The use of endophyte fungal isolates in controlling *Fusarium oxysporum*, the causal agent of wilt disease on chilli (*Capsicum annum*)

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Abstract. *Fusarium oxysporum* as the causal agent of wilt disease infected systemically on the chili plant (*Capsicum annum*) and cause a significant loss on its production. To control the pathogenic fungi, we isolated and identified the beneficial fungi from stem, leaf, and fruit tissues of healthy plants. Then, the work tested fungi capability to inhibit the pathogen in vitro and to enter plant tissues. The results of the work indicated that seven fungi isolates consisting of *Trichoderma* 1, *Trichoderma* 2, *Aspergillus*, *Fusarium* 1, *Fusarium* 2, *Lasiodiplodia* 1, and *Lasiodiplodia* 2 were identified. *Trichoderma* and *Lasiodiplodia* were found from leaf and fruit tissues, whereas *Aspergillus* and *Fusarium* were isolated from leaf and stem tissues. The inhibition of *F. oxysporum* by these seven fungi isolates in vitro about nine days after dual culture was 53.9%, 62.5%, 38.9%, 38.3%, 26.9%, 29.4%, and 34.7%, respectively. Endophyte study with *Trichoderma* morphospecies 2 showed that the isolate could colonize 84% of the root, 60% of the stem, and 80% of leaf tissues three weeks after inoculation through roots. Therefore, the research results demonstrate the presence of endophytic fungi derived from the chili plant that is potential to control wilt disease in vivo.

1. Introduction

Chilli (*Capsicum annum* L.) is one of horticultural and spice crops belonging to the family Solanaceae. This commodity is widely used in many cuisines as a spice to add heat to dishes. In addition, chilies contain several important compounds, including capsaicin having a benefit for health and appetite enhancer [1]. However, fusarium wilt disease’ causes a significant loss of yield caused by *Fusarium oxysporum* [2]. This disease produces morphological appearance such as wilting, chlorosis, necrosis, leaves fall, stunting and seedling crumbling. When the infected one is observed internally, the vascular system of the plant turns brown. Wilting symptoms in plants started from the old leaves at the bottom then spread to the younger leaves above. Advanced symptoms have the potential to cause the death of chilli seeds [1, 3].

*F. oxysporum* has more than 120 specialist forms (f.sp) [4]. The pathogenic fungi lives in the soil around the infested plant and survives in the form of mycelia and chlamydospores. This pathogen can infect more than 100 plant species and cause the main problem on chilli in Thailand, India, China, and Indonesia [5]. *F. oxysporum* belongs to the family: *Nectriaceae*, Genus: *Fusarium*, Species: *F. oxysporum* [6]. The fungus produces macroconidia, microconidia and chlamydospores. Macroconidia has a length of 25 - 35 µ and width of 3 - 5 µ. The macroconidia curve is like a canoe separated by 3-5
septa in thin walls and pointed tips. Microconidia are abundant with a length of 5 - 12 µ and a width 3 - 5 µ, elliptical in shape, appearing singly or in aggregates. Chlamydomospores or resting spores are also formed and abundant in the soil [1, 7]. Due to the fact that fungi can survive in the soil for several years, the disease is difficult to be controlled [8]. *Fusarium* sp. is a weak pathogen [9].

Normally, the control of the wilt disease in the farmers' field, generally uses synthetic fungicides. However, this chemical compound is hazardous for human health and the ecosystem [1, 3, 8]. Therefore, it needs more effort to solve this problem and apply an environmentally friendly alternative to control the field's organism. The one safety alternative as biological control of *F. oxysporum* is by using endophytic fungi. These endophytes colonize healthy plant tissues during at least part of their life cycle, without producing disease symptoms on the plant, and often develop a symbiotic relationship with the host [10]. Number of endophytes have the potential to be used as biological control agents [11]. They are capable of suppressing pathogen growth directly through parasitic mechanisms, antibiosis, and nutrition competition. Also, they can induce plant resistance mechanisms [12]. Based on the description, it is necessary to explore the endophytic fungi use to suppress the infestation of *F. oxysporum*, the causal agent of wilt disease on chili plants.

2. Materials and methods

2.1. The isolation and identification of endophytic fungi

Endophytic fungi isolation came from healthy chili plants grown at the Teaching Farm of Hasanuddin University, Makassar, South Sulawesi. The part of chili plants (root, stem and leaves) used running water, then cut into small pieces with a diameter of 0.5 cm. The surface of the plant part was gradually sterilized using 70% ethanol for a minute. Roots and leaves were sterilized, 1% NaOCl and stems used NaOCl (3%) for two minutes, then 70% ethanol for 30 seconds, respectively. Furthermore, each part of the plant was rinsed with aquadest three times and dried. The sterilized cut parts of the chili plant were transferred to the culture media of Potato Dextrose Agar (PDA) in several petri dishes and incubated into room temperature at 28°C. The isolated plant part sterile test was carried out by scraping the plant parts from the last rinse into culture media PDA. The results of endophytic fungi isolation cannot be used if contaminant microbes are found on the sterile test culture media. The mycelium that grows on the pieces of plant tissue is observed every day. Furthermore, the mycelium was transferred to the new culture media of PDA [13]. The growing endophytic fungi were observed macroscopically and microscopically using a digital microscope then identified using a classification literature [14].

2.2. The isolation and identification of pathogenic fungi

Isolation of pathogenic fungi originating from chili plants with symptoms of *F. oxysporum* attacks at the Teaching Farm, Hasanuddin University, Makassar, South Sulawesi. The plant part of the sample was cut into small pieces with a diameter of 1 cm. The sample pieces then sterilized in stages used 70% ethanol for a minute, then rinsed with 2.5% NaOCl solution for five minutes, rinsed again used steril aquadest three times and dried for a few minutes. After the sterilized process, the plant part was put in a petri dish containing culture media PDA and incubated at room temperature 28°C for three days. Culture of the pathogenic fungi *F. oxysporum* was purified by transferring it to new culture media of PDA [15]. The growing fungi pathogens were observed macroscopically and microscopically using a digital microscope then identified by comparing the pathogenic fungi *Fusarium* sp. morphology used literature [16].

2.3. The ability test of endophytic fungi

Endophytic fungi that have the highest value of PIGR (Percentage Inhibition of Radial Growth) were tested for their ability to colonize young plants. A forty of chili plants were planted in polybags containing about 250 g of soil. Endophytic fungi were taken as much as 1 x 10⁶ per ml of spores then inoculated through the soil into each of the ten chili plants. The presence of endophytic fungi in the chili
plant tissue was tested by taking root, leaf and stem tissue samples from five sample plants three weeks after inoculation. Roots, leaves and stems were cut along 1 cm, then sterilized each part of the plant for two minutes at 2% NaOCl, 70% ethanol for 1 minute, washed clean used sterile aquadest three times before placing it into a petri dish containing PDA. The five samples in the petri dish were filled with plant tissue, then incubated at room temperature. Observations were made on the presence of endophytic fungi and identified using the literature [14]. Endophytic fungi colonization was calculated using formula [17]:

\[ C = \frac{P}{5} \times 100\% \]

\( C \) = colonization
\( P \) = the number of parts or pieces that indicate the occurrence of colonization originating from endophytic fungi.

2.4. Pathogenicity test
The pathogenicity test of the isolates was carried out using the Koch Postulate test. This activity consists of inoculation of pathogenic fungi isolates on chili plants, re-isolating plant tissue that showed symptoms, and identified results of re-isolation. The sample used for inoculation was a 14 day old chili plant seed from the nursery. Each fungi isolate was inoculated on five chili seeds. Inoculation was performed using the plant root dipping method [18]. The chilli seeds were dipped in a conidia suspension of \( F. \) oxysporum with a concentration of \( 10^6 \) conidia/ml for 30 minutes. The inoculated chili seeds are then planted into a pot filled with sterilized soil. As a control, the chili seeds were dipped in aquadest for 30 minutes. Observation and evaluation of \( F. \) oxysporum every day until symptoms develop.

2.5. Dual culture test
The interaction between endophytic fungi and \( F. \) oxysporum was observed by placing endophytic and pathogenic fungi into one petri dish. Both types of fungi were grown side by side at a distance of about 3 cm in one place. The petri dishes were incubated at room temperature at 27°C then colony diameter was measured for seven days. Control was made with the same steps, but only \( F. \) oxysporum were grown. Each treatment was repeated four times. The data from observations were processed using one-way Anova SPSS 16 statistical analysis. The effect of endophytic fungi antagonism was calculated using PIGR (Percentage Inhibition of Radial Growth).

3. Results and discussion

3.1. The isolation and identification of endophytic fungi
Seven endophytic fungi were isolated from stem, leaf and fruit tissues of healthy chilli. The identification using digital microscope and references in the literature showed the fungus of \( Lasiodiplodia \) sp. (isolate 1 and 7), \( Trichoderma \) sp. (isolate 2 and 5), \( Aspergillus \) sp. (isolate 3) and \( Fusarium \) sp. (isolate 4 and 6) (table 1).
Table 1. The endophytic fungi characteristic from healthy chilli.

| Isolate/source | Upper surface | Lower surface | Color | Septum | Conidia/spore | Genus         |
|----------------|---------------|---------------|-------|--------|---------------|---------------|
| 1/1<sup>st</sup> chilli | Greyish white until blackish grey, having smooth surface | Greyish white until blackish grey | Hyaline | Septum | Elongated like a thread | Lasiodiplodia sp |
| 2/2<sup>nd</sup> chilli | Old green, white in the upper outer surface, solid and soft textures. | White yellowish | Hyaline | Septum | Round | Trichoderma sp. |
| 3/3<sup>rd</sup> leaf | Old green with white outer edge, soft and smooth textures. | Yellow brownish | Hyaline | Septum | Round | Aspergillus, sp |
| 4/2<sup>nd</sup> stem | White, similar color with outer edge, soft and smooth textures | White and Center has Yellow color | Hyaline | Septum | Ellips | Fusarium, sp |
| 5/2<sup>nd</sup> stem | Old green, white in the Outer surface, solid and soft texture | White Yellowish | Hyaline | Septum | Round | Trichoderma, sp |
| 6/1<sup>st</sup> stem | White similar the outer Surface, smooth texture | White and center has a yellow color | Hyaline | Septum | Elips | Fusarium, sp |
| 7/3<sup>rd</sup> leaf | Greyish white Until blackish grey Having smooth surface | Greyish white Until blackish grey | Hyaline | Septum | Elongated like a thread | Lasiodiplodia, sp |

Trichoderma was found to colonize leaf and fruit tissues and Aspergillus and Fusarium were in leaf and stem tissues. Lasiodiplodia sp. and another Trichoderma sp. were altogether within leaf and fruit tissues. The presence of beneficial fungi in roots play an essential role in increasing chilli plant health [19, 20, 21]. At the same time, the occurrence of endophytic fungi in leaf increases forestry and cacao plants against Melampsora rust and Phytophthora. Trichoderma in shallot tissues can reduce the incidence by basal bulb rot disease by F. oxysporum f.sp. lycopersici. Studies on Juniperus recurve indicate endophytic Fusarium produce toxins that can prevent viruses cells from dividing and multiplying. Lasiodiplodia theobromae (Dothideomycetes, Botryosphaeriaceae) has the ability to colonize many plants as both an endophyte and a pathogen [22].

3.2. Isolation of pathogenic fungi

Fusarium wilt disease of chili has symptoms of wilting, chlorosis, necrosis, leaf fall, brown discoloration in the vessel system and stunting. In general, wilting in chili seeds started from the old leaves at the bottom then spreads to the younger leaves above. Colony growth of F. oxysporum on PDA media showed the smooth texture of fungi in the above, then the upper surface is white and the bottom is pale to purplish blue. The shape is elliptical and conidia has 4 – 5 septa (figure 1).
Figure 1. The colony and conidia from *F. oxysporum*.

The pathogenic fungi *F. oxysporum* has many hosts from agriculture plants. Findings of [23] state that the infection started from chlamydospores that survive surrounding at host remains in the soil. This is an important part of the infection process of pathogenic fungi to their host. The synthetic fungicide application commonly used by farmers to control the presence of pathogenic fungi. The methods used for chemical material are very dangerous for the environment, human and organism surrounding the field.

3.3. Pathogenicity test
The symptom of wilting of chilli was observed two weeks after inoculation by *F. oxysporum* (figure 2). At this time, the fungus can be reisolated from all sample chilli plants.

![Figure 2](image)

**Figure 2.** The symptom of *F. oxysporum* caused wilt disease in chili (A= sample 1; B= sample 2; C= sample 3; D= sample 4; E= sample 5; F= Control).

*F. oxysporum* was found in the stem section of plant sample A, C, and E sample. While in the leaves, the fungus was identified on sample plant D and E. According to [24, 25], *F. oxysporum* infects plants and causes diseases in the stem, leaves and fruit of their host. The degree of pathogenicity depends on the ability of a pathogen to survive, environment, and availability of nutrition. Plants with sufficient nutrition can resist pathogens [26, 27].

3.4. Dual culture test
Observation of *F. oxysporum* inhibition (PIGR) by endophytic fungi was carried out nine days after dual culture in PDA medium. The highest inhibition was offered by *Trichoderma* derived from leaves and from fruit that was 62.5% and 53.9%, respectively. In comparison, the inhibition by *Aspergillus, Fusarium*, and *Lasiodiplodia* was 38.9%, 26.9%, and 29.4%, respectively. At nine days post-dual culture, the inhibition of *F. oxysporum* by the two isolates of *Trichoderma* was significantly different (*P* ≤ 0.05) with other endophytic fungi (figure 3). Based on these research results, all endophytic fungi can not entirely suppress the pathogenic fungus. It was likely due to rapid growth of *F. oxysporum*, so the endophytic fungi were unable to compete for space and nutrients.
Figure 3. Inhibition of *F. oxysporum* growth by endophytic fungi in culture PDA medium at three, six and nine days after application (HSA). Mean of inhibition followed by the same letter in the same column is not significantly different according to Tukey HSD (*p* ≤ 0.05).

*Trichoderma* live as saprophyte around plant roots, then spread to another part of the plant such as stem, leaves, roots, fruits and buds [28]. *Trichoderma* coils and lyses the hyphal wall of fungal pathogens by producing enzymes such as protease, cellulase, chitinase and 1.3-β-glucanase [29, 30, 31, 32, 33, 34].

3.5. Endophytic test
In the endophytic test, *Trichoderma* was presented with the highest inhibition *in vitro* against *F. oxysporum*. This fungus was inoculated into chilli seedlings of 14 days old through soil drenching. We could then observe its colonization in root, stem, and leaf by 100%, 48%, 52% one week post-inoculation. Further, the colonization was 84%, 60%, and 75%, respectively three weeks post-inoculation. In control, *Trichoderma* can be detected in stem and leaf tissues one week after treatment, but its colonization was very low (figure 4). Therefore, The *Trichoderma* isolated from chilli leaf was endophyte, and its ability to move from soil to, root, stem, and leaves was relatively fast.

Figure 4. *Trichoderma* sp. colonization of roots, stems and leaves. A, root; B, stem; D, leaf; KA, root control; KB, stem control; KD, leaf control; M1, colonization after one week; M3, colonization after three week. Mean of colonization in the plant part followed by the same letter in the same column is not significantly different according to Tukey HSD (*p* ≤ 0.05).

Several studies indicate that with its colonization in plant tissues, *Trichoderma* can improve plant health and produce secondary metabolites such as peroxidase and chitinase can effectively control systemic pathogens [35, 36, 37]. Endophytic fungi are microorganisms that live in plant tissue without causing apparent symptoms of the disease. Their occurrence was abundant in tropical areas [9, 37, 38]. Therefore, the results showed the presence of endophytic fungi in chilli plants can control wilt disease.
in vivo. For the future, both *Trichoderma* and their natural product can be recommended for use to reduce chemical fungicide application.

### 4. Conclusions

We conclude that seven isolates of fungi from four genera, e.g. *Trichoderma*, *Fusarium*, *Aspergillus* and *Lasiodiplodia* were determined in chilli plant tissues. *Trichoderma* of leaf tissue origin presented the highest inhibition against *Fusarium oxysporum*, the causal agent of chilli wilt. This isolate is identified as an endophyte that can race from the soil into root, stem, and leaf tissues when it is drenched through the soil. Therefore, the fungus could potentially be applied for controlling *Fusarium* wilt disease in the field.

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