University of Alberta

Effects of the endophyte *Piriformospora indica* on growth, physiology and water relations of tobacco (*Nicotiana tobacum*)

by

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ABSTRACT

In the current study, wildtype and transgenic tobacco constitutively over-expressing Arabidopsis aquaporins PIP 1;4 and PIP 2;5 were grown in association with the endophyte *Piriformospora indica*. Root hydraulic conductivity along with various physiological parameters and aquaporin expression were studied in conjunction with growth. Growth promotion was observed in inoculated wildtype and PIP 2;5 over-expression plants, while water relation parameters were not observed to change in any of the genotypes. Quantified expression levels of four aquaporins were not changed in inoculated wildtype plants; however immunolocalization techniques revealed a possible increase in PIP 1 aquaporins in exodermal tissues of the associated plants. It was concluded that the growth promoting benefits of the fungus were due to factors other than influencing water relations and modulation of aquaporins studied. Moreover, it was interpreted that PIP 1;4 over-expression may inhibit growth promotion in inoculated plants, although the mechanisms of that role remains unclear.

Key words: *Piriformospora indica*, aquaporins, hydraulic conductance, water relations mycorrhizae, growth

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS AND ABBREVIATIONS

A	Net Photosynthesis
A_c	Cell Area
ABA	Abscisic acid
AM	Arbuscular mycorrhizal
CPP	Cell pressure probe
ct	Cycle threshold
E	Net leaf transpiration rate
ECM	Ecotmycorrhizal
g_s	Stomatal gas exchange
K_r	Root hydraulic conductance
L_{pc}	Cell hydraulic conductivity
L_{pr}	Root hydraulic conductivity
P_t	Cell turgor pressure
PIP	Plasma intrinsic protein
PTS_3	Trisodium, 3-hydroxy- 5,8, 10- pyrenetrisulfonate
$T_{1/2}$	Halftime of water exchange
V	Cell volume
WT	Wild-type
ε	Cell elasticity
Ψ_{leaf}	Leaf water potential
π^i	Osmotic pressure of the cell
π^o	External osmotic pressure of the bathing solution used with the cell pressure probe

1 Introduction

In a world where plant biomass shortages are prevailing, demands for plant products are continually increasing, and land suitable for agriculture is limited, it is becoming evermore important that sustainable solutions are found to meet needs. Voiced concerns regarding current means to improve crop quality and yields are urging the desire for alternate, environmentally sound practices. Conventional agriculture employing mineral fertilizers contributes to eutrophication (Carpenter et al. 1998), whereas irrigation can induce soil salinization (Rengasamy 2006). Moreover, the use of genetically modified food is viewed as dangerous by 56.5% of Europeans surveyed (Eurobarometer 2001), indicating that a significant proportion of the consumer base can have an aversion to employing genetic tools to improve crops. With increased research devoted to understanding fungi, microbes, and plant physiology as a whole, the proposed use of natural tools to sustainably increase yields and augment plant tolerance to various stresses is becoming more practical.

It is estimated that approximately 80% of plants live in close association with mycorrhizal fungi (Wang and Qui 2006), and the nature of those associations can be quite complex, especially under various conditions. In spite of this, research into specific plant-fungi interactions is providing information into which associations prove to be beneficial and which do not in respect to improving plant quality. There is ample evidence suggesting some mycorrhizal associations can improve host tolerance to various stresses including cold stress (Zhu et al. 2010), drought (Porcel et al. 2006, Aroca et al. 2007), and salinity (Aroca et al. 2007). Some mycorrhizal symbioses have also been linked to improvement of root water uptake and/or flow (Kothari et al. 1990a, Muhsin and Zwiazek 2002, Siemens and Zwiazek 2008), uptake of nutrients (Kothari et al. 1990b, Hodge et al. 2001), and growth of the host (Ruiz-Lozano et al. 1995a, Ruiz-Lozano et al. 1995b). It is likely that these benefits are initiated by altered polypeptide patterns in the symbionts (Dumas-Gaudot et al. 1994,), and aquaporin protein expression patterns have been observed to change in some cases (Marjanović et al. 2005, Porcel et al. 2006, Aroca et al. 2007, Aroca et al. 2009).

The damaging effects of fertilizers and conventional farming on arbuscular fungi diversity (Oehl et al. 2004), the varied responses of different plant species to mycorrhizal fungi (Helgason et al. 2002), and the fact that arbuscular mycorrhizal fungi are obligate symbionts (Peterson and Bonfante 1994, Tunlid and Talbot 2002) making them difficult to propagate synthetically, have presented barriers to using fungi as possible biofertilizers. However, with the discovery of *Piriformospora indica* in the late 1990s (Verma et al. 1998), the use of an endophyte as a suitable biological tool has been revitalized. This endophytic fungus which displays characteristics most similar to arbuscular mycorrhizal fungi can be axenically cultured (Verma et al. 1998), associates with various plant species (see Varma et al. 1999, Rai et al. 2001, Deshmukh et al. 2006, Shende et al. 2006, Druege et al. 2007), promotes host growth (Varma et al. 1999, Rai et al. 2001, Peškan-Berghöfer et al. 2004, Druege et al. 2007), increases survival (Varma et al. 1999), and enhances plant tolerance to drought (Sherameti et al. 2008), salinity(Waller et al. 2005), and various pathogens (Waller et al. 2005, Schäfer et al. 2007, Serfling et al. 2007, Stein et al. 2008). Since mycorrhizal fungi can be involved in plant aquaporin modulation (Porcel et al. 2006, Aroca et al. 2007) and evidence suggests that *P. indica* is beneficial to many plant species in many regards, the goal of the current study was to see if the endophyte has a role in adjusting plant water relations as seen by various other mycorrhizal fungi (Kothari et al. 1990a, Muhsin and Zwiazek 2002, Siemens and Zwiazek 2008). It was hypothesized that *P. indica* would change aquaporin expression in tobacco roots and increase plant water uptake. It was hypothesized that the fungus would induce a greater alteration in aquaporin expression in wild-type tobacco than in PIP over-expression plants under favourable conditions, in turn, causing a greater increase in hydraulic conductivity of wild-types than transgenic plants. It was thought that the increase in hydraulic conductivity could then be a possible factor contributing to increases in overall plant biomass. A basic model of hypotheses and expected outcomes can be seen in Figure 1.1.

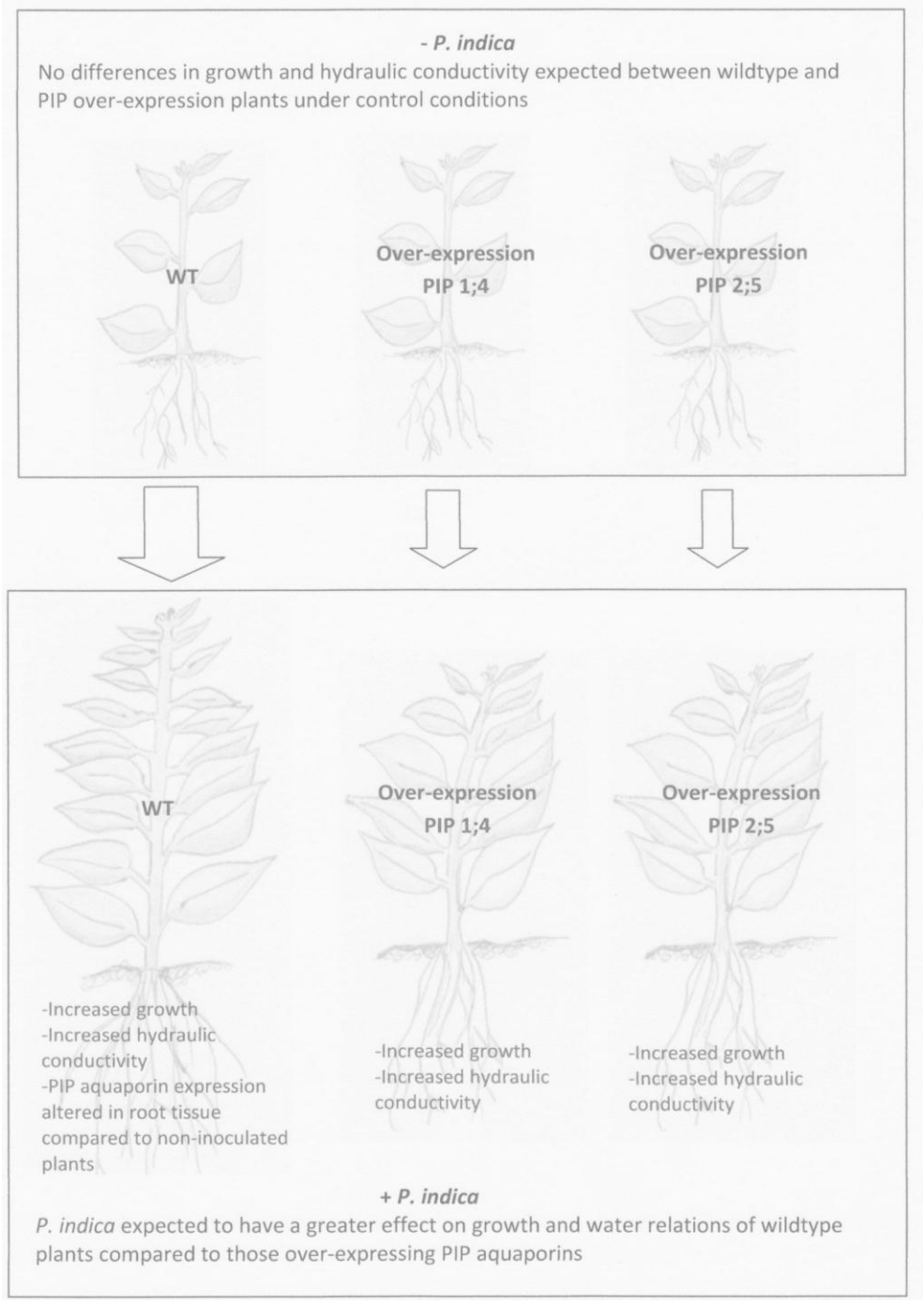

Figure 1.1: Model depicting hypotheses and expected outcomes when three *Nicotiana tobacum* lines are inoculated (+*P. indica*) or not inoculated (-*P. indica*) with *Piriformospora indica*. The three *N. tobacum* lines are wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5).

2 Literature Review

2.1 Water Relations

2.1.1 Root Anatomy

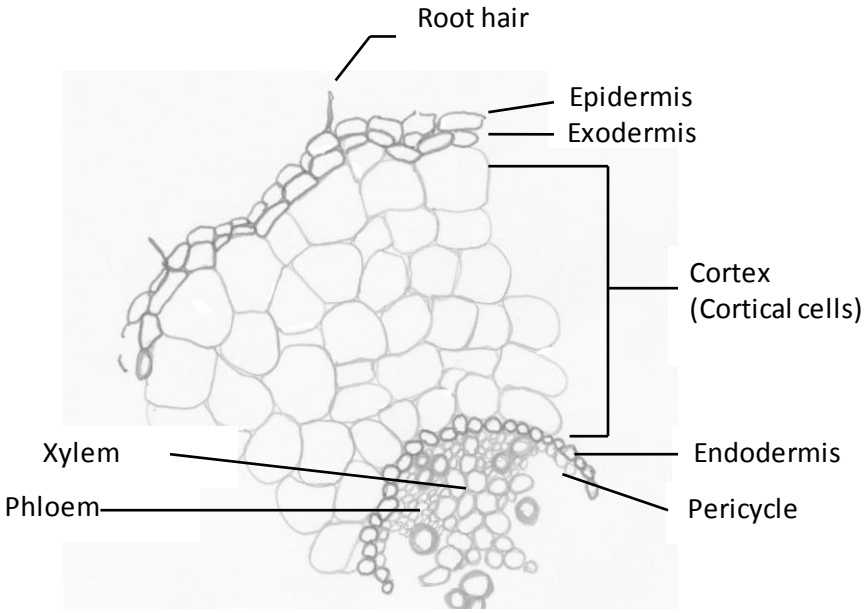

Figure 2.1.1: Cross section of an angiosperm root.

Knowledge of root anatomy allows one to develop a better understanding of how plants are able to procure water from the soil interface. Generally, the root hair zone is primarily responsible for water and nutrient absorption from the soil and this zone can normally be found within 10 mm of the root tip (Bramley et al. 2007, Segal et al. 2008). The pivotal role of root hairs in water uptake can be demonstrated through modeling and it has been estimated that root hairs 0.5 mm in length can increase soil water extraction by 30%, whereas root hairs 1 mm in length can increase extraction by 55% when compared to roots completely lacking hairs (Segal et al. 2008). Root hairs arise from individual epidermal cells and their overall quantity and longevity depends on both the plant species and the surrounding soil environment (Bramley et al. 2007). In many species of angiosperms, a layer of exodermal cells lines the interior of the epidermal cells. Following absorption through the epidermis, water flows radially through and around a series of cortical cells. The number of cortical cells can vary with species, stage

of root development and environmental conditions (Bramley et al. 2007). Previous to passing into the vascular tissue of the plant from the cortex, water must pass through the endodermis. Both the exodermis and endodermis act as an apoplastic barrier to water movement due to the Casparian strips found in these cellular layers (Zimmermann et al. 2000). Casparian strips may be described as band-like portions of cell walls that are saturated with suberin, where the suberin acts as a barrier to water movement because of its hydrophobic properties (Raven et al. 2005). Thus, water must penetrate the exodermis and endodermis through the cell-to-cell pathways (which will be discussed in Sections 2.1.3.2 and 2.1.3.3). It has been generally found that radial water movement encounters greater resistance than axial water movement, especially in smaller plants (Tyree 2007, Steudle and Peterson 1998).Hence, it can be said that whole root conductance is dependent on root surface area (Tyree 2007). Upon entering the vascular bundle, water is axially transported to the rest of the tissues via the xylem inside vessel or tracheid elements.

2.1.2 Water Properties

There are three important properties that have allowed water movement processes to take shape in plants (reviewed in Passioura 2001).

i. Water forms hydrogen bonds.
ii. Water is a polar molecule.
iii. Water has different viscosities at different temperatures which influences the rate of water flow.

The ability of water to form hydrogen bonds in combination with its polar nature renders it a good solvent for ions and molecules, in turn allowing osmotic pressures to arise inside cells with the formation of ion solutions (reviewed in Passioura 2001). In addition, hydrogen bond formation is responsible for high cohesive and adhesive strength of water, which makes it possible for water to be transported through the xylem elements due to the principle known as capillarity (Taiz and Zeiger 2010).

2.1.3 Transport Theories and Pathways in Plants

In general, water can be said to move through tracheary elements in passive, uninterrupted pathways characterized by the tensile strength of water due to its cohesive and adhesive properties (Mohr and Schoper 1995). The cohesion-tension theory substantiates that water is able to ascend in plants under this tensile strength and the driving force for the ascent is generated by surface tension at the evaporating surfaces of the plant (Tyree 1997). Because water movement is driven by surface tension, xylem tension will increase with drying conditions in turn triggering self-regulated oscillations in plant transpiration rates as a response to the gap between evaporative demand and water availability (Wallach et al. 2010). There is some discussion in literature, however, questioning the validity of the cohesion-tension theory. Benkert et al. (1995) observed tension gradients opposite to those expected from the cohesion-tension theory when measuring xylem pressures of *Tetrastigma voinierianum*. Xylem pressures measured 5 m above the ground were positive; whereas at the ground level, they were slightly negative (Benkert et al. 1995). In lieu of tension gradients driving water ascent, the authors suggested alternate theories to water movement including solute-supported water ascent, water transport in distinct vertical steps in a segmented xylem, and gel supported ascent. In gel supported ascent, it was suggested a water column could be established by a concentration gradient rather than a pressure gradient in stationary gel-like structures along the xylem conduit (Benkert et al. 1995). It was theorized that gel-like viscous material observed in cut leaf tissue would contain mucosubstances that would allow buoyancy driven water ascent through a density gradient (Benkert et al. 1995). Another theory for sap ascent explaining the development of low pressures in the xylem includes pressure from surrounding tissues driving sap ascent (reviewed in Tomos and Leigh 1999).

Despite alternate theories to sap ascent, the cohesion-tension theory appears to be sound when there is continuous transpiration under optimal conditions; yet one must ask what happens when transpiration diminishes to the point where tensile strength is no longer sufficient to maintain water ascent. Does water movement come to a

complete standstill? Plants are able to maintain water flow because ascent is not entirely dependent on the tensile strength of water alone. Some plants are able to maintain water ascent by the active pumping of solutes into root cells (Javot and Maurel 2002). When solute concentrations in the cytoplasm are greater than those found outside of the cell, water will osmotically enter the cell to try and balance out the solute gradients. The act of balancing out the gradients will thus drive water movement. Dehydration tolerance mechanisms, such as increasing potassium and sugar contents through osmotic adjustment, have been observed in sorghum, thus allowing plants to withstand dry soils and maintain water pressures necessary for water movement (Premachandra et al. 1992). Discussion of this osmotic gradient then brings forth the notion of various pressures inside the cell. As water osmotically enters the cell, the cell wall is stretched due to the water build-up and accordingly cell contents are pressed causing a positive pressure to develop in the cell (Boyer 1995). This build-up of pressure is called turgor pressure, whereas osmotic pressure can be described by the osmotic gradient formed. Tomos and Leigh (1999) discuss that higher plants have several mechanisms in place to adjust turgor pressure which include:

i. Exchanging solutes with other plant organs through plasmodesmata.
ii. Minimizing turgor changes by adjusting cell wall elasticity.
iii. Changing osmotic pressure through polymerization and de-polymerization reactions.

As stated above, the ability to adjust turgor pressure is important as it allows plants to continue water transport processes, to a certain extent, when transpiration ceases. It is also important to note that osmotic and turgor pressures come hand-in-hand, as osmotic pressure will generate the development of turgor pressure. This was demonstrated in transpiring wheat and maize plants, where both osmotic and turgor pressure gradients were observed to increase inwards in root cortical cells, with osmotic pressure values exceeding turgor pressure values (Rygol et al. 1993). When transpiration was prohibited, both gradients were no longer produced indicating to the authors that both active membrane transport and radial solvent drag are important in transpiring

roots (Rygol et al. 1993). Similar results were obtained by Zimmermann et al. (1992) with *Aster tripolium* roots.

The extent to which an osmotic pressure gradient will drive water flow across a membrane can be expressed by a reflection coefficient, which may have a value between 0 and 1 (Tomos and Leigh 1999). A gradient with a value of 0 indicates that an osmotic pressure gradient of a solute across a membrane will not drive water flow (Tomos and Leigh 1999), whereas a value of 1 will indicate a gradient will yield complete water flow across a membrane. It has been suggested that reflection coefficient values should always be around 1 (Knipfer and Fricke 2010). This is because the simple existence of pressure development in roots indicates at least one cellular membrane is crossed, and any value smaller than 1 is an experimental artifact (Knipfer and Fricke 2010).

When describing the development of osmotic pressure and complemental turgor pressure in plants, the idea of potential must be addressed. Potential represents the free energy per mol of a component in a mixture of many such components, and through the knowledge of water potential one can predict how water will move within a system (Mohr and Schoper 1995). Water potential is generated by the sum of three partial potentials: osmotic potential, turgor potential and gravitational potential (Taiz and Zeiger 2010). If water becomes diluted with the addition of particles, the mole fraction will decrease along with the associated energy content (Mohr and Schoper 1995). Thus, the active pumping of solutes across a membrane into a cell will result in a lower water potential. It is important to realize water will move from an area of high energy potential to an area of lower energy potential (Mohr and Schoper 1995, Tyree 2007). Hence, with the decrease in energy potential resulting from solute build-up across a membrane, water will move to the area of low energy where the solute concentration is higher. The idea of potentials helps to explain the mechanism behind osmosis and why the act of balancing out solute gradients will drive water movement.

As mentioned above, there are different pathways through which water can flow through tissues, and these pathways are largely driven by osmotic and turgor pressures. There are three pathways which will be discussed in detail: the apoplastic, the symplastic and the transmembrane pathways. Usually, the symplastic and the transmembrane pathways are grouped together and considered the cell-to-cell pathway as there few measurable ways to distinguish the two. However, for the purpose of this thesis, they will be separated and discussed as two distinct pathways. Being able to identify the contribution of different pathways to water movement is important for understanding the nature of resistances to root water transport (Bramley et al. 2007). Furthermore, being able to identify the type of pathway dominant in water transport within a plant provides the opportunity to regulate water flow by changing the influence of a pathway without anatomical changes (Bramley et al. 2007). Then again being able to identify the dominant pathway is rather difficult, as water flow follows different radial pathways when influenced by different driving forces (Bramley et al. 2009).

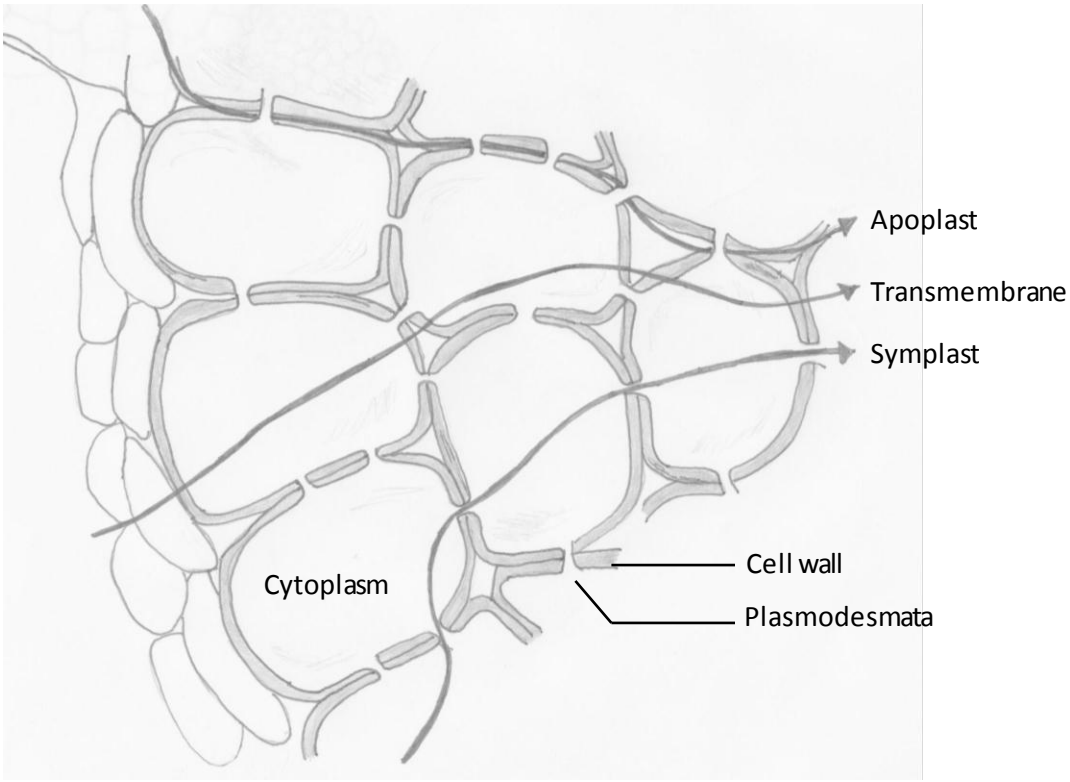

Figure 2.1.3: The apoplastic, the symplastic and the transmembrane pathways.

2.1.3.1 The Apoplastic Pathway

The apoplastic pathway (Figure 2) may be described as water movement restricted within intercellular spaces and cell walls. Apoplastic movement is believed to involve either a purely apoplastic pathway or a predominantly apoplastic pathway (Knipfer and Fricke 2010). In the former, water never crosses a cellular membrane whereas in the latter, water travels intercellularily to the endodermis where it passes through endodermal membranes (Knipfer and Fricke 2010). In the purely apoplastic pathway, membranes are not crossed, thus the reflection coefficient gradient of the root would be approximately 0 because osmotic gradients drive very little water transport (Steudle and Peterson 1998, Bramley et al. 2007). The possibility of a purely apoplastic pathway existing is questionable, as roots develop turgor pressure indicating that at least one cellular membrane is crossed (Knipfer and Fricke 2010). Furthermore hydrophobic Casparian strips in the exodermis and endodermis fine-tune the movement of water in the apoplastic pathway by inhibiting water advancement (Steudle and Peterson 1998), in turn forcing water through membranes. Thus reflection coefficients even through the apoplastic pathways should be closer to 1.

2.1.3.2 The Symplastic Pathway

The symplastic pathway may be described as the movement of water from cell-to-cell through the continuum of cytoplasm interconnected by plasmodesmata (Steudle and Peterson 1998). Plasmodesmata are described as narrow strands of cytoplasm that interconnect the protoplasts of neighbouring plant cells (Raven et al. 2005). In order for water to enter the symplast, one plasma membrane must be crossed, and in order for water to exit the symplast another plasma membrane must be traversed. Therefore, changes in plasma membrane water channel activity help to regulate water movement in the symplastic pathway (Steudle and Peterson 1998). Plasma membranes will be described in further detail in Section 2.1.5. Osmotic forces will drive water flow through the symplast, hence the reflection coefficient of the root would be around 1 (Steudle and Peterson 1998, Bramley et al. 2007). During transpiring conditions, it is argued that

water and solute flow cannot traverse plasmodesmata inwardly due to the development of increasing pressure gradients from the root surface to the vascular bundle (Rygol et al. 1993). Thus the mechanisms directing symplastic water flow require further study as the pressure gradients developed in transpiring roots would seem to direct flow outwards through plasmodesmata (Rygol et al. 1993).

2.1.3.3 The Transmembrane Pathway (The Transcellular Pathway)

Similarly to symplastic water movement, the transmembrane pathway refers to water passage through plasma membranes to get from one cell to the next; however unlike the symplastic pathway plasmodesmata are not required for this kind of water movement (Steudle and Peterson 1998). In some cases, the transcellular pathway is specifically referred to as the transport of water within the cytoplasm into a vacuole and then across the vacuole (Oertli 1991). Nonetheless, a multitude of membrane channels, termed aquaporins, are required so that water may pass from cell to cell, or across the tonoplast. Aquaporins will be described in further detail in Section 2.1.5. Like in the symplastic pathway, osmositic and turgor pressures are the primary forces driving water through the membrane, so the reflection coefficient of the root would be more or less equivalent to 1 (Steudle and Peterson 1998, Bramley et al. 2007).

2.1.4 Influences on Hydraulic Conductance

Water flow rates will fluctuate depending on plant structure and internal mechanisms as well as on external conditions. It has been demonstrated that various root tissues will have different radial hydraulic conductivity values depending on the conditions to which the plant is exposed and the age of the root. For instance, during wet conditions neither the endodermis nor the periderm of *Opuntia ficus-indica* roots were the main limitation to radial water movement (North and Nobel 1996). Contrarily, after 30 days of drought, radial hydraulic conductivity was most limited across endodermal, peridermal and conducting tissues; however decreases attributed to those tissues were offset by increases in radial conductivity across dead cortical tissue (North and Nobel 1996). Similarly, resistance to radial water flow is greatest in the cortex of grape root tips, but

resistance progressively decreases in the cortex further away from the apex due to cortical breakdown and increased suberization, consequently shifting resistance to endodermal cells (Mapfumo and Aspinall 1994). Even though suberization is more prevalent in the upper regions of the root, it is speculated that most radial water flow would occur in the upper regions because xylem tissues are more mature, therefore offering considerably less axial resistance which would offset the increased radial resistance (Mapfumo and Aspinall 1994). Not only is water flow dependant on the resistances of various tissues, it is highly dependent on diameter of the conducting channel. Hydraulic conductivity can differ immensely within a given plant species because large diameter stems are more conductive than smaller diameter stems due to the fact they can transport more volume (Tyree 1997). The ability of larger vessels to increase water flow was demonstrated when comparing axial conductivity of two lupin species to that of wheat (Bramley et al. 2009). The lupins had considerably higher axial flow and this was attributed to lupin xylem diameter and numbers increasing with root maturity, whereas wheat xylem area remained more or less constant along the whole root length with few increases (Bramley et al. 2009). Furthermore, most of the variability in hydraulic conductance in a plant is thought to be due to the switching between the cell-to-cell pathways (and the use of aquaporins) and the apoplastic pathway (Steudle 2000). Switching between pathways has been demonstrated in *Opuntia ficus-indica* roots, where lateral root emergence results in increased apoplastic uptake, in turn, increasing radial conductivity of tissues outside the vascular bundle (North and Nobel 1996). Furthermore, use of the symplastic tracer carboxyfluorescein has demonstrated that water movement can change from being predominantly symplastic (thus flowing through plasmodesmata) in developing maize roots to transmembranal (thus aquaporin mediated) in mature roots (Hukin et al. 2002). It is important to note that aquaporins can influence the hydraulic conductivity only if the cell-to-cell pathways offer less resistance than the apoplastic pathway (Bramley et al. 2007). The role of aquaporins in water flow is further discussed in detail in section 2.1.5.

Upon comparison of species, hydraulic conductivity can be more pronounced. When comparing a *Schefflera* stem, a *Thuja* stem, and an *Acer* stem, all of the same diameter,

the *Schefflera* stem can be 3 to 10 times more conductive than *Acer,* and *Thuja* can be 10 to 20 times lower than the other two species (Tyree et al. 1991). Hydraulics of species can be greatly different from one another because each species has its own mechanisms in place to achieve a balance between root diameter, hydraulic conductivity and the ability to adapt to changing conditions (Bramley et al. 2009).

2.1.5 Aquaporins

2.1.5.1 Aquaporin Classification and Structure

Generally speaking, aquaporins may be described as water channel proteins that mediate increases or decreases in water permeability (Javot and Maurel 2002) and are able to do this by providing a "molecular basis for the fast and reversible" transport of water across a membrane (Maurel and Chrispeels 2001). The capability of membrane channels to transport water across cellular membranes was first described in human erythrocyte and renal tubules in the early 1990s (Preston and Agre 1991, Preston et al. 1992). There is argument, however, that Benga et al. (1986) were actually first to suggest that either or both band 3 and 4.5 proteins had a role in forming channels for water transport across a membrane. To date, four major classifications of aquaporins have been described in plants: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs), and small basic intrinsic proteins (SIPs). There is also discussion of a newly discovered fifth aquaporin group referred to as X intrinsic proteins (XIPs) that appear to be absent in monocots (Danielson and Johanson 2008, Almeida Rodríguez 2009, Sade et al. 2009). The names of the proteins can be attuned to where they are located in the plant cell; for example PIPs are located in the plasma membrane, and TIPs are found in the tonoplast membrane. Aquaporin names can also be attuned to their organ of discovery (NIPs) or their relative molecular size (SIPs) (Fricke and Chaumont 2006). The major aquaporin classes can be further characterized into subclasses, for instance PIP aquaporins may be subdivided into two clades referred to as PIP1 and PIP2 (Wudick et al. 2009). An aquaporin

belonging to a PIP3 subfamily has also been described in the moss *Physcomitrella patens* (Danielson and Johanson 2008).

Generally, aquaporin proteins are formed by polypeptide subunits comprising of six linked α-helical transmembrane domains (Preston and Agre 1991, Preston et al. 1992, Murata et al. 2000) that comprise a monomer. Each monomer has an N terminus that is located on the cytoplasmic side of the membrane (Preston and Agre 1991, Murata et al. 2000). Monomers interact with the α-helices of neighbouring monomers to form tetramers which are stabilized by a network of hydrogen bonds in addition to interactions among loops of monomers (Murata et al. 2000). PIP1s and PIP2s structurally differ from one another in amino and carboxy termini length, in addition to some amino acid substitutions (Chaumont et al. 2000).

2.1.5.2 Aquaporin Function

Through the creation of transgenic plants either over-expressing or under-expressing selected aquaporins, scientists have been able to begin exploring the functional realm of water channels. Quantitative reverse transcription PCR has also allowed more insight into how aquaporins are regulated under various conditions. What is more, scientists have been able to study particular subgroups and/or individual aquaporins through the use of frog oocytes and yeast cells. Selected aquaporin RNA fragments are inserted into either oocytes or yeast cells, and transport capabilities are then observed. Through the advances of these technologies, among others, it has been proposed that aquaporins can belong to one of three functional groups: Those that transport only water, those that transport solutes and water, and those that transport only solutes (Reviewed in Chaumont et al. 2000). *Nicotiana tabacum Nt*AQP1 has the capability to transport water (Mahdeih et al. 2008) as well as CO_2, and it is able to switch its function from a water channel to a CO_2 channel depending on its location in the cell and on the membrane's permeability to CO_2 within which it is found (Uehlein et al. 2008, Sade et al. 2010). Aquaporins belonging to the first two above groups are required so that water may pass through plasma membranes in the symplastic and transmembrane pathways. Maurel

and Chrispeels (2001) discuss how studies have shown between 20 and 80% of root water transport is under metabolic control and likely involves the activity of aquaporins. Moreover, there is evidence that suggests aquaporins play an important role in plants' ability to respond to changing environmental conditions. Some aquaporin groups appear to be more involved in water transport when subjected to particular stimuli. This is demonstrated by aquaporin expression changing temporally with environmental conditions and aquaporin gating being initiated by phosphorylation (Johansson et al. 1998), cytoplasmic pH (Fischer and Kaldenhoff 2008) in addition to calcium (Alleva et al. 2006), and heavy metals such as gold and silver(Niemietz and Tyerman 2002). Thus, the study of aquaporin function is vital to understanding how plants are able to maintain water flow and overcome stresses.

Water molecules are believed to move through aquaporins in a single chain due to the nature of the narrow pore and it has been shown that mercury can block the water channels by binding to cysteine residue in the pore (Preston et al. 1992, reviewed in Vandeleur et al. 2005). Knowledge of aquaporin blocking has led scientists to gain a better understanding of the role of aquaporins in water movement, although definitive roles of individual water channels still remain unclear. It has been proposed that aquaporins can considerably affect water flow at the cellular level, however at the root level, control of conductivity by aquaporins in some plant species can be limited due to water predominantly following the apoplastic pathway (Bramley et al. 2009). Moreover, control of water movement by aquaporins may be limited to certain root tissues, for instance endodermal tissues in wheat (Bramley et al. 2009). This having been said, there are two general ways aquaporins can control flow across a cell membrane (Bramley et al. 2007):

1. The abundance of aquaporins can be changed within a membrane.
2. The rate of flow through the aquaporin can be changed.

The conductance of aquaporins is additive, therefore more aquaporins will create more parallel pathways for water flow and effectively hydraulic conductance should theoretically be increased (Bramley et al. 2007).

Availability of complete genome sequences makes it possible to identify all the aquaporin genes for a number of plant species, and the pioneering study for plant aquaporin identification was that of Johanson et al. (2001) where 35 aquaporins were found in *Arabidopsis.* The existence of large aquaporin families, as seen in *Arabidopsis*, have led Bots et al. (2005) to theorize that the redundancy of aquaporins in large families assures a favourable water balance at the cellular level. It has however, been concluded that the two PIP subgroups are not necessarily redundant since temporal and spatial expression patterns of *Nt*PIP1; 1 and *Nt*PIP2; 1 are not identical in reproductive tissues (Bots et al. 2005). Alternatively, it has been proposed that individual aquaporins in large families may functionally transport water differently or may be functionally identical, but are rather expressed differently in time and space allowing the plant to better adapt to stimuli (Bots et al. 2005). This notion is supported by findings where *Zea mays* PIP expression appears to be developmentally regulated, where some isoforms are more expressed in developing tissues and others are more prominent in mature tissues (Hachez et al. 2006, Hachez et al. 2008).

Oocyte studies have demonstrated that PIP2s have the ability to enhance membrane permeability more than PIP1s, further enforcing the theory that aquaporins have different functions or are differently regulated (Chaumont et al. 2000, Bots et al. 2005, Mahdieh et al. 2008). To further complicate matters, when PIP1s and PIP2s are expressed in conjunction with one another, a co-operative effect has been described (Fetter et al. 2004, Mahdieh et al. 2008). It has been suggested that heterotetramers result in better stability and/ or folding of the proteins, in turn resulting in more efficient transport of water across a membrane (Fetter et al. 2004). However, it has been found that not all aquaporin subgroups are activated by forming heterotetramers (Fetter et al. 2004). This may be demonstrated by analysis of aquaporin expression patterns where PIP2s are not found in all tissues of plants. Chaumont et al. (2000) found that *Zea*

*ma*ys *Zm*PIP2a (in a subsequent paper referred to as *Zm*PIP2; 5, see Fetter et al. 2004) was lacking in shoot tissues of maize, and Bots et al. (2005) found *Nt*PIP2 was lacking in tobacco stigmas. This lack of expression makes it appear heterotetramers may not be absolutely necessary for efficient water transport in all plant tissues.

As stated before, regulation of water flow appears to be a complex process that involves meticulous synchronization of aquaporin expression, particularly when plants are subjected to various stresses. To illustrate, a decline in tobacco *Nt*PIP1; 1 and *Nt*PIP2; 1 transcripts during severe drought was observed, however *Nt*AQP1 expression was increased during the stress (Mahdieh et al. 2008). Despite the increase in *Nt*AQP1 expression, the water permeability of roots at the cellular level still decreased as a response to drought (Mahdieh et al. 2008). Over-expression of *Nt*AQP1 in tomato plants, however, resulted in an increased photosynthetic rate and increased stomatal aperture, while root hydraulic conductivity was maintained during salt stress (Sade et al. 2010). Under favorable conditions, antisense tobacco plants exhibiting a decrease in the aquaporin *Nt*AQP1 had a hydraulic conductance 42% of that of controls (Siefritz et al. 2002). In all studies, hydraulic conductance was either decreased or maintained, yet *Nt*AQP1 expression was very different. Regarding *Nt*AQP1 as an example demonstrates the complex role individual aquaporins play in water regulation, and how that role becomes further complicated under various conditions in tandem with expression of other aquaporin types. Over-expression of PIP1b aquaporin in tobacco was found to be disadvantageous during drought and salt stress (Arahon et al. 2003). Contrarily, over-expression of *Brassica napus Bn*PIP1 in tobacco proved to be beneficial during drought (Yu et al. 2005). Moreover, broccoli plants subjected to high salinity stress were able to modify lipid membrane composition, in turn increasing the accumulation of PIP1 and PIP2 aquaporins (López-Pérez et al. 2009). Consequently, the broccoli plants were able to control membrane permeability and better tolerate salinity (López-Pérez et al. 2009). In oxygen deficient environments, such as during water logging, an increase in aquaporins has been found to generate faster water transport to shoots and to contribute to changes in plant growth during the recovery period (Bramely et al. 2007). The results of the previously mentioned studies illustrate how over-expression of some

aquaporins may become detrimental in some plants likely due of the risk of water backflow into soil (Bramely et al. 2007) while in other plants the same aquaporin type appears to be involved in mechanisms of stress resistance. That being said, there are two schools of thought of the function of aquaporins during water stress (Yu et al. 2005): Aquaporin levels increase in order to provide plants an additional ability to withstand water deficiencies, or alternatively plants are able to avoid excessive water loss through the down regulation of some aquaporins. Arahon et al. (2003) suggest that enhanced water transport through over-expressed aquaporins found in cells that do not normally contain aquaporins expressed at higher levels may result in detrimental effects, especially during drought. One must always take into account that the function of one aquaporin studied in transgenic plants will be influenced by its interaction with many other processes (Arahon et al. 2003).

2.1.6 Measuring Hydraulic Conductivity

2.1.6.1 Hydrostatic Method

Root hydraulic conductivity (L_p) may be measured on excised root systems through means of the hydrostatic pressure method (Markhart et al. 1979, Wan and Zwiazek 1999, Voicu and Zwiazek 2004). Roots are immersed in aerated nutrient solution in a pressure chamber and the pressure is increased at predetermined intervals from 0 to up to 0.6 MPa. The steady-state flow rate (Q_v) is measured at the increasing pressures. The volume flow density (J_v) may be calculated by dividing Q_v by either root volume (Voicu and Zwiazek 2004), root surface area (Wan and Zwiazek 1999), or root weight (Vysotskaya et al. 2010). Scaling Q_v to obtain J_v is necessary in order to normalize for plant size (Tyree 2007). L_p may then be calculated from the slope of J_v versus the pressure.

2.1.6.2 PTS_3

Apoplastic flux through a root system has been quantified through the use of fluorescent dyes such as rhodamine B (RB) (Skinner and Radin 1994, Wan and Zwiazek 1999), trisodium, 3-hydroxy- 5,8, 10- pyrenetrisulfonate (PTS_3) (Hanson et al. 1985, Skinner and Radin 1994, Voicu and Zwiazek 2004, Siemens 2008), rhodamine 6G (RG), 1,3,6,8 pyrenetetrasulphonic acid trisodium salt (PTS_4), lucifer yellow CH dipotassium salt (LY), and 4- acetoamido 4'- isothiocyanostilbene-2,2'- disalphonic acid (SITS) (Skinner and Radin 1994). Additionaly, the non-fluorescent tracer light green SF yellowish can be used to measure water movement in the apoplastic pathway (López-Pérez et al. 2007). As reviewed by Hanson et al. (1985), PTS_3 is a water soluble marker that is excluded from the symplast. The contribution of the apoplastic pathway may be quantified by comparing the PTS_3 concentration of the exudates of a pressurized root system to the known PTS_3 concentration of the bathing solution. There is debate, however, that apoplastic barriers in the roots may retain dyes more than water which would lead to a substantial underestimation of apoplastic water flow (Zimmermann and Steudle 1998). Additionally, PTS tracers have been found to enter the cytoplasm and/ or vacuoles of cortical cells in *Opuntia ficus-indica* plants (North and Nobel 1996), thus prompting further questions regarding the ability of these dyes to truly trace apoplastic flow.

2.1.6.3 Cell Pressure Probe (CPP)

Direct measurements of cell turgor, cell wall elasticity, and hydraulic conductivity of single cells are fundamental for the evaluation of water relations in plants (Husken et al. 1978) and these measurements may be accomplished with the aid of the cell pressure probe (CPP). The theory underlying the CPP relies on the notion that in order to understand the functioning of the whole organism, it is necessary to have a description of the activities of its component cells (Tomos and Leigh 1999). The CPP can measure the cell-to-cell component of water movement (Steudle and Peterson 1998), meaning it can measure water flow through the symplast and transmembrane pathways, but it

cannot distinguish between the two. Additionally, the location of principal resistances to water movement through roots can be estimated since the cell pressure probe provides real-time measurements of hydraulic conductivity (Bramley et al. 2007).

The pressure probe consists of a microcapillary, a pressure chamber containing the pressure transducer, and a motor driven metal rod (Husken et al. 1978). Manufacturing microcapillaries involves pulling borosilicate glass capillaries into a fine point with a pipette puller (Franks 2003). The diameter of the microcapillary's opening will be governed by the plant species used, the person using the instrument, and the desired cells to be punctured. The probe is filled with silicone oil and the movement of the metal piston within the probe allows pressure to be adjusted in the probe (Franks 2003). The electric transducer allows pressure within the probe to be directly measured and recorded by a computer.

CPP measurements can be performed on a variety of cells, depending on the objectives of the experiment. For example, Tyerman et al. (1989) conducted measurements on excised roots of *Nicotinana tobacum* where measurements were taken on the root hair zone on either the cortical or epidermal cells; whereas Lee et al. (2009) conducted measurements on the bundle sheath cells of intact and excised leaves of *N. tobacum* plants. With the objective of measuring aquaporins' role in root hydraulic conductivity, it can be speculated that measurement of endodermal cells in roots will provide the most accurate measurement as water from the apoplastic pathway is completely annulled. The probe is used to puncture a desired cell, and the turgor pressure inside the cell pushes cytoplasm into the probe (Boyer 1995), in turn causing formation of a meniscus. The meniscus can be brought close to the cell wall by pushing the piston into the oil reservoir which allows the original cell volume to be restored (Tomos and Leigh 1999), and thus allows half-times of water exchange ($T_{1/2}$), turgor pressure (P_t) and cell elasticity (ε) to be measured (Lee et al. 2005). An electric pressure transducer converts the pressure signal into a voltage that can then be read by the computer to allow calculation of the aforementioned variables (Lee et al. 2005). It must be noted, that the pressure in the cell is measured by creating an opposing pressure in the silicone oil of

the probe and that measured pressures are relative to atmospheric pressure (Boyer 1995). These three measured variables can consequently be used to estimate hydraulic conductivity of the cell if cell volume (V) and cell surface area (A_c) are also known, according to Azaizeh et al. (1992):

$$L_{pc} = V \times \mathrm{Ln}(2)/[\, A_c \times T_{1/2} \times (\varepsilon + \pi^i) \,] \qquad (1)$$

2.2 Mycorrhizal Associations

2.2.1 Types of Mycorrhizal Associations

Approximately 10% of fungi live in close associations with other organisms and some of these associations may be parasitic, leading to disease, or mutualistic, procuring benefits for both fungi and host (Tunlid and Talbot 2002). In some cases, the association may be described as neutral when neither harm nor benefit to either symbiont is observed. For instance, the associations between a mycorrhizal *Glomus* sp. and perennials grown under low nitrogen supply have been described as neutral, notably because little effect on biomass and nitrogen acquisition was observed (Reynolds et al. 2005). Plant-fungus interactions are thought to have commenced when plants first originated and to date it is estimated that approximately 80% of plant species and 92% of plant families are mycorrhizal (Wang and Qiu 2006). There are various kinds of fungi that can form associations with plants and mycorrhizal organization is divided on the basis of fungal associates: There are aseptate fungi in Glomeromycota, and septate fungi that can be found in either Ascomycetes or Basidiomycetes phyla (Smith and Read 2008). Arbuscular mycorrhizal (AM) fungi are members of Glomeromycota, whereas most ectomycorrhizal (ECM), Ericoid, Orchid, Ectendomycorrhizal, Arbutoid, and Monotropoid fungi belong to the latter group of septate mycorrhizal fungi mentioned above (Tunlid and Talbot 2002, Wang and Qui 2006, Smith and Read 2008). ECM fungi are thought to have evolved from AM fungi, whereas Ericoid and Orchid mycorrhizal fungi are believed to have been derived from ECM fungi (Wang and Qiu 2006).

Plants can be further described as being obligately mycorrhizal, implying they have been found to always form associations, facultatively mycorrhizal if associations can be formed in one habitat but not another, or nonmycorrhizal (Wang and Qiu 2006, Smith and Read 2008). Some families that are considered to be nonmycorrhizal include Chenopodiaceae, Polygonaceae, Juncaceae, Proteaceae, and Caryophyllaceae (Smith and Read 2008), while roughly half of the Brassicaceae and Crassulaceae species are believed to be nonmycorrhizal (Wang and Qiu 2006).

Plant-fungi interactions have proven to be quite complex and their ecology is not fully understood. There is evidence to suggest, however, fungi-host associations may lead to a cascade of events, including possible alterations at the cell and tissue level in both symbionts (Gianinazzi-Peason and Gianinazzi 1989). AM and ECM interactions will be discussed in further detail.

2.*2.1.1 Ectomycorrhizae*

Ectomycorrhizal roots are characterized by fungi that structurally form a mantle and a Hartig net around the plant's root while not physically penetrating cortical cells (Peterson and Bonfante 1994, Smith and Read 2008). It can be said that the interaction between the environment, the fungal genome, and the host genome control mantle formation (Peterson and Bonfante 1994). The Hartig net is a network of hyphae that are usually confined to either the epidermis, as in the case of most angiosperms, or permeate the cortex without penetrating the cells, as in the case of some conifer species (Peterson and Bonfante 1994). Due to the branching nature of the Hartig net, symplastic and apoplastic exchange between the plant roots and the fungus is thought to increase (Peterson and Bonfante 1994). The transfer of material within the symplastic and apoplastic regions is viewed to be facilitated by the permeability of the symbionts' cell walls in the Hartig net region (Peterson and Bonfante 1994).

2.2.1.2 Arbuscular Mycorrhizae

AM fungi may be described as endophytes due to the nature of their interaction with plant hosts. Endophytes are broadly referred to as "fungi that live for all, or at least a significant part, of their life cycle internally and asymptomically in plant parts" (Saikkonen et al. 1998). Endophytes are usually found in the above-ground tissues, or occasionally in the roots of plants, and they may be distinguished from ECM fungi by their lack of external hyphae and mantles (Saikkonen et al. 1998). Two major groups of endophytes have been recognized: 1) clavicipitaceous endophytes that infect the shoots of some grasses, and 2) non-clavicipitaceous endophytes that infect above and below ground tissue of non-vascular plants, ferns, conifers, and angiosperms (Rodriguez et al 2009). Based on the aforementioned, AM fungi may specifically be considered non-clavicipitaceous endophytes as the hyphae penetrate roots, growing within and between cortical cells. As previously stated, AM fungi are members of Glomeromycota, and they likely are asexual organisms able to acquire variation from mutations and possibly heterokaryosis (Smith and Read 2008).

When fungal spores contact the root, they germinate to form appressoria which give rise to inter and intracellular hyphae (Bonfante et al. 1996). As intracellular hyphae develop, branched structures called arbuscules are formed in the cortical cells (Peterson and Bonfante 1994). Due to this growth pattern, a very large and dynamic interface is formed between the two symbionts (Duan et al. 1996). The ability to form arbuscules while maintaining mutual symbiosis with a host plant differentiates AM fungi from all other described mycorrhizal associations (Smith and Read 2008). Some AM fungi also have the ability to form vesicles, and are therefore often referred to as vesicular-arbuscular mycorrhizal fungi. In this thesis, all AM fungi, whether they form vesicles or not, will be referred to as AM fungi. Arbuscules are regarded as major sites for nutrient exchange (Peterson and Bonfante 1994), specifically being sites of carbon acquisition by fungi (Smith and Read 2008). While fungi rely on carbon and photosynthates from plant hosts, external hyphal mycelium in the soil increase plants' availability to

macronutrients such as phosphorus and nitrogen and the micronutrients copper and zinc (Kothari et al. 1990b, Smith and Read 2008).

It is understood that AM fungi are obligate symbionts (Peterson and Bonfante 1994, Tunlid and Talbot 2002) meaning they require a host plant for survival. Most AM fungi that have been studied show little or no specificity to plant host (Kapulnik et al. 1996) likely because they are obligate symbionts. It is believed that AM fungi are unable to grow on synthetic media for one of two reasons (Reviewed by Singh et al. 2000 with references therein):

i) They have nutritional requirements that are not fulfilled by media due to our lack of knowledge, or
ii) They have lost a significant part of their genomic material thus requiring their host to supply the inducer for nucleic acid translation.

2.2.2 Effects of Mycorrhizal Associations on Plants

It has been found that endophytes can bring upon benefits to the host species including drought tolerance, growth enhancement, adaptations to pH, temperature and salinity (Rodriguez et al. 2009), and defenses against herbivores and plant pathogens by altering host physiology, morphology and allelochemistry (Saikkonen et al. 1998). Specific modifications in host cell morphology have been observed in arbuscular mycorrhizal plants including invagination of plant plasmalemma, fragmentation of vacuoles, and increases in organelles (Peterson and Bonfante 1994). Moreover, stomatal gas exchange in cowpea (*Vigna unguiculata*) inoculated with the endophytic mycorrhiza *Glomus intradices* tended to be higher in inoculated plants during drought because it was thought that concentration and delivery rates of abscisic acid to leaves were lower in mycorrhizal plants (Duan et al. 1996). In another experiment, *Zea mays* plants inoculated with *Glomus etunicatum* had higher water conservation and water use efficiency under cold stress (Zhu et al. 2010). The authors suggest that the improvements in water status due to AM symbiosis could, in turn, cause indirect

increases in nutrient uptake, osmotic adjustment, gas exchange and photosynthetic capacity. In a different study, infection with mycorrhizae was found to alter plant fitness by influencing reproductive components, in turn, increasing fecundity, and improving seed quality and seedling vigor (Koide 2000).

It is important to note that the interaction may not always be beneficial to both symbionts, and can vary along a continuum from pathogenic to mutualistic (Saikkonen et al. 1998). Modjo and Hendrix (1986) found that the AM fungus *Glomus macrocarpum* can cause tobacco stunt disease, and there was evidence that *G. microcarpum* can cause slight stunting as well. Further, in a case study performed by Kageyama et al. (2008), it was found that, most often, randomly collected endophytes tend to have a negative effect on plant host biomass. This being said, the endophytes may procure other benefits to the host.

2.2.3 Biology of *Piriformospora indica*

The characteristics of *Piriformospora indica* render it an intriguing mycorrhizal fungus, as it does not clearly fit into a pre-described mycorrhizal group. *P. indica* is an endophyte that was discovered by Verma et al. (1998) in the sandy desert soils of Rajasthan, in northwest India. *P. indica* belongs to the Hymenomycetes (Basidiomycota) (Verma et al. 1998), while further phylogenetic analyses have shown that the fungus belongs to the order Sebacinales (Schäfer et al. 2007). *P. indica* is very closely related to the endophyte *Sebacina vermifera* (Barazani et al. 2005, Desmukh et al. 2006), while deduced amino acid sequences of the *Pit*EF1 gene from *P. indica* were found to be significantly similar to the elongation factor-1α of *Schizophyllum commune* and *Filobasideilla neoformans* of the Hymenocytes and *Puccinia graminis* of the Urediniomycetes (Bütehorn et al. 2000).

P. indica asexually forms pear-shaped thick walled spores called chlamydospores at the tips of hyphae (Verma et al. 1998). Hyphae are thin-walled and range in diameter from 0.7-3.5 µm, whereas the chlamydospores range between 16-25 µm in length and 10-17 µm in width (Verma et al. 1998). It is unknown whether *P. indica* has a sexual state

(Shende et al. 2006). Due to the lack of host specificity, the endophytic and asexual nature of *P. indica*, it is tempting to prescribe the fungus as a member of the AM group. However, the endophyte differs from AM fungi in the following ways (Shende et al. 2006):

i) *P. indica* spores and hyphae are four to five times smaller and thinner in diameter.
ii) *P. indica* can be easily cultured while arbuscular mycorrhizal fungi are obligate endosymbionts, rendering them uncultivable in laboratory.

This being said, the fungus is able to grow on a wide range of synthetic and complex media such as MMN1, modified aspergillus, aspergillus, malt-yeast extract, and MS medium (Verma et al. 1998, Pham et al. 2004). As mentioned before, the endophyte is able to form associations with a wide variety of plant hosts including *Zea mays*, *Nicotiana tobacum*, *Populus tremula*, *Petroselinum crispum, Bacopa monnieri* (Varma et al. 1999), *Pisum sativum*, *Artemesia annua* (Shende et al. 2006), *Hordeum vulgare* (Deshmukh et al. 2006), *Pelargonium sp.*, *Euphorbia pulcherrima*, *Petunia hybrida* (Druege et al. 2007), *Spilanthes calva* and *Withania somnifera* (Rai et al. 2001). It has also been found to form associations with plant species that were not previously thought to be able to form associations, for instance *Arabidopsis*, a member of the Brassicaceae (Deshmukh 2006). Once colonizing the plant, hyphae do not traverse the endodermis, nor do they penetrate the aerial tissues of the plant; the hyphae rather enter and traverse cortical cells (Shende et al. 2006). In order to form associations with the host plant, it is reported that *P. indica* interferes with host cell death so as to penetrate cells (Deshmukh et al. 2006). It is reported that *P. indica* causes reduced expression of BI-I, a cell death suppressor protein which is often activated during various stresses (Deshmukh et al. 2006). Reduction of this protein allows the endophyte to proliferate dead cells while causing no stress to the host plant (Deshmukh et al. 2006).

2.2.4 Effects of *P. indica* on Plants

Potential use for the root colonizing endophyte *P. indica* in tissue culture industries as well as in agriculture looks promising (Shende et al. 2006). Co-culture with the fungus has been found to increase plant biomass in most cases. In a study conducted by Varma et al. (1999), the root and shoot biomasses of plants treated with *P. indica* were roughly twice of those of the controls. Similarly, *Arabidopsis* seedlings were found to have slightly larger leaves, to produce more leaves, to grow faster and to flower earlier when inoculated with the fungus (Peškan-Berghöfer et al. 2004). Inoculation with *P. indica* was found to increase shoot lengths, basal stem, leaf areas, number of flowers, seed count, as well as root and shoot fresh and dry weights of both *Spilanthes calva* and *Withania somnifera* plants (Rai et al. 2001). It was not clear however, if the mentioned increases in yield of the Rai et al. study were significant. Growth promotion was found to occur before the fungus grew around or inside the root system, thus it has been hypothesized that the growth promoting effect is due to early signaling events by the endophyte (Peškan-Berghöfer et al. 2004). It has been specifically found that ethylene signaling components are required to induce growth promotion in *P. indica* associated *Arabidopsis* (Camehl et al. 2010). It has also been found that auxin diffused by the fungus contributes to the host's increased growth, while the production of other growth factors by the fungus should not be overlooked (Sirrenberg et al. 2007). Moreover, ethylene signaling components may be required to balance the beneficial and non beneficial traits of the symbiosis while also being involved in repression of defense responses in the plant. The authors suggest the control of the defense gene plays a critical role in establishments and/or maintenance of the endophytic symbiosis.

Contrary to the above studies, Stein et al. (2008) found that *P. indica*-colonized *Arabidopsis* plants had reduced main root lengths and no visible differences in shoot development or weight. It was proposed that this reduction in root length may be due to the ability of colonized plants to acquire nutrients and water more effectively than the controls, in turn requiring less root tissue.

Additionally, the endophyte has been found to increase overall plant survival, notably during adverse conditions. After transplantation, the survival rate of plants colonized with the fungus was 95%, whereas the controls only had a survival rate of 57% (Varma et al. 1999). Furthermore, *P. indica* was found to improve barley's tolerance to mild salt stress in addition to conferring resistance to some root and leaf pathogens (Waller et al. 2005). The mutualistic interaction is believed to induce systemic protection against leaf pathogens by restricting microbial invasions to early infection sites, in turn protecting unaffected plant tissues (Schäfer et al. 2007). It has also been found that the endophyte can confer drought tolerance in *Arabidopsis* by priming the aerial parts of the plants for the expression of stress-related genes, and this mechanism is likely not plant-specific due to the fact the fungus forms associations with many plant species (Sherameti et al. 2008). Despite all the evidence, *P. indica* associations may not always prove beneficial for a plant. It has been found that the fitness benefit acquired by the plant host during association with *P. indica* may come at the cost of decreased resistance against herbivores (Barazani et al. 2005). Furthermore, mycorrhizae have been found to parasitize important agricultural plants such as citrus, wheat and maize in phosphorous-rich soils (Pham et al. 2004), thus one should regard the benefits of *P. indica* with caution. That being said, since *P. indica* associations have been found to be advantageous under many circumstances, one must not ignore the possible use of *P. indica* as a model to help clarify the mechanisms of host growth and fitness promotion (Kageyama et al. 2008).

It has been suggested time and time again that this endophyte may be used as a tool in sustainable agriculture (Waller et al. 2005, Deshmukh et al. 2006, Shende et al. 2006, Kageyama et al. 2008), in agro-forestry (Varma et al. 1999, Singh et al. 2000), and in horticulture applications (Sahay and Varma 1999, Singh et al. 2000, Druege et al. 2007). Kageyama et al. (2008) offer a precautionary note stating that although *P. indica* appears to be the best example of a truly beneficial endophyte, it is difficult to predict how an inoculated plant will compete with native flora. What is more, the possibility that the growth promotion of the endophytic biofertilizer will become short lived and may even become invasive if introduced under natural conditions is of concern.

2.3 Biology of Tobacco

2.3.1 Wild-type Varieties

It can be said that tobacco is the most studied species in plant sciences, physics, chemistry, and bioengineering technologies (Tso 1999). Tobacco is a member of Solanaceae, commonly referred to as the potato or nightshade family, and within the *Nicotiana* genus established by Linnaeus in 1753 (Tso 1972, Akehurst 1981). Depending on taxonomy and nomenclature, the number of regarded tobacco species varies. Tso (1972) states that there are 63 different species of tobacco, whereas Akehurst (1981, with references therein) divides *Nicotiana* into three species: 1) *N. rustica*, 2) *N. tobacum*, and 3) *N. petuniodes*. Akehurst (1981) goes on to explain that *N. tobacum* may further be subdivided into several varieties instead of considering all the varieties as separate species due *N. tobacum*'s ability to produce different products under a variety of environmental conditions. With this in consideration, during the year 1999, there were approximately 1500 tobacco varieties in the United States Department of Agriculture's inventory (Tso 1999). Varieties differ in the shape, texture, size, colour and number of leaves, in the length of the internodes, in the size, shape, and colour of the plant, and finally the shape of the flower (Tso 1972). *N. tobacum*, the species commonly used in tobacco production, has never been found growing naturally in the wild, thus it is assumed that it arose as a natural hybrid (Akehurst 1981).

Tobacco most often produces an extremely fibrous root system with many horizontal lateral roots (Tso 1972, Akehurst 1981, Tso 1999). Leaf shapes can be ovate to oblong-lanceolate with a matt surface (Tso 1999, Akehurst 1981). Atomically, a single layer of epidermal cells are found on the upper and lower surfaces of the leaves where stomata may be found on both leaf surfaces (Akehusrt 1981). Often, there are between one and two layers of palisade cells below the upper epidermis with spongy mesophyll cells filling the remaining space to the lower epidermis (Akehusrt 1981). The leaves and stems of *N. tobacum* plants are covered with gland-like trichomes that usually secrete a sticky liquid (Tso 1999, Akehurst 1981). The sticky liquid contains precursors to the

compounds that contribute to tobacco's aroma and taste and may also play a part in insect susceptibility and resistance (Tso 1999).

Flowers are terminally located at the end of the stalk, borne in panicles (Tso 1972, Akehurst 1981, Tso 1999). Flowers are pink, with the calyx divided into five lobes and an asympetalous, five-lobed corolla (Tso 1972, Akehurst 1981, Tso 1999). The five stamens are approximately the same length as the style, lending themselves to self-fertilization (Tso 1972, Akehurst 1981, Tso 1999).

The growth curve of tobacco (total mass plotted against time) is sigmoidal (Tso 1972). Reports of environmental effects on growth vary, stating soil, light, temperature and moisture have significant sway (Tso 1972) to reports where various environmental conditions have little effect (Akehurst 1981). There is general consensus that further physiological research is required to fully understand influences on growth. A large amount of research has been completed on the effects of irrigation on tobacco growth; however, physiological plant-water relations have not been fully investigated (Flower 1999). Despite this, it is known tobacco is sensitive to water logging as gas exchange is inhibited causing a decrease in available oxygen to root surfaces (Flower 1999).

2.3.2 Transgenic Varieties

Tobacco can be used as a good model plant for studies on aquaporins because of its large size and relative ease with which it can be transformed (Siefritz et al. 2001).

2.4 Objectives and Hypotheses

With increasing agricultural demands and decreasing available landbase with which to meet those demands, it is becoming more important to find means to maximize plant biomass production. Due to growth promoting benefits, it has suggested that the endophyte *Piriformospora indica* can be used as a possible tool in sustainable agriculture (Waller et al. 2005, Deshmukh et al. 2006, Shende et al. 2006, Kageyama et

al. 2008), in agro-forestry (Varma et al. 1999, Singh et al. 2000), and in horticulture (Sahay and Varma 1999, Singh et al. 2000, Druege et al. 2007).To gain a better understanding of how this particular endophyte procures benefits to its host, growth, physiology and water relations of wild-type and transgenic tobacco (*Nicotiana tobacum*) grown in association with the fungus were observed. Transgenic plants constitutively over-expressed plasma intrinsic proteins PIP 1;4 and PIP2;5 from *Arabidopsis*. Specific objectives of the studies were to:

1. Study the effects of *Piriformospora indica* on leaf water relations of tobacco by examining transpiration, net photosynthesis, stomatal conductance, and leaf water potential.
2. Study the effects of *Piriformospora indica* on root water relations of tobacco by tracing apoplastic flow, measuring root hydrostatic hydraulic conductivity and hydraulic conductivity at the cellular level.
3. Study PIP aquaporin expression through RT-PCR and immunolocalization techniques.
4. Study the effects of *Piriformospora indica* on plant growth by measuring root and shoot mass, plant height and root system length.

The following hypotheses were examined:

1. *Piriformospora indica* has a greater effect on water relations and growth of the wild-type plants compared with those over-expressing the aquaporins.
2. *Piriformospora indica* alters PIP aquaporin expression in wildtype plants.
3. Plant biomass increases with increasing hydraulic conductivity of the roots.

For further clarification, a basic model depicting hypotheses and expected outcomes can be seen in Figure 1.1.

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3 Materials and Methods

3.1 Study 1: Effects of Piriformospora indica on Biomass and Water Relations of Tobacco

3.1.1 Plant Materials and Growth Conditions

Previously described tobacco (*Nicotiana tobacum* cv. *Xanthi*) genotypes generously donated by Dr. H. Kang (Department of Plant Biotechnology, Agricultural Plant Stress Research Center and Biotechnology Research Institute, College of Agriculture and Life Sciences, Chonnam National University) consisting of a wild-type variety and two T_3 generation to T_4 generation homozygous transgenic varieties constitutively over-expressing PIP 1;4 and PIP 2;5 from *Arabidopsis thaliana* were used. Transformation procedures used *A. tumefaciens* GV3101 and are described in detail by Jang et al. (2007).

Seeds were surface-sterilized by soaking in 1 mL 70% ethanol for 2 minutes, followed by 10 minutes in 1 mL 2.5% sodium hypochlorite, and then 2 x 1 minute in 1mL autoclaved milliQ water. Seeds were subsequently soaked in milliQ water at 4°C for 48 hours in order to induce stratification. Following sterilization, seeds were germinated on half strength MS medium (Pham et al. 2004) in 9 cm Petri dishes and kept in the dark for 48 hours at room temperature. Germinates were then submitted to the following controlled growth conditions: 16 hour photoperiod, 22°C/18°C day/night temperature, relative humidity of 65 ±10%, and an irradiance level of 300± 50 µmol m^{-2} s^{-1} at the seedling level . After two weeks, seedlings were transplanted to 3'' pots (Kord Products, Toronto, Canada) with sterilized filter sand (Target Silica Sand Products, Morinville, Alberta, Canada). The sand was sterilized by autoclaving for 30 to 60 minutes at 121°C. Plants were watered twice a week with 50% Hoagland's nutrient solution (Epstein 1972) and once weekly with distilled water to flush out accumulated ions.

3.1.2 Fungal Inoculation

Pure culture of *Piriformospora indica* was obtained from Dr. K-H. Kogel (Justus Liebig University, Geissen, Germany). The endophyte was cultured on an adapted solid modified Melin-Norkans (MMN) medium (Mason 1980) in 9 centimeter Petri dishes and in adapted MMN liquid medium in 500mL Erlenmeyer flasks. The MMN medium composition was as follows per 1 liter of milliQ water: 2.0 g malt extract, 1.0 g yeast extract, 10.0 g glucose, , 0.5 g KH_2PO_4, 0.25 g $(NH_4)_2HPO_4$, 0.15 g $MgSO_4 \cdot 7H_20$, 5 mL $CaCl_2$ (1% solution), 2.5 ml NaCl (1% solution), 1.2 mL $FeCl_3$ (1% solution), and 15.0 g agar (for solid media). The pH of the media was not adjusted.

Seven days after germination on the half-strength MS medium, a 2-4 mm MMN plug containing *P. indica* was placed at the root base of treated plants and a blank MMN plug was placed at the base of control plants. Following transplantation into the pots, the plants were re-inoculated by applying 0.5-1 mL of liquid MMN slurry with the fungus into the sand. The MMN slurry was homogenized in a blender prior to application.

3.1.3 Methods

3.1.3.1 Mycorrhizal Staining and Quantification

Detection of *P. indica* was performed by dying the roots using a Shaeffer ink and vinegar staining technique described by Veirheilig et al. (1998). This particular staining method was modified for tobacco. Six samples were randomly taken from each plant in order to get a good representation of infection across an individual root system and fixed in ethanol: glycerol: H_2O (4:3:3, by volume). Roots were then rinsed for 20 minutes to remove the fixative and cleared by boiling in 10% (weight/volume) KOH for 3 minutes. The roots were subsequently rinsed in distilled water for two minutes and placed in a 5% ink-vinegar solution for eight minutes. Destaining was accomplished by rinsing in distilled water for 20 minutes. Following staining, roots were mounted on microscope slides in lactoglycerol (lactic acid: glycerol: H_2O, 1:1:1, by volume) and observed under a

light microscope (CH30RF100, Olympus Optical Co., Japan). Fungal colonization was quantified using the slide method described by Giovannetti and Mosse (1980). Root samples from six to ten plants per treatment were used to determine root colonization rates. In order to meet assumptions of ANOVA, data were transformed so that Colonization'=SQRT(Colonization).

3.1.3.2 Root Hydraulic Conductivity

Root hydraulic conductivity using the hydrostatic pressure technique has previously been described by Wan and Zwiazek (1999), and Voicu and Zwiazek (2004). The excised root system of a plant was sealed in a pressure chamber (PMS Instrument Co. Corvalis OR. USA) while immersed in 50% Hoagland nutrient solution. The entire pressure chamber was then placed on a stirrer to rotate a stir bar in the chamber in order to continually aerate the nutrient solution.

Initially, chamber pressure was increased and maintained at 0.3 MPa for five minutes to flush water flow through the system. Pressure was next decreased to 0.10 MPa and incrementally augmented to 0.5 MPa in 0.1MPa increases. At each pressure, flow was allowed to stabilize for 5 minutes, and then steady-state flow rate (Q_v) was measured for 15 minutes, every five minutes. Water flux density (J_v) was then determined at each pressure by dividing Q_v by the root volume. Root volume in m^3 was determined by the volume displacement method as described by Kamaluddin and Zwiazek (2002). The slope of the regression line of J_v versus pressure is equal to the hydraulic conductivity (L_p) of the root system. Hydraulic conductance (K_r) was also calculated from the slope of Q_v versus the pressure. Six to seven plants per treatment were used for all measurements.

3.1.3.3 Apoplastic Water Movement

Apoplastic water movement was quantified through the use of the tracer dye trisodium, 3-hydroxy- 5,8, 10- pyrenetrisulfonate (PTS_3). PTS_3 is a fluorescent dye believed to travel exclusively through the apoplastic pathway (Hanson et al. 1985).

Excised roots were attached to the pressure chamber in the same manner in which they were attached for hydrostatic pressure measurements. The bathing solution comprised of 0.01% (wt/vol) of PTS_3. Root systems were then pressurized and maintained at 0.3 MPa for 0.5 to 1.5 hours. PTS_3 concentrations from the exudates were collected, appropriately diluted and analyzed with a Sequoia-Turner 450 fluorometer (Apple Scientific, Chesterland, OH, U.S.A.) and compared to known standardized PTS_3 concentrations of the original bathing solution. An excitation wavelength of 405 nm and an emission wavelength of 515 nm were used. Six to seven plants per treatment were used for all measurements.

3.1.3.4 Cell Hydraulic Conductivity

Half-times of water exchange ($T_{1/2}$), turgor pressure (P_t) and cell elasticity (ε) of individual cells can all the measured with the aid of a cell pressure probe (CPP). In order to determine the previously mentioned parameters, a probe was filled with silicone oil (Type AS4, Wacker, München, Germany). Microcapillaries pulled to a fine point using a pipette puller (Kopf Vertical Puller, Model 72, Tujunga, California, USA) and subsequently ground to openings ranging from of 8-10 µm were attached to the probe. A root section attached to a metal sledge was covered with paper towel and bathed in one-half strength Hoaglands solution (Epstein1972). The probe was inserted 20 mm from the root apex into the third to fifth cortical layers of eight to ten week old plants. The distance of exposed microcapillary was subtracted from the total distance of a previously marked reference point on the microcapillary to determine what depth the capillary was inserted into the root. Knowing the distance inserted in combination with the average cell diameter of each cortical layer of the root, it was possible to estimate which cortical layer was punctured. Upon insertion, a meniscus was formed in the capillary, and the meniscus position could be adjusted through pressurization and depressurization of the probe. Resultantly, the hydraulic parameters of the cell could be determined as described previously (Zimmermann et al. 2000). Turgor pressure was recorded after eight minutes once it became stable. Freehand cross and longitudinal

sections were taken 20 mm from the root apex so that mean dimensions of each cortical layer could be determined. The hydraulic conductivity (L_{pc}) of an individual cell may be calculated as follows (Azaizeh et al. 1992):

$$L_{pc} = V \times \mathrm{Ln}(2)/[\, A_c \times T_{1/2} \times (\varepsilon + \pi^i)\,] \quad (1)$$

The osmotic pressure of the cell (π^i) can be estimated by the relationship $\pi^i = P_t + \pi^o$, where π^o is equal to the external osmotic pressure of the growth solution (Azaizeh et al. 1992). With π^o attaining small values ranging between 0.02 and 0.04 MPa (Lee et al. 2010), it can be assumed that $P_t \approx \pi^i$. Cell elastic modulus (ε) can be estimated from V and changes in cell volume caused by pressurization of the probe, so that:

$$\varepsilon = V \times \Delta P_t / \Delta V \quad (2)$$

Four to six plants per treatment were used for all measurements and eight to ten week old plants were used for measurement.

3.1.3.5 Plant Biomass

Fresh root and shoot weights were measured immediately following hydrostatic and apoplastic movement measurements. Shoot height was also measured from the root-stem interface to the top of the main stem. Dry root and shoot weights were measured following drying of plant tissue in a 60°C oven. Six to ten plants per treatment were used for all measurements and plants were between 9 and ten weeks old. In order to meet assumptions of ANOVA, root to shoot data were transformed so that root : shoot' = 1 / (root : shoot + 0.75).

3.1.3.6 Transpiration and Leaf Water Potential

Leaf transpiration rates (E) were measured with the aid of an infrared gas analyzer (LCA-4, ADC Bioscientific, England) on a fully expanded leaf located in the midregion of the

stem. Measurements were taken between 8:30 and 11:00 in the morning at room temperature with an irradiance level of 450± 50 µmol m^{-2} s^{-1}. Leaf areas were determined using Sigma Scan 5.0 scanning software (Systat Software Inc., San Jose, California) so that measurements could be adjusted accordingly. Measured plants were between 9 and 10 weeks old. In order to meet assumptions of ANOVA, data were transformed so that Transpiration'= Log (Transpiration +0.0514).

To measure leaf water potential, a fully expanded leaf located in the midregion of the stem was excised and was sealed in a pressure chamber (PMS Instrument Co. Corvalis OR. USA) so that the end of the petiole was easily visible from the exterior of the chamber. Chamber pressure was then increased to the point sap exuded from the excised petiole and balance pressure was recorded. The leaf potential measured in this described manner would be an average of the total potential throughout the whole leaf (Reviewed by Tuner 1988 with references therein). In order to meet assumptions of ANOVA, data were transformed to Potential'=Log (Potential).

3.1.3.7 Net Photosynthesis and Stomatal Conductance

Net photosynthesis (A) and stomatal conductance (g_s) were measured with the aid of a infrared gas analyzer (LCA-4, ADC Bioscientific, England) on the same fully expanded leaf located on the midregion of the stem that was used for transpiration measurement mentioned in section 3.1.3.6. Both measurements were taken between 8:30 and 11:00 in the morning at room temperature with an irradiance level of 450± 50 µmol m^{-2} s^{-1}. In order to meet assumptions of ANOVA, stomatal conductance values were transformed so that stomatal conductance' = (1/Stomatal conductance + 0.05).

3.1.4 Statistical Analysis

Two-way analysis of variances (ANOVA) using Proc Mixed in the statistical program SAS 9.2 (SAS, Toronto, Ontario, Canada) were performed to determine if significant

differences existed between treatments. The following model was used to analyze the data:

$$Y_{ijk} = \mu + Genotype_i + Inoculation_j + Genotype_i \times Inoculation_j + Day_k + e_{ijkl}$$

Where:

Y_{ijk}= Value of interest
μ= mean value
$Genotype_i$ (Fixed effect) = Effect of the genotype (Wild-type, PIP 1;4, or PIP 2;5).
Inoculation (Fixed effect) = Effect of inoculation with *P. indica* (Yes or no).
$Genotype_i \times Inoculation_j$ (Fixed effect) = Effect of the interaction between genotype and inoculation.
Day_k (Random effect) = Effect of the day of measurement.
e_{ijkl} = Random error

Differences between means were considered significant at $p \leq 0.05$.

3.2 Study 2: Effects of Piriformospora indica on tobacco aquaporin expression and localization

3.2.1 Plant Materials and Growth Conditions

Wild-type tobacco (*Nicotiana tobacum* cv. *Xanthi*) generously donated by Dr. H. Kang (Department of Plant Biotechnology, Agricultural Plant Stress Research Center and Biotechnology Research Institute, College of Agriculture and Life Sciences, Chonnam National University) was used to evaluate known PIP aquaporin expression patterns. Growth conditions for the tobacco are already described in Study 1 of this thesis.

3.2.2 Fungal Inoculation

Treated plants were inoculated in the same manner as described for Study 1 (Section 3.1.2).

3.2.3 Methods

3.2.3.1 Primer Design for Real-time PCR

Complete coding sequences (cds) of *N. tobacum* aquaporin and reference genes were acquired from the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Sequence homogeneities were compared using NCBI BLAST and aquaporin specific primers were then developed using the online tool Primer3 (http://frodo.wi.mit.edu/primer3/). The online program Clustal W2 was used to perform multiple sequence alignment of developed primers to ensure selected sequences did not overlap. Primer pairs may be found in Table 2.2.3.1.

Table 3.2.3.1 Primers used for quantitative RT PCR. Selected aquaporin transcripts are as follows: aquaporins belonging to the PIP1 clade (*Nt*PIP1a and *Nt*PIP1a), aquaporin PIP 1;1 (*Nt*PIP1;1), and aquaporin PIP 2;1 (*Nt*PIP2;1). *Nt*TUB2 depicts the tobacco reference gene alpha-tubulin 2

Gene	Primer	Size of cDNA Fragment
*Nt*PIP1a (AF024511)	Forward 5'- GTT TCC TCA AGA AGC CTT AAT C-3' Reverse 5'- GAC ATT TGA ACA CAA GAA AAT CC-3'	121 bp
*Nt*PIP1b (U62280)	Forward 5'- ATA ATC ATC AGA GCC ATT GCA TTC-3' Reverse 5' - GGT ACA GGA GTC TTG AAA TAT AAC-3'	102 bp
*Nt*PIP1;1 (AF440271)	Forward 5'- GCT AAG ATT CTC CTG CCA TTT GC-3' Reverse 5'- GAA ATT ACA CAT TTG ACA GAC ACC-3'	84 bp
*Nt*PIP2;1 (AF440272)	Forward 5'-GCA TTC ATC TGT CCA AAT TAT C-3' Reverse 5'-ATA CAT CCA AGG TTA ACA TTA AGG-3'	110 bp
*Nt*TUB2 (AJ421412)	Forward 5'-GTG TTT GTT TTT GTG TTG TTT GG-3' Reverse 5'- ACA GCA TAC TAC AGT TTA GAA G-3'	99 bp

*Nt*PIP1a and *Nt*PIP1b are undefined aquaporins belonging to the PIP1 clade of tobacco. Alpha-tubulin 2 *(Nt*TUB2) is a reference gene against which the relative expression of selected aquaporins can be compared. Specific primers for tobacco actin (forward: 5' TGG ACT CTG GTG ATG GTG TC-3', and reverse: 5' CCT CCA ATC CAA ACA CTG TA-3') described by Jang at al. (2007) were also used to check the validity of actin as a possible

reference gene. Guidelines used in primer design as well as specific properties of each primer can be viewed in Appendix B.

3.2.3.2 Tissue Harvesting, cDNA Synthesis and Real-time Quantitative RT PCR

Root tissue was harvested from 4-week-old seedlings and immediately frozen with liquid nitrogen. Approximately 100 mg of frozen tissue was subsequently ground using a mortar and pestle. Total plant and fungal RNA was extracted using the RNAeasy Plant Mini Kit (Product number 74904, Qiagen Inc. Germantown, MD, USA) according to the manufacturer's instructions. Specifically, buffer RLT was added to the ground tissue to produce a lysate solution and two 20 µl washes with RNAase –free water were performed during the elution step. RNA extracts were checked for quality spectrophotometrically using the nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and by electrophoresis at 80 volts for 20 minutes, followed by storage at -80°C until further use.

cDNA was synthesized from extracted RNA and genomic DNA was removed using a QuantiTect reverse transcription kit (Product number 205311, Qiagen Inc. Germantown, MD, USA) according to manufacturer's guidelines. Reverse transcription mastermix was incubated for 30 minutes to increase cDNA yields. The concentrations of cDNA were determined through the use of a nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

In order to evaluate optimal PCR conditions, the validity of developed aquaporin primers, and the validity of possible reference genes, standard curves were developed using assay cDNA concentrations diluted from 10^{-1} to 10^{-5} ng/µl. In accordance to the assumptions of the $2^{-\Delta ct}$ method of calculating gene expression, it was determined if amplification of the reference genes were approximately equal to the amplification efficiencies of the PIP genes (Livak and Schmittgen 2001). Details envolving the calculations to compare the amplifications can be found in Appendix C. An electrophoresis using PCR products of diluted cDNA concentrations was also run at 50

volts for 40 minutes on a 1.6% agarose gel in 1x TAE buffer to determine validity of candidate reference genes.

A mastermix of 120 ng of cDNA, 10 µl of Quantitect SYBRgreen (Product number 1017340, Qiagen Inc., Germantown, MD, USA), 10 pmol of each primer, and RNase free water adding to a final volume of 20 µl was used for amplification. Two replications were performed per sample, three samples per treatment. The 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA) was programmed to the following conditions: 95°C for 15 minutes, 50 cycles (95°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds), 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. Following amplification, standard curves were analyzed to determine cycle threshold (ct) and mean ct-values were determined for each sample. Δct was then calculated by subtracting the mean ct-value of the control samples from the ct-value of inoculated samples. Expression levels were determined by calculating $2^{-\Delta ct}$ and data were normalized with the actin and *Nt*TUB2 housekeeping gene.

3.2.3.3 Checking Inoculation with PCR

Presence or absence of *P. indica* in roots was monitored using the *Pitef*1 gene which encodes for the elongation factor EF-1α of the endophyte (Bütehorn et al. 2000). The equivalent of 200 ng of synthesized cDNA from extracted RNA was used for PCR with 7µl Redtract-N-Amp PCR readymix (Product number R4775, Sigma-Aldrich, St, Louis, MO, USA), 10 pmol each of *Pitef*1 specific primers (tefGSPa: 5'- CTT GAC CTC CTT CGA CCA TC-3' and tefGSPd: 5'- AAC ATG ATT ACC GGT ACC TCG CAG-3') described by Bütehorn et al. (2000), 0.5 µl Tween20 1%, and 2 µl of RNase free water. Amplification was carried out using a Mastercycler Pro S (Eppendorf AG, Hamburg, Germany) under the following conditions: 95°C for 1 minute, 55°C for 90 seconds, 72°C for 2 minutes, 35 cycles (94°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute), 72°C for 15 minutes. PCR conditions were modified from those described by Kawasaki (1990). An electrophoresis was run on reamplified PCR products on a 1% agarose gel using 1x TAE buffer and 1.5 µl SYBRsafe (Product number S33102, Sigma-Aldrich, St, Louis, MO, USA). Electrophoresis

was conducted at a constant 80 volts for 40 minutes in 1x TAE buffer. Bands were viewed under UV light.

Remaining primers and nucleotides were removed from PCR products using ExoSAP-it (Affymetrix/USB Corporation, Santa Clara, CA, USA) by incubating at 37°C for 15 minutes. Enzymes were then inactivated by incubating cleaned products at 80°C for 15 minutes. Following cleanup, 40 to 100 ng of cDNA, 5 pMol of *Pitef*1 specific primers (5' and 3' in separate reactions), 2 µl of Big Dye, 4µl of 3x Big Dye sequencing buffer (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) and RNase free water to total 20 µl were combined for sequencing. The mixture was incubated at 96°C for 1 minute, 35 cycles (96°C for 15 seconds, 50°C for 20 seconds, 60°C for 2 minutes), and held at 10°C for 10 minutes. A final clean-up of samples was performed using ethanol precipitation according to the Molecular Biology Services Unit (MBSU, Biosciences, University of Alberta) protocol. Samples were sequenced by MBSU and compared to the *Pitef*1 cDNA sequence (Bütehorn et al. 2000) using Clustal W2 and NCBI BLAST.

3.2.3.4 Immunofluorescence

Localization of the expression of PIP aquaporins was performed by immodetection approaches described by Hachez et al. (2006). Free hand cross sections were taken 20 mm from the root apex of six week old seedlings and PIP1 (*Zm*PIP 1, R-4445) and PIP2 (*Zm*PIP 2, R-2493) aquaporins were subsequently localized through the use of a diluted 1/100 anti-PIP antiserum (primary antibody) developed by Hachez et al.(2006). After several washes in blocking solution, sections were incubated in diluted 1/100 fluorescein-coupled goat anti-rabbit IgG antibodies (Secondary antibody; Product number F2765, Molecular Probes, Eugen, Oregon, USA). Sections were viewed under UV light (Excitation: 450-4905nm, Emission: 510 nm) at at the same exposure using a Leica DMR RXA microscope (Leica Microsystems, Concord, Ontario, Canada). Controls included untreated sections, sections incubated in only pre-immune serum, and sections incubated in only the secondary antibody. More details concerning the development of antibodies and the role of controls in immunofluorescence can be found in Appendix E.

3.2.4 Statistical Analysis

One-way analysis of variances (ANOVA) using Proc Mixed in the statistical program SAS 9.2 (SAS, Toronto, Ontario, Canada) were performed to determine if significant differences existed between treatments. The following model was used to analyze the data:

$Y_{ijk} = \mu + \text{Inoculation}_j + e_{ijkl}$

Where:

Y_{ijk}= Value of interest
μ= mean value
Inoculation (Fixed effect) = Effect of inoculation with *P. indica* (Yes or no).
e_{ijkl} = Random error

Differences between means were considered significant at $p \leq 0.05$.

4 Results

4.1 *Study 1: Effects of Piriformospora indica on Biomass and Water Relations of Tobacco*

4.1.1 Root Colonization

Control plants had mean colonization rates ranging between 2.03% and 4.23% of the root length, whereas inoculated plants had colonization rates ranging between 22.29% and 24.65% of the root length (Fig. 4.1.1). Despite the highest precautions, sand of the control treatments could not remain sterile throughout the entire experiment and some plants became infected with fungi, although it was not determined if contamination was *P. indica* or another fungus. Significant differences were found for the overall inoculation effect, however, no significant differences were found when looking at interaction or at genotypic effects.

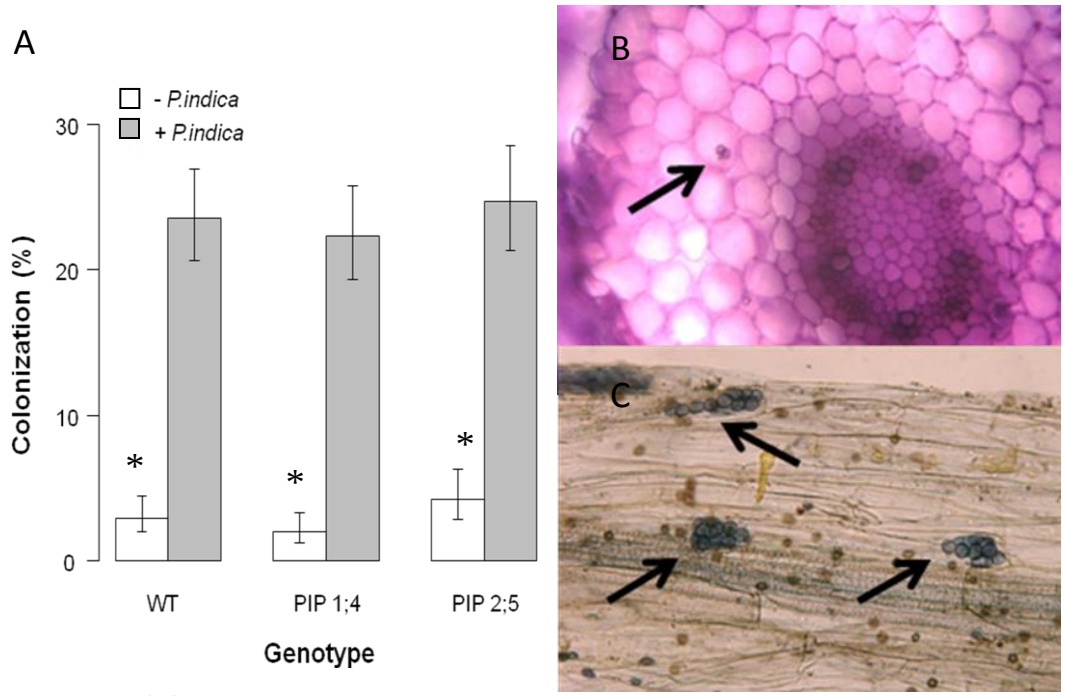

Figure 4.1.1: (A) Mean percent colonization of *Piriformospora indica* roots in three *Nicotiana tobacum* lines. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Significant differences between controls and inoculated plants are indicated by the asterisk ($P \leq 0.05$) and error bars represent the standard error of the mean (n= 6-10). **(B)** Cross section stained with toluidine blue, and **(C)** lateral section stained with Schaeffer ink. *P. indica* chlamydospores are indicated by arrows.

4.1.2 Root Hydraulic Conductivity (L_{pr})

Combined control plants had a greater mean hydrostatic L_{pr} value of 4.12 x 10^{-10} m^3 s^{-1} cm^{-3} MPa^{-1}, compared to combined inoculated plants with a value of 2.87 x 10^{-10} m^3 s^{-1} cm^{-3} MPa^{-1} (Figure 4.1.2B). Significant differences were found for the overall inoculation effect (p-value of 0.0272); however no significant differences were found when looking at interaction or at genotypic effects (p-values of 0.7503 and 0.9212 respectively). No significant differences were found between Kr values when looking at inoculation, genotypic, or interaction effects (p-values ranged between 0.2506 and 0.4436). Figure A.4.1.2. of Appendix A depicts mean values of K_r.

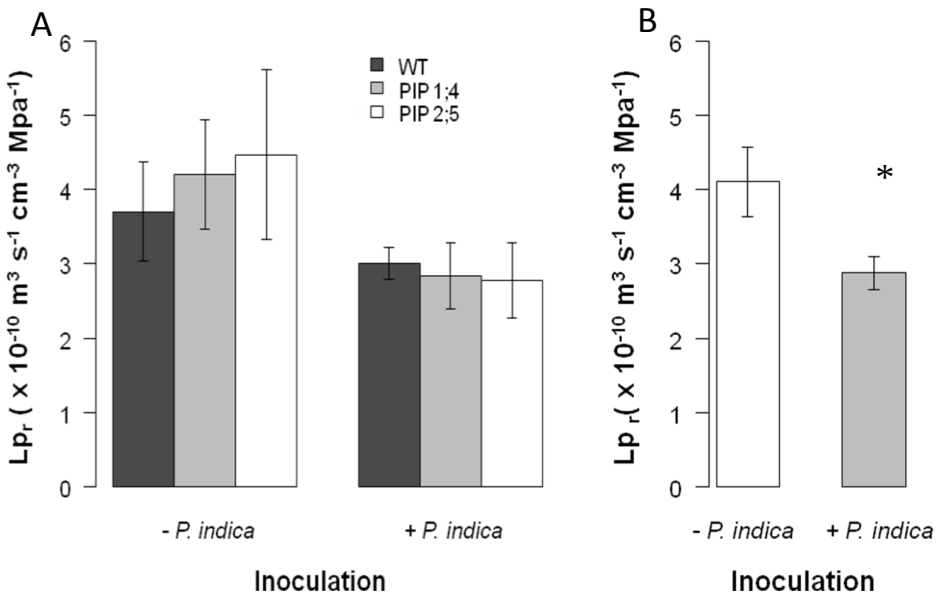

Figure 4.1.2: Root hydraulic conductivity (L_{Pr}) determined by the hydrostatic method in *Nicotiana tobacum* lines inoculated (+*P. indica*) and not inoculated (-*P. indica*) with *Piriformospora indica*. L_{pr} was determined for (**A)** all treatment groups, and **(B)** combined +*P. indica* and -*P. indica* plants regardless of genotype. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Significant differences are indicated by the asterisk ($P \leq 0.05$) and error bars represent the standard error of the mean (n= 6-7).

4.1.3 Xylem Sap PTS_3 Concentrations

Means and standard errors of % PTS_3 (trisodium, 3-hydroxy-5,8, 10- pyrenetrisulfonate) are depicted in Figure 4.1.3. The percentage represents the concentration of apoplastic dye exuded through the root system compared to that of the bathing solution, where a greater percentage indicates more water movement within the apoplastic pathway. Significant differences were not found between treatments when comparing Tukey adjusted p-values to an alpha of 0.05.

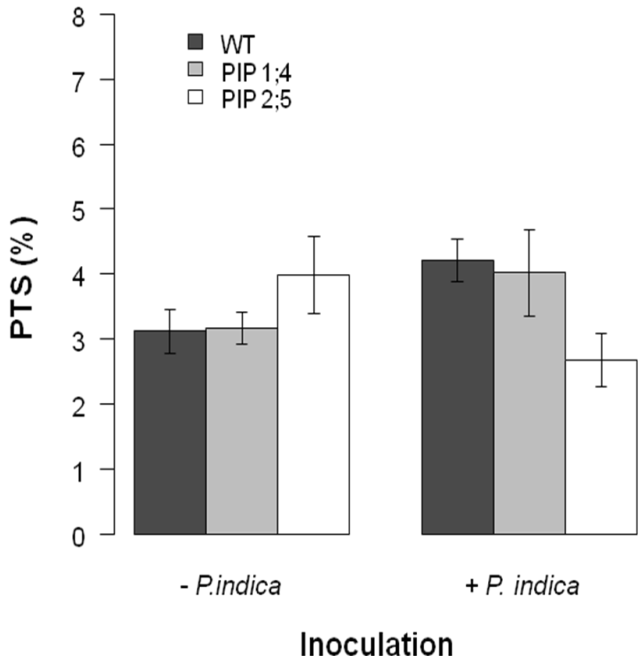

Figure 4.1.3: Effects of *Piriformospora indica* on trisodium, 3-hydroxy- 5, 8, 10-pyrenetrisulfonate (PTS_3) concentration in the xylem sap of three *Nicotiana tobacum* lines. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Significant differences are indicated by lower case letters ($P \leq 0.05$) and error bars represent the standard error of the mean (n= 6-7).

4.1.4 Cell Water Relations

An example of the pressure output from pressure relaxations, and rapid pressurizations and depressurizations is shown in Figure 4.1.4.1. No significant differences in cell turgor pressure (P) (p-values ranging between 0.5784 and 0.6699) or half-times of water exchange ($T_{1/2}$) (p-values ranging between 0.4904 and 0.7660) were found. Mean P and $T_{1/2}$ values can be seen in Figure 4.1.4.2. There were no significant differences in cell hydraulic conductivity (L_{pc}) for the inoculation, genotypic, or interaction effects with p-values ranging between 0.7512 and 8826.

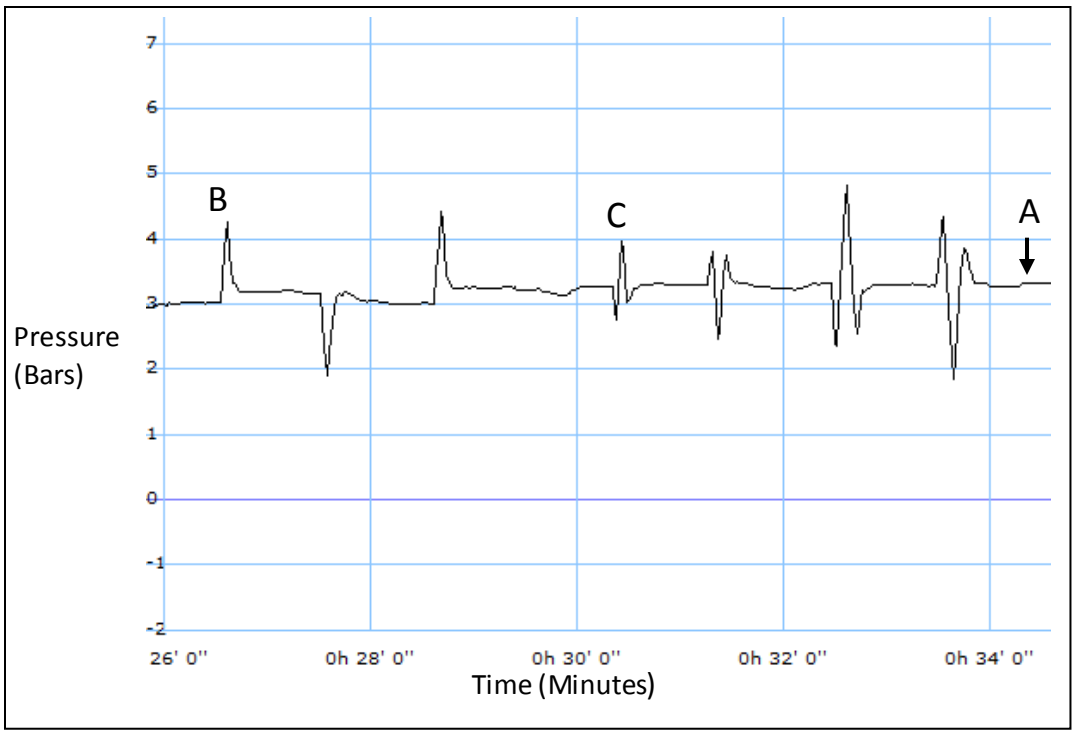

Figure 4.1.4.1: (A) Constant cell turgor. **(B)** Half-times of water exchange were determined by rapid pressurization or depressurization of the cell pressure probe and observing the resulting half-time it took for cell turgor to revert to normal levels. **(C)** Cell elasticity determined from rapidly changing the cell volume and observing corresponding changes in pressure.

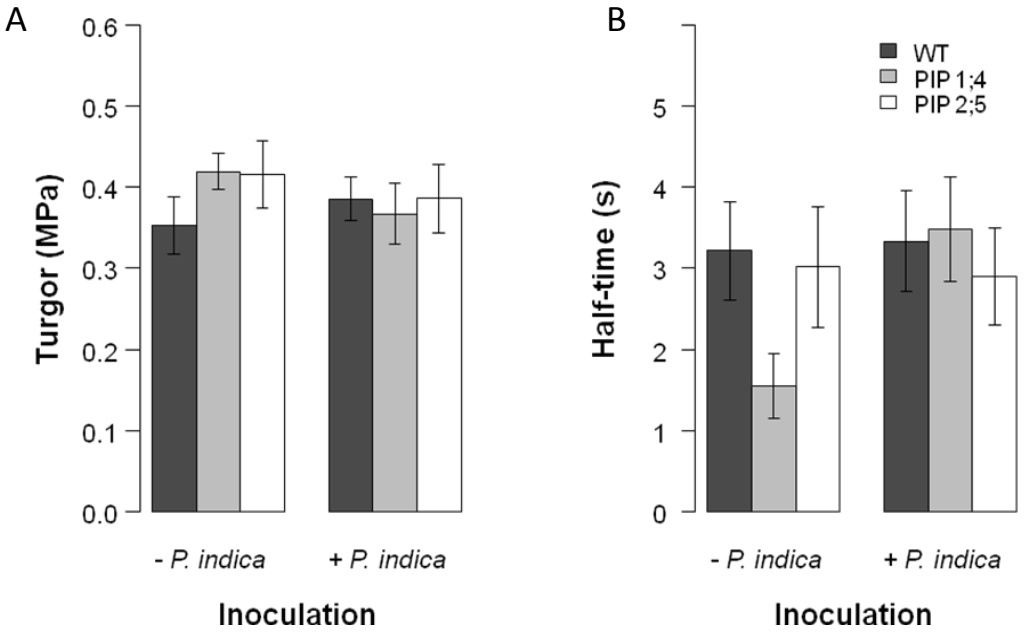

Figure 4.1.4.2: (**A**) Cell turgor pressure (P),and (**B**) half-times of cell water exchange ($T_{1/2}$) in root cortex cells of *Nicotiana tobacum* lines inoculated and not inoculated with *Piriformospora indica*. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP 1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Error bars represent standard error of the mean (n=4-6).

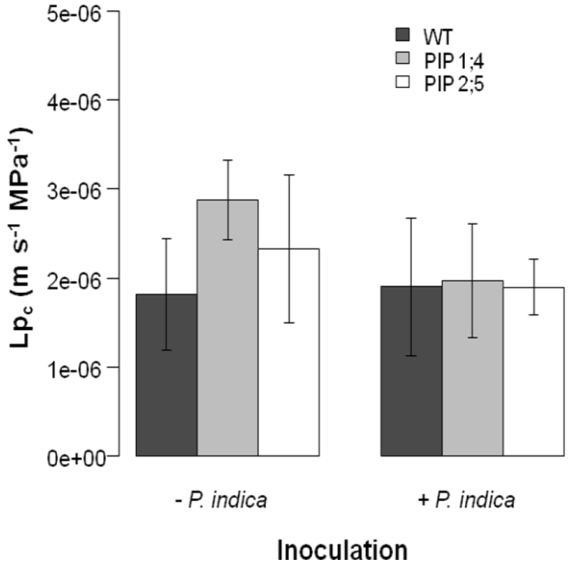

Figure 4.1.4.3: Effects of *Piriformospora indica* on root cell hydraulic conductivity (L_{pc}) of three *Nicotiana tobacum* lines. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Parameters used to calculate L_{pc} were measured with the cell pressure probe. No significant differences were found ($P \leq 0.05$), n=4-6. Error bars represent standard error of the mean.

Table 4.1.4: Significance values for cell turgor pressure (Turgor), half-times of water exchange ($T_{½}$), and cellular hydraulic conductivity (L_{pc}). ANOVA results indicate p-values for the genotypic, inoculation and interaction effects where three *N. tobacum* genotypes were inoculated and not inoculated with *Piriformospora indica.* No significant differences were found ($P \leq 0.05$)

Effect	Turgor (Pf > F)	$T_{1/2}$ (Pf > F)	L_p (Pf > F)
Genotype	0.6505	0.5454	0.7512
Inoculation	0.6699	0.4904	0.8333
Genotype * Inoculation	0.5784	0.766	0.8826

4.1.5 Plant Biomass

An inoculation effect was found for mean fresh and dry root weights, mean fresh and dry shoot weights, and mean shoot height. There was a general trend that *P. indica* increased root and shoot dry weights in both wild-type and PIP 2;5 over-expression plants, but not in PIP1;4 over-expression plants. There was a respective 28.7% and 36.8% increase in mean fresh root weight of inoculated wild-type and PIP 2;5 plants compared to controls. A 40.2% increase in mean fresh shoot weight of PIP 2;5 inoculated plants was observed when compared to the controls. Similarly, dry root weight of inoculated wild-type plants was increased by 53.4% and dry shoot weight was increased by 29.6% and 47.1% in wild-type and PIP 2;5 plants respectively. Mean shoot height of PIP 2;5 overexpression plants was increased by 35.0% upon inoculation. No significant differences were found for the inoculation, genotypic, or interaction effects for mean root system length and mean root to shoot ratio.

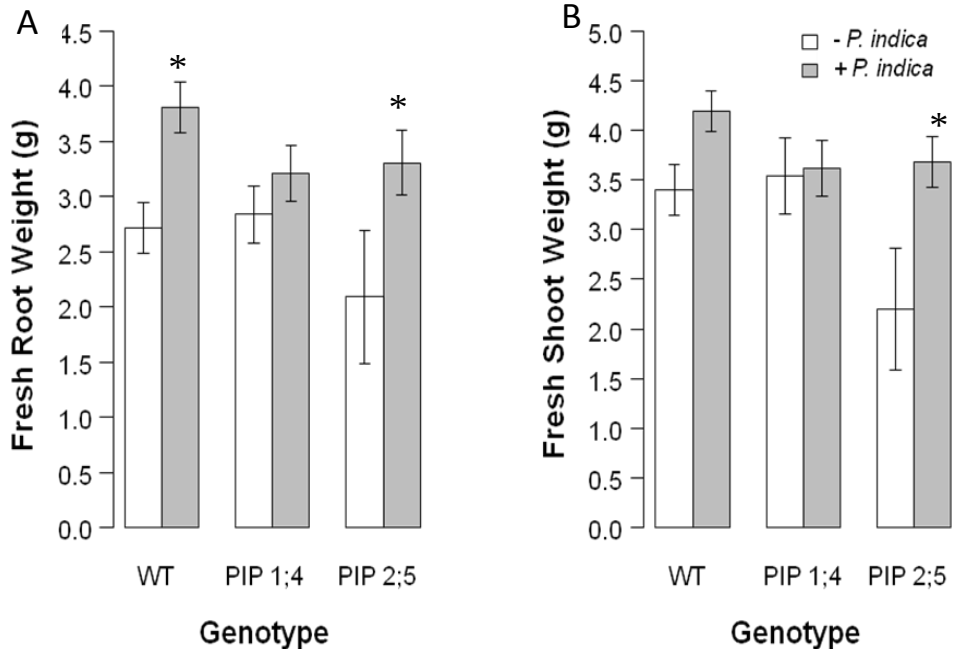

Figure 4.1.5.1: Effects of *Piriformospora indica* on (**A**) fresh root weight (g), and (**B**) fresh shoot weight (g) of three *Nicotiana tobacum* lines. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1; 4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Significant differences within genotypes are indicated by the asterisk ($P \leq 0.05$). Error bars represent standard error of the mean (n = 6-10).

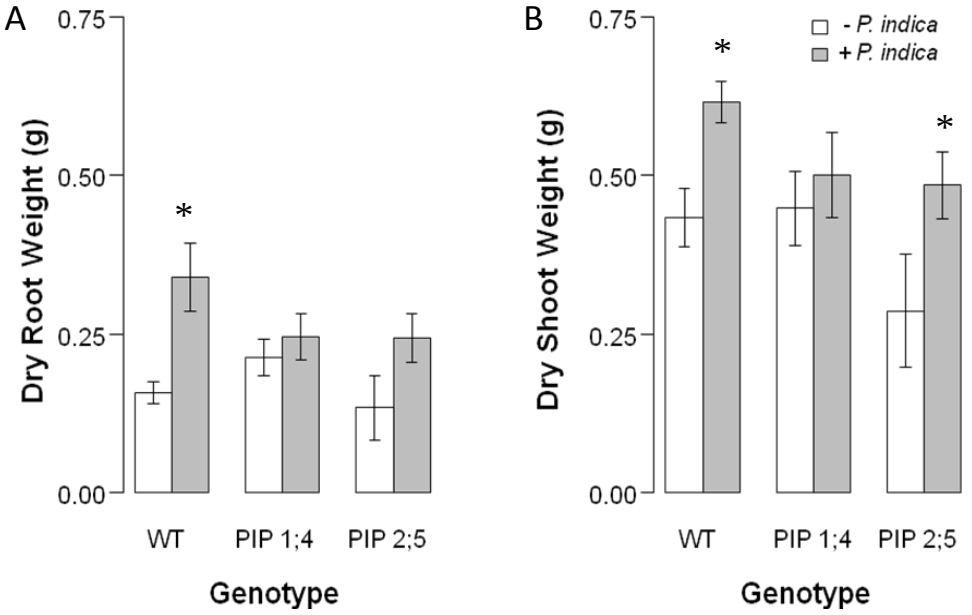

Figure 4.5.1.2: Effects of *Piriformospora indica* on (**A**) dry shoot weight (g), and (**B**) dry root weight (g) of three *Nicotiana tobacum* lines. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Significant differences within genotypes are indicated by the asterisk ($P \leq 0.05$). Error bars represent standard error of the mean (n=6-10).

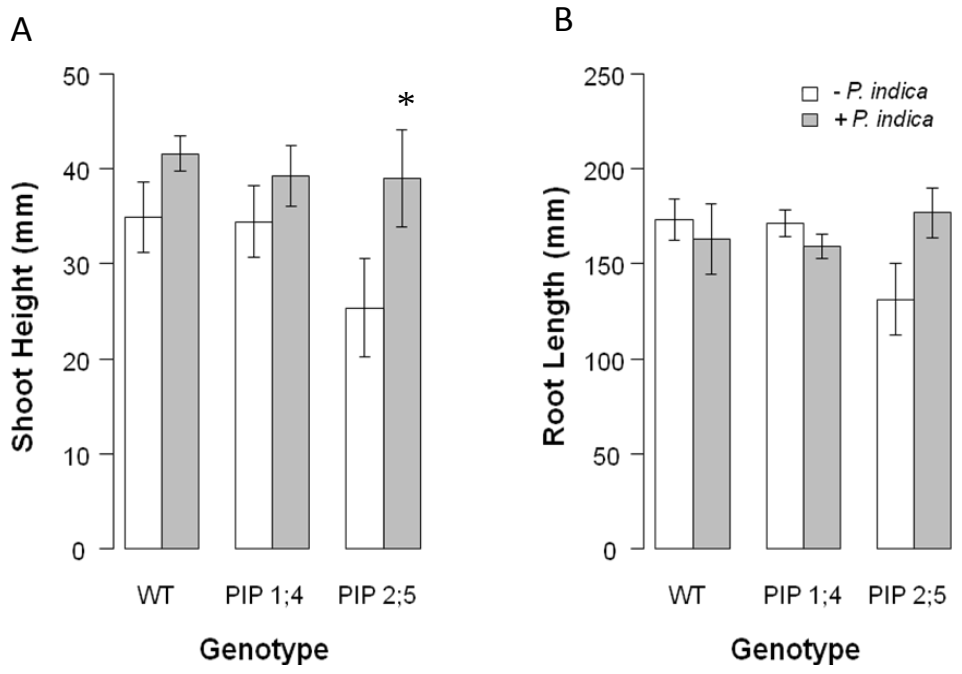

Figure 4.5.1.3: Effects of *Piriformospora indica* on (**A**) shoot height (mm), and (**B**) root length (mm) of three *Nicotiana tobacum* lines. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Significant differences within genotypes are indicated by the asterisk ($P \leq 0.05$). Error bars represent standard error of the mean (n=6-10).

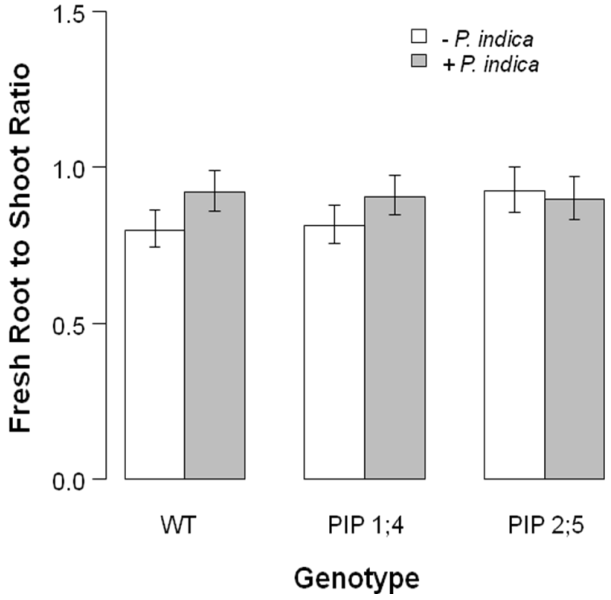

Figure 4.5.1.4: Effects of *Piriformospora indica* on root to shoot ratio of three *Nicotiana tobacum* lines. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Error bars represent standard error of the mean (n=6-10).

Table 4.1.5: Significance values for dry root weight, dry shoot weight, fresh root weight, fresh shoot weight, shoot height, root system length, and root to shoot ratio. ANOVA results indicate p-values for the genotypic, inoculation and interaction effects where three *N. tobacum* genotypes were inoculated and not inoculated with *Piriformospora indica.* Significant differences are indicated by asterisks ($P \leq 0.05$); "n/a" indicates comparison was not possible due to a lack of significant difference of the main fixed effect

Effect	Dry Root Weight (Pf > F)	Dry Shoot Weight (Pf > F)	Fresh Root Weight (Pf > F)	Fresh Shoot Weight (Pf > F)	Shoot Height (Pf > F)	Root Length (Pf > F)	Root : Shoot (P> F)
Genotype	0.3796	0.0768	0.2228	0.0501	0.2202	0.5744	0.5856
Inoculation	0.0024*	0.0034*	0.0011*	0.0075*	0.0060*	0.4816	0.1652
Genotype * Inoculation	0.1679	0.3291	0.3495	0.1083	0.3435	0.0784	0.3839
WT Control vs. WT Inoculated	0.0024*	0.0264*	0.0148*	0.1006	0.1673	n/a	n/a
PIP 1;4 Control vs. PIP 1;4 Inoculated	0.5615	0.5252	0.3932	0.9353	0.4004	n/a	n/a
PIP 2;5 Control vs. PIP2;5 Inoculated	0.0802	0.0220*	0.0129*	0.0045*	0.0100*	n/a	n/a

4.1.6 Transpiration and Leaf Water Potential

Transpiration rates were found to be higher in control plants at 0.3857 mmol $m^{-2}s^{-1}$ versus 0.2689 mmol $m^{-2}s^{-1}$ in inoculated plants (Figure 4.1.6.1). The inoculation effect on leaf transpiration was significant with a p-value of 0.0413, whereas the genotypic and interaction effects were not significant with respective p-values of 0.2106 and 0.8050. Differences in transpiration rates were not significant when comparing controls and inoculated plants within the same geneotype.

Leaf water potential and standard errors of the mean are presented in Figure 4.1.6.2. P-values of 0.8154 for the genotypic effect, 0.3242 for the inoculation effect, and 0.1840 for the interaction effect indicate that no significant differences were found between leaf water potentials of all treatment levels.

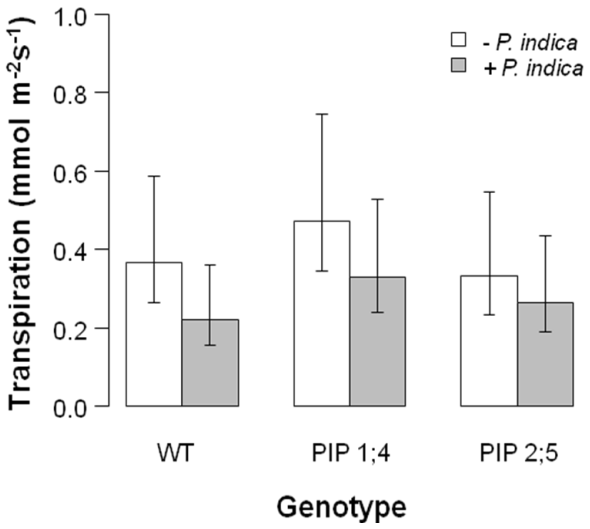

Figure 4.1.6.1: Effects of *Piriformospora indica* on leaf transpiration rate (mmol $m^{-2}s^{-1}$) of three *Nicotiana tobacum* lines. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Significant differences are indicated by the asterisk ($P \leq 0.05$). Means (n= 6-10) ± standard errors are shown.

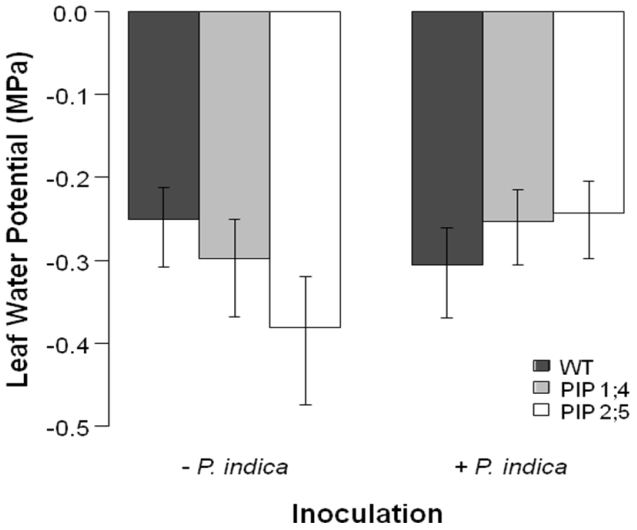

Figure 4.1.6.2: Effects of *Piriformospora indica* on leaf water potential (MPa) of three *Nicotiana tobacum* lines. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Means (n= 6-10) ± standard errors are shown.

Table 4.1.6: Significance values for leaf transpiration rates (E) and leaf water potential (Ψl_{eaf}). ANOVA results indicate p-values for the genotypic, inoculation and interaction effects where three *N. tobacum* genotypes (wild-type (WT), _over-expression of aquaporin PIP 1;4 (PIP1;4), and _over-expression of aquaporin PIP2;5 (PIP2;5)) were inoculated and not inoculated (control) with *Piriformospora indica.* Significant differences are represented by asterisks ($P \leq 0.05$); "n/a" indicates comparison was not possible due to a lack of significant difference of the main fixed effect

Effect	E (Pf > F)	Ψ_{leaf} (Pf > F)
Genotype	0.2106	0.8154
Inoculation	0.0413*	0.3242
Genotype * Inoculation	0.8050	0.1840
WT Control vs. WT Inoculated	0.0855	n/a
PIP 1;4 Control vs. PIP 1;4 Inoculated	0.1921	n/a
PIP 2;5 Control vs. PIP2;5 Inoculated	0.5071	n/a

4.1.7 Net Photosynthesis and Stomatal Conductance

Means and standard errors for net photosynthesis and stomatal conductance are in Figure 4.1.7. The interaction effect for net photosynthesis was found to be significant with a p-value of 0.0089. Control PIP 2;5 over-expression plants were observed to have

a net photosynthetic rate 2.94 times higher than its inoculated counterpart, whereas all other treatment levels were not found to be significantly different from each other.

For the stomatal conductance, the inoculation effect was significant with a p-value of 0.0045, whereas the genotypic and interaction effects were not significant with p-values of 0.1415 and 0.6563 respectively. *P. indica* had an effect on stomatal conductance only when comparing PIP 2;5 over-expression controls to treated PIP 2;5 over-expression plants. In general, stomatal conductance was higher in control plants than in inoculated plants.

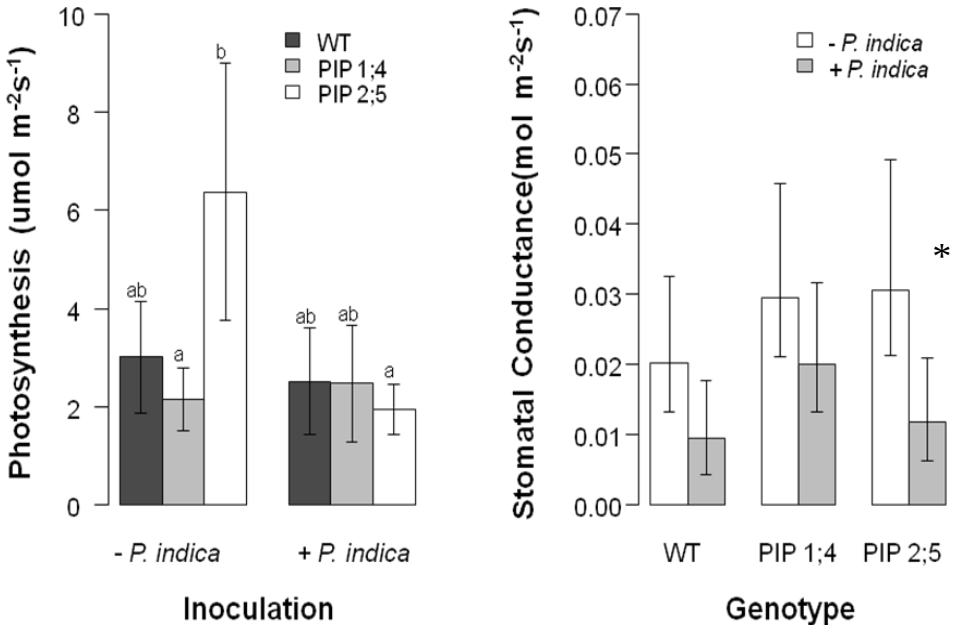

Figure 4.1.7: Effects of *Piriformospora indica* on **(A)** net photosynthesis (µmol $m^{-2}s^{-1}$) and, **(B)** stomatal conductance (mol $m^{-2}s^{-1}$) of three *Nicotiana tobacum* lines. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Significant differences between all treatment levels are indicated by lowercase letters and significant differences within genotype are indicated by the asterisk ($P \leq 0.05$). Means (n= 6-10) ± standard errors are shown.

Table 4.1.7: Significance values for leaf net photosynthesis (A) and stomatal conductance (g_s). ANOVA results indicate p-values for the genotypic, inoculation and interaction effects where three *N. tobacum* genotypes (wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5)) were inoculated and not inoculated with *Piriformospora indica.* Only significant differences are shown and are represented by the asterisk ($P \leq 0.05$); "n/a" indicates comparison was not possible due to a lack of significant difference of the main fixed effect

Effect	A (Pf > F)	g_s (Pf > F)
Genotype	0.1217	0.1415
Inoculation	0.0791	0.0045*
Genotype * Inoculation	0.0089*	0.6563
WT Control vs. WT Inoculated	1.000	0.0982
PIP 1;4 Control vs. PIP 1;4 Inoculated	0.9683	0.2288
PIP 2;5 Control vs. PIP2;5 Inoculated	0.0130*	0.0312*
PIP 1;4 Control vs. PIP 2;5 Control	0.0133*	n/a

4.2 Study 2: Effects of Piriformospora indica on Tobacco Aquaporin Expression and Localization

4.2.1 Primer Design for Real-Time RT PCR

Standard curves and electrophoresis using assay cDNA concentrations diluted from 10^{-1} to 10^{-5} depicted *Nt*Actin as a suitable reference gene due to its amplification efficiency being approximately equal to that of the target genes. Assumptions of the $2^{-\Delta ct}$ method for determining gene expression levels as well as comparison of amplification efficiencies can be found in Appendix C. Assay PCR also confirmed primers were functional.

4.2.2 Tissue Harvesting, cDNA Synthesis, and Quantitative Real-time PCR

Total RNA was successfully extracted from tobacco roots and RNA concentrations ranged between 57.85 ng μl^{-1} and 419.22 ng μl^{-1}. Electrophoresis of RNA products revealed two distinct bands, a heavier band depicting plant RNA, and a lighter band depicting fungal RNA (Data not shown). Genomic DNA was removed from the RNA product and cDNA was synthesized to a total of 500 ng.

Quantitative real-time PCR was thus performed and relative abundances of four *N. tobacum* aquaporins compared to *Nt*Actin are in Figure 4.2.2. No significant differences in individual aquaporin expression levels were observed upon inoculation as p-values between mycorrhizal and nonmycorrhizal plants ranged between 0.1328 and 0.6832. Surprisingly *Nt*PIP1;1 levels were found to be quite high with relative expression levels of 754.82 in control plants and 954.77 in inoculated plants, whereas relative expression levels of *Nt*PIP2;1were low with levels below 13. Relative abundances of the four *N. tobacum* aquaporins compared to the reference *Nt*TUB2 can be seen in Figure D.4.2.2. of Appendix D.

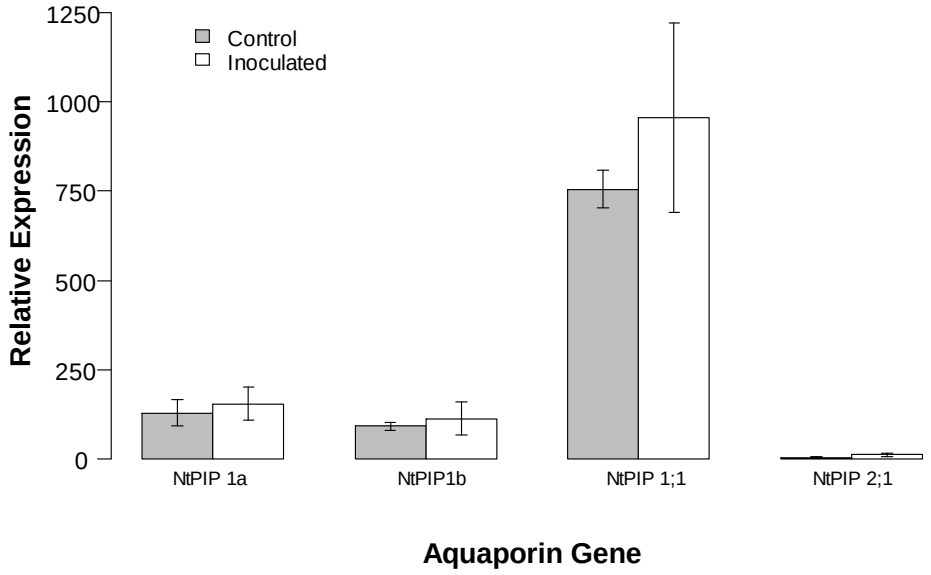

Figure 4.2.2: Effects of *Piriformospora indica* on relative expression patterns of four aquaporin transcripts in wild-type *Nicotiana tobacum* roots. Selected aquaporin transcripts are as follows: aquaporins belonging to the PIP1 clade (*Nt*PIP1a and *Nt*PIP1a), aquaporin PIP 1;1 (*Nt*PIP1;1), and aquaporin PIP 2;1 (*Nt*PIP2;1). Relative transcript abundance is described as the fold change of mean cDNA expression of the aquaporin gene relative to the reference gene (Actin, *Nt*Actin). Quantification was determined by the standard curve of quantitative RT PCR. There were three plants per treatment and two replicates per plant. No significant differences were found between aquaporin expression patterns when comparing inoculated plants to control plants ($P \leq 0.05$). Error bars represent the standard error of the mean.

Table 4.2.2: Significance values for aquaporin relative expression levels between control wild-type *N. tobacum* roots and roots inoculated with *Piriformospora indica*. Fold change of mean cDNA expression of the aquaporin gene was relative to the reference gene actin (*Nt*Actin). ANOVA results indicate p-values of the inoculation effect

Aquaporin	(Pf > F)
*Nt*PIP 1a	0.6724
*Nt*PIP 1b	0.6832
*Nt*PIP 1;1	0.5012
*Nt*PIP 2;1	0.1328

4.2.3 Fungal DNA in Roots

Synthesized cDNA from two inoculated samples were sent to MBSU for sequencing in order to check the validity of the *P. indica* reverse (tefGSPa) and forward (tefGSPd) primers used. Sequenced cDNA strands selected by the primers from the two samples can be observed in Figure 4.2.3.1. Homogeneity of selected cDNA sequences from the roots compared to the coding sequence (cds) of the *Piriformospora indica* EF1-α gene indicated that the primers were correctly selecting for the endophyte as queried sequences were between 97 and 100% homologous to the EF1-α gene (Figures 4.2.3.2 and 4.2.3.3). Furthermore, electrophoresis of PCR samples indicated the controls did not contain any *P. indica* in their roots whereas a band of approximately 350bp indicated the treated roots were inoculated with the fungus.

A

Sample 1 forward sequence:

NNNNNNNNNNNNNNNNNNNNNNNNNNNCCNGNGGNNNNGTGAGTTCGAGGNTNGTNNCTCCANGA
TGGCCAGACTNNNNNNNNNNNNNNGCTCGCCTTTACCCTCGGTGTCCGACAGCTCNNCNTNNCTG
TCAACAAGATGGACACCACCAACTGGTCTGAGGCCCGCTTCAACGAAATCGTCAAGGAAACCTCC
AACTTCATCAAGAAGGTCGGATACAACCCCAAGACGGTCGCCTTCGTCCCCATCTCTGGCTGGCA
CGGTGACAACATGCTCGAGCCCTCCACCAACATGCCCTGGTACAAGGGATGGTCGAAGGAGGTNN
AGNNNNNNNNGAAGGAGGTCAAGATGGTCGAAGGAGGTCAAGATGGTCGAAGGAGGTCAAGATGG
TCNAANGANGTCAAGATGNNCNANNNNNNNNNNNNNNNNNNNNNTTTTTTNTNN

Sample 1 reverse sequence:

NNNNNNNNNNNNNNNNNNNNGGNTCGNGCATGTTGTCACCGTGCCNGCCAGAGATGGGGACGAAG
GCGACCGTCTNNGNNNNGTATCCGACCTTCTTGATGAAGTTGGAGGTTTCCTTGACGATTTCGTT
GAAGCGGGCCTCANACCAGTTGGTGGTGTCCATCTTGTTGACAGCGACGATGAGCTGTCGGACAC
CGAGGGTAAAGGCGAGCAAAGCATGCTCACGAGTCTGGCCATCCTTGGAGATACCAGCCTCGAAC
TCACCGGTACCACCGGCGATGATGAGGATAGCGCAATCAGCCTGCGAGGTACNNNNNNNCATNNN
NNNNNNGAAGGAGGTCAAGATGGTCGAAGGAGGTCAAGATGGTCGAAGGAGGTNAAGANGGTCGA
AGGAGGTNNNGATGGTCGAAGGAGGTCNNGANNNCCGNNNNNNGTNNNTANNNNNNNTNNNANNN
NNNNNNCNNNNTNNNNNGNNNGTNNNNANNNNCNCNNNNNTN

B

Sample 2 forward sequence:

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGNNNNGNNNNTTNNNNNNNNNNTCNCCNNGATGG
CCAGACNNNNNNNNNNNNNNGCTCGCCTTTACCCTCGGTGTCCGACAGCTCNNNNNNNCTGTCAAC
AAGATGGACACCACCAACTGGTCTGAGGCCCGCTTCAACGAAATCGTCAAGGAAACCTCCAACTT
CATCAAGAAGGTCGGATACAACCCCAAGACGGTCGCCTTCGTCCCCATCTCTGGCTGGCACGGTG
ACAACATGCTCGAGCCCTCCACCAACATGCCCTGGTACAAGGGATGGTCGAAGGAGGTCAAGAGT
TAA

Sample 2 reverse sequence:

NNNNNNNNNNNNNNNNNNNGNNANNNNNNNNNNGNNNNNNNNGNNNNNNGATGGGGACGAAGGNN
ANGNNNNNNNNNNNNNNACCTTNTNGATGAAGTTGGAGGTTTNNNNNNNNNNNNNNTTGAAGCGG
GCCTCAGACCAGTTGGTGGTGTCCATCTTGTTGACAGCGACGATGAGCTGTCGGACACCGAGGGT
AAAGGCGAGCAAAGCATGCTCACGAGTCTGGCCATCCTTGGAGATACCAGCCTCGAACTCACCGG
TACCACCGGCGATGATGAGGATAGCGCAATCAGCCTGCGAGGTACCGGTAATCATNNNNNN

Figure 4.2.3.1: Sequenced cDNA from the EF1-α gene of *Piriformospora indica* using extracted RNA from **(A)** sample1 and, **(B)** sample 2 inoculated with the fungus. The forward primer (tefGSPd) and reverse primer (tefGSPa) used were those described by Bütehorn et al. (2000).

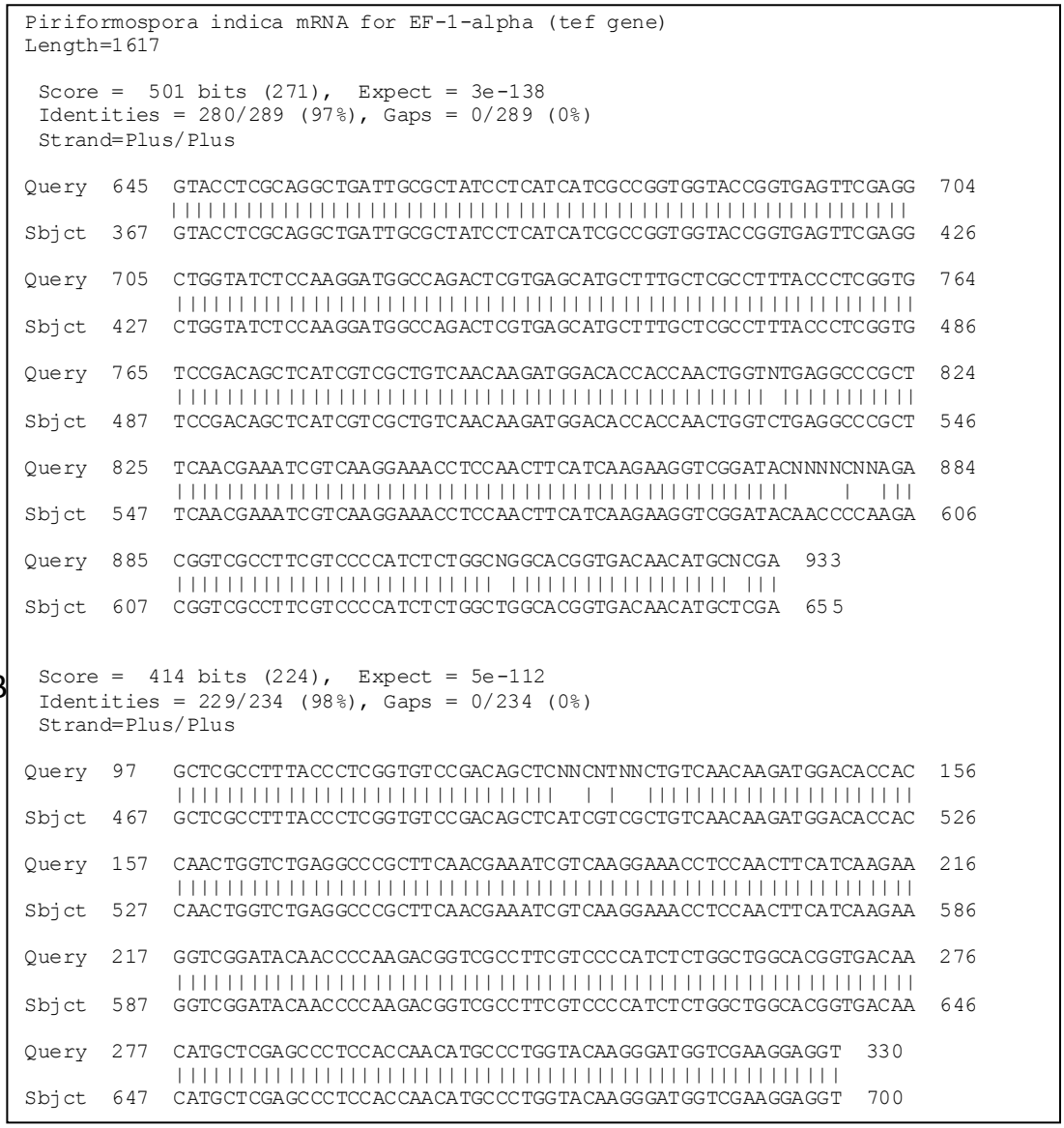

Figure 4.2.3.2: Nucleotide sequence homogeneities of sample 1 were compared to the complete coding sequence (cds) of the *Piriformospora indica* EF1-α gene using **(A)** the complementary reverse sequence of cDNA selected with the reverse primer (tefGSPa), and **(B)** cDNA selected using the forward primer (tefGSPd). Nucleotides were aligned using the NCBI BLAST tool (NCBI, http://www.ncbi.nlm.nih.gov/).

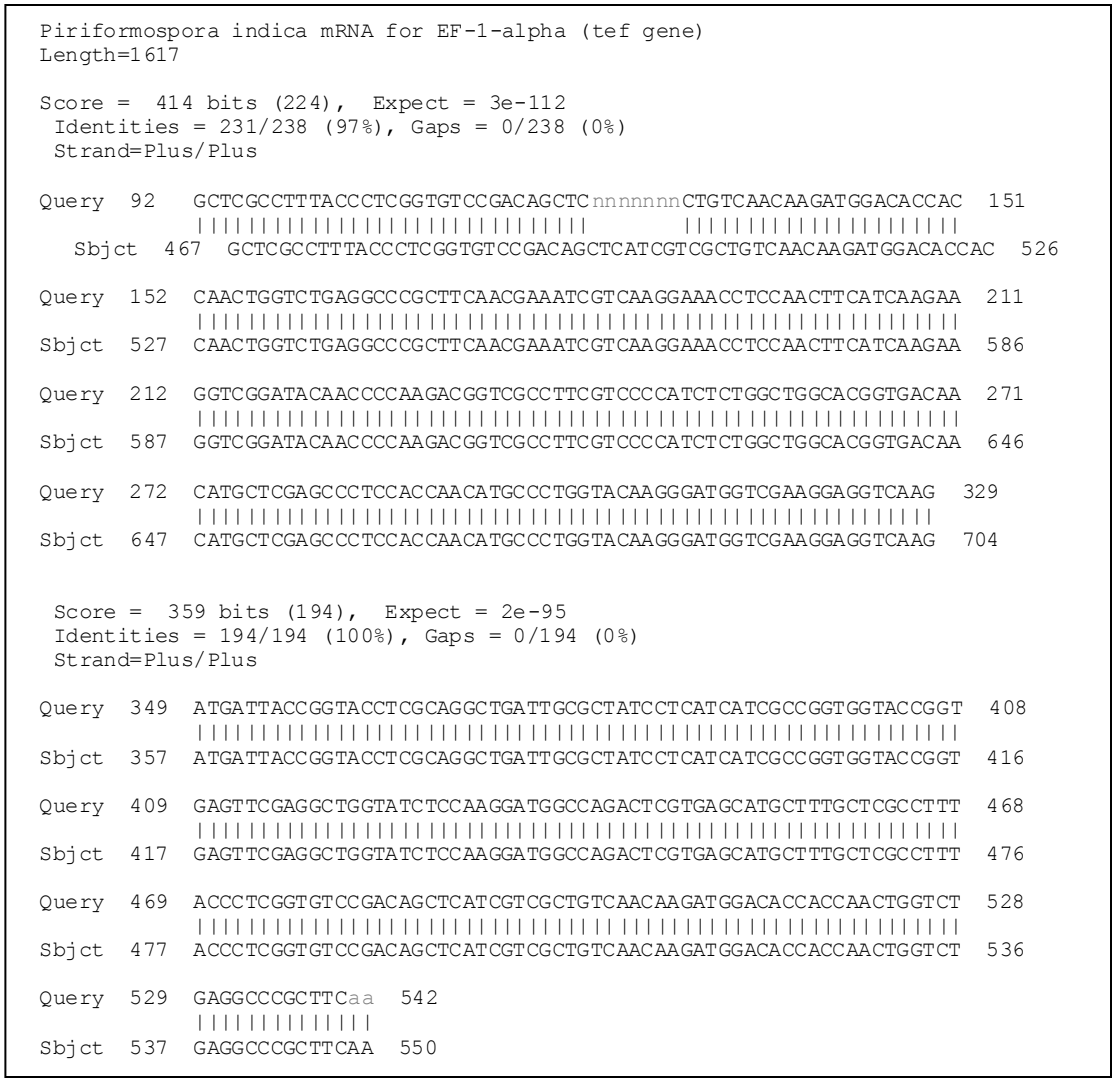

Figure 4.2.3.3: Nucleotide sequence homogeneities of sample 2 were compared to the complete coding sequence (cds) of the *Piriformospora indica* EF1-α gene using **(A)** cDNA selected with the forward primer (tefGSPd), and **(B)** the complemented reverse cDNA sequence selected using the reverse primer (tefGSPa). Nucleotides were aligned using the NCBI BLAST tool (NCBI, http://www.ncbi.nlm.nih.gov/).

4.2.4 Immunolocalization

Plate 4.2.4 (C through F) depicts localization of PIP1 and PIP2 aquaporins in wild-type control and inoculated roots. Plates 4.2.4 A and B demonstrate the antibody controls of the procedure, showing no fluorescence when either the primary or secondary antibodies were used alone. In control roots, PIP1 and PIP 2 aquaporins (Plates 4.2.4, C and D respectively) were found in the exodermis, throughout the root cortex, and in the

stele tissue. Upon inoculation with *P. indica*, localization of PIP 1 aquaporins (Plates 4.2.4, E) appeared to shift with a concentration of the aquaporins increasing in the root exodermal and endodermal cells and low localization patterns in the cortical cells. In inoculated wild-type roots, PIP 2 aquaporins (Plates 4.2.4, F) still spanned the entire exodermal, cortical, and endodermal regions, however, with less intensity as in control roots. There was a slightly higher concentration of PIP 2 aquaporins within the Casparian strips between exodermal cells in inoculated roots. The intensity of PIP signals differed between samples from each treatment, but expression patterns remained similar within treatment groups and within aquaporin clade. Differences in aquaporin intensities may be attributed to maintaining exposure levels and variations in root cross-section thicknesses.

Plates E.4.2.4.1 and E.4.2.4.2 in Appendix E depict respective localization of PIP aquaporins in PIP 1; 4 and PIP 2;5 over-expression plants with similar results.

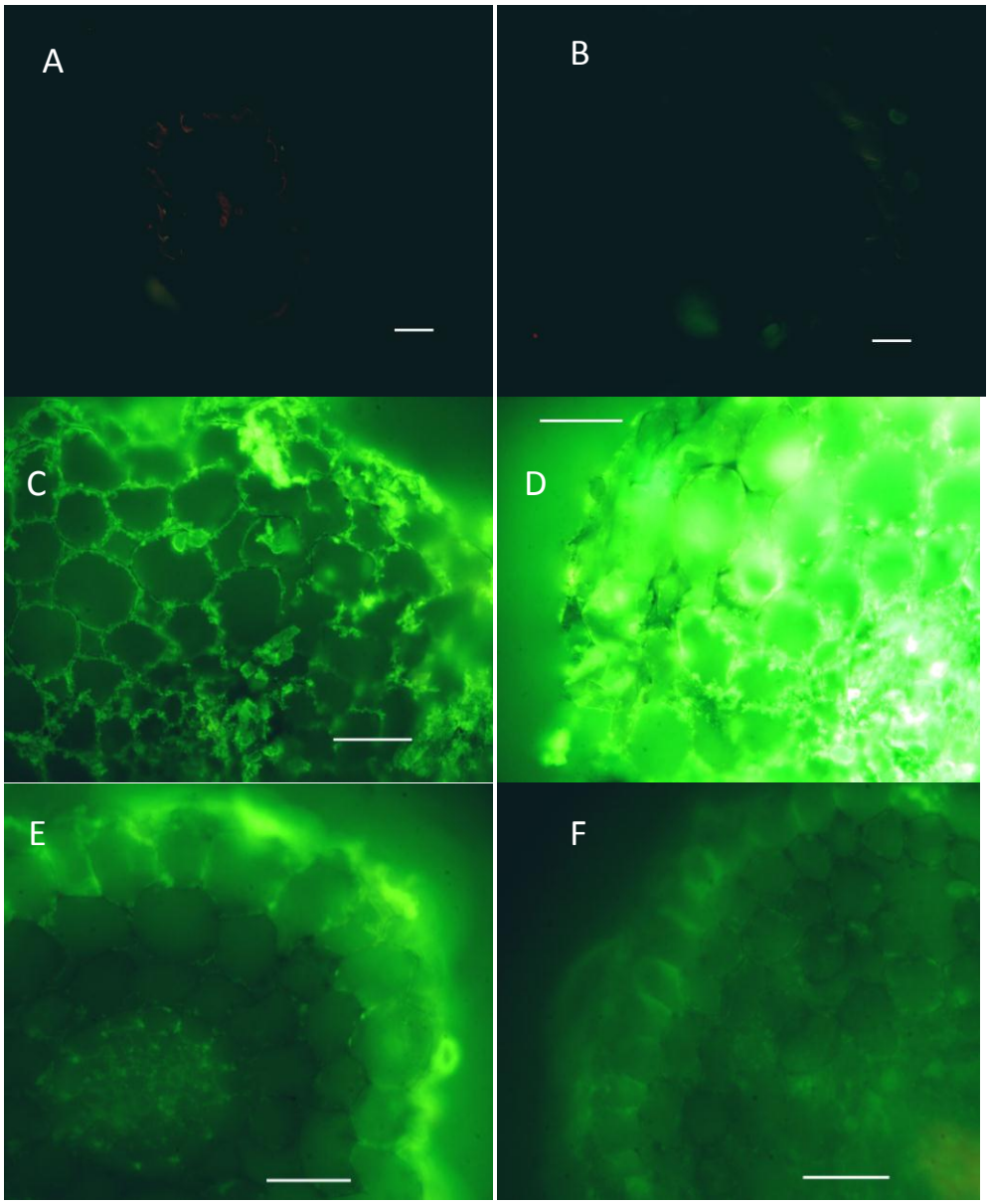

Plate 4.2.4: Expression patterns of PIP 1 and PIP 2 aquaporin subgroups in wild-type *Nicotiana tobacum* 20 mm from the root apex. **(A)** Primary antibody control displaying a lack of autofluorescence from the pre-immune serum (x 20), **(B)** Secondary antibody control demonstrating no autofluorescence from the secondary antibody, **(C)** PIP 1 localization in control plants, **(D)** Localization of PIP 2 aquaporins in control plants (x 40), **(E)** PIP 1 localization in wild-type inoculated with *Piriformospora indica*, **(F)** Localization of PIP 2 aquaporins in inoculated roots. Scale bars represent 50 µm.

5 Discussion

5.1 Expression and Localization of Tobacco Aquaporins in Tobacco Plants Inoculated with Piriformospora indica

Using quantitative RT PCR approaches, individual aquaporin expression was not observed to change in wild-type plants upon inoculation with *P. indica*. A means of communication between plant host and mycorrhizal fungi can bring upon changes in gene expression in both symbionts under various conditions (Dumas-Gaudot et al. 1994, Porcel et al. 2006, Aroca et al. 2009), however, it appears *P. indica* did not affect expression of the aquaporins studied here. Lack of plant aquaporin modulation by AM fungi has been previously reported, where no differences were found between *Daucus carota* TIP1; 1 gene expression in AM and non AM roots under control conditions (Aroca et al. 2009). However, *Dc*TIP2; 1 expression was down-regulated with inoculation (Aroca et al. 2009). In many AM associations, it is thought specific proteins are produced either as a stress reaction of the roots to inoculation, or as a mechanism of symbiotic mycorrhizal establishment (Dumas-Gaudot et al. 1994). Since aquaporin expression was not observed to change using quantitative RT PCR techniques, it can be interpreted that modulation of these aquaporins as a stress reaction did not occur. This is supported by evidence that *P. indica* reduces expression of BI-I, a cell death suppressor protein which is often activated during plant stress (Deshmukh et al. 2006). Reduction of this protein allows endophytic establishment while causing no stress to the host plant (Deshmukh et al. 2006), which would explain no need for changes in aquaporin patterns by tobacco as stress is theoretically not perceived by the plant upon inoculation. Moreover, barley roots inoculated with *P. indica* have not displayed induction of other defence-related genes (Waller et al. 2007), further supporting the notion that *P. indica* causes little stress to its host plant. It may be further interpreted that alteration of aquaporin abundance was not required for mycorrhizal establishment in tobacco roots under favorable conditions since no changes were observed. That being said, modulation of other tobacco aquaporins not studied here may occur, as synthesis and modification of various membrane proteins, though not all identified, has been observed in *Arabidopsis*

in response to inoculation with *P. indica* (Peškan-Berghöfer et al. 2004). In addition, PIP 1;3 and PIP 1;2 have been observed to be down-regulated in barley roots inoculated with the endophyte, and the regulation was speculated to be either a cause or consequence of faster root development in inoculated plants (Waller et al. 2007). Furthermore, alteration of aquaporin expression during other growth conditions is feasible, particularly since different fungal species alter aquaporin expression in contrasting ways which allows opposite strategies to be adopted during symbiosis. For instance, *Glomus intraradices* increases the rate of water uptake during drought by increasing the expression of plant PIP genes, whereas G*lomus mosseae* directs water conservation during drought by causing a down regulation of plant PIP genes (Porcel et al. 2006). Knowing the different ways fungi can affect aquaporin expression, as demonstrated by the two *Glomus* species, further research is required to determine if and how *P. indica* alters the expression of aquaporins under less than optimal conditions as a means to adapt to stress.

Alternatively, it is possible that that aquaporin expression and function reached a state of acclimation by the time roots were sampled for quantitative RT PCR, resulting in no detectable differences in expression in the root system. Aquaporin acclimation has been previously observed by Lee and Chung (2005) following cold stress. Even though expression was not studied, it was interpreted that activity of aquaporins in cold stressed figleaf gourd roots was able to return to normal levels within one day after the stress (Lee and Chung 2005). It is viable to speculate that wild-type tobacco roots had become acclimatized to the fungal presence, and if expression and function of aquaporins changed upon inoculation, they were able to return to control levels. When considering fungal mycorrhizal effects on host plants, it is crucial to remember that expression, modulation and function of aquaporins is a complex process still little understood and will vary when plants are submitted to various external stimuli (Jang et al. 2004). Studying the expression levels of some but not all aquaporin isoforms can lead to difficulty in interpreting results and the endophyte's role in modulation of tobacco aquaporins cannot be fully understood until the tobacco genome is mapped allowing a complete analysis of aquaporin expression levels during symbiosis. Moreover, analysis

of aquaporin expression in different root zones may have provided better insight into the involvement of *P. indica* in aquaporin modulation. It is possible that differences were not detected due to the fact that root tissues were analyzed collectively in turn masking possible expression differences in individual zones.

On another note, it was surprising that *Nt*PIP 1; 1 expression was higher than *Nt*PIP 2; 1 expression for both control and inoculated wild-type plants. It has been demonstrated that PIP2s have the ability to enhance membrane permeability more than PIP1s (Chaumont et al. 2000, Bots et al. 2005, Mahdieh et al. 2008), and that stability of formed heterotetramers results in more efficient transport of water across a membrane (Fetter et al. 2004). Previous research has demonstrated PIP 2;1, PIP 2;2, and PIP2;4 to be among the most highly expressed isoforms in *Arabidopsis* roots (Javot et al. 2003), and PIP 2;5 to account for 30% of PIP expression in primary maize roots (Hachez et al. 2006). Thus, higher N*t*PIP 2; 1 expression compared to *Nt*PIP 1;1 was expected. The higher *Nt*PIP 1;1 expression found in this particular study, however, is in agreement with results obtained by Chaumont et al. (2000) where transcripts for *Zm*PIP 1 (later to be identified *Zm*PIP1;1 in a subsequent publication, see Fetter et al. 2004) were found to be highly expressed in the roots, more so than *Zm*PIP 2 (later to be identified as *Zm*PIP 2;5). Again, PIP 1;1 was found to belong to the high expression group in the aerial parts and roots of *Arabidopsis*, whereas PIP 2;1 belonged to the low expression group (Jang et al. 2004). Similarly, a very weak signal was obtained from a PIP 2 probe when studying the cells of the circular cell cluster in the anther of tobacco; whereas signal with the PIP 1 probe was stronger (Bots et al. 2005). One must not ignore that expression of PIP isoforms not studied in this experiment may follow expected trends where PIP 2 aquaporins dominated expression patterns. If the abundance of *N. tobacum* aquaporin isoforms is anything like *Arabidopsis*, there is likely to be upwards of at least two dozen tobacco PIP aquaporins where some could follow the above mentioned expected expression levels. With the tobacco genome not being completely mapped, I studied expression levels of four aquaporins that had been characterized to some extent.

Regarding immunolocalization results, the role of tobacco PIP 2 aquaporins appears to follow expected trends under both control and inoculation treatments. Upon looking at Plate 4.2.4 (C through F) PIP 2 aquaporins were found to be pronounced in exodermal, cortical and endodermal cells regardless of treatment. Comparatively, PIP 1 expression patterns changed in inoculated roots to an increased localization along the root exodermis and endodermis, with less expression in cortical cells. Taking these results into account with quantitative RT PCR results, it appears that *P. indica* could modulate the expression of some aquaporins, but not NtPIP 1;1 nor *Nt*PIP2;1. Alternatively it can be interpreted that aquaporin expression may not have changed as a whole, but rather there was a simple relocalization of aquaporins in cells of exodermal and endodermal tissues. Aquaporins allow plants to alter water permeability, particularly to overcome apoplastic bottlenecks (Hachez et al. 2006). The localization pattern of PIP 1 aquaporins in *P. indica* associated roots suggests that PIP 1 proteins may have some role in bypasses to apoplastic barriers in exodermal cells, perhaps more so than PIP 2s. It is possible that the exodermal cells become more suberized in the associated roots, in turn requiring enhanced PIP 1 expression or relocalization to overcome these apoplastic barriers. There are two possible explanations for the apparent increase of apoplastic barriers in mycorrhizal tobacco. The first explanation is suberization of cells as a plant response to the fungus. Suberization of exodermal cells has been shown to be either lacking (Massicotte et al. 2010) or enhanced (Massicotte et al. 1993) in various kinds of mycorrhizal symbioses, respectively promoting or discouraging hyphal penetration into cortical cells. In regards to AM hyphal penetration, suberization of radial cell walls acts as a barrier forcing hyphae to enter the root complex via passage cells (Smith and Read 2008). It is possible that suberization of *P. indica* associated tobacco roots, if it did occur, was initiated by indirect hormonal regulation. SDIR1, an ubiquitin E3 ligase that positively regulates abscisic acid signaling (Zhang et al. 2007), has been shown to be upregulated in *P. indica* associated roots during drought and even slightly upregulated before the onslaught of drought (Sherameti et al. 2008). Increased ABA levels have been shown to result in augmented suberization of both potato tubers and *Arabidopsis* roots (Cottle and Kolattukudy 1982, Efetova et al. 2007), particularly upon wounding (Lulai et al. 2008). Since *P. indica* induces host cell death by interfering with the host cell death

program (Deshmukh et al. 2006) ABA levels in *P. indica* associated plants could increase due to SDIR1 over-expression and cell death. Moreover, PIP aquaporin upregulation has been linked to ABA dependent pathways (Mariaux et al. 1998). Hence suberization of root cells triggered by death of neighbouring cells may be feasible and consequent upregulation of PIP1 proteins as a plant response to overcome the apoplastic barriers is also feasible.

A second possible explanation for the apparent formation of more apoplastic barriers in mycorrhizal roots is the presence of hydrophobins found in the cell wall of fungal mycelia. Hydrophobin genes have been isolated from Ascomycetes, Deuteromycetes and Basidiomycetes, and it has been suggested that they play a role in attachment of ectomycorrhizal hyphae to plant hosts (Reviewed in Wösten and Wessels 1997). The hydrophilic sides of hydrophobin membranes face the fungal cell wall, whereas the hydrophobic side is exposed bestowing hydrophobicity to hyphae (Reviewed in Wösten an Wessels 1997). Though water movement has not been directly observed to change across the fugal sheaths of ectomycorrhizal roots, movement of certain ions such as calcium, magnesium, and potassium can be hindered across sheaths, hypothetically by hydrophobins (Bucking et al. 2002). Hydrophobins have been speculated to exclude water from lichen surfaces during cycles of wetting and drying (Reviewed in Wösten an Wessels 1997) and from mycorrhizal roots developed in the air (Vesk et al. 2001). Since *P. indica* displays some characteristics attributed to ectomycorrhizal fungi, such as the ability to be axenically cultured (Verma et al. 1998), there is the possibility hydrophobins in *P. indica* mycelia prevent solute and water flow into the apoplast of the root, in much the same manner described above. Upregulation or relocalization of PIP 1 isoforms in the plant exodermal cells may have occurred in order for water to overcome the apoplastic barriers caused by *P. indica* hydrophobins and to ultimately allow maintenance of the symbiosis. One must note that these hypotheses are speculative, as ABA levels, the permeability of *P. indica* hydrophobins if present, and analyses of exodermal cell walls to determine suberization were not performed to either affirm or revoke these presumptions. Moreover, interpretation of immunolocalization results is

prone to error as handcut cross-sections were not consistent in thickness and exposure levels were controlled, ultimately leading to over-exposure of one of the photographs.

5.2 Biomass and Water Relations of Tobacco Grown in Association with Piriformospora indica

In the current study, inoculation of tobacco plants with the endophyte was found to increase shoot and root weights by 29.6% to 53.4% in both wild-type and PIP 2;5 over-expression plants. This increase in overall plant biomass as result of inoculation with *P. indica* confirms the results of other studies (Varma et al. 1999, Bütehorn et al. 2000, Rai et al. 2001, Waller et al. 2005). It is speculated that no observable differences in root to shoot ratios were due to roughly proportional increases in root and shoot biomass from inoculation effects. It was interesting to note however, that none of the biomass parameters were increased in PIP 1; 4 over-expression plants upon inoculation.

Cortical hydraulic conductivity, as determined by the cell pressure probe, was not found to be significantly different between both inoculated and non-inoculated plants, nor between genotypes. Genotypic effects were not expected under control circumstances as growth conditions did not direct a water use beyond that which is normal. Previous studies using the exact genotypes found similar results where no differences in growth and hydraulic conductivity were observed between genotypes under optimal conditions (Jang et al. 2007, Lee et al. 2009). Differences in L_{pc} may not have been detected due to the fact cell pressure probe measurements were performed on cortical cells rather than exodermal or endodermal cells where symplastic movement would theoretically be truly represented. Alternatively, lack of detectable differences in L_{pc} may be due to acclimation of aquaporin activity and/or expression to the fungal presence, as previously discussed in Section 5.1. Though L_{pc} remained the same between all treatment levels, hydrostatic L_{pr} was lower in inoculated plants regardless of genotype and, and stomatal conductance was lower in inoculated PIP 2;5 over-expression plants. Looking at hydraulic conductivity data alone, assuming L_{pc} remained unchanged between mycorrhizal and non mycorrhizal plants, it can be interpreted that apoplastic

water flow was decreased in associated plants since L_{pr} decreased. In contrast to this interpretation, PTS_3 results do not indicate any differences in apoplastic flow. One must consider the drawback of apoplastic dyes adhering to cell walls (Zimmermann and Steudle 1998), thus it is possible apoplastic flow was not truly represented by the dye in this particular experiment. The interpreted lower apoplastic rates contradict previous research where water transport through the apoplastic pathway has been found to be larger in both ecto and arbuscular mycorrhizal roots (Muhsin and Zwiazek 2002, Aroca et al. 2007) or unchanged (Siemens and Zwiazek 2008). However, one must take into consideration that Aroca et al. (2007) observed a decrease in both exuded sap flow rates and in osmotic root hydraulic conductance in mycorrhizal roots of bean plants while transpiration rates remained constant between treatments, thus eliciting an interpretation of higher apoplastic rates. Comparatively, it has been found that AM associations can have no effect on water uptake rates or on conductance of root tissue and exuded sap flow rates (Bryla and Duniway 1997, Ruiz-Lozano et al. 2009) which is more along the lines of the results presented here. Likewise, hyphal penetration of cortical cells by ectoendomycorrhizae, fungi that display characteristics to AM fungi, have been observed to have little effect on L_{pr} (Siemens and Zwiazek 2008). Thus the lack of differences found in L_{pc} between mycorrhizal and nonmycorrhizal tobacco roots are within the range of normal trends. Lack of differences detected in hydraulic conductivity can be attributed to the idea that under saturated conditions it is unlikely mycorrhizae would directly increase plant water transport as surface area for hyphal transport is small in comparison to total root surface area (Allen 2007).

P. indica inoculation brought upon a decrease in stomatal conductance of PIP 2;5 over-expression plants. It has been suggested that underlying biochemical and molecular mechanisms may be responsible for the differences in effects of various fungi on plant responses (Siemens and Zwiazek 2008). The decrease in stomatal conductance could possibly be explained by *P. indica* indirectly modulating hormones in the PIP 2;5 over-expression plants. Sherameti et al. (2008) observed a relative 2-fold increase in SDIR1 expression in *P. indica* associated plants even before induction of drought. As plants underwent drought stress, the relative levels of SDIR1 were much higher in a shorter

period of time in inoculated plants than in controls (Sherameti et al. 2008). SDIR1 is a ubiquitin E3 ligase involved in abscisic acid (ABA) stress signal transduction and its over-expression leads to enhanced drought tolerance and induced stomatal closure (Zhang et al. 2007). Since the tobacco plants were grown under well-watered conditions, the upregulation of the stress induced SDIR1 seems conflicting. It is possible the fungus' affinity to program cell death in the host by reducing expression of BI-I (Deshmukh et al. 2006) or possible competition with the plant for nutrients and water results in stress-induced upregulation of SDIR1. Reducing expression of BI-I in the plant by the fungus is believed to cause no stress to the plant upon establishment (Deshmukh et al. 2006), so programming cell death should not be stressful to the host plant. What is more, typical defense related genes have not been observed to be up-regulated in *P. indica* established plants (Waller et al. 2007). Nonetheless, increases in ABA levels would help explain lower stomatal conductance in inoculated PIP 2;5 over-expression plants, while cellular L_{pc} was maintained. Knowing ABA levels have been observed to remain constant (Coleman et al. 1990, Ruiz-Lozano et al. 2009), to decrease (Allen et al. 1982), or to increase (Murakami-Mizukami et al. 1990, Danneberg et al. 1992, Schmidt et al. 2008) in fungal-associated plants, the indirect involvement of *P. indica* in ABA regulation requires study and should not be overlooked.

Collectively, the abovementioned results indicate that biomass in *P. indica* associated tobacco does not exclusively increase with increasing hydraulic conductivity of the roots. It has been suggested that *P. indica* is able to promote plant growth either by influencing ethylene signalling components (Camehl et al. 2010), by increasing an unidentified active compound in the fungal cell wall which initiates a signaling cascade by augmenting cystolic and nuclear calcium levels (Vadassery et al. 2009), or by influencing N-linked glycosylation in the endoplasmic reticulum (Peškan-Berghöfer et al. 2004). Additionally, there is evidence that the growth promotion is linked to auxin, but it is unclear whether *P. indica* interferes with expression of auxin related genes in the plant (Lee et al. 2011) or if diffused auxin by the endophyte promotes growth (Sirrenberg et al. 2007). Nonetheless, it appears that the fungus could be involved in many mechanisms that encourage growth, and one or a combination of the

abovementioned mechanisms may explain the increases in growth observed here. Furthermore, Sirrenberg et al. (2007) imply other growth promoting factors produced by the fungus that have not yet been studied should be considered as well. It has been shown that mycorrhizal fungi can increase uptake of macro and micro-nutrients such as phosphorous, zinc, copper, and nitrogen in exchange for some of the host plant's carbon (Kothari et al. 1990, Smith and Read 2008, Allen 2007), and the possibility of *P. indica* increasing plant availability to certain nutrients may explain the observed increases in growth parameters, though this matter requires further study. It has already been observed that *P. indica* can increase the uptake of iron, although results have not been published (Peškan-Berghöfer et al. 2004). Increases in nitrogen content in the aerial parts of *N. tobacum* plants have also been observed (Sherameti et al. 2005); while shoot nitrogen and phosphorous status were unchanged in *N. atenuata* associated with *P. indica* (Barazani et al. 2005). It appears the fungus' role in plant water relations, if any role, did not contribute to growth promoting effects; however future research concerning the endopyte's role in nutrient uptake may prove particularly beneficial to unraveling the mechanism(s) behind its growth promoting properties.

It seems puzzling that photosynthetic rates were not observed to be higher in inoculated plants even though increases in growth were observed in wild-type and PIP 2;5 over-expression plants. Increases in photosynthetic rates should be required to fulfill energy and carbohydrate demands necessary for increases in growth. One may argue that no increases in photosynthetic rates were observed here because conditions during photosynthetic measurement were slightly better than actual growing conditions. Consequently a stress incurred during growing conditions may have been better tolerated by inoculated plants resulting in higher yields, and at the time of measurement true differences in photosynthesis may not have been detected due to better conditions. Had tobacco plants been submitted to less than optimal growing conditions, a genotypic effect among unassociated plants likely would have been observed. Tobacco plants overexpressing PIP 1;4 and PIP 2;5 have been shown to have different water flow compared to wildtype plants during various stresses including rapid water loss during drought and increased water flow during cold stress (Jang et al. 2007).

No genotypic differences were observed here for any of the measured parameters in unassociated plants, giving some indication that plants were not stressed. Moreover, *P. indica* has been documented several times to increase growth in host plants during favorable controlled conditions (Varma et al. 1999, Bütehorn et al. 2000), while Fv/Fm values, which reflect photosynthetic efficiency of photosystem II, have not been observed to differ between associated and control plants during stress-free conditions (Peškan-Berghöfer et al. 2004, Sherameti et al.2008). Increases in growth while no significant change in photosynthetic activity have been previously reported in other mycorrhizal associations. *Lactuca sativa* inoculated with *Glomus occultum* were found to have increased root and shoot dry weights (Ruiz-Lozano et al. 1995a), while inoculation with either *G. occultum* , *G. mosseae*, or *G. caledonium* increased leaf area and total root length (Ruiz-Lozano et al. 1995b) with no increases in photosynthetic efficiency. No explanation was provided by the authors as to why this physiological response occurred. As in the case of the *Glomus* species described above, it appears that the growth promoting benefits of *P. indica* are not produced specifically during stress and higher photosynthetic rates may not be continually required for the increases in growth, particularly in older plants. Root colonization by *P. indica* has been characterized by faster root development in young barley plants (Waller et al. 2007), so it can be speculated photosynthetic rates return to control levels once the initial boost in tissue development occurred. Moreover, it appears that the growth promoting effect of the fungus is complemented by stimulation of the starch breakdown enzyme glucan-water dikinase (Sherameti et al. 2005). Thus the endophyte appears to have some involvement in supplying carbohydrates essential for increased growth by enhancing breakdown of stored starch, rather than increasing photosynthetic rates continuously over time. More studies will be required to explain the relationship between the photosynthetic rates and increased growth rates observed here, as well as provide insight in to the starch breakdown pathways that occur in mycorrhizal tobacco. To add, photosynthesis per unit leaf area may not vary much under controlled light conditions as seen here, but photosynthesis per unit leaf mass may. Hence, differences in photosynthesis per unit mass would provide better insight into photosynthetic return on biomass invested in leaf tissues.

The fact that wild-type and PIP 2;5 plants showed increases in growth, but PIP 1;4 plants did not indicates that upregulation of the PIP 1;4 aquaporin possibly negates the growth promoting qualities of the fungus in some manner. Then again, over-expression of PIP 1;4 may have caused alterations in expression patterns of other genes that could have affected fungal growth promotion. Jang et al. (2007) observed this sort of event when transcript levels of 13 PIP genes were varied by the over-expression of PIP 1;4 and PIP 2;5 under diverse stress conditions. What is more, major intrinsic proteins (MIPs) can be permeable to solutes other than water including boric acid, hydrogen peroxide, ammonia and carbon dioxide (reviewed by Tyerman et al. 2002, Jahn et al. 2004, Loqué et al. 2005), and transport of nitrous oxide, ethylene and undissociated organic acids through MIPs should also be considered (Tyerman et al. 2002). It is possible that the over-expression of PIP 1;4 either directly or indirectly interfered with solute transport which led to interference of the growth promoting effects of the fungus. One must note that this theory is entirely speculative as solute transport across PIP 1; 4 has not been studied to the knowledge of the author. All in all, either direct over-expression of PIP 1;4 or indirect change in expression of other genes as a result of PIP 1;4 over-expression appears to have halted of the growth inducing effects of the fungus.

6 Conclusion

6.1 Review of Results in Relation to Tested Hypotheses

P. indica has demonstrated in numerous regards that it is a unique mycorrhizal fungus. It does fit into a previously described mycorrhizal classification group due to its combined characteristics of forming endophytic associations while being capable of growing in axenic culture. *P. indica* is able to procure many benefits to the host plant, including growth promotion (Varma et al. 1999, Bütehorn et al. 2000, Rai et al. 2001, Peškan-Berghöfer et al. 2004, Waller et al. 2005), resistance to pathogens, and tolerance to salinity (Waller et al. 2005) and drought (Sherameti et al.2008). The fungus is able to form a mutualistic relationship with the plant by interfering with the host cell death program (Deshmukh et al. 2006), an interaction attributed exclusively to *P. indica*

(Schäfer et al. 2007). Since the combined characteristics of *P. indica* are not typical of any one classified mycorrhizal group, its effects on the physiology and growth of tobacco do not seem representative of any one mycorrhizal association type.

This study demonstrates that *P. indica* appears to either have little effect or to reduce water relation parameters of tobacco given cortical hydraulic conductance remained unchanged while whole root hydraulic conductivity decreased regardless of genotype and stomatal conductance decreased upon inoculation in PIP 2;5 over-expression plants. This indicates that over-expression of PIP 1;4 and PIP 2;5 are not required by either the fungus or the tobacco host to maintain water status. Furthermore, symbiosis with the fungus did not change aquaporin expression levels of *Nt*PIP 1;1, nor *Nt*PIP 2;1 in wild-type plants, indicating the endophyte either does not modulate those particular aquaporins under favorable conditions, or aquaporin expression becomes acclimatized after inoculation. In spite of this, expression and/or localization of at least one PIP1 isoform changed in wild-type plants. Due to possible suberization and decrease in the above mentioned water status parameters, it was inferred that the fungus interferes with the phytohomone balance in the plant which could change PIP1 expression patterns as a means to overcome apoplastic barriers. The apparent influence of the endophyte decreasing some of the measured water relation parameters did not seem to influence growth promotion in at least wild-type and PIP 2;5 over-expression plants. Growth promotion was not observed in PIP 1;4 plants indicating that the over-expression of PIP 1;4 may interfere with the growth promoting mechanism of the fungus, though it remains unclear in what manner the mechanism is impeded. Since the water relation parameters in associated PIP 1;4 plants were not different from associated wildtype and PIP 2;5 plants, it is speculated that lack of growth promotion in PIP 1;4 plants is not linked to changed water relations resulting from PIP 1;4 over-expression.

In conclusion, *P. indica* did not have a greater effect on water relations of wild-type plants compared to the plants over-expressing aquaporins. Furthermore, expression of PIP aquaporins studied was not altered in associated wild-type plants, indicating those aquaporins were not involved in maintaining water status during favorable conditions.

Biomass increases may not be directly linked to increases water transport since cell hydraulic conductivity remained unchanged and root hydraulic conductivity decreased with inoculation. Taking into consideration results published in previous literature, it appears other mechanisms are involved in the growth promotion by the fungus and further study is needed.

6.2 Suggested Future Research

Transcript levels of *Arabidopsis* PIP 1; 4 and PIP 2; 5 have been shown to increase up to five-fold during drought treatment (Jang et al. 2004). Furthermore, transgenic plants over-expressing PIP 1; 4 and PIP 2; 5 have displayed rapid water loss during drought stress (Jang et al. 2007). Knowing that *P. indica* can confer drought tolerance in *Arabidopsis* (Sherameti et al. 2008), it would be interesting to note if the fungus could induce water conservation in the transgenic plants during drought by down-regulating PIP genes as observed by Porcel et al. (2006) in *Glomus mosseae* inoculated plants. This experiment would allow for better interpretation of the role that the fungus plays in drought resistance in plants.

Since an increase in growth was observed upon inoculation in both wild-type and PIP 2; 5 plants and the exact mechanism causing the increase remains unclear, the fungus' contribution to nutrient uptake is worth investigating. Moreover, it appears the aquaporin PIP 1;4 is inhibitory to the growth promoting process, possibly as a direct transporter of solute(s) that inhibit the fugal benefits, or by promoting alterated expression of another gene involved in the growth promoting mechanism.

Finally, since it was inferred that an apoplastic barrier was formed in the exodermis of mycorrhizal roots, investigation into this apparent barrier is worth merit. Future research can reveal whether or not an apoplastic barrier is actually formed upon inoculation with *P. indica*, and if so whether the barrier is due to suberization of exodermal cells as a protective response from the plant, or if hydrophobins present in mycelia block apoplastic flow. Looking at ABA levels in various tissues will also shed

some light onto the plant's response to the endophyte and whether or not increased ABA levels contribute to the formation of the perceivable apoplastic barriers.

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Appendix A

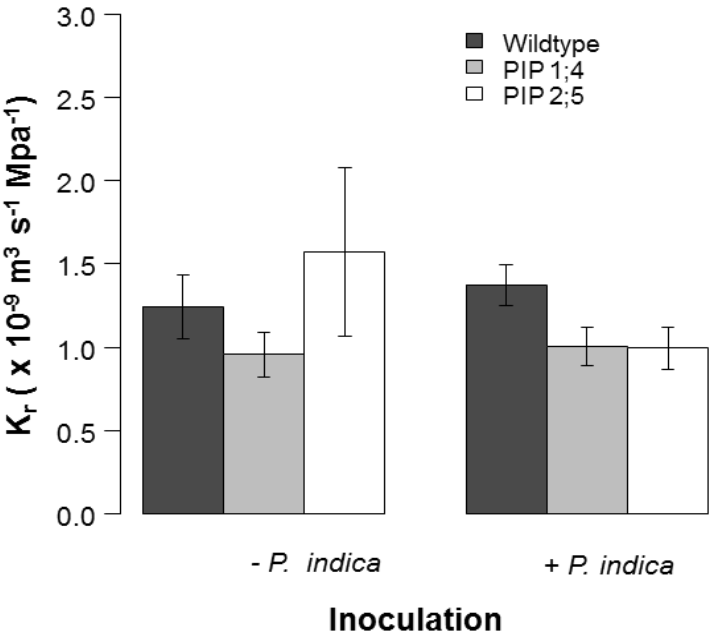

Figure A.4.1.2: Root hydraulic conductance (K_r) determined by the hydrostatic method in *Nicotiana tobacum* lines inoculated (+*P. indica*) and not inoculated (-*P. indica*) with *Piriformospora indica*. The three *N. tobacum* lines were wild-type (WT), over-expression of aquaporin PIP 1;4 (PIP1;4), and over-expression of aquaporin PIP2;5 (PIP2;5). Means (n= 6-7) ± standard errors are shown.

Appendix B

Primer Design Guidelines

When designing primers for tobacco aquaporin genes and the reference gene *Nt*TUB2, the following recommendations (Premier Biosoft International 2012) were considered:

- The primers were gene specific to avoid mistakenly amplifying other genes.
- Primer length should ideally be between 18 and 22 base pairs. Some of the designed primers had lengths up to 24 base pairs.
- The primer melting temperatures (T_m) were no higher than 65°C in order to avoid secondary annealing. Paired forward and reverse primers had closely matched melting points.
- GC content of primers should ideally be between 40% and 60%. GC content of the designed primers ranged between 33.3% and 47.8%. *Nt*Actin (Designed by Jang et al. 2007) had a GC content of 55.0%.
- G or C bases within the last five bases from the 3' end were present in order to promote specific binding. More than three G or C bases were avoided in the last five bases.
- Repeats of dinucleotides occurring consecutively many times as well as runs of single bases were avoided where possible.
- The target length of the cDNA fragment amplified was 100 base pairs. Amplified lengths ranged between 84 base pairs for *Nt*PIP1;1 and 120 base pairs for *Nt*PIP1a.
- Possible formation of secondary structures within and between paired primers (such as hairpins, self dimmers, and cross dimmers) was analyzed. The lowest folding free energies (ΔG) of secondary structures were calculated using Oligoanalyzer 3.1 (Integrated DNA Technologies, Coralville, Iowa, USA, http://www.idtdna.com) to ensure they were not too negative.

Specific properties and analysis output regarding each of the designed primers may be seen in Table B.3.2.3.1

Table B.3.2.3.1 Primers used for quantitative RT PCR. Selected aquaporin transcripts are as follows: Aquaporins belonging to the PIP1 clade (*Nt*PIP1a and *Nt*PIP1a), aquaporin PIP 1;1 (*Nt*PIP1;1), and aquaporin PIP 2;1 (*Nt*PIP2;1). *Nt*TUB2 depicts the tobacco reference gene alpha-tubulin 2 and *Nt*Actin depicts the tobacco reference gene actin. Properties such as primer length, percent GC content, and melting temperatures are depicted, as well as lowest folding free energy (ΔG) of secondary structures. "n/a" indicates no value was calculated

Gene	Primer	Primer Length (bases)	GC Content (%)	Melting Temperature (°C)	ΔG Hairpins (kcal/mole)	ΔG Self Dimers (kcal/mole)	ΔG Cross Dimers (kcal/mole)
*Nt*PIP1a	Forward 5'- GTT TCC TCA AGA AGC CTT AAT C-3'	22	40.9	51.5	-0.01	-4.85	-5.47
(AF024511)	Reverse 5'- GAC ATT TGA ACA CAA GAA AAT CC-3'	23	34.7	50.9	0.73	-5.36	
*Nt*PIP1b	Forward 5'- ATA ATC ATC AGA GCC ATT GCA TTC-3'	24	37.5	53.7	-0.99	-7.05	-3.53
(U62280)	Reverse 5' - GGT ACA GGA GTC TTG AAA TAT AAC-3'	24	37.5	50.9	-0.07	-3.91	
*Nt*PIP1;1	Forward 5'- GCT AAG ATT CTC CTG CCA TTT GC-3'	23	47.8	56.4	-0.56	-5.36	-5.36
(AF440271)	Reverse 5'- GAA ATT ACA CAT TTG ACA GAC ACC-3'	24	37.5	52.5	-0.41	-3.17	
*Nt*PIP2;1	Forward 5'-GCA TTC ATC TGT CCA AAT TAT C-3'	22	36.3	49.8	0.99	-5.36	-3.42
(AF440272)	Reverse 5'-ATA CAT CCA AGG TTA ACA TTA AGG-3'	24	33.3	50.8	0.99	-7.53	
*Nt*TUB2	Forward 5'-GTG TTT GTT TTT GTG TTG TTT GG-3'	23	34.8	52.3	2.71	n/a	-3.30
(AJ421412)	Reverse 5'- ACA GCA TAC TAC AGT TTA GAA G-3'	22	36.4	49.7	-0.68	-3.14	
*Nt*Actin	Forward 5' TGG ACT CTG GTG ATG GTG TC-3',	20	55.0	56.5	0.02	-2.92	-6.60
	Reverse 5' CCT CCA ATC CAA ACA CTG TA-3'	20	45.0	52.1	0.61	-1.95	

Appendix C

Assumptions of the $2^{-\Delta ct}$ method when determining gene expression levels

In order for the $2^{-\Delta ct}$ calculation of relative gene expressions to be valid, the efficiency of the target amplification and the reference amplification should be approximately equal (Livak and Schmittgen 2001). To asses if the amplifications have the same efficiency, cycle thresholds (ct) were first determined for each target and reference gene at different diluted cDNA concentrations (diluted from 10^{-1} to 10^{-5} ng/µl). The differences between ct values of the target and reference amplifications at each dilution were calculated (Δct). A plot of the log cDNA dilution versus Δct was made for each target and reference comparison and the absolute value of the slope was determined. If the absolute value of the slope was close to zero, the efficiencies of the target and reference genes were considered similar (Livak and Schmittgen 2001). Further details concerning assumptions and calculations of the $2^{-\Delta ct}$ method are descrbed in Livak and Schmittgen (2001). Absolute values of slopes from plots of the log cDNA dilution versus Δ ct for each target and actin reference comparison can be seen in Table C.4.2.1. All slopes had absolute values close to zero indicating the efficiencies of the target genes were similar to the efficiency of the actin gene.

Table C.4.2.1: Absolute values of slopes from plots of the log cDNA dilution versus Δ ct(ct_{target}- $ct_{reference}$). Selected aquaporin transcripts of target genes are as follows: aquaporins belonging to the PIP1 clade (*Nt*PIP1a and *Nt*PIP1a), aquaporin PIP 1;1 (*Nt*PIP1;1), and aquaporin PIP 2;1 (*Nt*PIP2;1). Amplification efficiencies of the target genes were compared to that of the reference gene actin (*Nt*Actin)

Aquaporin	Absolute slope
*Nt*PIP 1a	0.0371
*Nt*PIP 1b	0.0622
*Nt*PIP 1;1	0.0562
*Nt*PIP 2;1	0.0527

Appendix D

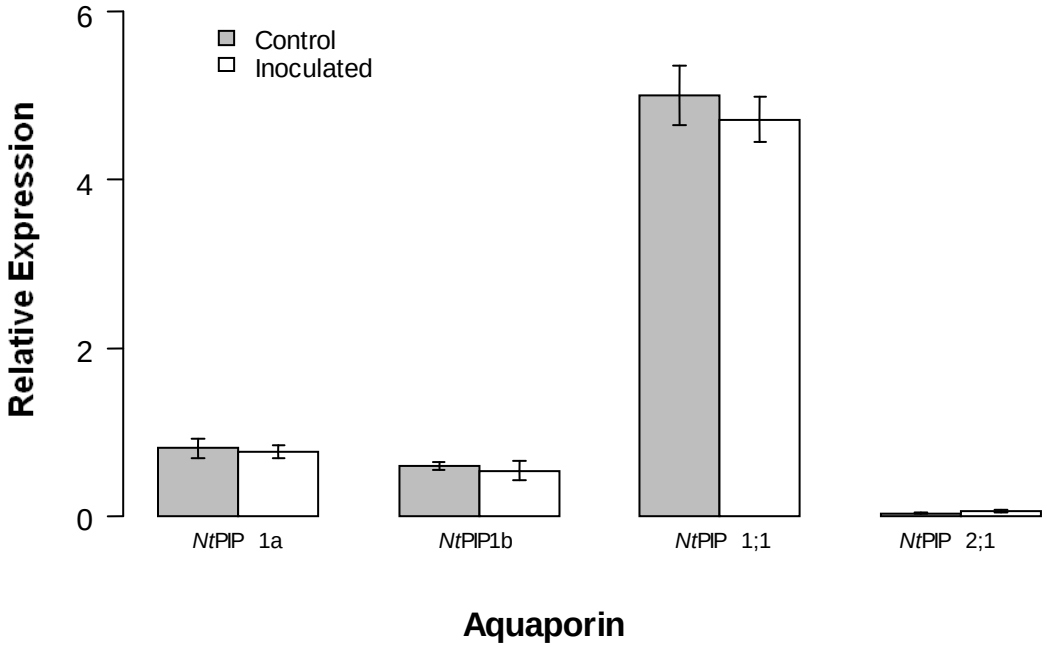

Figure D.4.2.2: Effects of *Piriformospora indica* on relative expression patterns of four aquaporin transcripts in wild-type *Nicotiana tobacum* roots. Selected aquaporin transcripts are as follows: aquaporins belonging to the PIP1 clade (*Nt*PIP1a and *Nt*PIP1a), aquaporin PIP 1;1 (*Nt*PIP1;1), and aquaporin PIP 2;1 (*Nt*PIP2;1). Relative transcript abundance is described as the fold change of mean cDNA expression of the aquaporin gene relative to the reference gene (Tubulin 2-α, *Nt*Tub). Quantification was determined by the standard curve of quantitative RT PCR. There were three plants per treatment and two replicates per plant. No significant differences were found between aquaporin expression patterns when comparing inoculated plants to control plants ($P \leq 0.05$). Error bars represent the standard error of the mean.

Table D.4.2.2: Significance values for aquaporin relative expression levels between control wild-type *N. tobacum* roots and roots inoculated with *Piriformospora indica*. Fold change of mean cDNA expression of the aquaporin gene was relative to the reference gene tubulin 2-α (*Nt*Tub). ANOVA results indicate p-values of the inoculation effect

Aquaporin	(Pf > F)
*Nt*PIP 1a	0.8074
*Nt*PIP 1b	0.6506
*Nt*PIP 1;1	0.5535
*Nt*PIP 2;1	0.2057

Appendix E

Negative Controls for Immunolocalization

In this particular experiment, primary and secondary antibodies were employed to depict the localization of PIP 1 and PIP2 aquaporins in fresh root cross sections of tobacco. An antibody that recognized the target antigen (the antigen being the PIP aquaporins in this case) is termed the primary antibody (Thermo Fisher Scientific Inc. 2011). The primary antibody was developed by Hachez et al (2006) by immunizing rabbits with prepared forms of PIP aquaporins from maize. A serum containing specific antibodies to the PIP aquaporins could then be harvested from the rabbits (anti-PIP antiserum). In order to view the localization patterns of the primary antibody attached to the antigen, a secondary antibody labeled with a detectable tag which probes for the primary antibody is required (Thermo Fisher Scientific Inc. 2011). The secondary antibody (goat anti-rabbit IgG antibody) was developed in the same manner as the primary antibody. Antibodies produced by a goat in response to some injected rabbit protein was harvested and then labelled with fluorescent molecules in order to be viewed under a fluorescent microscope.

For immunolocalization it is important that negative controls are performed to demonstrate that neither of the antibodies cause positive false signals. Pre-imune serum was employed in this experiment to ensure that the primary antibody was probing for PIP aquaporins and not other plant tissues. The pre-imune serum does not contain the specific antibodies to the PIP aquaporins, therefore it should not fix to the aquaporins upon incubation. Rather, the pre-imune serum should be entirely washed away with blocking solution prior to incubation with the secondary antibody. To ensure the secondary antibody does not have an affinity to attach itself to proteins other than the primary antibody, a control involving incubation of the sections without the primary antibody must be performed. In both controls, there should be no fluorescence as PIP aquaporins are not perceived by either of the antibodies.

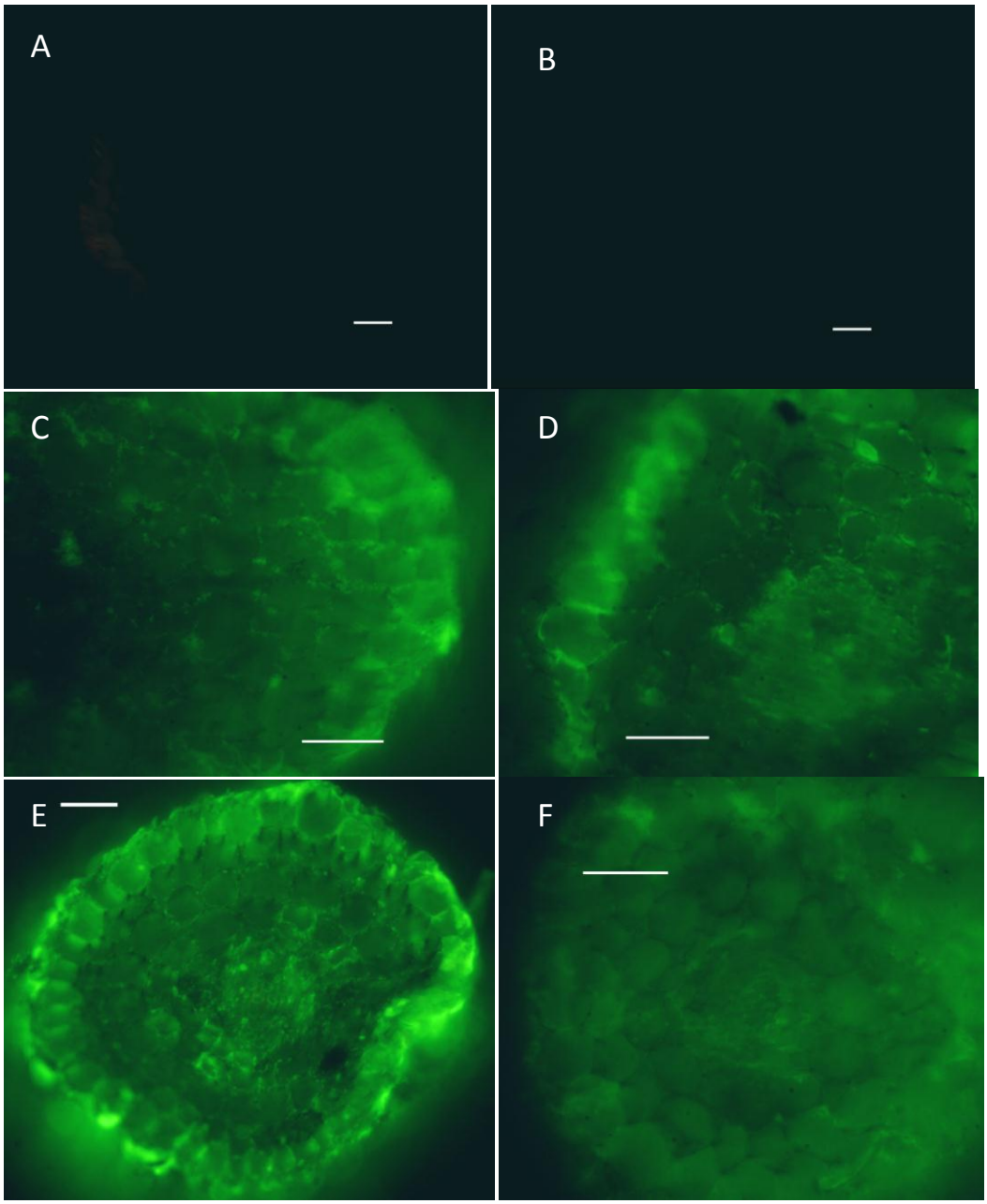

Plate E.4.2.4.1: Expression patterns of PIP 1 and PIP 2 aquaporin subgroups in PIP 1;4 over-expression *Nicotiana tobacum* 20 mm from the root apex. **(A)** Primary antibody control displaying a lack of autofluorescence from the pre-imune serum, **(B)** Secondary antibody control demonstrating a lack of autofluorescence from the secondary antibody, **(C)** PIP 1 localization in wild-type control plants, **(D)** Localization of PIP 2 aquaporins in wild-type control plants, **(E)** PIP 1 localization in wild-type inoculated with *Piriformospora indica,* **(F)** Localization of PIP 2 aquaporins in inoculated wild-type plants. Scale bars represent a distance of 50 µm.

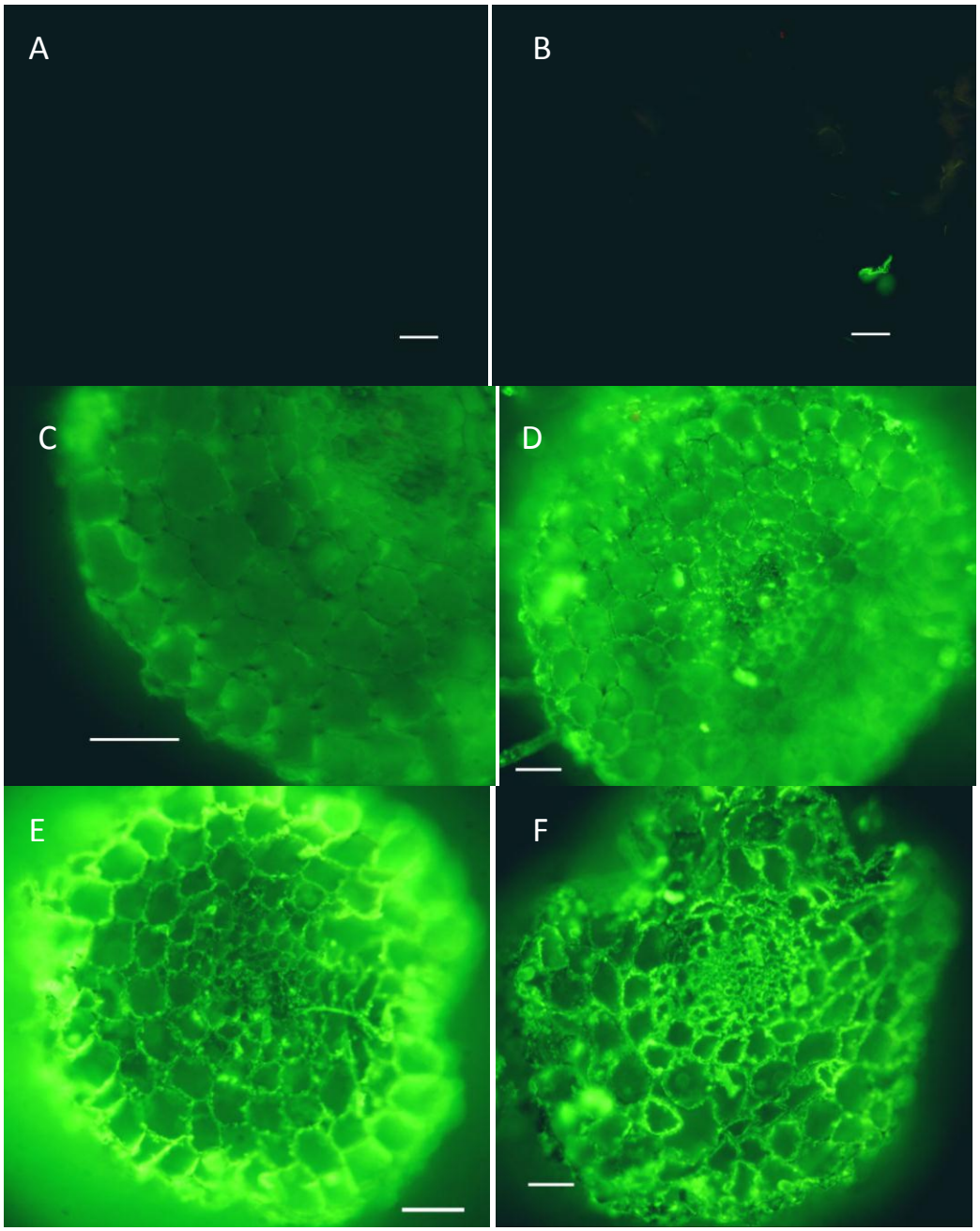

Plate E.4.2.4.2: Expression patterns of PIP 1 and PIP 2 aquaporin subgroups in PIP 2;5 over-expression *Nicotiana tobacum* 20 mm from the root apex. **(A)** Primary antibody control displaying a lack of autofluorescence from the pre-imune serum, **(B)** Secondary antibody control demonstrating a lack of autofluorescence from the secondary antibody, **(C)** PIP 1 localization in wild-type control plants, **(D)** Localization of PIP 2 aquaporins in wild-type control plants, **(E)** PIP 1 localization in wild-type inoculated with *Piriformospora indica,* **(F)** Localization of PIP 2 aquaporins in inoculated wild-type plants. Scales represent distances of 50 µm.