The Pennsylvania State University

The Graduate School

# IMPACTS OF THE BACTERIAL ENDOPHYTE *GLUCONACETOBACTER DIAZOTROPHICUS* ON THREE AGRICULTURALLY IMPORTANT CROPS

A Dissertation in

Horticulture

by

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**ABSTRACT**

As farmers strive to meet the challenges of an ever-growing populace, pressures seem to be emerging from every angle to limit traditional avenues of intensification. Application of synthetic fertilizers is non-sustainable and needs to be curtailed, fewer acres per capita are available each year for crop production as suburbia expands into farmland, and crop genetic modification approaches are embattled with regulation and public resistance. Enter the possibility of the biologicals, where synergisms between microbes and plants can help meet the needs of agricultural output. This application of mutualism is not new, but only recently have certain bacteria emerged that show potential to not just boost growth of a select few plant families, but myriad species across the globes' diverse cadre of agronomically important crops.

This work investigates one such bacterium, *Gluconacetobacter diazotrophicus* (*G. diaz*), in three crops. This bacterium has demonstrated the ability to fix atmospheric nitrogen ($N_2$), produce plant growth regulating hormones (PGRHs), solubilize otherwise inaccessible soil minerals, and bolster plant defenses and tolerances to external stressors. Using hydroponic techniques that alleviate arable land and seasonal pressures, the interaction of the bacterium and three cultivars of lettuce was determined with a focus on yield and bacterial N fixation. Hydroponic systems are growing in popularity and usage but are fully reliant on exogenous fertilization, often sourced synthetically. *G. diaz* inoculation increased leaf biomass yield in all three cultivars selected but plant root response varied by cultivar, and there was no solid evidence of N fixation. Leaf harvest increase with an alteration in root production due to *G. diaz* will allow producers to select varieties that will respond to fit their particular production systems.

Main season crops receive the lion's share of nutrient additions, with short-term plantings sown and harvested pre- or post- the main crop often relying on residual nutrients for growth. Maximizing yield of these 'shoulder crops' increases production on a static agricultural footprint. *G. diaz* did not have consistent benefits for the growth of three spring type radishes sown in typical off-season conditions. Some evidence emerged that one or more chemical adjuvants used to ensure bacteria-seed adhesion may impart growth changes, but inoculation itself had no or negative effects.

Lastly, *G. diaz's* impact on hot pepper production revealed that although differences were readily exhibited in the numbers of peppers produced, those peppers were concomitantly smaller and thus total biomass yield was unaffected. The quality of peppers produced was also negatively impacted at typical fertilization levels, questioning the benefit of inoculation in hot pepper production. Although hot peppers have nutritional value comparable to bell peppers, their primary reason for cultivation is their piquancy, imparted from production of capsaicinoids in the fruiting bodies and impacted by N stress and plant PGRH levels, so the potential for *G. diaz* in hot pepper may be in other metrics than yield. More research is needed into this aspect of hot pepper production. Thereafter, a short concluding chapter rounds out the dissertation, describing what was learned, thoughts on strengths and weaknesses of the experiments, and avenues for future research where warranted.

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# Chapter 1

## Introduction to the Research

### 1.1 Background and Rationale

Sustainable intensification of food production is rapidly approaching the threshold where action *needs* to be taken rather than *should be*, as current agricultural practices come under constricting pressures from multiple avenues, all while trying to feed increasing populations. Estimates are that food production will need to increase by 60 to 100% (Da Silva, 2012; United Nations, 2009) by 2050 to feed the estimated 10 billion people in the world at that time. All the while, crop responses to synthetic fertilizers are plateauing and those same fertilizers are increasingly under scrutiny for consuming considerable energy resources and leading to pollution and water impairment (FAO 2010; Good and Beatty, 2011). Soil erosion and arable cropland degradation threatens to reduce the productivity of the land we have today, and human habitat encroachment routinely claims farmland for non-food production usage, leaving the remaining land the burden of providing more food with less footprint (FAO 2003). Add to this the burdens of affluence, where farm products may be siphoned off for non-food uses such as ethanol or fiber production, and the increasing demand of consumers for varieties of food from low producing cultivars, and the need for revolutionary modes of thought and action in agricultural systems becomes readily apparent.

In this dissertation, I present my interest in using a novel plant endophyte to address some of the concerns facing agriculture today. My motivation for this research is utilizing extant plant-microbe synergisms on previously unassociated species, all for the benefit of human nutrition.

Current agricultural practices play a rates and ratios game where the trade-offs and opportunity costs increasingly come at environmental expense, and I seek to investigate a technology that can change the math while increasing stewardship. Whether by boosting the numerator through increased yields, calorie density, or crop quality, or by decreasing the denominator by reducing footprint and/or exogenous fertilization needs, bacterial mutualism may provide one pathway towards meeting agricultural demands for future generations. To that end the impact of the bacterial endophyte *Gluconacetobacter diazotrophicus* was investigated on three agronomically important crops.

The use of biological synergists in agriculture is not new technology; man was employing them long before he understood the mechanisms behind their mutualisms. The three-sisters cropping systems of the indigenous peoples of the Americas has long been lauded for the macroscale shading and trellising effects of the crops, but a greater contributor may have been the bacterial nitrogen (N) fixation of the legumes providing critical N for all three plants (Mt. Pleasant, 2006). That intentional inoculation of plants could provide them with a N nutritive advantage was only discovered in the 1880s (Hellriegel and Wilfarth, 1888), and the understanding of the mechanisms behind bacterial N fixation weren't elucidated until the early twentieth century (Beijerinck, 1901). Early attempts at inoculation of different plant species revealed that these microbes (now known to be *Rhizobia* and *Bradyrhizobia*) only colonized leguminous crops. Research may have continued but World Wars I and II and the Great Depression stalled advances, and from the 1950s onward the Haber-Bosch process proved a source of relatively cheap, reliable N, partly fueling the first 'Green Revolution' but shelving other research. It wasn't until the 1970s when the implicit trade-off costs of such grand-scale synthetic N synthesis and usage were realized that serious interest began again in biologicals,

and very often it focused solely on N fixers (Hardy and Helvelka, 1975; Pingali, 2012). Since then, the breadth of microbial enhanced agriculture has expanded considerably, with multiple genera of bacteria (*Azotobacter, Azospirillum, Bacillus, Pseudomonas, Flavobacterium, Pediococcus, Lactobacillus*, *Rhizobia*, *Frankia*, etc.) and fungi (*Glomus, Rhizopogon, Scleroderma*, etc.) utilized for disease resistance, drought tolerance, N fixation, mineral acquisition, plant growth regulating hormone (PGRH) production, secondary metabolite formation, and more.

In selecting an inoculant for this doctoral research, ten general microbial traits emerged as potentially important criteria to best meet the demands set forth in the individual hypotheses and experimental designs. They were:

1. Readily available, culturable, and isolable. Niche microbes that could not be maintained locally or readily sourced when needed would make conducting investigative pre-trials and replications more difficult, not to mention hindering usage in cropping systems.
2. Host generalist. Although cereals and grains provide the lion's share of world calories, bolstering productivity among myriad crops would allow many different agricultural sectors to benefit from this technology.
3. Production of PGRHs. Modulation of crop response and growth patterns can afford induced resistance, increase biomass accumulation or nutrient density, decrease non-harvestable material, increase seedling vigor, etc., some of which were addressed in these studies.
4. N fixation. N is commonly a limiting factor to production.

5. Inhibition resistant N fixation proteins. Microbial N fixation is often strongly inhibited by N presence or oxygen. A bacterium that circumvents this inhibition via genetic, chemical, or physical means would allow greater N capture potential.
6. Ease of plant incorporation. Multi-channel introduction (seed coating, in-furrow application, foliar sprays) allows for implementation in different cropping systems.
7. Solubilization of immobilized soil nutrients. Many microbes exhibit phosphate, zinc (Zn), or iron (Fe) solubilization allowing for increased plant uptake. Phosphorus (P) especially suffers from poor extractability and is increasingly subject to unsustainably sourced, exogenous application.
8. Amenable to multiple cropping systems. Field, soilless, and hydroponic production systems all contribute to food security.
9. Non-pathogenic. The microbe should not negatively impact plant growth, either by life stage alteration or under adverse environment condition changes.
10. Transient in the environment. Introduction of foreign species, no matter how beneficial the intent, has the possibility of upsetting extant microbiomes. The microbe ideally would not readily persist once the host crop was terminated.

Similar to other microbial candidates like the oft-studied *Azotobacter*, *G. diaz* is able to fix atmospheric N, forms non-pathogenic associations with host plants, and can solubilize soil bound Zn, Fe, and P (Aasfar et al., 2021; Jnawali et al., 2015). However, common *Azotobacter* strains do not thrive under acidic soil conditions commonly encountered in vegetable cultivation, aren't resistant to nitrogenase inhibition by N fertilization, and don't specifically provide N to intended plants because they are free-living soil bacteria (Aasfar et al., 2021; Banik et al., 2019; Jnawali et al., 2015). This last trait also means that *Azotobacter* has no protections against

becoming an introduced invasive species (Jnawali et al., 2015), unlike *G. diaz,* which is an obligate endophyte and cannot survive outside of a host plant for more than 48 hours (Jimenez-Salgado et al., 1997).

*Rhizobia* are also commonly researched bacteria that meet some of these criteria and has established trust in the agricultural community, but to date the range of agricultural host plants available for inoculation are limited to mostly leguminous crops. A similar host-interactive limitation is exhibited by *Frankia*, which while capable of colonizing dicots is restricted to actinorhizal plants of limited food system use (Santi et al., 2013). *G. diaz* contrasts these by being very host nonspecific, capable of innocuously infecting myriad monocot and dicot species. As an added aside, *G. diaz* does not require plant-microbe interaction mediated nodule formation to house the endophyte, instead living both inter- and intracellularly within plant tissues (Eskin et al., 2014; Cavalcante and Dobereiner, 1988) unlike *Frankia* and *Rhizobia* (Santi et al., 2013).

Among the bacteria that share *G. diaz's* trait of not needing specialized tissues for incorporation are several species of *Azospirillum* and *Flavobacterium.* Both have been shown to produce PGRHs that modulate plant growth characteristics; solubilize inorganic soil P, increasing its availability to the plant; and prime a host plant's induced systemic tolerance to abiotic and resistance to biotic stressors (Fukami et al., 2018; Menon et al., 2020; Soltani et al., 2010; Steenhoudt and Vanderleyden, 2000; Wisniewski-Dyé et al., 2013). However, *Azospirillum* (like *Azotobacter*) exists as a free-living bacterium, its N fixation activity is highly susceptible to feedback inhibition from environmental N (Fukami et al., 2018; Steenhoudt and Vanderleyden, 2000), and it does not share *G. diaz*'s ability to also solubilize Zn and Fe (Crespo et al., 2011; Sarathambal et al., 2010). N fixation in *Flavobacterium* is also to date poorly

understood despite some similarities to other *Rhizobial* family members, and samples were only isolated from gramineous plants (Giri and Pati, 2004; Kämpfer et al., 2015).

*Bacillus* and *Pseudomonas* emerged as potential candidates when assessing the ability of a microbe to solubilize Zn and Fe, and these two genera have long been established as effective phosphate solubilizers (Rashid et al., 2004; Ding et al., 2005; Yan et al., 2008). Additionally, they are able to fix N, tolerate acidic soils, produce PGRHs, and induce plant defenses. However, *Bacillus* often vacillates between parasitic and mutualistic heterotrophy even in supposedly beneficial species, while the mechanism driving switching is not well understood. Also, isolation of particular strains is very difficult and routinely fails due to the ubiquitous presence of *Bacillus* and relative ease of horizontal gene transfer in this species (Ding et al., 2005, Singh et al., 2020). *Pseudomonas* only partially evades or inhibits plant defense responses, even while inducing plant defenses against other pathogens (Preston 2003), and *nif* containing strains have the added detriment of also containing active denitrification genes (Yan et al., 2008), which would exacerbate N deficiency *in planta. Pediococcus* and *Lactobacillus* have shown some promise in plant growth promotion (Cai et al., 1999, Kang et al., 2014), specifically with the latter in *Cucurbita* (Radhakrishnan and Khan 2014), but are more often used as inoculants for controlled decomposition of plant material, not active phytostimulants in live tissue (Cai et. al 1999), and neither demonstrate N fixation. Lastly, although the fungal candidates listed have plant beneficial attributes meeting some of the inclusion criteria such as soilless cropping, mineral solubilization, and PGRH production (Hawkins and George 1997; Dasgan et al., 2008), they also fail to fix atmospheric N without commensal bacterial interaction, necessitating co-inoculation at a minimum (Lopes et al., 2019; Paula et al., 1991). Unlike all of the preceding candidates, *G. diaz* exhibited all of these beneficial factors and led to its selection. Although only recently

discovered (Cavalcante and Dobereiner 1988), a significant amount of preliminary research has been conducted into these unique properties and efficacies, which are further detailed across the individual chapters. This list of potential plant inoculants is not all inclusive, but allowed for rapid determination that *G. diaz* was the microbial candidate of choice for the applied approach of the experiments contained herein.

The choice of crops tested with *G. diaz* was guided by existing literature and a qualitative screening trial conducted in the fall of 2017 on 79 potential floral and vegetable candidates ranging from Arugula to Zinnia. Criteria included ease of seed soaking, no evident suppression of germination, and visual health of seedlings. Lettuce (*Lactuca sativa)* demonstrated no suppression and/or positive responses in the cultivars screened (Bibb, Nevada, Iceberg, Black Seeded Simpson, Grand Rapids Tipburn Resistant, Red Lollo), so three with similar growth habits and maturation dates were chosen for a hydroponic N fixation study. Similarly, of the seven radish cultivars screened (Cherry Belle, White Icicle, Sora, Rover, French Breakfast, Bacchus and Pearl), five demonstrated nascent responses to inoculation and three were ultimately chosen based on days to maturity. These would be used for the shoulders experiment, as radishes can easily be employed as a value-added off-season crop. For these two experiments, the short life cycle of the crop meant multiple types could be tested for variation in inoculation response, as had been shown to occur in corn (Bidarkar and Murumkar 2020; Tian et al., 2009), sorghum (Yoon et al., 2016), tomato (El-Shouny et al., 2020), and root vegetable (Rocafull et al., 2016). Lastly, jalapeño pepper (*C. annuum* var. 'Jalapeño Early') was selected for a large fruit study as it demonstrated both positive inoculation tolerance and is prone to produce 'corked' fruit, a phenotype that is characterized as damaged by the USDA and may be a response to growth hormones in the maturing fruit. Inclusion of a self-damaging cultivar highlighted the

need to assess all metrics of increased crop production using *G. diaz* and not just focusing on typical numerator increases, as tradeoffs may not be apparent with only partial assessment.

## 1.2 Funding for the Research

This research was conducted through a partnership between the USDA-ARS Pasture Systems and Watershed Management Research Unit and the Pennsylvania State University, who funded the majority of graduate research assistantship and tuition during my PhD tenure January 2018 – August 2021. Additional funding was sourced from the Northeast Sustainable Agriculture Research and Extension program through an awarded grant, and from the Jeanne and Charles Rider Endowment for Support of Research on the Biotechnology of Food Crops. *G. diaz* stocks were provided gratis from Azotic Technologies, LLC (Nottingham, U.K.) but beyond providing the bacterium they had no input on the studies herein. Lowes Home Improvement also donated materials and seeds to the research.

## 1.3 Organization of the Dissertation

This dissertation contains six chapters: a general introduction (Chapter 1), an in-depth literature review of *G. diaz* (Chapter 2), three manuscripts detailing unique experiments (Chapters 3-5), and a conclusion (Chapter 6). The introduction provides the groundwork for the what and why of the research and the conclusion briefly details what was learned, what could have been done differently, and the directions this research could possibly head.

In the first manuscript (Chapter 3), the use of *G. diaz* is investigated in hydroponic lettuce, a high-value commodity that is already subject to intensification and cultivation de-centralization in the United States. Controlled environment hydroponic systems have been touted

as one potential means towards "improving food supply, health conditions, local economy, social integration, and environmental sustainability altogether" in a rapidly urbanizing world (Orsini et al., 2013), and as these systems are employable irrespective of latitude, season, or soil access, they are gaining popularity as a means for production of local, perishable, year-round crops (Gomez et al., 2019). Many crops are already greenhouse cultivated at distance from centers of consumption, but the recent pandemic highlights how transportation interruptions can couple with shipping costs to hamper cost-effectiveness of these systems. The method employed for this study is a non-circulating, electricity free Kratky jar set-up (Kratky 2004), which will allow for implementation of findings from technology poor, subsistence, off-grid, or economically challenged systems all the way up to advanced and integrated, near-industrial controlled environmental systems. As the bacteria itself can readily be cultured on any of dozens of simple sugar substrates (Eskin et al., 2014), it is not unreasonable to assume that realizable benefits of inoculation could be made available across different cropping systems. The jars additionally excluded confounding covariates of P, Zn, and Fe solubilization, limited cross microbial interaction, and permitted easier root access and measurement. This design thus allowed better inference of bacterial effect from N fixation and PGRH production.

The second manuscript (Chapter 4) investigated the use of *G. diaz* as a means to bolster total land productivity in seasonal shoulders; the times pre- and post- a main crop harvest. In the rates and ratios model of agriculture this expands the per footprint production gains during times when exogenous fertilization is often ill-advised. Spring applications of fertilizer are more prone to leaching and run-off as water input often exceeds plant uptake (EPA.gov 2017, Sawyer 2021). Soil microbial processes that prevent loss by conversion of N into soil stable forms, or that liberate N from protected formulations, are also inhibited by colder temperatures in the spring

(Sawyer 2021). Crops sown at these times also have shallower root systems limiting the effective N capture depth. In the fall, residual soil N that was not captured by the main crop exacerbates water degradation concerns if additional N is added to a short-term crop. Soil microbial activity is typically high but the degradation of main crop residues shifts the C/N balance towards increased microbial uptake and N immobilization, potentially robbing planted secondary crops of available N (USDA NRCS 2011). A common shoulder crop is radish, and utilizing *G. diaz* as a bacterial endophyte in stands could ameliorate these N concerns by sequestering any fixed N *in planta* where losses or competition are negligible. Although a niche crop in the United States, radish is a staple in the eastern hemisphere, accounting for 2% of world agricultural production (Schippers 2004).

In the third manuscript (Chapter 5) an oft overlooked aspect of new technologies in agriculture is investigated: the impact on quality of the crop produced. The annals of agricultural history are rich with deleterious trade-offs that only became apparent long after damage had been done, such as soil erosion from tillage, water eutrophication from fertilization, or insect control resistance from pesticide use. In investigating the impact of *G. diaz* on a cultivar of jalapeño pepper known for routinely producing mildly damaged fruit, trade-offs between quality and quantity are investigated. Boosting the numerator can actually decrease net food production when the crop is deemed substandard and may not be entered into the food system at all. To a world population that may teeter on the brink of global food insecurity in the next 30 years (Da Silva, 2012; FAO, 2019) this may seem a trivial concern, but before any new technology can be employed at scale the subjectivity of quality assessment and fate of crops must be considered.

# Chapter 2

# Review of Current Literature

## 2.1 General Characteristics of *Gluconacetobacter diazotrophicus*

*G. diaz* is a plant-associated bacterium first isolated and characterized from sugarcane in 1988 (Cavalcante and Dobereiner, 1988) living in non-pathogenic mutualism either rhizospherically or endophytically within plant tissues. *G. diaz* is phylogenetically indexed within the phylum Proteobacteria, class Alphaproteobacteria, order Rhodospirillales, family Acetobacteraceae, and genus *Gluconacetobacter* (Kersters et al., 2006). A gram-negative, non-spore forming obligate endophyte, it propagates primarily through cuttings (Madhaiyan et al., 2004), insect-mediated transfer (Muthukumarasamy et al., 2002), and transiently in *Glomus* associations between closely spaced root tissues (Lopes et al., 2019; Paula et al., 1991). Agricultural techniques have demonstrated that while it cannot exist as a free-living endophyte, it will survive in nutrient broth allowing laboratory isolation, culture, and storage (Cavalcante and Dobereiner, 1988; Eskin et al., 2014; Saravanan et al., 2008). Since its discovery it has been sourced from five continents and 13 variant crop families as seen in Table 2.1. The bacterium possesses 1-3 flagella allowing for self-effected motility (Cavalcante and Dobereiner, 1988) and proliferation throughout plant tissues, including roots (Kumar et al., 2015; Rangel de Souza et al., 2015), stems and leaves (Cocking et al., 2006; Luna et al., 2010; Paredes-Villanueva et al., 2020).

Table 2.1 Native Host Plants for *G. diaz* , by Family and Location

| Family / Crop | Location | Ref. |
|---|---|---|
| Amaranthaceæ | | |
| Beet | India | Madhaiyan et al., 2004 |
| Anacardiaceae | | |
| Mango | Cuba | Rocafull et al., 2016 |
| Apiaceæ | | |
| Carrot | India | Madhaiyan et al., 2004 |
| Asparagaceae | | |
| Yucca | Cuba | Rocafull et al., 2016 |
| Brassicaceæ | | |
| Radish | India | Madhaiyan et al., 2004 |
| Bromeliaceæ | | |
| Pineapple | Mexico | Tapia-Hernández et al., 2000 |
| Convulvulaceæ | | |
| Sweet Potato (+VAM)* | Brazil | Paula et al., 1991 |
| Sweet Potato | Brazil | Dobereiner et al., 1993 |
| Musaceæ | | |
| Banana | Kenya | Matiru and Thompson 1998 |
| Myrtaceae | | |
| Guava | Cuba | Rocafull et al., 2016 |
| Poaceæ | | |
| Sugarcane | Brazil | Cavalcante and Dobereiner 1988 |
| " | Uruguay | Dobereiner et al., 1993 |
| " | Cuba | Dobereiner et al., 1993 |
| " | Mexico | Dobereiner et al., 1993 |
| " | Australia | Li and Macrae 1991 |
| " | S. Africa | Dobereiner et al., 1993 |
| " | Colombia | Restrepo et al., 2017 |
| " | Phillipines | Asis et al., 2000a |
| " | Japan | Asis et al., 2000b |
| " | Egypt | Youssef et al., 2004 |
| Cameroon Grass | Brazil | Dobereiner et al., 1993 |
| Finger Millet | India | Loganathan et al., 2001 |
| Rice | India | Muthukumarasamy et al., 2005 |
| Rice | Rep. Korea | Muthukumarasamy et al., 2007 |
| Rubiaceæ | | |
| Coffee | Mexico | Jimenez-Salgado et al., 1997 |
| Coffee | India | Madhaiyan et al., 2004 |
| Coffee | Kenya | Matiru and Thompson 1998 |
| Solanaceæ | | |
| Tomato | Colombia | Restrepo et al., 2017 |
| Theaceæ | | |
| Tea | Kenya | Matiru and Thompson 1998 |

*Vesicular-arbuscular mycorrhiza, Initially only found in three way synergism

As a relatively host non-specific endophyte, *G. diaz* has been successfully inoculated into myriad important agronomic and horticultural crops, including maize (Bidarkar and Murumkar, 2020, Riggs et al., 2001; Tufail et al., 2021), wheat (Kumar et al., 2015b; Luna et al., 2010; Youssef et al., 2004), potato (Pelligrini et al., 2020), cassava (Lopes et al., 2019), strawberry (Delaporte-Quintana et al., 2020), tomato (El-Shouny et al., 2020; Luna et al., 2012; Pelligrini et al., 2020), snap beans (de Oliveira et al., 2019), rice (Filgueiras et al., 2020; Silva et al., 2020) and forage grasses (Sebring 2017, Unpublished data; Dobereiner et al., 1993). Effects of inoculation are often co-factored with abiotic and biotic stressors such as salt or water stress (Filgueiras et al., 2020; Leandro et al., 2021; Tufail et al., 2021), nutrient deficiency (Delaporte-Quintana et al., 2020; de Oliveira et al., 2019) or phytopathogen presence (Oliveira et al., 2018; Rodriguez et al., 2019), but generally range from neutral (no evidence of impact from inoculation) to beneficial. Often plant responses to inoculation will vary by plant hybrid or with additional stress co-factors applied as mentioned in section 1.5, making specific response predictions difficult.

## 2.2 Nitrogen Fixation

Global agricultural N demand is expected to approach 125 million metric tons in 2021 with supplies nearing 180 million metric tons (FAO, 2017, forward projections). Although the N production balance will remain positive throughout 2021, considerable disparities in access to N fertilizers exist due to economic, geopolitical, and fossil fuel limitations. Macroscale farming operations in developed countries with working capital are likely to not be as impacted, but marginal farmers in emerging or subsistence systems cannot afford to fertilize at recommended

rates and may suffer reduced yields. (FAO, 2017; Williamson, 2011). *G. diaz* has demonstrated considerable N-fixation in some crops that can be used to offset these deficiencies.

In sugarcane, *G. diaz* has been shown to fix up to 200 kg N/ha/year, between half (Cavalcante and Dobereiner, 1988) and 80% (Boddey et al., 2003) of total crop need. The bacterium does so even in the presence of up to 30% saccharide concentration, its osmotolerance protecting itself from desiccation and damage via the formation of extracellular, microenvironmental biofilms (Eskin et al., 2014; Fisher and Newton, 2005; Muthukumarasamy et al., 2002). These biofilms also allow *G. diaz* to effectively contain and cleave (via excreted levansucrases, Hernandez et al., 1995) plant produced disaccharides such as sucrose which it cannot otherwise metabolize (Martínez-Fleites et al., 2005; Velázquez-Hernández et al., 2011). By sequestering oxidative processes such as ATP generation in extracellular biofilms and/or the periplasm, *G. diaz* affords itself a level of oxygen tolerance as N-fixing Fe and FeMo based enzymes are physically separated (Dong et al., 2002; Muthukumarasamy et al., 2002) away from $O_2$ inhibition that plagues other diazotrophs (Boddey et al., 2003; Mitchell and Silhavy, 2019). Additionally, *G. diaz* has been shown to colonize both apoplastic vascular tissues allowing for direct access to phloem or xylem transported plant constituents (Paredes-Villanueva et al., 2020), as well as intracellularly near photosynthetic tissues where synthates can be directly intercepted and catabolized (Cocking et al., 2006) for cleavage of high energy dinitrogen triple bonds.

Unique to date among diazotrophs, *G. diaz* does not contain a nitrate reductase enzyme, which typically serves as an inhibitory feedback component against N fixation. (Stephan et al., 1991; Soares et al., 2015; Wang et al., 2018). In practice, low to moderate levels of nitrate do not inhibit *G. diaz* N fixation, allowing for continuation of *in planta* fixed N production, without the risk of nitrite accumulation and subsequent nitric oxide formation causing plant damage (Wang

et al., 2018). This has important agronomic considerations, as it allows partial supplementation of crop nitrate N requirements to meet what *G. diaz* alone cannot provide (Fisher and Newton, 2005; Fuentes-Ramirez et al., 1999). Synergism between endogenous N fixation and applied nitrate-N can allow farmers to reduce applications and maintain yield, or co-apply *G. diaz* and typical N levels to increase yield. In sugarcane, Murumkar et al. (2016) found that *G. diaz* + 75% recommended N surpassed 100% mineral N applications for yield. Similar results have been demonstrated in beet (Kumar et al., 2015a), tomato (Fernández-Delgado et al., 2019), snap bean (de Oliveira et al., 2019), cassava (Lopes et al., 2019), wheat (Kumar et al., 2015b) and corn (Bidarkar and Murumkar, 2020; Murumkar et al., 2016; Tufail et al., 2021). However, bacterial N fixation is inhibited by environmental ammoniacal N, so synergisms when sought should avoid this N source (Medeiros et al., 2006; Rodríguez-Andrade et al., 2015). The experiments detailed in this dissertation that include a N rate component utilize calcium ammonium nitrate hydrated double salt, $5(Ca[NO_3]_2)NH_4NO_3 \cdot 10H_2O$, comprised of only 8.33% $NH_4$, so suppression should be minimal.

### 2.2.1 Isolation and Identification of *G. diaz* in vitro

The ability of *G. diaz* to fix atmospheric N, coupled with its saccharide osmotolerance and oxygen tolerance, provides a phenotypic method for isolation and identification of the bacterium from plant tissues. Plating of dilute extracts onto N free, saccharide-enriched media in oxygen presence yields yellowish-orange colonies with zones of acidification from build-up of gluconate (Cavalcante and Dobereiner, 1988; de Oliveira et al., 2016). Further verification can be obtained through genetic analysis as detailed by Tian et al. (2009).

### 2.2.2 Detection of *G. diaz* for this research

*G. diaz* sampling from plant tissues was not carried out during the duration of the experiments contained in this dissertation. Instead, inoculum and seed surfaces post bacterial application were sampled. Recent publications have divided localization and enumeration of bacterial colonies from investigation of long-term effects of inoculation, based on previous findings. Kumar et al., (2015a) noted that during the first 7 days post germination *G. diaz* was isolable from all tissues of wheat seedlings, but receded sporadically from aerial tissues until about day 21-28, with high variability of detection both between plants and within similar tissue types of the same plant. Net effect on plant growth did not corroborate to detectability but plants were not grown to maturity. Cocking et al., (2006) noted that *G. diaz* colonizes plant tissues very heterogeneously, a result mirrored in recent findings (Restrepo et. al, 2017, Vargas et al., 2014), leading to potential false negative tests for inoculation when non-destructive sampling techniques that necessitate removing only miniscule plant sections are employed. Multiple authors have established that activity and/or detectability of *G. diaz* from plant tissues varies with fertilization level. (Dobereiner et al., 1993; Fuentes-Ramirez et al., 1999; Muthukumarasamy et al., 2006; Paredes-Villanueva et al., 2020; Rodriguez-Andrade et al., 2015), so lack of detection at high N levels would not be unexpected. A net long-term effect of initial colonization with subsequent loss of detectability has been shown (Sebring et al., 2017) where despite lack of bacterial detection after 24 months, net beneficial impacts of inoculation existed. The experiments herein were conducted to full harvest, in line with established research that investigated effects of inoculation and not necessarily bacterial localization or retention, so although samples were stored for subsequent analysis, COVID interruptions precluded their assessment and inclusion here.

**2.3 Phosphorous Solubilization**

After N, phosphorus (P) is often a limiting factor in agricultural systems due to immobilization, adsorption to clay, iron, or aluminum, and/or precipitation to phosphates inaccessible to plant tissues. Conversion to accessible forms can occur through microbial communities including arbuscular mycorrhizae, mineral weathering, and/or pH modulation, but often at timescales too slow for a rapidly growing crop, necessitating exogenous applications even when soil P is high (Prasad and Chakraborty, 2019). *G. diaz* can mitigate some P fertilization requirements as it can readily mineralize soil P through the excretion of gluconic acid into the rhizosphere (Crespo et al., 2011; Delaporte-Quintana et al., 2017; Nieto-Penalver e. al., 2014). Unlike soil liming which seeks to correct field-wide imbalances and can lead to calcium phosphate precipitation, the effects of *G. diaz* are limited to the immediate root zone, protecting non-proximate P from inadvertent solubilization and losses to leaching or run-off (Crespo et al., 2011; Prasad and Chakraborty, 2019). Increases in plant available P from *G. diaz* effected mineralization have been demonstrated both in sugarcane (Murumkar et al., 2016; Natheer and Muthukkaruppan, 2012) and strawberry (Delaporte-Quintana et al., 2017). For all three experiments detailed in this dissertation, P, if provided, is in a plant available form eliminating confounding interactions. However, knowledge of potential solubilization is important for other applications.

**2.4 Zinc and Iron Solubilization**

In a global study for the FAO, Sillanpää (1990) noted that roughly 49% of the world's agricultural soils are wholly or partially deficient in plant available Zn, making this the most deficient plant required micronutrient. Fe, although much more environmentally prevalent, is not always present in plant available forms due to being oxidized in the ferric form (hematite being very common), imbalances with other cations, or interactions with soil phosphate (Prasad and Chakraborty 2019). *G. diaz* has the ability to solubilize Zn in the rhizosphere through the same gluconic acid excretory mechanism implicated in P solubilization (Nieto-Penalver et al., 2014), liberating elemental Zn from oxides (Paredes-Villanueva et al., 2020), carbonates, (Sarathamabl et al., 2010) or sulfates (Natheer and Muthukkaruppan, 2012). *G. diaz* liberation of Fe is via production and excretion of mineral chelating siderophores into the rhizospshere. As *G. diaz* utilizes hydroxamate sidephores that are water-soluble and relatively stable (Logeshwaran et al., 2009), transportation into plant tissues and subsequent reduction to the more bioactive ferrous form is possible. In one study involving strawberry inoculated with *G. diaz*, control plants provided with readily available, EDTA-bound ferrous Fe had the same *in planta* accumulation of Fe as plants inoculated with *G. diaz* but only supplied with oxidized ferric Fe, demonstrating high efficacy of bacterial siderophore production (Delaporte-Quintana et al., 2020). The bacterium may have evolved this mechanism of Fe uptake to secure sufficient availability for its Fe-Molybdenum based nitrogenase enzymes, allowing hydrophilic channel transport through cell membranes to the cytoplasm, as mutants lacking these siderophores exhibit significantly diminished N fixation. (Soares et al., 2015; de Oliveira et al., 2016). For all three experiments detailed in this dissertation, Fe was either not provided (radish) or was provided as a bioavailable chelate (lettuce, pepper).

## 2.5 Plant Growth Regulating Hormones (PGRHs)

Besides N fixation, *G. diaz* has the potential to alter plant growth via the production of plant growth hormones, Indole-3-Acetic Acid (IAA), an auxin, and Gibberellins A1 and A3. Plant response to endogenous IAA production is relatively well understood, with mechanisms for apical dominance, photo, and gravitropism attributed to absolute or gradated, localized concentrations in plant tissues (Friml, 2003; Zhao, 2010), among others. What is less well understood is effects of exogenous IAA produced from endophytic bacteria co-located, proximal, or even distal to normal plant production areas, especially considering the genetic expression cascade induced by this family of hormones (Maheshwari et al., 2015). In rice, Rodriguez et al. (2016) demonstrated that *G. diaz* excreted IAA increased root length, surface area, forking and number of tips, a finding corroborated by Silva et al. (2020) in drought stressed rice. Royer and Sebring (2017, unpublished data) showed that *G. diaz* interrupts apical dominance in cosmos and sunflower, leading to side branching with secondary flower development. Significant *in planta* IAA production has been also documented in tomato stem (Restrepo et al., 2017), an atypical location for IAA concentration, but growth metrics of plants specific to IAA were not assessed.

## 2.6 Induced Plant Resistances

Although they were not a factor in any of the three experiments presented in this dissertation (due to careful experimental design), mention should also be made of the ability of *G. diaz* to modify plant responses to biotic (induced plant defense) and abiotic (induced plant

tolerance) stressors. A common abiotic stressor in this era of climate uncertainly is drought and/or impaired water quality, and *G. diaz* inoculation has been shown to significantly improve drought tolerance in rice (Filgueiras et al., 2020, Silva et al., 2020), corn (Tufail et al., 2021), and sugarcane (Vargas et al., 2014).

*G. diaz* evades plant defense responses for successful colonization by strategies including secretion of hydrolytic enzymes to penetrate cell walls (Adriano-Anaya et al., 2005) and an active quorum sensing mechanism to prevent excessive proliferation (Bertini et al., 2014). However, once established inside plant tissues, *G. diaz* effects significant changes in plant defense hormone production through as yet unknown mechanisms, but putatively through auxin mediated abscisic and jasmonic acid production (Rangel de Souza et al., 2015). The net effect is resistance to subsequent infection by other microbes, imparting resistance to *Xanthamonas* (Arencibia et al., 2006; Blanco et al., 2005; Oliveira et al., 2018), *Ralstonia* (Rodriguez et al., 2019), *Fusarium* (Logeshwarn et al., 2011; Pelligrini et al., 2020), *Rhizoctonia* (Pelligrini et al., 2020), *Acidovorax* (Oliveira et al., 2018), and even *Meloidogyne*, a nematode (Chawla et al., 2013).

# Chapter 3

## Yield Promotion and Nitrogen Fixation in *Gluconacetobacter diazotrophicus* Inoculated Lettuces

### 3.0 Abstract

Nitrogen (N) fixation and growth-promoting effects of *Gluconacetobacter diazotrophicus* inoculation of leaf Lettuce (*Lactuca sativa* L.), vars. 'Black Seeded Simpson', 'Bibb', and 'Grand Rapids' were investigated. Plants were grown hydroponically in Kratky jars in growth chambers over a range of N fertilization levels (37.5-172.5 ppm N). Experiments were conducted twice. Aerial, root and total biomass production was measured, as well as N fixation via mass balance, mass fraction, and C(carbon)/N ratios. *G. diaz* in the first Black Seeded Simpson trial effected greater leaf and reduced root production but did not increase total plant N content. Bacterial treatments did not affect Black Seeded Simpson in the second trial. *G. diaz* in Bibb resulted in greater leaf and total biomass in both experiments and with greater root biomass in one repeat. Total N in inoculated Bibb lettuce was greater than in controls suggesting *G. diaz* fixed atmospheric N. Due to unknown reasons, total N recaptured from lettuce and Kratky jars was often more than what was originally supplied even in controls, making it impossible to use mass balance to confirm N-fixation. Results from the first Grand Rapids trial could not be used due to watering challenges due to COVID-19. *G. diaz* in the second Grand Rapids trial, however, caused an increase in root, shoot, and total biomass, and no effect on total plant N content. *G.diaz* did, however, cause diversion of N from roots to aerial tissues resulting in a C/N increase in root tissues. That *G.diaz* inoculation of lettuce increased shoot and root production but the

differences in responses varied between lettuce cultivars and under similar environmental conditions needs to be resolved for practical application.

## 3.1 Introduction

Lettuce is one of the most important vegetable crops in the United States, ranking third in total acreage (106,027 hectares) and first in economic value at USD 3.493 billion in 2019 (USDA-NASS, 2020). Over 97% of domestic production is concentrated in California and Arizona where environmental conditions allow multiple crops per season and year-round production (Agricultural Marketing Resource Center, 2018; USDA-NASS, 2020). Loss of arable farmland (Thompson 2009), and high N and irrigation water requirements threaten lettuce production in the U.S. Water demands place a burden on crop production, while chronic drought and increased irrigation salinity hinder yields (Smith et. al., 2011; AMRC 2018). High N fertilizer applications exacerbate these concerns as leaching and run-off impair water quality while increasing costs. Unfortunately, environmental conditions in agricultural areas elsewhere in the U.S. are often unsuitable for year-round production.

Hydroponic cultivation, a form of controlled environmental agriculture, has arisen as a means to localize and intensify lettuce production in areas otherwise unsuitable for continual, field-based sowing. In hydroponic production, light, temperature and nutrient fertilization are all monitored and adjusted to maximize crop output. Turnaround time from planting to harvest can be reduced by half in hydroponic systems and yields can increase 11-fold per unit area, increasing yields per annum and helping offset the costs of hydroponic production (Barbosa et. al., 2015; Cometti et. al. 2008). Additionally, hydroponic systems can be located in population centers, reducing transportation costs, loss of nutrition, and contact contamination risks. Further,

water salinity can be remediated and drainage water recycled improving water use efficiency (WUE) and mitigating water quality degradation concerns.

Nonetheless, 100% of plant nutrients are required as applied fertilizer in hydroponic production. Synthetic N provided has high energy and environmental costs so if lettuce could fix atmospheric N the requirement of exogenous fertilizer N could be reduced or eliminated. *G. diaz.* has shown potential to fix atmospheric N in non-legumes (Boddey et. al., 2001; Cavalcante and Dobereiner, 1988; Stephen, et. al., 1991; Youssef et. al., 2004). In sugarcane, *G. diaz* can fix up to 200 kg N/ha year (Boddey et. al., 2001) meeting nearly half of the crops' N requirement. This bacterium is an obligate endophyte that is able to colonize species of numerous plant families (Dobereiner et.al., 1993; Madhaiyan et. al., 2004). *G. diaz* can not only fix atmospheric N but also produces Indole-3-Acetic acid (IAA) and Gibberellins A1 and A3, important plant growth regulating hormones (PGRHs) (Bastián et. al., 1998). In carrot and beetroot, PGRH production by *G. diaz* increased leaf and taproot biomass (Pelligrini et. al., 2021; Rios et. al., 2016). Crop production increases due to *G. diaz* inoculation have also been reported in tomato (El-Shouny et. al, 2020; Fernández-Delgado et. al, 2019), snap bean under low N conditions (de Oliveira et. al., 2019), cassava (Lopes et. al., 2019) and corn (Bidarkar and Murumkar, 2020; Riggs et. al., 2001). In lettuce, increased concentrations of IAA in aerial tissues reduce root formation (Vysotskaya et. al., 2017). Gibberellins at low concentrations increase lettuce leaf production, but at high concentrations actually impede growth (Miceli et. al., 2019). As no prior research with *G. diaz* inoculation in lettuce has been performed, the impacts of this bacterium on lettuce were unknown prior to this research.

Unlike most N-fixing bacteria, *G. diaz's* nitrogenase is not wholly suppressed by added N and can tolerate high nitrate levels as it lacks nitrate reductase genes (Fisher and Newton, 2005;

Stephan, et. al. 1991). At high N levels, lettuce often exhibits luxury consumption with an accumulation of nitrate that is categorized as an anti-nutrient (Di Gioia et. al., 2017), but previous studies on *Poa pratensis*, have demonstrated that *G. diaz* can increase plant production at high levels of N fertilization (Sebring et. al. 2017). Conversely, under low N conditions, bacterial N fixation may alleviate N-deficiency similar to *Rahnella* in aquaponic lettuce (Day et. al., 2021). One important aspect of incorporating *G. diaz* into cropping systems has been a reported high variability of inoculation effect between species and even plant cultivars (Riggs et. al. 2016; Rocafull et. al. 2016).

The objective of this study was to determine the effects of *G. diaz* inoculation on lettuce leaf and root growth and examine N fixation at different fertilization levels. The study was done with three different cultivars to determine if responses are variety-dependent.

### 3.2 Materials and Methods

This experiment was carried out at the USDA-ARS Pasture Systems & Watershed Management Research Unit in University Park, PA, under controlled environmental conditions. First, six cultivars (Bibb/Limestone, Nevada, Iceberg, Black Seeded Simpson, Grand Rapids Tipburn Resistant, Red Lollo) were qualitatively screened based on ease of on-seed inoculation, no evidence of germination suppression, and normal seedling growth. All visually demonstrated tolerance to *G. diaz* inoculation and were inoculable using a recommended protocol (Azotic Technologies internal operating protocol, 2016). For this experiment, three lettuce cultivars, Black Seeded Simpson (BSS), Bibb/Limestone (BIB), and Grand Rapids Tipburn Resistant (GRR) were chosen based on similar days to maturity, compact stature, and disease resistance, in order to facilitate efficient use of growth chamber time and space. The three-letter designator

assigned to each cultivar was followed by a numeric digit indicating the first or second trial, e.g. BIB2 is the second (repeat) trial of BIB.

### 3.2.1 Experimental Design and Randomization

The experiment was conducted as a two-factor fixed-effect completely randomized design (CRD) with three or four replications in order to maximize usage of space and resources (Table 3.1). The first factor was the presence/absence of *G. diaz* from the seed soaking solution. The second factor was N fertilization levels: deficient (37.5 - 105 ppm N), sufficient (127.5 – 150 ppm N) and excessive (172.5 ppm N). Total number of experimental/sampling units was limited by space constraints to 44 plants sown in individual jars, so initial experiments attempted to retain a balanced design with N = 42, 2 levels of bacteria, 7 levels of fertilization, and 3 replicates (2x7x3 = 42; BSS1, Table 3.1). Once earlier occurring experiments revealed 37.5ppm N was too deficient and variability was high between the other fertilization levels, 37.5ppm N was dropped and other N level replicates increased to maximize assessment potential. An unbalanced CRD with 44 units was thus selected (Table 3.1). GRR1 started immediately before a building shutdown, so N levels for that experiment were further curtailed to expedite successful initiation, with 172.5ppm N removed. Placement of jars was randomized via the RAND() function in Excel (Microsoft, 2013). Jars were re-randomized once per week.

Table 3.1: Fertlizer levels included and total experimental units, by experiment trial designations

| | Inoculation (INOC) Treatment Level |
|---|---|
| Inoculation Status | |
| *G. diaz* applied (INOC) | 50% of all experiment units, see below |
| No bacteria (NON) | 50% of all experiment units, see below |

| | Fertilizer (FERT) Treatment Level (ppm)* | | | | | | | Total *N* |
|---|---|---|---|---|---|---|---|---|
| Variety and Trial | **37.5** | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** | |
| Bibb/Limestone | | | | | | | | |
| Trial #1 (BIB1) | ** | 6/3 | 8/4 | 8/4 | 8/4 | 8/4 | 6/3 | *44* |
| Trial #2 (BIB2) | ** | 6/3 | 8/4 | 8/4 | 8/4 | 8/4 | 6/3 | *44* |
| Black Seeded Simpson | | | | | | | | |
| Trial #1 (BSS1) | 6/3 | 6/3 | 6/3 | 6/3 | 6/3 | 6/3 | 6/3 | *42* |
| Trial #2 (BSS2) | ** | 6/3 | 8/4 | 8/4 | 8/4 | 8/4 | 6/3 | *44* |
| Grand Rapids Tipburn Res. | | | | | | | | |
| Trial #1 (GRR1) | ** | 8/4(2) | 8/4(3) | 8/4(1) | 8/4(1) | 8/4(3) | ** | *40**** |
| Trial #2 (GRR2) | ** | 6/3 | 8/4 | 8/4 | 8/4 | 8/4 | 6/3 | *44* |

*NOTE*: X/Y designates X total jars getting that FERT, with Y total replicates per inoculation status
*X/Y(#) designates only # of the NON treatment survived. All INOC plants (4) survived*
* Half received inoculated seed, half control seed
** Level not used in this experiment or replication.
*** Several failed to grow, reducing net experimental units to 30

The general model is given by the equation:

Response = [Inoculation Status] + [Fertilization Level] + [Interaction] + Error

Responses to be measured were dry weight biomass, N mass by location *in planta*, N mass fraction, C/N ratios, and total N within each experimental unit.

### 3.2.2 Plant and Bacterial Material

The three cultivars of lettuce selected for this trial, BSS, BIB and GRR, exhibit different leaf growth patterns (savoyed looseleaf, bunched rosette, or frilled looseleaf, resp.) but similar overall non-heading structure and suitability for hydroponic production. Estimated days to maturity of all three varieties was 43-45 days after planting. *G. diaz* was provided by Azotic

Technologies, LLC (Nottingham, UK) and is an intracellular, endophytic strain of the bacterium developed for delivery through their commercialized Envita$^{TM}$ product (Azotic-na.com 2019). Pure strain stocks of bacterium were maintained in 25% glycerol at -80°C until inoculum preparation.

### 3.2.3 Inoculant Preparation

*G. diaz* was cultured from glycerol preserved stock in a modified MESMA/ATGUS medium [2.7 g $L^{-1}$ glucose; 4.8 g $L^{-1}$ dipotassium phosphate (K2HPO4); 0.65 g $L^{-1}$ monopotassium phosphate (KH2PO4); 1.8 g $L^{-1}$ mannitol; 4.4 g $L^{-1}$ 2-(N-morpholino)ethanesulfonic acid (MES hydrate); 2.7 g $L^{-1}$ yeast extract] (Cocking et. al. 2006). Nutrients were combined in deionized water and autoclaved prior to aseptic bacterial introduction to ensure pure, single-strain culture. Inoculated media and bacteria free controls were isolated and incubated in a positive-pressure, enclosed shaker table (30°C, 120 rpm) for five days. Colony forming units (CFU) per milliliter solution was determined by serial dilution and standard plate counts on semi-solid ATGUS media (same as above, adding 0.8% w/v agar) in triplicate, corroborated with OD600 based estimation (NanoDrop One, Thermo Scientific) against standard curves. For this experiment achieved CFU in concentrate was $4.0 \times 10^8$ $mL^{-1}$ for BSS1, BSS2, and BIB1, $1.8 \times 10^9$ $mL^{-1}$ for BIB2 and GRR2, and $2.8 \times 10^8$ $mL^{-1}$ for GRR1. Differences in CFU were attributed to random variation inherent in bacterial culturing and were all much higher than the recommended application dose of $1.0 \times 10^7$ $mL^{-1}$ (Azotic-na.com/envita-label 2019).

Liquid media for seed soak inoculation was prepared from a proprietary blend of nutritive and adhesive adjuvants (sucrose, gum arabic, and Tween 80) in distilled water, as described by

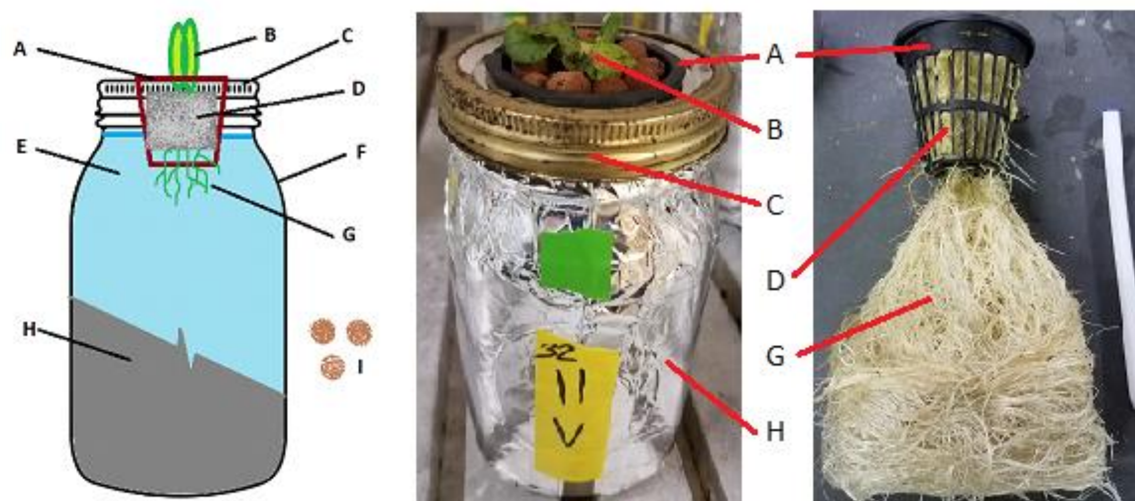
an operating protocol provided by Azotic Technologies (Internal document dated 2016) and reported on the safety data sheet (Azotic 2017, since deprecated). For each trial cluster, two equal 50ml portions of media were aseptically aliquoted into sterilized flasks. One portion received an equivalent amount of cultured bacterial concentrate and the second portion received an equivalent amount of deionized water. The portion containing bacterium, now at ½ cultured CFU/ml, became the inoculation treatment (INOC), while the adjuvant and water only solution became the un-inoculated control (NON). Both treatment stocks were assessed for equal pH and EC to ensure seeds soaked would experience identical conditions save for bacterial presence. Thus BSS1, BSS2, and BIB1 received 2.0 x $10^8$ CFU $mL^{-1}$ on seed, BIB2 and GRR2 received 9.0 x $10^8$ CFU $mL^{-1}$ on seed, and GRR1 received 1.4 x $10^8$ CFU/ $mL^{-1}$ on seed at inoculation.

### 3.2.4 Seed inoculation, planting, and growing conditions

For seed treatment application, 130 seeds each were distributed to two 60mm x 15mm sterile petri dishes, and 250µl of solution (INOC or NON) was added via pipette to each dish. Dishes were immediately sealed and gently agitated. After five minutes the solutions were pipetted out and a second 250µl portion of the same treatment applied as before to ensure saturation. After a second five-minute soak all residual solution was removed. Seeds were then stored for 10-30 minutes at 4°C and 65% relative humidity until planting. Three seeds were sown in 3.6 × 3.6 × 4 cm rockwool cubes (Grodan A036/40; Roxul Inc., Milton, ON, Canada) that were nested into 51mm circular net cups (Hummert Int'l) placed into foil-wrapped Kratky hydroponic jars (Figure 3.1; Kratky, 2004). By using Kratky jars PGRH residues from soil/media are excluded, plant nutrient and water supply is controlled, and all roots can be recovered. Jars were prefilled with 800ml of nutrient solution as outlined in Appendix A or Appendix B, with

the bottom 6mm of rockwool immersed to allow for nutrient solution wicking onto the seeds. Extruded horticultural clay pebbles (Hydroton 8-16mm, Hummert Int'l) were placed on top of the rockwool to reduce evaporation and limit algal growth. No further nutrient solution was added after the beginning of the trial.

Plants were grown in temperature, light and humidity-controlled growth chambers (Conviron MTR30, Conviron Environmentals Limited) for the duration of the experiment. Conditions were a 16/8 hour day/night cycle under metal halide and high-pressure sodium lamps providing photosynthetic photon flux density (PPFD) of 210 μmol $m^{-2}$ $s^{-1}$ and 24ºC/19ºC say and night temperatures, respectively. Relative humidity was maintained at 65% at all times. Plants were re-randomized once per week when jars were topped off with ultra-pure distilled water to a minimum of 400ml and maximum of 600ml solution. Each jar had 0.11 $m^2$ of growing area and was thinned to a single plant for trials BSS1, BSS2, BIB1, and GRR1. Due to the COVID-19 pandemic trials BIB2 and GRR2 were not thinned.




A: 50.8mm circular net cup, used to suspend the plant above the nutrient solution through a hole cut in the center of the Mason jar lid.

B: Plant aerial tissues, emerging between Hydroton pellets

C: Wide-mouth 946ml jar metal lid and lid collar. Center of metal lid is cut out to allow net cup to rest inside on the top lip.

D: A036/40 rockwool, used as the seedling and plant support media. Inert, pH neutral, high wicking growth media with no nutrient load. Bottom 6mm of rockwool extends into nutrient solution to allow wicking. Pre-drilled 10-11mm hole in top of each to accept seed.

E: Nutrient solution, 800ml for each sampling unit, per treatment level assigned.

F: 946ml wide-mouth glass Mason jar. At initiation 800ml was filled with nutrient solution and 146mm was moist airspace and the seated cube/net cup.

G: Roots extend down into solution for nutrient/water uptake, or laterally into moist air gap space once water levels drop (not shown) for oxygen.

H: Aluminum foil completely wrapping the sides and bottom of jar, to prevent light inclusion. (Light discourages root growth and fosters algal contamination.) Foil not shown to neck of jar.

I: Hydroton clay pebbles (Not shown on model for clarity). These were place atop the rockwool, around the seedling, to limit moisture loss and block light from reaching the rockwool.

*Figure 3.1: B. A. Kratky type, soilless hydroponic growing method*

### 3.2.5 Harvesting and Analysis

After 30/31 days all plant material was harvested, sectioned, and dried at 60°C until stable weights were achieved to yield shoot dry matter (SDM) and root dry matter (RDM). The dry weight of root material that could not be removed from the rockwool cubes was determined by subtracting pre-recorded mass of the dried cubes. After harvest, residual fluid in each jar was recorded (RES) and samples aliquoted and sealed for total N content analysis using alkaline persulfate digestion (USGS National Water Quality laboratory 2003). After dry matter mass determination, roots and shoots were finely ground to 0.5mm and elemental combustion analysis performed (Elementar, Thermo Fisher Scientific) to determine N and C content. N content (mg) in tissues was calculated by multiplying total tissue mass by determined N mass fraction percentage, N%. Residual solution N concentration was multiplied by volume of solution retained at the end of the trial. N mass balancing could then be performed using the equation:

$$N_{diff}\,(mg) = N_{provided}\,(mg) - N_{SDM}\,(mg) - N_{RDM}\,(mg) - N_{RES}\,(mg)$$

Where N sources or recovery/captures are identified as:

$N_{diff}$ = N gained by or lost from the system

$N_{provided}$ = mg of N provided in fertilizer solution

$N_{SDM}$ = N contained in the SDM, [$SDM_{mass}$ (mg) x $N\%_{SDM}$]

$N_{RDM}$ = N contained in the RDM, [$RDM_{mass}$ (mg) x $N\%_{RDM}$]

$N_{RES}$ = N remaining in Residual solution [$RES_{volume}$ (ml) x $N_{RES}$(ppm)]

And:

$N_{SDM} + N_{RDM} + N_{RES}$ = Recovered N, which with no loss or gain should equal $N_{provided}$

The N in individual seeds and background levels in the distilled water used in this experiment (reported as singular ppb) was equal across treatments, orders of magnitude lower, and not included in this analysis. If $N_{diff}$ is positive, more N was recovered from the sampling unit and evidences N fixation. Conversely, if $N_{diff}$ is negative it indicates N loss from the system.

### 3.2.6 Statistical Analysis

The data analysis for this paper was done using SAS 9.4 software for Windows. (Copyright2016 by SAS Institute Inc., Cary, NC, USA.) All significance tests were performed at alpha < 0.05 using PROC MIXED and PROC GLM for multi-factor ANOVA with type 3 tests of fixed effects and Tukey-Kramer HSD for least squares means differences. For dependent variable data exhibiting violations of heterogeneity of variance, PROC TRANSREG was used to generate BOX-COX lambdas for dependent variable transformation/normalization. Lambdas were chosen by *convenience* (SAS Institute 2016) and are listed in Appendix C if employed. For trials BIB2 and GRR2 where pots were not thinned, the number of plants present was included in the analysis as a discrete covariate in ANCOVA if indicated. Significance data is reported with standard errors of the means. Sample ANOVA tables by trial designator are in Appendix D.

In trial GRR1, a single objective outlier was visually identified using histograms and PROC UNIVARIATE indicating negative root biomass harvested (Obs #22, -0.054 g RDM). This data point was removed from analyses of RDM and calculations involving RDM; SDM for this unit was not adversely affected. This trial used an averaged weight for sample bags, net cups and rockwool cubes, and the possibility exists a low RDM plant coupled with one or more above average weights for the subtracted portions netted a negative measure. Whether this was a

measurement error or from averages is unknown, but as SDM cannot exist without RDM, this point was removed.

## 3.3 Results

Full results of ANOVA/ANCOVA analyses with degrees of freedom and p-values for all experiments can be found in Appendix D.

### 3.3.1 Black Seeded Simpson trials BSS1 and BSS2

BSS1 exhibited several effects from inoculation that will be presented in this section. BSS2, however, did not present any differences due to inoculation status so will not be presented here. Data for that trial is retained in Appendices D, E, F, and G.

#### 3.3.1.1 BSS1, BSS2 Biomass Accumulation

In BSS1, inoculation and N-fertilization had a significant effect on SDM and RDM and exhibited no interaction. Inoculation resulted in an average 8.19% increase in SDM over control (Figure 3.2). RDM in inoculated lettuce was on average 41.0% lower than in control (Figure

3.3). TDM was not impacted by inoculation.

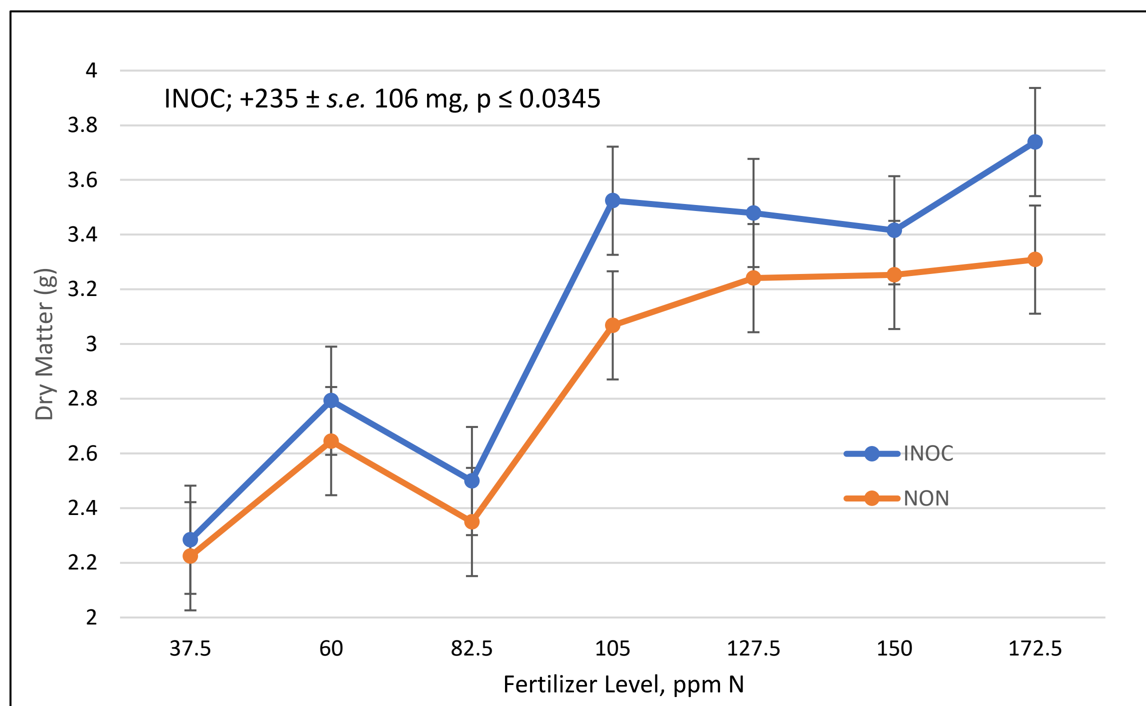



*Figure 3.2: BSS1 Mean Shoot Dry Matter. Note n=3 all Fertilizer levels, 37.5-172.5 ppm N*

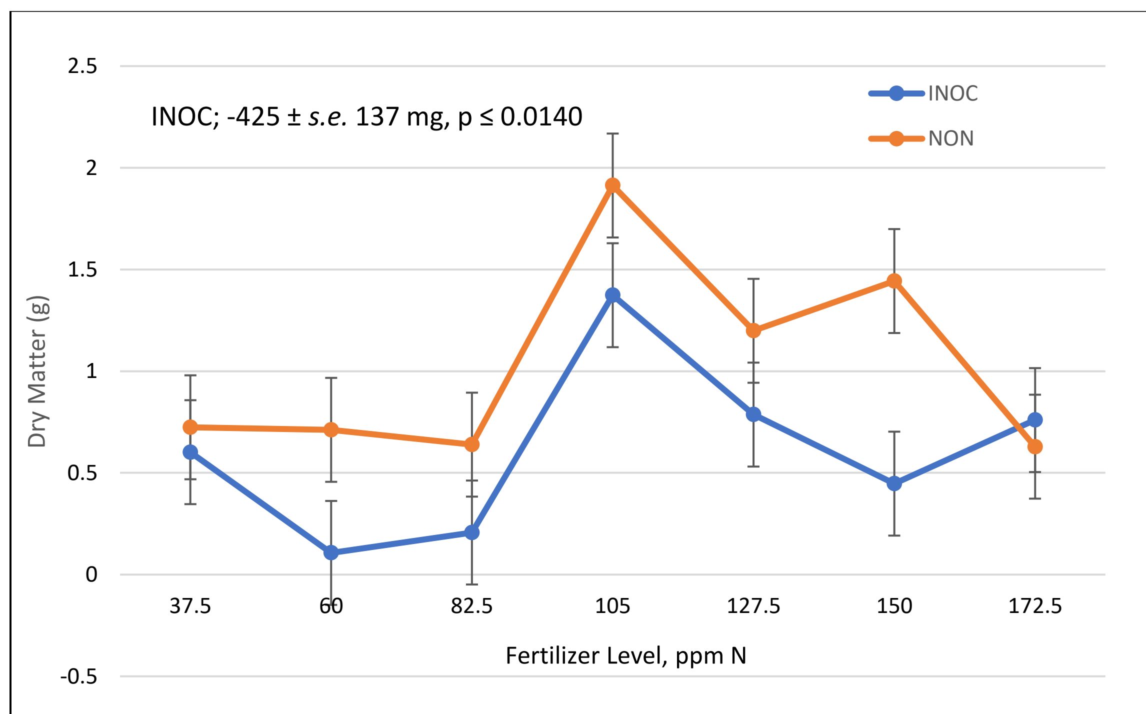



*Figure 3.3: BSS1 Mean Root Dry Matter. Note n=3 all Fertilizer levels, 37.5-172.5 ppm N*

### 3.3.1.2 BSS1, BSS2 C/N Ratios and N Content

Inoculation had a significant impact on SDM N mass fraction, SDM C/N ratio, N captured from RDM and from TDM, with no interactions. Fertilization was significant for all responses. In BSS1, pooled SDM N mass fraction was reduced from 2.21% to 1.98% (Table 3.2) but the total recovered N in shoots was not impacted, indicating no increased N concentration in leaves, either from fixation or mobilization. A subsequent increase in C/N ratios occurred in inoculated SDM tissues averaging 10.57% greater than control. The increased C capture in aerial tissues corroborates Figure 3.2 above.

Table 3.2: BSS1 N mass fraction, C/N ratio, and N content

| | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | | | *Average* |
|---|---|---|---|---|---|---|---|---|
| Trial and Measure | **37.5** | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** | ***105*** |
| BSS1 | | | | | | | | |
| SDM Nitrogen % ** | *LSD: -0.2328 s.e.: 0.06591 df=28 $p \leq 0.0015$* | | | | | | | |
| INOC | 1.2897 | 1.4647 | 1.9297 | 1.898 | 2.268 | 2.596 | 2.39 | *1.9766* |
| **NON** | 1.337 | 1.828 | 2.326 | 2.1103 | 2.276 | 2.6483 | 2.9397 | *2.2093* |
| *Difference* | *-0.0473* | *-0.3633* | *-0.3963* | *-0.2123* | *-0.008* | *-0.0523* | *-0.5497* | *-0.2327* |
| SDM N Content (mg) | *n/s* | | | | | | | |
| INOC | 29.0304 | 40.7487 | 48.375 | 66.337 | 79.012 | 86.6046 | 89.3205 | *62.7755* |
| NON | 29.347 | 48.2788 | 54.4208 | 64.3062 | 73.7542 | 85.8727 | 96.2369 | *64.6024* |
| *Difference* | *-0.3166* | *-7.5301* | *-6.0458* | *2.0308* | *5.2578* | *0.7319* | *-6.9164* | *-1.8269* |
| SDM C/N Ratio ** | *LSD: 2.2234 s.e.: 0.7305 df=28 $p \leq 0.0050$* | | | | | | | |
| **INOC** | 30.4953 | 26.4959 | 20.144 | 20.8206 | 17.3701 | 15.4389 | 16.5131 | *21.0397* |
| NON | 29.2597 | 21.1431 | 16.4662 | 18.783 | 17.4194 | 15.0597 | 13.583 | *18.8163* |
| *Difference* | *1.2356* | *5.3528* | *3.6778* | *2.0376* | *-0.0493* | *0.3792* | *2.9301* | *2.2234* |
| RDM N Content (mg) ** | *LSD: -7.6737 s.e.: 2.1391 df=28 $p \leq 0.0013$* | | | | | | | |
| INOC | 6.7533 | 1.3809 | 2.7716 | 18.2631 | 12.3212 | 11.4651 | 17.986 | *10.1345* |
| **NON** | 9.597 | 10.4535 | 10.1552 | 28.1261 | 19.9262 | 31.6756 | 14.7235 | *17.8082* |
| *Difference* | *-2.8437* | *-9.0726* | *-7.3836* | *-9.863* | *-7.605* | *-20.2105* | *3.2625* | *-7.6737* |

** = significant at alpha < 0.01, * = < 0.05, n/s = not significant
Note: Bolded main effect level shows which treatment was significantly greater

Inoculation had no effect on N mass fraction or C/N ratios in RDM (Appendix F), but resulted in reduced recovered N averaging 7.67mg, a 43.1% decrease (Table 3.2). Coupled with

no gain/loss in aerial tissues, total N recovered was reduced by an average of 9.5mg (Figure 3.4). No evidence of N fixation was found in this trial, but at several N levels more N was recovered than what was supplied. This occurred in both NON and INOC.

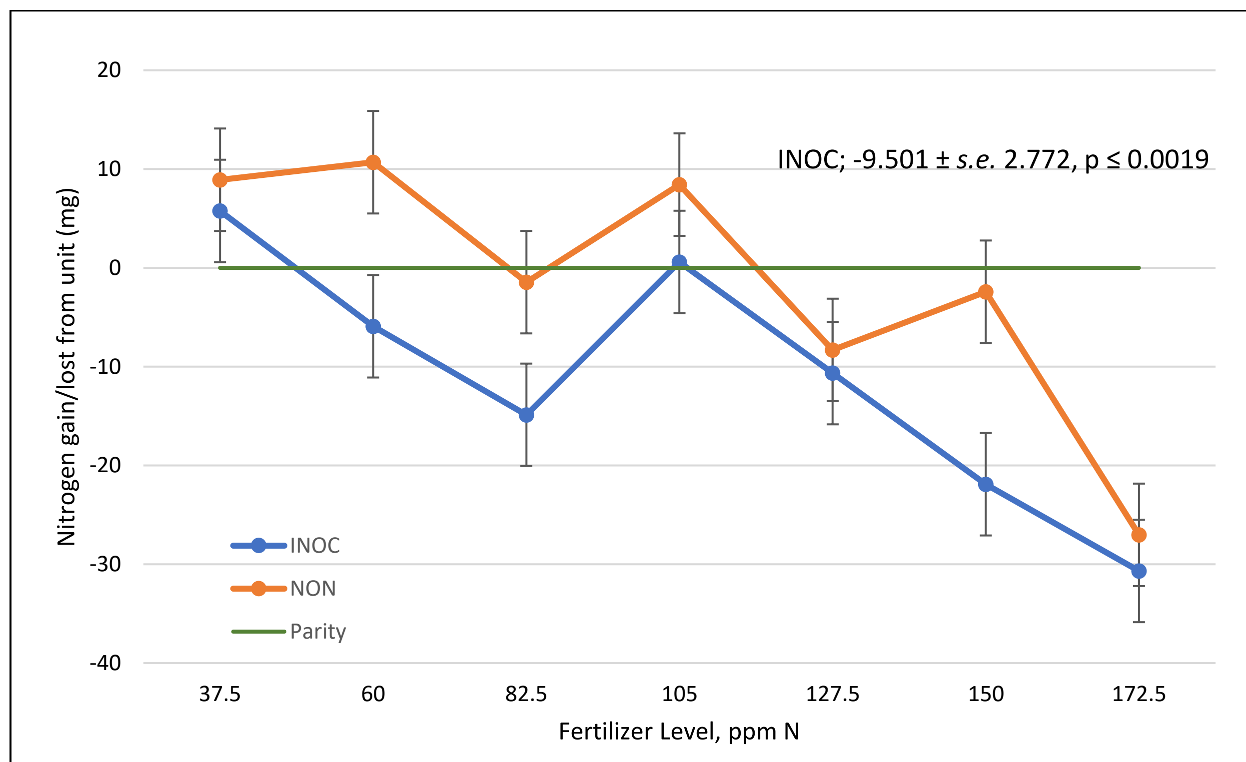



*Figure 3.4: BSS1 Mean N gains/losses from Jars. Note n=3 all Fertilizer levels, 37.5-172.5 ppm N*

### 3.3.2 Bibb trials BIB1 and BIB2

#### 3.3.2.1 BIB1, BIB2 Biomass Accumulation

In BIB1, inoculation and fertilization had significant effects on SDM and TDM with no interaction, however, RDM was not affected by inoculation but only by fertilization with no interaction effect. In BIB1 inoculation resulted in an average 10.9% increase in SDM (Figure 3.5).

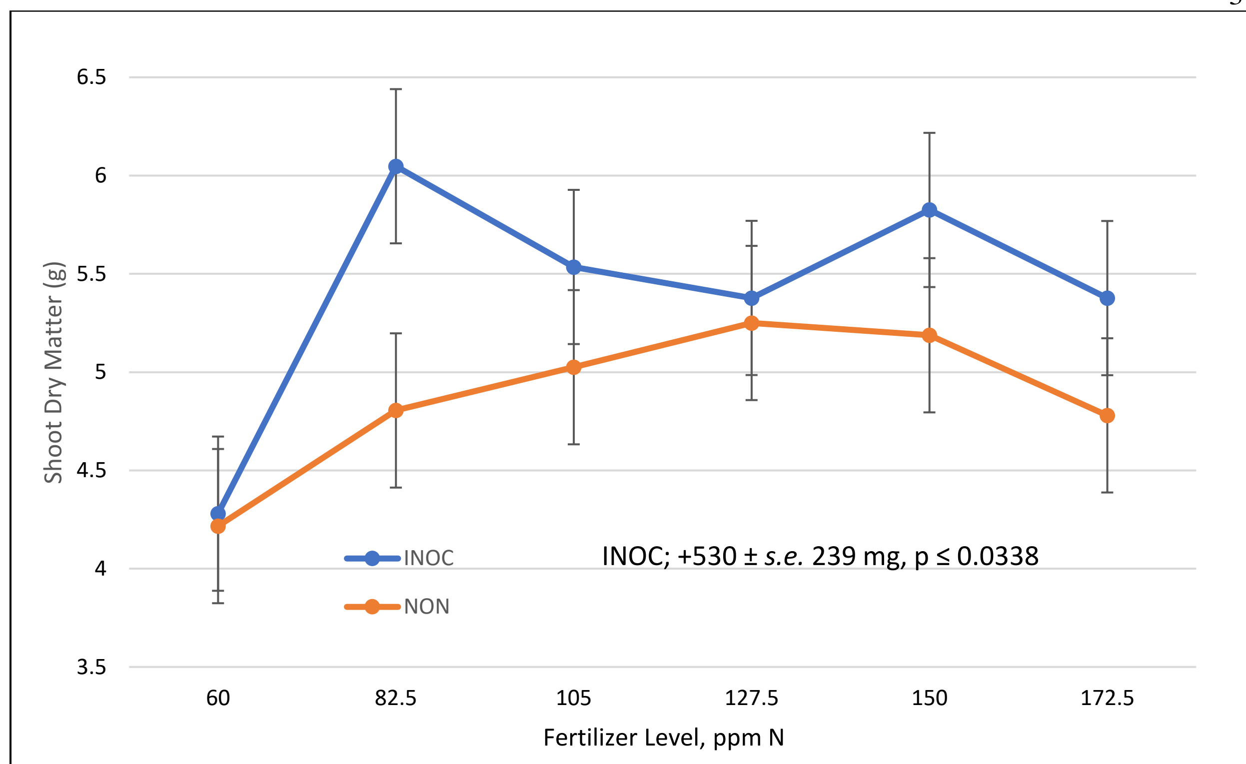



*Figure 3.5: BIB1 Mean Shoot Dry Matter. Note: n=3 for 60 and 172.5 ppm N. n=4 for all other Fertilization levels.*

RDM was not affected by inoculation. Inoculation therefore resulted in a similar response of TDM (10.04% increase) as in SDM (Figure 3.6).

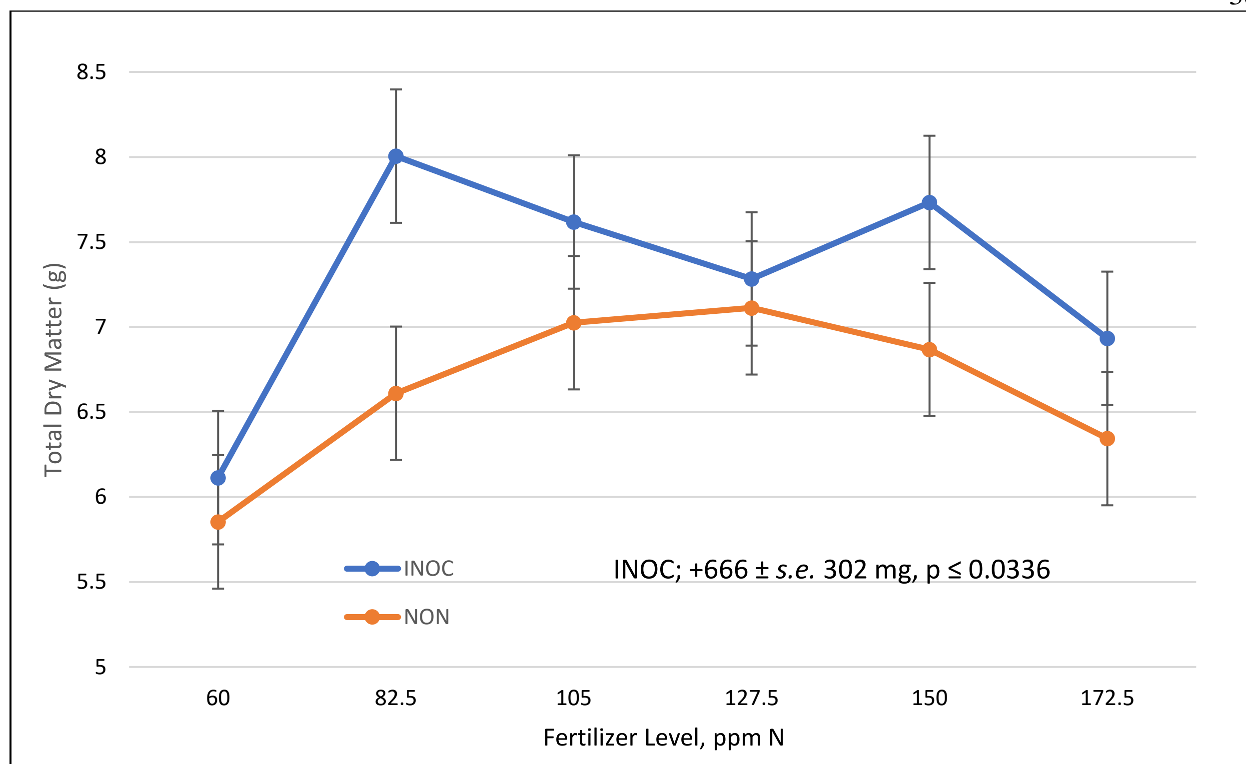



*Figure 3.6: BIB1 Mean Total Dry Matter. Note: n=3 for 60 and 172.5 ppm N. n=4 for all other Fertilization levels.*

In BIB2, inoculation, fertilization, and number of plants per jar had a significant effect on SDM, RDM and TDM with no interactions. Inoculation resulted in an average 16.2% increase in SDM over the control (Figure 3.7). The response curves were different in BIB2 than in BIB1, with greater SDM at low N-levels than at high N-levels (Figs 3.7 and 3.8). Further, SDM averaged 18.4% less in BIB2 than in BIB1. In BIB2 inoculation increased RDM 18.7% over controls averaged over all fertilization levels (Figure 3.8), contrary to BIB1 which saw no increase. TDM in BIB2 was 17.2% greater due to inoculation across all fertilization levels (Figure 3.9).

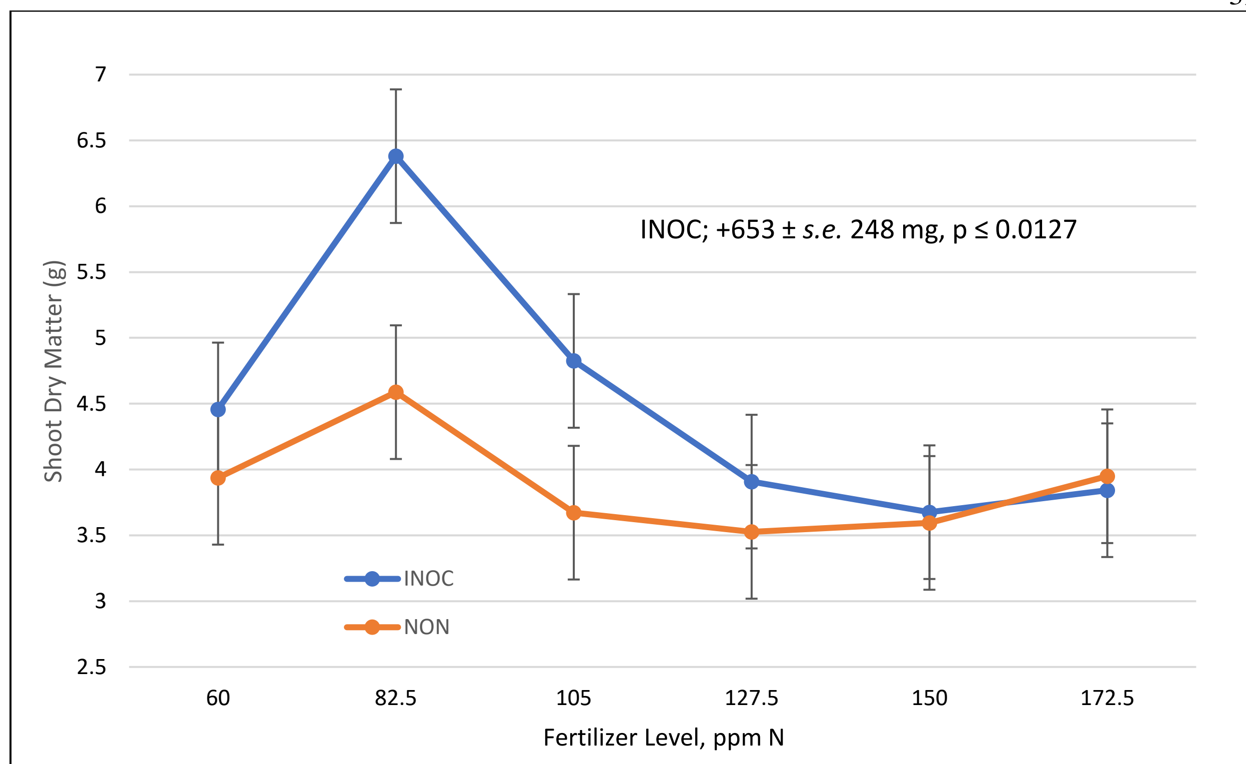



*Figure 3.7: BIB2 Mean Shoot Dry Matter. Note: n=3 for 60 and 172.5 ppm N. n=4 for all other Fertilization levels.*

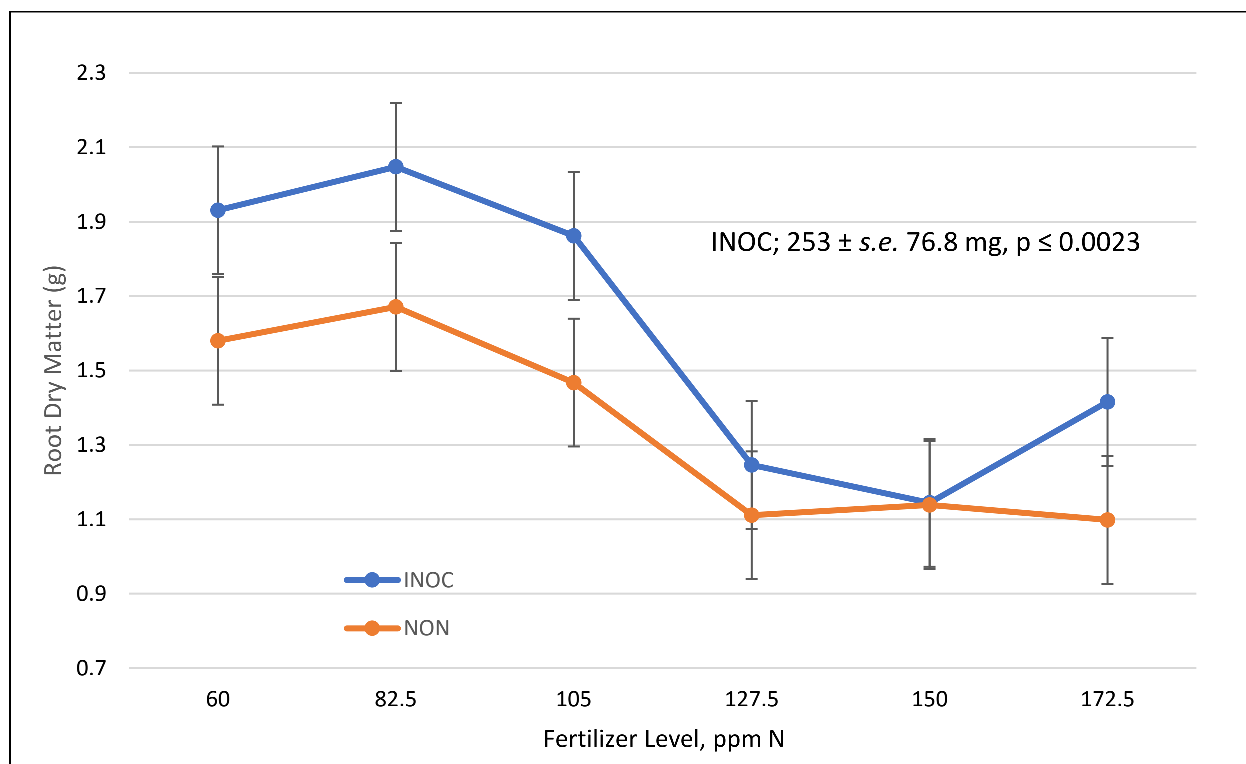



*Figure 3.8: BIB2 Mean Root Dry Matter. Note: n=3 for 60 and 172.5 ppm N. n=4 for all other Fertilization levels.*

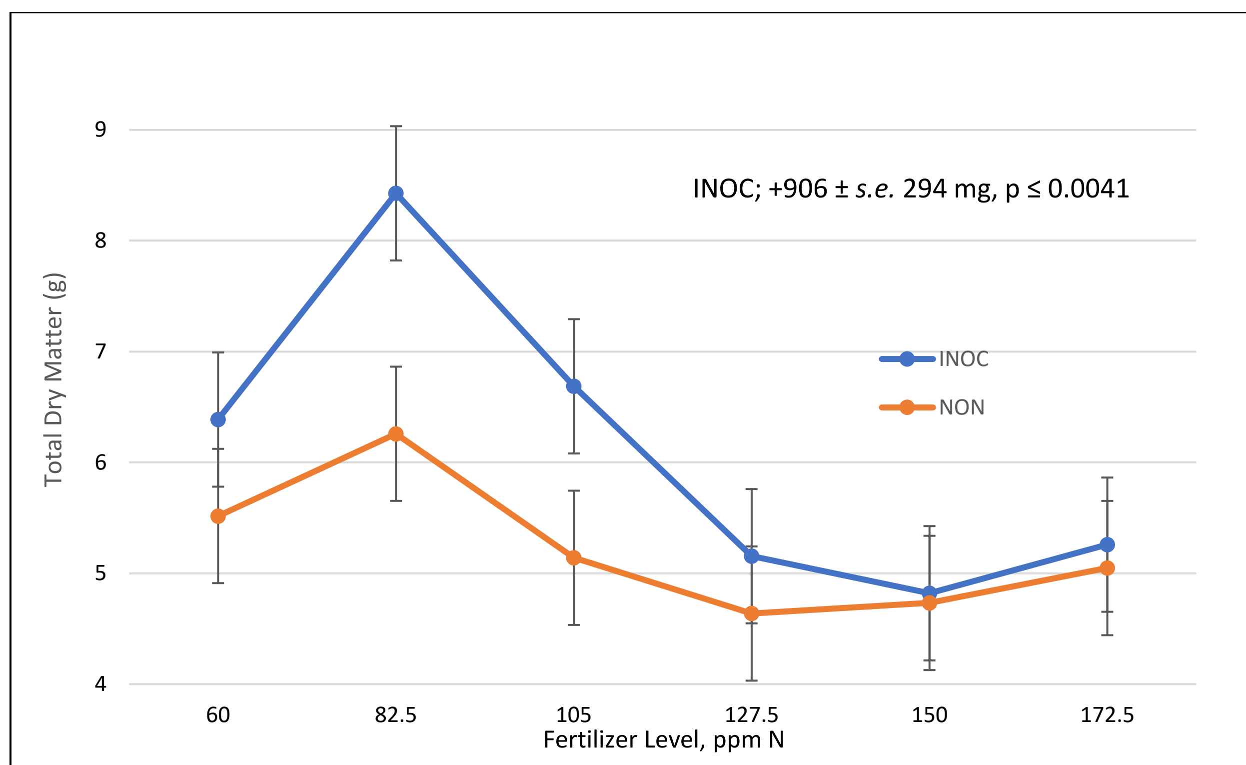



*Figure 3.9: BIB2 Mean Total Dry Matter. Note: n=3 for 60 and 172.5 ppm N. n=4 for all other Fertilization levels.*

#### 3.3.2.2 BIB1, BIB2 C/N Ratios and N Content

In BIB1, inoculation and N fertilization had a significant effect on N recovered from SDM, RDM, and TDM but did not interact. In this trial, N mass fraction and C/N ratios were only impacted by fertilization level; inoculation responses can be found in Appendix I. Recovered N from SDM was on average 6.04 mg higher in inoculated plants than in controls, and 2.04mg higher in RDM (Table 3.3), with N recovered in TDM shown in Figure 3.10.

Table 3.3: BIB1 N content

| Trial and Measure | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) 60 | 82.5 | 105 | 127.5 | 150 | 172.5 | *Average* *116.25* |
|---|---|---|---|---|---|---|---|
| BIB1 | | | | | | | |
| SDM N Content (mg) * | *LSD: 6.0360* | *s.e.: 2.2797* | *df=32* | *p ≤ 0.0125* | | | |
| **INOC** | 45.9767 | 70.3502 | 83.254 | 93.5854 | 107.04 | 120.28 | *86.7477* |
| NON | 46.0363 | 61.3058 | 80.6421 | 90.4181 | 104.75 | 101.12 | *80.7121* |
| *Difference* | *-0.0596* | *9.0444* | *2.6119* | *3.1673* | *2.29* | *19.16* | *6.0357* |
| RDM N Content (mg) * | *LSD: 2.0425* | *s.e.: 0.9315* | *df=32* | *p ≤ 0.0357* | | | |
| **INOC** | 16.9569 | 19.8892 | 28.3854 | 29.7192 | 32.1173 | 34.8268 | *26.9825* |
| NON | 15.6819 | 19.607 | 25.2417 | 29.4837 | 27.5801 | 32.0457 | *24.9400* |
| *Difference* | *1.275* | *0.2822* | *3.1437* | *0.2355* | *4.5372* | *2.7811* | *2.0425* |

** = significant at alpha < 0.01, * = < 0.05, n/s = not significant
Note: Bolded main effect level shows which treatment was significantly greater

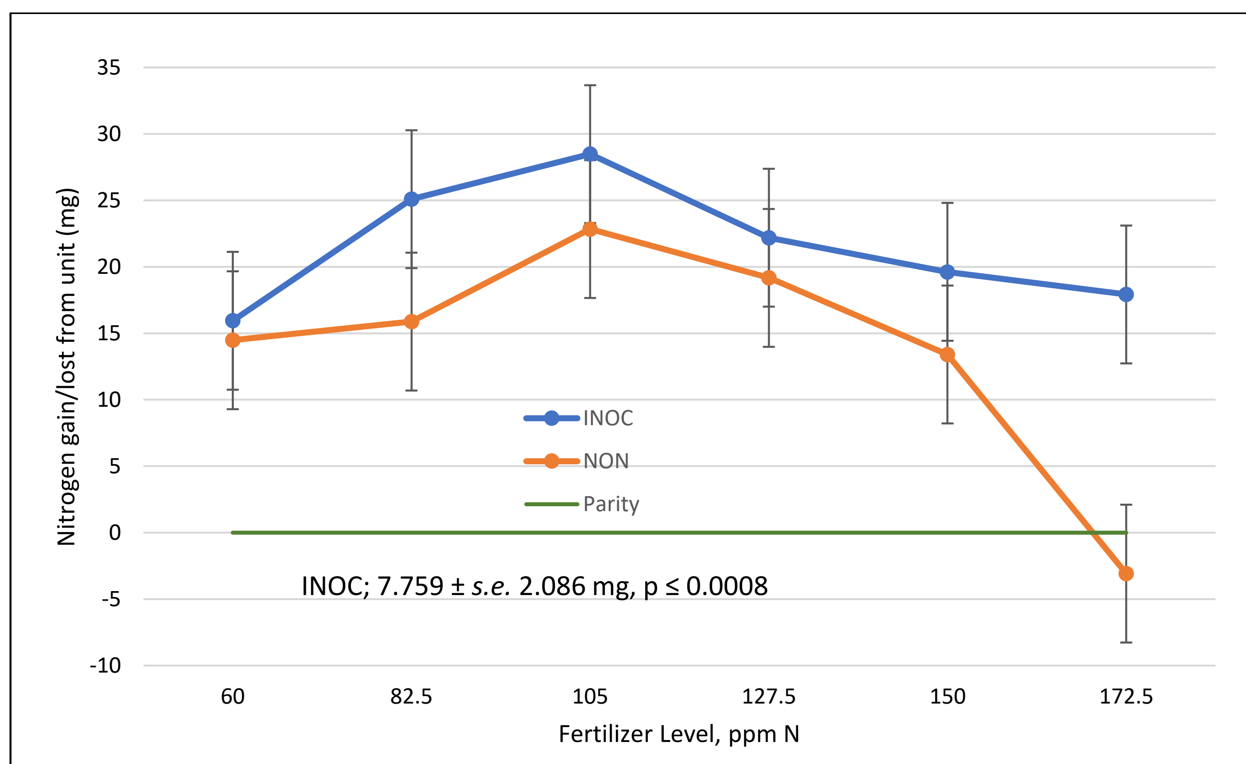



*Figure 3.10: BIB1 Mean N gains/losses from Jars. Note: n=3 for 60 and 172.5 ppm N. n=4 for all other Fertilization levels.*

Inoculated BIB1 gained on average 7.76mg N over control. However, more N was also recovered from control plants than what was supplied at almost all N levels (Fig. 3.10).

In BIB2, SDM N mass fraction, N captured in RDM and TDM, and SDM C/N ratio were significant with inoculation. Fertilization level was significant for all measures without interactions save for a fertilization*number (of plants) interaction in SDM N mass fraction. Number was additionally significant for all root measures and SDM N/C ratio, but not SDM or RDM N capture. No higher order interactions involving inoculation were significant.

For BIB2, N mass fraction in SDM was lower in inoculated lettuce compared with control (Table 3.4). SDM N content was not impacted by inoculation. Consequently, C/N ratio in SDM was greater in inoculated than control lettuce. As with the first trial, Total N recovered from all sources was greater in the inoculated treatment, by an average of 3.31mg N (Figure 3.11), but results show greater N recovery than what was supplied in the control as well. Full data for this set of trials can be found in Appendices D, H, I, and J, and supplied N amounts in Appendix B.

Table 3.4: BIB2 N mass fraction, C/N ratio, and N content

| Trial and Measure | Fertilizer (FERT) Treatment Provided N Concentration (ppm) | | | | | | *Average* |
|---|---|---|---|---|---|---|---|
| | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** | ***116.25*** |
| BIB2 | | | | | | | |
| SDM Nitrogen % * | *LSD: -0.2767 s.e.: 0.1311 df=29 p ≤ 0.0436* | | | | | | |
| INOC | 0.821 | 0.5246 | 1.347 | 1.9444 | 2.5782 | 2.7108 | *1.6543* |
| **NON** | 1.166 | 1.0179 | 1.7939 | 2.2655 | 2.5171 | 2.5981 | *1.8931* |
| *Difference* | *-0.345* | *-0.4933* | *-0.4469* | *-0.3211* | *0.0611* | *0.1127* | *-0.2388#* |
| SDM C/N Ratio * | *LSD: 4.3861 s.e.: 1.8028 df=29 p ≤ 0.0214* | | | | | | |
| **INOC** | 46.7108 | 54.0375 | 34.718 | 21.9845 | 18.1597 | 15.7018 | *31.8854* |
| NON | 45.5982 | 42.9597 | 24.1428 | 20.4196 | 16.3293 | 15.5464 | *27.4993* |
| *Difference* | *1.1126* | *11.0778* | *10.5752* | *1.5649* | *1.8304* | *0.1554* | *4.3861* |
| SDM N Content (mg) | *n/s* | | | | | | |
| INOC | 39.2079 | 47.9869 | 60.0839 | 76.0494 | 92.4076 | 100.44 | *69.3626* |
| NON | 38.9244 | 45.1925 | 63.517 | 75.3747 | 91.7889 | 103.84 | *69.7729* |
| *Difference* | *0.2835* | *2.7944* | *-3.4331* | *0.6747* | *0.6187* | *-3.4* | *-0.4103* |
| RDM N Content (mg) * | *LSD: 3.7552 s.e.: 1.3795 df=29 p ≤ 0.0109* | | | | | | |
| **INOC** | 20.5902 | 26.7523 | 28.8405 | 35.7133 | 30.441 | 38.1569 | *30.0824* |
| NON | 21.454 | 24.0066 | 26.911 | 24.7012 | 31.8548 | 29.0352 | *26.3271* |
| *Difference* | *-0.8638* | *2.7457* | *1.9295* | *11.0121* | *-1.4138* | *9.1217* | *3.7552* |

** = significant at alpha < 0.01, * = < 0.05, n/s = not significant

Note: Bolded main effect level shows which treatment was significantly greater

'#' indicates value different from SAS due to significant, non-INOC interaction (See Appendix D)

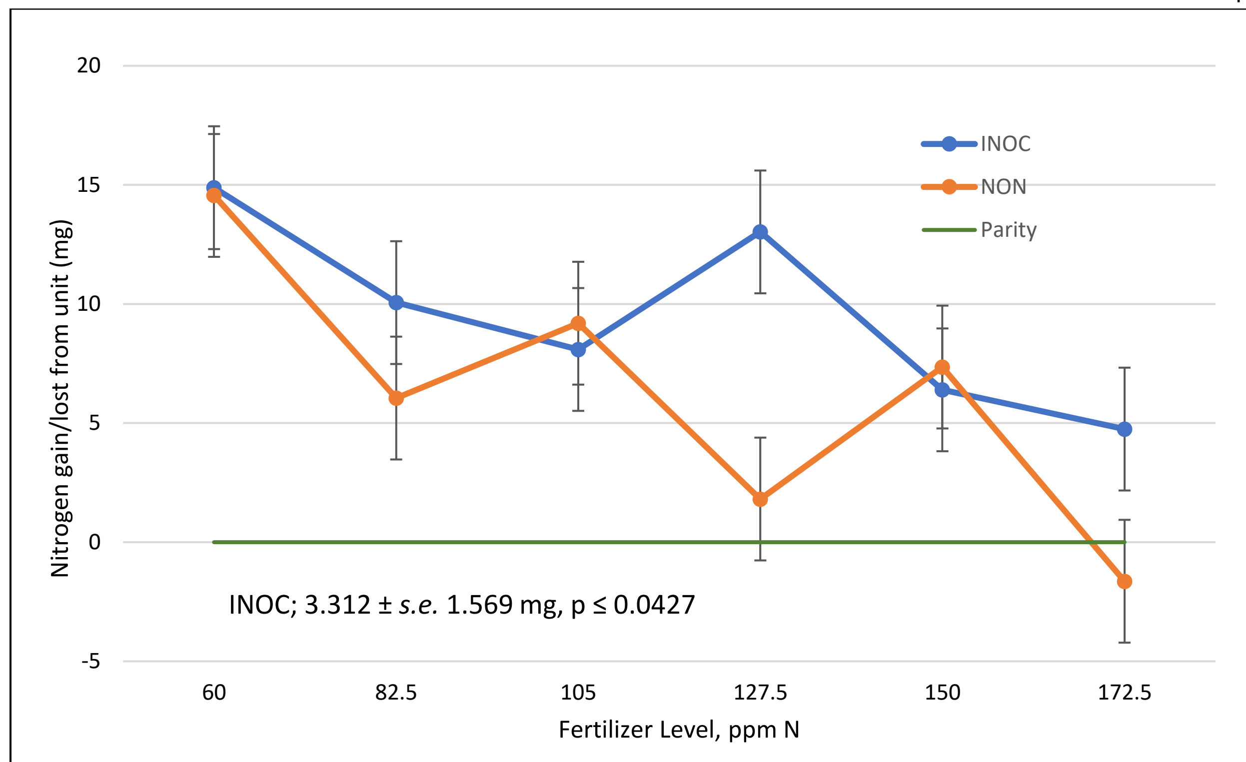



*Figure 3.11: BIB2 Mean N gains/losses from Jars. Note: n=3 for 60 and 172.5 ppm N. n=4 for all other Fertilization levels.*

### 3.3.3 Grand Rapids Tipburn Resistant trials GRR1 and GRR2

GRR1 underwent significant water stress as building access was restricted in the middle of the trial. As a result, 10 of the 40 plants died as seedlings, all from the control group. No inoculated plants died as a result of the three-week lapse in watering. Data for GRR1 can be found in Appendices K, L, and M but will not be discussed here due to the watering problems. Only trial GRR2 data will be presented here.

#### 3.3.3.1 GRR2 Biomass Accumulation

In GRR2, inoculation and fertilization had significant or very nearly significant (alpha = 0.1) effects on SDM, RDM, and TDM with no interaction. Inoculation resulted in a 12.7% average increase in SDM (Fig. 3.12) while RDM increased on average 9.12% (Fig. 3.13).

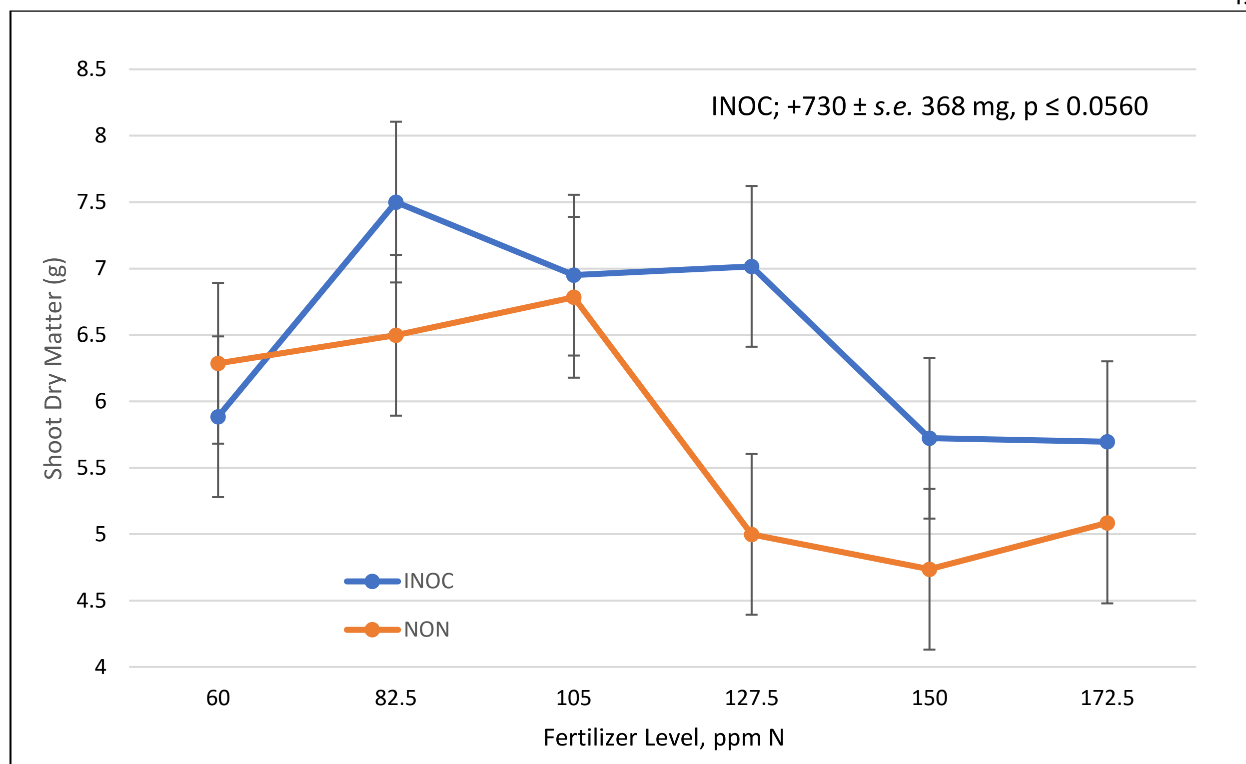



*Figure 3.12: GRR2 Mean Shoot Dry Matter. Note: n=3 for 60 and 172.5 ppm N. n=4 for all other Fertilization levels.*

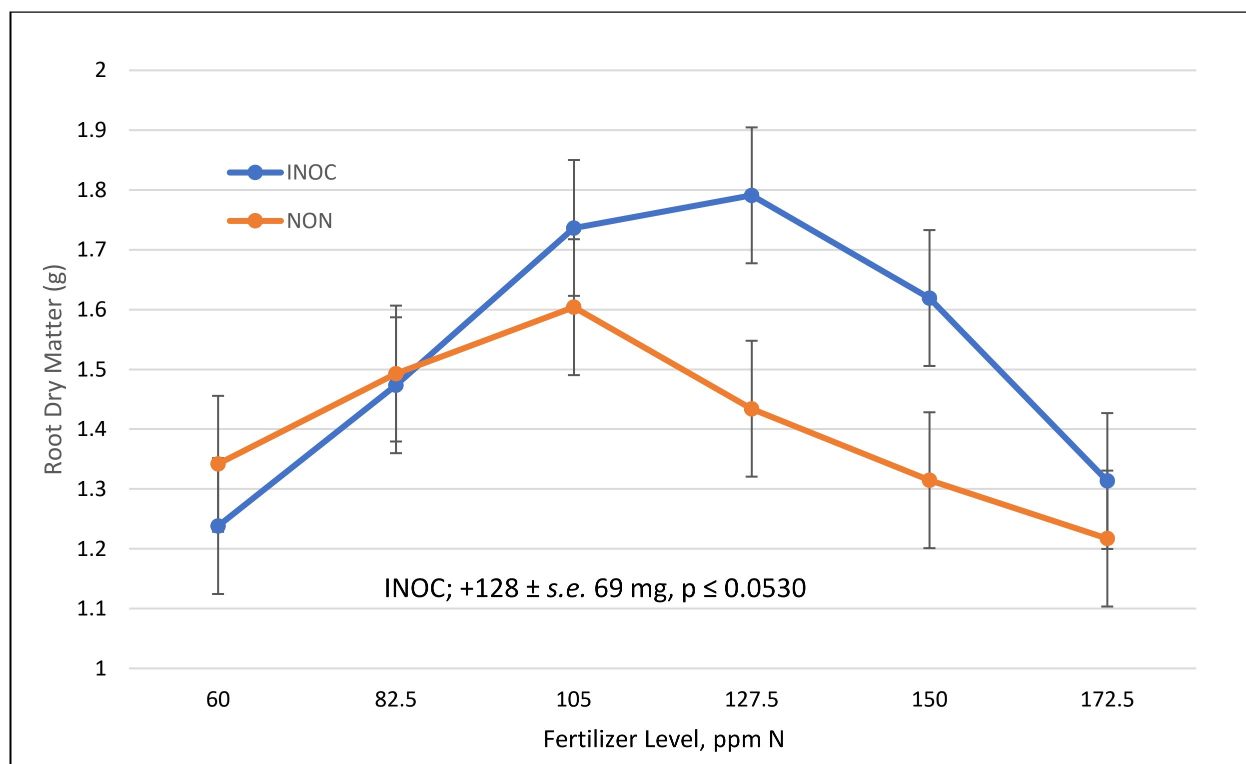



*Figure 3.13: GRR2 Mean Root Dry Matter. Note: n=3 for 60 and 172.5 ppm N. n=4 for all other Fertilization levels.*

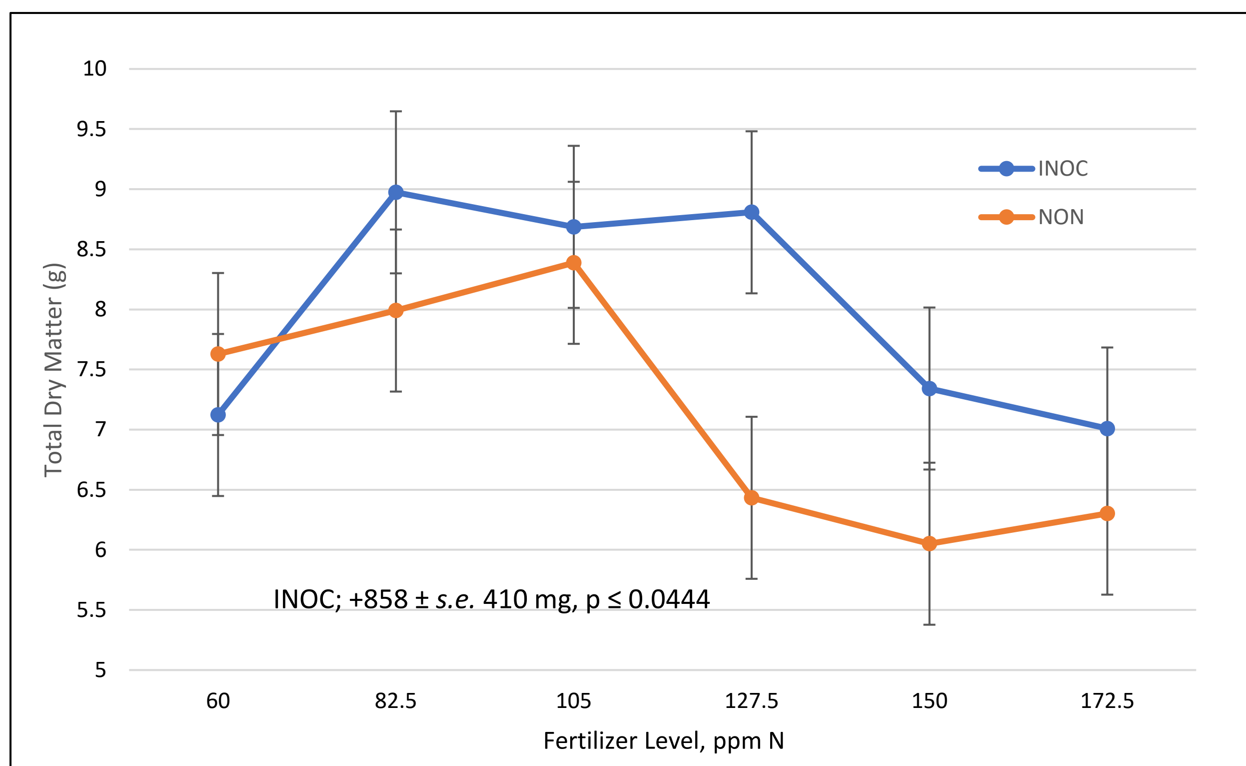



*Figure 3.14: GRR2 Mean Total Dry Matter. Note: n=3 for 60 and 172.5 ppm N. n=4 for all other Fertilization levels.*

Combining these for TDM, Figure 3.14 presents a 12.0% increase in pooled total plant production as a result of bacterial treatment.

### 3.3.3.2 GRR2 C/N Ratios and N Content

Inoculation had a significant or near-significant (alpha = 0.1) effect on N captured in SDM, N mass fraction in RDM, and RDM C/N, with no interaction with fertilization or number. Fertilization was significant for all responses and number for none. N content of SDM averaged 5.2% higher in inoculated lettuce than control (Table 3.5). Inoculation did not affect average N content of RDM but had lower N% and higher C/N ratio than control (Table 3.5).

Table 3.5: GRR2 N mass fraction, C/N ratio, and N content

| Trial and Measure | Fertilizer (FERT) Treatment Provided N Concentration (ppm) 60 | 82.5 | 105 | 127.5 | 150 | 172.5 | *Average* ***116.25*** |
|---|---|---|---|---|---|---|---|
| GRR2 | | | | | | | |
| SDM N Content (mg) ^ | *LSD: 3.4816* | *s.e.: 1.8768* | *df=32* | *p ≤ 0.0722* | | | |
| **INOC** | 38.8907 | 52.8927 | 58.1746 | 72.7094 | 91.3439 | 106.62 | *70.1052* |
| NON | 38.7317 | 44.1265 | 57.9122 | 77.8501 | 84.2805 | 96.8407 | *66.6236* |
| *Difference* | *0.159* | *8.7662* | *0.2624* | *-5.1407* | *7.0634* | *9.7793* | *3.4816* |
| RDM N Content (mg) | *n/s* | | | | | | |
| INOC | 17.7429 | 21.2196 | 29.7702 | 32.8626 | 37.6939 | 40.5481 | *29.9729* |
| NON | 20.4433 | 26.602 | 29.7517 | 32.2857 | 35.7068 | 38.8031 | *30.5988* |
| *Difference* | *-2.7004* | *-5.3824* | *0.0185* | *0.5769* | *1.9871* | *1.745* | *-0.6259* |
| RDM Nitrogen % * | *LSD: -0.2684* | *s.e.: 0.1164* | *df=32* | *p ≤ 0.0277* | | | |
| INOC | 1.4367 | 1.447 | 1.7123 | 1.8375 | 2.3502 | 3.2043 | *1.9980* |
| **NON** | 1.5265 | 1.7825 | 1.8568 | 2.383 | 2.8361 | 3.2133 | *2.2664* |
| *Difference* | *-0.0898* | *-0.3355* | *-0.1445* | *-0.5455* | *-0.4859* | *-0.009* | *-0.2684* |
| RDM C/N Ratio ** | *LSD: 2.5480* | *s.e.: 0.7095* | *df=32* | *p ≤ 0.0011* | | | |
| **INOC** | 28.6904 | 28.1277 | 22.4426 | 21.9732 | 18.7002 | 14.5575 | *22.4153* |
| NON | 26.45 | 23.7318 | 21.205 | 18.5039 | 15.2442 | 14.0689 | *19.8673* |
| *Difference* | 2.2404 | 4.3959 | 1.2376 | 3.4693 | 3.456 | 0.4886 | *2.5480* |

** = significant at alpha < 0.01, * = < 0.05, ^ = 0.1, n/s = not significant
Note: Bolded main effect level shows which treatment was significantly greater

Total N captured did not vary between inoculation and control in GRR2 (Figure 3.15, Appendix M). More N was recovered than what was supplied in both inoculated and control lettuce, except at the highest N-rates where the amount of recovered N in control equaled that supplied.

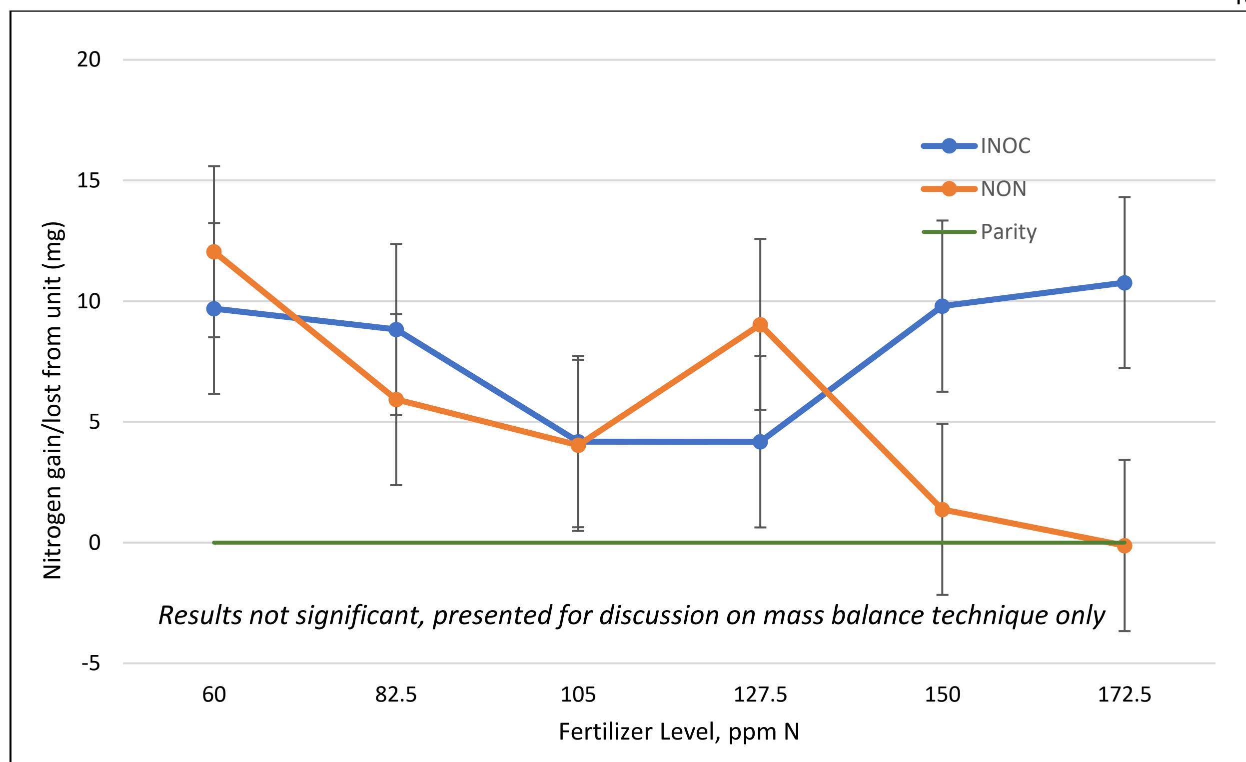



*Figure 3.15: GRR2 Mean N gains/losses from Jars. Note: n=3 for 60 and 172.5 ppm N. n=4 for all other Fertilization levels.*

## 3.4 Discussion

### 3.4.1 BSS1 and BSS2

For BSS, only the first trial yielded significant results for any of the responses measured. For BSS1, SDM increased due to inoculation with *G. diaz* but reached a plateau at 105 ppm N both in inoculated and control treatments, with a higher plateau for the inoculated treatment. In effect, [*G. diaz* + 105 ppm N] yielded equivalent or more plant biomass than [NON + 127.5 ppm N] or [NON + 150 ppm N]. This increase in SDM, however, corresponded to a decrease in N concentration and an increased C/N ratio. The *G. diaz* + 105 ppm N level corresponds to a 15-30% reduction in required N for the same biomass production. At the same time, RDM decreased 41.0%. As an aggregate construct, TDM was therefore not affected by *G. diaz*. Inoculated plants shifted biomass synthesis away from root tissues and into shoot tissues over

controls. Because only leaves are harvested, this apparent shift in photosynthate allocation from root to shoot would be beneficial as long as water and nutrients are not limited, as is normally the case in hydroponic lettuce production, but may not be the case in field lettuce production. A shift from root to shoot growth in lettuce was also reported by Vysotskaya et. al. (2017) in response to high levels of IAA in aerial tissues, suggesting that *G. diaz* produces IAA in BSS lettuce shoots. Gibberellins at moderate levels can also effect this morphologic response (Miceli et. al. 2019), another potential explanation for observed effects of *G. diaz*. The total amount of N in SDM inoculated with *G. diaz* was not significantly different from control plants, showing that the inoculated plants produced more shoot biomass with the same amount of N as the control, indicating increased use efficiency of absorbed N. (Amirouche et. al., 2020, Di Gioia et. al., 2013). Both hormone families produced by *G. diaz* have been implicated in increased N use efficiency in other plant-microbe synergisms (Gang, et. al., 2021). This effect appeared more pronounced at the lower levels of N, as at these levels N would be the limiting growth factor. Greater biomass and more C assimilation with the same amount of N align with goals of increasing plant productivity with the same inputs.

Evidence of N fixation in BSS1 was not found (Figure 3.4). Recovered N from SDM was not different from controls as mentioned previously, but the recovered N from RDM actually decreased in INOC plants and TDM N recovery was also significantly lower in INOC than NON. That 100% of supplied N would not be recovered experiment wide could be attributed to sample handling losses, fine root breakage, leaf drop and conservative recovery estimation, but explaining relative and significant N loss from a single treatment level, especially one that is reported to fix and add N, is more difficult. Neither *G. diaz* nor BSS have known denitrifying pathways, controls and standards were routinely employed to check N concentrations of

fertilizers, plants were not collected, moved, harvested or weighed by any treatment level or predetermined order, and ammoniacal N contributions from fertilizers were consistent and minor for all fertilization levels excluding substantial pre-plant-uptake volatilization. One possibility resides in N loss to adhesive biofilms produced by *G. diaz* (Velázquez-Hernández 2011) that were not recoverable from the interior of the Kratky jars, but their presence was not noted at harvest. The presence of denitrifying bacteria was determined to be extremely unlikely.

The reason for lack of response in the BSS2 is not clear. Conditions were held consistent between trials and seed treatments contained the same bacterial CFU concentration. Seed for the second trial was held over from the first trial but maintained in the dark at 4°C and 70% relative humidity for the 11 months between BSS1 and BSS2, excluding heat-induced thermal dormancy. As the seed was commercially sourced, age could not be determined prior to receipt, so may still have been a factor, as older lettuce seed may germinate but have different response (Ching and Danielson 1972). That BSS1 produced overall less biomass than BSS2 may be explained by calcium sourcing between trials, the only condition not held constant. BSS1 used a baked anhydrous calcium sulfate (Appendix A) whereas BSS2 has calcium sulfate dihydrate (Appendix B). Although equivalent ppm Ca was supplied, baked anhydrous $CaSO_4$ may become insoluble if heated above 370°C during formulation. Although no residual powder was witnessed in the fertilizer solution mixing buckets, time to dissolution was very long and required agitation, so incomplete hydration may have led to calcium deficiency in BSS1 plants.

### 3.4.2 BIB1, BIB2, and comparisons

Due to competition of multiple plants per jar in BIB2, the number of plants was included as a covariate in statistical analysis and helped explain the results seen. Maboko and Plooy

(2009) found intraspecific competition in densely sown lettuce crops reduced cumulative yield below that of single plants, possibly from diversion of synthates towards allelopathic hormones as seen by Kula et al. (2020). As the seedlings in this experiment were sown mere mm away from each other (Figure 3.1) interaction and competition would be unavoidable.

Comparing BIB lettuce trials, both exhibited similar responses to inoculation, with greater SDM and TDM. Additionally, BIB2 exhibited an increase in inoculated RDM as well. As has been exhibited in other multi-cultivar *G. diaz* experiments (Eskin at al., 2014; Riggs et. al., 2001; Yoon et. al. 2016) response to inoculation is not consistent between even closely related varieties, as is seen here comparing BSS to BIB. Both cultivars exhibit an improvement in SDM production with inoculation, yet a reversal in RDM response. For the BIB trials, inoculated BIB1 saw a pooled SDM increase of 10.9% (Figure 3.5) and for BIB2, 16.2% (Figure 3.7), compared to BSS1's increase of 8.2% (Figure 3.2). Even with the significant reduction in root mass in BSS1 (Figure 3.3) and possible delivery of synthates to aerial tissues, it was not able to match the percent increases in either BIB trial. BIB2 additionally produced 18.7% greater RDM biomass when inoculated, besting both BIB1 and BSS1. BSS1 did have anhydrous calcium sulfate as previously mentioned, so the potential exists that the glacially slow dissolution of $CaSO_4$ in water may have led to calcium deficiencies in that trial, reducing yield. Therefore, BIB lettuce biomass responds to inoculation with greater TDM and does not employ any root/shoot tradeoffs as seen in BSS. This will be important where water and nutrients are limited or roots are not fully submerged (Kerns et. al., 1999; Smith et., al. 2011).

N mass balancing presented the possibility of N fixation in both BIB1 and BIB2 trials, but as previously mentioned the control treatments also exhibit increased amounts of recovered N (Figures 3.10 and 3.11, Appendices I and J). The mass balancing design and equation

employed is standard across multiple disciplines and makes logical and mathematical sense, but the calculated values for recovered N indicate additions to the sampling units in both inoculated and bacteria free jars. Common forms of N contamination such as N fixation from cyanobacteria, impure water, or soil-borne organic or inorganic N sources were controlled and not a likely source of added N, so greater N recovery above parity and across inoculation treatments eludes explanation by the authors and complicates inferences about N-fixation in inoculated BIB.

### 3.4.3 GRR1, GRR2, and comparisons

As previously mentioned, GRR1 experienced significant water stress during cultivation, leading to the death of 25% of plants, all from the NON treatment group (10/20 perished). A concurrent run of BIB lettuce (not reported in this manuscript) similarly evidenced the drought stress death of 13/20 NON plants. In both trials, all of the INOC plants survived, albeit with highly variable growth and often an insufficient number of control plants remaining for statistical analysis. Therefore, GRR1 was not discussed in any detail in this paper, yet this trial indicates the (coincidental) discovery of drought stress tolerance and increased WUE in *G. diaz* inoculated lettuce, as has been reported in other crops such as rice (Filgueiras et. al., 2020; Silva et. al., 2020), corn (Tufail et. al., 2021) and sugarcane (Vargas et. al., 2014). GRR1 data are included in full in Appendices D, K, L and M for completeness.

For GRR2, response was very similar to BIB2 with INOC treatment effecting an increase in SDM, RDM, and TDM, albeit the former two were only significant at an alpha $< 0.1$ (Figures 3.12, 3.13, and 3.14, respectively). This consistency is important as these two cultivars represent different general phenotypes, GRR being a frilled looseleaf variety and BIB being a bunched rosette, that may have different suitability across different cropping systems. Being able to

employ a single bacterial inoculant over different varieties and effect the same growth would be important for widespread use of *G.diaz* in lettuce production. The importance of cultivar selection and understanding of bacterial-plant synergism is highlighted in GRR2, and this cultivar had an inoculated SDM percent increase (+12.7%) between the two values of BIB1 (+10.9%) and BIB2 (+16.2%), but only half the percent increase in INOC root biomass as BIB2 (GRR2; +9.1% vs. BIB2; +18.7%), demonstrating yet another possible tissue interaction between *G. diaz* and cultivar.

Like BIB1 and BIB2, GRR2 mass balancing (Figure 3.15) showed increased N recovered from control and inoculated treatments above what was supplied. This again leaves the question as to from where this exogenous N entered the system. NUE alone could account for a large portion of the increased biomass responses, as seen in Table 3.5.

## 3.5 Conclusions

For growers looking for new techniques to improve lettuce yield, *G. diaz* inoculation may present a beneficial additive. The bacteria's ability to thrive under oxygenated conditions and avoid suppression by high N salt presence make it especially suitable for hydroponic and/or aeroponic setups. For the three cultivars tested in this study, harvestable biomass was increased in all but one of the trials, with increases ranging from +8.19% in BSS to +16.16% in BIB. For BIB and GRR lettuce, the highest biomass yield was realized with inoculation and an N fertilization level well below established requirements, showing the potential for growers to reduce N fertilizer applications and still maintain production. *G. diaz* also reduced root production in one cultivar, allowing custom combinations of bacterium and lettuce variety to match cultivation techniques, such as nutrient film techniques or floating beds. Luxury

consumption trends were not seen in this study, partly because of the non-replenishment fertilization regime employed in Kratky jars, and partly because N fixation could not be definitely ascertained, being plagued by unexplained recovery of N that was not supplied, even in control treatments. As this is the first published report of *G. diaz* inoculation of lettuce, further research will need to be performed to elucidate the exact mechanisms behind the plant responses. Whether from PGRH production, N fixation, NUE or altering N translocation *in planta*, *G. diaz* has the potential to increase yields of this valuable crop.

# Chapter 4

## Radish Response to *Gluconacetobacter diazotrophicus* Inoculation

### 4.0 Abstract

*Gluconacetobacter diazotrophicus* is a non-host specific bacterial endophyte capable of affecting plant growth through N-fixation, plant growth regulating hormone (PGRH) production, mineral solubilization, and inducing resistance to biotic and abiotic stressors. The bacterium includes winter-type radish among its native host range, but looking to find an endophyte for use in spring varieties, radish seeds from three different cultivars were inoculated with *G. diaz* to assess impact on leaf and bulb yield. Plants were grown in N-poor soilless media to simulate deficiency that may occur when plants are sown as pre- or post- main season crops. Results demonstrated inconsistent responses to inoculation, both across cultivars and within trials of the same radish type grown under different conditions. The adjuvant material used as a bacterial binding agent also significantly impacted response. For these spring-type varieties under the cultivation conditions herein, no net beneficial synergism with *G. diaz* was realized.

### 4.1 Introduction

Modern agriculture has been highly successful to provide sufficient foodstuffs for a growing populace - at a cost. Environmental stewardship needs to be improved and one of the urgent needs to improve sustainability of agriculture is to reduce fertilizer inputs. Currently, production of synthetic fertilizer represents 3% of global energy consumption, excessive

fertilizer applications lead to nutrient losses resulting in eutrophication and other pollution, and the cost of fertilizers is an obstacle to yield improvement, particularly in smallholder agriculture in developing countries (FAO, 2010; Good and Beatty, 2011). Some techniques, such as no-till and residue management have arisen to ameliorate these environmental impacts, but for current planting and harvesting techniques of many root crops these approaches are untenable. This is especially true for very small root crops such as radishes, which require very even, tilled soils, cannot be effectively harvested with excessive residue present on the field, and produce in such a short timeframe that relying on natural breakdown processes to release nutrients from the decomposition of prior crop residuals is not achievable.

Radishes are an economically important spring and fall crop that have a very short growing life cycle. Worldwide, the cultivation of radish is estimated at 7 million tons per year, about 2% of the total world vegetable production (Schippers, 2004), with approximately 7.5% of U.S. vegetable farms producing the crop each year (USDA-NASS, 2019). The ‘Spring’ type radish specifically can produce a mature crop in as little as 3.5 weeks from sowing to harvest, adding a valuable commodity prior to spring plantings and after fall harvests, known as ‘shoulder’ cropping. This short time frame in the ground, coupled with temperature-inhibited bacterial processes of decomposition during their growing times, means that high yields of radishes may be highly reliant on exogenous fertilizer applications that the marginal farmer cannot apply. Recommended fertilization regimes call for 45-67 kg $ha^{-1}$ of applied N (Masabni, 2011) with fertilization imbalances negatively impacting both growth and utility of the crop (Zhang et al, 2019), but smallholders and subsistence farmers often cannot afford recommended rates. One approach to alleviate fertilizer demand is incorporation of biologic fertilizing agents such as bacterial inoculants, which can meet crop demands through rhizospheric or endophytic

fixation of N and solubilization of immobilized soil nutrients, thus creating a more sustainable, crop-localized fertilization regime.

One potential bacterial endophyte for use in radishes is *Gluconacetobacter diazotrophicus,* a species originally isolated from sugarcane (Cavalcante and Dobereiner, 1988). This bacterium has the ability to non-pathogenically infect myriad plant species including winter radish (Madhaiyan et al., 2004) with mutualistic effects observed in some situations, such as N fixation (Fuentes-Ramirez et al., 1999, Cocking et al., 2006, Eskin et. al, 2014, Saravanan et al., 2008), PGRH production (Bastian et al., 1998; Fuentes-Ramirez et al., 1999; Kumar et al., 2015), soil phosphate and zinc solubilization (Crespo et al., 2011, Delaporte-Quintana et al., 2017; Silva et al., 2020 ) and increased plant water use efficiency (Rangel de Souza et al., 2015; Tufail et al., 2021). Bacterial inoculation of radish with increased crop production has already been established with select strains of *Azotobacter spp. (*Ziaf, et al 2016; Basavaraju et al., 2002), *Azospirillum spp.* (Kumar et al., 2016), *Bacillus,* and *Stenotrophomonas* (Akhtar et al., 2018), but many inoculant candidates have also shown inhibitory effects (Antoun et al., 1998; Kumar et al., 2016; Ziaf et al., 2016). One hindrance to successful bacterial promotion is radish's production of raphanin, a potent anti-bacterial and anti-fungal component present in varying amounts in different cultivars. Mitigating the energetically costly plant defense responses to endophyte penetration into plant tissues is one challenge to the use of biologicals. Important for use in radish, this bacterium is gram-negative and may avoid this strong anti-bacterial agent as raphanin has predominantly anti-gram-positive inhibitory effects. Also, *G. diaz's* ability to produce plant defense response modulating hormones may provide a means to sidestep this inhibition and reduce plant metabolite diversion towards defense responses (Bastian et al., 1998; Arencibia et. al, 2006; Adriano-Anaya et al., 2005). Although *G. diaz* has been shown to

successfully inoculate various winter radish cultivars and varieties, little research has explored the use of this endophyte in growth and yield promotion from a horticultural, production-based perspective in spring radish.

This research project aimed to assess the biomass accumulation, bulb metrics, and leaf production effects of *G. diaz* seed inoculation on several common spring radish cultivars and varieties. Agronomically, bulb yield is of the highest economic importance, but other measures such as root length and leaf production will be assessed to uncover potential mechanisms of action for this yet unstudied plant-microbe synergism. Given the myriad mechanisms of plant growth promotion exhibited between *G. diaz* and winter radish varieties, any net positive or negative impact on spring-type growth will be assessed and if present, mechanisms impacting growth elucidated. Under nutrient sufficiency differences would likely be to a combinatory effect of PGRH production, N-fixation, WUE, or secondary nutrient mineralization, so this research seeks to investigate agronomic impacts of inoculation in a simulated shoulders planting scheme. Homogenized soilless media allowed for control of Zn, P, and Fe solubilization, and careful watering eliminated water stress, allowing better research inferences from N fixation and PGRH impacts, if any.

## 4.2 Materials and Methods

### 4.2.1 Plant species and bacterial stocks

Three different cultivars and/or varieties of radish, (*Raphanus sativus* L. 'Bacchus F1', 'Rover F1' and 'Sora OG') were acquired from commercial seed sources. Cultivars were chosen for tight germination windows, rapidity of growth, ease of sowing, and suitability for greenhouse

as well as external production. Upon receipt, all seed was sorted for size consistency, damaged/malformed seed was removed, and the remainder was stored at 4 $^{o}$C and ~65% relative humidity. *G. diazotrophicus* was provided by Azotic Technologies, LLC (Nottingham, UK) and is a commercially available strain of the bacterium sold under their Envita$^{TM}$ brand name (Azotic-na.com 2019). Pure strain stocks were maintained in 25% glycerol at -80 $^{o}$C.

### 4.2.2 Bacterial culture conditions

For each planting, the bacterium was grown from glycerol preserved stock in a modified MESMA/ATGUS medium [2.7 g $L^{-1}$ glucose; 4.8 g $L^{-1}$ dipotassium phosphate ($K_2HPO_4$); 0.65 g $L^{-1}$ monopotassium phosphate ($KH_2PO_4$); 1.8 g $L^{-1}$ mannitol; 4.4 g $L^{-1}$ 2-(N-morpholino)ethanesulfonic acid (MES hydrate); 2.7 g $L^{-1}$ yeast extract] (Cocking et al., 2006). All materials were mixed and autoclaved prior to bacterial introduction to ensure single culture stock. The medium was incubated in a positive pressure, enclosed shaker table (30$^{o}$C, 120 rpm) for five days. Serial plating and counts on semi-solid ATGUS media (same as above, adding 0.8% w/v agar) in triplicate, corroborated with OD600 based estimation (NanoDrop One, Thermo Scientific), was used to determine final colony forming units (CFU) of prepared culture. For the first experiment, achieved CFU was 5.2 x $10^{8}$ $mL^{-1}$ and for the second, 1.8 x $10^{9}$ $mL^{-1}$.

### 4.2.3 Inoculum preparation

To prepare the seed inoculation medium, a proprietary blend of sucrose, gum arabic, and Tween 80 was added to distilled water, autoclaved for sterility, cooled to 25$^{o}$C, and then

portioned into two equal aliquots (Azotic Technologies, Internal Standard Operating procedure 2017, since deprecated). One portion received an equivalent amount of cultured bacterial concentrate, forming the inoculate working stock 'INOC', while the other received only distilled water, becoming an adjuvant only, bacteria-free treatment 'NON'. This treatment was included as the process of seed coating using liquid formulas has been shown to impact germination rates and success (Murphy, 2017). Finally, an additional distilled water only solution was used as an additional bacteria-free negative control, 'Water'. The final components of the seed inocula can be found in Table 4.1.

Table 4.1: Prepared Inoculum

| Treatment | Experiment #1, 'Summer' | Experiment #2, 'Fall' |
|---|---|---|
| 'INOC' | $2.6 \times 10^8$ CFU $ml^{-1}$ *G. diaz* + adjuvants in distilled water | $9.0 \times 10^8$ CFU $ml^{-1}$ *G. diaz* + adjuvants, in distilled water |
| 'NON' | Adjuvants only, in distilled water | Adjuvants only, in distilled water |
| 'Water' | Distilled water only | Distilled water only |

### 4.2.4 Location

This experiment was conducted at two locations and during two plantings on the campus of The Pennsylvania State University, University Park, Pennsylvania. During the fall of 2019 a concurrent greenhouse and raised bed trial was conducted, with a subsequent replication in the late spring/ early summer of 2020. For all experiments and during each planting, three types of spring-style radish (*Raphanus sativus* L. 'Bacchus F1', 'Rover F1' and 'Sora OG') were grown. All varieties were grown in soilless media either under controlled greenhouse conditions or in a

raised crop-exclusive bed. During the fall planting the radishes were protected from extreme low temperatures with a cold-frame enclosure. For all planting, the distance between planting nodes and subsequent plants was maintained at 10 cm.

### 4.2.5 Bacterial application verification

Successful bacterial transfer to seed surfaces was assessed via nested PCR analysis post-inocula application on 'INOC' seeds. Unplanted seeds were resubmerged in phosphate buffered saline and lightly agitated to resuspend the dried inoculum, with 'NON', 'Water' and seeds not subjected to any treatment included as a complement of controls to analyze false positives. Solutions were maintained at 4$^{o}$C prior to nucleic acid extraction using an automated DNA isolater (MPure-12 Plant Nucleic Acid Extraction Kit, on MPure-12 Magnetic Nucleic Acid Purification System, MPBiomedicals). 16S rRNA primers GDI25F (5'-TAGTGGCGGACGGGTGAGTAACG-3') and GDI923R (5'-CCTTGCGGGAAACAGCCATCTC-3') (Tian et al., 2009) were used in the initial amplification, using Phire Plant Direct polymerase (ThermoFisher F160S). These primers target an 899-bp product of the *G. diazotrophicus* genome (Bertalan et al., 2009). Amplification parameters were an initial denaturation/inhibitory agent removal step (98$^{o}$C for 5 min) followed by 40 cycles of amplification (denature 98$^{o}$C for 5 s, anneal 62$^{o}$C for 5 s, extend 72$^{o}$C for 20 s). After 40 cycles, a final extension of 1 minute at 72$^{o}$C was performed before samples were held at 4 $^{o}$C pending subsequent amplification. One microliter of PCR product from this round served as the template in the second round of PCR, which used internally targeting primers GDI39F (5'-TGAGTAACGCGTAGGGATCTG-3') and GDI916R (5'-GGAAACAGCCATCTCTGACTG-

3') (Franke-Whittle et al., 2005). The same PCR enzyme chemical complement was employed in the second round to further remove PCR inhibitors, with the same parameters save the final hold was 30 minutes @ 23°C. The nested PCR amplification products, 878 bp in length, were visualized on 0.75% agarose gels containing SYBR™ Safe nucleic acid stain.

### 4.2.6 Seed inoculation and sowing, Fall 2019 experiment

From a sorted 360 seed selection for each radish type, 60 randomly chosen seeds were divided into each of six sterile 60mm petri dishes. Two dishes were randomly assigned to the 'INOC' treatment, two to adjuvant only solution 'NON', and two to 'Water'. Each petri dish received a 2ml aliquot of the appropriate, pre-prepared solution. Dishes were then lidded and swirled to fully immerse all seeds and allowed to soak for 10 minutes, with light agitation after 5 minutes to redistribute the solution. After 10 minutes, the excess solution was pipetted off and discarded. The dishes were then loosely covered with paper towels and allowed to air dry for 60 minutes before being covered and sealed. These steps were repeated by cultivar using the same solutions, applying the same bacterial CFU to each seed type. One set of dishes containing a full complement of 'INOC', 'NON' and 'Water' seeds was then chosen for the greenhouse experiment and sown immediately, with the other used for the raised bed portion and being sown within 30 hours post inoculation.

For the greenhouse experiment and by radish variety, 75 9cm x 9cm square black plastic pots [524cc (SVD-350; Greenhouse Megastore, Danville, IL)] were filled with lightly packed, low N perlite and peat moss media (Sunshine Mix #4; Sun Gro Horticulture, Agawam, MA) and pre-wetted with distilled water. A 15mm hole was pressed into the center of each pot with a

wooden dibbler, and pots were placed in a 5x15 grid on greenhouse benches under controlled temperature (23ºC day – 15.5ºC night) and ambient sunlight (23 - 50 mol $m^{-2}$ $day^{-1}$). Pots were assigned treatments using RAND() in Excel (Microsoft, 2007) with 25 pots per treatment, creating a 75 unit Completely Randomized Design (CRD). Into each pre-dibbled hole two seeds were sown, with the media pressed closed over top by pinching. In all, 225 pots were sown, and pots were thinned to a single plant 10 days after planting. Pots were watered once per week as needed and rotated randomly once per week for five weeks ('Rover F1'), six weeks ('Bacchus F1'), or 6.5 weeks ('Sora OG'), until the radishes were ready for harvest.

For the raised bed experiments and again by cultivar, 117 15mm holes were dibbled into firmly packed, nutrient enriched (Osmocote 19-6-12, 5.1 g $ft^{-2}$, Scotts Company LLC) perlite and peat moss media (Sunshine Mix #4; Sun Gro Horticulture, Agawam, MA) in a 4m x 2.7m grid in a cold-frame enclosed raised bed. The central 77 holes were assigned using RAND() in Excel to one of the three treatments (26 bacteria + adjuvant 'INOC', 26 adjuvant only 'NON', 25 water only 'Water') in a CRD. Plants were spaced 9.5cm apart in all directions, yielding ~100 plants per square meter. The remaining 30 holes were planted with untreated seed to provide a border between radish types and the edge of the walls of the bed. Into each pre-dibbled hole two seeds of the appropriate type were sown, with the media pressed closed over top by pinching. Plants were thinned to a single seedling per planting hole 12 days after planting and were watered twice over the duration of the experiment with tap water. Supplemental lighting was not provided, and low temperatures were modulated by the closing and sealing of the cold frame. All three cultivars were sown and grown side by side in the same raised bed.

**4.2.7 Seed inoculation and sowing, Spring/Summer 2020 experiment**

The same procedures were employed for this replication as in the Fall 2019 experiment, with the following changes. First, the CFU was different as noted in Table 4.1. Secondly, the raised bed protective cold frame was removed for this experiment. Lastly, watering of the raised bed took place intermittently as needed opposite natural rain events to maintain moisture levels.

**4.2.8 Harvest and measurements**

Plants were harvested when visual inspection demonstrated at least 66% percent of plants had fully formed, or by recommended guidelines from the seed supplier plus one week if growth was not progressing normally. Plants were carefully removed from the surrounding media and the roots washed under low pressure to remove adhering media. Plants were sectioned into aerial tissues (APP) or root/bulb tissues (BPP) and dried @ 140ºC for 48 hours to determine root dry matter (RDM), shoot dry matter (SDM) and total dry matter (TDM). Prior to drying, total leaf number and maximal leaf length was recorded when possible, and taproot length and bulb cross-section diameter was also recorded.

**4.2.9 Statistical analysis**

The data were analyzed using SAS software, Version 9.4 of the SAS System for Windows, (Copyright 2016 SAS Institute Inc., Cary, NC, USA), using ANOVA in PROC MIXED to generate F-tests with an associated critical value $p < 0.05$ and a Tukey-Kramer HSD

mean separation at 0.05. PROC UNIVARIATE was employed to visually determine outliers, if any exhibited heterogeneity of variance in histogram plots of residuals.

## 4.3 Results

### 4.3.1 Inoculum bacterial detection

Bands corresponding to the anticipated 878 bp nested PCR product were detected in all samples subjected to amplification and visualization. No bands were evident in 'NON', 'Water', or untreated seed samples. Given the specificity of nested PCR this indicates a high likelihood of successful bulking out and application of *G. diaz* at the seed surface with no cross or extant contamination. *In planta* bacterial detection was not performed in these trials.

### 4.3.2 Greenhouse Trials

Growth of the 'Rover F1' cultivar was significantly suppressed across both trials when inoculated with *G. diaz* (Table 4.2). In the first trial, inoculated 'Rover' plants produced 34.2% less RDM, 30.8% less TDM, and had bulbs 23.3% smaller in diameter than the water control. SDM was not significantly different according to Tukey-Kramer analysis but inoculated plants exhibited a 17.8% decrease. Compared to the bacteria-free adjuvant treatment, inoculated plants exhibited 31.8% decreased SDM, 36.4% lower RDM with a net 35.3% TDM reduction. Bulb diameter was reduced 29.5%, over 8mm, between 'NON' and 'INOC'. No significant differences presented between the water treatment and the adjuvant, save for in SDM where Non was 20.5% greater than Water. In the second trial inoculated 'Rover' plants exhibited reductions of 34.4% in

SDM, 34.8% in RDM, 34.7% in TDM, 18.4% in bulb diameter, 12.8% in leaf length, and 16.5% in the number of leaves as compared to water only. The reductions compared to the adjuvant treatment were again significant, with SDM decreased by 22.7%, RDM by 30.3%, TDM by 28.5%, BDIAM by 19.1% and leaf length by 12.7%. Water and adjuvant groups did not significantly differ for most measures save SDM, where water averaged 573.0 mg per plant to 'NON's 486.2 mg, an increase of 17.9%.

In table 4.2, least attributable contributor significances are indicated by colored boxes, with criterion based on largest value recorded for the response measured. Blue boxes evidence differences attributable to 'INOC' treatment, orange to the 'NON' control, and grey boxes to 'WATER' control. No box indicates no significance was present. Note that for the greenhouse trials, only 'NON' and 'WATER' had significant positive impacts on responses.

Table 4.2: Mean dry matter of leaves (SDM), radish bulb and attached root (RDM), total plant (TDM), bulb diameter (BDIAM), root length (RTL), leaf length (LFL), and total true leaf number (LFNUM) of radism crops by cultivar and trial number.

| Greenhouse Trial #1 | | [1]Dry Matter (mg) | | | [1]Measures (mm) | | | [1]Leaf Number |
|---|---|---|---|---|---|---|---|---|
| Cultivar | Treatment | SDM | RDM | TDM | BDIAM | RTL | LFL | LFNUM |
| Rover F1 | | ** | ** | ** | ** | | | |
| | INOC | 268.5 a | 810.5 a | 1079.0 a | 20.7 a | 116.9 | *N/R* | *N/R* |
| | NON | 393.9 b | 1274.3 b | 1668.2 b | 29.3 b | 106.2 | *N/R* | *N/R* |
| | Water | 326.8 a | 1232.7 b | 1559.5 b | 27.0 b | 109.2 | *N/R* | *N/R* |
| Bacchus F1 | | ** | ** | ** | * | * | * | |
| | INOC | 480.4 a | 1222.2 a | 1702.6 a | 26.4 ab | 95.1 ab | 148.2 ab | 7.6 |
| | NON | 509.1 a | 1214.6 a | 1723.8 a | 27.2 b | 110.0 b | 154.1 b | 8.0 |
| | Water | 354.2 b | 1026.0 b | 1380.2 b | 25.0 a | 91.6 a | 139.4 a | 7.3 |
| Sora | | | * | * | | | | |
| | INOC | 550.1 | 824.6 a | 1374.7 a | 19.6 | 163.7 | *N/R* | *N/R* |
| | NON | 561.3 | 1061.5 ab | 1622.8 ab | 23.4 | 150.7 | *N/R* | *N/R* |
| | Water | 542.3 | 1185.9 b | 1728.2 b | 23.2 | 162.6 | *N/R* | *N/R* |
| **Greenhouse Trial #2** | | | | | | | | |
| Rover F1 | | ** | ** | ** | ** | | ** | ** |
| | INOC | 375.6 a | 1031.6 a | 1407.2 a | 22.6 a | 131.5 | 130.4 a | 4.21 a |
| | NON | 486.2 b | 1481.0 b | 1967.3 b | 27.9 b | 136.6 | 149.4 b | 4.57 a |
| | Water | 573.0 c | 1581.8 b | 2154.8 b | 27.7 b | 148.4 | 149.5 b | 5.04 b |
| Bacchus F1 | | | ** | ** | | * | | * |
| | INOC | 519.1 | 1052.9 a | 1572.0 a | 22.6 | 132.4 ab | 135.8 | 5.96 ab |
| | NON | 513.1 | 920.3 a | 1433.5 a | 20.2 | 124.3 a | 135.0 | 5.43 a |
| | Water | 549.7 | 1319.6 b | 1869.3 b | 23.2 | 158.1 b | 141.0 | 6.17 b |
| Sora | | | * | * | * | | | |
| | INOC | 659.9 | 983.5 ab | 1643.4 ab | 18.9 ab | 129.7 | 150.4 | 5.80 |
| | NON | 653.9 | 849.3 a | 1503.2 a | 15.2 a | 153.9 | 149.1 | 5.77 |
| | Water | 741.2 | 1196.3 b | 1937.5 b | 20.1 b | 147.9 | 153.1 | 6.05 |

[1]Average values within a column differ when letters are different. *N/R* denotes not recorded

*, ** Significant at $p < 0.05$ or 0.01, respectively, for that column.

By Least Attrib. Contrib. Theory, values in grey are effected by Water treatment, orange by NON, and blue, INOC.

The ‘Bacchus F1’ cultivar yielded different results per trial despite conditions being nearly identical, but the effects were only due to the adjuvant. In trial #1 none of the metrics of ‘INOC’ vs ‘NON’ were significantly different, and differences in growth parameters between ‘Water’ and ‘INOC’ or ‘NON’ can therefore be attributed to the carrier solution (Table 4.2). Under the effects of the ‘INOC’ treatment, SDM was up 35.6%, RDM 19.1%, and TDM 23.4% over the Water control, with adjuvant increased 43.7%, 18.4%, and 24.9% over the same respective categories. Cumulatively, plants subject to the adjuvants (both with and without bacteria, weighted) exhibited increases of 39.9% SDM, 19.1% RDM and 24.2% TDM over those receiving only the $dH_2O$ treatment. The effect of adjuvant was also significant with respects to the linear measurements with increases in bulb diameter (+9.0%), root length (+20.0%) and leaf length (+10.6%) with adjuvant than with water. However, ‘INOC’ plants did not differ significantly from either ‘NON’ or ‘Water’. No differences were detected in leaf number between any of the treatments.

Similar to the first experiment, no significant differences were found in SDM, RDM or TDM between ‘INOC’ or ‘NON’ treatments in the second trial, but they were both mean separated from ‘Water’. However, in contrast adjuvant treated ‘Bacchus’ plants (‘INOC’ plus ‘NON’) exhibited a weighted cumulative 24.9% reduction in RDM and 19.3% in TDM, opposing the prior experiments increases. ‘Water’ also recorded a significant ~34mm maximal leaf length increase and averaged ~0.75 more true leaves than ‘NON’, although neither treatment was different than ‘INOC’ which fell between them. Bulb diameter, leaf length, and SDM were not significant between any treatments in this replication.

The Sora OG variety exhibited significant differences in the fewest measurements, but again there were no significant differences between 'INOC' and 'NON' in any of the metrics. In the first experiment there did seem to be a cumulative suppressive effect when both bacteria and adjuvant chemicals were applied though, as 'INOC' was significantly different than 'Water' for RDM (-30.4%) and TDM (-20.4%). None of the other measures proved significant. In the second trial the addition of bacteria seemed to have a phytoprotective rather than suppressive effect, as significant differences were found between 'NON' and 'Water' only, with 'INOC' reporting between them and not different from either. Shoot dry matter, root length, leaf length, and number of true leaves was not significantly different for any Sora OG greenhouse trial.

### 4.3.3 Raised bed trials

The first raised bed trial, conducted in the Fall of 2019, yielded no significant differences between any of the treatments over the six measures recorded (Table 4.3). The two F1 hybrids yielded radishes that met the USDA size requirement of 16mm minimum under all treatments, with none of the Sora radish treatments averaging over 15mm.

In the second trial, Rover F1 exhibited no differences in SDM, RDM, TDM or BDIAM, and a small but significant increase in root length of 35mm (+24.0%) of 'INOC' over the 'Water' control (Table 4.3). Adjuvant treated 'Rover F1' seed averaged significantly greater leaf length than Water (+11.5%), but was not different from 'INOC'. For Bacchus F1, shoot dry matter in inoculated plants was 40.3% greater than 'NON', and 25.7% greater than 'Water', but only the former proved significant. Leaf length was significantly greater between 'INOC' and both 'NON' and 'Water' at +45.6% and +41.8%, respectively. Leaf length between 'Water' and

‘NON’ was not different. For Sora, leaf length was again the only statistic to exhibit statistical difference, with ‘INOC’ showing nearly 100mm longer maximal leaf growth (465mm vs 367mm, +26.7%) over ’NON’. ‘Water’ was intermediate of these and was not significantly different from either.

In table 4.3, least attributable contributor significances are indicated by colored boxes, with criterion based on largest value recorded for the response measured. Blue boxes evidence differences attributable to ‘INOC’ treatment, orange to the ‘NON’ control, and grey boxes to ‘WATER’ control. No box indicates no significance was present. Note that for the raised bed trials, ‘INOC’ effected the significant leaf length gain in Bacchus F1 trial #2, the only time bacterial presence was the primary component of significant increase.

Table 4.3: Mean dry matter of leaves (SDM), radish bulb and attached root (RDM), total plant (TDM), bulb diameter (BDIAM), root length (RTL), leaf length (LFL), and total true leaf number (LFNUM) of radism crops by cultivar and trial number.

| Raised Beds Trial #1 | | [1]Dry Matter (mg) | | | [1]Measures (mm) | | | [1]Leaf Number |
|---|---|---|---|---|---|---|---|---|
| Cultivar | Treatment | SDM | RDM | TDM | BDIAM | RTL | LFL | LFNUM |
| Rover F1 | | | | | | | | |
| | INOC | 389.7 | 457.4 | 847.1 | 22.0 | 138.8 | 102.8 | N/R |
| | NON | 387.8 | 464.5 | 852.3 | 20.5 | 145.3 | 102.1 | N/R |
| | Water | 397.2 | 432.4 | 829.6 | 20.5 | 147.4 | 104.1 | N/R |
| Bacchus F1 | | | | | | | | |
| | INOC | 406.9 | 328.9 | 735.8 | 18.3 | 116.5 | 95.7 | N/R |
| | NON | 422.3 | 330.8 | 753.1 | 17.9 | 109.5 | 99.3 | N/R |
| | Water | 403.6 | 310 | 713.6 | 17.2 | 119.1 | 93.9 | N/R |
| Sora | | | | | | | | |
| | INOC | 387.5 | 204.1 | 591.6 | 13.9 | 120.9 | 106.1 | N/R |
| | NON | 366 | 221.5 | 587.6 | 14.5 | 121.6 | 104.7 | N/R |
| | Water | 381.4 | 225.3 | 606.7 | 15.0 | 121.7 | 102.8 | N/R |
| **Raised Beds Trial #2** | | | | | | | | |
| Rover F1 | | | | | | * | * | |
| | INOC | 3166.7 | 2941.9 | 6108.6 | 45.0 | 180.8 a | 385.2 ab | N/R |
| | NON | 2934.8 | 2581.6 | 5516.4 | 43.5 | 152.4 ab | 401.2 a | N/R |
| | Water | 2682.1 | 2508.4 | 5190.5 | 43.8 | 145.8 b | 359.7 b | N/R |
| Bacchus F1 | | * | | | | | ** | |
| | INOC | 8551.7 a | 4314.3 | 12866.1 | 46.4 | 197.9 | 560.5 a | N/R |
| | NON | 6094.9 b | 4152.7 | 10247.6 | 48.5 | 211.1 | 385.0 b | N/R |
| | Water | 6803.8 ab | 4556.6 | 11360.4 | 49.7 | 220.3 | 395.4 b | N/R |
| Sora | | | | | *note 1* | | ** | |
| | INOC | 6167.5 | 2966.4 | 9133.8 | 41.1 a | 174.7 | 464.9 a | N/R |
| | NON | 4184.4 | 3215.2 | 7399.6 | 49.0 b | 173.5 | 367.0 b | N/R |
| | Water | 6239.2 | 3183 | 9422.2 | 45.6 ab | 195.1 | 416.9 ab | N/R |

[1]Average values within a column differ when letters are different, by Tukey-Kramer 5% test. note 1 = $p < 0.0565$

*, ** Significant at $p < 0.05$ or 0.01, respectively, for that column. N/R denotes not recorded

By Least Attrib. Contrib. Theory, values in grey are effected by Water treatment, orange by NON, and blue, INOC.

**4.4 Discussion and Conclusions**

*Gluconacetobacter diazotrophicus* inoculation as a potential yield promoting biostimulant for these three radish types did not improve yield and sometimes reduced bulb yield. In a few cases, leaf length was increased due to the inoculation. In many cases, treatment effects were due to the adjuvant used, but sometimes this effect was positive, and in other cases it was negative. The greenhouse trials simulate low available N conditions, similar to what could be found if *R. sativa* is sown as an interim, non-fertilized shoulder crop between primary plantings. 'Rover F1' produced both smaller bulbs by weight and diameter and reduced TDM due to inoculation in both trials, and the suppression cannot be attributed to the chemicals present in the adjuvant as 'NON' and 'Water' were not significantly different. Rover also demonstrated significantly shorter leaves in the trial where that information was recorded. Sora OG was similarly suppressed in both trials although to a lesser extent, but a potential inhibitory factor of the adjuvants cannot be excluded because the differences between inoculated and adjuvant-only plants were not significantly different. As seen in Table 4.2, the lowest yields for Sora RDM and TDM were from the INOC (bacteria + adjuvant) treatment in trial #1, but were in just the adjuvant in trial #2. It cannot be excluded that the bacterial CFU between the trials, $2.6 \times 10^8$ CFU $ml^{-1}$ versus $9.0 \times 10^8$ CFU $ml^{-1}$ in #1 vs. #2, respective, may have altered plant defense responses to bacterial penetration of root tissues and thus may account for the differences. However, CFU was aligned well above the sufficiency recommendations for this bacterium at the time this research was conducted (Erika Wagner, Azotic-NA, personal communication, Sept 2018). Effects of treatments on Bacchus F1 seemed to be all due to the adjuvant because there were no significant differences between 'INOC' and 'NON', but there were significant effects due to the adjuvant compared with water only.

The raised bed experiments revealed even fewer significant differences than the greenhouse trials, and those that were revealed are of more dubious importance. Trial #1 simulated a shoulders planting regime, where radish would be sown either before or after a long-season main crop into a nutrient rich but microbially inactive soil. That no differences emerged may be attributed to *G. diaz's* sourcing, as it was initially discovered in and is native to tropical and sub-tropical soils (Cavalcante and Dobereiner, 1987), and the USDA 6B growing zone in central Pennsylvania may not provide an ideal environment, at least in a small vegetable crop such as radish. For fall shouldering, although *G. diaz* can survive cold temperatures *in planta* (Sebring et al., 2017), bacterial activity may be significantly suppressed. The second trial demonstrated increased root length in the Rover cultivar and increased SDM in Bacchus, both of which are agronomically unimportant for a crop grown primarily for its bulb. Some varieties of radish are prone to heat effected bolting, regulated by PGRHs that may be mimicked by the bacterium under nutrient sufficient conditions (Bastian et al., 1998; Fuentes-Ramirez et al., 1993), but as these varieties were chosen for summer heat tolerance, altered plant expression of these hormones due to micro-environmental differences may also be excluded.

The significant results of this experiment reveal an interaction between the bacterium and at least some aspects of each of the three cultivars tested, but do not predict a clear synergism for increasing agronomic output. As reported previously (Akhtar at al, 2013; Kumar, et al 2016; Ziaf, et al 2016), radish produces the potent anti-bacterial compound raphanin which has broad spectrum inhibitory effects (Lim et al., 2019, Rani et al., 2008). The possibility exists that this bacterium is only partially capable of eluding the plant defense responses, despite being gram-negative, and the ensuing plant responses impart a significant opportunity cost to growth. In any case, the potential for *G. diaz* to form beneficial plant-microbe mutualisms in these three spring

radish varieties was not demonstrated. That ADJ treatment had a positive impact in some instances may indicate that one or more of the chemical components contained therein may be a better fit for increasing yields in this crop.

## Chapter 5

## Biomass and Quality Impacts of *Gluconacetobacter diazotrophicus* in Jalapeño Pepper

### 5.0 ABSTRACT

Among plant growth modulating microorganisms, the non-pathogenic bacterium *Gluconacetobacter diazotrophicus* has emerged as a potential inoculant for enhancing crop production as it fixes atmospheric N, produces plant growth regulating hormones (PGRHs), and can solubilize immobilized minerals. Jalapeño pepper exhibits considerable external scarring of fruits, known as 'corking', which has been linked to nutrient deficiency, calcium uptake, and auxin mediated fruit fill, all stressors or mechanisms this bacterium may impact. To assess effect and usefulness of this bacterium on Jalapeño pepper, a greenhouse N rate trial was conducted testing effects on fruit quantity, quality, and coloration. Plants were grown hydroponically in deep water culture buckets over a range of N fertilization levels (37.5-172.5 ppm N) and either inoculated with *G. diaz* or bacteria free. Inoculated plants demonstrated an increase in fruit number at levels of moderate N deficiency, but no gain in total fruit biomass per plant. Inoculated plants also exhibited a greater number of red peppers under significant N stress and at optimal nutritional levels, with a general trend of more red peppers throughout. High levels of N fertilization led to significantly more corked peppers under inoculation than controls. All increases or decreases in fruit number were met with equivalent but opposite changes in fruit weight, yielding no net biomass gain. The use of *G. diaz* in jalapeño can confer differences in fruit set but the mechanisms are not consistent or clearly defined across N levels, and quality

defects increased at standard fertilization rates limit the usefulness of this plant-microbe synergism.

## 5.1. Introduction

*G. diaz* is an N-fixing obligate endophyte first isolated from Brazilian sugarcane in 1988 (Cavalcante and Döbereiner 1988) but subsequently discovered worldwide in myriad unrelated species such as mango from Cuba (Rocafull et al., 2016), carrot from India (Madhaiyan et al., 2004), banana from Kenya (Matiru and Thompson 1998), and tomato from Colombia (Restrepo et al., 2017). As a motile, aerotolerant bacteria that excretes a sucrose metabolizing biofilm, *G. diaz* is able to colonize not only rhizospherically within root hairs and among arbuscular endomycorrhizae (Lopes et al., 2019; Paula et al., 1991), but also within apoplastic and intracellular spaces throughout root, shoot, leaf, and fruit tissues depending on host species (Cavalcante and Dobereiner, 1988; Cocking et al., 2006; Eskin et al., 2014; Luna et al., 2010). N-fixation aside, *G. diaz* exhibits the ability to produce PGHRs such as Indole-3-Acetic acid (IAA), Gibberellins A1, A3, and gluconic acid, all of which have putative plant growth regulatory and improvement potential (Bastián et al., 1998; Restrepo et al., 2017; Rodriguez et al., 2016; Silva et al., 2020). Gluconic acid production and excretion also effects Zn and P solubilization in the rhizosphere, which coupled with bacterial siderophore production liberates three important yet often inaccessible mineral nutrients for plant growth (Crespo et al., 2011; Delaporte-Quintana et al., 2020; Eskin et al., 2014; Nieto-Penalver et al., 2014). *G. diaz* inoculation also imparts osmotic abiotic tolerance in rice and sugarcane (Filgueiras et al., 2020; Tufail et al., 2021; Vargas et al., 2014) and induces biotic resistance against pathogens in multiple crops including tomato, potato, (Pelligrini et al., 2020) and cotton (Chawla et al., 2011).

*G. diaz* inoculation tests in other non-native host crop species have been conducted in order to test colonization efficiency and net impact on plant growth. Among gramineous plants, many of which are important food cereals, *G. diaz* has been found to colonize and increase productivity in wheat (Youssef et al., 2004, Kumar et al., 2015b), dent corn (Cocking et al., 2006; Riggs et al., 2001; Tian et al., 2009), sweet corn (Bidarkar and Murumkar, 2020), sorghum (Luna et al., 2010; Yoon et al., 2016), and wetland rice (Rodrigues et al., 2016; Silva et al., 2020). Outside of cereals, *G. diaz* inoculation has been observed to increase Fe and P uptake in strawberry (Delaporte-Quintana et al., 2017; Delaporte-Quintana et al., 2020) improve N use efficiency in tomato (El-Shouny et al., 2020; Fernández-Delgado et al., 2019), cassava (Lopes et al., 2019) and snap bean (de Oliveira et al., 2019), and improve tuber and taproot formation in beet, carrot, potato and sweet potato (Kumar et al., 2015a; Logeshwarn et al., 2011; Pelligrini et al., 2020; Pelligrini et al., 2021) placed under biotic and abiotic stress. Noting the ability of *G. diaz* to colonize and positively impact growth tomato (El-Shouny et al., 2020, Luna et. al 2012), in this study seeds of its close relative Jalapeño pepper (*C. annuum* var “Jalapeño Early”) were inoculated with *G. diaz* to test bacterial impact on alleviating N stress, fruit quantity and fruit quality. This cultivar is prone to producing damaged fruit which may be exacerbated by bacterial auxin during fruit filling, and like most peppers is plagued by PGRH mediated fruit and flower abscission (Huberman et al., 1997; Marcelis et al., 2004). A range of N fertilizations was employed over two inoculation levels, and USDA-AMS grading metrics were employed to quantify fruit quality (USDA 2016).

## 5.2. Materials and Methods

### 5.2.1 Experimental design and randomization

This experiment was conducted as a 2x6 factorial under 4 replications, for a total of 48 experimental units in randomized complete block design. The binary factor is the presence/absence of *G. diaz* from the initial seed soaking solution, with the six-level factor being different levels of N fertilization ranging from severely deficient to excessive (Table 5.2.1), with 190 ppm N being recommended (Mattson and Peters 2014). Blocking was used perpendicular to air flow from unidirectional evaporative cooling vents to control error. All randomization was carried out using the RAND() function in Excel (Microsoft, 2013).

### 5.2.2 Plant and bacterial materials

Jalapeño pepper (*Capsicum annum* var. 'Jalapeño Early') was sourced from Burpee Seeds, LLC. This cultivar is open-pollinated and exhibits a compact growing habit making it suitable for greenhouse production. Common to this cultivar are fine white scarring lines on the surface of the fruit (Figure 5.1), called 'corking'; it produces both corked and uncorked peppers under normal growing conditions. Plants produce an initial crop of green fruit 60 days after transplanting which typically take an additional 20 days to set red. Harvesting encourages continual production. This cultivar is graded under USDA-AMS guidelines for both coloration and corking, which is considered damage although it does not alter fruit shelf-life or nutrition. Seeds were dry sorted to remove discolored, misshapen, or otherwise damaged units and stored under refrigeration (4.4$^{o}$C, 65% RH) until seed treatments were applied. *G. diaz* was provided by Azotic Technologies, LLC (Nottingham, UK) and is a commercially available strain of the

bacterium marketed under their Envita™ product line (Azotic-na.com 2019). Pure strain stocks were maintained in 25% glycerol at -80°C.

*Figure 5.1: Pepper Quality. Uncorked green (left), corked green (center), and corked red (right)*

### 5.2.3 Location

This experiment was conducted at The Pennsylvania State University College of Agricultural Sciences in a temperature and light controlled greenhouse. Conditions were a 16/8 hour light/dark photoperiod, light intensity of 165-215 µmol $m^{-2}$ $s^{-1}$ via natural sunlight and/or

high-pressure sodium discharge bulbs and/or shade screens, and temperature range of 16-24°C. Humidity was maintained via microenvironmental evaporation from the hydroponic deep-water culture buckets in which the plants grew, as well as transpiration. Airflow for evaporative cooling, when necessary, was perpendicular to block arrangement.

### 5.2.4 Inoculant preparation

Bacterium was grown from glycerol preserved stock in a modified MESMA/ATGUS medium [2.7 g $L^{-1}$ glucose; 4.8 g $L^{-1}$ dipotassium phosphate (K2HPO4); 0.65 g $L^{-1}$ monopotassium phosphate (KH2PO4); 1.8 g $L^{-1}$ mannitol; 4.4 g $L^{-1}$ 2-(N-morpholino)ethanesulfonic acid (MES hydrate); 2.7 g $L^{-1}$ yeast extract] (Cocking, et al. 2006). Media and deionized water were mixed and autoclaved prior to aseptic bacterial introduction to ensure single strain stocks. The media was incubated in a positive-pressure, enclosed shaker table (30°C, 120 rpm) for five days. Standard plate count and dilution series on semi-solid ATGUS media (same as above, adding 0.8% weight/volume agar) in triplicate, corroborated with OD600 based estimation (NanoDrop One, Thermo Scientific) was used to determine final colony forming units (CFU) of prepared culture. For this experiment achieved CFU was $1.8 \times 10^9$ $mL^{-1}$.

Positive *G. diaz* presence in inoculated broth was determined via PCR analysis on residual stock solution post seed immersion. Solution fractions of both bacterial containing and un-inoculated control treatments that immersed the seed during inoculation were retained for analysis. Nucleic acid extraction was performed using an automated DNA/RNA isolater (MPure-12 Plant Nucleic Acid Extraction Kit, on MPure-12 Magnetic Nucleic Acid Purification System, MPBiomedicals). For amplification, 16S rRNA primers GDI25F (5'-TAGTGGCGGACGGGTGAGTAACG-3') and GDI923R (5'-

CCTTGCGGGAAACAGCCATCTC-3’) (Tian et al., 2009) were used with Phire Plant Direct polymerase (ThermoFisher F160S). These primers target an 899-bp product of the *G. diaz* genome (Bertalan et al., 2009). Amplification parameters were an initial denaturation/inhibitory agent removal step (98ºC for 5min) followed by 40 cycles of amplification (denature 98ºC for 5s, anneal 62ºC for 5s, extend 72ºC for 20s). After 40 cycles, a final extension of 1 minute at 72ºC was performed before samples were held at 4ºC pending analysis. The PCR amplification rRNA fragments were UV-visualized on 0.75% agarose gels containing SYBR™ Safe nucleic acid stain.

Seed inoculation media was prepared from a sterilized proprietary blend of adjuvants (sucrose, gum arabic, and Tween 80) in distilled water (Azotic Technologies, trade name: *N-fix;* Internal Standard Operating Proceduce document, 2017*).* Two equal 100ml portions of media were aliquoted into sterilized flasks under aseptic conditions. One portion received an equivalent amount of $1.8 \times 10^9$ $mL^{-1}$ CFU cultured bacterial concentrate forming the inoculate working stock, which as diluted contained $9.0 \times 10^8$ $mL^{-1}$ CFU *G. diaz*. The second portion received an equivalent amount of distilled water to become the adjuvant only, bacteria-free control stock. Both treatment stocks (‘INOC’, containing *G. diaz* in adjuvant; and ’NON’, only containing water and adjuvant) were assessed for equal pH and EC to ensure seeds soaked would experience identical conditions save for bacterial presence.

### 5.2.5 Seed inoculation and sowing

For both inoculation factors 70 seeds were placed in a 60mm x 15mm sterile petri dish and 2ml of solution (INOC or NON) was added via pipette. Dishes were immediately swirled to provide all seeds with uniform solution contact. After five minutes, unabsorbed solution was

pipetted out and a second 2ml portion of the same treatment was added, with repeated swirling and immersion. After a second five-minute soak any remaining solution was removed and the seeds were allowed to air dry for one hour. Seeds were sown in 3.6 × 3.6 × 4 cm rockwool cubes (Grodan A036/40; Roxul Inc., Milton, ON, Canada) with two seeds per cube, 24 cubes total per treatment, in the same greenhouse described prior. Once seedlings reached about 5 cm in height and had their first true leaves they and their cubes were pressed into trimmed and hollowed 76mm rockwool bricks (Grodan Delta4; Roxul Inc., Milton, ON, Canada) set into 95mm round plastic net pots (Gro Pro, Hawthorne GC, Vancouver WA). These net pots were placed into 92mm holes drilled centrally through opaque 19L bucket lids, distributed across 48 buckets each containing 18L of pre-assigned starter nutrient solution (Table 5.1 at ½ strength all nutrients). Each bucket additionally had a 10L/min air feed through the lid into a 40.6mm air bubble diffuser for aeration (Pawfly ASD-040, Amazon.com). Each 19L bucket was fitted with a clear plastic external standpipe to allow draining and observation of water level and was raised 40cm above the floor (Figure 5.2). The bottom 2/3rds of each net pot was thus submerged in oxygenated nutrient solution. The exposed top of each rockwool brick was covered with black plastic for algae control. After one week all buckets were thinned to a single plant.

*Figure 5.2: Buckets used as experimental units. Visible are the airlines, seated 76mm rockwool blocks in net pots, and standpipes. View is down a block (I20, V12, etc..) placed perpendicular to the airflow from cooling fans seen in background.*

Table 5.2.1: Details of nutrients and effective ppm for fertilizer stock solns

| | Fertilizer (FERT) Treatment Level (ppm)* | | | | | |
|---|---|---|---|---|---|---|
| | **47.5** | **83** | **119** | **154** | **190** | **226** |
| Nutrient & Source | *(N levels rounded for clarity)* | | | | | |
| Nitrogen (N) | | | | | | |
| Jack's 5-12-26 | 47.5 | 47.5 | 47.5 | 47.5 | 47.5 | 47.5 |
| Calcium Nitrate** | 0 | 35.7 | 71.2 | 106.8 | 142.5 | 147 |
| $KNO_3$ | 0 | 0 | 0 | 0 | 0 | 31.1 |
| ***TOTAL N (ppm)*** | *47.5* | *83.2* | *118.7* | *154.3* | *190* | *225.6* |
| ***TOTAL N (mg) /Bucket*** | *855.0* | *1497.6* | *2136.6* | *2777.4* | *3420.0* | *4060.8* |
| Phosphorus (P) | | | | | | |
| Jack's 5-12-26 | 49.8 | 49.8 | 49.8 | 49.8 | 49.8 | 49.8 |
| ***TOTAL P (ppm)*** | *49.8* | *49.8* | *49.8* | *49.8* | *49.8* | *49.8* |
| Potassium (K) | | | | | | |
| Jack's 5-12-26 | 205 | 205 | 205 | 205 | 205 | 205 |
| $KNO_3$ | 0 | 0 | 0 | 0 | 0 | 86.8 |
| $K_2SO_4$ | 86.5 | 86.5 | 86.5 | 86.5 | 86.5 | 0 |
| ***TOTAL K (ppm)*** | *291.5* | *291.5* | *291.5* | *291.5* | *291.5* | *291.8* |
| Calcium (Ca) | | | | | | |
| Calcium Sulfate*** | 175.4 | 132.7 | 90.4 | 48.03 | 5.35 | 0 |
| Calcium Nitrate** | 0 | 42.62 | 85 | 127.37 | 169.99 | 175.38 |
| ***TOTAL Ca (ppm)*** | *175.4* | *175.32* | *175.4* | *175.4* | *175.34* | *175.38* |
| Other Minerals**** | | | | | | |
| Magnesium (Mg) | *59.5 ppm for all fertilizer levels* | | | | | |
| Iron (Fe) | *2.91 ppm for all fertilizer levels* | | | | | |
| Manganese (Mn) | *0.48 ppm for all fertilizer levels* | | | | | |
| Boron (B) | *0.48 ppm for all fertilizer levels* | | | | | |
| Copper (Cu) | *0.143 ppm for all fertilizer levels* | | | | | |
| Zinc (Zn) | *0.139 ppm for all fertilizer levels* | | | | | |
| Molybdenum (Mo) | *0.095 ppm for all fertilizer levels* | | | | | |

* Recipe taken from Mattson and Peters (2014).

** As hydrated calcium ammonium nitrate double salt, $5[Ca(NO_3)_2].NH_4NO_3.10[H_2O]$

*** As calcium sulfate dihydrate

**** Included as a micro suite in Jack's 5-12-26

### 5.2.6 Fertilization and maintenance

Plants were grown for two months with no nutrient additions other than the fertilizer supplied at the start of the experiment (Figure 5.3). Water was added as needed to maintain a min/max volume of 15L/18L in each bucket. After two months all remaining solution was drained and replaced with 18L of full production strength nutrient solution (Table 5.1) according to the treatment assignment. Insect control was achieved through a mixture of parasitic insectivores (*Aphidius colemani, A. ervi, A. abdominalis, Neoseiulus cucumeris)* and abamectin pesticide, as needed. Once flowers appeared they were sonicated with a battery powered toothbrush twice a week to ensure pollination. Upright plant stature was maintained with tomahooks strung from the ceiling, and all dropped leaves and damaged plant tissues, if any, were collected and retained for biomass.

*Figure 5.3: Jalapeño in 19L bucket with IPM and Standpipe visible.*

### 5.2.7 Harvesting and plant cull

Harvesting began 90 days after transplant with any fruit greater than 4.5cm in length, and/or showing corking above 10% surface area, and/or showing red coloration above 10% surface area harvested. Fruit was cut ½ centimeter above the calyx and sorted into one of four categories based on aggregate surface area. Figure 5.4 details the USDA-AMS guidelines for assessment of aggregate external scar damage herein referred to as corking (USDA-AMS 2016). USDA guidelines place peppers into four categories but for the purpose of this experiment bimodal assignments were employed to prevent excessive null values in ANOVA. ‘Uninjured’ (scarring < 5%) and injured (scarring ≥ 5% but < 10%) were binned as Uncorked, whereas ‘Damaged’ (scarring ≥ 10% but < 15%) and ‘Seriously Damaged’ (scarring ≥ 15%) were binned as Corked. Pepper coloration has no strict rigidity per these published guidelines so an equivalent bimodality of < 10% red or reddening was binned as Green, ≥ 10% red or reddening was binned as Red. Each fruit was individually weighed before being halved, comingled and dried at 60$^{o}$C until they reached a stable weight, yielding fruit dry matter (FDM).

**SCARS (Q)**

There are varieties of Other Peppers, particularly the Jalapeno, that tend to develop what the trade calls “dry lines” or “heat marks.” These longitudinal striations or corky cracks resemble skin checks and are actually scars. The scars may be an indication of heat intensity, but the data is inconclusive. In the market there are those who desire the presence of these scars and those who do not. However, the standards for Other Peppers consider scars a defect and provide the following scoring guide:

**Scoring Guide**

**Injury:** When aggregate area exceeds 5% of the surface.

**Damage:** When aggregate area exceeds 10% of the surface.

**Serious damage:** When aggregate area exceeds 15% of the surface.

*Figure 5.4: USDA-AMS Grading descriptions for 'corking', a (Q)ualitative defect*

Thirty days after the first harvest, a second harvest was performed using the same parameters, except that no minimum length requirement was enforced; all fruit were harvested and graded. Counts and fresh weight were obtained by quality category before all fruits were dried as before. Concurrent with the second harvest, all aerial plant matter was cut and all root matter was removed from the buckets and rinsed. These portions were individually dried at $60^{o}$C until stable weights were achieved, to yield shoot dry matter (SDM) and root dry matter (RDM).

### 5.2.8 Statistical analysis

The data analysis for this paper was generated using SAS 9.4 software for Windows. (Copyright 2016 by SAS Institute Inc., Cary, NC, USA.), and all significance tests were performed at alpha < 0.1 as this was a novel, previously unassessed plant-microbe interaction. ANOVA for continuous data were performed with PROC MIXED with type 3 tests of fixed

effects used, block being assigned as random, and Tukey-Kramer HSD employed to compare least squares means. For dependent variable data exhibiting graphical violations of homogeneity, PROC TRANSREG was used to generate BOX-COX lambdas for data normalization. Lambdas were chosen by *convenience* (SAS Institute 2016) but only used for analysis; data presented in graphs and tables are true means. For integer counts, data were analyzed with PROC GENMOD using Poisson distribution and Quasi-Newton optimization, with Bonferroni correction ($T$=6, adjusted alpha=0.0167) employed for post-hoc LSD when interactions were present. Count data exhibiting a large number of 0 values alternately used PROC GENMOD using Zero-Inflated Poisson distribution, with *zeromodel* set equivalent to the test parameters.

## 5.3. Results and Discussion

### 5.3.1 Biomass accumulation

Total fruit biomass was unaffected by inoculation with *G. diaz* (Table 5.2). Response to N fertilization followed the patterns demonstrated for jalapeño pepper found by Johnson and Decoteau (1996). Increases in numbers of peppers were met with concomitant decreases in the average size of those peppers as indicated by no net biomass change with inoculation. Non-fruit biomass was also not impacted by inoculation status, as again fertilization level was found to be significant (FERT; $p \leq 0.05$, all dependent variables), but no significant bacterial impact was evident on root tissue, non-fruit aerial tissue, total aerial tissue, or total plant biomass (Table 5.2).

Table 5.2: Jalapeno Early Biomass accumulation by Fertilization level (g), by ANOVA. Tests are for Inoculation Main Effect

| Trial and Measure | | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | **47.5** | **83.125** | **118.75** | **154.375** | **190** | **225.625** |
| (Fert Identifier)* | | *47.5* | *83* | *119* | *154* | *190* | *226* |
| Dry Matter All Fruit | *n/s* | | | | | | |
| INOC | | 25.598 | 52.493 | 64.816 | 82.551 | 86.419 | 105.392 |
| NON | | 27.519 | 40.544 | 60.796 | 86.931 | 79.427 | 96.148 |
| *Difference* | | *-1.922* | *11.949* | *4.020* | *-4.380* | *6.993* | *9.243* |
| DM Green Fruit | *n/s* | | | | | | |
| INOC | | 7.824 | 23.850 | 25.661 | 29.898 | 47.736 | 58.300 |
| NON | | 12.162 | 25.175 | 28.620 | 39.271 | 36.615 | 55.683 |
| *Difference* | | *-4.338* | *-1.325* | *-2.959* | *-9.373* | *11.121* | *2.617* |
| DM Red Fruit | *n/s* | | | | | | |
| INOC | | 6.262 | 12.596 | 9.731 | 17.579 | 18.148 | 16.561 |
| NON | | 3.003 | 5.329 | 9.594 | 21.231 | 17.079 | 12.678 |
| *Difference* | | 3.259 | 7.267 | 0.137 | -3.652 | 1.069 | 3.883 |
| DM Uncorked Fruit | *n/s* | | | | | | |
| INOC | | 2.463 | 5.670 | 2.481 | 4.616 | 0.273 | 1.413 |
| NON | | 0.361 | 0.469 | 2.753 | 0.307 | 6.385 | 12.640 |
| *Difference* | | 2.102 | 5.201 | -0.272 | 4.308 | -6.112 | -11.227 |
| DM Corked Fruit | *n/s* | | | | | | |
| INOC | | 11.623 | 30.776 | 32.911 | 42.861 | 65.612 | 73.448 |
| NON | | 14.805 | 30.035 | 35.461 | 60.194 | 47.309 | 55.721 |
| *Difference* | | -3.181 | 0.740 | -2.551 | -17.333 | 18.302 | 17.727 |
| DM Shoots/Leaves | *n/s* | | | | | | |
| INOC | | 65.733 | 105.893 | 150.743 | 162.018 | 183.393 | 214.750 |
| NON | | 68.500 | 123.070 | 134.680 | 170.348 | 194.023 | 236.643 |
| *Difference* | | -2.768 | -17.178 | 16.063 | -8.330 | -10.630 | -21.893 |
| DM Aerial Tissues** | *n/s* | | | | | | |
| INOC | | 91.330 | 158.386 | 215.558 | 244.569 | 269.812 | 320.142 |
| NON | | 96.019 | 163.614 | 195.476 | 257.279 | 273.449 | 332.791 |
| *Difference* | | -4.689 | -5.228 | 20.082 | -12.710 | -3.637 | -12.649 |
| DM Roots | *n/s* | | | | | | |
| INOC | | 41.975 | 44.513 | 64.890 | 63.743 | 73.768 | 87.115 |
| NON | | 38.513 | 59.273 | 44.140 | 67.648 | 70.028 | 87.195 |
| *Difference* | | 3.463 | -14.760 | 20.750 | -3.905 | 3.740 | -0.080 |
| DM Whole Plant*** | *n/s* | | | | | | |
| INOC | | 133.305 | 202.898 | 280.448 | 308.311 | 343.579 | 407.257 |
| NON | | 134.532 | 222.886 | 239.616 | 324.926 | 343.477 | 419.986 |
| *Difference* | | -1.227 | -19.988 | 40.832 | -16.615 | 0.103 | -12.729 |

* As reported in the text, for ease of cross-referencing ** Shoots/Leaves (SDM) + All Fruits (FDM)

*** Shoots/Leaves (SDM) + All Fruits (FDM) + Roots (RDM)

Note: All responses other than DM Red Fruit (p<0.1171) were highly signif (p <0.01) for N level. No interactions occurred.

### 5.3.2 Fruit number and quality

Inoculation with *G. diaz* increased the total number of fruit produced (Table 5.3) with increases evident at the 119ppm and 83ppm N fertilization levels (Figure 5.5), which correspond to slight and moderate N deficiency, respectively. The increase at 119ppm N was driven by an increase in green peppers produced (Figure 5.6), whereas 83ppm N was bolstered by an increase in red peppers (Figure 5.7). Earlier ripening in N starved plants is indicative of chlorophyll destruction and N resorption from fruit due to lack of incorporable assimilates (Chaki et al., 2015) and relative concentrations of auxins and ethylene are speculated to drive this response in Solanaceae in general (Su et al., 2015), but this has not been thoroughly investigated in Jalapeño. Red pepper number per plant also increased at 190ppm N (Figure 5.7) but not at the intermediate fertilization levels, detailing a bimodality of early ripening caused by *G. diaz* inoculation. At this latter fertilization level, sufficient plant synthate may have been available as relative N per pepper ratios were similar (Red @ 83ppm N, 7.7ppm N/fruit; 190ppm N, 7.4ppm N/fruit; Table 5.3), suggesting the possibility of exogenous PGRH alteration of plant gene expression.

Table 5.3: Jalapeno Early total fruit counts by Fertilization level, Poisson distributed counts*

| | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|
| Trial and Measure | **47.5** | **83.125** | **118.75** | **154.375** | **190** | **225.625** |
| (Fert Identifier)** | *47.5* | *83* | *119* | *154* | *190* | *226* |
| Total Number of Fruit | *INOC p ≤ 0.0006* | *INTX INOC*FERT p ≤ 0.0044* | | | | |
| **INOC** | 17.25 | **35.75** | **66.5** | 68.5 | 66.25 | 78.25 |
| NON | 16.25 | 26.25 | 44 | 59.75 | 59.25 | 83.75 |
| *Difference* | *1* | *9.5* | *22.5* | *8.75* | *7* | *-5.5* |
| | *n/s* | *p ≤ 0.0975 +30.9% ± 12.8%* | *p ≤ 0.0001 +41.3% ± 9.7%* | *n/s* | *n/s* | *n/s* |
| Total Green Fruit | *INOC p ≤ 0.0215* | *INTX INOC*FERT p ≤ 0.0039* | | | | |
| **INOC** | 14.25 | 25.00 | **53.00** | 52.00 | 40.50 | 63.00 |
| NON | 13.50 | 20.75 | 33.25 | 44.75 | 45.00 | 64.50 |
| *Difference* | *0.75* | *4.25* | *19.75* | *7.25* | *-4.5* | *-1.5* |
| | *n/s* | *n/s* | *p ≤ 0.0001 +46.6% ± 11.1%* | *n/s* | *n/s* | *n/s* |
| Total Red Fruit | *INOC p ≤ 0.0184* | *INTX INOC*FERT p ≤ 0.0071* | | | | |
| **INOC** | 3.00 | **10.75** | 13.50 | 16.50 | **25.75** | 15.25 |
| NON | 2.75 | 5.50 | 10.75 | 15.00 | 14.25 | 19.25 |
| *Difference* | 0.25 | 5.25 | 2.75 | 1.5 | 11.5 | -4 |
| | *n/s* | *p ≤ 0.0634* +67.0% ± 26.2% | *n/s* | *n/s* | *p ≤ 0.0020* +59.2% ± 16.5% | *n/s* |
| Total Uncorked Fruit | *n/s**** | | | | | |
| INOC | 1.50 | 4.00 | 1.50 | 2.00 | 0.25 | 1.00 |
| NON | 0.25 | 0.25 | 1.00 | 0.25 | 2.50 | 9.00 |
| *Difference* | 1.25 | 3.75 | 0.5 | 1.75 | -2.25 | -8 |
| | *n/s* | *n/s* | *n/s* | *n/s* | *n/s* | *n/s* |
| Total Corked Fruit | *INOC p ≤ 0.2001* | *INTX INOC*FERT p ≤ 0.0004* | | | | |
| INOC | 5.25 | 17.00 | 22.50 | 22.25 | **40.00** | **45.50** |
| NON | 6.25 | 15.00 | 18.50 | **31.75** | 25.50 | 33.00 |
| *Difference* | -1 | 2 | 4 | -9.5 | 14.5 | 12.5 |
| | *n/s* | *n/s* | *n/s* | *p ≤ 0.0607 -35.6% ± 13.8%* | *p ≤ 0.0023 +45.0% ± 12.7%* | *p ≤ 0.0298 +32.1% ± 11.4%* |

* Integer collected data adhering to Poisson distribution, no non-zero values
** As reported in the text, for ease of cross-referencing
*** Excessive 0 values even for truncpoisson or ZIP
Note: Bolded main effect level shows which treatment was significantly greater, if M.E. p ≤ 0.1
Note: Increases are relative to a single integer increase in NON, reported as ± Change% ± s.e. of Change

However, this hypothesis is contradicted by findings that IAA, when externally applied, increases fruit number, average weight, and total biomass accumulation in pepper under normal fertilization (Simarjeet et al., 2017). This research did not show the result, but any additional

IAA, if present, would have been sourced *in planta.* Hasanuzzaman et al., (2007) demonstrated that pepper under influence of exogenous IAA and Gibberellin A3 will increase fruit set, a shared finding, but again found a concomitant increase in total biomass accumulation and individual fruit weight. Das et al., (2015) investigated just Gibberellin A3 and found similar results to Simarjeet et al. (2017) and Hasanuzzaman et al., (2007), with hormone application increasing total fruit biomass per plant and individual fruit weights. None of these researchers assessed fruit ripening/reddening, but they all determined fruit biomass increases as a definitive result of PGRH applications. It is important to note though, these studies investigated PGRHs on bell pepper, and although jalapeño is of the same species, *C. annuum,* physiological responses may be wholly different given morphological and gene expression variations between piquant and non-piquant species. Also, localization of PGRHs may be more important than concentrations, especially in processes implicated by gradated or relative ratios like ripening (Chaki et al., 2015; Su et al., 2015). Lastly, the number of corked peppers exhibited a fertilization interaction as well (Figure 5.8), with no difference at severe N deficiency (47.5-119ppm N), a significant decrease in corking at moderate deficiency (154ppm N), and then a reversal to more corked peppers at optimal and excessive N levels (190ppm + N). Little research has focused on hot pepper response to hormones and it has been postulated that N fixation may discouraged at these fertilization levels, when N is no longer the limiting factor (Eskin et al., 2014, Stephan et al., 1991). Therefore, explaining the mechanism behind the decrease in corking at moderate N-levels but an increase at high N-levels is difficult.

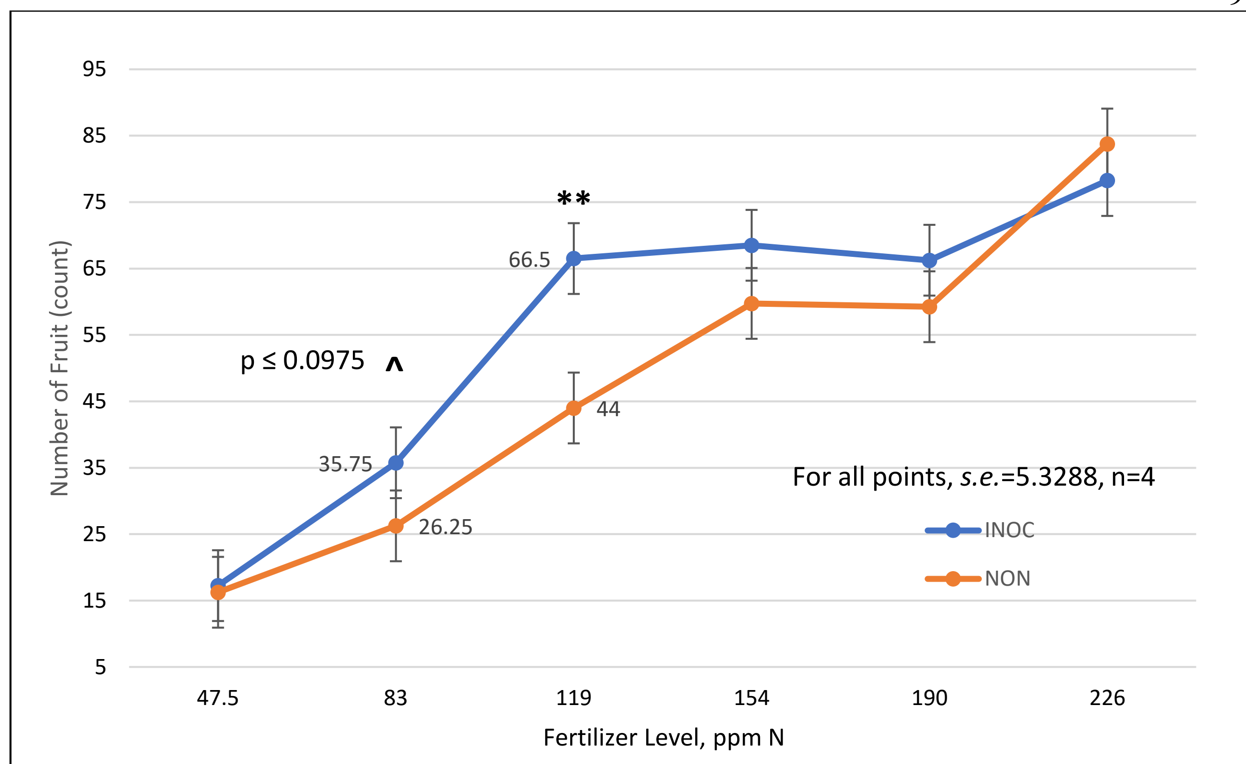



*Figure 5.5: Total number of fruit produced by FERT level. Signif: ^ <0.1, * <0.05, ** <0.01*

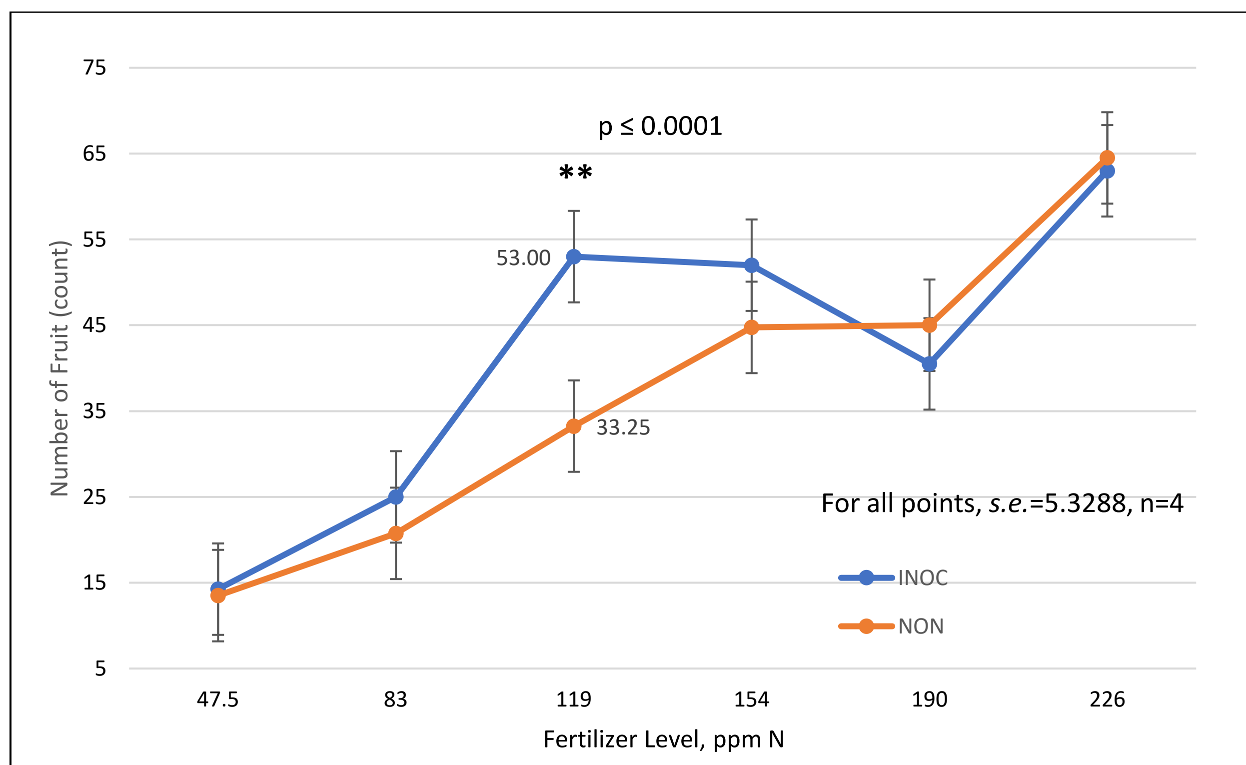



*Figure 5.6: Total number of green fruit produced by FERT level. Signif: ^ <0.1, * <0.05, ** <0.01*

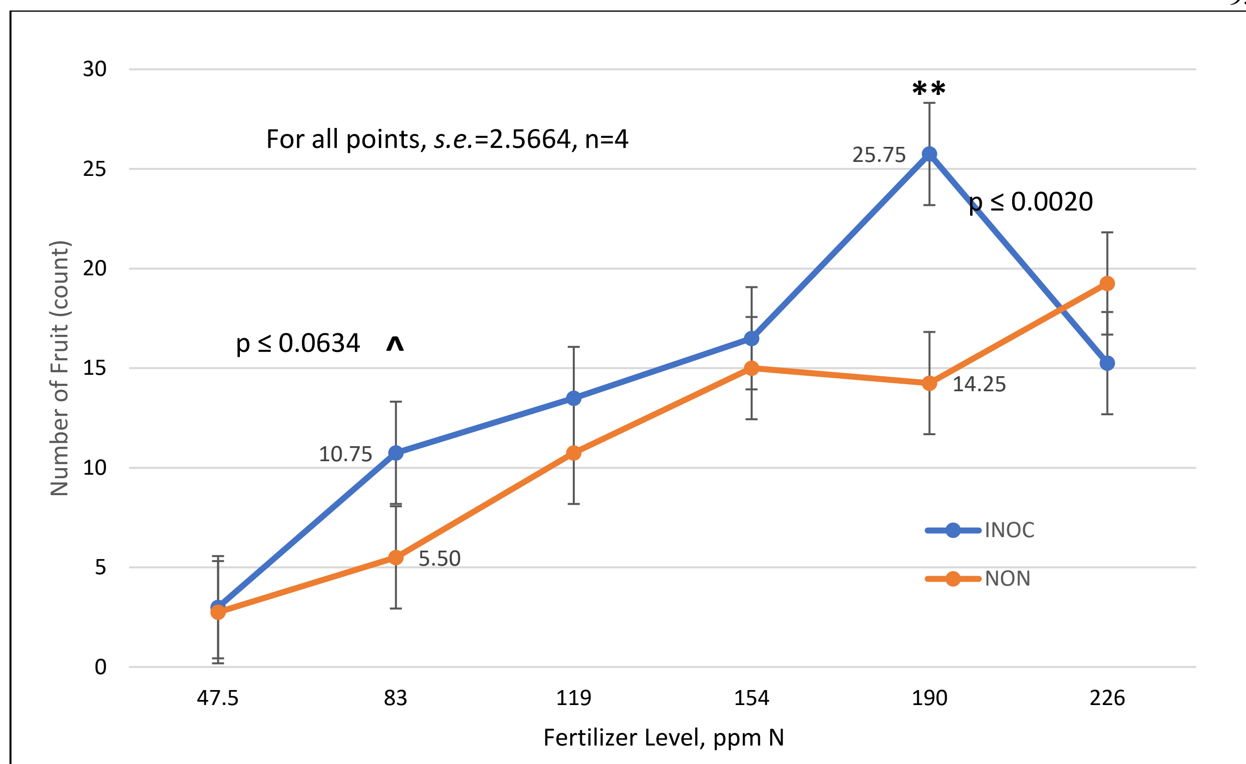



*Figure 5.7: Total number of red fruit produced by FERT level. Signif: ^ <0.1, * <0.05, ** <0.01*

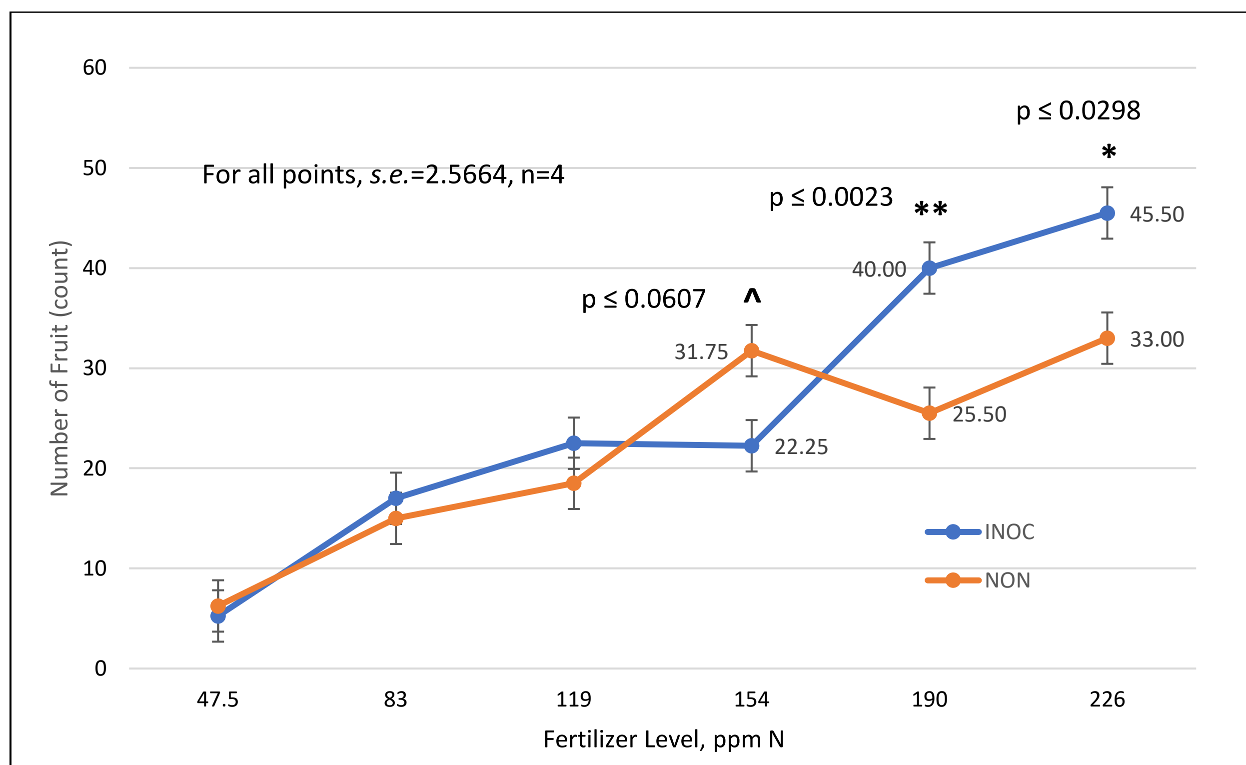



*Figure 5.8: Total number of corked fruit produced by FERT level. Signif: ^ <0.1, * <0.05, ** <0.01*

**5.4 Conclusion**

*G. diaz* inoculation of Jalapeño pepper does alter fruit set, fruit corking and fruit color, but it does so at fertilization levels that would impart yield reductions to producers or increase the fraction of damaged crop produced. Still, the quality assessments investigated provide valuable insight into the dangers of only focusing on the traditional measures of mass or counts. How *G. diaz* effected these changes was not clear from the data, as interactions occurred for every significant result and crossings were not consistent. Without further research to discover the exact mechanisms of altered morphology this research remains mainly academic, but yields definitive insights on how research designs into inoculation of non-tomato members of Solanaceae should be carried out. Future studies utilizing *G. diaz* mutants deficient in specific PGRH production should be employed alongside wild-type bacteria to elucidate which plant responses, if any, are hormone modulated and which can be attributed to N fixation or as yet undiscovered mechanisms (Eskin et al., 2014; Restrepo et al., 2017). Also, bacterial loading in specific tissues, during plant life stage changes, and as an effect of initial bacterial CFU/ml$^{-1}$ exposure should be assessed, as positive bacterial presence in Solanaceae does not always equate to altered plant response (El-Shouny et al., 2020).

# Chapter 6

## Conclusions and Final Remarks

*Gluconacetobacter diazotrophicus* as an agricultural enhancement provides definite benefits to a select cadre of crops, and from the experiments contained in the chapters of this dissertation lettuce emerges as one possible beneficial synergist. Leaf biomass yield increases between 8% and 16% depending on cultivar demonstrate potential benefits from bacterial inoculation, and may be of interest to those running hydroponic systems which have higher operating costs and slimmer profit margins than traditional field operations. The range of nutrient solution provided added insight to mechanisms across a range of fertilizations, but more repetitions at typical fertilization levels would have allowed for better clarity of plant-microbe interaction. That additional N fixation was both suggested (BIB1, BIB2, GRR2) yet also contradicted (BSS1, which seemed to show denitrification) is perplexing. Further, the increases of N above what was originally supplied in the control as well as in inoculated lettuce treatments could not be explained. Future studies of *G. diaz* in lettuce may want to employ acetylene reduction or N15 isotope analysis for definitive proof of N fixation. Recent investigations in *G. diaz* research have strayed away from N fixation and now focus on water use efficiency, micronutrient solubilization, and phytopathogen protection. The drought stressed plants from the partially failed GRR1 trial offer just as much insight into bacterial mechanisms as the yield boosts, and provide yet another approach for research into proving lettuce and *G.diaz* have potential.

The radish trials conducted for this dissertation did not reveal benefits of *G. diaz* inoculation of spring radish. Winter radish was one of the native associations discovered more recently, yet research routinely demonstrated inoculation benefits across multiple cultivars. Those same successes were not realized here. As the world's population grows and global calorie needs increase, we'll obviously need to increase crop production and make use of non-traditional timeslots, so the theoretical foundation of increasing crop production during the shoulders seasons was sound. However, a pilot project demonstrating inoculation effects of *G. diaz* on radish under optimal conditions would have been beneficial and may have identified potential hindrances. With so many growth limiting factors applied to one experiment, including N deficiency, cold temperatures, *in planta* bacteriosuppressants, determining why this experiment did not yield more positive results is difficult.

The radish and lettuce experiments do outline one important component of *G. diaz* use in agricultural systems, namely the wide variance in effect between closely related cultivars. Although genotypic differences obviously exist between the varieties of lettuce and radish tested, the results demonstrated herein are sometimes radically different and polarly opposite of each other. In the first greenhouse trial, Bacchus F1 radish growth was enhanced significantly in the presence of the adjuvants and/or water, but Rover F1 was wholly suppressed. Sora aligned more with Rover, which may indicate that the coloration of radish (Rover/Sora are red, Bacchus is purple) and the underlying pigments that provide coloration can change how the seeds respond to surface treatments. That neither responded to *G. diaz* may be of academic importance only, but the seed surface adjuvants and/or water certainly impacted yield, so in the overall perspective of increasing yields this may be a post-hoc success story.

For lettuce, the cross-cultivar responses to *G. diaz* are also of important note, as examining the underlying physiological and morphological differences between the cultivars may shed light on how and why the bacterium effected such different responses. *G. diaz* is known to alter its proliferation due to plant life cycle stage, but these were all grown to the same life stage and had equal maturation dates. Does the bacterium alter PGRH distribution in the plant, causing the shifting of synthates and biomass out of the ground and into the aerial portions, as seen in Black Seeded Simpson? If so, then why do Bibb and Grand Rapids both also increase their root production concomitant with shoot production. We could be seeing an N shuttling effect in BSS, evidenced by the C/N ratios and mass density, but this N movement response is also hinted to be occurring in trial BIB2, but not GRR. There may be an interplay here of both N distribution *and* hormones. Quantifying the bacterial load in each tissue type may even add a third interacting factor, if *G. diaz* does not colonize equally across tissues. Future research may look at quantifying these differences to give a better picture of why intra-special variances exist.

The hot pepper trial was a new application of *G. diaz*. One detriment discovered; the range of fertilization levels employed only served to reduce replications at each individual level, but were employed to stress plants effecting differences in vitamin, mineral, and secondary metabolites. The lattermost group containing the capsaicinoids are what give peppers their fiery bite and really drove the selection process and experimental design of this project, yet this portion could not be completed due to COVID. That effects in pepper numbers and quality did occur with inoculation and at fertilization levels that were not just on the extremes, suggest complex interactions within the plant in response to *G. diaz* presence. Publications more recent than the inception of any of these trials indicate bacterial CFU plays a much greater role in

determining impacts of mutualism (El Shouny et al., 2020). One suggestion would be to repeat the hot pepper trial, reducing fertilization levels and adding higher bacterial loading.

Over the course of these three experiments, numerous peculiar responses emerged to *G. diaz* inoculation that were not anticipated from the literature research of previous experiments and/or observations. In these crops, this bacterium does not seem to interact strongly with N fertilization levels, which was not wholly un-anticipated as nitrate N was supplied, but cultivar interactions and the odd responses such as the bimodal increase in red pepper numbers bring up even more questions. That utility exists with this bacterium I have no doubt, but the lack of *any* N interaction or evidence of fixation leads me to speculate that agronomic impacts of *G. diaz* may lie in one of the other principal modes of activity outlined in section 1.1. Water shortages are gaining significant interest in the United States' western agricultural areas as climate change devastates both terrestrial and aquifer-sourced water reserves. One rule of nature is that the shorter the lifecycle of an organism, the more rapidly it can adapt to a new environment, so we may see pathogens and plant pests expanding their ranges into once inhospitable agricultural areas as areas warm up or dry out. Or, we may see typic crops for an area suddenly show decreased performance due to fluctuating temperature, salinity, or other factors. *Gluconacetobacter diazotrophicus* may then prove more important for its positive impacts on water use efficiency, induced plant defenses, or imparted abiotic tolerances, helping address these three concerns, with potential N fixation being secondary or even a non-entity. Being a multi-action mutualist might allow for crop and/or environmental specific tailoring, and certainly opens up many avenues for research and exploration.

# Appendix A

## Nutrients added and effective ppm for fertilizer stock solutions, BSS1 GRR1

Appendix A: Nutrients added and effective ppm for fertilizer stock solns, BSS1 GRR1

| | Fertilizer (FERT) Treatment Desired Nitrogen Concentration (ppm)* | | | | | | |
|---|---|---|---|---|---|---|---|
| | **37.5** | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** |
| Nutrient & Source | | | | | | | |
| Nitrogen (N) | | | | | | | |
| Jack's 5-12-26 | 37.5 | 37.5 | 37.5 | 37.5 | 37.5 | 37.5 | 37.5 |
| Calcium Nitrate** | 0 | 5.14 | 27.61 | 50.09 | 72.56 | 95.04 | 117.56 |
| $KNO_3$ | 0 | 17.38 | 17.38 | 17.38 | 17.38 | 17.38 | 17.38 |
| ***TOTAL N (ppm)*** | *37.5* | *60.02* | *82.49* | *104.97* | *127.44* | *149.92* | *172.44* |
| ***TOTAL N (mg) /Jar*** | *30.00* | *48.02* | *65.99* | *83.98* | *101.95* | *119.94* | *137.95* |
| Phosphorus (P) | | | | | | | |
| Jack's 5-12-26 | 39.2 | 39.2 | 39.2 | 39.2 | 39.2 | 39.2 | 39.2 |
| ***TOTAL P (ppm)*** | *39.2* | *39.2* | *39.2* | *39.2* | *39.2* | *39.2* | *39.2* |
| Potassium (K) | | | | | | | |
| Jack's 5-12-26 | 161.7 | 161.7 | 161.7 | 161.7 | 161.7 | 161.7 | 161.7 |
| $KNO_3$ | 0 | 48.5 | 48.5 | 48.5 | 48.5 | 48.5 | 48.5 |
| $K_2SO_4$ | 48.6 | 0 | 0 | 0 | 0 | 0 | 0 |
| ***TOTAL K (ppm)*** | *210.3* | *210.2* | *210.2* | *210.2* | *210.2* | *210.2* | *210.2* |
| Calcium (Ca) | | | | | | | |
| Calcium Sulfate*** | 134.2 | 127.9 | 101.2 | 74.4 | 47.7 | 20.9 | 0 |
| Calcium Nitrate** | 0 | 6.1 | 32.9 | 59.7 | 86.5 | 113.3 | 140.2 |
| ***TOTAL Ca (ppm)*** | *134.2* | *134* | *134.1* | *134.1* | *134.2* | *134.2* | *140.2* |
| Other Minerals**** | | | | | | | |
| Magnesium (Mg) | *47 ppm for all fertilizer levels* | | | | | | |
| Iron (Fe) | *2.3 ppm for all fertilizer levels* | | | | | | |
| Manganese (Mn) | *0.38 ppm for all fertilizer levels* | | | | | | |
| Boron (B) | *0.38 ppm for all fertilizer levels* | | | | | | |
| Copper (Cu) | *0.113 ppm for all fertilizer levels* | | | | | | |
| Zinc (Zn) | *0.11 ppm for all fertilizer levels* | | | | | | |
| Molybdenum (Mo) | *0.075 ppm for all fertilizer levels* | | | | | | |

* Recipe taken from Mattson and Peters (2014).

** As hydrated calcium ammonium nitrate double salt, $5[Ca(NO_3)_2].NH_4NO_3.10[H_2O]$

*** As anhydrous calcium sulfate

**** Included as a micro suite in Jack's 5-12-26

## Appendix B

### Nutrients and effective ppm for fertilizer stock solns, BSS2 GRR2 BIB1 BIB2

Appendix B: Details of nutrients and effective ppm for fertilizer stock solns, BSS2 GRR2 BIB1 BIB2

| | Fertilizer (FERT) Treatment Desired Nitrogen Concentration (ppm)* | | | | | |
|---|---|---|---|---|---|---|
| | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** |
| Nutrient & Source | | | | | | |
| Nitrogen (N) | | | | | | |
| Jack's 5-12-26 | 37.5 | 37.5 | 37.5 | 37.5 | 37.5 | 37.5 |
| Calcium Nitrate** | 22.5 | 45 | 67.5 | 90 | 112.5 | 112.5 |
| $KNO_3$ | 0 | 0 | 0 | 0 | 0 | 22.4 |
| ***TOTAL N (ppm)*** | *60* | *82.5* | *105* | *127.5* | *150* | *172.4* |
| ***TOTAL N (mg) /Jar*** | *48.00* | *66.00* | *84.00* | *102.00* | *120.00* | *137.92* |
| Phosphorus (P) | | | | | | |
| Jack's 5-12-26 | 39.2 | 39.2 | 39.2 | 39.2 | 39.2 | 39.2 |
| ***TOTAL P (ppm)*** | *39.2* | *39.2* | *39.2* | *39.2* | *39.2* | *39.2* |
| Potassium (K) | | | | | | |
| Jack's 5-12-26 | 161.7 | 161.7 | 161.7 | 161.7 | 161.7 | 161.7 |
| $KNO_3$ | 0 | 0 | 0 | 0 | 0 | 62.6 |
| $K_2SO_4$ | 62.8 | 62.8 | 62.8 | 62.8 | 62.8 | 0 |
| ***TOTAL K (ppm)*** | *224.5* | *224.5* | *224.5* | *224.5* | *224.5* | *224.3* |
| Calcium (Ca) | | | | | | |
| Calcium Sulfate*** | 107.3 | 80.4 | 54.6 | 26.8 | 0 | 0 |
| Calcium Nitrate** | 26.8 | 53.7 | 80.5 | 107.4 | 134.2 | 134.2 |
| ***TOTAL Ca (ppm)*** | *134.1* | *134.1* | *135.1* | *134.2* | *134.2* | *134.2* |
| Other Minerals**** | | | | | | |
| Magnesium (Mg) | *47 ppm for all fertilizer levels* | | | | | |
| Iron (Fe) | *2.3 ppm for all fertilizer levels* | | | | | |
| Manganese (Mn) | *0.38 ppm for all fertilizer levels* | | | | | |
| Boron (B) | *0.38 ppm for all fertilizer levels* | | | | | |
| Copper (Cu) | *0.113 ppm for all fertilizer levels* | | | | | |
| Zinc (Zn) | *0.11 ppm for all fertilizer levels* | | | | | |
| Molybdenum (Mo) | *0.075 ppm for all fertilizer levels* | | | | | |

* Recipe taken from Mattson and Peters (2014).

** As hydrated calcium ammonium nitrate double salt, $5[Ca(NO_3)_2].NH_4NO_3.10[H_2O]$

*** As calcium sulfate dihydrate

**** Included as a micro suite in Jack's 5-12-26

# Appendix C

## Statistical analysis Box-Cox lambdas identified for response normalization

Appendix C: Statistical analysis Box-Cox lambdas identified for response normalization

| | Trial Designator | | | | | |
|---|---|---|---|---|---|---|
| Measurement | BIB1 | BIB2 | BSS1 | BSS2 | GRR1 | GRR2 |
| Shoot Dry Matter (RDM) | - | - | - | - | - | - |
| Root Dry Matter (RDM) | - | - | 0.5 | - | - | 2 |
| Total Dry Matter (TDM) | - | - | - | - | - | - |
| SDM Nitrogen % | -1 | 0 | - | -1 | -1 | -1 |
| SDM Nitrogen Recovered | 0 | 0 | - | 0 | 2 | 0 |
| RDM Nitrogen % | -1 | 0 | -1 | 0 | -2 | -1 |
| RDM Nitrogen Recovered | 0 | 0 | 0.5 | - | - | - |
| Total Nitrogen Recovered | - | 0 | - | 0 | -1 | 0 |
| SDM C/N Ratio | - | 0 | 0 | - | - | - |
| RDM C/N Ratio | - | 0 | - | - | - | - |

*- designates no correction indicated*

# Appendix D

## Type 3 Tests of Fixed Effects ANOVA Results and Pr >F values

Appendix D: Type 3 Tests of Fixed Effects ANOVA Results, Pr > F values

| | Effect | Num DF | Den DF | | Dry Matter Biomass | | | Mass Fractions | | C/N Ratios | | Nitrogen Recovery/Capture | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | SDM | RDM | TDM | SDM_NPC | RDM_NPC | SDM_CN | RDM_CN | SDM_CAP | RDM_CAP | TOT_CAP |
| BSS1 | INOC | 1 | 28 | | 0.0345 | 0.0042 | 0.3453 | 0.0015 | 0.6488 | 0.0050 | 0.1795 | 0.1788 | 0.0013 | 0.0019 |
| | FERT | 6 | 28 | | <0.0001 | 0.0007 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0002 | <.0001 |
| | INOC*FERT | 6 | 28 | | 0.9355 | 0.4539 | 0.6836 | 0.2362 | 0.2658 | 0.4566 | 0.4332 | 0.0939 | 0.1850 | 0.5018 |
| BSS2 | INOC | 1 | 32 | | 0.8416 | 0.5768 | 0.9764 | 0.8459 | 0.8094 | 0.9848 | 0.6817 | 0.6482 | 0.2489 | 0.8597 |
| | FERT | 5 | 32 | | 0.0002 | 0.3648 | 0.0004 | <.0001 | 0.0014 | <.0001 | 0.0015 | <.0001 | <.0001 | <.0001 |
| | INOC*FERT | 5 | 32 | | 0.2632 | 0.5245 | 0.2301 | 0.3627 | 0.5676 | 0.5185 | 0.5518 | 0.8717 | 0.6318 | 0.8801 |
| BIB1 | INOC | 1 | 32 | | 0.0338 | 0.2020 | 0.0336 | 0.6147 | 0.6138 | 0.3400 | 0.1272 | 0.0125 | 0.0357 | 0.0008 |
| | FERT | 5 | 32 | | 0.0766 | 0.0890 | 0.1149 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | INOC*FERT | 5 | 32 | | 0.7370 | 0.9716 | note 1 | 0.5880 | 0.9546 | 0.6820 | 0.6188 | 0.2432 | 0.6816 | 0.1791 |
| BIB2 | INOC | 1 | 29 | | 0.0151 | 0.0028 | 0.0047 | 0.0436 | 0.5394 | 0.0214 | 0.0987 | 0.7498 | 0.0109 | 0.0483 |
| | FERT | 5 | 29 | | 0.0012 | <.0001 | <.0001 | 0.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0005 | <.0001 |
| | INOC*FERT | 5 | 29 | | 0.2219 | 0.6112 | 0.2507 | note 2 | 0.0735 | 0.2561 | 0.1472 | 0.6787 | 0.0836 | note 3 |
| | NUMBER | 3 | 29 | | 0.0022 | 0.0002 | 0.0004 | 0.0001 | 0.0005 | 0.0079 | 0.0022 | 0.5033 | 0.4548 | 0.7170 |
| GRR1 | INOC | 1 | 20 | | <.0001 | 0.0128 | <.0001 | 0.0016 | 0.0061 | 0.0004 | 0.0005 | <.0001 | 0.0006 | 0.8421 |
| | FERT | 4 | 20 | note 4 | 0.0522 | <.0001 | 0.0356 | 0.0708 | 0.4097 | 0.0049 | 0.1674 | <.0001 | <.0001 | <.0001 |
| | INOC*FERT | 4 | 20 | | 0.1053 | 0.0020 | 0.1109 | 0.7603 | 0.1037 | 0.9237 | 0.0325 | 0.0203 | 0.0959 | 0.3518 |
| GRR2 | INOC | 1 | 32 | | 0.0560 | *0.0530* | 0.0444 | 0.1016 | 0.0277 | 0.2600 | 0.0011 | 0.0728 | 0.5846 | 0.2506 |
| | FERT | 5 | 32 | note 5 | 0.0345 | *0.0036* | 0.0330 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| | INOC*FERT | 5 | 32 | note 6 | 0.5323 | *0.3417* | 0.4773 | 0.4252 | 0.7068 | 0.1912 | 0.6025 | 0.1588 | 0.3708 | 0.2826 |

SDM = Shoot Dry Matter, RDM = Root Dry Matter, TDM = Total Dry Matter, NPC = Mass Fraction, CN = C/N Ratio, CAP = N Recovered/Captured

note 1: INOC*FERT was not significant and dropped from the model. Num DF = 1, 5; Den DF = 37

note 2: INOC*FERT was not significant and dropped from the model. FERT*NUMBER was significant and remained (DF=5, P<0.0296). Den DF = 29.

note 3: INOC*FERT was not significant and dropped from the model. Num DF = 1, 5, 3; Den DF = 34

note 4: For responses involving roots (RDM, TDM, RDM_NPC, RDM_CAP, RDM_CN) Den DF was 16-19 with removed outliers and by SAS exclusion

note 5: Values in Italics used the Box-Cox lambda adjusted values in Appendix C. Means in the text are unadjusted values.

note 6: NUMBER, alone or interacting, was never significant in GRR2 and was dropped as a covariate

# Appendix E

## BSS1, BSS2 Biomass dry matter

| Appendix E: BSS1, BSS2 Biomass dry matter | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | | |
| Trial and Measure | **37.5** | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** |
| BSS1 | | | | | | | |
| Shoot Dry Matter (g) * | *LSD: 0.235 s.e.: 0.1057 df=28 p ≤ 0.0345* | | | | | | |
| **INOC** | 2.2843 | 2.7927 | 2.499 | 3.524 | 3.4793 | 3.416 | 3.7387 |
| NON | 2.224 | 2.645 | 2.3493 | 3.0683 | 3.241 | 3.2527 | 3.3087 |
| *Difference* | *0.0603* | *0.1477* | *0.1497* | *0.4557* | *0.2383* | *0.1633* | *0.43* |
| Root Dry Matter (g) ** | *LSD: -0.4251 s.e.: 0.1366 df=28 p ≤ 0.0042* | | | | | | |
| INOC | 0.602 | 0.1067 | 0.207 | 1.3737 | 0.7867 | 0.4473 | 0.7597 |
| **NON** | 0.7243 | 0.7117 | 0.639 | 1.913 | 1.1987 | 1.4433 | 0.629 |
| *Difference* | *-0.1223* | *-0.605* | *-0.432* | *-0.5393* | *-0.412* | *-0.996* | *0.1307* |
| Total Dry Matter (g) | *n/s* | | | | | | |
| INOC | 2.8863 | 2.8993 | 2.706 | 4.8977 | 4.266 | 3.8633 | 4.4983 |
| NON | 2.9483 | 3.3567 | 2.9883 | 4.9813 | 4.4397 | 4.696 | 3.9377 |
| *Difference* | *-0.062* | *-0.4574* | *-0.2823* | *-0.0836* | *-0.1737* | *-0.8327* | *0.5606* |
| BSS2 | | | | | | | |
| Shoot Dry Matter (g) | *n/s* | | | | | | |
| INOC | - | 4.7867 | 5.7825 | 5.705 | 6.3525 | 6.6475 | 6.2067 |
| NON | - | 4.47 | 6 | 5.6875 | 7.0975 | 5.715 | 6.7767 |
| *Difference* | - | *0.3167* | *-0.2175* | *0.0175* | *-0.745* | *0.9325* | *-0.57* |
| Root Dry Matter (g) | *n/s* | | | | | | |
| INOC | - | 1.78 | 2.0675 | 2.135 | 2.0425 | 2.2175 | 2.2333 |
| NON | - | 1.89 | 1.92 | 2.0525 | 2.285 | 1.885 | 2.13 |
| *Difference* | - | *-0.11* | *0.1475* | *0.0825* | *-0.2425* | *0.3325* | *0.1033* |
| Total Dry Matter (g) | *n/s* | | | | | | |
| INOC | - | 6.5667 | 7.85 | 7.84 | 8.395 | 8.865 | 8.44 |
| NON | - | 6.36 | 7.92 | 7.74 | 9.3825 | 7.6 | 8.9067 |
| *Difference* | - | 0.2067 | -0.07 | 0.1 | -0.9875 | 1.265 | -0.4667 |

** = significant at alpha < 0.01, * = < 0.05, n/s = not significant

Note: Bolded main effect level shows which treatment was significantly greater

'-' indicates this fertilization (FERT) level was not used in this experiment

# Appendix F - BSS1, BSS2 N mass fraction and C/N ratios

Appendix F: BSS1, BSS2 N mass fraction and C/N ratios

| | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| Trial and Measure | **37.5** | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** |
| BSS1 | | | | | | | |
| SDM Nitrogen % ** | *LSD: -0.2328 s.e.: 0.06591 df=28 p ≤ 0.0015* | | | | | | |
| INOC | 1.2897 | 1.4647 | 1.9297 | 1.898 | 2.268 | 2.596 | 2.39 |
| **NON** | 1.337 | 1.828 | 2.326 | 2.1103 | 2.276 | 2.6483 | 2.9397 |
| *Difference* | *-0.0473* | *-0.3633* | *-0.3963* | *-0.2123* | *-0.008* | *-0.0523* | *-0.5497* |
| SDM C/N Ratio ** | *LSD: 2.2234 s.e.: 0.7305 df=28 p ≤ 0.0050* | | | | | | |
| **INOC** | 30.4953 | 26.4959 | 20.144 | 20.8206 | 17.3701 | 15.4389 | 16.5131 |
| NON | 29.2597 | 21.1431 | 16.4662 | 18.783 | 17.4194 | 15.0597 | 13.583 |
| *Difference* | *1.2356* | *5.3528* | *3.6778* | *2.0376* | *-0.0493* | *0.3792* | *2.9301* |
| RDM Nitrogen % | *n/s* | | | | | | |
| INOC | 1.191 | 1.3223 | 2.078 | 1.3597 | 1.5603 | 2.507 | 2.3823 |
| NON | 1.3393 | 1.475 | 1.5893 | 1.4753 | 1.6563 | 2.193 | 2.404 |
| *Difference* | -0.1483 | -0.1527 | 0.4887 | -0.1156 | -0.096 | 0.314 | -0.0217 |
| RDM C/N Ratio | *n/s* | | | | | | |
| INOC | 31.8388 | 25.6109 | 16.8458 | 19.2237 | 17.8979 | 14.0193 | 14.0467 |
| NON | 27.8539 | 22.8029 | 18.1328 | 18.0645 | 17.514 | 14.8858 | 13.4121 |
| *Difference* | 3.9849 | 2.808 | -1.287 | 1.1592 | 0.3839 | -0.8665 | 0.6346 |
| BSS2 | | | | | | | |
| SDM Nitrogen % | *n/s* | | | | | | |
| INOC | - | 0.9813 | 1.0985 | 1.2818 | 1.4158 | 1.4545 | 1.6703 |
| NON | - | 0.989 | 1.051 | 1.3025 | 1.2833 | 1.7218 | 1.6193 |
| *Difference* | - | *-0.0077* | *0.0475* | *-0.0207* | *0.1325* | *-0.2673* | *0.051* |
| SDM C/N Ratio | *n/s* | | | | | | |
| INOC | - | 40.3442 | 36.2582 | 31.0331 | 28.4246 | 27.4436 | 23.9174 |
| NON | - | 39.7888 | 37.9595 | 30.4981 | 30.9767 | 23.4604 | 24.8602 |
| *Difference* | - | *0.5554* | *-1.7013* | *0.535* | *-2.5521* | *3.9832* | *-0.9428* |
| RDM Nitrogen % | *n/s* | | | | | | |
| INOC | - | 1.135 | 1.092 | 1.5373 | 1.69 | 1.8 | 1.7483 |
| NON | - | 1.0993 | 1.3198 | 1.3795 | 1.3263 | 1.985 | 1.7373 |
| *Difference* | - | *0.0357* | *-0.2278* | *0.1578* | *0.3637* | *-0.185* | *0.011* |
| RDM C/N Ratio | *n/s* | | | | | | |
| INOC | - | 32.1686 | 35.6685 | 25.1305 | 24.014 | 21.3367 | 22.515 |
| NON | - | 36.0394 | 29.3506 | 28.0213 | 28.0778 | 21.4379 | 22.4662 |
| *Difference* | - | -3.8708 | 6.3179 | -2.8908 | -4.0638 | -0.1012 | 0.0488 |

** = significant at alpha < 0.01, * = < 0.05, n/s = not significant

Note: Bolded main effect level shows which treatment was significantly greater

'-' indicates this fertilization (FERT) level was not used in this experiment

# Appendix G – BSS1, BSS2 Nitrogen content

Appendix G: BSS1, BSS2 Nitrogen content

| | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| Trial and Measure | **37.5** | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** |
| BSS1 | | | | | | | |
| SDM N Content (mg) | *n/s* | | | | | | |
| INOC | 29.0304 | 40.7487 | 48.375 | 66.337 | 79.012 | 86.6046 | 89.3205 |
| NON | 29.347 | 48.2788 | 54.4208 | 64.3062 | 73.7542 | 85.8727 | 96.2369 |
| *Difference* | *-0.3166* | *-7.5301* | *-6.0458* | *2.0308* | *5.2578* | *0.7319* | *-6.9164* |
| RDM N Content (mg) ** | *LSD: -7.6737* | *s.e.: 2.1391* | *df=28* | *p ≤ 0.0013* | | | |
| INOC | 6.7533 | 1.3809 | 2.7716 | 18.2631 | 12.3212 | 11.4651 | 17.986 |
| **NON** | 9.597 | 10.4535 | 10.1552 | 28.1261 | 19.9262 | 31.6756 | 14.7235 |
| *Difference* | *-2.8437* | *-9.0726* | *-7.3836* | *-9.863* | *-7.605* | *-20.2105* | *3.2625* |
| TDM N Content (mg) ** | *LSD: -9.5007* | *s.e.: 2.7719* | *df=28* | *p ≤ 0.0019* | | | |
| INOC | 35.7837 | 42.1296 | 51.1466 | 84.6 | 91.3332 | 98.0697 | 107.31 |
| **NON** | 38.944 | 58.7324 | 64.576 | 92.4324 | 93.6804 | 117.55 | 110.96 |
| *Difference* | -3.1603 | -16.6028 | -13.4294 | -7.8324 | -2.3472 | -19.4803 | -3.65 |
| Gain/Loss N (mg)‡ | | | | | | | |
| Supplied N (mg) | 30.00 | 48.00 | 66.00 | 84.00 | 102.00 | 120.00 | 138.00 |
| INOC gain/loss | 5.7837 | -5.8704 | -14.8534 | 0.6 | -10.6668 | -21.9303 | -30.69 |
| NON gain/loss | 8.944 | 10.7324 | -1.424 | 8.4324 | -8.3196 | -2.45 | -27.04 |
| BSS2 | | | | | | | |
| SDM N Content (mg) | *n/s* | | | | | | |
| INOC | - | 46.9533 | 63.2495 | 72.7381 | 88.1324 | 95.8797 | 103.09 |
| NON | - | 44.0911 | 62.716 | 73.8477 | 90.5689 | 95.4324 | 108.3 |
| *Difference* | - | *2.8622* | *0.5335* | *-1.1096* | *-2.4365* | *0.4473* | *-5.21* |
| RDM N Content (mg) | *n/s* | | | | | | |
| INOC | - | 19.9036 | 22.028 | 32.2527 | 32.8469 | 39.7779 | 38.7949 |
| NON | - | 20.4017 | 24.5736 | 28.0432 | 30.2118 | 35.9125 | 37.0391 |
| *Difference* | - | *-0.4981* | *-2.5456* | *4.2095* | *2.6351* | *3.8654* | *1.7558* |
| TDM N Content (mg) | *n/s* | | | | | | |
| INOC | - | 67.8486 | 86.1856 | 105.78 | 121.73 | 135.97 | 142.02 |
| NON | - | 65.6696 | 88.5805 | 103.41 | 121.03 | 132.99 | 145.81 |
| *Difference* | - | *2.179* | *-2.3949* | *2.37* | *0.7* | *2.98* | *-3.79* |
| Gain/Loss N (mg)‡ | | | | | | | |
| Supplied N (mg) | - | 48.00 | 66.00 | 84.00 | 102.00 | 120.00 | 138.00 |
| INOC gain/loss | - | 19.85 | 20.19 | 21.78 | 19.73 | 15.97 | 4.02 |
| NON gain/loss | - | 17.67 | 22.58 | 19.41 | 19.03 | 12.99 | 7.81 |

** = significant at alpha < 0.01, * = < 0.05, n/s = not significant

Note: Bolded main effect level shows which treatment was significantly greater

'-' indicates this fertilization (FERT) level was not used in this experiment

‡ grams of N above or below the quantity provided via fertilization (TDM N - Supplied N)

# Appendix H – BIB1, BIB2 Biomass dry matter

Appendix H: BIB1, BIB2 Biomass dry matter

| Trial and Measure | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **37.5** | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** |
| BIB1 | | | | | | | |
| Shoot Dry Matter (g) * | *LSD: 0.5296* | *s.e.: 0.2387* | *df=32* | *p ≤ 0.0338* | | | |
| **INOC** | - | 4.28 | 6.0475 | 5.535 | 5.3775 | 5.825 | 5.3767 |
| NON | - | 4.2167 | 4.805 | 5.025 | 5.25 | 5.1875 | 4.78 |
| *Difference* | - | *0.0633* | *1.2425* | *0.51* | *0.1275* | *0.6375* | *0.5967* |
| Root Dry Matter (g) | *n/s* | | | | | | |
| INOC | - | 1.8333 | 1.9575 | 2.0825 | 1.905 | 1.9075 | 1.5567 |
| NON | - | 1.6367 | 1.805 | 2 | 1.8625 | 1.68 | 1.5633 |
| *Difference* | - | *0.1966* | *0.1525* | *0.0825* | *0.0425* | *0.2275* | *-0.0066* |
| Total Dry Matter (g) * | *LSD: 0.6655* | *s.e.: 0.3015* | *df=37* | *p ≤ 0.0336* | | | |
| **INOC** | - | 6.1133 | 8.005 | 7.6175 | 7.2825 | 7.7325 | 6.9333 |
| NON | - | 5.8533 | 6.61 | 7.025 | 7.1125 | 6.8675 | 6.3433 |
| *Difference* | - | *0.26* | *1.395* | *0.5925* | *0.17* | *0.865* | *0.59* |
| BIB2 | | | | | | | |
| Shoot Dry Matter (g) * | *LSD: 0.6371* | *s.e.: 0.2468* | *df=29* | *p ≤ 0.0151* | | | |
| **INOC** | - | 4.4565 | 6.3805 | 4.8252 | 3.9082 | 3.6759 | 3.8428 |
| NON | - | 3.9371 | 4.5877 | 3.6722 | 3.5264 | 3.5944 | 3.9488 |
| *Difference* | - | *0.5194* | *1.7928* | *1.153* | *0.3818* | *0.0815* | *-0.106* |
| Root Dry Matter (g) ** | *LSD: 0.2632* | *s.e.: 0.08075* | *df=29* | *p ≤ 0.0028* | | | |
| **INOC** | - | 1.9303 | 2.0471 | 1.8617 | 1.246 | 1.1443 | 1.4155 |
| NON | - | 1.5798 | 1.6708 | 1.4674 | 1.1108 | 1.1382 | 1.0985 |
| *Difference* | - | *0.3505* | *0.3763* | *0.3943* | *0.1352* | *0.0061* | *0.317* |
| Total Dry Matter (g) ** | *LSD: 0.9003* | *s.e.: 0.2943* | *df=29* | *p ≤ 0.0047* | | | |
| **INOC** | - | 6.3868 | 8.4276 | 6.6868 | 5.1542 | 4.8202 | 5.2583 |
| NON | - | 5.5169 | 6.2585 | 5.1396 | 4.6372 | 4.7326 | 5.0473 |
| *Difference* | - | 0.8699 | 2.1691 | 1.5472 | 0.517 | 0.0876 | 0.211 |

** = significant at alpha < 0.01, * = < 0.05, n/s = not significant

Note: Bolded main effect level shows which treatment was significantly greater

'-' indicates this fertilization (FERT) level was not used in this experiment

# Appendix I – BIB1, BIB2 N mass fractions and C/N ratios

Appendix I: BIB1, BIB2 N mass fraction and C/N ratios

| | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| Trial and Measure | **37.5** | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** |
| BIB1 | | | | | | | |
| SDM Nitrogen % ** | *n/s* | | | | | | |
| INOC | - | 1.078 | 1.1665 | 1.5253 | 1.813 | 1.8525 | 2.2753 |
| NON | - | 1.095 | 1.284 | 1.6123 | 1.7285 | 2.0593 | 2.1223 |
| *Difference* | - | *-0.017* | *-0.1175* | *-0.087* | *0.0845* | *-0.2068* | *0.153* |
| SDM C/N Ratio ** | *n/s* | | | | | | |
| INOC | - | 36.9902 | 34.4436 | 26.5337 | 22.6695 | 21.7115 | 17.9375 |
| NON | - | 36.4424 | 31.3367 | 25.2257 | 23.5292 | 19.697 | 19.0513 |
| *Difference* | - | *0.5478* | *3.1069* | *1.308* | *-0.8597* | *2.0145* | *-1.1138* |
| RDM Nitrogen % | *n/s* | | | | | | |
| INOC | - | 0.9293 | 1.0158 | 1.3957 | 1.6613 | 1.7115 | 2.2933 |
| NON | - | 0.9607 | 1.1185 | 1.2647 | 1.5828 | 1.6933 | 2.0897 |
| *Difference* | - | -0.0314 | -0.1027 | 0.131 | 0.0785 | 0.0182 | 0.2036 |
| RDM C/N Ratio | *n/s* | | | | | | |
| INOC | - | 38.1369 | 37.7678 | 29.1059 | 23.4494 | 22.3931 | 17.5548 |
| NON | - | 36.5926 | 30.9461 | 29.3563 | 23.1466 | 17.5784 | 17.1694 |
| *Difference* | - | 1.5443 | 6.8217 | -0.2504 | 0.3028 | 4.8147 | 0.3854 |
| BIB2 | | | | | | | |
| SDM Nitrogen % * | *LSD: -0.2767 s.e.: 0.1311 df=29 p ≤ 0.0436* | | | | | | |
| INOC | - | 0.821 | 0.5246 | 1.347 | 1.9444 | 2.5782 | 2.7108 |
| **NON** | - | 1.166 | 1.0179 | 1.7939 | 2.2655 | 2.5171 | 2.5981 |
| *Difference* | - | *-0.345* | *-0.4933* | *-0.4469* | *-0.3211* | *0.0611* | *0.1127* |
| SDM C/N Ratio * | *LSD: 4.3860 s.e.: 1.8028 df=29 p ≤ 0.0214* | | | | | | |
| **INOC** | - | 46.7108 | 54.0375 | 34.718 | 21.9845 | 18.1597 | 15.7018 |
| NON | - | 45.5982 | 42.9597 | 24.1428 | 20.4196 | 16.3293 | 15.5464 |
| *Difference* | - | *1.1126* | *11.0778* | *10.5752* | *1.5649* | *1.8304* | *0.1554* |
| RDM Nitrogen % | *n/s* | | | | | | |
| INOC | - | 0.9756 | 1.086 | 1.7114 | 3.1717 | 3.2466 | 2.8538 |
| NON | - | 1.6021 | 1.4698 | 1.9259 | 2.6806 | 2.901 | 2.9008 |
| *Difference* | - | *-0.6265* | *-0.3838* | *-0.2145* | *0.4911* | *0.3456* | *-0.047* |
| RDM C/N Ratio | *n/s* | | | | | | |
| INOC | - | 37.729 | 30.6127 | 25.9418 | 14.9565 | 16.6319 | 16.155 |
| NON | - | 29.5646 | 28.2079 | 22.6923 | 18.6733 | 15.365 | 16.0318 |
| *Difference* | - | 8.1644 | 2.4048 | 3.2495 | -3.7168 | 1.2669 | 0.1232 |

** = significant at alpha < 0.01, * = < 0.05, n/s = not significant

Note: Bolded main effect level shows which treatment was significantly greater

'-' indicates this fertilization (FERT) level was not used in this experiment

# Appendix J – BIB1, BIB2 Nitrogen content

Appendix J: BIB1, BIB2 Nitrogen content

| | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| Trial and Measure | 37.5 | 60 | 82.5 | 105 | 127.5 | 150 | 172.5 |
| BIB1 | | | | | | | |
| SDM N Content (mg) * | *LSD: 6.0360* | *s.e.: 2.2797* | *df=32* | *p ≤ 0.0125* | | | |
| **INOC** | - | 45.9767 | 70.3502 | 83.254 | 93.5854 | 107.04 | 120.28 |
| NON | - | 46.0363 | 61.3058 | 80.6421 | 90.4181 | 104.75 | 101.12 |
| *Difference* | - | *-0.0596* | *9.0444* | *2.6119* | *3.1673* | *2.29* | *19.16* |
| RDM N Content (mg) * | *LSD: 2.0424* | *s.e.: 0.9315* | *df=32* | *p ≤ 0.0357* | | | |
| **INOC** | - | 16.9569 | 19.8892 | 28.3854 | 29.7192 | 32.1173 | 34.8268 |
| NON | - | 15.6819 | 19.607 | 25.2417 | 29.4837 | 27.5801 | 32.0457 |
| *Difference* | - | *1.275* | *0.2822* | *3.1437* | *0.2355* | *4.5372* | *2.7811* |
| TDM N Content (mg) ** | *LSD: 7.7588* | *s.e.: 2.0855* | *df=32* | *p ≤ 0.0008* | | | |
| **INOC** | - | 63.9581 | 91.1108 | 112.5 | 124.22 | 139.66 | 155.9 |
| NON | - | 62.4907 | 81.9006 | 106.87 | 121.2 | 133.44 | 134.9 |
| *Difference* | - | 1.4674 | 9.2102 | 5.63 | 3.02 | 6.22 | 21 |
| Gain/Loss N (mg)‡ | | | | | | | |
| Supplied N (mg) | - | 48.00 | 66.00 | 84.00 | 102.00 | 120.00 | 138.00 |
| INOC gain/loss | - | 15.9581 | 25.1108 | 28.5000 | 22.2200 | 19.6600 | 17.9000 |
| NON gain/loss | - | 14.4907 | 15.9006 | 22.8700 | 19.2000 | 13.4400 | -3.1000 |
| BIB2 | | | | | | | |
| SDM N Content (mg) | *n/s* | | | | | | |
| INOC | - | 39.2079 | 47.9869 | 60.0839 | 76.0494 | 92.4076 | 100.44 |
| NON | - | 38.9244 | 45.1925 | 63.517 | 75.3747 | 91.7889 | 103.84 |
| *Difference* | - | *0.2835* | *2.7944* | *-3.4331* | *0.6747* | *0.6187* | *-3.4* |
| RDM N Content (mg) * | *LSD: 3.7552* | *s.e.: 1.3795* | *df=29* | *p ≤ 0.0109* | | | |
| **INOC** | - | 20.5902 | 26.7523 | 28.8405 | 35.7133 | 30.441 | 38.1569 |
| NON | - | 21.454 | 24.0066 | 26.911 | 24.7012 | 31.8548 | 29.0352 |
| *Difference* | - | *-0.8638* | *2.7457* | *1.9295* | *11.0121* | *-1.4138* | *9.1217* |
| TDM N Content (mg) * | *LSD: 3.5310* | *s.e.: 1.72419* | *df=34* | *p ≤ 0.0483* | | | |
| **INOC** | - | 62.8987 | 76.0797 | 92.1186 | 115.06 | 126.43 | 142.73 |
| NON | - | 62.5734 | 72.0726 | 93.2214 | 103.85 | 127.39 | 136.34 |
| *Difference* | - | *0.3253* | *4.0071* | *-1.1028* | *11.21* | *-0.96* | *6.39* |
| Gain/Loss N (mg)‡ | | | | | | | |
| Supplied N (mg) | - | 48.00 | 66.00 | 84.00 | 102.00 | 120.00 | 138.00 |
| INOC gain/loss | - | 14.90 | 10.08 | 8.12 | 13.06 | 6.43 | 4.73 |
| NON gain/loss | - | 14.57 | 6.07 | 9.22 | 1.85 | 7.39 | -1.66 |

** = significant at alpha < 0.01, * = < 0.05, n/s = not significant
Note: Bolded main effect level shows which treatment was significantly greater
'-' indicates this fertilization (FERT) level was not used in this experiment
‡ grams of N above or below the quantity provided via fertilization (TDM N - Supplied N)

# Appendix K - GRR1, GRR2 Biomass dry matter

Appendix K: Grand Rapids Tipburn Resistant Trial Data, Biomass Measures (GRR1, GRR2)

| | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| Trial and Measure | **37.5** | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** |
| GRR1 | | | | | | | |
| Shoot Dry Matter (g) ** | *LSD: 2.8550* | *s.e.: 0.4328* | *df=20* | *p ≤ 0.0001* | | | |
| **INOC** | - | 3.9585 | 3.436 | 4.911 | 4.411 | 6.6385 | - |
| NON | - | 2.311 | 1.4193 | 1.596 | 1.836 | 1.916 | - |
| *Difference* | - | *1.6475* | *2.0167* | *3.315* | *2.575* | *4.7225* | - |
| Root Dry Matter (g) * | *LSD: 0.3667* | *s.e.: 0.1335* | *df=19* | *p ≤ 0.0128* | *INTX INOC*FERT p≤ 0.0020* | | |
| **INOC** | - | 1.2535 | 1.126 | 1.646 | 1.7093 | 1.8335 | - |
| NON | - | 0.701 | 0.5793 | 0.616 | 2.866 | 0.9727 | - |
| *Difference* | - | *0.5525* | *0.5467* | *1.03* | *-1.1567* | *0.8608* | - |
| Total Dry Matter (g) ** | *LSD: 3.1940* | *s.e.: 0.5640* | *df=19* | *p ≤ 0.0001* | | | |
| **INOC** | - | 5.212 | 4.562 | 6.557 | 5.982 | 8.472 | - |
| NON | - | 3.012 | 1.9987 | 2.212 | 4.702 | 2.8887 | - |
| *Difference* | - | *2.2* | *2.5633* | *4.345* | *1.28* | *5.5833* | - |
| GRR2 | | | | | | | |
| Shoot Dry Matter (g) ^ | *LSD: 0.7304* | *s.e.: 0.3683* | *df=32* | *p ≤ 0.0560* | | | |
| **INOC** | - | 5.8837 | 7.5002 | 6.95 | 7.0165 | 5.7225 | 5.696 |
| NON | - | 6.2867 | 6.4975 | 6.7835 | 4.9988 | 4.7362 | 5.084 |
| *Difference* | - | *-0.403* | *1.0027* | *0.1665* | *2.0177* | *0.9863* | *0.612* |
| Root Dry Matter (g) ^ | *LSD: 0.1277* | *s.e.: 0.06913* | *df=20* | *p ≤ 0.0530* | | | |
| **INOC** | - | 1.238 | 1.4735 | 1.7365 | 1.791 | 1.6193 | 1.3133 |
| NON | - | 1.3421 | 1.493 | 1.604 | 1.4342 | 1.3147 | 1.2171 |
| *Difference* | - | *-0.1041* | *-0.0195* | *0.1325* | *0.3568* | *0.3046* | *0.0962* |
| Total Dry Matter (g) * | *LSD: 0.8581* | *s.e.: 0.4101* | *df=32* | *p ≤ 0.0444* | | | |
| **INOC** | - | 7.1217 | 8.9737 | 8.6865 | 8.8075 | 7.3418 | 7.0093 |
| NON | - | 7.6288 | 7.9905 | 8.3875 | 6.433 | 6.051 | 6.3011 |
| *Difference* | - | -0.5071 | 0.9832 | 0.299 | 2.3745 | 1.2908 | 0.7082 |

** = significant at alpha < 0.01, * = < 0.05, ^ = 0.1, n/s = not significant
Note: Bolded main effect level shows which treatment was significantly greater
'-' indicates this fertilization (FERT) level was not used in this experiment

# Appendix L - GRR1, GRR2 N mass fraction and C/N ratios

Appendix L: GRR1, GRR2 N mass fraction and C/N ratios

| | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| Trial and Measure | **37.5** | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** |
| GRR1 | | | | | | | |
| SDM Nitrogen % ** | *LSD: -1.2900 s.e.: 0.3512 df=19 p ≤ 0.0016* | | | | | | |
| INOC | - | 0.8343 | 1.8043 | 1.1553 | 1.7913 | 1.408 | - |
| **NON** | - | 1.3765 | 3.1387 | 2.626 | 3.008 | 3.294 | - |
| *Difference* | - | *-0.5422* | *-1.3344* | *-1.4707* | *-1.2167* | *-1.886* | - |
| SDM C/N Ratio ** | *LSD: 14.9447 s.e.: 3.4995 df=19 p ≤ 0.0004* | | | | | | |
| **INOC** | - | 48.2158 | 30.2649 | 34.6922 | 22.751 | 28.6315 | - |
| NON | - | 31.2955 | 17.9489 | 15.2784 | 13.1489 | 12.1602 | - |
| *Difference* | - | *16.9203* | *12.316* | *19.4138* | *9.6021* | *16.4713* | - |
| RDM Nitrogen % ** | *LSD: -0.9207 s.e.: 0.2914 df=16 p ≤ 0.0002 INTX INOC*FERT p ≤ 0.0263* | | | | | | |
| INOC | - | 1.192 | 1.9553 | 1.2558 | 1.4747 | 1.5762 | - |
| **NON** | - | 2.671 | 1.752 | 3.506 | 1.376 | 2.7525 | - |
| *Difference* | - | -1.479 | 0.2033 | -2.2502 | 0.0987 | -1.1763 | - |
| RDM C/N Ratio ** | *LSD: 7.9231 s.e.: 1.8289 df=16 p ≤ 0.0005 INTX INOC*FERT p ≤ 0.0325* | | | | | | |
| **INOC** | - | 29.4237 | 20.5117 | 25.3071 | 23.2271 | 22.8038 | - |
| NON | - | 14.3472 | 19.0411 | 10.4124 | 25.2943 | 12.563 | - |
| *Difference* | - | 15.0765 | 1.4706 | 14.8947 | -2.0672 | 10.2408 | - |
| GRR2 | | | | | | | |
| SDM Nitrogen % | *n/s* | | | | | | |
| INOC | - | 0.6617 | 0.7058 | 0.8675 | 1.0398 | 1.6433 | 1.876 |
| NON | - | 0.6157 | 0.6863 | 0.9119 | 1.6749 | 2.007 | 2.0187 |
| *Difference* | - | *0.046* | *0.0195* | *-0.0444* | *-0.6351* | *-0.3637* | *-0.1427* |
| SDM C/N Ratio | *n/s* | | | | | | |
| INOC | - | 63.0715 | 60.0939 | 50.0805 | 41.2942 | 25.962 | 22.083 |
| NON | - | 67.3202 | 61.9588 | 47.5469 | 26.0135 | 22.7375 | 21.4849 |
| *Difference* | - | *-4.2487* | *-1.8649* | *2.5336* | *15.2807* | *3.2245* | *0.5981* |
| RDM Nitrogen % * | *LSD: -0.2684 s.e.: 0.1164 df=32 p ≤ 0.0277* | | | | | | |
| INOC | - | 1.4367 | 1.447 | 1.7123 | 1.8375 | 2.3502 | 3.2043 |
| **NON** | - | 1.5265 | 1.7825 | 1.8568 | 2.383 | 2.8361 | 3.2133 |
| *Difference* | - | *-0.0898* | *-0.3355* | *-0.1445* | *-0.5455* | *-0.4859* | *-0.009* |
| RDM C/N Ratio ** | *LSD: 2.5480 s.e.: 0.7095 df=32 p ≤ 0.0011* | | | | | | |
| **INOC** | - | 28.6904 | 28.1277 | 22.4426 | 21.9732 | 18.7002 | 14.5575 |
| NON | - | 26.45 | 23.7318 | 21.205 | 18.5039 | 15.2442 | 14.0689 |
| *Difference* | - | 2.2404 | 4.3959 | 1.2376 | 3.4693 | 3.456 | 0.4886 |

** = significant at alpha < 0.01, * = < 0.05, n/s = not significant

Note: Bolded main effect level shows which treatment was significantly greater

'-' indicates this fertilization (FERT) level was not used in this experiment

# Appendix M - GRR1, GRR2 N content

Appendix M: GRR1, GRR2 N Content

| Trial and Measure | Fertilizer (FERT) Treatment Provided Nitrogen Concentration (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **37.5** | **60** | **82.5** | **105** | **127.5** | **150** | **172.5** |
| GRR1 | | | | | | | |
| SDM N Content (mg) ** | *LSD: 13.7486 s.e.: 2.4176 df=19 p ≤ 0.0001 INTX INOC*FERT p ≤ 0.0203* | | | | | | |
| **INOC** | - | 32.8968 | 46.0576 | 56.4364 | 78.2723 | 92.9913 | - |
| NON | - | 30.2415 | 22.8708 | 41.911 | 55.2269 | 87.6611 | - |
| *Difference* | - | *2.6553* | *23.1868* | *14.5254* | *23.0454* | *5.3302* | - |
| RDM N Content (mg) ** | *LSD: -5.7772 s.e.: 1.3591 df=16 p ≤ 0.0006* | | | | | | |
| INOC | - | 14.9236 | 18.8868 | 20.6669 | 25.2276 | 28.8531 | - |
| **NON** | - | 18.5664 | 22.5307 | 21.597 | 39.4362 | 35.3139 | - |
| *Difference* | - | *-3.6428* | *-3.6439* | *-0.9301* | *-14.2086* | *-6.4608* | - |
| TDM N Content (mg) | *n/s* | | | | | | |
| INOC | - | 48.3522 | 66.577 | 77.336 | 103.15 | 121.88 | - |
| NON | - | 49.8836 | 60.1337 | 77.5378 | 114.89 | 112.61 | - |
| *Difference* | - | -1.5314 | 6.4433 | -0.2018 | -11.74 | 9.27 | - |
| Gain/Loss N (mg)‡ | | | | | | | |
| Supplied N (mg) | - | 48.00 | 66.00 | 84.00 | 102.00 | 120.00 | - |
| INOC gain/loss | - | 0.3522 | 0.5770 | -6.6640 | 1.1500 | 1.8800 | - |
| NON gain/loss | - | 1.8836 | -5.8663 | -6.4622 | 12.8900 | -7.3900 | - |
| GRR2 | | | | | | | |
| SDM N Content (mg) ^ | *LSD: 3.4819 s.e.: 1.8768 df=32 p ≤ 0.0722* | | | | | | |
| **INOC** | - | 38.8907 | 52.8927 | 58.1746 | 72.7094 | 91.3439 | 106.62 |
| NON | - | 38.7317 | 44.1265 | 57.9122 | 77.8501 | 84.2805 | 96.8407 |
| *Difference* | - | *0.159* | *8.7662* | *0.2624* | *-5.1407* | *7.0634* | *9.7793* |
| RDM N Content (mg) | *n/s* | | | | | | |
| INOC | - | 17.7429 | 21.2196 | 29.7702 | 32.8626 | 37.6939 | 40.5481 |
| NON | - | 20.4433 | 26.602 | 29.7517 | 32.2857 | 35.7068 | 38.8031 |
| *Difference* | - | *-2.7004* | *-5.3824* | *0.0185* | *0.5769* | *1.9871* | *1.745* |
| TDM N Content (mg) | *n/s* | | | | | | |
| INOC | - | 57.7097 | 74.8473 | 88.209 | 106.21 | 129.83 | 148.75 |
| NON | - | 60.0653 | 71.9451 | 88.0562 | 111.07 | 121.42 | 137.86 |
| *Difference* | - | *-2.3556* | *2.9022* | *0.1528* | *-4.86* | *8.41* | *10.89* |
| Gain/Loss N (mg)‡ | | | | | | | |
| Supplied N (mg) | - | 48.00 | 66.00 | 84.00 | 102.00 | 120.00 | 138.00 |
| INOC gain/loss | - | 9.71 | 8.85 | 4.21 | 4.21 | 9.83 | 10.75 |
| NON gain/loss | - | 12.07 | 5.95 | 4.06 | 9.07 | 1.42 | -0.14 |

** = significant at alpha < 0.01, * = < 0.05, ^ = < 0.1, n/s = not significant
Note: Bolded main effect level shows which treatment was significantly greater
'-' indicates this fertilization (FERT) level was not used in this experiment
‡ grams of N above or below the quantity provided via fertilization (TDM N - Supplied N)

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**VITA**

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## Education

**Ph.D. – Horticulture** (2021) The Pennsylvania State University, University Park, PA
**M.Sc. – Agronomy** (2017) The Pennsylvania State University, University Park, PA
**B.Sc. – Biology** (2014) The Pennsylvania State University, University Park, PA

## Teaching Experience

**Teaching Assistant** – Hort 150N: Plants in the Human Context (2018); PSU
**Teaching Assistant** – Hort 497: Hydroponic and Aquaponics (2018-2019); PSU

## Awards/Grants

**NE SARE Competitive Graduate Student Grants -** GNE18-186: Symbiotic Nitrogen Fixation in Non-legumes: N-supply, Yield and Quality Effects in Tomato and Pepper. (2018-2021) *$13,366*
**Jeanne and Charles Rider Endowment for Support of Research on the Biotechnology of Food Crop** – Hot Pepper Secondary Metabolite Synthesis under Inoculation Effects of a Novel N-fixing Bacterium (2020-2021) *$5500*