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Exploring sterile pollen technique as a novel tool for management of Palmer amaranth (*Amaranthus palmeri*)

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Research Article

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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 with open pollination when irradiated pollen had prior access to the stigma through artificial pollination before open pollination. In 2021, to simulate real field conditions, three additional treatments were introduced into 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.

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, while herbicide resistance is increasing (MacLaren et al. 2020). An integrated approach to weed management that 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 that 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 (Alsamir et al. 2021; Zhang and Lespinasse 1991). 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

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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 (*Malus pumila* Mill.) (Zhang and Lespinasse 1991), pear (*Pyrus communis* L.) (Bouvier et al. 1993), citrus (*Citrus* L.) (Froelicher et al. 2007), cacao (*Theobroma cacao* L.) (Falque et al. 1992), and melon (*Cucumis melo* L.) (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 pests. 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 easily 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 United States. It was ranked as the worst weed in U.S. cornfields (*Zea mays* L.) in a survey by the Weed Science Society of America (Van Wychen et al. 2017). Furthermore, it has evolved resistance to nine herbicide classes (Heap 2022) and is able to produce up to 1 million seeds per plant (Ward et al. 2013). This weed is a particularly suitable candidate for exploration of the SPT for weed control. Being a dioecious species with separate male and female plants, it relies heavily 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 the 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 its preventative effects on fertile pollen, while low doses may allow the treated pollen to maintain its fertility. Accordingly, a broad range of irradiation doses were 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 to 15) of *A. palmeri* collected from Kansas were planted in May 2020 into 3-L pots filled with UC Davis potting medium containing (sand:redwood sawdust:peat, 1:1:1) in a greenhouse set at a 24/32 night/day temperature regime and extended photoperiod (14 h 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 2 min twice per day. Seedlings were periodically randomly thinned to 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 material. 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 Gy (Godbole and Murthy 2012; Košmrlj et al. 2013) at the UC Davis Center for Health and the Environment (https://che.ucdavis.edu). Irradiated and untreated pollen was immediately used for pollen viability tests and hand-pollination experiments as described in the following sections.

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 nonviable pollen did not stain at all (see Figure 1A). Viability of 100 pollen grains for 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 v. 1.46r (Schneider et al. 2012). Gray values were used to indicate the brightness of a pixel. Because the range for gray values is 0 to 255, gray value percentages (%) were calculated by dividing the recorded gray values by 255 and multiplying by 100. Higher gray value percentages indicated lower pollen viability. The effects of irradiation dosage on gray value percentages were analyzed using ANOVA with a 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 enclosed in a paper bag immediately with the exception of inflorescences receiving treatment 6 (i.e., open pollination). Hand pollination was conducted through a onetime application. About 6 wk 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

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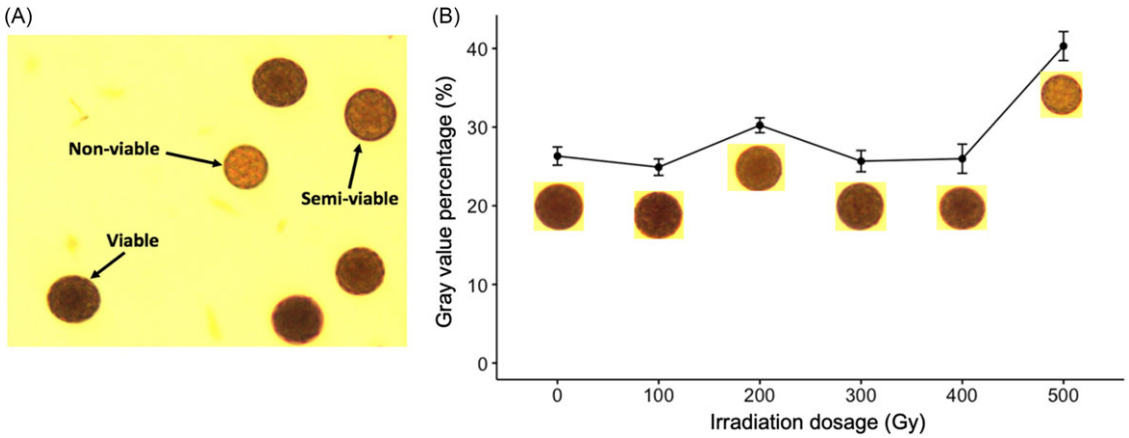

Figure 1. Viability of pollen grains stained with 2,5-diphenyl monotetrazolium bromide (MTT) showing differing intensities (A). Effect of irradiation dosages of gamma rays on pollen viability as quantified by mean gray value percentages from 100 pollen grains (B). Error bars indicate SE.

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 better 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 2 wk followed by hand pollination with irradiated pollen (with bagging), and (3) open pollination for 2 wk 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 the 2020 experiment, the hand pollination was performed as a single, onetime application.

Data from each year of study were analyzed separately because hand-pollination treatments differed slightly across the 2 yr of experiment. Before 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 first 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 2020). 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 the *lm()* function, followed by slicing each level of irradiation doses with the *SLICE()* function in the SASLM package (Bae 2020), to perform the *F*-test for the effect of hand pollination on seed set at each level of irradiation dose. Finally, seed set data were back-transformed using the *re_grid()* function in the EMMEANS package (Lenth 2020), and confidence intervals were constructed using the *confint()* function in R (R Core Team 2020).

Results and Discussion

The Effect of Irradiation on Pollen Viability

Pollen irradiated at the lowest dose (100 Gy) exhibited the lowest gray value percentage (highest viability), while pollen irradiated at 500 Gy had the highest gray value (lowest viability) (Figure 1B). The mean gray 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, because 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 (*Cucurbita pepo* L.) and winter squash (*Cucurbita moschata* Duchesne) (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 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 a 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 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

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Table 1. Dunnett's test for pair-wise comparison of gray value percentage between different irradiation doses and the control (0)

Irradiation dose comparisons gy	Gray 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 gray value percentages indicate greater brightness but lower pollen viability.

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 can 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 (Supplementary Appendices 1 and 2). Additionally, the effect of different irradiation doses, application treatments, and their interaction on seed set was significant in both years (Supplementary Appendices 3 and 4). Female plants that received no pollination produced no seed both years, so this treatment will not be discussed further. 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 significantly lower seed sets compared with open pollination (Figures 2 and 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 (Figures 2 and 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 accompanied an untreated 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 (Figures 2 and 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 (Tables 1 and 2). The seed set from pollination with irradiated pollen after non-irradiated pollen (NI+I) with bagging was lower than pollination with non-irradiated pollen after irradiated pollen (I+NI) with bagging (Figures 2 and 3). This finding shows that pollination with irradiated pollen is most effective in reducing seed set when it is applied before any fertile pollen reaches the stigma. Furthermore, applying irradiated pollen before non-irradiated pollen across all irradiation doses significantly reduced the seed set compared with open pollination (Figures 2 and 3), even though it may not completely disrupt double fertilization under some doses. Irradiated but nonviable 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 of inflorescences that received open pollination after irradiated pollen (I+Open) 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 (Figures 2 and 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 currently 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 the SPT

Introducing irradiated pollen for pollination before 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 a achieve desirable level of reduction in seed output. Even with multiple applications, questions remain about how many applications should be made and at 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 d) 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

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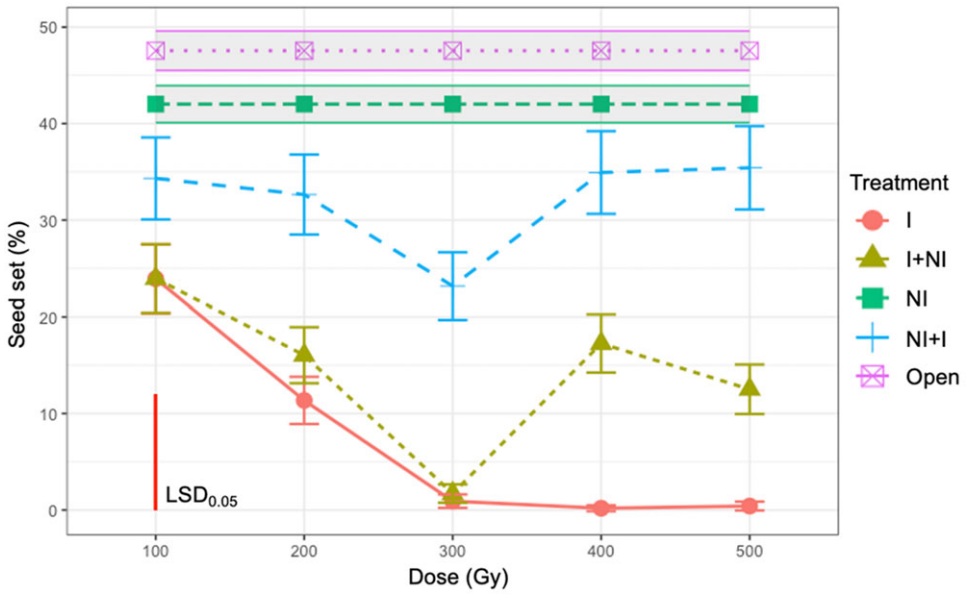

Figure 2. Effect of different irradiation doses on seed set of *Amaranthus palmeri* inflorescences in 2020 with back-transformed means and SE. Abbreviations: I, irradiated pollen; I+NI, irradiated pollen followed by hand pollination with non-irradiated pollen; NI+I, non-irradiated pollen followed by 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 SE; shaded areas for Open and NI are mean ± SE.

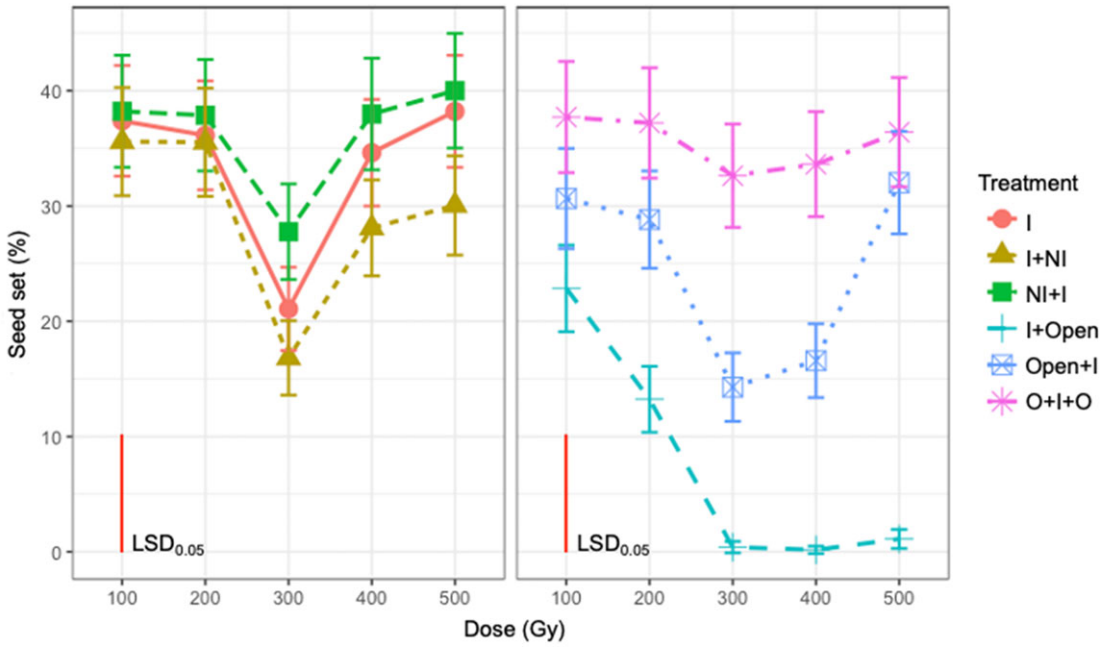

Figure 3. Effect of different irradiation doses on seed set of *Amaranthus palmeri* inflorescences in 2021 with mean and SE. Abbreviations: I, irradiated pollen; I+NI, irradiated pollen followed by hand pollination with non-irradiated pollen; NI+I, non-irradiated pollen followed by hand pollination with 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 SE. (The mean and SE are 0.3935% and 0.0220% for open pollination and 0.3790% and 0.0216% for non-irradiated pollen treatment, respectively.)

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 interplant distances and heat stress experienced during summer. The sensitivity of pollen to temperatures has been established through studies in tomato (*Solanum lycopersicum* L.) (Alsamir et al. 2021), maize (Begcy et al. 2019), and soybean [*Glycine max* (L.) Merr.] (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 SPT over short distances to the target with high efficiency will be crucial for success.

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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 wk followed by hand pollination with irradiated pollen (with bagging), and (3) open pollination for 2 wk followed by hand pollination with irradiated pollen, then open pollination with no bagging.

In conclusion, we tested the possibility of using sterile irradiated pollen as a means of disrupting seed production in *A. palmeri*, much as IST is applied in insect pests. 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 before 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), with sterile pollen from multiple weed species mixed and released in a single application. The SPT could be particularly helpful for managing herbicide-resistant weeds that have withstood in-season control and have reached a reproductive stage.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2024.7

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