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Application of Meadowfoam (Limnanthes alba) Seed Meal as a Soil Amendment for Management of *Pythium irregulare*

Yurdagul SIMSEK ERSAHIN[a], Jerry E. WEILAND[b], Ralph REED[c], and J. Fred STEVENS[d]

[a]Corresponding author; Çankiri Karatekin University, Çankiri, Turkey
[2]USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR, USA
[3]Department of Pharmaceutical Sciences, Oregon State University, Corvallis, OR, USA
Corressponding author: yurdagulersahin@gmail.com

Abstract

Meadowfoam (*Limnanthes alba* Hartw. ex Benth.) is a member of the order Brassicales and has been grown as a commercial oil seed annual crop in the Willamette Valley of Oregon, USA since the 1980's. After harvest, the seed is pressed to yield oil containing unique long chain fatty acids (20:1 and 22:1) of high quality and commercial value in cosmetics and lubricants, making meadowfoam a high-value oilseed crop. After the oil has been extracted from seed, the remaining seed meal contains the glucosinolate glucolimnanthin. When plant cells containing glucolimnanthin are physically damaged and exposed to moisture and the enzyme myrosinase, this secondary plant metobolite degrades into toxic breakdown products. In a previous study, we demonstrated the toxicity of the glucolimnanthin degradation products nitrile, thioamide, and isothiocyanage (ITC) to the plant pathogen *Pythium irregulare*. The ITC was the most toxic to this pathogen while glucolimnanthin and its degradation product acetamide were not toxic to the pathogen. This research demonstrated the potential to utilize meadowfoam seed meal (MSM) as a soil amendment to manage this soilborne pathogen.

Key words: Soil amendment, plant disease management, Pythium

Introduction

Meadowfoam (*Limnanthes alba* Hartw. ex Benth.) is a member of the order Brassicales and has a high commercial value for farmers in the Willamette Valley of Oregon starting the 1980's. After harvest, the seed is pressed to yield oil containing unique long chain fatty acids (20:1 and 22:1) of high quality and commercial value in cosmetics and lubricants. After the oil has been extracted from seed, the remaining Meadowfoam seed meal (MSM) contains the glucosinolate glucolimnanthin. When plant cells (Meadowfoam seed; MS) containing glucolimnanthin are physically damaged and exposed to moisture and the enzyme myrosinase, this secondary plant metobolite degrades into toxic breakdown products; including 3-methoxybenzyl isothiocyanate (ITC), 3-methoxyphenyl ethanethioamide (thioamide), 2-(3-methoxyphenyl)-acetamide (acetamide), and additional 3-methoxyphenylacetonitrile (nitrile) (Fig. 1) (27). In a previous study (33), we demonstrated the toxicity of the glucolimnanthin degradation products nitrile, thioamide, and ITC to the plant-parasitic nematode *Meloidogyne hapla* Chitwood and the plant pathogen *Pythium irregulare* Buisman. The ITC was the most toxic to the pathogen while glucolimnanthin and its degradation product acetamide were not toxic it. Many different glucosinolate-containing plants and seed meals have previously been evaluated and proven effective for the suppression of weeds, soilborne pathogens, and plant-parasitic nematodes (4,5,15,19–21).

The goal of this research was to generate information to facilitate the implementation of MSM into crop production systems for soilborne pathogen management; *P. İrregulare* which has over 200 host plant species,including cereals and grasses, vegetables, fruit and nut crops, forest tree seedlings, and a number of floricultural crops (9,31). The objectives of this study were to (i) determine the necessity of adding MS to MSM to achieve *P. irregulare* suppression; (ii) identify effective amendment rates of MSM+MS that suppress *P. irregulare* in soil; (iii) determine whether effective rates are phytotoxic to tomato, cucumber, and wheat, and; (iv) measure the generation of glucolimnanthin degradation compounds in MSM+MS-amended soil and

determine the relationship between compound production and soilborne pathogen suppression.

Materials and Methods

Materials. MSM and MS were provided by Natural Plant Products, Inc. MSM was produced in 2010 and MS was harvested in 2010 and 2011. *P. irregulare* isolate PR155d, originally isolated from a forest nursery field soil (30) and was used to produce inoculum according to methods Modified from Weiland et al. (31). Briefly, colonized agar was used to infest fungal spawn bags (Fungi Perfecti) containing 1.25 liter of dilute, clarified V8 juice and 3 liters of coarse vermiculite that had been autoclaved three times for 1 h at 48-h intervals. Spawn bags were then incubated in the dark at 20°C with weekly mixing by hand. After 30 days, inoculum was removed from the bags, air dried for 3 days, then stored in resealable polyethylene bags at 20°C until use.

MSM+MS pathogen toxicity assays. Soil (1:1vol/vol washed sand and Willamette loam) that had been steam pasteurized for 3 h, dried for 24 h at 70°C, and then passed through a 2-mm-diameter sieve was amended with one of 12 different MSM+MS formulations. For each formulation, MS was first ground with an electric coffee grinder (Proctor Silex E160BY; Hamilton Beach Appliances) for approximately 1 min, then mixed by hand with MSM at 0, 1.0, 2.0, or 3.0% (dry wt/wt) in 12 separate resealable plastic bags. Each MSM+MS formulation was then incorporated into 300 g of soil at 0.5, 1.0, or 2.0% (dry wt/wt)using an 8-liter, stainless-steel, twin-shell blender (Patterson Kelley) for 10 min to yield 12 MSM+MS soil amendment treatments. Soil that was not amended with either MSM or MS was also prepared. For convenience, these treatments will be abbreviated throughout this manuscript as soil amendment rate/seed formulation MSM+MS. Finally, soil for each of the 13 treatments was then moistened with nonsterile distilled water (0.12 ml/g of dry soil) and infested immediately with the pathogen as described. For P. irregulare, infested vermiculite inoculum was added to moistened soil in resealable plastic bags to achieve 100 propagules/g (ppg) of moist soil and then mixed by hand. Infested soil treatments were then distributed into polyethylene 50-ml tubes (56 g/tube) and 2 ml of nonsterile distilled water was added to the soil surface. Tubes were left uncapped to allow air circulation. Tubes (*P. irregulare*) were then incubated at 20°C in the dark for 4 days. The soil surface was monitored daily and approximately 1 to 2 ml of nonsterile distilled water was added as necessary to remoisten the soil surface. Individual tubes were arranged according to a completely randomized design (CRD), with five replicate tubes. At the end of the assay, mortality of *P. irregulare* was evaluated with two concurrent methods used previously (30,32): by dilution plating onto a semiselective medium (PARP) for Pythiaceous species (14) to quantify soil populations and by baiting the remaining soil with rhododendron leaf disks to confirm mortality. For the plating method, soil was placed into separate 10-by-10-cm resealable plastic bags and mixed thoroughly. A 10-g subsample was removed and mixed with 90 ml of 0.2% water agar in a 250-ml Erlenmeyer flask on a rotary shaker set at 300 rpm for 30 min. A 500-µl aliquot of the suspension was then spread with a sterile glass rod on each of five petri plates containing 20 ml of PARP agar. Plates were incubated for 2 days in the dark at 20°C before washing off the soil under running tap water and counting the number of *P. irregulare* colonies. For the baiting method, the remaining soil in each bag (approximately 45 g) was placed into a 150-ml wax paper cup and baited with five 5-mm diameter freshly collected, field-grown Rhododendron 'Unique' leaf disks from leaves that were approximately 4 months old, according to the double-cup leaf disk baiting method (16). After 2 days of incubation in the dark at 20°C, the leaf disks were removed, blotted dry, and plated on PARP agar medium to evaluate for the presence or absence of *P. irregulare* colonies after two further days of incubation in the dark at 20°C (30,32).

MSM+MS phytotoxicity assay. The same MSM+MS and con-trol treatments used in pathogen toxicity assays were also evaluated for their potential phytotoxic effects on three agronomic plant hosts. Approximately 400 g of dry amended or nonamended soil (prepared as above) was distributed into five replicate 9-cm2 pots for each host and planted immediately. Ten seeds of cucumber (Cucumis sativus L.) 'Straight Eight' or wintered wheat (Triticum aestivum L.), or a single 18- to 25-day-old seedling of Rutgers tomato washed free of soilless media (SunGrow Horticulture), were then planted into each replicate pot. The pots were watered to field capacity and placed on a greenhouse bench in a randomized complete block design (RCBD). Each MSM+MS and control treatment–plant species combination was replicated five times. The greenhouse was maintained at 23 and 18°C (day and night, respectively) for 2 weeks and supplemental light was provided to achieve a 16-h photoperiod. Plants were monitored daily and watered as necessary to maintain a slightly moist soil surface. At the end of the assay (15 days), observations regarding the number of germinated seed (cucumber and wheat), seedling height

(cucumber and wheat), and wilt symptoms (tomato) rated on a scale of 0 (no wilt) to 3 (severe wilt) were recorded. Wilt symptoms were not observed on either wheat or cucumber plants and no effects on height were observed on tomato plants for this assay; therefore, these symptoms were not recorded for those host plants. The entire assay was conducted twice.

MSM+MS time-course phyto toxicity assay. Because of the phytotoxicity observed at the minimum amendment rates needed to kill either M. hapla (0.5/1.0 MSM+MS) or P. irregulare (1.0/1.0 MSM+MS), a time-course assay was established to determine whether the symptoms of phytotoxicity could be alleviated by increasing the amount of time between soil amendment with MSM+MS and the planting date. For each treatment (0.5/1.0 MSM+MS, 1.0/1.0 MSM+MS, plus a nonamended control) and host (cucumber and tomato), approximately 400 g of dry amended or nonamended soil was distributed into five replicate 9-cm2 pots and watered to field capacity; then, assay plants were planted into the soil at 0-, 2-, 4-, 6-, and 8-day intervals after amendment. At each time interval, 10 seeds of Straight Eight cucumber or a single 18- to 25-day old seedling of Rutgers tomato, prepared as above, were planted into each replicate pot. Plants were monitored Daily and all pots were watered as necessary to maintain a slightly moist soil surface, regardless of whether a plant was present or not (i.e., regardless of the time interval between MSM+MS amendment and planting date). Pots were arranged on a greenhouse bench in an RCBD with five replicate blocks and maintained under the same greenhouse conditions as described above. Plants were allowed to grow for 2 weeks following each planting interval and then evalu- ated for the number of germinated seed (cucumber only), seedling height (cucumber and tomato), wilt or chlorosis rated on a 0-to-3 scale (tomato), and stem damage rated as present or absent (tomato). This latter symptom was characterized by a region of chlorotic, mainly hairless, hyperplastic stem tissue that extended for 1 to 2 cm above the soil line. Symptoms of leaf chlorosis were constrained to the leaf margin and were primarily interveinal; eventually, these regions turned necrotic. Wilt, chlorosis, and stem damage were not observed on cucumber; therefore, these symptomswere not recorded for that plant host. The entire assay was conducted twice.

Assay for glucolimnanthin degradation compounds and pathogen mortality over time in MSM+MS-amended soil. The two minimum amendment rates identified in and pathogen toxicity assay (above) needed to kill *P. irregulare* (1.0/1.0 MSM+MS) were assessed for the presence of glucolimnanthin as well as the production of the glucolimnanthin degradation compounds ITC and nitrile, and the concurrent suppression of P. *irregulare* at 10 time intervals after incorporation of MSM+MS into soil (0, 2,4, 8, 12, 24, 48, 72, 96, and 144 h). Thioamide, a glucolimnanthin degradation product which was also toxic to *P. irregulare* (33), was not investigated because it is only produced when MSM+MS is further amended with iron (27) and it was less toxic than either ITC or nitrile (33). MSM+MS soil treatments were prepared as described above (1.0/1.0 MSM+MS for P. irregulare, plus a nonamended control), moistened with nonsterile distilled water (0.12ml/g of dry soil), and infested immediately with the pathogen. For *P. irregulare*, amended and nonamended soil was infested as described in the pathogen toxicity assay. All tubes were incubated at 20°C. The contents of each tube were examined daily and remoistened with 1 to 2 ml of nonsterile distilled water if the soil surface appeared dry. Each time interval–MSM+MS treatment (amended or nonamended)–organism combination was replicated three times, and the entire assay was conducted twice.

Chemical and pathogen extractions for time 0 began immediately after inoculation and distribution of soil into the 50-ml tubes and occurred at the time intervals indicated above. For all of the time intervals except 12 h, chemical extractions of soil were only conducted on amended treatments; at 12 h, a set of three nonamended soil replicates was also extracted as a negative control to confirm the absence of glucolimnanthin and its degradation products. At each extraction time, the contents of each tube were emptied into a resealable plastic bag and mixed thoroughly. A 5-g aliquot of soil was then removed from the bag or tissue and placed in a clean 50-ml tube, and 15 ml of 94% methanol was added. The contents were vortexed for 10 s,sonicated for 10 min (Branson 2510 Ultrasonic Cleaner), then incubated for 1 h at 20°C. Each tube was then centrifuged for 5min at 3,000 rpm and 1 ml of the supernatant was transferred to a 1.5-ml Eppendorf tube before centrifuging for 10 min at 13,000 rpm. Finally, 900 µl of the solution was removed and placed in an 8-by-40-mm glass vial and stored at –20°C until chemical analyses could be conducted (see below). The remaining 45 g of amended or nonamended soil at each time interval was assayed for *P. irregulare* mortality, as described above.

Statistical analysis. To evaluate pathogen mortality, mean counts of *P. irregulare* were separated using Tukey's test (P = 0.05). All analyses

were performed with Minitab Statistical Software or SAS 9.2.

Results

MSM+MS pathogen toxicity assays. The soil population of *P. irregulare* in nonamended soil averaged 21 ± 1.5 ppg. Amending soil with MSM alone at 0.5, 1.0, or 2.0% caused soil populations to increase approximately 25-fold over the nonamended population level ($P < 0.001$), regardless of the amendment rate (555 ± 75.0, 543 ± 65.9, and 522 ± 64.7 ppg, respectively). However, once soil was amended with MSM+MS, soil populations decreased to 0 ppg for all but the lowest soil amendment rate and formulation (0.5/1.0 MSM+MS), for which the soil population (20 ± 6.9 ppg) did not differ from that found in the nonamended soil ($P = 0.911$). Although a trial effect was observed ($P < 0.001$), results were similar when each trial was analyzed separately (data not shown).

MSM+MS phytotoxicity assay. Except for the 2.0% amendment rate ($P < 0.001$), MSM alone (0.5 and 1.0%) did not cause tomato plants to wilt and yielded results similar to those in the nonamended control ($P \geq 0.984$) (Fig. 1A). Wilt symptoms with 2.0% MSM alone were not immediately apparent and took approximately 1 week to manifest. However, once soil was amended with the low amendment rate (0.5/1.0 MSM+MS), glucolimnanthin decreased from its initial concentration of 0.36 ± 0.032 µmol/g of soil to nondetectable levels within 24 h of amendment (Fig. 4A). At the high amendment rate (1.0/1.0 MSM+MS), glucolimnanthin decreased more rapidly from its initial concentration of 0.77 ± 0.036 µmol/g of soil to nondetectable levels by 12 h postamendment. Nitrile was present throughout the duration of the experiment for both amendment rates (0.08 ± 0.002 and 0.15 ± 0.002 µmol/g of soil for 0.5/1.0 and 1.0/1.0 MSM+MS, respectively), and remained relatively stable before declining on the sixth day. Production of ITC was rapid and peaked at 24 h with the lower amendment rate (0.32 ± 0.024 µmol/g of soil) but peaked earlier at 12 h for the higher amendment rate (0.65 ± 0.040 µmol/g of soil). Thereafter, ITC concentrations rapidly declined to at or near 0 µmol/g of soil at 6 days. Trial effects were observed for both nitrile and ITC ($P \leq 0.005$). However, results were similar for each trial separately (data not shown). No trial effect was observed for glucolimnanthin ($P = 0.145$) (Figure).

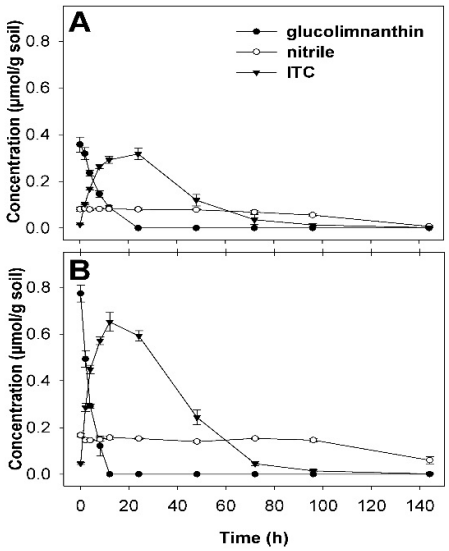

Figure. 2. Concentrations of glucolimnanthin, 3-methoxyphenylacetonitrile (nitrile), and 3-methoxybenzyl isothiocyanate (ITC) at 0, 2, 4, 8, 12, 24, 48, 72, 96, and 144 hours after soil amendment with 0.5% w/w meadowfoam seed meal formulated with 1.0% meadowfoam seed (A) or 1.0% w/w meadowfoam seed meal formulated with 1.0% meadowfoam seed (B). n = 6 replicate extractions. Error bars indicate standard error.

Discussion

To achieve consistent suppression of *P. irregulare* in soil, it was necessary to combine MSM with MS. The effective rate identified for suppression of *P. irregulare* was 1.0/1.0 MSM+MS. In our study, the soil amendment rates tested equate to field amendment rates of 10 t/ha for 0.5/1.0 MSM+MS and 20 t/ha for 1.0/1.0 MSM+MS. At these rates, there is also the potential for MSM+MS to be phytotoxic to the plant species evaluated in this study, indicating that caution is warranted when establishing plants in soil treated with MSM+MS, and delayed planting after amendment might be required. In our assay for glucolimnanthin degradation compounds produced in MSM+MS-amended soils, ITC was the most likely compound responsible for pathogen mortality, given that most mortality occurred within 2 h after amending soil with MSM+MS during the period of time that concentrations of this compound were increasing rapidly. Because of the inactivation of myrosinase by heat during the oil extraction process (26), there is a unique opportunity to direct the production of glucolimnanthin degradation products from MSM. The addition of enzyme-active MS to MSM results

in complete degradation of glucolimnanthin and formation of ITC and nitrile, while incubation of MSM and MS in an aqueous solution of $FeSO_4$ favors the formation of ITC, nitrile, and thioamide (26). Because we previously observed that ITC was the glucolimnanthin degradation product most toxic to M. hapla and Pythium irregulare (33), we chose to forgo the inclusion of FeSO4 and combine MSM solely with MS to promote ITC production. The lowest tested MS formulation evaluated (1.0%) was more than enough to stimulate the glucolimnanthin degradation pathway; whether even lower formulations of MS would achieve a similar effect is not known.

When MSM was applied to soil alone, there was a significant increase in population densities of *P. irregulare*; a 25-fold increase in populations occurred regardless of the amendment rate. In our previous study, the concentration of nitrile needed to cause 50% mortality of *P. irregulare* after a 24-h exposure was 0.9 mg/ml (33), which was much higher than the concentrations found in this study at the 0.5/1.0 and 1.0/1.0 MSM+MS amendment rates (nitrile at 0.077 and 0.147 mg/ml, respectively). In addition, *P. irregulare* is a facultative saprophyte and has the ability to survive and increase in the presences of organic substrates (28). Others have also observed an increase in Pythium spp. populations in soils amended with brassicaceous seed meals. These observations led to the conclusion that certain brassicaceous amendments may actually exacerbate disease severity (17). Based on our study, it appears that this would also be true if MSM was applied alone to *P. irregulare* infested soil. It has been demonstrated that MSM has herbicidal properties; therefore, the phytotoxicity observed here is not unexpected. MSM+MS had increased herbicidal potency compared with MSM alone, resulting in decreased downy brome (*Bromus tectorum*) coleoptile emergence (27). The glucolimnanthin degradation compound nitrile was implicated as the active compound causing herbicidal activity in ethyl ether and ethanol extracts of MSM (29). In this study, therefore, it is likely that nitrile played a role in wilt of tomato transplants and decreased germination and height of direct seeded cucumber and wheat when MSM was applied alone at rates of 1.0% or greater. As stated above, nitrile is the only toxic compound that would be present in significant quantities in MSM amended soil. Impacts on seed germination and plant growth were further exacerbated when MS was added to MSM, indicating that ITC also contributed to herbicidal activity.

Our data clearly demonstrate that planting tomato, cucumber, and wheat will have to be delayed after MSM+MS is amended to soil. Our data indicate that this period of time may need to be longer when transplants are used instead of crops that are direct seeded. The necessity for delayed planting after the incorporation of brassicaceous seed meals into soil has also been reported for carrot (15 to 36 days) (23) and strawberry (14 days) (1). Environmental conditions at soil amendment and planting can also play a role in phytotoxicity and duration of the phytotoxic effect, something that was not considered in this study. One strategy that could be used to avoid crop phytotoxicity by MSM+MS would be to amend soil in the fall and then plant the crop in the following spring.

A significant fertilizer effect was also observed in these experiments. When cucumber was seeded into soils amended with MSM+MS 6 to 8 days earlier, the plants were taller at the end of the experiment compared with those grown in nonamended soil. Similar observations have been made with other brassicaceous seed meals. Brassicaceous seed meals contain 5 to 6% N by weight and have C/N ratios of approximately 8:1 (11). Depending on the treatment, the duration of our time-course phytotoxicity assay extended from 14 to 22 days, which would have been adequate time for N mineralization to occur. It would be expected that MSM would have characteristics similar to these other related seed meals and that the growth response of cucumber observed in this study was due to a greater availability of N being provided to the plant from the MSM.

In our assay, where glucolimnanthin degradation products in soil were monitored in relation to pathogen suppression,*P. irregulare* were completely killed within 2 h after amending soil with MSM+MS. This was likely due to the activity of ITC rather than nitrile. At 2 h after amendment, ITC at an average of 0.12 and 0.28 mg/ml and nitrile at 0.077 and 0.147 mg/ml were recovered from soil amended with 0.5/1.0 MSM+MS and 1.0/1.0 MSM+MS, respectively. *P. irregulare* was not completely killed in our previous study, even with the highest concentrations of ITC (0.2 mg/ml) and nitrile (1.0 mg/ml); only 88 and 71% mortality was observed with each compound, respectively (33). Regardless of the amendment rate used in the present study, the 1.0 mg/ml concentration of nitrile required to kill 71% of *P. irregulare* was not achieved. However, within 2 h after incorporating the 1.0/1.0 MSM+MS amendment, the concentration of ITC was 0.28 mg/ml, which exceeded the highest concentration of ITC tested in our previous study. Concentrations of ITC continued to rise thereafter until they peaked at 24 h at 0.71 mg/ml. Therefore, the

results also indicate that ITC was the primary glucolimnanthin degradation product responsible for the mortality of *P. irregulare*.

Our research indicates the potential of MSM formulated with MS as a soil amendment to control *P. irregulare*. The soil amendment rates determined to cause complete mortality of *P. irregulare* (0.5/1.0 MSM + MS and 1.0/1.0 MSM + MS, respectively) are achievable in the field but a delayed planting of crops into amended soils will be necessary to prevent unintended crop phytotoxicity. However, once the toxic glucolimnanthin degradation products have dissipated, the remaining MSM product also has a fertilizer effect, making it a dual-use product. As we found in our previous study, both target organisms were the most sensitive to ITC, which was produced rapidly and peaked within 24 h after MSM+MS amendment into soil. However, the complete mortality of P. irregulare observed in the study could not be explained by the concentration of either ITC or nitrile alone; the dose of each compound that the *P. irregulare* was exposed to should have been incompletely lethal according to our previous study.

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