2014

In Vitro Mycelial Growth And Root Infection Of Loblolly Pine Seedlings By Bianchetto Truffle (Tuber Borchii)

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In vitro Mycelial Growth and Root Infection of Loblolly Pine Seedlings by Bianchetto Truffle (*Tuber borchii*)

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A thesis submitted to the graduate faculty

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department: Natural Resources and Environmental Systems

Major Professor: Dr. Omoanghe Isikhuemhen

Greensboro, North Carolina

2014

The Graduate School

North Carolina Agricultural and Technical State University

This is to certify that the Master's Thesis of

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North Carolina Agricultural and Technical State University

Greensboro, North Carolina

2014

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Biographical Sketch

Osejie F. Oriaifo was born in Kano, Nigeria in 1987. His primary education was at Federal Staff School, Benin City from 1990 to 1998. His secondary school education was obtained from 1998 to 2004 at UNIBEN Demonstration Secondary School, Benin City. He obtained his Bachelor of Science Degree in Microbiology in March, 2010 from Obafemi Awolowo University, Ile-Ife. In spring, 2012 he was admitted into the M.S. program of the School of Agriculture and Environmental Sciences, North Carolina A&T State University. His M.S. research focused on Mushrooms and Truffles under the tutelage of Dr Omoanghe Isikhuemhen. He is a member of the honor society of Gamma Sigma Delta.

Dedication

I dedicate this work to Almighty God who has given me grace and life.

Acknowledgements

I appreciate the Lord, who has given me grace and strength. I thank my advisor, Dr Omoanghe S. Isikhuemhen for his advice, guidance, teaching and leadership. I thank my thesis committee members; Dr. Charles W. Raczkowski and Dr. Antoine J. Alston, for helping me shape this work from the science to the structure. My committee members made the learning process more rewarding with their advice and input. I appreciate the MBFBL staff and students whom I have worked with, all have significantly contributed to my success thus far. I want to also thank Dr. Anike for her unflinching support and encouragement. I thank the chairman, Dr. Louis Jackai for his leadership. I also want to thank Ms. Farmer, and Ms. Dixon who were always available and willing to assist me with matters pertaining to administration.

To my friends, Dietrich Blum, kingley Ekwemalor and Emmanuel Asiamah, I say thank you for your input in this work. I want to acknowledge the love and care of my Uncle, Prince R. Okojie, who has stood by me all through my academic journey. Special thanks to my parents, siblings and my wife, Juliet, who daily encourages me to dream and strive for more.

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Abstract

Decline in *Tuber borchii* production has led to exploration of new cultivation strategies. Contemporary techniques including the use of spore slurry and root induction are both marred with low success rates, contamination, and are costly. Mycelia inoculation avoids the challenges of contemporary techniques, increases colonization rates, and increases the levels of mycorrization. In order to develop an optimized medium to culture *T. borchii* and use same for onward infection of *Pinus taeda* seedling, this research was conducted with two main intents: (1.) seeking the optimum growth conditions and concentrations of six strains of *T. borchii* on solid Modified Melin-Norkrans (MMN) media and (2.) assessing effect and mycorrization of mycelia generated *T. borchii* strains on *P. taeda* seedlings.

For growth conditions studies, the optimum pH for most strains was basic (7.5 and 9.0), incubation at 20^{O}C was optimum for most of the given strains, optimum carbohydrate source was starch in all strains and the nitrogen source preference varied. Starch and yeast extract concentration studies revealed that at 10 g/L Starch, the optimum concentration of yeast extract was 0.25 g/L for most strains and at 10 g/L of yeast extract, optimum starch concentration was strain dependent. In the *T. borchii* mycelia infection of *Pinus taeda* seedlings, morphological examination revealed positive mycorrhization. Statistical difference in shoot-root length ratio was observed at post infection sampling of three months. This was also revealed in the shoot-root weight ratio at four months sampling.

CHAPTER 1

Introduction

1.1 Overview

Current cultivation techniques have not been able to meet the rising demand of *Tuber borchii*. These current techniques; root induction and spore inoculation do not require laboratory techniques, and are fairly easy to carry out but are marred with low success rate, high cost, and high rate of contamination. Mycelia based inoculation, which this research is geared towards, will produce cheaper inoculated seedling, which will have higher success rates and *T. borchii* infection that will less likely be replaced by other ectomycorrhizae. *T. borchii*, like most other highbrow truffles grow slowly and are difficult to maintain pure culture (Iotti, Piattoni, & Zambonelli, 2012). In order to accelerate the growth of *T. borchii* axenically, there is need to seek optimum conditions and compositions for the growth media for *T. borchii*. Alternative inoculation technique, mycelia based inoculation requires further testing as it promises better rates at a cheaper cost.

Truffles are an ectomycorrhizal ascomycete characterized by the succession of three distinct phases including the vegetative (mycelia), symbiotic (ectomycorrhizal) and reproductive (sporic) stages (Saltarelli et al., 1998). With the high organoleptic properties of the truffles' fruit bodies, truffles are greatly demanded as gourmet delicacy and truffles are also of interest to those in the forestry and agronomy businesses (Pierleoni et al., 2004). Truffles are economically important costing $2300/lb for *T. magnatum, T. melanosporum* costing $800/lb, *T. indicum* costing $50/lb and *T. borchii* $200/lb (Bonito, 2009).

Tuber borchii, commonly known as Bianchetto is a member of the Puberulum group, Clade V, and Subclade V-4 (Jeandroz, Murat, Wang, Bonfante, & Tacon, 2008). *T borchii Vittad*

is found in the wild in most of Europe (Jeandroz et al., 2008; Kües & Martin, 2011). Bonito (2009) stated that attempts to cultivate European truffle species in North America and other parts of the world have not been successful. Problems associated with truffles in general includes: (a.) cost and ready availability; (b.) disease and pest; (c.) deforestation; and (d.) global warming (Hall, Yun, & Amicucci, 2003). Other causes of decline includes introduction of exotic forest species, which do not have symbiotic compatibility with edible mycorrhizae, indiscriminate harvest of truffle fruit bodies, pollution and acid rain (Giomaro, Sisti, & Zambonelli, 2005).

Tuber borchii has not been a favorite because it has been confused with the morphologically similar species which have poorer flavors such as *T. maculatum, T. foetidum, T. dryophilum* and *T. puberulum.* Another cause for the insufficient appreciation of European truffles is the invasion of Chinese variants under fraudulent means (García-Montero, Díaz, Massimo, & García-Abril, 2010). Presently, the decline of natural truffle production and increase in demand has resulted in the quest for development of technologies for fructification and conservation.

T. borchii can be found in association with both angiosperm and gymnosperm hosts including broad leave trees like Oaks, Hazel, Poplar, Linden, Chestnut and alder and coniferous species like Pine and Cedar (Bonito., 2009). Calcareous soils with pH between 7 and 8 have been found to be more supporting of this truffle's growth but they have also be found in acidic soils (Zambonelli, Lotti, Giomaro, Hall, & Stocchi, 2002).

Studies have demonstrated that *T. borchii* undergoes self-anastomosis within same isolates (by both plate-pairing and microscopic analysis) with evidence of protoplasmic flow and presence of nuclei in fusion bridges (Giovannetti, Roth-Bejerano, Zanini, & Kagan-Zur, 1994; Sbrana, Nuti, & Giovannetti, 2007). Most of the ongoing research are tied by strict confidential

agreement and are not under permit to publish/patent their findings (Hall et al., 2003). Further research needs includes efficient techniques for its culture, host inoculation, and mass production. This study was conducted with the following three main objectives: (1). to determine the best media condition- pH and temperature for in-vitro cultivation of *T. borchii*. (2). to determine the optimum nutrient requirement and concentrations for in-vitro cultivation of *T.borchii* and (3). to determine the presence and effect of mycelia based inoculation of *T. borchii* on *P. taeda* seedling. The primary goals of this research were to obtain an optimized medium and *in-vitro* condition for the mycelia generation of *T. borchii* and also the successful mycorrization of optimally generated *T. borchii* mycelia and *P. taeda* seedling.

1.2 Objectives

1. To determine the optimum pH level for the *in-vitro* cultivation of *T. borchii*.
2. To determine the optimum temperature for the *in-vitro* cultivation of *T. borchii*.
3. To determine the best carbohydrate source for the in-vitro cultivation of *T. borchii*.
4. To determine the best nitrogen source for the in-vitro cultivation of *T. borchii*.
5. To determine the optimum concentration of the best carbohydrate source for in-vitro cultivation of *T. borchii*.
6. To determine the optimum concentration of the best nitrogen source for in-vitro cultivation of *T. borchii*.
7. To determine the presence and effect of *T. borchii* mycelial based inoculation on mycorrization of *P. taeda* seedling.

1.3 Hypotheses

1. Optimum pH of MMN media will be basic; 7.0 to 8.0. *T. aestivum* has been reported to have an optimum pH of 7.51 in natural fields while T. melanosporum is found between

pH 7.0 and 8.0 (Chevalier, Gregori, Frochot, & Zambonelli, 2002; Thomas, 2012). Also, the pH of the locations where these truffles are commonly found are fairly acidic to basic (De Bellis et al., 1998).

2. The optimum incubating temperature of *T. borchii* will be 20^{O}C considering the average temperature of Italy, where T.borchii originates from.
3. We hypothesize that at optimal media condition, the preferred carbon sources will be glucose. *T. borchii* mycelia is reported to perform better in glucose and fructose as against sucrose as carbohydrate source, and of the three carbon sources, glucose containing media produced optimum for hypha growth (Saltarelli et al., 1998).
4. At optimal media condition, the preferred nitrogen source will be ammonium chloride. MMN media, which is known to isolate and sustain the growth of fungi, has ammonium chloride as its nitrogen source.
5. 10 g/L would be the optimum concentration of the preferred carbohydrate source. In MMN media composition, the amount of glucose is 10 g/L.
6. The preferred nitrogen source would be optimum at a concentration of 0.25 g/L. In MMN media, the amount of glucose added per liter is 0.25 g.
7. We hypothesize that *P. taeda* root tips will be positively colonized by *T. borchii* mycelia and the presence of mycorrization will affect the shoot-root ratio. Some mycelia strains of *T. borchii* have been reported to infect *Querscus robur* L. (Personal communication)(Iotti et al., 2012).

1.4 Justification

P. taeda L is a principal economically viable tree species in the United States (USDA newsroom, 2013(Moorhead & David Dickens, 2012). It is found in abundance in the South-Eastern U.S.A from Texas to District of Columbia (USDA Plant database). 58% percent of timber in the U.S and 15% of global timber are from southern pines (USDA newsroom, 2013). According to the USDA-NRCS database, loblolly pine is adapted to a wide range of textured soil from fine to coarse, and can survive at soil pH ranging from 4.0 to 7.0. Lowest acceptable temperature for survival of loblolly pine is -8^{O}F while acceptable precipitation is between ranges of 35 to 65. The economic importance of loblolly pines plantations can be stretched by alley cropping and truffle production (Benucci, Bonito, Falini, Bencivenga, & Donnini, 2012; Susaeta, Lal, Alavalapati, Mercer, & Carter, 2012).

T. borchii is the third most demanded truffle after *T. melanosporum* and *T. magnatum*. Current international price of *T.borchii* is about $200 with online/ virtual market rate between $19 - 20 an ounce. Unlike most Tuber species that are found in association with either angiosperms or gymnosperms, *T. borchii* can be found on both angiosperm and gymnosperm hosts including association with broad leave trees like Oaks, Hazel, Poplar, Linden, Chestnut and alder and coniferous species like Pine and Cedar (Bonito., 2009). *T. borchii* is commonly found in calcareous soils with pH between 7 and 8 but can also be found in acidic soils (Zambonelli et al., 2002). Presently, the decline of natural truffle production and increased demand for truffles has resulted in the search for new technologies for the fructification and conservation of truffles.

Truffle cultivation is done either by spores, roots induction and use of mycelial pure cultures (Iotti et al., 2012). Root induction is the oldest technique while spore inoculation is the most used and reported technique. Both the root induction and the spore inoculation techniques

are known to have low success rates, high rate of contamination by undesired species, and are costly as they require mature truffle fruit bodies or mycorrhized roots. Mycelia based inoculation avoids the challenges of the other techniques while providing trace to high performing strains, ability to preserve these strains. The challenges of mycelia based inoculation includes difficulty to culture and sustain these pure cultures of truffles (Iotti et al., 2012). Mycelia based inoculation requires further study, including the nutrients and conditions necessary for the optimum growth. There is also need to access the effect of mycelia based inoculation on the plant shoot and root ratios as well as the amount of mycorrhization. Most available works on *T.borchii* are not targeted at the cultivation while works done on the optimum nutrients and conditions are not extensive to include multiple strains and treatments.

Co-farming *T. borchii* and *P. taeda* would among numerous benefits, increase reforestation, and provide farmers with multiple sources of income while producing healthier trees. Successfully infecting *P. taeda* with mycelia generated *T.borchii* would translate to less production cost for farmers and higher colonization of roots by *T. borchii*, and higher production rates. In the *in-vitro* section of this study, six strains from diverse sources have been used and in the four parameters investigated, no single work has been as extensive to include the range of treatments designed in each parameter. With multiple strains tested and a robust treatment range, this study would yield more specific results pertaining to the preferences of pH and temperature ranges, and the preferences and concentrations of carbohydrate and nitrogen sources.

CHAPTER 2

Literature Review

2.1 Brief History and Cultivation

The word *truffle* may have been derived from the Latin term *tuber*, meaning "lump". The Latin word may have sprung other common European terms including: French *Truffe*, and Dutch *Truffel.* Prehistorically, truffles have been mentioned severally; Plutarch thought truffles resulted from lighting strikes, soil water and warmth as no obvious stem or root was found, making their origin elusive. Juvenal also assumed that thunder and rain were instrumental to their origin while Cicero regarded them as the children of the earth and Pedanius Dioscorides (40 to 90 AD) suggested that they were tuberous roots. Since truffles became more popular, availability moved from hand picking from natural fields to organized cultivation. Potential cultivators searched for means to domesticate these truffles. By 1808, the French term *trufficulture* was coined after Joseph Talon planted acorns picked from oak-truffle plantations and discovered that the truffles were found growing around the newly planted oak. Joseph talon is thus recognized as the first to cultivate truffles (Hall, Brown, & Zambonelli, 2007). This success led to wide spread cultivation of truffles in 19th century France and peaked at the end of the 19th century. By the 20th century however, there was a great decline in truffle production in France due to the French industrialization and move of farmers to urban areas. Industrialization matched with the World War caused a sharp decline in male work force due to death and handicap (Hall et al., 2007). Owing to these events, the acquired *trufficulture* skills were lost. The coming of the Second World War brought about the loss of more man power and experts. By the 19th century, truffle plantations had become none reproductive due to age, soil deteriorated, disease-forces and use of pesticides. As a result, since the 20th century, truffle production has plummeted drastically to

about 50 tons from approximately 1000 tons per year, this has further led to a sharp increase in demand and price (Hall, Zambonelli, & Wang, 2005).

Talon's inoculation method is still used today with a measure of success though the method is marred by irregularities including a harvest of mixed mycorrhizae population, transfer of pest, diseases and pathogens (Hall & Zambonelli, 2012). Other inoculation methods exists, including use of a piece of truffle of interest for inoculation, use of pieces of infected roots, truffle puree and spore inoculation are also employed today (Hall et al., 2005). All these techniques can be summarized as two main techniques; root induction and spore inoculation. Both techniques share similar disadvantages which this thesis intends to address. Mycelia based inoculation would provide tractability, increase infection, reduce contamination and be cheaper to use. *T. borchii* like other truffles grow slowly in-vitro, making it imperative to develop optimized growth medium and conditions. Achieving this will foster faster growth with thicker mycelia.

T. borchii is found in association with a wide range of host trees including oak, hazel, poplar, linden, chestnut, alder, cedar and pines (Iotti, Lancellotti, Hall, & Zambonelli, 2010). The size varies from a pea to an egg while color could be pale yellow to reddish brown. It has a smooth peridium and is usually found and collected in soils of varying pH though most common pH is basic; between 7 and 8 (Iotti et al., 2010; Zambonelli et al., 2002). *T. borchii* has also been shown to prefer natural soils to potting mix and harvest is usually from winter to spring (mid-January to late April) usually as early as four years post inoculation in pines and harvest can continue for decades, usually 30-80 years (Donnini, Baciarelli Falini, Di Massimo, Benucci, & Bencivenga, 2009; Shaw, 1995). In the US, an Idaho farmer planted Hazelnut inoculated with *T.*

borchii in 2008 and harvested a single truffle in 2012, fifty in 2013 and is currently harvesting more this season in 2014 (Personal communications)

2.2 Taxonomy and Species Description

The order Pezizales includes the following families; Ascobolaceae, Morchellaceae, Helvellaceae, Pyronemataceae, Sarcosomataceae, Pezizaceae, and Tuberaceae. Members of Tuberaceae are popular and expensive fungi. Truffles are members of the Tuberaceae family as their fruiting bodies are produced subterranean (underground or hypogenous). *Tuber*s are referred to as "true truffles". True truffles are found only in symbiotic mycorrhizae association with several varieties of deciduous trees including oak, poplar, hazel and pine (Iotti et al., 2010). The word truffle is used to refer to fruiting body produced by the fungi. They are collected year round with various species fruiting at specific seasons of the year. Those of common interest are *Tuber melanosporum* Vitt (Black perigord truffle), *Tuber magnatum* (Italian white), *Tuber aestivum* (summer or burgundy truffle), *Tuber borchii* (Bianchetto), and *Tuber brumale* Vitt (Black winter truffle). The fruit body (the truffle), which is protected from the weather slowly matures within the soil. Once mature, it gives out its unique species-dependent perfume/aroma which attracts fungivores for dispersal. If they are not harvested, after about ten days they become poisonous and rot (Jepson, 2008).

Truffles are usually harvested by trained dogs, pigs, wild boars or by humans who can either sniff or watch for columns of fungivorous flies (Jeandroz et al., 2008; Ramsbottom, 1953; Shaw, 1995; Talou, Gaset, Delmas, Kulifaj, & Montant, 1990). Of Hogs and dogs, the latter is preferred although both of them have strong sense of smell. Hogs usually eat the fruit bodies as it contains a compound similar to androstenol, the sex pheromone of boar saliva which keenly attracts the sow.

Tuber borchii closely resembles *Tuber magnatum*, which is commonly known as "white truffle". The differentiation is not helped with *T. borchii* also commonly referred to as "whitish truffle". *Tuber borchii* also has an aroma close to *Tuber magnatum*, although a bit more garlicky. *T.borchii* is spring harvested while *T. magnatum* is harvested in autumn and early winter. *T .borchii* has been wild collected in a wide range of edaphic conditions but is more pronounced in well-drained, sandy calcareous soils (Iotti et al., 2010). Natural Italian collection sites include Tuscany, Romagna and the Marche. Natural hosts include hazelnut, and oaks.

The outer surface of Bianchetto has a slightly hairy appearance, irregularly shaped, 2-3 cm in size, pubescent initially but at maturity becomes glabrous. The Gleba is firm, which is whitish initially then becomes beige briefly before maturation into reddish brown. It has a somewhat garlicky aroma, which is pleasant initially but becomes unpleasant with age. The strong garlicky taste is rich and gratifying. The ascospore of *T. borchii* is globose to subglobose, possessing 1 to 3 spores (Chen, Wang, & Liu, 2008). Peridium of *T. borchii* can measure up to 500 μm while the hyphae of the inner peridium measures 9 μm in diameter (García-Montero et al., 2010). The current taxonomic position of *T.borchii* is shown in Figure 2.1

2.3 Biology and cology of Tuber borchii

As an ectomycorrhizae; a form of fungus-root symbiosis, it is found forming a symbiotic association with the roots of plants (both angiosperms and gymnosperms) where they provide their host plant with trace minerals and protection and receive organic carbon from their hosts (Pruett, 2008). Ectomycorrhizae increases the area in contact with soil compared with non-mycorrhizal conditions (Rygiewicz & Andersen, 1994). *T. borchii* is not an obligate ectomycorrhizal fungi and can grow in axenic mycelia culture by taking advantage of their restricted saprotrophic capabilities (Soragni et al., 2001). The production of volatile organic

compounds (VOCs) appears to be the source of the flavorful aroma which is characteristic to each species. It is also suspected that the flavorful aroma of truffles is also contributed by yeast present and do this by "the independent synthesis of yeast-specific volatile constituents" (Ijah & Antai, 2003).

Mycorrhiza fungi are particularly of interest to forestry as they are involved in carbohydrate, water and mineral exchange while increasing resistance of the host plant (Alvarado, Honrubia, & Manjén, 2013). Like other ectomycorrhizae symbiotic relationships, truffles adsorb water and mineral nutrients from the soil and transfer them to the plant. In return the mycelium receives carbohydrate from its host plant. This specialization association reduces the function of the roots to merely transport, consequently, causing the root hairs to disappear (Giovannetti et al., 1994).

Like other mutualistic symbiotic filamentous fungi, truffles life cycle involves the sequence of three developmental phases; firstly, the vegetative growth phase where the mycelia develops without coming in contact with the roots. Secondly, the mycelia form ectomycorrhizae with the root (this is referred to as the symbiotic phase). The third and final phase, the development of a fruit body which contains sexual spores that are ready for dispersal (Zeppa et al., 2003). These fruit bodies possess a complex combination of several types of specialized tissues, containing the sexual spores and emit volatile sulphuric hydrocarbons (Soragni et al., 2001; Zeppa et al., 2003).

Fungal hyphae are repaired, and mycelia colonies get intertwined to form hyphal networks by a process known as, Anastomosis (connection of two structures) (Sbrana et al., 2007). In their work, Sbrana et al., 2007 showed that Self-anastomosing Isolates (SAIs) and Non-self-anastomosing Isolates (NSAIs) occurred within *Tuber borchii* species. SAIs produced larger

growth than NSAIs. “Compatibility either self- or non-self could play important role in the life circle of *Tuber borchii*, by permitting hyphal interconnection and protoplasm mingling” (Sbrana et al., 2007).

2.3.1 Cultivation and production. As other mycorrizal fungi, *T. borchii* extends the absorptive surface area of the plant root. Thus, increasing efficient uptake of nutrients and in return receives metabolites, carbohydrate and a place to live. Apart from wild cultivation, the first recorded organized cultivation of truffles was done by Joseph Talon. Around year 1810, Talon observed that when he transferred oak seedling found under Périgord black truffle producing oaks, the resulting trees also produced périgord black truffle. Though he kept this as a secret for years but his explanation of the concept later made it the main production strategy till the 1970s. This production method came with various challenges including low inoculation-success rate, transplanting pathogens and insect, and offsetting the microbial species in the plantation.

Currently, cultivation is done either by spores (gamic inoculum), mycelial pure cultures (vegetative inoculum) and colonized roots (symbiotic inoculum) (Iotti et al., 2012). Mycelia based inoculation has great advantages over other inoculation methods as its availability would not be seasonal, less prone to contamination, increased rate of infection, and may also provide uniformity of growth and development since they are from pure cultures. According to Iotti et al. (2012), mycorrhizae from pure culture colonize faster than spore inoculation, since there is less incidence of contamination during plant growth as seen in *T. borchii* forming mature mycorrhizae on *Quercus robur* L in less than a month (Iotti et al., 2012).

2.4 *T. borchii* Truffiere (Plantation)

Since the 20th century, there has been a call for nursery inoculation of truffles (Trappe, 1977). To achieve this, two major steps are required: seed stratification and seedling inoculation. Mycorrhiza fungi are particularly of interest to forestry as they are involved in carbohydrate, water and mineral exchange while increasing resistance of the host plant (Alvarado et al., 2013). *T. borchii* is known to be adapted to a wide array of soil conditions but are found in calcareous soils with pH ranging from 7-8.

The problems of establishing a truffle plantation includes availability of certified inoculated seedlings, mycorrhizae monitoring to ensure target truffle are still present in the roots and has not been overthrown by other mycorrhizae fungi. Poaching and influx of poor flavored truffles also pose big concern in the truffle industry. During a truffiere establishment, ectomycorrizal plants/seedlings with competing ectomycorrizal fungi should be avoided as they could displace target truffle (Hall et al., 2005). According to Hall et al. (2005), some possible problems associated in setting up a truffiere includes exposing inoculated seedlings to direct sunlight, soil temperatures above 40 ^{O}C, competition with other ectomycorrizal fungi, insufficient organic and inorganic requirements for truffle cultivation. In the event of contamination with a non-target ectomycorrhizae, the plant should be removed immediately and the soil should be fumigated with methyl bromide, methyl isothiocyanate or Basamid granules (Hall, Brown, & Zambonelli, 2008). In stabilizing a truffle orchard, many have mentioned the possibility of helper bacteria contributing to the dynamics and stabilization the system (Aspray, Eirian Jones, Whipps, & Bending, 2006; Citterio, Malatesta, Battistelli, Marcheggiani, & et al., 2001; Frey-Klett, Garbaye, & Tarkka, 2007).

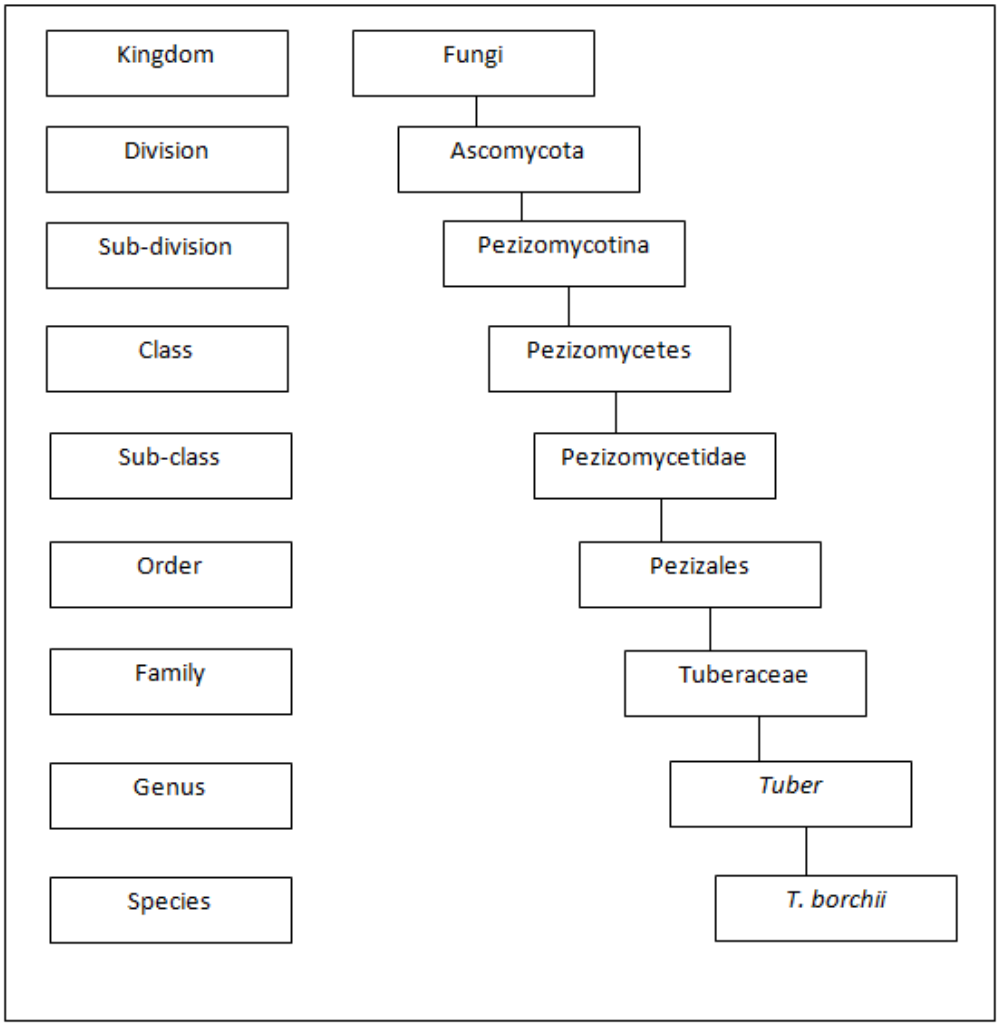


Figure 2.1. Taxonomic position of *T. borchii*

Different standards have been setup to guide the sale of truffles. Similarities exist in the 2004 United Nations publication which seems to have stemmed from the earlier versions of the 2006 French standards and the Australia-New Zealand standards for truffle (Hall & Nelson, 2008; United Nations, 2004; Vignaud, 2006). These standards dealt on collection practices, grading and packaging for sales. These standards were geared towards protecting the reputation and value of prized truffles. These standards vehemently kicked against adding a lower valued truffle to priced species.

2.5 Truffle, Inoculation and Identification

Inoculation methods include spore slurry, root induced inoculation and mycelia based inoculation. In order to grow the inoculum, a good mycorrhizal development must be obtained in the nursery after inoculation. To obtain good seedling survival and growth post planting, understanding of the temperature requirement must be known (Trappe, 1977). Guaranteed methods need to be developed to inoculate these truffles commercially.

For spore inoculation, inoculum is prepared by blending truffle in a sterile warring blender. To prevent the heat from destroying the spores, blending is done with distilled water and crushed ice for 5 minutes. The spore-slurry is then be mixed with sterile potting mix of crushed limestone, peat, vermiculite and perlite (0.5:1:2:2) and each Seedling then inoculated with 1g *Tuber borchii* fruit body (Bonito, Trappe, Donovan, & Vilgalys, 2011; Pinkas et al., 2000). Seedling cells is then filled with the potting mix and each cell will have a seedling in it through origin of seeds and container diameter (volume and depth) affects rooting quality (Lebude, 2005). On *Pinus taeda*, *Tuber indicum* was detected after five months (Bonito et al., 2011). According to Iotti et al. (2012), mycorrhizae from pure culture colonize faster than spore inoculation, since there is less incidence of contamination during plant growth as seen in *T. borchii* forming mature mycorrhizae on *Quercus robur* L in less than a month (Iotti et al., 2012).

Conventional identification- size and shape of their spores, wall ornamentation, structure of the peridium and gleba may not suffice when hypha of the mantle are developed at the area of contact with plant root (Mello, Nosenzo, Meotto, & Bonfante, 1996). Internal Transcribed Spacers (ITS) is the most popular locus for taxonomic characterization of ectomycorrhizal mycobionts (Iotti et al., 2010). The nuclear region lies between the small subunit (SSU) and the large subunit (LSU) ribosomal RNA (rRNA) genes and contains two noncoding spacer regions

separated by the 5.8S rRNA gene (Horton & Bruns, 2001). The amplification of the ITS1-4 region together with Restriction Fragment Length Polymorphism (RFLP) show a distinct pattern for *T. borchii* on *Quercus robur* against the interaction between other ectomycorrhizae and their host plants (Mello et al., 1996). Before and after planting, it is advisable to check for the presence of the mycorrhization of the *tuber borchii* and its interaction with pre-existing ectomycorrhizae (Gandeboeuf, Dupre, Chevalier, Nicolas, & Roeckel-Drevet, 1997).

2.5.1. Growth condition: pH *Tuber aestivum* syn. *T. uncinatum* requires a pH of 7.0 to 8.0 or higher but in peat based medium, the optimum pH for is between 6.7 to 7.5 (Chevalier et al., 2002; Pruett, Bruhn, & Mihail, 2009). In a study including natural and artificial *Tuber melanosporum* fields, the average soil pH was 8.0. Most truffles require calcareous soils with pH between 7 and 8 with the exception of *T. borchii,* which can be found in acidic soils (Mello, Murat, & Bonfante, 2006). *T. borchii*, has been cultured at a range from 6.5 to 6.6 (Ceccaroli, Saltarelli, Cesari, Zambonelli, & Stocchi, 2001; Pierleoni et al., 2001; Saltarelli et al., 1999). *T. aestivum* has an optimum pH of 6.7 and 7.5 in peat based potting-mix and at a pH of 7.51 in natural fields (Thomas, 2012). *T. melanosporum* is found between pH 7.0 to 8.0 (Chevalier et al., 2002). The pH of the locations where these truffles are commonly found, which range from fairly acidic in some regions but mainly basic soil (De Bellis et al., 1998).

2.5.2 Growth condition: temperature Truffles can withstand extreme temperatures but the Mediterranean climate seems ideal (Bonet et al., 2009). In controlled conditions, optimized rhizosphere temperature for *T. melanosporum* is between 20-25 ^{O}C while in-vitro, 25 ^{O}C is optimal (Bustan, Ventura, Kagan-Zur, & Roth-Bejerano, 2006). Optimum temperature of *T. melanosporum* is about 20 ^{O}C with decline at 26.5 ^{O}C (Michaels, 1982). In the production of *T. olbiensis*, the fermentor is kept at 23 ^{O}C (Morte, Honrubia, & Gutiérrez, 2008). Minimum

temperature for fungi is between 2-5 ^{O}C, with maximum temperature of 35-40 ^{O}C and optimum within the range of 22-27 ^{O}C (Ingold & Hudson, 1993). Also, *in-vitro*, the optimum pH for fungi is between 5-6.5, with only a few developing below 3 and above 9 (Ingold & Hudson, 1993).

2.5.3. Carbohydrate source Simple monosaccharaides; glucose and fructose have been observed to be preferred to sucrose by *T. borchii* while another report showed that mannose was preferred to mannitol and glucose (Ceccaroli et al., 2001; Saltarelli et al., 2003). In another work, glucose was preferred to sucrose and maltose which produced thinner and less branched hyphae. It is also reported by Saltarelli et al. (2003) that high glucose concentration inhibits mycelia growth of *T.borchii*, and high concentration of fructose or mannitol do not influence growth of *T. borchii*. A high sucrose concentration (80 g/ L) is unsuitable for polysaccharide accumulation in *T. melanosporum* (Liu et al., 2009). Sucrose is utilized by *Hymenoscyphus ericae*, an ectomycorrhizae though same carbohydrate source supports *T. borchii* poorly, if at all (Hughes & Mitchell, 1995; Saltarelli et al., 1998). Hughes and Mitchell (1995) showed that a 1:1 combination of glucose and fructose doubled the mycelial growth as against when grown solely than in either. *T. melanosporum* growth is supported by sucrose and mannose (Mamoun & Olivier, 1991).

Mannose was preferred to mannitol and glucose in *T. borchii* culture media (Ceccaroli et al., 2001). Mannitol has also been reported to be present in high quantities after high exposure of *T. borchii* to glucose suggesting that mannitol may be a storage carbon form for *T. borchii* as formation is via a direct channel after absorbing glucose (Ceccaroli et al., 2003). In another report, *T. borchii* mycelia performed better in glucose and fructose as against sucrose as carbohydrate source, and of the three sources, glucose containing media produced healthiest hypha (Saltarelli et al., 1998). According to Nehls (2004), glucose, fructose and mannose confer

best growth to ectomycorrhizae fungi. Though high glucose concentrations inhibit *T. borchii* growth and that *T. borchii* exhibits "intraspecific variability", slight variations in optimum conditions is expected within rational limits (Saltarelli et al., 1999; Saltarelli et al., 2003). Glucose and fructose are known to be the major sources of carbonhydrate supporting mycelia growth of most ectomycorrhizae though ectomycorrhizae fungi are also known to absorb and convert glucose and fructose from their host into mannitol, glycogen and trehalose.

2.5.4. Nitrogen source *T. melanosporum* growth was stimulated more by ammonium than nitrate though the combination of both yielded the best result (Mamoun & Olivier, 1991). Ammonium and nitrate are usually preferred by ectomycorrhizae (France & Reid, 1983). Organic nitrogen sources, yeast extract followed by peptone were preferred nitrogen source for the Chinese truffle, *T. sinense* (Liu, Li, Li, & Tang, 2008). Soils supporting the growth of *T. uncinatum* have a C/N ratio of 18-20 (Chevalier & Frochot, 1990). For liquid fermentation, introduction of nitrogen thrice is optimal for *T. melanosporum* (Liu et al., 2009). It has been noted that increase in soil nitrogen also changes the diversity and dynamics of ectomycorrhizal communities (Avis, McLaughlin, Dentinger, & Reich, 2003). *Cantharellus cibarius*, a basidiomycetes has been shown to selectively prefer ammonium to nitrate and bovine serum albumin (Rangel-Castro, Danell, & Taylor, 2002). According to Gobert and Plassard (2008),selection of ammonium over nitrate is a common phenomenon. In another report where ammonium sulphate (0.28 g l^{-1}), calcium nitrate (0.5 g l^{-1}) and bovine serum albumen (0.375 g l^{-1}) were compared, ammonium support growth of all ten species (Finlay, Frostegard, & Sonnerfeldt, 1992).

2.6 *Pinus taeda* Seed, Seedling and Stratification

Pinus taeda, Loblolly pine, was prehistorically a minor species in the south, with those growing in swamps surviving fires and pure *P. taeda* stands were established only after the fire control efforts of 1900s (Schultz, 1997). Loblolly pine also has the ability to grow rapidly, reproduce in large quantities and provide many market-ready produce at an early age. Loblolly pines have been found performing well on diverse sites, and reaching maturity in 80 years with an average life span of 300 years (Schultz, 1997). *Pinus taeda* is the principal economically viable tree species in the United States, (Moorhead and Dicksens, 2012; USDA newsroom, 2013). According to the plant database, *P. taeda* is found in abundance in the South-Eastern U.S.A from Texas to District of Columbia. 58% percent of timber in the U.S and 15% of global timber are from southern pines (USDA newsroom, 2013). Economically, loblolly pines plantations can be made more viable by alley cropping and truffle production (Truffiere) (Benucci et al., 2012; Susaeta et al., 2012). Mycorrhizal formation of *tuber borchii* on *Pinus taeda*, like other mycorrhizal association, would also likely improve the mineral adsorption, drought resistance and pathogen defense of *P. taeda* (Mitchell, 1993).

The embryo's ability to mobilize seed storage protein, which is a biochemical marker of early seedling growth is not affected by moist chilling (Cooke, Cooke, & Gifford, 2002). Moist chilling only minimally affects gene expression in the embryo or germinant and also alleviates some factors in the seed coat that significantly inhibits germination. Seed dormancy is a key limitation to immediate and synchronous germination, early seedling growth and of all the southern pine species, loblolly pine produces the most dormant seeds (Barnett, 1996; Bonner, 1991). Thus, overcoming seed dormancy is an important component of efficient and cost-

effective seedling production. Loblolly pines and other conifers are usually stratified by moist chilling at 1 to 5 ^{O}C for at least 35 days (Cooke et al., 2002).

It is generally accepted that the hard, thick loblolly pine seed coat mechanically restrains swelling of the embryo and mega gametophyte, and thus restricting water uptake by these tissues. There is circumstantial evidence that moist chilling-induced changes in the embryo at the cellular level may contribute to dormancy breaking and germination in loblolly pine.

2.7 Percentage Success

In a report by Alvarado et al. (2013), two years old *Quercus ilex* infected with *T. melanosporum*, above 40 percent mycorrhization is expected with the lowest acceptable threshold being 30 percent. 30 percent mycorrization is acceptable although an increased percentage would in turn encourage persistence of the truffle in the field (Iotti et al., 2012). According to Morte, Andrino, Honrubia, and Navarro-Ródenas (2012), sampling roots of 12 seedling in a lot of 1,000 and achieving 33 percent is a good success.

CHAPTER 3

Materials and Methods

3.1 *Tuber borchii* Strain History and Isolation

Six strains of *T. borchii* isolates were obtained from the Mushroom Biology and Fungal Biotechnology Laboratory (MBFBL), North Carolina A&T State University. The MBFBL IDs were MBFBL 1320, 1321, 1322, 1323, 1324 and 1325. MBFBL 1320 and 1321 were isolated from fruit bodies, MBFBL 1322 1323 and 1324 were derived from plate cultures (obtained from Bologna University, Italy) and MBFBL1325 was obtained from ATCC (America Type Culture Collection) in Rockville, MD, USA. Isolation from fruiting bodies was done by excising <1mm of the gleba, preferable vein (Iotti et al., 2012). In a bid to rid the cultures and isolates of contaminants, inoculation was done on four sets of MMN media; MMN alone; MMN and benomyl; MMN, benomyl, chlortetracycline and chloramphenicol. The composition of MMN was 10 g glucose, 0.25 g NH_4Cl, 0.025 g NaCl, 0.5 g KH_2PO_4, 0.05 g $CaCl_2$, 0.15 g $MgSO_4$.7 H_2O, 0.012g $FeCl_3$. 6 H_2O, 0.001 g thiamine (filter sterilized after autoclaving), 15 g agar, per 1L distilled water.

After the MMN media is prepared and autoclaved for 15 minutes at 121^{O}C. On cooling to about 55^{O}C, thiamine chloride was introduced using a sterile filter. For MMN media containing antibacterial and/or antifungal agents, they were also added to the warm media by sterile filter. Then, the media was then poured into 100 X 15mm Petri dishes (8.5mm in diameter) under the. laminar flow hood. On cooling and setting, inoculation was done using 4mm cork borer and incubation was done at 25 ^{O}C. All plates were observed daily and when pure cultures were seen growing from both the treated and untreated plates, subcultures were made immediately unto 100 X 15mm Petri dishes. These subcultures were then monitored weekly to ascertain the time length

it took the fastest growing inoculum to fully colonize a plate. A 35 day period incubation was hence selected.

3.2 Mycelia Growth

After the first pilot experiment was conducted, it was derived that the fastest growing strains colonized 100 X 15mm Petri dishes in five weeks while most were fully colonized in six-seven weeks on average. Thus, each study was conducted for five weeks and mycelia growth was measured weekly. To take a reading, the reverse of each plate was divided into two axis in the form of a "+". The vertical axis was labeled 'A', while the horizontal labeled 'B'. At weekly intervals, the growth was measured using mycelia length on each axis and the average taken (Miles & Chang, 2004; Weitz, Ballard, Campbell, & Killham, 2001). Though data was collated on weekly basis, only data from the final reading at day 35 is shown in this work.

3.2.1 pH experiment. For identification of optimal pH, the six given *T. borchii* strains were grown in a range of eleven treatment values i.e. from pH of 4.0 to 9.0 at 0.5 increments. The six given strains had five replicates each. MMN media was prepared without inclusion of agar. The pH was then adjusted 0.2 above the desired treatment pH by adding drops of 2M Calcium carbonate, CaCO3 while the media was stirring. The 0.2 adjustment was done as it was observed that after pH adjustment, inclusion of agar, heating and autoclaving, the pH level reduced by 0.2. Inoculation was then carried out from actively growing mycelia using 4mm cork borers. After inoculation, plates were sealed and labeled accordingly and incubation was done at 25 ^{O}C. The inoculated plates were read weekly for mycelia growth. After 35 days of incubation, mycelia growth was taken. With 11 treatments, 6 strains and 5 replicates, a total of 330 petri dishes were used per run for the pH studies.

3.2.2 Temperature experiment. Five incubating temperature treatments were used; 15 ^{O}C, 20 ^{O}C, 25 ^{O}C, 30 ^{O}C and 35 ^{O}C. MMN media was prepared and the pH was altered to 8.0 as the pH studies showed that MMN media at a pH level of 8.0 supported the mycelia growth of all strains without statistical difference from their individual optimum pH value. Inoculation was then done from actively growing mycelia using sterile 4 mm cork borer from the six given strains and five replications were made per strain. The plates were randomized and incubated at different incubators depending on the temperature treatment. The inoculated plates were read weekly for mycelia growth. With 5 treatments, 6 strains and 5 replicates, a total of 150 petri dishes were used per run for the temperature studies.

3.2.3 Carbohydrate studies. To identify the best carbon source used by *T. borchii*, nine carbohydrate sources were used including four monosaccharides (Glucose, Fructose, Xylose and Mannose), one disaccharide (Sucrose), one polysaccharide (soluble starch) and three sugar alcohols (Sorbitol, Mannitol and Glycerol). In the MMN media for the carbohydrate studies, 10 g of the treatment sugar replaced the conventional 10 g of glucose. The pH of the media was adjusted to 8.0 and after inoculating from actively growing mycelia using 4 mm cork borer, incubation was done at 20 ^{O}C. The inoculated plates were read weekly for mycelia growth. With 9 treatments, 6 strains and 5 replicates, a total of 270 petri dishes were used per run for the carbohydrate studies.

3.2.4 Nitrogen studies. To determine the preferred nitrogen source, five nitrogen sources including yeast extract, peptone, corn steep liquor, ammonium chloride and ammonium nitrate. The MMN media was prepared using soluble starch as carbohydrate source, and the pH was adjusted to 8.0. In preparing this treatment, the 0.25 g of Ammonium chloride (NH_4Cl) in MMN media was replaced with equal amount of the treatments; nitrogen sources. After inoculating

from actively growing mycelia using 4 mm cork borer, incubation was done at 20 ^{O}C. The inoculated plates were read weekly for mycelia growth. With 5 treatments, 6 strains and 5 replicates, a total of 150 petri dishes were used per run for the nitrogen studies.

3.2.5 Nitrogen (yeast extract) concentration. In determining the appropriate amount of nitrogen (yeast extract) in MMN media required by *T. borchii*, the amount of yeast extract per liter of MMN was varied i.e. 0.25, 0.5,1,5,10,15,20,25,30,35,40 g/L. MMN media was prepared using 10 g of starch and the pH was adjusted to 8.0. After inoculating from actively growing *T. borchii* mycelia using sterile 4 mm cork borer, incubation was done at 20 ^{O}C. The inoculated plates were read weekly for mycelia growth. With 11 yeast extract concentrations, 6 strains and 5 replicates, a total of 330 petri dishes were used per run for the nitrogen concentration studies.

3.2.6 Carbohydrate (starch) concentration. In determining the suitable quantity of carbohydrate (starch) in MMN media required by *T. borchii*, the amount of starch per liter of MMN was varied i.e. 5, 10, 15, 20, 25, and 30 g/L. MMN media was prepared using 10 g of yeast extract and the pH was adjusted to 8.0. After inoculating from actively growing *T. borchii* mycelia using sterile 4 mm cork borer, incubation was done at 20 ^{O}C. The inoculated plates were read weekly for mycelia growth. With 8 starch concentration treatments, 6 strains and 5 replicates, a total of 240 petri dishes were used per run for the carbohydrate concentration studies.

3.3 Loblolly Pine, *Pinus taeda* Infection by Bianchetto Truffle, *Tuber borchii* Mycelia

3.3.1 Seed stratification and germination. The seeds of *Pinus taeda* were surface sterilized in a solution of 0.005% (V/V) tween 20 with agitation for 30 minutes. The tween 20 was rinsed off the seeds and then surface sterilized for 10 minutes in 1% (V/V) hypochlorite, and rinsed thoroughly the second time with deionized water. The seeds were then subjected to 35

days stratification between layers of sterile moist paper towels placed in petri dishes. The petri dishes were wrapped with aluminum foil and kept to stratify at 2 ^{O}C for 35 days. Moist chilling, imbibition, stratification is done to break seed dormancy. Germination was carried out in the growth chambers at 30 ^{O}C under constant light of 19 $\mu mol^{-2}s^{-1}$ (Cooke et al., 2002) as seen in figure 2. The seed were spread evenly and the paper towel was kept moist.

Figure 3.1 Germinating seedling after stratification

3.3.2 Potting mix. After a series of potting mix were studied (data not shown) to determine a suitable medium capable of holding pH levels. The potting mix selected consisted of Lime: Peat: Sand: Vermiculite: Perlite in the ratio of 0.055:1:1:2:2. 350 g of potting mix was introduced into Microbox® micropropagation containers (transparent polypropylene containers with covers having filters). The potting mix contained in the Microbox® were then autoclaved twice; first time, for 15 minutes at 121 ^{O}C and then, for 1 hour at 121 ^{O}C. The Microbox® was then transferred to the laminar flow hood.

3.3.3 Mycelia infection of *T. borchii* on *P. taeda*. Two fully *T borchii* colonized 8.5 mm agar plates were cut into pieces under the laminar flow hood using sterile a blade. The pieces

were then introduced into the Microbox® containing the potting mix. The Microbox® was then thoroughly shaken to evenly spread the inoculum. Seedlings were then carefully planted allowing about 2 inches between seedlings. After the inoculation and planting, the Microbox® was covered and sealed with parafilm wax. Each Microbox® was then separated in autoclave bags and heat-sealed individually as seen in figure 3. The Microbox® were kept at room temperature for two weeks and then transferred to the greenhouse. After two months of close observation in the greenhouse, the autoclave bags were unsealed, the lids taken off, the plants watered and 250 mls of water was introduced between the autoclave bag and the Microbox® to improve humidity. Sampling was done at three months and four months post cultivation.

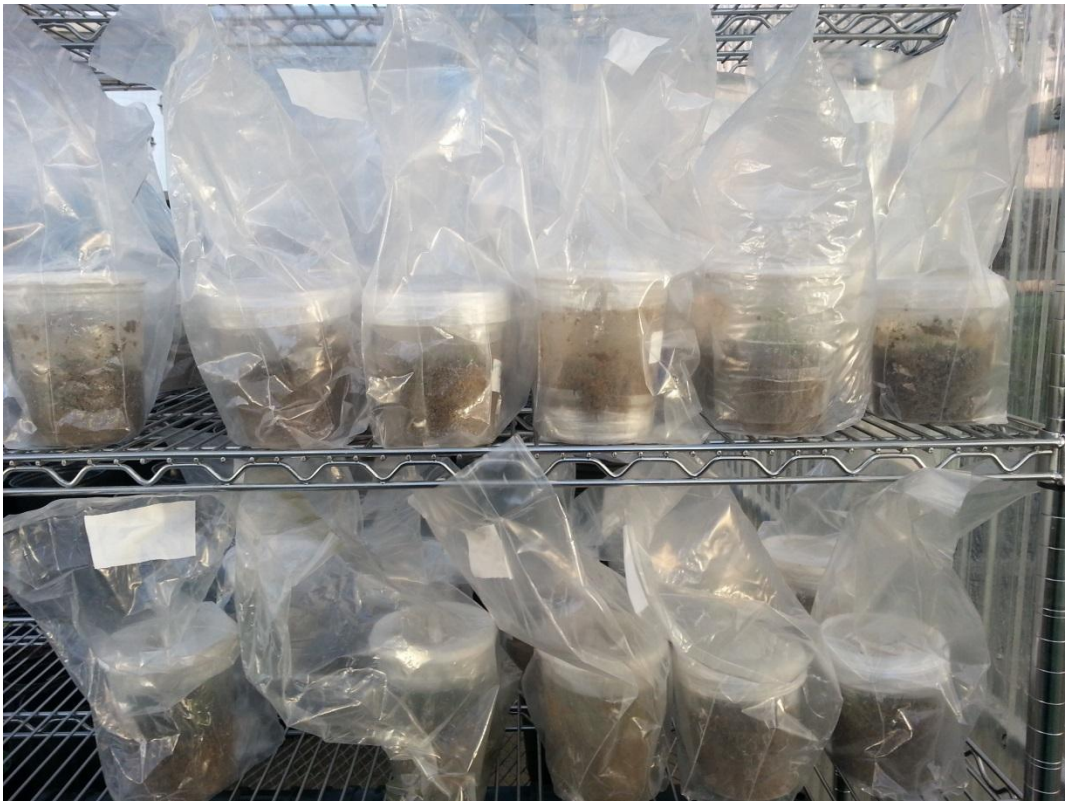


Figure 3.2. Infected pine seedlings in Microbox®

3.3.4 Morphological examination and characterization. To ascertain if *T. borchii* persisted as ectomycorrhizas on roots of seedlings inoculated with *T. borchii*, root samples were

taken. The Microbox® was gently shaken to detach the root system from the potting mix. The detached root systems were put into petri-dishes containing water to loosen attached soil debris. Morphological examination was done under a stereomicroscope, total number of root tips was counted and then the number of mycorrhized root tips was also counted as seen in figure 4. After morphological characterization, the seedling was cut into two; above ground and below ground and measurements were taken. The parameters measured included; shoot weight, root weight, shoot height and root height were measured from the above ground and below ground sections.

Figure 3.3 Roots of Loblolly pines being observed under the stereomicroscope

3.4 Experimental Design and Statistical Analysis

All statistical interpretations were made at the 5% level of significance. All experiments were conducted using a completely randomized design. Inoculated Petri dishes of each treatment were completely randomized before placing them in the incubator while in the greenhouse studies, all Microbox® were also completely randomized. The entire study was conducted is a

step wise manner with the pH study completed first, then the temperature study was conducted using the results of the pH study and this included altering the pH level of MMN to the preferred level. With the completion of both pH and Temperature studies, the optimum pH and temperature requirements were used to obtain the preferred carbohydrate source. With the completion of the carbohydrate source studies, the preferred and cheapest source of carbohydrate, starch was used to conduct the nitrogen study. Starch of the same quantity, 10 g, replaced glucose in MMN media composition and the derived media were then used with the optimum conditions to obtain the optimum nitrogen source. On obtaining the optimum nitrogen source, a new media composition was generated, further modifying the MMN media. The new media composed of starch as carbohydrate source and yeast extract as nitrogen source. Optimum starch and yeast extract concentrations were then evaluated. The nitrogen level was kept constant at 10g/L while the carbohydrate level was varied. After results were obtained, starch was kept at 10 g/l while the concentration of yeast extract was varied. With the optimized media condition and nutrient requirements obtained, it was then used to generate *Tuber borchii* mycelia for infecting *Pinus taeda*.

Regression analysis was conducted to model the function of pH and temperature on mycelia growth. For nutrient requirements, statistical analysis were performed using SAS 9.2 (32) (English) software for analysis of general linear model (GLM) procedures and paired comparisons were performed using Duncan's multiple range test (Institute, 1990). GLM procedure was used when testing between groups as data points per treatment were not equal as contamination of some replicates led to losses. For uniformity, all data were analyzed and reported with GLM. Duncan's multiple range tests were used to determine significant differences between treatment means.

CHAPTER 4

Results and Discussion

4.1 Mycelia Growth

After five weeks of incubation, radial mycelia growth was used to measure the effect of each treatment (Fenn & Coffey, 1984) in all *in-vitro* studies; pH, temperature, carbohydrate, and nitrogen studies.

Table 4.1

Analysis of Variance (P>F) for Mycelia Growth for In-vitro Study

Study	Source of variation[1] /P>F			CV, %
pH	S/0.0001	p/0.0001	S*p/0.0001	16.1
Temperature	S/0.0001	T/0.0001	S*T/0.0001	24.2
Carbohydrate	S/0.0002	C/0.0001	S*C/0.4068	29.6
Nitrogen	S/0.0001	N/0.0001	S*N/0.0001	11.4

[1]Sources of variation: pH study: S= strain; p=pH; S*p= strain*pH; temperature study: S= strain; T=Temperature; S*T= strain*temperature; carbohydrate study: S= strain; C=carbohydrate; S*C=strain*carbohydrate; nitrogen study: S= strain; N=nitrogen, S*N= strain*nitrogen.
CV= coefficient of variation

ANOVA test was performed to determine if there was interaction between the given *Tuber borchii* strains and each of the *in-vitro* treatments (pH, temperature, carbohydrate and nitrogen). As shown in Table 1, there was interaction between the given strain and all treatments except for carbohydrate. Also, the table shows that the strain effect and all the treatment effect were highly significant.

4.2 pH Studies

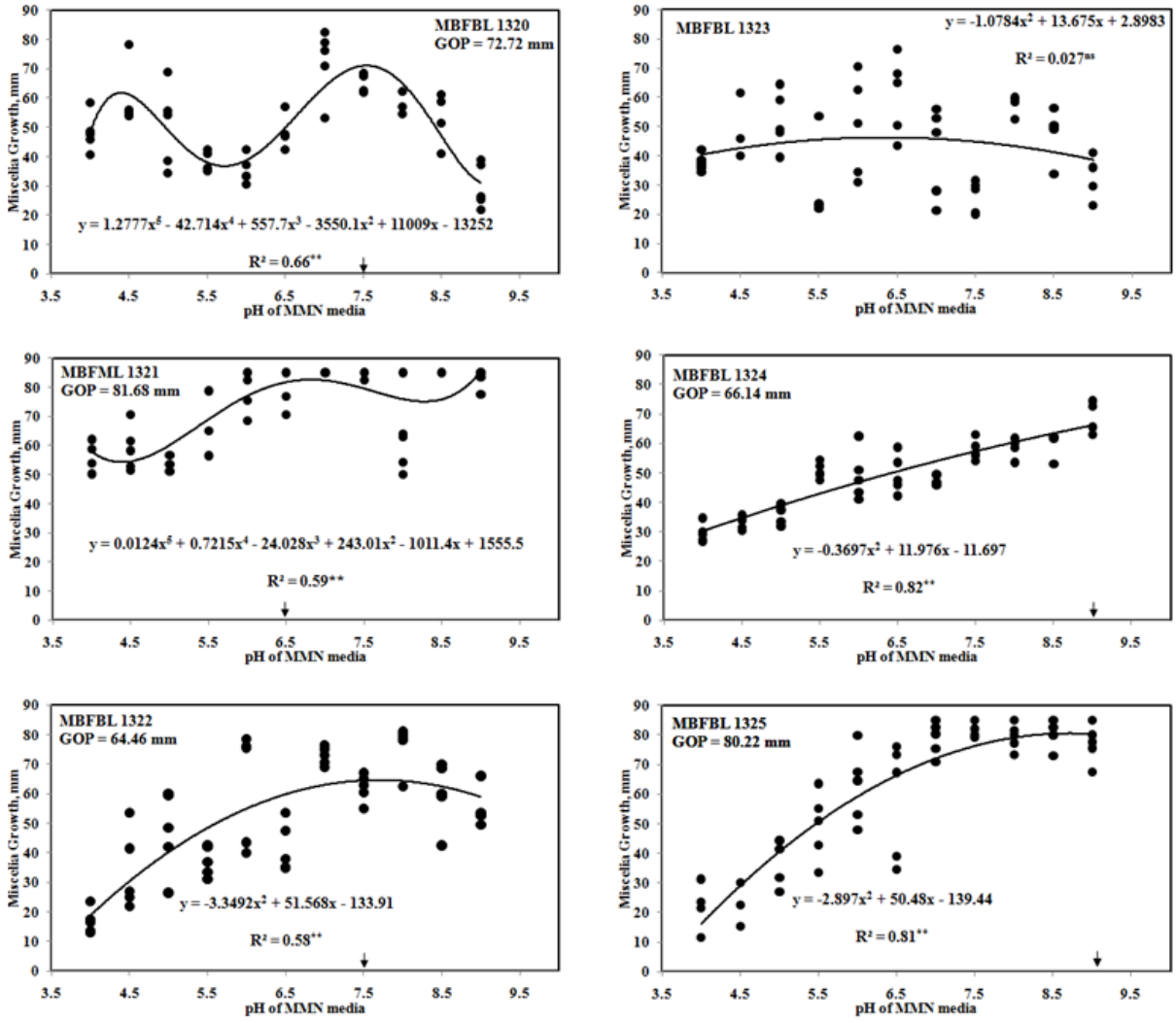


Figure 4.1 Mycelia growth, in millimeters on a polynomial regression with pH of Modified Melin-Norkrans media

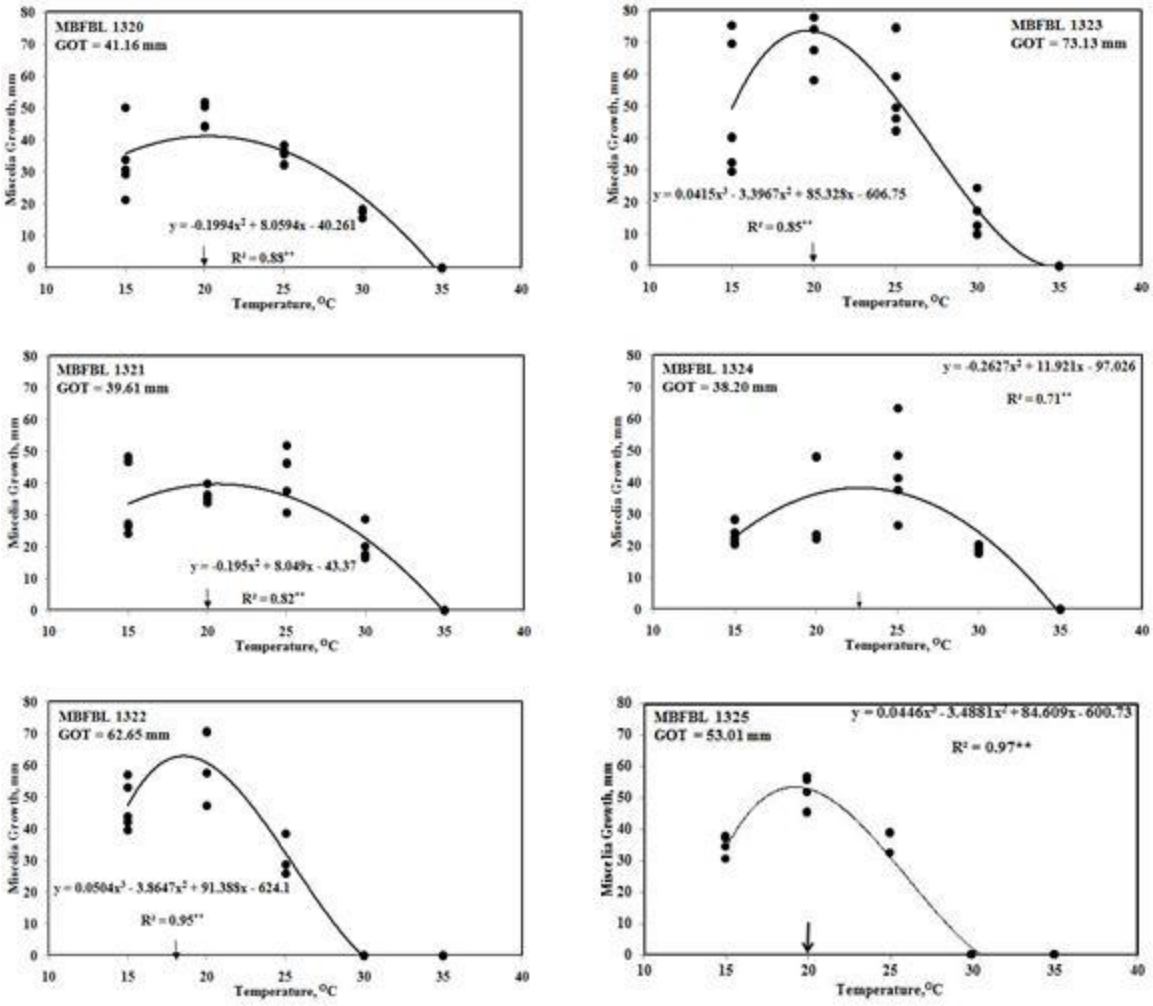
In order to investigate the effect of initial pH on mycelial growth, *T borchii* was cultivated in MMN media with different initial pH values (pH 4.0- pH 9.0 with 0.5 increments). According to fig1.1, the pH effect for all strains was highly significant except for MBFBL 1323.The optimal pH for mycelial growth was 7.5 for MBFBL 1320, and 1322 while it was 9.0 for MBFBL 1324 and 1325. Results show that regardless of the pH between 4.0 and 9.0, the mycelia growth of MBFBL 1323 was the same. Fig 1.1 also shows a steady progression for

MBFBL 1224 and 1325, which both peak at pH of 9.0. MBFBL 1322 steadily increased, peaking at pH level of 7.5 and steadily decreases.

The results obtained were similar to those for Pezizales, which are found in neutral to basic soils (Bonito, Smith, Brenneman, & Vilgalys, 2012). Iotti et al. (2012) suggested a pH of 8.0 was appropriate when preparing potting mixture for truffles. *T. melanosporum* is also favored by moderately basic pH (García-Montero, Díaz, Martín-Fernández, & Casermeiro, 2008; García-Montero, Quintana, Valverde-Asenjo, & Díaz, 2009). With French truffiere producing *T. melanosporum* having soil pH of between 7.7 and 8.35 while those of Italy have a pH level of 7.05 to 8.25 (Chevalier & Sourzat, 2012), probably explains the affinity of most of the given strains to pH of 7.5 and 9.0. *Tuber sinense* have also been found in broad pH range of 5.5 to 8.5 (Wang, 2012). *Terfezia*, a member of the *Pezizales* is found in soils ranging from pH level of 6.58 to 8.7 (Morte et al., 2012). Soils supporting truffles range from are mostly alkaline, from 7.5 to 8.0 (Benucci et al., 2012). Tuber borchii is found in calcareous soils having pH between 7 and 8, but are also present in acidic soils (Iotti et al., 2010).

4.3 Temperature Studies

According to fig 1.2, the temperature effect for all strains is highly significant. The optimum temperature for mycelial growth was 20 ^{O}C for MBFBL 1320, 1321, 1323 and 1325 while the optimum for MBFBL 1322 and 1324 was 17.5 and 22.5 ^{O}C respectively. For all strains, no growth was recorded at 35 ^{O}C while at 30 ^{O}C, only MBFBL 1322 and 1325 showed no growth.



*Arrows pointing to temperature of maximum mycelia growth: GOT; growth at optimum temperature

Figure 4.2 Mycelia growth, in millimeters on a polynomial regression with temperature of incubation, in degree Celsius (^{O}C)

After 9 years post cultivation in New Zealand, *T. melanosporum* was found on warm sites (Hall & Haslam, 2012). Mean daily temperature for truffle producing areas in the summer months are 15.3 to 25.5 (Hall, Frith, & Haslam, 2008). *Terfezia*, a member of the *Pezizales* has been prescribed to culture at a temperature between 16 and 26 ^{O}C (Morte et al., 2012).

4.4 Carbohydrate Studies

Table 4.2

Mycelia Growth from Nine Carbohydrate Sources on Six Strains of Tuber borchii

Carbohydrate source	Strains						Average[†]
	1320	1321	1322	1323	1324	1325	
	----------mm----------						
Fructose	47.4	37.8	50.6	40.1	33.8	45.2	42.7[b]
Sorbitol	49.3	47.6	51.6	37.9	38.9	53.5	46.3[b]
Mannitol	50.8	43.2	44.3	51.8	47.3	33.3	45.3[b]
Sucrose	42.9	53.3	52.3	46.7	44.9	50.3	48.4[b]
Glucose	51.9	47.3	45.6	48.2	30.3	46.4	45.5[b]
Starch	68.8	65.4	72.4	67.8	57.6	68.3	67.7[a]
Mannose	56.8	51.9	49.7	32.9	42.2	55.9	49.4[b]
Glycerol	46.8	44.3	54.9	50.5	52.9	55.0	50.3[b]
Xylose	0.00	0.00	30.2	0.00	0.00	0.00	05.0[c]
Average[†]	45.7[ab]	43.4[b]	50.2[a]	39.7[bc]	36.0[c]	42.7[b]	

[†]Within individual averages, means with a letter in common are not significantly different at 5% level of significance as indicated by Duncan's multiple range test for mycelia growth.

To find a suitable carbohydrate source for *T. borchii* mycelial growth, various carbohydrate sources were provided at a concentration of 10 g/L in MMN media. According to Table 1, there was no interaction between strain and carbohydrate source hence Table 2 reports the strain effect and the carbohydrate effect separately. As shown in Table 1, among the carbohydrate sources tested, the highest mycelial growth for all strains was obtained in starch

medium. The starch containing medium had the highest mean of 67.7 mm and was statistically different from all other tested carbohydrate sources.

4.5 Nitrogen Studies

To investigate the effect of nitrogen sources on mycelial growth, *T. borchii* strains were cultivated in media containing various nitrogen sources, where each nitrogen source was added at concentration of 0.25 g/L.

Table 4.3

Mycelia Growth from Five Nitrogen Sources on Six Strains of Tuber borchii

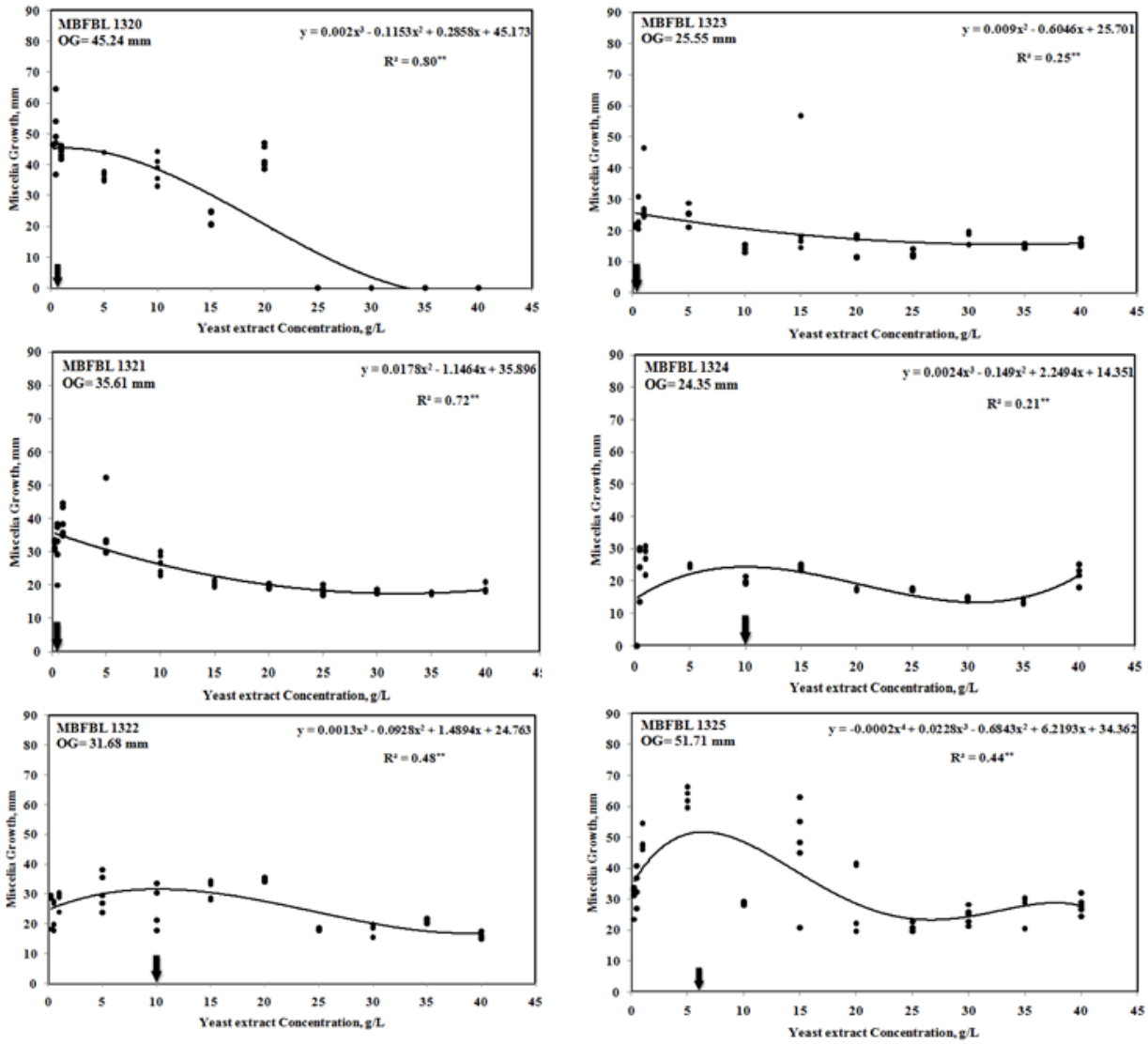
Nitrogen source	Strains					
	1320	1321	1322	1323	1324	1325
	----------mm[†]--------					
Yeast extract	43.1[a]	62.0[a]	60.4[a]	42.2[a]	44.7[a]	45.6[a]
Corn steep liquor	45.1[a]	61.0[a]	46.1[b]	46.5[a]	51.5[a]	53.8[a]
Ammonium chloride	42.2[a]	54.7[a]	59.9[a]	41.8[a]	29.5[c]	39.1[b]
Ammonium nitrate	43.8[a]	38.7[b]	34.1[c]	43.0[a]	45.9[a]	41.7[b]
Peptone	41.3[a]	56.4[a]	44.2[b]	40.9[a]	39.0[b]	42.6[b]

[†]Within each strain, means with a letter in common are not significantly different at 5% level of significance as indicated by Duncan's multiple range test for mycelia growth.

According to Table 1, there was highly significant interaction between strain and nitrogen source, hence Table 3 reports the strain effect and the nitrogen effect. As shown in table 3, all nitrogen treatments effect was the same for MBFBL 1320, and 1323 with no statistical difference between the treatments. For MBFBL 1321, all treatments but ammonium nitrate was preferred. For MBFBL 1322, yeast extract, and ammonium chloride had the highest mean and thus were preferred. MBFBL 1324 preferred yeast extract, corn steep liquor and ammonium nitrate while

MBFBL 1325 preferred yeast extract and corn steep liquor. Though each *Tuber borchii* strain had specific nitrogen preference, yeast extract was the only nitrogen source present as optimum for all strains.

4.6 Nitrogen (Yeast Extract) Concentration Studies

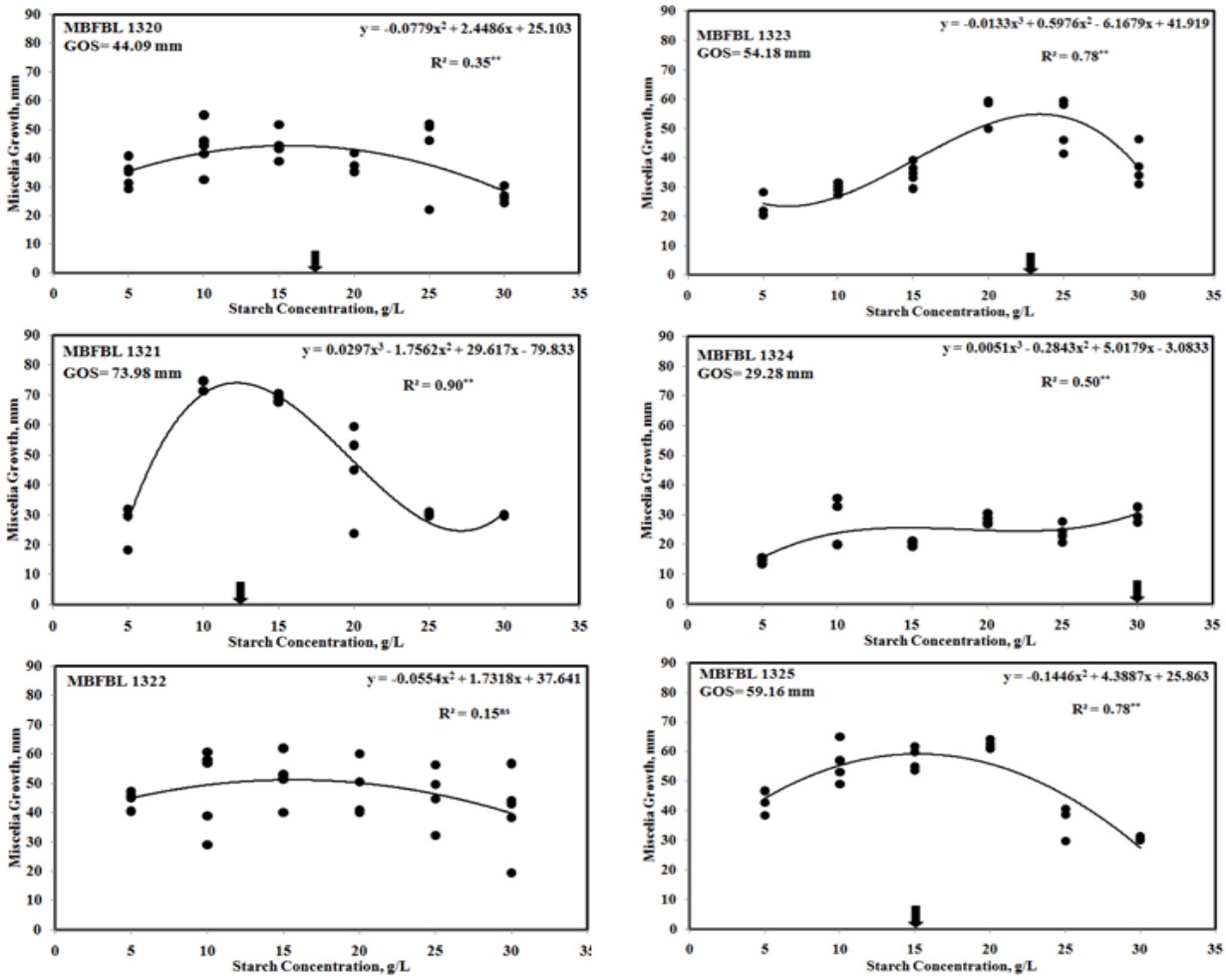


*Arrows pointing to nitrogen concentration: OG; optimum growth.

Figure 4.3 Mycelia growth, in millimeters on a polynomial regression with yeast extracts concentration, in grams per liter.

To find a suitable yeast extract concentration for *T. borchii* mycelial growth, various yeast extract concentrations were provided at concentrations of 0.25, 0.5, 1,5,10,15,20,25,30,35,40 g/L in MMN media. According to fig1.3, the yeast extract concentration effect for all strains is highly significant. The optimal yeast extract concentration for mycelial growth was 0.25 for MBFBL 1320, 1321, and 1323 while 10 g/L was optimum for MBFBL 1322 and 1324 and 6 g/L for 1325.

4.7 Carbohydrate (Starch) Concentration Studies



*Arrows pointing to optimum nitrogen concentration: GOS; growth at optimum starch concentration

Figure 4.4 Mycelia growth, in millimeters on a polynomial regression with starch concentration, in grams per liter

In order to find suitable starch concentrations for mycelial growth, *T borchii* was cultivated in MMN media with different starch concentrations 5,10,15,20,25, and 30 g/L. According to fig 1.4, the starch concentrations effect for all strains is significant except for MBFBL 1322. The optimal starch concentration level for mycelial growth was different for each strain 17.5 g/L for MBFBL 1320, 12.5 g/L for MBFBL 1321, 22.5 g/L for MBFBL 1323, 30 g/L for MBFBL 1324, and 15 g/L for MBFBL 1325.

4.8 Loblolly pine, *Pinus taeda* Infection by Bianchetto Truffle, *Tuber borchii* Mycelia

Table 4.4

Comparison of seedling morphological parameters

Source of variation	P>F	CV, %
-------90 days sampling-------		
Shoot length	0.1576	10.0
Root length	0.0678	28.5
Shoot: Root Length	0.0426*	24.4
Shoot weight	0.1215	20.7
Root weight	0.3078	40.3
Shoot: Root Weight	0.2782	50.2
-------120 days sampling--------		
Shoot length	0.4353	24.3
Root length	0.5295	46.0
Shoot: Root Length	0.5095	37.3
Shoot weight	0.5370	45.9
Root weight	0.3425	59.5
Shoot: Root Weight	0.0110*	19.0

*denotes significance at 5% rejection level

To determine the effect of *tuber borchii* mycelial inoculation on the roots of *Pinus taeda*, morphological parameters were measured at three months and four months post inoculation. As shown in Table 4, there was no statistic difference for all parameters tested except for shoot to root length at 90 days and shoot to root weight at 120 days post inoculation, which were both significant at the 5% rejection level.

Table 4.5

Comparison between the three months and four months sampling data, using individual seedling

Strain	Shoot : Root ratios (Standard error)[t]		Percent mycorrhization
	Length	Weight	
	----------90 days----------		
1320	0.8 (0.1)[ab]	3.5 (0.8)	25.1 (1.9)
1322	0.6 (0.1)[ab]	1.9 (0.1)	18.3 (1.3)
1323	0.4 (0.04)[b]	1.6 (0.1)	16.0 (1.5)
1324	0.9 (0.1)[a]	1.8 (0.2)	24.8 (3.8)
1325	0.4 (0.05)[b]	1.6 (0.1)	14.4 (0.9)
Control	0.5 (0.1)[b]	1.3 (0.3)	16.3 (1.9)
	----------120 days----------		
1320	0.7 (0.1)	2.3 (0.3)[a]	16.7 (2.5)
1322	0.7 (0.1)	2.2 (0.1)[a]	18.2 (8.3)
1323	0.5 (0.1)	1.5 (0.2)[b]	18.0 (4.3)
1325	0.6 (0.1)	1.4 (0.1)[b]	27.2 (8.1)

[t]means with a letter in common are not significantly different at 5% level of significance as indicated by Duncan's multiple range test for mycelia growth.

With shoot: root ratios being significant both at the 90 days and 120 days, Table 5 shows the means and the standard errors in parenthesis, measuring how accurate and precise the sample is an estimate of the population parameter. As shown in table 4, shoot to root length ratio and shoot to root weight ratio are significant at 90 and 120 post inoculation days respectively. At 90 days of post inoculation, MBFBL 1324 produced the highest shoot to root ratio which was not statistically different from 1320 and 1322 while MBFBL 1323 and 1325 was not statistically different form the control. At 120 days of post inoculation, 1320 and 1322 produced the highest shoot to root weight ratio means of 2.3 and 2.2 mm respectively which was statistically different from 1.5 and 1.4 mm for MBFBL 1323 and 1325.

Figure 4.5. Tuber borchii mycorrhized root tips of *Pinus taeda*

Usually, level of mycorrhization is checked after 6 months of inoculation while mature *T. borchii* mycorrhizae have been seen on *Quercus robur* L. in less than a month (Boutahir, Iotti,

Piattoni, & Zambonelli, 2013; Iotti et al., 2012). Figure 9 shows *T. borchii* mycorrhized root tips of Loblolly pine seedlings.

CHAPTER 5

Conclusion and Recommendations

In the *in-vitro* mycelia growth study, the optimum pH of solid MMN is between 7.5 and 9.0 for most strains with only one strain, MBFBL 1321 producing highest growth at pH 6.5. According to Figure 4.1, growth pattern for each strain was different. This signifies that though final pH preferences may be similar, there was difference in response at each pH level. The intermittent increase and decrease in growth present in all given strains but prominent in MBFBL 1320 and 1321 may have been due to unavailability of some ions at certain pH levels. Both MBFBL 1320 and 1321 were derived from similar sources; fruit bodies, thus, may have not been adapted to artificial medium conditions. Of the given strains, MBFBL 1323 showed capability of growing in MMN media irrespective of the pH of the medium.

Temperature studies revealed that 35 ^{O}C is unsuitable for the growth of *T. borchii* as none of the strains grew at this temperature. 30 ^{O}C supported the growth of *T. borchii* but very poorly for all strains except MBFBL 1322 and 1325 which showed no growth at 30 ^{O}C. Incubating at 20 ^{O}C was optimum for MBFBL 1320, 1321, 1323 and 1325. Using the model as shown in figure 4.2, the optimum temperature for MBFBL 1322 and 1324 are 17.5 and 22.5 ^{O}C respectively. With the average of 17.5 and 22.5 is 20, it can be deduced that 20 ^{O}C is the optimum incubating temperature for *T. borchii*. This compares to previous works, expectations and average temperatures of natural *T. borchii* fields.

Of the nine carbohydrate sources tested, starch was preferentially selected by all the given *T. borchii* strains. Comparing the averages of the other carbohydrate sources shows that all other sources except xylose were comparable to glucose. Though glucose is a regular constituent of MMN media, *T. borchii* has been shown to yield better growth in alternate carbohydrate

sources. This result also shows that starch is preferred to mannose which has been shown to be preferred to glucose for *T. borchii* and *T. melanosporum*. Xylose did not produce any growth for all strains except for MBFBL 1322, which had poorly grown and sparing mycelia. Examination of the concentration of starch revealed that the amount of starch required for the growth of *T. borchii* is strain dependent as no two strains required the same amount of starch.

Nitrogen source preference and utilization varied amongst the strains of *T. borchii* as each strain showed different trends. Each strain had a selection of sources which were optimum. Yeast extract was the only nitrogen source present in the selected optimums for each strain. With yeast extract being the only reoccurring optimum nitrogen source for all strains, it is the recommended nitrogen source in MMN media for culturing *T. borchii*. For MBFBL 1320 and 1323, nitrogen source did not have any effect. In the concentration examination carried out to determine the appropriate amount of yeast extract required to culture *T. borchii* in MMN media, 0.25 g/L of yeast extract was optimum for MBFBL 1320, 1321 and 1323 while MBFBL 1322 and 1324 showed preference for high concentrations of yeast extract at 10 g/L. Yeast extract provides the best source of nitrogen for *T. borchii* but the appropriate amount is strain dependent.

Loblolly pine, *Pinus taeda* infection by Bianchetto truffle, *T. borchii* generated on solid MMN media produced mycorrization at both the 90 and 120 days post infection and also the presence of mycorrhization caused increase in the shoot: root length ratios at 90 days post infection. This difference in shoot: root length ratio was not seen at the 120 days sampling but rather a difference was seen in the shoot: root weight as MBFBL 1320 and 1322 produced higher fractions than 1323 and 1325. These same strains, MBFBL 1323 and 1325 had the similar ratios with the control at 90 days of sampling.

More work needs to be done to develop new inoculation techniques and also to understand the role played by other nutrients including calcium, iron and thiamine in mycelia generation. The knowledge herein should be considered when selecting strains and appropriate media. The root infection results in this thesis serves as prove that *T. borchii* mycelia generated on solid MMN can positively infect roots of *P. taeda*.

In repeating this work to achieve optimized medium, the temperature treatments should be increased with closer treatment points; 15, 17, 19, etc. In repeating the infection of *T.borchii* mycelia and roots of *P. taeda*, seedlings should not be destroyed during sampling rather, only a fraction of the root should be examined. This will enable the same seedling be observed over a period of time. Also, the sampling time should be increased, extending up to a year and the presence and abundance of *T.borchii* should be confirmed with molecular techniques.

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Glossary

Ascomycete. Any fungus of the phylum (or Ascomycetes), including the molds and truffles. They bear their sexual spores in a sac.

Agar. A gelatinous substance obtained from seaweed and used in biological culture media as a thickener.

Anastomosis. A cross-connection between adjacent fungi mycelia.

Ascospore. A spore contained in an ascus. This kind of spore is specific is characteristic for ascomycetes (Ascomycota).

Axenic. Relating to, or signifying a culture medium that is free from living organisms other than the organism of interest

Ectomycorrhizae. A type of symbiotic mycorrhizae association composed of a fungus sheath around the outside of root tips.

Fungal hyphae. Fungi hypha is the branching filaments that make up the mycelium. Hyphae are divided into cells by internal cross-walls called "septa" (singular septum).

Fungivores. Fungi eating organism including birds, mammals, insects, plants, amoeba, gastropods, nematodes and bacteria.

Gleba. The fleshy spore bearing inter mass of a certain fungi

In-vitro. Taking place outside a living organism. Studies undergone in a test tube, culture dish, or flasks though these conditions attempt to mimic living organisms.

Peridium. This is the outer skin of a sporangium or other fruiting body (truffle) of a fungus

Rhizosphere. The region of soil in the vicinity of plant roots.

Saprotroph. An organism that feeds on or derives nourishment from decaying organic matter.

Subclade. A subgroup of a haplogroup

Subglobose. Imperfectly or nearly globose

Trufficulture. The cultivation of truffles

Truffle. A strong-smelling underground fungus that resembles an irregular, rough-skinned potato. They are prized by the amount of flavors they produce.