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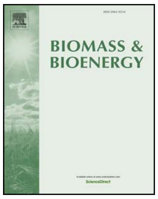

Research paper

# Extent of pollen-mediated gene flow and seed longevity in switchgrass (*Panicum virgatum* L.): Implications for biosafety procedures

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## ABSTRACT

New switchgrass (*Panicum virgatum* L.) bioenergy cultivars are being bred through genetic engineering; however, baseline information is urgently needed to establish guidelines for small-scale field trials prior to commercialization. In this study, we documented the pattern of pollen-mediated gene flow and the extent of seed longevity in field experiments. To mimic crop-to-wild, pollen-mediated gene flow, we planted wild recipient switchgrass ramets at various distances away from cultivar donor ramets at two sites in Ohio. Percent hybridization at each distance was estimated from seed set on recipient ramets, which were self-incompatible clones. The pattern of gene flow was best described by negative exponential models, and the minimum isolation distance for a 0.01% gene flow threshold was predicted to be 69 m and 109 m away from the pollen source at the two sites. To investigate seed longevity, we buried seeds of six cultivars and ten wild biotypes in Ohio and Iowa in 2011. A subset of the seeds were exhumed, germinated, and tested for dormancy over three years. Cultivars lost seed viability and dormancy significantly sooner than wild biotypes at both locations in the first year, and most biotypes lost dormancy by the second year. Cultivar seeds buried in the cooler, drier Iowa site had an overall greater longevity than those buried in Ohio. Our findings suggest that substantial amounts of pollen-mediated gene flow could occur in the immediate vicinity of switchgrass pollen sources, and current switchgrass cultivars are unlikely to persist in the seed bank for more than three years.

## 1. Introduction

Lignocellulosic "second-generation (2G)" bioenergy crops, for instance, switchgrass (*Panicum virgatum* L.) and miscanthus (*Miscanthus* spp.), have attracted much attention because they (a) utilize the more efficient $C_4$ photosynthetic pathway, (b) have a perennial growth habit, extensive root systems, and abundant, harvestable biomass, and (c) tolerate poor growing conditions by having relatively high nutrient and water use efficiency [1–3]. These traits combined with other considerations, such as providing ecosystem service, offer advantages while minimizing competition with major food crop production [4–6].

Switchgrass has become a potential bioenergy crop in the U.S. in part because of its native origin, minimal pest and disease problems, and great adaptability to a broad range of environments. It is a perennial $C_4$ grass that was commonly found throughout the U.S. east of the Rocky Mountains before European settlement [7]. While unique wild switchgrass populations are still present and scattered across the landscape [8], domesticated switchgrass has been in demand as a multipurpose crop across the U.S. Since the 1980s, switchgrass has been planted for animal feedstock, wildlife habitat, and soil restoration [9–12]. With the need to develop renewable energy sources, it has been studied as a model bioenergy species in the U.S. during the past three decades, alongside multimillion dollar investments in breeding and cultivar development [13–15].

In the last decade, genetic engineering has been used to expedite switchgrass breeding programs [16,17]. For instance, by overexpressing a transcription factor gene, Baxter et al. [18] created a switchgrass line with lower lignin content, resulting in significantly higher biofuel and biomass production (32% and 63%, respectively) compared to the non-transgenic control group. In another study, a sucrose synthase gene was overexpressed, which produced greater plant height, tiller number, and biomass relative to control plants [19]. Insertion and overexpression of a maize microRNA into switchgrass improved starch content and digestibility, and it also prevented switchgrass from flowering, therefore avoiding potential gene flow through pollen [20].

The development of transgenic switchgrass for bioenergy has

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generated discussion of the potential evolutionary consequences if transgenes are introduced into wild populations through gene flow. For instance, prolific aboveground production and fast growth are commonly found in weeds [21,22]. If new switchgrass cultivars possess significant fitness advantages and escape from cultivation, they could establish and possibly change the dynamics of local ecosystems in which they are planted, or to which they are dispersed [22]. In addition, hybridization between advanced cultivars and wild relatives could potentially replace local gene pools through pollen, seeds, or vegetative propagules [23–26]. Furthermore, if the introduced transgenes provide wild relatives with more advantageous traits, weed management could become more difficult and costly [24]. As of October 2017, the U.S. Department of Agriculture (USDA) has granted permission for thirty-five controlled open air field release involving transgenic switchgrass [27]. However, the extent of gene flow in switchgrass under field conditions, whether through pollen or seed, has not been well characterized. Without such information, efforts to minimize potential ecological impacts of transgenes are compromised. The same information is also urgently needed for commercialization of future transgenic switchgrass and conservation of wild switchgrass populations [23].

Despite interest in breeding 2G bioenergy crops, existing gene flow studies have mainly focused on food crops, and our understanding of gene flow in emerging bioenergy crops remains limited [21,23]. Moreover, while multiple switchgrass cultivars have been developed for forage and energy, ecological characteristics of new cultivars have not been well characterized. For example, the extent and variability of pollen-mediated gene flow in switchgrass under field conditions have only been recently described in the literature [28]. The pattern of pollen-mediated gene flow is dictated by numerous factors, for instance, climatic conditions, pollination biology, and size and proximity of source and recipient populations; therefore, patterns of gene flow are expected to vary in different landscapes [29–31]. Seed dispersal also contributes to gene flow. Switchgrass seeds are lightweight and can easily disperse from planted fields through wind, water movement, or during harvest and transportation. Furthermore, dispersed seeds emerging in the subsequent year, i.e., as volunteers, could act as a secondary source of gene flow [32]. Nevertheless, we are not aware of a systematic comparison of seed persistence between domesticated and wild switchgrass.

As 2G bioenergy crops go forward, the USDA has demanded more thorough ecological studies for making regulatory decisions [22,23,28,29,33]. Specifically, data are needed to characterize the minimum isolation distance between switchgrass carrying novel transgenes and sexually compatible relatives, and the longevity of these seeds when left in the soil. One of the goals of this study was to measure the frequencies of pollen-mediated gene flow in switchgrass from a small donor source to recipient plants at different distances from the source, inferring the characteristic gene-flow radius using the frequency of crop-wild hybridization versus distance in two locations. In addition, this study compared seed viability and dormancy in cultivated and wild switchgrass in two common garden experiments over the span of three years. To our knowledge, this study is the first to document pollen-mediated gene flow in switchgrass under field conditions and to compare seed longevity in switchgrass cultivars and wild biotypes over a period of several years. This study aimed to provide guidance for future regulation of transgenic switchgrass developed for bioenergy, especially for field trials prior to deregulation and commercialization. It also aimed to shed light on the long-term conservation of remnant switchgrass populations, which often occur near agricultural land.

## 2. Materials and methods

### 2.1. Pollen-mediated gene flow

To track pollen movement from cultivated to wild populations, we recorded the frequencies of crop-wild hybridization in experimental arrays at two sites in Ohio (Fig. 1). We used clonally propagated ramets from one pair of three-year-old, non-transgenic switchgrass clones at each site, with cultivar ramets as the pollen donors and wild ramets as the pollen recipients. Because switchgrass is self-incompatible and the ramets were genetically identical [34], they could only hybridize with genetically distinct individuals present in the field. Therefore, any seeds present on the recipient plants should be crop-wild hybrids, provided that no other pollen sources occur nearby. Note that switchgrass is generally believed to be an obligate outcrossing species; however, it is possible that an isolated individual could self in rare circumstances. Hence, we included molecular analysis to check for paternity status of the putative hybrid seeds using microsatellite DNA markers.

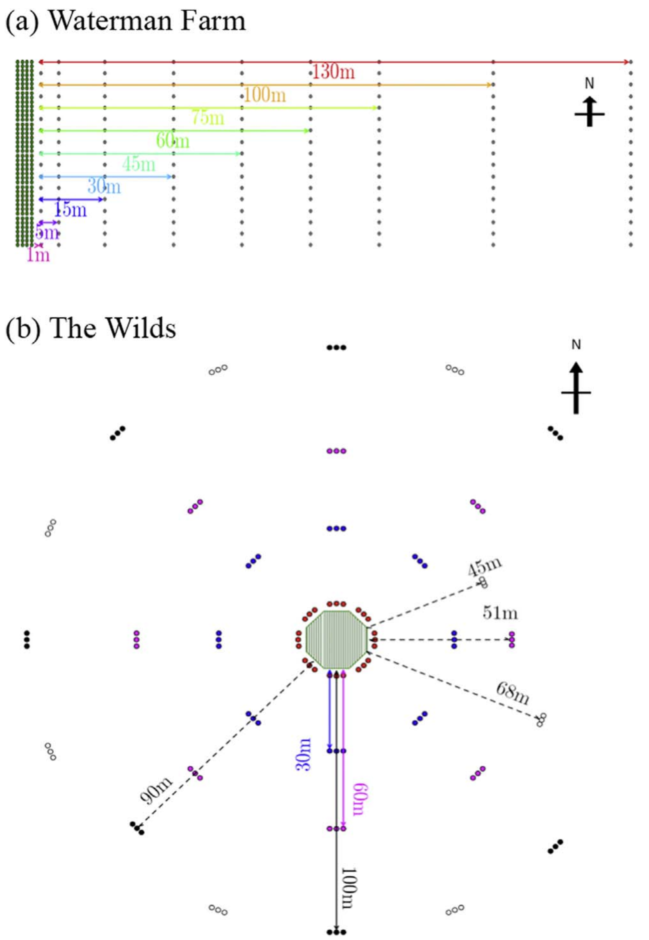

**Fig. 1.** Layouts of the experimental plots at (a) Waterman Farm and (b) The Wilds. At Waterman Farm, donors (cultivar ramets) were planted in four rows (green dots), and the recipients (wild ramets) were planted to the east side of the donors (gray dots). At The Wilds, donors were planted in the center (green octagon), and recipients were planted in triplets. Trees were present on the east side; therefore, planting of recipients was restricted. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

#### 2.1.1. Selection of pollen donors and recipients

First, we selected crop donors and wild recipients with the same ploidy level, because hybridization between different ploidy levels rarely occurs in switchgrass [34–36]. We selected two wild clones that were originally collected from a restored prairie in Marion, Ohio, and maintained as accessions in a common garden study [35]. We determined their ploidy levels using a *BD*™ LSR II flow cytometer at the University Cell Analysis and Sorting Core at the Ohio State University (OSU), Columbus, OH [37]. Following the protocol in Galbraith et al. [38], the genome size of each sample was estimated based on a minimum of 3000 nuclei count using *BD™ FACSDiva*, and ploidy was