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TITLE: The Efficacy of Oyster Mushroom, *Pleurotus ostreatus*forControlling Root-knotNematode, *Meloidogyne incognita*

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THESIS

THE EFFICACY OF OYSTER MUSHROOM, *PLEUROTUS OSTREATUS* FOR CONTROLLING ROOT-KNOT NEMATODE, *MELOIDOGYNE INCOGNITA*

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Amornsri Khun-in 2014: The Efficiency of Oyster Mushroom, *Pleurotus ostreatus* for Controlling Root-knot Nematode, *Meloidogyne incognita*. Doctor of Philosophy (Agriculture Research and Development), Major Field: Agriculture Research and Development, Faculty of Agriculture at Kamphaeng Saen. Thesis Advisor: Associate Professor Somchai Sukhakul, M.S. 100 pages.

Eighteen mushroom isolates were obtained from different growing sites in Thailand. The efficiency of mushroom cultures on egg mass infection of root knot nematode, *Meloidogyne incognita* indicated that *Pleurotus ostreatus* isolate Poa3 provided significantly higher colonized efficiency than other isolates. The infectivity (parasitism) of *P. ostreatus* isolate Poa3 on juvenile 2 was 37.25% after exposure for 96 h. The effect of culture filtrate obtained from *P. ostreatus* isolate Poa3 on egg mass was tested. The result showed that the culture filtrate at 25/100 dilution could completely kill (100%) *M. incognita* at 72 to 120 h after incubation. Application of culture filtrate onto tomato plants root effectively decreased galling inside roots and inhibited the hatching of nematode egg masses.

The media optimization on radial growth of *P. ostreatus* isolate Poa3 showed that PDA + 1% yeast extract medium provided the highest mycelial growth (9.00 cm at 6 days). Crude extract of *P. ostreatus* isolate Poa3 from column chromatography, and thin layer chromatography (TLC) gave 14 fractions. Each fraction was tested for egg mass hatching and mortality rate of *M. incognita*. Fractions 12 and 13 could decrease egg mass hatching and increase mortality rate of *M. incognitain vitro*.

Fractions 12 and 13 showed one spot on normal phase TLC with the R_f values of 0.52 and 0.58, respectively. Constituent analysis of fractions 12 and 13, the ^{1}H NMR data showed signal glucose and long chain hydrocarbon.

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Student's signature Thesis Advisor's signature

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Amornsri Khun-in
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LIST OF ABBREVIATIONS

ml	=	Milliliter
L	=	Liter
mm	=	Millimeter
cm	=	Centimeter
PDA	=	Potato dextrose agar
PDB	=	Potato dextrose broth
DNA	=	Deoxy nucleic acid
rpm	=	Revolutions per minute
AFLP	=	Amplified fragment length polymorphism
PCR	=	Polymeres chain reaction
PDYB	=	Potato dextrose yeast broth
EtOAc	=	Ethyl acetate
TLC	=	Thin layer chromatography
NMR	=	Nuclear magnetic resonance spectroscopy
FTIR	=	*Fourier transform infrared spectroscopy*
ppm	=	Part per million
LC_{50}	=	Lethal concentration fifty
NaOCl	=	Sodium hypochlorite
MeOH	=	Methanol
h	=	hour
UV	=	Ultra violet
%	=	Percent

THE EFFICACY OF OYSTER MUSHROOM, *PLEUROTUS OSTREATUS* FOR CONTROLLING ROOT-KNOT NEMATODE, *MELOIDOGYNE INCOGNITA*

INTRODUCTION

In Thailand, the most important group of plant parasitic nematodes is root-knot nematodes, *Meloidogyne* spp., which exist in soils from temperate to tropical region. The most popular root-knot nematode in northern part of Thailand is *Meloidogyne javanica* (*Mj*), whereas in central part are*M. incognita* (*Mi*) and *M. javanica* (*Mj*). The*Mi* is the most widespread one, which attacks more than 2,000 species of plants, including Solanaceae, Cucurbitaceae, Leguminosae, Poaceae and many others. Every year *Meloidogyne* spp. causes severe yield losses in vegetables and fruit trees. Root-knot nematodes belong to the genus *Meloidogyne* which are one of the most economically important pests causing severe damages to wide varieties of crops (Siddiqui and Shaukat, 2003). They cause the loss of economic crops by infecting root systems, decreasing and interfering the water and nutrients uptake. *Meloidogyne* spp., are very successful parasites which cause serious problems as global importance. In the evolution process, they have been evolved very interesting and highly specialized relationships with the plants. Especially, in various kinds of greenhouses, plant parasitic nematodes have longer lifetime, higher population, and can cause severe damage to horticultural plants. It is also recognized as a cause of soil-sickness. Plant parasitic nematodes cause drastically losses in agricultural production, therefore, the control of those nematodes had become an important matter in plant protection of the world.

Root-knot nematodes cause various symptoms on the infected plants, such as stunting, yellowing, stubby root and nutrient deficiencies. They also have rapid multiplication rates in good hosts, therefore, small population increases the difficulty in preventing during the growing season (Trudgill and Block, 2001). *Meloidogyne* species modify some root cells in feeding site, and this phenomenon is absolutely

necessary for the development and reproduction of the parasites (Hussey, 1985). The second stage juvenile 2 (J2) invade the host root cells (usually five to seven) by injecting special protein secreted from esophageal glands. In this way, J2 transforms these cambial root cells to highly specialized cells named “giant cells”. These transformed cells are the permanent feeding site for the parasite (Hussey *et al*., 1994). The feeding cells become multinucleate due to the karyokinesis without cytokinesis.

The morphology among *Meloidogyne* spp. are very similar, the species identification is difficult and has different methods. One method is the utilization of specimens from a single egg mass or from field population. The utilization of specimens from a single egg mass is very convenient and all of them belong to one species, which can reduce genetic variability.

Effective nematicides used up to now, such as carbofuran and others are going to be prohibited because of the environmental pollution. In Thailand has been prohibited in vegetable and many fruit crops production. On the other hand, the effective of newly provided chemicals are usually not satisfied on their activities of protection. Therefore, a lot of farmers have no effective chemicals for controlling nematode. Furthermore, the various troubles were found in the greenhouses after applying continuouslywith large amount of those harmful chemicals. There are not only food safety problem, but also the environmental pollution which decreases the bio-diversity in the field. Therefore, a new effective and safety use nematicide is desired. Nowadays this trend is also recognized all over the world. Trials of biological control of plant parasitic nematodes are practiced throughout the world. A large number of natural enemies, including parasitic and trapping fungi, mycorrhizal fungi, bacteria, viruses, nematodes, insects, mites, invertebrates, are investigated as possible biological control agents for nematode protection. The existing management procedures could be enhanced by the biological control development strategies (Siddiqui and Shaukat, 2003). An alternative measure to control this disease is performed using mushroom fungi, which has been reported to produce nematotoxin and directly infects some nematodes by their mycelia. *Pleurotusostreatus* possesses attributes of a successful biological control agent against nematode. It immobilizes second stage juveniles of *M. incognita* by nematotoxin and parasitizes fungal mycelia (Khun-in, 2005; Heydari *et al*., 2006). Barron and Thorn (1987) reported that nematode touching such droplets, showed a sudden response with the head region shrinked, hyphae infected to the body orifices and homed.

OBJECTIVES

1. To determine morphological characteristics and molecular phylogenetic relationships of *Pleurotus* species.

2. To evaluate the ability of oyster mushroom species, *Pleurotus ostreatus* and their toxins in suppressing root-knot nematode.

3. To identify and study the efficiency of nematicidal activity of secondary metabolites to egg mass and juvenile of root-knot nematode.

4. To isolate and identify the mushroom compounds.

LITERATURE REVIEW

1. Root-knot nematodes

Root-knot nematodes, *Meloidogyne* spp. are the sedentary endoparasitic nematodes, parasitize thousands of higher plant species including monocotyledons, dicotyledons, woody and herbaceous plants and distribute all over the world (Eisenback and Hirschmann, 1991). It is estimated about 11% loss for other vegetables, fruits and non-edible plantation 14% loss for total of over 80 US dollar annually (Agrios, 1997). Handoo *et al*. (2005) and Ruanpanum *et al*. (2010) reported that in Thailand, *Meloidogyne* spp. spread in the north, north-eastern and central region.

Meloidogyne spp. fed and reproduced in the roots, usually caused galls and formed knots on roots of susceptible host plants. After infection, the infected plantsare weak because roots cannot utilize water and fertilizers effectively, resulted in disordered, physiology of infected plants decreased crop yield quality. Moreover, plantsare susceptible to other pathogens (Back, 2002; Castello, 2003; Manzanilla-Lopez and Bridge, 2004).

About 10 species of *Meloidogyne* spp. are agricultural pests. Four species:*Meloidogyne incognita, M. javanica, M. arenaria and M. hapla* are the major crop pests and distribute worldwide ranging from temperate to tropical zone (Karrsen, 2002). Especially, *M. incognita* is the most widespread species accounting for 95% of genus *Meloidogyne* to infestation in agricultural area (Wondirad and Kifle, 2000).

Meloidogyne incognita is able to parasitize about 700 plant hosts, affects the quality and quantity of crop production in several annual and perennial crops. Infected plants show typical symptoms, stunting, root galling and nutrient deficiency (particularly nitrogen deficiency). Davis and May (2002) informed that crop yield of cotton production was losses caused by *M. incognita* were estimated between 18.0-47.3%. Wondirad and Tesfamariam (2002) reported that *M. incognita* is the major

problem in tomato cultivation in the central and western parts of Ethiopia. In 2003, *M. incognita* severely infested pepper plantation (Anonymous, 2004). Saša *et al*., 2004 were isolated *M. incognita* from root of hot peppers (*Capsicum annum* L.) from greenhouse situated in Portorož at the Adriatic Coast, Slovenia which is the first report of *M. incognita* in Slovenia. Rich *et al*. (2009) was found that *M. incognita* reproduce on over 138 weed plant hosts throughout the world. Ibrahim (2011) reported that *Meloidogyne* spp. are becoming a real threat to almost all vegetable crops and they have been considered as limiting factors in crop production in Egypt. Abbas and Mirinejad (2013) investigated susceptibility 20 species of weeds from 11 families and 4 species of vegetable plants to *M. incognita*, the resulted was found *Solanum nigrum*, followed by *Chenopodium album*, *Verbena officinalis*, *Atriplex persicum*, *Atriplex tartaric*, *Amaranthus chlorostachys*, *Physalis alkekengi* and *Amaranthus reteroflexus* was maximum susceptibility to *M. incognita*. Among vegetable crops, only *Lepidium sativum* was infected by nematode.

1.1 Life cycle

The life cycle of *Meloidogyne incognita* was shown in figure 1 and described as infective second-stage juveniles (J2) penetrate the root and migrate between plant cells and vascular cylinder. The stylet connected to the esophagus is used to pierce plant cell walls, release esophageal secretions and take up nutrients. Each J2 induces the dedifferentiation of five to seven root cells into multinucleate and hypertrophied feeding cells (*). These giant cells supply nutrients to the nematode (N). The nematode becomes sedentary and goes through three molts (J3, J4 and adult). Occasionally, males develop and migrate out of the roots. However, it is believed that they play no role in reproduction. The pear-shaped female produces eggs that are released on the root surface. Embryogenesis within the egg is followed by the first molt, generating second-stage juveniles (J2). The entire life cycle complete in 20-25 days at 27^{o}C, but it takes longer time at lower or higher temperature. When the eggs hatch, the infective second-stage juveniles migrate to adjacent parts of the root and cause new infections in the same root or other roots of the same plants or other plants. Most root-knot nematodes are found in the root zone from 5 to 25 centimeters below

the surface. They primarily spread uninfested areas by water or soil clinging to farm equipment or infected propagating (Agrios, 2005). Males are vermiform, and are not required in reproduction by parthenogenesis.

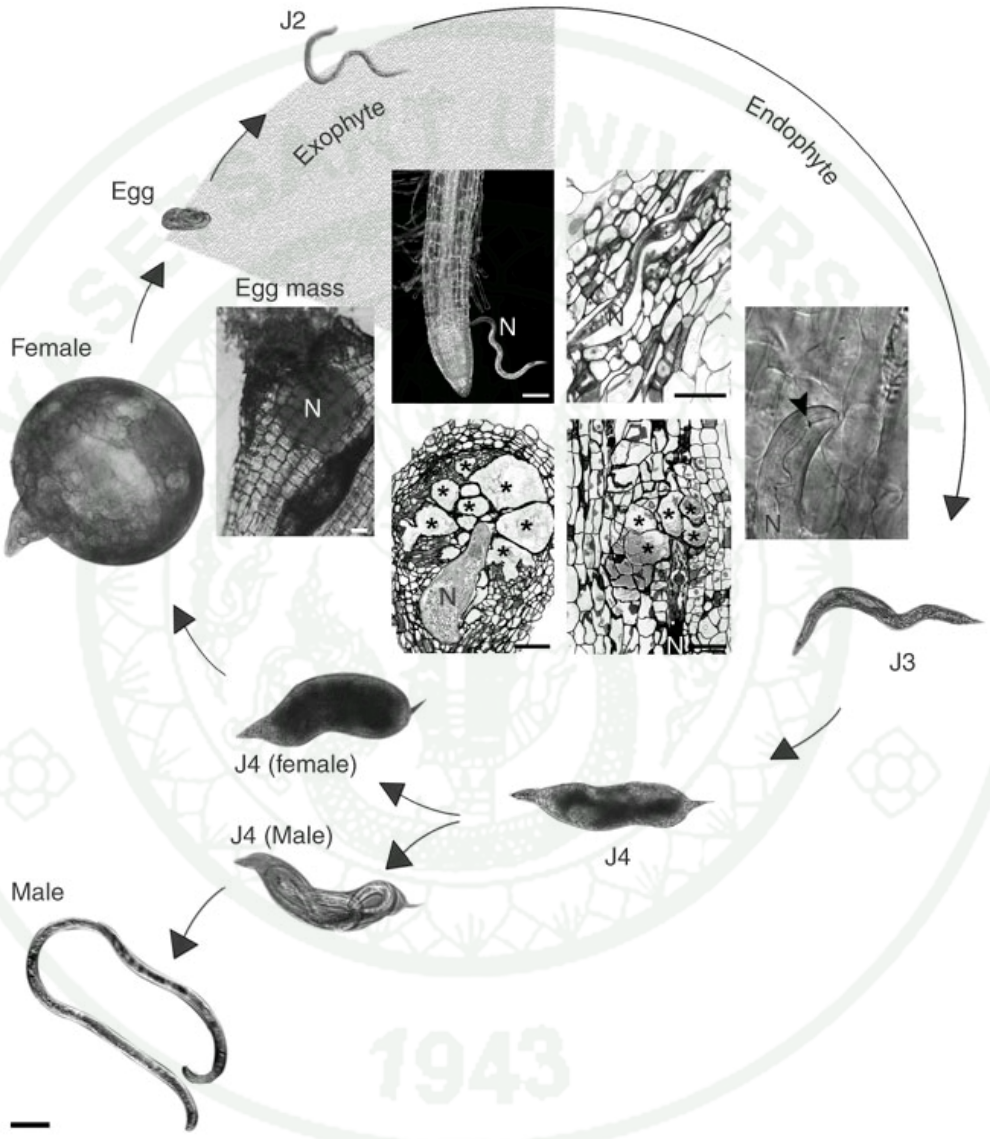


Figure 1 Life cycle of *Meloidogyne incognita*

Source: Abad *et al*., 2003

1.2 Symptoms

Aboveground symptoms are reduced growth and fewer, small, pale green, or yellowish leaves, that tend to wilt in warm weather. Blossoms and fruits are few and poor quality. Affected plants usually linger through the growing season and are seldom killed prematurely. Characteristic symptoms of the disease appear at the underground parts of the plants. Infected roots develop the typical root-knot galls that are two to several times as large in diameter as the healthy root. Several infections along the root give a rough root, clubbed appearance. Roots infected by certain species of the nematode also develop a bushy root system. Usually, infected roots remain smaller and show necrosis and rotting, particularly in the late season. When roots and tubers were attacked, they produce galling and small swellings over their surface (figure 2), which become quite prominent and cause distortion or cracking. According to that roots of plants are also attacked by the root-knot nematode and develop galls roughly proportional in size to the length of time since infection (Agrios, 2005). The top growth is poor and the foliage is frequently chlorotic (yellow), because essential elements are not taken in and transported by the impaired root system. Severe infections cause of wilting of the foliage and the plants require more frequent irrigations (Nickle, 1991).

Figure 2 The symptom of plants root infected by *Meloidogyne incognita*.

1.3 Classification and identification of *Meloidogyne* spp.

1.3.1 Classification of *Meloidogyne* spp.

In 1996, Kleyhens *et al*. had classified *M. incognita* as below

Phylum Nemata
Class Secernentea
Order Tylenchida
Suborder Tylenchina
Superfamily Tylenchoidea
Family Heteroderidae
Subfamily Meloidogyninae
Genus *Meloidogyne*

1.3.2 Identification of *Meloidogyne* spp.

Many factors that accurate identification of *Meloidogyne* spp. difficult include; inadequate funding to carry out research, limited number of nematology taxonomist and training of young scientists, sexual dimorphisms, polyploidy, overlapping morphological characters and wide host ranges (Oliveira *et al*., 2011). However, different approaches had devised for improved accurate identification of several nematodes species (Blok and Powers, 2009). Identification methods for *Meloidogyne* spp. are besed on either morphological, biochemical and/or molecular approaches.

1.3.2.1 Morphological identification

The male and female of root-knot nematode are easily distinguishes the morphological characteristics. The males are worm-liked and about 1.2 to 1.5 millimeters long, 30 to 36 micrometers in diameter. The females are pear shaped and about 0.40 to 1.30 millimeters in length, 0.27 to 0.75 millimeters in width.

Each female lays approximately 500 eggs in a gelatinous substance. The first and second-stage juveniles are worm-liked and develop inside each egg. The second-stage juvenile the only infective stage of the nematode, emerges from the egg into the soil. When the juvenile reaches a susceptible host, it enters the root, becomes sedentary, and grows thick like a sausage. The nematode feeds on the cells around its head by inserting its stylet and secreting saliva into the cell. The saliva stimulates cell enlargement and also liquefies part of the contents of the cell, which is then withdrawn through its stylet. The nematode then undergoes a second molt and gives rise to the third-stage juvenile, which is stouter and goes through the third molt and gives rise to the fourth-stage juvenile. This juvenile stage can be distinguished either male or female. These undergo the fourth and final molt, and the male emerges from the root as the worm-liked adult male, which becomes a free-living in the soil, while the female continues growing in thickness, somewhat in length and appears pear shaped. Moreover the female continues swelling with or without fertilization, producing eggs that are laid in a gelatinous protective coat inside or outside the root tissues, depending on the position of the female. Egg may hatch immediately or a few of them may overwinter and hatch in the spring (Agrios, 2005).

Sexually dimorphic, female saccate is globose, 0.4-1.3 mm long, usually embedded in root tissue which is often swollen or galled, body soft, pearl-white in color and does not form a cyst; the neck protrudes anteriorly and the excretory pore is anterior to the median bulb often near the stylet base. The vulva and anus are terminal, flush with or slightly raise from the body contour; the cuticle of the terminal region forms a characteristic pattern called the perineal pattern, which is made up of the stunted tail terminus, plasmids, lateral lines, vulva and anus surrounded by cuticular striae. This pattern is often characterized for individual species. The female stylet is shorter about 10-24 um, usually 14-15 μm, and more delicate than that in *Heterodera* with small basal knobs, which are the characteristic shape of some species. The paired gonads have extensive convuluted ovaries that fill most of the swollen body cavity. There are six large unicellular rectal glands in the posterior body, which produce and excrete a gelatinous matrix via the rectum to form an egg sac in which many eggs are deposited. Males vermiform, similar to

Heterodera, but the lip region has a distinct head cap, which includes a labial disc surrounded by lateral and medial lips. The head skeleton of many species is usually weaker than *Heterodera*, and the stylet is less robust and shorter, 18-24 μm long. Infective second stage juveniles are often free in the soil, and usually 0.3-9.5 mm long. They are less robust than *Heterodera* juveniles. Moreover, the stylet is delicate with small basal knobs, under 20 μm long, and the head skeleton is weak. The median esophageal bulb is well developed and the esophageal glands are extensive, overlapping the intestine for several body widths mainly ventrally; the tail is conoid, often ending in a narrow rounded terminus, but the tail length is variable, 1.5-7 anal body widths, between species, it often ends in a clear hyaline region, which the extent can help to distinguish species (Radewald, 1978).

1.3.2.2 Biochemical identification

More than 50 years, various biochemical methods have been applied for identification of several plant-parasitic nematodes. These biochemical methods are categorized into 2 broad groups; protein and serology separation. Protein characterization is based on unique separations for each species, including use of one-dimensional gel electrophoresis, two-dimensional gel electrophoresis (2-DGE), isoelectric focusing (IEF) and Sodium doedecyl sulphate capillary gel electrophoresis (SDS-CGE). In addition, Serological methods include use of monoclonal and polyclonal antibodies (Abrantes *et al*., 2004).

1.3.2.3 Molecular identification

Nowadays, molecular equipments have been developed (Powers, 2004; Blok and Powers, 2009). Several molecular approaches have been designed for accurate identification of many members of Meloidogyne genus. This is primarily because DNA-based methods are plausible and rapid compared to morphological or biochemical methods (Powers *et al*., 2005). The most extensively used DNA-based methods, include mitochondrial DNA (mtDNA), amplified fragment length polymorphisms (AFLP), microsatellite DNA (satDNA), random amplified

polymorphisms DNA (RAPD), restriction fragment length polymorphisms (RFLPs), sequence characterized amplified region markers (SCAR-PCR), ribosomal DNA (rDNA), microarrays and real time PCR (qPCR).

In addition, morphological analyses of several developmental stages of the nematode combined with host plant response evaluate and molecular analyses should help in the correct identification of a nematode population (Hunt and Handoo, 2009; Perichi and Crozzoli, 2010).

1.3.3 Nematode control

Currently, methods for the management are not effective against *Meloidogyne* spp. as they are soil inhabiting. Usually farmers use chemicals to soil which is harmful to other micro flora as well as to the environment. Further, these chemicals pollute ground water. Cultural practices such as crop rotation are commonly used, but such practices are not effective as root knot nematodes remain in soil for years and they have a wide host range. Even weeds are hosts and attacked are available in every season. Further, farmers cannot fallow rotation or afford rotation. Due to their wide host range they are difficult to control by other strategies like resistant cultivars and rotation because of species mixtures and virulent strains (Roberts, 1992). The existing management procedures could be improved by the development of organic strategies (Siddiqui and Shaukat, 2003). Nematicides are used to control root knot nematodes but difficult for subsistence farmers at small scale in developing countries. Since most nematicides are not only expensive but dangerous to human health and environment. The addition of organic matter in form of manure or compost will decrease nematode population and damage to crops (Walker, 2004). This could be a result of improved fertility and soil structure, release of anti-nematode-toxins, increase of plant resistance or increased populations of pathogens and other nematode-antagonistic agents (Akhtar and Malik, 2000).

Biological control using the *Paecilomyces lilacinus* affected *Mi* egg and *Pleurotus ostreatus* affected *Mi* juvenile and decreased 40-80% root-knot disease (Khun-in, 2005).

2. Oyster mushroom *(Pleurotus* spp.)

Pleurotus species (oyster mushrooms) are edible fungi and rich source of nutritional, and medicinal value that will sustain food security for people in developing countries. It is cultivated worldwide especially in South East Asia, India, Europe and Africa (Sanchez *et al*., 2002; Mandeel *et al*., 2005). In addition, *Pleurotus* spp. has important to environmental and biotechnological properties applications (Cohen *et al*., 2002).

2.1 Morphological identification of *Pleurotus* spp.

The genus *Pleurotus* is one of the most taxonomically challenging groups of macrofungi comprising many species and subspecific entities with complex affinities, whose delimitation or discrimination is in several cases problematic (Zervakis, 2004). *Pleurotus*species represent a well-defined group of Basidiomyceteous fungi of the order Agaricales and family Tricholomataceae. They are characterized by the production of fruit bodies with an eccentric stalk and a wide cap shaped like an oyster shell, with the widest portion of the cap being away from the stalk.

Pleurotus species are characterized by a white spore print, attached to decurrent gills, often with an eccentric (off center) stipe, or no stipe at all. They always grow on wood by nature, usually dead standing trees or fallen logs. The common name "oyster mushroom" comes from the white shell-like appearance of the fruiting body, not from the taste. The taste of the oyster mushroom varies from very mild to very strong, sometimes sweet with the smell of anise (licorice). It varies in texture from very soft to very chewy, depending on the strain and what time of the year you pick it, they tend to be chewier (and thus more interesting) during the colder

months of the year. You can make a delicious "Oyster Mushrooms Rockefeller" and a variety of stir-fry dishes.

The oyster mushroom, or *P. ostreatus*, is a common mushroom prized for its edibility and lack of confusing look-alikes. It is related to the "king oyster mushroom".Both the latin and common name refer to the shape of the fruiting body. The latin Pleurotus(sideways) refers to the sideways-growth of the stem with respect to the cap while the latin ostreatus (and the English common name, oyster) refers to the shape of the cap which resembles the bi-valve of the same name.

Cap is smooth; oblong and often convex with age; 50-200 mm in diameter; and ranges from white to brown to blue-gray. The margin can be smooth with a slight wave. Flesh of the most common variety is white and can be thin or thick. A range of different colors can be found in the wild and can be cultivated are yellow, pink, blue, and gray. Gills are decurrent (descend down the stem) and attached and white to light yellow. The stem is short, often horizontal and emerging from wood. Spore colors are white to lilac-gray print on dark media. Mycelium is white and grows rapidly.

Several agricultural by-products are being used as substrates for cultivation the oyster mushroom. Some of these wastes include sugarcane leaves, peanut hull, corn leaves, banana leaves, mango fruits and seeds, wheat and rice straw (Cangy and Peerally, 1995). In addition, rice straw is the widely used substrate for cultivation of the oyster mushroom in Asia (Thomas *et al*. 1998).

2.2 Molecular identification of *Pleurotus* spp.

To overcome this limitation, molecular /DNA based markers have proved very useful in investigating the genetic diversity of fungal organisms. Molecular markers being enormous in number have many properties over the morphological and biochemical markers viz. codominant inheritance, even and frequent distribution in the genome, high reproducibility, environmentally stable and ineffective from epistasis and pleiotropy (Williams *et al*.1990; Vilarinhos *et al*. 1995). Zervakis *et al*.,

2004 informed that molecular phylogenetic analysis provides a good tool to understand the systematics and species boundaries in *Pleurotus*.

Hadeel (2013) used RAPD-PCR technique to reveal DNA polymorphism in DNA of *Pleurotus ostreatus* and *Pleurotus sapidus* in order to search for the sources of differences that could be used as DNA marker represent the differentiation between this 2 species.

2.3 Phytochemical characteristics and medicinal properties of *Pleurotus* substance.

Pleurotus spp. should not consider simply as food. But it is contains of various substances and many of them are valuable source of biologically activity compounds (Barros *et al*., 2007; Patel *et al*., 2012) such as alkaloids, phenolics, polysaccharides, proteins (fungal immuno-modulating proteins-FIPs, glycoproteins, lectins and peptides and non-glycosylated proteins), polysaccharide–protein complexes, lipid components (ergosterol) and terpenoids, nucleotides and nucleosides, small peptides and amino acids. The great assortment of biological properties which include antimicrobial (Barros *et al*., 2007; Akyuz and Kirbag, 2009), antioxidant (Peralta*et al*., 2008; Puttaraju *et al*., 2006; Ferreira *et al*., 2009), antitumor/anticancer (Moradali*et al.*, 2007; Maness *et al*., 2011), immunomodulatory (Borchers *et al*., 2004), antiatherogenic (Mori*et al*., 2008), hypoglycemic actions (Hu *et al*., 2006) and anti-inflammatory (Padilha*et al*., 2009; Moro *et al*., 2012). In addition, also found that octan-3-one, oct-1-en-3-ol, oct-1-en,2-methylbutanol, and α-pinene indicated that the main aromatic compounds identified volatile compounds fruit body and mycelium of *P. ostreatus* JMO 95 submerged and solid-state cultures of by gas chromatography/mass spectrometry (GC/MS) and gas chromatography sniffing (GC/sniffing) (Abbaj, 2002).

2.3.1 Nematicidial structural formular

The genus *Pleurotus* is one of the most worldwide commercialised mushroom (Rosado *et al*., 2003). Several species of the genus were reported to be able to capture and consume nematode (Barron and Thorn, 1987). At least 10 species of gilled fungi belonging to the genera *Hohenbuehelia*, *Pleurotus* and *Resupinatus* can attack nematode by adhesion or toxins (Tzen and Liou, 1993.) Thorn and Barron (1984) also found that when nematodes contact with the hyphae of *Pleurotus*, they became inactive very quickly and were subsequently colonized and digested by the fungus.*Pleurotus* species display a kind of nematode capture that appears to be unique to this genus. They produce tiny appendages on the vegetative hyphae and these secrete droplets of a potent toxin (Thorn and Barron, 1984; Barron and Thorn, 1987; Petersen, 1993; Thorn and Tsuneda 1993). The toxin produced by *P. ostreatus* has been identified as trans-2-decenedioic acid (Kwock *et al*., 1992) and from another specie *P. pulmonarius*, as s-coriolic acid, linoletic acid,*p*-anisaldehyde, *p*-anisyl alcohol, 1-(4-methooxyphenyl)-1,2-propanediol and 2-hydroxy-(4-methoxy)-propiophenone were obtained as nematicidal components (Stadler *et al*., 1994). These studies suggested that more nematicidal metabolites maybe exist in the fungi of the genus *Pleurotus*. Oyster mushrooms are a source of statin drugs, and are also one of a few known carnivorous mushrooms. Its mycelia can kill and digest nematodes. This is believed to be a way to obtain nitrogen (Barron, 2003; Nordbring-Hertz *et al*., 1995). *P. ostreatus* immobilizes the nematode host by producing a toxin on specialized hyphal stalks and the hyphal tips grown chemotropically through the mouth of their victims and digest the contents (Satou *et al*., 2008). Many detrimental nematodes exist, including parasitic plant and animal nematodes. The *P. ostreatus*is a famous mushroom that preys upon live nematodes. However, there have been no details reported on the mechanism of this predatory activity. Therefore, this mushroom is the predatory relationship between the nematode and *P. ostreatus* as a potential way of exterminating other various detrimental nematodes. Upon invasion by the nematode, the mushroom defends itself by causing the nematode is head to shrink in size (anti-nematode activity). Our data suggest that this anti-nematode mechanism is associated with the peroxide of linoleic acid.

2.3.2 Nematicidial properties

Several researches have attempted probiotic properties and several interesting biological functions of polysaccharides complexes produced by *P. ostreatus* such as immunomodulatory, hypoglycemic and anti-tumoral effects, anti-inflammatory and cholesterol lowering properties or antiviral, antibiotic and antioxidant activities (Tavares *et al*., 2005; Sarangi *et al*., 2006; Li *et al*., 2008; Synytsya *et al*., 2009; Palacios, 2012).

Yang *et al*. (2002) found that *P. ostreatus* and *P. cystidiosus*fruiting extracted by methanol had antioxidant, reducing power, radical scavenging and iron chelating activities that were higher than those of other commercial mushrooms.

Alarcon *et al*. (2002) reported that fruiting bodies of *P. ostreatus* cultivated in conservatory on straw of wheat content of lovastatin by GC-MS and HPLC analysis, and lovastatin molecular structure was shown in Fig. 3. Lovastatin has been extracted from *P. pulmonarus* could be anti-cancerigenous properties on the same molecule. (Gunde-Cimerman and Cimerman, 1995; Kurashige *et al*., 1997)

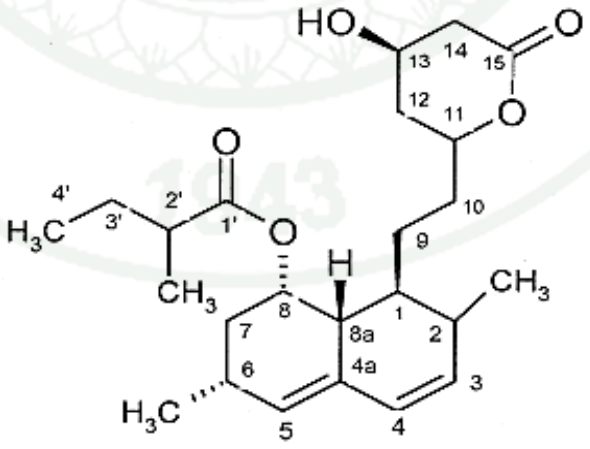


Figure 3 Lovastatin molecular structure

Source: Alarcon *et al*. (2002)

Zhan *et al*. (2003) isolated 12 steroids include 8 ergostane-type sterols and 4 mono-glucosides of ergostane-type sterols from ethyl soluble fraction of *P. ostreatus* (Jacq.: Fr.) Kummer by ^{1}H-NMR and ^{13}C-NMR analysis.

Gu and Sivam (2006) detected the active compounds (water-soluble proteins or polypeptides) in water extract of *P. ostreatus*. It exhibited the most cytotoxicity by inducing apoptosis of human carcinoma cells, when compared to several other mushroom extracts.

Jayakumar *et al*. (2006) displayed that extract from *P. ostreatus* was able to inhibit the hepatotoxicity induced by CCl_4 in rats. Jayakumar *et al*. (2007) also indicated that the extract of *P. ostreatus* emerged to protect major organs such as liver, heart, and brain of aged rats against oxidative stress.

Iwalokun *et al*. (2007) examined phytochemistry, antioxidant and antimicrobial potencies of petroleum ether and acetone extract of *P. ostreatus*. Petroleum ether (PE) extracts were exhibited greater to inhibit *Bacillus subtilis, Escherichia coli* and *Saccharomycee cerevisae* by agar well diffusion method than acetone extract (AE). In addition, PE also stronger inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa* by broth dilution method. Phytochemical analyses of both extracts revealed the presence terpenoids, tannins, steroidal glycosides and carbohydrates.

Satou *et al*. (2008) reported that fresh whole *P. ostreatus* extract with 50% methanol could be reduced the shrinkage of the head of the nematode (anti-nematode activity).

Akyuz and Sevda (2009) studied the antimicrobial activity of *P. eryngii* var. *ferulae* grown on various agro-wastes. *P. eryngii* var. *ferulae* were inhibition *Bacillus megaterium* DSM 32, *Staphylococcus aureus* COWAN 1, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* FMC 5, *Candida albicans* FMC 17, *Candida glabrata* ATCC 66032, *Trichophyton* spp., and *Epidermophyton*

spp. by disk diffusion method.

Kanagasabapathy *et al*. (2011) extracted chemical composition of *P. sajor-caju* by aqueous butanol and ethyl acetate. In ethyl acetate extract was found 22 compounds comprising methyl esters, hydrocarbon fatty acids, ethyl esters and sterols, while cinnamic acid, nicotinamide, benzeneacetamide and 4-hydroxybenzaldyhde were identified in butanol extracts by gas chromatography-mass spectrometry (GC/MS) and nuclear magnetic resonance (NMR) analysis.

Sathyaprabha *et al*. (2011) attempted that *P. platypus* and *P. eous*, extracted with 99% of ethanol. Extracted sample was identified bioactive compounds by gas chromatography – Mass spectrum technique (GC/MS). According to the results, in *P. platypus* Pyridine-3-carboxamide,4-dimethylamino-N-(2,4-difluorophenyl), Piperidin-4-carboxylic acid, Aspidofractinine-3-methanol, (2à, 3á, 5à), Indolizine, and 2-(4-methylphenyl). *P. eous* shows that Imidazolidine, 1, 3-dinitro, Phenol, 2-methyl-4-(1, 1, 3, 3-tetramethylbutyl), Aspidofractinine-3-methanol, (2à, 3á, 5à) and Squalene.

Polysaccharide extracted from *P. ostreatus* is β-D-glucan known as pleuran. Polysaccharide composed of (1→3)-linked *β*-D-glucopyranosyl residues, which contains of glucose, galactose and mannose in a molar ratio of 8:2:1 (Maity, 2011; Palacios, 2012).

Cholestane-3, 7, 1, 25-tetrol tetraacetate, (3a, 5a, 7a, 12a)-55.20, 9, 12-octadecadienoic acid, methyl ester (E, E)-18.55, 14, 17-octadecadienoic acid, methyl ester (E, E)-5.59, pentadecanoic acid and ethyl ester-3.84 were found from alcoholic extract of *P. ostreatus* by gas chromatography/mass spectrometry (GC/MS) analysis (Priya *et al*., 2012).

Papaspyridi *et al*. (2012) isolated 17 metabolites (e.g., fatty acids, phenolic metabolites, nucleotides and alkaloids) from dichloromethane (DCM) and methanol (MeOH) extract of *P. ostreatus* that produced by submerged fermentation in

a batch stirred tank bioreactor. The compounds were elucidated with 1D/2D NMR-spectroscopic analyses, and chemical correlations combined with GC/MS and LC/MS analysis were shown in Fig. 4 and 5.

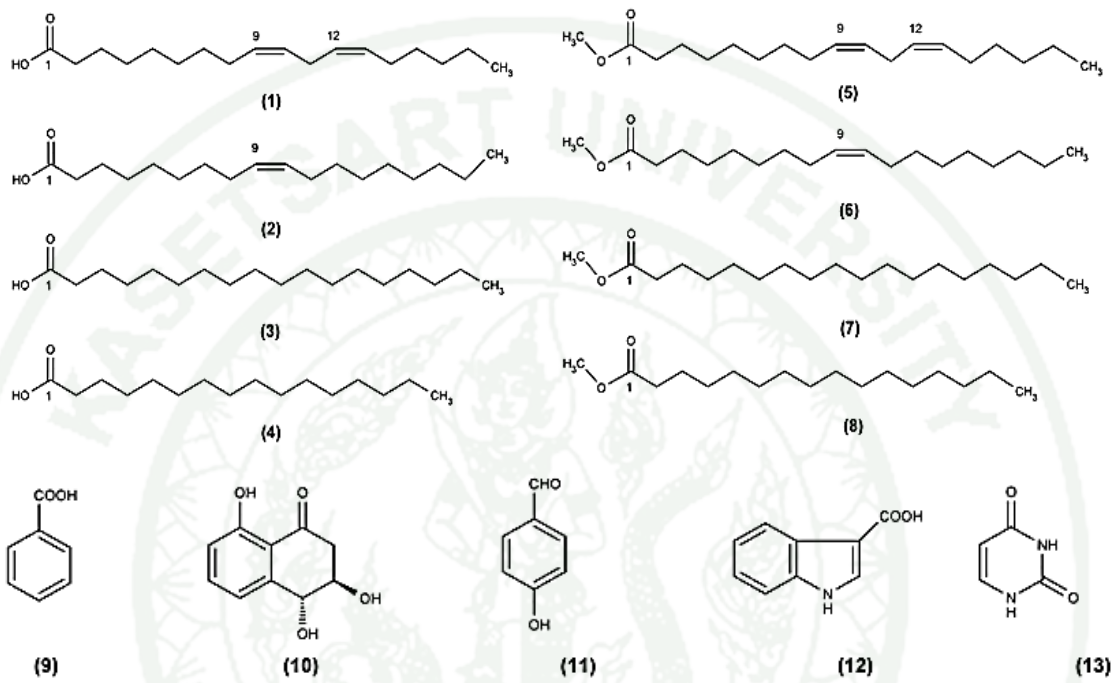
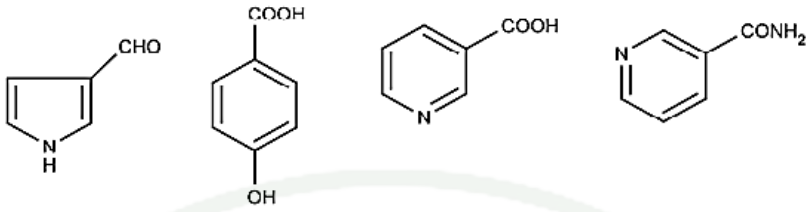


Figure 4 Structures of metabolites isolated from the dichloromethane extract (DCM) of biomass derived from *P. ostreatus* grown in submerged culture in a batch stirred tank bioreactor. The above investigation afforded linoleic acid(1), oleic acid(2), stearic acid(3), palmitic acid(4), methyl esters(5, 6, 7 and 8, respectively), benzoic acid (9) (Sato *et al*., 1999), trans 3,4-dihydro-3,4,8-trihydroxynapthalen-1(2H)-one (10) (Couché *et al*. 2009), 4-hydroxy-benzaldehyde (11) (Tan *et al*., 2004), indolo-3-carboxylic acid (12) (Bano *et al*., 1987) and uracil (13)

Source: Bednareka *et al*. (2000).



(14) **(15)** **(16)** **(17)**

Figure 5 Structures of metabolites isolated from of the methanolic extract (MeOH) derived from *P. ostreatus* grown in submerged culture in a batch stirred tank bioreactor. The above investigation afforded 3-formylpyrrole(14), 4-hydroxybenzoic acid (15) (Gutiérrez *et al*., 1994), nicotinic acid (16) and nicotinamide (17)

Source: Çağlarirmak, (2007)and Kanagasabapathy *et al*.(2011)

Oyetayo and Ariyo (2013) studied 3 different woody substrate such as *Canarium* sp., *Pycnanthus ongoleubis* (Welw.) and *Ceiba pentandra* (L.) Gaertn. used in cultivating. *P. ostreatus* has effect on antimicrobial and antioxidant properties. *P. ostreatus* cultivated on P. *ongoleubis* exhibited the best antioxidant activities and inhibitory effect against *Staphylococcus aureus*.

Pauliuc *et al*. (2013) revealed that gemmotherapic extracts of *P. ostreatus* had inhibitory activity with HCT-116 cell line (colorectal carcinoma cell line) by MTT assay and can also be used in combination with traditional chemotherapy.

Antifungal and antibacterial activities have been distinguished in *Pleurotus* spp. extracts and isolated compounds, presumably produced as defense mechanism against other organisms that shown in Table 1.

Table 1 Summarize reported antimicrobial activities of *Pleurotus* spp.

Species		Effective against	References
P. eryngii	Eryngin an antifungal peptide	*Fusarium oxysporum*, and *Mycosphaerella arachidicola*	Wang and Ng, 2004
	Eryngeolysin a haemolysin	*Bacillus* spp.	Ngai and Ng, 2006
P. ostreatus	Crude extracts from fermentation broth	Gram-positive and Gram-negative Bacteria and *Aspergillus niger*	Gerasimenya *et al*., 2002
	Haxane-dicholomethane extract containing *p*-anisaldehyde	*Bacillus subtilis*, *Pseudomonas aeruginosa* *Aspergillus niger* and *F. oxysporum*	Okamoto *et al*., 2002
	Various extracts; two main unidentified compounds	*Bacillus* spp., *Escherichia coli,* *Vibrio cholerae* and *Samonella typhi*	Periasamy, 2005
P.sajor-caju	12 kDa ribonuclease	*F. oxysporum, Mycosphaerella arachidicola, Pseudomonas aeruginosa* and *Staphylococcus aureus*	Ngai and Ng, 2004

3. Extraction, isolation and identification method of *Pleurotus* spp.

3.1 Extraction

Plants are producing a range of secondary metabolites with different polarities and functional groups. Categories of natural products commonly encountered include waxes and fatty acids, polyacetylenes, terpenoids (e.g., monoterpenoids, iridoids, sesquiterpenoids, diterpenoids, triterpenoids), steroids, essential oils (lower terpenoids and phenylpropanoids), phenolics (simple phenolics, phenylpropanoids, flavonoids, tannins, anthocyanins, quinones, coumarins, lignans), alkaloids, and glycosidic derivatives (e.g., saponins, cardiac glycosides, flavonoid glycosides). Several methods can be employed to extract the plant material (Sarker *et al*., 2006).

3.1.1 Maceration

The method is suitable for initial and bulk extraction. The extraction ultimately stops when equilibrium is attained between the concentration of metabolites in the extract and that in the plant material. After extraction, the residual plant material has to be separated from the solvent. This involves rough clasification by decanting, which is usually followed by filtration step. Centrifugation may be necessary if the powder is too fine to be filtered. To ensure exhaustive extraction, it is common to carry out an initial maceration, followed by clasification, and an addition of fresh solvent to the residual plant material. This can be performed periodically with all filtrates pooled together(Veronique, 2006).

3.1.2 Ultrasound-assisted solvent extraction

This is modified maceration method where the extraction is facilitated by use of ultrasound. The plant powder is placed in a vial. The vial is placed in ultrasonic bath, and ultrasound is used to induce mechanical stress on the cells through the production of cavitations in the sample. The cellular breakdown

increases the solubilization of metabolites in the solvent and improves extraction yields. The efficiency of the extraction depends on the instrument frequency, and length and temperature of sonication(Veronique, 2006).

3.1.3 Percolation

The powdered plant material is soaked initially in solvent in percolator. In addition, solvent is then poured on top of the plant material and allowed to percolate slowly (dropwise) out of the bottom of the percolator. Percolation is adequate for both initial and large-scale extraction. As for maceration, successive percolations can be performed to extract the plant material exhaustively by refilling the percolator with fresh solvent and pooling all extracts together. The percolate can be tested for the presence of metabolites with specific reagents(Veronique, 2006).

3.1.4 Soxhlet extraction

Soxhlet extraction is used widely in the extraction of plant metabolites because of its convenience. The plant powder is placed in cellulose thimble in extraction chamber, which is placed on top of collecting flask beneath reflux condenser. A suitable solvent is added to the flask, and set up is heated under reflux. When certain level of condensed solvent has accumulated in the thimble, it is siphoned into the flask beneath(Veronique, 2006).

3.1.5 Pressurized solvent extraction

Pressurized solvent extraction, also called ‘‘accelerated solvent extraction,’’ employs temperatures that are higher than those used in other methods of extraction, and requires high pressures to maintain the solvent in liquid state at high temperatures. It is best suited for the rapid and reproducible initial extraction of number of samples. The powdered plant material is loaded into extraction cell, which is placed in oven. The solvent is then pumped from reservoir to fill the cell, which is heated and pressurized at programmed levels for set period of time. The cell is flushed

with nitrogen gas, and the extract, which is automatically filtered, is collected in flask. Fresh solvent is used to rinse the cell and to solubilize the remaining components. A final purge with nitrogen gas is performed to dry the material. High temperatures and pressures increase the penetration of solvent into the material and improve metabolite solubilization, enhancing extraction speed and yield(Veronique, 2006).

3.1.6 Extraction under reflux and steam distillation

In extraction under reflux, plant material is immersed in solvent in round-bottomed flask, which is connected to condenser. The solvent is heated until it reaches its boiling point. As the vapor is condensed, the solvent is recycled to the flask. Steam distillation is similar process and is commonly applied to the extraction of plant essential oils. Plant is covered with water in flask connected to condenser. Upon heating, the vapors condense and the distillate is collected in graduated tube connected to the condenser. The aqueous phase is recirculated into the flask, while the volatile oil is collected separately(Veronique, 2006).

3.2 Isolation and identification

The choice of extraction procedure depends on the nature of the source material and compounds to be isolated.

3.2.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a solid-liquid technique in which two phases are solid (stationary phase) and liquid (moving phase). Solids most commonly used in chromatography are silica gel (SiO_2 x H_2O) and alumina (Al_2O_3 x H_2O). Both of these adsorbents are polar. Silica is acidic and alumina is available in neutral, basic, or acidic forms. Thin layer chromatography (TLC) is sensitive, fast, simple and inexpensive analytical technique. It is a micro technique as little as 10^{-9} g of material can be detected, although the sample size is from 1 to $100x10^{-6}$ g. TLC involves spotting the sample to be analyzed near one end of plastic or a sheet of glass

that is coated with thin layer of an adsorbent. The sheet, which can be the size of a microscope slide, is placed on end in covered jar containing a shallow layer of solvent. As the solvent rises by capillary action up through the adsorbent, differential partitioning occurs between the components of the mixture dissolved in the solvent the stationary adsorbent phase. The more strongly given component of a mixture is adsorbed onto stationary phase, the less time it will spend in the mobile phase and the more slowly it will migrate up the plate (Veronique, 2006).

3.2.2 Column Chromatography (CC)

Column chromatography (CC) is common can be used on both a large and small scale, and useful separation technique in organic chemistry. This separation method involves the same principles as TLC, but can applied to separate larger quantities than TLC. The applications of this technique are cross many disciplines and wide reaching including biochemistry, microbiology, medicine and biology. Column chromatography (CC) can be separate and collect the compounds individually. Column chromatography (CC) will be used to separate the starting material from the product in the oxidation of fluorene to flourenone and TLC will be used to monitor the effectiveness of this separation(Veronique, 2006).

3.2.3 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (NMR) is the most powerful technique available for determining the structure of organic compounds. This technique relies on ability of atomic nuclei to behave like small magnet and align themselves with external magnetic field. When irradiated with radio frequency signal the nuclei in molecule can change from being aligned with the magnetic field to being opposed to it. Therefore, it is called “nuclear” for instrument works on stimulating the “nuclei” of atoms to absorb radio waves. The energy frequency at which this occurs can be measured and is displayed as NMR spectrum. The most common nuclei observed using this technique are available for ^{1}H and ^{13}C, but also ^{31}P, ^{19}F, ^{29}Si and ^{77}Se (William, 2013).

3.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infraredspectroscopy(FTIR) is the preferred method of infrared spectroscopy. In infrared spectroscopy or IR radiation is passed through sample. Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting of spectrum represents the molecular transmission and absorption, creating molecular fingerprint of the sample. Like fingerprint No. 2 unique molecular structures produce the same infrared spectrum. This makes infrared spectroscopy useful for identify unknown materials, determine the quality or consistency of sample and determine the amount of components in mixture (Thermo Nicolet, 2001).

MATERIALS AND METHODS

Materials

1. Glassware
2. Hot air oven
3. Autoclave
4. Laminar flow
5. Analytical balance (4 digits)
6. Glass bottles (330 ml)
7. Rotary shaker
8. Lyophilizer
9. Electron microscope
10. Rotary evaporator
11. Refrigerator
12. Separatory funnel
13. Thermocycler
14. Sieve mesh
15. 24 well plates
16. Baerman funnel
17. Thin layer chromatography (TLC) tank
18. Column chromatography
19. UV light
20. Nuclear Resonance Spectrometry
21. Plate sheet silica gel 60 F 254
22. Capillary tube
23. Electrophoresis
24. Reagents: Ethyl acetate, Hexane, Dichloromethane, Methanol, Petroleum ether, Acetone
25. Chemicals: Liquid nitrogen, Silica gel (200-300 mesh), Potato dextrose agar

METHODS

1. Genetic diversity of *Pleurotus* spp.

1.1 Mushroom isolation and culture

Nine samples of mushroom were isolated from eccentric stalk (five isolates from Ratchaburi and four isolates from Loei farm), an eccentric stalk is stalk that produces a wide cap, likes an oyster shell. An eccentric stalk of mushroom was isolated that produced a wide cap on the top. This tissue is pure culture for mushroom isolation. Then, the tissue was placed on potato-dextrose-agar (PDA) in a petri dish 9 mm in diameter, incubated at room temperature (25±3ºC)for 7 days and then stored at 4 ºC. Another isolates were supported from Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. Sixisolates were obtained from Assoc. Prof. Dr. Prapaporn Tangkijchote, Department of Horticulture, Kasetsart University, Kamphaeng-saen campus and two isolated were obtained from Mr.Chaichana Noonseang, M.S. student, Department of Horticulture, Kasetsart University, Kamphaeng-saen campus (Table 2).

Table 2 Description of mushroom isolates used in this study

Isolate	Mushroom species	Isolate code	Latitude	Location
1	*Pleurotus* spp.	PH-U	14º01'54.5"N99º58'11.6"E	Prapaporn
2	*Lentinus squarrosulas*	LS	14º01'54.5"N99º58'11.6"E	Prapaporn
3	*Pleurotus* spp.	PH-D1	14º01'54.5"N99º58'11.6"E	Prapaporn
4	*Lentinus polychrous*	LP	14º01'54.5"N99º58'11.6"E	Prapaporn
5	*Lentinus edodes*	LE3	17º30'11.9"N101º21'04.1"E	Loei fram
6	*Pleurotus eous*	PE2	13º51'00.9"N100º34'21.7"E	Departmentof Agriculture
7	*P. ostreatus*	Po (R) 8.1	15º00'52.9"N98º26'08.1"E	Ratchaburi farm
8	*P. sajor-cajou*	PC2x	13º51'00.9"N100º34'21.7"E	Departmentof Agriculture
9	*P. ostreatus*	Po (R) 9.1	15º00'52.9"N98º26'08.1"E	Ratchaburi farm
10	*L. edodes*	LE5	17º30'11.9"N101º21'04.1"E	Loei fram
11	*L. edodes*	LE2	17º30'11.9"N101º21'04.1"E	Loei fram
12	*P. eous*	PE1	13º51'00.9"N100º34'21.7"E	Departmentof Agriculture
13	*P. sajor-cajou*	PC1	13º51'00.9"N100º34'21.7"E	Departmentof Agriculture
14	*P. ostreatus*	Po (R) 9.2	15º00'52.9"N98º26'08.1"E	Ratchaburi farm
15	*P. eous*	Pea2	14º01'54.5"N99º58'11.6"E	Prapaporn
16	*P. ostreatus*	Poa	14º01'54.5"N99º58'11.6"E	Prapaporn
17	*P. ostreatus*	Po (R)	15º00'52.9"N98º26'08.1"E	Ratchaburi farm
18	*L. edodes*	LE1	17º30'11.9"N101º21'04.1"E	Loei farm
19	*P. ostreatus*	Po R*	15º00'52.9"N98º26'08.1"E	Ratchaburi farm
20	*P. ostreatus*	Poa3X2	14º01'54.5"N99º58'11.6"E	Chaichana
21	*P. ostreatus*	Poa3 (2)	14º01'54.5"N99º58'11.6"E	Chaichana

1.2 DNA extraction and analysis

1.2.1 Mycelia preparation and DNA extraction

Twenty one mushroom isolates were initially cultured on potato dextrose agar medium (PDA) in a petri dish, then transferred to a potato dextrose broth medium (PDB) by punching out 5 mm of the agar plate culture with sterilized cork borer, then shaking at 27°C for 3 days. Excess broth was decanted and transferred the remaining mycelia to a sterile tube to lyophilize for 8-10 h, prior to DNA extraction. The isolates of *Lentinus* spp. were used to compare with the *Pluerotus* mushroom.

The mycelia of mushroom were ground in liquid nitrogenand put in 1.5 mL eppendrof tube. The 0.5 mL of extraction buffer (50 mM Tris-HCL, 850 mM NaCl, 100 mM EDTA, 1% SDS) was added. The sample was incubated at 65ºC for 30 min, then ½ volume of phenol and ½ volume of chloroform were added, mixed, and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a new tube and extracted with an equal volume of chloroform: IAA (ratio 1:1). The contents were centrifuged at 13,000 rpm for 10 min. The genomic DNA was precipitated with 2 mL ethanol and incubated at -20 °C. After 1 h, the contents were centrifuged at 13,000 rpm for 10 min. The pellet was washed with 50-100 mL ethanol. The contents were centrifuged at 13,000 rpm for 10 min, repeated for 2 times and allowed to dry in the air. The pellet was resuspended in TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA). The genomic DNA was verified by 0.8% agarose gel electrophoresis for 45 min and viewed on UV-light after ethidium bromide staining. DNA concentrations were estimated and standardized against the known concentrations of DNA on 0.8 % (w/v) agarose gels. Aliquots from the DNA preparations were used for AFLP analyses.

1.2.2 AFLP marker

The AFLP analysis was according to previously reported data (Vos *et al*., 1995). The 5 µL of DNA were digested at 37°for 3 h with 5 U of *Eco*RI. Following complete digestion, the sample volume was filled up to 4 µL with buffer A (1X) and DNA was additionally digested with 5 U of *Mse*I for 3 h. The 1 µL of mixture (50 ρmol *Mse*I adaptator, 5 ρmol *Eco*RI adaptator and 1 U of T4 DNA ligase in buffer A) was added to each double digested DNA. Ligation products were diluted 10 times with sterile distilled water. The adapter molecules and nucleotides adjacent to the site of ligation serve as templates for the annealing of specific oligonucleotide primers used for polymerase chain reaction (PCR) amplifications.An initial amplification was carried out using the following:

*Eco*R I primer^{+}A: 5'-GACTGCGTACCAATTCA-3'
Mse I primer^{+}C: 5'-GATGAGTCCTGAGTAAC-3',

Amplifications were carried out in Applied thermal cycle polymers chain reaction (PCR) using the program adopted byLueangpraplut (2011). Amplification products were diluted 10 times in sterile distilled water and conserved at 4 °C. Pre-amplifications were conducted in 25 µL containing 0.7 µL of *Taq* polymerase, 7 µL of each *Mse* I and *EcoR* I primer, 17.5 µL dNTP, 1 U of *Taq* polymerase and 5 µl of ligated DNA. The pre-amplification cycle profile was as follows: incubated at 72 °C for 2 min, then cycled 20 times (denatured at 94 °C for 30 s, annealed at 56 °C for 1 min, extended at 72 °C for 1 min). The PCR product of pre-amplification was then used as template for selective amplification using 2 AFLP primers.

Selective amplifications were conducted in 20 µL containing 0.2 µL 1 U Taq polymearase, 1 µL of each Mse I and EcoR I primer, 2 µL dNTP, 1 U of Taq polymearase and 5 µl of DNA. Reactions were carried out in applied thermal cycler(Table 2)(PCR) using the program adopted. Selective amplification was performed as follows: one cycle of 94 °C for 2 min, 65 °C for 30 s and 72 °C for 2

min, followed by 23 cycles with annealing temperature decreasing by 1 °C each cycle starting with 94 °C for 30 s, annealed at 56 °C for 30 s, extended at 72 °C for 30 s and ended with 23 cycles of 65 °C for 1 min until 56 °C 1 min. Amplification products were subjected to electrophoresis on 5% denaturizing polyacrylamide gels using BioRad sequencing gel system. Gels were run in 1X TBE buffer at 2000 V for 2-3 h. Amplified fragments were visualized after silver staining.

1.2.4 Data analysis

For the diversity analysis, the data of binary data were converted as present (1) or absent (0) and used as a raw data matrix. A square symmetric matrix of genetic distance was obtained using Jaccard's coeffient. The dendrogram was then generated by UPGMA (Unweighted pair-group method using arithmetic average) with software program NTSYS V2.2.

Table 3 The primer combinations of PCR interaction (Selective amplification) by AFLP technique

***Mse*I**	***EcoR*I**
5'-GATGAGTCCTGTAGTA**G**-3'	5'-GACTGCGTACCAATTC**ACT**-3'
5'-GATGAGTCCTGTAGTA**GT**-3'	5'-GACTGCGTACCAATTCC**AC**-3'
5'-GATGAGTCCTGTAGTA**GT**-3'	5'-GACTGCGTACCAATTC**AG**-3'
5'-GATGAGTCCTGTAGTA**GTA**-3'	5'-GACTGCGTACCAATTC**A**-3'

2. Efficacy of mushroom mycelia, *P. ostreatus* isolate Poa3 for parasitizing egg mass of *Meloidogyne incognita*

2.1 Single egg mass and infective secondary juvenile stage (J2) of *M. incognita* preparation

The culturing method of the root-knot nematode,*M. incognita* was based on the references (Li *et al*., 2005; Dong *et al*., 2006). Root-knot nematode were collected from tomato field in Cha-am district, Phetchaburi province (12º41′49.5″N) (99º54′15.1″E) and were identified using the perennial pattern of female cuticle as described by Taylor and Netscher (1974). The root-knot nematode (*M. incognita*) was cultured on tomato seedlings in a greenhouse from a single egg mass for 45 days. Nematode eggs were extracted from infested roots using a 0.1% NaOCl (sodium hypochlorite) solution and the released eggs from the roots were collected using the modified technique described by McClure *et al*., (1973).

2.2 Egg parasitism test *invitro*

Mushroom isolates used in this study were the same as in item 1.1.

Pure culture of *Pleurotus ostreatus* isolate Poa3 was maintained on PDAat room temperature for 7 days. The margin of colonies of each Poa3isolate was cut by 5 mm cork-borer. One plug of each Poa3 isolate was placed at the center of water agar (WA) plates and grown for 7 days at room temperature. By this time, the fungi had radiated distance 3-3.5 cm from the plug. Four egg masses of *M. incognita* were placed at the edge of the colonies 1 cm apart. The plate was incubated at room temperature and periodically observed on egg mass infection and number of hatched J2 under a compound microscope at 6, 12, 24, 48, 72, 96 and 120 h compared with negative control.

3. Efficacy of *P. ostreatus* isolate Poa3 culture filtrates on egg mass

3.1 Media optimization

Four different media including potato dextrose agar or PDA (potato 200 g, dextrose 20 g and agar 15 g), PDA + 1% yeast (potato 200 g, dextrose 20 g, agar 15 g and 1% yeast), PDA + 1% malt extract (potato 200 g, dextrose 20g, agar 15 g and 1% malt extract) and Malt extract agar or MEA (malt extract 20 g, dextrose 20 g, peptone 1 g and agar 15 g) were preliminary used to determine the optimal nutrient for mushroom growth and production of bioactive compounds at 25ºC in petri-dishes. The results are reported as a relation radial growth of the hyphae. The radius was measured daily from the 2 to 6 days of incubation.

3.2 Preparation of *P. ostreatus* isolate Poa3 culture filtrate

From item 2.2 selected the best effective of Poa3 isolate maintained on PDA at room temperature for 7 days. The margin of colonies of Poa3 isolate was cut by 5 mm of cork-borer. One plug of Poa3 isolate was inoculated into 3 L of flask that containing 1L of 1/5 strength potato dextrose yeast broth PDYB and then incubated at room temperature for 3 and 12 months. In addition, the fungus in PDYB was maintained with shaking condition at 120 rpm only in the first month. After 3 and 12 months, the filtrates were first passed through 0.45 μm filter. The filtrates were used at concentrations of the original preparation (1X) and diluted 25/100, 50/100, 75/100 and 100 (Heydari *et al*., 2006).

3.3 Effect of isolate Poa3 culture filtrate on *M. incognita* egg mass

Egg mass of *M. incognita*, 1 group surface sterilized egg mass was seeded into 24 wells tissue culture plate and treated with 1 mL/well of culture filtrated dilution 25/100, 50/100, 75/100 and 100, respectively, compared with control (sterile distilled water) and abamectin® (10 ppm).The plate was incubated at room temperature and checked number of egg hatching at 6, 12, 24, 48, 72 and 96 h.

Percentage of egg hatching per egg masswere counted (Heydari *et al*., 2006). Seven replication of each treatment was calculated.

Based on the above studies, the best concentration of Poa3 isolates atvarious exposure times to infection *M. incognita* egg mass and was selected for next experiments.

3.4 Effect of culture filtrate of *P. ostreatus* isolate Poa3 on *M. incognita* infection of tomato roots.

Initial inoculum of nematodes and eggs of *M. incognita* was prepared after checked the results from 3.3 and inoculating a single egg-mass into a pot of tomatoes. Two months later, galls from tomato roots will be chopped to small pieces and put in pot-grown tomatoes for mass production of inoculum. After inoculation for 45 days, egg masses were investigated on root surface. The inoculated plants were uprooted and their roots are gently washed to free of soil and fine chopped. To estimate the number of eggs + J2 in the chopped tissue, all the eggs and J2 in the suspension will be counted. Data on the appearance of symptoms of nematode attack (root gall) are recorded during the experiment. Plants were uprooted and the roots are washed free of soil. Root infection by the nematode is assessed by estimating root gall severity (RGS) on a 0-5 scale: 0 = no galls; 1 = 1 to 5 galls; 2 = 6 to 20 small galls; 3 = more than 20 galls homogeneously distributed in the root system; 4 = reduce and deform root system with some large galls; and 5 = completely deformed root system with few but large galls (Di *et al*., 1979). Eggs and J2 in the egg-mass in roots will be extracted by sodium hypochlorite and counted. Final nematode population density will be calculated as the total number of nematodes from tomato roots.

In this experiment, each plate at 6, 12, 24, 48, 72, 96 and 120 h after inoculation egg mass and J2 was transferred to 10 days of tomato seedling. Seven replications for each treatment were performed.

3.5 To study the effect of *P. ostreatus* isolate Poa3 culture extract on *M. incognita* egg mass.

3.5.1 Preparation of *P. ostreatus* isolate Poa3crude extract

The best effective of Poa3 isolate from 2.2 was maintained on Potato dextrose agar (PDA) at room temperature for 7 days. The margin of colonies of Poa3 isolate was cut by 5 mm of cork-borer. One agar disk of Poa3 isolate was inoculated into 3 L of flask that containing 1L of 1/5 strength PDYB and then incubated at room temperature for 3 months.

The broth culture of Poa3isolate was extracted with 350 mL of ethyl acetate (EtOAc). The extraction process was repeated three times and combined. The excess solvent was evaporated using a rotary vacuum evaporator at 32-35 °C. Afterwards, samples were dried, and then kept at -4°Cuntil used for analysis.

3.5.2 Effect of *P. ostreatus* isolate Poa3crude extract on *M. incognita* egg mass

In this study, the culture method of *M. incognita*single egg-mass were used same as 2.1.

Crude extract of the best effective of Poa3 isolate was dissolved with acetone, diluted with distilled water, the concentration at 10, 100, 500 and 1000 ppm. Each treatment was replicated seven times, compared with sterile distilled water as control and nematicide. Dead and active nematodes were counted after 48, 72 and 96 h. Mean percentage mortality and the LC_{50} values will be calculated to quantify the nematcidal effects against nematode. The immobile, malformed or motionless juvenile when probed with a fine needle were considered to be dead.

4.2 Relationship between treated inoculum density and plant growth after applyingthe secondary metabolite of *P. ostreatus* isolate Poa3 compounds

In this study, the method was applied same as 3.4. Galling was investigated on root surface after inoculation for 45 days by treated inoculum with secondary compound.

4.3 Thin Layer Chromatography (TLC)

The fractions of Poa3extract were determined using TLC method with some modification (Kanagasabapathy *et al*., 2011). The separation was carried out at room temperature (25±3ºC). Crude extract of Poa3isolate 1 mg was dissolved in 1 mL of 95% ethanol. TLC separations were performed on silica gel 60 F254 (20 × 20 cm) covered plates with concentrating zone of 10 × 2.5 cm (Merck, Darmstadt, Germany). The solvent used was methanol and acetone. Chromatograms were run in glass tanks with chromatography paper equilibrated with the running solvent. The developed plates were dried at room temperature. TLC plate was visualized in UV light 254 nm.The identification of secondary metabolites parameter often used for qualitative evaluation is the R_f value. The R_f value can be calculated as:

$$R_f = \frac{\text{distance spot travels}}{\text{distance solvent travels}}$$

Where, the $R_f$value is the “retardation factor” or the “ratio-to-front” value expressed as a decimal fraction.

Fractions were pooled according to the spots on TLC plates. The excess solvent in the pooled fractions was evaporated under reduced pressure using a rotary evaporator. Components in the isolated fractions were identified using nuclear magnetic resonance (NMR) and fourier transform infrared spectroscopy (FTIR).

4. Isolation and identification of nematicidal substances from *P. ostreatus* isolate Poa3 extract

4.1 Isolation of nematicidal substances

The nematicidal substances of Poa3 wereisolated from crude extract by column chromatography (CC) method.The best isolate of Poa3 extractwas mixed with silica gel (0.063-0.200 nm; 200-300 mesh; 10 g) (Merck). This mixture was dried at room temperature to yield a powdery consistency

The powdered mixture ofPoa3 isolate and silica were subjected to column chromatography initially eluting with 100% petroleum ether followed by petroleum ether enriched with increasing percentages of acetone ratio 100:0 to 20:80 v/v, then followed by acetone enriched with increasing percentages of methanol ratio 95:5 to 100:0 v/v. Fractions of 300 mL volume were collected in numbered bottles.

Each fraction of Poa3 compound was test by nematicidal activity assay.The excess solvent in the pooled fractions was evaporated under reduced pressure using a rotary evaporator. Crude extract of each fraction was dissolved with acetone, diluted with distilled water, the concentration at 100, 500 and 1000 ppm. Each treatment was replicated seven times, compared with sterile distilled water and abamectin (nematicide)as control. The plate was incubated at room temperature and check number of dead juvenile at 24, 48, 72, 96 and 120 h.

To evaluate the nematicidal substances, the three best fractions on inhibiting egg mass hatching were selected as representative substance for nematicidal substances which had highest effect to inhibit egg mass hatching of *M. incognita*. The study on *M. incognita* egg mass hatching were tested on J2 in 24 well tissue culture plate at the method described in 4.2 concentration at 100, 500 and 1000 ppm. A 100 µL of J2 suspensions (200 J2/100 µL).

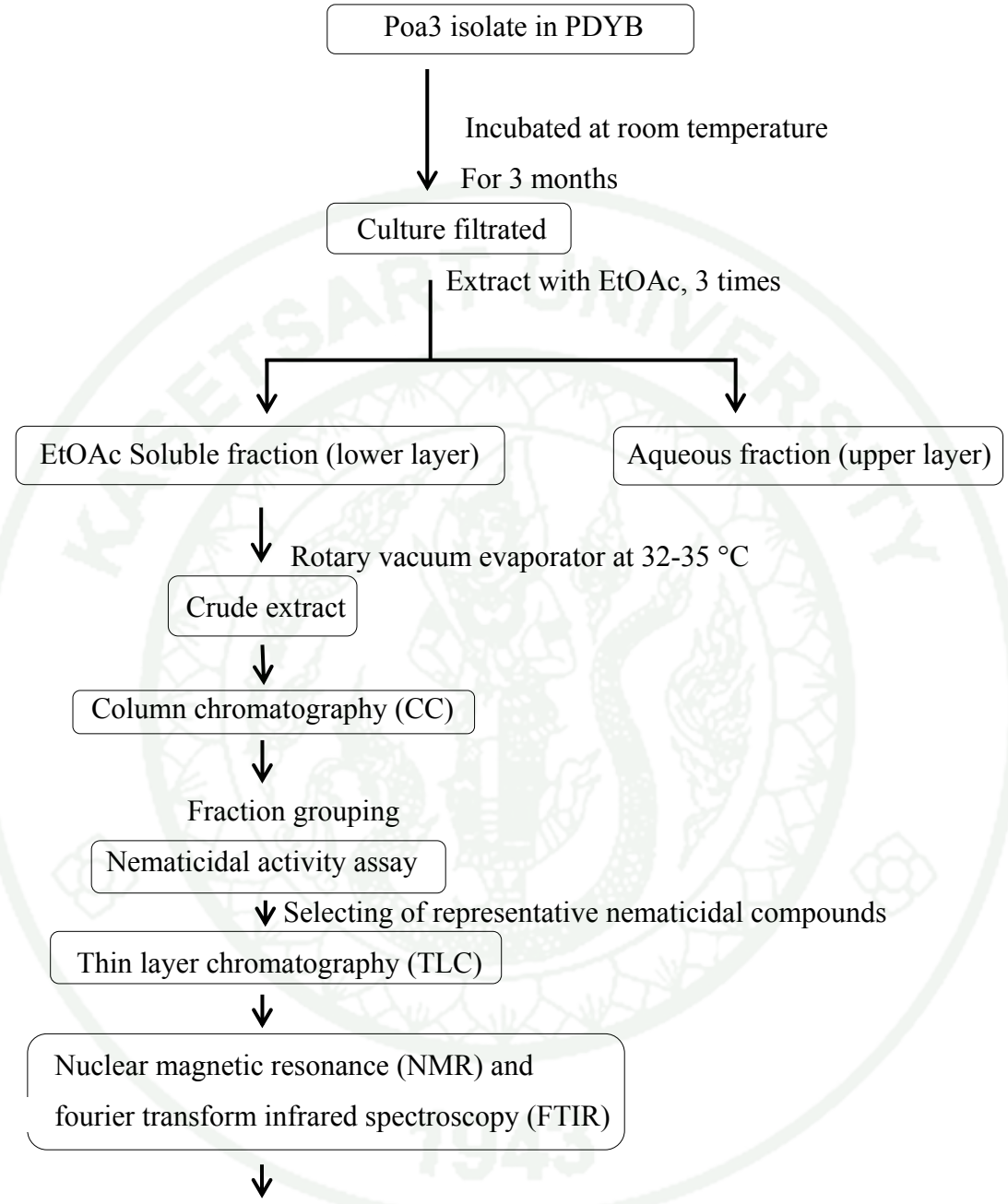


Figure 6 Schematic representative of Poa3 compounds isolation and identification

RESULTS AND DISCUSSION

Results

1. Genetic diversity of *Pleurotus* spp.

1.1 Mushroom species

Twenty-one isolates of *Pleurotus* spp. and *Lentinus* spp. were isolated from 5 locations samples from locations with 2 different genus of mushroom (Table 3) on Potato dextrose agar, all isolates grew slowly and produced white colony but after conidia produced they show yellow drop on top colony (Fig. 7).

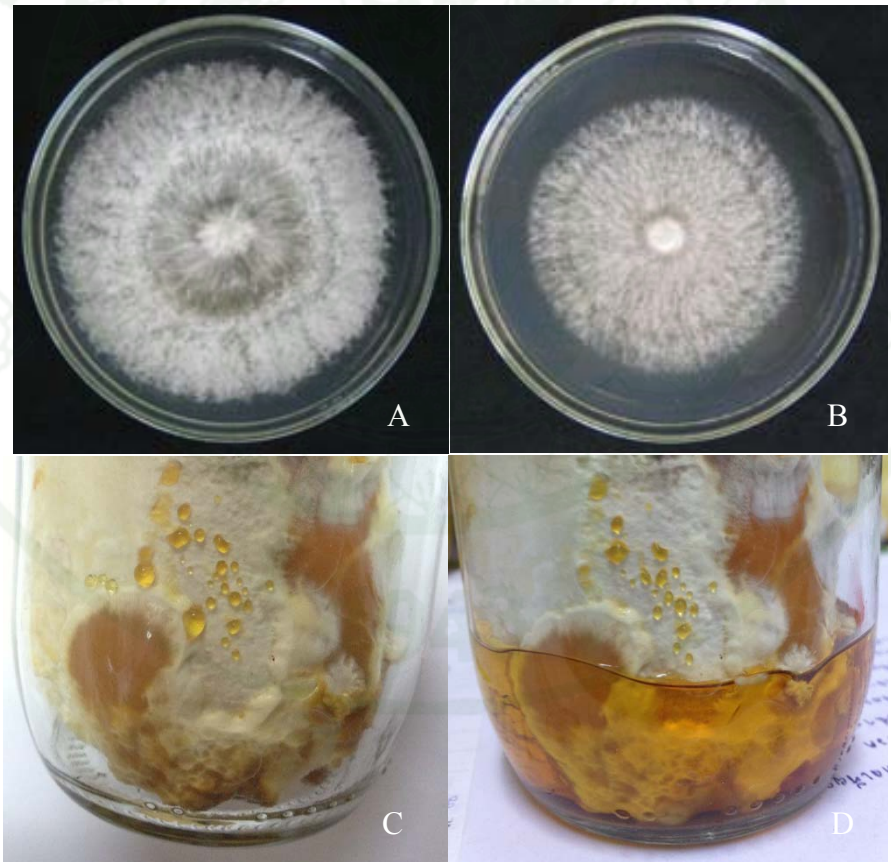


Figure7 Colony and droplet of *Pleurotus ostreatus* and *Lentinus edodes*on potato dextrose agar (PDA) for 7 days, A: Colony of *Pleurotus ostreatus*, B: Colony of*Lentinus edodes*, C and D:yellow droplet on top colony.

1.2 DNA extraction and analysis

Twenty one mushroom isolates were used in this study, as described in Table 3. Genetic diversity was studied by AFLP marker with four primer combinations as E+GTC/M+A, E+ACG/M+G, E+GTC/M+A and E+G/M+ACG (table 4 of polymorphic band). 112 polymorphic bands were observed (Fig 8-13).

The result of cluster analysis was shown obvious difference among isolates. 112 polymorphic bands were observed. The UPGMA dendrogram was divided into eleven groups at 55% similarity coefficient with Dice similarity coefficient and matrix correlation (r) value = 0.83817. First group was genus *Pleurotus* spp. as PH-u isolate and PH-ud isolate from Prapaporn, Second group was genus *P. ostreatus* as Po3x2 and Po3 (2) from Chichana, Third group was genus*P. sajor-cajou* as PC2x, PC1 isolate and *P. eous* as Pea2 and PE 2 isolate from Department of Agriculture, Fouth groups were genera*Lentinus edoles* as LE 5 isolate and LE 2 isolate from Loei farm, Fifth group was genus *P. ostreatus* as PO isolate from Prapaporn, Six and Seven group were genus *L. edodes* as LE1 and LE2 isolate fromLoei farm, Eight groups were*P. ostreatus* as POr isolate from Ratchaburi farm, Nine group is *L. edodes* as LE3 isolate fromLoei farm, Ten groups were POr 9.2 isolated from Ratchaburi farm and finally group is *Lentinus polychrous* as LP isolate and *P.ostreatus* as POr 9.1 from Ratchaburi farm.

Table 4 The total of polymorphic bands and total bands which analysis from AFLP with 4 primer combinations

Primer recombination	Total bands	Polymorphic bands
E+GTC/M+A	35	32
E+ACG/M+G	39	36
E+A/M+GTC	31	29
E+G/M+ACG	41	38

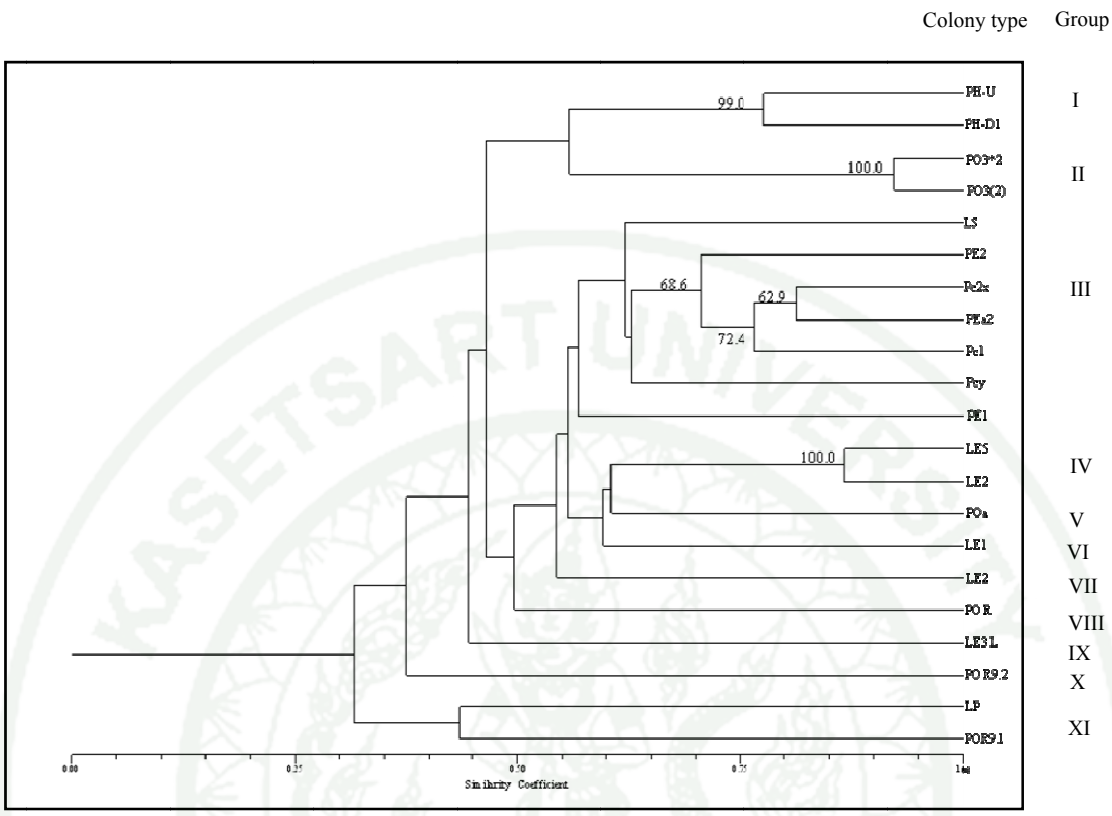


Figure 8 UPGMA dendrogram of 21 mushroom isolates constructed from the analysis of AFLP data obtained from *Eco*RI - *Mse*I primers[1]R: isolate

Figure 9 AFLP fragments obtained from 21 mushroom isolates from PCR with *Mse* I and *EcoR* I primers (E+G/M+ACG primer recombination)

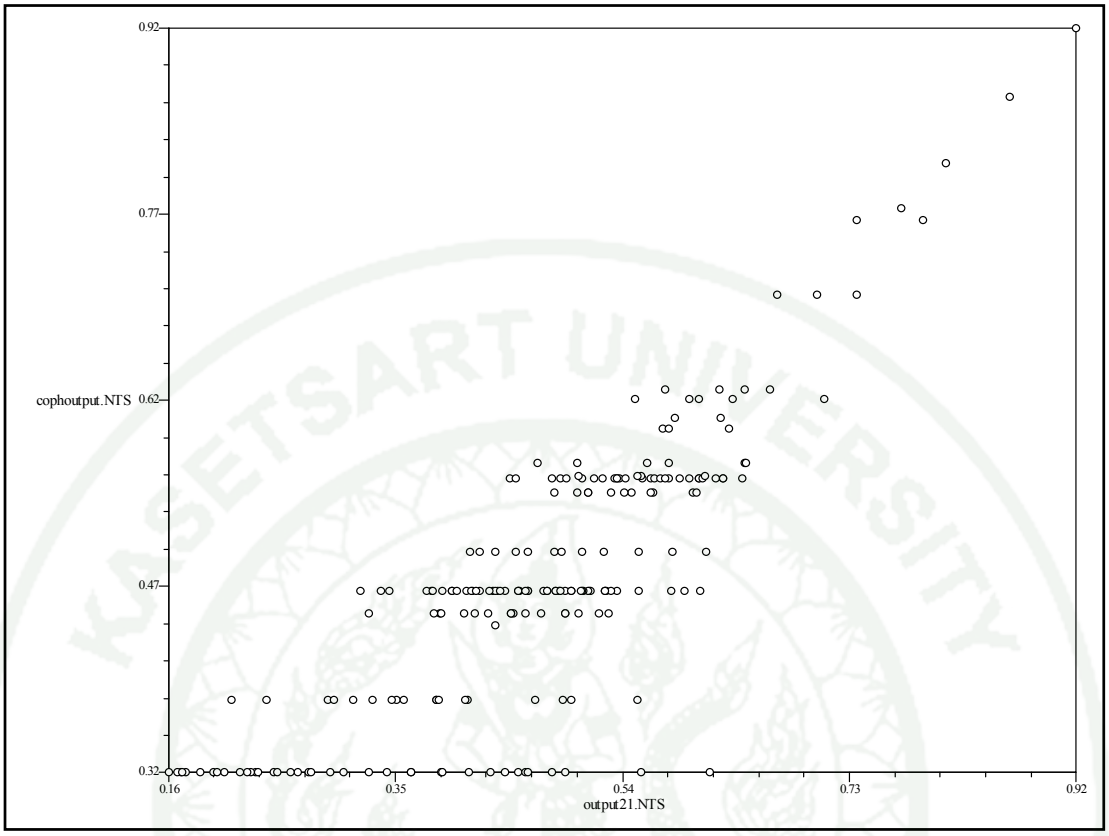


Figure 10 Distribution of data to group the cophenetic correlation 0.83817, the similarity and clustering were measured with NTSYS pc version 2.2 program

2. Efficacy of Poa3 mycelia for parasiting egg mass of *Meloidogyne incognita*

2.1 Single species and infective secondary juvenile stage (J2) of *M. incognita* preparation

The *M. incognita* was originally obtained from Phetchaburi province. They originated from secondary stage juvenile and single egg masses. Their species designation was confirmed using morphological characters of adult female perineal pattern (Fig. 14 A). The single egg massesof *M. incognita* pattern have a distinct high squarous dorsal arch (Fig. 14B). Some striae fork near the lateral lines but distinct lateral incisures are not present. *M. incognita* second stage juveniles have a dumbbell shaped labial disc and medial lips in face view.

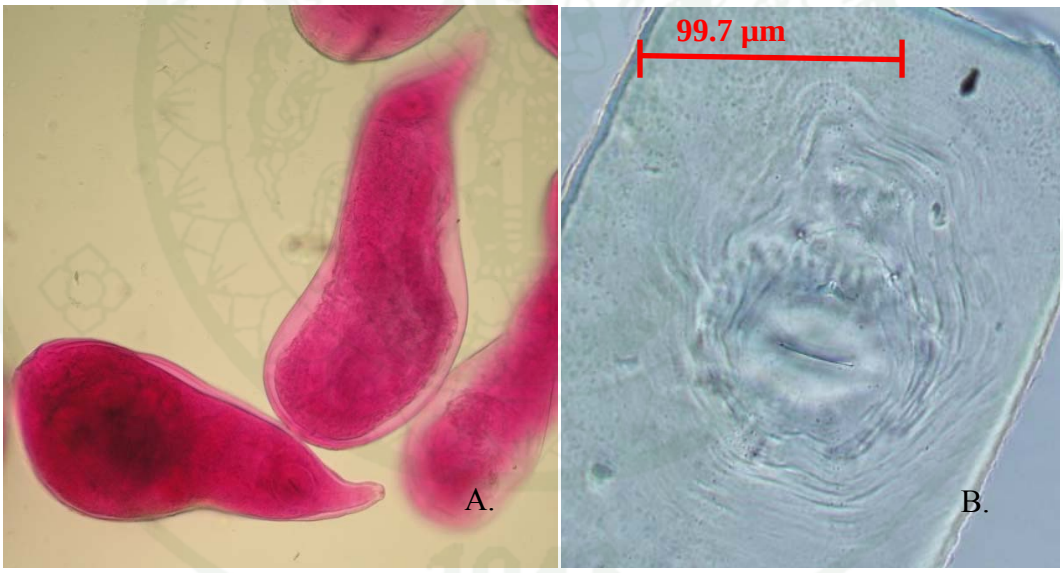


Figure 11 The *Meloidogyne incognita*morphometric characters of perineal pattern**,** A: secondary J4, B: perineal pattern.

2.2Egg parasitism efficacy of 18 mushroom isolates *in vitro*

Eighteen mushroom isolates were used for infectivity test *in vitro*. The efficacy of mushroom cultures on *M. incognita* J2 infection at different times was observed(as shown in Table 5). Results showed that after 96 h *P. ostreatus*; Poa2 isolate had the maximum infectivity on *M. incognita*J2 37.37%, while Poa3 and Poa1 isolate were infected on *M. incognita*J2 37.25 and 35.75%, respectively. For *L. edodes*; Le1, Le2, Le5, Le6, Le4 and Le3 isolate were infected on *M. incognita*J2 36.87, 34.62, 34.12, 32.37 and 28.50%, respectively. *P. sajor-caja, P. cystidious* and *P. eous* were infected on *M. incognita*J2 ranged from 0-2.12%.

In addition, after 24, 48 and 72 h of mushroom cultures had the most effective to infected *M. incognita* J2 is Poa3 isolate following Poa1, Poa2, Le1, Le2, Le3, Le4, Le5 and Le6 isolate, respectively ranged from 3.25-28.00%. All infectivity test, mushrooms hyphae had the most damaged on *M. incognita*J2 after 96 h following 72, 48 and 24 h, respectively. While, after 5 and 12 h mushrooms hyphae could not be colonized *M. incognita*.

Moreover, secretory processes were observed on aerial hyphae of all tested mushroom isolates. Only the round outlines of the toxin droplets were visible (Fig. 14). *M. incognita* moved without any damaged over the young hyphae at the periphery of the colony, where there were no toxin droplets were absent. After nematodes touched droplets of *P. ostreatus* Poa3 on the older parts of the colony. They were usually shrinkage immediately. By one or more contact, *M. incognita* became inactive and after 24-96 h. It was noticed that the hyphae of the mushrooms had grown toward the nematodes penetrating them through body orifices and their mouth (Fig. 15A – 15H). The body contents were digested within 3-4 days after infection (Fig. 15I and Fig. 15J). All mushroom isolates tested caused similar symptoms on nematodes, but length of exposure time and the number of contacts required for appearance of these different symptoms were varied between mushrooms tested.

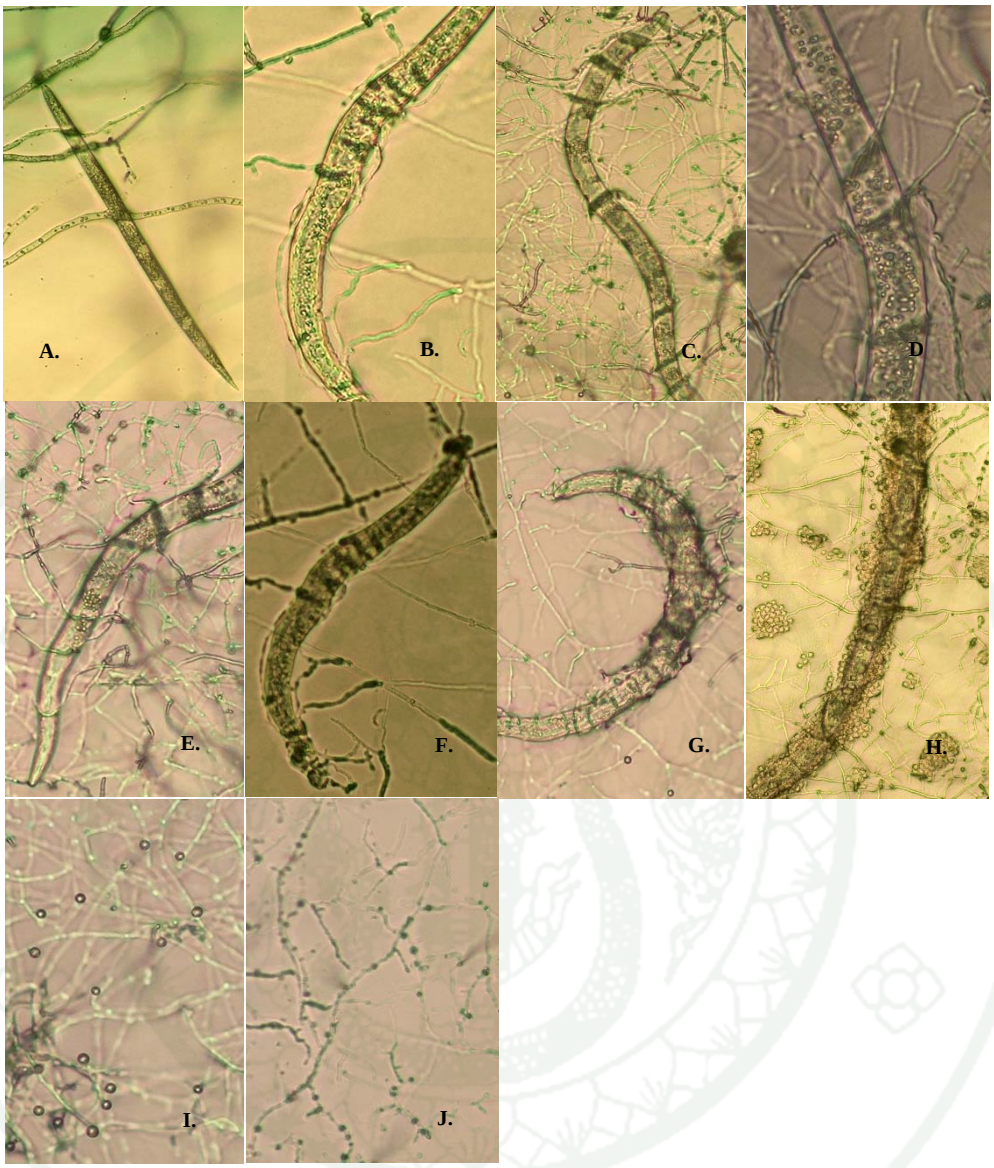


Figure 12 Nematode trapping structure of *P. ostreatus* and nematodes on water agar (WA) cultures,A, B, C, D, E and F:*M. incognita* J2 infected and colonized by hyphae of *P. ostreatus*, Gand H:*M. incognita* J2 lysis after infected by hyphae of *P. ostreatus*, I and J: toxin droplets on aerial hyphae, viewed with cover slip under compound microscope.

Table 5 Infection of percentage on2nd stage juvenile of root-knot nematode, *Meloidogyne incognita* byisolates of *Pleurotaceae* at 24, 48 72 and 96 h after inoculation with root-knot nematode

Isolate	Percentage of mushrooms mycelia infectivity at various incubation periods (h)					
	5 h	12 h	24 h	48 h	72 h	96 h
Le1	0.00 n[1/]	0.00 n	3.25 m	12.37 j	25.37 ef	36.87 a
Le2	0.00 n	0.00 n	4.37 k	11.00 j	22.50 g	34.62 b
Le3	0.00 n	0.00 n	4.62 k	11.12 j	19.75 h	28.50 d
Le4	0.00 n	0.00 n	5.00 k	10.87 j	22.25 g	32.37 c
Le5	0.00 n	0.00 n	5.00 k	11.00 j	23.50 fg	34.12 bc
Pe1	0.00 n	0.00 n	0.00 n	0.00 n	0.00 n	0.87 n
Pe2	0.00 n	0.00 n	0.00 n	0.00 n	0.00 n	1.62 mn
Pe3	0.00 n	0.00 n	0.00 n	0.00 n	0.12 n	1.00 n
Pc1	0.00 n	0.00 n	0.00 n	0.00 n	0.25 n	0.87 n
Pc2	0.00 n	0.00 n	0.00 n	0.00 n	0.12 n	0.87 n
Pcy1	0.00 n	0.00 n	0.00 n	0.00 n	0.00 n	0.00 n
Pcy2	0.00 n	0.00 n	0.00 n	0.00 n	0.00 n	0.00 n
Pcy3	0.00 n	0.00 n	0.00 n	0.00 n	0.00 n	0.00 n
Pea1	0.00 n	0.00 n	0.00 n	0.00 n	0.00 n	2.12 mn
Poa1	0.00 n	0.00 n	5.37 k	16.87 i	26.75 de	35.75 ab
Le6	0.00 n	0.00 n	4.87 k	11.37 j	22.50 g	34.12 bc
Poa2	0.00 n	0.00 n	6.25 k	12.75 j	23.75 fg	37.37 a
Poa3	0.00 n	0.00 n	5.75 k	17.37 i	28.00 d	37.25 a

[1/]Mean followed by different letters in row and column are significantly ($p< 0.05$) different from each other.

2.3 Egg parasitism test of Poa3 *in vitro*

P. ostreatus isolate Poa3 were selected from mortality test of *M. incognita* second juveniles stage (J2) onPoa3 cultures. Isolate Poa3 showed activity to increase J2 mortality lower than Poa2 but grew well and time to culture in medium faster than other isolate. In this study, the results showed that there were significant differences ofhatching of *M. incognita* eggs at different time intervals. Root-knot nematode,*M. incognita* was infected by the mycelium of *P. ostreatus*Poa3 after 12 hours of inoculating the fungal culture with egg suspension. The fungus infected eggs with its hyphal tips and colonized them and then digested their contents completely, thereafter it was able to form new hyphae on other distant parts of the mycelium (Fig. 16). The Poa3 isolate tested parasitized *M. incognita* eggs *in vitro*. The fungi were also observed as developing in egg masses.The *M. incognita*eggswere rapidly invaded by Poa3 hyphae, and themycelium was proliferating within eggs 12-120 h after first contact (Fig. 16), compared with water control (Fig. 15A). On the transfer of nematodes egg mass to Poa3 culture. There was only 14% of infection of *M. incognita* eggs after 12 h exposure to Poa3 culture. After 72-120 h exposure time 100% Poa3 of *M. incognita* eggs were completely infection when compared to control(Table6).

The *M. incognita* egg mass were hatching after infection by *P. ostreatus* Poa3 measured at different times (Table 6). After exposure for 6 to 24 h exposure time, the fungal cultures of *P. ostreatus* Poa3 inoculated with the *M. incognita* had to increased egg mass hatchingand after 48 to 96 h hatch egg mass were decreased, when compared to control.

Table 6 Percentage of infectiononegg mass of root-knot nematode,*Meloidogyne incognita* by *Pleurotus ostreatus* isolate Po3 at 12, 24, 48 72 96 and 120 h after inoculation with root-knot nematode

Treatment	Period (h)	Percentage of infection	
		Mycelial infectivity	Egg mass hatching
Control	12	0 e[1/]	3 h
	24	0 e	28 e
	48	0 e	60 d
	72	0 e	80 c
	96	0 e	96 b
	120	0 e	135 a
Poa3	12	0 e	1 h
	24	14 d	15 g
	48	43 c	22 f
	72	75 b	16 g
	96	100 a	15 g
	120	100 a	13 g

[1/]Mean followed by different letters in each column are significantly ($p< 0.05$) different from each other.

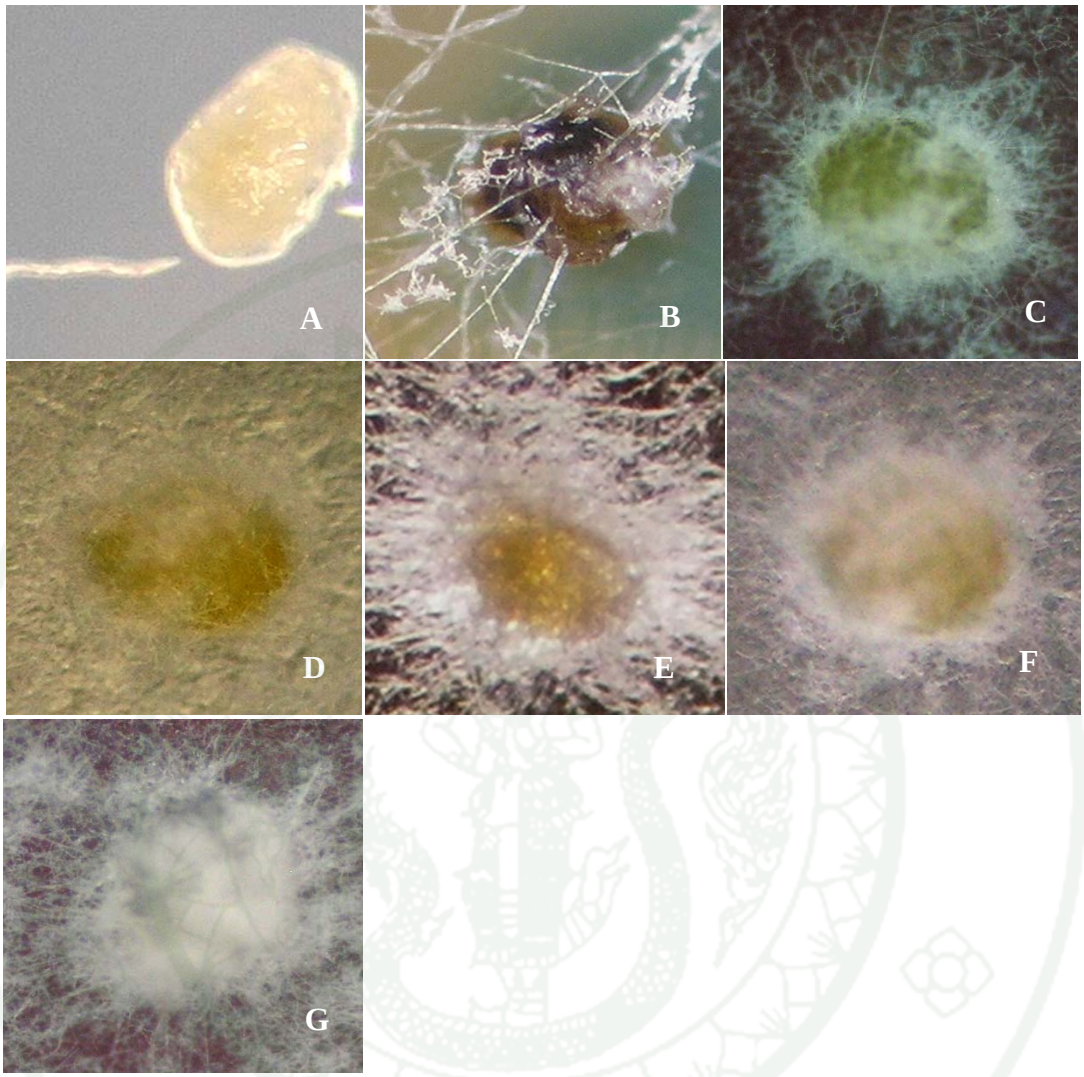


Figure 13 Effect of *Pleurotus ostreatus* cultures on *Meloidogyne incognita* egg mass infection at different incubation periods, A: Control, B, C, D, E, F and G: penetration of Po3 hyphal inside the *M. incognita* egg mass after 12, 24, 48, 72, 96 and 120 h.

3. Efficacy of *P. ostreatus* isolate Poa3 culture filtrates on egg mass

3.1 Media optimization

In this study was observed the radial growth rate of *P. ostreatus* isolate Poa3 on different media (Table 7). This data showed that Poa3 had the highest mycelial growth rate on media containing PDA + 1% yeast extract (9.00 cm at 6 days), following PDA + 1%malt extract, PDA and malt extract, respectively.

Table 7 Radial growth rate (cm/d) of filamentous fungi grown from1 to 6 days at 25ºC on different culture media

Medium	Radial growth rate (cm/d) of filamentous fungi					
	1	2	3	4	5	6
PDA	1.19 ab[1/]	2.40 ab	3.74 b	5.58 ab	7.50 b	8.53 b
PDA+1% yeast extract	1.23 a	2.57 a	4.23 a	6.11 a	7.73 a	9.00 a
PDA+1% malt extract	1.08 b	2.23 b	3.63 b	5.13 b	6.93 b	8.88 a
Malt extract	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c

[1/] Mean followed by different letters in each columnare significantly ($p< 0.05$) different from each other.

3.2 Effect of *P. ostreatus* isolate Poa3 culture filtrate on *M. incognita* egg mass

The hatchability test (as shown in Table 8) indicated that Poa3 culture filtrates strongly suppressed hatching of *M. incognita* egg masses in various times. The culture filtrate of Poa3 at 25/100 dilution; exhibited hatching of *M. incognita* egg masses 0-70 and nematicide at concentration 10-1000 ppm, exhibited similar toxic activities to hatching of *M. incognita* egg masses 0-52 after an exposure period of 12 h and all of them after 96 h. The different concentrations of culture filtrate exhibited significant differences compared to water control. After 12-96 h, abamectin showed the number of egg hatched less than *P. ostreatus* Poa3 filtrate and control.

Table 8 Effect of *Pleurotus ostreatus* isolate Poa3 culture filtrates on *M. incognita*J2hatching at various exposure times

Treatment	Dilution	Egg hatch rate					
		6 h	12 h	24 h	48 h	72 h	96 h
Control	0	0.0 a[1/]	10.7 a	20.1 a	40.5 a	71 a	107 a
abamectin	10 ppm	0.0 a	4.1 b	12.8 c	29.1 c	42.7 c	52 c
	50 ppm	0.0 a	1.4 cd	7.8 d	20.4 e	29.8 e	35.4 e
	100 ppm	0.0 a	0.5 de	3.4 e	10.4 f	19.4 f	27.2 f
	500 ppm	0.0 a	0.1 e	0.4 f	4.2 g	10 g	18.7 h
	1000 ppm	0.0 a	0 e	0 f	1 h	5.1 h	9.8 i
Poa3	25/100	0.0 a	5 b	16.8 b	34.2 b	56.4 b	70 b
	50/100	0.0 a	2.1 c	9.4 d	25.7 d	34.5 d	43.5 d
	75/100	0.0 a	1 cde	4.2 e	12.5 f	22.1 f	32.1 e
	100	0.0 a	0.4 de	1.1 f	5.2 g	11 g	23.4 g

[1/]Mean followed by different letters in each columnare significantly ($p< 0.05$) different from each other.

3.3 Effect of culture filtrate of *Pleurotus ostreatus* on *Meloidogyne incognita* infection of tomato rootsat different times

Forty-five days after inoculation with *M. incognita*, the culture filtrates of Poa3 reduced the gall index and number of nematodes egg mass in the root after exposure each period compared with the water control. The results showed that Poa3 filtrate reduced number of root galls and nematodes egg number after exposure period of 72 to 120 h when compared with other period and control (Table 9). There was significance between control and other period. The effect on nematode egg mass and galling inside roots clearly demonstrated that Poa3 filtrate inhibited the hatching of nematode egg mass.

Table 9 Effect of *Pleurotus ostreatus* isolate Poa3cultures on *Meloidogyne incognita* infection at different incubation periods

Treatment	Incubation (h)	Index of investigation	
		Number of galls	Egg mass in roots
Control	12	98 ab[1/]	21 ab
(inoculate)	24	98 ab	22 a
	48	104 a	20 abc
	72	97 b	19 bc
	96	99 ab	18 c
	120	100 ab	10 abc
Poa3	12	48 c	4 d
	24	51 c	4 d
	48	28 d	2 e
	72	1 e	0 e
	96	0 e	1 e
	120	0 e	2 e
Control (healthy)	0	0 e	3 e

[1/] Mean followed by different letters in each column are significantly ($p< 0.05$) differentfrom each other.

3.4 Effect of *P. ostreatus* isolate Poa3 extract on *Meloidogyne incognita* egg mass hatching

The effect of *Pleurotus ostreatus* isolate Poa3 extract on hatching of *M. incognita* egg mass between water and abamectin control. The result showed that the egg mass of *M. incognita* when treated with Poa3 extract after 6-96 h the nematode hatching was increased (Table 10). In contrast, after treated nematode with Poa3 extract at concentration 50, 100, 500 and 1000 ppm nematode egg mass hatching was decreased. A similar toxicity was observed in abamectin control.

Table 10 Effect of *P. ostreatus* isolate Poa3 extract on hatching of *Meloidogyne incognita* egg massat various exposure times

Treatment	Concentration (ppm)	Nematode hatching					
		6 h	12 h	24 h	48 h	72 h	96 h
Control	0	0.0 f	7.8 e[1/]	16.6 d	40.4 c	67.6 b	105.0 a
abamectin	50	0.0 d	0.0 d	1.0 d	13.2 c	21.2 b	25.2 a
	100	0.0 c	0.0 c	0.4 c	3.0 b	4.0 b	13.0 a
	500	0.0 c	0.0 c	0.2 c	1.4 b	2.2 b	7.0 a
	1000	0.0 d	0.0 d	0.0 d	0.6 c	1.2 b	3.0 a
Poa3	50	0.0 e	0.8 e	11.6 d	28.0 c	41.0 b	57.4 a
	100	0.0 e	0.2 e	1.6 d	17.8 c	27.8 b	35.0 a
	500	0.0 d	0.0 d	0.2 d	6.0 c	15.4 b	18.2 a
	1000	0.0 d	0.0 d	0.0 d	2.0 c	6.0 b	11.2 a

[1/] Mean followed by different letters in each row are significantly ($p< 0.05$) different from each other.

4. Isolation and identification of nematicidal substances from mushroom

4.1 Isolation of nematicidal substances

Cultivation of *P. ostreatus* isolate Poa3 on PDA+ 1%yeast extract medium. isolation the nematicidal organic compounds by column chromatography (CC) were delivered 14 fractions (Table 11). All components present in fractions were evaporated to dryness and tested for nematicidal activity. The result showed that *M. incognita* had highest mortality after treated J2 with abamectin at several times.While, fractions 12 at 100 ppm after exposure period from 24-120 h had more effects on mortality of *M. incognita*, following fractions 11 and 13, respectively (Table 11).

Table 11 Mortality of juvenile secondary stage (J2) of *Meloidogyne incognita* from eachfraction of nematicidal organic compound

Treatment	Mortality of *M. incognita*J2				
	24 h	48 h	72 h	96 h	120 h
Control	0.0 i[1/]	0.0 j	0.0 k	0.0 l	0.0 k
Abamectin	45.6 a	58.4a	72.4 a	95.0 a	119.2 a
Fr 1	0.0 i	0.0 j	0.4 jk	0.8 kl	1.8 jk
Fr 2	0.2 i	0.8 ij	0.8 jk	2.2 jkl	2.6 ijk
Fr 3	0.4 i	1.0 ij	1.4 ijk	2.8 ijkl	3.2 ijk
Fr 4	0.8 hi	1.4 hij	2.0mijk	3.6 ijk	4.0 hij
Fr 5	1.2 hi	1.6 hij	2.8 hij	4.4 hij	5.0 hij
Fr 6	1.8 hi	2.6 hi	4.0 hi	5.6 ghi	6.0 hi
Fr 7	2.8 gh	3.4 h	5.0 gh	6.8 gh	6.8 h
Fr 8	4.6 g	6.0 g	6.8 g	8.0 g	10.6 g
Fr 9	7.6 f	10.8 f	13.4 f	15.0 f	20.6 f
Fr 10	11.0 e	15.2 e	21.8 e	24.8 e	29.0 e
Fr 11	18.0 d	22.2 d	30.2 d	37.6 d	42.8 d
Fr 12	28.2 b	36.0 b	43.0 b	62.2 b	80.0 b
Fr 13	24.0 c	32.6 c	38.8 c	45.0 c	68.6 c
Fr 14	9.4 ef	11.0 f	12.6 f	17.2 f	21.8 f

[1/] Mean followed by different superscript letters in column are significantly ($p< 0.05$) different from each other.

4.2 Effect of *P. ostreatus* isolate Poa3secondary compounds on juvenile secondary stage (J2)of *M. incognita*

The three best fractions (Fr. 11, 12 and 13) from above studied were tested for toxicity. The results showed that every treatment at several periods of experiments abamectin concentration 100-1000 ppm had the highest effects on mortality of *M. incognita* J2 following fractions 12, Poa3crude extract, Poa3culture filtrate, fraction 11 and 13, respectively at 20-120 h (Table 12 and Fig. 17).

Table 12 Mortality of juvenile secondary stage (J2) of *Meloidogyne incognita* from column chromatography (CC)fractions.

Treatment	Concentration (ppm)	Mortality of *M. incognita* J2				
		24	48	72	96	120
Control	0	0 q	0 q	0 q	0 q	0 q
Abamectin	100	18 i	31 h	41 e	54 b	74 a
	500	23 k	40 hi	54 g	73 c	85 a
	1000	47 o	66 l	88 h	108 d	128 a
Fr. 11	100	3 o	10 mn	14 jkl	32 gh	44 d
(10%MeOH/Acetone)	500	17 lm	28 j	41 hi	66 e	72 c
	1000	37 p	51 o	68 l	82 i	100 f
Fr.12	100	4 o	12 klm	15 ij	36 f	48 c
(30%MeOH/Acetone)	500	20 kl	31 j	44 h	70 cd	81 b
	1000	40 p	55 n	72 k	95 g	122 b
Fr.13	100	3 o	9 n	11 lmn	30 h	40 e
(50%MeOH/Acetone)	500	15 m	31 j	38 i	62 f	72 c
	1000	36 p	49 o	60 m	77 j	36 fg
Poa3 (Crude extract)	100	3 o	10 mn	14 jk	34 fg	46 d
	500	16 m	29 j	42 hi	67 de	79 b
	1000	36 p	49 o	70 kl	90 h	117 c
Poa3 (Culture filtrate)	100	3 o	11 klmn	14 jkl	34 fg	46 cd
	500	16 m	28 j	40 hi	67 de	79 b
	1000	36 p	50 o	69 kl	89 h	115 c

[1/] Mean followed by different letters in each column are significantly ($p< 0.05$) different from each other.

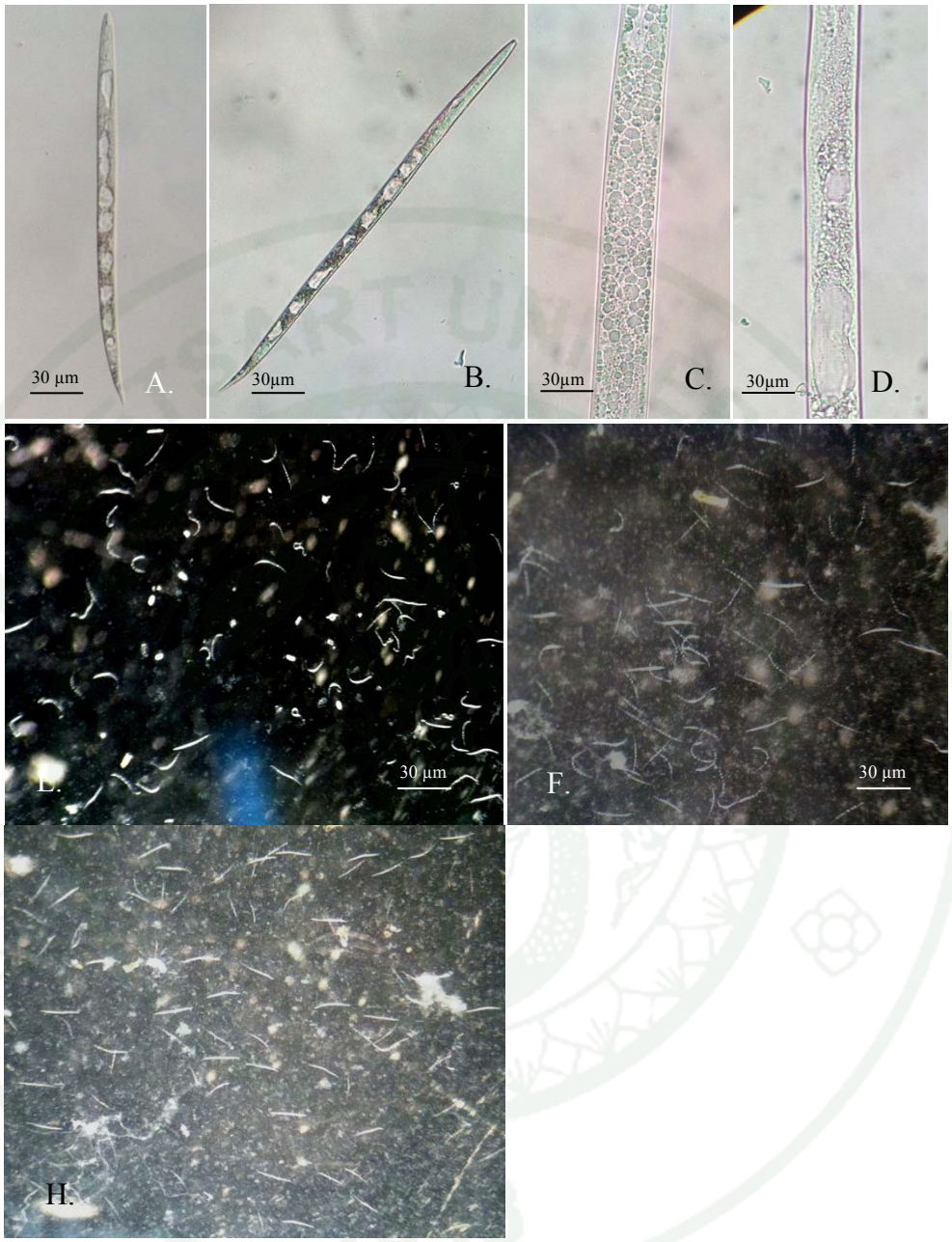


Figure 14 The *Meloidogyne incognita* juvenile 2 after treated with *P. ostreatus* Isolate Poa3 extract, A: Dead J2 of *M. incognita* caused by lacking nutrient, B, C and D: Dead of J2 of *M. incognita*caused by Poa3 extract, E: Non treated, F and H: Treated with Poa3 extract.

4.3 Nematicidal efficacy assay on infection

The bio-control efficiency of *P. ostreatus* isolate Poa3 fractions 11, 12 and 13 had high parasitism and strong nematicidal activity *in vitro* compared with control (untreated) were observed in greenhouse. The comparison secondary metabolite products of nematicidial Poa3 to management of *M. incognita* was accessed from the reduction in root galling expressed in terms of galling index. The result showed that both of fractions 12 and 13 could be reduced number of root galls (23.6 and 27.4) and number of egg mass in plant roots (4.2 and 6.0) compared with control (untreated nematode) (Table 13 and Fig. 18G and 18H). Moreover, activity of fractions 12 and 13 had efficiency less than abamectin compared among treatment.

Table 13 The efficacies of nematicidal activity of each concentration of fractions from Poa3on controlling root-knot formation of tomato at 45-day- old, under screenhouse condition.

Treatment	Investigation index	
	Number of gall	Number of egg mass
Abamectin	13.0g	6.4e
Culture filtrate	29.4cd	11.0c
Crude extract	29.8 c	11.8b
Fr. 11	37.0 b	8.4d
Fr. 12	23.6f	4.2g
Fr. 13	27.4e	6.0f
Control (nematode treated)	80.4a	30.8a
Control (nematode untreated)	0.0 h	0.0 h

[1/] Mean followed by different superscript letters in each column are significantly ($p< 0.05$)different from each other.

Figure 15 Galling index of 45-day-old tomato root,A: control non-inoculated J2, B: control inoculated with J2 of root-knot nematode, C: treated with abamectin, D: Poa3 crude extract E: Poa3 filtrate, F: fractions 11 G: fractions 12 H: fractions 13.

4.4 Relationship between inoculum density and plant growth after submerge egg mass in the secondary metabolite of *P. ostreatus* isolate Poa3

Based on above studies in 4.1, the Poa3 concentration at 1000 ppm at various times to infection *M. incognita* egg mass was selected. In this study indicated that the applicationof the secondary metabolite of Poa3, fractions 12 from column chromatography (CC) could reduce activity of *M. incognita* hatch egg compared with control, which is one of the important indices in disease diagnosis. The different time from 24-120 h after treated J2 with Poa3 fractions 12 we found an increasing of egg mass hatching. A linear relationship was proved between increasing concentration and the percentage of paralyzed nematodes (Table 14 and Fig. 19).

Table 14 Percentage of egg mass hatching and infection of plant of *Meloidogyne incognita*

Treatment	Period (h)	Investigation index	
		Egg mass hatching	Infection of plant roots
Abamectin	24	3 i[1/]	23 efg
	48	9 h	17 gh
	72	26 g	13 hi
	96	36 f	9 ij
	120	45 e	3 j
Fractions 12	24	4 i	30 e
	48	10 h	23 efg
	72	26 g	18 fgh
	96	39 f	14 hi
	120	58 d	5 j
Control	24	6 h	24 ef
	48	37 f	39 d
	72	67 c	49 c
	96	81 b	63 b
	120	115 a	83 a

[1/] Mean followed by different letters in each row and column are significantly *p* (< 0.05) different from each other.

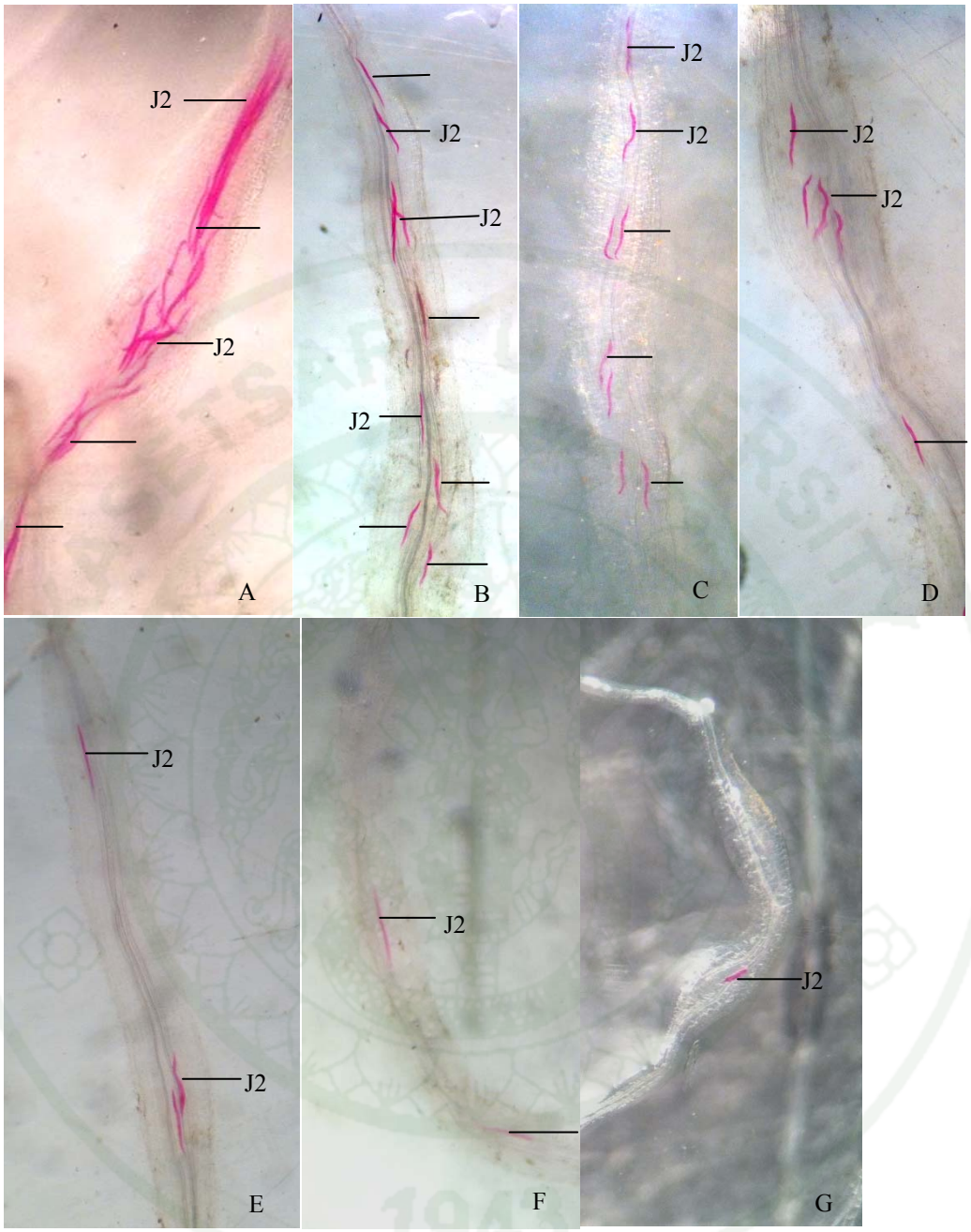


Figure 16 Secondary stage juvenile of *Meloidogyne incognita* in root plants in seven treatments, A: Control, B, C, D, E and F: fractions 12 after exposure of 24, 48, 72, 96 and 120h and G: Abamectin.

4.5Identification of *P. ostreatus* isolate Poa3 compounds

Fractions 12 and 13 were identified by spectroscopic data. Fractions 12 and 13 showed one spot on normal phase TLC with the R_f values of 0.52 and 0.58.

Structure determination of fractions 12 and 13 by nuclear magnetic resonance spectroscopy (NMR).Fractions 12 the signal was showed in Fig. 21, ^{1}H NMR (DMSO-d_6) (δ_{ppm}) (300 MHz): 6.18 (*d*, *J* = 4.0 Hz, 1H), 4.88 (*t*, *J* = 4.0 Hz, 1H), 4.77 (*d*, *J* = 5.4 Hz, 1H), 4.64 (*d*, *J* =4.5 Hz, 1H), 4.46 (*d*, *J* = 6.6 Hz, 1H), 4.37(*d*, *J* = 5.7 Hz, 1H), 3.55 (*m*, 1H), 3.52 (*m*, 1H), 3.43 (*m*, 1H), 3.40 (*m*, 1H), 3.12 (*m*, 1H), 3.02 (*m*, 1H). ^{13}C NMR (DMSO-d_6) (δ_{ppm})(75 MHz): 92.6, 73.5, 72.8, 72.3, 70.7, 61.6. The data showed structure of compound as a glucose (Fig. 20).

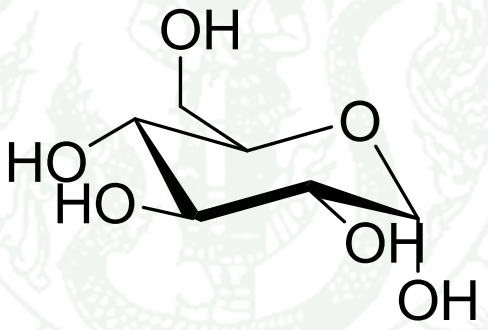


Figure 17 Structure of pure compound isolated *P. ostretus* Poa3 by spectroscopic data.

Fractions 13, the proton NMR spectrum at 300 MHz ^{1}H NMRshowed several peak two singlets at δ =0.8-2.5 ppm andδ = 5.3-5.5 ppm were assigned long chain hydrocarbon (Fig. 23), but could not identified compound. Therefore, fourier transform infrared spectroscopy (FTIR) was determine the amount of components in it. The results showed that IR spectrum range 4000 – 500 cm^{-1} of *P. ostreatus* Poa3 (Fig. 30) exhibited bands in common with spectrum, namely at 3392.19 cm^{-1} due to -OH stretching vibration is evident. The band observed at 2955.86 cm^{-1} is associated to $–CH_2$ extension. The band at 2851.89 cm^{-1} corresponds to vibration of the methyl group ($-CH_3$), while the band at 1712.37 cm^{-1} is due to C=O bonds in plane bending.

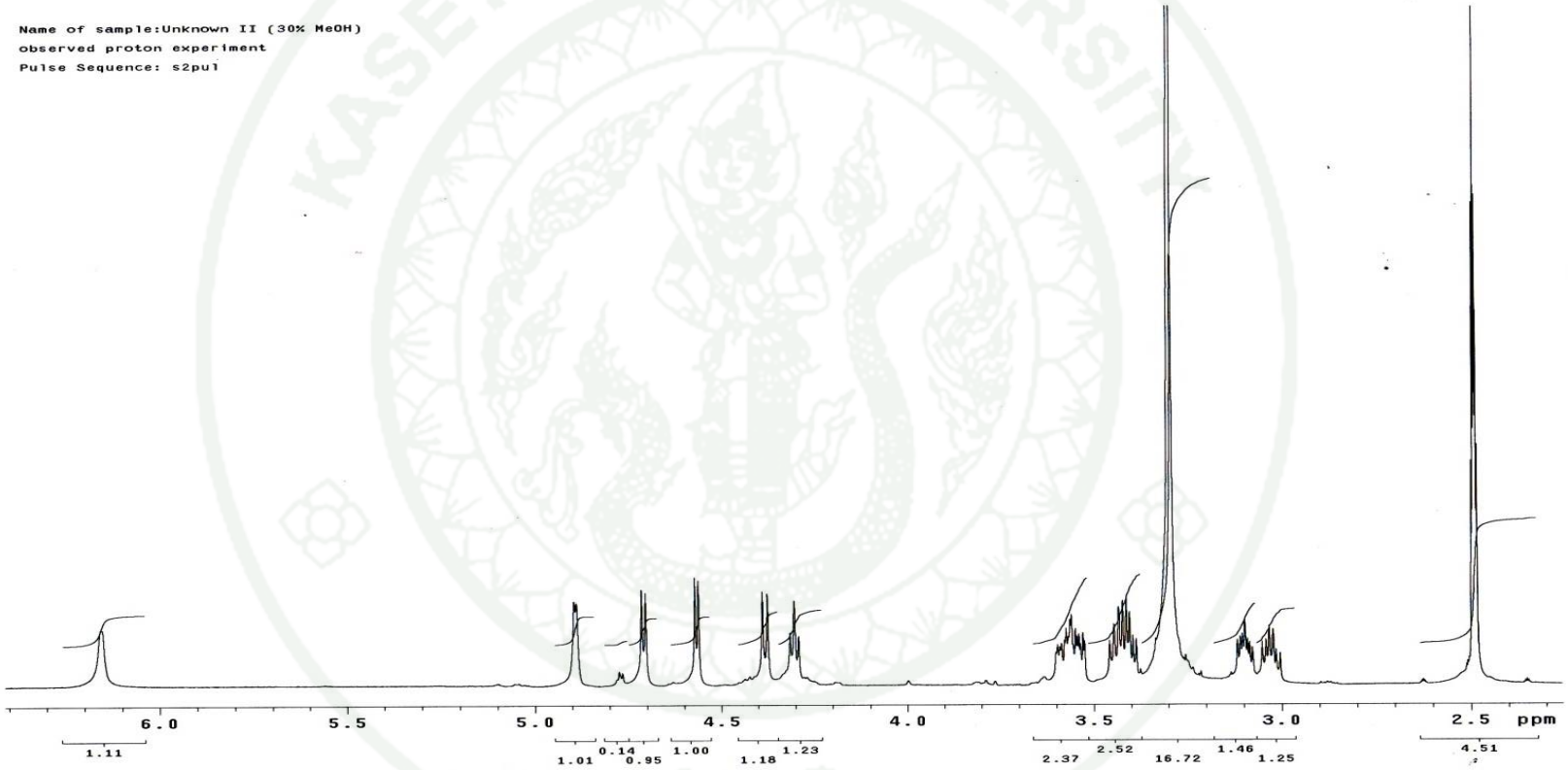


Figure 18 ^{1}H-NMR spectrum of purified *P. ostreatus* Poa3 fractions 12 compound

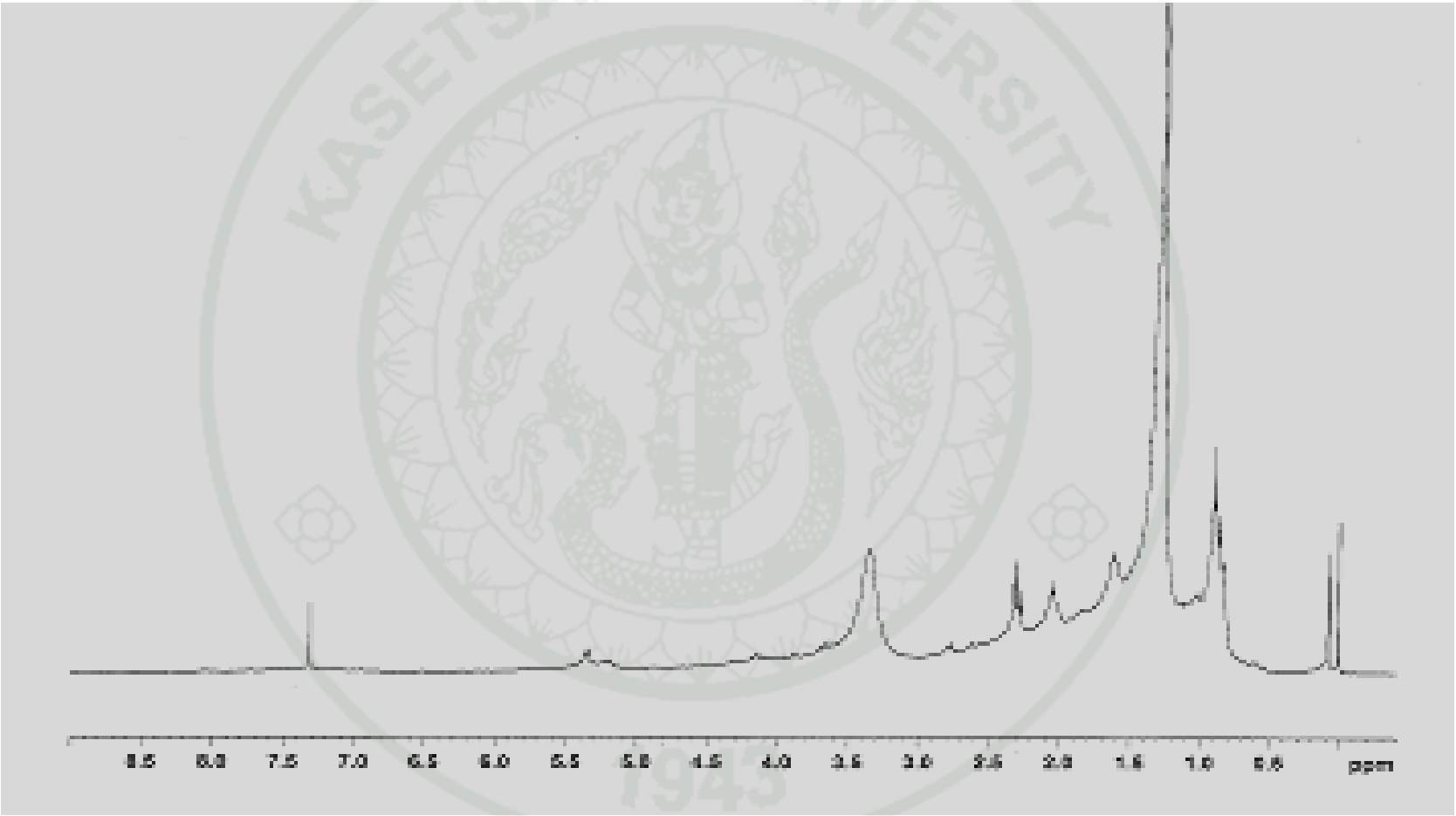


Figure 19 ^{1}H-NMR spectrum of purified *P. ostreatus* Poa3fractions 13 compound

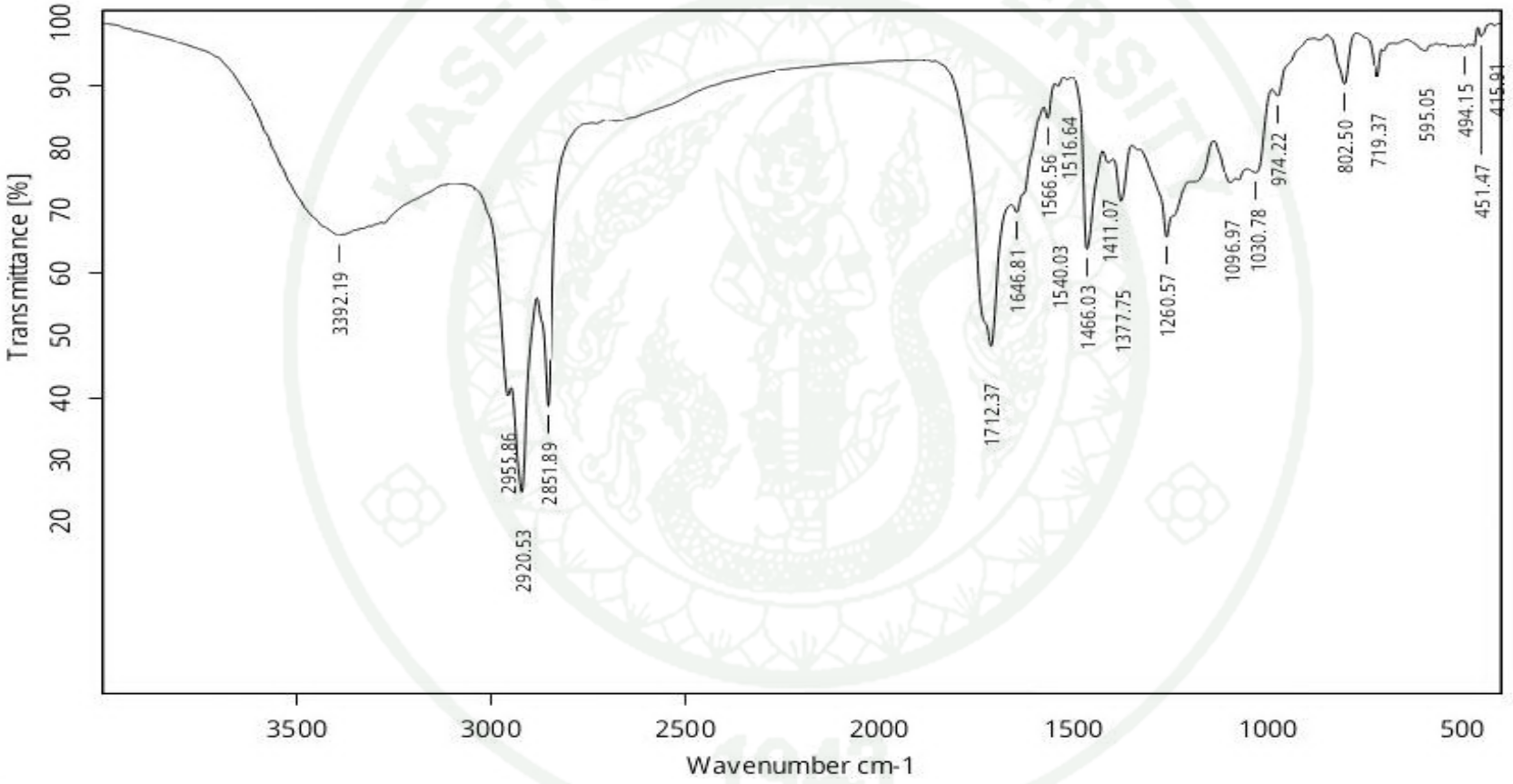


Figure 20 FTIR-spectra of compounds from *P. ostreatus* Poa3 compound

Discussion

Morphological characterization of females: Species of *Meloidogyne* can be identified based on female adult morphology, including head structures, stylet, and perineal patterns. The perineal pattern of nematode genus *Meloidogyne* is a valuable morphological feature used in the species identification (Eisenback and Hirschmann, 1991; Hirschmann, 1985). Significant perineal pattern variability observed in females from a single-egg mass population undermines the value of this character for comparing *Meloidogyne* species (Karssen and van Aelst, 2001; Carneiro *et al*., 2004).

Several mushrooms, previously considered only as saprobes, are actually considered capable of taking advantage of other organisms present in their microcosm, exploited for nutritional purposes (including nematodes, plants, other fungi, and bacteria). There is a special group of nematophagous basidiomycetous fungi provide with hyphal appendages that are indispensable for their nematode-attacking ability (Kong *et al*., 2013). In present study, mycelial of *Pluerotus ostreatus* Poa3 isolate had highest ability arrest, kill and digest *M. incognita*J2 at various times than other isolate. Except, after 5 and 12 h in each mushroom isolate mushroom hyphae could not be colonized *M. incognita*. Sikora (1992) showed the effect of fungi on improving growth characteristics of plants infected with nematode. The mode of infecting nematodes by all of *Pleurotus* species was consistent with that of *P. ostreatus* (Thorn and Barron, 1984; Hibbet and Thorn 1994; Sharma, 1994; Thorn *et al*. 2000). The secretory processes were observed on aerial hyphae of all mushroom isolates tested. Hallman and Sikora (1996) observed variation of toxin production among strains of mushroom species. Fungal products are very promising potential sources of new chemicals to manage plant-parasitic nematodes (Anke and Sterner, 1997). According to Barron and Thorn (1987) and Chitwood (2004) find that small secrete droplets are observe on hyphae of all *Pleurotus* species on water agar, and confirm in their study that the ability to infect nematodes is because of a toxin release as droplets by secretory hyphal cells, there is no relationship between nematicidal activity and droplet density. Heydari *et al*. (2006) also report that the toxin produce by *Pleurotus* species have different concentrations or several of *Pleurotu*s species have different

toxin structurally. After nematodes touched droplets of *P. ostreatus* Poa3 on parts of the mat. They were usually shrunk immediately. Khun-in *et al*. (2007) report that the efficiency of *P. ostreatus* and *Lentinus edodes* on killing 2nd juvenile stage of *M. incognita* provid significantly higher infective efficiency than the control. Palizi *et al*. (2008) investigate that *Pleurotus* spp. and *Hypsizygus ulmarius* (Bull.:Fr.) redhead kill the *Heterodera Schachtii* (cyst nematode) after only a short period of exposure to their hyphae.

Egg parasitism of *P. ostreatus* Poa3 were tested, *in vitro*. The mycelia of *P. ostreatus* Poa3 were completely infection to *M. incognita* egg mass at 120 h, and after infection, egg mass hatching were decrease compared with the control. The activity appeared similar to that of other fungal egg-parasites, through a mechanisms causing death by halting the embryo development, thus reducing host population growth (Gortari and Hours 2008).

Growing fungi on laboratory media is costly and tedious process. In this study, we carried out to determine the performance of growth of *P. ostreatus* Poa3 isolates on different media. From the observations, it was clearly evident that the PDA + 1% yeast extract medium has produced highest mycelial. Dudka *et al.* (1979) reported PDA medium is suitable for fungal growth and can use as growth stimulator to *P. ostreatus*. In this respect, Santiago (1983) investigate that mycelial development is marginally better on medium containing glucose and sucrose than other carbon sources. Gibriel *et al.* (1996) report potato dextrose extract as solid or liquid is the best medium test for fungal growth of *Pleurotus* strains. Czapeck's medium and glucose yeast extract is the best medium for produced mycelial, respectively.

The efficacy of *P. ostreatus* Poa3 culture filtrate on egg mass hatch rate was tested. The concentration of *P. ostreatus* Poa3 culture filtrate at 100 dilution had more effective to egg mass hatch rate of *M. incognita* at 12-96 h than other dilution, but less than abamectin compared with control. Nitao *et al*. (1999) investigated that *Fusarium equiseti* broth extracts inhibited root-knot nematode egg hatch. Regaieg *et al*., 2010 confirm the effects of *Verticillium leptobactrum* culture filtrates against *M. incognita*

and the fungi secrete nematode-antagonistic metabolites. Namanusart *et al*. (2013) find that the culture filtrates of *Neonothopanus nambi* Speg inhibit the egg hatching and cause mortality on J2 of *M. incognita* compare to sterile distill water and YMB medium. The infectivity of *M. incognita*J2 on tomato roots were tested in screenhouse to evaluate the effective of *P. ostreatus* Poa3 culture filtrate. The experiment was conducted by measuring number of galls and egg mass in plant roots. The result showed that after exposure period of 72 to 120 h *P. ostreatus* Poa3 culture filtrate could be reduced the number of nematode egg mass in tomato roots and number of galls compared with control. Nitao *et al*. (1999) investigated that *Fusarium equiseti* broth extracts immobilised second-stage juveniles that did hatch.Kong *et al.*(2006) applicate that *Stropharia* sp. to infest tomato plants in greenhouse to reduce the number of galls and nematode population of *M. incognita* with highest efficacy either at one month 68.16 to 84.19% and 45.28 to 88.24% two months after treatment, respectively. The results are supporting to Okorie *et al*. (2011) study the effects mix inculcations of *P. ostreatus* and *P. tuberregium*, fungal species on the spread of plant parasitic nematode *M. incognita* and growth of soybean cultivars, they find thatdecrease galling incidence,increasing growth and yields than only single inoculations of *M. incognita* in greenhouse.

In this study, the effect of ethyl acetate crude extract from Poa3 on egg mass of *M. incognita* were investigated. Crude extract from this mushroom isolate and abamectin could be decreased the egg hatch, when the concentration were decreased compared with control. According to Xiang and Feng (2001) study the nematicidal activity of acetate and water crude extract from *Pleurotus ostreatus* can kill nematodes.Satou *et al*. (2008) report that fresh whole *P. ostreatus* extract with 50% methanol can be reduce the shrinkage of the head of the nematode

The Poa3 were isolated by column chromatography (CC) and delivered to 14 fractions. When the fractions of compound were tested, the results showed fractions 11, 12 and 13 had more effect to mortality rate of *M. incognita* J2. The bio-control efficiency of 3 best fractions (fractions 11, 12 and 13) from *P. ostreatus* Poa3 with *M. incognita* J2 were tested, *in vitro*. The result showed fractions 12 had highest mortality rate than control, but lower than abamectin. When the experiment was repeated after taking J2 treated with *P. ostreatus* Poa3 fractions 12, the results

determined from egg mass hatching in tomato roots which increased while infection of tomato roots decreased. Waller and Bridge (1984) combined effect of nematode and fungi as result of either of the pathogen acting antagonistically could cause changes in host physiology of test plant.The variables evaluated included eggparasitism, egg hatch infection rate could relate to other mechanisms like antibiosis, competition and predation besides parasitism (Cayrol 1983; Kwork *et al*., 1992; Zaki 1994).

The secondary compound from fractions 12 and 13 were identified as glucose and long chain hydrocarbon, respectively. Maity (2011) and Palacios (2012) report polysaccharide extract from *P. ostreatus* is *β*-D-glucan. Polysaccharide compose of (1→3)-linked *β*-D-glucopyranosyl residues, which contains of glucose, galactose and mannose in a molar ratio of 8:2:1. Even though the two compounds have been described from other microorganism and their biological activities have been studies. It should be further developed to control *M. incognita* in actual agricultural applications.

CONCLUSIONS

For 18 Pleurutaceae isolated obtained from various locations, isolate Poa3 was selected as the most promising isolate which provided high infectivity percentage onn juvenile 2 (J2) of root-knot nematode, *Meloidogyne incognita*. This mushroom fungus effectively parasitized eggs, J2 inside egg masses as well as J2 after hatching. The isolate Poa3 producted toxic droplets which cauased the J2 became in active and shrank immediately after touching. Culture filtrates of isolate Poa3 showed the highest nematicidal activity toward J2, suppressed hatchinf of *M. incognita* egg masses and cause mortality. The filtrate also reduced the numbers of galls formation in tomato roots as well as J2 density.

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Appendix

Appendix Table 1 A square symmetric matrix of genetic distanceresult of 21 mushroom isolates

Isolates	Score																								
PH-u	0	1	0	0	0	1	0	0	0	1	1	1	1	0	1	1	0	1	0	0	0	0	0	0	1
LS	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PH-D1	0	0	0	1	1	1	0	0	1	1	0	1	1	0	1	1	0	1	0	0	0	0	0	0	1
LP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
LE3	0	0	0	1	1	1	0	1	0	1	1	0	1	1	0	1	0	0	1	0	1	1	1	0	1
PE2	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1
PO (R)8.1	0	1	1	1	1	0	1	0	0	0	1	1	1	0	0	0	0	1	0	1	1	1	0	1	1
PC2x	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	0	1	1	1
PO (R)9.1	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
LE5	0	0	1	0	1	1	0	0	0	1	0	0	1	1	1	1	1	0	1	1	1	1	0	1	1
LE2	0	1	1	0	1	1	0	0	0	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1
PE1	0	1	1	1	0	1	0	0	0	1	0	1	0	1	1	1	1	0	0	0	0	1	0	1	0
PC1	0	1	0	0	0	1	1	0	1	0	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1
PO (R)9.2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1
PEa2	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Poa	0	0	0	0	0	1	1	0	0	1	0	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0
PO(KPS)	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0
LE1	0	1	1	0	1	0	1	0	0	1	0	1	1	1	1	0	1	1	1	0	1	0	1	1	0
PO R*	0	0	1	1	1	0	0	1	1	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	1
PO3x2	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	1	1	1
PO3(2)	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	1	1	1

Appendix Table 1(Continued)

Isolates	Score																								
PH-u	1	1	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1	1
LS	1	1	0	1	1	0	1	0	1	0	0	1	1	0	0	1	1	1	1	1	0	0	0	0	0
PH-D1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1	1
LP	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LE3	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
PE2	0	1	1	1	1	1	0	1	1	1	0	0	1	1	1	0	0	1	1	1	1	1	1	0	0
PO (R)8.1	1	1	0	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	1	1	1
PC2x	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	1	1	0	0	1	0	0	0
PO (R)9.1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
LE5	0	0	1	0	0	0	1	0	1	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0
LE2	0	0	1	0	0	0	1	0	1	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0
PE1	1	1	1	1	1	0	0	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0
PC1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1	1	1	0	0	0	1	0	0
PO (R)9.2	1	1	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1	1	1
PEa2	1	1	1	1	1	0	1	1	1	1	0	1	1	0	1	0	0	1	1	1	0	0	1	0	0
Poa	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	1	1	1
PO(KPS)	1	0	0	0	0	1	1	1	1	1	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1
LE1	0	1	0	1	1	0	0	1	0	1	0	1	1	0	1	0	0	1	1	1	0	0	0	0	0
PO R*	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0
PO3x2	1	0	1	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	0	0
PO3(2)	1	0	1	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	0	0

Appendix Table 1(Continued)

Isolates	Score																								
PH-u	1	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0
LS	0	1	0	1	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
PH-D1	1	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
LP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LE3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PE2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0
PO (R)8.1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	1	1	1	0	0	0
PC2x	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0	1	1	0	1	1	1	1	0	0	0
PO (R)9.1	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
LE5	1	1	1	1	0	0	0	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	0	0	0
LE2	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	0	0	0
PE1	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	0	1
PC1	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0
PO (R)9.2	1	1	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0
PEa2	0	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0
Poa	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1	0	1	0	0	0
PO(KPS)	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	1	1	1	0	1	1	1	0	1	0
LE1	1	0	1	1	0	0	1	1	1	1	0	1	0	1	1	1	1	0	0	1	0	0	0	0	0
PO R*	1	0	0	0	1	1	1	1	1	1	0	1	0	0	0	0	0	0	1	1	1	1	0	0	0
PO3x2	1	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	1	1	0	0
PO3(2)	1	1	1	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	1	1	0	0

Appendix Table 1(Continued)

Isolate	Score																								
PH-u	1	0	0	0	0	0	0	1	0	0	1	1	0	1	1	1	0	0	1	0	1	0	1	1	0
LS	0	1	1	0	0	1	0	0	0	0	1	0	1	0	1	1	0	1	0	0	1	0	0	0	0
PH-D1	1	0	0	0	0	0	0	1	0	0	0	1	1	1	1	1	0	0	1	1	0	1	1	0	0
LP	0	1	1	1	0	0	1	1	0	1	1	1	0	0	0	0	0	1	0	1	0	0	1	0	1
LE3	0	1	0	1	1	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0	1	0	0	1
PE2	0	0	0	1	1	0	0	0	1	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	1
PO (R)8.1	0	1	1	1	1	1	0	1	0	0	1	1	0	1	0	1	0	0	0	1	1	0	0	0	0
PC2x	0	1	1	1	0	1	0	0	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1
PO (R)9.1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
LE5	0	1	1	1	1	1	0	0	1	0	0	1	1	1	0	1	1	1	0	0	1	0	0	0	1
LE2	0	1	1	1	1	1	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	0	1	0
PE1	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0	1	0	0	1
PC1	0	1	1	1	0	1	1	0	1	1	1	1	0	0	1	1	0	1	1	1	0	1	0	0	0
PO (R)9.2	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0
PEa2	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
Poa	1	1	0	1	1	1	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	1	1
PO(KPS)	0	1	1	1	1	1	1	0	0	0	1	0	0	1	1	1	0	0	1	0	1	1	1	1	0
LE1	0	1	1	0	1	1	0	0	1	1	0	0	1	1	0	0	0	1	0	0	0	1	0	0	1
PO R*	1	1	0	0	1	1	0	1	0	1	1	1	0	1	0	0	1	1	0	0	1	0	0	1	1
PO3x2	1	1	0	0	0	0	1	1	0	0	1	0	0	1	1	1	0	0	1	0	1	1	1	1	0
PO3(2)	1	1	1	1	1	1	1	0	0	0	1	0	0	1	1	1	0	1	1	0	1	1	1	1	0

Appendix Table 1(Continued)

Isolates	Score											
PH-u	0	1	0	1	0	0	0	0	0	1	1	0
LS	0	1	0	0	0	0	0	0	0	1	0	0
PH-D1	0	1	1	0	0	0	0	0	0	1	0	1
LP	0	0	0	0	0	0	1	0	0	1	0	0
LE3	0	1	0	0	1	0	0	0	1	0	1	1
PE2	0	1	0	1	0	0	0	0	0	0	0	1
PO (R)8.1	0	1	1	0	0	1	0	1	0	0	1	0
PC2x	1	1	1	0	1	1	1	1	0	1	1	1
PO (R)9.1	1	1	1	0	1	0	0	1	0	1	1	1
LE5	1	1	1	0	0	1	0	0	0	1	0	1
LE2	0	0	0	1	0	1	0	0	0	0	0	1
PE1	1	0	1	0	1	0	0	1	1	0	0	1
PC1	1	1	0	0	0	1	0	1	1	0	0	1
PO (R)9.2	0	1	1	0	0	0	0	0	0	0	1	0
PEa2	1	1	1	1	1	0	0	1	1	1	1	1
Poa	0	1	0	1	0	0	1	0	0	0	0	0
PO(KPS)	0	1	0	1	0	0	1	0	0	1	0	1
LE1	0	0	0	1	0	1	1	1	0	1	1	0
PO R*	1	1	0	0	1	1	1	0	0	0	0	1
PO3x2	0	1	0	1	0	0	1	0	0	1	0	1
PO3(2)	0	1	0	1	0	0	1	0	0	1	0	1

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