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Biological Control of Grape Ripe Rot and Bitter Rot

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ABSTRACT

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Among 46 isolated microorganisms, F3 and M5 were two bacterial isolates that had the greatest capability to inhibit growth of *Colletotrichum gloeosporioides* and *Melanconium fuligineum* on potato dextrose agar. The affected hyphae of the two pathogens malformed, shrank, and lysed. Whereas normal hyphae had dense cytoplasm with sound organells and structures. M5 was identified to be a species of *Bacillus*, denoted *Bacillus* sp M5. This isolate was significantly (P=0.05) able to control ripe and bitter rot of grapes at 28 C after inoculation of the studied pathogens for 14 days. The same capability was shown in the vineyard. Although effectiveness of *Bacillus* sp. M5 to control ripe and bitter rot was worse than spraying Dithane M-45, *Bacillus* sp. M5 was significantly able to control natural occurring ripe and bitter rot in the field. *Bacillus* sp. M5 sprayed on grapes was evidently able to disturb the germination and growth of the studied pathogens. Furthermore *Bacillus* sp. M5 produced unidentified antibiotic substance(s) to inhibit significantly (P=0.05) the growth of these two studied pathogens.

Key words: grape, biocontrol, ripe rot, bitter rot.

INTRODUCTION

Grape is an important fresh fruit and source of wine products in Taiwan. Currently, the cultivated area of vineyard is about 5,000 hectares. Disease therefore became a nonnegligible problem. More than 10 different fungal diseases were reported happened in local farms (4). Among them, ripe rot caused by *Colletotrichum gloeosporioides* Penz. (= *Glomerella cingulata* (Ston.) Spauld et Schr.) was the most serious disease which may cause more than 40% loss. Bitter rot caused by *Melanconium fuligineum* (Scrib. & Viala) Cav. (Syn. *Greeneria viticola* (Bert. & Curt.) Punithalingam) was another important disease common in the field (3). In order to derive greater benefit from marketing sound fresh fruit, farmers generally spray chemicals. To reduce side effects of sprayed chemicals, biological control is an attractive alternative. Although biological control of other fruit diseases has been studied (11,12), there were no reports of tests on grapes, which was the main purpose of this work.

MATERIALS AND METHODS

C. gloeosporioides and *M. fuligineum* were isolated from diseased fruits and cultured on potato dextrose agar (PDA) (8). The tested cultivar of grape was Kyoho that was collected directly from the vineyard.

Various microorganisms were isolated from grape tissues either by placing fruits, twigs, or leaves directly on 2% water agar, or the dilution plate method by shaking the tissues with sterilized distilled water. Those tissues were incubated in moist chamber at 28 C for 3 days. Microorganisms were then plucked and spread on a PDA plate. A single colony was transferred and cultured on PDA slants. The isolated microorganisms were then cultured dually with either tested pathogens on PDA. The most inhibited colonies of tested pathogens were observed under an optical microscope and a transmission electron microscope (Hitachi, H-600). An agar disc (0.6 cm in diam.) covered with a pathogen was immersed in suspension of selected antagonist (10^7 spores/ml) for 3–5 min and transferred to a PDA plate for 3 days.

F3 and M5 were the two most effective isolates among the microorganisms used for bioassay. F3 and M5 were identified according to the following tests: Gram's and endospore stains, responses to heat and the catalase test. A bacterial suspension (10^8 cfu/ml) of F3 or M5 was sprayed on grapes 24 hr before inoculation of either pathogen (10^6 spores/ml). Spraying sterilized distilled water instead of antagonist was used as control.

Each treatment consisted of detached clusters containing 100 grapes with four replicates. These treated grapes were incubated at 28 C for 14 days before recording the disease index. The disease index was classified according to five degrees (0, no symptom appeared on fruit; 1, 25%; 2, 50%; 3, 75% and 4, more than 75% of fruit expressed symptoms). This test was repeated once but F3 was not tested in the second trial.

A field trial was to test the effectiveness of M5 to control the pathogens. Two hundred and forty plants growing at Ta-Nan Villa (大南村) were divided into 12 equal blocks. Spraying M5 suspension (10^8 cfu/ml), water, and Dithane M-45 (800 ppm/a.i.) were carried out every ten days started on April 4, 1990. There were ten sprays in total and a completely randomized design was applied. Each treatment consisted of 80 plants and had four replicates. At the time of harvest (July 13, 1990) the percentage of sound fruit was recorded. The temperature was measured by thermometer (Suntex ST-52).

To understand the capability of M5 to control the studied pathogens, we peeled off the outer tissues of grape after inoculating M5 and pathogen for 0, 48 and 72 hr. These tissues were observed with a scanning electron microscope (JEOL T330) after treatment according to a series of procedures (15). Cultural filtrates of M5 in potato dextrose broth (PDB) which was incubated for 0, 24, 48, 72 or 96 hr were collected by passage though sterilized filter (Millipore, diameter 0.45 μm). 50, 100, or 150 μl of these filtrates was added in the center of PDA that had been covered with 0.1 μl of spore suspension (10^7 spore/ml) of tested pathogen. The size of the inhibition zone was recorded after incubation for three days.

RESULTS

Forty-six isolates of microorganisms were isolated from grape tissues by various methods. Among them, 15, 11 and 20 isolates were fungi, yeasts, and bacteria, respectively. After screening on PDA plates by means of dual and concomitant cultures, F3 and M5 were the most effective bacterial antagonists to inhibit the growth of *C. gloeosporioides* and *M. fuligineum*. The affected hyphae of these two pathogens became swollen, malformed and changed to a bead mostly. The affected hyphae generally lost their cytoplasm, had the cell wall lysed, formed abnormal vesicles, had the cytoplasmic membrane distorted or were even empty in the cells (Fig. 1). In contrast, the cytoplasm of normal hyphae of tested pathogens was dense; the cell wall and cytoplasmic membrane were sound.

Both F3 and M5 were Gram positive, whereas F3 had no endospore and was susceptible to 80 C/20 min treatment, while M5 had endospore and was able to tolerate to 80 C for 20 min. The catalase test was positive. M5 grew well in PDB within the pH range 5–8. The isolate M5 was identified as a species of *Bacillus*, denoted *Bacillus* sp. M5.

Both F3 and M5 were able to control ripe rot and bitter rot significantly on the detached cluster (P=0.05) (Table 1). These diseases were found even on fresh fruit treated with only sterilized distilled water. M5 was significantly (P=0.05) more efficient to control ripe rot than bitter rot.

Spraying M5 in the vineyard was significantly (P=0.05) able to control bitter and ripe rot (Table 2). The effectiveness of M5 to control diseases was worse than spraying Dithane M-45. At harvest, leaves after treatment of Dithane M-45 remained on the vine and

TABLE 1. The effectiveness of antagonists to control grape ripe and bitter rot on fresh fruits

Pathogen	Antagonist	Disease index[1]	
(10^7–10^8 spores/ml)	(10^8 cfu/ml)	I[2]	II
C. gloeosporioides	F3	2.63 c[3]	–
	M5	2.16 d	1.59 d
	St.dist.H_2O	3.63 a	3.05 b
M. fuligineum	F3	1.77 e	–
	M5	2.63 c	2.21 c
	St.dist.H_2O	3.08 b	3.80 a
CK	F3	1.75 ef	–
	M5	1.49 f	1.33 de
	St.dist.H_2O	1.01 g	1.07 e

[1] Disease was classified into 5 degrees. 0, no symptom; 1, 25%; 2, 50%; 3, 75%; and 4, 100% of fruits exposed rot symptom.

[2] I, the first trial; II, the second trial. Each treatment of every trial consisted of 100 grapes.

[3] Data, average of 4 replicates recorded after 14 days of inoculating pathogen, followed by the same letter in each column differed no significantly (p=0.05) by Duncan's multiple range test.

TABLE 2. The effectiveness of M5 to control grape ripe and bitter rot in the field

Treatment	% intact fruits[1]	Temperature (C)[2]
M5	77.00 b	31.41
Dithane M-45	92.25 a	31.02
CK	31.67 c	31.65

[1] Data, mean of 80 tested plants, followed by the same letter was not significantly (p=0.05) different by Duncan's multiple range test.

[2] Temperature was measured by thermometer (Suntex ST-52) when fruit was harvested.

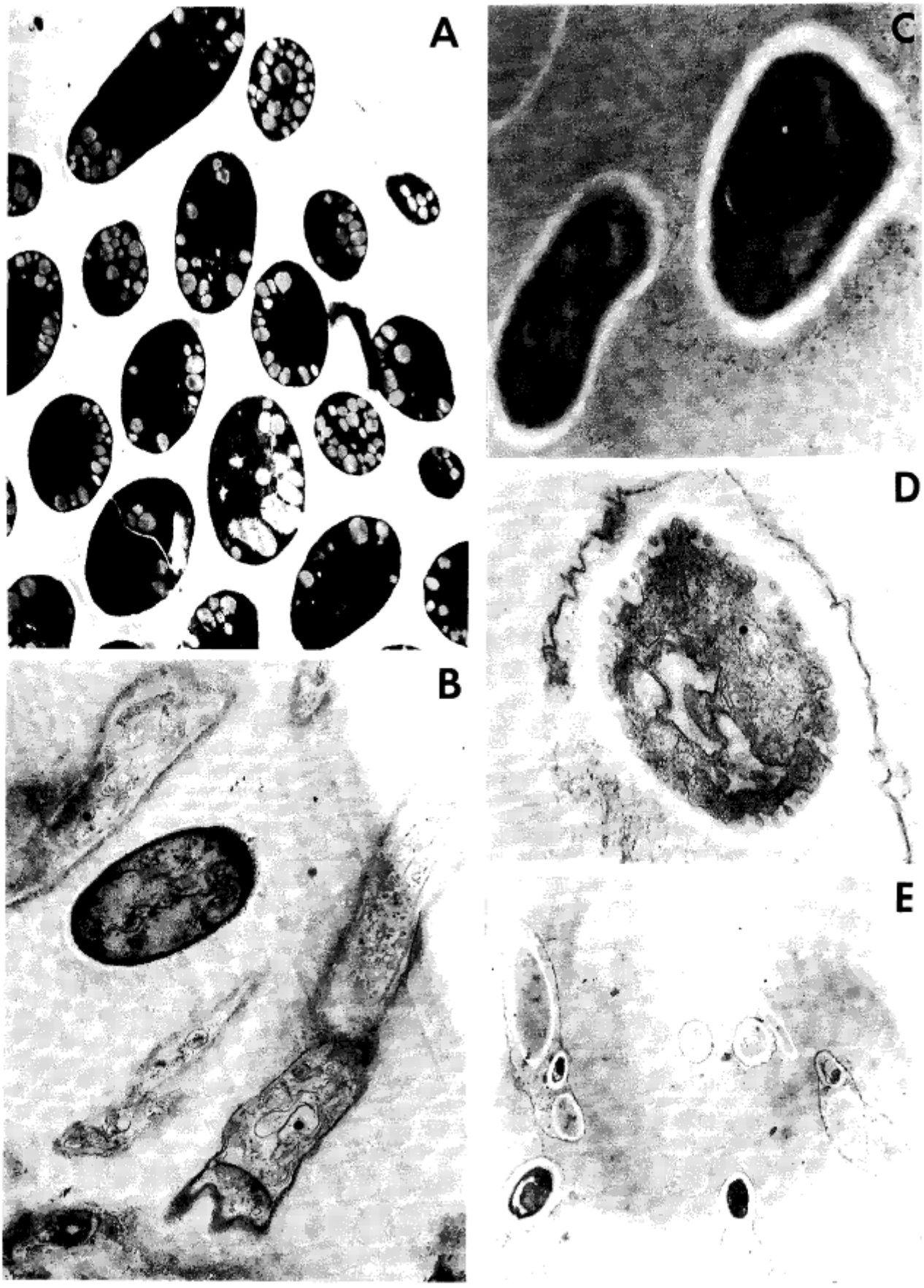

Fig. 1. Influence of *Bacillus* sp. M5 on hyphae of *Colletotrichum gloeosporioides* (A,B) and *Melanconium fuligineum* (C,D,E) on cultural medium. (A,C) healthy controls. (B,D,E) Affected hypha having loose cytoplasm and irregular structures. (A, 4,000 × ; B, 5,600 × ; C, 17,500 × ; D, 34,000 × ; E, 5,000 ×)

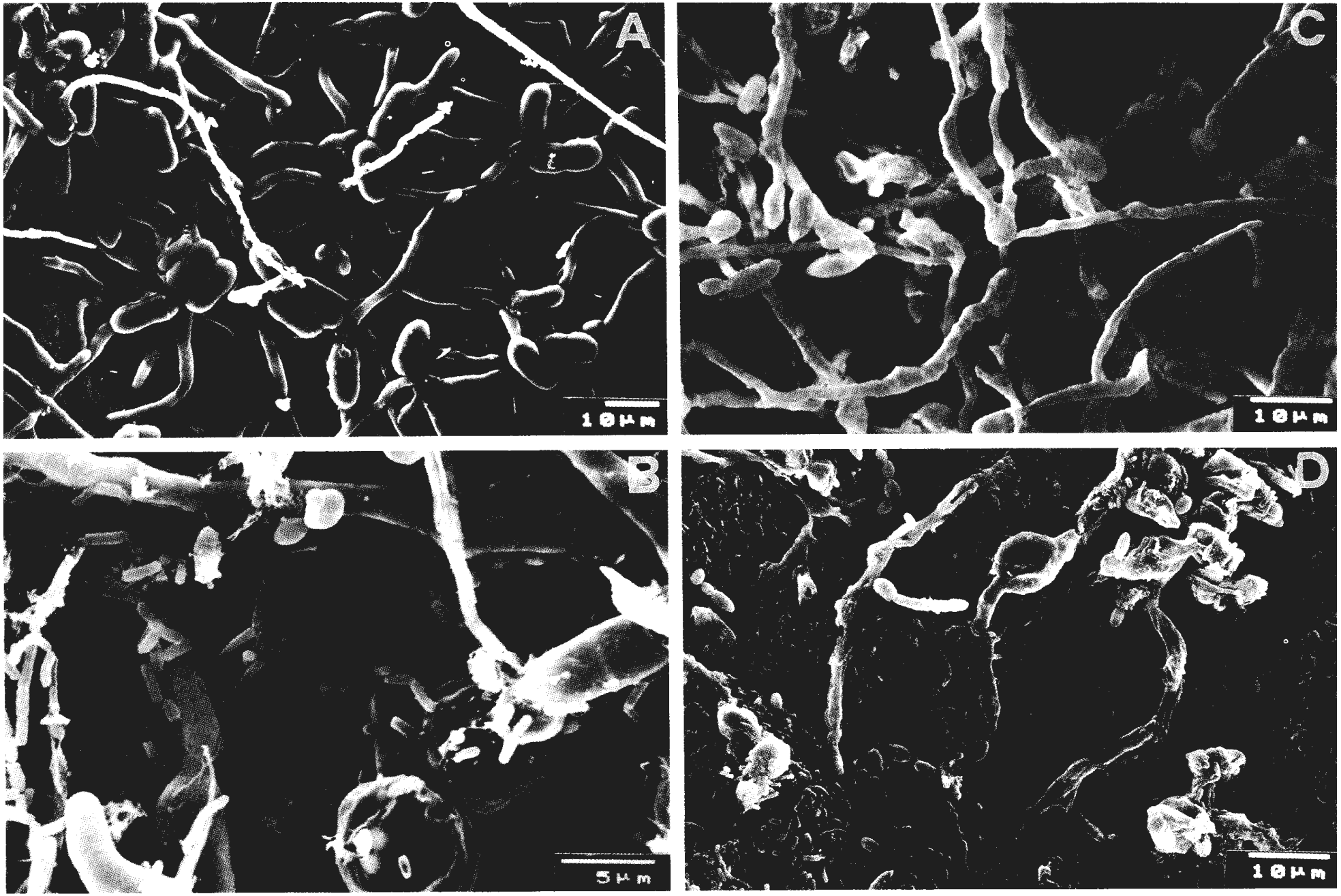

Fig. 2. The growth of *Colletotrichum gloeosporioides* (A,B) and *Melanconium fuligineum* (C,D) on grapes incubated under 28 C for 72 hr. Grapes were treated with *Bacillus* sp. M5 (B,D) and untreated controls (A,C).

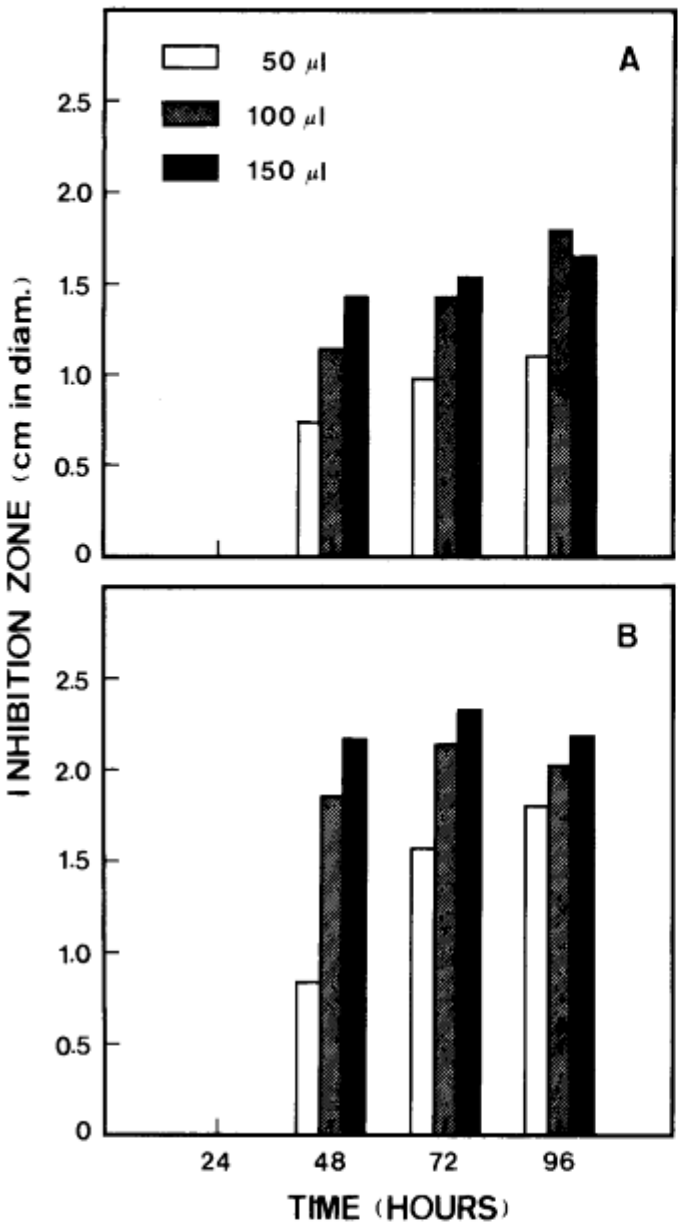

Fig. 3. The effectiveness of different amount of the cultural filtrate produced by *Bacillus* sp. M5 cultured in potato dextrose broth for different period of time to inhibit the growth of *Colletotrichum gloeosporioides* (A) and *Melanconium fuligineum* (B).

looked green. However, fewer leaves after treatment of M5 remained on the vine and almost no leaf was left on vines of the untreated control. Besides, fruit fell commonly among untreated control plants. Generally, ripe rot occurred to a much greater extent than bitter rot during the field trial between March and July. The temperature of untreated controls was the greatest among the treatments.

After inoculation for 72 hr, *C. gloeosporioides* and *M. fuligineum* germinated and grew normally over the grape (Fig. 2). When M5 was present on the grape, some pathogens germinated but bulbing, knotting and shrinkage occurred along the hyphae. Even lysed hyphae were observed within three days of treatment.

The filtrate collected from the culture of M5 after incubation for 24 hr had no capability to inhibit the growth of pathogens. After incubation for 48 hr, the cultural filtrates of M5 showed significant (P=0.05) capability to inhibit the growth of the pathogens (Fig. 3). Generally, the capability of inhibition increased with the period of incubation of M5 and the amount of cultural filtrate.

DISCUSSION

Grape is an important fruit in Taiwan, and some plant pathologists devoted attention to its diseases (3,4). Powdery and downy mildews, rust, ripe and bitter rots are prevalent in vineyards. Among them, ripe and bitter rot affected the grape directly and severely. Probably due to the favorable local environment (*i.e.* high temperature and humidity), these two diseases occur every year, as the optimum temperatures for *C. gloeosporioides* and *M. fuligenum* to germinate were 28–30 C and 28 C respectively (4,6). For this reason, grapes were stored under 28 C after inoculation with these pathogens. After inoculation for 14 days, grapes rotted badly. Hence the effectiveness of antagonist was readily determined under these conditions.

When grape ripened, some glucose covered the fruit. For this reason, bacteria and yeast are expected to be the predominant microorganisms. To reduce the handicap of introduced foreign microorganisms established at a specific niche, the original colonizer was considered to have priority of trial in this study. Yeasts successfully controlled several diseases (5,7), and were able to tolerate protracted dryness, to have good survivability, to reproduce rapidly, and to be capable to colonize the infection site of pathogens (1,2). We isolated 11 isolates of yeasts from grapes. However, their antibiotic and competitive abilities were not distinctive and they failed to control the two pathogens.

Bacillus sp. M5 was shown to have antibiotic, and probably hyperparasitic and competitive abilities to affect *C. gloeosporioides* and *M. fuligineum.* As a result, *Bacillus* sp. M5 was able to control ripe and bitter rot at pre- and postharvest stages. Due to the latent infection habitat of these two pathogens (3,4,6), some uninoculated grapes rotted during bioassay tests. Similarly, *B. cereus* had antibiotic effect and was able to control *Rhizoctonia solani* (13,14). *B. subtilis* showed its capability to inhibit the pathogenicity of *R. solani* (9) and controlled basal stem rot of *chrysanthemum.* *B. subtilis* F-29-3 was reported to produce fengycin which caused hyphal bulbing and emptying (10). This phenomenon happened on *C. gloeosporioides* and *M. fuligineum* when they approached *Bacillus* sp. M5. Whether the inhibitory substance(s) produced by *Bacillus* sp. M5 was fengycin or other antibiotic requires further investigation.

ACKNOWLEDGEMENTS

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摘　　要

吳文希、張琳．1993．葡萄晚腐病及苦腐病的生物防治．植病會刊 2:20-25．(台北市　國立台灣大學植物病蟲害學系)

自葡萄組織上分離出的 46 株菌種中，篩選出 F3 及 M5 兩株細菌，它們在馬鈴薯葡萄糖洋菜上表現出最具抑制 *Colletotrichum gloeosporioides* 及 *Melanconium fuligineum* 生長的能力，受影響的此兩種病原的菌絲呈現出變形、萎縮及溶解的現象；而正常的病原菌菌絲則具有緻密的細胞質及完整的胞器。經初步的鑑定 M5 是一種屬 *Bacillus* 的細菌，而暫稱之爲 *Bacillus* sp. M5; *Bacillus* sp. M5 在葡萄果實接種了病原 14 天之內，並存放在 28 C 的環境下，可以顯著地 (p=0.05) 有效防治晚腐病及苦腐病；此等防治效果在葡萄園中亦然彰顯，雖然防治效果不如噴灑大生 45 藥劑者，但是 *Bacillus* sp. M5 已能顯著地 (p=0.05) 在田間防治自然發生的晚腐及苦腐病；此乃可能由於 *Bacillus* sp. M5 在葡萄果實上可以顯著地干擾此二種病原的發芽及生長，同時它尙可產生一些尙未鑑定之抗生物質，而顯著地 (p=0.05) 抑制這二種病原的生長之故。

關鍵詞：葡萄、生物防治、晚腐病、苦腐病。