The potential of endophytic fungi as biodegradation of chlorpyrifos in shallots

R Fauriah¹, N Amin¹, I D Daud¹ and E S Harsanti²

¹Pest and Plant Diseases, Faculty of Agriculture, Hasanuddin University, Makassar, South Sulawesi 90245, Indonesia.
²Indonesian Agricultural Environment Research Institute, Pati, Central Java 59182, Indonesia.

E-mail: riafauriah@gmail.com

Abstract. Chlorpyrifos is one of the broad-spectrum organophosphate insecticides in controlling plant pests. They can be absorbed into agricultural products and in the long term can have negative effects on human health. The use of endophytic fungi for biodegradation of chlorpyrifos is one of the technologies to support food safety. Therefore, this study was aimed to determine the potential of the isolated endophytic fungi and identify it from shallot plantations as biodegradation of chlorpyrifos. The research is conducted in three stages; (1) isolation of endophytic fungi on shallot plantations in Bantaeng Regency, (2) macroscopic and microscopic identification, and (3) test of the degradation potential of chlorpyrifos by growing fungi on PDA medium containing chlorpyrifos with concentrations 600 ppm, 300 ppm, and 150 ppm. Furthermore, the results showed there are 47 fungal isolates from the roots, stems and tubers of shallots, and some were identified as Trichoderma sp. and Fusarium sp. Several isolates tested have the potential to degrade chlorpyrifos, where the percentage of growth inhibition below 50% is considered tolerant of chlorpyrifos. Considering the results, Trichoderma sp. and Fusarium sp. are potential degrading residual insecticide chlorpyrifos.

1. Introductions
Shallot is one of the horticultural commodities that become the focus of the Indonesian government today. Sulawesi is one of the provinces prioritized in the development of shallot production land with productivity up to 8.8 tons/ha in 2019. Various programs are carried out to increase the production of shallots including the provision of superior seeds that are disease free and high in production, facilities and infrastructure that accelerate plant cultivation, the use of organic fertilizers, and technology for pest and plant diseases control. This is inseparable from the many obstacles found in the land that have caused the decline in shallot production. One of the big problems is the pest attack on the plants. Although many pest controls have been found to be more environmentally friendly, the use of synthetic pesticides is still the main thing to control pests quickly and accurately. One of them is the use of insecticides to control pest attacks in plantations.

Chlorpyrifos is one of the active ingredients of broad-spectrum organophosphate insecticides often used to control plant pests. The data states that this pesticide class is the most widely used pesticide worldwide, with sales percentage reaching 36% [2]. Various pests can be controlled by using chlorpyrifos such as Myzus persicae, Conopomorpha cramerella, Plutella xylostella, Crocidolomia binotalis, Agrotis sp., and Heliothis armigera. Chlorpyrifos is generally considered safe for agriculture.
because of its relatively fast degradation process. This results in the high use of chlorpyrifos in crops every growing season. However, these residues are then absorbed by plants and into agricultural products such as shallot bulbs which are then consumed. This can lead to various health problems in the long run. Exposure to these insecticides can affect humans, animals and plants [3]. Chlorpyrifos can inhibit acetylcholinesterase activity which can cause respiratory, reproductive, nervous, liver, and kidney disorders [3, 4]. In addition, this insecticide can interfere with the growth-enhancing mechanism by inhibiting various enzymes and permeabilities which are important for the plant’s growth. The accumulation of pesticide residues in the soil also increases with continuous use at an increased dose. The use of organophosphate insecticides can reduce the population of microorganisms and reduce soil fertility [3].

Therefore, efforts to degrade the chlorpyrifos residue contained in soil and plants are needed. Some of the existing technologies are the use of activated charcoal, biochar, etc. The use of microorganisms such as fungi can also be an alternative to degrade pesticide residues in the soil. Pesticide degradation by utilizing microorganisms has advantages, i.e. highly efficient to use, easy to get, harmless to the environment, and has relatively low cost [5]. Previous studies have shown that several fungi such as Verticillium sp., Trichosporon spp., Verticillius sp., Cladosporium cladosporioides, Aspergillus sp., Penicillium sp., Eurotium sp., Emericella sp., and Aspergillus terreus are known to degrade chlorpyrifos [4]. So, the use of endophytic fungi (Aspergillus flavus, A. niger, Fusarium spp., Trichoderma spp., T. harzianum, Curvularia, Penicillium, Gilmaniella, and Beauveria bassiana) can be considered and have the potential to degrade pesticide residues that are safe for plants and can control pests and diseases [6, 7, 8]. This study shows the potential of endophytic fungi to degrade chlorpyrifos.

2. Methodology

2.1. Source of isolates
Endophytic fungi isolates were taken from shallot plantations in Loka, Bantaeng Regency. Bantaeng Regency is one of the locations for the development of shallot production in South Sulawesi. And Loka is one of the districts where shallots are planted.

Sampling was done by using purposive sample method. The selection of sub-locations was based on differences in the application of the active ingredient chlorpyrifos pesticide in shallot plantations. Based on the results of interviews with farmers, there is 1 planting location that uses chlorpyrifos insecticide with the trademark “Dursban” and there are 4 locations that do not use this insecticide. One sampling location point consists of 5 plant samples with a distance range of 25-50 meters. The taken plant samples came from healthy plants and had entered the generative phase. The samples were then put into sterile plastic bags and coded to be brought to the laboratory for processing within 24 hours [9, 10].

2.2. Endophytic fungi isolation
The obtained plant samples were washed once with running water and three times with distilled water that has been sterilized using autoclaving. Surface sterilization was carried out by first cutting the plant parts (roots, tubers, and leaves) from 0.5 to 1 cm long. Then, the samples were sequentially sterilized with 70% ethanol for 60 seconds, 1% sodium hypochlorite (NaOCl) for 60 seconds, and 70% ethanol for 30 seconds. Each sample was then rinsed with sterile distilled water 3 times. The sample was placed on sterilized filter paper to remove the remaining water in the sample. The sterilized samples were placed in Petri dishes containing Potato Dextrose Agar (PDA) media and added 50 μg/ml chloramphenicol. After that, the samples were incubated at 25 ± 2°C for 5-7 days until the mycelium emerged from the inoculated plant samples. As a control, the part of the plant to be inoculated was sterilized using autoclaving. If there’s no fungi grew on the control media, the sterilization was declared successful. Hyphae arising from the inoculated samples were purified by taking and transferring to a new PDA medium and incubated at 25 ± 2°C [11, 12].
2.3. **Endophytic fungi morphology identification**

After incubation, pure isolates were observed. Then, the macroscopic (mycelial color) and microscopic (conidia form) morphological identifications were carried out to determine the genus or species of the fungus [9]. Identification was carried out by comparing the results obtained from a microscope with a reference book [13, 14].

2.4. **Potential testing of fungi on chlorpyrifos**

Endophytic fungi selected in the previous stage were then tested on a commercial insecticide containing the active ingredient chlorpyrifos. Tests were carried out on solid media PDA [15]. On solid media, qualitative testing was carried out on Petri dishes containing media without pesticides as control and media with pesticides in three different concentrations: 150, 300, and 600 mg/L per plate (10 mL). The isolates in the previous pure culture were transferred to the testing medium and incubated for 7 days at room temperature (32-35°C). The growth of the isolates was observed by measuring the radius of the isolates on the media which was carried out at 3 days after incubation ( dai) and 7 dai. Each treatment was repeated 3 times. The tolerance of fungi to pesticides was determined by the size of the colonies that grew on the plates compared to controls [16, 17; with modifications to the media composition].

The growth observation data of the isolates were then processed, and the percentage of growth inhibition was calculated on media containing chlorpyrifos compared to the control. The percentage inhibition of mycelia growth of the fungus was calculated using the following equation [18, 19]:

\[
PIMG = \frac{(A - B)}{A} \times 100
\]

A is the average growth radius of the isolate in the control, and B is the average growth radius of the isolate on media with pesticides. Isolates showing an inhibitory value below 50% were considered tolerant of chlorpyrifos [19].

2.5. **Data analysis**

Statistical analysis of the results was performed using the apps Statistical Tool for Agricultural Research (STAR) version 2.0.1. One way ANOVA (Analysis of Variance) and continued with the DMRT test to determine significant differences between the results obtained in each experiment.

3. **Results and discussion**

3.1. **Endophytic fungi isolation and identification**

From the isolated shallot plants on the roots, leaves and tubers, 47 isolates of endophytic fungi were obtained from 5 different locations (table 1). Based on the results of interviews with farmers, Location 3 uses insecticides with active chlorpyrifos in the planting while Location 1, Location 2, Location 4, and Location 5 do not. Based on the parts of the plant, the most endophytic fungi (23 isolates) were obtained in the tuber part, while 17 isolates were on the leaves. At the root, there were less endophytic fungi (7 isolates). In terms of location and differences in the use of chlorpyrifos in the land, there was no significant difference between the presence of endophytes in the land using chlorpyrifos and in those that don’t, except at the second location.

Furthermore, the purified isolates were identified for their morphology. Based on the morphological identification carried out, 4 genera of fungi were detected. They are 2 *Trichoderma*, 5 *Aspergillus*, and 16 *Fusarium*. Meanwhile, 24 isolates were undetected (table 2).
### Table 1. Distribution of the presence of endophytic fungi at 5 shallot planting locations.

| Location | Roots | Tubes | Leaves | Total |
|----------|-------|-------|--------|-------|
| 1        | 0     | 5     | 0      |  5    |
| 2        | 5     | 9     | 6      | 20    |
| 3        | 1     | 3     | 5      |  9    |
| 4        | 1     | 4     | 2      |  7    |
| 5        | 0     | 2     | 4      |  6    |
| **Total**| **7** | **23**| **17** | **47**|

### Table 2. Description of endophytic fungi identified from the roots, leaves and tubers of shallots.

| No | Fungus genus | Colony color | Macroscopic | Microscopic | Total |
|----|--------------|--------------|-------------|-------------|-------|
| 1  | *Trichoderma*| Light to dark green | ![Macroscopic Image](image1.png) | ![Microscopic Image](image2.png) | 2     |
| 2  | *Aspergillus*| Light green | ![Macroscopic Image](image3.png) | ![Microscopic Image](image4.png) | 5     |
| 3  | *Fusarium*   | Pink to purple with a white border | ![Macroscopic Image](image5.png) | ![Microscopic Image](image6.png) | 16    |
| 4  | Undetected   | Yellow to light brown | ![Macroscopic Image](image7.png) | -          | 24    |

3.2. **Testing of fungal isolates on solid media**

Of the 47 isolates found, isolates from different genera and different plant parts were selected. A total of 24 isolates were then tested for their growth ability on PDA media that had been given chlorpyrifos at different concentrations. The results showed that almost all isolates showed values below 50% which indicated that these isolates were tolerant of chlorpyrifos treatment [19]. At 3 dai, there were 9 isolates showing a lower percentage value of inhibition than the control (figure 1a). Isolate 2D12 did not experience growth inhibition and grew better than the control at a concentration of 600 ppm, 300 ppm, and 150 ppm, with a value of -6.98%; -8.10%; and -35.00% respectively. At a concentration of 300 ppm, isolate 1B12 showed the lowest growth inhibition value of -16.26%. At a concentration of 150 ppm, isolates 2D12, 5B11, and 5D11 showed the lowest growth inhibition values (-35.00%; -29.27%; and -26.63%, respectively). The minus value on the percentage of growth inhibition showed
that the isolates in chlorpyrifos treatment were able to grow bigger than the control. Isolates 1B12 and 5B11 were detected as *Fusarium* sp. while 2D12 and 5D11 isolates were undetected. This indicates that *Fusarium* sp. can potentially degrade chlorpyrifos. Apart from being a pesticide degrading agent, previous studies have stated that *Fusarium solani* can degrade methane gas [20].

On the 7 dai observation, 14 isolates showed no growth inhibition (figure 1b). At 600 ppm treatment, isolates 2A13, 1B12, and 1B23 showed the lowest inhibition values (10.24%, 9.63%, and 8.60% respectively). At 300 ppm treatment, isolates 2B24, 4B21, and 3A11 showed the lowest growth inhibition values compared to the control (-21.90%, -20.88%, and -8.33% respectively). Whereas, at a concentration of 150 ppm, isolates 2B24, 2A11, and 4D21 showed the lowest inhibition values of -22.46%, -10.05%, and -4.59% respectively. Isolates 1B12, 2B24, 1B23, and 2A11 were detected as *Fusarium* sp. Meanwhile, isolates 4B21, 3A11, 2A13, and 4D21 were undetected. In addition, isolate 2D11 and isolate 3B21 showed a low inhibition value of 0% at 7 dai. The two isolates were detected to be *Trichoderma* sp.

![Graph of the percentage of the fungal growth inhibition in observations (a) 3 dai and (b) 7 dai.](image-url)
The growth of different isolates that have the potential to degrade chlorpyrifos in each treatment compared to the control at each observation can be seen more clearly in figures 2 a and b. From a qualitative perspective, the isolates in the pesticide treatment showed almost the same growth as the control. Isolates 1B23 and 2B24 were isolates of Fusarium sp., Isolates 3B21 and 2D11 were isolates of Trichoderma sp., and isolates 2D12 and 5D11 were undetectable.

The previous researches using PDA media showed that Fusarium oxysporum, F. gramen and Trichoderma harzianum have the potential to degrade fungicides, insecticides, and herbicides. Moreover, in sterilized soil and then added with Topramezone, Trichoderma was able to degrade 85% within 30 days of incubation. This shows that the fungus has a suitable substrate recognition mechanism and metabolic response system in Trichoderma isolates so that it can effectively degrade pesticides in the soil [21]. Other studies have shown that Aspergillus fumigatus can degrade chlorpyrifos in the temperature ranging 25-35°C. In the Gas-Chromatography Mass Spectrometer (GC-MS) method, the optimum degradation of chlorpyrifos was achieved at 25°C with a concentration of 1.5% for 5 days on a liquid medium of potato dextrose broth (PDB). A. fumigatus was able to degrade chlorpyrifos by 95.92% [22].

| Isolate | Control | 150 ppm | 300 ppm | 600 ppm |
|---------|---------|---------|---------|---------|
| 1B23    | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| 2B24    | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| 3B21    | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| 2D11    | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |
| 2D12    | ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) | ![Image](image20.png) |
| 5D11    | ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |

(a) (b)

**Figure 2.** The growth of isolates on PDA media was compared with the control in (a) 3 dai and (b) 7 dai observations.

The degradation process carried out by microorganisms such as fungi can be derived from enzymes or fungi that can use pesticides as a source of nutrients which are then converted into inorganic materials, water, and carbon dioxide that do not harm the environment. The group and molecular structure of each pesticide will show different degradation reactions to certain microorganism species [23]. It is more fully described that the general mechanisms of degradation are mineralization, co-metabolism, bio-concentration or cumulative effects, and microbial effects on pesticides [24]. Further, a more in-depth research is needed regarding the mechanism of fungi to degrade pesticides.
This study provides information for further research related to isolates that have the potential to degrade chlorpyrifos. In this case, these isolates need to be tested on different media such as liquid or soil media to determine the amount of reduction in chlorpyrifos concentration.

4. Conclusions
In this study, endophytic fungi isolated from shallot plants on the roots, leaves, and tubers were identified as Trichoderma sp., Aspergillus sp., and Fusarium sp. Several isolates tested had the potential to degrade insecticides with active chlorpyrifos with a growth inhibition percentage below 50% which were considered tolerant of chlorpyrifos, where at a concentration of 600 ppm ranging from 8.60% to 10.24%, at a concentration of 300 ppm -21.90% to 0%, and at concentration of 150 ppm ranging from -22.46% to 0%. This study shows the potential of Trichoderma sp. and Fusarium sp. as a chlorpyrifos degrading agent.

Acknowledgments
The authors would like to thank the Indonesian Agency for Agricultural Research and Development for providing scholarships and the Indonesian Cereals Research Institute for their support throughout the research.

References
[1] Kementan 2020 Kebijakan dan Program Kementerian Pertanian dalam Menjamin Ketahanan Pangan di Era New Normal Pandemi Covid 19 (Jakarta: Kementerian Pertanian)
[2] Chawla N, Bhardwaj J and Singh L 2020 Bioremediation of organophosphate pesticides: current status and future prospective Plant Arch. 20 3405-3412
[3] Sidhu G K, Singh S, Kumar V, Dhanjal D S, Datta S and Singh J 2019 Toxicity, monitoring and biodegradation of organophosphate pesticides: A review Crit. Rev. Env. Sci. Tec. 49 1135-1187
[4] Alizadeh R, Rafati, Ebrahimi A A and Sedighi K S 2018 Chlorpyrifos bioremediation in the environment: A review article J. Environ. Health Sustain. Dev. 3 606-61
[5] Zhang H, Yuan X, Xiong T, Wang H and Jiang L 2020 Bioremediation of co-contaminated soil with heavy metals and pesticides: influence factors, mechanisms and evaluation methods Chem. Eng. J. 398 125657
[6] Suswanto I, Simamora C J K and Anggorowati D 2018 Penggunaan cendawan endofit sebagai agens pengendali hayati pada lada (Piper nigrum L.) J. Agroqua 16 143-151
[7] Zakaria L, Yaakop A S, Salleh B and Zakaria M 2010 Endophytic fungi from paddy Trop. Life Sci. Res. 21 101-107
[8] Daud I D, Junaid M and Tuwo M 2020 Endophytic seed with Beauveria bassiana and liquid compost: control of pest stem borer of corn, Ostrinia furnacalis and increase yield resilient in marginal land IOP Conference Series: Earth and Environmental Science 486 12-14
[9] Rakshith D, Santosh P and Satish S 2013 Isolation and characterization of antimicrobial metabolite producing endophytic Phomopsis sp. from Ficus pumila Linn. (Moraceae). International Journal of Chemical and Analytical Science 4 156-160
[10] Sharma D, Pramanik A and Agrawal P K 2016 Evaluation of bioactive secondary metabolites from endophytic fungus Pestalotiopsis neglecta BAB-5510 isolated from leaves of Cupressus torulosa D. Don J Biotech. 6 209-223
[11] Arora D, Sharma N, Singamaneni V, Sharma V, Kushwaha M, Abrol V, Guru S, Sharma S, Gupta A P, Bhushan S and Jaglan 2016 Isolation and characterization of bioactive metabolites from Xylaria psidii, an endophytic fungus of the medicinal plant Aegle marmelos and their role in mitochondrial dependent apoptosis against pancreatic cancer cells Phytomedicine 23 1312-1320
[12] Choirani N A, Sunarto S and Baroroh H N 2018 Eksplorasi fungi endofit umbi lapis bawang merah (Allium cepa) sebagai antifungi dan antikolesterol Acta Pharmaciae Indonesia: Acta
Pharm. Indo. 6 12-19

[13] Barnett H L and Barry B H 1972 Illustrated Genera of Imperfect Fungi (United States of America Burgess Publishing Company)

[14] Kuwant S, Frisvad J C, Thrane U, Mathur S B 1991 An Illustrated Manual On Identification Of Some Seed-Borne Aspergilli, Fusaria, Penicillia, and Their Mycotoxins (Denmark: Danish Institute of Seed Pathology for Developing Countries)

[15] Allwbawi, Salwan A A J, Kadhum J H, Ghitheeth H H and Alshafiee A K 2019 Potential of using two Fusarium species and Trichoderma harzianum biodegrading factors of some pesticides in soil and organic compost Plant Arch. 19 756-760

[16] Alvarenga N, Birolli W G, Seleghim M H and Porto A L 2014 Biodegradation of methyl parathion by whole cells of marine-derived fungi Aspergillus sydowii and Penicillium decaturense Chemosphere 117 47-52

[17] Alvarenga N, Birolli W G, Nitschke M, de O Rezende M O, Seleghim M H R and Porto A L M 2015 Biodegradation of chlorpyrifos by whole cells of marine-derived fungi Aspergillus sydowii and Trichoderma sp. J. Microb. Biochem. Technol. 7 133-139

[18] Senhaji B, Ben Hmamou D, Salghi R, Zarrour A, Chebli B, Zarrok H, Warad , Hammouti B and Al-Deyab S S 2013 Asteriscus imbricatus extracts: Antifungal activity and anticorrosion inhibition Int. J. Electrochem. Sci. 8 6033-6046

[19] Camacho-Morales R L, Guillén-Navarro K and Sánchez J E 2017 Degradation of the herbicide paraquat by macrofungi isolated from southeastern Mexico Biotech. 7 324

[20] Vergara-Fernández A, Morales P, Scott F, Guerrero S, Yañez L, Mau S and Aroca G 2019 Methane biodegradation and enhanced methane solubilization by the filamentous fungi Fusarium solani Chemosphere 226 24-35

[21] Choudhury P P, Singh A and Singh R 2019 Biodegradation of topramezone by a Trichoderma isolate in soil J. Asian-Pacific Weed Sci. Soc. 1 43-54

[22] Anggreini C D, Tazkiaturrizki T and Rinanti A 2019 The effect of temperature and concentration of Aspergillus fumigatus on chlorpyrifos removal J. Phys. Conf. Ser. 1402 033004

[23] Huang Y, Xiao L, Li F, Xiao M, Lin D, Long X and Wu Z 2018 Microbial degradation of pesticide residues and an emphasis on the degradation of cypermethrin and 3-phenoxo benzoic acid: a review Molecules 23 2313

[24] Ye X, Dong F and Lei X 2018 Microbial resources and ecology-microbial degradation of pesticides Nat. Resourc. Cons. Res. 1 22-28