# Microbial Interactions in Tomato *Solanum lycopersicum* for Health, Growth, and Pathogen Defence

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Thesis submitted for the degree of
**Doctor of Philosophy (PhD)**

Faculty of Science, Agriculture and Engineering – School of Natural and Environmental Science

September 2021

**Abstracts**

*Solanum lycopersicum* is an important vegetable high in vitamin A and C and minerals such as phosphorus, iron and high in lycopene and beta-carotene. It is considered the favourite in the food processing and cosmetics industries. *S. lycopersicum* current global production is concentrated in the United States of America, China, and India. The production of *S. lycopersicum* depends on the application of chemical fertiliser; however, ecological damages caused by chemical fertiliser far outweigh its benefits. Thus, there is a need to initiate and adopt eco-friendly cultivation of *S. lycopersicum* using vertically transmitted endophytes. In this study, different strains of vertically transmitted endophytic bacteria were isolated from eight different cultivars of *S. lycopersicum*. The findings show that vertically transmitted endophytes are host specific and display various phenotypes that produce diverse metabolites with different concentrations. It also demonstrated that treated *S. lycopersicum* under fertilised microbial communities performed significantly better than those under the manure microbial community, untreated microbial community, and the control tank. The finding also shows that vertically transmitted endophytes in the *S. lycopersicum* failed to stimulate interaction between *S. lycopersicum* and its surrounding soil microbial communities, which promotes plant growth, increase chlorophyll content, increase fresh and dried biomass of the plant. Our result further demonstrated no significant difference when the isolated vertically transmitted endophytes Bb-B-1 was inoculated on *S. lycopersicum* under the optimum nutrient condition and deprived nutrient condition. Finally, the study demonstrates that microbial communities from fertilised treated soil, manure treated soil, and untreated microbial communities are not involved in inducing or suppressing Auxin, LelRT1, FER, FROS2 and LeNRT2;3 genes in *S. lycopersicum*. It further shows that the *S. lycopersicum* vertically transmitted endophytes are not involved in regulating these genes. Whilst no significant result to demonstrate the possible role of vertically transmitted endophytes the study demonstrated that *S. lycopersicum* vertically transmitted endophytes are host specific and display various phenotypes that produce diverse metabolites with different concentrations. Further investigation is required to focus on the isolated vertically transmitted endophytes precisely to understand their possible roles in the plant host. Additional studies investigating the role of different microbial communities on the host plant required more time to monitor the suitable duration needed by the microbial communities to be established in the new environment.

**Acknowledgements**

Glory and honour to my heavily Father for the gift of life, grace, and mercy. Thanks to my supervisors, Prof. Angharad Gatehouse, Dr Jem Stach and Dr Martin Edwards, this project would not have been possible without your guidance, motivation, and constructive suggestions. I also appreciate Gillian Division, who is always there for me when ordering reagents and other lab utensils. Thanks to my progression panel members, Thomas Howard, and Mark Shirley, for their constructive suggestions and positive comments. Thanks to the NYSC Nigeria for nominating me to be a recipient of the National Merit Awards; I am most grateful to Goodluck Ebele Azikiwe Jonathan GCFR, GCON, for giving me the scholarship, and big thanks to PTDF Nigeria for funding the project.

My heartfelt gratitude to my friends and colleagues, the plant-insect molecular interaction group. Whose companies have made my PhD journey stressless, Names like Jui, Will, Abdul, Feras, Chloe, Cahyo, Robert, Mohamed 1, Mohamed 2, and Mohemed 3, Habiba, Solafa, Jessica, and Sadam Baba. Also, appreciate my friends on the 5th floor Devonshire Building, Yanhua, Adnan and Albar.

Thanks to my Father Irimiya Ajuji Danladi, Mother Rose Ajuji, and my siblings Victoria, Charles, Amin and Benjamen.

Save last is my lovely wife, Ayalwace, my two fantastic boys Zadok, the *priest*, and Salem, *Aye!! Captain*, you guys are amazing; you filled my home with positive energy. Noise, love, and sound of laughter, love you guys to the moon and back.

Contents

Microbial Interactions in Tomato Solanum lycopersicum for Health, Growth and Pathogen Defence ..... 1

**Abstracts** ..... 2

**Acknowledgements** ..... 3

*Chapter one* ..... 7

*1. General introduction* ..... 7

*1.2 Effect of Nitrogen on the environment* ..... 10

*1.3 Organic fertiliser* ..... 11

*1.6 Plant growth-promoting bacteria (PGPB)* ..... 17

*1.7 Endophytes* ..... 18

*Vertically transmitted endophytes (VTE)* ..... 20

*1.8 Project Aim and Objectives* ..... 21

*The overarching aim of the project is to identify vertically transmitted endophytes (VTEs) and better understand their modes of action for improved health, growth, and pathogen defence in Tomato Solanum lycopersicum.* ..... 21

*1.9 This will be addressed through the following specific objectives, which are to:* ..... 21

*Chapter two* ..... 22

*2.1 Isolation and identification of vertically transmitted endophytes from tomato seeds* ..... 22

*2.2 Material and methodology* ..... 24

*2.3 Nutrient agar preparation and other tool use in the isolation and identification of VTE* ..... 24

*2.5 Investing the isolated plant growth-promoting potentials:* ..... 25

*2.6 Bacteria colony incubation* ..... 26

*2.7 Phosphate solubilising and Nitrogen fixation activity assay* ..... 26

*2.8 Siderophores activity assay* ..... 27

*2.9 Phylogeny three construction* ..... 27

*2.11 Phytohormones* ..... 29

*2.12 Results* ..... 29

*2.12 PCR product used for the identification of the VTE species* ..... 33

*2.13 Phylogenetic tree analysis for the VTE isolates* ..... 34

*2.14 Plant growth metabolites produced by the vertical transmitted endophyte using plate assay* ..... 35

*Phosphate solubility* ..... 35

*2.15 plant growth metabolites produced by the vertical transmitted endophyte using spectrophotometry assay* ..... 38
*2.16 Discussion* ..... 40
*3.1 Introduction* ..... 45
*3.2 Plant growth-promoting bacteria* ..... 46
*3.3 Objectives* ..... 46
*3.4 Material and method* ..... 47
*Hydroponic system experiments* ..... 47
*3.5 Isolation of VTE from the cultivars* ..... 48
*3.6 Hydroponic system* ..... 48
*3.7 The phytohormone measurement:* ..... 49
*3.9 Physiological measurement:* ..... 50
*3.10 Statistical tools used for analysis* ..... 50
*3.11 Results and interpretation of plant response to plant-microbe interaction* ..... 51
Measurement of CFU of the three different soil sample microbial communities ... 51
*3.13 Evaluating the improved chlorophyll content of the cultivars* ..... 52
*3.14 Evaluating the improved growth of the cultivars* ..... 53
*3.15 Evaluating fresh biomass of the cultivars* ..... 54
*3.16 Evaluating dried biomass of the cultivars:* ..... 55
*3.17 Evaluating increase root development:* ..... 56
*3.18 Evaluating phytohormone:* ..... 57
*3.19 Discussion* ..... 58
*3.20 Increase in cultivars growth* ..... 59
*3.21 Increase biomass and roots growth.* ..... 60
*3.22 Stimulation of phytohormones* ..... 60
*3.23 conclusion* ..... 61
*Chapter four* ..... 62
*4.1 Exogenously applied vertically transmissible endophytes alter development and disease signalling parameters in tomato* ..... 62
*Introduction* ..... 62
*4.2 Hypothesis* ..... 63
*4.3 Materials and methods* ..... 64
*Media preparation and inoculation with Bb-B-1 and Pseudomonas sp. CT364 strains* ..... 64
*4.4 Evaluating changes in Phenotype and concentration of phytohormone in the treatment* 65
*4.5 Sample data analysis* ..... 65
*4.6 Results* ..... 66
*Evaluating the response of Elegance (tomato cultivar void of VTE) cultivated under four differential microbial communities* ..... 66

*4.7 Exogenous application of VTE Bb-B-1 and known PGPB (Pseudomonas sp. CT364) has less effect on tomato growth and development than increased nitrogen availability ..... 70*
*4.8 Effect of VTE on leaf number in tomato ..... 71*
*4.9 The shoot length development of the inoculated samples ..... 72*
*4.10 The fresh biomass of the inoculated samples ..... 73*
*4.11 Dried biomass of the inoculated samples ..... 74*
*4.12 The concentration of jasmonic acid in the inoculated samples ..... 75*
*4.14 The concentration of 3-indole acetic acid in the inoculated samples ..... 76*
*4.15 The concentration of gibberellic acid in the inoculated samples ..... 77*
*4.16 Discussion ..... 78*
*4.17 Evaluating an increased number of leaves, biomass, and the phytohormones ..... 79*
*Chapter five ..... 82*
*The effects of differential microbial communities on solanum lycopersicum genes involved in the biosynthesis of key plant phytohormones ..... 82*
*5.1 introduction ..... 82*
*5.2 Materials and methods ..... 84*
*bHLH protein ..... 86*
*5.3 Results ..... 87*
*Validation and optimisation of genes of interest using differential temperature ..... 87*
*5.4 Gene expression of genes regulating the biosynthesis of plant phytohormones ..... 88*
*5.5 LeNRT2.3 gene expression ..... 88*
*5.6 Auxin gene expression ..... 90*
*5.7 FRO2 gene expression ..... 91*
*5.8 LelRT1 gene expression ..... 93*
*5.8 FER gene expression ..... 94*
*5.9 Discussion ..... 95*
*5.10 Conclusions ..... 100*
*Summary of the project findings and future perspectives ..... 101*
*6.2 General conclusions ..... 103*
*References ..... 105*

## Chapter one

### 1.1 General introduction

Global population growth is currently 1.1% per year, and if its continues at this rate by 2050 global population growth would reach 9.7billion (Desa 2019), as shown in figure 1.1(DESA 2015). Despite errors and challenges that are involved in population growth projections, with an example of overestimation in the late 90s (Keilman 1998), current technology has made it possible to estimate population growth with 98% to 99% accuracy. An estimation by (Desa 2019) forecasts a population growth of 9.4 to 10.1 billion, with over 50% of this growth coming from sub-Sahara Africa between 2019 to 2050. These forecasts suggest an additional 1.05 billion people to the global population (Desa 2019). According to the estimate, approximately two-thirds of the global population are young people (age 30 or less). Therefore, even a decline in fertility alone may not affect the projected population growth of 9.4 to 10 billion by 2050. 90% of the projected global population growth would come from fewer countries(Desa 2019) such as Asia (China, India, Pakistan and Indonesia), Africa (Nigeria, DR Congo, Egypt, Ethiopia and Tanzania) and North America (USA) (Desa 2019) as shown in table 1.1 (DESA 2015)

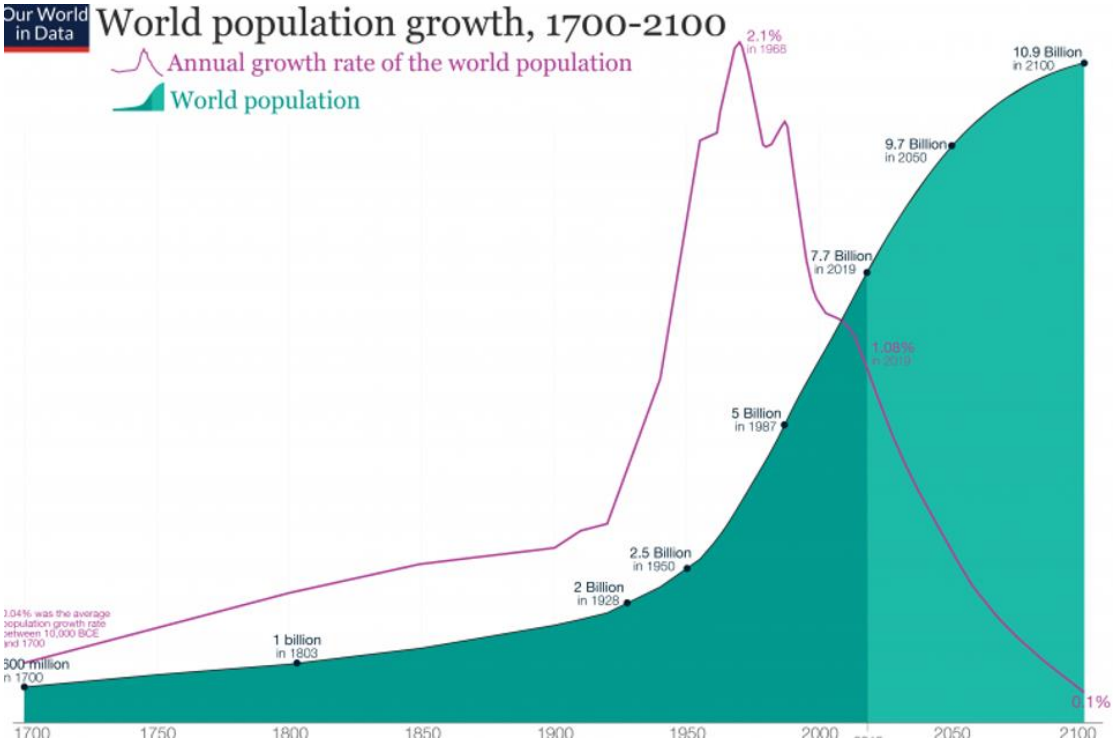


Figure 1.1

Global population growth is estimated to be 9.7 billion people by 2050 estimates from 1950-2015, medium-variant projection and 80 and 95% confidence intervals from 2015-2100. Source: United Nations, Department of Economic and Social Affairs, Population Division (2015). World Population Prospects: The 2015 Revision. New York: United Nations

| Rank | Country | Population 1990 | Population 2010 | Estimated Population 2020 | Growth (%) 1990-2010 | Growth (%) 2010-2020 |
|---|---|---|---|---|---|---|
| | World | 5,306,425,000 | 6,895,889,000 | 7,503,828,180 | | |
| 1 | China | 1,139,060,000 | 1,341,335,000 | 1,384,688,986 | 17.1% | 3.23% |
| 2 | India | 873,785,000 | 1,224,614,000 | 1,296,834,042 | 40.2% | 5.90% |
| 3 | United States | 253,339,000 | 310,384,000 | 329,256,465 | 22.5% | 6.08% |
| 4 | Indonesia | 184,346,000 | 239,871,000 | 262,787,403 | 30.1% | 9.55% |
| 5 | Brazil | 149,650,000 | 194,946,000 | 208,846,892 | 30.3% | 7.13% |
| 6 | Pakistan | 111,845,000 | 173,593,000 | 207,862,518 | 55.3% | 19.74% |
| 7 | Nigeria | 97,552,000 | 158,423,000 | 203,452,505 | 55.3% | 28.42% |
| 8 | Bangladesh | 105,256,000 | 148,692,000 | 159,453,001 | 62.4% | 7.24% |
| 9 | Russia | 148,244,000 | 142,958,000 | 142,122,176 | -3.6% | -0.58% |
| 10 | Japan | 122,251,000 | 128,057,000 | 126,168,156 | 4.7% | -1.48% |

Table 1.1 Estimates global population growth with a given percentage from name countries (UN, 2015)

While there is a significant increase in the global investment towards agricultural technology with 3.25% of spending coming from high-income countries (Molotoks, Smith et al. 2021) however, research spending towards agriculture is currently at 0.52%, despite evidence that research can effectively provide a solution to alleviate the impending food scarcity (Fuglie, Gautam et al. 2019). There is a need for robust investment in technology to curtail the current challenges of climate change, if not an estimated third of the earth land surface may have to be used for agriculture (FAOSTAT 2018) to cope with the food demand in the near future. There are intensified management plans to improve crop production through increased chemical fertiliser application (FAOSTAT 2018), however, this would be at the expense of a

healthy environment. Research evidence has demonstrated that population growth and increasing demand for food exert pressure on the use of chemical fertiliser in agriculture with the desire to increase crop yield and improve global food security (Duan, Xu et al. 2016).

Notable the advent of chemical fertiliser application in agriculture has dramatically increased global food production from 30% to 50%. The increase is attributed to Nitrogen fertiliser (Stewart, Dibb et al. 2005) which had led to high demand for the production of nitrogen fertiliser, as shown in table 1.2. Although different types of fertilisers are used by the farmers, such as phosphorus fertilisers and potassium fertilisers, nitrogen fertilisers are commonly used by farmers. It is estimated 123 Tg nitrogen fertiliser is used per year, giving a 9.5-fold increase from its start production in 1961 (Food 2018). The impact of nitrogen fertiliser to increase food supply through agricultural production is undeniable and reliance on nitrogen fertiliser is evident with 50% of the global population depending on it for agriculture production (Erisman, Sutton et al. 2008).

| Year | 2015 | 2016 | 2017 | 2018 | 2019 | 2020 |
|---|---|---|---|---|---|---|
| Nitrogen (N) | 1100027 | 111575 | 113607 | 115376 | 117116 | 118763 |
| Phosphates ($P_2O_5$) | 41151 | 41945 | 43194 | 44120 | 45013 | 45858 |
| Potash ($K_2O$) | 32838 | 33149 | 34894 | 34894 | 35978 | 37042 |
| Total ($N+P_2O_5+K_2O$) | 184017 | 186668 | 190850 | 194390 | 198107 | 201663 |

Table 1.2 Global demand for fertiliser nutrient use, 2015-2020 (1000 tonnes). World fertiliser outlook to 2020

### *1.2 Effect of Nitrogen on the environment*

Synthetic nitrogen fertiliser has been used in agricultural practice for many decades due to the poor nitrogen content in soil (Fagodiya, Pathak et al. 2020). It is an essential component of fertiliser used to stimulate crop growth and yield (Huang, Zhang et al. 2007, Galloway, Dentener et al. 2008). Nitrogen fertiliser is highly soluble in water and vulnerable to loss when not managed correctly (Pathak, Jain et al. 2016). Continual chemical fertiliser application with inefficient usage is the leading cause of nitrogen

cycle imbalance (Galloway, Aber et al. 2003). It causes the accumulation of many reduced irons, such as nitrate, nitrous oxide, nitrate oxide, and ammonia (Galloway, Aber et al. 2003), leading to groundwater pollution, negatively affecting climate change (Ladha, Tirol-Padre et al. 2016). The nitrogen cycle in the soil influences atmospheric gases such as nitrous oxide, methane, and carbon dioxide (Velthof, Oudendag et al. 2009, de Vries, Lesschen et al. 2010). Nitrous oxide is a major factor that plays a role in global warming been a potent greenhouse gas (Bhattacharyya, Bhatia et al. 2018). Many studies demonstrate that the overuse of chemical fertilisers in the agricultural field reduces crop yield and air quality (Tilman, Cassman et al. 2002, Sutton, Oenema et al. 2011) and increases atmospheric carbon dioxide (Tilman, Cassman et al. 2002). Furthermore, the efficiency of the fertiliser keeps decreasing as the usage intensified, with a decline from 68% to 47% between 1961 to 2010 (Lassaletta, Billen et al. 2014). As mentioned earlier, its application comes with soil deteriorating consequences of acidification, nutrient loss (Cai, Wang et al., 2015), greenhouse gas emission, and groundwater contamination (Uphoff and Dazzo, 2016). Studies by (Mózner, Tabi et al. 2012) demonstrated that plants captured only about 35-50% of the applied fertiliser, and 50 or 65% are usually washed. Thus, synthetic fertiliser has been implicated as the primary cause of groundwater pollution, with pollutants such as nitrogen due to excess fertiliser saturation (Galloway, Dentener et al. 2008, Press 2016, Wang, Zhu et al. 2018).

### 1.3 Organic fertiliser

To provide a more lasting solution that can curb synthetic fertiliser use and supply the crops with the required nutrient. Organic fertiliser has been reputed to be the option to reduce the use of synthetic fertilisers. The common organic fertiliser used is animal manure, which may be solid, semisolid or liquid, usually produced during growing animals for meat and milk or eggs (Sims and Maguire 2005). This manure is made of animal waste feed, faeces, urine and bedding materials as well as any other material used during the handling of the manuring process, these mixtures, when added to the soil, improve soil properties by adding nutrients to its organic matter(Sims and Maguire 2005). Research has shown that China produces an estimated 4.6 billion tonnes of manure annually. The United States of America generates approximately 24billion

tonnes of animal manure, whilst the United Kingdom produces 35.7 million tonnes (Carlin, Annamalai et al. 2009, Smith and Williams 2016, Niu and Ju 2017).

Organic fertiliser has demonstrated improved crop yield, improved soil organic carbon stock, restore and maintain soil structure and significantly influences the mitigation of negative climate changes ((Diacono and Montemurro 2011, Lu, Chadwick et al. 2015, Abdalla, Hastings et al. 2018, de Melo, Pereira et al. 2019). Among the different types of organic fertiliser used, animal manure is the popular organic fertiliser use, rich in plant nutrients with evidence to increase soil organic carbon stock and sequestration efficiency, thus, providing the plant with more nutrients (Du, Cui et al. 2020), (Zhang, Wang et al. 2010, Wang, Lu et al. 2016). Moreover, it improved phenolic compounds, antioxidants, carotenoids, and certain vitamins that have been found in crops cultivated under organically treated soil (Sharpe, Gustafson et al. 2020).

### 1.4 Organic farmland area in the United Kingdom

The United Kingdom in 2018 reported 474000 hectares of land area used for cultivation of organic products, which was a decrease by 8.4% total land area (fully converted and under conversion) used for an organically grown product in 2017 figure 1.3. The decrease was due to a decline in plots of land with low production value, such as temporary pasture and woodland. The reports show that in 2010 organic farming reach its peak however, since then, it has declined with a 36% decrease in production by 2018.

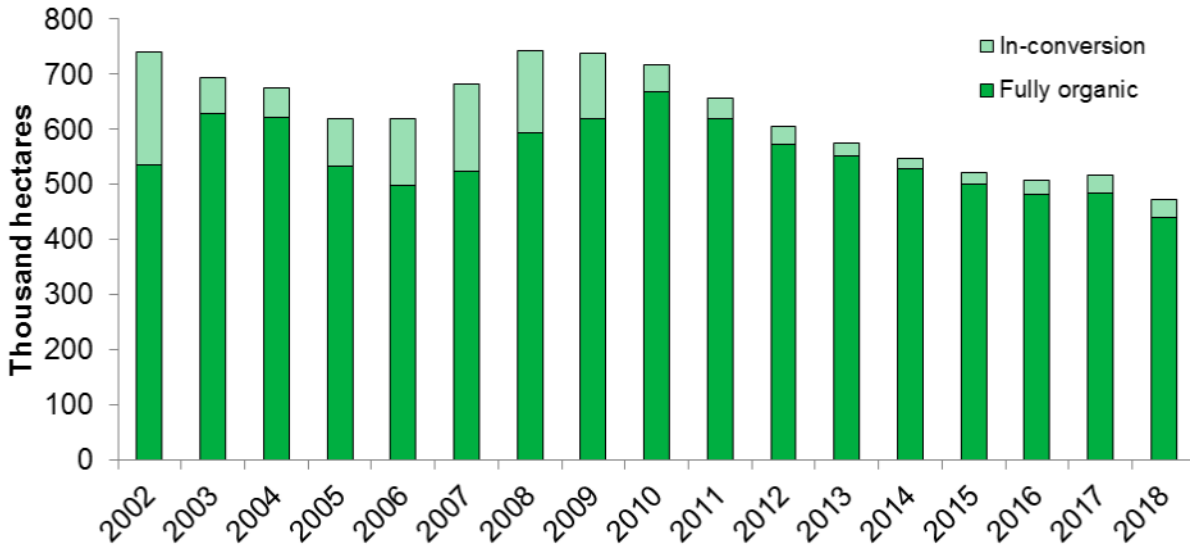


**Figure 1.2 Organically cultivated land area in the United Kingdom 2002-2018**

The graph demonstrates a decline in organic farming due to decreases in the total area under (fully converted) for organic farming 2008-2018. Source: Department for Environment, Food & Rural Affair 16 May 2019

### 1.5 The decline in yield of organically grown crops in the United Kingdom

The main crops (Cereals, arable crops, and vegetables) which are grown through organic farming in the United Kingdom reach their peak in 2008-2009. However, the cereal and arable crops yield shows a drastic decline in yield from 2010 to 2018 as result of reduction in land area used for organic farming figure 1.3 above. Source: Department for Environment, Food & Rural Affair 16 May 2019.

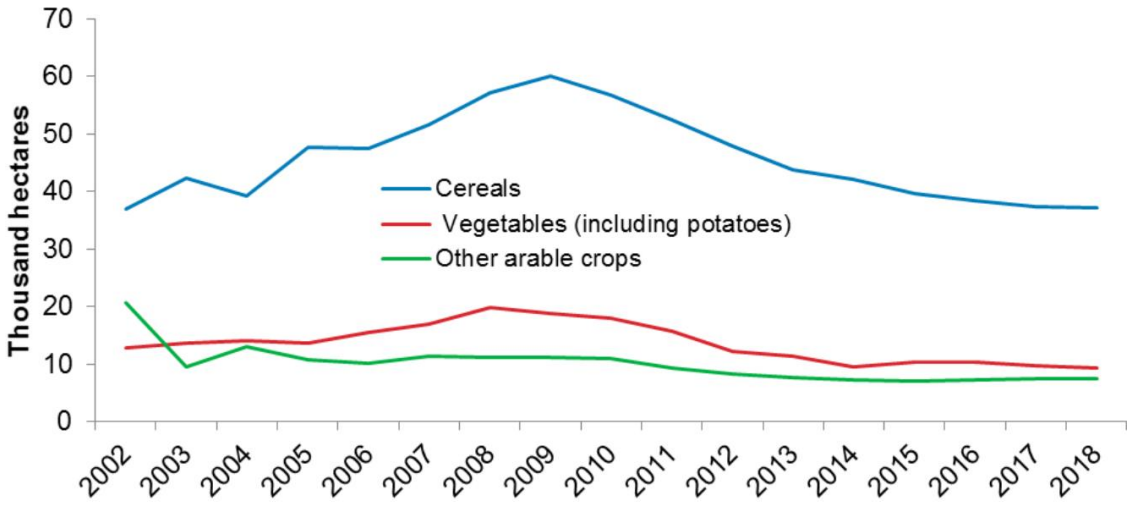


**Figure 1.3 United Kingdom graphical representation of decline in yield of organically grown crops 2002-2018**

The graph demonstrates a decline in the United Kingdom organic farming crops yield, reflecting the decrease in farmland use organically from 2008. Source: Department for Environment, Food & Rural Affair 16 May 2019.

Reports from the department for environment food and rural affairs National Statistics United Kingdom figure 1.5 shows that 489000 hectares of (fully converted and under conversion) area cultivated organically in 2020, an increase of 0.8% than 2019. The increase was attributed to a 12% rise in fully organically converted land area in 2020. Source: Department for Environment, Food & Rural Affair 24 June 2021. Furthermore, the data shows that all crops, i.e., cereals, vegetables, and arable crops, contributed to this slight increase for 2020.

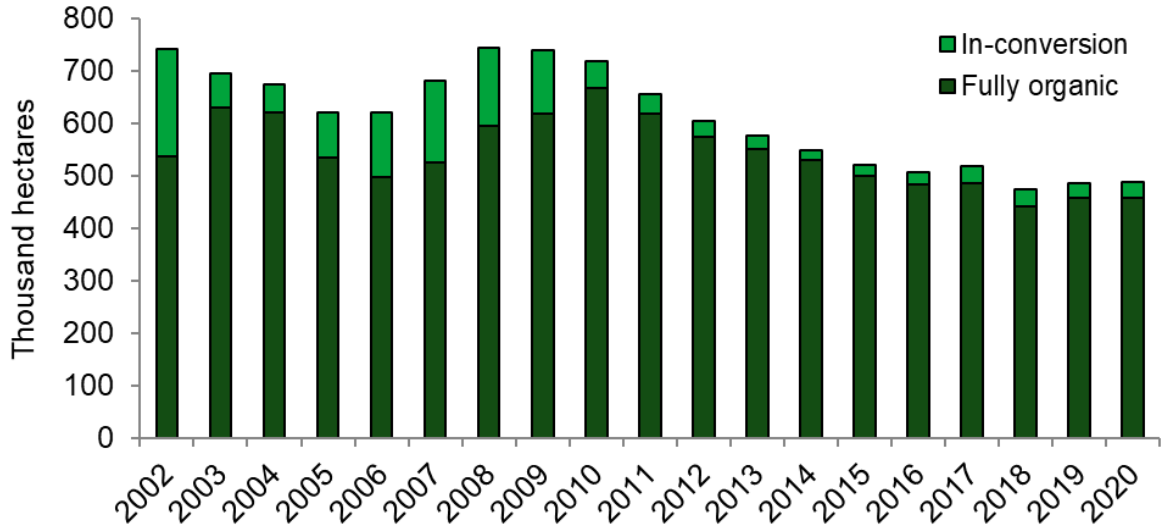


**Figure 1.4 Organically cultivated land area in the United Kingdom 2002-2020**

The graph demonstrates an increase in the United Kingdom farmland used for organic farming, reflecting an increase in crop yield under fully converted for organic farming 2020 as shown in figure 1.6 below. Source: Department for Environment, Food & Rural Affair 24 June 2021. As a result of increase in farmland used for organic farming figure 1.5, there was an increase in the yield of cereal and vegetables in 2020, as seen in figure 1.6. However, there was no significant increase in other arable crops even with an increase in organic farmland

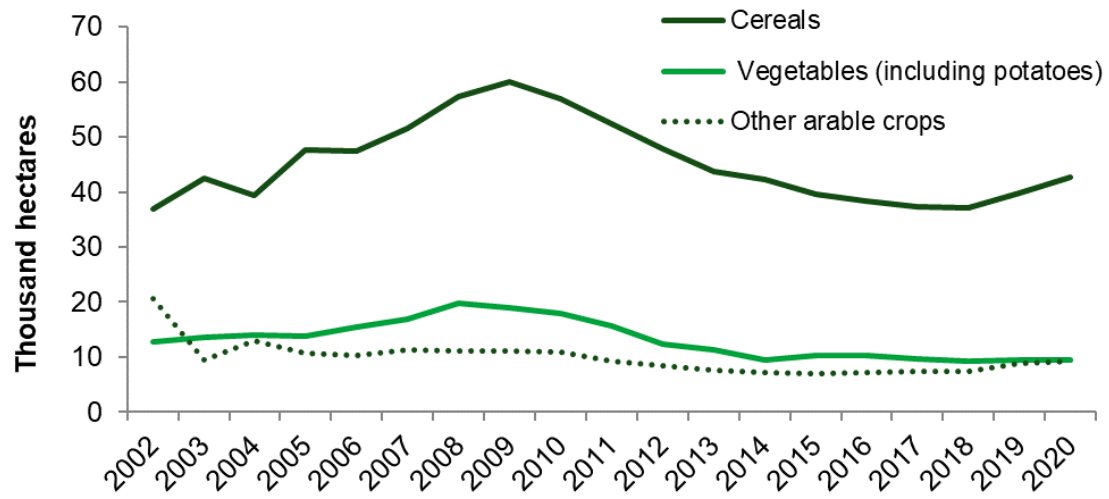


**Figure 1.5 United Kingdom graphical representation increase in yield of organically grown crops 2002-2020**

The graph demonstrates an increased yield of organic crops as a result of increasing the size of organic farmland in the United Kingdom in 2020 as shown in figure 1.5 above. Source: Department for Environment, Food & Rural Affair 24 June 2021

Global organic farming is met with many challenges due to a lot of factors as demonstrated in the United Kingdom, indicating that organic fertiliser application on agricultural soil has either a low or marginalised effect on crop yield and growth (Seufert, Ramankutty et al. 2012, Wei, Yan et al. 2016) compared to synthetic fertiliser application. Moreover, applying organic manure, which is usually directly spreading to the soil, often becomes a source of water and air pollution (Font-Palma 2019). Due to washing off the manure into the river, with over 78% of it, N excreta lost, therefore, increasing the biochemical oxygen demand in the water, causing an imbalance in the aquatic life and encouraging algae blooms in freshwater due to dissolved phosphorus and ammonia (Kesaano and Sims 2014, Bai, Ma et al. 2016, Font-Palma 2019). Approximately 130Tg of N is produced by manure annually, and only 20 to 40% fertilises our cropland. The rest is wasted in the atmosphere and washed off into ground or surface water (Bai, Ma et al. 2016). An additional challenge to using organic fertiliser is the production process, which is laborious and water consumption, with roughly 435liters of water per cow per day (Font-Palma 2019). Manure also contains traces of heavy metals, antibiotics, pathogens and other toxic constituents that may threaten human health and the environment (Zhou, Hao et al. 2005, Leclerc and Laurent 2017)

Our study focuses on tomato (*Solanum lycopersicum*), a versatile vegetable consumed raw or processed (Story, Kopec et al. 2010). it is an exciting plant for dietitians, biotech companies, farmers, and scientific research (Mehrin, Zakir et al. 2020). Tomato is an important vegetable globally due it high vitamin A and C and in minerals such as iron and phosphorus, as well as high in lycopene and beta-carotene (Melomey, Danquah et al. 2019). Epidemiological data have demonstrated an inverse relationship between tomato consumption and the risk of cardiovascular disease (Lazarus and Garg 2004). Also dermatological research has demonstrated that adding Aloe vera gel and tomato powder can improve the efficacy of a body lotion (Ijaz, Durrani et al. 2021). Tomato is also considered the favourite in food processing industries available in paste, puree and juice (Willcox, Catignani et al. 2003). Currently, tomato is a global product, and the primary producers are China, India, and the United States of America (Faostat 2017). 2014 tomato production recorded 171 million tonnes with an estimated yield of 37 tonnes per/hectare (Faostat 2017)

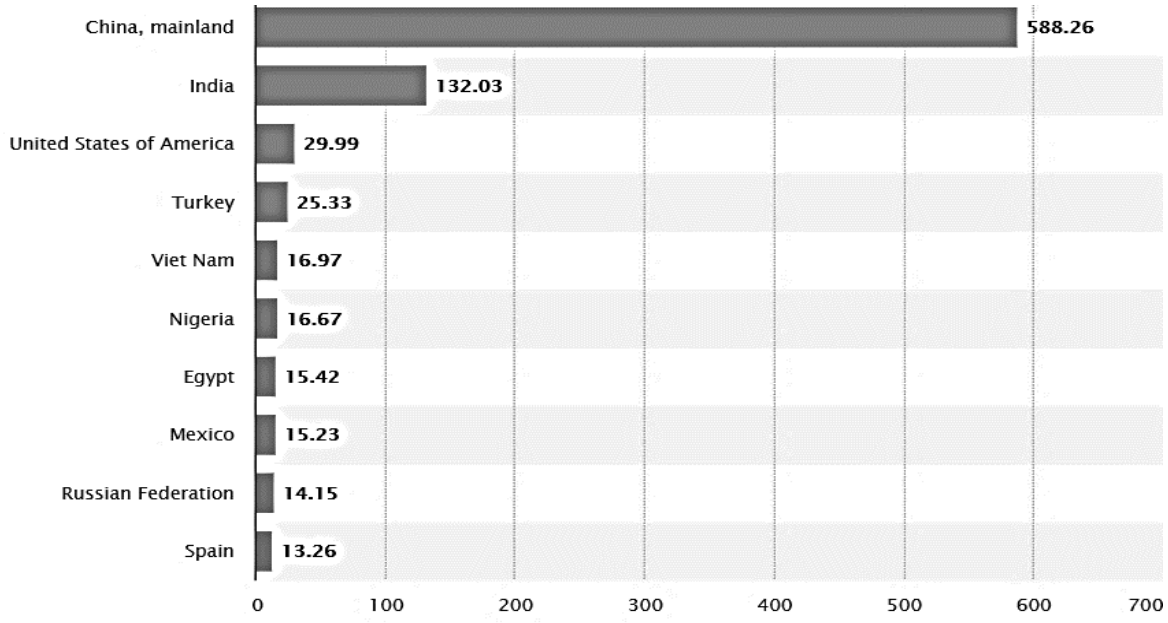


**Figure 1.6 Top global producers of fresh vegetable (tomato) worldwide 2019**

The figure above shows statistics of the top global producer of fresh vegetable (tomato) in the year 2019, China as the leading producer, followed by India and the United States of America (Shahbandeh 2019)

Tomato crop depends on the application of fertiliser, more specifically nitrogen (Liang, Ridoutt et al. 2019) for their growth and development. Studies using manure and chemical fertiliser have shown that tomatoes under chemical fertiliser produce a better yield than tomatoes under manure treated (Ghorbani, Koocheki et al. 2006). Many claims have been attributed to the effects of the moderate application of nitrogen fertiliser on the yield and growth of tomatoes, with a reported increase in tomato yield with an optimum rate of 75-100kg/N/ha. More evidence has shown that there is a correlation between tomato growth rate and the concentration of nitrogen. Also, Potassium fertiliser has been implicated for better fruit quality and chemical composition of tomatoes (Gupta and Sengar 2000, Ddamulira, Idd et al. 2019). Potassium is also considered a crucial element in tomato development that influences physiological and biochemical processes involving enzyme activation, such as the metabolism of carbohydrate and proteins compounds (Zhende, Junjie et al. 1996). Potassium has also been demonstrated to stimulate root, enhance growth, and

increase fruit size and quality, and additional finding shows that nitrogen, potassium and phosphorus increase tomato productivity (Marschner 1995, EL BASSIONY 2006, ORTAS 2013). Despite all the boosts given tomato production by chemical fertiliser, studies have demonstrated that the hazard associated with chemical fertiliser far outweigh its benefits. It is causing a range of challenges, from ecological imbalance to decline in quality of the tomato fruits (Mehdizadeh, Darbandi et al. 2013). Initiative to encourage adaptation of eco-friendly cultivation practice suitable for sustainable development and to curtail or eliminate the adverse disadvantage caused by the chemical fertiliser requires a suitable system to initiate the use of plant growth-promoting bacteria.

### 1.6 Plant growth-promoting bacteria (PGPB)

The interaction between a plant and its microbial community has been the subject of interest, potentially changing global agriculture dramatically (Podile and Kishore 2007, Antoun 2013, Kashyap, Solanki et al. 2019). Plant coexists with various taxa of microbial communities that play a vital role in plant growth, resistance to pathogens and tolerance to environmental stress. The microbial strains are numerous such as the bio fertiliser promoting host plant growth through nutrient supply and fixed nitrogen, the rhizoremediator that degraded soil pollutants, and the photostimulation that stimulate the release of phytohormone. They are commonly called plant growth-promoting bacterial (PGPB) (Kloepper and Schroth 1981, Kashyap, Solanki et al. 2019). Usually found in the plant's rhizospheres, phyllosphere, and endosphere (Brimecombe, De Leij et al. 2007). Numerous studies have been conducted to elucidate the role, function, and effectiveness of PGPB interaction with a plant under laboratory settings with positive results (Lugtenberg and Kamilova 2009, Ahemad and Kibret 2014). Example under the laboratory settings addition of *P.putida* strain PCL1760 to *Forl* spores control (Validov, Kamilova et al. 2007). Also, *D. salina* extract when inoculated on young *S. lycopersicum* seedlings infected with bacterial speck spot disease, reduced 65% -77.0% severity of the bacterial speck spot disease (Ambrico, Trupo et al. 2020). Evidence from various studies has shown that differential soil treatments have a tremendous influence on the structure and function of the soil microbiome (Wang, Niu et al. 2011, Zhang, Shamsi et al. 2012). Research on the application of organic fertiliser has been demonstrated to enhance soil microbiome

activities (Jilani, Akram et al. 2007, Francis, Holsters et al. 2010). Furthermore, organic fertiliser has significan impact soil carbon and nitrogen cycle compared to chemical fertiliser. The organic fertiliser improved microbial biomass and thus, enhanced organic Nitrogen decomposition more than the chemical fertiliser which changed the microbial community structure (Ma, Wen et al. 2020). Organic fertilisers are nutrient-rich and stay longer in the soil than chemical fertiliser, and they are potential carbon and nitrogen sources suitable for microbial reproduction and growth (Zeng, Liao et al. 2007). Also, chemical fertiliser has been shown to improve the soil microbial population and activities compared to the control soil, demonstrating that both organic fertiliser and inorganic chemical fertilisers can be used to exploit the soil microbiome. However, when the overall bacterial population and the microbiome activities between the organic manure treated soil with inorganic chemical fertiliser treated soil was compared, microbial communities and activities under organic manure treated soil are higher (Zhang, Shamsi et al. 2012). Up to date research is ongoing to establish reliable evidence thus, PGPB technology results in a field setting have not provided consistent findings (Bashan, de-Bashan et al. 2014). Due to the difference in types of plant, cultivar, weather, climate, and soil type (Bashan, de-Bashan et al. 2014), which play a significant role in the effectiveness and prevalence of the PGPB (Kloepper 1981, Bashan, de-Bashan et al. 2014, Kumari, Mallick et al. 2019). Studies have demonstrated that microbes introduced to a new environment need time to adapt to the new change before colonising and competing with the indigenous microbiome (Michalet, Rohr et al. 2013, Thijs, Sillen et al. 2016). With the inconsistency in using organic fertiliser and soil bacterial, another option that may be suitable is the vertically transmitted endophytes. That is, microbial communities transferred from parent to offspring.

### *1.7 Endophytes*

Endophytes referred to as those microbial communities colonising the intracellular spaces of plant tissues but not exerting pathogenic or any negative effects on the hostplant (Adeleke and Babalola 2021). These endophytes inhabit different populations occupying different domains within the host plant, actively contributing to the host. They are also a prolific source of bioactive metabolites that are distinctively different from plants with unique characteristics (Aly, Debbab et al. 2011). Endophytes

have shown to mediate the host plant tolerance under salinity stress, downregulates endogenous hormones to enhance tolerance and increase host plant resistance to drought (Bilal, Shahzad et al. 2020, Kushwaha, Kashyap et al. 2020, Sadeghi, Samsampour et al. 2020). Furthermore, endophytes have been demonstrated to enhance host tolerance to oxidative stress through increased release of antioxidants such as phenolic and flavonoids (Herrera-Carillo, Torres et al. 2009, Torres, Singh et al. 2009). They also produce phenolic acids and their derivatives, such as isobenzofuranones, isobenzofurans, and mannitol, enhancing host tolerance to stress (Richardson, Chapman Jr et al. 1992, Strobel 2002, Harper, Arif et al. 2003, Huang, Cai et al. 2007). Furthermore, they regulate secondary metabolites using sexual and asexual development pathways and their vegetative growth (Tag, Hicks et al. 2000, Shwab and Keller 2008). Apart from producing specific bacterial/fungal-related metabolites, endophytes can produce plant-related metabolites. Thus, they can be a potential candidate for alternative plant metabolites source (Priti, Ramesha et al. 2009) by providing the host plant with its supportive produce metabolite and enhancing the host chemical defences (Wink 2008).

In contrast, a study by Rodriguez and Redman suggests that endophytes may switch to pathogens due to a change in a host or growing conditions (Rodriguez and Redman 2008). The study further noted that some endophytes might be mutualistic or commensal (Schulz, Römmert et al. 1999). An example is *Colletotrichum magna* endophytes demonstrate a dynamic lifestyle when inoculated in different tomato cultivars. Three different expressions observed were mutualistic interaction, commensal interaction, or parasitic interaction (Redman, Dunigan et al. 2001). Therefore, the initial notion that all endophytes are microbial communities beneficial to the host plant needs to be re-evaluated, it also shows that there is still a need for more research to explore this exciting frontier. Furthermore, it is arguable that some of these endophytes have a very negligible impact on the host communities.

## Vertically transmitted endophytes (VTE)

Vertically transmitted endophytes (VTE) interact with plants, contributing to preserving the seeds during the dormant state and helping growth and development under suitable conditions (Adams and Kloepper 2002). The VTEs are transferred from the germinating seedlings, usually during the release of organic molecules by the developing seedlings tissue (Kaga, Mano et al. 2009). The release of the organic molecules such as amino acids, fatty acids and sugars (Vives-Peris, de Ollas et al. 2020), citric acid, malic acid and fumaric acid (Jin, Zhu et al. 2019) by the host plant, recruits and encourages the population growth of the VTE (Baker and Cook 1974), allowing the VTE to dominate the plant and, subsequently, the seeds. In contrast, the seeds provide protection and nutrition for the microbes (VTE) (Kaga, Mano et al. 2009). The microbes (VTE) offer nutritional support and encourage the growth and development of the hostplant by releasing various metabolites that stimulate plant growth and development (Ek-Ramos, Gomez-Flores et al. 2019, Omomowo and Babalola 2019). Specific study on vertically transmitted fungal endophytes *Penicillium janthinellum* LK5 in *S. lycopersicum* has demonstrated to stimulate growth, improve plant defences, and mitigate the host plant against aluminum induce stress (Khan, Hussain et al. 2015). The PJLK5 demonstrate to reduce *S. lycopersicum* root structure damage and the essential lipid membrane by regulating antioxidants and endogenous salicylic acid during aluminum induce stress (Khan, Hussain et al. 2015). Also facilitate host plant development by activating supplements nutrient in the soil, delivering and controlling phytohormones and conferring the hostplant with resistance to pathogens(Shahzad, Khan et al. 2018). The reports by Shahzad and colleagues show that VTE can enhance the soil structure and serve in bioremediating contaminated soil through sequestering and degrading hazardous iron and xenobiotic mixes(Shahzad, Waqas et al. 2016). Also, vertically transmitted bacteria have been reported to lower the level of ethylene using ACC deaminase enzyme catalytic activities (Luo, Xu et al. 2012, Rashid, Charles et al. 2012, Pandya, Rajput et al. 2015). Currently, host-associated microbes have been demonstrated to effectively confer stress tolerance, improve nutrient uptake, and facilitate phytoremediation of heavy metals and hydrocarbons (Busby, Soman et al. 2017).

Therefore, we recommend more research to identify the suitable candidate and best inoculation techniques for the desired result output. These project experiments are

tailor to understand how the VTE encourage plant growth and development and whether differential treatment on microbial communities can influence plant growth and health. We use series of experiments following a guided key plan in our aim and objective, as stated at the general summary of the thesis.

### 1.8 Project Aim and Objectives

The overarching aim of the project is to identify vertically transmitted endophytes (VTEs) and better understand their modes of action for improved health, growth, and pathogen defence in Tomato *Solanum lycopersicum*.

### 1.9 This will be addressed through the following specific objectives, which are to:

1. Identify and characterise vertically transmitted endophytes (VTE) in tomato *S. lycopersicum*.
2. Elucidate the influence of fertilised, manured, and untreated microbial communities on the growth and health of *S. lycopersicum*
3. Investigate the influence of vertically transmitted endophytes on the growth and health of *S. lycopersicum*
4. Investigate whether exogenous application of vertically transmitted endophytes influence the expression of plant transcription factors
5. Elucidate the role of exogenously applied vertically transmitted endophytes on expression of genes encoding plant transcription factors in *S. lycopersicum*
6. Evaluate the effects of differential microbial communities on expression of genes involved in the biosynthesis of key phytohormone *S. lycopersicum*.

## Chapter two

### *2.1 Isolation and identification of vertically transmitted endophytes from tomato seeds*

Plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria that colonise the host plant root zone and provide support by stimulating the plant roots to access nutrients in the soil (Sharma, Kumar, et al., 2012). Evidence from several PGPR isolated strains shows that they can produce secondary metabolites such as auxin, siderophores, and phosphorus solubiliser (Pii, Mimmo, et al. 2015, Shabanamol, Divya, et al. 2018, Szymańska, Borruso, et al. 2018, Stefanowicz, Stanek, et al. 2019) which enhance plant growth, improved health, and improved crop yield. It is important to note that the rhizosphere bacteria are the first bacteria to be identified as plant growth-promoting bacteria (PGPB), with species capable of enhancing plant growth and providing defence against the pathogenic microorganisms (Akintokun and Taiwo 2016). However, previous experiment by Ningthoujam showed that many of the various isolates used in inoculation on different fields outside the region of their origin performed poorly with different outcomes, which led to inconsistent field results (Ningthoujam, Sanasam, et al. 2009). Therefore, to ensure consistency from applying the PGPB on a different field outside their origin region an innovative solution is required alternatively we can use an isolate common to each specific indigenous soil (Salantur, Ozturk, et al. 2005, Ningthoujam, Sanasam, et al. 2009) or use an endophyte that is common to the plant. It is interesting because endophytic microorganisms dwell within the plant tissues, interacting with the plant. They initially access the plant by penetrating the tissues through the root zone, usually targeting the junction between epidermal cell and the cell wall of the root hair, flowers, and stems (Kobayashi and Palumbo 2000). Or by releasing enzymes that degrade the plant cell wall polysaccharides (Huang 1986). once inside the tissues, most strains may remain at localised entry points like strain *Pseudomonas fluorescens* 89B-61 that demonstrate colonised cotton root tissues however, they failed to move beyond the root's zones (Quadt-Hallmann, Hallmann et al. 1997). While some variants may quickly spread throughout the whole plant tissue (Hallmann, Quadt-Hallmann, et al. 1997).

A good example are other species of *P. fluorescens* which colonised their host by moving from the roots to the stem and up to the leaves (Quadt-Hallmann, Kloepper et al. 1997) to support the host by improving the nutrient uptake. In addition to acquiring endophytes from the soil, the bacteria can be vertically transmitted via the seeds. Vertically transmitted endophyte (VTE) are microorganisms that move from one parent cultivar to the next generation. This is a novel approach at a developing stage and a promising natural source for biofertiliser. However, the successful transmission, abundance, and the rate of microbial (vertically transmitted endophyte) interaction within the seed of a crop depend on the plant requirement. Its subsequent transmission to the next generation is mainly controlled by selective plant release of its allelochemicals with little interference from other factors (Grayston, Wang, et al., 1998). The plant is the architecture of their microbial community, the selective release of exudes can either nurture or inhibit microbe (Luo, Tao et al. 2019, Trivedi, Leach et al. 2020). Thus, the colonisation and the adaptation of specific vertically transmitted endophytes depend on the cultivar. Vertically transmitted endophytes are beneficial to their host plant by producing metabolites such as phosphate solubilisers, auxin release, and siderophores potentials (Sgroy, Cassán, et al. 2009, Ji, Gururani, et al. 2014), also conferred tolerance/resistance to the host plant (Huo, Zhuang et al. 2012), and are sensitivity to antibiotics (Rashid, Charles, et al. 2012). Plant-microbes interaction is a reciprocal relation in return, the endophytes use the plant for protection and nutrition, benefiting from a nutrient source with less competition than outside tissues (Bacon and Hinton 2007). Evidence of endophytes utilised plant nutrient as a carbon source has been demonstrated by *Pseudomonas spp* which employed L-arabinose as a carbon source (Malfanova, Kamilova et al., 2013). Therefore, since the VTE are inside the plant tissues, we hypothesised that any change in the external environment would not influence the VTEs PGP potentials, unlike the rhizospheres PGPB that reside within the soil environment and easily affected by influence of the external environmental conditions. We aim to test this hypothesis in the subsequent chapters as we progress with the project. The objective of this chapter is to isolate VTE from the cultivars of *S. lycopersicum* seeds. Also, to identify and characterised these isolates on a plate media and a phylogenetic tree.

Hypothesis

1. Tomato seeds is habitat of different types of vertically transmitted endophytes
2. Vertically transmitted endophytes release secondary metabolites that are beneficial to the host plant
3. All vertically transmitted endophytes are beneficial to their host plant

## 2.2 Material and methodology

All seeds were purchase from magic Garden Seeds GmbH, Junkersstr 7,93055 Regensburg (seeds are open-pollinated, pure breed), except for the cultivar Elegance (hybrid seeds from De Ruiter). Seeds such as Roma (*Solanum lycopersicum)* organic, Ananas (Pineapple) (*Solanum lycopersicum*) organic, Beate'Blanche' (*Solanum lycopersicum*) organic, Zuckertraube (*Solanum lycopersicum*) organic, Wild type (*Solanum pimpinlliforlium*), Matina (*Solanum lycopersicum*) organic, Rose de Beme (*Solanum lycopersicum*) organic, yellow pear (*Solanum lycopersicum*) organic and Elegance (*Solanum lycopersicum*) hybrid.

## 2.3 Nutrient agar preparation and other tool use in the isolation and identification of VTE

NaOCl, Ethanol, Petri-Dish, inoculation loop, and reagents for preparing nutrient agar (peptone 5g, sodium chloride 5g, HM peptone B 1.5g, Yeast extract 1.5g, and agar 15g). All reagents are suspended in 1000 ml distilled water dissolved by heating in a microwave. The media solution was further sterilised at 121$^{o}$C for 15 min in an autoclave. In a Laminar hood, solution cool 40-55$^{o}$C before aliquoting in petri-dish (for selective bacterial growth). The seeds were placed on a sterile petri-dish, an ethanol 95% added agitate slowly for ten (10) sec and drained the 95% ethanol. The seeds were washed with sterile water, and 0.53% NaOCl was added, agitate slowly for two minutes, and the NaOCl drained, followed by seed was washed with distilled water, 70% ethanol was added mix for 2 minutes. The ethanol was removed from the petri dish, and seeds were washed three times with sterile water; seeds were spread on

sterile filter paper in a sterile petri dish and allowed to be dried. 10ul of an aliquot from the last seeds washed were pipetted on nutrient agar and Rose Bengal media as a control. The surface sterile seeds were added to nutrient agar for selective bacteria isolation and Rose Bengal media for selective fungal isolation.

### 2.5 Investing the isolated plant growth-promoting potentials:

*Pikovskaya's agar with slight modification* Yeast extract 0.5g, dextrose 10.0g, calcium phosphate 5.0g, ammonium sulphate 0.5g, potassium chloride 0.2g, magnesium sulphate 0.1g, manganese sulphate 0.0001g, ferrous sulphate 0.0001g and agar 25.0g. Reagents were dissolved in 1000ml sterile distilled water by microwave to boiled and autoclaved at 121$^{o}$C for 15min, allowed to cool 40 $^{o}$C to 55$^{o}$C in a luminal hood. The semi-cool media is aliquoted into sterile petri-dish and allowed to solidify.

*Jensen's nitrogen-free media* Sucrose 20g, dipotassium phosphate 1.0g, magnesium sulphate 0.5g, sodium chloride 0.5, ferrous sulphate 0.1g, sodium molybdate 0.005g, calcium carbonate 2.0g, agar 15.0g. Dissolved in 1000ml of distilled water by microwave to boiled, autoclaved at 121$^{o}$C and allowed to cool 40-55$^{o}$C in the luminal hood, aliquot into the petri-dish, and allowed to solidify.

*Norris glucose nitrogen-free media* glucose 10.0g, dipotassium phosphate 1.0g, magnesium sulphate 0.2g, calcium carbonate 1.0g, sodium chloride 0.2g, sodium molybdate 0.005g, ferrous sulphate 0.1g and agar 10.0g. all the reagents dissolved in 1000ml of sterile distilled water, microwave to boiled and autoclaved at 121$^{o}$C and allowed to be cool at 45 to 50$^{o}$C in a luminal hood, aliquot into sterile petri-dish, and allowed to solidify.

*Blue Agar, chrome azurol s (CAS)* for siderophore detection assay, media was collected from study lab Newcastle University where it was prepared as instructed in (Louden, 2011) with modification to suit our desired experiment: blue dye preparation solution 1: 0.08 g Fluka chemicals was dissolve in 50ml distil water. Solution 2: 0.0027 g FeCl3-6H20 was dissolved in 10 ml mM HCL. Solution 3: 0.073 g HDTMA was dissolved in 40 ml distil water. 9 ml solution 2 was added to solution 1 and mixed thoroughly before adding solution 3, and autoclave and store. Solution B minimal

media solution was prepared by making solution (i): Minimal Media 9 salt solution stock (dissolved 15 g KH2PO4, 25 g NaCl, 50 g NH4Cl in 500ml of distil water). (ii): 20% Glucose Stock (dissolved 20 g glucose in 100nl distil water). (iii): NaOH Stock (25 g NaOH was dissolved in 150 ml of distil water) (iv): Casamino Acid Solution (3 g Casamino acid was dissolved in 27 ml distil water, it was then extracted with 3% 8-hydroxylquinoline in chloroform to removed trace iron. (v) CAS agar Preparation: 100 ml MM9 salt was added to 750 ml of distil water, and 16 g Bacto agar was added the mixture was autoclave allowed cool to 50$^{o}$c before adding 33 ml of sterile Casamino acid solution and 12 ml of 20% glucose solution to MM9/PIPE mixture. Slowly 110 ml of blue dye solution added and agitate before poured into a plate. Bacteria used for siderophores assay was from pure culture colony where 10µl aliquots were added to 990µl of distilled water, and a serial dilution was performed. The strain with lower bacteria optical density (OD) which was 8x108 nm/ml was used to set a standard for the other strains.

### 2.6 Bacteria colony incubation

From the aliquot, 10µl of the dilute was inoculated to a segment on the petri-dish. The plate was stored in the dark at 37$^{o}$C for 48hrs assay phosphate solubilising bacteria on Pikovskaya's media and another set of media inoculated and incubated at 25$^{o}$C for 72hrs for nitrogen fixation bacteria on Norris glucose Agar. Also, sets of plates for siderophores detection on CAS media was inoculant, incubated in the dark at 25$^{o}$C for 72 to 96 hrs.

### 2.7 Phosphate solubilising and Nitrogen fixation activity assay

The phosphate-solubilising potential strains produced a clearing zone around the colony, solubilising the chemical tricalcium orthophosphate on the Pikovskaya's media. While the strain with a nitrogen fixation potential was incubated on nitrogen-free media (Jensen's media) and examined, the strain efficiency was established by taking the index. (index=A/B where A is (colony + halo zone diameter) and B is the colony diameter on the media. Each strain used for inoculation was replicated four times, recorded, and the data plotted using sigma plot to run an analysis of variance and subsequently plot a bar graph.

## 2.8 Siderophores activity assay

Strains with the ability to produce discolouration on CAS media around the colony indicate a potent iron chelator (siderophore); the strains index taken as A/B, where A is (colony + halo zone) diameter, B is the (diameter of the colony). For each strain, the plate's inoculation was replicated four times, recorded, and the data used in sigma plot to run an analysis of variance and subsequently plot the graph.

## 2.9 Phylogeny three construction

Genomic deoxyribonucleic acid (gDNA) extraction, amplification, sequencing, and bioinformatic analysis for phylogeny tree analysis. Protocol was based on sigma Ltd procedure with modifications.

gDNA extraction was performed with a GenElute Bacterial genomic kit purchase from sigma Ltd. Sample from a fresh prepared pure colony culture 0.2-0.3g of biomass directly collected using an inoculation loop into a 2ml centrifuge tube, 180µl of lysin solution T/buffer was added into the centrifuge tube, 20µl of RNAase solution was added into the centrifuge tube, mixed, and incubated for 2min at room temperature. 20µl of proteinase K was added, mixed thoroughly 15 secs on vortex, and incubate in a water bath at 60$^{o}$C for 1hr· 200µl of lysis solution C, vortex thoroughly, and incubate for 60$^{o}$C for 30min.

2ml binding column was prepared by adding 500µl column preparation solution, centrifuge at 12000xg for 1min, the supernatant was decanted and a 200µl of ethanol (95%) was then added to the lysate from the above samples and vortex thoroughly for 5-10 secs. The content was transfer into the treated binding column, the binding column in the centrifuge tube was ran in centrifuged at 6500xg for 1 min. The was supernatant decanted with the collection tube, binding was column placed on a new collection tube and 500µl wash buffer1 that had previously been mixed with 95% ethanol was added to the column, and centrifuge at 6500xg for 1 min. The inflow was descanted, but the centrifuge tube was retained. An additional 500µl wash solution1 previously mixed with 90% ethanol was added to the column and centrifuge for 3min at the maximum speed of 14500xg to dry column. The tube centrifuged for 1 minute

dried the column at maximum speed and finally, the collection tube with the inflow liquid was decanted, and a 100µl of elution buffer was directly added into the gDNA binding tube, incubate at room temperature for 6minutes before centrifuged at 6500xg for 1minutes. The gDNA concentration, its purity and protein content were quantified with a Nanodrop™ 2000/2000c spectrophotometer. gDNA integrity was also checked on agarose electrophoresis gel before the concentration from all the different strains were optimised, and the ribosomal subunit of the bacterial gene 16s was amplified, using the universal bacterial primers (27F sequence 5' AGAGTTTGATCCTGGCTAG 3', 1525R sequence, and 1492R sequence 5' GGTTACCTTGTTGTTACGACTT 3'). PCR (Polymerase Chain Reaction) setting was 95ºC for 5:00min, 95ºC for 0.15sec, 46ºC for 0.30sec,72ºC for 0.30sec and 72ºC for 5min finally hold at 4ºC.

After the amplification of the 16s RNA (ribonucleic acid) of the bacterial strains were confirmed on an agarose gel, and Exonuclease I- Shrimp Alkaline Phosphatase clean kits, was used to clean the PCR product. 10µl of PCR product was incubated with 4µl of Exonuclease I- Shrimp Alkaline Phosphatase; and incubated at 37ºC for 15min, and additional incubation at 80ºC for 15min, and all the process uses the PCR thermal circle. The clean PCR product was sent to Durham University for sequencing. The forward and reversed sequences were view with Finch TV chromatogram viewer used for viewing trace data from sanger DNA sequencing, edit, and create contigs with Bioedit, a biological sequence alignment tool. The consensus sequence of each strain was compared to the online sequences on the EZBio-cloud database (Yoon, 2017) to get the strains with sequence similarities. Strain similarities were copied in FASTA format and align in MegaX with muscle Alignment option; the align strains used to construct a tree was with maximum likelihood, model method Kimura 2-parameter was used for the tree-bootstrap.

The estimated Transition/Transversion bias (*R*) is 1.52 substitution patterns, and rates were estimated under the Kimura (1980) 2-parameter model [1]. The nucleotide frequencies are A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. For estimating Machine Learning (ML) values, a tree topology was automatically computed. The maximum Log-likelihood for this computation was -6098.588. This analysis involved 32 nucleotide sequences. There were a total of 1675 positions in the

final dataset. Evolutionary analyses were conducted in version 10 MEGAX Molecular Evolutionary Genetics Analysis across computing platforms that run on a window.

### 2.11 Phytohormones

The strains were inoculated into a lysogeny broth (LB) with the addition of tryptophan 0.1%. For 1-aminocyclopropane-1-carboxylate (ACC) assay, it was a culture in tryptic soy broth (TSB) incubate on a shaker at 200rpm and 28$^{o}$C for 72hrs while for IAA assay, incubate on a shaker at 170rpm and 28$^{o}$C for 48hrs to obtain the log phase, none inoculate culture serves as a control. The collected pellet from the culture medium was used for the IAA assay, with Salkowski Reagent for the sample preparation. In contrast, Dworkin, and Foster (DF) media were used to prepare the ACC assays sample. Both assays were in replicate three-time

### 2.12 Results

After surface sterilisation, seeds from the nine cultivars were incubated on a selective media of nutrient agar for bacterial growth and Rose Bengal agar to grow fungi. Table 1.1 demonstrated the isolate results from the different cultivar after 72hrs of incubation at 25$^{o}$C in a darkroom to encourage the growth and development of the vertically transmitted endophyte. Cultivars such as Wild type, Zuckertraube, Berner Rose, and Ananas produced two vertically transmitted endophytes per cultivar and cultivars such as Roma, and Beaute Blanch Matina Yellowpear had one isolate per cultivar.

| *Cultivar* | *Accession Number* | *Bacteria Isolate* | *Accession Number* | *Fungal Isolate* |
|---|---|---|---|---|
| *Roma* | *Ro-B-1* | *One* | *Ro-F-1* | *One* |
| *Wild Type* | *Wt-B-1, Wt-B-2* | *Two* | *Wt-F-0* | ----- |
| *Beaute Blanch* | *Bb-B-1* | *One* | *Bb-F-1, Bb-F-2* | *Two* |
| *Zuckertraube* | *Zt-B-1, Zt-B-2* | *Two* | *Zt-F-0* | ----- |
| *Matina* | *Mt-B-1* | *One* | *Mt-F-0* | ----- |
| *Berner Rose* | *Br-B-1, Br-B-2* | *Two* | *Br-F-0* | ----- |
| *Ananas* | *An-B-1, An-B-2* | *Two* | *An-F-1, An-F-2* | *Two* |
| *Yellow Pear* | *Yp-B-1* | *One* | *Yp-B-0* | ----- |
| *Elegance* | *Eg-B-0* | ----- | *Eg-F-0* | |

*Table 2.1 Demonstrating the number of vertically transmitted endophyte isolated per cultivar where first and second alphabet respresnt isolate name third alphabet represent (B) bacteria/ (F) fungi and the number represent accession number of the isolate*

One bacterium and one fungus were isolated on a nutrient, and Rose Bengal agar from Roma cultivar. The wild type has two bacteria isolated, Beaute Blanch has one bacterium, and two fungi were isolated. The cultivar Zuckertraube has two bacteria isolated. One bacterium was isolated from the Matina cultivar, and from Berner rose cultivar, two bacteria were isolated. The Ananas cultivar has two bacteria and two fungi isolated. One bacterium was isolated from the yellow pear cultivar, and no endophytes were isolated from the Elegance cultivar.

| Isolate | Texture | Form | Elevation | Margin |
|---|---|---|---|---|
| *Mt-B-1* | Flat,shiny surface, butterlike to touch | Irregular/punctiform | Convex | undulate |
| *An-B-1* | Brawny white | Punctiform | Raise | Entire |
| *An-B-2* | Filamentous, whitish | Circular | Raise | Undulate |
| *Zt-B-1* | Rough whitish milky | Circular | Convex | Entire |
| *Zt-B-2* | Whitish/grey butterlike | Punctiform | Convex | Undulate |
| *Ro-B-1* | Slippery/pale white | Irregular | Flat | Lobate |
| *Yp-B-1* | Mucoid/milky white | Circular | Convex | Lobate |
| *Bb-B-1* | Mucoid/milky white sticky | Punctiform | Raise | Undulate |
| *Wt-B-1* | Rough/whitish grey | punctiform | Raise | Undulate |
| *Wt-B-2* | Tough/ rough | Punctiform | Raise | Undulate |
| *Br-B-1* | Rough/whitish | Punctiform | Raise | Undulate |
| *Br-B-2* | Sticky/mucoid yellowish/white | Punctiform | Filamentous | Undulate |

*Table.2.1 vertically transmitted endophyte bacteria colony appearance and description on the agar media where the first and second alphabet represent isolate name third alphabet represent bacteria and the number represent accession number of the isolate*

The VTE isolates were inoculated on Nutrient agar and incubated in the dark for 72hrs at 25$^{o}$C. The bacteria isolated from the Matina cultivar has a flat glistening appearance with an irregular form with convex elevation and an entire margin. The bacteria isolated from Ananas cultivar appear brown, white punctiform. Filamentous white with two

forms of punctiform and circular with rise elevation on margin of an entire. Zuckertraube cultivar isolates appearance is from whitish milky rough to whitish-grey butter-like with circle and punctiform formation and convex elevation and undulated and entire margin. The isolates from Roma cultivars appear slippery pale white with an irregular form and flat elevation lobate margin. Yellow-pear isolates appear mucoid milky white with a circular form and convex elevation and lobate margin. Beauteblanch isolates appear mucoid milky white with a punctiform formation and raise elevation and undulate margin. The wild type isolates appear rough whitish and whitish gray with a punctiform formation raise elevation and undulate margin. Bermer rose isolates appear rough whitish and sticky mucoid with a filamentous form and pulvinate elevation and undulate margin.

## 2.12 PCR product used for the identification of the VTE species.

Before the downstream application in figure 1.1, the gDNA integrity was checked on an agarose gel. Line 2 has a concentration of 218.4ng and purity of 1.81. Line 3 has 147.7ng, with purity of 1.79. Line 4 has 122.9ng with a purity of 1.76. Line 5 has 373.5ng with a purity of 1.79. Line 7 has 297.6ng with a purity of 1.78. Line 8 has 267.8ng with a purity of 1.72. Line 9 has 213ng with a purity of 1.80. The PCR product was ran on gel electrophoresis, where the sizes of the target gene were identified to be around 1500 bp long on the gel. These base pair lengths correlate with 16s rRNA base pair for the suitable sequencing that would allow species identification (Clarridge 2004).

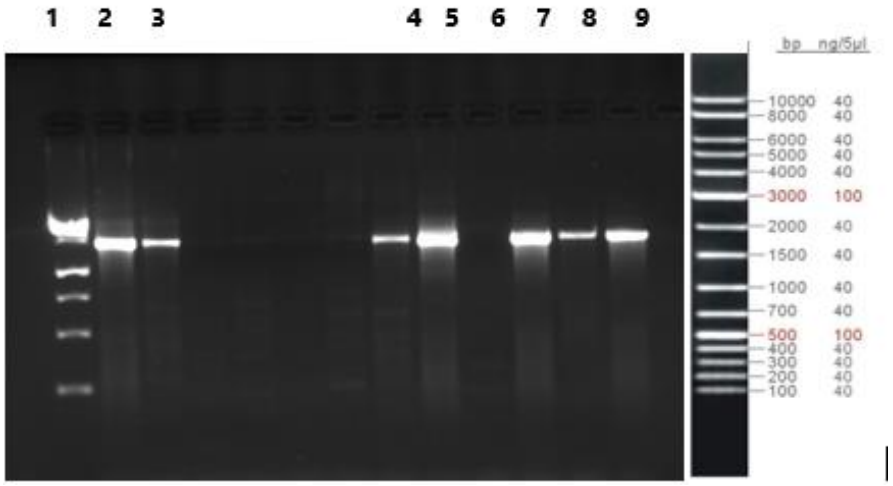


***Fig.2.1 gel electrophoresis of 16s CPR product of VTE***

Gel electrophoresis presenting the PCR products of isolated bacterial strains from the seeds of tomato seeds

1= ladder(1kb), 2 = isolate strain from Matina seed cultivar, 3 = isolate strain from Ananas seed cultivar, 4 = isolate strain from Zackertraube seed cultivar, 5 =isolate from Roma seed cultivar, 6 = isolate strain from yellow pear seed cultivar, 7 = isolate from Beauteblache seed variety, 8= isolate from Berner Rose seed cultivar1 and 9= isolate from Berner Rose seed cultivar2.

### 2.13 Phylogenetic tree analysis for the VTE isolates

From the sequence's fragments, curated sequences were deposited into the Ezbiocloud.net to identify the sequence similarities. Sequences were further deposited into NCBI to validate the exported sequence from Ezbiocloud.net before constructing the phylogenetic tree with MEGA-X. The estimated Transition/Transversion bias (*R*) is 1.52. Substitution pattern and rates were estimated under the Kimura (1980) 2-parameter model [1]. The nucleotide frequencies are A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. For estimating ML values, a tree topology was automatically computed. The maximum Log-likelihood for this computation was -6098.588. This analysis involved 32 nucleotide sequences. There were a total of 1675 positions in the final dataset—evolutionary analyses with MEGA-X.

From figure 2.2, two bacterial genera were identified by the phylogenetic tree from the sequenced strain analyses as the co-habitat of the different *Solanum lycopersicum* cultivars, Bacillus, and Paenibacillus, a crucial step in the establishment of the strain identities and their functional role in their host plant. The 16s rRNA was used as the targeted gene based on its ubiquity within most bacterial species. Yet, diverse, and specific, only the same bacteria species have similar 16s rRNA, making it a reliable genetic marker among the different bacterial species.

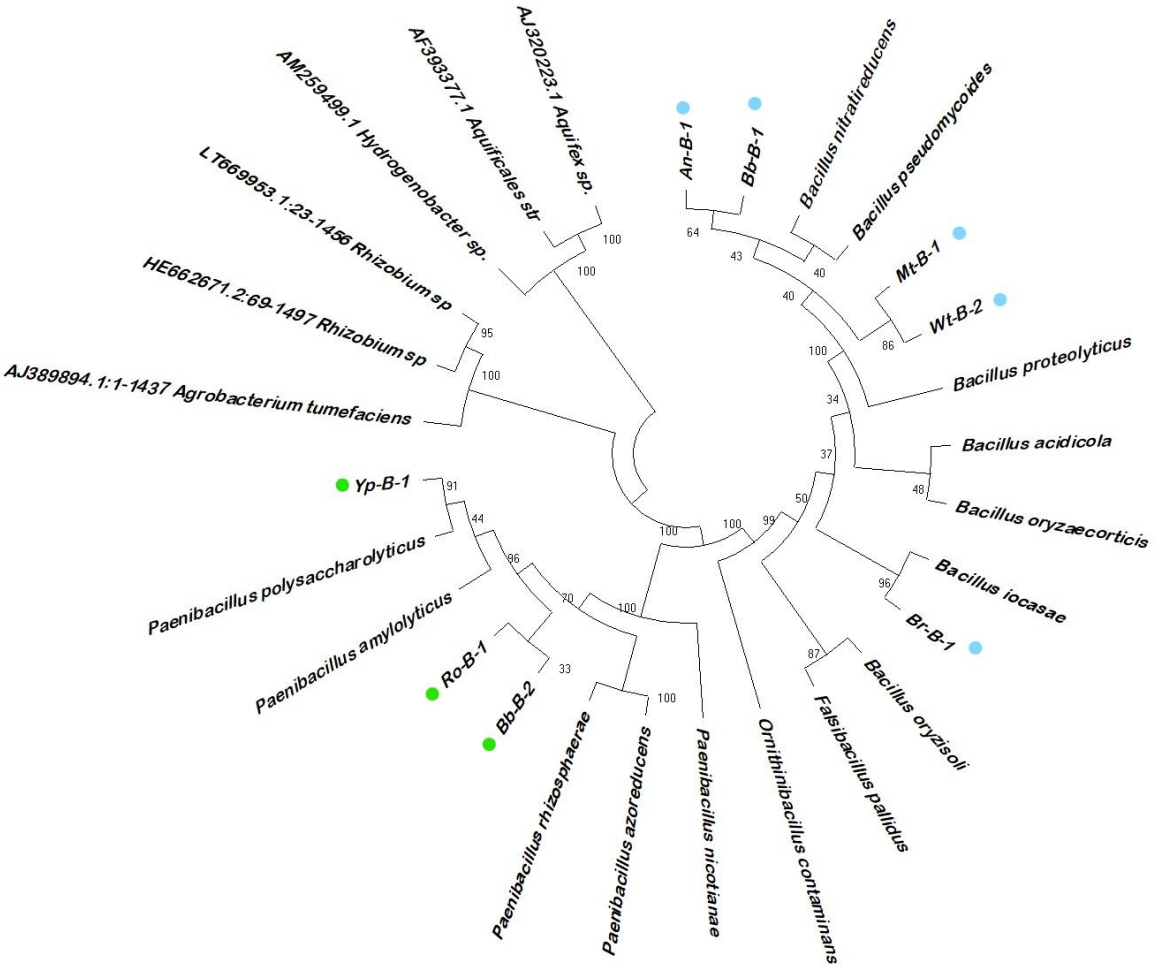


Figure. 2.2 Phylogeny tree of isolates VTE from *S. lycopersicum* cultivars showing their genus and ancestral linkages: An-B-1, Mt-B-1, Br-B-1, Bb-B-1, Wt-B-2 are drive from the genus *Bacillus*. Isolates Ro-B-1, and Yp-B-1 and Bb-B-2 are from *Paenibacillus*.

## 2.14 Plant growth metabolites produced by the vertical transmitted endophyte using plate assay

### Phosphate solubility

All strains were screen for their plant growth promoting (PGP) potential, and isolates with phosphate solubility potential was analysed using the Pikovskaya media. The strains from An-B-2, Bb-B-1(Bacillus), Br-B-1and Br-B-2(Bacillus), Mt-B-1 (Bacillus), and Ro-B-1 (Paenibacillus) demonstrates the same phosphate solubility potentials. While An-B-1, Wt-B-2(Paenibacillus), Yp-B-1(Paenibacillus), and Zt-B-2 isolates have the same phosphate solubility potential, the Strain from Br-B-1(Bacillus) and Zt-B-1

Isolate have demonstrated the same phosphate solubility potential. However, a strain isolated from the Wt-B-1 (Paenibacillus) demonstrates evidence of higher phosphate solubility potential. Strain with a phosphate solubility potential is a beneficial trait that the host plant need, especially in phosphate scares environmental conditions.

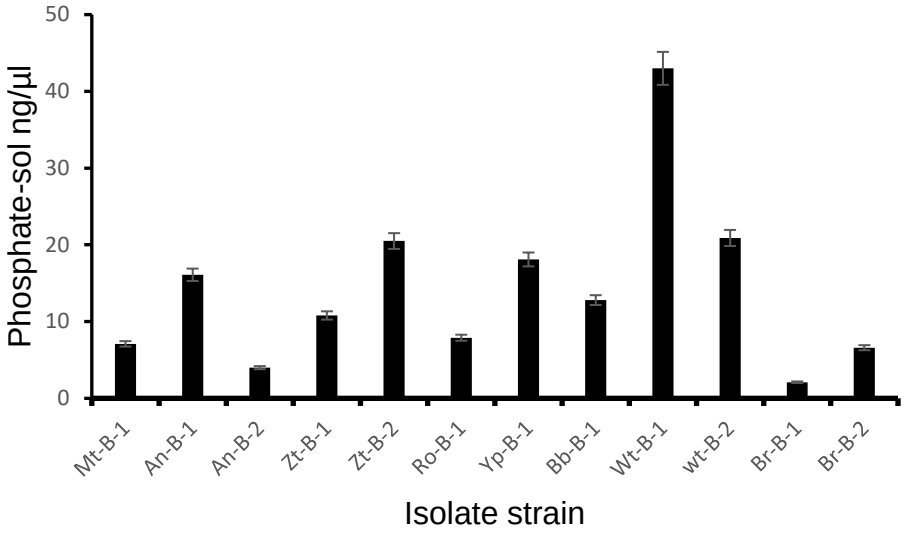


*Figure 2.2 phosphates solubility potential of the isolated strains using the Pikovskaya media to analyse the isolate vertically transmitted endophyte metabolite potential at α=0.05 with a confidence level of 95%. Isolates demonstrate significant different release of phosphate solubilising metabolites.*

**Siderophores potential**

Another assay to determine the siderophore potential of the vertically transmitted endophytes from the *S. lycopersicum* showed that not all isolates were able to release siderophores, strains such as An-B-1= and An-B-2 demonstrate siderophore potential whilst in contrast, the strains from Bb-B-1 (Bacillus),Br-B-1(Bacillus),Bb-B-2(Bacillus), Mt-B-1 (Bacillus), Ro-B-1(Paenibacillus), Wt-B-1(Paenibacillus), Wt-B-2(Paenibacillus), Yp-B-1 (Paenibacillus), Zt-B-1, and Zt-B-2 did not demonstrate any siderophores potential. As demonstrated in previous research strain with evidence to release siderophores are considered potential bio-fertilisers. Here the samples will be tested in the subsequent experiment.

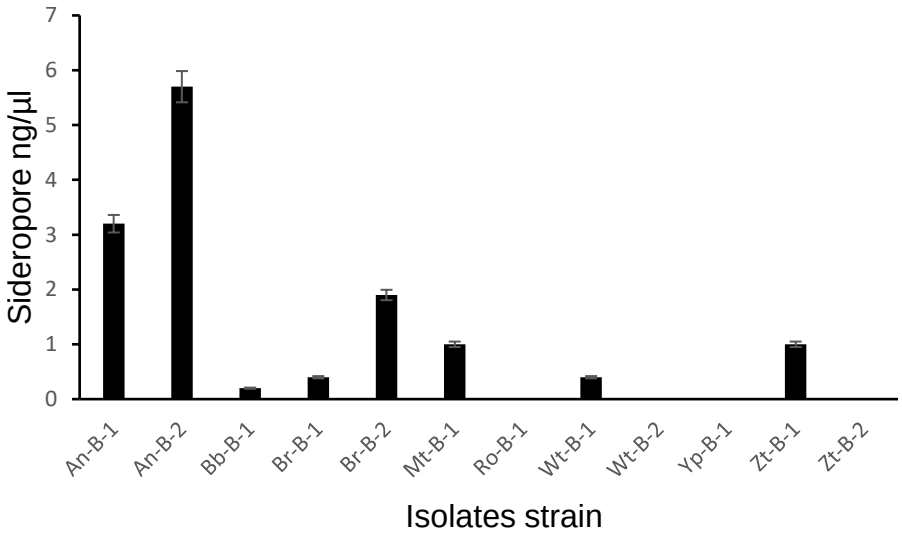


*Figure. 2.3 Strains of isolated vertically transmitted endophytes demonstrate a significantly different release of siderophores within the different isolates on CAS at α=0.05 with a 95% level.*

The ability for the different strain to fixed nitrogen was tested on Jensen media. The strains demonstrated variable potential in the nitrogen fixation: From An-B-1, Bb-B-1 (Bacillus), Ro-B-1 (Paenibacillus), Yp-B-1 (Paenibacillus), and Zt-B-1 demonstrate the same nitrogen fixation. In contrast, strains An-B-2, Br-B-1(Bacillus), and Mt-B-1 (Bacillus) show the same nitrogen fixation potential whilst strains Br-B-2(Bacillus), Wt-B-1(Paenibacillus), and Zt-B-2 demonstrate better nitrogen fixation potential.

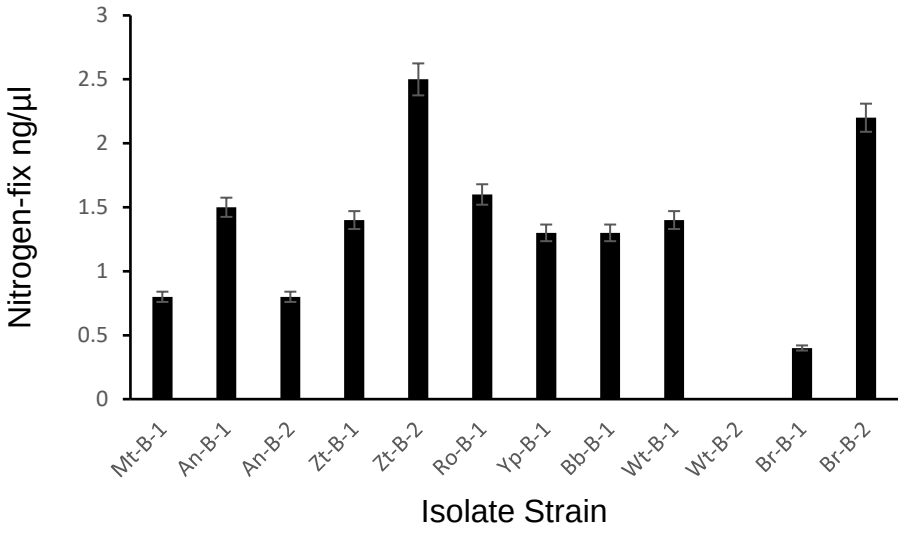


*Figure. 2.4 Strain of isolated vertical transmitted endophyte release metabolites for Nitrogen fix on Jensen media. Selective growth and clear zone by nitrogen fixation bacteria after incubation at 25$^{oc}$ for 72hrs in the dark growth room at α=0.05 with a confidence level of 95% has the P-Value of 0.000.*

## 2.15 plant growth metabolites produced by the vertical transmitted endophyte using spectrophotometry assay

### 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme

Using a spectrophotometer, we analysed the potential of the isolated strains to produce ACC deaminase enzymes, our rational is based on previous studies that demonstrates bacteria and fungi influence plant growth regulators and inhibitors by releasing secondary metabolite which significantly influence plant responses (Frankenberger Jr and Arshad 2020). The measured 1-aminocyclopropane-1-carboxylate ACC deaminase enzyme concentration in all the strain was assayed. However, there is no significant difference at $p<0.05$ compared to the positive control Pseudomonas sp. CT364 (Montero-Calasanz, 2013) which has demonstrate to release IAA, siderophore and phosphate solubilisation metabolites responsible to stimulate plant grow. The strains ability to produce a 1-aminocyclopropane-1-carboxylate ACC deaminase enzyme is beneficial to the host plant essential in reducing excess ACC production which may lead to stress plant.

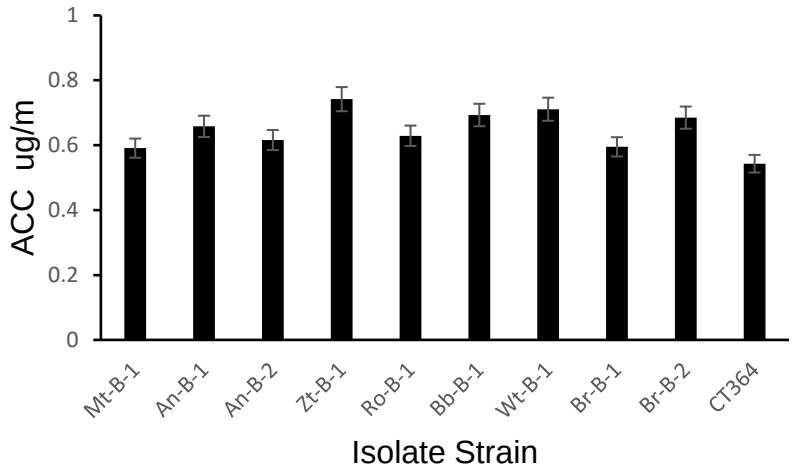


*Figure 2.5 concentration of 1-aminocyclopopane-1-carboxylate release by isolate of vertically transmitted endophytes at α=0.05 with confident level 99.80% within the isolates is not significantly difference.*

**indole-3-acetic acid**

Furthermore, the indole-3-acetic acid concentration produced by the strains was analysed with the following results. An-B-1, An-B-2, Bt-B-1(Bacillus), Br-B-2(Bacillus), Mt-B,Ro-B-1(Paenibacillus),Wt-B-1(Paenibacillus),Wt-B-2(Paenibacillus), Yp-B-1(Paenibacillus), Zt-B-1, and Zt-B-2 strains is not significantly different at $p<0.05$ compared to the reference strain CT364. CT364 strain has been previously proving to produce IAA to promote plant growth hence, any strain that can produce an equal concentration of IAA can confer advantageous traits on the host plant.

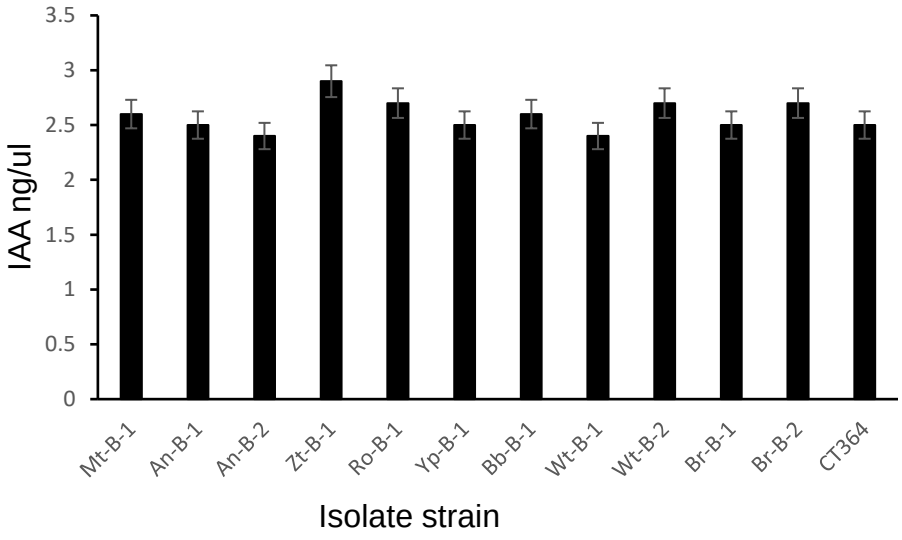


*Figure 2.6 concentration of indole-3-acetic acid release by isolate of vertically transmitted endophytes at α= 0.05 with confidence level = 99.88% has the P-Value of 0.169 shows no significant difference within isolate*

### 2.16 Discussion

Various bacterial strains have demonstrated over time to be a useful tool in the pharmaceutical, food, and agricultural sectors (Caplice and Fitzgerald 1999, Schwarz, Kehrenberg, et al. 2001, Babalola 2010, Rattanachaikunsopon and Phumkhachorn 2010). The potential of microbial to benefit industrial application is often investigated before proceeding with the downstream applications. The strains identification and characterisation are crucial in all the downstream applications. Here vertically transmitted endophytes have been isolated from different tomato cultivars twelve bacterial strains were isolated and incubated in various microbial enriched media. The incubated isolate tested for the plant growth-promoting potentials shows various morphological identities ranging from the flat shiny glistening surface, butter-like, and punctiform to filamentous and mucoid milky white recorded table 2.1. Despites several attempts to extract gDNA from all the samples VTE, only few strains eight were successfully isolated. Strains were subjected to genus identification and ancestral linkage with a suitable primer that binds to the 16S rRNA mitochondrial gene. To determine the position of the sequence and to establish the identities of the isolated strains, 16S rRNA sequence was amplified with the universal primer 1429R and 27F

primers. The sequence sizes of the amplified genes were 1500bp Figure 2.1, which aligns with the standard length of the bacterial mitochondrial gene 16S rRNA sequence. The results of the PCR products were sequenced, and comparisons were made for the sequence similarity. The results demonstrate that only two bacterial genus and fungal endophytes (fungi which we have not identified) co-inhabit the seed of the *S. lycopersicum*, and these genera share ancestral linkage, one genus evolving from the other genus, i.e., *Bacillus* and *Paenibacillus* genera.

The isolate sequence from Mt-B-1 and Wt-B-2 figure 2.2, demonstrates that the strain shares a clade with *Bacillus Pseudomycoides*, *Bacillus toyonnensis, Bacillus proteolyticus*, and *Bacillus pacificus* with bootstrap value of 86%, confirming its status as a *Bacillus* moreover, based on the EZBioCloud database, the sequence had a 99.30% sequence similarity with *Bacillus* and a top hit strain number 0711P9-1. The strains on this clade are novel strains drawn from common ancestor *Bacillus cereus* a subdivision of the genus bacillus with 12 closely related species (Liu, Du et al. 2017). Furthermore, the strains were characterised by spore-forming and facultative anaerobic. They are also ubiquitously distributed in various environments with diverse conditions (Rasko, Altherr, et al. 2005). These strains grow under the optimum temperature of 30$^{o}$C and pH range of 7-8(Dufrenne, Soentoro, et al. 1994, Dufrenne, Bijwaard, et al. 1995, Liu, Du et al. 2017). Another comparison is the sequence of an isolate from Bb-B-1 figure 2.2 with rough surface, whitish and punctiform morphological features, and raised elevation table 2.2. confirmed that the strain shares a clade with *Bacillus nitratireducens,* a gram-positive non-motile rod-shaped, aerobic, and endospore-forming bacterium. The strain closes relative is *Bacillus aryabhatta* with 97.4% sequence similarity and *Bacillus megaterium* with 97.1% sequence similarity, a clade that evolves from the genus bacillus. This clade is characterised by its optimum growth temperature of 30$^{0}$C and pH7.0 (Xi, He et al. 2014). Also, the 16S rRNA sequence of the isolate from Br-B-1 was analysed and deposited into the EZBioCloud database where it was identified 96% similarity with *Bacillus oicasae* phylogeny tree analysis with nucleotide frequencies A = 25.00%, T/U = 25.00%, C = 25.00%. G = 25.00% showed that it shares clade with *Bacillus altitudinis* with 99% bootstrap value, *Bacillus xiamenensis* with 99% bootstrap value, and *Bacillus safensis* with 86% bootstrap value. This clade is a novel *Bacillus* species characterised with an optimum temperature of 300$^{0}$C, with a pH of 6.8-7.0, rod-shape, and spore-forming

motile gram-positive bacteria (Elbanna, Elnaggar, et al. 2014). In contrast, some of the isolates were identified as the genus paenibacillus, with an example of isolates from the Wt-B-1 when trim and align with Finch TV and deposited in the EZBioCloud database showed 91% sequence similarity to *Paenibacillus amylolyticus*. The phylogeny tree analysis shows that it shares clade with *P. amylolyticus* at 98% bootstrap value and *Paenibacillus tundrae* 87% bootstrap value. This clade is characterised by its endospore-forming potential, facultative aerobic, and growth at the optimum temperature of 30$^{o}$C (Kämpfer, Busse, et al. 2016). Similarly, the sequence of an isolate Ro-B-1 in EZBioCloud database shows that the strain shares similarity with *Paenibacillus seodonensis* with 98.92% and a top hit DCT19. And share clade with *Paenibacillus silvae* at 99% bootstrap value, 60% bootstrap value with an isolate from a yellow-pear cultivar, and 72% bootstrap value *Paenibacillus polysaccharo lyticus*, 62% bootstrap value with *Paenibacillus intestinii* and *Paenibacillus cucumis*. This taxon's characteristics include having a rod-like shape, gram-positive bacteria, with an optimum temperature of 27-30$^{OC}$ and a pH 7.0 (Kang, Lee, et al. 2018). Additionally, strains in this taxon live and survive various environmental conditions, with plant growth-promoting potentials such as nitrogen fixation traits and the ability to degrade the host plant's xylan (Montealegre, Herrera et al. 2005, Han, Agarwal, et al. 2012, Kang, Lee, et al. 2018). An additional sequence from the yellowpear cultivar was also analysed; with MEGA and the sequence deposited into the EZBioCloud database, the sequence shows 84.88% similarity with *Paenibacillus amylolyticus*. It also shares taxon with *Paenibacillus polysaccharo* lyticus at 62% bootstrap value with *Paenibacillus cucumis* 62% bootstrap value, and with 72% bootstrap value with Ro-B-1 isolates**.** This taxon is characterised by a spore-forming, motile, milky white colour, punctiform, and a noticeable odour. They can degrade lignocellulose, biodegradable polyesters, and produce antimicrobial peptides (Teeraphatpornchai, Nakajima-Kambe, et al. 2003, Lyoo, Lee, et al. 2004, DeCrescenzo Henriksen, Phillips et al. 2007, Boland, Henriksen, et al. 2010). The phylogenetic tree analysis of the isolated sequences identified two genera, *Bacillus* and *Paenibacillus,* as the *S. lycopersicum* seeds co-habitat. It is important to note that these genera are well documented to promote growth, yield, and confer resistance to their host plant against pathogens (Kloepper, Ryu et al. 2004, Asghari, Khademian et al. 2020, del Carmen Orozco-Mosqueda, Glick et al. 2020). *Bacillus* genus is known

for its various activities, ranging from releasing protease enzymes by alkaliphilic Bacillus (Saeki, Ozaki, et al. 2007). The release of peptide antibiotics with various chemical structures (Bizani, Motta, et al. 2005) and the formation of endospores are to their advantage to survive extreme conditions. These genera have been implicated in conferring pathogenic resistance traits to their host, thus serving as biocontrol agents (Bais, Fall, et al. 2004). In contrast, Paenibacillus is characterised by spore-forming and facultatively anaerobic bacteria. It is also known for certain enzymatic activities such as polysaccharides hydrolysis (Wang, Shyu, et al. 2008), extracellular cellulolytic, and proteolytic enzyme activities (Budi, Van Tuinen, et al. 1999). Additional traits with *Paenibacillus* are resistance to heat, desiccation, and other organic solvents (Beatty and Jensen 2002). This factor may allow the successful transmission and survival of these vertically transmitted endophytes from one generation to another within their host's seeds.

Furthermore, the isolates possess plant growth-promoting potentials such as phosphorus solubility Figure 2.3, as demonstrated when incubated on the Pikovskayas selective media. The strains release a low molecular weight organic acid that uses its hydroxyl and carboxyl groups to chelate the cation bound to the phosphate, creating a clearing zone on the media (Stella and Halimi, Malboobi, Owlia et al. 2009). This mechanism is that the strains used to hydrolyse the organic or inorganic immobilised phosphates in the soil and convert them to an available and usable form for plant use. Evidence of siderophores potential was also demonstrated Figure 2. 4 when incubated on Chrome Azurol S agar. The isolate strains release siderophores that change the agar colour from blue to orange, the change of colour in the media was the result of hexadentate and octahedral iron complex formation by the excreted siderophore.

In addition to siderophores producing potential, these strains demonstrated nitrogen fixation potentials Figure 2.5, creating a clearing zone on Norris agar. While in the field, these strains convert atmospheric nitrogen into ammonia, a bioavailable form suitable for plant absorption. Furthermore, it demonstrates evidence of 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme activities when incubated and assayed. A spectrophotometer was used to measure ACC deaminase concentration in each strain and to confirm that the strains can release the ACC deaminase enzyme, which reduces ethylene production by cleaving the plant exuded ACC, a precursor for ethylene synthesis ammonia α-ketobutyrate. Therefore, inhibiting

the excessive ethylene production to its minimum. Subsequently, these strains' exuded plant ACC cleavage is expected to promote long/more roots branching and shoots in the host plant (Penrose and Glick 2001).

Further investigation has confirmed that these strains can also release indole-3-acetic acid (IAA) when incubated in the Salkowski reagent. Previous research had shown that the exogenous produce IAA by the bacteria and the endogenous synthesis IAA by the plant. Stimulate the host plant cell proliferation and subsequently induces the transcription of ACC synthesis precursors for the synthesis of ethylene in the host plant, which at a relative amount is essential for roots and shoots elongation (Glick 2014). Even though all the isolated vertically transmitted endophytes (VTE) have demonstrated PGPT potentials. Here we recognised existing diversity within the various isolates of the VTE, displaying various phenotype, metabolite, and other beneficial traits. Thus, it should be expected at the downstream application for some strains not to have a measurable effect on plant growth, health, and development.

# Chapter three

# Investigating plant response to plant-microbe interaction

## 3.1 Introduction

Tomato (*Solanum lycopersicum*) is an important vegetable fruit used in the cosmetic and food industries globally (Saini, Moon et al. 2018). It is also the most consumed vegetable after *Solanum tuberosum* (Costa and Heuvelink 2018), a plant with interesting features and diverse varieties of phenotypic traits that can be cross-bred with its wild relatives (Chime, Aiwansoba et al. 2017). Its fruit developmental stages (Fei, Tang et al. 2004) and the whole genome sequence have been well documented (Fei, Tang et al. 2004, Ashrafi-Dehkordi, Alemzadeh et al. 2018). Its short life cycle, well-regulated pollination, and smaller genome size (Budiman, Mao et al. 2000, Menda, Semel et al. 2004, Giovannoni 2007) are few among the numerous characteristics of *S. lycopersicum* that makes it an attractive plant to study. While *S. lycopersicum* data is robust, it has led to the elucidation of its basic physiology under biotic and abiotic stresses (Ashrafi-Dehkordi, Alemzadeh et al. 2018). It had attracted attention that led to the development of various techniques, which have used to engineering beneficial foreign genes that confers either resistance to pathogens or tolerance to drought and salinity to the host (McCormick, Niedermeyer et al. 1986). However, due to over cultivation of farmland, *S. Lycopersicum* and other agricultural crops lacks the optimum nutrient from the soil, which is currently supported by applying chemical fertilizer, leading to increase demand for chemical fertilizer consumption. Previous studies have demonstrated that chemical fertiliser harms the ecosystem, such as ground and surface water contamination, negatively influencing our aquatic environment and wildlife growth and development. It also directly or indirectly negatively affects human health (Shorette 2012, Brusseau and Artiola 2019). Thus, to curtail the use of chemical fertilizer and harness a potential eco-friendly bio fertiliser, there is a need for an innovative solution by investigating how VTE relates and influences plant-microbial interaction to promote plant growth, and yield.

## 3.2 Plant growth-promoting bacteria

To elucidate a novel way to substitute chemical fertilizer application and provide the plants with the required nutrient, there is a need to explore the already established research evidence. Particularly the plant growth-promoting bacteria (PGPB), which has been identified as a potential bio fertiliser, eco-friendly, renewable, and has demonstrated to actively restored soil fertility (Chaurasia and Apte 2011, Jain and Khichi 2014). Even though data on how bacteria improved growth, yield, and resistance to pathogens has been documented (Souza, Ambrosini et al. 2015), the inconsistent results obtained from field trials need to be addressed. Thus, to harness this potential eco-friendly bio fertiliser, there is a need for an innovative solution through investigating the interaction between the plant and its microbial communities. Previous studies have implicated plant growth-promoting bacteria for protecting the plant from pathogenic invasion through direct or indirect stimulation of antipathogenic peptides such as thionine and lipid transfer protein (Compant, Duffy et al. 2005). Herewith the aim to elucidate how the vertically transmitted endophytes (VTE) prime the rhizosphere to enhance plant nutrient uptake for optimum growth and development and investigate the influence of differential microbial community on *Solanum lycopersicum* cultivars. This study focused on plant microbial interaction and the influence of VTE to promote growth and development on the host cultivar.

## 3.3 Objectives

This chapter investigates influence of VTE in priming it host plant to obtaining the required nutrient for optimum growth and development by quantitative analysis of the tomato response in terms of chlorophyl content, fresh and dried biomass, and change in hight of cultivars with the VTE and the cultivar without the VTE. Also, the influence of different microbial communities (fertilised microbial community, manure microbial community and untreated microbial community) on the growth, biomass, and advantageous roots is also quantified of the cultivars is measure. We hypothesis that 1. Manure microbial community increase plant chlorophyll content which can lead to increase plant growth and increase plant fresh and dried biomass compared to plant

cultivated under fertilised microbial community regime, untreated microbial regime, and the control.

2. tomato cultivar with vertically transmitted endophytes can be prime the host plant for better plant microbial interaction with it rhizobacterial communities than tomato cultivars with no vertically transmitted endophytes

## 3.4 Material and method

### Hydroponic system experiments

Soil samples 5-8cm deep (rhizosphere layer of fertilised treated plot soil, manure treated plot soil, and untreated plot soil) were collected from the Palace Leas farm. 1g of soil sample was added into 100 ml of sterile water shake thoroughly using a sieve, and the sample was transferred into a beaker. The flow-through solution was used to perform a serial dilution. 100µl of 0.001 ml of the dilute at OD600 was taken, using Haemocytometer microscope slide on a fluorescent light microscope. A strain with the lowest OD ($9.8x10^4$) was used as a standard to optimise the other Strain's OD, 210ml of the optimised microbial aliquot was added to the 21L hydroponic solution. The pH of the hydroponic was optimised to PH 5.5, and the room temperature was $19^oC$. The experimental design was two factorial designs (factors: three (3) microbial community and nine (9) tomato cultivars). Four hydroponic systems were set up (control tank, microbe from fertilised treated soil background, microbes from untreated soil background, and microbes from manure treated soil background). Each tank consists of 36 Rockwool; on each Rockwool, four cultivars were planted and replicated four times per hydroponic system. A total of nine cultivars plated per hydroponic system in a completely randomised design, here the experiment 9 units are place randomly, run 4 time with possibly placement in 36 places as show in figure 2.1. Statistical tool SPSS was used to analyse the generated data.

Reagents used to prepare nutrient agar (peptone 5g sodium chloride 5g, HM peptone B1.5g, Yeast extract1.5g, and aga 15g) for bacteria growth

All reagents were suspended in 1000 ml distilled water dissolved by heating in a microwave. The media solution was further sterilised at 121$^{o}$C for 15 min in an autoclave. In a Lumina hood, solution cool 40-55$^{o}$C before aliquoting in petri-dish. (Selective bacterial growth)

Rose Bengal media: Papaic digest of soyabean meal (a media meal to encourage the growth of bacteria and fungi), Dextrose, Monopotassium phosphate, magnesium sulphate, Rose Bengal, and agar (at 25$^{o}$C solution final pH 7.2 -/+0.2). The solution was suspended in 1000ml distilled water, dissolved in a microwave, and autoclave at 121$^{o}$C for 15. Allowed to cool 40-50$^{o}$C Rose Bengal and chloramphenicol added before aliquoting into petri-dish. (For selective fungal growth)

### 3.5 Isolation of VTE from the cultivars

The seeds used for the VTE isolation were placed on a sterile petri-dish, with 95% ethanol added. The Petri dish was stirred slowly for ten seconds before draining the 95% ethanol and the seed rinse with distilled water. 0.53% NaOCl was added into the petri dish and stirred slowly for two minutes. The 0.53%NaOCl was drained, and the seeds rinse with distilled water. Further, 70% ethanol was added and agitated for two minutes. The ethanol was drained, and seeds washed three times with distilled water; seeds were spread on filter paper in a petri dish and dried. The last rinse distilled water was aliquoted on nutrient agar and Rose Bengal media as a control. Seeds were added to nutrient agar for selective bacteria isolation and on Rose Bengal media for selective fungal isolation.

Five *S. lycopersicum* cultivars were used in this project. Elegance (cultivar that demonstrates to be void of VTE), Yellow-Pear, Ananas, Matina, and Zuckertroube (cultivar that shows to possess the VTE).

### 3.6 Hydroponic system

Four hydroponic tanks were used, each tank containing 42L of water, 40g of soluble nutrient at the temperature of 25$^{o}$C  daylight, and darkness were alternate at the

interval of 12hrs. Each tank was inoculated with 1L of soil microbial community (fertilized, untreated, and manure) at day zero.

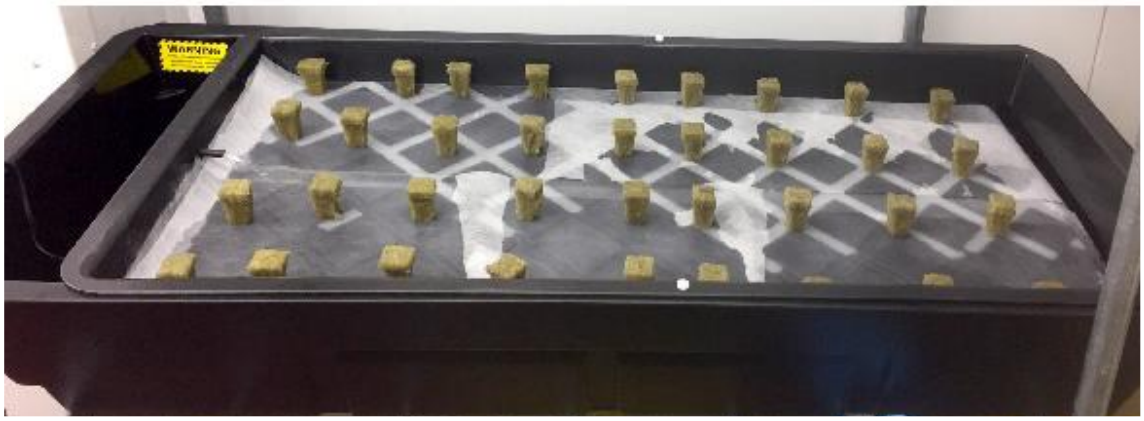

*Figure 2.1 experimental arrangement of the 9 tomato cultivars on hydroponic system, each cultivar is replicated 4 time. Elegance cultivar (cultivar void of VTE) is the control sample for the experiment.*

### 3.7 The phytohormone measurement:

Plant samples harvested from the hydroponic system grown under different microbial communities (control, fertilised, untreated, and manure microbial communities) were freeze-dried overnight; the samples were collected and place in a 2ml microfuge tube containing 3mm Qiagen tungsten beads. The tubes are then placed in a Qiagen tissueLyser II at 27 Hz/s for 5 min. 10mg of powder tissue was weighed into a 2ml microfuge tube with 400µl of 10% methanol mix with 1% acetic acid and internal standards (13.8 ng $^{2}H_{4}$ salicylic 1ng of $^{2}H_{4}$ abscisic acid, and 1ng $^{2}H_{4}$ indoleacetic acid). The sample pool extracted from each plant treatment was replicated four times per sample, and each extracted sample also has negative control with no plant material. Sample extracted by adding a 3mm tungsten bead into each tube, the tube was placed into the Qiagen tissueLyser II for 5min at 27Hz/s. The sample was removed from the Qiagen tissueLyser II and immediately placed on ice for 25min and centrifuge at 4$^{o}$C for 10 min. The extract is removed from the centrifuge and placed on the ice. Each tube was carefully tilted to remove the supernatant. The pellet re-extracted by the addition of 400µl of 10% methanol mix 1% acetic acid and placed on

ice for another 25min before centrifuged. The pooled supernatant was added to the first supernatant providing ~ 95% recovery of analytes. The collected sample was analysed with high-performance liquid chromatography (HPLC)

### 3.9 Physiological measurement:

Chlorophyll content was taken from the fresh plant using a SPAD instrument measuring µmol of chlorophyll per/$m^2$ of a leaf. The plant growth was measured using the table ruler, plant fresh and dried biomass measure using meter Toledo analytical balance.

### 3.10 Statistical tools used for analysis

Statistical analysis was carried out using SPSS, and graphs were plotted with excel. Data were subjected to the general linear model, under the analysis of variance at P-Value 0.05 with the level of significance of 95% to determine the effect of the difference in cultivar, the difference in microbial communities and to determine if there is any effect of plant-microbe interaction concerning the following parameters: chlorophyll, growth, fresh biomass and dried biomass and the concentration of phytohormone.

### 3.11 Results and interpretation of plant response to plant-microbe interaction

Three different soil samples (fertilised treated soil, untreated soil, and manure treated soil) were collected from Palace Leas Hay Meadow plots, Newcastle University research plot. Soil samples were store in the cool room at 4$^{o}$C until the time for the experiment. 5g of the soil sample was weighed into a beaker containing 250ml of distilling water, covered, vortex briefly, and kept at room temperature for 10mins for the sediment to settle before collecting the supernatant into a fresh beaker. The process was repeated six times up to a pooled 1.5L aliquot from each treated soil microbial community collected from each microbial community a tenfold serial dilusion was made and the CFU of the first dilute was calculated for each microbial community. The CFU from fertilised microbial community was 5.667x10$^{-5}$/ml, untreated soil microbial community was 7.9x10$^{-1}$/ml, and the manure microbial community was 3.57 x10$^{-1}$ /ml, aliquot from each microbial community was incubated on nutrient agar

A B C

Measurement of CFU of the three different soil sample microbial communities

*Fig. 3.A Fertilised soil microbes Fig. 3.B Untreated soil microbes Fig. 3.C Manure soil microbes. Due to few CFU of the microbial community found in the three-soil sample, the first serial dilution was used to aliquot into each of the nutrient agar plate and incubate at 27$^{o}$C for 72hrs in the darkroom at Devonshire laboratory 1.7*

### 3.13 Evaluating the improved chlorophyll content of the cultivars

Our Initial investigation on the seeds of five different *S. lycopersicum* cultivars demonstrated four cultivars out of the five cultivars possessed endophytes from two bacteria genera (*Bacillus* and *Paenibacillus).* These genera are known to release plant growth-promoting metabolites when incubated on selected media. Thus, it is expected that they may confer the beneficial traits to their hosts by improving the physiology of the plant by improving the chlorophyll content, water, and mineral uptake. Using the SPSS statistical tool under the univariate post hoc test, Tukey, the result in figure 3.2 recorded and evaluated the chlorophyll content of the different *S.lycopersicum* cultivars. The cultivar demonstrated to possess VTE, and the cultivar void of VTE (see chapter 1) are grown under differential microbial communities.

We assessed three different microbial community influences on the chlorophyll content of the *S. lycopersicum*. The study showed the cultivars under the fertilise microbial community, Ananas with a mean 9.5nM, Matina with a mean of 10.1nM and Yellowpear with a mean of 9.5nM, are significantly different from the control. However, in the cultivar, under manure and untreated microbial communities are not significantly different from the control.

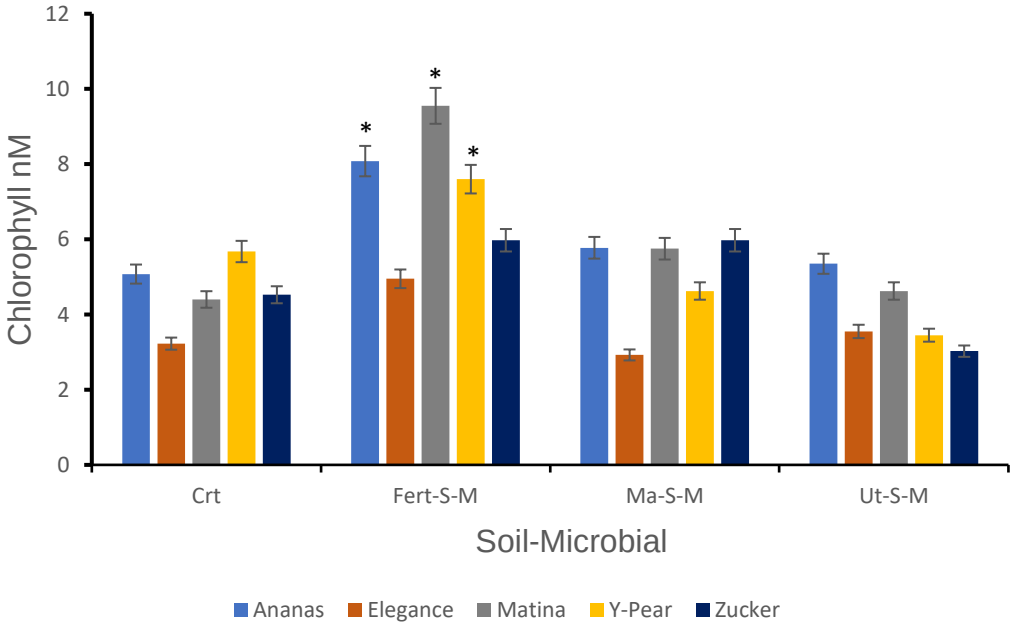


Figure 3.2. Chlorophyll content of *Solanum lycopersicum* grown in the presence of three different microbial communities (Fert-S-M=Fertilised soil microbial community, Ut-S-M=Untreated soil microbial community, and Ma-S-M=Manure soil microbial community) and

Control treatment (water mix with soluble fertilizer without any microbial community) in five cultivars: Ananas cultivar, Elegance cultivar, Matina cultivar, Y-Pear cultivar, and Zucker cultivar. Asterisks indicate a statistically significant difference in the microbial community from the control at the ($p<0.05$), the number of replicates per sample is (N=3). Error bar 1.0%, Ananas cultivar, Matina cultivar, Yellowpear cultivar, and Zuckertroube cultivar are the cultivars that possessed the VTE, and Elegance is a cultivar that is void of the VTE. The samples were grown under 25$^{o}$c with light alternating after 12hours in hydroponic tanks.

### 3.14 Evaluating the improved growth of the cultivars

The variation in the growth rate of samples under differential microbial community can indicate the influence of the microbial community on the sample. Here an SPSS statistical tool under the univariate post hoc test Tukey showed in figure 3.3 the growth of five different cultivars of S. *lycopersicum,* grown under three different microbial communities with one control. From each sample, measurement was carried out randomly and replicated three times. The microbial fertiliser community, manure microbial community, untreated microbial community, and the control (aliquot of soluble fertiliser without any microbial community) was evaluated. Also, the growth rate of the cultivars with VTE and the cultivar without the VTE was assessed. The increased growth of VTE cultivar Ananas under the fertilise, manure and untreated microbial community with a mean of 4.7cm are significantly different from the control. Also, the cultivar Elegance under untreated microbial community with a mean of 5.0cm, the cultivar Matina under manure microbial community with a mean of 5.0cm, untreated microbial with a mean of 4.7cm are significantly different from the control. The cultivar Yellowpear under manure microbial community with a mean of 4.5cm and untreated microbial community with a mean of 4.8cm is significantly different from the control. However, the cultivar Zucker under fertilise, manure and untreated microbial communities is not significantly different from the control.

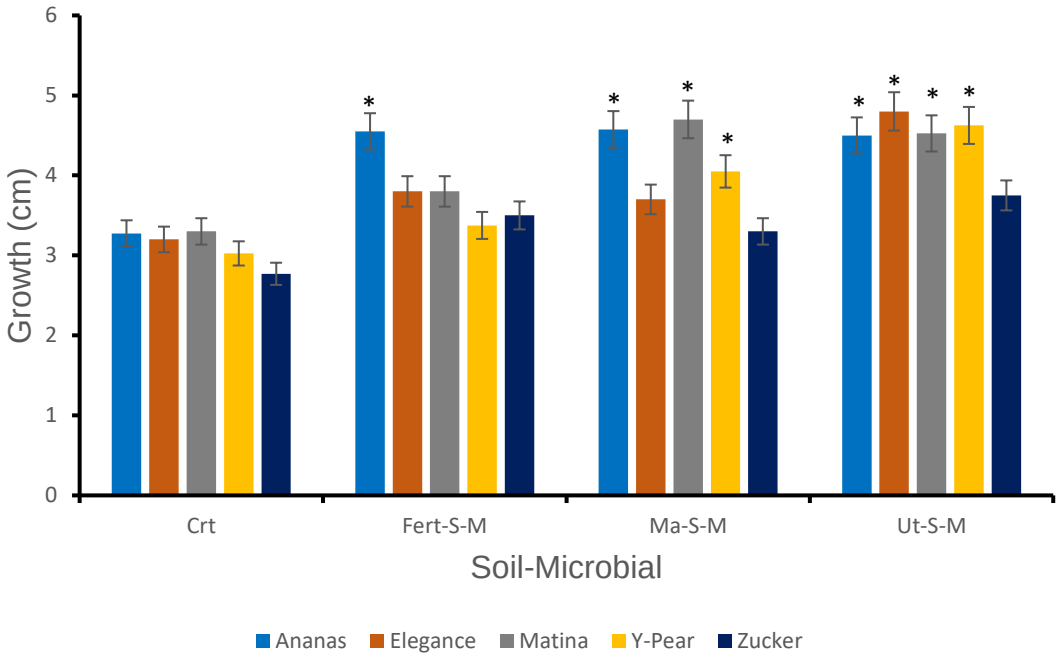


Fig 3.3 *Solanum lycopersicum* grown in different microbial communities, express in % to control in five cultivars: Ananas, Elegance, Matina, Y-Pear, and Zucker under four different microbial treatments: (Fert-S-M=Fertilised soil microbial community, Ut-S-M=Untreated soil microbial community, and Ma-S-M=Manure soil microbial community. Asterisks indicate a statistically significant difference in microbial community from the control ($p<0.05$, the number of replicate N=3. Error bar 1.0%, Ananas, Matina, Yellow pear, and Zuckertroube are cultivars possessing the VTE. Elegance is a cultivar void of the VTE, samples grown under 25$^{o}$C with light alternating after 24hours in hydroponic tanks.

### 3.15 Evaluating fresh biomass of the cultivars

The difference in biomass of the same sample grown under differential microbial communities can serve as a parameter indicating the influence of a microbial community. Figure 3.4 shows the fresh biomass of five different cultivars under three differential microbial communities and a control. The fresh biomass of the cultivar Ananas under the fertilised microbial community with a mean of 1.1g and cultivar Yellowpear with a mean of 1.4g are significantly different at $p<0.05$ compared to the control. Also, the fresh biomass of the cultivar Yellowpear under the manure microbial community with a mean of 1.1g is significantly different at $p<0.05$ from the control. While the cultivar Elegance, Matina and Zucker under the fertilise, manure and

untreated microbial communities are not significantly different at p<0.05 from the control

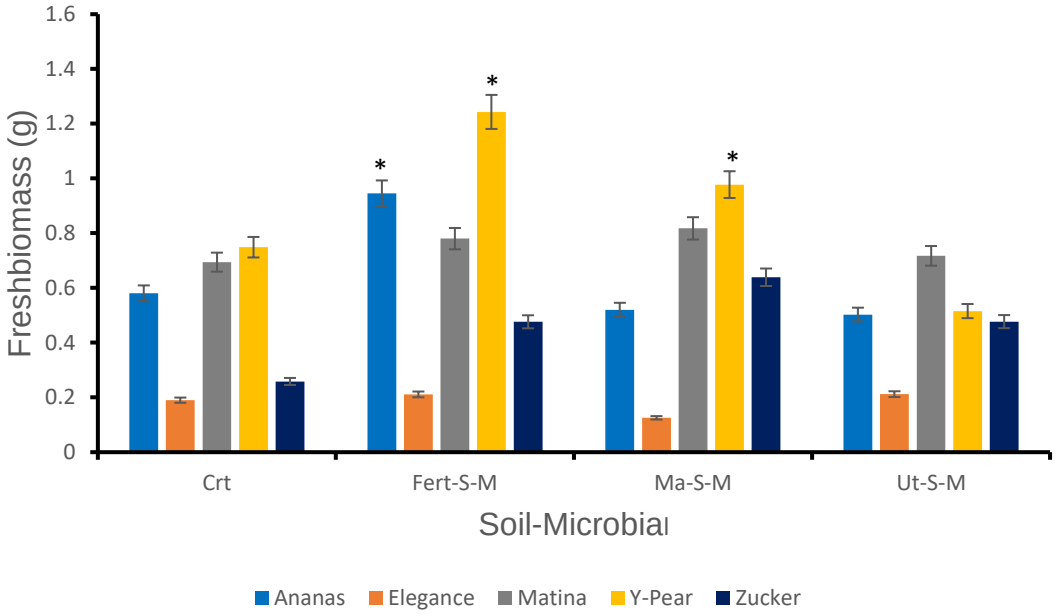


Figure 3.4 Fresh biomass response of *Solanum Lycopersicum* grown in different microbial communities, express in % to control in five cultivars: Ananas, Elegance, Matina, Y-Pear, and Zucker under four different microbial treatments: (Fert-S-M=Fertilised soil microbial community, Ut-S-M=Untreated soil microbial community, and Ma-S-M=Manure soil microbial community. Asterisks indicate a statistically significant difference in microbial community from the control at the (p<0.05), longline with asterisks indicate a significant difference in cultivars at p<0.05, the number of replicate N=3. Error bar 1.0%, Ananas, Matina, Yellow pear, and Zuckertroube are cultivars possessing the VTE. Elegance is a cultivar that is void of the VTE grown under 25$^{o}$c with light alternating after 24hours in hydroponic tanks.

### 3.16 Evaluating dried biomass of the cultivars:

Figure 3.5 demonstrates the dried biomass of the cultivar after removing water from the tissues of the samples. The dried biomass of the cultivar Ananas (cultivar with VTE) under fertilizer with a mean of 0.07g and untreated microbial communities with a mean of 0.05g is significantly different at p<0.05g from the control. The dried biomass of the cultivar Matina (cultivar with VTE) under the manure with a mean of 0.06g and untreated microbial community with a mean of 0.06g is significantly different from the control. In contrast, the cultivar Yellowpear (with VTE) and Elegance (without

VTE) under fertilize, manure and untreated microbial communities are not significantly different at p<0.05 from the control.

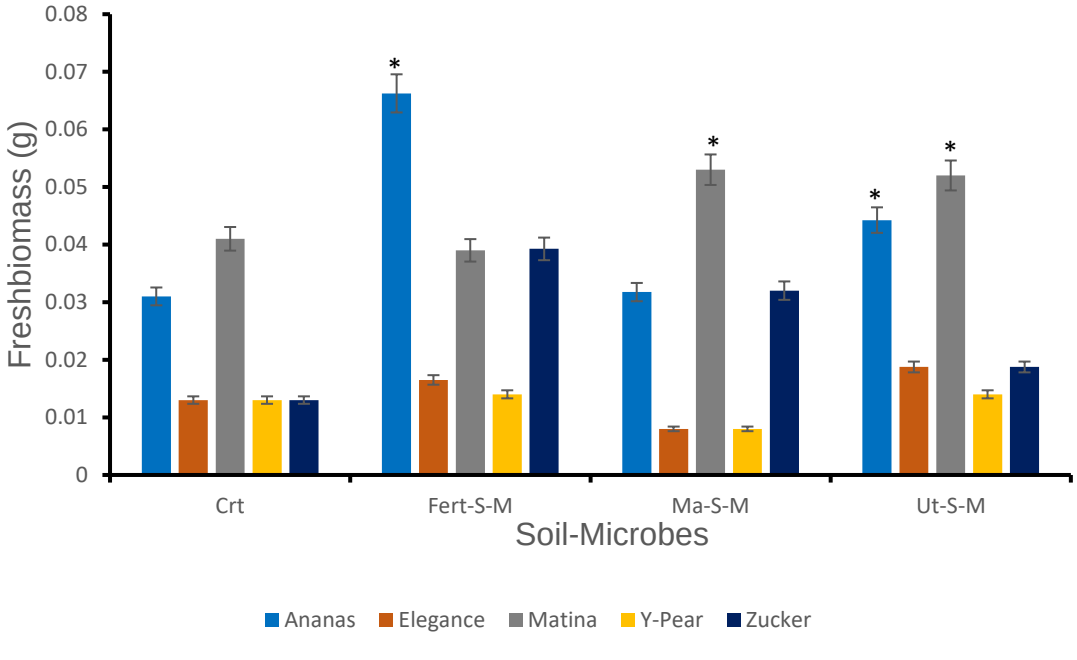


Fig 3.5. dried biomass response of *Solanum lycopersicum* grown in different microbial communities, express in % to control in five cultivars: Ananas, Elegance (control cultivar), Matina, Y-Pear, and Zucker under four different microbial treatments: (Fert-S-M=Fertilised soil microbial community, Ut-S-M=Untreated soil microbial community, and Ma-S-M=Manure soil microbial community. Asterisks indicate a statistically significant difference in microbial community from the control at the (p<0.05), the number of replicate N=3. Error bar 1.0%, Ananas, Matina, Yellow pear, and Zuckertroube are cultivars possessing the VTE. Elegance is a cultivar that is void of the VTE, samples grown under 25$^{o}$c with light alternating after 24hours in hydroponic tanks

### 3.17 Evaluating increase root development:

An advantageous root in a plant enables it to obtain more water and nutrients from the soil. Thus, any microbial community with the potential to confer increase root would be a potential bioinoculant candidate. Figure 3.6 demonstrate the influence of three different microbial communities in developing advantageous root in the cultivar Elegance, a cultivar that has demonstrated to be void of VTE. We investigate how the three different microbial communities can influence root growth in this cultivar without any interference of endophytes. The finding shows that the cultivar Elegance under the fertilise microbial community with a mean of 8.1 cm is significantly different from

the control. In contrast, the cultivar under the manure microbial community with a mean of 4.2cm and the untreated microbial community with a mean of 3.1cm are not significantly different from the control.

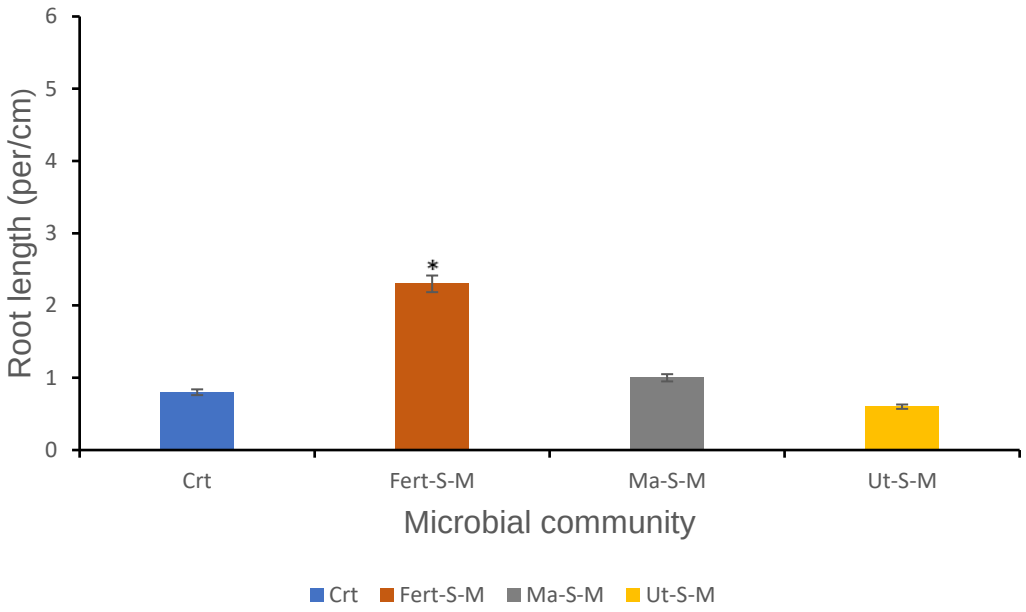


Fig.3.6 Elegance root development from four different microbial (Fert-S-M=Fertilised soil microbial community, Ut-S-M=Untreated soil microbial community, and Ma-S-M=Manure soil microbial community. The roots length at $p<0.05$ with a confident level of 95%, the sample with an asterisk is significantly different from the control, the cultivar void of VTE). n=3. Error bar 1.0%, samples were grown under 25$^{o}$c with light alternating after 24hours in hydroponic tanks

### *3.18 Evaluating phytohormone:*

The difference in the concentration of phytohormones in a plant from different cultivar can be used to understand its response to differential treatments. The concentration of abscisic acid table 3.1 and indole acetic acid table 3.2 from three different cultivars of *S. lycopersicum*, Ananas and Yellowpear (cultivar with VTE) and Elegance (cultivar with no VTE) grown under the same nutrient condition was evaluated. These cultivars from the previous experiment chapter 2 were shown each to possession different type of VTE. The concentration of abscisic acid in the cultivar Elegance with mean of 139nM, Yellowpear 140nM, and the Ananas 113nM were not significantly different at a $P<0.05$ see below table 3.1 The concentration of indole acetic acid in the cultivar

Elegance with mean of 37.3nM, cultivar Yellowpear with mean of 22.4nM and the cultivar Ananas with mean of 28.8nM are not significantly different at P-Value 0.05 see table 3.2. Similarly, the concentration of salicylic acid in Elegance with mean of 56.7nM, Yellowpear with mean of 23.1nM and Ananas with mean of 27.1nM are not significantly different at p<0.05


Figure 3.7A

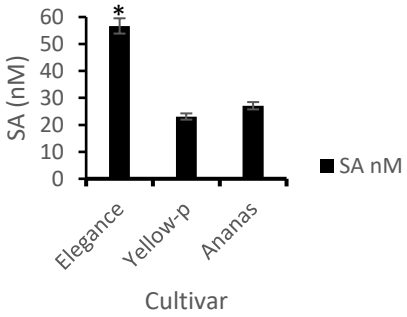


Figure 3.7B

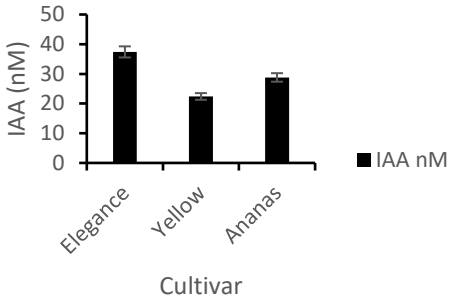


Figure 3.7C

Figure. 3.7A Abscisic acid concentration from the three cultivars grown hydroponically under fertilised microbial community, Ananas (cultivar with VTE), Elegance (cultivar void VTE) and Yellowpear. figure 3.7B Indole acetic acid from three cultivars Ananas, Elegance and Yellowpear and figure 3.C Salicylic acid from the three cultivars Ananas, Elegance and Yellowpear. Number of replicates N=4, level of significance at p<0.05. Numbers in the same subset are not significantly different from each other. Numbers in different subset are significantly different from each other. All samples were grown under 25$^{o}$C with light alternating after 24hrs in hydroponic tanks

### 3.19 Discussion

Plant phenotypic parameters are clear and strong indicators of a plant capacity to respond to different environmental conditions, the plant phenotypic response results from phytohormone stimuli initiated by internal or external factors. Critical factors in plant phenotypic development are photosynthesis, in which the plant generates its

energy and assimilates it for its growth and development (Lawlor 2009). Therefore, the chlorophyll content of the *S. lycopersicum* grown under the four differential microbial communities was investigated Figure 3.2. The results demonstrate an increased chlorophyll content significantly different in samples under fertilised microbial community compared to the control. However, there was no significant difference at $p<0.05$ between plants grown under the untreated microbial community, manure microbial community and the control. These findings are consistent with Mikanová, Šimon, et al. (2015), who demonstrate that the function and community structure of manure treated soil and the control soil were the same. The manure microbial community may have an overall increased population size than the untreated soil microbial community and the control. However, the microbial community structure, diversity and function of these communities are the same. Thus, no significant difference in chlorophyll content between these samples.

Furthermore, in Figure 3.2 the VTE cultivars chlorophyll content and the cultivar void of VTE show no significant difference. It demonstrates that the VTE was not involved in stimulating the plant-rhizobacteria interaction as initially hypotheses that vertically transmitted endophyte stimulates plant-rhizosphere bacteria interaction to improve plant growth, health, and development in the cultivar with the VTE.

### 3.20 Increase in cultivars growth

Increased growth of the *S. lycopersicum* demonstrated in figures 3.2 a significant difference in the stem length under a fertilised microbial community, manure microbial community, and untreated microbial community compared to the control. Consistent with previous studies (Abdykadyrova, Aipova et al. 2020), that showed PGPB expedites plant growth in various ways. Through direct stimulation of growth-related phytohormones or enhancing the availability of nutrients(Pathania, Rajta et al. 2020). However, in contrast to growth between the differential microbial communities, the VTE cultivar and cultivar void of VTE, there is no significant difference suggesting the VTE is not involved in stimulating the growth relating to phytohormone.

### 3.21 Increase biomass and roots growth.

Figure 3.4 shows that the fresh biomass of cultivars Ananas and Yellowpear under fertilised microbial community are significantly different from the control ($p<0.05$). In contrast, the fresh biomass of cultivars Elegance, Matina and Zucker were not significantly different under the differential microbial communities. It is an indication that the increase in fresh biomass is due to water retention in the cultivar under the fertilise microbial community. The dried biomass in figure 3.5 cultivars grown under the microbial fertiliser communities, manure microbial community, and untreated microbial community is significantly different compared to the control. Demonstrating that the plant-microbial interaction improves the water content of the *S. lycopersicum;* and, therefore, can be used to improve plant water absorption in plants under water stress conditions. This can consequently influence the growth and yield of the plant (Hao, Cao et al. 2019). However, due to a lack of consistency in the response of the cultivars to the differential microbial communities. Additional parameters were studied, such as the increased root growth in the cultivar void of VTE.

Assessment of increased root growth in figure 3.6, for cultivar void of VTE grown under the fertilised microbial community, improved significantly different compared manure and untreated microbial communities at $p<0.05$. The increase can be attributed to fertilised microorganism community such as firmicutes and proteobacteria (Haldar and Sengupta 2015). This result further reiterates the finding on the role of the fertilised microbial community to improve plant in figure 3.2, 3.3 and 3.4. Further investigation may be needed to confirm these findings.

### 3.22 Stimulation of phytohormones

Here we measured the concentration of phytohormones from cultivars with VTE and the cultivar void of the VTE to evaluate the response of these cultivars. Plant growth regulators, such as abscisic acid, promote root hair (Vivanco 2000), improving water, mineral, and carbon dioxide uptake by regulating the stomata opening. Table 1.2A shows the abscisic acid concentration was no significant difference between Ananas (VTE), Yellowpear (VTE), and Elegance (void of VTE) at $p<0.05$. Suggesting the possession of VTE by the cultivar does not induce stimulation of abscisic acid. Also, salicylic acid regulates the biochemical responses in plant health when at the optimum concentration. It promotes vegetative growth, flower formation, and seed germination

(Rivas-San Vicente and Plasencia 2011). However, higher, or lower concentrations inhibit seed germination and promote leaf senescence, not significantly different between Ananas, Yellowpear, and Elegance. The same pattern is observed in indole-3-acetic acid (IAA), a phytohormone responsible for stimulating biosynthesis, promoting phloem formation, and shooting elongation (Teale, Paponov et al. 2006, Vanneste and Friml 2009). Table 1.2B at $p<0.05$ was not significantly different in IAA accumulation between cultivar Elegance and Ananas and Yellowpear, demonstrating that VTE is not involved in IAA regulation in *S. lycopersicum.*

### 3.23 conclusion

The findings are divergent to previous studies demonstrating that an inoculated plant with growth-promoting bacteria produces a metabolite that promotes water and minerals uptake and stimulates phytohormones release (Glick 1995, Fürnkranz, Adam, et al. 2012, Kumari, Mallick, et al. 2016). Our result showed that samples grown under fertilised microbial communities significantly perform better compared to the control. However, the samples under the manure and untreated microbial communities were not significantly different from the control, consistent with the field trial (Bainard, Koch, et al. 2013) and other plant inoculants (Jensen 1984). Which demonstrates no significant difference when samples are grown in differential microbial treatments with the same nutrient supplied. This is because the bacterial strain potential is only effective under experimental soil with limited nutrient or adverse environmental conditions (Glick 2014), as demonstrated in other studies investigate plant stress and defence against pathogens. Though we need to consider PGPB needs time to grow and multiply before it would be effective, once established can be a promising bioinoculant (Patel, Mistry et al. 2017). The results further demonstrate that VTE in seeds of *S. lycopersicum* failed to stimulate *S. lycopersicum*-microbial interaction that promotes growth, chlorophyll increases and plant fresh and dried biomass. It also failed to stimulate phytohormone release in the *S. lycopersicum.* However, previous studies noted that these microbial communities potentials are ineffective when the experimental soil nutrient requirement is optimum (Egamberdiyeva 2007).

# Chapter four

## *4.1* Exogenously applied vertically transmissible endophytes alter development and disease signalling parameters in tomato

### *Introduction*

Global tomato production is faced a series of challenges ranging from biotics to abiotic stresses, of which each need urgent attention and action (Yousaf, Li et al. 2016). There have been many approaches to improve crop yield and production, such as the application of synthetic fertilisers, pesticides, and herbicides, together these dramatically changed the course of agroindustry for over many decades (Tilman, Cassman et al. 2002). The development of phosphate fertiliser in 1861 and nitrogen fertiliser in 1903 (Russel and Williams 1977), within a short period improved crop yield (Hirzel and Rodríguez 2013, Wang, Lu et al. 2017, Yousaf, Li et al. 2017). However, the application of these synthetic compounds has resulted in the accumulation of toxic metalloids contaminants. Some of the heavy metals that contaminate the soil and affect the ecosystem are Ion, Chromium, and Lead and Manganese (Kloke, Sauerbeck, et al. 1984, Wang, Cui et al. 2003, Singh, Singh, et al. 2018). These contaminants pollute the environment and adversely affect ecosystems. In addition, they also negatively impacting the climate, with consequences such as greenhouse gas emissions contributing to global warming (Bitew and Alemayehu 2017, Wei, Hu et al. 2017, Davydov, Sokolov, et al. 2018, Zhang, Chen, et al. 2018).

Thus, the need for an alternative to curtail these chemical compounds effects needs to be implemented. Previous studies have demonstrated that plant growth-promoting bacteria (PGPB) can be used in forestry regeneration and soil contaminant phytoremediation (Lucy, Reed, et al. 2004, Cakmakçi, Dönmez, et al. 2006). It has also been demonstrated to provides bioavailable nutrients for plant growth via the production or stimulation of nutrient-related hormones (Santos, Berlitz et al. 2018). Although most PGPB has been shown to fix nitrogen and solubilise phosphate, most of these are likely to be in a small amount with a minimal measurable impact on the plant growth requirement (Yang, Kloepper et al. 2009, Souza, Ambrosini et al. 2015). The production and availability of these nutrients likely vary depending on the soil nutrient and composition (Holguin and Patten 1999). The most common parameters often invoked to demonstrate the various potential of PGPB on crops are their ability

to stimulate the production of abscisic acid, gibberellins, auxin and salicylic acid and supply nutrients to the host plant. PGPB are known to promote growth and enhance resistance/ or tolerance of the host plant (Vessey 2003), via the direct provision of required mineral nutrients or indirectly by stimulating the synthesis of phytohormones (Costacurta and Vanderleyden 1995, Fernández, Zalba, et al. 2007).

The most used PGPB in agriculture are those isolated from the rhizosphere; these bacteria have been used to demonstrate the potential to replace synthetic fertilisers conventional use. (Egamberdieva 2013, Numan, Bashir, et al., 2018). However, a general lack of reproducibility in the effectiveness of PGPB when used in different soil conditions and field locations (Ferreira, Pires, et al. 2013, Inagaki, Guimarães, et al. 2015) has resulted in reduced uptake of PGPB to substitute for synthetic fertilisers at a commercial scale.

This study investigates the physiological and biochemical effects of inoculating *S. lycopersicum* cv elegance, cultivar void of endophytes as demonstrated in chapter 1. Here we inoculated Elegance with *Bb-B-1* strain which had shown to release relative level of metabolite and compared the plant response to a positive control *Pseudomonas sp.* CT364 (Montero-Calasanz, 2013) which has demonstrated to release IAA, siderophore and phosphate solubilisation metabolites responsible to stimulate plant grow. Also, the effects of different microbial community (fertilized microbial community, manure microbial community and untreated microbial community on plant development will be investigated.

### 4.2 Hypothesis

1.Exogenous inoculation of vertically transmitted endophytes can prime plant interactions with its rhizosphere microbial community and positively improved the plant growth

2. Vertically transmitted endophytes confers plant growth and increase both fresh and dried biomass of the host plant

## 4.3 Materials and methods

### Media preparation and inoculation with Bb-B-1 and *Pseudomonas sp*. CT364 strains

2.0% Media was prepared by adding 8.0g of agar into a 1000ml beaker, 400ml of distilled water was poured into the beaker, shake thoroughly by hand, and heat by microwaving at 100$^{o}$C for 4min to dissolve the mixture and sterilise by autoclaving at 15lbs pressure (121$^{o}$C) for 15min. The mixture was kept at ambient temperature to cool in the laminar hood. After the aliquot media has cool, tomato Elegance cultivar seedlings were grown in a 280ml aliquot of agar inoculated with 140ml isolates of *Bb-B-1* or *Pseudomonas sp. CT364* strain in a magenta vessel and incubated in a growth cabinet with a fluorescent lighting temperature range of 25$^{o}$C to 19$^{o}$C alternating at 12 hours intervals for 14 days. The control contains 320ml of aliquot agar with no added nutrients and microbes. Seedling of elegance cultivar was grown in magenta vessels in the experimental design order as shown in table 1.3. 100ml of water was added to each magenta vessel every 4days for 14days.

### 4.5 Arrangement of the experimental treatment replicates three times per sample treatment

Bb-B-1 is a bacterium isolated strain from Beauteblanch tomato cultivar, and positive control *Pseudomonas sp*. CT364 strain known to confer PGPB trait. Sample Bb-B-1 +N or *Pseudomonas sp*. CT364+N mean Bb-B-1 or *Pseudomonas sp*. CT364 strain grown under optimum nutrient concentration, where sample Bb-B-1 or sample CT364 are inoculated sample with no added nutrient, sample grown under deprive nutrient condition.

| **isolates** | **Bb-1-B** | **CT-364** | **Nutrient** | **Bb-1-B +N** | **CT-364+N** | **CTRL** |
|---|---|---|---|---|---|---|
| **samples** | **Bb-1-B inoculated Sample** | **Ct-364 inoculated Sample** | **Nutrient inoculated Sample** | **Bb-1-B inoculated N+ Sample** | **CT-364 inoculated N+ Sample** | **CTRL no inoculation Sample** |

Table 4.1 Sample inoculation with Bb-B-1 isolate with no nutrient added to aliquot, sample inoculated with positive control *Pseudomonas sp*. CT364 with no nutrient added to solution, and CTRL control with no strain in the solution, sample inoculated with Bb-B-1+ Nutrient solution with nutrient solution added to solution, sample inoculated with nutrient added to aliquot *Pseudomonas sp*. CT364+N, all samples incubation in growth cabinet:

### 4.4 Evaluating changes in Phenotype and concentration of phytohormone in the treatment

Samples were harvested after two weeks and weighed to record the fresh biomass of the samples. While the plant stem length was measure with a table ruler and immediately immersed in liquid nitrogen to preserve the biochemical properties at the point of harvest. The tissues were freeze-dried and ground to powder as described by Forcat, Bennett, et al. (2008) with slight modifications to suit our experiment. For the rapid quantification of targeted phytohormones such: as salicylic acid, jasmonic acid, and indole acetic acid from each single sample crude. 2.5mm beat beater in 2ml microfuge tubes at 30 $H_z$/s for 15 minutes. Approximately 100mg fresh weight tissue was weighed into a new 2ml centrifuge tube, an aliquot of 400 µl of 1% acetic acid in 10%methanol containing 1ng of $^2H_6$ ABA, 10ng of $^2H_2$ JA, and 13.8 ng $^2H_4$ SA, an internal control. Tomato leaves were extracted in triplicate, a control extraction consisting of the three phytohormone standards with no leaf tissues was also generated. The 2ml tubes with the 2.5µl tungsten beat were used to extract the samples at 30$H_z$/s for 15 minutes. The samples were placed on ice for 45 minutes and centrifuged at 4$^o$c for 15 minutes at 8,000 x g. The supernatant was carefully transferred into a fresh tube, and the pellets re-extracted in 400 µl of 1% acetic acid in 10% methanol. The process was repeated by placing the sample on ice for 45 minutes and centrifuged using the parameters above, making two extracts with aliquot approximately 90-95% analyte recovery. The samples were analysed using HPLC electrospray ionisation/MS-MS, (SAgE Mass Spectrometry Facility, Newcastle University).

## 4.5 Sample data analysis

The data from SPAD non-destructive chlorophyll meter, HPLC-electrospray ionisation/MS-MS and the phenotypic parameter recorded using scales and ruler were analysed using excel and SPSS under general linear model subsection univariate for ANOVA with a significant level of 0.05 p-value and 95% confidence level

## 4.6 Results

### *Evaluating the response of Elegance (tomato cultivar void of VTE) cultivated under four differential microbial communities*

This chapter evaluate the response of Elegance a tomato cultivar void of VTE demonstrated in chapter 2 table 2.1, cultivated under three different microbial communities i.e., fertilise microbial community, manure microbial community and untreated soil microbial community. The chlorophyll concentration, fresh biomass and shoot length from each sample cultivated under the three microbial communities were evaluated. Figure 4.1 demonstrate significant increase chlorophyll content in sample under fertilised microbial community with mean of 3.8 nmol/cm of leave area and manure microbial community with mean of 4.6 nmol/cm of leave area compared to the control at $p<0.05$. However, the sample under untreated microbial community with mean of 2.3 nmol/cm of leave area is not significant difference at $p<0.05$ with the control sample with mean of 1.1 nmol/cm of leave area. The fresh biomass of the aerial tissue figure 4.2 weighed immediately after harvest with a precision mini scale, indicates that the mean biomass of the samples under fertilised microbial community mean 0.1cm, manure microbial community with mean 0.2, untreated microbial community with mean 0.2 and the control with mean 0.2 are not significantly different at $p<0.05$. Also, the shoot length of the sample under fertilised microbial community with mean of 3.5 cm, manure microbial community with mean of 4.6 cm, untreated microbial community with mean of 4.4, control with mean of 5.2 cm is not significant different at $p<0.05$. All analysis was carried out using ANOVA and Tukey Post Hoc test.

The rationale behind this experiment is to establish the response of Elegance cultivar under the differential microbial communities before inoculation of the cultivar. This can clearly show whether there are any changes in the response of the cultivar after inoculation and cultivation under the same differential microbial communities.

**Chlorophyll concentration of samples cultivated under three different microbial community**

Tɵmato cultivar Elegance (cultivar void of VTE) cultivated under three differential microbial community and one control. Sample is replicated four times, and chlorophyll concentration was analysed using SPSS ANOVA general linear model with Tukey post hoc test at $p<0.05$.

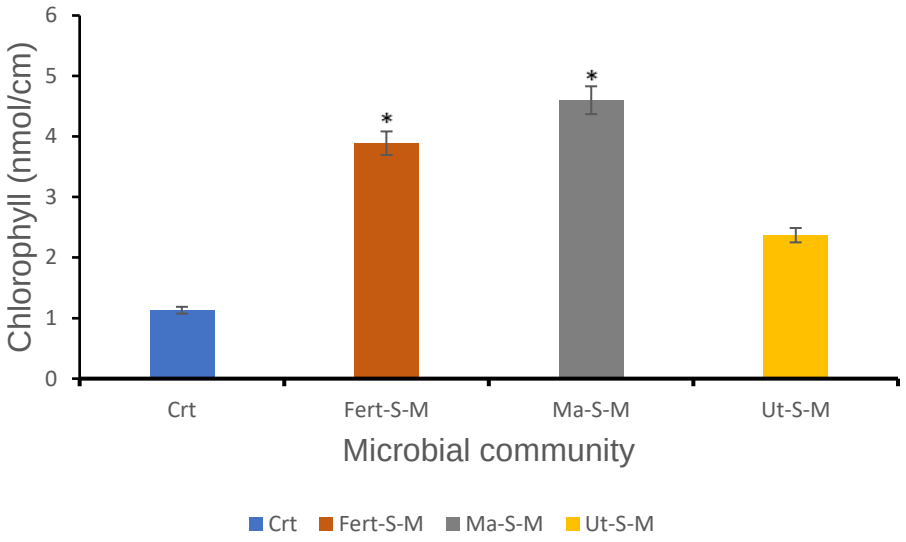


Figure 4.1 The chlorophyll concentration of sample under three differential microbial communities where Crt is the control sample (distil H20), Fert-S-M is fertilised soil microbial community, Ma-S-M is manure soil microbial community and Ut-S-M is untreated soil microbial community. Sample with asterisk is significant different from the control at $p<0.05$

**The fresh biomass of Elegance under three different microbial community**

Fresh biomass of Elegance cultivar cultivated under three differential microbial communities and one control, the fertilised microbial community (Fert-S-M), manure microbial community (Ma-S-M), untreated microbial community (Ut-S-M), and control (Ctr), analysed using SPSS ANOVA general linear model with Tukey post hoc test at $p<0.05$.

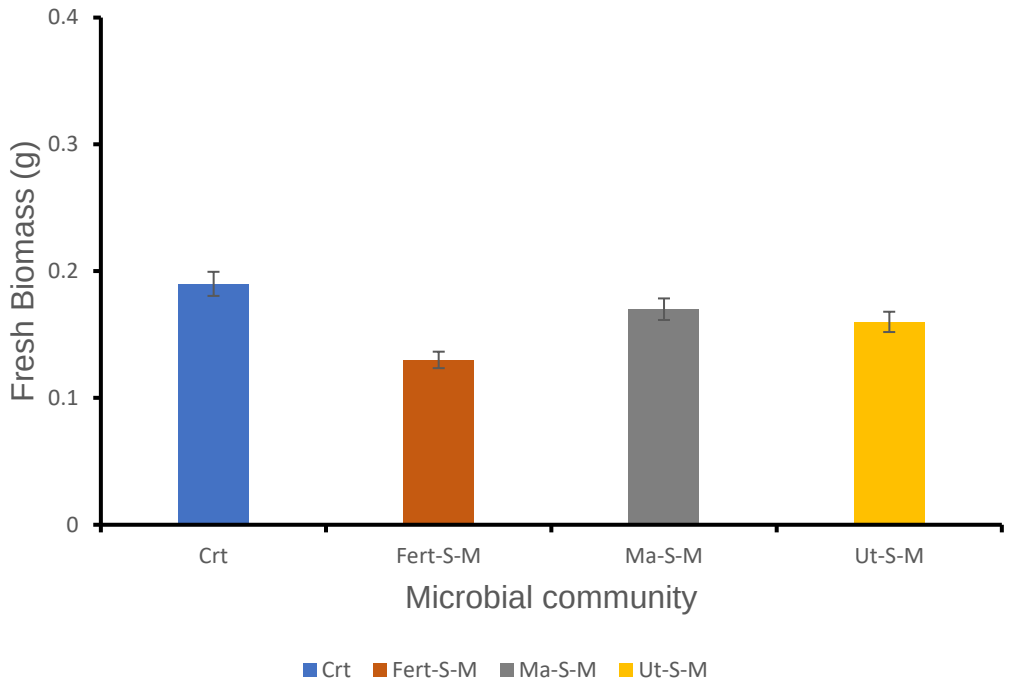


Figure 4.2 The fresh biomass of *S. lycopersicum* under three differential microbial communities of sample under three differential microbial communities where Crt is the control sample (distil H20), Fert-S-M is fertilised soil microbial community, Ma-S-M is manure soil microbial community and Ut-S-M is untreated soil microbial community. Sample with asterisk is significant different from the control at $p<0.05$

**Shoot length of samples cultivated under three different microbial community**

Tomato cultivar Elegance cultivated under three differential microbial communities, fertilised soil microbial community (Fert-S-M), untreated soil microbial community (Ut-S-M), manure soil microbial community (Ma-S-M), and the control (distil H20) at $p<0.05$. Each sample shoot length was analysed using SPSS ANOVA general linear model with Tukey post hoc test at $p<0.05$

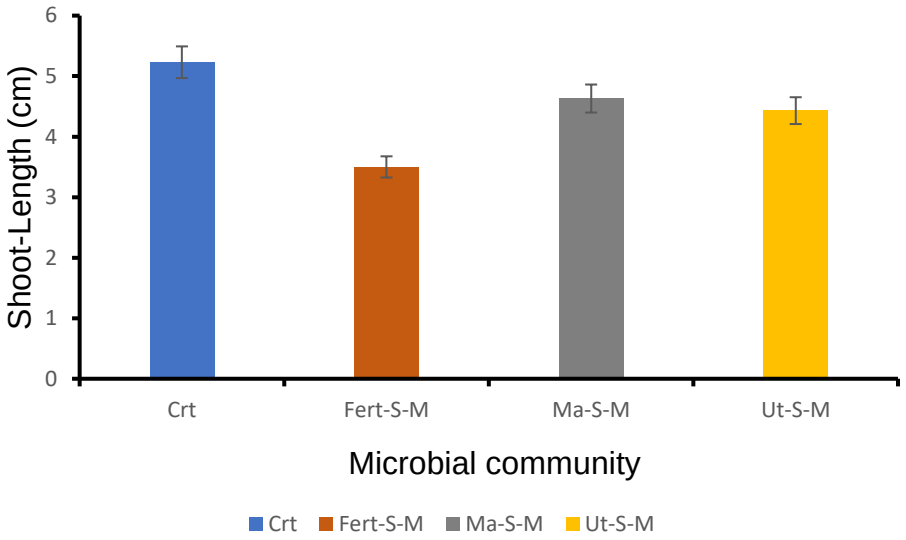


Figure 4.3 Increased shoot length of samples under three differential microbial communities and one control where Crt is the control sample (distil H20), Fert-S-M is fertilised soil microbial community, Ma-S-M is manure soil microbial community and Ut-S-M is untreated soil microbial community. Sample with asterisk is significant different from the control at $p<0.05$

### 4.7 Exogenous application of VTE Bb-B-1 and known PGPB (*Pseudomonas sp.* CT364) has less effect on tomato growth and development than increased nitrogen availability

The *S. lycopersicum* were grown in small magenta vessels in randomised design place in a plant growth incubator. Some magenta vessels containing plants inoculated with a single strain of either *Bb-B-1* or *CT364* strain and divided into two sets; one set was grown under optimum nutrient conditions while other sets were grown without nutrients. Each plant treatment was replicated four times and allowed to grow for two weeks. The difference in the number of leaves was taken immediately before harvesting the treated plants. The stem shoot length was measured using a table ruler. Fresh and dried biomass was taken using a precisions mini scale, and chlorophyll content was measured using a SPAD non-destructive chlorophyll meter. The amount of three phytohormones (JA, IAA, and GA) in the tomato leaves was evaluated with HPLC-electrospray ionisation/MS-MS, and the final data were analysed with SPSS statistical analysis.

### 4.8 Effect of VTE on leaf number in tomato

The number of leaves from two weeks Elegance cultivar exogenously inoculated with vertically transmitted endophyte. Samples are divided into two sets Nutrient, Bb-+N, CT364+N, grown under optimum nutrient condition, and another set of samples, Be, CT364 and CTR, under nutrient-deprived conditions. All data analyse at $p<0.05$. Error bars represent at standard error. The number of leaves on inoculated Elegance cultivar grown under nutrient-deprived conditions was not significantly different from the control at $p<0.05$, with an Elegance cultivar inoculated with VTE *Bb-B-1* strain, has a mean of 4.0, and an Elegance cultivar inoculated with CT364 strain has a mean of 4.0. Similarly, no significant difference was recorded between inoculated Elegance cultivar grown under the optimum nutrient condition with an Elegance cultivar with Be+N has a mean of 6.3 and an Elegance cultivar with strain CT364+N mean of 6.3 at $p<0.05$

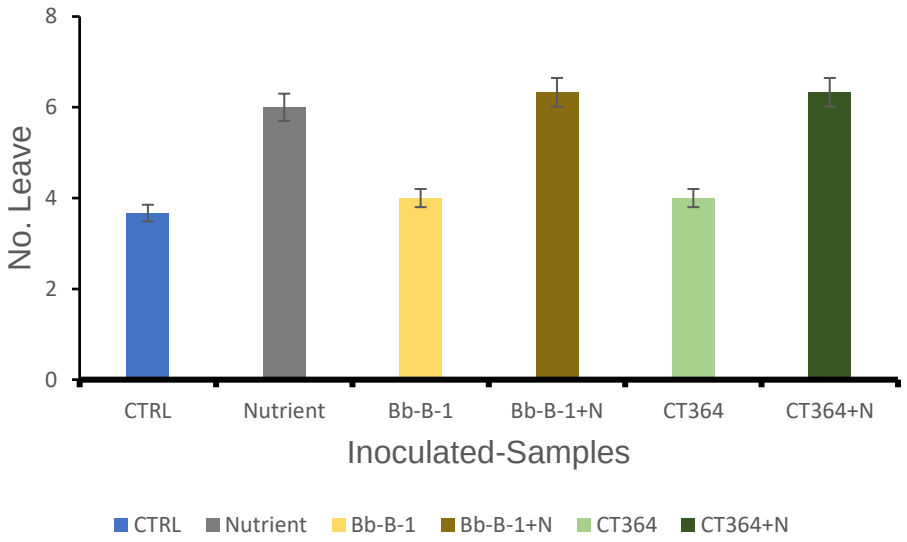


Fig. 4.4 Evaluating increased number of leaves on un-inoculated tomato (Elegance cultivar) and inoculated Elegance cultivar. Elegance one-week seedlings are cultivated for two weeks in magenta vessel, leaves were counted immediately after harvest. CRT is sample grown under deprive nutrient and soil microbial community, Nutrient is sample grown under added optimum nutrient condition but deprive of any soil microbial community, Bb-B-1 and CT364 are samples inoculated with Bb-B-1 and CT364 strain but deprive of nutrient condition, Bb-B-1+N and CT364+N are samples inoculated with Bb-B-1 and CT364 under optimum nutrient condition.

### 4.9 The shoot length development of the inoculated samples

Evaluating increase shoot length of a two-week seedlings of Elegance cultivar inoculated with vertically transmitted endophyte strain and CT364 strain. The samples were divided into sets Nutrient, *Bb-B-1* +N, CT364+N grown under optimum nutrient conditions, while the other set with *Bb-B-1*, CT364, and CTR grown under nutrient-deprived conditions. All samples were analyses at $p<0.05$. Error bars represent at standard deviation. The increase in shoot length on the inoculated sample with VTE *Bb-B-1* strain has a mean of 2.0, and CT364 strain with a mean of 1.8 grown under the deprived nutrient condition are not significantly different from the control at $p<0.05$. Similarly, the growth rate of samples treated with Bb-B-1+N strain, mean of 3.2 and CT364+N strain with a mean of 3.2 are not significantly different from the control (nutrient) at $p<0.05$.

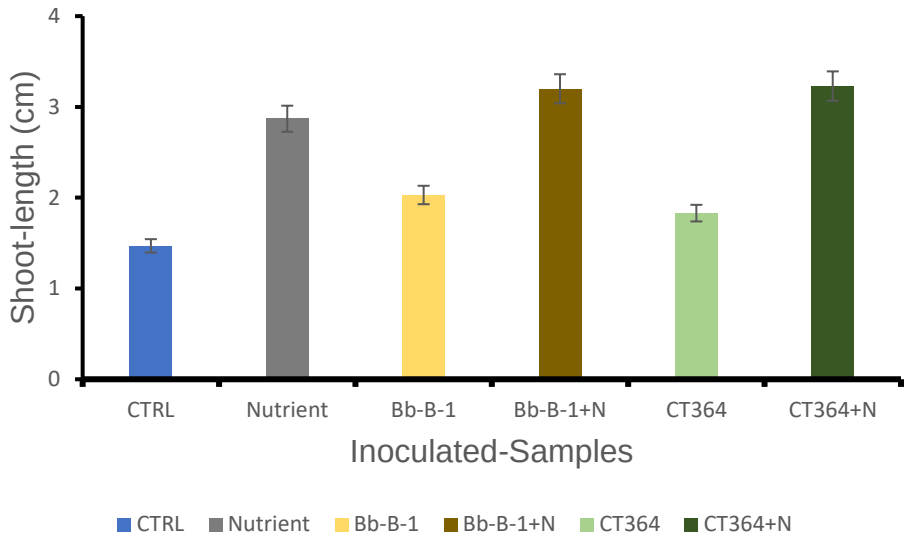


Fig.4.5 Evaluating shoot length of not-inoculated tomato (Elegance cultivar) and inoculated Elegance cultivar. Samples are one-week seedlings transfer into magenta vessel cultivated for two weeks, shoot length are immediately measure after harvest. CRT is sample grown under deprive nutrient and soil microbial community, Nutrient is sample grown under added optimum nutrient condition but deprive of any soil microbial community, Bb-B-1 and CT364 are samples inoculated with Bb-B-1 and CT364 strain but deprive of nutrient condition, Bb-B-1+N and CT364+N are samples inoculated with Bb-B-1 and CT364 under optimum nutrient condition.

### 4.10 The fresh biomass of the inoculated samples

Evaluating fresh biomass of a two-week seedlings of Elegance cultivar inoculated with vertically transmitted endophyte strain and CT364 strain under a deprived nutrient condition and in optimum nutrient condition. The samples were divided into *Bb-B-1* +N, CT364+N grown under optimum nutrient condition, while the other set *Bb-B-1*, CT364, and CRT grown under nutrient deprive condition. All data analyses at $p<0.05$. Error bars represent standard deviation. The fresh biomass of sample under nutrient deprived condition, control CRT with mean of 0.1, sample nutrient with mean 0.1, sample inoculated with Bb-B-1 with mean of 0.1, and sample inoculated with CT364 with mean 0.1 are not significantly different at $p<0.05$. However, they are significantly different from the samples in optimum nutrient condition inoculated with Bb-B-1+N with mean 0.2 and samples inoculated with CT364 with mean of 0.2.

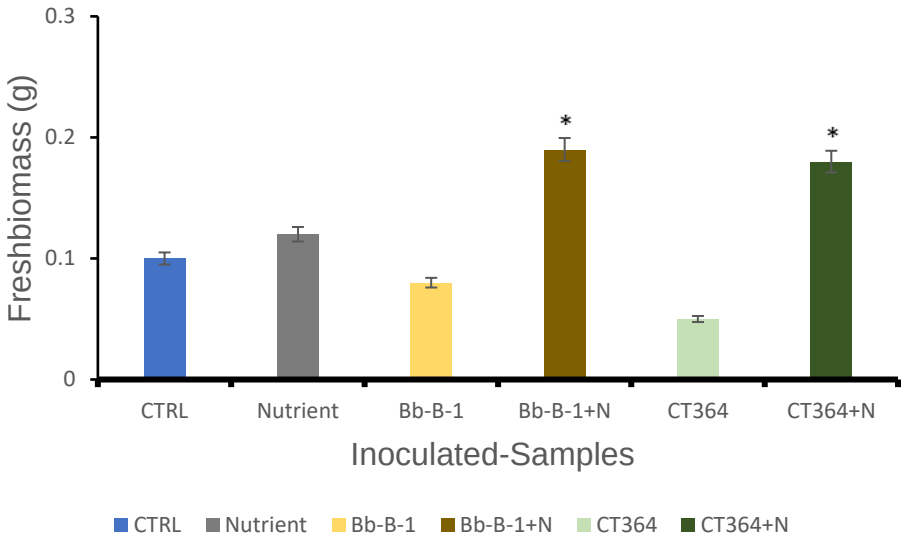


Fig.4.6 Fresh biomass of Elegance cultivar two weeks after inoculation, sample with asterisk is significantly different at $p<0.05$. CRT is sample grown under deprive nutrient and soil microbial community, Nutrient is sample grown under added optimum nutrient condition but deprive of any soil microbial community, Bb-B-1 and CT364 are samples inoculated with Bb-B-1 and CT364 strain but deprive of nutrient condition, Bb-B-1+N and CT364+N are samples inoculated with Bb-B-1 and CT364 under optimum nutrient condition.

### 4.11 Dried biomass of the inoculated samples

Evaluating dried biomass of a two-week seedlings of Elegance cultivar inoculated with vertically transmitted endophyte strain. The samples were divided into sets *Bb-B-1* +N, CT364+N grown under optimum nutrient conditions, while the other set with *Bb-B-1*, CT364, and CTR grown under nutrient-deprived conditions, all data analyses at $p<0.05$ error bars represent at standard error. The dried biomass in the inoculated sample with *Bb-B-1* strain has a mean of 0.014g and CT364 strain with a mean of 0.015 grown under the deprived nutrient condition are not significantly different from the control with mean of 0.17g at $p<0.05$. Likewise, the dried biomass of samples treated with *Bb-B-1* +N strain mean of 0.033g and CT364 strain with a mean of 0.034g is not significantly different from the control (nutrient) with mean of 0.34 at $p<0.05$.

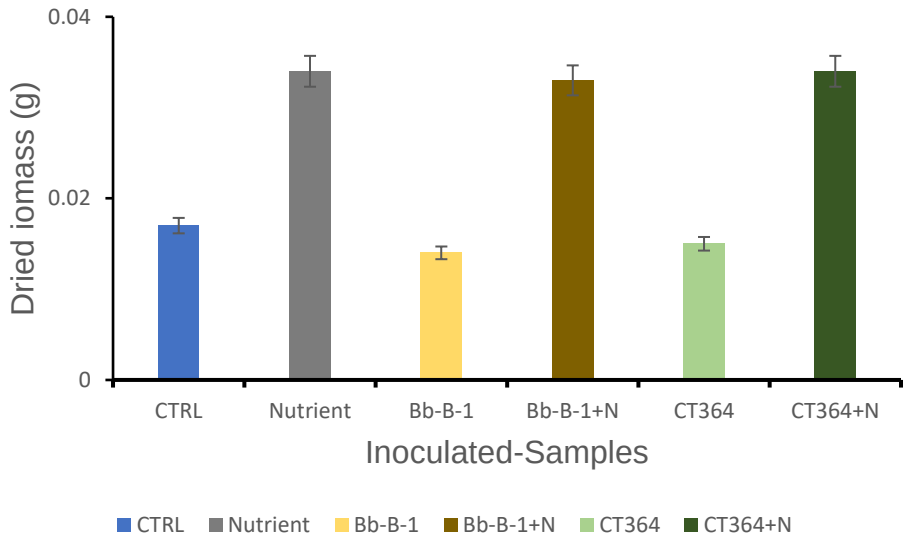


Fig.4.7 Dried biomass of Elegance cultivar two weeks after inoculation, sample with asterisk is significantly different at $p<0.05$. CRT is sample grown under deprive nutrient and soil microbial community, Nutrient is sample grown under added optimum nutrient condition but deprive of any soil microbial community, Bb-B-1 and CT364 are samples inoculated with Bb-B-1 and CT364 strain but deprive of nutrient condition, Bb-B-1+N and CT364+N are samples inoculated with Bb-B-1 and CT364 under optimum nutrient condition.

### 4.12 The concentration of jasmonic acid in the inoculated samples

Evaluating concentration of Jasmonic acid of a two-week seedlings of Elegance cultivar inoculated with vertically transmitted endophyte strain. The samples were divided into sets *Bb-B-1* +N, CT364+N grown under optimum nutrient conditions, while the other set with *Bb-B-1*, CT364, and CTR grown under nutrient-deprived conditions. All data analyses at $p<0.05$. Error bars represent at standard error. The concentration of jasmonic acid in a sample grown under a deprived nutrient condition and inoculated with *Bb-B-1* strain has a mean of 33.2nM, and CT364 strain has a mean of 13.8nM at $p<0.05$, indicating that the concentration between the samples treated with the two strains is not significantly different from the control. Also, the concentration of jasmonic acid in the samples grown under optimum nutrient, inoculated with *Bb-B-1* +N, has a mean of 37.6nM, and CT364+N has a mean of 37.4nM at $p<0.05$ are not significantly different from the control.

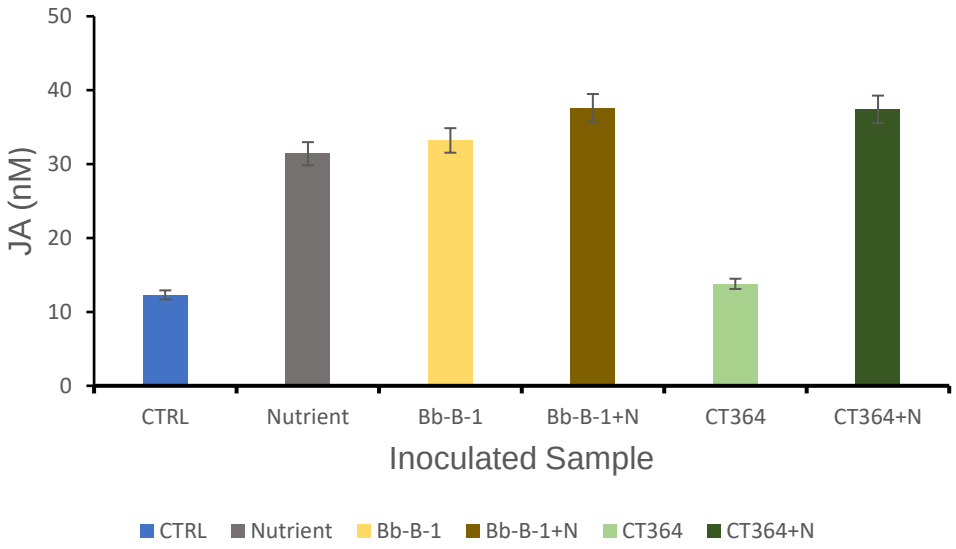


Fig.4.8 Concentration of Jasmonic acid in Elegance cultivar two weeks after inoculation, sample with asterisk is significantly different at $p<0.05$. CRT is sample grown under deprive nutrient and soil microbial community, Nutrient is sample grown under added optimum nutrient condition but deprive of any soil microbial community, Bb-B-1 and CT364 are samples inoculated with Bb-B-1 and CT364 strain but deprive of nutrient condition, Bb-B-1+N and CT364+N are samples inoculated with Bb-B-1 and CT364 under optimum nutrient condition.

### 4.14 The concentration of 3-indole acetic acid in the inoculated samples

Evaluating 3-indole acetic acid concentration of a two-week seedlings of Elegance cultivar inoculated with vertically transmitted endophyte strain. The samples were divided into sets *Bb-B-1* +N, CT364+N grown under optimum nutrient condition, while the other set with *Bb-B-1*, CT364, and CTR grown under nutrient deprive condition all data analyses at $p<0.05$. Error bars represent at standard error. The concentration of 3-indole acetic acid in a sample grown under a deprived nutrient condition and inoculated with *Bb-B-1* strain has a mean of 218.5nM, and CT364 strain has a mean of 181.5nM at $p<0.05$, indicating that the concentration between the samples treated with the two strains is significantly different from the control. Also, the concentration of 3-indole acetic acid of the samples grown under optimum nutrient, inoculated with *Bb-B-1* +N, has a mean of 38.9nM, and CT364+N has a mean of 12.4nM at $p<0.05$ are significantly different from the control.

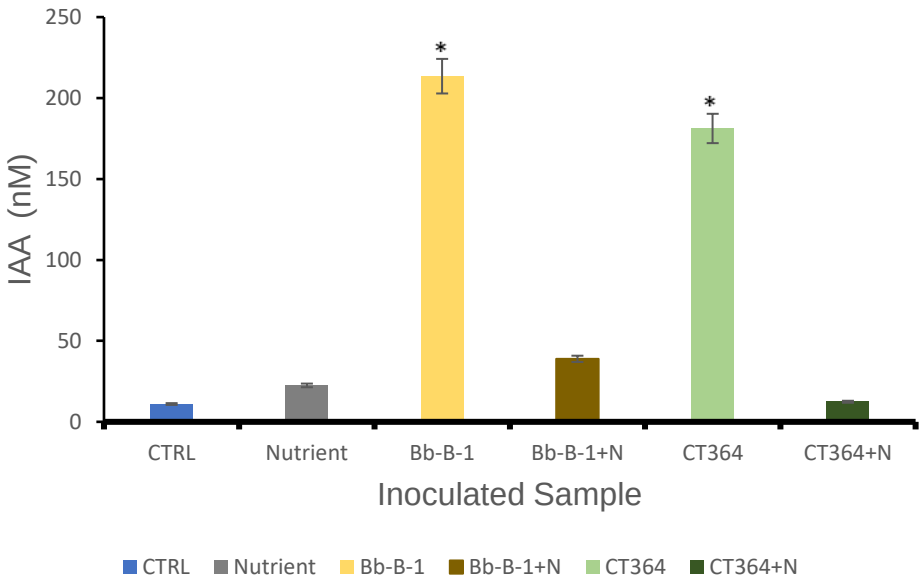


Fig.4.9 Evaluating influence of VTE inoculant to regulate 3-indole acetic acid in Elegance cultivar two weeks after inoculation, sample with asterisk is significantly different at $p<0.05$. CRT is sample grown under deprive nutrient and soil microbial community, Nutrient is sample grown under added optimum nutrient condition but deprive of any soil microbial community, Bb-B-1 and CT364 are samples inoculated with Bb-B-1 and CT364 strain but deprive of nutrient condition, Bb-B-1+N and CT364+N are samples inoculated with Bb-B-1 and CT364 under optimum nutrient condition.

### *4.15* The concentration of gibberellic acid *in the inoculated samples*

Evaluating the concentration of gibberellic acid of two-week seedlings of Elegance cultivar inoculated with vertically transmitted endophyte strain. The samples were divided into sets *Bb-B-1* +N, CT364+N grown under optimum nutrient conditions, while the other set with *Bb-B-1*, CT364, and CTR grown under nutrient-deprived conditions. All data analyses at $p<0.05$. Error bars represent at standard error. The concentration of gibberellic acid in a sample grown under a deprived nutrient condition and inoculated with *Bb-B-1* strain has a mean of 3.7nM, and CT364 strain has a mean of 3.4nM at $p<0.05$. indicated that the concentration between the samples treated with the two strains is significantly different from the control. The concentration of gibberellic acid of the samples grown under optimum nutrient, inoculated with *Bb-B-1* +N, with a mean of 4.5nM, and CT364+N a mean of 6.2nM at $p<0.05$ are significantly different from the control.

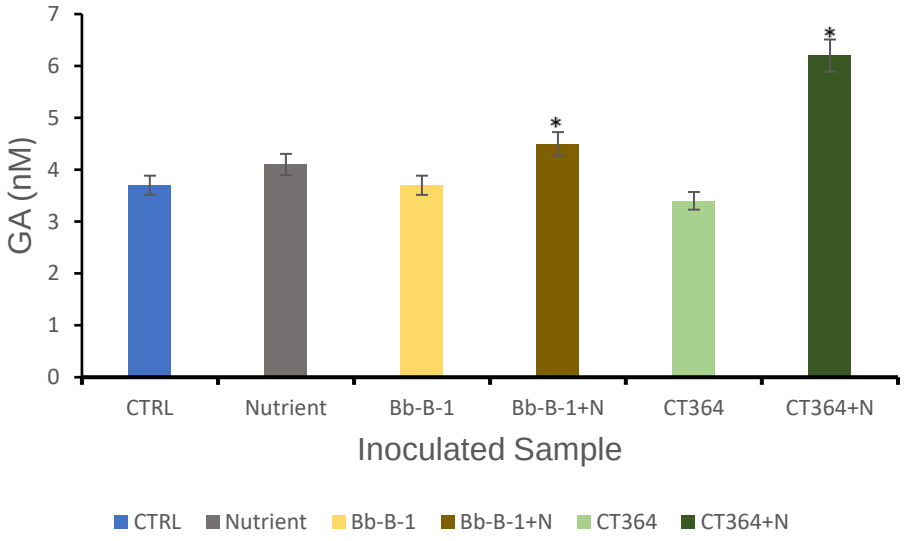


Fig.4.10 Concentration of gibberellic acid in Elegance cultivar two weeks after inoculation, sample with asterisk is significantly different at $p<0.05$. CRT is sample grown under deprive nutrient and soil microbial community, Nutrient is sample grown under added optimum nutrient condition but deprive of any soil microbial community, Bb-B-1 and CT364 are samples inoculated with Bb-B-1 and CT364 strain but deprive of nutrient condition, Bb-B-1+N and CT364+N are samples inoculated with Bb-B-1 and CT364 under optimum nutrient condition.

## 4.16 Discussion

The application of gnotobiotic or single bacteria to seeds, seedlings, or the soil is a frequent technique in scientific research as a searchlight to identify the suitable strain to replace synthetic fertiliser. Evidence of bioinoculant improving soil fertility, providing nutrients, and conferring resistance/tolerance traits to the host plant has been demonstrated (Ma, Látr, et al. 2019, de Souza, Rocha, et al. 2020). However, based on the public domain available information, no investigation has been performed related to vertically transmitted endophyte potentials. Therefore, for the vertically transmitted endophytes to serve as an inoculant for improving plant growth, yield, and health. This study evaluates an isolated VTE *Bb-B-1* influence on the Elegance tomato cultivar, which has demonstrated to be void of any vertically transmitted endophyte in our previous experiment chapter 1. The treated elegance cultivar was randomly placed in a plant growth incubator treated with *Be* isolate or without to investigate the influence of the vertically transmitted endophytes on tomato plant growth.

Furthermore, the experiments investigate the effect of the four microbial communities on the growth of the Elegance cultivar. Plants cultivated under manure microbial community, untreated microbial community, and control conditions were not significant differences between the shoot length $p<0.05$ and Table 1.3. However, the plant under fertilised microbial community was significantly different from the control at a p-value of 0.05 with reduced shoot length compared to all other treatments.

The results indicated that the shoot length of the cultivar under fertilising microbial community is significant less than shoot length in manure and untreated microbial communities, as shown in figure 4.3. The fertilise microbial community demonstrated has less microbial CFU in figure 2.2, chapter 2 than the manure and untreated microbial communities therefore, it is expected that the shoot length of samples under the manure and untreated microbial communities would have high shoot length increase than the fertilised microbial community since microbial community structure plays a crucial role in improving the nutrient cycle and soil aggregates formation (Stark, Condron, et al. 2008). The previous experimental results, figure 2.2, chapter 2 of this thesis, show that microbial community colony-forming unit under fertilised microbial

community has less CFU than the manure microbial community and untreated microbial community.

The chlorophyll content of the cultivars under the differential microbial communities table 2.3 demonstrates that the fertilised microbial community, manure microbial community, and the untreated microbial community at $p<0.05$ are not significantly different. Likewise, the fresh and dried biomass of the cultivar under the fertilised microbial community, manure microbial community, and untreated microbial community is not significantly different at $p<0.05$ from the control.

After evaluating parameters such as the shoot length, chlorophyll content, fresh and dried biomass of the cultivars, it established that without the addition of the vertically transmitted endophyte. All the samples except the cultivars grown under the fertilised microbial community were not significantly different from the control samples at $p<0.05$. Furthermore, the experiment evaluated the effects of the *Bb-B-1 isolate* and *CT364* (a known PGPB) on tomato phenotypic and phytohormone.

### 4.17 Evaluating an increased number of leaves, biomass, and the phytohormones

The evaluation of the number of leaves on experimental samples is an important morphological parameter influenced by the interaction between the plant and its external factors (Giuffrida, Minervini et al. 2016). The number of leaves on a plant indicates the plant growth stage, demonstrating the level of growth in treated samples. Our quest to investigate the influence of exogenous inoculation of VTE on the plant cultivated under optimum nutrient condition demonstrates that treated samples with Bb-B-1+ N mean of 6.3 (Figure 2.3) is not significant difference in the number of leaves compared to plant inoculants with *CT364+N* mean of 6.3. It also showed that plants cultivated under the nutrient-deprived condition, inoculated with strain *Bb-B-1* with a mean of 4.0 and *CT364* with a mean of 4.0, were not significantly different compared to the control. Also, fresh plant biomass is a parameter used to measure the level of water content in inoculated plant samples compared to the non-inoculated samples (Huang, de-Bashan et al. 2017). It is a determining factor of influenced plant growth by plant growth-promoting inoculant. The fresh biomass of plants cultivated under the optimum nutrient condition figure 2.3 shows that between plants inoculated with *Bb-B-1 +N* means 0.2g and CT*364+N,* a mean of 0.2g is not significantly different from

the control at p<0.05. Similarly, a plant cultivated under nutrient-deprived conditions shows is no significant difference between the plant inoculated with strain *Bb-B-1* and strain *CT364* compared to the control.

As one may argue, dry biomass of plant samples gives reliable results regarding the plant tissue's actual biomass(Bashan and de-Bashan 2005). Here we consider evaluating both the fresh biomass and the dry biomass of our samples to avoid bias. The evaluation of plant dried biomass cultivated under optimum nutrient condition was not significantly different between plant inoculant with strain *Bb-B-1 +N* means of 0.03g, strain *CT364+N with a* mean of 0.03g and the plant cultivated under nutrient condition but not inoculated with any strain, with a mean of 0.03g. Also, the plants cultivated under nutrient-deprived conditions, inoculated with *Bb-B-1* and *CT364,* were not significantly different at p<0.05 from the control. Thus, it demonstrated that the exogenous inoculation of these strains under these conditions failed to increase the number of leaves and increase growth; it also failed to improve the fresh and dried biomass compared to the control group.

Investigation on the influence of the strain to regulate and release phytohormones such as Jasmonic acid figure 4.8 demonstrate that the plant inoculated with strain *CT364*, *Bb-B-1*, *Bb-B-1+N*, *CT364+N*, and the plant under the nutrient are not significantly different at p<0.05 from the control. Also, the potentials of the strains to regulate the release of gibberellic acid figure 4.10 was also analysed. The cultivar inoculated with *CT364*, *Bb-B-1*, *Bb-B-1 +N* strains are not significantly different at p<0.05 from the control. However, strain *CT364+N* shows an increased concentration significantly different from the control at p<0.05.

Further investigation figure 4.9 on the role of the strains to influence the release of indole acetic acid demonstrates that the plant inoculated with the VTE strain *Bb-B-1* and the *CT364* strain was significantly different at p<0.05 from the control. However, the plant inoculated with *Bb-B-1+N*, *CT364+N*, and the plant under the nutrient is not significantly different at p<0.05 from the control.

The finding demonstrated that the exogenous application of the isolated vertically transmitted endophytes at the giving condition did not confer the plant with PGP traits and could not stimulate growth and development of the *S. lycopersicum* as demonstrated in the number of leaves in figure 4.4, increase shoot length figure 4.5

the fresh and dried biomass of the plant the *S. lycopersicum*. Further evidence demonstrates the lack of involvement of these VTE in stimulating the release of phytohormone jasmonic acid figure 5.3. The acid is known for its role in defence, also served as a plant growth regulator that regulates plant reproductive growth and plays a role in nutrient storage (Alisofi, Einali, et al., 2020). Through regulating various biochemical processes relating to plant growth and development (Raza, Charagh, et al. 2020). Jasmonic acid, networking with other growth regulators to mediate environmental stresses, assists plants in developing tolerance under environmental stress conditions. This study also demonstrates that vertically transmitted endophyte did not stimulate indole acetic acid produced by the plant through an indole-3-pyruvic acid pathway as a hormone responsible for promoting plant cell division and elongation (Gallei, Luschnig, et al. 2020) figure 6.3. furthermore, gibberellic acid concentration is another plant hormone regulator that control cell division, stem elongation, shortening seeds dormancy, promoting germination and stimulate seedlings (Chen, Kuo, et al., 2008). Figure 7.3 demonstrates no improved gibberellic acid concentration in the inoculated plants compared to the control. The finding shows that exogenous application of the isolated vertically transmitted endophyte *Bb-B-1* under these conditions did not result in a measurable increase in the parameters measured. Contrary to the hypothesis that the exogenous application of vertically transmitted endophytes to the plant can confer plant growth-promoting traits.

## Chapter five

## The effects of differential microbial communities on *solanum lycopersicum* genes involved in the biosynthesis of key plant phytohormones

### *5.1 introduction*

Gene expression is a process through which encoded information stored in genes within the genome of the organism is used to generate functional products such as proteins or non-coding RNA (Schwanhäusser, Busse et al. 2011). Multiple steps are involved with protein expressions, such as transcription, messenger RNA splicing, translation, and posttranslational protein modification (Volgin 2014). Overall, multiple processes are employed to regulate the quantity of the functional protein in the system, which are crucial for maintaining the normal cellular structure and function. The temporal and spatial regulation of gene expression is required for developmental changes such as differentiation and morphogenesis within the organism. Furthermore, the regulation of gene expression is essential to enable a coordinated response to biotic and abiotic factors. In these cases, gene expression is regulated by one or more transcription factors that respond to the stressor and activate or deactivate downstream gene expression to provide a mechanism to overcome the impact of the stress. This system is particularly important for plants due to their sessile nature plants cannot move from their site of initial germination thus, evolved highly refined mechanisms for the rapid production of protective molecules to enable survival in rapidly changing environments (Li and Loake 2016).

Therefore, the ability of transcription factors to regulate a particular gene expression at a given location and time allows the delivery or accumulation of a functional protein at a specific location to maintain plant normal function or survival. It is important to note that the exogenous application of microbial organisms and synthetic substances can influence gene expression in plants. Particularly, tomato has been used in several scientific research to elucidate the effect of external stimulants on the control of gene expression and resulting phenotype. A good example of such an experiment by Porcel and Evelin demonstrated how exogenous inoculation of AM fungi on a plant regulate the plant gene mediating Na+/H20 influx in the plant(Porcel, Aroca et al. 2012, Evelin, Devi et al. 2019). Further evidence demonstrates that the expression of the SOS1

gene in rice shoots was altered in AM fungi inoculated rice plants (Porcel, Aroca et al. 2016, Chen, Zhang et al. 2017). As well as the expression of PIP1 in tomato root was altered when the tomato plant was inoculated with the AM fungi(Ouziad, Wilde et al. 2006)

This chapter reports the influence of different microbial consortia on regulating the expression of transcription factor genes and genes that control iron homeostasis in tomato plants. The *LeNRT2.3*, a nitrated transport gene, induced nitrate in the plasma membrane and involved mediating low-affinity nitrate transport from root to the shoot and involved in the long-distant transport of nitrate within the plant (Fu, Yi et al. 2015). Nitrate in the soil has different concentrations, and the plant usually adopts two types of nitrate transport: *NPF/NRT1* and *NRT2,* to transport the nitrate to the plant various tissue. The *NPF/NRT1* has a dual iron affinity transport role due to phosphorylation and dephosphorylation (Liu, Huang, et al. 1999).

In contrast, the *NRT2* family members such as 4 *AtNRT2.1, AtNRT2.2, AtNRT2.*, and *AtNRT2.5* are involved in higher affinity nitrate transport (Lezhneva, Kiba et al. 20), with each having a specific role to play. Here we investigate the influence of the differential microbial communities on the gene expression of *LeNRT2.3* in two tomato cultivars. The *LeNRT2.3* is known to initiate embryogenesis development, tissues, organs, roots, flowering, and fruit enhancement.

Another gene of interest is the auxin response factors due to its vital role as the plant hormone. Auxin phytohormones are essential for plant growth and development; they initiate the development of embryogenesis, tissues, organs, root, flowering, and fruit. Auxin-response factors (*ARF*s) are well known for binding to their target (TGT CTC), an auxin response element with specificity, positioned in the promoters of primary auxin response gene elements (*AuxRE*s) (Ulmasov, Hagen, et al. 1999). These *ARF*s play an essential role in activating or repressing transcription by binding to the *AuxREs* directly. Previous findings have demonstrated that exogenous application of auxin to the plant cell can bring about overexpression of this gene (Ulmasov, Hagen, et al. 1999). Therefore, with this notion, we evaluate the influence of the differential microbial communities on *Auxin ARF's* response in the two *S. lycopersicum* cultivars and investigate the vertically transmitted role of endophyte on the *Auxin ARF* of the cultivars. Other genes of interest are the iron transporters, knowing that iron is an

essential component in plant nutrition. And the common source of plant iron is the rhizosphere, usually in its inactive form. Converting the solid iron into a suitable and usable form, the plant induces a strategy I response involving the *FROS2* gene, the ferric chelate reductase enzyme. Reduces $Fe^{3+}$ to a soluble form, $Fe^{2+}$, transporting iron from the root to the shoots and other parts of the plant (Robinson, Procter, et al. 1999), playing an essential role in the plant growth and development. Previously research has demonstrated that the *FROS* gene comprises seven family members, playing the functional role of iron homeostasis within the plant. In addition to the *FROS2* gene, *LeIRT1* and *FER* gene also plays a role in iron transport within the plant. This project design experiment to identify the influence of different microbial communities on the gene expression of three tomato cultivars and evaluate their effects.

**Hypothesis**

4. Different type of microbial communities can induce host plant *Auxin* ARF gene, *Actin* gen, LeNRT2.3, FROS2 FER gene expression

5. Vertically transmitted endophytes can regulate *Auxin* ARF gene, *Actin* gen, LeNRT2.3, FROS2 FER gene expression in the host plant

## 5.2 Materials and methods

Two *Solanum lycopersicum* cultivars: Elegance and Yellowpear seedlings, were grown in the presence of microbial communities isolated from soils extracted from the long-term hay meadow plots at Palace Leas, Newcastle University. Plots were chosen that have been continually maintained with the following amendments chemical fertiliser, farmyard manure, or receive no treatment. Randomised experimental design with each cultivar per microbial community replicated four times. Seeds were germinated, and seedlings were grown for two weeks in magenta vessels containing solidified agar with or without microbial inoculum. All treatments were cultured in a growth chamber with cool-white, fluorescent light at 25°C and 19°C on a 12-hour day: night cycle. Control seeds were germinated in 280 ml of 2.0 % agar (w/v) in distilled

water. Microbial community inoculum was prepared as described in Chapter Three, and 140 mL was used to inoculate the agar of the experimental group

Total RNA extracted seedlings following two weeks of growth. 15 mg of fresh leave tissue was harvested, and RNA was extracted using the Pure Link RNA Mini Kit (Invitrogen) following the manufacturer's instructions. RNA yield and quality were determined by UV spectrophotometry (NanoDrop) with a concentration of 137.7ng/µl. cDNA was synthesised using a total mRNA of 275.4 ng, 4µl of 5xtransAmpbuffer, 1µl of reverse transcriptase, 13µl of RNase free water. The synthesis run was set at 25°C for 10 minutes, 37°C for 2hrs, 85°C for 5minutes and hold at 4°C. Primers specific to the *Auxin* ARF gene, *Actin* gen, LeNRT2.3, FROS2 FER, and LelRT1 genes were designed using Thermo fisher Oligo design tools below table give the primer sequence and the protein it regulated and µM per reaction.

| Primer | Sequence | µMper reaction | Target |
|---|---|---|---|
| *actin* | | | G-actin pool And F-actin |
| Forwards | ACTGGTATTGTGCTGGACCTG | 1pmol/µl | |
| Reverse | TCGGCTGTGGTGGTGAAG | 1pmol/µl | |
| *Auxin (ARF)* | | | AuxRE |
| Forwards | GGTTTGCCTGGGATAATAAAC | 1pmol/µl | |
| Reverse | TTTGGTGCAGGAGCTGAGTC | 1pmol/µl | |
| *FROS2* | | | IRT1 and FIT1 |
| Forwards | GCTTGCATCATGCTTCCTGG | 1pmol/µl | |
| Reverse | GCAGAACACGAGCAGAGACT | 1pmol/µl | |
| *LeIRT1* | | | bHLH protein |
| Forwards | TGGCACTTGTGGATCTCCTT | 1pmol/µl | |
| Reverse | TAGACATTCCACCAGCACCT | 1pmol/µl | |
| *LeNRT2.3* | | | NRT2 transporters |
| Forwards | GGAXCACCAGGAXGTTCAATG | 1pmol/µl | |
| Reverse | AAACAGAGGCAACTCCAGCX | 1pmol/µl | |
| *FER* | | | bHLH |
| Forwards | GAGXAGCTGCAATXTGTCGC | 1pmol/µl | |
| Reverse | GAATCTTCXGGCACAACGGX | 1pmol/µl | |

Table 5.1 Lists of selective candidate genes regulating the biosynthesis of these phytohormones used in qPCR reactions.

## 5.3 Results

### Validation and optimisation of genes of interest using differential temperature

Validation and optimisation of target gene before the downstream application is crucial for accurate and robust data generation and analysis. This chapter focuses on the expression of genes involved in the biosynthesis of key plant phytohormones. It investigates the role of differential microbial communities influence on *S. lycopersicum* genes regulating the biosynthesis of these phytohormones. After the extraction of mRNA and quantification using the Nanodrop, the pure mRNA was synthesis into a cDNA. The synthesised cDNA was used as the template to set up a PCR to an amplified gene of interest. Where 4µl of cDNA was mixed with 6µl of RNA free water, 2µl of forwarding primer, 2µl of reverse primer and 11ul of master mix. The mixed reagents were used for PCR with and subsequent agarose gel to separate the different genes of interest using the respective primers of *Actin*, *LeNRT2.3*, Auxin, *FRO2*, *LelRT1* and *FER*. Various band sizes were obtained to demonstrate the presence of these genes in the plant. Figure 4.1 A using ladder 100bp, Actin gene, *LeNRT2.3*, *Auxin* and *FRO2* and figure 4.1 B *LelRT1* and Fer gene. The band sizes obtained from Actin =210 base pair, *LeNRT2.3*=310 base pair, *Auxin* =125 base pair, *FOR2* with =103 base pair amplified at 65ºC and *LelRT1*=154bp, *FER* Gene=195bp amplified at 55°C.

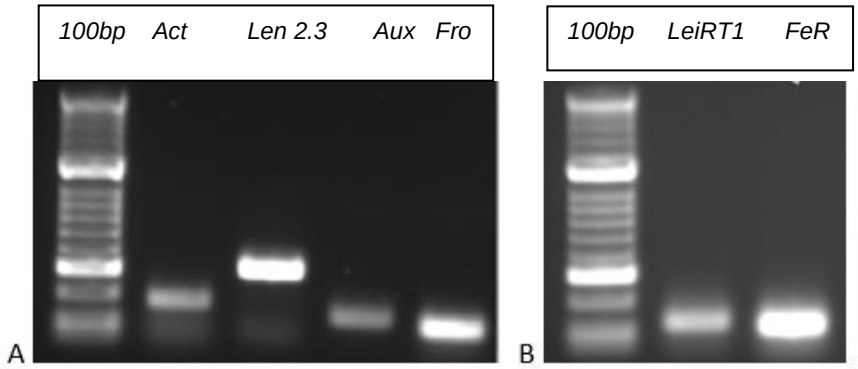


Figure 5.1 PCR amplification of gene (A) Act, LeNRT2.3, Aux, FRO, and (B) LeIRT1 and FER gene in tomato plants cultivated in three different microbial communities. Seedlings harvested after two weeks, tissues from the leaves were used to extracts the mRNA for the PCR amplification with the various primers. The PCR product was run on agarose 2% electrophoresis with a 100bp ladder.

### *5.4 Gene expression of genes* regulating the biosynthesis of plant phytohormones

The gene expression assay used in this study evaluates the relative abundance of genes of interest (LeNT2.3, *FRO2, Auxin, LelRT1, and FER)*. The experiment consists of two tomato cultivars grown under differential microbial communities with the same nutrient condition. The changes in the molecular regulation in these genes were evaluated using quantitative reverse transcription qPCR. The relative abundance of the transcript gene was normalised against housekeeping actin gene, as preferential reference gene because of its reliability in expression at different stage of plant growth (Barros Rodrigues, 2014) which is suitable for our research because we used similar tissue in all samples i.e. the leave of young plant. Although may not be suitable reference gene in experiment that uses comparison expression among different tissues (Barros Rodrigues, 2014).

### 5.5 LeNRT2.3 gene expression

The relative fold changes in expression of *LeNRT2.3* regulating NRT2 transporters that increased nitrate uptake in root to the shoot of *S. lycopersicum* (Fu, Yi et al. 2015) in the two cultivars were calculated using comparative threshold cycle techniques in which the relative abundance of the transcript gene was normalised against the internal reference actin gene. Figure 5.2 gene expression assay of *LeNRT2.3* genes from the treated plant of two cultivars, Elegance and Yellowpear, represented as 1 on the log 2 y-axis. Bars extending above the x-axis represent a relative increase in gene expression, and those below the x-axis indicate a decrease in transcript abundance. The result shows a significant difference between cultivar with vertically transmitted endophytes (VTE) and cultivar without VTE with the relatives 2fold change in the expression of *LeNRT2.3* gene between cultivar with VTE and the cultivar without VTE. It also showed that there is significantly different in the plant grown under the differential microbial communities furthermore, figure 5.2 show that the expression of the *LeNRT2.3* genes was upregulated in Elegance cultivar under the fertilised microbial community with a 1.2-fold change and 1.1-fold change in an untreated microbial community, and 1.0-fold change in manure microbial community. In contrast to the yellowpear cultivar that is downregulated under the fertilised microbial community with 0.7-fold change but upregulated under the untreated microbial

community with a 1.0-fold change and downregulated with 0.6-fold change. The results showed relative of 2fold significant difference in the expression of *LeNRT2.3* in Elegance cultivar compared to the downregulated *LeNRT2.3* genes in Yellowpear cultivar. The finding demonstrates that differential microbial communities affect the expression of *LeNRT2.3* of the plant.

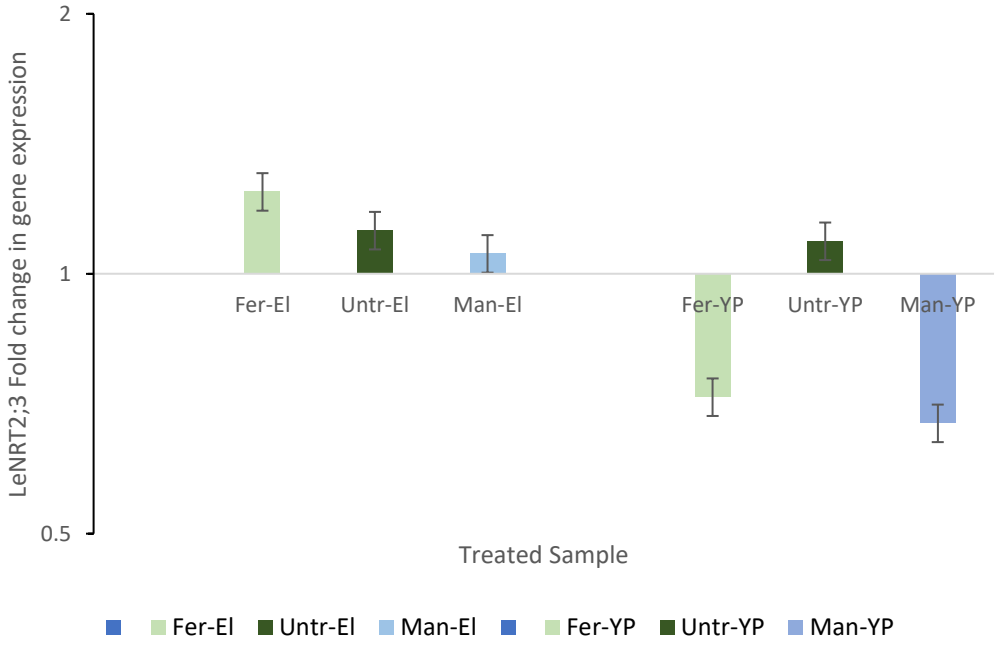


Figure 5.2 gene expression assay for LeNRT2;3 genes in the leave tissues of two tomato cultivars Elegance (void of VTE) and Yellowpear (possessing VTE). E-Ctr=Elegance control, E-Fer=Elegance Fertilized soil microbe, E-Utr=Elegance+ Untreated soil microbes, E-Mn=Elegance+manure soil microbes, Y-Ctr=Yellow+cont roll, Y-Fer=Yellowpear+Fertilize soil microbes, Y-Untr=Yellowpear+Untreated soil microbes, Y-Mn=Yellowpear+Manure soil microbes. LeNRT2;3 gene expression in the cultivar Elegance and Yellowpear, the cultivar seedlings are grown in the differential microbial community in an incubator with variable temperature. The sample was harvested after two weeks, and mRNA was extracted from the leave of the seedlings. The relative change in the transcript gene was normalised against the internal reference gene. Error bars indicate a standard error at ($p<0.05$) confidence level of 95%

## 5.6 Auxin gene expression

The gene expression showed the change in relative auxin gene abundance in plants grown under the differential microbial communities. It also showed changes in Elegance cultivar and Yellowpear cultivar grown under differential microbial communities, change in auxin gene expression was calculated using the comparative threshold cycle technique, in which the relative abundance of the transcript gene was normalised against the *Actin* gene. Figure 5.3 shows the fold-change observed in the *Auxin* gene expression for the two cultivars that grow under the differential microbial communities after two weeks. The gene expression assay from the treated plant of two cultivars Elegance and Yellowpear is represented as 1 on the log 2 y-axis. Bars extending above the x-axis represent a relative increase in gene expression, and those below the x-axis indicate a decrease in transcript abundance. Auxin gene expression in both cultivars was downregulated with a fold change of 0.9 in the Elegance cultivar under fertilising microbial community, a 0.8-fold change in the untreated microbial community, and a 0.7-fold change in manure microbial community. This demonstrated a significant fold change of 2 and 3-fold change of the auxin gene in the Elegance cultivar. The expression of auxin in cultivar Yellowpear was also downregulated under the fertilised microbial community with a fold change of 0.6 and 0.9-fold change in untreated microbial community and 0.8-fold change in manure microbial community. likewise, there is 0.6-fold change downregulated in fertilised microbial community, and 0.9-fold change downregulated in the untreated microbial community and 0.8-fold change in manure microbial community. The results show a significant difference in plant growth under the differential microbial communities, with >2-fold change as seen below.

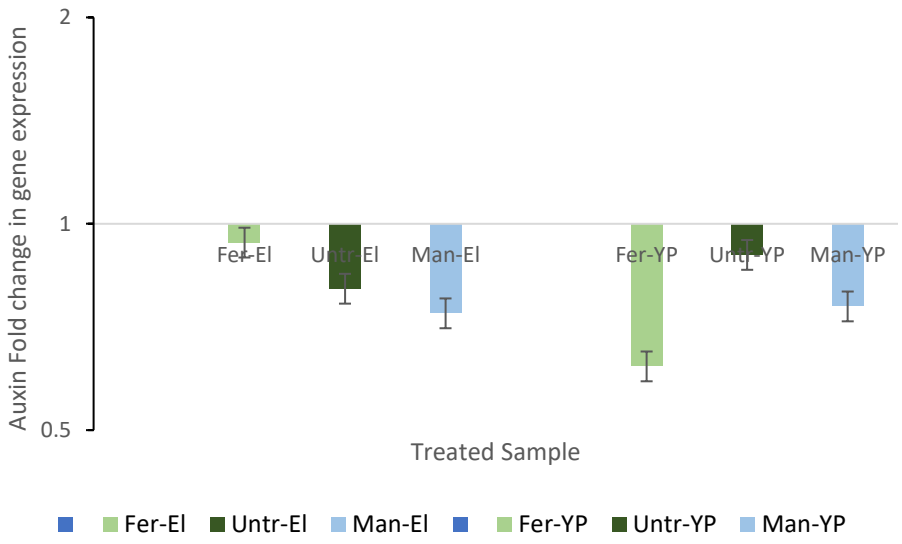


Figure 5.3 gene expression assay for Auxin gene in the tissues of two tomato cultivars, Elegance (void of VTE) and Yellowpear (possessing VTE). E-Ctr=Elegance control, E-Fer=Elegance Fertilized microbial community E-Utr=Elegance+ Untreated microbial community, E-Mn=Elegance+manure microbial community, Y-Ctr=Yellowpear+controll, Y-Fer=Yellowpear+Fertilize soil microbes, Y-Untr=Yellowpear+Untreated soil microbes, Y-Mn=Yellowpear+Manure soil microbes.

Auxin gene expression in the cultivar Elegance and Yellowpear, the cultivar seedlings are grown in the differential microbial community in an incubator with variable temperature. The sample was harvested after two weeks. The tissues from the leave the seedlings used to extract mRNA. The relative change in the transcript gene was normalised against the endogenous gene. Error bars indicate standard deviation ($p<0.05$) with a confidence level of 95%

### 5.7 FRO2 gene expression

Expression of *FRO2* gene in the two cultivars Elegance and Yellowpear vary from one treatment to another, gene expression assay from the treated plant of two cultivars, Elegance and Yellowpear are represented as 1 on the log 2 y-axis. The bars extending above x-axis represent a relative increase in gene expression, and those below the x-axis indicate a decrease in transcript abundance. The expression between the cultivars was significantly different figure 5.4 showed that the Elegance under the fertilised microbial community is upregulated with 1.0-fold change and downregulated under the untreated microbial community with 0.9-fold change and 0.7-fold change

under the manure microbial community. Also, the expression of the Yellowpear cultivar under the fertilised microbial community is downregulated with 0.8-fold change and upregulated under the untreated microbial community with 1.0-fold change and downregulated with 0.9-fold change under the manure microbial community. The results show significant changes in the gene expression of the *FRO2* gene under the differential microbial communities treatments. It also demonstrates a significantly difference in *FRO2* gene expression in the two cultivars.

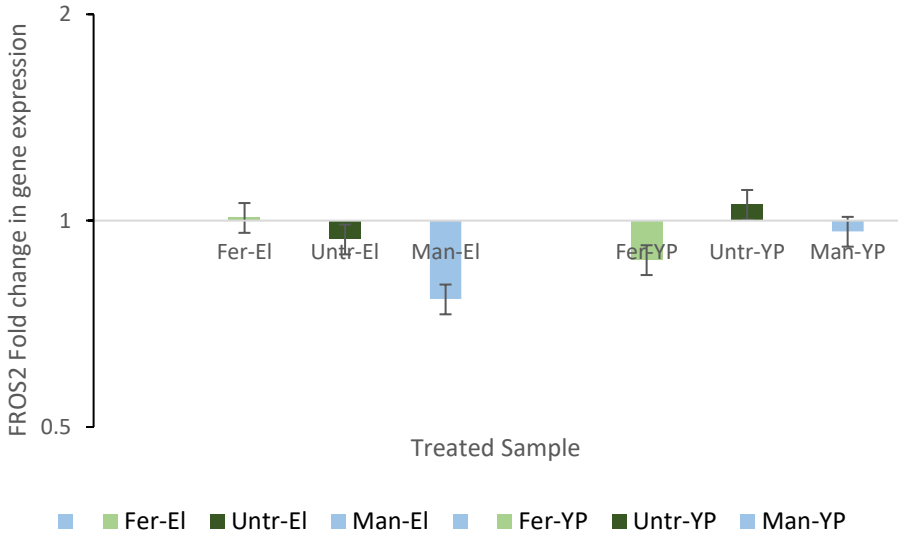


Figure 5.4 gene expression assay for FROS2 gene in the tissues of two tomato cultivars Elegance void of VTE and Yellow pear possessing VTE. E-Ctr=Elegance control, E-Fer=Elegance Fertilized microbial community, E-Utr=Elegance+ Untreated soil microbes, E-Mn=Elegance+manure soil microbes, Y-Ctr=Yellow+control, Y-Fer=Yellowpear+Fertilize microbial community, Y-Untr=Yellowpear+Untreated microbial community, Y-Mn=Yellowpear+Manure soil microbial community.

FROS2 gene expression in the cultivar Elegance and Yellowpear, the cultivar seedlings are grown in the differential microbial community in an incubator with variable temperature and sample was harvested after two weeks. mRNA was extracted from the leaves of the seedlings the relative change in transcript gene was normalised against endogenous gene Actin. Error bars indicate confidence interval ($p<0.05$)

### 5.8 LelRT1 gene expression

The gene expression of *LelRT1* in two cultivars shows that the expression of *LelRT1* in both cultivars was evaluated using comparative threshold cycle techniques, where the relative abundance of the transcript gene was normalised against the Actin gene. The gene expression assay from a plant grown under the differential microbial communities is represented as 1 on the log 2 y-axis. Bars extending above the x-axis represent a relative increase in gene expression, and those below the x-axis indicate a decrease in transcript abundance. Figure 5.4 shows that there was a significant difference in Elegance cultivar grown under the fertilised microbial community with LelRT1 downregulated with 0.7-fold change and 0.3-fold change in untreated microbial community and 0.9-fold change in manure microbial community. The LelRT1 gene expression in Yellowpear cultivar under the fertilised microbial community was also downregulated with 0.7-fold change, and under the untreated microbial community with 0.9-fold change and 0.9-fold change under the manure microbial community. this result demonstrates that differential microbial community effect changes in the LelRT1 gene expression of the plant.

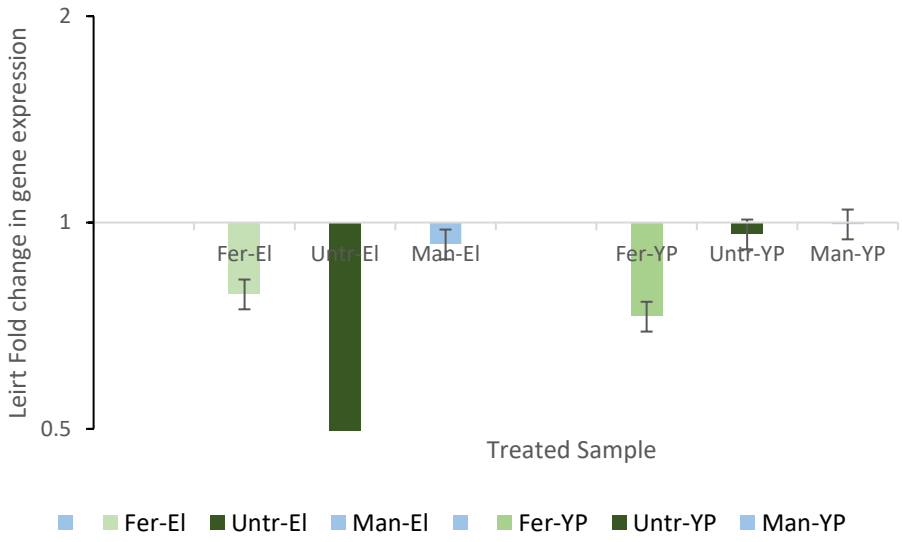


Figure 5.5 gene expression assay for LelRT1 gene in the tissues of two tomato cultivars Elegance void of VTE and VTE cultivar Yellowpear.

E-Ctr=Elegance control, E-Fer=Elegance+fertilisedmicrobe, E-Utr=Elegance untreated, Y-Fer=Yellowpear +fertilised microbes Y-Untr=Yellowpear+Untreated soil microbes-Mn=Yellowpear+Manure soil microbes.

Lelrt1 gene expression in the cultivar Elegance and Yellowpear, the cultivar seedlings were cultivated in the differential microbial community in an incubator with alternating temperature the sample were harvested after two weeks. mRNA was extracted from the leaves of the seedlings and the relative change in the transcript gene was normalised against the endogenous gene. All measurements are at ($p<0.05$) level of significant.

### 5.8 FER gene expression

*FER* gene expression in the two cultivars Elegance and Yellowpear grown under differential microbial communities. The expression between the cultivars was calculated using comparative threshold cycle techniques, in which the relative concentration of the transcript gene was normalised against the internal reference gene, Actin. The gene expression assay from the differential microbial communities treated plant, Elegance and Yellowpear is represented as 1 on the log 2 y-axis. Bars extending above the x-axis represent a relative increase in gene expression, and those below the x-axis indicate a decrease in transcript abundance. Figure 5.6 shows that the Elegance cultivar is downregulated under the fertilised microbial community with 0.9-fold change, and 0.2-fold change under the untreated microbial community and 0.9-fold change under the manure microbial community. Likewise, in Yellowpear cultivar is downregulated under fertilised microbial community with 0.7-fold change, and 0.8-fold change under the untreated microbial community and 0.8-fold change under the manure microbial community. The folds change within-sample under the differential microbial communities were significantly different at $p<0.05$.

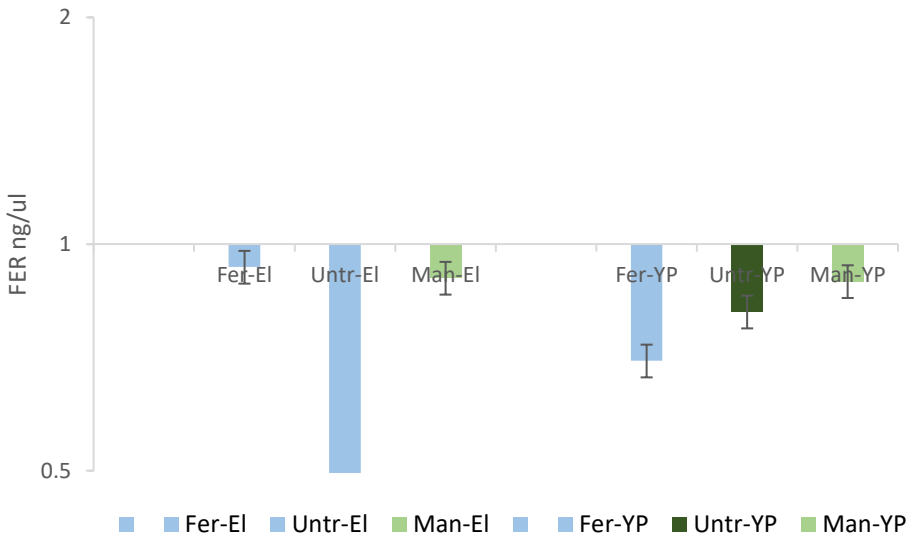


Figure 5.6 gene expression assay for FER gene in the tissues of two tomato cultivars Elegance void of VTE and Yellow pear possessing VTE. E-Ctr=Elegance control, E-Fer=Elegance Fertilised soil microbe, E-Utr=Elegance+ Untreated soil microbes, E-Mn=Elegance+manuresoilmicrobes,YCtr=Yellow+controll,YFer=Yellowpear+Fertilizesoilmicrobes, YUntr=Yellowpear+Untreated soil microbes, Y-Mn=Yellowpear+Manure soil microbes. FER gene expression in the cultivar Elegance and Yellowpear shows that the cultivar seedlings are grown in an incubator with alternative temperatures in the differential microbial community. The sample was harvested after two weeks. mRNA was extracted from the leaves of seedlings. The relative change in the transcript gene was normalised against the endogenous gene. Error bars indicate confidence interval ($p<0.05$)

## 5.9 Discussion

The results from the study demonstrated that genes regulating the release of plant phytohormone are affected when plants are grown under differential microbial communities. The findings show that expression of *Auxin ARF, LeNRT2.3, FROS2 FER, and LelRT1* genes in the two tomato varieties Elegance and Yellowpear are affected when grown in the presence of microbial communities isolated from soils under different fertiliser regimes with fold changes that vary from one cultivar to another as well as the difference in the microbial community.

The results in chapter five demonstrate a significant change in the expression of the various genes even though, chapter 3 has demonstrated that no changes in the

accumulation of the plant phytohormones recorded. The relative changes observed in chapter 5 from the different treatments are significantly different as shown in the expression of *LeNRT2.3* nitrate transporter gene, which is a gene among a cluster of nitrate genes with encoding protein found in tomatoes located at the plasma membrane and confined to tomato root hair alongside protein encoded by *LeNRT2.2* and *LeNRT2.1* (lower affinity for nitrate) (Fu, Yi et al. 2015). Previous research has demonstrated that they can be induced by external factors (Fu, Yi et al., 2015). However, no research evidence to date indicates plant growth-promoting bacterial (PGPB) have a role in inducing *LeNRT2.3* gene expression. However, there is evidence on the potential role of PGPB to stimulate root and shoot growth, which in turn can lead to an increase in nitrate uptake (Dobbelaere, Croonenborghs et al. 2002). Research by Dobbelaere et al. has demonstrated that arbuscular mycorrhiza fungi (AMF) induced the synthesis of *LeNRT2.3* transcripts (Dobbelaere, Croonenborghs, et al. 2002) thus, proving fungal-induced action on the synthesis of the plant gene *LeNRT2.3.* Here, with qPCR, we attempt to elucidate the expression of *LeNRT2.3* in tomato cultivars Elegance and YellowPear, both grown in the presence of soil microbial communities isolated from the long-term hay meadow trial at Newcastle University (Palace Leas). These soils have been continually amended with either chemical fertiliser, farmyard manure or no treatment. The result demonstrates that in the cultivar Elegance, the expression of *LeNRT2.3* was upregulated in the seedlings grown under the fertilised microbial community with an increased 2-fold change in gene expression and increased 1-fold change in the untreated microbial community, and an increased 05-fold change in the manure microbial community. In contrast the Yellowpear cultivar demonstrated that *LeNRT2.3* genes are downregulated in the fertilised microbial community with a decreased 3-fold change, untreated microbes with an increased 1-fold change, and manure microbial community with a decreased 4-fold change. In both microbial communities, the fold change in the expression of *LeNRT2.3* is significantly different. Moreover, the gene expression of the *LeNRT2.3* between the VTE cultivars and the cultivars void of the VTE are significantly different from one cultivar to the other. The results show that the differential microbial communities did influence *LeNRT2.3,* and the VTE were involved in the stimulation of *LeNRT2.3* in Yellowpear cultivar.

### Auxin gene (*ARF)*

Another important gene is the auxin gene *ARF* very crucial for plant growth and development it regulates auxin release, control bud dormancy, promotes root, and shoots elongation (Demeulenaere and Beeckman 2014). Maintaining a stable plant phytohormone level is crucial for plant health and growth, and development. Previous studies have shown evidence indicating that microbial inoculants can influence plant auxin homeostasis (Tsukanova, Meyer, et al. 2017) and its subsequent phenotypic effects. However, our initial studies in chapter 4 demonstrate that exogenous inoculation of VTE on the plant does not influence changes in the accumulation of the plant phytohormones. Chapter 5 look at whether a microbial community can effect changes in gene regulation of the plant, the plant samples cultivated under three differential microbial communities were subjected to a gene expression assay to investigate the auxin *ARF* gene expression response. The findings showed that the auxin *ARF* gene in Elegance was downregulated with a decreased 1-fold change in the fertilised microbial community, decreased 2-fold changes in the untreated microbial community, and decreased 3-fold change in the manure microbial community. Also, in yellowpear cultivar the auxin *ARF* gene expression was down-regulated in the yellowpear cultivar under a fertilised microbial community with a decreased 4-fold change and down-regulated under the manure microbial community decreased 3-fold change. The expression of the auxin *ARF* gene in both cultivars are significantly different from one other. The finding is consistent with previous studies that showed bacterial strain used as inoculant could stimulate the release of auxin in the host plant (Spaepen, Bossuyt, et al. 2014). This finding indicates that both differential microbial communities and vertically transmitted endophytes affect stimulating plant host auxin *ARF* gene.

### *FROS2* gene

*FROS2* gene is known to encode low iron inducible ferric chelate reductase responsible for reducing excess iron from the root surface (Connolly, Campbell, et al., 2003). The *FROS2* and *LeIRT1* are two major Arabidopsis high-affinity iron uptake genes and are regulated at transcriptional and posttranscriptional levels. Thus, elucidating the expression of the *FROS2* gene in the two cultivars under the differential microbial treatment would provide information on whether they can stimulate the expression of the *FROS2* gene and indirectly regulating a balance iron flow in the host plant. Consequently, understanding *FROS2* gene expression status in the two cultivars would guide how to mitigate its effect and serve as a novel bio-stimulant that can enhance iron uptake in a plant under iron stress. The current study showed that *FROS2* gene expression in the cultivar void of VTE (Elegance) under a fertilised microbial community had increased 01-fold change, and under the untreated microbial community had a fold change decreased of 1-fold change and under the manure microbial community have a decreased 4-fold change.

Similarly, in cultivar yellow pear, the *FROS2* gene expression is also downregulated with a fold change decreased of 1-fold change in the fertilised microbes samples, increased 1-fold change under the untreated microbial community, and decreased 1-fold change under the manure microbial community. Both cultivars grown in different microbial communities show a significant difference in the expression of the *FROS2* gene. Notably, there is no significant difference between the VTE cultivar and the cultivar void of VTE.

### *FER* gene

Another gene of interest is the *FER* gene, which encodes the basic helix-loop-helix domain (bHLH) Protein, a crucial protein for plant cellular activity, acting alongside other iron transcriptional factors to regulate iron uptake (Ling, Bauer, et al., 2002). The bHLH domain indicates *FER* is a putative transcription factor that controls root iron uptake by regulating the expression of root ferric chelate reductase genes *FROS* and *LeIRT1* (Connolly, Campbell, et al. 2003). Thus, we evaluate *FER* gene expression to elucidate the functional role of vertically transmitted endophytes and how the differential microbial communities influence the expression of *FER* in the two cultivars. In cultivar Elegance, *FER* is downregulated with a decreased 1-fold change

in the fertilised microbial community, a decreased 7-fold change in untreated soil microbial community, and a decreased 1-fold change in manure microbial community. It is also downregulated in yellowpear with a decreased 3-fold change in the fertilised soil microbe samples, decreased 2-fold change in the untreated soil microbes' samples, and decreased 1-fold changes in manure soil microbes. A significant difference in the FER gene expression in both cultivars is grown under the differential microbial communities.

### ***LeIRT1* gene**

The expression of the *LeIRT1* gene in the cultivar Elegance and Yellowpear demonstrated that the *LeIRT1* gene is regulated by the *FER* gene as demonstrated in (Ling, Bauer, et al. 2002). The results show that the *LeIRT1* gene in the cultivar elegance is down-regulated with a decreased 2-fold change in the fertilised microbial community, decreased 1-fold change in the manure microbial community, and decreased 6-fold change in an untreated microbial community. The *LeIRT1* gene in yellowpear cultivar under the fertilised microbial community is decreased with a 3-fold change. Under the untreated microbial community is decreased with a 1-fold change and decreased under the manure microbial community 1-fold change. The results show that there is a significant difference between the different cultivars under the differential microbial community. Findings across the treatment demonstrate that the *LeIRT1* gene has the same expression pattern as the *FER* gene except in the cultivar elegance grown under the untreated microbial community with fold change decrease across the treated plant of the two cultivars. The result consent with (Ling, Bauer, et al. 2002), which show that the knock off *FER* gene can eliminate LeIRT1 and *FOR* gene in all the plant tissue. It further demonstrates that differential microbial communities can affect changes in the expression of genes regulating the biosynthesis of phytohormones in *S. lycopersicum.* Thus, the use of different microbial communities can be regarded as suitable candidates for phytohormone stimulation. The experiment showed that the differential microbial communities, fertilised, manure and untreated microbial communities induce or suppress *Auxin, LeIRT1, FER, FROS2*, and *LeNRT2.3* genes. Furthermore, the plant genotype is a key factor in regulating plant gene expression.

## 5.10 Conclusions

The findings undoubtedly demonstrate that a community of soil microbes can affect changes in the expression of plant *Auxin, LelRT1, FER, FROS2*, and *LeNRT2.3* genes regulating the biosynthesis of phytohormones. Furthermore, it demonstrates the role of plant genotype in the expression of these genes, although these effects may not appear visibly in the phenotype of the affected plant, however, may have long term effects at the molecular levels.

## Chapter 6

### Summary of the project findings and future perspectives

To summarise the project findings in Chapter two, the focus was on identifying and characterising the isolates from the seeds of *S. lycopersicum*, the results demonstrated that all the isolated vertically transmitted endophytes (VTE) have potential of PGP traits. We recognised existing diversity within the various isolates of the VTE, displaying range of phenotypes such as browny white punctiform, whitish grey punctiform, mucoid milky white circular, and slippery pale white irregular shapes and textures. The sequences of the isolates showed that the isolated bacteria belong to two bacterial genera, i.e., *Bacillus* and *Paenibacillus,* which share ancestral linkages with each other. Further assays showed that these isolates release different types of metabolites, such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme, which inhibit excessive ethylene production by cleaving the plant exuded ACC and indole-3-acetic acid (IAA), which stimulates plant cell proliferation and induces the transcription of ACC synthesis. These assays also showed other beneficial traits such as phosphate-solubilizing on Pikovskaya media and the release of siderophores on blue CAS agar. The release of these metabolites and the plant growth-enhancing traits vary between the different isolates. Both bacillus and paenibacillus strains have demonstrate the same potential to release plant growth promoting metabolites, except in the release of siderophores and on nitrogen fixation assays where paenibacillus showed no release of siderophores and less nitrogen fixation activity by the strain. Whilst most of these strains such as Ananas 1, Ananas 2, BeauteB, BernerR 2, Matina, and Zuckert 1would be expected to have significant beneficial effects on plant growth and development as shown in figure 2.3, 2.4, 2.5, 2.6 and 2.7 having potential as plant growth promoting agents, however, others strains such as the Wild type strain, Zuckert 2, Roma and Yellowpear in figure 2.4 and figure 2.5 would be expected to have a negligible measurable effect on plant growth, health, and development.

Chapter three attempted to investigate whether the interaction of specific microbial communities can affect *S. lycopersicum* growth and health. Three different microbial communities were used in a hydroponic system, where three tanks containing water with the same nutrient concentration in each tank. From each tank one of the three

microbial communities was added, and a two-week-old *S. lycopersicum* seedling was transferred into the tank and allowed to grow for two weeks. The findings showed that *S. lycopersicum* grown under fertilised microbial communities performed significantly better than the control. We also showed that *S. lycopersicum* under manure treatment and untreated microbial communities were not significantly different from the control (sterile water mix with soluble fertiliser). These findings are consistent with field trials (Bainard, Koch, et al. 2013) where vesicular arbuscular mycorrhizal fungi were inoculated on barley, pea, and maize (Jensen 1984). The finding showed that there was no significant difference in plant growth and development, when the plants were grown in differential microbial treatments with the same nutrient supplied. This is because the potential of a given bacterial strain is only manifested under experimental conditions when nutrients are limited, or under adverse environmental conditions (Glick 2014), as demonstrated in other studies that investigating plant stress and defence against pathogens. However, it is important to recognise that PGPB needs time to grow and multiply before their efficacy can be determined, but once established, they can act as effective bioinoculants (Patel, Mistry et al. 2017). The results presented here did not demonstrate that VTE in seeds of *S. lycopersicum* stimulates *S. lycopersicum*-microbial interactions that promote growth, increase chlorophyll content and plant fresh and dried biomass. The present project also did not show that the PGPB stimulated the release of the phytohormones such as abscisic acid, indole acetic acid and salicylic acid in the *S. lycopersicum.* However, these results are consistent with previous studies that noted that these microbial communities were ineffective as PGP agents when the nutrient content present in the experimental soil is optimal (Egamberdiyeva 2007).

Further experiments to investigate whether vertically transmitted endophytes in *S. lycopersicum* stimulate the release of phytohormones resulting in improved growth was carried out using the isolate *Bb-B-1* strain in chapter 5. The strain *Bb-B-1* in chapter 3 was shown to release an increase in metabolites, as seen in figure 3.6 IAA and figure 3.5 ACC. However, our findings in chapter 4 showed that exogenous application of the isolated vertically transmitted endophyte *Bb-B-1* under optimal nutrient or deprived nutrient conditions does not have a notable increase in the parameters measured, contrary to the hypothesis that the exogenous application of vertically transmitted endophytes to the plant can confer plant growth-promoting traits.

The final experimental chapter of the project investigated the role of microbial communities on the expression of selected genes in *S. lycopersicum*. The findings showed that the different microbial communities, fertilised, manure and untreated microbial communities did induce or suppress *ARF, LelRT1, FER, FROS2,* or *LeNRT2.3* gene expression in *S. lycopersicum*. However, it failed to demonstrate that VTE in the yellowpear cultivar can confer beneficial traits on the cultivar in comparison to the Elegance cultivar void of VTE.

## 6.2 General conclusions

The project findings demonstrate that vertically transmitted endophytes (VTE) are host specific, as shown in the different tomato cultivars it also demonstrates that when fertilised with appropriate microbial communities, plant growth is enhanced compared to plants under manure microbial community or untreated microbial community. However, exogenous inoculation of *S. lycopersicum* with VTE under similar nutrient conditions demonstrate that there were not significantly different compared to the control (Elegance seedling not inoculated with VTE). In a separate experiment *S. lycopersicum* seedlings grown under different microbial communities show relative changes in gene expression significantly different changes in expression of the following genes: *ARF, LelRT1, FER, FROS2, and LeNRT2.3*. These research findings contribute towards a greater understanding of vertically transmitted endophytic bacterial genera in *S. lycopersicum*. The project has also demonstrated that these isolates vary phenotypically and release various metabolites and other plant beneficial traits. Additionally, it showed that fertilised microbial communities increase *S. lycopersicum* growth and chlorophyll content whilst demonstrating that vertically transmitted endophytes under the given experiment conditions (i.e., the same nutrient, temperature, and alternating light) did not improve *S. lycopersicum* growth and health. The present study also attempted to elucidate the role of vertically transmitted endophytes and microbial communities in regulating key genes involved in the biosynthesis of phytohormones in *S. lycopersicum*.

## Future perspectives

Future investigation is needed to understand the role of vertically transmitted endophytes within the host, focusing on specific bacterial and fungal vertically transmitted strains that have been shown to confer increased beneficial plant traits. Based on the findings of this project, we recommend that for successful research to understand the role of differential microbial communities on *S. lycopersicum.* More time is required to monitor the period needed and conditions the different microbial communities establish when introduced into a new environment.

Successful identification and integration of eco-friendly alternative biofertilizer would provide the plants with the required nutrients with minimal to zero environmental hazards. Further thorough screening of the isolated vertically transmitted endophytes is needed and, there is a need for understanding the suitable concentration and optimise the required concentration of vertically transmitted endophytes on the host plant for efficient application. Recommended experiments to selectively screen and identify suitable strains are

1. Perform specific plant growth-promoting straits test for the isolated vertically transmitted endophytes that have demonstrated PGRB potentials.

2. perform an anti-pathogen assay on specific pathogens such a *Fusarium oxysporum* an S. lycopersicum root rot pathogen

3. perform a whole-genome sequence on the library of isolated vertically transmitted endophytes that demonstrates promising plant growth-promoting traits.

4. Run a growth room experiment for the vertically transmitted endophytes inoculated S. lycopersicum

5. PCR and qPCR assay to identify the role of the selected vertically transmitted endophytes on crucial S. lycopersicum growth-related transcription factors

6. Using the discover information from the experiments mentioned above, design a definite and clear road map to achieve an eco-friendly biofertilizer that will provide the plant with all the required nutrients with no threads to the environment.

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