

**INTERVENTION OF THE RICE MICROBIOME
IN ABATING ARSENIC TOXICITY IN RICE**

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment
of the requirements for the degree of Master of Science in Plant and Soil Sciences

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“For I know the plans I have for you declares the Lord, plans to prosper you and not to harm you, plans to give you hope and a future.” – Jeremiah 29:11

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ABSTRACT

Food security and crop protection are of vital importance as the world is challenged with supplying enough food for the growing population. Arsenite (As (III)) is a naturally occurring, inorganic, form of Arsenic (As), and is responsible for polluting groundwater in areas of South East Asia, India, and Bangladesh. This contamination poses a serious health concern to people using As(III)- polluted water for drinking and irrigation. Rice (*Oryza sativa*) is among one of the largest agronomic crops in Asia and provides essential caloric intake to much of the world's population. Rice plants exposed to inorganic As(III) suffer from low grain yields and overall poor plant health as a result of increased As concentrations in the plant grains and tissues. It is hypothesized that plant growth promoting rhizobacteria (PGPR) can be used to ameliorate the effects of As contamination by limiting uptake into the rice plant. There has been minimal research done to identify the effects of As or bio-inoculants on plant hormone expression levels, which play an active role in plant growth, development, and defense. Through a series of hydroponic and gene expression assays I analyzed the effects of varying As toxicity and bacterial inoculants on rice plant development. As- tolerant Nipponbare varieties show grain production at high As(III) concentrations compared to As- susceptible IR66 variety. Treatment of bio-inoculum to both susceptible and tolerant varieties under moderate As environment led to upregulation of auxin and brassinosteroids biosynthetic genes. Future work includes a detailed

genetic and biochemical mapping in rice to understand As toxicity, uptake, and the potential use of bio-inoculants in As alleviation.

Chapter 1

INTRODUCTION

The need for nutritious food has become extremely important as the world population exponentially increases. The Food and Agriculture Organization (FAO) of the United Nations predicts that by the year 2050 the human population will surpass 10 billion. This increase will raise the population's demand for maize, rice and wheat by more than 33 percent (FAO, 2018). Rice is a staple crop feeding more people than any other agricultural crop, and nearly 85 percent of all rice produced goes towards consumption (CGIAR Research Program on Rice, 2015). Millions of people depend on its nutritional benefits to meet their daily caloric needs (Yadav, S., & Kumar, V., 2019). Thus, increasing rice production and yields will not only help meet the population demand for food, but also keep the cost of rice down.

Asian countries are amongst the largest producers and consumers of rice. They also embody a bulk of the world's poor (Yadav, S., & Kumar, V., 2019). Shortages in the rice industry do not only increase product pricing but could also lead to increased malnutrition and starvation for those that depend on it the most. In light of this situation, it is necessary to find innovative ways to maximize grain yields and reduce crop loss due to disease and abiotic stressors (Kumarathilaka et al., 2018).

1.1 Heavy metal toxicity

There are many biotic and abiotic stressors that impact rice production and yields. Some biotic stresses include insect damage, plant pathogenic fungus, viruses,

and bacteria (Ansari et al., 2015). Abiotic stressors affecting production are climate changes, drought, fertilizers, and heavy metals. Out of all pollutants, heavy metals are considered the second most dangerous to human health (Sytar et al., 2018). One heavy metal in particular, arsenic (As) [hereafter As] is a huge risk to human health because it is easily integrated into the food chain by indirect or direct consumption (Sytar et al., 2018).

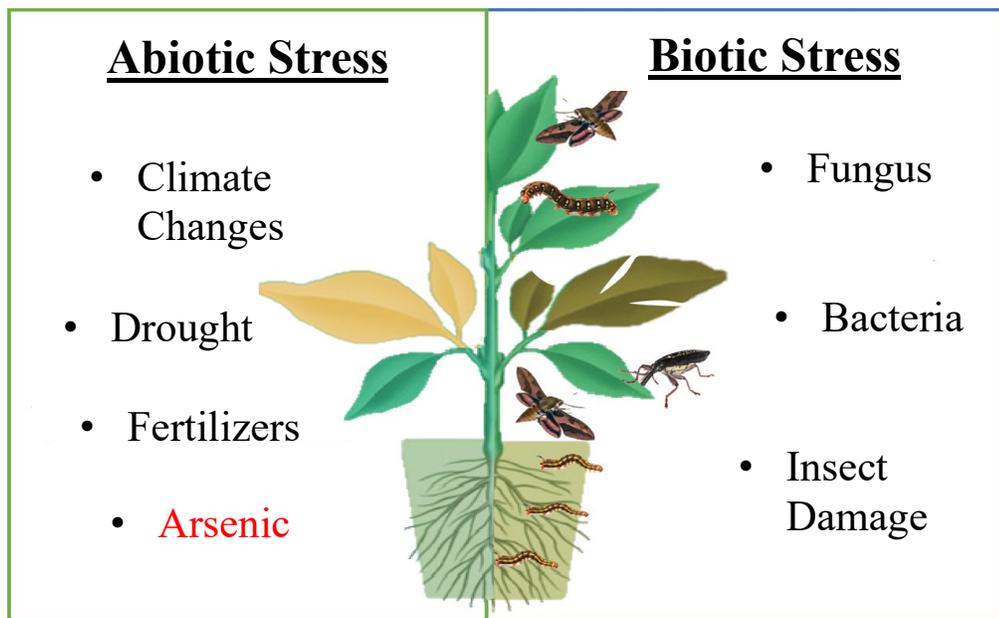


Figure 1.1 Panel representing different biotic and abiotic stress regimes on plants. The stressors are representation of a plant response at both belowground and aboveground level.

Contamination of surface and ground water has become an agricultural concern in many parts of the world (Raessler et al., 2018). Contamination occurs when dissolved chemicals, heavy metals, and non-aqueous phase liquids run-off and pollute

natural water resources. The soil contamination can be a result of poorly managed industrial and agricultural wastes and byproducts that leak into the soils and surrounding water sources (Sharma et al., 2014). It can also occur when heavy metals in the soil interact with surrounding sulfide minerals and enter the groundwater and surrounding sediments (Awasthi et al., 2017).

Plants grown in contaminated soil or irrigated with contaminated water can suffer from heavy metal toxicity. There are 17 mineral elements required for proper plant growth comprised of macro and micro-nutrients recovered from the soil. While required for plant growth at normal concentrations, excessive concentrations of sodium (Na), chlorine (Cl), boron (B), iron (Fe), manganese (Mn), and aluminum (Al) can be toxic to plants (Kalisz et al., 2019 and White and Brown., 2010). Depending on the toxic element, plants can display symptoms such as, by not limited to, inhibited seed germination, chlorosis, decreased photosynthetic efficiency, browning of leaves and roots, inhibited growth, decreased water uptake, decreased nutrient uptake, reduced yields, and plant death (Moulick et al., 2018).

The people who live in areas with highly contaminated soils and water sources, like China, India, Bangladesh, and regions of South-East Asia, are at risk for developing As related health problems. Studies have shown more than 70% of all heavy metals in the human body come from the food we eat (Sytar et al., 2018). Direct As contamination occurs when a person drinks or cooks with contaminated water. Contamination occurs indirectly when a person ingests food, plants or animals, that were exposed to heavy metals and are passed through the food chain (Sharma et

al., 2014). In the indirect case, heavy metals are taken up by the plant and stored in the roots, stems, leaves, fruit, and grains that are eaten by animals or people (Raessler et al., 2018). Constant consumption of contaminated food and water can lead to serious health problems. In many cases the toxin accumulates within the human body and digestive system (Syta et al., 2018). Some health concerns include dermal lesions; gastrointestinal, renal, and cardiovascular issues; neurological deterioration; reproductive problems; and cancer (CDC-ATSDR., 2009 and Das and Sarkar, 2017).

1.2 Arsenic (As) contamination

One of the most notable heavy metals affecting agriculture and human health is As. Arsenic is a naturally occurring, non-essential, metalloid found widespread throughout the world. Areas of southeast Asia, Bangladesh, and India experience the highest levels of As contamination in the world (Awasthi et al., 2017). Over 120 million people are reported to be affected by As-related diseases in these regions (Sharma et al., 2014).

Arsenic can be found naturally or be introduced through anthropogenic processes (Sharma et al, 2014). Natural contamination occurs when rocks containing high levels of sulfide minerals and coal seams are disturbed and deposited into a lowland environment (Kumarathilaka et al., 2018). The deposited minerals are oxidized to form iron oxides. Microbes in the ground water interact with the iron oxides and release the As into the water (Awasthi et al., 2017 and Kumarathilaka et

al., 2018). Human contamination occurs when they ingest As contaminated water or food exposed to As.

The types of As can be divided into two categories, organic and inorganic. The most toxic and prevalent forms of As are the inorganic arsenate [As(V)] and arsenite [As(III)] (Awasthi et al., 2017). Redox potential plays a big factor into which form of As is found in the ground water and surrounding sediments (Kumarathilaka et al., 2018). As(V) is abundant in surface waters that are more oxygenated. The reduced form, As(III) is more abundant in groundwater and can be sixty times more toxic and generally more available in paddy soil than As(V) (Sharma et al., 2014 and Seyfferth et al., 2017). In my studies I used As(III) as the most relevant source to mimic the groundwater used to irrigate rice paddies.

1.3 Rice and Arsenic interactions

One of the crops most affected by As toxicity is rice. This is due to both the geographical location of As contamination where large volumes of rice are grown, as well as the crop growing conditions (Kumarathilaka et al., 2018). Rice is grown in a paddy environment where it can be planted in As contaminated soil and irrigated, by flooding, with As contaminated water. This type of farming exposes the rice plants to As throughout the entire growth period (Sharma et al., 2014). In addition, the concentration of As in the rice grains, which are the primary source of human consumption and ingestion, can differ between rice variety (Althobiti et al., 2018). For example, brown rice typically has a higher As content because the same levels of As

can be stored in the bran layer as in the rice grain itself. Removing the bran layer to reduce As abundance in the grain is not a good solution because the bran layer houses the essential vitamins and minerals people depend on to meet their daily needs (Kumarathilaka et al., 2018).

The maximum level of As permitted in rice by the World Health Organization (WHO) is 0.2 mgkg^{-1} . However, As contaminated areas, like Bangladesh, have recorded As levels as high as 0.68 mgkg^{-1} in rice (Raessler et al., 2018). The mean As concentration for the contaminated areas is 0.451 mgkg^{-1} , well above the recommended limit (Sharma et al., 2014). Beyond the human health concerns, heavy metal As toxicity in the soil is a leading cause of yield loss in rice (Sharma et al., 2014). In the past, several approaches have been studied to mitigate As contamination in rice seeds. These methods include, salicylic acid, calcium, inorganic phosphate, silicon fertilizers, and the role of iron plaques (Moulick et al., 2018).

Transport of As from paddy soil to rice grains is not fully known. However, it is shown that inorganic species are less mobile in the plant than organic As species (Awasthi et al., 2017 and Hashimoto and Kanke, 2018). In fact, it is predicted that 10% of As(III) will reach the shoot and 3.3% of As(III) will reach the grains (Zhao et al., 2012). At first glance these numbers may not seem significant but when the starting soil concentrations are as high as those in Bangladesh, the final As concentration in the grains can become quite high. It is well accepted that inorganic As species enter rice roots due to structural similarities they share with the phosphorous and silicic acid transport pathways. The structure of As(V) is analogous to inorganic

phosphate. Whereas, As(III) mimics the structure of silicic acid and is thought to use the low silicon 1 (Lsi1) and low silicon 2 (Lsi2) silica transporter pathways to enter the plant (Seyfferth et al., 2017).

The Lsi1 protein is located on the outer side of the plasma membrane. It is a passive Aquaporin channel permeable to both silica and As. The Lsi2 protein is an active transporter polarly located to the inner side of the plasma membrane (Sun et al., 2018). Both are highly expressed in the exo and endodermis of rice root cells where Casparian strips are formed and nutrient uptake is regulated (Seyfferth et al., 2017 and Sun et al., 2018). Their strategic locations are vital for As transport to the xylem and into the rest of the plant (Sun et al., 2018). As(III) is the dominant form of As loaded into the xylem. High concentrations of As can be found in the lateral root junctions of roots and nodes in shoots; these are regions where the Casparian strips have not fully formed (Seyfferth et al., 2017 and Sun et al., 2018).

1.4 The Functional Microbiome

The rhizosphere is the defined region of soil surrounding a plant's roots, which is influenced by root exudates and the soil microbiome (Osman et al., 2017). The microbiome consists of consortium of diverse microbial species found in the soil rhizosphere (Lakshmanan, et al., 2015). The soil microbiome is comprised of many different groups of microorganisms; fungi, nematodes, protozoa, algae, and bacteria. Bacteria is the largest most plentiful group consisting of food contaminates, bacteria that cause plant disease, and plant beneficial bacteria. Food contaminates are bacteria

like *Salmonella* and *Listeria* that can cause widespread illness when ingested. The second group of microbes are those that cause plant disease. The final group of bacteria found in the rhizosphere microbiome consists of bacteria that positively benefit the plant by influencing plant health and productivity (Li et al, 2015) It is important to know the composition of the rhizospheric microbiome. Previous studies have shown that on a whole the microbiome functions to positively influence the plants they surround (Osman et al., 2017).

The beneficial bacteria found in the rhizosphere are termed plant growth promoting rhizobacteria (PGPR) (Lakshmanan et al., 2015 and Osman et al., 2017). They are known to promote nutrient acquisition, positively influence growth and development, influence plant physiology and metabolism, protection against pathogens, play a role in immune response, and play a role in tolerating abiotic stressors (Osman et al., 2017 and Busby et al., 2017). For example, previous studies where bacteria were isolated from the rhizosphere of M-104 rice; a japonica cultivar grown abundantly in California, have shown a beneficial response in rice (Lakshmanan et al., 2015; Spence et al., 2014). Spence et al., 2014 isolated more than eight phyla of bacteria using 16S rDNA sequencing from the M-104 rhizospheric soil sample. The experiments speculated that microbes found and associated with field rice, may offer more protection than bioinoculants from another plant species due to their ability to survive and compete with other agents in the rice rhizosphere (Spence et al., 2014).

It has been shown that the plant-associated microbiome positively impacts how plants interact with both biotic and abiotic stressors (Busby et al. 2017). Previously, Bais lab members showed that natural rice rhizospheric microbes impact rice growth and improve rice response against both biotic and abiotic stresses (Spence et al., 2014). Using bioinoculants is a cost effective and eco-friendly way to combat problems reducing rice yields (Sytar et al., 2018). In this study, both natural rice rhizospheric microbes and a Gram-positive, soil associated, *Bacillus subtilis* UD1022 strain were used to evaluate if bioinoculants mitigate the impacts and accumulation of As toxicity in rice. These strains were selected on the basis on increased iron (Fe)-siderophore activity. It is known that both Fe and As compete in the soil (Gustave et al., 2018). Thus, using microbes that mobilize Fe in soil may abate As uptake. Strains such as, *Pantoea* sp. EA106 has been shown to increase iron siderophore in culture (Lakshmanan et al., 2015 and Spence et al., 2014). This is important because siderophore production positively influences Fe uptake benefiting plant growth (Lakshmanan et al., 2015). Strains such as EA104, a *Pseudomonas* sp., have been found to produce antimicrobial secondary metabolites and are used as biocontrol bacteria (Spence et al., 2014). The EA201 strain, an *Anthrobacter* sp., is a gram-positive bacterium commonly found in soil. Previous studies have shown *Anthrobacter* may be useful for bioremediation of contaminated soils (Dsouza et al., 2015). The non-rice rhizospheric strain UD1022, a *Bacillus subtilis* strain, has shown to be a potent PGPR involved in elevating plant response against multiple stress regimes (Zheng et al., 2018). Previous studies have shown that *B. subtilis* can

produce surfactants inducing antifungal activity (Lakshmanan et al., 2015). Looking at each isolate individually in association with As can help us further understand their potential to abate As uptake and As toxicity in rice plants.

1.5 Phytohormones and their role in arsenic signaling

Phytohormones are plant hormones well known for their regulation of plant growth and development (Sytar et al., 2018). They can also act as signaling compounds for the plants affected by biotic and abiotic stressors (Spence and Bais, 2015). Currently there are nine known phytohormones; auxin, cytokinin, ethylene, abscisic acid, gibberellin, brassinosteroid, salicylic acid, jasmonic acid, and strigolactone (Sytar et al., 2018). Two of these are thought to play a key role in the response to heavy metal toxicity- auxin and brassinosteroid (Tong and Chu, 2012 and Yamamoto et al., 2007).

Auxin is a plant hormone that plays a vital role in cell division, differentiation, and elongation. It is also involved with development of roots, flowers and vascular systems, root hairs, and tropism (Praveen and Gupta, 2018 and Yamamoto et al., 2007). The predominant form of auxin found in plants is indole-3-acetic acid (IAA) (Yamamoto et al., 2007). This type of auxin, IAA, is involved in development of plants under both normal and stressed conditions. Levels of IAA are thought to increase under heavy metal stress (Sytar et al., 2018). However, the response of IAA specifically to As is unknown. One common way to monitor the levels of IAA produced is to measure the expression of YUCCA, a key gene in the IAA biosynthesis

pathway (Yamamoto et al., 2007). Expression of the YUCCA gene is not ubiquitous but rather localized to roots, leaves, and vascular stem tissues. When YUCCA genes are over expressed, plant demonstrate phenotypes of auxin over production. Rice plants suppressing YUCCA will have an auxin insensitive phenotype (Yamamoto et al., 2007).

Brassinosteroids (BR) are plant hormones that regulate grain size, leaf angle, and yield potential in rice (Feng et al., 2016 and Tong and Chu, 2012). Acute leaf angles result in a compact plant that allows for more efficient photosynthesis and better nitrogen storage for grain filling (Feng et al., 2016). Brassinosteroids are synthesized in roots, leaves, shoots, seeds, flowers, pollen, and fruits (Feng et al., 2016 and Sytar et al., 2018). They are expressed in the same tissue they are synthesized and do not move around freely (Feng et al., 2016). In the presence of heavy metals, BRs support plant growth processes and potentially play a role in reducing the toxin (Sytar et al., 2018).

Measuring YUCCA and BR expression in different tissue types after exposing rice to As and/or bacteria for different lengths of time will provide insight into the plant's reaction and signaling response. Understanding how plants use phytohormones to signal behavioral changes from abiotic stresses can help us better research solutions to reduce symptoms from the toxins.

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Chapter 2

THE ROLE OF DIFFERENT ARSENIC ENVIRONMENTS IN RICE SUPPLEMENTED WITH BENIGN MICROBES UNDER HYDROPONIC CONDITIONS

2.1 Introduction

Exposure to As causes acute toxicity in rice, influencing rice growth and yields (Awasthi et al., 2017). One method to reduce heavy metals in soil is through the use of bioinoculants. These are benign bacteria found naturally in the root rhizosphere (Lakshmanan et al., 2015). They benefit plant health through enhancing nutrient efficiency, plant growth, disease tolerance, and abiotic stress tolerance (Busby et al., 2017 and Lakshmanan et al., 2016). Studies have shown that bacteria taken from the rice microbiome play a unique role in defending the plant against abiotic stressors (Spence et al., 2014). I hypothesize that bioinoculants can be used to mitigate the effects and uptake of As in rice.

In the present chapter, the effects of four different PGPRs are observed on As toxicity in rice are quantified. A hydroponic approach was used to control nutrients, As, and bacteria supplementation to each plant. Submerging the roots in nutrient solution, rather than soil, allowed me to easily track morphometric and phenotypic traits throughout the experiment. I applied moderate and high As toxicity conditions to two rice cultivars (Nipponbare, spp. *japonica* and IR66, spp. *indica*). I measured root and shoot dry mass at different developmental stages for plant growth under high and moderate As toxicity environments with different bioinoculants. I also measured total

grain weight at harvest. Together, these results provide the basis for future studies to refine the hypothesis that PGPRs can be used to ameliorate As uptake in rice plants.

2.2 Materials and Methods

2.2.1 Set up of Hydroponic rice plants in Fischer Greenhouse Growth Chamber

2.2.1.1 Preparing Rice Seeds

The outer husk layer of each rice seed was removed, and each seed was inspected for maturity and fungus. Seeds that appeared mature and fungus-free were added to a 50 mL conical tube, in a biosafety cabinet, and soaked in 30 mL of 50% bleach solution for ten minutes; swirling occasionally. After ten minutes the bleach was drained into a beaker and the seeds were washed with autoclaved deionized water three times. Standard petri dishes were prepared with one piece of autoclaved chromatography paper and 3.5 mL of autoclaved deionized water. Sterilized seeds were transferred to the prepared petri dishes using sterile forceps. Five to seven seeds were added to each dish before sealing the plates with parafilm and labeling the dish with seed type, name, and date. Labeled petri dishes were placed under growth lights in the lab set to a 16-hour photoperiod, for seven to ten days until germinated. This procedure was used to prepare all rice seeds used in greenhouse and lab experiments, both Nipponbare and IR66 rice varieties.

2.2.1.2 Transferring Germinated Rice to the Greenhouse

After the rice seeds had germinated the seedlings were transferred to the hydroponic nursery system in the greenhouse growth chambers. Before transfer, foam plugs (Identi-Plug® Plastic Foam Plugs, Jaece) were prepared by cutting them in half once, cutting a slit into one side, and then autoclaving. Once all of the supplies were prepared, germinated seeds and supplies were transferred to the campus greenhouse. Seedlings were individually removed from the petri dishes and inserted into a foam plug. The plug, with seedling, was then inserted into an opening in the hydroponic nursery system filled with rice nutrient solution (Table 2.1). The rice stays in the nursery system for 7 days before being transferred to the large hydroponic buckets.

After seven days, the black hydroponic buckets were prepared to transfer the rice seedlings. To prepare the 2-gallon buckets; first a 1^{1/4} inch hole was drilled into the center of the lid. Then each bucket and lid were washed with soap and water. They were filled with one liter of 8x concentrated rice nutrient media and filled the rest of the way with water from the greenhouse faucet. Buckets were then transferred into the growth chamber with a daily cycle of 14 hr light (28°C, 70% RH), and 10 hr dark (26°C, 60% RH) where the remainder of the experiment would be run. A rice seedling was taken from the nursery system and carefully inserted into the hole of the lid and placed on the 2-gallon bucket. For each experiment there was a total of thirty-two buckets, broken down to eight replicates per treatment group.



Figure 2.1. Representation of rice growth from seed to mature plant in the controlled environment hydroponic system.

2.2.2 Supplementation of bacteria and arsenic to hydroponic buckets

2.2.2.1 Preparing bacterium for inoculation

Under sterile conditions a loop of bacteria was removed from the working glycerol stock of the bacteria of interest (EA106, EA104, EA201, UD1022). It was then struck out on a LB agar plate using the four-quadrant technique. The plate was sealed with parafilm and placed in a 30°C incubator (EA106/UD1022 16-24 h, EA104/EA201 24-48 h) until single colonies were formed. Once the bacteria had grown, in a sterile hood, a single colony was selected and transferred to a flask with LB liquid media. The flask was put into a 30°C shaking (200 rpm) incubator overnight.

After the bacteria culture was grown overnight, 45 mL of culture was distributed into 50 mL conical tubes and centrifuged down (4°C, 3000 rpm) to a pellet with an initial spin of 25 minutes. The supernatant was poured off and the pellet was

washed with deionized water and then spun down for 10 minutes (4°C, 3000 rpm). The pellet was washed two additional times. Next, 10-15 mL of deionized water was added to each conical tube and the pellet was resuspended. The resuspended liquid culture from each tube was added to an autoclaved flask and the volume was brought to 200 mL using more deionized water. The cell count and OD were determined using a spectrophotometer (BIORAD Spectrophotometer). The number from the spectrophotometer was used to calculate the amount of culture needed to inoculate 1×10^6 cell/mL into the appropriate hydroponic buckets. The culture was brought to the greenhouse and bacteria was added to the hydroponic solution.

2.2.2.2 Preparing Arsenic for experimental treatment

A stock solution of 0.01 M sodium (meta) arsenite (NaAsO_2) was made to be used in all lab experiments containing As. The Sigma-Aldrich Mass Molarity calculator was used calculate the mass of NaAsO_2 (M.W. 129.91 g/mol) that should be measured out for a 0.01 M stock solution. The NaAsO_2 was measured while wearing a mask as to not breath in the powder. Sterile deionized water was added to the measured reagent to bring to the final volume. Once dissolved, the solution was filter sterilized. For greenhouse use 3.7 mL aliquots were made. One aliquot would be added to one hydroponic bucket (8 L) for a final As concentration of 5 μM NaAsO_2 . For treatment groups that called for As, the As would be added 48 hours after bacterium. For high toxicity experiments, plants were irrigated with the rice nutrient

solution containing 5 μM As. For moderate toxicity experiments, plants were irrigated with rice nutrient solution alone.

2.2.3 Plant Materials, growth materials and conditions

Oryza sativa Nipponbare seeds were taken from stocks bulked by previous lab members in Dr. Harsh Bais's lab. *Oryza sativa* IR66-103-2 seeds were obtained from the National Small Grains Collection in Aberdeen Idaho and then bulked in the greenhouse. All plants were grown in the growth chambers in Fischer Greenhouse with a daily cycle of 14 hr light (28°C, 70% RH), and 10 hr dark (26°C, 60% RH). The rice nutrient solution was obtained from Hoagland, 1950. It is comprised of eight stock solutions named A to H. The chemical makeup of each stock solution can be found in Table 2.1. Hydroponic plants were topped off with nutrient solution every 4 to 6 days.

Table 2.1. Rice nutrient media used in hydroponic experiments.

Salt	Stock Number	MW	Stock Solution (g/L)	Stock Solution (mol/L)	Aliquot (mL) for 8L
Calcium nitrate tetrahydrate Ca(NO ₃) ₂ 4H ₂ O	a	236.15	224.34	0.95	16
Ammonium nitrate H ₄ NO ₃		80.06	4.005	0.05	
Potassium nitrate KNO ₃	b	101.11	50.555	0.5	16
Magnesium sulfate heptahydrate MgSO ₄ 7H ₂ O	c	246.48	123.24	0.5	8
Potassium dihydrogen phosphate KH ₂ PO ₄	d	136.09	32.66	0.24	2.667
Boric Acid H ₃ BO ₃	e	61.83	0.618	0.01	8
Sodium molybdate dehydrate Na ₂ MoO ₄ 2H ₂ O		241.95	0.024	0.0001	
Zinc chloride ZnCl ₂	f	136.28	1.0902	0.008	8
Manganese (II) chloride tetrahydrate MnCl ₂ 4H ₂ O		197.91	0.119	0.0006	
Copper (II) chloride dihydrate CuCl ₂ 2H ₂ O		170.48	0.341	0.002	
Nickel (II) chloride hexahydrate NiCl ₂ 6H ₂ O		237.71	0.024	0.0001	
Iron (III) chloride hexahydrate FeCl ₃ 6H ₂ O		270.3	5.41	0.02	
HEDTA		344.2	19.86	0.0577	
Hydrochloric acid HCl			150 mL		
MES monohydrate	g	213.24	106.62	0.5	16
Sodium hydroxide NAOH		40	10	0.25	
1M Sodium Hydroxide NaOH	h		40 g dissolved in 1L of H ₂ O	1	3.5

2.2.4 Hydroponic plant imaging and harvest

Throughout the experiments, hydroponic plants were imaged at different stages of growth. Photos were taken of both shoots and roots against a black background; using a representative plant from each treatment group. Once the hydroponic plants

reached maturity they were harvested for further analysis. The timeline to maturity differed between Nipponbare and IR66 varieties. Once ready, all plants in the trial were removed from the growth chambers and photographed. As the experiments progressed, a plant imaging box was made to take pictures of submerged roots.

After imaging, plants were cut at the foam plug and roots and shoots were separated into paper bags labeled with trial, treatment, plant number, and tissue type. Nutrient solution containing arsenic was disposed in chemical waste streams compliant with the University of Delaware Environmental Health and Safety guidelines. The buckets and lids were cleaned and stored in the greenhouse. Plants were brought back to the lab where grain heads were removed and separated into conical tubes to dry. The fresh weight was taken for the remaining shoot and the root tissues. Plant samples were sent to the soil testing lab where they were dried overnight at 60°C. The dry weight was taken for dried tissue samples. Dried grains were removed from the pinnacles and weighed. The total plant dry weight was calculated by combining the measurements of each tissue type.

After obtaining all weight measurements, three plants were selected from each of the treatment groups. The shoot, root, and grain for the selected samples were ground down separately and sent to the soil testing lab to test for total elemental analysis and As concentration in the different tissue types. All results were graphed using Prism GraphPad.

2.3 Results

The microbiome plays an important role in plant growth and defense. It is shown that plant specific microbiome may provide more benefits to the host plant compared to the non-specific microbiome (Busby et al., 2017). Chapter 2 focuses on how individual rice and natural PGPRs isolates impact the plant's ability to reduce As toxicity in plants. This chapter explains the hydroponic approach used to evaluate phenotypic and morphometric traits in two different rice cultivars (Nipponbare, As-tolerant line, and IR66, As-susceptible lines) when treated with toxic and moderate concentrations of As.

The Nipponbare rice variety is of the *japonica* sub-species, and has a fully sequenced genome (Kawahar et al., 2015). The *japonica* sub-species is short grained and typically grown in the dry uplands. Upland rice varieties, like Nipponbare, have genes that are triggered to provide a tolerance against drought (Matsumoto, et al., 2016). It is therefore speculated that it may be equipped with mechanisms to combat other abiotic stressors such as As toxicity (Matsumoto, et al., 2016). In contrast, the IR66 rice cultivar used in my experiments derives from the *indica* sub-species (Khush, 2005). This is a long-grained variety, typically grown in lowland country, where plants are grown submerged in a paddy. Previous studies have shown the IR66 rice variety to be susceptible to salt stress and therefore may not have an efficient mechanism to overcome abiotic stress (Khush, 2005). The rhizospheric isolates used in these experiments were isolated from japonica sub-species M-104 rhizospheric soil samples from California (EA106, EA104, EA201) (Spence et al., 2014). The UD1022

isolate is a common *Bacillus* sp., soil associate bacteria, found on the surface of plant roots and in surrounding soils (Bishnoi et al., 2015).

2.3.1 High Toxicity (HT) As environment under hydroponic setup

In the high toxicity As experiments, treatment groups treated with As were topped off with 5 μ M of 0.01 M NaAsO₂ every time the rice plants were watered. This resulted in As concentrations greater than 50 μ M As by the end of the experiment. Plant images show the difference in phenotype between treatment groups within rice variety as well as differences between Nipponbare and IR66 varieties.

2.3.1.1 Supplementation of *Pantoea* sps. (EA106) under HT As environment:

Results for Nipponbare rice treated with EA106 bacteria under highly toxic conditions can be seen in Figure 2.2. and Figure 2.4. Nipponbare treated with HT As displayed signs of abiotic stress in the form of chlorosis and reduced growth. In order to maintain the highest level of photosynthetic ability, the leaves should be tall and upright. At harvest, the As treated plants showed disorganized shoot structure (Figure 2.2). Both the control and EA106 bacteria supplemented plants produced grains (Figure 2.2). The Nipponbare roots treated with highly toxic levels of As showed reduced biomass (Figure 2.3). However, root length does not appear to be affected post As treatment (Figure 2.3). The IR66 rice treated with EA106 bacteria under highly toxic As conditions displays similar results as Nipponbare plants though plant shoot organization is not defined until later in developmental phase (Figure 2.3,

harvest). The biomass of IR66 roots treated with As has less biomass than the plants treated with As and EA106 bacteria (Figure 2.5, harvest).

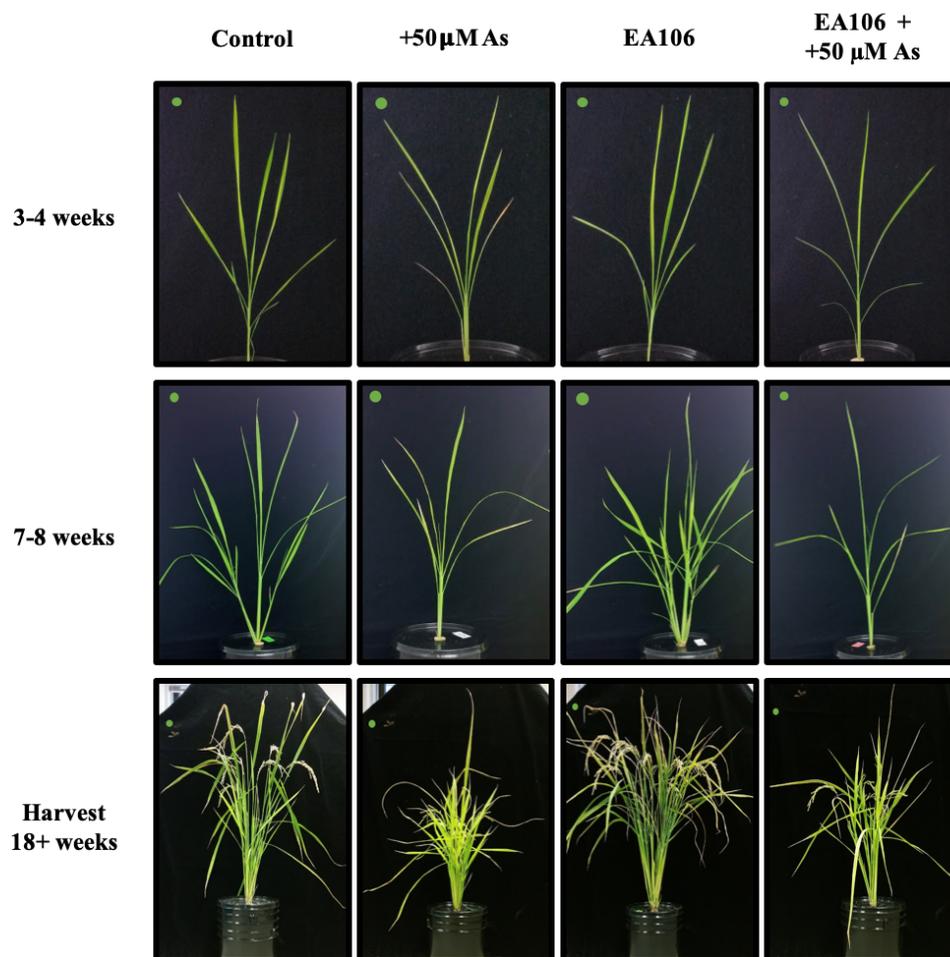


Figure 2.2. Nipponbare rice plants inoculated with EA106 bacteria and treated with a high concentration of As (~50 μ M). Images show phenotypic differences in the shoots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment. The green dot scales to 0.75 inches in diameter.

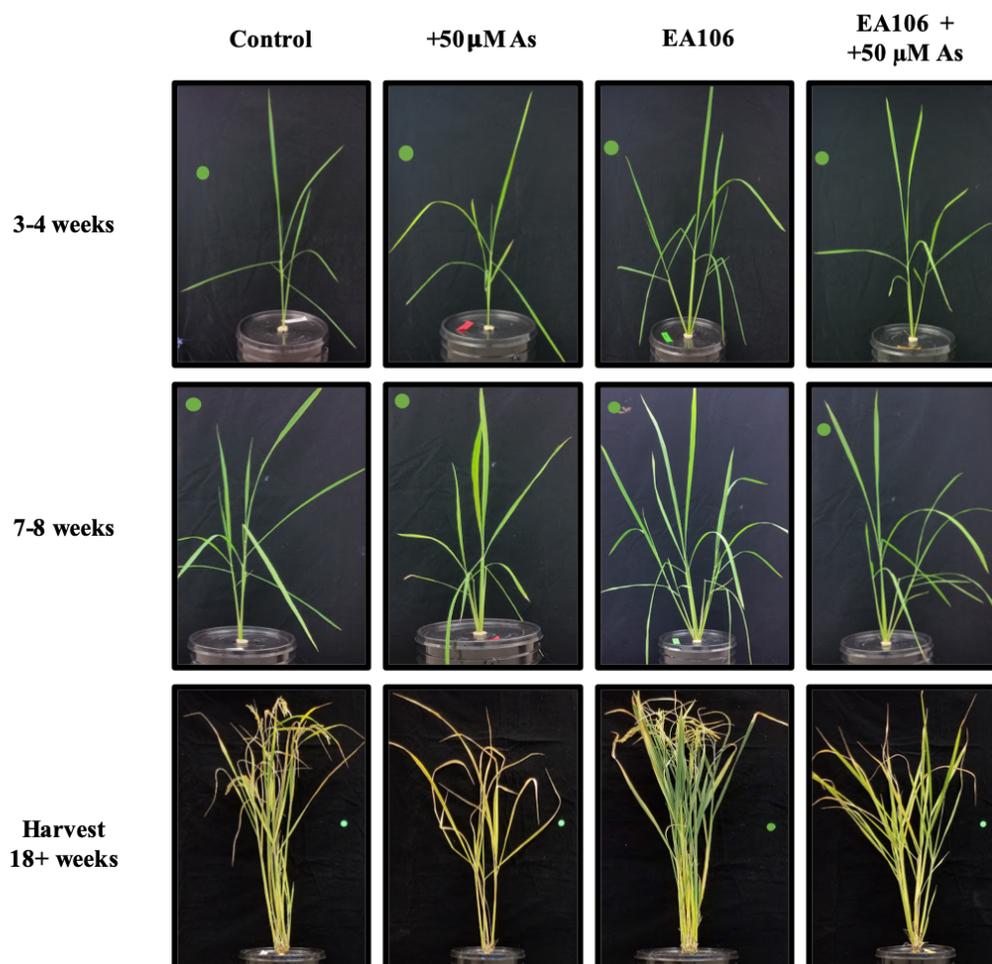


Figure 2.3. IR66 rice plants inoculated with EA106 bacteria and treated with a high concentration of As (~50 μ M). Images show phenotypic differences in the shoots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

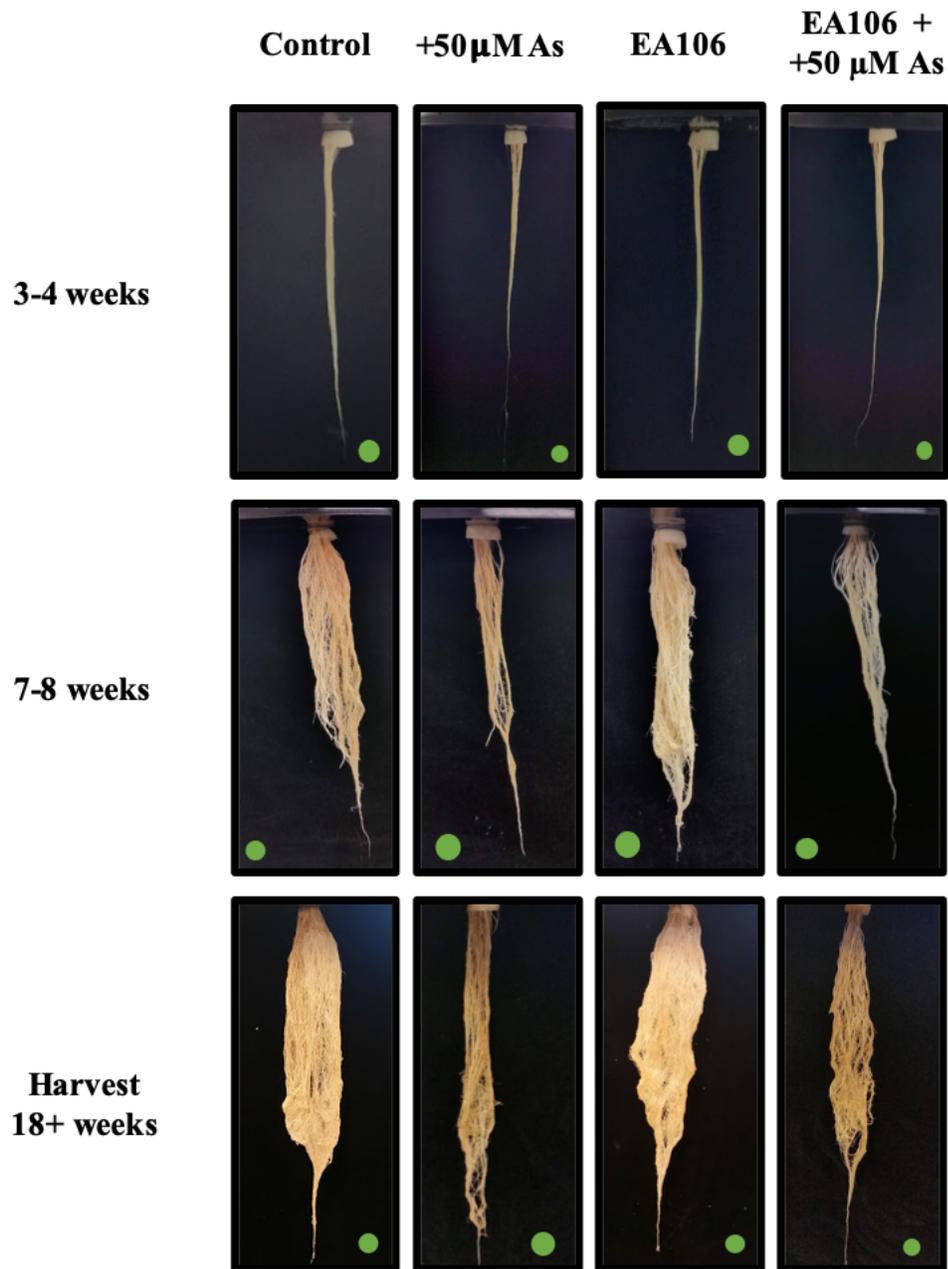


Figure 2.4. Nipponbare rice plants inoculated with EA106 bacteria and treated with a high concentration of As (~50 μ M). Images show phenotypic differences in the roots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.



Figure 2.5. IR66 rice plants inoculated with EA106 bacteria and treated with a high concentration of As (~50 μ M). Images show phenotypic differences in the roots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

Statistical analyses comparing treatment groups within Nipponbare and IR66 shows no significance in total plant biomass between control and EA106 treatments compared to the arsenic treatments in both rice cultivars (Figure 2.6). The same for grain mass. Though Nipponbare rice is more tolerant to As toxicity, the Nipponbare plants exposed to As still display the correct phenotypes for As toxicity. In IR66 rice there is no significance between and treatment group when comparing grain mass. However, the Nipponbare rice plants produced significantly more grains than IR66 plants under As conditions. In the IR66 trial with EA106 bacteria and HT As concentrations, no grains were produced in treatment groups exposed to As (Figure 2.7). There is no significance between the treatment groups for either cultivar when analyzing harvest index ($HI = \text{total grain mass} / \text{total plant biomass}$) (Figure 2.8). The distribution of results for total shoot biomass are comparable to those in total plant biomass and grain mass (Figure 2.9).

Arsenic concentrations in each tissue type; root, shoot, and grain were analyzed. As expected, overall concentration of As reduced as it traveled upward in the plant, where the roots that were directly exposed with As had higher concentrations than the grains. This is consistent with Zhoa et al (2012) paper where the roots contain ~89% of total arsenic, shoots retain ~10%, and grains retained ~3%. The Nipponbare plants had far less As uptake into the tissues than the IR66 plants (Figure 2.10 and Figure 2.11). This is consistent with the suggestion that the Nipponbare cultivar is tolerant to As.

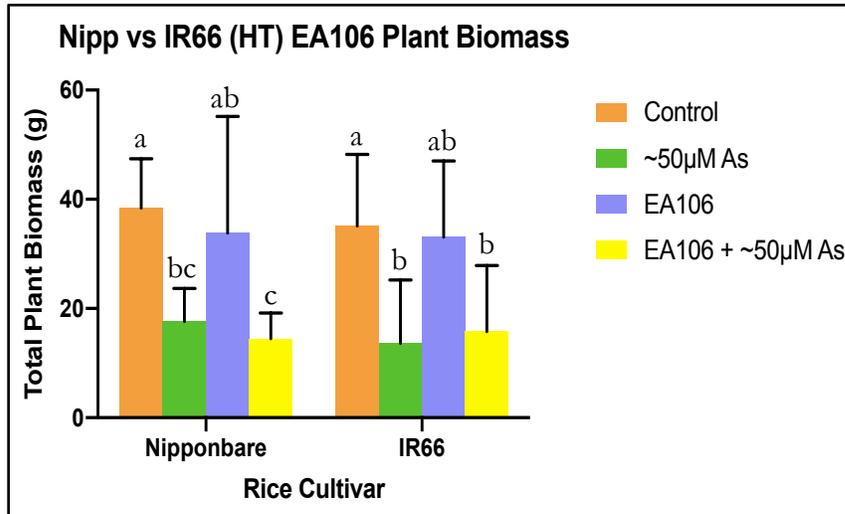


Figure 2.6. Total plant biomass for Nipponbare and IR66 rice exposed to HT As and EA106 bacteria. Significance is measured between treatment groups in each rice cultivar; “a” being more significant compared to “b”.

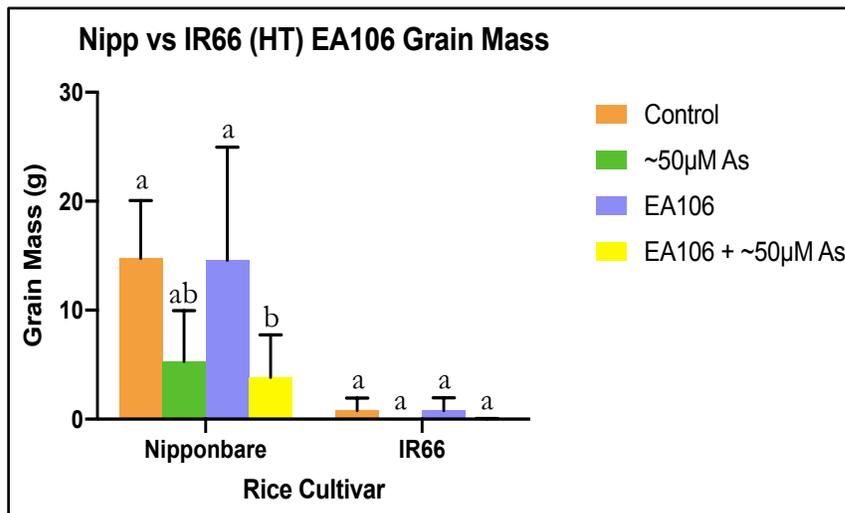


Figure 2.7. Total grain mass for Nipponbare and IR66 rice exposed to HT As and EA106 bacteria. Significance is measured between treatment groups in each rice cultivar.

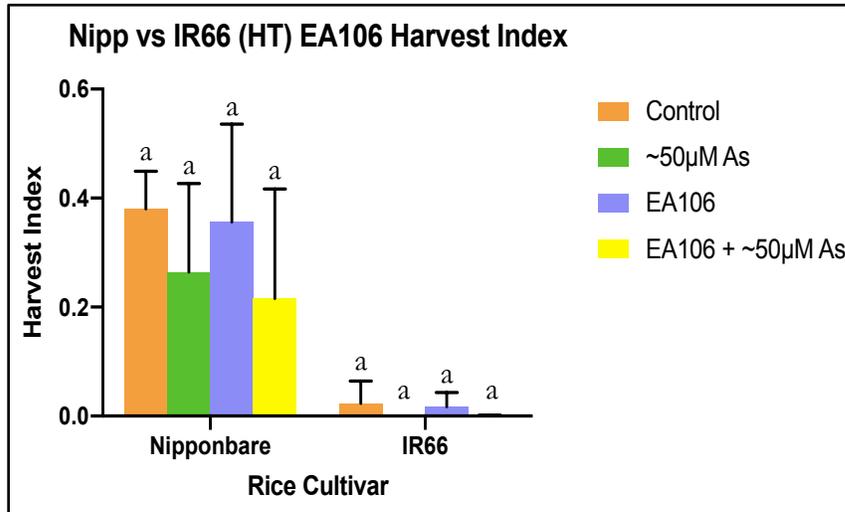


Figure 2.8. Harvest index for Nipponbare and IR66 rice exposed to HT As and EA106 bacteria. Significance is measured between treatment groups in each rice cultivar

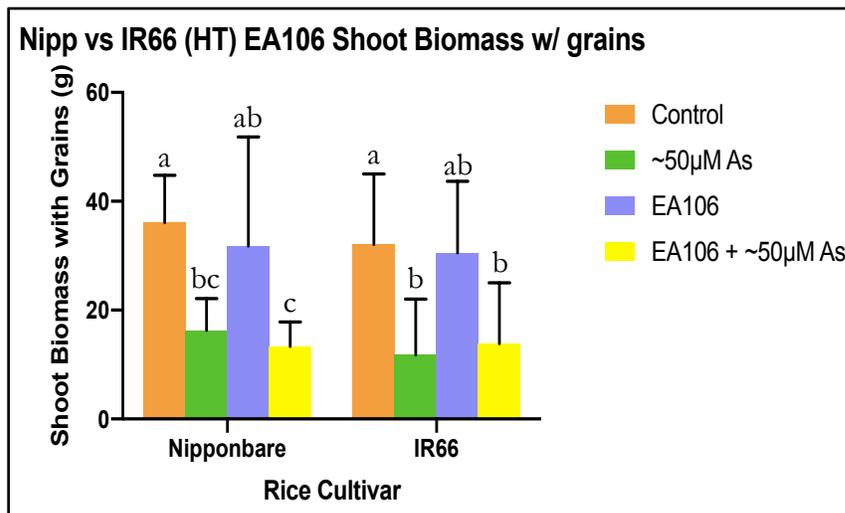


Figure 2.9. Total shoot biomass for Nipponbare and IR66 rice exposed to HT As and EA106 bacteria. Significance is measured between treatment groups in each rice cultivar

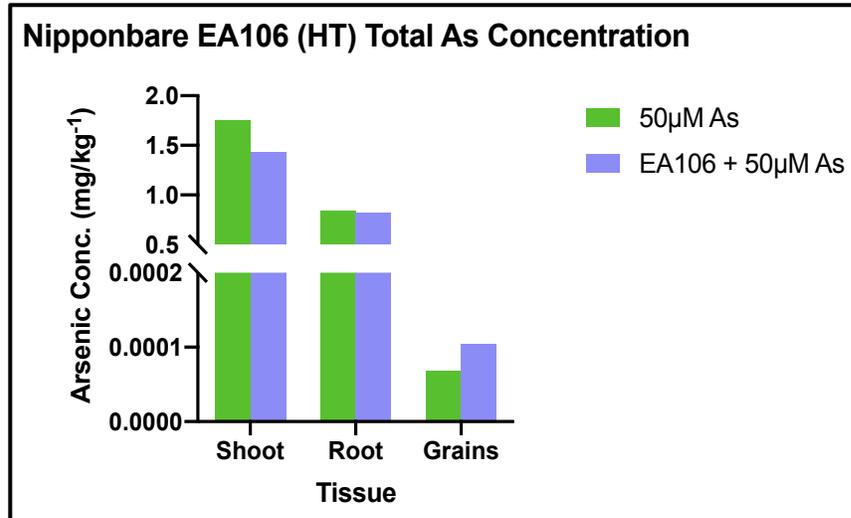


Figure 2.10. Arsenic concentration in Nipponbare rice shoot, root, and grains exposed to HT As and EA106 bacteria.

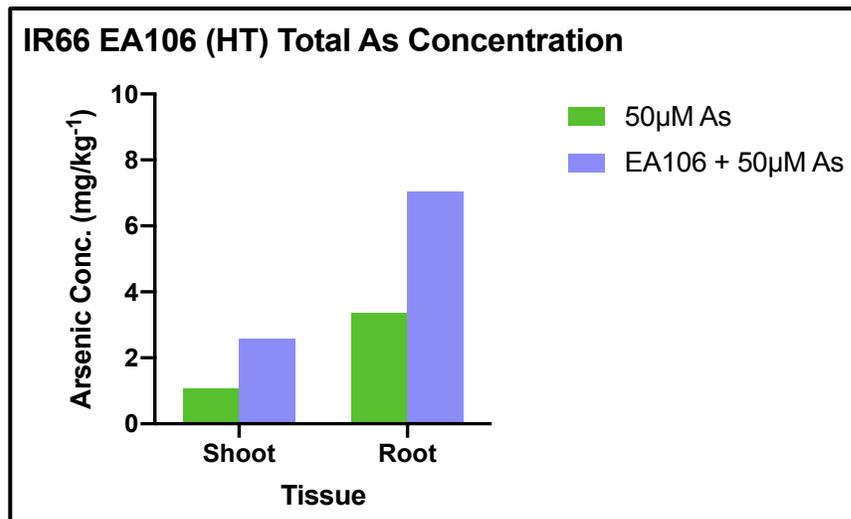


Figure 2.11. Arsenic concentration in IR66 rice shoot, root, and grains exposed to HT As and EA106 bacteria.

2.3.1.2 Supplementation of *Pseudomonas* sps. (EA104) under HT As environment:

Results for Nipponbare rice treated with EA104 bacteria under highly toxic conditions can be seen in Figures 2.12. and 2.14. Here the EA104 treated plants displayed more efficient shoot organization from the other treatment groups as well as more grain production (Figure 2.12). The Nipponbare roots treated with highly toxic levels of As showed a reduced biomass but again no truncation in root length (Figure 2.13). In IR66 rice treated with EA104 bacteria under HT conditions, As stress at week seven and eight is visible where is it no as obvious in Nipponbare rice until a later growth stage. The As treated plants were not as full as the control and EA104 only plants and the leaves were more chlorotic and yellow. The biomass of IR66 roots treated with As only, appeared to have less biomass than the plants treated with As and EA104 bacteria (Figure 2.14).

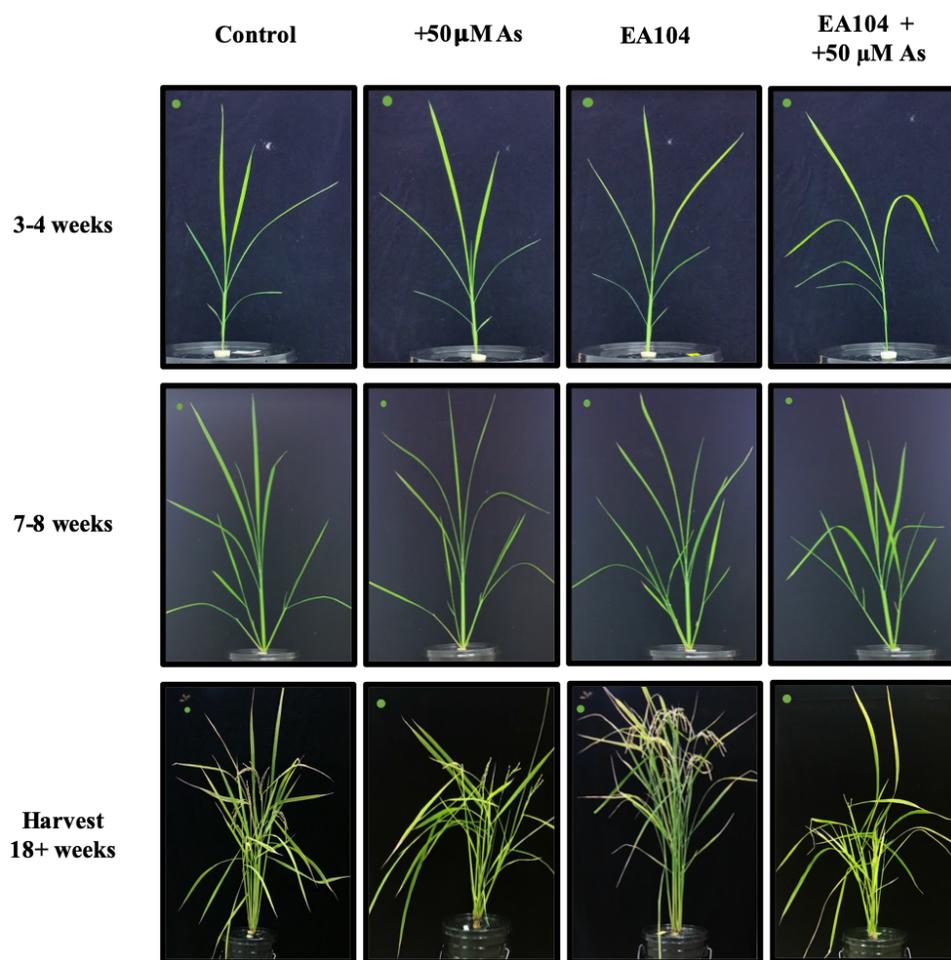


Figure 2.12. Nipponbare rice plants inoculated with EA104 bacteria and treated with a HT concentration of As (~50 μ M). Images show phenotypic differences in the shoots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

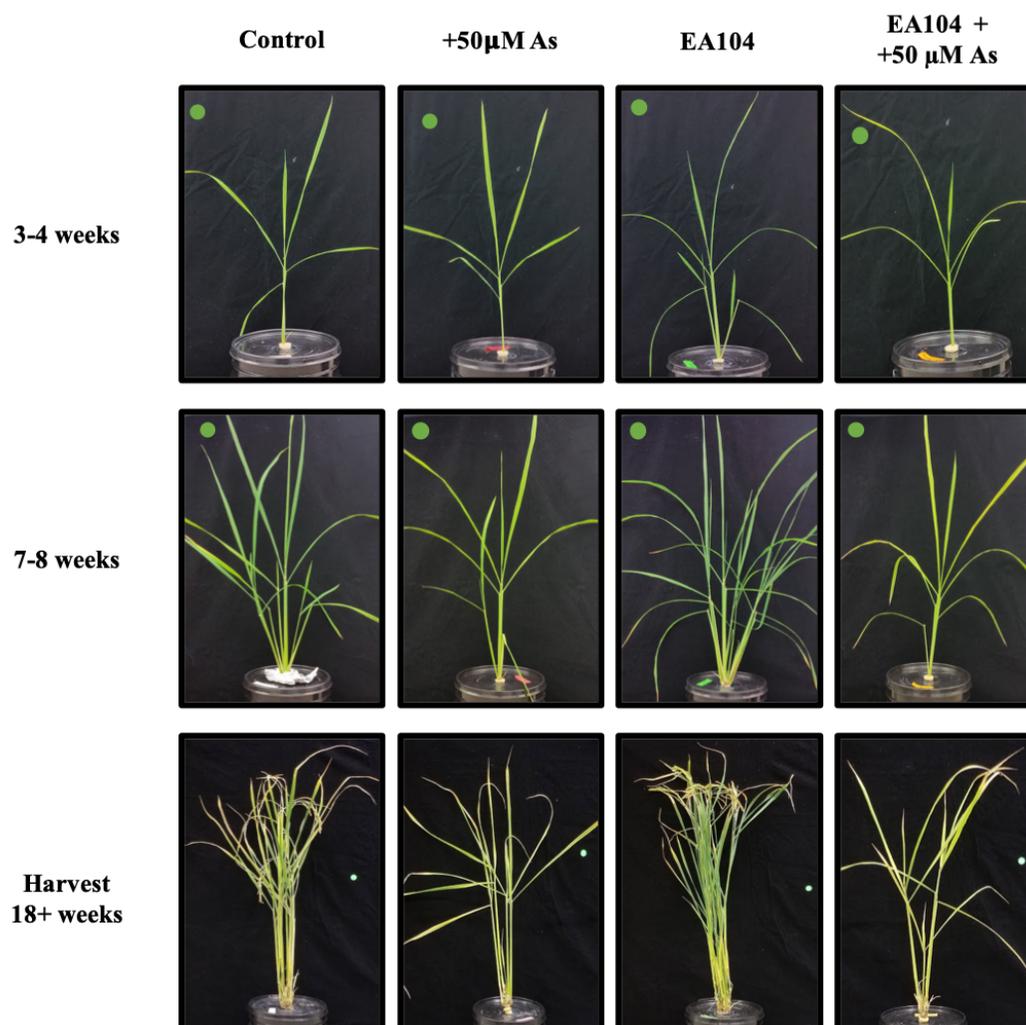


Figure 2.13. IR66 rice plants inoculated with EA104 bacteria and treated with a HT concentration of As (~50 μ M). Images show phenotypic differences in the shoots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.



Figure 2.14. Nipponbare rice plants inoculated with EA104 bacteria and treated with a HT concentration of As (~50 μ M). Images show phenotypic differences in the roots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.



Figure 2.15. IR66 rice plants inoculated with EA104 bacteria and treated with a HT concentration of As (~50 μ M). Images show phenotypic differences in the roots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

Statistical analysis for Nipponbare shows significance in total plant biomass for EA104 only bacterial treatment when compared to the other treatment groups. In IR66 rice there appears to be significance between the EA104 only bacteria treated group compared to the other treatments, however the control group and As only group have some statistical similarities (Figure 2.16). Grain mass is higher for all treatment groups in the Nipponbare cultivar with EA104 only being slightly more significant than control and the As treated plants. IR66 again has little to no grain yield for all treatments and there is no statistical significance (Figure 2.17). Harvest index for Nipponbare EA104 only and control groups is statistically significant over the As treated groups. There is no statistical significance between the IR66 rice plants (Figure 2.18). Total shoot biomass shows the EA104 treatment group to be statistically more significant in both rice cultivars (Figure 2.19). Arsenic concentrations in both cultivars yielded results comparable to those for the EA106 HT trial. Again, the Nipponbare plants display overall lower concentrations of As in the plant tissues than the IR66 cultivar (Figure 2.20 and Figure 2.21). These results are consistent with those from the EA106 HT trial further supporting Nipponbare to be an As tolerant cultivar.

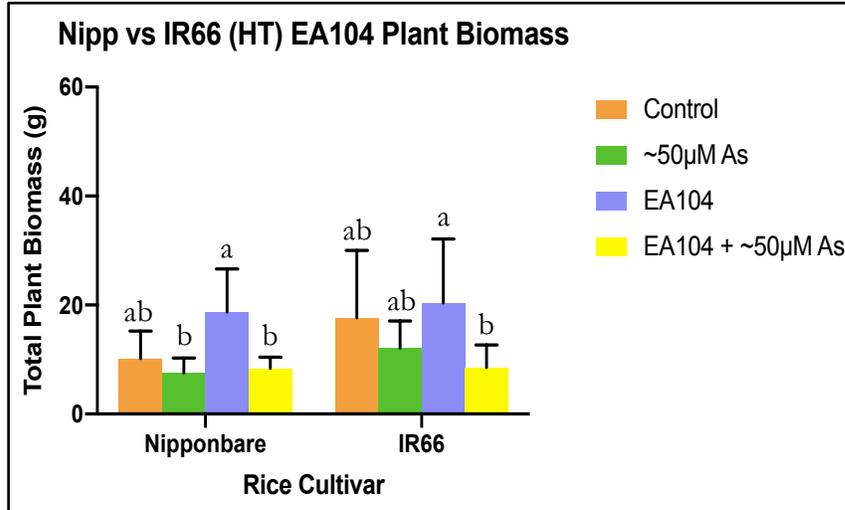


Figure 2.16. Total plant biomass of Nipponbare and IR66 rice exposed to HT As and EA104 bacteria. Significance is measured between treatment groups in each rice cultivar; where “a” is more significant compared to “b”.

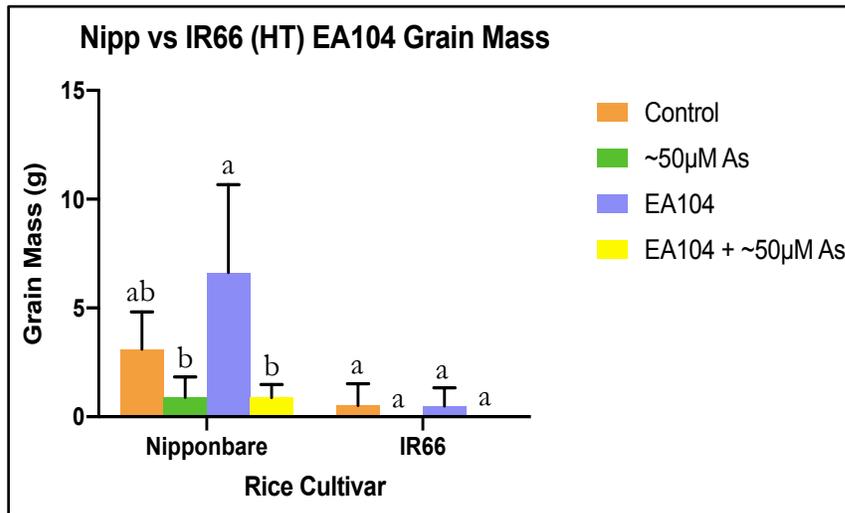


Figure 2.17. Grain mass of Nipponbare and IR66 rice exposed to HT As and EA104 bacteria. Significance is measured between treatment groups in each rice cultivar.

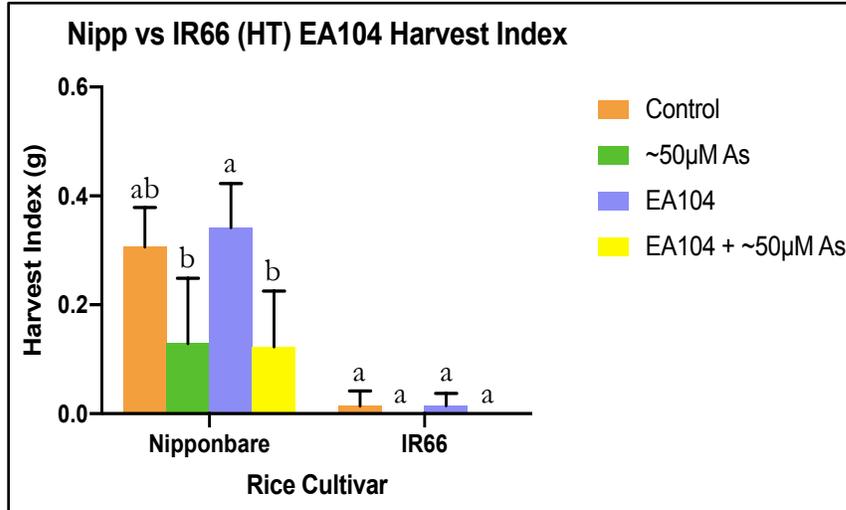


Figure 2.18. Harvest index of Nipponbare and IR66 rice exposed to HT As and EA104 bacteria. Significance is measured between treatment groups in each rice cultivar.

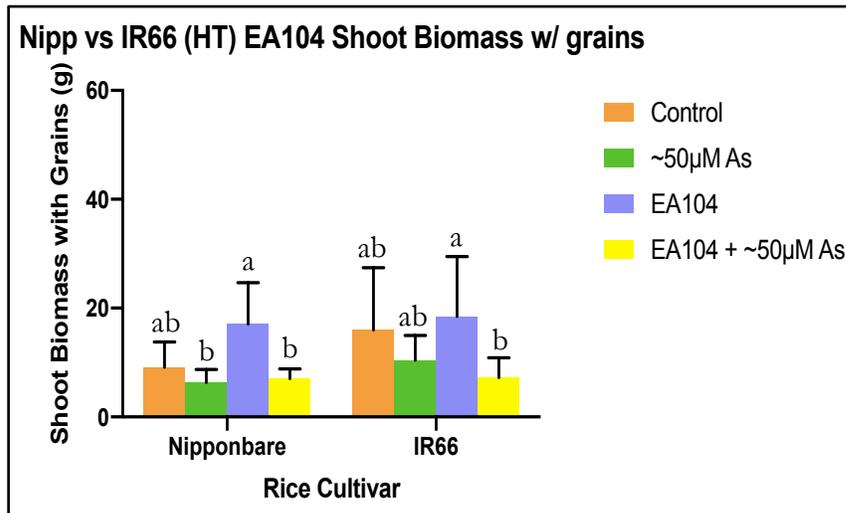


Figure 2.19. Total shoot biomass of Nipponbare and IR66 rice exposed to HT As and EA104 bacteria. Significance is measured between treatment groups in each rice cultivar.

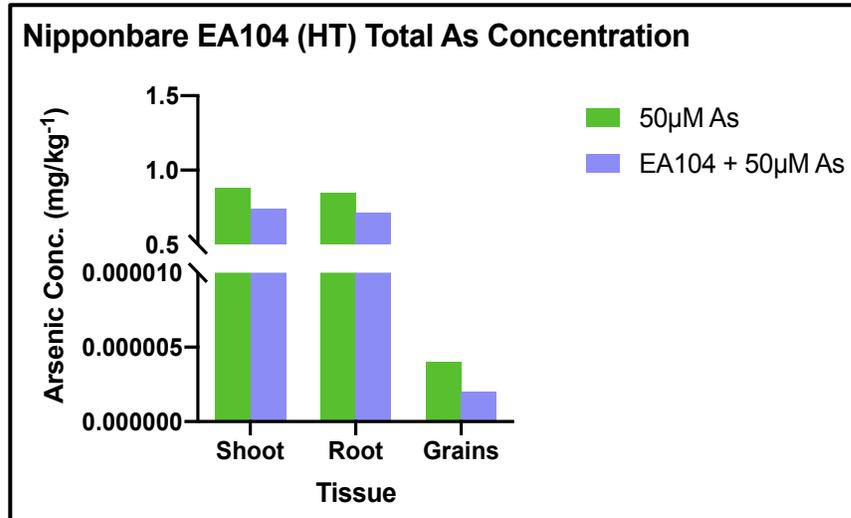


Figure 2.20. Arsenic concentration in Nipponbare rice in shoot, root, and grains exposed to EA104 bacteria and HT concentrations of As.

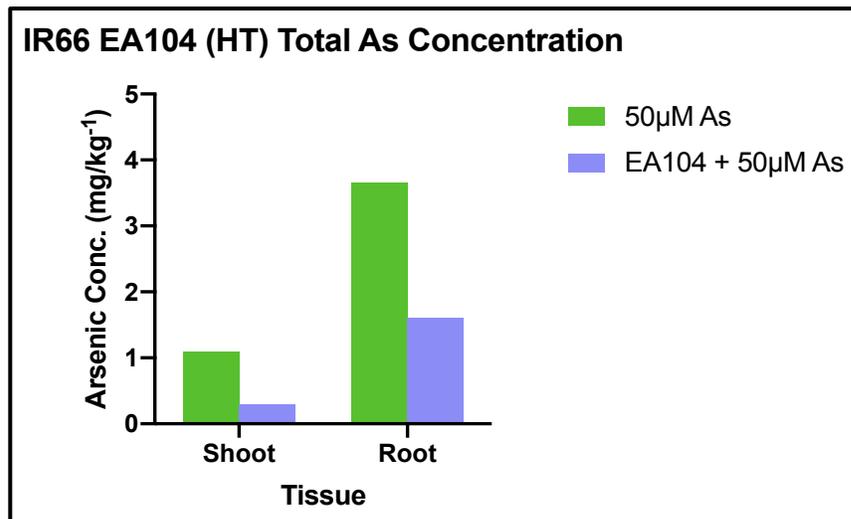


Figure 2.21. Arsenic concentration in IR66 rice in shoot, root, and grain tissues exposed to EA104 bacteria and HT concentrations of As.

2.3.1.3 Supplementation of *Anthrobacter* sps. (EA201) under HT As environment:

In the EA201 trial, at harvest, all Nipponbare rice plants except those treated with only As produced grain (Figure 2.22). Consistent with other bacteria trials, the control and EA201 only roots have an increased biomass compared to the As treated plants (Fig 2.24). In the IR66 rice trial with EA201 bacteria, the phenotype of the As only plant is consistent with other trials. Unlike other trials, the EA201 plus As treatment plants produced grains (Figure 2.23).

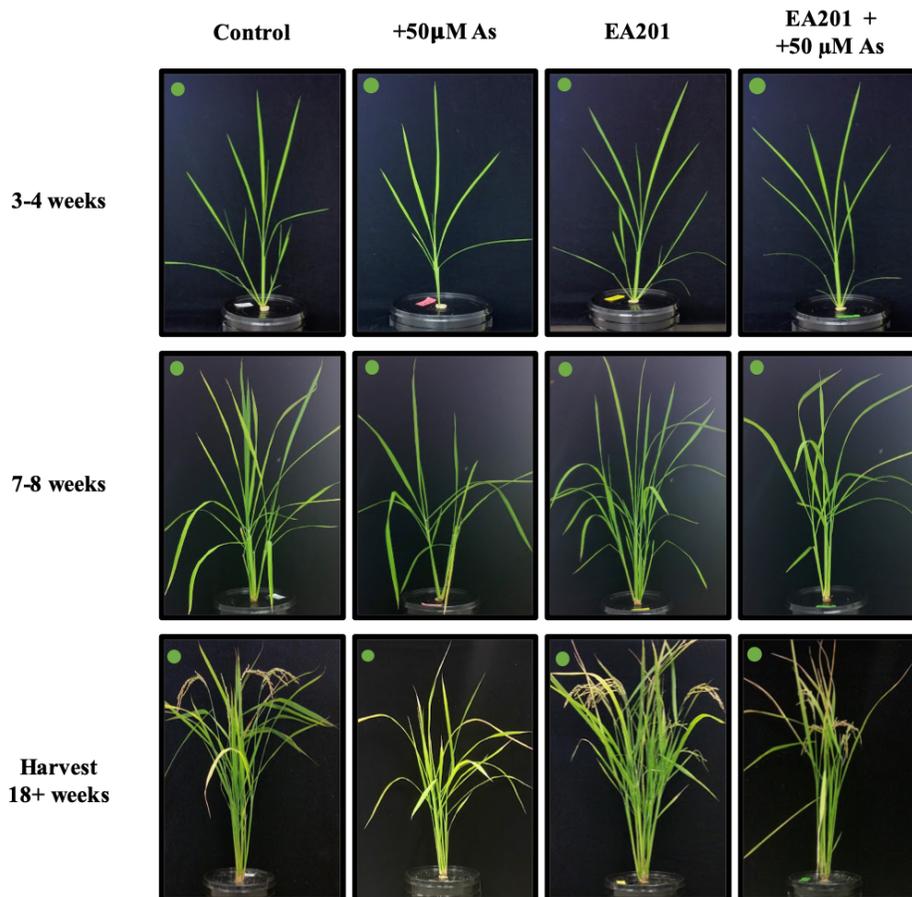


Figure 2.22. Nipponbare rice plants inoculated with EA201 bacteria and treated with a HT concentration of As ($\sim 50 \mu\text{M}$). Images show phenotypic differences in the shoots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

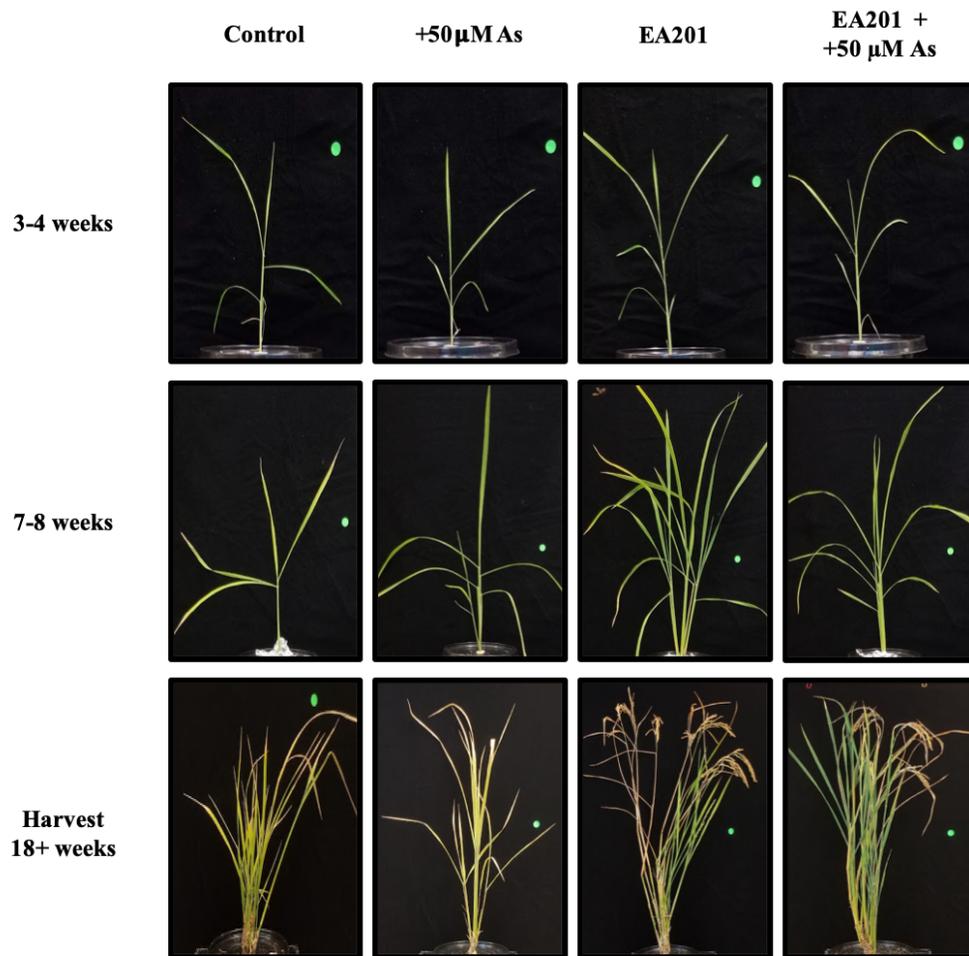


Figure 2.23. IR66 rice plants inoculated with EA201 bacteria and treated with a HT concentration of As (~ 50 μ M). Images show phenotypic differences in the shoots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

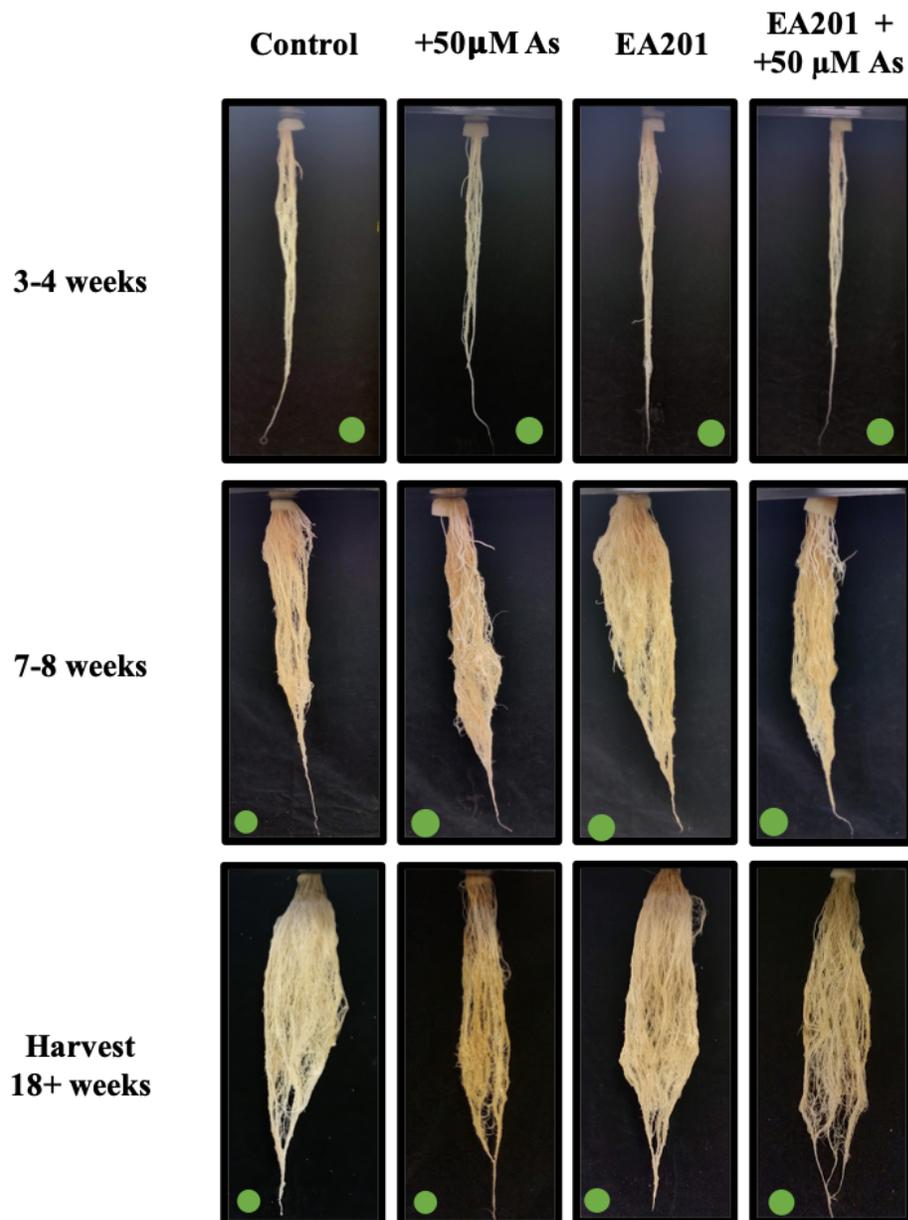


Figure 2.24. Nipponbare rice plants inoculated with EA201 bacteria and treated with a HT concentration of As ($\sim 50 \mu\text{M}$). Images show phenotypic differences in the roots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

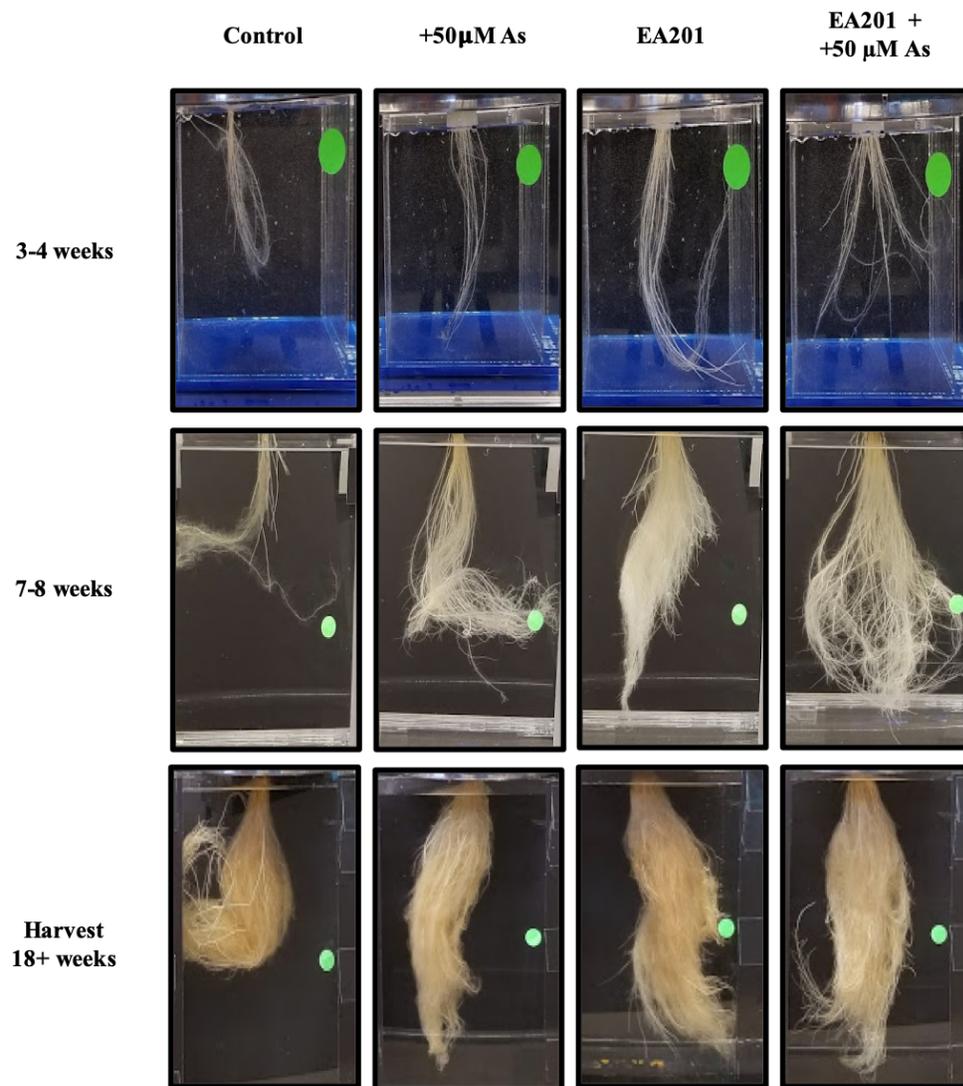


Figure 2.25. IR66 rice plants inoculated with EA201 bacteria and treated with a HT concentration of As ($\sim 50 \mu\text{M}$). Images show phenotypic differences in the roots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

In the statistics for the Nipponbare EA201 bacteria trial, the total plant biomass for the EA201 only treatment is significant over the other treatment groups. Due to variation, statistically, the IR66 treatment groups has the same significance when compared to each other (Figure 2.26). In both cultivars, the EA201 only treatment produced significantly more grains than the other treatment groups (Figure 2.27). The harvest index is statistically the same across all treatments for the Nipponbare rice plants but the EA201 only treatment group is significant over the rest for the IR66 cultivar (Figure 2.28). In total shoot biomass EA201 only is significant over other treatments in the Nipponbare cultivar but not in the IR66 cultivar (Figure 2.29).

The progression of As concentrations in the Nipponbare tissues is comparable to other Nipponbare trials but overall concentration was slightly elevated (Figure 2.30). Also, there is less of a step in As accumulation between the shoots and roots of the Nipponbare plants. Unlike the other HT trials, the total As concentration in IR66 rice plants is similar to the concentrations found in Nipponbare plants (Figure 2.31). In other trials As accumulation in the tissues of IR66 plants is much higher than concentrations in Nipponbare tissues. In this case As transport acted similarly in both cultivars.

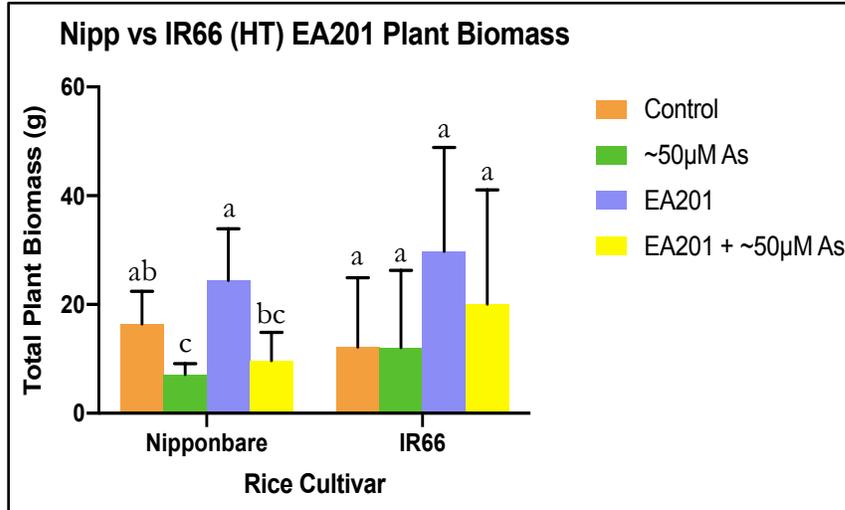


Figure 2.26. Total plant biomass of Nipponbare and IR66 rice exposed to HT As and EA201 bacteria. Significance is measured between treatment groups in each rice cultivar; where “a” is more significant compared to “b”.

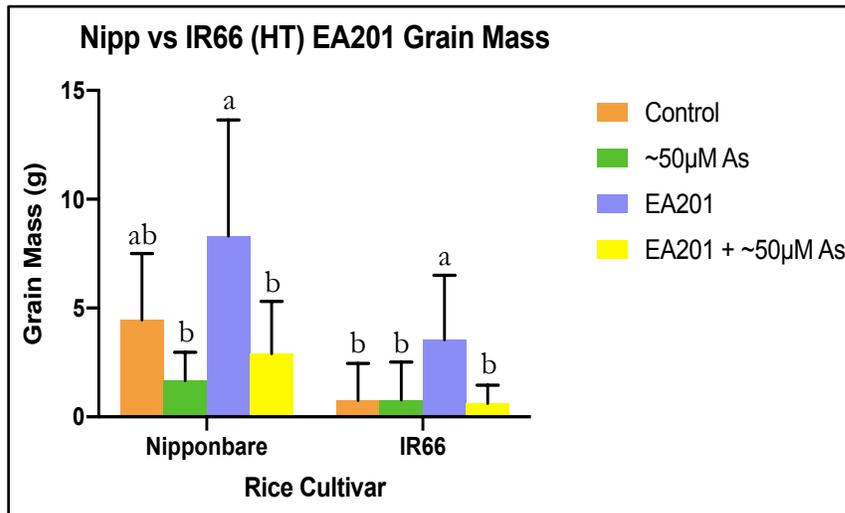


Figure 2.27. Grain mass of Nipponbare and IR66 rice exposed to HT As and EA201 bacteria. Significance is measured between treatment groups in each rice cultivar.

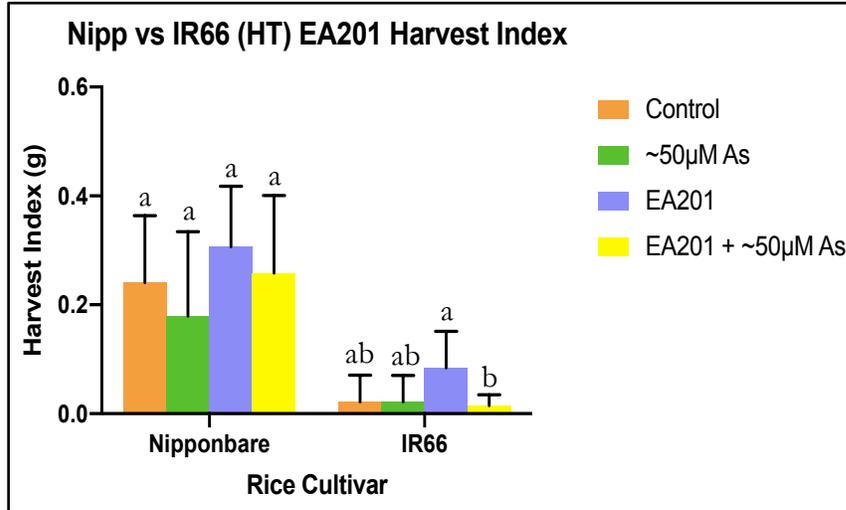


Figure 2.28. Harvest index ratio of Nipponbare and IR66 rice exposed to HT As and EA201 bacteria. Significance is measured between treatment groups in each rice cultivar.

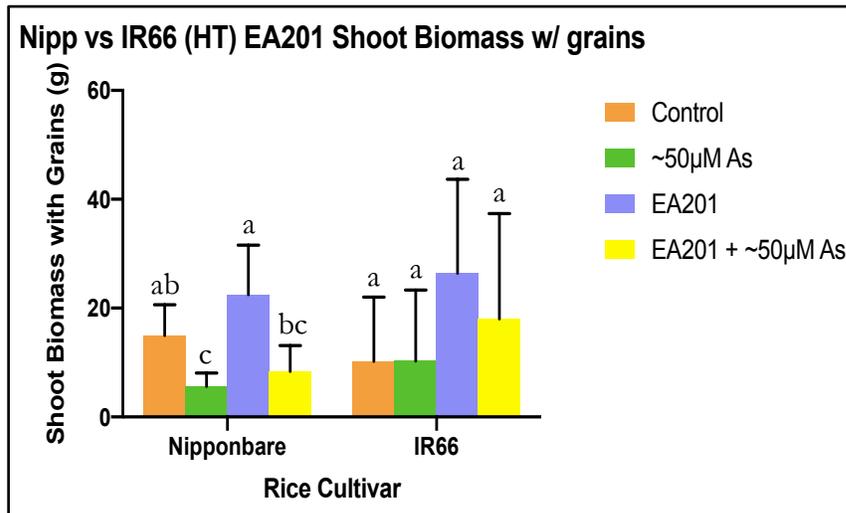


Figure 2.29. Total shoot biomass of Nipponbare and IR66 rice exposed to HT As and EA201 bacteria. Significance is measured between treatment groups in each rice cultivar.

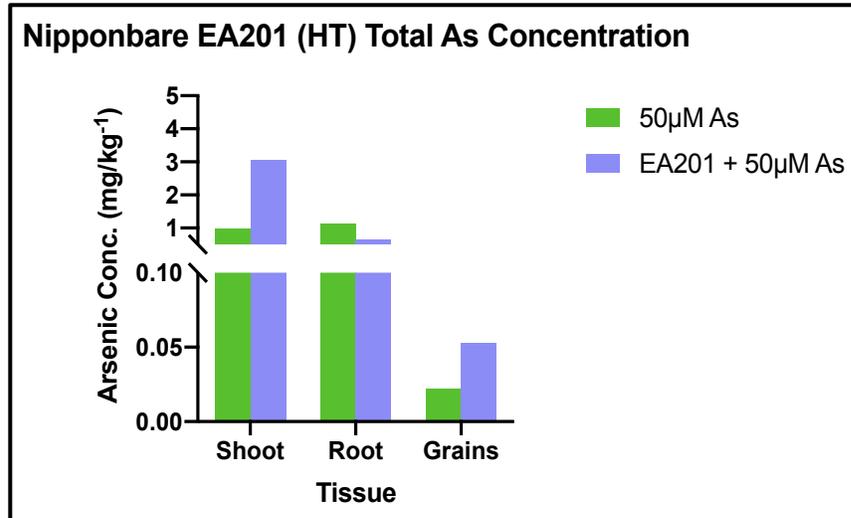


Figure 2.30. Arsenic concentration in Nipponbare rice in shoot, root, and grains exposed to EA201 bacteria and HT concentrations of As.

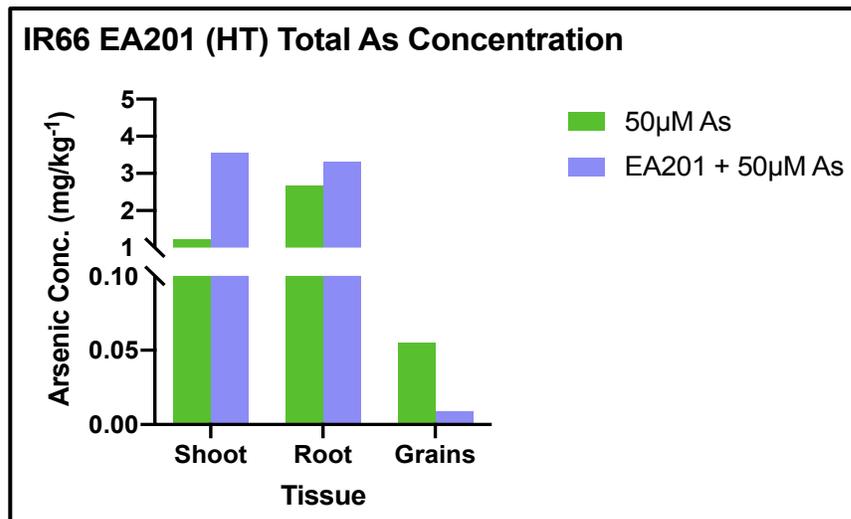


Figure 2.31. Arsenic concentration in IR66 rice in shoot, root, and grains exposed to EA201 bacteria and HT concentrations of As.

2.3.1.4 Supplementation of *Bacillus subtilis* (UD1022) under HT As environment:

In the UD1022 trial, at harvest, all Nipponbare rice plants except those treated with only As produced grain (Figure 2.32). Here the control Nipponbare rice plants appear to produce more grains than the UD1022 only plants. Plants treated with As display similar phenotypes as seen in previous bacteria trials. The root biomass of the control and UD1022 only Nipponbare rice plants is more abundant than the As treated plants, this is seen as early as 3 to 4 weeks (Figure 2.34). IR66 rice plants treated with UD1022 only have a better shoot structure than any other treatment groups. The As only plants looked severely inhibited in comparison (Figure 2.33). This trend is carried on into the IR66 plant roots, where the As only plants have much less biomass than the other treatment groups (Figure 2.35).

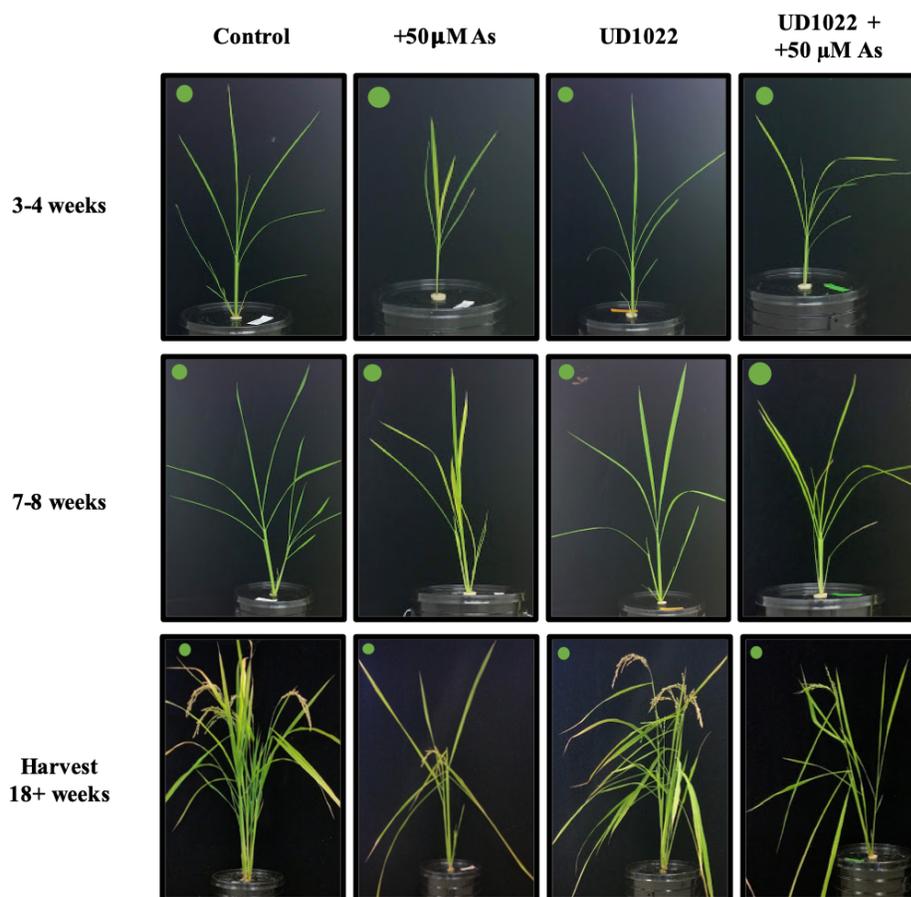


Figure 2.32. Nipponbare rice plants inoculated with UD1022 bacteria and treated with HT concentration of As ($\sim 50 \mu\text{M}$). Images show phenotypic differences in the shoots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

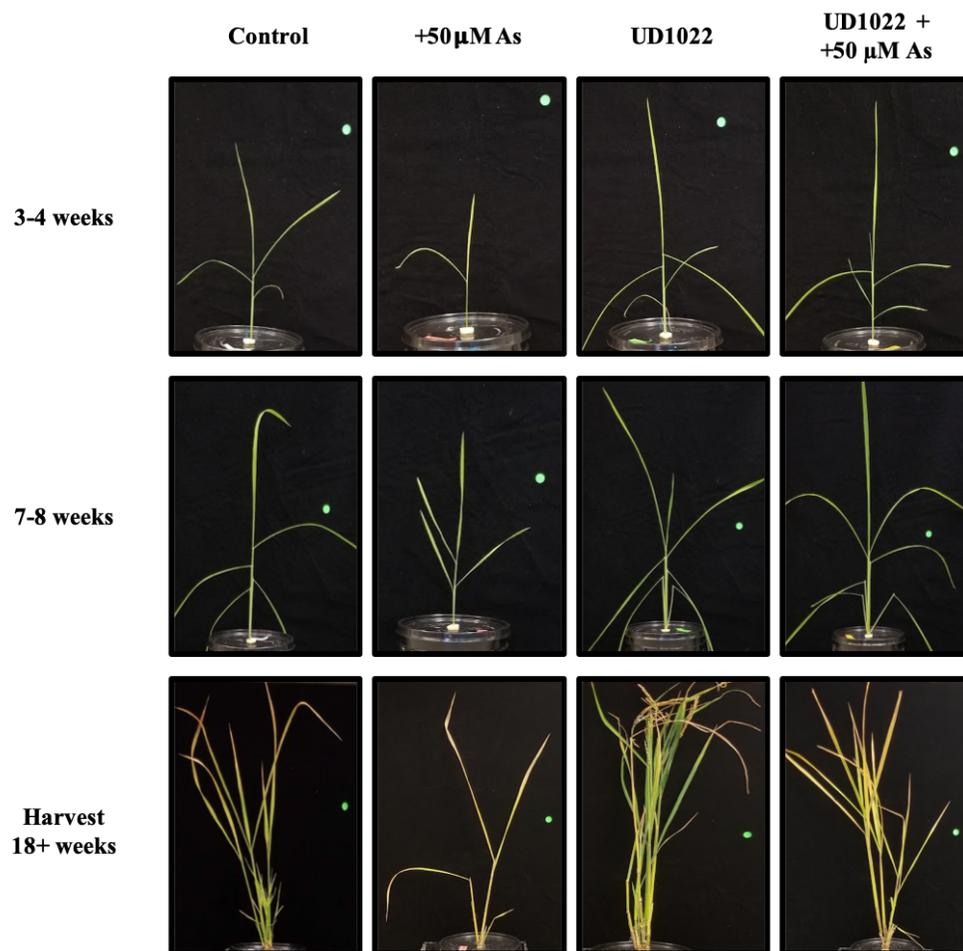


Figure 2.33. IR66 rice plants inoculated with UD1022 bacteria and treated with HT concentration of As ($\sim 50 \mu\text{M}$). Images show phenotypic differences in the shoots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.



Figure 2.34. Nipponbare rice plants inoculated with UD1022 bacteria and treated with HT concentration of As ($\sim 50 \mu\text{M}$). Images show phenotypic differences in the roots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

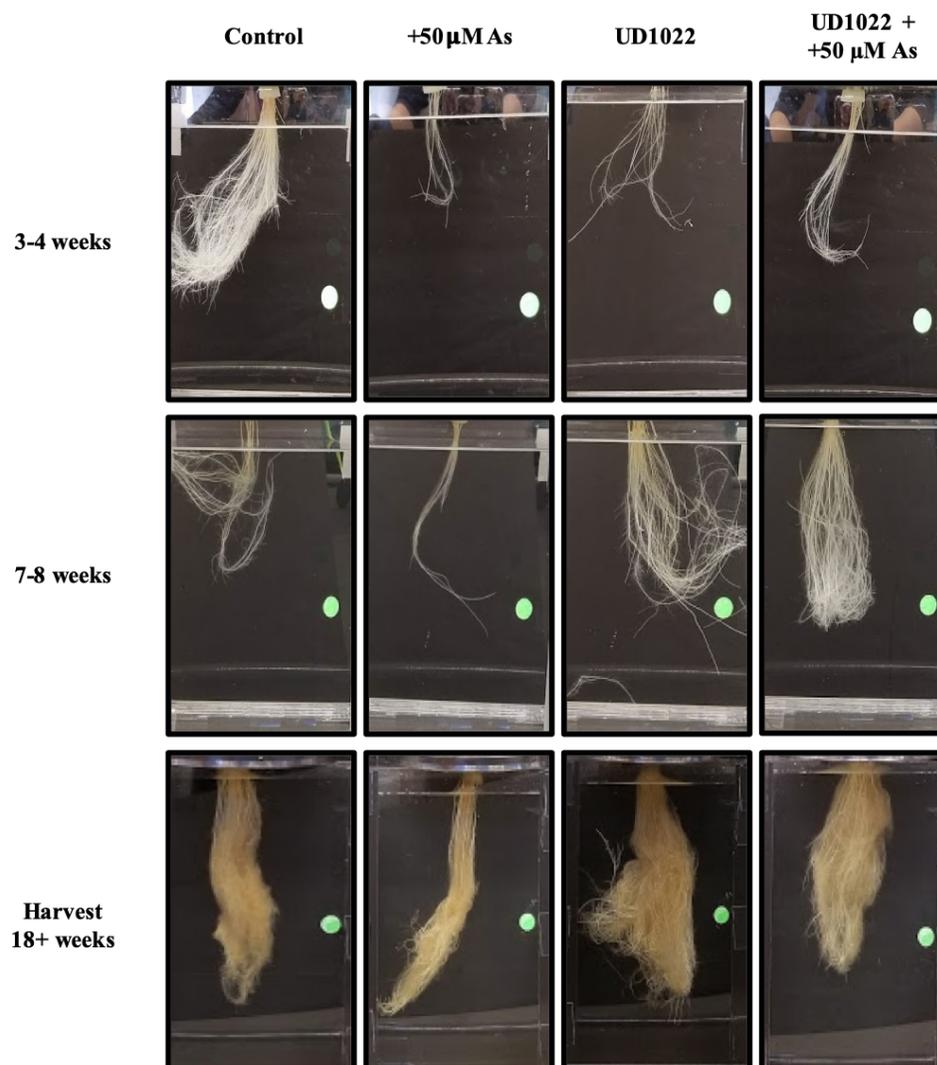


Figure 2.35. IR66 rice plants inoculated with UD1022 bacteria and treated with HT concentration of As ($\sim 50 \mu\text{M}$). Images show phenotypic differences in the roots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

Statistically, the total plant biomass of control Nipponbare rice plants is significantly higher than the other treatment groups but has similarities to the UD1022 only group. The UD1022 only and the UD1022 plus As Nipponbare IR66 rice plants have significantly higher total plant biomass than the other treatment groups (Figure 2.36). Nipponbare grain mass shows the control group again is more significant than the other treatments where in the IR66 cultivar all the treatments are statistically similar (Figure 2.37). The harvest index shows no significance between treatment groups within cultivars (Figure 2.38). The results for total shoot biomass are comparable to those for total plant biomass between treatments within rice cultivars (Figure 2.39). The progression of As in the Nipponbare tissues was comparable to other Nipponbare trials but overall concentration was slightly elevated (Figure 2.40). Surprisingly the As concentrations in the IR66 plant tissues are decreased from the Nipponbare plants (Figure 2.41). These results do not support Nipponbare as tolerant to As.

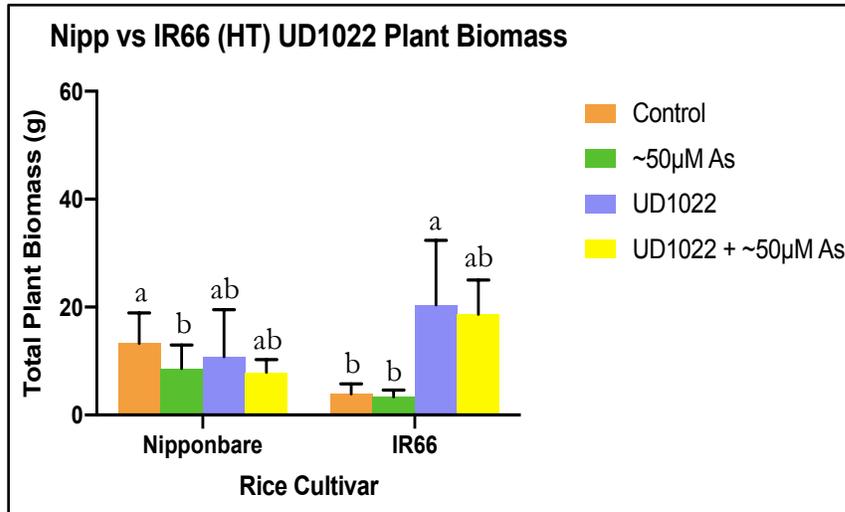


Figure 2.36. Total plant biomass of Nipponbare and IR66 rice exposed to HT As and UD1022 bacteria.

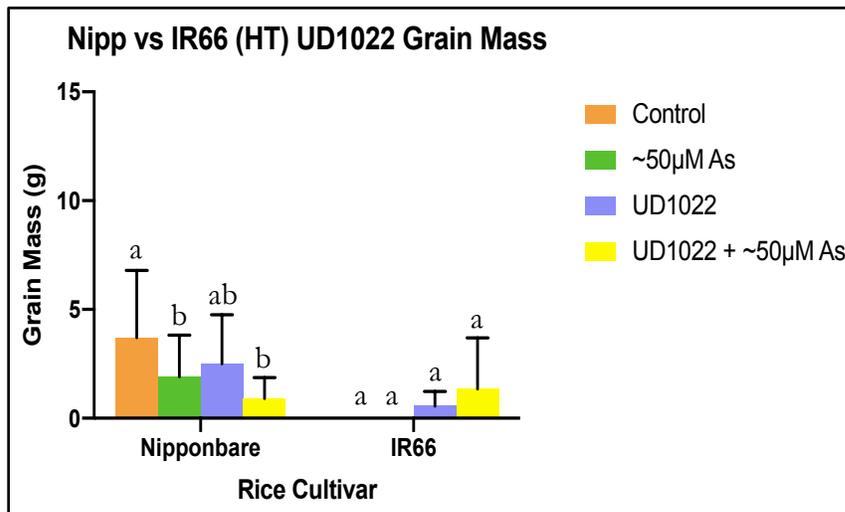


Figure 2.37. Grain mass of Nipponbare and IR66 rice exposed to HT As and UD1022 bacteria.

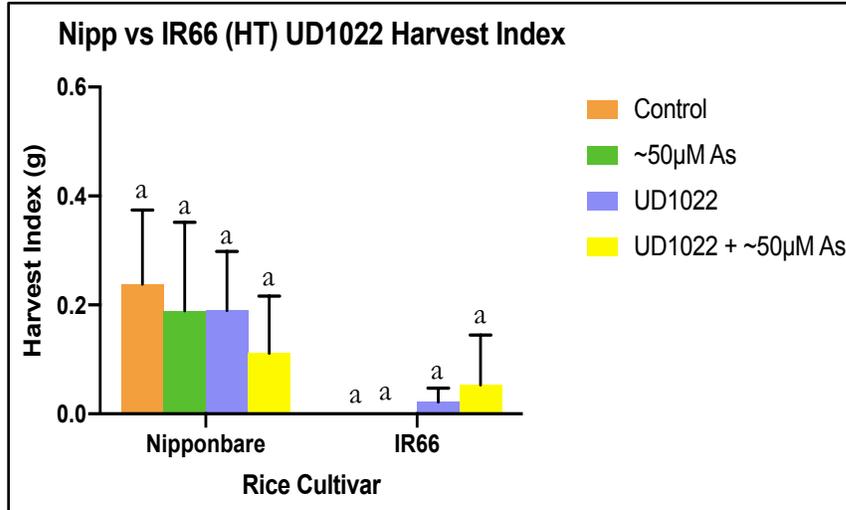


Figure 2.38. Harvest index of Nipponbare and IR66 rice exposed to HT As and UD1022 bacteria.

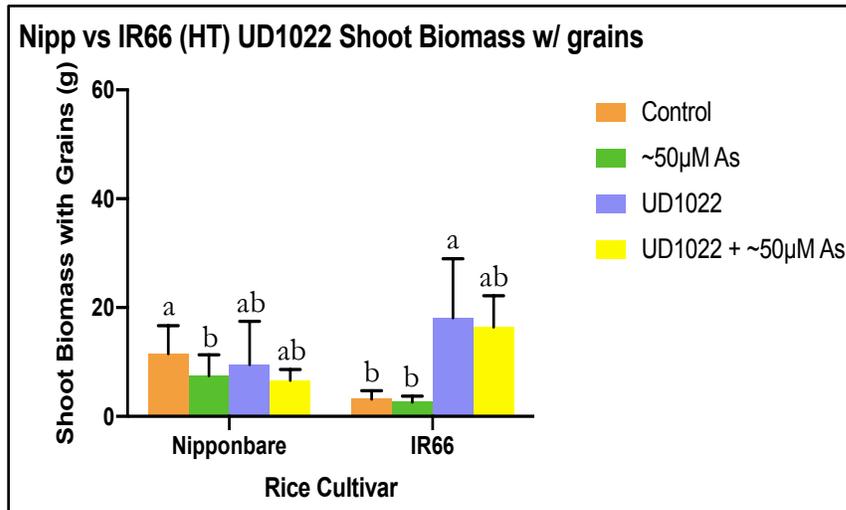


Figure 2.39. Total shoot biomass of Nipponbare and IR66 rice exposed to HT As and UD1022 bacteria.

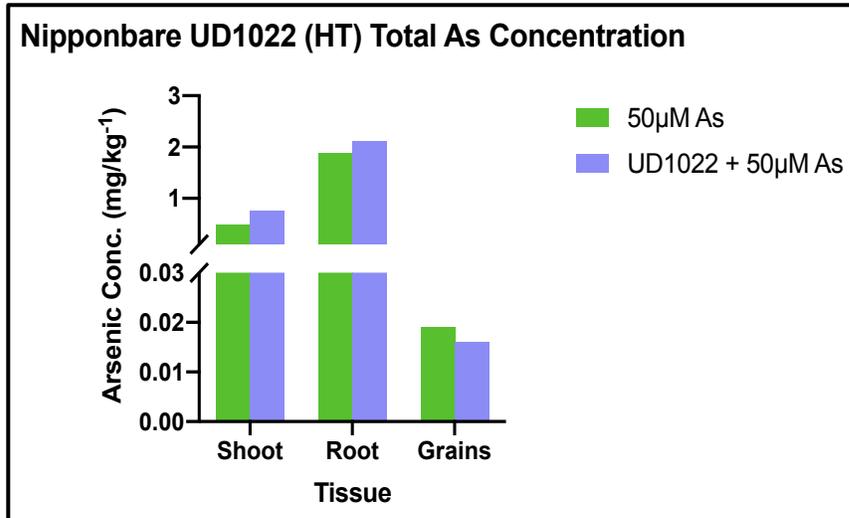


Figure 2.40. Arsenic concentration in Nipponbare rice exposed to HT As and UD1022 bacteria.

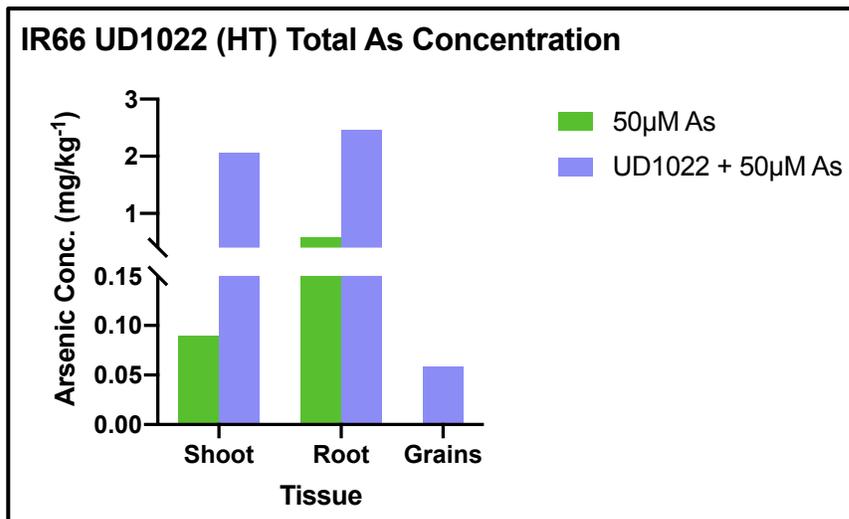


Figure 2.41. Arsenic concentration in IR66 rice exposed to HT As and UD1022 bacteria.

2.3.2 Moderate Toxicity Hydroponics

2.3.2.1 Supplementation of *Pantoea* sps. (EA106) under MT As environment:

In the EA106 trial under MT As concentrations in all Nipponbare treatment groups except the As only group visibly show more shoot biomass compared to the HT EA106 experiment. This is clearly visible at 10 weeks (Figure 2.42). The Nipponbare roots display a similar trend, however the EA106 plus As treatment looks to have less biomass when compared to the control and EA106 only groups (Figure 2.44). The IR66 plants also appear to be less affected by the lower concentration of As. This is seen in the more abundant shoot biomass in the EA106 plus As treated plants (Figure 2.43). The biomass of IR66 rice plant roots do not appear to be affected by the As treatment (Figure 2.45).

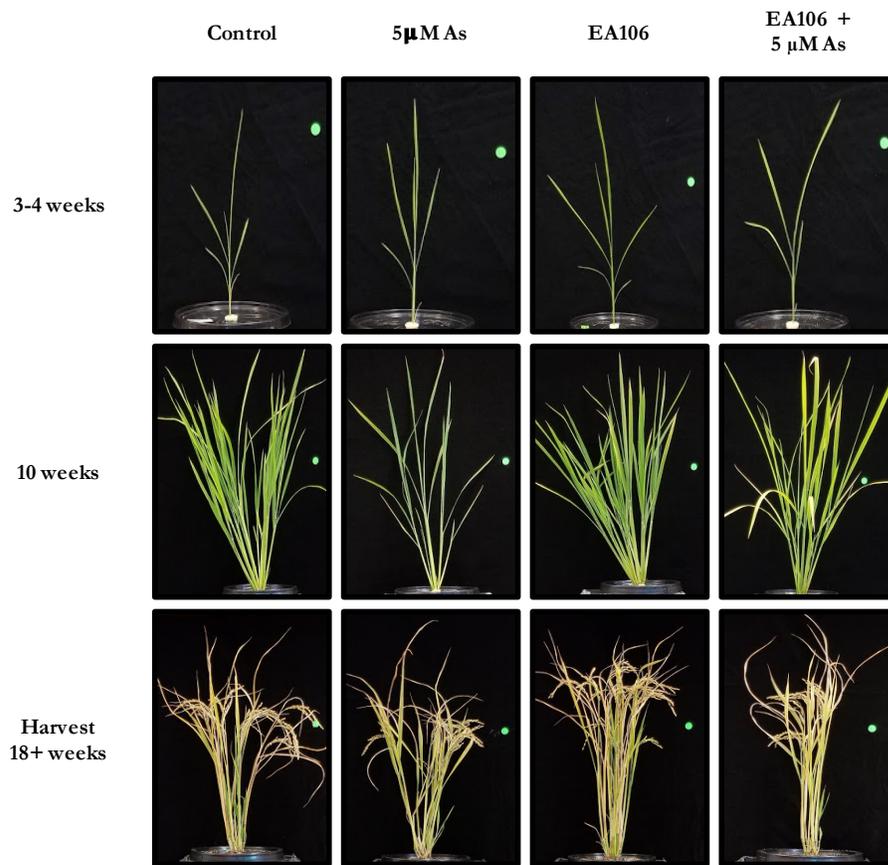


Figure 2.42. Nipponbare rice plants inoculated with EA106 bacteria and treated with a MT As concentration ($\sim 5 \mu\text{M As}$). Images show phenotypic differences in the shoots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

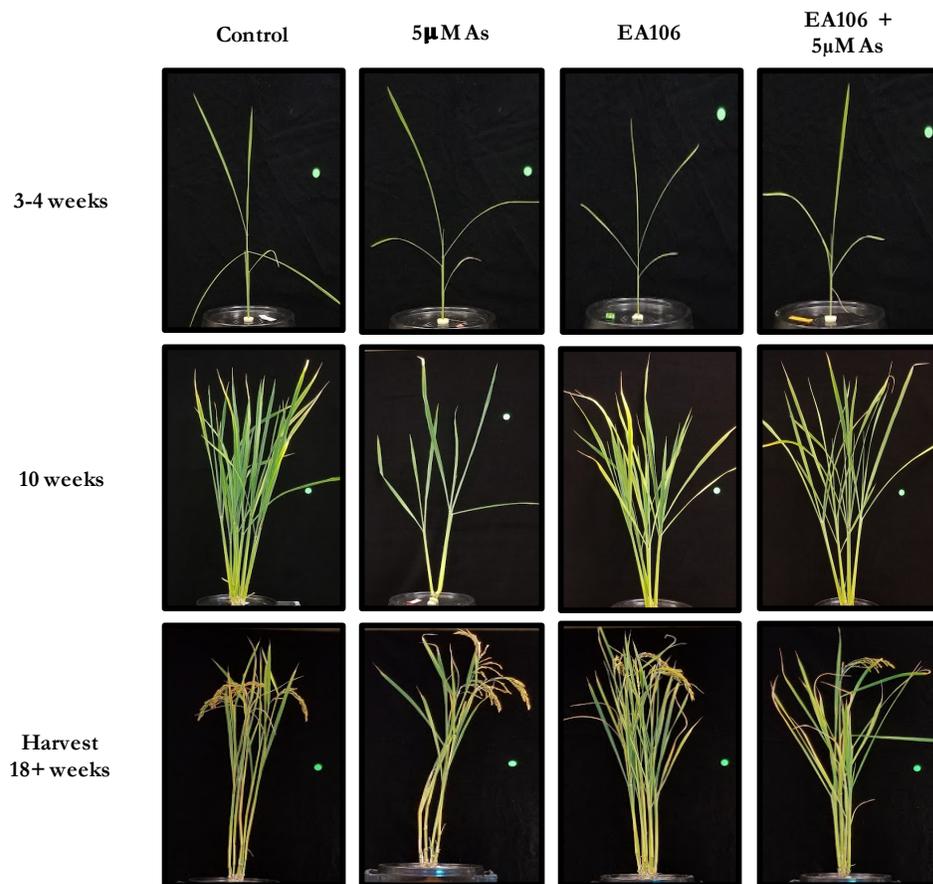


Figure 2.43. IR66 rice plants inoculated with EA106 bacteria and treated with a MT As concentration (~5 μ M). Images show phenotypic differences in the shoots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

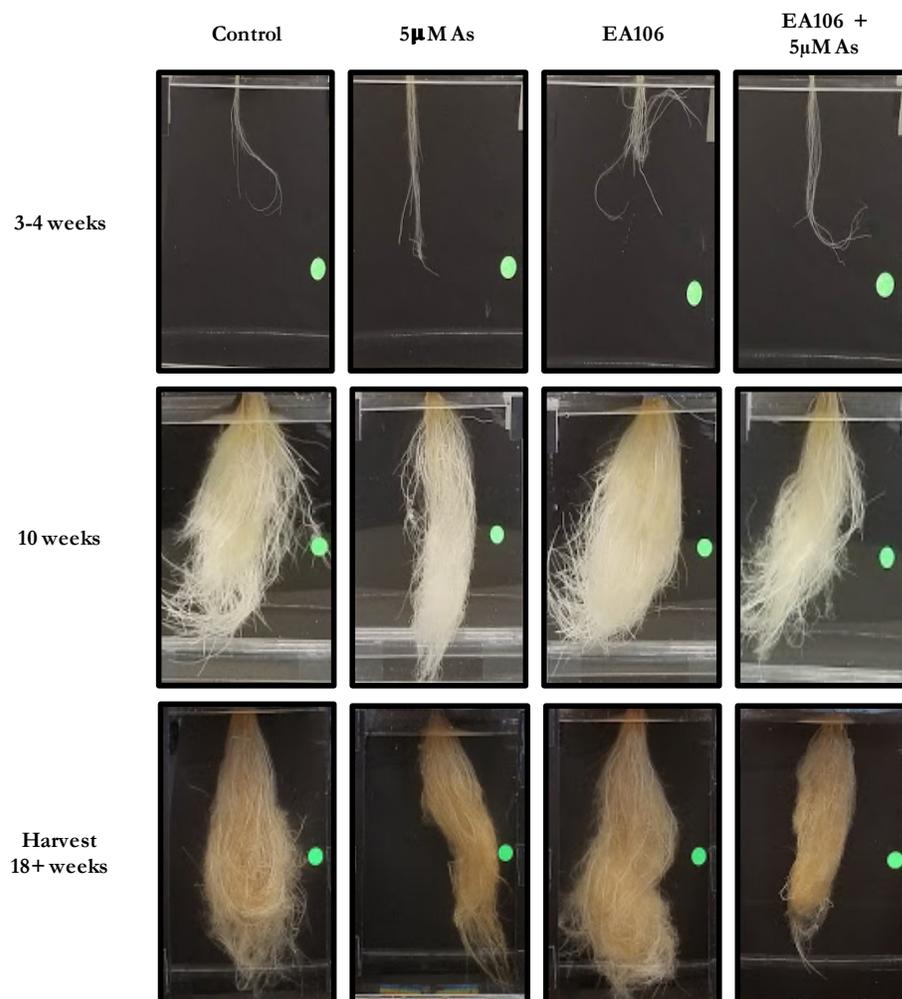


Figure 2.44. Nipponbare rice plants inoculated with EA106 bacteria and treated with a MT As concentration ($\sim 5 \mu\text{M}$). Images show phenotypic differences in the roots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

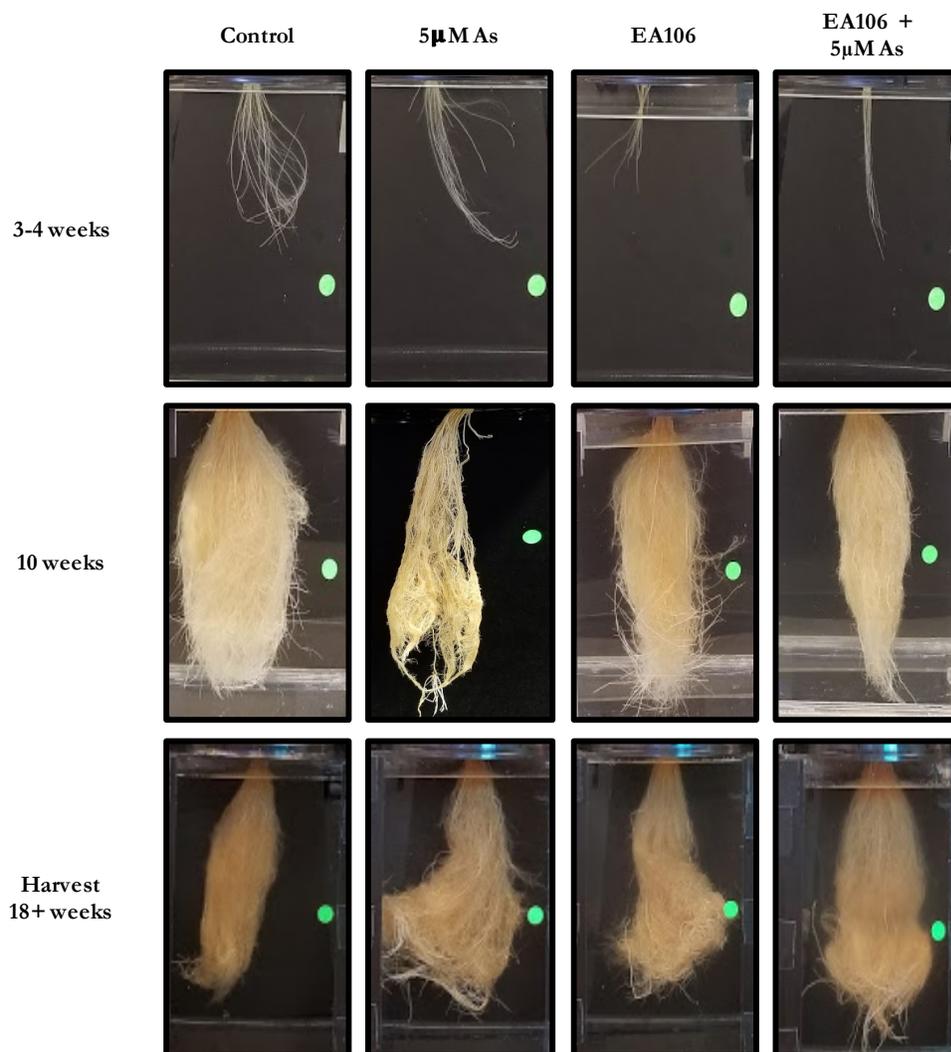


Figure 2.45. IR66 rice plants inoculated with EA106 bacteria and treated with a MT As concentration ($\sim 5 \mu\text{M}$). Images show phenotypic differences in the roots of each plant. Representatives are shown from four treatment groups at three different timepoints over the experiment.

Moderate toxicity statistics for total plant biomass in both Nipponbare and IR66 cultivars show significant differences between the control and EA106 only treatment groups when compared to the As only treatment groups (Figure 2.46). The grain mass is also significantly higher in the Nipponbare and IR66 treatment groups without As exposure (Figure 2.47). The harvest index in the Nipponbare rice plants is significantly higher for the EA106 only and control groups. In IR66 plants, the EA106 plus As treatment group is more statistically similar to the control and EA106 only group than the As alone group (Figure 2.48). For total shoot biomass in the Nipponbare cultivar, the control and EA106 only treatments are significant over the As treatments. In IR66 cultivar, all treatments are statistically similar and significant over the As only treatment group (Figure 2.49). Total As concentrations for As accumulated in Nipponbare and IR66 rice tissues for MT trials is similar to values seen in HT experiments. Again, As concentrations are higher in Nipponbare compared to IR66 tissues (Figure 2.50 and Figure 2.51). While concentrations are also higher in the grain for both cultivars, under HT As no grains were produced in the IR66 cultivar but are produced in the MT trial.

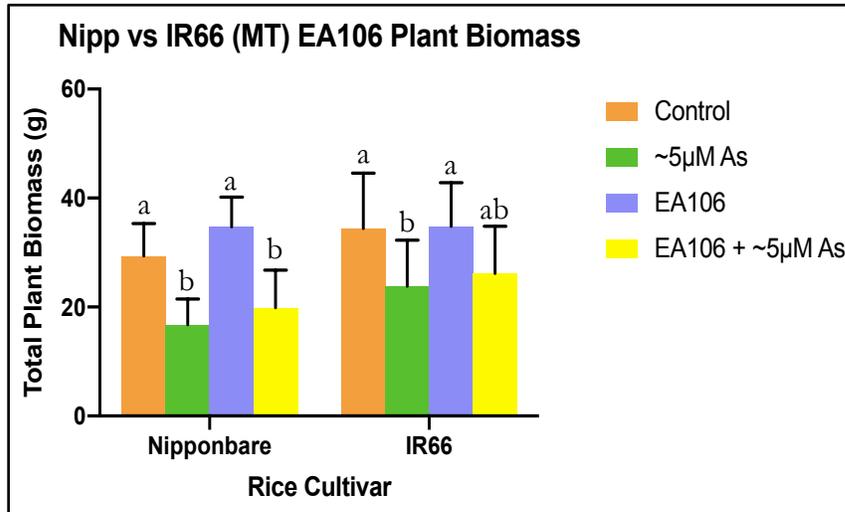


Figure 2.46. Total plant biomass of Nipponbare and IR66 rice plants inoculated with EA106 bacteria and treated with a MT As concentration (~5 µM).

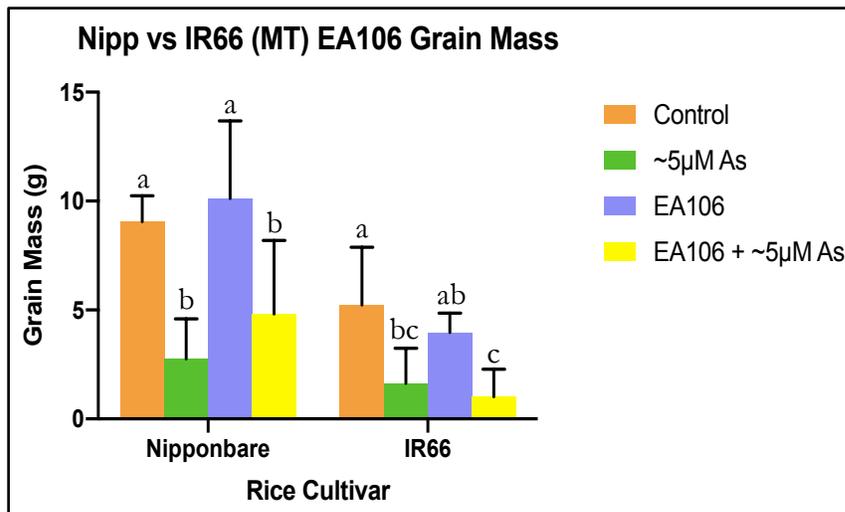


Figure 2.47. Grain mass of Nipponbare and IR66 rice plants inoculated with EA106 bacteria and treated with a MT As concentration (~5 µM).

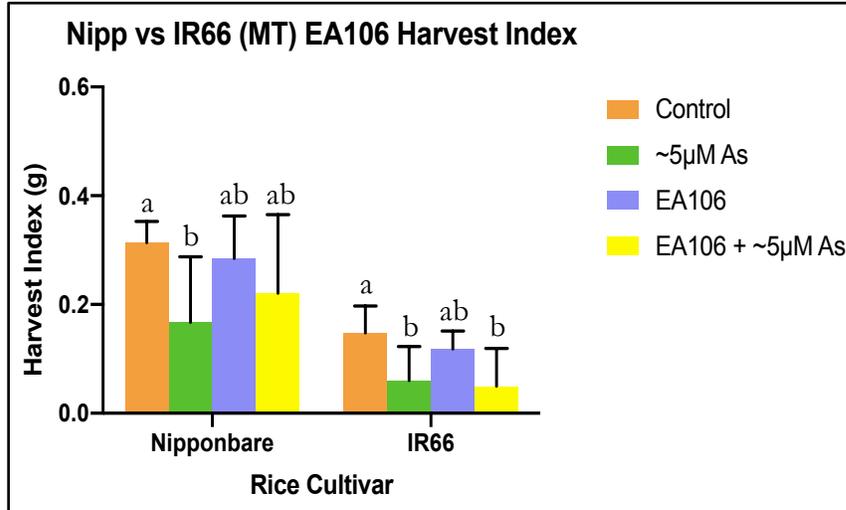


Figure 2.48. Harvest index of Nipponbare and IR66 rice plants inoculated with EA106 bacteria and treated with a MT As concentration (~5 µM).

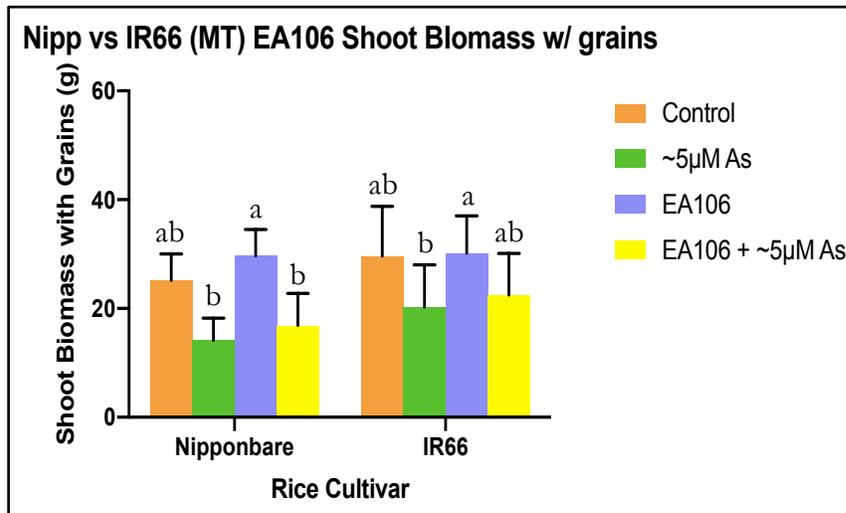


Figure 2.49. Total shoot biomass of Nipponbare and IR66 rice plants inoculated with EA106 bacteria and treated with a MT As concentration (~5 µM).

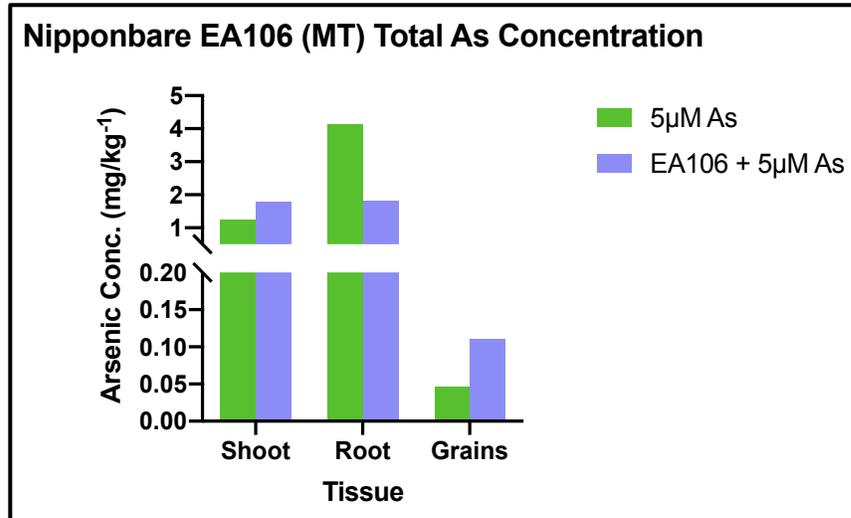


Figure 2.50. Arsenic concentration in Nipponbare rice exposed to MT As and EA106 bacteria.

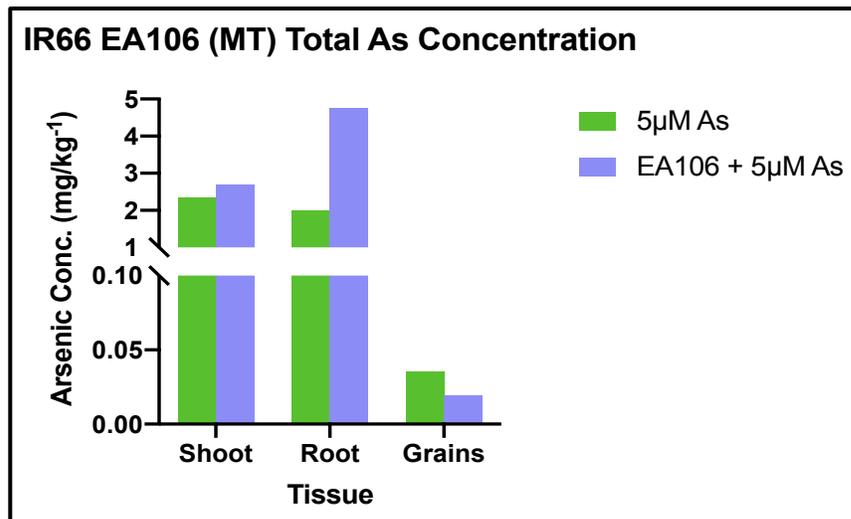


Figure 2.51. Arsenic concentration in IR66 rice exposed to MT As and EA106 bacteria.

2.4 Conclusions and Future Perspectives

In conclusion, As has detrimental phytotoxic impacts on the above ground physiology in rice despite As being added directly to the roots. This supports previous

data showing abiotic stress, no matter where it is applied, impacts the whole plant system (Lakshmanan et al., 2015 and Spence et al., 2014). Photographs of the rice plants, taken throughout growth, visibly show the impacts of As on rice in HT and MT environments. For both tolerant Nipponbare and susceptible IR66, the plant representative for the arsenic treatment displayed signs of heavy metal As toxicity. These signs were visible earlier in the IR66 cultivar compared to Nipponbare. Not only did root health and biomass suffer but the health of the aboveground plant also suffered. Symptoms included stunted growth, chlorosis of leaves, disorganized plant structure, and reduced number of rice panicles. The shoot and root phenotypes for Nipponbare and IR66 rice plants treated with HT As and EA201 bacteria isolate presented signs of As recovery. These plants had relatively normal plant structure and the presence of grains compared to some HT trials where As treated plants did not yield grain.

When comparing the two cultivars statistically, Nipponbare displays more resistance against As stress than IR66 in the EA106 and EA104 HT trials. Here Nipponbare plants has less As accumulation in each tissue type. Surprisingly in the EA201 and UD1022 trials, Nipponbare plants had an equal or increased amount of As in each tissue type compared to the IR66 plants in the trial. This could be a result of several factors; first, As uptake can be dictated by species availability and growth stage (Awasthi et al., 2017). If plants were topped off with As at a critical developmental stage more As could be transported into the plant. Because the As was

added as As(III) it was more mobile and available for transport into the plant. Second there could be error in the ICP analysis protocol.

The significant increase in total plant biomass and total grain mass in Nipponbare and IR66 rice plants exposed to EA104 and EA201 only could be attributed to the bacteria having better association with these rice varieties than the other isolates. Though EA104 and EA201 were both isolated from M-104 rhizospheric soil, they may have attributes that positively benefit Nipponbare and IR66 cultivars. The lack of microbe specificity to Nipponbare and IR66 could account for the variability between the cultivars and the isolate trials. In the future it would be interesting to isolate and test bacteria naturally occurring in the rhizosphere of Nipponbare and IR66 cultivars to see if they have greater impacts on plant growth and abiotic resistance. Following the notion that As uptake can be greater at different times during rice development, perhaps adding additional doses of PGPRs near these developmental stages will mitigate As uptake into the plants (Awasthi et al., 2017). Further experimentation should be done to observe bacteria root colonization and to test for quantity of viable bacteria after exposure to MT and HT As environments.

The MT toxicity experimental trials were performed to yield results for inorganic As concentrations commonly found in nature. The phenotypes of the As only plants for both cultivars is similar to what is seen in the HT experiments but appears to not be as extreme. These plants have greater shoot biomass and more visible lamina than the HT EA106 trial. The statistics were surprising in that the values and distributions of significance aligne closely to those in the EA106 HT trials.

It was also surprising to see As tissue concentrations, for both Nipponbare and IR66 cultivars, that are the same or similar to the HT trials. Even in the most toxic As contaminated areas of the world, As concentrations in rice tissue does not exceed 50 mgkg⁻¹ (Kumarathilaka et al., 2018). To rule out error in the ICP nutrient testing protocol samples should be rerun using ICP-MS and speciation should be done to determine what species of As is abundant in each of the tissue types.

To end, microbial intervention improves growth and yield in Nipponbare and IR66 rice cultivars. Each rhizospheric isolate used in these experiments behaved differently and thus have varying effects on abating As toxicity in rice. There is no isolate in particular that presents as an obvious As mitigator. Therefore, future work should surround finding a PGPRs specific to the rhizosphere of Nipponbare and IR66 cultivars and their effects on reducing As uptake.

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Chapter 3

PLANT GROWTH REGULATORS WORKING AS SIGNALING COMPOUNDS TO INDICATE ARSENIC EXPOSURE

3.1 Introduction

Abiotic stressors can negatively impact plant health and development. Arsenic (As) is one of the most detrimental abiotic stressors affecting both plant and human health. Arsenic and other heavy metals are introduced into the environment through natural geochemical processes or human induced pollutants (Thongnok et al., 2018). Rice is particularly susceptible to As toxicity because of the unique cultivation method used to irrigate. Unlike other grain crops, rice is irrigated by flooding the fields at different plant developmental growth stages. In flooded paddy conditions, the oxidation state of As is altered changing its bioavailability and mobility, therefore allowing it to enter the plant more easily (Kumarathilaka et al., 2018 and Sun et al., 2019). A plant's continuous exposure to high As concentrations will alter normal development and yields.

Plant hormones play an important role in plant growth and development under abiotic stress environments (Feng et al., 2016). Of these, auxin is an important plant hormone responsible for cell division, cell differentiation, elongation, flowering and vascular development in plants (Yamamoto et al., 2007). Brassinosteroids are another group of phytohormones that control plant features such as plant height, leaf erectness, flowering, seed germination, and stress tolerance (Tong and Chu, 2011). Thus, plant

growth regulators likely play a pivotal role in plant response to abiotic stress (Spence and Bais, 2015).

In this chapter I hypothesize that the changes in plant architecture observed in response to As and PGPR treatments is mediated by plant hormone pathways. To test this hypothesis, I look at the expression of growth regulators in response to arsenic toxicity. This is done by looking at expression of IAA auxin gene *OsYUCCA1* and brassinosteroid *qOsBZR1* gene in root and shoot tissues after treatment with As and different putative PGPRs.

3.2 Materials and Methods

3.2.1 Seed preparation and plant set up

The Nipponbare and IR66 seeds were sterilized as in chapter two section

2.3.1.1. After germinating on the petri dishes for 7 to 10 days, the seedlings were transferred to sterile test tubes. Prior to transfer, test tubes were cleaned with Alconox solution and autoclaved for sterility. Foam plugs were cut, slit and autoclaved, similar to what was done for the hydroponic experiments. Rice nutrient solution was made and autoclaved.

Once the reagents and materials were ready, in a sterile hood, 15 mL of autoclaved rice nutrient solution was distributed into the clean test tubes. More nutrient solution was poured into a reservoir. Each foam plug was dipped into the reservoir of nutrient solution and a germinated rice seedling was inserted in the foam plug. Using sterile forceps, the foam plug was inserted into the test tube so that the

bottom of the plug was touching the nutrient solution. Special attention was taken to be sure there were no air bubbles between the nutrient solution and the plug.

Each experimental trial consisted of 4 treatment groups with 6 plants per treatment group. The treatment groups were as follows: control, arsenic treatment (5 μM for moderate toxicity, 50 μM for high toxicity), bacteria only (1×10^6 cells/mL), arsenic and bacteria. Tissue from two plants was collected for each condition to achieve the appropriate amount of starting material. Once all of the plants were transferred, they were set under growth lights with a 16-hour photoperiod. Arsenic and bacteria were added 48 hours after seedling transfer.

3.2.2 Adding Arsenic

Arsenic was added to the appropriate treatment groups via pipette. For the moderate toxicity experiments 7.9 μL of .01 M NaAsO_2 was added to each test tube to achieve a final concentration of 5 μM As. For the high toxicity experiments 49.9 μL of .01 M NaAsO_2 was added to each test tube to achieve a final concentration of 50 μM As. Arsenic was added directly into the nutrient solution below the foam plug. Special attention was made to be sure no bubble where introduced when adding arsenic.

3.2.3 Preparing and inoculating bacteria

A bacterial culture was grown as for the hydroponics experiment with the following modifications. First a plate was struck, and the plate was incubated until single colonies were formed. Less bacteria were needed for the test tube assay therefore, 40 mL of LB media was added to two conical tubes. A single colony was

selected and added to each conical tube. The lid was taped to allow air ventilation and prevent spilling. The tubes were placed in the shaking incubator overnight so the culture could grow. Conical tubes were placed in the centrifuge and the culture was spun down to pellet. The supernatant was poured off and the pellet was washed 3 times with autoclaved M9 media (1x concentration, Table 3.2). After the final wash, 10 mL of M9 media was added to the conical tubes and the pellets were resuspended. The resuspended culture was added to a 25 mL autoclaved flask and brought up to 20 mL final culture with M9 media. A spectrophotometer was used to determine the OD and cell count per mL. The amount of bacteria culture to inoculate into the test tubes was determined using the Sigma Cell Dilution calculator. The final bacteria concentration in the test tube was 1×10^6 cell/mL.

The bacteria culture was added to the appropriate treatment groups using a pipette. Special attention was made not to introduce bubbles while inoculating plants. The bacteria were added at the same time as the arsenic.

Table 3.1. 5x M9 Salt Solution

Reagent	Amount of Reagent
1.5% Potassium Phosphate	15.0 g/L
6.4% Sodium Phosphate	64.0 g/L
0.25% Sodium Chloride	2.5 g/L
0.5% Ammonium Chloride	5.0 g/L

Table 3.2. 1x M9 Media

Reagent	Amount of Reagent
1.5% Potassium Phosphate	15.0 g/L
6.4% Sodium Phosphate	64.0 g/L
0.25% Sodium Chloride	2.5 g/L
0.5% Ammonium Chloride	5.0 g/L

3.2.4 Gene Expression

RNA was extracted from leaf and root tissue at 0, 12, and 24-hour timepoints following inoculation with bacteria and/or addition of arsenic. Each biological replicate was pooled from two plants. Tissue samples were homogenized in liquid nitrogen using mortar and pestle. RNA was extracted using the Qiagen RNeasy Plant Mini Kit and samples were treated with DNaseI (Qiagen). The cDNA was synthesized

from 1000 ng of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA samples were used to run reverse transcription quantitative polymerase chain reaction (RT-qPCR). The gene specific primers are listed in Table 3.3.

Table 3.3. Primer Sequences

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
UBQ5	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT
qOsBZR1	AGATGGTTCCTTTCGTGGAC	AGAATGAAATCGCCCAAATC
OsYUCCA1	TCATCGGACGCCCTCAACGTCGC	GGCAGAGCAAGATTATCAGTC

3.2.5 Primer Tests

Before proceeding with qRT-PCR, the primers for housekeeping and the two target genes were tested for amplification efficiency. Serial dilutions were made from each target gene (1 → 0.5 → 0.25 → 0.125) and run on a 96 well plate, in triplicate, on the BioRad CFX96 qPCR machine. The measured Ct values were plotted on a logarithmic scale and a linear regression curve was generated. The efficiency of each primer was calculated using the following equation: $E = -1 + 10^{(-1/\text{slope})}$. All primers yielded 100% efficiency.

3.2.6 qRT-PCR Tests

qRT-PCR was used to determine the expression of brassinosteroid and auxin in shoot and root tissue of Nipponbare and IR66 rice exposed to HT and MT As environments with or without the presence of a PGPR. Each 96 well plate consisted of three rows of target gene samples run with the housekeeping gene for corresponding samples. Each sample was run in triplicate. A mix of SYBR green, molecular grade water, primers, and cDNA was loaded into each well. Plates were labeled according to rice cultivar, bacteria isolate, toxicity level, and target gene. They were run on the BioRad CFX96 qPCR machine in the DBI common equipment room. Results were uploaded as Microsoft Excel files and saved for statistical analysis.

3.2.7 Statistical Analysis

The Ct values were obtained and normalized to expression of the ubiquitin housekeeping gene and the control (value =1). Microsoft Excel was used to run calculations and results were graphed using Prism GraphPad.

3.3 Results

3.3.1 High Toxicity Gene Expression EA106

The qRT-PCR results were normalized to ubiquitin 5 and the control for each target gene and sample group. In Nipponbare rice roots exposed to HT As environments, there is a significant up regulation of brassinosteroids 12 hours post inoculation (hpi) with EA106 only. There is also a significant fold change for YUCCA1 expression in HT As Nipponbare roots at 12 and 24 hpi in both EA106 only

and EA106 + As treatment groups (Figure 3.1). In IR66 rice plants, exposed to HT As environments, there is an increase in expression for brassinosteroids at 12 and 24 hpi 50 μ M As shoot samples as well as at 24 hpi in EA106 + 50 μ M As shoot samples. Looking at the results for YUCCA1 expression, IR66 rice plants exposed to HT As conditions, show increased fold change at 12 and 24 hpi in 50 μ M As and EA106 + 50 μ M As shoot samples. IR66 root samples show elevated YUCCA1 expression at 12 and 24 hpi for 50 μ M As treated plants; 0 and 12 hpi in EA106 only and EA106 + 50 μ M As treated plants (Figure 3.2).

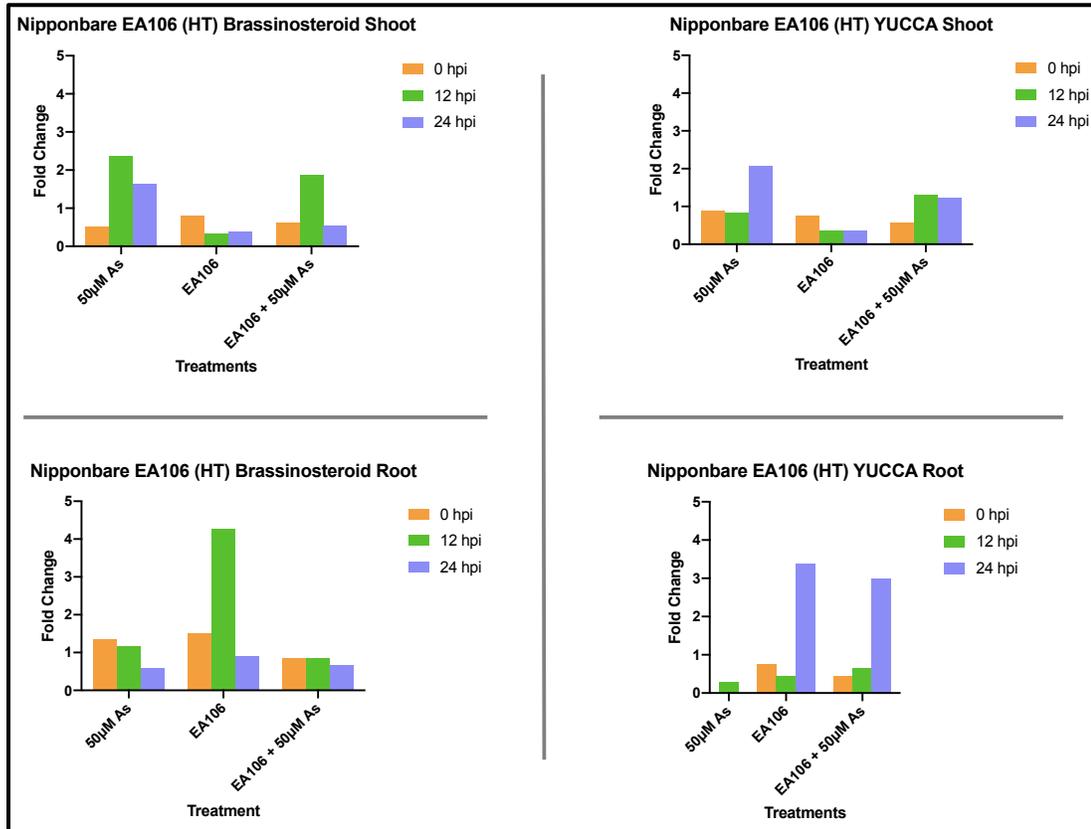


Figure 3.1. Results for Nipponbare rice gene expression of brassinosteroid and auxin hormones when normalized to ubiquitin in plants exposed to HT As environment.

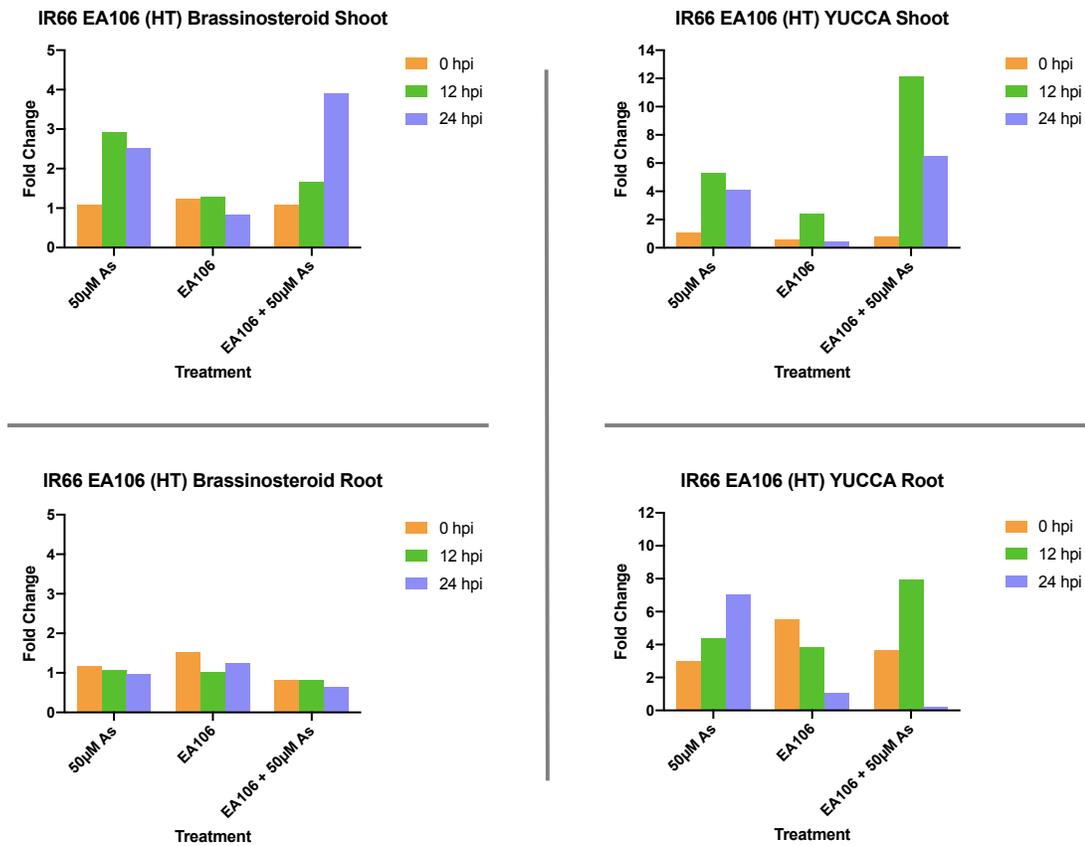
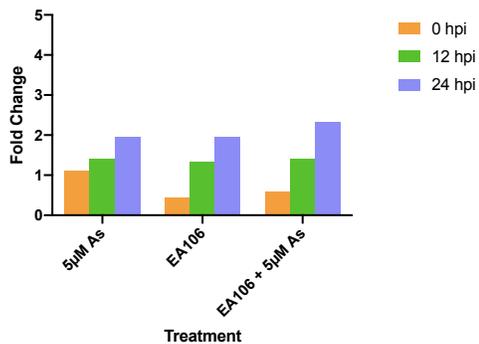


Figure 3.2. Results for IR66 rice gene expression of brassinosteroid and auxin hormones when normalized to ubiquitin in plants exposed to HT As environment.

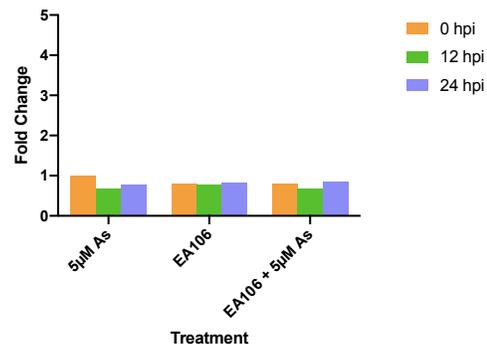
3.3.2 Moderate Toxicity Gene Expression EA106

Gene expression results for Nipponbare and IR66 rice plants exposed to a MT As environment is as follows. There is no significant fold change, over control (value = 1), in brassinosteroid or YUCCA1 genes for either shoot or root tissues (Figure 3.3). In IR66 plants there is increased expression of brassinosteroids at 12 and 24 hpi in 5 μ M As and EA106 + 5 μ M As shoot samples; as well as 24 hpi in the EA106 only shoot treatment group. Expression of YUCCA1 in IR66 shoot is increased at 24 hpi in the EA106 + 5 μ M As treatment group only. There is no significant fold change for brassinosteroids or YUCCA1 in IR66 roots exposed to a MT As environment.

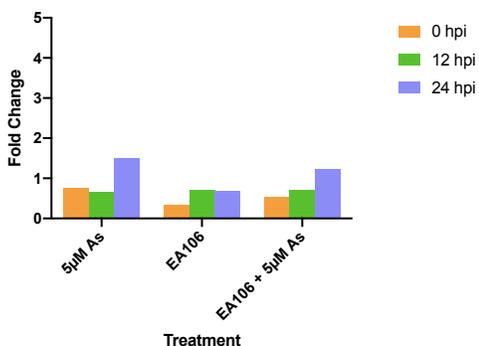
Nipponbare EA106 (MT) Brassinosteroid Shoot



Nipponbare EA106 (MT) Brassinosteroid Root



Nipponbare EA106 (MT) YUCCA Shoot



Nipponbare EA106 (MT) YUCCA Root

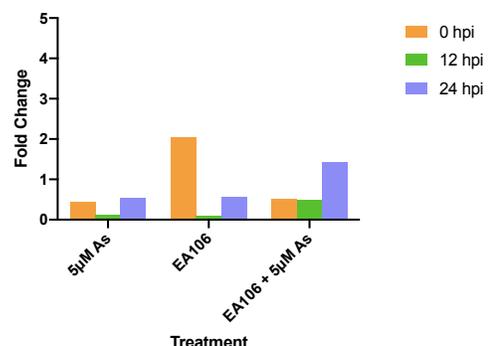


Figure 3.3. Results for Nipponbare rice gene expression of brassinosteroid and auxin hormones when normalized to ubiquitin in plants exposed to MT As environment.

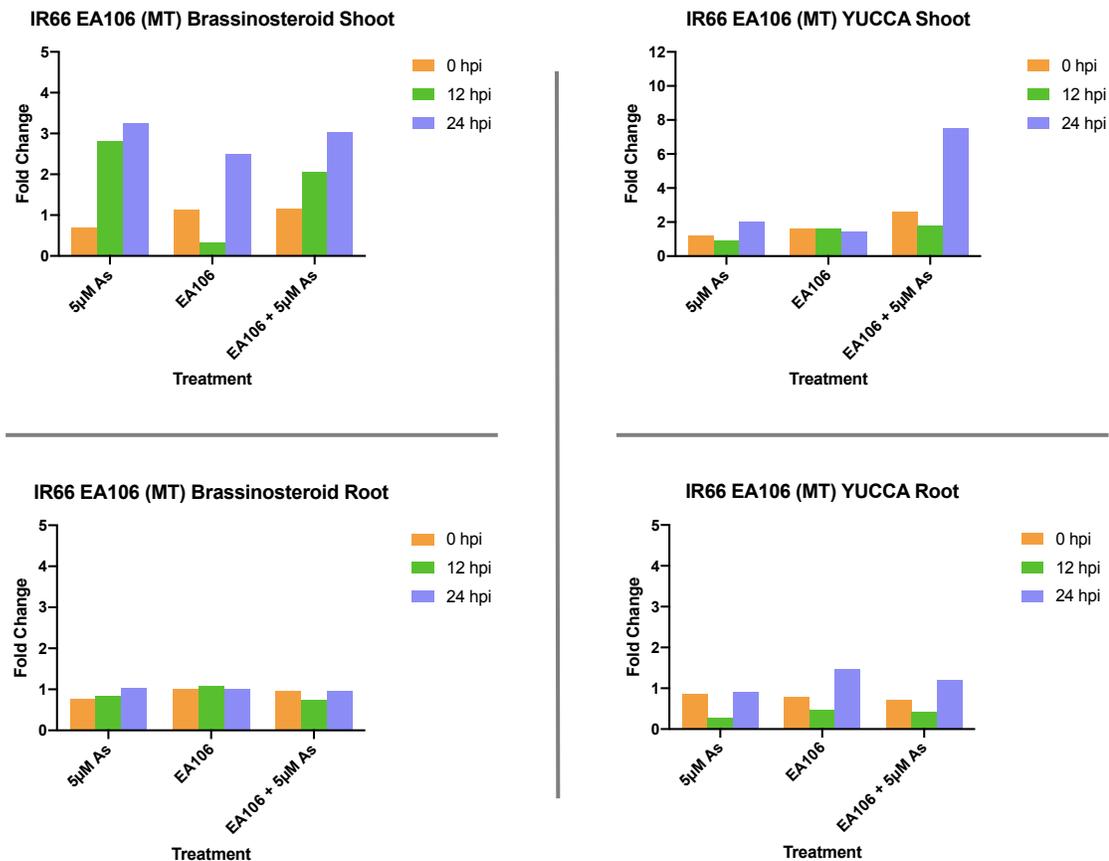


Figure 3.4. Results for IR66 rice gene expression of brassinosteroid and auxin hormones when normalized to ubiquitin in plants exposed to MT As environment.

3.4 Conclusions

Plant hormones play a significant role in plant defense (Feng et al., 2016). Specifically, brassinosteroids and auxin, in the form of YUCCA1, play a role in signaling abiotic stress. In this chapter it is seen how plant hormones respond to the same treatments and As environments but in different rice cultivars.

Overall expression levels of brassinosteroid and YUCCA1 were increased and showed more response in the HT As environment compared to the MT As

environment. Increased expression of brassinosteroid is observed at 12 hpi for EA106 only in Nipponbare roots but for both As treatment groups at the same timepoint in Nipponbare shoots. Here it is assumed that brassinosteroids are signaling the movement of As into the rice plant. To test this, an additional experiment can be done by adding As to the roots at the 12-hour timepoint. If the brassinosteroid gene is expressed in the roots at 12 hours it would be a good indication that brassinosteroids are acting to signal As exposure in different tissues. Results suggest that YUCCA1 works differently than brassinosteroid because there is an increase in fold change in Nipponbare roots at 24 hpi for the plants treated with EA106 and EA106 + 50 μ M As. Unlike Nipponbare, both brassinosteroid and YUCCA1 genes have increased expression for As treated groups in IR66 rice shoots. This suggested that YUCCA1 may not act as a signaling molecule for As in Nipponbare but may be in IR66. This would not be abnormal because Nipponbare and IR66 are very different cultivars. The trends in the MT environment are not as obvious as to the role of these plant hormones in signaling As movement.

In the future the qRT-PCR experiment should be run with more biological replicates to determine if the trends for brassinosteroid and YUCCA expression are consistent. Finally, the qRT-PCR experiment should be repeated using the EA104, EA201 and UD1022 rhizospheric isolates to determine how different PGPRs impact hormone signaling.

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