**Title**: Nitrogen fertilizer, arbuscular mycorrhizal fungi, and soil nematodes affect lignin quality and quantity in switchgrass (*Panicum virgatum* L.)

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**Abstract**

Plant lignin content and composition, which limit cell wall digestibility and efficiency of cellulose conversion to bioethanol, can be influenced by belowground biotic and abiotic factors. Switchgrass (*Panicum virgatum L.*) is a leading lignocellulosic biofuel crop and forms strong belowground associations with arbuscular mycorrhizal fungi (AMF), is susceptible to belowground plant-parasitic nematodes (PPN), and when grown in monoculture generally requires nitrogen (N) fertilization. The main objectives of the study were to investigate the effects of N fertilizer and belowground organisms on lignin content and composition of switchgrass. Leaf, stem, and root tissues were evaluated separately to test whether these factors had varying belowground (local) or aboveground (systemic) effects on plants. These factors were manipulated in a field study in 2017 using biocide applications to reduce soil fungi and nematodes. Combined biocide application reduced p-hydroxyphenyl (H) unit abundance in the leaves by 14% and increased the syringyl:guaiacyl (S:G) ratio in stems by 2%. Application of fungicide alone increased stem syringyl (S) unit by 12.4% as compared with control plots, and 11.1% as compared to nematicide plots. Overall, fertilizer increased total stem lignin by 3%, stem S unit by 6.7% and stem S:G ratio by 10%, whereas it reduced the amount of H-unit in the roots by 11%. While the effects of N fertilizer were more pronounced in this study, changes to soil organisms had similar magnitudes of effect for some measures of lignin, indicating that these belowground interactions may be important for growers to consider in the future.

**Key Words:** Lignin, Lignocellulosic Biofuel, Switchgrass, Arbuscular Mycorrhizal Fungi, Soil Nematodes, Nitrogen

## Introduction

Lignocellulosic biofuels such as switchgrass (*Panicum virgatum*) are gaining traction as sustainable and alternative energy sources as the economic and environmental costs of conventional fossil fuels and grain-based biofuels have risen [1]. However, an important challenge to the use of bioenergy crops as fuels is efficiently converting cellulose from plant biomass into bioethanol. Acid pretreatment and enzymatic digestion, the two main steps of the conversion, are limited by total lignin content in plant cell walls [2]. The monomeric composition of lignin in plants can also affect the efficiency of cellulose conversion to bioethanol [3]. Biomass with higher syringyl : guaiacyl (S:G) ratio is desirable from a bioenergy standpoint because S-rich lignin has predominantly linear chains with less crosslinking than G-rich lignin and as a result lower recalcitrance to enzymatic degradation [4]. This importance of S:G ratio in lignin composition for efficient energy conversion has been clearly demonstrated in wood pulp production research [5]. Grasses differ from woody dicots by having a significant proportion (4-15%) of p-hydroxyphenyl (H) lignin, and very little is known about what regulates the H unit in plants [6]. For bioenergy purposes, any significant alteration in the proportion of all these lignin units can have important implications for enzymatic digestion.

Environmental factors and growing conditions are known to affect plant lignin content and monomeric composition. Soil nitrogen (N), an important abiotic factor in both natural and agricultural systems, also increases plant lignin content [7], although the mechanism by which fertilization affects lignin composition is unclear. In one study conducted in a *Populus tremuloides* Michx. system, high N fertilizer increased the H unit, but decreased the S:G ratio [8]. However, the relationship between N fertilizer and lignin may not be straightforward, as the effects of N on lignin varies across plant tissues and with age [9-11]. For switchgrass grown as bioenergy feedstock, N fertilizers can significantly increase plant biomass [12]. However, this may have unintended effects on the digestibility of biomass via effects on lignin content and composition [12].

Interactions between plants and belowground organisms have also been shown to affect plant lignin content and composition. For example, 6% to 21% of the variation in lignin in *Hordeum vulgare* L. cultivars was attributed to belowground biotic interactions [13]. Pathogens and herbivores such as plant-parasitic nematodes (PPN) generally trigger an increase in lignin plant content as a defense against damage and infection [14]. Some beneficial symbiotic interactions can also increase plant lignin. For example, arbuscular mycorrhizal fungi (AMF) are ubiquitous in soils and have been shown to increase lignin deposition in *Capsicum annuum* L. xylem vessels [15]. However, AMF have also been found to have ameliorative effects under stressful environmental conditions, thus reducing plant lignin content [16].

Lignin content also varies across organs within a plant. Some plant-soil interactions may have only localized effects on plants, increasing lignin in roots but not in aboveground tissues. For example, in pine seedlings, high N fertilizer reduced root lignin content without affecting shoot lignin [10]. Such differential accumulation of lignin in roots and shoots can indicate whether plant responses are local or systemic. This is important because most of the cellulose and hemicellulose used in bioethanol conversion occurs in the stem parenchyma tissues of grasses such as switchgrass. Thus, it is desirable to have low lignin levels in the stem of a bioenergy feedstock [17].

The goal of this study was to examine the effects of belowground abiotic and biotic factors on lignin content and composition in above- and belowground plant tissues of switchgrass. Switchgrass forms strong associations with AMF [18] and is susceptible to several belowground plant-parasitic nematodes such as *Pratylenchus penetrans*, *Helicotylenchus pseudorobustus*, and *Hoplolaimus galeatus* when grown in monocultures [19]. Additionally, N fertilization is recommended for growers of switchgrass and other lignocellulosic bioenergy crops [20] and so it is critical to understand how both biotic and abiotic factors belowground may influence lignin in bioenergy crops. However, to date, no studies have documented whole-plant lignin responses to soil factors in switchgrass. The primary objective of this study was to

investigate the effects of N fertilizer application and plant interactions with AMF and nematodes on lignin content and monomeric composition. A secondary goal was to evaluate whether these factors had varying belowground (local) or aboveground (systemic) effects on plants by analyzing lignin composition of leaves, stem, and roots separately. Decline in lignin content in roots with reduced AMF and nematode abundance is predicted. Similarly, increased lignin at the whole-plant level in response to N fertilizer addition is expected because N quickly becomes part of the soil cation exchange complex and is readily assimilated by plants [21]. A higher S:G ratio and lower p-hydroxyphenyl levels in response to N fertilizer [22], but lower S:G ratios corresponding to higher rigidity with AMF colonization and nematode herbivory is also expected to be observed.

## Materials and Methods

### Study Site

The experiment was conducted at the W.K. Kellogg Biological Station Long-Term Ecological Research (KBS LTER) site of Michigan State University in Michigan, USA (42°23047″N, 85°22026″W) as part of the Cellulosic Biofuels Diversity Experiment. Average precipitation at this site is 810 mm $yr^{-1}$ and soils are Kalamazoo series fine loamy, mixed, mesic Typic Hapludalfs [23]. The Cellulosic Biofuels Diversity Experiment was established in 2008 to compare different biofuel cropping systems, including four switchgrass monoculture systems: two cultivars of switchgrass ("Cave-in-Rock" and "Southlow"; each planted at a rate of 3.9 kg $ha^{-1}$ pure live seed) grown at two levels of fertilization (56 kg N $ha^{-1}$ and unfertilized). The four switchgrass treatment combinations were planted in randomly assigned 9 m × 27m plots, replicated four times in randomized blocks. Fertilizer treatments (28%N, Urea + Ammonium nitrate, at a rate of 56 kg N $ha^{-1}$) were applied to the relevant switchgrass treatment plots every year in the spring. No additional nutrients were added. For the control of broadleaf weeds, the herbicide *Drive* (quinclorac-$C_{10}H_5Cl_2NO_2$; BASF Corp, NC) was applied at the rate of 0.56 kg commercial product per ha following planting in 2009-2010. Harvesting of the switchgrass plots was done annually at the end of

each growing season (Oct/Nov). Additional details about the experiment, including yield data, are available elsewhere [24].

**Biocide Experiment**

To manipulate switchgrass associations with AMF and nematodes, a biocide experiment within each of the 16 switchgrass plots of the LTER Cellulosic Biofuel Diversity Experiment site at KBS was set up in June of 2017. In each 9 m × 27 m plot, four 2 m × 2 m microplots along the west side of the plot were set up, with each microplot receiving one of four treatments: 1) control, 2) fungicide, 3) nematicide, and 4) fungicide + nematicide. Buffer strips (0.5m wide) were established between the microplots and the four biocide treatments were randomly assigned to the microplots in each plot. A commercial fungicide Topsin (Thiophanate-Methyl Fungicide, $C_{14}H_{18}N_4O_4S_2$, Nippon Soda Company, Ltd) was used to reduce soil fungi, particularly AMF. Topsin is widely used in field studies of mycorrhizal fungi [25]. The fungicide was applied by hand in 3.7-7.5 L of water as a soil drench every 2 weeks throughout the growing season (June to August) of 2017 at the rate of 4.4 g $m^{-2}$. A commercial nematicide, Nimitz (Fluensulfone, $C_7H_5ClF_3NO_2S_2$, ADAMA, USA) was used to reduce soil nematodes. Nimitz is a low toxicity and narrow spectrum nematicide and previous studies have shown that it is effective in the control of both migratory and sedentary nematodes [26]. Nimitz was applied to plots by hand twice during the growing season at a rate of 0.3 g $m^{-2}$ (June) and 0.5 g $m^{-2}$ (July) active ingredient, mixed in 100 mL of silica sand. The applications of both biocides were made prior to rainfall events. Control plots received equal amounts of silica sand and/or water without biocides.

**Soil Organisms**

To evaluate the effectiveness of the biocides on AMF and nematodes, soil and root samples were collected in October 2017. Ten soil cores (2 cm x 15 cm) were collected in each microplot and composited for analyses. Additionally, 4-5 individual tillers per microplot were dug up to collect roots for AMF analyses. To characterize AMF activity, soils were processed for extra-radical hyphae (ERH) by

vacuum-filtering 20 g subsamples through 45 μm filters and mounting filters on slides following methods described by Sun et al. [27]. Hyphal length was estimated using the grid intersection method [27]. Root colonization by AMF was determined using the wet sieve method [28]. Briefly, ten 3-cm fine root subsamples were clipped and cleared in 10% potassium hydroxide solution and stained using the ink-vinegar method [29] and scored as a percentage of fields of view at 200X magnification based on the presence of fungal hyphae, arbuscules or vesicles. Soil nematode abundance was measured by sieving and centrifuging 100 g soil subsamples from each microplot following protocols described by Hallmann and Subbotin, 2018 [30]. The extracted nematodes were counted and preserved in 3% formalin solution for storage.

**Plant responses:**

Samples for lignin analyses were collected from 5 whole tillers and associated roots in each microplot at two times in 2017: mid-growing season (August) and just prior to plot harvest (October). Tillers (separated into leaf and stem tissues) and roots were dried at 65°C for 24 h. After drying, leaf, stem, and root samples from each microplot were combined and were ground using a Thomas Wiley Mill (Thomas Scientific, NJ) with a 40 μm mesh screen. Subsamples (100 mg) of the ground tissue were sent to the Great Lake Bioenergy Research Center (GLBRC) Cell Wall Facility at Michigan State University for analysis of lignin monomeric composition using the thioacidolysis method [31]. Additionally, acid detergent lignin (ADL) was determined using an ANKOM$^{200}$ fiber analyzer (Macedon, NY) to assess total lignin content.

**Statistical analysis**

Data analysis was done using two-factor linear mixed effect models with biocide treatment (4 levels) and N fertilizer (2 levels) as the main factors and switchgrass cultivar and sampling date as covariates. Experimental block was also included in the models as a random effect. A significance level of 5% was used to make statistical inference. Tukey HSD was used to perform post-hoc analyses when overall

models were significant. All statistical analysis was performed using the *lme4* and *lmerTest* packages in R version 3.6.1 [32]. While switchgrass cultivar and sampling dates were often significant in the models (Tables 1-3), they were not specifically relevant to the study's outlined research objectives and thus are not discussed here.

**Results and Discussion**

At harvest time, fungicide application had reduced soil ERH by 43% and AMF root colonization by 32% compared with the control. Nematicide application reduced total nematode abundance by 47% compared to the control. When applied in combination, the biocides reduced nematodes by 42%, soil ERH by 51%, and root AMF colonization by 38%. Nitrogen fertilization and the reduction in soil organisms affected different aspects of switchgrass lignin content and composition both above- and belowground.

In stem and root tissue, several components of lignin differed in response to N fertilization whereas there was no significant effect of N fertilization on leaf lignin (Fig.1-2, Table 1-3). Total stem lignin increased by 3% in N fertilized plots (Fig. 2a). The amount of stem S unit increased by 6.7% in plants from N fertilized plots (Fig. 2b). This consequently resulted in an increase in stem S:G ratio of about 10% (Fig. 2c). The increase in stem lignin is likely due to the increased availability of phenolic amino acid precursors such as phenylalanine and tyrosine, which are rate-limiting resources in lignin metabolic pathway at cellular level [33]. Thus, added N fertilization might potentially enhance lignification by removing the limitation of such rate-limiting resources. The increase in stem lignin content in this study was mostly due to increases in the S unit, which may improve lignin conversion efficiency because S-rich lignin or lignin with higher S:G ratio is structurally more linear and chemically more susceptible to enzymatic digestion [4, 34].

A decrease in the H unit was the only significant root lignin response to N fertilizer in this study. The root H unit decreased by 11.1% (Fig. 2d) in plants in fertilized plots compared with non-fertilized plots. H-rich

lignin has a lower degree of polymerization and forms shorter chains which results in increased extractability and digestibility [35]. Grasses like switchgrass contain significant amounts of H unit (~15%) [36] and changes in its proportion can have important implication for enzymatic digestion of the biomass. The lack of response in leaves and roots to N fertilization (except for H unit) may be due to the inhibitory effects of soil nitrates on the phenylpropanoid pathway-one of the major steps in lignin biosynthesis [37]. While nitrate is the form of nitrogen that plants prefer for uptake, it is known to inhibit large sectors of lignin biosynthetic pathway [37].

High lignin content in aboveground tissues is not generally desirable from a grower's perspective [2], and because N fertilization is generally recommended in switchgrass production for higher yield [38], increase in stem lignin content could be an unintended negative consequence of standard switchgrass management practices. However, increase in stem S unit abundance and S:G ratio upon N fertilization might potentially offset biomass recalcitrance caused by increase in lignin content to some degree. In general, any belowground effects of N fertilization are unlikely to have significant economic consequences for growers because only aboveground structures are harvested for bioenergy conversion, although, this can be an important consequence in bioenergy crops where roots are harvested such as *Manihot esculenta* [39]. However, changes in root lignin may affect plant susceptibility to belowground pathogens, which in turn could affect the sustainability of these perennial systems [40]. The results of this study generally support the findings of other studies that have shown that N fertilization increases plant lignin content [41] but more work is needed on the economics of increasing total lignin abundance versus changes in composition due to N fertilization.

Despite the known importance of belowground community interactions on switchgrass production [42], no effects on the total amount of lignin in switchgrass in response to AMF and nematode reductions either above- or belowground was observed in this study. Manipulation of AMF and nematodes via biocide application had significant effects on both leaf and stem lignin monomeric composition, but not on root

lignin composition (Tables 1-3). Reducing AMF and soil nematodes together decreased the abundance of the H unit in leaf lignin by 14% and increased the stem S:G ratio by 2% compared with the control (Fig. 1, 2c). Reducing AMF alone increased the amount of S unit in stem lignin by 12.4% as compared with control plots and 11.1% compared with nematicide plots (Fig. 2b). Several previous studies in other plant systems have demonstrated an increase in lignification in response to symbiotic organisms such as AMF and nematodes [13]. For example, AMF penetrate the root cortex of the host plant and in doing so are known to prime the salicylic acid pathway which is widely known to induce plant defensive responses such as lignin biosynthesis [43]. Arbuscular mycorrhizal fungi have also been found to prime genes involved in lignin biosynthesis as a response to attack by plant parasitic nematodes [40]. Thus, it was expected that biocides would reduce lignification, especially in roots where symbiotic organisms were directly reduced. The lack of effects of soil organism manipulation on total lignin in this study may be a result of the potential interactions between AMF and nematodes belowground. Plant parasitic nematodes are reported to enhance lignification in host plants [44] and AMF are known to provide protection against such nematodes via wide array of mechanisms [45,40]. Thus interactions among soil organisms may cancel out each other in terms of their effects on plant lignin. Previous studies report positive, neutral, and negative effects of AMF on lignin indicating a complicated context dependent role of the soil microbe on lignification [15,16,46]. For example, a study that looked at the effects of AMF on *Medicago sativa* L. lignin found that mycorrhization increases total lignin under ambient $CO_2$ conditions [16]. However, the same study found that AMF reduces plant lignin when $CO_2$ concentration increases from 392 µmol $mol^{-1}$ to 700 µmol $mol^{-1}$.

Almost no previous studies have attempted to explore the effects of soil organisms on lignin composition, but at least one previous study documented an increase in the S unit upon infection by *Puccinia graminis,* a fungal causative agent of stem rust in wheat [47]. If reducing AMF increased plant susceptibility to pathogenic fungi [48], this could explain the observed increase in S unit abundance. The mechanism for the observed effects of biocides on the H unit of leaves remains unclear, however. Grasses like

switchgrass typically have relatively high proportion of H unit (about 15%), and results of this study support the assumption that plant elicitor responses to symbionts alter the monomeric composition of lignin by depositing more H units [49]. However, given that the response in this study was observed in leaves only, other unknown mechanisms may be operating. Regardless, it is clear that AMF and nematodes do not have only localized effects on plant lignin, but instead have effects on important aboveground tissues at a systemic scale. Because enzymatic recalcitrance comes mainly from stem tissues which contain more lignin than leaves [16], soil organisms may indeed have economic consequences in terms of bioenergy conversion.

Importantly, while N fertilization effects on lignin generally were more common than effects of reduced biotic interactions, both N fertilization and reduced soil organisms had similar effects on S:G ratio in stems. Because the biotic manipulations occurred only during a single field season, while the N treatment was long-term over a 10-year period, this indicates that plants are able to respond quickly to changes in belowground conditions. It is also notable that there were no interactive effects between N fertilization and biocide applications especially given that increased N availability in grassland soil can alter nutritional and energy dynamics between plants and AMF and typically decreases the abundance of AMF in soil [50] with potential implication to lignin quality and quantity. There can be considerable variation in the effects of AMF on lignin as demonstrated by previous studies and much of the effects seem to be dictated by the surrounding abiotic factors [16]. However, evidence for this was not detected in this study.

The specific focus of this study was on manipulating AMF with fungicide applications; however, other pathogenic or non-pathogenic fungi also may have been reduced by this treatment. Because biocides are widely used by growers to control pathogens in a variety of different agronomic systems, it is important to understand the effects of such treatments on biofuel crops. The results of this study indicate that there may be complex multitrophic interactions in the soil among AMF, PPNs, and pathogenic fungi which could explain the lack of fungicide-only or nematicide only responses [15,40]. While the focus of this

study was on S/G/H units, other important lignin components such as coumaric acid, ferulic acid and sinapic acid should also be evaluated for a better understanding of how lignin biosynthetic pathways respond to N fertilization and soil organisms. Lignin synthesized in response to biotic or abiotic stress display a distinct structural signature when compared with normal developmental lignin [51]. Assessment of such structural features in the future studies can provide additional information on whether the changes in lignin quality observed in this study could have been stress responses to AMF or nematodes.

**Conclusions**

Overall, the manipulation of the biotic and abiotic soil factors had stronger effects on lignin composition than on total lignin abundance. Nitrogen fertilization was a more important regulator of lignin than the reduction of soil organisms, though for some responses such as S:G in stem tissues, these factors had similar effect sizes. Even given the relatively minor effects on total lignin, changes in lignin composition may greatly affect biomass digestibility. The economic importance of the observed effects needs to be explored in more detail, and under a wider range of conditions, especially given that bioenergy crops are likely to be grown on marginal lands where environmental stresses and biotic interactions are likely to be important controllers of production. The recent focus on lignin bioengineering as one of the major strategies to improve biomass conversion to biofuel warrants a deeper evaluation of the effects of soil organisms and crop fertilization on lignin. Ultimately, trade-offs between digestibility and plant yield involved with having altered lignin should be evaluated carefully against any potential biofuel gains.

***Table 1. Results of two-way general linear models testing for effects of biocide and N fertilization treatments on lignin content and composition in switchgrass leaf tissue. Abbreviations: N (nitrogen), SE (standard error), P (p-value)***

| Source of Variation | Total lignin (ug/ml) | | | Syringyl unit (S) (ug/ml) | | | Guaiacyl unit (G) (ug/ml) | | | p-hydroxyphenyl unit (H) (ug/ml) | | | S:G | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F | SE | P | F | SE | P | F | SE | P | F | SE | P | F | SE | P |
| N-Fertilizer | 0.95 | 0.60 | 0.33 | 2.01 | 0.15 | 0.15 | 0.62 | 0.46 | 0.43 | 0.01 | 0.01 | 0.91 | 2.68 | 0.015 | 0.10 |
| Biocides | 1.17 | 0.59 | 0.32 | 1.54 | 0.15 | 0.20 | 1.02 | 0.45 | 0.38 | 2.91 | 0.01 | 0.03 | 0.93 | 0.019 | 0.42 |
| N-Fertilizer × Biocides | 0.34 | 0.85 | 0.79 | 0.42 | 0.22 | 0.73 | 0.29 | 0.66 | 0.82 | 0.48 | 0.01 | 0.76 | 0.38 | 0.016 | 0.76 |
| Variety | 6.82 | 0.30 | 0.01 | 18.67 | 0.07 | <0.01 | 3.73 | 0.23 | 0.60 | 1.18 | 0.005 | 0.27 | 22.40 | 0.01 | <0.01 |
| Sampling date | 189.23 | 0.30 | <0.01 | 143.68 | 0.07 | <0.01 | 1.788.5 | 0.23 | <0.01 | 172.85 | 0.005 | <0.01 | 31.32 | 0.01 | <0.01 |

***Table 2. Results of two-way general linear models testing for effects of biocide and N fertilization treatments on lignin content and composition in switchgrass stem tissue. Abbreviations: N (nitrogen), SE (standard error), P (p-value)***

| Source of Variation | Total lignin (ug/ml) | | | Syringyl unit (S) (ug/ml) | | | Guaiacyl unit (G) (ug/ml) | | | p-hydroxyphenyl unit (H) (ug/ml) | | | S:G | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F | SE | P | F | SE | P | F | SE | P | F | SE | P | F | SE | P |
| N-Fertilizer | 4.24 | 1.48 | 0.04 | 7.34 | 0.85 | <0.01 | 0.64 | 1.07 | 0.42 | 1.78 | 0.06 | 0.18 | 4.79 | 0.03 | 0.03 |
| Biocides | 1.46 | 1.45 | 0.22 | 4.27 | 0.84 | <0.01 | 0.79 | 1.05 | 0.49 | 0.35 | 0.06 | 0.78 | 3.80 | 0.02 | 0.01 |
| N-Fertilizer × Biocides | 0.21 | 2.07 | 0.88 | 1.36 | 1.26 | 0.25 | 0.75 | 1.50 | 0.52 | 1.77 | 0.09 | 0.15 | 2.30 | 0.03 | 0.80 |
| Variety | 0.22 | 0.72 | 0.63 | 6.14 | 0.42 | 0.01 | 5.84 | 0.52 | 0.01 | 13.40 | 0.01 | <0.01 | 14.42 | 0.01 | <0.01 |
| Sampling date | 32.40 | 0.72 | <0.01 | 12.55 | 0.42 | <0.01 | 24.27 | 0.52 | <0.01 | 2.07 | 0.01 | 0.15 | 0.27 | 0.01 | 0.59 |

346 ***Table 3. Results of two-way general linear models testing for effects of biocide and N fertilization***

347 ***treatments on lignin content and composition in switchgrass root tissue. Abbreviations: N (nitrogen),***

348 ***SE (standard error), P (p-value)***

| Source of Variation | Total lignin (ug/ml) | | | Syringyl unit (S) (ug/ml) | | | Guaiacyl unit (G) (ug/ml) | | | p-hydroxy phenyl unit (H) (ug/ml) | | | S:G | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F | SE | P | F | SE | P | F | SE | P | F | SE | P | F | SE | P |
| N-Fertilizer | 1.74 | 2.76 | 0.18 | 0.80 | 1.01 | 0.37 | 1.86 | 1.88 | 0.17 | 8.36 | 0.04 | <0.01 | 0.009 | 0.02 | 0.95 |
| Biocides | 0.83 | 2.11 | 0.47 | 0.45 | 0.78 | 0.71 | 1.25 | 1.44 | 0.29 | 0.44 | 0.04 | 0.71 | 1.33 | 0.02 | 0.26 |
| N-Fertilizer × Biocides | 1.51 | 2.99 | 0.21 | 1.02 | 1.10 | 0.38 | 1.61 | 2.04 | 0.19 | 1.93 | 0.05 | 0.12 | 0.27 | 0.03 | 0.84 |
| Variety | 0.02 | 1.05 | 0.87 | 0,01 | 0.39 | 0.91 | 0.08 | 0.72 | 0.77 | 0.03 | 0.02 | 0.84 | 0.02 | 0.01 | 0.88 |
| Sampling date | 74.02 | 1.05 | <0.01 | 110.27 | 0.39 | <0.01 | 46.33 | 0.72 | <0.01 | 20.50 | 0.02 | <0.01 | 81.01 | 0.01 | <0.01 |

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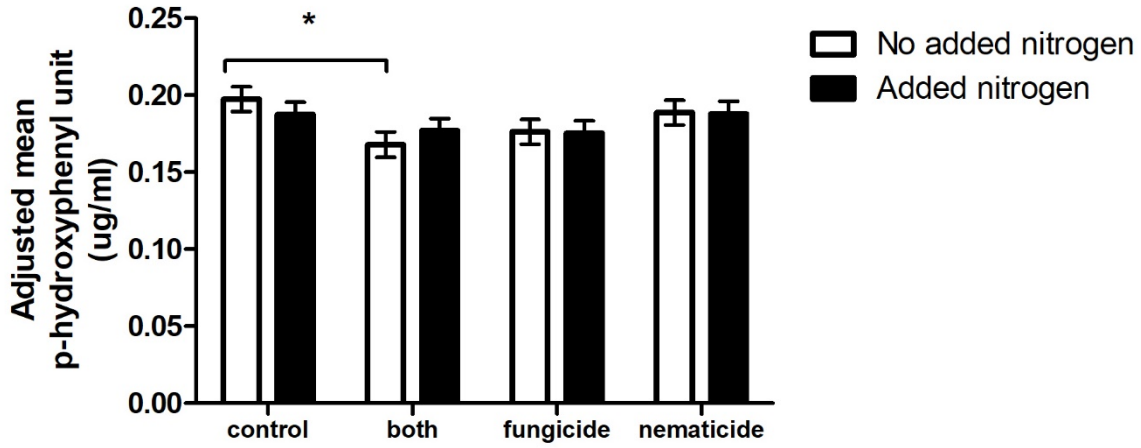
**Figure Legends**

Figure 1: Adjusted mean of leaf p-hydroxyphenyl (H) unit response to biocide and N fertilizer application. Statistically different average values found between biocide treatments with Tukey's HSD ($p < 0.05$) test are indicated by a horizontal bar with an asterisk above. Vertical bars show the standard error of the adjusted mean value. Open bars are unfertilized treatments, black bars are N fertilized treatments. 'Both' denotes combined fungicide and nematicide treatment. Main biocide effects were significant at p=0.03.

Figure 2: Adjusted means of stem and root lignin responses: (a) total lignin, (b) S unit (c) S:G (d) H unit to biocide and N fertilizer application. Statistically different average values between biocide treatments found with Tukey's HSD ($p < 0.05$) tests are indicated by horizontal bars with asterisks above. Vertical bars show the standard errors of the adjusted mean values. Open bars are unfertilized treatments, black bars are N fertilized treatments. 'Both' denotes combined fungicide and nematicide treatment.

392 Figure 1

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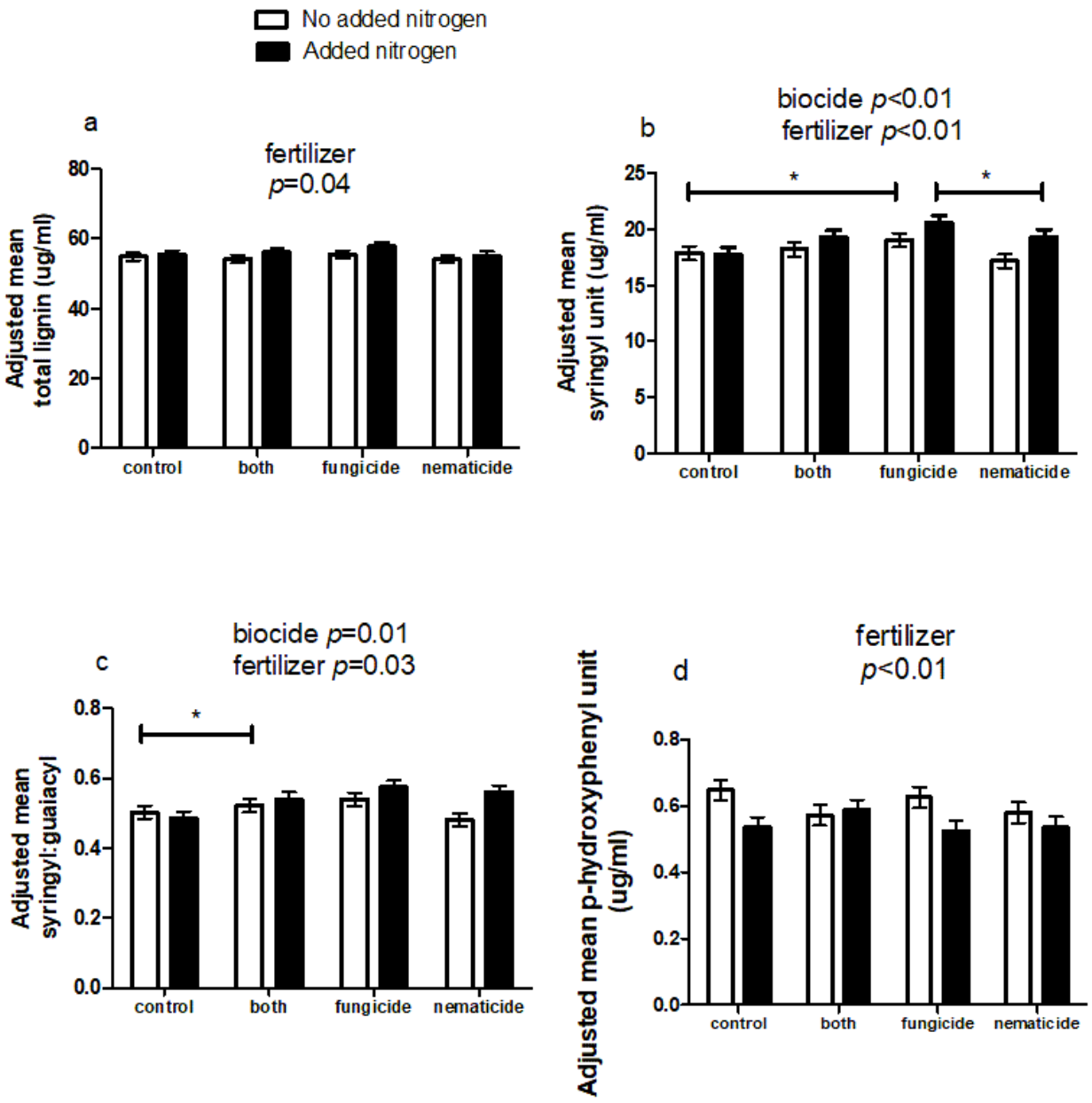
411 Figure 2

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## Declarations

### Conflict of Interest

The authors declare that they have no conflict of interest.

### Ethical Approval

Not applicable.

### Consent to Participate

All the authors consent to participate.

### Consent to Publication

All the authors consent to publication.

### Data Availability

Data are available on request from authors.

## References


1. Scarlat N, Dallemand J-F (2011) Recent developments of biofuels/bioenergy sustainability certification: A global overview. Energy policy 39 (3):1630-1646. https://doi.org/10.1016/j.enpol.2010.12.039

2. Chen F, Dixon RA (2007) Lignin modification improves fermentable sugar yields for biofuel production. Nat Biotechnol 25 (7):759. https://doi.org/10.1038/nbt1316

3. Li X, Ximenes E, Kim Y, Slininger M, Meilan R, Ladisch M, Chapple C (2010) Lignin monomer composition affects *Arabidopsis* cell-wall degradability after liquid hot water pretreatment. Biotechnol Biofuels 3 (1):1-7. https://doi.org/10.1186/1754-6834-3-27

4. Studer MH, DeMartini JD, Davis MF, Sykes RW, Davison B, Keller M, Tuskan GA, Wyman CE (2011) Lignin content in natural Populus variants affects sugar release. Proc Natl Acad Sci 108 (15):6300-6305. https://doi.org/10.1073/pnas.1009252108

5. Huntley SK, Ellis D, Gilbert M, Chapple C, Mansfield SD (2003) Significant increases in pulping efficiency in C4H-F5H-transformed poplars: improved chemical savings and reduced environmental toxins. J Agric Food Chem 51 (21):6178-6183. https://doi.org/10.1021/jf034320o

6. Barrière Y, Ralph J, Méchin V, Guillaumie S, Grabber JH, Argillier O, Chabbert B, Lapierre C (2004) Genetic and molecular basis of grass cell wall biosynthesis and degradability. II. Lessons from brown-midrib mutants. Comptes Rendus Biologies 327 (9-10):847-860. https://doi.org/10.1016/j.crvi.2004.05.010

494 7. Liu J, Wu N, Wang H, Sun J, Peng B, Jiang P, Bai E (2016) Nitrogen addition affects chemical
495 compositions of plant tissues, litter and soil organic matter. Ecol 97 (7):1796-1806.
496 https://doi.org/10.1890/15-1683.1
497
498 8. Moura JCMS, Bonine CAV, de Oliveira Fernandes Viana J, Dornelas MC, Mazzafera P (2010) Abiotic
499 and biotic stresses and changes in the lignin content and composition in plants. J Integr Plant Biol 52
500 (4):360-376. https://doi.org/10.1111/j.1744-7909.2010.00892.x
501
502 9. Blodgett JT, Herms DA, Bonello P (2005) Effects of fertilization on red pine defense chemistry and
503 resistance to *Sphaeropsis sapinea*. For Ecol Manage 208 (1-3):373-382.
504 https://doi.org/10.1016/j.foreco.2005.01.014
505
506 10. Entry JA, Runion GB, Prior SA, Mitchell RJ, Rogers HH (1998) Influence of CO 2 enrichment and
507 nitrogen fertilization on tissue chemistry and carbon allocation in longleaf pine seedlings. Root Demogr
508 Effic Sustain Agric, Grassl and For Ecosyst pp 3-18. https://doi.org/10.1007/978-94-011-5270-9_1
509
510 11. Kostiainen K, Kaakinen S, Saranpää P, Sigurdsson BD, Linder S, Vapaavuori E (2004) Effect of
511 elevated $CO_2$ on stem wood properties of mature Norway spruce grown at different soil nutrient
512 availability. Glob Change Biol 10 (9):1526-1538. https://doi.org/10.1111/j.1365-2486.2004.00821.x
513
514 12. Lemus R, Brummer EC, Burras CL, Moore KJ, Barker MF, Molstad NE (2008) Effects of nitrogen
515 fertilization on biomass yield and quality in large fields of established switchgrass in southern Iowa,
516 USA. Biomass and Bioenergy 32 (12):1187-1194. https://doi.org/10.1016/j.biombioe.2008.02.016
517
518 13. Bennett AE, Grussu D, Kam J, Caul S, Halpin C (2015) Plant lignin content altered by soil microbial
519 community. New Phytol 206 (1):166-174. https://doi.org/10.1111/nph.13171

14. Bonello P, Blodgett JT (2003) *Pinus nigra–Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. Physiol Mol Plant Pathol 63 (5):249-261. https://doi.org/10.1016/j.pmpp.2004.02.002

15. Goicoechea-Preboste N, Garmendia I, Sanchez-Diaz M, Aguirreolea J (2010) Arbuscular mycorrhizal fungi (AMF) as bioprotector agents against wilt induced by *Verticillium spp*. in pepper. Span J Agric Res 8:25-42. https://doi.org/10.5424/sjar/201008s1-5300

16. Baslam M, Antolín MC, Gogorcena Y, Muñoz F, Goicoechea N (2014) Changes in alfalfa forage quality and stem carbohydrates induced by arbuscular mycorrhizal fungi and elevated atmospheric CO2. Ann Appl Biol 164 (2):190-199. https://doi.org/10.1111/aab.12092

17. Byrt CS, Grof CP, Furbank RT (2011) C4 Plants as biofuel feedstocks: optimising biomass production and feedstock quality from a lignocellulosic perspective free access. J Integr Plant Biol 53 (2):120-135. https://doi.org/10.1111/j.1744-7909.2010.01023.x

18. Emery SM, Kinnetz ER, Bell-Dereske L, Stahlheber KA, Gross KL, Pennington D. (2018). Low variation in arbuscular mycorrhizal fungal associations and effects on biomass among switchgrass cultivars. Biomass and Bioenergy, *119*, 503-508. https://doi.org/10.1016/j.biombioe.2018.10.012

19. Mekete T, Reynolds K, Lopez-Nicora HD, Gray ME, Niblack TL (2011) Plant-parasitic nematodes are potential pathogens of *Miscanthus* × *giganteus* and *Panicum virgatum* used for biofuels. Plant Dis 95 (4):413-418. https://doi.org/10.1094/PDIS-05-10-0335

20. Engbers HM (2012) Evaluation of nitrogen fertilization in C4 grasses grown for bioenergy. Dissertation, University of Guelph

21. Aquilina L, Poszwa A, Walter C, Vergnaud V, Pierson-Wickmann AC, & Ruiz L (2012). Long-term effects of high nitrogen loads on cation and carbon riverine export in agricultural catchments. Environ Sci Technol *46*(17), 9447-9455. https://doi.org/10.1021/es301715t

22. Pitre FE, Cooke JE, Mackay JJ (2007) Short-term effects of nitrogen availability on wood formation and fibre properties in hybrid poplar. Trees 21 (2):249-259. https://doi/10.1007/s00468-007-0123-5

23. Muñoz JD, Kravchenko A (2011) Soil carbon mapping using on-the-go near infrared spectroscopy, topography and aerial photographs. Geoderma 166 (1):102-110. https://doi.org/10.1016/j.geoderma.2011.07.017

24. Michigan State University (2020) M Kellogg Biological Station LTER Cellulosic Biofuels Diversity Experiment. https://lter.kbs.msu.edu/research/long-term-experiments/cellulosic-biofuels-experiment/. Accessed 16 March 2021

25. Wilson G, Williamson M (2008) Topsin-M: the new benomyl for mycorrhizal-suppression experiments. Mycol 100 (4):548-554. https://doi.org/10.3852/08-024R

26. Jones RK (2017). Nematode control and nematicides: developments since 1982 and future trends. Nematology in South Africa: A view from the 21st century. Springer, Berlin, pp 129-150. https://doi.org/10.1007/978-3-319-44210-5_6_

570 27. Sun XG, Tang M (2012). Comparison of four routinely used methods for assessing root colonization
571 by arbuscular mycorrhizal fungi. Botany *90*(11):1073-1083. https://doi.org/10.1139/b2012-084
572
573 28. Vierheilig H, Schweiger P, Brundrett M (2005) An overview of methods for the detection and
574 observation of arbuscular mycorrhizal fungi in roots. Physiol Plant 125 (4):393-404.
575 https://doi.org/10.1111/j.1399-3054.2005.00564.x
576
577 29. Vierheilig H, Coughlan AP, Wyss U, Piché Y (1998) Ink and vinegar, a simple staining technique for
578 arbuscular mycorrhizal fungi. Appl Environ Microbiol 64 (12):5004-5007.
579 https://doi.org/10.1128/AEM.64.12.5004-5007.1998
580
581 30. Hallmann J, Subbotin, SA (2018). Methods for extraction, processing and detection of plant and soil
582 nematodes. Plant Parasitic Nematodes in Subtropical and Tropical Agriculture. CABI, England.
583 https://doi.org/10.1079/9781786391247.0087
584
585 31. Harman-Ware AE, Foster C, Happs RM, Doeppke C, Meunier K, Gehan J, Yue F, Lu F, Davis MF
586 (2016) A thioacidolysis method tailored for higher-throughput quantitative analysis of lignin monomers.
587 Biotechnol J 11 (10):1268-1273. https://doi.org/10.1002/biot.201600266
588
589 32. R Core Team (2017) R: A language and environment for statistical computing. R Foundation for
590 Statistical Computing, Vienna, Austria URL https://www.r-project.org/
591
592 33. Glasser WG, Northey RA, Schultz TP (1999) Lignin: historical, biological, and materials
593 perspectives. ACS Publ 742. https/doi.org/10.1021/bk-2000-0742
594

595 34. Cesarino, Igor, Araújo, Pedro, Domingues Júnior, Adilson Pereira, & Mazzafera, Paulo. (2012). An
596 overview of lignin metabolism and its effect on biomass recalcitrance. Braz J Botany 35(4), 303-
597 311. https://dx.doi.org/10.1590/S0100-84042012000400003
598
599 35. Ziebell A, Gracom K, Katahira R, Chen F, Pu Y, Ragauskas A, Dixon RA, Davis M (2010) Increase
600 in 4-coumaryl alcohol units during lignification in alfalfa (*Medicago sativa*) alters the extractability and
601 molecular weight of lignin. J Biol Chem 285 (50):38961-38968. https://doi.org/10.1074/jbc.M110.137315
602
603 36. Vogel J (2008) Unique aspects of the grass cell wall. Curr Opin Plant Biol 11 (3):301-307.
604 https://doi.org/10.1016/j.pbi.2008.03.002
605
606 37. Fritz C, Palacios-Rojas N, Feil R, Stitt M (2006) Regulation of secondary metabolism by the carbon–
607 nitrogen status in tobacco: nitrate inhibits large sectors of phenylpropanoid metabolism. Plant J 46
608 (4):533-548. https://doi.org/10.1111/j.1365-313X.2006.02715.x
609
610 38. Sanderson MA, Adler PR, Boateng AA, Casler MD, Sarath G (2006) Switchgrass as a biofuel
611 feedstock in the USA. Can J Plant Sci 86 (Special Issue):1315-1325. https://doi.org/10.4141/P06-136
612
613 39. Jansson C, Westerbergh A, Zhang J, Hu X, Sun C (2009) Cassava, a potential biofuel crop in (the)
614 People's Republic of China. Appl Energy 86:S95-S99. https://doi.org/10.1016/j.apenergy.2009.05.011
615
616 40. Schouteden N, De Waele D, Panis B, Vos CM (2015) Arbuscular mycorrhizal fungi for the biocontrol
617 of plant-parasitic nematodes: a review of the mechanisms involved. Front Microbiol 6:1280.
618 https://doi.org/10.3389/fmicb.2015.01280
619

620 41. Emery SM, Stahlheber KA, Gross KL (2020) Drought minimized nitrogen fertilization effects on
621 bioenergy feedstock quality. Biomass and Bioenergy133:105452.
622 https://doi.org/10.1016/j.biombioe.2019.105452
623
624 42. Ghimire SR, Charlton ND, Craven KD (2009) The mycorrhizal fungus, *Sebacina vermifera*, enhances
625 seed germination and biomass production in switchgrass (*Panicum virgatum* L). BioEnergy Res 2 (1-
626 2):51-58. https://doi.org/10.1007/s12155-009-9033-2
627
628 43. Zhang R-Q, Zhu H-H, Zhao H-Q, Yao Q (2013) Arbuscular mycorrhizal fungal inoculation increases
629 phenolic synthesis in clover roots via hydrogen peroxide, salicylic acid and nitric oxide signaling
630 pathways. J Plant Physiol170 (1):74-79. https://doi.org/10.1016/j.jplph.2012.08.022
631
632 44. Huang WK, Ji HL, Gheysen G, Kyndt T (2016) Thiamine-induced priming against root-knot
633 nematode infection in rice involves lignification and hydrogen peroxide generation. Mol Plant Pathol 17
634 (4):614-624. https://doi.org/10.1111/mpp.12316
635
636 45. Basyal B (2020) Aboveground-belowground interactions: roles of soil biotic and abiotic factors on
637 switchgrass's (*panicum virgatum*) growth, defense against herbivory and cell wall chemistry. Dissertation,
638 University of Louisville
639
640 46. Basyal B, Emery SM (2020) An arbuscular mycorrhizal fungus alters switchgrass growth, root
641 architecture, and cell wall chemistry across a soil moisture gradient. Mycorrhiza 31:251–258.
642 https://doi.org/10.1007/s00572-020-00992-6
643

644 47. Menden B, Kohlhoff M, Moerschbacher BM (2007) Wheat cells accumulate a syringyl-rich lignin
645 during the hypersensitive resistance response. Phytochem 68 (4):513-520.
646 https://doi.org/10.1016/j.phytochem.2006.11.011
647
648 48. Akhtar MS, Siddiqui ZA (2008) Arbuscular mycorrhizal fungi as potential bioprotectants against
649 plant pathogens. Mycorrhizae: Sustain Agric For pp 61-97. https://doi.org/10.1007/978-1-4020-8770-7_3
650
651 49. Gallego-Giraldo L, Posé S, Pattathil S, Peralta AG, Hahn MG, Ayre BG, Dixon RA (2018). Elicitors
652 and defense gene induction in plants with altered lignin compositions. New Phytol 219(4):1235-1251.
653 https://doi.org/10.1111/nph.15258
654
655 50. Bradley K, Drijber RA, Knops J (2006) Increased N availability in grassland soils modifies their
656 microbial communities and decreases the abundance of arbuscular mycorrhizal fungi. Soil Biol Biochem
657 38 (7):1583-1595. https://doi.org/10.1016/j.soilbio.2005.11.011
658
659 51. Cesarino I (2019) Structural features and regulation of lignin deposited upon biotic and abiotic
660 stresses. Curr Opin Biotechnol 56:209-214. https://doi.org/10.1016/j.copbio.2018.12.012
661
662
663
664
665
666
667
668
669