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Research Article

# Studies on endophytic fungi associated with medicinally important aromatic plant *Artemisia nilagirica* (C.B. Clarke) Pamp. and their antagonistic activity against *Phytophthora infestans*

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**Abstract:** Antagonistic activity of endophytic fungi associated with medicinally important aromatic plant *Artemisia nilagirica* was studied against the pathogen *Phytophthora infestans* that causes late blight of potato. The study has a dual purpose, firstly identification of endophytic fungi isolated from *Artemisia nilagirica*; secondly, to evaluate their antagonism against *Phytophthora infestans* using the dual culture method. Altogether 23 fungal endophytes were isolated from root, stem and leaf of which 14 fungal endophytes were isolated from roots, 10 from stem and 6 from leaf. Among the isolates, 4 fungal species, namely *Trichoderma viride*, *Penicillium atrovenetum*, *Aspergillus fumigatus* and *Cladosporium cladosporioides* were selected to study the antagonistic effect against *Phytophthora infestans*. *T. viride* was found to have the highest percentage of inhibition of 67.0% followed by *A. fumigates* (59.6%), *P. atrovenetum* (56.7%) and *C. cladosporioides* (33.0%). Among the test organisms, a zone of inhibition was produced only by *T. viride* and *P. atrovenetum*. *T. viride* showed the maximum inhibition zone of 1cm against *P. infestans* while that of *P. atrovenetum* was 0.4cm. This study shows that out of the four test organisms, *Trichoderma viride* may be recommended as a good source of biocontrol agent against *P. infestans* the causal organism of potato late blight.

**Keywords:** Fungal endophytes, Antagonistic effect, Dual culture, *Artemisia nilagirica*, *Phytophthora infestans*.

## 1. Introduction

Endophytic fungi are capable of living in host plants without causing any symptoms (Petrini *et al*., 1992). These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Carroll, 1978; Azevedo *et al*., 2000; Strobel, 2003).

Many plants and algae have been reported as hosts of fungal endophytes (Davis *et al*., 2003). Among the host plants, the medicinal herbs are one of the important groups of hosts for endophytic fungi (Li *et al*., 2004; Yan *et al*., 2007; Huang *et al*., 2008; Xu *et al*., 2008). Previous reports have demonstrated that fungal endophytes from medicinal herbs show efficacy as pharmaceutical and agricultural compounds, especially from Chinese herbs (Yi, 2003; Li *et al*., 2004; Shentu *et al*., 2007; Yan *et al*., 2007; Kusari *et al*., 2008). Recently, certain isolates of endophytic fungi from Chinese herbs have been used as biocontrol agents for agricultural crops (Redman *et al*., 1999; Schulz *et al*., 2002; Kunkel and Grewal, 2003; Backman and Sikora, 2008; Mejía *et al*., 2008; Maciá-Vicente *et al*., 2009; Mercier and Jiménez, 2009; Gabler *et al*., 2010).

*Artemisia nilagirica* commonly called Indian wormwood is an aromatic shrub, 1-2m high and found throughout the mountain districts of India. It is said to be anthelmintic, antiseptic and expectorant, leaves and flowering tops are bitter, astringent, aromatic, anti-inflammatory, appetizer, digestive and diuretic. Also use in cough, asthma, nervous and leprosy. It is considered to produce most medicinally important secondary metabolite and essential oils. There has been an increased interest in looking at antimicrobial properties of extracts from aromatic plants, particularly essential oils. In recent years there has been extensive research to explore and determine the antimicrobial activity of essential oils.

The present study focuses on screening and identification of endophytic fungi isolated from *Artemisia nilagirica* collected from North Eastern Hill University Campus, Shillong, India and to assess the

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antagonistic effect of certain selected endophytes against *Phytophthora infestans* the causal organism of potato late blight.

## 2. Materials and Method

### 2.1 Isolation and Identification of Endophytic Fungi

#### 2.1.1 Collection of plant material

The medicinal plant *Artemisia nilagirica* (C.B. Clarke) Pamp. was collected from the campus of North Eastern Hill University, Shillong, India in the months of October-December (2013) for the investigation of endophytic fungal communities. The disease free parts of the plant that is mature photosynthetic leaves, stems, and roots were collected and brought to the laboratory in sterile bags. The samples were processed immediately to reduce the risk of contamination.

#### 2.1.2 Isolation of endophytic fungi

The samples were processed following the method of Suryanarayanan *et al*., (2003) using potato dextrose agar (PDA) medium. Endophytic isolation was carried out under aseptic conditions. Asymptomatic healthy part of the plant such as stem cuttings, leaves, and roots were used for the isolation of endophytes. Following are the different steps followed:

a. Plant material was first cleaned by washing several times under running tap water to remove dust and debris adhering to them.
b. All the samples were surface sterilized following the method of Fisher *et al*., (1994). Surface sterilization was done by soaking the samples in 70% ethyl alcohol for 1-3 minute and 4% sodium hypochlorite (NaClO) solution for 3-5 minutes.
c. Then rinsed with 70% ethyl alcohol for 2-5 seconds and finally with deionized sterile distilled water to remove the sterilants.
d. The surface sterilized plant material, i.e. stems, leaves and roots were cut into small pieces. The stem and root were cut into 1 x 1mm in size and the leaves were cut into 5 x 5mm size with and without the midrib under aseptic conditions using a sterile scalpel. Each sampled was blot dried under aseptic conditions.
e. 4-6 small pieces of the plant materials, i.e. root, stem and leaf were cultured in Petri dishes containing potato dextrose agar medium (PDA) supplemented with streptomycin (100mg/l).
f. The Petri dishes were sealed with parafilm and incubated at 25°C for 5 days in BOD and were regularly monitored for any microbial growth.

#### 2.1.3 Identification of endophytic fungi

For the characterization of the morphology of fungal isolates, slides were prepared from cultures using lactophenol cotton blue stain and examined under the light microscope. The fungal colonies were identified based on their morphological and reproductive structures using standard manuals (Domsch *et al*., 1980; Subramanian, 1981; Ellis, 1993 and Barnett and Hunter, 1998). The fungal isolates were then subcultured in order to get pure cultures using Czapek Dox agar (CDA) medium.

### 2.2 Isolation of Fungal Pathogen, *Phytophthora infestans*

#### 2.2.1 Method of sample collection

Potato leaves with typical symptoms of late blight were collected from a major potato growing area. Then kept in a plastic bag or paper bag and taken to the laboratory.

**Plate 1.** ***Aspergillus fumigatus*** **(A) vs. Pathogen (P).**

**Plate 2.** ***Cladosporium cladosporioides*** **(C) vs. Pathogen (P).**

#### 2.2.2 Isolation of *Phytophthora infestans*

*Phytophthora infestans* was isolated from the potato leaves with typical symptoms (the lesion was small and a white mildew was visible). The steps involved in isolation are as follows:

**a.** The leaves with initially infected single lesion were cut into small pieces (5-10mm).

**b.** Segments (4 to 6 per Petri-plates) of each sample were placed on potato dextrose agar (PDA) medium amended with streptomycin (100mg/l).
**c.** The plates were then sealed with paraffin and incubated at 25°C for 5 days in BOD. After 5 days, colonies were formed on the Petri-plates.
**d.** Pure cultures of the pathogen isolated were maintained using the Czapek Dox agar medium.

**Plate 3. *Penicillium atrovenetum* (P) vs. Pathogen (P).**

**Plate 4. *Trichoderma viride* (T) vs. Pathogen (P).**

**Plate 5. Control (only the pathogen).**

### 2.2.3 Identification of *Phytophthora infestans*

The colony was morphologically and reproductively identified by staining with lactophenol cotton blue and observing under microscope. *Phytophthora infestans* which belong to the class Oomycetes consist of mycelium which is characterized by the absence of cross walls, among which both asexual and sexual reproduction occurs. The sporangiophores and sporangia emerge at asexual reproduction phase. The sporangia are lemon shape, measurement of 21-38µm x 12-23µm. Sporangia develop at the end of these sporangiophores.

## 2.3 Study of Antagonistic Effects

The antagonistic activity of selected endophytic fungi was studied following the method of Skidmore and Dickinson (1976) by the presence or absence of inhibition zone observed in dual cultures. Four endophytic fungi such as *Trichoderma viride, Aspergillus fumigatus, Penicillium atrovenetum* and *Cladosporium cladosporioides* were tested for antagonism against the selected phytopathogen, *Phytophthora infestans,* by using dual culture techniques. The steps followed are:

**a.** In this method, the mycelial bits of 5mm diameter of the endophytic fungi and pathogen were placed on the opposite end of the Czapek Dox (CDA) plate.
**b.** The plates were run in triplicates with one control set in which only the pathogen was inoculated.
**c.** The plates were incubated in BOD at 25°C for one week.
**d.** The growth of pathogen was tested against all the 4 fungi and the data were recorded regularly on the growth of the pathogen and the endophytic fungi.
**e.** The antimicrobial activity is assessed by the colony interactions or by the presence or the absence of the inhibition zone, which was measured as percentage of inhibition of radial growth of *Phytophthora infestans.*

Percentage of inhibition was calculated using the formula:

$$\textbf{Percentage (\%) of inhibition} = \frac{R1 - R2}{R1} X100$$

Where R1 = radius of the radial growth of the pathogen towards the opposite side in control plate;
R2 = radius of the radial growth of the pathogen towards the opponent antagonist in test plate.

## 3. Results

### 3.1 Isolation and Identification of Endophytic Fungi

A total of 23 endophytic fungi was isolated from samples of roots, leaves and stem of *Artemisia nilagirica.* Amongst the fungal species, isolated 21 species belonged to the Ascomycota, 1 species belonged to Oomycota and 1 species belonged to Zygomycota. The class Ascomycota was represented by

9 genera and 21 species, Oomycota by 1 genus and 1 species and Zygomycota by 1 genus and 1 species (Table 1).

The maximum number of species was isolated from the roots and most of them belonged to Ascomycota. A total number of 14, 10 and 6 fungal species were isolated from the roots, stem and leaf respectively.

Species such as *Aspergillus fumigatus, A. flavus, Acremonium murorum, Arthroderma insingulare, Fusarium oxyspoium, Penicillium brevicompactum, P. canescens, P. citrinum, P. expansum, Phoma eupyrena and Trichoderma viride* were isolated only from roots. *Acremonium kiliense, Penicillium citrinum, P. chrysogenum, P. spinulosum, Phoma eupyrena, Pythium intermedium* and *Verticillium chlamydosporium* were isolated only from the stem. Whereas, species such as *Acremonium strictum, Cladosporium cladosporioides, Penicillium expansum, P. funiculosum, P. lanosum* and *Phoma eupyrena* were isolated only from the leaves.

*Cladosporium herbarum* and *Penicillium atrovenetum* were isolated from both root and stem, while *C. cladosporioides* was isolated from both stem and leaves. From this study, it was found that *Phoma eupyrena* were found to be a common occurrence in all the plant samples.

All these fungi exhibited characteristic colony and microscopic morphology that could be used to differentiate them. Out of the total 23 endophytic fungi isolated, 4 endophytic fungi were selected and inoculated on CDA media to maintain as a pure culture for further study. These isolates were tested for antagonism against plant pathogen (*Phytophthora infestans*).

## 3.2 Study of Antagonistic Effects

### 3.2.1 Antagonism in dual culture

Results showed that all the four endophytic fungi tested in this study exhibited antagonistic activities against *P. infestans*, the pathogen of potato. Radial growth of the pathogen was considerably hindered by all the test antagonists under the conditions of this study. In a control petri dish (without endophytes) the pathogen *P. infestans* grew at a faster rate and covered the whole Petri dish within 8 days whereas *P. infestans* showed comparatively a slower growth in the petri dish with dual culture. There was a significant difference in percentage inhibition of radial growth of pathogen by all the test antagonists, *Trichoderma viride* was found to be the most antagonistic and inhibited the radial growth of the pathogen while *Cladosporium cladosporioides* was found to be the least antagonistic (Fig. 1). The percentage of inhibition increases with the increase in the number of days of incubation (Table 3). Among the test organisms, zones of inhibition were produced only by *T. viride and P. atrovenetum* (Table 2) and there was significantly different in the zones of inhibition between them. The intermingle zone between *A. fumigatus* and *C. cladosporioides* was also found to be significantly different.

**Table 1. List of Fungal species isolated from different parts of *Artemisia nilagirica* (C.B. Clarke) Pamp.**

| S. No. | Fungal species isolated | Root | Stem | Leaf |
|---|---|---|---|---|
| | **Oomycota (1 genus, 1 species)** | | | |
| **1** | *Pythium intermedium* | - | + | - |
| | **Zygomycota (1 genus, 1 species)** | | | |
| **2** | *Rhizopus oryzae* | + | - | - |
| | **Ascomycota ( 10 genera, 21 species)** | | | |
| **3** | *Aspergillus fumigatus* | + | - | - |
| **4** | *A. flavus* | + | - | - |
| **5** | *Acremonium kiliense* | - | + | - |
| **6** | *A. murorum* | + | - | - |
| **7** | *A. strictum* | - | - | + |
| **8** | *Arthroderma insingulare* | + | - | - |
| **9** | *Cladosporium cladosporioides* | - | + | + |
| **10** | *C. herbarum* | + | + | - |
| **11** | *Fusarium oxysporum* | + | - | - |
| **12** | *Penicillium atrovenetum* | + | + | - |
| **13** | *P. brevicompactum* | + | - | - |
| **14** | *P. canescens* | + | - | - |
| **15** | *P. citrinum* | + | + | - |
| **16** | *P. chrysogenum* | - | + | - |
| **17** | *P. expansum* | + | - | + |
| **18** | *P. funiculosum* | - | - | + |
| **19** | *P. lanosum* | - | - | + |
| **20** | *P. spinulosum* | - | + | - |
| **21** | *Phoma eupyrena* | + | + | + |
| **22** | *Trichoderma viride* | + | - | - |
| **23** | *Verticillium chlamydosporium* | - | + | - |
| **Total** | | 14 | 10 | 6 |

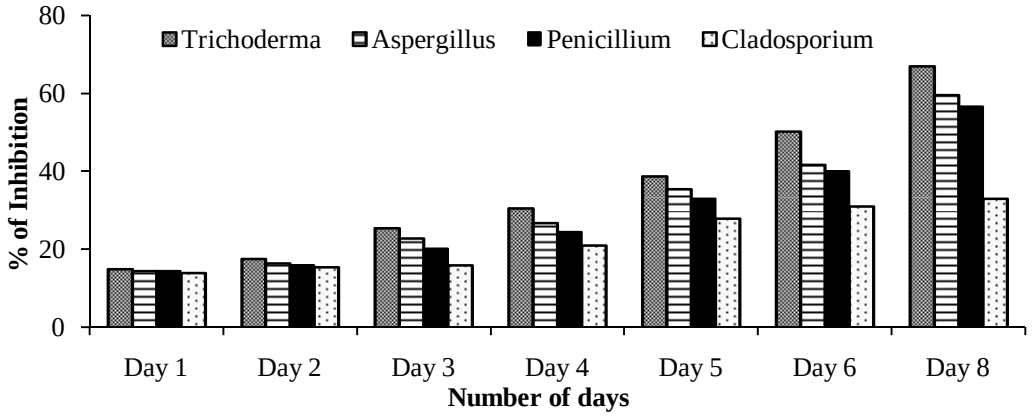


**Fig. 1. Graphical representation of antagonistic activity of different fungal isolates showing the % of growth inhibition of the fungal pathogen *(Phytophthora infestans)*.**

**Table 2. Showing the zone of inhibition of the pathogen by the different fungal species.**

| Fungal antagonist | Inhibition zone (cm) | Intermingle zone (cm) |
|---|---|---|
| ***Trichoderma viride*** | 1.0 | - |
| ***Penicillium atrovenetum*** | 0.4 | - |
| ***Aspergillus fumigatus*** | - | 0.5 |
| ***Cladosporium cladosporioides*** | - | 1.2 |

**a. Effect of *Trichoderma viride* on the growth of *Phytophthora infestans*:** *T. viride* inhibits the maximum growth of inhibition of *P. infestans* as compared to the other three test endophytes i.e. *A. fumigatus*, *P. atrovenetum* and *C. cladosporioides* (Table 3). The percentage (%) of maximum growth of inhibition of *P. infestans* in presence of *T. viride* was 67.0% and a clear zone of inhibition measuring 1.0cm was observed exhibiting antibiosis between pathogen and the antagonist, *T. viride* (Table 3).

**b. Effect of *Aspergillus fumigatus* on the growth of *Phytophthora infestans*:** The percentage (%) of maximum growth of inhibition of *P. infestans* in presence of *A. fumigatus* was 59.6% and the intermingle zone between the pathogen and the antagonist was found to be 0.5cm. This shows that there is growth inhibition of *P. infestans* in presence of *A. fumigatus*.

**c. Effect of *Penicillium atrovenetum* on the growth of *Phytophthora infestans*:** The percentage (%) of maximum growth inhibition of *P. infestans* in presence of *P. atrovenetum* was 56.7% and the zone of inhibition between the pathogen and the antagonist was found to be 0.4cm. This shows that there is growth inhibition of *P. infestans* in presence of *P. atrovenetum.*

**d. Effect of *Cladosporium cladosporioides* on the growth of *Phytophthora infestans*:** The percentage (%) of maximum growth inhibition of *P. infestans* in presence of *C. cladosporioides* was 33.0% and the intermingle zone between the pathogen and the antagonist was found to be 1.2cm. *C. cladosporioides* shows the least inhibition on the growth of the pathogen.

**Table 3. Percentage (%) of growth inhibition of the fungal pathogen (*Phytophthora infestans*) by dual culture.**

| Fungal antagonists | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 8 |
|---|---|---|---|---|---|---|---|
| ***Trichoderma viride*** | 15.0 | 17.6 | 25.5 | 32.8 | 38.8 | 47.6 | 67.0 |
| ***Aspergillus fumigatus*** | 14.5 | 16.5 | 22.8 | 26.7 | 35.4 | 44.8 | 59.6 |
| ***Penicillium atrovenetum*** | 14.5 | 16.0 | 20.2 | 24.5 | 33.0 | 40.0 | 56.7 |
| ***Cladosporium cladosporioides*** | 14.0 | 14.2 | 16.0 | 21.0 | 28.0 | 31.0 | 33.0 |

## 4. Discussions

The study shows that most of the endophytic fungi isolated from *Artemisia nilagirica* belonged to Ascomycota, similar findings were also reported by Goveas *et al.*, (2011) from *Coscinium fenestratum* –a red listed endangered medicinal plant. It was probably due to the medium requirement in the exertion having to be specified as the fungal needed. With the exception of *Cladosporium cladosporioides*, *C. herbarum*, *Penicillium atrovenetum* and *Phoma eupyrena* all of them are organ-specific. However, *Cladosporium herberum* and *Penicillium atrovenetum* was found in both root and stem, while *Cladosporium cladosporioides* was isolated from stems and leaf. *Phoma eupyrena* was found to be common in all the samples. There is sufficient evidence that endophytic fungi play an important role in host-plant physiology. They receive nutrition, protection and propagation opportunities from their hosts (Thrower and Lewis, 1973; Clay and Schardl, 2002), while host plants are also benefited from this symbiosis. Endophytes provide

protection to their hosts from insects, pests, and herbivore, and help their hosts to adapt in different stress conditions (Clay and Schardl, 2002; Clay *et al*., 2005; Malinowski and Belesky, 2006; Knop *et al*., 2007). However, endophytes also act as opportunistic microorganisms under some conditions (Faeth *et al.*, 2004; Saikkonen *et al*., 1998). Endophytic fungi from tropical plants have recently gained importance in biological control of plant diseases and also as a source of pharmacologically active compounds. Only a few plant species have been investigated for their endophytic fungal population (Strobel and Daisy, 2003). Therefore, any information and/or research on endophyte-plant symbiosis, such as in this study is of value. Effective extracts could provide potential leads towards the development of novel and environmentally friendly biologically active agents.

Endophytic microorganisms are excellent sources of bioactive natural products that can be used to satisfy demand of pharmaceutical, medical, agriculture and industries. Much more work is essential to understand endophytes physiology, biochemical pathways, defensive role, secondary metabolite production, motivation and encouragement of researcher from life sciences to contribute research related to endophytes.

Mycelial growth inhibition of the target pathogen revealed that the suppression rate was highly reciprocal, with a wider inhibition zone. That isolates the most effectively inhibited fungal pathogen growth in the dual culture experiment generated such a large zone of inhibition, indicating that the fungi produce certain non-volatile antibiotics and antifungal metabolites. A microbial biological control agent may express different mechanisms against pathogens during their antagonistic activity. There are at least three primary mechanisms by which endophytes can improve host resistance to pathogens (Mandyam and Jumpponen, 2005); it weakens or destroying the pathogen by parasitize the pathogen directly, by producing fumigants or other antimicrobial compound or by produce phytoalexins, and/or biocidal compounds, by compete for space and nutrients, by producing enzymes that attack the cell components of the pathogens. Antibiosis, production of antibiotic compounds and inhibition of other microbes, is the most important mechanism expressed by the antagonistic organism. The antagonistic effect expressed by the *T. viride, A. fumigatus, P. atrovenetum* in dual culture method might be due to the one or combination of all the above mechanisms.

In the present study, *Trichoderma viride* is the best antagonists for growth inhibition of several soils and seed borne plant pathogens as well as plant growth enhancers and comprises a great number of fungal strains that act as biological control agents. However, the results for *Trichoderma* sp. may also depend on the ability of producing antimicrobial compounds and degradative enzymes by the tested antagonistic organisms. Chet *et al*., (1997) reported that *Trichoderma* species are common inhabitants of rhizosphere and contribute to the control of many soil borne plant diseases caused by fungi. It produces antibiotics and toxins such as trichothecin, sesquiterpene and trichodermin, which have a direct effect on other organisms. *Trichoderma* hyphae either grow along the host hyphae or coil around it and secrete different lytic enzymes such as chitinase, glucanase and pectinase that are involved in the process of mycoparasitism.

These indirect and direct mechanisms may act coordinately, and their importance in the biocontrol process depends on the *Trichoderma* strain, the antagonized fungus, the crop plant, and the environmental conditions, including nutrient availability, pH, temperature, and iron concentration.

In dual culture, all the test organisms except *Cladosporium* sp. grew faster than the pathogen. A zone of inhibition was observed on a dual culture plate of *Trichoderma* and *Penicillium,* this is due to the production of antifungal metabolites, volatile as well as nonvolatile antifungal agents by the test antagonists (Shanker *et al.*, 1993; Adejumo *et al*., 1999). In the dual culture plate, the percentage of inhibition of radial growth of pathogen increases with the increase in number of days of incubation. This is due to an increase in the production or concentration of the antifungal metabolites (Odigie and Ikotun, 1982).

The dual culture technique reveals that *Aspergillus fumigatus* also has an antagonist effect against *P. infestans*. *A. fumigatus* inhibit the growth of the pathogen in a dual culture due to the production secondary metabolites such as Aflatoxin which is carcinogenic and mutagenic metabolites. Similar findings were also reported by Adebola and Amadi (2010) in which they tested three *Aspergillus* species, *A. fumigatus, A. repens* and *A. niger* as biological control agents against *Phytophthora palmivora*, the pathogen of cocoa black pod disease.

*Penicillium atrovenetum* secretes some secondary metabolites such as Penicillin and enzyme like β-1-3-glucanase, a cell wall lytic enzyme which inhibits the growth of the pathogen (Druvefors *et al.*, 2002.).

The successful in suppressions of *P. infestans* in dual culture provide useful information on the potential use of these isolates as biocontrol agents against late blight disease (Lamsal, 2013). Therefore, they may be utilized as biofertilizer and biological control agents for fruit and vegetable production in sustainable and ecological agricultural systems.

## 5. Conclusion

It was observed that *Trichoderma viride* has the highest percentage of inhibition, followed by *Aspergillus fumigatus* and *Penicillium atrovenetum,* while *Cladosporium cladosporioides* had the least effect against *Phytophthora infestans.* Therefore, it can

be concluded that out of the four test organisms, *Trichoderma viride*, *Aspergillus fumigatus* and *Penicillium atrovenetum* may be recommended as good biocontrol agents of *P. infestans* the pathogen of potato as all the three fungi showed inhibition of the growth of the pathogen.

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