Title: Developing a Soil Bioassay for Alfalfa Autotoxicity

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Abstract:

Alfalfa autotoxicity is believed to be a form of allelopathy but has never been fully explained. It can lead to direct failure of germination in some cases, but the most damaging effect is damage to surviving root systems that reduces persistence and lifetime yield of stand that appear visibly normal. The duration and extent of autotoxicity is influenced by a complex mix of environmental, genetic, and management factors. Current best management practices recommend a waiting period up to two years before replanting alfalfa in the same field. Initial work in our lab indicated that a soil bioassay could detect inhibition of alfalfa seedling growth in soil. With refinement and validation, this bioassay might be used to inform replanting decisions. Our objective for the current work were to: 1) Refine bioassay methodology in the laboratory, 2) Compare autotoxicity in soils obtained from a diversity of alfalfa stands varying in environmental, genetic, and management factors, 3) Evaluate ability of bioassay to identify autotoxicity response to alfalfa termination date, and 4) Compare autotoxicity in farm fields before and after planting of new alfalfa seedings. Changes to the bioassay protocol include improved growth medium, control growth medium, container, standardization of soil moisture, and best sample storage practices. No autotoxicity differences were detected among the alfalfa varieties tested. Longer rotation intervals generally resulted in better performance of reseeded alfalfa. Soil fertility was identified as a possible factor in autotoxicity. Seedling growth in field plots was accurately predicted when reseeding bioassays were positive for autotoxicity, but the bioassay had an unacceptable rate of false negative results and is not yet suitable for commercial use. Causes of autotoxicity will be explored in a new NIFA trial successfully leveraged from this funding.

Introduction:

Alfalfa autotoxicity is a well-known phenomenon that has never been fully explained. The problem has been attributed to a water-soluble compound or compounds that are allelopathic to new alfalfa seedlings. While there are several leading candidates, the specific chemical entities have never been definitively identified, thus confounding any attempt to measure them directly.

Autotoxicity can cause direct failure of germination and seedling establishment in some cases, but the most damaging effect is permanent damage to root systems on seedlings that appear to have established successfully. This root damage causes reduced persistence and lifetime yield for the stand, a phenomenon called autosuppression or autoconditioning.

The degree and duration of autotoxicity and autosuppression have been attributed to a complex mix of environmental, genetic, and management factors. The problem is documented to increase with age and density of the alfalfa stand, dissipate over time after alfalfa stand termination, dissipate faster from sandy than fine-textured soils, wash out of soil with precipitation, and be reduced by tillage after alfalfa termination (Undersander et al., 2015). Response to these factors is not always evident in field studies (Seguin et al., 2002). Alfalfa varieties differ in autotoxicity potential in vitro (Chung and Miller, 1995), but it is not clear whether this effect is related to reduced toxin concentration, increased tolerance, or both.

Field studies on autotoxicity are challenging because it can take several years simply to set up field plots with a range of stand ages, and it is difficult to control all the possible interactions of environmental and management factors. Much of our knowledge on autotoxicity is obtained from laboratory bioassays using extracts of plant material. Best management practices for the appropriate planting delay after termination of an existing alfalfa stand range from two weeks (Tesar et al., 1993) to two years (Undersander et al., 2015). This large range in waiting period leaves growers in limbo, reluctant to risk expensive seed on trial and error, and may contribute to decline in alfalfa acreage if it seems less risky to just grow something else. Producers need an answer to the question, "is it safe to replant alfalfa, right now?"

We conducted a pilot study (Cassida and Hill, 2020) funded by USAFRI to determine whether a bioassay could be used to identify inhibition of growth in alfalfa seedlings grown in presumably autotoxic soils. We adapted a soil-on-agar (SOA) method (Voight et al., 1997) and demonstrated differences in alfalfa seedling establishment and morphology when growing seed in soils potentially affected by autotoxicity, thus establishing proof of concept. The current study extends that work to develop a soil bioassay for potential use by MSU Plant Diagnostic Services. This test could be offered to farmers across the country or adopted by other diagnostic services but requires validation to ensure that results are reliable. An accurate bioassay will help reduce risk of establishment failure and lifetime stand productivity losses to autosuppression, while reducing rotation time between alfalfa plantings. All these should improve profitability of growing alfalfa, thus encouraging its use. The bioassay will also provide a research tool that can assist in finally identifying the compounds responsible for autotoxicity. It can assess soils across a wide range of conditions, allowing faster progress in identifying trends than conducting controlled field experiments for every possible combination of factors. The second goal of this project was to obtain further preliminary data to support a larger proposal to USDA NIFA targeted at identifying the causes of autotoxicity.

Materials and Methods:

Objective 1. Refine bioassay methodology in the laboratory. We conducted dozens of individual trials aimed at specific refinements identified below as sub-objectives. All trials were replicated within or among growth chamber runs. Growth chamber settings were 16-h photoperiod and

constant 72°F temperature for all trials. All statistical analyses are conducted using mixed models or regression in SAS (SAS, Inc, Cary, NC).

Sub-objective 1.a. Refine the growth medium. While the SOA method allows us to visualize seedling roots as they grew, it proved challenging to regulate soil moisture content. We tested standardization of soil water holding capacity (WHC; Haney and Haney, 2010) in two SOA trials using 1) 30, 40, or 50% WHC or 2) 50, 60, and 70% WHC. We also tested alternate growth media including placing our field soil layer on washed sand (soil-on-sand, SOS) or filling the entire container with field soil (all soil).

Sub-objective 1.b. Identify control growth medium. The bioassay requires a valid comparison of unknown field soil to a control growth medium where alfalfa seedling growth is not restricted. Tested candidates for control soil included non-alfalfa field soils, washed sand, and nutrient-enriched peat-based potting mix.

Sub-objective 1.c. Identify acceptable soil storage parameters. Soil samples will need to be shipped and stored prior to any commercial bioassay or research work. Multiple trials were conducted to determine best storage practices for soil samples. Treatments included storage of moist soil samples at room temperature in plastic bags, air-dried and stored in plastic bags, or frozen at -17.8°C (home freezer) or -80°C (ultralow freezer) for up to 4 weeks.

Sub-objective 1.d. Identify ideal soil sampling depth. Autotoxins are water soluble and move through soil horizons with water (Jennings and Nelson, 1998). Therefore, soil sampling depth may influence bioassay results. We evaluated soil samples taken from various soil depths in two trials. Trial 1 soil treatments were: 0 to 1, 0 to 2, 0 to 3, 0 to 4, 0 to 5, and 0 to 6-inch depths. Trial 2 treatments were: 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, and 5 to 6-inch soil horizons.

Sub-objective 1.e. Determine threshold levels for biomass. We evaluated seedling growth in soils treated with alfalfa tissue extracts to begin the process of determining the threshold for autotoxicity. Alfalfa top growth was dried in a forced air oven at 150°F and ground sequentially through a 4-mm screen in a Wiley mill and then through a 1-mm screen in a cyclone mill. Tissue extracts were prepared as described by Chon et al. (2023), added to potting mix in the SOA assay, and grown for 4 d in the growth chamber. Germination tests with the same extract concentrations were also conducted on filter paper in petri dishes.

Objective 2. Compare autotoxicity in soils obtained from a diversity of alfalfa stands varying in environmental, genetic, and management factors. Under this objective we planned to expand scope of autotoxicity evaluation using soil samples from existing replicated alfalfa research plots at MSU and University of Wisconsin. However, because this objective was partly contingent on successful validation of the bioassay in Objective 3, we did not pursue collecting samples from UW. We conducted bioassays on soil from alfalfa variety test plots from two five-year-old tests at MSU (Cassida et al., 2022). Selected varieties represented FD2 through FD5 with four replications for each variety.

Objective 3. Evaluate ability of bioassay to identify autotoxicity response to alfalfa termination date. Two alfalfa reseeding trials were conducted in East Lansing, MI using small plots from two public variety tests running 2018-2021 and 2019-2022 (Cassida et al., 2022; 2023). Soil types for the two trials were Marlette fine sandy loam (2022) and Conover loan (2023). In the fifth year of each set of plots, existing alfalfa was terminated with glyphosate (2 qt/acre plus ammonium sulfate) in a randomized strip block design at two times prior to a late summer reseeding. The main treatment was three rotation intervals (RI): 1) control, previously in perennial grass (terminated 20 and 13 d before alfalfa seeding), 2) long RI (terminated 109 and 99 d before reseeding in 2022 and 2023, respectively), and 3) short RI (20 and 13 d before reseeding). An alfalfa variety with autotoxicity susceptibility indicated by prior bioassay was no-tilled at right angles to the original alfalfa rows and the adjacent control grass strip on 8/16/22 and 8/21/23. Permanent sampling sites consisting of two adjacent 1.5-ft sections of row were marked in each plot for measurement of seedling counts (14, 30, and 60 days after planting and at first cut the following spring). Seedling height was measured from 10 shoots per plot on the same dates. Alfalfa dry yield was determined four times in 2023 (May 31, July 7, Aug. 11, and Sep. 15) by hand-clipping to 2-inch height from the marked rows, and remaining biomass was mechanically cleared from plots. The same measurements will be collected from the plots reseeded in 2023 during the 2024 growing season, and we anticipate repeating this trial on a third set of plots to be reseeded in 2024.

Objective 4. Compare autotoxicity in farm fields before and after planting of new alfalfa seedings. To rapidly collect preliminary data on the timing of planting relative to termination, we proposed to recruit growers to collect soil samples from new alfalfa fields being replanted into an old alfalfa field and an adjacent non-alfalfa field edge at planting and send those to MSU for bioassay. Growers would be trained to monitor seedling emergence and performance in resulting stands.

Objectives and Corresponding Results:

Project Objective:	**Project Results:**
1. Refine bioassay methodology in the laboratory	Changes to the bioassay protocol include improved growth medium, control growth medium, container, standardization of soil moisture, and best sample storage practices.
2. Compare autotoxicity in soils obtained from a diversity of alfalfa stands varying in environmental, genetic, and management factors.	No autotoxicity differences were detected among the alfalfa varieties tested. Longer rotation intervals generally resulted in better performance of reseeded alfalfa. Soil fertility was identified as a factor in reseeding success.
3. Evaluate ability of bioassay to identify autotoxicity response to alfalfa termination date.	Seedling growth in field plots was accurately predicted when reseeding bioassays were positive for autotoxicity, but the bioassay had an unacceptable rate of false negative results.

4. Compare autotoxicity in farm fields before and after planting of new alfalfa seedings.	The project was unable to complete this objective because of the false negative finding in Obj. 3. We will continue to work to on this past the end date of the funding.

Results and Discussion:

Objective 1. Refine bioassay methodology in the laboratory.

*Sub-objective 1.a. Refine the growth medium***.** Results of WHC trials indicated that soil moisture over 60% resulted in very poor seed germination presumably due to anoxia. Soil in subsequent trials was standardized to 60% WHC for the bioassay but excess moisture still unpredictably wicked into the soil layer from the agar, frequently resulting in soil becoming too wet for alfalfa. Consequently, we conducted trials to look at alternate growth media to SOA. Bioassays using soil-on-sand or all soil were more likely to detect differences among soils than SOA (Figure 1). We selected all soil as our preferred growth medium due to simplicity of assay set-up.

Figure 1. Alfalfa seedlings growing in potting soil (left) and all soil (right) bioassays. Photo: Paige Baisley

Sub-objective 1.b. Identify control growth medium. The bioassay requires a valid comparison of unknown field soil to a control growth medium where alfalfa seedling growth is not restricted. Candidates for control soil included non-alfalfa field soils, washed sand, and potting mix. Comparisons to non-alfalfa field soil were generally unsatisfactory. Possible reasons include uncontrolled allelopathic compounds from other plants or herbicide carryover in row crop soils. Ultimately, we selected nutrient-enriched, peat-based potting soil as the bioassay control (Figure 1).

Sub-objective 1.c. Identify acceptable soil storage parameters. The ideal storage method was defined as one that resulted in no post-storage change from a pre-storage baseline bioassay but results across multiple trials were inconsistent (data not shown). The "best" storage method differed among runs. Bioassay response sometimes changed over time with storage up to 4 w and sometimes did not. We concluded that the optimum scenario would be analyzing commercial samples as soon as possible after collection. For research samples when running all samples immediately is limited by growth chamber space, freezing as-collected or after rapid air-drying are both acceptable. Repeated freezing and thawing of samples may contribute to instability and should be avoided.

Sub-objective 1.d. Identify ideal soil sampling depth. In a series of trials, we found no consistent differences in autotoxicity related to soil depth down to 8 inches. Presence of concentrated bands of autotoxic activity within the measured horizons were random among

runs and possibly related to movement of autotoxins through the soil profile after rainfall events. We conclude that a sampling depth of 4 to 6 inches will be acceptable for the bioassay. This is relevant also relates to the soil volume explored by alfalfa seedlings that are up to 4 d old.

Sub-objective 1.e. Determine threshold levels for biomass. Alfalfa root growth was reduced at tissue extract concentrations greater than 10 g/L (Figure 3). Converted to field scale, 10 g/L of tissue extract is roughly equivalent to 1 inch of rain percolating through 1 ton/acre of dry alfalfa residue from the previous stand.

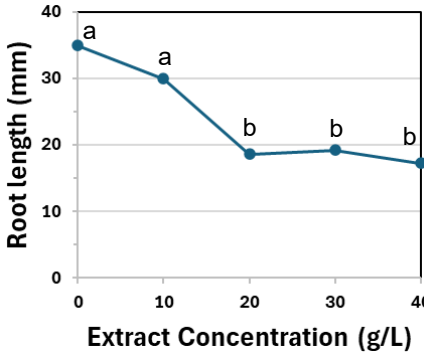

Figure 3. Alfalfa seedling root growth is reduced by addition of extracts of dry alfalfa tissue to soil. Different letters for data points indicate differences ($P < 0.05$).

One challenge to the bioassay that was not resolved revolves around the synthetic nature of alfalfa varieties. The bioassay concept requires that the alfalfa seeds grown are sensitive to autotoxins and the variety used across this work displayed such sensitivity in the pilot study. However, all seeds in a variety are not genetically identical and a 10-seed bioassay may have too much genetic variation to give repeatable assessments of autotoxicity. Genetic differences in susceptibility to autotoxicity have been previously reported (Zhang et al., 2021) and were found in our pilot study (Cassida and Hill, 2020). We observed considerable variation in seedling performance even within potting soil control bioassays. We hesitated to adjust results for germination percentage or use pre-germinated seeds because germination can be directly affected by autotoxicity. One possible solution to be explored in future is to conduct a 100-seed germination test in test soil or test soil extract as well as the 10-seed bioassay to examine root growth.

The current bioassay is conducted using field soil collected to 6-inch depth. Moist soil is sieved through a ¼-inch screen and stored at -80°C until bioassays are conducted. Ninety grams of soil is moistened to 60% WHC and placed in a thin rectangular petri dish (4 x 3 x 0.5 inches). All bioassays are replicated (at least 4 replications) within and/or among growth chamber runs. Control growth media is a comparable volume of nutrient-enriched peat-based potting soil. Ten fungicide-treated seeds of a susceptible alfalfa variety are planted ¼-inch deep. Petri dishes are taped shut and stand upright in a growth chamber set at 16-h photoperiod and constant 72°F temperature. Four days after planting, seedlings are removed from growth media and washed. Percentage germination and emergence are recorded. Root radicles are separated from hypocotyls, suspended in water, and scanned for analysis of root length and diameter using WinRhizo (Quebec City, Quebec). Reduced root length and increased root diameter are the most consistent markers of autotoxicity, with few differences observed for germination and emergence. Abnormal root orientation cannot be accurately assessed in an all-soil bioassay and is no longer measured. To adjust for variation among individual seeds and growth chamber runs, all root data is expressed as a percentage of the potting soil control. Statistical analyses are conducted using mixed models or regression in SAS (SAS, Inc, Cary, NC).

Objective 2. Compare autotoxicity in soils obtained from a diversity of alfalfa stands varying in environmental, genetic, and management factors. We evaluated autotoxicity potential of preceding alfalfa varieties for reseeded alfalfa in the plots described in Objective 3. The soil bioassay did not detect autotoxicity for the seven varieties compared in 2022 or five compared in 2023 (data not shown). We have found variety differences using the bioassay in previous work (Cassida and Hill, 2020). However, without a better understanding of what can make a variety more or less autotoxic, we are limited to testing the varieties on hand which possibly are not varieties exhibiting high levels of autotoxicity.

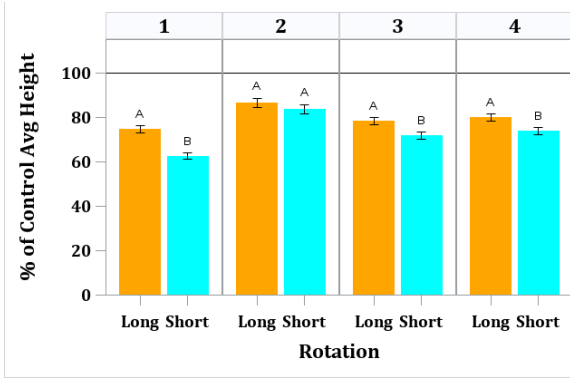

Figure 4. 2022 field trial alfalfa yield as a percent of the alfalfa yield in control plots during each year 1 cutting. Letters indicate difference between treatments in each cut ($P < 0.05$). Error bars

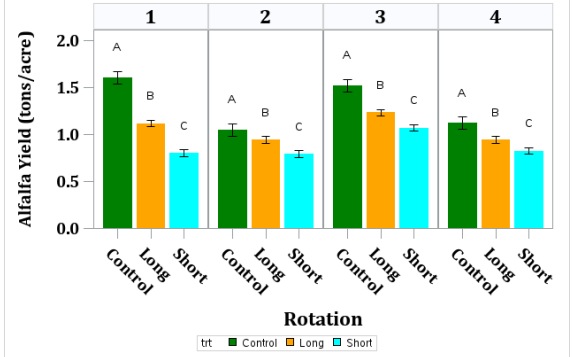

Figure 5. 2022 field trial alfalfa yield during year 1 harvest by treatment for each cut. Letters indicate difference between treatments ($P < 0.05$). Error bars represent standard error of the ls-means.

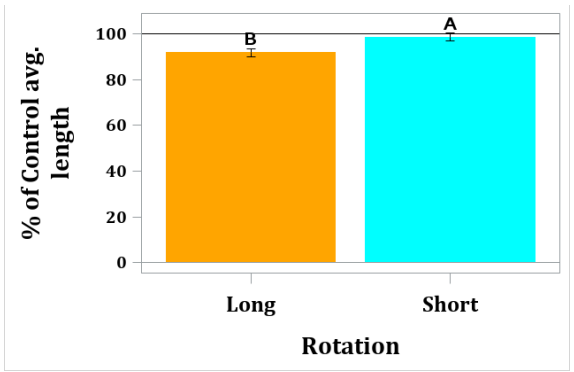

Figure 6. 2023 field trial bioassay. Root length per seedling as a percent of the root length per seedling in field control plots. Letters indicate difference between treatments ($P < 0.05$). Error bars represent standard error of the ls-means.

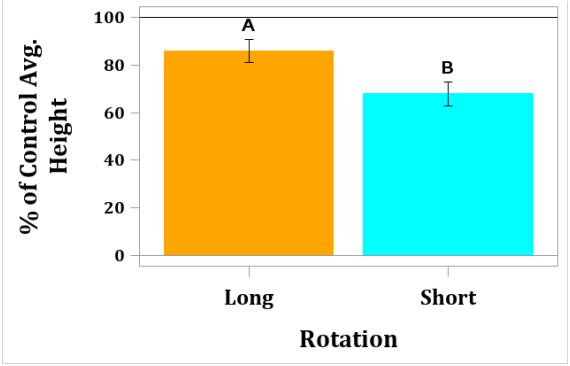

Figure 7. 2023 field trial average plant height as a percent of the average plant height in control plots. Letters indicate difference between treatments ($P < 0.05$). Error bars represent standard error of the ls-means.

Objective 3. Evaluate ability of bioassay to identify autotoxicity response to alfalfa termination date. Bioassay root length and diameter did not differ among RI treatments in the 2022 trial (data not shown, $P > 0.05$), nor did we detect differences in fall seedling count or height. These results indicate an absence of autotoxicity at seeding for both RI. However, in the following production

year there were clear differences among treatments in shoot height (Figure 5, 3 of 4 cuts, $P < 0.001$) and dry matter yield (Figure 4, all cuts, $P < 0.001$) that were consistent with a reduction in autotoxicity with lengthening RI or absence of alfalfa in the previous rotation.

Routine soil testing in summer 2023 indicated that the plot site was lower than desirable in soil P and K. Because of this we conducted mineral analysis at a commercial laboratory for all harvested alfalfa tissue. Almost all tissue samples were deficient in K. Across all cuttings, tissue K differed as control > long RI > short RI (2.49, 1.75, 1.62 mg/kg, $P < 0.001$). This unintended confounding of RI with soil nutrient concentration makes it impossible to determine whether autotoxicity or soil fertility were causing depressed alfalfa growth, but also sparked the question of whether infertile soils may be a factor affecting development of autotoxicity. Aging alfalfa stands may well also be low in K, and plants often respond to nutrient deficiency by excreting compounds into the rhizosphere to aid nutrient acquisition (Sardans et al., 2023).

In the 2023 trial, bioassay root lengths decreased (Figure 6, $P < 0.05$) and diameters increased (data not shown, $P < 0.05$) for long versus short RI, a finding opposite of our expectation that longer RI would reduce autotoxicity. Seedling heights varied in the expected direction with greater growth inhibition for short than long RI (Figure 7, $P < 0.05$). Yields for 2024 have not yet been harvested at the time of this report.

Objective 4. Compare autotoxicity in farm fields before and after planting of new alfalfa seedings. This objective cannot be completed until we have a reliably validated bioassay. Moreover, discussions with producers indicated this approach is not likely to provide useful information on autotoxicity. Extension and industry have done an excellent job teaching them to wait at least one year for autotoxicity to dissipate before replanting alfalfa. We will need to devise a more controlled method for making this evaluation where we deliberately plant alflafa earlier than recommended with compensation to producers for potential income losses.

Outreach. Scientific results from this project were presented at the XXV International Grasslands Congress (Baisley et al., 2023c) and the 2023 ASA/CSSA/SSSA Conference (Baisley & Cassida, 2023b). A refereed review paper on autotoxicity is in preparation for submission in 2024 and a paper on the bioassay will be forthcoming once validation trials have been completed. A PhD dissertation at MSU by Paige Baisley will be based in part on this funding. Results were presented to producers and industry at the Great Lakes Forage & Grazing Conference in 2022, 2023, and 2024, and at the 2024 Midwest Forage Symposium. The project has been featured in *Hay and Forage Grower* magazine (Rankin, 2021), the MSUE Virtual Breakfast webinar/podcast (Cassida, 2020), and the UW Focus on Alfalfa webinar (Cassida, 2024).

CONCLUSIONS

This project is our first effort to validate bioassay results against alfalfa performance in the field. Our overall conclusion is that we can be confident of the existence of autotoxicity as a binary yes/no choice if the bioassay gives a positive result. However, we cannot be confident that we do *not* have autotoxicity if the bioassay results are negative. More validation work is needed to determine if this false negative obstacle can be overcome. Our serendipitous finding on

involvement of soil fertility suggested a new line of investigation on the possible interaction of soil fertility with autotoxicity. This will be explored in our new NIFA-ASAFS project leveraged by this funding (Cameron, 2023).

Acknowledgements: Funding for this study was provided by the U.S. Alfalfa Farmer Research Initiative of the National Alfalfa & Forage Alliance and Project GREEEN of Michigan.

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Keywords: alfalfa, autotoxicity, establishment