เชื้อรา ASCHERSONIA PLACENTA BERK. ที่พบในแปลงหม่อนเป็นครั้งแรกในไทยและผลของสารสกัดจากพืชต่อการเจริญของเชื้อรา

THE FIRST REPORT OF ASCHERSONIA PLACENTA BERK. ON MULBERRY FIELD IN THAILAND AND EFFECT OF PLANT EXTRACTS TO THE GROWTH OF THE FUNGUS

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บทคัดย่อ

เชื้อราทำลายแมลง Aschersonia sp. ถูกพบบนใบหม่อนจากแปลงหม่อนที่ปลูกไว้เพื่อใช้เลี้ยงไหม ซึ่งพบว่าเมื่อหนอนไหมกินใบหม่อนที่มีเชื้อรานี้เข้าไปจะท้องเสียและตายในที่สุด ได้เก็บตัวอย่างใบหม่อนที่มีเชื้อราจากแปลงหม่อนของเกษตรกรในจังหวัดกำแพงเพชร มาศึกษาลักษณะสัณฐานวิทยาของเชื้อราและแยกเชื้อจนได้เชื้อบริสุทธิ์ แล้วจึงทำการศึกษาลักษณะทางสัณฐานวิทยาของเชื้อที่แยกได้ การระบุชื่อเชื้อโดยอาศัยลักษณะทางสัณฐานวิทยาเป็นหลักพบว่าคือเชื้อรา A. placenta Berk. ทำการทดสอบการยับยั้งการเจริญของเชื้อราในระดับห้องปฏิบัติการโดยใช้สารสกัดเมทานอลจากข่า ผักโขม บอระเพ็ด และขิงผสมลงในอาหารเลี้ยงเชื้อรา ผลการศึกษาพบว่าทุกสารสกัดสามารถยับยั้งการเจริญของเชื้อรา A. placenta ได้ ยกเว้นสารสกัดจากผักโขม โดยสารสกัดจากบอระเพ็ดให้ผลยับยั้งการเจริญของเชื้อราได้ดีที่สุด และพบว่าสารออกฤทธิ์ต้านเชื้อราของสารสกัดบอระเพ็ดมีคุณสมบัติทนต่อความร้อนสูงแม้จะผ่านการนึ่งในหม้อนึ่งอัดความดันไอ

คำสำคัญ: Aschersonia การควบคุมโดยชีววิธี สารสกัดพืช อนุกรมวิธาน

Abstract

An entomopathogenic fungus, Aschersonia sp., found on mulberry leaves (Morus alba L.) was investigated during cultivation of mulberry leaves for silkworm-rearing in Kamphaeng Phet province, Thailand. The silkworms had diarrhea and subsequent mortality after consumption of leaves colonized by this fungus. Microscopic and macroscopic characteristics of the fungus on substrate were examined and pure culture was isolated. Colony morphology of the pure culture obtained then has been investigated. Identification of the fungus based on morphological characteristics suggesting it is A. placenta Berk. Attempt to inhibit the growth of the fungus using plant extracts in a laboratory scale has been done. Methanol extracts of

Alpinia galanga (L.) Willd., Amaranthus viridis L., Tinospora crispa (L.) Miers ex Hook.f. & Thomson and Zingiber officinale Roscoe were added to the medium and tested for their ability to control fungal growth in the Petri dish. All methanol extracts, except for A. viridis, inhibited the growth of Aschersonia placenta, where methanol extracts from T. crispa showed the highest inhibition against the growth of this fungus with heat resistant properties after sterilization via autoclaving.

Keywords: Aschersonia, biocontrol, plant extracts, taxonomy

Introduction

Fungi in the genus Aschersonia (Mont. s. l.) are insect pathogens (teleomorph: Hypocrella, Clavicipitaceae, Hypocreales), characterized by bright-colored stromata, pycnidial to acervular fruiting bodies (Chaverri et al., 2008). The genus was established and typified with A. tahitensis Mont. from the tropics by Montagne in 1848 (Chaverri et al., 2008; Qiu & Guan, 2010). Chaverri et al. (2005) accepted forty-four species in the genus based on morphological and molecular data. Recently, eight more new species; A. calendulina, A. conica, A. fusispora, A. insperata, A. luteola, A. macrostromatica, A. minutispora, and A. narathiwatensis, have been reported and currently indexed in Index Fungorum (Indexfungorum, 2016). According to several reports, Aschersonia can cause disease in whiteflies (Aleyrodidae, Homoptera) and several scale insects (Coccidae and Lecaniidae, Homoptera) (Liu et al., 2006; Chaverri et al., 2008; Wang et al., 2013a, b; Homrahud et al., 2016). Even the genus is common in tropical regions but a few species can also be found in subtropical areas (Petch, 1921; Mains, 1959a, b; Evans & Hywel-Jones, 1990; Hywel-Jones & Evans, 1993; Liu et al., 2006). The importance of this genus, especially A. aleyrodis, is a well-known entomopathogenic fungus using in biocontrol of whitefly pests worldwide (Cohen & Yasnosh, 2001; Liu et al., 2006; Wang et al., 2013b). In Thailand, there are several new species reports on Aschersonia, its teleomorph and allied genera (Mongkolsamrit et al., 2009; 2011; 2014). Most new species are obtained from the host collecting from natural forests and far from human disturbance.

Currently, plant pathologists have focused their attention to develop environmentally safe and effective biocontrol methods for the management of fungi. Natural plant extracts are of interest as a source of antimicrobial agents that may prevent plant from fungal colonization (Harish et al., 2008). Crude extracts of various plants have been shown to have good antimicrobial property (Yin & Cheng, 1998; Mahmoud, 1999; Fiori et al., 2000; Al-Mughrabi, 2003; Odedina et al., 2015). In Thailand, several plant extracts used in biocontrol of fungi have been also increasing recently (Thanaboripat et al., 2006; Udomsilp et al., 2009; Jantasorn et al., 2016).

During the cultivation of mulberry for silkworm rearing in Kamphaeng Phet province of Thailand (in December 2008), an entomopathogenic fungus, Aschersonia sp., was found on leaves of mulberry in the field. Observation on growth of silkworm feeding by colonized leaf showed that it was causing diarrhea in silkworm and lead to their mortality (ca 80%) subsequently. It is therefore necessary to find out the species identity of the Aschersonia sp., how to prevent the occurrence and control this fungus if an outbreak already occurred in the mulberry field which would have least effect to silkworm rearing. Thus, the aims of this study are to identify the Aschersonia sp. and evaluate the inhibitory efficacy of plant extract against the growth of Aschersonia sp. in laboratory condition. Four different plants were chosen, collected, extracted and studied for their fungal suppression activity against the growth of Aschersonia under in vitro condition. The result obtained from this preliminary study could be used as primary information for further studies.

Materials and Methods

Identification of Aschersonia fungus

1. Morphological characteristics of the fungus on leaf

Mulberry leaves colonized by the fungus were collected from the field and were then examined for overall morphology under stereo microscope and photographed at the laboratory of Microbiology Program, Faculty of Science and Technology, Pibulsongkram Rajabhat University. Cross section of the fruiting body was prepared and mounted with water. Microscopic characteristics of fungi were observed and measured under compound microscope. All observed data were recorded and photographed.

2. Isolation of living culture of the fungus

The living culture of the fungus was isolated from the colonized leaf using isolation method modified from Choi et al. (1999). The fungus attached to the leaf surface was scraped from the leaf and turn the inside surface out then used sterile fine forceps to pick a tiny amount of fruiting body/conidia from the colony and pointed it on the half strength potato dextrose agar (½ PDA) plate. Observation was done after one day of incubation to check the germination of conidia. Once it was germinated, hyphal tips of the mycelia were cut and transferred to a new Petri dish of PDA and re-checked for purification from the forming colony under stereo and compound microscope after 5-7 days of incubation.

3. Identification of Aschersonia fungus

Identification of Aschersonia based on the morphological characters of fungus on substrate and from living culture has been done using relevant texts and

references available (Liu et al., 2006; Luangsa-ard et al., 2007; Chaverri et al., 2008; Wang et al., 2013b; Homrahud et al., 2016).

In vitro study on effect of plant extracts against the growth of A. placenta

Based on the result of previous studies, four plant species (Figure 1) i.e. Alpinia galanga (L.) Willd. (rhizome), Amaranthus viridis L. (leaves), Tinospora crispa (L.) Miers ex Hook.f. & Thomson (stem) and Zingiber officinale Roscoe (rhizome) were collected from Subsomboon village in Phitsanulok province and then brought back to the laboratory. The plant materials were then washed in running water for 1 hour and cut into small pieces after let the water dried out. Thirty grams of each plant tissue was soaked in 300 ml of 95% methanol at room temperature for 7 days. The extracts were then filtered through cotton cheesecloth and centrifuged at 4,000 g for 30 minutes. The filtrates were then collected by filter through Whatman No. 1 filter paper and evaporated using vacuum rotary evaporator at 40 degree Celsius to get rid of the solvent. The extracts were collected and kept at 4 degree Celsius for further investigation.

Figure 1 Four selected plants used in this study.
A) Al. galanga rhizome. B) Am. viridis leaves. C) T. crispa stem. D) Z. officinale rhizome.

1. Cultivation of fungi on mixture of plant extracts and ½ PDA

The effect of plant extracts on the growth of Aschersonia was determined by the poisoned food technique (Schmitz, 1930). The plant extract solutions were mixed with ½ PDA medium to obtain the final concentrations of 5% (equal to 50,000 ppm). Plant extracts were mixed with medium before sterilization via autoclaving, while the second set was aseptically added plant extracts to sterilized ½ PDA. The assay was carried out in Petri dishes (9 cm diam) containing 19 ml PDA medium and 1 ml of plant extracts. An actively growing PDA culture disc of Aschersonia was cut using a sterilized yakult's straw (ca 3 mm diam) and transferred to the center of the new PDA plate. The plates were incubated at room temperature (28 ± 2 degree Celsius). Half-strength PDA without plant extract served as control. For comparison, ½ PDA mixed with 0.025% benomyl was prepared as positive control by following the same procedure. Three replications were maintained for individual treatment. The growth of the mycelium in each treatment was measured at day 7. The results were exhibited as percent growth inhibition compared to the control.

2. Experimental design and statistical analysis

The experiment was designed by completely randomized design with ten treatments (four plant extracts; add before sterilization and add after sterilization, and control; negative and positive control) and three replications. The results obtained then statistically analyzed by the analysis of variance (ANOVA) and the significance of the difference between the mean of the control and treated groups was considered at $p < 0.05$.

Results

Morphological characteristics of A. placenta on mulberry leaves

Stromata variable, usually circular, flattened to convex pulvinate, 0.75-3.00 mm diam, white to yellowish white, base surrounded by a thin-white hypothallus (a distinct thin layer of hyphae surrounding the base of the stroma and appressed to the plant surface), stroma surface minutely tomentose, the center covered with confluent orange conidial masses, some stroma exhibited a shape similar to a fried egg with the conidial mass in the top and center of the colony (Figure 2). Conidiomata occurs as simple depressions in the stroma surface, irregular, widely open. Approximate number of locules in conidioma is fewer than ten (mostly 8). Paraphyses present, linear, filiform, up to 90 µm long and ca 3 µm wide. Conidia hyaline, fusoid, sometimes narrowly fusiform, with acute ends, (7.5-)10-13 x 1.5-2.5 µm, smooth, one-celled with guttules.

Specimen Examined: THAILAND; Kamphaeng Phet province, Khlonglan district, Sak Ngam subdistrict, Tah Ma Kua village, on leaves of Morus alba L. (Buriram 60 cultivar), 15 December 2008, S. Thongdaengsee. RKPP001.

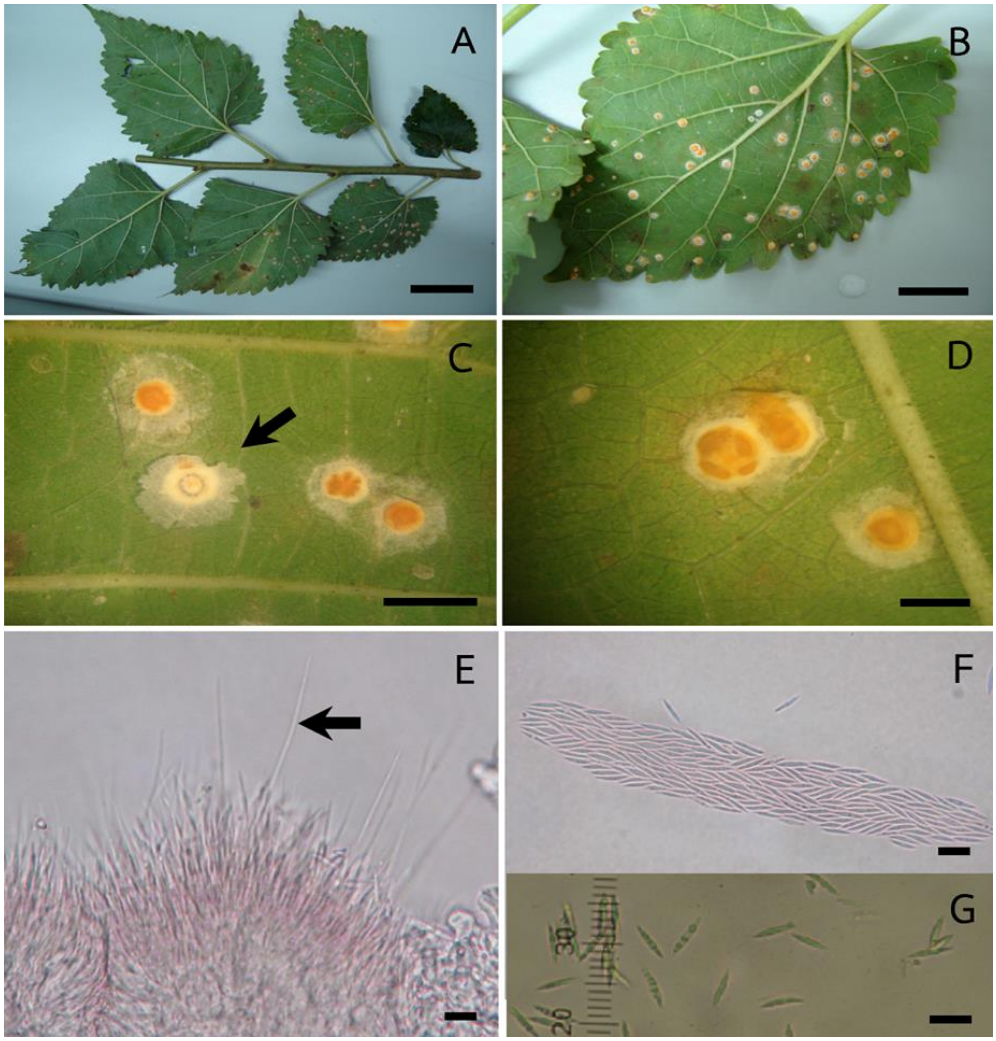

Figure 2 Morphological characteristics of A. placenta collected from the mulberry's field. A-D. Gross morphology of fungus on insect host on substrata. E. Conidioma, conidiogenous cells and paraphyses. F-G. Conidia of A. placenta seen under microscope (400X).

Remarks Scale bars; A = 5 cm, B = 2 cm, C = 5 mm, D = 2.5 mm, E-G = 10 µm. Aschersonia stromata attached to the leaf surface without invasion into the plant tissue (arrowed). Paraphyses shown in E (arrowed). E-F mounted in Lactoglycerol, G mounted in water.

Morphological Characteristics of Aschersonia's living culture in Petri dish

Colonies effused, white, and fluffy at the center of colony. The colony had a convex and spherical surface with smooth edge. Colonies produced light yellow viscous conidial masses, concentrically arranged appearing as abundant slimy masses (Figure 3). Growth rate on PDA is moderate, between 20 mm and 25 mm diameter in 3 weeks at 28 degree Celsius. Conidioma produced all over the colony. Old cultures developed a pale brown pigment in media.

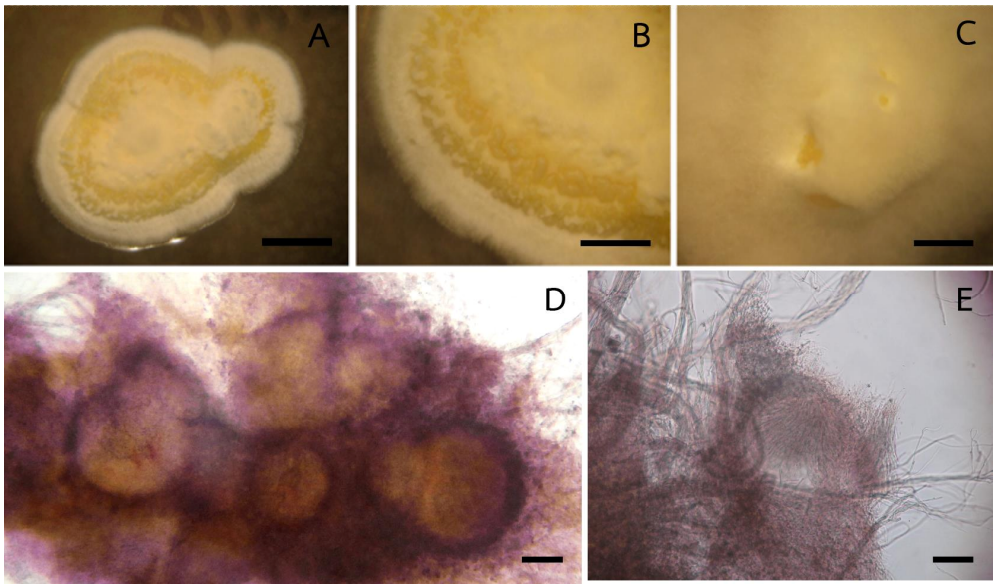

Figure 3 Morphological characteristics of A. placenta's living culture. A-C. Morphology on PDA Petri dish. D-E. Conidioma formed in living culture seen under microscope.

Remarks Scale bars; A = 1 cm, B = 5 mm, C = 3 mm, D = 150 µm, E = 100 µm.

Effect of plant extracts on the growth of A. placenta

The effect of methanol extracts of A. galanga rhizome (AGR), A. viridis leaf (AVL) T. crispa stem (TCS) and Z. officinale rhizome (ZOR) on the growth of A. placenta are given in Table 1. The results showed that only AGR and ZOR which were aseptically added into sterilized ½ PDA that completely inhibited the growth of A. placenta, where no growth is seen on day 7 of incubation, and vice versa for the set that added extracts before sterilization. While in the case of TCS extract, both set which were aseptically added into ½ PDA before and after sterilization, is completely inhibited the growth of A. placenta, where no growth could be seen on day 7 of incubation. Statistical analyses showed that each methanol extract added to sterilized ½ PDA was

significantly inhibited the growth of A. placenta at $p < 0.05$. Only methanol extract of AVL was not inhibited the growth of A. placenta where the growth of the fungus is decreased less than 5% (see Table 1). For the control set, the diameter of A. placenta colony in negative control plate was 9.23 mm while no growth could be observed in the positive control plate mixed with 0.025% benomyl fungicide.

Table 1 Effects of plant extracts on growth of A. placenta

Plant extracts	Diameter of A. placenta colony (mm)*	
	Added before media sterilization	Added after media sterilization
Alpinia galanga rhizome	8.14±0.06 ^{c} (11.80)	0.00±0.00 ^{d} (100)
Amaranthus viridis leaves	9.10±0.10 ^{b} (1.41)	9.09±0.11 ^{b} (1.52)
Tinospora crispa stem	0.00±0.00 ^{d} (100)	0.00±0.00 ^{d} (100)
Zingiber officinale rhizome	8.23±0.05 ^{c} (10.83)	0.00±0.00 ^{d} (100)
Negative Control (½ PDA)	9.23±0.05 ^{a}	
Positive control (0.025% benomyl)	0.00±0.00 ^{d}(100)	

Remark * Mean of three replications. Percent growth inhibition is indicated in the bracket. Different letters on the same column indicate significant differences according to Duncan's test ($p < 0.05$).

Discussion

A. placenta is morphologically similar to A. aleyrodis. Not only similar in morphology but it also has potential as biocontrol agent against whitefly and scale insects as A. aleyrodis (Wang et al., 2013b). Even these two Aschersonia resemble each other but they both have different geographical range where A. aleyrodis is very common and only reported from neotropical and subtropical habitats while A. placenta is common in the Eastern hemisphere/tropics (Mains, 1959b; Brady, 1984). The fungal species A. placenta is characterized by flattened stromata composed of loose hyphal tissue and wide hypothallus, possesses conidiomata with very wide openings and confluent conidial masses (Wang et al., 2013a). The typical character of the fungus found in this study is the production of conidial masses on the surface of stroma and with fusoid conidia [(7.5-)10-13 x 1.5-2.5 µm in this study vs. (9-)11-14(-16) x 1.5-2 µm from Petch (1921)], suggesting it is A. placenta. Liu et al. (2006) mentioned the morphological variation among A. placenta found during their studies, the variation includes color and size of stromata, color and the arrangement of conidial masses on the stromata, and size of conidia. Based on the descriptions in their study, A. placenta exhibited a large degree of phenotypic plasticity within individual isolates (Wang et al., 2013a). A. placenta used to be common species in Thailand. However, this fungal

species is currently become complex species and intensive taxonomic identification based on comparison of the morphological characters and molecular data with the type species is required to make sure about taxonomy of this fungus (Mongkolsamrit, unpublished data).

In Thailand, the natural occurrence of Aschersonia found on crops is mostly reported from citrus orchards that it has been used as a biocontrol agent for whitefly pests (Homrahud et al., 2016). The first study of occurrence of Aschersonia sp. on mulberry field in Thailand was reported on October 1976, where Aschersonia sp. has been examined from dead whiteflies (Pleleus mori Takabashi) collected from the mulberry field in Nong Hiang, Huan Hin, Prachuap Khiri Khan province and later on November 1979 from the same field area (Panyarachoon, 1981). Apart from Prachuap Khiri Khan, Aschersonia was also reported from mulberry fields in several provinces in Northeastern Thailand e.g. Nachon Ratchasima, Roi Et and Ubon Ratchathani (Panyarachoon, 1981). When comparing the identity of Aschersonia from Panyarachoon (1981) with Aschersonia placenta of this study, the conidial size of these two isolates is similar (10-12 x 3 µm vs. (7.5-)10-13 x 1.5-2.5 µm). However, there is inadequate information on typical characters of the fungus in Panyarachoon (1981). Thus, it could not be concluded that the fungus belongs to A. placenta.

It is also worth noting that since 1981 there is no report of Aschersonia found on mulberry filed in Thailand. The report of Aschersonia found on mulberry worldwide is also scarce. There was a record of Aschersonia collected from Amani, Berlin, that found on Morus indica (Petch, 1921). Dutta et al. (2012) reported the finding of A. aleyrodis infected to mulberry aphids from north east of India. Recently in 2015, there was a report of Aschersonia infected on unknown scale insect found on branches of mulberry (Morus spp.) from India according to the question asked in Research Gate website (Research Gate.net, 2015). Generally speaking, the occurrence of Aschersonia on cultivated crops could exist if the plant is the host of whitefly/scale insects since these two insects are the host of Aschersonia (Homrahud et al., 2016). This fungus was scarcely reported on cultivated crops in Thailand might be because the fungus itself has not affect or harm to plant or plant's products so farmers do not pay attention to or, just ignore the occurrence. In the current study, since farmers used the infected leaves for silkworm rearing so they noticed the effect of the fungus on the silkworm mortality leading them to report and seek for the solution. In this study the coincidence of whitefly, the Aschersonia fungus and the environment at that time may take into account for fungal infection and colonization. Improper pest management of whitefly is the main key for the infection since there is the report that spores/conidia of Aschersonia could survive and last for a month on plant leaf surface (Meekes et al.,

2000), providing spore a chance to disperse by wind and germinate where fallen to whitefly during high-moisture season.

In the point of biocontrol consideration, this isolate of A. placenta could be a good candidate to be developed into commercial product for whitefly (and may be for scale insects and other caterpillars as well) biocontrol since the uses of other species of Aschersonia, besides A. aleyrodis, as biological agent is currently limited and not provided consistent efficacy in several isolates (Wang et al., 2013b). Homrahud et al. (2016) have been isolated A. placenta from infected whiteflies found in citrus orchard and tested for its efficacy to control the scale insect (Parlotoria ziziphi), however, low control efficiency was obtained suggesting that the isolate of A. placenta Asp001 might not be an efficient control agent. For the isolate obtained from this study since it caused mortality in silkworm so it is high possibility that this isolate could be used as biocontrol agent for other caterpillar pests as well. However, since in this study we have not done on isolation of the fungus directly from the dead silkworm and we also have not try to reinoculate the fungus isolated from the substrate back to silkworm so further studies are necessary to confirm the efficacy of this A. placenta in terms of caterpillars biocontrol agent. It is worth noting that awareness on using some species as biocontrol agent needs more concern/carefulness since the species which once used as a biological control agent could become an invader (Tayeh et al., 2015) if occurrence/distribution is not in the right place and right time. So in the early step of experiment, it is important to avoid and limit the use of Aschersonia only in the greenhouse as suggested in Evans & Hywel-Jones (1990).

The evaluation on effect of various plant extracts on growth of A. placenta showed significant inhibitory effect ($p < 0.05$) against fungal growth. Plant extract from stem of T. crispa was the most efficient candidate against the Aschersonia growth either adding to the media before or after sterilization. These can be concluded that antifungal property of active ingredients in the extract is heat resistant, and vice versa for the extracts of A. galanga rhizome and Z. officinale rhizome which were only inhibited the growth of Aschersonia where the plant extracts were aseptically added into the media after sterilization. In the case of T. crispa, extracts from this plant has been reported to actively against the growth of several bacteria and some fungi (mainly Candida albicans; Warsinah et al., 2015, Islam et al., 2014; Aspergillus niger and Saccharomyces cerevacae; Islam et al., 2014). Phytochemical analyses of T. crispa revealed the presence of several compound i.e. alkaloids, flavonoids, and flavone glycosides, triterpenes, diterpenes and diterpene glycosides, cis clerodane-type furanoditerpenoids, lactones, sterols, lignans, and nucleosides (Ahmad et al., 2016), further studies is needed to clarify that which compound is acted as the active

ingredient against the growth of A. placenta in this study. In the case of A. galanga similarly to T. crispa, there were several studies mentioned about the activity of crude extract against pathogenic fungi (Phongpaichit et al., 2005; Chudiwal et al., 2010; Avasthi et al., 2015). From the result of Avasthi et al. (2015), the presence of flavonoids and cardiac glycosides is obtained in qualitative phytochemical analysis of the methanolic extract of the A. galanga. Not only those two compounds mentioned but steroids, triterpenoides and alkaloids were also detected from methanol extract of A. galanga. Z. officinale is one of the most medicinal plants used in indigenous medicine. It's essential oils comprises of several active compounds (e.g. α-zingiberene, citral, β-phellandrene). Yamamoto-Ribeiro et al. (2013) evaluated the effect of Z. officinale essential oil on Fusarium verticillioides growth and found that the essential oil could control the presence of F. verticillioides. The ethanolic extract of ginger powder has also showed inhibitory activities against Candida albicans in the study of Supreetha et al. (2011). Further studies are needed to clarify that which compound in extracts of A. galanga and Z. officinale is acted as the active ingredient against the growth of A. placenta in this study. In the case of A. viridis, this plant extracts have been reported to have antifungal property against several fungi (Carminate et al., 2012; Colletotrichum musae and Fusarium solani f. sp. piperis, Ahmed et al., 2013; Fusarium solani and Rhizopus oligosporus) but in this study just provided only little inhibition against Aschersonia placenta. Variation on the inhibition result might be caused by difference in extraction solvent, plant parts and species of test organisms and so on.

Conclusions

In conclusion, the fungus found on leaves of mulberry (Morus alba) is morphologically similar to A. placenta however since this species is now a complex species in Thailand so identification based on molecular data comparing with the sequence of type species is needed. The best way for farmer to avoid the occurrence of this fungus in similar occasion that may occur in the future is try to protect the mulberry from the whitefly attack. However, if the outbreak of the fungus already occurred in the field then just avoids choosing the infected leaf for silkworm rearing. In terms of biological agent selection, we could try to develop this fungus for controlling other caterpillars in vegetable field e.g. cabbage looper or diamondback moth but some intensive investigation in further studies is needed.

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