Title: Developing a Soil Bioassay for Alfalfa Autotoxicity

Dr. Kimberly A. Cassida (Project Leader)
Assistant Professor – Fixed Term
Plant, Soil and Microbial Sciences
1066 Bogue St. R, A486
East Lansing, MI 48824
517-353-2078
cassida@msu.edu

Dr. Erin Hill
Academic Specialist- Weed Science Diagnostician
Plant, Soil and Microbial Sciences
578 Wilson Rd., Room 113
East Lansing, MI 48824
517-432-9693
hiller12@msu.edu

Abstract:

Alfalfa autotoxicity is a well-known phenomenon that has never been fully explained. What we know is that alfalfa contains an unidentified water-soluble compound or compounds that are allelopathic to new alfalfa seedlings. Autotoxicity causes direct failure of germination and seedling establishment in some cases, but the most damaging effect is permanent damage to surviving root systems that causes reduced persistence and lifetime yield for the stand. The duration and extent of autotoxicity is influenced by a complex mix of environmental, genetic, and management factors. Current best management practices recommend waiting two weeks to two years after stand termination before planting a new stand. Our objective was to initiate development of a bioassay with field soil samples that can be used to predict whether it is safe to replant. Simple germination tests with water extracts from soil were not sensitive enough to be useful. A soil-on-agar bioassay was able to detect differences in root development between alfalfa varieties grown in the field soil, seed varieties used in the bioassay, stand age, and time since termination. Greenhouse-grown plants confirmed root development differences predicted by SOA assay. These results provide proof of concept that a soil bioassay can quickly detect differences in alfalfa seedling development. Longer-term research is required to validate the bioassay before commercial deployment is possible.

Introduction:

Alfalfa autotoxicity is a well-known phenomenon that has never been fully explained. What we know is that alfalfa contains 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 causes 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.

The degree and duration of autoxicity and autosuppression are influenced by a complex mix of environmental, genetic, and management factors. The problem is believed 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). Genotype influences toxicity of alfalfa plant extracts in vitro (Chung and Miller, 1995), but it is not clear whether this effect is related to reduced toxin concentration, increased tolerance, or both.

Much of our knowledge on autotoxicity is obtained from laboratory bioassays using extracts of plant material. 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 for all the possible interactions of environmental and management factors. In some cases, no autotoxicity was observed (Sequin et al., 2002). Best management practices for the appropriate planting delay after termination of an existing alfalfa stand are based on relatively few field studies and range from two weeks (Tesar et al., 1993) to two years (Undersander et al., 2015). This large range of 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. Growers need an answer to the question, "is it safe to replant alfalfa, right now?" From this practical perspective, it is not necessary to quantify all the factors that contributed to autotoxicity status of particular soil, but simply to answer "yes" or "no" to alfalfa planting decisions. With this one-year funding, we propose to develop and begin validation of an affordable, rapid bioassay for field soil that can be used to evaluate the autotoxicity status of a field and assist farmers with planting decisions for alfalfa. This assay can be offered through MSU Plant Diagnostic Services to farmers across the country or adopted by other diagnostic services.

Materials and Methods:

Task 1. Develop a bioassay protocol for measuring alfalfa seed germination in soil affected by autotoxicity. Established in vitro procedures for measuring seed germination in the presence of alfalfa autotoxins are based on germinating seeds on paper or agar moistened with water extracts from herbage (Chon et al., 2000; 2001). We investigated adapting these methods to water extracts from field soils that had grown alfalfa for 0, 1, 2, 3, 4, or 5 years. All tests under this task were conducted using unscarified, uncoated seeds of a VNS variety or scarified uncoated seeds of uncertified Vernal. Soils were extracted by vortexing screened, airdried soil with deionized water at a 1:3 ratio (w/v), gravity or vacuum filtered through Whatman filter paper, and stored in a refrigerator if not used immediately. Sterilization of extracts by autoclaving was investigated to destroy alfalfa pathogens and stop possible degradation of autotoxins by microbes.

Best results were obtained with seeds sandwiched between two layers of germination paper saturated with 8-10 ml of soil extract in a petri dish. This was a large amount of extract to prepare when samples were run with 4 to 6 replications. Petri dishes were stood on their sides to keep roots all growing in one direction and incubated in the dark at room temperature. When

radicles were at least 1 mm long, the seed was counted as germinated and removed from the dish.

Task 2. Develop a bioassay protocol for measuring alfalfa root growth and structure in soil affected by autotoxicity. The soil-on-agar method (SOA) was developed to test the effects of soil chemistry on seedling germination (Voight et al., 1997). The method requires minimal soil processing and consists of growing seeds in a half-inch layer of field soil on top of clear agar in an upright rectangular culture flask. This enables roots to grow in a normal orientation with good visibility for evaluating root length and morphology. Incubation flasks were made from plastic tissue culture flasks with the tops cut off, resulting in dimensions of 8.3 x 8.3 x 3.4 mm. Flasks were sanitized with 10% bleach solution, filled with approximately 200 ml of freshly made hot agar (7 g agar/L distilled water), and agar was allowed to set overnight. Fifteen grams of screened (quarter inch mesh) field soil was spread to a uniform depth over the agar, 15 alfalfa seeds were placed down the center line, and seeds were covered with 15 g additional soil. Water from the agar moistened the soil via capillary action. The sides of the flasks were covered with black material (paper or felt) to exclude light. Flasks were randomly arranged on trays and incubated up to 7 days in a germination chamber (Seedboro Equipment Co., Chicago, IL) with a temperature of 25 C and a light:dark cycle of 12:12 hours. Ultimately, runs of 4-5 days were adequate to detect differences, and by the end of this time the longest roots were nearing the bottom of the flask. At the end of the incubation, flasks were scanned to provide images for root length measurement using ImageJ, with root length recorded as the length visible below the soil layer. Seedlings were removed from agar, cut at the top of the radicle, and shoots and roots were dried at 100 C.

Task 3. Compare autotoxicity in soils obtained from a range of alfalfa stand ages, soil types, time since termination, and genetics. We tested different alfalfa seed sources for response to soils of different autotoxicity potential. Multiple test runs were made to evaluate soils differing in variety of alfalfa grown, age of stand, soil type, or termination status. All soil samples were obtained as 6-inch-depth cores from MSU variety test plots in East Lansing (south central region of Michigan) and Chatham (Upper Peninsula). All control soils were obtained from long-term grass plots. Measurements included number of emerged shoots, number of visible roots, number of abnormal roots (not growing straight down), shoot and root dry weight, and root length. Root length was measured using ImageJ. Each run lasted 4 to 5 days with 2 to 4 replications of each treatment combination within runs. Treatment combinations were selected to test specific limited hypotheses for each run. Three runs are highlighted here. *Run 1* compared a fall dormancy 3 (AFX 429) variety and a FD4 variety (AFX 469) across the same three sandy l from Chatham plus three soils from East Lansing: control, 5th year living alfalfa, and 5th year alfalfa terminated with glyphosate 3 wk before sampling. *Run 2* compared three test seed varieties (Vernal, AFX429, AFX469) across the same three loam soils used in Run 1. *Run 3* compared the two AFX test seed varieties with 12 soil treatments selected from a range of varieties and stand ages. Vernal and Hybriforce 4400 are used as check varieties in public tests and therefore plots with multiple stand ages were available. Plots of AFX 429 and AFX 469 were used because they are the test seed varieties, and 54R02 was selected because it was the oldest research plot available. Means were separated using LSD or single-degree of freedom contrasts as appropriate.

Task 4. Validate bioassay results using potted alfalfa plants in a greenhouse. Alfalfa seedlings were grown in replicated (n=4) cone-tainers in a greenhouse with 16:8 hours light:dark periods, and day/night temperature of 70/65^{o}F. Field soils were obtained from the East Lansing site. Treatments were field soil (orchardgrass control and a five-year-old stand of 54R02 that had inhibited alfalfa seedling development in previous SOA runs) and time after planting (7, 14, and 21 days).

Three seeds were planted in each cone. Cones were sub-irrigated with tap water and were not fertilized. At weekly intervals, seedling counts, developmental stage and height were recorded. Each week, one set of cones was destructively sampled, roots were washed free of soil and pictures were taken using a scanner. Images were analyzed for root length using WinRhizo. Shoot and root mass were recorded after drying at 100 C.

Fig.1. Alfalfa seedlings growing in soil from grass (control) or alfalfa plots in Task 4. Photo: Kim Cassida

Project Objectives and Corresponding Results:

Project Objective:	**Project Results:**
1. Develop a bioassay protocol for measuring alfalfa seed germination in soil affected by autotoxicity.	Germination assays using soil extracts were not sensitive enough to detect differences.
2. Develop a bioassay protocol for measuring alfalfa root growth and structure in soil affected by autotoxicity.	The soil-on-agar (SOA) assay was able to detect soil-based differences in root development of alfalfa seedlings.
3. Compare autotoxicity in soils obtained from a range of alfalfa stand ages, soil types, time since termination, and genetics.	The SOA bioassay was able to detect differences in root development between alfalfa varieties grown in the field soil, seed varieties used in the bioassay, stand age, and time since termination.
4. Validate bioassay results using potted alfalfa plants in a greenhouse	Greenhouse-grown plants confirmed root development trends predicted by SOA assay.

Results and Discussion:

Task 1. Develop a bioassay protocol for measuring alfalfa seed germination in soil affected by autotoxicity. No differences in germination were detected among soils (data not shown) when soil extracts were used to dampen germination paper in petri dishes. Chung and Miller (1995) found water extracts of soil inhibitory to alfalfa germination, but they used a longer extraction time. It is possible our soil extracts were too dilute in comparison to what would be present in soil solution. Longer extraction times with multiple filtration and centrifugation steps as described by Chung and Miller (1995) could be useful for research but is problematic in a fast-turnover laboratory testing scenario because it would greatly increase assay time requirement and cost. Extraction of pure soil solution (Reynolds, 1983) would avoid dilution questions, but is time-consuming and would require submission of a large volume of soil in order to give enough solution to run duplicate assays. For these logistical reasons, we concluded that a simple germination assay is unlikely to be the desired diagnostic tool and therefore, we focused our attention on Task 2.

Task 2. Develop a bioassay protocol for measuring alfalfa root growth and structure in soil affected by autotoxicity. The SOA assay allows germination in soil plus the ability to visualize root growth. Most consistent results were obtained when field soil was screened through ¼-inch mesh, frozen if not assayed immediately, and used without air-drying. Because moisture may allow microbes to break down the toxins during storage, future work will need to investigate the impact of soil sample storage conditions. To be useful to growers, samples sent to a diagnostic lab would need to be run immediately thus reducing this potential problem.

Figure.1. Many seedlings exhibited abnormal growth orientation and this may be an indicator of the problems in autotoxic soils. Photo: Kim Cassida

We observed a high rate of abnormal seedling root morphology in the bioassay (Fig. 1). Abnormalities included taproots growing sideways or up, bending abruptly, seedlings that developed a visible shoot with no root extending into the agar, and seedlings where the junction between hypocotyl and radicle was pushed far out of the soil. In some runs, incidence of abnormal roots was significantly greater in autotoxic soil, but this effect was not consistent across all runs.

Initially we used untreated seed to avoid possible confounding of results due to fungicide effects on beneficial soil microbiota. However, field soil samples differed greatly in degree of soil borne-disease and many early runs were completely lost to *Pythium.*

Sterilization of soil samples might have prevented this problem, but would add expense to the assay, prevent beneficial biological processes and possibly alter autotoxin activity. This needs further investigation. Meanwhile, using commercially fungicide-treated (mefenoxam) seeds of WL354HQ gave better survival (12.8 vs. 4.2 seedlings, $P < 0.001$) and longer radicals (14 vs 10 mm, $P < 0.01$) than untreated seeds of 5301. Because the treated and untreated seeds were not the same variety, it is not possible to attribute this solely to seed treatment, but we consequently changed to using only fungicide-treated seeds in the assay and thereafter lost few seedlings to *Pythium.*

The SOA bioassay could detect differences in alfalfa seedling development in field soils within four days after planting. In general, the number of developed roots and root length were more sensitive indicators of differences among soils than shoot emergence or shoot and root weight. ImageJ was better suited than WinRhizo to measure root length in scans from the SOA assay because scan photo contrast was not sharp enough between roots and agar for WinRhizo to detect interfaces.

Task 3. Compare autotoxicity in soils obtained from a range of alfalfa stand ages, soil types, time since termination, and genetics.

Table 1. Effect of soil treatment on bioassay shoot emergence, root emergence, and root length for two alfalfa varieties grown in soils differing in soil texture, stand age, or termination management.

Soil treatment	Shoot emergence, %	Radicle count, %		Radical length, mm	
		AFX 429	AFX 469	AFX 429	AFX 469
loam, orchardgrass	93 a	91 a	87 ab	36 abc	42 a
loam, alfalfa 5th yr	75 c	70 cd	87 ab	37 abc	31 c
loam, alfalfa 5th yr, killed‡	77 bc	63 d	93 a	37 abc	33 bc
sand, orchardgrass	87 abc	87 ab	83 ab	36 abc	40 ab
sand, alfalfa 2nd yr	85 abc	80 bc	93 a	36 abc	35 abc
sand, alfalfa 5th yr	88 ab	80 bc	93 a	31 c	42 a
LSD	8.3	--------8.5--------		--------5.7--------	

abcd Values with different letters within variable groups are different (P< 0.05).
‡ Killed with glyphosate 3 wk before sampling.

Run 1. Table 1 summarizes a comparison of two soil types (loam, sandy loam) with an orchardgrass control versus alfalfa stands with different stand age (2nd or 5th year of stand) or termination management (5th year alfalfa either live or three weeks after termination with glyphosate). Two seed varieties were selected for the bioassay based on fall dormancy (FD3-AFX 429, and FD4-AFX 469). Compared to orchardgrass, shoot emergence was reduced up to 19% by alfalfa on loam (P<0.05), but there was no difference on sand. There was an interaction between test seed and field soil for radicle emergence and length (P< 0.05). Interactions occurred because the two test seed varieties did not respond uniformly to soil treatment. Radicle emergence by AFX 429 was reduced (P<0.05) by alfalfa on the loam soil only while AFX 469 was not affected by soil treatment. For radicle length, 5th year alfalfa reduced radicle length over orchardgrass for AFX469, but not AFX 429. However it is not clear at this time whether short

radicles are beneficial or detrimental in autotoxicity because twisted radicles were often longer than straight ones. Long-term root evaluations are needed to determine if twisted radicles can form normal taproots.

Run 2. Shoot emergence and radical length were not different among soils and there were no interactions (P>0.05)(data not shown). Radical counts were reduced in 5th yr killed alfalfa soil compared to 5th yr live alfalfa or orchardgrass soils (68 vs 77% , respectively, $P < 0.06$) and there were more than twice as many abnormal radicals in autotoxic soils as in control soils (45 vs 20% of the radicals, $P < 0.05$).

Table 2. Main effect means for alfalfa seedling development after a four-day bioassay in soils from grass and five alfalfa varieties planted in 2017.

Soil	Shoot emergence, %	Radical length, mm	Shoot weight, mg	Root weight, mg
Seed				
AFX 429	70 b	29 a	21.4	5.1
AFX 469	85 a	26 b	18.6	5.4
LSD	5.9	5.4	2.1	-
Soil				
Orchardgrass	78	28 abc	21.1	4.8
AFX 429-Yr 3	83	25 cd	20.3	5.3
AFX 469-Yr 3	78	26 bcd	20.1	5.5
54R02-Yr 5	73	31 ab	20.0	5.5
Vernal-Yr 1	79	28 bc	20.4	5.2
Vernal-Yr 2	79	31 ab	19.4	5.2
Vernal-Yr 3	78	33 a	20.1	4.2
Vernal-Yr 4	77	25 cd	21.0	5.6
Hybriforce 4400-Yr 1	73	27 bc	18.2	3.6
Hybriforce 4400-Yr 2	74	27 bc	18.5	5.9
Hybriforce 4400-Yr 3	78	22 d	20.5	5.4
Hybriforce 4400-Yr 4	78	23 cd	20.5	6.7
LSD	-	6.6	-	-
Significance P<				
seed	0.001	0.01	0.01	ns
soil	ns	0.01	ns	ns
seed*soil	ns	ns	ns	ns

abcd Values with different letters are different (P< 0.05).

Run 3. Table 2 summarizes results comparing the two AFX test seed varieties with 12 soil treatments selected from a range of varieties and stand ages. AFX 429 had reduced shoot emergence but greater radicle length and weight than AFX469. There were no differences among soil treatments for shoot emergence or shoot and root weights. Soil from the third year stand of Hybriforce 4400 had shorter radicles than many other soil treatments. Figure 2 illustrates the interaction (P< 0.05) between test seed variety and soil treatment for radicle count. Test seeds

did not respond consistently across soil treatments. AFX 469 had greater radicle counts than AFX 429 in soil from first year stands.

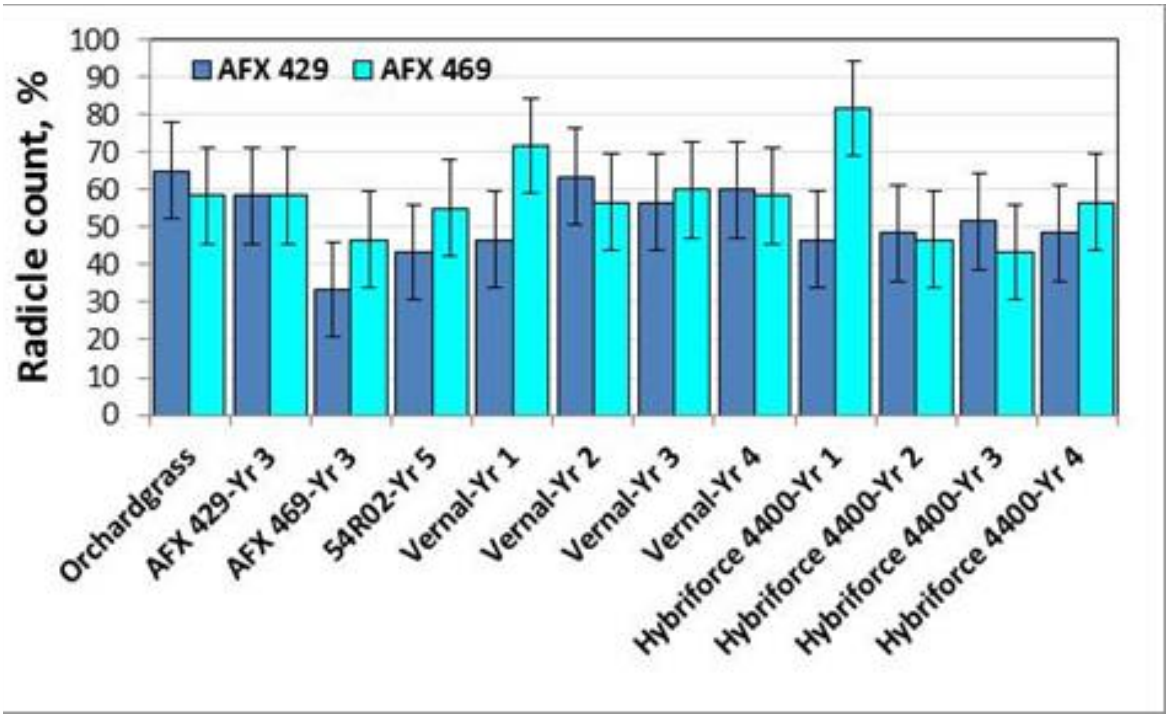

Figure 2. Seed by soil treatment interaction for radicle emergence four days after planting in soil from two alfalfa varieties of differing stand ages. Error bars represent LSD such that values with no overlap between bars are different ($P < 0.05$).

Task 4. Validate bioassay results using potted alfalfa plants in a greenhouse. The greenhouse trial used seeds treated with mefoxonam to reduce soil-borne disease. Compared to orchardgrass soil, seedlings grown in the alfalfa soil had reduced shoot height (42 vs 33 mm, $P<0.01$). Emergence counts were less (seed x soil interaction, $P < 0.01$) in alfalfa soil for AFX 469 test seed (2.2 vs 1.6) but not for AFX 429 (1.1 vs 0.9). Root length per seedling did not differ ($P>0.05$) at 7 (mean 14.4 cm) or 14 (67.3 cm) days after planting, but at 21 days after planting, root length in the alfalfa soil was reduced by 42% over grass control (183 vs 106 cm for control and alfalfa, $P<0.05$).

CONCLUSIONS. These results provide proof of concept that a laboratory bioassay can detect differences in alfalfa seedling development from field soils. Variety of alfalfa seed used in the bioassay played a greater than expected role in results, indicating varieties differ in their tolerance to autotoxicity. An effective bioassay may require use of test seeds from the same variety that will be planted. Variety of alfalfa grown in the field soils also had a strong impact on results. However, alfalfa varieties and field soils tested here were selected from available variety test plots based on hypotheses that furthered development of the bioassay and should not be construed as a systematic evaluation of autotoxicity or a definitive statement on variety differences.

This pilot project does not and was not intended to provide a final answer to the problem of autotoxicity. While we can detect differences in alfalfa seedling development among soils that are consistent with autotoxicity, there are still many questions to answer. We did not detect as many differences as expected and it is not clear whether this indicates the bioassay still needs refinement or that our soil samples were simply not as toxic as expected. The unprecedented rainfall in Michigan during this funding period may have reduced our ability to detect autotoxic effects because the compound(s) are known to leach out of soil with rainfall (Undersander et al., 2015). Testing of many more soils from a variety of environments, including dry ones, is required before the bioassay can be considered to be fully validated and used for planting decisions. Field-scale validation will require a timeframe of years. We have already used the USAFRI data to leverage additional funding from Michigan's Project GREEEN (2019-2021, $79,892) and these funds are being used to recruit a graduate student for the project. A proposal submission to the next cycle of the USDA-NIFA alfalfa research program is planned using this preliminary data. We also plan to request additional USAFRI funds to help move us into the next phase of the project.

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

References:

Cassida, K., J. Paling, and C. Kapp. 2018. 2017 Michigan Forage Variety Test Report. MSU Forage Factsheet 18-01, 37 pages. https://forage.msu.edu/publications/ (Online.) https://forage.msu.edu/wp-content/uploads/2018/05/2017-Michigan-Forage-Variety-Test-Report-FINAL.pdf. Published Feb. 1, 2018. Verified 5/1/18.

Chon, S.U., J.H. Coutts, and C.J. Nelson. 2000. Effects of light, growth media, and seedlings orientation on bioassays of alfalfa autotoxicity. *Agronomy Journal* 92:715-720.

Chon, S.U., and C.J. Nelson. 2001. Effects of experimental procedures and conditions on bioassay sensitivity of alfalfa autotoxicity. *Communications in Soil Science & Plant Analysis* 32:1607-1619.

Chung, I.M., and D.M. Miller. 1995. Differences in autotoxicity among seven alfalfa cultivars. *Agronomy Journal* 87:596-600.

Reynolds, B. 1984. A simple method for the extraction of soil solution by high speed centrifugation. *Plant and Soil* 78:437-440.

Seguin, P., C.C. Sheaffer, M.A. Schmitt, M.P. Russelle, G.W. Randall, P.R. Peterson, T.R. Hoverstad, S.R. Quiring, and D.R. Swanson. 2002. Alfalfa autotoxicity: effects of reseeding delay, original stand age. and cultivar. *Agronomy Journal* 94:775-781.

Tesar, M.B. 1993. Delayed seeding of alfalfa avoids autotoxicity after plowing or glyphosate treatment of established stands. *Agronomy Journal* 85:256-263.

Undersander, D., M. Renz, C. Sheaffer, G. Shewmaker, and M. Sulc. 2015. *Alfalfa Management Guide.* ASA/CSSA/SSSA, Madison, WI.

USDA-NASS. 2017. Statistics by State. https://www.nass.usda.gov/Statistics_by_State/ Verified 5/1/18.

Voight, P.W., D.R. Morris, and H.W. Godwin. 1997. A soil-on-agar method to evaluate acid-soil resistance in white clover. *Crop Science* 37:1493-1496.

Keywords: alfalfa, autotoxicity, establishment