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EFFICACY OF SOME PLANT EXTRACTS AGAINST ROOT ROT DISEASE OF GREEN OAK LETTUCE (*Lactuca sativa* var. *Crispa*) CAUSED BY *Pythium sp.* GROWN IN A HYDROPONIC SYSTEM

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ABSTRACT

An antifungal activity of the twelve plant extracts were tested against Pythium sp., the causal agents of root rot diseases grown in hydroponic systems. The crude extracts of Acorus calamus and Syzygium aromaticum completely inhibited the mycelial growth of Pythium sp. in the in vitro condition at 5,000 ppm, whilst Curcuma zedoaria and other plant extracts had show lesser inhibitory activity. Out of these twelve plant extracts selecting A. calamus, S. aromaticum and C. zedoaria for further in vivo test in nursery raising seedling in plastic tray of hydroponic system by inoculating Pythium sp. mycelial developed on agar medium, A. calamus rhizome extract exhibited highest inhibitory effect against % root tip colonized by Pythium sp. of 5.85 to 48 followed by S. aromaticum floral bud 9.97 to 60 and rhizome of C. zedoaria extract 28.32 to 90 at 2000 ppm on seven and twenty one days after inoculation respectively. The concentrations and application methods of these extracts should be further investigated to control the root rot disease of lettuce in the hydroponic system.

1. INTRODUCTION

Vegetable cultivation in hydroponic system is considered as growing plants with or without use of substrate (e.g. gravel, sand, sawdust, vermiculite, rockwool, sponge or peatmoss) to provide mechanical support to the plants where nutrients are supplied through solution (Jensen, 1997; Jensen & Collins, 1985). Mostly, hydroponic systems are enclosed inside greenhouses or shade nets to control temperature, reduce water loss from evaporation water loss and to protect from diseases, pest and against the elements of weather such as wind and rain. Besides the advantages of hydroponics growing system through avoidance of soil borne pathogen, there might be introduction of pathogen through people moving in and out of the greenhouse, dust on the uncovered system, infected seeds, propagation materials, pests etc. (Paulitz, 1997).

For higher production and productivity, application of chemical fungicides is not an option for disease control in vegetables grown hydroponically as it would result in the rejection of the produce by the consumers who perceive vegetables grown hydroponically as a pesticide-free produce and are willing to pay higher prices for these commodities (Kanjanamaneesathian, 2015).

The negative environmental impact of pesticides used for pest and disease control is intensively increasing every day, especially in vegetable production system and the consumer are now getting curious about fresh and organic products for their better health and sustainable development. For this reason, several alternative methods of reducing pesticide are being developed among which one of the effective methods

is to use plant extracts which incorporating natural antifungal substances. Antifungal effects of plant and plant products are emerging every day. As antifungal substances, obtained from plants have no side effect against environment, they are showing significant advantages to the environment and organic management system for pest and disease. So, conducting a research of alternative control methods based on plant extract comes into prominence for minimizing use of commercial pesticide and development of sustainable agriculture production in hydroponics.

Plant extracts, essential oils, gums, resins etc. have been shown to exert biological activity against plant fungal pathogens *in vitro* and *in vivo* and can be used as bio-fungicidal products (Fawzi *et al*., 2009; Jalili *et al*., 2010; Romanazzi *et al*., 2012). These products are generally assumed to be more acceptable and less hazardous for the ecosystems and could be used as alternative remedies for treatment of plant diseases (Chuang *et al*., 2007). Natural plant products have a narrow target range with specific mode of action, therefore are suitable for a specific target, mostly nontoxic for antagonistic microorganisms, show limited field persistence and have a shorter shelf life and no residual threats.

Antifungal activity of plant extracts has been reported (Cowan, 1999; Grainge & Ahmed, 1988; Kurita *et al*., 1981; Wilson *et al*., 1997). These researches have indicated that antimicrobial components of the plant extracts act by crossing the cell membrane and interacting with the enzymes and proteins of the membrane, leading to produce a flux of protons towards the cell exterior which induces change in the cell and ultimately their death (Omidbeygi *et al*., 2007; Pane *et al*., 2011). Other researcher attributed the inhibitory effect of these plant extracts to hydrophobicity characters of these plant extracts and their components. This enables them to make partition in the lipids of the cell wall and cell membrane, causing leakage of ions and other cell contents which may lead to cell death (Burt, 2004).

Plant extracts are generally prepared by using alcohol or other aqueous solutions, either by hot or cold extraction methods. Crude and alcohol extract of several plants have been screened and tested for their potential antimicrobial activities against plant pathogenic microbes such as virus, bacteria, fungi and protozoa (Abere *et al*., 2007; Afolayan, 2003).

This research study was aimed at evaluating the potentials plant extracts of *S. aromaticum, C.zedoaria, A.calamus* in the biological control of Pythium sp. causing root rot in hydroponic system. This study pursues highlights on useful and sustainable alternative of costly and non-eco-friendly synthetic fungicides to control of fungal diseases of lettuce grown hydroponically.

2. MATERIALS AND METHODS

2.1. Collection and Preparation of Plant Extracts

Plant materials used for tests were either collected from Hua-Hin district, Prachub Khiri khan province and Cha-Am district, Phetchaburi province or purchased from "Tai-Hua-Chan" medical plant shops, Bangkok, Thailand in 2018 (Table 1). Most of the collected plants were locally and widely available in Nepal and Thailand. The fresh plant parts were washed with tap water, air dried and then weighted. These plant parts were cut into small pieces, packed to aluminum trays and placed in the oven at 60°C for 24 hours. Each dried plant part was weighted and then grinded into powder which was used to prepare the crude extract.

The powdered plant parts (such as flowers, leaves, rhizomes and buds) were separately soaked in 95% ethanol in the glass bottles for maceration. These mixtures were incubated at room temperature (26-32°C) for three days. After incubation, the powdered plant parts were filtered with the muslin cloth and the liquid part was collected in the round bottom flasks. The liquid was then evaporated by a rotary evaporator at 100 rpm for 20 min (at 50-56°C) to obtain the crude extracts. These crude extracts were weighted. The stock solutions of each crude extract were prepared by dissolving 0.5 g of each crude extract in one mL of acetone to obtain 500,000 ppm stock solution for further use (Figure 1)

Table 1. List of plant species for extraction and efficacy testing against fungi.

Family	Scientific name	Common name	Nepali name	Part of plant	Collected place
Acoraceae	*Acoros calamus*	Sweet flag	Bojo	Rhizome	Medicinal plant shop, Bangkok
Asteraceae	*Azeratum conizoides*	Goatweed	Gande	Aerial part	Cha-Am, Phetchaburi
Asteraceae	*Eupatorium odoratum*	Siam weed	Aule Banmara	Aerial part	Hua-Hin, Prachuabkhirikhan
Asteraceae	*Tagetus erecta*	Marigold	Saypatri	Aerial part Inflorescence	Local market, Phetchaburi
Clusiaceae	*Garcinia mangostana*	Mangosteem	Mangosteem	Fruit pericarp	Local market, Phetchaburi
Fabaceae	*Cassia spectabilis*	Yellow cassia	Rajbrikshya	Flower	Hua-Hin, Prachuabkhirikhan
Meliaceae	*Azadiracta indica*	Neem	Neem	Leaf	Hua-Hin, Prachuabkhirikhan
Myrtaceae	*Syzygium aromaticum*	Clove	Lwang	Floral bud	Medicinal plant shop, Bangkok
Plantaginaceae	*Scoparia dulcis*	Sweet-broom	Patal Mishri	Whole plant	Hua-Hin, Prachuabkhirikhan
Verbenaceae	*Lantana camara*	*Lantana* weed	Banmara	Aerial part	Cha-Am, Phetchaburi
Zingiberaceae	*Alpinia galangal*	Galangal	Haledo	Rhizome	Cha-Am, Phetchaburi
Zingiberaceae	*Curcuma zedoaria*	*Zedoary*	Seto Besar	Rhizome	Cha-Am, Phetchaburi

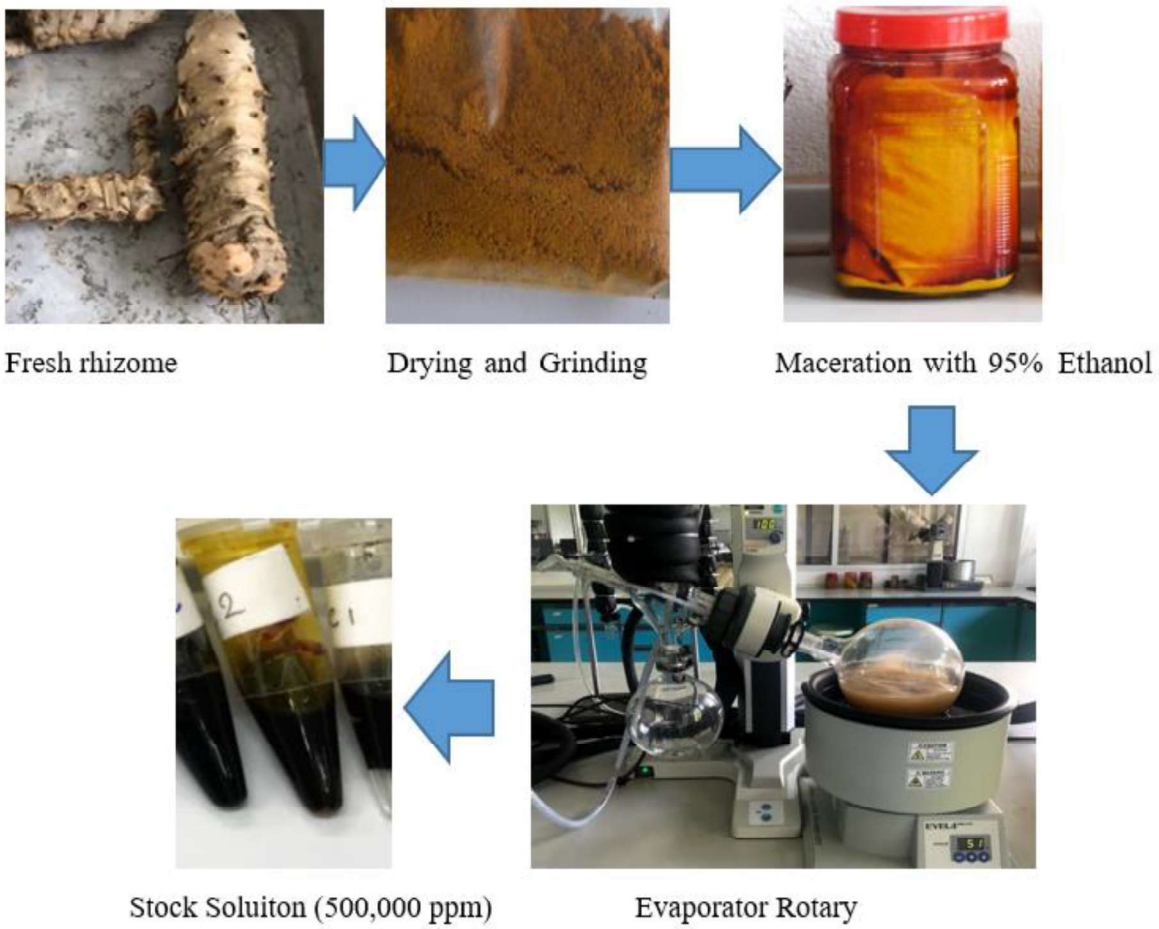

Figure 1. Preparation of crude extract by maceration technique

2.2. Laboratory screening of the antifungal activity of plant extract

An antifungal activity of the selected plant extracts was tested against both *Pythium* sp., using food poisoning technique (Kumar *et al*., 2008). Each plant extract stock solution (0.1 mL) was added to 9.9 mL of sterile PDA at 55°C. The mixture prepared was poured into the petri dishes to attain final concentration of 5,000 ppm. Nil control medium was prepared by mixing acetone (0.1 mL) in the sterile PDA (9.9 mL). The growing mycelia from the seven-day-old culture of both pathogens on PDA were cut and placed onto the centre of petri-plates filled with each plant extract individually. Sterile cork borer (6mm in diameter) was used to cut the growing mycelia and then transferred aseptically onto the centre of PDA which had been mixed the crude extracts. The plates were then incubated at room temperature (at 26-32°C) for seven days. The diameter of the fungal colony of the treatments and nil control were measured and the percentage of mycelial growth inhibition (M) was calculated (Wang *et al*., 2005).

$$M = [(A-B) / A] \times 100$$

Where A is the colony diameter of the control treatment and B is the colony diameter of the treated crude extracts.

2.3. Efficacy testing of the plant extracts to suppress root colonization by Pythium sp. *in the plastic tray*

A. calamus, S. aromaticum and *C. zedoaria* were selected to test their efficacy against *Pythium* sp. The lettuce seedlings were raised in the seed beds which had been drenched with the mixture of extract solution and nutrients (at 2,000 ppm) for seven days. After seven days, the seedlings were transplanted to the hydroponic unit.

One hundred lettuce seeds were germinated in polyurethane sponge (23x25x2.5 cm in dimension) which had been moisten with distilled water. After 12 days, these seedlings were transferred to plastic tray which contained the mixture of distilled water (4 L) and nutrient solution A (3 mL L-1 of water) and B 3 mL L-1 of water). Twenty seedlings were planted in each plastic tray in the experiment which consisted of eight treatments with four replications and each trays contains 20 lettuce seedlings (Table 2)(Kobayashi & Yamaji, 2022; Lee *et al*., 2006).

Table 2. Lettuce seedlings treated with different plant extracts solution

Name of treatment	Replication	Number of plants / tray	Total plants/ treatment
A. calamus+ *Pythium sp.*	4	20	80
A. calamus	4	20	80
S. aromaticum + *Pythium sp.*	4	20	80
S. aromaticum	4	20	80
C. zedoaria + *Pythium sp.*	4	20	80
C. zedoaria	4	20	80
Pythium sp. (Control)	4	20	80
Nutrient (Nil Control)	4	20	80

Two days after the seedlings were transferred to the hydroponic unit in plastic tray, eight grams of plant extracts were added to the nutrient solution 2,000 ml to maintain 2,000 ppm. Twelve agar plugs of the 5-day-old of the mycelia of *Pythium* sp. growing on PDA were inoculated into the plastic tray. Root tip of the lettuce which had been colonized with *Pythium* sp. was assessed. Three lettuce seedlings from each plastic tray were sampled and the root was cleaned with tap water and distilled water twice. The root was subsequently dipped into 1% sodium hypochlorite solution for 40 seconds and then rinsed twice with distilled water. Ten root tips (2-4 mm in length) were selected randomly from seedlings by cutting the secondary root tips and

they were placed onto the Petri plates containing 10 mL PDA. The growing mycelia of *Pythium* sp. from the root tissues on PDA were observed in 7 days and 21 days of inocualtion. Percentage root colonization per plant were calculated (Bhale, 2018):

$$\% \text{ Root colonization} = \frac{\text{Total } \textit{Pythium} \text{ sp. mycelial colony developed on petri plate}}{\text{Total root tips placed on the petri plates}} \text{x100}$$

2.4. Statistical Analysis

Data collected from this research were analyzed using Microsoft excel 2013 and R-Program software. Duncan Multiple Range Test (DMRT) analysis was used to compare the means using R-Program version 3.5.1.

3. RESULTS AND DISCUSSION

3.1. In vitro inhibition of Pythium sp. by plant extracts on PDA

In vitro efficacy of plant extracts were examined against *Pythium* sp. using poison food technique. Among 13 ethanol crude extracts from 12 plant species, rhizome extract of *A. calamus* and floral bud of *S. aromaticum* completely inhibited *Pythium* sp., followed by rhizome of *C. zedoaria* (54.15%), aerial part of *E. odoratum* (40.62%), rhizome of *A. galanga* (40%) and fruit pericarp of *G. mangostana* (32.92%), compared with acetone only as control treatment. The other ethanol crude extracts with lesser than 10% inhibition were flower of *C. spectabilis* (8%), whole plant of *S. dulcis* (5.54%), aerial part of *A. conizoides* (2.77%) and aerial part of *L. camara* (1.23%). Some crude extracts such as aerial part and inflorescence of *T. erecta* and leaf of *A. indica,* did not inhibit the mycelial growth of *Pythium* sp. (Table 3 and Figure 2).

Table 3. Efficacy of crude extracts against mycelial growth of *Pythium sp*. on PDA

Family	Scientific name	Plant parts	Inhibition (%)± SD
Acoraceae	*A. calamus*	Rhizome	100±0.00a
Asteraceae	*A. conizoides*	Aerial part	2.77±1.05d
Asteraceae	*E. odoratum*	Aerial part	40.62±0.64bc
Asteraceae	*T. erecta*	Aerial part	-47.69±0.90e
		Inflorescence	-54.15±0.61e
Clusiaceae	*G. mangostana*	Fruit pericarp	32.92±0.39bcd
Fabaceae	*C. spectabilis*	Flower	8.00±0.91cd
Meliaceae	*A. indica*	Leaf	-46.46±0.58e
Myrtaceae	*S. aromaticum*	Floral bud	100±0.00a
Plantaginaceae	*S. dulcis*	Whole plant	5.54±1.18d
Verbenaceae	*L. camara*	Aerial part	1.23±0.28d
Zingiberaceae	*A. galangal*	Rhizome	40±0.83bc
Zingiberaceae	*C. zedoaria*	Rhizome	54.15±0.03b
Control			0.00±0.00
CV			16.09
$LSD_{0.05}$			1.211

*Indicate significant effects at $p<0.01$ (DMRT) within a column.

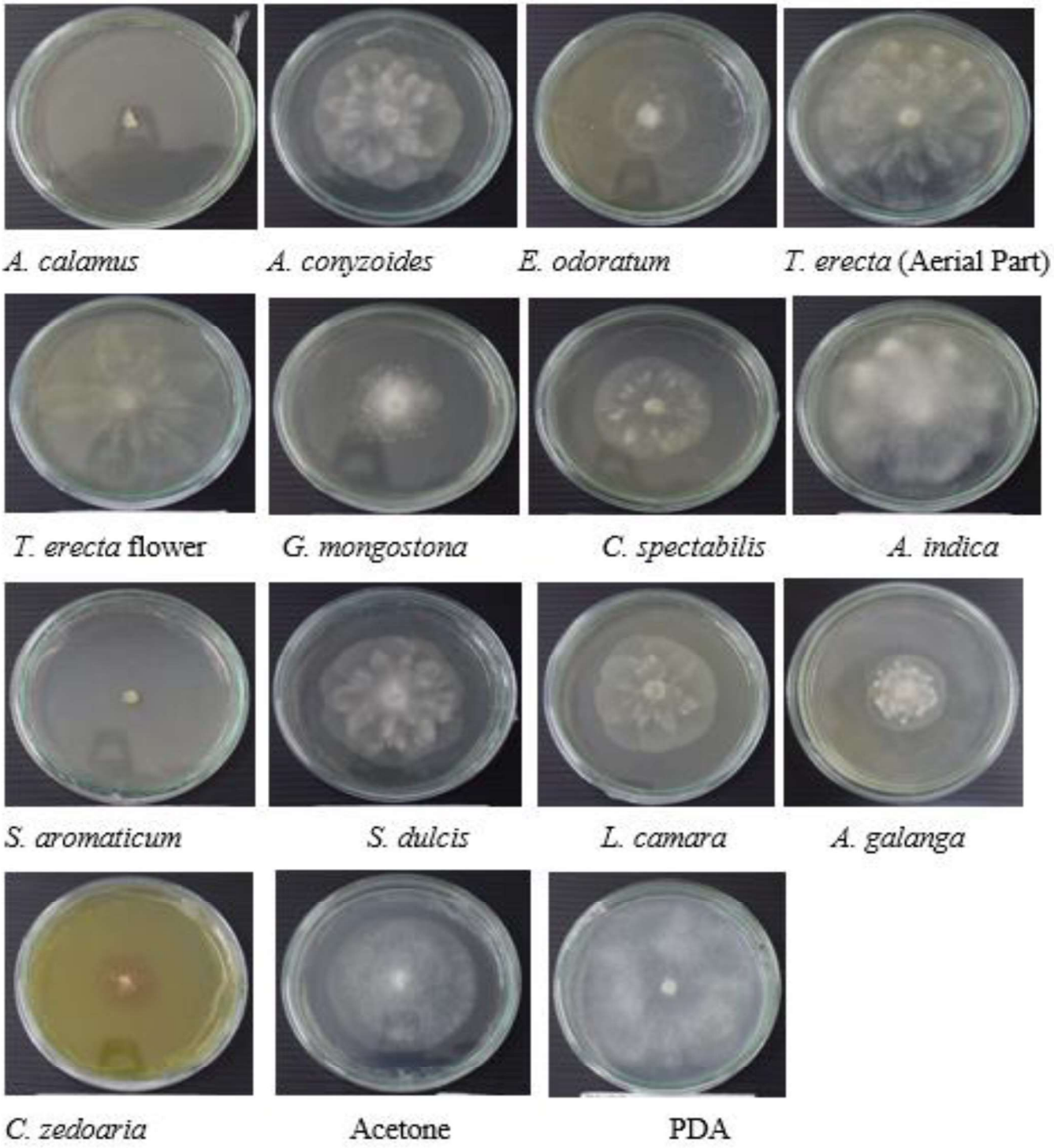

Figure 2. *In vitro* efficacy of different plant crude extracts against *Pythium* sp. at 5,000 ppm

3.2. Efficacy of plant extracts in reducing the capacity of Pythium sp. to colonize lettuce root

Based on the percentage of root tips which were colonized by *Pythium sp., A. calamus* was effective in reducing the percentage of root colonization followed by *S. aromaticum* and *C. zedoaria* as shown in table 4 and Figure 3.

Table 4. Percent root tip of lettuce colonized by *Pythium* sp.

Name of Treatments	% Colonization on 7 Day	% Colonization on 21 Day
A. calamus +Pythium sp.	5.825±0.56c	48±29.49b
S. aromaticum +Pythium sp.	9.975±1.053c	60±21.21b
C. zedoaria +Pythium sp.	28.32±0.794b	90±14.14a
Pythium sp.	90+0.901a	90+10.00a
Nutrient	6.67±0.722c	10±14.14
A. calamus	0±00c	0±00c
S. aromaticum	0±00c	0±00c
Cu. zedoaria	0±00c	0±00c

**indicate significant effects at p<0.01 (by Duncan multiple range test) within a column.

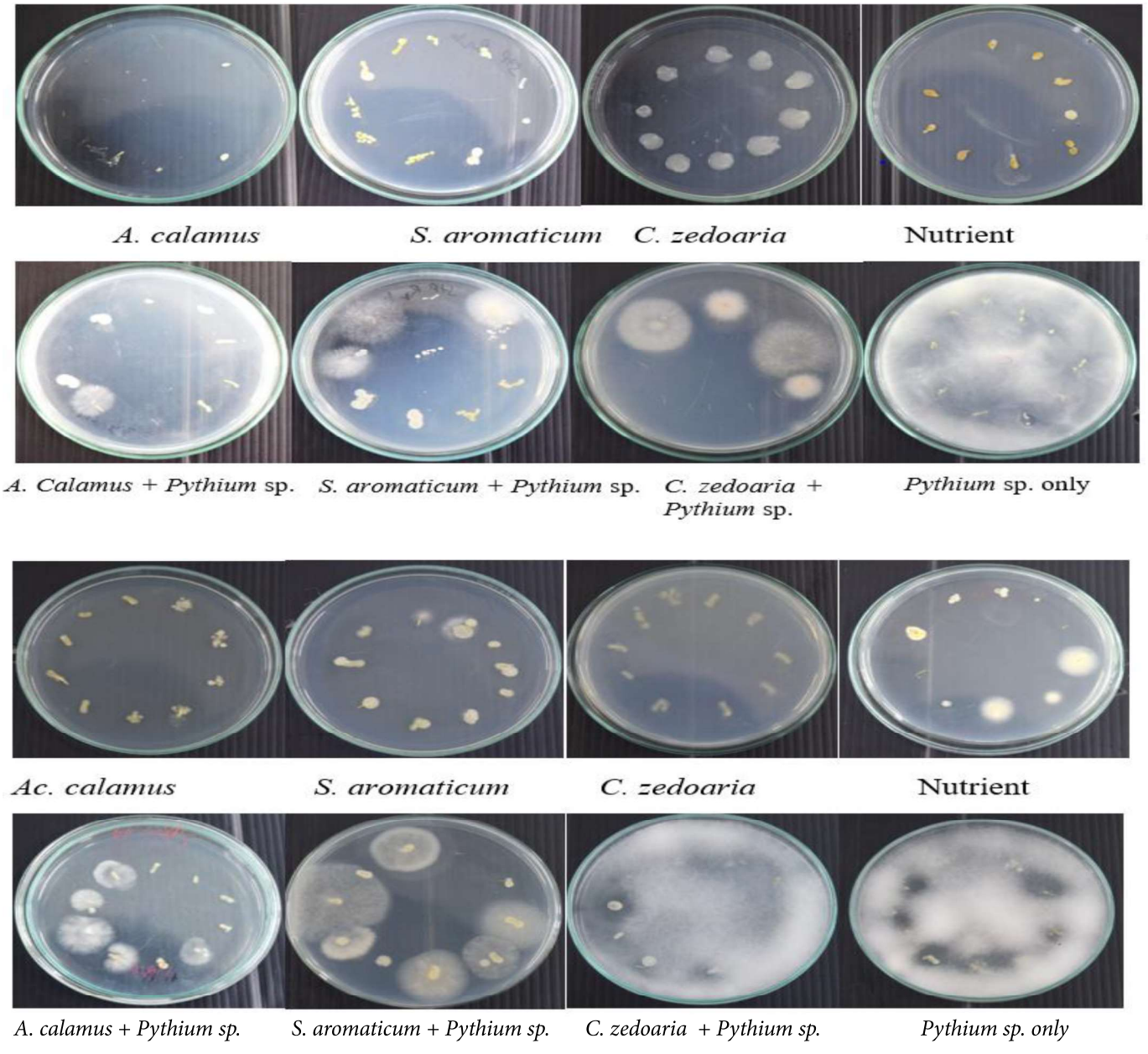

Figure 3. Percentage colonized by Pythium sp. on root tips of lettuce at 7 and 21 Day

3.3 Discussion

Antifungal activity of plant extracts has been reported (Cowan, 1999; Grainge & Ahmed, 1988; Kurita *et al*., 1981; Wilson *et al*., 1997). These researches have indicated that antimicrobial components of the plant extracts act by crossing the cell membrane and interacting with the enzymes and proteins of the membrane, leading to produce a flux of protons towards the cell exterior which induces change in the cell and ultimately their death (Omidbeygi *et al*., 2007; Pane *et al*., 2011). Other researcher attributed the inhibitory effect of these plant extracts to hydrophobicity characters of these plant extracts and their components. This enables them to make partition in the lipids of the cell wall and cell membrane, causing leakage of ions and other cell contents which may lead to cell death (Burt, 2004).

A. calamus, C. spectablilis, C. zedoaria, S. aromaticum were reported to possess the antimicrobial, anti-bacterial, antifungal, anti-protozoal, anti-inflammatory and antioxidant activities (Ayoola *et al*., 2008; Latha *et al*., 2009; Sangetha *et al*., 2008; Umamaheshwari & Rekha, 2018; Yusuf *et al*., 2001). Plant extracts could also be used against plant pathogenic fungi (Yulia, 2005).

In this study, twelve plants were selected for screening to determine their efficacy against Pythium sp. an important causal agents of root rot diseases of lettuce grown hydroponically. The results showed that the crude ethanol extracts of rhizome of *A. calamus, C. zedoaria* and *A. galanga*; floral bud of *S. aromaticum*, and fruit pericarp of *G. mangostana* have potential plant for farmers to use in hydroponic system to control root rot. Rhizome extract of *A. calamus* completely inhibited *Pythium sp.* in vitro at 5,000 ppm. Koohakan (2009) also reported that ethanol extract of rhizome of *A. calamus* showed the efficacy to completely suppress both *P. aphanidermatum* and *P. myriotylum* at 5,000 ppm on agar tests. Thus, the extract of *A. calamus* at 5,000 ppm was confirmed to be the appropriate choice for use in further research. The antifungal compound present in the crude extract may cause fluid leakage from cells leading to fungal cell death and the overall suppression to the fungal colony (Phongpaichit *et al*., 2005). However, various factors such as the choice of solvents, concentrations of the solvent and extraction methods may have contributed to the quality, quantity and efficacy of the yield. Khruasanit (2004) reported that dichloromethane was found to be an effective solvent for extracting the active compounds from *A. calamus*, whilst methanol was unable to extract the active compounds and the product was not inhibitory to *Pythium sp.*

The inhibitory activity of plant extracts observed in the study could be attributed to the presence of biologically active anti-fungal compounds. The active compounds from ethanolic extracts of S. aromaticum and *A. galanga* were characterized as eugenol and 1′-acetoxychavical acetate (ACA) respectively against the mycelial growth and conidial germination of *Curvularia lunata* and *Fusarium sacchari* (Madi *et al*., 2020). 1′-Acetoxychavicol acetate is identified as active compound from *A. galangal* that inhibited the growth of *Phytophthora nicotianae* and *Alternaria porri*, with minimum inhibition concentration values of 15.6 and 31.5 μg mL−1, respectively (Mongkol *et al*., 2015).

Nevertheless, in this study the ethanol crude extract of *A. calamus*, *S. aromaticum* and *C. zedoaria* were found to be effective to suppress *Pythium sp.* both *in vitro* and *in vivo* conditions.

4. CONCLUSION

The results are showed that the crude ethanol extracts of rhizome of *A. calamus, C. zedoaria* and floral bud of *S. aromaticum*, have potential plant for farmers to use in hydroponic system to control root rot. Rhizome extract of *A. calamus* completely inhibited *Pythium* sp. in vitro at 5,000 ppm. Ethanol crude extract of *A. calamus, S. aromaticum* and *C. zedoaria* was found to be effective to suppress both *Pythium* sp. in the laboratory tests as well as in root tips of nursery seeding before transplanting it into main hydroponic systems. Based on the both *in vitro* and *in vivo* results of selected plant extracts these plant extract could be potential bio-fungicide for hydroponic system while phytotoxicity and compatibility to the plant of these potential bio-fungicides should be considered for further research before applying it in the system.

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