The Effects of Exogenously Applied L-Tryptophan on the Nodulation and Overall Development of Soybeans Grown in Acidic Soil

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Abstract

Acidic soil severely inhibits legume nodulation and devastates crop yields worldwide. Among the many phytohormones that regulate nodulation, indole-3-acetic acid is known to improve the nodulation and stress tolerance between specific legumes and their symbiotic rhizobacteria. Importantly, Bradyrhizobium Japonicum, a symbiotic soybean bacteria, has been observed to synthesize indole-3-acetic acid from l-tryptophan. This experiment tested the hypothesis that exogenously applied l-tryptophan could improve the nodulation and overall development of soybeans grown in acidic soils with Bradyrhizobium Japonicum. Six trials were conducted, each involving three soybeans: a control, a soybean treated with an inoculant, and a soybean treated with an inoculant along with l-tryptophan. Treatments were applied prior to sowing, and the pH of sterile potting soil was adjusted to 4.9 with aluminum sulfate. Soybeans ($n=18$) were grown in a controlled environment for 23 days before harvesting. Student's t-tests ($p<0.05$) were conducted using measurements regarding overall plant development and observed nodulation to gauge the significance of the experimental treatment. Although the limited sample set yielded results which fell short of statistical significance, weighing of dry samples and observations of nodulation generally suggested that the experimental treatment improved overall development. These initial results may show promise in the development of an inexpensive method farmers can emulate to overcome the effects of acidic soil on soybean production.

Introduction

Legumes embody some of the most important crop species worldwide. In 2019, ~334,000,000 metric tonnes of soybeans alone were produced (FAO, 2020). In addition to providing over 35% of the world's protein intake (Jaiswal et al., 2018), legumes also utilize the unique ability to form novel root organs called "nodules" with symbiotic bacteria collectively known as "rhizobia". In exchange for nutrients, the rhizobia fixes atmospheric di-nitrogen (N_2) into ammonium (NH_3) for the legumes. Biological Nitrogen Fixation (BNF) is a sustainable process that is agriculturally and ecologically critical. The legume-rhizobia symbiosis is estimated to produce approximately 80% of the biologically fixed nitrogen in agricultural systems (Jaiswal et al., 2018), establishing legumes as both a major food source and a crucial nitrogen fertilizer. The evolutionary ability for legumes to form nodules is key to the viability of legume crops.

Despite the enormous benefits of legume BNF, its exploitation is severely inhibited by soil acidity (Ferguson et al., 2013; Jaiswal et al., 2018; Taylor et al., 1991; Lin et al., 2012). It is estimated that 30% of the world's ice-free land is acidic ($pH<5.5$) with up to 40% of the world's potential arable land affected (Von Uexküll & Mutert, 1995). Soil acidity greatly inhibits legume production, and nodulation, in general, has been reported to be more sensitive to acidic pH than other aspects of legume growth (Evans et al, 1990). Acidic pH has been reported to inhibit nodule formation in common legumes by more than 90% and nodule dry weight by more than 50% (Lin et al., 2012; Alva et al., 1990; Vargas & Graham, 1989, Evans et al., 1990). Of the 3.6 million tons of nitrogen fertilizer applied worldwide each year, 75% of the fertilizers are used in major soybean production lands where acidic soils are prevalent (Ferguson et al., 2013). In comparison to BNF, chemical nitrogen fertilizers are more expensive, less readily available, and more damaging to the environment (Ferguson, 2013). Soil acidification, mainly due to soil leaching, inefficient nutrient cycling, and the use of chemical nitrogen fertilisers, continues to worsen today and devastates crop yields worldwide (Von Uexküll & Mutert, 1995; Ferguson et al., 2013; Ferguson, 2013). Current methods employed to battle soil acidification include the application of lime or organic acids to soils. These methods, however, are costly and inconvenient when considering the vast and expensive amounts of material needed to ameliorate acidic soil (Jaiswal et al., 2018). To make matters worse, many rural farmers struggle to invest in liming practices. One study reports that returns on liming investments pay off only after at least 2 years following optimal liming practices (Hijbeek et al., 2021). It is essential to find a way to boost legume nodulation and production in acidic soils, but more research is needed to balance the demands for practical treatments, sustainable practices, costs, and legume production.

One way legumes are able to control nodulation is via a systemic mechanism labeled the "autoregulation of nodulation" (AON). In soybeans, the AON pathway inhibits nodulation by releasing CLE peptides via root exudates that travel to the shoot where the peptides interact with the Nodulation Autoregulation Receptor Kinase (NARK) and produces nodulation-inhibitory compounds (Lin et al., 2012). In acidic pH, long-distance root-shoot signaling through root-derived signals initiates early on in nodule development as a response to stress signals and severely decreases flavonoid synthesis in legumes (McKay & Djordjevic 1993; Hungria & Stacey, 1997). Rhizobia also has been reported to decrease Nod gene induction and Nod metabolite production in acidic soils (McKAY & Djordjevic, 1993; Miransari et al., 2006). Numerous hormones are linked to the regulation of nodulation, including cytokinins, auxins, ethylene, gibberellins, and peptide hormones (Lin et al., 2020). The theory for this study experiments with a well known auxin that has been an important focus of study in relation to nodulation: indole-3-acetic acid (IAA).

Indole-3-acetic acid is the most common plant hormone of the auxin class and is known to control various aspects of legume development and interactions. The main precursor for the synthesis of IAA is tryptophan, and at least 4 different pathways in plants have been proposed in tryptophan-dependent IAA biosynthesis: indole-3-pyruvic acid (IPA), indole-3-acetamide (IAM), tryptamine (TRM), and the indole-3-acetaldoxime pathways (Mano & Nemoto, 2012; Fu et al., 2015; Spaepen & Vanderleyden 2011). The application of exogenous tryptophan to various bacteria, including rhizobium, has been reported to enhance IAA production (Spaepen et al., 2007; Broek et al., 2005). Plant growth promoting rhizobacteria (PGPR) have been observed to produce IAA as an effector molecule to influence interactions between microbes and plants, establishing IAA as an essential point of study in relation to the legume-rhizobia symbiosis. It is widely speculated that auxins produced from PGPR can regulate legume development by influencing the auxin balance of legumes. In general, nodulated roots contain more auxin than non-nodulated roots, and morphological changes to legume roots upon inoculation with PGPR have been attributed to the production of auxins from PGPR (Spaepen & Vanderleyden 2011). Nodules colonized with mutant IAA overproducing strains have reported to have increased nitrogen fixation capacity (Camerini et al., 2008). One study reported that *Medicago truncatula* had an increased tolerance to salt stress when inoculated with a mutant IAA-overproducing *Sinorhizobium meliloti* strain (Bianco & Defez, 2009). Many more studies have reported similar benefits to stress tolerance, root development, and overall nodulation from an increase in IAA production by PGPR.

In light of the reported benefits of IAA production by PGPR to legumes, this study aims to observe the effects of exogenously applied l-tryptophan on the nodulation and overall development of soybeans grown in acidic soil. Soybeans were inoculated with compatible *Bradyrhizobium Japonicum,* and a commercially-available dry inoculant was bought to mimic a contingency that farmers could emulate. Soybeans were chosen because of their massive production and importance worldwide. Important to this study, *B. Japonicum* has been reported to produce IAA as a bacteroid within the soybean nodule, and *B. Japonicum* has also been reported to accumulate more IAA with an exogenous supply of l-tryptophan (Donati et al., 2013; Mubarik et al., 2013; Hunter, 1987). In theory, if the soybeans successfully release enough signals, mostly flavonoids, to trigger the transcription of bacterial genes, then nodule organogenesis will commence, and the bacteroids may use the l-tryptophan to produce more IAA to ameliorate oxidative stress. The hypothesis for this study is that if l-tryptophan is applied to soybeans with *Bradyrhizobium Japonicum*, then the soybeans will exhibit improved nodulation and overall development in acidic soils when compared to soybeans grown in acidic soil that did not receive exogenous l-tryptophan.

This soybean-rhizobacteria project was chosen because it is believed that this idea shows potential and is yet to be experimented with after extensive research. The logic seems straightforward, but there is much that seems out of the bounds of tested experimentation that makes the experiment exciting. The results can be predicted to be many different outcomes because of the vague fog that covers the notions of how the soybeans and *B. Japonicum* will interact in such inhibitory settings. Could it be that they will use the l-tryptophan to benefit each other? Or perhaps they may completely ignore each other? What messages are they secretly exchanging underground? What survival plans are strategized in such labyrinthine pathways? Only experimentation will tell the story.

Materials

1. L-tryptophan (purchased as a supplement-pills)
2. Soybean seeds (*Glycine Max*)
3. 10 pots (4.5" diameter, 5" height)
4. Aluminum sulfate (purchased as pure powder)
5. Soybean Inoculant (N-Dure Dry inoculant: 2*10^9 CFU/g *Bradyrhizobium Japonicum*)
6. 3% Hydrogen Peroxide
7. Distilled Water
8. Two 24 Watt growth lights
9. High-accuracy (0.001 gram) weigher (calibrated before measuring)
10. Drying oven with convection fan
11. Calibrated pH meter

Methods

Soil Preparation

1. pH of sterile and dry potting soil was lowered to 4.9 by homogeneously incorporating aluminum sulfate at a rate of 1 gram aluminum sulfate per 9.9 cubic inches. Soil pH was double checked to be 4.9 with a pH meter after preparation.

Seed Sterilization

1. Soybean seeds were sterilized by soaking in a 1:10 solution of 3% Hydrogen Peroxide and clean distilled water for 30 minutes.
1. Soybean seeds were thoroughly rinsed 5 times after sterilization with clean distilled water

Seed Preparation

1. After sterilizing soybean seeds, seeds were prepared with a dry inoculant by integrating inoculant onto the moist seed surface at a rate of 1.8 grams of inoculant per seed. The amount of inoculant per soybean was calculated based on instructions from the purchased inoculant and adjusted through trial and error for necessary surface coating.
2. Soybean seeds were prepared with l-tryptophan by integrating l-tryptophan onto surface of seeds at a rate of 0.005 grams l-tryptophan per seed (these seeds should receive the inoculant treatment first and then the l-tryptophan treatments). The amount of l-tryptophan applied per soybean was based on previous studies on legumes and adjusted through trial and error for necessary surface coating.

Set Up

1. A growth-chamber was established by constructing a 3 layered enclosure structured with pvc pipes (13.25” * 27.5” * 38”). The layers integrated plastic vinyl, styrofoam, and cardboard in order to trap heat/moisture.

2. A digital heater was recovered from an egg-incubator and was installed to control and measure temperature and humidity (Temperature was maintained at 29±1℃ in the day, and 28±2℃ at night. Humidity was measured to be 80±10%)
3. Two 24 Watt growth lights were installed to be able to be raised/lowered as needed. Lights were hung so that every soybean received equal lighting.

Trial (1-6)

1. 9 seeds that have received no treatments, 9 seeds that have received the inoculant treatment only, and 9 seeds that have received the inoculant and l-tryptophan treatment were planted into jiffy pots with acidic soil (pH=4.9) to germinate. Soybeans were watered with distilled water as needed
2. After 7 days, three sprouted individuals of each seed-treatment were transferred into the bigger containers with the same acidic soil. It is important to note that three trials happened concurrently in two “rounds” such that n=6 trials involving 18 plants.
2. Soybeans were harvested 23 days after sowing.
    a. Pictures of soybeans were taken before collecting data
    b. Soybean roots were gently washed to remove dirt. The stem of the soybean was cut right above the roots
    c. Fine tweezers/needles were used to thoroughly remove dirt clumps and visible nodules were recorded
    d. The lengths of the trifoliate leaf groups, stem, and tap root were measured
    e. The trifoliate leaf groups were cut off and the weight of all trifoliate, root, stem, and nodule samples were recorded
    f. Pictures of soybean roots were taken with 1cm^2 unit grid paper

g. Nodules were carefully sliced open to check color

h. Samples were dried at 70℃ until constant weight. Dry weight of all samples were recorded

Data Analysis

Data were collected and expressed through grouped column charts (infogram.com and Excel 2006). Data from individual trials as well as overall averages were recorded. Statistical differences in treatment groups were analyzed using Student's t-tests to create *p* values. If a significant statistical difference was identified in root, stem, or leaves dry weight, then the soybean group demonstrated significantly improved development. Furthermore, the variable X was defined in order to further assess nodulation: X=(active nodules/ nodule number) * nodule weight. If two statistically significant differences were identified in nodule number, nodule weight, active nodule number, nodule number per mg root dry weight, or X, then the soybean group demonstrated significant statistical improvement in nodulation. For each test, the null hypothesis (H_0) was set to $\mu_1=\mu_2$ for the mean of the l-tryptophan data set and the mean of another data set respectively, and the alternative hypothesis (H_a) was set to $\mu_1>\mu_2$. Statistical significance was set to $p<0.05$ when n=6 trials. All statistical tests were performed on Excel 2006.

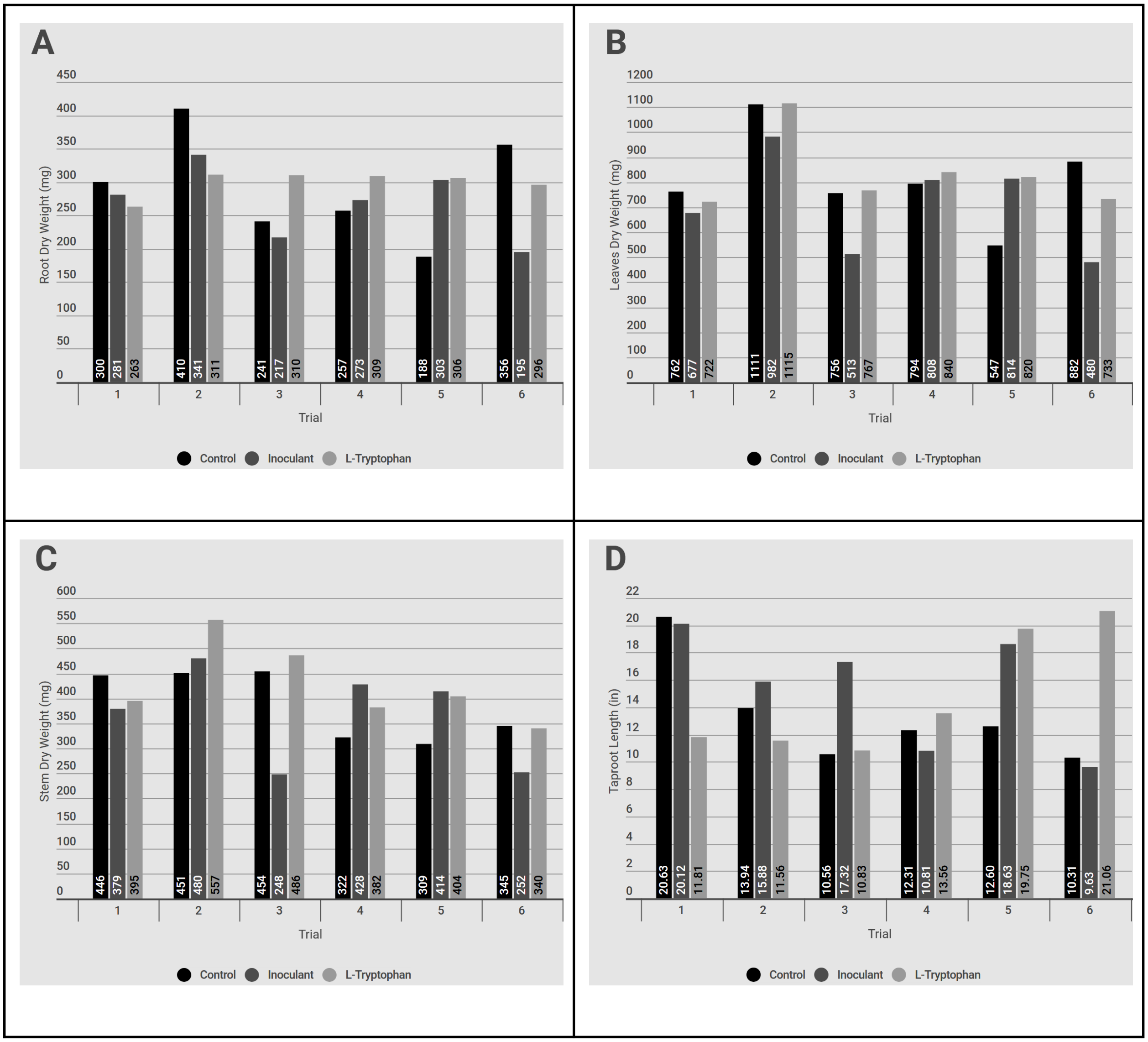
Results

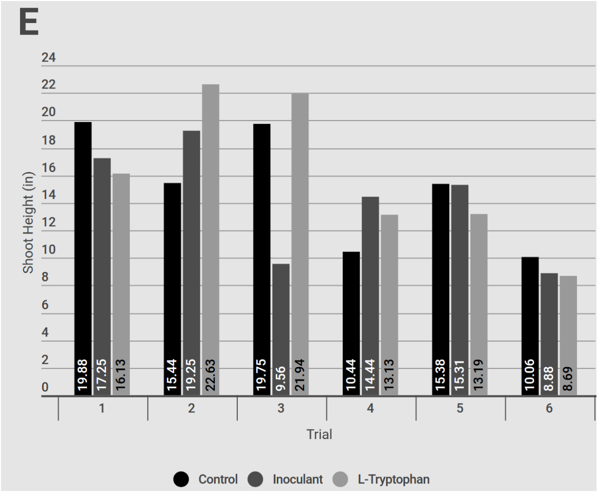
In order to eliminate ambiguity, it is important to note that all soybeans that received the l-tryptophan will be referred to as "l-tryptophan treated soybeans," and all soybeans that only received the inoculant will be referred to as "inoculant-treated soybeans." Soybeans that did not receive any treatment will simply be referred to as "control soybeans"

Acidic Soil Inhibits Soybean Development

To assess soybean development under acidic conditions, a variety of measurements were taken after 23 days of growth (Figure 1). Data were analyzed by calculating the averages for each measurement over 6 trials (Figure 3), and Student's t-tests were taken between each set of measurements in order to gauge statistical significance (Figure 6 and 7). In order to gain a deeper insight into the development of the soybeans, two soybeans were grown in un-adjusted potting soil (pH=6.5) without any treatments. The same measurements were collected in order to compare results (Figure 2). As expected, the two soybeans demonstrated improved development in all measurements when compared to the soybeans grown in acidic conditions. For example, the soybeans grown in pH=6.5 soil sustained an average root dry weight of 331 mg (Figure 2), which is 31.83 mg greater than the average root dry weight of the l-tryptophan soybeans. Based on this data, it is determined that the acidic conditions inhibited the soybeans.

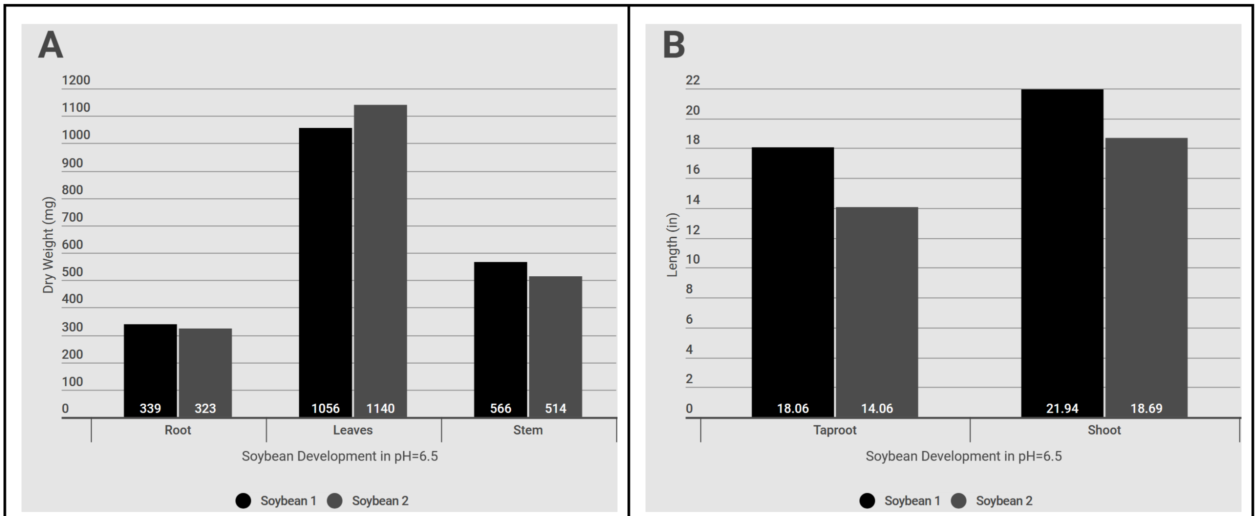
Figure 1.

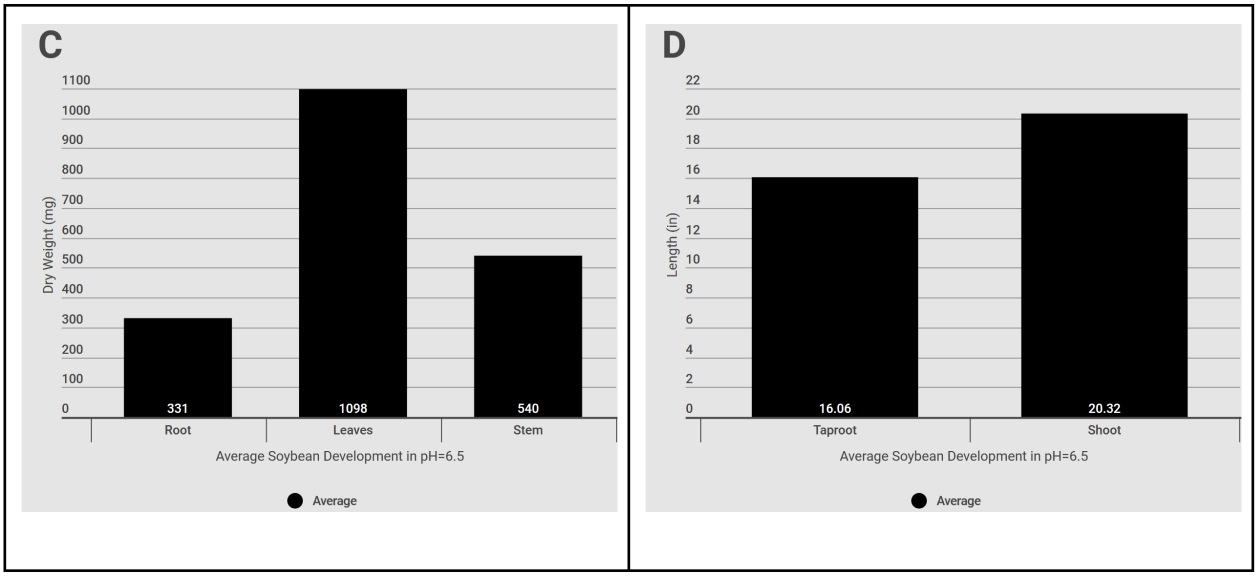

A
Root Dry Weight (mg)
450
400
350
300
250
200
150
100
50
0
300 281 263
410 341 311
241 217 310
257 273 309
188 303 306
356 195 296
1 2 3 4 5 6
Trial
Control Inoculant L-Tryptophan
B
Leaves Dry Weight (mg)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
762 677 722
1111 982 1115
756 513 767
794 808 840
547 814 820
882 480 733
1 2 3 4 5 6
Trial
Control Inoculant L-Tryptophan
C
Stem Dry Weight (mg)
600
550
500
450
400
350
300
250
200
150
100
50
0
446 379 395
451 480 557
454 248 486
322 428 382
309 414 404
345 252 340
1 2 3 4 5 6
Trial
Control Inoculant L-Tryptophan
D
Taproot Length (in)
22
20
18
16
14
12
10
8
6
4
2
0
20.63 20.12 11.81
13.94 15.88 11.56
10.56 17.32 10.83
12.31 10.81 13.56
12.60 18.63 19.75
10.31 9.63 21.06
1 2 3 4 5 6
Trial
Control Inoculant L-Tryptophan



Measurements collected after 23 days of growth in pH=4.9 soil to assess soybean development. A, Root Dry Weight. B, Leaves Dry Weight. C, Stem Dry Weight. D, Taproot Length. E, Shoot Height. Data represents 6 trials (n=6). Charts were made on infogram.com

Figure 2.



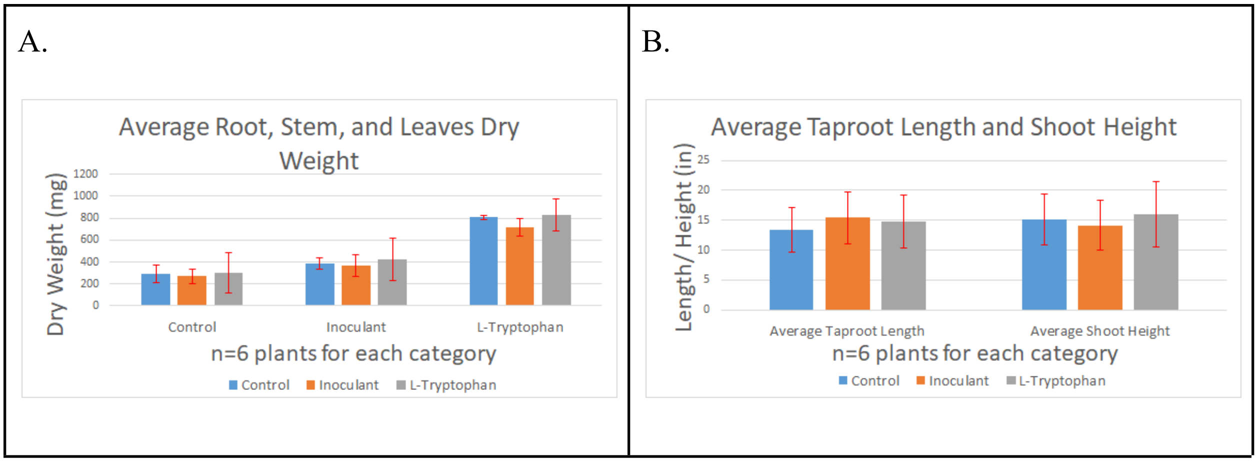


Charts represent data from two soybeans grown in un-adjusted (pH=6.5) soil. Measurements were collected after 23 days of growth. A, Dry Weights of Roots, Leaves, and Stems. B, Lengths of Taproots and Shoots. C, Average Dry Weights of Root, Leaves, and Stem. D, Average Lengths of Taproot and Shoot. Charts were made on infogram.com.

L-Tryptophan Treated Soybeans Do Not Exhibit Statistically Significant Improvement in Development when Compared to other Soybeans Grown in Acidic Soil

When comparing average dry weights, the l-tryptophan-treated soybeans exhibited the highest averages with 299.17 mg for roots, 832.83 mg for leaves, and 427.83 mg for stems (Figure 3). These averages were always followed by the control soybean measurements, and the inoculant soybeans developed the worst averages. Although the l-tryptophan treated soybeans yielded the highest averages in all dry weight measurements (Figure 3), there were no statistically significant improvements in any of these measurements (Figure 6). The *p* values were closer to the significant threshold when comparing the l-tryptophan and inoculant data sets, but they did not meet the threshold of $p<0.05$. Because none of the null hypotheses were rejected, the data did not support the hypothesis.

Figure 3.

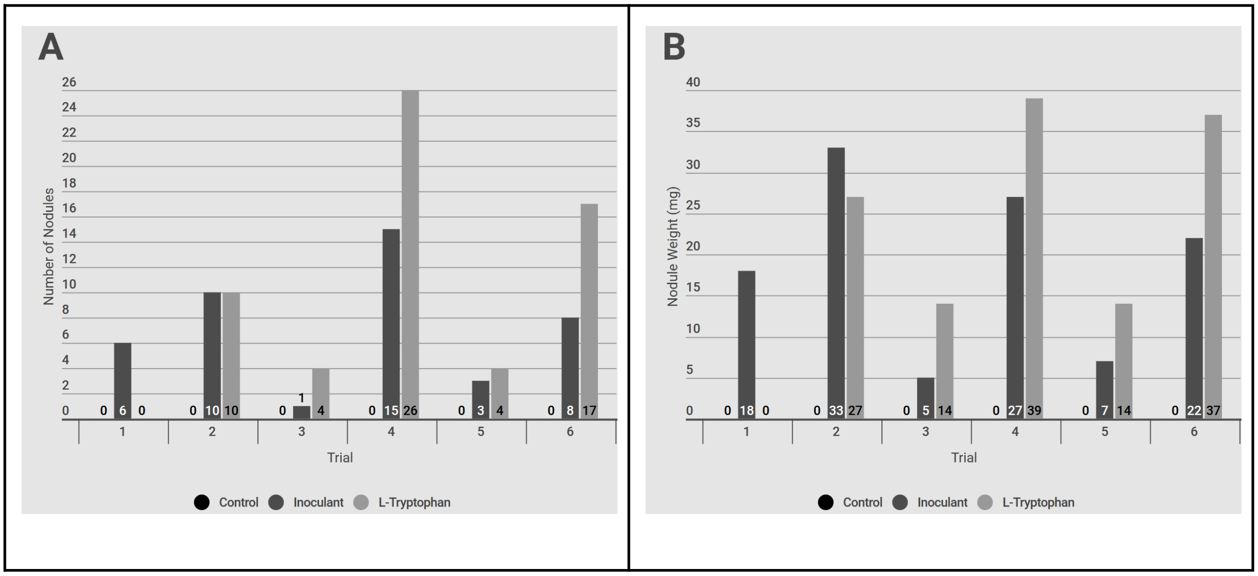


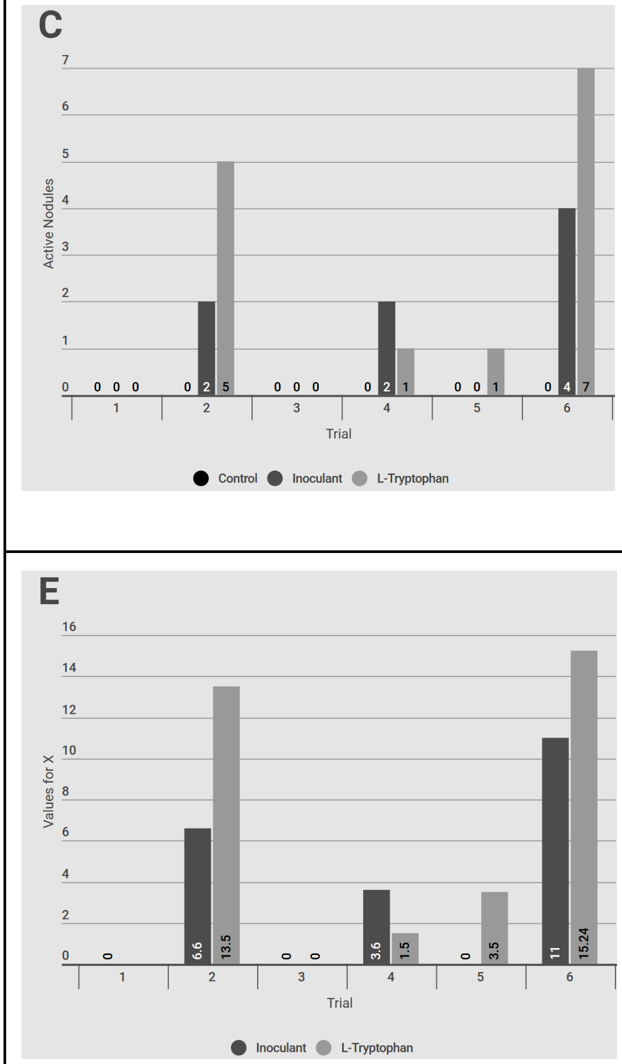
Average assessment of soybean development (n=6 trials). A, Average Dry Weights of Roots, Leaves, and Stems. B, Average Lengths of Taproot and Shoot. Error Bars represent standard deviation (SD). Charts were made on Excel 2006.

L-Tryptophan Treated Soybeans Do not Exhibit Statistically Significant Improvement in Nodulation when Compared To Other Soybeans Grown in Acidic Soil

In order to assess soybean nodulation, the number of nodules, number of nodules per mg root dry weight, nodule weight, number of active nodules, and computations for the value X were recorded (Figure 4). The value X was calculated by dividing the number of nodules by the number of active nodules and then multiplying the product by the weight of the nodules. X was calculated in order to represent three variables, but the assessment of nodulation also took into consideration other measurements (Figure 7). As expected, the control soybeans did not nodulate. Nodulation was inhibited under acidic conditions with the greatest number of nodules being only 26 nodules with the corresponding greatest total nodule weight of 39 mg (Figure 4). Furthermore, active nodules were scarce with some nodulated soybeans displaying no active nodules at all. In all nodulation-related measurements, the l-tryptophan treated soybeans displayed the greatest average results (Figure 5). On average, the l-tryptophan treated soybeans displayed three more nodules and one more active nodule when compared to the results of the inoculant-treated soybeans (Figure 5). Furthermore, the l-tryptophan treated soybeans developed an average nodule weight of 21.83 mg, which is about 16.93% higher than the average nodule weight of the inoculant treated soybeans (Figure 5). However, none of the *p* values in comparing the l-tryptophan and inoculant data sets were statistically significant (Figure 7); these results fail to reject the null hypothesis that l-tryptophan will not affect the nodulation of soybeans. It is also important to note that the l-tryptophan treated soybean in trial 1 failed to nodulate (Figure 4). This failure to nodulate nullifies the predicted benefits of the l-tryptophan on the soybean/ *B.Japonicum* symbiosis. The results of this data do not support the hypothesis.

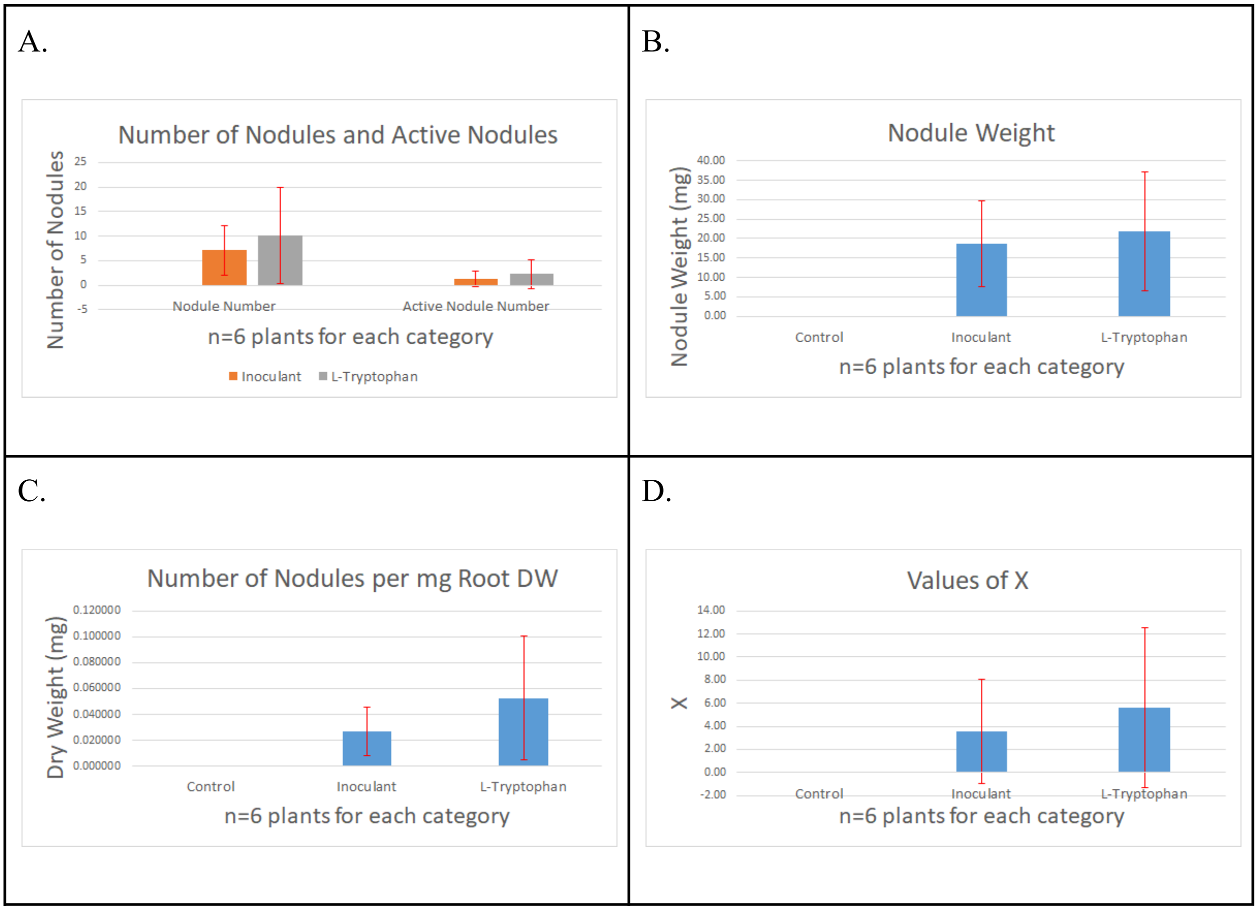
Figure 4.





*Soybean nodulation following 23 days of growth in pH=4.9 soil. A, Total Nodule Number. B, Total Nodule Weight. C, Active Nodules. D, Number of Nodules per milligram Root Dry Weight. E, Values for X (X=(active nodules/nodule number)*nodule weight). Data represents 6 trials (n=6). Charts were made on infogram.com*

Figure 5.



Average assessment of soybean nodulation (n=6) . A, Average Number of Nodules and Active Nodules. B, Average Weight of Nodules. C, Average Number of Nodules per mg Root Dry Weight. D, Average Values of X. Error Bars represent SD. Charts were made on Excel 2006.

Figure 6

	Root DW	Leaves DW	Stem DW
L-tryptophan/Control	p=0.42004	p=0.403367	p=0.190544
L-tryptophan/Inoculant	p=0.117074	p=0.1266	p=0.13155

This table shows the p values for the Student's t-test between the l-tryptophan measurement sets and control/inoculant measurement sets. The columns indicate what set of measurements is being statistically compared, while the rows indicate which group is being compared to the l-tryptophan treated soybean measurements. For each Student's t-test, the Null Hypothesis was set to "L-Tryptophan will not affect the growth of soybeans" (H_0: $\mu_1=\mu_2$; $p\geq 0.05$ when $n=6$ trials), and the Alternative Hypothesis was set to "L-Tryptophan will positively affect the growth of soybeans" (H_a: $\mu_1>\mu_2$; $p<0.05$ when $n=6$ trials). DW= dry weight.

Figure 7

	Nodule Number	Active Nodule Number	Nodule Weight	Nodule Weight per mg Root DW	X
L-Tryptophan/Inoculant	p=0.261861	p=0.244044	p=0.344419	p=0.13254	p=0.275926

*This table shows the p value for the Student's t-test between the l-tryptophan and inoculant data sets. The columns indicate what data set is being compared. For each Student's t-test, the Null Hypothesis was set to "L-Tryptophan will not affect the nodulation of soybeans" (H_0: $\mu_1=\mu_2$; $p\geq 0.05$ when $n=6$ trials), and the Alternative Hypothesis was set to "L-Tryptophan will positively affect the nodulation of soybeans" (H_a: $\mu_1>\mu_2$; $p<0.05$ when $n=6$ trials). DW =dry weight. X = (active nodule/nodule number)*nodule weight.*

Discussion

The results of the experiment did not support the hypothesis that l-tryptophan will improve soybean development and nodulation in acidic soil. Although the l-tryptophan treated soybeans exhibited the highest averages in almost all of the measurements (Figures 3 and 5), the Student's t-tests failed to reject any of the null hypotheses (Figure 6 and 7). When examining the tests, the *p* values closest to the threshold value of 0.05 are the tests between the l-tryptophan and inoculant dry weights (Figure 6). Not surprisingly, the inoculant-treated soybeans exhibited the worst dry weight averages (Figure 5). This supports the widely observed sensitivity of nodulated soybeans to acidic soil (Ferguson et al., 2013; Jaiswal et al., 2018; Lin et al., 2012; Taylor et al., 1991).

Despite the lack of statistically significant improvements, the data encourages the further examination of the hypothesis; the average measurements support the hypothesis, but the variances in the samples introduces an ambiguity to the data. Although the hypothesis was not supported, there are still a few inferences that can be made. For example, the results that the l-tryptophan treated soybeans exhibited higher averages than other soybeans grown in acidic soil but lower averages than the unadjusted soybeans supports the idea that plants possess numerous mechanisms involved in acidic stress response (Shavrukov & Hirai, 2016). If the mean differences found in this study hold true, it can be speculated that the application of l-tryptophan improves the stress response of the soybean/*B.Japonicum* symbiosis, but it does not completely ameliorate acidic stress. This corroborates the idea of the function of IAA in the legume-microbe symbiosis (Camerini et al., 2008; Donati et al., 2013; Spaepen & Vanderleyden, 2011; Spaepen et al., 2007). Furthermore, the failure of the l-tryptophan treated soybean in trial 1 to nodulate contradicts the idea that the exogenous application of auxins increases bacterial colonization of plants (Spapen et al., 2007). This implies that the application of exogenous l-tryptophan does not initiate rapid regulations in gene expression like the direct application of IAA does; the synthesization of IAA from l-tryptophan occurs only after initial nodulation is established. This is corroborated by the observation that acidic soil inhibits nodulation early in development (Lin et al., 2012). Furthermore, the idea that rhizobia depend on Ca^{2+} to attach to the surface of legume root cells can account for the failure to nodulate (Smit et al., 1992). Not surprisingly, the l-tryptophan treated soybean that failed to nodulate exhibited worse root, leaves, and stem dry weights when compared to the averages for all l-tryptophan treated soybeans. This skewed the averages for the l-tryptophan treated soybeans and worsened the ambiguity.

Furthermore, it is worth noting that the soybeans in the un-adjusted soil had an average taproot length of about 16 inches, which is only 0.6 inches greater than the average taproot length of the inoculant treated soybeans. This supports the idea that taproots are less sensitive to acidic soils than lateral roots (Ferrufino et al., 2000). Finally, it is important to note that some soybeans grown in acidic conditions exhibited improved development in specific measurements when compared to the soybeans grown in un-adjusted soil. This may be due to the partial buffering capacity/ ion exchange effect of the peat in the potting soil. After experimentation, the pH of the potting soils were measured to be 5.5±0.5. This is higher than the pH originally measured, but it is still inhibitory to soybeans (Lin et al., 2012).

In conclusion, the application of exogenous l-tryptophan was not statistically proven to improve the growth of soybeans in acidic soil. The failure of the l-tryptophan treated soybean in trial 1 to nodulate demonstrates how the direct application of l-tryptophan differentiates from the direct application of IAA. While the direct application of IAA forces changes in gene expression, leading to increased susceptibility of legumes to infection by rhizobacteria, it can be inferred that l-tryptophan-induced development depends on the manner in which the soybeans/*B.Japonicum* choose to utilize l-tryptophan. For example, the free-living *B.Japonicum* in trial 1 may have synthesized IAA after initial nodulation was inhibited and catabolized the IAA as a carbon source (Egebo et al., 1991; Jensen et al., 1995). Unfortunately, the presence of an apparent outlier in trial 1 further exacerbates the statistical uncertainty of the hypothesis. In the end, it can be concluded that the application of l-tryptophan to soybeans and *B.Japonicum* does not statistically ($p<0.05$) improve the overall development and nodulation of soybeans grown in acidic soil for this experiment.

It is recognized that the ambiguity of the Student's t-test demands more trials in order to collect more sample data. However, it is certain that such ambiguities will be eliminated in the future when time constraints are not present. Although the p values did not meet the threshold, it is still believed that the hypothesis may be valid because the l-tryptophan treated soybeans always surpassed the assessed averages when compared to the other soybeans grown in acidic soil. Furthermore, it is recognized that two soybeans can not fully represent the development of soybeans grown in un-adjusted potting soil. However, this study was focused on the comparison of soybeans grown in acidic soil, and the soybeans grown in un-adjusted potting soil were merely utilized to gain a vague understanding of how soybeans should optimally develop. The soybeans grown in un-adjusted potting soil were also utilized to confirm the inhibition of soybeans grown in acidic soils.

In the future, it would be of great interest to work in labs in order to achieve a higher sample size and more advanced measurements. Future research may also consider measuring the timing of initial nodulation and the amount of IAA present in the soybeans throughout development in order to gain a deeper insight into the effects of exogenously applied l-tryptophan on the legume/microbe symbiosis. It is understood that there are numerous treatments available to ameliorate acidic stress, but this experiment is focused on developing a practical treatment that is accessible to farmers worldwide. Currently, 120,000 mg of l-tryptophan can be purchased on Amazon for $18.95. Estimating that up to 140,000 soybean seeds are planted per acre, the price to treat an acre of soybeans with l-tryptophan would be approximately $110.54 per acre. Numerous factors affect the cost of liming soil, but although liming soils may be less costly in long-term cases, it is important to recognize that liming is not practical for many remote small-scale farmers who lack the capital or optimal geography to invest in lime. Furthermore, optimal liming requires lab-testing of soil, shipping of numerous tons of lime, machinery/labor to successfully spread lime, and access to quality limestone. In the end, providing farmers with a few bottles of l-tryptophan to be applied along with an inoculant seems to be more practical than providing farmers with many tons of limestone that they must apply with machinery. Hopefully, this study encourages future researchers to consider the use of l-tryptophan as a practical and perhaps inexpensive constituent of a treatment for soybeans grown in acidic soil.

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Supplementary Photos

https://web.kamihq.com/web/viewer.html?state=%7B%22ids%22%3A%5B%221MLIhuVtgWjc4QoQZeM7OFdG2Nr_YRrUK%22%5D%2C%22action%22%3A%22open%22%2C%22userId%22%3A%22116690596764027667505%22%2C%22resourceKeys%22%3A%7B%7D%7D&kami_user_id=9048128

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