

***Myrothecium verrucaria* isolates and formulations as bioherbicide agents for kudzu**

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Abstract

The fungus *Myrothecium verrucaria* (MV) has previously been shown to have potential as a bioherbicide for kudzu (*Pueraria lobata*) control. It has also been shown that MV wild-type (MV-wt) often forms sectors, when grown on various nutrient media. Experiments compared MV-wt and MV sector efficacy when grown on agar or on rice grains. In greenhouse evaluations of sectors, applied as foliar sprays in water or in other formulations (corn oil, surfactant, and corn oil plus surfactant) for efficacy against kudzu seedlings, some sectors possessed bioherbicidal activity equal that of MV-wt, but others exhibited lower activity. Without a dew period, aqueous formulations of MV-wt, a yellow sector, and a white sector provided zero control, but all three isolates were active without a dew period when formulated in corn oil, Silwet L-77 surfactant, and in surfactant plus corn oil. Generally, the yellow sector was less effective than the other two isolates in any formulations, and the MV-wt and white sector provided approximately 100% mortality of the test plants. Dew (10 h) increased weed control to 100, 33, and 65%, respectively, for MV-wt, the yellow sector and the white sector. All isolates provided nearly 100% control in the oil and surfactant formulations with a dew period compared to treatments receiving no dew. Soil incorporation studies were also performed to compare MV-wt efficacy of preparations grown on agar versus growth on rice grains. Higher efficacies (1.75–3.3-fold increase) were obtained from rice grain preparations compared to preparations grown on agar, when preparations were incorporated at several rates into soil prior to planting. Cell-free extracts of the MV-rice cultures were also phytotoxic to kudzu seedlings up to the eight- to 10-leaf growth stage. Thus, formulation, growth media, and the application method are important determinants in the efficacy of MV and MV sectors on kudzu seedlings.

Keywords: *Myrothecium verrucaria*, bioherbicide, biological weed control, fungal phytopathogen, kudzu, [*Pueraria lobata* (Willd.) Ohwi], surfactant

Introduction

Kudzu [*Pueraria lobata* (Willd.) Ohwi] is a perennial, leguminous vine native to east Asia, introduced into the eastern and southern US in the late 1800s (McKee & Stephens 1943). In the early 1900s, kudzu was promoted as an inexpensive pasture forage, and for erosion control in the southern US (Piper 1920). The US Soil Erosion Service supplied millions of kudzu seedlings to this region for erosion control and land

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revitalization, and the federal government offered monetary incentives for farmers to plant kudzu (Bailey 1944). By 1946, kudzu had been planted on nearly 1.25 million ha and by the early 1950s, it had spread throughout the southeastern US and was delisted as a permissible cover plant by the Agricultural Conservation Program. By 1970, the US Department of Agriculture had listed kudzu as a common weed in the southern US (Everest et al. 1994), and in 1993, a Congressional report (US Congress, Office of Technology Assessment 1993) deemed kudzu as one of the most harmful, non-indigenous plants in the US. Presently, kudzu occurs in the US from Florida to New York, westward to central Oklahoma and Texas, with the heaviest infestations in Alabama, Georgia, and Mississippi (Miller 1997).

Most of the monetary losses (approximately \$20 ha⁻¹ year⁻¹) from kudzu are due to reduced land productivity, primarily in forested areas (Beckwith & Dangerfield 1996). These productivity losses have been estimated at \$336 million year⁻¹ for 2.84 million infested ha, but expanding kudzu infestations have been estimated at 48 600 ha year⁻¹. Such estimates, however, do not include costs for weed control, estimated at \$81 ha⁻¹ year⁻¹ (Miller 1997). Foliar sprays of synthetic herbicides such as picloram, picloram plus 2,4-D, dicamba plus 2,4-D, and tebuthiuron can be effective after full leaf expansion occurs, but some of these herbicides cannot be applied near streams, ditches, etc., and repeat applications may be required annually up to 10 years for complete control (Everest et al. 1999). Burning and grazing may also be effective, but are impractical in most heavily infested areas such as urban areas, and highway rights-of-way (Everest et al. 1994). Additionally kudzu causes losses in biodiversity and reduced aesthetic values in areas such as national parks, forests, and scenic highways.

Bioherbicides are phytopathogenic fungi and bacteria that offer promise for the control of certain weeds. One example of a fungal bioherbicide is *Myrothecium verrucaria* (Alb. & Schwein.) Ditmar:Fr., originally isolated from diseased sicklepod (*Senna obtusifolia* L.). Host range tests with this fungus (IMI 361690) indicated it possessed a broad host range, but many plant species were not significantly affected (Walker & Tilley 1997; Anderson & Hallett 2004; Hoagland et al. 2007). Other studies reported that this bioherbicide could control kudzu (Boyette et al. 2001; Boyette et al. 2002; Hoagland et al. 2007), and that combination of the bioherbicide with the herbicide glyphosate resulted in a synergistic interaction against kudzu (Boyette et al. 2006a). Evaluation of this bioherbicide against some problematic weeds in tomato crops indicated that several weeds were controlled and that transplanted tomato plants into these areas were unaffected (Boyette et al. 2006b).

Many microbial bioherbicides require free moisture or a dew period for propagule germination, penetration, and infection of host targets, and various formulation ingredients have been used in attempts to replace such requirements (Charudattan 2005; Ghosheh 2005; Hallett 2005; Weideman et al. 1995). However, the wild-type *M. verrucaria* (MV-wt) exhibited excellent biocontrol potential for several weed species, including the legumes sicklepod and hemp sesbania [*Sesbania exaltata* (Raf.) Rydb. ex A.W. Hill], when formulated with the silicone-poly-ether surfactant, Silwet L-77 (OSi Specialties, Inc., Charlotte, NC) in the absence of dew (Walker & Tilley 1997). MV-wt, when formulated in Silwet L-77 was also highly virulent to kudzu in the absence of dew (Boyette et al. 2002). The objectives of the current studies were to: (1) comparatively examine MV-wt efficacy on kudzu when the organism was grown on agar or on a solid substrate (rice grains) and applied pre-plant incorporated (PPI) or as

a soil drench; (2) examine the efficacy of various formulated MV sectors compared to the MV-wt isolate on kudzu, with and without a dew period; (3) compare the efficacy of crude and cell-free MV-wt-rice grain extracts on kudzu seedlings at different growth stages; and (4) to examine the effects of Silwet-77 and corn oil alone or combined, on the efficacies of some sectors on kudzu seedlings.

Materials and methods

Media and chemical sources

Potato dextrose agar (PDA), Czapek-Dox agar, and Emerson's agar were purchased from Difco Laboratories, Inc. (Detroit, MI).¹ The surfactant, Silwet L-77 was obtained from Lovelace Industries (Greely, CO). Unrefined corn oil was from Spectrum's Naturals Co. (Petaluma, CA). Rice grain was obtained from Uncle Ben's, Co., Inc. (Houston, TX).

MV source and culture

MV cultures (IMI 361690) were obtained from H.L. Walker, Louisiana Tech University (Ruston, LA). MV conidial preparations were produced in Petri dishes containing PDA. For tests on different nutrient agars, inoculation was achieved by placing a 2-mm section from the edge of an actively growing MV-wt culture from PDA plates. Plates were then inverted on open-mesh wire shelves and incubated (25°C, 10 days) in lighted incubators. Twelve-hour photoperiods were provided by cool-white fluorescent lamps at an intensity of approximately 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR). Conidia were rinsed from plates with sterile distilled H₂O, conidia densities estimated with the aid of a hemacytometer, followed by adjustment with distilled H₂O to obtain the desired concentrations. Occasionally, sectors spontaneously formed on culture plates. Then spores of these sectors were selected and cultured on PDA or other media as described above. Sector isolates were stored on PDA slants at 4°C until cultured as described above.

Kudzu propagation

Kudzu seeds (Adams-Briscoe Seed Co., Jackson, GA 30233, USA) were placed on moistened filter paper in Petri dishes, and incubated at 28°C for 3 days in the dark. Germinated seeds were then planted in 7.6-cm plastic pots (one seed per pot) containing a 1:1 commercial potting mix (Jiffy Products of America, Inc., Batavia, IL 60510, USA):sandy loam soil combination, supplemented with a controlled-release (13:13:13, N:P:K) fertilizer (Grace Sierra Horticultural Products, Milpitas, CA 95035, USA). After placement on greenhouse benches, the plants were sub-irrigated daily. Greenhouse temperatures ranged from 28 to 32°C at 40–60% RH with a photoperiod of approximately 14 h, at 1600–1800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR as measured at midday.

¹ Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by USDA-ARS and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Fungal growth on solid substrate (rice grains)

Long-grain, converted, enriched rice grains (200 g) were mixed with 120 mL H₂O in 1-L flasks and allowed to stand for approximately 1 h until the water was absorbed. Flasks were plugged with cotton and autoclaved 60 min. After cooling, the flasks were shaken to break up clumps, allowed to stand 24 h, and then autoclaved, shaken again and cooled. The flasks were inoculated with MV spores (10 mL at 2.0×10^7 mL⁻¹) and incubated 3–4 weeks at 22°C with a 12-h photoperiod (approximately 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, PAR). Daily shaking of the flasks for the first several days assured uniform distribution/penetration of the fungus into the rice grains. At the end of the incubation, the fungal–rice mixture was spread onto screen-bottomed trays and air-dried. Dried samples were ground to a flour-like consistency (30–60 mesh) using a mill grinder (Stein Mill, Model M-2; F. Stein Laboratories, Inc., Atchiso, KS) housed in a biosafety cabinet. Crude extracts were made by stirring various amounts of this MV-rice mixture in 100 mL distilled water, for 15 min and then filtered through cheesecloth to remove larger particulates. These cheesecloth-filtered crude extracts were used directly in the pre-plant incorporated (PPI) and soil drench tests. Cell-free preparations were prepared and designated as the filtrates of crude extracts that were vacuumed through a micro-pore filter (0.45 μm) to remove cells and cellular debris.

Soil drench and PPI experiments

The PPI and soil drench experiments were performed under greenhouse conditions (28–32°C, 40–60% RH, photoperiod ca. 14 h, at 1600–1800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR at midday). PPI treatments consisted of the addition of a standard volume (15 mL) of crude filtrates from 0.25, 0.50, 0.75, and 1.00 g the MV-rice grain mixture, to soil (ca. 300 g) contained in 7.6-cm (diameter) pots. Control treatments received crude filtrates from equivalent amounts of uninoculated rice grains. Kudzu seeds were then planted into the soil, and emergence and growth was recorded 14 days after treatment (DAT). Each treatment consisted of five plants, with three replicates, and the experiment was repeated in time.

Soil drench treatments consisted of additions of a volume of crude or cell-free preparations to the soil containing kudzu plants in 7.6-cm pots. Plants were treated at the three- to five- and eight- to 10-leaf growth stages. Control treatments received crude filtrates or cell-free extracts prepared from equivalent amounts of un-inoculated rice grains. Plant growth (diameter of radiating leaf arrangement) was measured prior to treatment, and at 21 DAT. Reductions in growth of the treated plants were compared to those of untreated control plants and controls receiving crude filtrates or cell-free extracts from un-inoculated rice grains. Each treatment consisted of five plants, with three replications, and the experiment was repeated in time.

Spray applications of MV-wt and MV sectors to kudzu seedlings

Kudzu plants (two- to four-leaf growth stage) were inoculated by spraying (hand-held compressed air sprayer) suspensions containing 2.0×10^7 conidia mL⁻¹ plus 0.2% SW until the foliage was fully wetted (ca. 500 L ha⁻¹). Control plants received 0.2% Silwet L-77 surfactant only. Each replicate contained 10 pots, with three replications. The experiment was repeated in time. After inoculation, plants were placed either in a dew chamber (28°C, 100% RH) or in a dark chamber (ca. 50% RH) without dew for

8 h, and then transferred to greenhouse benches under conditions described above. Disease development was monitored, and at 14 days after inoculation, the percentage control was determined based on plant mortality. Data received an *arc sin* transformation prior to analysis (Steele & Torrey 1980). Experimental units consisted of 10 plants, treatments were replicated three times, and the experiment was repeated in time. All experiments in these studies were arranged in randomized complete block designs and means were separated using Fisher's LSD (0.05).

Results and discussion

MV and sector growth on various media

MV-wt occasionally formed sectors when cultured on media such as PDA. The morphology of MV-wt was altered, and in some cases sectors formed when the fungus was grown on several other nutrient media, e.g. Emerson's yeast peptone soluble starch (YpSs) agar, and Czapek-Dox agar and broth, and Czapek agar plus peptone (Figure 1). After sectors formed, isolates were collected, cataloged, and cultured on PDA for further studies. Several such sectors and MV-wt were cultured on autoclaved rice grains (solid substrate) in attempts to find improved bioherbicidal formulations of the parent fungus and/or its sectors (Figure 2). In the present studies, we report on MV-wt, a white sector (Figures 1 and 2) and a yellow sector (Figure 2). Morphological variants or sectors are sometimes observed when fungi are cultured on artificial media (Jennings & Lysek 1996; Vannacci & Cristani 1998). This phenomenon has been attributed to genomic rearrangement, mutation, mycoviruses, and transposons (Flower & Milton 2000; Chu et al. 2002; Firon et al. 2002; Becker et al. 2003). Sectors can exhibit morphological and physiological traits that differ from their parent

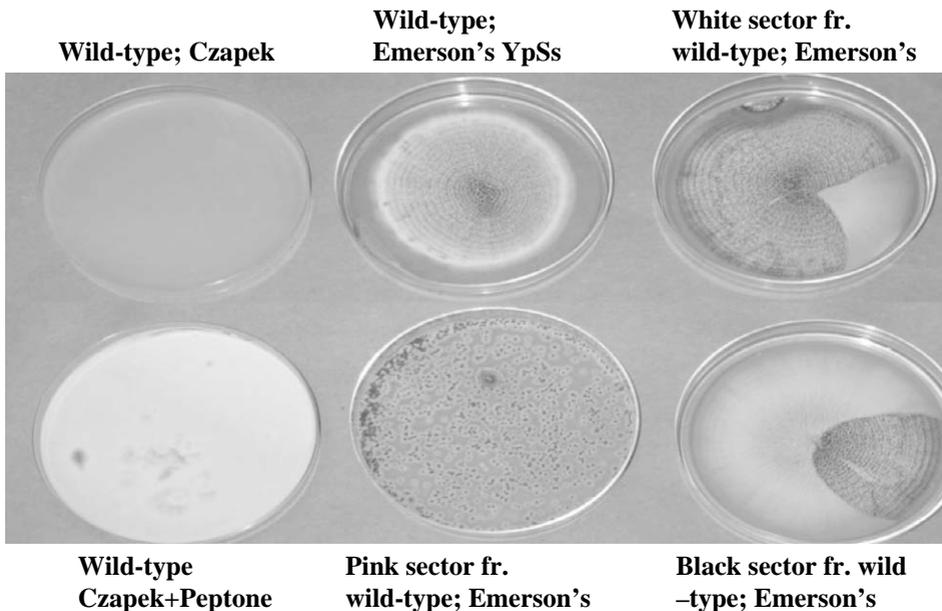


Figure 1. Photograph of selected MV-wt and sectors grown on various media.

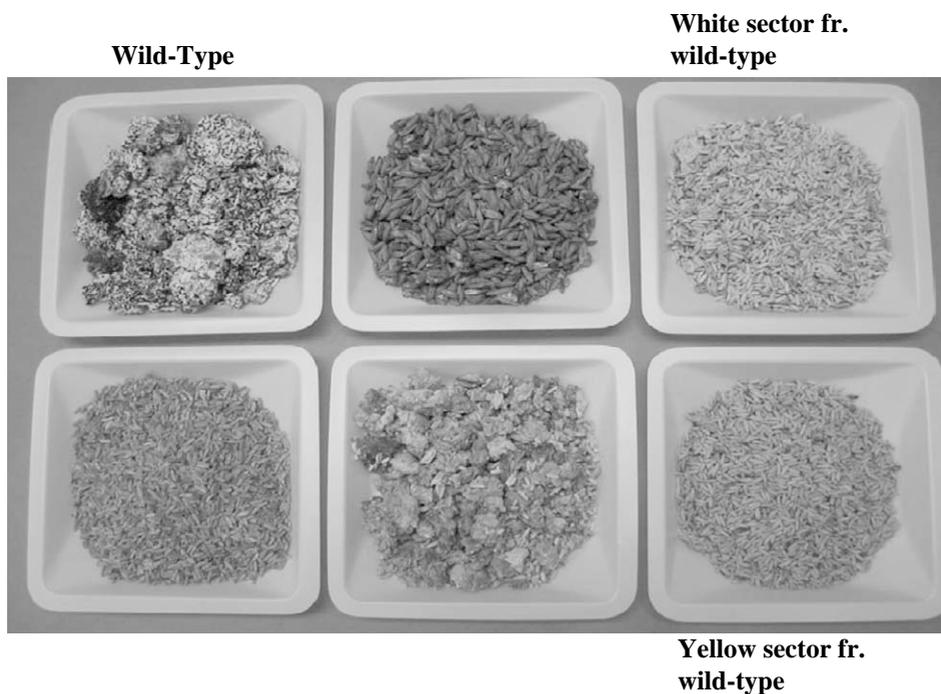


Figure 2. Photograph of various MV sectors grown on solid substrate (rice grains). Rice grain was autoclaved and then inoculated as described in the Materials and methods section.

cultures, such as spore production and metabolite production (Guzman-de-Pena & Ruiz-Herra 1997; Chu et al. 2002). Some phytopathogenic fungi that contain mycoviruses have reduced virulence (Chu et al. 2002).

Effects of dew on the efficacy of MV and MV sector formulations

Two sectors were comparatively tested for efficacy on kudzu seedlings versus MV-wt, with and without a dew period. When kudzu seedlings at the three- to four-leaf stage were individually sprayed with either, MV-wt, a yellow sector, or a white sector, in the absence of a dew period, their aqueous formulations did not infect or provide any control of this weed (Figure 3). However, formulating these isolates with a surfactant (Silwet L-77), corn oil, or the combination of corn oil and L-77, vastly improved efficacy, even without dew (Figure 3). Nearly 100% control was found in the MV-wt and white sector formulated in L-77 or corn oil. The efficacy of the yellow sector was increased above that of its aqueous formulation by formulating with L-77 and corn oil, but still this surfactant or adjuvant provided only approximately 65 and 32% control, respectively. L-77 can enhance the wetting of plant foliage and increase stomatal penetration of aqueous solutions (Field & Bishop 1988). This surfactant has also been reported to increase infection of kudzu by a bacteria pathogen (*Pseudomonas syringae* pv. *phaseolicola* van Hall) via facilitating direct penetration of bacterial cells through stomata (Zidak et al. 1992). Contrary to this, we observed some antagonism of MV-wt efficacy in the combined L-77 and corn oil formulation since this treatment provided

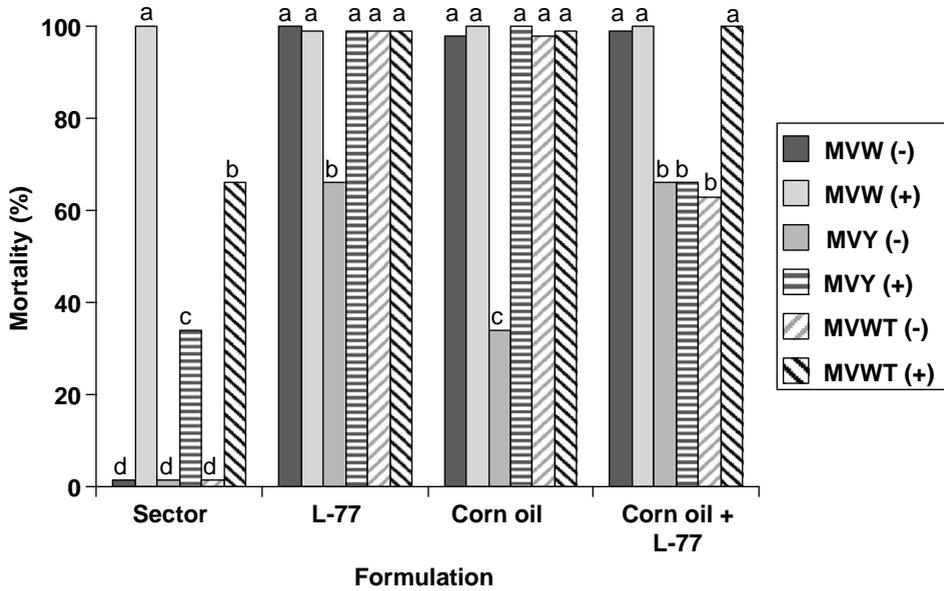


Figure 3. Kudzu control with MV-wt and two sectors with and without a dew period. Column means with the same letter are not statistically different according to Fisher’s LSD test (0.05).

control equal to that when the sector was applied with L-77 alone, i.e. approximately 65% (Figure 3).

A dew period of 10 h increased efficacy of the aqueous formulations of these three isolates, but only the white sector provided 100% control, while the yellow sector and MV-wt isolate provided 30 and 65% control, respectively (Figure 3). All other isolates and formulations tested, except the yellow sector in corn oil plus L-77, provided 100% control. Adequate dew or free moisture to allow spore germination, mycelial growth and development, and infectivity is one of the most critical factors in bacterial and fungal bioherbicide effectiveness (Weidemann et al. 1995; Boyette et al. 1996), but various formulations such as used in these present studies can overcome a lack of moisture and provide sufficient weed control.

Efficacy of MV and sector grown on solid substrate (rice grains)

As an alternative to growing MV-wt on agar, the efficacy and formulation utility of growing the fungus on a solid substrate such as rice grains MV for kudzu seedling control was examined. When aqueous extracts of ground fungus–rice grain cultures were incorporated at several rates into soil prior to planting kudzu seeds, the efficacy at all application rates was higher than for MV-wt cultured on agar (Figure 4). The average percentage control or mortality caused by agar formulations were between 32 and 38%, regardless of application rate, however, extracts from the rice formulation increased as the rate increased, and ranged from approximately 70–100%. Solid substrate formulations of other bioherbicide preparations have also been found useful (Abbas & Boyette 2000).

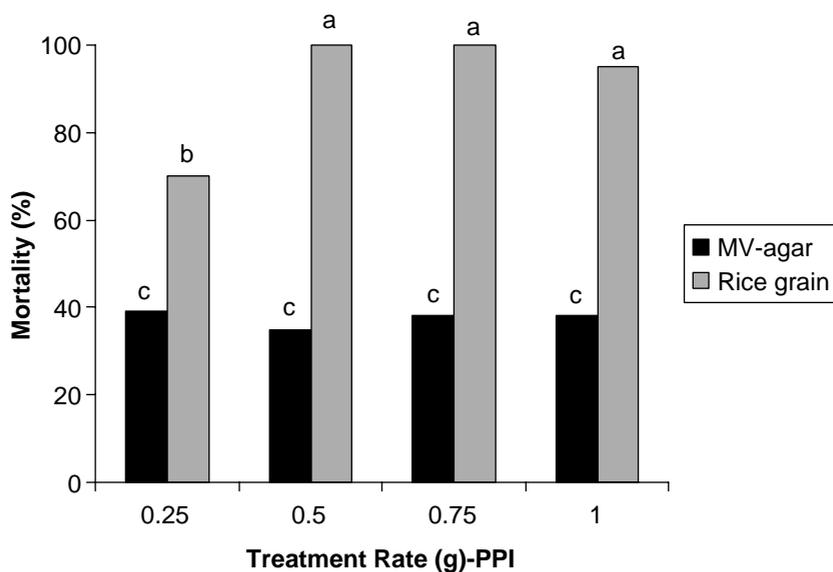


Figure 4. Kudzu control with MV-wt grown on agar or on solid substrate (rice grains). Column means with the same letter are not significantly different according to Fisher's LSD test (0.05).

Soil drench versus pre-plant incorporation of MV-rice extracts

Other experiments compared the efficacy of crude and cell-free extracts of MV-rice cultures on kudzu seedlings at two different growth stages, in soil drench applications rated at 21 DAT (Figure 5). At the time of treatment, the means of the overall maximal widths (diameters) of radiating expanding leaf arrangements of plants at the three- to five-leaf stage and eight- to ten-leaf stages were 4.9 and 14.6 cm, respectively.

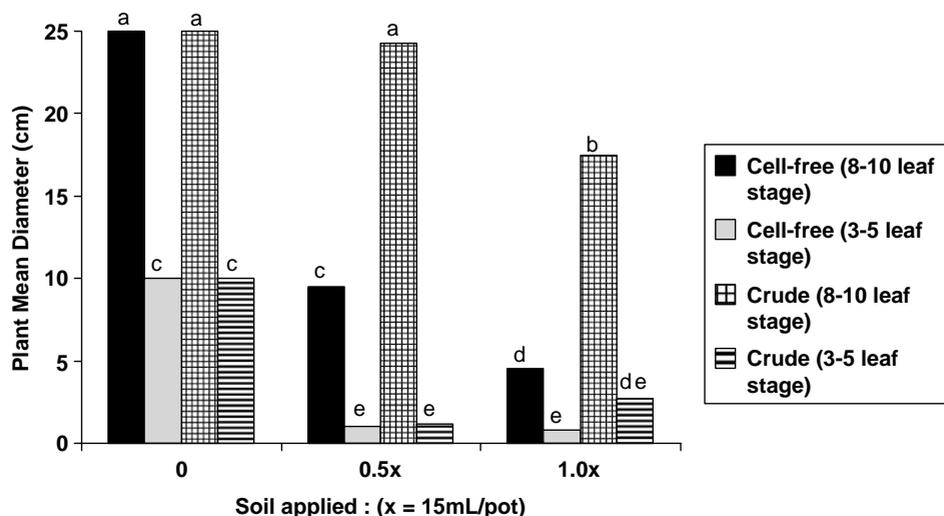


Figure 5. Bioherbicidal activity of crude and cell-free MV-rice extracts on kudzu seedlings at different growth stages. Column means with the same letter are not significantly different according to Fisher's LSD test (0.05).

The crude extracts of MV-rice cultures reduced growth an average of 80% for the three- to five-leaf seedlings, but only approximately 4 and 30% when 7.5 or 15 mL extract was applied to the soil of eight- to 10-leaf seedlings. Cell-free extracts prepared from the crude preparations caused approximately a 90% growth reduction of the younger seedlings at the two application rates, but approximately a 60 and 80% reduction of the older plants at these two rates, respectively. That cell-free extracts caused growth reductions indicates the presence of an elicitor, or phytotoxin that passed through the microbiological membrane filter. The fact that the cell-free treatments caused greater growth reductions than the crude extracts suggests that some the rice grain particulate material may somehow interact with possible elicitors or phytotoxins, preventing their uptake and/or expression by plant tissues.

The procedure of applying MV via soil drench or PPI, although not necessarily applicable in the case of kudzu control, may have more utility in crops. One example is commercially grown tomatoes, because MV can also control several common weeds occurring in this crop with little or no effect on transplanted tomato plants (Boyette et al. 2006b).

Some isolates of MV are pathogenic to several ornamental and crop plants (Cunfer et al. 1969; Nguyen et al. 1973; Leath & Kendall 1983) and virulence and host range differences of MV isolates are evident (Cunfer et al. 1969; Nguyen et al. 1973; Yang & Jong 1995a). Still other MV isolates have bioherbicidal activity on thistle (*Carduus acanthoides* L.), morningglories (Millhollon et al. 2003), and leafy spurge (*Euphorbia esula* L.) (Yang & Jong 1995a,b). There are, however, concerns of safety because some species of *Myrothecium* including MV (but not limited to MV) produce mycotoxins (trichothecenes, e.g. the roridins and verrucarins) (Jarvis et al. 1985). Trichothecenes are a large class of secondary metabolites exhibiting a wide range of acute and chronic effects in mammals (D'Mello et al. 1999). Although this MV-wt strain produces these compounds, trichothecenes were not detected in infected sicklepod and kudzu plants inoculated with this fungus (Abbas et al. 2001). Future research in our laboratory will focus on the elimination or reduction of trichothecene levels in MV cultures using inhibitors, mutant selection, and cultural methods. Another possibility would be to discover an MV sector or mutant that is highly efficacious as a bioherbicide, but lacks the ability to produce these mycotoxins. If successful, MV could become a valuable bioherbicide for the control of several economically important weeds.

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