TESTING THE EFFICACY OF A POLYESTER BAGGING METHOD FOR SELFING SWITCHGRASS, AND CHARACTERIZING MALE STERILITY IN UPLAND-LOWLAND SWITCHGRASS HYBRIDS AND THEIR S1 PROGENIES

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Abstract: The efficacy of a polyester bagging method required for inbred (selfed seed) development in switchgrass (*Panicum virgatum* L.) was determined. The inflorescences of a total of 26 genotypes in the field, and 14 F1 hybrids in the greenhouse were bagged and putative selfed seeds were collected. The efficacy of the bags was determined based on the genetic origin of the progeny seedlings via eight to 10 SSRs. Two kinds of contaminants: physical (PC) and outcrossing (OC) were observed based on amplified alleles of progeny and their seed parents. Of 39 polyester bags tested in 2012 in the field, 35 bags generated 100 % selfed while the other four generated five PCs. Similarly, of 61 bags tested in 2013 in two field plots, 50 bags produced 100% selfed, four bags produced OCs, five produced PCs and the other two produced both OCs and PCs. No contaminant was identified in the progeny of 18 bags in the greenhouse. High wind speed and accidental errors were detrimental for bagging in the field. In another experiment, pollen viability of the S1 plants developed via selfing of the upland-lowland F1 was tested. Nine upland and nine lowland switchgrass were synchronized by trimming and crossed in isolation to develop F1. Forty nine F1 in the greenhouse and 71 F1 in the field were bagged and produced seeds. Genetic origin was confirmed with two SSRs for the F1 hybrids, and with eight to 10 SSRs for the S1 progeny of the F1 hybrids. About 64 S1 seedlings were potted in the greenhouse to determine pollen viability. The Lugol solution staining did not ratify the pollen viability. Of 64 S1, fresh pollen was collected from 47 and pollen germination was observed in 37. Selfed and open pollinated seeds of the same genotypes were compared. Based on the pollen germination and selfing assessment tests, it was identified that three S1 genotypes had male sterility, seven displayed both male and female sterility, five showed extremely low (G% $\leq$ 2) male fertility, four with female sterility, and 28 had normal male and female fertility under greenhouse conditions.

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CHAPTER I

GENERAL INTRODUCTION

Switchgrass (*Panicum virgatum* L.) is a warm-season perennial grass that has been identified as a potential herbaceous bioenergy species native to the North American prairies [1,2]. Decade long investigations funded by the United States Department of Energy (USDOE) on evaluating potential bioenergy feedstock sources selected switchgrass from screening trials, and recommended it for further investigation as a bioenergy feedstock species [3]. Recommending switchgrass as a bioenergy crop was primarily based on its attributes like wide adaptation, relatively high yield, suitability to marginal land, and low input requirements [4]. Switchgrass is environmentally beneficial, owing to soil erosion protection and greenhouse gas mitigation via carbon sequestration [5]. The ethanol extracted from switchgrass feedstock emits much lower greenhouse gases than by non-renewable gasoline [6]. Nonrenewable energy (petroleum) needed to produce renewable energy from switchgrass feedstock is one fifth of the net energy production from switchgrass [6]. Hence, improvement of switchgrass cultivars for enhanced biomass and chemical composition is pivotal for environmental protection, reduction of fuel cost, and eco-sustainability.

Increased productivity in agronomic crops can be achieved either by exploiting improved cultivars and hybrids, or by adjusting management practices and inputs. Unlike other crops

[rice (*Oryza, sativa* L.), maize (*Zea mays* L.) etc.], adequate breeding methods have not been explored for improving switchgrass. The phenotypic and genotypic recurrent selection that leads to the development of synthetic cultivars has become a common method in switchgrass breeding [7]. However, different methods have been reported for effective selection [8]. Despite an increase in switchgrass breeding and genetics efforts in the last two decades, additional work is required for high biomass yielding cultivars. Single-cross hybrids, which have been the major reason behind increased yield in maize (*Zea mays* L.), have not been reported in switchgrass to date. The single cross hybrid exploits hybrid vigor or heterosis from its homozygous inbred parents and exhibits superior performance. However, inbred development in switchgrass is hindered by its natural mode of sexual reproduction because it is a predominantly out-crossing species.

Switchgrass, a highly self-incompatible species [9], exhibits the anemophilous type of pollination with a low self-pollination tendency. Self-compatibility in tetraploid and octaploid switchgrass has been reported as low as 0.35 % and 1.39 %, respectively [10]. Two types of self-incompatibility (SI), pre-fertilization and post-fertilization are reported as major barriers in self-pollination [10]. The pre-fertilization SI mechanism in switchgrass is similar to the S-Z allelic system that occurs in other members of *Poaceae* [11,10]. The post-fertilization incompatibility is independent of the pre-fertilization one, the former type highly expresses during selfing and the later one manifests during interploidy mating. However, both types of SI are involved in some extent in suppressing self-fertilization [10]. Inbreeding depression is another phenomenon that stifles self-fertilization and subsequent inbreeding [7].

Despite the natural interferences linked to the crop's reproductive system and mode of pollination, recent studies indicated that conditional self-compatibility exists in switchgrass;

thereby the species has potential to develop inbred lines and heterotic F1 in the long term [12,13]. Those studies reported that controlled self-pollination would facilitate the switchgrass genotypes to be self-fertilized. The potentiality of a switchgrass genotype for producing selfed seeds under controlled environments can be regarded as the conditional self-compatibility of the species. The self-pollination can be controlled by providing proper isolation to a parent plant in a field, placing the parent in a growth chamber, bagging its inflorescences etc. However, only the bagging method seems a reasonable choice for breeders considering cost and the practicality of producing selfed seeds for multiple genotypes. However, information on a bagging system that effectively prohibits the extraneous switchgrass pollen is limited. In this study, the efficacy of a polyester bagging method in switchgrass in the field and greenhouse was measured via determining the genetic origin of the progeny obtained from bagged seedheads in 2012 and 2013. The genetic origins of the seeds collected from bagged parents were identified using SSR molecular markers. Knowledge of the bagging methods in regard to their reliability and necessary precautions will minimize the possible contamination in selfed seeds and widen the possibility of commercial inbreds in switchgrass.

Male sterile (MS) breeding lines are another breeding tool used for the successful development of hybrids in a large scale with predetermined parents. Dramatic yield increases in maize in the last 80 years [14] has been contributed by the use of heterosis via incorporation of inbreds and MS mechanism in the breeding system. In the earlier days, before the identification of the MS systems, hand emasculation was a common practice to create female lines in hybrid breeding. Such manipulation was costly and laborious for large scale breeding. Because of the smaller florets size, hand emasculation is challenging in grasses, and no easy method of emasculation has yet been developed for large-scale population [8]. To date, little information is

available on male sterility of the grasses, which could be due to the prevalence of the self-incompatibility in them [12]. No report has been documented on MS in switchgrass so far. Understanding the basis of MS in annual and perennial plants of *Poaceae* is critical in developing MS in switchgrass.

Male sterility (MS) has three common types: cytoplasmic, genetic and cytoplasmic-genetic, of which, the cytoplasmic male sterility (CMS) is widely used in hybrid breeding [15]. The fertility of the CMS lines can be restored by certain nuclear genes known as fertility restorer gene(s) (Rf) [16,17]. CMS has been developed and commercially used in other crops like rice, wheat (*Triticum aestivum* L.), sunflower (*Helianthus annuus* L.), sorghum (*Sorghum bicolor* L.), onion (*Allium cepa* L.), pearl millets (*Pennisetum glaucum* L.), oilseed rape (*Brassica napus* L.), and *Petunia* [15,17]. There are different origins of CMS in plants; it can evolve spontaneously in a population, or by mutations, diseases and stresses, as well as in the progeny derived from wide crosses [17-19]. In perennial ryegrass (*Lolium* spp.), the CMS was identified in intergeneric crosses [20]. Similarly, CMS in the sunflower was discovered in interspecific hybrids of a cross between *Helianthus petiolaris Nutt*. X *Helianthus annus L.* [21,22]. Moreover, Edwardson [23] indicated intergeneric, interspecific, and intraspecific crosses as possible origins of CMS in plants. Therefore, wide crosses are important to create male sterile cytoplasms in plants.

Achieving wide crosses in switchgrass is limited by differential ploidy levels. The interploidy mating in switchgrass is hindered by the post-fertilization incompatibility [10]. However, mating between two ecotypes within the same ploidy level is possible [24]. Since the two ecotypes are genetically diverse [25] and normally represents different heterotic groups [24], the crosses made between two ecotypes may be wide. Hence, the segregation of interecotypic hybrid F1 and their S1 progeny for male sterility is possible. Although upland-lowland

(interecotypic) crosses within the same ploidy (intraploidy) level have been in report, male fertility of the F1 hybrids and their S1 progeny has not been documented.

LITERATURE REVIEW

Switchgrass before 1990s (species for forage and soil conservation)

The history of studies on breeding and genetics of switchgrass is more than a half century long. Previously scientists working on switchgrass aimed at improving the species for livestock and conservation purposes [7]. Exploring locally adapted cultivars in the Great Plains and Midwest states begun in early 1940s [26,24]. Cornelius and Johnston [27] studied the type and rust resistance potentiality of 34 switchgrass accessions collected from four different states: Nebraska, North Dakota, Kansas and Texas in the Great Plains. They observed considerable differences among the accessions morphology and their ability to resist rust. The accessions collected from Nebraska and North Dakota showed lower yield of forage and seed, and matured earlier, whereas the accessions collected from the lowlands of Oklahoma and Texas showed opposing characters; leafy, high seed yield, late maturity and rust resistance [27]. Preliminary evaluation on the variation present in endemic switchgrass strains of Nebraska was made [28]. The substantial phenotypic variations that were observed due largely to genetic differences, so that the crop can be improved by selection [28]. Porter [29] observed the morphological differences in upland and lowland populations. He described the differences in clonal habit, clone size and vegetative growth between the plants of two ecotypes. Physiological differences of water and nitrogen requirements were also detected between the two ecotypes. A flooded

condition and lesser nitrogen seemed better for lowland types, whereas uplands plants grew better on moderate soil water and higher nitrogen [29]. Barnett and Carver [25] observed meiosis in two morphologically distinct groups (upland and lowland). They examined chromosome pairing, presence of off-plate bodies, occurrence of meiotic irregularities and quartet micronuclei. The meiosis in tetraploids was found to be more regular than in octaploids. The ploidy level observed via stained pollen indicated that all the tested lowland plants were tetraploids (2n=36), and most of the uplands were octaploids [25]. Brunken and Estes [30] detected uniformity in tetraploidy of lowland plants, however, the upland plants were octaploids and aneuploids. The cytological and morphological investigations of the plants in sympatric population inferred no genetic interactions between the two ecotypes. The authors also indicated that the natural hybridization between two ecotypes was not common. Therefore, there were no common hexaploids (2n=2x=72), decaploids (2n=2x=90) and duodecaploids (2n=2x=108) [30]. All these studies support the notion of diversity and distinctness that existed between two ecotypes of the switchgrass.

A recurrent restricted phenotypic selection (RRPS) method investigated by Burton [31] in a perennial species *Paspalum notatum* has also become a common breeding method for switchgrass [7]. Vogel et al. [1] effectively applied RRPS selection method on switchgrass to improve in vitro dry matter digestibility and suggested that the Burton's RRPS method can be used for improving the forage quality in switchgrass. Talbert et al. [32] measured variance components and narrow-sense heritabilities for height, maturity, dry weight, in vitro dry matter disappearance (IVDMD), and nitrogen contents in a population. They also estimated phenotypic and genotypic correlation for those traits in the population developed from 33 half-sib families [32]. Moreover, the author also calculated predicted gains for three traits, dry weight, in vitro dry

matter disappearance and nitrogen content. Index selection method was found effective to increase the switchgrass growth yield and IVDMD [33]. Forage quality of the switchgrass was estimated by the grazing performance of the animals like yearling cattle, steers and sheep and by in vitro digestibility [34,35].

Switchgrass after 1990s (Dedicated bioenergy crop)

In 1992, USDOE selected switchgrass as a model herbaceous cellulosic bioenergy crop [7]. Of the 34 herbaceous species screened by seven institutions (1985-1992), six institutions recommended switchgrass as a model bioenergy crop [4]. In recent years, several studies on switchgrass have been conducted focusing on breeding, genetics and genomics approaches targeting the enhanced biomass and other traits relevant to ethanol production and conversion.

Four methods with relevance to the perennial grass breeding: recurrent restricted phenotypic selection (RRPS), half-sib progeny test (HSPT), between and within family selection (BWFS), and recurrent multiple family selection (RMFS) were described taking switchgrass as the model [8]. DNA contents and ploidy level in switchgrass were measured using flow cytometry [36]. On average, 3.1 pg. DNA/nucleus in a haploid genome was found in tetraploid lowlands, whereas DNA content in upland octaploids was about 5.3 pg/nucleus [36]. Another study on the DNA contents and chromosome number in switchgrass reported 3.1 pg/nucleus average DNA in tetraploids and 6.1 pg/nucleus in octaploids [37]. The authors indicated that the tetraploids of the both upland and lowland had similar DNA contents. They further indicated that doubling of chromosomes of the tetraploid might result into evolution of the octaploids; DNA content of octaploids was twice the DNA content of tetraploids and had same basic genome (x=9) [37].

Early molecular characterization began with RAPD markers. Gunter et al. [38] worked on 14 switchgrass populations and revealed the usefulness of markers for understanding the genetic diversity and relatedness in populations. The dendrogram obtained by RAPD-PCR separated two ecotypes into distinct clusters [38]. Casler et al. [39] conducted RAPD analysis on 818 switchgrass genotypes originally developed from 46 prairie-remnant and 11 switchgrass cultivars. The authors reported a similar structural pattern in the populations of prairie-remnant and switchgrass cultivars as defined by the marker variability. Further, spatial variations among the prairie remnants populations had a significant pattern [39]. In an earlier study on genomic organization in switchgrass, Missaoui et al. [40] determined the linkage association in two parents, lowland (Alamo) and upland (Summer) using the single dose restriction fragments (SDRF) segregation in their full sib progeny. The authors estimated the switchgrass genome recombinational length and observed the degree of preferential pairing during segregation of chromosome [40]. Eleven cosegregation groups in Alamo and 16 cosegregation groups in Summer were reported by using 45 SDRF and 57 SDRF, respectively. These markers were later used for finding homology groups by combining two maps [40]. Tobias et al.[41] generated 61,585 expressed sequence tag (EST) from 36,565 clones, which have been used for marker development and producing gene inventories. The analysis of molecular variance (AMOVA) characterized by EST-SSR showed 80 % variability within population and 20 % variability among populations [42]. Cluster analysis also separated the uplands and lowlands ecotypes into separate clusters [42]. Okada et al. [43] constructed linkage maps in a full-sib population developed from a cross between lowland tetraploids, Kanlow x Alamo. The authors used SSR and STS markers to construct the map in nine homologous chromosomes. They also indicated

the disomic inheritance pattern in switchgrass based on the ratio of coupling to repulsion phase linkages [43].

Very recent studies on switchgrass have been focused on the development of molecular and genomic tools. Wang et al. [44] developed 1030 highly polymorphic genomic SSR markers in switchgrass from four enriched genomic SSR libraries. The first switchgrass bacterial artificial chromosome (BAC) library was constructed using a tetraploid switchgrass by partial digestion of genomic DNA with EcoRI [45]. The BAC library made up of 147, 456 clones was constructed which had 120 kb insert size. A genome wide multiple duplex-SSR protocol for genotyping switchgrass has been considered as a quick and less costly method to identify selfed progeny [46]. Wang et al. [47] explored switchgrass transcriptomes using next generation sequencing, where they sequenced the cDNA libraries constructed from the tissues of dormant seeds, germinating seedlings, emerging tillers and flowers. They generated 243, 600 contigs having average length of 535 base pairs. Ersoz et al. [48] discovered single nucleotide polymorphisms (SNP) using EST libraries in switchgrass [48]. Genome-wide association study (GWAS) and genomic selection (GS) were supported by developing full-sib linkage populations and association panels based on SNP [49]. The authors also [49] described the diversity in switchgrass according to distance and ploidy level, which was not identified by RAPD analysis. The authors further reported the reproductively isolated nature of the two ecotypes; distinct clades of different ploidy levels appeared in a Neighbor- Joining (NJ) tree of 29,221 markers. Diploidized nature of tetraploid switchgrass was also observed [49]. Vogel [50] evaluated the two breeding methods: between and within family selection (BWFS) and multistep family selection (MFS) in three switchgrass populations and indicated that the former method is best to increase biomass of the switchgrass.

Heterosis, inbreeding and bagging in switchgrass

Superiority of hybrid performance compared to its parents is referred to as heterosis or hybrid vigor [51]. By far maize hybrids are the best example of the exploitation of heterosis. Taliaferro and Das [51] estimated high and mid parent heterosis in switchgrass based on yield for three years in three populations: NU 94, NU 93 and SL 93. The heterosis was not consistent in three hybrids and also differed year to year. The highest average mid parent (56%) and high parent (39%) heterosis in three years were observed in NU 94. Studies on heterosis in the hybrids of the lowland tetraploid (Kanlow) and the upland tetraploid (Summer) distinctly separated the two parents into two heterotic groups [24]. Development of F1 hybrids in switchgrass using self-incompatibility and conserving the parent plants asexually was suggested [24]. Following this study, Vogel and Mitchell [52] calculated the high parent heterosis in the F1 hybrids obtained from the reciprocal crosses between Kanlow and Summer in swards as 30 to 38%.

Inbreeding in switchgrass has been restricted by self-incompatibility [10]. However, switchgrass sets some selfed seeds upon bagging. Taliaferro and Das [51] bagged 45 S1 plants and obtained S2 seeds from 13 parents in which seven parents produced more than 100 seeds. However, the authors also indicated the possible risk of inbreeding depression on the advanced inbred (S1, S2.) targeted for homozygous lines. A highly self-compatible (61.2%) lowland switchgrass genotype NL94 was reported, of which, 279 seedlings out of 456 as assessed by 12 SSRs were selfed [13]. The author also observed the inheritance pattern among the progeny of NL94 was disomic [13]. However, Liu et al. [12] in another study observed no significant number of selfed seeds from the NL94 genotype when it was grown in a field with other populations. Albeit the switchgrass is highly cross-pollinated in the field, yet it can produce

selfed seeds in the control environment. Thus, conditional self-incompatibility exists in switchgrass [12]. The duplex PCR protocol described by Liu and Wu [46] in switchgrass, which was believed to reduce cost and time for PCR, is effectively used in our Grass Breeding and Genetics Laboratory at Oklahoma State University (OSU).

There is limited information on the performances of bags for selfing switchgrass. Our lab tested four bagging methods previously: paper bags (Lawson paper bags), muslin cloth bags, pillow cases and microfiber bags in switchgrass [53]. However, significant outcrossing contaminants in the progeny seedlings were observed; the paper bags performed best with 61.5% selfed and the microfiber produced the worst with only 20 % selfed seeds [53]. Recently, Vogel et al. (2014) reported the Micro-mesh Fabric pollination bags for controlling pollination in crosses between switchgrass genotypes. However, they did not report the efficacy of the bagging method for avoiding extraneous pollen. Furthermore, the study was conducted in a greenhouse and the bags were not used for selfing [54].

Male sterility in switchgrass

No study has been reported on switchgrass male sterility [55]. However, male sterility is a very common maternally inherent biological phenomenon in many crop species and has been used to generate female lines. The detail use of cytoplasmic male sterility (CMS) for hybrid seed production is described by Duvick [56]. Until 1959, CMS has been established and commercially used in maize, sorghum, onion, petunia and sugar beet (*Beta vulgaris* L.) [56]. The CMS was firstly used in onion for hybridization [56]. CMS has been extensively used in hybrid seed productions of maize [57]. Laughnan and Gabay-Laughnan [57] explained the types, cytology

and ultrastructure of CMS in maize. In maize, CMS types: cms-T, cms-S, cms-C and cms-EP have been recognized in accordance with the restoration pattern [57].

There are limited information regarding MS in perennial grasses [55]. However, some MS strains were identified in perennial ryegrass through intergeneric and interspecific crosses [20,55]. Witt [20] identified large number of MS ryegrass (*Lolium perenne*) genotypes that were developed from a cross between F4 hybrid developed from *L. perenne* x *L. multiforum* and *Festuca pratensis*. The MS plants developed in ryegrass via intergeneric crosses was based on the interaction of a sterile cytoplasm and two recessive genes in the nucleus [20]. MS in annual diploid cereal rye (S*ecale cerale* L.) was also identified in S1 of hybrid between Argentinian 'Pampa' rye (*Secale cerale* L.) and Hohenheim inbred line [58]. Geiger and Schnell [58] concluded that MS occurred in the cereal rye was due to the interaction of cytoplasm of 'Pampa' and nuclear factor of 'Hohenheim line'. Chemical induction has also been considered as a method of creating male sterile lines. Burton and Hanna [59] found male sterile mutants in Pearl millet (*Pennisatum americanum* L.), which was induced by ethidium bromide [59]. Once a male sterile line is created, it can be exploited to develop genetic systems like CMS and nuclear male sterility [17]. The MS lines can also be utilized to control gene flow that occurs via pollen transfer, and to create knockout mutation for the genes involved in floral development [17].

OBJECTIVES

I) To test the efficacy of the polyester bagging method for selfing switchgrass.

II) To characterize male sterility of switchgrass in upland-lowland F1 hybrids and their S1 progenies.

RFERENCES

1. Vogel KP, Haskins FA, Gorz HJ (1981) Divergent selection for in vitro dry matter digestibility in switchgrass. Crop Sci 21:39-41. doi:10.2135/cropsci1981.0011183X002100010011x

2. Sanderson MA, Reed RL, McLaughlin SB, Wullschleger SD, Conger BV, Parrish DJ, Wolf DD, Taliaferro C, Hopkins AA, Ocumpaugh WR, Hussey MA, Read JC, Tischler CR (1996) Switchgrass as a sustainable bioenergy crop. Bioresource Technology 56:83-93. doi:http://dx.doi.org/10.1016/0960-8524(95)00176-X

3. McLaughlin SB, Adams Kszos L (2005) Development of switchgrass (*Panicum virgatum* L.) as a bioenergy feedstock in the United States. Biomass and Bioenergy 28 :515-535. doi:http://dx.doi.org/10.1016/j.biombioe.2004.05.006

4. Wright L, Turhollow A (2010) Switchgrass selection as a "model" bioenergy crop: A history of the process. Biomass and Bioenergy 34:851-868. doi:http://dx.doi.org/10.1016/j.biombioe.2010.01.030

5. Liebig MA, Schmer MR, Vogel KP, Mitchell RB (2008) Soil carbon storage by switchgrass grown for bioenergy. Bioenerg Res 1:215-222. doi:10.1007/s12155-008-9019-5

6. Schmer MR, Vogel KP, Mitchell RB, Perrin RK (2008) Net energy of cellulosic ethanol from switchgrass. Pnas 105:464-469. doi:10.1073/pnas.0704767105

7. Casler M (2012) Switchgrass breeding, genetics, and genomics. In: Monti A (ed) Switchgrass. green energy and technology. Springer London, pp 29-53. doi:10.1007/978-1-4471-2903-5_2

8. Vogel KP and Pedersen JF (1993) Breeding systems for cross-pollinated perennial grasses. Plant Breeding Reviews 11:251-274. Edt. by Jules Janick

9. Bouton JH (2007) Molecular breeding of switchgrass for use as a biofuel crop. Current opinion in genetics & development 17:553-558. doi:http://dx.doi.org/10.1016/j.gde.2007.08.012

10. Martínez-Reyna JM, Vogel KP (2002) Incompatibility systems in switchgrass. Crop Sci 42:1800-1805. doi:10.2135/cropsci2002.1800

11. Lundqvist A (1956) Self-incompatibility in rye. Hereditas 42:293-348. doi:10.1111/j.1601-5223.1956.tb03021.x

12. Liu L, Thames S, Wu Y (2013) Lowland switchgrass plants in populations set completely outcrossed seeds under field conditions as assessed with ssr markers. Bioenerg Res:1-7. doi:10.1007/s12155-013-9367-7

13. Liu L, Wu Y (2012) Identification of a selfing compatible genotype and mode of inheritance in switchgrass. Bioenergy Res, vol 5. Springer-Verlag. doi:10.1007/s12155-011-9173-z

14. Jauhar PP (2006) Modern biotechnology as an integral supplement to conventional plant breeding: The prospects and challenges. Crop Sci 46 :1841-1859. doi:10.2135/cropsci2005.07-0223

15. McVetty PB (1997) Cytoplasmic male sterility. Pollen biotechnology and crop prodcution and improvement. Cambridge University Press Cambridge University.

16. Eckardt NA (2006) Cytoplasmic male sterility and fertility restoration. The plant cell online 18:515-517

17. Kausch AP, Hague J, Deresienski A, Tilelli M, Longo Jr C, Nelson K, Oliver M, Li Y (2012) Male sterility and hybrid plant systems for gene confinement. Plant gene containment:83-98. Edited by Melvin J. Oliver and Yi Li. A john wiley & sons, inc., publication.

18. Schnable PS, Wise RP (1998) The molecular basis of cytoplasmic male sterility and fertility restoration. Trends in Plant Science 3:175-180

19. Budar F, Pelletier G (2001) Male sterility in plants: occurrence, determinism, significance and use. Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie 324 :543-550. doi:http://dx.doi.org/10.1016/S0764-4469(01)01324-5

20. Wit F (1974) Cytoplasmic male sterility in ryegrasses (*Lolium spp*.) detected after intergeneric hybridization. Euphytica 23:31-38. doi:10.1007/BF00032737

21. Leclercq P Cytoplasmic male-sterility in sunflower. 2. Genetic and environmental-effects on fertility restoration. Annales de l amelioration des plantes, ate 1979.vol 2. Inst natl Recherche Agronomique 147 rue de l Universite, 75338 Paris cedex 07, France, pp 201-212

22. Hans Köhler R, Horn R, Lössl A, Zetsche K (1991) Cytoplasmic male sterility in sunflower is correlated with the co-transcription of a new open reading frame with the atpA gene. Molec Gen Genet 227:369-376. doi:10.1007/BF00273925

23. Edwardson J (1970) Cytoplasmic male sterility. Bot Rev 36:341-420. doi:10.1007/BF02859253

24. Martinez-Reyna JM, Vogel KP (2008) Heterosis in switchgrass: spaced plants. Crop Sci., vol 48. doi:10.2135/cropsci2007.12.0695

25. Barnett F, Carver R (1967) Meiosis and pollen stainability in switchgrass, *Panicum virgatum* L. Crop Sci 7:301-304

26. Vogel KP (2004) Switchgrass. In: Moser LE, Burson BL, Sollenberger LE (eds) warm-season (C4) grasses. ASA, CSSA, SSSA, pp 561-588. doi:10.2134/agronmonogr 45.c16

27. Cornelius DR, Johnston CO (1941) Differences in plant type and reaction to rust among several collections of *panicum virgatum* L.1. Agron J 33:115-124. doi:10.2134/agronj1941.00021962003300020003x

28. Eberhart SA, Newell LC (1959) Variation in domestic collections of switchgrass, *panicum virgatum L*[1]. Agron J 51:613-616. doi:10.2134/agronj1959.00021962005100100015x

29. Porter CL (1966) An analysis of variation between upland and lowland switchgrass, *panicum virgatum* L., in central oklahoma. Ecology 47:980-992. doi:10.2307/1935646

30. Brunken JN, Estes JR (1975) Cytological and morphological variation in *panicum virgatum* L. The southwestern naturalist 19:379-385. doi:10.2307/3670396

31. Burton GW (1974) Recurrent restricted phenotypic selection increases forage yields of pensacola bahiagrass1. Crop Sci 14:831-835. doi:10.2135/cropsci1974.0011183X001400060016x

32. Talbert LE, Timothy DH, Burns JC, Rawlings JO, Moll RH (1983) Estimates of genetic parameters in switchgrass. Crop Sci 23:725-728. doi:10.2135/cropsci1983.0011183X002300040029x

33. Godshalk EB, Timothy DH, Burns JC (1988) Effectiveness of index selection for switchgrass forage yield and quality. Crop Sci 28:825-830. doi:10.2135/cropsci1988.0011183X002800050021x

34. Anderson B, Ward J, Vogel K, Ward M, Gorz H, Haskins F (1988) Forage quality and performance of yearlings grazing switchgrass strains selected for differing digestibility. Journal of animal science 66

35. Ward M, Ward J, Anderson B, Vogel K (1989) Grazing selectivity and in vivo digestibility of switchgrass strains selected for differing digestibility. Journal of animal science 67:1418-1424

36. Hopkins AA, Taliaferro CM, Murphy CD, Christian DA (1996) Chromosome number and nuclear DNA content of several switchgrass populations. Crop Sci 36:1192-1195. doi:10.2135/cropsci1996.0011183X003600050021x

37. Lu K, Kaeppler SW, Vogel K, Arumuganathan K, Lee D (1998) Nuclear DNA content and chromosome numbers in switchgrass. Great plains research: A journal of natural and social sciences:396

38. Gunter LE, Tuskan GA, Wullschleger SD (1996) Diversity among populations of switchgrass based on rapd markers. Crop Sci 36:1017-1022. doi:10.2135/cropsci1996.0011183X003600040034x

39. Casler MD, Stendal CA, Kapich L, Vogel KP (2007) Genetic diversity, plant adaptation regions, and gene pools for switchgrass. Crop Sci 47:2261-2273

40. Missaoui AM, Paterson AH, Bouton JH (2005) Investigation of genomic organization in switchgrass (*Panicum virgatum* L.) using DNA markers. Theor Appl Genet 110:1372-1383. doi:10.1007/s00122-005-1935-6

41. Tobias C, Gautam S, Twigg P, Lindquist E, Pangilinan J, Penning B, Barry K, McCann M, Carpita N, Lazo G (2008) Comparative genomics in switchgrass using 61585 high-quality expressed sequence tags. The Plant Genome 1:111 - 124

42. Narasimhamoorthy B, Saha MC, Swaller T, Bouton JH (2008) Genetic diversity in switchgrass collections assessed by EST-SSR markers. Bioenerg Res 1:136-146. doi:10.1007/s12155-008-9011-0

43. Okada M, Lanzatella C, Saha M, Bouton J, Wu R, Tobias C (2010) Complete switchgrass genetic maps reveal subgenome collinearity, preferential pairing, and multilocus interaction. Genetics 185:745 - 760

44. Wang Y, Samuels T, Wu Y (2011) Development of 1,030 genomic SSR markers in switchgrass. Theor Appl Genet 122 :677-686. doi:10.1007/s00122-010-1477-4

45. Saski C, Li Z, Feltus F, Luo H (2011) New genomic resources for switchgrass: a BAC library and comparative analysis of homoeologous genomic regions harboring bioenergy traits. BMC Genomics 12:369

46. Liu L, Wu Y (2012) Development of a genome-wide multiple duplex-SSR protocol and its applications for the identification of selfed progeny in switchgrass. BMC *Genomics* 13:1-13. doi:10.1186/1471-2164-13-522

47. Wang Y, Zeng X, Iyer NJ, Bryant DW, Mockler TC, Mahalingam R (2012) Exploring the switchgrass transcriptome using second-generation sequencing technology. PLoS ONE 7 :e34225. doi:10.1371/journal.pone.0034225

48. Ersoz ES, Wright MH, Pangilinan JL, Sheehan MJ, Tobias C, Casler MD, Buckler ES, Costich DE (2012) SNP discovery with EST and nextgen sequencing in switchgrass (*Panicum virgatum* L.). PLoS ONE 7:e44112. doi:10.1371/journal.pone.0044112

49. Lu F, Lipka AE, Glaubitz J, Elshire R, Cherney JH, Casler MD, Buckler ES, Costich DE (2013) Switchgrass genomic diversity, ploidy, and evolution: Novel insights from a network-based snp discovery protocol. PLoS Genet 9:e1003215. doi:10.1371/journal.pgen.1003215

50. Vogel K (2013) Comparison of two perennial grass breeding systems with switchgrass. Crop Sci 53:863-870

51. Taliaferro CM, Das M (2002) Breeding and selection of new switchgrass varieties for increased biomass production. ORNL/SUB-02-19XSY162C/01 Oak Ridge National Laboratory, Oak Ridge, Tennessee

52. Vogel KP, Mitchell RB (2008) Heterosis in switchgrass: biomass yield in swards. Crop Sci 48:2159-2164. doi:10.2135/cropsci2008.02.0117

53. Todd J (2011) Study of the diversity, inbreeding potential, and sward heritability of switchgrass. Proquest dissertations and theses 147. Retrieved from http://search.proquest.com/docview/926208151?accountid=4117. (926208151). (Order No. 3498112, Oklahoma State University)

54. Vogel K, Sarath G, Mitchell R (2014) Micro-mesh fabric pollination bags for switchgrass. Crop Sci.doi:10.2135/cropsci2013.09.064

55. Arias Aguirre A, Studer B, Frei U, Lübberstedt T (2012) Prospects for hybrid breeding in bioenergy grasses. Bioenerg Res 5:10-19. doi:10.1007/s12155-011-9166-y

56. Duvick DN (1959) The use of cytoplasmic male-sterility in hybrid seed production. Economic Botany 13:167-195. doi:10.2307/4288018

57. Laughnan JR, Gabay-Laughnan S (1983) Cytoplasmic male sterility in maize. Annual review of genetics 17:27-48. doi:doi:10.1146/annurev.ge.17.120183.000331

58. Geiger HH, Schnell FW (1970) Cytoplasmic male sterility in rye (*Secale cereale* L.)1. Crop Sci 10:590-593. doi:10.2135/cropsci1970.0011183X001000050043x

59. Burton GW, Hanna WW (1976) Ethidium bromide induced cytoplasmic male sterility in pearl millet1. Crop Sci 16:731-732. doi:10.2135/cropsci1976.0011183X001600050035x

CHAPTER II

TESTING THE EFFICACY OF A POLYESTER BAGGING METHOD FOR SELFING SWITCHGRASS

ABSTRACT

Switchgrass (*Panicum virgatum* L.) is a naturally allogamous species. Recent studies indicated conditional self-compatibility exists in the species and can be used to produce inbreds, which provides potential for exploiting heterosis in biomass production. However, efficient and reliable bagging methods are unavailable for breeders to develop inbreds in switchgrass. This study was designed to determine the efficacy of a polyester bagging method to prevent extraneous pollen while selfing switchgrass plants. In this experiment, four northern lowland (NL) inbreds, four NL non-inbreds, two southern lowland (SL) non-inbreds, and 16 upland-lowland (interecotypic) F1 hybrids were self-pollinated by enclosing their inflorescences into polyester bags in the field. The bagging method was also tested on 14 F1 interecotypic hybrid plants potted in a greenhouse. The reliability of the bags was determined based on the genetic origin of the bagged progeny if they were selfed using eight to 10 SSR markers. Contaminants were identified in two groups: outcrossing contaminants (OCs) and physical contaminants (PCs) based on amplified alleles of progeny and their seed parents. Of 39 polyester bags tested in 2012 in the field, 35 bags generated 100 % selfed progeny while the other four generated five PCs, however, no OCs were identified from these bags. Similarly, of 61 bags tested in 2013 in two field plots, 50 bags

produced 100% selfed progeny while four bags produced OCs, five produced PCs and the other two produced both OCs and PCs. No contaminant was identified from the progeny of 18 bags used in the greenhouse in 2013. It appeared that high wind speed and/or accidental errors resulted contaminations of bagged progeny in the field. The results of this experiment provide valuable insights into the reliability of the polyester bags and the bagging method for selfing switchgrass under field as well as greenhouse conditions. Additionally, S1, S2 and S3 inbreds produced in the study will be valuable to develop complete or near complete homozygous inbred lines in the future.

Keywords: Switchgrass, Inbred, Polyester bagging, Self-incompatibility, Simple sequence repeat

ABBREVIATIONS

bp	base pair
CTAB	Cetyltrimethyl ammonium bromide
F	Field
G	Greenhouse
NL	Northern lowland switchgrass
OC	Outcrossing contaminant
PC	Physical contaminant
PCR	Polymerase chain reaction
PP	Primer Pair

SI	Self-incompatibility
SL	Southern lowland switchgrass
SSR	Simple sequence repeat

INTRODUCTION

Switchgrass (*Panicum virgatum* L.), a warm-season perennial grass, is native to the central and eastern US and is identified as a model herbaceous cellulosic bioenergy crop [1]. The ploidy level in switchgrass ranges from diploid (2n = 2x = 18) to duodecaploid (2n= 12x= 108) [2]. The lowland genotypes are usually tetraploid (2n= 4X=36) and most of the upland genotypes are octaploid. However, tetraploid uplands are also common [2]. The lowland ecotypes mostly originate from bottomlands, flood-plains and river side habitats in the south, whereas upland ecotypes are found at higher elevations and relatively dry soils of the more northern latitudes [3]. Lowland ecotypes are comparatively taller and coarser than upland ecotypes, and they also differ in their nutrient and water requirements [4]. Further, F1 hybrids developed from crosses and reciprocal crosses of upland 'Summer' and lowland 'Kanlow' switchgrass, identified as different heterotic groups, were reported to have significant high parent heterosis (30 to 38 %) [5,6]. Increased biomass and other valuable traits in switchgrass could be obtained by improved cultivars including hybrids [7]. Heterosis has been widely exploited to cause a dramatic rise in the yield of major crops like maize (*Zea mays* L.), rice (*Oryza sativa* L.), and sorghum (*Sorghum bicolor* L.). Development of inbreds is basic for hybrid breeding in major crop species. However, to date, little information is available on inbred and heterosis breeding in switchgrass. So far,

switchgrass has been improved as populations via recurrent selection, resulting in synthetic cultivars [2].

Switchgrass is a naturally out-crossing species [8]. The taxon is a highly self-incompatible (SI) species exhibiting a low tendency to produce selfed seeds [2]. Self-compatibility in tetraploid and octaploid switchgrass has been reported as low as 0.35 % and 1.39 %, respectively [2]. Pre-fertilization and post fertilization SI hinder the plants to be self-fertilized, while the former one shows extreme expression during selfing, and expression of the latter type occurs in interploidy mating [2]. Recently, a few reports indicated that some genotypes are relatively self-fertile and can produce more than 60 % selfed seed under a controlled environment [9]. However, the same genotypes failed to produce any selfed seed in open pollination conditions due largely to conditional self-compatibility [10]. Casler et al. [11] also reported that some genotypes possess self-fertility up to 50%, and that heterosis breeding in switchgrass is theoretically feasible. Identification of the genetic origin of selfed progeny in switchgrass has become easier in terms of cost and time after the development of a protocol of a genome-wide multiple duplex-SSR by Liu and Wu [12].

Pollen is a key form facilitating gene flow in outcrossing species [13]. A recent study about switchgrass pollen indicates that the average pollen diameter of transgenic and control switchgrass plants ranges from 42.5 to 54.0 μm [14], and that there are no significant differences in average pollen sizes of other genotypes. Longevity of pollen viability in switchgrass depends on the weather conditions and can persist up to more than an hour [14,15]. The viable pollen can disperse to a distance of 3.5 km under light wind conditions [15]. Hence, the chance of foreign pollen contamination in switchgrass is high. So, highly secured bags that are supposed to have pores smaller than switchgrass pollen are desirable.

In fact, it is difficult to obtain selfed seeds in switchgrass unless an accurate extraneous pollen control mechanism is developed. In previous years, the Grass Breeding and Genetics Lab at Oklahoma State University (OSU) attempted to develop switchgrass advanced inbreds using four different bagging methods: lawson paper bags, microfiber bags, muslin cloth bags and cages (cylindrical frames made with chicken wire) each covered with a cotton pillowcase. However, these methods resulted in significant outcrossing contaminations, 39 to 80 % [16]. Among those four bagging methods, paper bagging performed relatively better with a higher inbreeding average (61.2%) [16]. Nevertheless, this method is not suitable for selfing switchgrass in the field due to size, strength, durability and environmental tolerance [16]. Recently, Vogel et al. [17] introduced micro-mesh fabric pollination bags for crossing switchgrass genotypes, nonetheless, the effectiveness of the bagging method in excluding extraneous pollen was not reported. Moreover, the bags were used to make crosses and tested in a greenhouse. Knowledge about pollination bags that effectively exclude extraneous pollen is required for breeders to develop inbreds for breeding hybrid cultivars. Further, an effective bagging method would reduce the extent of molecular assessment required to verify genetic origin of the progeny. It is our understanding that limited information has been documented on developing reliable bagging methods for selfing switchgrass under field as well as greenhouse conditions. Accordingly, the major objective of this study was to test and report the efficacy of a polyester bagging method for selfing switchgrass plants.

MATERIAL AND METHODS

Plant materials, plant growth and management

Lowland inbreds (S1 and S2) and non-inbreds, and interecotypic F1 hybrids were used as parents for the study and associated information is given in Table 1. Four of the six lowland genotypes bagged in 2012 were inbreds and two of them were non-inbreds derived from a conditionally self-compatible NL genotype [9,10]. The interecotypic F1 hybrids were developed by crossing between tetraploid upland and lowland plants in isolation after synchronization in two greenhouses. They were synchronized by trimming early flowered upland plants. Isolation of the synchronized crossing pairs was begun on 8th March, and continued to the second week of April in 2012. Crosses were facilitated by the manual shaking of flowering panicles before noon. The genetic origins of the F1 progeny were confirmed by two of four SSRs: PVAAG-2895/6, PVAAG-3051/2, PVAAG-3311/2, PVGA-1143/4 [18]. The F1 progeny thus developed were given names with the number of corresponding crosses and reciprocal crosses. For example, C-1-33 represented progeny No. 33 of a cross between upland (♀) 'Dacotah-13' and lowland (♂) 'NL313/9' genotypes, which was given a crossing pair number 'C-1'. Similarly, RC-6-6 was given to the progeny No. 6 of the reciprocal cross No. 6. All the crosses and reciprocals associated with the F1 hybrids used in this study are presented in Table 1.

On the 1st August, 2011, the lowland switchgrass seedlings were transplanted into a field plot on the Agronomy Research Farm at OSU, Stillwater, OK. Plants were transplanted on a clean seed bed, spaced 1.07 m between each plant on a row. After transplanting, 0.0105 kg a.i. /ha Cimarron plus herbicide (Metsulfuron Methyl 48%, Chlosulfuron 15 %, and inert ingredients 15 %) was sprayed for broadleaf weed control, and 0.091 kg a.i. /ha Dual and 0.07 kg a.i. /ha

atrazine were applied to control pre-emergent weeds. Weekly irrigation was provided to overcome the 2012 prevailing drought during heading to maximum flowering stages. Similar growing conditions were maintained for the F1 plants bagged in the field in 2013, but no periodic irrigation was applied because of adequate rainfall in 2013. Daily watering, necessary fertilization and controlled temperatures of 20-30 ^{0}C were maintained for potted plants in the greenhouse.

Bagging, seedhead harvesting, seed processing and germination

Inflorescences of the selected parents were bagged before anthesis using three-dimensional polyester bags, type PBS3D/75F (PBS International, North Yorkshire, UK). The bags were made up of traditional non-oven polyester materials with external dimensions of 0.75 x 0.158 x 0.158 m^3. These white colored bags included one clear PVC observation window 0.10 x 0.25 m^2 in the front with a flap (Fig. 1) [19]. A square shaped flap, attached to the bag top, was used to label parent genotypes. In the field the bags were tied on T-posts while bags were attached to bamboo sticks in the greenhouse. Early developed flowers were removed before bagging to avoid unwanted fertilization. All the bags used in 2012 were new, and bagging was initiated on 29th July and completed on 29th August. Each bag often enclosed four inflorescences. In 2013, the F1 hybrids developed inflorescences relatively earlier than lowland switchgrass in the field, so bagging was started on 10th July 2013 and completed by 28th July 2013. For F1 parents, 37 new, and 20 previously used bags were installed after vacuum followed by washing. The reused bags were bag No. 4 and 5 of C-1-33; 5 and 6 of C-3-8; 1 of C-4-17; 2 of C-5-27; 1, 2 and 3 of C-7-9; 1, 2 and 3 of C-7-10; 1 of C-8-22; 1 and 2 of C-9-5; 5 and 6 of RC-1-12; and 1, 2, and 3 of RC-6-

5. The four lowland non-inbreds were bagged on 18th August 2013 using new bags. Further, 14 different F1 parents (Table 1) were bagged in the greenhouse with 18 reused bags.

In 2012, mature seed heads were harvested on 17th October and were allowed to dry for a week or longer before processing. Seed heads were hand rubbed in a pan, and then were processed using a South Dakota Seed Blower (Seedburo Equipment Company), and clean seeds were collected and counted. The seeds were scarified before germination using 50 % H_2SO_4 and water [20], and were kept at 4 ^{0}C for two weeks for stratification. In this year, seeds were germinated inside a growth chamber under 15 ^{0}C for 16 h with darkness, and 30 ^{0}C for 8 h with light.

In 2013, seedheads were harvested on 27th September. Seeds were processed using the same method described for 2012. This time only 34 to 61 seeds per bag were germinated for genotyping since a large number of seeds were harvested from some bagged plants (Table 1). However, all the seeds obtained from bags in the greenhouse were germinated. In 2013, seeds were germinated in the greenhouse.

DNA isolation and PCR amplification

Genomic DNA of all parents and selected progeny was isolated using a cetyltrimethyl ammonium bromide (CTAB) method as described [9]. Concentration of isolated DNA was measured using a Nano Drop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE) [9]. Progeny obtained from field plants in both years, were genotyped with 10 polymorphic SSRs, whereas the progeny obtained from parents in the greenhouse were tested with eight SSRs. SSR primer pairs (PPs) were selected from 1030 switchgrass SSR PPs developed in our lab [18],

and two other single PPs were used from Okada et al. [21]. Selected PPs were screened for polymorphism as described [9]. Three duplex SSRs used were Sets: 12, 14 and 23 of the 24 duplexes reported [12]. The single SSR PPs selected were PVAAG-2895/6, PVAAG-3051/2, PVAAG-3311/2, PVGA-1143/4, PVGA-1549/50, PVGA-1813/4 [18], SWW-125 and SWW-1889 [21]. The volume of chemicals and the processes in thermal cycles for duplex SSR and single SSR PCR followed the procedures in our lab [12].

The amplified SSR products were separated using 6.5% KB plus polyacrylamide gels on a LI-COR 4300 DNA Analyzer (LI-COR Biosciences, Lincoln, NE, USA) [9]. A standard sized DNA marker of 50-350 base pairs (bp) (LI-COR Biosciences, Lincoln, NE, USA) was used to locate amplified alleles (Fig. 2), which were visually observed and scored. Samples generating stutter bands and no proper amplification on target loci were genotyped and checked twice. Visual scoring and size measurement of the bands were performed using Saga Generation 2 software, version 3.3 (LI-COR Biosciences, Lincoln, NE, USA) [10,9] .

Determination of progeny breeding origin

Genetic origin for each progeny derived from bagged plants was determined using the progeny array approach, i.e., SSR amplified DNA bands of progeny and respective seed parents were compared. The progeny with the band(s) of the same size as their seed parents across all SSR markers were regarded as selfed progeny, and the progeny having different sized band(s) than its seed parent but containing at least one parent band were regarded as OCs. Further, the samples which did not show any bands as expected from the seed parent, in at least one of the tested SSRs, were recognized as PCs (Fig. 3).

RESULTS AND DISCUSSIONS

Numbers of putative inbred seeds obtained per bag varied from genotype to genotype. In the field, the average seeds per bag, in two years, in all 26 genotypes were 176. However, the mean number of putative seeds per bag in three S2 parents was 33; the S2 parent NL94/85/5 produced only 19 seeds per bag on average (Table 1). Similarly, the average number of seeds per bag in a S1 parent NL94/85 was 73. Fewer seeds were obtained from the parents in the greenhouse relative to the field; the average seeds per bag in 14 F1 hybrids in the greenhouse was 31 (Table 1).

A total of 291 seedlings from seeds of 39 bags on six parents in 2012 were genotyped (Table 1). In 2013, 328 progeny of 16 F1 parents obtained from 57 bags in the interecotypic hybrid field and 56 progeny of four lowland parents in another field, were tested with SSR markers (Table 1). One-hundred seedlings from seeds of 18 bags in greenhouse were also genotyped in 2013 (Table 1).

One electrophoresis-gel image, amplified by a duplex SSR Set 23 (PVCAG-2361/2 and PVCAG-2269/70), is given in Fig 2. Amplified alleles of five different parents (P1, P2, P2, P4 and P5) and their progenies in the image indicated the breeding origin of the respective progeny. Progeny No. 5 and 11 of C-4-17 (P3) each labeled with an asterisk (*) were grouped into OCs, because two bands of No. 5 and two bands of No. 11 progeny were not from the seed parent, albeit three bands of the two progeny were the same as the parent. However, the progeny No. 11 was later identified as PC, due to lack of parent bands amplified with SSR PVGA 1549/50.

SSR testing of seedlings from bagged seeds harvested in 2012 indicated that the polyester bagging method for selfing switchgrass was impeccable because no OC progeny was identified (Table 1). Upon parentage analysis, three progenies of NL94/85/1: NL94/85/1-6, NL94/85/1-27

and NL94/85/1-29; and two progenies of NL94/85/5: NL94/85/5-13 and NL94/85/5-17 were identified as PCs (Fig. 3). All remaining 286 seedlings tested this year were selfed, and were consistent across all 10 SSR markers genotyped. Those PCs were observed in four different bags in 2012 (Table 1). Accordingly, in 2012, lowland inbreds were developed, including 41 S3 progeny of NL94/85/1, 86 S3 progeny of NL94/85/3, 15 S3 progeny of NL94/85/5, four S2 progeny of NL 94/85, and 70 S1 progeny of each NL94/81/6 and NL94/81/7. Of the total 291 progeny tested from 39 bags, 286 were inbred (98.3 %) and 5 (1.7 %) were PCs.

In 2013, in the first field, of the 16 F1 interecotypic parents, the seedlings from nine parents were 100 % selfed and seedlings from seven parents were mostly selfed (Table 1). Of the 328 progeny plants, one seedling of C-1-33, two of C-4-17, one of C-5-20 and one of C-5-27 were OCs, while one seedling each of C-4-10, C-4-17 and C-7-10 and three of C-8-43 were genotyped as PCs (Table 1). Careful examination of the individual bags generating contaminants indicated that bag No. 4 used for C-1-33 had a circular hole of 2 cm diameter. This hole probably was a pathway to allow extraneous pollen inside. The eight bags, with minor contamination of one or two seedlings, did not have any sign of physical damages. Despite the slight contamination, of the 328 progeny tested, 317 (96. 64 %) were selfed, five were (1. 52 %) OCs and six (1.83 %) were PCs. In this field, of the 57 tested bags, 50 bags produced 100 % selfed.

Seemingly ambiguous results were obtained from bagging of four lowland non-inbred genotypes in 2013 in the second field, with variation from 100 % inbreds to 100 % contaminants (Table 1). Out of 56 seedlings of four parents, 14 (100 %) of NLH2x12, 14 (100 %) of SL10x30 and 5 (35.71 %) of SL32x25 were selfed, and no selfed progeny was obtain from NLH39x12. The bag of NLH39x12 was checked to have a circular hole of 4 cm diameter observed in the middle of the bag. The hole was likely responsible for this severe contamination. However, no

hole was observed on the bag of SL32x25. Substantial contaminants were identified in seedlings of SL32x25, suggesting that either some florets had already flowered before bagging or another unknown reason contributed the contamination. This was the only bag in the entire study producing a large number of contaminants without any observable reasons. Genotyping of the 100 progenies obtained from 14 genotypes grown in the greenhouse in 2013, showed no contamination, indicating the polyester bags were fully reliable for selfing switchgrass under greenhouse conditions (Table 1).

In this study, it appeared that two important issues, affecting the effectiveness of the polyester bagging method used in the field, were the high wind speed and the accidental operational error. Wind blowing occurred regularly but was not favorable to bags on the T-post since winds twisted bags, forced them to move away from original positions causing inflorescences, partially or wholly to come out of the bags. Occasionally, winds blew bags off from the plants. Winds could cause bags to be repetitively contacted with T-posts, metal cords and other possible objects that could be a reason for the physical breakdown on bags. In 2012, two bags of each of NL94/85/1 and NL94/85/5 parents were blown off overnight on 7th September, 2012 (max wind speed at 71.94 km/hr from due north) [22], but those bags were fixed early the next morning. Since no inflorescence was left unbagged in both genotypes, the blown bags were reestablished randomly within the opened panicles. Getting no outcrossing contaminant from these two bags might be due to timing. Jones and Brown [8] reported that pollen shedding in switchgrass begins from 9:30 a.m., the shedding rate increases till noon, and declines continuously until 4:0 p.m. and almost zero after 5:0 p.m. Another possibility is, the panicles enclosed by the blown bags could have set the seeds before, or its female reproductive

part lost receptivity due to maturity. To secure the purity of seeds using this polyester bagging method a careful, daily monitoring and checking of the bags is necessary.

Accidental errors can occur from bagging, harvesting, seed processing to germination and genotyping. OCs could arise from incomplete removing of blooming florets before bagging. Similar reasons may have caused contamination observed in lowland parents, NLH39x12 and SL32x25, bagged on 18th August 2013. Thus, this study suggested that bagging just after emergence of an inflorescence from the flag leaf sheath and no sign of any florets flowering would be optimum.

There was no significant effect of the reuse of bags in greenhouse as all tested seedlings were selfed progeny. In the field in 2013, of 20 reused bags, three generated OCs: one OC in bag No. 4 of C-1-33, two OCs in bag 1 of C-4-17 and one OC in bag 2 of C-5-27. Thus, no major difference was observed among the new and older bags in the field. However, using the same bags for multiple seasons may not be effective due to possible physical breakdown in the bag.

Liu and Wu [9] reported conditional self-compatibility of switchgrass grown in a growth chamber. However, selfing several hundred plants normally needed in a breeding program annually is impractical using growth chambers. Since the enforced environment is required for selfing switchgrass [10], the polyester bagging method is a better option than other bagging methods tested in our lab.

CONCLUSIONS

This study reports the efficacy of a polyester bagging method for selfing switchgrass in the field as well as under greenhouse conditions. The bagging method was fully reliable in the greenhouse. Most progeny were selfed from bagged plants in the field indicating the most efficacious bagging method for selfing switchgrass in the field. However, cautions must be

exercised when the method is used for selfing switchgrass plants under field conditions. Proper and careful use of the bagging method would accelerate the development of inbreds in switchgrass.

REFERENCES

1. Sanderson MA, Reed RL, McLaughlin SB, Wullschleger SD, Conger BV, Parrish DJ, Wolf DD, Taliaferro C, Hopkins AA, Ocumpaugh WR, Hussey MA, Read JC, Tischler CR (1996) Switchgrass as a sustainable bioenergy crop. Bioresource Technology 56: 83-93. doi:http://dx.doi.org/10.1016/0960-8524(95)00176-X

2. Martínez-Reyna JM, Vogel KP (2002) Incompatibility systems in switchgrass. Crop Sci 42 :1800-1805. doi:10.2135/cropsci2002.1800

3. Porter CL, Jr. (1966) An analysis of variation between upland and lowland switchgrass, *Panicum Virgatum* L., in Central Oklahoma. Ecology 47 :980-992. doi:10.2307/1935646

4. Thomson AM, Izaurralde RC, West T, Parrish DJ, Tyler DD, Williams JR (2009) Simulating potential switchgrass production in the United States. Pacific Northwest National Laboratory Richland, WA

5. Martinez-Reyna JM, Vogel KP (2008) Heterosis in switchgrass: spaced plants. Crop Sci., vol 48. doi:10.2135/cropsci2007.12.0695

6. Vogel KP, Mitchell RB (2008) Heterosis in switchgrass: Biomass yield in swards. Crop Sci 48:2159-2164. doi:10.2135/cropsci2008.02.0117

7. Schmer MR, Vogel KP, Mitchell RB, Perrin RK (2008) Net energy of cellulosic ethanol from switchgrass. Pnas 105:464-469. doi:10.1073/pnas.0704767105

8. Jones MD, Brown JG (1951) Pollination cycles of some grasses in Oklahoma. Agron J 43:218-222. doi:10.2134/agronj1951.00021962004300050003x

9. Liu L, Wu Y (2012) Identification of a selfing compatible genotype and mode of inheritance in switchgrass. BioEnergy Res, vol 5. Springer-Verlag. doi:10.1007/s12155-011-9173-z

10. Liu L, Thames S, Wu Y (2013) Lowland switchgrass plants in populations set completely outcrossed seeds under field conditions as assessed with SSR markers. Bioenerg Res:1-7. doi:10.1007/s12155-013-9367-7

11. Casler M (2012) Switchgrass breeding, genetics, and genomics. In: Monti A (ed) Switchgrass. Green Energy and Technology. Springer London, pp 29-53. doi:10.1007/978-1-4471-2903-5_2

12. Liu L, Wu Y (2012) Development of a genome-wide multiple duplex-SSR protocol and its applications for the identification of selfed progeny in switchgrass. BMC Genomics 13:1-13. doi:10.1186/1471-2164-13-522

13. Wang Z-Y, Ge Y, Scott M, Spangenberg G (2004) Viability and longevity of pollen from transgenic and nontransgenic tall fescue (*Festuca arundinacea*)(*Poaceae*) plants. American Journal of Botany 91:523-530

14. Ge Y, Fu C, Bhandari H, Bouton J, Brummer EC, Wang Z-Y (2011) Pollen viability and longevity of switchgrass (L.). Crop Sci 51:2698-2705

15. Ecker G, Meyer T, Auer C (2013) Pollen longevity and dispersion models for switchgrass. Crop Sci 53:1120-1127. doi:10.2135/cropsci2012.06.0382

16. Todd J (2011) Study of the diversity, inbreeding potential, and sward heritability of switchgrass. ProQuest Dissertations and Theses 147. Retrieved from http://search.proquest.com/docview/926208151?accountid=4117. (926208151). (Order No. 3498112, Oklahoma State University)

17. Vogel K, Sarath G, Mitchell R (2014) Micro-mesh fabric pollination bags for switchgrass. Crop Sci. doi:10.2135/cropsci2013.09.064

18. Wang YW, Samuels TD, Wu YQ (2011) Development of 1,030 genomic SSR markers in switchgrass. Theor Appl Genet 122 :677-686. doi:10.1007/s00122-010-1477-4

19. pbsinternational why our bags are best http://www.pbsinternational.com/our-products/. Accessed 3/2 2014

20. Bhandari H, Saha M, Bouton J (2010) Genetic variation in lowland switchgrass (*Panicum virgatum* L.). In: Huyghe C (ed) Sustainable use of genetic diversity in forage and turf breeding. Springer Netherlands, pp 67-71. doi:10.1007/978-90-481-8706-5_7

21. Okada M, Lanzatella C, Tobias C (2011) Single-locus EST-SSR markers for characterization of population genetic diversity and structure across ploidy levels in switchgrass (*Panicum virgatum L.*). Genet Resour Crop Evol 58:919-931. doi:10.1007/s10722-010-9631-z

22. Mesonet (2012) Mesonet climatological data summary. http://www.mesonet.org/common/db/library/functions/mcd/mcd.php?ver=2&year=2012&month=9&format=pdf&stid=STIL. Accessed 6/27 201

Table 1 Parent genotypes and their description, experimental sites, number of bags, harvested seeds, and seedlings and their genetic origin.

Plant ID	Description	Site ǂ	Bags	Seeds	Seedlings genotyped	Selfed Seedlings	Contamination notes §
		----------2012----------					
NL94/85/1	S2 of NL94	F	11	491	44	41	1 PC in bag 2, and 2 PCs in bag 6
NL94/85/3	S2 of NL94	F	13	404	86	86	
NL94/85/5	S2 of NL94	F	7	134	17	15	1 PC in bag 6, and 1 PC in bag 7
NL94/85	S1 of NL94	F	2	146	4	4	
NL94/81-6	Lowland non-inbred	F	3	158	70	70	

NL94/81-7	Lowland non-inbred	F	3	253	70	70	
	----------2013----------						
C-1-33	F1, Dacotah-13(♀) x NL313/9 (♂)	F	5	1,350	30	29	1 OC in bag 4
C-3-8	F1, Dacotah-15 (♀) x NL313/10 (♂)	F	6	602	37	37	
C-3-30	F1, Dacotah-15 (♀) x NL313/10 (♂)	F	4	100	28	28	
C-4-10	F1, Summer-14 (♀) x SL34/13 (♂)	F	2	71	10	9	1 PC in bag 2
C-4-17	F1, Summer-14 (♀) x SL34/13 (♂)	F	3	143	18	15	2 OCs in bag 1, and 1 PC in bag 2
C-5-20	F1, Summer-15 (♀) x SL6/10 (♂)	F	3	75	20	19	1 OC in bag 2
C-5-26	F1, Summer-15 (♀) x SL6/10 (♂)	F	2	55	10	10	
C-5-27	F1, Summer-15 (♀) x SL6/10 (♂)	F	3	85	18	17	1 OC in bag 2
C-7-9	F1, WS4U-19 (♀) x NL387/8 (♂)	F	4	774	20	20	
C-7-10	F1, WS4U-19 (♀) x NL387/8 (♂)	F	4	270	20	19	1 PC in bag 2
C-8-22	F1, WS4U-20 (♀) x NL33/7 (♂)	F	2	184	12	12	
C-8-43	F1,WS4U-20 (♀) x NL33/7 (♂)	F	3	550	20	17	1 PC in bag 1, and 2 PCs in bag 2
C-9-5	F1, WS4U-21 (♀) x NL81/8 (♂)	F	4	122	20	20	

RC-1-12	F1, NL313/9 (♀) x Dacotah-13 (♂)	F	6	2,766	29	29	
RC-6-5	F1, NL387/6 (♀) x Summer-16 (♂)	F	5	759	30	30	
RC-6-6	F1, NL387/6 (♀) x Summer-16 (♂)	F	1	150	6	6	
NLH2x12	Northern lowland non-inbred	F	1	576	14	14	
NLH39x12	Northern lowland non-inbred	F	1	549	14	0	13 OCs and 1 PC in bag 1
SL10x30	Southern lowland non-inbred	F	1	2,898	14	14	
SL32x25	Southern lowland non-inbred	F	1	3,938	14	5	7 OCs and 2 PCs in bag 1
C-2-3	F1, Dacotah-14 (♀) x NL225/7 (♂)	G	1	19	5	5	
C-2-6	F1, Dacotah-14 (♀) x NL225/7 (♂)	G	1	49	8	8	
C-2-14	F1, Dacotah-14 (♀) x NL225/7 (♂)	G	1	34	5	5	
C-3-5	F1, Dacotah-15 (♀) x NL313/10(♂)	G	1	53	8	8	
C-4-3	F1, Summer-14 (♀) x SL34/13 (♂)	G	1	44	6	6	
RC-2-16	F1, NL225/7 (♀) x Dacotah-14 (♂)	G	1	42	10	10	
RC-2-17	F1, NL225/7 (♀) x Dacotah-14 (♂)	G	1	17	5	5	

RC-2-20	F1, NL225/7 (♀) x Dacotah-14 (♂)	G	1	19	5	5	
RC-3-4	F1, NL313/10 (♀) x Dacotah-15 (♂)	G	1	102	10	10	
RC-7-2	F1, NL387/8 (♀) x WS4U-19 (♂)	G	1	19	5	5	
RC-7-3	F1, NL387/8 (♀) x WS4U-19 (♂)	G	2	21	5	5	
RC-7-8	F1, NL387/8 (♀) x WS4U-19 (♂)	G	2	56	10	10	
RC-8-1	F1, NL33/7 (♀) x WS4U-20 (♂)	G	3	73	13	13	
RC-8-2	F1, NL33/7 (♀) x WS4U-20 (♂)	G	1	21	5	5	
Total: 40 genotypes			118	18,172	775	736	25 OCs and 14 PCs in 15 bags

ǂ F= field and G= greenhouse; § OC= outcrossing contaminants; and PC= physical contaminants;

Figure 1. A polyester bag attached on a T-post in a field, the lowermost opening was tied by using a metal cord and the top portion was tied with two strips of the bag. Switchgrass inflorescences inside the bag can be seen from the observation window in front.

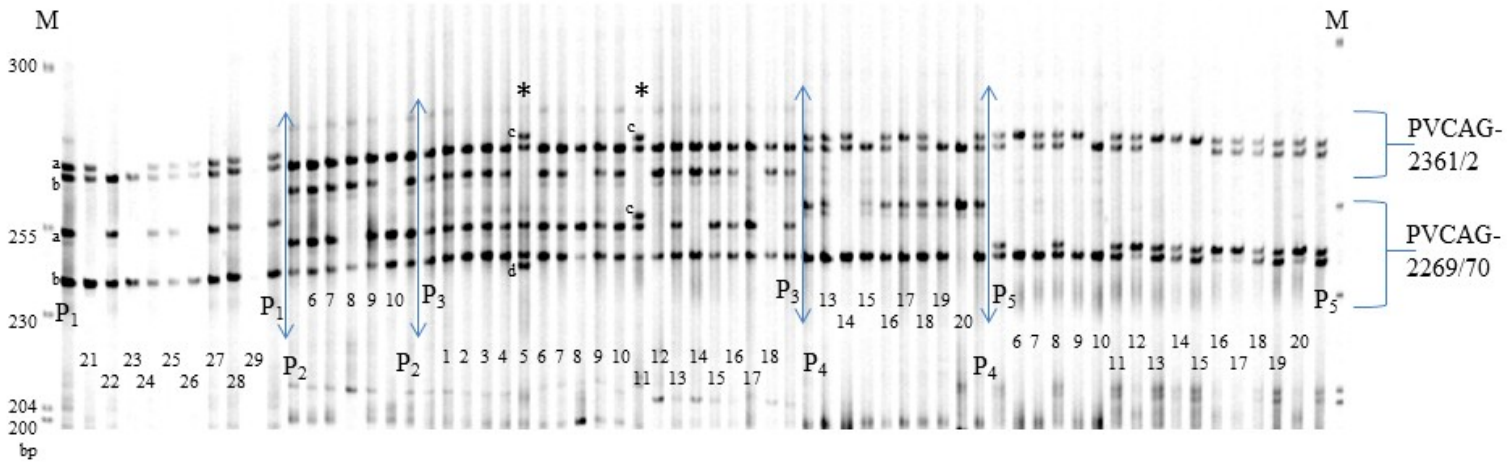

Figure 2 . DNA profiles of the female parents RC-1-12 (P1), C-4-10 (P2), C-4-17 (P3), C-7-9 (P4) and C-9-5 (P5) and their respective progeny (indicated by numbers below bands) genotypes amplified by a duplex SSR Set 23 (PVCAG-2361/2 and PVCAG-2269/70).The marker bands of the known parent was encoded as “a” and “b” for both markers to all five female parents. The foreign alleles were encoded by “c” for PVCAG-2361/2 and by “c” and “d” for PVCAG-2269/70. The asterisk indicates contaminant progeny, No. 5 and No.11 of P_3. The first and last lanes with M indicate the standard DNA markers and their sizes in base pair.

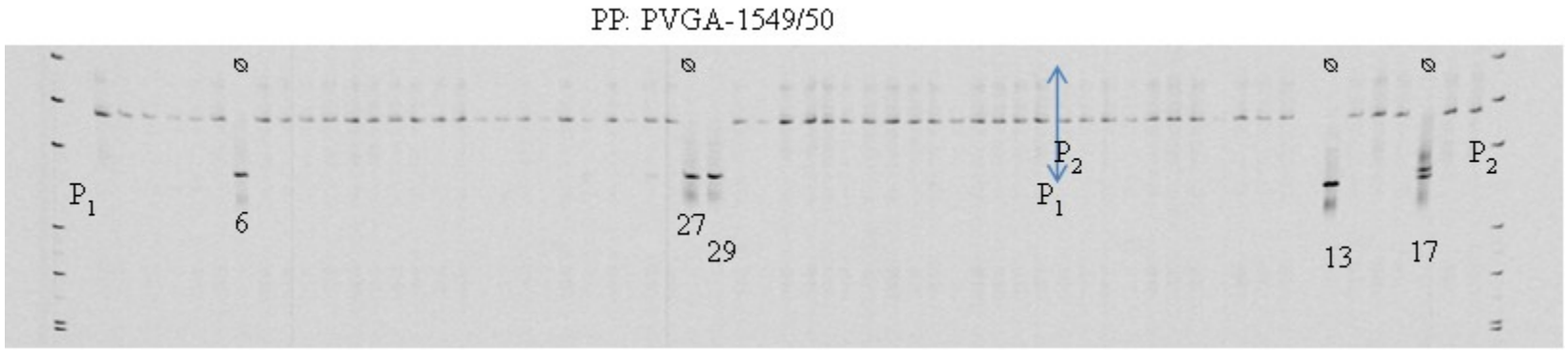

Figure 3. DNA profiles of two female parents NL94/85/1 (P1), and NL94/85/5 (P2) and their progenies amplified with the SSR marker PVGA 1549/50. The gel band of a physical contaminant (PC) is labeled as "ꝏ". The numbers below bands indicate the progeny number of P_1 and P_2 as separated by arrowhead. Standard molecular markers are given at the two ends of the gel.

CHAPTER III

CHARACTERIZING MALE STERILITY IN UPLAND-LOWLAND SWITCHGRASS HYBRIDS AND THEIR S1 PROGENIES

ABSTRACT

Male sterility (MS) has long been identified as a biological system to generate female breeding lines for field-scale hybrid seed production in several major crops. However, information on male sterility of switchgrass is currently unavailable. This research was based on a hypothesis that S1 plants developed via selfing of upland-lowland (intraploid-interecotypic) F1 hybrids would segregate for male sterility in switchgrass. Nine upland and nine lowland switchgrass genotypes were synchronized by trimming, which was followed by isolation and crossing in two greenhouses. Seeds were collected from all 18 seed parents of the crosses. A total of 63 F1 hybrids verified with two SSR markers were potted in the greenhouse and 79 F1 were transplanted in the field, of which, 49 in the greenhouse and 71 of the field plants were bagged with polyester bags. All the bagged F1 plants in both sites produced seeds. Genetic origin of the S1 seedlings were examined with eight to 10 SSRs. Sixty-four S1 seedlings were potted in the greenhouse to test their pollen viability. Male fertility of the selected S1 plants was determined based on pollen viability through pollen germination, pollen grain staining, and seed set of the bagged panicles. However, the Lugol solution staining method did not ratify the pollen viability. Of the 64 S1 genotypes, pollen was collected from 47 and pollen germination was observed in 37

plants. Pollen germination, selfed seed numbers, and open pollinated seed numbers in individual S1 parents were compared. Based on the pollen germination and selfing assessment tests, three S1 genotypes appeared to be male sterile, five had extremely low male fertility i.e. low pollen germination (G% ≤ 2 %,), seven exhibited both male and female sterilities, four showed female sterility and 28 plants had normal male (G% > 2 %) and female fertility under the greenhouse conditions. Since the reproductive potentiality of switchgrass for selfed seeds production is complicated and the study was conducted in the greenhouse, two methods for further confirmation of male fertility of the identified sterile lines are proposed. This experiment added valuable information to the knowledge pool of switchgrass reproductive biology.

Keywords: Male Sterility (MS). Cytoplasmic male sterility (CMS). Synchronization. Interecotypic hybrid. Simple sequence repeats (SSR). Switchgrass. Pollen Viability

ABBREVIATIONS

C-1	Cross (upland x lowland) no. 1
CMS	Cytoplasmic male sterility
CTAB	Cetyltrimethyl ammonium bromide
DB	Dark Brown
LY	Light Yellow
MS	Male sterility
NL	Northern lowland switchgrass

OC	Contaminant seed
OPS	Open pollinated seed
OSH	Open pollinated seedhead
PCR	Polymerase chain reaction
PP	Primer Pair
RC-1	Reciprocal cross (lowland x upland) no. 1
SI	Self-incompatibility
SL	Southern lowland switchgrass
SSR	Simple sequence repeat

INTRODUCTION

Switchgrass (*Panicum virgatum* L.), a potential bioenergy crop, is a cross pollinated, warm-season, perennial grass native to North America [1]. To date, switchgrass cultivars have often been developed using breeding methods like recurrent selection. F1 hybrid development in switchgrass has been proposed based on crossing of two vegetatively propagated heterozygous parents [2,3]. Pre-fertilization self-incompatibility (SI), which exists in the species, prohibits self-fertilization in switchgrass [4]. Since proper mechanisms to breakdown SI are unknown; no homozygous inbred lines have been reported. Moreover, inbreeding depression also suppresses self-fertilization in switchgrass [2]. Recent reports have indicated that inbred lines and heterotic F1 hybrids in switchgrass are possible as the species exhibits conditional self-compatibility [5]. Inbred lines and male sterile lines are often important parts of heterotic hybrid breeding in major

crops like rice (*Oryza sativa* L.), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.) etc. For large scale hybrid seed production, male sterility (MS) mechanisms provide female lines and eliminate the obligation of tedious emasculation. Moreover, the male sterility can be used in other breeding processes such as creating nuclear and cytoplasmic male sterility and the control pollen gene flow, which would be possible by modifying or incorporating the sterile genes [6].

Male sterility (MS) is the inability of a genotype to produce functional anthers, pollen grains or male gametes. MS is common in most of the bisexual plants [7]. MS has evolved naturally in plants due to mutation, diseases or extreme growth conditions [8]. Emasculation was a common process for creating female parents by the earlier breeders working on cereals like maize and wheat (*Triticum aestivum* L.). In grasses, hand emasculation is tedious and no field scale emasculation method has been discovered. MS has been recognized only in a handful of annual and diploid forages [9].

The cytoplasmic male sterility system (CMS) is the most common MS system identified in 150 species, and is inherited maternally [10,6,11]. Like other types of MS, the CMS can be evolved spontaneously, from wide crosses, or due to the effects of mutagens and antibiotics on mitochondrial genes and/or from interspecific recombinations [12,6]. In rye (*Secale cereale* L.), CMS was reported in 1966 in S1 lines derived from wide crosses of Argentinian 'Pampa rye' (*Secale cereale* L.) and Hohenheim inbred lines [13]. The mitochondrial genes encode the CMS [14], which is comprised of a binary genetic system, i.e. controlled by maternally inherited cytoplasmic genes and paternal nuclear genes [15]. The CMS plants are normal isogenic lines but differ in functional pollen. Sterile cytoplasm can cause abnormalities like infertile pollen, or flowers without stamen etc. [15].

Wide crosses normally produce sterile hybrids and other mutants different from the existing one [16]. Pelletier and Budar [15] indicated that CMS can be introduced in a species without normal form of CMS, by sexual or somatic hybridization between different, but related, species. Furthermore, Kausch et al.[6] indicated that sterile hybrids can be developed in switchgrass and other polyploids through wide crosses. To date, several studies have been reported on the genetic and morphological variations between the upland and lowland switchgrass ecotypes. The two ecotypes are physiologically and morphologically diverse [17] and genetically distinct [18]. Hultquist et al. [1] identified restriction fragment length polymorphism (RFLP) in chloroplast DNA (cpDNA) and grouped switchgrass germplasms into two cytotypes: U and L, for upland and lowland ecotypes, indicating each group as a unique pool. Analysis in molecular, morphological and both data sets also revealed the genetic differences between two ecotypes [19]. Variations between two ecotypes have been studied from the phenotypic to the genomic level. Young et al. [20] sequenced the entire chloroplast genome of one lowland (Kanlow Lin 1) and one upland (Summer Lin 2) plant and observed the variations that occur between the members of the two ecotypes at the genomic level. The authors observed significant variations in the sizes, quality and content of genes, and in the organization of the genomes. Thus, upland-lowland (intraploid-interecotypic) crosses in switchgrass would be wide to produce male sterile plants in F1 generation or S1 progeny of F1 hybrids.

Understanding pollen viability is essential to determine the male fertility in switchgrass genotypes. In vitro pollen germination has been considered as the most effective method to determine the pollen viability of perennial grasses like switchgrass and tall fescue (*Festuca arundinacea* Schreb.), which has also been successfully tested in other grasses like perennial ryegrass (*Lolium perenne* L.) and Italian ryegrass (*Lolium multiflorum* Lam.) [21, 22]. However,

the pollen viability and longevity in switchgrass differs according to physical factors such as irradiation, humidity and other weather conditions [21]

Assessment of the male fertility of interecotypic hybrids and their S1 progenies, and the detection of possible male sterile lines in switchgrass could be useful in hybrid breeding. To our knowledge, this is the first experiment which was focused on male fertility and sterility in switchgrass. Accordingly, the major objective of this study was to characterize male sterility in upland-lowland switchgrass hybrids and their S1 progenies.

MATERIALS AND METHODS

Plant materials, synchronization and hybridization, and F1 seed collection

Switchgrass genotypes used in this study were tetraploid upland and lowland plants, their F1 hybrids and S1 progenies of the F1 plants. The upland genotypes were random plants derived from seed of cultivars Dacotah, Summer and WS4U, whereas lowlands parents were breeding lines of the sub-ecotypes: northern and southern lowlands (NL and SL). Nine upland and nine lowland plants potted in a greenhouse were selected as the original parents for nine crosses and their reciprocals (Table 2). Each crossing pair was monitored for four months to judge their heading and flowering time. The upland parents, which headed earlier than their lowland counterparts, were trimmed just after completion of heading until flowering was synchronized. Isolation of the synchronized crossing pairs in two greenhouses was begun on 8th March, 2012 and completed on the 11th April, 2012 (Fig. 4). Five crossing pairs were placed in a greenhouse and four in another. Crosses were facilitated by manual shaking of flowering panicles before noon. Because of differential plant heights of upland and lowland parents, external supports were

provided under the bases of most of the upland pots, so that panicles of both parents came closer for crossing (Fig. 4). Seeds were collected on each parent plant during late May to early July, 2012. A South Dakota seed blower was used to clean seeds [23]. Seeds were treated with 50 % H_2SO_4 and pre-chilled for two weeks at 4 °C before germination [24]. All seeds of C-9, RC-1, RC-4, RC-5, RC-6, and RC-9, and about 155 seeds of the rest of the parents were germinated.

The F1 progeny seedlings were given their IDs with respect to their cross number. The nine crosses and reciprocal crosses as in the order given in Table 2 were named from C-1 to C-9 for crosses, and RC-1 to RC-9 for reciprocal crosses. Upland (♀) x Lowland (♂) was considered as a cross, and Lowland (♀) x Upland (♂) was considered as a reciprocal cross. Thus, in this study, C-1-5 represents the F1 seedling No. 5 derived from a seed obtained from a cross: Dacotah-13 x NL313/9. Similarly, RC-7-2 represents the F1 seedling No. 2 obtained from NL387/8 x WS4U-19. No adequate seedlings developed from RC-5 and RC-9 parents and were excluded from the study (Table 2).

Transplantation of F1 hybrids, and bagging of F1 and their S1 genotypes

A total of 63 F1 hybrids: 35 derived from crosses and 28 derived from the reciprocal crosses were immediately potted in the greenhouse during the 2nd week of December, 2012 after testing their genetic origin using two SSRs. Daily watering and essential fertilization with controlled temperatures of 20-30 °C were provided to the potted plants. Similarly, a total of 79 F1 (49 obtained from upland seed parents and 30 derived from lowland seed parents) were transplanted into a field, spaced 1.07 m between each plant on a row, on the Agronomy Research Farm at OSU, Stillwater, OK, on 8th May, 2013.

The inflorescences of 49 of the 63 F1 genotypes in the greenhouse were bagged in mid-March, 2013. Similarly, a total of 71 of the 79 F1 plants in the field were bagged between 10th and 28th July, 2013 using polyester bags (Fig. 6). The remaining 14 plants in the greenhouse and 8 plants in the field were not bagged due to poor growth. Often two to four inflorescences were enclosed in a bag. T-posts and bamboo sticks were used to attach bags in the field and greenhouse, respectively. Florets developed before bagging were removed to prevent unnecessary fertilization.

From F1 plants in the greenhouse, bagged seeds were collected during the 1st week of May, 2013 (Table 3), and their seedlings were tested with 8 SSRs. Of 64 S1 progenies, 17 from four different F1 hybrids of three original crosses and 47 from 10 different F1 hybrids of 6 original reciprocal crosses (Table 4) were instantly potted in the greenhouse during the 2nd week of the July, 2013. All 64 S1 were bagged for their self-fertility assessment in September/October in 2013.

The seedheads of the bagged F1 plants in the field were harvested on 27th September, 2013 and their seeds were processed, and counted (Table 3). Of the 71 F1 bagged parents in the field, 328 seedlings, of the 16 random parents, were germinated to test their genetic origin.

DNA isolation and PCR amplification of F1 hybrids and their S1 progenies

Fresh leaf tissue of each parent and progeny was separately collected in order to extract their genomic DNA samples and the extraction was completed using a cetyltrimethyl ammonium bromide (CTAB) method described previously [23]. Genetic origins of the 505 putative F1 seedlings of 16 parents (Table 2) were identified using two of the four single SSR primer pairs

(PPs): PVAAG-2895/6, PVAAG-3051/2, PVAAG-3311/2, PVGA-1143/4. One hundred S1 seedlings from 14 different F1 plants in the greenhouse and 328 seedlings of 16 F1 plants in the field were genotyped with eight and 10 polymorphic SSRs, respectively (this research part has been described in chapter II while estimating bagging efficacy in detail). Of the 100 seedlings in the greenhouse, 64 confirmed S1 plants were selected and grown to test pollen viability. Both single and duplex SSR PPs, used for genotyping S1, were selected from [25] and [26] studies. Additionally, other single SSRs: PVGA-1549/50, PVGA-1813/4, and SWW-125 and SWW-1889, and three duplex SSR Sets: 12, 14 and 23 [27] were used for genotyping S1 DNA samples. The processes of single and duplex PCR amplifications, with required volume of chemicals for thermal cycles and separation of PCR products, were followed as described [23,5]. DNA bands were visually scored and measured using Saga Generation 2 software, version 3.3 (LI-COR Biosciences, Lincoln, NE, USA) [5,23].

Sizes of the DNA bands of the parents and respective progenies were compared to determine the breeding origins of the progeny. The DNA band(s) of an F1 seedling were compared with the band (s) of both seed and male parents (Fig. 5). Similarly, the band (s) produced by selfed progeny were compared to its seed parent. Seedlings with DNA band(s) sized different from either parent were regarded as contaminants (Fig. 5).

Pollen stainability and germination test for S1 plants in the greenhouse

Pollen stainability test for all 64 S1 switchgrass genotypes in the greenhouse followed the Lugol solution method with slight modifications as described in rice (*Oryza sativa* L.) [28]. The solution (1 % I_2 – KI solution) was made by adding 1 gm-I_2 and 1 gm- KI to 100 ml distilled

water. Three spikelets per plant were randomly collected just before extrusion of their anthers and stored at 70% ethanol. Anthers of each spikelet were dissected and pollen grains were smashed within the Lugol solution, and 100 stained pollen grains per spikelet were counted. Based on the stained color, two types of pollen: dark brown (DB) and light yellow (LY) were grouped (Fig. 7). Mean values of the percentage of each type of the pollen from three spikelets were calculated (Table 4).

Pollen germination was performed as described [21,22]. Growing media was prepared by mixing the components: 1 % agar, 0.8 M sucrose, 1.28 mM boric acid and 1.27 mM calcium nitrate, and the medium was sterilized by autoclaving for 20 min at 121 ^{0}C. Once the sterilized media cooled to 65 ^{0}C, it was poured into petri dishes and the dishes with solid medium were stored at 4 ^{0}C. For pollen harvest, flowering panicles were brought to the lab with their peduncle placed in water, and stored in the growth chamber at 24 ^{0}C [21]. The in vitro pollen germination conditions were optimized as described [21]. The pollen grains were shed on the medium, incubated for 20 min, and then observed using light microscopy. Pollen tube length was taken as a visible marker for germination; pollen germination was considered successful when the pollen tube length was greater than the pollen diameter [29,21]. Pollen germination percentage (G %) was calculated by counting No. of germinated pollen in a group of 100 pollen grains into the medium after an hour of pollen shedding. The images were visualized using a microscope camera from Leica, and LAS EZ software (Fig. 8). Of the 64 S1 plants, fresh pollen grains were collected from 47 genotypes. The panicles of the remaining 17 plants were sparsely developed; nonetheless, the panicles were bagged for selfing assessment.

RESULTS

All 18 parents of the nine original crossing combinations produced seeds, but seed numbers varied from parent to parent (Table 2). The upland seed parents produced relatively more seeds than their lowland counterparts. A total of 505 genotyped progeny seedlings of the 16 parents were categorized into hybrid (F1), selfed (S1) and outcrossed contaminants (OC). One PCR image (Fig. 5) includes DNA bands of 45 progeny of the C-2 cross amplified by a single-SSR PVAAG-3051/2, where 41 were F1 and four (progeny No. 11, 18, 40 and 41) were OCs, as indicated by "C" in the image (Fig. 5). Similarly, the seedlings of other crosses tested with two SSRs indicated their different genetic origins; C-1, 32 F1 and 13 S1; C-3, 36 F1, 4 S1 and 5 OC; C-4, 35 F1, 9 S1 and 1 OC; C-5, 26 F1, 2 S1 and 17 OC; C-6, 30 F1 and 15 S1; C-7, 42 F1 and 3 OC; C-8, 40 F1 and 5 S1; and C-9, 7 F1 and 3 S1. Progeny seedlings of the reciprocal crosses also showed three different genetic origins; RC-1, all (14) F1; RC-2, 19 F1, 5 S1 and 1 OC; RC-3, 24 F1 and 1 S1; RC-4, 14 F1 and 1 S1; RC-6 all (6) F1; RC-7, 15 F1 and 10 S1; and RC-8 all (25) F1. The contaminants were all outcrossing types, rather than physical contaminants in the seeds.

Seeds were obtained from all 49 bagged F1 in the greenhouse and 71 parents in the field. However, seed numbers per bagged seedhead varied with genotypes as well as bagging sites (Table 3). In the greenhouse, the F1 parent C-2-14 generated a maximum, 38 seeds per seedhead, whereas some plants in the greenhouse produced a little seed (Table 3). No contaminant was observed in 100 S1 seedlings collected from greenhouse parents when tested with 8 SSRs. In the field, the F1 hybrids started heading earlier than other plants in breeding populations. The highest number of seed per bagged seedhead in the field was 452, which were produced by RC-7-13, whereas a parent RC-3-18 produced a minimum seeds per bagged seedhead (Table 3).

Three-hundred-twenty-eight progeny seedlings of the field F1 parents were examined with 10 SSRs showing 317 (96.6 %) true S1, and the remaining 11 were OCs or PCs.

Average dark brown (DB) stained pollen grains from three spikelets of each of S1 plants in the greenhouse ranged from 1 % of RC-1-3/5 to 91.7 % in RC-2-20/3 (Table 4). However, the proportion of stained pollen also varied within the spikelets of the same inflorescences. About 100 % light yellow (LY) pollen grains were observed in a spikelet of the C-2-14/2 (Fig. 7), however, the anthers of another spikelet of the same plant possessed higher amount of DB pollen grains than LY.

Pollen germination was taken as a vital process to determine the male fertility of the S1 genotypes, which was further verified by self-fertility assessment. Pollen germination was observed in 37 S1 genotypes (Table 4) and no pollen was germinated in 10 genotypes grown in the greenhouse. Pollen germination percentage (G %) varied from 1 % (RC-7-8/2) to 67 % (RC-3-4/5). Of the 37 genotypes with viable pollen, extremely low G % (≤ 2) was observed in five genotypes: C-3-5/3 (2 %), RC-3-4/4 (2%), RC-7-2/4 (2%), RC-7-8/2 (1%) and RC-8-2/5 (1%). Four genotypes: C-2-14/1, RC-7-8/1, RC-7-8/4, RC-8-2/1 with viable pollen did not produce selfed and open pollinated seeds. Nine other genotypes: C-2-14/2, RC-1-3/4, RC-2-17/4, RC-2-17/5, RC-4-2/1, RC-4-3/1, RC-7-2/1, RC-7-2/3 and RC-7-2/5 with viable pollen (G % >2 %) did not produce selfed seeds but generated a few seeds under open pollination (Table 4). Nineteen S1: C-2-3/2, C-8-10/4, RC-1-3/1, RC-1-3/2, RC-1-3/6, RC-2-17/3, RC-2-20/1, RC-2-20/2, RC-2-20/3, RC-3-4/1, RC-3-4/3, RC-3-4/5, RC-3-4/7, RC-4-2/2, RC-4-2/5, RC-4-3/3, RC-7-2/2, RC-7-8/3, and RC-8-1/5 with viable pollen successfully produced selfed (S2) and open pollinated seeds in the greenhouse (Table 4).

Of the 10 genotypes without pollen germination, seven plants: C-2-14/3, C-2-14/5, C-3-5/4, C-8-10/2, C-8-10/3, C-8-10/5, and RC-4-3/5 did not produce any seed under both bagged and open pollination conditions in the greenhouse. The three other genotypes: C-2-3/1, C-2-3/5 and RC-8-1/4 produced a few seeds under open pollination and no seed was observed in bagging (Table 4). Seven plants of the former types appeared to be both male and female sterile, and the remaining three displayed male sterility under the greenhouse conditions.

Ten of the 17 S1 plants from which we failed to collect pollen, also did not produce any seeds under both pollination conditions. The remaining seven: RC-1-3/8, RC-2-17/1, RC-2-17/2, RC-2-20/4, RC-4-3/2, RC-8-1/1, and RC-8-1/2 produced some seeds under open pollination (Table 4). Because the pollen germination test was not successful in these 17 S1, no further analysis was made on their male fertility.

DISCUSSIONS

This study further endorses the compatibility of intraploidy crossing between two ecotypes of switchgrass, which was previously reported [30,31]. The tetraploid upland and lowland ecotypes produced small to a substantial amount of F1 seeds. However, timely and skillful trimming is required for flowering synchronization. Six of the nine upland parents were trimmed once, and remaining three parents were trimmed twice because of delayed-flowering of their lowland counterparts. Furthermore, obtaining fewer F1 seeds from lowland parents could be affected by the maturity of the upland (male) parent, because they matured a few days earlier than the corresponding lowland parents. Contaminants (OCs) found among the F1 progeny indicated that the isolation provided to their parents was not enough. Contaminants might also arise during

shaking of the crossing panicles, since the same hand was used for different crossing pairs. However, both parents of some crossing combinations produced no contaminant and mostly F1 seeds, *e.g*. C-1 and RC-1, C-6 and RC-6, and C-8 and RC-8. Hence, proper synchronization and isolation is crucial to produce interecotypic hybrid seeds while crossing between upland and lowland switchgrass.

The earlier (in early July) heading of the interecotypic F1 hybrid population in the field was interesting. Their heading time, which occurred at the middle of the normal heading time of the upland and the lowland, suggests the heading date in switchgrass could be controlled by the additive effects of the genes from both parents. Figure 6 shows bagged F1 plants in the field where the panicle inflorescences of other populations in near field had not been developed up to their bagging period. It is therefore, study on physio-morphological and reproductive biology of the upland-lowland F1 hybrids would be necessary to understand their phenotypic and genotypic differences with the normal upland and lowland populations.

Since all the bagged F1 at both environments produced S1 seeds, they should be normal male and female fertile plants. Obtaining relatively fewer number of S1 in the greenhouse may be due to growing conditions, plant health, panicle sizes, and pollen flow. Most of the putative S1 tested within the experiment remained true S1 indicating that the bagging system worked effectively.

Earlier studies have indicated that the Lugol solution method for pollen staining simply detects starch contents and is not effective in estimating the viability of pollen in switchgrass [21]. This study also indicates no direct relation among pollen germination, selfed seed generation and proportion of the dark brown (DB) pollen grains of the switchgrass. The dark DB

grains are often considered viable in the staining test of the other crops like rice. In an S1 genotype C-2-14/1, pollen germination (11 %) was observed whereas the DB stained pollen was only about 8 %. Similarly, the opposite result was observed in RC-4-3/5. A genotype C-2-14/2, consisting completely LY pollen grains (Fig. 7) in a spikelet, had 38 % average DB pollen grains as well as 6% pollen germination. It is therefore, the color of the stained pollen in switchgrass varies not only genotype to genotype but also spikelet to spikelet. However, most of the S1 parents which produced substantial selfed seeds (S2) had relatively higher DB pollen *e.g.* RC-1-3/1, RC-2-20/2, RC-3-4/5, etc. Nonetheless, the relation was not consistent, and thus the Lugol pollen staining does not ratify the pollen viability in switchgrass.

Pollen germination up to 67 % indicated that the protocol we followed **[**21**]** was effective to detect pollen fertility through viability under the optimal conditions (Fig. 8). The S1 genotypes with higher proportion of viable pollen often produced a higher number of selfed seeds, *e.g.* RC-3-4/5, RC-2-20/2, RC-3-4/7 etc. Despite having viable pollen, nine genotypes did not produce selfed seeds, which may be due to the self-incompatibility or another reason [4] (Table 4). Similarly, some genotypes with viable pollen also did not generate selfed as well as open pollinated seeds. These genotypes would be female sterile; C-2-14/1, RC-7-8/1, RC-7-8/4, RC-8-2/1 under greenhouse conditions.

Male and female fertilities of seven of the 10 genotypes with null germination (0 G %) seemed ambiguous. Producing no open pollinated seeds and no viable pollen possibly indicates that the genotypes may have both male and female sterilities. Three of those 10, (C-2-3/1, C-2-3/5, RC-8-1/4) would be male sterile under the greenhouse conditions since they produced few open pollinated seeds. Thus, future research on these 10 genotypes would be valuable for confirmation of their male fertilities. In addition to these, the five genotypes (C-3-5/3, RC-3-4/4,

RC-7-2/4, RC-7-8/2, RC-8-2/5) with extremely low pollen germination (G % ≤ 2) could also be male sterile, they set significantly higher No. of seeds under open pollination than with selfing. And two of them (C-3-5/3 and RC-7-2/4) did not generate any selfed seeds (Table 4). Four other genotypes with viable pollen but no seeds may be female sterile. Twenty eight male fertile plants with viable pollen (G % >2) observed in this study most likely indicates that male sterility in the S1 of the interecotypic F1 hybrids in switchgrass is not common.

Two methods for future breeding work are suggested to validate the data of this study. It is possible to grow the genotypes possessing male sterility, and both male and female sterility that were recognized in this experiment under field conditions. Subsequently, the pollen germination and bagging can be carried out. The experiment can be repeated for two years if results are positive. Secondly, we can cross the male sterile genotypes with known male fertile parents in isolation for which growth chambers could be appropriate. Seeds can be collected from both parents and genotyping of the seeds collected from both the crossing partners can be conducted to determine their parental history. If no seedling is identified with the paternal history of the male sterile parent, then the male sterile parent will be treated as a true male sterile under all growing conditions. Both tests can also be performed at the same time.

CONCLUSIONS

This is the first experiment for the assessment of male sterility in switchgrass which reporting the development of some male sterile plants under greenhouse conditions. All F1 hybrids tested within the experiment were fertile, and can be used for further breeding works. The substantial

amounts of S1 seeds obtained from selfing of F1 hybrids are potential breeding materials for the future. The S1 of the F1 genotypes showed varied pollen viability in the greenhouse. Field based experiments are recommended on the potential male sterile plants for detecting their performance under natural conditions.

REFERENCES

1. Hultquist SJ, Vogel K, Lee D, Arumuganathan K, Kaeppler S (1996) Chloroplast DNA and nuclear DNA content variations among cultivars of switchgrass, *Panicum virgatum* L. Crop Sci 36:1049-1052

2. Casler M (2012) Switchgrass breeding, genetics, and genomics. In: Monti A (ed) Switchgrass. Green Energy and Technology. Springer London, pp 29-53. doi:10.1007/978-1-4471-2903-5_2

3. McLaughlin SB, Adams Kszos L (2005) Development of switchgrass (*Panicum virgatum* L.) as a bioenergy feedstock in the United States. Biomass and Bioenergy 28:515-535. doi:http://dx.doi.org/10.1016/j.biombioe.2004.05.006

4. Martínez-Reyna JM, Vogel KP (2002) Incompatibility systems in switchgrass. Crop Sci 42:1800-1805. doi:10.2135/cropsci2002.1800

5. Liu L, Thames S, Wu Y (2013) Lowland switchgrass plants in populations set completely outcrossed seeds under field conditions as assessed with ssr markers. Bioenerg Res:1-7. doi:10.1007/s12155-013-9367-7

6. Kausch AP, Hague J, Deresienski A, Tilelli M, Longo Jr C, Nelson K, Oliver M, Li Y (2012) Male sterility and hybrid plant systems for gene confinement. Plant Gene Containment:83-98

7. Lewis D (1941) Male sterility in natural populations of hermaphrodite plants the equilibrium between females and hermaphrodites to be expected with different types of inheritance. New Phytologist 40:56-63. doi:10.1111/j.1469-8137.1941.tb07028.x

8. Budar F, Pelletier G (2001) Male sterility in plants: occurrence, determinism, significance and use. Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie 324:543-550. doi:http://dx.doi.org/10.1016/S0764-4469(01)01324-5

9. Vogel KP and Pedersen JF (1993) Breeding systems for cross-pollinated perennial grasses. Plant Breeding Reviews 11:251

10. Williams ME, Leemans J, Michiels F, Shivanna K, Sawhney V (1997) Male sterility through recombinant DNA technology. Pollen biotechnology for crop production and improvement:237-257

11. McVetty PBE (1997) Cytoplasmic male sterility. Pollen biotechnology and crop production and improvement. (eds KR Shivanna and VK Sawhney) 155-182, Cambridge University Press, Cambridge

12. Schnable PS, Wise RP (1998) The molecular basis of cytoplasmic male sterility and fertility restoration. Trends in plant science 3:175-180

13. Geiger H, Schnell F (1970) Cytoplasmic male sterility in rye (*Secale cereale* L.). Crop Sci 10:590-593

14. Bentolila S, Alfonso AA, Hanson MR (2002) A pentatricopeptide repeat-containing gene restores fertility to cytoplasmic male-sterile plants. Pnas 99:10887-10892

15. Pelletier G, Budar F (2007) The molecular biology of cytoplasmically inherited male sterility and prospects for its engineering. Current opinion in biotechnology 18:121-125. doi:http://dx.doi.org/10.1016/j.copbio.2006.12.002

16. Clark J (1936) Improvement in wheat. Year book of the United States department of agriculture US Department of agriculture

17. Casler M (2005) Ecotypic variation among switchgrass populations from the northern USA. Crop Sci 45:388-398

18. Barnett F, Carver R (1967) Meiosis and pollen stainability in switchgrass, *Panicum virgatum* L. Crop Sci 7:301-304

19. Cortese L, Honig J, Miller C, Bonos S (2010) Genetic diversity of twelve switchgrass populations using molecular and morphological markers. Bioenerg Res 3:262-271. doi:10.1007/s12155-010-9078-2

20. Young HA, Lanzatella CL, Sarath G, Tobias CM (2011) Chloroplast genome variation in upland and lowland switchgrass. PloS one 6 :e23980

21. Ge Y, Fu C, Bhandari H, Bouton J, Brummer EC, Wang Z-Y (2011) Pollen viability and longevity of switchgrass (L.). Crop Sci 51:2698-2705

22. Wang Z-Y, Ge Y, Scott M, Spangenberg G (2004) Viability and longevity of pollen from transgenic and nontransgenic tall fescue (*Festuca arundinacea*)(*Poaceae*) plants. American Journal of Botany 91:523-530

23. Liu L, Wu Y (2012) Identification of a selfing compatible genotype and mode of inheritance in switchgrass. BioEnergy Res., vol 5. Springer-Verlag. doi:10.1007/s12155-011-9173-z

24. Bhandari HS, Saha MC, Fasoula VA, Bouton JH (2011) Estimation of genetic parameters for biomass yield in lowland switchgrass (*Panicum virgatum* L.). Crop Sci 51:1525-1533. doi:10.2135/cropsci2010.10.0588

25. Wang YW, Samuels TD, Wu YQ (2011) Development of 1,030 genomic SSR markers in switchgrass. Theor Appl Genet 122 :677-686. doi:10.1007/s00122-010-1477-4

26. Okada M, Lanzatella C, Tobias C (2011) Single-locus EST-SSR markers for characterization of population genetic diversity and structure across ploidy levels in switchgrass (*Panicum virgatum* L.). Genet Resour Crop Evol 58 :919-931. doi:10.1007/s10722-010-9631-z

27. Liu L, Wu Y (2012) Development of a genome-wide multiple duplex-SSR protocol and its applications for the identification of selfed progeny in switchgrass. BMC Genomics 13:1-13. doi:10.1186/1471-2164-13-522

28. Li W, Jiang L, Zhou S, Wang C, Liu L, Chen L, Ikehashi H, Wan J (2007) Fine mapping of pss1, a pollen semi-sterile gene in rice (*Oryza sativa* L.). Theor Appl Genet 114:939-946. doi:10.1007/s00122-006-0491-z

29. Tuinstra M, Wedel J (2000) Estimation of pollen viability in grain sorghum. Crop Sci 40:968-970

30. Martinez-Reyna JM, Vogel KP (2008) Heterosis in switchgrass: spaced plants. Crop Sci., vol 48. doi:10.2135/cropsci2007.12.0695

31. Vogel KP, Mitchell RB (2008) Heterosis in switchgrass: biomass yield in swards. Crop Sci 48:2159-2164

Table 2 Nine upland and lowland genotypes selected for hybridization, their crosses and reciprocal IDs, number of seeds produced, and seedlings tested.

S.N	Upland x Lowland parents (9 parental combinations)	Crosses ID	No. seeds upland(♀)	Tested F1 Seedlings	Reciprocal cross ID	No. seeds Lowland (♀)	Tested F1 seedlings
1	Dacotah-13 x NL313/9	C-1	1184	45	RC-1	49	14
2	Dacotah-14 x NL225/7	C-2	2589	45	RC-2	791	25
3	Dacotah-15 x NL313/10	C-3	2403	45	RC-3	939	25
4	Summer-14 x SL34/13	C-4	206	45	RC-4	67	15
5	Summer-15 x SL6/10	C-5	648	45	RC-5	11	1
6	Summer-16 x NL387/6	C-6	672	45	RC-6	63	6
7	WS4U-19 x NL387/8	C-7	1449	45	RC-7	353	25
8	WS4U-20 x NL33/7	C-8	923	45	RC-8	507	25
9	WS4U-21 x NL81/8	C-9	47	10	RC-9	6	0

Table 3 F1 genotypes bagged in the field and greenhouse with the number of bagged seedheads and seeds generated by each parent in 2013.

Genotypes	No. BSH Ъ	No. putative Selfed seeds	Genotypes	No. BSH Ъ	No. putative Selfed seeds
---------- F1 hybrids bagged in a greenhouse ----------					
C-1-5	10	5	RC-1-1	7	4
C-1-9	12	27	RC-1-3	8	204
C-1-14	8	52	RC-1-4	3	5
C-2-3	16	355	RC-2-16	5	42
C-2-6	4	49	RC-2-17	9	88
C-2-9	6	5	RC-2-20	2	19
C-2-14	14	528	RC-2-21	9	41
C-3-3	7	14	RC-3-4	5	102
C-3-4	10	77	RC-3-7	6	7
C-3-5	5	53	RC-4-1	3	16
C-3-10	2	10	RC-4-2	8	16
C-4-1	4	38	RC-4-3	11	29
C-4-2	3	10	RC-4-4	4	7
C-4-3	12	110	RC-6-1	5	35
C-4-4	2	1	RC-6-2	5	2
C-6-8	4	5	RC-6-3	4	3
C-7-4	5	17	RC-6-4	1	2

C-7-5	9	12	RC-7-2	3	19
C-7-6	4	20	RC-7-3	6	21
C-8-2	2	1	RC-7-5	7	42
C-8-10	3	16	RC-7-8	8	56
C-8-11	3	11	RC-8-1	9	73
C-8-17	1	1	RC-8-2	9	61
C-9-3	1	5	RC-8-3	10	24
			RC-8-4	5	8

---------- F1 hybrid bagged in a field ----------

C-1-15	14	3471	C-8-28	6	76
C-1-22	7	155	C-8-33	11	225
C-1-33	12	1350	C-8-42	6	202
C-1-37	4	378	C-8-43	10	550
C-1-44	9	1352	C-8-45	11	421
C-2-1	9	877	C-9-5	8	122
C-2-15	6	681	RC-1-5	4	97
C-2-16	15	3659	RC-1-6	14	1281
C-2-21	6	79	RC-1-7	23	2487
C-2-23	4	70	RC-1-8	5	150
C-2-24	3	172	RC-1-12	13	2766
C-3-8	19	602	RC-2-10	4	85
C-3-15	10	131	RC-2-11	6	242
C-3-16	10	1302	RC-2-12	5	941
C-3-19	2	65	RC-3 -9	11	156
C-3-20	38	6285	RC-3-13	8	152
C-3-30	9	100	RC-3-18	3	12
C-4 -5	5	1057	RC-3-19	6	71
C-4-9	4	27	RC-3-21	20	85
C-4-10	7	71	RC-4-5	2	10

C-4-11	6	1902	RC-4-6	6	1004
C-4-17	5	143	RC-4-12	5	39
C-4-18	7	191	RC-4-13	7	165
C-5-9	8	698	RC-6-5	14	759
C-5-20	7	75	RC-6-6	5	150
C-5-26	10	55	RC-7-13	15	6785
C-5-27	6	85	RC-7-14	10	2047
C-5-30	9	388	RC-7-15	14	391
C-6-29	6	35	RC-7-16	4	255
C-7-7	6	93	RC-7-19	3	88
C-7-8	5	248	RC-8-8	8	961
C-7-9	12	774	RC-8-9	5	145
C-7-10	8	270	RC-8-10	14	261
C-7-35	6	435	RC-8-12	6	378
C-7-40	14	535	RC-8-14	20	944
C-8-22	9	184			

Ъ BSH = No. of bagged seedheads

Table 4 S1 progeny of interecotypic F1 tested for their male fertility via pollen staining, pollen germination and self-fertility assessment.

S1 genotypes	DB pollen % Ψ	LY pollen % ¥	G % ±	No. BSH Λ	No. putative selfed seeds	No. OSH β	No. OPS Ж
C-2-3/1	44.0	56.0	0	6	0	3	2
C-2-3/2	49.7	50.3	21	7	69	2	45
C-2-3/4	76.3	23.7	ND	3	0	4	0
C-2-3/5	45.4	54.6	0	4	0	3	2
C-2-14/1	8.0	92.0	11	3	0	2	0

C-2-14/2	38.0	62.0	12	6	0	4	12
C-2-14/3	42.4	57.4	0	11	0	5	0
C-2-14/5	39.3	60.7	0	4	0	3	0
C-3-5/1	78.67	21.33	ND	6	0	2	0
C-3-5/2	41.67	58.33		3	0	2	0
C-3-5/3	89.7	10.3	2	3	0	1	2
C-3-5/4	79.0	21.0	0	4	0	2	0
C-3-5/5	80.67	19.33	ND	7	0	3	0
C-8-10/2	79.67	20.33	0	4	0	3	0
C-8-10/3	47.33	52.67	0	5	0	4	0
C-8-10/4	46.0	54.0	6	1	3	1	5
C-8-10/5	49.0	51.0	0	7	0	3	0
RC-1-3/1	89.0	11.0	56	5	118	3	94
RC-1-3/2	88.7	11.3	4	9	14	2	4
RC-1-3/3	56.0	44.0	ND	7	0	3	0

RC-1-3/4	76.0	24.0	33	6	0	5	10
RC-1-3/5	1.0	99.0	ND	3	0	4	0
RC-1-3/6	87.3	12.7	53	4	33	2	71
RC-1-3/7	65.0	35.0	ND	4	0	3	0
RC-1-3/8	41.8	58.2	ND	3	0	2	4
RC-2-17/1	76.8	23.2	ND	9	0	3	2
RC-2-17/2	84.0	16.0	ND	8	0	3	18
RC-2-17/3	86.6	13.4	34	7	404	1	58
RC-2-17/4	56.0	44.0	32	10	0	7	4
RC-2-17/5	62.7	37.3	43	6	0	4	2
RC-2-20/1	67.0	33.0	47	8	18	4	76
RC-2-20/2	83.0	17.0	46	12	1178	4	488
RC-2-20/3	91.7	8.3	47	6	6	4	81
RC-2-20/4	68.0	32.0	ND	7	0	6	11
RC-3-4/1	80.4	19.6	19	4	20	2	16

RC-3-4/3	63.7	36.3	31	5	49	3	85
RC-3-4/4	46.7	53.3	2	6	4	5	61
RC-3-4/5	90.0	10.0	67	12	1172	6	1344
RC-3-4/7	86.4	13.6	43	7	541	4	150
RC-4-2/1	64.3	35.7	23	5	0	6	47
RC-4-2/2	54.7	45.3	38	3	5	4	42
RC-4-2/5	52.6	47.4	46	3	10	4	22
RC-4-3/1	32.0	68.0	23	6	0	3	5
RC-4-3/2	79.3	20.7	ND	2	0	2	2
RC-4-3/3	76.5	23.5	24	2	11	7	27
RC-4-3/5	85.0	15.0	0	2	0	3	0
RC-7-2/1	49.0	51.0	12	3	0	3	2
RC-7-2/2	45.7	54.3	19	4	2	3	46
RC-7-2/3	75.7	24.3	20	7	0	3	1
RC-7-2/4	85.0	15.0	2	7	0	5	13

RC-7-2/5	74.0	26.0	22	3	0	5	3
RC-7-8/1	41.0	59.0	23	9	0	2	0
RC-7-8/2	86.4	13.6	1	5	2	4	62
RC-7-8/3	56.7	43.3	31	7	1	3	5
RC-7-8/4	74.0	26.0	14	3	0	1	0
RC-8-1/1	57.3	42.7	ND	3	0	3	7
RC-8-1/2	69.7	33.3	ND	3	0	4	9
RC-8-1/3	46.0	54.0	ND	6	0	4	0
RC-8-1/4	23.0	77.0	0	7	0	2	15
RC-8-1/5	79.7	20.3	39	5	17	4	21
RC-8-2/1	54.6	45.4	10	4	0	3	0
RC-8-2/3	34.3	65.7	ND	5	0	3	0
RC-8-2/4	56.4	43.6	ND	1	1	2	0
RC-8-2/5	47.0	53.0	1	4	0	4	14

Ψ DB = average dark brown pollen grains from 3 spikelets each measured from a group of 100 pollen grains;

¥ LY= average light Yellow pollens from 3 spikelets each measured from a group of 100 pollen grains;

± G % =Pollen germination percentage;

Λ BSH= No. of bagged seedheads;

β OSH= No. of open pollinated seedheads;

Ж OPS = No. of open pollinated seeds;

ND= no data available

Figure 4. Synchronized upland (Summer-16) and lowland (NL387/6) switchgrass parents isolated in a greenhouse for crossing. Because of the shorter plant height, a support was provided under the base of the container for the upland parent to equalize their panicles height.

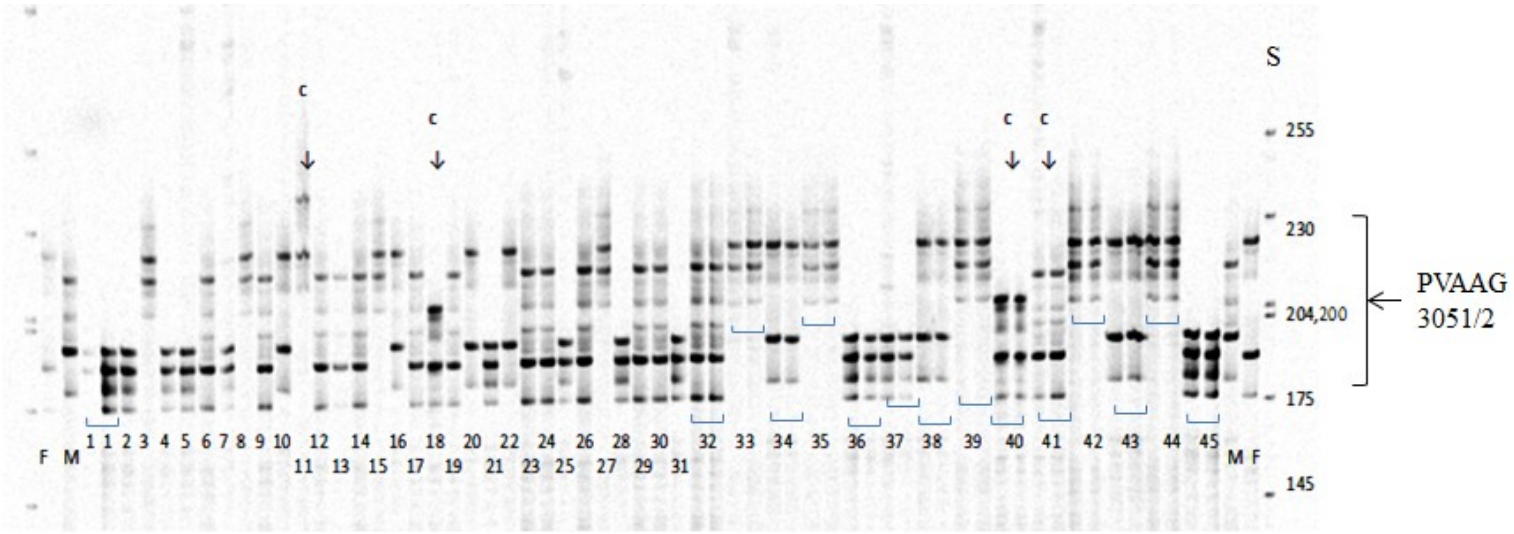

Figure 5. DNA profiles of a female parent Dacotah-14 (F), male parent NL225/7 (M) and their 45 F1 progeny (indicated by numbers 1-45 below bands) genotypes amplified by a single-SSR PVAAG 3051/2. DNAs of parents and some progenies were replicated to use all 64 wells in the panel. Parents DNA lanes were indicated as 'M' (male parent) and 'F' (female parent) at two ends with standard DNA marker 'S'. The scale given at the right-end gives the molecular weight of DNA fragments in base pair unit. The foreign alleles were encoded by 'C'.

Figure 6. Bagged interecotypic F1 switchgrass plants in the field. Their panicles were developed about four weeks earlier than the other lowland plants in the field

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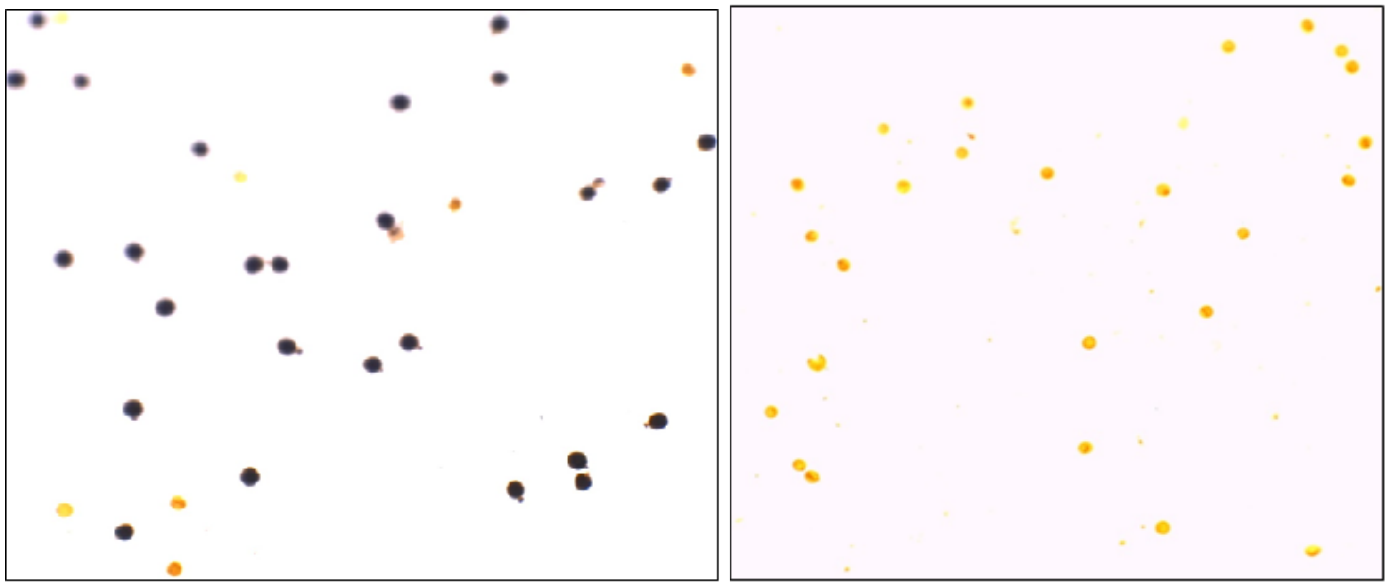

Figure 7. Stained pollen grains of two S1 plants: the left box consists of the dark brown and light yellow pollen of a switchgrass genotype C-2-3/4, and right box includes completely light yellow pollen grains of a spikelet of C-2-14/2 genotype.

Figure 8. Germination of pollen grains of an S1 plant RC-2-20/1 in a growing media observed after an hour of pollen shedding. Three of twelve pollen grains were already germinated with a distinct pollen tube, two just protruded pollen tubes, and remaining six remained ungerminated. The image was taken using a microscope camera from Leica, and LAS EZ software.

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CHAPTER IV

GENERAL CONCLUSIONS

The objective of the first experiment was to determine the efficacy of the polyester bagging method required for selfing switchgrass. Selfed seed production in switchgrass is critical because of the crop's sexual mode of reproduction and its reproductive feature which includes self-incompatibility. The conditional self-compatibility in switchgrass is useful in developing inbreds, and thus an efficient bagging method is necessary to exclude extraneous pollen and provide a secure selfing environment. Of the total 100 bags tested in the field, in two years, 85 bags produced 100 % selfed seeds. In 13 bags out of the remaining 15, a very low number of physical and outcrossing contaminants were observed. The contaminants that are believed to have originated from the operational accidental errors and physical breakdown in the bags can be minimized by careful monitoring of the bagged parents in the field. Therefore, the polyester bagging method is the most efficacious bagging method in switchgrass reported to date and a valuable tool for switchgrass breeders.

The second experiment investigated male sterility in the greenhouse which would form the basis for the establishment of commercial male sterile lines under field condition. The substantial amount of intraploid-interecotypic hybrid seeds generated from all the crossing pairs of upland-lowland hybridization indicated that the two ecotypes are crossable if flowering could be

synchronized. Hence, any breeding and genetics activities on either of the ecotypes that result in their heading and flowering synchronization would be effective for hybridizing the two ecotypes at the farm level. Once male sterile lines are developed, they can be incorporated into the breeding system to generate female breeding lines. The value of male sterile lines will further increase as a part of heterotic hybrids with successful development of homozygous inbreds. The complementary inbreds consisting of an ideally male sterile parent could be useful for single-cross hybrid development.

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Candidate for the Degree of

Master of Science

Thesis: TESTING THE EFFICACY OF A POLYESTER BAGGING METHOD FOR SELFING SWITCHGRASS, AND CHARACTERIZING MALE STERILITY IN UPLAND-LOWLAND SWITCHGRASS HYBRIDS AND THEIR S1 PROGENIES

Major Field: Plant and Soil Sciences

Biographical

Educations:

Completed the requirements for the Master of Science in plant and soil sciences at Oklahoma State University, Stillwater, Oklahoma (May, 2014).

Completed the requirements for the Bachelor of Science in Agriculture at Tribhuvan University, Nepal (August, 2009).

Experiences:

Employed by Department of Plant and Soil Sciences at Oklahoma State University as a Graduate Research Assistant (August, 2011-May, 2014).

Employed by Arava International Center for Agricultural Training (AICAT), Israel as an intern (September, 2009-July, 2010).

Professional and Honorary Memberships:

The Honor Society of Phi Kappa Phi (2012)
American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America (2013)