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Morphological and Physiological Comparisons between Yellow Toadflax (*Linaria vulgaris*) Individuals Exposed to 2,4-Dichloro-phenoxyacetic Acid (2,4-D)

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Abstract

Current management strategies for the containment of the invasive plant populations of yellow toadflax using auxin-based herbicides have been known to induce evolutionary modifications within the plant species. For the past three years, several 20-week block experiments were conducted throughout the various growth stages (2-week intervals) of yellow toadflax's first-year life cycle. Individuals were grown from seed under greenhouse conditions and treated with a single application of 2,4-D solution set at 386.9 ppm during one of several predetermined stages. Collections of data regarding growth rates between soil-only (indirect) applications, shoot-only (direct) applications, and untreated individuals were then documented. Additionally, individuals were either grown in sandy-loam or medium-loam soil-types as a matter of collecting comparative measurements in plant growth and herbicide absorption, degradation and/or leaching potential. Our results have successfully demonstrated that the yellow toadflax species has the ability to withstand various 2,4-D applications (41% survivability), and 75% of the treatment-surviving individuals were asexual, sucker shoots (ramets). The auxin-based herbicide treatments appear to promote an increase in asexual, vegetative reproduction through root budding compared to the majority of untreated, yellow toadflax individuals which focused on sexual reproduction via flowering.

Keywords: 2,4-D; Asexual Reproduction; Invasive Plant

Introduction

Yellow toadflax (*Linaria vulgaris*) is a short-lived perennial herb native to the steppes of southeastern Europe and southwestern Asia (Eurasia) [1]. It is believed to have been introduced into North America prior to the year 1672 [2] and continued to spread as European settlement expanded westward. By the 1950s, yellow toadflax became present across much of North America [3] and, as of 2018, the species is still considered an aggressive, noxious weed throughout a majority of the western United States [4]. Yellow toadflax produces many secondary compounds found in the foliage and stems (linarin and choline), and in the flowers and the seeds (saponins), serving primarily as defensive functions. Yellow toadflax has been used as an insecticide in animal bedding, as a yellow dye, and is regarded as a plant imbued with culturally significant properties.

Despite relentless management strategies and containment efforts, yellow toadflax remains persistent in its ability to adapt and survive. The plant is mostly a cross pollinating, herbaceous perennial with an adventitious root system, characterized as a root sucker system capable of producing both a woody stem [5] and a taproot [6]. The asexual, clonal root spread is often regarded as the species' primary means of reproduction for creating new individuals. The classification of separation between the parent shoots (genets) and the offspring shoots (ramets) is based on root morphology and characteristics among several perennials, vegetative plant species [6-9]. However, seed production, dispersal, and dormancy are equally as beneficial for the plant's successful establishment and distribution [10]. Recent genetical analyses were conducted among various clonal patches of yellow toadflax found throughout the Rocky Mountain region of the United States, and the results indicate the likelihood that there have been multiple introductions which have promoted high levels of diversity throughout yellow toadflax populations [11].

There have been several methods and management strategies used in order to prevent the spread of yellow toadflax. Herbicides, tillage, and biological control are among the most common practices utilized. Tillage is thought to be the most successful method, especially during the fall after crop production [5]; however, tillage is not easily manageable in certain locations, such as wilderness areas in the intermountain West where yellow toadflax invasions have started to spread [12]. Several attempts using biological agents such as the two-seed, capsule-feeding weevils (*Gymnaetron antirrhini*) and the ovary-feeding beetle (*Brachypterolus pulicarius*) during the 1950s in Canada showed a large decrease across several yellow toadflax populations [13-14]; however, recent attempts with biologic introductions have met with minimal to no success [15].

The most widely used technique for controlling the spread of invasive and weedy plants has been with the applications of herbicides. Herbicides are easily transportable and relatively efficient, depending on the proper strategies regarding use. Remedies for the control of weeds have likely been practiced for thousands of years, yet the first commercialized production of herbicides didn't start until the late 19th and early 20th centuries, especially during the 1940s with the discovery of phenoxyacetic acids [16]. 2,4-Dichlorophenoxyacetic acid (2,4- D) is regarded as the prototype for effective herbicide treatments [17]. By the end of the 20th century, the United States alone used approximately 46 million pounds of 2,4- D in agricultural and non-agricultural settings for weed control [18], and as of 2015, there have been an additional 410 classified active ingredients within 119 chemical families created for the purpose of herbicide application [19]. However, despite the current advancements in chemical technologies, targeted plant species have continually demonstrated their abilities to develop resistances to herbicide treatments. Although there has been some success in reducing yellow toadflax populations, the species continues to remain inconsistently controlled with the use of current herbicide applications under regulated standards [20-21]. In light of these perplexities, recent advancements in the molecular sciences have begun to pinpoint the causes of herbicidal resistances that may be found in mutations within the genetic loci of the target organism. The objectives of this research were designed to compare the physiological and morphological effects of 2,4-D on various growth-stage developments in European wild types of *Linaria vulgaris* under greenhouse conditions.

Materials and Methods

Imported yellow toadflax seeds from Hungary were purchased from Sheffield's Seed Company (U.S.A.). Seeds were germinated from sets grown in medium-loam and sandy-loam soils. For each soil type, fifty pots each containing a single seedling were placed in the greenhouse at 22°C with relative humidity fluctuating between 15% and 50%, and a 12-h photoperiod. Each container was watered to field capacity every other day using unfiltered spring water and continued throughout the first two weeks after seedling emergence (EW). Two-week old seedlings were transplanted into cylindrical pots containing medium-loam and sandy-loam soils with volumes (V= π r^2 h) of 19.5 cm (h) x 11.25 cm (r) in order to minimize root damage and to provide each yellow toadflax seedling an adequate, uncompetitive environment for the duration of each experiment. 200 mL of unfiltered spring water (on an every-other-day basis) were applied per pot.

The experimental layouts in regard to 2,4-D application for each 20-wk block experiment consisted of treating various age groups of yellow toadflax individuals in their first-year life cycle. Collectively, groups of individuals were treated at 4wks, 6wks, 8wks, 10wks, and 12wks of age, and each age group was subdivided further into a factorial category: groups were either treated directly (aboveground/ shoot only) or indirectly (soil-only treatment). 2,4-D treatments were applied from a standard, concentrated solution based on mixing methods provided by Sigma-Aldrich, Inc. Equal mixing portions of 95% ethanol (500 mL) and water (500 mL) were added with 3.87 g of 98% 2,4-D concentrated dry powder in order to obtain a standard solution set at 3869.71 ppm. Herbicide applications were then administered using a pressurized hand sprayer at a rate of 89 mL per direct or indirect treatment. Following the application of each individual, a 72-hour window was allotted without any added precipitation. Afterwards, 200 mL of unfiltered spring water commenced at the every-other-day rate.

Biomasses of treated and untreated yellow toadflax individuals were recorded throughout each 20-week experiment. Shoot measurements were taken on a biweekly basis, beginning at the two-week age mark. Prior to the production of additional genets (typically, starting at 3 wks to 4 wks of age), small wire bands were placed on the first genet of each plant to track shoot growth rates. Additionally, the number of genets produced throughout each individual's development were recorded to help establish values for aboveground biomass. Flowering events were also recorded. Aboveground-ramet shoot heights (cm) were measured upon their appearance as they were considered individuals apart from the parent cluster of genets.

Complete plant measurements were obtained at the beginning of two-weeks of age and continued biweekly throughout the 20-week experiments using cautious excavation methods. Yellow toadflax individuals of each treatment and control relative to soil-type were excavated and measured to document the overall spread of an individual (length and width of roots and shoots) within an area of occupancy. Stems and roots were separated at the stem base section where the root pigmentation begins, and wet weights were obtained in both roots and shoots. Dry weight was obtained for both stems and roots at 22˚C for 72 hours. Stems and roots were stored in a deep freeze at -60˚C for later analysis. Soil samples were collected based upon two distinctions: surface samples (depth of 2 cm) and subsurface samples (within 2 cm of the roots' surrounding rhizosphere). Soil samples were air-dried to determine the moister content. Dry soil samples were sieved using 2 mm sieve and stored in -60°C deep freeze for later analysis.

Results

Growth and survivorship comparisons between the Fall 2016 (Table 1) and Spring 2017 (Table 2) common-garden experimental blocks demonstrate slightly significant differences for successful adaptability within genet-to-ramet clonal integration regarding herbicide applications and nutrient availability found between sandy-loam and medium-loam soil types. First and foremost, direct applications of 2,4-D per plant were very effective at eliminating first-year yellow toadflax generation (98.6% control). Out of both Fall 2016 and Spring 2017 direct treatments, only one individual survived in sandy-loam soil while there were no survivors in medium-loam soils at the end of the 20-wk experiment (Table 2). 43% of the single, indirectly-treated individuals were able to survive the application, not including the 26 ramets of the 12wk indirect treatment in medium-loam soil (table 1). As expected, indirectly-treated, sandy-loam individuals had a higher success rate of survival and reproduction (94% in Fall and 29% in Spring) than indirectly treated, medium-loam individuals (20% in Fall and 18% in Spring) because of soil-texture and particle size in correspondence with 2,4-D leaching potentials. Using Liquid Chromatography and Mass Spectrometry (LC-MS), monitoring the movement of 2,4-D throughout plant and soil systems has allowed us to gather more background information for determining the cause of increased resource translocations among clonal species (data not shown).

Treatment period	Soil type	Treatment	# of individuals prior to 2,4-D application	# of ramets prior to 2,4-D application	# of flowering individuals prior to 2,4-D application	# of flowering ramets prior to 2,4-D application	# of individuals at 20 wks	# of ramets at 20 wks	# of flowering individuals at 20 wks	# of flowering ramets at 20 wks
12 wk	sandy-loam	direct treatment	9	3	1	0	0	0	0	0
		indirect treatment	6	0	3	0	3	2	1	1
	med.-loam	direct treatment	7	2	1	0	0	0	0	0
		indirect treatment	3	0	0	0	28*	26*	11*	10*
8 wk	sandy-loam	direct treatment	6	0	0	0	0	0	0	0
		indirect treatment	6	0	0	0	11	7	2	0
	med.-loam	direct treatment	6	0	0	0	0	0	0	0
		indirect treatment	6	0	0	0	4	3	0	0

6 wk	sandy-loam	direct treatment	6	0	0	0	0	0	0	0
		indirect treatment	6	0	0	0	3	1	1	0
	med.-loam	direct treatment	6	0	0	0	0	0	0	0
		indirect treatment	6	0	0	0	0	0	0	0
	sandy-loam	controls	-	-	-	-	3	0	3	0
	med.-loam	controls	-	-	-	-	7	3	3	0
*One 12-wk, indirect-treated individual in medium-loam soil did not appear to be affected by the 2,4-D										

Table 1: Summary of the Fall 2016 2,4-D experiment comparing 12, 8, and 6 weeks aged individuals with controls.

Treatment period	Soil type	Treatment	# of individuals prior to 2,4-D application	# of ramets prior to 2,4-D application	# of flowering individuals prior to 2,4-D application	# of flowering ramets prior to 2,4-D application	# of individuals at 20 wks	# of ramets at 20 wks2	# of flowering individuals at 20 wks	# of flowering ramets at 20 wks
10 wk	sandy-loam	direct treatment	6	0	1	0	1*	1*	0	0
		indirect treatment	6	0	1	0	0	0	0	0
	med.-loam	direct treatment	6	1	0	0	0	0	0	0
		indirect treatment	6	2	0	0	2	1	0	0
8 wk	sandy-loam	direct treatment	6	1	0	0	0	0	0	0
		indirect treatment	6	0	2	0	5	3	0	0
	med.-loam	direct treatment	6	0	0	0	0	0	0	0
		indirect treatment	6	2	0	0	1	0	1	0
4 wk	sandy-loam	direct treatment	5	0	0	0	0	0	0	0
		indirect treatment	5	0	0	0	0	0	0	0
	med.-loam	direct treatment	5	0	0	0	0	0	0	0
		indirect treatment	5	0	0	0	0	0	0	0

	sandy-loam	controls	-	-	-	-	6	0	6	0
	med.-loam	controls	-	-	-	-	6	0	4	0
*One 10-wk, direct-treated individual in sandy-loam produced a single offspring.										

Table 2: Summary of the Spring 2017 2,4-D experiment comparing 10, 8, and 4 weeks aged individuals with controls.

Regardless of the method of treatment, 2,4-D exposed individuals displayed the classic symptoms of plants exposed to high levels of auxins. Nearly all directly and indirectly treated demonstrated signs of early uncontrolled tissue growth within the shoot and root sections. Some individuals even began to produce over-abundances of flowers. Yet, for the majority of the treated yellow toadflax, excessive cellular growth led to programmed cell death as nutrients could not be efficiently taken-up and transported quick enough by the plant's roots and vascular system. The effects are visibly noticeable as discoloration in the leaves and stem, beginning at the apical meristem of the shoot apex, as leaves began to dry and wilt.

Yellow toadflax's root system typically follows a similar fate to the auxin-herbicide as with its shoot system; however, survivorship rates throughout the 20-wk experiments were mostly contributed with the adventitious root budding of sucker shoots (ramets). 75% of the surviving individuals were offspring that appeared after the 2,4-D treatments. The few ramets that appeared prior to treatment did not survive. Compared to the untreated controls, ramet production was larger within individuals exposed to 2,4-D. (Figure 1)

Figure 1: 12-wk, indirectly treated individual (parent genets are dead) in sandy-loam soil had asexually produced a ramet 2-weeks after 2,4-D exposure.

Discussion

The 20-week layouts of this experiment were simulated to follow similar environmental conditions found throughout the intermountain, western United States in order to gain insight into why the yellow toadflax species is so successful in establishing populations across the region. It is not uncommon for the parent individuals of yellow toadflax to asexually produce offspring during the first-year cycle; however, the use of synthetic auxin herbicides such as 2,4-D have the potential to increase sexual and asexual reproductive responses. Yellow toadflax has several defensive responses due to its ability to allocate resources rather quickly and efficiently. By treating the species with both direct and indirect applications, our results indicate that improper management strategies could lead to larger populations the following year with potential increases in resistance, especially within asexual, clonal-patch populations. Several grasses and weed species have the natural ability to resist auxin-type herbicides through several non-target site mechanisms, including cellular structural barriers, reduced translocation, and detoxification [22]. Clonal integration between ramets and genets of yellow toadflax and other rhizomatous clonal species have been reported to increase nutrient availability and to help aid in defense against external disturbances [23-24]. The vegetative growth (ramets and genets) from severely damaged plants can cause the roots to undergo successful root-budding processes, depending on the age of the plant [9]. Although increased asexual, clonal reproduction can lead to reduced genetic diversity within populations, many clonal patch populations of yellow toadflax have been reported to contain highly differentiated, genetical backgrounds due to the likely possibility of multiple introductions in the United States throughout the past couple of hundred years [11]. Yellow toadflax is also known to be an outcrossing species and has hybridized with Dalmatian toadflax throughout the intermountain West [25]. The taxonomic group *Linaria* is very large and complicatedly diverse [26], which may be the underlying cause of why it is so difficult to control the spread of the species.

Our results reveal that direct spot-treatments would be highly recommended for any herbicide management strategy needing to reduce the spread of yellow toadflax. Out of the 74 directly-treated yellow toadflax individuals, the experiments revealed a 98.6% control in first-year individuals using 2,4-D. Similar reports of management strategies using herbicides to control yellow toadflax recommend using repeated treatments with adequate concentrations to ensure significant reductions in yellow toadflax populations. Additionally, applications during the initial flower-production stage resulted in higher percentages of control [6,20-21]. Our data also shows that the most effective period for control occurs between 8 to 12 weeks of growth when flower blooming begins. Flower production is known to cause susceptibilities to external stresses in many plant species due to the lowered amount of reserved resources available; however, in the field, large deposits of nutrients may be circulated through the plant's root system, depending on size, age, competition, and nutrient availability [27]. Indirect applications, as may be the case with broadcast-spraying, may not be effective enough in eliminating the species entirely. The control of invasive yellow toadflax populations has to be based on how well its root system can be eliminated.

Soil-types play a large role in an herbicide's ability to become effectively absorbed into the target species; however, the sorption time of an herbicide in soil is susceptible to biodegradation and/or leaching. We tested plant growth and 2,4-D applications in both sandy-loam and medium-loam soil-types as a matter of demonstrating the effectiveness of herbicide and plant interactions. Loamy, nutrient-rich soils can limit the amount of herbicide movement throughout the soil, but these types of soils usually contain more bacteria capable of degrading the herbicide. The optimum soil-type for the yellow toadflax species is considered to be sandy-loam soil [5], and sandy soils tend to create more leaching potential. In fact, 2,4-D is very degradable and is vulnerable to leaching [28]. 2,4-D analysis from our experiment using Liquid Chromatography and Mass Spectrometry (LC-MS) has demonstrated that on average there were higher concentrations of the herbicide being retained in medium-loam soil-types than in sandy-loam soil-types. Herbicides in the form of amine salts can be used to reduce degradation rates in the soil; however, the concentrations will likely be lowered due to increased amount of leaching potential and location of susceptible waterways or water tables [18]. There have been several attempts to reduce the environmental impact by using single high-rate herbicide treatments, but yellow toadflax control remains inconsistent with these methods thus far [21]. Advancements in molecular plant sciences have begun to shed light on the mechanisms involved with plant resistances associated with developments in plant growth and defense, and *Linaria vulgaris* could be a valuable species used for providing substantial data in the future.

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