Bioenerg. Res.
DOI 10.1007/s12155-012-9263-6

Characterization of the Rust Fungus, *Puccinia emaculata*, and Evaluation of Genetic Variability for Rust Resistance in Switchgrass Populations

Srinivasa Rao Uppalapati · Desalegn D. Serba · Yasuhiro Ishiga · Les J. Szabo · Shipra Mittal · Hem S. Bhandari · Joseph H. Bouton · Kirankumar S. Mysore · Malay C. Saha

Abstract Several fungal pathogens have been identified on ornamental and native stands of switchgrass (*Panicum virgatum* L.). Diseases of switchgrass, particularly rust, have been largely neglected and are likely to become the major limiting factor to biomass yield and quality, especially when monocultured over a large acreage. Based on teliospore morphology and internal transcribed spacer-based diagnostic primers, the rust pathogen collected from switchgrass research fields in Oklahoma was identified as *Puccinia emaculata*. Furthermore, to identify genetically diverse source(s) of rust resistance, several switchgrass genotypes from both upland (cv. 'Summer' and 'Cave-in-Rock') and lowland (cv. 'Alamo' and 'Kanlow') ecotypes were evaluated in Ardmore, Oklahoma during 2008 and 2009 and in growth chamber assays. Field and growth chamber evaluations revealed a high degree of genetic variation within and among switchgrass cultivars. In general, Alamo and Kanlow showed moderate resistance to *P. emaculata*, while Summer was highly susceptible. Distinct ecotypic variations for reactions to rust were also prevalent with the lowlands maintaining a high level of resistance. These results suggest the potential for improvement of rust resistance via the selection of resistant individuals from currently available cultivars. Further, the selection pressure on the pathogen would also be reduced by employing several rust resistant cultivars in production-scale situations.

Keywords Switchgrass rust · *Puccinia emaculata* · Host resistance · Genetic variability · Internal transcribed spacer (ITS) · *Panicum virgatum*

Electronic supplementary material The online version of this article (doi:10.1007/s12155-012-9263-6) contains supplementary material, which is available to authorized users.

D. D. Serba · H. S. Bhandari · J. H. Bouton · M. C. Saha
Forage Improvement Division, Samuel Roberts Noble Foundation, Ardmore 73401, USA

Y. Ishiga · S. Mittal · K. S. Mysore
Plant Biology Division, Samuel Roberts Noble Foundation, Ardmore, OK 73401, USA

L. J. Szabo
Cereal Disease Laboratory, Agricultural Research Service, U.S. Department of Agriculture, St. Paul, MN 55108, USA

M. C. Saha (✉)
Forage Improvement Division, Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA
e-mail: mcsaha@noble.org

Present Address:
S. R. Uppalapati (✉)
Trait Discovery & Technologies, E.I. Dupont India Private Ltd., Hyderabad, A.P. 500078, India
e-mail: Srinivasa.R.Uppalapati@ind.dupont.com

Present Address:
H. S. Bhandari
Department of Plant Sciences, University of Tennessee, 346 Plant Biotech Building, Knoxville, TN 37996, USA

Introduction

Switchgrass (*Panicum virgatum* L.) has been identified as a dedicated herbaceous bioenergy crop for cellulosic biofuel production [1–3]. Switchgrass is a C4 perennial grass which is native to North American grasslands and tall grass prairies. Over the past 15 years, research has been initiated to improve yields and feedstock conversion properties as well as to enable synthesis of valuable co-products [2, 3]. However, efforts to improve genetic resistance to diseases to switchgrass and other bioenergy crops are limited. Stewart and Cromey [4]

Published online: 14 October 2012

encountered the scarcity of references on bioenergy crop diseases, accentuated the threats disease may pose on the economics of bioenergy crops production, and suggested the development of resistant cultivars and application of integrated agronomic management strategy to control diseases.

High genetic diversity is apparent in switchgrass and has resulted in the emergence of two distinct ecotypes (lowland and upland) [5]. Lowland ecotypes are predominantly tetraploids with $2n=4x=36$ and well adapted to the southern parts of the USA [6]. These ecotypes have been reported to grow faster and yield higher dry mass than the upland ecotypes in the southern and central USA. Although the upland types produce less biomass, they are well-adapted to semi-arid climates and are predominately octaploids with $2n=8x=72$ [7]. Commercial cultivars suitable for each US geographic zone have been released [8]. The lowland cultivars, Alamo and Kanlow, were selected for deeper south and mid-latitudes, respectively. Conversely, the upland commercial cultivars Summer, Trailblazer, Sunburst, Blackwell, Pathfinder, and Cave-in-Rock (CIR) were developed for northern regions of the USA. These research efforts indicated the need for deployment of switchgrass cultivars for specific geographic niches [9].

Diseases of switchgrass have been largely neglected, but could be a major limiting factor with respect to seedling establishment, biomass quality, and yield, especially if planted in monocultures. Several different fungal pathogens have been recorded on switchgrass [10–12]. There is limited knowledge on the potential impact of these diseases on biomass yield on switchgrass grown in large acreages. Preliminary studies have shown that potentially damaging diseases include rust caused by *Puccinia emaculata* [13], leaf spot caused by *Bipolaris oryzae* [14], and smut caused by *Tilletia maclaganii* [11, 12]. Biomass losses of 1.7–40.1 % to smut in Iowa have been reported [15]. More recently, anthracnose caused by *Colletotrichum navitas* has been reported on selected upland and lowland switchgrass cultivars [16]. Three rust pathogens, *P. emaculata*, *Puccinia graminis*, and *Uromyces graminicola*, are reported on switchgrass [10]. Of these three fungi, *P. emaculata* is most widely distributed causal agent of rust in switchgrass [11, 12, 17, 18]. Cornelius and Johnston [17] have conducted the earliest studies on switchgrass rust and classified the lowland cultivars as rust resistant and the upland cultivars as rust susceptible. High incidence of *P. emaculata* infection was reported in year 2000 and 2001 in cultivated or research fields in Iowa and North Dakota [12, 13] and more recently in Arkansas and Tennessee [18, 19]. This present study conducted in Oklahoma, one of the major states earmarked for cellulosic bioenergy production, was also severely impacted by switchgrass rust in the past 5 years.

As experience from wheat breeding indicates, the dynamics of rust pathogen due to genetic recombination and long distance travel of spores by wind poses a constant threat to the resistant genes with reports of crop failures in different parts of the world [20–22]. Developing durable resistance through breeding [21] is a viable strategy which needs to be adopted for switchgrass improvement.

The large acreage of switchgrass monoculture which is expected in southern USA may emerge as hotspots for evolution of more virulent forms of *P. emaculata*. There is an urgent need for development of a breeding program to develop and deploy durable disease resistant switchgrass cultivars. More detailed pathogen surveys and a national program to screen local populations are required to identify cultivars that are resistant to rusts. In addition, breeding efforts are required to develop synthetic cultivars for regional deployment with increased biomass and rust resistance. It is not clear if new virulent strains have emerged with increased plantings of switchgrass in experimental and large-scale pilot plantations in southern states of USA since the studies conducted by Gustafson et al. [13] in South Dakota. Furthermore, it is not clear if upland and lowland cultivars planted in a different geographical location (South Dakota vs. Oklahoma) have different outcomes in rust reactions. Thus, the objectives of this investigation were to identify the pathogen of importance, develop diagnostic markers, and evaluate the variability in switchgrass cultivars including the commercially important lowland cultivars, Alamo and Kanlow, and two upland cultivars (Cave-in-Rock and Summer) that were also evaluated earlier by Gustafson et al. [13] in a different location.

Materials and Methods

Switchgrass Rust Maintenance and Inoculation Procedures

Several isolates of the switchgrass rust were collected from Ardmore, Oklahoma from 2007 to 2011 (isolates named 4-3, 6-2, 6-3, 2-2, and 2-3) and were maintained on a susceptible upland switchgrass genotype. The rust pathogen isolates named above were based on single site collection and no single urediniospore isolation culture conducted. A mixture of rust spores collected from the field plants were used as inoculum in our growth chamber studies. Fresh urediniospores were collected using a gelatin capsule spore collector designed by the Cereal Disease Laboratory, St. Paul, MN, USA and suspended in distilled water with 0.001 % Tween 20. The seedling leaves were spray inoculated with 1×10^5 spores ml^{-1} (0.001 % Tween 20) using an artist air-brush (Paasche Airbrush Co., Chicago, IL, USA) set at 2 PSI with a portable air-pump (Gast Mfd Co., Benton Harbor, MI, USA) for uniform spore deposition. The spray-inoculated plants were incubated in the dark for 18 h at 100 % humidity in a dew chamber. The plants were subsequently moved to a growth chamber maintained at 80 % relative humidity, 29:22 °C day/night temperature, 16 h photoperiod, and photon flux density 150–200 μmol m^{-2} s^{-1}.

DNA Extraction, Recombinant DNA Techniques, and DNA Sequencing

For genomic DNA preparations, the fungal material from infected switchgrass plants, approximately 10 mg of urediniospores of each isolate, was incubated in 200 μl of DNA extraction buffer containing 100 mM Tris–HCl (pH 8.0), 20 mM ethylenediaminetetraacetic acid (EDTA), 0.5 M NaCl, 1 % sodium dodecyl sulfate, and 1.0 % β-mercaptoethanol at 75 °C for 1 h in microcentrifuge tubes. Samples were mixed with equal volume of Tris-saturated phenol and centrifuged at 12,000×*g* for 10 min to pellet debris. The supernatants were transferred to new tubes and equal volume of chloroform/isoamyl alcohol (24:1, vol/vol) was added, and the samples were centrifuged as above. The aqueous phase was transferred to a new tube, and 1/10 volume of 3 M NaOAc (pH 5.5) was added with equal volumes of isopropanol. Samples were mixed and centrifuged at 5,000×*g* for 10 min, and the supernatant was decanted. After washing with 70 % EtOH, the pellets were allowed to air dry. DNA pellets were resuspended in 50 to 100 μl of Tris–EDTA buffer. The amount of DNA was quantified by spectrophotometry using a NanoDrop® ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) and confirmed by agarose gel electrophoresis.

The nuclear ribosomal internal transcribed spacer (ITS) region and the 5′-end of the large subunit were amplified from *P. emaculata* Oklahoma isolates by polymerase chain reaction (PCR) in 50 μl of reaction mixture with *Takara Ex Taq* Polymerase (Takara, Kyoto, Japan). Primer pairs used for amplification were ITS1rustF10d [23] and RUST1 [24]. The PCR products were cloned into the TA cloning vector pGEM T-easy (Promega, Madison, WI, USA). Four to six clones from independent PCR amplifications were sequenced for each *P. emaculata* Oklahoma isolate. Plasmid DNA was extracted with a plasmid isolation kit (Wizard; Promega) according to the manufacturer's guide. DNA was labeled with a sequencing kit (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction; Applied Biosystems, Inc., Foster City, CA, USA), and nucleotide sequence was determined by capillary electrophoresis with a genetic analyzer (ABI Prism 3100; Applied Biosystems).

P. emaculata-Specific Primer Design and PCR

The primer sets of ITS1rustF10d and RUST1 were used for amplification of ITS fragments and ITS1rustF10d and ITS1-rustR3c to amplify rust-specific fragments in ITS1 region [23]. Sequence alignments of ITS region of *P. emaculata* Oklahoma isolates collected from 2007 to 2010 and other *Puccinia* spp. were used to design primer sets for amplification of *P. emaculata*-specific fragments. The primer sets of SGR-SP1-FW and SGR-SP1-RV amplify *P. emaculata*-specific fragments. Primers used in this study are shown in Table 1. The DNA isolated from the germinating urediniospores of different rust pathogens collected from respective host species were used to design and check the specificity of *P. emaculata*-specific primers. These included *Puccinia andropogonis* on comandra (*Comandra umbellata* (L.) Nutt.); *P. andropogonis* on buckeye (*Aesculus* spp.); *P. andropogonis* on lupine (*Lupinus perennis* L.); *P. andropogonis* on prickley ash (*Aralia spinosa* L.), penstemon (*Penstemon digitalis* Nutt. ex Sims.), and big bluestem (*Andropogon gerardii* Vitman); *U. graminicola* and *P. emaculata* on switchgrass (*P. virgatum* L.); *P. graminis*, *Puccinia striiformis*, and *Puccinia triticina* on wheat (*Triticum aestivum* L.); and *Puccinia coronata* on oat (*Avena sativa* L.). The DNA was isolated as described [23].

For PCR with individual primer pairs, each reaction mixture contained 2 μl (~1 ng) of diluted genomic DNA template, 1× *Takara Ex Taq* buffer, 250 μM dNTP, 0.5 μM each primer, and 0.5 U *Takara Ex Taq* Polymerase in a total volume of 50 μl. PCR amplification conditions were 5 min of denaturation at 95 °C, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 m and a final extension step of 72 °C for 10 min. A sample of 10 μl of product from each PCR was confirmed by agarose gel electrophoresis.

Field Experiment

Two lowland cultivars, Alamo and Kanlow, and two upland cultivars, Cave-in-Rock and Summer, were selected from switchgrass diversity collections. Ten seedlings from each cultivar were randomly selected from germinated seedlings pool. Seed treatment, germination, and seedling management were as described in Bhandari et al. [25]. Each seedling was grown in a 3.78-L (one gallon) pot in a greenhouse

Table 1 Primers used for polymerase chain reaction in this study

Primer	Sequence	Target species	Reference
ITS1rustF10d	5′-TGAACCTGCAGAAGGATCATTA-3′	All rust	[23]
RUST1	5′-GCTTACTGCCTTCCTCAATC-3′	All rust	[24]
ITS1rustR3c	5′-TGAGAGCCTAGAGATCCATTGTTA-3′	All rust	[23]
SGR-SP1-FW	5′-TTACCCTCCCCTTTTATTCTTAAA-3′	*P. emaculata*	This study
SGR-SP1-RV	5′-GAAGTCTCTTTCTCAACAACAAAATTTTAC-3′	*P. emaculata*	This study

chamber at 32:21 °C day/night temperature and 16 h photoperiod. After 150 days of seedling growth, each genotype was clonally split into five ramets, four for planting in field and one to maintain in greenhouse. The ramets were planted in Normangee clay loam soil with pH=5.7 in Ardmore, OK (34°11′N; 97°05′W) on August 1, 2007 following a randomized complete block design (RCBD) with four replications. Standard plant management practices were carried out as described in Bhandari et al. [25]. The field rust infection was visually scored on a scale of 1 to 9, 1=resistant and 9=susceptible, originally described by McNeal et al. [26]. Rust data were collected for each of the genotypes from all four replications at post-heading stage on July 19 and July 12 in 2008 and 2009, respectively.

Growth-Chamber Experiments

To validate the field rust evaluation results, two independent experiments were conducted with seedlings germinated at two time intervals (labeled as experiment 1 and experiment 2) and were challenged with the same mixed isolate of rust spores collected from the Ardmore fields by artificial inoculation under growth chamber conditions.

Experiment 1

Seeds of four cultivars (Alamo, Kanlow, Cave-in-Rock, and Summer) were obtained from commercial sources. For each cultivar, 40 seedlings were randomly selected from germinating seeds and grown in 15 cm plastic cones (model D16; Stuewe and Sons, Inc., Tangent, OR, USA) in a greenhouse chamber for 30 days under the conditions described above. After 30 days of growth, each cone was arranged in 50-hole plastic trays (model D50T; Stuewe and Sons, Inc., Tangent, OR, USA) following a RCBD with two replications. A tray of 20 seedlings of each cultivar was considered as the experimental unit. The trays were randomized following the experimental design and spray-inoculated with rust urediniospores collected from the Ardmore field. Disease symptoms on fully expanded leaves were scored at 7, 10, 14, and 20 days post-inoculation (pi). Each individual genotype was scored following the 1–9 scale (Supplementary Fig. 1). Symptoms at 14 days pi produced the most reliable disease scoring, and therefore, all the subsequent disease scorings in the growth chamber were carried out at 14 days pi. After screening these seedlings, the selected resistant/susceptible genotypes were sprayed with a fungicide (Terraguard SC, Chemtura USA Corporation, Middlebury, CT, USA) to rescue these genotypes for future use.

Experiment 2

For the second growth-chamber experiment, 100 seedlings were raised from commercial seed of each of the four cultivars (Alamo, Kanlow, Summer, and Cave-in-Rock). The 100 genotypes were divided into four groups of 25 random genotypes that were used as replicates. Conditions for seedling growth, experimental design, and screening protocols were similar as described in experiment 1. Switchgrass seedlings were grown in 36 celled (9×4) plastic trays, and the cultivars were arranged in a randomized block design. Five-week-old seedlings at E2 growth stage [27] were sprayed with rust urediniospores as described above, and disease symptoms on fully expanded leaves were scored at 14 days pi.

Data Analyses

For the field data analysis, we used a randomized complete block design with four replications where 10 genotypes of each cultivar were assigned to block. Prior to rust data analysis, we created a new response variable that was continuous in nature that described the total rust response for each combination of cultivar, environment, and block. This approach used each genotype (n=10) within a cultivar, environment, and block as the sampling unit from which we developed our composite response variable (i.e., total rust). The first step to calculating total rust score was to multiply each level of rust score (1 to 9 in increments of 2) by the number of genotypes with that level of rust score. Next, we summed the five values of rust for each environment, cultivar, and block combination. We used a general linear mixed model (GLMM) in SAS 9.2 ® (SAS Institute, Inc., Cary, NC, USA), which was carried out using the MIXED procedure. The GLMM was used to test for the effects of cultivar, environment, and a cultivar×environment interaction (independent variables) on total rust (response variable). We accounted for the block design by specifying block as a random effect. We also used a Kenward–Roger denominator degrees of freedom adjustment to account for random effects and correlated errors [28, 29]. We visually examined plots of residuals, which indicated that the data were approximately normally distributed. When we observed a significant F test for the cultivar×environment interaction, we partitioned the data by environment using a one-way analysis of variance to examine the influence of cultivar on total rust (similar to the GLMM described above). When a significant F test occurred for cultivar, we used least squares means when conducting multiple comparison tests among cultivars within environment. Last, a variance component analysis and a completely random effects model (generalized linear mixed model) were used to partition the variance attributable to year, block, cultivar, year×cultivar, genotype

within cultivar, and extraneous factors (i.e., residual error). To assess the contribution of genotype within cultivar, we used the original raw data that described the level of rust for each individual genotype within cultivar. We report the percentage contribution of each variable to total rust production. The growth-chamber experiments data were analyzed using each genotype score as an independent sample of the cultivars. DNA sequence alignment and phylogenetic analysis of *Puccinia* spp. was performed as described in Jafary et al. [30].

Results and Discussion

General Observations of Rust Occurrence and Severity Prior to Present Study

Severe rust outbreaks were observed in a diverse switchgrass collection being studied at Noble Foundation Research Fields in Ardmore (34°11′N, 97°05′W) during 2007–2010. Several switchgrass accessions obtained from USDA-GRIN were evaluated as part of the feedstock improvement program at Ardmore, Oklahoma, and tremendous variation with respect to rust resistance or susceptibility was observed. In general, upland germplasms were more susceptible to rust than lowland germplasms. Although biomass yield loss was not quantified, several genotypes of Alamo also showed a high degree of susceptibility, and almost all the foliage was severely affected by rust resulting in early leaf senescence (Fig. 1a, b). Highly resistant plants without any pustule formation on leaves were also found in Alamo (Fig. 1c, d).

Fig. 1 Genetic diversity with respect to rust resistance or susceptibility observed in Oklahoma field. **a**, **b** A field picture of an Alamo genotype showing early senescence due to severe infection (**a**) and heavy sporulation on leaf (**b**). **c**, **d** An Alamo genotype showing significant resistance (**c**), with healthy leaf without any sporulation on surface (**d**). Photos were taken on August 4, 2009

Morphological and Molecular Characterization of *P. emaculata*

Multiple collections of switchgrass rust urediniospores from different locations in Oklahoma were made in the past 5 years. A single site collection was maintained as an isolate, since single spore isolation was not conducted yet. As part of preliminary observations, different preservation and dormancy-revision procedures were tested. Dehydrated (overnight) spores flash-frozen and stored at −80 °C were found to show the highest viability (data not shown). Dormancy-revision and infection rates were high from the spores that were given a brief thermal shock (42°C, 5 min) and rehydrated for 12 h. Uredinia collection were isolated from the field as described in materials and methods, and a protocol developed with suitable temperature and humidity for rust development in growth chamber. Diseased switchgrass produced numerous uredinia containing single-celled urediniospores on the leaf and stem within 14 days pi (Fig. 2a, b). The urediniospores collected from Oklahoma on switchgrass were similar in size and morphological characters to those of *P. emaculata* Schw., reported on *Panicum* spp. [31] and on switchgrass agronomic fields more recently in Tennessee [19] and Arkansas [18]. Uredinia were caulicolous and cinnamon, whereas the urediniospores were ornate (Fig. 2c), ellipsoidal or globoid with thick cell walls (Fig. 2c, d). Abundant teliospores were isolated from Oklahoma fields in late August to September and after long incubation in growth chamber experiments. Teliospores were two-celled, narrowly obovoid, and were dark brown

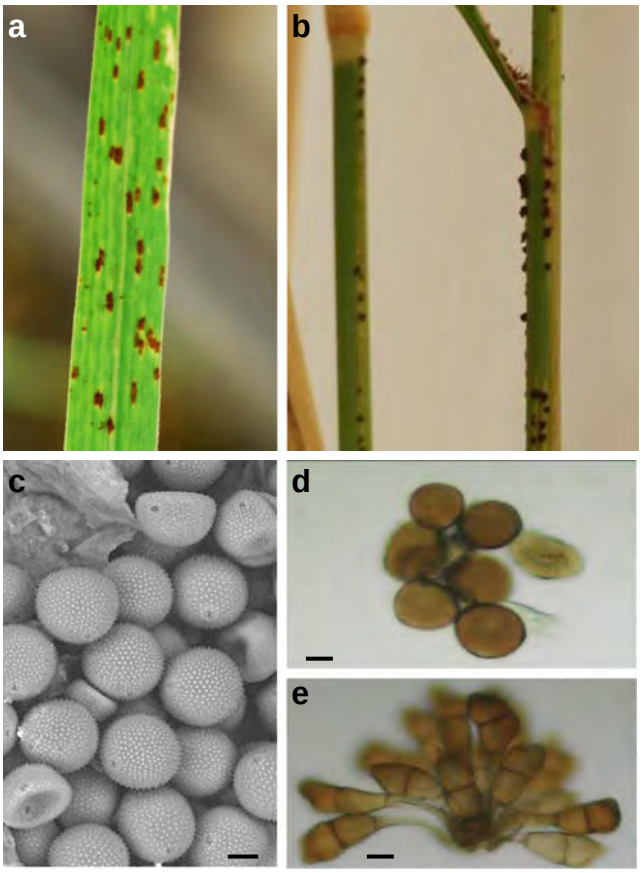

Fig. 2 Morphological characterization of switchgrass rust caused by *P. emaculata* in Oklahoma. **a**, **b** Rust symptoms from the field isolate in the lab. Notice the high rate of rust infection on both leaf (**a**) and stem (**b**). **c**, **d** Morphology of urediniospores observed using SEM (**c**) and light microscope (**d**). **e** Two-celled ellipsoid or obovoid, pigmented telia of *P. emaculata*. *Scale bar* (**c**–**e**)=10 μM

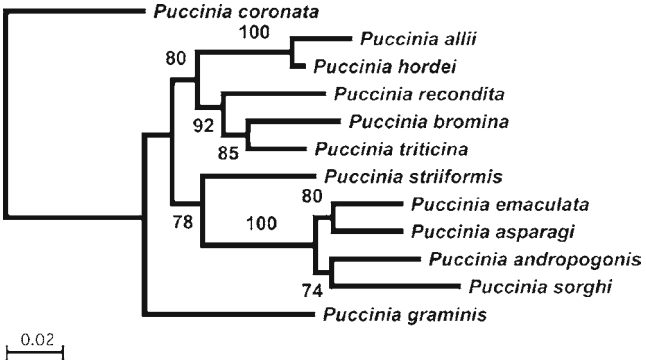

Fig. 3 Neighbor joining dendogram based on the analysis of nuclear rDNA ITS sequences of *P. emaculata* and selected rust fungi. *Numbers* alongside branches indicate percentage of congruent clusters in 1,000 bootstrap trials. Only bootstrap values above 75 % are shown. *P. coronata* was used as an out-group

in color (Fig. 2e). The two-celled teliospore morphology confirmed the identification of the rust fungus on switchgrass in Oklahoma as *P. emaculata* [31].

The ITS sequence from the collections made in Oklahoma was nearly identical to the ITS sequences from other collections of *P. emaculata* but distinct from sequences for *P. graminis* and *U. graminicola* (data not shown). Phylogenetic analysis compared the ITS sequence of *P. emaculata* with sequences from a selected set of other rust fungi of grasses (Fig. 3). *P. emaculata* fell into a highly supported cluster (100 % bootstrap) containing *Puccinia asparagi*, *P. andropogonis*, and *Puccinia sorghi*, all three of which have hosts that are native to the New World. It is interesting to note that the closest phylogenetic relative to the switchgrass isolates is *P. asparagi*, a rust pathogen of asparagus. At present, only a limited number of ITS sequences of rust fungi that infect grasses native to North and Central America are available. Additional phylogenetic work needs to be done on rust fungi in order to get a better understanding of their evolution. Furthermore, very little is known about the genetic diversity and population structure of *P. emaculata*. An important component of understanding the population genetics of this rust fungus will be to better define the role that asexual and sexual reproduction plays. *P. emaculata* has a complex life cycle (macrocyclic and heteroecious); however, the aecial host range within *Euphorbia* or other species is not known [32, 33].

PCR-Based Assay for the Detection of *P. emaculata*

Current morphological methods for identification of *P. emaculata* and *U. graminicola* depend on the presence of teliospores (Fig. 2e; Gustafson et al. [13]). It is common to make collections of rust infected switchgrass that contains only urediniospores and not teliospores, therefore making positive identification based on morphology difficult. Taking advantage of the highly variable ITS regions between species, we designed specific diagnostic primers and developed a sensitive PCR-based method which would also allow the quantification of the pathogen load in the infected tissues. A DNA fragment of approximately 1,250 bp spanning 3′ end of the 18S rDNA, ITS1, the 5.8S rDNA, ITS2, and the 5′ end of 28S rDNA was amplified using primer sets of ITS1-rustF10d and RUST1 (Fig. 4a, upper panel, c). The Oklahoma isolates of *P. emaculata* shared greater than 95 % identity in ITS regions, and they were separated into five non-redundant groups. An alignment of the five *P. emaculata* Oklahoma isolates and *P. andropogonis* ITS regions demonstrated the level of divergence between two species (Supplementary Fig. 2).

Primer sets for PCR amplification of *P. emaculata*-specific fragments were designed using sequence alignments of ITS regions of Oklahoma isolates of *P. emaculata* and *P. andropogonis* as a guide to maximize the specificity of the diagnostics assay (Table 1, Supplementary Fig. 2). The rust-specific primer sets ITS1rustF10d and RUST1 amplified a single band of approximately 1,250 bp using DNA isolated

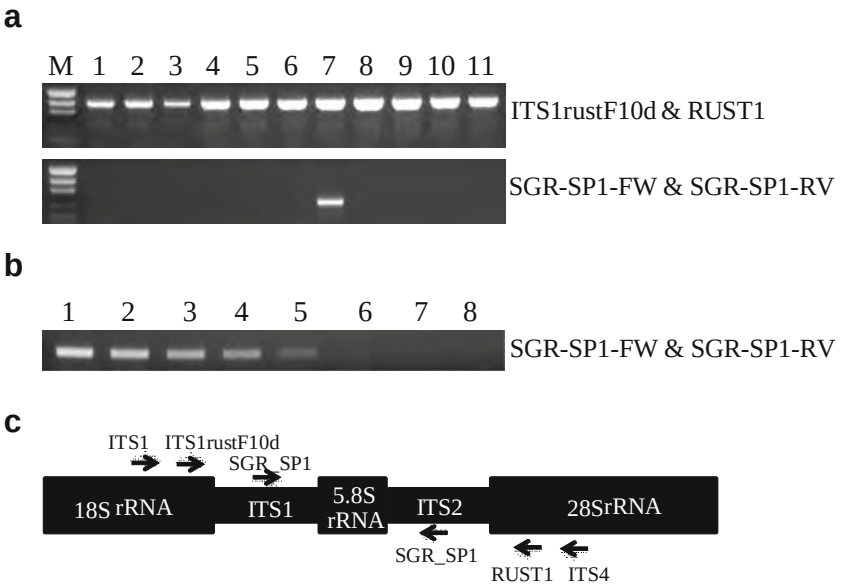

Fig. 4 Amplification of internal transcribed spacer (ITS) primers in rust DNA. **a** Amplicon from RUST1 and ITS1rustF10d primers in DNA from the germinating urediniospores of different rust pathogens collected from respective host species (*top*). *Lane 1*: *P. andropogonis* on comandra, *lane 2*: *P. andropogonis* on buckeye, *lane 3*: *P. andropogonis* on lupine, *lane 4*: *P. andropogonis* on prickley ash, *lane 5*: *P. andropogonis* on penstemon, *lane 6*: *U. graminicola* and *lane 7*: *P. emaculata* on switchgrass, *lane 8*: *P. andropogonis* on big bluestem, *lane 9*: *P. graminis* on wheat, *lane 10*: *P. coronata* on oat, and *lane 11*: *P. triticina* on wheat. *M* represents the lane with size marker. *P. emaculata*-specific primers, SGR-SP, amplified only in rust spores collected from switchgrass (*lane 7*, *lower*). **b** A 10-fold serial dilution of the DNA isolated from urediniospores of *P. emaculata*. As little as 10 pg of template DNA can amplify the desired fragment in 35 PCR cycles. **c** A schematic showing the ITS and locations of the primers used for amplification of rust pathogens

not only from switchgrass infected with *P. emaculata* but also from comandra, buckeye, lupine, prickly ash, and penstemon infected with *P. andropogonis*, *U. graminicola*, *P. graminis*, *P. coronata*, and *P. triticina*, respectively (Fig. 4a, upper panel). As expected, the primer sets SGR-SP1-FW and SGR-SP1-RV designed specifically to detect *P. emaculata* Oklahoma isolates only amplified a single band of approximately 470 bp in a rust-infected switchgrass sample (Fig. 4a, lower panel). To test the sensitivity of the assay, a 10-fold serial dilution of the DNA isolated from urediniospores of *P. emaculata* was used as the template in conventional PCR reactions. The *P. emaculata*-specific primer sets amplified the ITS target sequence in reactions containing as little as 10 pg of template DNA through 35 PCR cycles (Fig. 4b). These primers could be used for a more sensitive and rapid assays using real-time PCR [34]. The specific primers developed in this study are derived from *P. emaculata* isolates collected from limited geographical area. As the acreage in switchgrass cultivation increases, it is possible that additional isolates of *P. emaculata* will be identified and divergent or distinct ITS sequence types may emerge from these isolates demanding a need for developing new primers. However, the primers developed in this study will be helpful for facilitating a highly sensitive PCR-based method for molecular identification, early detection, and understanding of epidemiology of switchgrass rust pathogens.

Evaluations of Switchgrass Cultivars

The analysis of variance using the calculated total rust value found statistically significant among the cultivars, years, and year-by-cultivar interactions (Table 2, Suppl. Fig. 3). The pairwise comparison of least square means showed significant

Table 2 ANOVA for rust scores of four switchgrass cultivars each with 10 genotypes evaluated at Ardmore in 2008 and 2009

Source	2008[a]	2009	Combined	Variance component (%)[b]
Year	–	–	860.81*	6
Replication	9.06*	5.28*	6.38[ns]	1
Cultivar	1,172.17*	189.66*	1,027.07*	14 (39)
Genotype (Cultivar)	–	–	–	23 (61)
Year × cultivar	–	–	334.34*	15
Error	–	–	–	41

ns not significant

*p=0.01

[a] Mean square values calculated for F-statistics

[b] Variance component was calculated using the total variance (5.25) as a denominator for each of the variances components and converted to percentage: figures in parenthesis indicate the percent variability explained by among and within cultivars

differences among cultivars except between Alamo and CIR. The pairwise least square means comparison of the cultivars was significant between all pairs in 2008. In 2009, significant differences were also observed between pairs of cultivars except between Alamo and CIR, Alamo and Summer, and CIR and Summer. Kanlow had the lowest calculated total rust value (composite rust score) both in 2008 (41) and 2009 (62), while higher value was observed in Summer (83) in 2008. In addition to genetics, other factors such as temperature and humidity also played a significant role on rust disease development in switchgrass. In general, scores in 2009 were higher than the 2008 field evaluations (data not presented) which may be due to a favorable weather condition (more rainfall and cooler temperature in 2009 vs. 2008, Supplementary Fig. 4) and greater availability of initial inoculum in 2009. Growth chamber experiments 1 and 2 also showed significant differences in rust severity among the cultivars tested. The average disease score of Summer was higher than the other cultivars in experiment 1 but similar to Cave-in-Rock and Kanlow in experiment 2 (Table 3). Alamo had the lowest disease scores in both experiments.

Frequency distributions of stem rust resistance in switchgrass cultivars obtained through field and growth chamber experiments are presented in Fig. 5. Average rust scores of each genotype obtained in growth chamber experiments 1 and 2 and field experiments in 2008 and 2009 were considered for the frequency distribution. Disease severity was greater on plants grown in the field than the more controlled conditions of the growth chamber, perhaps due to unidentified weather conditions or differences in inoculum density. Several resistant genotypes (scores 1–3) were identified in all cultivars in the growth chamber studies. The frequency distribution of the growth chamber experiments data (Fig. 5a) showed that most of Alamo and Kanlow genotypes had scores ranging from 3 to 6 and had no score of 9. Most Cave-in-Rock genotypes scored as 3–6, with a few genotypes scored from 7 to 9. Summer had the most genotypes with scores of 5 and above. Field evaluation frequency graphs (Fig. 5b) indicated two distinct classes in Alamo (<4 and >6). Majority of the Kanlow genotypes were in 3–4 and 6–7 classes. Cave-in-Rock genotypes had scores 3 and above with the majority having scores of 7–9. All the Summer genotypes had scores of 5 and above. None of the Summer and a single genotype of Cave-in-Rock were found resistant under field evaluations. Conversely, several of the Alamo and Kanlow genotypes demonstrated high levels of rust resistance in the field evaluations.

Table 3 Mean rust scores for four switchgrass cultivars evaluated under growth-chamber with artificial inoculation of the rust spores

Cultivar	Mean rust score	
	Experiment 1[a]	Experiment 2[b]
Alamo	4.2a	4.7a
Kanlow	4.6a	5.0ba
Cave-in-Rock	4.2a	5.4ba
Summer	7.3b	6.2b

[a] The mean is calculated from two replications of 20 random genotypes raised from seeds of each cultivar

[b] The mean is calculated from four replications of 25 random genotypes raised from seeds of each cultivar

Variance component analysis suggested that the variance within cultivars accounted for 61 % of the total variance, while among cultivars variance contributed only 39 %. The differences among the genotypes within each cultivar for rust resistance could be attributable to the heterozygous and heterogeneous nature of the crop. The extensive variation among these genotypes within cultivars for rust resistance was consistent with the large within cultivar variation observed for several other agronomic traits. Significant variation for rust resistance within and among switchgrass populations was also reported in eastern South Dakota [13]. Studies on molecular marker diversity demonstrated wider within population than among population variations in several outcrossing polyploid species, including switchgrass [35], Harding grass (*Phalaris aquatica* L.) [36], orchardgrass (*Dactylis glomerata* L.) [37], and perennial ryegrass (*Lolium perenne* L.) [38]. In switchgrass, within population variation was estimated 79.6 % while among population variation was only 20.4 % [35]. These findings suggested that selecting resistant individuals from commercial cultivars can be an effective approach in developing rust resistant switchgrass cultivar(s).

As genetic uniformity increases vulnerability to diseases [39], multi-lines and cultivar mixtures were advocated for disease management in crop plants [40, 41]. This variation among the genotypes of a switchgrass cultivar is a genetic advantage for buffering the effect of rust on the crop and reduces the selection pressure on the pathogen. The spatial effect and efficacy of genotypic admixtures has been suggested to be greater in production-scale situations than in small-scale experimental plots [40]. This analogy is applicable in switchgrass by synthesizing cultivars from several heterozygous genotypes with different levels of resistance. This will help to keep the disease below threshold for significant yield loss and will insure durability.

Combined analysis across environments demonstrated that Summer is the most susceptible of the four cultivars (Fig. 6a). The average rust score of Summer across years in the field was 7.9, and its composite rust score (83) was significantly higher than the other three cultivars ($p<0.01$). Alamo and Cave-in-Rock had average rust scores of 6.4 (composite rust score of 59.5) and 7.2 (composite rust score of 65.5), respectively. Kanlow had the average rust score of 5.2 (composite rust score of 41.5) which was significantly

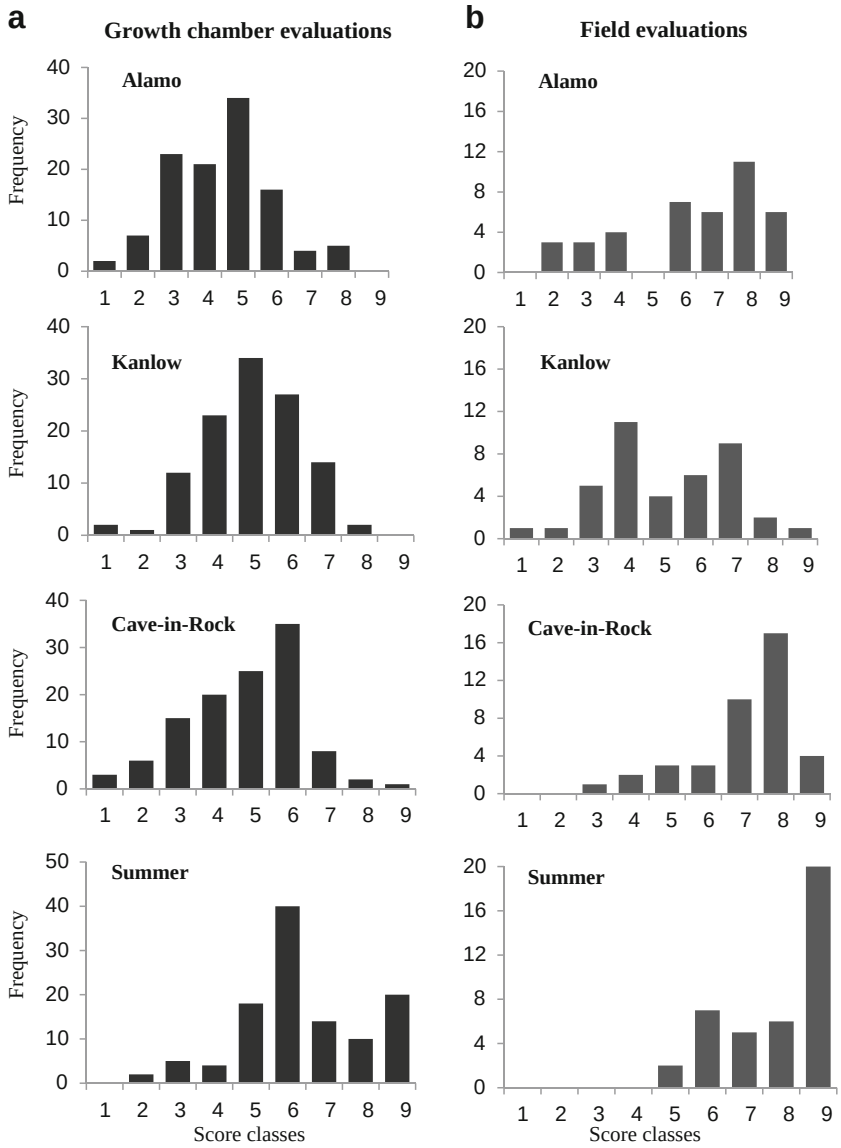

Fig. 5 Distribution of stem rust reactions in switchgrass cultivars evaluated in growth chamber (**a**) and field conditions (**b**). Growth chamber data were summarized across experiment 1 and experiment 2. Field data were summarized across 2008 and 2009 evaluations

lower than the other cultivars (Fig. 6a). In growth chamber experiments, Summer had the highest disease score (6.7), while other three cultivars had lower mean rust scores (Fig. 6a). Some genotypes within Cave-in-Rock demonstrated good rust resistance (Fig. 5 and Supplementary Fig. 5).

There are two ecotypes in switchgrass: upland and lowland [42]. Lowland ecotypes are robust and very productive in the southern USA [43]. Distinct ecotypic variations for rust resistance were observed in the current study. The lowland ecotypes are more resistant than the upland ecotypes (Fig. 6b). Rust reaction in the lowland and upland ecotypes showed similar trends in both the field (disease score 5.79 vs. 7.52) and growth chamber (disease score 4.63 vs. 5.87) studies. Gustafson et al. [13] found that upland collections from Dakota and Nebraska were susceptible while collections from Oklahoma and southern Texas were highly resistant to rust.

Challenges and Prospects for Mitigating (Rust) Disease in Switchgrass

Our results clearly demonstrate the potential disease threat to cellulosic bioenergy crops like switchgrass. The need for developing strategies including integrated pest management (IPM) practices, studies supporting basic and applied plant-microbe interactions, and breeding research to incorporate durable resistance to multiple pathogens in these crops is paramount to their success. Knowledge of existing disease threats on native and commercial cultivars/genotypes provides the knowledge base to hopefully prevent more severe epidemics when propagated in large-scale monoculture that could cause severe economic damage and hamper the sustained supply of quality biomass to local refineries. Plant pathogenic fungi and insect pests could cause severe economic damage to the developing biofuel industry [10, 44,

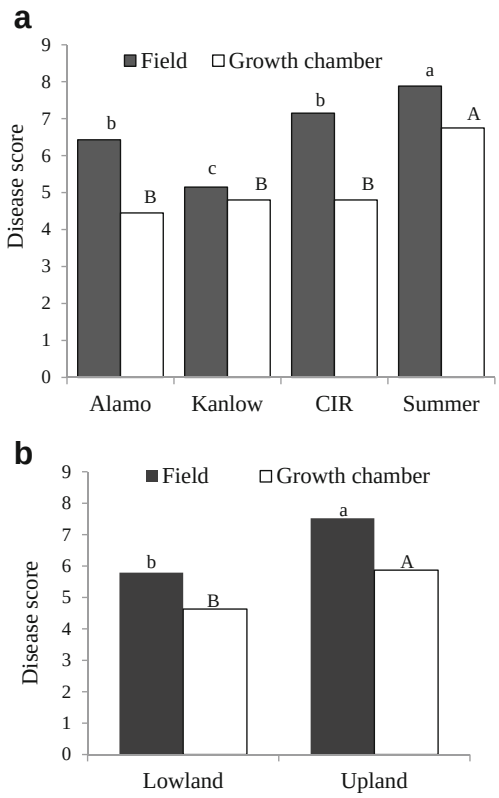

Fig. 6 Mean rust scores of switchgrass cultivars (**a**) and ecotypes (**b**) evaluated at Ardmore field condition during the 2008 and 2009 and two independent growth chamber studies. Means with *the same letter* within a given evaluation are not significantly different ($p<0.05$)

45], thus hampering its success and negating the promise of alternative fuel technologies. Therefore, efforts to integrate existing management practices developed for other crops and new management strategies specific for bioenergy crops and switchgrass have to be developed [4]. The more exhaustive screening conducted in this study and the earlier reports by Gustafson et al. [13] clearly indicated the potential of exploiting genetic variation for switchgrass rust management. Our results suggest the potential for improvement of rust resistance via the selection of plants with different levels of resistance for buffering the population against the effect of rust on the crop. This approach should enhance durability of rust resistance in switchgrass by employing heterogeneous populations in production scale situations by reducing selection pressure on the pathogen.

Alamo and Kanlow showed superior rust resistance. This information has a paramount importance to initiate a proactive approach in the breeding program to incorporate resistance sources in the new cultivars to ensure genetic resistance to longstanding large-scale switchgrass cultivation. This outcome will also help initiate studies to identify the resistance genes involved and their mode of inheritance. Recurrent phenotypic selection procedure and/or intermating of genotypes with contrasting response to disease and subsequent marker-assisted selection can also be implemented to improve rust resistance in switchgrass. The identification of the genes involved would lead to the development of molecular markers that can be used as an aid for indirect selection for rust resistance.

Research should also be focused on robust pathogen surveys to understand population structures and race dynamics. Development of molecular diagnostic markers will help to better understand the epidemiology of fungal diseases of switchgrass and help with the implementation of IPM strategies for control of fungal diseases by early detection and identification. We believe that the present study will improve the research infrastructure to protect the regional bioenergy system (switchgrass) of southern and central USA from rust and emerging diseases. Furthermore, the genotypes with different levels of disease resistance within a given cultivar identified in this study will be a good source for cultivar synthesis to suppress rust disease. In a perennial crop like switchgrass, slow rusting or suppression of rust by planting diverse cultivars is the preferred control strategy. Cultivar mixtures will also provide dilution and barrier effects and keep the selection pressure low and slow the selection of virulent rust races. The deployment of resistant cultivars may help to reduce the inoculum load that may delay disease spread throughout the future switchgrass production regions where there is significant disease pressure.

Acknowledgments We thank Dr. Mark Sorrells for helpful discussions and encouragement and Drs. Kelly Craven and Seonghee Lee for critical reading of the manuscript. We also thank Ms. Karen Flower for fungicide treatments and plant rescue following rust infections. We are very grateful to Dr. Stephen Webb for his help in statistical analysis. This work was supported through funding support from The Samuel Roberts Noble Foundation, Ceres Inc., CA, and Bioenergy Science Center were supported by the Department of Energy. S.R.U also acknowledges financial support through a grant from National Science Foundation-Experimental Program to Stimulate Competitive Research (EPS-0814361) funded to K.S.M.

References

1. Bouton J (2008) Improvement of switchgrass as a bioenergy crop. In: Vermerris W (ed) Genetic improvement of bioenergy crops. Springer, New York, pp 309–345
2. Bouton JH (2007) Molecular breeding of switchgrass for use as a biofuel crop. Curr Opin Genet Dev 17(6):553–558
3. McLaughlin SB, Adams Kszos L (2005) Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. Biomass Bioenergy 28(6):515–535
4. Stewart A, Cromey M (2011) Identifying disease threats and management practices for bioenergy crops. Curr Opin Environ Sustain 3:75–80

5. Vogel KP (2004) Switchgrass. In: Moser LE, Burson BL, Sollenberger LE (eds) Warm-season (C4) grasses. ASA-CSSA-SSSA, Madison, pp 561–588
6. Casler MD (2005) Ecotypic variation among switchgrass populations from the Northern USA. Crop Sci 45(1):388–398
7. Berdahl JD, Frank AB, Krupinsky JM, Carr PM, Hanson JD, Johnson HA (2005) Biomass yield, phenology, and survival of diverse switchgrass cultivars and experimental strains in Western North Dakota. Agron J 97(2):549–555
8. McLaughlin SBJ, Bransby D, Conger B, Ocumpaugh W, Parrish D, Taliaferro C et al (eds) (1999) Developing switchgrass as a bioenergy crop. Perspectives on new crops and new uses. ASHS, Alexandria
9. Casler MD, Vogel KP, Taliaferro CM, Ehlke NJ, Berdahl JD, Brummer EC et al (2007) Latitudinal and longitudinal adaptation of switchgrass populations. Crop Sci 47(6):2249–2260
10. Farr DF, Bills GF, Chamuris GP, Rossman AY (1989) Fungi on plants and plant products in the United States. APS, St. Paul
11. Tiffany LH, Shearer JF, Knaphus G (1990) Plant parasitic fungi of four tallgrass prairies of northern Iowa: distribution and prevalence. J Iowa Acad Sci 97:157–166
12. Gravert CE, Munkvold GP (2002) Fungi and diseases associated with cultivated switchgrass in Iowa. J Iowa Acad Sci 109:30–33
13. Gustafson DM, Boe A, Jin Y (2003) Genetic variation for *Puccinia emaculata* infection in switchgrass. Crop Sci 43(3):755–759
14. Krupinsky JM, Berdahl JD, Schoch CL, Rossman AY (2004) Leaf spot on switch grass (*Panicum virgatum*), symptoms of a new disease caused by *Bipolaris oryzae*. Can J Plant Pathol 26:371–378
15. Thomsen PM, Brummer EC, Shriver J, Munkvold GP (2008) Biomass yield reductions in switchgrass due to smut caused by *Tilletia maclaganii*. Plant Health Prog. doi:10.1094/PHP-2008-0317-01-RS
16. Crouch JA, Beirn LA, Cortese LM, Bonos SA, Clarke BB (2009) Anthracnose disease of switchgrass caused by the novel fungal species *Colletotrichum navitas*. Mycol Res 113:1411–1421
17. Cornelius DR, Johnston CO (1941) Differences in plant type and reaction to rust among several collections of *Panicum virgatum* L. J Am Soc Agron 33:115–124
18. Hirsch RL, TeBeest DO, Bluhm BH, West CP (2010) First report of rust caused by *Puccinia emaculata* on switchgrass in Arkansas. Plant Dis 94(3):381
19. Zale J, Freshour L, Agarwal S, Sorochan J, Ownley BH, Gwinn KD et al (2008) First report of rust on switchgrass (*Panicum virgatum*) caused by *Puccinia emaculata* in Tennessee. Plant Dis 92(12):1710
20. Dean R, Van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD et al (2012) The top 10 fungal pathogens. Mol Plant Pathol 13(4):414–430
21. Leonard KJ, Szabo LJ (2005) Stem rust of small grains and grasses caused by *Puccinia graminis*. Mol Plant Pathol 6:99–111
22. Hovmoller MS, Sorensen CK, Walter S, Justesen AF (2011) Diversity of *Puccinia striiformis* on cereals and grasses. Annu Rev Phytopathol 49:197–217
23. Barnes CW, Szabo LJ (2007) Detection and identification of four common rust pathogens of cereals and grasses using real-time polymerase chain reaction. Phytopathological 97:717–727
24. Kropp BR, Albee S, Flint KM, Zambino P, Szabo L, Thomson SV (1995) Early detection of systemic rust infections of dyers woad (*Isatis tinctoria*) using the polymerase chain reaction. Weed Sci 43 (3):467–472
25. Bhandari HS, Saha MC, Fasoula VA, Bouton JH (2011) Estimation of genetic parameters for biomass yield in lowland switchgrass (*Panicum virgatum* L.). Crop Sci 51(4):1525–1533
26. McNeal FH, Konzak CF, Smith EP, Tate WS, Russel TS (1971) A uniform system for recording and processing cereal research data. Agricultural research service bulletin. USDA, Washington, DC, pp 1–42
27. Shen H, Fu C, Xiao X, Ray T, Tang Y, Wang Z et al (2009) Developmental control of lignification in stems of lowland switchgrass variety Alamo and the effects on saccharification efficiency. BioEnergy Res 2(4):233–245
28. Kenward MG, Roger JH (1997) Small sample inference for fixed effects from restricted maximum likelihood. Biometrics 53(3):983–997
29. Littell RC, Milliken GA, Stroup W, Wolfinger RD (2006) SAS system for mixed models (2nd edition). SAS Institute, Cary
30. Jafary H, Szabo LJ, Niks RE (2006) Innate nonhost immunity in barley to different heterologous rust fungi is controlled by sets of resistance genes with different and overlapping specificities. Mol Plant Microbe Interact 19(11):1270–1279
31. Ramachar P, Cummins GB (1965) The species of *Puccinia* on the *Paniceae*. Mycopathologia 25(1):7–60
32. Cummins GB (1971) The rust fungi of cereals, grasses and bamboos. Springer, Berlin
33. Arthur JC (1934) Manual of the rusts in United States and Canada. The Science Press Printing Co., Lancaster
34. Schaad NW, Frederick RD (2002) Real-time PCR and its application for rapid plant disease diagnostics. Can J Plant Pathol 24:250–508
35. Narasimhamoorthy B, Saha M, Swaller T, Bouton J (2008) Genetic diversity in switchgrass collections assessed by EST-SSR markers. BioEnergy Res 1:136–146
36. Mian MAR, Zwonitzer JC, Chen Y, Saha MC, Hopkins AA (2005) AFLP diversity within and among hardinggrass populations. Crop Sci 45(6):2591–2597
37. Ubi BE, Kolliker R, Fujimori M, Komatsu T (2003) Genetic diversity in diploid cultivars of rhodesgrass determined on the basis of amplified fragment length polymorphism markers. Crop Sci 43(4):1516–1522
38. Huff DR (1997) RAPD characterization of heterogenous perennial ryegrass cultivars. Crop Sci 37(2):557–564
39. Castro A (2001) Cultivar mixtures. Plant Health Instr. doi:10.1094/PHI-A-2001-1230-01
40. Mundt CC (2002) Use of multiline cultivars and cultivar mixtures for disease management. Annu Rev Phytopathol 40:381–410
41. Wolfe MS (1985) The current status and prospects of multiline cultivars and variety mixtures for disease resistance. Annu Rev Phytopathol 23(1):251–273
42. Hultquist SJ, Vogel KP, Lee DJ, Arumuganathan K, Kaeppler S (1996) Chloroplast DNA and nuclear DNA content variations among cultivars of switchgrass, *Panicum virgatum* L. Crop Sci 36(4):1049–1052
43. Casler MD, Vogel KP, Taliaferro CM, Wynia RL (2004) Latitudinal adaptation of switchgrass populations. Crop Sci 44(1):293–303
44. Boe A, Gagne RJ (2011) A new species of gall midge (Diptera: Cecidomyiidae) infesting switchgrass in the Northern Great Plains. BioEnergy Res 4(2):77–84
45. Prasifka JR, Bradshaw JD, Boe AA, Lee D, Adamski D, Gray ME (2010) Symptoms, distribution and abundance of the stem-boring caterpillar, *Blastobasis repartella* (Dietz), in switchgrass. BioEnergy Res 3(3):238–242