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Analysis of β-tubulin gene from carbendazim resistant isolates of *Cercospora lactucae-sativae* on Lettuce in Thailand

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The sensitivity of *Cercospora* spp causing leaf spot to carbendazim was tested by plating assays. Out of 60 isolates, 48 were highly resistant which identified as *Cercospora lactucae-sativae* isolated from leaf spot of lettuces and 12 were sensitive isolates; one from lettuce and other isolates from other host plants. Analysis of the β-tubulin gene (*TUB1*) from representative isolates of *C. lactucae-sativae* showed a single nucleotide mutation (A to C) at the predicted codon, codon 198, which encodes glutamic acid (GAC) in sensitive isolates that caused codon 198 to encode alanine (GCG) in highly resistant isolates. Moreover, highly resistant isolates showed the mutations e.g., from leucine (CTC) to histidine (CAC) and valine (GTC, GTT) to alanine (GCC) at codons 139 and 189, respectively, without conferring a phenotype. The detection of such point mutations in the β-tubulin gene allows the rapid screening to detect carbendazim resistant isolates in the field.

Key words: β-tubulin gene, carbendazim resistant, *Cercospora lactucae-sativae*, Lettuce

Introduction

Lettuce (*Lactuca sativa* L.) is the most popular leafy salad vegetable (Raid, 2004) which has grown steadily during the last two decades (Hospido *et al*., 2009) but it is subjected to many diseases (Savary, 1983). Cercospora leaf spot caused by *Cercospora* spp is one of the most important foliar disease of lettuce in many regions (Koohakan *et al*., 2008; Savary, 1983; Hotegni *et al*., 2011). The variable symptoms (Chupp, 1954) may be presented as small, circular lesions with tan, gray or white in centers and later turned to blight. *Cercospora* spp. cause leaf spot on numerous host plants in tropical–regions

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(Agrios, 2005). The damaged leaves are usually lost in weight and market value due to their low quality (Koohakan *et al*., 2008).

Disease management relies on a foliar fungicides (Davis *et al*., 1997) which usually apply carbendazim which it is a benzimidazole, a broad spectrum chemical fungicide (Davidse, 1986). It is affected to mycelia growth and distorted germ tubes (Leroux, 2007) by specifically inhibiting microtubule assembly and mitosis (Davidse, 1986; Steffens *et al*., 1996; Ma and Michailides, 2005). A single nucleotide mutation in β-tubulin genes at either codon 198 or 200 and associated with amino acid substitutions (Buhr and Dickman, 1994) reduces the binding affinity of benzimidazole to β-tubulin, conferring sensitivity among fungal isolates (Davidson *et al*., 2006). Benzimidazoles can quickly control disease which essential for disease management (Davidson *et al*., 2006; Gado, 2007). It is continuously applied for disease control for long periods which leading to the emergence of carbendazim-resistant fungi. Fungicide resistance has been reported in apple scab fungus *Venturia inaequalis* and grey mold fungus *Botrytis cinerea* after two years of intensive benzimidazole application in the field (Deising *et al*., 2008), and a high risk for resistance to benzimidazoles has been predicted (Brent and Hollomon, 1998; Damicone and Smith, 2009). The resistance in *Cercospora* spp., especially *C. beticola*, causal agent of sugar beet leaf spot, have been reported in many countries (Georgopoulos and Dovas, 1973; Ruppel and Scott, 1974; Dexter and Luecke, 1999; Weiland and Smith, 1999; Weiland and Halloin, 2001; Davidson *et al*., 2006; Imazaki *et al*., 2006; Piszczek and Czekalska, 2006; Gado, 2007) after a few years of extended application and the fungicides lost their effectiveness due to a mutation in a single gene (Ma and Michailides, 2005).

However, the investigation of carbendazim-resistant isolates of *Cercospora lactucae-sativae* isolated from leaf spot of lettuce in Thailand has not been previously described . The aims of this study were to evaluate and characterize the level of carbendazim resistant isolates of *C. lactucae-sativae*. The nucleotide sequences of the partial β–tubulin gene fragment from resistant and sensitive isolates of *C. lactucae-sativae* were compared to understand the mechanism of fungicide resistance and strategies for disease management

Materials and methods

Survey, sample collection and identification of Cercospora species

Cercospora leaf spot disease was surveyed during 2008-2009, the leaf spot samples were collected from twelve cultivated fields mostly planted to lettuces (*Lactuca sativa* L.) and other host plants *Coffea arabica, Eupatorium*

adenophorum, Lantana camara, Jatropha curcus, Vigna unguiculata, Capsicum annuum, Hydrangea macrophylla, Zinna elegans, Areca catechu, Capsicum frutescens and Cocconia grandis and some samples from markets in the Chiang Mai and Chian Rai provinces, Thailand. The leaf spot samples were made moist chamber and incubated at room temperature, then periodically observed under microscope. The single conidium was picked up from a necrotic lesion using a stereomicroscope and suspended in 50–70 µl of sterile distilled water, spread over the surface of water agar (WA) in a Petri dish and incubated at 25^{o}C for 24 hr. Single colony was then transferred to potato dextrose agar (PDA) plates to be pure culture for further experiment.

Screening for carbendazim resistant isolates of Cercospora lactucae-sativae on Lettuce

All isolates were tested to screen carbendazim sensitivity to *Cercospora* spp. The experiment was done by using Completely Randomized Design (CRD) with three replications. Treatments were different concentrations of carbendazim of 0, 1, 10, 50, 100, 500 and 1000 µg/ml amended in PDA before autoclaved at 121 °C, 15 lbs/inch^2 for 20 min. A mycelia plug of *Cercospora* spp. was cut from peripheral of colony by sterilized cock borer and transferred into the middle of PDA amended with carbendazim in each concentration and incubated for 14 days at 25 °C. Data were collected as colony diameter (cm) and computed to percent inhibition as colony diameter in control – colony diameter in treatment/ colony diameter in control X 100.

Colony growth diameter of each fungal isolate was measured and expressed as percent inhibition. Percentage of inhibition was equivalent compared to the level of carbendazim resistant by phenotype reactions modified from Farungsang and Farungsang (1992); Farungsang *et al*. (1994); Koenraadt *et al*. (1992) and Peres *et al*. (2004) as follows:-

Percent inhibition (%)	phenotype reactions
<10%	sensitive (Car^S) at ≤1 µg/ml
≥10–35%	weakly resistant (Car^{WR}) at ≤10 µg/ml;
>35–65%	moderately resistant (Car^{MR}) at ≤100 µg/ml
>65–90%	highly resistant (Car^{HR}) at ≥500 µg/ml

The representative isolates were selected from highly resistant isolates (Car^{HR}) and sensitive isolates (Car^S) for further experiment.

Analysis of β-tubulin gene from carbendazim resistant isolates of Cercospora lactucae-sativae

The representative isolates of Car^S and Car^{HR} were selected to analyze β-tubulin gene from carbendazim sensitive or resistant isolates. DNA extraction: all selected isolates of *Cercospora* spp were separately grown on PDA for 14 days at room temperature (28-30°C) under 12-h photoperiod. Five hundred milligrams of the mycelia from the agar surface were collected and ground to a fine powder in liquid nitrogen using a mortar and pestle. The nucleic acids were then extracted from the powdered mycelia using a NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The genomic DNA pellets were resuspended and separated by 1% agarose gel electrophoresis and visualised with 0.7% ethidium bromide staining and UV illumination. Fragments were compared with a 100-bp molecular weight marker (RBC Bioscience).

Polymerase chain reaction amplification of β-tubulin gene from *Cercospora* species: The representative isolates of *Cercospora* species were then analysed using a polymerase chain reaction and a set of β-tubulin gene species-specific primers TB2L (5′-GTT TCC AGA TCA CCC ACT CC-3') and CER2R (5′-TGA GCT CAG GAA CAC TGA CG-3′) were designed from the sequence of the *TUB2* region (Peres *et al*. 2004). The total 50-µl volume for the PCR reactions contained 10 ng of genomic DNA, 5 µl of 10× PCR buffer (iNtRON Biotechnology), 25 mM of $MgCl_2$ (iNtRON Biotechnology), 10 mM of dNTPs (iNtRON Biotechnology), 50 pmol of each primer, and 1 unit of *Taq* polymerase (Fermentas). All PCR reactions were carried out in a PTC-100 programmable thermal controller (MJ Research), with a hold of 5 min at 95°C; followed by 30 cycles of 1 min at 95°C, 1 min at 35°C (T_m), and 1 min at 72°C; and a final extension of 5 min at 72°C. The amplification products were separated by 1% agarose gel (Research Organics) electrophoresis. Products were visualised with 0.7% ethidium bromide staining and UV illumination, and sizes were determined by comparison with a 100-bp molecular weight marker (RBC Bioscience).

Cloning and sequencing: the amplification products were extracted from 1% low melting point agarose gel and cloned into pGEM-T Easy Vector Systems (Promega, WI, USA) using the manufacturer's instructions. The sequences were obtained from both strands, using the dideoxy chain termination method with an ABI Prism Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) and an automated fluorescent DNA Sequencer (Model 310, Applied Biosystems). Sequence similarity and alignment analyses were performed using BLAST in GenBank or

the NCBI database with the implemented CLUSTALW algorithm and BioEdit program.

Results

Survey, sample collection and identification of Cercospora species

Result showed that 60 isolates were morphologically identified as *Cercospora* spp. Withthis, 49 isolates were from *Lactuca sativa* and the rest isolates were from *Coffea arabica , Eupatorium adenophorum, Lantana camara, Jatropha curcus, Vigna unguiculata, Capsicum annuum, Hydrangea macrophylla, Zinna elegans, Areca catechu, Capsicum frutescens* and *Cocconia grandis* as seen in Table 1.

Screening for carbendazim resistant isolates of Cercospora lactucae-sativae on Lettuce

Out of 60 isolates, 48 were highly resistant to carbendazim which identified as *Cercospora lactucae-sativae* isolated from leaf spot of lettuces and 12 were sensitive isolates; one from lettuce and other isolates from other host plants. It is observed that carbendazim-sensitive isolates could not grow at the lowest concentration (1 µg/ml) (Table 1). All 48 highly resistant isolates grew well at all concentrations of carbendazim (Fig. 1).

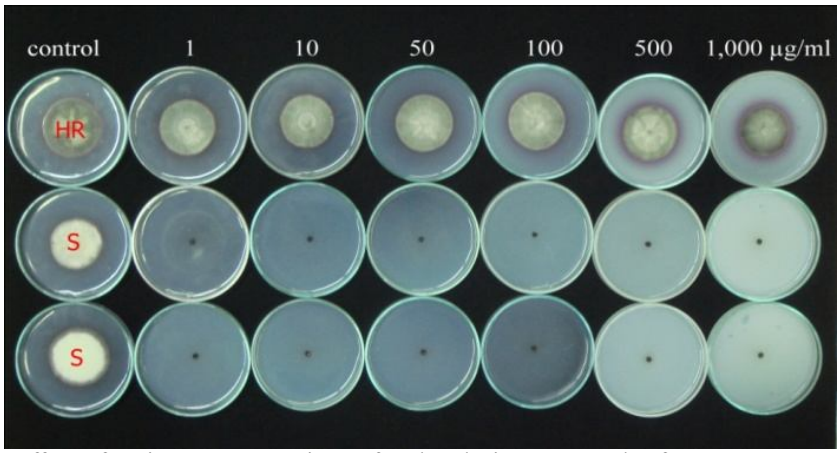

Fig. 1. Effect of various concentrations of carbendazim on growth of *Cercspora* spp after 14 days at 25°C on PDA, S = Car^{S}, sensitive isolates; HR = Car^{HR}, highly resistant isolates

Table 1. *Cercspora* spp used in this study, the site of origin, pigment on PDA, and carbendazim phenotype.

No.	Isolate	Host	Location	Phenotype
1	CCR01	*Lactuca sativa* var. *longifolia*	Municipal market,Muang,CM[1/]	Car^{S}
2	CCR02	*Coffea arabica*	CMU, Muang, CM	Car^{S}
3	CCR03	*Eupatorium adenophorum*	Queen Sirikit Botanical garden, Mae-rim, CM	Car^{S}
4	CCR04	*Lantana camara*	Wieng-pa-pao,CR[2/]	Car^{S}
5	CCR05	*Jatropha curcas*	Suthep-pui forest, Muang, CM	Car^{S}
6	CCR06	*Vigna unguiculata*	Wieng-pa-pao, CR	Car^{S}
7	CCR07	*Capsicum annuum*	Suthep-pui forest, Muang, CM	Car^{S}
8	CCR10	*Lactuca sativa* var. *crispa*	Municipal market, Muang, CM	Car^{HR}
9	CCR11	*Hydrangea macrophylla*	Suthep-pui forest, Muang, CM	Car^{S}
10	CCR12	*Zinnia elegans*	Municipal market, Muang, CM	Car^{S}
11	CCR13	*Areca catechu*	Mushroom Research Center, Mae-tang, CM	Car^{S}
12	CCR14	*Coccinia grandis*	CMU, Muang, CM	Car^{S}
13	CCR15	*Lactuca sativa* L.	Maesapok, Mae-wang, CM	Car^{HR}
14	CCR18	*Lactuca sativa* var. *longifolia*	Pangda, Sa-meung, CM	Car^{HR}
15	CCR19	*Lactuca sativa* var. *capitata*	Huayluk, Chiang-dao, CM	Car^{HR}
16	CCR20	*Lactuca sativa* L.	Pangda, Sa-meung, CM	Car^{HR}
17	CCR21	*Capsicum frutescens*	CMU, Muang, CM	Car^{S}
18	CL01	*Lactuca sativa* var. *capitata*	MaeHae, Mae-wang, CM	Car^{HR}
19	CL02	*Lactuca sativa* var. *capitata*	MaeHae, Mae-wang, CM	Car^{HR}
20	CL03	*Lactuca sativa* var. *capitata*	MaeHae, Mae-wang, CM	Car^{HR}
21	CL04	*Lactuca sativa* var. *capitata*	MaeHae, Mae-wang, CM	Car^{HR}
22	CL05	*Lactuca sativa* L.	Nonghoi, Mae-rim, CM	Car^{HR}
23	CL06	*Lactuca sativa* L.	Nonghoi, Mae-rim, CM	Car^{HR}
24	CL07	*Lactuca sativa* var. *crispa*	MJU, San-sai, CM	Car^{HR}
25	CL08	*Lactuca sativa* var. *crispa*	MJU, San-sai, CM	Car^{HR}
26	CL09	*Lactuca sativa* var. *crispa*	MJU, San-sai, CM	Car^{HR}
27	CL10	*Lactuca sativa* var. *crispa*	MJU, San-sai, CM	Car^{HR}
28	CL11	*Lactuca sativa* var. *crispa*	MJU, San-sai, CM	Car^{HR}
29	CL12	*Lactuca sativa* L.	MJU, San-sai, CM	Car^{HR}
30	CL13	*Lactuca sativa* L.	MJU, San-sai, CM	Car^{HR}
31	CL14	*Lactuca sativa* L.	MJU, San-sai, CM	Car^{HR}
32	CL15	*Lactuca sativa* L.	MJU, San-sai, CM	Car^{HR}
33	CL16	*Lactuca sativa* L.	MJU, San-sai, CM	Car^{HR}
34	CL17	*Lactuca sativa* L.	MJU, San-sai, CM	Car^{HR}
35	CL18	*Lactuca sativa* L.	MJU, San-sai, CM	Car^{HR}
36	CL19	*Lactuca sativa* var. *longifolia*	MJU, San-sai, CM	Car^{HR}
37	CL20	*Lactuca sativa* var. *longifolia*	MJU, San-sai, CM	Car^{HR}
38	CL21	*Lactuca sativa* var. *longifolia*	MJU, San-sai, CM	Car^{HR}
39	CL22	*Lactuca sativa* var. *longifolia*	MJU, San-sai, CM	Car^{HR}
40	CL23	*Lactuca sativa* var. *longifolia*	MJU, San-sai, CM	Car^{HR}
41	CL24	*Lactuca sativa* var. *longifolia*	MJU, San-sai, CM	Car^{HR}
42	CL25	*Lactuca sativa* var. *longifolia*	MJU, San-sai, CM	Car^{HR}
43	CL26	*Lactuca sativa* var. *longifolia*	MJU, San-sai, CM	Car^{HR}
44	CL27	*Lactuca sativa* var. *longifolia*	MJU, San-sai, CM	Car^{HR}
45	CL28	*Lactuca sativa* var. *longifolia*	MJU, San-sai, CM	Car^{HR}
46	CL29	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
47	CL30	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
48	CL31	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
49	CL32	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
50	CL33	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
51	CL34	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
52	CL35	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
53	CL36	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
54	CL37	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
55	CL38	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}

56	CL39	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
57	CL40	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
58	CL41	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
59	CL42	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}
60	CL43	*Lactuca sativa* var. *capitata*	MJU, San-sai, CM	Car^{HR}

[1/]CM = Chiang Mai, [2/]CM = Chiang Rai, Car^{s} = sensitive to carbendazim, Car^{HR} = highly resistant to carbendazim

Analysis of β-tubulin gene from carbendazim resistant isolates of Cercospora lactucae-sativae

Of the 21 representative isolates of *Cercospora lactucae-sativae* from lettuces (*Lactuca sativa*) used for further genomic DNA analysis, 17 isolates were Car^{HR} and three isolates were Car^{S} which 2 isolates from lettuce and one isolate from *Capsicum annuum* including C-3 isolate which is *Cercospora beticola*) accession no. AY856373 (used for comparison. Because, there are no reports on genomic ANA analysis on *C. lactucae-sativae*. The amplicon of the partial β–tubulin gene fragment with the specific primer pair produced a 474-bp product for all isolates. No nucleotide mutations were found in the Car^{S} isolates, CCR01, CCR07 and CCR21. The Car^{HR} strains expressed a single nucleotide mutation in the fragment at the codon 198 when compared with the nucleotide sequence of the *TUB1* gene fragment retrieved from GenBank, *C. beticola* (accession AY856373), the sensitive strain. Subsequently, a single amino acid substitution for glutamic acid (GAG) by alanine (GCG) at the codon 198 was found, but no substitutions were found at codon 200 in any isolates. Moreover, deduced amino acid substitution for leucine (CTC) by histidine (CAC) was demonstrated at another codon in isolate CL06 (Car^{HR}), and valine (GTC, GTT) was substituted by alanine (GCG) at codon 189 in isolate CL43. (Table 2 and Figure 2)

Table 2. Amino acid and nucleotide substitutions in partial sequence of the β-tubulin (*TUB1*) gene in *Cercospora* isolates with different resistance levels to carbendazim

Isolate	Resistant level	Substitution		
		Codon 139	Codon 189	Codon 198
C-3[1/]	Car^{s}[2/]	L (CTC)[/3]	V (GTT)	E (GAG)
CCR01	Car^{s}	L (CTC)	V (GTC)	E (GAG)
CCR21	Car^{s}	L (CTC)	V (GTC)	E (GAG)
CCR19	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL02	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL05	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL06	Car^{HR}	H (CAC)	V (GTC)	A (GCG)
CL07	Car^{HR}	L (CTC)	V (GTC)	A (GCG)

CL08	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL10	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL15	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL16	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL18	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL19	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL23	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL24	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL28	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL32	Car^{HR}	L (CTC)	V (GTT)	A (GCG)
CL40	Car^{HR}	L (CTC)	V (GTC)	A (GCG)
CL43	Car^{HR}	L (CTC)	A (GCC)	A (GCG)

[1/]C-3 = *Cercospora beticola*)accession no. AY856373(, [2/]Car^{S} = sensitive to carbendazim, Car^{HR} = highly resistant to carbendazim, [3/]A = alanine, E = glutamic acid, H = histidine, L = leucine, V = valine

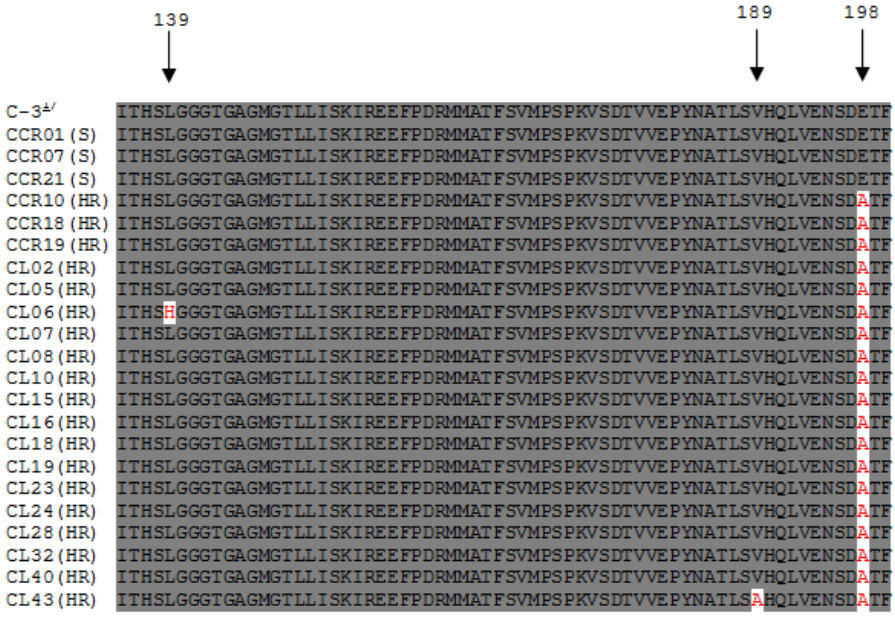

Fig. 2. Comparison of deduced nucleotide and amino acid sequences of the β-tubulin (*TUB1*) from highly resistant (HR) or sensitive (S) *Cercospora* isolates to carbendazim with *Cercospora beticola*)[1/]C-3: accession AY856373(. A = alanine, E = glutamic acid, H = histidine, L = leucine and V = valine

Discussion

Of 60 isolates of *Cercospora* spp that were classified as either sensitive or highly resistant according to their response to carbendazim fungicide sensitivity assays, 48 (80%) isolates were defined as highly resistant and 12 (20%) were sensitive. In this study, *C. lactucae-sativae* from lettuce was only highly

resistant phenotype which Briere *et al*. (2001) and Weiland and Halloin (2001) pointed out that mostly due to the extensive, continuously field application of the systemic fungicide. Moreover, *Cercospora kikuchii* isolates were also resistant to carbendazim at high concentrations (Imazaki *et al*., 2006), and *C. beticola* isolates exhibited benzimidazole resistance (Davidson *et al*., 2006).

Fungal resistance to benzimidazoles has been studied for decades. The resistance is associated with the benzimidazole target site, the β-tubulin (*TUB1*) gene, and a single nucleotide mutation and distorted amino acid sequence translation that changes glutamic acid to glycine, lysine, alanine, or valine (Koenraadt *et al*., 1992; Buhr and Dickman, 1994; Peres *et al*., 2004; Sholberg *et al*., 2005; Chung *et al*., 2006; Ru and Sheng, 2007), subsequently conferring various resistance levels appeared in the field isolates (Orbach *et al*., 1986; Fujimura *et al*., 1992; Koenraadt *et al*., 1992; Yan and Dickman, 1996; Yarden and Katan, 1993; Buhr and Dickman, 1994; Gafur *et al*., 1998; Albertini *et al*., 1999; Peres *et al*., 2004; Chung *et al*., 2006; Davidson *et al*., 2006; Ziogas *et al*., 2009).

In this study, isolates of *Cercospora lactucae-sativae* were highly resistant to carbendazim, and all isolates obtained from lettuce leaves. A single nucleotide mutation at codon 198 in the β-tubulin gene was observed in all highly resistant isolates but not in the sensitive isolates. The partial *TUB1* genes sequence analyses of *Cercosprora* sp. isolates showed a nucleotide mutation at codon 198 with adenine (A) changed to cytosine (C), resulting in the amino acid substitution from glutamic acid (GAG) in the Car^{S} phenotype to alanine (GCG) in the Car^{HR} phenotype. This mutation was correlated with the Car^{HR} phenotype in all tested isolates, agreeing with previous reports on benomyl tolerance in citrus with *Mycospaerella citri*, which causes greasy spot disease (Whiteside, 1980) and post-bloom fruit drop disease of citrus in the United States and Brazil (Peres *et al*., 2004).

The mutation of amino acid at codon 198 in the β -tubulin gene has been reported in others fungi-*Botrytis cinerea* (Yarden and Katan, 1993; Ziogas *et al*., 2009), *Monilinia fructicola* (Koenraadt *et al*., 1992; Ma *et al*., 2003), *Mycosphaerella fijiensis* (Cañas-Gutiérrez *et al*., 2006), *Neurospora crassa* (Fujimura *et al*., 1992), *Penicillium* spp. (Koenraadt *et al*., 1992; Baraldi *et al*., 2003; Sholberg *et al*., 2005), *Sclerotinia homoeocarpa* (Koenraadt *et al*., 1992), *Tapesia yallundae, Tapesia acuformis* (Albertini *et al*., 1999), *Venturia inaeqalis* and *Venturia pirina* (Koenraadt *et al*., 1992), *Cladosporium fulvum* (Yan *et al*., 2008), *Colletotrichum gloeosporioides* (Kongtragoul *et al*., 2011). Major binding sites for carbendazim involve codon 198 and 200, localized in *TUB1* gene. However, different mutation points in others codon have been discovered, e.g., in *M. fructicola* at codon 6 (Ma *et al*., 2003); *Fusarium*

moniliforme at codon 50 (Yan and Dickman, 1996); *Cochliobolus heterostrophus* (Gafur *et al*., 1998), *P. expansum* (Baraldi *et al*., 2003) and *N. crassa* (Orbach *et al*., 1986) at codon 167; and *T. yallundae* and *T. acuformis* at codon 240 (Albertini *et al*., 1999). In the present study, mutations were also found at different codons, resulting in substitution for the deduced amino acids of the isolates CL06 (Car^{HR}) and CL43 (Car^{HR}) at codon 139 (leucine replaced by histidine) and 189 (valine replaced by alanine); the mutation at different codons resulted in different levels of resistance level as reported previously for to benzimidazole (Ma *et al*., 2003).

However, the notable findings in this study, the deduced amino acid at codon 134 and 189 did not confer benzimidazole resistance in *Cercospora lactucae-sativae*; the amino acid at the oblique target site within the β-tubulin gene fragment was not associated or involved directly in the carbendazim-response phenotype.

When a large number of isolates need to be tested, the planting assay to characterize the resistance phenotype is labor-intensive and time-consuming (Yan *et al*., 2008). On the contrary, detecting a single nucleotide to genotype fungicide sensitivity is a rapid screening method to monitor and understand fungicide resistance in fields. Hence, the investigation of *Cercospora lactucae-sativae* resistant to carbendazim in Thailand has elucidated the mechanism of fungicide resistance associated with extensive use of a fungicide and such evaluation would help in control strategies involving fungicides.

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