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The Inhibitory Effect of the Various Seed Coating Substances Against Rice Seed Borne Fungi and their Shelf-Life during Storage

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Abstract: Presently, chemical seed treatments are in discussion due to their directly or indirectly impacts on human health or other living organisms. They may also negatively affect the ecosystem and the food chain. In rice seeds, chemicals may cause phytotoxic effects including seed degradation. Eugenol is the main component of clove (*Eugenia caryophillis*) oil, which was proved to act simultaneously as bactericide, virocide and especially fungicide. The *in vitro* study was aimed to compare the inhibitory effect of the following seed treatment substances against seed borne fungi and their shelf-life during 12 months of storage; conventional captan (CA), chitosan-lignosulphonate polymer (CL), eugenol incorporated into chitosan-lignosulphonate polymer (E+CL) and control (CO). The obtained results of fungi inhibition were classified in three groups, which showed at first that CA treatment led to a better, i.e., longer, inhibitory effect on *Alternaria padwickii*, *Rhizoctonia solani*, *Curvularia* sp., *Aspergillus flavus* and *Aspergillus niger* than E+CL. Secondly, E+CL coating polymer showed the longest inhibitory effect against *Bipolaris oryzae* and *Nigrospora oryzae* compared to CA and CL coating polymer. Finally, both CA and E+CL coating polymer had non-significant difference inhibitory effect on *Fusarium moniliforme*. The variant of CL coating polymer for seed coating was only during the first 6 months of storage able to inhibit all species of the observed seed borne fungi, whereas CA and E+CL coating polymer were capable to inhibit most of the fungi until 9 months of storage.

Key words: Seed coating technology, eugenol, rice seed borne fungi, antifungal activity, shelf-life

INTRODUCTION

More than 90% of the field crops grown in the world are propagated via the seed and all of them are attacked by devastating seed born pathogens. The rice crop (*Oryza sativa*) is known to be attacked by many pathogenic seed borne fungi; e.g., *Bipolaris* sp. (brown spot), *Alternaria* sp. (stackburn), *Fusarium* sp. (bakanae), *Rhizoctonia* sp. (sheath blight), *Nigrospora* sp. (kernel smut), *Curvularia* sp. (blast). Important storage fungi are *Aspergillus flavus* and *Aspergillus niger*. Fungi contamination is a major cause leading to seed deterioration and degradation of rice grain qualities (Hewett, 1987).

The fungicide seed treatments are the most used traditional application to protect the seeds and young-seedlings from many seed-and soil-borne pathogens. However, the uses and expectations of chemical seed treatment have raised the concern due to the impact on environment, which caused either direct or indirect impacts on human or other living organisms. It can also negatively affect the food chain, the ecosystem at all and may increase fungicide resistance problems (Ester *et al*., 2003). Moreover, the fungal pathogen normally attached to the seed, so it is difficult to find chemical substances that will destroy the fungus without harming the seeds, because many fungicides were developed from bromine, iodine, sulfur, copper and chloride compounds. Since, the toxicity of fungicide has been ascribed to produce phytotoxic compounds induced seeds deterioration (Han, 2000).

The application of medical plant extracts to control plant pathogenic fungi is a key way in the chemical-free agricultural system as an alternative for the toxic synthetic fungicides (Burt and Reinders, 2003). Several antifungal compounds of plant origin are known to control the seed borne infection, phenolic compounds, as eugenol and eugenol acetate, are the main components of clove (*Eugenia caryophillis*) (Velluti *et al*., 2004). They were proved to have bactericide (Pawar and Thaker, 2006), virocide (Tullio *et al*., 2006) and fungicide effects and act against many seed borne fungi (Chami *et al*., 2005a). Additionally, they can easily be obtained from readily available plant (Mungkornasawakul *et al*., 2003) and possess no residues and phytotoxic effects (Chami *et al*., 2004).

The application of coating the seeds with chemicals or alternative bioactive compound substances

significantly may reduce the percentage of plant damage by pest and the level of pesticide application (Badei *et al*., 1996). In the modern era of seed treatment, compared with direct drench application methods, the seed coating technology could decrease 85% of pesticide utilization (Chami *et al*., 2005b). Moreover, the conventional seed coating substances can be mixed with natural products, which have protective effects on seeds. Another advantage of seed coating is the preservation of the quality of seeds and the seed components, which may not easily be deteriorated (Ester *et al*., 2003). These new uses often require improved application systems for better established dosages and coverage of materials (Chami *et al*., 2005a). However, there is a paucity of information on the comparison of inhibitory effect of the eugenol bioactive compounds and the synthetic chemical seed treatment to eradicate seed born fungi during storage. Thus, the *in vitro* experiment was aimed to compare the inhibitory effect of traditional chemical fungicide (captan) and botanical fungicide (eugenol) seed coating substances against rice seed borne fungi. Rice seeds will be also stored before sowing; therefore, the effectiveness and their shelf-life of the different seed coating substances during storage were also studied.

MATERIALS AND METHODS

Seed materials preparation: The study was conducted at Department of Agricultural Technology, Faculty of Technology, Maha Sarakham University, Thailand and the Seed Science and Technology Laboratory, Faculty of Agriculture, Department of Agronomy, Chiang Mai University, Thailand in the year 2007 - 2008. Dry graded rice seeds (*Oryza sativa* L. cv. KDML 105) from one seed lot were supplied from the Bureau of Seed Multiplication of Thailand. The initial seed moisture content and germination percentage were 10.65 and 96.00%, respectively. The split-plot design with four replications was applied. The main plot was seed coating substances, which were captan (CA), only chitosan lignosulphonate polymer (CL) eugenol incorporated into chitosan lignosulphonate polymer (E+CL) and control (CO) (Fig. 1a-d). The sub-plot was the 12-month storage period, seeds were randomly stored in a plastic bag sealed and then kept in an incubation chamber, (KPB6395FL, Termaks, S/N 2-858 Germany). The storage was carried out at controlled temperature of 30±2°C and relative humidity of 40±5%. Seeds were sampled immediately and then, every month for seed borne fungi determination collected.

Traditional captan treatment preparation (CA): Captan fungicide (1, 2, 3 and 6-tetrahydro-N-(trichloromethyl thio) phthalimide was applied as slurry dust in a solution of polyethylene glycol (PEG 8000 at -2 MPa) (4 g of captan per 1 kg of the seeds). After that, seeds were dried to 10±2% of moisture content (MC) at 35°C to obtain a similar MC as the control (untreated) seeds.

Chitosan-lignosulphonate coating polymer preparation (CL): Three percentage of chitosan-lignosulphonate coating polymer was prepared by adding 3 g chitosan (Fluka, Germany) into 100 mL of 1% v/v acetic acid combined with 1% w/v sodium lignosulphonate (Fluka, Germany) in distilled water. Then, the chitosan-lignosulphonate polymer was sprayed and mixed well into 500 g seeds. The seed MC of 10±2 % was obtained after drieing at 35°C.

Fig. 1: Rice seed coated with various seed coating substances; (a) CO, (b) CA, (c) CL and (d) E+CL

Table 1: The analysis of variance (ANOVA) for seed treatments and storage time on seed borne fungi control

		Statistic significant (P)							
SOV	df	*A. solani*	*R. solani*	*Curvularia* sp.	*A. flavus*	*A. niger*	*B. oryzae*	*N. oryzae*	*Fusarium* sp.
Treatment (T)	2	*	*	*	*	*	*	*	*
Storage time (S)	12	*	*	*	*	*	*	*	*
T×S	24	*	*	*	*	*	*	*	*

*Significant different at the 0.05 level of probability

Table 2: The 50% inhibitory effect enhancement of various seed coating substances against rice seed borne fungi during storage

	Treatment		
Storage (Months)	CA	CL	E+CL
0	-	-	-
1	-	*Curvularia* sp.	-
2	-	*Nigrospora oryzae.*	-
3	*Nigrospora oryzae*	*Fusarium moniliforme, Rhizoctonia solani*	-
4	-	*Alternaria solani, Aspergillus flavus, Aspergillus niger*	*Nigrospora oryzae* .
5	-	-	*Aspergillus flavus, Aspergillus niger, Curvularia* sp.
6	*Aspergillus flavus, Aspergillus niger, Curvularia* sp.	*Bipolaris oryzae*	-
7	*Bipolaris oryzae, Fusarium moniliforme*	-	*Alternaria solani, Fusarium moniliforme, Rhizoctonia solani*
8	*Rhizoctonia solani*	-	-
9	*Alternaria solani*	-	-
10	-	-	-
11	-	-	*Bipolaris oryzae*
12	-	-	-

Eugenol incorporated into chitosan-lignosulphonate coating polymer preparation (E+CL): One percentage of eugenol incorporated into chitosan-lignosulphonate coating polymer was prepared by adding 0.5 mL eugenol active compound solution (Fluka, Germany) into 50 mL of 3% chitosan-lignosulphonate coating polymer binder. Then, it was sprayed onto mixed-well 500 g seeds samples. The seeds were dried at 35°C to obtain MC of 10±2%.

Seed health testing: According to ISTA (2006), the Blotter method is the testing method recommended for seed borne fungi detection. The procedure started with the test of a 400 seeds working sample in four replications and 25 seeds per dish were placed on three filter papers (blotters) which were soaked well in sterilized water. The seeds were later incubated at 20-25°C in 12 h light for 14 days. Seed borne fungi infection was recorded under a stereoscopic microscope (Olympia-SZ61). Then, the inhibition percentage of each seed born species was calculated, based on control seed, with the following equation:

$$\text{Inhibition percentage} = \frac{\text{Infection control (\%)-Infection sample (\%)}}{\text{Infection control (\%)}} \times 100$$

Calculation and statistical analysis: The data are presented as Mean±Standard deviation. The analysis of variance was performed for data analysis and differentiated with LSD test at $p<0.05$ using the software SX release 8.0 (Analytical software, Tallahassee, USA).

RESULTS AND DISCUSSION

The ANOVA results indicated that the effectiveness of seed borne fungi control were significantly affected by seed treatment variants, storage duration as well as interaction between them (Table 1). As shown in Fig. 2, E+CL showed a strong inhibitory effect on seed borne fungi depending on storage period, especially the early storage period. Within 5 months storage there was no significant difference of the inhibitory effects in E+CL and CA. The inhibitory effects differences of both treatments became significant when the seeds were stored longer than 6 months ($p\leq0.05$). CL showed slightly inhibitory effect, prior to 3 months storage.

During storage, the results are expressed the effectiveness of each seed treatment variants to control seed borne fungi. The results of 50% inhibitory effect (IE_{50}) could be classified into three groups. Firstly, CA had more capability to inhibit *Alternaria solani*, *Rhizoctonia solani*, *Curvularia* sp., *Aspergillus flavus* and *Aspergillus niger* than CL and E+CL (Table 2).

For *A. solani*, CA could maintain the 50% inhibitory effect for 9 months. The E+CL kept the inhibitory effect for 7 months, whereas CL inhibited this fungus only for 4 months (Fig. 3, Table 2).

Comparing the inhibitory effects on *R. solani*, the CA and E+CL had the 50% inhibitory effect at 8 and 7 months respectively, but the CL inhibited *R. solani* for only 3 months (Fig. 4, Table 2). CA and E+CL could remain inhibitory effect on *Curvularia* sp. for at least 6 and 5 months, respectively (Fig. 5, Table 2).

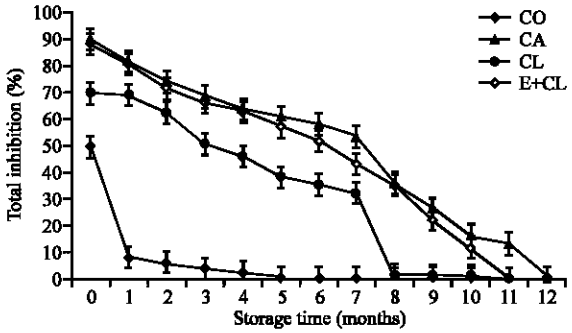

Fig. 2: The effect of various seed coating substances on the total fungi inhibition percentage during 12 months of storage (The results indicated in term of Mean±SD)

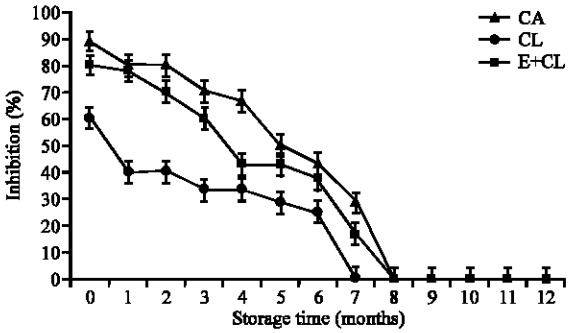

Fig. 5: The inhibitory effect of various seed coating substances on *Curvularia* sp. during 12 months of storage (The results indicated in term of Mean±SD)

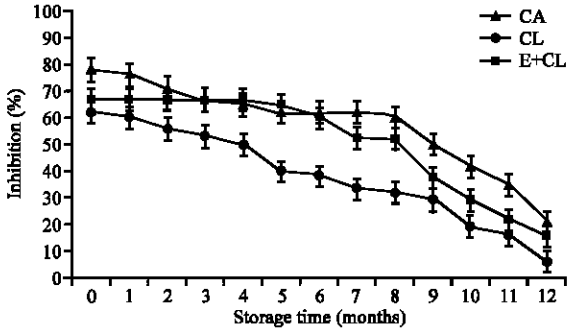

Fig. 3: The inhibitory effect of various seed coating substances on *Alternaria solani* during 12 months of storage (The results indicated in term of Mean±SD)

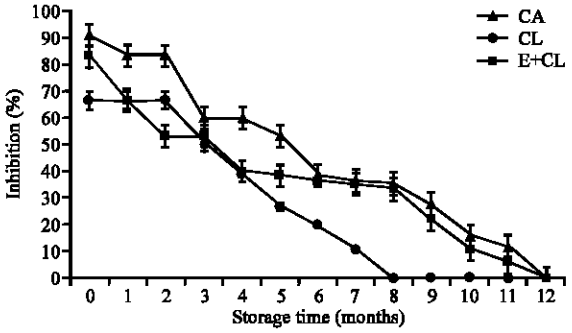

Fig. 6: The inhibitory effect of various seed coating substances on *Aspergillus flavus* during 12 months of storage (The results indicated in term of Mean±SD)

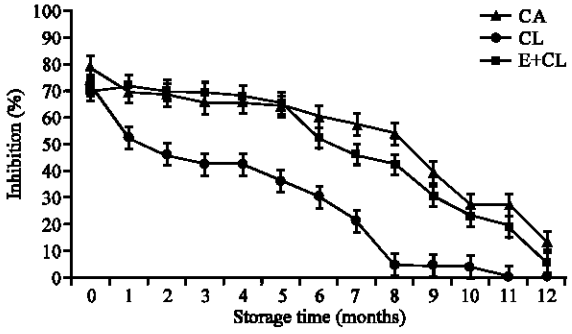

Fig. 4: The inhibitory effect of various seed coating substances on *Rhizoctonia solani* during 12 months of storage (The results indicated in term of Mean±SD)

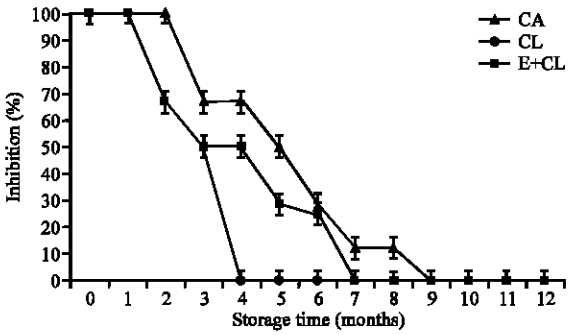

Fig. 7: The inhibitory effect of various seed coating substances on *Aspergillus niger* during 12 months of storage (The results indicated in term of Mean±SD)

Figure 6 and 7 demonstrate the antifungal activity of various seed coating substances on *A. flavus* and *A. niger*. CA could enhance 50% inhibitory effect up to 6 months for *A. flavus* and 5 months for *A. niger*. E+CL could enhance inhibitory effect to 3 months for *A. flavus* and 4 months for *A. niger*. CL showed the highest susceptibility and could inhibit both fungi for only 3 months.

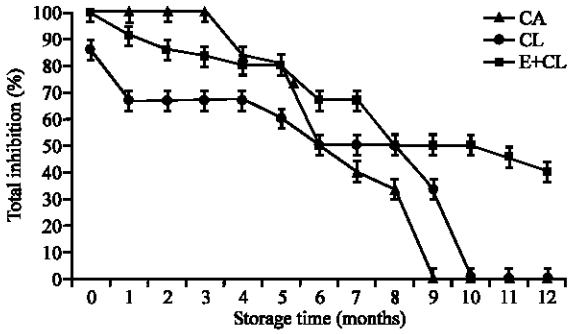

Fig. 8: The inhibitory effect of various seed coating substances on *Bipolaris oryzae* during 12 months of storage (The results indicated in term of Mean±SD)

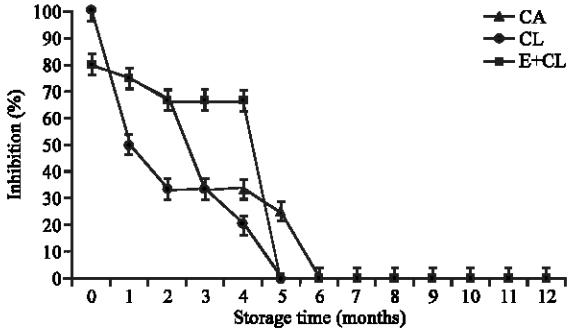

Fig. 9: The inhibitory effect of various seed coating substances on *Nigrospora oryzae* during 12 months of storage (The results indicated in term of Mean±SD)

In the second group, E+CL showed stronger inhibitory effect on *Bipolaris oryzae* (11 months) than CA (7 months) and CL (6 months) (Fig. 8, Table 2). This treatment had 4 months an inhibitory effect on *N. oryzae*. CA inhibited *N. oryzae* for 3 only months and CL for less than 2 months (Fig. 9, Table 2).

For the last group, as showed in Fig. 10, both CA and E+CL showed no significant difference of inhibitory effect on *F. moniliforme*. The E+CL coating polymer and CA could control *F. moniliforme* within 7 months of storage. With CL, *F. moniliforme* could be generated after 3 months (Table 2).

The present study demonstrated the antifungal activity of E+CL treatment. Moreover, the combination of E+CL might be useful as part of a strategy to reduce losses caused by fungi infection, declined resistant of current use of chemical fungicide, but offered equivalent inhibition of seed borne fungi to the commercial recommended captan.

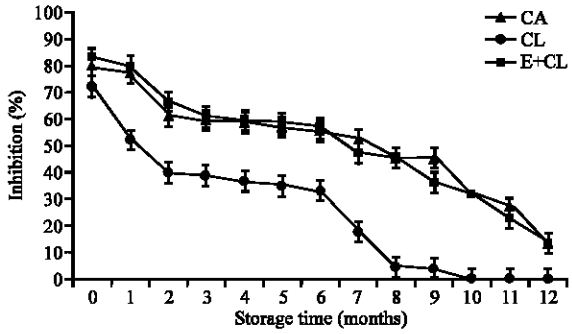

Fig. 10: The inhibitory effect of various seed coating substances on *Fusarium moniliforme* during 12 months of storage (The results indicated in term of Mean±SD)

This study has indicated that a bioactive coating polymer consisting of a combination of eugenol with chitosan polymer. This combination makes it possible to exploit both antifungal and eliciting properties of chitosan polymer, as well as the biological activity of the eugenol.

The study result suggested that the effectiveness of E+CL coating polymer might be related to the interaction of the antifungal activity of eugenol as active compound and chitosan polymer. Consistent with other scientific data, Don *et al.* (2001) reported that chitosan polymer had inhibitory effect against 16 different fungi, especially on *Fusarium* sp., *R. solani* and *Phomopsis* sp. Han (2000) supported that chitosan is bio-polymer, which provided antifungal activity against *A. solani* and *F. moniliforme*, although antifungal activity of chitosan polymer on other fungi was reported by Roller and Covil (1999). Moreover, the result of the previous experiment indicated that eugenol had a strong antifungal activity. Furthermore, number of studies supported that eugenol from clove oils was effective against seed borne fungi (Chami *et al.*, 2005b; Evandro *et al.*, 2005).

The HPTLC results indicated that eugenol essential oil was persisted well on the E+CL coated seeds (Fig. 11a-c). From the literature, it is known that chitosan polymer was act to provide carrier, barrier and protective functions of eugenol, which could be incorporated into the coating material to improve its general functions. Labuza and Breene (1988) found that when antifungal agents are incorporated into the chitosan polymer, the coating polymer could either inhibit or prevent microbial, especially on fungal growth, because chitosan polymer can be prolong the shelf life of antifungal agent and maintain the effectiveness (Ouattara *et al.*, 1999).

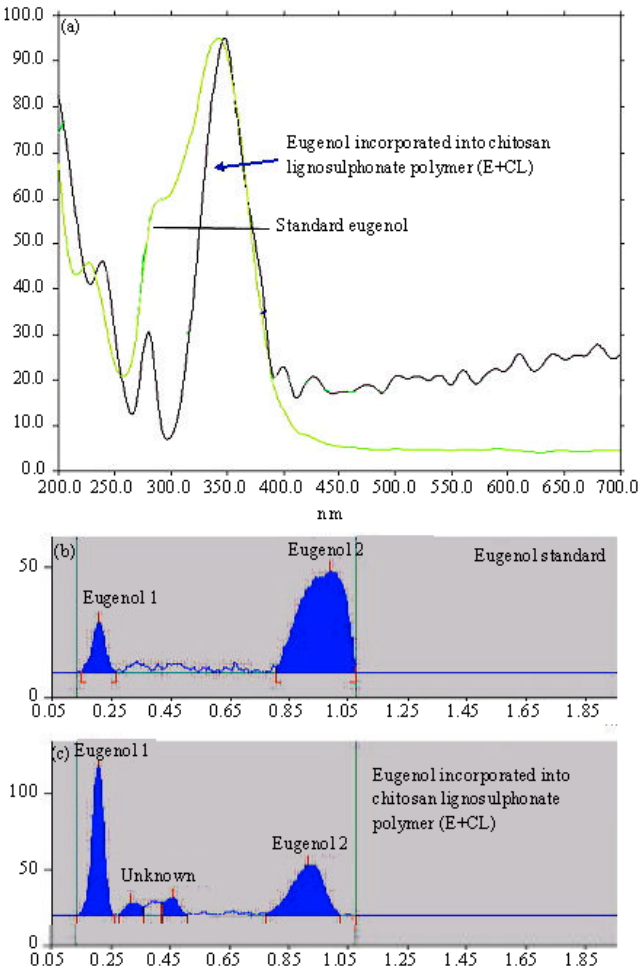

Fig. 11: (a-c) The persistence of eugenol active compound on the E+CL coated seeds compared with standard eugenol, established by HPTLC with the solvent system of C_7H_8-EtO

A major consideration for the study is how to achieve and maintain the antifungal activity of active compounds. The experimental results suggested that CL polymer could control and enhance the releasing rate of antifungal agent for the long time. Consistent with Smith *et al*. (1990) reported that antifungal agents once incorporated into CL polymer could provide antifungal activity by releasing the active compound at a controlled rate. Moreover, Ngah and Liang (1999) found that the use of chitosan coating polymer is better than the direct drench application, because it can control and enhance the releasing rate of antifungal agent for the long time. Normally, when antifungal agent is added into the coating polymer, it affects general physical and chemical properties of coating polymer substances such as elongation, oxygen and water permeability and water absorptiveness (Han, 1996). The adequate solubility of the eugenol is an important point to indicate this antifungal agent can be incorporated well into chitosan polymer (Padgett *et al*., 1998).

The experiment suggested that the diffusion between chitosan coating polymer, antifungal agent and partitioning at the interface is the main migration of active compound involved contacting with the tested microorganism on the surface seed. According to Jun *et al*. (2005), antifungal agent can initially be incorporated into the chitosan coating polymer and migrate to the seed surface through diffusion and partitioning. The controlling of release rate and migration of antifungal agent from chitosan coating polymer to contact target microorganism is a very important factor in establishing the effectiveness of antifungal agent (Roller and Covil, 1999). However, the experiment results showed the reduction of antifungal activity due to the

deterioration of antifungal agents during long-term storage. According to Horita and Kodama (1996) reported that the deterioration of antifungal activity is among others related to high temperatures and high relative humidity, which affect the chemical stability of incorporated antifungal agent and reduce so their activity (Lee *et al*., 2004). Besides chemical degradation, loss of volatile property of essential oil is a reason for the loss of antifungal activity during the storage (Han and Floros, 1999). Moreover, their efficacy is often limited by poor retention at the site of action due to the self-cleansing action of eugenol (Don *et al*., 2001).

In addition, the experiments suggested the loss of antifungal activity of eugenol during storage due to the differences of the chemical and biological characteristics between seed and coating substances such as pH, water activity, temperature, oxygen availability, carbon dioxide levels, presence of antimicrobial and nutrient content availability provide different environmental effect on microorganisms and antifungal agent to act. Nah and Jang (2002) found that the lipids components of the seed might affect the activity of essential oils because of the hydrophobic properties of their active compounds. This minor activity *in vivo* could be because of interactions of essential oils active compounds (known to be lipophilic) with chemical components, such as proteins and lipids, decreasing their effective level (Nugraha *et al*., 2004). Water activity, storage temperature and their interactions alter the antimicrobial activity and chemical stability of incorporated active substance can decrease the antifungal activity (Han, 2000).

Moreover, most species of fungi were growth in wide values of temperature and water activity (Dix and Webster, 1995). The experiment suggested that the rates of inhibition of fungal growth differ at various water activities, depending upon the species, type of cell and factors associated with the environment in which cells are suspended. Beyond some optimum concentration of essential oil, e.g. below some optimum water activities, solutes may act fungi. This might be the penetration of eugenol into the cells of the pathogen is improved in the presence of water activity and therefore pathogens could be more easily controlled (Paster *et al*., 1995; Zambonelli *et al*., 1996). However, the interaction of rice seed, essential oil, coating substances and pathogen was very complex that why the result *in vivo* was different from that *in vitro*. According to the present study, the antifungal mechanisms of essential oils combined with chitosan coating polymer were remain unclear. More work on synergistic action of essential oils and coating material *in vitro* and *in vivo* conditions is required.

The eugenol (the main component of clove oil) is a phenolic compound. The antimicrobial activity of this essential oil can be attributed to the presence of an aromatic nucleus and a phenolic -OH group that is known to be reactive and to form hydrogen bonds with active sites of target enzymes (Farag *et al*., 1989). It was described that the hydroxyl group (bound to a benzene ring) is important for the activities of antifungal compounds that these activities are enhanced by the presence of a-b double bonds (Ultee *et al*., 2002). The experiment suggested that the acidic pH conditions from CL polymer could alter the ionization (dissociation/association) of hydroxyl group, which can change the antimicrobial activity of eugenol (Wang *et al*., 2006). Moreover, this condition affects on the growth rate of target microorganisms because fungi generally grow well in acidic conditions. Furthermore, Dix and Webster (1995) pointed out that the H^+ concentration has direct effect on fungal metabolism due to the buffering system in hyphae, may influence the ionization of salts in solution and the permeability of the plasmalemma of the hyphae. Additionally, enzyme activity is also affected by H^+ concentration (Enokibara *et al*., 1993).

The results of our experiments reported that during the storage time, the antifungal activities of captan fungicide were not long lasting because captan has low antifungal level. Moreover, the experiment investigation suggested that the resistance of certain insolated fungus to the captan fungicide found in this study could be caused by the exposure of the fungus to the intensive chemical control, which leads to the pre-resistant to the fungicide condition.

CONCLUSION

This study demonstrated that eugenol is very active to inhibit rice seed borne fungi and could be an alternative replacement for the synthetic chemical seed treatment. E+CL coating polymer had a potential antifungal activity against seed borne fungi as *F. moniliforme*, *A. solani*, *B. oryzae*, *R. solani*, *Curvularia* sp., *A. flavus* and *A. niger* which are often resistant to available antifungal agents. However, the inhibitory effects *in vivo* were not as strong as those *in vitro*. Further studies are required to emphasize on the effects and toxicity of E+CL coating polymer in rice seed and examining their safety for using as antifungal agents against rice seed borne fungi.

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