

Effects of *Myrothecium verrucaria* on Two Glyphosate-Resistant *Amaranthus palmeri* Biotypes Differing in Betacyanin Content

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Abstract

Previously we found two biotypes of *Amaranthus palmeri* (Palmer amaranth) in a population of this economically important weed that were resistant to glyphosate but differed with respect to pigmentation. One biotype was typically red-pigmented (betacyanin) while the other was green, with no visual appearance of red hue on any plant part at any growth stage. We have also reported that a strain of *Myrothecium verrucaria* (MV) exhibited bioherbicide activity against several important weeds including glyphosate-resistant Palmer amaranth. In greenhouse tests, MV was applied to these two biotypes (red and green) at two ages (3-week- and 6-week-old) and effects of this fungus monitored over a 5-day time course. Initial symptoms of MV (16 to 24 h after inoculation) were: epinastic curvature, wilting and development of lesions on leaves and stems. Generally, the younger plants tended to be more sensitive to MV than older plants. Bioherbicide damage increased with time leading to necrosis and plant mortality and increasing disease progress. Severe loss of fresh weight occurred in both biotypes as compared to untreated plants. Results indicated that MV was effective on both biotypes, but effects on growth reduction and disease progression were more rapid and generally greater in the green biotype, suggesting that compounds responsible for red pigmentation may be more potent as defense against pathogen attack.

Keywords

Betalain, Bioherbicide, Biological Weed Control, Palmer Amaranth, Pigweed

1. Introduction

Palmer amaranth (*Amaranthus palmeri* S. Wats.) is an important weed that has

spread from its origin in southwestern North American, to eastern North America, Europe, Asia and Australia [1] [2]. It has been reported to be resistant to several classes of herbicides including the triazines, acetolactate-synthase inhibitors, dinitroaniline, protoporphyrinogen oxidase inhibitors and glyphosate [3]-[9]. The molecular mode of action of glyphosate is inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in the shikimate pathway [10], responsible for the synthesis of aromatic amino acids (tyrosine, phenylalanine and tryptophan) and phenolic compounds, some of which are related to plant defense [11]. Currently, 44 weed species have been found resistant to glyphosate [9]. Glyphosate resistance in Palmer amaranth plants has been attributed to high copy numbers of the EPSPS gene, compared to glyphosate-susceptible plants [12] and high EPSPS copy numbers enable adequate EPSPS production and the concomitant synthesis of required aromatic amino acids even when high levels of glyphosate exist in the resistant plant tissues. The high EPSPS gene copy number trait is heritable when plants are cross-bred [12]. The transfer of resistance through cross-breeding, its aggressive nature and the prolific seed-producing capacity of Palmer amaranth [13] have intensified its spread.

Previously we found two biotypes of Palmer amaranth in a population of this economically important weed that were resistant to glyphosate but differed with respect to pigmentation [14]. One biotype was typically red-pigmented while the other was green, with no visual appearance of red hue on any plant part at any growth stage. The compounds responsible for these pigmentations are betalains, a small group of in dole-derived glycoside pigments. Betalains are water-soluble nitrogen-containing compounds derived from tyrosine and have important functions in plants. They are distributed among ten plant families belonging to the order, Caryophyllales (and in some fungi) and are divided into two groups: red betacyanins and yellow betaxanthins [15] [16] [17]. The most commonly occurring betacyanin, *i.e.*, betanin, occurs mainly in red beet (*Beta vulgaris* L.) [15]. Numerous betalains have been identified and these compounds possess strong antioxidant properties [18]. Reactive oxygen species (ROS) have been implicated in plant-pathogen interactions with respect to tissue damage and defense responses via plant cell wall strengthening and/or toxicity to pathogens. A protective ROS scavenging role has been implicated for betalains during stress [19] and betalains play a role in plant defense [20]. A recent example was increased resistance to gray mold disease (*Botrytis cinerea*), attributed to ROS in transgenic betalain-producing tobacco seedlings [21]. ROS activity related to betalain synthesis was induced in red beet leaves after infection by *Agrobacterium tumefaciens* or *Pseudomonas syringae* [22]. Defense against pathogenic fungi has also been implicated as a factor in betalain evolution [23].

Biological control initiatives using plant pathogens as bioherbicides for weed control have been studied since the early 1970s, as exemplified in recent reviews [24] [25] [26]. The fungus *Myrothecium verrucaria* (Alb. and Schwein.) Ditmar: Fr. (strain IMI 368023) (MV) exhibits bioherbicidal activity on several weeds [27] [28] [29]. Other studies in our laboratory demonstrated that MV was bio-

herbicidal against economically important weeds including: kudzu (*Pueraria lobata* var. *montana*) [30], purslanes (*Portulaca* spp.) and spurge (*Euphorbia* spp.) [31], *Morning glory* spp. (*Ipomoea* spp.) [32], hemp sesbania (*Sesbania exaltata*) [33], and Palmer amaranth [34] [35]. Synergistic interactions have occurred when MV and glyphosate were applied to control some weeds [33] [36] [37] [38] and other bioherbicidal plant pathogens have also been reported to exhibit synergistic interactions with glyphosate [39] [40].

Nearly 20 betacyanin (red-violet) and betaxanthin (yellow) pigments have been identified in Amaranthaceae plants [41] and marked differences in antioxidant activity among these compounds have been reported. Palmer amaranth belongs to the Amaranthaceae family (order Caryophyllales and contains betalains (rather than anthocyanins). Due to the involvement of plant pigments such as betalains in plant-pathogen interactions and the severe economic importance of Palmer amaranth and glyphosate-resistant Palmer amaranth, we wished to investigate the effects of the bioherbicidal fungus, *Myrothecium verrucaria* (MV) on our two biotypes (red-pigmented and green-pigmented). Heretofore we showed that these biotypes possessed high EPSPS copy numbers and were resistant to glyphosate [42] [43]. In more recent research with other biotypes of Palmer amaranth, we demonstrated that MV exhibited bioherbicidal potential for Palmer amaranth control in greenhouse tests [34] [35]. However, this paper is the first report of the interactions of MV on these two differentially-pigmented Palmer amaranth biotypes.

2. Materials and Methods

2.1. Plant Source and Culture

A population of seeds of the weed, Palmer amaranth, was collected from a site near Stoneville, MS, USA [14]. This population contained two distinct biotypes that exhibited resistance to the herbicide glyphosate, one biotype that produced the typical red-pigmented Palmer amaranth plants (Red biotype), and one biotype (Green biotype) that produced no visible red pigment as reported earlier [14]. Seeds were planted in a commercial potting soil under greenhouse conditions (22°C - 25°C; 16 h photoperiod). When seedlings were about 30 mm tall (cotyledon: first true-leaf stage), they were transplanted into individual pots (~75 L × 55 W × 50 D mm) containing potting soil and grown under greenhouse conditions to various growth stages for testing. Plants were watered with de-ionized water and fertilizer [N:P:K (13:13:13)] was provided routinely.

2.2. *M. verrucaria* Cultures

M. verrucaria (IMI 368023) originally isolated from sicklepod (*Senna obtusifolia* (L.) H.S. Irwin & Barneby) was cultured in Petri dishes on potato dextrose agar (PDA) (Difco Laboratories, Inc., Detroit, MI, USA). Inoculum from these dishes was used to generate mycelial cultures in a fermenter as described elsewhere [44]. This fungal product, consisting of mycelium and unspent growth medium

without spores, was substantially lower in trichothecene content compared to spore cultures of this fungus [44]. The mycelial formulation from the fermenter was used full-strength (typically $\sim 1.0 \times 10^7$ cfu·mL⁻¹) and a surfactant (Silwet L-77) was added to achieve a formulation containing 0.20% (v/v) surfactant.

2.3. Spray Application of *M. verrucaria* to Palmer Amaranth Biotypes

Ten to twelve uniform-sized seedlings of each biotype at two growth stages (3-week and 6-week-old) were sprayed with MV plus Silwet (0.20%) mycelial formulation using compressed air spray canisters (Crown Spra-Tool, North American Professional Products, Woodstock, IL, USA). Plant leaves were sprayed until run-off, corresponding to an application rate of ~ 300 L·ha⁻¹. Control plants received applications of Silwet only (0.20% in H₂O). All spray applications were performed in a biosafety cabinet (NuAire, Model No. NU-425-400, Plymouth, MN, USA). Immediately following treatment, plants were moved to a dew chamber (Percival Scientific, Model No. 1-35 DL, Boone, IA, USA) maintained at 25°C for 16 h. At the end of the dew period, plants were placed under greenhouse conditions [27°C - 31°C, 45% - 65% RH, and a 14 h day, at 1650 - 1825 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (photosynthetically active radiation) PAR] and observed and/or sampled as required for measurements.

2.4. Effects of MV on Growth of Palmer Amaranth Biotypes

Plants were visually examined for injury symptoms at various times after MV application. The fresh weights and dry weights of plant shoots (excised at the soil surface) were measured 5 days after MV application to assess MV effects on growth. Dry weight determinations were made on shoot tissue placed in paper bags and dried in an oven (90°C to 98°C, 48 h).

2.5. Disease Progression of MV on Palmer Amaranth Biotypes

Progression or severity of the disease incited by MV spray application [MV mycelial fermentation product containing $\sim 1.0 \times 10^7$ cfu·mL⁻¹] to these biotypes (6-week-old) after MV was applied as a spray prepared in 0.20% Silwet was monitored at several intervals over a 5-day period. A disease rating scale (modified from Horsfall and Barratt [45]) was used to visually evaluate bioherbicide efficacy. The numerical scale defined 0 as equivalent to no infection, and values of 1.0, 2.0, 3.0 and 4.0 represented 20%, 40%, 60%, and 80% leaf/stem lesion coverage and/or injury, respectively. Plant mortality was set at a value of 5.0. Disease ratings of 3.0 to 5.0 were considered “severe”. Standard errors of means and regression analyses were used to evaluate and pattern the data.

2.6. Statistical Considerations

The experimental design was a randomized complete block and each treatment comprised 10 to 12 plants of each age. The treatments were performed in triplicate and all experiments were repeated in time. The data were compared using

analysis of variance ($P = 0.05\%$). Values shown are means of replicated experiments. When significant differences were detected (F-test), means were separated (Fisher's protected LSD; $P = 0.05$). Error bars presented in graphics are ± 1 SEM (standard error of the mean).

3. Results and Discussion

3.1. Red and Green Biotypes of Palmer Amaranth Resistant to Glyphosate

Visual appearance of the two Palmer amaranth biotypes used in this study is strikingly different (**Figure 1**). Pigmentation differences in the biotypes are discernable upon emergence of seedlings from the soil and at about 6 to 8 days after emergence (2- to 3-leaf stage; **Figure 1**, top) red pigmentation typically occurs in stems and leaf petioles of the red biotype, while no red pigments are visibly apparent in the green biotype. Examination of abaxial surfaces of these young leaves and cotyledons, again demonstrates red pigmentation in red biotype, but green biotype tissues are green (**Figure 1**, bottom). At later growth stages (**Figure 2**) and through seed production, each biotype retained their respective colorations, with the red usually deepening in color and the green often becoming more yellowish-green with time as shown in **Figure 2** (bottom), comprised of excised stems from several more mature plants. The upper portion of the red biotype has much less red pigmentation than in its stems and petioles. Examination of mature roots of the green biotype also indicated no appearance of red pigmentation (data not presented). The red pigment is a betalain (betacyanin) and the green biotype may be void of betalains (red and yellow classes), or it may produce only betalain(s) belonging to the yellow class. Concurrent studies on the comparative characterization of these two biotypes are underway that will be published elsewhere.

3.2. Effects of MV on Glyphosate-Resistant Red and Green Biotypes of Palmer Amaranth

Both Palmer amaranth biotypes exhibited some bioherbicidal effects of MV inoculation as early as 16 h after inoculation, when plants were removed from a high humidity cabinet and placed in a greenhouse (data not shown). These early effects were exemplified as some epinastic effects, slight wilting and initial development of lesions on leaf and stems. Generally, the younger (3-week-old) plants tended to be more sensitive to the MV inoculation than the older plants (6-week-old). Much more bioherbicidal damage occurred with time and at 48 h after inoculation with this fungus, effects were more severe (**Figure 3**). Epanastic curvature of stems, wilting/dehydration, necrosis and leave-drop were apparent effects in both biotypes. At this time point, the green biotype appeared slightly more damaged by the fungus than the red biotype. This effect was supported by fresh weight analysis from plants at the 48-h time point (**Figure 4**). Severe loss of fresh weight occurred in both biotypes compared to that of untreated their respective control plant shoots. The green biotype was slightly more damaged with



Figure 1. Visualization of young seedlings of Red and Green Palmer amaranth biotypes. Top: Photograph of seedling biotypes in the second-third-leaf growth stage, grown under greenhouse conditions. Bottom: Photograph of adaxil surface of leaf and cotyledon of Red and Green biotypes.



Figure 2. Visualization of seedlings of Red and Green Palmer amaranth biotypes. Top: Photograph of seedling biotypes after 5-week growth under greenhouse conditions. Bottom: Photograph of cut stems of several Red and Green biotypes.

respect to fresh weight biomass.

3.3. Disease Progression of MV on Glyphosate-Resistant Red and Green Biotypes of Palmer Amaranth

Three-week-old green biotypes were generally more susceptible to infection by

MV than were red biotype plants at this age (Figure 5). Severe infection of 3-week-old biotypes (disease rating of 3.8) occurred after 48 h, and increased to 4.5 after 96 - 120 h. In comparison, a disease rating of only 2.6 occurred on 3-week-old red biotype plants after 96 h, but this increased to a disease rating of 3.5 after 120 h (Figure 5). In the biotypes, MV disease progression and severity were generally greater on 3-week-old plants as compared to 6-week-old plants (Figure 5).



Figure 3. Bioherbicidal effects of *Myrothecium verrucaria* mycelial formulation on 3-week-old seedlings of Red (R) and Green (G) Palmer amaranth biotypes 48 h after spray inoculation under greenhouse conditions. Control plants received Silwet (0.20%, v/v) only.

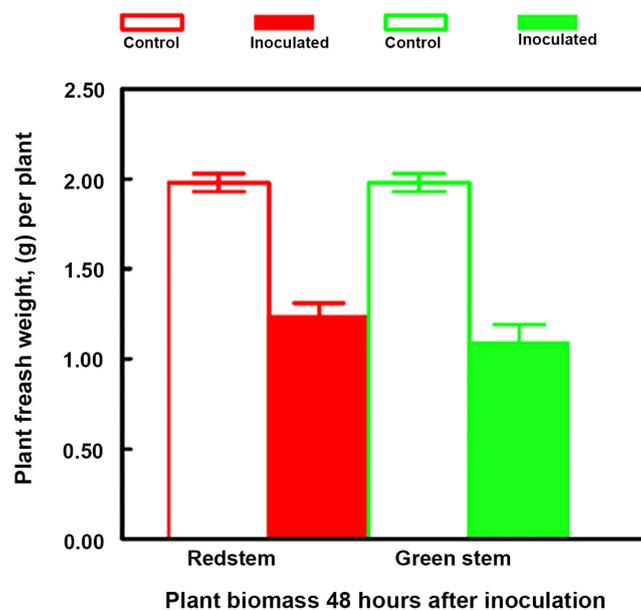


Figure 4. Effects of *Myrothecium verrucaria* mycelial formulation on fresh weight accumulation of 3-week-old Red stem and Green stem Palmer amaranth seedlings, 48 h after spray inoculation under greenhouse conditions. Control plants received Silwet (0.20%, v/v) only.

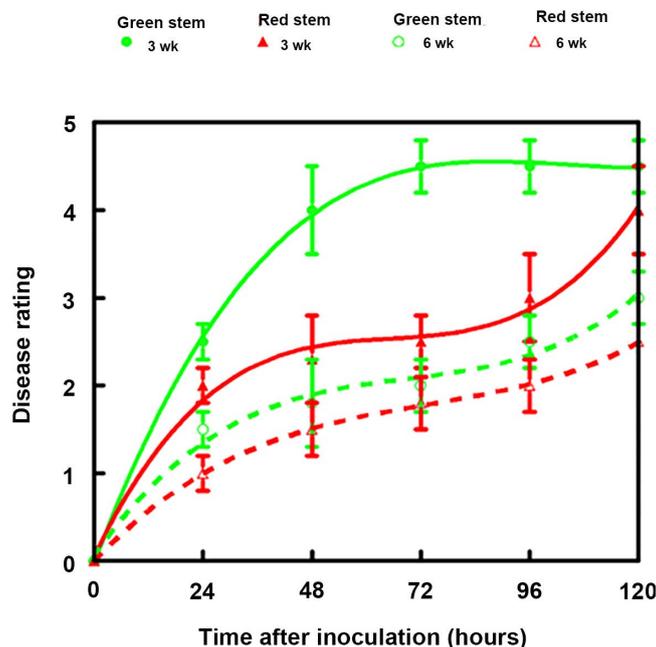


Figure 5. Disease progression effects of *Myrothecium verrucaria* mycelial formulation on 3-week-old and 6-week-old Red stem and Green stem Palmer amaranth seedlings over a 5-day time course. Solid lines represent 3-week-old plants; dashed lines represent 6-week-old plants. Regression equations relative to the data are as follows: 3-week-old Green: $Y = -0.02 + 0.14X - 0.001X^2$, $R^2 = 0.98$; 3-week-old Red: $Y = 0.05 + 0.11X - 0.002X^2$, $R^2 = 0.98$; 6-week-old Green: $Y = 0.05 + 0.08X - 0.001X^2$, $R^2 = 0.98$; 6-week-old Red: $Y = -0.02 + 0.06X - 0.006X^2$, $R^2 = 0.98$.

The green biotype might be compromised under natural environmental conditions and stress since betalains have been shown to be involved in plant photoprotection mechanisms. For example, photosynthetic capacity damage was reduced in red-pigmented versus green leaves after exposure to excess light [46] [47] [48]. Betalain synthesis in a related plant, *Amaranthus tricolor*, has been shown to be under photocontrol [49]. Other reports show that betalain production in plants is upregulated after exposure to light or UV radiation [50] [51] [52].

There was a definite trend that the green biotype was somewhat more susceptible to MV when treated under greenhouse conditions. This may suggest that lack of the red betacyanin pigment renders the green biotype less resistant to pathogen attack. As outlined and presented earlier, betacyanin has been implicated in disease resistance. Further testing of the effects of MV on these two biotypes under field conditions and expanded characterization of the pigment contents and traits of these two biotypes (work in progress) will help to clarify the interaction of the bioherbicide, *Myrothecium verrucaria* on these two glyphosate-resistant Palmer amaranth biotypes.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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