
Effect of Different Factors on Sporulation of *Colletotrichum coffeanum*

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Abstract The effect of different factors on sporulation of *Colletotrichum coffeanum* was investigated. Result showed that V8 and PDA media were suitable for the growth of *C. coffeanum*. On the contrary, the content of the media gave a significant impact on sporulation. The media content increased then it would greatly increase the sporulation. 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp) was the most suitable light condition for *C. coffeanum* sporulation, compared with constant darkness (continuous darkness; CD) and constant illumination (continuous light; CL; 10000 lux, fluorescent lamp). Moreover, the growth rate in the 28th day showed the fastest growing culture. It is concluded 10 ml PDA medium and incubated 28 days under natural light conditions was the best time of sporulation.

Keywords: Sporulation, *Colletotrichum coffeanum*

Introduction

Coffee belongs to Rubiaceae, is a perennial evergreen shrub and it is a perennial horticultural crop. Leaves which are opposite elongated oval, glossy, at the end of a long branches, small branches, and flowers are white, open branches in the base of the petiole link. Once ripe, coffee "berries" are picked, processed, and dried to yield the seeds inside. The seeds are then roasted to varying degrees, depending on the desired flavor, before being ground and brewed to create coffee. The main active ingredient caffeine of coffee, have a strong central stimulant effect. People taking caffeine or caffeinated beverages often disappears drowsiness, fatigue mitigation, quick thinking. Dose increased, the central stimulant effects more obvious tensions, anxiety, restlessness, insomnia and tremor. Larger doses produce local or systemic spasm (Frey and Rebecca, 2003).

Coffee anthracnose which caused by *Colletotrichum coffeanum* is very common and one of the important disease of coffee trees. It causes

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economically important losses in coffee plantation. The symptom of coffee anthracnose disease is appeared irregular light brown to dark brown spots; lesion central white, yellow edge, later gray, many small black dots are arranged in concentric ring pattern in leaves. Ripe berries and green berries show the symptom: water-soaked spots, lesions become sunken, dark brown to gray-black big spots (Brooklyn Botanic Garden, 2000). Therefore, the study of the use of bioactive substances to control coffee anthracnose has been interestingly increased. Ajith (2010) reported that all the selected *Trichoderma harzianum*, *Trichoderma saturnisporum* and *Trichoderma reesei* has potential to control growth of *Colletotrichum capsici* causing chilli anthracnose by 21 to 68% at a concentration of 50% culture filtrate. Meanwhile, Vilavong and Soyong (2013) reported that *Chaetomium cupreum* can be antagonized *C. coffeanum* in dual culture plate after incubation for 30 days. The colony of *Ch.cupreum* grew over pathogen colony implies competition mechanism of control. This research finding proved that *C. coffeanum* causing anthracnose shown to be infected leaves and beans. *Ch.cupreum* proved to be effective antagonist against anthracnose pathogen.

Under certain conditions some fungi undergo microcycle conidiation (Smith, 1981), whereby sporulation occurs directly after spore germination without, or with greatly reduced, mycelia growth. Microcycle conidiation of certain fungi may be induce by high-temperature stress (Anderson *et al.*, 1971; Anderson *et al.*, 1972; Sekiguchi *et al.*, 1975), nutrient depletion (Boosalis *et al.*, 1962; Mangenot *et al.*, 1976), or other factors inhibiting vegetative development (Park *et al.*, 1970; Rotem *et al.*, 1970). In the report of Slade (1987) mentioned that the effects of inoculum density, medium composition and concentration, and temperature on spore-carrying capacity (SCC) and microcycle conidiation by *Collectotrichum gloeosporioides* that was studied on solid media. For this fungus, spore production on solid media was similar to that in liquid media, so relationships found with the microplate method should provide useful information for spore production in analogous liquid systems.

The objective of this research finding was to study on the type of media, volume of media, light condition and incubation time for sporulation of *Colletotrichum coffeanum*.

Material and methods

Isolation of pathogen and pathogenecity test

Colletotrichum coffeanum was isolated by using tissue transplanting technique from coffee anthracnose on leaves var Arabica. The advanced margin of lesion was surface disinfected with sodium hypocholite 10 %, then cut with

sterilized blade into small piece of 0.5 X 0.5 cm between advanced margin of healthy and infested tissues on symptom of leaf, then soaked into 10 % sodium hypochlorite for a few minutes, and moved to sterilize distilled water, then placed in sterilized tissue paper to dry out, thereafter picked up with needle and placed onto water agar (WA), then incubated at room temperature approximately 27-30 °C. The hyphal tip isolation was done by cutting with needle into small piece of hyphal tip and transferred onto potato dextrose agar (PDA), incubated at room temperature and observed growing colony until getting pure cultures. All isolates were morphologically identified into species by using binocular compound microscope.

The experiment was conducted by using 3 factors factorial in Completely Randomized Design (CRD) with four replications. Factor A represented the different media which give effect on spore production: A1= PDA media (potatoes, 200 g; dextrose, 15 g; agar, 20 g; H₂O, 1 L); A2= V8 juice media (V8 Juice, 200.0 ml; CaCO₃, 3.0 g; Agar, 15.0 g; Tap water to 1.0 L; PH, 7.2). Factor B represented different light conditions: B1= constant illumination (continuous light; CL; 10000 lux, fluorescent lamp), B2= constant darkness (continuous darkness; CD), B3= 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp). Factor C represented the different volume of media: C1= 5ml media, C2=10ml media. 3 mm-diameter agar plug cut by sterilized cork borer from the pure cultures of *Colletotrichum coffeanum* placed in the center of 5 cm petri dishes which contained 5 ml of PDA media, 10 ml of PDA media, 5 ml of V8 media, 10 ml of V8 media, respectively.

Data were collected and observed for sporulation every 7 days. The culture surface was washed 3 times with 10ml tap water to remove the fungal spores for counting. Spore production was quantified using a hemacytometer. Data was statistically computed analysis of variance. Treatment means were computed by Duncan Multiple's Range Test (DMRT) at P=0.05 and P=0.01. The comparison between normal and abnormal grew on corneal dual-culture were observed under compound microscope.

Pathogenicity test was conducted using detached leaf inoculated method. The experiment was done using Completely Randomized Design (CRD) with four replications. Treatments were inoculated into wounded leaves surface with an agar plug of pathogen and placed in moist chamber done in Petri dishes. Control treatment was done by transferring an agar plug of PDA alone onto wounded surface leaf.

Results

Colletotrichum coffeanum was isolated and identified causing anthracnose on coffee leaves var. Arabica (Fig.1).



Figure 1. *Colletotrichum coffeanum* isolated from coffee leaves anthracnose, a, b = Pure cultures of *C.coffeanum*. c = conidia. Bar. c= 10 μ m

The results showed that the lesion size developed by *C. coffeanum* isolate was 27.25 mm, which gave the high virulent for disease incidence to prove pathogenicity test.

Two kinds of media and two kinds of volume of media were tested to determine the effect of nutrients on sporulation. That was 5 ml of PDA media, 10 ml of PDA media, 5 ml of V8 media, 10 ml of V8 media, respectively. All of plates were incubated under constant illumination (continuous light; CL; 10000 lux, fluorescent lamp), constant darkness (continuous darkness; CD) and a 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp), respectively. In these three different lighting conditions, continuous cultured 35 days. Spore production was quantified using a hemacytometer at each stage. Specific data are shown in Table 1.

Table 1. Effect of different factors on sporulation of *Colletotrichum coffeanum*

Media	Light conditions ^{1/}	volume of media	Spore production at different times (10 ⁶)			
			14 days	21 days	28 days	35 days
PDA	CL	5ml	3.75 ^{d1}	11.00 ^d	34.00 ^h	90.50 ^{fg}
		10ml	5.75 ^{bc}	18.00 ^{bc}	55.25 ^f	111.50 ^e
	12:12 L:D	5ml	5.50 ^{bcd}	16.75 ^{bcd}	78.50 ^{cde}	175.25 ^c
		10ml	9.50 ^a	30.50 ^a	155.50 ^b	231.00 ^a
V8	CD	5ml	5.00 ^{bcd}	13.50 ^{cd}	41.25 ^{gh}	83.25 ^g
		10ml	6.50 ^b	22.75 ^b	75.75 ^{de}	152.25 ^d
	CL	5ml	4.25 ^{cd}	13.00 ^{cd}	44.00 ^g	100.00 ^{ef}
		10ml	5.75 ^{bc}	17.50 ^{bcd}	75.00 ^e	111.50 ^e
V8	12:12 L:D	5ml	5.75 ^{bc}	18.00 ^{bc}	86.25 ^c	192.50 ^b
		10ml	9.00 ^a	33.50 ^a	163.50 ^a	231.00 ^a
	CD	5ml	5.25 ^{bcd}	16.50 ^{bcd}	53.00 ^f	88.25 ^{fg}
		10ml	6.00 ^{bc}	23.25 ^b	83.25 ^{cd}	169.75 ^c

^{1/} CL=continuous light, 10000 lux, fluorescent lamp; CD=constant darkness, continuous darkness; 12:12 L:D= 12 h light/dark photoperiod, 10000 lux, fluorescent lamp

^{2/} Average of four replications, Mean followed by the same letters are not significantly different by Duncan's multiple range test at P= 0.05.

The type of Media

Result showed that V8 juice agar was the best media for inducing sporulation (up to 2.31×10^8) at 35th day, but it was not significantly different from PDA media, and followed by media in different volume constant illumination (continuous light; CL; 10000 lux, fluorescent lamp), respectively (Fig.2). The best one was 10ml V8 juice agar under constant illumination. Under the same conditions as the volume, the spore production of V8 was higher than PDA. The effects of 5 ml PDA, 10 ml PDA, 5 ml V8, 10 ml V8, two different media in different volume under the same 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp) were shown in Fig. 3. The best one was 10ml V8 juice agar. Under the same conditions as the volume, the spore production of V8 was higher than PDA, but not significantly. The effects of 5 ml PDA, 10 ml PDA, 5 ml V8, 10 ml V8, two different media in different volume under constant darkness (continuous darkness; CD) were shown in Fig. 4. The best one was also 10ml V8 juice agar. The sporulation of V8 was higher than PDA. In short, PDA and V8 were able to induce the spore production of *Colletotrichum coffeanum*. But, V8 was better than others.

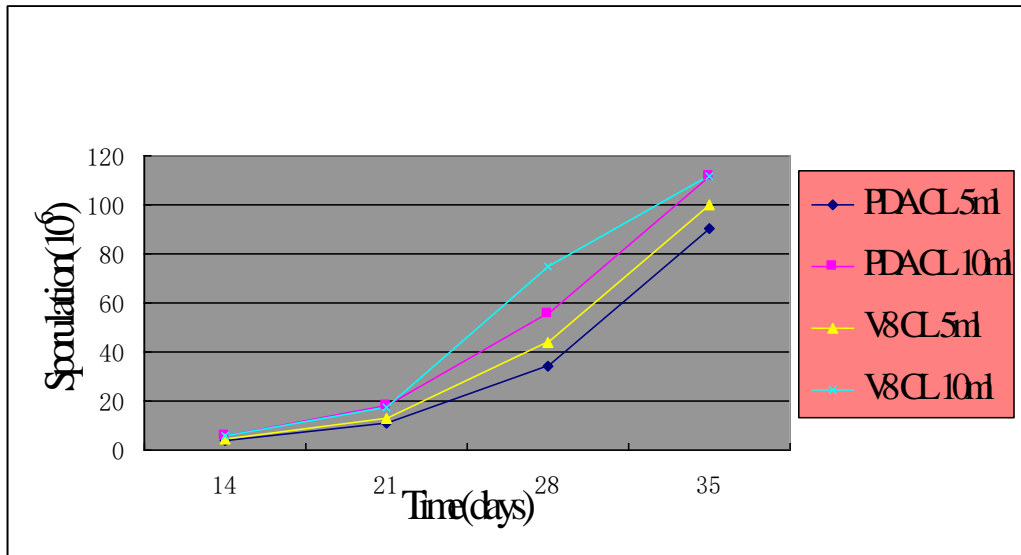


Figure 2. Effect of two kind of media of different volume under constant illumination (continuous light; CL; 10000 lux, fluorescent lamp) on spore production

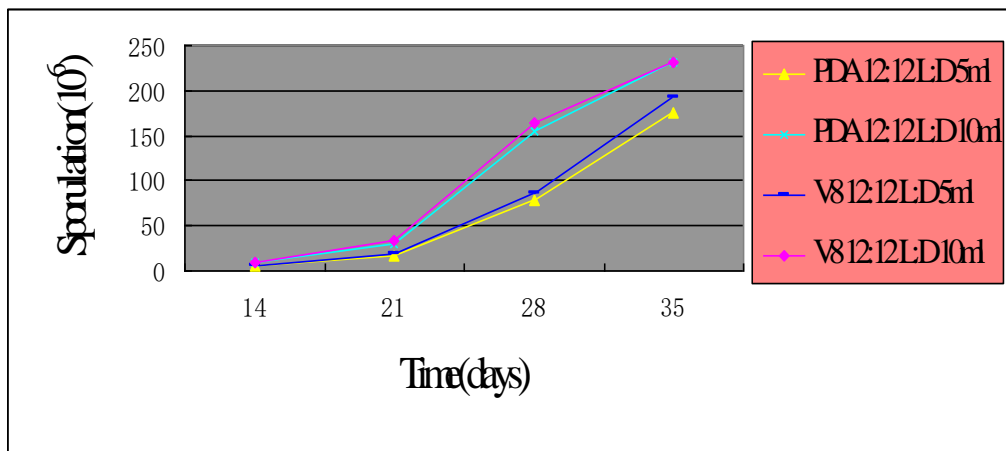


Figure 3. Effect of two kinds of media of different volume under 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp) on spore production

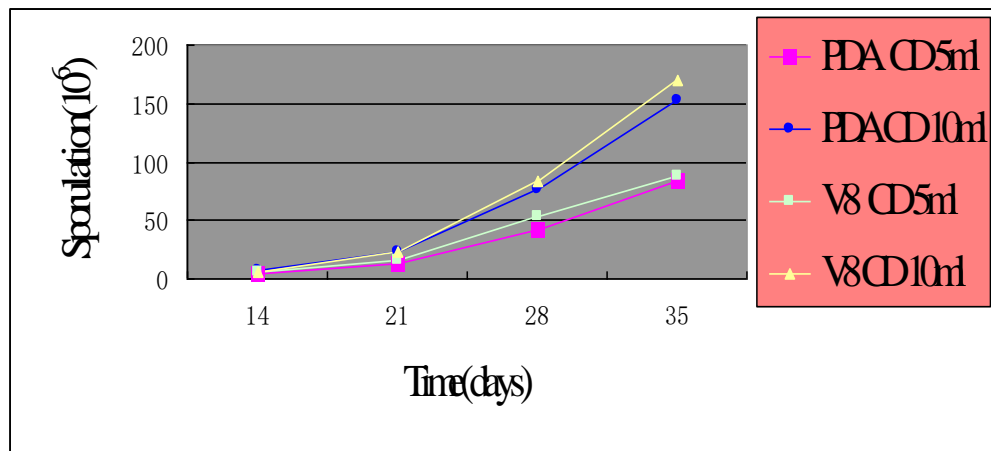


Figure 4. Effect of two kinds of media of different volume under constant darkness (continuous darkness; CD) on spore production

The volume of media

Result showed the effect of 5 ml and 10 ml PDA under constant illumination (continuous light; CL; 10000 lux, fluorescent lamp) on sporulation were differed to each other (Fig.5). The best one was 10ml V8 juice agar under constant illumination. At the same time, 10 ml of the PDA medium gave a greater degree of sporulation of *Colletotrichum coffeanum* than other treatments when compared with 5ml PDA medium. The result of constant darkness (continuous darkness; CD) and a 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp) were shown in Figs. 6 and 7.

The effect of different volume V8 under constant illumination (continuous light; CL; 10000 lux, fluorescent lamp) on spore production, effect of different volume PDA under 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp) on spore production and effect of different volume PDA under constant darkness (continuous darkness; CD) on spore production were shown in Figs. 8, 9 and 10.

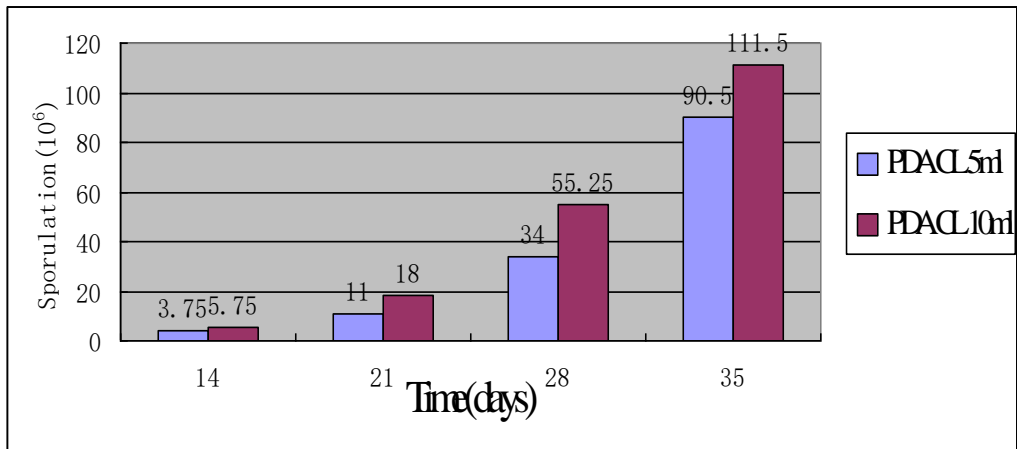


Figure 5. Effect of different volume PDA under constant illumination (continuous light; CL; 10000 lux, fluorescent lamp) on spore production

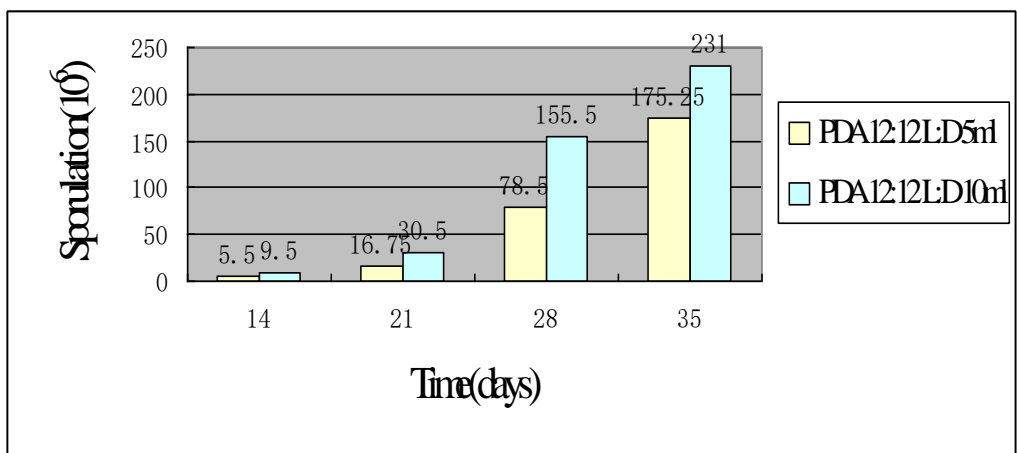


Figure 6. Effect of different volume PDA under 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp) on spore production

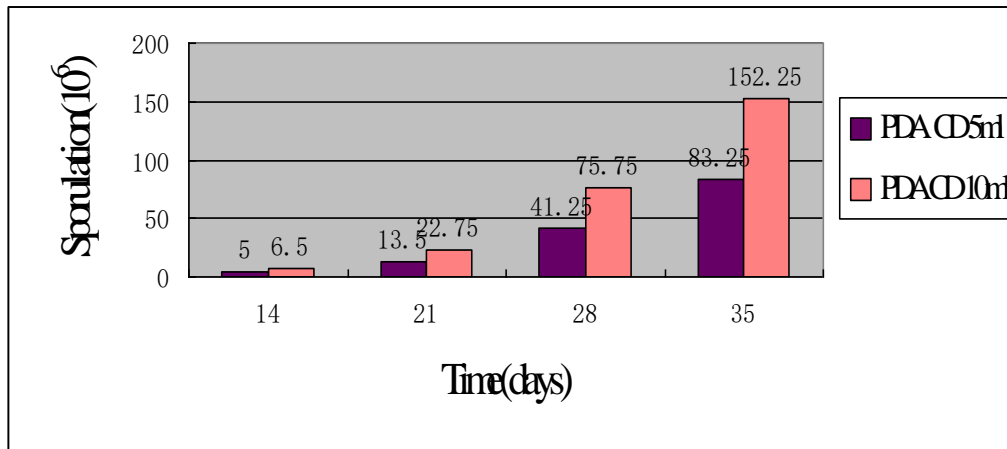


Figure 7. Effect of different volume PDA under constant darkness (continuous darkness; CD) on spore production

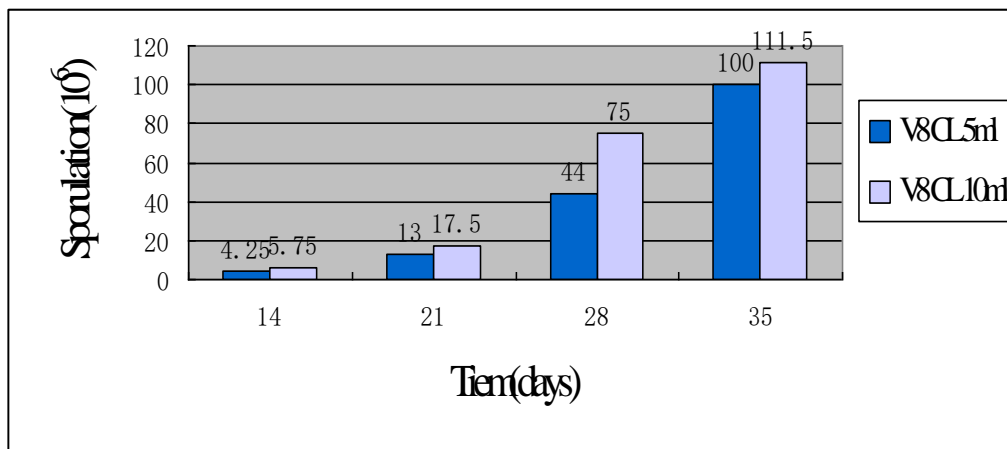


Figure 8. Effect of different volume V8 under constant illumination (continuous light; CL; 10000 lux, fluorescent lamp) on spore production

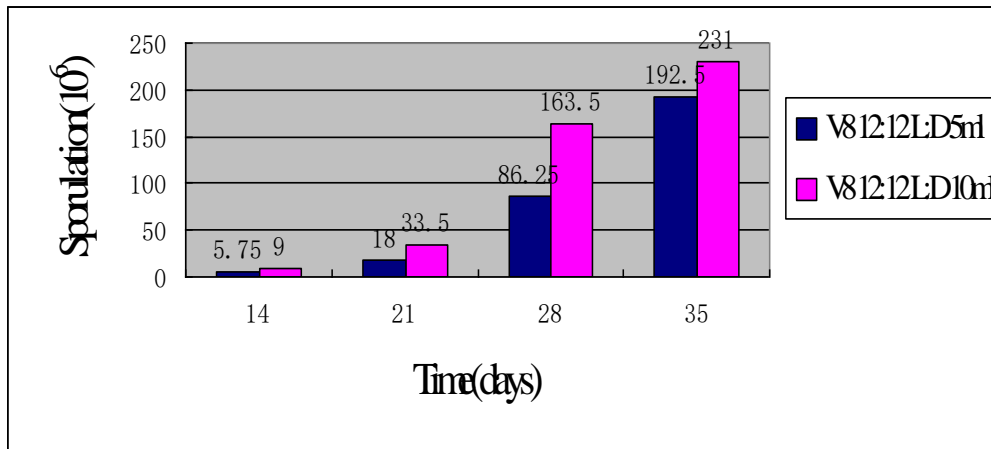


Figure 9. Effect of different volume PDA under 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp) on spore production

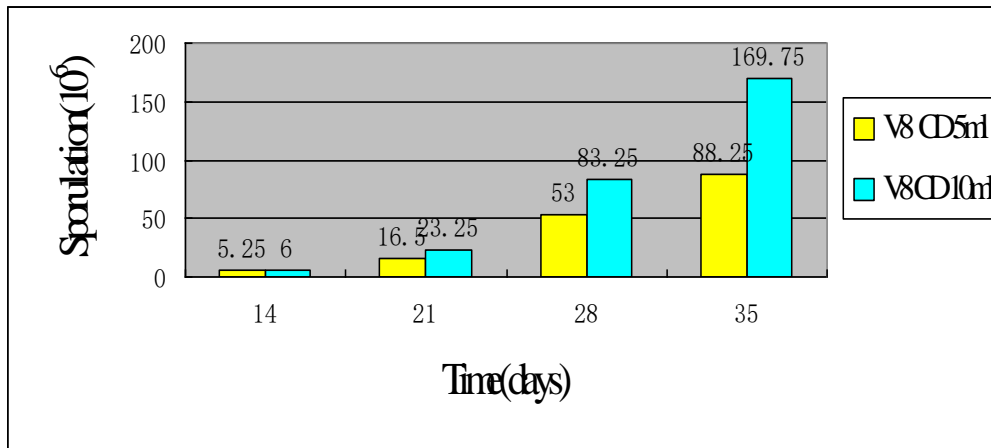


Figure 10. Effect of different volume PDA under constant darkness (continuous darkness; CD) on spore production

Light conditions

Result showed the effect of different light conditions in same volume PDA medium on spore production were differed (Figs. 11 and 12). At early observation (before 21st day) found that there was no significantly affected on

sporulation. However, at the 28th days observation found that only under 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp) would significantly promote the induction of sporulation. Another two groups were still no significantly differed. The effect of different light conditions in 5ml and 10 ml V8 medium on sporulation was shown in Figs. 13 and 14.

In conclusion, the illumination time, there was no significantly affected on sporulation, but in the late of growth stage, light time would impact the sporulation. 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp) gave the highest sporulation when compared with constant darkness (continuous darkness; CD) and constant illumination (continuous light; CL; 10000 lux, fluorescent lamp). The 28th day incubation, the sporulation was 155.5×10^6 and 35th day, the sporulation was 231×10^6 .

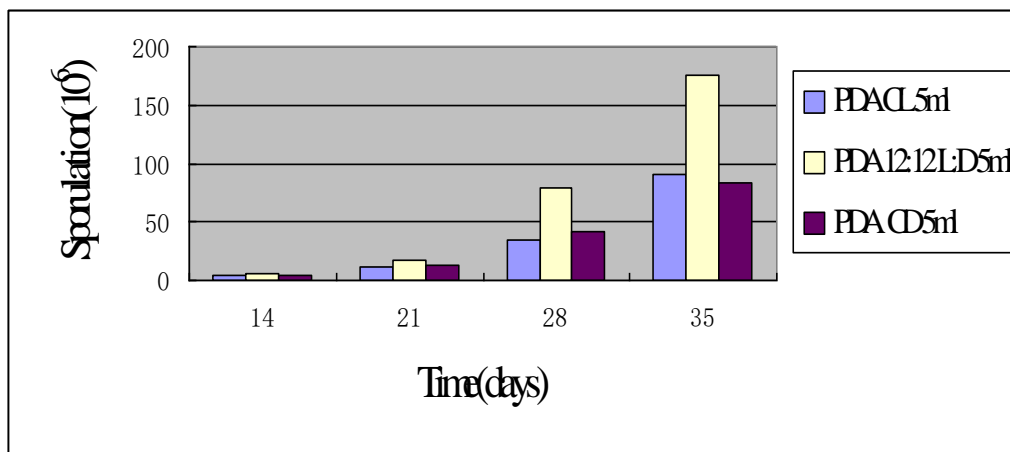


Figure 11. Effect of different light conditions in 5ml PDA medium on spore production

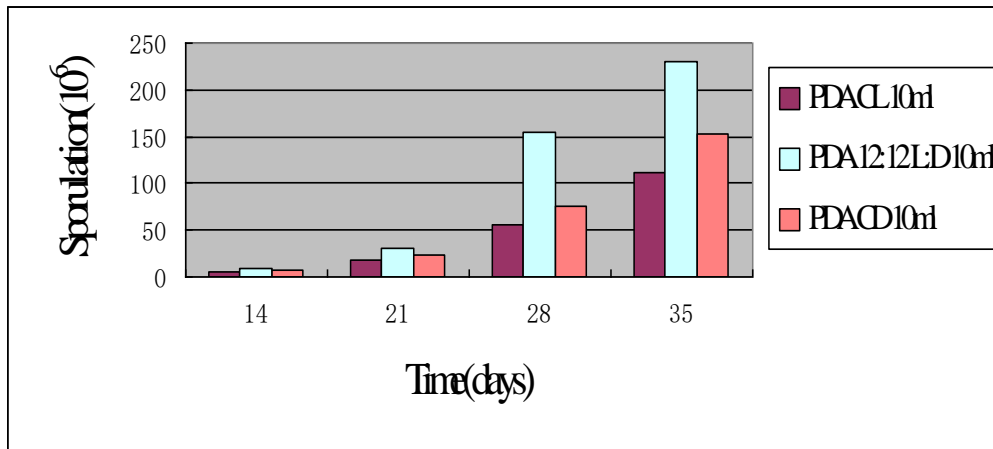


Figure 12. Effect of different light conditions in 10ml PDA medium on spore production

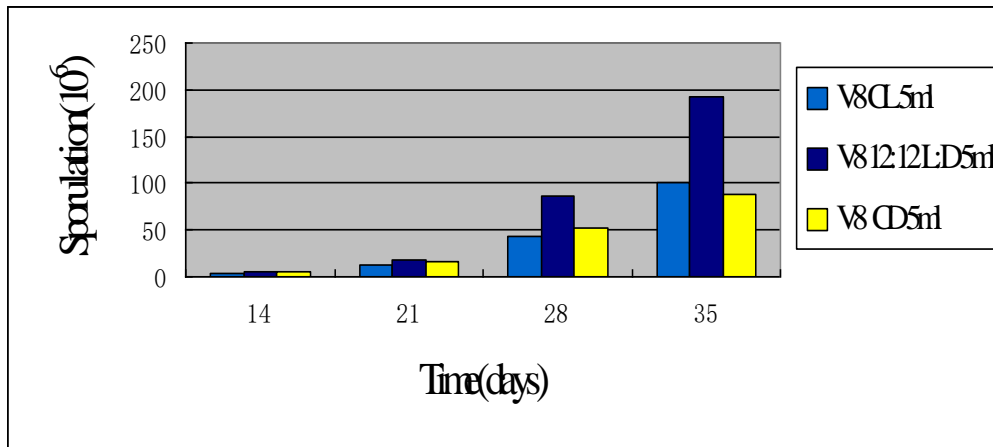


Figure 13. Effect of different light conditions in 5ml V8 medium on spore production

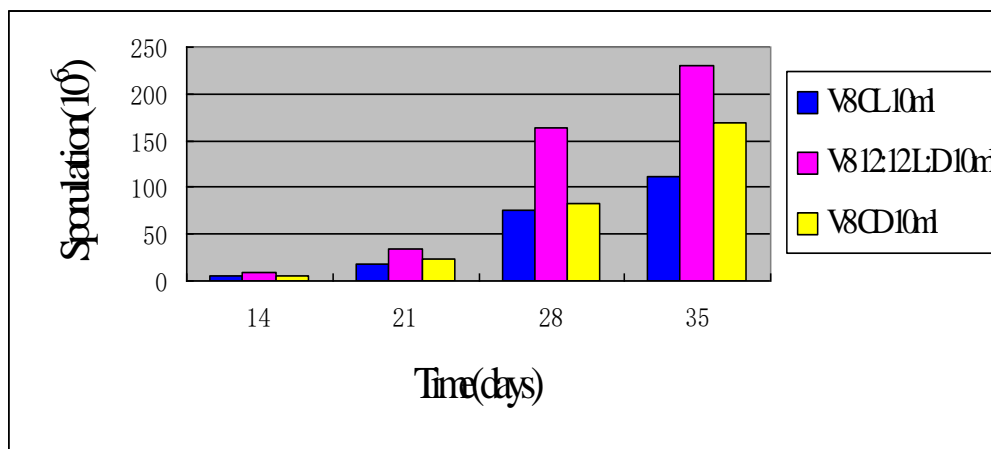


Figure 14. Effect of different light conditions in 10ml V8 medium on spore production

Incubation time

The highest growth rate of *Colletotrichum coffeanum* was shown on the 28th day incubation. With this, 10ml PDA medium gave the highest growth rate. At the 28th day, the growth rate started to decrease (Table 2, Fig.15).

Table 2. Growth rate of sporulation of *Colletotrichum coffeanum*

Media	Light conditions ^{/1}	The volume of media	Growth rate		
			21 st day ^{/2}	28 th day ^{/3}	35 th day ^{/4}
PDA	CL	5ml	1.93	2.09	1.66
PDA	CL	10ml	2.13	2.07	1.02
PDA	12:12 L:D	5ml	2.05	3.69	1.23
PDA	12:12 L:D	10ml	2.21	4.10	0.49
PDA	CD	5ml	1.7	2.06	1.02
PDA	CD	10ml	2.5	2.33	1.01
V8	CL	5ml	2.06	2.38	1.27

V8	CL	10ml	2.04	3.29	0.49
V8	12:12 L:D	5ml	2.13	3.79	1.23
V8	12:12 L:D	10ml	2.72	3.88	0.41
V8	CD	5ml	2.14	2.21	0.66
V8	CD	10ml	2.88	2.58	1.03

^{1/} CL=continuous light, 10000 lux, fluorescent lamp; CD=constant darkness, continuous darkness; 12:12 L:D= 12 h light/dark photoperiod , 10000 lux, fluorescent lamp

^{2/} Growth Rate=(Sporulation at 21st day - Sporulation at 14th day)/ Sporulation at 14th day

^{3/} Growth Rate=(Sporulation at 28th day - Sporulation at 21st day)/ Sporulation at 21st day

^{4/} Growth Rate=(Sporulation at 35th day - Sporulation at 28th day)/ Sporulation at 28th day

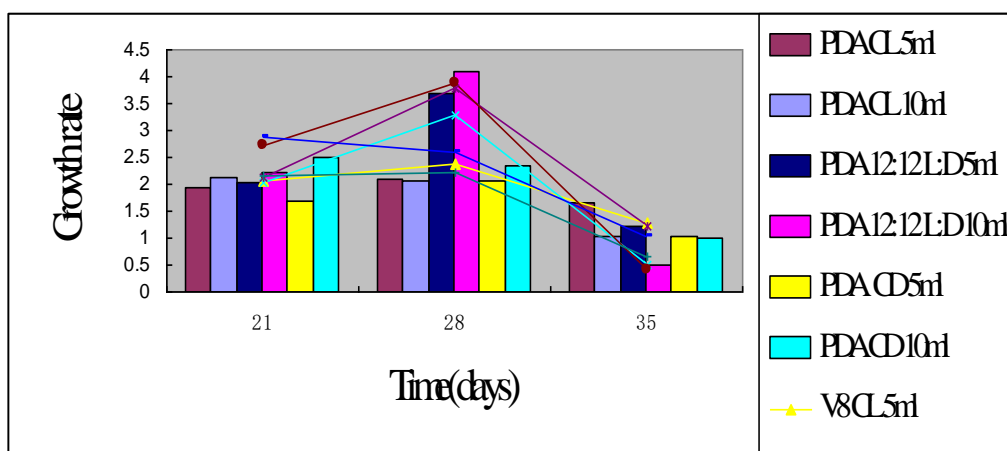


Figure 15. Effect of incubation time on spore production

Result showed that V8 and PDA were suitable for the growth of *Colletotrichum coffeanum* and not significantly differed when compare to the control. The media content gave significantly difference on sporulation. The increased media content, it also would increase sporulation in this research findings. 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp) was the most suitable light condition for the growth of *Colletotrichum coffeanum* sporulation, compared with constant darkness (continuous darkness,CD) and followed by the constant illumination (continuous light; CL; 10000 lux, fluorescent lamp). Although with the growth of time, spore production was also growing. However, the growth rate in the 28th day was the

fastest sporulation with 10 ml PDA medium and incubated 28 days under natural light condition showed the best sporulation.

Discussion

In this study showed that V8 juice agar was the more suitable medium for sporulation of *C. coffeanum*, compared with the PDA agar but not significant. The content of the medium gave a significant impact on spore yield. Increase media content, also would greatly improve the spore production. 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp) was the most suitable light condition for *Colletotrichum coffeanum* sporulation. Slade *et al.* (1987) reported that the effect of spore inoculum density, medium concentration, and temperature on slime-spot formation, spore yield, and mycelium production by *Colletotrichum gloeosporioides* on agar media were studied with a simple microplate assay. A steady-state spore yield (spore-carrying capacity) independent of inoculum density was reached only on media that supported good fungal growth and sporulation. The spore-carrying capacity was reached earlier, the denser the inoculum. On standard mycological media a high inoculum density (2.5×10^6 spores per ml) resulted in a slimy mass of conidia forming a slime spot, a phenomenon associated with greatly reduced mycelium formation and indicative of microcycle conidiation. In contrast, for a similar inoculum density, enhanced mycelial growth preceded sporulation and overrode slime-spot formation on highly concentrated media; a very low medium concentration resulted in much less mycelium, but spore production was also decreased. Exposure to suboptimal growth temperatures of 36 to 48 °C for up to 8 days did not induce microcycle conidiation from inocula that did not form a slime spot at 28 °C. Microcycle conidiation, as indicated by rapid development of a slime-covered colony after fungal spore inoculation of solid media, occurred on diverse commonly used microbial media but only under conditions of a high inoculum density (25, μ l, 2.5×10^6 spores per ml). Microcycle conidiation was most pronounced on media highly favorable for fungal growth, such as V8 and RV8 (both of which contain 20% V8 juice) and PDA. However, increasing the V8 concentration beyond the normal 20% level caused microcycle conidiation to be replaced by dense vegetative mycelial development. These data are consistent with microcycle induction being a function of (i) diffusion-restricted nutrient availability to the fungal colony (resulting from nutrient demand by a rapidly developing high inoculum colony outstripping nutrient resupply to the colony by diffusion from the surrounding agar), (ii) spore density-dependent accumulation of microcycle-inducing fungal metabolites, or both. High nutrient concentrations in the media (e.g., >20% V8) would attenuate diffusion-restricted nutrient availability and might also

override metabolite induction of microcycle conidiation. The critical nutrient factor promoting mycelial growth rather than microcycle conidiation at high inoculum densities is not simply organic content, since for similar inocula slime spots are formed on RV8 (56 g of organic matter liter⁻¹) and on 20% V8 (8 g liter⁻¹) but are substantially reduced on 40% V8 (16 g liter⁻¹) and are not formed at all on 60% V8 (24 g liter⁻¹) (Slade *et al.*, 1987).

References

- Ajith, P. S. and Lakshmidēvi, N. (2010). Effect of volatile and non-volatile compounds from *Trichoderma* spp. against *Colletotrichum capsici* incitant of Anthracnose on Bell peppers. *Nature and Science* 8.
- Anderson, J. G. and Smith, J. E. (1971). The production of conidiophores and conidia by newly germinated conidia of *Aspergillus niger* (microcycle conidiation). *Journal of General Microbiology* 69:185-197.
- Anderson, J. G. and Smith, J. E. (1972). The effect of elevated temperature on spore swelling and germination in *Aspergillus niger*. *Journal of General Microbiology* 18:289-297.
- Boosalis, M. G. (1962). Precocious sporulation and longevity of conidia of *Helminthosporium sativum* in soil. *Phytopathology* 52:1172-1177.
- Brooklyn Botanic Garden (2000). *Natural disease control: A common-sense approach to plant first aid. Handbook*. Washington Avenue, Brooklyn, NY: Brooklyn Botanic Garden, Inc. 1000.
- Mangenot, F. and Reisinger, O. (1976). Form and function of conidia as related to their development. In Weber, D.J. and Hess, W.H. (Eds.), *The fungal spore: form and function*. New York: John Wiley and Sons, Inc. pp. 789-847.
- Park, D. and Bashi, E. (1970). Gemination studies with *Geotrichum candidum*. *Transactions of the British Mycological Society* 53:83-92.
- Rotem, J. and Bashi, E. (1969). Induction of sporulation of *Alternaria porri* f.sp. *solani* by inhibition of its vegetative development. *Transactions of the British Mycological Society* 53:433-439.
- Sekiguchi, J., Gaucher, G. M. and Costerton, J. M. (1975). Microcycle conidiation in *Percillium urticae*: an ultrastructural investigation of spherical spore growth. *Canadian Journal of Microbiology* 21:2048-2058.
- Smith, J. G., Anderson, J. G., Deans, S. G. and Berry, D. R. (1981). Biochemistry of microcycle conidiation. In Cole, G.T. and Kendrick, B. (Eds.), *Biology of conidia fungi*, vol 2. New York: Academic Press Inc. pp. 329-356.
- Vilavong, S. and Soyong, K. (2013). Plant pathogenic fungi and pathogenicity test on Arabica coffee plantation at Paksong in Lao PDR and preliminary biocontrol test in vitro. ICIST 2013, KMITL, Bangkok, Thailand, 28-29 November 2013. pp. 56-92.
- Frey, R. J. (2003). Caffeine-related disorders. *Gale Encyclopedia of Mental Disorders*. Retrieved from <http://www.encyclopedia.com/doc/1G2-3405700066.html>.

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