

OPTIMIZATION OF *PIRIFORMOSPORA INDICA* APPLICATION AND
OBSERVATION OF *PIRIFORMOSPORA INDICA* INDUCED DISEASE RESISTANCE
AGAINST VARIOUS PLANT PATHOGENS IN WHEAT AND BARLEY

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**OPTIMIZATION OF PIRIFORMOSPORA INDICA APPLICATION AND
OBSERVATION OF PIRIFORMOSPORA INDICA INDUCED DISEASE
RESISTANCE AGAINST VARIOUS PLANT PATHOGENS IN WHEAT AND
BARLEY**

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ABSTRACT

OPTIMIZATION OF *PIRIFORMOSPORA INDICA* APPLICATION AND OBSERVATION OF *PIRIFORMOSPORA INDICA* INDUCED DISEASE RESISTANCE AGAINST VARIOUS PLANT PATHOGENS IN WHEAT AND BARLEY.

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Piriformospora indica is an endophyte with the ability to colonize roots and it is cultivable. It forms a specific type of mycorrhiza symbiosis with a broad spectrum of plant species, promoting plant growth and biomass production and also induction of resistance against pathogens. In this study our aim is to test *Piriformospora indica* against powdery mildew in barley and *Puccinia striiformis* in wheat to determine best application method of *P. indica* to assess the induction of resistance. Also we aimed to develop a procedure that enables the usage *P. indica* in large scale agricultural applications. To achieve our goals, we applied *P. indica* in different spore concentrations and in different form as powder and liquid cultures. After establishing symbiosis with *P. indica* on plants, the plant leaves were infected with pathogens. The disease formations were evaluated in comparison with the control group of plant samples (without *Piriformospora indica*).

Key words: *Piriformospora Indica*, wheat and barley pathogens, mycorrhiza symbiosis.

ÖZ

PIRIFORMOSPORA INDICA UYGULAMASININ OPTİMİZASYONU VE *PIRIFORMOSPORA INDICA*'NİN FARKLI BİTKİ PATOJENLERİNE KARŞI BUĞDAY VE ARPADA OLUŞTURDUĞU HASTALIK DİRENCİNİN GÖZLENMESİ.

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Piriformospora indica bitki köklerini kolonize edebilme yeteneğine sahip kültüre alınabilen bir çeşit endofittir. Çok geniş sayıda bitki türüyle simbiyotik ilişki kurabilir ve bu simbiyotik ilişki bitkinin büyümesinde ve hastalıklara karşı direnç geliştirilmesinde etkilidir. Bu çalışmada amacımız *Piriformospora indica*'yı külleme hastalığına karşı arpa ve buğdayda test etmek ve en iyi uygulama tekniğini daha iyi direnç için belirlemektir. Ayrıca bu mantarın büyük çaplı tarım uygulamalarında kullanılabilmesine imkan sağlayacak bir prosedür geliştirmek amaçlanmıştır. Bu amaçlarımıza ulaşmak için değişik spor konsantrasyonlarında, toz ve sıvı olmak üzere farklı formlarda *Piriformospora indica*'nın uygulaması yapıldı ve sonrasında bitkiler patojenlerle infekte edildi. Bitki köklerinin *P. indica* ile kolonizasyonu sağlandıktan sonra bitki yaprakları patojenler ile infekte edildi. Hastalık oluşumunun değerlendirilmesi kontrol grubu bitki örnekleri (*Piriformospora indica* içermeyen) ile karşılaştırılarak yapıldı.

Anahtar Kelimeler: *Piriformospora indica*, buğday ve arpa patojenleri, mycorrhiza simbiyosis.

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LIST OF ABBREVIATIONS

B	Boron
<i>Bgh</i>	<i>Blumeria graminis</i> f.sp. <i>hordei</i>
Ca	Calcium
Cu	Copper
dpi	Days post inoculation
Fe	Iron
g	gram
hpi	Hour post inoculation
K	Potassium
m	meter
Mg	Magnesium
mg/L	milligram per liter
mm	millimeter
mM	miliMolar
Mn	Manganese
Mo	Molybdenum
N	Nitrogen
P	Phosphorus
<i>Pst</i>	<i>Puccinia striiformis</i> f.sp. <i>tritici</i>
Zn	Zinc

CHAPTER 1

INTRODUCTION

1.1 *Piriformospora indica*

Piriformospora indica is a basidiomycete of the order Sebaciniales, serving as a model organism for mutualistic symbiosis studies and further as a plant promoter for many vascular plants. Germinating arbuscular mycorrhizal fungal spore, the fungus colonizes the roots of a wide range of vascular plants, increasing the host growth, seed yield and adaptation to abiotic and biotic stresses such as disease resistance or tolerance to physiological factors.

1.1.1 General Properties of *Piriformospora indica*

The term mycorrhiza refers to the association between fungi and roots of higher plants. This association is usually considered a mutualistic symbiosis because of the highly beneficial relationships established between both partners, in which the host plants receive mineral nutrients *via* the fungal mycelium (mycotrophism), while the heterotrophic fungi obtain carbon compounds from the host plants [1-3]. Arbuscular mycorrhizal (AM) fungi belong to nine genera: *Gigaspora*, *Scutellospora*, *Glomus*, *Acaulospora*, *Entrophospora*, *Archaeospora*, *Gerdemannia*, *Paraglomus* and *Geosiphon*.

Scientists from the School of Life Sciences, Jawaharlal Nehru University, New Delhi, have screened a novel endophytic fungus, *Piriformospora indica*, which mimics the functional capabilities of typical AM fungi [4]. The properties of this fungus have been patented [5]. Recent studies have indicated that *P. indica* belongs to Sebacinaceae and is closely related to *Sebacina vermifera* [6,7].

Sebacinaceae members *P. indica* and *S. vermifera* form chlamydospores or vesicle-like structures within or between the cortical cells. Like AM fungi, hyphae multiply within the host cortical tissues and never traverse through the endodermis. Likewise, they also do not invade the aerial portion of the plant (stem and leaves).

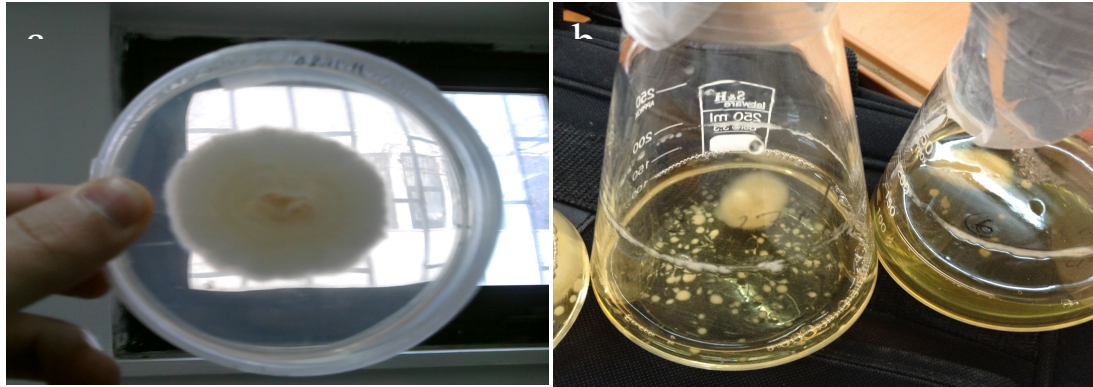


Figure 1.1 *Piriformospora indica* grown in solid and liquid culture: **a)** Fungus grown for 60 days on agar medium at 30°C. **b)** Fungus grown for 30 days in dark in Erlenmeyer flask containing broth medium. These figures are taken during thesis study while optimization the growth conditions of *P.indica* (pictures taken in lab).

One of the most striking features of *P. indica* is that they are cultivable on synthetic media as shown in Figure 1.1. As these fungi can be grown in culture, it becomes a model for mutualistic symbiosis studies. Furthermore, they have the potential for seed and plant growth stimulator.

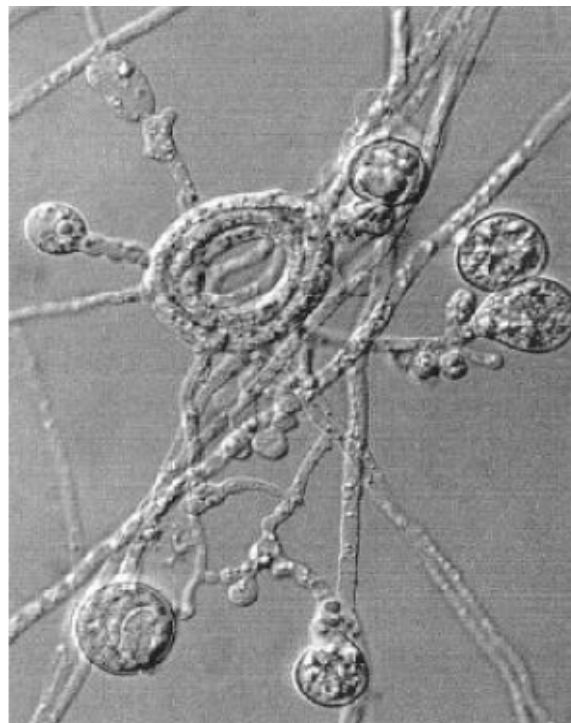


Figure 1.2 *Piriformospora indica* hyphae and chlamydospore (taken from [9]). Hyphae are the extensions from chlamydospore.

P. indica anatomically differs from other fungus. They lack clamp connections and hyphal knots. There is high frequency of anastomosis formation and hypha-hypha aggregation is often observed. Investigations on the ultrastructure of the mycelium showed multilayered cell walls and dolipores with continuous and straight parentheses. Chlamydospores occur 16–25 μm in length and 10–17 μm in width [10]. Figure 1.2 shows the structure of chlamydospores and hyphae. There are 8-25 nuclei located in spores as shown in Figure 1.3 [10].

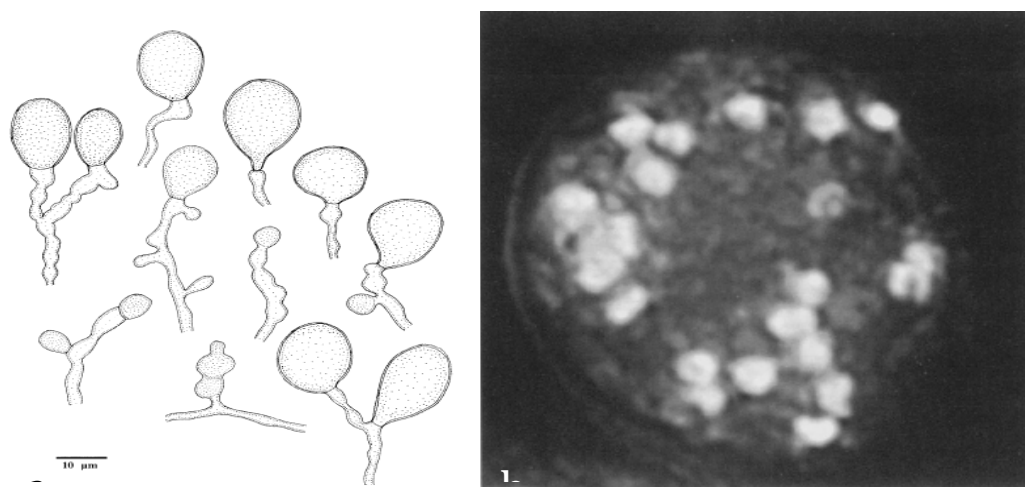


Figure 1.3 *Piriformospora indica* spores: **a)** Drawings of Chlamydospores of *Piriformospora indica*. **b)** Nuclei in a chlamydospore. Chlamydospores were stained with DAPI and observed by epifluorescence microscopy (taken from [10]).

Arbuscules is a unique feature of Arbuscular Mycorrhizal (AM). This organ is responsible for bidirectional flux for the transport of photosynthates to the mycelium and *vice versa* nutrient and water to the plants. *P. indica* presents some functional similarities with AM fungi[8]. *P. indica* has broad and diverse host spectrum (as will be mentioned in section 1.1.3). Hyphae never invade the endodermis and can be located extramatrically, inter- and intracellular. Chlamydospores are located in soil and within cortical tissues. No sexual stages for *P. indica* has ever been reported.

Apart from these anatomical similarities with Arbuscular Mycorrhizal fungus, *P. indica* has positive effects on host organism by promoting the plant growth. It can also affect phosphorus translocation. Moreover, it can both induce the hardening of micropropagated plantlets and induce resistant against root pathogens and physiological stress condition [3,4,9].

1.1.2 Root Colonization by *Piriformospora indica*

P. indica colonize the root cortex and form inter- and intracellular hyphae. Within the cortical cells, the fungus often forms (intracellularly) dense hyphal coils or branched structures. How the fungus penetrates plant roots, how roots are eventually colonized, or whether the mutualistic fungus has a facultative biotrophic or a necrotrophic lifestyle are issues that have not yet been studied.

There are several root colonization examples by *P. indica* for different plant species. In one study *P. indica* was successfully associated with root organ cultures of transformed maize. This association was materialized by a marked root surface colonization by the fungus and its establishment into the cortical tissues (inter- and intracellular). Characteristic pear-shaped spore formation occurred in the cortical regions of the root as well as in the extramatrix environment [2]. Figure 1.4 shows the association of *P. indica* with maize.

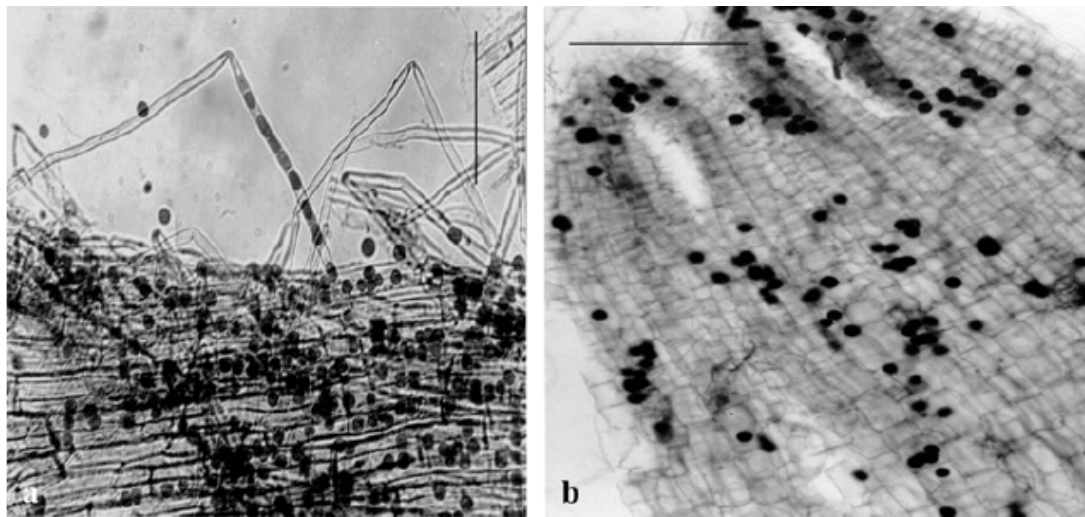


Figure 1.4 Maize root colonization by *P. indica*: **a)** A view of the root tip showing extensive inter-/intracellular fungus colonization. **b)** Colonized root tip. Figure shows the interaction of *P. indica* and maize root (taken from [2]).

In another study for root colonization, mycelium covers the surface of the roots of *Arabidopsis*. Hyphae penetrate root hairs and rhizodermis cells and eventually form chlamydospores in these cells [11]. In a more detailed study for root hyphae interaction [12], it was shown that *P. indica* hyphae run parallel to the root axis of *C. asiatica* and produces coiled hyphae intracellularly. By using scanning electron microscopy, the penetration of fungal hyphae into the root epidermis, apart from forming a mesh of hyphael networks and profuse chlamydospores on the surface were shown. Figure 1.5 shows another study for root colonization by *P. indica* in barley.

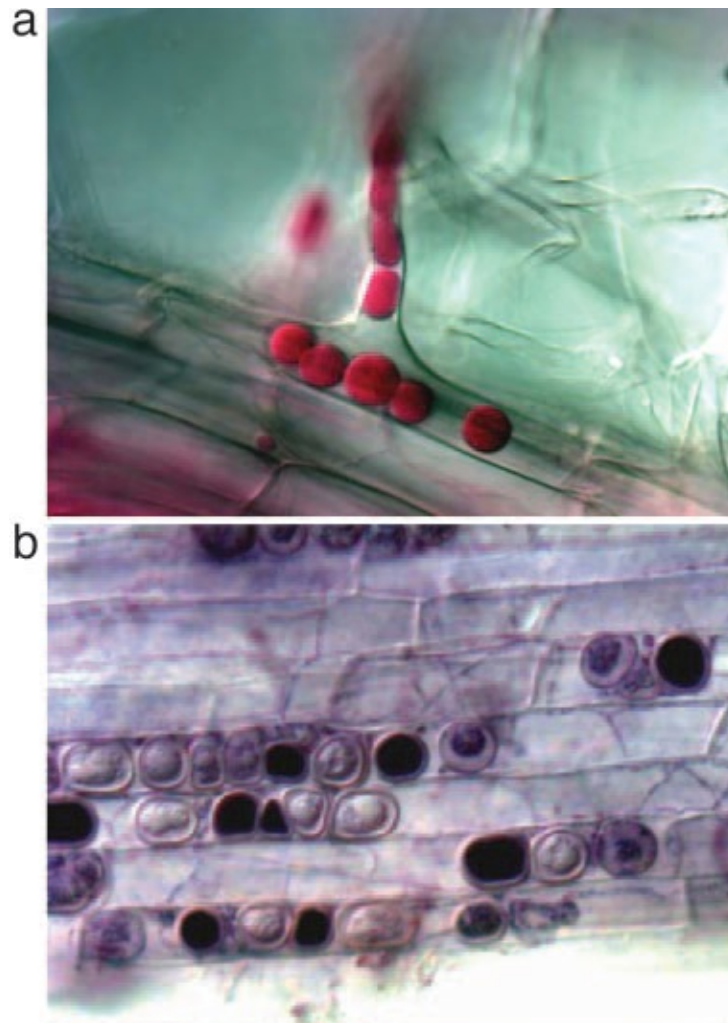


Figure 1.5 Colonization Pattern of *P. indica* in barley roots. **a)** Fungal hyphae enter roots *via* root hairs from 10-day-old plants. The fungus forms pearshaped chlamydozoospores within root hairs and proceeds into rhizodermis cells. **b)** The fungus grows into the root cortex tissue (taken from [13]).

1.1.3 Effects of *Piriformospora indica* on Plant Growth

Unlike mycorrhizal fungi, which cannot be cultured axenically, *P. indica* can be easily grown both on various substrates ranging among mono- and dicotyledonous plants and on solid or liquid culture [2,11,14, 15]. It has been found to promote plant growth during its mutualistic symbiotic relationship with a wide variety of plants. Experiments have shown that *P. indica* increases the resistance of colonized plants against fungal pathogens. It has also been found in experiments with barley that *P. indica*-inoculated plants are tolerant to salt stress and more resistant to root pathogens. *P. indica*-infested roots also show antioxidant capacity. The fungus also induces systemic disease resistance in plants. In spring

barley, *P. indica* colonization enhanced plant biomass, which was accompanied by grain yield increases of up to 11% [13]. *P. indica* stimulates adventitious root formation in ornamental cuttings [4], while enhanced salt tolerance has been observed in barley [13]. The growth promotion effect of *P. indica* on the medicinal plant *Adhatoda vasica* has been reported [16], and recently, Oelmüller *et al.* [17] demonstrated an enhanced production of podophyllotoxin in cell suspension cultures of *Linum album* by elicitation with culture filtrate and cell extract of *P. indica*.

Figure 1.6 shows some examples of the effect of *P. indica* on various plants. Figure 1.6-a shows the effect of symbiotic fungi on maize (*Zea mays white*) growth. *P. indica* stimulates the growth of maize compared to controls [18]. In the next figure, in b, both growth-promoting effect of *P. indica* on tomato plants and protection of tomatoes from *Fusarium oxysporum* by the aid of *P. indica* were shown [19]. In Figure 1.6 c, protective potential of *P. indica* toward *F. culmorum* in barley was shown [13]. Finally in Figure 1.6 d, protection against *Golovinomyces orontii* (*Go*) in *Arabidopsis* by the help of *P. indica* was shown [15].

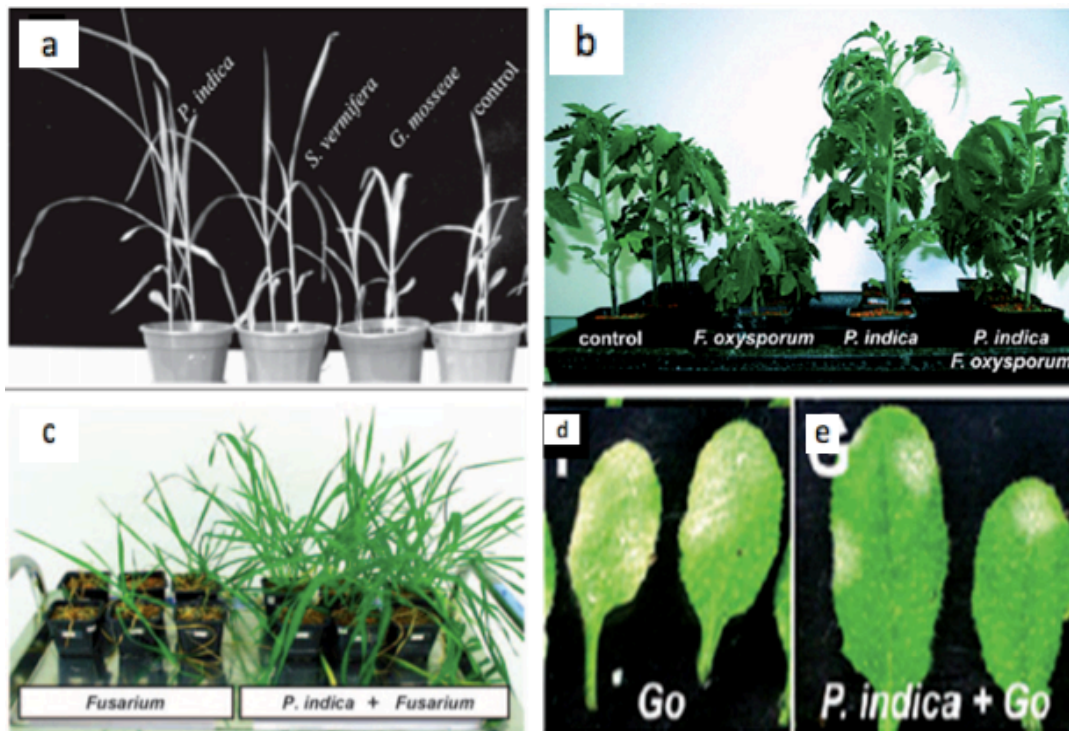


Figure 1.6 Effects of *P. indica* on various plants: a) maize b) tomato c) barley d) *Arabidopsis*. In all four cases *P. indica* enhances the growth of various plant species and induces the resistance against species specific pathogens. Figures taken from [18,19,13,15] respectively.

1.2 Wheat and Barley Production

Wheat and barley are two major crops highly produced in Turkey. Wheat is mainly used for bread making whereas barley is mainly used for feeding the animals. Wheat and barley can be produced in every region of Turkey except Black sea shores. In Turkey 55% of fertilizers used are used for wheat and barley production. Nevertheless due to inadequate development and improper fertilization methods, highest quality and quantity cannot be achieved for wheat and barley production. On the other hand, it is not difficult to achieve high yield in production of wheat and barley.

Apart from preparing the soil for plant growth and struggle against pathogens and wild weed, certified seed usage and proper fertilization can enhance the wheat and barley production by increasing the quality and quantity.

According to Turkey Statistics Institution (TÜİK) data, for the last 4 years (2009-2012), Turkey produced on average 20 million tons of wheat and 7 million tons of barley while in the same time, world production for wheat is around 654 million tons and for barley is around 135 million tons. Although Turkey is one of the most important grain producers in the world, with the efficiency of 1.95 tons of wheat per hectare, still it is behind the average of European Union with the efficiency of 5.66 tons of wheat. The reason for this low efficiency mostly depends on low scale production in field, low quality of seed sow and inadequate usage of fertilizers.

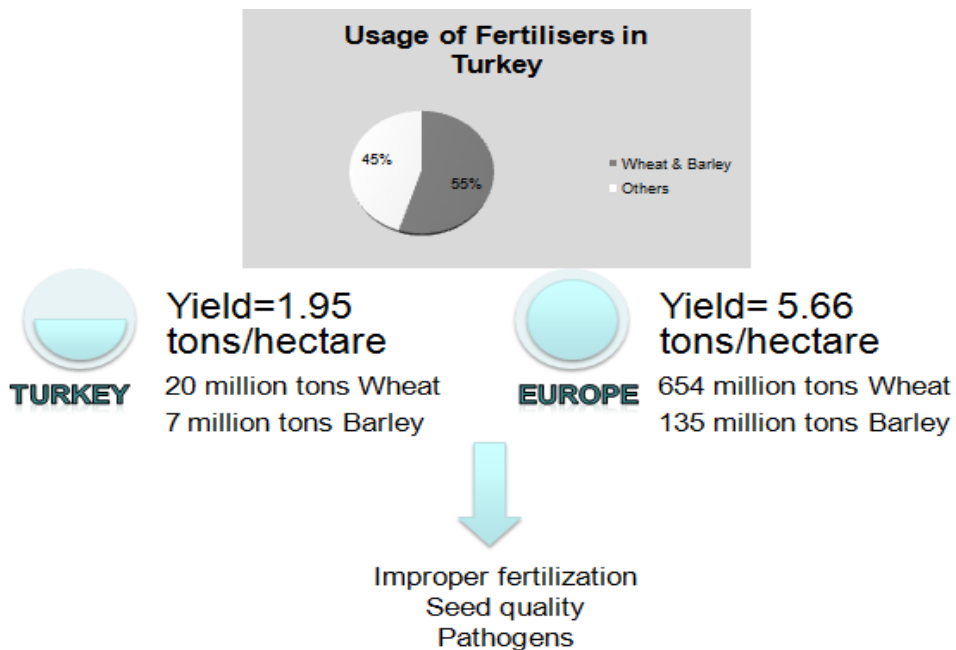


Figure 1.7 Usage of fertilizers and yield in Turkey in comparison with Europe (TUIK data 2009-2012).

At this point, more developed technologies for fertilizers for phyto-stimulation and resistance to pathogens and stress conditions are required. Being a cultivable mycorrhizal endophyte, *P. indica* can be included in the growth process of wheat and barley as plant growth promoter and pathogen resistant inducer.

1.3 Barley Disease Powdery Mildew Caused by *Blumeria graminis*

Powdery mildews are one of the most widespread and easily recognized plant diseases. It is the serious fungal disease, induced by *Blumeria graminis* [20]. Pathogen affect virtually all kinds of plants: cereals and grasses, vegetables, ornamentals, weeds, shrubs, fruit trees, and broad-leaved shade and forest trees. Powdery mildew diseases are caused by many different species of fungi. *Blumeria graminis* is the fungus that causes powdery mildew on plants.

The most characteristic symptom of powdery mildew is its white spots, occurring on a leaf surface. In addition to the leaf surface, this disease also infects other side of the leaf, bud, flower, young stem and young fruit. Powdery mildew will begin as discrete circular, powdery white spots. As these spots expand they will run together, producing a continuous matt of mildew which to the casual observer would appear similar to dirt or dust. Powdery mildews are characterized by spots or patches of white to grayish, talcum-powder-like growth.as in Figure 1.8 and the leaf may become deformed and fall prematurely. The degree of the disease rests on many factors such as the host plant variety, age of the host and weather during the growing season [21].



Figure 1.8 *Blumeria graminis* infected barley plant. Black spots in white regions indicate the infection *Bgt* in barley species (taken from [21]).

Tiny, pinhead-sized, spherical fruiting structures that are first white, later yellow-brown and finally black may be present singly or in a group. These are the cleistothecia or overwintering bodies of the fungus. The disease is most commonly observed on the upper sides of the leaves. It also affects the bottom sides of leaves, young stems, buds, flowers, and young fruit. Infected leaves may become distorted, turn yellow with small patches of green, and fall prematurely. Infected buds may fail to open.

Normally, symptoms will appear late in the growing season on outdoor crops. The fungus is favored by high relative humidity periods or site conditions that promote this type of environment, such as close spacing of plants, densely growing plants, or shade. Indoors, symptoms may occur at any time of year, but the rate of spread and development will be affected by the relative humidity and temperature. Injury due to powdery mildews may include stunting and distortion of leaves, buds, growing tips, and fruit. The fungus may cause death of invaded tissue. Yellowing of leaves and death of tissue may result in premature leaf drop. Nutrients are removed from the plant by the fungus during infection and may result in a general decline in growth and vigor of the plant. The seriousness of the disease will depend on the extent of these various types of injury.

1.3.1 *Blumeria graminis* f.sp. *hordei*

Blumeria graminis is a fungus that belongs to ascomycota the biggest division of fungi. Ascomycota has several subspecies and a specific host. *B. graminis* f.sp. *hordei* is one example infecting barley and *B. graminis* f.sp. *tritici* infecting wheat and *B. graminis* f.sp. *secalis* infecting rye [22, 23]. *Blumeria graminis* as a pathogen causes serious problems as it causes yield and quality losses [24]. Pathogen cannot survive without its host as it is an obligate biotrophic parasite [25]. For spore germination, pathogen requires cool and humid environment [26]. That is the reason of widespread property of disease in higher number of plants in small areas where the environment favors the spore germination under the conditions of insufficient circulation.

Blumeria graminis f.sp. *hordei* have cylindrical-shaped conidia structures in chains that are spread by blow of wind and cause of the disease on plant leaf surface to infect [27]. If a host has *Mlo* (*Mildew Locus O*) gene, the pathogen can invade most efficiently [28]. *Mlo* gene produces plant specific integral membrane protein, whose biochemical role is still unknown [29]. *Mlo* has a negative regulator function on cell death; thus, mutations at this locus, causing loss of function, result in resistance to powdery mildew disease *via* the cell-death response mechanism and the accumulation of a callose rich barrier at infection site [30].

The asexual life cycle of *Blumeria graminis* proceeds in a highly synchronous way [31]. When wind-dispersed "conidia of the pathogen reach to a leaf surface, the process of infection begins. After infection, an extracellular matrix is instantly produced. The function of the extracellular matrix is thought that it helps fungus to bind the leaf surface and to get

signal cues of surface [27]. An hour post inoculation (hpi), a short primary germ tube occurs from a conidium. It provides the perception of the surface nature where the conidia proliferate [32, 33]. After a short time, appressorium germ tube, also called secondary germ tube, appears. Then, appressorium germ tube forms the appressorium, obviously noticeable at 8 hpi. This swollen-hooked structure adheres to the surface. At 15 hpi, a penetration peg occurs from appressorium. This hyphal strand destroys the epidermal cell wall by turgor pressure and enzymatic activity. The penetration peg does not invade the plasmalemma; therefore, a haustorium, forming from penetration peg, develops in between the inner membrane and the cell wall, called periplasmic space. The function of the haustorium is to feed *Blumeria graminis* by providing nutrients from epidermis cells of the host plant. It allows the pathogen to pullulate rapidly on the leaf surface. The pathogen colonies can be observed by naked eye at approximately 3 days post inoculation (dpi). Then, the colonies start to generate numerous conidiospores. At about 5 dpi, conidiospores produce conidia", asexual and non-motile spores. These can spread by wind to infect the other host plants [31]. The whole life cycle of the pathogen development is briefly described in Figure 1.9.

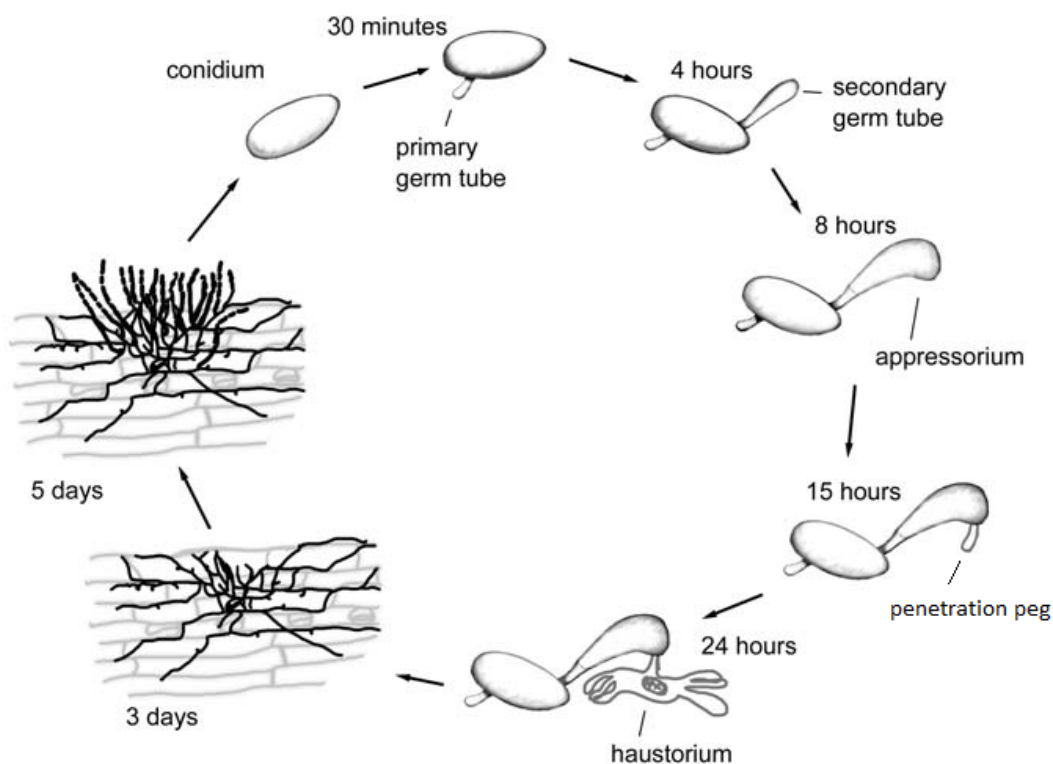


Figure 1.9 Asexual life cycle of *Blumeria graminis* f.sp. *hordei* (*Bgh*). Only after the third day, *Bgh* shows the pathogenic effect on target plant leaves by forming hyphae as can be seen from the figure, 3rd day drawing (taken from [31]).

1.4 Wheat Disease Stripe Rust Caused by *Puccinia striiformis* f.sp. *tritici*

The wheat rusts are caused by three species of the fungal genus *Puccinia*: stripe rust by *Puccinia striiformis* f.sp. *tritici*; leaf rust by *Puccinia triticina*; and stem rust by *Puccinia graminis* f.sp. *tritici*. Generally, these pathogens are confined to wheat but can occur to a small extent on other cereals and grasses.

Puccinia striiformis f. sp. *hordei*, is an obligate parasite that overseasons on volunteer barley or rye, certain wild barleys such as *Hordeum jubatum* (foxtail barley), wheat, and numerous perennial grass species. The disease begins from a very small number of infections that are difficult or impossible to detect in the field. Spread of the pathogen can be explosive and cause significant losses, especially in wet weather, which greatly favors disease development.



Figure 1.10 *Puccinia striiformis* f.sp. *tritici* (*Pst*) causing stripes on wheat. Yellow stripes resembling the rust can be observed after *Pst* infection (taken from [20]).

Stripe rust survives year-round on living wheat. Some other cereals and grasses can play a minor role in its survival. Wind spreads spores within crops, and over long distances. The rate of development of stripe rust depends on moisture and temperature: it is most active in autumn and spring.

The reaction of a wheat variety to stripe rust depends on two forms of resistance. The first, seedling resistance, operates throughout the life of the plant. The second, adult plant resistance (APR), develops as the plant matures. APR usually develops some time during the booting stage, but can develop earlier during stem elongation, or is delayed until early head emergence, depending on variety and growing conditions.



Resistant to moderately resistant



Moderately resistant



Moderately susceptible



Moderately susceptible to susceptible



Susceptible



Very susceptible

Figure 1.11 Resistance against yellow rust in wheat. Varying levels of adult plant reaction to stripe rust (taken from [20]).

1.5 Aim of the Study

P. indica is recently explored fungus that can be cultivated. It has the potential to be plant promoter, biofertilizer, bioregulator, bio-herbicide, immunomodulator, phytoremediator, regulator against insects and pathogens, tolerance inducer against temperature and salt. With these potential properties and cultivable ability, *P. indica* becomes the ideal fertilizer for many crop products.

Wheat and barley are two main grains that are highly produced in Turkey. Nevertheless due to inadequate development and improper fertilization methods, highest quality and quantity cannot be achieved for wheat and barley production. More developed technologies for fertilizers for phyto-stimulation and resistance to pathogens and stress conditions are required. Being a cultivable mycorrhizal endophyte, *P. indica* can be included in the growth process of wheat and barley as plant growth promoter and pathogen resistant inducer.

In this study, our aim is to test *Piriformospora indica* against powdery mildew in barley and *Puccinia striiformis* in wheat to determine best application method of *P. indica* to assess the induction of resistance, growth and yield. Also we are aiming to develop a procedure that enables usage *P. indica* in large-scale agricultural applications. Although there are specific chemicals and/or drugs that can be used to cure the barley and wheat diseases, usage of *Piriformospora indica* against the diseases not only induce the resistance and enhance the growth but also worldwide classified as organic agricultural application. In other words, if *Piriformospora indica* is used against the pathogens, then still the product will be classified as organic product. To achieve our goals, we applied *P. indica* in different spore concentrations and in different form as powder and liquid culture. Then we infected plants with pathogens to evaluate the disease level in comparison with control group of plants (without *Piriformospora indica*).

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant and Fungi Materials

Barley (*Hordeum vulgare* ssp. *vulgare*) differential line Bülbül seeds were amplified by Assist. Prof. Dr. Mehmet Aybeke, Trakya University. The Bülbül is well characterized differential line produced by James Kolmer and provided by ICARDA. Wheat (*Triticum* spp.) differential line Michigan Amber used as plant material for *P. indica* and pathogen infections experiments as well as Bülbül for barley.

Fungal pathogen *Blumeria graminis* f.sp. *hordei*, *Bgh95*, was kindly obtained from Prof. Dr. Mogens Stovring Hovmoller of Aarhus University, Demark (2008), and used as pathogen materials to inoculate barley leaves in order to assess *P. indica* effects on plants resistance against powdery mildew by trypan blue staining.

Fungal pathogen *Puccinia striiformis* f.sp. *tritici*, *TR-09/97*, was obtained from Turkey and was characterized by Prof. Dr. Claude Pope of INRA, France, and used as pathogen materials to inoculate wheat leaves in order to assess *P. indica* effects on plants resistance against yellow rust.

Root endophyte *Piriformospora indica* was obtained in solid stock culture, from Prof. Dr. Ralf Oelmüller who works as the head Faculty of Biology and pharmacy at Jena University, Germany.

2.2 *P. indica* Growth Conditions

The stock culture was maintained on slants containing Kaefer medium supplemented with 15 g/L agar. Slants were inoculated, incubated at 30 °C for 10 days and then stored at 4°C. For the preparation of inoculums, *P. indica* was initially grown on Kaefer medium, detailed ingredients were given in Table 2.1, in a petri-dish and then transferred to the seed culture medium by punching out 8 mm of agar discs with a sterilized cork-borer in a sterile hood. The seed culture was grown in a 500 mL flask containing 100 ml potato dextrose broth at 30 °C on a rotary shaker at 200 rpm for 14 days. Alternatively, instead of potato extract, Kaefer medium without agar was also tried to optimize the maximum the *P. indica* spore

Table 2.1: Kaefer medium ingredients:

Constituent	Concentration
Glucose	20.0 g/L
Peptone	2.0 g/L
Yeast extract	1.0 g/L
Casamino acid	1.0 g/L
Vitamin stock solution	1.0 mL
Macroelements from stock	50 mL
Microelements from stock	2.5 mL
Agar	10 g/L
CaCl ₂ , 0.1 M	1.0 mL
FeCl ₃ , 0.1 M	1.0 mL
pH:6.5	
Macroelements Stock Solution (major elements)	
NaNO ₃	120.0 g/L
KCl	10.4 g/L
MgSO ₄ · 7H ₂ O	10.4 g/L
KH ₂ PO ₄	30.4 g/L
Microelements Stock Solutions (trace elements)	
ZnSO ₄ · 7H ₂ O	22.0 g/L
H ₃ BO ₃	11.0 g/L
MnCl ₂ · 4H ₂ O	5.0 g/L
FeSO ₄ · 7H ₂ O	5.0 g/L
CoCl ₂ · 6H ₂ O	1.6 g/L
CuSO ₄ · 5H ₂ O	1.6 g/L
(NH ₄) ₆ Mo ₇ O ₂₇ · 4H ₂ O	1.1 g/L
Na ₂ EDTA	50.0 g/L
Vitamin Stock Solution (%)	
Biotin	0.05
Nicotinamide	0.5
Pyridoxal phosphate	0.1
Amino benzoic acid	0.1
Riboflavin	0.25

number in the growth medium. Potato weight was measured and different amount of potato was extracted and was examined to get the maximum number of spores. Basically, potato weight was measured, was sliced in small pieces and was boiled. Different volumes of extract was added to water and *P. indica* was grown in these different amount containing potato extracts such as; 100 g/L, 150 g/L, 200 g/L, 250 g/L and finally 300 g/L. There was no difference between in both broth culture conditions. Passage of *P. indica* was done basically changing the petri dish for growing by punching out about 8 mm of agar discs with a sterilized cork-borer in a sterile hood.

After growing *P.indica* in different media conditions, spores were dislodged by adding 1 ml of Tween 80 to 100 ml of culture broth, vortexing, grinding in a mixer-grinder. After their detachment, the spores were counted with a hemacytometer by applying 10 µl of the cells to the area of 1 mm² in the hemacytometer. Effectiveness of growth medium by means of spore production was done by counting the spores by hemacytometer.

2.3 *P. indica* Inoculation

P. indica was applied to plant material by different ways. For all different ways, first spore numbers were detected by hemacytometer and then according to the pot volume that the plant will be grown, a certain number was set. In fact, optimum spore numbers per soil volume or perlite volume was also optimized. By that way, optimum number and optimum conditions for *P. indica* inoculation was set. *P. indica* was applied to plants either in liquid form just after growing in the culture and grinding the spores from fungus main body or mixing with gum to enhance the attachment of spores to target plant root. To summarize, simply *P. indica* inoculation was done adding proper amount of spores in plant pot.

Effectiveness of inoculation was set by trypan blue staining of the plant roots. After inoculation of the plants, at some certain time points, roots were stained with trypan and inoculated spore number in the root was determined. Time period for optimum inoculation was also optimized. Basic protocol for root staining includes; root clearing, washing, staining and finally again washing steps. First root tips were isolated from the plant and cleared from soil and other support material such as perlite. Then roots were dipped into 2 % KOH solution for clearing. This step was done in autoclave at 121 °C for 15 minutes to fasten the clearing process. After this clearing step, roots were cleaned with water. Prior to staining with trypan, HCl was applied to roots to soften the roots in order to enhance the stain penetration to inner layers of root structure. 30 minutes of 2 % HCl treatment, again roots were washed to get rid of acid. Finally again in autoclave at 121 °C for 15 minutes, roots were treated with 0.05 % (w/v) trypan blue solution (1:1:1- lactic acid:glycerol:water). After staining process final washing was done to roots to get rid of excess stain. Observations were done with light microscope and spore numbers were counted to determine the effectiveness of *P.indica* inoculation.

2.4 Maintenance of Pathogens and Inoculation of Pathogens

Bülbül 89 is a cultivar of barley and is susceptible to all the known *Blumeria graminis* f.sp. *hordei* races; thus, Bülbül 89 was used as plant material for maintenance of *Bgh95*. Bülbül 89 seeds were planted on soil into 650 cm³ pot and grown for 10 days in a growth chamber, Sanyo Versatile Environmental Test Chamber (Model MLR-351H), (conditions; at 18 °C light-period for 16 hr and dark-period for 8 hr). After 10 days, first leaves were cut and approximately 5 cm long pieces were put onto water-agar plates (Agar plates were prepared as follows: 7 g agar (LabM, Lot#: Q34567/129) was dissolved in 450 ml of ddH₂O and the mixture was sterilized in autoclave. After cooling to 50 °C, 50 mL of

Benzimidazole (Aldrich, Lot#: 23968 222) (1 g/L, in 0.1 % DMSO) was added.). Then, they were inoculated with pathogens by blowing. The leaves were incubated in a growth chamber at the same conditions as above. This process was repeated every 10-12 days to keep pathogens alive.

Puccinia striiformis which is the causative agent of yellow rust cells can be kept alive only on barley (*Triticum aestivum*), Michigan Amber and the maintenance of them requires frequent subculturing of these rust cells on new plant leaves. Michigan Amber is a cultivar of wheat and is susceptible to all the known *Puccinia striiformis* f.sp. *tritici* races; Michigan Amber was used as plant material for maintenance of TR09-97 of *Puccinia striiformis*.

When the seedlings were reached to 3-4 cm, which corresponds to 5 days of growth in the same conditions, 0.25 mg/L maleic hydrazine was added. Maleic hydrazine prevents overgrowing of wheat seedlings. Moreover, it leads to all the seedlings in one pot stay at the same height. Maleic hydrazine solutions were prepared by mixing 0.25 mg of maleic hydrazine with 1L distilled water. It was prepared and mixed in a bottle by using a magnetic stirrer at 250 rpm for 1 hour. 20 ml of this solution was poured to each pot having 20 seeds in each one. Seedlings were left to grow at 20 °C under light, with moisture. After 2 days of maleic hydrazine treatment, the plants were prepared for infection. Spores that were kept at -80 °C were placed into 4 °C for 10 minutes. After that, they were put in tubes and oil was added on spores. The spores were sprayed onto plants using equipment similar to atomizer in the sterile hood. For equal dispersion, it was sprayed on every side of the pot. After spraying, they were kept in the sterile hood for 10 minutes to let them dry. Then, pots were put in glass chambers with steam engine overnight at dark and at 10 °C. After 16-18 hours, steam engine was taken out and the lights were turned out. About 10-15 days after infection, the spores were observed on leaves of the plants and they were collected.

For collection, plants from glass chambers were taken out and spores were collected by slowly touching and tapping to the leaves of the seedlings. After collecting the spores, they were put into glass tubes. The collected spores were put into the desiccators filled with CaCl₂ to keep moist away from the spores. Collected spores were either stored at -80 °C or used for inoculation.

2.5 Determination the Effect of *P. indica* on Plant Resistance Against Pathogen

As mentioned above for maintenance of the pathogen, inoculation was done in the same way. In leaf segment test, secondary and tertiary leaves of the barley plants, which were seeded with *P. indica* spores, were collected and *Bgh* was inoculated to the plants. At different time points; 8 hours, 12 hours, 24 hours and finally 72 hours, leaf samples were collected and were stained with trypan blue as mentioned above. This time during staining no clearing or cleaning steps were done. Leaves were just put in staining solution and *Bgh* were tried to be observed. There are different growth conditions for *Bgh* species as explained

in introduction. The reason why leaf samples were taken at different time points is to observe the different growth stages of *Bgh*. According to staining procedure, effectiveness of pathogen inoculation and effectiveness of *P. indica* to plant by means of resistance can be measured.

Trypan blue staining was performed as Vogel and Somerville's procedure (2000). The leaf segments from the plant which were silenced and control treated, 3 and 5 days after inoculation were collected from water-agar plates and put into 95 % ethanol for overnight to bleach green background. Bleaching leaves were stained with trypan blue solution for 15 min at room temperature (250 µg/mL trypan blue (Applichem; Lot#: 7D008258) in a solution of lactic acid (Fluka; Lot#: BCBC5008): 87 % glycerol: ddH₂O (1:1:1)). Then, samples were transferred to rinsing solution (Lactic acid: 87 % glycerol: ddH₂O (1:1:1)) and kept for approximately 4 min at room temperature. Afterwards, leaf segments were placed on glass slides and the structures was observed under light microscope (Leica, DFC 280).

For *Puccinia striiformis* f.sp. *tritici* inoculation to wheat, spraying was done to the plants as mentioned in previous section. Pathogen solution was basically sprayed to the leaves of the wheat. Determination of the effect of pathogen and *P. indica* on the leaf conditions was empirically determined. Generally resistance to yellow rust in wheat can be classified into 4 groups: Resistant, moderately resistant, moderately susceptible and susceptible. According to literature, resistance level and effect of *P.indica* to yellow rust resistance was determined.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 *P. indica* Growth Condition Optimization

P. indica is recently explored fungus that can be cultivated. It has the potential to be plant promoter, biofertilizer, bioregulator, bio-herbicide, immunomodulator, phytoremediator, regulator against insects and pathogens, tolerance inducer against temperature and salt. With these potential properties and cultivable ability, *P. indica* becomes the ideal fertilizer for many crop products.

In order to obtain the ideal growth conditions for *P. indica* in culture, several optimization studies were conducted. Ideal conditions were set as growing the fungi culture at 30 °C on a rotary shaker at 200 rpm for 14 days.

To obtain the maximum numbers of spores that will be used in plant inoculation studies, different amount of potato extract were tested as growth medium. Under the given conditions and with different amount of additions potato extract, the spore numbers were determined by using heamocytometer after 14 days. For this purpose, different potato broth extract concentration was set as 100 g/L, 150 g/L, 200 g/L, 250 g/L and finally 300 g/L. These values were determined by measuring the solid potato weighted and boiled the solid potato in given amount of dH₂O. As mentioned in materials and method section, after growing *P. indica* in different media conditions, spores were dislodged by adding 1 mL of Tween-80 to 100 mL of culture broth, vortexing, grinding in a mixer-grinder. After their detachment, they were counted with a heamocytometer by applying 10 µL of the cells to the area of 1 mm² in the heamocytometer.

Growth media optimization trials to obtain the highest number of spores were repeated three times from each of the repeat. Four samples were taken from 100 mL culture and each sample was counted twice in heamocytometer. Totally, 18 numbers of counts were performed. The values of the spore numbers are presented below as the average values of these 18 counting events.

The highest yield for spore number was obtained from 250 g/L of potato amount culture as 2.9x10⁶ spores/mL, and the closest value to 250 g/L was set in 300 g/L potato containing media, as 2.6x10⁶ spores/mL. For these two values non-parametric t test of Mann-Whitney test was applied to determine the significance of the closest values obtained (n=18, p<0.0001) and the difference was found to be significant.

As can be observed from Figure 3.1, for 100 g/L concentration of potato extract 8.6×10^5 spores/mL was measured. For other concentrations of solid potato weight the values calculated are: for 150 g/L; 9.9×10^5 spores/mL, for 200 g/L potato; 2.3×10^6 spores/mL, for 250 g/L potato 2.9×10^6 spores/mL and finally for 300 g/L potato 2.6×10^6 spores/mL values were obtained in these growth media optimization studies.

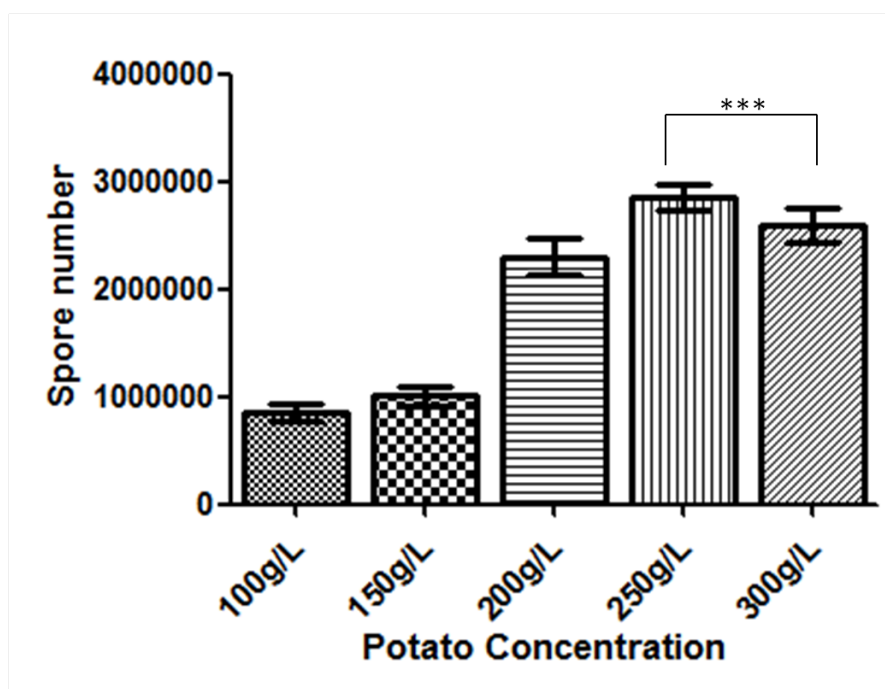


Figure 3.1 *Piriformospora indica* Growth medium optimization. Comparison of different amount of potato extracts concentrations on spore production (30 °C, 200 rpm, 14 days). *** Significant at $p < 0.0001$ according to Mann Whitney test.

While in some studies to obtain the maximum number of spores potato extract was used [2,13,11] to increase the yield, here we found that different medium such as Kaefer was better to obtain highest number of spores. In one study, authors claimed that by using Kaefer media, they get 9.25×10^7 spores/mL [4]. For that purpose, the best spore yielding potato concentration containing media was compared with Kaefer medium in this study. Again same experimental procedure was followed and spore numbers were counted after 14 days. Figure 3.2 summarizes the comparison of potato broth medium and Kaefer medium. Potato broth medium with 250 g/L potato concentration produced around 3 million spores per mL while Kaefer medium increased the spore production yield to around 6 million spores, doubling the spore number. This difference was again tested statistically and was found to be significant with a p value of $p < 0.0001$. Although Kaefer medium significantly enhances the spore production compared to potato broth, still it is 1/15 of the given values in Kumar's paper [4]. This situation was found to be contradictory. Nevertheless, there is only one publication about Kaefer medium giving such a high yield. Thus, due to this increased yield in spore number by using Kaefer media, it was decided to use Kaefer media to produce spores for further plant inoculation studies.

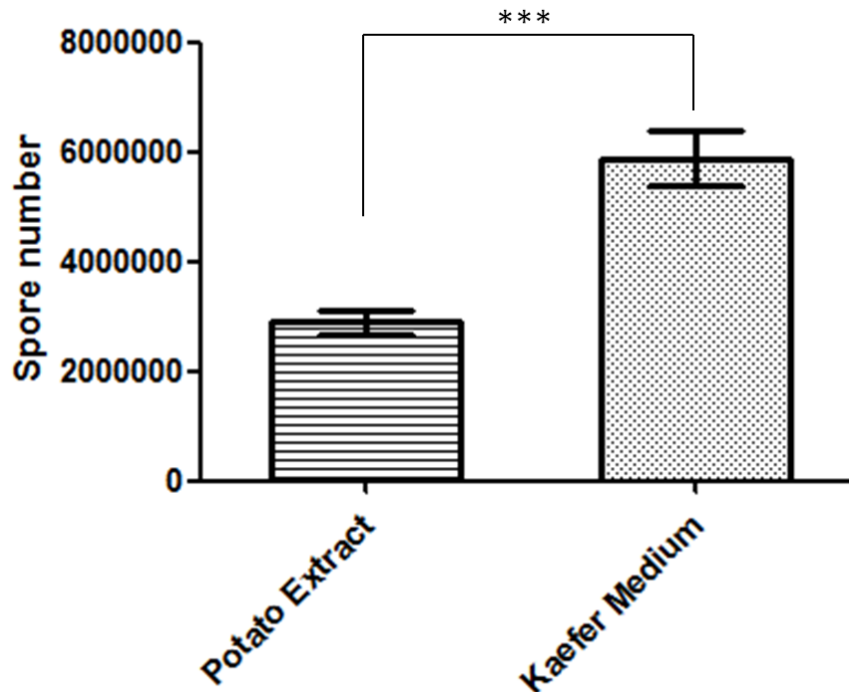


Figure 3.2 Comparison of Kaefer medium and potato broth (250 g/L). The growth conditions are 30 °C, 200 rpm, and 14 days. *** Significant at $p < 0.0001$ according to Mann Whitney test.

3.2 *P. indica* Inoculation Optimization

In order to see the effects of *P. indica* on plant growth or to observe induced resistance response in plants, first of all the endopyhte *P.indica* must be inoculated to the plant of interest. There are several different ways of inoculation procedures for each plant species. In some other cases, plant seeds were sown in the soil containing *P. indica* spores. In some cases plant seeds were washed with *P. indica* spore culture. If the plant of interest will be inoculated at a later stage, then the roots of the plant will be dipped into spore solution [11].

For *P. indica* inoculation of barley and wheat species, in order to set a standard for all experiments, a certain method discussed above was decided with a fixed amount of fungi spores. For that purpose, different numbers of spores were tested to observe the effectiveness of the inoculation of *P. indica* to wheat and barley. 100,000 spores, 500,000 spores and finally 1,000,000 spores per plant seed were tested. Basically, a decided number spores per seed was arranged and pour into to pot that contains plant seed and perlite and/or soil. For instance, if a pot contains 5 barley seeds, 100,000 spores per seed was used for inoculation, thus 500,000 spores were added.

Detection of the infected plants (barley and wheat) was performed by staining the roots of the plants with trypan blue staining so that chlamydo spores can be visualized and counted as discussed in materials and methods section. Although successful inoculations could be detected by this staining, quantification could not be done by simple staining. This method indicated whether the fungus was successfully inoculated or not. However, as can be seen from the Figure 3.3, increased number of spores used in inoculation optimization, gave clues about the number of spores that should be used in other experiments. 100,000 spore per plant seed caused very few numbers of spores located at the root of plants while 1,000,000 spores per plant seed caused crowdedness in the plant root and affecting the growth of plant. Optimum number for plant inoculation with *P. indica* was set to be 500,000 spores per plant seed as can be seen from figure 3.3.

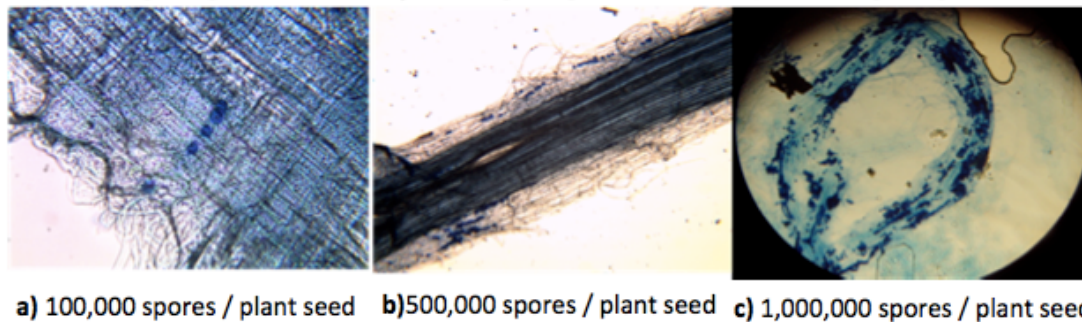


Figure 3.3 Comparison of spores occupying the barley roots: a) 100,000 b) 500,000 c) 1,000,000 spores in Kaefer media per barley seed (cv. Bülbül). Trypan blue staining was performed (28 dpi). The chlamydo spores were observed under light microscope at 10X magnification (Leica, DFC 280).

Another trial was conducted to optimize the method of inoculation. In some studies, for different plants [19,9,13], to enhance the binding of spores to the seeds and thus to enhance the localization of spores in the roots of plants, additive materials in spore culture was used. For that purpose, additive gums were mixed with certain number of spores in culture. To see the effects of gums to spore localization, again roots of plants were stained with trypan blue to observe the *P. indica* spores in root. Figure 3.4 shows the results of this trial. Although same number of *P. indica* spores was used in both methods, spores without additive material better colonized in the roots of plants. Based on these trials, *P. indica* spores were inoculated without any mixing or additive material with the number of 500,000 spores in Kaefer per seed for further experiments.

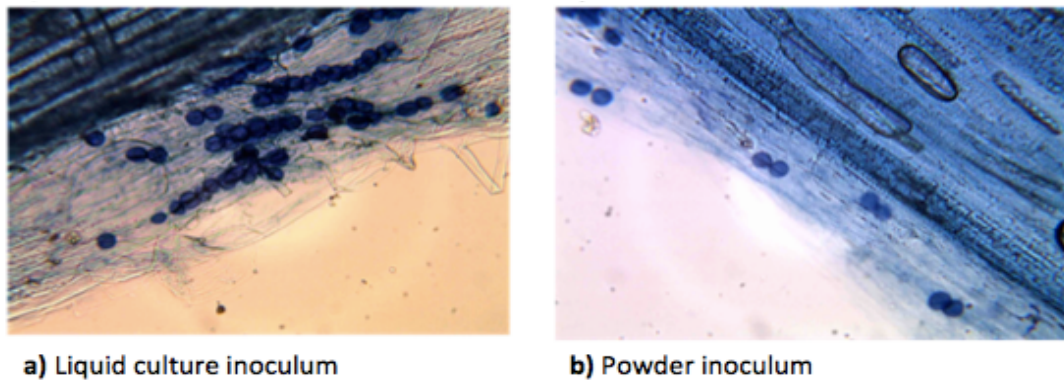


Figure 3.4 Effect of additive material for spore colonization in plant roots: Same numbers of *P. indica* spores (500,000 per barley seed (cv. Bülbül)) were used in both methods; trypan blue staining was performed to plant roots (28 dpi). The chlamydospores were observed under light microscope at 10X magnification (Leica, DFC 280).

3.3 Determination of Induced Resistance Against Powdery Mildew in Barley

Powdery mildews are one of the most widespread and easily recognized plant diseases. They affect virtually all kinds of plants: cereals and grasses, vegetables, ornamentals, weeds, shrubs, fruit trees, and broad-leaved shade and forest trees. Powdery mildew diseases are caused by many different species of fungi. *Blumeria graminis* is the fungus that causes powdery mildew on plants. *P. indica* is the endophyte and it was shown to increase the resistance of colonized plants against fungal pathogens [15].

Completing the optimization of growth conditions of *P. indica* and inoculation conditions to plant species, then it was tested to determine the induced resistance levels of barley species against powdery mildew.

As shown in Figure 1.8, *Blumeria graminis* f.sp. *hordei* has asexual life cycle. At the 8th hour appressium appears. Then sequentially at the 12th hour penetration peg and at the 24th-hour haustorium appears. Finally after 72nd hour, hyphae appear and pathogen begins to colonize the plant leaves. Resistance of the barley was determined by tracking the sexual life cycle of pathogen during this 72 hours and sampling from the leaves at 8th, 12th, 24th and 72nd hours during the development. Final determination of the resistance of barley upon *P. indica* inoculation was determined by comparison of the primary and secondary hyphae length, namely, leaf segment test. Experiment was performed in three repeats and for each time point 5 different leaf samples were used to inoculation with *Bgh*. From each 5 leaf 3 slides were prepared. From each slide 10 infection sides were measured for the hyphae length. Totally around 450 spores were examined and hyphae extending from the spores were measured. Figure 3.4 represents the slides that were prepared for leaf segment test at different time points. Figure 3.5 shows the details of slide that was prepared for hyphae length analysis. Finally, Figure 3.6 summarizes the statistical analysis conducted for primary and secondary hyphae length measurements.

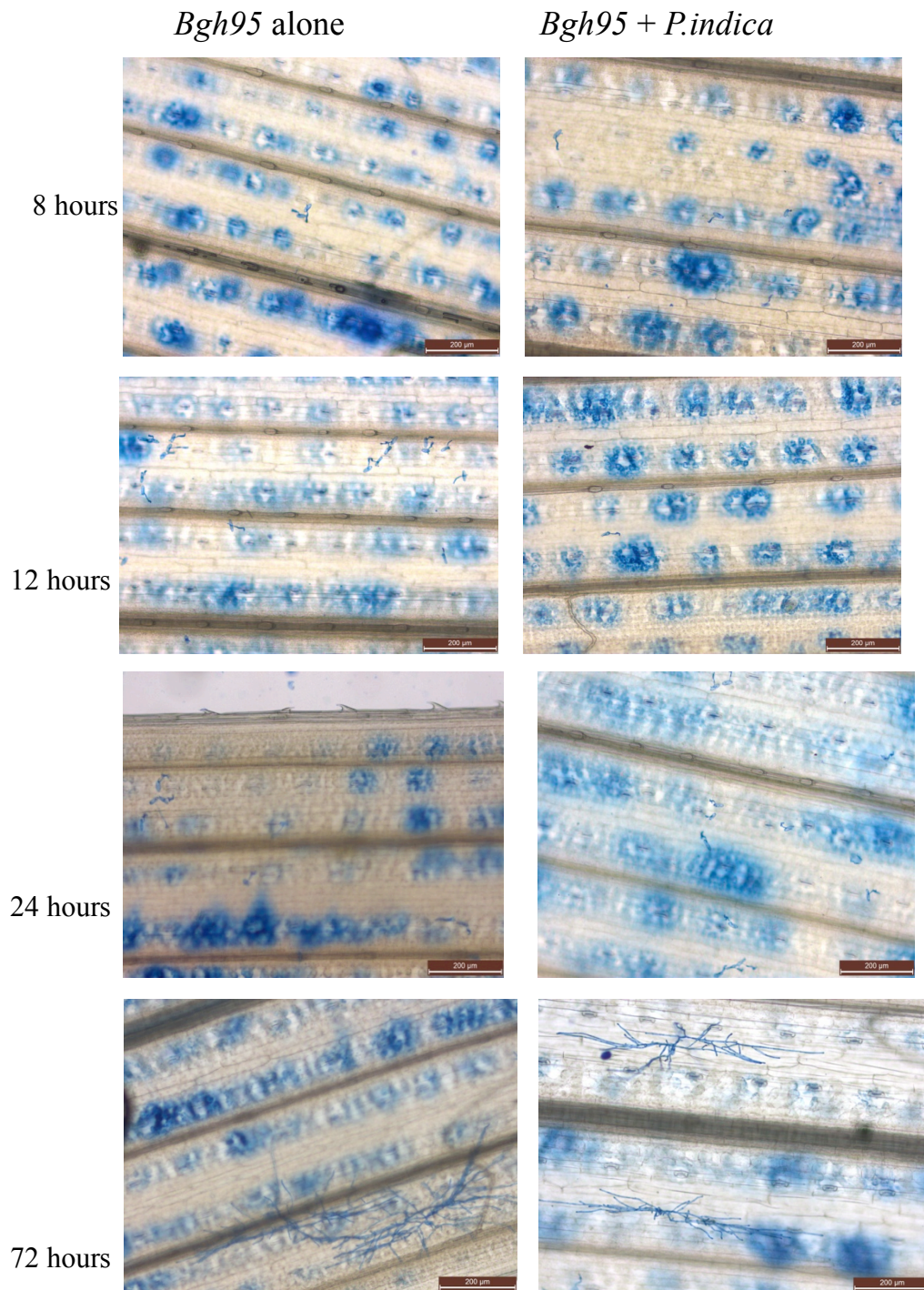
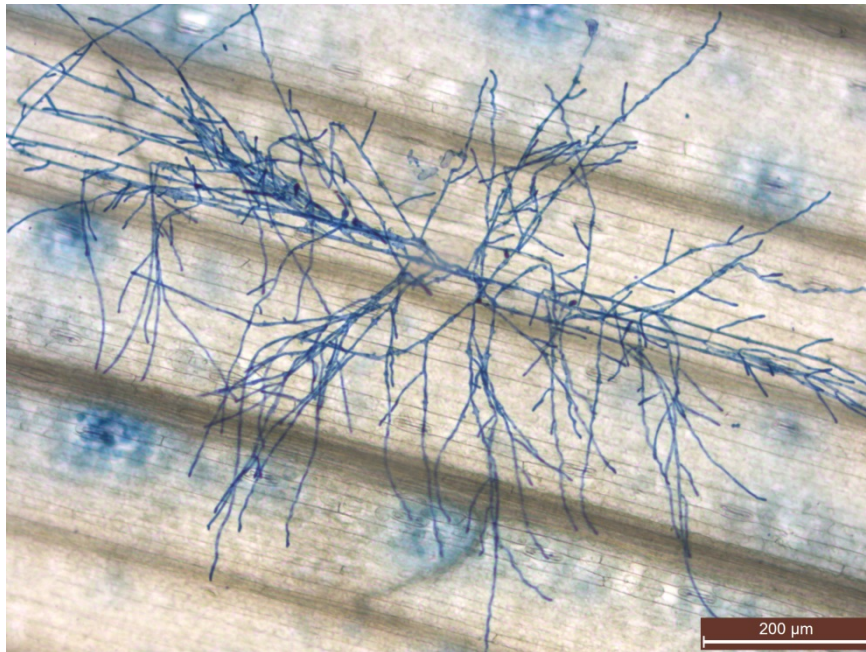


Figure 3.5 Representative leaf segment test analysis: For barley leaves after inoculation with pathogen fungi *Blumeria graminis* and endophyte fungi *P. indica*. Life cycle of pathogen was tracked during 72 hours in the plant leaf (Bülül, *Bgh95*). The stages were observed under light microscope at 10X magnification (Leica, DFC 280).

a



b

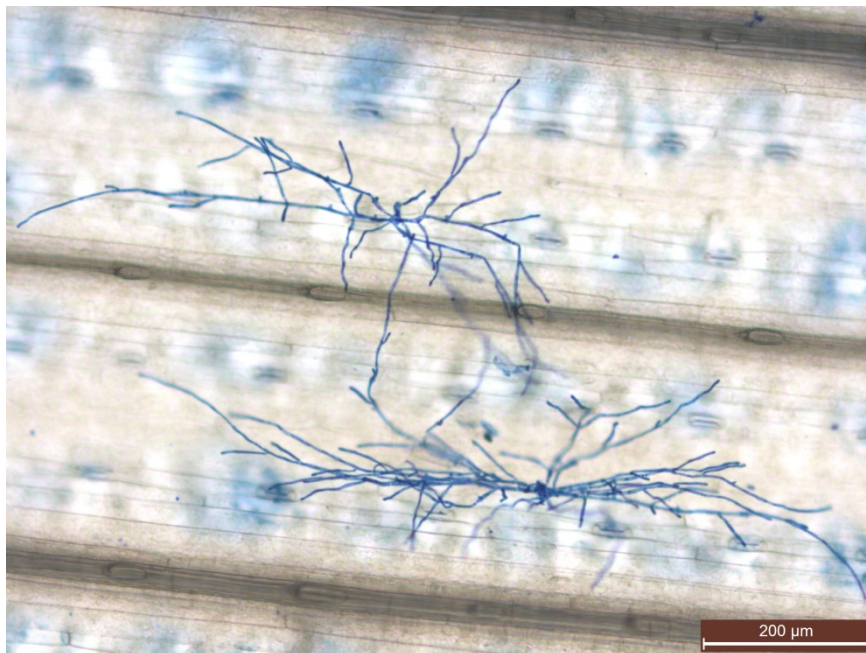


Figure 3.6 *Blumeria graminis* infection in barley leaves (72 hpi): **a)** Only *Bgt* infected barley plants **b)** *Bgt* and *P. indica* infection together. The 3rd leaves of barley plants were taken and stained with trypan blue and placed on glass slides. The primary and secondary hyphae were observed under light microscope at 10X magnification (Leica, DFC 280).

As mentioned in Figure 1.9, *Blumeria graminis* f. sp. *hordei* hyphae forms spores in the leaves after infecting the plant. To determine the hyphae length trypan blue stained leaves of 72 hpi were investigated. Figure 3.5 shows the effect of *P. indica* on resistance against powdery mildew in barley plant. The experiment was performed in three repeats and for each time point 5 different leaf samples were used. From each 5 leaf 3 slides were prepared. From each slide 1 conidium infection site was picked in order to measure the primary and secondary hyphae lengths. Totally around 30 conidium infection sites (15 for *P. indica*+*Bgh95* and 15 for *Bgh95* alone) were examined and hyphae extending from the conidium infection sites were measured. Average primary hyphae length was found to be around 180 μm for *Bgh95* only inoculations while it was 110 μm for *Bgh95* inoculation with *P.indica*. For average secondary hyphae length, it was 90 μm and 60.6 μm respectively. All data was statistically tested by non-parametric t test; Mann Whitney, and both hyphae lengths were found to be significantly differed from each other. For primary hyphae the reduction in length was around 40% while for secondary hyphae, it was found to be 30%.

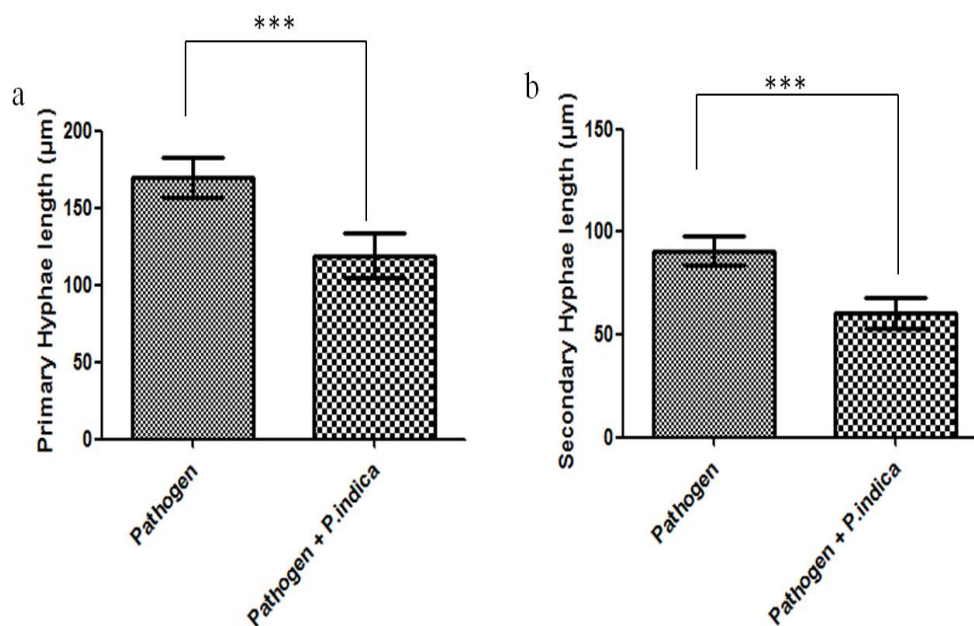


Figure 3.7 Statistical representation of effect of *P.indica* on the growth of primary and secondary hyphae at plant leaf in barley. **a)** Primary hyphae lengths; **b)** Secondary hyphae lengths. *** Significant at $p < 0.0001$ according to Mann Whitney test.

Table 3.1: Primary and secondary hyphae lengths: Lengths of hyphae were measured from the figures taken during the experiment.

	Primary Hyphae Lengths (µm)		Secondary Hyphae Length (µm)	
	<i>Bgh95</i> only	<i>Bgh95</i> + <i>P.indica</i>	<i>Bgh95</i> only	<i>Bgh95</i> + <i>P.indica</i>
Sample 1	180	110	90.0	65.0
Sample 2	165	105	90.0	56.0
Sample 3	189	100	90.0	70.0
Sample 4	170	95.0	90.0	70.0
Sample 5	145	134	95.0	60.0
Sample 6	163	106	96.0	60.0
Sample 7	159	143	97.0	60.0
Sample 8	176	132	80.0	60.0
Sample 9	182	127	85.0	45.0
Sample 10	153	123	89.0	67.0
Sample 11	189	119	83.0	49.0
Sample 12	176	113	96.0	53.0
Sample 13	155	127	98.0	67.0
Sample 14	169	137	79.0	69.0
Sample 15	177	113	103	58.0
Averages:	170	119	90.0	60.6

Representative slide photos and statistical analysis showed that *P.indica* reduced both the primary and secondary hyphae lengths that are extending from infection site. Decrease in the length of hyphae showed that, *P.indica* induce resistance in barley against *Blumeria graminis* to a certain extent.

Before starting to each experiment, in order to confirm the correct endophyte inoculation, plant roots were stained to observe *P.indica* spores. Figure 3.8 shows the results for *P.indica* spore staining with trypan blue.

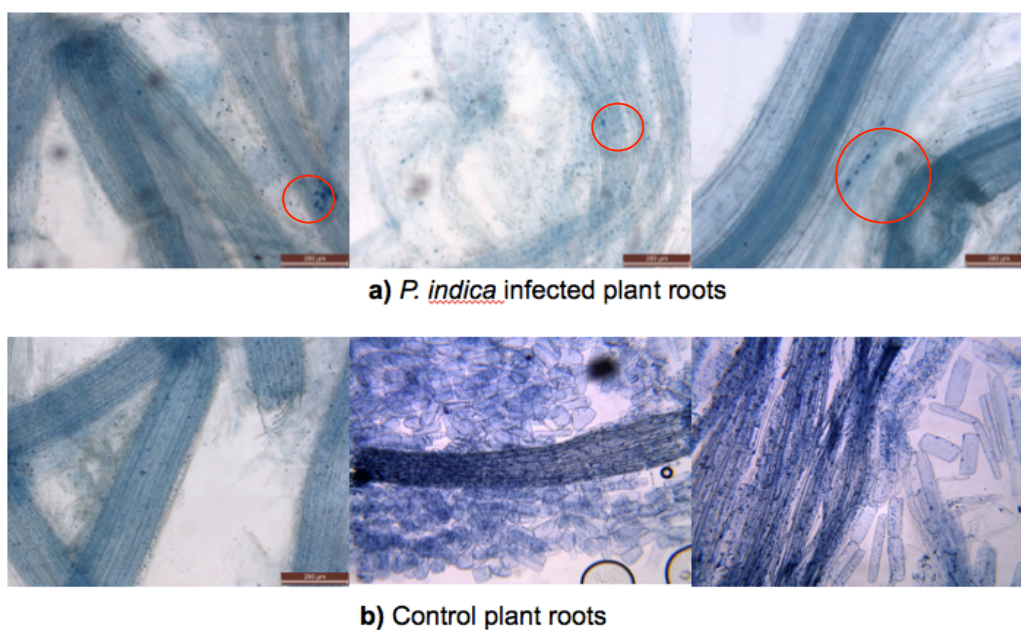


Figure 3.8 Confirmation of *P.indica* inoculation in barley: **a)** *P.indica* infected barley roots (chlamydospores are inside the red circle) **b)** Control group barley root (without *P. indica*). Root segments were stained with trypan blue (28 dpi) and placed on glass slides and the chlamydospores were observed under light microscope at 10X magnification (Leica, DFC 280).

3.4 Determination of Induced Resistance Against Yellow Rust in Wheat

The yellow rust also known as stripe rust can be caused by several species of the fungal genus *Puccinia*: stripe rust by *Puccinia striiformis* f.sp. *tritici* (*Pst*). The disease begins from a very small number of infections that are difficult or impossible to detect in the field. Spread of the pathogen can be explosive and cause significant losses, especially in wet weather, which greatly favors disease development.

For yellow rust disease, there is no specific *Pst* level determination method to test the induced resistance against yellow rust as it was conducted by powdery mildew. Molecular techniques can be applied to confirm the disease. However, in this thesis study, a number of plants were evaluated to determine the level of resistance against yellow rust in wheat. Empirical methods were used to determine the possible decrease in disease formation in the presence of *P. indica*. After inoculation of plant pathogen and endophyte, plants were tracked and categorized according to *Pst* disease level.

1

2

3

4



Pst –TR09-97

Pst –TR09-97
+ *P.indica*

Figure 3.9 Representative pictures of wheat after pathogen and endophyte inoculation (Michigan Amber). The pictures were taken at 20 dpi of *Pst*. The leaf segments from the 2nd leaves of wheat (**1** and **2**) were taken from control plants (*Pst* alone) and (**3** and **4**) were taken from *P. indica* infected plants (*Pst*+*P. indica*).

Experiments were performed three times independently. For each treatment 5 plants were used and 5 leaves from each wheat plant were evaluated and categorized into 4 groups after pathogen inoculation: Resistant, moderately resistant, moderately susceptible and susceptible. In the study, no wheat plant was grouped into resistant or susceptible categories. Totally observed 75 Michigan Amber leaf samples were all formed spores both in controlled sets and *P. indica* pre-treated ones. Figure 3.8 and Figure 3.9 show the representative photos of wheat leaves that are analyzed after *Puccinia striiformis* and *Puccinia striiformis* with *P. indica* inoculations.

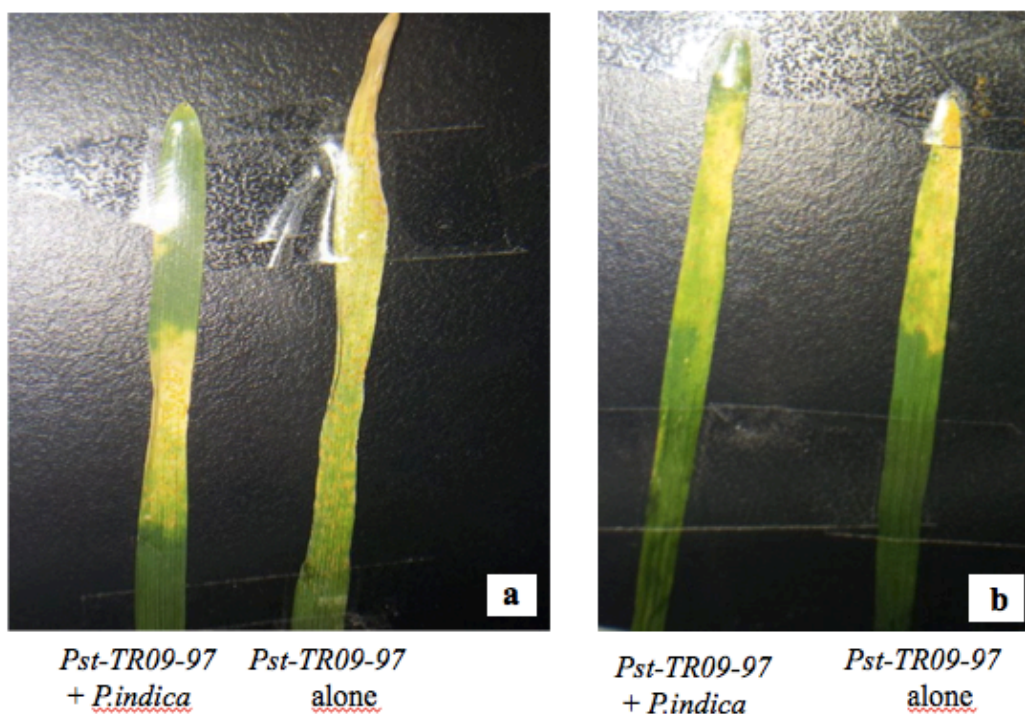


Figure 3.10 Limiting effect of *P. indica* on spreading of *Pst* in wheat leaves (Michigan Amber). **a)** 2nd leaves of wheat, **b)** 3rd leaves of wheat. The pictures were taken at 20 dpi of *Pst*.

Table 3.1: Observed disease levels after pathogen and endophyte inoculation

	Heavily Infected	Lightly Infected
<i>Puccinia striiformis</i> only	63	12
<i>Puccinia striiformis</i> & <i>P. indica</i>	37	38

As mentioned above, totally 75 samples were analyzed and categorized. As can be measured in Table 3.1, *P.indica* inoculation increased the number of moderately resistant sample number. When only pathogen was applied, 63 samples were found to be heavily infected the other 12 were lightly infected while *P. indica* increased the number of samples with lightly infected to 38. Thus, *P. indica* inoculation limits the spreading of yellow rust in leaves up to 3 times. According to representative Figures, 3.8 and 3.9, *P. indica* inoculation limits the spreading of yellow rust in leaf structure.

Before starting to each experiment, in order to confirm the correct endophyte inoculation, plant roots were stained to observe *P.indica* spores. Figure 3.11 shows the results for *P.indica* spore staining with trypan blue.

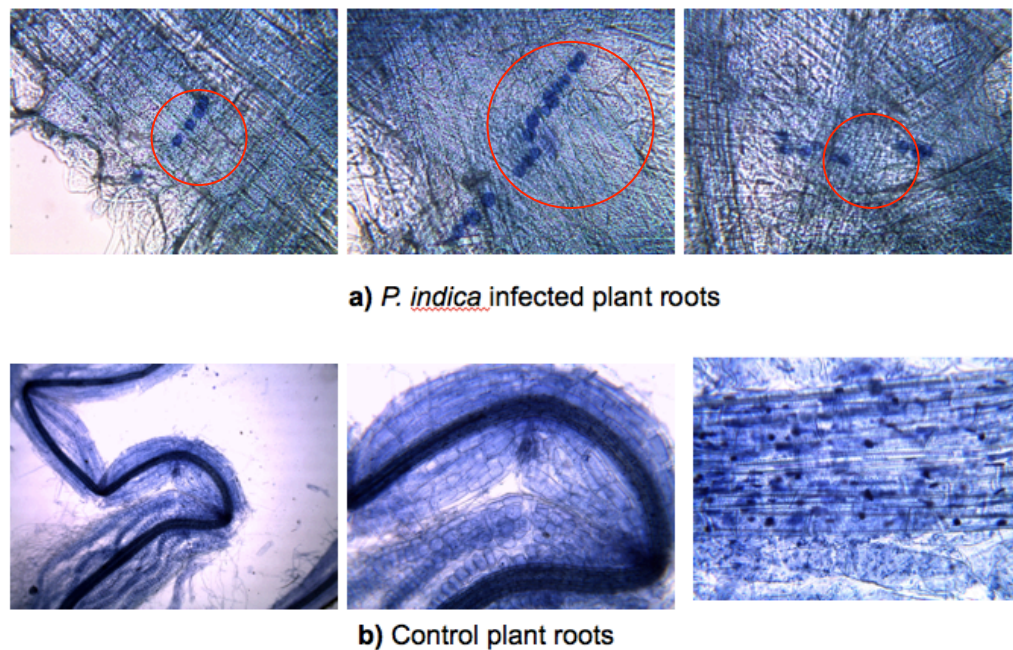


Figure 3.11 Confirmation of *P.indica* inoculation in wheat: **a)** *P.indica* infected wheat roots (chlamydospores are inside the red circle) **b)** Control group wheat roots (without *P. indica*). Root segments were stained with trypan blue (35 dpi) and placed on glass slides and the chlamydospores were observed under light microscope at 10X magnification (Leica, DFC 280).

According to these studies, it can be concluded that, although it is not a complete resistance in wheat plant, *P. indica* can reduce the level of disease formation.

CHAPTER 4

CONCLUSION

P. indica is recently explored fungus that can be cultivated. It has the potential to be plant promoter, biofertilizer, bioregulator, bio-herbicide, immunomodulator, phytoremediator, regulator against insects and pathogens, tolerance inducer against temperature and salt. With these potential properties and cultivable ability, *P. indica* becomes the ideal fertilizer for many crop products.

Wheat and barley are two main grains that are highly produced in Turkey. Nevertheless, due to inadequate plant development and improper fertilization methods, highest quality and quantity cannot be achieved for wheat and barley production. More developed technologies for fertilizers for phyto-stimulation and resistance to pathogens and stress conditions are required. Being a cultivable mycorrhizal endophyte, *P. indica* can be included in the growth process of wheat and barley as plant growth promoter and pathogen resistant inducer.

According to results obtained during this study, growth condition for plant endophyte, *P. indica* was first optimized. Then different inoculation methods to barley and wheat species tested to decide the best method. After obtaining the best possible conditions for growth of *P. indica* and inoculation method, the effects of *P. indica* against plant pathogens were tested. *Blumeria graminis* causing powdery mildew in barley and *Puccinia striiformis* causing yellow rust in wheat were selected to determine *P. indica* induced resistance against these diseases. Although, in both cases there were no complete resistance induced by *P. indica*; it reduced the disease development and increased the plants' resistance response to pathogens. For powdery mildew, *P. indica* indicated a decrease in the length of hyphae and for yellow rust; *P. indica* prevented the spreading of *Puccinia striiformis* on wheat leaves and systemic infection.

All these studies conducted in this thesis are preliminary studies about optimizing the ideal conditions for *P. indica* growth, maintenance and the inoculation methods and about the effects of *P. indica* on different pathogens. More studies are required to understand the exact mechanism of the effects of *P. indica* on plants' disease resistance. In general what is the mechanism of symbiotic mycorrhizae against biotic stresses is not known. Furthermore, molecular effects of *P. indica* can be addressed in the future.

Successful applications *P. indica* in agriculture may prevent usage of fungicides and may enhance chemical free farming.

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