Novel Approaches to Weed Management: Exploiting Breeding System Handicaps in Dioecious Species with a Focus on Palmer Amaranth (*Amaranthus palmeri)*

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Abstract

The success of the Insect Sterile Technique (IST) in managing insect pests raised the hypothesis that a similar approach could be employed to control weed populations. This research delved into the potential of using sterile pollen technique (SPT) to disrupt seed production in dioecious weeds, with a focus on Palmer amaranth (*Amaranthus palmeri* S. Watson). The overall objective this project is to understand the reproductive biology of dioecious weeds and to examine the possibility of using sterile pollen to disrupt seed production in dioecious weeds. In ***Chapter 1***, we characterized phases of flower development in *A. palmeri* and compared organogenesis of flower development in female and male plants. Understanding the reproductive biology of this species is crucial for guiding the development of novel weed management strategies, as it enables the identification of specific vulnerabilities that can be targeted to disrupt seed production. Results showed the distinction between the two flower types became apparent at stage four by the formation of stamen primordia in staminate flowers, which developed both the female and male reproductive organs initially, as contrasted to pistillate flowers which produced carpel primordia only. Our study suggests that the evolution of *A. palmeri* from a cosexual ancestral to complete dioecy is still in progress. ***Chapter 2*** examined the optimal pollen irradiation dose to reduce seed production in *A. palmeri.* An irradiation dose of 300 Gy seems to be the most effective in reducing seed set in Palmer amaranth. This effectiveness is attributed to the ability of pollen irradiated at 300 Gy to generate pollen tubes, yet they are incapable of successful fertilization with egg cells. Furthermore, the greatest reduction in seed set was achieved when irradiated pollen was introduced to the stigma through artificial pollination prior to open pollination. It appears that irradiated pollen exerts a preventive effect on naturally occurring pollen that arrives later. Furthermore, in order to increase the efficiency of SPT applications, ***Chapter 3*** focused on investigating an ideal dry (inert) diluent and a most effective mix ratio of pollen/diluent and identifying the optimal

strategy of sterile pollen applications to minimize seed production in Palmer amaranth. The findings showed that the optimal formulation was a 25%/75% v/v mixture of irradiated pollen and talc powder, successfully reducing seed set in *A. palmeri* while efficiently utilizing the limited resource of irradiated pollen. The most effective application strategy was initiating the application 7 days after anthesis and repeating it three times at 7-day intervals. This study also addressed the potential trade-off between inflorescence growth and fertilization rate, hypothesizing that high fertilization could divert resources away from inflorescence development to seed production. We found massive pollination of irradiated pollen or non-irradiated pollen did not have an effect on inflorescence growth, but it did affect the sex ratio in the progeny population, resulting in female-biased progeny as predicted by certation theory.

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Introduction

Current agricultural systems rely heavily on the use of herbicides and tillage for weed management, but both have negative impacts on the environment and farm productivity in long-term use (Maclaren et al. 2020). An integrated approach to weed management which incorporates ecological principles and involves using multiple tactics that vary in timing and type of control is needed to reduce the probability of rapid weed adaptation to management practices (Harker and O'Donovan 2013). Moreover, weed management decisions should aim to prevent soil seedbank inputs rather than just minimize current yield loss for agricultural profitability (Swanton et al. 2008). While there have been many studies focused on weed seed biology and seedbank management (Buhler et al. 1998), research focused on reducing weed seed production by manipulating flowering and seed set is lacking.

Flowering plants have developed a wide range of reproductive systems. Most flowering plants are hermaphrodites, having both functional sex organs in the same flower, which is believed to be the ancestral form (Darwin 1876; Charlesworth 1984). Plants have evolved various mechanisms to deviate from this ancestral form such as production of unisexual staminate or pistillate flowers to avoid inbreeding (Charlesworth 1984). However, the adoption of any sexual strategy is often accompanied by genetic and demographic trade-offs (Baker 1955). For example, dioecy (distinct male and female individuals) maximizes outcrossing and thereby reduces the likelihood of inbreeding depression (Thomson and Brunet 1990). It further allows for more efficient resource allocation between male and female roles by reducing competition between these two functions through sexual specialization (Charnov 1982). However, successful fertilization in dioecious species relies on proximity and synchronization of male and female flowers. These limitations present an opportunity for the development of novel management strategies for dioecious weeds.

Insect sterile technique (IST), an environmentally-friendly and biologically-based method for controlling insect pest, involves sterilizing male insects by irradiation and subsequently releasing the sterile males to mate with wild females (Parker and Mehta 2007), resulting in infertile eggs and reduced insect pest population sizes. Drawing from the success of the IST in controlling insect pests, a similar approach could be effective for weed populations. There are several methods to make pollen grains functionally deficient and thereby reduce seed set (Zhang and Lespinasse 1991; Alsamir et al. 2021). The most commonly used is ionizing irradiation with X-rays or gamma rays due to their ease of use, effective penetration, consistent results, and minimal disposal issues (Chahal and Gosal 2002). Irradiated pollen can germinate and grow pollen tubes but fails to fertilize egg cells (Musial and Pzrywara 1998). Moreover, when sterile pollen is artificially applied to stigmas, it can obstruct the fertilization process by fertile pollen, thus disrupting seed production. Pollinating female plants of *Amaranthus palmeri* with sterile pollen (irradiated) resulted in 40% reduction in the number of newly formed seeds (Efrat et al. 2020). This also has been demonstrated in various plants like apple, pear, citrus, cacao, and melon (Zhang and Lespinasse 1991; Bouvier et al. 1993; Froelicher et al. 2007; Falque et al. 1992; Lotfi et al. 2003).

The genus *Amaranthus* (Amaranthaceae) contains about 75 species worldwide (Ward et al. 2013). Most *Amaranthus* species are monoecious whereas a few species are dioecious (Trucco and Tranel 2011). Dioecious *Amaranthus* were originally placed into a single subgenus *(Acnida*) due to dioecy (Mosyakin and Robertson, 1996) but in recent phylogenetic studies have been grouped with monoecious species in various clades (Stetter and Schmid, 2017). Palmer amaranth (*Amaranthus palmeri* S. Wats.) is indigenous to the southwestern United States and northern Mexico (Sauer 1957). Initially considered an insignificant weed in fields of cotton, soybean, and maize in 1974, it is now one of the top 10 most troublesome weeds in the US (Webster and Coble 1997). This is due to a combination of human-assisted seed

dispersal and new habitat creation through agricultural expansion (Ward et al. 2013). *Amaranthus palmeri* has emerged as one of the most economically damaging weeds in the United States, causing yield losses of up to 91% in corn (Massinga et al. 2001), 79% in soybean (Bensch et al. 2003), and 65% in cotton (Rowland et al. 1999). Furthermore, *A. palmeri* has evolved resistance to nine herbicide classes (Heap 2022) and is able to produce up to one million seeds per plant (Ward et al. 2013). This weed is a particularly suitable candidate for exploration of the sterile pollen technique for weed control. As a dioecious species with separate male and female plants, it relies heavily on cross-pollination for successful seed production. Theoretically, this makes it feasible to collect pollen grains from male plants, sterilize them, and subsequently release pollen onto female plants.

The overall goal of this dissertation research was to examine the possibility of using sterile pollen as means of disrupting seed production in dioecious weeds. To accomplish this goal, the research focused on *Amaranthus palmeri* as a model dioecious weed species and consisted of three specific studies described in the three chapters of the dissertation:

Chapter 1 characterized phases of flower development in *A. palmeri* and compared organogenesis of flower development in female and male plants. Understanding the reproductive biology of this species is crucial for guiding the development of novel weed management strategies, as it enables the identification of specific vulnerabilities that can be targeted to disrupt seed production.

Chapter 2 aimed to determine the optimal irradiation dose that strikes a balance between inducing pollen sterility (allowing for pollen germination and tube growth but no fertilization) and preserving competitiveness, as excessive doses could result in pollen mortality (no germination and no pollen tube growth), while low doses may allow pollen to retain fertility.

Chapter 3 detailed how to improve the efficiency of the sterile pollen technique for reducing the seed production in *A. palmeri.* It firstly identified an ideal dry (inert) diluent and a most effective mix ratio of irradiated pollen and diluent for large scale application. The chapter then established the optimal application strategy, experimenting with different starting times, frequencies, and number of sterile pollen applications to effectively minimize seed production in Palmer amaranth. Additionally, it investigated the effects of massive pollination on inflorescence growth and seed production.

Although the research focused on a single weed species, the Sterile Pollen Technique (SPT) has broad-spectrum potential for weed control; sterile pollen from multiple weed species can be mixed and released in a single application. The SPT could be particularly helpful for managing herbicide-resistant weeds which have withstood in-season control and have reached the reproductive stage.

References

Alsamir M, Mahmood T, Trethowan R, Ahmad N (2021) An overview of heat stress in tomato (*Solanum lycopersicum* L.). *Saudi J Biol Sci* **28**:1654-1663

Baker HG. 1955. Self-compatibility and establishment after 'long-distance' dispersal. *Evolution* **9(3)**: 347-349.

Bensch CN, Horak MJ, Peterson D (2003) Interference of redroot pigweed (*Amaranthus retroflexus*), Palmer amaranth (*A. palmeri*), and common waterhemp (*A. rudis*) in soybean. *Weed Sci.* **51**: 37–43.

Bouvier L, Zhang YX, Lespinasse Y (1993) Two methods of haploidization in pear, Pyrus communis L.: greenhouse seedling selection and in situ parthenogenesis induced by irradiated pollen. *Theor Appl Genet* **87(1):**229–232.

Buhler DD, Hartzler RG, Forcella F (1998) Weed seed bank dynamics: Implications to weed management. *Journal of Crop Production* **1(1):**145–168.

Chahal GS, Gosal SS (2002) Principles and procedures of plant breeding: biotechnological and conventional approaches. Oxford: Alpha Science Int'l Ltd. Pp 399–412.

Charlesworth D (1984) Androdioecy and the evolution of dioecy. *Biological Journal of the Linnean Society* **22(4)**: 333–348.

Charnov EL (1982) The Theory of Sex Allocation. Princeton University Press, Princeton,

Darwin C (1876) The Effects of Cross and Self Fertilization in the Vegetable Kingdom. John Murray, London, UK.

Efrat LN, Ido S, Herve H, Miriam A, Micheal DO, Jonathan G, Orly NB (2020) Inhibiting herbicide-resistant Amaranthus by suppressing reproduction. Pages 87 in Proceedings of the 75th annual meeting of the north central weed science society. Virtual: North

Falque M, Kodia AA, Sounigo O, Eskes AB, Charrier A (1992) Gamma-irradiation of cacao (*Theobroma cacao* L.) pollen: Effect on pollen grain viability, germination and mitosis and on fruit set. *Euphytica* **64(3):**167–172.

Froelicher Y, Bassene JB, Jedidi-Neji E, Dambier D, Morillon R, Bernardini G, Costantino G, Ollitrault P (2007) Induced parthenogenesis in mandarin for haploid production: induction procedures and genetic analysis of plantlets. *Plant Cell Rep* **26(7):**937–944.

Harker KN, O'Donovan JT (2013) Recent weed control, weed management, and integrated weed management. *Weed Technol* **27(1)**:1–11.

Heap, I (2022) The International Herbicide-Resistant Weed Database [Online]. Website www.weedscience.org [accessed 02 November 2022]

Lotfi M, Alan AR, Henning MJ, Jahn MM, Earle ED (2003) Production of haploid and doubled haploid plants of melon (*Cucumis mel*o L) for use in breeding for multiple virus resistance. *Plant Cell Rep* **21(11):**1121–1128.

MacLaren C, Storkey J, Menegat A, Metcalfe H, Dehnen-Schmutz K (2020) An ecological future for weed science to sustain crop production and the environment. A review. *Agron Sustainable Dev* **40(4**): 1–29.

Massinga RA, Currie RS, Horak MJ, Boyer J (2001) Interference of Palmer amaranth in corn. *Weed Sci.* **49**: 202–208.

Mosyakin SL, Robertson KR (1996) New infrageneric taxa and combinations in Amaranthus (Amaranthaceae). *Annales Botanici Fennici* 33:275–281.

Musial K, Przywara L (1998) Influence of irradiated pollen on embryo and endosperm development in kiwifruit. *Ann. Bot.* **82**:747–756.

Parker A, Mehta K (2007) Sterile insect technique: A model for dose optimization for improved sterile insect quality. Florida Entomologist 90:88–95

Rowland MW, Murray DS, Verhalen LM (1999) Full-season Palmer amaranth (*Amaranthus palmeri*) interference with cotton (*Gossypium hirsutum*). *Weed Sci.* **47**: 305–309.

Sauer, JD (1957) Recent migration and evolution of the dioecious amaranths. *Evolution* **11**: 11–31

Stetter MG, Schmid KG (2017) Analysis of phylogenetic relationships and genome size evolution of the Amaranthus genus using GBS indicates the ancestors of an ancient crop. *Molecular Phylogenetics and Evolution* 109:80–92.

Swanton CJ, Mahoney KJ, Chandler K, Gulden RH (2008) Integrated weed management: knowledge-based weed management systems. *Weed Sci* 56(1): 168–172.

Thomson JD, Brunet J (1990) Hypotheses for the evolution of dioecy in seed plants. *Trends in Ecology & Evolution* **5(1)**: 11-16.

Trucco F, Tranel PJ (201). Amaranthus. *In* Kole, C. [eds.], Wild crop relatives: genomic and breeding resources. 11-21. Springer, Berlin, Heidelberg, Germany.

Ward SM, Webster TM, Steckel LE (2013) Palmer amaranth (*Amaranthus palmeri*): A review. *Weed Technol.* **27**: 12–27.

Webster TM, Coble HD (1997) Changes in the weed species composition of the southern United States: 1974 to 1995. *Weed Technol.* **11**:308–317.

Zhang YX, Lespinasse Y (1991) Pollination with gamma-irradiated pollen and development of fruits, seeds and parthenogenetic plants in apple. *Euphytica* **54**(1):101–109.

Chapter 1: Comparative Floral Development in Male and Female Plants of Palmer Amaranth (*Amaranthus palmeri*)

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Abstract

- **Premise of the study** Characterizing the developmental processes in the hermaphroditism-to-unisexuality transition is crucial for understanding floral evolution. *Amaranthus palmeri*, one of the most devastating weeds in the US, is an emerging model system for studying a dioecious breeding system and understanding the biological traits of this invasive rapidly spreading weed. The objectives of this study were to characterize phases of flower development in *A. palmeri* and compare organogenesis of flower development in female and male plants.
- **Methods** Flower buds from male and female plants were dissected for light microscopy. Segments of male and female inflorescences at different stages of development were cut longitudinally and visualized using scanning electron microscopy.
- **Key Results** Pistillate flowers have two to three styles, a single ovary with a single ovule and five obtuse tepals. Staminate flowers have five stamens with five acute tepals. Floral development was classified into ten stages. The distinction between the two flower types became apparent at stage four by the formation of stamen primordia in staminate flowers, which developed both the female and male reproductive organs initially, as contrasted to

pistillate flowers which produced carpel primordia only. In staminate flowers the putative carpel primordia showed little change in size and remained undeveloped.

- **Conclusions** Timing of inappropriate organ termination varies across the two sexes in *A. palmeri.* Our study suggests that the evolution of *A. palmeri* from a cosexual ancestral to complete dioecy is still in progress since males exhibited transient hermaphroditism whilst females produced strictly pistillate flowers.

Key words: Reproductive biology, mating systems, dioecy, pistillate, staminate, weediness, invasive plants, sex determination; Amaranthaceae

Introduction

The separation of sexes is the norm in animals, but it is rare in plants (Bachtrog et al., 2014). Most angiospermous plants are hermaphrodites, having both functional sex organs in the same flower, which is believed to be the ancestral form (Darwin 1876; Charlesworth 1984). Plants have evolved various mechanisms to deviate from this ancestral form such as production of unisexual staminate or pistillate flowers to avoid inbreeding (Charlesworth 1984). Only about 6% of plant species are dioecious (Renner 2014), i.e. having male and female reproductive structures on separate individual plants. Despite its rarity, dioecy has developed from hermaphroditic ancestors independently in numerous taxonomic groups, with at least one dioecious species found in 50% of angiosperm families (Charlesworth 1999; Renner 2014).

Historically, two types of unisexual flowers have been defined based on the developmental mechanisms of the evolution from hermaphroditism. In type I, flowers initiate reproductive structures of both sexes but ultimately become unisexual by arresting the development of the inappropriate sex, such as most dioecious Anacardiaceae (Wannan and Quinn 1991). In type II, unisexual flowers have only one set of sex organ primordia that emerges from the floral meristem, such as spinach (*Spinacia oleracea*) (Grant et al., 1994). It is believed that sex determination of type II occurs before initiation of stamens or carpels (Sherry et al., 1993); hence, flowers of either sex exhibit no hermaphrodite stage. Most species can be characterized as type I where the arrest of development of stamens or carpels can be triggered by diverse mechanisms at any point from initiation of stamen and carpel primodia to post-meiosis (Diggle et al., 2011). For example, the development of male and female structures in pistillate flowers resembles hermaphroditic flowers until the end of stage seven (total of 12) in *Silene latifolia* when stamen development is terminated after anther differentiation (Grant et al., 1994b). In *Celtis iguanaea*, gynoecium development arrests before carpal elongation in staminate flowers whereas androecium development in pistillate flowers terminates before

pollen maturation (Leme et al., 2020). Thus, it has been speculated that the termination of carpels and stamens rarely happens by a typical procedure or at a certain stage (Diggle et al., 2011).

In angiosperms, sex chromosomes have been identified in about 150 species, with half the species having visible heteromorphic chromosomes (Baránková et al., 2020). In species with no sex chromosomes, there are often sex-determining genes that either suppress maleness or femaleness (Louis, 1989). For instance, in *Silene latifolia* (type I species), there are two important regions in sex determination: one inhibits gynoecium growth and the other supports androecium development (Grant et al., 1994a; Juarez and Banks 1998). Additionally, programmed cell death (PCD) has been proposed as a main force in driving the development of unisexuality in some angiosperms (type I); PCD terminates the gynoecial development in male flowers of *Zea mays* (Cheng et al., 1983) and anther development in *Opuntia robusta* (Hernández-Cruz et al., 2019).

The genus *Amaranthus* (Amaranthaceae) contains about 75 species worldwide and includes crops as well as invasive weeds (Ward et al., 2013). Most *Amaranthus* species have a monoecious breeding system whereas a few species are dioecious (Trucco and Tranel, 2011). Dioecious *Amaranthus* were originally placed into a single subgenus *(Acnida*) due to dioecy (Mosyakin and Robertson, 1996) but in recent phylogenetic studies have been grouped with monoecious species in various clades (Stetter and Schmid, 2017). Because of the widespread evolution of herbicide resistance, several weedy *Amaranthus* species have received increasing attention recently.

Palmer amaranth (*Amaranthus palmeri*), one of the most devastating weeds in the US, is a dioecious summer annual weed (see Fig. 1). It ranked as the worst weed in US corn fields in a survey conducted by the Weed Science Society of America (Van et al., 2017). Furthermore,

A. palmeri has evolved resistance to nine herbicide classes (Heap, 2022) and is able to produce up to one million seeds per plant (Ward et al., 2013). To control this noxious weed, we must adopt a multi-tactic approach that incorporates ecological principles into weed management practices. Understanding the reproductive biology of this species can aid in the development of agronomic strategies for management and mitigation of herbicide resistance (Mesgaran et al., 2021).

Only a few species have been studied for the potential loss of sexual function in pistillate or staminate organs during floral morphogenesis (Diggle et al., 2011) and we are aware of no study of *A. palmeri.* Characterizing floral development in *A. palmeri* is extremely difficult due to the minute flower buds size (about 1 mm diameter). The objectives of this study were twofold: (1) to compare floral organogenesis in pistillate and staminate flowers of *A. palmeri* using scanning electron microscopy and (2) to define stages of floral development in *A. palmeri*. Considering the higher prevalence of type I (where both reproductive structures are initiated but the inappropriate one is arrested), our hypothesis was that *A. palmeri* flowers produce structures of both sexes during the early stages of development and then the vestigial organs from the opposite sex are aborted as development continues.

Material and Methods

Plant material—

Seeds (10-15) of *Amaranthus palmeri* (originating from Kansas) were planted into 3-L pots filled with UC Davis potting medium (1 sand:1 redwood sawdust:1 peat) in a greenhouse set at 24/32 °C night/day temperature regime and extended photoperiod (14 hours of lighting). A general-purpose fertilizer solution (Jack's Professional General Purpose 20–20–20, Allentown, Pennsylvania) was applied at 80 ml weekly starting from 2-true leaves thorough

drip irrigation with 65 mL/min flow rate for two minutes twice per day. Seedlings were periodically and randomly thinned to one plant per pot. The sex of plants was determined by visual inspection of flowers. After sex determination, samples were collected from plants and prepared for visualization using a light microscope and a scanning electron microscope.

Sample collection and preparation—

Flower buds from both male and female plants were dissected for light microscopy (Leica M205A; Leica Microsystems AG, Wetzlar, Germany). The inflorescence axes from male and female plants were cut into approximately 1cm segments and these segments were further sliced longitudinally. The cut surface was visualized under scanning electron microscopy (Quattro ESEM, ThermoFisher Scientific, FEI Deutschland GmbH, Germany) to locate axillary floral structures. Multiple buds at different angles from male and female plants were prepared to increase throughput and the likelihood of successful imaging. Bracts and tepals of flowers were removed as much as possible without damaging the reproductive structures. Following dissection, tissues were transferred into 70% Formalin-acetic acid-alcohol (FAA); 10:1:2:7 Ethanol (95%): Glacial acetic acid: Formalin (37% formaldehyde): Distilled H_2O (Ruzin, 1999) to fix for 24 hours. Samples were transferred through a dehydration series of 70%, 80%, 90%, 95%, 100%, and another 100% ethanol for at least 10 minutes each step. The tissue in 100% ethanol was critical point dried to preserve the surface structure which could be damaged due to surface tension when changing from the liquid state to gas state. Dried samples were mounted on aluminium stubs with adhesive tabs. Samples were coated with a thin gold-palladium layer by sputter coating (Bio-Rad E5100, Microscience Division, Hemel Hempstead, UK) for 60 seconds at 12mA. Lastly, samples were visualized with a ThermoFisher Quattro ESEM scanning electron microscopy operating at 5 kV and digital images captured as TIFF files.

Results

Images from Light Microscopy—

Flowers of both sexes are about 1 mm diameter, white or whitish green and are produced in dense and compact clusters on cylindrical inflorescences with smaller axillary spikes at the base (Fig. 2A-C; Fig. 3A-C). Each pistillate or staminate flower is enclosed by 1 to 3 stiff awl-shaped bracts (Fig. 2B-D; Fig. 3B, 3D). The bracts are green and extend beyond the tepals. Notably, pistillate flowers possess bracts that are comparatively longer than those of staminate flowers (Fig. 2B; Fig. 3B). Pistillate flowers have five tepals, which are rounded or blunt with an apical notch (Fig. 2F). Each pistillate flower has one superior gynoecium. Fruit are single-seeded utricles and become wrinkled when dry (Fig. 2E, 2F). Seeds are dark reddish-brown to black, lens-shaped, and 1.0 to 1.3 mm long. Staminate flowers have five stamens and five acute tepals with a dark green midrib (Fig 3D-F).

Based on these observations, the floral diagrams and formulae for *Amaranthus palmeri* pistillate and staminate flowers are shown in Fig. 4. In summary, a staminate flower has one to three bracts, five tepals in a quincuncial sequence, and five stamens. A pistillate flower has one to three bracts, five tepals in a quincuncial sequence and one superior gynoecium made of a single ovary containing one ovule.

Staminate flower development from SEM—

In the axils of the young leaves, inflorescence primordia appear soon after the reproductive transition. The initiation of tepal primordia is the first indication of floral organogenesis (Fig. 5A). The diameter of the central meristem above the tepal primordia is about 30 µm. The meristem broadens, and five stamen primordia are initiated as hemispherical mounds in a spiral pattern along the outer rim of the floral meristem (Fig. 5B). A protuberance forms at the center of the meristem shortly thereafter (Fig. 5C). This protuberance has similar

morphology and position to the early stages of carpel initiation in pistillate flowers. Stamen primordia soon become elliptical, and anthers have elongated sufficiently to arch inward and cover the floral apex (Fig. 5D-5G). Differentiation into anther and filament is obvious when the stamen primordia broaden, which is accompanied by a change of shape (anther primordia become four-lobed) (Fig. 5H-J). The lobes will later develop into the pollen sacs. The filament remains short and starts to elongate just prior to anthesis. Mature pollen grains are spherical-shaped with a golf ball-like exine and numerous apertures (Fig. 5K). The diameter of *A. palmeri* pollen is about 31 µm and the number of apertures per grain is about 25. The centrally-located putative carpel primordium shows little change in size, with the approximate diameter ranging between 45 µm and 70 µm, and the approximate height between 30 µm and 50 µm (Fig 5C-H; Fig. 6). This structure remains undeveloped and surrounded by the bases of the filaments rather than progressing to form a fertile mature gynoecium (Fig. 6).

Pistillate flower development from SEM—

With the transition to flowering, five tepal primordia are initiated first, in a weakly spiral pattern on the flower apical meristem (Fig. 7A-B). At the center of the flower meristem, the gynoecium arises, consisting of one carpel primordium. The primary carpel primordium is differentiated into an annular ovary wall primordium around a central single ovule primordium (Fig. 7C-E). The ovary wall grows up from the ring primordium, forming two (or three) style primordia (Fig. 7F-H). Each style primordium elongates and forms one long style. The gynoecium elongates by intercalary growth and forms the ovary that later closes post-genitally at the top (Fig. 7I-K). Styles elongate and a stigmatic region differentiates along the adaxial surface. The stigmatic branches are unifacial and papillate. After fertilization and seed maturation, stigmas and styles desiccate (Fig. 7L). The single ovule of *A. palmeri* is anatropous (inverted ovule with the micropyle tip facing the placenta) or campylotropous (curved ovule and micropyle with chalaza not lying in the same straight line) (Fig. 7J). We also found some

mature pistillate flowers with three styles (Fig. 8). We speculate that the protuberance (Fig. 8C) located near the inwardly elongating two styles may develop and become the third style. Further research is needed to confirm this.

Flower development stages of Amaranthus palmeri—

Based on the study of organogenesis, floral development of *A. palmeri* was categorized into ten distinct stages and the differences between staminate flower and pistillate flower were noted (Table 1). The first morphological feature distinguishing staminate and pistillate flower development is the initiation of stamen primordia (stage 4) found only in staminate flowers. Results show staminate flowers initially develop both androecia and gynoecia, but eventually become functionally male with a central bulge instead of a fully differentiated and fertile gynoecium (Fig. 6) whereas pistillate flowers do not develop an androecium.

Discussion

This is the first study of floral development in Palmer amaranth (*A. palmeri*) and documents the developmental differences between staminate and pistillate flowers. Flower and plant sex in *A. palmeri* can be distinguished morphologically by sharpness of bracts and shape of tepals prior to anthesis. Bracts are sharper in female plants than those in male plants. Tepals are of obtuse shape (rounded or blunt) in pistillate plants while tepals are acutely pointed in staminate plants. Based on the progression of organogenesis in flower development, we also classified floral development of *A. palmeri* into ten stages. The distinction between the two flower types only became apparent at stage 4 with the formation of stamen primordia (Fig. 5B and Table 1). Pistillate flowers only develop female reproductive structures whereas staminate flowers develop both female and male reproductive organs initially. This finding (i.e. presence of rudiments of opposite sex) places *A. palmeri* in the type I group of unisexual species.

Unisexual species are believed to have evolved from hermaphroditic ancestors through a variety of evolutionary processes (Charlesworth 1985). Both androecial and gynoecial primordia are often initiated in the unisexual flower of dioecious plants. The developmental halt of the organs of the opposing sex, which occurs at different phases of development in different species, gives rise to unisexual flowers (Dellaporta and Calderon-Urrea, 1993). In *Silene latifolia* (white campion), the development of male and female organs is identical until stage 5 (total of 12) when stamen development is terminated after anther differentiation in pistillate flowers, but in staminate flowers rudimentary gynoecia continue to grow throughout flower development (Grant et al., 1994a). In *Celtis iguanaea*, termination of gynoecium development in staminate flowers happens earlier than the arrest of androecium development in pistillate flowers (Leme et al., 2020). Results of *A. palmeri* flower development show staminate flowers initially develop both androecium and gynoecium, but eventually become functionally male with a central bulge instead of a fertile gynoecium (Fig. 6) whereas pistillate flowers do not go through a hermaphroditic stage. This implies that the sex of pistillate flowers is determined earlier than staminate flowers. Sex determination of pistillate flowers may occur before flower initiation or even before floral evocation because pistillate flowers only develop a fertile gynoecium with no anther primordia at any stage. Differences in the timing of residual organ termination suggests that the developmental program that suppresses gynoecium development in staminate flowers is probably independent from the program that stops stamen growth in pistillate flowers (Diggle et al., 2011). This observation implies the existence of different mechanisms involving different genes, which does not corroborate the hypothesis that stage of organ abortion in male and female flowers is temporally correlated within species (Diggle et al., 2011). Similarly, Grant et al. (1994a) found that genetic lesions in the Y-linked genes which prevent gynoecium growth have no effect on stamen development in *S. latifolia*.

It is interesting that *S. latifolia* with heteromorphic sex chromosomes (XY system) exhibits a similar pattern of late floral sex differentiation as *A. palmeri* which lacks visually distinct sex chromosomes. Sex in *A. palmeri* is controlled by a male specific genome region perhaps with an XY system without dimorphism between X and Y (Neves et al., 2020). However, sex-determination loci have not been identified in these taxa. Early cytogenetic studies in *S. latifolia* show that there are three regions identified on the Y chromosome related to sex expression: a gynoecium suppression region and two promotion regions of stamen development (Westergaard 1946, 1958). The speculation was made that the male Y chromosome linked genes with gynoecium-suppressing functions are expressed in staminate flowers before the first sex-specific difference appears (Grant et al., 1994a). Miller and Kesseli (2011) suggests the Y chromosome in *S. latifolia* remains quite similar to the X chromosome, probably with the main differences in the primary sex determination regions.

The slow differentiation of staminate flowers in *A. palmeri* may relate to the allocation of resources to male compared with female reproductive functions (Delph, 1999). According to Bateman's Principle (Bateman, 1948), female fitness tends to be limited by resources needed to fill seeds and fruits, whereas male fitness is more likely to be limited by mating opportunities. Reproduction is more costly to females than males; sex allocation theory therefore predicts that the environmental conditions favorable for plant growth should induce femaleness whereas resource-poor environments induce maleness (Meagher, 1988). This strategy is very important for *A. palmeri* to establish a population after colonizing a new habitat.

In expressing an initial stage of hermaphroditism, the *A. palmeri* pattern seems to differ from a few other species studied in this family. For example, in dioecious *Spinacia oleracea* L. flowers appear to be unisexual from inception and only initiate development of either stamens or pistils (Sherry et al., 1993). In *Amaranthus hybridus*, a monoecious species, flowers bear only either a primary gynoecium primordium or stamen primordia at early stages

(Sanchez-Del Pino et al., 2019). Our result suggests the evolution of *A. palmeri* from a cosexual ancestral state to dioecy is at an early or intermediate stage, which is in line with the findings from the whole-genome sequencing analysis (Neves et al., 2020). By identifying a potential Y chromosome in the *A. palmeri* draft genome sequence, Neves et al. (2020) suggested that dioecy in *A. palmeri* is at an intermediate evolutionary state with a young Y chromosome.

Our study is the only detailed floral developmental study of a dioecious member of the Amaranth genus and may offer unique insights into the evolution of sex determination in plants and into the development of novel control strategies to control dioecious weeds such as changing the sex through manipulation of the environment (Mesgaran et al., 2021). *Amaranthus palmeri* is of major interest because it represents one of the most important agronomic weeds in North America. The species can evolve resistance rapidly and repeatedly to herbicides (Adhikary and Pratt 2015). Understanding the reproductive biology of weeds can aid in the development of agronomic strategies and to reduce herbicide resistance and weed populations. Mesgaran et al. (2021) found water stress induced a female sex expression of Palmer amaranth resulting in female to male ratio of 1.78:1, which was significantly different from 1:1 sex ratio. This finding is consistent with our observation that males produce rudimentary gynoecia and can be potentially bipotent (i.e., capable of producing either sex). If there are more females than expected due to water stress, they could have been produced from males since males initially produce female organs. Further, water stress reduced the synchrony in anthesis between the two sexes of *A. palmeri* mainly through a delay in male anthesis whilst females were almost unaffected (Mesgaran et al., 2021). To better decipher the above observations, future research should investigate the floral development in male and female plants under water stress conditions.

Conclusion

Comparing floral organogenesis of both sexual types, we found that *Amaranthus palmeri* staminate flowers initially develop both androecium and gynoecium, but eventually become functionally male with a central bulge instead of a fertile gynoecium, whereas pistillate flowers only develop a fertile gynoecium with no anther primordia at any stage. Timing of residual organ termination varies across the two sexes in *A. palmeri.* Sex determination in pistillate flowers probably occurs before flower initiation or even before floral evocation, which is much earlier than staminate flowers. The results of our floral development study suggests that the evolution of *A. palmeri* from a cosexual ancestral to complete dioecy is still in progress since males exhibited transient hermaphroditism whilst females produced strictly pistillate flowers. It is very important to understand the reproductive biology of this species as it can help develop weed control methods and reduce herbicide resistance. Techniques such as skewing the sex ratio and reducing the flowering overlap between the two sexes have been offered to reduce seed output in *A. palmeri* and perhaps other dioecious weeds for which a better understanding of weed reproductive biology is required.

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Author Contributions

MBM conceived the idea, all authors designed the experiments, WW conducted the work, collected the data, and drafted the manuscript. JJ and MBM reviewed all drafts of the manuscripts. All authors approved the final version of the manuscript.

Data Availability

All data are provided in the manuscript.

Literature Cited

Adhikary, D., and D.B. Pratt, 2015. Morphologic and taxonomic analysis of the weedy and cultivated *Amaranthus hybridus* species complex. *Systematic Botany* 40(2): 604–610.Bachtrog, D., J. E. Mank, C.L. Peichel, M. Kirkpatrick, S. P. Otto, T.L. Ashman, M.W. Hahn, J. Kitano, and et al. 2014. Sex determination: why so many ways of doing it? *PLoS Biology* 12:e1001899.

Baránková, S., J. P. Pascual-Díaz, N. Sultana, M. P. Alonso-Lifante, M. Balant, K. Barros, U. D'Ambrosio and et al. 2020. Sex-chrom, a database on plant sex chromosomes. *New Phytologist*, 227(6), 1594-1604.

Bateman, A. J. 1948. Intra-sexual selection in Drosophila. *Heredity*, *2*(Pt. 3): 349–368.

Charlesworth, D. 1984. Androdioecy and the evolution of dioecy. *Biological Journal of the Linnean Society* 22(4): 333–348.

Charlesworth, D. 1999. Theories of the evolution of dioecy. *In* Geber M.A. Dawson T. E. and Delph L.F. [eds.] Gender and sexual dimorphism in flowering plants, 33-60. Springer-Verlag, Berlin, Germany.

Charlesworth, D. and D.S. Guttman, 1999. The evolution of dioecy and plant sex chromosome systems. *In* C.C. Ainsworth [ed.], Sex Determination in Plants. 25–49. BIOSIS, Oxford, UK.

Cheng, P.C., R.I. Greyson, and D.B. Walden,. 1983.Organ initiation and the development of unisexual flowers in the tassel and ear of *Zea mays*. *American Journal of Botany* 70: 450–462.

Darwin, C. 1876. The Effects of Cross and Self Fertilization in the Vegetable Kingdom. John Murray, London, UK.

Dellaporta, S. L., and A. Calderon-Urrea, 1993. Sex determination in flowering plants. *Plant Cell* 5: 1241–1251.

Delph, L. F. 1999. Sexual Dimorphism in Life History. *In* Geber, M.A., T.E. Dawson, and L.F Delph [eds.], Gender and Sexual Dimorphism in Flowering Plants, 149-173. Springer, Heidelberg, Heidelberg, Germany.

Diggle, P. K., V. S. Di Stillo, A.R. Gschwend, E.M. Goldenberg, R. C. Moore, J. RW. Ruseell and J.P. Sinclair. 2011. Multiple developmental processes underlie sex differentiation in angiosperms. *Trends in Genetics* 27: 368–376Grant S., A. Houben, B. Vyskot, J. Siroky, W. H. Pan., J. Macas and H. Saedler. 1994a. Genetics of sex determination in flowering plants. *Developmental Genetics* 15: 214–230.

Grant, S., B. Hunkirchen and H. Saedler. 1994b. Developmental differences between male and female flowers in the dioecious plant Silene latifolia. *The Plant Journal* 6(4): 471-480.

Heap, I. 2022. The International Herbicide-Resistant Weed Database [Online]. Website www.weedscience.org [accessed 02 November 2022]

Hernández-Cruz, R., J. Silva-Martínez, , F. García-Campusano, , F. Cruz-García, G. Orozco-Arroyo, I. Alfaro and S. Vázquez-Santana. 2019. Comparative development of staminate and pistillate flowers in the dioecious cactus Opuntia robusta. *Plant Reproduction* 32(3): 257-273.

Juarez C. and J. A. Banks 1998. Sex determination in plants. *Current Opinion in Plant* 1:68–72.

Leme F.M., J. Schönenberger, Y.M. Staedler and S.P. Teixeira. 2020. Comparative floral development reveals novel aspects of structure and diversity of flowers in Cannabaceae. *Botanical Journal of the Linnean Society* 193:64–83.

Louis, J. P. 1989. Genes for the regulation of sex differentiation and male fertility in Mercurialis annua L. *Journal of Heredity* 80(2): 104-111.

Meagher, T. R. 1988. Sex determination in plants. *In* Lovett Doust J. and L. Lovett Doust [eds.] Plant reproductive ecology: patterns and strategies, 125-138. Oxford University Press, New York, New York, USA.

Mesgaran, M.B., M. Matzrafi and S. Ohadi. 2021. Sex dimorphism in dioecious Palmer amaranth (*Amaranthus palmeri*) in response to water stress. *Planta* 254(1): 17.

Miller, P.M. and R.V. Kesseli. 2011.A sex-chromosome mutation in *Silene latifolia. Sexual plant reproduction* 24(3): 211–217.

Ming, R., A. Bendahmane and S. S. Renner. 2011. Sex chromosomes in land plants. *Annual review of plant biology* 62: 485-514.

Mosyakin S.L. and K.R. Robertson. 1996. New infrageneric taxa and combinations in Amaranthus (Amaranthaceae). *Annales Botanici Fennici* 33:275–281.

Neves, C. J., M. Matzrafi, , M. Thiele, A. Lorant, M. B. Mesgaran and M. G. Stetter. 2020. Male Linked Genomic Region Determines Sex in Dioecious Amaranthus palmeri. *The Journal of heredity* 111(7): 606–612.

Renner, S. S., 2014 The relative and absolute frequencies of angiosperm sexual systems: dioecy, monoecy, gynodioecy, and an updated online database. *American Journal of botany* 101: 1588–1596.

Ruzin S. E. 1999. Plant Microtechnique and Microscopy. Oxford University Press, New York, New York, USA.

Sánchez-Del Pino I., A. Vrijdaghs, P. De Block, H. Flores-Olvera, E. Smets and U. Eliasson. 2019. Floral development in Gomphrenoideae (Amaranthaceae) with a focus on androecial tube and appendages. *Botanical Journal of the Linnean Society.*190: 315–332.

Sherry, R. A., K. J. Eckard and E. M. Lord. 1993. Flower development in dioecious Spinacia oleracea (Chenopodiaceae). *American Journal of Botany* 80: 283–291.

Stetter M. G. and K. J. Schmid. 2017. Analysis of phylogenetic relationships and genome size evolution of the Amaranthus genus using GBS indicates the ancestors of an ancient crop. *Molecular Phylogenetics and Evolution* 109:80–92.

Trucco, F. and P. J. Tranel. 2011. Amaranthus. *In* Kole, C. [eds.], Wild crop relatives: genomic and breeding resources. 11-21. Springer, Berlin, Heidelberg, Germany.

Van Wychen, L. 2017. Survey of the Most Common and Troublesome Weeds in grass crops, pasture and turf in the United States and Canada. Weed Science Society of America National Weed Survey Dataset [online]. Website: https://wssa.net/wp-content/uploads/WSSA-Weed-Survey-2017-05-19.pdf [accessed 04 January 2023]

Wannan, B. S. and C. J. Quinn, 1991, Floral structure and evolution in the Anacardiaceae. *Botanical Journal of the Linnean Society* 107: 349-385.

Ward, S., T. Webster and L. Steckel, 2013. Palmer amaranth (*Amaranthus palmeri*): A Review. *Weed Technology* 27(1): 12-27.

Westergaard M. 1946. Structural changes of the Y-chromosomes in the offspring of polyploid Melandrium. *Hereditas* 32: 60–64.

Westergaard M., 1958. The mechanism of sex determination in dioecious plants. *Advanced Genetics* 9: 217–281

Tables

Table 1. **Summary of the phenology of pistillate and staminate flower development in *Amaranthus palmeri* with reference to respective figures.**

Stage	Staminate flowers	Pistillate flowers
1	Formation of floral apical meristem	
2	Tepal primordia appear (Fig. 5A)	
3	Tepal primordia well-established	
4	Stamen primordia arise	Carpel primordium arises (Fig. 7A)
5	Five stamen primordia well-established (Fig. 5B)	Single carpel primordium enlarges (Fig. 7B)
6	Primary gynoecium primordium initiates (Fig. 5C)	Ovule and ovary wall primordium initiate and develop (Fig. 7C-7E)
7	Five stamen primodia develop with vestigial carpel remnants remaining undeveloped and surrounded by stamen bases (Fig. 5D-5H)	Two (to three) style primordia form and develop (Fig. 7F-7H)
8	Stamens become four-lobed (Fig. 5I, J)	Stigmatic region forms along adaxial surface of styles (Fig. 7I)
9	Stamen filaments elongate and pollen matures	Carpel differentiation complete (Fig. 7J-7K)
10	Anthesis	Anthesis

Figures

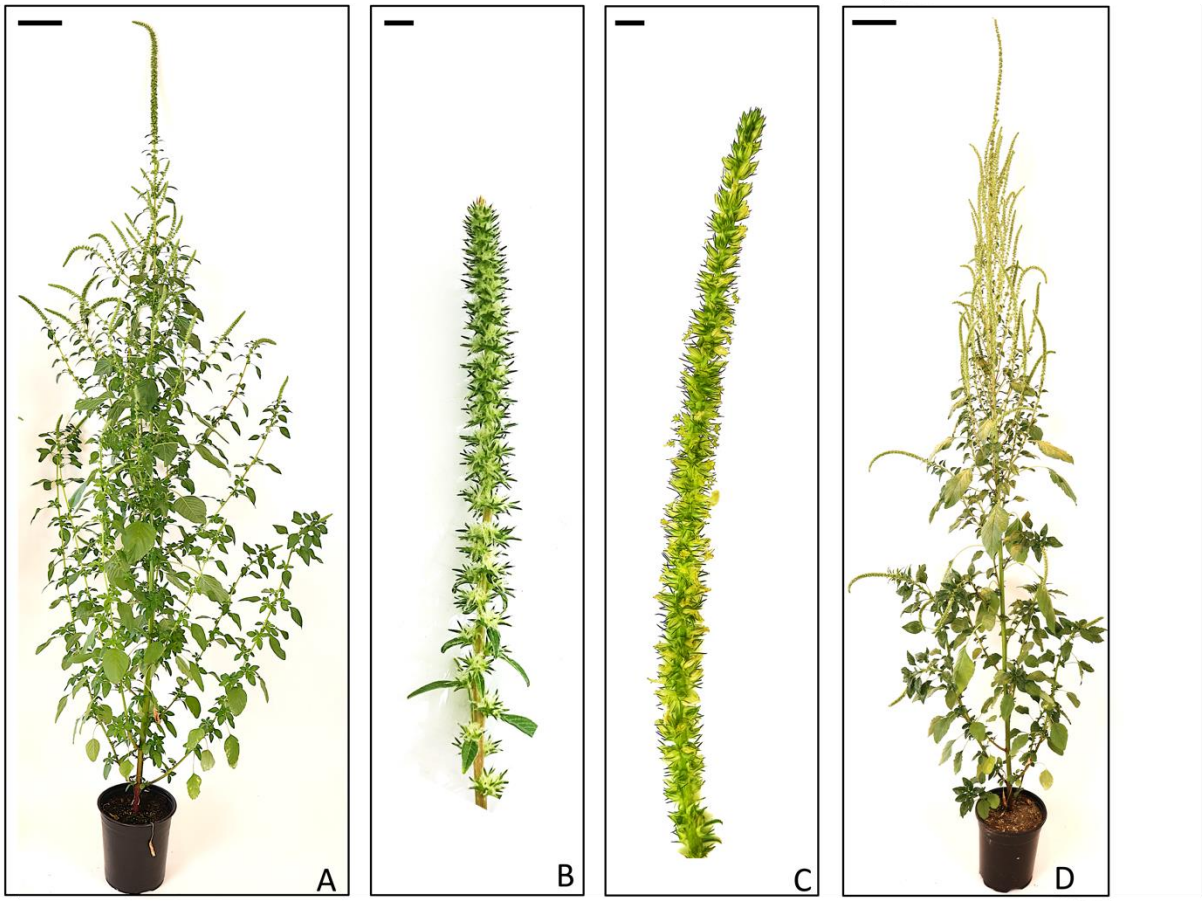

Figure 1. Pistillate and staminate plants in *Amaranthus palmeri.* A. Pistillate plant at anthesis. Note highly branched inflorescence axis. B. Pistillate inflorescence at anthesis. Note alternating long and short internodes. C. Staminate inflorescence at anthesis. D. Staminate plant at anthesis. Note highly branched upper inflorescence axis. Scale bars: A,D = 10cm; B-C=1cm.

Figure 2. Pistillate flowers in *Amaranthus palmeri*. A. Segment of inflorescence. B. Two pistillate flowers enclosed by three bracts. C. One pistillate flower with two styles, five tepals and one bract. D. Single dissected pistillate flower with two bracts, five tepals, two styles, one dehisced utricle and one mature seed. E. Immature and mature seed enclosed by utricle. F. Five tepals and one utricle (bracts, styles and seed removed from one pistillate flower). Labels: B = bract, S = seed, Sy = style, T = tepal, U= utricle. Scale bars: A-F = 1000 µm.

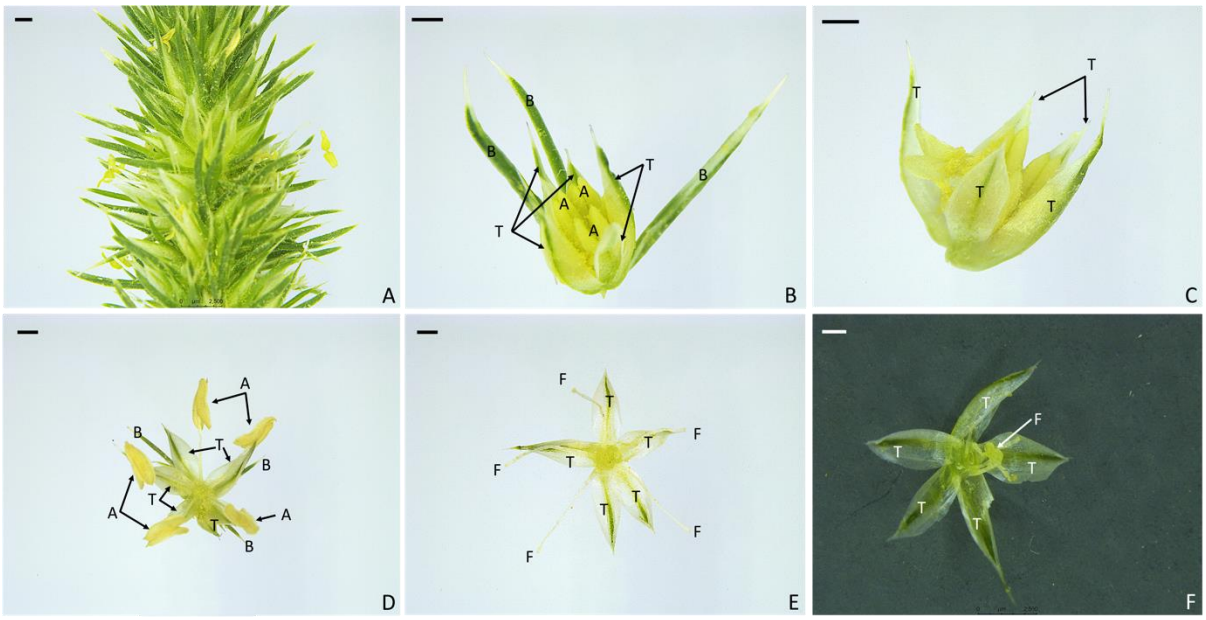

Figure 3. Staminate flowers in *Amaranthus palmeri.* A. Segment of inflorescence. B. Staminate flower before anthesis with three bracts, five tepals and three visible anthers. C. Staminate flower before anthesis with five tepals (bracts removed). D. Staminate flower at anthesis with three bracts, five tepals, and five anthers. E-F. Staminate flower at anthesis with five tepals and five filaments (in E), (bracts and anthers removed). Labels: A = anther, B = bract, F = filament, T = tepal. Scale bars: A-F = 1000 µm.

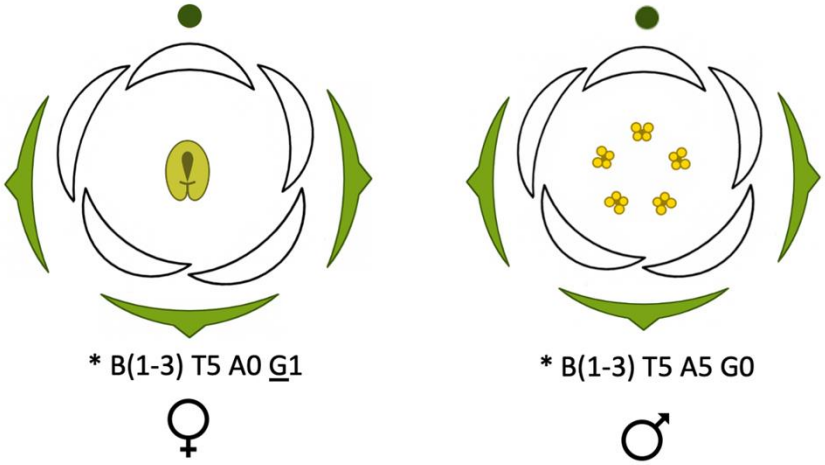

Figure 4. Floral diagrams and formulae for *Amaranthus palmeri* pistillate and staminate flowers at anthesis.

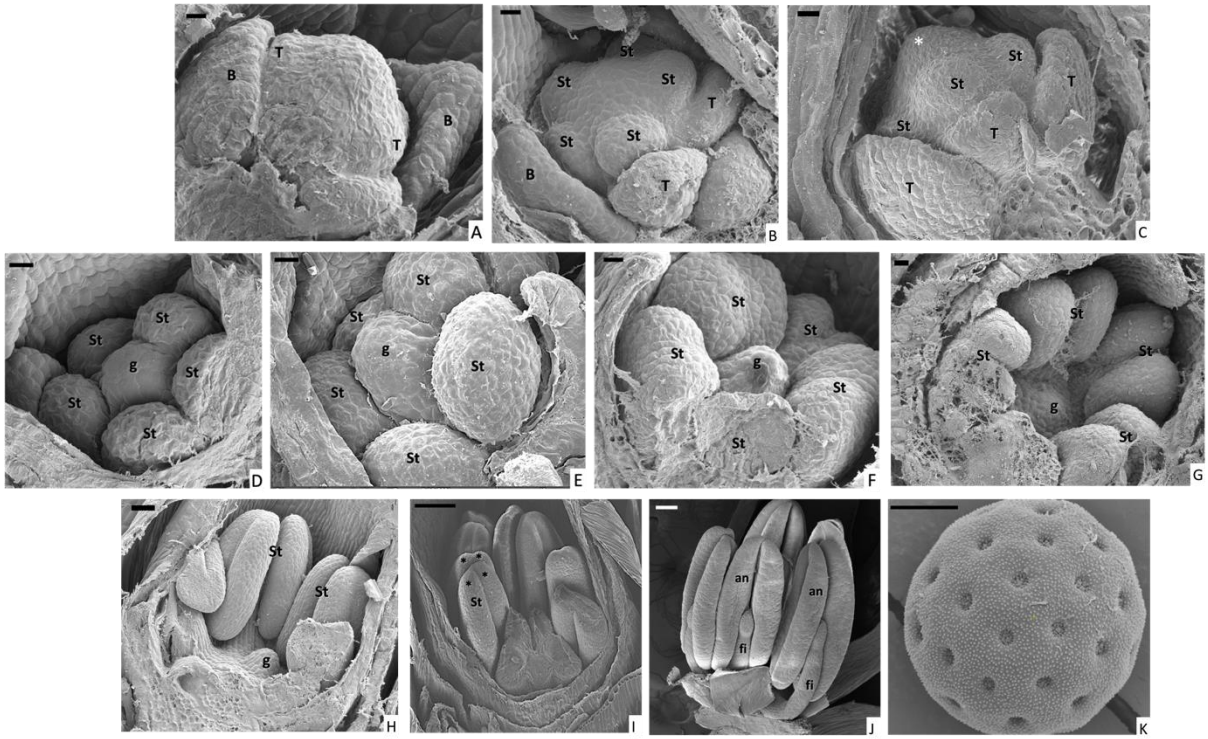

Figure 5. Scanning electron micrographs of *Amaranthus palmeri* staminate flower development. A. Tepal initiation with two bracts. B. Five stamen primordia developing with two visible tepal primordia and one visible bract. C. Floral apex enlarges and is recognized as a primary gynoecium primordium. D-H. Development of five stamen primordia with one putative gynoecium. I-J. Male flower at anthesis with anther lobes and filaments. K. Mature pollen grain with mean diameter of 31 µm and numerous apertures. Labels: an = anther, B = bract, fi = filament, g = putative gynoecium, St = stamen primordium, T = tepal, *(white asterisk) = floral apex, *(black asterisk) = anther lobes. Scale bars: A-G, K= 10 µm; H-J= 20 µm.

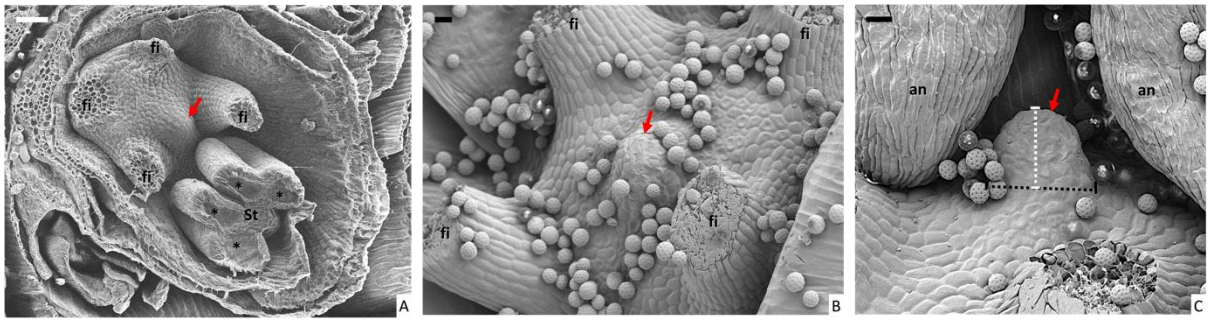

Figure 6. Putative carpel primordium (red arrow) at the base of filaments in *Amaranthus palmeri* at anthesis stage. A. Apical view of a putative gynoecium primordium surrounded by four severed filaments and one anther with four lobes. B. One putative gynoecium primordium surrounded by four severed filaments. C. Lateral view of a putative gynoecium primordium with two anthers. Black dotted line measures the diameter of the dome while white dotted line measures the height of the dome. Labels: an = anther, fi = filament, St = stamen. Scale bars: A-C = 20 µm.

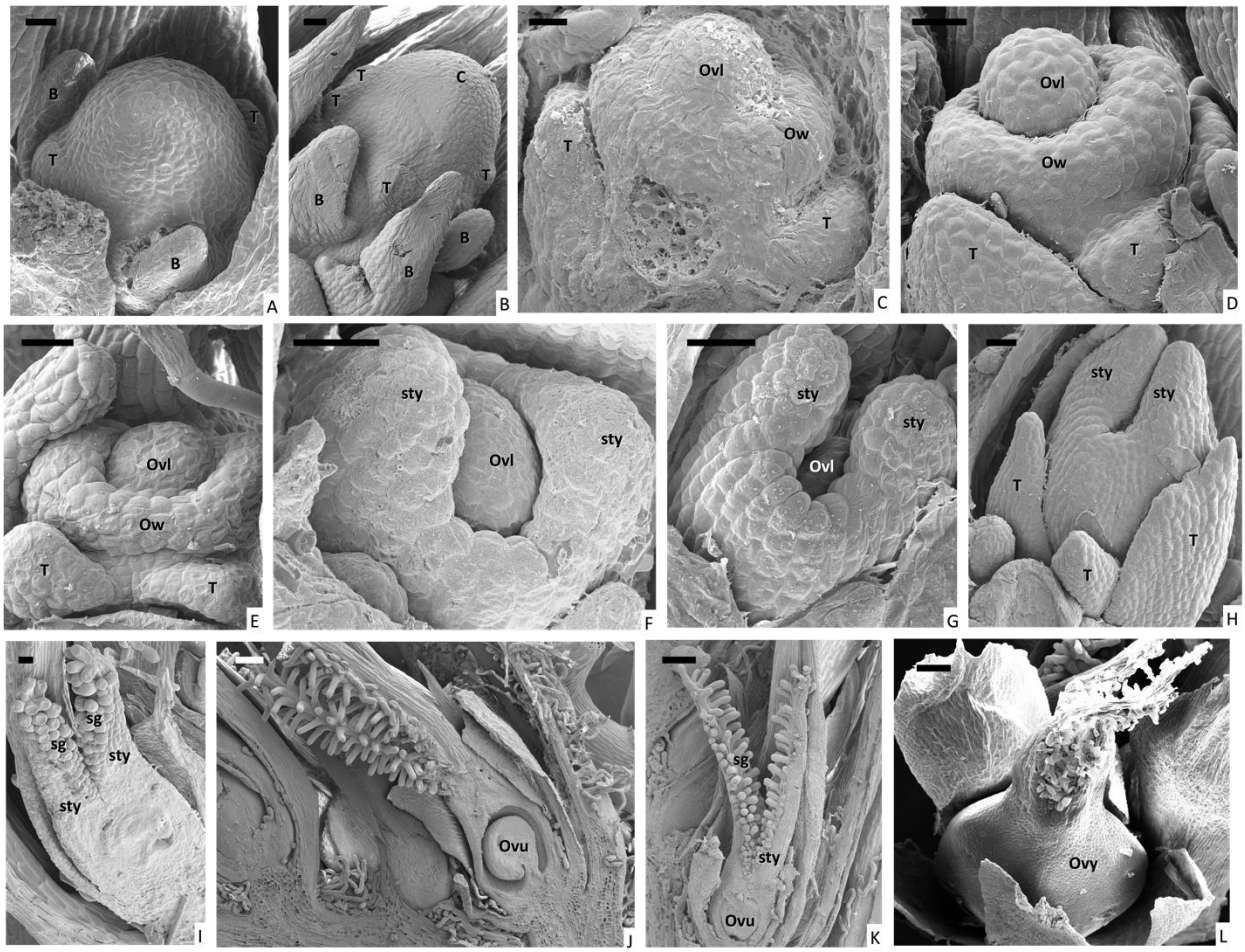

Figure 7. Scanning electron micrographs of *Amaranthus palmeri* pistillate flower development. A-B. Tepal and carpel primordia initiation. C-E. Ovule and ovary wall primordia development. F-H. Two style primodia development and style elongation. I-K. Stigma development. L. Single ovary seed maturation. Labels: B = bract, C= carpel primordium, Ovu = ovule, Ovl = ovule primordium, Ovy = ovary, Ow = ovary wall primordium, sg = stigma, sty= style, T = tepal. Scale bars: A-I = 20 µm; J-L, = 100 µm.

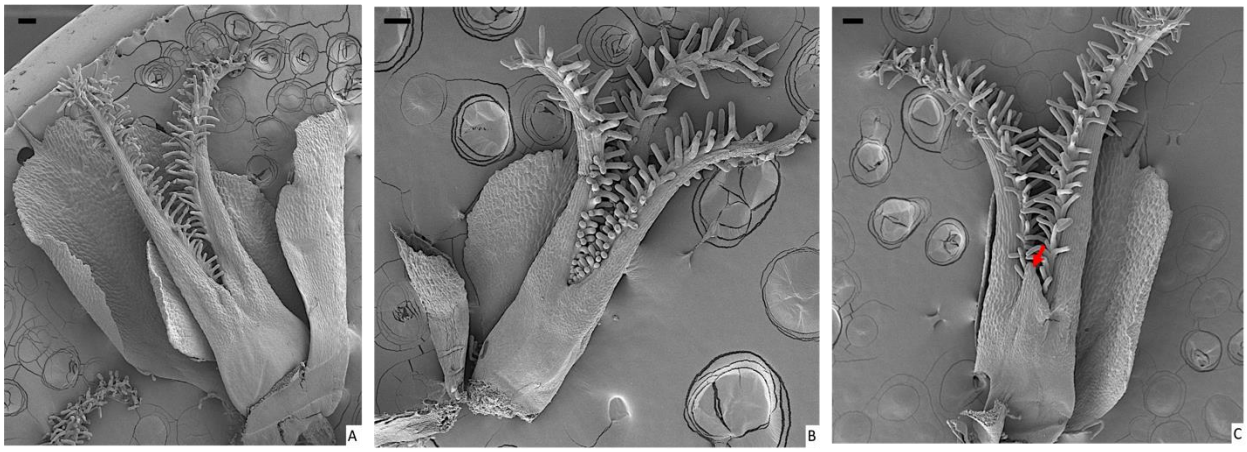

Figure 8. Mature pistillate flowers in *Amaranthus palmeri***.** A. Mature pistillate flowers with two styles, B. Mature pistillate flowers with three styles. C. Underdeveloped style (red arrow) near the base of two developing styles. Scale bars: A-C = 100 µm.

Chapter 2: Exploring Sterile Pollen Technique as a Novel Tool for Management of Palmer Amaranth (*Amaranthus palmeri*)

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Abstract

The success of the Insect Sterile Technique (IST) in managing insect pests raised the hypothesis that a similar approach could be employed to control weed populations. Here, we investigated the feasibility of employing irradiated sterile pollen as a means to disrupt seed production in dioecious weeds, specifically focusing on Palmer amaranth (*Amaranthus palmeri* S. Watson). Our goal was to determine the optimal irradiation dose that strikes a balance between inducing sterility and preserving competitiveness, as excessive doses could result in pollen mortality, while low doses may retain fertility. Plants were grown in a greenhouse during the summer of 2020 and spring of 2021. Once they reached the flowering stage, male and female individuals were isolated. Mature pollen samples were collected and exposed to varying dosages (0, 100, 200, 300, 400, and 500 Gy) of gamma rays. These irradiated and non-irradiated pollen samples were used in pollen viability assessments and hand-pollination experiments. In the hand-pollination study conducted in 2020, we employed six pollination treatments using different irradiation doses. The results showed that 300 Gy was the most effective dose, resulting in a maximum reduction of 30% in seed set compared to open pollination when irradiated pollen

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had prior access to the stigma through artificial pollination before open pollination. In 2021, to simulate field conditions, three additional treatments were introduced in the study, further confirming the effectiveness of the optimal 300 Gy dose. Our findings indicate that the sterile pollen technique (SPT) using irradiated pollen can be a valuable approach for reducing weed seed production. SPT also holds potential for broad-spectrum weed control by mixing sterile pollen from multiple weed species in a single application. Additionally, it could aid in managing herbicide-resistant weeds that have survived in-season control efforts. This research contributes to the development of novel and sustainable weed management strategies.

Keywords: Dioecy, Double fertilization, Ecological method, X-ray, Insect Sterile Technique, Irradiation, Pollination, Sexual reproduction

Introduction

Current agriculture systems rely heavily on the use of herbicides and tillage for weed management but both have negative impacts on the environment and farm productivity in long-term use while herbicide-resistance is increasing (Maclaren et al. 2020). An integrated approach to weed management which incorporates ecological principles and involves using multiple tactics that vary in timing and type of control is needed to reduce the probability of rapid weed adaptation to management practices (Harker and O'Donovan 2013). Moreover, weed management decisions should aim to prevent soil seedbank inputs rather than just minimize current yield loss for agricultural profitability (Swanton et al. 2008).

While there have been many studies focused on weed seed biology and seedbank management (Buhler et al. 1998), research focused on reducing weed seed production by manipulating flowering and seed set is lacking. At anthesis, pollen grains landing on a compatible stigma may germinate and produce pollen tubes which grow through the style to fertilize the ovules. Later pollen tubes are unable to enter fertilized ovules as the first pollen tube's sperm cell delivery causes an immediate block to further fertilizations (Beale et al. 2012). During its journey inside the pollen tube, the generative cell of a pollen grain divides into two male gametes. One gamete fuses with the egg cell nucleus and the other fuses with the pair of central cell nuclei. Together, these two fertilization processes are referred to as double fertilization (Edlund et al. 2004), which is unique to angiosperms (Russell 1992). The fertilized egg cell will give rise to an embryo while the fertilized central cell will give rise to the endosperm (Baroux et al. 2002).

There are several methods to make pollen grains functionally deficient and thereby reduce seed set (Zhang and Lespinasse 1991; Alsamir et al. 2021). The most commonly used

is ionizing irradiation with X-rays or gamma rays due to their ease of use, effective penetration, consistent results, and minimal disposal issues (Chahal and Gosal 2002). Irradiated pollen grains can be physiologically alive, depending on the irradiation dosage, but are infertile. Irradiated grains can germinate on the stigma and even produce pollen tubes but cannot fertilize egg cells to produce embryos (Musial and Pzrywara 1998). Further, when sterile pollen grains are deposited on a stigma through artificial pollination, they can interfere with fertile pollen in the process of fertilization and disrupt seed production, as has been shown in apple (Zhang and Lespinasse 1991), pear (Bouvier et al. 1993), citrus (Froelicher et al. 2007), cacao (Falque et al. 1992), and melon (Lotfi et al. 2003).

The use of sterile pollen to reduce weed seed production is similar to the insect sterile technique (IST), an environmentally-friendly and biologically-based method for controlling insect pest. This technique involves sterilizing male insects by irradiation and subsequently releasing the sterile males to mate with wild females (Parker and Mehta 2007), resulting in infertile eggs and reduced insect pest population sizes. Pollinating the female plants of Palmer amaranth (*Amaranthus palmeri* S.Watson) with sterile pollen (irradiated) resulted in 40% reduction in the number of newly formed seeds (Efrat et al. 2020). However, the sterile pollen technique (SPT) has been rarely used as a weed control technique but could potentially be effective on dioecious weedy species because female and male flowers are in separate plants and pollen grains can be collected from male plants, sterilized and then released on female plants.

The summer annual dioecious weed *A. palmeri* is one of the most devastating weeds in the US. It was ranked as the worst weed in US corn fields in a survey by the Weed Science Society of America (Van Wychen et al. 2017). Furthermore, it has evolved resistance to nine herbicide classes used (Heap 2022) and is able to produce up to one million seeds per plant (Ward et al. 2013). This weed is a particularly suitable candidate for exploration of the sterile

pollen technique for weed control. Being a dioecious species with separate male and female plants, it relies on cross-pollination for successful seed production. This makes it feasible to collect pollen grains from male plants, sterilize them, and subsequently release them onto female plants.

The primary goal of this research was to examine the effectiveness of sterile pollen technique, SPT, as a means of disrupting seed production in *A. palmeri*. To this end, it was necessary to determine the optimal irradiation dose for pollen sterilization as excessively high doses may kill the pollen entirely, thereby eliminating their preventative effects on fertile pollen, while low doses may allow the treated pollen to maintain its fertility. Accordingly, a broad range of irradiance doses was tested in combination with an extensive array of artificial pollination treatments to fully explore the potential effects of the SPT on seed production in *A. palmeri*. Our hypothesis is that pollinating with sterile pollen, irradiated at an optimal dose, could reduce seed production in this weed. Furthermore, we speculated that the maximum reduction in seed output could be achieved when pollination with sterile pollen precedes open pollination.

Materials and Methods

Plant material

Seeds (10-15) of *A. palmeri* collected from Kansas were planted in May 2020 into 3-L pots filled with UC Davis potting medium containing (1 sand:1 redwood sawdust:1 peat) in a greenhouse set at a 24/32 ℃ night/day temperature regime and extended photoperiod (14 hours of lighting). Fertilizers were applied as 80 ml of a general-purpose fertilizer solution (Jack's Professional General Purpose 20–20–20, Allentown, PA) weekly at 350 ppm N starting from the 2-true leaf plant stage with drip irrigation applied at 65 mL/min for two minutes twice per day. Seedlings were periodically thinned to maintain one plant per pot. Once plants reached

the flowering stage, 50 male and 50 female plants were isolated and grown in separate greenhouses (males often flower first).

Pollen collection and irradiation

Pollen collections were made from male plants by gentle tapping or shaking of the inflorescence. Pollen grains from all male plants were pooled and released onto aluminum foil held beneath the inflorescence. The collected pollen was then sieved through 250-mm mesh to remove large floral materials. Pollen was placed in Petri dishes covered with parafilm and then irradiated immediately with gamma rays from Cesium-137 at six dosages of 0 (no irradiation), 100, 200, 300, 400 and 500 Gray (Gy) (Košmrlj et al. 2013; Godbole and Murthy 2012) at the UC Davis Center for Health & the Environment (https://che.ucdavis.edu). Irradiated and untreated pollen were immediately used for pollen viability tests and hand-pollination experiments as described below.

Pollen viability tests

Pollen viability was assessed immediately after irradiation by using a test solution consisting of a 1% concentration of the substrate 2,5-diphenyl tetrazolium bromide (MTT) in 5% sucrose. The MTT assay measures cellular metabolic activity as an indicator of cell viability and cytotoxicity (Karakaş et al. 2017). In this assay, viable pollen appears dark violet and non-viable pollen did not stain at all (See Figure 1a). Viability of 100 pollen grains for each dose at each irradiation dose was assessed by analyzing the brightness of the resulting tetrazolium stain using a digital camera (Leica MC190 HD; Leica Microsystems AG, Wetzlar, Germany) and ImageJ software Version 1.46r (Schneider et al. 2012). Grey values were used to indicate the brightness of a pixel. Because the range for grey values is 0-255, grey value percentages (%) were calculated by dividing the recorded grey values by 255 and multiplying by 100. Higher grey value percentages indicated lower pollen viability. The

effects of irradiation dosage on grey value percentages were analyzed using ANOVA with Dunnett's test.

Hand pollination experiments

In the 2020 experiment, six lateral inflorescences of similar size from each female plant were selected to receive the following treatments: hand pollination with 1) non-irradiated pollen only, 2) irradiated pollen only, 3) non-irradiated pollen followed by irradiated pollen, 4) irradiated pollen followed by non-irradiated pollen, 5) no pollination, or 6) open pollination (no bagging). Each inflorescence was meticulously dusted with 1 ml of pollen, ensuring even and gentle distribution using a paintbrush. Thereafter, the inflorescence was immediately enclosed in a paper bag with the exception of inflorescences receiving the open pollination treatment. Hand pollination was conducted through a one-time application. About 6 weeks after pollination, inflorescences were harvested. Flower and seed numbers were measured on the abovementioned six inflorescences for each of five plants (replicates) at each irradiation dosage. For each replicate, six 1-cm sections of plant branches were dissected and measured for flower and seed numbers. Two categories of seeds were identified and recorded: abnormal seeds (undeveloped ovules or empty seeds) and normal full seeds. Seed set was calculated by using the number of viable and full seeds divided by the number of flowers and expressed as percentage.

To more closely simulate field conditions, this experiment was repeated in 2021 with three additional treatments: 1) hand pollination with irradiated pollen followed by open pollination (without bagging), 2) open pollination for two weeks followed by hand pollination with irradiated pollen (with bagging), and 3) open pollination for two weeks followed by hand pollination with irradiated pollen with no bagging, i.e., open pollination. These treatments began simultaneously when nine lateral inflorescences of similar size from five female plants

(serving as replicates) reached full anthesis. As with 2020 experiment, the hand pollination was performed as a single, one-time application.

Data from each year of study was analyzed separately because hand-pollination treatments differed slightly across the two years of experiment. Prior to ANOVA, in order to reduce heteroscedasticity of the residuals, seed set values were transformed using a square root transformation. Two factors, irradiation dose and pollination treatment, were firstly combined into a single factor and a one-way ANOVA was performed on seed set measurements by using aov() functions in R (R Core Team 2020a). To better explore the interaction between the two factors, the non-crossed treatments (treatment 1: non-irradiated pollen, treatment 5: no pollination, and treatment 6: open pollination) were removed to obtain a full-factorial design for a two-way ANOVA. The two-way factorial ANOVA was conducted using lm() function followed by slicing each level of irradiation doses, with SLICE() function in sasLM package (Littell et al. 2015), to perform the F-test for the effect of hand-pollination on seed set at each level of irradiation dose. Lastly, seed set data was back-transformed using the re_grid() function in the emmeans package (Lenth and Lenth 2017) and confidence intervals were constructed using confint() function in R (R Core Team 2020b).

Results and Discussion

The effect of irradiation on pollen viability

Pollen irradiated at the lowest dose (100 Gy) exhibited the lowest grey value percentage (highest viability) while pollen irradiated at 500 Gy had the highest grey value (lowest viability) (Figure 1b). The mean grey value of pollen irradiated at 500 Gy was significantly different from the other doses, which indicates this highest irradiation dose reduced pollen viability to a greater degree than the other doses (Table 1). Under this high irradiation dose, pollen will likely be unable to produce a pollen tube and disrupt the process of double fertilization since it has lost its viability as determined by MTT staining.

The viability of pollen is affected by factors such as genotype, pollen maturity, growth media composition (Ferri et al. 2008), and environmental variables such as air temperature and humidity (Pacini 1996). Gamma ray irradiation can decrease water content in pollen, reducing the ability to transfer carbohydrate reserves, leading to changes in the cytoplasmic water, abnormal meiosis, irregular gamete formation, and ultimately decreased viability, which has been supported in studies on apples (Zhang and Lespinasse 1991), pumpkins and winter squash (Kurtar 2009), and citrus (Kundu et al. 2014).

The effect of radiation dose on pollen viability is species dependent. In some species irradiation effect is limited. For example, melon (*Cucumis melo* L.) pollen can tolerate gamma-irradiation doses up to 3,600 Gy (Cuny et al. 1992) whereas in winter squash a 300 Gy dose reduced pollen viability by almost 80% (Kurtar 2009). We found significantly reduced viability of *A. palmeri* pollen irradiated at 500 Gy compared with non-irradiated pollen, which indicates that seed production in this weed is sensitive to ionizing irradiation. However, our goal in the practice of irradiation is not the complete loss of viability. For effective implementation of SPT, it is essential to have semi-functional pollen that can outcompete and displace wild pollen while remaining incapable of fertilizing the ovule.

Understanding the sexual reproduction process is important to gain insight about how to increase the competitiveness of irradiated pollen. When the pollen tube enters the female reproductive tissue, intensive communication occurs between the pollen tube and one synergid cell. After the contact of pollen tube and synergid cell, the receptive synergid degenerates (Leydon et al. 2015). Following release of the two sperm cells from the pollen tube, they interact and fuse with the egg cell nucleus and the central cell nuclei, forming the major seed components embryo and endosperm, respectively. Any of the steps involved in double fertilization or a subsequent event could trigger the block of attraction of multiple pollen tubes to a single ovule (Beale et al. 2012). If fusion fails, one synergid can persist and continue to

attract multiple pollen tubes until fertile sperm are delivered or the synergid senesces. The recovery of fertilization is limited to the second pollen tube, indicating that there is no third chance for fertilization in two synergid celled plants. The optimal irradiation dosage to sterilize pollen should maintain the function of the vegetative cell but induce failure in cell fusion. If the irradiated pollen can disrupt fertilization twice, there is no third chance for this ovule to produce a seed (Kasahara et al. 2012), thereby reducing overall seed production.

Effect of irradiation dose on seed production

Both in 2020 and 2021, the combined effect of irradiation dose and application treatment had a significantly different effect on seed set (Appendix 1 and 2). Additionally, the effect of different irradiation doses, application treatments, and their interaction on seed set was significant in both years (Appendix 3 and 4). Female plants that received no pollination did not produce seed in either year so this treatment will not be discussed further in the results. However, in contrast to this observation, a study has proposed apomixis as a potential mechanism for seed production in isolated female plants (Ribeiro et al. 2014).

In both years, regardless of the irradiation dose, all pollination treatments involving irradiated pollen consistently resulted in lower seed sets compared to open pollination (Figure 2-3). The mean seed set obtained from pollination treatments involving irradiated pollen never exceeded 35% and decreased to nearly 0% when using only irradiated pollen at doses of 300, 400, and 500 Gy (Figure 2-3). Seed set decreased with increasing irradiation dose up to 300 Gy in all pollination treatments with irradiated pollen. However, there was an increase in seed set beyond the 300 Gy dose when pollination with irradiated pollen followed by hand pollination with non-irradiated pollen. This suggests that pollen irradiated at 100 Gy and 200 Gy maintained some ability to fertilize egg cells and produce seeds, while pollen irradiated at the higher doses of 300 Gy to 500 Gy were functionally deficient and unable to complete sexual reproduction. The 300 Gy dose seems to be the optimal dose for disrupting seed production in

A. palmeri as it produced the lowest seed set when interfering with non-irradiated pollen (Figure 2-3). Irradiation of pollen has also decreased seed production and seed set in other species. A study of *Arabidopsis* found a 50% reduction in seed set when pollen was irradiated at 400 Gy and less than 10% seed set with pollen irradiated at 800 Gy (Yang et al. 2004).

The effects of different irradiation doses on seed set varied depending on the sequence of pollination treatments applied (Table 1-2). The seed set from pollination with irradiated pollen after non-irradiated pollen (NI+I) with bagging was higher than pollination with non-irradiated pollen after irradiated pollen (I+NI) with bagging (Figure 2-3). This finding shows that pollination with irradiated pollen is most effective in reducing seed set when it is applied before any fertile and fully functional pollen reaching the stigma. Furthermore, applying irradiated pollen before non-irradiated pollen across all irradiation doses significantly reduced seed set compared to open pollination (Figure 2-3), even though it may not completely disrupt double fertilization under some doses. Irradiated but non-viable pollen may act as a physical barrier covering the stigma and preventing viable non-irradiated pollen from fertilizing ovules and producing seeds.

The additional treatments in 2021 which combined different sequences of pollinating with irradiated pollen and open pollination produced lower seed set than open pollination (Figure 3). However, the timing of the pollination with irradiated pollen is critical as it can greatly affect the reduction of the seed set. Seed set on inflorescences that received open pollination after irradiated pollen (I+O) was lower than that of the inflorescences pollinated with irradiated pollen after open pollination (without bagging: O+I+O) for all irradiation doses (Figure 3). This suggests that irradiated pollen has a preventive effect on subsequently arriving, naturally occurring pollen when it has initial access to the stigma and that it successfully disrupts fertilization twice in two synergid-celled plants.

Based on the results of hand pollination (Figure 2-3) and pollen viability (Figure 1)

experiments, a radiation dose of 300 Gy is deemed to be the optimal balance between sterility and interference. However, irradiated pollen is less competitive than naturally occurring pollen, presenting a challenge in terms of efficiency improvement. To address this issue, additional measures such as dispersing irradiated pollen in the field before male anthesis and releasing it multiple times may be necessary.

Enhancing efficiency of sterile pollen technique

Introducing irradiated pollen for pollination prior to the arrival of naturally occurring pollen on the stigmas of female plants can potentially create a temporal advantage, enhancing the efficiency of SPT. However, determining the optimal timing for dispersing irradiated pollen remains challenging. Due to the indeterminate nature of *A. palmeri* inflorescences (Tranel et al. 2002), flowers will be at various ages when irradiated pollen is applied (within-individual variation). Furthermore, not all plants will flower simultaneously; consequently, a proportion of plants in a population will not have flowers exposed to sterile irradiated pollen when it is applied (between-individual variation). Because of within- and between-individual variations in the timing of flowering, multiple bouts of pollination with irradiated pollen are necessary to achieve desirable level of reduction in seed output. Even with multiple applications, there remain the questions about how many applications should be made and what time intervals. To address these questions a thorough analysis of the flowering phenology of *A. palmeri* is required. Our previous study has shown that male plants of *A. palmeri* enter the flowering stage earlier than females but anthesis happens earlier (~4 days) in females than males (Mesgaran et al., 2021). The earlier anthesis in female plants of *A. palmeri* can make the SPT more effective. Early pollination with irradiated pollen, such as at the first occurrence of female anthesis, can block or interfere with these receptive stigmas and prevent fertilization with fertile pollen from naturally occurring males.

Under field conditions, pollen viability was reduced within 30 min of anthesis and approached nonviability at 240 min following anthesis under field conditions (Sosnoskie et al. 2012). As non-irradiated pollen moves from male plants to female plants, its viability may rapidly decrease due to long inter-plant distances and heat stress experienced during summer. The sensitivity of pollen to temperatures has been established through studies in tomato (Alsamir et al. 2021), maize (Begcy et al. 2019), and soybean (Djanaguiraman et al. 2018). High temperatures can lead to increased respiration and metabolism, water loss, and a rapid decrease in vitality, while low temperatures can slow down metabolism and respiration, reducing the rate of pollen viability loss (Du et al. 2019; Paupière et al. 2014). Even if the pollen lands on a compatible stigma, it may have reduced viability and vigor and be unable to germinate. Thus, developing a mechanism to preserve irradiated pollen viability and implement the sterile pollen technique over short distances to the target with high efficiency will be crucial for success of SPT.

In conclusion, we tested the possibility of using sterile irradiated pollen as means of disrupting seed production in *A. palmeri* in a similar way to the Insect Sterile Technique (IST). Results demonstrated that an irradiation dose of 300 Gy seems to be the most effective in reducing seed set in *A. palmeri*. Furthermore, we observed that the greatest reduction in seed set was achieved when irradiated pollen was introduced to the stigma through artificial pollination prior to open pollination. It appears that irradiated pollen exerts a preventive effect on naturally occurring pollen that arrives later. Although the focus of this project was a single weed species, the method can be extended to the control of seed production for multiple weed species simultaneously (broad-spectrum weed control) where sterile pollen from multiple weed species can be mixed and released in a single application. The sterile pollen technique could be particularly helpful for managing herbicide-resistant weeds which have withstood in-season control and have reached a reproductive stage.

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References

Alsamir M, Mahmood T, Trethowan R, Ahmad N (2021) An overview of heat stress in tomato (*Solanum lycopersicum* L.). Saudi J Biol Sci 28:1654-1663

Baroux C, Spillane C, Grossniklaus U (2002) Evolutionary origins of the endosperm in flowering plants. Genome Biol 3(9):reviews1026

Beale KM, Leydon AR, Johnson MA (2012) Gamete fusion is required to block multiple pollen tubes from entering an Arabidopsis ovule. Curr Biol 22:1090–1094

Begcy K, Nosenko T, Zhou LZ, Fragner L, Weckwerth W, Dresselhaus T (2019) Male sterility in maize after transient heat stress during the tetrad stage of pollen development. Plant Physiol 181:683–700

Bouvier L, Zhang YX, Lespinasse Y (1993) Two methods of haploidization in pear, *Pyrus communis* L.: greenhouse seedling selection and in situ parthenogenesis induced by irradiated pollen. Theor Appl Genet 87:229–232

Buhler DD, Hartzler RG, Forcella F (1998) Weed seed bank dynamics: Implications to weed management. J Crop Prod 1:145–168

Calabrese JM, Fagan WF (2004) Lost in time, lonely, and single: reproductive asynchrony and the Allee effect. Am Nat 164:25–37

Chahal GS, Gosal SS (2002) Principles and procedures of plant breeding: biotechnological and conventional approaches. Oxford: Alpha Science Int'l Ltd. Pp 399–412

Cuny F, Devaulx RD, Longhi B, Siadous R (1992) Analysis of muskmelon plants (*Cucumis melo* L.) obtained after pollination with gamma-irradiated pollen-effect of different doses. Agronomie 12:623–630

Djanaguiraman M, Perumal R, Jagadish SVK, Ciampitti IA, Welti R, Prasad PVV (2018) Sensitivity of sorghum pollen and pistil to high-temperature stress. Plant Cell Environ 41:1065-1082

Du G, Xu J, Gao C, Lu J, Li Q, Du J, Lv M, Sun X. (2019) Effect of low storage temperature on pollen viability of fifteen herbaceous peonies. Biotechnol Rep 21

Edlund AF, Swanson R, Preuss D (2004) Pollen and stigma structure and function: the role of diversity in pollination. The Plant Cell 16(Suppl):S84–S97

Efrat LN, Ido S, Herve H, Miriam A, Micheal DO, Jonathan G, Orly NB (2020) Inhibiting herbicide-resistant Amaranthus by suppressing reproduction. Pages 87 in Proceedings of the 75th annual meeting of the north central weed science society. Virtual: North Central Weed Science Society

Falque M, Kodia AA, Sounigo O, Eskes AB, Charrier A (1992) Gamma-irradiation of cacao (*Theobroma cacao* L.) pollen: Effect on pollen grain viability, germination and mitosis and on fruit set. Euphytica 64:167–172

Ferri A, Giordani E, Padula G, Bellini E (2008) Viability and in vitro germinability of pollen grains of olive cultivars and advanced selections obtained in Italy. Adv in Hortic Sci 22:116–22

Firon N, Nepi M, Pacini E (2012) Water status and associated processes mark critical stages in pollen development and functioning. Ann Bot 109:1201–1213

Forero-Montaña J, Zimmerman JK (2010) Sexual dimorphism in the timing of flowering in

two dioecious trees in a subtropical wet forest, Puerto Rico. Caribb J Sci 46:88-95

Forrest JR (2014) Plant size, sexual selection, and the evolution of protandry in dioecious plants. Am Nat 184:338–35.

Froelicher Y, Bassene JB, Jedidi-Neji E, Dambier D, Morillon R, Bernardini G, Costantino G, Ollitrault P (2007) Induced parthenogenesis in mandarin for haploid production: induction procedures and genetic analysis of plantlets. Plant Cell Rep 26:937–944

Georgieva D, Kruleva MM (1994) Cytochemical investigation of long-term stored maize pollen. Euphytica 72:87-94

Germanà MA (2011) Anther culture for haploid and doubled haploid production. Plant Cell Tissue Organ Cult 104:283–300

Godbole M, Murthy HN (2012). Parthenogenetic haploid plants using gamma irradiated pollen in snapmelon (*Cucumis melo* var. momordica). Plant Cell Tissue Organ Cult 109: 167-170

Harker KN, O'Donovan JT (2013) Recent weed control, weed management, and integrated weed management. Weed Technol 27:1–11

Heap I (2022) The International Herbicide-Resistant Weed Database. www.weedscience.org. Accessed November 02, 2022

Kasahara RD, Maruyama D, Hamamura Y, Sakakibara T, Twell D, Higashiyama T (2012) Fertilization recovery after defective sperm cell release in arabidopsis. Curr Biol 22:1084–1089

Korres N, Norsworthy J, FitzSimons T, Roberts T, Oosterhuis D (2017) Differential Response of Palmer Amaranth (*Amaranthus palmeri*) Gender to Abiotic Stress. Weed Sci 65:213-227

Košmrlj K, Murovec J, Bohanec B (2013) Haploid induction in hull-less seed pumpkin through parthenogenesis induced by X-ray-irradiated pollen. J Am Soc for Hortic Sci

138:310-316

Kurtar ES (2009) Influence of gamma irradiation on pollen viability, germination ability, and fruit and seed-set of pumpkin and winter squash. Afr J Biotechnol 8:918– 26

Lenth R, Lenth MR. Package 'emmeans'. Statistician. 2017 Nov 4;34:216-21 https://cran.microsoft.com/snapshot/2017-11-19/web/packages/emmeans/emmeans.pdf

Leydon AR, Tsukamoto T, Dunatunga D, Qin Y, Johnson MA, Palanivelu R (2015) Pollen tube discharge completes the process of synergid degeneration that is initiated by pollen tube-synergid interaction in Arabidopsis. Plant Physiol 169:485–496

Li YM, Chen LB (1998) Vigor change of several grasses pollen stored in different temperature and humidity conditions. Plant Physiol 01:35-37

Littell RC, Stroup WW, Freund RJ (2015) SAS System for Linear Models (SAS LM) User's Guide. Version 2.4. Retrieved from https://cran.r-project.org/web/packages/sasLM/sasLM.pdf

Lloyd DG, Webb CJ (1977) Secondary sex characters in plants. Bot Rev 1977 43:177–216

Lotfi M, Alan AR, Henning MJ, Jahn MM, Earle ED (2003) Production of haploid and doubled haploid plants of melon (*Cucumis mel*o L) for use in breeding for multiple virus resistance. Plant Cell Rep 21:1121–1128

MacLaren C, Storkey J, Menegat A, Metcalfe H, Dehnen-Schmutz K (2020) An ecological future for weed science to sustain crop production and the environment. A review. Agron Sustainable Dev 40: 1–29

Mesgaran MB, Matzrafi M, Ohadi S (2021) Sex dimorphism in dioecious Palmer amaranth (*Amaranthus palmeri*) in response to water stress. Planta 254: 17

Musial K, Przywara L (1998) Influence of irradiated pollen on embryo and endosperm development in kiwifruit. Ann. Bot. 82:747–756

Oerke E (2006) Crop losses to pests. J Agric Sci 144:31 - 43

Pacini E (1996) Types and meaning of pollen carbohydrate reserves. Sexual Plant Reprod 22:362–366

Parker A, Mehta K (2007) Sterile insect technique: A model for dose optimization for improved sterile insect quality. Florida Entomologist 90:88–95

Paupière M, van Heusden A, Bovy A (2014) The metabolic basis of pollen thermo-tolerance: perspectives for breeding. Metabolites 4:889–920

Pressman E, Peet MM, Pharr DM (2002) The effect of heat stress on tomato pollen characteristics is associated with changes in carbohydrate concentration in the developing anthers. Ann Bot 90:631-636

Purrington CB, Schmitt J (1998) Consequences of sexually dimorphic timing of emergence and flowering in *Silene latifolia*. J Ecol 86: 397–404

R Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

R Core Team. (2020a). stats: The R Stats Package. R Foundation for Statistical ComputingRetrieved from https://www.rdocumentation.org/packages/stats/versions/3.6.2/topics/glm

R Core Team. (2020b). stats: The R Stats Package. R Foundation for Statistical Computing. Retrieved from https://www.rdocumentation.org/packages/stats/versions/3.6.2/topics/aov

Ribeiro D N, Pan Z, Duke SO, Nandula VK, Baldwin BS, Shaw DR, Dayan FE. (2014) Involvement of facultative apomixis in inheritance of EPSPS gene amplification in glyphosate-resistant Amaranthus palmeri. Planta 239: 199-212

Russell SD (1992) Double fertilization. International review of cytology 140: 357-88.

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675

Smith JD, Doe JK (2019) Effects of A, B, and C on Y: A three-way ANOVA. J ExpPsychol: General 148:333-355

Sosnoskie LM, Luschei EC, Fanning MA (2007) Field margin weed-species diversity in relation to landscape attributes and adjacent land use. Weed Sci *55*:129-136

Sosnoskie LM, Webster TM, Dales D, Rains GC, Grey TL, Culpepper AS (2009) Pollen grain size, density, and settling velocity for Palmer amaranth (*Amaranthus palmeri*). Weed Sci *57*: 404-409

Sosnoskie LM, Webster TM, Kichler JM, MacRae AW, Grey TL, Culpepper AS (2012) Pollen-mediated dispersal of glyphosate-resistance in Palmer amaranth under field conditions. Weed Sci 60:366-73

Swanton CJ, Mahoney KJ, Chandler K, Gulden RH (2008) Integrated weed management: knowledge-based weed management systems. Weed Sci 56: 168–172

Tranel P, Wasson J, Jeschke M, Rayburn A (2002) Transmission of herbicide resistance from a monoecious to dioecious weedy amaranth species. Theor Appl Genet 105:674 – 679

Van Wychen L (2017) Survey of the Most Common and Troublesome Weeds in grass crops, pasture and turf in the United States and Canada. Weed Science Society of America National Weed Survey Dataset. https://wssa.net/wp-content/uploads/WSSA-Weed-Survey-2017-05-19.pdf. Accessed January 04, 2023

Wang J, Wang D, Luo Q, Xiao H, Zhang X, Fang X, Gao Z, Han S (2017) Lipid oxidation and color degradation kinetics under different storage conditions of pollen. Trans Chin Soc Agric Eng 33(S1): 367-373

Ward S, Webster T, Steckel L (2013) Palmer Amaranth (*Amaranthus palmeri*): A Review. Weed Technol 27:12-27

Yang C, Mulligan BJ, Wilson ZA (2004) Molecular genetic analysis of pollen irradiation mutagenesis in Arabidopsis. New Phytol 164: 279–288

Yanmaz R, Elliatioglu S, Taner KY (1999) The effects of gamma irradiation on pollen viability and haploid plant formation in snake cucumber (*Cucumis melo L. var. flexuosus* Naud) Acta Horticulturae 492:307-310

Zhang YX, Lespinasse Y (1991) Pollination with gamma-irradiated pollen and development of fruits, seeds and parthenogenetic plants in apple. Euphytica 54:101–109

Tables

Table 1. Dunnett's test for pair-wise comparison of grey value percentage between different dose in Gy unit with control (0).

Irradiation doses comparison	Grey value percentage difference[a]	Pr(>F)
100-0	-0.0141	0.9282
200-0	0.0392	0.2136
300-0	-0.0064	0.9975
400-0	-0.0033	0.9999
500-0	0.1399	<0.0001

[a]Higher grey value percentages indicated greater brightness and, thus, lower pollen viability.

Table 2. The results of slicing the interactive effects of pollination treatments and irradiation dose.[a]

2020			2021		
Dose	**DF**	**Pr(>F)**	**Dose**	**DF**	**Pr(>F)**
100	2	0.1929	100	5	0.0364
200	2	0.0008	200	5	<0.0001
300	2	<0.0001	300	5	<0.0001
400	2	<0.0001	400	5	<0.0001
500	2	<0.0001	500	5	<0.0001

[a]This post hoc ANOVA tests for differences between pollination treatments at each level of irradiation dose, as indicated by the p-values. Fully crossed hand-pollination treatments in 2020 included pollination with irradiated pollen only (treatment 3), with non-irradiated pollen followed by irradiated pollen (treatment 4), and with irradiated pollen followed by non-irradiated pollen (treatment 5). There were three additional treatments in 2021, including (1) irradiated pollen followed by open pollination (without bagging), (2) open pollination for 2 weeks followed by hand pollination with irradiated pollen (with bagging), and (3) open pollination for 2 weeks followed by hand pollination with irradiated pollen, then open pollination with no bagging.

Figures

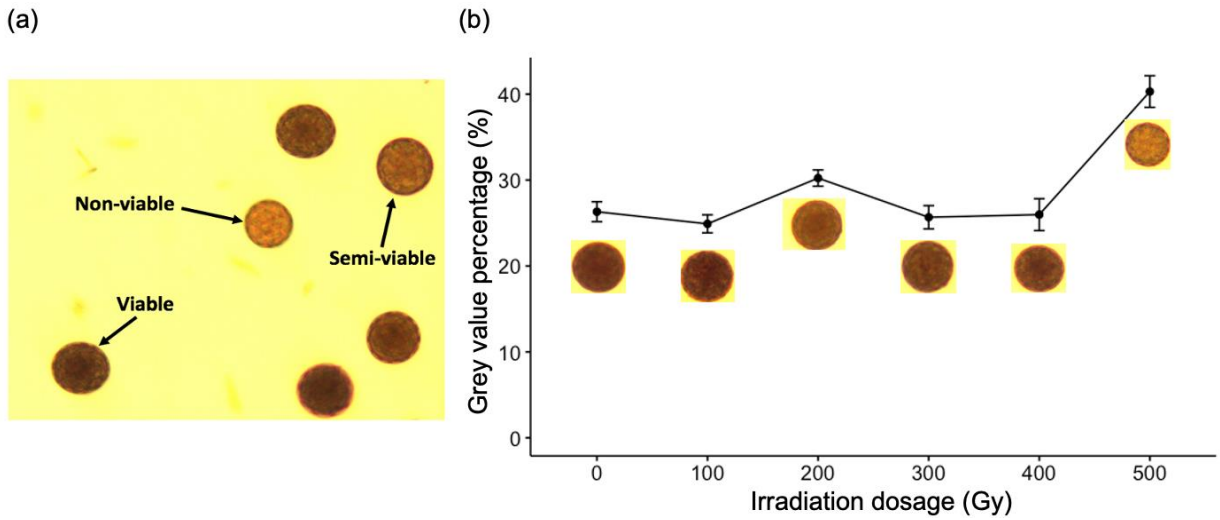

Figure 1. Viability of pollen grains stained with 2,5-diphenyl tetrazolium bromide (MTT) showing differing intensities (a). Effect of irradiation dosages of Gamma rays on pollen viability as quantified by mean grey value percentages from 100 pollen grains (b); error bars indicate standard error.

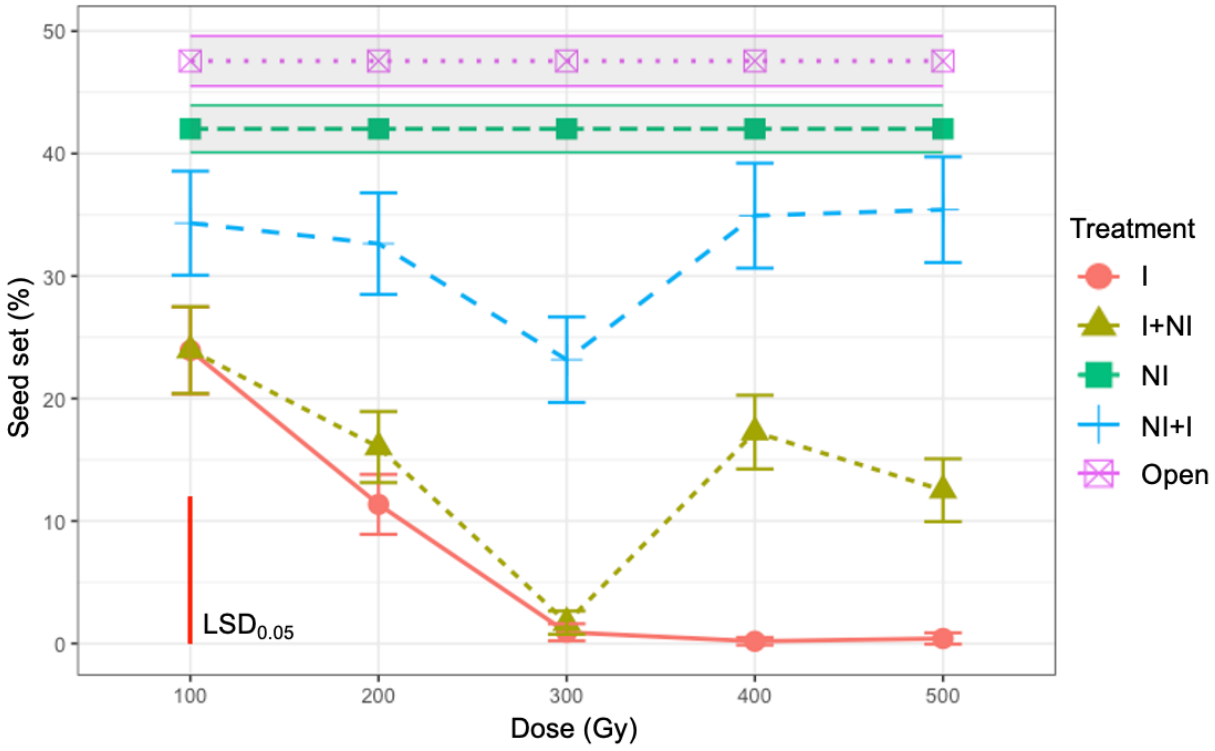

Figure 2. Effect of different pollen irradiation doses on seed set of *A. palmeri* inflorescences in 2020 with back-transformed means and standard errors. Abbreviations: I: irradiated pollen, I+NI: irradiated pollen followed by hand pollination with non-irradiated pollen, NI+I: non-irradiated pollen followed by hand pollination with irradiated pollen, NI: non-irradiated pollen, Open: open pollination). $LSD_{0.05}$ is the least square distance for significance level 0.05; error bars indicate standard error; shaded area for Open (open pollination) and NI (non-irradiated pollen) are mean ± standard error.

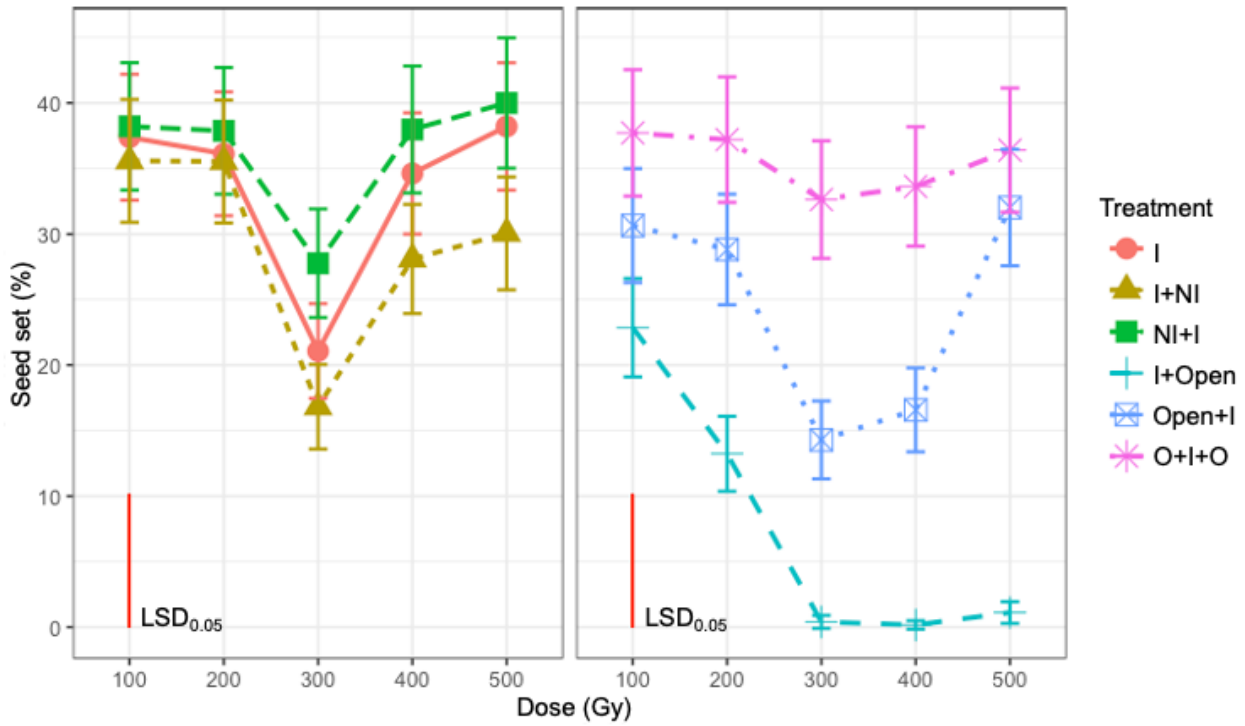

Figure 3. Effect of different pollen irradiation doses on seed set of *A. palmeri* inflorescences in 2021 with mean and standard error. Abbreviations: I: irradiated pollen, I+NI: irradiated pollen followed by hand pollination with non-irradiated pollen, NI+I: non-irradiated pollen followed by hand pollination with irradiated pollen, I+O: Irradiated pollen followed by open pollination, O+I: open pollination followed by hand pollination with irradiated pollen, O+I+O: open pollination followed by hand pollination with Irradiated pollen followed by open pollination). $LSD_{0.05}$ is the least square distance for significance level 0.05; error bars indicate standard error (The mean and standard error for open pollination is 0.3935% and 0.0220%; the mean and standard error for non-irradiated pollen treatment is 0. 3790% and 0.0216%).

Appendix

Appendix 1. ANOVA table of the effect of combined factors on square root transformed seed set in 2020.

Analysis of Variance				
Source	**DF**	**Mean Square**	**F Ratio**	**Pr (>F)**
treat	17	3505.1	42.916	<0.0001***
Residuals	147	81.7		

Appendix 2. ANOVA table of the effect of combined factors on square root transformed seed set in 2021.

Analysis of Variance				
Source	**DF**	**Mean Square**	**F Ratio**	**Pr (>F)**
treat	32	33.657	43.602	<0.0001***
Residuals	192	0.772		

Appendix 3. Analysis of Variance for all crossed treatments of hand pollination experiments on square root transformed seed set in 2020.

Analysis of Variance					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**	**Pr (>F)**
Treatment	2	162.666	81.333	88.528	<0.0001***
Dose	4	69.925	17.481	19.027	<0.0001***
Treatment: Dose	8	49.927	6.241	6.793	<0.0001***
Residuals	60	55.123	0.919		

Appendix 4. Analysis of Variance for all crossed treatments of hand pollination experiments on square root transformed seed set in 2021.

Analysis of Variance					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**	**Pr (>F)**
Treatment	5	276.539	55.308	91.2214	<0.0001***
Dose	4	61.534	15.383	25.3726	<0.0001***
Treatment: Dose	20	57.161	2.858	4.7139	<0.0001***
Residuals	120	72.756	0.606		

Chapter 3: Exploring the efficacy of the sterile pollen technique and the effect of massive pollination

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Abstract

Seed production by dioecious weeds, like Palmer amaranth (*Amaranthus palmeri* S. Wats.), can be disturbed through artificial pollination using irradiated pollen, as demonstrated experimentally. Although irradiated pollen is capable of germinating on the stigma, it fails to fertilize the egg cell and produce a viable seed. The effectiveness of the sterile pollen technique in reducing Palmer amaranth seed production has been demonstrated and showed that 300 Gy was the most effective irradiation dose to balance induced sterility and mating competitiveness (Wu and Mesgaran, 2021). In order to increase the efficiency of sterile pollen application which is essential for this practice to be utilized for weed control, the objectives of this research were to 1) determine an ideal dry (inert) diluent and a most effective mix ratio of pollen and diluent for larger scale application and to 2) identify the optimal combination of starting time, frequency, and number of sterile pollen applications to minimize seed production in Palmer amaranth. Plants were grown in the greenhouse in summer 2021. Sterilized pollen irradiated at 300 Gy was mixed with talc or wheat powder flour, respectively, at six v/v ratios (pollen%/powder%) of 0/100 (powder alone), 5/95, 10/90, 25/75, 50/50, or 100/0 (pure irradiated pollen). An equal amount of the pollen-diluent powder mixture was brushed onto standardized lengths of inflorescence of receptive female plants with three replications. Flower and seed number on each inflorescence was counted and used to calculate seed set in each treatment. The findings showed that the optimal formulation was a 25% mixture of irradiated pollen and talc powder, successfully reducing seed set in *A. palmeri* while efficiently utilizing the limited resource of irradiated pollen. Subsequently, during the fall of 2021, we conducted

another round of greenhouse experiments in which female plants were pollinated with a 25% mixture of irradiated pollen and talc powder by using a powder duster. Pollination treatments were initiated at different times after anthesis (7, 14, and 21 days). The pollination treatments included several combinations of the number of applications (once, twice, and three times) and interval between applications (one week, two weeks, or three weeks). Each combination of treatments was replicated three times. After seed maturation, plants were crushed and sieved to collect seeds. In this experiment, we identified that the optimal application strategy for minimizing seed production was initiating the application of irradiated pollen 7 days after anthesis and repeating the artificial pollination three times at 7-day intervals.

A scientific unknown is whether there exists a trade-off between inflorescence growth and fertilization rate. This question arises by the possibility that when fertilization rates are high, the allocation of resources to seed production may be resource-intensive, potentially diverting resources that could be used for inflorescence development. If such a trade-off exists, massive pollination with sterile pollen may have the additional benefit of reducing inflorescence growth. This, in turn, might lead to a female-biased offspring population, as anticipated by certation theory. In our research, where we conducted massive pollinations using irradiated and non-irradiated pollen separately, we observed that the assumed trade-off was not statistically significant in either treatment when compared to open pollination. However, this approach did result in a slightly female-biased progeny population.

Keywords: Palmer amaranth, *Amaranthus palmeri*, sterile pollen, effectiveness, diluent, application frequency, massive pollination, inflorescence outgrowth, certation, sex ratio

Introduction

Weeds can cause considerable damage to agricultural production (Oerke 2006). While herbicides and tillage have been the primary tools used in controlling weeds in modern agricultural systems, over-reliance on these tactics has resulted in negative impacts on both the environment and crop productivity (MacLaren et al. 2020). The breeding system of a weed species plays a critical role in its ability to invade and establish in new environments (Rambuda and Johnson 2004). However, research on reducing seed production in weedy species through exploitation of breeding system limitations has received little attention. Palmer amaranth (*Amaranthus palmeri* S. Wats.), a dioecious weed species with male and female reproductive organs on separate plants, is a highly aggressive and invasive weed species. It is ranked as the worst weed in US corn fields in a survey conducted by the Weed Science Society of America and is now a serious threat to agricultural production systems in over 40 countries (Van Wychen 2020; Webster and Nichols 2012). Dioecy enforces outcrossing, which minimizes inbreeding depression and increases genetic variation within populations through pollen dispersal by male plants (Thomson and Barrett 1981; Charlesworth et al. 1999). Studies have shown that the long-distance dispersal of pollen in *Amaranthus palmeri* has facilitated the transfer of herbicide resistance genes to susceptible female plants, resulting in offspring with acquired herbicide resistance (Oliveira et al. 2018; Sosnoskie et al. 2012). However, successful fertilization in dioecious species relies on proximity and synchronization of male and female flowers. These limitations present an opportunity for the development of novel management strategies for *A. palmeri.*

One possible strategy for controlling dioecious weed species is to induce pollen sterility, whereby a plant becomes incapable of producing viable seeds. At certain doses, irradiated pollen retains physiological viability and can germinate on the stigma to produce a pollen tube but is genetically inactive and cannot fertilize the egg cell to form seeds (Musial and Przywara 1998). The effectiveness of the sterile pollen technique in managing Palmer amaranth has been

shown experimentally, as results showed 300 Gy is the most effective irradiation dose to balance induced sterility and mating competitiveness (Chapter 2). This dosage significantly reduced seed set by at least 50% when compared to open pollination. This method is similar to the sterile insect technique, which utilizes eco-friendly and species-specific methods to decrease mosquito populations by releasing a large number of sterile male mosquitoes into the environment to mate with females (Parker and Mehta 2007).

This study aimed to explore how to increase the effectiveness of sterile pollen application to reduce seed production in Palmer amaranth. Under field conditions, it can be challenging to uniformly apply small volumes of pollen to stigmas. Dry particulates used as pollen diluents can improve the flow and uniformity of pollen distribution (Desai et al. 1997). Thus, the first objective was to determine an ideal dry (inert) diluent at the most effective mixed ratio for large scale application. Furthermore, due to the indeterminate nature of Palmer amaranth inflorescences (Tranel et al., 2002), flowers are likely to be at various ages when irradiated and sterile pollen is applied (within-individual variation). In addition, not all plants will flower simultaneously, meaning that a proportion of the population will not be exposed to sterile pollen with a single pollination event (between-individual variation). These within-individual and between-individual variations in flowering pattern make it challenging to maximize the efficiency of this technique. Therefore, our second objective was to identify the optimal combination of starting time, frequency, and number of sterile pollen applications to minimize seed production of Palmer amaranth. The aim is to apply the sterile pollen technique with the ideal timing, frequency, and number of applications to result in the minimum possible seed production.

To better understand the ecological implications of sterile pollen technique, we further investigated the effects of massive sterile pollen application. Flower production and seed output are influenced by the development of the inflorescence (Kirchoff et al. 2017). Maximal

reproductive success depends on the timing of flowering and on balancing the number of seeds produced with resources allocated to individual seeds (Benlloch et al. 2007). The production of large inflorescences itself can be costly as it may deplete resources that could otherwise be allocated to other plant organs (Suetsugu et al. 2015). A scientific unknown is whether there exists a trade-off between inflorescence growth and fertilization rate. If a trade-off exists, massive pollination with sterile pollen may have the additional benefit of reducing inflorescence growth. Consequently, our next objective was to investigate the effect of massive pollination on inflorescence growth and seed output. We hypothesized that inflorescence growth and total seed output should be reduced by artificial massive pollination (sterile pollen) in Palmer amaranth. Massive pollination may also influence sex ratio due to certation, a prezygotic mechanism of sex determination hypothesized to originate from the competition between a female-determining gamete and a male-determining gamete (Correns 1928). As a result, when a heavy load of pollen is dusted on female flowers, the female-determining gamete would rapidly reach and sire more than half the ovules and leave a smaller proportion of ovules available to male-determining gametes as was found in *Rumex* species (Conn et al. 1981). We hypothesized that massive pollination would change the sex ratio in the progeny population resulting in a female-biased progeny as predicted by certation theory.

Materials and Methods

Section 1 - The optimal formulation of the sterile pollen technique

Seeds (10-15) of *Amaranthus palmeri* were planted into 3-L pots filled with UC Davis potting medium containing a ratio of 1 sand:1 redwood sawdust:1 peat in a greenhouse set at 24/32 C night/day temperature regime and extended photoperiod (13-14 hours of lighting). Fertilizers were applied as 80 ml of a general-purpose fertilizer solution (Jack's Professional General Purpose 20–20–20, Allentown, PA) weekly at 350 ppm N starting from 2-true leaves

with drip irrigation for two minutes and twice per day. Seedlings were thinned to one plant per pot (300 pots in total). Once plants reached the flowering stage, 100 male and 100 female plants were grown in the same greenhouses to simulate mixed population conditions in the field.

Pollen was collected by gently tapping or shaking the male inflorescence, causing the pollen grains to be released onto aluminum foil placed beneath the inflorescence. The collected pollen was then sieved through a 250-mm mesh opening to remove large floral materials and stored in polyvinyl containers at 95 to 100% relative humidity until needed. To sterilize the pollen, fresh and mature pollen was placed in Petri dishes with parafilm and irradiated with gamma-rays from Cesium-137 at the UC Davis Center for Health & the Environment (https://che.ucdavis.edu). The irradiation was delivered at a dosage of 300 Grey (Gy), which was determined to be the most effective irradiation dosage in previous tests (Chapter 2).

Two types of diluent powders, wheat flour and talc powder, were evaluated for their effectiveness in diluting the sterilized pollen. Previous work has shown that these compounds do not change the biological properties of pollen and can be mixed and applied with pollen uniformly (Wetzstein and Law 1999; Vaknin et al. 1999). The sterilized pollen was mixed with each powder at six v/v ratios (pollen%/powder%) of 0/100 (powder alone), 5/95, 10/90, 25/75, 50/50, or 100/0 (pure irradiated pollen). The pollen-powder mixture was then uniformly applied to the inflorescence, each about 18 cm long, of receptive female plants using the same amount of pollen-diluent mixture. Each treatment had three replications. For each replicate, five 1 cm sections of treated inflorescences were dissected and analyzed to determine the number of flowers, normal full seeds and abnormal seeds (undeveloped ovules or empty seeds). Seed set was calculated by using the number of viable seeds divided by the number of flowers and expressed as percentage. The optimal pollen-powder mixture was determined based on the formulation that resulted in the lowest seed set.

Seed set data from the optimal diluent at the most effective mixed ratio experiment were subjected to dose-response analysis using the DRC package (Ritz et al., 2015) in R software (R Core Team 2019). Because the maximum pollen share in a mixture (%) is 100, a four-parametric log-logistic function (Equation 1; (Streibig et al. 1993) best describes seed set in relation to the pollen share in mixture (%) of talc and wheat powder :

$$Y = l + \frac{u-l}{1+\exp[b(log(x)-\log(e))]} \quad (1)$$

where Y is seed set, e is the effective pollen share in a mixture producing a seed set halfway between the u and l parameters, u is the upper limit, l is the lower limit of the curve and b is the relative slope around the inflection point (e). Data from the two different powders were pooled because there was no difference between the full model and the reduced (pooled) model.

Section 2 - The optimal application of the sterile pollen technique

The optimal sterile pollen-powder mixture identified in section one was used for this subsequent experiment. Plant material and pollen collection remained consistent with the methods previously described. Based on preliminary studies, it was estimated that 1 ml of pollen contains approximately 5,000,000 individual grains. Furthermore, our observations indicate that one centimeter of inflorescence contains approximately 100 flowers on average. Assuming that approximately 20% of the pollen is expected to be distributed onto female plants, the volume of pollen needed for each experiment was calculated as follows:

Volume of pollen (ml) = $\frac{\text{length of inflorescence (cm) x 100}}{5 \text{ x } 10^6 \text{ x } 20\%}$.

Female plants were pollinated with sterile pollen and powder mixture at different starting times after anthesis (when we can visually identify the sex of plants), which includes 7 days, 14 days, and 21 days. The number of applications was once, twice or three times. Application time intervals were one week, two weeks and three weeks. Open pollination was used as control. Each combination of treatments (See Figure 1) had three replicates. Pollination was done with a powder duster. The duster was squeezed to release the powder and then the

nozzle applicator guided the powder to targeted female flowers. After seeds matured, plants were crushed and sieved to collect seeds. The total amount of seeds was weighed; five groups of 100 seeds were weighed and averaged to calculate the total number of seeds produced.

Since the optimal application of the sterile pollen technique experiment is not a full-factorial design, three factors: application starting time, the number of applications and application intervals were combined into a single factor and a one-way ANOVA was performed by using aov() functions in R (R Core Team 2019). Number of seeds estimated per plant from the treatments was compared to the seed production from open pollination using ANOVA with Dunnett's test.

Section 3 - The effect of massive pollination on inflorescence growth and certation theory

In this experiment, we employed the optimal application scheme identified in Section 2 but with massive pollen. Plant material and pollen collection are the same as described previously. To achieve a massive pollination effect, we utilized a pollen quantity that was one hundred times greater than the standard amount:

$$\text{Volume of massive pollen (ml)} = \frac{\text{length of inflorescence (cm) x 100}}{5 \text{ x } 10^6 \text{ x } 20\%} * 100.$$

After anthesis, male and female plants were randomly separated into three different greenhouses for specific treatments: open pollination (control), pure massive irradiated pollen and pure massive non-irradiated pollen. Each treatment was replicated 5 times and applied using brushes. Greenhouse 1 served as the control with five females and five males (open pollination). Greenhouse 2 had five females and five males with females receiving massive sterile pollen based on the optimal application scheme identified in Section 2. Similarly, greenhouse 3 had five females and five males, with females receiving massive fertile pollen following the same optimal application scheme. These two greenhouses had both female and male plants to simulate the condition in the field. An additional 50 male plants were kept outdoors for pollen collection.

Once seeds reached maturity, we assessed various aspects of inflorescence outgrowth, including plant height, the number of branches per plant, length of the main inflorescence, total length of all inflorescences, and dry weight. Following these measurements, the plants were crushed and sieved to collect the seeds. The total seed weight was recorded, and we calculated the total seed production by weighing and averaging five groups of 100 seeds. A t-test was used to compare the differences in inflorescence growth and seed production for treatments using massive, irradiated pollen or non-irradiated pollen against the results from open pollination. This analysis was conducted using the R software.

Lastly, seeds from the massive pollination experiment were used for a certation experiment. Two hundred seeds were randomly selected from female plants within the same treatment and were then planted in the greenhouse under the previously described conditions. Plant sex was recorded after anthesis. Finally, the sex ratio observed in both the irradiated massive pollination group and the non-irradiated massive pollination group was subjected to a statistical analysis for comparison against the sex ratio found in the open pollination group using a chi-square test.

Results and Discussion

Section 1 - Optimal formulation of the sterile pollen technique

Two powder types were effective in reducing the seed set in *Amaranthus palmeri* as shown by our dose-response analysis (Figure 2). Data from the two tested powder types were pooled because there was no difference between the full model (with the powder type as a covariate) and the reduced model (without the powder type as a covariate), as shown in Appendix 1. This suggests four parameters (e, l, u, b) can be fixed across curves of talc powder and wheat powder (flour) without significantly reducing the goodness of fit. Under the competition with naturally-occurring pollen, seed set was 50.73% and 28.03% when pure

powder and pure irradiated pollen were applied, respectively, as indicated by the values of parameters u and l (Table 1). The seed set in both treatments was lower than the seed set from open pollination (64.15% ± 4.52%). The effective pollen share in the mixture was 4.81 (Table 1), producing a seed set halfway between the lower limit and upper limit. The ED_{50}, the ratio reducing seed set by 50%, was not estimable because the lower limit of the model was greater than half the maximum response (i.e. 50.73% × 0.5 = 25.365%) (Keshtkar et al. 2021). To minimize seed set and conserve irradiated pollen, a mixture ratio of 25%:75% is recommended; this is the smallest ratio that yielded seed set close to the lower limit (28.03%) while minimizing the amount of irradiated pollen required (Figure 1). This ratio can effectively reduce seed set in *A. palmeri* while conserving the limited resources of irradiated pollen.

The lower seed set observed with pure powder application compared to open pollination can be attributed to the physical barrier created by the powder, which covers the stigma and prevents non-irradiated pollen from fertilizing the ovule and producing seeds (Vaknin et al 1999). With an increase in the proportion of irradiated pollen in the mixture, there is a decrease in the seed set. This is due to the fact that while the irradiated pollen can germinate on the stigma and produce a pollen tube, it is incapable of fertilizing the egg cell, thereby failing to produce any seeds (Musial and Przywara 1998).

When selecting an optimal diluent for pollen application, it is crucial to take into account factors such as non-toxicity and preventing any disruption to pollen-stigma interactions. Artificial supplementary application of pollen using non-toxic diluents like wheat flour and talc powder, has shown positive results in various plants. For instance, in raspberry (*Rubus idaeus* L.), talc-diluted pollen is employed to enhance fruit production (Jennings and Topham 1971). Similarly, in *Cannabis sativa*, the use of cryopreserved pollen mixed with wheat flour yields seeds of comparable number, size, and morphology to those produced with untreated fresh pollen (Gaudet et al 2020). However, when wheat flour is combined with

irradiated pollen of Palmer amaranth, it tends to clump and degrade pollen flow more than talc powder. This is due to wheat flour's higher water absorption capacity ($\frac{\text{Weight of water absorbed (gram)}}{\text{Initial powder weight (gram)}}$), which is approximately 80%, compared to the 1.5% for talc powder (Vaknin et al. 1999; Yi et al. 2003). Additionally, the size of the diluent particles should be similar to that of the pollen to achieve consistent mixing and uniform application. Talc powder has a median diameter of 26.57 µm whereas milling produces wheat flour particles with a median diameter of about 120 µm (Gilbert et al 2018; Liu et al 2016). The diameter of Palmer amaranth pollen is approximately 31 µm (Wu et al. 2023). Therefore, talc powder is a more suitable diluent than wheat flour.

Section 2 - Optimal application of the sterile pollen technique

The combined effect of sterile pollen application starting time, application frequency and application interval had a statistically significant impact on seed production (Figure 3, Appendix 2). Initiating application 7 days after anthesis consistently resulted in the lowest calculated seed production per plant, reduced by about 50% relative to the open pollination ($P<0.001$) as shown in Figure 3. Estimated seed production from single applications of sterile pollen at 14 or 21 days after anthesis did not show a significant difference compared to open pollination (Figure 3). Although not statistically significant, a trend was observed where increasing the number of applications or decreasing the interval between them tended to reduce seed production per plant. Based on these findings, the optimal application strategy for the sterile pollen technique is to begin at 7 days after anthesis and apply three times at 7-day intervals.

Due to the indeterminate nature of Palmer amaranth inflorescences (Tranel et al. 2002), flowers varied in age at the time we applied irradiated and sterile pollen, resulting in within-individual variation. Additionally, not all plants within the population flower simultaneously, leading to between-individual variation where a portion of the population may not be exposed

to sterile pollen. Those flowering variations among female flowers present challenges in terms of the timing for the application of the sterile pollen technique. Furthermore, the initiation of flowering occurred earlier in males than females under both water stress and control conditions (Mesgaran et al. 2021). When applying a pollen-powder mixture to female flowers, our aim is to cover as many stigmas as possible while minimizing the influence of naturally occurring pollen on seed development, considering the earlier flowering of males compared to females. Through our experiments, the optimal application strategy we identified above allows us to achieve minimal seed production while using a reduced amount of irradiated pollen.

The time of flower opening in Palmer amaranth marks the onset of a period in which pollen will be released from male flowers and when pollination, fertilization, and seed production occur in female flowers. Therefore, the timing of flower opening in females plays a significant role in determining the appropriate initiation and interval for application of the sterile pollen technique. In Palmer amaranth, flowers on the same plant have a continuous opening sequence, with varying opening times among the flowers. Flower opening behavior in Palmer amaranth can be influenced by factors such as the time of day and the position of the flower within the inflorescence (van Doorn and Van Meeteren 2003). Based on our observations, it appears that flowers situated in the middle lower part of the inflorescence tend to open first. In many species, including Palmer amaranth, flower opening occurs in the morning, correlated with an increase in temperature and light intensity, and with a decrease in ambient humidity (Mondo et al 2022).

The majority (62 to 73%) of plant species, as indicated by studies utilizing the GloPL Dataset (Lebot et al. 2019), the Konstanz Breeding System Dataset (Agre et al. 2020), and the Stellenbosch Breeding System Dataset (Mondo et al. 2021), demonstrate pollination frequency plays a critical role in determining seed production (Ashman et al 2004; Bennett et al 2020). The reduced seed-set rate in early-flowering plants was associated with pollen limitation, as

observed in species like *Peucedanum multivittatum* and *Rhododendron aureum* (Kudo, 1993; Kudo and Hirao, 2006). While the failure of pollen tubes to enter ovules is a common cause of reduced seed production, it is not the sole factor contributing to low fertility, as post-fertilization ovule abortion has been observed to decrease fertility in alfalfa (Sayers and Murphy, 1966). Additionally, reports indicate that embryo abortion can also lead to reduced seed production in various plant species, including red clover (*Trifolium pratense*) and garden pea (*Pisum sativum*), as well as in plant families other than the Fabaceae (Sato 1956; Linck 1961; Brink and Cooper 1947).

Section 3 - The effect of massive pollination on inflorescence outgrowth and certation theory

Regarding massive pollination with non-irradiated and fertile pollen in Palmer amaranth, there were no statistically significant differences in plant height, branch number, inflorescence length, dry weight, seed weight, and seed production per plant compared to open pollination (Appendix 3). This indicates our assumed tradeoff between inflorescence outgrowth and fertilization rate is not significant. These results suggest that when plants receive a substantial amount of sterile pollen, the presumed trade-off between inflorescence growth and fertilization rate is not significant either.

The activity and development of apical and lateral buds, as well as fruits, are controlled by light, temperature, hormone, carbohydrate, and nutrient signaling (Montgomery, 2008; Barbier et al., 2019). These signals enable communication between the shoot apex and lateral sinks (meristems or fruits), ensuring the plant's architecture and reproductive capacity align with available resources (Walker and Bennett, 2018; Barbier et al. 2019). In annual plants, the suppression of inflorescence growth due to fruit load typically occurs at the late stage of inflorescence development, referred to as the end of the flowering transition (Gonzalez-Suarez et al. 2020; Ware et al. 2020). For instance, in Arabidopsis (*Arabidopsis thaliana*), during this

phase, the inhibition of inflorescence shoot growth by fruit load is regulated by auxin and carbohydrate signaling (Goetz et al. 2021). Our findings suggest that the rate of fertilization has a minor effect on inflorescence outgrowth in Palmer amaranth, likely because the development of fruit in Palmer amaranth, which is a thin membranous structure known as an utricle, has relatively low costs.

Pollen limitation has two aspects: quality limitation and quantity limitation. Quality limitation refers to the reduced effectiveness of pollination due to the inferior quality of pollen. In Palmer amaranth, irradiated pollen under 300 Gy doses is genetically inactive and cannot fertilize the egg cell to form seeds (Wu and Mesgaran 2021). In addition, much literature on inbreeding depression has shown that pollen quality effects associated with both self-fertilization and mating between related plants (Herlihy and Eckert 2004) can also reduce seed production (Charlesworth and Charlesworth 1987). This reduction is likely because embryos homozygous for deleterious alleles die during development. On the other hand, traditional pollen limitation is typically associated with plants receiving an insufficient quantity of pollen grains to fertilize all their ovules (quantity limitation). Extensive reviews show that supplemental pollination often increases (Burd 1994, Ashman et al. 2004), and rarely decreases (Young and Young 1992), seed or fruit production.

Regarding sex ratio in offspring after massive pollination in Palmer amaranth, results from massive pollination with irradiated and non-irradiated consistently showed the sex ratio in the progeny population is female dominant as predicted by certation theory (Table 2). It is a prezygotic mechanism of sex determination hypothesized to originate from the competition between a female-determining gamete and a male-determining gamete (Correns, 1928). As a result, when a heavy load of pollen is dusted on female flowers, the female-determining gamete would rapidly reach and sire more than half the ovules and leave a small proportion of ovules available to male-determining gametes as was found in *Silene alba* (Taylor, 1996) and *Rumex*

species (Conn et al. 1981). Several other mechanisms have been proposed to account for female bias. In species with sex chromosomes where males are heterogametic, Y-chromosome degeneration may lead to female-biased populations (Smith 1963). This is due to sex viability differences and sex-chromosomal genotype performance during pollination and fertilization. Studies on *Rumex nivalis*, a species with heteromorphic sex chromosomes (XX in females, XY_1Y_2 in males), show that both certation and gender-based mortality contribute to female-biased sex ratios (Stehlik et al. 2007, 2008). Research using sex-specific markers across different life stages revealed that female bias starts in pollen and intensifies from seeds to flowering. Environmental factors, like proximity of females to males, affect these ratios (Stehlik et al. 2008); females nearer to males capture more pollen, resulting in more female-biased ratios. Experiments confirm that higher pollen loads intensify this bias (Stehlik and Barrett 2006), supporting Correns' certation hypothesis that larger pollen loads increase gametophytic competition, favoring fertilization by female-determining pollen tubes.

In conclusion, we investigated how to improve the efficiency of the sterile pollen technique for reducing seed production in *A. palmeri* under greenhouse conditions. The results demonstrated that talc powder is a more suitable diluent than wheat flour. The optimal formulation is to utilize a mixture of 25% irradiated pollen to 75% talc powder by volume, which enhances pollen distribution by improving flow and uniformity. Furthermore, the efficiency of the sterile pollen technique is affected by variations in the timing of female flower opening and interference from naturally-occurring pollen. These factors make it challenging to further enhance the technique's efficiency. However, through our investigations, we identified the optimal sterile pollen application strategy: initiating application 7 days after anthesis and repeating it three times at 7-day intervals. This strategy allows us to achieve reduction in seed production while also minimizing the amount of irradiated pollen required. Lastly, we found massive pollination of irradiated pollen or non-irradiated pollen did not have an effect on

inflorescence growth, but it did affect sex ratio in the progeny population, resulting in slightly female-biased progeny as predicted by certation theory.

Tables

Table 1. Estimated parameter values for the four-parameter (Equation 1) log-logistic models used to describe seed set in Palmer amaranth in response to increasing irradiated pollen share in mixture with talc powder and wheat powder in greenhouse conditions.

Parameter	Estimate	Std. Error	t-value	p-value[b]
b[a]	0.9434	1.5329	0.6155	0.5626
l[a]	28.0285	8.9542	3.1302	0.0037**
u[a]	50.7323	3.4407	14.7448	<0.0001***
e[a]	4.8075	3.8732	1.2412	0.2235

Residual standard error: 0.0844 (32 degrees of freedom).

[a]The parameters are fixed across different diluents. e is the effective pollen share in a mixture producing a seed set halfway between the u and l parameters, where u is the upper limit, l is the lower limit of the curve and b is the relative slope around the inflection point (e).

[b]Statistical significance is denoted by asterisks (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$).

Table 2. Observed counts of female and male Palmer amaranth plants in the progeny resulting from open pollination, non-irradiated massive pollination, and irradiated massive pollination. We conducted chi-square tests to compare each of the two treatments with open pollination separately.

	Female	Male	Treatment comparison	Chi-Square value	P-value[a]
Open pollination	94	106			
Non-irradiated massive pollination	117	83	Open vs non-irradiated	4.8547	0.0276*
Irradiated massive pollination	113	87	Open vs irradiated	3.244	0.0717

[a]Statistical significance is denoted by asterisks (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$).

Figures

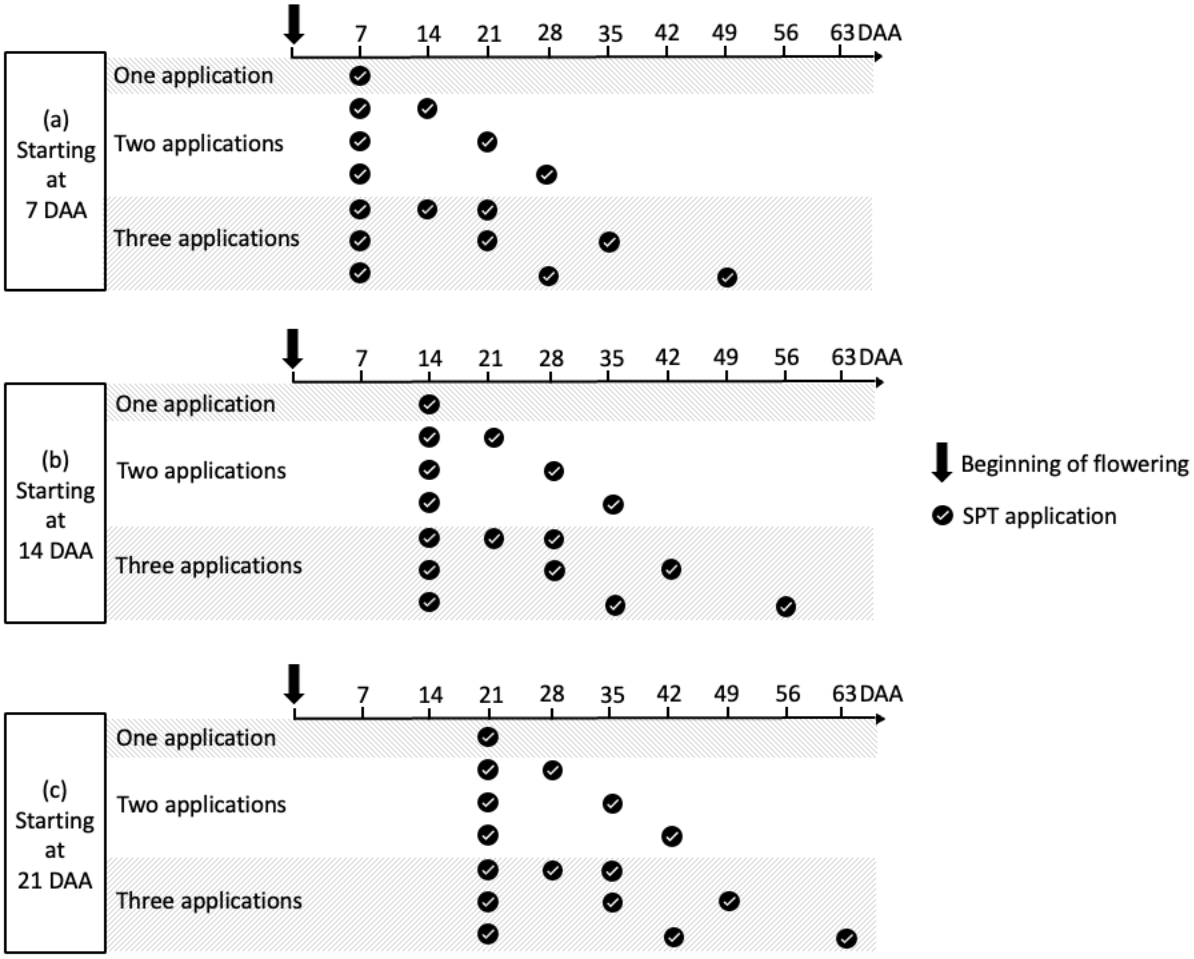
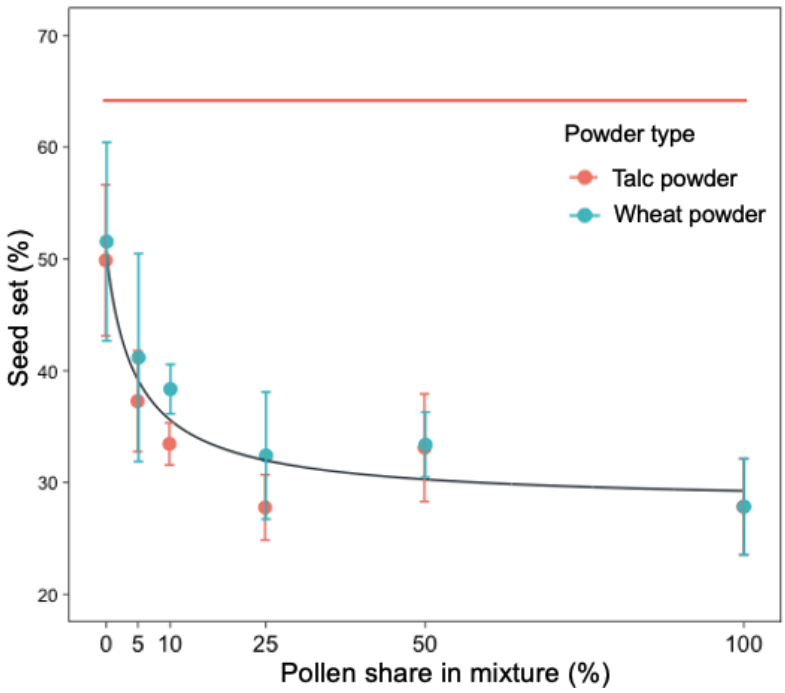


Figure 1. Application plan of sterile pollen technique (SPT). DAA = days after anthesis

(a) When the application starts at 7 DAA, it was applied once, twice at 7-day, 14-day, and 21-day intervals, and three times with 7-day, 14-day, and 21-day intervals.

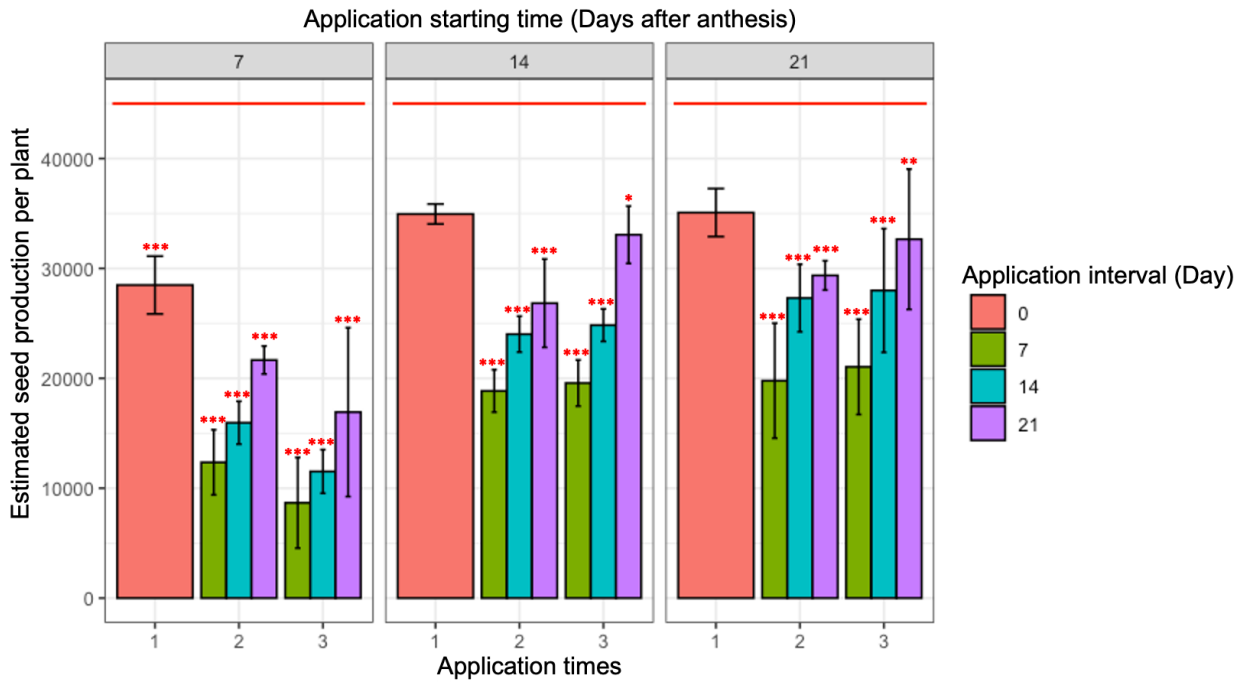
(b) When the application starts at 14 DAA, it was applied once, twice at 7-day, 14-day, and 21-day intervals, and three times with 7-day, 14-day, and 21-day intervals.

(c) When the application starts at 21 DAA, it was applied once, twice at 7-day, 14-day, and 21-day intervals, and three times with 7-day, 14-day, and 21-day intervals.



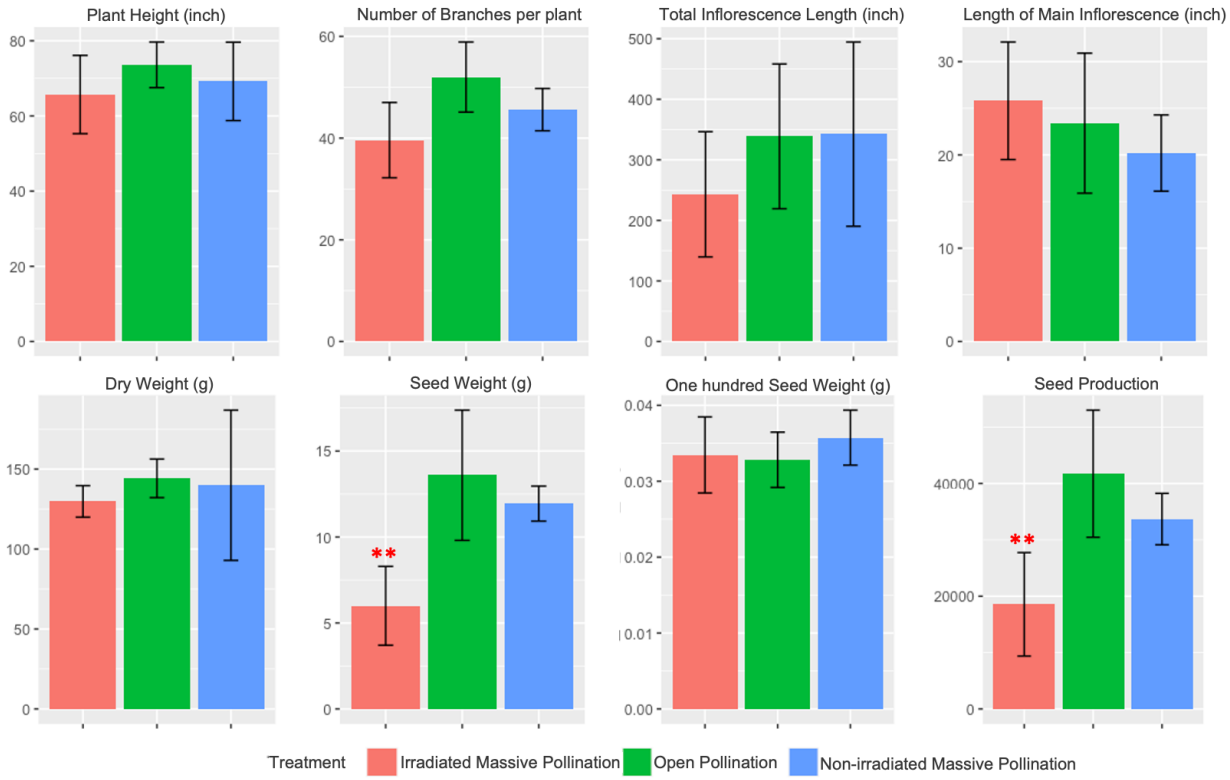
Note: To minimize seed set and conserve irradiated pollen, a pollen share in mixture of 25% should be used; this is the smallest ratio that yields a seed set close to the lower limit (28.03%) while minimizing the amount of irradiated pollen required.

Figure 2. Dose response curve showing seed set in Palmer amaranth at different pollen shares in mixture (%) with talc powder and wheat powder flour (Equation 1) under greenhouse conditions. Black line is fitted value of pooled data, and red flat line represents the average seed set for open pollination. Solid circles indicate observed seed set averaged with three replications each. Error bars indicate standard error. Model parameter estimates are shown in Table 1.



Note: Seed count per plant was significantly reduced in sterile pollen application compared to open pollination, with the exception of single applications made at either 14 or 21 days after anthesis.

Figure 3. Average seed production per plant in Palmer amaranth, measured across three replications for each combination of application starting time, interval, and times, in greenhouse condition. Error bars indicate standard errors. The red solid line represents the average seed production per plant for open pollination. Significance levels are denoted by asterisks (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$) based on Dunnett's test for comparing treatments with open pollination (control).



Note: Seed weight and seed count significantly decreased following massive pollination with irradiated and sterile pollen, in comparison to open pollination.

Figure 4. Average values for inflorescence growth measurements and seed production per plant of palmer amaranth, with five replications**.** Error bars represent standard errors. Significance levels are indicated by asterisks (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$), based on t-tests comparing each treatment with open pollination (control).

Appendix

Appendix 1. Comparison of full model (with powder type as a covariate) with reduced model (without powder type as a covariate) using anova() functions in R for dose response of seed set in Palmer amaranth at different pollen share in mixture (%) with talc powder and wheat powder under greenhouse conditions.

Model	Model-DF	RSS	DF	F-value	p-value
Full model	32	2277.6			
Reduced model	28	2183	4	0.3032	0.8733

Appendix 2. ANOVA table of the effect of combined factors of application starting time, interval, and times on seed production per plant in Palmer amaranth. The three factors, application starting time, the number of applications and application intervals were combined into a single factor and a one-way ANOVA was performed by using aov() functions in R.

Analysis of Variance				
Source	**DF**	**Mean Square**	**F Ratio**	**Pr (>F)**
Combined treatment	21	253856	17.249	<0.0001***
Residuals	44	14717		

Appendix 3. T-tests were performed to compare inflorescence outgrowth measurements and seed set per plant of Palmer amaranth, with five replications, between each treatment (irradiated massive pollination and non-irradiated massive pollination) and open pollination (control).

Comparison	Plant Features	T-Statistic	P-Value[a]
Irradiated massive pollination vs. Open pollination	Plant height	-1.46	0.189
	Total length of inflorescence (inch)	-0.26	0.801
	Length of main inflorescence (inch)	1.08	0.313
	Number of branches	-1.06	0.801
	Dry weight (g)	-2.07	0.073
	Seed weight (g)	-3.83	0.007**
	One hundred seed weight (g)	0.23	0.823
	Seed production	-3.56	0.007**
Non-irradiated massive pollination vs. Open pollination	Plant height	-0.81	0.443
	Total length of inflorescence (inch)	1.07	0.325
	Length of main inflorescence (inch)	-0.31	0.762
	Number of branches	-1.77	0.121
	Dry weight (g)	-0.20	0.850
	Seed weight (g)	-0.94	0.393
	One hundred seed weight (g)	1.27	0.239
	Seed production	-1.47	0.196

[a]Significance levels are indicated by asterisks (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$). The decrease in seed weight and seed production were statistically significant in massive pollination with irradiated and sterile pollen, compared to open pollination.

References

Agre P, Nwachukwu C, Olasanmi B, Obidiegwu J, Nwachukwu E, Adebola P, Koeyer DD, Asfaw A (2020) Sample preservation and plant sex prediction in white guinea yam (*Dioscorea rotundata Poir.*). J Appl Biotechnol Rep 7(3):145–151.

Ashman TL, Knight TM, Steets JA, Amarasekare P, Burd M, Campbell DR, Dudash MR, Johnston MO, Mazer SJ, Mitchell RJ, Morgan MT (2004) Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. Ecology. 85(9):2408-2421.

Barbier FF , Dun EA, Kerr SC, Chabikwa TG, Beveridge CA (2019) An update on the signals controlling shoot branching. Trends Plant Sci24: 220–236.

Benlloch R, Berbel A, Serrano-Mislata A, Madueño F. (2007) Floral initiation and inflorescence architecture: a comparative view. Ann Bot. 100(3):659-676.

Bennett JM, Steets JA, Burns JH, Burkle LA, Vamosi JC, Wolowski M, Arceo-Gómez G, Burd M, Durka W, Ellis AG, Freitas L (2020) Land use and pollinator dependency drives global patterns of pollen limitation in the Anthropocene. Nat Commun 11(1):3999.

Brink RA, Cooper DC (1947) The endosperm in seed development. Bot Rev 13: 423–477.

Burd, M. 1995. Ovule packaging in stochastic pollination and fertilization environments. Evolution 49: 100–109.

Charlesworth D Geber MA, Dawson TE, Delph LF (1999) Theories of the evolution of dioecy. Pages 33-60 *in* Gender and sexual dimorphism in flowering plants. Berlin: Springer.

Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. Annual Review of Ecology and Systematics 18:237–268.

Conn JS and Blum U (1981) Sex ratio of *Rumex hastatulus*: the effect of environmental factors and certation. Evolution 35:1108–1116.

Correns C (1928) Bestimmung, Vererbung und Verteilung des Geschlechtes bei den höheren Pflanzen ("Inheritance and distribution of sex in higher plants"). Bornträger, Berlin, Germany.

Desai BB, Kotecha PM, Salunkhe DK (1997) Seeds handbook. Dekker, New York, Pp 361–362

Gaudet D, Yadav NS, Sorokin A, Bilichak A, Kovalchuk I (2020) Development and optimization of a germination assay and long-term storage for *Cannabis sativa* pollen. Plants 9(5):665.

Gilbert CR, Furman BR, Feller-Kopman DJ, Haouzi P (2018) Description of particle size, distribution, and behavior of talc preparations commercially available within the United States. Journal of Bronchology & Interventional Pulmonology 25(1):25-30.

Goetz M, Rabinovich M, Smith H (2021). The role of auxin and sugar signaling in dominance inhibition of inflorescence growth by fruit load. Plant Physiol. 187, 1189–1201

Gonzalez-Suarez P , Walker CH, Bennett T (2020) Bloom and bust: understanding the nature and regulation of the end of flowering. Curr Opin Plant Biol57: 24–30

Herlihy CR, Eckert CG. (2004) Experimental dissection of inbreeding and its adaptive significance in a flowering plant, *Aquilegia canadensis* (Ranunculaceae). Evolution 58:2693–2703.

Jennings DL, Topham PB (1971) Some consequences of raspberry pollen dilution for its germination and for fruit development. New Phytol 70:371–380

Keshtkar E, Kudsk P, Mesgaran MB (2021) Perspective: common errors in dose–response analysis and how to avoid them. Pest Manag Sci 77:2599–2608

Kirchoff BK, Claßen-Bockhoff R. Inflorescences: concepts, function, development and evolution. Ann Bot. 2013;112(8):1471-1476.

Kudo G (1993) Relationship between flowering time and fruit set of the entomophilous alpine shrub, *Rhododendron aureum* (Ericaceae), inhabiting snow patches, Am J Bot 80: 1300-1304.

Kudo G, Hirao AS (2006) Habitat-specific responses in the flowering phenology and seed set of alpine plants to climate variation: implications for global-change impacts, Popul Ecol 48: 49-58.

Lebot V, Abraham K, Kaoh J, Rogers C, Molisalé T (2019) Development of anthracnose resistant hybrids of the Greater Yam (*Dioscorea alata L.*) and interspecific hybrids with *D. nummularia* Lam. Genet Resour Crop Evol 66: 871–883.

Linck AJ (1961) The morphological development of the fruit of *Pisum sativum*, var. Alaska. Phytomorphology 11(20):79.

Liu T, Hou GG, Lee B, Marquart L, Dubat A (2016) Effects of particle size on the quality attributes of reconstituted whole-wheat flour and tortillas made from it. J Cereal Sci 71:145-152.

MacLaren C, Storkey J, Menegat A, Metcalfe H, Dehnen-Schmutz K (2020) An ecological future for weed science to sustain crop production and the environment. A review. Agron Sustainable Dev 40(4): 1–29.

Mesgaran MB, Matzrafi M, Ohadi S (2021) Sex dimorphism in dioecious palmer amaranth (*Amaranthus palmeri*) in response to water stress. Planta 254(1): 17.

Mondo JM, Agre PA, Asiedu R, Akoroda MO, Asfaw A (2021) Genome-wide association studies for sex determination and cross-compatibility in water yam (*Dioscorea alata* L.). Plants 10(7):1412.

Mondo JM, Agre PA, Asiedu R, Akoroda MO, Asfaw A (2022) Optimum time for hand pollination in yam *(Dioscorea* spp.). PloS one 17(8):e0269670.

Montgomery BL (2008) Right place, right time: spatiotemporal light regulation of plant growth and development. Plant Signal Behav3: 1053–1060

Musial K. and Przywara L (1998) Influence of irradiated pollen on embryo and endosperm development in kiwifruit. Ann. Bot. 82: 747–756.

Oerke E (2006) Crop losses to pests. J Agric Sci 144:31- 43.

Oliveira MC, Gaines TA, Patterson EL, Jhala AJ, Irmak S, Amundsen K, Knezevic SZ (2018) Interspecific and intraspecific transference of metabolism-based mesotrione resistance in dioecious weedy *Amaranthus.* Plant J 96(5):1051–1063.

Parker A, Mehta K (2007) Sterile insect technique: A model for dose optimization for improved sterile insect quality. Florida Entomologist 90(1):88–95.

R Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Rambuda TD, Johnson SD (2004) Breeding systems of invasive alien plants in South Africa: does Baker's rule apply? Divers and Distrib 10: 409-416.

Ritz C, Baty F, Streibig JC, Gerhard D (2015) Dose-response analysis using R. PLoS One 10:e0146021

Sato H (1956) Morphological and physiological studies on occurrence of abortive seeds in leguminous forage crops: II. Formation of one-seeded pod in red clover. Jpn J Crop Sci 24(3):224-226.

Sayers ER, Murphy RP (1966) Seed set in alfalfa as related to pollen tube growth, fertilization frequency, and post-fertilization ovule abortion 1. Crop Sci 6(4):365-368.

Smith BW. 1963 The mechanism of sex determination in *Rumex hastatulus*. Genetics 48:1265–1288.

Sosnoskie LM, Webster TM, Kichler JM, MacRae AW, Grey TL, Culpepper AS (2012) Pollen-mediated dispersal of glyphosate- resistance in Palmer amaranth under field conditions. Weed Sci 60(3):366–373.

Stehlik I., Barrett SCH. 2006 Pollination intensity influences sex ratios in dioecious *Rumex nivalis*, a wind-pollinated plant. Evolution 60: 1207–1214.

Stehlik I., Friedman J. Barrett SCH. 2008 Environmental influence on primary sex ratio in a dioecious plant. Proc. Natl Acad. Sci. USA 105,10: 847–10 852.

Stehlik I., Kron P., Barrett SCH., Husband BC. 2007. Sexing pollen reveals female bias in a dioecious plant. New Phytol.175:185–194

Streibig JC, Rudemo M, Jensen JE (1993) Dose-response curves and statistical models. Pages 29–55 in: Streibig, JC, Kudsk, P, eds. Herbicide Bioassays. Boca Raton, FL: CRC Press

Suetsugu K, Naito RS, Fukushima S. (2015) Pollination system and the effect of inflorescence size on fruit set in the deceptive orchid *Cephalanthera falcata*. J Plant Res 128, 585–594.

Taylor, D. 1996. Parental expenditure and offspring sex ratios in the dioecious plant *Silene alba* (= *Silene latifolia*). The American Naturalist, 147(5), 870-879.

Thomson JD, Barrett SC (1981) Selection for outcrossing, sexual selection, and the evolution of dioecy in plants. Am Nat 118:443–449.

Tranel P, Wasson J, Jeschke M, Rayburn A (2002) Transmission of herbicide resistance from a monoecious to dioecious weedy amaranth species. Theor Appl Genet 105:674 – 679.

Vaknin Y, Gan-Mor S, Bechar A, Ronen B, Eisikowitch D (1999) Effects of desiccation and dilution on germinability of almond pollen. J Hortic Sci Biotechnol 74(3):321-327.

van Doorn WG, Van Meeteren U (2003) Flower opening and closure: a review. J Exp Bot 54(389):1801-1812.

Van Wychen, LR (2017) Survey of the most common and troublesome weeds in grass crops, pasture and turf in the United States and Canada. Weed Science Society of America National Weed Survey Dataset. https://wssa.net/wp-content/uploads/WSSA-Weed-Survey-2017-05-19.pdf. Accessed: January 01, 2023

Van Wychen L (2020) 2020 Survey of the most common and troublesome weeds in grass crops, pasture, and turf in the United States and Canada. Weed Science Society of America National Weed Survey Dataset. https://wssa.net/wpcontent/uploads/2020-Weed-Survey_grass-crops.xlsx. Accessed: February 20, 2021

Walker CH ,Bennett T (2018) Forbidden fruit: dominance relationships and the control of shoot architecture. Annual Plant Reviews1: 217–254

Ware A ,Walker CH, Simura J, Gonzalez-Suarez P, Ljung K, Bishopp A, Wilson ZA, Bennett T (2020) Auxin export from proximal fruits drives arrest in temporally competent inflorescences. Nat Plants6: 699–707

Webster TM, Nichols RL (2012) Changes in the prevalence of weed species in the major agronomic crops of the Southern United States: 1994/1995 to 2008/2009. Weed Sci 60:145–157.

Wetzstein HY, Law SE (1999). An evaluation of dry particulates as pollen diluents for supplemental mass pollination. HortSci, 34(3), 529E-530.

Wu W, Jernstedt J, and Mesgaran MB (2023) Comparative floral development in male and female plants of Palmer amaranth (*Amaranthus palmeri*). American Journal of Botany 110(8): e16212.

Wu W, Mesgaran M (2021) Sterile Pollen Technique as a Novel Weed Management Tool. [Online poster]. [Accessed 17 April 2023]. Available from: https://ucanr.edu/sites/lassen/files/343051.pdf

Yi W, Law ES, Wetzstein HY (2003) Polyester and nylon powders used as pollen diluents preserve pollen germination and tube growth in controlled pollinations. Sexual Plant Reproduction 15:265-269.

Young HJ, Young TP (1992) Alternative outcomes of natural and experimental high pollen loads. Ecology 73:639-647.

Concluding Remarks

Floral development of *Amaranthus palmeri* S. Wats was categorized into ten distinct stages. The first morphological feature distinguishing staminate and pistillate flower development is the initiation of stamen primordia (stage 4) found only in staminate flowers. The evolution of *Amaranthus palmeri* from a cosexual ancestor to complete dioecy is still in progress since males exhibited transient hermaphroditism while females produced strictly pistillate flowers. The detailed study of this species' reproductive biology is crucial for guiding the development of novel weed management especially strategies aimed at the flowering stage to disrupt seed production.

The possibility of using sterile irradiated pollen as means of disrupting seed production in *Amaranthus palmeri* in a similar way to the Insect Sterile Technique (IST) was tested. Results demonstrated that an irradiation dose of 300 Gy seems to be the most effective in reducing seed set in *A. palmeri*. Furthermore, we observed that the greatest reduction in seed set was achieved when irradiated pollen was introduced to the stigma through artificial pollination prior to open pollination. It appears that irradiated pollen exerts a preventive effect on naturally occurring pollen that arrives later.

For large scale application of SPT, this research suggests that the optimal formulation is a 25%:75% mixture of irradiated pollen and talc powder, which not only reduces seed set in *A. palmeri* but also enhances pollen distribution by improving flow and uniformity. Furthermore, the efficiency of the sterile pollen technique is affected by variations in the timing of female flower opening and interference from naturally-occurring pollen. These factors make it challenging to further enhance the technique's efficiency. However, through our investigations, we identified the optimal sterile pollen application strategy: initiating application 7 days after anthesis and repeating it three times at 7-day intervals. This strategy

allowed us to achieve minimal seed production while also minimizing the amount of irradiated pollen required.

A scientific unknown is whether there exists a trade-off between inflorescence growth and fertilization rate. This question arises because of the possibility that when fertilization rates are high, allocation of resources to seed production may be resource-intensive, potentially diverting resources that could be used for inflorescence development. We found massive pollination of irradiated pollen or non-irradiated pollen did not have an effect on inflorescence growth, but it did affect the sex ratio in the progeny population, resulting in female-biased progeny as predicted by certation theory.

All experiments in this research were conducted in the greenhouse, but situations in the field are more complicated. Weed growth and distribution would need to be monitored more frequently and optimal patterns for monitoring will need to be determined. Adapting this approach for field use will likely require a drone-mounted dispenser for the pollen-diluent mixture. Future studies will be needed to explore how different flight parameters affect the dispersion efficacy of this mixture. Integrating these techniques will make sterile pollen technique more practical and feasible for field use.