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Grasspea (*Lathyrus sativus* L.) as a Green N Source Reduces the Effects of 2,4 D in Susceptible Cotton (*Gossypium hirsutum* L.) Cultivars

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Abstract

Cotton (*Gossypium hirsutum* L.) production in Oklahoma has more than tripled in the last ten years. This increase in cotton acreage in Oklahoma, a region that traditionally produces winter wheat, has led to increased incidences of 2,4 D contamination in sprayers that are used to spray cotton crops. Cotton is extremely sensitive to 2,4 D, with losses of yield and ultimately profit to the cotton producer in cases of drift or tank carryover. In this study, six cotton cultivars (three 2,4 D susceptible and three 2,4 D tolerant) were grown in combination with four N treatments to determine the benefits or drawbacks of using a green manure as N source. Seedcotton, seed, and lint yield were all negatively impacted by 2,4 D in the susceptible cultivars. Additionally, water use was increased in the susceptible cultivars compared to the tolerant cultivars. The negative effects of 2,4 D on cotton growth were partially mitigated by grasspea, though the supply of too much nitrogen increased the negative effects of 2,4 D. Applying nitrogen to a susceptible cultivar of cotton contaminated by 2,4 D is not recommended for short season environments like Oklahoma.

Keywords

Gpssypium Hirsutum, 2,4 D, Green Manure

1. Introduction

Winter wheat has long been the primary grain and forage crop in the southern Great Plains (SGP) of the U.S. [1]. Wheat is capable of producing multiple saleable commodities (grain, hay, grazing) from one planting [2] [3] [4] [5]. However, wheat is susceptible to regular cycles of over-production, resulting in low prices for grain and pasture [2]. In response to recent downturns in the value of wheat as grain, producers in Oklahoma have begun researching other crops with potential for producing a better economic response [4] [6]. One such candidate is cotton (*Gossypium hirsutum* L.), an important fiber and seed crop grown throughout the world.

In Oklahoma, cotton production has increased from ~60,000 hectares to ~260,000 hectares from 2008-2018 [7]. Although cotton is again becoming popular in Oklahoma as a fiber crop, there is little information in the region about nitrogen and water use efficiency of cotton when grown within cropping systems. In particular, there is a shortage of information on the function of cotton in rotation with green manures, an area of current interest of Oklahoma producers for most cash crops. In Australia, and in other parts of the U.S., green manures such as field peas (*Pisum* spp.), clovers (*Trifolium* spp.), and lablab (*Lablab purpureus*) have been grown to reduce synthetic nitrogen fertilizers in a subsequent cotton crop [8] [9].

One annual legume with potential as a green manure is grasspea (*Lathyrus sativus* L.). Grasspea is an under-utilized old-world cool-season species that originated in the highlands regions of Ethiopia. It is tolerant of drought and low-fertility soils, and has largely been grown for grain in northern Africa, the southern Mediterranean, and the Indian sub-continent [10]. Though under-utilized globally, the promise of grasspea as a grain, forage, or green manure for more arid regions has resulted in continual development and testing of new cultivars in many countries, including Canada [11], and the Mediterranean region [12]. Studies in Oklahoma [13] reported grasspea produces 6500 (±1200) kg ha^{-1} yr^{-1} of aboveground biomass by early May (75 days after planting) containing 174 (±32) kg N ha^{-1} yr^{-1}. These N levels approximate the upper range of nitrogen applications (112 - 224 kg ha^{-1}) required for optimum cotton production in the United States [14]. Additionally, legumes such as hairy vetch (*Vicia villosa* Roth) or faba beans (*Vicia faba* L.) could also reduce the need to apply inorganic N to cotton to 0 kg ha^{-1} [15]. However, lint yield and fiber quality of cotton have shown mixed responses to some legume-based sources of green N [9].

One additional challenge to cotton production in an area traditionally associated with wheat is the potential for 2,4 D (2,4 dichlorophenoxyacetic acid) drift or tank carryover from applications applied for broadleaf weed control in cereals. Cotton is extremely sensitive to 2,4 D [16] [17] [18]. However, new Enlist™ cottons from Corteva have a gene (aad-12), which provides tolerance to 2,4 D. Cultivars with tolerance to 2,4 D can help to effectively control weeds, thus improving cotton production systems [19].

The objective of this study was to use soil and plant measurements of nitrogen to investigate the impact of 2,4 D on tolerant and sensitive cotton plants in a green manure and synthetic nitrogen based cotton production system in Oklahoma.

2. Materials and Methods

2.1. Study Site

The study was undertaken at the USDA-ARS Grazinglands Research Laboratory, near El Reno, OK (35˚34'N; 98˚2'W, 414 m a.s.l.) during the March to October time periods of 2019 and 2020. The study was organized in a randomized complete block design, with three blocks based on different positions along a continuous slope (tread slope, upper riser, and middle riser positions). Within blocks, 24 combinations of amount and form of N ($n = 4$) and cotton cultivars ($n = 6$) were randomly assigned to 2-row plots, 9.1 m in length with 0.76 m between rows and ~0.08 m inter-row spacing of plants within rows, with rows oriented in an East-West configuration.

Soils at the study site were described as members of the Norge silt loam (fine-silty, mixed themic, Udic Paleustolls) series, with slopes ranging from 1% to 5% [20]. These soils evolved from parent material defined as Permian-aged Dog Creek shale, a reddish-brown shale with thin inter-beds of reddish-brown sandstone and siltstone [21]. The surface (0 - 30 cm) layers of these soils have low permeability [33 (±25) $mm \cdot h^{-1}$], water-holding capacities of 0.15 (±0.12) $cm \cdot cm^{-1}$ soil, and moist bulk densities of 1.36 (±0.12) $g \cdot cm^{-3}$.

Initial soil tests for pH, NO_3-N, P, and K in the 0 to 15 cm and 15 to 30 cm depths were conducted prior to grasspea planting in March 2019 and March 2020. Samples were collected from plots using soil probes, and evaluated at the Oklahoma State University Soil, Water and Forage Analytical Laboratory. Samples were also evaluated in-house for percentage of total C and N in soils with a LECO CN 928 (St. Joseph, MI, USA). In addition, amounts of gravimetric water in samples were assessed. Initial 2019 soil tests indicated: pH 5.8 (±0.4), NO_3-N 5.7 (±2.4) kg ha^{-1}, P 339.6 (±117.0) kg ha^{-1}, K 651.7 (±149.3). Based on these data, a producer would need to apply 4.3 Mg ECCE ha^{-1}, 100.7 (±4.9) kg ha^{-1} N and 2.2 kg ha^{-1} P for a 435 kg per hectare lint yield (Oklahoma average lint yield). Initial 2020 soil tests indicated: pH 6.4 (±0.3), NO_3-N 9.6 (±2.5) kg ha^{-1}, P 127.4 (±63.6) kg ha^{-1}, K 560.7 (±56.3). Based on these data, a producer would need to apply 92.8 (±4.9) kg ha^{-1} N and 38.9 (±17.1) kg ha^{-1} P for a 435 kg per hectare lint yield (Oklahoma average lint yield).

2.2. N Treatments

In both years, the experiment consisted of four organic and inorganic nitrogen treatment combinations. The treatment combinations were 1) unfertilized (no grasspea or inorganic nitrogen applied nitrogen), 2) grasspea + 0 kg ha^{-1} inorganic nitrogen, 3) grasspea + 30 kg ha^{-1} inorganic nitrogen, 4) grasspea + 60 kg ha^{-1} inorganic nitrogen. Inorganic nitrogen was applied to the cotton approximately two weeks prior to flowering. In 2019, seed from grasspea was sown at 67 kg ha^{-1} on 19 March and terminated on 16 May by a vertical tillage implement and lime was applied at a rate of 4.3 Mg ha^{-1} effective calcium carbonate equivalence (ECCE). In 2020, grasspea was sown at 67 kg ha^{-1} on 25 February and over-seeded on 04 April due to poor responses to growing conditions. On May 13th, the grasspea was terminated by a vertical tillage implement and phosphorous was applied to all plots to amend phosphorous deficiencies identified by soil testing.

2.3. Cotton Treatments

In 2019 and 2020, cotton was planted on May 28th and May 18th respectively. Seed of six commercial cultivars of cotton (FiberMax 1830 GLT, FiberMax 1888 GL, FiberMax 2498 GLT, Phytogen 300 W3FE, Phytogen 350 W3FE, and Phytogen 490 W3FE) were planted. The FiberMax cultivars were 2,4 D susceptible while the Phytogen cultivars were 2,4 D tolerant. All plots were sprayed with Staple (Pyrithiobac sodium Sodium 2-chloro-6-[(4,6-dimethoxypyrimidin-2-yl) thio] benzoate) to control broadleaf weeds approximately four weeks after planting. The sprayer was previously used for applying 2,4D to winter wheat thus providing 2,4 D carry over. At the end of each growing season, Folex (Tribufos) and Super boll (Ethephon) were applied at recommended rates when 60 to 70 percent of harvestable bolls were open, to increase defoliation and boll opening.

2.4. Data Collection

Measurements of plant height, number of nodes, leaf area index (LAI), fractional coverage by green canopy, chlorophyll content, leaf moisture, and percent N and C in leaves were collected at critical stages of growth during the growing season. Stages included flower initiation, peak bloom, cutout (five nodes above white flower), and first open boll. Plant height and number of nodes was taken from three randomly selected plants within each replication. The LAI was measured with a Li-Cor LAI 2200C (Lincoln, NE, USA). Fractional coverage by green canopy (% ground cover) was obtained with the canopeo application [22] on a Samsung Galaxy S10+, at a height approximately 1.0 m above the plant canopy. Chlorophyll content was obtained using an Opti-Sciences CCM 300 (Hudson, NH, USA) on the most recently matured leaf from the apical meristem (fifth leaf from the terminal) from five randomly selected plants within each plot. These same five leaves were then removed from the plant, weighed, and oven dried at 65˚C to determine moisture content of leaves. The leaves were then ground to a 2.0 mm particle size using a Thomas Scientific Wiley mill for laboratory analysis (Swedesboro, NJ, USA). The leaf samples were then evaluated for C and N concentrations with a Vario Macro Cube Organic Elemental Organizer (Langenselbold, Germany).

After all mature bolls were opened at the end of growing seasons, 1.0 m of row lengths were hand harvested from each plot. Samples were then ginned on a Dennis Manufacturing 10-saw gin (Athens, TX, USA). Measurements for seed-cotton weight, seed weight, lint (fiber) weight, 25 seed weight, and lint percent were obtained for all samples. Fiber quality was analyzed by High Volume Instrument (HVI) [Uster HVI 1000 using a 1-2-2 protocol for fiber quality measurements (micronaire, length, uniformity, strength, elongation, leaf trash)]. The 1-2-2 protocol is the USDA standard for testing all U.S. cotton, it measures each sample twice for color, once for micronaire, two times for length, and two times for strength.

2.5. Soil Moisture

Amounts of soil moisture present during the growing seasons was monitored by neutron density gage (Campbell Pacific Nuclear International, model 503 DR, Martinez, CA, USA). Approximately four weeks after planting the cotton in 2019, access tubes for moisture measurements were installed. Aluminum access tubes to 1.2 m depths were inserted into soil of two replicate plots per treatment at mid-points in plots, at locations that were equal distance between rows. The tubes were covered with PVC covers when not in use to prevent water and debris entry. Moisture readings were monitored at depths between 0.1 to 0.9 m in depth, in 0.2 m increments. Readings by neutron moisture gage began approximately two weeks prior to flowering and were continued at the growth stages of peak bloom, cutout, and first open boll.

2.6. Data Analysis

Data were analyzed using the GLIMMIX procedure in SAS 9.4. The nitrogen rate by 2,4 D tolerance main effects and interaction were considered the fixed effects while the intercept was considered a random effect with year as the subject (level). Mean separation and determination of least significant differences were evaluated using the Tukey adjustment or Tukey-Kramer adjustment for unbalanced designs. When data did not follow a normal distribution, the link/ilink functions (link the data scale to the model scale) were used. For all statistical analyses, effects were declared significant at the 0.05 probability level.

3. Results

3.1. Impact of Nitrogen Rate

The two growing seasons of 2019 and 2020 were different in terms of rainfall distribution and amounts. In 2019, the rainfall was poorly distributed with most rainfall occurring April to May (**Table 1**). In 2020, the rainfall was better distributed with good levels of precipitation during cotton flowering. Water use by cotton decreased with increases in nitrogen applied, with the highest water use in treatment 1 (310 mm for tolerant and 312 mm for susceptible) and the smallest water use in treatment 4 (301 mm for tolerant and 308 mm for susceptible). The F-test showed significant ($p \le 0.05$) differences in water use between nitrogen rates. However, the post-hoc test was non-significant ($p \le 0.05$).

Nitrogen rate also significantly affected ($p \le 0.05$) seedcotton yield, 25 seed weight, seed yield, lint yield, and fiber micronaire (**Table 2**). Seedcotton yield decreased by 606 kg ha^{-1} for the tolerant cultivars and 266 kg ha^{-1} for the susceptible cultivars with change in N rate from treatment 1 to treatment 4. The 25 seed

Table 1. Temperatures and amounts of precipitation recorded during different proportions of the March to October periods of the study; LTA were long-term averages (±1 std. dev.) for 1990 to 2020.

	Temperature (˚C)			Precipitation (mm)		
Time Periods	2019	2020	LTA	2019	2020	LTA
Mar-May	13.8	14.8	15.3 (±6.4)	602	190	312 (±217)
Jun-Aug	26.0	25.8	26.7 (±3.3)	217	338	314 (±190)
Sep-Oct	19.2	16.4	19.3 (±5.8)	114	62	172 (±78)
Annual	14.7	14.9	15.7 (±9.8)	933	590	942 (±407)

Table 2. Yield and fiber quality traits of 2,4 D tolerant (tol) or susceptible (sus) cotton plants at El Reno, OK 2019 and 2020 under multiple N rates.

		Tol	Sus	Tol	Sus	Tol	Sus	Tol	Sus	SE	Tolerance	N rate
		0 N		Grasspea only		Grasspea + 30 kg ha^{-1} inorganic N		Grasspea + 60 kg ha^{-1} inorganic N			p-value	
Seedcotton yield	kg ha^{-1}	1657	784	1141	835	1375	719	1051	518	168	<0.01	<0.01
25 seed weight	g	2.20	2.20	2.10	2.23	2.08	2.31	1.92	2.09	0.1	<0.01	0.01
Seed yield	kg ha^{-1}	776	368	548	399	630	323	501	227	79	<0.01	<0.01
Lint yield	kg ha^{-1}	865	410	588	429	733	390	536	287	93	<0.01	<0.01
Fiber micronaire		3.8	3.8	3.5	3.7	3.4	3.3	3.1	3.2	0.2	0.55	<0.01
Fiber length	mm	27.4	26.9	27.0	27.7	27.5	28.0	27.1	27.9	0.5	0.07	0.28
Fiber uniformity	%	81.1	80.1	80.8	81.1	80.7	80.0	79.8	79.6	0.1	0.55	0.64
Fiber strength	kN, m·kg^{-1}	294	285	288	300	291	294	284	288	11	0.60	0.70
Fiber elongation	%	6.1	5.7	6.2	5.8	6.3	5.7	6.1	5.6	0.2	0.27	0.99

Note: for all statistical analyses, effects were declared significant at the 0.05 probability level. Tolerance x N rate interactions were not significant. SE is the standard error for the Tolerance x N rate interaction.

weight also decreased with increasing nitrogen rate for the tolerant cultivars (2.20 g to 1.92 g). However, the 25 seed weight increased for the susceptible cultivars as nitrogen increased, except for the highest applied rate. The tolerant cultivars showed a decrease in seed yield as nitrogen rate increased, except for a slight increase for treatment 3 compared to treatment 2. For the susceptible cultivars, the seed yield was greatest in response to treatment 2 (399 kg ha^{-1}) and the least in response to treatment 4 (277 kg ha^{-1}). Lint yield followed the same trend as seed yield of both tolerant and susceptible cultivars, resulting in a decrease of 329 kg ha^{-1} for the tolerant cultivars and 142 kg ha^{-1} for the susceptible cultivars. Both tolerant and susceptible cultivars showed decreases in fiber micronaire with increase in nitrogen rate. Both tolerant and susceptible cultivars were in the premium price range for cotton fiber in response to treatment 1, while all cultivars were in the discount price range in response to treatment 4. Overall, the traits that were significantly impacted by nitrogen rates, with the largest effects noted for treatment 1 and lowest for treatment 4. This response was likely related to delays in maturity caused by increasing amounts of applied nitrogen.

3.2 Impact of 2,4 D Tolerance

The traits of fresh weights of leaves, dry weights of leaves, and percent moisture were significantly impacted ($p \le 0.05$) by tolerance to 2,4 D (**Table 3**). Fresh weight was always highest in the tolerant cultivars, with ranges of 8.04 g to 7.40 g, while the susceptible cultivars ranged from 7.43 g to 6.41 g. Dry weight of leaves, were also higher in the tolerant cultivars with ranges of 2.19 g to 2.03 g, while susceptible cultivars ranged from 1.85 g to 1.60 g. Percent moisture was highest in the susceptible cultivars (76.85 percent to 75.86 percent), and lowest in the tolerant cultivars (75.11 percent to 73.16 percent).

Seedcotton yield, 25 seed weight, seed yield, and lint yield were all impacted by the tolerance of cultivars to 2,4 D. When averaged across all nitrogen rates, 2, 4 D susceptibility resulted in a decrease in seedcotton yield of 592 kg ha^{-1}. However, 2,4 D susceptibility resulted in an average increase of 0.13 g for 25 seed weights. The seed yield of 2,4 D susceptible cultivars were, averaged across nitrogen rates, 285 kg ha^{-1} lower than the tolerant cultivars, and lint yield was 302 kgha^{-1} lower. Overall, 2,4 D susceptibility resulted in a 9% to 46% decrease in the traits that were significantly affected. Surprisingly, water use and 25 seed weight increased in cultivars that were susceptible to 2,4 D.

Table 3. Vegetative measurements of 2,4 D tolerant (tol) or susceptible (sus) cotton plants at El Reno, OK 2019 and 2020 under multiple N rates.

		Tol	Sus	Tol	Sus	Tol	Sus	Tol	Sus	SE	Tolerance	N rate
		0 N		Grasspea only		Grasspea + 30 kg ha^{-1} inorganic N		Grasspea + 60 kg ha^{-1} inorganic N			*p*-value	
Chlorophyll content	m·gm^{-2}	458	463	458	451	469	465	472	482	33	0.95	0.79
Leaf fresh weight	g	8.04	6.41	7.40	728	7.69	6.94	7.73	7.43	0.68	0.04	0.90
Leaf dry weight	g	2.19	1.60	2.03	1.82	2.03	1.72	2.16	1.85	0.20	<0.01	0.80
Percent moisture	%	74.61	76.85	74.64	76.47	75.11	76.16	73.16	75.86	0.51	<0.01	0.53
Plant height	cm	52.39	50.70	53.12	51.46	52.51	51.56	54.42	54.37	4.20	0.60	0.78
Number of nodes		10	10	9	10	10	10	11	11	1	0.69	0.17
LAI	m^{2}m^{-2}	2.18	2.30	2.42	2.41	2.46	2.81	2.33	3.02	0.33	0.08	0.20
Canopeo	%	48.92	47.23	51.36	51.95	51.52	52.38	50.40	52.96	0.05	0.33	0.23
Leaf C	%	39.98	39.18	40.44	39.40	40.41	39.93	39.98	39.53	0.04	0.17	0.86
Leaf N	%	4.14	4.20	4.17	4.23	4.14	4.17	3.97	4.12	0.10	0.70	0.94
Water use	mm	310	312	308	312	304	305	301	308	4	0.09	0.03

Note: For all statistical analyses, effects were declared significant at the 0.05 probability level. Tolerance x N rate interactions were not significant. SE is the standard error for the Tolerance x N rate interaction.

4. Discussion

Although cotton has been evaluated under different green manures and amounts of applied synthetic N fertilizer in Oklahoma and around the world, few studies have examined the effect of 2,4 D tolerance or susceptibility. This is likely related to the relatively new development of 2,4 D tolerance in cotton, combined with the sensitivity of cotton to 2,4 D [16] [17] [18] [19] [23]. In this study, both nitrogen rate and 2,4 D tolerance impacted seedcotton yield, 25 seed weight, seed yield, and lint yield of the cultivars. Nitrogen rate additionally affected water use, while 2,4 D tolerance also affected leaf fresh weight, leaf dry weight, and leaf moisture percentage. However, many of the vegetative traits (plant height, number of nodes, etc.) were not affected by 2,4 D or nitrogen rate.

Nitrogen rate impacted water use, seedcotton yield, 25 seed weight, seed yield, lint yield, and fiber micronaire in this study. In general, the water use decreased as nitrogen rate increased. This was counter intuitive, as increased growth often leads to increased water use. Additionally, cover crops have been shown to increase water storage for following cotton crops [24]. However, other studies have shown a reduction in soil water following cover crops, due to water use by the covers [25] [26]. Although these studies are contradictory, cotton normally uses more soil water with increasing availability [27]. Results of the current study indicate, that grasspea reduced available soil water for the subsequent cotton crop.

Nitrogen from grasspea had a negative impact on lint yield for 2,4 D tolerant cultivars. This may be due to excess nitrogen, which can delay maturation of cotton plants by three to eleven days, and decrease lint yields [28] [29] [30]. A delay in maturity would be an especially difficult challenge for Oklahoma due to the short growing season that is available for cotton. For example, dates of first significant freezes for Fall in central Oklahoma can occur by mid-October [20]. Although grasspea negatively impacted performance of the 2,4 D tolerant plants, some benefit was observed for 2,4 D susceptible cotton compared to the control (treatment 1). This indicates that some amount, or form, of nitrogen may reduce the effects of 2,4 D in susceptible plants. However, synthetic nitrogen appeared to harm 2,4 D susceptible plants, so an unmeasured benefit of the legume may have benefitted the 2,4 D susceptible plants. The harm from synthetic nitrogen may simply be due to a delay in maturity. Further studies in areas with longer growing seasons for cotton and/or different green manures will help define the extent of the benefits of green manures to 2,4 D susceptible plants.

Fiber micronaire was reduced as nitrogen rate increased. Similar results were observed in Australia, when cotton was grown after a variety of legumes grown for green N [31]. The increased nitrogen availability delayed maturity, which exposed the forming fiber to increased total cool hours, resulting in micronaire values in the discount price range [32] [33]. Current nitrogen rates for cotton are 56 kg ha^{-1} per bale (218 kg) of fiber [34]. However, this current study suggests that new recommendations may be needed for the northern U.S. cotton belt to account for cotton following green manure crops.

In this study, 2,4 D tolerant cultivars produced plants with heavier leaves and greater seedcotton, seed, and lint yields, while using less water and maintaining better fiber micronaire. The greater levels of seedcotton, seed, and lint yields is due to the negative effect of 2,4 D on the reproductive parts of the plant, which caused a reduction in the number of bolls per plant and delayed boll maturity [24]. Although the 2,4 D susceptible cultivars had lower seed yields, the individual seeds (25 seed weight) were heavier. The 25 seed weight is mostly affected by the environment, but it is also affected by boll size and seed number within the boll [35] [36]. Thus, the greater 25 seed weight may be due to inherent differences between the FiberMax and Phytogen cultivars. For all quality traits of fiber there was no impact of 2,4 D tolerance, except for fiber micronaire; these results were similar to findings by other studies [37] [38]. These studies suggested that 2,4 D does not negatively affect fiber quality because more bolls were aborted, allowing full development of remaining bolls.

In the current study, 2,4 D had no impact on plant height, number of nodes, LAI, and canopy coverage (canopeo). Similar observations were made for plant height and node number in other studies that evaluated cotton's response to simulated 2,4 D drift, or varying rates of 2,4 D exposure [39] [40]. Not surprisingly, leaf weights in the current study were positively associated with 2,4 D tolerance, as leaf epinasty and leaf strapping caused by 2,4 D injury were visible early in the growing season of both years. The ability of leaves to recover (outgrow) 2,4 D injury resulted in LAI and canopeo observations in this study being similar between tolerant and susceptible cultivars. Many authors have noted that visual ratings of leaf injury do not correlate well to yield [16] [24] [38] [41].

5. Conclusion

The 2,4 D tolerant and susceptible cultivars responded differently to nitrogen provided by grasspea. The 2,4 D susceptible cultivars used more water and had less yield than the 2,4 D tolerant cultivars. Grasspea helped susceptible cultivars overcome some of the damage caused by 2,4 D exposure. However, too much nitrogen magnifies the effects of 2,4 D on delaying plant maturity. Further studies need to examine the effect of 2,4 D and green manures on cotton with different maturity lengths. Additionally, a wider range of green manures should be included in studies, to define potential benefits of green N crops to 2,4 D susceptible cultivars.

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Conflicts of Interest

The authors declare no conflict of interest.

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