Nutritional characteristics of *Hydrilla verticillata* and its effect on two biological control agents

J.F. Shearer, M.J. Grodowitz and J.E. Freedman

Summary

A complex of abiotic and biotic factors is known to impact the establishment and success of biological control agents. Experiments using the ephydrid fly *Hydrellia pakistanae* Deonier have demonstrated that hydrilla, *Hydrilla verticillata* (L.f.) Royle, containing low protein content appears to impact larval development time and the number of eggs oviposited per female. Eggs per female were over twofold higher for larvae reared on hydrilla containing 2.4-fold more protein. Mean adult female fly weight peaked when emergence is low (i.e. low crowding) and leaf protein content is high. The hydrilla biological control pathogen *Mycoleptodiscus terrestris* (Gerd.) Ostazeski also responds to plant nutritional condition. The nutritional status of hydrilla shoots affects *M. terrestris* vegetative growth, disease development and conidia and microsclerotia production. High protein content in shoot tissues was associated with a more than threefold increase in conidia production and maximum disease severity. In contrast, low protein content in shoot tissues stimulated a 3.7-fold increase in melanized microsclerotia, reproductive structures that are more persistent in the environment than conidia. These studies suggest that the nutritional condition of target plants cannot be excluded as an important factor in efficacy of biological control agents. Both agents responded to favorable conditions by reproducing prolifically, which ultimately resulted in increased host damage.

Keywords: *Hydrellia pakistanae*, *Mycoleptodiscus terrestris*, evaluation.

Introduction

In aquatic systems, there is scant information on the impact of biological control agents relative to the physical and nutritional characteristics of submersed aquatic macrophytes. Two agents of hydrilla, *Hydrilla verticillata* (L.f.) Royle, the Asian leaf-mining fly *Hydrellia pakistanae* Deonier (Diptera: Ephydridae) and the pathogenic fungus *Mycoleptodiscus terrestris* (Gerd.) Ostazeski (Ascomycota: Magnaporthaceae), perform extremely well under laboratory, greenhouse and experimental conditions (Doyle *et al.*, 2002; Shearer, 2002; Shearer and Nelson, 2002; Grodowitz *et al.*, 2003a,b; Owens *et al.*, 2006; Shearer and Jackson, 2006) but at times are inconsistent in their ability to successfully reduce hydrilla populations under field conditions. Since 1987, more than 20 million *H. pakistanae* individuals have been released with established populations occurring in Florida, Arkansas, Alabama, Georgia and Texas (Center *et al.*, 1997; Julien and Griffiths, 1998; Grodowitz *et al.*, 1999). Field establishment has generally been excellent with close to 90% establishment observed (Center *et al.*, 1997; T. Center, unpublished data). Populations are now found far removed from their original release sites, indicating the fly is spreading naturally throughout the southeastern United States. Significant *Hydrellia* spp. impact has been observed at sites in Texas, Florida and Georgia (Grodowitz *et al.*, 2003a,b) but significant increases in fly populations and subsequent impact have not occurred at many sites. Reasons are not completely understood.

Mycoleptodiscus terrestris reproduces asexually by thin-walled conidia and by melanized survival structures called microsclerotia. To date, sexual reproduction of the fungus has not been observed, therefore sexual spores were not an issue in this study. Conidia develop from spore-producing structures called sporodochia following ingress by the pathogen. The sporodochia form on tissue surfaces within 5 to 7 days postinoculation

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followed within a day by commencement of spore production. It has been documented that spore production may vary in relation to the substrata available and to environmental variables such as stress or disturbance (Dix and Webster, 1995). Under optimum conditions in greenhouse studies, the hydrilla pathogen *M. terrestris* is consistently pathogenic to hydrilla and can reduce shoot biomass by 97% to 99% (Shearer, 2002). However, subjecting field populations of hydrilla to similar rates of *M. terrestris* inoculum has often produced inconsistent results.

Potential factors that might limit agent performance on hydrilla include parasites, predators, temperature, water flow, turbidity, plant density, age and plant nutritional status. To better understand the importance of plant nutrition on agent performance, hydrilla plants of

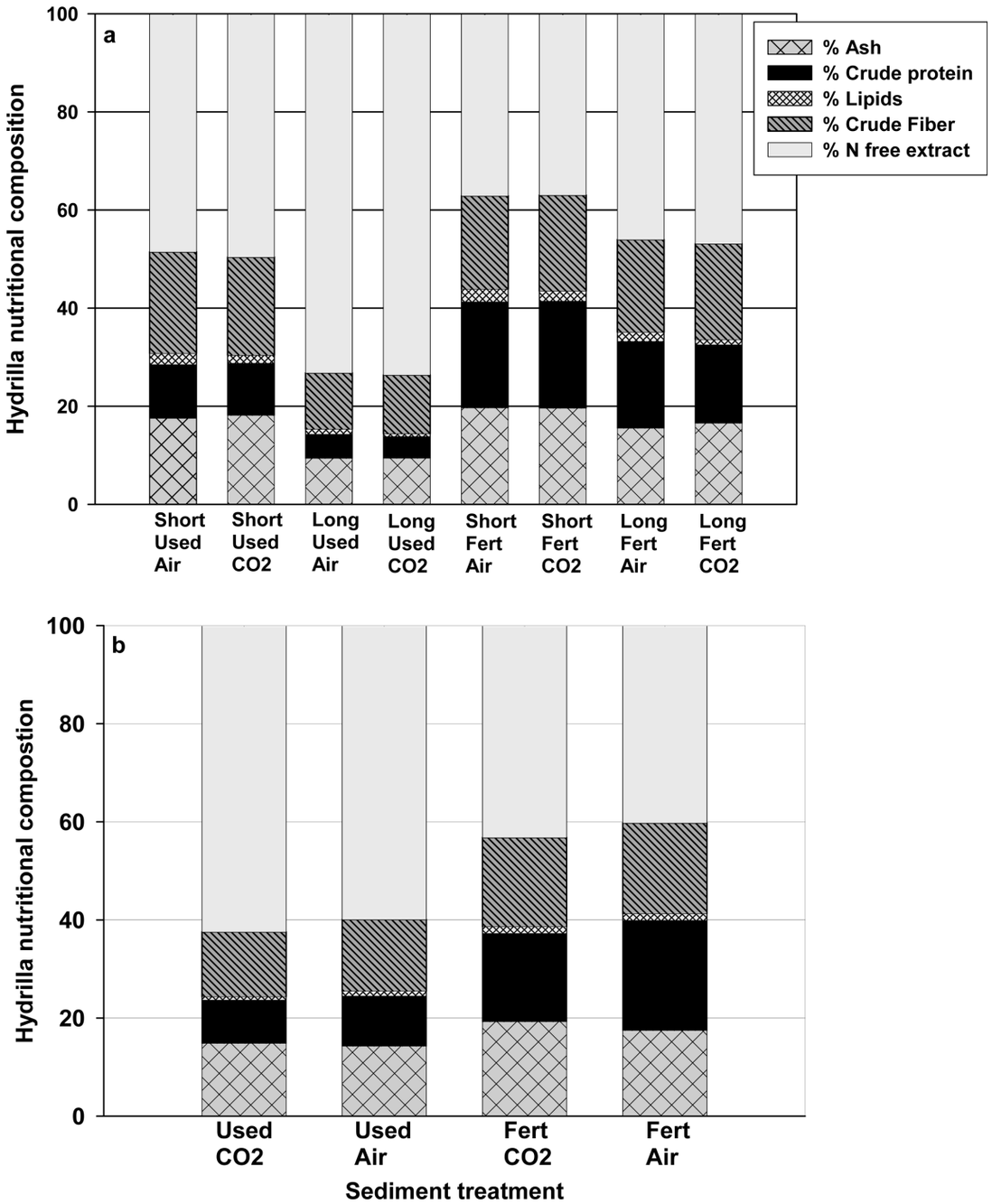

Figure 1. Proximate analysis of hydrilla shoot tissues for (a) insect and (b) pathogen study. Sediments were nutrient-deficient (Used) or nutrient-enriched (Fert = fertilized). Plants received ambient air (Air) or air enriched with carbon dioxide (CO_2). Growth periods were 4 weeks (Short) or 10 weeks (Long).

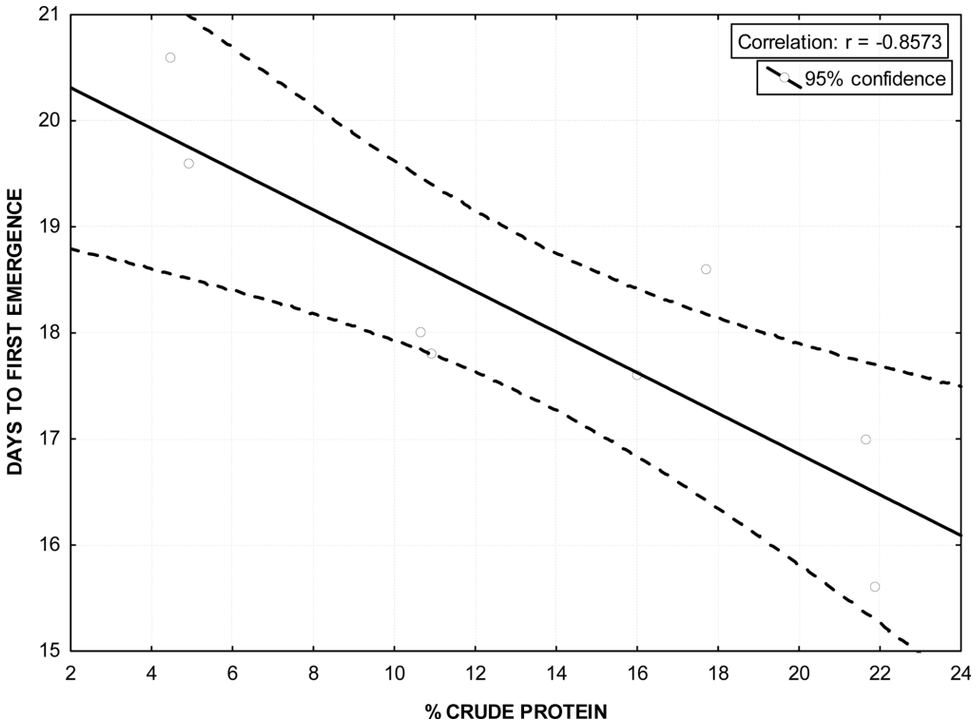

Figure 2. Correlation between crude protein and days to first emergence of *Hydrellia pakistanae*.

varying nutritional status were challenged with *H. pakistanae* and *M. terrestris*.

Materials and methods

Plant growth

Hydrilla plants of known nutritional composition were produced by growing them in used or fertilized sediments under different aeration conditions (high or low CO_2) using procedures described by Grodowitz and McFarland (2002) and Shearer *et al.* (2007). The used sediment was rendered nitrogen-poor because of previous growth of submersed macrophytes. Fertilized sediments were amended with 0.7 g NH_4Cl per liter of wet sediment. Additionally, for the insect experiment, period of growth was varied (long vs. short) to produce plants having varying degrees of leaf hardness as measured by a penetrometer. Nutritional parameters, including percent ash, crude protein, ether-extractable compounds, crude fiber and nitrogen-free extract, were determined using a standard feed analysis known as a proximate analysis described in detail by Grodowitz and McFarland (2002). Phosphorous concentration was determined using atomic absorption techniques.

Insect biological control agent

Insects were reared in a greenhouse, beginning with 50 eggs per container, on hydrilla plants of varying nutritional composition in 3.5-l containers in a water bath maintained at 22–25°C (Freedman *et al.*, 2001). Emerged adults were removed from the containers daily and released into oviposition chambers (30.5 × 30.5 × 30.5 cm). Percent emergence was calculated. Each treatment was replicated five times.

Within the oviposition chambers, females were allowed to oviposit freely onto five to seven hydrilla apical shoots held within an open 100 × 15-mm (d × h) Petri dish containing deionized water. After the adults died, the sex ratio was recorded and dessicated females were weighed. Hydrilla shoots were removed from the oviposition chambers every 3 to 5 days, eggs were identified and counted and number of eggs per female was calculated.

Pathogen biological control agent

Hydrilla apical shoots (5 cm) of variable nutritional compositions were placed in 250-ml Erlenmeyer flasks containing 150 ml sterile water and 20 µl wet inoculum. Inoculum was prepared as described by Shearer *et al.* (2007). Control flasks received an additional 20 µl of sterile water. Each treatment was replicated five times. The flasks were randomly arranged on a rotary shaker (Innova 2300, New Brunswick Scientific, Edison, NJ) set at 50 rpm and incubated at room temperature for 2 weeks.

At 7 and 14 days postinoculation, the hydrilla shoots were visually assessed for disease development based

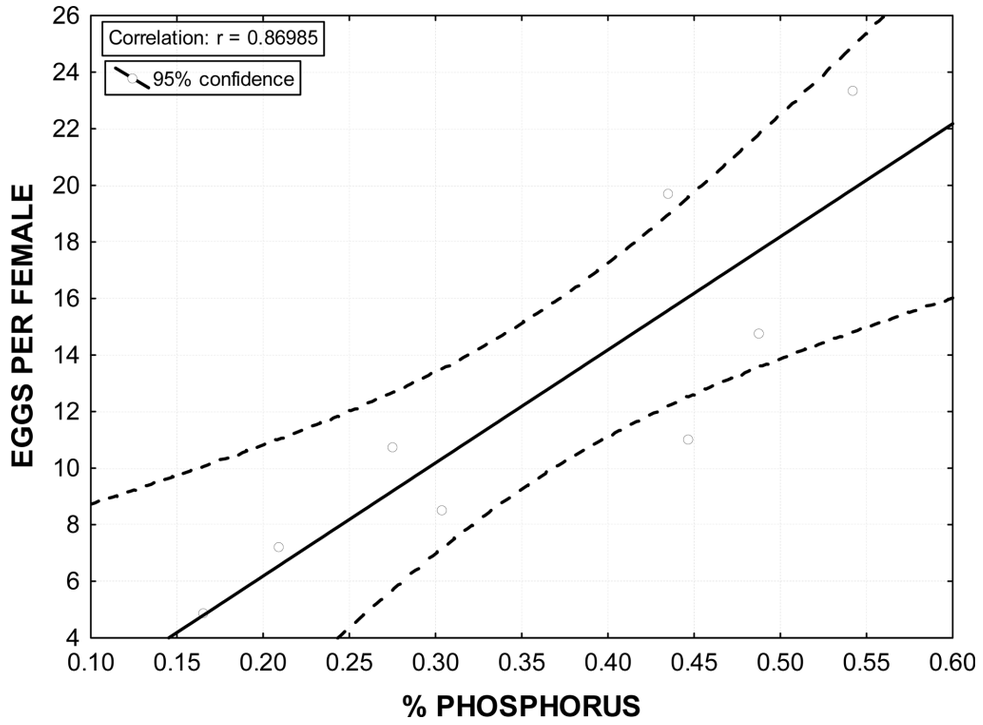

Figure 3. Correlation between hydrilla phosphorous content and eggs per female for *Hydrellia pakistanae*.

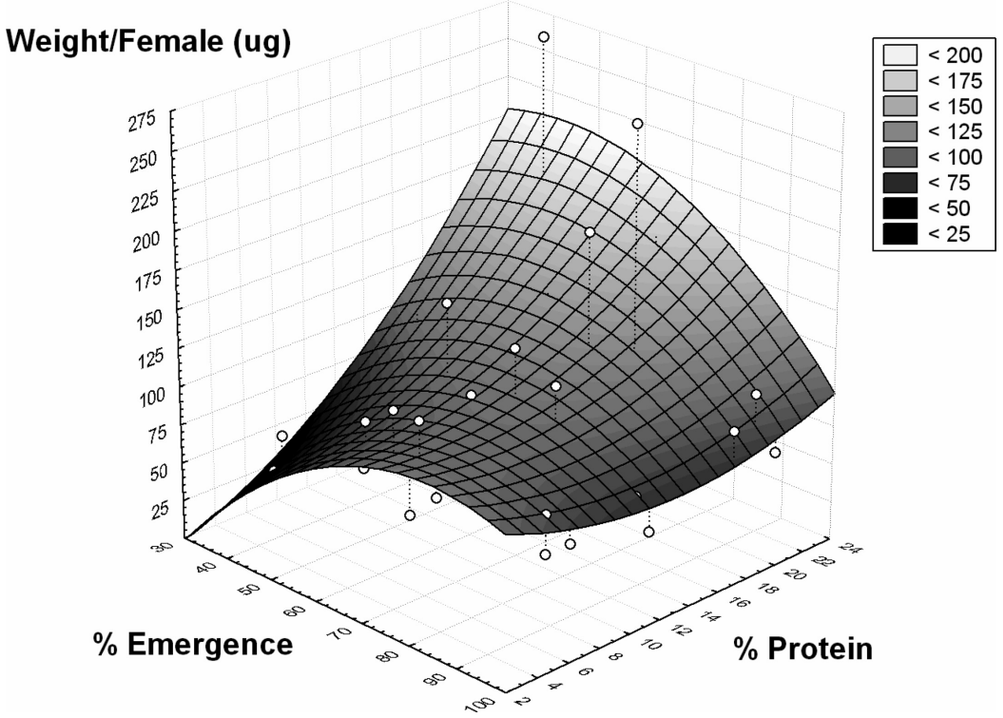

Figure 4. 3-D surface plot with data points marked for percent emergence of *Hydrellia pakistanae* vs weight per female and leaf protein content.

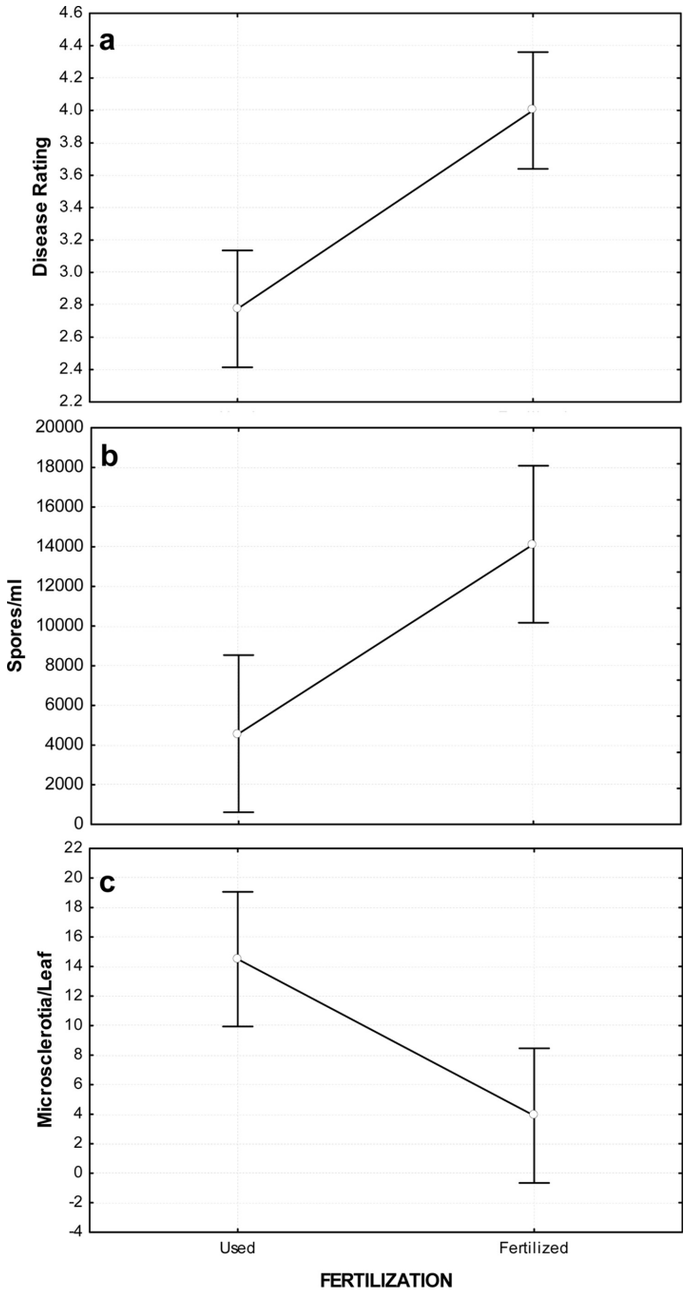

Figure 5. Effects of hydrilla fertilization levels on *Mycoleptodiscus terrestris* (a) disease development, (b) asexual spore production in the form of conidia and (c) production of survival structures or microsclerotia.

on a disease rating scale from 0 to 4, where 0 = green and healthy, 1 = slight chlorosis, 2 = general chlorosis, 3 = tissues flaccid and disarticulating and 4 = complete tissue collapse. At 14 days postinoculation, the flasks were gently shaken to dislodge any spores that had developed on infected tissue surfaces. The number of spores released into the water was then determined using a hemacytometer. Three leaves were randomly retrieved from each flask to count microsclerotia that had developed within leaf tissues.

Statistical analysis

Statistical analyses were performed using Statistica version 7.1 (Statsoft, 2005) and included ANOVA, correlation analysis and a distance-weighted least square means graphing technique to visualize three-dimensional trends with corresponding measures of the amount of variance explained (i.e. R). Statistical significance was assumed at or below $P = 0.05$, unless otherwise noted.

Results and discussion

Plant nutritional status

By manipulating growing conditions, hydrilla plants were produced with significant differences in nutritional composition for percent nitrogen-free extract (soluble sugar, starch and some hemicelluloses), crude fiber (cellulose and some lignin), ether-extractable compounds (lipids and fats), crude protein (total nitrogen) and ash (mineral content) (Fig. 1). Of particular note was that crude protein, as a measure of total nitrogen, was approximately twofold higher in plants grown in fertilized sediments compared with plants cultured in used or nutrient-depleted sediments. Protein levels were similar for corresponding treatments for plants used for both the insect and pathogen experiments.

Insect response to plant nutrition

Significant difference in days to first emergence (an indication of development time) was noted for both the fertilized (df = 1, 32, P = 0.0009) and growth period (df = 1, 32, P = 0.001) main effects only. Time to first emergence was 2 days shorter in fertilized sediments as compared with used sediments and 2 days longer for plants grown for longer periods under cooler temperatures compared with shorter growth periods at higher temperatures. As expected, a significant correlation between percent crude protein and days to first emergence was observed where higher crude protein values were associated with fewer days to first emergence (Fig. 2). This was not surprising, as similar results were noted in experiments conducted by Wheeler and Center (1996), where larvae reared on harder hydrilla leaves (and lower protein) resulted in longer developmental times.

Plants grown in fertilized sediments gave rise to female flies that laid more than twice as many eggs (df = 1, 32, $P < 0.00017$), as female flies that were reared on plants in used sediments. Mean number of eggs per female was 7.8 for used sediments compared with 17.2 for fertilized sediments. Although there was a strong linear relationship between phosphorous and protein content in plant tissues, egg production appeared to be more strongly correlated with phosphorous than protein. The r values were higher when egg numbers were correlated with phosphorous (Fig. 3, r = 0.87) than with crude protein (r = 0.65).

There is an interesting relationship among weight per female (an indication of fecundity), crude protein and percent emergence as an indicator of crowding (Fig. 4). Female fly weight peaks when emergence is low (i.e. low crowding) and protein is high. However, as percent emergence increases, leading to increased crowding and competition amongst larvae, the emerging female weight remains low even at high protein levels. Hence, crowding strongly influences female weight and most likely fecundity.

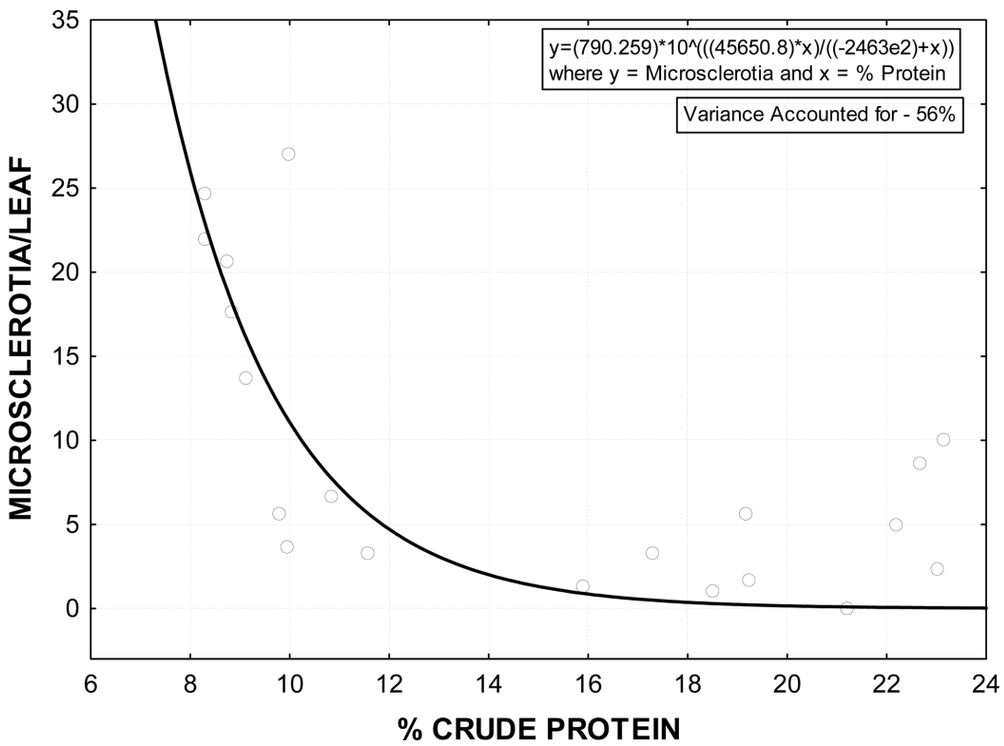

Figure 6. Relationship between percent crude protein in hydrilla leaf tissues and production of *Mycoleptodiscus terrestris* microsclerotia.

Pathogen response to plant nutrition

Fourteen days postinoculation with *M. terrestris*, disease ratings between plants grown in fertilized and used sediments were significantly higher ($df = 1, 16$, $P = 0.0001$; Fig. 5a) than for plants grown in used sediments. Although the leaves of plants grown in low-fertility sediment were chlorotic and becoming flaccid, the stems remained intact. In field situations, such plants would probably recover and regrow from undamaged root crowns (Netherland and Shearer, 1996). The highest disease severity rating (Fig. 5a) was consistently found on inoculated hydrilla that had high leaf-protein content. These plants collapsed to the bottom of the flasks and, lacking cell integrity, would have had no possibility of recovery. Other studies have documented that high leaf-protein content is often associated with increases in disease severity (Ghorbani *et al.*, 2002; Latty *et al.*, 2002).

Plant nutritional status also affected the pathogen's reproductive ability. *Mycoleptodiscus terrestris* conidial production appeared to be influenced by the substrate. This is indicated by significantly higher numbers of spores produced in flasks containing hydrilla plants grown in high-fertility sediments ($df = 1, 16, P = 0.0021$) (Fig. 5b). In contrast to conidia, significantly higher numbers of vegetative reproductive structures, microsclerotia, were present in leaves of hydrilla plants grown in low-fertility or used sediment at 14 days postinoculation ($df = 1, 16$, $P = 0.0028$) (Fig. 5c). Lacking nutrients for continued mycelial growth, *M. terrestris*, in all likelihood, used the available nutrients in plant tissues and mycelium for production of survival structures.

The highest number of microsclerotia developed in leaves from plants that had the lowest available nitrogen. The response was strongly curvilinear, suggesting that microsclerotia production may be triggered by some threshold level of leaf protein, perhaps <9% (Fig. 6). Limited nitrogen availability apparently induced changes in the pathogen that altered growth from active proliferation, i.e. conidial production, to preparation for a period of dormancy or lack of resources, i.e. microsclerotia production. A similar curvilinear response for microsclerotia numbers and spore production was observed, indicating that such a shift had occurred (Fig. 7).

Implications for biological control

Hydrilla plant nutrition affected the two very different biological control agents in similar ways. High hydrilla leaf-protein content stimulated agent reproduction as indicated by increased *H. pakistanae* female weight and fecundity and increased conidial production and disease severity in the case of *M. terrestris*. Low leaf-protein content in hydrilla negatively affected reproduction of both biological control agents, resulting in increased developmental times and reduced fecundity for the insect and a shift by the pathogen to a higher

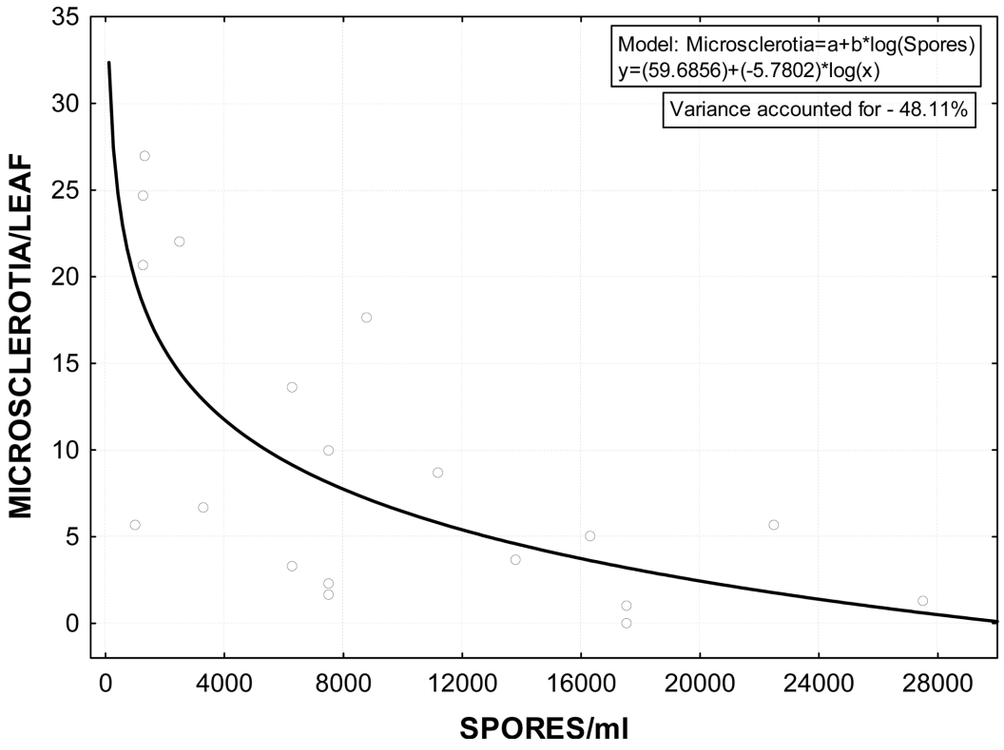

Figure 7. Relationship between *Mycoleptodiscus terrestris* microsclerotia and spore production.

production of microsclerotia. Based on the case studies, it appears that plant nutritional quality could have been a factor in inconsistent field results in the past and that higher quality plants should lead to increased agent establishment and impact. Support for this conclusion can be found in a study using the salvinia weevil, *Cyrtobagous salviniae* Calder and Sands, where fertilization of giant salvinia plants, *Salvinia molesta* D.S. Mitchell, in the field substantially aided the establishment of the agent and ultimately lead to a successful biological control effort (Room and Thomas, 1985).

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