Developing a Simple Bioassay for Detection of Alfalfa Autotoxicity in Field Soils

Baisley, P; Cassida, K; Gruss, S; Hill, E
Michigan State University, East Lansing, MI US

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Abstract. Alfalfa autotoxicity causes yield reductions in alfalfa production by inhibiting plant establishment and decreasing plant productivity. Accurate predictions regarding autotoxic potential of the soil in a given field at a given time are an essential tool for alfalfa growers to make appropriate planting decisions. To address this issue, we are developing a soil bioassay that can be conducted as a mail-in soil test for alfalfa growers through plant diagnostic service laboratories. We hypothesize that we will detect differences in seed germination, emergence, root length, and root morphology between control and autotoxic soils. A preliminary trial testing alfalfa field soils and fallow field soils against a potting soil control found significant response of percent abnormal roots ($P<0.001$) and average root length ($P<0.05$) to soil variety. There were significantly lower percentages of abnormal roots in the control and fallow soils than in alfalfa soils ($P<0.05$) and significantly longer roots in the control soil than in alfalfa soils ($P<0.05$). An ongoing field trial encompassing multiple alfalfa varieties and termination dates will be used to validate the bioassay methodology for detecting autotoxicity. Implementing this bioassay could inform alfalfa establishment decisions, reduce yield losses from autotoxicity, and allow collection of data that can be used to further understand alfalfa autotoxicity.

Introduction

Alfalfa is an important forage crop for its high crude protein and energy (Undersander 2011). Alfalfa and alfalfa mixture production was estimated at 15.46 million acres in the U.S. in 2022 (USDA-NASS, 2022). The importance of alfalfa in U.S. forage production drives research efforts to increase yield, reduce disease, and improve overall management. One ongoing challenge to alfalfa production is the unexplained phenomenon of alfalfa autotoxicity. Alfalfa autotoxicity occurs when alfalfa releases compounds that are allelopathic to new alfalfa seedlings, causing poor germination and seedling death. Less severe cases of autotoxicity may not kill seedlings, but instead inhibit normal taproot development. This phenomenon, known as autoconditioning or autosuppression, leads to yield loss and decreased stand persistence.

Our understanding of alfalfa autotoxicity is largely based on laboratory work examining compounds present in plant tissues and their effect on germination. The compounds causing autotoxicity remain unidentified but are agreed to be water-soluble (Undersander 2011). Genotype influences autotoxicity, but the mechanism is unknown: autotoxin production, autotoxin tolerance, or both (Chung and Miller 1995a). The presence and persistence of autotoxins in the soil is influenced by soil type (Jennings and Nelson 1998), decomposition conditions (Bonanomi et al. 2011), rotation interval (Tesar 1993; Seguin et al. 2002; Jennings and Nelson 2002), tillage (Undersander 2011), original stand age (Seguin et al. 2002), and irrigation (Chon et al. 2006). Controlling all of the environmental and management factors influencing autotoxicity is challenging in field studies. Attempts to induce autotoxicity in a field setting have been mostly unsuccessful, with autotoxicity effects unobserved or inconsistent in multiple studies (Tesar 1993; Seguin et al. 2002). Because of these complexities, best management practices for avoiding autotoxicity in a production field remain ambiguous. The current recommendation is based on rotating out of alfalfa for a period long enough to ensure natural dissipation of autotoxicity. While autotoxicity may dissipate in as little as two weeks (Tesar 1993), it may take as long as two years (Undersander 2011). This uncertainty is unacceptable to some producers who may opt out of growing alfalfa altogether in favor of more predictable crops. This is concerning because alfalfa provides many useful ecosystem services in addition to yield.

Our objective is to develop a soil bioassay that can be offered through plant diagnostic service laboratories to directly answer producers' questions about the viability of planting alfalfa in a particular field. Such diagnostic services would reduce producer uncertainty associated with the current best management recommendations. Widespread adoption of the bioassay will also facilitate the collection of autotoxicity data from diverse sites for future research into the phenomenon of alfalfa autotoxicity. The soil-on-agar method bypasses time intensive and yet unsuccessful toxin compound identification by focusing on plant response parameters to detect autotoxicity in soils. Based on previous research on seedling response to autotoxicity (Chon et al. 2000; Chon and Nelson 2001; Chung and Miller 1995b; Hedge and Miller 1990; Jennings and Nelson 1998) we hypothesize that autotoxicity will manifest as lower germination, lower emergence, shorter root length, and more abnormal roots in autotoxic soil compared to control soil. An effective bioassay will produce results that can be validated in greenhouse and field trials.

Methods

Bioassay

The autotoxicity bioassay is based on the soil-on-agar method (Voigt et al. 1997; Chon and Nelson 2001) using 200ml of agar in clear sanitized plastic flasks measuring 8 x 2.5 x 8-cm. One cm of test soil is placed on top of agar and ten alfalfa seeds are planted in the soil layer in an evenly spaced line at 0.5-cm depth. Preliminary trials (data not shown) refined individual components of the methodology including: agar concentration, identification of a consistent control soil, length of growth period, growth chamber temperature and light conditions, identification of an autotoxin-sensitive alfalfa variety as seed source, relative importance of shoot versus root measurements, and soil sample handling and storage between acquisition and testing. During the preliminary testing, soils were obtained to a 15-cm depth from a variety of existing research plots with or without alfalfa of varying genotypes and stand histories using a 2.5-cm-diameter soil probe or shovel. We continue to refine the bioassay but results reported here use agar concentration of 5 mg/L, soil sample storage temperature of 0˚C, a peat-based commercial potting soil as a control, and 3-d growth period in a growth chamber set at 22C with 16:8 h light/dark period. A commercial seed treatment with fungicide for our indicator alfalfa variety proved essential to prevent seedling loss from phytopthera "damping off" in many test soils. Root measurements proved more useful than shoot measurements and include: total emergence (number of shoots visible above the soil surface); visible roots (number of roots visible in the agar below the soil); root morphology (described as normal or abnormal, with normal roots growing straight down and abnormal roots growing with curves, curls, kinks, or bends); and percent germination (destructively sampled to identify germinated seeds that have not left the soil layer). Roots were separated from shoots and then scanned with WinRhizo software (Regent Instruments, Sainte Foy, Quebec) to measure total root length and root diameter.

A preliminary trial using the refined methodology tested three alfalfa field soils from a two-year-old stand (from three alfalfa varieties coded: A, B, and C) and three fallow field soils (F1: previously in grass; F2: previously in soybeans; F3: previously in red clover) against the potting soil control. Each field soil or control was replicated with four flasks in the growth chamber and location of individual flasks within the growth chamber was completely randomized. All analysis for the preliminary trial was generated using PROC MIXED in SAS (SAS, Inc, Cary, NC). Statistical significance was declared at $P \leq 0.05$.

Validation Trial

A field trial is underway to begin the process of validating the soil bioassay for detecting alfalfa autotoxicity. Plots were established as a public alfalfa variety trial with fifteen entries run from 2018 to 2021 East Lansing, MI (Cassida et al. 2022). The variety trial was designed as a completely randomized block design with four replicates. After the variety test was completed, plots were divided in half in a strip

block design with factors of alfalfa variety (15 varieties) and alfalfa kill timing (terminated with glyphosate at: 1) early kill, four months before planting, and 2) late kill, one month before planting). Plot size for each variety-kill date combination is 1 x 3.5-m. Alfalfa was no-till seeded across all plots on August 16, 2022. Soil samples for the autotoxicity bioassay were taken prior to seeding with ten to twelve soil cores at 15-cm sampling depth from each plot and results are incomplete.

To begin the process of correlating bioassay results with field performance of alfalfa, seedling counts and percentage ground cover were measured at two, four, and eight weeks after planting. Average height was measured eight weeks after planting. Seedling counts were conducted along two rows in 45-cm strips, centered 15-cm in from the edges of each plot. Percentage ground cover was measured with Canopeo from 30.5 x 30.5-cm quadrats, centered in the plots. Average height was calculated from measuring ten seedlings per a plot along the seedling count strips. In spring 2023, seedling performance will be measured as stem counts, first cutting forage yield, and forage nutritive value.

Results and Discussion

Soil samples differed by percent abnormal roots (P=0.001) and average root length (P<0.05). Figure 1 shows lower percentages of abnormal roots in the control and all fallow soils than in all alfalfa soils. Figure 2 shows significantly longer roots in the control soil than in all alfalfa soils and one fallow soil. Roots in fallow soil F2 were also significantly longer than in all alfalfa soils. These results are consistent with our hypothesis that seedlings in autotoxic soils will have more abnormal roots and shorter roots than control soils and supports previous research suggesting that root growth is inhibited by exposure to autotoxic compounds (Chon et al. 2000; Chon and Nelson 2001; Chung and Miller 1995b; Hedge and Miller 1990; Jennings and Nelson 1998). This suggests that the refined components of the methodology together create an effective bioassay. The significance of visibly abnormal roots as an indicator of autotoxicity expands on existing literature and may be important for identifying autotoxicty when root scanning software is unavailable or cost-prohibitive.

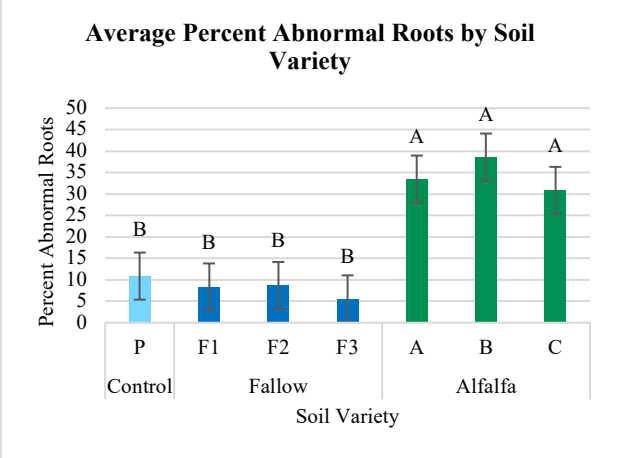

Figure 1. Bioassay measurements. Error bars indicate standard error. Different letters indicate significance at P<0.05.

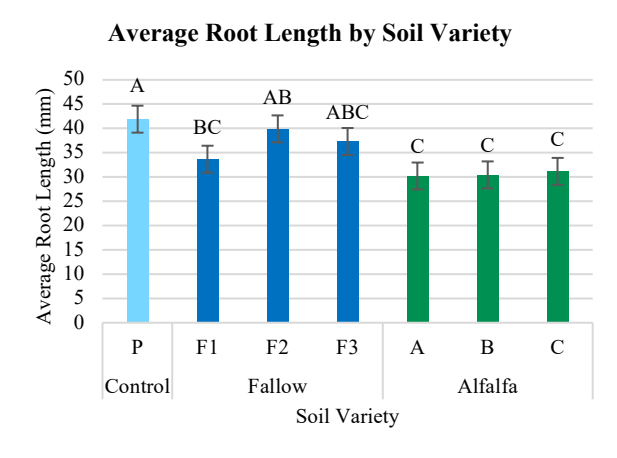

Figure 2. Bioassay measurements. Error bars indicate standard error. Different letters indicate significance at P<0.05.

Conclusions

The soil-on-agar bioassay can detect differences between seedling performance in control soils, non-alfalfa field soils, and alfalfa soils. Percent abnormal roots and average root length were the most reliable parameters for detecting differences. Results from the field trials is necessary to confirm whether differences in seedling performance in bioassays accurately predicts performance in field settings.

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