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Screening of fungal strains isolated from dead insects for production of plant litter-degrading enzymes

A. Armand
S. A. Khodaparast ✉
H. Masigol
Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran

H. P. Grossart
Department of Experimental Limnology, Leibniz Institute of Freshwater Ecology and Inland Fisheries, Stechlin, Germany

Abstract: Exploring the enzymatic capabilities of fungi is of great importance to better understand their ecological roles and improving their manifold usage in industry. Therefore, rapid and reliable screening methods to evaluate fungal enzymatic potentials are needed. In this study, 76 fungal strains were isolated and identified from insect pests from citrus plantations in Guilan province, northern Iran. All the strains belonged to *Akanthomyces* and *Lecanicillium* (*Ascomycota*: *Hypocreales*). Plate assay methods were applied to investigate fungal ability to produce the following enzymes: Endo-1,4-*β*-glucanase (CM-cellulase, endoglucanase and endocellulase), *β*-Glucosidase, Cellobiodydrolase, pectinase and laccase. The results showed that the majority of the strains were able to produce at least one of these enzymes. Cellulolytic activity was found in 33 % and laccase activity was detected in 11 % of all strains tested. Interestingly, the enzymatic ability of strains identified as *A. muscarius* and *A. lecanii*, were generally different, likely confirming their taxonomic position.

Keywords: Enzymatic potential, *Akanthomyces*, *Lecanicillium*, citrus plantations

INTRODUCTION

Recently, the enzymatic potential of fungi has gained great attention for their manifold applications in different industries such as paper production (Witayakran & Ragauskas 2009), biofuel generation (Wilson 2009), bioremediation (Strong & Claus 2011), phytopathogen management (Kikot et al. 2009), etc. For instance, fungal strains including *Cryphonectria parasitica* (Murrill) M.E. Barr, *Thermomyces lanuginosus* Tsikl., and *Rhizopus oryzae* Went & Prins. Geerl. are frequently used in food processing (Jensen et al. 2006), detergent production (Johnsen et al. 1997), and pharmaceutical industries (Loureiro et al. 2009), respectively. One of the most predominant fungi used in large-scale production of enzymes is *Aspergillus*, from which 12 different industrial enzymes are currently used in various industrial sectors, including production of rubber, milk and other dairy products, as well as bread (Park et al. 2017).

In addition to their industrial applications, fungi possess an enormous ecological role being parasitic or saprophytic degraders of plant lignocellulosic materials in either aquatic or terrestrial ecosystems (Lundell et al. 2010). Moreover, pectin can be found in lignocellulosic residues to varying degrees based on their original source (Sánchez 2009). The lignocellulolytic enzymes-producing fungi are more prevalent in the phylum Basidiomycota than Ascomycota, in particular, white-rot (e.g., *Phanerochaete chrysosporium* Burds.) (Leonowicz et al. 1999) and brown-rot fungi (e.g., *Fomitopsis palustris* (Berk. & M.A. Curtis) Gilb. & Ryvarden) (Yoon & Kim 2005). However, both Dikarya phyla (Ascomycota and Basidiomycota) are differently involved in lignocellulose degradation processes. Ascomycota dominates during the first stages of litter degradation (Koide et al. 2005) by hydrolyzing cellulose (Osono et al. 2003), while Basidiomycota are considered to be prevalent in the later stages of plant litter decomposition (Osono 2007).

Cordycipitaceae belong to the order *Hypocreales* (Sung et al. 2007) and members of this family are mainly associated with arthropods (Chen et al. 2019), plants (Avery et al. 2011), etc. In particular, some genera such as *Akanthomyces* Lebert and *Lecanicillium* W. Gams, H.C. Evans & Zare have been applied as

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✉ Corresponding Author E-mail: khodaparast@guilan.ac.ir

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biopesticides because of their well-known entomopathogenecity (Goettel et al. 2008, Helaly et al. 2017) and their inhibitory activity against fungal plant pathogens (Ownley et al. 2010). In this context, the production of cuticle-degrading enzymes has been the subject of many studies (Ali & Moharram 2014; Gandarilla-Pacheco et al. 2015). However, the ability of these fungi to produce plant litter-degrading enzymes has been rarely investigated.

The main goal of this study was to evaluate the production of plant litter-degrading enzymes in fungi associated with insects collected from citrus plantations in Guilan province. This study can improve our knowledge about the enzymatic activities of fungi which is important for understanding their role in soil and forest ecosystems.

MATERIALS AND METHODS

Isolation and Identification of Fungi

Plant sucking insects (scales and aphids) infested with fungi were collected from citrus orchards (sweet orange, sour orange, mandarin) and transformed to the laboratory. Insects' exoskeleton was disinfected using 1 % sodium hypochlorite for three min and then thoroughly rinsed with distilled water. Afterward, the exoskeleton was smashed and small parts were cultivated in potato dextrose agar (PDA). The plates were incubated at 25 °C in a 12:12 light-dark regime.

In samples with fungal structures, the direct culturing method was applied for isolation (Stone et al. 2004). In these cases, a small piece of mycelium or conidial mass on the top of insect bodies was removed using a sterile needle and transferred to or streaked out on agar containing media. After five days of incubation, growing colonies were sub-cultured by transferring a small piece of mycelia to a new PDA medium and incubated at the same conditions for three days. Finally, the hyphal tip technique was used to obtain pure cultures. All cultures were kept at 4 °C for further studies. The fungal strains were identified based on their morphological (Zare & Gams 2004, 2008) as well as molecular features including growth rate, characteristics of the colonies such as shape, color, and pigment production (on PDA medium), the structure of conidia, conidiophore branches, phialides and crystal formation (on PCA medium) that have been already described in Armand (2020). Twelve representative strains were deposited in the Fungal Collection of the Iranian Research Institute of Plant Protection, Iran (refer to Armand 2020 for further information).

Enzymatic screening

Cellulolytic and pectinolytic activities were evaluated using the MEA medium (30 g of malt extract with 20 g of agar in 1 L H_2O) amended separately with either 7.5 g carboxymethyl cellulose (CMC), 7.5 g cellobiose (CEL), 5 g Avicel (AVL) and 5 g pectic acid (PGA) per liter. CMC, cellobiose, AVL and PGA were considered as indicators of Endo-1,4-*β*-glucanases (CM-cellulase, endoglucanae and endocellulase), *β*-Glucosidases and Cellobiodydrolase (exocellobiohydrolase, exocellulase and Avicelase) and pectinases, respectively. Seven-day-old mycelia were transferred to the medium and kept at room temperature for three weeks. To estimate the enzyme activity, 15 ml of Congo Red (1 mg ml^{-1}) was poured into the medium as an indicator. Subsequently, petri dishes were gently shaken for 15 min and finally rinsed with distilled water. Thereafter, 30 ml of 1 M NaCl were added (Pointing 1990).

Ligninolytic activity was also measured using the mentioned medium amended with 0.1 % wt/vol 2,2′--Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS). Seven-day-old mycelia were transferred to the medium, kept at room temperature for three weeks, and checked daily (Masigol et al. 2019). The results were considered positive (production of laccases) when the area around the colonies turned blue.

Removal of color from the medium by 0-33, 33-66 and 66-100% in all tested plates were detected as weak, medium and strong reactions, respectively (Masigol et al. 2019). This considers the different growth rates of the tested strains for the five different substrates to cluster the strains by using hierarchical cluster analysis in SPSS 16.0 according to the Ward method (Ward 1963).

RESULTS

Considering all five substrates testing for Endo-1,4-*β*-glucanase, *β*-Glucosidase, Cellobiodydrolase, pectinase and laccase activities of the fungal strains, nearly 35.52 % of all strains did not show any positive reactions. In addition, 35.52 %, 1.52 %, 6.57 %, 9.21 % and 2.63 % of the strains revealed one, two, three, four and five positive reactions, respectively. Based on the hierarchical clustering analysis of five enzymatic activities, the strains were placed in two clades. One clade mainly consists of *A. lecanii* and *Akanthomyces* sp. strains, while the other contains *A. muscarius* with some minor exceptions (Fig.1a).

About 71 %, 76 % and 86 % of all strains did not reveal any cellulo-, pectino- and lignolytic activities, respectively. On average, 13 %, 5 %, and 11 % of the strains showed high, medium and low cellulolytic activity, respectively. In addition, high, medium and low pectinase activity was observed in 9 %, 3 %, and 12 % of the tested strains, respectively. Only 7.89 %, 2.63 %, and 3.94 % of the strains showed a low, medium and high ligninolytic activity, respectively (Fig. 1b).

A Venn diagram (Fig. 1c) shows all possible logical combinations between different enzymes. Regarding cellulolytic activity, 12 strains (nearly 16 %) produced Endo-1,4-*β*-glucanase, *β*-Glucosidase as well as Cellobiodydrolase, including O1103-1, R128-1, R128-2 and Sh901-3 strains with strong activities

for all mentioned enzymes. In addition, two strains, including F901-2 and 243 were able to produce cellulolytic enzymes, pectinase, as well as laccase (Fig. 1c).

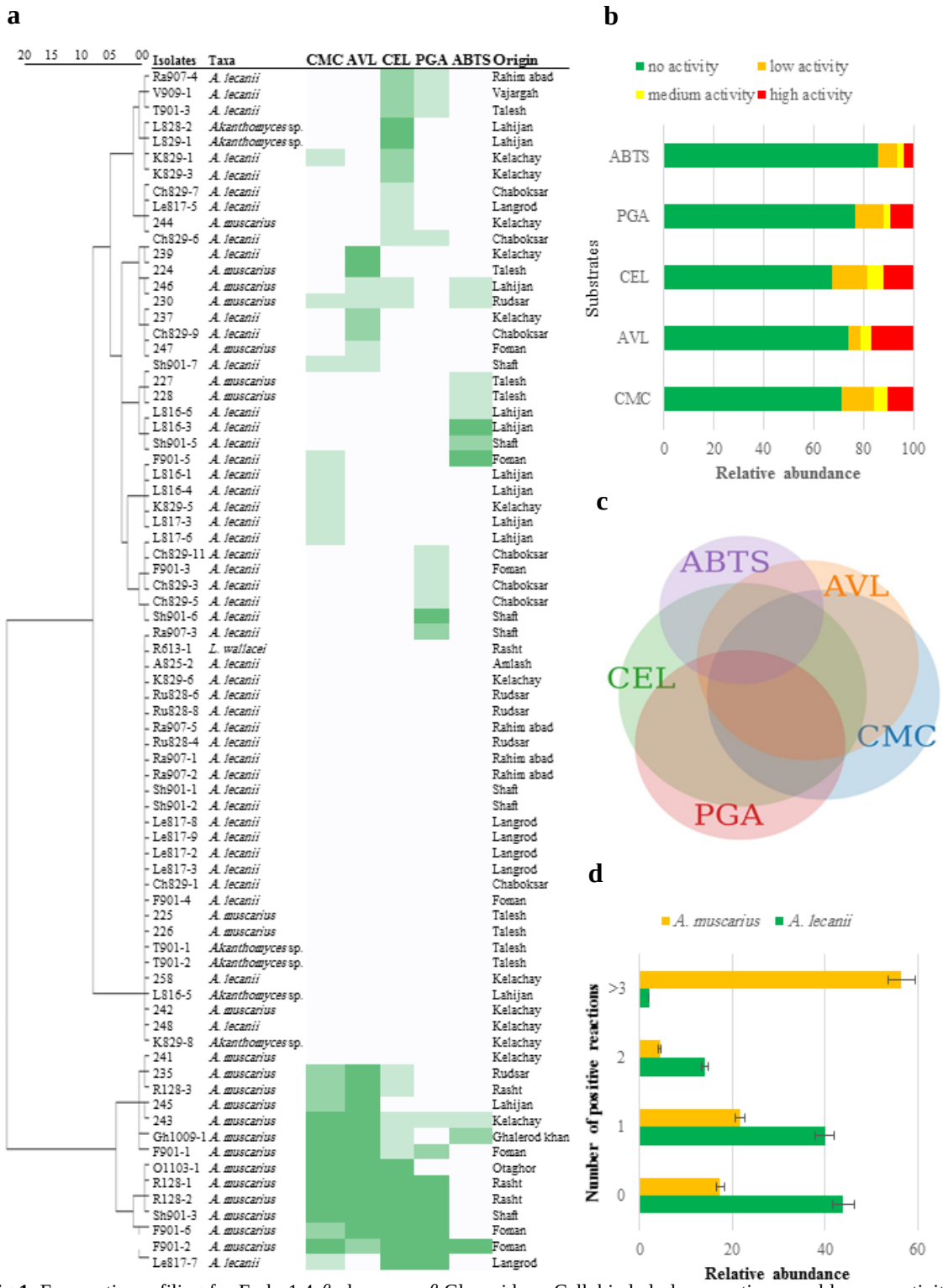

Fig 1. Enzymatic profiling for Endo-1,4-*β*-glucanase, *β*-Glucosidase, Cellobiodydrolase, pectinase and laccase activities and the dendrogram inferred from hierarchical cluster analysis of 76 *Akanthomyces* strains isolated from Guilan, Iran. Positive degradation activities are shown in a semi-quantitative scale: white represents no activity, whereas light to dark green squares represent low, medium and high activities, respectively, a. Composition of enzymatic activities, b. A Venn diagram showing overlapping enzymatic activities with respect to all tested strains in this study, c. Enzymatic ability of *A. muscarius* and *A. lecanii* (d) (ABTS: Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, AVL: Avicel, CMC: Carboxymethyl cellulose, CEL: Cellobiose and PGA: Polygalacturonase A).

Interestingly, *A. muscarius* and *A. lecanii* showed different enzymatic activities (Fig. 1d). In general, enzymatic diversity of *A. muscarius* strains were higher than *A. lecanii* strains.

To illustrate, nearly 60% of *A. muscarius* strains showed positive activities in at least three cultures amended with the abovementioned substrates. For instance, *A. muscarius* strain F901-2 showed enzymatic activities in all five cultures amended with ABTS, AVL, CEL, CMC, and PGA. In contrast, 46 % of *A. lecanii* strains showed no enzymatic activities at all.

DISCUSSION

In this study, the majority of our strains did not show any activities for the tested enzymes (Fig. 1b). We showed that even though the isolated fungi are usually associated with insects, some isolates were also able to produce enzymes to degrade plant litter. Fungal strains from insects have been previously tested for their high chitinase potential (Charnley & Leger 1991, Charnley 2003, Charnley & Collins 2007), therefore, the focus of this study was to analyze the activity of cellulolytic- ligninolytic and pectinolytic enzymes.

The findings of this study are interesting from a taxonomic point of view since *A. muscarius* and *A. lecanii* species were generally differentiated by analyzing their enzymatic abilities (Fig. 1a). This differentiation is promising because there has been confusion over species delineation in the members of *Cordycipitaceae*, leaving morphological characters unreliable to distinguish a natural taxonomic group (Hodge et al. 2005). Therefore, a combination of morphological, molecular as well as ecological characterizations seems to offer an effective approach to overcome difficulties in species identifications.

In this study, we evaluated the capacity of *Akanthomyces* spp. and one *Lecanicillium* sp. associated with dead insect bodies for the production of cellulo-, ligno- and pectinolytic enzymes (Fig. 1). Based on the results, no basidiomycetes were isolated. This result is generally in accordance with our current knowledge, i.e., a dominance of *Ascomycota* and *Entomophthoromycota* associated with insects, since until now only three genera from basidiomycetes have been reported to infect insects (Araújo & Hughes 2016). The fact that *Entomophthoromycota* usually grows slowly might explain why we could not isolate any of these members. In this study, the fungal strains were found on dead insects in citrus plantations which might indicate the tight association of these fungi with chitin-based substrates. In fact, *Cordycipitaceae* (including the genus *Akanthomyces*) are generally considered as entomopathogens (Chiriví-Salomón et al. 2015, Vinit et al. 2018) with a high ability to produce chitinases (Ali et al. 2011, Wang et al. 2012), making them perfect targets for biocontrol approaches (Ali et al. 2009, Wu et al. 2010). Aside from their entomopathogenic affinities, producing enzymes involved in plant litter degradation by the obtained strains is a matter of interest. The fact that insects collected in our study are all plant suckers and *Akanthomyces* species have been previously reported as common plant endophytes (Vega 2018, Vinit et al. 2018) might suggests they could constantly move from plant hosts to the insects' body and contribute to their digestion processes.

In addition to their enzymatic properties, other potential applications of *Akanthomyces* species should not be ignored. Several strains have shown antimicrobial activity against several human bacterial pathogens (*Staphylococcus aureus* and *Cryptococcus neoformans*) (Kuephadungphan et al. 2014). Wagenaar et al. (2002) also introduced a new antibiotic called Akanthomycin from *A. gracilis*. Moreover, they have been accepted as a rich source for a wide range of bioactive secondary metabolites (Helaly et al. 2017), which calls upon scholars to elucidate their unknown diversity.

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ارزیابی جدایه های قارچی به دست آمده از حشرات مرده برای تولید آنزیمهای تجزیه کننده بقایای گیاهی

علیرضا آرمند[1]، سیداکبر خداپرست[1]✉، حسین مسیگل[1]، هانس-پیتر گروسارت[2]

۱- گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه گیلان، رشت، ایران

۲- دپارتمان آب شیرین شناسی تجربی، موسسه آی جی بی، برلین، آلمان

چکیده: بررسی خصوصیات آنزیمی قارچها دارای اهمیت زیادی است چرا که منجر به درک بهتر نقش بوم‌شناختی آن‌ها و بهبود عملکردشان در صنعت می‌شود. بنابراین معرفی (شناسایی) روش‌های غربالگری سریع و مطمئن برای ارزیابی توانایی آنزیمی قارچها ضرورت دارد. در این مطالعه، ۷۶ جدایه قارچی از حشرات آفت روی گیاهان مرکبات در استان گیلان که قبلا جداسازی و شناسایی شده بودند مورد استفاده قرار گرفتند. تمامی جدایه‌ها متعلق به جنس‌های *Akanthomyces* و *Lecanicillium* (*Ascomycota*: *Hypocreales*) بودند. توانایی جدایه‌ها در تولید آنزیم‌های اِندو-یک و چهار-بتا-گلوکاناز (سی ام-سلولاز، اندوگلوکاناز و اندوسلولاز)، بتاگلوکوزیداز، سلوبیوهیدرولاز، پکتیناز و لاکاز با روش‌های ارزیابی وابسته به محیط‌کشت بررسی شد. نتایج نشان داد که بیشتر جدایه‌ها توانایی تولید حداقل یک آنزیم را دارا بودند. فعالیت سلولولایتیکی در ۳۳٪ و فعالیت لاکازی در ۱۱٪ از جدایه ها مشاهده شد. هم‌چنین، به طور کلی توانمندی آنزیمی جدایه‌های دو گونه *A. muscarius* و *A. lecanii* با هم متفاوت بود که احتمالا تأییدکننده جایگاه تاکسونومی آن‌هاست.

کلمات کلیدی: پتانسیل آنزیمی، *Akanthomyces* و *Lecanicillium*، باغات مرکبات

مکاتبه کننده: سید اکبر خداپرست. Email: khodaparast@guilan.ac.ir

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