

Effects of *Myrothecium verrucaria* on Ultrastructural Integrity of Kudzu (*Pueraria montana* var. *lobata*) and Phytotoxin Implications

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ABSTRACT

The fungus *Myrothecium verrucaria* (Alb. & Schwein.) (MV), originally isolated from diseased sicklepod (*Senna obtusifolia* L.), has bioherbical activity against kudzu and several other weeds when applied with low concentrations of the surfactant Silwet L-77. To more fully understand the initial events of MV infection or disease progression, and to improve knowledge related to its mechanism of action, the effects of MV and its product (roridin A) on kudzu seedlings were examined at the ultrastructural level. Ultrastructural analysis of MV effects on kudzu seedlings revealed a rapid (~1 h after treatment) detachment of the protoplast from the cell wall and plasmodesmata appeared to be broken off and retained in the wall. These symptoms occurred well in advance of the appearance of any fungal growth structures. Some fungal growth was observed after severe tissue degeneration (24 to 48 h after treatment), but this occurred primarily at the extra-cellular location with respect to the kudzu tissues. Kudzu seedlings treated with roridin A, a trichothecene produced by the fungus, exhibited some symptoms similar to those induced by the fungus applied in spore formulations with surfactant. The overall results are the first to report the ultrastructural effects of this bioherbicide on plants and suggest that penetration of a phytotoxic substance(s) in the fungal formulation was facilitated by the surfactant, and that roridin A exerts phytotoxicity toward kudzu.

Keywords: Bioherbicide; Biological Weed Control; Kudzu; *Myrothecium verrucaria*; Ultrastructure; Trichothecene

1. Introduction

Kudzu [*Pueraria lobata* (Willd.) Ohwi], a perennial leguminous vine native to eastern Asia, was introduced into the US in the late 1800's [1] and now occurs from Florida to New York, westward to central Oklahoma and Texas, with the heaviest infestations in Alabama, Georgia, and Mississippi causing over \$340 million yr⁻¹ in losses [2]. Cited in a 1993 Congressional Report as one of the most harmful non-indigenous plants in the U. S., kudzu was labeled a federal noxious weed in 1998. This aggressive weed is very difficult to control using synthetic chemical herbicides [3] and has been identified as an over-wintering host of Asian soybean rust (*Phakop-sora pachyrhizi* Syd. & P.) [4].

Bioherbicides (microorganisms and/or their products) can cause injury and/or mortality to weeds. One example of a fungal bioherbicide is *Myrothecium verrucaria* (Alb. & Schwein.) Ditmar:Fr., originally isolated from diseased sicklepod (*Senna obtusifolia* L.). This wild-type *M. verrucaria* (MV) 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-polyether surfactant Silwet L-77 (OSi Specialties, Inc., Charlotte, NC) [5]. A patent for use of MV as a biological control agent on kudzu was issued [6], and this pathogen was found to be highly virulent to kudzu in the absence of dew [7].

We are evaluating MV as a potential bioherbicide for kudzu [7] and several other economically important weeds [8-10]. MV effectively controls kudzu in the absence of a dew treatment over a wide range of physical, environmental, and field conditions [7]. Reports indicate significant performance improvements of several bioherbicides through additive or synergistic effects of chemicals [11] and we reported synergistic interactions of MV and glyphosate on kudzu and some other weeds [12-14]. MV host range tests on various tree species commonly found in natural kudzu infestations indicated most trees were not susceptible to MV [15].

Despite the positive bioherbicidal aspects of MV, one negative factor is its production of macrocyclic trichothecenes which are known to be mycotoxins [16]. Being aware of the risks associated with this trait, we have discussed and presented the risk factors associated with bioherbicides and outlined strategies to overcome or circumvent this problem in MV [13,17,18]. Methodologies have been developed to isolate and quantify trichothecenes produced by MV [19,20]. Several reports indicate that some macrocyclic trichothecenes are phytotoxic and a non-specific biological assay was developed to measure the presence of trichothecenes based on their effects on an alga (*Chlorella vulgaris*) and on two fungi (*Ustilago maydis* and *Trichoderma viride*) [21]. The phytotoxicity of some trichothecenes has also been examined using another alga, *Chlamydomonas reinhardtii*, as a model system [22]. Furthermore, some reports suggest that macrocyclic and non-macrocyclic compounds act as virulence factors of the phytopathogenic organisms that produce them [23-25]. Despite these findings, there are few reports on the effects of these mycotoxins at the ultrastructural level in plants. Ultrastructural analysis and cytological methods have been very useful in identifying the effects of other compounds designed to injure plants (synthetic herbicides), and some of these results are paramount to determining and understanding the uptake and translocation, and the molecular mode of action of such compounds in plant tissues.

The primary mode of trichothecene action in eukaryotic cells is inhibition of the synthesis of protein, DNA, and RNA [26,27]. Phytotoxicity of trichothecenes was first reported in the early 1960s, e.g., [28]. Later, other trichothecenes were found to be phytotoxic, e.g., one report [27] found 15 trichothecenes with phytotoxic activity and that the macrocyclic trichothecenes were more potent. Ultrastructural analysis of some *Stachybotrys chartarum* isolates with or without the gene producing satratoxin (macrocyclic trichothecene) have been reported [29].

Even though substantial information has been amassed concerning the utility of MV as a bioherbicide, little is known about its infectivity mechanism(s) and virulence factors. Thus the objectives of the present experiments were to examine the ultrastructural effects of high purity roridin A and MV spores on kudzu tissues early after application to seedlings, in order to glean more precise information on the action of this macrocyclic trichothecene alone, compared to that of MV spores. This would provide important information related to the primary or initial events of MV infection or disease manifestation, and aid in the elucidation of possible mechanisms of action of MV and of one of its metabolites (roridin A) on host plant organelles and structural constituents.

2. Materials and Methods

2.1. Inoculum Production

Myrothecium verrucaria (MV) spores (IMI 361690) were grown in Petri dishes containing potato dextrose agar (PDA). The plates were incubated (28°C, fluorescently-lighted incubator, 7 - 10 days) and spores were washed from the agar surfaces with sterile distilled water and filtered through two layers of cheesecloth to remove clumps of agar. The concentration of the spore suspension thus obtained was adjusted using a hemacytometer to yield an inoculum containing 1×10^6 spores·ml⁻¹. The surfactant Silwet L-77 (Silwet) (Osi Specialties, Inc.; Charlotte, NC, USA) was added to the final inoculum to attain a concentration of 0.20% (v/v).

2.2. Test Plant 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°C - 32°C at 40% - 60% RH with a photoperiod of about 14 h, at 1600 - 1800 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR as measured at midday.

2.3. Test Plant Inoculation

Kudzu seedlings at the 1 - 2 leaf stage, grown as described above, were inoculated by brushing (small artist's brush) with Silwet, MV plus Silwet, or roridin A at 10^{-4} M (Sigma Chemical Co, St. Louis, MO) prepared in Silwet. The treated seedlings were then placed in a dew chamber (28°C, 12 h), and incubated in a greenhouse as previously described above. Leaf and stem tissues were collected at 1 to 48 h after treatment (HAT). Control plants received 0.2% Silwet surfactant only. Each replicate contained five seedlings, with three replications.

2.4. Electron Microscopy

The kudzu tissues were excised at the appropriate sample times after treatment, and several seedlings from each treatment were harvested at each time point. The tissues sections were fixed (6% glutaraldehyde), post-fixed (2% osmium tetroxide), stained (2% uranyl acetate), dehydrated (acetone), embedded (epoxy resin), sectioned with

a Reichert UltraCut E microtome (Vienna, Austria), and examined using a Zeiss EM10CR transmission electron microscope.

3. Results and Discussion

3.1. Disease Symptomatology at the Ultrastructural Level

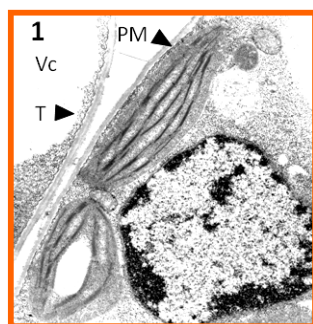
3.1.1. Visual Symptoms

Treatment of kudzu seedling leaflets with MV spore preparations resulted in disease symptomatology characterized by necrotic flecking which occurred within 2 h following treatment. Disease symptoms progressed from the cotyledons and leaves producing stem lesions within about 48 h (data not shown).

3.1.2. Ultrastructural Symptoms

At the ultrastructural level, MV treatment caused rapid effects associated with detachment of the protoplast from cell walls, rapidly resulting in cell death. There were no visual or ultrastructural effects caused by MV spores alone, or by the surfactant Silwet alone in sections from 6 and from 24 h after treatment (**Figure 1**). All organelles are well preserved and the tonoplast and plasmamembrane are intact. In fact, the ultrastructural preservation obtained from the Silwet samples was actually superior to the untreated controls (not shown) probably because of the facilitation of the penetration of the fixatives into the plant tissue (**Figure 1**). Previous studies have indicated a lack of infectivity by MV unless the surfactant Silwet was used [5,6].

There was a rapid (~1 - 6 HAT) protoplast detachment from cell walls (**Figure 2**), and although the cells were mostly intact, small perturbations were evident at the wall-plasma membrane interface. By 12 HAT (**Figure 3**), the cell at the top of the figure is affected only slightly more than the ones after 6 HAT (**Figure 2**), while the

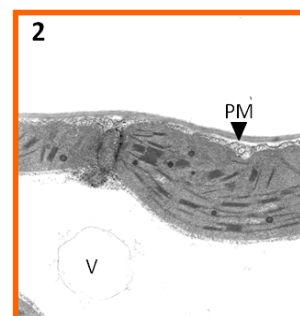


Silwet Control

Figure 1. Electron micrograph of kudzu mesophyll cells from seedlings treated with 0.2 % Silwet L-77 (control), 6 HAT. Vc = vacuole; PM = plasma membrane; T = tonoplast.

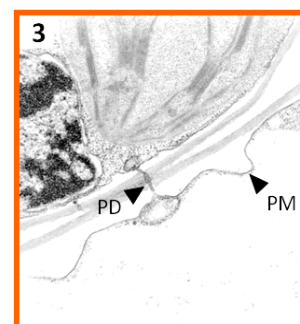
cell at the bottom (**Figure 3**) has nearly separated its plasma membrane from the cell wall. As a consequence, the plasmodesmata has been stretched. Also many plasmodesmata appeared to be broken off and retained in cell walls (not shown). It should be noted that all of these ultrastructural symptoms occurred prior to the appearance of fungal growth structures. However, fungal growth was observed following severe tissue degeneration (24 to 48 HAT), but growth occurred primarily on the leaf surfaces, rather than within the plant tissue (not shown). There was also a complete displacement of plasma membranes from the wall surfaces and distortion of many of the cells (**Figure 4**).

Roridin A in surfactant treatment caused symptoms similar to those induced by spores formulated in surfactant (**Figure 5(A)**). This trichothecene is present in unwashed MV spores, but can be removed by washing [30]. In contrast to the treatment with MV spores alone, treatment of kudzu leaves with roridin A gives only patchy perturbations of cell structure. In this low magnification electron micrograph (**Figure 5(A)**), the cell in the center and the 2 adjacent cells in the bottom-left quadrant have lost their tonoplasts and their cellular contents are dis-



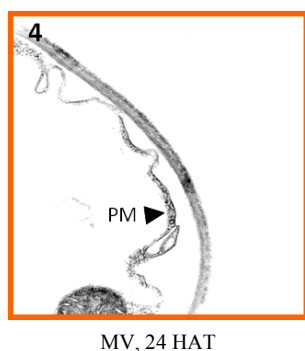
MV, 6 HAT

Figure 2. Electron micrograph of kudzu cells 6 HAT of *M. verrucaria* spores in Silwet L-77 (0.2%). V = vesicle; PM = plasma membrane.



MV, 12 HAT

Figure 3. Electron micrograph of kudzu mesophyll cells treated with *M. verrucaria* spores in Silwet L-77 (0.2%), 12 HAT. PD = plasmodesmata; PM = plasma membrane.



MV, 24 HAT

Figure 4. Electron micrograph of kudzu mesophyll cells treated with *M. verrucaria* spores in Silwet L-77 (0.2%), 24 HAT. PM = plasma membrane.



Roridin A, 24 HAT



Roridin A, 24 HAT

Figure 5. Electron micrograph of kudzu leaf tissue, 24 HAT with roridin A (10^{-4} M) in Silwet L-77 (0.2%). (A) = relatively low magnification showing patchy effects occurring on only a few cells; (B) = largest patch of cells found to be affected in roridin A treated leaf sections examined.

rupted. However, most of the remaining cells in the section, are apparently only moderately affected or unaffected.

Kudzu treated with roridin A 24 HAT (**Figure 5(B)**) also exhibited detachment of the plasma membrane from the cell wall. This is the largest patch of cells found to be affected in any roridin A treated leaf sections examined and even they are flanked by essentially unaffected cells in the upper-right and bottom-left quadrants. This local-

ized damage may indicate poor translocation or movement of this mycotoxin within the plant tissues. Previous research in our laboratory has suggested that roridin A is not rapidly translocated in kudzu seedlings [20].

Treatment with the MV toxin roridin A plus Silwet however gave a number of unique effects upon the tissue. Most striking was the apparent detachment of the protoplast from the cell wall. At early stages of this process, organelles within the cell appeared normal but increasingly the protoplast exhibited symptoms of necrosis and leakage of phenolics and other substances from the vacuole. Plasmodesmata, that normally would connect the protoplasts of adjacent cells are either stretched or detached during this process of protoplast detachment. Despite this dramatic effect on the cells, there was no indication of either hyphae or spores even in these severely disrupted cells. Thus, it is likely that this disruption is due to some diffusible substance, perhaps a phytotoxin, that causes the plant tissue injury, rather than to fungal invasion of the cell.

These data suggest that penetration of a phytotoxic substance(s) in the fungal formulation is facilitated by the surfactant, and clearly show that the phytotoxic action of roridin A on kudzu tissues that are similar to MV toxin(s). Some ultrastructural studies on the effects of non-macrocytic and macrocytic trichothecenes have been published, but most have dealt with mammalian systems, e.g., effects on mice tissues [31], myocardial microvasculature [32], and effects on sperm motility and changes in the plasma membrane [29]. With regard to pathogen-crop plant interactions, the infection process of *Fusarium graminearum* in wheat plants has been investigated using ultrastructural and immunocytological methodologies [33]. Deoxynivalenol and its acetylated derivatives are the most commonly found non-macrocytic trichothecenes in *F. graminearum*-infected plant tissues. Scanning and transmission electron microscopy were also used to study the infection process, the spread of hyphae of *F. culmorum*, and the localization of trichothecenes in wheat tissues [34]. These authors found that the fungus developed dense mycelium on the inner plant tissue surfaces with invasion into the lemma, glume, palea and ovary by penetration pegs. During spreading of the fungus, alterations in host tissues including degeneration of cytoplasm, cell organelles, and deposition of electron dense material between cell wall and plasmalemma were observed. Ultrastructural studies revealed that host cell walls in proximity to the fungal penetration pegs, and in contact with hyphae, were less dense or transparent suggesting involvement of cell wall degrading enzymes. Enzyme- and immunogold-labelling confirmed the presence of cellulases, xylanases and pectinases. Hydrolytic (proteolytic) activity has been detected and as-

sayed in MV [35]. Localization studies also demonstrated the presence of trichothecenes in host tissues early after infection. *F. culmorum* and other *Fusarium* spp. produce several trichothecenes: nivalenol, deoxynivalenol, fusarenon X, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol, and zearalenone, a macrocyclic lactone derivative of resorcinic acid [36]. We are unaware of any reports suggesting that *F. culmorum* synthesizes macrocyclic trichothecenes as do *Myrothecium* spp. However, the phytotoxicity of many non-macrocyclic trichothecenes has been documented [37].

4. Conclusions

Our results are the first to report on the ultrastructure effects of a high purity macrocyclic trichothecene (roridin A) on plant tissue. This also appears to be the first report of ultrastructural studies of MV on plant tissues. Overall, the results suggest that the effects of MV spores cannot be explained solely by presence of roridin A. This point is strongly supported by recent findings in our laboratory that indicate washed MV spores that are void of trichothecenes, remain highly efficacious against kudzu [36], and that mycelia preparations of MV (also void of trichothecenes) are as phytotoxic as unwashed MV spore preparations when applied to plants [12]. These latter two points would tend to rule out macrocyclic trichothecenes (roridin A) as a virulence factor of MV against kudzu. The overall ultrastructural effects of MV spores applied in Silwet also appear unique among those caused by identified phytotoxins or mycotoxins.

Knowledge of MV virulence factors would greatly aid the development and improvement of a commercial bioherbicide product. Cytological investigations at the ultrastructural level will yield invaluable information on the mechanism(s) of action of MV. Although we have recently shown that mycelial formulations of MV are highly efficacious in controlling kudzu, and do not produce roridin A or other trichothecenes at detectable levels via HPLC analysis [19], further investigations are necessary to assess the bioherbicide effects of mycelial preparations and washed spore formulations of MV on plant tissues of various weeds. Research is also in progress to assess the role of hydrolytic enzymes in the virulence of MV and to discover the nature of possible non-trichothecene phytotoxins produced by MV. Advances in the understanding of bioherbicide mechanisms of action and the elimination of mycotoxins in MV formulations will help promote this fungus as a safe and effective bioherbicide.

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