Modification of native grasses for biofuel production may increase virus susceptibility

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Modification of native grasses for biofuel production may increase virus susceptibility

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Abstract

Bioenergy production is driving modifications to native plant species for use as novel biofuel crops. Key aims are to increase crop growth rates and to enhance conversion efficiency by reducing biomass recalcitrance to digestion. However, selection for these biofuel-valuable traits has potential to compromise plant defenses and alter interactions with pests and pathogens. Insect-vectored plant viruses are of particular concern because perennial crops have potential to serve as virus reservoirs that influence regional disease dynamics. In this study, we examined relationships between growth rates and biomass recalcitrance in five switchgrass (*Panicum virgatum*) populations, ranging from near-wildtype to highly selected cultivars, in a common garden trial. We measured biomass accumulation rates and assayed foliage for acid detergent lignin, neutral detergent fiber, *in vitro* neutral detergent fiber digestibility and *in vitro* true dry matter digestibility. We then evaluated relationships between these traits and susceptibility to a widely distributed group of aphid-transmitted Poaceae viruses (Luteoviridae: *Barley and cereal yellow dwarf viruses*, B/CYDVs). Virus infection rates and prevalence were assayed with RT-PCR in the common garden, in greenhouse inoculation trials, and in previously established switchgrass stands across a 300-km transect in Michigan, USA. Aphid host preferences were quantified in a series of arena host choice tests with field-grown foliage. Contrary to expectations, biomass accumulation rates and foliar digestibility were not strongly linked in switchgrass populations we examined, and largely represented two different trait axes. Natural B/CYDV prevalence in established switchgrass stands ranged from 0% to 28%. In experiments, susceptibility varied notably among switchgrass populations and was more strongly predicted by potential biomass accumulation rates than by foliar digestibility; highly selected, productive cultivars were most virus-susceptible and most preferred by aphids. Evaluation and mitigation of virus susceptibility of new biofuel crops is recommended to avert possible unintended consequences of biofuel production on regional pathogen dynamics.

Keywords: Barley yellow dwarf virus, biofuel, grass, pathogen, *Rhopalosiphum padi*, selection, switchgrass, trait, vector, virus

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Introduction

At present, the North American biofuel industry relies heavily on ethanol-based fuels produced through maize

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grain fermentation (de Vries *et al.*, 2010). To broaden the plant materials available for ethanol production, new methods for converting plant lignocellulosic material to ethanol are being developed for deployment in the coming decade (Gomez *et al.*, 2008). Much attention is focused on developing perennial grasses as novel second-generation biofuel crops that could provide multiple environmental advantages, such as increased soil carbon storage, while supporting reliable bioenergy

production (Lemus & Lal, 2005; Heaton *et al.*, 2008). Switchgrass (*Panicum virgatum* L.) is one of the strongest contenders as a perennial biofuel crop in the United States and is also being considered for use in Canada and Europe (Samson & Omielan, 1992; Smeets *et al.*, 2009). Switchgrass is a warm-season native prairie grass from North America and was historically used for forage and erosion control (Vogel, 2004).

In modifying switchgrass and other grasses for biofuel production, two key aims are to increase crop productivity (Karp & Shield, 2008) and to improve conversion efficiency by reducing biomass recalcitrance to enzymatic and microbial digestion (Himmel *et al.*, 2007). Here we examine the extent of such trait modifications in switchgrass, evident in near-wildtype populations and developed cultivars, and evaluate relationships between these traits and virus susceptibility. Whereas pathogens of maize and other food crops are largely well understood, pathogen interactions with novel bioenergy crops have been less well explored and deserve significant attention. Unless mitigated, pathogens have the potential to depress feedstock yields and even spill over into other crops in the region (Spencer & Raghu, 2009; Agindotan *et al.*, 2010). Previous anthropogenic changes to plant traits and distributions have driven pathogen emergence and development of disease outbreaks (Kennedy & Barbour, 1992; Webster *et al.*, 2007; Jones, 2009), so potential consequences of biofuel crop deployment for pathogen dynamics merit investigation.

A major aim of increasing the productivity of novel biofuel crops, such as perennial grasses, is to make their expected profitability competitive with first-generation crops, such as maize (James *et al.*, 2010). However, plant allocation theory predicts that increases in growth rates often come at the cost of reductions in defense (Herms & Mattson, 1992); as a result, selection for increased productivity may inadvertently increase plant disease susceptibility of these new crops. Likewise, efforts to reduce lignin in crop species have raised concern about the potential for increased pest and pathogen pressure (Li *et al.*, 2008).

Although links between reduced lignin and increased herbivore pressure remain unclear (Pedersen *et al.*, 2005), some evidence suggests that chewing herbivores (mammals or insects) may sometimes prefer or benefit from low lignin plants. For example, brown midrib lines of sorghum-sudangrass and pearl millet, which have reduced lignin, were preferred over wildtype varieties by grazing lambs (Cherney *et al.*, 1990; Li *et al.*, 2008). The effects of lignin on insect herbivores are complex. For example, across a broad spectrum of sorghum lines, high lignin concentration was associated with resistance to fall armyworms, as measured by duration of larval

development. However, among the most resistant sorghum lines, larvae developed more rapidly on lines with the highest lignin levels (Diawara *et al.*, 1991). In more recent work with transgenic aspen and silver birch lines with altered lignin content, differences in growth rates and preferences among lepidopterans and coleopterans were not clearly attributable to lignin (Tiimonen *et al.*, 2005; Brodeur-Campbell *et al.*, 2006). The complexity of these interactions may be explained in part by plant allocation to other defensive pathways, such as production of phenolic compounds (Brodeur-Campbell *et al.*, 2006), in addition to or in place of lignin biosynthetic pathways. The response of sucking insects, like aphids and thrips, to changes in lignin in biofuel feedstocks has not been previously addressed.

Numerous microbes and insect pests have potential to cause damage in biofuel crops. Highly visible pathogens, such as fungal diseases, are among the first to have been identified as possible problems in switchgrass (Gustafson *et al.*, 2003; Crouch *et al.*, 2009). Less visible pathogens, such as viruses, may be more easily overlooked in field trials and thus merit deliberate investigation. Viruses have likely influenced crops since the dawn of early agriculture (Gibbs *et al.*, 2008, 2010), and their potential interactions with novel biofuel crops cannot be ignored.

Our long-term goal is to assess how trait changes associated with bioenergy crop development could alter ecological interactions between Poaceae species and Poaceae-infecting viruses at multiple scales. In the study reported here, we examine the influence of human selection pressure on susceptibility of switchgrass populations to infection by *Barley and cereal yellow dwarf viruses* (B/CYDVs), a key group of globally important pathogens. Our specific objectives are (1) to examine the influence of anthropogenic selection pressures on key biofuel-valued traits (biomass recalcitrance and growth rates) in existing switchgrass populations; (2) to quantify the susceptibility of these same populations to virus infection; and (3) to investigate the nature of any relationships between biofuel-valued traits and virus susceptibility.

Materials and methods

Common garden experiment

For this study, we conducted a suite of field and greenhouse experiments. To evaluate differences in growth traits among switchgrass populations, we first established a common garden experiment in the field at Michigan State University's Horticulture Teaching and Research Center in East Lansing, MI (42.7°N, 84.5°W). Soils at the site are loamy alfisols and mollisols (Aquic

Glossudalfs, Typic Endoaquolls, and Typic Argiaquolls). East Lansing averages 796 mm of precipitation per year and has an average maximum daily temperature of 13.9 °C and average minimum daily temperature of 2.7 °C (Michigan State Climatologist's Office, http://climate.geo.msu.edu).

To compare effects of different selection pressures on switchgrass traits and performance, we chose five commercially available populations, either native Michigan genotypes or cultivars suitable for use in Michigan (Michigan Department of Natural Resources), which represented a spectrum from near-wildtype populations (Michigan Wildflower Farm, Southlow) to cultivars developed for use as forage grasses ('Nebraska 28', 'Blackwell', 'Trailblazer') (Table 1). 'Trailblazer' is the most intensively selected cultivar, having been selected initially for vigor and then for tissue digestibility (Vogel & Moore, 1993; Vogel & Pedersen, 1993). Second-generation switchgrass cultivars currently in development for biofuel purposes are likely to further extend the trajectory of this spectrum towards greater productivity and digestibility.

Switchgrass individuals were started from seed in a virus-free greenhouse in April 2008. They were planted into the field as plugs (set 1 m apart) in June 2008 in a completely randomized design ($n = 9$–10 per population after minimal initial mortality). Plants were open-grown with minimal competition from neighbors; weeds were removed by hand. No fertilizer or pesticides were applied, and plants were irrigated only during the first week after transplanting.

Growth traits

At the end of the growing season, we counted tillers per plant in the field and harvested all aboveground biomass in October 2008 after a hard frost. We dried biomass for 3 days with forced air, separated panicles, and weighed both panicles and aboveground vegetative portions.

Foliar tissue chemistry and digestibility

To assess traits associated with biomass recalcitrance and conversion efficiency, we compared tissue chemistry and digestibility among switchgrass populations sampled from the common garden experiment. For large herbivores and mechanical harvests that consume a mixture of stems and foliage, these parameters are determined both by leaf-to-stem biomass ratios as well as by tissue constituents (Twidwell *et al.*, 1988). However, because virus-transmitting aphids are small, they can sample only one tissue type at a time. We therefore focused our analysis on foliar tissue because in our

experience cereal aphids prefer leaves over stems in experimental situations (data not shown).

To evaluate tissue chemistry, we measured two aspects of cell wall components from leaf blade tissue: (1) acid detergent lignin (ADL) and (2) neutral detergent fiber (NDF). To determine lignin concentration, we followed the methods of Van Soest (1973). In brief, ground tissue samples were heated in acid-detergent (containing cetyl trimethylammonium bromide and sulfuric acid) and treated with 72% sulfuric acid. Silica content was accounted for by ashing at 550 °C for 6 h. To determine NDF, we followed the methods of Mertens *et al.* (2002). Samples were refluxed in neutral-detergent solution (containing sodium hydroxide, EDTA, dibasic sodium phosphate, sodium borate decahydrate, and sodium lauryl sulfate) and α-amylase solution, and afterwards, remaining residues were washed with boiling water and then acetone. In biofuel feedstock development, reduction of lignin is predicted to increase conversion efficiency because lignin can block enzymatic digestion by encasing cell wall polysaccharides (Gomez *et al.*, 2008); similarly, in forage crop development, reduced lignin can improve forage quality (Li *et al.*, 2008). NDF is a broader measure of total insoluble fiber that includes cellulose, hemicellulose, as well as lignin. The cellulose and hemicellulose components of NDF provide the substrate for enzymatic hydrolysis, which then provides sugars for fermentation in biofuel production (Gomez *et al.*, 2008).

As direct measures of biomass recalcitrance, we quantified both *in vitro* neutral detergent fiber digestibility (IVNDFD) and *in vitro* true dry matter digestibility (IVTDMD) of leaf tissue following the methods of Goering & Van Soest (1970). In brief, ground foliar tissue (0.5 g dry weight) was placed in a flask with a rumen buffer and mineral solution (containing rumen fluid collected from a rumen-fistulated dairy cow) and allowed to incubate in a shaking water bath at 40 °C under carbon dioxide. The sample was then rinsed with neutral-detergent, treated with decahydronapthalene, and washed with boiling water and then acetone. *In vitro* TDM digestibility was calculated as 100 – percent dry residue; *in vitro* NDF digestibility, as percent NDF – percent dry residue. *In vitro* NDF digestibility measures how easily NDF is hydrolyzed by ruminal microbes over the expected retention time in the rumen (here, 48 h). High *in vitro* NDF digestibility is associated with low lignification, improved forage quality, and greater cellulosic ethanol production (Allen, 2000; Lorenz *et al.*, 2009; Bals *et al.*, 2010). *In vitro* TDM digestibility assesses to what extent total dry matter (which includes both cell contents and NDF) can be broken down by ruminal microbes.

Tissue chemistry and digestibility measures were conducted on leaf blade tissue from common garden

Table 1 Characteristics of switchgrass populations included in the common garden experiment

	Population	Level of selection	Geographic origin	Release date	Population type	Characteristics selected for	Other notable characteristics	Disease characteristics
Common garden populations	Michigan Wildflower Farm (MWF)	Near-wildtype	Michigan	–	Near-wildtype. Propagated for native restoration	–	–	–
	Southlow	Near-wildtype	Lower Michigan	2001	Registered germplasm. Multi-origin G1 seed assembled from 11 native G0 stands*	Native diversity	Mixed ploidy†	–
	Nebraska 28	Moderately selected	Nebraska	1949	Registered variety	–	Leafy, medium height, early maturing‡, octoploid¶, upland‖	Rust susceptible‡
	Blackwell	Moderately selected	Oklahoma	1944	Registered variety. Single-plant origin	–	Leafy, medium height, good forage production‡, octoploid§‖, upland¶	Good stem rust resistance‡
	Trailblazer	Intensively selected	Kansas and Nebraska	1984	Registered variety	Forage quality, high *in vitro* dry matter digestibility**	Leafy, good forage production, late maturing‡, octoploid‖††, upland¶	Rust susceptible in a field study‡‡
Additional populations for aphid preference tests	Dacotah	Moderately selected	North Dakota	1989	Registered variety	Leafiness, vigor, seed production, winter hardiness‡	Short height, drought tolerance‡, tetraploid§§	Leaf spot susceptible in a field study¶¶
	Cave-in-Rock	Moderately selected	Illinois	1973	Registered variety	–	Vigorous, high seed yield, resistant to lodging‡, octoploid‖‖‖	Resistant to leaf spot‡
	Pathfinder	Moderately selected	Kansas and Nebraska	1967	Registered variety	–	Vigorous, leafy, late maturing‡, octoploid§‖	Rust resistant‡
	Shawnee	Intensively selected	Illinois	1995	Registered variety. Cave-in-Rock used as base population***	High *in vitro* dry matter digestibility, yield, leafiness***	Octoploid***	Disease resistant compared to Cave-in-Rock***

*Durling et al. (2008).
†D. Schemske (unpublished results).
‡Alderson & Sharp (1995).
§Riley & Vogel (1982).
¶Vogel (2004).
‖Hopkins et al. (1996).
**Vogel et al. (1981).
††Vogel et al. (1991).
‡‡Redfearn et al. (1999).
§§Narasimhamoorthy et al. (2008).
¶¶Krupinsky et al. (2004).
‖‖Wullschleger et al. (1996).
***Vogel et al. (1996).
–, data not applicable or available.

plants harvested in 2008 ($n = 9$–10 per population). However, limits in foliar tissue from small plants reduced the number of individuals tested for lignin concentration in the MWF population ($n = 7$).

Susceptibility to Barley and cereal yellow dwarf viruses

Barley and cereal yellow dwarf viruses (*Luteoviridae*: BYDVs and CYDVs; hereafter B/CYDVs) are a group of aphid-transmitted + ssRNA viruses that infect wild and cultivated Poaceae species worldwide (Lister & Ranieri, 1995). B/CYDVs are sometimes called the 'yellow plague of cereals' (Conti *et al.*, 1990) because of stunting and yield loss they cause in cereal crops (Jensen & D'Arcy, 1995; McKirdy & Jones, 2002), and their influence on wild grasses is of increasing interest. These viruses are emerging as key model systems in plant virus ecology (Power, 1991; Malmstrom *et al.*, 2006; Borer *et al.*, 2007). Several virus species have been recognized, including BYDV-PAV and CYDV-RPV (Lister & Ranieri, 1995).

The few previous studies investigating switchgrass susceptibility to B/CYDVs reported conflicting results. An early study did not find switchgrass to be susceptible to a B/CYDV vectored by *Rhopalosiphum padi* L. (bird cherry-oat aphid) (Stoner, 1976), but more recent work using enzyme-linked immunosorbent assays (ELISA) found switchgrass plants infected with BYDV-MAV and BYDV-SGV in the tallgrass prairie in Kansas, USA (Garrett *et al.*, 2004). Like other perennial grasses, switchgrass has the potential to serve as a long-term reservoir for B/CYDVs and as a host for aphid vectors.

To better understand switchgrass susceptibility to B/CYDVs, we conducted three studies. First, to evaluate the extent to which switchgrass in Michigan develops B/CYDV infection under contemporary virus pressure, we used molecular diagnostics to quantify B/CYDV infection in six established switchgrass fields in Southern Michigan across a 300-km transect (Table 2). We harvested foliar tissue in September and early

October 2008 from 30 switchgrass individuals along two 70 m transects through each field. We stored tissue samples at $-20\,^{\circ}\text{C}$ until processing. Total RNA was extracted from 75 mg subsamples of tissue using Tri-Reagent (Sigma-Aldrich, Inc., St. Louis, MO, USA) and chloroform according to the manufacturer's instructions. To identify viruses, we used 1 µg of RNA in multiplexed reverse transcription polymerase chain reaction (RT-PCR) with primers that detect a wide range of B/CYDVs (following protocols in Malmstrom & Shu, 2004). As per this protocol, we used SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) for RT and AmpliTaq Gold DNA Polymerase (Applied Biosystems, Carlsbad, CA, USA) for PCR.

Second, we evaluated infection rates from natural virus pressure in our common garden experiment. We sampled fully expanded leaves near the tops of tillers from individuals ($n = 9$–10 per population; 48 total samples) in the common garden experiment to compare field infection rates among varieties. Because virus analysis is best conducted on samples from physiologically active tissue, we sampled plants while they were still green in September 2008, after about three months of field exposure to natural aphid and virus populations. Samples were processed as above.

Following the discovery of B/CYDV infection in the field study, we decided to further quantify virus interactions by assessing rates of virus acquisition under uniformly high virus pressure. To do this, we caged viruliferous *R. padi* on individual greenhouse-grown switchgrass plants for six days and measured subsequent development of infection. *R. padi* is the most common B/CYDV vector in our region (D. Voegtlin, North Central Regional Suction Trap Network, unpublished results) and can acquire and transmit multiple B/CYDV species (Irwin & Thresh, 1990). We tested infection rates in the same switchgrass populations used in the common garden experiment, except for Southlow, which was dropped due to low germination rates.

For the inoculation tests, we cold-stratified switchgrass seeds for 2 weeks, planted multiple seeds into

Table 2 B/CYDV prevalence in established switchgrass fields in Southern Michigan in 2008 as quantified with RT-PCR

County	Cultivar	Stand age (years)	Field size (ha)	B/CYDV prevalence	
				n/n	*%*
Allegan	Unknown	8	2.4	0/30	0
Tuscola	Unknown	6	14.2	0/27	0
Cass	Forestburg	8	2.8	3/30	10
Saginaw	Unknown	8–18	4.0	3/30	10
Barry	Cave-in-Rock	Unknown	6.1	8/30	27
Kalamazoo	Cave-in-Rock	7	3.2	8/29	28

12.7 cm plugs, and thinned seedlings to one individual per plug approximately 1.5 weeks later. Before inoculation, nonviruliferous *R. padi* were allowed to acquire BYDV-PAV from the local Great Lakes region by feeding for 24 h on infected *Avena sativa* leaves (Gray *et al.*, 1991) from plants that had tested positive for infection using the RT-PCR assay. Next, five viruliferous aphids were caged on each switchgrass seedling ($n = 28$–38 per population) within 10.5 cm tall transparent cages, which covered each plant completely. Aphids had access to seedlings for 6 days [a long inoculation access period (Power *et al.*, 1991)] before being killed with Astro insecticide (FMC Corporation, Philadelphia, PA, USA). Ten days later, we harvested the three youngest, fully developed leaves from each plant. Plants from all populations were at similar developmental stages for inoculation and when tissue was harvested. Tissues were stored and processed as above.

Aphid host preferences

To assess whether aphid host preferences explained differences in virus acquisition rates among switchgrass populations, we conducted a series of host choice tests in arenas in the laboratory. In these tests, 15 or 20 adult apterae (wingless aphids) were placed in a Petri dish containing up to four equal-size pieces of tissue from leaves of different host populations and left in the dark for 24 h at 22 °C, after which the number of aphids on each leaf type was counted (following methods in Malmstrom *et al.*, 2005b). Tissue samples were taken from fully expanded, physiologically active leaves from the upper half of the larger tillers of each plant and were placed on moist filter paper in the Petri dish in randomized order. Aphids were placed on the filter paper, not on leaves.

For context, we first compared *R. padi*'s relative preference between switchgrass and maize, using representative cultivars [switchgrass 'Dacotah' (Table 1) and maize hybrid 36R19]. We used 4 cm × 0.5 cm leaf portions from 4-week-old maize and 5-week-old switchgrass, due to the slower nature of switchgrass growth. There were 15 replicate arenas containing 15 *R. padi* each.

Next, we compared *R. padi* feeding preferences among the field-grown switchgrass populations from the common garden experiment. In June 2009, we used 0.5 cm × 2 cm samples of green leaves from field-grown individuals from each of the four populations tested in the inoculation study. Aphid preferences were tested using 20 apterae per arena. The experiment was structured as a complete block design with 40 replicates.

Third, we used an additional arena study with 20 replicates to compare the attractiveness of 'Trailblazer' to that of three other switchgrass types commonly planted in our region: 'Cave-in-Rock', 'Pathfinder',

and 'Shawnee' (Table 1). We obtained field-grown tissue for this study from a common garden established in 2006 by Suleiman Bughrara at Michigan State University's Crop and Soil Teaching and Research Center (East Lansing, MI, USA). Tissue was collected from the second leaf from the top of tillers.

Analysis

We compared growth parameters, tissue chemistry, and measures of biomass recalcitrance among switchgrass populations with ANOVA in STATISTIX 9.0 (Analytical Software, Tallahassee, FL, USA). Lignin concentration and IVNDFD were log transformed to meet assumptions of normality for all analyses. To reduce redundancy among switchgrass traits, we used principal components analysis (PCA) (R 2.11.1, R Foundation for Statistical Computing, Vienna, Austria). PCA is useful for assessing multiple plant traits (Oyarzabal *et al.*, 2008), and we used it to compress seven trait variables: vegetative aboveground biomass, panicle biomass, number of tillers, NDF, IVNDFD, IVTDMD, and lignin concentration. As noted, some natural infection occurred in the common garden experiment during the course of the experiment. However, virus-infected plants did not differ significantly from uninfected plants for any of the traits measured (ANOVA, $P > 0.05$), so analyses reported include data for all plants.

To compare infection rates among populations, we used Fisher's exact test for data from the common garden study and a Chi-Square test in STATISTIX 9.0 for greenhouse inoculation data. We used ANOVA, also in STATISTIX 9.0, to evaluate aphid host preferences in arena tests.

Given the population differences that became evident in the trait analyses, we then explored potential relationships between biofuel-valuable traits and susceptibility. To assess which individual traits or which trait-space (based on principal components) may be associated with susceptibility to infection and attractiveness to aphid vectors, we used linear regression in R 2.11.1. Because virus and aphid interactions were assessed at the population level, we used population averages of trait values for regression. We then ranked traits based on their R^2 value, excluding traits that explained <10% of variation in the data.

Results

Effects of selection on switchgrass traits

Contrary to our expectations, foliar digestibility and growth rates of switchgrass populations were not strongly associated and largely represented different trait axes. For example, principal component analysis
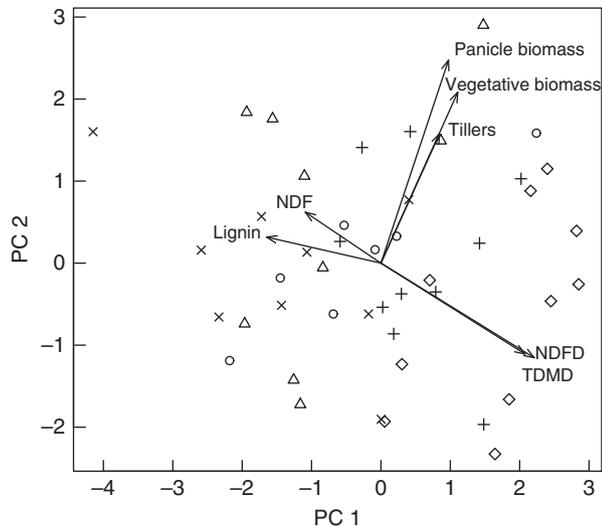
Fig. 1 Distribution of switchgrass individuals according to principal components 1 and 2. Each point marks an individual plant (○ , MWF; △, Southlow; + , 'Nebraska 28'; x, 'Blackwell'; and ◇ 'Trailblazer'). Arrows represent relative loadings of trait values on the principal component axes.

of all measured traits found foliar digestibility measures to be the primary loading factors for PC1 (loading values: IVTDMD = 0.55; IVNDFD = 0.52; lignin = −0.41) whereas growth traits were the primary loading factors for the orthogonal PC2 (loading values: vegetative biomass = 0.52; panicle biomass = 0.62) (Fig. 1). With respect to the first principal component, the two near-wildtype populations (MWF, Southlow) grouped separately from the intensively selected cultivar, 'Trailblazer', while the two moderately selected varieties, 'Blackwell' and 'Nebraska 28,' occupied intermediate positions (Fig. 1).

Two-dimensional trait diagrams further highlight distinctions between digestibility and growth traits and reveal how switchgrass cultivars have been shaped along these axes by human selection. As expected, lignin concentration was a significant predictor of *in vitro* TDM digestibility (linear regression, $R^2 = 0.22$, $P = 0.001$), but lignin is relatively poorly predicted by aboveground biomass (linear regression, $R^2 = 0.09$, $P = 0.045$) (Fig. 2a and b). The trait spaces of the two near-wildtype populations (MWF, Southlow) were broad but centered in zones that represent less desirable values for biofuels (less biomass, lower digestibility, more lignin) (Fig. 2b and c). Selection pressure for increased yield and forage value were evident in the shift of the trait space of cultivar 'Nebraska 28' towards the upper right in comparison with the near-wildtype populations (Fig. 2c). This shift continues further with 'Trailblazer', a cultivar subject to intense selection for forage quality, and in which foliar digestibility was

uniformly high. Biomass accumulation rates in 'Trailblazer' were also high but more variable, suggesting primacy of selection for tissue digestibility. In contrast, 'Blackwell' diverges from this selection trajectory. This cultivar appears to have been shaped for increased forage value along a different pathway: through increased 'leafiness' and reduction of tiller number. Thus, these two cultivars inhabit nearly orthogonal spaces in an *in vitro* TDM digestibility × tiller number comparison: 'Trailblazer' is most uniform in *in vitro* TDM digestibility, 'Blackwell' in tiller number (Fig. 2d). Both selection pathways lead to increased forage value, but by different means.

On a single trait basis, selection pressures have created continuums of trait differences from wildtype to highly selected, as evidenced by accumulation rates of aboveground vegetative biomass during the first growing season (ANOVA, $F_{4,43} = 3.25$, $P = 0.02$) (Fig. 3a). Populations also differed significantly in tiller number (ANOVA, $F_{4,43} = 4.1$, $P = 0.007$) (Fig. 3b), foliar lignin concentration (ANOVA, $F_{4,40} = 6.8$, $P = 0.0003$) (Fig. 3c), foliar NDF concentration (ANOVA, $F_{4,43} = 2.8$, $P = 0.04$) (Fig. 3d), *in vitro* NDF digestibility (ANOVA, $F_{4,43} = 2.8$, $P = 0.002$), and *in vitro* TDM digestibility (ANOVA, $F_{4,43} = 5.2$, $P = 0.0004$) (Fig. 3e and f). On average, 'Trailblazer' was more digestible than both near-wild-type populations and 'Blackwell', but not 'Nebraska 28' (Tukey HSD $P < 0.05$).

In tiller and digestibility measures, the differing nature of selection pathways used to improve forage quality is evident in the divergence of 'Blackwell's values from trends among the other populations (Fig. 3).

Susceptibility to virus infection

Our findings indicate that switchgrass can accumulate B/CYDV infection quickly under natural virus pressure. In fields (ca. 6–18 years old) across a transect in southern Michigan, B/CYDV prevalence ranged from 0% to 28% (Table 2). On a shorter timescale, the initially virus-free switchgrass individuals in our common garden accumulated 10.6% incidence of B/CYDV infection (5/47 plants sampled) in just 3 months of field exposure in 2008 (Table 3). Infected plants showed few signs of the B/CYDV-induced discoloration typically evident in cultivated cereals. Cultivars appeared to be more susceptible to virus infection than near-wild-type populations. In our common garden experiment, none of the near-wildtype plants were infected in the first year, whereas five cultivar individuals were (Fisher's exact test, $P = 0.07$). Of these, two were infected with BYDV-PAVs and three with CYDV-RPVs.

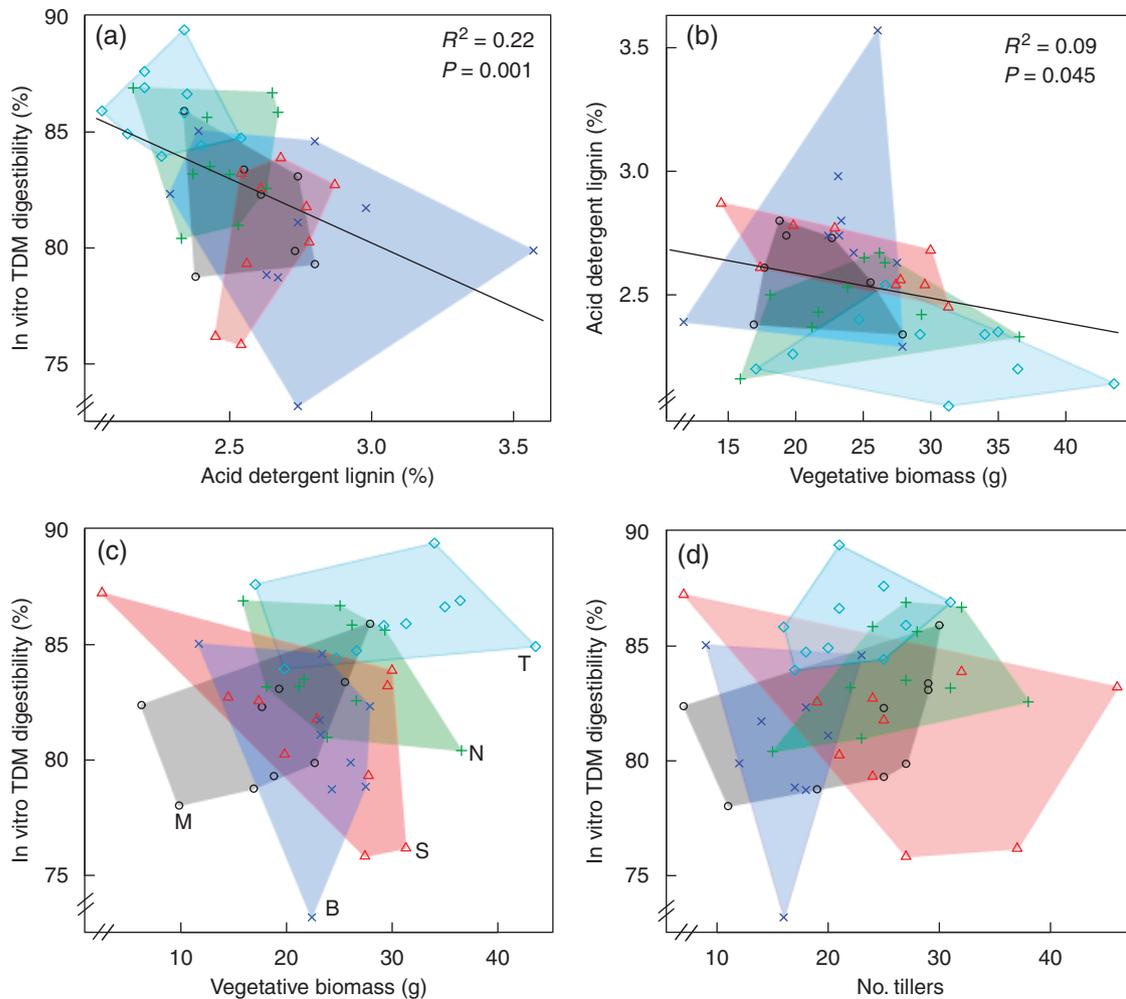In the greenhouse trial, infection rates were higher overall, averaging 38% incidence across all populations.

Fig. 2 Trait associations between (a) lignin and IVTDMD, (b) vegetative biomass and lignin, (c) vegetative biomass and IVTDMD, and (d) tiller number and IVTDMD. Each point designates an individual switchgrass plant (○, MWF; △, Southlow; +, 'Nebraska 28'; x, 'Blackwell'; and ◇, 'Trailblazer'). Trait space of each population is shown in shaded minimum convex polygons. Lignin data was unavailable for three individuals, resulting in fewer points in (a) and (b).

As in the field trials, near-wildtype plants (MWF) experienced the lowest infection rates (12%), and were less likely to be infected than cultivars ($\chi^2 = 10.9$, $df = 1$, $P = 0.0009$) (Table 3). At the other extreme, BYDV incidence in the fast-growing, highly digestible forage cultivar 'Trailblazer' was 68% – 5.5 times greater than in MWF ($P < 0.05$) and 2.1 times greater than in 'Nebraska 28' (32% infection incidence) ($P < 0.05$).

Aphid host preferences

R. padi, the primary vector of several B/CYDVs in our region, will feed on switchgrass but strongly prefers maize when given a choice of foliar tissue. For example, in our arena tests, approximately 3.5 times more *R. padi* preferred maize tissue (hybrid 36R19) over switchgrass ('Dacotah'; ANOVA, $F_{1,28} = 51.3$, $P < 0.0001$).

In arena comparisons among switchgrass populations examined here, *R. padi* strongly preferred leaves from two cultivars noted for good forage production ('Trailblazer' and 'Blackwell'; Table 1) over leaves from 'Nebraska 28' or the near-wildtype population (MWF) (Tukey's HSD $P < 0.05$) (Fig. 4a). In comparisons with other widely planted switchgrass cultivars, 'Trailblazer' was comparable in preference to 'Shawnee', 'Pathfinder,' and 'Cave-in-Rock'. Interestingly, 'Shawnee', which was developed for increased tissue digestibility from 'Cave-in-Rock' (Vogel *et al.*, 1996), attracted three times more aphids than 'Cave-in-Rock' (Tukey's HSD, $P < 0.05$). Since our field surveys detected notable B/CYDV prevalence in 'Cave-In-Rock' under natural virus pressure (Table 2), this aphid preference suggests that 'Shawnee' might suffer similar or greater pressure in the field.
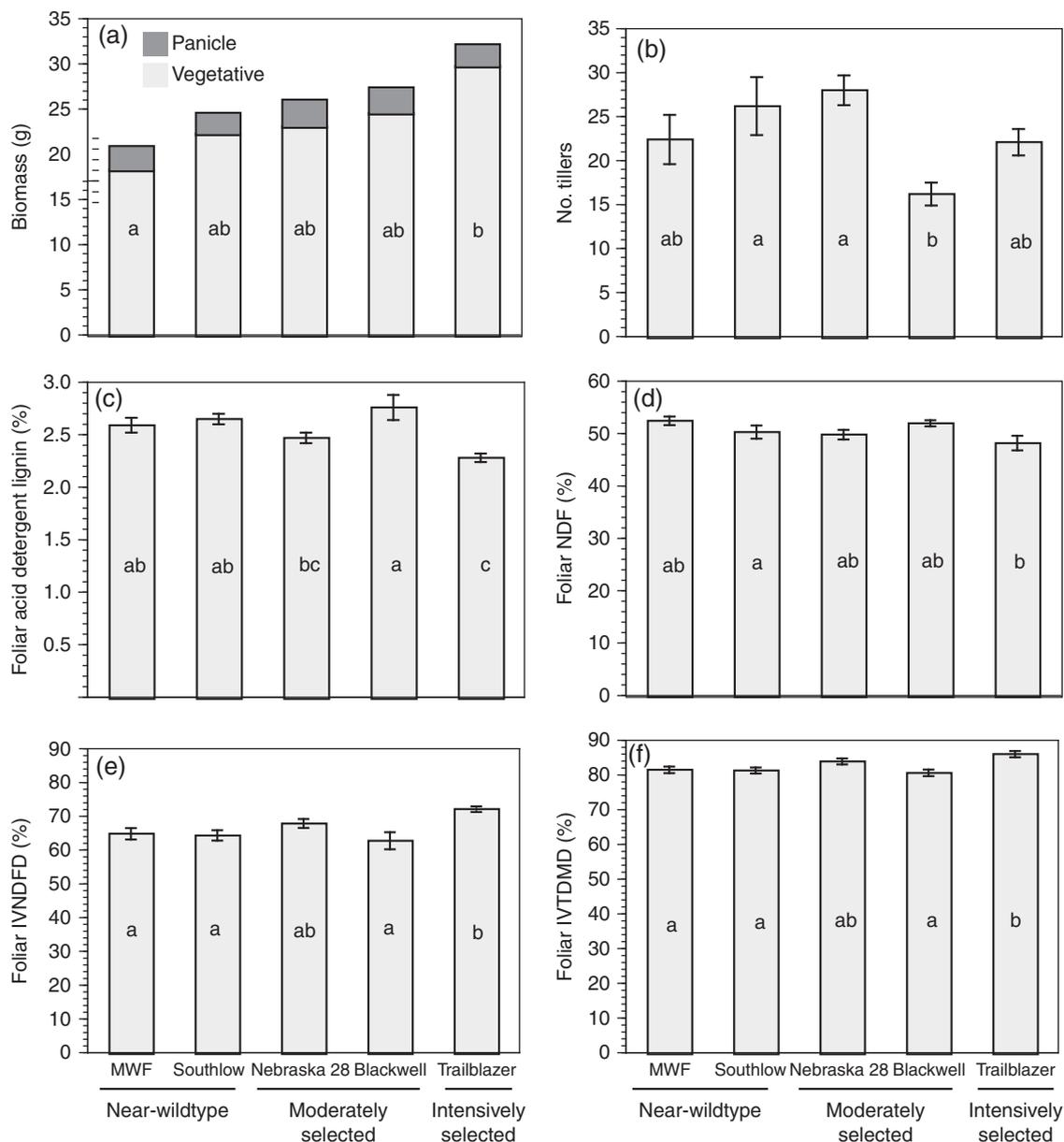
Fig. 3 Growth trait differences among populations of switchgrass. (a) Aboveground biomass, both vegetative mass (light bars) and reproductive panicles (dark bars). Significant differences are given for vegetative biomass only; panicle biomass differences were not significant. (b) Tiller number. (c) Foliar lignin concentration. (d) Foliar NDF concentration. (e) Leaf tissue digestibility as measured by IVNDFD and (f) IVTDMD. Different letters indicate significant differences at $\alpha = 0.05$. Error bars indicate one standard error.

Predictors of virus and aphid susceptibility in switchgrass

Among the plant traits measured, mean vegetative biomass accumulation per individual in the field was the best predictor of population-level susceptibility to virus in the greenhouse inoculation (linear regression, $R^2 = 0.99$, $P = 0.006$) (Fig. 5; Table 4). While other traits may also influence susceptibility, none were significant within the limits of detection. Lignin concentration, for example, was not significantly related to popula-

tion-level susceptibility (linear regression, $R^2 = 0.46$, $P = 0.32$) (Fig. 5).

When traits were ranked according to the amount of variability they explained, rate of vegetative biomass accumulation, followed by measures of tissue digestibility and cell wall traits, explained the most variability in BYDV-PAV susceptibility (Table 4). Differences in tillering strategies explained the most variability among switchgrass populations in attractiveness to aphids, with *R. padi* tending to prefer populations that pro-

duced fewer tillers per individual. Digestibility and cell wall traits had essentially no influence on aphid preferences (Table 4). Principal component values, although they incorporated multiple traits, were not significantly associated with virus and aphid interactions (linear regression, $P > 0.05$).

Discussion

Viruses likely have infected crops since the beginning of agriculture (Stukenbrock & McDonald, 2008; Gibbs *et al.*, 2010). In many locations and periods, humans have sparked serious crop epidemics through intensification of agriculture (Thresh, 1982), transport of crops into new regions (Thresh, 1980; Jones, 2009), and specific modifications of plant traits (Thresh, 1982). Our findings here suggest that, without mitigation, deployment of new bioenergy crops could perturb virus epidemiology and possibly increase risk of regional

virus spread. Of particular concern is the indication that selection for biofuel-valuable traits could increase disease susceptibility in perennial species that already have the potential to serve as long-term pathogen reservoirs. Our study suggests a framework for risk assessment of pathogen interactions with novel biofuel crops; future population-level studies will expand understanding of these interactions and provide a basis for mitigation strategies.

In the last century, humans began a renewed campaign of selecting native species for use in working landscapes and for ecological restoration (Vogel, 2000; Sanderson *et al.*, 2004, A. Schrotenboer and C. Malmstrom, unpublished results). The effort to domesticate wild plants is now intensifying to meet human demand for renewable energy sources (Casler, 2010). As in past domestication events (Vasey, 1992; Ladizinsky, 1998), a substantial effort is focused on increasing plant growth rates and improving the quality of tissue for consumption. Historically, the consumers for which new crops were selected were humans and livestock, but changes in crop traits that benefited these target consumers often inadvertently benefited other consumers, including pathogens and insect pests (Thresh, 1982). At present, the target consumers for cellulosic biofuels are combustion engines and the upstream feedstock processing units needed to recreate or replace the digestion of cellulosic material by ruminal microbes. Like ruminants, these industrial consumers will benefit from highly productive, easily digestible feedstocks (Lorenz *et al.*, 2009). Thus, selection of new biofuel crops retraces or extends in many aspects the trajectory of past domestication events and may exert analogous influences on pathogen communities.

To date, work on pathogens in biofuel crops has focused on disease identification (Crouch *et al.*, 2009; Agindotan *et al.*, 2010; Gao *et al.*, 2010). Our findings

Table 3 B/CYDV prevalence in switchgrass populations (1) in the field under natural virus pressure and (2) in the greenhouse inoculation, as quantified by RT-PCR

| | | B/CYDV prevalence | | | |
| | | Field experiment | | Greenhouse inoculation | |
Population	Type	n/n	%	n/n	%
MWF	Near-wildtype	0/9	0	4/33	12[a]
Southlow	Near-wildtype	0/10	0	–	–
Nebraska 28	Cultivar	3/8	37.5	12/38	32[a]
Blackwell	Cultivar	1/10	10	11/29	38[a,b]
Trailblazer	Cultivar	1/10	10	25/37	68[b]

Different letters indicate significant differences for percent infection in the greenhouse inoculation.
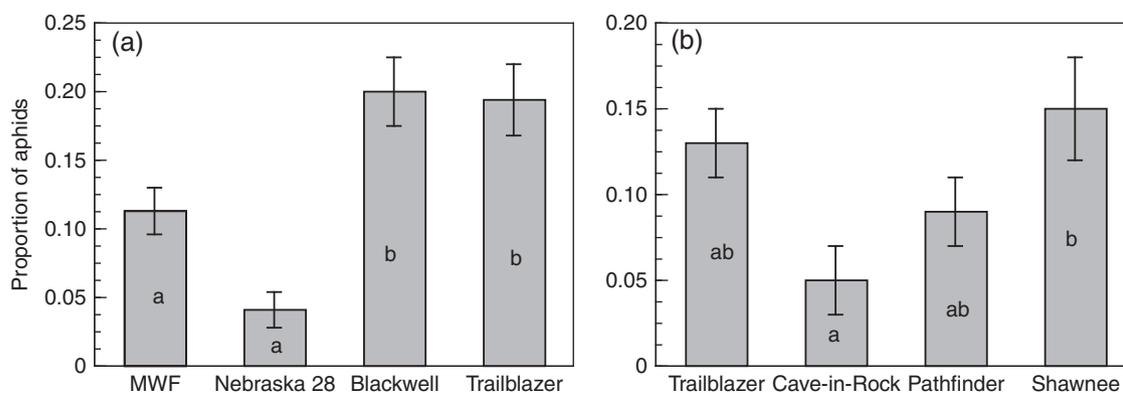

Fig. 4 Aphid host preferences (a) among leaf tissues of field-grown switchgrass populations from our common garden experiment and (b) among field-grown tissue of four common cultivars of switchgrass. Different letters indicate significant differences at $\alpha = 0.05$. Error bars indicate one standard error.
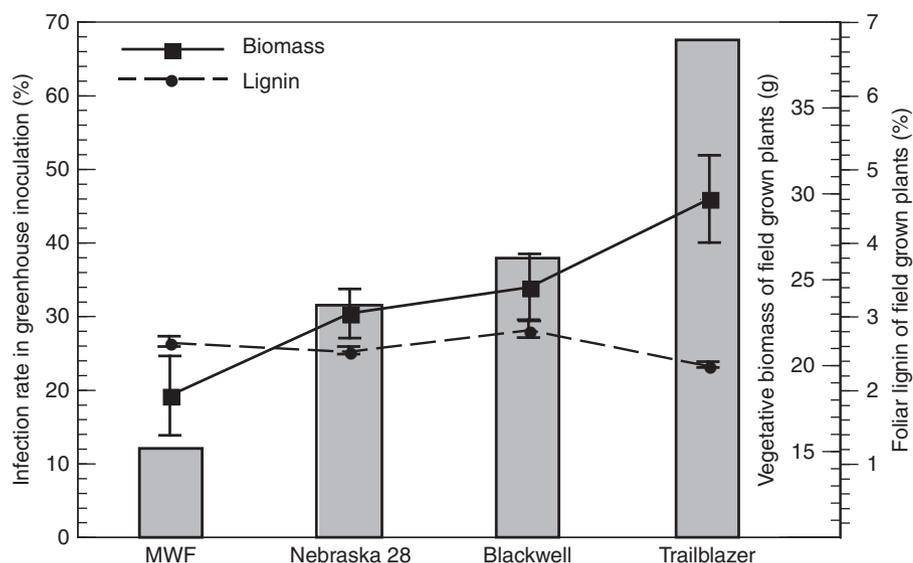
Fig. 5 Relationship between switchgrass virus susceptibility in greenhouse inoculation trials and biofuel-valuable traits as measured in the common garden (see results for R^2 values). Biomass and lignin values are population averages, and error bars indicate one standard error.

Table 4 Predictors of population-level virus suscepti-bility and aphid preferences, ranked by R^2 values in linear regression

	Virus susceptibility	Aphid preference
Vegetative biomass accumulation	1*	3
IVNDFD	2	–
IVTDMD	3	–
Lignin	4	–
NDF	5	–
Panicle biomass	6	2
No. tillers	–	1

Traits are described from field-grown populations.
*Significant regression.
–, traits with $R^2 < 0.1$ are not ranked.

demonstrate the need to quantify how different selec-tion pressures aimed at reduced biomass recalcitrance or increased productivity will influence a broad range of pathogen types, specifically including viruses and the phloem-feeding insects that transmit many of them.

In selection of biofuel crops, the aim of reducing lignin and other constituents that contribute to biomass recalcitrance has spurred conversations about potential consequences for stem strength and plant hardiness (Casler *et al.*, 2002; Pedersen *et al.*, 2005). High lignin levels are associated with decreased forage intake (For-bes & Garrigus, 1950) and decreased attractiveness to grazers (Cherney *et al.*, 1990; Aregheore *et al.*, 2006). Thus, reducing lignin might increase plant vulnerability

to mammalian and other grazers. In switchgrass, how-ever, we found that growth rates better predicted aphid preferences and virus susceptibility than did lignin levels. Similarly, foliar lignin levels did not explain plant resistance to another phloem-feeder, *Blissus insu-laris* (Hemiptera: southern chinch bug), in St. Augusti-negrass (Rangasamy *et al.*, 2009).

The linkage we found in switchgrass between fast growth rates, virus susceptibility, and aphid attractive-ness may be only partly explained by an understanding of trade-offs between growth and defense in plant allocation. In general, fast growth is associated with short life-cycles and reduced investment in defense, such as lignin and polyphenols, whereas slow growth is associated with longevity and greater investment in defenses (Coley *et al.*, 1985). In *Arabidopsis thaliana*, for example, individuals with natural constitutive expres-sion of a defense pathway were slower growing than plants lacking this allele (Todesco *et al.*, 2010). In grasses, fast growth is one hallmark of a 'quick return' species that may disproportionately serve as effective pathogen hosts (Cronin *et al.*, 2010). However, the trait analysis presented here demonstrates that, in switch-grass, growth rates and foliar digestibility measures (including lignin concentration) are not strongly linked and, in fact, represent dominant components on two separate axes that are orthogonal to each other. Selec-tion pressures have acted on both these axes to varying degrees in different cultivars. A key priority for future research, therefore, is to elucidate mechanisms that underlie the linkages between growth rates and differ-ent types of plant defense, both physical and chemical.

That growth rates per se appear to influence virus and aphid susceptibility may be best understood in the context of how these organisms interact with hosts. Whereas lignification strongly influences the quality of tissue that grazers must chew and digest, phloem-feeding insects, such as aphids, must only puncture cell walls to gain access to phloem (Tjallingii & Hogen Esch, 1993) and then digest liquid phloem contents. As a result, fast host growth rates may be important promoters of fitness in phloem-sucking insects (Grechi *et al.*, 2008; Sauge *et al.*, 2010) that can benefit from increased rates of nutrient translocation (White, 1993). In parallel, increased host growth rates may benefit viruses by providing more opportunities for replication and expression as a function of their dependence on the host's cellular machinery (Whitham & Wang, 2004). Plant resistance to B/CYDV infection may be a function of interactions with aphid vectors, through effects on virus transmission, and with viruses, through interference with viral replication (Qualset *et al.*, 1990).

Risk assessment and importance of landscape context

In our study, the majority of the switchgrass fields surveyed showed some level of B/CYDV infection, suggesting that these fields have potential to serve as virus reservoirs. This potential is underscored by recent discoveries of novel marafi-like viruses (Tymoviridae) in similar fields (Agindotan *et al.*, 2010, A. Schrotenboer & C. Malmstrom, unpublished results). The effects of these viruses on biofuel crop yield and their potential for spillover into other regional crops are therefore of great interest.

B/CYDVs have been shown to significantly reduce biomass production in California native perennial grasses (Malmstrom *et al.*, 2005a) and in *Miscanthus sinensis*, an Asian perennial grass under consideration as a biofuel feedstock (Huggett *et al.*, 1999), and marafiviruses can cause substantial yield loss in maize (Gámez, 1976; Gámez, 1983). However, the influence of these species on tallgrass prairie species is poorly understood. Based on current knowledge, it is reasonable to predict that consequences of virus interactions with native prairie grasses may decrease productivity as well. Consistent with this prediction, insecticide application to switchgrass increased yields by 11% (C. Gratton, unpublished results). Nonetheless, it is important to consider the alternative that virus influence on native grasses might be neutral to positive. Although little is understood about plant–virus interactions in nature, a few studies indicate that some viruses promote fitness in stressful environments (Gibbs, 1980; Márquez *et al.*, 2007; Xu *et al.*, 2008).

The larger concern is the potential for spillover from biofuel grasses to food crops and other vegetation types that are virus-susceptible. Because B/CYDVs and many other plant viruses are transmitted by sucking insects that can travel long distances (Irwin & Thresh, 1990), the development of biofuel grasses as virus reservoirs could change pathogen dynamics in a broad area. The likelihood of this occurring will be determined by a series of interacting factors, including vector transmission efficiency and dispersal patterns and the counteracting influence of biocontrol services in perennial stands (Gardiner *et al.*, 2010; Landis & Werling, 2010), which merit further investigation.

Here, our finding of linkage between growth rates and virus susceptibility in switchgrass highlights the potential for biofuel cultivar selection to inadvertently increase virus reservoir capacity. Disease amplification by fast-growing plant species has been documented in other ecosystems. For example, fast-growing, susceptible annual hosts can increase B/CYDV incidence in other, less susceptible species (Power & Mitchell, 2004; Malmstrom *et al.*, 2005a, b). Proactive steps to prevent or mitigate pathogen susceptibility before widespread use of newly developed biofuel crops could aid in averting potential unintended consequences of biofuel production on pathogen dynamics.

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