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Effects of Neonicotinoid Seed Treatments on Cotton Seedling Physiology, Nutrition, and Growth

Aqeela Sehrish [1], Megha Parajulee [2], Suhas Vyavhare [2], Cade Coldren [3], Haydee Laza [1] and Catherine R. Simpson [1,*]

[1] Department of Plant and Soil Sciences, Texas Tech University, Lubbock, TX 79409, USA; aqeela.sehrish@ttu.edu (A.S.); haydee.laza@ttu.edu (H.L.)
[2] Texas A&M AgriLife Research and Extension Center, Lubbock, TX 79403, USA; m-parajulee@tamu.edu (M.P.); suhas.vyavhare@ag.tamu.edu (S.V.)
[3] Department of Natural Resources Management, Texas Tech University, Lubbock, TX 79409, USA; cade.coldren@ttu.edu
* Correspondence: catherine.simpson@ttu.edu

Abstract: Plant growth and physiology can be affected by environmental and chemical factors that have the potential to influence yields. Among the factors that influence plant growth, neonicotinoid seed treatments have shown significant effects on plant growth, particularly in cotton. The dual benefits seen from neonicotinoids on plant growth and insect control show promise in improving cotton yields but little is known about how different seed treatments affect seedling physiology. A greenhouse experiment was undertaken to investigate how three neonicotinoid seed treatments (clothianidin, thiamethoxam, and imidacloprid) affect the physiology and growth of cotton seedlings in controlled environmental conditions. A randomized complete block design was used to examine seed treatments and an untreated control. Cotton seeds were treated, grown, and evaluated for physiological changes until the fifth true leaf-stage and measurements were taken at each of these stages. Data were collected on plant height, shoot fresh weight, leaf area, root length, and root biomass. In addition, chlorophyll pigments and nutrient analysis were performed on cotton seedlings. The seedlings of imidacloprid treated seeds had greater height, shoot fresh mass, leaf area, and relative growth rate by the fifth true leaf stage compared to other treated plants; however, clothianidin showed comparative performance at earlier stages in plant development that equilibrated over time. While all neonicotinoid seed treatments showed positive effects, imidacloprid showed the most potential as a bioactivator on plant growth.

Keywords: neonicotinoids; seed treatments; bio-activators; true leaf stage; physiology

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1. Introduction

Cotton (*Gossypium hirsutum* L.) is one of the most significant textile fibers in the world, cultivated in more than 100 countries. The U.S. ranks third in production, yielding 4336 thousand metric tons each year or about 25% of total world fiber production [1]. Within the U.S., cotton is primarily produced in 17 southern states from Virginia to California. The Texas High Plains comprises 60% of the total cotton cultivated area of Texas with >1.6 million hectares planted each year [1]. However, despite intensive chemical control, cotton losses caused by insects, weeds, and pathogens can reach around 30% of total yields [2].

Cotton encounters a variety of arthropod pests throughout its growth and development stages, affecting both vegetative and fruiting structures. The severity of arthropod pest infestations and the resulting damage are typically influenced by the crop's developmental stage [3]. In Texas, early-season cotton pests include thrips, wireworms, and cotton fleahoppers (*Pseudatomoscelis seriatus*) [4]. Among these pests, thrips are particularly problematic for cotton plants during the seedling stages [3]. Currently, western flower

thrips (*Frankliniella occidentalis*) are a significant cotton pest not only in Texas but also in other regions within the United States cotton belt [5]. Because most early stages of plants are very susceptible to pest damage and biological or cultural controls are often inadequate, the most common method used for thrips control is through insecticides [6]. Neonicotinoid insecticides are currently one of two classes of chemicals available as a seed treatment for growers to manage early-season insect pests of cotton [7]. The most commonly used neonicotinoid seed treatments are imidacloprid, clothianidin, and thiamethoxam, which offer systemic insecticidal protection to cotton plants and have been shown to significantly reduce thrips infestation rates, damage, and increase yields [8].

Neonicotinoids have traditionally been studied for their effectiveness against early-season thrips in cotton. However, recent research has revealed that these chemicals also have physiological effects on plants, influencing their metabolism and morphology, and ultimately impacting their growth and productivity [9]. When an agrochemical substance exhibits such effects on plant metabolism and morphology, it is categorized as a bio-activator [10]. Research has indicated that neonicotinoid systemic pesticides may enhance plant vigor and provide protection under drought conditions [11]. The potential for neonicotinoids to be bioactivators has increased interest in using neonicotinoids, particularly to promote growth during the seedlings' most vulnerable stages [12]. For instance, thiamethoxam has been reported to act as a bioactivator in rice plants (*Oryza sativa*), altering photosynthetic pigments, phenylalanine ammonia-lyase activity, root development, and nitrate reductase activity [13]. Additionally, thiamethoxam seed treatments in soybean (*Glycine max* L.) were shown to enhance germination under water deficit conditions [11]. Thiamethoxam and clothianidin are reported to be effective in reducing drought stress effects on sugarcane (*Saccharum* spp.), leading to greater total plant weight, increased plant height, and improved photosynthetic activity with lower proline accumulation in drought-stressed plants [14]. Furthermore, it is reported that thiamethoxam seed treatments increased root development, protein content, shoot dry matter, and ear dry weight in the spring wheat crop [9].

These observations highlight the potential for neonicotinoids to act as bio-activators affecting important growth aspects in various crops. However, these findings were not always consistent. For example, the impact of thiamethoxam seed treatment on sunflower fields was investigated over a span of three years and it was reported that they may not consistently yield economic benefits for farmers, particularly in terms of reducing pests or improving yields [15]. Yet, there is some evidence that there are variabilities in bioactivator effects based on chemical formulation, environment, crop species, and cultivar. In the case of crops like cotton and okra, the use of neonicotinoid pesticides, specifically, imidacloprid, thiamethoxam, acetamiprid, and thiacloprid, has resulted in increased plant height and higher levels of soluble protein content [16]. Interestingly, imidacloprid has been found to enhance cotton yield even in the absence of insects [17]. Field studies carried out on cotton in Arkansas in 2004 further highlighted the positive impact of imidacloprid, revealing enhanced growth and increased yield [18]. Furthermore, the superior effects of imidacloprid found by [17] contrast with previous studies that have shown better effects of thiamethoxam or clothianidin on cotton yields in comparison to imidacloprid [18]. This indicates that neonicotinoid seed treatments must be selected based on environmental conditions, pests, and crops to achieve beneficial bioactivator effects.

While neonicotinoids are known to be effective in inhibiting insect pest damage, more needs to be learned on how to optimize their growth promotion potential in different crops. This is particularly true for cotton due to its established use in cotton production. Furthermore, information on physiological performance measurements such as growth, pigments, and nutrient status of tissues is lacking. For example, monitoring leaf chlorophyll content is crucial in assessing cotton growth post-neonicotinoid seed treatments [19]. Chlorophyll, as a key indicator of photosynthetic efficiency, reflects the plant's ability to convert sunlight into energy for growth [8]. Following neonicotinoid seed treatment applications, changes in chlorophyll levels may signal alterations in photosynthesis, impacting overall growth [19].

Chlorophyll content serves as a crucial indicator of nitrogen (N) status and, consequently, potential crop yield. These factors collectively signify distinctive attributes of a thriving and robust plant [18]. Analysis of leaf tissue nutrient levels is also important in evaluating cotton growth, providing insights into the plant's nutritional status and availability of nutrients in the environment [8]. Leaf tissue nutrient analysis identifies deficiencies or excesses, impacting boll production and vegetative growth [20]. Research on the impact of neonicotinoid seed treatments on plant nutrients is an evolving field and findings may vary based on specific conditions. Studies have indicated potential effects on plant physiology and nutrient uptake due to neonicotinoids [21]. For instance, neonicotinoids may alter root systems, leading to changes in nutrient absorption and their systemic movement within plants might influence nutrient distribution [22]. It is important to note that the body of research in this area is expanding and new studies, data, and findings are continuously being added to the existing knowledge base. The concentration of neonicotinoids, the duration of exposure, and the plant species involved can all contribute to varying outcomes [8]. In addition to chlorophyll content and leaf tissue nutrient analysis, examining the relative growth rate (RGR) and its response ratio (RGR RR) helps us understand the influence of various factors on plant growth, especially in cotton. RGR quantifies the plant's growth rate over time, providing insights into overall performance and productivity [23]. Analyzing RGR RR allows for a comparative assessment of growth, such as exposure to neonicotinoid seed treatments during the initial true leaf stages, offering valuable information on the long-term impact of these treatments on cotton physiology [24]. Because these factors have been underexplored with regard to cotton, it is imperative that controlled environment research is conducted to determine how various neonicotinoid seed treatments may impact the growth of cotton plants at different developmental stages.

Therefore, the objectives of this study were to determine how cotton seedling growth is affected by neonicotinoid seed treatments, including imidacloprid, thiamethoxam, and clothianidin, up until the fifth true-leaf stage. The study also examined variations in the concentrations of nutrients and chlorophyll in leaf tissue with the goal of evaluating the overall effect of these neonicotinoids on cotton plant growth.

2. Materials and Methods

2.1. Experimental Setup

Two separate replicated greenhouse experiments were conducted from 11 September to 12 October 2022 (Trial 1) and 19 September to 20 October 2022 (Trial 2), at Texas Tech University in Lubbock, Texas, USA (33.58324249° N, 101.88206800° W). Environmental conditions were maintained in the greenhouse and ranged from 20 to 35 °C throughout both experiments. Supplemental LED lighting was set at 14/10 h on/off every day, respectively. Environmental data (temperature, relative humidity, and photosynthetically active radiation (PAR) were collected periodically throughout the experiments. Both experiments were set up in a randomized complete block design, with three seed treatments (clothianidin, imidacloprid, and thiamethoxam) and a control with 5 replications for each treatment. Five replications were grown for each treatment so that 20 plants could be harvested at each of the first five leaf stages for a total of 100 plants. Cotton seeds (DP 1646 B2XF) (Bayer CropScience, Creve Coeur, MO, USA) were treated with each neonicotinoid treatment prior to planting in pots (3 × 5″) filled with all-purpose soilless media (Berger BM6Ò). Seed treatments were applied by adding the recommended rate of each neonicotinoid chemical (12.04 mL/kg seed for clothianidin (Poncho Votivo®), 9.04 mL/kg seed for imidacloprid (Gaucho®), and 8.20 mL/kg seed for thiamethoxam (Cruiser®) (Bayer CropScience, Creve Coeur, MO, USA)) in a seed treatment applicator to distribute the products evenly [16].

The cotton seedlings were irrigated two or three times per weekend, allowing the top layer of potting mixture to dry slightly between each watering throughout the experiments. Plants were inspected every other day and yellow sticky traps were strategically positioned throughout the interior of the greenhouse to monitor possible pests such as aphids and

whiteflies. This approach significantly curbed the colonization and growth of non-target pest populations in the greenhouse.

Five plants from each treatment were destructively sampled at the emergence of each true leaf from the 1st to 5th true-leaf stages of cotton. The sampling stage was determined when 75–80% of plants reached each true leaf stage and plants were cut at the base of the stem, just above the soil. Fresh weight, plant height, and leaf area were measured immediately after collecting each plant. Image J (version 1.54f, 29 June 2023) was used to determine leaf area by uploading photographs of each leaf of harvested plants [25]. Photographs were taken by keeping the camera horizontal, the leaf flat on the work surface with a white background, and a ruler was used for reference.

Plant height was measured by using a standard ruler from the base of the stem to the tip of the apical meristem. Once the stems were cut, the roots were collected from the pots, washed, and weighed and the length of the taproot was measured using a ruler. Following this, leaves, stems, and roots were carefully bagged and frozen at −80 °C for subsequent processing. Plants were freeze-dried using a Harvest Right Freeze Dryer (Harvest Right, Salt Lake City, UT, USA) and the dry biomass of the shoot (stems and leaves) was recorded. Freeze-dried materials were then stored frozen until further analysis.

Relative growth rate (RGR) and RGR RR were calculated for plant height and leaf area for the 5th true-leaf stage (Equations (1) and (2), respectively) to normalize growth data in relation to initial plant height [23]. This provided relative plant heights for comparison between treatments.

$$\text{RGR} = ((\text{PH (T2)} - \text{PH (T1)})/(\text{DAG}) \times 1000 \quad (1)$$

* DAG (days after germination)
* PH = Plant Height (cm)
* T2 = plants at 5th true-leaf stage; T1 = plants at 1st true-leaf stage

$$\text{RGR RR} = \text{RGR (Treated seedlings)}/\text{RGR (Untreated seedlings)} \quad (2)$$

2.2. Chemical Analysis

2.2.1. Chlorophylls

The analysis of chlorophylls (Chl) was conducted following [26] with some modifications for the microplate spectrophotometer (SpectroMax iD3, San Jose, CA, USA). The chlorophyll analysis in this study utilized freeze-dried leaf samples from seedlings collected at the 1st, 2nd, 3rd, 4th, and 5th true-leaf stages during the trial. These samples were stored at −80 °F after initial processing. Briefly, 100 mg of ground cotton leaves were placed into 5 mL test tubes and then 1 mL of 100% methanol was added to each sample. The resulting mixture was homogenized to aid in the extraction of chlorophylls and carotenoids, followed by centrifugation at 10,000 rpm for 15 min. Next, 200 µL of the supernatant was transferred to a microplate and absorbance was measured at 663 and 646 nm and recorded for Chl a and Chl b, respectively, using a microplate spectrophotometer. Subsequently, concentrations of Chl a and Chl b were determined using the following formulas [26]:

$$\text{Chl a } (\text{mg} \cdot \text{g}^{-1}\text{FW}) = (12.25\ (\text{A663}) - 2.79\ (\text{A646})) \times \text{V}/100 \times \text{W}$$

$$\text{Chl b } (\text{mg} \cdot \text{g}^{-1}\text{FW}) = (25.51\ (\text{A646}) - 5.10\ (\text{A663})) \times \text{V}/100 \times \text{W}$$

* A: Absorbance (as 646 and 663).
* V: final volume of chlorophyll extracted in 80% acetone.
* W: fresh weight of the sample used

2.2.2. Nutrient Analysis

Subsamples of freeze-dried leaf tissues (15 g) from the 5th true-leaf stage were analyzed by Waters Agricultural Laboratories, Inc. (Camilla, GA, USA) using ICP-OES (iCAP 7600, Thermo Fisher, Waltham, MA, USA) for a comprehensive nutrient analysis encompassing nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sulfur (S), boron (B), zinc (Zn), iron (Fe), copper (Cu), and manganese (Mn).

2.3. Statistical Analysis

Statistical analysis was performed using JMP Pro 16.0.0 (SAS Institute, Cary, NC, USA) software. Analysis of variance (ANOVA) was used to determine significant differences between treatments at $p < 0.05$. Where significant differences were found, Tukey's HSD was used to separate means at $p < 0.05$. Both trials were combined for analysis as there were no differences in growing conditions between the trials.

3. Results

3.1. Plant Physiological Data

Plant height, shoot fresh mass, root mass, and leaf area increased significantly at each successive leaf stage ($p < 0.001$) (Figure 1). Root fresh mass was the only factor not significantly affected by leaf stage, treatment, or their interactions. Significant differences between treatments varied by crop stage. The imidacloprid-treated seedlings were significantly taller than the clothianidin and thiamethoxam treatments at the fourth and fifth true-leaf stages (Figure 1A; $p \leq 0.0001$). There were no significant differences among seed treatments for seedling height at the first, second, and third true-leaf stages. At the first, second, third, and fifth true-leaf stages, there were no significant differences in shoot fresh weight within neonicotinoid-treated seedlings. However, at the fourth true leaf stage, the imidacloprid, clothianidin, and control seedlings had significantly greater shoot mass ($p \leq 0.0001$) than the thiamethoxam-treated seedlings (Figure 1B). At the first, second, third, and fifth true-leaf stages, there were no significant differences in shoot fresh weight within neonicotinoid-treated seedlings; however, control seedlings were the heaviest followed by clothianidin, imidacloprid, and thiamethoxam. Leaf area was also affected by seed treatments and significant differences were found between treatments at the first, second, third, and fifth true-leaf stages (Figure 1D). In the first three leaf stages, imidacloprid and clothianidin performed similarly while thiamethoxam and untreated control had smaller leaf areas compared to imidacloprid and clothianidin. However, at the fifth true-leaf stage, clothianidin, thiamethoxam, and imidacloprid were not significantly different from each other; the control treatment had significantly less leaf area than clothianidin and thiamethoxam (Figure 1D).

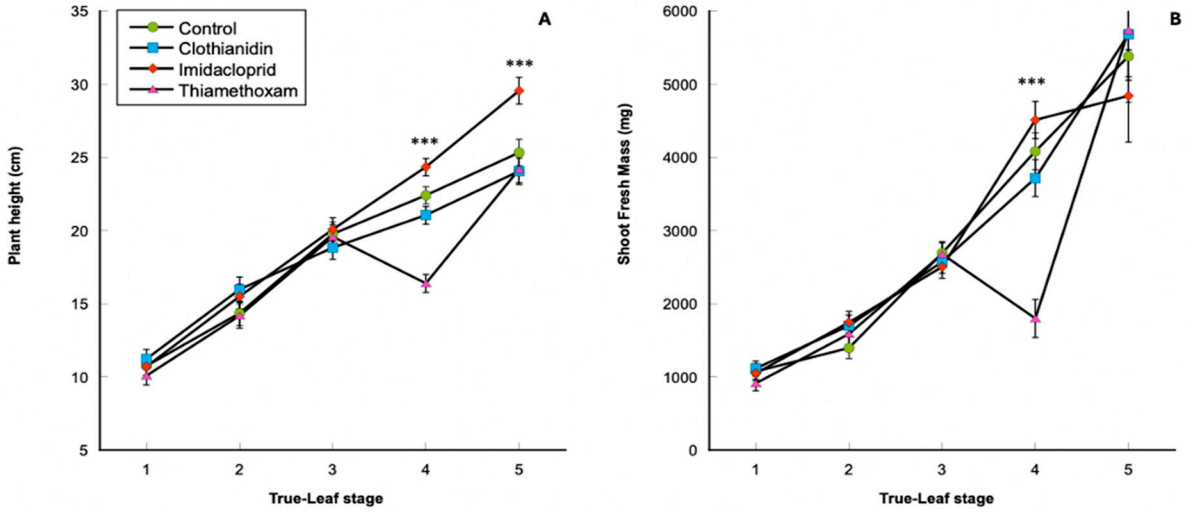

Figure 1. *Cont.*

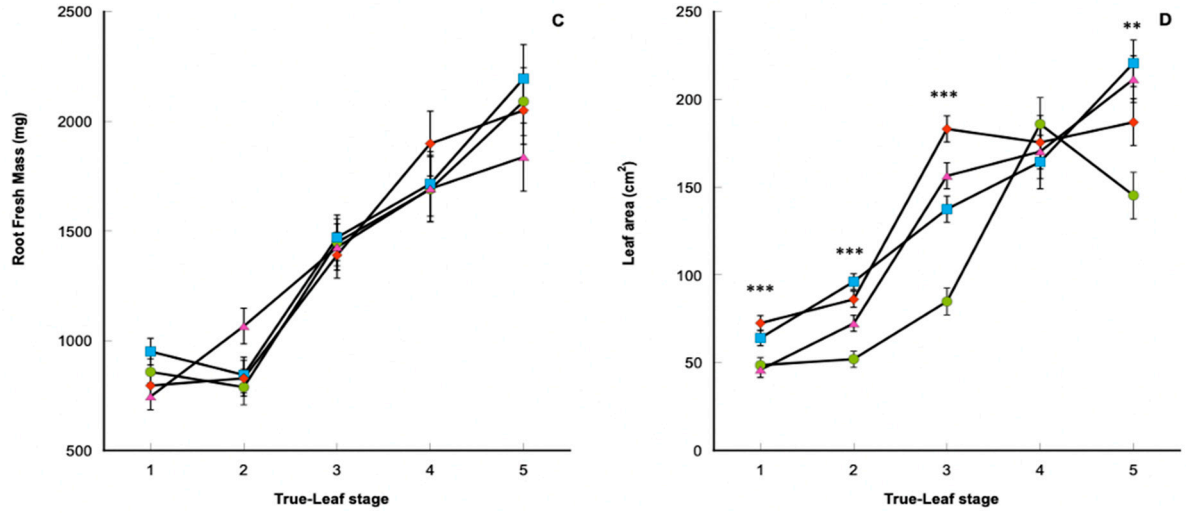

Figure 1. Cotton seedling physiological parameters for both trials, leaf stage, and treatment. (**A**) Plant height; (**B**) Shoot fresh mass; (**C**) Root fresh mass; (**D**) Leaf area. Significant differences were determined at $p \leq 0.05$. Significance differences between treatments at each leaf stage are indicated by asterisks, where ** indicates the significance of $p < 0.01$ and *** indicates significance at $p \leq 0.0001$. Bars indicate ± 1 standard error of the mean.

3.2. Relative Growth Rate and Response Ratio

Relative growth rate (RGR) and relative growth rate and response ratio (RGR RR) for plant height were significantly affected by treatment in both trials (Figure 2). Plant height RGR was significantly greater for imidacloprid-treated seedlings compared to all other treatments and controls; however, no significant difference was observed between clothianidin, thiamethoxam, and the control (Figure 2A). Plant height RGR RR also followed similar trends as RGR, where imidacloprid-treated seeds were taller than clothianidin and thiamethoxam-treated seedlings. There were no significant differences in response ratios between clothianidin and thiamethoxam (Figure 2B). Similarly, no significant differences in leaf area RGR and RGR RR were observed between treatments.

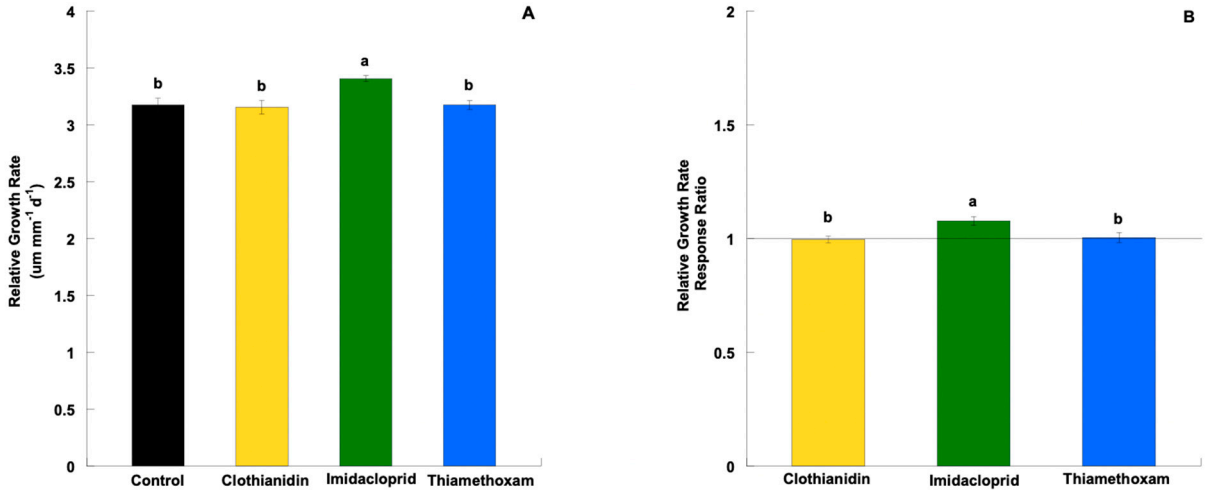

Figure 2. Relative growth rate (RGR) and relative growth rate response ratio (RGR RR) for plant height. (**A**) RGR of plant height; (**B**) RGR RR of plant height. Different lowercase letters indicate significant differences between seed treatments at $p \leq 0.05$. Bars represent ± 1 standard error of the mean.

3.3. Chlorophyll Content in Cotton Seedlings

In addition to growth factors, there was a significant effect of leaf stage on Chl a ($p < 0.0001$), with the lowest value of Chl a observed at leaf stage 5. There were no significant effects of neonicotinoids or interaction of neonicotinoid treatments and leaf stages on Chl a. Conversely, seedling stage significantly influenced the amount of Chl b in cotton seedlings (Figure 3B); Chl b peaked at the third leaf-stage but declined by the fifth leaf-stage ($p < 0.0001$). Significant effects of seed treatments were only found for Chl b ($p = 0.014$). Chl b was greatest in the control and imidacloprid treatments while clothianidin and thiamethoxam treatments had significantly lower Chl b values (Figure 3B).

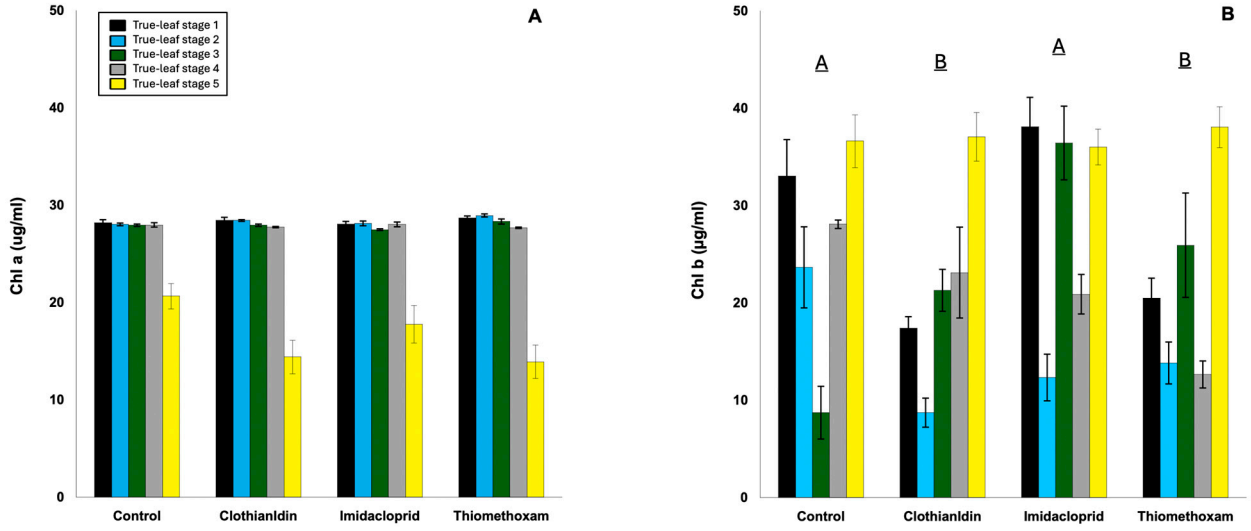

Figure 3. Chlorophyll a and b concentrations in different stages of leaves in both trials. (**A**) Chlorophyll a and (**B**) chlorophyll b. Different uppercase letters with brackets indicate significant effects of seed treatment at $p \leq 0.05$. Bars represent ± 1 standard error of the mean.

3.4. Nutrient Analysis

There were no significant effects of seed treatments on nutrient concentrations of leaves at the fifth leaf stage (Table 1). Furthermore, no nutrient deficiencies were seen in

the concentration of the essential plant-nutrient elements in the cotton leaf tissue except Cu, which was deficient (<4 ppm) [27].

Table 1. Average (±SEM) concentrations of leaf nutrient constituents of cotton seedlings at fifth true leaf stage, Lubbock, Texas, 2022. Significant differences were determined at $p \leq 0.05$.

Seed Treatment	N (%)	P (%)	K (%)	Mg (%)	Ca(%)	S (%)	B (ppm)	Zn (ppm)	Mn (ppm)	Fe (ppm)	Cu (ppm)
Control	2.48	0.42	2.66	0.64	2.79	1.05	50.50	30.67	97.50	58.83	3.83
Clothianidin	2.55	0.42	2.27	0.62	2.94	1.02	52.00	30.50	105.17	57.00	3.67
Imidacloprid	2.29	0.43	2.44	0.59	2.60	1.05	46.33	32.83	100.50	53.00	3.50
Thiamethoxam	2.39	0.38	2.18	0.62	2.70	0.96	46.00	28.33	114.67	54.50	3.33
DF	3	3	3	3	3	3	3	3	3	3	3
F ratio	0.442	1.480	2.53	0.443	0.83	0.160	0.691	1.162	0.366	0.438	0.505
p-value	0.725	0.250	0.086	0.725	0.493	0.922	0.568	0.349	0.778	0.728	0.683

DF represents degrees of freedom.

These results illustrate the differential effects of neonicotinoids on plant physiology. In summary, imidacloprid had positive impacts on plant growth, biomass, and leaf area at later true leaf stages, while clothianidin had more impact at younger stages (Figure 4). While roots were not affected in this study, this could be due to the limitations of using containers.

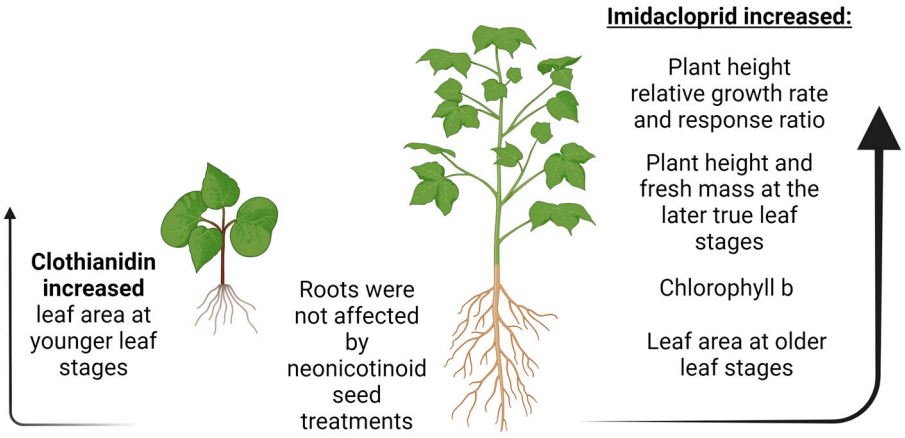

Figure 4. Diagram of the effects of neonicotinoids on cotton plant physiology. Image generated using Biorender.com (accessed on 5 March 2024). Arrows represent increases in plant growth parameters.

4. Discussion

Neonicotinoids have been found to trigger a variety of physiological processes, which can occasionally increase plant stand density and yield [28]. Furthermore, neonicotinoid pesticides (imidacloprid, thiamethoxam, acetamiprid, and thiacloprid) have increased plant height and soluble protein content in crops such as cotton and okra [16]. These findings have led to the assumption that some neonicotinoids may act as physiological bioactivators. However, this phenomenon has not been studied to a great extent and it is not known if all neonicotinoids induce these effects or if they are limited to a certain growth stage or other factors [29]. The present study quantified the effects of neonicotinoid insecticides across the entire thrips susceptible cotton growth stages.

Differences between seed treatments occurred at different seedling growth stages. For example, imidacloprid-treated seedlings had greater values for plant height and shoot weight at the fourth and fifth true-leaf stage as well as the fourth true-leaf stage, respectively. This is similar to the study by [30] that found that imidacloprid-treated cotton plants were taller than those treated with clothianidin. Furthermore, [17] found that, in comparison to untreated cotton seedlings, imidacloprid treatments were effective at enhancing seedling vigor and growth and decreasing the prevalence of early-sucking pests.

Yet, the results of [17] contrasted with findings by [16] who found that thiamethoxam was the only neonicotinoid that showed significantly greater cotton plant height, while clothianidin, imidacloprid, acetamiprid, and thiacloprid did not. Furthermore, [17] found that imidacloprid treatments were effective at enhancing seedling vigor and growth and decreasing the prevalence of early sucking pests compared to untreated control cotton seedlings. Cotton plant response to foliar-applied imidacloprid was investigated and found no significant influence on plant growth parameters [18]. This demonstrates the inconsistency in bioactivator effects amongst neonicotinoid treatments. For example, the bioactivation effect of thiamethoxam-treated soybean seeds was limited to leaf area and root dry mass; it did not extend to other parameters or plant yield. Consequently, even though thiamethoxam-treated soybean seeds had a favorable effect on leaf area, these benefits may not be translatable to bioactivation [31]. The impacts of bioactivator effects of neonicotinoids can also vary based on measured physical factors [17,18]. Leaf area is an important indicator of plant health and photosynthetic activity potential and is impacted by biotic and abiotic stressors [32]. Results from this study demonstrated that the neonicotinoid seed treatments exerted varying temporal effects on total leaf area as cotton seedlings advanced through growth stages. Overall, imidacloprid and clothianidin treatments resulted in similar effects on leaf area, especially at the initial leaf stages. However, at the fourth stage, there were no significant differences between the treatments or control and, at the 5th stage, clothianidin, thiamethoxam, and imidacloprid were similar. Yet, when comparing these results to those of previous studies, similar variable impacts of neonicotinoid seed treatments were evident. Our results at early seedling stages are similar to the findings of [14] who showed that clothianidin had greater impacts on the leaf area of sugarcane crops in comparison to imidacloprid. Similarly, our findings from this study are in agreement with the findings of [18] that the effect of neonicotinoid seed treatments dissipated at later seedling stages (the fourth and fifth leaf stages).

Root fresh weight was not significantly affected by neonicotinoid seed treatment in either trial, which indicated that the effect of neonicotinoid is more pronounced in shoot growth. The impacts of imidacloprid and other neonicotinoids on root physiology and the underlying causal mechanisms are not well known [17] found that imidacloprid-treated seedlings had greater root mass but no mechanisms were given to explain this occurrence. [22] also reported that increasing thiamethoxam doses led to increased root development, resulting in gains in root volume and linear area. When thiamethoxam was used, similar responses were seen for roots in wheat, soybeans [11,22], and sugarcane [33]. However, these beneficial effects may not be seen until later stages of maturity.

Due to the high variability across treatments and growth parameters, it was necessary to normalize plant growth data to elucidate true impacts over the course of the experiment. Therefore, RGR and RGR RR were calculated to determine temporal effects of neonicotinoid seed treatments prior to squaring and flowering as described in [14]. Cotton seeds treated with imidacloprid resulted in higher RGR and RGR RR in comparison to other neonicotinoid treatments and controls. This indicates that over the most vulnerable stages of cotton growth, imidacloprid had noticeable plant growth impacts on cotton seedlings as seedling heights increased compared to other treatments. Imidacloprid-treated cotton plants were significantly taller than thiamethoxam and clothianidin-treated plants [17]. This provides concurring evidence that imidacloprid likely provides longer-term bioactivator impacts on cotton; however, this was not directly measured. Because plant height and growth parameters can vary if initial parameters are not controlled, (i.e., uniform cutting in horticultural plants), which may not be possible for many experiments, RGR and RGR RR can serve as better indicators of treatment impacts [34]. For example, in our experiments, the impacts of clothianidin and imidacloprid were similar for many of the measured growth parameters, showing no clear indication of better performance between treatments. Thus, based on RGR and RGR RR, imidacloprid treatments more clearly influence plant height compared to other treatments and the control [30].

While our study showed greater bioactivator effects of imidacloprid than other neonicotinoids, several other studies showed thiamethoxam as more effective in affecting plant growth in field and greenhouse experiments with maize and cotton [14,18,35]. This apparent discrepancy is likely due to factors such as different genotypes and abiotic stresses between these studies. Also, physical measurements are not the only indicators of plant stress or health. Plant pigments and nutrient status are important indicators of plant health and potential performance [36]. While we studied the effects of neonicotinoid seed treatments on chlorophyll in these studies, they varied greatly by growth stage. However, Chl b showed greater differences between treatments than Chl a. Plants treated with clothianidin and thiamethoxam contained lower concentrations of Chl b in this study compared to the imidacloprid and control treatments, indicating changes in pigments related to nitrogen content. Neonicotinoid insecticides can increase the pigment content in cotton leaves but chlorophylls can be decreased under pest pressure [37]. A drop in chlorophyll content may stem from either the degradation of chlorophyll or a reduction in its synthesis. The transformation of Chl b into Chl a during the chlorophyll degradation process [38] could be responsible for the elevated Chl a/Chl b ratio in stressed leaves, contributing to the overall decline in chlorophyll content.

In addition to plant pigments, nutritional status can also impact plant physiology and overall plant health and, in turn, nutrient deficiencies can impact growth and yields [16]. In this study, we saw no impact of neonicotinoid seed treatments on nutrients in leaves collected at the fifth leaf stage. However, this is not consistent with other studies. For example, different rates of imidacloprid affected nutrient content in cucumber [39] as well as an increase in cotton plant height, chlorophyll content, and nitrogen content of leaves with increases in imidacloprid seed treatment concentration [16,40]. It is important to note that the nutrients were only analyzed in plants in the fifth true leaf stage in our study and nutrient status at earlier seedling stages could have been impacted. Nevertheless, the lack of a significant effect on leaf nutrient constituents at the fifth true-leaf stage suggests that the effect of neonicotinoids on leaf nutrient profile is either non-existent or short-lived.

5. Conclusions

In this study, we examined how neonicotinoid seed treatments affected the plant height, shoot and root fresh weight, leaf area, chlorophyll pigments, and nutrient status of cotton seedlings. These results demonstrated the variable effects of neonicotinoid seed treatments on cotton seedling growth and physiology. Overall, imidacloprid appeared to have the strongest positive bioactivator effects on seedlings, which also lasted until the fifth leaf stage. However, clothianidin and imidacloprid performed similarly at younger leaf stages, indicating that clothianidin could be a good option if imidacloprid is not available. These findings also lead us to believe that the effects of imidacloprid are longer-lived than those of clothianidin or thiamethoxam. Overall, the variability between neonicotinoid seed treatments on plant physiology is concerning and further underscores that this is an understudied area that should be explored to a greater extent to fully clarify the mechanisms of neonicotinoid seed treatments as bioactivators. It should also be noted that these results were found in greenhouse conditions, which may not translate to field-based systems. Further research should be conducted on field systems over long-term growing conditions while ecological implications are also studied. Ultimately, these results demonstrate the need for additional research exploring and comparing neonicotinoid pesticides that have the potential to act as bioactivators.

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