ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

In-vitro bioassays of culturefiltrate of endophytic fungi *Trichoderma sp.* In different medium against root-Knot Nematode *Meloidogyne* spp.

Nur Amin*.

Dept of Plant Protection, Faculty of Agriculture, Hasanuddin University, Makassar 90245, South Sulawesi–Indonesia.

ABSTRACT

Trichoderma sp. is one of the endophytic fungi, which is produce toxic culturefiltrate against plant pathogen e.g plant parasitic nematode. Experiments were conducted to *in vitro* bioassays of the antagonistic effects of culturefiltrate produced by endophytic fungi *Trichoderma* sp. in different medium on mobility and mortality of *Meloidogyne*-J2. Three different medium of culturfiltrate were investigated for the *in vitro* bioassays against *Meloidogyne*-J2. Inactivation/paralysis and mortality were recorded at 1, 3 and 24 hours of exposure to culturefiltrates. All of treatment of three different medium were statistically different on inactivation/paralysis of root-knot nematode *Meloidogyne*-J2 to compare with control (H_2O and H_2O + *Trichoderma* sp). Mortality of root-knot nematode *Meloidogyne*-J2 after 24 hrs exposed to culturefiltrate, followed by rinsing with sterile distilled water, demonstrated that the effects of culturefiltrates of the treatment of Czapeks-Dox + *Trichoderma* sp and GFM + *Trichoderma* sp were irreversible, as nematodes did not recover and were considered dead.

Keywords: Bioassays, endophytic fungi, culturfiltrate, *Meloidogyne* spp., *Trichoderma* sp.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Plant parasitic nematodes, are among the most widespread and important pathogens causing crop loss. Plant parasitic nematodes cause serious damages to agriculture and forestry (Siddiqui and Mehmood, 1996; Li *et al.,* 2007). Among the plant parasitic nematodes, the root knot nematodes attack wide range of host plants (Regaieg *et al.,* 2010). About 2000 plants are susceptible to their infection and they cause approximately 5% of global crop loss (Hussey and Janssen, 2002). The damages to global agricultural crops due to root knot nematodes is estimated around US$ 80 billion annually (Li *et al*, 2007; Rodrigue-Kabana and Canullo,1992). Root-knot nematode damage results in poor growth, a decline in quality and yield of the crop and reduced resistance to other stresses (e.g. drought, other diseases). A high level of root-knot nematode damage can lead to total crop loss. Nematode damaged roots do not utilise water and fertilisers effectively, leading to additional losses. Infection of young plants may be lethal, while infection of mature plants causes decreased yield. *Meloidogyne* species constitute the major nematode problem in developing countries. Infected plants show growth of reduction, swollen roots which develop into the typical root-knot galls, are two, or three times larger in diameter as healthy root. Root-knot nematodes are very difficult to control because they are polyphagous, where its over 2000 plants species is a highly specialized and complex feeding relationship with their host (Hussey and Janssen, 2002). The life cycle is almost completely confined inside the host plant and high reproductive capacity. Although chemical control is still a common method for reducing nematode population, there is a considerable public pressure to limit or even ban the use of nematicides. Many nematicides are highly toxic and sometimes very mobile in the soil because of their Solubility in water Concern over these chemicals has led to an increased interest in biological control in order to achieve more environmentally friendly methods of reducing nematode damage.

One of the alternative of chemical pesticides for controlling the parasitic nematodes is the use of beneficial or antagonistic microorganisms which can suppress soilborne pathogens in rhizosphere (Berg *et al*., 2005). Several fungi are known to regulate the nematode densities in soil by exhibiting a range of antagonistic activity including production of nematoxic compounds (Kerry, 2000; Lopez-Llorca and Jansson, 2006). There are several reports available about the production of nematicidal compounds by the fungi active against plant parasitic nematodes (Anke*,* 2010; Hallmann and Sikora, 1996; Chen *et al.,* 2000; Meyer *et al.,* 2000; Meyer *et al.,* 2004).

Culturefiltrates from fungi have shown various levels of nematicidal activity on different trophic groups of nematodes (Hallmann and Sikora, 1996; Park *et al.,* 2004). It has been reported that large quantities of secondary metabolites of antagonistic fungi are not produced during normal vegetative growth, but occur in circumstances where mycelia growth has ceased (Faull, 1988). Possibly the metabolites are remaining in the mycelium. The objective of the present work was to observe and evaluate In Vitro of the culture filtrate of endophytic fungi *Trichoderma* sp. in different medium on the mobility and mortality of *Meloidogyne*-J2.

MATERIALS AND METHODS

Nematode Species Origins

The root-knot nematode *Meloidogyne* spp. was originally isolated from an infested field on tomato plant in distric Barombong, South Sulawesi, Indonesia. The extraction of *Meloidogyne*-J2 was obtained by using the modified extraction technique of Hooper *et al* (2005). Roots were washed free from soil under tap water, cut into 1 cm pieces and macerated in a warring blender at high speed for 20 s and collected in a glass bottle. Sodium hypochloride (NaOCl) was added to obtain a final concentration of 1.5% active Chlorine and the bottle was shaken vigorously for 3 min. The suspension was then thoroughly washed with tap water through a sieve combination 250, 100, 45 and 25 µm mesh to remove the NaOCl. Eggs were collected on the 25 µm sieve and then transferred to a glass bottle. The egg suspension was supplied with oxygen from an aquarium pump over 10 days to induce juvenile hatching. To separate active J2 from unhatched eggs or dead J2, a modified Baermann technique over 24 hours was used. The collected active J2 were adjusted to 1000 J2 ml-1 and used immediately as source of inoculum

Preparation of Culturefiltrates of Endophytic Fungi *Trichoderma* sp. In Different Medium

Endophytic fungi *Trichoderma* sp. were cultured in autoclaved 100 ml Erlenmeyer flasks containing 50 ml different medium (Potato Dextrose Broth, Czapeks-Dox and Gliotoxin Fermentation Medium). Each flask received 5 piece in a 1 cm disk from a ten days old *Trichoderma* sp. The flasks were shaken at 80 rpm for 10 days at room temperature 25°C on rotary shakers. Non-cultured flasks were also prepared served as control. The fungal filtrates were obtained by filtering the culture broth firstly through a 0.2µm sterile filtration unit (Nalgene), afterwards the fungal culture filtrates in the bottom of the filter were re-filtered through 0.22 µm filter to remove bacteria and fungal spores and mycelium. The filtrates were later used at concentrations of the original preparation.

Investigation of Culturefiltrate *Trichoderma* sp. in Different Medium on Inactivation and Mortality of *Meloidogyne*-J2

One milliliter of 10 days undiluted culturefiltrates transferred to 30-mm-diameter sterile glass petri dishes. The filtrates were inoculated with approximately 100 nematode of *Meloidogyne*-J2. Control petri dishes had pure sterile destilled water (SDW) and SDW + *Trichoderma* sp. as control positif inoculated with the nematode of *Meloidogyne*-J2. The investigation was laid in a completely randomized design on a laboratory bench. All investigation were repeated thrice over time, with three replicates per medium. Inactivation and Mortality was determined by counting the number of active, inactive and dead nematode of *Meloidogyne*-J2 after 1, 3 and 24 hours of exposure to culture filtrate of endophytic fungi *Trichoderma* sp. in different medium. Individual counts of each category were recorded for each time interval. The activity (mobility) of nematodes immersed in diluted culture filtrates was determined by counting the number of active and inactive nematodes after an exposure time of 1, 3 and 24 hrs. After 24 hours, nematodes from each replication were rinsed with sterile distilled water after concentrating them using 28-µm sieve and transferring them back into the petri dishes containing sterile distilled water. The petri dishes were left on the bench under laboratory conditions for extra 24 hours. Nematodes were probed with a fine needle under the microscope and those which were straight in shape and remained immotile even after probing were considered dead (Nur Amin, 1994; Nur Amin, 2013).

Data Analysis

Abott's corrected mortality formula was used to calculate nematode inactivation. Mortality was calculated as the percentage of mortalited nematodes out of the total original number of nematodes, at each time interval. Corrected inactivation and percentage mortality data was used to evaluate the effects of individual of the different medium. Data were arcsine-square root transformed before analysis of variance (ANOVA). Laverne test was used to test for normality of distribution and homogeneity of variances. Analysis of Variance was carried to determine single factor effects and factor interactions. Where evident, effects of one factor were analysed at each time of the interacting factor. Effects of the factors were significant, means were separated using the Tukey's test.

RESULT AND DISCUSSION

Investigation of Culturefiltrate *Trichoderma* sp. in Different Medium on Inactivation and Mortality of *Meloidogyne*-J2

After time exposure of one hours inactivated Meloidogyne-J2 on the treatment of Czapeks-Dox + Trichoderma and GFM + Trichoderma more than 90 % and significantly different with the othe treatment (Table 1). Likewise after time exposure of three hours inactivated Meloidogyne-J2 100 % on the treatment of Czapeks-Dox + Trichoderma and GFM + Trichoderma.

ISSN: 0975-8585

Table 1: Percentage Inactivation of *Meloidogyne*-J2 In Different Culture filtrate Media of Endophytic *Trichoderma* sp. after 1, 3 and 24 hours- exposure.

Treatment	Hours after Treatments Inactivation (%) Meloidogyne-J2		
	1	3	24
SDW	5 c	5 c	7
SDW + *Trichoderma*	6 c	5 c	9
PDB + *Trichoderma*	24 b	27 b	100*
Czapeks-Dox + *Trichoderma*	90 a	97 a	100*
GFM + *Trichoderma*	92 c	96 a	17.0 b

Means followed by the same letter for each Columm are not significantly different at P=0.05 according to Duncan's Multiple Range Test, n = 3; SDW = sterile destilled water * values could not be statistically calculated

Significant differences in the percentage of dead nematodes were evident all of filtrate medium. In the the treatment of Czapeks-Dox + *Trichoderma* and GFM + *Trichoderma* and 24 hours exposure, followed by rinsing with distilled water still 100% nematode *Meloidogyne*-J2 dead (Figure 1).

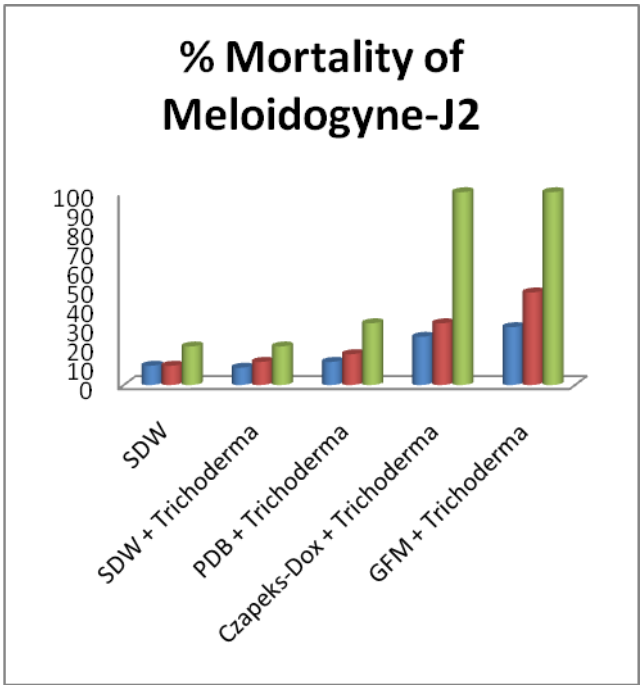

Figure 1: Percentage Mortality of *Meloidogyne*-J2. In Different Culture filtrate Medium of Endophytic *Trichoderma* sp. after 1, 3 and 24 hours- exposure.

DISCUSSION

Fungal natural products are very promising potential sources of new chemicals to manage plant-parasitic nematodes (Anke and Sterner, 1997). In this study, the treatment of the fermentation medium czapeks-Dox and GFM inactivated more than 90 % Meloidogyne-J2 after time exposure of one hours (Table 1). Significant differences in the percentage of dead nematodes were evident all of filtrate medium. In the the treatment of Czapeks-Dox + *Trichoderma* and GFM + *Trichoderma* and 24 hours exposure, followed by rinsing with distilled water still 100% nematode *Meloidogyne*-J2 dead (Figure 1). *Trichoderma* sp. formed various kind of toxic metabolites as nematicidal. Acetic acid was identified as nematicidal principle in the culturefiltrate *Trichoderma longibrachiatum* (Djian et al, 1991). Gliotoxin has been isolated from a large number of fungi including a strain of *Trichoderma virens* which show nematicidal activity (Anitha and Murugesan, 2005). A peptide cyclosporin A possessing nematicidal activity against *M. incognita* was obtained from *Trichoderma polysporum* (Li et al, 2007). Viridin was obtained from *Trichoderma* spp. which was found to possess weal

activity against *Anguillula aceti* (Watanabe *et al*, 2004; Anitha and Murugesan, 2005). According to (Yang et al, 2010) reported trichodermin as nemacidal activity against nemaodes *Panagrellus redivivus* and *Caenorhabditis elegans*, which could kill more than 95% both *P. redivivus* and *C. elegans* in 72 h at 0.4 g l.

ACKNOWLEDGEMENTS

I am deeply thankful to the Minister of Education and Culture, Directorat General of Higher and Education (DIKTI), Directur of Research and Public Extension Service for providing fund for the current study, under the project of Hibah Bersaing.

REFERENCES

[1] Anitha, R and Murugesan, K. 2005. Production of Gliotoxin on natural substrates by *Trichoderma virens*. J. Basic Microbiol. 45 : 12 - 19

[2] ANKE, H., STERNER, O. (1997): Nematicidal metabolites from higher fungi. Current Organic Chemistry 1: 361-374.

[3] Anke, H. 2010. Insecticidal and nematicidal metabolites from fungi. In: *The Mycota: Industrial Applications*. 10 : 2nd (Ed.): M. Hotrichter. Springer- Verlag, Berlin.

[4] Berg, G., C. Zachow, J. Lottmann, M. Gotz, R. Costa and K. Smalla. 2005. Impact of plant species and site on rhizosphere associated fungi antagonistic to *Verticillium dahlia* Kleb. Appl. Environ. Microbiol., 71: 4203-4213.

[5] Chen, S.Y., Dickson, D.W., Mitchell, D.J. (2000) : Viability of *Heterodera glycines* exposed to fungal filtrates. Journal of Nematology 32: 190-197.

[6] Djian, C., L. Pijarowski, M. Ponchet, N. Arpin and J. Favre-Bonvin. 1991. Acetic acid: a selective nematicidal metabolites from culture filtrates of *Paecilomyces lilacinus* (Thom) Samson and *Trichoderma longibrachiatum* Rifai. Nematologica, 37: 101-112.

[7] Faull, J.L. (1988): Competitive antagonism of soil-borne plant pathogens. In: M.N. Burge (Ed.) Fungi in Biological Control Systems. Manchester University Press, Manchester, UK. 129 p.

[8] Hallmann, J and Sikora, R.A. (1996) : Toxicity of fungal endophyte secondary metabolites to plant parasitic nematodes and soil-borne plant pathogenic fungi. European Journal of Plant Pathology 102: 155-162.

[9] Hooper, D.J., Hallmann, J. and Subbotin, S. 2005. Methods for Extraction, Processing and Detection of Plant and Soil Nematodes In: Luc, M., Sikora, R.A. and Bridge, J. (Eds). Plant parasitic nematodes in subtropical and tropical Agriculture. CAB International, Wallingford, UK. pp. 53-86.

[10] Hussey, R.S. and G.J.W. Janssen. 2002. Root-knot nematodes: *Meloidogyne* species. pp. 43-70. In: *Plant Resistance to Parasitic Nematodes.* (Eds.): J. L. Starr, R. Cook and J.Bridge. CAB International, United Kingdom

[11] Kerry, B.R. 2000. Rhizosphere interactions and exploitation of microbial agents for the biological control of plant-parasitic nematodes. Annu. Rev. Phytopathol., 38: 423-441.

[12] Li., G., K. Zhang, J. Xu, J. Dong and Y. Liu. 2007. Nematicidal substances from fungi. Recent patents on Biotechnology, 1: 1-22.

[13] Lopez-Llorca, L.V. and H.B. Jansson. 2006. Fungal parasites of invertebrates : multimodal biocontrol agents. pp. 310-335. In: *Exploitation of f Fungi.* (Eds.): G.D. Robson, P. vanWest and G.M. Gadd. Cambridge University Press, Cambridge.

[14] Meyer, S. L. F., Massoud, S.I., Chitwood, D.J., Roberts, D.P. 2000. Evaluation of *Trichoderma virens* and *Burkholderia cepacia* for antagonistic activity against root-knot nematode, *Meloidogyne incognita*. Journal of Nematology 2: 871-879.

[15] Meyer, S.L.F., Huettel, R.N., Liu, X.Z., Humber, R.A., Juba, J., Nitao, J.K. 2004. Activity of fungal culture filtrates against soybean cyst nematode and root-knot nematode egg hatch and juvenile motility. Journal of Nematology 6: 23-32.

[16] Nur Amin. 1994. Untersuchungen über die Bedeutung endophytischer Pilze für die biologische Bekämpfung des wandernden Endoparasiten *Radopholus similis* (Cobb) Thorne an Bananen. PhD-Thesis, 112 p. Bonn University.

[17] Nur Amin. 2013. Investigation of Culture Filtrate of Endophytic Fungi *Nigrospora* sp. Isolate RS10 in Different Concentrations towards Root-Knot Nematode *Meloidogyne* spp. Indian Journal of Science and Technology, Vol 6, (9) : 5177 – 5181

[18] Park, J-O., Hargreaves. J.R., Mc Conville, E.J., Stirling, G.R., Ghisalberti, E.L., Sivasithamparam, K. (2004): Production of leucinostatins and nematicidal activity of Australian isolates of *Paecilomyces lilacinus* (Thom) Samson. Letters in Applied Microbiology 38: 271-275.

[19] Regaieg, H., A. Ciancio, N.H. Horrigue, G. Brasso and L. Rosso. 2010. Effects of culture filtrates from the nematyophagous fungus *Verticillium leptobactrum* on viability of the root-knot nematode *Meloidogyne incognita.* World J. Microbiol. Biotechnol*.,* 26: 2285-2289.

[20] Rodrigue-Kabana, R. and G.H. Canullo. 1992. Cropping systems for the management of phytonematodes. Phytoparasitica, 20: 211-224.

[21] Siddiqui, Z.A. and I. Mehmood. 1996. Biological control of plant parasitic nematodes by fungi: A review. Biores..

[22] Technol., 58: 229-239.

[23] Watanabe, A; Kamei, K; Sekine, T; Waku, M; Nishimura, K; Miyaji, M; Tatsumi, K and Kuriyama, T. 2004. Effect of aeratrion on gliotoxin production by *Aspergillus fumigates* in its culturefiltrate. Mycophatologia 157 : 19-27

[24] Yang, Z.S; Guo-Hong Li; Pei-Ji Zhao; Xi Zheng; Shao-Liu Luo; Lei Li; Xue-Mei Niu; Ke-Qin Zhang. Nematicidal activity of *Trichoderma* spp. and isolation of an active compound. World Journal of Microbiology & Biotechnology; Dec 2010, Vol. 26 Issue 12. p 2297