QUARTERLY REPORT

January 1, 2021 – March 31, 2021

Kentucky Tobacco
Research & Development Center

College of Agriculture,
Food and Environment

UNIVERSITY OF KENTUCKY
College of Agriculture, Food and Environment

MEMORANDUM

DATE: April 27, 2021

TO: Kentucky Tobacco Research Board Members
Legislative Research Commission

FROM: Dr. Ling Yuan
Director, KTRDC

SUBJECT: Kentucky Tobacco Research & Development Center
Quarterly Report for January 1, 2021 – March 31, 2021

Enclosed is a copy of the Kentucky Tobacco Research & Development Center's Quarterly Report for January 1, 2021 – March 31, 2021.

If you have any questions, please feel welcome to contact me at (859) 257-5798 or email lyuan3@uky.edu.

Enc.

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EXECUTIVE SUMMARY

Introduction

The legislation (KRS 248.510 - 248.580) which provides funds in support of the research programs at the Kentucky Tobacco Research and Development Center (KTRDC) requires that a quarterly research report be submitted to the Kentucky Tobacco Research Board (KTRB) and the Legislative Research Commission.

The overall reporting plan is:

January 1	-	March 31:	Selected topics
April 1	-	June 30:	Selected topics
July 1	-	September 30:	Selected topics
October 1	-	December 31:	Annual comprehensive report

As required by KRS 248.570, a financial report covering expenditures for the relevant proportion of the January 1, 2021 – March 31, 2021 fiscal year is included in this report.

The news and research publications provided in this quarterly report are a representative selection of the Center's output. For a full description of all KTRDC research and activities please refer to the KTRDC Annual Report.

Quarterly News

- Calls for papers for the two main tobacco conferences have gone out.
  - The CORESTA call for papers was sent out in February for the conference to be held October 4th-8th. The conference will again be virtual, but with live question and answer sessions.
  - The 74th TSRC conference will be held in Boston, Massachusetts, August 29th-September 1st. This conference will be in-person, and is rescheduled from 2020: the 2020 74th conference was cancelled.
- KTRDC staff have continued to work through the pandemic, with strict precautions in place, as there are ongoing grants and many research projects that cannot be put on hold. Those who can work from home have been doing so, which means that the number of people in the building is greatly reduced. Social distancing is observed, workstations and public areas are regularly sanitized, face masks are mandatory, and lab staff are carefully spread out, rotating their hours and locations.

- Activity at the farm this quarter has been limited to stripping and seedling production.
  - Stripping finished much later than usual this year, because prolonged dry periods made it impossible to take down the tobacco sooner. The last of the experimental tobacco was stripped and sampled in mid-March.
  - The first tobacco seeding was on March 12. The seedlings are growing well.
  - A high cannabinoid population of hemp mother plants has been started from commercial feminized seed. These plants will be maintained through sequential cloning, and will be a resource for laboratory work.
- The March board meeting was held via Zoom, because of the pandemic.
  - Dr. Yuan informed the Board that income was on track through December, although January's income was less than projected.
  - KTRDC's financial reserve continues to be healthy. This is a result of moving personnel from KTRDC to FDA funds. However, two of the FDA grants have ended, and KTRDC is currently in year two of a five-year FDA grant. Once this grant has ended it will be necessary to support personnel with KTRDC funds.
  - Dr. Yuan requested a release of unallocated funds, which was approved by the Board for the 2020-2021 budget. The KTRDC farm headhouse and greenhouse need significant improvements and there is also a need for both a farm truck and a utility vehicle. The estimated cost for these items is $79,200. Dr. Yuan requested that $71,000 be released from unallocated funds with the remainder of the funds coming from existing funds in the greenhouse (1235411410) account. The release of $71,000 from unallocated funds was approved.
  - Anne Fisher gave a presentation entitled "Reducing nicotine in burley tobacco by combining low alkaloid varieties and agronomic practices – two very different years". To investigate the feasibility of achieving nicotine levels of 0.3-0.5 mg/g (the non-addictive levels suggested by the FDA), a two-year experiment was done combining three low alkaloid (LA) varieties (two French varieties and LA TN 90) and a check, with all known nicotine-reducing agronomic practices (and the check, standard practices). The two seasons, 2018 and 2019, were very different. In 2018, there was excessive rain, and nicotine levels were unusually low. In contrast, 2019 was very hot and dry, and nicotine levels were high. In 2018, everything in the check (standard practices) block was above the 0.5 mg/g limit, in all stalk positions. In the nicotine-reducing practices block, the flyings of all three LA varieties were

varieties; the leaf and tips of only one French variety were. Although these were below the 0.5 mg/g limit, they were very close to it. In 2019, everything was well above the 0.5 mg/g limit, even the flyings in the nicotine-reducing practices block. Nicotine levels in the LA varieties in the nicotine-reducing practices block were three to five times the suggested limit. This work illustrates the impossibility of consistently achieving a specified nicotine target level. The effect of seasonal variability is clearly demonstrated by these two extreme years; in addition, there are decades of nicotine data from the Minimum Standards Program showing the variability due to season and location. With the currently available varieties, growers could not consistently achieve 0.3 – 0.5 mg/g nicotine, especially over the whole plant.

- The proficiency testing (PT) program continues to be well patronized.
  - The CIG-2020C round opening was delayed until mid-June due to the pandemic, which resulted in a delay of the final report from November 2020 to January 2021.
  - The SMK-2020D was scheduled to open at the end of August but was delayed until September. This was the first smokeless PT round that CTRP offered, following the A2LA scope expansion in March of 2020. The results for this round of testing had good agreement within all of the data submitted, which were included in the final report released March 2021.
- The four certified reference smokeless tobacco products were used in a CORESTA collaborative study, which was delayed from 2020 due to the COVID-19 pandemic. The results are currently being reviewed by CORESTA Tobacco and Tobacco Products Analysis (TTPA) members.
- CTRP introduced four reference cigars for research purposes in October 2019 to facilitate research and method development for cigars. The preliminary data from these test materials is being used to identify design parameters for the production of three certified reference cigar products as part of a cooperative agreement with the FDA.
- To date, the University of Kentucky, Center for Tobacco Reference Products (CTRP) has been awarded three FDA (Food & Drug Administration) grants to produce and distribute reference products, the first of which is now complete.
  - The second grant, to produce four smokeless reference products, should be completed in 2021. The four products have been produced, certified and are being sold to research institutions around the world. The CTRP recently

completed its first smokeless proficiency testing round in March 2021. Our scientists are working to finalize several research projects on these products.

- The major activity during this period was focused on the cigar grant, which was funded to produce three certified cigar reference products: a filtered little cigar, a cigarillo, and a large, non-premium cigar. All of these products will be machine-made with an HTL (homogenized tobacco leaf) wrapper. The main achievements for this reporting period focus on the analysis of commercial cigar products, including: 25 filtered little cigars; 17 cigarillos, and 11 large cigars, to support background research to guide the design specifications for these reference products. Our efforts have been slowed due to university research restrictions resulting from the COVID-19 pandemic. Additionally, the CTRP has hosted many teleconferences with the FDA, cigar manufacturers who are part of the Cigar Working Group, and other stakeholders. Through detailed discussions with these groups, the CTRP is working to finalized product specifications for the filtered little cigar and cigarillo during the second quarter of 2021. Unfortunately, all of our prospective cigar manufacturing partners have been significantly impacted by COVID, reducing their manufacturing capabilities. The project continues to make good progress and our plan is to finalize the design of all three reference cigars and produce the filtered little cigar, and possibly the cigarillo during 2021.

The KTRDC Quarterly Reports include copies and brief summaries of work published by KTRDC scientists and scientists partly funded by KTRDC, as well as work arising from KTRDC summit grants. I would like to thank Andrea Keeney and Dr. Andy Bailey; Will Barlow, Bob Pearce and Emily Pfeufer; and Dr. Colin Fisher for their help with writing the summaries.

Summary of Selected Research Topics

Report #1 "*Soil test potassium changes over time following fall potassium application in three western Kentucky soils*." Andrea Keeney, Edwin Ritchey and Andy Bailey

Field experiments were done on bare-ground fields to investigate changes in soil test potassium levels over two years following fall potassium applications to Crider, Grenada, and Zanesville soils in western Kentucky. Understanding the relationship between potassium application and environmental conditions in different soil types is important in the management of potassium applications. This research shows

how seasonal fluctuations in precipitation and clay content may influence potassium soil test fluctuations, as a high clay content can trap potassium during dry conditions, therefore lowering potassium levels available for plant uptake. These results are important in understanding why spring soil tests may still call for additional potassium, even when potassium applications were made the previous fall based on fall soil tests.

Although potassium sulfate has been the potassium source of choice for spring applications to tobacco fields in Kentucky, many tobacco growers have begun using the more economical potassium chloride in fall applications, prior to tobacco the following spring. However, some growers have noticed that when potassium chloride applications were made in the fall based on soil test recommendations, a spring soil test may still recommend additional potassium. To investigate these apparent fluctuations in soil test potassium, field experiments were done on bare-ground areas from 2016 to 2018 to monitor changes in soil potassium levels over a two-year time period on three western Kentucky soil types: Crider, Grenada, and Zanesville. Potassium applications were done in December 2016 and soil samples were taken every three months thereafter for two years. Laboratory studies were also done to investigate particle size and clay mineralogy in these soil types. Seasonal fluctuations in soil test potassium levels occurred in all three soils, and generally followed fluctuations in precipitation. The laboratory studies showed that the Crider and Zanesville soils had a higher clay content than the Grenada soil type. Soils containing a higher amount of clay particles such as vermiculite have a greater potential to trap potassium during dry periods, potentially making soil test potassium levels lower than they might be under higher moisture conditions. The Crider soil type was more responsive to the fall potassium application compared to the other two soil types, as it had the greatest increase in soil test potassium levels in the spring following potassium application.

Report #2 *"Cytochrome b Mutations F129L and G143A Confer Resistance to Azoxystrobin in Cercospora nicotianae, the Frogeye Leaf Spot Pathogen of Tobacco*." Ed Dixon, Will Barlow, Grant Walles, Bernadette Amsden, Robert Louis Hirsch, Bob Pearce and Emily Pfeufer

*This paper identifies azoxystrobin (Quadris) sensitivity distributions from frogeye leafspot (*Cercospora nicotianae*) isolates collected throughout Kentucky, the specific mutations that confer resistance to azoxystrobin, and differences between*

sensitivity patterns of isolates as a result of management factors. Azoxystrobin is the only synthetic systemic fungicide currently labeled for tobacco frogeye leaf spot disease, and widespread resistance could render this tool ineffective. Sensitivity values of isolates collected indicated qualitative resistance to three different concentrations of azoxystrobin, allowing documentation of the specific gene mutations that result in azoxystrobin resistance for this organism. Few, if any, alternatives to azoxystrobin are likely to be labeled for use in tobacco, so identification of these mutations will allow for more rapid screening of C. nicotianae*, to enable growers to make better informed disease management decisions.*

Azoxystrobin, the active ingredient in Quadris and some other FRAC 11 (Fungicide Resistance Action Committee: group 11 – strobilurins) fungicides, is the only synthetic systemic fungicide labeled in the United States for management of frogeye leaf spot (FLS) of tobacco, caused by *Cercospora nicotianae*. Though traditionally considered a minor disease in the United States, FLS has recently become yield- and quality-limiting. In 2016 and 2017, 100 *C. nicotianae* isolates were collected from symptomatic tobacco in eight counties in Kentucky. Spores from each isolate were placed on a gel plate containing increasing concentrations of azoxystrobin. After counting the number of spores that germinated at each concentration, the effective concentration to inhibit 50% germination (EC50) was calculated. The distribution of *C. nicotianae* EC_{50} values indicated three qualitative groupings (high, moderate, and low) of sensitivity to azoxystrobin. DNA testing of the isolates revealed that highly sensitive individuals had the expected wild-type cytochrome b gene DNA sequence. Individuals that were moderately sensitive were found to have a mutation designated as F129L while individuals with low sensitivity (spores germinated at higher concentrations of Azoxystrobin) were found to have a mutation designated as G143A. Low sensitivity isolates were found at higher frequencies from greenhouse transplants (4 of 17) as compared to mature field-grown tobacco (<4 weeks prior to harvest; 4 of 62). Moderately sensitive isolates were found more frequently in fields with azoxystrobin applications (58 of 62) samples, than in fields with organic management (1 of 7). Together, these results suggest that resistance to azoxystrobin in *C. nicotianae* occurs broadly in tobacco throughout Kentucky, and they generate new hypotheses about how selection pressure (spraying Azoxystrobin) affects resistance mutations in fungal populations. DNA testing of populations of *C. nicotianae* can potentially be used to track the occurrence of Azoxystrobin resistance in fields, and to assist in the

development of appropriate management to slow the development of resistance and help tobacco growers better utilize the limited tools available to control FLS.

Report #3 *"What happens to in situ net soil nitrogen mineralization when nitrogen fertility changes?"* Congming Zou, John Grove, Bob Pearce, Mark Coyne and Ke Ren

This research investigates how natural nitrogen in the soil is affected by applied inorganic nitrogen fertilizer. Applied nitrogen fertilizer runs off the fields and pollutes water. Applied fertilizer nitrogen levels could be reduced, if the environmental conditions and interacting factors involved in the natural production of nitrogen from organic matter is better understood. This work cannot be simulated in a laboratory, and this is one of the few studies that has investigated several different factors at the same time, and how they interact.

There is considerable effort around the world to reduce the amount of fertilizer applied to crops, because a large proportion of this leaches and pollutes both surface and groundwater. One possible way of achieving this is to make better use of the nitrogen mineralization, which is the natural process of nitrogen production from the degradation of soil organic matter. Studies to understand this system are difficult, because the complexity of the natural soil environment cannot be simulated in the laboratory. This work took advantage of a study where the same fertilizer rates have been applied to the same plots every year for the past 43 years. Three hundred and twenty-four soil cores were collected by removing the 12-inch long, 2-inch dimeter tubes that were punched into the soil, placing a small bag of resin in the bottom of each tube, and replacing these back into the field. The results of this study showed that, with the higher soil organic matter in the plots that had the high nitrogen rate every year, a higher rate of applied nitrogen was needed in the current season in these plots to initiate the mineralization process (because of the higher organic matter). Most of the mineralization occurred in the earlier part of the season, and was affected by total rainfall and temperature, with too much soil moisture causing nitrogen loss from denitrification. This study demonstrated that there are several factors that must be known when trying to estimate the level of natural soil nitrogen available to the crop, not just the level of organic matter.

Received: 9 January 2020 | Revised: 8 April 2020 | Accepted: 25 April 2020

DOI: 10.1002/agg2.20058

Agrosystems, Geosciences & Environment OPEN ACCESS

ORIGINAL RESEARCH ARTICLE

Agrosystems

Soil test potassium changes over time following fall potassium application in three western Kentucky soils

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Abstract

Field experiments were conducted under bare-ground conditions to investigate changes in soil test K levels over 2 yr following fall K applications to Crider, Zanesville, and Grenada soils in western Kentucky. Additional laboratory studies investigated particle size and clay mineralogy of these soils. In field experiments, highest soil test K in the Crider and Zanesville soils occurred 12–15 mo after fall K application. However, seasonal fluctuations in soil test K occurred in all three soils, likely due to fluctuations in precipitation prior to sampling. The Crider and Zanesville soils showed a much more substantial drop in soil test K at 9 and 21 mo after December K application than did the Grenada soil. The 9- and 21-mo samplings were both preceded by 1–2 mo of dry conditions where precipitation was well below the 30-yr average at the Crider and Zanesville sites. Particle size analysis showed that the Crider and Zanesville soils had a higher clay content than the Grenada soil. Soils with higher amounts of clay particles such as vermiculite have greater potential to trap K during dry periods, making soil test K levels lower than they might be under better soil moisture conditions. Over the course of these experiments, the Crider soil appeared to be more responsive to fall K application than the other soils, showing the greatest increase in soil test K by the following spring. This research illustrates the seasonal fluctuations in soil test K levels that occur naturally in these soils and how precipitation and clay content may influence these fluctuations.

1 | INTRODUCTION

Muriate-of-potash (0–0–60, KCl) applied at more than 56 kg Cl ha^{-1} can lead to high levels of chloride in cured tobacco leaves. These high chloride levels can lead to reduced cured leaf quality and decreased combustibility of the leaf (Ritchey, Pearce, & Reed, 2019). The Kentucky Fertilizer Law (12 KY Admin. Regs. 4:170) limits the amount of Cl that can be applied to tobacco fields after 1 January of the current crop year. Therefore, sulfate-of-potash (0–0–50, K_2SO_4) should be the primary source of K applied to tobacco fields in spring applications in the current crop year. However, more tobacco growers are using KCl as their primary K source and applying it in the fall due to this restriction against spring applications and also because K_2SO_4 is approximately 2.5 times more expensive than KCl (Ritchey et al., 2019).

Some western Kentucky tobacco farmers have applied KCl in the fall at the amount recommended from a fall soil test that same year. These farmers sampled the soil again the following spring only to find that the spring soil test results still recommend K. For this reason, field experiments were established to monitor how soil test K levels change over time following fall

TABLE 1 Recommended potassium fertilizer application rates for each field site based on initial composite soil sample taken in November 2016 at each site

Soil type	Location	Previous crop	K recommendation[a]
			kg ha^{-1}
Crider	Princeton, KY	corn	224
Zanesville	Princeton, KY	fescue	247
Grenada	Murray, KY	bermudagrass	171

[a]Potassium recommendation based on soil test K levels from November 2016 composite soil sample at each site and University of Kentucky potassium recommendations for burley tobacco production.

Core Ideas

- Highest soil test K occurred 12–15 mo after fall K application in Crider and Zanesville silt loam soils.
- Seasonal fluctuations in soil test K occur naturally in Crider, Zanesville, and Grenada soils.
- Precipitation patterns and clay content likely influence fluctuations in soil test K levels.
- Crider soil type appear to be more responsive to fall K application than Zanesville or Grenada soil types.

K applications on three distinct soil types common to western Kentucky. Additional laboratory studies were conducted to investigate soil physical properties of these soils, including particle size and clay mineralogy analyses.

2 | MATERIALS AND METHODS

In November 2016, soil samples were taken from several potential sites to determine locations for a K bare-ground study. Sites were established to evaluate long-term changes in soil K levels following fall K application. The Mehlich III soil test extractant was used to establish soil test K levels. Three field sites were established in December 2016 on contrasting soil types where initial soil test K levels were low (<224 kg ha^{-1} soil test K). Two of the sites were located at the University of Kentucky Research and Education Center in Princeton, KY, and a third site was located at the West Farm of Murray State University in Murray, KY. The two Princeton sites were previously in corn or fescue, and the Murray site was previously in bermudagrass (Table 1). Any existing vegetation was killed with glyphosate, and bare-ground conditions were maintained throughout the 2-yr experiment with additional glyphosate applications at each site.

Soil types at Princeton were Crider silt loam (fine-silty, mixed, active Typic Paleudalfs) at the site previously in corn and Zanesville silt loam (fine-silty, mixed, active, mesic Oxyaquic Fragiudalfs) at the site previously in fescue. Soil type at the Murray location previously in bermudagrass was Grenada silt loam (fine-silty, mixed, active, thermic Oxyaquic Fraglossudalfs) (Web Soil Survey, 2016). Initial soil pH was 5.7 at the Crider site, 6.7 at the Zanesville site, and 6.4 at the Grenada site.

2.1 | Soil physical properties study

A composite 30-cm soil sample was collected from each site in November 2016 and segmented into 0- to 15-cm and 15- to 30-cm sections for particle size analysis and clay mineralogy analysis for each site. Particle size analysis was conducted using the pipet method described by Gee and Bauder (1986) and Green (1981). Clay mineralogy analysis was conducted using sample preparation and x-ray diffraction techniques described by Whittig and Allardice (1986) and Jackson (1969).

2.2 | Potassium field study

Field trials were established at each site to evaluate changes in soil test K levels over time following fall K application. Field trials at each site were arranged in a randomized complete block design with four replications of treatments. Individual plots at all locations were 1.5 m in width and 1.5 m in length. There was a 1.5 m by 1.5 m border area around each plot and replication to ensure no overlaps in K application between plots.

Potassium rates used were calculated from soil test K levels from the initial composite soil sample taken at each site in November 2016 for University of Kentucky K recommendations for burley tobacco production (Ritchey & McGrath, 2018). Potassium sources used at all locations were potassium sulfate (0–0–50, K_2SO_4) or potassium chloride (0–0–60, KCl). Potassium sulfate and KCl were each applied at the rate recommended by the initial composite soil test taken at each site in November 2016 (Table 1). Potassium chloride was also applied at two times the recommended application rate. An untreated control that received no K was included.

Potassium treatments were broadcast applied by hand to individual plots in early December 2016. Potassium treatments were not incorporated after application. Soil samples were taken from each plot just prior to K treatment applications and then every 3 mo following K application for 2 yr (Table 2). All samples consisted of five 15-cm-deep soil cores that were taken and homogenized from each plot.

The last sampling event was collected in December 2018 at the conclusion of the 2-yr monitoring period. This sample was segmented by soil depth into two soil samples per plot

TABLE 2 Potassium application and soil sample dates

Location[a]	Pre-application soil sample and K application date	3-mo sample	6-mo sample	9-mo sample	1-yr sample	1-yr 3-mo sample	1-yr 6-mo sample	1-yr 9-mo sample	2-yr sample[b]
Princeton	5 Dec. 2016	9 Mar. 2017	7 June 2017	11 Sept. 2017	5 Dec. 2018	6 Apr. 2018	9 July 2018	12 Sept. 2018	17–19 Dec. 2018
Murray	6 Dec. 2016	8 Mar. 2017	8 June 2017	14 Sept. 2017	7 Dec. 2018	5 Apr. 2018	12 July 2018	11 Sept. 2018	19 Dec. 2018

[a]The Princeton location included the Crider and Zanesville field sites; the Murray location included the Grenada site. [b]The Crider site was sampled on 17 Dec. 2018 and the Zanesville site was sampled on 19 Dec. 2018 due to saturated soils from excess rainfall at the Zanesville site.

(one sample at the 0- to 7.5-cm depth and another sample at the 7.5- to 15-cm depth).

Data were analyzed using Statistical Analysis Software version 9.4. PROC GLIMMIX (SAS Institute, 2004) was used to conduct an ANOVA for a repeated-measures analysis over the first 1 yr and 9 mo of the experiment. An $\alpha < .10$ was used to indicate statistical significance, and means were separated using least square means. The model consisted of soil type, treatment, and soil sampling time.

Data for the 2-yr sampling date were analyzed separately from the other sampling dates due to being split between two sample depths. PROC GLIMMIX was used for ANOVA with a model consisting of soil type, treatment, and depth. Significant treatment means were separated using least square means at $\alpha < .10$.

3 | RESULTS AND DISCUSSION

3.1 | Particle size analysis

Particle size analysis determined the percentage of sand, silt, and clay for each soil type at two depths (0–15 cm and 15–30 cm) (Table 3). The amount of clay increased as soil depth increased for all three soil types. The Grenada soil was the only soil type where the percentage of silt increased with depth. The Grenada soil also contained a lower percentage of clay and a higher percentage of sand than the other soil types. The textural class for all three soil types at both depths was silt loam (Table 3).

3.2 | Clay mineralogy analysis

Clay minerals varied within each soil type and by soil depth (Table 4). All three soil types contained vermiculite/hydroxy interlayered vermiculite, mica, quartz, and kaolinite at both depths. All soil types also contained feldspar at the 15- to 30-cm depth, but only the Zanesville soil type contained feldspar at the 0- to 15-cm depth. Smectite only appeared in the Crider soil type and was within both sampling depths (Table 4).

3.3 | Changes in soil potassium levels over time

The ANOVA showed significant main effects of soil type, treatment, and sampling time ($p \leq .0001$ for each) for soil test K in samples collected between 0 and 21 mo after application. There were also significant interactions for soil type × treatment ($p = .0002$), soil type × sampling time ($p \leq .0001$), and treatment × sampling time ($p = .0011$). When data were analyzed by soil type, treatments were different from each other over time within each soil type: Crider ($p \leq .0001$; Figure 1),

TABLE 3 Particle size analysis for each soil type

Location	Soil type	Sample depth	Clay	Sand	Silt	Textural class
		cm		%		
Princeton	Zanesville	0–15	16.4	7.7	75.9	silt loam
		15–30	23.7	3.9	72.4	silt loam
	Crider	0–15	17.2	2.8	80	silt loam
		15–30	23.1	1.7	75.2	silt loam
Murray	Grenada	0–15	12	12.4	75.6	silt loam
		15–30	14.5	8	77.5	silt loam

TABLE 4 Clay mineralogy analysis for each soil type

Location	Soil type	Prior crop	Depth	Minerals[a]
			cm	
Princeton	Crider	corn	0–15	smectite
				vermiculite/HIV[b]
				mica
				interstratified[c]
				kaolinite
				quartz
			15–30	vermiculite/HIV
				smectite
				interstratified
				mica
				quartz
				kaolinite
				feldspar
Princeton	Zanesville	fescue	0–15	vermiculite/HIV
				mica
				kaolinite
				quartz
				feldspar
			15–30	vermiculite/HIV
				interstratified
				mica
				kaolinite
				quartz
				feldspar
Murray	Grenada	bermudagrass	0–15	vermiculite/HIV
				interstratified
				mica
				kaolinite
				quartz
			15–30	vermiculite/HIV
				mica
				kaolinite
				quartz
				feldspar

[a]Minerals are not listed in order of prevalence. [b]Hydroxyinterlayered vermiculite. [c]Interstratified indicates a mixture of two or more minerals.

Zanesville ($p = .0308$; Figure 2), and Grenada ($p = .0002$; Figure 3).

Seasonal fluctuations in soil test K levels were evident for each K treatment for all three soil types. The Crider and Zanesville soil types showed drastic decreases in soil K at 9 mo after application (September 2017) and showed another decrease at 18–21 mo after application (June–September 2018) (Figures 1 and 2). The Grenada soil type showed more variation in soil K than the Crider or Zanesville. The Grenada soil type showed a major decrease at 9 mo after application and showed smaller decreases at 15 and 21 mo after application (Figure 3).

At the 9-mo sampling date, the month prior to sampling had rainfall well below average in Caldwell County (Figures 1 and 2). This could have had an effect on the lower soil test K levels that were identified in the 9-mo sampling data. The K could have been trapped between clay layers due to interlayer collapse, preventing the K from being extracted in the soil sample. For the 18- and 21-mo samplings, rainfall in the month prior to the 18-mo sampling was slightly (0.16 cm) above average but was below average in the 2 mo prior to the 21-mo sampling (2.2 and 4 cm below average at 19 and 20 mo, respectively). Lower soil test K levels at 18 and 21 mo in the Crider and Zanesville sites may also be attributed to drier conditions in the months prior to sampling. The 9-mo sampling data and the 21-mo sampling data were from September of 2017 and 2018. September is historically one of the driest, if not the driest, month of the year in both Caldwell and Calloway County.

In the Crider and Zanesville soil types, the KCl × 2 treatment had the largest effect on soil test K levels and showed the greatest fluctuations in soil test K. The KCl × 2 treatment had higher soil test K levels compared with the other two K treatments for the majority of the sampling dates. However, the KCl treatment had the highest K levels in the majority of the sampling times for the Grenada soil type. Interestingly, the untreated control that received no K also showed variation in soil test K levels over time, particularly in the Grenada soil type. Again, this effect could have been due to precipitation fluctuations that occurred in 2017 and 2018 in the months prior to each sampling. The Grenada soil type generally

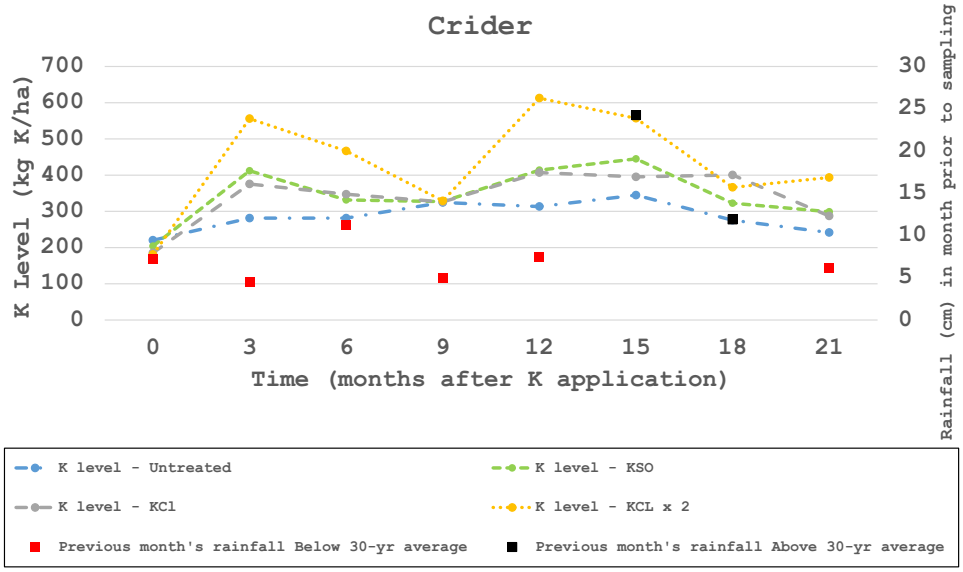

FIGURE 1 Soil K levels (kg ha^{-1}) in the Crider soil type for potassium sulfate (KSO), potassium chloride (KCl), double potassium chloride (KCl × 2), and untreated plots over a 21-mo period following application. Rainfall levels in the month prior to each sampling are indicated by red squares if rainfall was below the 30-yr average or black squares if rainfall was above the 30-yr average

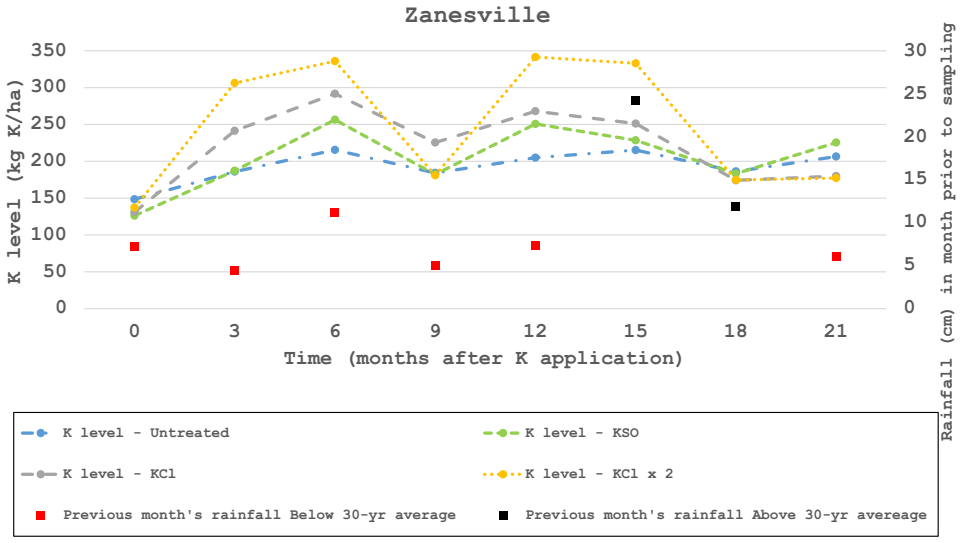

FIGURE 2 Soil K levels (kg ha^{-1}) in the Zanesville soil type for potassium sulfate (KSO), potassium chloride (KCl), double potassium chloride (KCl × 2), and untreated plots over a 21-mo period following application. Rainfall levels in the month prior to each sampling are indicated by red squares if rainfall was below the 30-yr average or black squares if rainfall was above the 30-yr average

showed more variation in K levels between each sampling and showed more deviation from average rainfall compared with the Crider and Zanesville sites. Murdock and Call (2006) state that seasonal fluctuations in nutrient levels are heavily controlled by the patterns of when nutrients are taken up or released by the current crop or by environmental conditions if there is no crop, as in these experiments. Particularly in the Crider and Zanesville soil types, there is a trend toward lower soil test K levels when below-average rainfall occurred in a 1- to 2-mo period prior to sampling (Figures 1 and 2).

The ANOVA for the 2-yr (24-mo) sampling that was split into two different depths (0–7.5 cm and 7.5–15 cm) showed significant main effects of soil type ($p = .0015$) and depth ($p \leq .0001$) on soil test K levels. There was also a significant interaction between soil type and depth ($p \leq .0001$) on soil test K levels (Table 5). There were no significant

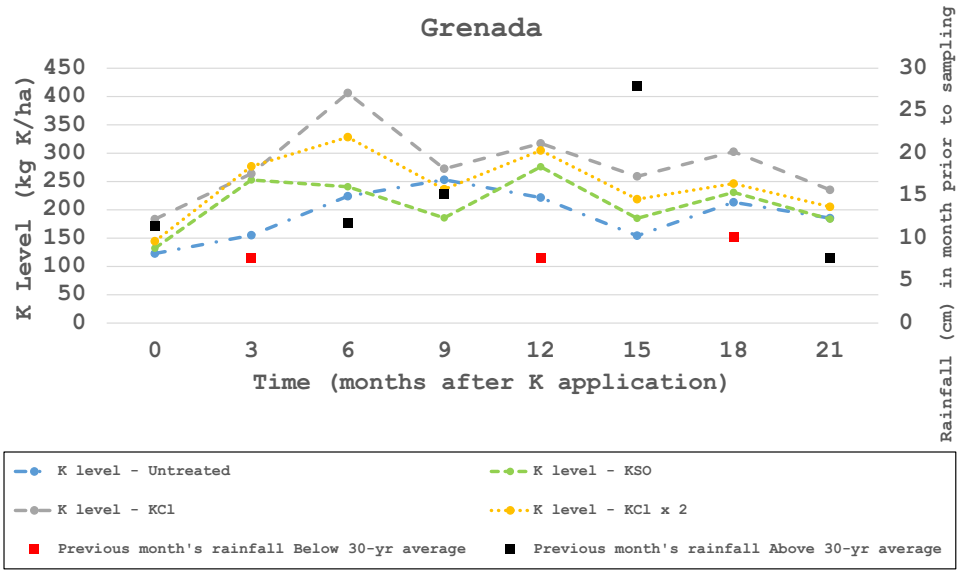

FIGURE 3 Soil K levels (kg ha^{-1}) in the Grenada soil type for potassium sulfate (KSO), potassium chloride (KCl), double potassium chloride (KCl × 2), and untreated plots over a 21-m period following application. Rainfall levels in the month prior to each sampling are indicated by red squares if rainfall was below the 30-yr average or black squares if rainfall was above the 30-yr average

TABLE 5 Soil type by depth interaction for soil test K levels in the 2-yr sampling data where the 15 cm core was split between 0- to 7.5-cm and 7.5- to 15-cm depths

Soil type	Depth		*P* value
	0–7.5 cm	7.5–15 cm	
	kg ha^{-1}		
Crider	306a[a]	269a	<.0001
Grenada	217b	105c	<.0001
Zanesville	143c	233b	<.0001
P value	<.0001	<.0001	

Note. Soil test K levels are averaged over potassium treatment.

[a]Soil test K levels within a depth column followed by different letters are statistically different.

differences in soil test K levels between K treatments in any of the three soil types at either depth in the 2-yr sampling. Therefore, soil test K data from the 2-yr sampling were averaged over treatment. Potassium levels were different between soil types within each depth and between depths within each soil type (Table 5). Two-year soil test K levels were highest at both depths in the Crider soil compared with the other soil types and higher in the top 7.5 cm in both the Crider and Grenada compared with the deeper 7.5- to 15-cm soil depth. Soil test K levels were higher in the 7.5- to 15-cm depth in the Zanesville soil 2 yr after application. The majority of the K was in the top 7.5 cm of soil in two of the three soils at 2 yr after K application. This makes sense because the K was placed on the soil surface and not incorporated. Total soil test K from both depths showed another major increase at 24 mo (December 2018), similar to the increase seen at 12 mo (December 2017) in the Crider and Zanesville soils.

These data show that the highest soil test K levels generally occur between 12 and 15 mo after K application in the Crider and Zanesville soil types, although smaller increases in soil test K are seen at 3 and 6 mo after K application. Both Crider and Zanesville soils showed a substantial decrease in soil test K levels at 9 mo after K application. Soil test K levels in the Grenada soil type showed too much variation over time to determine when maximum K levels occur after K application. The Grenada soil type also showed more variation in soil test K over time in untreated plots that did not receive K.

Table 6 illustrates the changes in soil test K that occurred between fall K applications in December 2016 based on fall soil test recommendations from November 2016 and soil test results from the following spring (March 2017) in each soil type. The Crider soil type exhibited the greatest response to fall-applied K, having the highest soil test K values and the lowest K recommendations the following spring (Table 6). The Zanesville and Grenada sites responded similarly to fall-applied K. However, both the Zanesville and Grenada sites still required 130–195 kg K ha^{-1} the following spring compared with no more than 46 kg K ha^{-1} at the Crider site. These results suggest that fall K applications made to well-drained soils such as Crider silt loam may come much closer to satisfying the K requirements from fall soil test by the following spring than lesser well-drained soils, such as Zanesville or Grenada.

TABLE 6 Change in soil test K levels from fall (December 2016) to spring (March 2017) following fall potassium applications on Crider, Zanesville, and Grenada soil types in Princeton and Murray, KY

Treatment	Princeton								Murray			
	Crider				Zanesville				Grenada			
	Fall soil test K[a]	Fall K applied[b]	Spring soil test K	Spring K recommended	Fall soil test K	Fall K applied	Spring soil test K	Spring K recommended	Fall soil test K	Fall K applied	Spring soil test K	Spring K recommended
	kg ha^{-1}											
No fall K	220	–	281	111	149	–	186	195	123	–	165	214
Fall K_2SO_4	204	224	412	28	126	247	187	195	132	171	253	139
Fall KCl	196		376	46	131		241	149	194		264	130
Fall KCl × 2	181	448	556	0	137	495	306	93	145	342	277	111

[a]Initial composite soil samples taken from each site in November 2016 indicated low soil test K (<224 kg K ha^{-1}). Fall soil test K data are from samples collected from each individual plot in December 2016 just prior to K application. [b]Fall K application was based on K recommendation from initial soil test taken from each site in November 2016. Spring soil samples were collected in March 2017. Fall and spring soil test values are the mean of four replications of each treatment at each site.

4 | CONCLUSION

These data indicate that the highest soil test K levels in Crider and Zanesville soil types generally occur between 12 and 15 mo after fall (December) K application. For all three soil types, seasonal fluctuations in soil test K levels were evident for each of the K treatments and were likely influenced by seasonal fluctuations in precipitation. These fluctuations in precipitation could have influenced the soil test K levels that were observed in 2017 and 2018. Seasonal fluctuations in soil test K are observed almost every year (Murdock & Call, 2006), making K recommendations difficult. Within all three of these soil types, it appears the soils fix added fertilizer K over time.

When soils are dry, clay interlayers can collapse and trap K, resulting in lower soil test K values. Clay minerals such as vermiculite have greater potential to trap K when soils are dry, making soil test K lower than if soils had a higher moisture content (Karathanasis, 1987; Murdock & Wells, 1973). Precipitation at Princeton in the month prior to the 9- and 21-mo sampling was well below the 30-yr average in both 2017 and 2018. This may explain the lower K values in the Crider and Zanesville soil types at the 9- and 21-mo sampling dates (Figures 1 and 2). There was also a greater clay content in the Crider and Zanesville soil types (Table 3), which may have contributed to the greater changes in soil test K values over time compared with the Grenada soil type.

The Crider soil type is a well-drained soil with high amounts of total K present that appeared to be more responsive to fall K applications in this research. The less well-drained Grenada and Zanesville soil types still required 130–195 kg K ha^{-1} the following spring after a fall soil test and K application, compared with no more than 46 kg K ha^{-1} in the Crider soil type (Table 6).

These data illustrate the seasonal fluctuations in soil test K levels that occur naturally in these three soil types and how clay content and precipitation may influence these fluctuations.

ACKNOWLEDGMENTS

The authors thank Chris Rodgers and Vickie Witcher for technical assistance in the field study, Yvonne Thompson for technical assistance in the particle size and clay mineralogy analyses, and John Grove for constructive comments and suggestions.

CONFLICT OF INTEREST

There are no conflicts of interest.

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How to cite this article: Keeney AB, Ritchey EL, Bailey WA. Soil test potassium changes over time following fall potassium application in three Western Kentucky soils. *Agrosyst Geosci Environ*. 2020;3:e20058. https://doi.org/10.1002/agg2.20058

Plant Disease • 2020 • 104:1781-1785 • https://doi.org/10.1094/PDIS-02-19-0382-RE

e-Xtra*

Cytochrome b Mutations F129L and G143A Confer Resistance to Azoxystrobin in *Cercospora nicotianae*, the Frogeye Leaf Spot Pathogen of Tobacco

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Abstract

Azoxystrobin is the only synthetic, systemic fungicide labeled in the United States for management of frogeye leaf spot (FLS) of tobacco (*Nicotiana tabacum* L.), caused by *Cercospora nicotianae*. Though traditionally considered a minor disease in the United States, FLS has recently become yield and quality limiting. In 2016 and 2017, 100 *C. nicotianae* isolates were collected from symptomatic tobacco from eight counties in Kentucky, United States. Prior to azoxystrobin sensitivity testing, some *C. nicotianae* isolates were found to utilize the alternative oxidase pathway and, after assay comparisons, conidial germination was utilized to evaluate sensitivity in *C. nicotianae* as opposed to mycelial growth. Azoxystrobin sensitivity was determined by establishing the effective concentration to inhibit 50% conidial germination (EC_{50}) for 47 (in 2016) and 53 (in 2017) *C. nicotianae* isolates. Distributions of *C. nicotianae* EC_{50} values indicated three qualitative levels of sensitivity to azoxystrobin. Partial *cytochrome b* sequence, encompassing the F129L and G143A mutation sites, indicated single-nucleotide polymorphisms (SNPs) conferring the F129L mutation in *C. nicotianae* of moderate resistance (azoxystrobin at $0.177 \leq EC_{50} \leq 0.535$ μg/ml) and the G143A mutation in isolates with an azoxystrobin-resistant phenotype (azoxystrobin $EC_{50} > 1.15$ μg/ml). Higher frequencies of resistant isolates were identified from greenhouse transplant (4 of 17) and conventionally produced (58 of 62) tobacco samples, as compared with field-grown tobacco (<4 weeks prior to harvest; 4 of 62) or organically produced samples (1 of 7), respectively. Together, these results suggest that resistance to azoxystrobin in *C. nicotianae* occurs broadly in Kentucky, and generate new hypotheses about selection pressure affecting resistance mutation frequencies.

Keywords: chemical, field crops, disease management, fungi

Frogeye leaf spot (FLS), caused by *Cercospora nicotianae* Ellis & Everh., has historically been considered a minor foliar disease restricted to older tobacco (*Nicotiana tabacum* L.) tissue in the lower canopy (Lucas 1975). Lesions are typically 1 to 1.5 cm in diameter, with a chlorotic halo surrounding a red-brown necrotic ring. Lesion centers are tan and, under humid conditions, pseudostromata, short conidiophores, and conidia develop (Supplementary Fig. S1). The disease was traditionally considered so minor that FLS in lower-canopy leaves was desirable to some tobacco buyers, indicating that the crop reached full maturity in the field (Lucas 1975). However, since approximately 2015, FLS has vastly increased in incidence and severity, resulting in price dockage or outright rejection due to reduced quality (Bickers 2016a,b,c,d, 2017, 2018; Fowlkes 2016, 2017) (personal communication). This also coincided with the first documentation of FLS occurring in greenhouse-produced tobacco transplants (Dixon et al. 2018). Although demand for U.S.-produced tobacco has steadily decreased over the past few decades, the U.S. crop was still worth over $1.4 billion dollars in 2017 (USDA-NASS 2018). Kentucky is the leading U.S. producer of burley and dark tobacco, two subtypes of tobacco utilized in different consumer product blends. Although some growth in organic tobacco production has occurred in recent years, the authors estimate fewer than 10 organic tobacco producers in Kentucky at time of publication.

Azoxystrobin is the only systemic, synthetic fungicide labeled for tobacco production in the U.S. for the control of foliar, fungal diseases. It is among the oldest fungicides in the outer quinol oxidation site inhibitor (QoI) fungicide class and has been labeled for commercial agricultural use since 1994 (Fernández-Ortuño et al. 2010). Biological control products such as those with *Bacillus* spp. active ingredients and synthetic protectant fungicides, with copper or mancozeb as active ingredients, are labeled for tobacco; however, these are utilized by a minority of growers due to efficacy concerns or in avoidance of undesirable residues in cured leaves. Thus, some growers apply up to four sequential azoxystrobin applications in a given tobacco season (personal communication). Numerous aspects of the FLS pathosystem align with high risk factors associated with fungicide resistance by the Fungicide Resistance Action Committee (FRAC) (Brent and Holloman 2007; NAFRAC 2019), including azoxystrobin's single-site mode of action, multiple applications per year, exclusive usage without integration of other modes of action, frequent application for curative rather than preventative purposes, geographically large usage areas, and the potential for sublethal dosages applied to tobacco. As of 2019, the maximum rate of azoxystrobin products labeled for tobacco was 0.877 liter/ha; however, replicated trials indicated that an azoxystrobin rate of 0.585 liter/ha managed target spot (caused by *Thanatephorus cucumeris*) equally well (Seebold 2008; Seebold and Henderson 2010; Seebold and Palmer 2007; Seebold and Sizemore 2011). Target spot has historically been a more yield-limiting fungal leaf spot in tobacco compared with FLS. Azoxystrobin has been labeled for tobacco disease management in Kentucky since at least 2005, in which the only rate indicated was 0.585 liter/ha (FIFRA 2005).

Resistance to QoI fungicides has been documented in other agriculturally relevant *Cercospora* spp., including *C. sojina* (Standish et al. 2015; Zhang et al. 2012), *C. kikuchii* (Price et al. 2015), and *C. beticola* (Birla et al. 2012; Bolton et al. 2012; Vaghefi et al. 2016). In each of these pathosystems, the G143A mutation within the *cytochrome b* gene has been reported at higher prevalence than

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Funding: Funding was provided by the College of Agriculture, Food, and the Environment at the University of Kentucky as well as the Council for Burley Tobacco.

*The *e*-Xtra logo stands for "electronic extra" and indicates that one supplementary figure is published online.

The author(s) declare no conflict of interest.

Accepted for publication 13 January 2020.

the F129L mutation. Isolates with the G143A mutation, which results in an amino acid substitution from glycine to alanine, have effective concentration to inhibit 50% germination (EC_{50}) values ranging from 5- to 1,000-fold higher than the EC_{50} values of baseline isolates (Bradley and Pedersen 2011; Vaghefi et al. 2016; Zhang et al. 2012). Isolates with the F129L mutation, which results in an amino acid substitution from phenylalanine to leucine, have EC_{50} values 10- to 140-fold higher than baseline isolates when evaluated with conidial germination assays in *Pyricularia grisea*, *Alternaria solani*, and *C. beticola* (Kim et al. 2003; Pasche et al. 2005; Vaghefi et al. 2016). References from other *Cercospora* pathosystems detail baseline EC_{50} values in nonexposed isolates for azoxystrobin ranging from 0.002 to 0.16 μg/ml (Bradley and Pedersen 2011; Price et al. 2015; Vaghefi et al. 2016; Zhang et al. 2012).

Neither the alternative oxidase respiration pathway nor a comparison of QoI fungicide sensitivity assays has been previously completed in *C. nicotianae*. Several agriculturally relevant *Cercospora* spp. have been found to utilize the alternative oxidase pathway (Bradley and Pedersen 2011; Price et al. 2015; Vaghefi et al. 2016; Zhang et al. 2012), which has been described as a respiratory "rescue mechanism" deployed by some fungi in in vitro evaluations of QoI fungicide sensitivity (Fernández-Ortuño et al. 2010; Zhang et al. 2012). Amending fungicide sensitivity culture media with salicylhydroxamic acid (SHAM) has been used to eliminate potential spore germination via the alternative respiration pathway (Olaya and Koller 1999). For many studies evaluating sensitivity to QoIs, a conidial germination assay has been utilized (Bradley and Pedersen 2011; Secor et al. 2010; Vaghefi et al. 2016; Zhang et al. 2012), though mycelial growth assays were also used (Price et al. 2015). QoI fungicides inhibit adenosine-triphosphate production by binding the quinol oxidation site of *cytochrome* bc_1 (Fernández-Ortuño et al. 2010), which has been suggested to have greater influence over conidial germination than mycelial growth (Vaghefi et al. 2016).

Because azoxystrobin is the only currently labeled, systemic fungicide option for FLS management in tobacco, documentation of the sensitivity of *C. nicotianae* isolates to this active ingredient and correlations with production systems are critical to elucidating resistance prevention and mitigation strategies. Therefore, specific objectives of the study were to (i) establish azoxystrobin sensitivity distributions from *C. nicotianae* isolates collected from Kentucky tobacco in 2016 and 2017, (ii) identify point mutations conferring resistance to azoxystrobin in a priori phenotyped *C. nicotianae* isolates, and (iii) compare azoxystrobin sensitivity patterns in isolates from greenhouse transplants and field-grown tobacco approaching harvest, as well as organic and conventional management regimes.

Materials and Methods

Sampling. FLS samples were acquired from seven tobacco farms in each of 2016 and 2017, resulting in 47 and 53 *C. nicotianae* isolates, respectively, in each sample year. Information on collection year, Kentucky county of origin, age of sampled plant material, management system, and tobacco type is provided in Table 1. Field-grown tobacco was sampled within 1 month of harvest and had been treated with azoxystrobin in some cases. When multiple samples were collected per field, plants were located no less than 10 m apart. Samples collected in 2016 were from farms in which the grower suspected a loss of control (except for samples from the organic farm). *C. nicotianae* isolates collected prior to 2005 (marking azoxystrobin's first usage in tobacco) are not known to exist. Samples from 2017 were collected at the first suspected incidence of FLS in greenhouse transplants (Dixon et al. 2018), submitted to the University of Kentucky Plant Disease Diagnostic Laboratories, or collected during other visits to grower fields by extension agents or specialists.

Single-sporing and isolate storage. Single-conidium isolates were generated from lesions on tobacco leaves. In most cases, a single conidium was removed from a sporulating lesion with a scalpel blade as visualized under a dissecting microscope. Where sporulation was not present on the sample surface, a mycelial culture was initiated on one-quarter acidified potato dextrose agar (1/4 aPDA; Difco, Becton Dickinson and Company, MD, U.S.A.); then, the culture was transferred to clarified V8 juice agar to induce sporulation. Briefly, V8 juice agar was made by stirring commercial V8 juice with a final concentration of 1.5% $CaCO_3$ for 30 min, then centrifuging the solution for 10 min at 3,000 rpm. Clarified V8 suspension was combined

Table 1. *Cercospora nicotianae* isolates collected from Kentucky tobacco in 2016 and 2017 and included in azoxystrobin sensitivity assays

Isolate range[a]	Number of isolates	Year collected	County of origin	Host age	Management	Tobacco type
16Cn001-010	9	2016	Green	Mature	Conventional	Burley
16Cn011-020	9	2016[x]	Metcalfe	Mature	Conventional	Burley
16Cn022-029	8	2016	Barren	Mature	Conventional	Burley
16Cn031-040	9	2016	Metcalfe	Mature	Conventional	Burley
16Cn041-050	10	2016	Barren	Mature	Conventional	Burley
16Cn056-058	2	2016	Scott	Mature	Organic	Burley
17Cn001-006	6	2017[w]	Allen	Transplant	Conventional	Burley
17Cn007-010	4	2017[w,x]	Allen	Transplant	Conventional	Burley
17Cn011	1	2017	Barren	Transplant	Conventional	Burley
17Cn012-016	4	2017	Barren	Transplant	Conventional	Burley
17Cn017-021	5	2017[y]	Metcalfe	Transplant	Conventional	Burley
17JJ501–JJ614e	4	2017[x,y]	Metcalfe	Mature	Conventional	Burley
17D1–D7	7	2017	Christian	Mature	Organic	Burley
17H1a–H2e	4	2017[z]	Daviess	Mature	Conventional	Dark
17H1405a	1	2017	Metcalfe	Mature	Conventional	Burley
17L101–407	3	2017	Todd	Mature	Conventional	Dark
17P751a–d	3	2017[z]	Daviess	Mature	Conventional	Burley
17P752a–c	2	2017[z]	Daviess	Mature	Conventional	Burley
17P753b–d	3	2017[z]	Daviess	Mature	Conventional	Burley
17P754b–c	2	2017[z]	Daviess	Mature	Conventional	Burley
17P755a–d	3	2017[z]	Daviess	Mature	Conventional	Dark
17P846	1	2017[x]	Allen	Mature	Conventional	Burley

[a] Isolate ranges reported in groups by field or greenhouse sampled.
[x] Samples originated from mature tobacco from the same production field visited in 2016 and 2017.
[w] Samples were collected from the same general greenhouse area managed by the same grower but represented two different transplant lots started on 1 March (17Cn001 set) and 10 March (17Cn007 set).
[x] Samples originated from identical transplant lots gathered in April and August for transplant and mature tobacco, respectively.
[y] Samples originated from identical transplant lots gathered in April and July for transplant and mature tobacco, respectively.
[z] Samples collected from different fields managed by the same grower on the same farm.

with deionized (DI) water to a final concentration of 20%. After adding agar at 15 g/liter, the media was autoclaved and poured into Petri plates while still molten. A plug from the sporulating culture was transferred to 10 ml of sterile DI water and agitated with a vortex, and 0.1 ml of aliquot was spread on 1/4 aPDA. After 24 h, a single germinating spore was transferred to V8 juice agar and grown as a single conidium isolate. Each isolate was grown individually on V8 juice culture and placed under 12 h of black to blue light (370 to 400 nm wavelength) to induce sporulation; then, conidia were harvested and frozen in 15% glycerol at −80°C until further processing. Small aliquots of frozen conidial suspensions were utilized to revive isolates as needed.

Evaluation of the alternative oxidase pathway in *C. nicotianae*. To determine the presence of the alternative oxidase pathway in *C. nicotianae* (Fernández-Ortuño et al. 2010; Zhang et al. 2012), 12 isolates representative of sample years and locations were chosen to determine the effect of SHAM on conidial germination of *C. nicotianae*. SHAM (final concentration 0, 60, 70, or 100 ppm; dissolved in methanol) was included or not in the presence of six concentrations of technical-grade azoxystrobin (0, 0.001, 0.01, 0.1, 1, and 10 μg/ml) (Syngenta Crop Protection, Greensboro, NC, U.S.A.). This range of SHAM concentrations was selected because, in QoI sensitivity evaluations of *A. solani* (Rosenzweig et al. 2008), *C. zeae-maydis* (Bradley and Pedersen 2011), *C. sojina* (Zhang et al. 2012), *C. beticola* (Vaghefi et al. 2016), and *P. grisea* (Vincelli and Dixon 2002), SHAM concentrations used were 100, 60, 100, 60, and 100 ppm, respectively. Azoxystrobin was dissolved in acetone and all media contained a final concentration of 1% (vol/vol) acetone. Conidial suspension methods approximated Zhang et al. (2012), with slight modifications. Conidial suspensions of each isolate were made by removing 8 to 10 7-mm plugs from a 7- to 10-day-old, actively sporulating culture. Plugs were agitated with a vortex for approximately 30 s in 6 ml of sterile DI water. The suspension was filtered through two layers of cheesecloth; then, 75 μl was spread on each media plate. Three replicate plates of each SHAM–azoxystrobin combination were established for each isolate and stored in the dark at ambient temperature for 20 to 28 h. In all, 100 conidia were evaluated for 2016 isolates and 50 conidia were evaluated for 2017 isolates. A conidium was considered germinated if the germ tube was at least half the length of the spore. At each azoxystrobin concentration, the number of germinated conidia was determined as a percentage of the germinated conidia in the nonamended medium, completed in three replicate plates (Vaghefi et al. 2016; Zhang et al. 2012) for each *C. nicotianae* isolate at each SHAM concentration. Germination percentages and corresponding fungicide concentrations were utilized in PROC PROBIT in Statistical Analysis Software (SAS; v. 9.4; SAS Institute Inc., Cary, NC, U.S.A.) (Pfeufer and Ngugi 2012; Vaghefi et al. 2016) to calculate the specific azoxystrobin concentration expected to inhibit 50% of conidial germination. PROC PROBIT was used to construct a normal regression model for each *C. nicotianae* isolate, in which the response (percent germinated conidia compared with the untreated control) was modeled against discrete $\log_{10}$ concentrations (doses) of azoxystrobin. The azoxystrobin concentration at which 50% inhibition was modeled indicated the EC_{50} value for each isolate (Pfeufer and Ngugi 2012). Experimental replicates were separated for the SHAM and sensitivity assay comparisons in order to generate three separate EC_{50} values for each isolate tested; however, replicates were combined in the same analysis for each isolate represented in the EC_{50} distributions by year.

Fungicide sensitivity assays in *C. nicotianae*. Conidial germination assays were similar to those described by Secor et al. (2010) and Zhang et al. (2012), with the following modifications. *C. nicotianae* isolates were grown from frozen culture for 7 to 10 days under black to blue light (wavelength 370 to 400 nm) to induce sporulation. Conidia were dislodged from cultures, suspended in sterile DI water, and spread plated on amended media. Three replicate plates per isolate on each azoxystrobin concentration were prepared. Conidial germination was determined for 100 (2016 isolates) or 50 (2017 isolates) conidia per plate under a compound microscope at ×100 magnification after 20 to 28 h of incubation. EC_{50} values were calculated using the conidial germination assay described above for 47 and 53 isolates from 2016 and 2017, respectively.

To compare broadly utilized fungicide sensitivity assays, 14 *C. nicotianae* isolates were tested on PDA amended with azoxystrobin at 0, 0.001, 0.01, 0.1, 1.0, and 10 μg/ml in order to determine the EC_{50} for mycelial growth or conidial germination. These isolates were chosen to be representative of both sample years and unique sample sites, and based on results from initial azoxystrobin sensitivity evaluations completed using the conidial germination assay. For these comparisons, the conidial germination assay was repeated on identical media used for the mycelial growth assay. In both assays, PDA was amended with azoxystrobin to the final concentrations listed after media were sterilized and warm to touch but still molten. Based on results from the previously described experiments, both mycelial growth and conidial germination media were amended to a final concentration of SHAM at 70 ppm.

Mycelial growth evaluation assays were conducted using the methods described by Pfeufer and Ngugi (2012) and Smith et al. (1991). Mycelial plugs were removed from the leading edge of isolate culture plates using a number 3 cork borer (approximately 5 mm in diameter), and plugs were placed in plates with the same size area removed from the center. Each isolate–concentration combination was tested in three replicate plates. Using a handheld digital caliper, mycelial growth of the colony was measured on two perpendicular diameters at 3, 5, 7, 10, and 14 days postplug transfer. EC_{50} values were calculated using PROC PROBIT as described above, with the response being 14-day colony diameter as a percentage of the same isolate growth on nonamended media (Pfeufer and Ngugi 2012).

Amplification and sequencing of partial *cytochrome b* gene of selected *C. nicotianae* isolates. Twenty-two isolates were selected for PCR and subsequent sequencing based on sample year, sample location, and a priori phenotyped azoxystrobin EC_{50} value from conidial germination assays, as determined above. After the initial identification of the F129L mutation in isolate 16Cn004, the remaining seven isolates from that sample site were amplified and sequenced. Isolates were grown on clarified V8 juice medium for 8 to 10 days; then, mycelia and conidia were harvested, lyophilized, and extracted using the cetyltrimethylammonium bromide method of Li et al. (2008), with one modification. The single modification was an initial extraction with an equal volume of phenol/chloroform/isoamyl-alcohol (50:49:1) after the first heating step. DNA was quantified using the Qubit fluorometer (Invitrogen Technologies, Carlsbad, CA, U.S.A.) and stored at 4°C until further use.

Amplification of partial *cytochrome b* was accomplished using the *mut4* primer set, which flanks the region of *C. sojina cytochrome b* encompassing the F129L, G137R, and G143A mutation sites (Zeng et al. 2015). PCR cycling conditions were as described by Zeng et al. (2015); however, only 15 ng of template was used per reaction. Amplicons were approximately 380 bp when visualized on a 1× Tris-acetate-EDTA gel; then, PCR products were cleaned using ExoSAP-IT (Affymetrix, Inc., Cleveland, OH, U.S.A.). PCR products were Sanger sequenced by Eurofins Genomics (Eurofins Inc., Louisville, KY, U.S.A.). Forward and reverse sequences were trimmed of low-quality reads and aligned with ApE (A Plasmid Editor, v2.0.55; https://jorgensen.biology.utah.edu/wayned/ape/) to acquire consensus sequences. The consensus sequences for each isolate were then aligned with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) utilizing default settings. All consensus sequences exhibited homology to fungal *cytochrome b* genes in GenBank, including closely related species *C. beticola* and *C. kikuchii*. Sequences of selected isolates are available in GenBank (Table 2).

Data analysis. Distributions of EC_{50} values for *C. nicotianae* isolates for each sampling year were constructed by subtracting the largest EC_{50} (2.795) from the smallest EC_{50} (0.0187), dividing by 30 (each value referred to as a bin), and using the range around each bin to collapse values into bars. Normality of each year's distribution was assessed using the normaltest option in PROC UNIVARIATE in SAS. Azoxystrobin EC_{50} values in the presence of SHAM at 0, 60, 70, and 100 ppm were compared for selected isolates using PROC

GLM in SAS. If the experiment-wise F test indicated an experiment-wise difference among the treatments, means were separated using Fisher's least significant difference. After applying a $\log_{10}$ transformation, the Wilcoxon rank-sum test was conducted using PROC NPAR1WAY on EC_{50} values grouped by sampling location (transplant or field-grown) or management system (conventional or organic) and evaluating isolates from burley tobacco only. Transplant isolates were excluded from the management system analysis, and organic isolates were excluded from the sample location analysis.

For additional comparisons on the effect of sample location and production system on *C. nicotianae* azoxystrobin sensitivity, isolates were grouped by their conidial germination assay EC_{50} values, utilizing ranges indicative of sensitive, F129L, or G143A partial *cytochrome b* sequence results. Isolates with EC_{50} values for azoxystrobin at less than 0.038 μg/ml were considered sensitive for these analyses, 0.177 to 0.535 μg/ml were moderately resistant, and >1.150 μg/ml were highly resistant, corresponding to sensitivity patterns of assumed-sensitive isolates, those with an F129L mutation, or those with the G143A mutation, respectively. A two-by-three χ^2 comparison between greenhouse transplant and field-grown sample isolates from burley tobacco was completed using PROC NPAR1WAY. Similarly, a two-by-two analysis was completed between isolates from samples of organic and conventionally managed burley tobacco utilizing only the sensitive and moderately resistant isolates, due to small overall organic sample numbers. For all statistical tests, significant results were indicated by $\alpha = 0.05$.

Results

Comparisons of fungicide sensitivity assay methods and determination of an alternative oxidase pathway in selected isolates. The azoxystrobin EC_{50} values for 9 of 14 *C. nicotianae* isolates differed depending on the fungicide sensitivity assay used ($P < 0.05$; Table 2). The conidial germination assay clustered isolate EC_{50} values into single orders of magnitude, whereas the mycelial growth assay encompassed broader ranges of sensitivity. For example, EC_{50} values from highly resistant isolates ranged from 1.212 to 3.021 μg/ml utilizing the conidial germination assay but the same isolates had EC_{50} values for azoxystrobin at >10 μg/ml in the mycelial growth assay. Four isolates (16Cn004, 16Cn043, 17JJ614c, and 16Cn008) had significantly lower EC_{50} values when the conidial germination assay was conducted in the presence of SHAM at 70 ppm ($P < 0.05$; Table 2). In one of two experiments, there was no significant difference in azoxystrobin sensitivity between SHAM at 0 and 60 ppm for isolate 16Cn008 (Table 2). Based on these data, all remaining EC_{50} values were determined in media amended with SHAM at 70 ppm.

***C. nicotianae* isolate EC_{50} distributions from 2016 and 2017.** In 2016, EC_{50} values were determined for 47 *C. nicotianae* isolates originating from six tobacco fields located in four counties in Kentucky. The majority of isolates ($n = 40$) had EC_{50} values for azoxystrobin in the range of 0.178 to 0.360 μg/ml, with the overall isolate EC_{50} range spanning 0.0274 to 2.795 μg/ml. The 2016 distribution was nonnormal (Shapiro-Wilk test, P value ≤ 0.01; data not shown), with isolate EC_{50} values grouping roughly into three orders of magnitude for azoxystrobin at around 0.03, 0.3, and 2.0 μg/ml. Isolates with azoxystrobin EC_{50} values > 1.5 μg/ml originated from two different farms (Fig. 1A).

In 2017, EC_{50} values were determined for 53 *C. nicotianae* isolates originating from nine farms located in six counties in Kentucky (Fig. 1B). A more thorough sampling of an organically managed field (without exposure to azoxystrobin for a minimum of 3 years) and isolates from dark tobacco were included in 2017, which together accounted for 8 of the 11 isolates with $EC_{50} < 0.085$ μg/ml. As with the 2016 dataset, the 2017 EC_{50} values were nonnormally distributed (Shapiro-Wilk test, P value ≤ 0.01; data not shown). In 2017, 31 of 53 isolates had EC_{50} values in the range of 0.178 to 0.360 μg/ml, and EC_{50} distributions for azoxystrobin grouped around 0.03, 0.3, and 2.0 μg/ml. Six isolates with $EC_{50} > 1.150$ μg/ml were identified, which originated from four different farms in three counties (Fig. 1B). Mean and median *C. nicotianae* isolate EC_{50} values were 0.358 and 0.270 μg/ml, respectively, for 2016 and 0.451 and 0.271 μg/ml, respectively, for 2017 (Fig. 1A and B).

Effect of location and production system on *C. nicotianae* azoxystrobin sensitivity. *C. nicotianae* EC_{50} values grouped by plant sample location were not significantly different between isolates from transplant and field-grown burley tobacco samples when

Table 2. Effective azoxystrobin concentration to inhibit 50% of either conidial germination or mycelial growth (EC_{50}) and in the presence of salicylhydroxamic acid (SHAM) at 0, 60, 70, or 100 ppm for selected *Cercospora nicotianae* isolates

	Sensitivity assay EC_{50} comparison[a]			Azoxystrobin EC_{50} values at various SHAM concentrations[b]				
Isolate	Germ	Growth	P value[c]	0 ppm	60 ppm	70 ppm	100 ppm	Accession[d]
16Cn058	0.030	0.084	0.008	0.043	0.043	0.042	0.036	MK369760
16Cn056	0.036	0.061	0.340[w]	...	...	...	...	MK369759
17D1	0.038	0.777	0.018[w]	0.030	0.031	0.034	0.036	MK369764
16Cn026	0.245	3.324	0.011[w]	0.653	0.470	0.710	0.337	MK369756
16Cn004	0.283	4.030	0.001	3.933 a[x,y]	0.648 b	0.477 b	0.349 b	MK369750
16Cn009	0.292	2.431	0.017[w]	0.217	0.187	0.169	0.191	MK369754
17JJ501e	0.313	2.243	0.028[w]	...	...	...	...	MK369765
17P751a	0.332	7.237	0.036[w]	0.394	0.456	0.445	0.403	MK369767
16Cn043	0.340	2.431	0.019[w]	0.204 a	0.124 b	0.137 b	0.125 b	MK369758
17JJ614c	1.212	36.522	0.004[w]	7.479 a	3.976 b	4.118 b	3.470 b	MK369766
16Cn035	1.891	118.700	0.035[w]	6.350	4.102	5.700	5.564	MK369757
17Cn018	2.531	39.945	0.096[w]	3.815	2.692	3.390	2.990	MK369762
16Cn008	2.879	53.748	0.061[w]	3.177 a[x]	2.957 ab	2.700 b	2.590 b	MK369753
17Cn006	3.021	1,137.800	0.202[w]	4.080	2.107	2.797	2.046	MK369761

[a] Results from conidial germination (Germ) and mycelial growth (Growth) assays were compared using a t test; means are reported. Media were amended with SHAM at 70 ppm.

[b] EC_{50} values using different SHAM concentrations were compared using the conidial germination assay and a one-way analysis of variance; mean EC_{50} values are reported.

[c] Azoxystrobin EC_{50} values of each isolate compared using a t test.

[d] GenBank accessions. Partial *cytochrome b* sequence amplified using the *mus4* primer set (Zhang et al. 2012).

[w] A Satterthwaite correction to the P value is reported due to results from the Folded F test, which indicated unequal variances about the treatment mean.

[x] Means for each isolate by SHAM concentration were separated using Fisher's protected least significant difference; statistically significant differences ($P \leq 0.05$) are indicated across the row.

[y] Three replicate experiments were completed with similar results; means from one representative experiment shown.

[z] In one experiment, all SHAM concentrations were significantly different from the 0-ppm SHAM treatment; means shown are from a separate experiment in which the 60-ppm SHAM treatment was not significantly different from the 0-ppm SHAM treatment.

Wilcoxon signed-rank tests were utilized ($Z = -0.271$, $P = 0.788$; Table 3). Using the same signed-rank test, isolates from organic production systems had lower median EC_{50} values than isolates from samples from conventional production systems ($Z = -3.804$, $P < 0.001$; Table 3).

In χ^2 analyses, in which isolates were grouped according to threshold EC_{50} values indicated by partial *cytochrome b* sequence data (Fig. 2), significant differences in isolate frequencies were found according to the sampling location and production system. Proportionally more highly resistant *C. nicotianae* individuals were isolated from transplant samples as compared with field-grown tobacco ($\chi^2 = 8.283$, $P = 0.019$; Table 4). Proportionally more moderately resistant individuals were isolated from conventionally produced samples compared with organically produced samples ($\chi^2 = 55.770$, $P < 0.001$; Table 4).

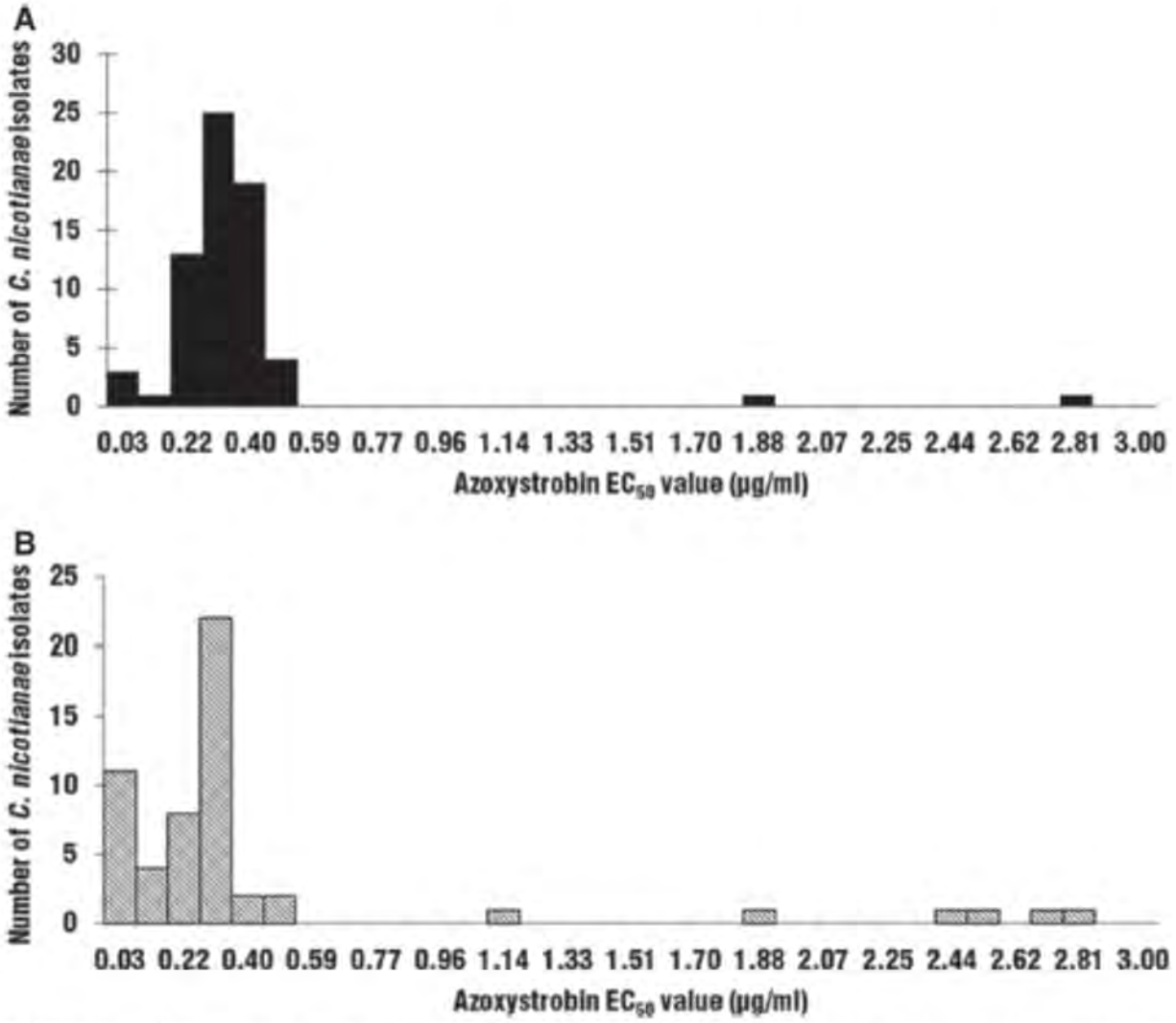

Fig. 1. Distributions of effective azoxystrobin concentration to inhibit 50% of conidial germination (EC_{50}) values of *Cercospora nicotianae* isolates from tobacco crops in Kentucky. *C. nicotianae* isolates were collected in A, 2016 and B, 2017. X-axes show every other bin value.

Table 3. Effect of sample location (transplant or field-grown) or production system (organic or conventional) on median *Cercospora nicotianae* azoxystrobin concentration to inhibit 50% of conidial germination (EC_{50}) values[x]

Sample descriptor	Sample detail	EC_{50} range	Wilcoxon signed rank test		
			Median EC_{50}	Z value	Two-tailed *t* *P* value
Location	Transplants ($n = 21$)	0.025–2.753	0.272	...	...
	Field-grown ($n = 65$)[y]	0.025–2.795	0.280	−0.271	0.788
Production system	Organic ($n = 9$)[z]	0.019–0.579	0.035	...	...
	Conventional ($n = 65$)	0.025–2.795	0.280	−3.804	<0.001

[x] Data were log-transformed prior to statistical tests; nontransformed values are shown.
[y] Isolates from conventionally produced burley tobacco only.
[z] Isolates from mature burley tobacco only.

Partial *cytochrome b* sequence comparisons of selected *C. nicotianae* isolates. Mutations demonstrated to confer resistance in other pathosystems were identified within *C. nicotianae cytochrome b* sequences, which corresponded to order-of-magnitude azoxystrobin sensitivity groupings. Isolates with azoxystrobin $EC_{50} \leq 0.038$ µg/ml were the presumed wild type at codons 129, 137, and 143 and considered fully sensitive. *C. nicotianae* samples isolated prior to the use of azoxystrobin on tobacco were not available, because this active ingredient has been labeled for the crop since at least 2005. Isolates with EC_{50} for azoxystrobin within the range 0.177 to 0.535 µg/ml carried a single-nucleotide polymorphism (SNP) conferring the F129L mutation, and were considered moderately resistant to azoxystrobin. Sequences of 11 of 17 moderately resistant isolates indicated a C to A SNP at this locus (Fig. 2). However, six isolates carried a C to G SNP in the same location (16Cn003, 16Cn004, 16Cn007, 17P753b, 17D7, and 17H1d; Fig. 2). At the codon level, these SNPs confer a synonymous amino acid change from phenylalanine to leucine. *C. nicotianae* isolates with azoxystrobin $EC_{50} > 1.150$ µg/ml carried a G to C SNP, which conferred the G143A mutation (Fig. 2). The G143A SNP results in an amino acid change from glycine to alanine, and these isolates were highly resistant to azoxystrobin. Both sample years were represented within the sensitive, moderately resistant, and highly resistant sequence groupings from four, eight, and seven unique sample sites, respectively (Table 1; Fig. 2). Sequences of selected isolates were deposited in GenBank, with accessions in Table 1.

Discussion

FLS now limits tobacco yield and quality in the United States, where previously it had been regarded as a minor disease with few to no economic implications. Azoxystrobin currently is the only systemic, synthetic fungicide labeled for U.S. tobacco production and, as such, it is heavily relied upon for FLS management. Loss of efficacy of this fungicide through the development of resistance has critical implications for U.S. tobacco growers.

EC_{50} values of *C. nicotianae* isolates from Kentucky in 2016 and 2017 indicated qualitative resistance around azoxystrobin concentrations of 0.03, 0.3, and 2.0 µg/ml (Fig. 1), which is the first documentation of fungicide resistance in this organism. The assumed sensitivity of wild-type *C. nicotianae* in Kentucky is azoxystrobin at 0.03 µg/ml, which is comparable with baseline levels in other *Cercospora* spp. evaluated with this fungicide (Bradley and Pedersen 2011; Price et al. 2015; Vaghefi et al. 2016; Zhang et al. 2012). Order-of-magnitude shifts in sensitivity are characteristic of resistance to QoI fungicides in general (FRAC 2018) and have been documented for *A. solani, C. kikuchii, C. beticola*, and *C. sojina* (Pasche et al. 2005; Price et al. 2015; Rosenzweig et al. 2008; Vaghefi et al. 2016; Zhang et al. 2012). Distributions around discrete EC_{50} values presented here suggested three qualitative levels of azoxystrobin sensitivity, which were further evaluated through partial *cytochrome b* sequence analysis to identify polymorphisms at mutation sites documented to confer resistance to azoxystrobin in *C. sojina* (Zeng et al. 2015).

Cytochrome b sequence data indicated phenotype-specific SNPs. *C. nicotianae* isolates inhibited at low levels of azoxystrobin (<0.038 µg/ml) were determined to be sensitive and wild type at the F129L and G143A loci. Nucleotide sequences shown for the five sensitive *C. nicotianae* isolates in Figure 2 were identical to those of baseline *C. beticola* (GenBank accession number MF327260) and *C. sojina* (GenBank accession numbers KJ566928 to KJ566930) (Standish et al. 2015). *Cytochrome b* sequence data generated for isolates with EC_{50} values ranging from 0.177 to 0.535 µg/ml carried a mutation at the F129L locus (Fig. 2). The more common point mutation found among these isolates, cytosine to adenine, was documented in QoI-resistant *P. grisea* (Kim et al. 2003) and *C. beticola* (Vaghefi et al. 2016), and was one of three SNPs conferring the F129L amino

Table 4. Effect of sample location (transplant or field-grown) or production system (organic or conventional) on *Cercospora nicotianae* isolates grouped by concentration to inhibit 50% conidial germination of a single-conidium *C. nicotianae* isolate (EC_{50}) values delineated based on partial *cytochrome b* sequence

	Number of isolates[y]					
Sample type	S	MR	HR	Total	χ^2 Statistic	Two-tailed *P* value
Transplants	1	12	4	17	…	…
Field	0	58	4	62	…	…
Totals	1	70	8	79	8.283	0.019
Organic	6	1	0[z]	7	…	…
Conventional	0	58	4[z]	62	…	…
Totals	6	59	4[z]	69	55.770[z]	<0.001

[y] Sensitive (S) isolates (EC_{50} < 0.038 µg/ml), moderately resistant (MR) isolates (EC_{50} 0.177 to 0.535 µg/ml), and highly resistant (HR) isolates ($EC_{50} \geq 1.187$ µg/ml).

[z] In production system comparisons, only S and MR isolate counts are compared, because sample numbers were too low to include the HR isolate grouping.

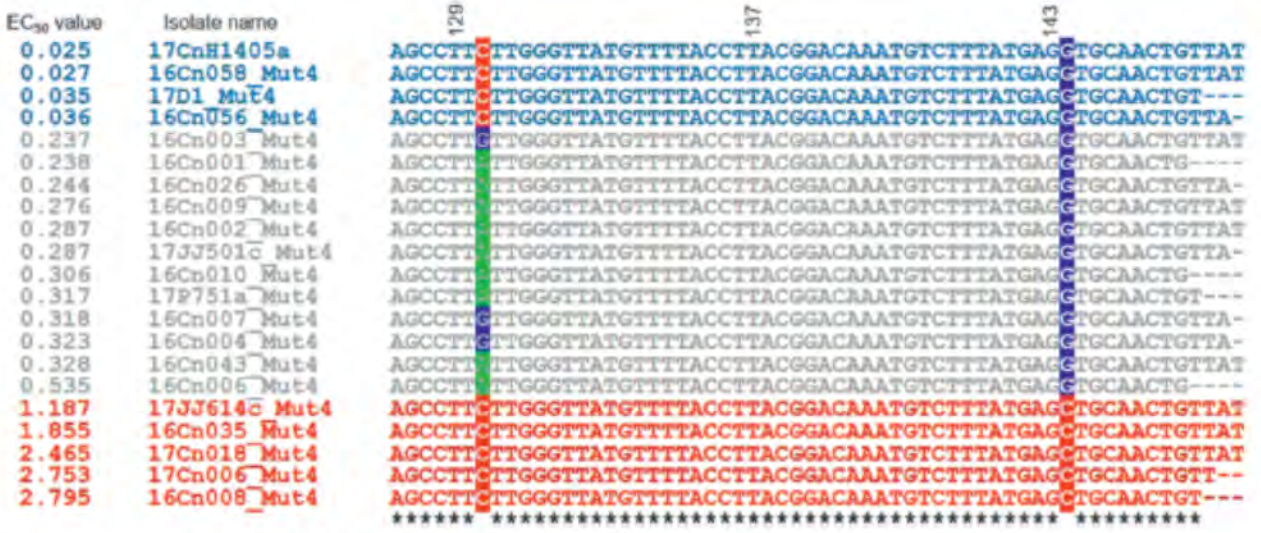

Fig. 2. Azoxystrobin effective concentration to inhibit 50% of conidial germination (EC_{50}) values and partial *cytochrome b* sequence from *Cercospora nicotianae* isolates collected from tobacco with frogeye leaf spot symptoms in Kentucky in 2016 and 2017. Nucleotide polymorphisms in codons 129 and 143 are highlighted.

acid substitution in *Pyrenophora teres* (Sierotzki et al. 2007) and *A. solani* (Rosenzweig et al. 2008). In the latter two pathogens, the less common *C. nicotianae* point mutation, cytosine to guanine, was also found (Rosenzweig et al. 2008; Sierotzki et al. 2007) although, similar to the data presented here, this SNP was less common in *A. solani* populations (Rosenzweig et al. 2008). The presence of multiple synonymous mutations conferring the F129L amino acid change has important implications for molecular identification of QoI resistance in *C. nicotianae*. From a grower perspective, commercially acceptable control of field populations composed of individuals with the F129L mutation has been reported by simply increasing the rate of fungicide applied (Fernández-Ortuño et al. 2010) but this has not yet been evaluated in the tobacco FLS pathosystem.

Unlike other *Cercospora* pathosystems where resistance to QoIs has been established (Price et al. 2015; Vaghefi et al. 2016; Zeng et al. 2015; Zhang et al. 2012), a minority (7 of 29) of sequenced *C. nicotianae* individuals carried the G143A mutation conferring high levels of resistance to azoxystrobin (Fig. 2). However, given limited sampling and the presence of high resistance phenotype isolates at seven different locations, individuals with this mutation should not be considered rare. Compared with the high frequency of *C. nicotianae* with moderate resistance (78 of 100, occurring at all sample sites; Fig. 1A and B), less common occurrences of individuals with the G143A mutation may be related to both FLS epidemiology and grower standard practices in tobacco production. FLS typically begins on leaves in the lower tobacco canopy, and lesions are often only visible when plants reach 1 m or more. Though Extension recommendations indicate that the use of drop-nozzles is optimal (Johnson et al. 2019; Thiessen 2018), the authors estimate that fewer than 20% of growers actually utilize these sprayer modifications due to crop injury from the apparatuses as the sprayer moves through the field. Combined with the standard practice of applying submaximal dosages of azoxystrobin (Johnson et al. 2019; Thiessen 2018), the dose typically encountered by *C. nicotianae* in the field ranges from 359 to 599 μg/ml under conditions of perfect coverage, assuming typical volumes of 77 and 46 liter/ha, respectively. Lower than maximum fungicide rates combined with poor coverage would exert weaker selection pressure on pathogen populations compared with systems where excellent coverage and maximum azoxystrobin dosages were utilized. Studies are underway to evaluate mutation frequency dynamics in mixed populations where optimal fungicide coverage and application timing are achieved. Conversely, competitive fitness among *C. nicotianae* isolates with varying azoxystrobin sensitivities has not yet been documented, and it is possible that the G143A mutation may confer a fitness cost in *C. nicotianae*, even though reductions in fitness were not documented in *C. sojina* carrying the G143A mutation (Zhang and Bradley 2017).

Frequency comparisons based on production system or tobacco sample location provide further correlative evidence for the influences of fungicide coverage and competitive fitness in production environments. In this study, proportionally more *C. nicotianae* isolates from greenhouse-sampled, transplant-age tobacco had EC_{50} values indicating that the G143A mutation was present than *C. nicotianae* from field-grown, mature tobacco (Table 4). Azoxystrobin is labeled for one application per year in greenhouse tobacco transplant production for target spot management, caused by *T. cucumeris* (anamorph *Rhizoctonia solani*). *C. nicotianae* has only recently been shown to occur in greenhouse transplant systems (Dixon et al. 2018) and, in this confined space with very small plants, thorough fungicide coverage is more easily achieved compared with spray applications in the field. Consequently, *C. nicotianae* would be exposed to higher doses and greater selection pressure in the greenhouse environment, possibly resulting in the higher frequency of G143A mutants.

In comparisons of *C. nicotianae* isolate EC_{50} values from organically and conventionally produced tobacco, it is not surprising that mean and median values were significantly lower (Table 3) and isolate EC_{50} frequencies shifted toward sensitive (Table 4) from organically produced tobacco. Organic certification requires that no azoxystrobin be used in that field for 3 years prior to actual marketing of the crop as organic, resulting in little to no selection pressure for several seasons. Though sample numbers were small, one individual carrying the F129L mutation was identified from organic production systems (17D7; Fig. 2), and another is likely from the same population based on its calculated azoxystrobin EC_{50} value of 0.579 μg/ml (17D6). Two hypotheses that would follow from these occurrences are that (i) the F129L mutation does not impose a comparative fitness cost on *C. nicotianae* individuals or (ii) the F129L mutation is relatively common in wild populations of *C. nicotianae*. Comparatively, *A. solani* with the F129L mutation has been shown to have lower in vitro conidial germination rates but induced greater disease severity in vivo (Pasche and Gudmestad 2008). Similar experiments are being undertaken in the FLS pathosystem.

Other results presented here, for the first time in *C. nicotianae*, detail evidence for the presence of the alternative oxidase respiration pathway and a comparison of fungicide sensitivity assays. In several *Cercospora* spp., the alternative oxidase pathway has been documented as a "rescue mechanism" for pathogens to continue to respire during in vitro tests of QoI fungicide sensitivity (Bradley and Pedersen 2011; Fernández-Ortuño et al. 2010; Price et al. 2015; Vaghefi et al. 2016; Zhang et al. 2012). Data presented here indicate that some but not all *C. nicotianae* isolates were also capable of alternative respiration (Table 2). The choice of fungicide sensitivity assessment method can be influenced by pathogen trophic level, ease of in vitro sporulation induction, equipment and space availability, fungicide target site, and numerous other factors. In the FRAC publication on Monitoring Methods, *A. solani*, *Mycosphaerella fijiensis*, *Phakopsora pachyrhizi*, and *Venturia inaequalis* were all pathogens for which a conidial germination assay was recommended for QoI sensitivity testing (FRAC 2012). However, the recommended method for *Sclerotinia sclerotiorum* and *M. graminicola* QoI sensitivity determination was a mycelial growth assay, which was also used in recent *C. kikuchii* QoI sensitivity evaluations (Price et al. 2015). Comparisons of the two sensitivity assays for a selection of *C. nicotianae* isolates indicated statistically significant differences between calculated EC_{50} values for most tested individuals (Table 2). The conidial germination assay resulted in qualitative, order-of-magnitude groupings of isolate EC_{50} values, which is consistent with QoI fungicide sensitivity patterns recognized in other pathosystems (FRAC 2018; Sierotzki et al. 2007). More broadly, results presented here suggest that fungicide EC_{50} values determined using different evaluation assays may not be directly comparable, though this could be pathogen specific.

Contemporary increases in FLS severity may not solely be due to shifts in *C. nicotianae* sensitivity to azoxystrobin. For instance, rainfall data from one Kentucky county where FLS is particularly severe (Metcalfe), indicated that, in July and August 2015, 2016, and 2017, there were one, three, and one periods, respectively, of six or more consecutive days of rain (Kentucky Mesonet 2019). Compared with the earliest available rainfall records in Mesonet, in July and August 2010 to 2014, no 6-consecutive-day rainy period occurred (Kentucky Mesonet 2019). Environmental conditions notwithstanding, data presented here indicate that *C. nicotianae* populations from Kentucky tobacco are broadly shifted away from wild-type azoxystrobin sensitivity. With few, if any, azoxystrobin alternatives likely to be labeled for use in tobacco in the short term, practical extensions of this work include optimization of spray intervals and evaluation of variety susceptibility to FLS. Rapid-throughput resistance identification protocols are also needed so that commercial tobacco growers can make informed management decisions about fungicide applications. Though moderately resistant *C. nicotianae* plants were most common in this study, practical loss-of-control in tobacco fields has not yet been empirically attributed to the resistance status of pathogen populations, which will be a focus of future investigation. Furthermore, elucidation of strength of selection pressure will improve understanding of how *C. nicotianae* populations may change under different management regimes.

Acknowledgments

We thank S. Osborne and C. Hardy, University of Kentucky Cooperative Extension Agents, as well as 10 tobacco growers for site visit assistance; H. Li, research

technologist, for submitting *cytochrome b* sequence results to GenBank on behalf of B. Amsden and E. Pfeufer; B. Kennedy and J. Beale, University of Kentucky Plant Disease Diagnosticians, for assistance with sample maintenance; and an anonymous reviewer for her excellent presubmission feedback on the manuscript.

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What happens to *in situ* net soil nitrogen mineralization when nitrogen fertility changes?

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Abstract

Does net soil nitrogen (N) mineralization change if N-fertility management is suddenly altered? This study, conducted in a long-term no-tillage maize (*Zea mays* L.) fertility experiment (established 1970), evaluated how changing previous fertilizer N (PN) management influenced *in situ* net soil N mineralization (NSNM). Net soil N mineralization was measured by incubating undisturbed soil cores with anion and cation exchange resins. In each of three PN fertilizer application plots (0, 84, and 336 kg N ha^{-1}), another three fertilizer application rates (0, 84, and 336 kg N ha^{-1}) were imposed and considered the current fertilizer N (CN) management. Generally, PN-336 (336 kg N ha^{-1}) had significantly greater NSNM than PN-0 (0 kg N ha^{-1}) or PN-84 (84 kg N ha^{-1}), which reflected differences in soil organic-C (SOC) and soil total-N (STN). The three CN rates had no significant effect on NSNM when they were applied to PN-0 or PN-84, but CN-336 (336 kg N ha^{-1}) had significantly higher NSNM than CN-0 (0 kg N ha^{-1}) or CN-84 (84 kg N ha^{-1}) in the PN-336 plots. The CN or "added N interaction" used the indigenous soil organic matter (SOM) pool and the added sufficient fertilizer N. Environmental factors, including precipitation and mean air temperature, explained the most variability in average daily soil N mineralization rate during each incubation period. Soil water content at each sampling day could also explain NSNM loss *via* potential denitrification. We conclude that "added N interaction" in the field condition was the combined effect of SOM and sufficient fertilizer N input.

Key words: added nitrogen interaction / N management / *in situ* resin core method / priming effect

Accepted January 30, 2019

1 Introduction

When fertilizer N synthetized by the Haber–Bosch process began to be utilized in agricultural production in the 1910s (*Erisman* et al., 2008), the cycling of N in agroecosystems was dramatically modified. The contribution of fertilizer N to soil organic-N transformations in agriculture is still debated (*Powlson* et al., 2010; *Mulvaney* et al., 2009). One of the central points in this argument is whether fertilizer N application affects soil indigenous N mineralization. In the current drive to improve N use efficiency in crop production, and to reduce undesirable environmental effects, understanding this influence of fertilizer N on indigenous soil nitrogen mineralization has become increasingly important and demanded.

To address this question, two dimensions of fertilizer N management should be clarified. One is the effect of previous fertilizer N (PN) management on the soil N mineralization rate, which can provide insight into how the soil mineralizable N pool can be shaped by previous fertilizer N. The other dimension is the effect of current or "in-season" fertilizer N on net soil N mineralization rate, which can unveil the mechanism of the priming effect or added N interaction effects on indigenous soil N mineralization (*Jenkinson* et al., 1985). Most previous studies on this topic have solely focused on the effect of previous N fertility management on soil N mineralization because soil samples were taken either at spring (before) or fall (after) the growth season. Few studies have been conducted on both previous and current fertilizer N application for *in situ* conditions in agroecosystems.

Previous research focused on the effect of the previous fertilizer N fertilization on soil N mineralization show mixed or controversial results, regardless of *in situ* or laboratory incubation method (*Kuzyakov*, 2010). Several studies found a positive correlation between historical fertilizer N application and soil N mineralization in some agriculture production systems (*El-Haris* et al., 1983; *Singh* and *Singh*, 1994; *Kolberg* et al., 1997; *Rasmussen* et al., 1998; *Kanchikerimath* and *Singh*, 2001; *Graham* et al., 2002; *Jordan* et al., 2004). Other studies noted no effect of fertilization on soil N mineralization (*Franzluebbers* et al., 1994a; 1994b). Negative fertilizer–mineralization interaction has also been documented (*Wienhold* and *Halvorson*, 1999; *Carpenter-Boggs* et al., 2000). Although results have been mixed, the mechanism that is proposed to explain these effects is relatively similar. For a negative effect, lower soil N mineralization at higher historical N rates could be due to inorganic N immobilization because of greater plant

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biomass production at higher N application rates (*Wienhold* and *Halvorson*, 1999). However, mineralization with time can still release N retained in root biomass, plant residue, and the active soil organic pool, which can explain the positive effect of high N application on soil N mineralization (*El-Haris* et al., 1983; *Yan* et al., 2007). From this perspective, it seems critical to investigate the quality and status of the plant residue and SOM pool when evaluating the effect of PN fertilizer application on soil N mineralization.

Few studies have addressed the effect of current fertilizer N application on net soil N mineralization. Some reports called this added nitrogen interaction as one kind of priming effect (*Woods* et al., 1987; *Kuzyakov*, 2010). The main reason could be the limited technical approaches. Laboratory incubation methods based on Stanford and Smith's procedure (*Stanford* and *Smith*, 1972) is a classic way to estimate soil N mineralization. In this method, however, leaching soil inorganic-N with $CaCl_2$ is frequently used, which can make fertilizer N additions have a shorter retention period in soil samples than natural field leaching conditions. Isotope tracers might seem to be a direct way to test how fertilizer N interacts with soil indigenous N mineralization. However, ^{15}N-labelled fertilizers might have an "apparent" effect, caused by pool substitution or by isotope displacement reactions (*Jenkinson* et al., 1985). The biggest potential disadvantage for laboratory incubation and isotope methods is that neither reflects field conditions, including ambient temperature and moisture (*Carpenter-Boggs* et al., 2000). Temperature and moisture are important environmental factors affecting soil organic N microbial processes (*Sierra*, 1997). Therefore, to make observation relevant to actual production, *in situ* incubation methods in the field condition are needed (*Kuzyakov*, 2010).

There are three major *in situ* incubation methods frequently adopted by researchers to estimate soil N mineralization in the field: buried bags, covered cylinders, and resin-trap soil core methods. Among these methods, the resin-trap soil core method tends to have the closest relationship to field conditions in terms of fluctuations in temperature, moisture, and aeration (*Khanna* and *Raison*, 2013). Another advantage of the resin-trap method is the potential for use during in-season fertilizer N application studies because mineral N in the soil column from mineralization and/or fertilizer N addition can be leached and captured during rainfall or irrigation due to its open design (*Hanselman* et al., 2004). This may decrease the artificial stimulation of denitrification especially when addition of fertilizer N is heavily loaded on the surface (*Groffman* and *Tiedje*, 1988). Therefore, in this study, we used resin-trap methods to test the effect of current fertilizer N on soil N mineralization in the field conditions.

Temperature and moisture are primary environmental drivers of soil organic matter decomposition (*Kirschbaum*, 1995; *Gabriel* and *Kellman*, 2011). They can substantially affect soil N microbial processes. Unlike well-controlled conditions for laboratory incubation methods, *in situ* N mineralization studies can be hard to explain without considering these climate factors. Generally, within a reasonable range, moisture and temperature have a positive relationship to soil N mineralization. These two factors could also interact because there is a negative relationship between soil moisture and temperature (*Sierra* et al., 2015). In this case, N mineralization might be more responsive to one factor when the level of the other is more favorable for activity. Additionally, except for direct effects, temperature and moisture can have indirect effects related to soil aeration, which affects aerobic microbial activity (*Sierra*, 1997). Previous *in situ* incubation N mineralization studies showed high correlation between climate factors with N mineralization rate. *Singh* and *Singh* (1994) reported that as much as 80% of the variability in N-mineralization rate was explained by soil moisture content. *Kolberg* et al. (1997) reported that precipitation in combination with mean air temperature and their interaction gave the best prediction of N mineralization daily rate across study sites. Besides, the relationship between air temperature and soil temperature was well correlated (*Song* and *Wang*, 2006).

To sum up, the present study tested the effects of the N fertilizer management on *in situ* net soil N mineralization during a maize growing season by separating previous and current fertilizer effects. Precipitation and temperature data were included to help explain N mineralization in field conditions. Specifically, the objectives of this study were to: (1) measure the influence of previous N fertilizer application on net soil N mineralization; (2) evaluate the effect of current N fertilizer rate on net soil N mineralization; (3) correlate precipitation, air temperature, and soil moisture content with average daily net soil N mineralization rate.

2 Material and methods

2.1 Field sites and climatic information

This study was conducted at the University of Kentucky Spindletop Research Farm, near Lexington, Kentucky, USA (38°07′18.9′′N 84°29′10.6′′W). This site has been a long-term maize N fertilizer and tillage study since 1970. This site was continuous maize and fertilizer applications were broadcast by hand. The soil is a Bluegrass-Maury silt loam (fine, mixed, active, mesic Typic Paleudalf) with a 2 to 4 percent slope. This region is characterized by a wide variation in mean monthly air temperature from 0°C in January to 24°C in July and August, and a relatively even distribution in mean monthly precipitation with a total of 1159 mm in average annual precipitation level.

2.2 Experiment design

For this long-term study site, two factors were included in the existing field experiment design since 1970: (1) fertilizer N rate (0, 84, 168, and 336 kg N ha^{-1}); (2) tillage method (tillage and no tillage) (*Stoddard* et al., 2005). Maize plots (11.9 m width × 12.2 m length) were arranged in a randomized complete block design with four replications. For this *in situ* study, only no tillage practices and three field fertilizer N rates (0, 84, and 336 kg N ha^{-1}) combinations were investigated. The existing field fertilizer N rates were considered background or previous N fertilizer rates (PN). Three current fertilizer N rate (0, 84, and 336 kg N ha^{-1}) treatments in the incubation samples were introduced to each PN rate plot. For example, the incubation samples in the main plot of NT-0 kg N ha^{-1} had treatments of 0 kg N ha^{-1}, but also of 84 and 336 kg N ha^{-1}.

2.3 Field incubation procedure

In situ soil N mineralization analysis with 20.3 cm deep soil cores was performed by a modified procedure (*Kolberg* et al., 1997; *Zou* et al., 2017). The incubation tubes were kept plant-free by applying herbicide and hand removal. The cores were prepared by driving and removing aluminum conduit (4.8 cm inner diameter by 25.4 cm long) into the ground between non-traffic crop rows. The bottom 2.5 cm of soil was removed from each soil core and replaced with a nylon bag filled with resin beads. After the resin bag was placed, 1-mm sized nylon mesh was used to wrap the bottom of the tube. The details of the soil resin cores are in Fig. 1. The resin bag (exactly 35 g total based on original product moisture condition) consisted of equal amounts of cation and anion exchangeable resins (Lanxess Sybron, Birmingham, NJ). In a preliminary study, the resin was evaluated for retention of absorbed inorganic N with highly intensive water irrigation (5.9 mm d^{-1}) during a 30-d period, and less than 1% of the inorganic N was observed in the leachate. For CN rate treatment application, N fertilizer solution (5 mL NH_4NO_3) was evenly sprayed into the inner soil surface of tubes at rates of 0, 84, and 336 kg N ha^{-1} equivalent based on the inner area (20.26 cm^2) of the tubes. To avoid contamination from field N fertilizer, the CN treatments were applied to the incubation tubes before field N application and covered until field N application was finished.

To remove intact soil columns and avoid bypass contamination we took the following procedures: (1) a hydraulic soil sampler (Giddings 6S RPS) was used to remove the soil cores with as little disturbance as possible to the inside and surrounding soil; (2) when the entire core assembly was returned to the original hole, a rubber washer and some soil was used to surround the tube to avoid preferential bypass flow; (3) the original plant residue on soil surface was kept in the tube to account for the contribution of N mineralization from this fresh organic matter pool and microbial communities; (4) the top of soil core was exposed to the atmosphere to simulate natural field conditions, which allows mineralized soil N to leach from soil column into the resin bags. It was assumed that N introduction from precipitation was small and the same for all treatments (4.7 kg^{-1} ha^{-1} y^{-1} average for Kentucky, National Atmospheric Deposition Program, 2013; http://nadp.sws.uiuc.edu/data/ntn/).

The tubes were installed immediately before fertilizer application for the following reasons: (1) the main research purpose was to check the effects of synthetic N fertilizer application on indigenous soil N mineralization, which required the precise application of synthetic N fertilizer in the tubes. This requirement could only be fulfilled before fertilizer application. (2) The tube installation was operated by hydraulic soil sampling equipment. Therefore, it would be impossible to perform when the crop grew bigger. Given the limitation of resin bag durability in field conditions, the replacement of resin bags occurred at longer than 60 d intervals (*Wienhold* et al., 2009). Replacement of the resin bags at the bottom of the tube did not not severely disturb soil structure in the tubes. The final inorganic N absorbed by resin was the sum of two resins bags.

To account for suspected high variance in field condition, three soil resin cores were composited into one observation. Each plot had 27 cores (3 current fertilizer rates × 3 sampling dates × 3 replicates) installed. Each plot had four rows and all soil tubes were randomly assigned to between the rows in the field. Sampling times were intended to occur one month and two months after installation, and at crop harvest. Actual sampling dates were adjusted due to weather and soil moisture conditions (Tab. 1). Composite soil samples (20 cores $plot^{-1}$, 0–20 cm depth) were taken upon initiating the study to obtain the baseline concentrations of soil NO_3-N and NH_4-N. On each sampling day, resin bags and soil cores were removed from the field, immediately transported to the laboratory, mixed thoroughly, and frozen to stop further mineralization until analysis.

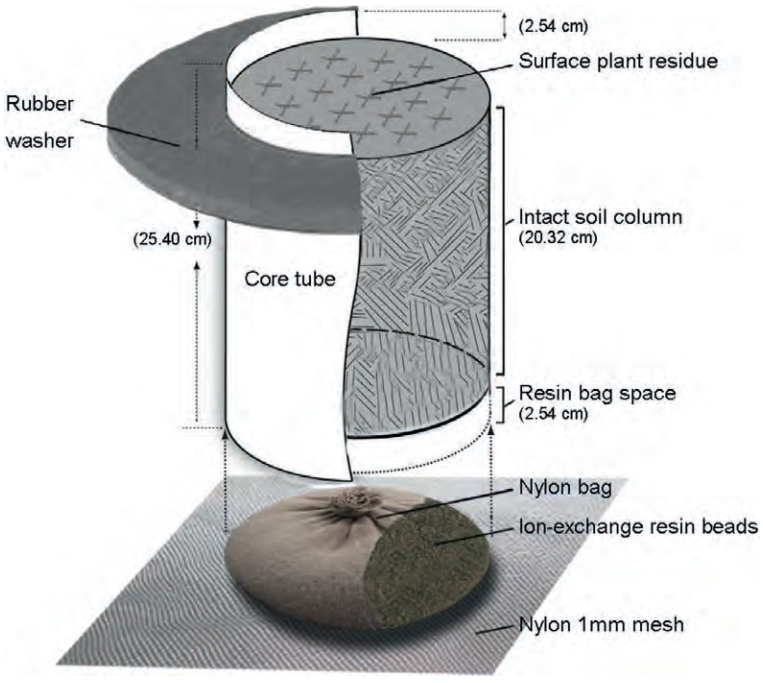

Figure 1: Cutaway diagram of soil resin core, in which surficial plant residue and intact soil column (0–20.32 cm) and one mixed-media ion-exchange resin bag (35 g) are incubated inside a core tube. A transparent example of an ion-exchange resin nylon bag is shown with 1 mm size nylon mesh as cove.

2.4 Laboratory analyses

2.4.1 Soil sample analysis

Each composite (three tubes) of field soil sample dry weight was determined by weighing the field moist sample and determining the gravimetric moisture content of a subsample. A 10-g field moist subsample was chemically extracted with 25 mL 1 M KCl for 1 h shaking. A 1-mL aliquot was centrifuged at 3700 rpm (2250 *g*) for 27 min using the Eppendorf model 5810 centrifuge with an A-2-DWP head. The extracted filtrates were ready for NO_3-N and NH_4-N analysis. Nitrate-N was determined by cadmium reduction method (Paratech, Lexington, KY) (*Crutchfield* and *Grove*, 2011). Ammonium-N was determined by phenol-

Table 1: Sampling dates for soil N mineralization in 2013.

Operation	Date	Day of year
Initiation	22-June-2013	172
Sampling date 1	26-July-2013	206
Sampling date 2	6-Sept-2013	248
Sampling date 3	3-Oct-2013	275

hypochlorite reaction (*Weatherburn*, 1967; *Ngo* et al., 1981). All soil chemical analyses for each sample was duplicated. The final inorganic N concentration for soil sample was represented in N μg kg^{-1} oven-dried soil (105°C) by adjusting gravimetric water content for each soil sample.

2.4.2 Resin extraction procedure

Each composite resin sample was thoroughly mixed by shaking. A subsample, equal to 20 g based on original product moisture content, was weighted and re-packed into a new nylon bag. The subsample resin bags were serially extracted by shaking the bags in three separate volumes of 50 mL each of 1 M KCl for three different intervals (15, 30, and 60 min). The three liquid samples were composited together. All inorganic N analyses of liquid samples followed the same analytical method as soil sample. Resin chemical analysis for each sample was duplicated. The final recovery of NO_3-N and NH_4-N were calculated by recovery rate using the equation of the standard curve [Eqs. (1) and (2)], which came from previous extraction studies following the same and standard extraction protocol above (Figs. 2 and 3).

$$NO_3 - N(\textit{absorbed by resin}) = NO_3 - N(\textit{recovered from resin}) \times 1.27 + 258.5, \quad (1)$$

$$NH_4 - N(\textit{absorbed by resin}) = NH_4 - N(\textit{recovered from resin}) \times 1.12 - 45.42, \quad (2)$$

$$\textit{Net soil N mineralization} = \textit{inorganic N}\ (t_i) - \textit{inorganic N}\ (t_0) - \textit{fertilizer N}, \quad (3)$$

where t_i is sampling date and t_0 is the initial day in Eq. (3).

2.5 Calculation

Net soil mineralized N at each sampling day was determined by using the combined NO_3-N and NH_4-N in both soil and resin analysis after correction for initial soil inorganic N amount and synthetic N fertilizer input [Eq. (3)]. The inorganic N concentration in the soil samples (dry soil weight basis) and the inorganic N concentration in resin samples were converted into μg N $tube^{-1}$ for each tube. Net soil mineralized N at each sampling day equaled the sum of the total inorganic N from soil and resin at each sampling day less the sum of the initial soil inorganic N and synthetic N fertilizer input. The unit of net soil N mineralization was converted into

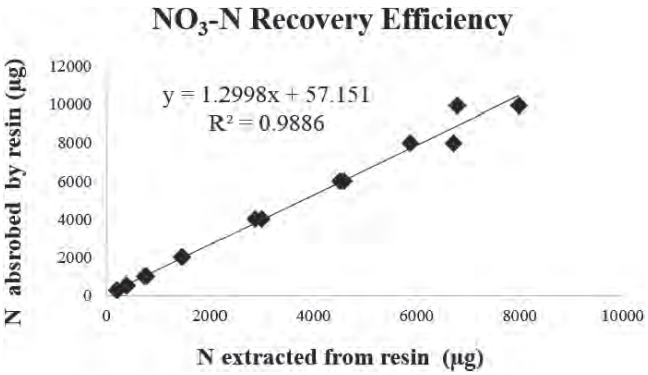

Figure 2: NO_3-N recovery efficiency by the standard extraction protocol in this study.

mg N kg^{-1} dry soil according to the oven dry soil weight for each composite sample. Average daily net soil N mineralization in each incubation period was calculate by total net soil mineralized N divided by the number of days during that period.

Bulk SOC and TN were analyzed on an Elementar Vario MAX CNS Analyzer (Elementar Americas Inc., Mount Laurel, NJ) with air-dried soil subsamples. The visible shoot and root residue was hand removed with forceps and the subsamples were ground with a mortar and pestle before any chemical analysis.

2.6 Statistical analysis

Data were statistically analyzed using the General Linear Model (GLM) procedure of the SAS 9.3 computer package (SAS Institute Inc., Cary, NC). PN plots were considered as main plots and the tubes with different current N rates were considered subplots. Duplicate measurements of composite soil and resin samples were averaged for statistical analysis of treatments effects. Treatment and their effects were considered significant at alpha equals 0.05 in ANOVA table. Mean separation was done using the Tukey's honest significant difference (HSD) test at an alpha level of 0.05. The regression analyses in this study compared the relative effect of precipitation (cumulative amount during each incubation period), temperature (mean air temperature during each incubation period), and soil water content (soil gravimetric moisture as

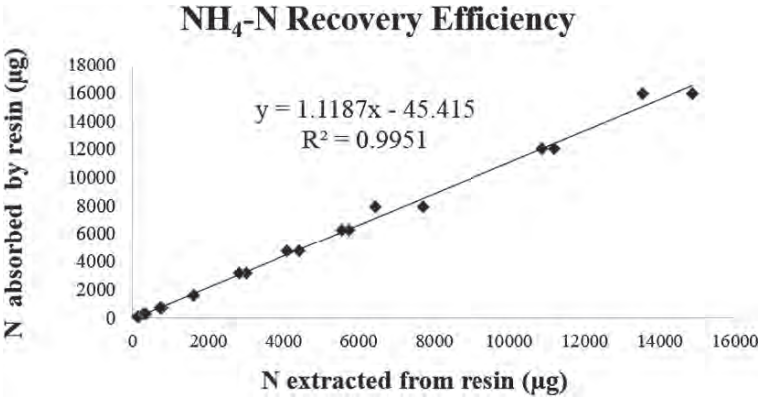

Figure 3: NH_4-N recovery efficiency by the standard extraction protocol in this study.

measured in the soil cores at each sampling date) on average daily net soil N mineralization. Regression analyses were performed using different combinations of these independent variables and comparing their predictive ability as measured by the corresponding correlation coefficient (R^2) and p values in the selected models

3 Results

3.1 Effect of long-term fertilizer applications on soil organic carbon and total nitrogen

Soil organic C and STN in the soil surface were significantly affected by the 43-year-long differences in fertilizer N rate (Tab. 2). Mineral-N includes NH_4^+-N and NO_3^--N. The PN-336 (336 kg N ha^{-1}) plot had significantly higher SOC, STN, and C : N ratio and significantly lower pH than PN-0 (0 kg N ha^{-1}) and PN-84 (84 kg N ha^{-1}) plots. On the initial day of field incubation, there was no difference in inorganic N between the three PN fertilizer rate plots.

3.2 Effect of previous and current nitrogen rate and sampling date on net soil N mineralization

Net soil N mineralization was significantly influenced by CN rate, PN rate, sampling day, and their three-way interaction (α = 0.05, Tab. 3). Within the same PN rate, NSNM was unaffected between CN-0 (0 kg N ha^{-1}) and CN-84 (84 kg N ha^{-1}). When the CN rate increased to 336 kg N ha^{-1}, NSNM numerically increased within each PN rate. However, only in the PN-336 plot did CN-336 (336 kg N ha^{-1}) significantly increase NSNM on Day 206 and Day 248, but NSNM declined on Day 275 (Fig. 4).

For the PN effect, NSNM was not different between PN-0 and PN-84 at any CN rate treatment and on any sampling day. On Day 206 and Day 248, when CN increased to 336 (kg N ha^{-1}), PN-336 had significantly higher NSNM than PN-0 and PN-84. However, on Day 248, PN-336 had no significantly higher NSNM than PN-0 and PN-84 because NSNM at PN-336 and CN-336 declined 29% on Day 275 compared to Day 248.

3.3 Effect of previous and current nitrogen rates and incubation period on average daily net soil N mineralization

Average daily net soil N mineralization was significantly influenced by previous N fertilizer rate, current N fertilizer rate, and incubation period; their three-way interaction was also significant (α = 0.05, Tabs. 3 and 4). Only at PN-84 and PN-336 during the first incubation period did CN-336 have a significantly higher average daily net soil N mineralization

Table 2: Selected soil properties at initiation of *in situ* incubation at the experiment site for the 0–20 cm depth.

Previous fertilizer N rate	pH[a]	C : N	Organic C	Total N	Mineral-N
(kg N ha^{-1})			(g kg^{-1})		(mg kg^{-1})
0	6.57A[b]	8.83B	13.71B	1.55B	4.11A
84	6.88A	9.06B	13.98B	1.54B	4.63A
336	6.09B	9.38A	17.13A	1.83A	4.78A

[a]pH here refers to soil pH_{water} (1:1, w/v).
[b]Values followed by the same uppercase letter within a column are not significantly different at $p > 0.05$ according to Tukey's HSD mean separation test.

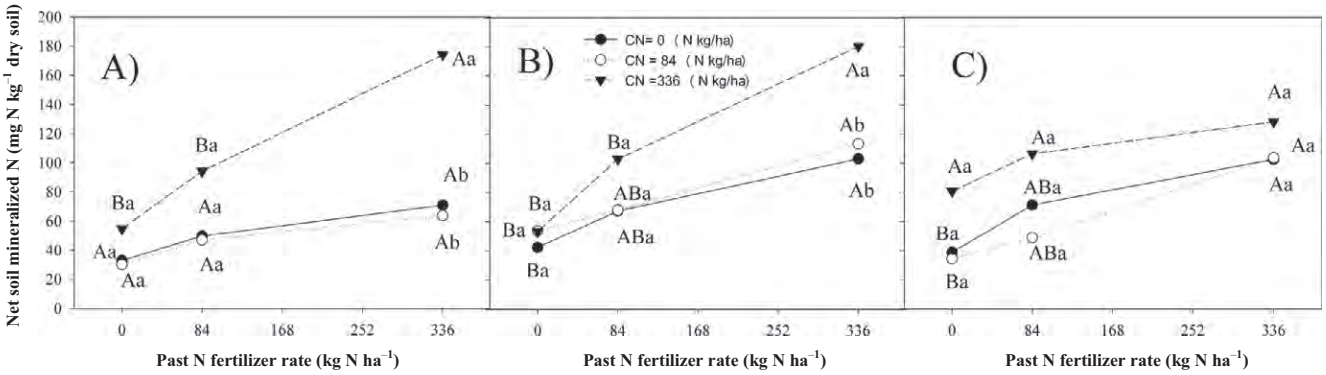

Figure 4: The effect of past and current fertilizer rate on the net soil mineralized N for three sampling days: (A) Day of Year 206 (July 26, 2013), (B) Day of Year 248 (Sept. 09, 2013), (C) Day of Year 275 (Oct. 03, 2013). Values followed by the same lowercase letter within the same past N fertilizer rate and between current N fertilizer rates are not significantly different at P > 5% according to Tukey's HSD mean separation test. Values followed by the same uppercase letter within the same current N fertilizer rate and between past N fertilizer rates are not significantly different at P > 5% according to Tukey's HSD mean separation test.

Table 3: Analysis of variance summary for the effects of previous N fertilizer rate, current N fertilizer rate, sampling day or incubation period on net soil nitrogen mineralized (NSNM) and average daily net soil N mineralization rate.[a]

Source of Variation	NSNM	Average daily net soil N mineralization
	Pr > F	Pr > F
Previous N fertilizer rate (PN)	< 0.001	< 0.001
Current N fertilizer rate (CN)	< 0.001	< 0.001
PN × CN	0.003	0.249
Sampling Day (SD)	0.002	N/A
SD × PN	0.407	N/A
SD × CN	0.168	N/A
SD × PN × CN	0.018	N/A
Incubation Period (IP)	N/A	< 0.001
IP × PN	N/A	< 0.001
IP × CN	N/A	< 0.001
IP × PN × CN	N/A	< 0.001
Coefficient of variance	27.500	64.300

[a]N/A in this table indicates not applicable.

than CN-0 and CN-84. Only at CN-336 during the first incubation period did PN-336 have a significantly higher average daily net soil N mineralization than PN-0 and PN-84. Except for PN-0 with CN-0 and CN-84, the average daily net soil N mineralization rate in the first incubation period (IP) was significantly higher than that in the third incubation period. The average mean for each IP at each PN rate also showed a trend of average daily net soil N mineralization: IP 1 (Day 173–206) > IP 2 (Day 206–248) > IP 3 (Day 248–275).

3.4 Effect of precipitation, temperature and soil water content on average daily net soil N mineralization

Regression analysis for each PN and CN combination showed that NSNM was more strongly driven by precipitation and temperature than soil water content in the tube at each sampling day (Tab. 5). Overall, precipitation alone was the largest influence at this study site for each PN and CN combination, accounting for 56% of the variability. The second largest independent variable was temperature, accounting for 40% of the variability. Soil water content only accounted for 8% of the variability at the experiment site. Cumulative precipitation and mean air temperature decreased from incubation period 1 to 3 (Fig. 5). Cumulative precipitation for incubation period IP 1 (Day 173–206), IP 2 (Day 206–248), and IP 3 (Day 248–275) were 309, 104, and 55 mm, respectively; mean air temperature for IP 1, IP 2, and IP 3 were 23.0°C, 21.7°C, and 19.2°C, respectively.

Table 4: Average daily net soil N mineralization for the three-way interaction of incubation period (IP), previous N fertilizer rate (PN), and current N fertilizer rate (CN).

IP	PN = 0			Avg.[b]	PN = 84			Avg.	PN = 336			Avg.
	CN = 0[a]	CN = 84	CN = 336		CN = 0	CN = 84	CN = 336		CN = 0	CN = 84	CN = 336	
Day of year												
173–206	0.94A[c]a[1]	0.86Aa[d1]	1.56Ba[1e]	1.12	1.42Ab[1]	1.34Ab[1]	2.69Ba[1]	1.82	2.03Ab[1]	1.82Ab[1]	4.98Aa[1]	2.94
206–248	0.21Aa[1]	0.44Aa[1]	–0.03Aa[3]	0.21	0.21Aa[2]	0.27Aa[1,2]	0.05Aa[2]	0.17	0.25Aa[2]	0.46Aa[1,2]	0.04Aa[1,2]	0.25
248–275	–0.03Aa[1]	–0.19Aa[1]	0.31Aa[2]	0.03	0.04Aa[3]	–0.19Aa[2]	0.03Aa[3]	–0.04	–0.01Aa[3]	–0.10Aa[2]	–0.51Aa[2]	–0.20

[a]The unit of N fertilizer rate for both PN and CN is kg N ha^{-1}.
[b]The mean for daily net soil N mineralization between the same previous fertilizer rate and the same incubation period.
[c]Values followed by the same uppercase letter within the same current N fertilizer rate and incubation period and between previous N fertilizer rates are not significantly different at $p > 0.05$ according to Tukey's HSD mean separation test.
[d]Values followed by the same lowercase within the same previous N fertilizer rate and incubation period and between current N fertilizer rates are not significantly different at $p > 0.05$ according to Tukey's HSD mean separation test.
[e]Values followed by a different superscript number within the same previous N fertilizer rate and current N fertilizer rate and between incubation periods are significantly different at $p < 0.05$ according to Tukey's HSD mean separation test.

Table 5: Correlation coefficients (R^2) of regression analysis using average daily net soil N mineralization of three incubation periods for three previous N (PN) fertilizer rates and current N (TN) fertilizer rates.

Independent variables in model	PN = 0[a]			PN = 84			PN = 336			Overall
	CN = 0[a]	CN = 84	CN = 336	CN = 0	CN = 84	CN = 336	CN = 0	CN = 84	CN = 336	
	R^2									
Precip[b]	0.81**	0.68**	0.85**	0.82**	0.87**	0.79**	0.87**	0.70**	0.95**	0.56**
Temp	0.67**	0.84**	0.37*	0.57**	0.75**	0.49*	0.61**	0.60**	0.65**	0.40**
SWC	0.39*	0.29	0.12	0.27	0.51**	0.40*	0.08	0.00	0.03	0.08**
Precip,Temp	0.81**	0.85**	0.94**	0.82**	0.88**	0.82**	0.87**	0.72**	0.96**	0.56**
Precip,SWC	0.86**	0.75**	0.82**	0.82**	0.88**	0.81**	0.89**	0.75**	0.96**	0.56**
Temp,SWC	0.70**	0.85**	0.37	0.57*	0.75**	0.56*	0.75**	0.65*	0.69**	0.40**
Precip,Temp, Precip × Temp	0.81**	0.85**	0.94**	0.82**	0.88**	0.82**	0.87**	0.72**	0.96**	0.56**
Precip,SWC, Precip × SWC	0.89**	0.89**	0.82**	0.83**	0.88**	0.85**	0.91**	0.75**	0.96**	0.56**
Temp,SWC,Temp × SWC	0.70**	0.91**	0.47	0.62*	0.85**	0.73*	0.75**	0.65*	0.69*	0.42**
Precip,Temp,SWC	0.78**	0.85**	0.94**	0.82**	0.88**	0.84**	0.89**	0.76**	0.96**	0.56**
Precip,Temp,SWC, Precip × Temp × SWC	0.86**	0.94**	0.95**	0.83**	0.90**	0.86**	0.91**	0.76*	0.96**	0.56**

[a]The unit of N fertilizer rate for both PN and CN are kg N ha^{-1}.
[b]Precip = precipitation received during each incubation period; Temp = mean air temperature during each incubation period; SWC = soil water content measured at each sampling date.
*, ** significant at the 0.05 and 0.01 probability levels, respectively.

4 Discussion

4.1 Effect of long-term fertilizer applications on soil organic carbon and total nitrogen

Soil N mineralization has been reported to be highly correlated with SOC and TN (*Schomberg* et al., 2009). *Zou* et al. (2018) reported that the SOC and N fractions could be stable indicators for net soil N mineralization for the same soil type in this study. Therefore, quantifying the SOC and STN pools is useful in predicting soil N mineralization. High PN-336 had significantly higher SOC and STN among the three PN rates. This result is consistent with other long-term fertilizer N studies (*El-Haris* et al., 1983; *Rasmussen* et al., 1998; *Graham* et al., 2002). The primary reason for building up a high SOC pool with annual high fertilizer N use could be greater crop production, resulting in increased plant residue, including roots returned to soil, although *Six* et al. (2002) suggested other possible stabilization mechanisms. While PN-84 is considered insufficient for optimal maize production in this soil and region, PN-84 N usually had higher yield and grain N removal than PN-0 N. Slightly higher plant residue might be a tradeoff for higher grain N removal in PN-84 plots, resulting in similar SOC and TN pools in PN-84 and PN-0.

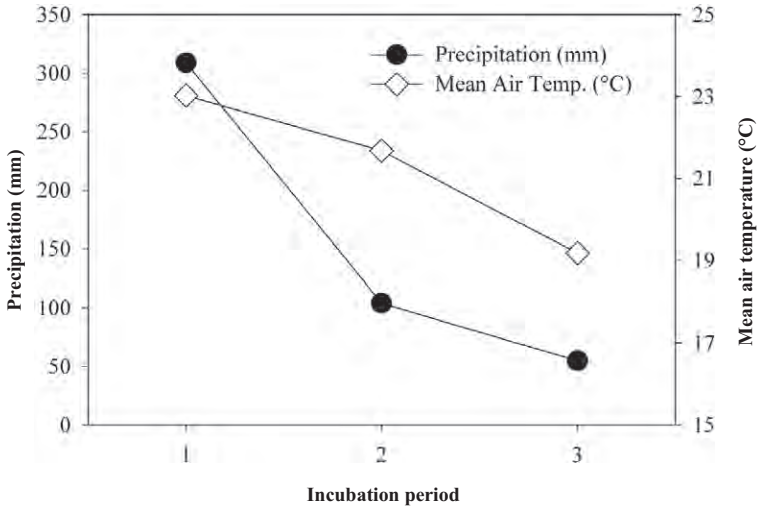

Figure 5: Precipitation and mean air temperature of three incubation periods at the experiment site.

Soil N mineralization could also be influenced by pH (*Curtin* et al., 1998) and substrate C : N ratio (*Janssen*, 1996). pH was lower in PN-336 compared to PN-0 and PN-84 due to the acidification induced by higher N input (*Dancer* et al., 1973). However, agricultural limestone was commonly used to adjust pH at this study site and the difference between fertilizer rates was unlikely to cause soil N mineralization differences (*Dancer* et al., 1973). The difference in C : N ratio between the

three PN rates was relatively narrow as well. This result makes sense because after 43 years of the same crop and the same tillage management, the microbial populations in the soil have essentially stabilized. Therefore, pH and C : N in this study might not explain the difference of NSNM between different PN rates.

4.2 Effect of previous nitrogen rate and sampling date on net soil N mineralization

The effect of previous fertilizer N application on soil N mineralization reflected the SOC and STN pools well. At any current fertilizer N application rate and any sampling day, there was no difference in NSNM between PN-0 and PN-84, which can be explained by the same soil organic carbon and nitrogen pool between these two historical N application rates. Increased SOM due to long-term fertilizer N application in this study could play a role in *in situ* net soil N mineralization. These findings might explain why there have been mixed results on the effect of previous N application on soil N mineralization, because there also have been mixed results on whether fertilizer N application can have higher SOC pools than unfertilized controls (*Yan* et al., 2007; *Liu* et al., 2013; *Brown* et al., 2014). Furthermore, in contrast to fertilizer N application, most studies report that manure or straw amendment increases the SOC pool (*Sommerfeldt* et al., 1988; *Haynes* and *Naidu*, 1998; *Yan* et al., 2007). Unsurprisingly, most of studies show that the effect of these amendment increases soil N mineralization (*Singh* and *Singh*, 1994; *Ma* et al., 1999; *Jordan* et al., 2004; *Zhang* et al., 2009). A potential hypothesis is that the historical N application affects net soil N mineralization and depends on whether these previous N applications significantly built up different SOC levels. From this perspective, we could better understand why there have been such diverse results on the effect of historical N application on soil N mineralization (*Franzluebbers* et al., 1994a; 1994b; *Carpenter-Boggs* et al., 2000; *Wienhold* et al., 2009), and even the same N rate studies but different cropping management systems can have different NSNM responses to previous N fertilizer application (*Kolberg* et al., 1999).

4.3 Effect of current nitrogen rate and sampling date on net soil N mineralization

The influence of current (or in-season) fertilizer N application on soil N mineralization usually refers to the "priming effect." *Jenkinson* et al. (1985) reported that fertilizer N can promote plant growth, thereby increasing the volume of soil explored by roots, resulting in the priming effect or "added nitrogen interaction." Our incubation system excluded plant growth by herbicide application and discarding tubes with weeds. Therefore, in our case, the current fertilizer N effect was strictly an interaction between the inorganic N with soil microorganisms. Our *in situ* study showed that the effect of current N application on NSNM or the "priming effect" has two requirements. First, the priming effect needs a sufficient SOM pool because CN application significantly affected NSNM only in PN-336 and the SOM in the PN-336 treatment was significantly higher than treatments with less fertilizer N. This result is consistent with an isotope study of soil SOM decomposition (*Chen* et al., 2014) in which added sucrose or maize straw promoted additional CO_2 respiration (carbon mineralization) from soil relative to soil alone. Second, the priming effect needs sufficient inorganic N because increasing the CN from 0 to 84 kg N ha^{-1} in this study did not cause significantly increased NSNM. This result is also consistent with *Chen* et al. (2014). In their study, when inorganic N was added to sucrose or maize straw, the exogenous organic C and inorganic N caused a synergistic effect on SOM decomposition. *Woods* et al. (1987) in an isotope study also suggested that the "priming effect" resulted from increased net N mineralization accompanied by increased N fertilization as long as mineral N concentrations remained low enough to limit soil microbial activity. Therefore, our result suggests that it is necessary to separately study PN and CN when we study the effect of fertilizer N management on indigenous net soil N mineralization in an agroecosystem because they might affect soil N mineralization due to different mechanisms.

4.4 Effect of previous and current nitrogen rate and incubation period on average daily Net soil N mineralization

The effect of previous and current nitrogen on average daily net soil N mineralization rate could provide more detailed information on each incubation period than cumulative NSNM on each sampling day. During the first incubation period, only at CN-336 did PN-336 have a significantly higher average daily net soil N mineralization rate than PN-0 and PN-84. This result confirmed that to have a significant priming effect on soil N mineralization, higher CN and higher SOM induced by high PN are both necessarily required. In a contrast to cumulative NSNM on Day 206 and Day 248, in which CN-336 had higher NSNM than CN-0 and CN-84 only in the plots of PN-336 did, CN-336 have a significantly higher average daily net soil N mineralization rates than CN-0 and CN-84 in both PN-84 and PN-336. This result demonstrated that PN-84 could have more readily labile organic pool or higher quality of SOM than PN-0, even though the total quantity of soil organic carbon and nitrogen pool and accumulative NSNM were similar (*Doran* and *Parkin*, 1994).

Average daily net soil N mineralization in the first period was significantly higher than the following incubation periods in our study. The mean for each incubation period at each PN rate also showed the trend of average daily net soil N mineralization: IP (1) > IP (2) > IP (3) (*Powlson* et al., 2010). This trend was consistent with *El-Haris* et al. (1983) and *Zhang* et al. (2009). This change in N mineralization rate could be attributed to the greater readily mineralizable N during the initial incubation period. In *Zhang*'s (2009) study, potentially mineralizable N in annually fertilized N treatments was significantly higher at the rice (*Oryza sativa* L.) transplanting stage than the later stages. In *El-Haris*'s (1983) study, soil N mineralization rate was higher in plots with higher historical fertilizer N than plots with lower historical fertilizer rates in the first 4 weeks. However, after that, soil N mineralization rates were relatively unchanged by different previous fertilizer N rates, thus, they argued that the fertilizer N application might contribute largely to the readily available N pool with little

effect on the intermediately available N pool. This phenomenon was also observed in Tab. 3, which the interaction of the previous nitrogen rate × current nitrogen rate × sampling date (or incubation period) was significant. In the first and second incubation period, the differences of net soil N mineralization, caused by previous and current nitrogen rate, was larger than third incubation period. Other reasons for explaining different soil N mineralization rates during different incubation periods might be the environmental conditions during each incubation period.

4.5 Effect of precipitation and temperature and soil water content on net soil N mineralization

Soil N mineralization can be affected by soil moisture content and temperature (*Kirschbaum*, 1995; *Sierra*, 1997). Compared to well-controlled laboratory conditions, estimating soil N mineralization from *in situ* incubation methods might be difficult to explain without consideration of weather data. Regression models showed most of variability can be explained by precipitation and moisture, which is consistent with *Kolberg* et al. (1997). Cumulative precipitation and mean air temperature gradually decreased from IP (1) to IP (3). Therefore, weather data also helped explain the different patterns of average daily net soil N mineralization rate during the three incubation periods.

Compared to precipitation and mean air temperature, soil water content measured at the end of each incubation period (which can be highly affected by rain events before each sampling day) accounted for less variability than precipitation and mean air temperature, however, including soil water content (SWC) into the regression models increased predictive capacity. Moreover, the SWC data might help explain why there was a more negative net soil N mineralization rate and NSNM declined at PN-336 and CN-336 on DAY 275. SWC on DAY 275 at the plots annually receiving PN-336 was significantly higher than other plots. The higher SWC might be due to shading caused by high biomass production induced by higher fertilizer N application and greater water field water holding capacity caused by higher SOM because the incubated tubes were plant-free by applying herbicide and hand removal of weeds Therefore, the incubation tubes are likely to have higher denitrification potential due to high soil water content and high inorganic N accumulation, especially in a no-till environment.

5 Conclusion

Understanding how fertilizer N application affects indigenous soil N mineralization will improve N fertilizer management in agro-ecosystems, resulting in improved soil productivity and reduced adverse environmental consequences. Also, it is important to separately clarify that the effects of previous and current N fertilizer application on soil N mineralization. In this present study, whether previous N fertilizer affects soil N mineralization depends on whether the SOM pool has been modified by this long-term effect. The priming effect of current fertilizer N on soil N mineralization depends on both amount of fertilizer N input and whether soil has enough SOM to support and promote soil microbial decomposition. This present study suggests that the effects of N fertility management on indigenous soil N mineralization should go beyond merely evaluating the effects of previous and current fertilizer rates. Total SOM and readily labile SOM pools induced by long term inorganic fertilizer or organic amendment application as well as controlling factors of the priming effect caused by in-season inorganic N fertilizer are important factors to evaluated the effect of fertilizer N on soil N mineralization.

Acknowledgments

We would like to express our appreciation to *Jack Zeleznik, Matthew Allen, Laura Mitchell*, *Andres Nunez* and *Antonio Marchi* for field management and collecting soil and resin samples for this study. We are grateful to *Tami Smith* and *Diane Hunter* for providing us with laboratory expertise and technical assistance for soil sample analysis. The information reported in this paper (*No.15-06-067*) is a product of the Kentucky Experiment Station and is published with the approval of the Director. Authors thank to *Yunnan Technology Innovation Program (2018-8)* and *Yunnan Ten Thousand People Program (2018-73)* for supporting Congming Zou.

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FISCAL YEAR 2020 – 2021

FINANCIAL REPORT

January 1, 2021 – March 31, 2021

QUARTERLY REPORT

TOBACCO RESEARCH INCOME
INCOME COMPARISON

Fiscal Years	2014-2015	2015-2016	2016-2017	2017-2018	2018-2019	2019-2020	2020-2021
July	$ 13,386.52	$ 139,619.47	$ 157,187.49	$ 2,459.48	$ 120,890.40	$ 141,864.01	$ 136,565.92
August	$ 301,292.71	$ 177,916.68	$ 137,652.03	$ 292,266.42	$ 126,982.37	$ 145,789.42	$ 11,873.82
September	$ 133,527.76	$ 47,768.58	$ 42,873.59	$ 139,414.92	$ 178,553.92	$ 132,169.60	$ 261,157.23
1st QUARTER	**$ 448,206.99**	**$ 365,304.73**	**$ 337,713.11**	**$ 434,140.82**	**$ 426,426.69**	**$ 419,823.03**	**$ 409,596.97**
October	$ 166,587.97	$ 255,006.22	$ 157,120.53	$ 126,862.91	$ 97,793.84	$ 150,849.00	$ 141,682.93
November	$ 74,462.42	$ 127,495.52	$ 251,055.77	$ 123,267.74	$ 128,963.50	$ 117,280.34	$ 135,157.14
December	$ 190,289.54	$ 26,196.02	$ 113,251.82	$ 135,314.04	$ 175,277.00	$ 151,323.23	$ 159,616.92
2nd QUARTER	**$ 431,339.93**	**$ 408,697.76**	**$ 521,428.12**	**$ 385,444.69**	**$ 402,034.34**	**$ 419,452.57**	**$ 436,456.99**
SIX MONTHS	**$ 879,546.92**	**$ 774,002.49**	**$ 859,141.23**	**$ 819,585.51**	**$ 828,461.03**	**$ 839,275.60**	**$ 846,053.96**
January	$ 44,597.62	$ 264,622.53	$ 109,584.57	$ 127,719.90	$ 564,217.88	$ 120,247.87	$ 93,056.96
February	$ 212,408.73	$ 10,472.72	$ 155,644.33	$ 114,047.53	$ 141,118.46	$ 114,095.14	$ 125,797.09
March	$ 133,593.90	$ 255,769.54	$ 159,012.56	$ 159,645.83	$ 122,472.86	$ 403,962.17	$ 143,903.75
3rd QUARTER	**$ 390,600.25**	**$ 530,864.79**	**$ 424,241.46**	**$ 401,413.26**	**$ 827,809.20**	**$ 638,305.18**	**$ 362,757.80**
NINE MONTHS	**$ 1,270,147.17**	**$ 1,304,867.28**	**$ 1,283,382.69**	**$ 1,220,998.77**	**$ 1,656,270.23**	**$ 1,477,580.78**	**$ 1,208,811.76**
April	$ 165,299.42	$ 20,461.50	$ 43,764.59	$ 65,036.15	$ 146,789.57	$ 117,862.64	
May	$ 183,052.97	$ 144,713.80	$ 174,933.87	$ 209,087.27	$ 63,797.02	$ 141,525.18	
June	$ 137,563.73	$ 288,160.23	$ 242,003.23	$ 168,621.20	$ 250,352.13	$ 138,849.18	
4th QUARTER	**$ 485,916.12**	**$ 453,335.53**	**$ 460,701.69**	**$ 442,744.62**	**$ 460,938.72**	**$ 398,237.00**	**$ -**
TOTAL INCOME	**$ 1,756,063.29**	**$ 1,758,202.81**	**$ 1,744,084.38**	**$ 1,663,743.39**	**$ 2,117,208.95**	**$ 1,875,817.78**	**$ 1,208,811.76**

FISCAL YEAR 2020-2021 **INCOME AND FINANCIAL REPORT** **KTRDC 3rd QUARTER REPORT**

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235410080	HOLDING ACCOUNT	All Budget Commitmen	$ (2,360,000.00)	$ (1,064,908.01)	$ (143,903.75)	$ (1,208,811.76)	$ (1,151,188.24)
1235410080	HOLDING ACCOUNT	All Revenue Commitme	$ (2,360,000.00)	$ (1,064,908.01)	$ (143,903.75)	$ (1,208,811.76)	$ (1,151,188.24)
1235410080	HOLDING ACCOUNT	TTL Revenue Excl Tra	$ (2,360,000.00)	$ (1,064,908.01)	$ (143,903.75)	$ (1,208,811.76)	$ (1,151,188.24)
1235410080	HOLDING ACCOUNT	State/Local Grants a	$ (1,900,000.00)	$ (1,064,908.01)	$ (143,903.75)	$ (1,208,811.76)	$ (691,188.24)
1235410080	HOLDING ACCOUNT	Fund Balance	$ (460,000.00)				$ (460,000.00)
1235410090	KENTUCKY TOBACCO RES	All Budget Commitmen	$ 1,000.00				$ 1,000.00
1235410090	KENTUCKY TOBACCO RES	All Expenses Commitm	$ 1,000.00				$ 1,000.00
1235410090	KENTUCKY TOBACCO RES	TTL Expense Excl Tra	$ 1,000.00				$ 1,000.00
1235410090	KENTUCKY TOBACCO RES	Operating Expense	$ 1,000.00				$ 1,000.00
1235410100	ADMINISTRATION	All Budget Commitmen	$ 260,000.00	$ 116,374.71	$ 13,494.86	$ 129,869.57	$ 89,766.09
1235410100	ADMINISTRATION	All Expenses Commitm	$ 260,000.00	$ 116,374.71	$ 13,494.86	$ 129,869.57	$ 89,766.09
1235410100	ADMINISTRATION	TTL Expense Excl Tra	$ 260,000.00	$ 116,374.71	$ 13,494.86	$ 129,869.57	$ 89,766.09
1235410100	ADMINISTRATION	Faculty		$ 53,500.24	$ 6,687.53	$ 60,187.77	$ (80,250.39)
1235410100	ADMINISTRATION	Staff		$ 30,453.26	$ 3,494.87	$ 33,948.13	$ (45,830.72)
1235410100	ADMINISTRATION	Other Personnel	$ -	$ 76.49		$ 76.49	$ (76.49)
1235410100	ADMINISTRATION	Fringe Benefits		$ 20,742.72	$ 2,649.03	$ 23,391.75	$ (31,754.50)
1235410100	ADMINISTRATION	Operating Expense	$ 260,000.00	$ 11,602.00	$ 663.43	$ 12,265.43	$ 247,678.19
1235410110	KTRDC PERSONNEL	All Budget Commitmen	$ 1,046,700.00	$ 640,803.49	$ 98,223.84	$ 739,027.33	$ 74,757.79
1235410110	KTRDC PERSONNEL	All Expenses Commitm	$ 1,046,700.00	$ 640,803.49	$ 98,223.84	$ 739,027.33	$ 74,757.79
1235410110	KTRDC PERSONNEL	TTL Expense Excl Tra	$ 1,046,700.00	$ 640,803.49	$ 98,223.84	$ 739,027.33	$ 74,757.79
1235410110	KTRDC PERSONNEL	Staff		$ 408,928.59	$ 53,074.86	$ 462,003.45	$ (641,287.83)
1235410110	KTRDC PERSONNEL	Other Personnel		$ 92,521.67	$ 19,961.16	$ 112,482.83	$ (112,482.83)
1235410110	KTRDC PERSONNEL	Fringe Benefits		$ 130,044.19	$ 18,031.09	$ 148,075.28	$ (201,705.78)
1235410110	KTRDC PERSONNEL	Operating Expense	$ 1,046,700.00	$ 9,309.04	$ 7,156.73	$ 16,465.77	$ 1,030,234.23

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235410120	PUBLICATIONS & TRAVE	All Expenses Commitm	$ 35,000.00	$ 10,420.23	$ 56.58	$ 10,476.81	$ 24,523.19
1235410120	PUBLICATIONS & TRAVE	TTL Expense Excl Tra	$ 35,000.00	$ 10,420.23	$ 56.58	$ 10,476.81	$ 24,523.19
1235410120	PUBLICATIONS & TRAVE	Operating Expense	$ 35,000.00	$ 10,420.23	$ 56.58	$ 10,476.81	$ 24,523.19
1235410130	BUILDING MAINTENANCE	All Budget Commitmen	$ 75,000.00	$ 23,343.81	$ 9,251.49	$ 32,595.30	$ 42,404.70
1235410130	BUILDING MAINTENANCE	All Expenses Commitm	$ 75,000.00	$ 23,343.81	$ 9,251.49	$ 32,595.30	$ 42,404.70
1235410130	BUILDING MAINTENANCE	TTL Expense Excl Tra	$ 75,000.00	$ 23,343.81	$ 9,251.49	$ 32,595.30	$ 42,404.70
1235410130	BUILDING MAINTENANCE	Operating Expense	$ 75,000.00	$ 23,343.81	$ 9,251.49	$ 32,595.30	$ 42,404.70
1235410180	SHOP	All Budget Commitmen	$ 2,000.00	$ 462.84		$ 462.84	$ 1,537.16
1235410180	SHOP	All Expenses Commitm	$ 2,000.00	$ 462.84		$ 462.84	$ 1,537.16
1235410180	SHOP	TTL Expense Excl Tra	$ 2,000.00	$ 462.84		$ 462.84	$ 1,537.16
1235410180	SHOP	Operating Expense	$ 2,000.00	$ 462.84		$ 462.84	$ 1,537.16
1235410240	LABORATORY EQUIPMENT	All Budget Commitmen	$ 240,300.00	$ 584,754.82	$ 44,174.98	$ 628,929.80	$ (426,491.23)
1235410240	LABORATORY EQUIPMENT	All Expenses Commitm	$ 240,300.00	$ 584,754.82	$ 44,174.98	$ 628,929.80	$ (426,491.23)
1235410240	LABORATORY EQUIPMENT	TTL Expense Excl Tra	$ 240,300.00	$ 584,754.82	$ 44,174.98	$ 628,929.80	$ (426,491.23)
1235410240	LABORATORY EQUIPMENT	Operating Expense	$ 240,300.00	$ 170,781.53	$ 986.58	$ 171,768.11	$ 61,918.57
1235410240	LABORATORY EQUIPMENT	Capital Outlay		$ 413,973.29	$ 43,188.40	$ 457,161.69	$ (488,409.80)
1235410250	UNALLOCATED RESERVE	All Budget Commitmen	$ 71,028.00				$ 71,028.00
1235410250	UNALLOCATED RESERVE	All Expenses Commitm	$ 71,028.00				$ 71,028.00
1235410250	UNALLOCATED RESERVE	TTL Expense Excl Tra	$ 71,028.00				$ 71,028.00
1235410250	UNALLOCATED RESERVE	Operating Expense	$ 71,028.00				$ 71,028.00
1235410280	GENERAL LABORATORY	All Budget Commitmen	$ 125,000.00	$ 31,172.96	$ 4,415.35	$ 35,588.31	$ 89,377.45
1235410280	GENERAL LABORATORY	All Expenses Commitm	$ 125,000.00	$ 31,172.96	$ 4,415.35	$ 35,588.31	$ 89,377.45
1235410280	GENERAL LABORATORY	TTL Expense Excl Tra	$ 125,000.00	$ 31,172.96	$ 4,415.35	$ 35,588.31	$ 89,377.45
1235410280	GENERAL LABORATORY	Operating Expense	$ 125,000.00	$ 31,172.96	$ 4,415.35	$ 35,588.31	$ 89,377.45

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235411040	DISCRETIONARY	All Budget Commitmen	$ 10,000.00	$ 1,504.85	$ 47.73	$ 1,552.58	$ 8,447.42
1235411040	DISCRETIONARY	All Expenses Commitm	$ 10,000.00	$ 1,504.85	$ 47.73	$ 1,552.58	$ 8,447.42
1235411040	DISCRETIONARY	TTL Expense Excl Tra	$ 10,000.00	$ 1,504.85	$ 47.73	$ 1,552.58	$ 8,447.42
1235411040	DISCRETIONARY	Operating Expense	$ 10,000.00	$ 1,504.85	$ 47.73	$ 1,552.58	$ 8,447.42
1235411310	OUTREACH & COMMUNICA	All Budget Commitmen	$ 30,000.00	$ 16,973.33	$ 366.95	$ 17,340.28	$ 12,659.72
1235411310	OUTREACH & COMMUNICA	All Expenses Commitm	$ 30,000.00	$ 16,973.33	$ 366.95	$ 17,340.28	$ 12,659.72
1235411310	OUTREACH & COMMUNICA	TTL Expense Excl Tra	$ 30,000.00	$ 16,973.33	$ 366.95	$ 17,340.28	$ 12,659.72
1235411310	OUTREACH & COMMUNICA	Operating Expense	$ 30,000.00	$ 16,973.33	$ 366.95	$ 17,340.28	$ 12,659.72
1235411320	PLANT GENETIC ENGR	All Budget Commitmen	$ 30,000.00	$ 31,925.97	$ 119.88	$ 32,045.85	$ (2,045.85)
1235411320	PLANT GENETIC ENGR	All Expenses Commitm	$ 30,000.00	$ 31,925.97	$ 119.88	$ 32,045.85	$ (2,045.85)
1235411320	PLANT GENETIC ENGR	TTL Expense Excl Tra	$ 30,000.00	$ 31,925.97	$ 119.88	$ 32,045.85	$ (2,045.85)
1235411320	PLANT GENETIC ENGR	Staff		$ 17,080.83		$ 17,080.83	$ (17,080.83)
1235411320	PLANT GENETIC ENGR	Other Personnel		$ 3,421.13		$ 3,421.13	$ (3,421.13)
1235411320	PLANT GENETIC ENGR	Fringe Benefits		$ 9,039.29		$ 9,039.29	$ (9,039.29)
1235411320	PLANT GENETIC ENGR	Operating Expense	$ 30,000.00	$ 2,384.72	$ 119.88	$ 2,504.60	$ 27,495.40
1235411340	GENETIC MANIPULATION	All Budget Commitmen	$ 30,000.00	$ 30,197.59	$ (193.84)	$ 30,003.75	$ (3.75)
1235411340	GENETIC MANIPULATION	All Expenses Commitm	$ 30,000.00	$ 30,197.59	$ (193.84)	$ 30,003.75	$ (3.75)
1235411340	GENETIC MANIPULATION	TTL Expense Excl Tra	$ 30,000.00	$ 30,197.59	$ (193.84)	$ 30,003.75	$ (3.75)
1235411340	GENETIC MANIPULATION	Staff		$ 22,207.04	$ (158.71)	$ 22,048.33	$ (22,048.33)
1235411340	GENETIC MANIPULATION	Fringe Benefits		$ 5,396.10	$ (38.57)	$ 5,357.53	$ (5,357.53)
1235411340	GENETIC MANIPULATION	Operating Expense	$ 30,000.00	$ 2,594.45	$ 3.44	$ 2,597.89	$ 27,402.11
1235411370	PLANT BIOTECH MOLECU	All Budget Commitmen	$ 30,000.00	$ 7,436.98	$ 2,670.87	$ 10,107.85	$ 19,892.15
1235411370	PLANT BIOTECH MOLECU	All Expenses Commitm	$ 30,000.00	$ 7,436.98	$ 2,670.87	$ 10,107.85	$ 19,892.15
1235411370	PLANT BIOTECH MOLECU	TTL Expense Excl Tra	$ 30,000.00	$ 7,436.98	$ 2,670.87	$ 10,107.85	$ 19,892.15
1235411370	PLANT BIOTECH MOLECU	Operating Expense	$ 30,000.00	$ 7,436.98	$ 2,670.87	$ 10,107.85	$ 19,892.15

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235411380	MOLECULAR GENETICS	All Budget Commitmen	$ 30,000.00	$ 6,384.84	$ 2,637.69	$ 9,022.53	$ 20,595.15
1235411380	MOLECULAR GENETICS	All Expenses Commitm	$ 30,000.00	$ 6,384.84	$ 2,637.69	$ 9,022.53	$ 20,595.15
1235411380	MOLECULAR GENETICS	TTL Expense Excl Tra	$ 30,000.00	$ 6,384.84	$ 2,637.69	$ 9,022.53	$ 20,595.15
1235411380	MOLECULAR GENETICS	Operating Expense	$ 30,000.00	$ 6,384.84	$ 2,637.69	$ 9,022.53	$ 20,595.15
1235411390	METABOLIC ENGR.	All Budget Commitmen	$ 30,000.00				$ 30,000.00
1235411390	METABOLIC ENGR.	All Expenses Commitm	$ 30,000.00				$ 30,000.00
1235411390	METABOLIC ENGR.	TTL Expense Excl Tra	$ 30,000.00				$ 30,000.00
1235411390	METABOLIC ENGR.	Operating Expense	$ 30,000.00				$ 30,000.00
1235411410	GREENHOUSE	All Budget Commitmen	$ 30,000.00	$ 8,551.10	$ 2,251.74	$ 10,802.84	$ 19,141.61
1235411410	GREENHOUSE	All Expenses Commitm	$ 30,000.00	$ 8,551.10	$ 2,251.74	$ 10,802.84	$ 19,141.61
1235411410	GREENHOUSE	TTL Expense Excl Tra	$ 30,000.00	$ 8,551.10	$ 2,251.74	$ 10,802.84	$ 19,141.61
1235411410	GREENHOUSE	Other Personnel		$ 726.44		$ 726.44	$ (726.44)
1235411410	GREENHOUSE	Fringe Benefits		$ 51.35		$ 51.35	$ (51.35)
1235411410	GREENHOUSE	Operating Expense	$ 30,000.00	$ 7,773.31	$ 2,251.74	$ 10,025.05	$ 19,919.40
1235411430	PLANT ANALYTIC	All Budget Commitmen	$ 30,000.00	$ 78.73	$ 70.80	$ 149.53	$ 29,850.47
1235411430	PLANT ANALYTIC	All Expenses Commitm	$ 30,000.00	$ 78.73	$ 70.80	$ 149.53	$ 29,850.47
1235411430	PLANT ANALYTIC	TTL Expense Excl Tra	$ 30,000.00	$ 78.73	$ 70.80	$ 149.53	$ 29,850.47
1235411430	PLANT ANALYTIC	Operating Expense	$ 30,000.00	$ 78.73	$ 70.80	$ 149.53	$ 29,850.47
1235411640	GENE DISCOVERY	All Budget Commitmen	$ 30,000.00	$ 17,103.16	$ 2,007.80	$ 19,110.96	$ 5,075.67
1235411640	GENE DISCOVERY	All Expenses Commitm	$ 30,000.00	$ 17,103.16	$ 2,007.80	$ 19,110.96	$ 5,075.67
1235411640	GENE DISCOVERY	TTL Expense Excl Tra	$ 30,000.00	$ 17,103.16	$ 2,007.80	$ 19,110.96	$ 5,075.67
1235411640	GENE DISCOVERY	Staff		$ 11,740.32	$ 1,467.54	$ 13,207.86	$ (17,610.48)
1235411640	GENE DISCOVERY	Fringe Benefits		$ 3,700.87	$ 462.61	$ 4,163.48	$ (5,574.23)
1235411640	GENE DISCOVERY	Operating Expense	$ 30,000.00	$ 1,661.97	$ 77.65	$ 1,739.62	$ 28,260.38

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235411750	REFERENCE CIGARETTES	All Budget Commitmen		$ 72,730.00		$ 72,730.00	$ (72,730.00)
1235411750	REFERENCE CIGARETTES	All Expenses Commitm		$ 72,730.00		$ 72,730.00	$ (72,730.00)
1235411750	REFERENCE CIGARETTES	TTL Expense Excl Tra		$ 72,730.00		$ 72,730.00	$ (72,730.00)
1235411750	REFERENCE CIGARETTES	Operating Expense		$ 72,730.00		$ 72,730.00	$ (72,730.00)
1235412240	GREENHOUSE EVALUATIO	All Budget Commitmen	$ 8,547.00	$ 5,971.18	$ 342.60	$ 6,313.78	$ 2,233.22
1235412240	GREENHOUSE EVALUATIO	All Expenses Commitm	$ 8,547.00	$ 5,971.18	$ 342.60	$ 6,313.78	$ 2,233.22
1235412240	GREENHOUSE EVALUATIO	TTL Expense Excl Tra	$ 8,547.00	$ 5,971.18	$ 342.60	$ 6,313.78	$ 2,233.22
1235412240	GREENHOUSE EVALUATIO	Other Personnel		$ 2,662.80	$ 315.00	$ 2,977.80	$ (2,977.80)
1235412240	GREENHOUSE EVALUATIO	Fringe Benefits		$ 179.22	$ 27.60	$ 206.82	$ (206.82)
1235412240	GREENHOUSE EVALUATIO	Operating Expense	$ 8,547.00	$ 3,129.16		$ 3,129.16	$ 5,417.84
1235412360	FLAVONOID - SMALLE	All Budget Commitmen	$ 30,000.00	$ 8,103.87	$ 1,535.38	$ 9,639.25	$ 15,751.22
1235412360	FLAVONOID - SMALLE	All Expenses Commitm	$ 30,000.00	$ 8,103.87	$ 1,535.38	$ 9,639.25	$ 15,751.22
1235412360	FLAVONOID - SMALLE	TTL Expense Excl Tra	$ 30,000.00	$ 8,103.87	$ 1,535.38	$ 9,639.25	$ 15,751.22
1235412360	FLAVONOID - SMALLE	Staff		$ 6,097.00	$ 1,219.40	$ 7,316.40	$ (10,974.60)
1235412360	FLAVONOID - SMALLE	Fringe Benefits		$ 1,579.90	$ 315.98	$ 1,895.88	$ (2,847.21)
1235412360	FLAVONOID - SMALLE	Operating Expense	$ 30,000.00	$ 426.97		$ 426.97	$ 29,573.03
1235412690	SMOKELESS TOBACCO	All Budget Commitmen	$ 37,069.00	$ 757.89		$ 757.89	$ 31,645.68
1235412690	SMOKELESS TOBACCO	All Expenses Commitm	$ 37,069.00	$ 757.89		$ 757.89	$ 31,645.68
1235412690	SMOKELESS TOBACCO	TTL Expense Excl Tra	$ 37,069.00	$ 757.89		$ 757.89	$ 31,645.68
1235412690	SMOKELESS TOBACCO	Operating Expense	$ 37,069.00	$ 757.89		$ 757.89	$ 31,645.68
1235412790	JI: PURIFICATION OF	All Budget Commitmen	$ 41,672.00	$ 13,649.90	$ 933.17	$ 14,583.07	$ 27,033.93
1235412790	JI: PURIFICATION OF	All Expenses Commitm	$ 41,672.00	$ 13,649.90	$ 933.17	$ 14,583.07	$ 27,033.93
1235412790	JI: PURIFICATION OF	TTL Expense Excl Tra	$ 41,672.00	$ 13,649.90	$ 933.17	$ 14,583.07	$ 27,033.93
1235412790	JI: PURIFICATION OF	Operating Expense	$ 41,672.00	$ 13,649.90	$ 933.17	$ 14,583.07	$ 27,033.93

FISCAL YEAR 2020-2021 **INCOME AND FINANCIAL REPORT** **KTRDC 3rd QUARTER REPORT**

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235412820	FISHER: FARMER BALES	All Budget Commitmen	$ 13,500.00	$ 11,231.01	$ 1,443.13	$ 12,674.14	$ 825.86
1235412820	FISHER: FARMER BALES	All Expenses Commitm	$ 13,500.00	$ 11,231.01	$ 1,443.13	$ 12,674.14	$ 825.86
1235412820	FISHER: FARMER BALES	TTL Expense Excl Tra	$ 13,500.00	$ 11,231.01	$ 1,443.13	$ 12,674.14	$ 825.86
1235412820	FISHER: FARMER BALES	Staff		$ 4,090.78	$ 1,500.19	$ 5,590.97	$ (5,590.97)
1235412820	FISHER: FARMER BALES	Other Personnel		$ 4,131.88	$ (776.44)	$ 3,355.44	$ (3,355.44)
1235412820	FISHER: FARMER BALES	Fringe Benefits		$ 2,421.16	$ 719.38	$ 3,140.54	$ (3,140.54)
1235412820	FISHER: FARMER BALES	Operating Expense	$ 13,500.00	$ 587.19		$ 587.19	$ 12,912.81
1235412840	MARTINEZ: GREENHOUSE	All Budget Commitmen	$ 5,000.00	$ 2,577.17	$ 324.54	$ 2,901.71	$ 2,098.29
1235412840	MARTINEZ: GREENHOUSE	All Expenses Commitm	$ 5,000.00	$ 2,577.17	$ 324.54	$ 2,901.71	$ 2,098.29
1235412840	MARTINEZ: GREENHOUSE	TTL Expense Excl Tra	$ 5,000.00	$ 2,577.17	$ 324.54	$ 2,901.71	$ 2,098.29
1235412840	MARTINEZ: GREENHOUSE	Operating Expense	$ 5,000.00	$ 2,577.17	$ 324.54	$ 2,901.71	$ 2,098.29
1235412940	ZAITLIN: INDUCE EARL	All Budget Commitmen	$ 20,502.00	$ 17,277.24	$ 280.24	$ 17,557.48	$ 2,101.01
1235412940	ZAITLIN: INDUCE EARL	All Expenses Commitm	$ 20,502.00	$ 17,277.24	$ 280.24	$ 17,557.48	$ 2,101.01
1235412940	ZAITLIN: INDUCE EARL	TTL Expense Excl Tra	$ 20,502.00	$ 17,277.24	$ 280.24	$ 17,557.48	$ 2,101.01
1235412940	ZAITLIN: INDUCE EARL	Staff		$ 3,201.60	$ 213.44	$ 3,415.04	$ (4,055.36)
1235412940	ZAITLIN: INDUCE EARL	Fringe Benefits		$ 1,001.98	$ 66.80	$ 1,068.78	$ (1,271.97)
1235412940	ZAITLIN: INDUCE EARL	Operating Expense	$ 20,502.00	$ 13,073.66		$ 13,073.66	$ 7,428.34
1235412950	BAILEY: BURNDOWN	All Budget Commitmen	$ 6,000.00				$ 5,003.05
1235412950	BAILEY: BURNDOWN	All Expenses Commitm	$ 6,000.00				$ 5,003.05
1235412950	BAILEY: BURNDOWN	TTL Expense Excl Tra	$ 6,000.00				$ 5,003.05
1235412950	BAILEY: BURNDOWN	Operating Expense	$ 6,000.00				$ 6,000.00
1235412950	BAILEY: BURNDOWN	Capital Outlay					$ (996.95)

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Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235413180	KROU: ABS CEMB	All Budget Commitmen	$ 20,000.00	$ 1,500.60	$ 3,001.20	$ 4,501.80	$ 15,498.20
1235413180	KROU: ABS CEMB	All Expenses Commitm	$ 20,000.00	$ 1,500.60	$ 3,001.20	$ 4,501.80	$ 15,498.20
1235413180	KROU: ABS CEMB	TTL Expense Excl Tra	$ 20,000.00	$ 1,500.60	$ 3,001.20	$ 4,501.80	$ 15,498.20
1235413180	KROU: ABS CEMB	Other Personnel		$ 1,500.60	$ 3,001.20	$ 4,501.80	$ (4,501.80)
1235413180	KROU: ABS CEMB	Operating Expense	$ 20,000.00				$ 20,000.00
1235413200	PERRY: PROD MAL	All Budget Commitmen	$ 22,173.00	$ 10,736.15	$ 3,910.99	$ 14,647.14	$ 7,525.86
1235413200	PERRY: PROD MAL	All Expenses Commitm	$ 22,173.00	$ 10,736.15	$ 3,910.99	$ 14,647.14	$ 7,525.86
1235413200	PERRY: PROD MAL	TTL Expense Excl Tra	$ 22,173.00	$ 10,736.15	$ 3,910.99	$ 14,647.14	$ 7,525.86
1235413200	PERRY: PROD MAL	Other Personnel		$ 9,363.39	$ 2,472.83	$ 11,836.22	$ (11,836.22)
1235413200	PERRY: PROD MAL	Fringe Benefits		$ 361.15	$ 27.45	$ 388.60	$ (388.60)
1235413200	PERRY: PROD MAL	Operating Expense	$ 22,173.00	$ 1,011.61	$ 1,410.71	$ 2,422.32	$ 19,750.68
1235413210	PATRA: ROOT TO LEAF	All Budget Commitmen	$ 19,509.00	$ 5,139.15		$ 5,139.15	$ 14,369.85
1235413210	PATRA: ROOT TO LEAF	All Expenses Commitm	$ 19,509.00	$ 5,139.15		$ 5,139.15	$ 14,369.85
1235413210	PATRA: ROOT TO LEAF	TTL Expense Excl Tra	$ 19,509.00	$ 5,139.15		$ 5,139.15	$ 14,369.85
1235413210	PATRA: ROOT TO LEAF	Staff		$ 3,878.96		$ 3,878.96	$ (3,878.96)
1235413210	PATRA: ROOT TO LEAF	Fringe Benefits		$ 1,227.90		$ 1,227.90	$ (1,227.90)
1235413210	PATRA: ROOT TO LEAF	Operating Expense	$ 19,509.00	$ 32.29		$ 32.29	$ 19,476.71