ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 10, pp. 13555-13559, October, 2016

International Journal of Recent Scientific Research

Research Article

HIGHLIGHTING OF ALTERNARIA TENUIS CONIDIA IN THE ROOTS OF EUCALYPTUS GOMPHOCEPHALA THROUGH THE TECHNIQUE OF CLEARING AND STAINING OF PHILLIPS AND HAYMANN (1970)

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ARTICLE INFO

Article History:

Received 20th June, 2016
Received in revised form 29th August, 2016
Accepted 30th September, 2016
Published online 28th October, 2016

Key Words:

Alternaria tenuis, *Eucalyptus*, stained roots, endophyte.

ABSTRACT

In this study, the *Alternaria tenuis* conidia was observed in the roots of *Eucalyptus* plants stained thanks to the technique of Philips and Haymann (1970), used to study the root colonization by endomycorrhizae. The stained roots are bearers of compartmentalized and melanized hyphae, internal and external, and multicellular conidia of *Alternaria* emanating from the root bark. Different stages of formation of these conidia were observed. Inoculation of *Eucalyptus* plants with the roots fragments holding *Alternaria tenuis* could induce root infection that became sporogenous, one month after inoculation, no disease symptoms were observed neither on the plant's roots or on the leaf systems.

INTRODUCTION

Alternaria genus representatives are cosmopolitan in nature. They are considering as important fungal pathogens that usually cause disease in the aerial parts of many plants (Macauley and Thrower, 1966; Lamb et Brown, 1970; Macauley, 1979; Upadhyay, 1961, 1989; Pati *et al.,* 2008). The *Alternaria* genus is characterized by the formation of polymorphic conidia that form short or long chains with longitudinal and transverse bulkheads, and a long or short beak (Joly, 1966). These conidia are frequently present in the atmosphere and also into the soil (Neeraj *et al.,* 2010).

On the other hand, the *Alternaria* genus causes several types of plant diseases; the most important is called *Alternaria* blight is also known as early blight, caused by two species: *Alternaria solani* and *Alternaria alternata*. The development of this disease is characterized by the appearance of rounded black spots, which requires a temperature of 20 to 30 °C but also a minimum moisture (Macdonald *et al*., 2007). Some *Alternaria* species can attack, in different growth stages, a large number of plant species (Cereals, Ornamental plants, Oilseeds, Cucurbitaceae, Brassicaceae, Solanaceae, Brassicaceae, vegetables and fruits, ...) (Srivastava *et al*., 1964; Lugauskas *et al*., 2002; Thomma, 2003; Survilienė *et al*, 2004; Mitakakis *et al*., 2001; Petraitienė, 2005). The *Alternaria* species have been also reported as pathogens of a high number of forest trees species around the world. It is the case of *Amaranthus curentus*, *A. paniculatus* and *A. retroflexus*, which are responsible for necrotic lesions observed on the stems and the leaves (Pusz, 2009). According to the literature, *Alternaria* species were occasionally reported as endophytic fungi. In fact, some researchers considered as a saprophytic endophyte, *A. alternata,* which is isolated from needles of pine, (Lu *et al*., 2000; Grunden *et al*., 2001; Tokumasu and Aoiki, 2002).

In the present study, we note the presence of *Alternaria tenuis* in the roots of young plants of *Eucalyptus gomphocephala*. Indeed, *Alternaria tenuis* is considered as agents of leaf spots disease (Mittal et Sharma, 1982; Cabral, 1985; Nounsi *et al.,* 2014). However, few *Alternaria* species are described as pathogens or endophyts of *Eucalyptus* roots.

MATERIALS AND METHODS

The healthy *Eucalyptus gomphocephala* roots are cleaned from soil particles by an abundant rinsing with tap water in a colander. Then, only the fine roots were selected.

A lot of fine roots were used for the isolation of fungi on agar medium. Fragments of these roots are deposited for 2 minutes in alcohol 90° solution then rinsed for several times with sterile

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water. By the end, the fragments are quickly dried on sterile filter paper and placed on water agar medium (20 g of agar per liter of distilled water). The observations are made after one-week incubation. Other root fragments were transferred into PSA medium (200 g of potatoes, 20 g of Sucrose, 15 g of agar-agar, and 1000 ml of distilled water). Consecutive picking out from colonies growing around the root fragments, accompanied by microscopic observations has yielded to get pure cultures of *Alternaria tenuis.*

Another lot of healthy roots were treated according to the technique of clearing and staining of Philips and Haymann (1970). The roots are cut into a length of 1 to 2 cm and placed into glass vials containing 10 ml of a potassium hydroxide (KOH) solution 10%. These glass vials are then placed in the water bath at 90 °C for 15 min. The Root fragments are then bleached by adding a few drops of H_2O_2 to the KOH solution. 15 min later, the fragments are rinsed with distilled water and then stained with a solution of cresyl blue (0.05%) for 15 min. The fragments were finally rinsed with sterile distilled water and observed under a microscope. Each fragment was carefully observed throughout its length, to the magnifications of ×100 and 400.

Several *Eucalyptus* plants roots, which have shown the presence of *Alternaria tenuis*, were selected and cut into fragments of 1 cm then mixed with disinfected sand and peat. Young seedlings of apparently healthy *Eucalyptus* roots, which contain no *Alternaria tenuis,* are prick out on the substrate containing the infected roots. These ones are observed one month after inoculation. The roots fragments are grown on water agar medium and continually checked to control the presence of fungi. Some other roots were used for observation under the microscope according to previously mentioned technique of Kormanik and McGraw (1982); Trouvelot *et al*. (1986) and Kormanik et Mc Graw (1982).

RESULTS

Microscopic observations revealed that the roots, stained by the technique of Philips and Haymann, Roots have septate and melanized, internal and external, mycelium filaments. In addition, we observe multicellular Alternaria conidia on the root bark (Fig.1). Different stages of conidia formation were observed (Figure.2). The mycelium is internal and developing external conidia. Functional cells, also called 'reproductive apical cells', emanate along the surface of the cortex. These cells get across the root cortex and bud normally to give daughter cells that will subsequently differentiate into multicellular conidia. At first, cells are formed with transverse bulkheads then longitudinal bulkheads appear. Mature conidia were observed on the surface of the root cortex. Sometimes the conidia germinate and produce filaments that grow from the dictyospore basal cells (Figure.3).

The identified species, according to the taxonomic key of Nees (1817), is *Alternaria tenuis*. We note the presence of a bulkheaded mycelium (Fig.1. A and B), as well as the formation of conidia inside the root cells (Fig.1. C and D).

After the roots inoculation, *Alternaria tenuis* is established at the fine roots a month later. Multicellular conidia emanating from the cells of the root cortex were observed as well as the different stages of dictyospores formation. (Fig.2.). *A. tenuis* was also isolated from the inoculated roots. At first, cultures obtained from PSA are white, by the time, the color changes to become olive green (Fig.4.A). We note that the mycelium is bulkheaded (Fig.4.B).

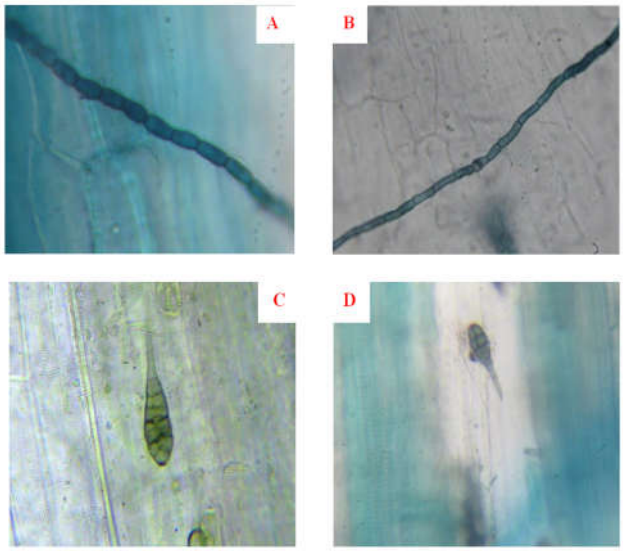

Figure.1 Microscopic observations of *Eucalyptus* fine roots, stained according to the technique of Philips and Haymann (1970), developing in nurseries: bulk headed and melanized Mycelial filaments : **A :** internal, **B :** external, **C** and **D :** Multicellular *Alternaria* conidia emanating from the root bark.

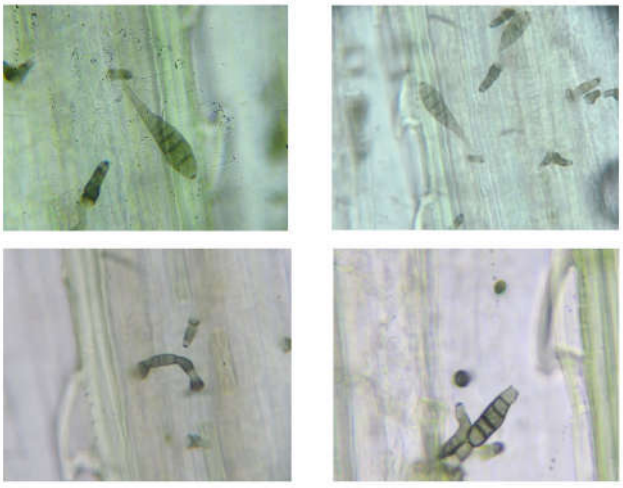

Figure 2 Different formation stages of *Alternaria tenuis* reproductive structures, conidia, asexual spores.

Figure 3 Mature germinating conidia observed at the surface of the root cortex, the filaments grow from basal dictyospore cells.

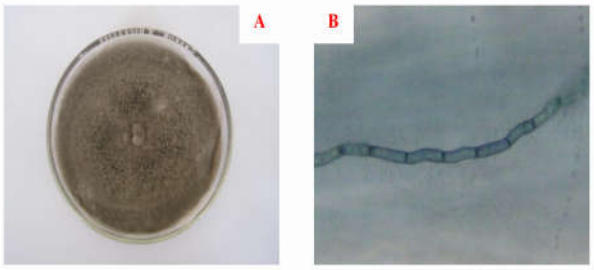

Figure 4 A Colony of *Alternaria tenuis* obtained from infected roots; **B:** bulk headed Mycelium; fluid mounting: cotton blue; M : × 400.

DISCUSSION AND CONCLUSION

Alternaria tenuis conidia have been highlighted in the *Eucalyptus* roots by the staining roots technique used to study endomycorrhizae. Different stages of conidia development were observed. The germination of mature conidia can be observed at the surface of the roots.

The roots, in which conidia are developed, can be called 'sporogenous roots ' show no signs of illness, they are healthy looking roots. In this sense, we can consider *Alternaria tenuis* as Eucalyptus roots endophytic fungus. It does not cause any damage to the hosted roots. Inoculation by *Eucalyptus* roots fragments, that hold *Alternaria,* could induce root infection, after one month of incubation. During the incubation period, no symptoms of the disease were identified either on the roots or on the leaf system of plants.

The isolation of endophytic fungi from different plant tissues has been achieved since the mid-1970s (Hata and Futai, 1996). Endophytes showed the power to protect their host plants from insects and coleopteran attacks (Webber and Gibbs, 1984; Petrini *et al.,* 1989), the stimulation of seed germination (Luginbühl and Müller, 1988), the growth (Leuchtmann and Clay, 1988) and reducing disease (Jhonson *et al.,* 1992; Smith *et al.,* 1996). *Alternaria* species are widely distributed in endophytic mycoflora of a wide range of plants (Rotem, 1994). In the studied *Eucalyptus* plants, *Alternaria tenuis* sporulates well in the cells of the root cortex. It was isolated from the culture medium and identified. In South Africa, *Alternaria* species were isolated from the roots and the seeds of *Eucalyptus* and *Pinus* (Viljoen *et al.,* 1992). In Iraq, *Alternaria* were also isolated from the roots of various types of plants, including the leaves and the roots of *Eucalyptus* (Ali Hassan, 2012). In Australia and England, a comparative study, of *Eucalyptus* leaves, bark and xylem endophytic fungi, cited the *Alternaria* genus as an endophytic fungi of *Eucalyptus* (Fisher *et al*., 1993). In India, Beena *et al*. (2000) noted the presence of *Alternaria* as the endophytic flora of the *Ipomoea pes-caprae, Launaea sarmentosa* and *Polycarpaea corymbosa* roots. In Argentina, *A. alternata* and *A. tenuissima* were cited as endophytes of Soybean (Larran *et al.,* 2002).

In Pakistan, *A.alternata* was identified as a causative agent of wheat and rice root rot (Iram and Ahmad, 2005), the fungus has demonstrated the ability to form conidia which infect roots. In Florida (United States), Alternaria was reported as pathogens of *Torreya taxifolia* roots (Alfieri *et al*. 1984; El Gholl 1985; USFWS, 1986; Alfieri *et al*., 1987; Schwartz *et al.,* 1995). Moreover, Kucharek (2000) and Laemmlen (2001) reported that the appearance of leaf lesions on the aerial part of turnips is the expression of root infection by a pathogenic *Alternaria* species.

A. tenuis is considered as a foliar pathogen of *Eucalyptus*, it induces sporulating lesions on leaves (Nounsi *et al.,* 2013). We noted also the *A. tenuis* spores presence and development on the root bark of the Eucalyptus plants. However, their presence did not induce necrotic lesions either on the roots or on the plant's leaves. The same observation was made after the inoculation of the of Eucalyptus plants roots. Indeed *A. tenuis* is well installed at the roots, it sporulates at this level but does not induce symptoms either on the root mass or on the aerial part. Inoculation of the leaf mass of different species of *Eucalyptus* with *A. tenuis*, isolated from roots, will help to clarify if those isolates are considered as *Eucalyptus* pathogenic endophytes or not.

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How to cite this article:

Anasse NOUNSI *et al*.2016, Highlighting of Alternaria Tenuis Conidia In The Roots of Eucalyptus Gomphocephala Through The Technique of Clearing and Staining of Phillips And Haymann (1970). *Int J Recent Sci Res.* 7(10), pp. 13555-13559.