Identification and Pathogenicity of *Pestalotiopsis chamaeropis*, Causal Agent of White Heather (*Erica arborea*) Dieback, and in vitro Biocontrol with the Antagonist *Trichoderma* sp.

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ABSTRACT

Hlaiem, S., Zouaoui-Boutiti, M., Ben Jemâa, M.L., Della Rocca, G., Barberini, S., and Danti, R. 2018. Identification and pathogenicity of *Pestalotiopsis chamaeropis*, causal agent of white heather (*Erica arborea*) dieback, and in vitro biocontrol with the antagonist *Trichoderma* sp. Tunisian Journal of Plant Protection 13 (si): 49-60.

Plant pathogenic fungi are one of the main causes of forest trees diseases. The symptoms of dieback include a foliage yellowing and fall, a drying and necrosis at branches, cankers, deformations, a blackish fluid and flow of rots at the level of the trunks. Symptoms of wilting were observed on one species of scrub: white heather (*Erica arborea*), located in the forest of "Henchir Kort" northeast of Tunisia. Isolations from the margins of these cankers revealed the fungal genus of *Pestalotiopsis*. Morphological and molecular analysis of the ITS allow to identify the pathogen as *Pestalotiopsis chamaeropis*. The Koch's rules have been verified. The antagonistic effect between *P. chamaeropis* and *Trichoderma* sp. was assessed in vitro. Tests of direct or remote confrontation on PDA medium revealed that *Trichoderma* sp. inhibited mycelial growth of the pathogen compared to the untreated control.

Keyswords: Antagonistic effect, dieback, *Pestalotiopsis chamaeropis*,*Trichoderma* sp., white heather

White heather (*Erica arborea*) is one of a common shrub in the Mediterranean region, North Africa and in Tunisia that can reach 4 m in height (Mezghani et al. 1992). The big woody root of this plant is used in the manufacture of pipes. The honey of this species is very rich in minerals and is an excellent restorative (Mezghani et al. 1992). The plant is known for its antiseptic, urinary, diuretic and astringent properties (Rejeb et al. 2006).There are a very few reports of plant pathogens causing diseases in white heather.

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Accepted for publication 30 March 2018

Pestalotiopsis species are common in tropical and temperate ecosystems (Bate-Smith et al. 1957) and may cause plant disease (Das et al. 2010).They were historically named according to the host on which they were first observed. In spite of this practice, many argued that *Pestalotiopsis* species are generally not host-specific and are found on a wide range of hosts and substrates (Jeewon et al. 2004; Lee et al. 2006). *Pestalotiopsis* species are common phytopathogens causing a variety of symptoms, including

canker lesions, shoot dieback, leaf spots, needle blight, tip blight, grey blight, scabby canker, severe chlorosis, fruit rots, and various post-harvest diseases(Crous et al. 2011;Maharachchikumbura et al. 2012, 2013a, 2013b;Zhang et al. 2012a, 2013). These pathogensare commonly isolated as endophytes (Kumar et al. 2004; Watanabe et al. 2010; Wei et al. 2004) and some species likely have endophytic and pathogenic phases during their life cycle (Tejesvi et al. 2009; Wei et al. 2007).

The purpose of the present study is to identify the causal agent of white heather dieback, presumed to be a species of *Pestalotiopsis*, and to investigate its possible biological control using a local species of *Trichoderma*. This antagonistic genus has proven efficient in controlling a wide range of phytopathogens (Dominguesa et al. 2000) of different crops through different modes of action (Elad et al. 1982; Papavizas 1985; Ridout et al. 1988; Taylor 1986) in eco-friendly manner.

MATERIALS AND METHODS

The forest of Henchir Kort, located in Northeastern Tunisia (36°.30'.406" N; 10°.38'.780"E) suffered from heavy disease infestations since 2012. In October 2016, twigs diebacks and cankers often associated with gummy exudates were observed in the white heather (Fig. 1). Attacked samples with symptoms of necrosis and dryness were collected from infected white heather plants.

Fig. 1. Disease symptoms observed in white heather (*Erica arborea*).

Isolation and identification of the pathogen.

The isolation of the pathogen was performed by adopting the technique of Franceschini et al. (2005). Samples of white heather were collected in a forest of Henchir Kort. Small woody pieces (2 × 2 mm) were taken from the margin between necrotic and healthy tissues of the twigs following surface sterilization as described by Alves et al. (2013)and incubated in the dark at 25°C for 3 days on Petri plates containing potato dextrose agar (PDA) added with streptomycin sulfate (0.05 g/l) antibiotic. Pure cultures were obtained by plating a small piece of

mycelium from the margin of each colony grown on PDA and incubating them under the same conditions described above.

Pestalotiopsis was identified based on its cultural traits, conidial morphological characteristics and molecular analysis.

Regarding the molecular identification, fungal DNA was directly extracted from mycelia growing on plates, using a commercial Kit Macherey-Nagel- 07/2014, Rev.09.PCR reactions were carried out with ITS1 and ITS4 primers (White et al. 1990) to amplify the ITS region of the ribosomal RNA as described by Alves et al. (2004) using the following conditions: 95°C for 3 min; 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; final extension at 72°C for 10 min. Products from PCR reactions were electrophoresed on a 1.5%agarose gel, then stained with GelRed, and visualized with UV transilluminator. The size of PCR products was estimated by comparison with a DNA ladder 100 bp plus, TransgenBiotech. Amplified products were sequenced and the sequences obtained were blasted in GenBank.

Pathogenicitytest.

Pathogenicity test was made according to the method of inoculation of Linaldeddu et al. (2014) by inoculating pathogen on five excised green white heather shoots (30 cm in length) from cv. Cannonau. A mycelia plug (3 to 4 mm^2) taken from the margin of an actively growing colony of 7 days on PDA was placed in a shallow wound (3mm) made by a scalpel on the middle of each shoot.

The inoculation point was covered with cotton wool soaked in sterile water and wrapped with Parafilm. The inoculated shoots were placed in a break containing 200 ml of sterile water, whereas the bottom and top end of each cane were sealed with a synthetic grafting resin to prevent drying and contamination and then enclosed in a transparent plastic bag at room temperature (20- 26°C).

Antagonist assay protocol.

The antagonistic agent used in this work is *Trichoderma* sp. which was isolated from asymptomatic tissues of white heather and grown on PDA at 25°C before use. The fungus was identified based on its morphological cultural traits and conidial characteristics.

In vitro antagonist activity of *Trichoderma* sp. against *Pestalotiopsis chamaeropis* was studied according to two methods.

Direct confrontation. This technique consists in placing, in the same plate of Petri dishes containing a PDA medium, two small pieces of mycelium of *Trichoderma* sp. and *Pestalotiopsis chamaeropis* cultures. The two fragments were placed along a diametrical axis 3 cm and equidistant of the center at the same time. Incubation was performed at 25°C for 7 days. Ratings on the inhibition of *P. chamaeropis* growth and invasion of its colony by *Trichoderma*mycelium are noted every day. Control plates were challenged with a transplant of the pathogen only which was placed at the center.

Remote confrontation. This method consists of transplanting the antagonist and pathogen in two separated plates. Later, an assembly was carried out by super-positioningthe two plates (*Trichoderma* downside and *P. chamaeropis* upside). The junction between the two plates was insured by a Parafilm in order to avoid any loss of volatile substances (Daami-Remadi and El Mahjoub 2001). Growing conditions

were identical to those of direct confrontation. The control was formed by stacking plates, the upper one contained a small piece of mycelium of *P. chamaeropis* and the bottom one contained only PDA.

The average diameter of treated colonies was noted when pathogen mycelium in control plates reached the periphery. The percentage of inhibition of pathogen mycelial growth was calculated based on the following formula (Hmouni et al. 1996): I (%) = (1- Cn /Co) × 100, where Cn is the average diameter of the colonies in the presence of the antagonist and Co the average diameter of control colonies.

RESULTS

Pathogen identification.

The culture characteristics were white fluffy aerial mycelium (Fig. 2.a). Black acervular conidiomata on the surface of mycelium were produced after about 10 days (Fig. 2.b). Conidia were 4-septate (5 cells) and were fusoid to ellipsoid, straight to slightly curved (Fig. 2.c), measured 22.5µm (16.9-28.0) × 6.5µm (4.8-8.5). There were three median cells dark brown. Both apical and basal cells were hyaline, the apical cell with 2 or 3 appendages (mostly 3). Morphological and cultural characteristics as well as type of conidia observed were very similar and typical of the genus *Pestalotiopsis.*

The sequence resulting from the PCR amplification of the nuclear rDNA operon using the primers ITS1 and ITS4 revealed a high degree of similarity (100%) with the 16S rRNA sequence of *P. chamaeropis*. The fungus was identified as *Pestalotiopsis chamaeropis* and the representative sequence was deposited in GenBank (ITS: KY996524).

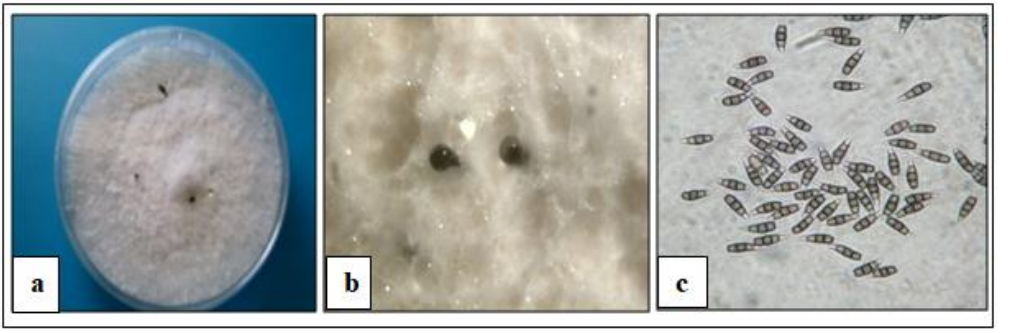

Fig.2.*Pestalotiopsis* colony cultured after 10 days on Potato-Dextrose-Agar at 25°C (a);Acervuli wih exuded conidia(b); Conidia with apical and basal appendages (c).

Morphological and cultural characterization of the antagonist.

On PDA medium, cultures were woolly initially white then green (Fig. 3.a), mycelial growth was rapidreaching more than 65mm after 3 days. Conidia were abundant gray-green, single-celled, smooth, round or ovoid (Fig. 3.b). Cultural characteristics as well as type of conidia observed were typical of the genus *Trichoderma.*

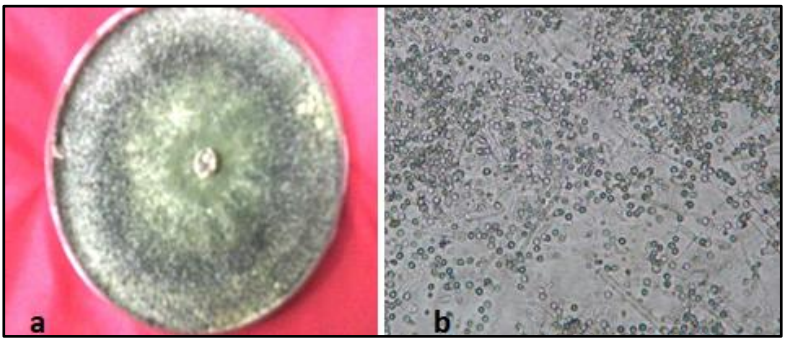

Fig. 3. *Trichoderma* colony growth on PDA medium noted after 7 days of incubation (a); Abundant gray-green conidia (b).

Pathogenicity test.

Five weeks after inoculation, all 5 shoots inoculated by *P. chamaeropis* showed a necrotic lesion around the inoculation point which measured 2.5 ± 0.5 cm. Control shoots, inoculated with sterile PDA plugs only, remained symptomless (Fig. 4).

The fungus was successfully re-isolated from necrotic bark and the margin of symptomatic wood tissues, thus fulfilling Koch's rules.

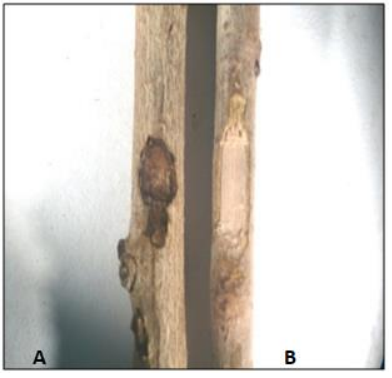

Fig. 4. White heather (*Erica arborea*) shoot showing brown lesion five weeks after inoculation with *Pestalotiopsis chamaeropis* (A). Asymtomatic control shoot (B).

Antagonist activity of *Trichoderma*. sp against *Pestalotiopsis chamaeropis*

Direct inhibition. The simultaneous growing of *Trichoderma* sp. and *P. chamaeropis* in the same plate showed faster growth of *Trichoderma* sp. In fact, after five days of incubation, the Petri plate was invaded by the antagonist, while the pathogen colony measured only 26 mm in diameter as expressed by a growth inhibition of 64% (Fig. 5). The cultivated *P. chamaeropis* control alone covers an area of about 69 mm in diameter. Beyond this period and at the end of seven days, *Trichoderma* invaded *P. chamaeropis* colonies and sporulated on.

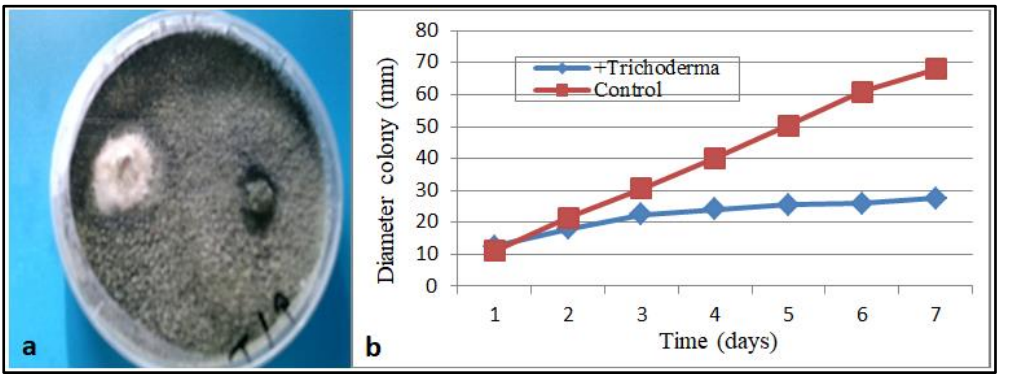

Fig. 5. Inhibition of pathogen mycelial growth in presence of *Trichoderma* sp (a). *Pestalotiopsis chamaeropis* growth in presence and in absence of *Trichoderma* sp. as compared to control (b).

Distant inhibition.This technique enabled us to highlight the inhibiting effect even remotely of the *Trichoderma* sp. on *P. chamaeropis*; this effect was assessed and the obtained results showed a significant reduction in the average diameter of *P. chamaeropis* colonies in presence of *Trichoderma* sp. compared to control. After six days of incubation at 25°C, this reduction reached 58% as compared to control (Fig. 6).

DISCUSSION

Morphological characters of *P. chamaeropis* showed similar characteristics to the ex-type isolate such as pigmentation of the median cells, number of apical appendages (2-3, mostly 3), basal appendages and septa (4-septate) (Maharachchikumbura et al. 2014). The length and width of median, basal and apical cells were shorter. Jeewon et al. (2003) reported that the length of spores, median cells and appendages were not phylogenetically important in identifying the species; but might be informative in identifying species groups. In contrast, pigmentation of the median cells and appendage tip morphology are important factors in phylogenetic identification of the species. *Pestalotiopsis* consists of approximately 205 described species that are easily identified by the presence of relatively fusiform conidia formed within compact acervuli (CABI Bioscience database 2001).

The results of our study indicated that *P. chamaeropis* is a pathogen that could cause canker lesions and dieback symptoms on white heather. To the best of our knowledge, this fungus is a new pathogen for white heather. Dieback of white heather is a serious ecologic problem in Tunisia. There are very few reports of plant pathogens causing diseases in white heather.

Several studies showed that the success of *Pestalotiopsis* infection requires an entry way in the host caused by injury (Espinosa et al. 2008; McQuilken et al. 2004). *Pestalotiopsis* is just one of a complex group of fungi. This fungus is also considered as a weak pathogen (Madar et al. 1991), which penetrates the host through natural openings such as stomata, lenticels and hydathodes (Agrios 2005). Many authors stated that *Pestalotiopsis* species infect only wounded or stressed plants which may be stressed due to insects, pesticides or sun damage. (Hopkins and McQuilken 2000; Wright et al. 1998).

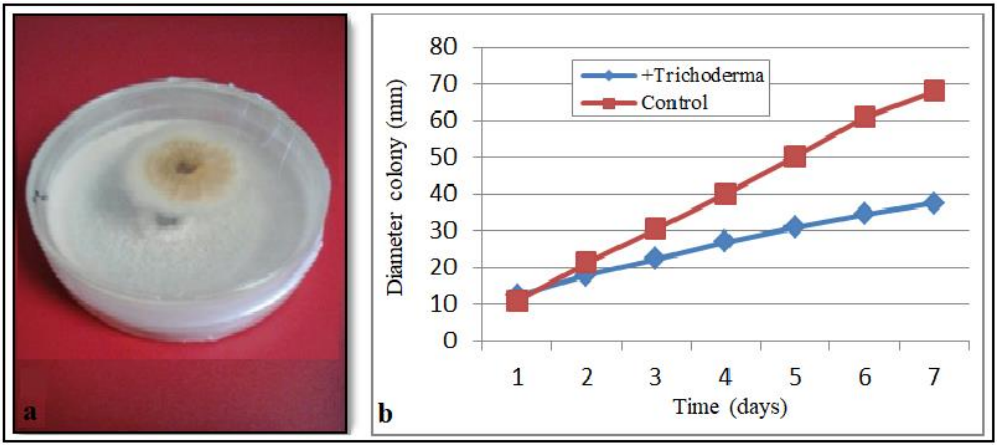

Fig. 6. Distant inhibition of pathogen growth by *Trichoderma* sp. (a); Antagonist effect remotely of *Trichoderma* sp. on *Pestalotiopsis chamaeropis* mycelia growth (b).

Studies undertaken in Australia and America showed that *Pestalotiopsis*-like fungi occurred not only leaves, but also on canes, wood, berries and flowers (Castillo-Pando et al. 2001;Deng et al. 2013; Sergeeva et al. 2001; Urbez-Torres et al. 2009, 2012).

Nowadays biological control is considered as one of the most interesting aspects of the science to find out the mechanisms employed by biocontrol agents for effective disease control (Howell 2003). Vidhya Pallavi et al. (2010) noted that under laboratory conditions, *Trichoderma* spp. exhibited a very good antagonistic potentiality against the grey blight (*Pestalotiopsis* sp.). Similarly, Naglot et al. (2015) tested several isolates of *Trichoderma* spp. and reported their antagonistic potency for the control of this pathogen and these findings are in agreement with our results.

Antagonistic microorganisms, such as *Trichoderma* species reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion (Cook et al. 1983). Several strains of the *Trichoderma* sp. are found to be effective biocontrol agents for the various plant pathogens (Amin et al. 2010) and they are characterized by rapid growth, abundant conidial formation and a high degree of ecological adaptability as reported by several researchers (Bissett 1991; Domsch et al. 1980; Papavizas 1985). *Trichoderma* sp. are capable to induce metabolic changes in plants that increases resistance to a wide range of plant pathogenic microorganisms (Harman et al. 2004).

This study was conducted in order to control the pathogenic plant fungus *P. chamaeropis*. The tests of direct and remote confrontation on culture between the target pathogen and *Trichoderma* sp. revealed the ability of the last to inhibit *P. chamaeropis* mycelial growth. Moreover, beyond this period and after six days, *Trichoderma* sp. invaded pathogen colonies and sporulated abundantly suggesting its highly mycoparasitic potential. Additionally, the results of the remote confrontation showed a significant reduction in the average diameter of pathogen colonies in presence of *Trichoderma* compared to control.

Similar effects of *Trichoderma* were also observed by Benhamou et al. (1997) following its direct confrontation with *Pythium ultimum*. This inhibitory action is due to substances, chemicals released by the strains of *Trichoderma* (antibiosis). Antifungal activity displayed by *Trichoderma* strain may be due to extracellular enzyme production (Davet 1983). These enzymes are responsible for the degradation of cell walls of target pathogenic agents (Lorito et al. 1993).The production of secondary metabolites by different *Trichoderma* species is well documented. It has been reported that *Trichoderma* spp. produce a wide range of volatile and non-volatile substances, antibiotics (Sivasithamparam et al. 1998). *Trichoderma* spp. are known to produce Volatile Organic Compounds(VOCs) that can inhibit fungal growth (Bruce et al. 2000;Wheatley et al. 1997). Specific VOCs, i.e. heptanal, octanal and 2-methyl-1-butanol, have previously been identified as compounds involved in the inhibition of fungal growth(Wheatley et al. 1997).Volatile secondary metabolites produced by *Trichoderma pseudo-koningii*, *T. viride* and *T. aureoviride* affected the growth of the mycelium and the synthesis of proteins in two isolates of *Serpula lacrymans* to various degrees (Humphris et al.2001).

RESUME

Hlaiem S., Zouaoui-Boutiti M., Ben Jemâa M.L., Della Rocca G., Barberini S. et Danti R. 2018. Identification et pathogénie de *Pestalotiopsis chamaeropis*, agent causal du dépérissement de la bruyère blanche (*Erica arborea*), et control biologique *in vitro* avec l'antagoniste *Trichoderma* sp. Tunisian Journal of Plant Protection 13 (si): 49-60.

Les champignons phytopathogènes sont l'une des causes principales des maladies des arbres. Les symptômes du dépérissement consistent à une chute et un jaunissement du feuillage, un dessèchement et des nécroses au niveau des branches, des chancres, des déformations, des écoulements d'un liquide noirâtre et des pourritures au niveau des troncs. Des symptômes de flétrissement ont été observés sur les espèces du maquis: bruyère blanche (*Erica arborea*), situé dans la forêt de Henchir Kort au Nord-est de la Tunisie. L'isolement à partir la bordure de ces chancres a révélé le genre de *Pestalotiopsis*. L'analyse morphologique et moléculaire de l'ITS a permis d'identifier l'agent pathogène comme étant *Pestalotiopsis chamaeropis*. Le postulat de Koch a été vérifié. L'effet antagoniste de *Trichoderma* contre *P. chamaeropis* a été évalué *in vitro*. Les tests de confrontation directe sur PDA ou à distance, ont révélé que *Trichoderma* sp. a inhibé la croissance mycélienne de l'agent pathogène par rapport au témoin non traité.

Mots clés: Bruyère blanche, dépérissement, effet antagoniste, *Pestalotiopsis chamaeropis*, *Trichoderma* sp.

ملخص

حليّم، سوسن ومريم زواوي-بوتيتي ومحمد الحبيب بن جامع وجياني ديلا روكا وسارة بربريني وروبيرتو دانتي. 2018. التشخيص والقدرة الإمراضية لفطر *Pestalotiopsis chamaeropis* العامل المسبب لمرض تدهور الخلنج الأبيض ودراسة المكافحة البيولوجية في الأنابيب باستعمال الفطر المضاد *Trichoderma* sp.

Tunisian Journal of Plant Protection 13 (si): 49-60.

تعتبر الفطريات الممرضة واحد من الأسباب الرئيسية لأمراض الأشجار. تشمل أعراض تدهور الأشجار تساقط واصفرار الأوراق وتجفف ونخر الاغصان والتقرح والتشوه وسيلان سوائل سوداء وتعفن على مستوى الجذع. لوحظت أعراض ذبول على أنواع الاحراش: الخلنج الأبيض (*Erica arborea*) المتواجد في غابة "هنشير الكرت" في شمال شرق تونس. مكن العزل من حواشي القروح من الحصول على فطر من جنس *Pestalotiopsis*. مكنت التحاليل المورفولوجية وكذلك الجزيئية للفواصل الداخلية المسجلة (ITS) من تشخيص الفطر الممرض بأنه *Pestalotiopsis chamaeropis*.

تم التثبت من قواعد "كوخ". تم تقييم التأثير العدائي للفطر المضاد *Trichoderma* على الفطر *P. chamaeropis* في الأنابيب. بينت اختبارات المواجهة المباشرة وعن بعد على المستنبت PDA، أن .sp *Trichoderma* منع النمو الغزلي للفطر الممرض مقارنة بالشاهد غير المعامل.

كلمات مفتاحية: تأثير مضاد، تدهور، خلنج أبيض، *Pestalotiopsis chamaeropis*، *Trichoderma* sp.

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