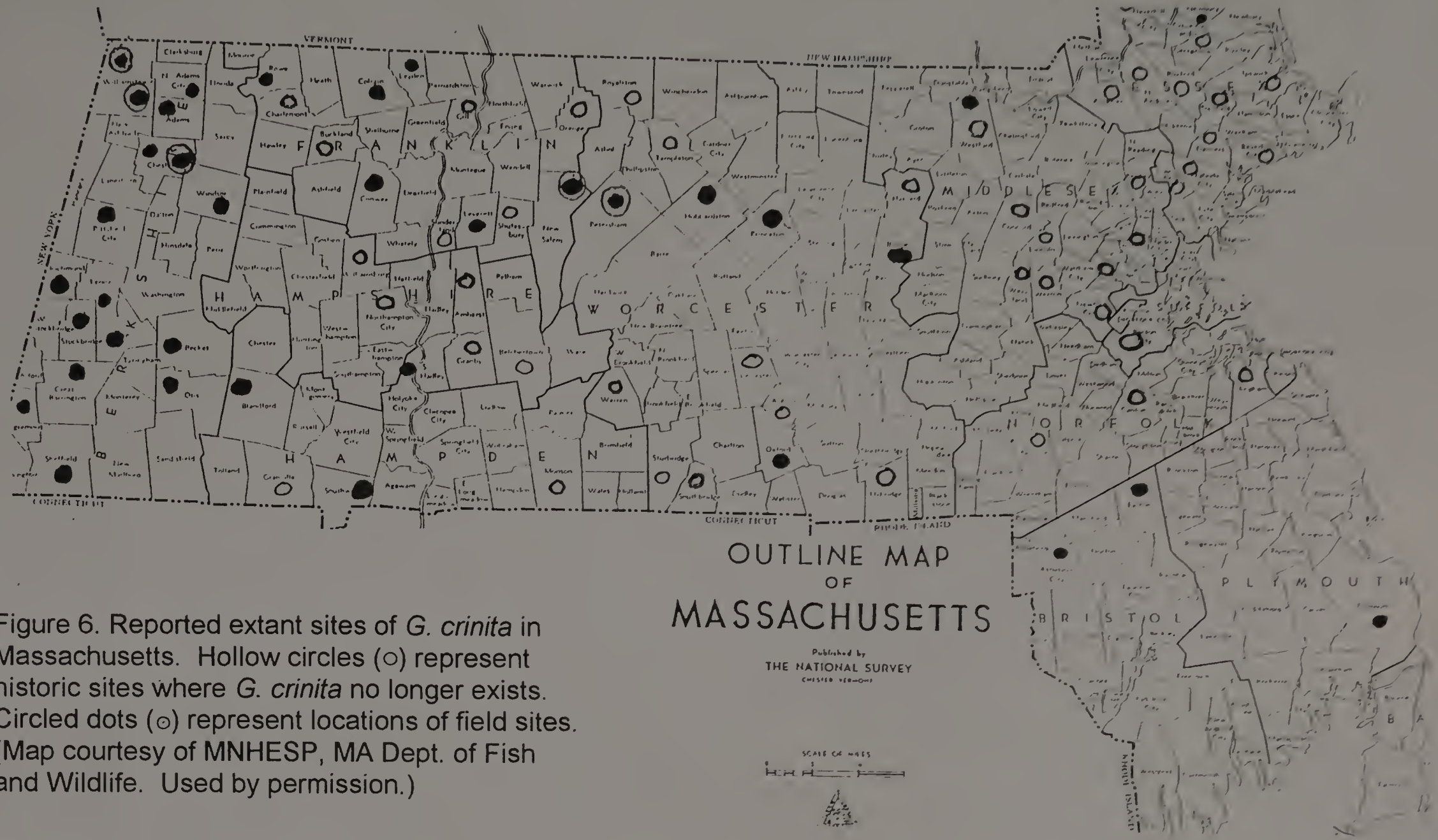


Figure 6. Reported extant sites of *G. crinita* in Massachusetts. Hollow circles (○) represent historic sites where *G. crinita* no longer exists. Circled dots (⊙) represent locations of field sites. (Map courtesy of MNHESP, MA Dept. of Fish and Wildlife. Used by permission.)

CHAPTER II

LITERATURE REVIEW

A. The importance of VAM in plant ecology

The vast majority of terrestrial plants in nature are mycorrhizal - that is, their roots live in mutualistic symbiotic association with one or more species of mycorrhizal fungi. Mycorrhiza (plural = mycorrhizae) is the term used to describe the root-fungus structures. Harley and Smith (1983) have described mycorrhizae as a "super-organism", much like lichens and root nodules in legumes. This relationship is such an integral part of the plant kingdom that it has been suggested that one cannot fully understand the physiology and ecology of most plants without studying their mycorrhizal associations as well (Jackson and Mason, 1984). Mosse (1975) suggests that mycorrhizae be considered a plant-fungus-soil partnership. Several researchers believe that for most plant species in natural ecosystems, mycorrhiza formation is at least as important as the other two primary root functions - water absorption and mineral uptake (Brundrett and Kendrick, 1988; Allen 1991).

Vesicular-arbuscular mycorrhizae (VAM), which are the most prevalent type of mycorrhizae in nature, is also the type found in most herbaceous plants. The fungi which form VAM (VAMF) are Zygomycetes in the order Glomales and have non-septate hyphae which penetrate the root cell wall (not the cell membrane) and occupy the cortical cells, forming structures known as vesicles and arbuscules, and extending out into the surrounding soil (see Harley and Smith, 1983). As mentioned, the benefits of this mycorrhizal association to plants have been well documented. These include enhancement of the following functions: growth

(Harley and Smith, l983; Powell and Bagyaraj, 1988), reproduction (Bryla and Koide, l990), uptake of nutrients (Cooper, l984) especially phosphorus (Sanders, et al., 1974), drought tolerance (Nelson and Saffir, l982; Sylvia, et al., 1988), pathogen resistance (Jalali and Jalali, 1991), water uptake (Ange, et al., 1986), and photosynthesis (Levy and Krikun, 1980; Paul and Kucey, 1981). Other indirect benefits include stimulation of nitrogen fixation in legumes (Hayman, 1987), actinorhizal associations (Gardner, et al., 1984), free-living bacterial associations (Pacovsky, et al., 1985; Azcon, et al, 1989), and the creation of a beneficial rhizosphere microflora (Linderman, 1994). Mycorrhizae have also been shown to play a role in plant resistance to heavy metal toxicity (Dehn and Schuepp, 1990), and to effect positive changes in plant morphology (Huang, et al., 1985; Krishna, l981) which help increase survival, especially under stressful conditions. In addition, VAM appear to lessen injury and enhance survival rate of transplants, both in field experiments (Menge, et al., 1980) and in habitat and degraded land restoration (Allen and Friese, 1990).

Mycorrhizae have been shown to function in plant population biology as well, in several ways. First, by allowing greater access to resources, they insure competitive advantage and thus survival of individuals within a particular habitat. Second, some studies have indicated that there is a correlation between abundance of mycorrhizae in the soil and increasing herbacous plant species diversity (Grime, et al., 1987; Newman and Reddell, 1988). Furthermore, mycorrhizae appear to be a critical component of ecosystem dynamics in many different biomes around the world. It has been demonstrated that they play a major role in nutrient and carbon cycling, breakdown of organic matter, species succession and diversity, soil structure and aggregation, plant-plant and plant-animal interactions, and colonization of harsh

environments (St. John, 1990; Allen, l991). It is now well-established that mycorrhizal symbiosis is an integral part of plant physiology and ecosystem ecology, and its applications to land reclamation and reforestation (Carpenter, et al, 1987; Allen and Friese, 1990; Wood, et al, 1991) and to agriculture (Sylva, et al., 1987) have been exhaustively documented.

Research into the distribution of mycorrhizae in natural ecosystems around the world has been less extensive (see Allen, 1991, and Harley and Harley, l987), and is generally considered to be incomplete from a scientific standpoint (Torrey and Berliner, 1989; St. John, 1990). Further work in this area is warranted in light of the fact that these studies have consistently shown the great majority of plant species to be mycorrhizal (Allen and Friese, 1990), and due to the importance of mycorrhizae in natural ecosystems (Allen, 1991). There is a lack of information regarding North American flora, and a compelling need for a comprehensive list of the mycorrhizal associations of this continents' plant species, much like the list for British flora compiled by Harley and Harley (1987).

B. Investigations of VAM in Eastern North American flora

There is good reason to believe that the great majority of New England plant species are mycorrhizal. Several studies which have explored the mycorrhizal status of various herbaceous species of eastern North American forests have found consistently high rates of mycorrhization. Virtually all of the species looked at in the Canadian forests are indigenous to New England as well, and so are included here. Based on the following studies, it is reasonable to conclude that 80-90% of New England's herbaceous flora is mycorrhizal at least at some point in its life cycle.

Torrey and Berliner (1988) examined the roots of 45 species of common understory plants in Harvard Forest, Massachusetts, and found 91% of them to be mycorrhizal. Brundrett and Kendrick (1988, 1990) examined 55 common herbacous species in a sugar maple forest in Ontario, Canada and found a 93% rate of VA mycorrhization. Malloch and Malloch (1981, 1982) came up with an 80% VAM infection rate in their two studies encompassing a total of 35 herbaceous species in a boreal forest in Northeastern Ontario. Other studies of New England coastal areas yielded similar findings (Gemma, et al., l989; Cooke and Lefor, 1990) . Clearly, a pattern of mycorrhization throughout the natural ecosystems of New England emerges from this research, and this has significant implications for endangered species conservation throughout the region.

C. Plant biodiversity conservation: Current strategies

The preservation of floral biodiversity was one of the main motivations behind this research. The importance of preserving plant biodiversity is still widely under-recognized, by society in general, by the scientific community in particular, and by governmental organizations around the world as well as in the United States. There is evidence, however, that the U.S. government began to realize its significance in the early 1990's and shift its environmental priorities and funding accordingly (Falk and Olwell, 1992; Schneider, 1993). In the most basic sense, plant life, in all its diversity, is fundamental to the survival of the animal life on earth, including human beings. More specifically, rare species are indicators of environmental change or stress within ecosystems, of rare and unique biotic communities, and of natural biodiversity (Longland, 1992). *Gentianopsis crinita* is considered to be an indicator of rare or unusual

wetland-associated habitats in New England, such as calcareous fens and seepage banks, which often contain many uncommon or endangered plant species (Weatherbee, 1991). Rare plant species are part of what is often called the "web of life", building blocks for a biological hierarchy of more complex communities upon which many other organisms depend (Falk and Holsinger, 1991). Science has yet to explore many of these species for their potential uses in horticulture, agriculture, forestry, industry, medicine and biotechnology (Longland, 1992). Furthermore, there are the moral, ethical and aesthetic aspects to be considered. The loss of these rare plants, many of which are beautiful, would deprive future generations the opportunity to know, study and appreciate them. Also, protecting, restoring, or enhancing native flora and fauna implies a healthy and productive relationship between human beings and the natural world which is all too uncommon in this day and age (Jordan, 1986). Finally, involvement with one's natural heritage and surroundings makes life richer and more rewarding (Jordan, 1986; Longland, 1992).

The current approach to endangered species preservation emphasizes an integrated conservation strategy (Falk and Olwell, 1992). This strategy proposes that the traditional approaches of habitat preservation through legal protection, land acquisition, and habitat management be combined with more recent strategies, collectively known as conservation horticulture - propagation and research, habitat restoration, and species reintroduction. It is likely, given current trends of habitat destruction and subsequent endangerment patterns, that conservation horticulture will come to play a crucial and ever-increasing role in plant conservation in the coming decades (Falk, 1990; Edwards, 1990; IUCN, 1992).

As pressures on biodiversity in New England increase, and more species become more highly imperiled, it is just such an approach that is being highlighted by the predominant conservation organizations throughout New England and the rest of the United States. Federal land management agencies as well as the Nature Conservancy and other conservation organizations are increasingly involved in various forms of restoration (Falk and Olwell, 1992). The formation of the Center for Plant Conservation (CPC) in 1985 was a significant step in creating scientifically credible plant conservation programs, by organizing a network of over 20 U.S. botanic gardens and arboreta to maintain offsite collections of endangered plants to use for conservation purposes. Various methods of conservation horticulture are employed by these network organizations - horticultural propagation, seed and germplasm banks, living collections, research, and re-establishment of depleted wild populations, as well as development of conservation policy (McMahan, 1990). In New England, NEPCOP (New England Plant Conservation Program) emerged in 1991 as a consortium of 65 organizations devoted to conserving endangered plants in the region (Longland, 1992). The New England Wildflower Society (NEWFS) headquartered at Garden in the Woods, Framingham, MA, developed the concept and practice of conservation horticulture, and has spearheaded these efforts. NEWFS maintains a sophisticated endangered species production and preservation facility, including a seed bank, a living collection garden of endangered New England flora, a 45-acre botanic garden, and a fully functioning greenhouse and nursery operation.

Despite the considerable progress that these efforts represent, there are still significant obstacles to be overcome in the production and restoration arm of the plant conservation community. Given the urgency of the task of maintaining plant biodiversity in New England

and elsewhere, and the primary role that conservation horticulture will play in these efforts, it is imperative that these issues be addressed.

D. VAM and conservation horticulture

While research into native plant propagation and reintroduction has progressed in recent years, there remains a surprising scarcity of scientific knowledge, as well as a lack of readily available information, on native plant species (McMahan, 1991). This is especially true for rare and endangered flora. Scientific data on the genetics, population biology, physiology, life history and ecological interactions of these species in their natural state is inadequate or absent altogether (Falk and Olwell, 1992). This lack of information has negative implications for rare plant conservation - that is, reintroductions of endangered plants involve species whose developmental, reproductive, and ecological characteristics, including mycorrhizal status, are little known, even though such information is often critical to the success of reintroduction efforts. One of the major principles of conservation horticulture is to replicate in cultivation the plant's natural environment. Falk and Olwell (1992) take this concept one step further, and assert that one of the major biological considerations in rare plant propagation and reintroduction is inclusion of the species' essential symbionts: pollinators, seed dispersants, and mycorrhizal and other endophytic fungi. Without such information, rare plant reintroductions will remain a hit-or-miss affair, based largely on informed speculation and anecdotal information rather than empirical knowledge, and involving a substantial element of trial and error (Griffith, et al., 1989). This is a serious concern, given the limited resources available to the plant conservation community, who can ill-afford such uncertainty.

This current situation can be remedied with basic and applied research into the mycorrhizal status of endangered species in the wild, and investigations into the use of mycorrhizae (mycorrhization) in the propagation and restoration of these species. At present, studies on the mycorrhizal status of rare New England plants is virtually non-existent, with two notable exceptions involving orchid and ericaceous species, which form a distinct, non-VA mycorrhizae (Zettler and McInnis, 1992; Ling, 1993). Accordingly, the potential utilization of mycorrhizae in native plant production in general, and conservation horticulture in particular, remains untried. There was not a single citation in the literature involving the use of mycorrhizal inoculation in the production or restoration of non-orchidaceous, non-ericaceous endangered or rare native plant species. This despite the fact that there is a very high probability that most, if not all, of these species are mycorrhizal, and that mycorrhization at the greenhouse level very well may determine the success or failure of such undertakings.

Mycorrhization of endangered species during propagation makes sense on many levels. Herbaceous plants growing in temperate deciduous forests and fields are exposed to seasonal extremes in environmental conditions, including low temperatures in the spring and autumn, periods of drought in the summer, and extreme cold during the winter months (Hicks and Chabot, 1985). Many of these plants have adapted to these environmental constraints by adapting various survival strategies (Brundrett and Kendrick, 1988), one of the most important being mycorrhization.

As mentioned earlier, mycorrhizal association would give the reintroduced plant a competitive advantage by allowing it greater access to resources, thus increasing its chances of survival under environmental pressures. Subtle and little-understood functions which contribute

to fitness and survival, such as rhizosphere ecology or perennialization, may be enhanced by mycorrhizal fungi. Increased phosphorus uptake (Mosse,1975), positive changes in root morphology (Schenck, 1981; Brundrett and Kendrick, 1990a and 1990b), enhanced drought resistance (Nelson and Saffir, 1982), and increases in lipid and carbohydrate storage (Maronek, et al., 1981) may also contribute to increased survival of herbaceous perennials during dormancy and overwintering periods. Maronek, et al (1981) concluded that low temperature tolerance by mycorrhizal fungi may be of critical importance in plant cold hardiness. In fact, mycorrhizal fungi may play a role in the perennialization of certain plant species (Magrou, 1921; Waksman, 1932), a hypothesis which is supported by the observations of this researcher. If this is so, it may contribute to the survival of these species in temperate environments, as well as aiding in the maintenance in living rare plant collections of some difficult- to-maintain species.

Other studies have noted that the roots of many herbaceous woodland perennials have root systems that are very inefficient at absorbing nutrients from the soil directly, and may therefore be dependent on their VA mycorrhizal symbiont to supply necessary nutrients. This is especially true regarding phosphorus, which is tightly conserved by living organisms in forest soils (Brundrett and Kendrick, 1988). The above evidence indicates that the symbiotic relationship with VAM is of vital importance to herbaceous plant survival in natural ecosystems. This information, coupled with the evidence that mycorrhization increases both transplant and restoration survival (St. John., 1990; Allen and Friese, 1990, Menge, et al., 1980) leads to the conclusion that mycorrhization during horticultural production can play a pivotal role in the successful reestablishment of endangered species in their natural habitats.

Some of the practical problems encountered by conservationists involved in the propagation and reintroduction of endangered species may be solved by the use of VAM. First, mycorrhization might confer survival advantages to reintroduced transplants, which have more difficulty establishing themselves under stressful environmental conditions than their natural counterparts. These transplants need to be cared for and maintained for at least the first year following reintroduction, which is often difficult or impossible, given the lack of accessibility and shortage of manpower (McMahan,1990). Mycorrhization might enable these transplants to survive without care. Second, mycorrhization would better enable propagators to recreate, in the greenhouse, nursery, or living collections garden, the natural conditions under which the plant grows in the wild (Brumback and Curtis,1986; Phillips, 1985; Brumback, 1989), which is very often a necessary prerequisite for endangered species propagation. Third, many of these plants have difficulty reproducing in the wild (Falk and Holsinger, 1991), and this difficulty usually carries over into greenhouse propagation. Even those species that reproduce adequately in the wild are often notoriously difficult to propagate or maintain in cultivation (Brumback, 1989, 1993, Carlock, 1991).

E. Gentians, VAM, and other root-associated fungi

Plants from the Gentianaceae, collectively known as gentians, are notable for their rather unusual and wide-ranging ecological, physiological, and biotrophic characteristics. Foremost among the latter are the gentians' mycotrophic relationships, that is, their affiliation with root-associated fungi. Gentians inhabit a remarkably diverse array of habitats, from wetlands to deserts, tropical rainforests to alpine tundra, and so have adapted an equally

diverse range of mycotrophic "lifestyles". These mycotrophic relationships run the gamut from mycorrhizal symbiosis to a saprotrophic or even parasitic relationship of the gentian host upon its fungal associate, and may be related to the particular habitat in which the plant resides (Karlsson, 1974, Weber, 1984).

This makes the cultivation of gentians quite challenging; many growers have found that plants in this family (Gentianaceae) are often difficult to maintain in cultivation (Phillips, 1985; Brumback, 1993). In the case of Asclepias, another member of the same order (Gentianales) with some endangerment status (MNHESP, 1993; Somers, 1993), seedlings often die shortly after germination for no apparent reason (Phillips, 1985). The same holds true for *Centaurium*, another species in the Gentianaceae; Mcgee (1985) attributed the mortality of his *C. erythraea* seedlings to a lack of mycorrhizae. Gentians, many of which have some endangerment status in New England (MNHESP, 1992; CPC, 1990) are known to be mycorrhizal in their natural state (Read and Haselwandter, 1981; Weber, 1984; Heymons, et al., 1986; Harley and Harley, 1987).

There have also been reports of other non-pathogenic endophytes both in and on the surface of gentian roots. These have been given the generic name DSE, or dark-septate endophytes, which refer to their color and morphology. Several investigators (Read and Hasselwandter 1981; Lesica and Antibus, 1985; Currah and Van Dyck, 1986; Bledsoe, et al, 1990) have reported the presence of these DSE's in gentians and other species in habitats whose soils contain insignificant levels of VAM fungi (as indicated by few spores and low levels of mycorrhizal root colonization), such as deserts, wetlands, heathlands, and alpine tundra. Some researchers believe that these root endophytes may be of ecological importance,

serving some of the same functions in these habitats as VAM fungi serve in most other habitats; e.g., uptake of scarce nutrients and stress and disease resistance for their plant hosts (Stoyke and Currah, 1990; Seeliger, 1996). DSE's have been reported to increase the growth of some host plants (Haselwandter and Read, 1982; Wang and Wilcox, 1987), but this phenomenon needs further investigation.

It is possible that the difficulties encountered in propagating gentians may be related to both their unusual mycotophic affiliations and the lack of indigenous mycorrhizal or other fungal endophytes which are involved in these associations. Incorporation of these root-associated fungi during the cultivation of gentians and other difficult-to-grow native plant species may contribute to their successful propagation, maintenance, reintroduction and survival. Native mycorrhizal endophytes have already been shown to substantially increase the successful outplanting of native trees and shrubs (St. John, 1990; Allen 1991), so there is reason to believe that the same results could be achieved with herbaceous plants. Other conservationists agree with this proposition (Falk and Olwell, 1992; Brumback, 1993; Somers, 1993).

F. VAM in commercial horticulture

The non-utilization of mycorrhizae in plant production is by no means limited to the plant conservation community. Predictions by Maronek et al. (1981), Linderman (1978), Verkade and Hamilton (1980) and others that the use of mycorrhizae would become a part of standard horticultural plant production have not been realized. Its use is very uncommon in commercial horticulture (Johnson, 1987; Linderman, 1993), though work is currently being

done to develop and refine the technology to identify, isolate, and characterize mycorrhizal fungi for horticultural production (Linderman, 1993). Thus far, use of mycorrhizae has largely been confined to the following: a) investigations into horticultural production of agricultural crops, such as strawberry (Kiernan, et al., 1984), citrus (Edriss, et al., 1984), apples (Geddeda, et al., 1983), and cereal grains (Sylva, et al., 1987; McGonigle, 1988; Bagyaraj, 1991); b) investigations into horticultural production of woody plant material (Maronek, 1981; Johnson, 1987; Wood, et al., 1991); c) production of woody material for reforestation (Wood, et al., 1991; St. John, 1990; Allen, 1991), and d) production of plant material for land reclamation (Maronek, et al., 1981; Allen and Friese, 1990; Wood, et al, 1991). Scientific experiments involving greenhouse trials have been extensive, and examples can be found in nearly every relevant citation at the end of this paper. However, this has not translated into its intentional use in either commercial or non-commercial horticultural production of herbaceous species.

According to Maronek, et al. (1981), mycorrhizal fungi may already be a factor in horticultural production, albeit unintentionally, but its contribution has yet to be acknowledged or explored. He goes so far as to say that if the mycorrhizal state is the normal one for horticultural crops, then much if not most of the investigative information on plant production is based on research into abnormal plants. The limited number of studies that have been done on the intentional use of mycorrhization for horticultural crops have consistently turned up positive results. Substantial growth increases were noted for such standard horticultural crops as geranium (Bierman and Linderman, 1983a, 1983b), chrysanthemum (Linderman, 1978), Easter lily (Ames and Linderman, 1978), and poinsettia (Barrows and Roncadori, 1977).

Linderman (1978) also found that VAM inoculation hastened the rooting as well as the growth of chrysanthemum cuttings. Ericoid mycorrhizae had the same beneficial effect on cuttings of *Arcostaphylos uva-ursi* and rhododendron, both of which are difficult to root (Holden, 1978; Linderman, 1978).

Research findings also indicate that mycorrhizae can be used as a biological control agent against various soil-borne pathogens in horticulturally-produced plants (Jalali and Jalali,1991; Feldman, et al., 1990), and this certainly warrants further study (Avidson, et al., 1990). Many of the fungicides normally used for this purpose are potentially toxic, particularly in the enclosed space of a greenhouse, and are often rendered ineffective due to the development of pathogen resistance (Olkowski, et al., 1991; Jarvis, 1992). The use of mycorrhizae as a nutrient-enhancement agent to increase phosphorus uptake (Bagyaraj, 1991), alone and in tandem with nitrogen-fixing bacteria (Punj and Gupta, 1988), has been proposed numerous times and is being actively investigated. Exploring alternatives to chemical fertilizers and pesticides seems especially important given the environmental contamination and degradation that has been associated with their production and use (Johnson, 1987; Olkowski, 1991). Furthermore, the wide availability and ready supply of cheap phosphorus may be coming to an end in the near future, due to expected declines in phosphate mining (Maronek, et al., 1981). Recent studies have indicated that an estimated 25% savings would be possible through utilization of VAM in a current commercial horticulture operation (Johnson, 1987). These factors may make the use of mycorrhizae in plant production economically feasible.

G. VAM and the control of soil-borne root pathogens

1. Introduction

The protection offered by VAM to the plant host from root-infecting pathogenic fungi can be characterized in three ways: a) induced resistance, b) pathogen suppression, and c) enhanced plant health. Induced resistance occurs when VAM colonization elicits a host response consisting of one or more resistance mechanisms which may inhibit subsequent infection by fungal pathogens (Lewis, et al, 1988). Pathogen suppression involves VAM induced alterations in both the root and the mycorrhizosphere which help to create a "zone of protection" by inhibiting the development or reproduction of the fungal pathogen, thereby suppressing subsequent infection.

The overall enhancement of plant health as a result of VAM colonization in and of itself confers protection to the host plant. Increased uptake of nutrients will most certainly contribute to a healthier plant, better able to resist or tolerate root disease. A mycorrhizal seedling whose root tissue is growing and developing faster will be less susceptible to root disease than its smaller, more vulnerable non-mycorrhizal counterpart. Improved phosphorus uptake decreases root cell membrane permeability (via the production of phospholipids). Enhanced nutrient status leads to a strengthened cell membrane and wall structure. In addition, VAM colonization results in enhanced growth linked to elevated phytohormone production levels (Linderman, 1994).

The relationship between VAM and root-infecting pathogens appears to be, at least in some instances, one of competitive exclusion. If VAMF are allowed to fully colonize the roots of its plant host, then in many cases the development of the pathogenic fungi will be inhibited

or reduced (Powell and Bagyaraj, 1984). The VAMF will essentially out-compete the pathogen for available infection sites in the root tissue, and in doing so will exclude the pathogen and prevail in the "race" for photosynthate and nutrients. Conversely, if the pathogen is allowed the upper hand in this rivalry for sustenance, it will predominate and suppress the mycorrhizal fungi. Because pathogenic fungi are generally much faster and more aggressive colonizers of roots, protection of plant roots by VAM against pathogens is generally much more effective where the host roots were pre-inoculated with VAMF and allowed to become fully colonized before exposure to the pathogen (Jalali and Jalali, 1991).

2. Physiological changes in the root tissues

The specific VAM-induced morphological and biochemical changes in roots as well as the related chemical and microbial changes in the mycorrhizosphere comprise the second major mechanisms of disease resistance and suppression. Plant roots are endowed with the ability to respond to invading organisms in a variety of ways aimed at limiting infection by the invading organism (Bonfante-Fasolo and Spanu, 1992). While colonization by VAM fungi does not elicit a defense response per se, the arbuscules formed within the root cells are eventually degraded (and the nutrients therein digested) by the plant host through the production of chitinase within the roots. The arbuscules are eventually reformed by the VAM fungi in the same and other root cells in an ongoing process which reflects the delicate symbiotic balance between the fungal symbiont and its plant host. The presence of chitinase also serves to create a protection zone both within and around the roots, which inhibits and in some cases attacks

pathogenic fungi (Campbell, 1989), whose cell walls are comprised mainly of chitin and polysaccharides.

Other VAM-induced anti-fungal compounds produced by plants include isoflavinoids, compounds which appear to stimulate VAMF spore germination and subsequent root colonization (Safir, 1992). These phytoalexin-like compounds have been implicated in enhanced disease-resistance capabilities in the plant host (Bonfante-Fasolo and Spanu, 1992), and disease suppression of pathogens in the mycorrhizosphere (Linderman, 1994). The increased production of amino-acids, especially arginine, in VAM-colonized roots has been shown to inhibit chlamydospore formation by both *Fusarium* and *Thielaviopsis*, resulting in a lower disease incidence in subsequent crops (Campbell, 1989).

Morphological changes in roots brought about by VAM appear to have a significant influence on enhancement of plant host disease resistance. As mentioned earlier, increased P uptake results in reduced root cell membrane permeability. Root growth and function, and subsequent cell wall development, increases overall as a result of improved nutrition and stimulation of growth hormones by VAM. Specific changes include increased production of lignin and other insoluble polysaccharides in VAM- colonized root cell walls (Dehne, 1982b), increased wound barrier formation in mycorrhizal roots challenged by *Thielaviopsis basicola* (Wick and Moore, 1984), and a greater transmembrane potential in mycorrhizal roots, which contributes to pathogen resistance (Bonfante-Fasolo and Spanu, 1992).

Decreased exudation of carbohydrates from VAM-colonized roots is a function of decreased membrane permeability brought about by enhanced P nutrition (Campbell, 1989); in any case, it robs pathogenic fungi of much of their nutrient base. This coupled with the differing

compounds that are released from mycorrhizal roots significantly alter the chemical and microbial environment of the mycorrhizosphere, which in turn influences the development of pathogens in the surrounding soil.

3. VAM-induced changes in the mycorrhizosphere

There is a profound difference between the structure, chemical constituents, and microflora of the mycorrhizosphere and the rhizosphere of non-mycorrhizal roots. Changes in root exudations, consisting of both a reduction of carbohydrates and the addition of VAM-specific secretions, contribute to these differences. The presence of a network of extra-radical VAM hyphae, which serves as an added substrate for differing microflora, further alters the micro-ecological makeup of this zone. Some of the VAM-related secretions are the anti-fungal compounds discussed earlier, which will obviously affect the populations of fungal pathogens. Also, the mycorrhizosphere has been shown to possess bacteria which are found only in association with VAM (Meyer and Linderman, 1986). Populations of nitrate-reducing, phosphate-solubilizing, and starch-hydrolyzing bacteria increase as well, possibly contributing to a greater availability of nutrients for the plant host and its fungal symbiont. Fluorescent pseudomonads, which have biocontrol potential, increased in number on the rhizoplane of mycorrhizal roots and hyphae, and were found on VAM spores (Powell and Bagyaraj, 1984). Populations of fungal antagonists, such as *Trichoderma*, *Actinomyces*, and *Rhizobacter* (all of which are currently being used or investigated as biological control agents) are greater in mycorrhizosphere soils than in non-mycorrhizal soils (Secilia and Bagyaraj, 1987), which may help to supplement the disease suppression activities of VAM.

The extra-radical VAM hyphae also have a profound influence on the soil structure and subsequently on the soil microflora as well. Increased aggregation has been observed near these hyphae, and these aggregates support a flourishing microbial community, including some of the previously noted antagonists. Studies indicate that VAM fungi are not only tolerant of such antagonists, but may actually function in compatible and even synergistic ways with them (Linderman, 1994). As mentioned, the altered root exudations and secretions of both mycorrhizal roots and hyphae may serve to stimulate the populations of these beneficial microorganisms.

4. Survey and analysis

Approximately 85% of the studies reviewed showed increased disease resistance or suppression by VAM (Table 1, following page). In a series of investigations by Caron and his colleagues (1986), the *Fusarium* population in the mycorrhizosphere of tomato roots was significantly reduced by the VAM fungus *Glomus intraradices*, demonstrating the principle of pathogen suppression. In studies by Wick and Moore (1984), Japanese holly roots which were colonized by *Glomus mosseae* or a "cocktail" of *Glomus* species exhibited increased wound barrier formation, effectively halting the progress of *Thielaviopsis basicola* within the cortex. This is an example of induced disease resistance stimulated by VAM.

Studies by Ross (1972) and Davis et al (1978) revealed increased incidence of *Phytophthora cinnamomi* in mycorrhizal soybean; however, this was probably due to the fact that these crops were inoculated with both the pathogen and VAMF simultaneously. Because *Phytophthora* is a much more aggressive root colonizer than VAM (as are most root-invading

fungal pathogens), it predominated under these circumstances and proceeded to exclude and actually suppress the VAMF. Davis and his colleagues (1978) reported similar results with soybean and alfalfa.

Table 1. Survey of investigations into VAM and disease suppression with various plant hosts.

+ = positive effect; reduced incidence of disease and/or increased yield
- = negative effect; increased incidence of disease and/or decreased yield

Pathogen	**Host**	**Result**	**Author**
Pythium ultimum	poinsettia	+	Chou and Schmitthenner (1974)
P. ultimum	cucumber	+	Rosendahl and Rosendahl (1990)
Phytophthora cinnamomi	soybean	-	Ross (1972)
P. cinnamomi	alfalfa	-	Davis, et al (1978)
P. megasperma	soybean	+	Chou and Schnitthenner (1974)
P. parasitica	citrus	+	Davis and Menge (1980)
Fusarium oxysporum	cucumber	+	Dehne (1977)
F. oxysporum	tomato	+	Caron, et al (1986)
F. vasinfectum	cotton	+	Zhengjia and Xiangdong (1991)
Thielaviopsis basicola	tobacco	+	Baltruschat and Schoenbec (1972)
T. basicola	Japanese holly	+	Wick and Moore (1984)
Verticillium dahliae	cotton	-	Davis, et al (1979)
Rhizoctonia solani, F.	cauliflower	+	Iqbal, et al (1988)
R.solani, Fusarium, Pythium	tomato	+	Nemec, et al (1991)

As mentioned earlier, the relationship between VAMF and pathogenic fungi is often one of competitive exclusion. It became clear in the course of this review that early

establishment of the mycorrhizal symbiosis was vital if this system is to be employed effectively against root disease. The negative results reported by Davis et al (1979) when mycorrhizal cotton was exposed to *Verticillium* were attributed to increased P uptake in the mycorrhizal plants; added fertilizer P also increased the incidence of disease. There were several studies in which disease complexes were employed to challenge mycorrhizal and non-mycorrhizal plants. Nemec, et al (1991) used a combined inoculum of *Rhizoctonia, Fusarium,* and *Pythium,* replicating circumstances that are often found in natural or artificial growth environments, including those in the present investigation. The study yielded positive results; mycorrhizal tomatoes exhibited a lower incidence of disease than non-mycorrhizal tomatoes. Iqbal, et al (1988) attempted to use pre-inoculation of cauliflower seedlings with VAM fungi as a biological control against damping-off caused by *Rhizoctonia* and *Fusarium* spp., and found that pre-inoculation caused a significant reduction in pathogenic infection of the seedlings.

Another important point to note is that enhanced disease resistance in mycorrhizal plants appears to be mostly a localized phenomenon; the morphological and biochemical changes in the roots which contribute to pathogen resistance are generally restricted to the colonized roots, which strengthens the argument for pre-inoculation with VAMF made earlier. In many cases, VAM colonization encompasses a significant portion of the root system, and colonization levels of 70-85% are not uncommon (Powell and Bagyaraj, 1984), thus affording protection to the greater part of the root complex. However, changes in the mycorrhizosphere attributed to VAM which may contribute to disease control are generally not localized, and occur throughout the entire zone of soil around the root/hyphae matrix. There are studies that

have found a systemic response, in which the entire root system exhibited protective mechanisms even if only one portion was colonized by VAM (Rosendahl and Rosendahl, 1990).

5. Summary

In general, mycorrhizal plants appear to exhibit a lower incidence of root disease, show greater resistance to infection by pathogens, inhibit or reduce pathogen development, and suffer less damage or have greater yield than non-mycorrhizal plants. While many researchers have implicated increased uptake of phosphorus (and subsequent decreased permeability in the root cell membranes and decreased carboydrate exudation) as the main mechanism of disease control, this has been largely speculative. In studies where the effects of P were excluded or controlled for, there was still disease suppression, suggesting the involvement of other mechanisms (see Jalali and Jalali, 1991, and Smith, 1988, for a review of these studies). Furthermore, many of the specific changes in root tissues attributed to VAM are direct responses to the fungal symbiont and are not related to P uptake (Bonfante-Fasolo and Spanu, 1992).

It is apparent that VAM induces significant changes in both the root physiology and the mycorrhizosphere of the host plant; while many of these changes result in an overall enhancement of plant health, many can be linked directly to disease control, particularly those involved in enhancing root tissue integrity and stimulating antagonistic microflora. Clearly the interactions between plant host roots, VA mycorrhizal fungi, plant pathogens, and mycorrhizosphere microflora, in conjunction with abiotic factors, are exceedingly complex, precluding the possibility of making straightforward predictions or

drawing simple conclusions regarding the results of thes studies. Wide variations in growth systems and environments, experimental design, VAM fungal symbiont, pathogens, and host plant warrant caution in drawing any definitive conclusions about the relationship between VAM and root-infecting fungal pathogens. However, it seems reasonably certain that VAM and associated antagonistic microorganisms can and should be part of a stable and effective biological control strategy in resource-sustainable plant production systems when such systems are managed properly to insure predominance of the VAM fungi and enhancement of the mutual symbiosis.

H. Other considerations

There are other facets of endangered plant production that are compatible with mycorrhization, and additional factors to consider in its utilization. Already mentioned is the necessity for increased knowledge and understanding of endangered plant species. Also mentioned was the necessity to artificially recreate in the greenhouse the conditions of the plant in its natural state, which includes its mycorrhizal associations. Another important point to note is that studies have indicated that pre-inoculation in the greenhouse, preferably at the germination stage before the plant has been transplanted into the field or reintroduced into its native habitat, has been shown to be the most efficient and most effective way to insure mycorrhization (Menge and Timmer, 1982; Biermann and Linderman, 1983; St. John, 1990). Furthermore, because mycorrhizae are already an unrecognized part of horticultural production, low-input practices currently being employed by many native plant propagators (Clark, 1993, McCargo 1994, Phillips, 1985), such as organic

and integrated pest management techniques and the use of pre-sterilized media, tend to augment naturally-occuring as well as non-indigenous introduced mycorrhizae (Maronek, et al., 1981, Verkade and Hamilton, 1980).

This distinction is significant because it is now well-established that indigenous VAM fungi may not always be the most effective or efficient at conferring the desired benefits. There are hundreds of isolates of VA mycorrhizal fungi (see Schenck and Perez, 1987, Allen, 1991, and INVAM, 1992), and they differ in their ability to colonize or infect their host (referred to as infectivity or efficiency), as well as in the benefits they provide (referred to as effectivity). Some non-indigenous VAM isolates have proven to be much more infective than their indigenous counterparts (Gianninazzi, et al., 1990). Likewise, some introduced strains of mycorrhizae have "performed" much better than indigenous types in terms of benefits provided to their host (Abbott and Robson, 1981; Harley, 1989). This is another reason why pre-inoculation is preferred over field inoculation ; the VAMF isolate found to be the most effective for a particular plant host species can be chosen and introduced. Such information is currently not available for any endangered species, demonstrating that further research is needed to make these determinations.

I. Summation of literature review

The benefits of mycorrhization to plant growth and health is an established fact. That this has not translated into its utilization in commercial or conservation horticulture is clear. The intentional use of mycorrhization in the propagation and reintroduction of rare and endangered species is virtually non-existent, although the preservation of plant biodiversity is

becoming an increasingly urgent matter. This apparent lapse on the part of conservationists is not at all surprising, and is in fact reflective of an overall lack of knowledge regarding both the biology and ecology of native plants (especially endangered species) and the use of VAM in horticultural production. Given the importance of the task at hand, the limited resources and lack of information available to the conservation community, and the practical problems that conservationists face in pursuit of rare species reintroduction, coupled with the compatibility of mycorrhization with the biological considerations of current conservation strategies, further research into the mycorrhizal status of imperiled plants and its applications to propagation and reintroduction efforts is warranted.

CHAPTER III

OBJECTIVES

To advance the preservation of New England's floral biodiversity.

- Develop efficient, cost-effective production of vigorous, healthy greenhouse stock of Fringed gentian and other endangered plant species for botanical gardens, native plant preserves, reestablishment projects, and possible commercial horticultural production.
- Contribute to the knowledge of endangered plant species, including the elucidation of previously unknown plant/fungus interactions.

To promote the use of sustainable plant production technologies as an alternative to high-input, chemically-based methods, particularly in the greenhouse/nursery industry.

- Encourage and expand the potential uses of VAM in sustainable plant production, by a) gaining information about the "behavior" of VA mycorrhizal fungi throughout plant production cycles (stratification, germination, overwintering, reemergence, etc.), and b) discovering, cultivating and testing effective indigenous VAM isolates for applications in New England plant production.
- Contribute to the knowledge of how rhizosphere interactions influence the growth of cultivated plants and how these can be manipulated or augmented for maximum benefit.

CHAPTER IV

1993-94 FIELD RESEARCH AND INOCULUM PRODUCTION

A. Field Research

1. Materials and Methods

Field investigations were undertaken to confirm the natural mycorrhizal status of *G. crinita* and to acquire propagules (indigenous soil) for VAM inoculum production. Three representative specimens of *G. crinita* were dug up in October 1993 from five field sites, each from within a different "eco-zone" of Massachusetts (Fig. 7, p. 46). This served to increase the diversity of sampled VAMF species, thus improving the chances that an effective indigenous isolate would be found via the pot culturing process (see Table 2, p. 45, for a list of the field sites, and Figs. 8-11, pp. 47-50, for field site photographs). Roots of excavated specimens were washed several times, cleared in 8% KOH, stained in aniline blue, destained in 50% glycerol and mounted on slides in lactoglycerol for assessment of root colonization by VAMF. Spores were extracted by a wet-sieving, decanting and centrifuge process, vacuum-filtered onto gridded filter paper, and counted in a petri dish under a dissecting microscope. Spores were seperated by color and morphology; from five to twenty-five spores of each morphological type were mounted on slides for VAMF species identification (Schenck and Perez, 1990). (The entire procedure for investigating VAM, including root preparation, spore extraction, and observation is provided in detail in the Appendix, pp. 187-190).

2. Results and Discussion

The field data was collected from excavated soil and plants from the five Fringed gentian field sites (Table 2, p. 45). There was a significant correlation ($R^2 = .84$, $p = .05$) between spore density at a given field site and the percentage of root colonization of the Fringed gentian samples excavated from that site. This has been seen in other native plants in other similar field studies (Vijayalakshmi and Rao, 1988; Koske and Halvorson, 1989). Also, wet meadow habitats had a significantly higher average spore density than calcareous fens (2417 vs. 1625), and subsequently supported a higher level of mycorrhizal colonization on average in gentian roots, 13% vs. 6%. In addition, wet meadows had a very different VAMF species composition than the fens; this was so pronounced that it can be seen in photographs of spores extracted directly from those habitats (Figs. 12 and 13, pp. 51-52). *Glomus geosporum* dominated the fen sites, while a range of isolates inhabited the wet meadows. Although *Glomus etunicatum* proved to be the most widespread isolate, inhabiting all five sites to varying degrees, it was only predominant at site 3. Nevertheless, it proved to be the most prolific VAM fungi in both its natural habitat and subsequently in the pot culturing process as well.

The gentians at site 3 (Field Farm), which had the highest spore density and rate of colonization of any site, were also by far the tallest and most vigorous field plants observed, reaching heights of 3½ ft. (The average height of the remaining field plants sampled was about 1.5 ft.). As mentioned, the predominant VAMF species from that site, *Glomus etunicatum*, which was the most prolific field isolate, emerged as the most prolific in pot

cultures. The second most prolific isolate, *G. geosporum*, from site 4, was also relatively easy to culture. Unfortunately, both pot cultures eventually proved to be contaminated with pathogens and were therefore ultimately inconsequential as far as data on their potential benefit to plants was concerned.

B. Inoculum production

1. Materials and Methods

In addition to the excavated plant samples, five soil subsamples were collected throughout each site (including from the root zone of the excavated specimen) during the first-year sampling (1993), batched, mixed 1:1 (v/v) with sterilized coarse sand, set up 11/1/93 in five 2-liter pot cultures, and seeded with a sorghum/sudan grass hybrid (Sudex) as a nurse plant to bait and trap the indigenous mycorrhizal fungi. The pot cultures, after five months of regular greenhouse care (with minimum fertilization), were dried down for one month (4/1 - 5/1/94) and sampled with a 25cc soil probe from each pot to examine for the type and number of VAMF spores. Only three of the five pot cultures (from sites 2, 3, and 4) resulted in mycorrhizal production. A 100cc sample from each of these "successful" pot cultures was sent to INVAM for VAMF species identification and to attempt to set up isolate cultures. The remaining pot culture inocula was stored away for future use. Also, fifty spores of each of the two predominant VAMF isolates from these three pot cultures were extracted from 5/1 - 5/15/94, separated by morphology, and used to set up six "isolate" cultures (each consisting of a single VAMF isolate) via the same pot-culturing process.

These isolate cultures were grown from 5/15 to 10/15/94, and dried down to 11/15/94. They were tested shortly thereafter for mycorrhizal activity (none was found).

Although Fringed gentian was found to be mycorrhizal in its natural habitat, this does not always translate into mycorrhization in the greenhouse. A highly infective "cocktail" starter culture (Morton, 1993), consisting primarily of a non-indigenous *Glomus etunicatum*, with lesser amounts of *G. clarum* and *G. claroides* (UT-316) was obtained from INVAM in August 1993 for use as a test inoculum for preliminary growth trials to ascertain whether Fringed gentian was able to undergo mycorrhization during greenhouse cultivation. In order to increase the volume of inoculum 15-20 fold for greenhouse experiments, this starter culture was used to inoculate six 2-liter pot cultures, which were then grown out as described above. The inoculum (referred to as "bulked-up inoculum) was ready for use by February of 1994, in time for the preliminary trials.

2. Results and Discussion

While three of the original five pot cultures (from sites 2, 3, and 4) started from field soils developed mycorrhizal activity, none of the isolate cultures grown from those three yielded any usable inoculum. It should be noted that the only isolate found in the pot culture from site 3 was *G. etunicatum*, despite the presence of several other VAMF isolates in the field soil; this was the only pot culture of the three which yielded only one isolate (Fig. 14, p. 53, is a picture of *G. etunicatum* spores after being extracted from a pot culture and separated out for isolate culture inoculation). Nevertheless, attempts to grow it as an isolate culture failed. However, INVAM had more success. Two of the three samples sent to them

did yield isolate cultures (MA 103 - *G. etunicatum*, and MA 104 - *G. geosporum*, from sites 3 and 4, respectively) which had spores and colonized roots, indicating mycorrhizal activity (Fig. 15, p. 54, is a photographs of vesicles in roots, which is typical of what would be seen from a successful and infective pot culture).

Neither of the indigenous isolate cultures grown here or by INVAM were ready at the start of the 1994 preliminary trials, so a portion of the "bulked-up" inoculum started from the INVAM cocktail starter culture was incorporated into the transplant medium. The remainder was stored under refrigeration for future use. This inoculum did eventually prove to be effective at enhancing gentian transplant survival in the 1994 preliminary trials (Table 5). It was therefore included in the 1995 growth and survival experiments for comparison to the indigenous isolate culture inocula which INVAM had grown (MA 103 and 104) as well as the two original pot culture inocula from which these isolate cultures were started.

The indigenous isolate cultures which INVAM grew out were received in late 1994, and stored along with the original pot culture inoculum produced from sites 3 and 4 until their use in the 1995 growth and survival experiments. These four inocula were used in experiment 2, transplant media 6-9 (TM 6 and 7 were the original stored pot cultures, TM 8 and 9 the INVAM isolate cultures from those), but 98% of the plants transplanted into these media died within three months after transplant and so were not used in data collection. Experiment 1 (TM 1-5) simply incorporated soil from the five field sites into the transplant media, but this also resulted in a 97% mortality rate, so neither of these experiments yielded usable data. TM 10-14 (experiment 3), which incorporated the bulked-up, non-indigenous *G. etunicatum* inoculum from INVAM, turned out to be the only "effective" transplant media,

and so provided most of the data for the 1995 growth and survival experiments (Table 6, p. 88, is a complete list of the treatment media used in the 1995 experiments).

The mycorrhizal pot culture inoculum produced in 1993-94 from the non-indigenous cocktail starter culture obtained from INVAM (UT-316) was tested for mycorrhizal activitity by counting extracted spores and assessing sampled Sudex roots for mycorrhizal colonization. High spore #'s (~ 20/cc^{-1} soil) and extensive root colonization (>70%) indicated significant mycorrhizal activity and a potentially infective inoculum (Fig. 15, p. 54). One of the purposes of the 1994 preliminary trials was to test the efficacy of the VAMF isolates in the inoculum regarding their ability to form mycorrhizae with the test plants. The cocktail starter culture originally contained three VAMF species (*Glomus etunicatum*, *G. clarum*, and *G. claroides*) but only one fungal species, *G. etunicatum*, was found in the final "bulked-up" inoculum. This paralleled the results seen in the original indigenous pot culture inoculum from site 3 as well, where *G. etunicatum* emerged as the predominant VAMF species, apparently out-competing the other fungal isolates. While not necessarily reflecting processes always found in nature, it was clear that *G. etunicatum* , as the most prolific field isolate and the one best adapted to the Sudex pot-culturing production process, was the best isolate to use in subsequent growth trials.

Table 2. *Gentianopsis crinita* (Fringed gentian) field sites in MA.

1) Women's Fed. State Forest, New Salem	4) Powerline, Cheshire
2) Mt. Hope Park, South Williamstown	5) North Common Meadow, Petersham
3) Field Farm, Williamstown	

Table 3. Field data from soil and plant excavations at five fields sites, 0ctober-November, 1993.

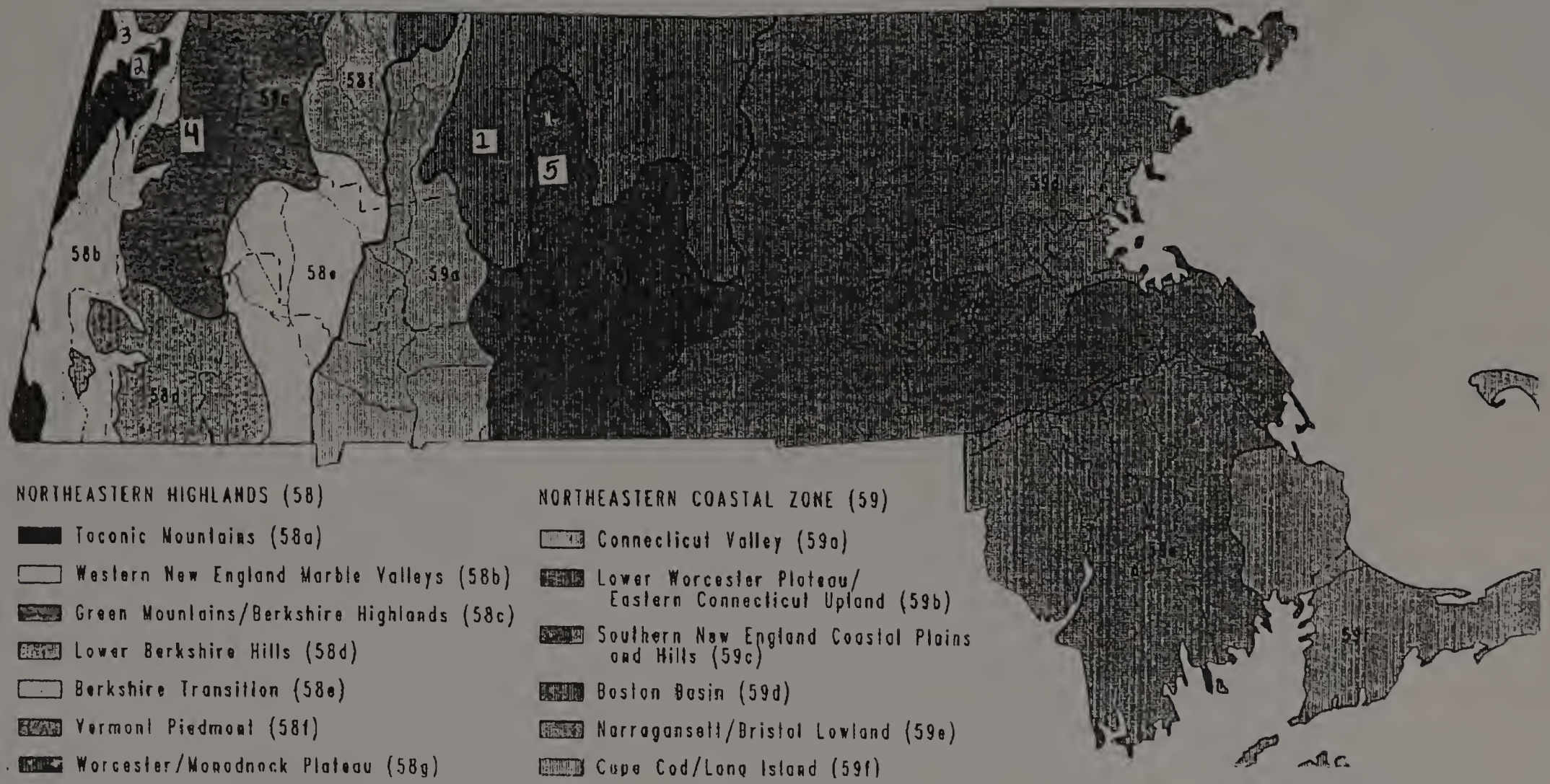
Field site	Habitat type	Mycorrhizal colonization (%)*	Spore density** (per 100 cc soil)	Predominant VAMF species	% of total	Plant height†
1	wet meadow	7	1750	*Glomus clarum*	35	1.25
2	calcareous fen	5	1275	*G. geosporum*	40	1.25
3	wet meadow	17	3200	*G. etunicatum*	65	3.5
4	calcareous fen	7	1975	*G. geosporum*	60	1.5
5	wet meadow	15	2300	*Scutellospora reticulata*	30	1.75

*mean of roots from three plants (gridline intersect method - Giovanetti and Mosse, 1980)
**mean of three extractions (from batched sample of five subsamples per site)
† mean of three plants, measured in feet.

Table 4. VAMF species extracted from field sites or pot cultures and identified.

Predominant species(>200/100cc)	Secondary species (<20/100 cc)
Glomus etunicatum	*Glomus boreale*
G. geosporum	*G. canadensis*
G. clarum	*G. radiatum*
*Entrophospora infrequens**	*G. pubescens*
*Scutellospora reticulata**	*Gigaspora sp*

*Difficult to grow in pot cultures



Field Sites:

1) Women's Fed. State Forest, New Salem (wet meadow)
2) Mt. Hope Park, South Williamstown (calcareous fen)
3) Field Farm, Williamstown (wet meadow)
4) Powerline, Cheshire (calcareous fen)
5) North Common Meadow, Petersham (wet meadow)

Figure 7. Map of "eco-zones" of each *G. crinita* field site. (Map courtesy of ManTech, Inc., EPA, Corvallis, OR. Used by permission.)

Figure 8. Fringed Gentian and associated vegetation in a calcareous fen, field site 2, Mt. Hope Park, South Williamstown, MA. Surrounding vegetation consists mostly of *Equisetum scirpoides* and *Potentilla fruticosa*. Gentian flower can be seen in the lower left hand section of the photograph.

Figure 9. Fringed Gentian in a wet meadow at the edge of a pond, field site 5, North Common Meadow, Petersham, MA.

Figure 10. Field site 1, Women's Federated State Forest, New Salem, MA. Body of water is part of the Quabbin Reservoir.

Figure 11. Field site 3, wet meadow at Field Farm, Williamstown, MA.

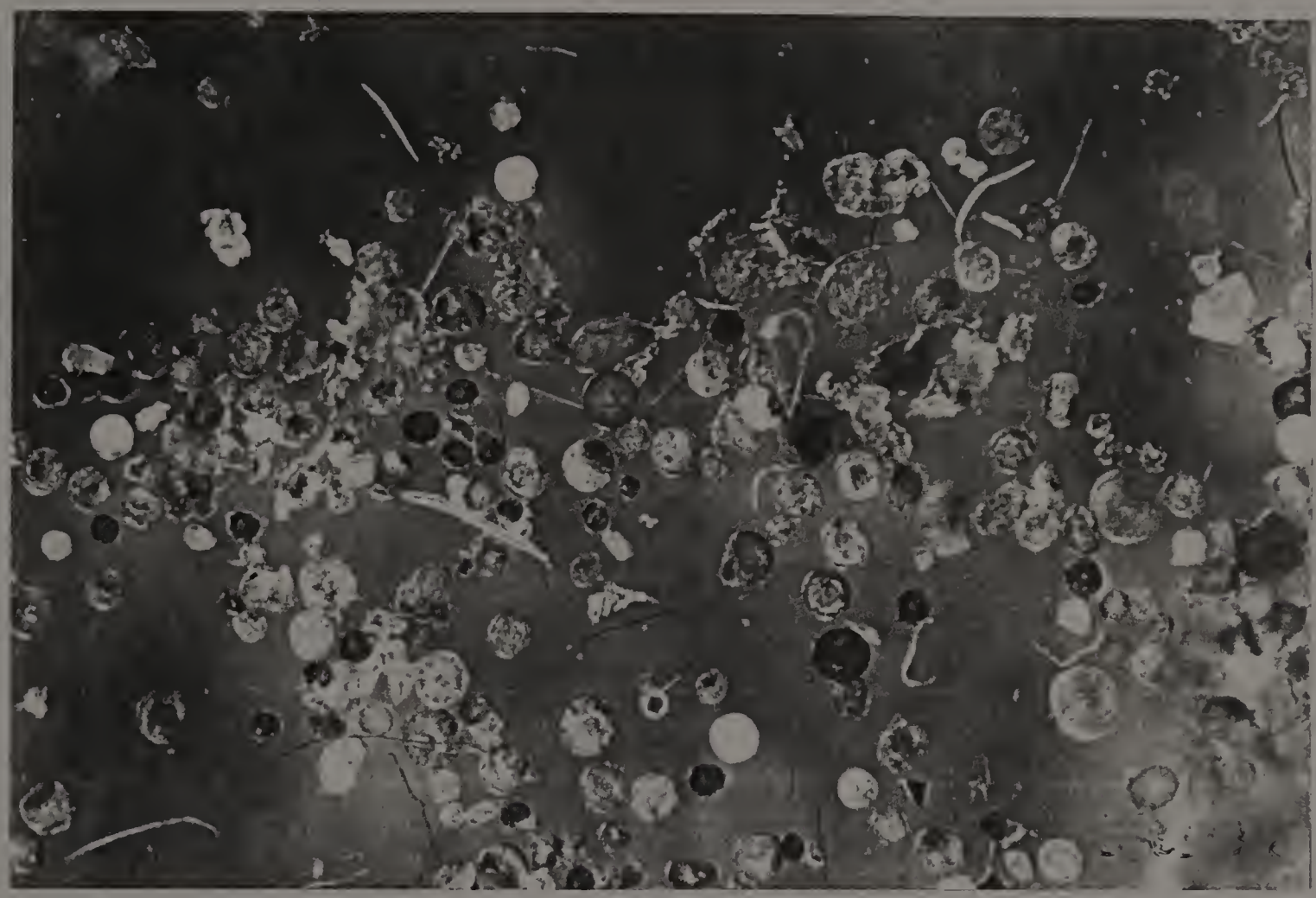


Figure 12. VAMF spores extracted from field site 5, North Common Meadow, Petersham, MA. "Donut-shaped" spores are *Scutellospora reticulata*, the predominant VAMF species at this site. The threadlike objects are free-living (non-pathogenic) nematodes.

Figure 13. VAMF spores extracted from field site 4, a calcareous fen in Cheshire, MA. Dark brown to black spores are *Glomus geosporum*, the predominant VAMF species at this site.

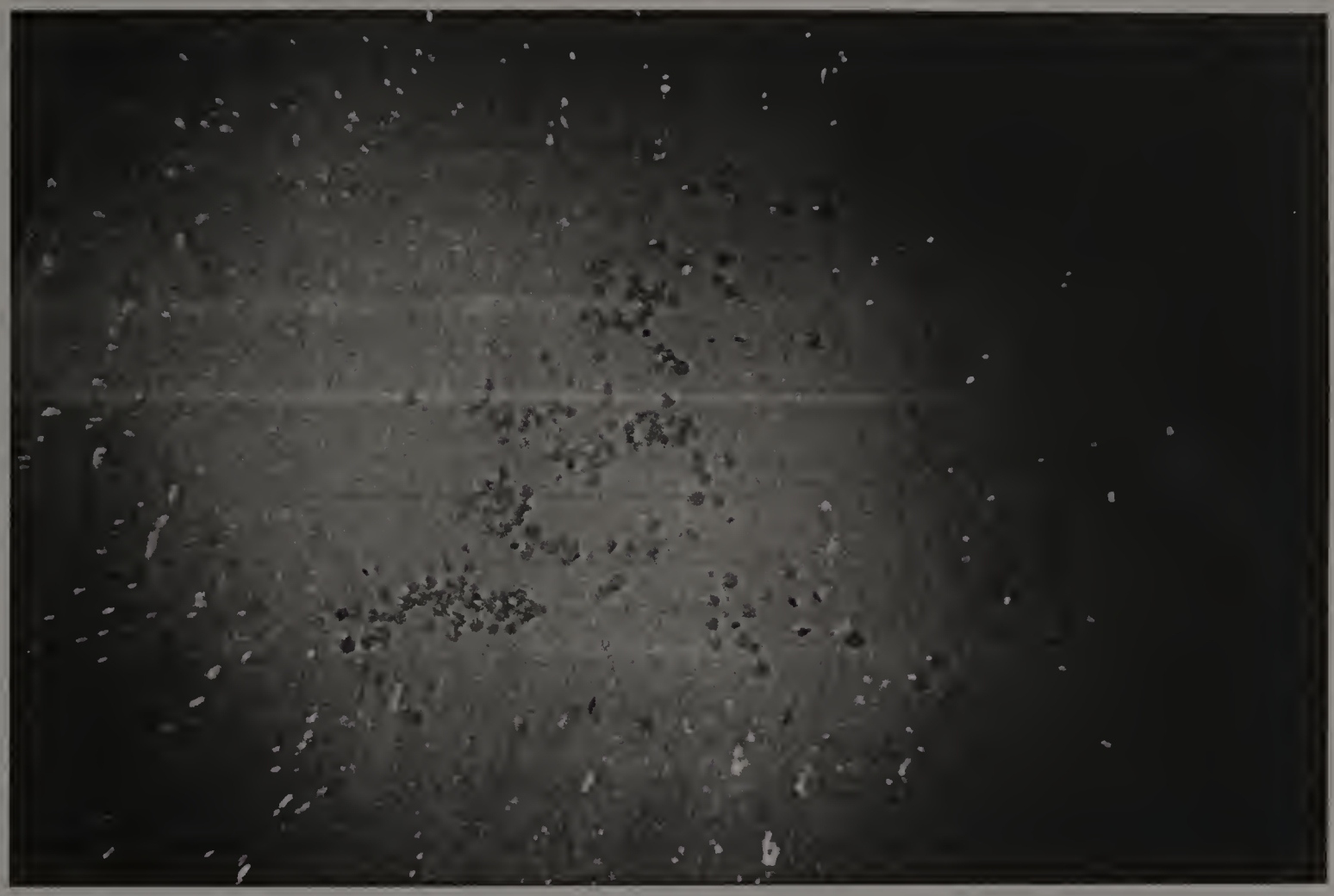


Figure 14. *Glomus etunicatum* spores extracted from a pot culture started with soil from field site 3, Field Farm, South Williamstown, MA. The spores were separated out on filter paper and viewed through a dissecting microscope before being used to inoculate an isolate culture.

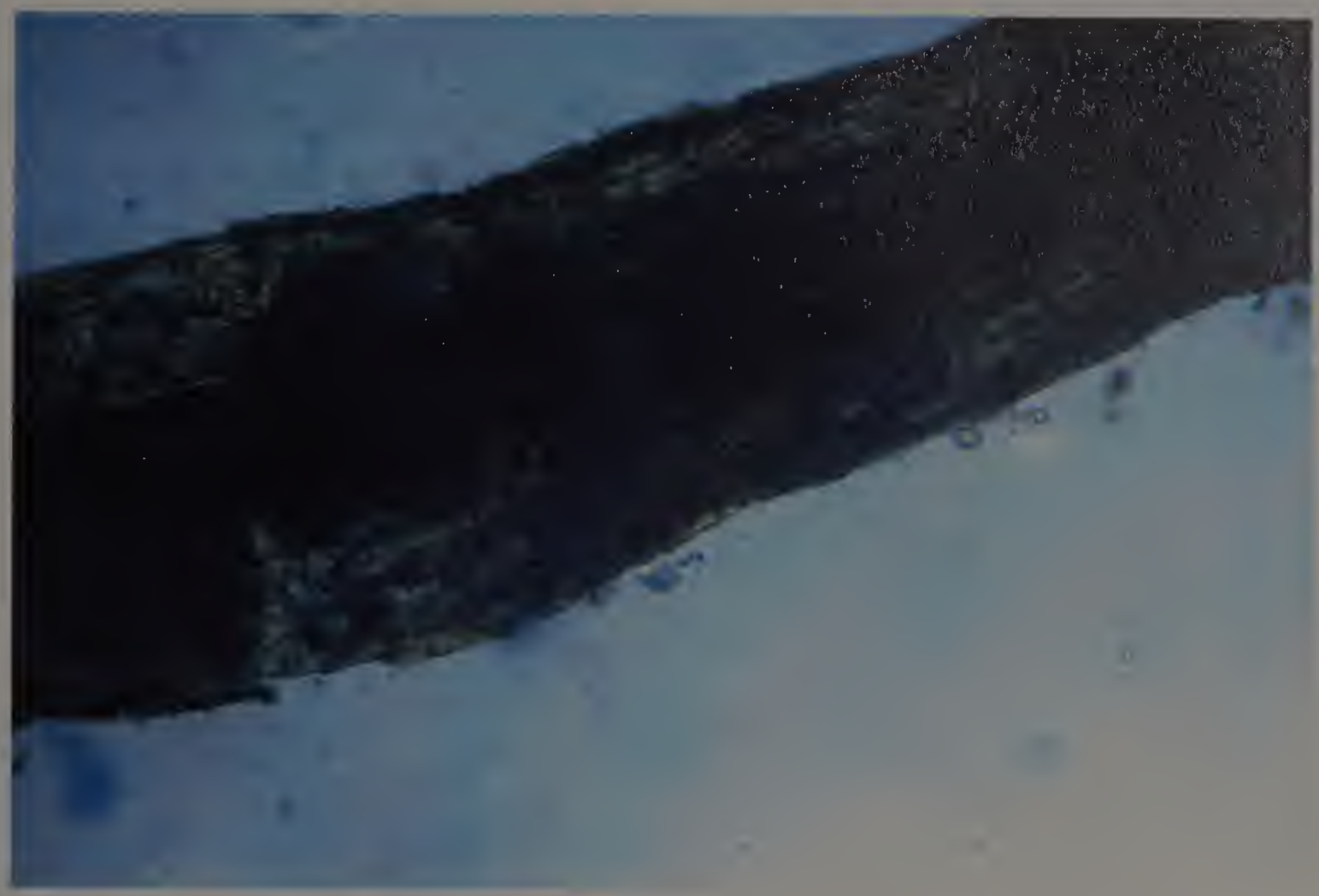


Figure 15. Vesicles from a Sudex (sorghum/sudangrass hybrid) pot culture, indicating a successful and potentially infective mycorrhizal inoculum.

CHAPTER V

1994 PRELIMINARY TRIALS

A. Materials and Methods

Preliminary greenhouse trials were undertaken to assess the infectivity and effectivity of the bulked-up INVAM inoculum and to establish basic cultural, fertility, and medium requirements for the cultivation of *Gentianopsis crinita* in the greenhouse. Gentian seeds were collected from field site 3 (Field Farm, Williamstown) and were dried and stored at 4°C until 1/2/94, when they were sown into sterile germination media and stratified for two months at 2°C. On 3/2/94, the trays were removed from refrigeration, uncovered, and placed in a mist house. Germination commenced approximately one month later. The germinated seedlings remained in the mist house for another two months (until 6/1/95) and were then transplanted and moved to a cool greenhouse (55-65°F) for use in preliminary growth trials.

Two other plant species were tested in these pilot studies in addition to *G. crinita*: *Penstemon hirsutus* (L.) Willd. and *Gentiana clausa* Raf. *Penstemon hirsutus* is easily started from seed (no stratification necessary), and seed was readily available (supplied by the New England Wildflower Society). This species, which is an endangered species in MA (MNHESP, 1992), is sometimes difficult to maintain in cultivation (Brumback, 1993) and has an unusual relationship with its mycorrhizal symbiont, much like gentians (Mcgee, 1995). *G. clausa*, which has Special Concern status in some of the New England States (CPC, 1990) is closely related to *G. crinita*,

both in terms of physiology and habitat, but is much easier to grow. It was chosen as a backup test plant in the event that no results were obtained from *G. crinita*. *Gentiana clausa* was measured and then harvested to test the validity of the longest leaf span as a correlative measure of total shoot biomass. This was necessary because the subsequent 1995 experiments with *G. crinita* would involve repeated measurements over time of the same leaves on the same plants, and so required a valid non-destructive measure of plant growth.

A portion of the inoculum produced from the "cocktail" supplied by INVAM (a non-indigenous *G. etunicatum*, UT-316) was subsequently used in 1994 preliminary growth experiments to determine whether this known infective VAM isolate would actually form mycorrhizae with *G. crinita* and the other test plants (indigenous isolate culture inoculum was not yet ready for use at this time). The remaining inoculum was stored under refrigeration until its use in the 1995 experiments. Initial fertilizer and media treatments for preliminary tests were derived from a combination of mycorrhizal research, gentian research, and low-input native plant production protocols (Giersbach, 1937; Brumback and Curtis, 1986; Millner and Kitt, 1992).

Penstemon hirsutus seed was sown into sterile germination medium in March 1994. One month later (April) a total of 60 seedlings were transplanted into 4" plastic pots containing either mycorrhizal or non-mycorrhizal medium (30 per treatment). The transplant medium consisted of 1:1:1:1 (v/v) unsterilized compost, sand, vermiculite, and commercial growing mix (Fafard II™ - Fafard, Inc. Agawam, MA). The mycorrhizal medium also had mycorrhizal pot culture inoculum containing the non-indigenous

Glomus etunicatum isolate mentioned above, incorporated at a 1:7 (v/v) ratio of inoculum to medium. In July, after twelve weeks of growth, leaf area measurements were taken and disease incidence was assessed. One month later the percentage of surviving plants was tallied and compared. *Gentiana clausa* underwent the same treatment as *G. crinita* except that the *G. clausa* plants were harvested twenty weeks after transplant so that they could be weighed for the leafspan/shoot biomass correlation. The *G. crinita* seedlings were transplanted a second time in early November to grow on and overwinter. *G. crinita* roots from both treatments were sampled during this transplant to assess mycorrhizal colonization.

B. Results and Discussion

1 *Penstemon hirsutus*

The overall beneficial influence of the mycorrhizal transplant medium on the growth and survival of *P. hirsutus* can be seen in Fig. 16 (p. 63). The data presented in Fig. 17, p. 64, demonstrates that the mycorrhizal transplant medium increased the growth and survival of *P. hirsutus* over the non-mycorrhizal medium. Average leaf area of the three largest leaves from each plant, which was the parameter used to assess growth, was significantly greater in plants from the mycorrhizal medium. Differences in growth, vigor, and survival between the two treatments can clearly be seen in Figs. 18 and 19 (pp. 65-66), which are photographs of the test plants three months after transplant into the treatment media.

The disease incidence and subsequent fatality were also significantly lower in the mycorrhizal transplant medium (Fig.20, p. 67). The disease which killed some of the transplants in the non-mycorrhizal transplant medium was a leaf and stem blight caused by *Sclerotinia* spp., a fairly common and widespread soil-borne fungal pathogen with a wide host range.

2. *Gentiana clausa*

The transplant medium used in preliminary trials with *G. clausa* was the same used for *P. hirsutus.* Results are summarized on p. 69, Fig. 22. As with *P. hirsutus*, the mycorrhizally-inoculated transplant medium increased the growth of *G. clausa,* as measured by longest leaf span (Fig. 23, p. 70). Fig. 24 (p. 71) shows the difference in shoot biomass between the two treatments; the mycorrhizal treatment yielded a significantly higher biomass than its non-mycorrhizal counterpart. In addition, the 80% correlation between the longest leaf span and the shoot biomass of *G. clausa* (Fig. 25, p. 71), which closely resembles *G. crinita* in growth and habit, indicated that leaf span was in fact a valid non-destructive correlative measure of plant growth and thus could be used as a legitimate parameter by which to compare the effects of different treatments on *G. crinita* in the subsequent 1995 experiments.

3. *Gentianopsis crinita*

Based on the results of the 1994 preliminary trials with this species, some basic cultivation guidelines were established. To begin with, the highest germination rate was

achieved by a two-month stratification (cold) period at 2° C in a controlled environment such as a refrigerator, rather than outside in a cold frame. *Gentianopsis crinita* does best in a germination and transplant medium containing about 25% organic matter in the form of compost. This had been noted and used by previous researchers (Giersbach, 1937; Phillips,1985) and was confirmed in these pilot trials. The medium ultimately chosen as both germination and transplant medium consisted of a 1:1:1:1 (v/v) compost/commercial growing mix/sand/vermiculite mix, hereafter referred to as "gentian medium" .

This plant species does not require high fertility levels; in fact, standard fertilization with a number of different fertilizers resulted in decline and death. The optimal nutritional regime was found to be fertilization once per month with a water soluble "fish" fertilizer at 1/3 strength (2-1-1). The low phosphorus levels (5-11 ppm) of the compost-based medium used in these and subsequent experiments approximated naturally-occuring P levels found in field site soils. Higher P levels inhibit mycorrhizal colonization, and therefore should be avoided (Powell and Bagyaraj, 1984).

G. crinita had to be transplanted from its germination tray within 4 months of germination or it died. However, transplantation also resulted in high mortality. Mortality was reduced when care was taken to transplant the entire root/soil mass into the new container, which was accomplished by transplanting the plants in groups of 3-5 as opposed to individually. This has also been noted by other researchers (Phillips, 1985). Plants were moved outdoors into a partially shaded cold frame for the summer season (June -September), as they did not respond well to the intense heat and sun in the

greenhouse. *Gentianopsis crinita* is a cool-weather plant, putting on most of its growth from September to November. Plants were transplanted a second time in November. Not a single *G. crinita* from these 1994 trials survived more than two months beyond this second transplant, although some of the transplants in the mycorrhizal medium survived several weeks longer than their non-mycorrhizal counterparts (data not shown).

These pilot studies revealed the non-indigenous *Glomus etunicatum* to be an infective isolate for *G. crinita* and the other test plants. Roots randomly sampled during the second transplant in early November revealed significant mycorrhizal development (25 - 40%). This was thought at the time to be related to the length of post-tranpslant time in the mycorrhizal medium. As a result, the test plants for the 1995 experiments were started three months earlier, so mycorrhization would occur while the plants were still actively growing, before the onset of dormancy. However, despite this earlier start, the 1995 test plants still formed mycorrhizae at the same time of year, early to mid-November. It was then hypothesized that mycorrhization in *G. crinita* was tied to phenological events in the plant's life cycle, such as the onset of dormancy, rather than the length of post-transplant time in the mycorrhizal medium. This is discussed in greater detail in the discussion section (pp. 147-172).

The 1994 results revealed that a light fertility regime in a compost-based gentian medium supported both healthy plant growth and significant mycorrhizal symbiosis. Furthermore, the viability of the mycorrhizal component of the gentian medium was maintained throughout the greater portion of the growing season, as the late mycorrhization demonstrates. This is significant in so far as mycorrhizal inoculum, when

incorporated into a growing medium for growth experiments, is usually "used up" by the third month, the point at which the vast majority of host plants would have formed mycorrhizae. In this case, the inoculum potential of the mycorrhizal component of the transplant medium remained viable into the fifth and sixth month after transplant. Based on these results, the mycorrhizal inoculum and the cultural techniques established in the 1994 trials were the same ones adopted for use in subsequent 1995 growth and survival experiments with *G. crinita*.

Table 5. Results of the 1994 preliminary experiments: Establishment of cultural guidelines for the propagation of *Gentianopsis crinita*. Seedlings were transplanted into gentian medium in 2" cubes twelve weeks after germination in a sterile medium, and were grown in a lightly shaded greenhouse. There were 15 plants per treatment, unless otherwise noted.

Treatment	**Survival % (20 weeks after transplant)**
1. <u>Fertilization</u>	
F1 (no fertilization)	**36***
F2 (Osmocote™)†	6
F3 (organic fertilizers**)	0
F4 (Peter's™ liquid soluble 5-10-5)†	0
2. <u>Growth medium</u>	
G1 (with compost)	**38**
G2 (without compost)	5
3. <u>Mycorrhizal inoculum</u> (30 plants per treatment)	
M1 (with mycorrhizal inoculum)	**41**
M2 (without mycorrhizal inoculum)	29
4. <u>Transplanting</u>	
T1 (not tranplanted)	0
T2 (transplanted individually)‡	8
T3 (transplanted in groups of 3-5)‡	**35**

*Bold text indicates a significant difference from the other treatments within the group, as per Z-test (p=.05).
**Combination of blood meal, rock phosphate, and greensand (12-6-4).
†W.R. Grace, Fogelsville, PA.
‡Plants roots were gently pulled apart for individual transplant. For group transplant, entire cells containing 3-5 plants were transplanted as a whole unit.

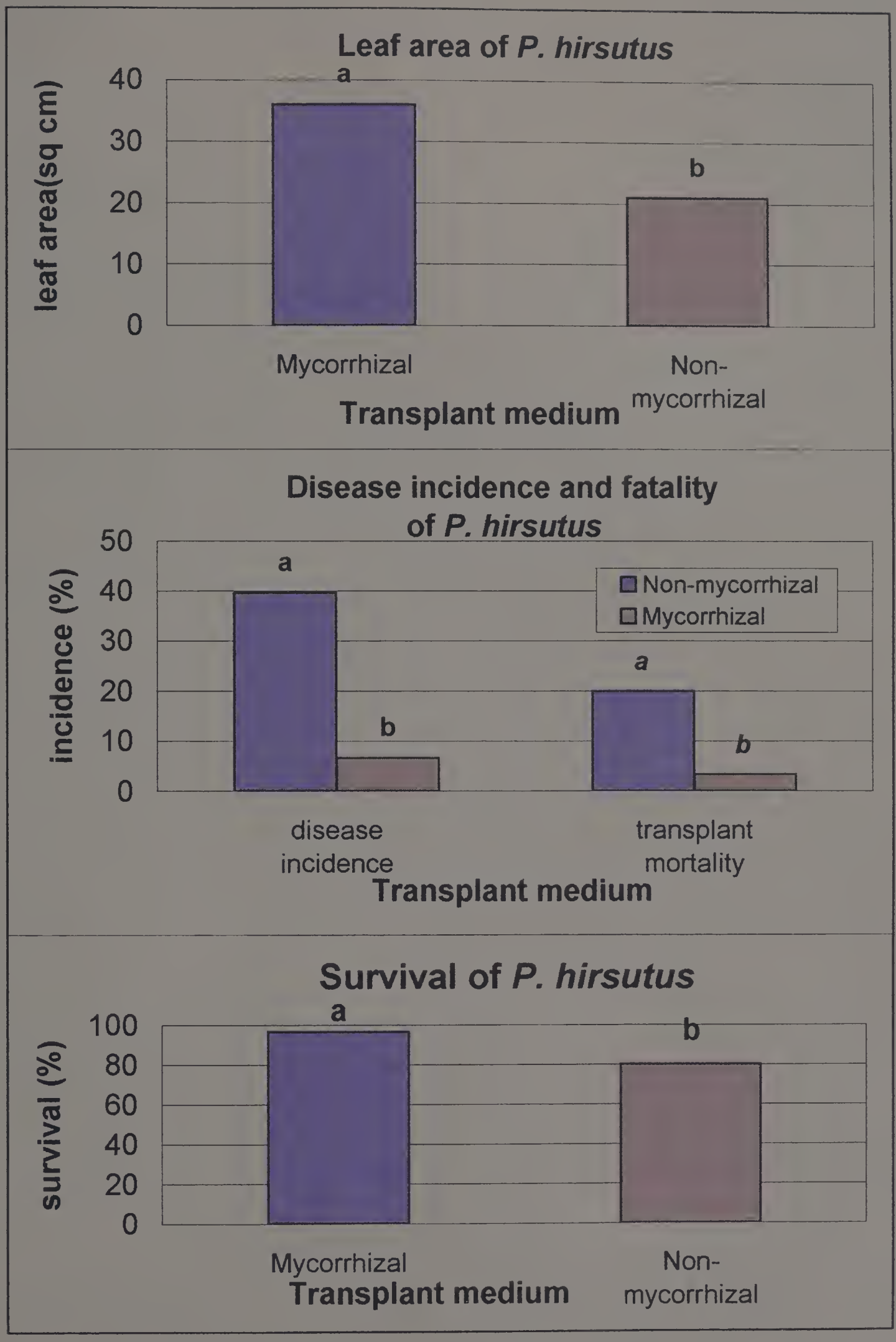


Figure 16. Beneficial effect of mycorrhizal inoculum on *Penstemon hirsutus*.

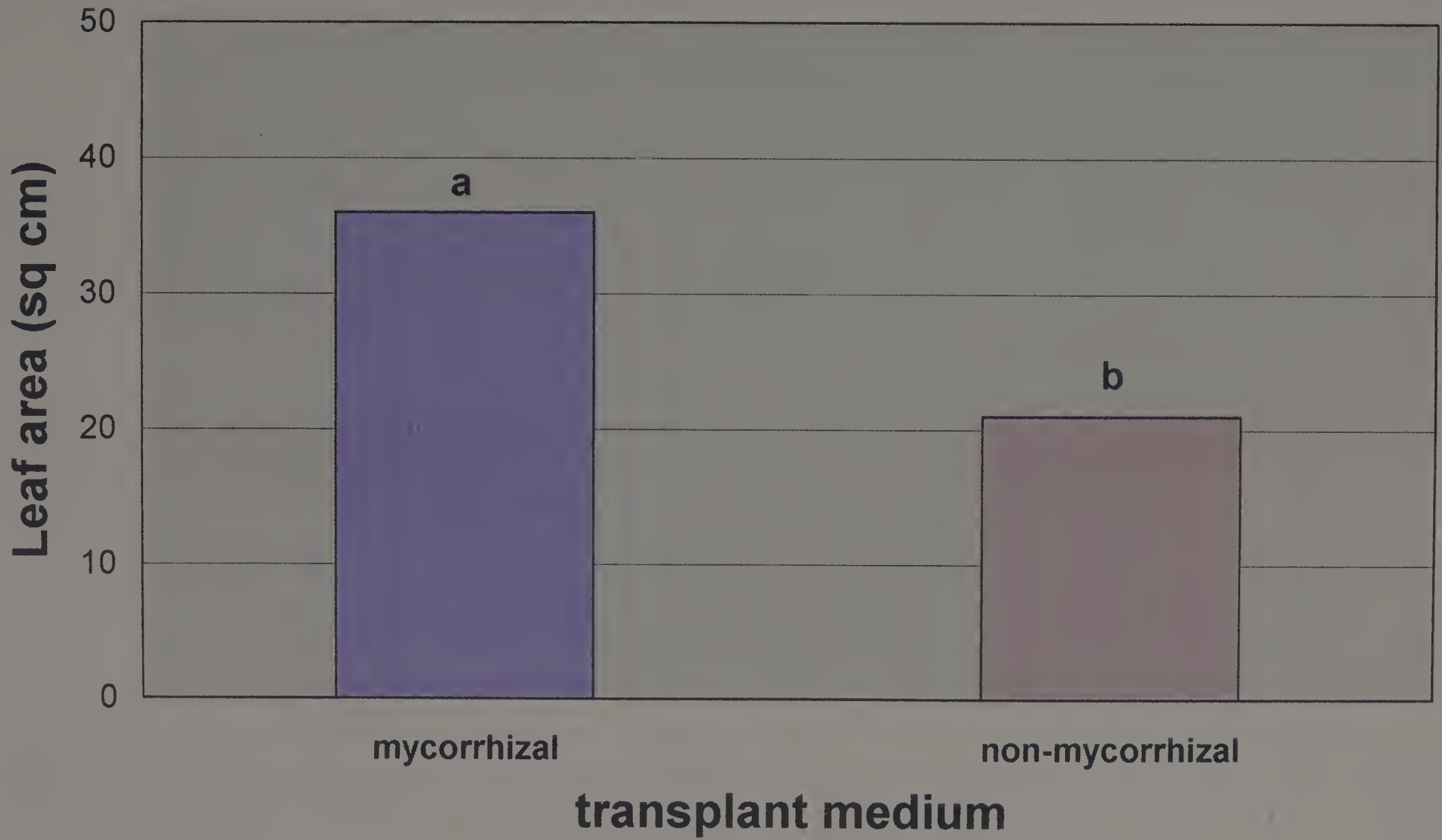


Figure 17. Influence of mycorrhizal inoculum on leaf surface area of *P. hirsutus* ten weeks after transplant. Data represent means of three largest leaves of each plant (n=30). Means are significantly different as per t-test (p =.01).

Figure 18. *Penstemon hirsutus* three months after transplant into a mycorrhizally-inoculated transplant medium.

Figure 19. *P. hirsutus* three months after transplant into a non-mycorrhizal transplant medium. The disease-causing fungal pathogen in these plants was *Sclerotinia* spp. Note the differences in size, vigor, and survival between these plants and those pictured in Fig. 18 (previous page).

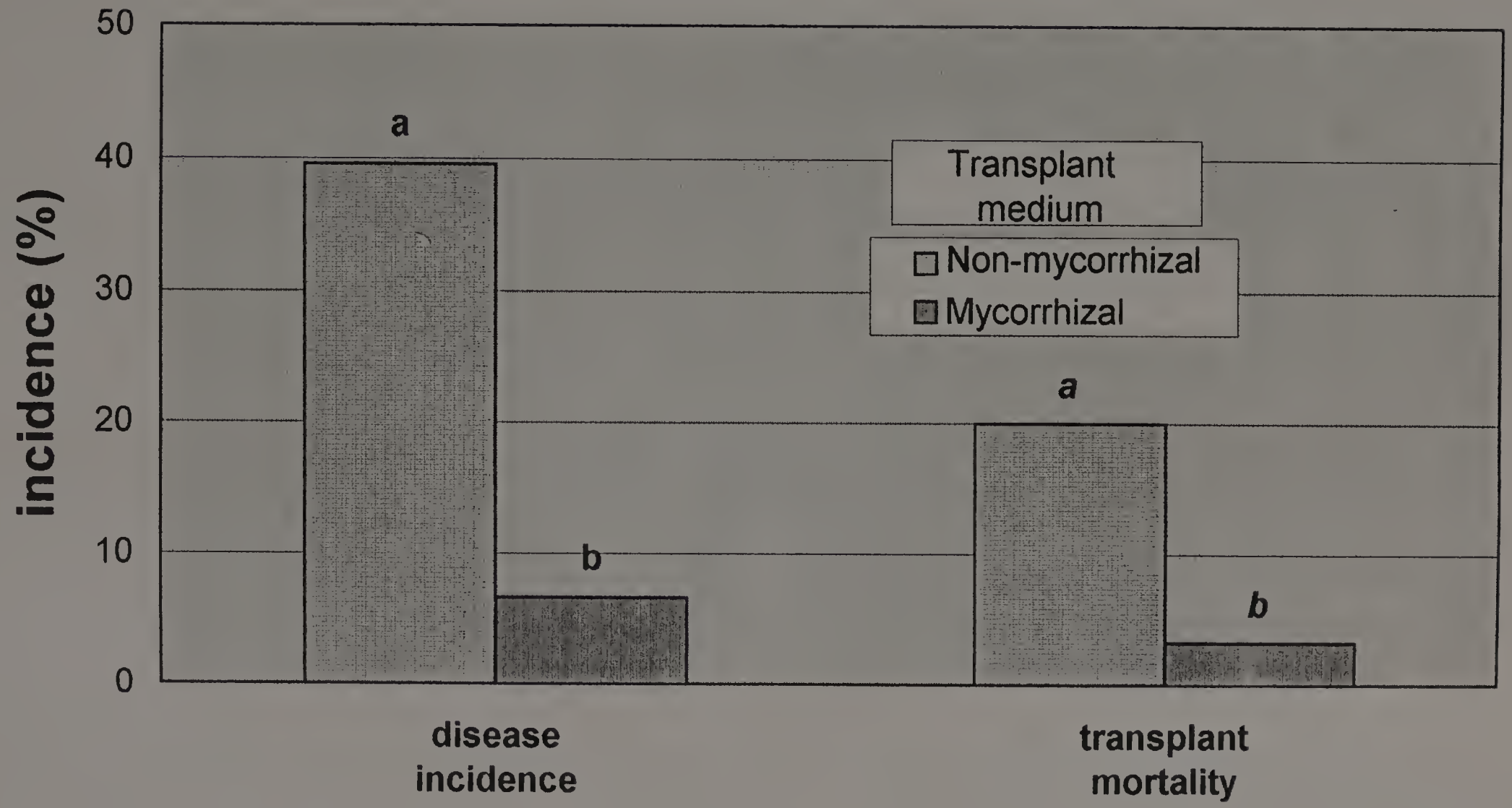


Figure 20. Post-transplant disease incidence and mortality of *P. hirsutus*. Pathogen which caused disease was *Sclerotinia* spp. Percentages are significantly different as per Z-test (p =.01).

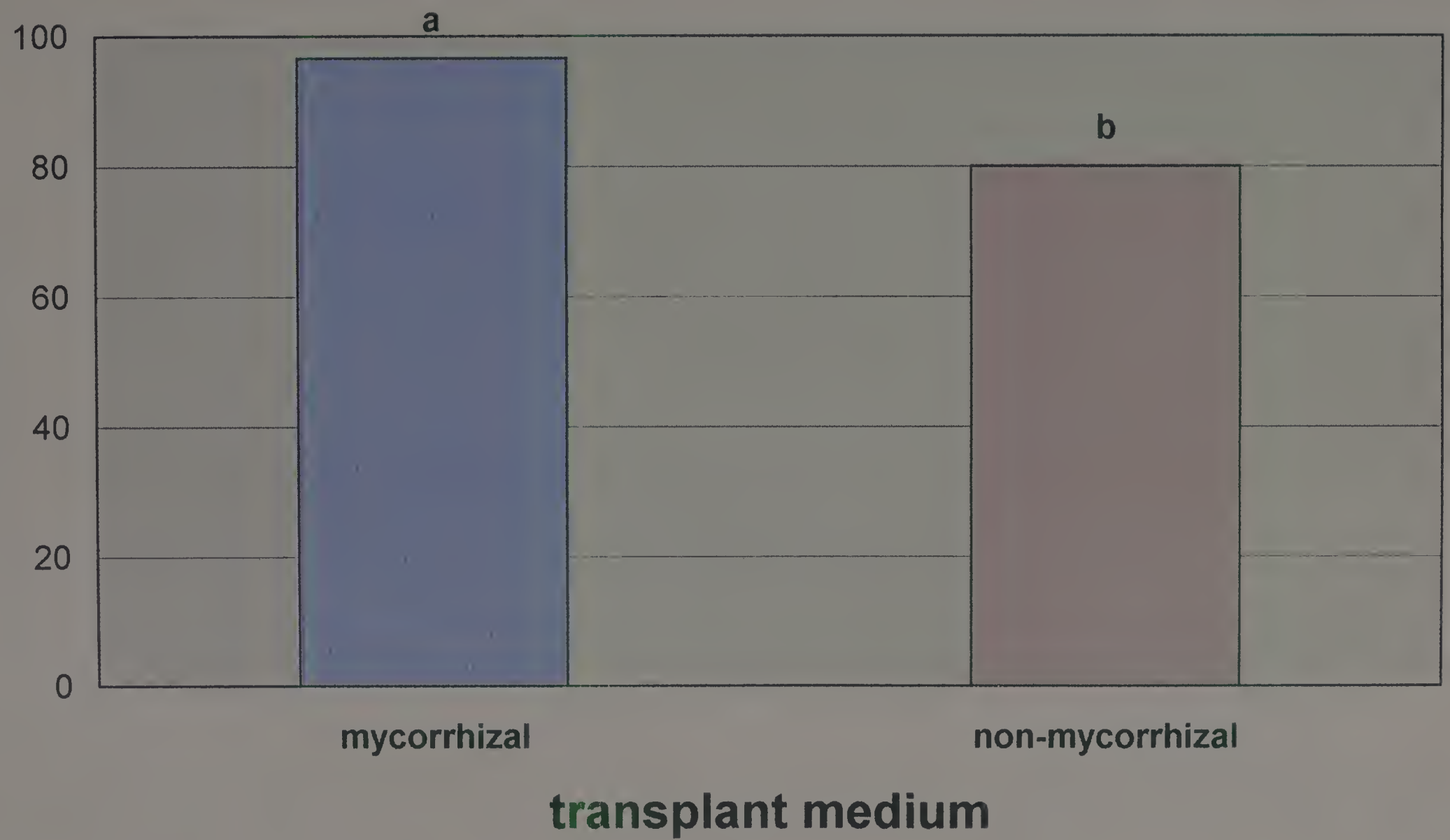


Figure 21. Post-transplant survival of *P. hirsutus.* Plants were germinated in sterile medium 3/94, transplanted 4/94, and surveyed 8/94. Means are significantly different as per Z-test (p =.01).

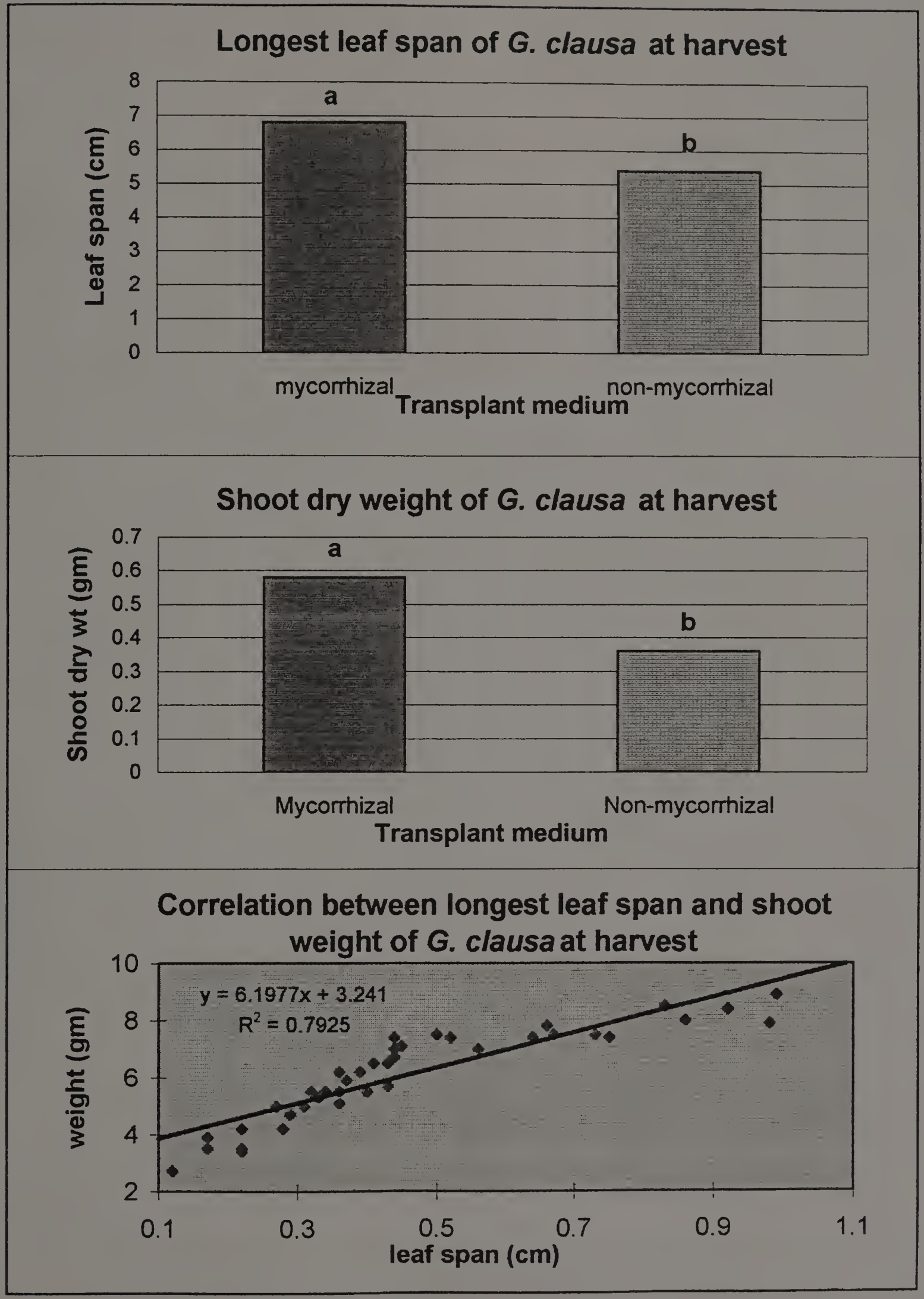


Figure 22. The beneficial effect of mycorrhizal inoculum on *G. clausa* and the correlation between longest leaf span and shoot dry weight.

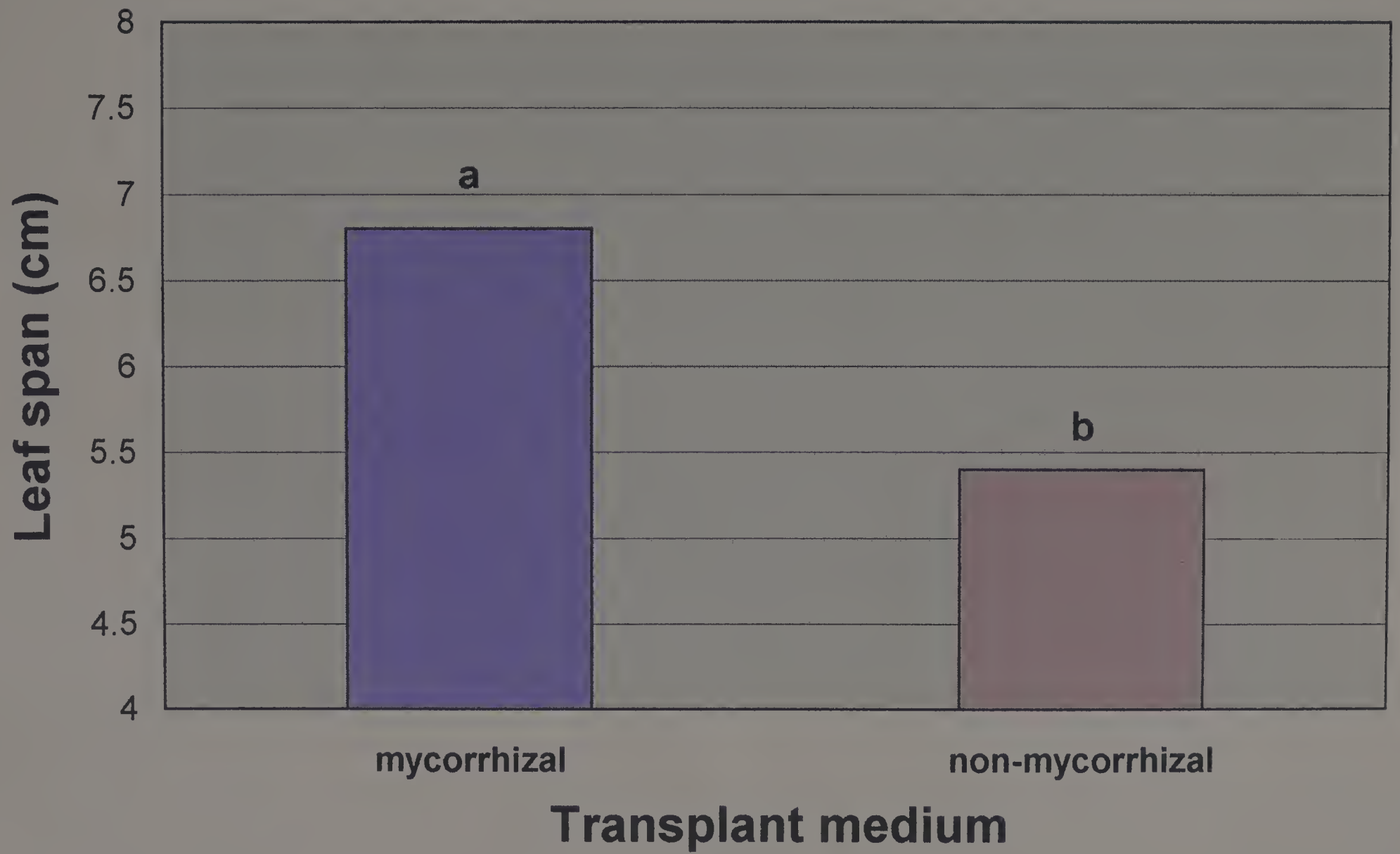


Figure 23. Influence of mycorrhizal inoculum on leaf span of *G. clausa* at harvest. Plants were germinated 4/94, transplanted 6/94, and harvested and measured 10/94. Means are significantly different as per t-test (p =.01).

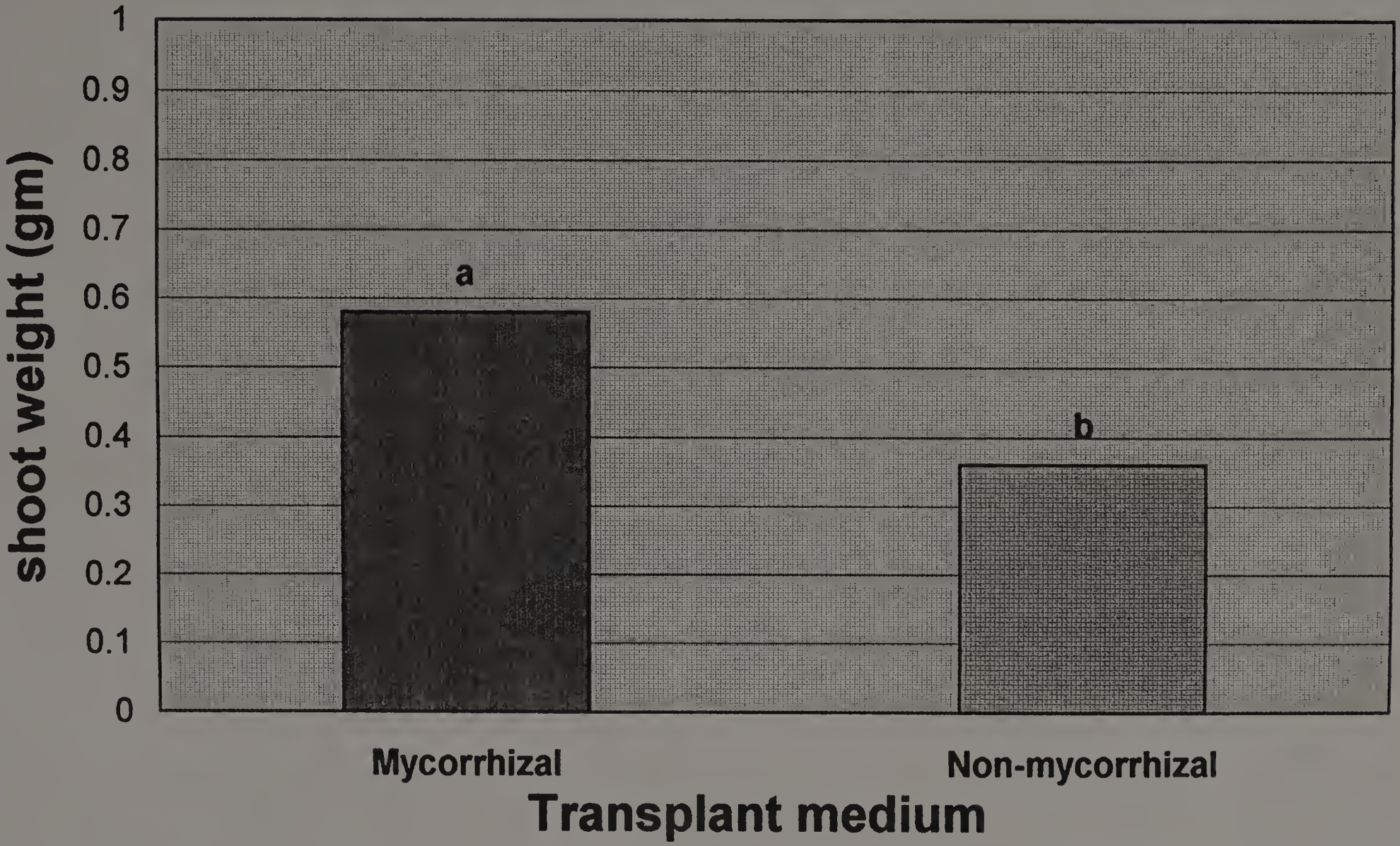


Figure 24. Mycorrhizal influence on shoot weight of *G. clausa* at harvest. Plants were germ. 4/94, tx'd 6/94, and harvested 10/94. Means are significantly different as per t-test (p=.01).

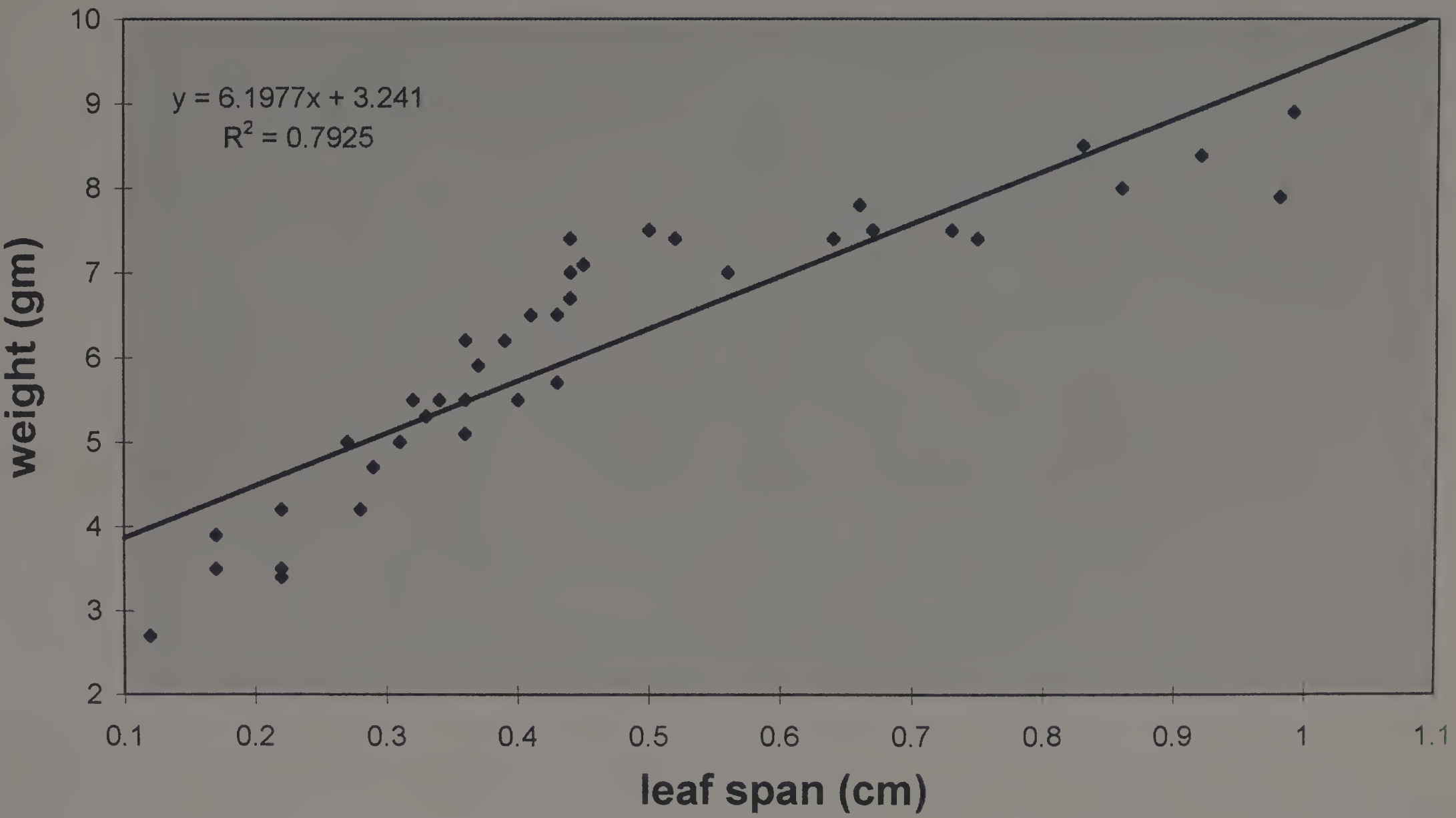


Figure 25. Correlation between longest leaf span and shoot weight of *G. clausa* at harvest. Plants for this analysis were from mycorrhizal and non-mycorrhizal transplant media.

CHAPTER VI

1995 GROWTH AND SURVIVAL EXPERIMENTS

A. Materials and Methods

Due to the realization that *Gentianopsis crinita* was extremely slow-growing, as well as the initial but ultimately incorrect assumption that mycorrhizal formation was based on length of post-transplant time, plants used in the 1995 experiments were started several months earlier than the previous year's crop. In addition, based on the observation that the incorporation of this infective mycorrhizal pot culture inoculum into the transplant medium (at a 1:7 v/v ratio) benefitted *G. crinita* as well as the other plant species that were being tested at the time (*Gentian clausa* and *Penstemon hirsutus*), it was realized that incorporating mycorrhizal inoculum into the germination medium might actually increase these enhancement effects by allowing them to begin from the outset rather than from the point of transplantation. Also, by using this approach, it was thought that useful information could be obtained about the behavior of mycorrhizal inoculum throughout the production life-cycle of perennials that require a stratification period after seeding.

Seeds that had been collected the previous year were sown on 10/1/94 into two separate cube trays - one with a mycorrhizally-inoculated gentian germination medium and the other a non-inoculated control. These were stratified and removed to the mist house on 12/7/94. Seedlings germinated approximately one month later. Three months after germination, these seedlings were large enough to be transplanted. Prior to transplant in April, germination data was collected. This seed-sowing, stratification and germination procedure was repeated again in 1996 to confirm the 1995 results.

Three-month-old *G. crinita* seedlings were randomly transplanted in April, 1995 from two germination media treatments (GM 1 = mycorrhizal, GM 2 = non-mycorrhizal) into one of 14 transplant media (TM 1-14), which had been categorized into three separate experimental sets. Seedlings were transplanted in groups of three from germination cubes with the root ball intact so as to minimize damage to their delicate roots. Only those cubes with at least three seedlings were used in the actual experimental measurements of growth and survival. The remainder were transplanted to separate pots containing same treatment media, to be used as sampling replicates for the purpose of monitoring mycorrhizal activity during the course of the experiment without disturbing the test plants themselves.

All treatment media (germination and transplant media are collectively referred to as "treatment" media) with the exception of TM 12, consisted of 7 parts gentian medium to 1 part inoculum (v/v); the inoculum portion of the media comprised the only difference between each treatment (Table 6, p. 89). The first set, referred to as experiment 1, consisted of five transplant media, inoculated with field soil from each site (TM 1- 5). The second set, experiment 2 (TM 6-9), consisted of inoculum from the two active pot cultures that were grown at UMass (TM 6-7), and the two isolate cultures grown by INVAM (TM 8-9) from these pot cultures (named MA 103 and 104, respectively). Experiment 3 (TM 10-14) utilized the effective non-indigenous *Glomus etunicatum* isolate (UT-316) mentioned earlier, plus two control media (sterilized and commercial greenhouse media) Samples of TM 7-14 were submitted for nutrient analysis prior to use and found to be virtually identical with regard to nutrient levels.

TM 14 was essentially the same as GM 1, the mycorrhizal germination medium; the gentian seedlings were simply transplanted back into the same medium from which they germinated. TM 10 is similar to TM 14, except that TM 10 did not undergo stratification (cold-period) as TM 14 had. The three remaining media (TM 11-13) were included as non-mycorrhizal controls, and TM 12 and 13 were steam-sterilized.

Throughout the experiments, soil and root samples from extra non-test replicates were harvested periodically and examined for mycorrhizal activity, via spores and root colonization, or for other fungal activity (including pathogens). The mycorrhizal activity was correlated with plant growth , assessed by measuring leaf span of the longest leaves once per month, to discern any differences among treatments, and to see if such differences were related to mycorrhizal formation. In addtion, dead and diseased plants were periodically removed, and their roots sampled and observed for mycorrhizae and/or plant pathogens.

Experiments had a randomized complete block design. Post-transplant survival data was collected throughout the 7-month growing season (April - November), and was statistically analyzed using repeated measures ANOVA, followed by a Z-test for proportions, which compared the *P*-value of each treatment. Duncan's MRT was also used where appropriate, to compare mean survival percentages across treatments.

The growth rate of *G. crinita* was determined by non-destructive repeated measurement of the span of longest leaf couplet. This data will be referred to heretofore as "growth rate". Growth data was collected monthly beginning the day after transplant

until dormancy in November. However, only the data collected subsequent to the initial two-month period following transplant were used for comparison and analysis. This was the point at which the downward slopes, representing post-transplant survival (Figure 26, p. 95), levelled off and the survival rate stabilized. This allowed statistically viable growth data to be collected from that point on. Before this stabilization point, growth data would have been confounded by the non-quantified (and non-quantifiable) pathogen effect, and differences could therefore not have been definitively attributed to growth stimulation per se.

Growth data, beginning two months after transplant, was analyzed using repeated measures ANOVA to discern the presence of treatment differences. The mean growth rate from each treatment was plotted on regression lines, which were then compared using a Z-test for slopes. Data collection for the experiment ended with the onset of dormancy in early November.

B. Results

1. Germination

Germination of *G. crinita* seedlings was significantly higher in the mycorrhizal germination medium (GM 1) than in the non-mycorrhizal germination medium (GM 2) for both 1995 and 1996 (Fig. 27, p. 96). In January 1995, 88% of the GM 1 seedlings emerged and survived the 3-month "incubation" period in the mist house prior to transplant, as opposed to 49% of those seedlings in the non-mycorrhizal GM 2. The

following year, in 1996, more *G. crinita* seeds from the same batch, which had been stored for one year at 3°C, were sown into the same two germination treatments in order to replicate and confirm the differences in germination. There was only a slight reduction in seed viability, but the results were similar: 75% of the GM 1 seedlings emerged and survived the 3-month incubation in the mist-house compared to only 23% of those from GM 2 (Figs. 28 and 29, pp. 97-98).

2. Survival and pathogen contamination

During the two-month period following the first transplant of *G. crinita* seedlings in April 1995, plants were dying off at a very high rate, and root and crown-rot pathogens were at least partly responsible for this high mortality (Fig. 30, p. 99). Microscopic examination of the roots and crowns of dead and dying plants from all transplant media during this time revealed the presence of root disease and crown rot in over 80% of the roots (Fig. 31, p. 100). Various soil-borne fungal pathogens were found and identified in 74% of diseased tissue (Table 7, p. 90, and Figs. 32-37, pp. 101-106). *Pythium* and *Rhizoctonia* were found in the majority of diseased roots in the first month after transplant, and *Rhizoctonia* continued to be a problem throughout the experiment (Figs. 32-34). *Alternaria* and *Fusarium* were also associated with mortality, though they were more numerous in diseased root tissue during the second and third month after transplant and appeared to be associated primarily with crown rot after that (Figs. 35 and 36). Also observed were the chlamydospores of an unidentified dematiaceous fungi (Fig. 37) which

was present in the roots at low levels during the early stages, but was more prevalent towards the end of the experiment and during the overwintering period, where it was associated with most of the overwintering losses (Fig. 39, p. 108).

The prevalence of these pathogens, including their presence in the roots of transplants from the control transplant media (commercial and sterilized, TM 12 and 13), suggests that the germination media (GM 1 and 2) was the source of contamination. This, coupled with the observed correlation between the presence of these pathogens and diseased tissue, implicate these pathogens as the main causal agent of transplant mortality of *G. crinita* during this experiment; in effect, these experiments became *de facto,* albeit non-quantifiable, pathogen suppression bioassays.

The high post-transplant mortality rate, conversely referred to as survival rate, began to stabilize in June, approximately two months after transplant (Fig. 26, p. 95). The second transplant (10/6/96, six months after the first transplant) did not appear to affect the survival rate, which remained essentially unchanged for the subsequent month, until the onset of dormancy in November. During the subsequent overwintering period (November to March), there was a decrease in the survival rate (Fig. 39, p.108). This was due to improper cultural conditions, such as excess warmth and humidity, in the greenhouse in which the plants overwintered. These conditions were conducive to disease development. The unidentified dematiaceous fungi mentioned earlier (Fig. 37) may have played a major role in overwintering mortality, as the chlamydospores of this fungus were found in the roots and crown of virtually every dying plant during this period.

Table 9 and Fig. 26 (pp. 91 and 95, respectively) compare the survival of transplanted gentian seedlings at monthly intervals throughout the 1995 growth and survival experiment, and are the only chart and table which include data from all three sets of transplant media (TM 1-14). From the period shortly after transplant to the final survey in November, just prior to dormancy, plants from the mycorrhizal germination medium (GM 1) achieved a higher survival rate than their non-mycorhhizal counterparts (GM 2). Especially significant is the decreased mortality rate in the first two months after transplant; this is reflected in the sharp divergence of the slopes of the two lines from 4/21 to 6/15 (Fig. 26). After 6/15, both lines level off, and the slopes are relatively equal from that point on, indicating that the disease pressure from root -infecting fungal pathogens began to wane by the third month after transplant.

The higher survival rate established soon after transplant translated into a greater percentage of surviving gentians at the end of the first growing season. Almost 25% of the mycorrhizally-germinated transplants from the original treatment set survived, as compared to only 6% of those from the non-mycorrhizal germination medium (Table 9). This was the time when these plants would normally have been outplanted, either into a cold frame, preservation garden or back into their native habitat as part of a reestablishment project. With about four times as many plants available from the mycorrhizal treatment set as from the non-mycorrhizal set, the chances are greatly increased that at least some of the fringed gentians would have survived the subsequent dormancy and overwintering period outside.

The transplant media used in experiment 3 (TM 10-14), referred to as "effective" media, supported a much higher survival rate overall than the media from experiments 1 and 2. Apparently, the field soil and mycorrhizal inoculum used in experiments 1 and 2 were contaminated with pathogens. Root-infecting pathogens are a common problem in mycorrhizal pot cultures started from field soil inoculum, and often cause disease and mortality in growth experiments. As a result, the focus turned to experiment 3 exclusively, which, due to this lower mortality rate, could be measured and surveyed for survival and growth in subsequent months. The results presented from this point on will be from experiment 3, TM 10-14.

Table 10 and Fig. 40 (pp. 91 and 109, respectively) include data from TM 10-14, spanning the first growing season from April to November. Once again, the survival rate was significantly greater in mycorrhizally-germinated plants, but the difference between the survival rates of the two germination treatment sets, as represented by the slope of the lines, is even greater in the TM 10-14 subset than it was when all transplant media were included. The difference between the slope of the lines in Fig. 40 (TM 10-14), 3.26, is much larger than the difference between the corresponding slopes seen in Fig. 26, p. 95 (all media) which is .96. Also, the slope of the lines in Figs. 26 and 40 diverge sharply in the two-month period after transplant, and then level off. In both cases, this resulted in a significantly higher number of plants from the mycorrhizally-germinated treatment for outplanting in November. Once again, the difference between the two germination treatments was much greater in TM 10-14 than it was when all transplant media were

included: 68.5% to 31.6%, a 37% difference, vs. 24.7% to 6.4%, an 18.3% difference (compare the bottom lines in Table 9 and Table 10).

If the survival data from TM 10-14 is broken down further, the influence of the mycorrhizal inoculum can be more finely discerned. Figs. 41 and 42 (pp. 110-111) represent the survival rate of transplants within each germination medium treatment set, differentiated by transplant medium . The two mycorrhizal transplant media, TM 14 and 10, supported a higher survival rate in both mycorrhizal and non-mycorrhizal germination treatment sets; when final survival was surveyed in November, TM 10 and 14 outperformed their non-mycorrhizal counterparts. This can be seen in Fig. 43 (p. 112), which is a bar chart showing the percentage of surviving plants just prior to dormancy from all ten treatment combinations (GM 1+2 x TM 10-14; 2 x 5 =10). This chart, as well as Table 11, p. 92 (the same data in tabular form) demonstrates that plants from GM 1 survived at a significantly higher rate than plants from GM 2 regardless of the medium into which they were transplanted. Among the set of plants germinated in the mycorrhizal germination medium, the transplant medium with the lowest survival percentage, TM 12 (50%), was still higher than the transplant media which supported the highest survival percentage (TM 10 and 14) among the non-mycorrhizally-germinated plants. It can be seen in the chart in Fig. 12 (p. 92) that the two mycorrhizal transplant media, TM 10 and 14, significantly increased survival of gentians from both mycorrhizal and non-mycorrhizal germination treatments.

If the means of the two survival percentages within each TM (one from each GM treatment set) are compared, a clearer picture emerges of the influence of transplant medium on final survival. Plants transplanted into the mycorrhizal transplant media (TM 10 and 14) emerged with much higher survival percentages than those transplanted into the non-mycorrhizal transplant media (TM 11-13). Fig. 44 (p. 113) and Table 12 illustrate this difference.

3. Growth

Fringed Gentian is a cool-weather species. The phenology of Fringed gentian , including the growth rate, is genetically controlled and dictated by decreasing day length and not air temperature. Most of its vegetative growth occurs in autumn, followed by either rosette formation or flowering in late September to mid-November. In these experiments, it followed the same growth pattern as well. The growth rate averaged approximately .5 cm/month from mid-April to mid-July, and then increased to slightly over 1 cm/month from mid-July to November.

The plants began to show signs of dormancy in early November (Fig. 30, p. 99), as indicated by several phenological events. Some of the lower foliage began to senesce, while the remaining foliage formed a rosette, as is typical of many biennials preparing to overwinter after their first season of growth. Also, a compact, thick-leaved apical bud structure began to form at the growing tip (Fig. 45, p. 114). At the same time, the stem and crown began to thicken, as did the tap root extension. Finally, leaf growth stopped.

Fig. 46 (p. 115) displays the cumulative differences in growth rate between the two germination treatment sets, GM 1 and GM 2, just prior to transplant in April 1995. This chart is presented to illustrate that the mycorrhizal germination medium had an effect on growth at the seedling stage as well as post-transplant. Seedlings from the mycorrhizal germination medium (GM 1) were larger than their non-mycorrhizal counterparts even at this early stage of growth (Fig. 28, p. 97). Once transplanted, the mycorrhizally-germinated plants continued to sustain a higher growth rate than plants from GM 2, as indicated by the slope of the lines in Fig. 47 (p. 116), which are significantly different from each other as per a Z-test for slopes (p=.05).

The next four graphs, Figs. 48-51 (pp. 117-120), represent the same growth data used to plot the lines in the previous graph (Fig. 47), but the data are further delineated. Each germination treatment set is represented by two graphs; each graph has five lines plotting growth in the five transplant media (TM 10-14). The blue and pink lines previously seen in Fig. 47 representsthe means of the five lines in Figs. 48 and 50, respectively. The first graph (Fig. 48) shows the growth of plants which were germinated in the mycorrhizal germination medium (GM 1) and transplanted to the five transplant media (TM 10-14). Fig. 49 is the same graph, but includes trendlines and their corresponding regression equations, which represent the growth rates of the various transplant media for purposes of comparison. Table 13 (p. 93) is a comparison of the slope of the growth lines in Fig. 49 using a Z-test for slopes. Of the two mycorrhizal transplant media (TM 10 and 14), only TM 10 supported a significantly higher growth rate

than the remaining three non-mycorrhizal media, TM 11-13; TM 14, though slightly higher, was not significantly so.

The second set of graphs (Figs. 50 and 51) contain the growth data of plants from the non-mycorrhizal germination medium (GM 2), which were transplanted into the same five transplant media (TM 10-14). Once again, trendlines and regression equations were included in the latter graph (Fig. 51) to compare the various growth rates; Table 14 (p. 93) compares the slopes of these trendlines. In this case, TM 10 and 14, as well as TM 13 (sterilized, non-mycorrhizal control) supported a significantly higher growth rate that the remaining two non-mycorrhizal media (TM 11 and 12).

Table 15 (p. 94) compares the growth rates of GM 1 plants vs. GM2 plants for each of the five transplant media. In three of five, there was a significant difference between mycorrhizally and non-mycorrhizally germinated plants. Furthermore, the overall growth rate, as represented by the mean of each of the two sets of plants, was significantly higher for plants from GM 1 than those from GM 2.

In addition to differences which were discernible through data collection and analysis, many of the differences were either not measurable or were seen in the second year of growth and therefore were not included in the growth data just presented. Figs. 52-55 (pp. 121-124) illustrate some of these differences. For example, plants from the mycorrhizal germination medium (GM 1) generally looked healthier and more vigorous than those from the non-mycorrhizal GM 2 (Fig. 52) throughout the course of the 1995 experiments. Fig. 53 shows examples of plants from each germination treatment set

emerging from dormancy in late February 1996; the mycorrhizal plant on the right reemerged from dormancy and "bolted" more quickly than its non-mycorrhizal counterpart of the left. After reemergence, the mycorrhizal plants continued to show stronger and more vigorous growth, as seen in Fig. 54, taken in May, 1996, which clearly illustrates this difference. Fig. 55 pictures a nearly full-grown Fringed gentian taken about one month later, with what appeared to be the beginnings of a flower bud forming at the growing tip. This plant went on to flower in October of 1996 in the greenhouse, about the same time as its counterpart in nature.

4. Mycorrhization

Mycorrhizal colonization levels were assessed by randomly sampling plant roots from the four mycorrhizally-inoculated media treatments, GM 1 + 2, TM 10 and 14. Differences in mycorrhizal colonization between these treatments was not discerned, and mycorrhization data represents estimated means from all four treatments.

Appressoria, which are pads from which fungal infection pegs develop, occur where the mycorrhizal fungus first penetrates the root endodermis. Appressoria were first observed in the roots of Fringed gentian from the four mycorrhizal treatments in July and August. However, these were not accompanied by any mycorrhizal colonization. In fact, there were no mycorrhizae observed during the entire course of the growth and survival experiments, from mid-April to early November, despite the apparent infectivity of the mycorrhizal treatment media. Infectivity was confirmed by sorghum pot cultures set up

with excess treatment media; the same process was used on non-mycorrhizal treatment media to confirm a lack of infectivity. The first appearance of mycorrhizae was in early November, concurrent with the onset of dormancy, beginning with low levels of colonization (1-2%), and reaching levels of 25-40% by late November and early December (timeline, Fig. 30). The beginning and peak of mycorrhizal colonization in gentian roots can be seen in Figs. 56 and 57, pp. 125 -126. In addition, it was observed that mycorrhizal roots often swelled to more than two times their normal diameter (Fig. 58, p. 127), from an average of 330 μm to more than 700 μm. In addition, roots of plants from the four mycorrhizal treatments sampled throughout the overwintering period, as well as those emerging from dormancy in late February and early March, 1996 had mycorrhizae as well, though the arbuscules at this point had a different appearance than those observed in the early dormancy period. They were difficult to distiguish in the cortical cells, and took on a dense, amorphous appearance, as opposed to the more finely discernible arbuscules seen in December, 1995.

5. Other root/fungus associations

Periodic plating of washed roots (both diseased and healthy) on culture media yielded *Pythium, Fusarium, Rhizoctonia, Alternaria, Trichoderma, Cladosporium, Mucor, Aspergillus, Penicillium, Geotrichum, Helminthosporium*, and other unidentified and assorted fungi, yeasts and bacteria, including so-called "black yeasts" (Summerbell,

1987). This data was qualitative only, but does indicate the presence of a wide range of fungal organisms associated with gentian roots.

Other unusual, apparently non-pathogenic root-inhabiting fungi were observed in Fringed gentian throughout the 1995 experiments (Table 8, p. 90, and Figs. 59-61, pp. 128-130). Unlike VA mycorrhizal fungi, these fungi colonized the roots and root surfaces throughout the active growing season, from May-October (timeline, Fig. 30, p. 99). They were found in the roots of healthy and diseased plants, but were never actually associated with diseased tissue, as the pathogenic fungi were. Most were endophytic, i.e., occupying the internal root tissue, but some, referred to in the literature as dark septate fungi, or DSF, were found on the surface of roots. Altogether, these non-mycorrhizal root-associated fungi were found in nearly 60% of the roots sampled during this period, and more than half of those had more than one type of fungus.

Nearly half of the root-inhabiting fungi were protoctistan fungi, with the majority resembling chytrids, or chytrid-like organisms. A small percentage of these were unidentified oomycetes characterized by fine, hyaline, aseptate mycelia (the latter may have been pathogenic in some cases; see Fig. 32, p. 101). The chytrid-like fungi had a holocarpic, endobiotic morphology, characterized by a lack of hyphae and one or multiple thalli occupying individual root cortical cells. These were observed in roots sampled throughout the experiment, and were often seen occupying a significant portion of the root tissue (up to 25%).

The remainder consisted of dematiaceous septate endophytes, but these were observed largely in the latter half of the growing season (July - October). For example, a *Rhizoctonia*-like fungus which formed pseudo-parenchymatous or sclerotia-like structures within the cortical cells was observed colonizing the roots of plants sampled in July and August of 1995 (Fig. 59). These were seen in healthy root tissue as well, unaccompanied by any rot or necrosis, suggesting a non-pathogenic or neutral relationship. They resembled the description of dematiaceous endophytic fungi seen by Neumann (1934), who identified them as *Mycelium radicis atrovirens* Melin. These and other similar fungi have been described in the literature as dark-septate endophytes (DSE) and dark septate fungi (DSF), found inside and outside the roots, respectively. This group of fungi were eventually identified and re-assigned the new species names *P. dimorphospora* by Richard and Fortin (1973) and *Phialocephala fortinii* by Wang and Wilcox (1985). Attempts at isolating these fungi from the roots were unsuccessful. These fungi, as well as a similar grey sterile fungus with dark, septate hyphae (Fig. 60), have been observed by other researchers in the roots of gentians and other plant species both in cultivation (Neumann, 1934) and from the habitats noted above (Read and Hasselwandter, 1981; Currah and Van Dyck, 1986; Stoyke and Currah, 1990).

Table 6. Treatment media used in 1995 growth and survival experiments.
All treatment media (except TM 12) consisted of a base medium (1:1:1:1 v/v compost/sand/vermiculite/Fafard II™) mixed 7:1 (v/v) with the listed component. This 1/8th component comprised the only difference between the treatment media. All media were tested and found to be identical in nutrient composition, except TM 12, which had a higher level of nutrients.

A. **Germination media** (GM)
1. Base medium with *Glomus etunicatum* (INVAM UT-316) inoculum (mycorrhizal)
2. Base medium without mycorrhizal inoculum (non-mycorrhizal)
B. **Transplant media** (TM)
<u>Experiment 1 - Field site soils</u>
1. Women's Federated State Forest - New Salem (wet meadow)
2. Mt. Hope Park - South Williamstown (calcareous fen)
3. Field Farm - Williamstown (wet meadow)
4. Powerline - Cheshire (calcareous fen)
5. North Common Meadow - Petersham (wet meadow)
<u>Experiment 2 - Indigenous pot and isolate cultures</u>
6. Field Farm pot culture (started with soil from site #3)
7. Powerline pot culture (started with soil from site # 4)
8. Field Farm isolate culture - *Glomus geosporum* (cultured by INVAM - MA-103)
9. Powerline isolate culture - *Glomus etunicatum* (cultured by INVAM - MA-104)
<u>Experiment 3 - Non-indigenous isolate cultures</u> ("effective media")
10. *Glomus etunicatum* (INVAM UT-316) isolate culture - same as GM 1 above
11. Base medium (referred to as "non-mycorrhizal gentian medium" - same as GM2 above)
12. Commercial growing mix (Fafard II™)
13. Sterilized non-mycorrhizal gentian medium
14. Mycorrhizal gentian medium (same as GM1 and TM10, but underwent stratification)

Table 7. Incidence of pathogenic fungi in roots or crowns of diseased *G. crinita* from the 1995 growth and survival experiments.

Pathogenic fungi (associated with diseased tissue)	**Diseased tissue***	**% of samples with pathogen**
*Pythium***	root	39
Rhizoctonia spp.	root and root surface	28
Unidentified dematiaceous fungi	root and crown	24
Fusarium spp.	root and crown	16
Alternaria spp.	crown	13
More than one pathogen	root	40
Unidentified or absent	root and crown	26

*81% of tissue samples from dying plants had diseased root or crown tissue.
Samples were taken from all transplant media (TM 1-14).
**Originally identified as *P. irregulare*, a common contaminant of greenhouse media.

Table 8. Incidence of non-pathogenic fungi in roots of healthy and diseased *G. crinita* from the 1995 growth and survival experiments.

Non-pathogenic fungi (associated with healthy tissue)	**Tissue**	**% of samples with fungi**
Chytrids	root	36
Unidentified protoctistan fungi	root	9
DSE*	root	13
DSF**	root surface	31
More than one fungi	root and root surface	53

Note: 58% of tissue samples from all plants had non-pathogenic root-associated fungi.
Samples were taken from all transplant media (TM 1-14).
*Probably *Phialocephala fortinii* Wang and Wilcox.
**Probably non-pathogenic *Rhizoctonia* or *Phialocephala dimorphospora* Richard&Fortin (formerly *Mycelium radicis atrovirens* Melin).

Table 9. Influence of germination medium on survival of all transplanted *G. crinita* (TM 1-14) from the 1995 experiments. Data represents survival percentages and corresponds to data in Fig. 26, p. 95.

	Germination medium		
Date	mycorrhizal	non-mycorrhizal	Z-test*
4/21/95	100	100	0
5/18/95	72	60.5	3.3
6/15/95	45.9	22.8	5.6
7/13/95	41.7	16.4	7.4
8/10/95	29.5	9.5	6.1
9/7/95	26.1	8.1	5.6
10/5/95	25.2	6.9	5.6
11/2/95	24.7	6.4	5.8

Table 10. Influence of germination medium on survival of *G. crinita* in "effective" transplant media (TM 10-14) from the 1995 experiments. Data represents survival percentages and corresponds to data in Fig. 40, p. 109.

	Germination medium		
Date	mycorrhizal	non-mycorrhizal	Z-test*
4/21/95	100	100	0
5/18/95	94.8	60.8	6.4
6/15/95	89.9	45.8	7.3
7/13/95	81.4	41.7	6.4
8/10/95	78.8	38.3	6.4
9/7/95	74.0	35.8	6.0
10/5/95	73.2	33.3	6.2
11/2/95	68.5	31.6	5.7

*All Z-test scores (except 0) indicate a significant difference between mycorrhizal and non-mycorrhizal germination media (p=.01).

Table 11. Influence of germination medium and transplant medium (TM 10-14) on survival of *G. crinita*. Data represents survival percentages as of 11/2/95, after the first season of growth, and corresponds to data in Fig. 43, p. 112.

	Germination medium		
Transplant medium	mycorrhizal	non-mycorrhizal	Z-test
14	95.8	45.8	4.23**
10	87.5	45.8	3.06**
13	54.2	25	2.08*
12	50	29.1	1.65*
11	58.3	12.5	3.32**

10 - gentian medium (M)† 11 - gentian medium (NM)
14 - germination medium (M) 12 - sterilized gentian medium (NM)
13 - commercial mix (NM)

†M = mycorrhizal, NM = non-mycorrhizal
*p=.05 **p=.01

Table 12. Influence of transplant medium (TM 10-14) on survival of *G. crinita*. Data represents mean survival percentages of plants from both mycorrhizal and non-mycorrhizal germination media as of 11/2/95, after the first season of growth. Data corresponds to data in Fig. 44, p. 113.

Transplant medium	Mean survival (%)	Duncan's grouping*
14 (M)	72.9	A
10 (M)	66.6	A
13 (NM)	39.6	B
12 (NM)	39.6	B
11 (NM)	35.4	B

10 - gentian medium (M)† 11 - gentian medium (NM)
14 - germination medium (M) 12 - sterilized gentian medium (NM)
13 - commercial mix (NM)

†M = mycorrhizal, NM = non-mycorrhizal
*Means with same letter not significantly different (p=.05).

Table 13. Growth of *G. crinita* transplanted into TM 10-14 from mycorrhizal germination medium (GM 1). Data is from 1995 experiments, and corresponds to data in Figs. 48 and 49, pp. 117 and 118. Slopes represent relative growth rates from two to seven months after transplant.

Transplant medium	Slope	Z-test grouping*
10 (M)	1.087	A
14 (M)	.87	A
13 (NM)	.84	B
11 (NM)	.83	B
12 (NM)	.80	B

10 - gentian medium (M)†
14 - germination medium (M)
13 - commercial mix (NM)
11 - gentian medium (NM)
12 - sterilized gentian medium (NM)

† M = mycorrhizal, NM= non-mycorrhizal.
* Z-test for slopes. Means with the same letter are not significantly different (p =.05).

Table 14. Growth of *G. crinita* transplanted into TM 10-14 from non-mycorrhizal germination medium (GM 2). Data is from 1995 experiments, and corresponds to data in Figs. 50 and 51, pp. 119 and 120. Slopes represent relative growth rates from two to seven months after transplant.

Transplant medium	Slope	Z-test grouping*
14 (M)	.88	A
10 (M)	.87	A
13 (NM)	.85	A
11 (NM)	.71	B
12 (NM)	.68	B

10 - gentian medium (M)†
14 - germination medium (M)
13 - commercial mix (NM)
11 - gentian medium (NM)
12 - sterilized gentian medium (NM)

† M = mycorrhizal, NM = non-mycorrhizal.
* Z-test for slopes. Means with the same letter are not significantly different (p =.05).

Table 15. Comparison of growth of *G. crinita* from mycorrhizal and non-mycorrhizal germination media in the "effective" transplant media (TM 10-14). Data is from the 1995 experiments. Slopes represent relative growth rates from two to seven months after transplant.

	Germination medium		
Transplant medium	mycorrhizal	non-mycorrhizal	Z-test
10	1.09	0.88	3.28*
14	0.87	0.87	---
13	0.84	0.85	---
11	0.83	0.71	1.72
12	0.80	0.68	1.88
mean	0.86	0.78	1.97**

10 - gentian medium (M)†
14 - germination medium (M)
13 - commercial mix (NM)
11 - gentian medium (NM)
12 - sterilized gentian medium (NM)

†M = mycorrhizal, NM = non-mycorrhizal.
*p=.01 **p=.05

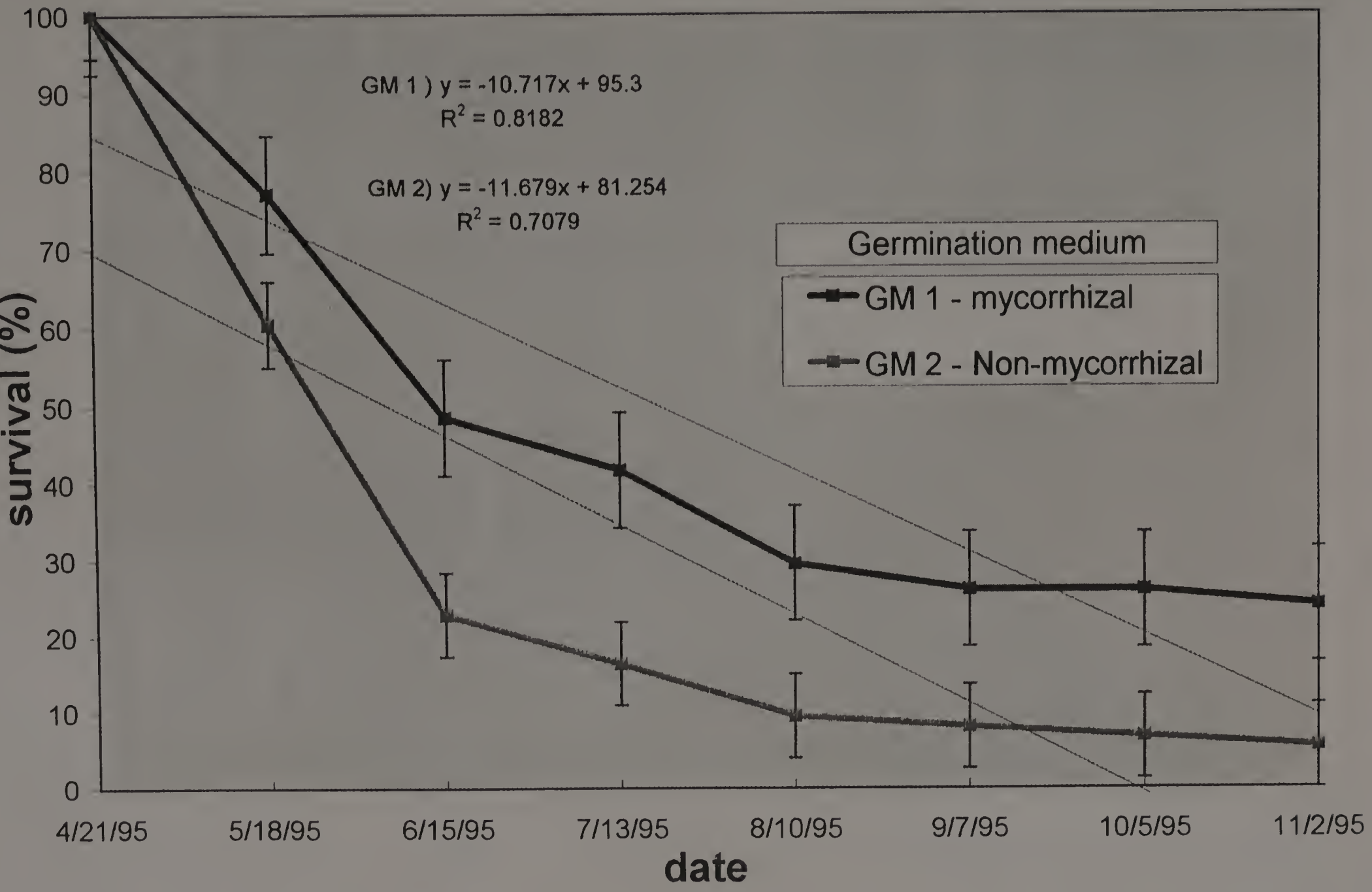


Figure 26. Influence of germination medium on survival of *G. crinita*. Data represents mean survival percentages of plants from all transplant media (TM 1-14). Trendlines and equations are included for purpose of comparing slope of lines, which are significantly different as per Z-test (p =.05). Bars equal one standard error. Plants were transplanted on 4/20/95, at three months old, and again on 10/6/95.

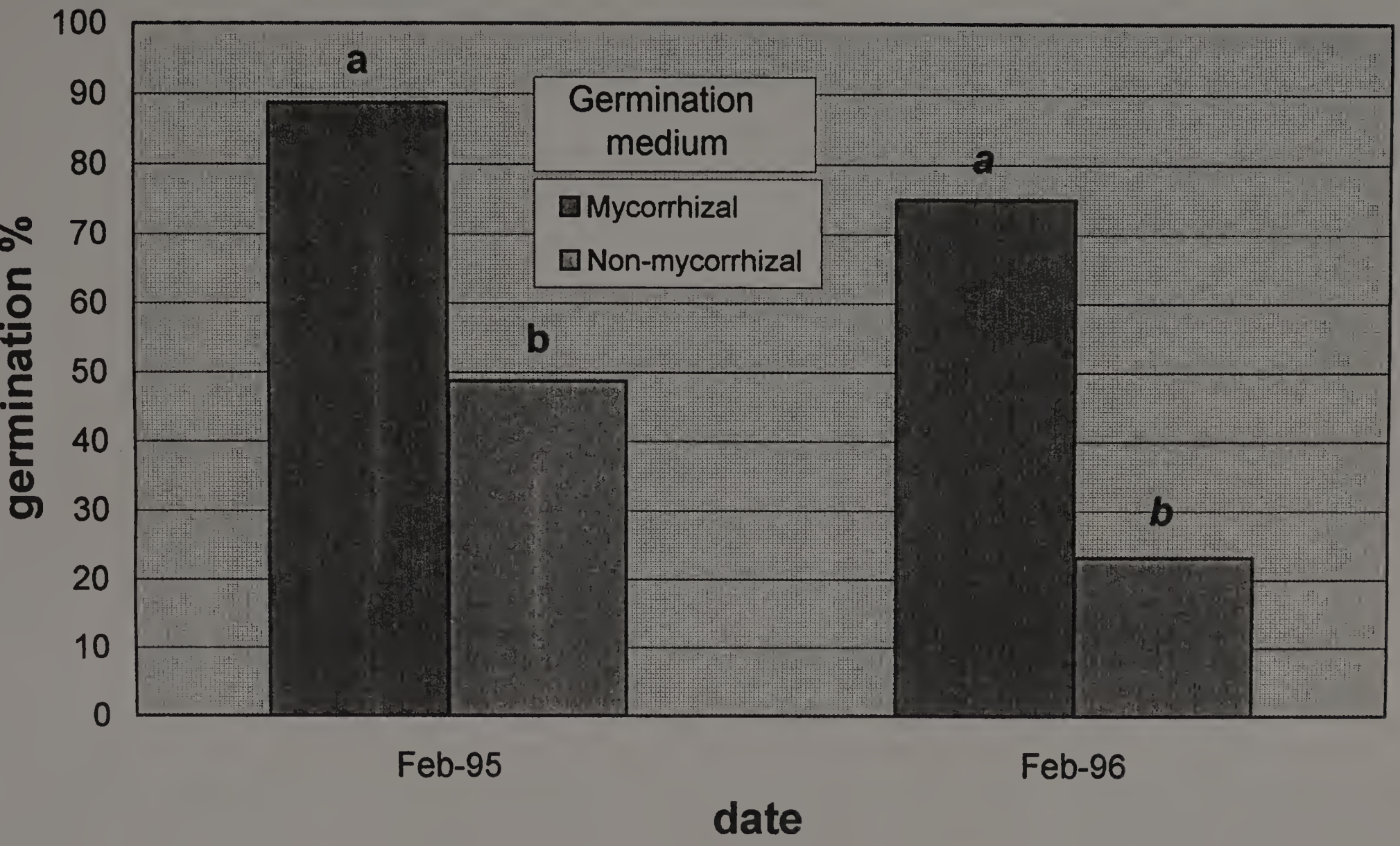


Figure 27. Influence of mycorrhizal inoculum on germination of *G. crinita.* Tests were done for two years. Differences are as per Z-test (p=.01).

Figure 28. *G. crinita* seedlings approximately eleven weeks after germination, March, 1995. Note higher number of germinated seedlings in the mycorrhizal germination medium on the right.

Figure 29. *G. crinita* seedlings approximately eleven weeks after germination, March, 1996. Note higher number of germinated seedlings in the mycorrhizal germination medium (top right).

10/1/94	12/1/94	1/1/95	**4/21/95****	**5/18/95**	**6/15/95**	**7/13/95**	**8/10/95**	**9/7/95**	**10/5/95**	**11/2/95**	12/1/95	1/1/96	2/1/ - 3/15/96
Phenology													
G. crinita seeds sown (GM 1 & 2) and stratified													
Seed flats removed from cooler, placed in mist house													
Seeds germinate, seedlings emerge and grow 3.5 months in mist house													
Seedlings transplanted to various transplant media (TM 1-14)													
Very slow vegetative growth	Gradually increasing growth rate												
Monthly measurements, sampling of roots from non-test replicates.													
2nd transplant													
Onset of dormancy -apical bud forms,etc.													
Dormancy													
Re-emergence													
Disease development													
Seedlings transplanted to various transplant media (TM 1-14)													
High transplant mortality, various pathogens found in roots of dead and dying transplants													
TM 1-9 die off (97% mortality) due to *Pythium, Rhizoctonia* and other pathogens													
Soil-borne fungal pathogens found associated with diseased roots and crown rot													
Pythium and *Rhizoctonia* prominent in diseased roots (some *Fusarium*)													
Non-pathogenic chytrids, and other protoctistan fungi observed													
Fusarium and UDF+ in diseased roots, these and *Alternaria* also associated with crown rot.													
Mortality rate decreases, survival increases for "effective" media only (TM 10-14)													
Unidentified dematiaceous fungi associated with root + crown rot during overwintering													
Fusarium sp. isolated from diseased roots, none on healthy roots													
Pythium sp. isolated from germination medium (GM1 and 2)													
Non-pathogenic DSF and DSE observed in healthy roots													
Mycorrhizal formation													
Viable VAMF spores found in monthly samples of mycorrhizal treatment media													
External hyphae and appressoria from VAMF observed													
No mycorrhizal root colonization observed													
Increasing mycorrhizal root colonization (to 40%)													
Colonized roots thicken (2x normal diameter)													
Arbuscular disintegration													

Figure 30. Timeline of 1995 growth and survival experiments. Gray areas denote period during which listed event occurred. Dates in bold text indicate experimental period.

Figure 31. Diseased *G. crinita* root (unstained) sampled June, 1995. This is typical of what was observed in the dead and dying plants from the 1995 growth and survival experiments. No identifiable pathogen was found in this root.

Figure 32. Fungal root pathogens invading a damaged *G. crinita* root (stained) sampled May, 1995. The darker, thicker fungal hyphae (along the top and center of root) was identified as *Rhizoctonia*, plasmolyzed by the clearing and staining procedure. The thinner, lighter mycelium belongs to an unidentified fungi, possibly an oomycete.

Figure 33. *Pythium* oospores in a diseased *G. crinita* root (unstained), sampled May, 1995.

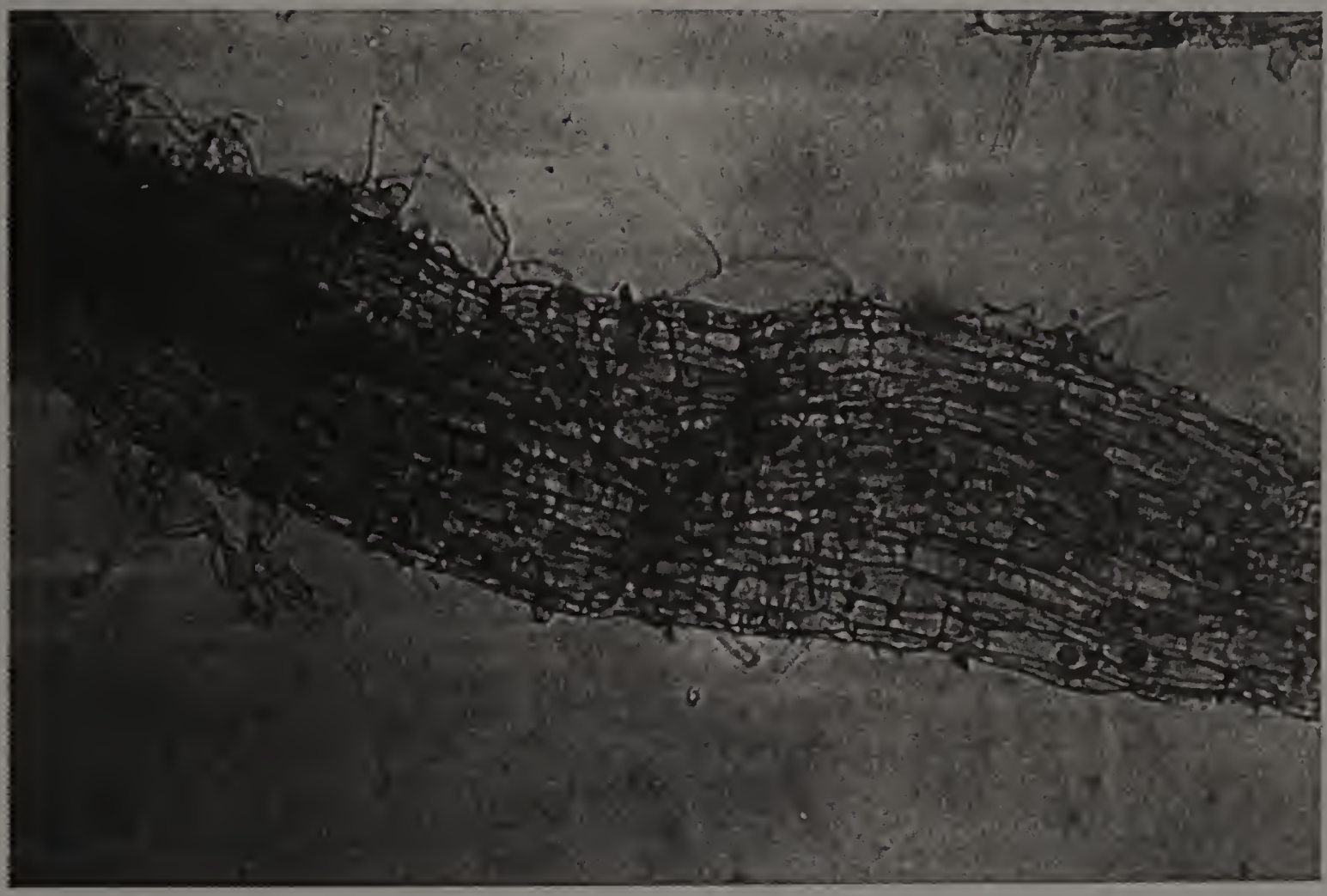


Figure 34. *Pythium* oospores in the root of *G. crinita*. This plant was sampled in May, 1995, shortly after it had died. Note the presence of fungal hyphae, made visible by staining.

Figure 35. Conidia of *Fusarium* from diseased root tissue of *G. crinita* (unstained), collected June, 1995. Conidia can be seen in the top right portion of the picture.

Figure 36. Conidia of *Alternaria* in diseased crown tissue of *G. crinita*, collected June, 1995.

Figure 37. Chlamydospores of an unidentified dematiaceous fungus from the roots of *G. crinita* shortly after it had died in November, 1995. This pathogen was first observed shortly after transplant (May) and did not become prevalent until late in the season, where it was found in the roots and crowns of almost every diseased plant. Circumstantial evidence suggests that it may have been responsible for significant losses during the overwintering period.

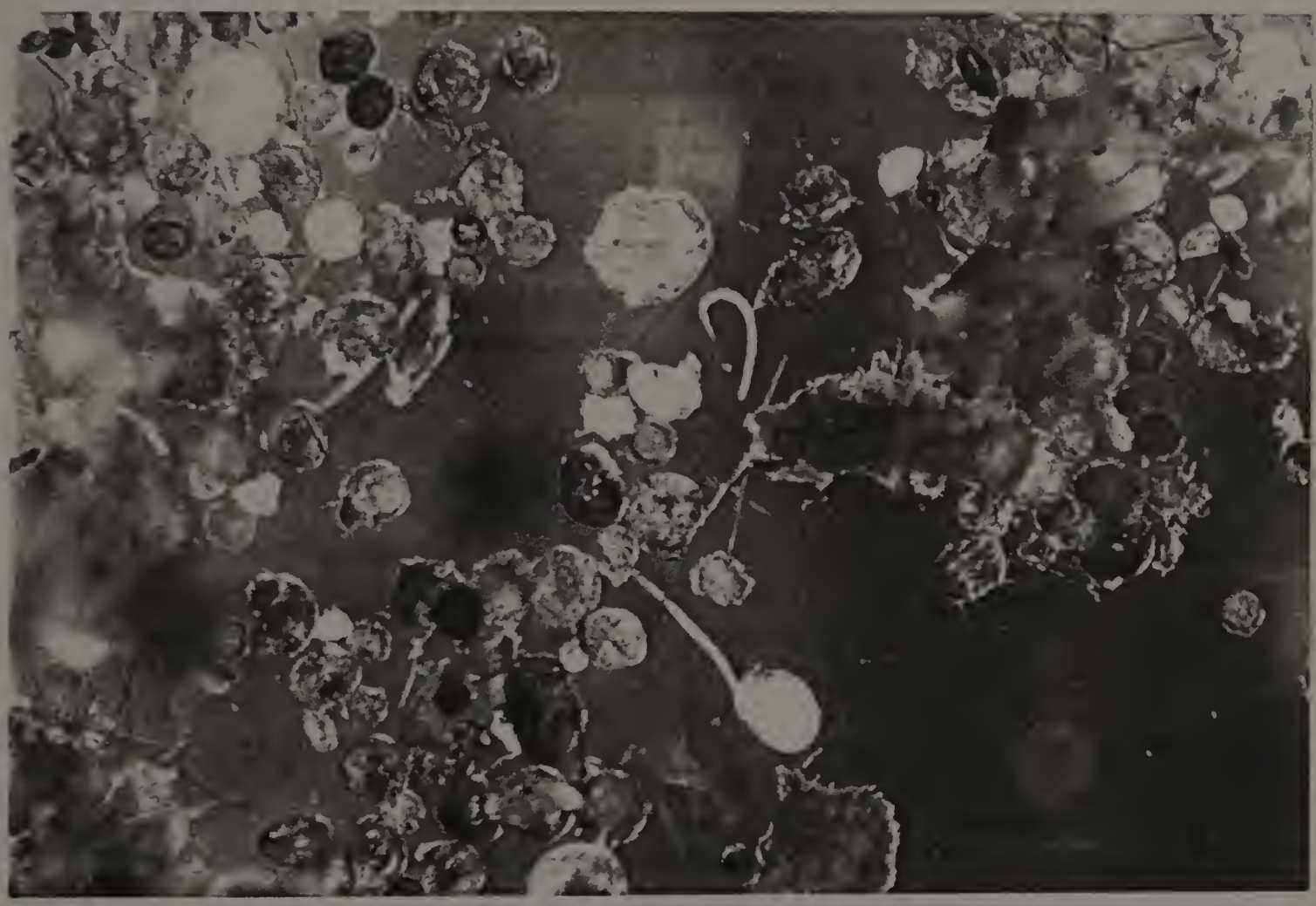


Figure 38. Close-up of VAMF spores from site 5, North Common Meadow, Petersham, MA, showing nematode eggs and nematodes (center). Nematodes were found in all of the indigenous pot cultures, and subsequently contaminated the growing media into which these cultures were incorporated. The "comma-shaped" nematodes may be *Paratylenchus,* though it is unlikely that they were pathogenic in greenhouse culture.

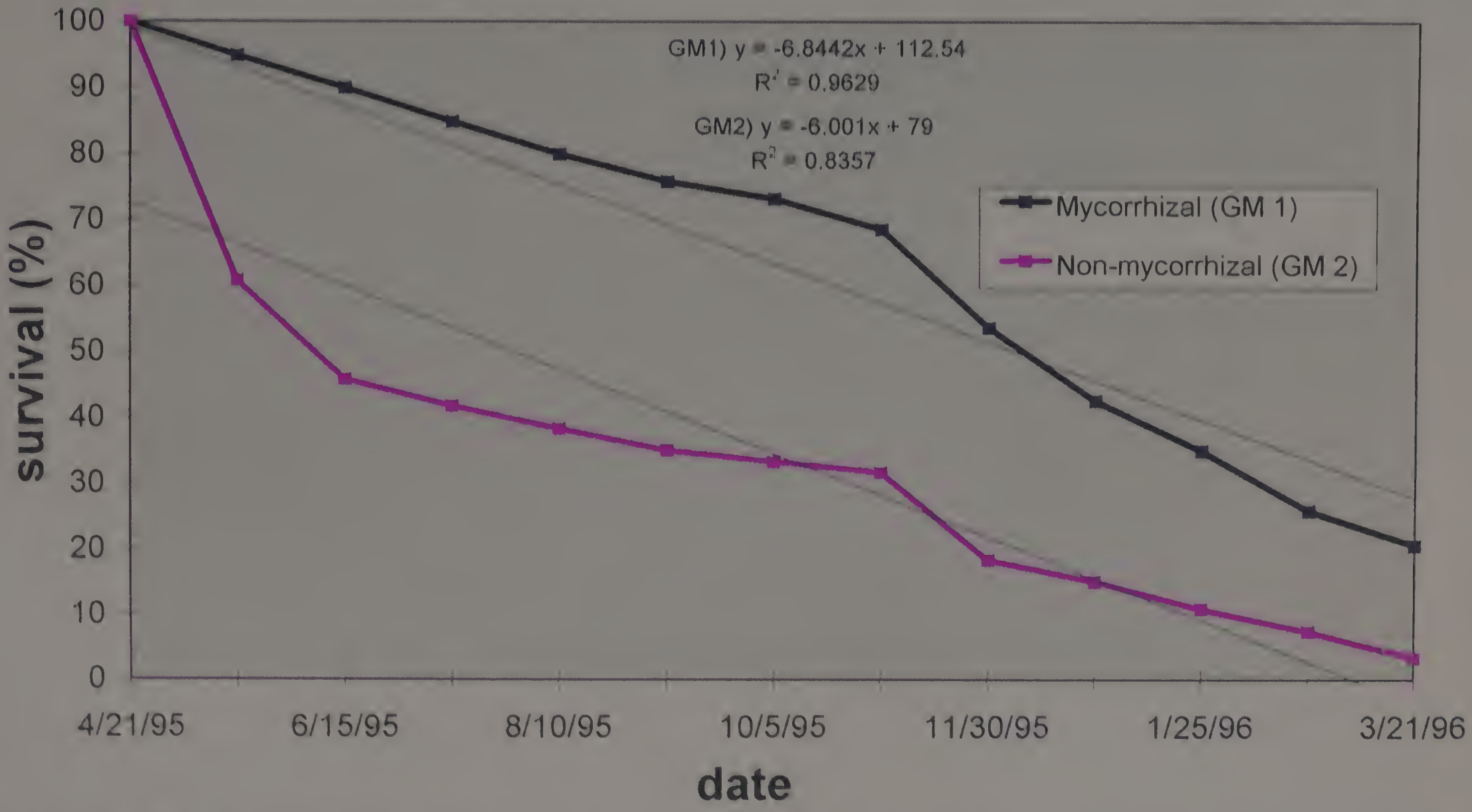


Figure 39. Influence of germination medium on survival of *G. crinita* through overwintering period. Data represents mean survival rate of plants from TM 10-14, from initial transplant to the end of dormancy. Trendlines and equations are included for purposes of comparing slope of lines, which are significantly different as per Z-test (p =.05). Plants were transplanted 4/20/95, and again on 10/6/95.

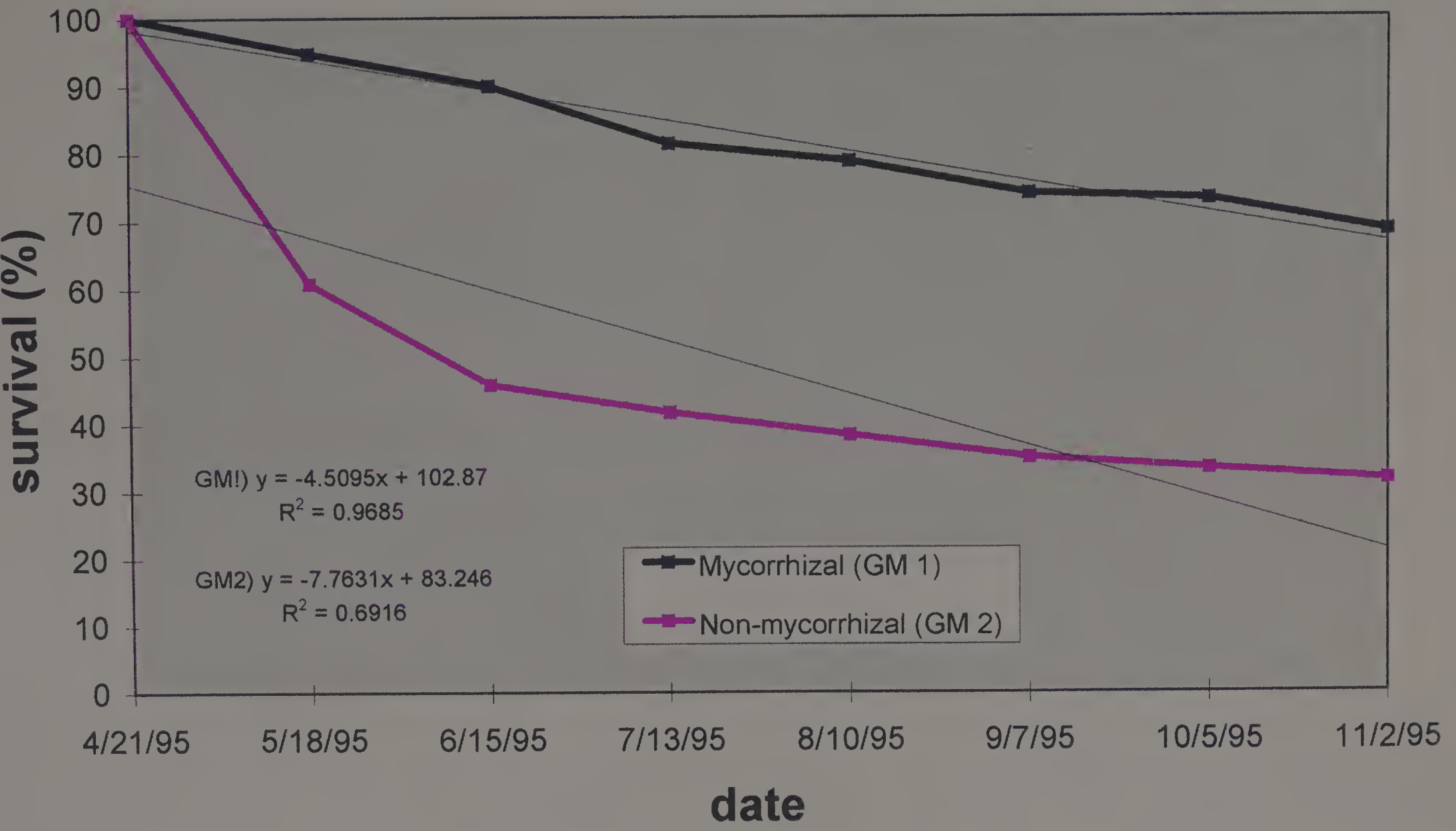


Figure 40. Influence of germination medium on survival of *G. crinita* throughout first-year growing season. Data represents mean survival rate of plants from TM 10-14. Trendlines and equations are included for purposes of comparing slope of lines, which are significantly different as per Z-test (p =.05). Plants were transplanted 4/20/95, and again on 10/6/95.

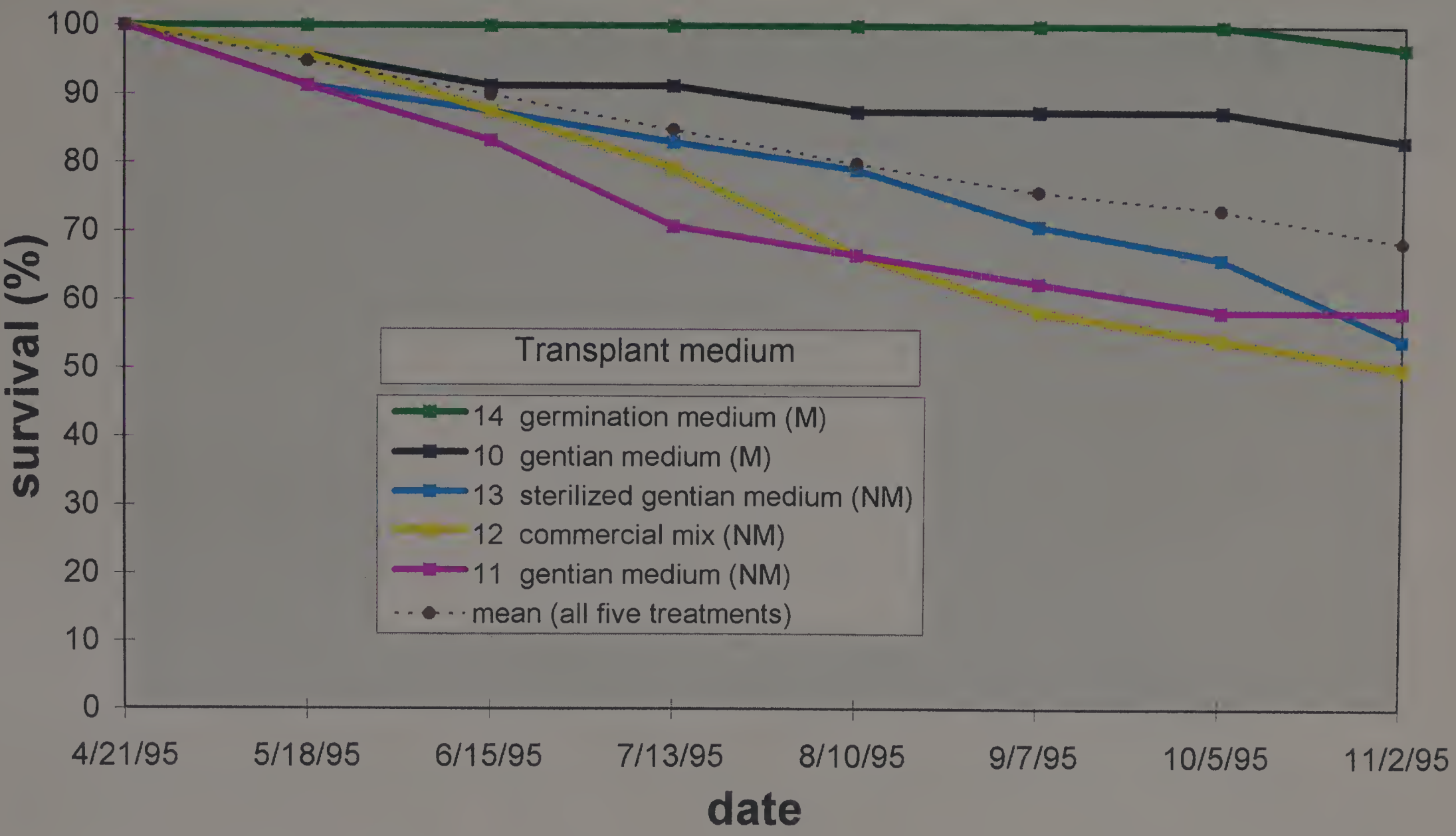


Figure 41. Influence of transplant media (TM 10-14) on survival of *G. crinita* from mycorrhizal germination medium (GM 1). Each solid color line represents one of the five transplant media. The top two lines are mycorrhizal media; the bottom three lines are non-mycorrhizal media. The brown dotted line in the center is the mean of all five lines, and corresponds to the thick black line in Fig. 40, p. 109. First date shown is date of first transplant.

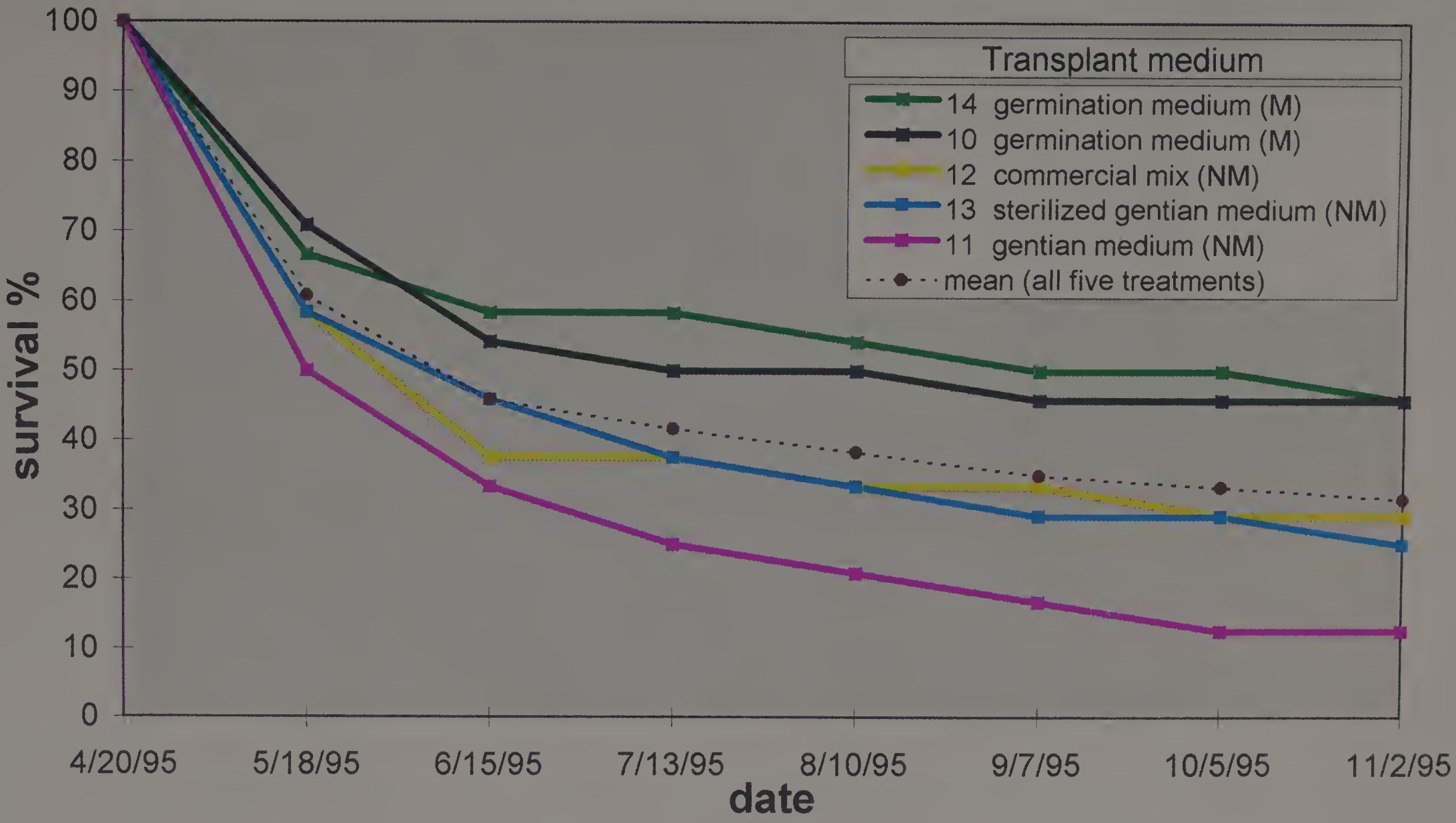


Figure 42. Influence of transplant media (TM 10-14) on survival of *G. crinita* from non-mycorrhizal germination medium (GM 2). Each solid color line represents one of the five transplant media. The top two lines are mycorrhizal media; the bottom three lines are non-mycorrhizal media. The brown dotted line in the center is the mean of all five lines, and corresponds to the thick pink line in Fig. 40, p. 109. First date shown is date of first transplant.

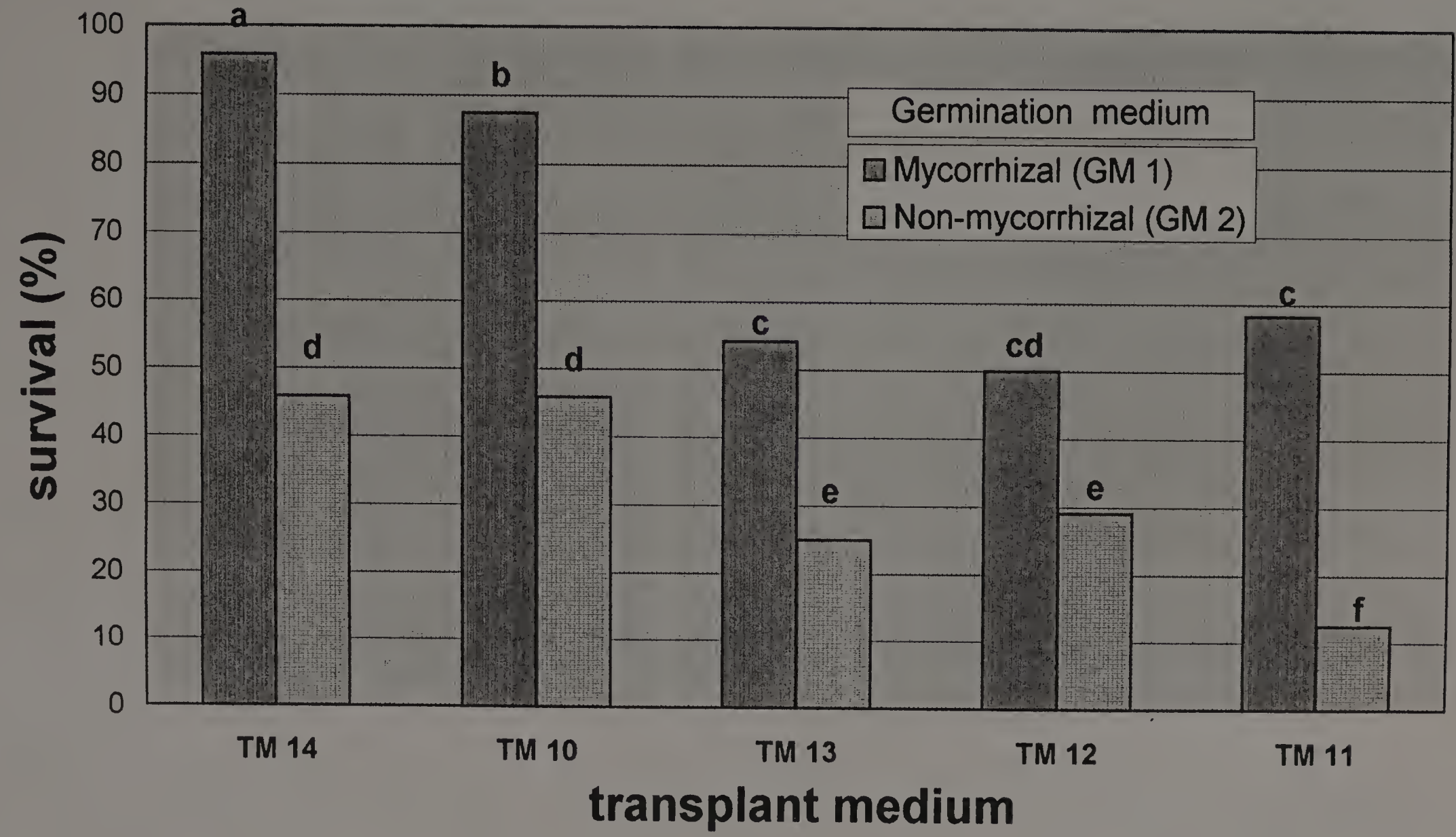


Figure 43. Survival of *G. crinita* from all ten treatments. Data is survival on 11/2/95, and corresponds to data in Table 11, p. 92. Bars with same letter not significantly different, as per Duncan's (p =.05).

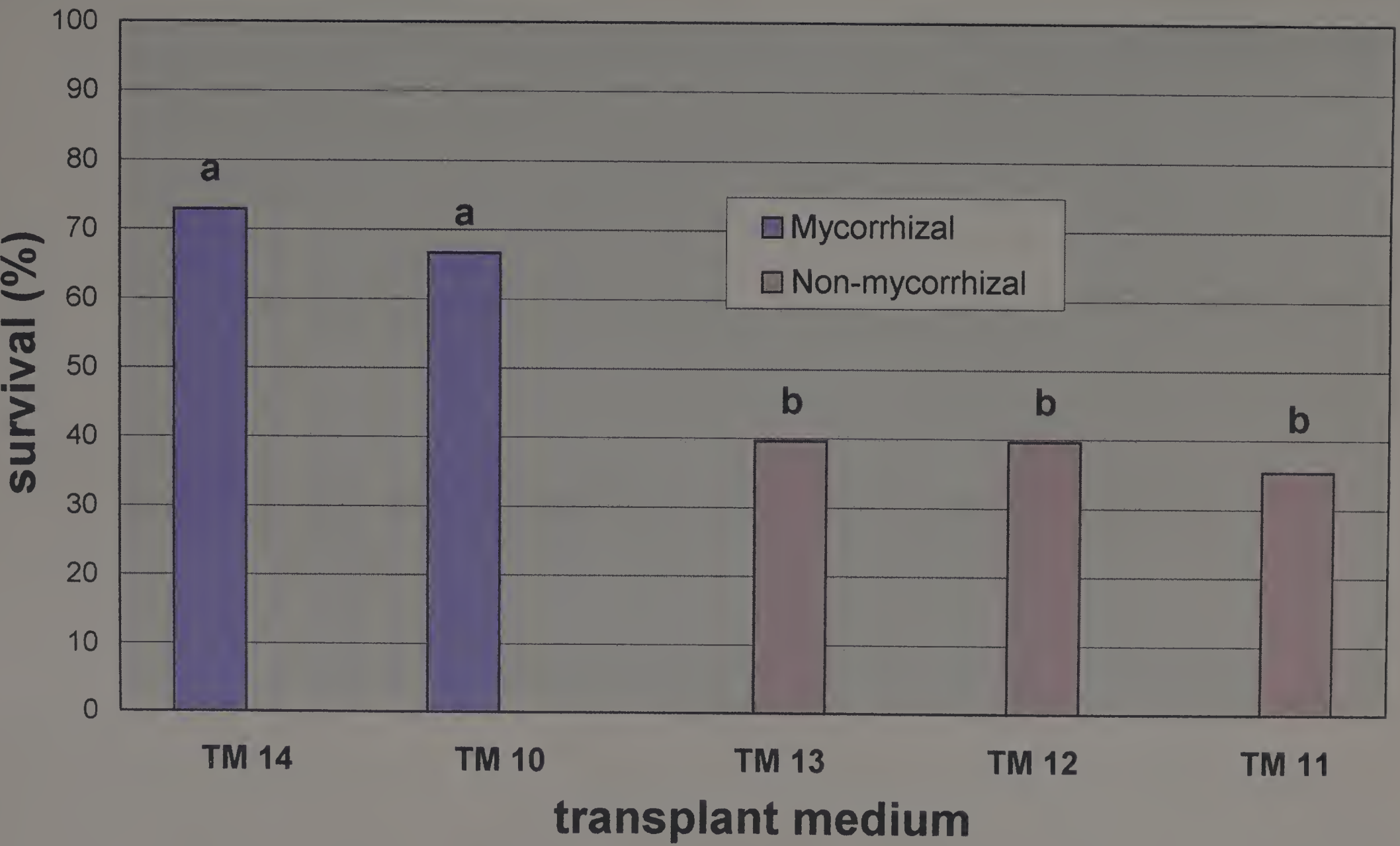


Figure 44. Influence of transplant media on survival of *G. crinita*. Data is mean survival percentage of plants from mycorrhizal and non-mycorrhizal germination media as of 11/2/95. Data corresponds to data in Table 12, p.92. Means with same letter not significantly different as per Duncan's MRT (p =.05).

Figure 45. *Gentianopsis crinita* in early November during onset of dormancy. Note the rosette formation of the leaves and the thick-leaved bud at the apical meristem.

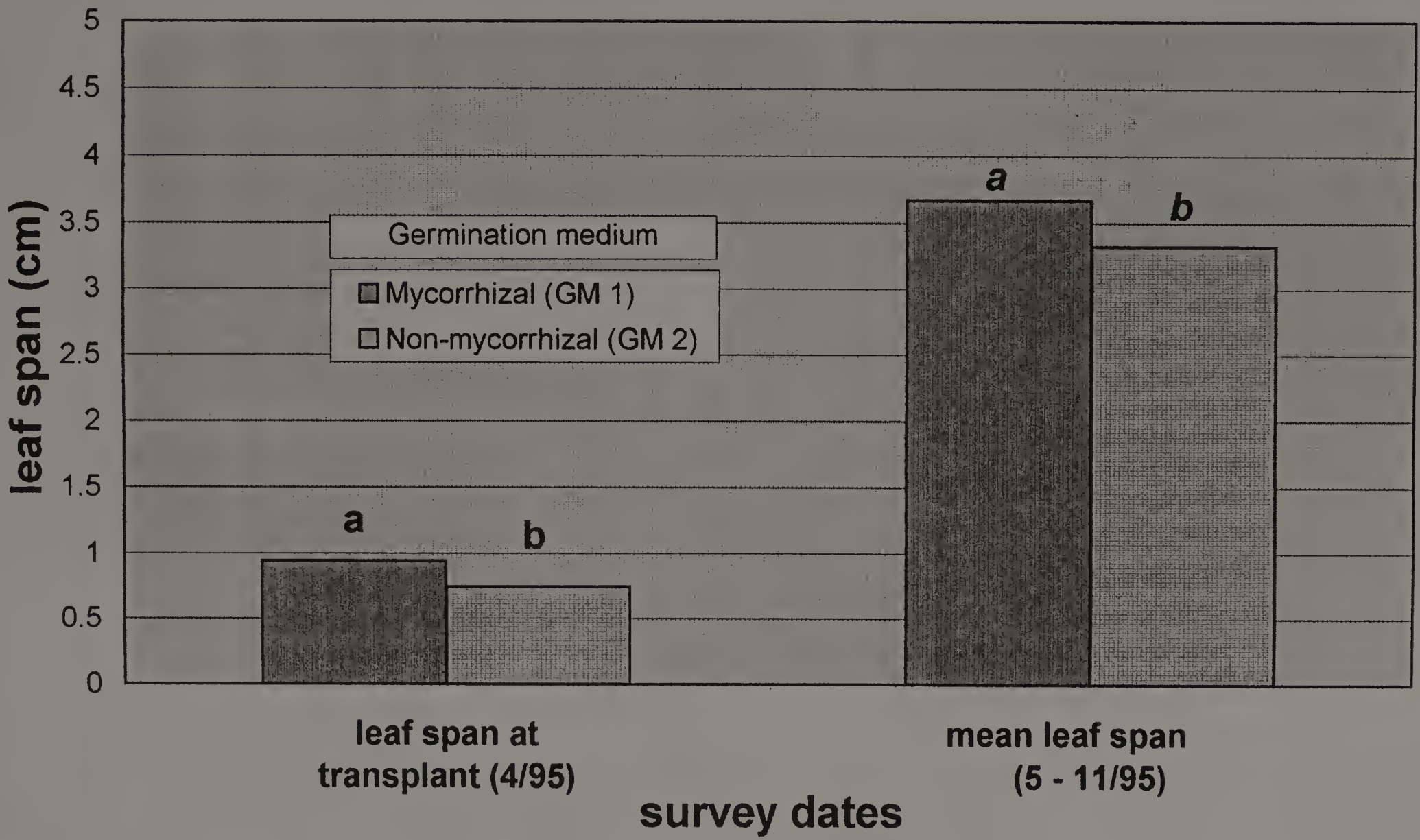


Figure 46. Influence of germination medium on size of *G. crinita,* as measured by leaf span. Bars on right are mean size over six months. Means are significantly different as per t-test (p =.05).

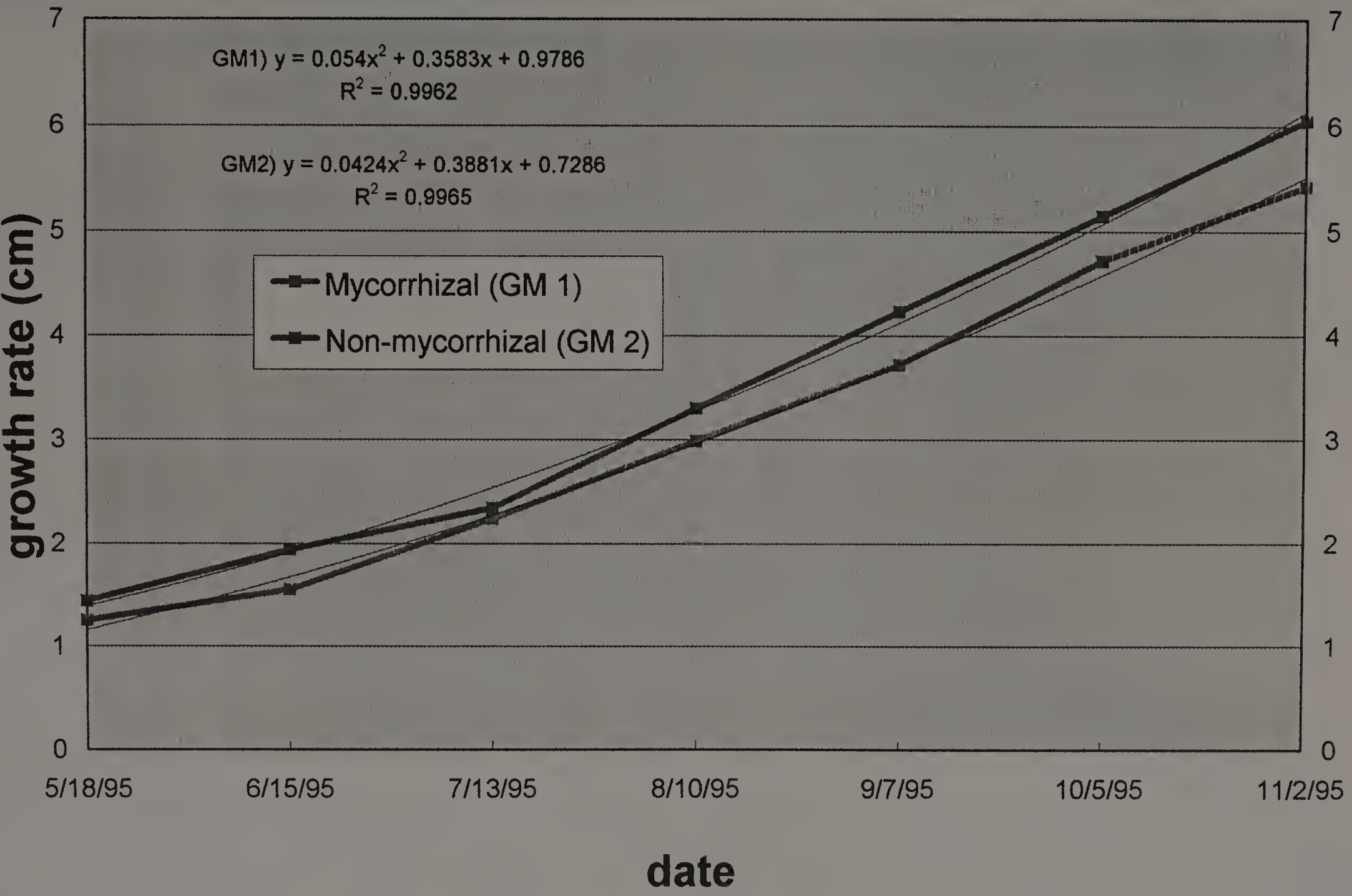


Figure 47. Influence of germination medium on growth of *G. crinita* . Slopes = relative growth rates, and are significantly different as per Z-test (p =.05). First date is one month after transplant.

Figure 48. Influence of transplant media on growth of *G. crinita* from mycorrhizal germination medium (GM 1). First date is 2 months after transplant, when survival rate stabilized after a steep decline.

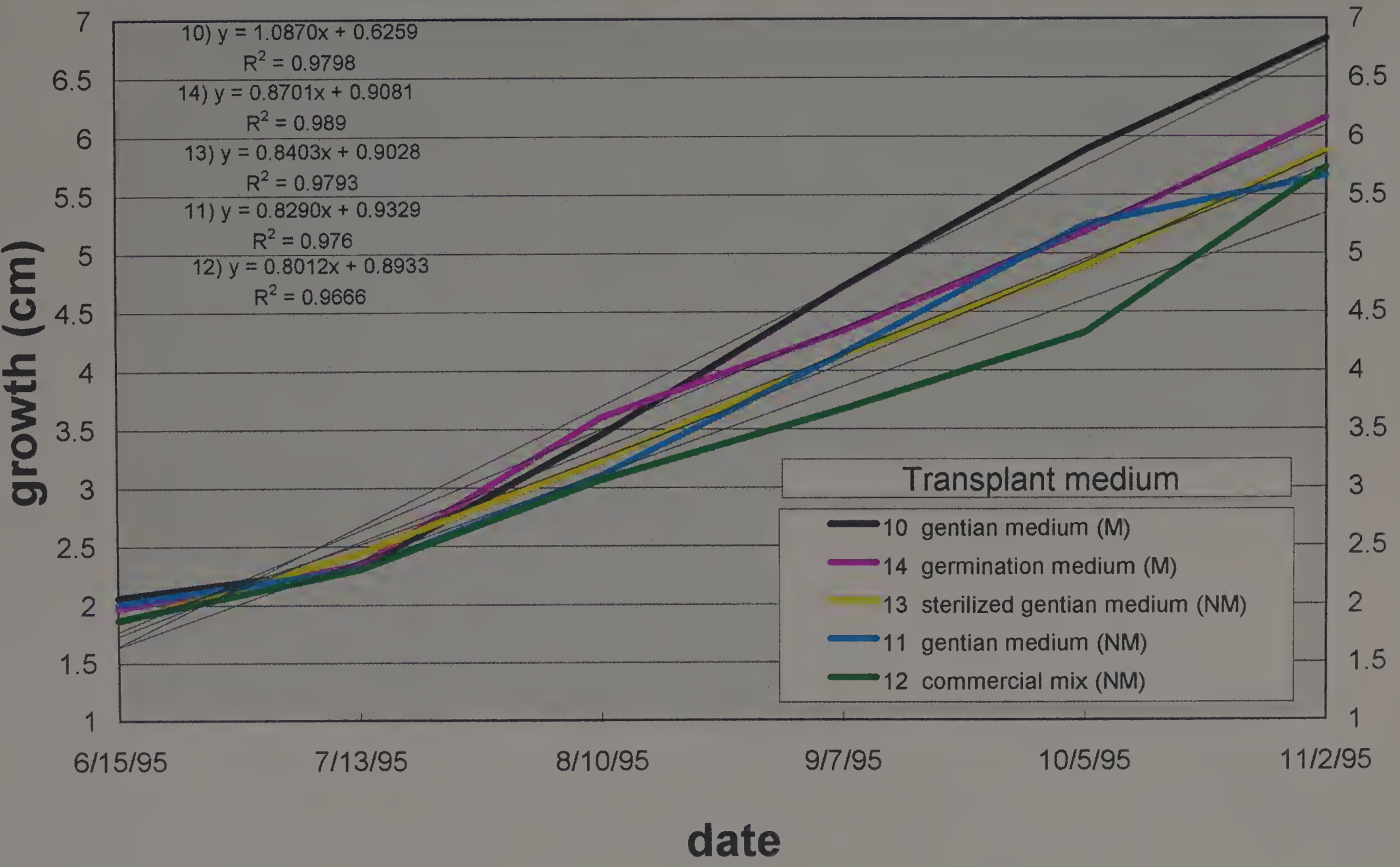


Figure 49. Influence of transplant media on growth of *G. crinita* from mycorrhizal germination medium (GM 1), with trendlines. First date is two months after transplant, point at which survival rate stabilized following a steep decline. Data corresponds to data in Table 13, p. 93.

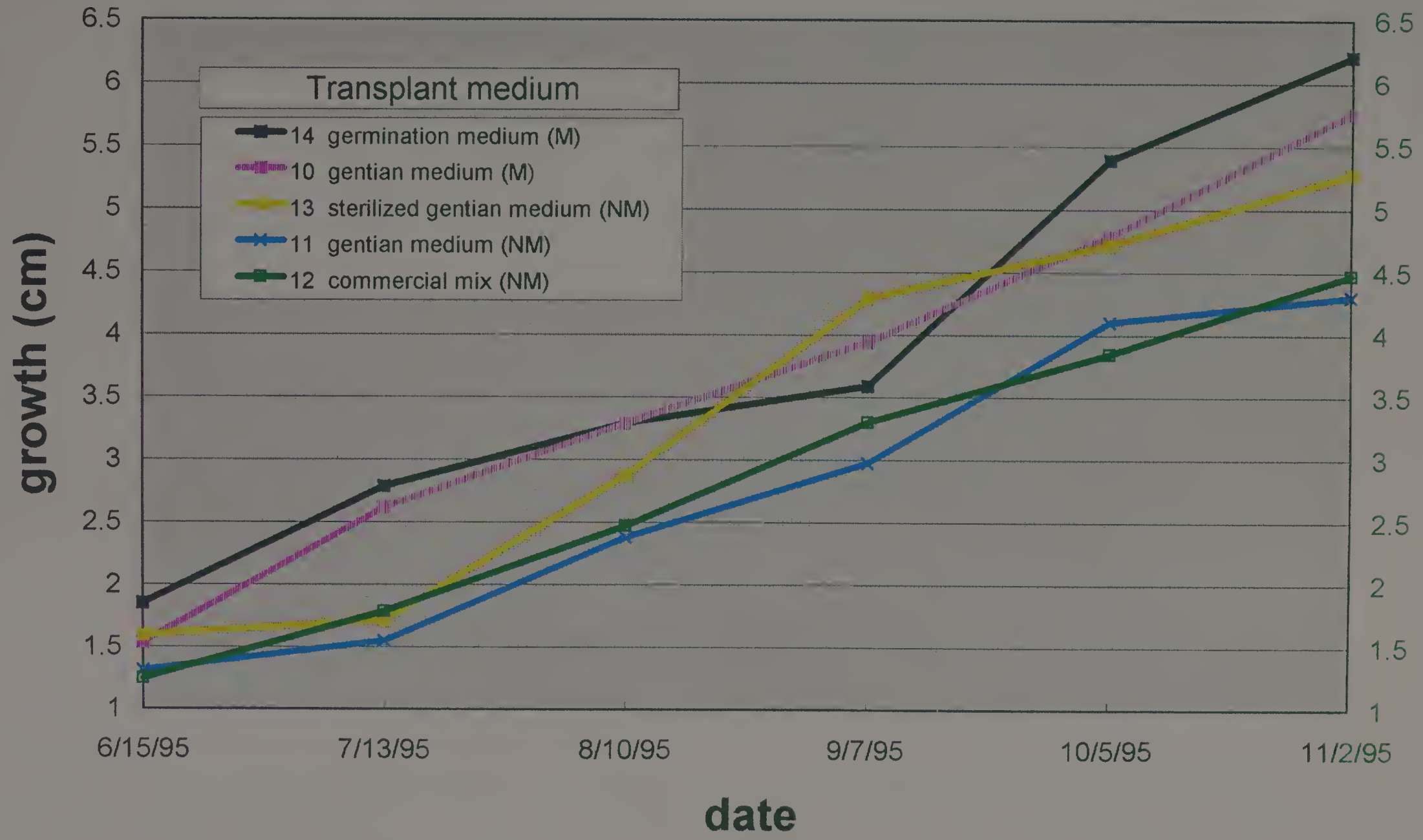


Figure 50. Influence of transplant media on growth of *G.crinita* from non-mycorrhizal germination medium (GM 2). First date is 2 months after transplant, when survival rate stabilized after a steep decline.

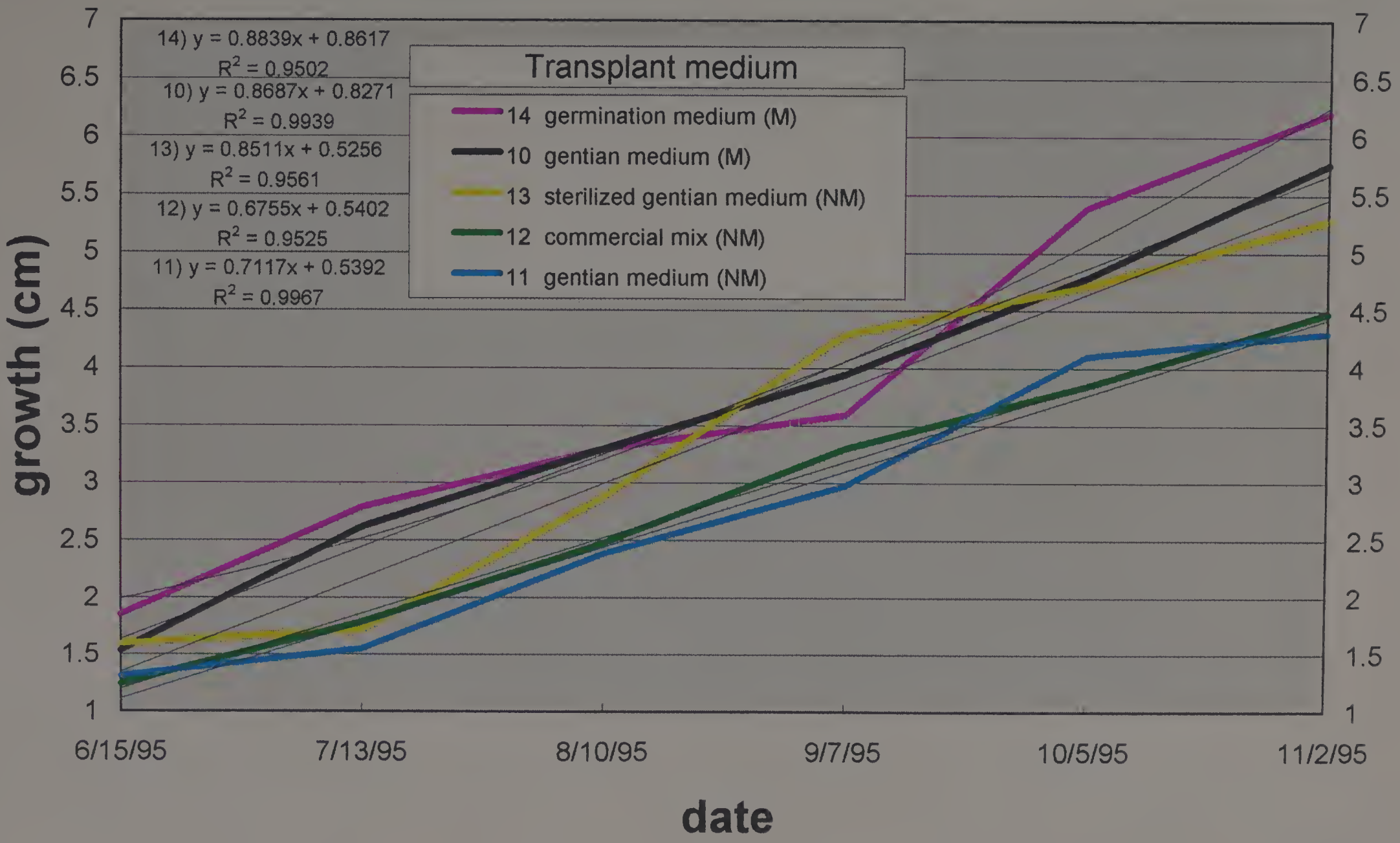


Figure 51. Influence of transplant media on growth of *G. crinita* from non-mycorrhizal germination medium (GM 2), with trendlines. First date is two months after transplant, point at which survival rate stabilized after a steep decline. Data corresponds to data in Table 14, p. 93.

Figure 52. *Gentianopsis crinita* plants approximately four months after transplant (August 1995) from each of the germination treatment sets. The plants from the mycorrhizal germination medium (GM 1) on the right are larger and more vigorous than those from the non-mycorrhizal germination medium (GM 2).

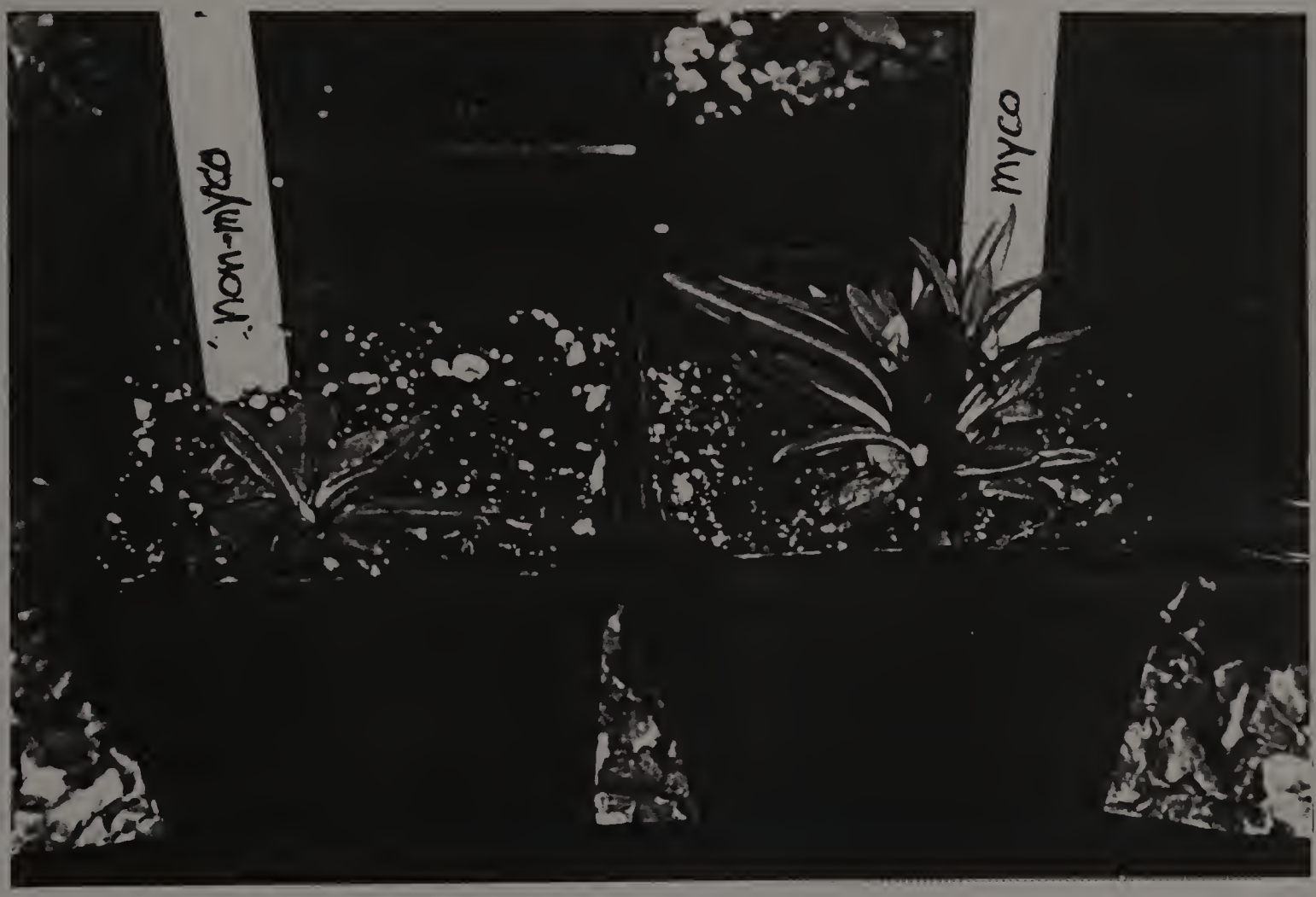


Figure 53. *G. crinita* emerging from dormancy in late February, 1996. Note that the non-mycorrhizal plant (on left) still has the thickened apical bud at its growing tip, and has not yet reemerged from dormancy, while the mycorrhizal plant on the right is reemerging faster and more vigorously.

Figure 54. Mycorrhizal *G. crinita* plants three months after reemergence (May, 1996). Mycorrhizal plants (on left) continued to exhibit more vigorous growth than their non-mycorrhizal counterparts (on right) throughout the second-year growing season.

Figure 55. A nearly full-grown *G. crinita*, June, 1996. A flower bud is beginning to form at the growing tip. These greenhouse-grown gentians went on to flower in October of that year.

Figure 56. The beginning of mycorrhizal colonization in the roots of *G. crinita*, early November, 1995. Note appressorium, or infection peg (arrow), where the extra-radical VAM fungal hyphae first penetrates the root endodermis.

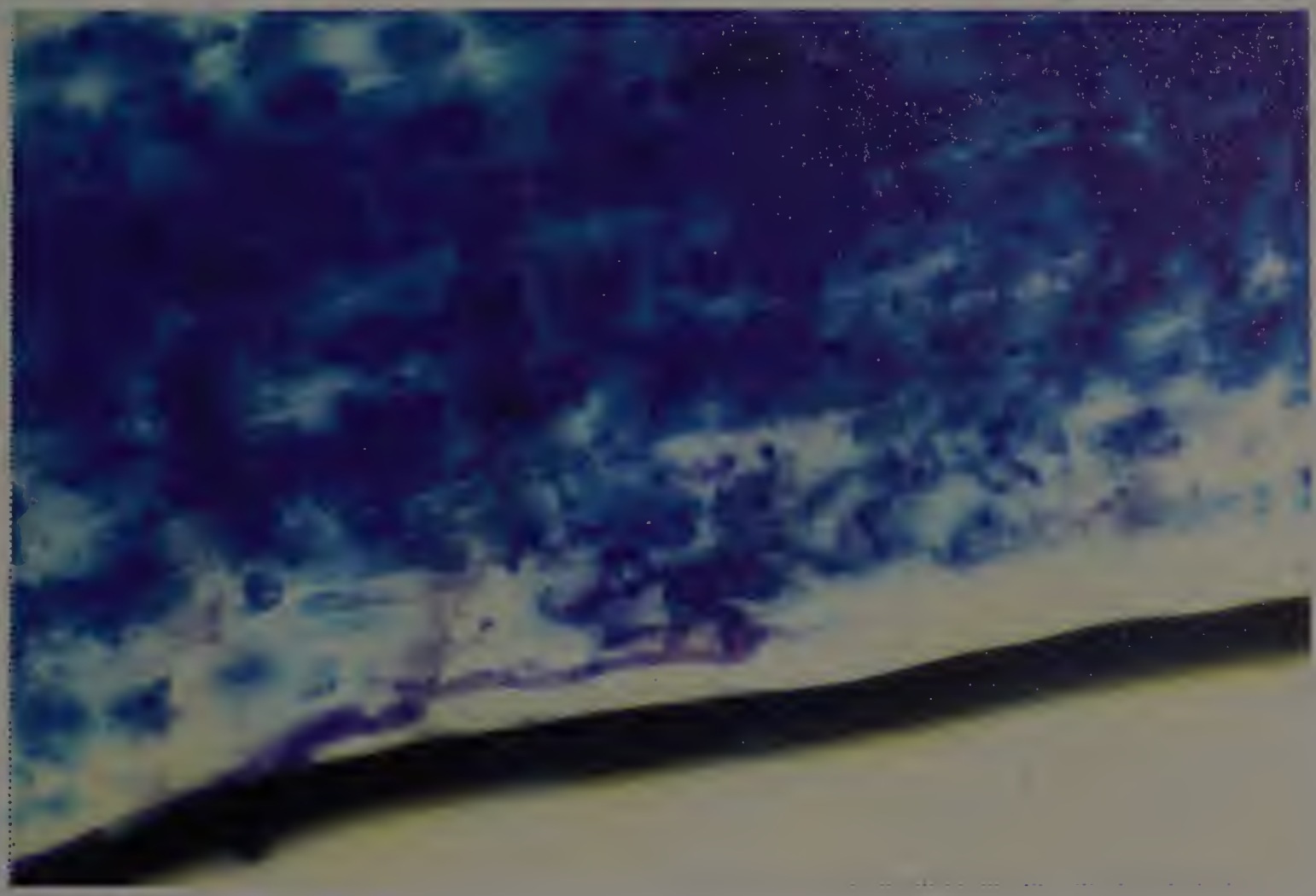


Figure 57. The peak of mycorrhization in *G. crinita* root, mid-December, 1995. Note VAM fungal hyphae (stained purple), and heavy colonization of root cortex by arbuscules.

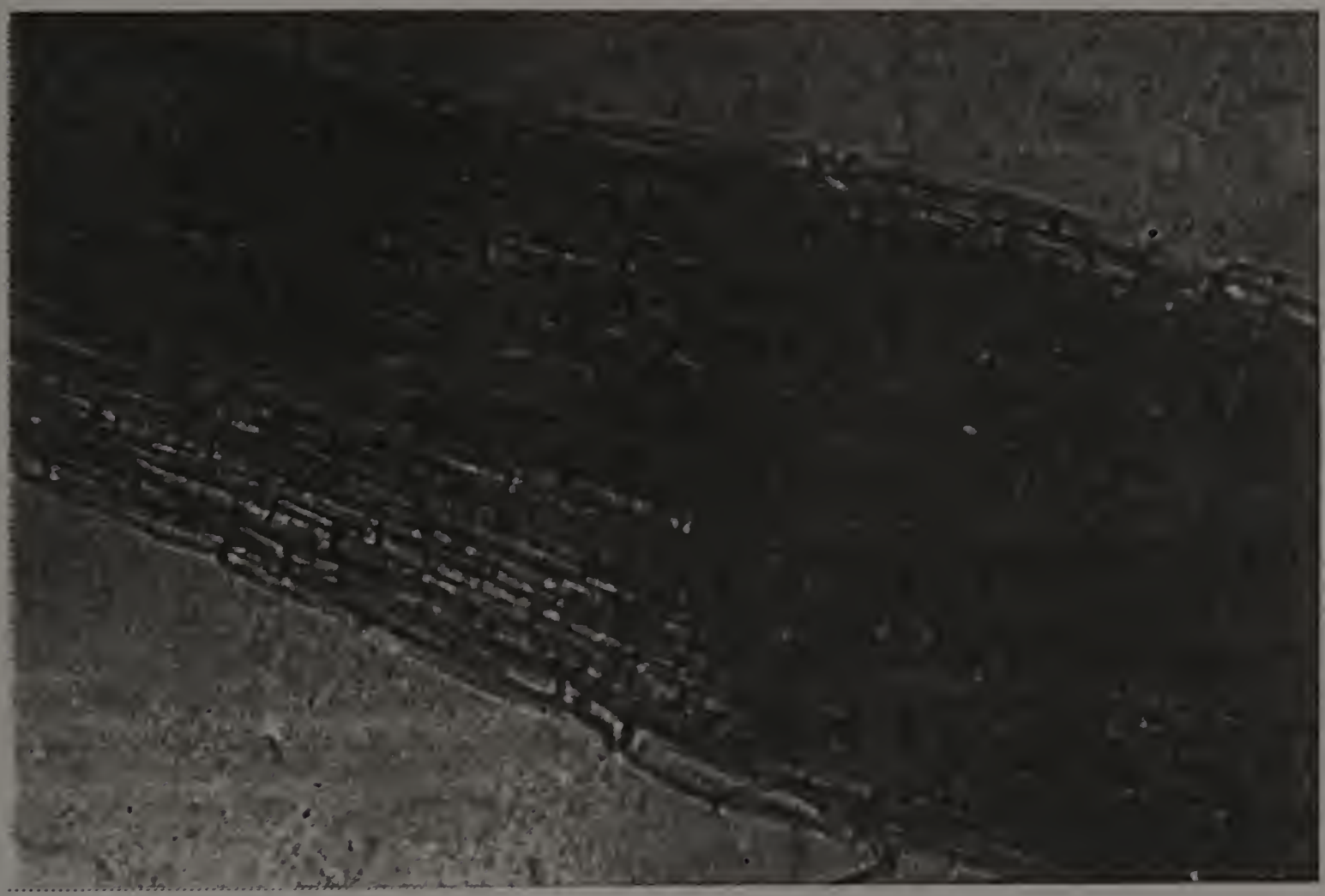


Figure 58. Swollen *G. crinita* root in response to heavy mycorrhizal colonization, mid-December, 1995. Mycorrhizal roots swelled to slightly more than 2x their normal diameter, from an average of 330 μm to more than 700 μm.

Figure 59. A dark-septate endophytic (DSE) fungus, probably *Phialocephala fortinii*, inhabiting the roots of a healthy *G. crinita*, August, 1995. Note the sclerotia-like structures. The absence of root disease suggests a non-pathogenic relationship with its plant host.

Figure 60. A dark-septate endophyte (DSE) inhabiting the roots of a healthy *G. crinita*, August, 1995. This fungus resembles the descriptions of a "grey-sterile endophyte" observed in gentian roots extracted from natural habitats by other field investigators.

Figure 61. Non-pathogenic chytrids in the roots of a healthy *G. crinita*. Dark areas are actually comprised of dozens of tiny circular bodies (not clearly visible in this photograph) packed together in the cortical cells. A DSE similar to the one pictured in Fig. 60 can be seen in the lower right portion of the root, just below the chytrids.

CHAPTER VII

1996 EXPERIMENTS

A. Pathogen suppression experiments

1. Materials and Methods

The hypothesis behind this series of experiments was that mycorrhizal fungi and the soil microflora associated with mycorrhizal pot cultures suppresses soil-borne fungal root pathogens, thus reducing incidence of damping-off disease among plants grown in a commercial growing mix into which pot culture inoculum has been incorporated. In order to test this hypothesis, a series of experiments were undertaken in December 1995 with root-infecting fungi found in the gentian experiments (*Rhizoctonia*, *Fusarium*, and *Pythium* spp.). Because of the long generation time of Fringed gentian, radish, basil, and lettuce were used as hosts. The purpose of this series of experiments was to confirm and elucidate, under controlled conditions, possible pathogen-suppressive properties of VAM inoculum as observed during the 1995 experiments.

Three different plant/pathogen combinations were used: Radish cv. Rabano White Icicle/*Rhizoctonia solani* (isolated from infected radishes), Lettuce cv. Black-seeded Simpson/*Pythium* spp. (isolated from infected lettuce seedlings), and Basil cv. Genova/*Fusarium oxysporum* f. sp. *basilicum* (isolated from basil seeds). All seeds used were hot-water treated, and were not treated with fungicides. The four treatment media (M1-4) used in the experiments were as follows:

- M1) Sterilized commercial growing mix - Fafard II™ , referred to as SM (sterilized medium)
- M2) SM + sterilized pot culture inoculum
- M3) SM + non-mycorrhizal pot culture inoculum
- M4) SM + mycorrhizal pot culture inoculum

Of these, only M4 contained mycorrhizal inoculum; the remaining three did not. The mycorrhizal pot culture inoculum was the same used in the 1995 experiments, INVAM UT- 316, containing *Glomus etunicatum*. The base media used was a sterilized commercial growing mix (Fafard II™); M1 was simply this base media with no additions. This, along with M2, were both sterilized by steam-sterilization, and were included as positive controls to test the pathogenicity of each pathogen. The pot culture in M2-M4 was incorporated into the growing mix at a 1:4 (v/v) ratio. Therefore, the treatment component of the media consisted of pot culture comprising 1/5 (or 20%) of the mix, slightly higher than the 1/8 (12.5%) used in the 1995 trials. This was an attempt to obtain a stronger and less ambiguous treatment effect. Treatment M3, which incorporated a non-mycorrhizal pot culture inoculum, was used as a contol to assess the effects of what was essentially a rhizosphere inoculum without its mycorrhizal component, in order to determine if there was something in the pot culture inoculum itself, apart from its mycorrhizal component, that was at least partially responsible for disease suppression.

The general procedure followed involved sowing five seeds into each of the 48 (2 square inch) cells per tray, at a depth of 1 cm. The trays were then placed in a mist house, where they remained until germination. In approximately twenty days, the test

plant seedlings germinated, and the appropriate cells were subject to the various inoculation treatments.

Radish/*Rhizoctonia* inoculation treatments:

- No inoculation
- Inoculation with one rice grain/plant colonized by *Rhizoctonia solani*
- Inoculation with rice grain without *Rhizoctonia* (rice grain control)

Inoculum was prepared by inoculating one autoclaved jar of brown rice (250 cc) with *Rhizoctonia solani.* This was incubated at room temperature in darkness for 14 days, until mycelia was visible throughout the jar. One autoclaved jar of non-inoculated brown rice was simultaneously prepared, for use as a control. Each cube which was inoculated received five grain of rice, one per seed, planted at a depth of two cm., .5 cm from each radish seedling (5 seedlings per cube).

Lettuce/*Pythium* inoculation treatments:

- No inoculation
- Inoculation with .5 cm. plug of *Pythium* culture on V8 medium
- Inoculation with .5 cm. plug of V8 media without *Pythium*

Five 0.5 cm plugs were placed into the appropriate cells at a depth of 1 cm., 0.5 cm from each lettuce seedling (5 seedlings per cube).

Basil/*Fusarium* inoculation treatments:

There was only one inoculation treatment in this experiment; the entire tray (48 cubes) was inoculated with *Fusarium oxysporum* f. sp. *basilicum.* The inoculum was prepared by transferring the original *Fusarium* maintenance culture to Komeda medium

and incubating it at 23°C until the fungal culture filled the entire plate. The *Fusarium* culture was then put into solution by scraping it from the Komeda medium with a sterile knife and mixing the fungus with sterilized water. This was mixed vigorously, until the culture was evenly dispersed throughout the water, and then poured directly over the basil seedlings. Each cell (2 square inch) received 30 ml of solution, @ 300,000 conidia per milliliter.

After inoculation, which was carried out under a flowhood, the trays were returned to the mist house until the conclusion of the experiments. Seedlings were germinated and grown in same treatment medium and were not transplanted. Seedling survival in the Radish/*Rhizoctonia* and Lettuce/*Pythium* experiments was measured 20 days after pathogen inoculation. For the Basil/*Fusarium* experiment, seedling survival was measured four months after inoculation (April 1996), as the *Fusarium* did not become actively pathogenic until that time.

The experimental design for the Radish/*Rhizoctonia solani* and Lettuce/*Pythium* experiments was a randomized complete block, with a 4x3 factorial of media and pathogen inoculation (4 x 3 = 12 treatments); this included pathogenicity and inoculation controls. The experimental unit consisted of one cube (each cube had five seedlings). There were four replications of each treatment, and twelve treatments (4x12=48 cubes per experiment). Because each tray had 48 cubes, there was one tray for each plant/pathogen combination. Inoculation treatments for the radish and lettuce experiments were randomly administered within each media block. The Basil/*Fusarium* experiment used a split plot, rather than randomized block design, meaning that all the seedlings in all

four treatment media (each containing12 replications) received the same pathogen inoculation. Pathogenicity and host susceptibility had already been established in previous greenhouse trials (Trueman, 1995), so non-pathogen controls were omitted.

2. Results

The lettuce plants inoculated with *Pythium* failed to result in disease, and so did not produce any usable data. The results of the other two experiments, using radish/*Rhizoctonia* and basil/*Fusarium*, are presented here.

Table 16 and Fig. 62 (pp. 139 and 141, respectively) show the results of the Radish/*Rhizoctonia* experiment, and list the four different media which were tested. Both M3 and M4 (the two media containing active pot culture inoculum, non-mycorrhizal and mycorrhizal, respectively) demonstrated a significantly enhanced disease suppression capability over the control media, M1 and M2. This was assessed by surveying the survival of radish seedlings twenty days after pathogen inoculation (Duncan's MRT, p=.05). The strain of *Rhizoctonia solani* used was a known pathogen of radish, causing a "collar" or lower stem rot, manifested as damping-off in newly-germinated seedlings. M3, which contained active non-mycorrizal pot culture inoculum, still exhibited a significantly greater disease-suppression capability than either of the two controls (M1 and M2), though it was still significantly less than that shown by M4, which contained mycorrhizal inoculum.

The second experiment tested basil inoculated with *Fusarium oxysporum* f. sp. *basilicum* in the same four treatment media. It took four months for the disease to

manifest itself, most likely because *Fusarium* is only pathogenic at soil temperatures greater than 60°F (16°C); it was not until the fourth month after germination (April 1996) that the greenhouse in which this experiment took place warmed up enough to sustain that soil temperature.

Table 17 and Fig. 63 (pp. 139 and 142, respectively) show the results of this experiment. The medium containing active non-mycorrhizal pot culture inoculum, M3, exerted a greater supressive effect on the pathogen than either of the controls (M1 and M2), but not nearly as much as M4, which contained mycorrhizal inoculum (Figs. 64 and 65, pp. 143-144, are photographs of the basil four months after pathogen inoculation; M4 is in the upper left corner of Fig. 64).

B. Soil dilution and plating experiment

1. Materials and Methods

The purpose of this experiment was to assess and compare microbial populations from mycorrhizal and non-mycorrhizal treatment media used in the 1995 experiments. The protocol involved a standard dilution and plating of the mycorrhizal medium which supported the highest percent survival of Fringed gentian (TM 14), the non-mycorrhizal medium which supported the lowest (TM 11), and a commercial growing mix as a control (TM 12). Three different selective culture media were used to isolate fungi, bacteria, and actinomycetes. Each dilution was plated onto three petri plates containing the particular culture medium, for a total of 9 plates per TM (9 x 3 TM = 27 plates total). Soil fungi were isolated on Rose Bengal, and a 1:10,000 (10^{-4}) dilution from that culture was plated

out. SCA (starch-casein agar) was used to isolate actinomycetes, and a 1:100,000 (10^{-5}) dilution was plated. For bacteria, TSA (trypticase-soy agar) was used, and was plated out at a 1:1,000,000(10^{-6}) dilution. After several weeks in an incubator (23°C), the colony forming units were counted and the results statistically analyzed using ANOVA, Duncan's MRT, and t-tests (p=.05). Data was log-transformed for analysis.

2. Results

The overall population of soil microflora from the different treatment media, as reflected in the soil dilution and plating, can be seen in Table 18 (p. 140) and Fig. 66 (p. 145). The mycorrhizal medium, TM 14, represented by the blue bar on the right of the chart, had lower populations of soil microorganisms, indicating a suppression of soil microflora when compared to the other two non-mycorrhizal media (TM 11 and 12). When this data is separated by type of microorganism (Fig. 67, p. 146), it is revealed that the only significant suppressive effect (as per t-test, p=.05) was on soil fungi (important since pathogens in the 1995 experiments were soil-borne fungal pathogens). The bacterial populations were suppressed in M14, though not significantly so, and the actinomycete population remained relatively unaffected.

One additional observation should be noted which may have had an influence on the outcome of the 1995 experiments, though this data was not counted or statistically analyzed. The TSA culture medium used for the isolation of soil bacteria contained potassium phosphate (K_2HP0_4), which resulted in a medium with a cloudy appearance. Surrounding several of the bacterial colonies on the dilution plate from TM 14, the

growing medium containing mycorrhizal inoculum, were "zones of clearing" in the cloudy medium, which resulted from phosphate-solubilization and is indicative of the presence of phosphate-solubilizing bacteria. These zones of clearing were not seen on the dilution plates from the other two non-mycorrhizal growing media, TM 11 and 12.

Table 16. Survival of radish (*Raphanus sativus*) seedlings in various media 20 days after inoculation with *Rhizoctonia solani*. Only data from *Rhizoctonia* inoculation treatment is shown; remaining inoculation treatments had no effect on survival. Data corresponds to data in Fig. 62, p. 141.

Media	Survival (%)	Duncan's grouping*
1**	0	A
2**	0	A
3	15	B
4	20	C

1 (NM) Sterilized commercial growing mix (SM)
2 (NM) SM + sterilized pot culture inoculum
3 (NM) SM + non-mycorrhizal pot culture inoculum
4 (M)† SM + mycorrhizal pot culture inoculum

*Letters indicate significant differences between percentages as per Duncan's MRT; percentages with same letter not significantly different (p=.05).
**Media 1 and 2 were included as controls to test for pathogenicity of *Rhizoctonia* strain. Zero percentage indicates that all of the plants died as a result of pathogen inoculation.

Table 17. Survival of basil (*Ocimum basilicum*) seedlings in various media four months after inoculation with *Fusarium oxysporum* f. sp. *basilicum*. Data corresponds to data in Fig. 63, p. 142.

Media	Survival (%)	Z-test grouping*
4	67.9	A
3	48.2	B
2	27.8	C
1	9.1	D

1 (NM) Sterilized commercial growing mix (SM)
2 (NM) SM + sterilized pot culture inoculum
3 (NM) SM + non-mycorrhizal pot culture inoculum
4 (M)† SM + mycorrhizal pot culture inoculum

*Letters indicate significant differences between percentages as per Z-test; percentages with same letter not significantly different (p=.05).
†M = mycorrhizal, NM = non-mycorrhizal.

Table 18. Influence of mycorrhizal inoculum on soil microorganism populations in various transplant media from the 1995 growth and survival experiments. Data represents colony forming units (CFU's) two weeks after plating, and was log-transformed for ANOVA and Duncan's MRT (p=.05). Letters indicate significant differences between media for each type of soil organism listed. Data corresponds to data in Figs. 66 and 67, pp. 145 and 146.

	Type of soil microorganism (CFU's)			
Media	Fungi*	Actinomycetes**	Bacteria†	mean
11	3.56a	4.63a	6.98a	5.06a
12	3.36a	4.42a	6.54a	4.78a
14	2.00b	4.10a	5.69ab	3.93b

11 - Gentian medium (NM) - compost-based potting mix
12 - Commercial mix (NM) - used as a control
14 - Gentian medium (M)‡ - compost based potting mix + mycorrhizal inoculum

*CFU's per 10^{-4} gram of growing medium.
**CFU's per 10^{-5} gram of growing medium.
†CFU's per 10^{-6} gram of growing medium.
‡M = mycorrhizal, NM = non-mycorrhizal.

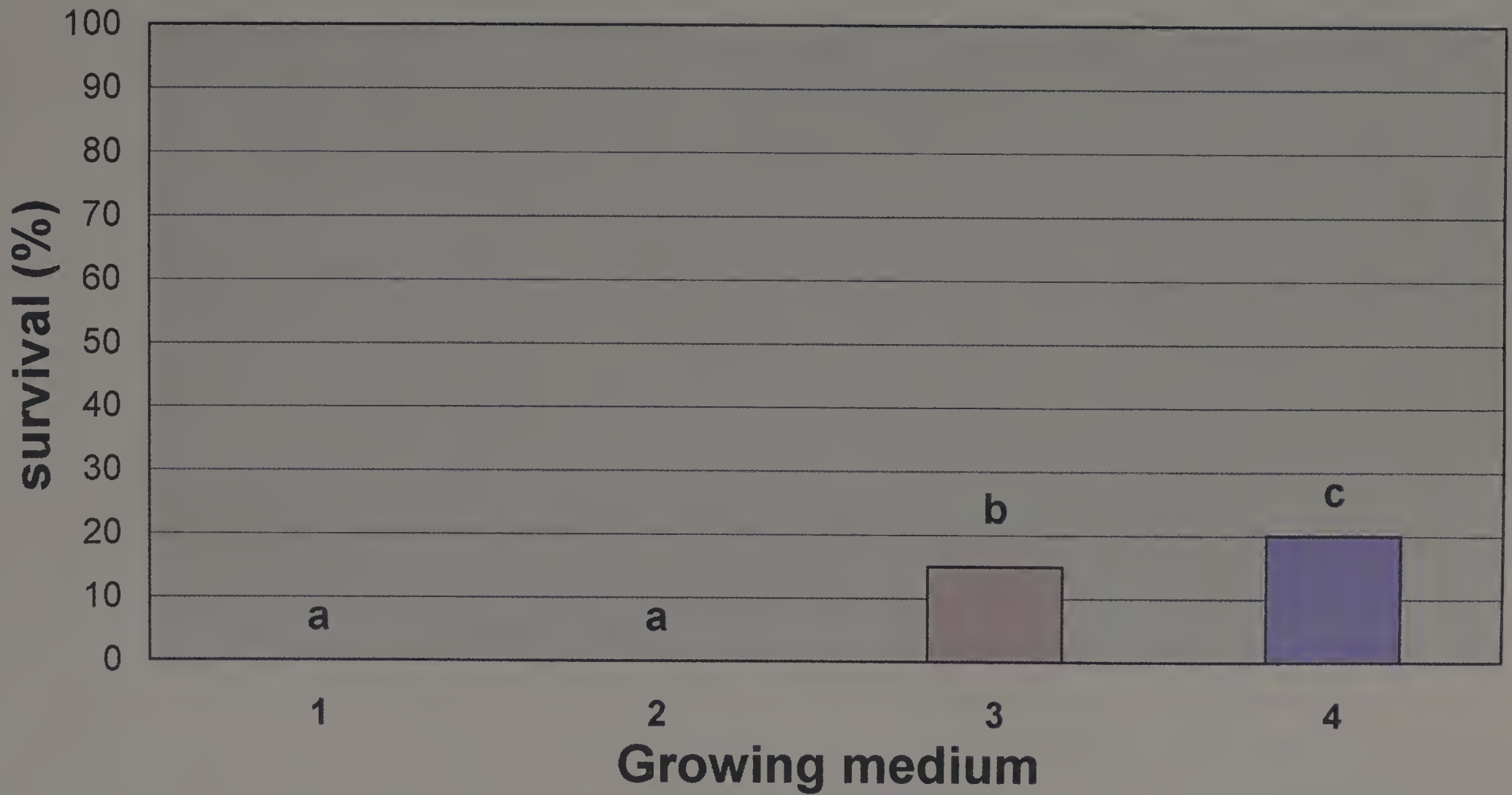


Figure 62. Survival of radish in various media twenty days after inoculation with *Rhizoctonia*. Data equals mean of four replications. Means separated by Duncan's MRT. Only data from *Rhizoctonia* inoculation treatment is shown; remaining inoculation control treatments had no effect on survival. Data corresponds to data in Table 16, p. 139.

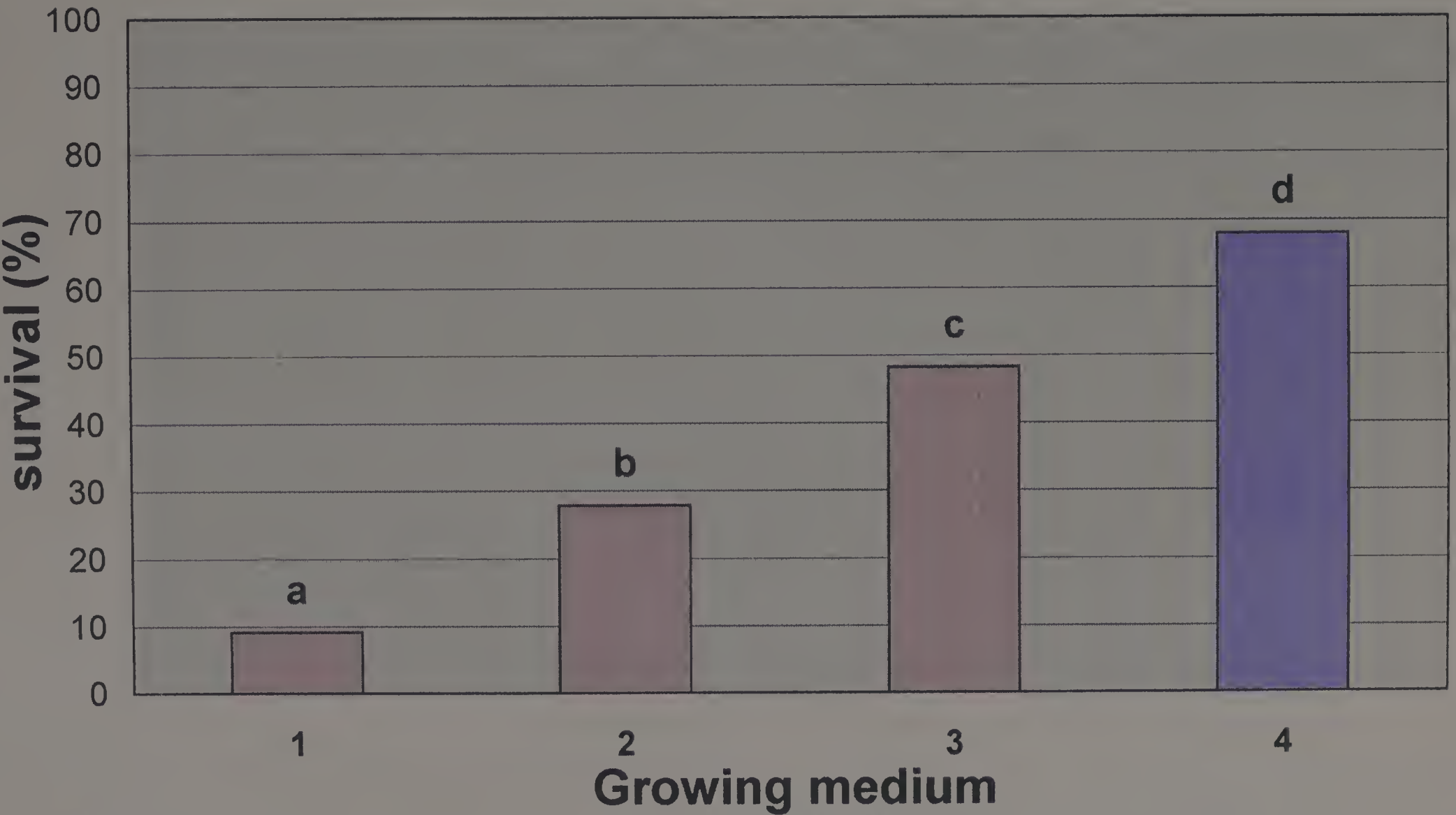


Figure 63. Survival of basil in various media four months after inoculation with *Fusarium*. Data equals mean of twelve replications. Means separated by Duncan's MRT (p =.05). Media 2, 3, and 4 mixed 5:1 (v/v) with pot culture inocula. Data corresponds to data in Table 17, p. 139.

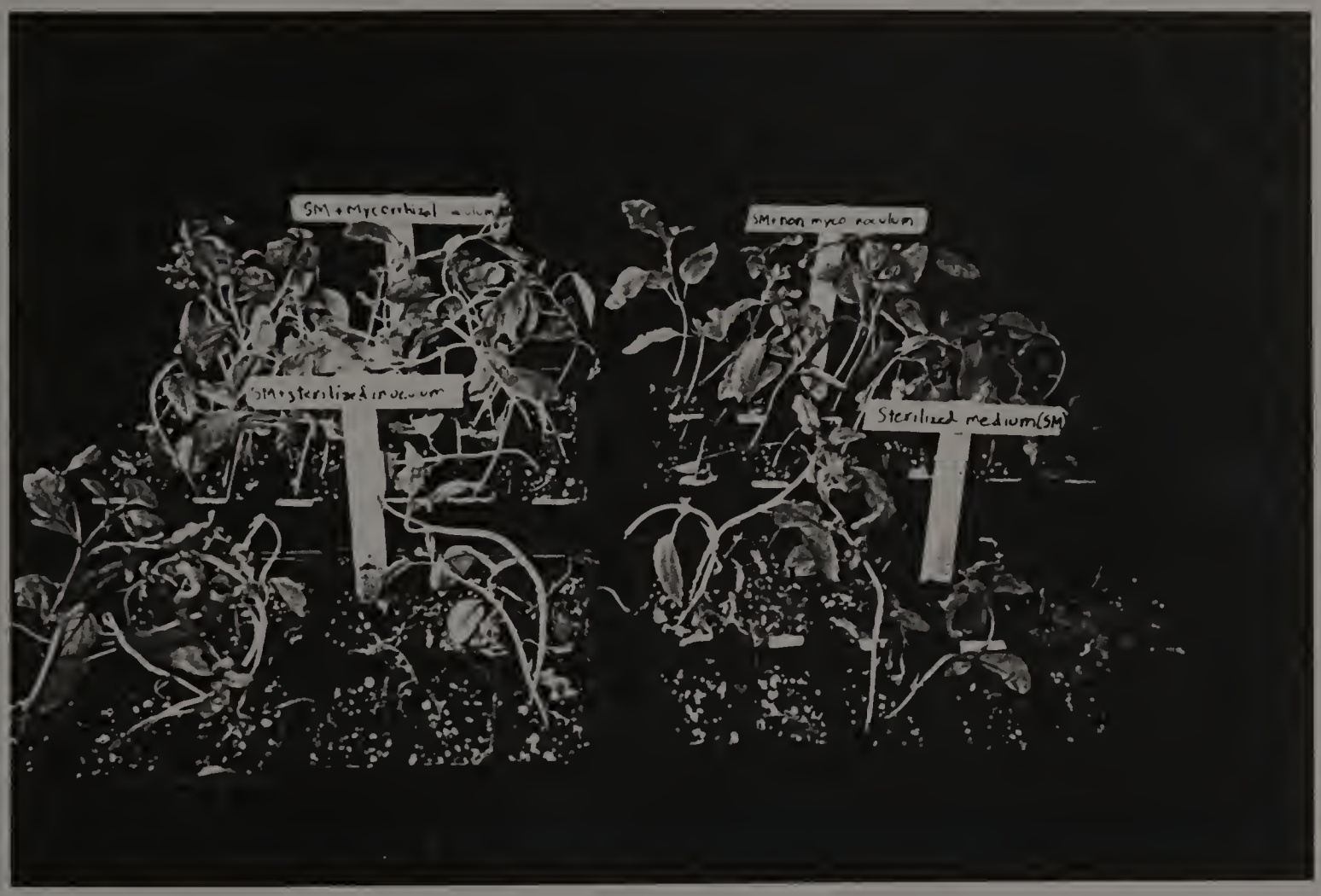


Figure 64. Basil from the 1996 disease suppression experiments, four months after inoculation with the pathogenic fungus *Fusarium oxysporum* f.sp. *basilicum*, April, 1996. The plants in the upper left hand block were grown in the medium containing the mycorrhizal pot culture inoculum; the remaining three blocks are various non-mycorrhizal controls.

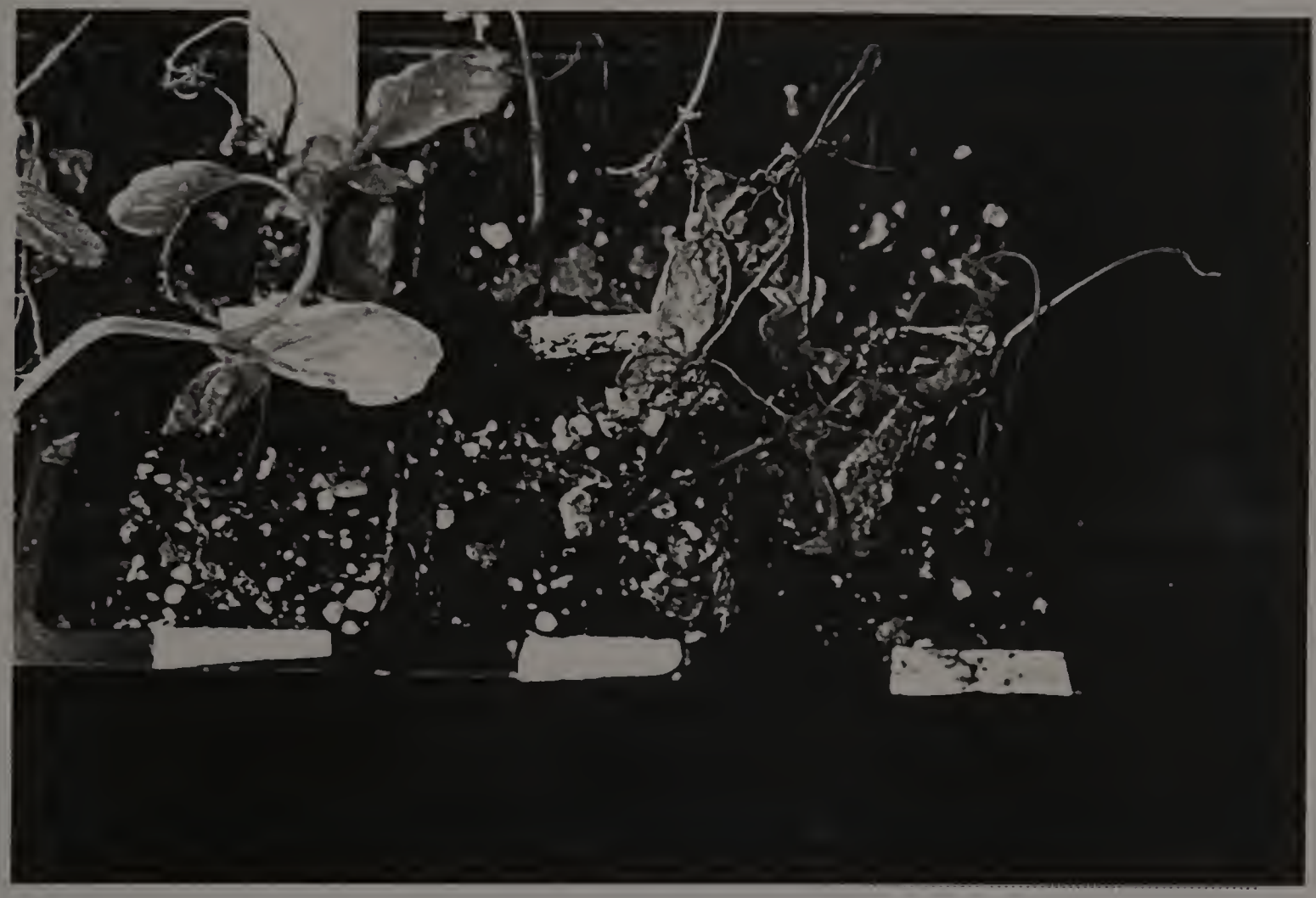


Figure 65. A close-up of a diseased basil plant inoculated with the *Fusarium* pathogen. This fungus enters through the roots, eventually causing a vascular wilt leading to the death of the plant.

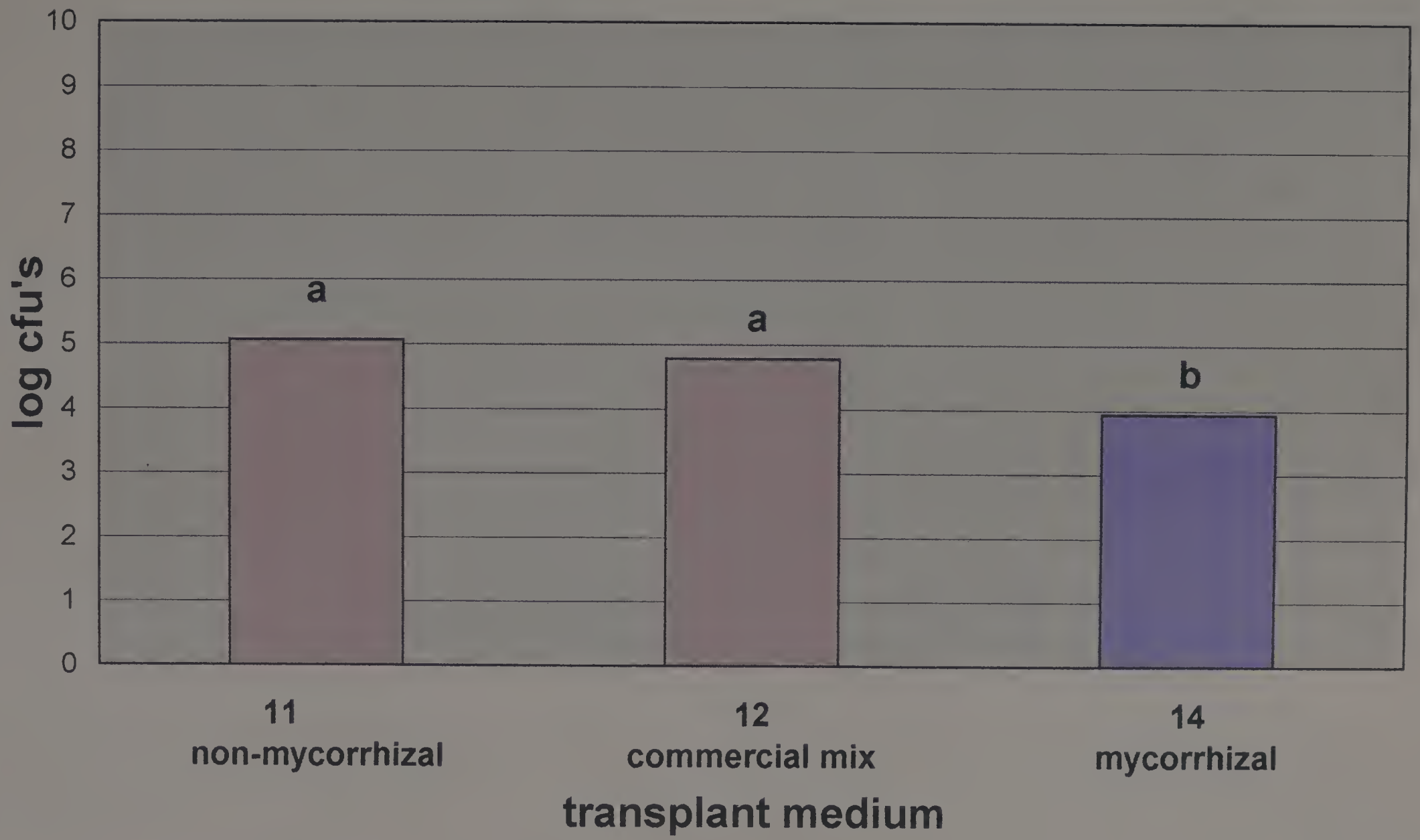


Figure 66. Influence of mycorrhizal inoculum on total soil microorganism populations of transplant media from 1995 experiments. Log cfu's = mean number of colony-forming units from all three dilutions, incorporating total populations of bacteria, actinomycetes, and fungi, log-transformed for ANOVA. Means separated by Duncan's MRT (p =.05).

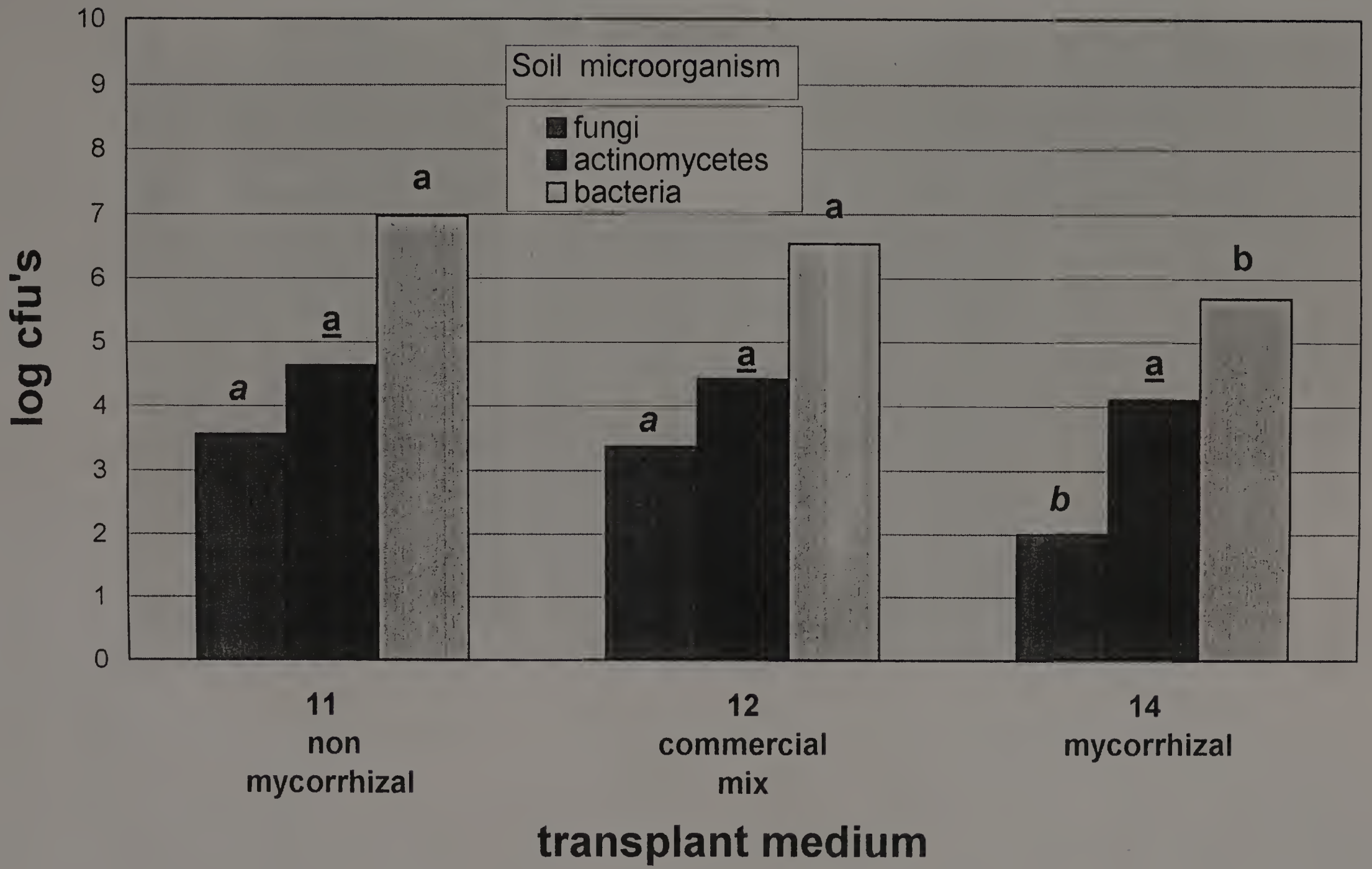


Figure 67. Influence of mycorrhizal inoculum on soil microorganism populations. Comparison is between same color bars; those with same letters not significantly different (Duncan's, p=.05). Data corresponds to data in Table 18, p.140.

CHAPTER VIII

DISCUSSION

A. 1995 growth and survival experiments

Although mycorrhizal inoculum enhanced the germination, growth and survival of *G. crinita*, interpretation of these findings was complicated by the unintentional contamination of the treatment media by root and crown infecting fungal pathogens, and the unusual timing of mycorrhization. Mycorrhizae were absent throughout the growing season, but mycorrhization finally occured at the onset of dormancy and persisted throughout the overwintering period.

Gentians have long had a reputation for being difficult to grow. This is especially true for Fringed gentian, which has frustrated growers and researchers from at least the turn of the century (Weiss 1933; Giersbach, 1937) to the present day (Brumback, 1993; Jacquelinet-Jeanmougin, 1996). While several researchers have looked into the possibility that this difficulty is due to a lack of mycorrhizal fungi (Weiss, 1933; Mcgee, 1985), none has reported the presence of root pathogens as a cause of their failure in culture, though one grower earlier in this century mentioned that transplanted gentians were highly vulnerable to "rot" (Hedden, 1931). Results reported here showed *G. crinita* to be susceptible to several more soil-borne pathogens than had been previously reported (see Farr, et al, 1989), including *Pythium*, *Rhizoctonia*, *Alternaria*, and several unidentified fungal pathogens. Because it is widely understood that *G. crinita* appears to have a requirement of at least some organic matter in the growing mix (Giersbach, 1937; Phillips, 1985; Brumback, 1986), the idea of growing them in a completely sterile medium is something of a contradiction. As could have been expected, efforts in this direction have not met with success

(Brumback, 1993; McCargo, 1994), nor have efforts at sowing or distributing Fringed gentian seeds directly into the soil at various habitat sites (Somers, 1993; Clark and Woolsey, 1995). Weatherbee (1994) has speculated that germination and survival of *G. crinita* in nature may rely to some degree on snow cover, and subsequent snow melt, the former of which is sometimes present when the seeds are released in late December. This may account for this species' irregular distribution and reproductive patterns. At present, offsite propagation remains the only viable method of insuring a healthy stock of this elusive and increasingly endangered plant.

1. Effect of mycorrhizal inoculum on growth and survival

Results from both the 1994 preliminary trials and the 1995 growth and survival experiments clearly indicate a beneficial effect from incorporation of mycorrhizal pot culture inoculum into the growing medium. Despite the lack of mycorrhizal colonization in the roots of *G. crinita* during the course of the 1995 experiments (which will be discussed later in greater detail), plants that were germinated and grown in the mycorrhizally-inoculated medium showed significantly higher transplant survival and growth rates than those from the non-mycorrhizal medium. When it was discovered that many of the gentians had died as a result of invasion by soil-borne root-infecting pathogens, and the roots of those gentians that had survived exhibited no mycorrhizal colonization, it was realized that something other than mycorrhizae in the gentian roots, some component in the mycorrhizal inoculum itself, was offering some degree of protection from these pathogens.

Because transplant mortality was attributed to root-infecting fungal pathogens, it is likely that the incorporation of mycorrhizal inoculum into the germination and growing medium

contributed to an overall reduction in disease incidence by creating a disease-suppressive medium in which pathogen populations were reduced, and/or a disease-resistance capability was conferred to the gentian host (systemic acquired resistance). In light of the absence of mycorrhizal formation in these gentians during the growing season, it is likely that the reduced disease incidence, as well as the enhanced growth, can be attributed to mycorrhizally-associated microflora in the pot culture substrate used as inoculum. It appears that the incorporation into the growing medium of what is essentially a beneficial rhizosphere microflora, present in compost, and perhaps even augmented or amplified by a mycorrhizal component (such as a pot culture inoculum), may have been responsible for enhancing the survival of *G. crinita* under these circumstances. This was achieved either by suppressing populations of soil-borne pathogenic fungi, or by inducing a systemic defense response in the gentian roots.

The existence of a rhizosphere microflora beneficial to specific plants is a well-recognized phenomenon, and has been for many years (Timonin, 1940 a, 1940b, 1941; Newman, 1978; Rovira, 1991). Recent attention has been paid to the enhancement of this beneficial microflora by mycorrhizal roots, hyphae and spores, particularly during the pot-culturing process (Linderman 1982, 1991, Garbaye, 1991) . Other researchers have noted increased levels of potentially antagonistic microflora in the "mycorrhizosphere" of mycorrhizal pot cultures (Secilia and Bagyaraj, 1987, Paulitz and Linderman, 1991, Linderman, 1994), in conjunction with a general suppressive effect on soil microorganism populations (George,et al, 1996).

There is circumstantial evidence that rhizosphere microflora played a significant role in enhancing the growth and survival of Fringed gentian. First, this plant species seems to require at least some non-sterilized compost or organic matter in its growing medium, and does very poorly

without it. Second, transplant survival was significantly increased when seedlings were transplanted in groups as opposed to individually, implying a possible benefit from the increased concentration of rhizosphere activity which would accompany an increased root matrix (Table 5, p. 62). Third, when the transplant medium containing VAM inoculum was sterilized (TM 13), it provided no greater benefit than the sterilized commercial transplant medium (TM 12) used as a control (Table 12, p. 92, and Fig. 44, p. 113) indicating that the benefits conferred to Fringed gentian in the mycorrhizally-inoculated treatment media were associated with the biotic or microbial component of the VAM inoculum. Finally, mycorrhizally-inoculated germination medium provided the greatest protection from root pathogens in the first three months after transplant (Fig 39, p.108), a benefit which appeared to level off after that and throughout the remainder of the first growing season. This may have reflected the strong initial influence of a beneficial rhizosphere microflora which was transferred in the soil of the root ball when the seedlings were first transplanted. As would be expected, these effects waned. One possible explanation is that the populations of these beneficial microflora levelled off and reached an equilibrium in relation to the extant microflora of the transplant media, a phenomenon which is known to occur in these situations (Jalali and Jalali, 1991). Nevertheless, the initial benefits conferred during this period continued to express themselves in terms of enhanced growth and survival throughout the course of the first growing season, as detailed below.

The mycorrhizal germination medium also increased the growth of the gentian seedlings during the initial three month "incubation" period in the mist house (Fig. 46, p. 115), suggesting an enhancement effect from beneficial microflora even at this early stage. Once the seedlings were transplanted into the various transplant media, the germination medium from which the plants

originated continued to exert beneficial effects on plant growth and survival. Although the positive effect on growth was observed throughout the course of the first-year growing season (4/21 - 11/2/95), it was during the three-month period after transplant that the germination medium exerted its greatest effect on survival. The germination medium was transferred intact during transplant as a cube-shaped root ball, creating a "zone of protection" around the root. If , as the evidence suggests, pathogen contamination and root and crown disease were primarily responsible for the steep decline in survival seen during this post-transplant period, then the protection conferred by the mycorrhizal inoculum to the gentian roots was at its peak during this time. It is important to note that the difference between the two treatments was even greater when the seedlings were germinated and incubated in the mycorrhizal medium (as in 1995) rather than simply transplanted into one (as in 1994), as reflected in the 20% increase in survival rate in 1995 (68.5% vs. 41% - see Table 10, p. 91 and Table 5, p. 62). Furthermore, in 1995 the difference between the survival rates of plants from the two germination treatments when all transplant media were included (TM 1-14), compared to the corresponding survival rates of the subset of effective media only (TM 10-14), indicate that under less disease pressure from soil-borne pathogens (which is presumably closer to normal growing conditions) the mycorrhizal germination medium conferred an even greater beneficial effect on survival.

As mentioned, growth enhancement in Fringed gentian was also most likely attributable to the associated microflora from the VAM inoculum, although the mechanisms may have been different than those involved in disease suppression. Enhanced growth may have been due to the suppression of major or minor plant pathogens. Alternately, it may have been due to the overall suppressive effect of the mycorrhizal inoculum on the rhizosphere microflora, hinted at by the

results of the 1996 disease suppression and soil dilution experiments (see pp. 135-138). This same phenomenon may have suppressed competitive saprophytic microflora in the rhizosphere of Fringed gentian and the other test plants, which would almost certainly have made a difference in a low-nutrient substrate such as the one in which these plants were growing. The last possibility is that VAM inoculum selected for certain beneficial rhizosphere microflora at the expense of other competitive or detrimental microorganisms (including pathogens). Other researchers have observed the emergence of a unique mycorrhizally-associated microflora from the pot-culturing process, which includes plant growth promoting rhizobacteria (PGPR), urea- and starch-hydrolizing bacteria, and phosphate-solubilizing bacteria (Barea, et al, 1975; Ames, et al, 1984; Secilia and Bagyaraj, 1987; Paulitz and Linderman, 1991). There was in fact some circumstantial evidence for this on the dilution plates in the 1996 experiments. On the starch-casein agar plates (which were for isolating actinomycetes, and which were "cloudy" from potassium phosphate) several bacterial colonies from the mycorrhizal transplant medium (TM 14) were surrounded by zones of clearing indicative of phosphate solubilization; these were absent from the SCA plates of the two non-mycorrhizal media (TM 11 and 12). There is some evidence that mycorrhizal fungi interact with phosphate-solubilizing organisms in ways which benefit the host plant (Barea, et al, 1974). This may have been a factor in the outcome of the 1995 experiments, though these experiments were not designed to answer these questions or elucidate these mechanisms, and a definitive assessment is beyond the scope of this project. Nevertheless it is clear that some component of the mycorrhizal inoculum other than nutrients (which were identical in the two germination media) stimulated growth and had a significant influence on the survival of Fringed gentian.

Growth difference were relatively small, albeit significant, amounting to approximately .5 cm of leaf span between the two germination treatments (Fig. 46, p. 115). A large stimulation of growth may not necessarily be advantageous for Fringed gentian during the growing season. *G. crinita* is a cool-weather species, putting on most of its growth towards the end of the growing season. Also, it is a plant which only grows under low nutrient conditions, both in cultivation and in nature, and has adapted to this scarcity of resources. In greenhouse culture, *G. crinita* was a biennial. As such, the plant's resources during its first year are directed towards root growth in preparation for dormancy and overwintering, with very little allocated for foliar development, as can be seen in the small size, relative to the full-grown plant, of the foliar rosette just prior to dormancy (Fig. 45, p. 114). Practically all of the foliar growth is exhibited during the second growing season as the plant develops towards flowering and reproduction (Fig. 55, p. 124). Too much leaf growth in the first year may be disadvantageous to the Fringed gentian, "stealing" from the pool of already scarce resources those necessary for root development and dormancy. Seen in the light of this particular phenological strategy, lack of mycorrhizal formation in the roots during the first year's growth makes ecological sense, as does the eventual development of mycorrhizae after dormancy.

The parameter used to measure success regarding the use of VAM with plants such as Fringed gentian would be survival rather than growth. Therefore, despite the lack of a strong growth response, the results of this experiment regarding survival can still be considered a successful application of VAM. The presence of VAM may increase growth or yield in crop plants (the parameter often used to measure success in VAM research), but increases in survival rate have a greater ecological significance for native plants, particularly endangered species. Although this may not be relevant or given much consideration in crop plant production, survival is clearly the

essential criteria for the success of any plant species in nature. Although mature gentians in the wild do not appear to be susceptible to pathogen attack, and no data is available regarding the vulnerability of gentian seedlings under these circumstances, the seedling stage is generally considered to be the most critical time in the life cycle of any plant species in nature (Swain, 1996). This is certainly the case with gentian seedlings in cultivation; there is no doubt after this study that greenhouse-grown gentian seedlings, once transplanted, are highly vulnerable to pathogens. Under greenhouse conditions, there are apparent as well as subtle benefits other than growth that mycorrhizal inoculum may confer to the gentian seedlings which result in protection from pathogens and increased survival. Janos (1988) addresses and elucidates these issues in the following statement:

> "In the natural habitats in which host and mycorrhizal fungi evolved, success is the avoidance of extinction. Success demands fitness, the capacity of individuals to survive and reproduce. In a competitive milieu, successful reproduction is not always correlated with growth, but humans often focus on biomass production.... the abilites of hosts and mycorrhizal fungi engendered by natural selection may not be consistent with human production goals.....Moreover, if host demand for mineral nutrients is greatest at the onset of reproduction, mycorrhizae might improve reproduction without stimulating early vegetative growth (McGraw and Schenck, 1980); such fungi would not seem effective in seedling trials. This disparity between human and natural selection contributes to the apparent "inefficiency" of mycorrhizal fungus species; all mycorrhizal fungi may favor host fitness in the appropriate environment. The difficulty of finding effective mycorrhizal fungi for introduction is increased in direct proportion to deviation of production systems from native hosts and soil environmental conditions. Several investigators..... have felt that survival is as important an index of mycorrhizal inoculation success as growth, expecially in highly stressed habitats. Fertilization likely can not substitute for protection from pathogens. Mycorrhizal inoculation that significantly improves host survival beyond that possible by other means can be judged a success." (p. 140).

2. Pathogen contamination of growing media

In the 1995 experiments, plants were dying in huge numbers after the first transplant. Experiments 1 and 2 (TM 1-9) were nearly a total loss due to plant pathogens, while experiment 3 (TM 10-14) was comparatively successful, achieving survival rates averaging 61%, which is very high for this particular plant species under cultivation. It is difficult to determine the source of contamination, or to quantify pathogen inoculum among the various media, but evidence points to the the unsterilized compost component of the germination medium as the likely source of contamination. In any case, diseased and infected roots were found in every transplant medium, indicating that pathogen contamination was widespread throughout the media. In light of these circumstances, it is safe to assume that survival data was in fact reflecting, at least to some extent, pathogen suppression, rather than differences in pathogen exposure between treatment media. This is demonstrated in the fact that approximately 80% of the roots sampled from dead and dying plants from both mycorrhizal and non-mycorrhizal germination media showed evidence of root disease (brown areas and necrosis) and in 74% of these, one or more of the crown or root-infecting fungal pathogens previously mentioned was observed and identified (Table 7, p. 90).

Analysis of the roots of dead and dying plants revealed the presence of pathogens and root disease, indicating contamination of the growing mix by soil-borne root-infecting and crown-rotting fungal pathogens (*Pythium, Rhizoctonia, Fusarium, Alternaria,* an unidentified dematiaceous fungi, and nematodes - see Table 7, p. 90, and Figs. 31-37, p. 100-106). The presence of diseased roots so soon after transplant into the sterilized transplant media used as controls (TM 12 and 13) provided evidence that the germination medium was the source of

pathogen contamination. Given these events, the 1995 experiments can be interpreted as pathogen-suppression bioassays which tested the suppressive capabilities of the mycorrhizal inoculum.

The primary evidence for the germination medium as the source of pathogen contamination was the consistent presence of these pathogens (in all fourteen transplant media) as well as in the two sterile control transplant media (TM 12 and 13) . In addition, under the circumstances described above, plants from non-mycorrhizal germination medium (GM 2) transplanted into TM 11 (which was same as GM 2) would have been exposed to the highest level of pathogen contamination without the suppressive benefit of the VAM inoculum. In fact, this turns out to be the case, as this treatment set had the lowest survival rate of any of the "effective" media. Conversely, those plants from the mycorrhizal germination medium (GM 1) which were transplanted back into the same medium would presumably been exposed to the lowest level of pathogens or had the highest level of protection from them, and this was also true (Fig. 43, p. 112).

It is likely that the combination of improper cultural conditions and the fine root morphology of the cultivated gentians contributed to this situation. The relative warmth and moisture in the greenhouse during the winter months, compared to natural conditions, were conducive to the development of root and crown rot. Furthermore, it is likely that the finer roots of the cultivated gentians, not having developed a more rhizomatous morphology in preparation for dormancy as the roots of gentians in nature would have, were more vulnerable to these diseases, resulting in a steady dieoff throughout this period.

Given the extended period of time which the seed flats spent in mist house during the pre- and post-germination periods, it is not surprising that pathogens emerged to cause root and crown disease. Both flats, which were rotated once a week to insure equal treatment, were subject to three to four months of moist, warm, waterlogged conditions, on a poorly drained bench-covering fabric which supported pools of water highly conducive to the emergence and spread of soil-borne fungal root pathogens which may have been in the mist-house or in the germination medium. There were other seed flats in the mist-house with damping-off disease; it is possible that some of these pathogens would have found their way to the *G. crinita* seed flats. It is also possible that under different conditions, with a less vulnerable plant host, these naturally-occurring soil-borne fungi would have been less pathogenic.

A comprehensive investigation into a wide range of gentians, including Fringed gentian, from natural habitats reported virtually no pathogenic infection in the roots, though there was frequent and widespread colonization by mycorrhizal and other root-associated fungi (Weber, 1984). It seems that it is only the broken gentian roots resulting from transplant during cultivation which are vulnerable to root-infecting fungal pathogens; the intact roots in nature appear to be impervious to pathogen invasion. Differences in root morphology between natural and cultivated plants only serve to underscore this dichotomy. Gentian roots in nature are sparse, thick and rhizomatous; in cultivation, they are thin, fibrous, delicate, and more plentiful. It is apparent that the cultivation of *G. crinita* has a detrimental influence on it's root morphology, rendering it more vulnerable to pathogen invasion. Many researchers have noted how easily these roots break during transplant, which by itself may result in high mortality

(Giersbach,1937; Phillips, 1985); however, none have noted that it may be opportunistic infection by widespread fungal pathogens that is the cause of their demise.

The second transplant in October 1995 did not result in the devastating effect on survival as the first tranplant had. Gentians which had survived up to that point were apparently less vulnerable to broken roots and subsequent pathogen invasion. It is possible that disease pressure in the growing media (from reduced pathogen populations) had been diminished, or that the gentian roots had undergone biochemical and/or morphological changes sufficient to protect them, such as suberization or the production of anti-fungal compounds. In fact, Neumann (1934) reported on the development of an outer "rind" layer (perhaps the periderm) in gentian roots after several months of growth in cultivation. In addition, the main alkaloid extracted from gentian roots, gentiopicrin, has anti-fungal activity (Van der Sluis, et al, 1983), and the concentration of this alkaloid increases in response to mycorrhizal colonization and as the plant matures (Heymons, et al, 1986).

Despite these factors, overwintering in the greenhouse had a detrimental effect on survival regardless of treatment (Fig. 39, p.108), due in large part to the unidentified dematiaceous root and crown-infecting fungal pathogen mentioned previously (Fig. 37, p. 106). The survival rate during the overwintering period was approximately the same for plants from both germination treatments, indicating that the germination medium from which the plants originated was not exercising the influence that it had in the earlier part of the experiment, in the months following transplant.

Nevertheless, the only plants which actually survived overwintering to any significant degree were those germinated in mycorrhizal germination medium. At the end of the

overwintering period, approximately 20% of the mycorrhizally-germinated plants survived, compared to only about 3% of the non-mycorrhizally germinated plants. This was due to the larger number of plants going into the overwintering period from the mycorrhizal treatment, which was a function of the greater survival rate exhibited in that treatment set during the active growing season. From a conservationist point of view, the number of surviving gentians at the end of the growing season was the most important result of the experiment. The final survival survey was done just prior to the onset of dormancy (November), and under normal circumstances, this is when these plants would have been outplanted, either into a cold frame, preservation garden, or back into their natural habitat at a reintroduction site as part of an effort to reestablish natural populations of this species.

The 1996 disease suppression and soil plating experiments, which are discussed later (pp. 173-181), were conceived and implemented in order to further explore the apparent disease-suppressing capabilities of the VAM inoculum under more controlled conditions. Experiments to test VAM inoculum for disease suppression capabilities against three genera of fungal root pathogens seen in the 1995 experiments were carried out with susceptible (and easier to grow) plant host in an attempt to confirm and elucidate the results of the 1995 experiments. The results of the 1996 disease suppression and soil plating experiments did in fact demonstrate these capabilities.

3. Mycorrhizal activity in Fringed gentian

G. crinita is mycorrhizal in its natural state, and the plants sampled from the field sites had low to moderate levels of mycorrhization, suggesting facultative mycotrophy (Table 3, p. 45).

Higher levels were achieved in greenhouse cultivation with an effective non-indigenous VAM isolates (*Glomus etunicatum*, UT-316), though this may have altered the mycotrophic relationship between the gentian host and its mycorrhizal symbiont. Actual mycorrhization seems to correspond to certain phenological events in the plant's life cycle, and occurs towards the end of each growing season (*G. crinita* is an annual or biennial, depending on its habitat or culture). In nature, mycorrhizal formation coincides with bud formation and flowering (September - November). In culture, it occured at the onset of perennialization and dormancy in early November. Mycorrhization lasted throughout dormancy, though the mycorrhizae observed in roots sampled during the latter part of the dormancy period (late February-early March) had a distinctively different appearance than those sampled at the beginning. The arbuscules had a more amorphous, non-defined structure, and the finger-like hyphae seen in the arbuscules in November and December were indistinguishable from eachother at this later date.

During the first part of the growing season (April - June), Fringed gentian is extremely slow-growing, almost dormant, and it follows that there is no mycorrhizal activity during this period, as slow growth precludes the need for mycorrhizae during this time. During the more active vegetative growth phase of the plant (July - October), growth rates rise slightly. External hyphae and appressoria were observed in roots sampled from the mycorrhizal treatment media in July and August at the start of this more active phase, but these were not accompanied by mycorrhizal colonization. This suggests that mycorrhizal fungi were suppressed once they entered the gentian root, a phenomenon noted by other researchers (Mcgee, 1985, Jacquelinet-Jeanmougin and Gianinazzi-Pearson, 1983).

The occurrence of mycorrhization at the onset of and throughout dormancy suggests that VAM play a role in the perennialization and dormancy of *G. crinita*. There are several possible explanations for why Fringed gentian would form mycorrhizae at this phenological stage of its life cycle. One possibility is that its roots continue to grow after the above-ground parts of the plant have ceased growth. This phenomenon is not unusual for native herbaceous biennials and perennials in temperate zones. Brundrett and Kendrick (1988) observed that many of the biennial and perennial species they investigated had peak periods of mycorrhizal development, and in some cases, root growth, in late autumn. Some of these species produced mycorrhizae after leaf growth had ceased, or even after they had lost their foliage. They attributed this to the competitive ecological advantage it gives these plants in their quest for scarce nutrients. Different root phenology and mycorrhization patterns, by staggering periods of nutrient uptake within a given plant/soil community, may reduce competition for limited resources, namely phosphorus, the main nutrient supplied by mycorrhizae (Mosse, 1973), and one which is almost always limited in these habitats (St. John and Coleman, 1983). This ultimately encourages higher species diversity in plant communities.

Late-season mycorrhization is an elegant adaptive strategy for *G. crinita*. It may be the only way that gentians (which lack root hairs) and other associated perennial plants can obtain sufficient phosphorus for perennialization and reemergence in resource-limited habitats. Janos (1988) refers to species which utilize this strategy as "ecologically-obligate" mycotrophs, distinguishing them from fully obligate mycotrophs which require mycorrhizae for all of their basic growth functions, and cannot survive without them. As with all biennials, *G. crinita* spends most of its resources in the first year of growth preparing its roots for perennialization.

During the normal growing season, when the majority of plant species adopt a mycorrhizal posture to obtain the phosphorus necessary for growth , foliar growth is restricted in Fringed gentian, and phosphorus would not be necessary at this time. Instead, it is needed more at the onset of dormancy and during reemergence in the spring. Mycorrhizae and the phosphorus they provide are required towards the end of the growing season, shortly after the onset of shoot dormancy, when starches, phospholipids and other materials necessary to the overwintering root are being formed.

Gentianaceae is one of only a few plant families whose members do not manufacture starch in their leaves (Miller, 1957), instead relying exclusively on roots and stems for its production. Phosphorus is required for the synthesis of starch compounds (Dodd, 1962) and starch is a necessary precursor for roots which are forming perenniating storage structures such as rhizomes in preparation for dormancy. Therefore, plants entering into dormancy would require a mechanism during this period for the acquisition of P, and mycorrhizae serve just such a function. It is reasonable to assume that gentians rely on late-season mycorrhization to supply phosphorus for the buildup of starch necessary for the formation of rhizomes crucial to overwintering survival.

There are several other ways in which mycorrhizae, both directly through physiological changes and indirectly through the acquisition of phosphorus, might benefit gentians entering into and reemerging from dormancy. According to Weber and Heymons (1987), mycorrhizal gentians produced three times more root dry weight than their non-mycorrhizal counterparts. In addition, root swelling, a phenomenon which was seen in the roots of mycorrhizal *G. crinita* in both the 1994 and 1995 experiments, was also observed by these and other

researchers (Neumann, 1934; Jacquelinet-Jeanmougin and Gianinazzi-Pearson, 1983). Both physiological changes would give mycorrhizal gentians a definite advantage going into dormancy.

4. The unusual nature of gentian mycorrhizae

The mycorrhizal symbiosis in Fringed gentian, as in other gentians, is unique. In *G. crinita*, as well as some other species in the Gentianaceae (Mcgee, 1985), mycorrhization is suppressed during the growing season, as evidenced in the present study by the appearance of appressoria in the root endodermis without actual root colonization. Eventually, towards the end of the growing season, mycorrhization occurs during the onset of and throughout the first part of dormancy, when the plant roots are preparing for overwintering. The mycorrhizae that are formed have a very unusual morphology. This is followed by the degradation of the fungal symbiont prior to reemergence, which was reflected in the dense grainy appearance of the mycorrhizae observed in roots sampled in late February, 1996.

The unusual nature of mycorrhizae in gentians have made it the focus of several investigations, some of which have attempted to elucidate the underlying basis for and mechanisms behind this symbiosis. Perhaps it can be partially accounted for by the peculiar qualities of the gentians themselves. Many species in the Gentianaceae have been shown to live mycotrophic lifestyles ranging from symbiotic to saprophytic to semi-parasitic (Neumann, 1934; Weber, 1984, Knobel and Weber, 1988). In addition, many gentians alter their morphology and life cycle (whether annual, biennial, perennial, etc.), exhibiting different ecotypes in response to habitat and the presence and type of mycotrophy established within

that habitat (Karlsson, 1974). Mycoheterotrophy and semi-parasitism, which is considered a rare occurrence in the plant kingdom, is actually a fairly common arrangement in the Gentianaceae.

Mycorrhizae in the Gentianaceae have a distintive morphology , which represents an extreme example of the more unusual "Paris" type of mycorrhizae. This type of mycorrhizae is characterized by thicker, periodically septate intra-radical hyphae, intracellular hyphal coils, gnarled finger-like arbuscule, and very infrequent vesicles, rather than the aseptate hyphae, typical tree-like arbuscules and frequent vesicles seen in the "Arum" type commonly observed in the majority of plant species (Fig. 3, p. 9). These two morphologically distinct mycorrhizae were first recognized and differentiated by Gallaud (1905); recently these distinctions have received renewed attention (Smith and Smith, 1986). Jacquelinet-Jeanmougin and Gianinazzi-Pearson (1983) demonstrated that the same VAM fungus, *Glomus mosseae*, which formed a typical "Arum" type of mycorrhizae in the roots of onion (*Allium cepa* L.), formed a "Paris" type of mycorrhizae in gentian roots.

The distinctive morphology of the "Paris" mycorrhizae, exemplified by the hugely reduced surface interface area between fungus and host, suggests more of a storage and embodiment function, and less of the nutrient exchange function which is so elegantly expressed by the typical tree-like arbuscular structure found in the majority of mycorrhizal plant species. The "Paris" morphology is thought by some researchers to indicate a mycoheterotrophic rather than mutualistic relationship, one in which it is likely that the fungus benefits little, if at all, or for a very limited period of time (Knobel and Weber, 1988; Smith and Smith, 1996).

Several researchers have noted that gentian mycorrhizae begin to degrade, or senesce, one to several months after the initial colonization (Neumann, 1934; McGee, 1985; Jacquelinet-Jeanmougin, et al, 1987). Jacquelinet-Jeanmougin and her colleagues observed that although there was a slight increase in the metabolism of the colonized gentian root cells during the initial phase of root colonization (most typical VAM colonization causes greatly increased metabolism at this initial stage of colonization; see Powell and Bagyaraj, 1984), metabolic activity was greatly enhanced during and after fungal senescence, as indicated by the presence of enlarged nuclei and numerous mitochondria, plastids, endoplasmic reticulum, and other organelles in the cytoplasm of the gentian host. When the VAM fungi is degraded, its contents are evacuated, releasing phophate-rich polyphosphate granules into the host cytoplasm, which is characterized at this point by high alkaline phosphatase activity, indicating a breakdown of these substances for use by the gentian host.

These investigators eventually reached the same conclusion as Smith and Smith (1990, 1996) and others, namely, that the morphology of the structures involved in root-fungus interactions evidence the nature of the symbiosis, and that the "Paris" type of VA mycorrhizae implies host control over the mycorrhizal symbiont. If this is the case, then this control reaches its apex in the Gentianaceae, which as mentioned contains the most extreme form of the "Paris" type of mycorrhizae. In fact, Weber and Heymons (1987), both of whom have done considerable work with gentians, consider Gentianaceae to be an excellent plant family with which to demonstrate the phylogenetic tendencies of mycorrhizae; i.e., how mycorrhizal morphology and functioning are under the genetic control of the host plant. Smith and Smith (1996) conclude that under this arrangement, the gentian host is living mycoheterotrophically,

even semi-parasitically, off of the fungal symbiont, at least for a portion of the symbiosis. The gentian host, by controlling the morphology of the mycorrhizal association, also seems to be exercising control over the nature and function of the symbiosis to its maximum advantage.

Smith and Smith (1996), in discussing what amounts to a parasitic relationship by certain plant species (including gentians) on their fungal symbiont, refer to these plants as "cheaters". The gentians engage in a symbiotic relationship with the mycorrhizal fungus for a short while, but ultimately "cheat" the fungus out of its expected benefit, parasitizing it in the end (at least the intraradical portion; the external hyphae remain viable, and the VAM fungi may even go on to recolonize the roots of other plant species and complete its life cycle, producing reproductive spores - see Mcgee, 1985). This "cheating" is precisely what appears to take place in the gentian roots. One possible explanation for the ability of gentians to exercise this kind of control over their fungal partner is the presence of anti-fungal compounds such as gentiopicrin and gentiopicrosidase in gentian roots (Van der Sluis, et al, 1983). As previously noted, the concentration of these alkaloids is greater in mycorrhizal roots, perhaps as an induced response to mycorrhizal colonization (Heymons, et al, 1986). These compounds may help to explain the unusual "Paris" morphology that typifies mycorrhizae in gentian roots, characterized by reduced surface area interface between the plant host and its fungal symbiont, and perhaps reflecting a defensive response on the part of the mycorrhizal fungus to these compounds.

The variations in this mycorrhizal interaction and the accompanying metabolic activity seem to correspond to various phenological stages in the gentian life cycle and to the physiological requirements of the gentian host at different points during and after dormancy.

Mycorrhization is initially suppressed, coinciding with little to no growth in the gentian host. Finally, towards the end of the season, mycorrhizal colonization occurs, precisely when the gentian can benefit from the materials which mycorrhization provides, in preparation for dormancy. The plant host then accomodates a mutualistic relationship for a short period of time; the gentian is most likely receiving phosphorus in exchange for some photosynthate (carbohydrates) proferred to the fungus. Finally, the intraradical portion of the fungus is suddenly and completely degraded, and the contents are released into the host cytoplasm. This is accompanied by the presence of alkaline phosphatase, indicating that the host is breaking down the released polyphosphates for use in biosynthesis just prior to the plant's reemergence in the spring.

5. The role of phosphorus in dormancy and reemergence

One of the primary functions of mycorrhizae is to supply phosphorus to the plant host. Phosphorus is vital to the entire perennialization and overwintering process, from dormancy in autumn to reemergence in the spring. The importance of phosphorus throughout dormancy and reemergence has been investigated by several researchers. Phosphorus has been shown to play an integral role in the following processes: a) the synthesis and function of abscisic acid (ABA) (Wareing, et al, 1983); b) the formation of starch in roots (Miller, 1957); c) the production of phospholipids, which function in root cell membranes; d) the protection of roots from freezing; and e) biosynthetic processes involved in new tissue development during reemergence.

Phosphorus is necessary for the synthesis and function of abscisic acid (ABA) implicated in the precipitation and maintenance of dormancy in virtually all herbaceous perennial plants (Wareing, et al, 1983). ABA production starts with pyrophosphate (Hirai, 1986); in addition, ABA functions by inhibiting the action of growth hormones and stimulating the production of storage proteins, a process which is catalyzed by phosphate kinases. Subsequently, there is a buildup of P and P compounds in root storage organs during the onset of dormancy , in the form of sugar phosphates, phytin, nucleoside phosphorus, and phospholipids (Appleman, 1914, Villiers, 1975). Another major process in preparing for dormancy is the build up of carbohydrates in the form of starch. Starches in perennial root structures such as rhizomes function primarily as a souce of stored energy to be tapped by the reemerging plant in the spring. In addition, starch formation in roots is essentially a condensation reaction which removes excess water from root cells, a critical step in the protection of roots from freezing temperatures (Dodd, 1962). Phosphorus is crucial to the formation of starch; the actual method of starch synthesis consists of a phophorylation process (Miller, 1957) involving the bridging of glucose-1-phosphate molecules and the removal of water and phosphoric acid by the enzyme alpha-glucosan phosphorylase.

Phosphorus is also involved in other physiological and biochemical processes which occur in perenniating roots. Phosphorus increases the strength and decreases the permeability of root cell membranes by augmenting phospholipids in these membranes (Raven, 1987). Phospholipids, along with glycogen and other lipids, may also help protect perennial roots from freezing by acting as a sort of anti-freeze (Villiers, 1975). It is possible that mycorrhizae, which consist largely of polyphosphates, glycogen, and phospholipids (Jacquelinet-Jeanmougin et al,

1987), and as such are tolerant of sub-zero temperatures, may actually embody these phosphorusreserves and anti-freeze compounds in the host plant roots, providing P as needed and helping to protect colonized root tissue from freezing. There is in fact some evidence that mycorrhizal roots play a role in herbaceous plant cold hardiness and overwintering survival (Hayman, 1979; Maronek, et al, 1981).

Perhaps the most important role of phosphorus is in the reemergence of the dormant plant in spring. Phosphorus is a fundamental part of the biosynthetic, tissue-building processes inherent in new spring growth. Phosphorus-based compounds such as ATP and $NADPH_2$ undergo phosphorylation reactions which provide the large amounts of energy necessary to fuel these biosynthetic processes. The pentose phosphate pathway (PPP), which is the main energy-yielding pathway in new tissue reemerging from dormancy (Raven, et al, 1987), produces $NADPH_2$, which provides energy directly to the cell for biosynthesis, and is also oxidized by mitochondria in the cell to yield ATP. The PPP also produces five-carbon sugars such as ribose-5-P and erythrose-4-P which , along with nucleoside phosphates, are precursors in the synthesis of nucleotides and the nucleic acids RNA and DNA. These nucleic acids are essential building blocks of life and function in the storage, transcription and translation of genetic information for cell reproduction and development integral to the production of new tissue.

6. Mycorrhizae and its role in the perennialization of root tissue

Mycorrhizae may also play a role in the formation of tubers, rhizomes and other perenniating structures in plants. This has been widely observed in orchidaceous species, but less so in non-orchid species. Magrou (1921, 1929) and Waksman (1929) reported

this phenomenon in several non-orchidaceous plant species, including *Lathyrus*, *Solanum*, and *Mercurialis*. Roots of these species which were normally fine and hair-like would thicken and form rhizomes in the presence of symbiotic fungi; conversely, plants that were rhizomatous formed fine roots in sterile soil in the absence of fungal symbionts. Neumann (1934) observed a similar phenomenon, and noted that in response to fungal infection, the root tissues of gentian "undergo an increase in growth so sudden and irregular that the position of the various elements is changed and an abnormal development of cambial tissue takes place." This may actually refer to the formation of perennial structures, which it seems to resemble; it also corresponds to the significant enlargement and thickening of *G. crinita* roots which were observed in response to mycorrhizal infection in late autumn of 1994 and 1995. As mentioned, these enlarged roots and their mycorrhizae may actually enhance overwintering survival of perennial plants (Hayman, 1979). The additional root growth observed by Weber and Heymons (1987) would almost certainly increase the survival of overwintering gentians, as some perennial roots invariably succumb to freezing temperatures, and a greater number of roots improves the chances that at least some will survive the overwintering period intact.

7. Fringed Gentian and other root-associated fungi

The non-glomalean , non-mycorrhizal endophytes found associated with the roots of Fringed gentian during the 1995 experiments were not anticipated, and were therefore not correlated with any survival or growth data. Other researchers have reported the presence of these unusual non-pathogenic root-inhabiting fungi in the roots

of gentians, variously referred to as dark-septate endophytes, or DSE (Read and Haselwandter, 1981; Stoyke and Currah, 1990), grey-septate or grey-sterile endophytes (Neumann, 1934; Harley, 1969), dark-septate fungi, or DSF (Currah and Van Dyck, 1986), and even pseudo-mycorrhizal fungi (Wang and Wilcox, 1985). Some of these fungi, which occur both inside and on the surface of roots, have been cultured and identified as various species of *Phialocephala* and *Phialophora*, formerly known collectively as *Mycelium radicis atrovirens (*Richard and Fortin, 1973, Wang and Wilcox, 1985). Experimental inoculations of indigenous plant hosts with these endophytes were shown to increase their growth (Hasselwandter and Read, 1982; Wang and Wilcox, 1987), but more research is needed to verify this phenomenon. It is not known what effect they may have on gentians.

These fungi are present in the roots of many alpine, wetland, heathland and desert species which live in habitats with low levels of VAM. In some of these habitats, such as alpine tundra, up to 75% of the plant species surveyed, including gentians, exhibit some form of mycotrophy other than VA mycorrhizal associations (Read and Haselwandter, 1981; Currah and Van Dyk, 1986; Stoyke and Currah, 1990). These other endophytic associations appear to be non-pathogenic, as they are rarely accompanied by diseased tissue. This suggests a possible ecological role; perhaps these fungi fill the niche in low-VAM habitats that would otherwise be occupied by VA mycorrhizal fungi. Many gentians, including Fringed gentian, originated in alpine floristic regions (Weatherbee and Crow, 1990) and evolved in conjunction with these fungal endophytes. As such, it is possible that these endophytes may play an ecological role in the growth and

development of gentians. Coincidentally, these root inhabiting fungi were found in *G. crinita* roots from July to October during the 1995 experiment (most likely originating from the unsterilized compost component of the growing medium) which represents the more active growth phase of the Fringed gentian. This suggests that these fungi may play some role in the growth of the gentian host, though these experiments were not designed to address this question..

Many of these endophytic fungi form sclerotia-like resting structures in the cortical cells of gentians, indicating a fungal response to an inhospitable environment. The presence of constituent and induced anti-fungal compounds in roots of gentians may elicit this response. These and other fungitoxic compounds often elicit similar hyphal distortions and the production of pseudoparenchymatous tissues in culture (Campbell, 1989). The unusual "Paris" formation of gentian mycorrhizae - thick, finger-like arbuscules and intracellular hyphal coils in contrast to the more common finely-branched arbuscular structure - reduces hyphal surface area, thereby minimizing contact with these anti-fungal compounds. It is possible that hese structures may reflect a defensive morphology in these fungal endophytes much as they do in the mycorrhizae.

It may be the lack of these root-associated fungi that accounts for the difficulties encountered during cultivation of Fringed gentian. The incorporation of these indigenous fungal endophytes may enhance the propagation and transplant survival of this and other plant species. This proposition , which has also been advanced by other researchers and conservationists (Falk and Olwell, 1992; Brumback, 1993; Somers, 1993), certainly deserves further study.

B. 1996 Experiments

1. Disease suppression experiments

The purpose of the 1996 experiments was to investigate, under controlled conditions, the possible pathogen-suppressive properties of the VAM inoculum used in the 1994 and 1995 experiments, and to confirm and elucidate the results obtained in those experiments. The underlying hypothesis was that some component of the mycorrhizal inoculum, perhaps a suppressive rhizosphere microflora associated with the pot-culturing process which produced the inoculum, had reduced the incidence of root and crown disease in *P. hirsutus* (1994) and *G. crinita* (1995). It was believed that the mechanisms behind this phenomenon might be further discerned through a series of disease suppression and soil dilution and plating experiments.

Several important features distinguished the 1996 disease suppression experiments from the 1995 trials. First, the three predominant genera of pathogenic fungi found infecting the roots of the 1995 transplants (*Rhizoctonia*, *Fusarium*, and *Pythium*) were paired with easy-to-grow plant hosts (radish, basil, and lettuce, respectively) known to be susceptible to these same pathogens. Second, the experiments were designed to test the effect of mycorrhizal inoculum without actual mycorrhizal root colonization of the plant hosts. This was attempted by using at least one plant host which was known to be non-mycorrhizal (radish) and surveying the plants ten to twenty days after pathogen inoculation, before mycorrhizal colonization could occur (as it turned out, the basil was measured four months after inoculation, but had little to no mycorrhizae at the time of survey). Third, the host plants were germinated and grown in the same medium (i.e., they

were not transplanted), to eliminate the effects of transplantation on the outcome, and to minimize the possibility of contamination. Fourth, the inoculum component of the treatment media was increased to 1/5th, or 20%, from the 1/8th, or 12% used in 1995, in an attempt to obtain less ambiguous results. Fifth, the remaining base component of the various treatment media, which was a commercial growing mix (Fafard II™), was steam-sterilized in order to eliminate other soil microflora, including pathogens, which could have potentially influenced the outcome of these experiments.

Finally, and perhaps most important, a non-mycorrhizal pot culture inoculum was included in one of the treatment media (M3). This is a relatively rare event in mycorrhizal research, and is considered by this author to be a significant oversight. Almost all mycorrhizal research is carried out with inoculum created via the pot-culturing process, a production system which involves a living plant host (known as a "nurse" plant) in an inherently non-sterile growing medium. The result is a product which is essentially a mycorrhizosphere-based inoculum, composed of a thick matrix of mycorrhizal roots, hyphae, spores and medium. Root exudates, as well as associated rhizosphere and mycorrhizosphere microflora (those found with non-mycorrhizal and mycorrhizal roots, respectively) are an integral and active component of this pot culture inoculum. When this inoculum is incorporated into a growing medium for mycorrhizal growth experiments, the entire array of components, including microflora, are transferred with the inoculum. However, the influence of this associated microflora is rarely considered in the outcome of these investigations, and it remains largely unaccounted for. While mycorrhizal pot culture inoculum may indeed exert a strong influence on test plants, the vast majority of studies focus only on the effects on the

host plant once it is mycorrhizal, and generally disregard the effects on host plants prior to mycorrhization, when the influence of this associated microflora would most likely predominate. Even after mycorrhization has occurred, associated microflora transferred from the pot culture inoculum could conceivably continue to influence the makeup of the microflora in the mycorrhizosphere. In any case, without treatments which control for this effect, the researcher can not be sure what portion of the results obtained is due to actual mycorrhizal colonization, to mycorrhizally-associated microflora, or to the root exudate/microflora component of the inoculum itself.

This is particularly true when Sudex (a sorghum/sudangrass hybrid) is used as the "nurse" plant in the pot culture, as was the case in the present study. The use of Sudex as a pre-plant cover crop has been associated with a reduced incidence of black root rot in field-grown strawberries (Cooley, 1995). Black root rot is a disease complex involving root infection by *Pythium*, *Rhizoctonia*, and nematodes, the first two of which also happened to be the two major fungal pathogens infecting *G. crinita* in the 1995 experiments. The mechanisms behind this disease reduction phenomenon in strawberries are not known. One possibility is that the Sudex, a highly mycorrhizal plant, forms mycorrhizae with the indigenous VAMF in the field, thereby creating a suppressive mycorrhizosphere microflora. Another possibility is that Sudex root exudates directly suppress these pathogens or do so indirectly by influencing the microflora towards this end. This phenomenon certainly warrants further investigation. In any case, it was important for the present study that a non-mycorrhizal pot culture inoculum was included as a control, in light of the fact that Sudex

was the nurse plant used and *Pythium* and *Rhizoctonia* were two of the pathogens being tested.

The inoculation of lettuce with *Pythium* failed to produce any disease, and so could not be used for data collection and analysis. However, the radish/*Rhizoctonia* and basil/*Fusarium* experiments yielded interesting results. In both experiments, the growing medium containing active pot culture inoculum (M3 and M4) exhibited a suppressive effect against their respective pathogens, as indicated by the enhanced survival of the pathogen-inoculated seedlings. Thus, the inclusion a non-mycorrhizal pot culture inoculum as one of the media (M3) would appear to be justified. This inoculum actually exhibited some suppressive effect, although suppression was significantly augmented by the mycorrhizal component included in M4 (Figs. 62 and 63, pp. 141-142).

In the radish/*Rhizoctonia* experiment (Table 16, p. 139, and Fig. 62), both M3 and M4 resulted in significantly greater survival than the two sterilized control media (M1 and M2). The pathogenicity of the particular *Rhizoctonia* strain being used was apparent in M1 and M2, where every seedling germinated in these media and inoculated with this pathogen was killed. The fact that M3 exhibited some suppression of *Rhizoctonia* suggests that the Sudex root/media matrix that comprised the inoculum was responsible for at least a significant portion of the suppressive effect. However, when the same inoculum was mycorrhizal, i.e., contained mycorrhizal roots, spores and associated mycorrhizosphere microflora (as in M4), pathogen suppression, and seedling survival, was significantly enhanced.

Since radish, as a member of the mustard family, Cruciferaceae, is non-mycorrhizal, the reduction in disease seen in M3 and M4 was most likely attributable to some component of the pot culture inoculum itself, which was transferred into these treatment media when the inoculum was incorporated. Evidence that a biotic factor in the inoculum, such as a suppressive microflora, rather than an abiotic factor such as root exudate, was responsible for the suppression observed is provided by the fact that M2, which contained sterilized pot culture inoculum, provided no suppressive effect whatsoever.

The creation of a beneficial microflora, including one that is suppressive to pathogens, associated with mycorrhizal roots and the pot-culturing process itself has been discussed previously (pp. 28-30 and p. 149). Iqbal, et al (1988) observed similar results when mycorrhizal inoculum was incorporated into the germination medium sown with *Brassica campestris* (field mustard) and *Brassica napus* (rapeseed). Though this is a non-mycorrhizal plant species, and no mycorrhizal colonization occurred, the medium into which the inoculum had been incorporated showed significantly greater resistance to damping-off caused by *Rhizoctonia solani*. The authors did not discuss the possibility that the mycorrhizal inoculum may have contained a suppressive microflora which protected the seedlings from *R. solani*; nevertheless, this was a likely scenario. The authors attributed the outcome to the "biological deterrence" effect of endomycorrhizas, but did not account for the fact that the seedlings were non-mycorrhizal. This is understandable, given the fact that at the time this study was undertaken, little to no work had been done on the creation of a beneficial mycorrhizosphere microflora during the pot culturing process.

The Basil/*Fusarium* results were comparable to the Radish/*Rhizoctonia* results (Table 17, p. 139, and Fig. 63), although it took four months for the *Fusarium* pathogen to cause disease in the basil (Figs. 64 and 65, pp. 143-144). Nevertheless, the treatment medium containing active pot culture inoculum displayed analogous suppression against this fungal pathogen, and once again, the presence of a mycorrhizal component in the inoculum significantly enhanced this suppressive effect. This echoes the results seen by Caron, et al (1986), who demonstrated a reduced population of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in the rhizosphere of mycorrhizal tomatoes when compared to non-mycorrhizal tomatoes. In the present study, the incorporation of mycorrhizal Sudex roots and associated hyphae and spores lowered the incidence of disease in basil, presumably by reducing the population of the *Fusarium* pathogen (mycorrhizal colonization was not detected in the basil roots, eliminating this as a factor in the outcome). Furthermore, the fact that both of the media containing active pot cultures (M3 and M4) were still exerting a suppressive effect after four months is consistent with the 1995 results, which also suggested a long-term effect resulting from germination in a mycorrhizally-inoculated medium, transferred with the roots during transplant. It is interesting to note that M2, which contained sterilized pot culture inoculum, did have a small, albeit significant, effect compared to the control medium (M1), implying an abiotic factor such as root exudates in the suppression of this particular pathogen. This also parallels the conclusions of Jalali and Jalali (1991), who in a review of a study by Caron, et al, (1986), attributed decreased *Fusarium* populations to the increased root exudation of arginine and other inhibitory amino acids in the mycorrhizosphere of mycorrhizal tomatoes.

The results of both disease suppression experiments indicate that the pot culture inoculum itself, even without a mycorrhizal component, confers some suppressive factor, whether biotic or abiotic, to the growing medium into which it is incorporated. Nevertheless, the presence of a mycorrhizal component in the pot culture inoculum (i.e., mycorrhizal Sudex roots and associated hyphae, spores and microflora) significantly enhances the suppression of these soil-borne root infecting fungal pathogens, and by extension, reduces the disease incidence of seedlings and transplants in these media. Based on the results of these 1996 disease suppression experiments, it seems likely that this is what occurred with the gentian seedlings in the 1995 experiments, resulting in their increased survival.

2. Soil dilution and plating

This 1996 soil dilution and plating series demonstrated that TM 14, the mycorrhizally-inoculated growing medium which supported the highest survival rate of *G. crinita* in the 1995 experiments, supported the lowest population levels of soil microflora when compared to the non-mycorrhizal media (TM 11) and a control (TM 12). When total soil microorganism populations (at least of the three types selected for in this study) were compared, the significantly lower population in TM 14 suggests that there was a generalized suppressive effect occurring in this medium (Fig. 66, p. 145). When each type of organism was looked at individually, it was revealed that the bulk of this difference was accounted for by the reduced population of soil fungi in TM 14. Bacterial and actinomycetes population levels were not affected. This fungal data are of particular significance, since the pathogens infecting the roots of *G. crinita* were soil-borne fungal pathogens. This provides additional

evidence that there was indeed a suppression of these pathogens in TM 14, the mycorrhizally-inoculated growing medium, which protected the *G. crinita* seedlings germinated and transplanted into TM 14 from disease during the 1995 experiments (It should be noted again that GM 1 and TM 14 were essentially the same medium).

The suppression of soil microflora may have influenced the 1995 results in other ways as well. Saprophytic rhizosphere microflora normally present in growing media compete with the plant root for resources (water and nutrients). By significantly suppressing their numbers, more resources are made available to the plant (Schippers, et al, 1987; Campbell, 1989; Christensen and Jakobsen, 1993). Particularly noteworthy was the presence of phophorus-solubilizing organisms on the TM 14 (mycorrhizal medium) dilution plate, as indicated by "zones of clearing" in the cloudy culture medium (caused by potassium phosphate), which were not present on the two control plates from the non-mycorrhizal media (TM 11 and 12). The phenomenon of a unique or altered microflora in the mycorrhizosphere (previously discussed on pp. 28-30, and again on p. 149) can partially explain this difference. The presence of phosphorus-solubilizing organisms in the rhizosphere make more phosphorus available to the plant by transforming the normally unavailable pool of phosphorus in the soil into a form available to the plant, one which is more readily available for uptake by plant roots (Okon and Hadar, 1987). This would almost certainly enhance the growth and survival of these plants. Increased P, in addition to enhancing growth, induces physiological changes in the root which would make it less susceptible to pathogen infection and disease (see p. 30). Reduced root cell membrane permeability through the augmentation of the phospholipid bilayer decreases root exudation, which diminishes populations of pathogenic fungi by

depleting their nutrient base. Also, increased P uptake is associated with increased production of lignin and other insoluble polysaccharides in root cell walls. Either or both of these may have played a role in the increased survival of Fringed gentian in the mycorrhizal medium, although this evidence remains circumstantial.

The soil dilution and plating results revealed that in the mycorrhizally-inoculated treatment medium (TM 14), soil microorganism populations, particularly of soil-borne fungi, were suppressed. This, combined with the results from the disease suppression experiments, provided further insights into the possible mechanisms by which the mycorrhizal inoculum may have reduced disease incidence and enhanced growth in *G. crinita* seedlings and transplants.

CHAPTER IX

SUMMARY AND CONCLUSIONS

VA mycorrhizal pot culture inoculum conferred a pathogen-suppressive influence to the treatment medium into which it was incorporated and protected plants germinated or transplanted into that medium. Increased transplant survival was most likely due to this suppressive effect, which reduced pathogen populations in the growing medium. This occurred in the 1994 preliminary trials with *Penstemon hirsutus* and *Gentiana clausa,* as well as in the 1995 and 1996 experiments, where the growth and survival of *G. crinita* and other test plants were significantly enhanced. These beneficial effects occurred despite the lack of actual mycorrhizal root colonization of the plant hosts, suggesting that the underlying basis of lower disease incidence and enhanced growth is the suppressive influence of mycorrhizally-associated microflora from the inoculum rather than the direct influence of mycorrhizal colonization of the host. While enhanced survival was due to pathogen suppression, the exact mechanism of growth enhancement in Fringed gentian and the 1994 test plants (*G. clausa* and *P. hirsutus*) remains undefined. It was most likely due to suppression of competitive saprophytic microflora and root-infecting pathogenic fungi.

Strong circumstantial evidence and observations indicated that the germination medium used in the 1995 experiments was the source of contamination by soil-borne root- and crown-infecting fungal pathogens which plagued *G. crinita* throughout the course of the experiment. Therefore, these *G. crinita* experiments can be interpreted as bioassays which tested the effectiveness of VAM inoculum as a suppressive agent against these pathogens. In order to

further test this hypothesis, pathogen suppression and soil dilution and plating experiments were set up to confirm and elucidate these results under controlled conditions.

The outcome of the 1996 pathogen suppression experiments with *Rhizoctonia* and *Fusarium* indicated that the mycorrhizal inoculum reduced the incidence of disease in susceptible plant hosts when these hosts were germinated and grown in a mycorrhizally-inoculated medium. The inclusion of a non-mycorrhizal pot culture inoculum in one of the treatments demonstrated that a significant portion of this pathogen suppression was attributable to the pot culture inoculum itself, even without its mycorrhizal component. Nevertheless, the addition of a mycorrhizal component significantly enhanced this suppressive effect.

The results of the 1996 soil dilution and plating series indicated that mycorrhizal inoculum had a general suppressive effect on soil microflora, particularly soil fungi. This was a very informative result, since the pathogens causing disease in the test plants from all three years (1994-96) were soil-borne fungi. These results provided strong evidence that the basis of disease suppression in the test plants was the incorporation of a mycorrhizal inoculum in the growing medium, and that this suppressive effect was most likely due to the associated microflora which developed during the pot-culturing process.

These findings are corroborated by numerous reports in the literature of a beneficial mycorrhizae-specific (or mycorrhizosphere) microflora. Also reported is the creation of an antagonistic microflora selected for by the pot-culturing process and suppressive against various pathogens, including those seen in the 1994-96 experiments. This applies to damping-off diseases of seedlings as well, which is caused by these same pathogens and which amounts to a pre-mycorrhization event under these circumstances.

The results of these experiments imply that rhizosphere microflora play a significant role in growth and survival of *G. crinita* , which probably ranks among the most horticulturally recalcitrant of plant species. VAM pot culture inoculum significantly enhances the growth and survival of Fringed gentian in culture by conferring to the medium into which it is incorporated a suppressive influence on soil microflora in general and soil-borne pathogens in particular. Fringed gentian appears to rely on a particular constellation of rhizosphere microflora for optimum growth and survival, and mycorrhizal inoculum added to a compost-based potting medium may provide just the right mix necessary to tilt the balance in favor of survival.

There is also ample evidence, both from the literature and from this study, that mycorrhization plays a role in the perennialization and dormancy of Fringed gentian. The primary evidence is the timing and development of mycorrhizal colonization. The gentian initially suppresses the mycorrhizal fungi throughout the growing season. During the onset of dormancy, mycorrhization occurs. In response colonization by mycorrhizal fungi, root "overgrowth", an abnormal development of root tissue, takes place. This may be a direct morphological response to colonization. It may also reflect the production of starch necessary for root perennialization, for which phosphorus provided by the mycorrhizae is required. In any case, this process, which appears to represent the formation of the root perennial structures (i.e. rhizomes) preceding dormancy, was exhibited in the root thickening (up to 2x normal width) observed by this author and other investigators, which accompanied mycorrhizal colonization.

Throughout the first part of the dormancy period, mycorrhizae serve as both a provider and embodiment of phophorus reserves, which function as a storage reservoir and in the protection of roots from freezing. Finally, towards the end of the overwintering period,

disintegration of arbuscules occurs, releasing phosphorus compounds which the plant utilizes for biosynthesis during reemergence. The unusual "Paris" morphology of mycorrhizae in the Gentianaceae, coupled with the correlation between the development of mycorrhization and the physiological needs of the gentian host at various phenological stages during the overwintering period from dormancy to reemergence may indicate host control over the association. These circumstances further suggest that the gentian is living mycoheterotrophically or semi-parasitically off of its fungal symbiont, at least for a portion of the symbiosis, obtaining both carbohydrates and phosphorus. This emerges as a fairly common arrangement among species in the Gentianaceae.

Gentians also form associations with other non-pathogenic root-associated fungi, and this was observed in the roots of Fringed gentian during the current investigation. There is evidence that these fungi, known as "pseudomycorrhizal" or dark-septate endophytes (DSE), may have some ecological significance and may be analagous to mycorrhizal fungi in habitats with low populations of VAM. These fungal endophytes may even provide benefits to their plant host, though this was not investigated in the present study.

From the observations of this author, in conjunction with a detailed review of the literature, a picture emerges which begins to shed light on the peculiar nature of the mycorrhizal symbiosis in Fringed gentian. Mycorrhization appears to be a phenologically-timed and functionally-defined symbiosis which is for the most part under the control of the gentian host. The resulting association is not the classic symbiosis seen in most plants, which typically involves nutrient exchange during active growth. Rather, it is one whose function is relegated to root development in preparation for dormancy, temporary symbiosis, phosphorus embodiment and storage, and finally, the dissolution of the fungal symbiont for its phosphorus reserves, to meet the specific needs of the gentian host at

various phenological stages throughout this portion of its life cycle. Given the integral role that mycorrhizal colonization, and the P it provides, play in perennialization, dormancy, overwintering survival and reemergence, the adoption of unusual mycorrhizal and "pseudomycorrhizal" associations makes ecological sense. Fringed gentian, which evolved in highly-stressed habitats under conditions of scarcity , needed to develop alternative phenological and mycotrophic strategies in order to survive.

These results demonstrate that VAM inoculum, when incorporated into a compost-based germination and growing medium, can significantly improve the propagation of Fringed gentian and other herbaceous perennials in the greenhouse. Nevertheless, cultivation protocols must be adapted to accommodate both the host plant and its fungal symbiont. Furthermore, the particular VAM isolate used must be selected on the basis of its ability to adapt to and function under the particular mycotrophic and cultivation requirements of the plant species being grown, no matter how unusual the plant host.

In conclusion, VAM inoculum holds great potential as a bio-suppressant, bio-protectant, and bio-enhancement agent in horticultural production systems. There is much room for improvement in this area, as production of herbaceous perennials and nursery stock is currently a very high-input system. The use of VAM and VAM inoculum may contribute to the ultimate goal of all ecologically-based plant enhancement and disease management strategies - an overall reduction in the use of chemical inputs, specifically fungicides and synthetic fertilizers - and help to advance the development of sustainable plant production systems.

APPENDIX

LAB TECHNIQUES FOR INVESTIGATING VAM

Materials:

Root staining

1 2g root sample
15 ml 10% KOH
15 ml 1% HCL
1 sampling bottle (small)
15 ml 0.05% methyl blue solution
1 petri dish
1 millipore filter disc (w/grid)
15 ml 50% glycerol
forceps
autoclave
dissecting scope
1 fine-tipped paintbrush or needle
1 vacuum filter pump
1 blue paper disc
50 micron nylon mesh
horticultural sand
Whatmans filter paper
35 ml 50% sucrose solution
3 50ml centrifuge tubes (with caps)
1 petri dish
parafilm
dissecting scope

Spore extraction

1 50g soil sample (from pot culture or field)
1 sampling bottle (large)
sieves (in microns)- 500m (no.35), 250m (no. 60), 125m, (no.120), and 53m (no. 270).
1 large-mouthed funnel
1 large beaker
1 fleaker (combination flask and beaker)
100 mg. kaolin clay
1 water spray bottle

Procedure:

Root staining

1. Washing - Wash root sample clear of soil by placing roots in small sampling bottle, filling with enough water to cover, and shaking bottle gently for 10 seconds. Pour off water.

2. Clearing - Pour 15ml of 10% KOH solution into bottle; autoclave for 15 minutes at 121 C (15 psi).

3. Rinsing - After autoclaving, pour off KOH solution, and refill bottle with water. Soak roots for 10 seconds, swirl gently for 10 seconds, then pour off water. Repeat this two more times.

4. Acidification - Refill bottle with 15 ml 1% HCL and soak 2-3 minutes. Pour off HCL.

5. Staining - Refill bottle with 15 ml methyl blue solution; autoclave roots in stain solution for 15 min. at 121 C (15 psi.).

6. Destaining - After autoclaving, pour off stain solution. Place roots on filter paper (grid side up) in petri dish filled with 50% glycerol. Cover, apply parafilm, and allow to soak for several days, for observation during the next lab period.

Procedure:

Spore extraction

1. Mix 50g soil sample in large sampling bottle with 200 ml water. Shake vigorously for 1 minute, until aggregates are dispersed evenly and suspended. Pour entire solution into fleaker, and allow heavier particles to settle for 15 seconds.

2. Pour solution slowly (decant) through series of sieves stacked on top of one another (see diagram); sieves are placed on top of funnel into large beaker to catch liquid. Refill fleaker with approximately 200 ml tap water.

3. Resuspend heavy particle residue by swirling fleaker gently. Allow to settle for 10 seconds. Decant again through sieves, catching liquid in bottom beaker.

4. Repeat above process until water in beaker is clear (2-3 times), and only pure sand is at bottom of fleaker. Set large beaker with drained liquid aside.

5. Discard sievate from first (500 m) sieve. Collect sievate from 250, 125 and 53 m sieves, and wash with spray bottle into separate 50 ml centrifuge tubes; fill to 2/3 capacity with water, add 100mg kaolin clay, and replace caps.

6. Centrifuge for 5 min. at 2000 rpm (make sure centrifuge is balanced with tube of equal volume water).

7. After centrifugation, pour off water and floating debris (supernatant).

8. Resuspend pellets (solid material in tube) in 35 ml 50% sucrose solution, and centrifuge again for 1 min. at 2000 rpm.

9. This time, after centrifugation, pour debris at top of each tube (supernatant) through 50 micron sieve, and wash with water sprayer to remove sucrose.

10. With water sprayer, wash sievate through vacuum filter pump fitted with glass fibre filter disc.

11. After filtering, transfer filter disc to petri dish for examination under dissecting scope.

Observation of spores

1. Observe spores under dissecting scope at 40x magnification; sketch and record observations, including spore size, color, and morphology. Estimate total # of spores by either counting entire petri dish or counting one quadrant and multiplying by 4. Record all data (# of spores per 50g. soil).

2. Several spores can be transferred with fine-tipped paintbrush or micropipette to slides with cover slips for closer observations. Spores under cover slips can be squashed for improved viewing by lightly pressing cover slip at spore point with fine-tipped needle or forceps.

3. Spores can also be germinated, both to test % of viability and to view emerging hyphae. (see Fig. 4.1). Fill the bottom of a petri dish with 1 cm layer of horticultural sand, moisten with water, cover with blue paper disc, and place 9mm millipore filter squares (cut from filter paper) over disc. Transfer individual spores from observation dish to germinating dish using fine-tipped paintbrush or inoculating needle (spores will adhere to and release from fine-tipped instrument with a light touch). Cover, seal with parafilm and place in the dark at 20 C for two days to two weeks (check daily) until germinated.

4. Once spores have germinated, they can be observed under dissecting or compound microscope, or placed in pots filled with sterile growth media and used in greenhouse pot cultures with appropriate host plant (eg. Sorghum/sudangrass, clover, etc.).

Observation of stained roots

Destained roots can be observed in petri dishes (with 50% glycerol) under a dissecting scope at 40x to 100x. A quick visual assay can determine percentage of roots infected by counting total # of intersections between grid lines and roots, and then # of intersections with mycorrhizal structures. Try to estimate % of roots infected as well as percent of colonization within infected roots. Sketch observations and record data. For closer examination, mount roots on slide under cover slip and view with compound microscope. Look for vesicles, arbuscules, intra-radical hyphae; sketch observations.

BIBLIOGRAPHY

Abbey, F.M. 1930. Habits of the fringed gentian. *Horticulture* 8:49

Abbott, L.K. and Robson, A.D. 1981. Infectivity and effectiveness of five endomycorrhizal fungi: competition with indigenous fungi in field soils. *Aust. J. Agric. Res.* 32:621-630.

Abbott, L.K. and Robson, A.D. 1984. The effect of root density, inoculum placement, and infectivity of inoculum on the development of vesicular-arbuscular mycorrhiza. *New Phytologist*, 97:285-299.

Addy, H. 1993. Extraction and staining of VAM hyphae, pp. 18-24. In: L. Melville (ed.), *Ninth North American Conference on Mycorrhizae - Anatomy Workshop Handbook.* University of Guelph, Ontario.

Allen, M.F. and Freise, C.F. 1990. Mycorrhiza and reclamation success: Importance and measurement. In: *Evaluating Reclamation Success: The Ecological Consideration -- Proceedings of a Symposium*. General Technical Report NE-164, Forest Service, USDA.

Allen, M.F. 1991. *The Ecology of Mycorrhizas.* Cambridge University Press, Cambridge, UK.

Ames, R. N., Reid, C.P.P., and Ingham, E.R. 1984. Rhizosphere bacterial populatión responses to root colonization by a vesicular-arbuscular mycorrhizal fungus. *New Phytologist* 96:555-563.

Ames, R.N., and Linderman, R.G. 1978. The growth of Easter Lily (*Lilium longiflorum*) as influenced by vesicular-arbuscular mycorrhizal fungi, *Fusarium oxysporum*, and fertility level. *Can. J. Bot.* 56:2773-80.

Amsler, M. 1933. Synonynity and culture of the commoner European gentians. *Gardener's Chronicle* 93:58.

Ange, R.M., Schenkel, K.A. and Womple, R.L. 1986. Greater leaf conductance of well watered vesicular mycorrhizal plants is not related to phosphorous nutrition. *New Phytologist*, 103:107-116.

Appleman, C.O. 1914. Biochemical and physiological study of the rest period in the tubers of *Solanum tuberosum*. *Maryland Agricultural Experiment Station*, Bulletin 183. pp. 181-226.

Avidson, J.M., 1990. Management of rhizosphere dynamics to control soilborne pathogens and promote plant productivity. *Grant Proposal for Regional Project* (# S-241).

Azcon, R., Rubio, R., Morales, C., and Tobar, R. 1989. Interactions between rhizosphere free-living microorganisms and VAM fungi. *Agriculture, Ecosystems, and Environment* 29:11-15.

Bagyaraj, D.J. 1991. Ecology of vesicular-arbuscular mycorrhizae. In: Arora, D.K., Rai, B., Mularji, K.G., and Knudsen, G.R.(eds.) *Handbook of Applied Mycology. Soil and Plants* 1:3-34.

Bagyaraj, D.J., and Menge, J.A. 1978. Interaction between a VA mycorrhiza and and their effects on rhizosphere microflora and plant growth. New Phytologist 80:567-573.

Baltruschat, H. and Schonbeck, F. 1972. The influence of endotrophic mycorrhiza on the infestation of tobacco by *Thielaviopsis basicola*. *Phytopathology* 84:172-188.

Barea, J.M., Azcon, R., and Hayman, D. 1975. Po ssible synergistic interactions between *Endogone* and phosphate-solubilizing bacteria in low-phosphate soil. In: B. Mosse, F.E. Sanders, and P.B. Tinker, (eds.) *Endomycorrhizas*. Academic Press, New York.

Barker, A.V., and Dery, J.C. 1986. *Assessment of Agricultural Activities on Cropland in Massachusetts*. The Environmental Institute, Univ. of Massachusetts, Amherst.

Barrows, J.B., and Roncadori, R.W. 1977. Endomycorrhizal synthesis by *Gigaspora margarita* in poinsettia. *Mycologia* 69:1173-84.

Bentivenga, S. 1995. Personal communication. INVAM, West Virginia University, Morgantown, WV.

Berger, J. J. 1990. *Environmental Restoration: Science and Strategies for Restoring the Earth*. Island Press, Washington, D.C.

Berthelin, J., and Leyval, C. 1982. Ability of symbiotic and non-symbiotic rhizosphere microflora of maize (*Zea mays*) to weather micas and to promote plant growth and plant nutrition. *Plant and Soil* 68:369-377.

Biermann, B.J. and Linderman, R.J. 1983a. Effect of container plant growth medium and fertilizer phosphorus on establishment and host growth response to vesicular-arbuscular mycorrhizae. *Journal of the American Society of Horticultural Science* 108(6):962-971.

Biermann, B.J. and Linderman, R.J. 1983b. Increased geranium growth using pretransplant inoculation with a mycorrhizal fungus. *Journal of the American Society of Horticultural Science* 108(6):972-976.

Bledsoe, C. Klein, P. and Bliss, L.C. 1990. A survey of mycorrhizal plants on Truelove Lowland, Devon Island, N.W.T., Canada. *Can. J. Bot.* 68:1848-1856.

Bonfante-Fasolo, P. and Spanu, P. 1992. Pathogenic and endomycorrhizal associations. In: *Methods in Microbiology*, Vol. 24, Academic Press, NY. pp. 141-167.

Bowen, G.D. 1978. Integrated and experimental approaches to the study of growth of organisms around roots. In: B. Schippers and W. Gams (eds.) *Soil-borne Plant Pathogens*. Academic Press, London. pp. 209-227.

Boyle, T. 1993. Personal communication. Dept. of Plant and Soil Sciences, University of Massachusetts, Amherst, MA.

Brumback, W.E. 1989. Notes on propagation of rare New England species. *Rhodora*, 91:154-162.

Brumback, W.E., 1993. Personal communication. New England Wild Flower Society, Garden in the Woods, Framingham, MA.

Brumback, W.E. and Curtis, W.C. 1986. *Garden in the Woods Cultivation Guide*. New England Wildflower Society, Framingham,MA.

Brundrett, M.C. 1993. Bioassay measurements of mycorrhizal inoculum in soils, pp.50-52. In: L. Melville (ed.) *Ninth North American Conference on Mycorrhizae - Anatomy Workshop Handbook*. University of Guelph, Ontario.

Brundrett, M.C., and Kendrick, B. 1988. The mycorrhizal status, root anatomy, and phenology of plants in a sugar maple forest. *Can. J. Bot.* 66:1153-73.

Brundrett, M.C., and Kendrick, B. 1990a. The roots and mycorrhizas of herbaceous woodland plants. I. Quantitative aspects of morphology. *New Phytologist* 114:457-468.

Brundrett, M.C. and Kendrick, B. 1990b. The roots of mycorrhizas of herbaceous woodland plants. II. Structural aspects of morphology. *New Phytologist* 114:469-479.

Bryla, D.R. and Koide, R.T. 1990. Regulation of reproduction in wild and cultivated *Lycopersicon esculentum* (Mill.) by vesicular arbuscular mycorrhizal infection. *Oecologia* 84:74-92.

Campbell, R. 1989. Biocontrol of diseases in roots. In: R. Campbell (ed.) *Biological Control of Microbial Plant Pathogens*. Cambridge University Press, UK. pp. 113-160.

Carling, D.E. and Brown, M.F. 1982. Anatomy and physiology of vesicular-arbuscular and non-mycorrhizal Roots. *Phytopathology* 72(8):1108-1114.

Carlock, M. 1991. Recipe for natives. *American Horticulturist.* Feb. 1991, pp. 19-24.

Caron, M., Fortin, J.A., and Richard, C. 1986. Effect of *G. intraradices* on infection by *F. oxysporum f.sp. radicis-lycopersici* in tomatoes over a 12-week period. *Can. J. Bot.* 64:552-6.

Carpenter, S.E. Trappe, J. M., and Ammirati, J. Jr. 1987. Observations of fungal succession in the Mount St. Helens devastation zone, 1980-83. *Can J. Bot.* 65:716-728.

Center for Plant Conservation (CPC). 1990. *Endangerment survey*. Jamaica Plain, MA.

Chou, L.G. and Schmitthenner, A.F. 1974. Effect of *Rhizobium japonicum* and *Endogone mossae* on soybean root rot caused by *Pythium ultimum* and *Phytophthora megasperma var. sojae. Plant Dis. Rep.* 58:221-5.

Christensen, H. and Jakobsen, I. 1993. Reduction of bacterial growth by a vesicular-arbuscular mycorrhizal fungus in the rhizosphere of cucumber *(Cucumis sativus* L.). *Biology and Fertility of Soils*15:253-258.

Clapperton, M.J.and Reid, D.M. 1992. A relationship between plant growth and increasing mycorrhizal density. *New Phytologist* 120:227-234.

Clark, F. 1993. Personal communication. New England Wild Flower Society, Garden in the Woods, Framingham, MA.

Clark, R.A., and Woolsey, H.L. 1995. Personal Communication. MNHESP, Mass. Div. of Fisheries and Wildlife, Westboro, MA.

Cooke, J.C., and Lefor, M.W. 1990. Comparison of vesicular-arbuscular mycorrhizae in plants from disturbed and adjacent regions of a coastal salt marsh in Clinton, Conneticut, USA. *Environmental Management* 14(1): 131-137.

Cooley, D. 1995. IPM for control of strawberry diseases. Departmental seminar, Dept. of Plant Pathology, Univ. of Massachusetts, Amherst.

Cooper, K.M. 1984. Physiology of vesicular-arbuscular mycorrhizal associates. In: C.L. Powell and D.J. Bagyaraj (eds.) *VA Mycorrhiza*; pp. 135-186. CRC Press, Boca Raton, FL.

Currah, R.S., and Van Dyk, M. 1986. A survey of some perennial vascular plant species native to Alberta for occurrence of mycorrhizal fungi. *Canadian Field Naturalist* 100:330-342.

Curtis, W.C., and Brumback, W.E. 1986. *Propagation of Wildflowers*. New England Wildflower Society, Framingham, MA.

Daniels, B.A., and Skipper, H.D. 1982. Methods for the recovery and quantitative estimation of propagules from soil. In: N.C. Schenck (ed.) *Methods and Principles of Mycorrhizal Research*; pp.29-35. American Phytopathological Society, St. Paul, MN.

Datnoff, L.E., Nemec, S., and Raid, R.N. 1991. Influence of various vegetable potting mixes on colonization of tomato by *Glomus intraradix*. *Proceedings of the Florida State Horticultural Society* 104:253-257.

Davis, R.M., Menge, J.A. and Zentmeyer, G.A. 1978. Influence of vesicular-arbuscular mycorrhizae on *Phytophthora* root rot of three crop plants. *Phytopathology* 68:1614-17.

Davis, R.M., Menge, J.A., and Erwin, D. 1979. Influence of *Glomus fasciculatus* and soil phosphorus on *Verticillium* wilt of cotton. *Phytopathology* 69:453-6.

Davis, R.M., and Menge, J.A. 1980. Influence of *Glomus fasciculatus* and soil phosphorus on Phytophthora root rot of citrus. *Phytopathology* 69. 453-456.

Deacon, J.W. 1973. *Phialophora radicicola* and *Gaeumannomyces graminis* on roots of grasses and cereals. *Trans. Br. Mycol. Soc*. 61:471-485.

Dehn, B. and Schuepp, H. 1990. Influence of VA mycorrhizae on the uptake and distribution of heavy metals in plants. In: V. Mejstrik (ed.) *Ecological and Applied Aspects of Ecto- and Endomycorrhizal Association*. *Agriculture, Ecosystems and Environment* 29(1-4):79-83.

Dehne, H.W., Schonbeck, F., and Baltruschat, H. 1978. The influence of endotrophic mycorrhizae on plant diseases: III. Chitinase activity and the ornithene cycle. *Z. Pflanzenkrankh.Pflanzenschuts* 85:666-678.

Dehne, H.W. and Schonbeck, F. 1979. The influence of endotrophic mycorrhiza on plant diseases: I. Colonization of tomato plants by *Fusarium oxysporum* f.sp. *lycopersici*. *Phytopathol. Z*. 95:105-110.

Dehne, H.W. 1982a. Interaction between vesicular-arbuscular mycorrhizal fungi and plant pathogens. *Phytopathology* 72:1115-9.

Dehne, H.W. 1982b. Morphological alterations under the influence of endotrophic mycorrhiza. *Beitrage Biologie der Pflanzen*. Vol. 56.

Dehne, H.W. 1982c. The use of vesicular-arbuscular mycorrhizal fungi in plant production. I. Inoculum production. *Journal of Plant Diseases and Protection* 93(4):415-24.

Devore, J., and Peck, R. 1993. *Statistics: The Exploration and Analysis of Data* (2nd ed.). Duxbury Press, Belmont, CA.

Dodd, J.D. 1962. *Form and Function in Plants*. Iowa State University Press, Ames, IA.

Douds, D.D., and Schenck, N.C. 1990. Increased sporulation of vesicular-arbuscular mycorrhizal fungi by manipulation of nutrient regimens. *Applied and Environmental Microbiology* 56(2):413-18.

Edriss, M.H., Davis, R.M. and Burger, D.W. 1984. Increased growth response of citrus to several species of mycorrhizal fungi. *Horticultural Science* 19(4):537-539.

Edwards, S.W. 1990. The role of historical gardens in a deteriorating global environment. *Fremontia*, 18:3-8.

Falk, D.A. 1990. Restoration of endangered species: A strategy for conservation, pp.328-334. In: J.Berger (ed.) *Environmental Restoration: Sciences and Strategies for Restoring the Earth*. Island Press, Washington, D.C.

Falk, D.A. and Olwell, P. 1992. Scientific policy considerations in restoration and reintroduction of endangered species. *Rhodora* 94:287-315.

Falk, D.A. and Holsinger, K.E. 1991. *Genetics and Conservation of Rare Plants*. Oxford University Press, New York.

Farr, D.F., Bills, G. F., Chamuris, G.P., and Rossman, A.Y., 1989. *Fungi on Plants and Plant Products in the United States*. APS Press, St. Paul, MN, pp 241-2.

Feldman, F. Junqueira, N.T.V., Lieberei, R. 1990. Utilization of VA mycorrhiza as a factor in intergrated plant protection. In V. Mejstrik (ed.) *Ecological and Applied Aspects of Ecto- and Endomycorrhizal Associations. Agriculture, Ecosystems and Environment* 29(1-4) pp.131-135.

Fitter, A.H. 1985. Functioning of vesicular-arbuscular mycorrhizas under field conditions. *New Phytologist* 99:257-265.

Gallaud, I. 1905. Etudes sur les mycorrhizes endotrophes. *Revue Generale de Botanique* 17:5-48.

Garbaye, J. 1991. Biological interactions in the mycorrhizosphere. *Experientia* 47:370-375.

Gardner, I.C., Clelland, D.M., and Scott, A. 1984. Mycorrhizal improvement in non-leguminous nitrogen fixing associations with particular reference to *Hippophae rhamnoides* L. *Plant and Soil* 78:189-199.

Gay, P. E., Grubb, P.J., and Hudson, H.J. 1982. Seasonal changes in the concentrations of nitrogen, phosphorus and potassium, and in the density of mycorrhiza, in biennial and matrix forming perennial species of closed chalkland turf. *Journal of Ecology* 70:571-593.

Geddeda, Y.I., Trappe, J.M., and Stebbins, R.L. 1983. Vesicular -arbuscular mycorrhizal fungi associated with apples grown in Oregon. *HortScience* 18(6):929-930.

Gemma, J.N., Koske, R.E., and Carreiro, M. 1989. Seasonal dynamics of selected species of V-A mycorrhizal fungi in a sand dune. *Mycol. Res.* 92(3):317-321.

George, E. Marschner, H., and Jakobsen, I. 1995. Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. *Critical Reviews in Biotechnology* 15(3/4):257-270.

Gianinazzi, S., Trouvelot, A., Gianinazzi-Pearson, V. 1990. Conceptual approaches for the rational use of VA endomycorrhizae in agriculture: Possibilities and limitations. In: V.Mejstrik (ed.) *Ecological and Applied Aspects of Ecto- and Endomycorrhizal Associations. Agriculture, Ecosystems, Environment* 29(1-4):153-161.

Giersbach, J. 1937. Some factors affecting germination and growth of gentian. *Contributions from the Boyce-Thompson Institute for Plant Research, Cornell University* 9:91-103.

Giovanetti, M. and Mosse, B. 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytologist* 84:485-500.

Graham, J.H. 1986. Citrus mycorrhizae: Potential benefits and interaction with pathogens. *Hortscience* 21(6) 1302-1305.

Griffith, B., Scott, J.M., Carpenter, J.W., and Reed, C. 1989. Translocation as a species conservation tool: status and strategy. *Science* 245: 477-480.

Grime, J.P., Mackey, J.M.L., Hillier, S.H., and Read, D.J. 1987. Floristic diversity in a modal system using experimental microcosms. *Nature* 328:420-422.

Harley, J.L., 1969. *The Biology of Mycorrhiza*. University Press, UK. pp. 235-6.

Harley, J.L. 1989. The significance of mycorrhiza. *Mycol. Res.* 92(2):129-139.

Harley, J.L. and S.E. Smith, 1983. *Mycorrhizal Symbiosis*. Academic Press, London.

Harley, J.L., and Harley, E.L. 1987. A checklist of mycorrhiza in the British flora. *New Phytologist* supplement to Vol. 103(2):1-102.

Haselwandter K, and Read, D.J. 1982. The significance of root-fungus associations in two *Carex* species of high-alpine plant communities. *Oecologia* 53:352-354.

Hayman, D.J. 1979. Mycorrhizae and its significance in horticulture. *The Plantsman* 2:214-224.

Hayman, D. 1982a. VA mycorrhizas: Their ecology and influence on rhizosphere interactions. Biological and Chemical Interactions in the Rhizosphere. *Symposia of the Ecological Research Committee.* Swedish Natural Science Council.

Hayman, D. 1982b. Influence of soils and fertility on activity and survival of vesicular-arbuscular mycorrhizal fungi. *Phytopathology* 72(8) 1982.

Hayman, D.J. 1987. VA mycorrhizas in field crop systems, pp.171-192. In G.R.Sohr (ed.) *Ecophysiology of VA Mycorrhizal Plants.* CRC Press, Boca Raton, FL.

Hedden, E. N. 1931. Pot-grown gentians from seed. *Horticulture* 9:80.

Hetrick, B.A. 1991. The influence of soil micro-organisms on mycorrhizal plant growth. Abstract of unpublished research, Kansas State University, Kansas (from *Current Research Information Systems*, USDA).

Heymons, S., Holzl, J., and Weber, H.C. 1986. VA-mycorrhiza in *Gentiana lutea*, the importance of cultivation and influence on constituents. *Planta Medica* 7:510.

Hicks, D.J., and Chabot, B.F. 1985. Deciduous forest. In: B.F. Chabot and H.A. Mooney (eds.) *Physiological Ecology of North American Plant Communities*; pp. 275-277. Chapman and Hall, New York.

Hirai, N. 1986. Biosynthesis and metabolism of abscisic acid. In Takahashi, N. (ed) *Chemistry of Plant Hormones.* CRC Press, Boca Raton, FL. pp. 223-241.

Hoflich, G., Wiehe, W., and Kuhn, G. 1994. Plant growth stimulation by inoculation with symbiotic and associative rhizosphere microorganisms. *Experientia* 50:897-905.

Hoitink, H.A.J., Herr, L.J., and Schmitthenner, A.F. 1976. Survival of some plant pathogens during composting of hardwood tree bark. *Phytopathology* 66:1359-1372.

Holden, V.L. 1978. The use of mycorrhizae in the propagation of *Arcostaphylos uva-ursi*. *Proc. Int'l. Pl. Prop. Soc.* 28:132-133.

Huang, R.S. Smith, W.K. and Yost, R.J. 1985. Influence of vesicular-arbuscular mycorrhizae on growth, water relations, and leaf orientation in *Leucaena leucocephala* (Lam) de Wit. *New Phytologist* 99:229-144.

International Union for the Conservation of Nature and Natural Resources (IUCN) 1992 -- *Guidelines for reintroductions.* Species Survival Commission, Reintroduction Specialist Group. Royal Botanical and Zoological Gardens. Kew, U.K.

International Culture Collection of Arbuscular and VA Mycorrhizal Fungi (INVAM), 1993. College of Agriculture and Forestry, West Virginia University, Morgantown, WV.

INVAM, 1992. Methods to trap indigenous fungi and establish single isolate cultures - revisited. *INVAM Newsletter* 2(1):2-3.

Iqbal, S.H., Nasim, G., and Niaz, M. 1988. Role of vesicular-arbuscular mycorrhiza as a deterrent to damping-off caused by *Rhizoctonia solani* in *Brassica oleracea. Biologia* 34(1):79-84.

Jackson, R.M., and Mason, P.A. 1984. *Mycorrhiza: Studies in Biology*. No. 159. Camelot Press, Southampton, U.K.

Jacquelinet-Jeanmougin, S., and Gianinazzi-Pearson, V. 1983. Endomycorrhizas in the Gentianaceae: I. The fungi associated with *Gentiana lutea* L. *New Phytologist* 95:663-6.

Jacquelinet-Jeanmougin, S., Gianinazzi-Pearson, V., and Gianinazzi, S. 1987. Endomycorrhizas in the Gentianaceae: II. Ultrastructural aspects of symbiont relationships in *Gentiana lutea* L. *Symbiosis* 3: 269-286.

Jacquelinet-Jeanmougin, S., 1996. Personal communication via the Myconet listserver. Laboratorie de Phytoparasitologie, Station d' Amelioration des Plantes, INRA, Dijon Cadox, France.

Jalali, B.L., Jalali, I. 1991. Mycorrhiza in plant disease control. In: D.K. Arora, B. Rai, K., Mukerji, K.G., Knudsen, G.R. (eds.) *Handbook of Applied Mycology. Soil and Plants* 1:131-154.

Janos, D.P. 1988. Mycorrhiza applications in tropical forestry: Are temperate-zone approaches appropriate? In F.S.P. Ng (ed) *Trees and Mycorrhiza. Proceedings of the Asian Seminar.* Forest Research Institute, Kuala Lumpur, Malaysia. pp. 133-188.

Janos, D.P. 1995. Personal communication. Harvard Forest, Petersham, MA.

Jarvis, W.R. 1992. *Managing Diseases in Greenhouse Crops.* APS, St. Paul, MN.

Johnson, C. 1987. Utilization of vesicular-arbuscular mycorrhizal fungi in greenhouse production of transplanted crops. In: D.M. Sylvia, D.M., J.H. Hung, and J.H.Graham, (eds.) *Mycorrhizae in the Next Decade: Practical Applications and Research Priorities*. IFAS, Gainesville, Fl. pp. 275-277.

Jordan, W.R. 1986. Restoration and the reentry of nature. *Orion* 5:14-25.

Karlsson, T. 1974. Recurrent ecotypic variation in Rhinantheae and Gentianaceae in relation to hemiparasitism and mycotrophy. *Botanika Notiser* 127:527-539.

Keister, D.L. and Cregan, P.B. (eds.) 1989. *The Rhizosphere and Plant Growth*. Beltsville Symposia in Agricultural Research, USDA. Klewer Pub. Boston.

Kiernan, J.M., Hendrix, J.W., Stoltz, L.P. and Maronek, D.M. 1983. Characterization of strawberry plants produced by tissue culture and infested with specific mycorrhizal fungi. *HortScience* 19(6):883-885.

Knobel, M. and Weber, H.C. 1988. Observations on mycorrhizae of *Gentiana verna* L. and *Voyria truncata* (Stand.) Stand. and Stey. (Gentianaceae). *Beitrage Biologie der Pflanzen* 63:463-77.

Koide, R.T. and Li, M. 1990. On host regulation of the vesicular-arbuscular mycorrhizal symbiosis. *New Phytologist* 114:59-74.

Koide, R.T., Li, M., Lewis, J. and Irby, C. 1988. Role of mycorrhizal infection in the growth and reproduction of wild vs. cultivated plants. I. Wild vs. cultivated oats. *Oecologia* 77:537-543.

Koide, R.T. and Li, M. 1989. Appropriate controls for vescicular-arbuscular mycorrhiza research. *New Phytologist* 111:35-44.

Kormanik, P.P. and McGraw, A.C. 1982. Quantification of vesicular-arbuscular mycorrhizae in plant roots. In: N.C. Schenck (ed.) *Methods and Principles of Mycorrhizal Research*; pp.37-45. American Phytopathological Society, St. Paul, MN.

Koske, R.E., and Halvorson, W.L. 1989. Mycorrhizal associations of selected plant species from San Miguel Island, Channel Islands National Park, CA. *Pacific Science* 43(1):32-40.

Krishna, K.R., Suresh, H.M., Syamsunder, J. and Bagyaraj, D.J. 1981. Changes in the course of finger millet due to mycorrhizal infection. *New Phytologist* 87:717-722.

Kuter, G.A., Nelson, E.B., Hoitink, H.A.J., and Madden, L.V. 1983. Fungal populations in container media amended with composted hardwood bark suppressive and conducive to *Rhizoctonia* damping-off. *Phytopathology* 73:1450-1456.

Lesica, P. and Antibus, K. 1985. Mycorrhizae of alpine fell-field communities on soils derived from crystalline and calcareous parent material. *Can. J. Bot.* 64:1691-1697.

Levy, Y. and Krikun, J. 1979. Effect of vesicular-arbuscular mycorrhiza in citrus jambhir water relations. *New Phytologist* 85:25-32.

Lewis, K., Whipps, J.M., and Cooke, R.C. 1988. Mechanisms of biological disease control. In: J.M. Whipps and R. D. Lumdsen (eds.) *Biotechnology of Fungi for Improving Plant Growth*. Cambridge Univ. Press, UK. pp.191-217.

Linderman, R.G. 1978. Mycorrhizae -- indispensible aids to profitable plant production. *American Nurseryman* 37:129-133, 38:70-75.

Linderman, R.G., and Hendrix, J.W. 1982. Evaluation of plant response to colonization by vesicular-arbuscular mycorrhizal fungi. A. Host Variables. In: N.C. Schenck (ed.) *Methods and Principles of Mycorrhizal Research*. APS, St. Paul, MN. pp. 69-76.

Linderman, R.G., 1982. Mycorrhizal interactions with the rhizosphere microflora: the mycorrhizosphere effect. *Phytopathology* 78:366-71.

Linderman, R. G., 1986. Managing rhizosphere microorganisms in the production of horticultural crops. *Hortscience* 21(6):1299-1306.

Linderman, R.G. 1991. Mycorrhizal interactions in the rhizosphere. In: D.L. Keister and P.B. Cregan (eds.) *The Rhizosphere and Plant Growth*. Kluwer Academic Publishers, Netherlands. pp. 343-348.

Linderman, R.G.. 1993. Effects of beneficial rhizosphere microorganisms on plant growth and health. Unpublished research, Agricultural Research Service, Corvallis, OR (From *Current Research Information Systems* - USDA).

Linderman, R.G., 1994. The Role of VAM Fungi in Biocontrol. In: Pfleger, F.L., and Linderman, R.G. (eds) *Mycorrhizae and Plant Health.* APS Press, St. Paul, MN. pp 1-25.

Ling, H. L. 1993. Personal communication. Dept. of Biology, County College of Morris, Randolph, N.J.

Longland, D.R., 1992. *New England Plant Conservation Program (NEPCOP). Wild Flower Notes* 7:9. New England Wildflower Sociey, Framingham, MA.

Magrou, J. 1921. Symbiose et tuberization. *Annales des Sciences Naturelles:Botanique* 10(4):181-187.

Magrou, J. 1929. Les champignons de mycorrhizes et leur role dand le developpement des plantes. *Proc. of the 1st International Congress of Soil Science* 3:72-91.

Malloch, D., and Malloch, B. 1981. The mycorrhizal status of boreal plants: species from northeastern Ontario. *Can. J. Bot.* 59:2167-2172.

Malloch, D., and Malloch, B. 1982. The mycorrhizal status of boreal plants: additional species from northeastern Ontario. *Can. J. Bot.* 60:1035-1040.

Maronek, D.M., Hendrix, J.W., and Kiernan, J. 1980. Mycorrhizal fungi and their importance in horticultural crop production. *Horticultural Reviews* 3:172-213.

Massachusetts Natural Heritage Endangered Species Program (MNHESP) 1992. *Massachusetts List of Endangered, Threatened, and Special Concern Species.* Div. of Fish and Wildlife, Westboro, MA.

Matschat, C. H. 1933. Raising fringed gentians from seed. *Garden Chronicle of America* 37:279-80.

McCargo, H. 1994. Personal communication. New England Wildflower Society, Garden in the Woods, Framingham, MA.

McCargo, H. 1996. Compost-based potting soils. *Horticulture* 74(3):27-30.

Mcgee, P.A. 1985. Lack of spread of endomycorrhizas of *Centaurium* (Gentianaceae). *New Phytologist* 101:451-458.

Mcgee, P. A. 1995. Personal communication. Dept. of Plant Pathology, Univ. of Adelaide, Waite Agricultural Research Institute, Glen Osmond, South Australia.

McGonigle, T.P. 1988. A numerical analysis of published field trials with vesicular-arbuscular mycorrhizal fungi. *Functional Ecology* 2(4):473-478.

McGraw, A.C., and Schenck, N.C. 1980. Growth stimulation of citrus, ornamental, and vegetable crops by select mycorrhizal fungi. *Proc. Fla. State Hort. Soc.* 93:201-205.

McMahan, L.R. 1990. Propagation and reintroduction of imperiled plants, and the role of botanical gardens and arboreta. *Endangered Species Update* 8(1):4-7.

Menge, J.A., LaRue, J., Labanauskas, C.K., and Johnson, E.L.V. 1980. The effect of two mycorrhizal fungi upon growth and nutrition of avocado seedlings grown in six fertilizer treatments. *J. of the Amer. Soc. of Hort. Sci.* 105:400-404.

Menge, J.A., and Timmer, L.W. 1982. Procedures for inoculation of plants with vesicular-arbuscular mycorrhizae in the laboratory, greenhouse, and field. In: N. C. Schenck (ed.) *Methods and Principles of Mycorrhizal Research.* APS, St. Paul, MN. pp. 59-68.

Meyer, J.R., and Linderman, R.G. 1986. Selective influence on populations of rhizosphere bacteria and actinomyces by mycorrhizas formed by *Glomus fasciculatum. Soil Biol. Biochem.* 18:191-6.

Miller, E.V. 1957. *The Chemistry of Plant*s. Reinhold Publishing, New York.

Millner, P.D., and Kitt, D.G. 1992. The Beltsville method for soilless production of vesicular-arbuscular mycorrhizal fungi. *Mycorrhiza* 2:9-15.

Morton, J. 1993. Personal Communication. INVAM, University of West Virginia, Morgantown, WV.

Mosse, B. 1973. Advances in the study of vesicular-arbuscular mycorrhiza. *Ann. Rev. Phytopath.* 11:171-196.

Mosse, B. 1975. Specificity in VA mycorrhizas. In: F.E. Sanders, B. Mosse, and P.B. Tinker, (eds.) *Endomycorrhizas.* Academic Press, New York. pp. 469-84.

National Research Council, 1995. *Ecologically-based Pest Management: New Solutions for a New Century.* Board On Agriculture, National Academy Press. Washington, D.C.

Nature Conservancy 1996. *Bulletin*, Massachusetts Chapter, Vol 6 (8), p. 6.

Nearing, G.G. 1932. Experimenting with fringed gentians. *Horticulture* 10:313.

Nelson, C.E., and Safir, G.R. 1982. Increased drought tolerance of mycorrhizal onion plants caused by improved phosphorus nutrition. *Planta* 154:407-413

Nemec, S. Datnoff, L., and Raid, R. 1991. Application of mycorrhizal fungi and biocontrol agents to control root diseases in horticultural crops. Agricultural Research Service, Univ. of Florida, Belleglade (From *Current Research Information Systems*, USDA).

Neumann, G. 1934. Uber die mykorrhiza in der gattung *Gentiana. Zbl. f. Bakt. etc. II. Abt. Bd* 89(21/24):433-58.

Newman, E.I. 1978. Root microorganisms: their significance in the ecosystem. *Biological Reviews, Cambridge Philosophical Society*: 53:511-554.

Newman, E.I., and Reddell, P. 1988. Relationship between mycorrhizal infection and diversity in vegetation: evidence from the Great Smoky Mountains. *Functional Ecology* 2(2): 259-262.

Newman, E.I., Heap, A.J., and Lawley, R.A. 1981. Abundance of mycorrhizas and root-surface micro-organisms of *Plantago lanceolata* in relation to soil and vegetation: a multi-variate approach. *New Phytologist* 89:95-108.

Norris, J.R., Read, D.J., and Varma, A.K. (eds.) 1992. *Techniques for the Study of Mycorrhiza. Methods in Microbiology*, Vol 24. Academic Press, London.

Norton, G.F. 1925. The fringed gentian. *Journal of the New York Botanical Garden* 26:38-40.

O'Dell, T.E., Massicotte, H.B. and Trappe, J.M. 1993. Root colonization of *Lupinus latifolius* Agardh. and *Pinus contorte* Dougl. by *Phialocephala fortinii* Wang & Wilcox. *New Phytologist* 124:93-100.

O'Dell, T.E., and Trappe, J.M. 1992. Root endophytes of lupin and some other legumes in Northwestern USA. *New Phytologist* 122:479-485.

Okon, Y., and Hadar, Y. 1987. Microbial inoculants as crop-yield enhancers. *CRC Critical Reviews in Biotechnology* 6(1):61-86.

Olkowski, W., Daar, S., and Olkowski, H. 1991. *Common Sense Pest Control.* Taunton Press, Newtown, CT.

Olsen, S.R., Cole, C.V., Watanabe, F.J., and Dean, L.A. 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. *Circ. 939.* Sup. of Documents, U.S. Government Printing Office, USDA, Washington, D.C.

Omernik, J.M., Griffith, G.E., Pierson, S.M., and Kiilsgaard, C.W. 1993. *Massachusetts Regionalization Project.* ManTech Environmental Technology, Inc., Environmental Research Lab, U.S. EPA, Corvallis, OR.

Pacovsky, R.S., Fuller, G., and Paul, E.A. 1980. Influence of soil on the interactions between endomycorrhizae and *Azospirillum* in sorghum. *Soil Biology and Biochemistry* 17:525-531.

Paul, E.A. and Kucey, R.M.N. 1981. Carbon flow in plant microbial associations. *Science* 213: 473-4.

Paulitz, T.C., and Linderman, R.G. 1991. Mycorrhizal interactions with soil organisms. In: Arora, D.K., Rai B., Mukerji K.G., Knudsen, G.R. (eds.) *Handbook of Applied Mycology. Soil and Plants* 1:77-129.

Phillips, J.M. and Hayman, D.S. 1970. Improved procedures for clearing and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Brit. Myc. Soc.* 55(l):157-162.

Phillips, H.R. 1985. *Growing and Propagating Wildflowers.* Univ. of North Carolina Press, Chapel Hill, NC.

Powell, C. Ll., and Bagyaraj, D.J. 1984. *VA Mycorrhiza.* CRC Press, Boca Raton, FL.

Punj, V., and Gupta, R.P. 1988. VA mycorrhizal fungi and *Rhizobium* as biological fertilizers for *Leucaena leucocephala. Acta Microbiol. Pol.* 37(3-4): 327-336.

Raven, P.H., Evert, R.F., and Eichhorn, S.E. 1987. *Biology of Plants.* Worth Publishers, New York, New York.

Read, D.J., and Haselwandter, K., 1981. Observations on the mycorrhizal status of some alpine plant communities. *New Phytologist* 88:341-352.

Read, D.J., Koucheki, H.K., and Hodgson, J. Vesicular-arbuscular mycorrhiza in natural vegetations systems. I: The occurrence of infection. *New Phytologist* 77:641-653.

Richard,C. and Fortin, J.A. 1973. The identification of *Mycelium radicis atrovirens* (*Phialocephala dimorphospora*). *Can. J. Bot.* 51:2247-8.

Rosendahl, C.N. and Rosendahl, S. 1990. The role of vesicular-arbuscular mycorrhiza in controlling damping-off and growth reduction in cucumber caused by *Pythium ultimum. Symbiosis* 9:363-6.

Ross, J.P. 1972. Influence of *Endogone* mycorrhizae on *Phytophthora* rot of soybean. *Phytopathology* 62:896-7.

Rovira, A.D. 1991. Rhizosphere research - 85 years of progress and frustration. In: D.L. Keister and P.B. Cregan (eds.) *The Rhizosphere and Plant Growth.* Kluwer Academic Publishers, Netherlands. pp. 3-13.

Safir, G. R. 1992. Environmental and chemical determinants of VA mycorrhizal root colonization. Hatch Grant # 01499, Dept. of Botany and Plant Pathology, Michigan State Univ., East Lansing MI. (from *Current Research Information Systems,* USDA).

Sanders, I.R. 1990. Seasonal patterns of vesicular-arbuscular mycorrhizal occurrence in grasslands. *Symbiosis* 9:315-320.

Sanders, F.E., Mosse, B., and Tinker, P.B. 1974. *Endomycorrhizas*. Academic Press, London.

Schenck, N.C. 1981. Can mycorrhizae control root disease? *Plant Disease* 65:230-234.

Schenck, N.C. 1982. *Methods and Principles of Mycorrhizal Research*. American Phytopathological Society, St. Paul, MN.

Schenck, N.C. and Perez, Y. 1987a. *Manual for the Identification of VA Mycorrhizal Fungi*. INVAM, Gainesville, FL.

Schenck, N.C. and Perez, Y. 1987b. The international culture collection of VA mycorrhiza (INVAM). In: D.M. Sylvia, L.L. Hung,and J.H. Graham (eds.) *Mycorrhizae in the Next Decade: Practical Applications and Research Priorities*. IFAS, Gainesville, FL. pp. 303-4.

Schippers, B., Bakker, A.W., and Bakker, P. A. H. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Ann. Rev. Phytopathol*. 25:339-58.

Schneider, K. 1993. Second chance on environment: Opportunity to redefine core of American policy on pollution. *NY Times*. March 26, Vol.CXLII, No. 49, 282, p. A17.

Schonbeck, F. 1978. Endomycorrhiza in relation to plant diseases. In: B. Schippers and W. Gams (eds.) *Soil-borne Plant Pathogens*. Academic Press, London.

Secilia, J. and Bagyaraj, D.J. 1987. Bacteria and Actinomyces associated with pot cultures of vesicular-arbuscular mycorrhizas. *Can J. Microbiol.* 33:1069-73.

Seeliger, C. 1996. Personal Communication. Humboldt State University, Arcata, CA

Smith, G.S. 1988. The role of phosphorus nutrition in interactions of vesicular-arbuscular mycorrhizal fungi with soil-borne nematodes and fungi. *Phytopathology* 78:371-4.

Smith, S.E. 1980. Mycorrhizae of autotrophic higher plants. *Biological Reviews* 55:475-510.

Smith, F.A, and Smith, S.E. 1996. Mutualism and parasitism: Diversity in function and structure in the "arbuscular" (VA) mycorrhizal symbiosis. *Advances in Bot. Research* 22:1-43.

Smith, F.A, and Smith, S.E. 1990. Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. *New Phytologist* 114:1-38.

Sneh, B., Burpee, P., and Ogoshi, R. (eds.) 1991. *Identification of Rhizoctonia Species.* APS press, St. Paul, MN

Somers, P. 1993. Personal communication. Massachusetts Natural Heritage and Endangered Species Program (MNHESP), Dept. of Fish and Wildlife Field Headquarters, Westboro, MA.

Sreenivasa, M.N., and Bagyaraj, D.J. 1989. Suitable form and level of phosphorus for mass production of the VA mycorrhizal fungus *Glomus fasciculatum*. *Zentral. Mikrobiol.* 144(l):33-36.

St. John, T.V. 1990. Mycorrhizal inoculation of container stock for restoration of self-sufficient vegetation. In: J.J. Berger (ed.) *Environmental Restoration: Science and Strategies for Restoring the Earth.*. Island Press, Washington, D.C. pp.103-112.

St. John, T.V., and Coleman, D.C. 1983. The role of mycorrhizae in plant ecology. *Can. J. Bot.* 61:1005-1014.

Stoyke, G. and Currah, R.S. 1991. Endophytic fungi from the mycorrhizae of alpine ericoid plants. *Can. J. Bot.* 69:347-352.

Summerbell, R.C. 1987. Microfungi associated with the mycorrhizal mantle and adjacent microhabitats within the rhizosphere of black spruce. *Can. J. Bot.* 67:1085-95.

Swain, P. 1996. Plant communities of New England. Class at the New England Wildflower Society, Garden in the Woods, Framingham, MA.

Sylva, D.M., Hung, L.L., and Graham, J.H.(eds.) 1987. *Mycorrhizae in the Next Decade: Practical Applications and Research Priorities.* IFAS, Gainesville, FL.

Sylvia, D.M., Hammond, L.C., and Bennett, J.M. 1988. Mycorrhizae improve drought resistance of maize. *Phytopathology* 78(12):1604.

Thomas, W.D. Jr. 1943. Mycorrhizae associated with some Colorado flora. *Phytopathology* 33:144-149.

Timonin, M.I. 1940a. The interaction of higher plants and soil micro-organisms. I. Microbial population of rhizosphere of seedlings of certain cultivated plants. *Canadian Journal of Research* C18:307-317.

Timonin, M.I. 1940b. The interaction of higher plants and soil micro-organisms. II. Study of the microbial population of the rhizosphere in relation to resistance of plants to soil-borne diseases. *Canadian Journal of Research* C18:444-455.

Timonin, M.I. 1941. The interaction of higher plants and soil micro-organisms. III. Effect of by-products of plant growth on activity of fungi and actinomycetes. *Soil Science* 52:395-413.

Torrey, J.G. and Berliner, R. 1989. Studies on mycorrhizal associations in Harvard Forest, Massachusetts. *Can. J. Bot.* 67:2245-2251.

Trueman, S.L. 1995. Personal communication. Dept. of Plant Pathology, Univ. of Massachusetts, Amherst.

Van der Sluis, W.G., Van der Nat, J.M., Spek, A.L., Ikeshiro, Y., and Labadie, R.P. 1983. Gentiogenal, a conversion product of Gentiopicrin (Gentiopicroside). *Plant Medica*, 49:211-215.

Varma, A. and Singh, K. 1986. Interaction between VA mycorrhizae, rock phosphate, and *Rhizobium* on tropical legume *Cicer arietinum* in semi-arid soils. *Transactions of the Int. Society of Soil Science*, Vol.II., pp. 654-5.

Verkade, S.D., and Hamilton, D.F. 1980. Mycorrhizae and their uses in the nursery. *Proc. Int'l. Plant Prop. Soc.* 30:353-63.

Vestberg, M. 1992. The effect of growth substrate and fertilizer on the growth and vesicular-arbuscular mycorrhizal infection of three plant hosts. *Agricultural Science in Finland* 1:95-105.

Vijayalakshmi, M. and Rao, A.S. 1988. Vesicular-arbuscular mycorrhizal associations of some Asteraceae and Amaranthaceae. *Acta Botanic Indica* 16:168-74.

Villiers, T.A. 1975. *Dormancy and the Survival of Plants.* Studies in Biology no. 57. Edward Arnold (Pub.) Ltd., London.

Waksman, S.A. 1927. *Principles of Soil Microbiology*. Williams and Wilkins, Baltimore, MD.

Wang, C.J.K., and Wilcox, H.E. 1985. New speicies of ectendomycorrhizal and pseudomycorrhizal fungi: *Phialophora finlandia*, *Chloridium paucisporum*, and *Phialocephala fortinii*. *Mycologia* 77:951-958.

Wang, C.J.K., and Wilcox, H.E., 1987. Mycorrhizal and pathological associations of dematiaceous fungi in roots of 7-month old tree seedlings. *Can. Jour. of Forestry Research* 17:884-889.

Wareing, P.F., Good, J. and Manuel, J. 1983. Some possible physiological roles of abscisic acid. In: Addicot, F.T. (ed.) *Abscisic Acid*. Praeger, New York.

Weatherbee, P.B. 1991. Calcareous habitats and their plant communities. In: *Special Habitats in New England. Wild Flower Notes*, Vol. 6(1):14-18. New England Wild Flower Society, Framingham, MA

Weatherbee, P.B. and Crow, G.E. 1990. Phytogeography of Berkshire County, Massachusetts. *Rhodora* 92(872):232-56.

Weatherbee, P.B. 1994. Personal Communication. Trustees of Reservations, Field Farm, Williamstown, MA.

Weber, H.C. 1984. "Radix Gentianae" (Roots of Gentian): from extracts of gentian roots to basic research. *Biol. Rdsch.* 22:379-81.

Weber, H.C. and Heymons, S. (1987). Gentianaceae: An excellent model to demonstrate the phylogenetic tendency of VA mycorrhiza. In: Sylva, D.M., Hung, L.L., and Graham, J.H.(eds.) 1987. *Mycorrhizae in the Next Decade: Practical Applications and Research Priorities*. IFAS, Gainesville, FL. p. 320.

Weiss, F. E. 1933. On the germination and the seedlings of gentians. *Journal of the Royal Horticultural Society* 58: 296-300.

Wick, R.L. and Moore, L.D. 1984. Histology of mycorrhizal and non-mycorrhizal *Ilex crenata* 'Helleri' challenged by *Thielaviopsis basicola. Can. J. Plant Pathology* 6:146-150.

Wilkie, D. 1977. *Gentians*. Theophrastus Pub., Little Compton, R.I.

Wilson, E.O. 1992. *The Diversity of Life*. Harvard University Press, Cambridge, MA

Wilson, G.W.T., Hetrick, B.D., and Kitt, D.G. 1989. Suppression of vesicular-arbuscular mycorrhizal fungus spore germination in non-sterile soil. *Can. J. Bot.* 67(2):18-23.

Wood, T., Nance, L., Jedrzejek, S., and Johnson, G. 1991. Use of VA mycorrhizal inoculum to improve growth of forest tree seedlings in fumigated soil. In: *Proceedings, Intermountain Forest and Nursery Association. USDA Gen. Tech. Report RM-211*. Rocky Mt. Forest and Range Exp. St., Ft. Collins, CO.

Zettler, L.W., and McInnis, T.M. Jr. 1992. Propagation of *Plantananthera integrilabia* (Correll) Luer, an endangered terrestrial orchid, through symbiotic seed germination. *Lindleyana* 7(3):154-161.

Zhengjia, H. and Xiangdong, G. 1991. Pre-transplant inoculation with VA mycorrhizal fungi and *Fusarium* blight of cotton. *Soil Biol. Biochem* 23(2):201-3.