CULTURAL CHARACTERISTICS OF *SPHACELOMA AMPELINUM*, CAUSAL PATHOGEN OF GRAPE ANTHRACNOSE ON DIFFERENT MEDIA

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Abstract

***Sphaceloma ampelinum*, the causal pathogen of grape anthracnose, is the anamorph stage of *Elsinoe ampelina.* To evaluate its variability, isolates of *S. ampelinum* were collected from diseased plants from the northeastern, northern, eastern, and western regions of Thailand. The pathogen was isolated by a tissue transplanting method on water agar (WA) and the growing mycelium was subsequently transferred onto cereal agar (CA) and susceptible grape leaves to induce sporulation. Single conidial isolates were obtained and 19 representatives from all regions were cultured on potato dextrose agar (PDA), CA, corn cereal agar (CCA) and Job's tear corn cereal agar (JCCA) for cultural characterization. It was found that the different culture media affected growth, colony color, appearance, and formation of aerial mycelium. The best culture media for surface mycelium growth was CA, while PDA led to the highest aerial mycelium formation. In addition, CA induced the highest morphological variability among isolates. Variation of isolates appeared to be more pronounced among different regions than that within the same regions, except isolates from the eastern region. By using cultural characteristics alone, some isolates within the same region cannot be fully differentiated. These results suggest that diversity exists among isolates of *S. ampelinum* in Thailand, particularly those from different regions.**

Keywords: *Elsinoe ampelina*, *Sphaceloma ampelinum*, culture medium, grape

Introduction

Grape (*Vitis vinifera*) grows well in the tropical areas, but it usually faces with numerous disease problems. Major grape diseases in Thailand are downy mildew, anthracnose, and rust. Particularly, anthracnose or scab caused by the fungus *Sphaceloma ampelinum* de Bary, a pathogen of European origin, is one of the most significant. In Thailand, this disease was first reported in 1990 (Peinpuck *et al*., 1993). It is widely dispersed in the rainy season when temperature and moisture are favorable for disease development (Koůdela and Krejzar,

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2006). Symptoms appear as small light brown spots on young tissues, and old leaves appear shot-hole and dry. On berries, severely affected berries shrivel and finally dry. Anthracnose is a serious disease that could cause as high as 50% crop losses in a season, thus the use of chemicals is usually required to allow sufficient protection, especially during the rainy season (CAB International, 2000). However, chemicals are detrimental to human health as well as to the environment. Therefore, a grape cultivar resistant to anthracnose is an alternative approach for efficient and environmentally friendly grapevine production in Thailand. As a first step for designing efficient breeding strategies and selecting suitable parents, knowledge of pathogen diversity is often required. Previous study on *S. ampelinum* diversity in Thailand was based on cultural characterization of isolates grown on PDA. However, this approach had been faced with very slow growth and limited variation of *S. ampelinum* (Makrung, 2005). In this study, the variability in cultural characteristics of *S. ampelinum* isolates from different regions of Thailand was evaluated using 3 new media developed in our laboratory in addition to PDA.

Materials and Methods

Pathogen Isolation

Isolates of *S. ampelinum* from 4 main grape-growing regions of Thailand, northeastern (Nakhon Ratchasima province), eastern (Chonburi province), northern (Chiang Rai and Prae provinces) and western (Ratchaburi province) regions were collected from leaves of susceptible grape varieties affected by the anthracnose disease in 2006. Leaf samples were surface sterilized in 1.5% (w/v) sodium hypochlorite for 3 - 5 min, rinsed 3 times in sterile distilled water for 30 s, and dried with sterile absorbent paper towels. Approximately 1 mm^2 of lesion samples were placed on water agar (WA; 2% (w/v) agar) with 25 mg/L streptomycin and 0.05% (v/v) lactic acid). The plates were incubated for 3 - 5 d at 28ºC in the dark. Subsequently, the hyphal tips of each isolate were transferred to cereal agar (CA; 2% (w/v) mixed cereal (Aharn Thammachart Thantan Roak®; 40% milled unpolished rice, 20% Job's tear, 20% lotus seed, 15% barley, and 5% mixture of different beans), 2% (w/v) dextrose, 1.5% (w/v) agar with 25 mg/L streptomycin). After 1 week, pieces of colony were placed on susceptible grape leaves to promote conidia formation. After 7 days, single conidium was isolated from each culture and transferred to the CA medium and incubated at 28ºC in the dark. A total of 19 single conidial isolates of *S. ampelinum* were obtained and used for cultural characterization.

Cultural Characterization

Of the 19 single conidial isolates characterized, 4 isolates were from the northeastern (Nk2-1, 3-1, 4-1 and 5-1), 5 isolates were from the eastern (Cb1-1, 2-1, 3-1, 4-1 and 5-1), 5 isolates were from the western (Rc1-1, 2-1, 3-1, 4-1 and 5-1) and 5 isolates were from the northern (Cr1-1, 2-1, 3-1, Pr4-1 and 5-1) regions. A 4-mm-diameter agar disk of each isolate was obtained by cutting with a sterile cork borer, and placed onto 4 different media: (1) PDA, (20% (w/v) potato, 2% (w/v) dextrose, 2% (w/v) agar); (2) CA; (3) corn cereal agar (CCA), (1% (w/v) corn grit, 1% (w/v) mixed cereal, 2% (w/v) dextrose, 1.5% (w/v) agar); and (4) Job's tear corn cereal agar (JCCA), (0.5% (w/v) Job's tear, 0.5% (w/v) corn grit, 0.5% (w/v) soybean, 1% (w/v) mixed cereal, 2% (w/v) dextrose, 1.5% (w/v) agar). The plates were incubated at 28ºC in the dark. Five replicates per isolate were made, and the following observations were taken at 2, 5, and 8 weeks after plating on the medium: (1) colony size (area = ¶ (width/2) (length/2)), (2) presence or absence of aerial mycelium by observation under stereo microscope (3) colony color, and (4) colony appearance (colonies were characterized based on height [flat, raised, and highly raised] and surface texture [smooth, wrinkled, and deeply wrinkled]). Analysis of variance was conducted using the statistical analysis system (SAS, 1987) to evaluate the differences in colony size among isolates and regions.

Results

Nineteen single conidial isolates were obtained from 4 geographical regions of Thailand, representing the main grape-growing areas with distinct climates. When these isolates were grown on PDA, CA, CCA, and JCCA, it was found that different cultural media affected growth of all 19 isolates of *S. ampelinum* significantly ($P < 0.001$; Table 1, Figure 1). Overall the best media for *S. ampelinum* growth were CA and CCA (Figure 1) and the best medium for aerial mycelium formation was PDA (Table 2). Maximum difference in colony size was observed at 5 weeks when colonies grown on CA and CCA were approximately 60% larger than those grown on PDA and JCCA (Figure 1). However, at 8 weeks the size of colonies grown on PDA was no longer significantly different from those grown on CA and CCA since colonies of most isolates had almost reached the edges of the petridishes. Growth variation also existed among isolates collected from different regions of Thailand. Significant differences in colony size ($P < 0.0001$) were observed at 5 and 8 weeks when average colony sizes from the eastern region were the largest (Table 1, Figure 2, data not shown).

The presence of aerial mycelium depended on the media used. Among the 4 media, PDA induced the highest aerial mycelium density at 5 weeks. In contrast, most isolates

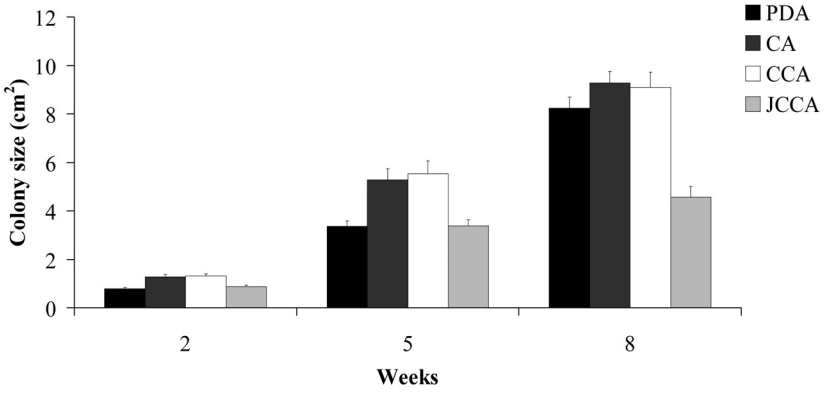

Figure 1. Effect of different media on growth of 19 *S. ampelinum* isolates at 2, 5, and 8 weeks

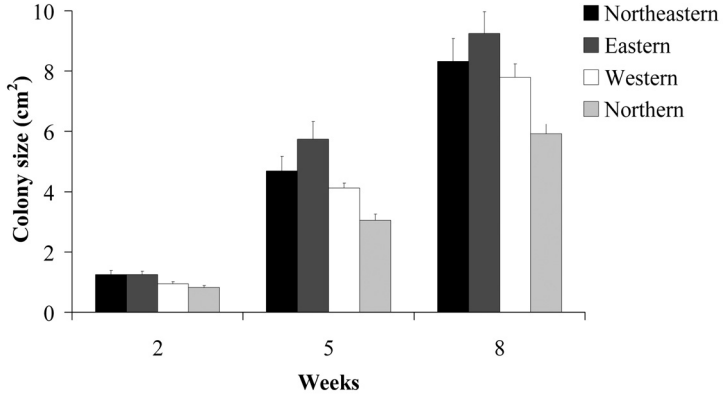

Figure 2. Growth of 19 *S. ampelinum* isolates from the northeastern, eastern, western, and northern regions at 2, 5, and 8 weeks

produced aerial mycelium poorly on CA and CCA even at 8 weeks (Table 2; data not shown). When sporulation of aerial mycelium was evaluated microscopically at 2 weeks, 2 isolates (Cb2-1 and Rc1-1) grown on the JCCA medium sporulated. However, at 5 weeks, both isolates sporulated to a greater extent on PDA than JCCA (data not shown). It appeared that the sporulation of this pathogen was correlated with aerial mycelium formation.

Different cultural media also affected cultural characteristics (colony color and appearance). These characteristics were more diverse among isolates on CA and CCA. It was found that the color was not a stable characteristic over time. At 2 weeks, all colonies had only 1-2 colors, especially those grown on PDA and JCCA (most colonies were brown and yellow, respectively). As colonies developed, more color was formed and the greatest color variation among isolates as well as among media was observed at 5 weeks. At this time colony color was distinctly different among isolates from different regions, especially on CA and CCA. At 8 weeks, the fungus began to stop growing and appeared darker and hence less variable (data not shown). For example, the colonies of Nk3-1 on CA appeared yellow at 2 weeks, turned yellow, orange, and white at 5 weeks, and were brown and black at 8 weeks. Likewise, Cr3-1 colonies appeared yellow and red at 2 weeks on CA, turned yellow, red, brown, and white at 5 weeks and at 8 weeks they were grey and brown (Figure 3; data not shown).

Colony appearance was characterized based on colony height and surface texture. In contrast to colony color which changed over time, colony appearance of each isolate was

Table 1. Colony size of 19 isolates of *S. ampelinum* grown on different media for 5 weeks

Region	Isolate	Colony size (cm^2)			
		PDA	CA	CCA	JCCA
Eastern	Cb1-1	3.53 ± 0.16 defg [1]	4.28 ± 0.26 ghij	4.11 ± 0.16 ghi	4.06 ± 0.12 bc
	Cb2-1	4.47 ± 0.24 abc	8.59 ± 0.28 b	7.01 ± 0.84 d	2.99 ± 0.25 def
	Cb3-1	4.03 ± 0.30 abcde	8.51 ± 0.40 b	8.13 ± 0.49 c	3.19 ± 0.25 cde
	Cb4-1	2.19 ± 0.25 hi	9.64 ± 0.25 a	11.11 ± 0.62 a	6.57 ± 0.45 a
	Cb5-1	3.86 ± 0.14 bcdef	6.13 ± 0.27 d	8.69 ± 0.26 c	3.81 ± 0.57 bcd
Mean		3.62 ± 0.39	7.43 ± 0.98	7.81 ± 1.14	4.12 ± 0.64
Northern	Cr1-1	1.43 ± 0.14 i	4.38 ± 0.24 fghi	3.58 ± 0.24 i	1.73 ± 0.26 g
	Cr2-1	1.73 ± 0.21 i	4.33 ± 0.61 fghij	4.23 ± 0.27 ghi	2.23 ± 0.26 fg
	Cr3-1	3.10 ± 0.15 fg	2.73 ± 0.15 k	3.48 ± 0.31 i	1.58 ± 0.14 g
	Pr4-1	1.98 ± 0.13 hi	3.27 ± 0.19 jk	3.58 ± 0.14 i	3.83 ± 0.17 bcd
	Pr5-1	3.74 ± 0.27 bcdef	3.80 ± 0.16 ij	3.81 ± 0.34 hi	2.37 ± 0.14 efg
Mean		2.40 ± 0.44	3.70 ± 0.32	3.74 ± 0.14	2.35 ± 0.40
Northeastern	Nk2-1	2.77 ± 0.16 gh	5.41 ± 0.20 def	5.05 ± 0.23 efg	3.46 ± 0.06 cd
	Nk3-1	3.71 ± 0.49 bcdef	3.95 ± 0.32 hij	3.92 ± 0.15 hi	2.40 ± 0.11 efg
	Nk4-1	4.09 ± 0.29 abcd	5.49 ± 0.64 de	5.63 ± 0.34 e	3.38 ± 0.38 cd
	Nk5-1	4.76 ± 0.29 a	7.49 ± 0.15 c	9.94 ± 0.30 b	4.65 ± 0.35 b
Mean		3.83 ± 0.42	5.34 ± 0.93	6.14 ± 1.32	3.47 ± 0.46
Western	Rc1-1	3.94 ± 0.30 abcdef	4.51 ± 0.55 efghi	4.50 ± 0.12 fghi	3.62 ± 0.19 cd
	Rc2-1	3.60 ± 0.23 cdef	4.74 ± 0.35 efghi	4.85 ± 0.23 efgh	3.69 ± 0.11 cd
	Rc3-1	3.12 ± 0.28 efg	5.26 ± 0.28 defg	5.43 ± 0.25 ef	3.43 ± 0.38 cd
	Rc4-1	3.24 ± 0.25 defg	5.03 ± 0.36 efgh	4.48 ± 0.21 fghi	3.86 ± 0.20 bcd
	Rc5-1	4.57 ± 0.54 ab	3.69 ± 0.04 ijk	3.71 ± 0.08 hi	3.22 ± 0.31 cde
Mean		3.69 ± 0.26	4.65 ± 0.27	4.59 ± 0.28	3.56 ± 0.11
Grand mean		**3.36 ± 0.22 b**	**5.28 ± 0.45 a**	**5.54 ± 0.53 a**	**3.37 ± 0.26 b**

[1] Data are presented as means ± SE. Means in the same column followed by different letters differ significantly at $P < 0.05$, based on the DMRT

Table 2. Colony color, aerial mycelium, and colony appearance of 19 *S. ampelinum* isolates grown on different media for 5 weeks

Isolate	Colony color [1]				Aerial mycelium [2]				Colony appearance			
	PDA	CA	CCA	JCCA	PDA	CA	CCA	JCCA	PDA	CA	CCA	JCCA
Cb1-1	TBr-W	Br-O-W-Y	Br-O-R	TBr	++	++	-	+	highly raised, deeply wrinkled	raised, wrinkled	raised, wrinkled	highly raised, deeply wrinkled
Cb2-1	G-W-Y	Y	Y	Br-W	++	-	-	++	highly raised, deeply wrinkled	flat, smooth	flat, smooth	highly raised, wrinkled
Cb3-1	G-W-Y	Y	Y	Br-W	++	-	-	++	highly raised, deeply wrinkled	flat, smooth	flat, smooth	highly raised, wrinkled
Cb4-1	Br-O-W	Br-W	Br-O-Y	TBr	++	++	-	++	highly raised, wrinkled	flat, smooth	flat, smooth	highly raised, deeply wrinkled
Cb5-1	Br-O-W	Br-G-O-W	Br-O-W-Y	TBr	++	++	-	+	highly raised, wrinkled	flat, smooth	flat, smooth	highly raised, wrinkled
Rc1-1	TBr-W	Br-O-R	Br-O-R	TBr-W	++	-	-	++	highly raised, deeply wrinkled	raised, smooth	raised, smooth	highly raised, deeply wrinkled
Rc2-1	TBr-W	Br-O-R	Br-O-R	TBr	++	+	+	+	highly raised, deeply wrinkled	raised, smooth	raised, smooth	highly raised, wrinkled
Rc3-1	TBr-W	Br-O-R	Br-O-R	G-TBr	++	-	+	++	highly raised, deeply wrinkled	flat, smooth	flat, smooth	highly raised, deeply wrinkled
Rc4-1	TBr-W	Br-O-R	Br-O-R-W	TBr	++	-	+	++	highly raised, deeply wrinkled	raised, smooth	raised, smooth	highly raised, deeply wrinkled
Rc5-1	TBr-W	Br-O-R	Br-O-R	TBr	++	-	+	++	highly raised, deeply wrinkled	raised, smooth	raised, smooth	highly raised, wrinkled
Nk2-1	TBr	O-W-Y	Br-O-Y	TBr	++	+	-	+	highly raised, deeply wrinkled	raised, wrinkled	raised, smooth	highly raised, deeply wrinkled
Nk3-1	TBr	O-W-Y	Br-O-Y	TBr	++	+	+	+	highly raised, deeply wrinkled	raised, wrinkled	flat, smooth	highly raised, wrinkled
Nk4-1	TBr	O-W-Y	Br-O-Y	TBr	++	++	+	+	highly raised, deeply wrinkled	flat, wrinkled	flat, wrinkled	highly raised, deeply wrinkled
Nk5-1	Bl-W-Y	Br-G-W-Y	TBr-Y	Br	++	++	+	++	highly raised, deeply wrinkled	flat, smooth	flat, smooth	highly raised, wrinkled
Cr1-1	TBr-W	R-Y	R-Y	Br	++	-	-	-	highly raised, deeply wrinkled	flat, smooth	flat, smooth	highly raised, deeply wrinkled
Cr2-1	TBr-W	Br-O-Y	R-Y	Br	++	-	+	-	highly raised, deeply wrinkled	raised, smooth	raised, wrinkled	highly raised, deeply wrinkled
Cr3-1	TBr-W	Br-R-W-Y	R-Y	Br	++	+	+	-	highly raised, deeply wrinkled	raised, wrinkled	flat, smooth	highly raised, deeply wrinkled
Pr4-1	TBr-W	Br-O-Y	R-Y	Br	++	-	-	+	highly raised, deeply wrinkled	flat, smooth	raised, smooth	highly raised, wrinkled
Pr5-1	TBr	TBr-Y	Br-W-Y	TBr	+	+	-	-	highly raised, deeply wrinkled	flat, smooth	raised, smooth	highly raised, wrinkled

[1] Color codes for fungal colonies: Bl = Black, Br = brown, G = gray, O = orange, R = red, TBr = different tones of brown, W = white, Y= yellow

[2] ++ = presence of aerial mycelium at high density; + = presence of aerial mycelium at low density; - = absence of aerial mycelium

stable from 2 - 8 weeks. Colony appearance of each isolate varied according to the media. All isolates grown on PDA and JCCA appeared highly raised with a deeply wrinkled or wrinkled surface. However, the colony appearance of isolates grown on CA and CCA were more variable in appearance; flat and smooth, flat and wrinkled, raised and smooth, and raised and wrinkled (Table 2) Only limited variability in colony appearance was observed among isolates and there was no association between this characteristic and regions.

Discussion

Cultural characteristics of *S. ampelinum* varied among isolates, especially those from different regions. These characteristics were also affected

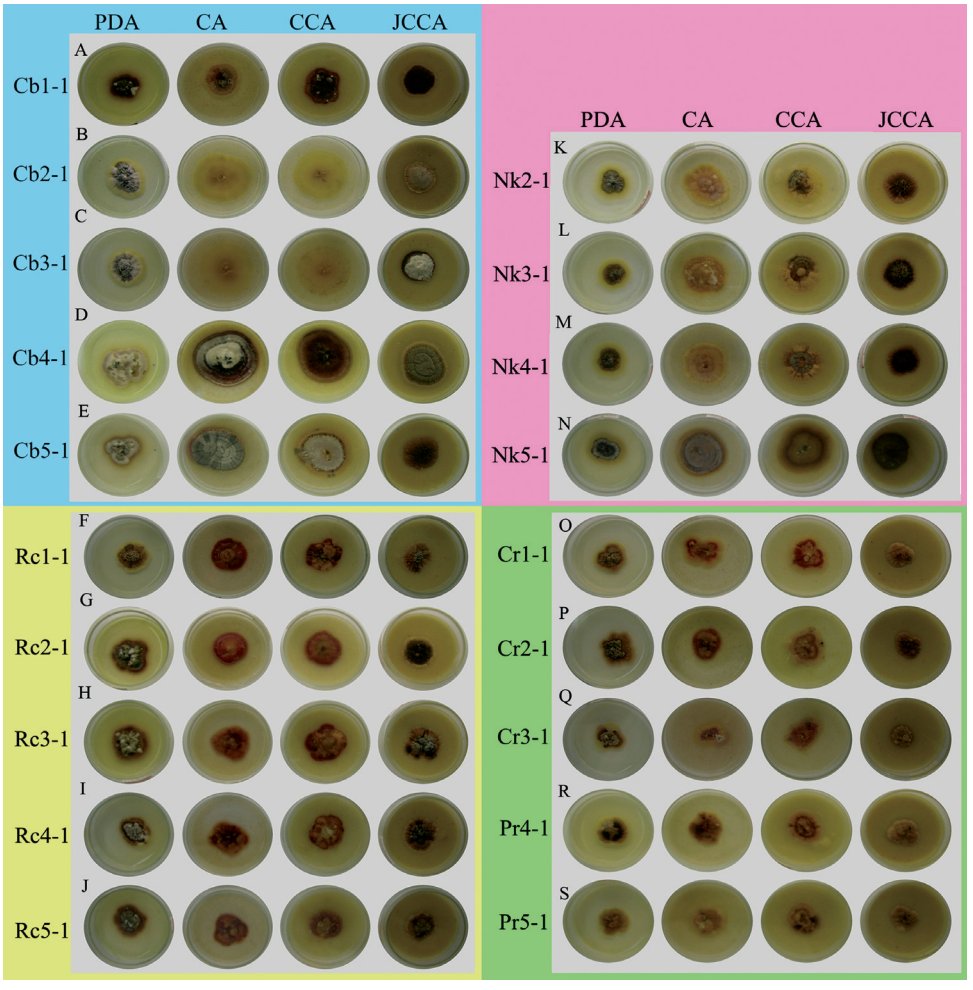

Figure 3. Colony morphology of *S. ampelinum* isolates grown on different media for 5 weeks. Isolates from eastern region (A-E): Cb1-1 (A), Cb2-1 (B), Cb3-1 (C), Cb4-1 (D), and Cb5-1 (E); western region (F-J): Rc1-1 (F), Rc2-1 (G), Rc3-1 (H), Rc4-1 (I), and Rc5-1 (J); northeastern region (K-N): Nk2-1 (K), Nk3-1 (L), Nk4-1 (M), and Nk5-1 (N); northern region (O-S): Cr1-1 (O), Cr2-1 (P), Cr3-1 (Q), Pr4-1 (R), and Pr5-1 (S)

by the different media used. Similarly, Cheema *et al.* (1978) reported variation in colony diameter of 9 *S. ampelinum* isolates grown on different media. In addition, Alvarez and Molina (2000) reported that colony growth of *S. manihoticola*, the causal pathogen of cassava superelongation, was irregular and highly variable among different isolates. All 19 isolates from the various regions grew slowly on PDA in agreement with those reported in Peinpuck *et al*. (1993) and Makrung (2005) for *S. ampelinum*, Alvarez and Molina (2000) for *S. manihoticola*, and Koŭdela and Krejzar (2006) for *S. symphoricarpi*. However, the 2 new media CA and CCA promoted 1.1- to 5-fold lateral colony growth in most isolates, except Rc5-1.

At 2 weeks, another new medium, JCCA, was the most favorable for rapid growth of aerial mycelium; however, at 5 weeks PDA promoted the greatest aerial mycelium formation, followed by JCCA. It should be noted that the media that were best for lateral growth (CA and CCA), on the other hand induced poor aerial mycelium formation and sporulation in most isolates. An inverse relationship between the production of ascospores and the production of hyphae was also observed in *Talaromyces flavus* grown on different media (Engelkes *et al*., 1997). The slow aerial mycelium development on PDA was previously reported by Alvarez and Molina (2000) in *S. manihoticola* which required at least 3 or 4 weeks of growth. Similarly, Makrung (2005) found that most *S. ampelinum* isolates sporulated after 6 - 8 weeks on PDA. From our observation, the formation of aerial mycelium appeared to be associated with sporulation in this pathogen. It is possible that high nutrient media may be unsuitable for inducing sporulation in some fungi. Instead they might promote high surface mycelium growth, but low aerial mycelium formation (Anonymus, 2007).

Engelkes *et al*. (1997) reported that the amount of carbon source in the culture medium is important for the growth of fungi. However, the C:N ratio appeared to be more influential for sporulation than the carbon concentration (Goa *et al*., 2007). It was found that ascospore production of *T. flavus* increased with an increasing C:N ratio. All media used in this experiment had the same concentration of sugar (2% dextrose in PDA, CA, CCA, and JCCA), but had various tuber/grain supplements. The C:N ratios of tuber/grain components in PDA, CA, CCA, and JCCA used in this study were 10.9:1, 5:1, 6.7:1, and 6.5:1, respectively. On PDA with the highest C:N ratio, *S. ampelinum* had the poorest surface mycelium growth but had the highest aerial mycelium growth and sporulation. On the contrary, media with a low C:N ratio like CA and CCA promoted the highest surface mycelium growth but suppressed formation of aerial mycelium and sporulation in most isolates. Our results are in agreement with previous reports showing that growth of aerial mycelium and sporulation on artificial media usually depends on media components, particularly the C:N ratio and the fungal species (Rajderkar, 1965; Jackson and Bothast, 1990; Goa *et al*., 2007). For example, the optimal condition for *Paccilomyces lilacinus* sporulation was a C:N ratio between 10:1 and 20:1, while a C:N ratio of 160:1 was optimal for *Metarhizium anisopliae*, *Lecanicillium lecanii*, and *Trichoderma viride* sporulation (Goa *et al*., 2007). In some fungi a too high or too low C:N ratio could suppress conidia production. *Colletotrichum truncatum* (Schwein.) produced significantly more conidia on a medium with a C:N ratio of 15:1 than media with C:N ratios of 40:1 and 5:1 (Jackson and Bothast, 1990). However, factors other than the C:N ratio must also have played an important role on growth of *S. ampelinum* since the effect of JCCA on surface mycelium growth was similar to PDA, although its C:N ratio was much lower. In fact the C:N ratio of JCCA was more similar to those of CA and CCA; however, this media contained Job's tear and soybean in addition to the cereal mix. Previous reports showed that deficiencies or excesses of mineral elements as well as variation in the protein and lipid content of the media could contribute to the quantity and quality of conidia production (Rajderkar, 1965; Jackson and Bothast, 1990). It is possible that the variation in aerial mycelium formation and

conidium yield of *S. ampelinum* observed here reflected the differences in essential nutrients among the media.

There was a wide diversity in colony color for *S. ampelinum* isolates and this characteristic was not stable over time. It was found that the best time for color diversity evaluation was 5 weeks. On PDA, Makrung (2005) reported that colony color of *S. ampelinum* had only 3 groups (light yellow, dark yellow or orange, and red). Similarly, we found 5 groups of colony color on PDA. Nevertheless, CA gave much higher color diversity (11 groups) which was more informative for characterization. Whether the variation in colony color among isolates reflected the underlying genetic variability in the *S. ampelinum* population remained to be determined using molecular analysis.

Similar to colony color, colony appearance was more diverse on CA and CCA (4 groups; flat and smooth, flat and wrinkled, raised and smooth, and raised and wrinkled) than on PDA and JCCA (2 groups; highly raised and deeply wrinkled or highly raised and wrinkled). Although we found new media with higher characterization efficiency than PDA, it was clear that cultural characterization alone still cannot fully differentiate all individual isolates of this pathogen. To gain a more complete illustration of the diversity in this pathogen population, a molecular genetic approach is currently under investigation in our laboratory.

Among the 4 media used in this study, CA was the best medium for cultural characterization of *S. ampelinum.* In addition, it promoted the highest surface mycelium growth that could be useful for multiplication. Our results indicated that diversity in cultural characteristics existed among *S. ampelinum* isolates, especially those from different geographical regions. This implied genetic variability within the *S. ampelinum* population, possibly due to pathogen adaptation to different environments and cultural practices, and suggested that breeding of grape for sustainable anthracnose resistance should utilize multiple resistance genes either pyramided into the same cultivar, or used individually to develop region-specific resistant cultivars.

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