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EFFECTS OF THE COPPER TOLERANCE PROTEIN BLL2211 (COPB) IN THE NODULATION PROCESS FOR SOYBEAN PLANTS AND NITROGEN-FIXING BACTERIA BRADYRHIZOBIUM JAPONICUM

Christina Koo

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by

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ABSTRACT

EFFECTS OF THE COPPER TOLERANCE PROTEIN BLL2211 (*COPB*)
IN THE NODULATION PROCESS FOR SOYBEAN PLANTS
AND NITROGEN-FIXING BACTERIA
BRADYRHIZOBIUM JAPONICUM

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The University of Texas at Arlington, 2017

Faculty Mentor: Woo-Suk Chang

Bradyrhizobium japonicum is a nitrogen-fixing bacterium which forms a symbiotic relationship with leguminous soybean plants, converting atmospheric nitrogen into ammonia. The process of nodulation involves signaling components from the plant and activation of genes promote nodule formation. Soybean lectin's current function in plants are unknown and may be a crucial component in nodulation. In previous studies, the copper tolerance protein, bll2211 (*copB*) was differentially expressed when exposed to soybean lectin. In this study, the role of environmental copper exposure was investigated to understand the role of soybean lectin in growth and nodulation of *B. japonicum*. We examined this by construction of a growth curve and performing a pouch experiment at various concentrations of copper for *B. japonicum* and its mutant Δ*copB*.

Overall, there was less growth, nodule formation, and nitrogen-fixation in Δ*copB*. Thus, soybean lectin and *copB* may play a role in nodulation between *B. japonicum* and soybean plants.

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CHAPTER 1

INTRODUCTION

Bacteria can form symbiotic relationships with their host plants. One model of this relationship is biological nitrogen fixation, or BNF. In this model, the bacteria will fix atmospheric nitrogen, N_2, into a more soluble, usable form of ammonia, NH_3. In return, the plant will provide essential nutrients and photosynthetic products for the bacteria, creating a mutual, beneficial relationship. One model of plant-microbe interactions of biological nitrogen fixation involves *Bradyrhizobium japonicum*, a Gram-negative, non-spore forming, and nitrogen-fixing bacterium found in soil, and soybean plants, *Glycine max*.

Biological nitrogen fixation is a beneficial model to understand, since it could potentially allow a more sustainable form of agriculture. In particular, plants require nutrients and specifically nitrogen to grow. Free forms of nitrogen, which reside as N_2 in the atmosphere, are not utilizable by plants. Thus, plants must be supplied with nitrogen from fertilizers to grow. However, fertilizers require immense heat and pressure to produce, making it very costly to grow crops. In addition, with the use of fertilizers, excess nitrogen that runs off into water systems, such as oceans and rivers, causes severe environmental impact, including the eutrophication of water systems. Eutrophication can lead to algal blooms, which can have detrimental impacts on the surrounding species by deoxygenating the water (6).

Understanding the role of environmental factors towards biological nitrogen-fixation is important to build a strong foundation towards optimizing agricultural crop yields, reducing costs of using expensive fertilizers, and reducing the environmental contamination of nitrogenous waste into water systems. With an increasing population, the demands for agricultural products and an increased crop yield are pertinent; biological nitrogen fixation can be used to help alleviate the increased demands of crop yields and costly, deleterious effects of fertilizers to provide a more sustainable form of agriculture.

The process in which bacteria enter the plant, or nodulation, is known to typically involve factors secreted by the plant that induces expression of nodulation genes in *B. japonicum* (1). The typical model of nodulation involves secretion of plant factors called flavonoids that are released from the plant roots into the rhizosphere (2, 3). These flavonoids will induce expression of *nod* factor genes in *B. japonicum* (4). These plant signaling factors and expression of the *nod* genes ultimately induce infection of *B. japonicum* into the soybean plants, creating an infection thread and root hair curling (3). Subsequently, the bacteria will form nodules, where they exists as bacteriods, and the bacteria fix atmospheric nitrogen into ammonia, while the plant provides essential nutrients for growth, such as photosynthetic products and adequate amounts of oxygen required for nitrogen fixation (3, Figure 1.1).

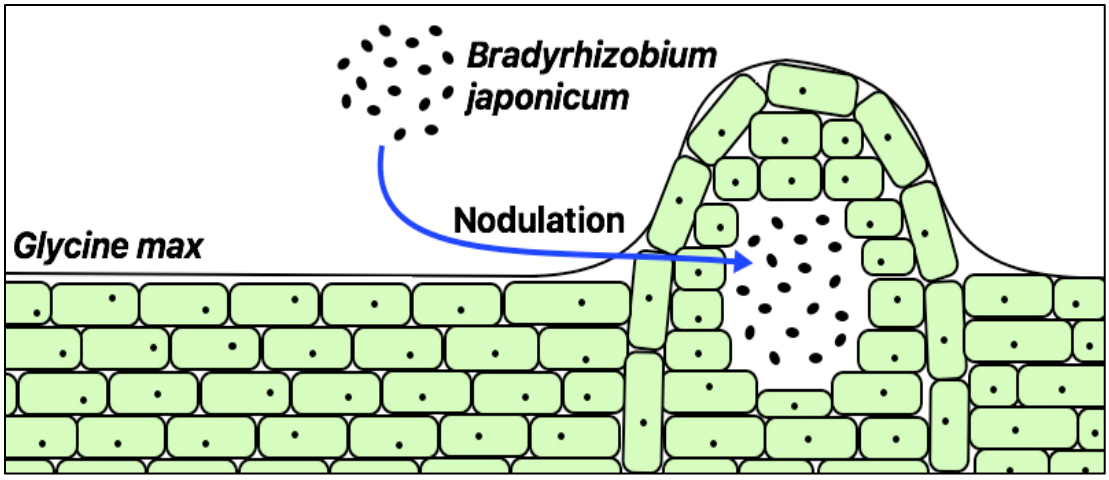

Figure 1.1: Symbiotic Model of Nodulation

However, there may be other factors involved in the overall process of nodulation. One potential molecule may be soybean lectin, a carbohydrate-binding protein excreted by soybean, as a signaling factor. Soybean lectin, a prevalent component in the environment for the nitrogen-fixing bacteria *B. japnocium*, may actually play a role in the nodulation process, as the current function of soybean lectin in plants and in its symbiotic relationship are currently unknown. Exposure to soybean lectin may potentially induce expression of other essential genes that play a role towards nodulation (5). In addition, it is thought that soybean lectin can aid in the nodulation process in root adsorption of bacteria and formation of the infection thread (7, 8).

In a previous study, *B. japonicum* USDA110 differentially expressed a copper tolerance protein gene, bll2211 (*copB*), when exposed to soybean lectin. Copper, usually present in trace elements for growth of USDA110, may affect nodulation when coupled with soybean lectin. We hypothesized *copB* plays a role in nodule formation in the presence of heavy metal stress. To examine this, a functional genomics study of the copper tolerance protein gene was conducted to observe how copper and *copB* impacted the overall nodulation process. By examining the role of *copB*, the functions of this gene and its

overall role and effect in the nodulation process of soybean plants and USDA110 bacteria can be better understood. Thus, this may broaden the current understanding of the model of biological nitrogen fixation.

CHAPTER 2

METHODOLOGY

2.1 Gene Selection

In a previous study, soybean lectin was exposed to *B. japonicum* strain USDA110 bacteria. Through DNA microarray, up-regulated and down-regulated genes were observed. Of the differentially expressed genes that were up-regulated, a copper tolerance protein gene, bll2211 (*copB*), was selected for mutant construction for gene knock-out. The mutant was mutagenized through double-homologous recombination, replacing the copper tolerance protein gene with a kanamycin cassette.

2.2 Growth Curve

In this ongoing study, the growth of the mutant Δ*copB* was observed by construction of a growth curve in comparison to the wild-type, *B. japonicum* strain USDA110. An initial culture of bacteria USDA110 and mutant were incubated in 10 ml of arabinose-gluconate (AG) media, adjusted to a pH of 6.8 at 30°C, and shaken at 200 rpm for 3 days. Then, 50 µl of each individual initial culture was inoculated into 50 ml of Bergersen's minimal media, Bergersen's minimal media supplemented with 10 µM of $CuSO_4 \cdot 7H_2O$, and Bergersen's minimal media supplemented with 50 µM of $CuSO_4 \cdot 7H_2O$. Three biological replicates for each condition for the wild-type and the mutant were observed. The optical density at 600 nm was recorded every 12h for 144h to examine growth.

2.3 Pouch Experiment

To examine the effect of environmental exposure of copper to the mutant's performance on nodulation and nitrogen fixation, a pouch experiment was conducted. Soybean seeds were sterilized by immersing seeds in 25% Clorox for 10 minutes and subsequent washing with autoclaved deionized H_2O. The seeds were then immersed in 0.1 M HCl for 10 minutes and additional washing was performed with autoclaved deionized H_2O. The seeds were germinated in a sterilized petri dish containing water agar and supplemented with 100 µl of autoclaved deionized H_2O. The seeds were kept in the dark in foil wrapped plates and incubated for 3 days until the seeds reached an inch of root sticking out.

After seeds were germinated, in a sterile environment, 3 germinated seeds were placed into a sterilized pouch, marking the positions of the root tips on each side, along with a straw. The pouches were then placed into a hanging folder, using paper clips to attach the pouch, in a folder rack. Using the straw, 20 ml of half-strength Broughton and Dilworth (B&D) medium, half-strength B&D medium with 10 µM of $CuSO_4 \cdot 7H_2O$, or half-strength B&D medium supplemented with 50 µM of $CuSO_4 \cdot 7H_2O$ was added to the pouch. For each bacterial strain, 1.0 ml (ca. $1x10^8$ cells/ml) of bacterial culture of 0.1 OD_{600} was inoculated on each seed. The cells were incubated in the pouches with a cycle of 15h of day and 9h of night at 27ºC for 30 days. All pouches were watered with its respective half-strength B&D medium, half-strength B&D medium supplemented with 10 µM of $CuSO_4 \cdot 7H_2O$, or half-strength B&D medium supplemente4d with 50 µM of $CuSO_4 \cdot 7H_2O$.

After incubation, the soybean plants were harvested, and the physical properties were examined, including average nodule size, nodule number, nodule dry weight, and plant dry weight. In a single pouch experiment, 6 pouches, each containing 3 seeds, were used for each strain per $CuSO_4$ concentration, for a total of 18 seeds per experimental condition.

2.4 Acetylene Reduction Assay

The overall activity of nitrogen fixation was measured by an acetylene reduction assay to quantify the activity of the nitrogenase enzyme. Using a Shimadzu GC-2014 machine, the ethylene production was measured through the reduction of acetylene. Samples from the soybean plants were prepared for gas chromatography after harvesting. Soybean roots and nodules were placed into an airtight flask comprising of 10% acetylene. 250 µl of the harvested soybean samples were placed into the GC chamber and run for 8 minutes. The area of the ethylene peak was recorded for each soybean seed used in the pouch experiment to indirectly quantify the nitrogenase activity through ethylene production. Ethylene production was measured at time intervals of 0h, 1h, and 2h for each treatment and replication.

2.5 Statistical Analysis

Statistical analysis of the pouch experiment was done by conducting a Student's t-test with a significance level of $\alpha=0.05$.

CHAPTER 3

RESULTS

3.1 Growth Curve

B. japonicum USDA110 grown in minimal media appeared to grow better than the mutant Δ*copB* at almost all concentrations of copper. The wild-type had almost the same amount of growth compared to the mutant at 0 µM of Cu (Figure 3.1). Although there was less growth in the mutant compared to the wild-type at 50 µM, there appeared to be almost no growth in both USDA110 and Δ*copB*, as optical density values never exceeded OD_{600}=0.1. In addition, at 10 µM of Cu, there was an apparent difference in growth from the wild-type compared to the mutant; there was also a slight difference in growth of the wild-type and mutant in 25 µM Cu.

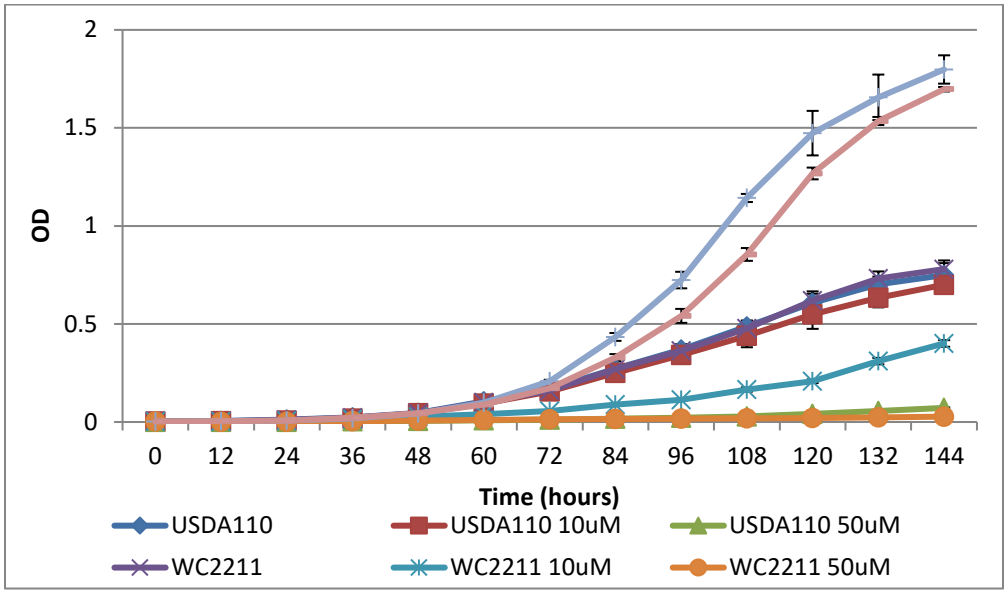

Figure 3.1: Growth Curve in Various Copper Concentrations

Since there was not a significant difference in the growth of the wild-type and the mutant, it appeared that the overall environmental exposure of copper at the tested concentrations did not affect the difference in the number of cells between the wild-type and mutant in the varying treatments of Cu.

3.2 Soybean Plant Dry Weight

Soybean plants inoculated with USDA110 and Δ*copB* overall showed no statistical difference in plant dry weight at 0 µM Cu treatment with ½ strength B&D media. In addition, this treatment showed the greatest plant dry weight. The treatments with 10 µM Cu with ½ strength B&D media showed no statistical difference between the wild-type and mutant, although displaying less growth than the treatment with 0 µM Cu. There was a statistical difference (p-value<0.01) at 25 µM of Cu in ½ strength B&D media. Thus, Δ*copB* showed less growth at the higher concentrations of Cu heavy metal stress. In addition, it is worth nothing that the wild-type maintained steady plant growth at 10 µM and 25 µM of Cu treatments, while the mutant displayed a decrease in plant dry weight as Cu treatment increased (Figure 3.2). Overall, plant dry weight decreased as the Cu treatment increased, but the wild-type maintained a steadier dry weight in comparison to the mutant.

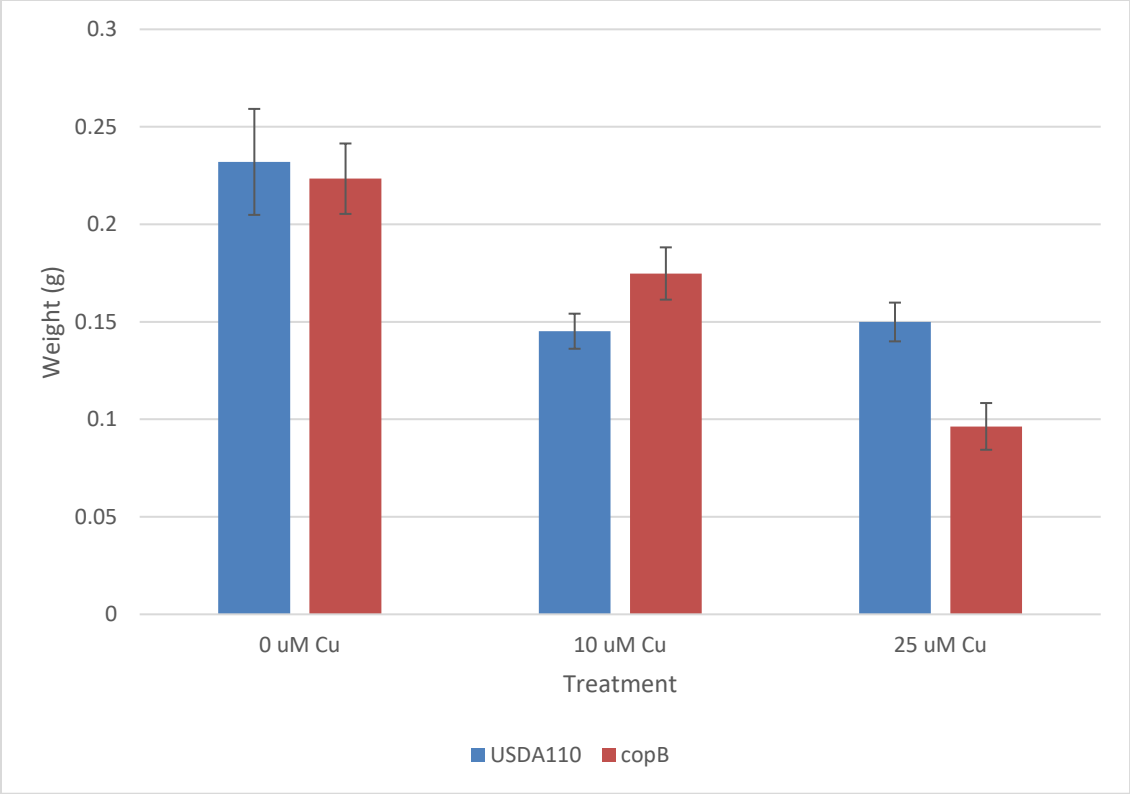

Figure 3.2: Plant Dry Weight

3.3 Soybean Nodules

The number of nodules showed no statistical difference in the treatments of 0 µM and 10 µM of Cu in USDA110 and Δ*copB*. At the treatment with 25 µM Cu, there was a statistical difference (p-value<0.01) in the nodules between USDA110 and Δ*copB*, with USDA110 producing more nodules than Δ*copB*. Overall, there was a decrease in the number of nodules in Δ*copB* with increasing Cu treatment, while USDA110 maintained a steady amount of nodules throughout treatments (Figure 3.3). It is also worth noting that both the wild-type and mutant produced the most nodules at 0 µM of Cu.

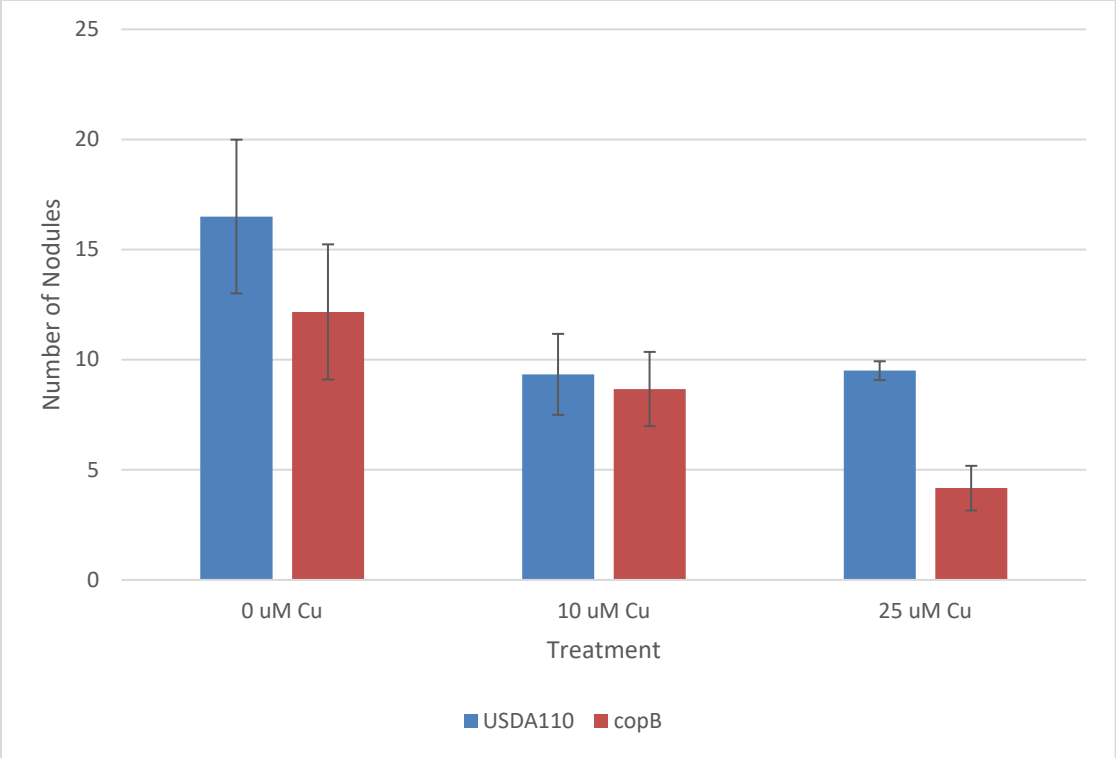

Figure 3.3: Nodule Number

The average nodule weight per nodule showed no statistical difference among both the wild-type and mutant in all different treatments (Figure 3.4). The nodules in the 0 µM Cu treatment showed the most weight per nodule, while the 10 µM and 25 µM Cu treatments showed the least weight per nodule. Overall, the nodules on the treatments with Cu were smaller than the treatment with no Cu on both the wild-type and mutant.

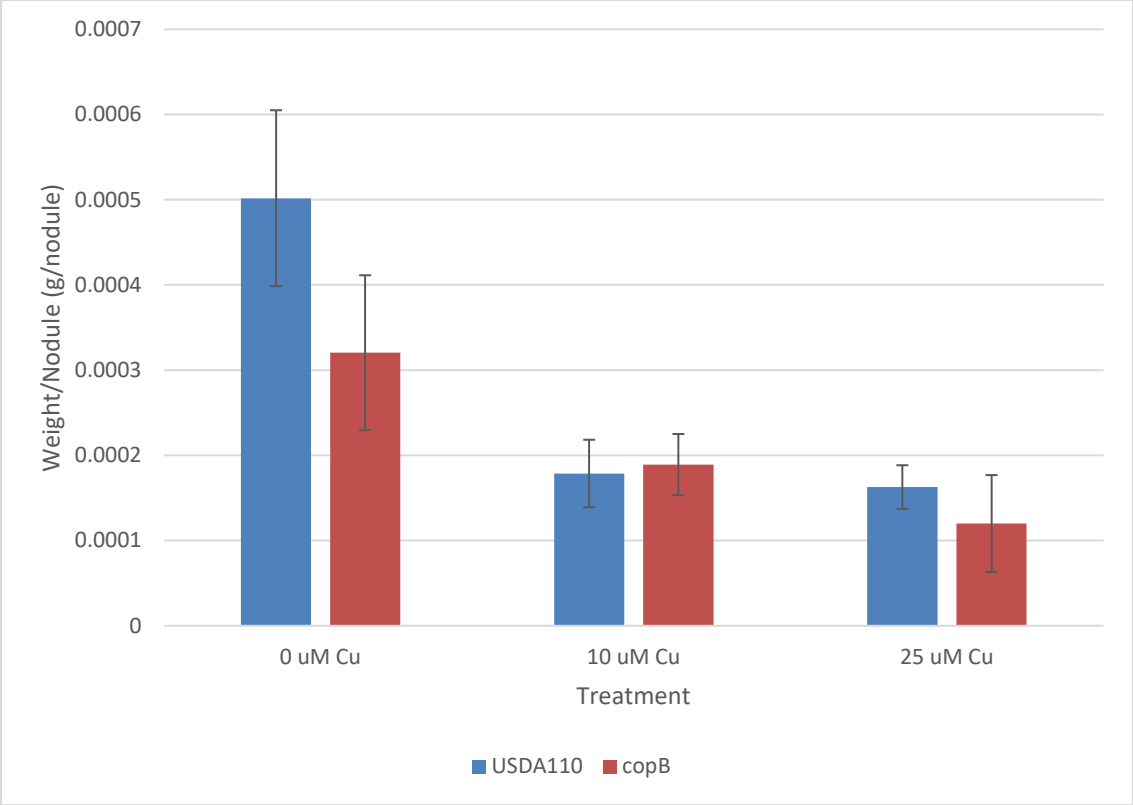

Figure 3.4: Nodule Dry Weight/Nodule Number

3.4 Nitrogenase Activity

The acetylene reduction assay quantified ethylene production, estimating the activity of nitrogenase. Overall ethylene production decreased as the Cu treatment increased, showing nitrogenase activity tampered due to heavy metal stress (Figure 3.5). Ethylene production at 0 µM and 10 µM treatments were not statistically different in USDA110 and Δ*copB*; although, it appeared that the wild-type produced more ethylene than the mutant. In addition, ethylene production at 25 µM Cu treatment showed statistical significance (p-value<0.01), showing less ethylene production in Δ*copB* compared to USDA110.

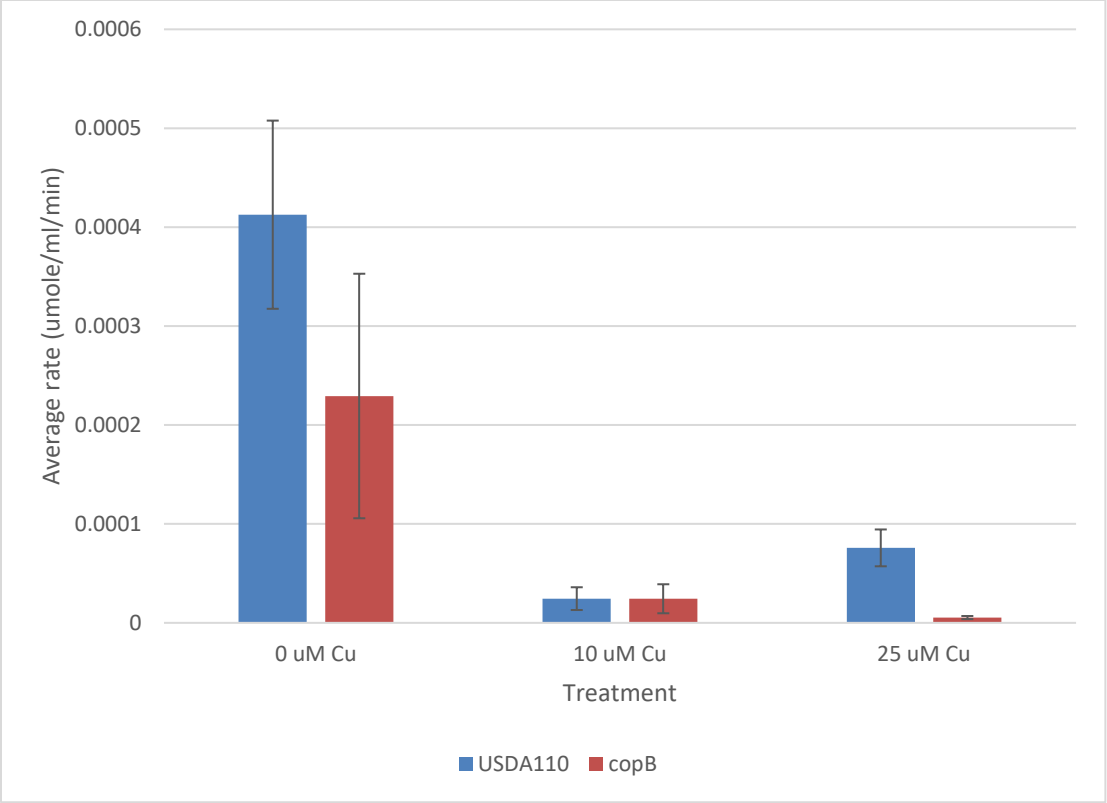

Figure 3.5: Ethylene Production

The overall ethylene production per dry nodule weight showed similar amounts of ethylene production in USDA110 at a treatment of 0 µM and 25 µM Cu (Figure 3.6); in addition, Δ*copB* appeared to show less ethylene production in the presence of Cu. The 10 µM treatment appeared overall to fix the least amount of nitrogen, while the 0 µM Cu treatment fixed the most. There was no statistical difference in the ethylene production per dry nodule weight between the wild-type and mutant in 0 µM and 10 µM Cu treatments. At the 25 µM Cu treatment, the amount of ethylene production per dry nodule weight was statistically significant (p-value<0.01) between the wild-type and mutant. Thus, there was less nitrogenase activity in treatment with a higher concentration of 25 µM Cu.

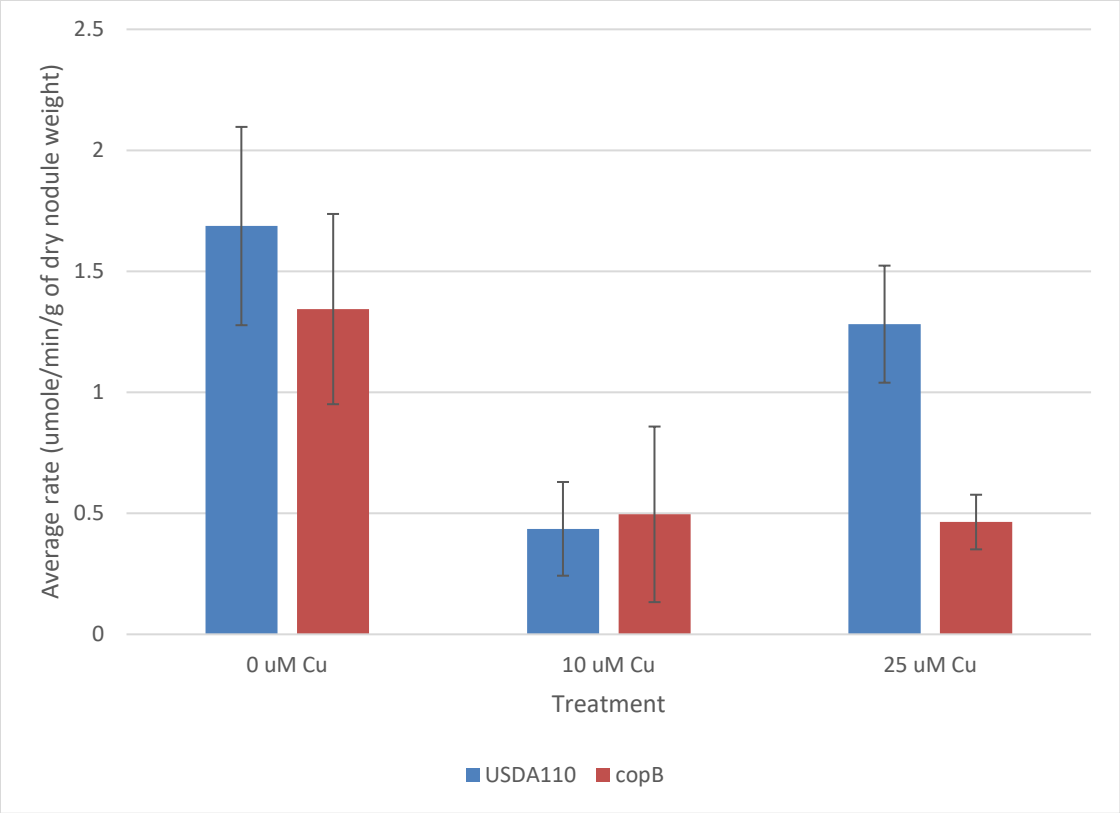

Figure 3.6: Ethylene Production/Dry Nodule Weight

CHAPTER 4

DISCUSSION

This study involved exploring the symbiotic relationship of *B. japonicum* USDA110 and soybean *G. max*. These nitrogen-fixing bacteria convert atmospheric nitrogen into ammonia for their host plants and receive photosynthetic products in return. This relationship is overall beneficial to understand as nitrogen-fixing bacteria could be a potential solution over using costly and environmentally hazardous synthetic nitrogen fertilizers. We believe that rhizobia can lead to a more eco-friendly and environmentally safe practice of agriculture for more sustainable living. Thus, understanding its relationship with its plant hosts on a molecular level is essential to optimizing biological nitrogen fixation. Here, we tested the function of bll2211, a copper tolerance protein, and its role in the nodulation process between *B. japonicum* USDA110 and *G. max*.

The copper tolerance protein, bll2211 or *copB*, was selected due to its differential expression in a DNA microarray experiment with exposure to soybean lectin in a previous experiment. Lectin, a carbohydrate-binding protein, is known to serve as a potential immune and stress response for the defense of the plant (11, 9). However, several studies have shown that soybean lectin could serve as an important molecule in the signaling and recognition of *B. japonicum* in the soil to the soybean roots (10). Although the current symbiotic function of soybean lectins are unknown, they are believed to aid in root adsorption of rhizobia and formation of the infection thread (12, 13). Since soybean lectin may be expressed for signaling and recognition to rhizobia, as well as serving as an immune

and stress response for soybeans, we believed that Cu may interact with soybean lectin and affect the symbiotic relationship with *B. japonicum* in the process of nodule formation. Currently, there are no functional genomic studies of *copB* in relation to soybean lectin or in heavy metal stress, but its annotated function is a transporter for atypical conditions and is about 1500 base pairs long (14). While *copB* was selected due to its up-regulation in gene expression, other copper tolerance genes around the same locus, such as *copA* and *copC*, and multioxidase proteins were also differentially expressed. Thus, we believed that the interaction with soybean lectin and heavy metals, such as Cu, may have played a role in this nodulation process.

From the growth curve of the wild-type, *B. japonicum* USDA110, and the mutant, Δ*copB*, there was only a slight difference in growth. It was expected that Δ*copB* would grow less than USDA110, due to the missing copper tolerance protein, but there was only a small difference. Both bacteria exhibited almost consistent growth in each concentration of Cu treatment, with the most growth at 25 μM Cu. Since there was not a true variation between USDA110 and Δ*copB*, the tested concentrations of Cu for heavy metal stress did not affect cell number or health of the cells overall. Thus, any differences observed within our tests for nodulation were not due to the ability of USDA110 and Δ*copB* to thrive in minimal media in 0 μM, 10 μM, and 25 μM of Cu. However, it appeared that 50 μM of Cu for heavy metal stress was too high, as OD_{600} never exceeded 0.1; as a result, further testing with this concentration was not performed.

The pouch experiment was conducted in order to assess the role of *copB* in nodulation and nitrogen fixation. The soybean plants inoculated with the wild-type, USDA110, showed better growth, more nodule formation, and more nitrogenase activity

than the mutant, Δ*copB*, especially at the highest tested concentration of 25 μM Cu with statistical significance (p-value<0.01). In plant growth and health, nodulation, and nitrogen-fixation, Δ*copB* did not perform as well compared to the wild-type, indicating the importance of the role of copper tolerance in heavy metal stress in *B. japonicum*'s symbiotic relationship with soybeans. Furthermore, upon addition of Cu, both USDA110 and Δ*copB* showed less plant growth, nodule formation, and nitrogenase activity. Thus, the presence of heavy metals appeared to affect the health of the soybean plant and nodule formation. However, we did not observe our expected results of a gradual decrease from 10 μM Cu treatment to 25 μM Cu in nodule formation and nitrogenase activity, but we observed similar results in both treatments, with the exception of USDA110 producing more ethylene at 25 μM Cu. Perhaps this observed increase of ethylene production at 25 μM Cu for USDA110 could be due to an up-regulation of other stress responses, but further testing of gene expression of *B. japonicum* in heavy metal stress needs to be examined. Our data was consistent with our hypothesis that *copB* played a role in the formation of nodules in heavy metal stress by disturbing plant growth and nitrogenase activity.

Heavy metal contamination is becoming an increasing problem. With industrialization, technology, and industrial agriculture, hazardous materials are continuously being released into the soil (15). It has been shown that heavy metals can affect physiological and biochemical processes in bacteria by disruption of growth, development, and enzyme and hormone production (16). In particular, Cu contamination may come from common products used in agriculture, such as insecticides, fungicides, fertilizers, and other forms of waste in sewage and landfills. The active ingredient Cu in these fungicides and insecticides are not only toxic to pathogens, but are toxic to soil

microbes as well (17). In addition, other heavy metals, such as nickel, zinc, cadmium, and copper, have deleterious and toxic effects on soil microbiota (18). These heavy metals are a cause of concern as they are not readily biodegradable, leading to bioaccumulation and downstream consequences of heavy metal contamination (19, 20).

Our data suggests that soybean lectin and *copB* play an important role in nodulation when subjected to heavy metal stress. Future studies in testing the role of *copB* in this symbiotic relationship would encompass a greenhouse experiment and soybean field study inoculated with Δ*copB*. By obtaining a better understanding of the molecular processes of nodule formation and signaling in *B. japonicum* and soybean plants, this symbiotic relationship could be optimized for greater utilization of biological nitrogen-fixation.

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BIOGRAPHICAL INFORMATION

Christina is currently a senior majoring in microbiology at UT Arlington and is a part of the Honors College. She is interested in exploring all facets of microbiology, which led her to research in environmental microbiology and microbial genetics in Dr. Woo-Suk Chang's laboratory. There, she primarily investigates the symbiotic relationship between nitrogen-fixing bacteria *Bradyrhizobium japonicum* and soybean plants. In Dr. Chang's laboratory, she has worked on several projects, including soil metagenomics, soybean lectin, and working with unusual models of biological nitrogen fixation with *Bradyrhizobium* BTAi1 and *Bradyrhizobium* ORS278. Although she loves environmental microbiology, she is also aspiring to pursue a career in medicine after obtaining her B.S. in Microbiology.