

## Leaf endophytic fungi of chili (*Capsicum annuum*) and their role in the protection against *Aphis gossypii* (Homoptera: Aphididae)

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### ABSTRACT

Hernawati H, Wiyono S, Santoso S (2011) Leaf endophytic fungi of chili (*Capsicum annuum*) and their role in the protection against *Aphis gossypii* (Homoptera: Aphididae). *Biodiversitas* 12: 187-191. The objectives of the research were to study the diversity of leaf endophytic fungi of chili, and investigate its potency in protecting host plants against *Aphis gossypii* Glov. Endophytic fungi were isolated from chili leaves with two categories: aphid infested plants and aphid-free plants, collected from farmer's field in Bogor, West Java. Abundance of each fungal species from leave samples was determined by calculating frequency of isolation. The isolated fungi were tested on population growth of *A. gossypii*. The fungal isolates showed suppressing effect in population growth test, was further tested on biology attributes i.e. life cycle, fecundity and body length. Five species of leaf endophytic fungi of chili were found i.e. *Aspergillus flavus*, *Nigrospora* sp., *Coniothyrium* sp., and SH1 (sterile hypha 1), SH2 (sterile hypha 2). Even though the number of endophytic fungi species in aphid-free and aphid-infested plant was same, the abundance of each species was different. *Nigrospora* sp., sterile hyphae 1 and sterile hyphae 2 was more abundant in aphid-free plants, but there was no difference in dominance of *Aspergillus flavus* and *Coniothyrium* sp. *Nigrospora* sp., SH1 and SH2 treatment reduced significantly fecundity of *A. gossypii*. Only SH2 treatment significantly prolonged life cycle and suppress body length, therefore the fungus had the strongest suppressing effect on population growth among fungi tested. The abundance and dominance of endophytic fungal species has relation with the infestation of *A. gossypii* in the field.

**Key words:** leaf endophytic fungi, chili, biological control, resistance, *Aphis gossypii*.

### INTRODUCTION

Endophytic fungi are fungi colonize internally plant tissue, without giving detrimental effect to the host plant (Petrini 1992; Avezedo 2000). They act as symbiont, mediated plant resistance against biotic stress i.e. pests and diseases and abiotic stress such as drought and extreme of temperature. The previous research in temperate region showed that endophytic fungi have detrimental effect on some insects from various taxonomic groups. For instance, endophytic fungi on grasses have been reported to inhibit the growth and development of the feeding insects. The colonization of an endophytic fungus *Acremonium coenophialum* Morgan-Jones et. Gams in tall fescue (*Festuca arundinacea* Schreb.) deterred the feeding of *Rhopalosiphum padi* Rondani and *Schizaphis graminum* Rondani (Johnson et al. 1985). In addition, Sabzalian et al. (2004) reported the significant inhibition of population growth of mealybug *Phenacoccus solani* Ferris and barley aphid, *Sipha maydis* Passerini, on fungal endophyte-infected tall and meadow fescues. Moreover, the larval growth of *Popillia japonica* beetle larvae also inhibited in infected *Taraxacum laxum* by an endophyte *Neotyphodium* sp. (Richmond et al. 2004).

However up to now, study on this field is conducted mostly in grasses and in some more recent research works,

are on trees. The research on dicotyl-annual plant such as chili, is not available. System chili-*Aphis gossypii* Glov. was chosen due to the importance of chili as main vegetable crops in Indonesia and *A. gossypii* is a vector of various viral diseases. The objectives of the research were to study the diversity of leaf endophytic fungi of chili, and to examine their effect on the population growth and some biological aspects of *Aphis gossypii*.

### MATERIALS AND METHODS

#### Location and time

The research was carried out in Laboratory of Plant Mycology and Laboratory of Insect Ecology, Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University on April-October 2007.

#### Isolation, identification and quantification of leaves fungal endophyte

Isolation leaf fungal-endophytes of was carried out by modified technique of Petrini (1992). Sample of chili leaves without necrotic symptom was obtained from two category i.e. aphids-free plant, and plant with aphids, each 40 samples, originated from farmers field in Cibungbulang, Bogor, West Java, Indonesia (ca. 150 m asl). The leaves

were disinfected two times with 70% ethanol and 1% sodium hypochloride, each for three minutes, then rinsed by sterilized water and excessive water tapped by towel paper and plated on medium potato dextrose agar (PDA) pH 5.5. Endophytic fungi were then purified by reculturing on PDA. After colony age of one week, the isolated fungi was purified and collected. The sporulated fungal isolates were directly identified. Non-sporulating fungal-isolates were induced the sporulation by growing in S-medium (CaCO<sub>3</sub>, sucrose 10 g/L, aquadest 1000 ml) (Hanada et al. 2010), and incubated under near ultra violet (NUV) for 14 days. Identification was conducted up to genus level using identification books of Barnett and Hunter (1988) and Hanlinn (1990).

Non sporulating endophytic fungi *i.e.* SH 1 and SH2 were molecular identified based on 18 S rDNA. Extraction of DNA was conducted based on methods of modified Orozco-Castillo et al. (1994). Amplification of fungal DNA using pair of primer ITS1 5' TCCGTAGGTGAACCTGCGG 3' and ITS4 5' TCCTCCGCTTATTGATATGC 3' that amplify region *internal transcribed spacer* (ITS) ribosomal DNA (rDNA) (White et al. 1990). DNA resulted from PCR then sequenced and examined the homology with reference collections of Genbank using BLAST program ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Species or genus was determined based on percentage of similarity (Arnold and Lutzoni 2007; Crozier et al. 2006).

Abundance of leaf endophytic fungi was depicted by frequency of isolation, in which calculated by the percentage of samples with certain endophyte. The frequency of isolation then compared between aphid free plants and plant with aphids. The collected endophytic fungi were stored on test tube containing PDA and store at 5°C. Propagation was carried out by reculturing this isolate on PDA. The 14-days old colony of endophyte was used for inoculation.

### Rearing of aphids

An adult of *A. gossypii* from the chili plant in the field in Bogor was kept on free insect potted chili plant. After species determined using identification book Blackman and Eastop (2000), the progeny was reared on chili plant to obtain homogenous population. First nymph of the population was then used for experiment of biology and also population growth.

### Inoculation of endophytic fungi

Suspension of conidia was used as inoculum for sporulating fungi, and mycelial fragment was applied for non-sporulating fungi. Conidia of fungi were harvested from 14-days old culture. A PDB-based 14-days old colony of non-sporulating fungi, filtered, washed with sterilized water then mixed with sterilized water and blended with medium speed for two minutes. Both are assessed the density by direct count with a haemocytometer under light microscope with 10 x 10 magnification. Both types of suspension were adjusted to 10<sup>4</sup> cfu/mL. Inoculation was done twice, first by seed treatment, second by propagules spraying. Before treatment the seed was treated with hot water at 52°C for 20 minutes to eliminate possible existing fungi on and inside the seeds. Seeds of chili *cv. Hot pepper*

was soaked by conidia suspension for 6 hours, then grown in sterilized soil in pot. Conidial spraying was conducted at 10 days after transplanting, aided by hand sprayer with application volume of 50 mL/individual plants. For control, seeds was only soaked and then the plants sprayed by sterilized water.

### Endophyte colonization study

The aim of this test was to investigate whether the isolated fungi are able to colonize leaves of chili. Endophyte treatment was carried out by seeds application and spraying plants leaves at 10 days after transplanting, each treatment consisted of ten plants. Leaves of each plant were plated on PDA pH 5.5 at 20 days after transplanting. The growth of the fungi the same as inoculated in media indicating that the tested fungi are able to colonize the leaves.

### The effect of leaf-endophytic fungi on the population growth of *A. gossypii*

Two first nymph of *A. gossypii* were inoculated on chili potted plant. The plant was grown in a cheesecloth cages to avoid migration and attack of natural enemies and laid under greenhouse. Five plants as replication were used in this study. Treatment consists of endophytic fungi *i.e. Aspergillus flavus*, *Nigrospora* sp., *Coniothyrium* sp., sterile hypha 1 (SH1) and sterile hypha 2 (SH2), control (water). One plant was considered as one replication. The observation was done each 3 days with aided by hand counter for 30 days.

### The effect of fungal endophyte on the biology of *A. gossypii*

Treatment consisted of endophytic fungus *i.e. Aspergillus flavus*, *Nigrospora* sp., *Coniothyrium* sp., sterile hypha 1 and sterile hypha 2, and control (water). The detached leaves of endophyte inoculated plants and control plants were laid on petridish diameter 9 cm and the basal of petiole was covered by moistened cotton. A first nymph of *A. gossypii* was laid on leaf, and each 3 days the leaf was replaced by the new and similar size from the same plants. The observation was made on the periods of each nymph, pre-natal periods, life cycle, and fecundity. In addition, body length was also measured microscopically using micrometer. If the insect produce progeny then its progenies was killed. The 20 petridishes were used; one petridish was considered one replication. The experiment was designed in randomized complete design.

### The effect of leaf-endophytic fungi on body size of *A. gossypii*

The aphid treatment was same as in biology experiment. Each instars of aphid's nymph was measured longitudinally using micrometer, under a compound microscope with 40 x 10 magnifications.

### Data analysis

Frequency of isolation of endophytic fungi was arranged in cross tabulation, and compared the value for assessing abundance of each fungus. Variables such as life cycle, fecundity and body length were statistically analyzed using analysis of variance (ANOVA). When ANOVA resulted significant different, Duncan Multiple Range Test (DMRT) was applied for comparing mean of each variables.

## RESULTS AND DISCUSSION

Based on the sample number used in the research (40 plants from the field of Bogor), the species diversity of fungal endophyte was low. Only five species found *i.e.* *Aspergillus flavus*, *Nigrospora* sp., *Coniothyrium* sp. and sterile hypha 1 (SH1), and sterile hypha 2 (SH2) (Table 1). SH1 and SH2 did not produce conidia to allow further species identification morphologically. Further molecular identification based on 18 S rDNA resulted that SH1 similar (99%) to Accession FJ524323 of GeneBank refer to Unculture endophytic fungus clone R3-63, obtained from wild rice root in China (Yuan et al. 2010) (Table 2). SH2 was similar to Accession No FJ612897 of GeneBank, Fungal sp ARIZ B031, endophytic fungus of tree *Cecropia insignis* (U'Ren et al. 2009). The rank of species from the most abundant to the least was *Nigrospora*, SH2, SH1, *Coniothyrium* sp. and *A. flavus* respectively. Low species diversity of chili plants may be related to high rate of fungicide frequency application in this area (once per week). Gaitan et al. (2005) noted that fungicide application reduced the diversity of endophytic leaf fungi of a tropical tree *Guarea guidonia* L.

**Table 1.** Frequency of isolation of leaf endophytic fungi on chili from bogor

Endophytic fungi	Isolation frequency (%)	
	Aphid-free plant	Plant with aphid
<i>Aspergillus flavus</i>	10	10
<i>Nigrospora</i> sp.	30	15
<i>Coniothyrium</i> sp.	25	20
SH1	55	25
SH2	60	15

Note: number of leaves with aphid and aphid-free, each 40 from 40 plants.

**Table 2.** Molecular identification of non-sporulating endophytic fungi

Isolate number	Category	GeneBank reference accession	Maximum identity (%)
SH1	Unculture endophytic fungus clone R3-63	FJ524323	99
SH2	Fungal species ARIZ B031	FJ612897	99

Even though there was no difference on the species number of fungi between aphid-infested and aphid-free plants, the abundance of each fungus was greatly different. Abundance (indicated by frequency of isolation) of *Nigrospora* sp., SH 1 and SH2 was higher in aphid-free plants than of aphid-infested plants (Table 1). Other endophytic fungi: *Aspergillus flavus*, *Coniothyrium* sp. has no different abundance between aphid-infested and aphid-free plants.

All isolated fungi can act as endophytes proven by their colonization ability-lowest frequency was *A. flavus* and

other fungi reached more than 80% frequency of reisolation (Table 3). All of isolated fungi have no potency to be pathogens, indicated by negative result of pathogenicity tests (data not shown). *Aspergillus* has rarely been reported as leaf endophyte, but this work resulted that this species as leaf endophyte of chili and proven by colonization test. The role of *Aspergillus* as leave endophyte has been reported in soybean and neem trees (Pimentel et al. 2006; Verma et al. 2007). Other fungal endophytes isolated in this study were *Coniothyrium* sp. and *Nigrospora* sp., the two genera had been reported as leave endophyte in various plants such as *Quercus alba* L, neem tree, banana tree *Ulmus davidiana* var. *japonica* and *Parthenium hysterophorus* (Fisher et al. 1994; Romero et al. 2001; Tomita et al. 2003; Photita et al. 2004; Verma et al. 2007). The presence of sterile hypha as endophyte in this research is also common in other endophyte research on various host plants (Fisher et al. 1994; Pimentel et al. 2006; Verma et al. 2007).

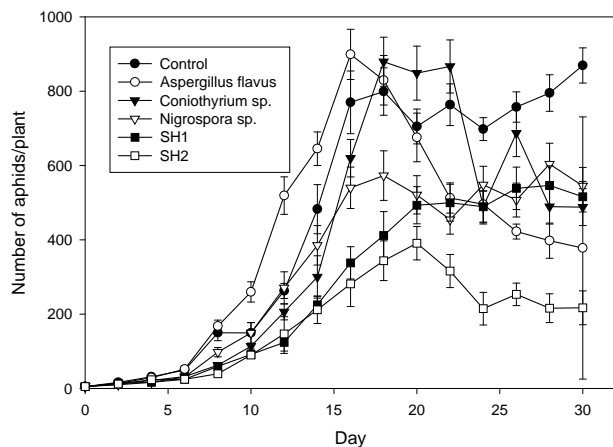
**Table 3.** Frequency of reisolation of leaf endophytic fungi on chili

Endophytic fungi	Frequency of reisolation
Control	0
<i>Aspergillus flavus</i> .	70
<i>Coniothyrium</i> sp.	80
<i>Nigrospora</i> sp.	90
SH1	80
SH2	80

Further test showed that *Nigrospora* sp., SH1 and SH2 reduce population growth of *A. gossypii*, with SH2 provide highest suppression (Figure 1). This was indicated by delaying peak of population growth curve and reducing population density by these fungi treatments. Untreated or control had peak of population growth at 18 days. Population growth curve reached a peak at 18, 20 and 20 days for *Nigrospora* sp. SH1 and SH2 respectively. *Nigrospora* sp. SH1 and SH2 suppressed population density at average rate of 29.05%, 40.36% and 54.37% respectively.. Population growth curve reach a peak at 16 and 18 days and suppressing rate of 0.00% and 19.23% for *Aspergillus flavus* and *Coniothyrium* sp. respectively. It can be said that *Aspergillus* and *Coniothyrium* sp. has minor effect on population growth of *A. gossypii*, consequently those fungi were not further used in life cycle and fecundity test.

In life cycle test, only SH2 showed the effect *i.e.* prolonging life cycle by 10.77%. The endophyte SH2 treatment prolonged significantly nymph periods, pre-oviposition periods and life cycle of *A. gossypii* (Table 4). Other tested endophytic fungi did not affect these parameters.

All of tested fungi reduced significantly the fecundity of *A. gossypii* (Table 5). The reduction of fecundity was 41.36%, 49.32%, and 53.11% for SH1, SH2 and *Nigrospora* sp. respectively. Aside from suppressing fecundity and prolonged life cycle, SH2 endophyte reduced *A. gossypii* body length. Other tested fungal endophyte, even though tended to reduce this parameter too, but not significant. Again, SH2 endophyte showed the strongest inhibitory effect on *A. gossypii*.



**Figure 1.** Population growth of *A. gossypii* on endophyte-infected chili plants.

**Table 4.** Life cycle of *A. gossypii* on endophyte-treated leaves

Treatment	Nymph periods	Pre-oviposition periods (days)	Life cycle (days)
Control	5.25±0.13 b	1.25±0.46 a	6.45±0.31 b
<i>Nigrospora</i> sp.	5.13±0.05 b	1.35±0.34 a	6.45±0.43 b
SH1	5.32±0.12 b	1.25±0.57 a	6.55±0.27 b
SH2	5.85±0.16 a	1.35±0.63 a	7.20±0.19 a

Note: number followed by different symbol in the same column is significantly different according DMRT test with  $P < 0.05$

**Table 5.** Fecundity of *A. gossypii* on endophyte-treated leaves

Treatment	Total
Control	29.62±3.58 a
<i>Nigrospora</i> sp.	13.89±5.84 b
SH1	17.37±3.88 b
SH2	15.31±4.65 b

Note: number followed by different symbol in the same column is significantly different according DMRT test with  $P < 0.05$

By comparing exploratory data and experimental data, it can be said that there is relation between the abundance of endophytic fungi and anti insect activity of fungi. Fungi having no different abundance between aphid-free and aphid-infested, such as *Aspergillus flavus* and *Coniothyrium* sp., have minor effect on the suppression of aphid population. On the contrary endophytic fungi with high dominance in aphid-free plant, have significant suppressing effect on aphid population, and inhibit some other aphid biological attributes *i.e.* fecundity, life cycle, body size, even though the inhibitory effect varied among species. Thus, the facts show that colonization of later groups of

endophytic fungi play important role on the protection of chili plant against aphid in the field.

Previous worker reported that some endophytic fungi has mediated plant resistance on phytophagous insects from various taxa *i.e.*, aphid, grasshopper, cotton ballworm and beetle (Johnson et al. 1985; McGee et al. 2003; Richmond et al. 2004; Sabzalian et al. 2004; Avezedo 2000). However, most of research was done with grasses in temperate region. Our finding show for the first time in cultivated annual crops *i.e.* chili that endophytic fungi is able to suppress the growth, development and population growth of *A. gossypii*. One isolate SH2, beside prolonged life cycle, also decreased fecundity, therefore had strongest effect on decreasing population growth of *A. gossypii*. Other tested endophytic fungi (SH1, SH2 and *Nigrospora* sp.) decreased fecundity but had no effect on life cycle. The other important point was some endophytic fungi *i.e.* *Nigrospora* sp. and SH2, suppressed the body length of aphids (Table 6). The reduction of body size of aphids due to endophyte treatment was also reported on aphid *Rhopalosiphum padi* on ryegrass inoculated by endophyte *Neotyphodium lolii* (Meister et al. 2006). Thus, the fungi affected not only the development but also growth of *A. gossypii*.

The experiment showed obviously that some fungal leaf endophyte treatments play a role in protecting chili against *A. gossypii*. It is known that non preference and antixenosis are main mechanism in increasing host resistance against insects mediated by fungal endophyte (Johnson et al. 1985; Faeth et al. 2002; Lehtonen et al. 2005). Antixenosis is proven in this research showed by suppression of fecundity, prolonged life cycle and decreased body size. Non-preference was not elaborated in this study, therefore needs further investigation.

Inhibitory effect of endophyte on feeding insects is mostly due to toxin produced by fungal endophyte. Endophytic fungi alone or in association with host plant are able to produce toxin (Petrini 1992; Sumarah and Miller 2009). Highly diverse groups of toxin produced by fungal endophyte *i.e.* alkaloids, terpenoid, steroid, quinone, and flavonoid, phenylpropanoids and lignans, peptides, phenol, phenolic acids and aliphatic compounds (Tan and Zou 2001). Siegel et al. (1990) stated that toxin produced in grasses infected by endophyte *Acremonium coenophialum* and *Epichloe typhina* is peramine, lolitrem B and ergovaline. Moreover reported that endophytic fungus *Phyllosticta* sp. and *Hormonema dematioides* in balsam fir

**Table 6.** Effect of endophyte-treated leaves on body length of *A. gossypii*

Treatment	Body length (mm)				
	Nymph-instar				Adults
	1	2	3	4	
Control	0.42±0.15 a	0.58±0.25 a	0.73±0.16 a	0.94±0.09 a	1.17±0.06 a
SH1	0.42±0.24 a	0.58±0.22 a	0.76±0.18 a	0.91±0.073 a	1.12±0.04 ab
SH2	0.41±0.22 a	0.57±0.23 a	0.71±0.11a	0.89±0.15 a	1.08±0.03 b
<i>Nigrospora</i> sp.	0.41±0.19 a	0.61±0.20 a	0.75±0.24 a	0.89±0.11 a	1.12±0.03 ab

Note: number followed by different symbol in the same column is significantly different according DMRT test with  $P < 0.05$

produce toxic compounds, mainly heptelidic acid and rugulosine (Avezedo 2000; Sumarah et al. 2008). *Nodulisporic acid*, benzofuran derivatives and naphthalene are also insecticidal substances produced by endophytic fungi (Sumarah and Miller 2009). Possible mechanism other than toxin production is the change of plant metabolism such as sterol metabolism which not favors the insects (Avezedo 2000). The exact mechanism and the type of toxin associated with the increasing chili resistance against *A. gossypii* mediated by fungal endophyte need further investigation.

## CONCLUSION

Endophytic fungi isolated from chili in Bogor are *Aspergillus favus*, *Coniothyrium* sp., *Nigrospora* sp., sterile hypha 1 (SH1) and sterile hypha 2 (SH2). Colonization of some endophytic fungi has important role in the protection of chili plants against *Aphis gossypii*. Some of those fungi i.e. SH1, SH2, and *Nigrospora* sp. are able to increase resistance chili against *A. gossypii*, in which SH2 has the strongest effect. This plays an initial basis for using fungal leaf endophytes as biocontrol agent against pests of chili. Better understanding on the aspects related to endophytic fungi of chili leaves, such as mechanism involve, type of produced toxin, mode of transmission, spectrum of affected insect pests, host-environment relation, should be furthermore elaborated to obtain appropriate strategy and technique for the use in biological control.

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