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Interaction between Likubin bacterium and *Phytophthora parasitica* in citrus hosts

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Abstract

Infection with Likubin bacterium (LB) followed by *Phytophthora parasitica* increased the mortality of sour orange and pummelo seedlings, and enhanced the *P. parasitica*-induced root rot in all the four types of citrus tested. The LB-induced enhancement of root infection by *P. parasitica* was apparent within 1 h of exposure to zoospore suspension. The enhancement of *P. parasitica*-induced root rot was affected by the infection sequence. Inoculation of sour orange seedlings with LB before *P. parasitica* was more effective in increasing *P. parasitica*-induced root rot than LB and *P. parasitica* concomitantly or LB after *P. parasitica*. Grafting *P. parasitica* susceptible scions of ponkan (*Citrus reticulata*) onto *P. parasitica*-tolerant rootstocks of sour orange greatly increased the susceptibility of rootstocks to *P. parasitica*. Results also demonstrate the enhancement of LB-induced symptoms by *P. parasitica* in citrus plants.

Introduction

Phytophthora species cause serious soilborne diseases of citrus in all citrus growing regions and bring about considerable yield losses worldwide (Graham and Menge, 1999). Among them, *Phytophthora parasitica* Dast. (syn. *P. nicotianae* Breda de Haan) is the most important and widespread causal organism of citrus root rot (Ann, 1984; Timmer and Menge, 1988). The fungus infects the fibrous roots and causes decay and sloughing of their cortex leaving only the white stele. In Taiwan, although most commonly planted scion cultivars such as ponkan (*Citrus reticulata*) are highly susceptible to infection by *P. parasitica*, most rootstocks used by growers, including sour orange (*C. aurantium*), are tolerant. However, when citrus orchards were surveyed, a number of citrus plants were unexpectedly found to be seriously affected by *Phytophthora* root rot (Ann, 1984).

Likubin disease, also called greening or Huanglongbing, is one of the most devastating diseases of citrus in Asia and Africa (Garnsey, 1999). It is caused by a phloem-limited, not yet culturable bacterium of *Liberobacter* (Jagoueix et al., 1994). In Taiwan, the disease is the most serious problem of the citrus industry, affecting almost all major citrus varieties (Huang et al., 1980). Since the disease is so widespread, it was considered possible that the serious *Phytophthora* root rot may have resulted from coinfection of citrus plants with Likubin bacterium (LB) and *P. parasitica*. A project was, therefore, initiated to investigate the interaction between these two pathogens in citrus.

Materials and methods

Plant material

Seeds of sour orange and pummelo (*C. grandis*) were sown in vermiculite and peat moss (1 : 1 v/v). One month after germination, seedlings were transplanted to black plastic pots ($35 \times 10 \times 10\,cm^3$) containing 60% sandy loam soil, 20% peat moss and 20% vermiculite. Soil was heat-treated at 80 °C for 5 min. At the age of 3–6 months, the sour orange and pummelo seedlings were grafted with buds of ponkan and grapefruit (*C. paradisi*), respectively. Plants were inoculated 3–4 months after grafting. Buds were obtained from budwood sources freed from Likubin by thermotherapy and shoot-tip grafting (Su and Chu, 1984). Budded seedlings were designated as ponkan/sour orange grafts and grapefruit/pummelo grafts, respectively.

Inoculation with LB

A strong pathogenic line of LB was maintained in 10 small ponkan trees in the greenhouse. Two slanted cuts were made by gently pressing a scalpel half way into the opposite sides of a stem about 5 cm apart. The inoculated sites were 15–20 cm above the ground. A small piece (ca. $2 \times 3\,cm^2$) of symptomatic leaf with main vein in the center was obtained from the LB-infected tree and inserted into each cut. The inoculated portion was then wrapped with parafilm and covered with a thin plastic sheet. Inoculation was judged successful if a leaf piece had remained alive on the inoculated stem when the plastic sheet was removed after 1 month.

Inoculation with P. parasitica

The isolate of *P. parasitica* used in this study was obtained from a diseased citrus root. It was strongly pathogenic to citrus (Ann, 1984). The method of Huang et al. (1976) was used to produce zoospores and the zoospore concentration was determined and adjusted with a Pipetman microliter pipette (Ko et al., 1973). Citrus seedlings were inoculated by removing the seedlings from the pots, gently washing the roots in water, dipping the roots in a zoospore suspension at a concentration of 3×10^3 zoospores ml^{-1} for 24 h, and planting the seedlings back in the pots. Detection of *P. parasitica* in roots was carried out by surface sterilization of root segments (1–1.5 cm long) in 0.5% NaClO for 3 min, and incubation of root segments on a selective medium (Masago et al., 1977). Sixty root segments from each seedling were used. *Phytophthora parasitica* mycelium growing from root segments was recorded after incubation at 24 °C for 5–7 days. All the experiments were carried out at least twice. Data from one experiment were presented.

Results

Response of citrus seedlings to infection with LB and/or P. parasitica

Double infection with LB and *P. parasitica* increased the mortality of 6-month-old sour orange and pummelo seedlings, but not ponkan/sour orange or grapefruit/pummelo grafts (Table 1). When citrus seedlings were inoculated with LB followed by *P. parasitica* 45 days later, about 36% of sour orange seedlings and 41% of pummelo seedlings died after 6 months. There was no mortality among those inoculated with only LB or *P. parasitica* or among those that were not inoculated. For ponkan/sour orange grafts, only one out of seven plants inoculated with LB and *P. parasitica*, and one out of eight plants inoculated with only *P. parasitica* died. No mortality occurred among uninoculated seedlings or seedlings inoculated with only LB. For grapefruit/pummelo grafts, none of the treatments caused death.

For comparing the average height and weight increase in 6 months after inoculation, seedlings that had been killed by the pathogens were excluded from the calculation. In general, infection of citrus seedlings with LB and/or *P. parasitica* reduced both the increase in height and weight. Growth reduction was most drastic when seedlings were infected with both LB and *P. parasitica* (Table 2). When height

Table 1. Mortality of citrus seedlings and grafts resulting from infection with LB and/or *P. parasitica* (PP)[a]

Infection sequence		No. killed/no. tested			
First	Second[b]	Sour orange[c]	Pummelo[c]	Ponkan/sour orange[d]	Grapefruit/pummelo[d]
LB	PP	5/14	7/17	1/17	0/6
LB	None	0/10	0/8	0/17	0/6
None	PP	0/14	0/12	1/8	0/5
None	None	0/12	0/12	0/7	0/5

[a]Mortality was recorded 6 months after second inoculation.
[b]Second inoculation was performed 45 days after first inoculation.
[c]First inoculation was performed when seedlings were 6 months old.
[d]Grafting was carried out when seedlings were 6 months old, and the first inoculation was performed 3–4 months after grafting.

and weight were combined in the consideration, LB plus *P. parasitica* was most effective in reducing the growth of sour orange and pummelo followed by LB or *P. parasitica* alone. In ponkan/sour orange grafts, LB plus *P. parasitica* and *P. parasitica* alone were most effective in reducing the growth, followed by LB alone. During the period of 6 months, weights were actually reduced when ponkan/sour orange grafts were infected with LB plus *P. parasitica* or *P. parasitica* alone. In grapefruit/pummelo grafts, height and weight increase were most severely reduced by infection with LB plus *P. parasitica* and *P. parasitica* alone, followed by LB alone. Infection with LB plus *P. parasitica* also reduced plant weights during the growth period.

Sour orange and pummelo seedlings did not show foliar symptoms typical of Likubin when they were inoculated with LB alone or LB plus *P. parasitica* (Table 3). Root rot caused by *P. parasitica* was mild when they were inoculated with *P. parasitica* alone, but the symptoms were severe when they were inoculated with LB plus *P. parasitica*. For ponkan/sour orange and grapefruit/pummelo grafts, symptoms caused by LB were mild when plants were inoculated with LB alone, but were severe and appeared 20–45 days earlier when they were inoculated with LB plus *P. parasitica*. Root rot caused by *P. parasitica* on ponkan/sour orange grafts was severe when plants were inoculated with LB plus *P. parasitica* or *P. parasitica* alone. On grapefruit/pummelo grafts, root rot was severe when plants were inoculated with LB plus *P. parasitica*, but was mild when they were inoculated with *P. parasitica* alone.

Effect of inoculation sequence on disease severity

When 3-month-old sour orange seedlings were concomitantly inoculated with LB and *P. parasitica*, foliar symptoms caused by LB were severe (Table 4). The symptoms were mild, when inoculation was with LB alone. Inoculation with *P. parasitica* alone did not cause seedlings to produce LB-induced symptoms. Root rot severity was increased from mild when seedlings were inoculated with *P. parasitica* alone, to intermediate when they were concomitantly

Table 2. Net growth of citrus seedlings and grafts 6 months after infection with LB and/or *P. parasitica* (PP)[a]

Infection sequence		Sour orange (%)		Pummelo (%)		Ponkan/sour orange (%)		Grapefruit/pummelo (%)	
First	Second	Ave. height increase[b]	Ave. weight increase[c]	Ave. height increase	Ave. weight increase	Ave. height increase	Ave. weight increase	Ave. height increase	Ave. weight increase
LB	PP	31 D[d]	22 C	23 D	31 B	23 C	−41 C	28 B	−18 C
LB	None	131 AB	309 A	93 B	223 A	71 B	69 B	189 A	114 B
None	PP	119 BC	128 B	70 C	28 B	32 C	−48 C	25 B	25 C
None	None	159 A	327 A	161 A	277 A	223 A	159 A	210 A	204 A

[a]Age of seedlings were the same as those in Table 1. An average of nine plants per treatment was used.
[b](Seedling height 6 months after inoculation with PP − seedling height before inoculation)/seedling height before inoculation × 100.
[c](Seedling weight 6 months after inoculation with PP − seedling weight before inoculation)/seedling weight before inoculation × 100.
[d]Values in the same column followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 3. Likubin symptoms and *Phytophthora* root rot of citrus seedlings and grafts resulting from infection with LB and/or *P. parasitica* (PP)[a]

Infection sequence		Sour orange		Pummelo		Ponkan/sour orange		Grapefruit/pummelo	
First	Second	Likubin symptoms[b]	*Phytophthora* root rot[c]	Likubin symptoms	*Phytophthora* root rot	Likubin symptoms	*Phytophthora* root rot	Likubin symptoms	*Phytophthora* root rot
LB	PP	N	S	N	S	S	S	S	S
LB	None	N	N	N	N	M	N	M	N
None	PP	N	M	N	M	N	S	N	M
None	None	N	N	N	N	N	N	N	N

[a]Ages of seedlings were the same as those in Table 1.
[b]N = no symptoms; S = severe, leaves turned chlorotic and became hardened 20–45 days earlier than those with mild symptoms (M).
[c]N = no symptoms; S = severe, roots turned brown and became rotten more quickly and in larger amount than those with mild symptoms (M).

Table 4. Disease severity of 3-month-old sour orange seedlings resulting from concomitant infection with LB and *P. parasitica* (PP) or infection with PP 45 days prior to infection with LB

Infection sequence		Mortality		Symptoms caused by		Infection sequence		Mortality		Symptoms caused by	
First	Second	No. tested	No. killed	LB[a]	PP[b]	First	Second	No. tested	No. killed	LB[a]	PP[b]
LB, PP	None	8	0	S	I	PP	LB	12	0	S	I
LB	None	8	0	M	N	None	LB	12	0	M	N
PP	None	8	0	N	M	PP	None	8	0	N	M
None	None	8	0	N	N	None	None	8	0	N	N

[a]N = no symptoms; S = severe, leaves turned chlorotic and became hardened 20–45 days earlier than those with mild (M) symptoms.
[b]N = no symptoms, I = intermediate, between severe (S) and mild (m) symptoms (see Table 3).

inoculated with LB and *P. parasitica*. Similar results were obtained when sour orange seedlings were inoculated with *P. parasitica*, 45 days prior to inoculation with LB (Table 4). Foliar symptoms caused by LB became severe and appeared earlier, when seedlings were inoculated with *P. parasitica* followed by LB. No LB-induced foliar symptoms were observed when they were inoculated with *P. parasitica* alone. Root rot caused by *P. parasitica* was increased from mild when seedlings were inoculated with *P. parasitica* alone to intermediate when they were inoculated with *P. parasitica* followed by LB.

Effect of LB on root colonization by P. parasitica *zoospores*

To determine the effect of LB on susceptibility of roots to infection by *P. parasitica*, 3-month-old sour orange seedlings were inoculated with LB and 7, 14, 21, 28 and 35 days later, their roots were dipped in *P. parasitica* zoospore suspension for 24 h and immediately assayed for the presence of *P. parasitica* in root tissues with the selective medium. Inoculation with LB for 7 days did not significantly increase root colonization by *P. parasitica*. However, percentages of root segments colonized by *P. parasitica* were significantly increased when zoospore inoculation was performed after seedlings were inoculated with LB for 14–35 days (Table 5).

To determine the time needed for citrus roots to be infected by *P. parasitica* zoospores, 3-month-old sour orange seedlings were inoculated with LB and 14 days later their roots were dipped in *P. parasitica* zoospore suspension for 1, 2, 4, 8 and 24 h and immediately assayed for successful infection by *P. parasitica* with the selective medium. Results showed that infection of roots by *P. parasitica* zoospores was accomplished within 1 h of exposure to zoospore suspension (Table 6). When seedlings were infected with LB, the percentage of root tissues infected with

Table 5. Root susceptibility to *P. parasitica* (PP) of 3-month-old sour orange seedlings at various times after inoculation with LB

Infection sequence		Root segments colonized by PP (%)[a]				
First	Second	7[b]	14	21	28	35
LB	PP	22 A[c]	34 A	46 A	41 A	44 A
None	PP	18 A	20 B	20 B	17 B	21 B
LB	None	0 C	0 C	0 C	0 C	0 C
None	None	0 C	0C	0 C	0 C	0 C

[a]Roots were placed in zoospore suspension for 24 h at 24 °C before root segments were cut, surface sterilized and placed on the selective medium.
[b]Days after inoculation with LB.
[c]Values in the same column followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 6. Root colonization by *P. parasitica* (PP) at various times of dipping in zoospore suspension, of 3-month-old sour orange seedlings inoculated with LB 14 days before

Infection sequence		Root segments colonized by PP (%)[a]				
First	Second	1[b]	2	4	8	24
LB	PP	35 A[c]	43 A	65 A	60 A	50 A
None	PP	15 B	31 B	49 B	35 B	38 B
LB	None	0 C	0 C	0 C	0 C	0 C
None	None	0 C	0 C	0 C	0 C	0 C

[a]Roots were dipped in zoospore suspension for various times at 24 °C before root segments were cut, surface sterilized and placed on the selective medium.
[b]Hours of dipping in zoospore suspension.
[c]Values in the same column followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

P. parasitica within 1 h of exposure to zoospore suspension was 130% higher than the root tissues of seedlings without prior inoculation with LB. Thereafter, prior inoculation of seedlings with LB increased root infection by *P. parasitica*, extents ranging from 32% to 71%.

Discussion

Results from this study demonstrate the enhancement of *P. parasitica*-induced root rot by LB in citrus plants. There have been a number of reports on the enhancement of root rot caused by various soilborne pathogens by different viruses (Farley and Lockwood, 1964; Beute and Lockwood, 1968; Thanassoulopoulos, 1976; Pieczarka and Zitter, 1981; Gbaja and Chant, 1983). It is, therefore, considered possible that root rot enhancement by a fastidious bacterium may also occur in fungus–bacterium interactions. The mechanism by which LB increases the severity of *P. parasitica*-induced root rot is not known. Since the LB-induced increase of root infection by *P. parasitica* was apparent within 1 h of exposure to a zoospore suspension (Table 6), root rot enhancement could be due to an increase in zoospore attraction or susceptibility to zoospore infection.

The sequence of inoculation with LB and/or *P. parasitica* appears to be important in LB-induced susceptibility of citrus plants to *P. parasitica*. In sour orange, severity of root rot caused by *P. parasitica* was increased from mild without LB to intermediate with LB and *P. parasitica* inoculated at the same time or with LB inoculated after *P. parasitica* (Table 4). However, when LB was inoculated prior to *P. parasitica*, the severity of root rot was increased from mild to severe (Table 3). Apparently, the later stage of LB development inside host plants is most effective in enhancing the severity of *P. parasitica*-induced root rot.

When *P. parasitica* susceptible scions of ponkan were grafted onto *P. parasitica*-tolerant rootstocks of sour orange, susceptibility of rootstocks to *P. parasitica* was greatly increased (Table 3). These results suggest that the seriousness of *Phytophthora* root rot among citrus trees with *P. parasitica*-tolerant rootstocks in Taiwan is due not only to infection with LB but also to grafting of rootstocks with root rot-susceptible scions.

During this study, we also unexpectedly discovered the enhancement of LB-induced symptoms by *P. parasitica* in citrus plants. In ponkan/sour orange and grapefruit/pummelo grafts, LB-induced symptoms were increased from mild when seedlings were inoculated with LB alone to severe when they were inoculated with LB followed by *P. parasitica* (Table 3). In sour orange, although LB-induced symptoms were mild when seedlings were inoculated with LB alone, the symptoms became severe when they were inoculated with LB and *P. parasitica* concomitantly or LB after *P. parasitica* (Table 4). The mechanism by which *P. parasitica* increases susceptibility of citrus plants to LB remains to be investigated.

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