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Field performance of a red clover germplasm selected for increased tolerance to 2,4-D

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Abstract

This study compared the field performance of red clover germplasm UK2014, selected for 2,4-D tolerance, to Kenland, a standard variety grown in the transition zone of the United States. UK2014 and Kenland were seeded in the spring of 2017 and 2018. Single applications of 0, 1.12, or 2.24 kg ae ha^{-1} 2,4-D-amine were made in June, August, or October. One week after the treatments, yields were determined. Visible herbicide injury ratings were made prior to harvest and regrowth was visibly assessed 1 wk after harvest. Red clover stands were visibly assessed the following spring. Kenland, across all application timings, was injured by 2,4-D more than UK2014, with mean injury ratings of 39% and 63% compared with 26% and 37% at 1.12 and 2.24 kg 2,4-D ae ha^{-1}, respectively. At equivalent rates, Kenland regrowth was less than UK2014 at all application timings. UK2014 regrowth after 2,4-D treatment ranged from 65% to 91%, whereas Kenland regrowth ranged from 12% to 72%. Applications of 2,4-D in October were the most damaging to stands of both UK2014 and Kenland the following spring, but Kenland stands were reduced much more than those of UK2014. Kenland and UK2014 had similar season total yields when not treated with 2,4-D (means of 7,550 and 7,880 dry matter kg ha^{-1}, respectively in 2017 and 5,280 dry matter kg ha^{-1} for both in 2018). Kenland season total yield in 2017 was reduced by both 2,4-D rates applied in June or August and at all timings in 2018. UK2014 season total yield in 2017 was reduced only when 2.24 kg 2,4-D ae ha^{-1} was applied in August. In 2018, 2.24 kg ae ha^{-1} 2,4-D resulted in reduced UK2014 season total yield across application timings. UK2014 has greater 2,4-D tolerance than Kenland, but additional selection might be beneficial.

Introduction

Red clover is a widely used legume species worldwide. Originating in southeastern Europe and southwestern Asia, it was adopted throughout northern Europe and the Americas by the end of the 17th century, becoming one of the most important cultivated forage legumes (Smith et al. 1985). The widespread use of red clover is due to its many beneficial properties and its adaptability to a wide range of soil types, pH levels, and adverse environmental conditions (Williams and Nichols 2011). Most of the red clover in the United States is grown in pastures and hayfields, although some attention has been given to its use as a cover crop or in rotation with cereal grains (Blaser et al. 2006). Most commonly, red clover is interseeded into grass pastures, either by frost seeding or spring planting, for pasture renovation (Ball et al. 2002; Undersander et al. 1990).

As a legume species, red clover is capable of fixing nitrogen through a symbiotic association with rhizobia (*Rhizobium leguminosarum* bv. *trifolii*; Smith et al. 1985; Williams and Nichols 2011). Nitrogen fixation by the rhizobia can provide more than 95% of the clover's nitrogen requirement. Additionally, when red clover is interseeded into grass pastures, it can provide up to 36% of the nitrogen requirement of the companion crop. This substantially reduces the need for synthetic nitrogen applications and increases the overall system profitability (Heichel and Henjum 1991). Red clover's deep tap root system and the enhanced microbiota in the rhizosphere also confer enhanced soil structure, porosity, increased organic matter, reduced erosion, and decreased soil pH (Berg et al. 1987; Frame et al. 1998; Nyatsanga and Pierre 1973). Compared to a grass monoculture, forage from a clover-grass mixture has higher protein content, which increases animal daily gains (Ball and Prevatt 2009, Nyfeler et al. 2011). Red clover also has a higher content of isoflavones relative to other forage legumes (Harlow et al. 2017). Isoflavones in a ruminant's diet improve both blood flow and nitrogen use efficiency. In tall fescue (*Festuca arundinacea* Schreb.) pastures, particularly common in the transition climatic zone of the United States, red clover reduces toxicity from endophyte-infected tall fescue (Aiken et al. 2016).

Unfortunately, mixing red clover into grass pastures complicates weed management. Red clover is sensitive to the herbicides used for broadleaf weed management in pastures (Ferrell and Sellers 2004; Isobe et al 2014; Robinson et al. 2014). The weed management guide for pastures in Kentucky (Green 2021) includes the statement "In grass pastures interseeded with clover or other forage legumes, selective herbicide options are not available for use as broadcast treatments". Thus, farmers who choose to interseed red clover into their pastures are forced to rely on other, often less effective, weed management strategies. Weeds can reduce both the stand and the quality of desirable forage. Many weed species are poisonous or induce selective grazing by the animals, which ultimately leads to the degradation of the pastures (Marten et al. 1987). Additionally, weed infestations severely decrease the economic and aesthetics value of pastures (Marten et al. 1987; Masters et al. 2020).

Herbicides commonly used in grass pastures are either acetolactate synthase inhibitors (a Group 2 herbicide as classified by the Weed Science Society of America [WSSA]) or synthetic auxins (WSSA group 4; Green 2021; Senseman 2007). Synthetic auxins have been the standard herbicide used for broadleaf weed management in grass pastures due to their affordability and selectivity to grasses. The herbicide 2,4-D is a common active ingredient in many of the labeled products for use in pastures (Robinson et al. 2014; Anderson 1996). Red clover has poor tolerance to 2,4-D. Fortunately, there is enough inherent variability in 2,4-D sensitivity in red clover to select for increased 2,4-D tolerance (Taylor et al. 1989b). A 2,4-D-tolerant red clover would allow farmers to gain the benefits of the legume and use this herbicide in their weed management programs.

There are three major efforts in the United States to increase red clover tolerance to 2,4-D. In 1989, a population of red clover, with greater tolerance to 2,4-D and adapted to the southern region of the United States, was developed at the University of Florida through recurrent selection (Taylor et al. 1989a). The parent material of this population was a mixture of two cultivars, Kenstar and Nolins, and an experimental population, QC5. A mixed red clover stand containing equivalent parts of these three red clovers was treated with 2,4-D, then individual plants that survived the 2,4-D treatment were selected for interbreeding in half-sib families (Quesenberry et al. 2015; Taylor et al. 1989a). The population selected from this study fostered two additional efforts to develop 2,4-D-resistant varieties better adapted to the northern United States. One approach, undertaken in Wisconsin, crossed a mixture of elite varieties from Wisconsin with the 2,4-D-tolerant material from the University of Florida (Riday 2014). The second, in Kentucky, began in 1989 by crossing the tolerant population from the University of Florida with Kenland, a red clover variety widely used by producers in the transition zone. After several cycles of selection for 2,4-D tolerance within the population resulting from the cross with Kenland, a red clover line, designated UK2014, was produced (Lewis 2015). The primary objective of this study was to compare the 2,4-D tolerance as well as the yield potential, with and without 2,4-D treatment, of UK2014 to Kenland under field conditions.

Material and Methods

Development of the Experimental Material

Six cycles of mass recurrent selection were conducted by Dr. Norman Taylor at the University of Kentucky following the initial cross in 1989 between a 2,4-D-tolerant germplasm developed for use in Florida (Taylor 1987) and Kenland, a cultivar adapted for use in Kentucky (Taylor et al. 1989a). The original 2,4-D-tolerant Florida population was subsequently registered as $FL_{24}D$ (Quesenberry et al. 2015). After the six cycles of selection, a 38% decrease in mean 2,4-D injury ratings compared to the cycle zero on the populations treated with 0.56 kg ae ha^{-1} of 2,4-D was observed (data not shown). An additional two cycles of recurrent selection were carried out using a more stringent selection (2.24 kg ae ha^{-1}) and included removal of any injured plants prior to flowering (Lewis 2015). The population from these selections was named UK2013. The red clover cultivar used in our studies, UK2014, was obtained from a seed increase from UK2013 plants.

Site Characteristics and Management

The study was conducted at the University of Kentucky's Spindletop Research Farm in Lexington (38.128889°N, 84.496111°W). The entire study consisted of a replicated experiment in two years, 2017 and 2018. Soil at the study site is a Maury silt loam (fine, mixed, active, mesic Typic Paleudalfs) with 2.6% organic matter and pH 6.5 to 7.0. No supplemental fertilization was applied during the study.

Weeds initially present in the area were controlled by two sequential applications of 1.12 kg ae ha^{-1} of glyphosate (Mad Dog® Plus; Loveland Products, Greeley, CO) on March 21 and March 28, 2017, and on March 8 and March 23, 2018. Due to higher weed pressure in the red clover stand in 2018, clethodim at 134 g ai ha^{-1} (Select Max®; Valent U.S.A. Corporation, Walnut Creek, CA) and imazethapyr at 105 g ai ha^{-1} (Pursuit®; BASF Corporation, Research Triangle Park, NC) were applied postemergence on June 27 and July 4, respectively. Both clethodim and imazethapyr were applied with 1% vol/vol crop oil concentrate (Maximizer®; Loveland Products Inc.). Herbicides were applied using an all-terrain vehicle sprayer, equipped with XR11004 flat fan nozzles (TeeJet®, Wheaton, IL), 243 L of spray solution per hectare, and 275 kPa pressure.

Red clover was seeded into prepared seedbeds at a rate of 13 kg seed ha^{-1} with a drill-disk plot planter. Two varieties were tested for their field performance: Kenland (Victory Seed Company, Molalla, OR) and UK2014 (University of Kentucky, Lexington, KY). Kenland is a red clover variety adapted to the transition zone and widely grown in Kentucky, but it is sensitive to 2,4-D. UK2014 is a cultivar that was bred at the University of Kentucky, and which has increased tolerance to 2,4-D in greenhouse studies (Lewis 2015). The 2017 study was seeded April 3. The 2018 study was initially seeded on April 5. A frost on April 10, 2018, caused significant injury to the newly germinated seedlings. Therefore, on April 13, the entire area for the 2018 study was treated with glyphosate at 1.12 kg ae ha^{-1} (Mad Dog® Plus) to eliminate existing red clover seedlings and was reseeded on the same date.

Experimental Design and Treatments

A randomized complete block design with four replicates was used for the experiments. Each experimental unit consisted of a 1.5-m × 6.0-m plot with six red clover rows spaced 0.20 m apart. The studies were arranged in a 3 × 2 × 2 factorial with the factors being three herbicide rates, three herbicide application timings, and the two red clover cultivars. Herbicide treatments consisted of 2,4-D-amine (Loveland Products Inc.) at a low and a high rate (1.12 and 2.24 ae kg ha^{-1}, respectively). The control (no 2,4-D treatment) was the third 2,4-D rate. The 2,4-D treatments were applied either early-season (June), mid-season (August), or late-season (October). Each plot received only one 2,4-D treatment during the growing season, and treated plots were compared with those

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in plots that were not treated with 2,4-D. No 2,4-D injury symptoms due to drift were observed on plants in the untreated plots. Treatments were applied when the red clover had at least 25% of the plants flowering across the entire population. As previously mentioned, two cultivars of red clover, UK2014 and Kenland, were used for comparison. Casual observations did not detect any relevant differences in the flowering pattern or timing between these two cultivars.

All 2,4-D treatments were applied using a CO_2-pressurized sprayer equipped with a 1.8-m wide boom and TeeJet® 8002 flat fan tip nozzles spaced 51 cm apart. The carrier volume was 243 L ha^{-1} at 207 kPa spray pressure.

Measurements

Plots were harvested approximately 1 wk after 2,4-D treatment. The red clover was at the bud to early-flowering stage at this point with approximately 25% of the plants flowering. Red clover biomass was harvested three times across the year after the early-season, mid-season, and late-season 2,4-D applications. All plots, whether treated with 2,4-D, or not, where harvested 1 wk after each 2,4-D treatment. A sickle-type forage harvester was used to harvest an area of 1.5 × 5.5 m of each plot. Both individual harvest and season total yields (dry matter in kilograms per hectare) were determined. Season total yield consisted of the sum of the three individual harvests across the season.

Visible herbicide injury was assessed before harvest as percentage damaged clover on a scale from 0% (no visibly observable injury) to 100% (death of all plants in the plot). Percent visible red clover regrowth was estimated 1 wk after each harvest using a similar scale from 0% (no regrowth) to 100% (equal to the untreated control). Red clover is considered a perennial forage legume, with a good production in the first year, and reasonable production in the second year (Olson et al. 2021). The red clover stand starts to decline beyond 2 yr of production, with both reduced yield and decreased protein content (Marshall et al. 2017; Olson et al. 2021). The following spring, 1 yr after planting each study (March 17, 2018, and April 3, 2019), the plots were visibly assessed for growth, using a scale from 0% (no growth) to 100% (growth equal to the untreated control). This measurement of spring growth in the second year is an indication of stand persistence.

Statistical Analysis

Data were subjected to ANOVA using the GLIMMIX procedure with SAS software (v9.4; SAS Institute Inc., Cary, NC). The assumptions of the variance analysis were assessed before the ANOVAs for herbicide injury, regrowth ratings, and season total yield. Normality was evaluated by the Shapiro-Wilk test (UNIVARIATE procedure, SAS software v9.4) and heterogeneity of variances was assessed by visible inspection of residual plots. To meet the assumptions of the ANOVA, visible injury data and percent regrowth ratings data were transformed to the arcsine square root of their respective values before analysis. For these variables, normality was met, and homogeneity of residuals was improved after transformation. Data for season total yield met the analysis criteria and was not transformed.

The full model in the GLIMMIX procedure included the fixed effects of red clover cultivar, timing of herbicide application, herbicide rate, and all possible combinations of these factors (concatenation in the model statement as cultivar|timing|rate). Block was considered a random effect. Means were separated with the LINES option of the LSMEANS statement adjusted with the Tukey-Kramer test ($\alpha = 0.05$). Multiple comparison tests were executed in the transformed scale, and means were converted back to the original scale for presentation of results. Years of the study were considered a fixed effect and analyzed separately if higher-order interactions between year and other fixed effects were significant ($P < 0.05$). When interactions with years were not significant, the year effect was pooled with the random variance.

Results and Discussion

Interactions with year and the main effects were not significant ($P < 0.05$) for herbicide injury and regrowth ratings. Thus, for these variables, data from the 2 yr of the study (2017 and 2018) were pooled. There was a significant ($P < 0.045$) four-way interaction for season total yield between year, cultivar, timing, and rate. Therefore, season total yield was analyzed separately for each year of the study.

Injury

There was a significant ($P < 0.035$) interaction between red clover cultivar and 2,4-D rate for herbicide injury assessed 1 wk after treatment for both 2017 and 2018. Both UK2014 and Kenland cultivars were injured by both rates of 2,4-D (Table 1). However, Kenland was injured more than UK2014 at each 2,4-D rate. For example, the injury observed on UK2014 following treatment with 2.24 kg ae ha^{-1} 2,4-D (37%) was equivalent to that observed on Kenland treated with 1.12 kg ae ha^{-1} 2,4-D (39%).

The relatively high 2,4-D tolerance of Kenland in our study was surprising based on previous observations by Lewis (2015) in greenhouse studies. The performance of red clover seeded in greenhouses is necessarily different from when it is broadcast or drilled in field conditions (Taylor and Quesenberry 1996). Individual plants were treated in a greenhouse setting in Lewis's study, whereas we treated populations in a field setting. We did find, as Lewis (2015) did, that the red clover selections, UK2013 in her study and UK2014 in this study, were more tolerant of 2,4-D than Kenland (Table 1). We did not find, however, as large a difference in the 2,4-D tolerance between Kenland and UK2014 as Lewis (2015) did between UK2013 and Kenland. Overlapped red clover foliage in our study may have reduced exposure of some Kenland plants to the 2,4-D treatment. This lower exposure may have, in turn, resulted in less injury to some of the Kenland plants in the stand compared with the injury to individual plants observed by Lewis (2015).

Injury to Kenland and UK2014 from 2,4-D was consistent across years and timings of application in our study. Regardless of what time of the year the 2,4-D was applied, both Kenland and UK2014 were injured by the herbicide. Although UK2014 was injured less than Kenland, the injury to UK2014 was still 26% at the 1.12 kg ae ha^{-1} 2,4-D rate (Table 1). In contrast to our results, Lewis (2015) observed only 25% injury to UK2013 from 2.24 kg ae ha^{-1} 2,4-D. In our study, we considered 1.12 kg ae ha^{-1} as a 2,4-D field rate that would manage most annual broadleaf weeds. The higher 2,4-D rate (2.24 kg ae ha^{-1}) was included to test the safety of a 2,4-D rate that is twice the standard 2,4-D (ester or amine) rate recommended for pastures (Green 2021). A rate of 2.24 kg ae ha^{-1} 2,4-D might also offer some utility for control of perennial weeds in a pasture. However, the high level of injury

Table 1. Effect of cultivar and 2,4-D rate on red clover injury 1 wk after treatment and cultivar, 2,4-D rate, and timing of 2,4-D application on red clover regrowth 1 wk after harvest.[a]

Cultivar	Application rate	Injury[b]	Regrowth: 2,4-D Application timing Early[c]	Mid	Late
	kg 2,4-D ae ha^{-1}	%	Visible rating 0 to 100[d]		
Kenland	0 (untreated)	0 a	100 a	100 a	100 a
	1.12	39 c	72 c	34 d	55 c
	2.24	63 b	31 d	12 e	12 d
UK2014	0 (untreated)	0 a	100 a	100 a	100 a
	1.12	26 d	91 b	84 b	93 b
	2.24	37 c	68 c	65 c	85 b
Standard error		3.15	2.41	3.59	3.23

[a]Means followed by a different letter within the same column represent significant differences by Tukey's honestly significant difference test ($P < 0.05$).
[b]Percent visible injury where 0% is no injury and 100% is dead plants.
[c]Early, Mid, and Late correspond to 2,4-D applications in June, August, and October, respectively.
[d]Visible regrowth rating where 100 is regrowth equal to the untreated control and 0 is no regrowth.

(37%) to UK2014, from 2.24 kg ae ha^{-1} 2,4-D, suggests that caution will be needed during application of a lower 2,4-D rate to avoid excessive injury.

Regrowth After Harvest

There was small but significant ($P < 0.045$), interaction between clover cultivar, 2,4-D application rate and 2,4-D application timing on red clover regrowth 1 wk after harvest in 2017 and 2018. Compared with the control plants, regrowth of Kenland was reduced by both 2,4-D rates (Table 1). UK2014 regrowth was also reduced by both 2,4-D rates compared with its respective controls. However, at the same rate of 2,4-D and at the same application timing, UK2014 regrowth was reduced less than that of Kenland. UK2014 regrowth following treatment with 2,4-D at 1.12 kg ae ha^{-1} ranged between 84% and 91% of the control. A further reduction of 32% to 35% in UK2014 regrowth was observed when the 2,4-D rate was increased to 2.24 kg ae ha^{-1} in the early-season and mid-season treatments. The high 2,4-D rate had less effect (15% reduction) on UK2014 regrowth at the late-season application.

Red clover yield and stand persistence are highly related to the regrowth capability of the crown (Taylor and Quesenberry 1996). Van Minnebruggen et al. (2014) demonstrated that regrowth is largely determined by both the number of meristems that remain after harvest and their capacity to grow out. We observed in greenhouse evaluations of red clover 2,4-D tolerance that regrowth of plants after harvest (clipping 2 wk after 2,4-D treatment) was a more sensitive measure of tolerance than initial injury (data not shown). Because of these observations, we believe that assessments of red clover regrowth 1 wk after harvest (2 wk after 2,4-D treatment) in the field is more an indicative measure of the red clover tolerance than initial injury.

There were large differences in regrowth between UK2014 and Kenland following 2,4-D application (Table 1). The 2,4-D tolerance of Kenland, as measured by regrowth, appeared to decrease later in the season. After the late application, the 2,4-D tolerance difference between UK2014 and Kenland, expressed as regrowth, was at its highest. Although specific data on plant architecture was not taken in our study, casual observations did not detect any phenotypic or architectural differences between Kenland and UK2014. It may be that the 2,4-D injured more meristems in Kenland than UK2014, which caused a slower regrowth rate in Kenland. Lewis (2015) demonstrated that UK2014 translocated less of the absorbed 2,4-D than Kenland from treated leaves to other tissues. It is well demonstrated that a lower translocation and concentration of leaf-applied 2,4-D to meristems is related to increased tolerance to the herbicide (Han et al. 2013; Hill et al. 1980; Riar et al. 2011).

The effects of the regrowth differences following 2,4-D treatment between Kenland and UK2014 on yield and stand persistence may be magnified in mixed red clover–grass pastures. In the mixed system, reduced regrowth would make the red clover less competitive with the companion grass species. Studies with red clover interseeded with annual ryegrass (*Lolium multiflorum* Lam.) suggest that the number of red clover basal growing points is a prime determinant of competitive ability with the grass companion (Van Minnebruggen et al. 2014). Riday (2016) used fine fescue sod (65% *Festuca rubra* L., 20% *F. trachyphylla* (Hack.) Krajina, and 15% *F. ovina* L.) to establish nurseries for the development of the 2,4-D-tolerant cultivar (WI-2,4D12) developed for use in Wisconsin. In that work, the WI-2,4D12 germplasm was more competitive with the companion grass following 2,4-D treatment compared with the 2,4-D sensitive germplasm. Although such a competitive advantage for UK2014 compared to Kenland is suggested from our results, further studies with UK2014 and Kenland in a grass-red clover mixed system are needed to confirm this.

Stand Persistence

The red clover stand in the spring of the second year from establishment potentially impacts the second-year yield, even though this parameter was not measured in this study. In addition, the vigor of a red clover stand in the spring stand could determine whether reseeding red clover was needed to renovate the pasture. There was a significant interaction ($P < 0.0014$) between clover cultivar, 2,4-D application rate, and 2,4-D application timing on stand persistence, as measured by red clover growth in the spring 1 yr after planting. Although some spring growth reductions were caused by the early-season and mid-season applications of 2,4-D the previous year, none were greater than 11% (Table 2). The high 2,4-D rate (2.24 kg ha^{-1}) applied at any of the timings reduced spring growth of both cultivars. The magnitude of the spring growth reductions, like the results for regrowth following harvest (Table 1), for both Kenland and UK2014, was much greater when 2,4-D applications took place late in the growing season. Although these reductions were greater for Kenland, 70% and 89% at 1.12 and 2.24 kg ha^{-1} 2,4-D, respectively, than for UK2014, 18% and

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Table 2. Effect of cultivar, 2,4-D rate, and timing of 2,4-D application on red clover stand persistence, as measured by growth in the Spring 1 yr following establishment.[a]

Cultivar	Application rate	Spring growth[b] 2,4-D Application timing: Early[c]	Mid	Late
	kg 2,4-D ae ha^{-1}	Visible rating 0 to 100[d]		
Kenland	0 (control)	100 a	100 a	100 a
	1.12	94 b	99 a	30 d
	2.24	93 b	89 c	11 e
UK2014	0 (control)	100 a	100 a	100 a
	1.12	99 ab	99 a	82 b
	2.24	94 b	92 b	68 c
Standard error		0.43	0.28	0.31

[a]Means followed by a different letter within the same column represent significant differences by Tukey's honestly significant difference test ($P < 0.05$).
[b]Spring growth ratings were taken in March 2018 and April 2019, for red clover planted in 2017 and 2018, respectively; 2,4-D was not applied in either 2018 or 2019.
[c]Early, Mid, and Late correspond to 2,4-D applications in June, August, and October the previous year.
[d]Visible spring growth rating where 100 is growth equal to the untreated control and 0 is no growth.

Table 3. Effects of 2,4-D rate, sliced by timing of 2,4-D application in the 2017 growing season, and across application 2,4-D timings in the 2018 growing season, on season total yields of Kenland and UK2014.[a].

Cultivar	Application rate	Growing season 2017, 2,4-D Application timing: Early[b]	Mid	Late	2018: Mean across 2,4-D application timings[c]
	kg 2,4-D ae ha^{-1}	Dry matter in kg ha^{-1}			
Kenland	0 (untreated)	7,320 a	7,730 a	7,610 a	5,280 a
	1.12	4,440 b	3,530 b	8,010 a	3,400 c
	2.24	3,870 b	36,50 b	8,350 a	3,130 c
UK2014	0 (untreated)	8,560 a	7,610 a	7,460 a	5,280 a
	1.12	7,510 a	7,090 a	7,040 a	5,010 ab
	2.24	7,300 a	4,950 b	8,440 a	3,930 b
Std Error		590	570	700	400

[a]Means followed by a different letter within the same column represent significant differences by Tukey's honestly significant difference test ($P < 0.05$).
[b]Early, Mid, and Late correspond to 2,4-D applications in June, August, and October 2017, respectively.
[c]The interaction cultivar*rate*timing was not significant for 2018 data; the next highest order significant interaction was cultivar*rate, thus results for 2018 are presented across application timing.

32% at the same 2,4-D rates, respectively, the reductions in spring regrowth are high enough for concern with applying 2,4-D late in the season to UK2014. As was hypothesized with regrowth following harvest, there may not be enough time for recovery from 2,4-D injury before winter with the late applications, which could also affect growth in the following spring.

Red clover is considered a short-lived perennial, mainly cultivated for 2 or 3 yr with acceptable yield (Boller et al. 2010; Smith et al. 1985). If the number of harvests is kept to two or three across the season in the second year after establishment, most modern red clover cultivars would yield comparably well in both the first year and second year after establishment (Wiersma et al. 1998). Crude protein levels, and the general quality of the red clover forage, are significantly lower in the second year compared with the first year after establishment (Cassida et al. 2000; Krawutschke et al. 2012). Even though forage quality diminishes, forage producers would likely maintain red clover in their pastures after the first year because plants still provide reasonable yield, and pasture renovation is costly. The reductions in stand persistence following 2,4-D use, especially with late-season applications, observed in this study, are therefore a matter of concern. Further efforts to develop a 2,4-D-tolerant red clover cultivar should focus on the impact of 2,4-D treatments on the red clover stand persistence.

Season Total Yield

Season total red clover yield in 2017 ranged from 3,530 kg ha^{-1} to 8,560 kg ha^{-1} and in 2018 from 3,130 to 5,280 kg ha^{-1} (Table 3). For comparison, Kenland yield in the University of Kentucky forage variety trials was 4,980, 12,100, and 8,100 kg ha^{-1} in 2019, 2020, and 2021, respectively (Olson et al. 2021). UK2014 and Kenland had equivalent season total yields in 2017 and 2018 without 2,4-D treatment (Table 3). There was a significant ($P < 0.003$) interaction between cultivar, 2,4-D treatment timing, and 2,4-D rate for season total yield in 2017. Both 2,4-D rates (1.12 and 2.24 kg ha^{-1}) at the early application timing caused similar reductions (39% and 47%, respectively) in Kenland season total. However, there was no effect of either 2,4-D rate on UK2014 season total yields in 2017 from the early applications.

Like the early treatments, both 2,4-D rates applied at mid-season in 2017 reduced Kenland season total yields (54% and 53%, respectively; Table 3). Mid-season application of 1.12 kg ae ha^{-1}

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2,4-D did not reduce UK2014 season total yield, but UK2014 yield was reduced by 34% by 2.24 kg ae ha^{-1} 2,4-D at the same time. Yield of either Kenland or UK2014 was not reduced by 2,4-D applied late-season in 2017. This is not surprising because the yield of these plots at the first and second harvests would have been the same as the untreated plots. In addition, it is unlikely that the biomass of the plants 1 wk following 2,4-D treatment, despite the apparent 2,4-D injury observed (epinasty), would have been significantly reduced.

In 2018, there was not a significant interaction between cultivar, 2,4-D treatment, and 2,4-D rate for season total red clover yields. The only significant ($P < 0.039$) interaction in 2018 for season total yield was between red clover genotypes and 2,4-D rate. Kenland season total yields were reduced by both 2,4-D rates (Table 3). UK2014 season total yields were reduced by 2.24 kg ae 2,4-D ha^{-1} but not 1.12 kg ha^{-1}. Kenland had a lower season total yield than UK2014 following treatment with 1.12 kg ae ha^{-1} or 2.24 kg ae ha^{-1} 2,4-D.

Yield, in absolute numbers, was higher in 2017 than in 2018. This could be related to the later planting in 2018 caused by the need to replant due to the poor stand after the first attempt at stand establishment in 2018. The poor stand was caused by a late-spring freeze that killed the newly emerged red clover seedlings. Thus, there was a 2-wk difference in the establishment time between the studies in 2017 and 2018. Essentially, 2018 had a shorter growing season than 2017, resulting in lower yields across the season.

Total yields with earlier 2,4-D application timings are a function of initial 2,4-D injury plus the regrowth rate after harvest. As previously noted, the regrowth of Kenland after individual harvests was substantially lower than UK2014 regrowth, particularly in plots treated mid-season (Table 1). This effect could result in season total yield reductions. The slower regrowth of Kenland when treated with either 2,4-D rate (at all application timings) and the slower regrowth of UK2014 treated with the high 2,4-D rate (at the early-season and mid-season applications) resulted in yield reductions for Kenland in 2017 and across application timings in 2018. Similarly, UK2014 yield was reduced by the high rate of 2,4-D in 2018.

The equivalent yields of Kenland and UK2014, when not treated with 2,4-D, is an important finding (Table 3). Apparently, the initial cross with the 2,4-D-tolerant germplasm developed for use in Florida to transfer of the 2,4-D tolerance and subsequent selection has not resulted in any yield penalty in UK2014 relative to Kenland.

Genetic mapping of the tolerance phenotype derived from the initial 2,4-D resistance in red clover by Taylor et al (1989b) demonstrated that inheritance of the trait is quantitative (Benevenuto et al. 2019) and selection is required to obtain adapted varieties. Our study demonstrated that significant progress has been made toward developing germplasm to transfer 2,4-D tolerance to a red clover cultivar that would be adapted to the transition zone of the United States as UK2014 produces similar yields to the well-adapted Kentucky cultivar, Kenland. Although we found a surprising level of tolerance to 2,4-D in Kenland, particularly in terms of surviving 2,4-D applications, there was, nevertheless, several indications of 2,4-D damage to Kenland. Kenland season total yield was reduced by application of 1.12 kg ae ha^{-1} 2,4-D at the early-season or mid-season timing (Table 3). The total season yield of UK2014 was less affected by 2,4-D treatments, indicating a higher tolerance to the herbicide. Most importantly, a substantial gain was obtained in improving 2,4-D tolerance in the UK2014 line, particularly in terms of regrowth after harvest, second-year spring regrowth, and season total yields (Tables 1, 2, and 3). Future efforts will be directed toward further selection of the UK2014 population and assessing the persistence of UK2014 under pasture-based settings.

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