Micropollen Formation in F$_1$ Hybrids and Backcrosses of Waterhemp (*Amaranthus tuberculatus*) x Smooth Pigweed (*A. hybridus*) as a Quick Method for Determination of Interspecific Hybridization Events

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Abstract

Various weedy amaranths have demonstrated the possibility of genetic exchange via interspecific hybridization. Normal meiotic events lead to the formation of normal tetrads and normal pollen while abnormal meiosis results in abnormal segregation of chromosomes in the tetrads resulting in macro and micropollen. Thus, pollen size can be used as an indirect measure of meiotic integrity. The purpose of this study was to examine the frequency of micropollen from putative F$_1$, BC$_1$, and BC$_2$ progeny of waterhemp and smooth pigweed crosses to determine if infertility in the hybrids could be attributed to abnormal meiosis. Significant variation was observed with respect to the occurrence of micropollen. The F$_1$ hybrids had the highest occurrence of micropollen, and highest percentage of abnormal tetrad formation. Problems in meiosis could be causing decreased fertility, but not complete sterility. However, backcrossing resulted in progeny with smaller percentages of micropollen, consistent with previous reports that fertility is restored in these progeny. This method allows for quick and cost effective method for determination of interspecific hybridization events.

Keywords: introgression identification, gene exchange, interspecific hybridization, tetrad

1. Introduction

Extensive research has been conducted to examine the ecological risks associated with weed hybridization events (Zhu et al., 2004). These hybridization events are mechanisms which not only play an important role in shaping evolutionary trajectories, but are also factors strongly implicated in the establishment, spread and ecological impact of a biological invasion (Ellstrad & Scherenbeck, 2000). Introgression of genomic elements across species boundaries has the potential to disrupt genetic complexes which have been generated through divergent adaptive evolution; leading to concerns regarding genetic integrity and evolutionary viability of native taxa, particularly those threatened by other stressors (Largiader, 2007; Levin et al., 1996; Rhymer & Simberloff, 1996; Vila et al., 2000). In disturbed habitats, species may come in contact with one another and form interspecific hybrids (Baker, 1974; Dowling et al., 1997; Scheffler & Dale, 1994; Lewis, 1969). Interspecific hybrids could act as a bridge for the exchange of genetic material, which could play an important role in the evolution of the parental species (Rayburn et al., 2005). However, both inter- and intraspecific hybridization may result in novel genetic types with the potential for increased invasiveness relative to parental populations (Vellend, 2007; Dlugosch & Parker, 2008; Prentis et al., 2008). For instance, if various weedy amaranths develop herbicide resistance, genetic exchange between species by interspecific hybridization could result in transfer of herbicide resistance among these species (Trucco et al., 2005a, 2005b, 2006; Wetzel et al., 1999a, 1999b). Among amaranths, waterhemp has been one of the most problematic in terms of multiple mode of action herbicide resistance (Heap, 2006). Resistance to Photosystem II-, acetolactate synthase- and protoporphyrinogen oxidase-inhibitors, are all important herbicides and have been observed in a single waterhemp biotype from Illinois (Patzoldt et al., 2005). More recently, waterhemp and Palmer amaranth (*A. palmeri*) have evolved resistance to glyphosate (Heap, 2015). Because of the importance of glyphosate to current weed management, there is concern that this resistance will be transferred to other *Amaranthus* species (Culpepper et al., 2006;

Nandula et al., 2014).

Examination of reproductive fitness of F_1 and first generation backcross (BC_1) hybrids between smooth pigweed and waterhemp (Trucco et al., 2005b, 2006) showed greatly reduced fitness of the females, with F_1 hybrid females only producing an estimated 800 seeds. This seed production is a small fraction found in the parental populations (Trucco et al., 2005b). With BC_1 hybrids, the seed production was almost 80 times greater than that of the F_1 hybrids and approximately 20% of that of the parents (Trucco et al., 2006).

One of the mechanisms that could be responsible for the decrease in fertility is abnormal meiosis. Chromosomal changes in hybrids can produce reproductive barriers, which cause problems at meiosis in the heterozygotes (Docker, 2003; Husband, 2004; Rieseberg et al., 2000). During normal meiosis, chromosomes pair and divide, resulting in the formation of tetrads that have the same number of chromosomes in each of the 4 daughter cells. Abnormal meiosis, however, will result in meiocytes with different numbers of chromosomes (de Oliveira et al., 2004).

It has been reported that pollen grains of a hybrid between spiny amaranth (*A. spinosus*) and spleen amaranth (*A. dubius*) have differing chromosome numbers (Pal & Khoshoo, 1972). The authors reported two sizes of pollen, which are the macropollen containing – 19 to 27 chromosomes and the micro-pollen containing – 1, 2, 3 or 4 chromosomes. Since normal meiotic events lead to the formation of normal tetrads, the presence of normal tetrads and subsequent pollen could be used as a measurement of normal meiosis (de Oliveria et al., 2004).

The objectives of this study were to examine the rates of micropollen and abnormal tetrad formation from hybrids produced by two weedy *Amaranthus* species (smooth pigweed and waterhemp), as well as BC_1 and BC_2 progeny and determine if these rates were comparable to the parental species. These species were selected because they contain the same number of chromosomes, increasing their ability to interbreed, and are often found together in agricultural fields. Tetrad and pollen formation was examined from both parents, as well as from F_1 hybrids and BC_1 and BC_2 progeny.

2. Materials and Methods

2.1 Plant Material

An accession of *A. tuberculatus* (LW22) and *A. hybridus* (LW1-1) were obtained from the weed science collection, University of Illinois. Seeds were planted in pots filled with soil, peat, and perlite growing medium (1:1:1 by wt). Two weeks after emergence individual seedlings were transplanted to 4 L pots. Plants were grown in a greenhouse with temperatures between 28 and 29 °C and supplemental lighting programmed (photon flux no less than 800 µmol m^{-2} s^{-1} at canopy) to achieve a 15 h photoperiod.

Interspecific hybrids were produced using smooth pigweed as the female parent and waterhemp as the male. Smooth pigweed is monoecious whereas waterhemp is dioecious. The F_1 progeny were dioecious as reported previously (Trucco et al., 2005b).

Hybrid females were backcrossed to either smooth pigweed or waterhemp to generate BC_1 progeny. BC_1 progeny were again crossed with their respective recurrent parent to generate second generation backcross (BC_2) progeny. The paternal waterhemp contained an ALS-inhibitor resistance gene, allowing for ease of hybrid screening via hybrid selection. While the full extent of micropollen events among smooth pigweed and waterhemp would be apparent by evaluating frequencies in the reciprocal direction, subsequent herbicide selection assays would overestimate hybridization frequencies from each plant. Also, if waterhemp were the female, all the resulting progeny would be female not allowing for the detection of micropollen events. All - these crosses were obtained using the methods as described by Tranel et al. (2002).

2.2 Flow Cytometry

Flow cytometric analysis was performed on all plants from which florets were collected. The nuclei for flow cytometric analysis were isolated following the methodology described by Rayburn et al. (1989). Two centimeter sections of leaf tissues (~ 1 cm^2 of each) from plant and inbred Zea mays corn line W-22 (used as an internal standard) were co-chopped and placed in 10 ml extraction buffer and 200 µl 25% Triton X. Extraction buffer consisted of 13% (v/v) hexylene glycol, 10 mM Tris-HCl [pH 8.0], and 10 mM MgCl$_2$.

The tissue was homogenized using a tissue grinder for 10 sec at 4,500 rpm, and samples were filtered through 250- and 53-µm nylon meshes. Samples were kept on ice throughout. Following filtration, samples were centrifuged for 15 min at 11,000 rpm at 4 °C. The supernatant was aspirated and the pellet was re-suspended in 300 µl of propidium iodine (PI) stain (Bashir et al., 1993). The samples were then placed in a 37 °C water bath for 20 min. After the incubation period, 300 µl of PI salt was added to each sample. The samples were then

briefly vortexed and placed on ice and kept in the refrigerator for at least one h.

Nuclei were analyzed using a laser Flow Cytometer-Cell Sorter Epics XL-MCL 4-color flow cytometer (Beckman Coulter (Note 1)). The excitation wavelength was set at 488 nm. A minimum of 8,000 nuclei per sample was analyzed. The mean of the G1 fluorescence peak of each sample was divided by the mean of the G1 fluorescence peak reading of the internal control, multiplied by nuclear DNA content of the internal standard, 5.35 pg per 2C (Rayburn & McMurphy, 1991), and expressed in pg per 2C nucleus. Readings on nuclear DNA content were used for GLM analysis, and LSD tests were conducted using SAS statistical analysis package (SAS version 8.0).

2.3 Tetrad Analysis

For tetrad analysis, florets 4 to 5 cm long were collected and stored in ethanol and glacial acetic acid (3:1 by vol). Florets were maintained at room temperature for 4 days and then stored at 4 °C. For analysis, the anthers were dissected from individual florets using an Olympus SZ60 (Note 2) dissecting microscope and two 28-gauge needles. Anthers were placed in a drop of 1% (w/v) acetocarmine and squashed. Five plants each of smooth pigweed, waterhemp and F_1 hybrids were examined. For each floret, a minimum of 300 tetrads were counted and recorded as either normal or abnormal. Tetrads were observed using an Olympus BX61 (Note 2) microscope at 20X magnification, and pictures were taken using an Olympus Magnafire digital camera and software. Normal tetrad formation was considered to be any tetrad that contained four nuclei of essentially the same size. Abnormal tetrad formation was any tetrad that had fewer or greater than four nuclei or nuclei of various sizes. Statistical analysis on tetrads was performed using SAS statistical package, version 8.0.

2.4 Micropollen Analysis

For micropollen analysis, florets 6 to 7 cm long were collected and stored in ethanol and glacial acetic acid (3:1 by vol). Florets were maintained at room temperature for 4 days and then stored at 4 °C. For analysis, the anthers were dissected from individual florets using an Olympus SZ60 dissecting microscope and two 28-gauge needles. Pollen was squeezed out of the anthers into a drop of 1% (w/v) acetocarmine and then squashed. At least 500 pollen grains were counted from each floret and two florets were examined from 12 plants each of smooth pigweed, waterhemp and F_1 hybrids. Two florets each from twenty BC_1 and thirty BC_2 plants were also examined. Due to the large number of plants, pollen grains were sampled on two different days. Pollen grains were observed using an Olympus BX61 microscope at 10X magnification and pictures were taken using an Olympus Magnafire digital camera and software. Pollen grains were counted and then classified as either normal or micropollen. Micropollen was considered to be any pollen grains that were less than 1/2 the diameter of the parental pollen grains. Statistical analysis on micropollen was performed using SAS statistical package, version 8.0.

3. Results

This study confirmed previous studies that demonstrated while genome size can be used to identify hybrids between smooth pigweed and waterhemp (Jeschke et al., 2003; Tranel et al., 2002), genome size of subsequent backcross progeny are similar to the recurrent parent genome size (Trucco et al., 2005a). In the plants used for tetrad analysis and micronuclei analysis, the total nuclear DNA content was as expected with waterhemp having the largest genome size, smooth pigweed having the smallest genome size and the hybrids having an intermediate genome size (Table 1).

Table 1. Nuclear DNA contents

Plant	No. of plants	pg DNA[1]
Waterhemp	5	1.40 ± 0.02
Smooth pigweed	5	1.10 ± 0.02
F_1	5	1.26 ± 0.02

Note. [1] ncDNA mean ± standard error.

In the backcross progeny, the nuclear DNA content was not different from that of the recurrent parent (Table 2). As expected, each backcrossing resulted in the recovery of parental genome. There were significant differences between the micropollen percentages (P < 0.01) with the F_1, BC_1, and BC_2 hybrids.

Table 2. Nuclear DNA contents and micropollen percentages for parental species, F_1 hybrid, and backcross progenies, where F_1 females were backcrossed to males of other species

Plant	# plants	pg DNA[1]	Micropollen (%)[2,3]
Waterhemp	12	1.40 ± 0.03	0.3 ± 0.15[e]
Smooth pigweed	12	1.11 ± 0.03	0.3 ± 0.05[e]
F_1	12	1.26 ± 0.01	23.4 ± 4.72[a]
BC_1 to waterhemp	10	1.39 ± 0.02	4.2 ± 0.89[b]
BC_1 to smooth pigweed	10	1.17 ± 0.02	3.9 ± 1.16[b]
BC_2 to waterhemp	20	1.38 ± 0.03	2.4 ± 2.64[c]
BC_2 to smooth pigweed	10	1.11 ± 0.14	1.2 ± 0.97[d]

Note. [1] Plant mean ± standard error; [2] Micropollen mean ± standard error; [3]500 pollen grains per plant analyzed.

Tetrad analysis revealed that in both waterhemp and smooth pigweed the majority of the tetrads had four nuclei of similar size (Figures 1A and 1B) with a low percentage of abnormal tetrads, 4.4% and 5.1% respectively (Table 3). The F1 hybrids had higher percentage of abnormal tetrads, 84.6% (Table 3). In the hybrids, tetrads that had five or more nuclei and or nuclei of various sizes were observed (Figure 1C).

Table 3. Abnormal tetrad percentages for parental lines and F1 hybrid

Plant	Tetrads counted	Abnormal tetrads (%)[1]
Waterhemp	1565	4.4 ± 0.88[b]
Smooth pigweed	1541	5.1 ± 0.65[b]
F_1	1558	84.6 ± 5.89[a]

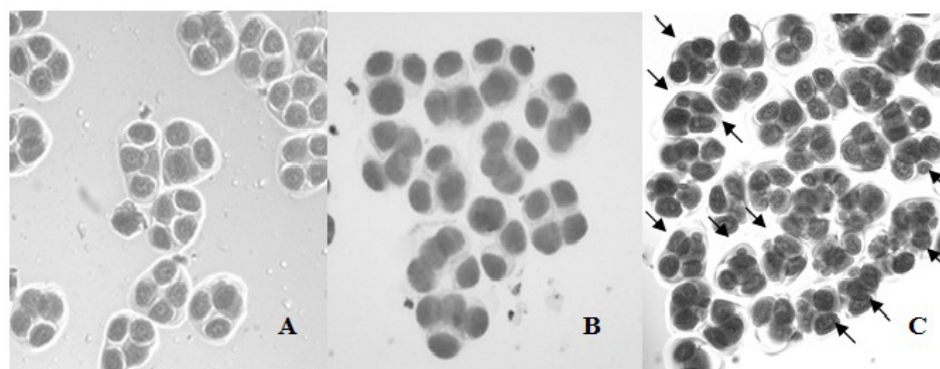*Note.* [1] Abnormal tetrad percentage ± standard error.

Figure 1. Tetrad formation in (A) waterhemp, (B) smooth pigweed, and (C) F_1 hybrids; arrows denote abnormal tetrad formation with abnormal amounts of nuclei or nuclei of various sizes

Mature pollen grains, while not providing information about chromosome size, can provide information regarding cyto-morphological changes, such as hybridization events (Srivastave et al., 1977). A pollen size of ~18.5 μm was observed for smooth pigweed pollen and ~20.1 μm for waterhemp, with both of these sizes corresponding with sizes reported by Franssen et al. (2001). Micropollen was defined as having a diameter of 9 μm or smaller. The two parental species contained a small number of micropollen (Table 2) while the hybrid was observed to have a significantly higher amount of micropollen, 23.4% (Figure 2 and Table 2). The waterhemp BC_1 progeny had a micropollen percentage of 4.2%, while the average micropollen percentage for the smooth pigweed BC_1 was 3.9% (Table 2). The waterhemp BC_2 progeny had a micropollen percentage of 2.4%, while the average micropollen percentage for the smooth pigweed BC_1 progeny was 1.2% (Table 2). There was a positive correlation between the total pg DNA and the percentage of micropollen of the F_1 and BC_1 and BC_2 progeny backcrossed to smooth pigweed (r = 0.94, p ≤ 0.01). In contrast, there was a negative correlation for the

corresponding data from F_1 and BC_1 and BC_2 progeny backcrossed to waterhemp ($r = 0.92$, $p < 0.01$). Statistical analysis showed no significance related to the day of pollen collection, therefore the remaining analyses disregard day effect.
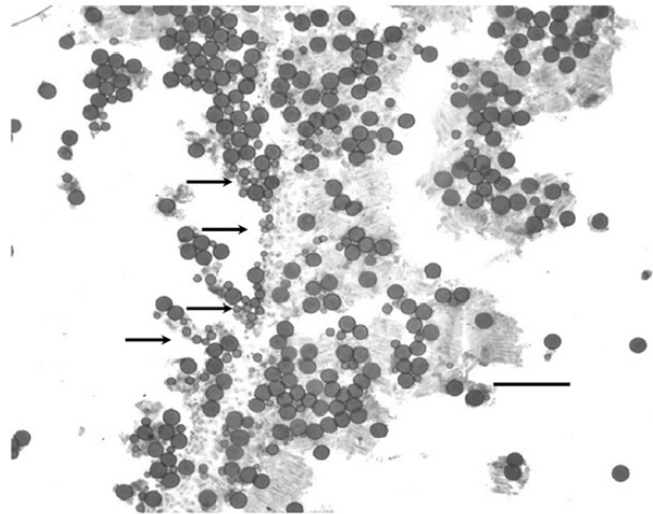

Figure 2. Pollen and micropollen in the F_1 hybrid. Arrows denote clusters of micropollen. Bar = 50 μm

4. Conclusion

We hypothesize that high frequency of abnormal tetrads in the hybrids is indicative of problems during meiosis, which subsequently resulted in increased micropollen formation. This increase in micropollen is similar to the decrease in hybrid seed production observed by Trucco et al. (2005b). The percentage of micropollen decreased through subsequent backcross generations, in a pattern similar to that of the recovery of seed production in backcrossed females seen by Trucco et al. (2006). Backcrossing results in plants containing more of the recurrent parent's DNA content and therefore its chromosomes, thus reducing the potential for problems at meiosis usually seen in hybrids (Docker, 2003). Despite problems with abnormal meiosis and subsequent decreases in fertility, these hybridization events between waterhemp and smooth pigweed could prove to be evolutionarily significant. There have been cases of the inheritance of a herbicide-resistance gene that remained functional through two backcrossing events (Tranel et al., 2002). This ability to inherit and maintain such genetic information could prove to be beneficial to plants, especially those found in environments under severe selection for herbicide resistance such as in the Midwestern US. The benefit of carrying the herbicide resistance gene could outweigh the effects of reduced fertility of the hybrids and subsequent backcrosses.

While these experiments were conducted under controlled, greenhouse conditions it is believed that the results can have 'real world' implications. The percentage of micropollen events remains high even through two backcross events, indicating that this could be a quick method of detection of hybridization events. This quick method would be beneficial in rapidly identifying appropriate weed control measures. This technique could even provide useful in the monitoring of introgression from GM crops to wild relatives. Future experiments will be conducted on research plots where a silicone grease covered rod will be rotated in the air to collect pollen. The rod will be rotated periodically during the collection period to gather samples through an entire 24 hour day, and micropollen events will be evaluated.

Acknowledgements

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Notes

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