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First Report of *Trichoderma harzianum* as an effective bio-control agent against Leaf Spot Disease of a Wonder Plant (Seabuckthorn) in Uttarakhand

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Abstract: Harnessing disease suppressive micro-organisms to improve plant health is bio-control. Due to the shortage of available fungicides for proper management of fungal diseases, alternative techniques such as biological control are increasingly being utilized for disease management. Future prospects of biocontrol of plant diseases are bright and promising and with the budding demand for biocontrol products among the growers, it is possible to use the biological control as an effective strategy to manage plant diseases. The present piece of work was carried out to study the potential of fungi *Trichoderma harzianum* isolated and screened from the rhizospheric soil of some *Hippophae salicifolia* D.Don (Seabuckthorn) growing areas of Uttarakhand. Antagonistic activity of *Trichoderma harzianum* was observed against *Alternaria alternata* causing leaf spot disease in seabuckthorn. Under *in vitro* conditions *Trichoderma harzianum* significantly reduced the growth of pathogenic *Alternaria alternata.* This is the first report of *Trichoderma harzianum*, isolated from the rhizosphere of the diseased plant i.e seabuckthorn itself and being utilized as bio control agent against leaf spot disease on seabuckthorn, which is considered as a magical plant of the Garhwal Himalayas, which open up the new horizons for eco-friendly method of disease management among seabuckthorn growers in Uttarakhand, India.

Keywords: Antagonistic, biocontrol, disease, isolate, rhizosphere, soil.

INTRODUCTION

Seabuckthorn, locally known as Chuk is one of the main plants collected and exploited unscientifically from Uttarakhand due to its use as food, medicines and cosmetics. It is also known as "wonder plant," "magical plant," and "cold desert gold" due to its multifarious benefits. But due to many pathogenic agents, the production and yield are affected considerably which is increasing gradually due to its scientific ignorance. Phytopathogenic microorganisms have a great impact on crop yields and can significantly reduce plant performance and crop quality. They are serious threats to crop production and ecosystem stability. Plant pathogens including fungi, bacteria, viruses and nematodes cause severe losses or damage to crops worldwide and thereby significantly reduce the quality and quantity of agricultural commodities (El Ghaonth et al., 2002; Dean et al., 2012; Singh, 2014; O' Brien, 2017). Leaf spot diseases are probably the most common types of plant diseases. Symptoms of leaf spot vary with the host plant and the pathogenic agent. Leaf spots seen on seabuckthorn have fairly defined margins and brown or black centres (Figure 1).

Spots are pin head to several centimeters in diameter and coalesce to encompass entire leaves. Leaves which are heavily infected turn yellow and drop prematurely. Leaf spots are especially problematic on new transplants or on weakened or stressed plants.

MATERIALS AND METHODS

Study location: The study was carried out in the different locations of Chamoli district in Uttarakhand, India.

Collection and Identification of plant species: The species of *Hippophae salicifolia* was identified using a dichotomous key for the genus *Hippophae*. A plant twig was exsiccate, herborized and deposited in the Herbarium of Botanical Survey of India, Northern Regional Centre, Dehradun, Uttarakhand, India with the accession number: 115802.

Isolation and Identification of Pathogen: Diseased leaf samples of *Hippophae salicifolia* D.Don (seabuckthorn) were taken into the laboratory and washed thoroughly with running tap water to remove the surface dirt. The diseased portions of the leaves were cut into small pieces using sterile scalpel blades and kept in sterile petri dishes after surface sterilizing with 0.1 percent mercuric chloride for about one minute followed by two changes of sterile water. Isolated fungi were identified to the genus level with the help of standard mycological reference publications. The plates were incubated at 25±2°C for 5 to 7 days, and the growth of fungal colonies were recorded every day. Fungal colonies were isolated after 5-7 days and pure cultures were transferred to Potato Dextrose Agar slant. Pure cultures of the fungi were preserved in PDA slants at 4oC as stock cultures for future use the mycelia and spore characters of the fungi were studied under microscopic examination. Fungal isolates were identified on the basis of cultural, morphological and microscopic viz., Mycelium, sporangiophore, spore bearing organ, spore structure etc. and were identified following Barnett and Hunter (1972) and Gilmann (1967). Pure culture of *Alternaria alternata* was stored at 5ºC in culture tubes and petri plates containing PDA. The fungal sample was compared by visual and microscopic analysis with a reference culture of fungus deposited at ITCC having reference no.1774.

Figure 1: Leaf spot symptoms on Seabuckthorn

Isolation of rhizosphere associated fungal biocontrol agent: Rhizosphere soil samples of seabuckthorn were collected from some selected sites of district Chamoli in Uttarakhand. Soil samples were collected in sterile polythene bags. The isolation of rhizosphere fungi from these was done by dilution plate method of Wakesman (1927) and Warcup (1955a). This method includes shaking of 1 g soil in 100 ml of distilled water followed by further dilutions under asceptic conditions. Various dilutions were cultured on Potato Dextrose Agar medium and Czapek's Dox medium (Raper and Thom, 1949). The colonies with characteristic growth of *Trichoderma* spp were observed under the microscope and growth from such colonies was sub cultured on agar slants and the fungi were identified following Barnett and Hunter (1972) and Gilmann (1967).

Morphological characterization: The morphological characterization of the spores was performed as described by Menezes Filho *et al.* (2020) from counting 60 spores with the help of Image J software. To verify the viability of the spores, a solution of Toluidine Blue 1% (m/v) was used. Microscopic observation was performed using an optical microscope at 4, 10 and 40 X magnification. The dimensions of *Alternaria alternata* conidiospores were evaluated in terms of length and width expressed in micrometer.

Inhibition (%) Effect of *T.harzianum* against *Alternaria alternata*: Antagonistic activity of *T. harzianum* was evaluated using dual culture method (Rao, 2003). Four mm diameter mycelial disc from the periphery of 07 day old culture of bioagent was placed on the petri plate at four locations, approximately 3cm from the center. A loopful of actively growing *Alternaria alternata* isolate (Fig. 2a) was placed opposite to *T.harzianum* isolate on the plate. Plates inoculated with *Alternaria alternata* but not with *T. harzianum* were used as control. All *in vitro* tests of antagonism were performed three times. All plates were incubated at 27^0 C for 7 days. Visual observations of growth inhibition were recorded every two days and the final measurements were recorded at the 7th day for incubation. Degree of antagonism was determined by measuring the radial growth of pathogen, (radial mycelia growth reduction) *Alternaria alternata* by *Trichoderma harzianum* in dual culture in relation to growth of the control. Three replications were maintained for each treatment. Percentage inhibition of pathogen was calculated by formula, given below (Fokkema, 1973).

Inhibition (%) = X-Y/ X × 100

Where X = mycelial growth (radial) of the pathogen on the control plate, and Y = mycelial growth (radial) of the pathogen on the dual culture plate.

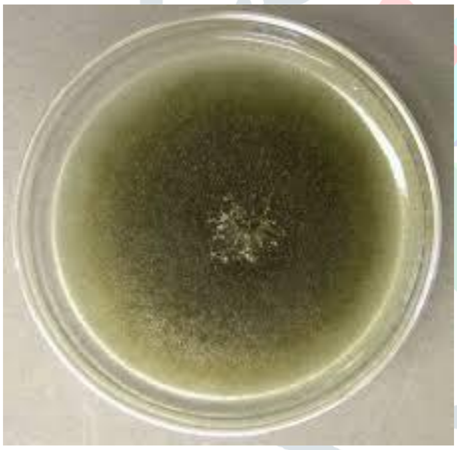

***Figure 2a Alternaria alternata*, 7-day-old colony on MEA**

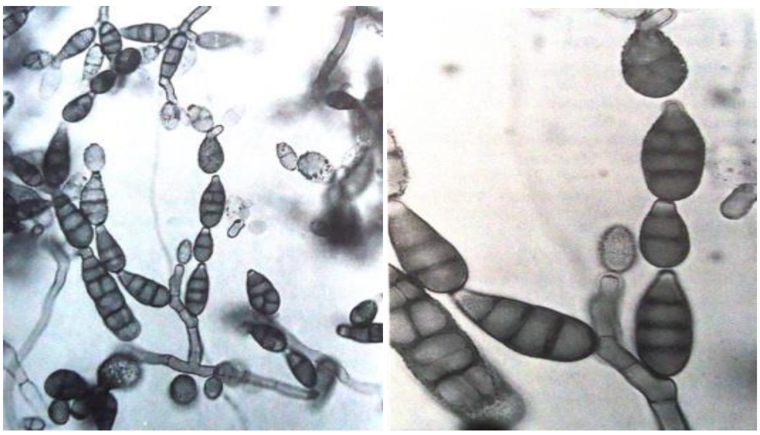

***Figure 2b: Alternaria alternata* (a) Conidiophores [x400] and (b) Conidia [x1000]**

RESULTS

Isolation of *Trichoderma harzianum*

A number of colonies were observed in PDA plate after 3-7 days when serially diluted samples were placed on PDA media. Colony that produced green colour conidia was picked, observed under microscope by staining with lactophenol cotton blue stain. The microscopic analysis of the mycelium with spore revealed that the isolate was *Trichoderma harzianum* (Fig. 3a) .The isolate was subcultured and stored in PDA slants at -20^{0}C. Fungal leaf spot is the most common and obvious disease present during crop production of seabuckthorn. In most cases, they are easily noticed, and the urge to guess specific causes is great. Pathogen such as *Alternaria* affects seabuckthorn plantations. Fungal leaf spots rarely kill a crop, but on rooted cuttings, *Alternaria* can result in massive losses. Huge loss also occurs by planting plugs contaminated with foliar diseases. *Trichoderma harizanum* was evaluated for its antagonistic activity against *Alternaria alternata* in Petri dishes containing PDA medium. Results showed that *T. harizanum* inhibited the growth of pathogen. Growth inhibition in the pathogen differed significantly. Seven days of incubation showed different degrees of mycelial growth inhibition of *Alternaria alternata* (Fig. 4). However, after 3 days of incubation there was suppression of growth on antagonized portion of hyphae. *T.harzianum* completely overgrew the pathogen with percentage inhibition of 67.6%. Coculture of the phytopathogen and *Trichoderma harzianum* under laboratory conditions has clearly demonstrated the dominance of *Trichoderma* species. The geunus, *Trichoderma* is ubiquitous which has been extensively studied for their biological control of plant diseases. Strains of *Trichoderma* are strong opportunistic invaders fast growing, prolific producers of spores and powerful antibiotic producers. The antagonistic activity of *Trichoderma* species depends on multiple synergistic mechanisms. According to Papavizas and Lumsden (1982); Devaki *et al.*, (1992), the mechanisms involved in the control of pathogens by *Trichoderma* spp. are probably due to antibiosis, lysis, competition and mycoparasitism. The species of *Trichoderma* have shown efficiency in the biological control of many foliar diseases. The *Trichoderma* may suppress the growth of the pathogen population in the rhizosphere through competition and thus reduce disease development. It produces antibiotics and toxins such as trichothecin and a sesquiterpine, trichodermin, which have a direct effect on other organisms. The antagonist (*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. Although it is a preliminary study on the antagonistic activity of *T. harzianum* against different pathogens associated with various diseases of Seabuckthorn but may become an instrumental tool in bio-controlling of the disease of other medicinal and ecological important plants of the fragile Himalayan region.

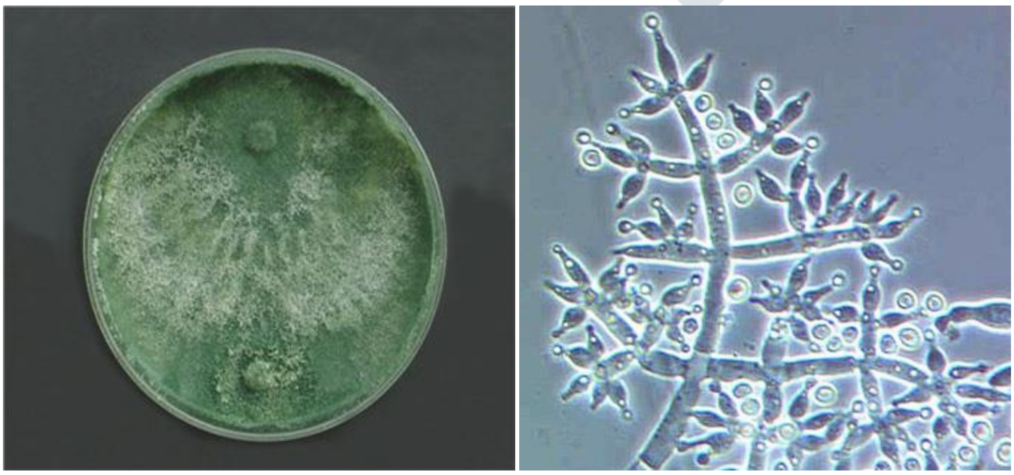

Figure 3 (a): Trichoderma harzianum, 10-day-old colony on CDA

(b): Trichoderma harzianum, Sporangiophore [x400]

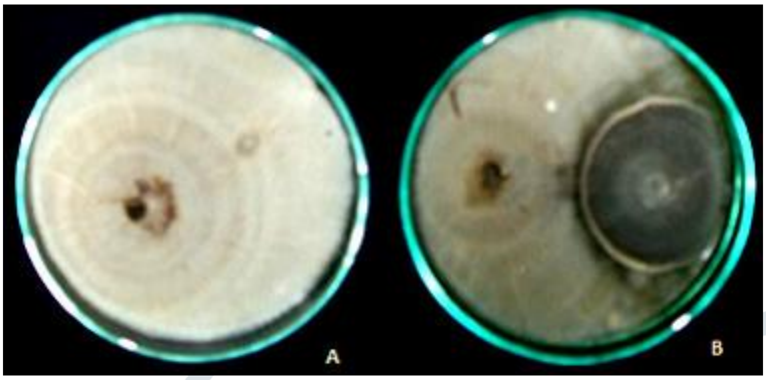

Figure 4: Antagonistic potential of T. harzianum against Alternaria alternata a) Control b) Dual Culture

DISCUSSION

Various pesticides, insecticides and other chemicals are used for the treatment of valuable plant wealth. The intensive use of fungicides for the control of diseases has resulted in the accumulation of toxic chemicals which are hazardous to human beings and to the environment; therefore, effective bio-control mechanisms have some hope to treat such plants in a healthy vision. The marketing potential of medicinal plants is also seriously affected due to accumulation of various residual chemical pesticides and their formulations. The increasing awareness of fungicide related hazards has emphasized the need for adopting a safer biological method but unfortunately very little work has been done to search for microbial antagonists which could serve as biocontrol agents against *Hippophae salicifolia* diseases. For this purpose *Trichoderma harzianum* was screened out for its antagonistic activity against *Alternaria alternata*. Fortunately, it proved to be a success acting as strong antagonist against the pathogen. To control a few post-harvest fungal as well as bacterial diseases antagonistic fungi have also been exploited. Composted form of biocontrol microorganisms are also being used in some plants. The antagonistic potential of *Trichoderma* spp. against plant disease was first recognized in the early 1930's (Harman, 2000). The antagonistic effect of *T. harzianum* on a range of phytopathogenic fungi including *Alternaria solani, Botrytis fabae, Cladosporium cucumerinum, F. oxysporum* and *F. tricinctum*, has been studied (Booth, 1971). Research data and observations in nurseries have shown that addition of composted organic matter to potting mixes results in suppression of leaf spot diseases. A significant improvement have been made in different aspects of biological control of fungal plant diseases, but this area still need much more development and investigations to solve the existing problems. It is critical to carry out more research studies on some less developed aspects of biocontrol including development of novel formulations, understanding the impact of environmental factors on biocontrol agents, mass production of bio control0 microorganisms and the use of biotechnology and nano-technology in improvement of bio control mechanisms and strategies.

CONCLUSION

Future prospects of biological control is bright and promising and with the growing demand for biocontrol products among the growers, it is possible to use the biological control as an effective plan to manage plant diseases, protect the environment, increase yield and production of biological resources and approach a sustainable agricultural system. Thus, biological control can be safer for humans, crops, and the environment. It also has the potential to be more stable and longer-lasting than other controls, and is compatible with the concepts and goals of integrated pest management and sustainable agriculture. Therefore, incorporation of biological control is of primary significance.

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