EFFECT OF *CLOSTRIDIUM* IN THE CONTROL OF FLAX *FUSARIUM*-WILT [1]

M. Chen, C. C. Tu, and L. H. Jwo [2]

ABSTRACT : Bacteria were examined in two soils similar except that one was conducive while the other one suppressive to flax *Fusarium*-wilt. Bacterial flora differed markedly between these two soils. The former had a higher count of cocci and actinomycetes whereas the latter had a higher count of anaerobic bacteria and gram-negative rods. Several *Clostridium* spp. isolated from the suppressive soil were inhibitory to various formae of *F. oxysporum* including *lini*, *pisi*, *niveum*, *lycopersici*, and *cubense* race 1 and race T. In the pot test *Fusarium*-wilt was controlled by inoculating *Clostridium* sp. into moist soil kept at a neutral pH condition.

Fusarium wilt is a major crop disease in Taiwan. More than a dozen crops are susceptible to *F. oxysporum* infection, and as a result loss to annual yield is heavy. At present, there is no practical mean to control the disease.

Biological control of *Fusarium* wilt has been rarely successful in the past. The reasons are mainly due to lack of information on the mechanism that the fungi are inhibited, and on the proper way to maintain the antagonistic microorganism in soil. In fact, the phenomenon that some soils are more resistant to *Fusarium* wilt than others has been known for many decades [9,15]. This phenomenon has also been found in Taiwan where flax is cultivated between two rice crops in the winter time [14]. Commonly, *Fusarium* wilt becomes prevalent in one field but in an adjacent field of same soil type and under similar agricultural system, the disease is not present. Such occurrence provides an excellent opportunity for studying the interaction between soil parameters and the disease severity.

This work was designed to study the bacterial populations of *Fusarium*-suppressive and -conducive soils and to note if any of the bacteria isolated possessed the potential on the control of the disease.

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MATERIALS AND METHODS

Bacteria were isolated from soils of Fou-chein Chuen, Show-shui Shian and of Fan-po Chuen, Fu-hsing Shian, respectively. Both are alluvial soil and belong to show-shui soil series. The former soil contained 3.8 percent organic matter, had a pH of 7.8, and contained sand 14.8 percent, silt 39.9 percent, clay 45.3 percent. Flax was cultivated in this soil for the past ten years and the wilt had not been found, though it did occur in the adjacent fields. The latter soil contained 3.2 percent organic matter, had a pH of 8.0, and consisted of sand 9.9 percent, silt 45.5 percent, clay 44.7 percent. Flax was culti vated in that soil for seven years and the *Fnsarium*-wilt, which gradually developed during the last two years, had completely destroyed the flax at the time of sampling.

The number of viable bacteria were determined by plate dilution on soil extract agar amended with 0.2 percent yeast extract [6], asparagine-mannitol agar [13] and nutrient agar containing 2 part/10^6 crystal violet [5]. Three replicate plates were poured for each sample, and random colonies were selected for 14 days at room temperature. Anaerobic bacteria were isolated with a 2×15 cm tube containing soil extract-yeast extract agar medium and then both ends stoppered. The medium was cooled immediately after autoclaving, and carbon dioxide was flushed over the tube to remove dissolved oxygen. Diluted soil was inoculated in the tube and incubated in CO_2 at room temperature for 10 days. Deep agar colonies were transferred and serially diluted into soil extract-yeast extract solution amended with 0.5 percent of glucose which was cooled in carbon dioxide immediately after autoclaving. The cultures were then incubated at room temperature in an atomosphere of carbon dioxide. Cellular morphology of these bacteria were examined and were categorized by the modified procedure of Stanier and van Niel [11].

To test the inhibitory effect of aerobic bacteria on the pathogenic *F. oxysporum*, the bacteria were inoculated as a circle (2 cm diameter) on soil extract-yeast extract agar. A 10-mm diameter disc of *F. oxysporum* grown on potato-glucose agar medium was placed in the circle one day after inoculation of the bacteria. The inhibitory effect of the anaerobic isolates was tested by first growing the bacteria in the glucose-soil extract-yeast extract medium in test tubes. Five-ml of the growth medium (ca. 5×10^8 bacteria) was mixed with 10-ml soil extract agar medium and poured in a petri dish. The fungal disc was then placed on the agar medium. Although the fungus was grown aerobically, the bacteria submerged within the agar, were under an environment approching anaerobiosis. Growth of the fungus was observed after incubation at 28°C under continuous light for at least 6 days.

Tam-shui River alluvial soil collected near the Taiwan Agricultural Research Institute, Taipei was used for the pot experiment. The soil contained 3. 4 percent organic matter, had a pH 5.4, and consisted of sand 68.6 percent, silt 20.8 percent, and clay 10.6 percent. After steam sterilization for 30 min., the soil was mixed with minced"diseased-flax" residue and kept moist for one week. Approximately 2,700 chlamydospores per gram of soil were counted with the plate-counting method [7]. Soil pH was then adjusted to 7.2 using 2—1 (w/w) $Ca(OH)_2$—$CaCO_3$. To create a wet and anaerobic condition, pots

were wrapped with plastic film and placed in a dish containing water. Approximately 10^7 axenic *Clostridium* sp. were inoculated in a gram of soil 7 days prior to the flax planting. Each 10.5—cm diam. clay pot was planted with 5 flax seedlings. Six replicates were made for each tested bacterium. Three series of control were used ; one with bacteria, one without soil pH adjustment, and one without the technique used to mimic moist anaerobic condition. However, sufficient water was added once every two days to the control treatments without the continuous water supply.

RESULTS

The type of bacteria differed markedly between the two soil samples (Table 1). In the suppressive soil, a greater number of anaerobic bacteria and gram-negative rods were counted, whereas in the conducive soil cocci and actinomycetes counts were higher. Aerobic spore formers, the asporogeneous gram-positive rods and total bacteria populations varied in size on the three agar media showing physiological selecting of the media.

The number of bacteria capable of producing antagonistic effect on *F. oxysporum* f. sp. *lini* in the soils are illustrated in Figure 1. Only a small portion of aerobic isolates could inhibit the fungi in petri dish and their numbers, including a few *Bacillus* and actinomycetes, were less in the suppressive soil. However, within the suppressive soil were a higher number of anaerobes, mainly *Clostridium*, that were capable of inhibiting the pathogenic *F. oxysporum*. Seven anaerobic isolates effectively inhibited *F. oxysporum* f. sp. *lini* in petri dish (Figure 2). Isolate a, also inhibited other formae of *F. oxysporum* including *pisi*, *niveum*, *lycopersici*, and *cubense* race 1 and race T (Figure 3).

Table 1. Viable bacterial counts ($\times 10^6$/g) in the suppressive (1) and the conducive soil (2).

Microbial group	Soil extract-yeast extract agar		Nutrient agar with crystal violet		Thornton's asparagine mannitol agar	
	1	2	1	2	1	2
Aerobic bacteria						
Total	442	386	67	43	211	260
Actinomycetes	52	94	—*	3	12	91
Spore formers	100	73	5	5	41	135
Gram-neg. rods	232	47	62	25	25	18
Asporogenous						
Gram-pos. rods & Arthrobacter	60	68	—	10	133	10
Cocci	—	82	—	—	—	5
Anaerobic bacteria			—	—	—	—
Total	72	50				

*Not detected

Table 2. Number of flax plant survived and of *Clostridium* sp. ($\times 10^4$) and *Fusarium oxysporum* f. sp. *lini* ($\times 10^2$) in per gram of soil (pot experiment).

Clostridium isolate	Intermittently wet			Continuously wet			
	Percent flax survived	Colony numbers of microorganism		Percent flax survived		Colony numbers of microorganism	
		Clostridium	*Fusarium*			*Clostridium*	*Fusarium*
	7 days	30 days	30 days	7 days	30 days	30 days	30 days
a	0	10.4	16	100	71	52.0	8
b-g	0	7.2-10.8	19-32	71-89	44-55	17.0-61.0	12-17
CK_1*	0	7.2	34	71	44	28.5	18
CK_2**	0	3.2	43	0	0	5.5	31

* soil pH 7.2, no external bacteria added.

** soil pH 5.4, no external bacteria added.

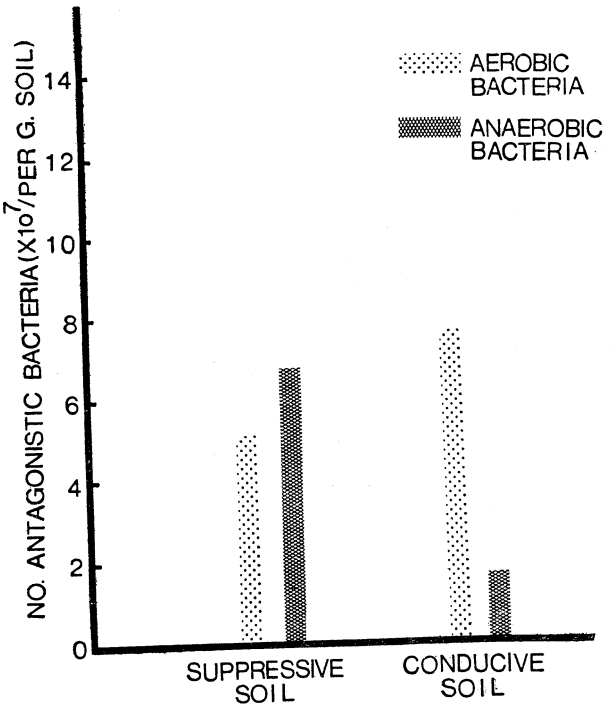


Figure 1. Number of antagonistic bacteria in the suppressive and conducive Soils.

Flax wilt was effectively prevented by all isolates of *Clostridium* sp. if the soil (pH 7.2) was kept continuosly wet in the pot experiment (Figure 4 upper row). Intermittently watering (supplied once every two days) allowed the pathogen to destroy seedlings, additionally, failure to maintain soil pH at 7.2 (Figure 4 lower row) allowed the wilt development. The aerobic isolates could not prevent wilt by maintaining the soil

(pH 7.2) in intermittently wet condition. Numbers of *Fusarium* propagules and *Clostridia* in the potted soil as well as surviving flax plants are summarized in Table 2 according to treatment. The results show a clear relationship among the pathogen, bacteria and surviving flax plants.

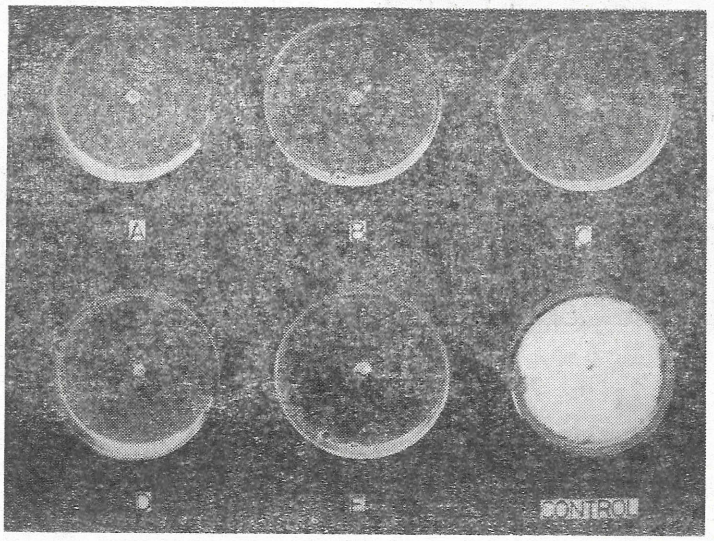


Figure 2. Inhibitory effect of five isolates of *Clostridium* sp. on *Fusarium oxysporum* f. sp. *lini*.

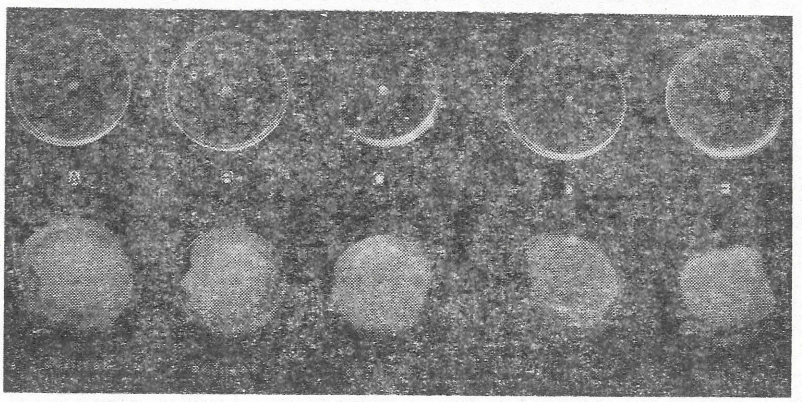


Figure 3. Inhibitory effect of *Clostrdium* sp. isolate *a* on various formae of *Fusarium oxysporum*. A. *lycopersici*, B. *cubense* race T. C. *Pisi*, D. *niveum*, E. *cubense* race 1.

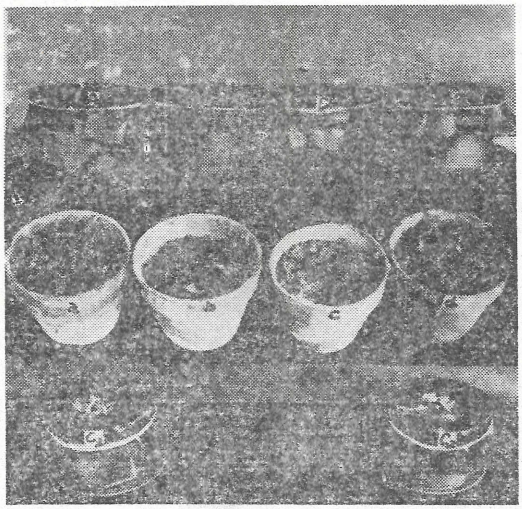


Figure 4. Pot experiment by using four isolates (isolate *a*, *b*, *c*, and *d*) of *Clostridium* to control flax *Fusarium*-wilt.
Upper row treatment: soil pH 7.2, with bacteria inoculated, continuous wet.
Middle row treatment: soil pH 7.2, with bacteria inoculated, intermittent wet.
Lower row treatment: soil pH 5.4, external bacteria not added, continuous wet.

DISCUSSION

Change in water content of soil results in many physical, chemical and biological alternations. Cook and Papendick [1] have shown that *Fusarium* activity in soil was changed markedly after only slight change of soil water content. Therefore, it is not surprising that arable soils with low moisture content are more prone to the wilt development. Supplying continual moisture to soil has been shown to suppress seedling blight of wheat caused by *F. roseum* f. sp. *cerealis* "*Graminearum*" [2], the damping-off of Ladino clover caused by *F. roseum* [3], and the stem rot of sweet potatoes caused by *F. solani* f. sp. *batatas* [4]. Flooding of banana field leads to eradication of *F. oxysporum* f. sp. *cubense* [12]. However, the reason behind the suppression of *Fusarium* in the wet soils has not been thoroughly investigated especially the role played by anaerobic bacteria.

Soil management and ground water level probably are two main factors affecting soil moisture. With regard to the soil management, application of a greater amount of water in one field could affect the soil water content thereafter. Tilling practice, variation in climate during rice harvest, shading, and soil percolation rate all affect water content and hence affect bacterial antagonism in the soil. Other physical and chemical parameters (e. g. organic matter) present in soils can be important in maintaining the wilt suppressive nature of soil. More research is required in these areas.

Although the anaerobic isolates have not yet been specifically identified, they were all spore formers. Their subsurface growth in agar media in air indicates that they belong to the oxygen tolerant *Clostridium* spp. These bacteria were present in both the conducive and suppressive soils, but their number were much less in the former soil type. In the pot experiment, *Fusarium* propagules increased remarkedly in wet soil with a pH of 5.4. This indicated that water alone did not suppress the *Fusarium*. Addition of *Clostridium* isolates to the potted soil increased the survival of flax plant, especially in the pot with isolate *a* added in continuous wet condition (Table 2 and

Figure 4). The exact nature of isolate *a* and whether the bacterium worked alone or in concert with other bacteria in the suppression of the *Fusarium* is not certain at the present time.

After passing the bacterial suspension (grown in one percent of peptone and glucose soil extract solution) through a 0.22 mu millipore filter, the inhibitory effect disappeared entirely. It appears, then, that the suppressive agent is either associated with the bacterial cell or an unstable extracellular product. Many of these bacteria isolates, including isolate *a*, produced abundant carbon dioxide, ethanol, ethylene and it's derivatives. These gaseous compounds are fungistatic(8, 10). Fungal growth was completely inhibited if the *Fusarium* was grown on the upper lid and the bacterial suspension on the lower lid of a petri dish. suppressive agents confined to the bacterial cells may inhibit the *Fusarium* in the area adjacent to bacterial colonies. The gases which permeate the soil are thought to be detrimental to the *Fusarium*.

Although other bacteria, including the gram-negative rods, showed no inhibitory effect on the *Fusarium* in petri dish, their function should not be overlooked. A few also produce gases. By virtue of their highly aerobic characteristics they could create an anaerobic condition in moist soil. Certainly, for the plant pathologist, the situation is further complicated by the necessity to consider the tolerance of higher plants to the anaerobiosis. The suppressive soil in the present study is a good example of interactions between host tolerance and pathogen suppression.

Bacteria inhibitory to one forma of *F. oxysporum* are also inhibitory to other formae. But the inhibitory effect could vary with the forma of the fungi and the type of the bacteria tested. Use of these bacteria to control *Fusarium*-wilt of various crops in the field is proceeding.

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嫌氣性芽孢桿菌(*Clostridium*)防治亞麻立枯病之效果

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本研究之目的爲比較亞麻立枯病（萎凋病）抑病土壤和導病土壤內細菌之分佈情形，並報導利用分離自抑病土壤之嫌氣性芽孢桿菌防治亞麻立枯病之盆栽試驗結果。導病土壤內以球菌和放射菌數量較多，而抑病土壤內則以革蘭陰性桿菌及嫌氣性芽孢桿菌居多。

從抑病土壤分離之嫌氣性芽孢桿菌能抑制爲害亞麻、豌豆、西瓜、番茄及香蕉（Race 1及T）等之萎凋病鐮刀菌菌絲之生長。盆栽試驗結果發現如接種此等細菌至病土內，並保持土壤酸鹼度爲中性及維持適當土壤水份，能有效防治亞麻立枯病之發生。

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