International Journal of Agricultural Technology 2017 Vol. 13(2): 153-162
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Controlling *Cercospora lactucae-sativae* causes Lettuce Leaf Spot Disease Using Antagonistic Yeasts

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Kham-un, P, Cheewangkoon, R. and To-anun, C. (2017). Controlling lettuce leaf spot disease using antagonistic yeasts. International Journal of Agricultural Technology 13(2):153-162.

Abstract The experiment was investigated the antagonistic yeasts to potential against leaf spot in lettuce disease by *Cercospora lactucae-sativae*. Use isolation of epiphytic yeasts was made from surfaces of healthy lettuce 12 isolate and vegetable and fruits 63 isolates were obtained. Preliminary test showed that 24 isolates gives percentage of growth inhibition higher than 30. Then 24 isolates were selected to do efficacy test on *Cercospora lactucae-sativae* using dual culture method. Results on percentage of growth inhibition are as 14.75 to 77.18. Fifth yeast isolates (CMY057, CMY050, CMY071, CMY027 and CMY035) were selected for their inhibitory effects on conidial and assayed by spore germination for their antagonistic. Then 5 isolates of higher inhibition were selected to test spore germination on agar to show inhibitory effect against *Cercospora lactucae-sativae,* the plant pathogen causing leaf spot disease on lettuce. Results Four yeast isolates (CMY050, CMY071, CMY027 and CMY035) the conidial germinate on inhibition at 12 hours.

Keywords: antagonistic yeasts, *Cercospora lactucae-sativae*, lettuce

Introduction

Lettuce is the world's most popular leafy salad vegetable (Raid, 2004). Lettuce probably originated from Asia, where it was grown for centuries and its early forms were used in Egypt around 4500 years ago (Lindqvist, 1960). Around 50 AD, the Romans cooked the leaves with oil and vinegar while smaller leaves were occasionally eaten raw. After the Romans, medieval Europe popularized poaching lettuce alongside mixing the leaves with hot oil and vinegar (Weaver 1997, 170-172). The Romans grew types of lettuce resembling the present romaine cultivars as early as the beginning of the Christian era. in 2006, China produced around 11,005,000 metric tonnes of lettuce on 500,250 hectares of land. In 2010, the FAO reported that some 12,574,500 tonnes (12,375,900 long tons; 13,861,000 short tons) of lettuce were produced during that year (FAO, 2012). There are several types of lettuce, but three (leaf, head and cos or

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romaine) are the most common (Bradley, 2009). Though low in calories it is a vitamin and mineral supplement. Stem lettuce provides vitamin C and also calcium (Simoons, 1991). Lettuce is now one of the world's most important salad crops and is grown worldwide.

Cercospora leaf spot caused by cercospora longissima is fairly common in china. It has been reported in North America. Itcan affect both cultivated and wild lettuce. (Chupp, 1954, Szeto and Bau, 1975 and Toro, 1929) Genus *Cercospora* was established by Fresenius in 1863 (Braun, 1995). The number of species has increased yearly, because most species are plant pathogenic and appear to be highly host-specific (Den Breeÿen *et al*., 2006; Hunter *et al*., 2006; Periera and Barreto, 2006) in Thailand has been reported Cercospora leaf spot cause by *Cercospora lactucae-sativae* (To-Anun *et al*., 2011). Leaf spots first appear as minute, water-soaked specks that gradually enlarge into circular to irregular spots that turn various shades of tan to brown. Sometime, spot centers are dingy gray. Occasionally, dead areas sufficiently numerous to kill an entire leaf. The disease progresses from older outer leaves to newer leaves (Szeto and Bau, 1975). commercial varieties generally have only moderate levels of resistance and require fungicide applications to obtain acceptable levels of protection against Cercospora leaf spot (Miller *et al*., 1994). application of fungicides is still the effective method to control these diseases. The wide spread use of the chemical fungicides has become a subject of research concern due to their harmful effect on non-target organisms as well as their possible carcinogenicity. Some reports, which have done, on field isolates of *C. beticola* and *E. betae* indicated that some of these isolates were fungicide resistant's (Weiland and Koch, 2004 and Fernández-Aparicio *et al*., 2009).

The use of biological control agents constitutes an important tool in meeting the growing demand for products that are less toxic to humans, animals and the environment (Jamalizadeh *et al*., 2011) A variety of microbial antagonists have been reported to control several different pathogens on various fruits and vegetables (Fravel, 2005; Mari and Guizzardi, 1998). Some yeasts have the capability to effectively compete with plant pathogens and can be used as biocontrol agents (Leibinger *et al*., 1997; Roberts, 1990; Yin, 2008). Saprophytic yeasts have been studied as biological control agents for the control of various fungal pathogens, including *Botrytis cinerea*, *Penicillium* spp., and *Monilinia fructicola* in apples, grapes, pears, peaches, sweet cherries and citrus fruit. Furthermore, selected yeast strains can also be applied in combination with suitable fungicides to maximize the efficacy of biocontrol while reducing the amount of fungicides on food products (Janisiewicz *et al*., 2002; Fravel, 2005; Droby *et al*., 2009). Saprophytic yeasts can be found on the surface of plant fruits and leaves. Under dry conditions, saprophytic yeasts colonize plant surfaces and produce extracellular polysaccharides to enhance their survivability. By producing extracellular polysaccharides, these yeasts

restrict nutrient flow and inhibit the colonization of other microorganisms. Some researchers have used saprophytic yeasts to control Aspergillus in nut trees (Hua *et al*., 1997; Hua, 2001; Palumbo *et al*., 2006). The aim of this study was to control cercospora leaf spot disease on lettuce cause by *cercospora lactucae-sativae.*

Material and methods

The fungal pathogen

Lettuce leaves and leaf litter showing signs of fungal colonization were chosen for study. Isolate *Cercospora lactucae-sativae* from by obtaining single conidial colonies as explained in Crous (2002). Colonies were subcultured onto potato-dextrose agar (PDA) plates. Incubated at 25 °C under continuous near nature light 8-10 hr, to promote sporulation.

Isolation of epiphytic yeasts

Yeast isolates were obtained from the healthy lettuce leaves epidermis following the methodology described by Rabosto *et al*. (2006). Five leaves from each cluster were washed for 30 minutes with water and Shake for 10 min of rose bengal solution (0.05 g rose bengal in 1 L sterile distilled water). The resulting solution was diluted (1:10), and 50 µL were spread on yeast-extract peptone dextrose (YPD) medium and incubated at 25 °C for 3-5 day. Selection different single colony of yeast was cultured in YPD. Finally, isolates were inoculated in tubes with inclined YPD medium and stored at 4 °C for subsequent analysis.

Screening for yeast with inhibitory activity on C. lactucae-sativae mycelial growth on PDA

Initial screening of yeast for maximum inhibitory activity against the mycelial growth of *C. lactucae-sativae* was performed on PDA on Petri dishes by the dual culture method Magnusson (2003). The mycelial plugs (5 mm diameter) of pathogens were placed on the same dish 6 cm from each other. To test for antagonistic yeast, a 5 mm of mycelia agar disc from pathogen cultures was placed on the one side of a Petri dish containing PDA medium. The dishes were incubated at 25 °C for for 3-5 day. A loop of yeast was then streaked 3 cm apart from the fungal pathogen and incubated at a temperature of 25 °C. A mycelial disk of the pathogen growing without yeast was the control. Cultures were incubated at 25 °C for 60 day. The experiment was arranged using a complete randomized design (CRD) with fifth replications. The percent growth inhibition (PGI) was calculated using the formula (((R1 - R2)/R1) x 100 – where equation R1 = radial growth of

the pathogen in control and R2 = radial growth of the pathogen in dual culture with antagonist yeast) (Korsten *et al*., 1995).

Inhibitory of yeast isolates on C. lactucae-sativae conidial germination on PDA culture

An assay was established to select yeasts with antagonistic activity on *C. lactucae-sativae* conidial germination at room temperatures (28 $^{\circ}$C). A volume of 100 µL of suspended yeast (1×10^8 cells mL^{-1}) and 100 µL suspended pathogen conidia (1×10^6 conidia mL^{-1}) were spread on PDA medium. The suspended pathogen conidia were spread on PDA medium in the control treatment. Records 100 conidia were counted for each replicate and the length of the germination tube was determined at time 0, 3, 6, 9 and 12 hr.

Results

The fungal pathogen

The symptom cercospora leaf spot: leaf spots circular or oval, up to 1 cm diam, sometimes coalescing to cover large areas of the leaf, grey or light brown with a small (0.5–1 mm) whitish centre. On the lesion found conidiophores single or in fascicles of 2–10, pale olivaceous brown, 25–90 × 4–6 µm, on both leaf surfaces. Conidiophores usually continue to elongate, producing successive conidia, and hence 2 or more scars. Conidia 20–220 × 3.5–5 µm, hyaline, 10–20-septate, smooth, ob clavate, straight or slightly curved, tip rounded, basal cell truncate with a distinct scar (McKenzie, 2013). On PDA media show the all most color of colony is dark-green and gray some time have white color.

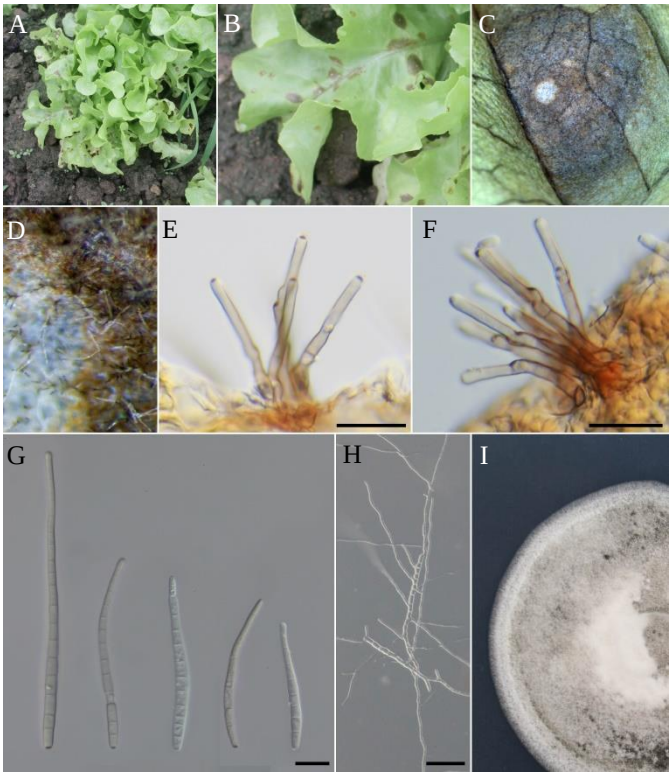

Figure 1. Morphology of leaf spot disease on lettuce., A–B: Symptoms/signs of leaf disease, C: Signs of leaf spot disease under stereo microscope (20X), D: Signs of leaf spot disease under stereo microscope (80X), E–F: Morphology of conidiophore–straight type, G: conidia–club, H: conidial germination and I: colony on PDA (Scale bar= 20 µm).

Isolation of epiphytic yeasts

12 isolates were obtained from the healthy lettuce leaves epidermis in Chiang Mai provincde: 3 isolate from red oak at Doi Saket, 1 isolate from green oak at Doi Saket, 2 isolate form green leaf at Doi Saket, 2 isolate from cos at Sanpatong, 1 isolate form green leaf at Muang, 2 isolate form green oak at Maejo and 1 isolate form green leaf at Maejo. Add 63 isolate epiphytic yeast from laboratory, Faculty of Agriculture, Chiang Mai University.

Screening for with inhibitory activity on C. lactucae-sativae mycelial growth on PDA

The results of the growth inhibition confirmed very significant differences between the isolate yeast with *C. lactucae-sativae* on PDA using the dual culture technique isolates. The culture *C. lactucae-sativae* grew actively and full colonized the plate in 60 days. Five out of the seventy-five

isolates tested were able to inhibit the growth of three pathogens each by more than 60% is CMY057, CMY050, CMY017, CMY027 and CMY035. In seventy-five isolates CMY117 gave the lowest percentage inhibition of 14.75% against while CMY057 and CMY050 gave the highest inhibition of 76.50-77.18%.(Fig. 2)

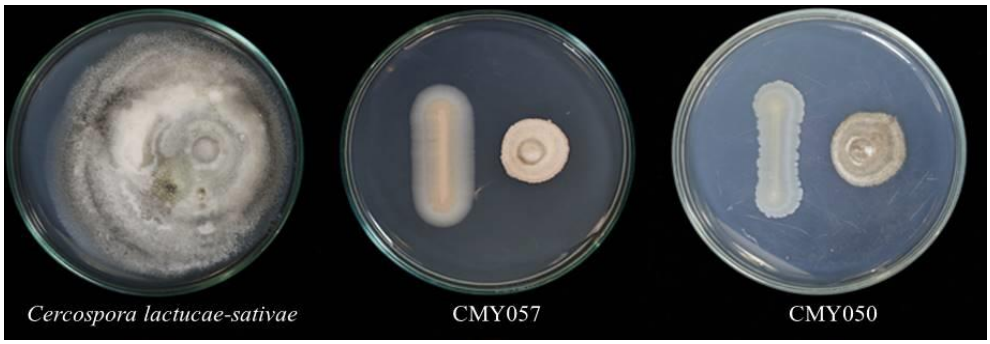

Figure 2. Screening for with inhibitory activity on C. lactucae-sativae mycelial growth on PDA by dual culture technique

Inhibitory of yeast isolates on C. lactucae-sativae conidial germination on PDA culture

Five yeast isolates (CMY057, CMY050, CMY071, CMY027 and CMY035) were selected for their inhibitory effects on conidial and assayed by spore germination for their antagonistic. Then 5 isolates of higher inhibition were selected to test spore germination on agar to show inhibitory effect against *Cercospora lactucae-sativae,* the plant pathogen causing leaf spot disease on lettuce. Results 4 yeast isolates (CMY050, CMY071, CMY027 and CMY035) the conidial germinate on inhibition at 12 hours. (Fig 3, Table 1)

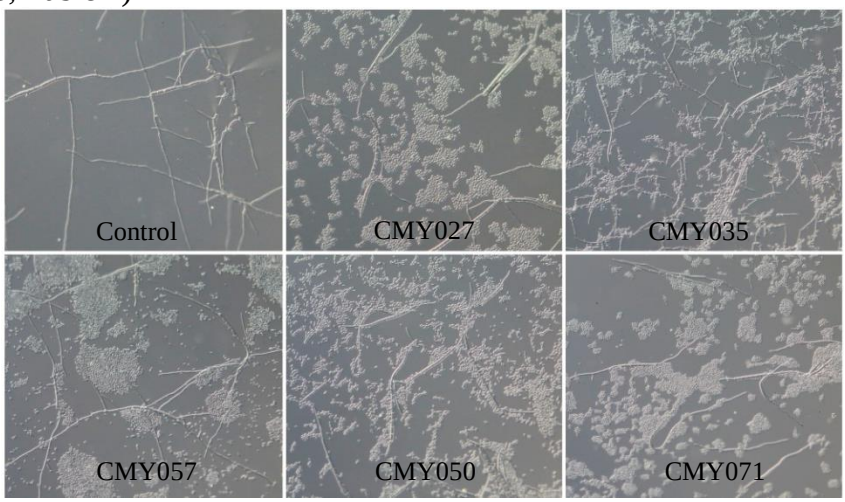

Figure 3. Inhibitory of yeast isolates on *C. lactucae-sativae* conidial germination on PDA culture at 12 hours.

Table 1. efficiency of yeast isolates to inhibit the *C. lactucae-sativae* conidial germination cause by leaf spot in lettuce

Treatment	Germtube length (µm)[1]						
	0	**3**	**6**	**9**	**12**	**CV%**	**$LSD_{p=0.05}$**
Control	1.47eA[2]	10.02dA	30.02cA	73.34bA	150.00aA	12.18	4.04
CMY027	1.04dAB	11.76cA	13.23cB	20.45bC	28.02aC	48.38	4.52
CMY035	0.88aAB	1.70aB	1.50aC	1.64aDC	2.10aD	169.00	1.66
CMY057	0.36dB	3.81dB	16.61cB	28.82bB	37.87aB	71.97	7.90
CMY050	1.07dAB	10.53cA	13.20bcB	18.10bC	25.76aC	57.79	4.98
CMY071	0.43cB	4.43cB	15.15bB	21.52aC	24.96aC	60.46	5.05
CV%	165.43	63.94	58.11	38.57	23.79		
$LSD_{p=0.05}$	0.91	2.82	5.44	6.60	6.67		

[1] The average was calculated using data from 100 replications.
[2] Values in the same column with different superscripts significantly differed at P=0.05.

Discussion

On lettuce, leaf spot disease caused by *Cercospora lactucae-sativae* was identified based on morphology with the help of literature (McKenzie, 2013). A variety of microbial antagonists have been reported to control several different pathogens on various fruits and vegetables (Fravel, 2005; Mari and Guizzardi, 1998). Some yeasts have the capability to effectively compete with plant pathogens and can be used as biocontrol agents (Leibinger *et al*., 1997; Roberts, 1990; Yin, 2008). This approach has become a positive alternative to chemical pesticides which is safe for humans, animals and the environment (Attyia and Youssry, 2001).

The laboratory experiments demonstrated the growth inhibition differences between the isolate yeast with *C. lactucae-sativae* on PDA using the dual culture technique isolates. The culture *C. lactucae-sativae* grew actively and full colonized the plate in sixty days. Fifth out of the seventy-fifth isolates tested were able to inhibit the growth of three pathogens each by more than 60%. Found that the isolate yeast can produce the secondary metabolite to control fungus agents as 2 types. Yeast products the secondary metabolite transfer in media to control fungus agent (Masih *et al*., 2001). This make inhibition zone between isolate yeast with fungus agents. And the isolate yeast product the secondary metabolite in the form of gas (volatile) (Höfte *et al*., 2004 and Andrews, 1992). This make the size colony is smaller than usual. In vitro experiments confirm that this yeast can be used as a biological control organism against (Masih *et al*., 2001). And experiments demonstrated the inhibitory effect of antagonistic yeast isolates on conidial germination of *C. lactucae-sativae* on PDA. Found that the isolate product volatile can,t control conidial germination of *C. lactucae-sativae* in open systems. Moreover, Several antagonistic yeasts have previously been isolated from fruits and vegetables and efficaciously used

as biocontrol agents as Alternaria rot in pair cause by *Alternaria alternata* (Wan and Tian, 2005), *Botrytis cinerea* causes rot in fruit (Mercier and Wilson, 1995), fruit rot in mango cause by *Botryodiplodia theobromae* (Sugiprihatini *et al*., 2011), Anthracnose in fruit and vegetable cause by *Colletotrichum capsici* (Chanchaichaovivat *et al*., 2007), damping off in suger beet young plant cause by *Fusarium oxysporum* (El-Sayed Shalaby and El-Nady, 2008), brown rot in stone fruits cause by *Monilinia laxa* (Bonaterra, 2003), blue mold in apple cause by *Penicillium expansum* (Vero *et al*., 2002), damping off cause by *Sclerotinia sclerotiorum* (Reeleder, 2004), root rot in chickpea cause by *Fusarium solani*, *Rhizoctonia solani*, *Macrophomina phaseolina* and *Sclerotium rolfsii* (Ali, 2001; Siddiqui, 2001; Abdel-Kader, 1997) Powdery mildew in grape cause by *Erysiphe necator* Schwein. var. *necator* (formerly *Uncinula necator*) (Pearson, 1988). This study presents that yeast strain CMY035 can slow and stop the conidial germinate. So, CMY035 can use to be a biocontrol agent against *C. lactucae-sativae* infection of lettuce leaves in greenhouse and field.

Acknowledgments

This research was supported by Bhubing Palace. The financial support from the Graduate School, Chiang Mai University, is also gratefully acknowledged.

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(Received: 23 January 2017, accepted: 26 February 2017)