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Glyphosate-Resistant Transgene Persistence in Feral Alfalfa Populations

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by

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ABSTRACT OF THE THESIS

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by

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Master of Science, Graduate Program in Plant Biology
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Studying transgene flow will enhance our understanding of the fate of crop alleles in nearby populations. In addition to the potential for weediness, gene flow from transgenic crops into neighboring fields could have severe economic consequences if those fields are destined for markets with strict thresholds for transgene detection. This study can serve as a model system for understanding transgene introgression in free-living populations.

Transgene escape has been documented for at least eight different species. I chose to work with Alfalfa (*Medicago sativa)* due to its proximity and because there was already previous data from a 2011 survey to allow for a greater time span analysis of results. Almost half of the populations at each of the three sites surveyed were still remaining in 2019, indicating that feral alfalfa populations are self-perpetuating. About half of the populations surveyed at each site were still remaining from 2019 to 2021.

The presence of the transgene increased at all three sites from 2011 to 2019, likely due to unilateral gene flow from nearby transgenic alfalfa production. It is of concern that feral populations may act as bridges to facilitate transgene movement, so the increase in transgene presence is not desirable.

Mowing, tilling, and spraying are sometimes used to manage feral roadside populations. Tilling was the most efficient method to eradicate feral alfalfa populations. No differences in effectiveness were observed between other management types.

This is the longest timespan data has been collected for transgene flow into feral populations. Having data that spans across a decade will enable a more complete understanding of the dynamics between population presence, transgene presence, management type, and other site characteristics.

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LIST OF ABBREVIATIONS/TERMINOLOGY

GR = Glyphosate Resistant

GS = Glyphosate Suseptible

GE = Genetic Engineering or Genetically Engineered

Free-living populations = Wild, weedy, or feral populations that persist without human management

Transgene escape = Pollen or seed-mediated gene flow of transgenes into populations they are not intended to reside in

AP = Adventitious Presence

Adventitious Presence = Incidental contamination of one food product with unintended material from another crop usually at a low level. In this study, AP refers to unwanted low-level transgene presence in non-transgenic seed or hay bound for consumer sensitive markets

CP4 EPSPS Transgene = 5-enolpyruvylshikimate-3-phosphate synthase gene from agrobacterium that confers resistance to glyphosate

MTA = Material Transfer Agreement

J101 = CP4 EPSPS transgene insertion event. Forage Genetics subcontracts this breeding material out to other alfalfa seed companies

J163 = CP4 EPSPS transgene insertion event that is the same as J101 but inserted into an unlinked locus.

GENERAL INTRODUCTION

OVERVIEW

Crop genes are known to sometimes cross the apparently strict borders that farmers impose on them (Ellstrand 2003). The transfer of crop alleles to nearby wild, weedy, and feral plant populations through seed or pollen dispersal (gene flow) can result in the establishment of crop alleles in those free-living populations (Ellstrand et al., 2010). Gene flow of crop alleles into nearby populations has been a driving force shaping the genetic composition of these populations throughout agricultural history, but it has gained increased attention recently with the commercialization of transgenic crops (Ellstrand et al., 1999).

The first genetically engineered genes were transgenes, which are genes originating from one organism that are introduced into a different, genetically distinct organism (NASEM, 2016). Nearly all transgenic crops grown commercially contain genes that code for herbicide or insect resistance, typically through incorporation of bacterial genes (NASEM, 2016). The broader term “genetic engineering (GE)” refers to all breeding methods that directly modify an organism's genes using biotechnology—transgenes are only one of the GE techniques.

Recent advances using GE have created synthetically edited genes, which offer increased precision, target more genes, and can be inserted into more species compared to previous GE techniques (NASEM, 2016). Studies of transgene flow in agricultural metapopulations (which include free-living plant populations) will both increase our

basic understanding of agricultural gene flow and provide useful data for biosafety risk assessments.

Three main factors make transgenes optimal for studying gene flow from crops to free-living populations. First, gene flow studies of conventional crop alleles into nearby populations are problematic due to the unknown origin of the allele. If a crop allele is found in a nearby population, it is difficult to determine whether the allele is present due to recent gene flow, or due to shared ancestry (Ellstrand et al., 2013). In contrast, transgenes provide a unique system for evolutionary and population genetic study because their creation, known sequence, time of release, and field locations have been fully documented (Ellstrand et al., 2013).

Second, widespread public apprehension about GE genes has led to strict regulations and bans in many countries (NASEM, 2016). One of the main concerns that has led to bans is uncertainty concerning environmental risks associated with gene flow (Christiansen et al., 2019). Directly studying transgene flow from crops to free-living plant populations will address the uncertainty.

Third, transgenic crops have been cultivated for longer and over more area than other types of GE crops, which makes assessment of potential environmental impacts feasible (NASEM, 2016).

Transgenes of eight different crops have been detected in hundreds of free-living populations (Ellstrand, 2018). The most prominent example of transgene escape is the case of glyphosate resistant (GR) creeping bentgrass developed by the Scotts company (Zapiola & Mallory-Smith, 2017). During a field trial strong winds dispersed many seeds

over long distances and subsequent gene flow and hybridization created novel GR weeds that posed a problem in irrigation canals (Zapiola & Mallory-Smith, 2017). As a result, the Scotts company was fined $500,000 and glufosinate, which was previously unregistered in the region, had to be used for eradication of the transgenic weedy plants (Doering, 2007; "Glufosinate 280 Herbicide").

Another major concern is transgene contamination of conventional and organic fields cultivated for consumer-sensitive markets, known as adventitious presence (AP), which can lead to the rejection of entire harvests ("Liability for GMOs," 2007). The knowledge gained through further study of transgenes in free-living populations can help companies and regulators make more accurate predictions about potential detrimental effects of GE crops prior to release.

STUDY SYSTEM

Feral alfalfa (*Medicago sativa* subsp. *sativa* L.) represents an ideal study system for investigating the impact of transgene flow into free-living populations. Transgenic alfalfa contains the CP4 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) transgene, which codes for glyphosate-resistance (GR) (Rogan & Fitzpatrick, 2004). Transgenes are a heavily politicized issue. After GR was initially deregulated in 2005, organic farmers were concerned about gene flow from GR alfalfa fields into their fields causing contamination of their product, which is bound for consumer-sensitive markets that will turn transgene contaminated products away. Even a low level of unwanted

presence can cause economic damage via adventitious presence. The organic farmers filed a lawsuit, and in 2007 GR alfalfa was regulated again until 2011 when it became deregulated for the second time (Stankiewics, 2010).

Distancing guidelines between GR alfalfa fields and non-GR alfalfa fields became the industry standard to prevent gene flow from GR fields into conventional fields. Alfalfa seed production is concentrated in the western United States in counties where alfalfa hay and seed production for both GR alfalfa and transgene-free glyphosate-susceptible (GS) alfalfa occurs ("Grower Zones," NAFA). The bees used for seed production are capable of traveling outside their intended field and pollinating GS plants (NAFA, 2015).

Feral alfalfa populations are much smaller than the surrounding alfalfa production fields, making them subject to strong unidirectional gene flow. As a result, feral alfalfa populations may be reservoirs that contain transgenes and are of concern to farmers because they may occur at intermediate distances and act as bridges to facilitate transgene movement between fields.

Because transgenes are inserted only into one copy of a chromosome, inbreeding is typically used to create homozygotes. This strategy cannot be used in alfalfa because alfalfa is highly outcrossing and subject to severe inbreeding depression. Alfalfa is an autotetraploid with tetrasomic inheritance in which the four homologous chromosomes pair randomly as bivalents (Dilkova & Bingham, 2017). Forage Genetics created a breeding strategy using two different transgenic events labeled J101 and J163 that were inserted at two different unlinked loci. They created glyphosate resistant (GR) alfalfa

varieties containing both insertions through a breeding strategy such that each locus may have zero to four copies of the transgene and alfalfa expressing glyphosate resistance may have between one and eight copies of the transgene (Rogan & Fitzpatrick, 2004). Only one copy of the transgene is needed to make the plant resistant, so the purpose of this breeding strategy is to reduce the number of non-GR seeds because even with this careful methodology non-GR seed will still occur at some level in the final product.

OBJECTIVES

Six months after the 2011 second deregulation of GR alfalfa, Greene et al. (2015) began surveying feral alfalfa populations in Canyon County, ID, Walla Walla County, WA (this region also includes parts of Umatilla County, OR, but for purposes of keeping this manuscript concise and understandable this region will just be referred to as Walla Walla), and Fresno County, CA (Greene et al., 2015). Their main findings were that 21.4% of ID populations contained the transgene, and 8.3% of WA populations, and 32.7% in CA populations contained the transgene (Greene et al., 2015). Because they surveyed just after the second deregulation, most of the transgene introgression probably occurred during the first deregulation. Most studies on transgene escape document the escape without any follow-up. In my study, the areas surveyed by Greene et al. (2015) were resurveyed in order to answer the following questions and to provide evidence in support, or not in support, of their subsequent hypotheses:

1. Are feral alfalfa populations self-perpetuating and able to persist throughout time despite farmer management?
    a. Prior research by Bagavathiannan et al. (2012) indicates alfalfa populations are self-sustaining and self-perpetuating, but due to farmer management and the length of time, I would expect about half of the populations present during the Greene et al. (2015) study to be extant during my surveys.
2. Has the percentage of GR populations significantly increased compared to 2011?
    b. Due to unilateral gene flow from transgenic fields into feral populations, I predict that the proportion of feral populations containing the transgene will increase over time.
3. Will the transgene presence differ for the two different insertion events (J101 vs. J163)?
    c. Because the same transgene insertion was used, I do not believe it will differ. However, if a selectively beneficial allele is in linkage with one of the transgenes it may become more prevalent over time.
4. What will be the most effective management practice to eradicate feral populations and reduce transgene presence? How will management change across years?
    d. Tilling would be expected to be the most effective for alfalfa removal because tilling tears up the earth and pours dirt over the plants. I think in

areas like Fresno where glyphosate is more common, it will be used less or in conjunction with other management needs.

5. In populations testing positive for the transgene, will there be more transgenic adults or seedlings
    e. More transgenic seedlings than adults can occur due to selection from glyphosate spraying.

MATERIALS AND METHODS

Field Surveys

In 2018, I conducted a preliminary visit to Fresno County, CA to determine if there were enough feral alfalfa populations remaining to allow a feasible study. Using the data and coordinates provided by Drs. Greene and Kesoju, I visited 48 sites and found feral populations at 28/48 of sites. I also discovered five additional feral sites. I recorded site characteristics including age class, management type, plant number, vegetation coverage, and other aspects I thought could be relevant. I collected 1-10 leaf samples depending on the size of the population and stored them in bags on dry ice until mowing them to a -80°C freezer. When present, I collected seeds from the plants.

In 2019, I surveyed the remaining locations in Fresno in late spring/early summer and then surveyed Canyon and Walla Walla counties in August and September (Figure 1). Using the previous coordinates and site information, I scheduled daily sampling of sites that were closest to one another. To answer question one, whether populations are

self-perpetuating, I recorded the presence or absence of plants at each site. In addition to recording the site characteristics I previously listed, I also recorded whether I could see an alfalfa seed or hay field nearby. I collected seeds from alfalfa plants when available.

In Walla Walla and Canyon counties, instead of using paper, I used the app KoboCollect to record data and take photographs and I recorded the coordinates of individual plants.

In Walla Walla and Canyon counties, some local residents conversed me as I was conducting my work. Two different farmers mentioned having difficulty with killing GR feral alfalfa and said they were already planning on switching herbicides from glyphosate to 24,D to solve their problem. I also had two different individuals tell me that in heavy windstorms they could see alfalfa seeds from nearby fields blow into their lawns and later saw alfalfa grow as weeds in their lawns and gardens.

In May of 2021, I revisited Fresno, Walla Walla, and Canyon counties for a final follow-up survey. Due to limited time, I restricted my surveys to sites that had approximately 20 or more plants in 2019. During this visit I collected additional bulk samples to increase the probability of locating the transgene. For Fresno and Walla Walla counties, when present, I collected 20 adults and 20 seedlings from sites that bulk tested as positive in order to determine if I would find differences in transgene presence between generations.

Lab Work

In order to answer question 2, whether there is a change in the percentage of

positive populations, I tested bulked population samples using lateral flow AgraStrip® RUR Seed and Leaf TraitChek™ test strips. (Romer Labs Inc, St Louis, MO, USA). These strips test for the presence of a protein produced by CP4 EPSPS-specific antibodies and are coupled to a color reagent which produces two lines when positive and one line when negative. Seeds positive and negative for the glyphosate-resistance transgene were obtained via an MTA with S&W seed company to serve as controls. GR and GS seeds were grown in a greenhouse at UCR and leaf tissue was extracted. I confirmed the accuracy of the test strips by using these known controls provided and found them to be as accurate as PCR. Prior research also provides evidence that these test strips are as accurate as PCR (Greene et al., 2015; Watrud et al., 2004).

To answer question 3, whether there was a difference in the abundance of either transgene insertion, I used PCR on a subset of individual samples collected. Leaf tissue from samples was ground in liquid nitrogen and DNA extractions were performed using a modified CTAB protocol (Doyle & Doyle, 1987; Cullings, 1992). DNA concentration was analyzed using a qubit fluorometer and diluted to 50ng/uL when applicable. Primers for both events were obtained from Patent US9,068,196 B2 (Beazley et al. 2015). Primers "A" and "Z" were used to detect event J101 while Primers "Y" and "F" were used to detect event J163. Reactions were completed using 25uL consisting of 14uL molecular grade H2O, 2.5uL Buffer, 2.5 uL 10mM dNTP, 1.5 uL MgCl2, 1uL of each primer, and 0.5 Biolase taq polymerase. The thermocycler was set to denature at 94°C for 3 minutes,

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32 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds, and a final extension of 72°C for 10 minutes before being held at 4°C.

When using control samples from S&W Seed Company, event J101 amplified while event J163 did not. After contacting S&W Seed company, I realized that the material obtained from the MTA contained event J101 without event J163. Through further research of patents, I discovered that Forage Genetics subcontracts out their transgenic breeding material to other companies, but often they only contract out event J101 without J163. I had initial interest in question 3 because I believed that if one transgene was more prominent in feral populations it could indicate linkage to a fitness gene. However, this new information suggests that event J101 should be more prevalent than J163 in feral populations based solely on probability that gene flow from fields will provide more of event J101 because it is the more prominent transgene of the two in the overall agricultural landscape.

Transgenic seed was procured from S&W Seed Company instead of Forage Genetics because the latter company would not provide seeds for this project. As a result, there to be no true control for J163. To circumvent this issue, I used PCR to amplify the J163 transgene on feral specimens until I found a few in which a band of the appropriate size amplified, indicating J163 transgene presence. I used DNA from the feral samples that amplified as controls for J163.

PCR for each transgene was performed on 76 individuals from Fresno County coming from 38 different populations. PCR for each transgene was also used on 17

individuals from 3 populations in Walla Walla County, and 6 individuals from 1 population in Canyon County.

To test for gene flow into GS populations, seeds from negative populations in Walla Walla and Canyon counties were ground for 5 pulses of 10 seconds each, using a spice grinder (Cuisinart, East Windsor, NJ).

RESULTS

Feral alfalfa populations appear to be self-perpetuating

For clarity, the 2011/2012 survey is called the 2011 survey and the 2018/2019 survey is called the 2019 survey. In Fresno County, 46.7% of the populations surveyed from 2011 coordinates were present in 2019 (Figure 2). In 2021, populations found in Fresno County in 2019 were resurveyed and 52.9% were present (Table 1). In Canyon County, 44.0% of the populations surveyed from 2011 coordinates were present in 2019 (Figure 3). In 2021, populations found in Canyon County in 2019 were resurveyed and 50% of the populations were present (Table 1). In Walla Walla County, 40.6% of the populations surveyed from 2011 coordinates were still present in 2019 (Figure 4). In 2021, populations located in 2019 were resurveyed and 55.6% of populations were present (Table 1). There were no significant differences found for population presence among counties in either 2019 or 2021.

Increase in transgene occurrence throughout time

The percentage of positive populations is significantly greater in 2019 compared to 2011 for all three regions (Table 1). When calculating the percentage of positive populations for 2019, new populations were included in the calculations in addition to the 2011 sites. A proportion test was used to compare the percentage of positive populations across years (one-sided and two-sided proportion tests both gave significant results). In Fresno County the percentage of positive populations increased from 42.0% to 78.6% (Figure 5). In Canyon County the percentage of positive populations increased from 17.9% to 49.0% (Figure 6). In the Walla Walla region the percentage of positive populations increased from 7.8% to 34.8% (Figure 7). Note that the percentages of positive populations for the original 2011 survey differ from those reported by Greene et al. (2015). This difference is due to the inclusion of additional study sites obtained from data provided by Drs. Greene and Kesoju.

In addition to locating new sites that tested positive, a portion of the increase was due to sites that were previously negative now testing positive for the transgene. In Fresno County, 65.8% of previously negative sites tested positive in 2019. In Canyon County, 36.0% of previously negative sites tested positive in 2019. In the Walla Walla region, 30.6% of previously negative sites tested positive in 2019. Two sites in Walla Walla tested negative in 2011 and 2019 but tested positive in 2021 (Table 2).

To determine whether pollen-mediated gene flow could have contributed to the increase in positive populations, seed from negative individuals in negative sites was tested for transgene presence. One negative plant out of the 30 analyzed from Canyon County had positive seed. Four negative plants out of the 24 analyzed from Walla Walla

had positive seed, with each individual belonging to a different population. Seed from Fresno County was not analyzed due to time constraints.

Transgene presence quantified for the two different insertion events

EPSPS transgene insertion event J101 was more frequent in each region tested than event J163. Out of the individuals from Fresno tested for the presence of event J101, 67.6% (48/71) tested positive for the transgene, while only 32.9% (24/73) tested positive for event J163. A two-sided proportion test was used to determine that this difference is significant. Ten individuals from ten different populations tested positive for both transgenes.

In Walla Walla, 19 individuals from 3 different populations were tested for presence of each event. Forty-seven percent (8/17) of individuals tested positive for event J101 while 25% (4/16) tested positive for event J163. Only one individual was positive for both events.

Six individuals from a single population were tested from Canyon County. All six contained event J101 while only one contained event J163.

Effect of management practices and other site characteristics

In Fresno, coordinates of sites from 2012 that were observed as being tilled in 2019 were significantly less likely to contain feral populations in 2019 (Figure 8).

The results were not significant for Canyon County. There were insufficient data to complete this analysis for Walla Walla.

A significant increase to no management from 2011 to 2019 was observed in each of the three populations. While this result could be due to less management efforts taking place, it could also be impacted by individual surveying technique or time of visit (mowing and spraying occur at specific times and could be impacted temporally). In order to increase accuracy of comparisons, I have removed "none" as a character option.

With "none" removed, Fresno had significantly less sprayed populations, significantly more tilled, and significantly more mowed populations in 2019 when compared to 2011. Walla Walla had significantly less mowed populations in 2019 compared to 2011. There are no significant differences in management between time frames in Canyon County.

In 2011, Walla Walla had significantly more populations managed by mowing compared to Canyon and Fresno counties when using two-sided proportion tests. In 2019, Canyon County managed significantly more populations with spraying compared to Fresno County, as determined using a two-tailed proportion test. Canyon County also managed significantly more populations with spraying compared to Walla Walla using a one-tailed proportion test.

In all three regions, average plant number per population was significantly greater in 2019 when compared to 2012 (Figure 9). There were no significant differences in average plant number between the three regions in 2019.

In Walla Walla, transgene presence in 2019 was significantly greater when an alfalfa field was visibly present nearby, compared to when there were no alfalfa fields visible nearby. Locations of visible alfalfa fields visible from feral sies remained significantly (76.5) the same in Walla Walla. However, neither correlation was observed for Fresno and Canyon counties.

No change observed in transgene presence between generations

Four sites in Fresno and four sites in Walla Walla were analyzed for differences in transgene presence between adult and seedling generations. Approximately 20 adults and 20 seedlings were collected and individually tested for transgene presence. There were no correlations between generations.

DISCUSSION

Despite intensive management practices, approximately 40- 47% of the 2011 feral alfalfa populations in all three regions were able to persist, indicating that feral alfalfa populations can be self-perpetuating. Alfalfa is able to grow well despite poor nutrient conditions along roadsides due to its deep tap root and ability to fix nitrogen (Bagavathiannan et al., 2010). Alfalfa's ability to produce thousands of seeds per plant, maintain a persistent seed bank, its perennial nature, quick regrowth, and drought and

cold tolerance also contributes to its ability to form self-perpetuating feral populations (Bagavathiannan et al., 2010; Bagavathiannan et al., 2012). Bagavathiannan et al. (2010) found that two years after seedling recruitment, 16% of seedlings remained, indicating that seedlings are capable of establishing new adult plants within their population. In the absence of management efforts, the average lifespan of feral plants is between 5-20 years (Dr. Daniel Putnam, personal communication, 2018-2019). However, population growth and seedling establishment is limited by alfalfa's auto-allelopathy (Bagavathiannan et al., 2012).

The significant increase in transgene presence in all three regions is primarily due to unilateral gene flow from transgenic alfalfa seed and, to a lesser extent, hay fields (Papa & Gepts, 2004). Unilateral gene flow may also explain why event J101 was more abundant in feral populations than event J163. To ensure proper distancing between fields, industry maps have been created that pinpoint the locations of transgenic and non-transgenic fields. Unfortunately, I was denied access to these maps along with other information, possibly due to industry apprehension that transgene flow research may have a negative impact on marketing. I called alfalfa farmers in Fresno to ask whether they grew RR alfalfa seed or hay and what the acreage was being grown, and while some answered that they were growing RR alfalfa or that they decided not to grow it, most farmers did not answer. One farmer that was known for growing substantial acres of RR alfalfa seed in the past consistently dodged my calls. Thus, industry and farmer apprehension limited the scope of what I was able to determine (which is understandable given that farmers may have concerns that they could be targeted by anti-transgene

organizations if their field locations became public).

Most farmers surveyed responded that they managed feral alfalfa populations but typically only within their property lines (Kesoju & Greene, 2017). In addition to farmer management, each county manages roadside weeds through planned herbicide applications along specific roads (personal communications from A. Gibson, Fresno County; A. White, Walla Walla County; P. Rhodes, Canyon County, 2018-2019). Herbicide mixtures used in Walla Walla and Canyon counties include a broad set of herbicides; glyphosate use varies. In California, different herbicides and herbicide mixtures are used; glyphosate is most common (Dolcini et al., 2019).

Since glyphosate is most commonly used in Fresno County, I hypothesized that selection for GR feral alfalfa populations would occur there. While Fresno County has significantly more positive populations than Canyon and Walla Walla counties, this does not provide evidence for selection because the ratios of GR alfalfa being grown is unknown between the three regions. However, there was a significant decrease in spraying in Fresno from 2011 to 2019, indicating that spraying might have decreased in effectiveness, so farmers switched to management via tilling and mowing.

There is some evidence that spraying may lead to increased transgene presence in Canyon County. Populations with visible spray damage observed in 2019 were significantly more likely to test positive for transgene presence compared to populations that were mowed or unmanaged.

Despite predicting that mowing would have a negative impact on population presence due to reducing racemes, this was not observed for any of the three sites.

Bagavathiannan et al. (2010) found that mowing did not have a significant impact on growth, reproductive attributes (such as the number of racemes), or mortality. Although fecundity of mowed plants was lower than un-mowed plants, this did not follow a consistent pattern (Bagavathiannan et al., 2010). Limited efficiency of mowing for controlling feral alfalfa populations could explain the significant decrease in mowing in Walla Walla from 2011 to 2019.

The increase in average population size in 2019 compared to 2011 could indicate that the average population size is increasing over time, but it could also be attributed to sampling bias towards larger populations.

Due to limited time constraints and difficulty locating suitable populations, I was not able to sample enough populations to draw accurate conclusions about whether there is a difference in transgene presence between generations. However, even if a larger data set was attainable, I would not recommend pursuing this question further. Alfalfa's seed bank can last decades (Bagavathiannan et al., 2012). Therefore, seedlings might not represent a future generation, but could be from the same or even a prior generation than the established adults.

Conclusion

A proportion of roadside feral alfalfa populations are self-perpetuating and capable of persisting throughout at least a ten-year time span despite farmer management practices. Transgene presence in feral populations significantly increased for all regions

between 2011 and 2012 and although the sample size collected in 2021 was much smaller, it also displayed a trend towards increased transgene presence. If GR alfalfa continues to be grown at a similar rate, I expect transgene incidence to increase, especially for feral populations located in closer proximity to GR production fields.

There was a significant correlation between tilling and population absence in Fresno County in 2019. Fresno significantly decreased their use of spray to manage feral alfalfa populations from 2011 to 2019 while Walla Walla significantly decreased mowing from 2011 to 2019. I suggest that farmers and other agricultural professionals increase their use of tilling when feral alfalfa extermination is their goal.

Future Directions

Many of the feral populations were extremely small in size, with many under 20 plants and few over 100 plants. Smaller feral alfalfa populations are unlikely to act as bridges between fields, so their importance is limited. Future studies should focus on more extensive sampling in populations that consist of 100 or more individuals.

Due to the limited scope of this project, I was only able to access transgene presence and could not quantify transgene frequency. Knowledge of allele frequencies is essential for population genetic analysis. However, since alfalfa is a tetraploid, assessing transgene frequency while ensuring accuracy may be difficult. For future studies on transgene escape, I suggest quantifying transgene frequencies in a diploid species with a

less persistent seed bank so that population genetic analysis can be used to make comparisons across generations.

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Table 1 Change in population presence and transgene presence across study timeframe. Population presence is documented for 2019 and 2021. The percentage of positive populations is different from the percentages in the Greene et al. (2015) study because I used additional datapoints compiled from datasheets provided by Drs. Greene and Kesoju. When testing for transgene presence in 2019, I included new sites I discovered in 2019 and excluded data that was not analyzed for transgene presence. Significance was determined using a two-sided proportion test.

Region	**Feral Populations in 2019 Remaining from 2012**	**2012 Positive Populations**	**2019 Positive Populations**	**2012 Negative Populations Testing Positive in 2019**	**2021 Feral populations remaining from 2019**
Fresno County, CA	46.7% (79/169)	42.0% (71/169)	78.6% (55/70) (Significant)	65.8% 27/41 (significant)	52.9% 18/34
Canyon County, ID	44.0% (59/134)	17.9% (24/134)	49.0% (47/96) (Significant)	36.0% 18/50	50% 15/30
Walla Walla County, WA	40.6% (52/128)	7.8% (10/128)	34.8% (23/66) (Significant)	30.6% 15/49	55.6% 35/63

Table 2 Transgene presence/absence data recorded at each of the three time frames including populations assessed in 2021. Fresno populations surveyed stayed positive in 2019 and 2021, as to be expected since Fresno has the greatest percentage of positive populations. The percentage of positive populations significantly increases in Walla Walla from 2011 to 2021 using a one-sided proportion test. Two populations in Walla Walla that tested negative in 2011 and 2019 tested positive in 2021, indicating gene flow is still occurring. Notably, one of the two had seed that tested positive in 2019, providing evidence that transgenic seed may establish in a previously negative population.

Site	2011	2019	2021
Walla_13	Neg	Neg	Neg
Walla_70	Neg	Pos	Pos
Walla_74	Neg	Neg	Neg
Walla_84	Neg	Neg, pos seed	Pos
Walla_88	Neg	Neg	Neg
Walla_89	Neg	Neg	Neg
Walla_90	Neg	Neg	Neg
Walla_91	Neg	Neg	Neg
Walla_94	Neg	Neg	Pos
Walla_FS_1	N/A	N/A	Pos
Walla_FS_4	N/A	N/A	Pos
Walla_my_2	N/A	Pos	Pos
Walla_my_4	N/A	Pos	Pos
Walla_my_7	N/A	Neg	Neg
Walla_103	Pos	Pos	Pos
Walla_FS_5	N/A	N/A	Neg

Site	2011	2019	2021
Fresno_3	Pos	Pos	Pos
Fresno_51	Pos	Pos	Pos
Fresno_67	Pos	Pos	Pos
Fresno_106	Neg	Pos	Pos
Fresno_107	Neg	Pos	Pos
Fresno_169	Neg	Pos	Pos
Fresno_New_2	N/A	Pos	Pos

Figure 1:

Google Earth map depicting the three study locations. The red boxes with an X indicate sites at a location. The northern most points depict study sites in Walla Walla. The points to the southeast represent Nampa. The southernmost points represent Fresno, CA.

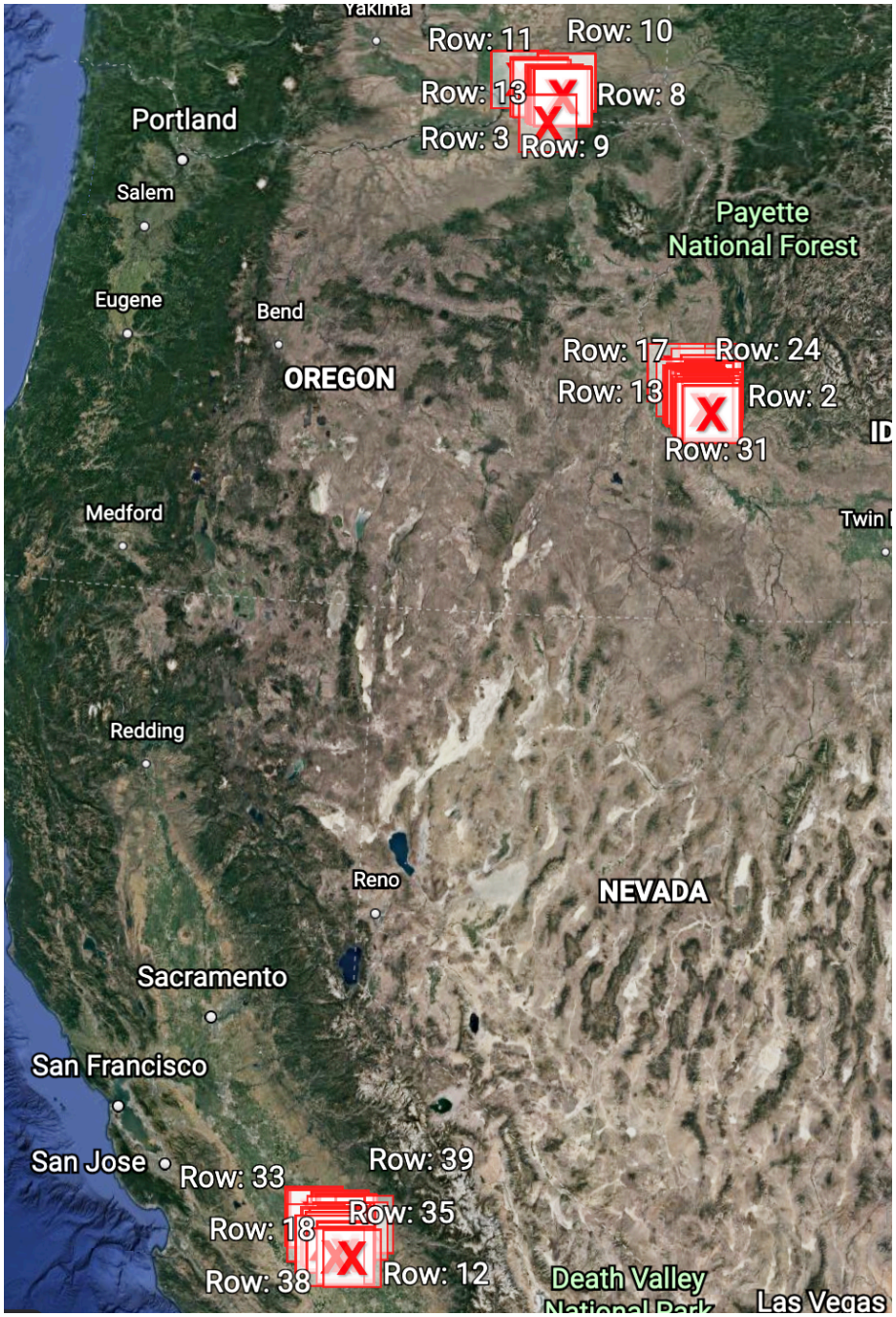

Figure 2: Feral alfalfa populations found in Fresno County a). 2012 and b). 2019. Maps were created by collaborator Dr. Sandya Kesoju in ARCGIS using our combined survey data.

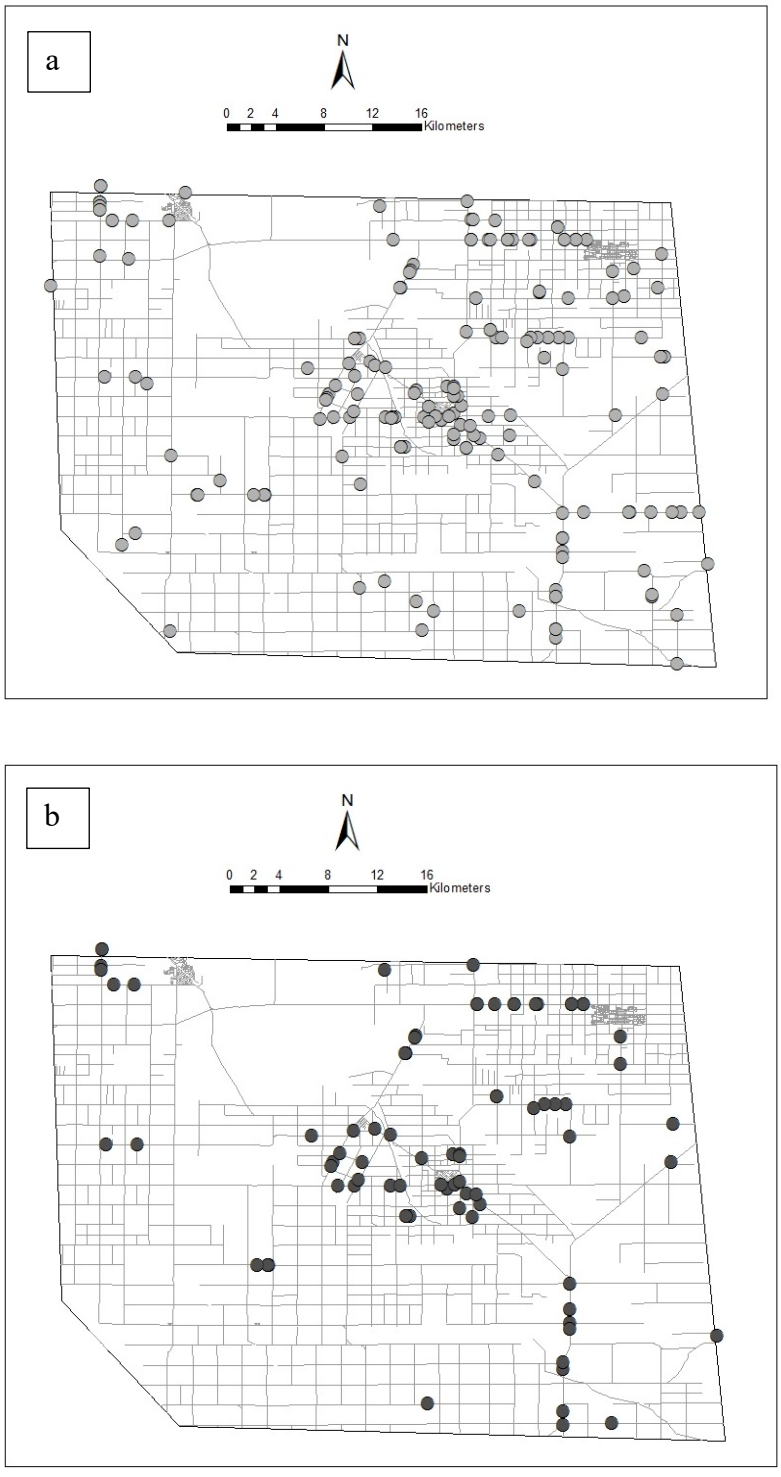

Figure 3: Feral alfalfa populations found in Canyon County a). 2012 and b). 2019. Maps were created by collaborator Dr. Sandya Kesoju in ARCGIS using our combined survey data.

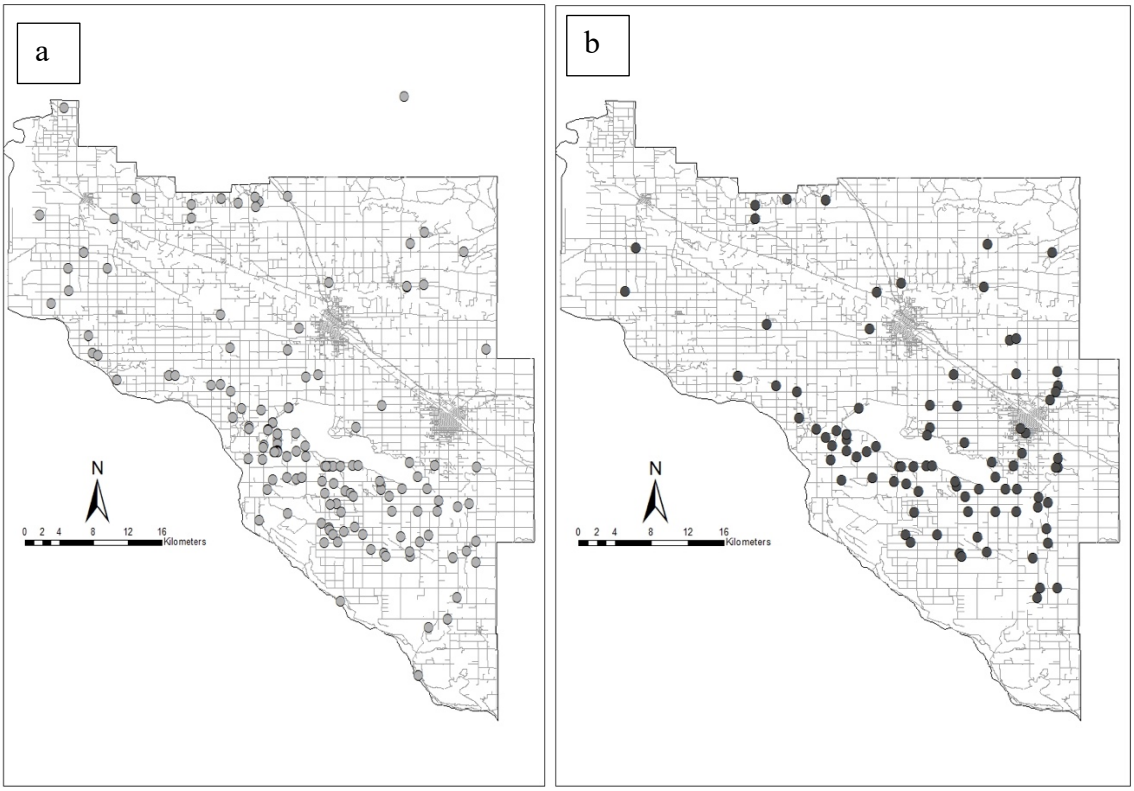

Figure 4: Feral alfalfa populations found in Walla Walla County a). 2012 and b). 2019. Maps were created by collaborator Dr. Sandya Kesoju in ARCGIS using our combined survey data.

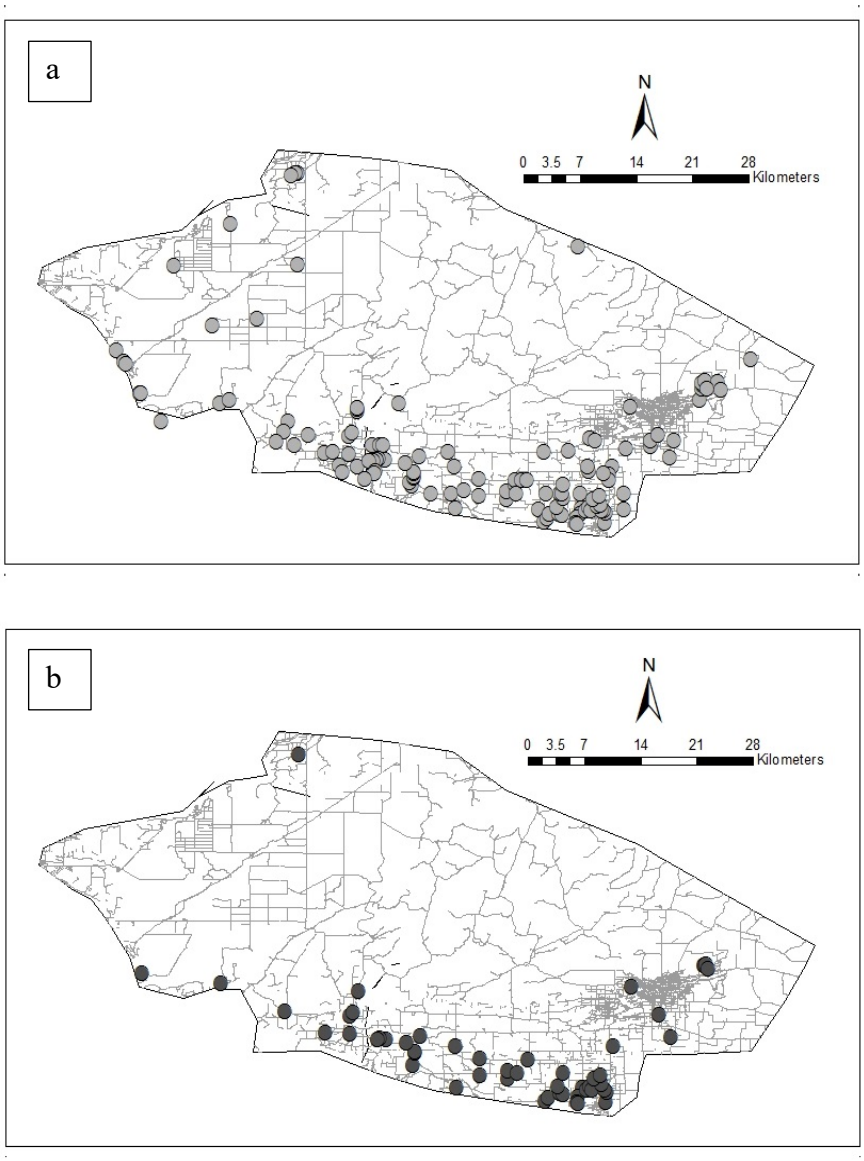

Figure 5: Transgene presence and absence in feral alfalfa populations found in Fresno County a). Light purple circles represent absence of transgene while dark purple circles represent presence of transgene in 2012 and b). Light pink circles represent absence of transgene while dark pink circles represent presence of transgene in 2019. Maps were created by collaborator Dr. Sandya Kesoju in ARCGIS using our combined survey data.

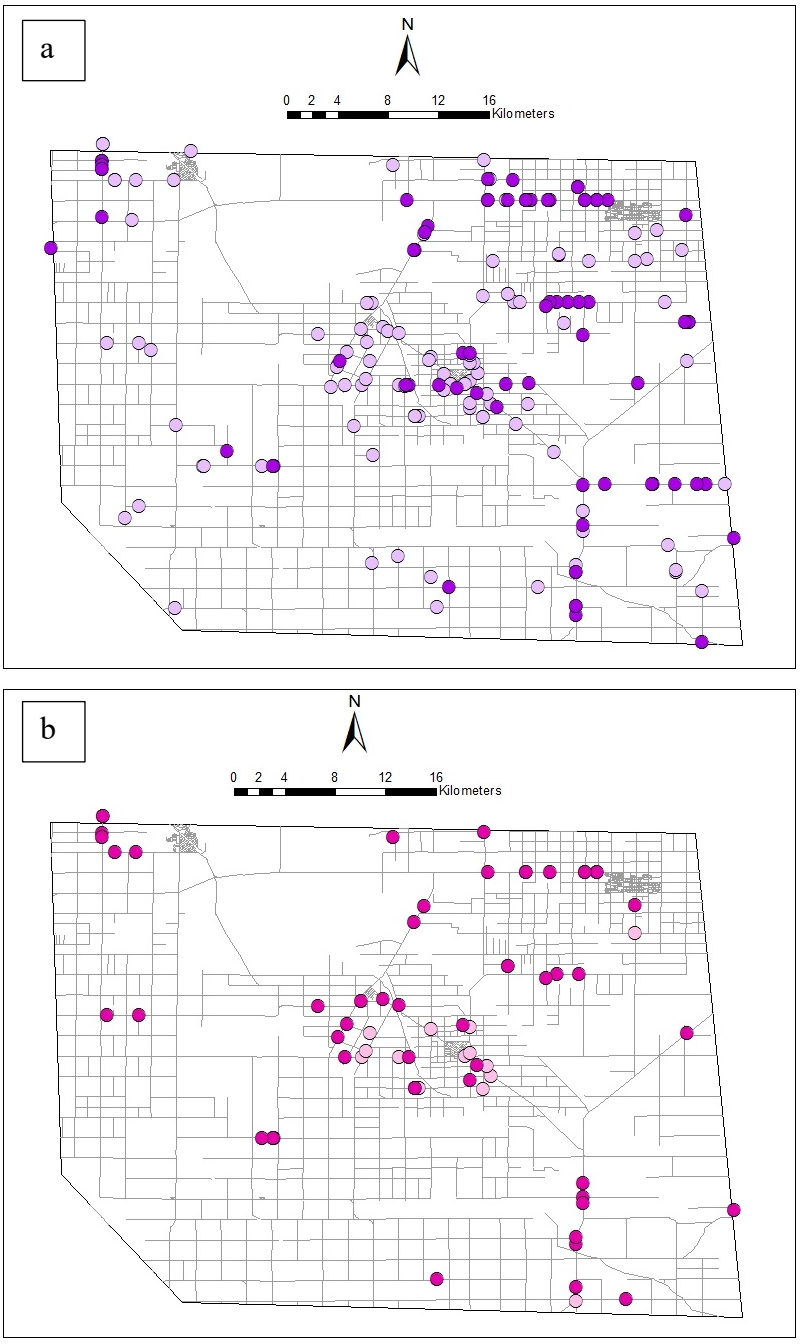

Figure 6: Transgene presence and absence in feral alfalfa populations found in Canyon County a). Light purple circles represent absence of transgene while dark purple circles represent presence of transgene in 2012 and b). Light pink circles represent absence of transgene while dark pink circles represent presence of transgene in 2019. Maps were created by collaborator Dr. Sandya Kesoju in ARCGIS using our combined survey data.

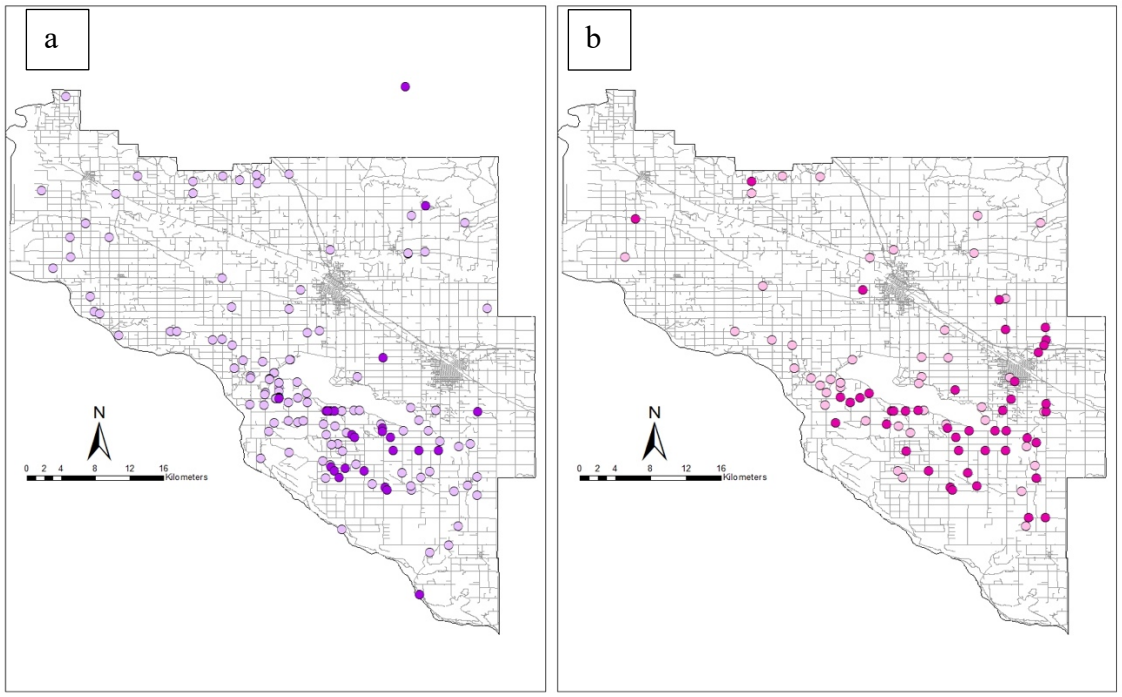

Figure 7: Transgene presence and absence in feral alfalfa populations found in Walla Walla County a). Light purple circles represent absence of transgene while dark purple circles represent presence of transgene in 2012 and b). Light pink circles represent absence of transgene while dark pink circles represent presence of transgene in 2019. Maps were created by collaborator Dr. Sandya Kesoju in ARCGIS using our combined survey data.

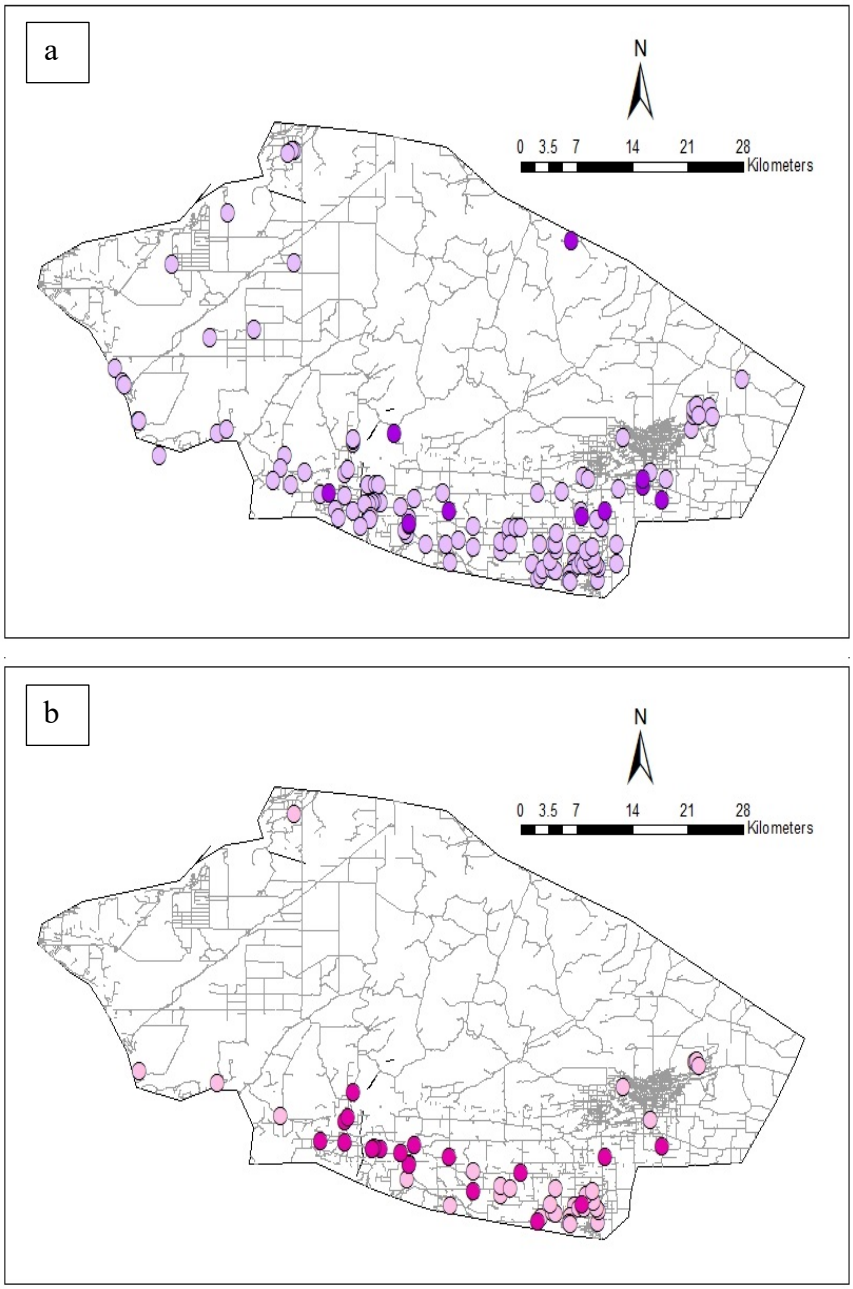

Figure 8: Tilling was the most effective method for eradicating feral alfalfa populations in Fresno in 2019. Using a pairwise proportion test, p values were found to be significant when comparing the average population presence (coded as 0 for absent, 1 for present) for tilling management compared to mowing, spraying, and none.

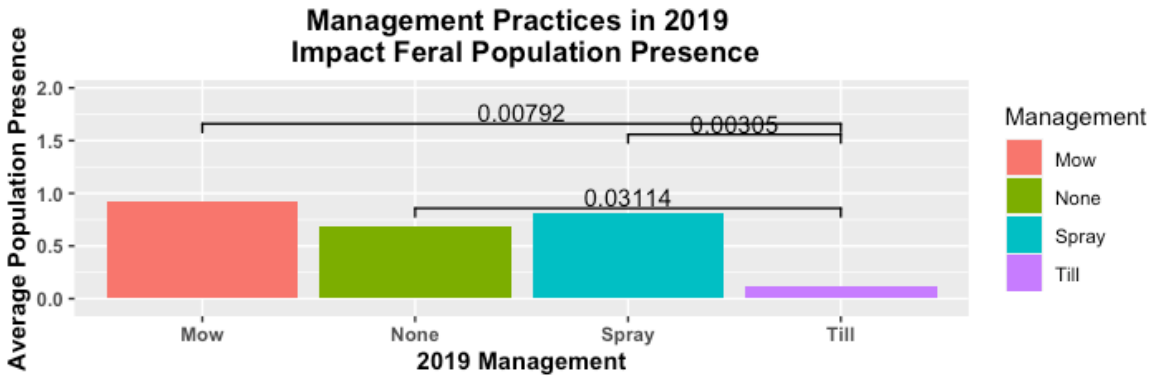

Figure 9: Average population number found for each of the three survey counties. Average population number for the 2011/2012 survey was compared to the 2018.2019 survey using a t test and p values were found to be significant in all three counties.