Characterizations of *Fusarium* Isolates Obtained from Tomato Plants in Taiwan

Summer Internship Final Report

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Abstract

In this study, we were using three variants of tomato plants to characterize the recent Fusarium isolates collected from Taiwan, which is Bonny Best as our susceptible check, UC-82L that have *I-1* resistance gene that makes them resistance towards race 1, and MH-1 that *I-1* and *I-2* resistance gene that makes them resistance towards race 1 and race 2 isolates. In this experiment, we divide the experiment into two parts bioassay and phenotyping, where for the bioassay using root dip technique for the inoculation of the pathogens, as for the phenotyping we use several primers to identify *Fusarium oxysporum* specific and determine the races of FOL isolates. With the help of NCBI BLAST, we can identify other several *fusarium* isolates.

Introduction

One of the most important vegetable crops in the world is the tomato (*S. lycopersicum*). The development of a tomato cultivar depends on breeding for the necessary disease resistances because tomato is vulnerable to more than 200 pests and diseases. Tomato breeders have used various methods for more than a century, the ability of wild tomato species' genetic variety to contribute desirable features, notably, disease resistance genes, by crossing wild tomatoes with cultivated tomato (Chitwood-Brown et al., 2021).

A wide variety of plant species, including bananas, cotton, tomatoes, and legumes, are susceptible to the vascular wilt disease caused by the ubiquitous species complex of fungi known as *Fusarium oxysporum*. Depending on the host species they infect, pathogenic strains of *F. oxysporum* are divided into *formae speciales* (ff. spp.). The virulence patterns on the resistant or susceptible variants of the host species can be used to classify a *forma specialis* (f. sp.) into races (Carmona et al.,2020). The infection enters the vascular tissue and spreads to the root epidermis. The xylem channels become colonized, and mycelium and conidia are produced. Severe water stress, primarily because of vessel obstruction, causes the recognizable wilt signs to manifest (Nirmaladevi et al. 2016). The first signs of the disease are an obvious chlorosis and wilting of the lower basal leaves, which spread acropetally to the upper leaves. These signs are frequently asymmetrical, confined to one or two plant branches or perhaps just one side of a leaf. Wilting can happen on the entire plant or just one side as symptoms worsen, and early wilting may be more obvious in the afternoon before the plant appears to recover overnight. As the pathogen colonizes the vascular system, browning is frequently seen in the stems' vascular tissue. The illness eventually causes a quick fall in the plant's health, faster fruit ripening, and plant death (Chitwood-Brown et al., 2021).

The purpose of this study is to characterize the recent Fusarium isolates, can identify and determine recent Fusarium oxysporum specific isolatesfrom tomato plants in Taiwan, and testing markers from previous study (Hirano and Arie, 2006) to determine the physiological races of FOL.

Materials and Methods

Host Plants and Propagation

For this study, we tested three varieties of tomato plants, Bonny Best, UC-82L, and MH-1. Bonny Best are susceptible to FOL since, it lacks any of the resistance gene, on the other hand, UC-82L have *I-1* resistance gene which means it have a resistance towards FOL race 1 pathogens, and for MH-1, it has *I-1* and *I-2* resistance gene which means it have a resistance towards FOL race 2 pathogens. In this study, we grow them in a peat-moss and let them grow for two weeks, after two weeks they are ready to be inoculated.

Fungal Culture

In this study, we use 40 *Fusarium oxysporum* (FO) isolates that are preserved in silica gel and kept in a 4ºC, using FOL-008 strains (race 1) and FOL 34-1 strains (race 2) as the control group. At first, each of the isolates' silica gel culture were transferred into PDA plates and incubated for one-week at 28ºC with 12 hours alternating light-dark periods. After one-week has passed, we transferred the isolates into a new PDA plates, preparing for the inoculation, which need 4~6 plates for each isolate, and incubated for one-week at 28ºC with 12 hours alternating light-dark periods. Table 1 below show the isolates that we used.

no.	Isolate no.	Date isolated	Host plant	County	Collected from
1	Fol-008	1993.12.13	Tomato	Nantou	Hsinyi
2	Fol-032	1996.02.14	Tomato	Tainan	Shanhua (AVRDC)
3	Fol-179	2005.04.26	Tomato	Ilan	Yuanshan
4	Fol-180	2005.04.26	Tomato	Ilan	
5	Fol-181	2005.07.15	Tomato	Nantou	Renai
6	Fol-185	2005.07.15	Tomato	Nantou	Hsinyi
7	Fol-207	2006.01.23	Tomato	Kaohsiung	Meinung
8	Fol-231	2006.08.09	Tomato	Nantou	Hsinyi
9	Fol-233	2006.08.09	Tomato	Nantou	Hsinyi
10	Fol-289	2008.04.02	Tomato	Chiayi	Shuishang
11	Fol-291	2008.04.02	Tomato	Tainan	Paiho
12	Fol-312	2009.06.02	Tomato	Yunlin	Siluo
13	Fol-315	2009.7.30	Tomato	Nantou	Hsinyi
14	Fol-316	2010.07.26	Tomato	Hualien	Sibao
15	Fol-322	2015.04.15	Tomato	Yunlin	Siluo
16	Fol-325	2018.05.30	Tomato	Tainan	Shanhua (AVRDC)/Entomo./OpenII
17	Fol-326	2019.02.21	Tomato	Tainan	Shanhua
18	Fol-327	2019.02.21	Tomato	Tainan	Shanhua
19	Fol-328	2019.02.21	Tomato	Tainan	Guantian
20	Fol-329	2019.02.21	Tomato	Tainan	Guantian
21	Fol-330	2019.02.25	嫁接Tomato	Yunlin	Yuanchang
22	Fol-331	2019.02.25	嫁接Tomato	Yunlin	Yuanchang
23	Fol-334	2019.02.21	Tomato	Tainan	Yanshuei
24	Fol-335	2019.02.21	Tomato	Tainan	Yanshuei
25	Fol-337-1	2019.03.13	Tomato	Yunlin	Yuanchang A
26	Fol-338-1	2019.03.13	Tomato 1	Yunlin	Yuanchang A
27	Fol-339-1	2019.03.13	Tomato 2	Yunlin	Yuanchang A
28	Fol-340-1	2019.03.13	嫁接Tomato	Yunlin	Yuanchang A
29	F.sp-172	2019. 07.26	Tomato	Miaoli	Nanjhuang
30	F.sp-191	2019.11.06	Tomato/茄子根砧	Miaoli	Tai-an
31	F.sp-196	2019.11.07	Tomato	Hsinchu	Jianshih
32	F.sp-199	2019.11.06	Tomato	Miaoli	Dahu
33	F.sp-213	2020.03.16	Tomato	Yunlin	Linnei
34	F.sp-214	2020.03.16	Tomato	Changhua	Ershuei
35	F.sp-224	2020.04.13	Tomato	Yilan	Jiaosi
36	F.sp-225	2020.04.13	Tomato	Yilan	Jhuangwei
37	F.sp-227	2020.04.13	Tomato	Yilan	Jhuangwei
38	F.sp-237	2020.05.15	Tomato	Yunlin	Siluo
39	F.sp-238	2020.05.15	Tomato	Yunlin	Siluo
40	F.sp-249	2020.06.18	Tomato	Miaoli	Jhuolan
41	FOL-34-1				

Table 1. *F. ozysporum* isolates used in this study, showing when, where, and which host plants the isolates were retrieved, with FOL-008 and FOL 34-1 as the group control.

Inoculum and Inoculation

Blend the entire contents of each plate (4 plates are enough) for about 10~30 seconds with 500ml of distilled water to form slurry. In this study, we used root-dipping technique to let the pathogens absorbed into the tomato plants. We immersed the roots into the slurry inoculum for three-minutes. Then, transplant inoculated plants into the plastic tray with 4.2cm x 4.2cm x 5.5cm cells, containing a soil mixture (1:1 v/v) of pasteurized sand and peat-moss. Inoculate 12 plants of each lines with each isolate and arrange in three replications of six plants each. Maintain the plants in the greenhouse or growth room with daytime temperature ≥25ºC. Provide plants with soluble fertilizer once a week after inoculation.

Disease Severity Rating

The disease severity of stem was evaluated three-weeks after inoculation by stem cutting. For the evaluation, a standardized 0-3 scale was used to score each plant, where 0 = no symptoms or healthy, 1 = stunted and vascular browning (when cutting the stem, we can see the browning on one of the side of the stem), 2 = severely stunted and vascular browning (browning on both sides of the stem), and 3 = wilted beyond recovery or dead (figure 1).

Figure 1. The rating scale of evaluation of plants 3 weeks after the inoculation with *Fusarium oxysporum* f. sp. *Lycopersici* (FOL), where 0 = 0 = no symptoms or healthy, 1 = stunted and vascular browning (when cutting the stem, we can see the browning on one of the side of the stem), 2 = severely stunted and vascular browning (browning on both sides of the stem), and 3 = wilted beyond recovery or dead.

Genotyping

Preparing sample materials

At first, each of the isolates' silica gel culture were transferred into PDA plates and incubated for one-week at 28ºC with 12 hours alternating light-dark periods. After one-week has passed, we transferred the isolates into a new PDA plates, preparing for the DNA extraction, which need 3~5 plates for each isolate, and incubated for one-week at 28ºC with 12 hours alternating light-dark periods. After one-week has passed, we scrape the mycelium using glass slide and put it into the 1.5ml Eppendorf tube, and grind until smooth. Add 400µl LB buffer and 5µl RNase A (10mg/ml), vortex and centrifuge. Incubate the isolates at 65ºC for 10 minutes, and gently mix the solution for every 2~3 minutes. Add 130µl BD buffer, mix and incubate on ice for 5 minutes. Centrifuge the solution at 12000 rpm for 10 minutes, the transfer 400µl suspension into a new centrifuge tube. Add 600µl PL buffer (1.5x of the suspension volume) and mix the sample immediately.

DNA Extraction

Combine the DNA extraction column and the collection tube, transfer the sample, including the pellet in a DNA extraction column, centrifuge at 12000 rpm for one minute. Remove the liquid, combine the column and the collection tube, add 600µl Wash buffer, then centrifuge at 12000 rpm for 30 seconds, remove the liquid, then repeat. Combine the column and the collection tube, centrifuge at 12000 rpm for 2 minutes. Combine the column with a new 1.5 ml centrifuge tube, air dry, add 50-100µl Elution buffer (TE), then centrifuge at 12000 rpm for one minute, and collect the liquid, store at -20ºC.

PCR and Primers

After DNA extraction, all isolates were ready to be used in PCR. In this study, we used five groups of primers (table 2).

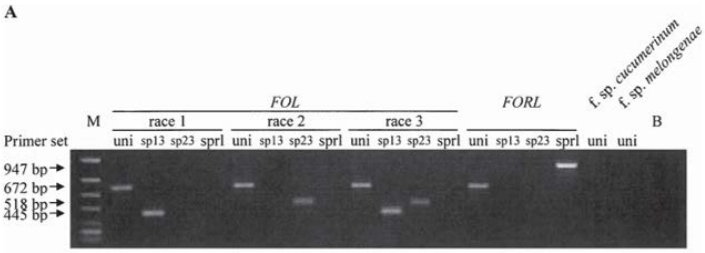
Purpose	Primer	Primer sequence (5'-> 3')	Reference
To amplify and sequence intervening region	ITS4	TCCTCCGCTTATTGATATGC	Mcbreen et al. (2003)
	ITS5	GGAAGTAAAAGTCGTAACAAGG	
Discrimination of *Fusarium oxysporum*	FOF1	ACATACCACTTGTTGCCTCG	Mishra et al. (2003)
	FOR1	CGCCAATCAATTTGAGGAACG	
Discrimination of physiological race 1, race 2, and race 3 of *F. oxysporum* f. sp *Lycopersici*	unif	ATCATCTTGTGCCAACTTCAG	Hirano and Arie (2006)
	unir	GTTTGTGATCTTTGAGTTGCCA	
	sp13f	GTCAGTCCATTGGCTCTCTC	
	sp13r	TCCTTGACACCATCACAGAG	
	sp23f	CCTCTTGTCTTTGTCTCACGA	
	sp23r	GCAACAGGTCGTGGGGAAAA	

Table 2. Primers that were used and their purpose in this study.

Amplifying and sequencing To amplify and sequence the intervening region that expected to contain much of the genetic region, we use ITS4 and ITS5. The PCR amplifications were performed in a 25µl mixture, containing DDH_2O, 10x Taq buffer, 25µm of $MgCl_2$, 10µm dNTP, 10µm ITS4/5 primers, 0.25 protaq and 1µm of DNA. Reactions were performed using the thermal cycler, with denaturation at 95ºC for five minutes, 30 seconds at 55ºC for annealing, and elongation at 72ºC for one minute, and repeated 35 times. The products will be tested in 1.5% agarose gel electrophoresis.

Identification of *Fusarium oxysporum* f. sp. *Lycopersici* In this study, we use FOF1/FOR1 primers to identify the specific of *F.oxysporum*. The PCR amplifications were performed in a 25µl mixture, containing DDH_2O, 10x Taq buffer, 25µm of $MgCl_2$, 10µm dNTP, 10µm FOF1/FOR1 primers, 0.25 protaq and 1µm of DNA. Reactions were performed using the thermal cycler, with denaturation at 95°C for one minute, 30 seconds at 52°C for annealing, and elongation at 72°C, and repeated 30 times. The products will be tested in 1.5% agarose gel electrophoresis.

Determining the physiological race of *F. oxysporum* f. sp. *Lycopersici* In this study, we use three groups of primers to identify the specific of *F.oxysporum*, uni, sp13, and sp23 (Hirano and Arie, 2006). The PCR amplifications were performed in a 25µl mixture, containing DDH_2O, 10x Taq buffer, 25µm of $MgCl_2$, 10µm dNTP, 10µm uni, sp13, and sp23 primers, 0.25 protaq and 1µm of DNA. Reactions were performed using the thermal cycler, with denaturation at 95°C for five minutes, one minute at 55~60°C for annealing, and elongation at 72°C for one minute, repeated 35 times. The products will be tested in 1.5% agarose gel electrophoresis. Following the previous study in Japan on 2006, we can determine FOL physiological races by following the figure 2 below.



B

	Primer set			
	uni	sp13	sp23	sprl
FOL race 1	+	+	-	-
FOL race 2	+	-	+	-
FOL race 3	+	+	+	-
FORL	+	-	-	+

Figure 2. Race identification of *F. oxysporum* f.sp. *lycopersici* based on previous experiment in Japan by PCR with the uni, sp13, sp23 and sprl primer sets, where (A) is showing the electrophoresed in 2.0% agarose gel and (B) is the PCR amplification pattern, where plus sign showing amplicon was present, meanwhile negative sign showing amplicon was absent. (Hirano and Arie,2006)

Electrophoresis and Molecular Marker Data Analysis

For electrophoresis, the agar was made from 0.5g of 1.5% agarose and 25ml of TBE buffer, mix it together, and put it in a microwave for at least 2 minutes to make sure there's no coagulation. Stir the agar until it's warm enough, and add 5µm of dye stain to help the agar become clearer when scanning. The PCR products were loaded and run for 33~34 minutes with 100V and 400mA. ProTech Bio-500bp was used as a marker and 6X DNA gel loading dye was used to dye the DNA. MICROTEK Bio-100F scanner and Mibio Fluo software were used to visualized the band.

Result and Discussion

Bioassay

The phenotypic response of *F. oxysporum* f. sp. *lycopersici* is shown in table 3. Obviously show that "Bonny Best" were susceptible, on the other hand, MH-1 were the hardest one to be infected due to its resistance gene. From the table 3, we see that most of the isolates were race 2, there is race 1 but it's more hard to find (Fol-008), and we can found some of the isolates showing no symptoms of *Fusarium* wilt, where it didn't show any yellowing or wilting, when we do the stem cutting, there's no browning on their vascular system. From this result, we also found the occurrence of race 3, therefore we do another re-isolation on the APDA plates and water agar plates for confirmation in the future (figure 4).

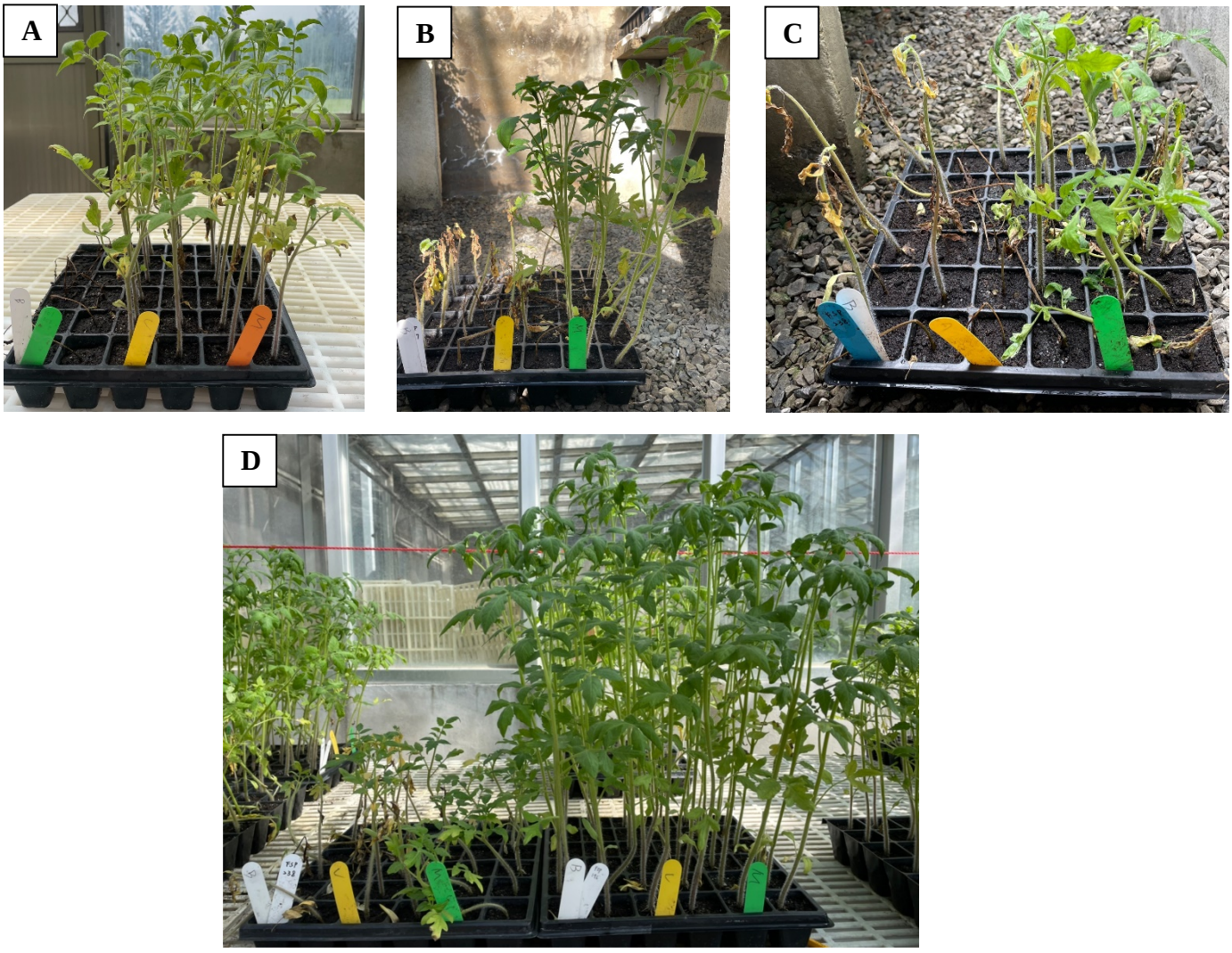


Figure 3. Result of bioassay, where we can see (**A**) is race 1 isolate, where Bonny Best showing severe symptoms of *Fusarium* wilt, meanwhile UC-82L and MH-1 showing no symptoms. (**B**) is race 2 isolate, where Bonny Best and UC-82L showing quite severe symptoms of *Fusarium* wilt, meanwhile MH-1 showing no symptoms. (**C**) is race 3 isolate, where Bonny Best, UC-82L, and MH-1 showing quite severe symptoms of *Fusarium* wilt, even though, for MH-1 not showing more serious wilting but from the stem-cutting can be seen the browning of vascular system. (**D**) is a figure comparing the length of tomato plants that got infected with race 3 isolates and isolates that showing no symptoms (healthy).

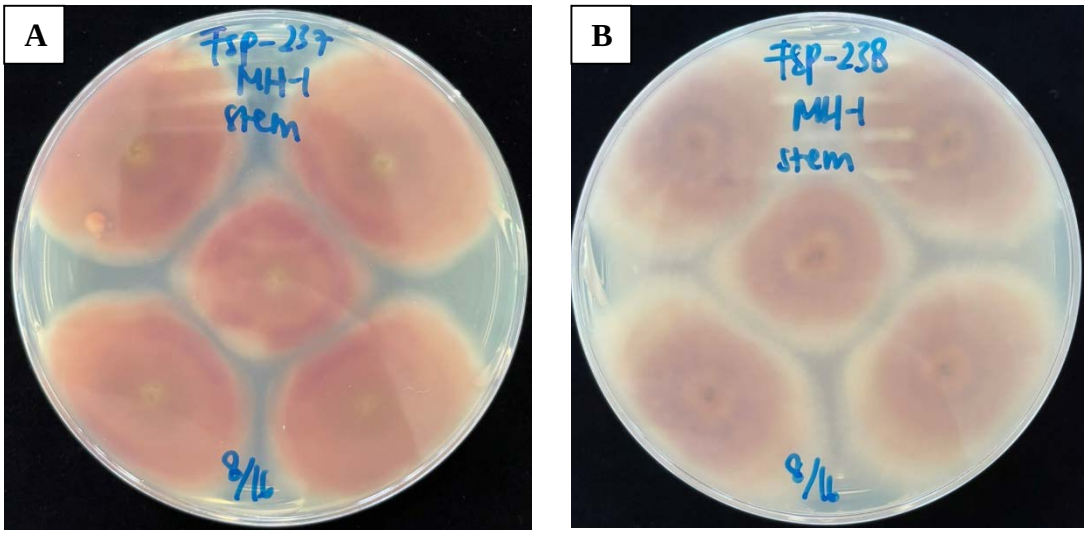


Figure 4. Result of race 3 re-isolation from the stem of MH-1 cultivar plants on APDA plates after 5 days of incubation at 28°C, showing mycelium growth on each stem pieces.

no.	Isolate no.	Plant Varieties			race 1/2/3 ?
		Bonny best	UC-82L	MH-1	
1	Fol-008	+	-	-	1
2	Fol-032	-	-	-	no sickness
3	Fol-179	-	-	-	no sickness
4	Fol-180	+	+	-	2
5	Fol-181	+	+	-	2
6	Fol-185	+	+	-	2
7	Fol-207	+	+	-	2
8	Fol-231	+	+	-	2
9	Fol-233	+	+	-	2
10	Fol-289	-	-	-	no sickness
11	Fol-291	-	-	-	no sickness
12	Fol-312	-	-	-	no sickness
13	Fol-315	-	-	-	no sickness
14	Fol-316	-	-	-	no sickness
15	Fol-322	+	+	-	2
16	Fol-325	-	-	-	no sickness
17	Fol-326	-	-	-	no sickness
18	Fol-327	-	-	-	no sickness
19	Fol-328	+	+	-	2
20	Fol-329	+	+	-	2
21	Fol-330	+	+	-	2
22	Fol-331	+	+	-	2
23	Fol-334	+	+	-	2
24	Fol-335	+	+	-	2
25	Fol-337-1	-	-	-	no sickness
26	Fol-338-1	-	-	-	no sickness
27	Fol-339-1	-	-	-	no sickness
28	Fol-340-1	-	-	-	no sickness
29	F.sp-172	-	-	-	no sickness
30	F.sp-191	-	-	-	no sickness
31	F.sp-196	-	-	-	no sickness
32	F.sp-199	-	-	-	no sickness
33	F.sp-213	-	-	-	no sickness
34	F.sp-214	-	-	-	no sickness
35	F.sp-224	+	+	-	2
36	F.sp-225	+	+	-	2
37	F.sp-227	+	+	-	2
38	F.sp-237	+	+	+	3
39	F.sp-238	+	+	+	3
40	F.sp-249	-	-	-	no sickness
41	FOL 34-1	+	+	-	2

Table 3. The result of bioassay; plus sign showing appearance of Fusarium wilt, meanwhile minus sign showing no symptoms of Fusarium wilting.

Optimization of PCR primers

Optimization were required for uni, sp13, and sp23 primers, since from the previous experiments that have been done in Japan (Hirano and Arie, 2006), we found that it is not very suitable for the isolates we found. We examined the annealing temperature, from 55°C~65°C for the three primer groups (uni, sp13, and sp23), with increments of 5°C. We set, 94°C for 5 minutes and 1 minute for the DNA to denature, and we found the most suitable annealing temperature for uni and sp13 primers, which at 60°C for 1 minute, but unfortunately for sp23 there's none of the band that can be seen. Therefore, we do another test, where we set the temperature lower than the first examination, 45°C~55°C, later we found that at 55°C we can see some bands showing, but not in every isolate where it should be seen.

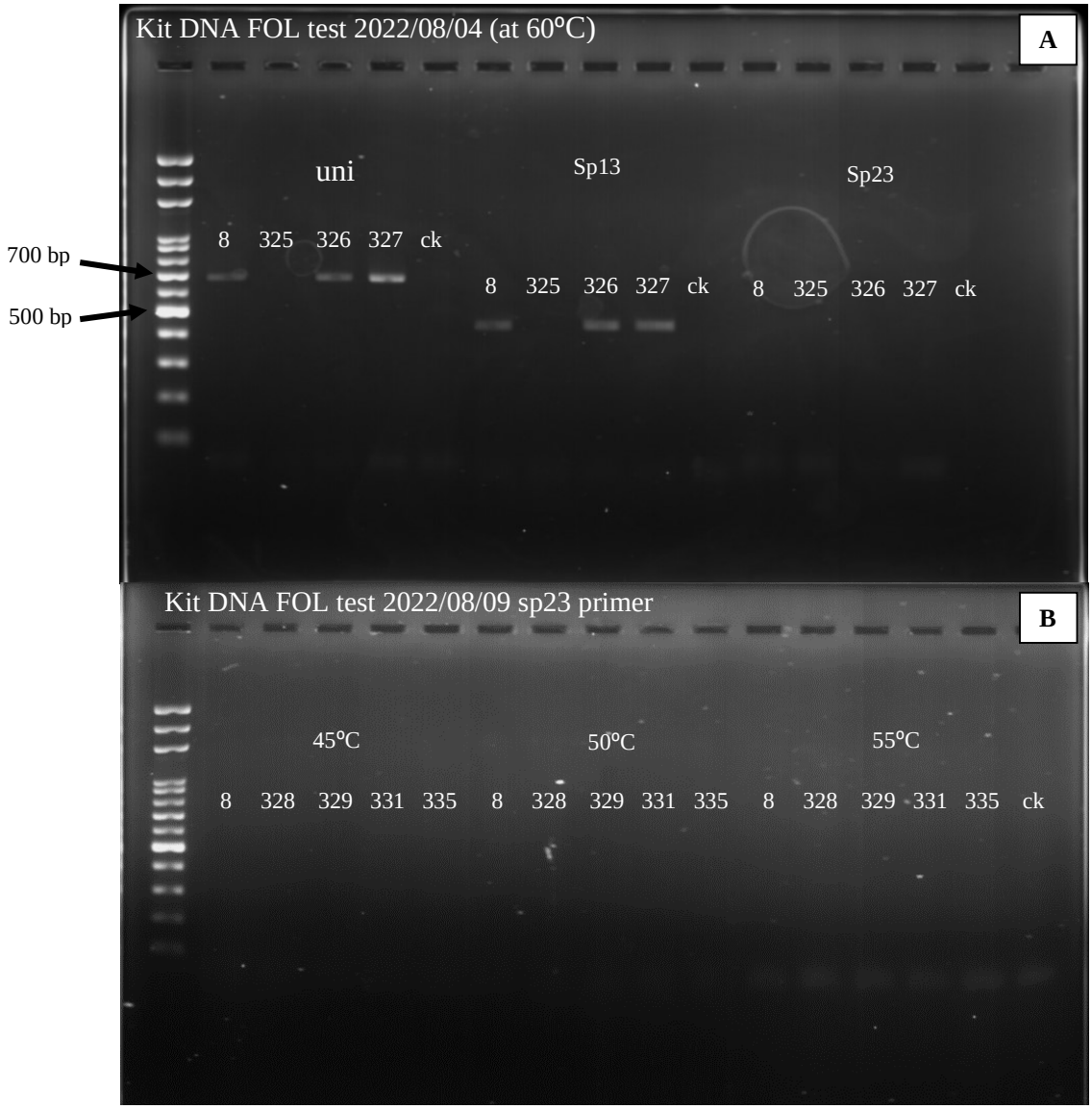


Figure 5. The result of PCR primer by adjusting their annealing temperature, for (A) showing the uni and sp13 pirmers at 60°C showing the molecular marker near 700bp and near 500bp respectively, where for uni the amplified region will be at 672bp, and for sp13 the amplified region will be at 445bp, unfortunately, sp23 showing

none bands can be seen from these FOL isolates. For (B) showing adjustment for sp23 at 45ºC, 50ºC, and 55ºC, and still none of the bands can be seen on the agarose gel.

Phenotyping

We can see from the result of ITS4 and ITS5 primers all are showing plus sign, which means that the ITS region of their DNA was successfully amplified. FOF1 and FOR1 primers were used to see the *F. oxysporum* specific isolates, and from the table 4, it shows the result based on the molecular marker that showed on the agarose gel (data not shown). Some isolates' molecular markers were not showing on the agarose gel, which means that they are not *F. oxysporum*, thus we can do the BLAST analysis of the ITS rDNA sequence data in the NCBI GenBank database, whereby the closest match between 98-100% similarity (Fol-032, Fol-291, Fol-325, Fol-337-1, Fol-338-1, Fol-339-1, Fol-340-1, and F.sp-196). From the NCBI BLAST result, we found some of the isolates that we have collected not F. oxysporum species, we have found another Fusarium species, such as *F. solani, F. proliferatum, F. acutatum, and F. croci* (table 6). As for the remaining isolates, we have found 31 isolates that are *F. oxysporum* specific. Comparing with the bioassay result and the FOF1/FOR1 primer result, we can see some of the "no symptoms" isolates are *F. oxysporum* species but unfortunately, they are avirulent isolates.

To determine the physiological races of FOL races, we are using the uni, sp13, and sp23 primers. Based on the previous study in Japan, the pattern of the result to determine the races is show in figure 2, and this will be our putative race result. If we compare from the bioassay result with the putative result we can see that these molecular markers are not suitable for the Taiwan's population, because from the table, only two isolates that are consistent with the putative result. This result might happen due to the location of the isolates; therefore, it is cannot be a reliable marker for the Taiwan's population (table 5).

no.	Isolate no.	Host plant	Collected from		ITS4/5	FOF1/FOR1	uni	sp13	sp 23	Plant Varieties			race 1/2/3 ?
			County	Area						Bonny best	UC-82L	MH-1	
1	Fol-008	Tomato	Nantou	Hsinyi	+	+	+	+	-	+	-	-	1
2	Fol-032	Tomato	Tainan	Shanhua (AVRDC)	+	-	-	-	-	-	-	-	not FOL
3	Fol-179	Tomato	Ilan	Yuanshan	+	+	+	+	-	-	-	-	AVR.
4	Fol-180	Tomato	Ilan		+	+	+	+	-	+	+	-	2
5	Fol-181	Tomato	Nantou	Renai	+	+	+	+	-	+	+	-	2
6	Fol-185	Tomato	Nantou	Hsinyi	+	+	+	+	-	+	+	-	2
7	Fol-207	Tomato	Kaohsiung	Meinung	+	+	+	+	-	+	+	-	2
8	Fol-231	Tomato	Nantou	Hsinyi	+	+	+	-	+	+	+	-	2
9	Fol-233	Tomato	Nantou	Hsinyi	+	+	+	-	+	+	+	-	2
10	Fol-289	Tomato	Chiayi	Shuishang	+	+	+	-	-	-	-	-	AVR.
11	Fol-291	Tomato	Tainan	Paiho	+	-	-	-	-	-	-	-	not FOL
12	Fol-312	Tomato	Yunlin	Siluo	+	+	+	+	-	-	-	-	AVR.
13	Fol-315	Tomato	Nantou	Hsinyi	+	+	+	+	-	-	-	-	AVR.
14	Fol-316	Tomato	Hualien	Sibao	+	+	+	+	-	-	-	-	AVR.
15	Fol-322	Tomato	Yunlin	Siluo	+	+	+	+	-	+	+	-	2
16	Fol-325	Tomato	Tainan	Shanhua (AVRDC)/Entomo./OpenII	+	-	-	-	-	-	-	-	not FOL
17	Fol-326	Tomato	Tainan	Shanhua	+	+	+	+	-	-	-	-	AVR.
18	Fol-327	Tomato	Tainan	Shanhua	+	+	+	+	-	-	-	-	AVR.
19	Fol-328	Tomato	Tainan	Guantian	+	+	+	+	-	+	+	-	2
20	Fol-329	Tomato	Tainan	Guantian	+	+	+	+	-	+	+	-	2
21	Fol-330	Tomato	Yunlin	Yuanchang	+	+	+	+	-	+	+	-	2
22	Fol-331	Tomato	Yunlin	Yuanchang	+	+	+	+	-	+	+	-	2
23	Fol-334	Tomato	Tainan	Yanshuei	+	+	+	+	-	+	+	-	2
24	Fol-335	Tomato	Tainan	Yanshuei	+	+	+	+	-	+	+	-	2
25	Fol-337-1	Tomato	Yunlin	Yuanchang A	+	-	-	-	-	-	-	-	not FOL
26	Fol-338-1	Tomato	Yunlin	Yuanchang A	+	-	-	-	-	-	-	-	not FOL
27	Fol-339-1	Tomato	Yunlin	Yuanchang A	+	-	-	-	-	-	-	-	not FOL
28	Fol-340-1	Tomato	Yunlin	Yuanchang A	+	-	-	-	-	-	-	-	not FOL
29	F.sp-172	Tomato	Miaoli	Nanjhuang	+	+	-	+	-	-	-	-	AVR.
30	F.sp-191	Tomato	Miaoli	Tai-an	+	+	-	+	-	-	-	-	AVR.
31	F.sp-196	Tomato	Hsinchu	Jianshih	+	-	-	-	-	-	-	-	not FOL
32	F.sp-199	Tomato	Miaoli	Dahu	+	+	+	+	-	-	-	-	AVR.
33	F.sp-213	Tomato	Yunlin	Linnei	+	+	+	+	-	-	-	-	AVR.
34	F.sp-214	Tomato	Changhua	Ershuei	+	+	+	+	-	-	-	-	AVR.
35	F.sp-224	Tomato	Yilan	Jiaosi	+	+	+	+	-	+	+	-	2
36	F.sp-225	Tomato	Yilan	Jhuangwei	+	+	+	+	-	+	+	-	2
37	F.sp-227	Tomato	Yilan	Jhuangwei	+	+	+	+	-	+	+	-	2
38	F.sp-237	Tomato	Yunlin	Siluo	+	+	+	-	-	+	+	+	3
39	F.sp-238	Tomato	Yunlin	Siluo	+	+	+	-	+	+	+	+	3
40	F.sp-249	Tomato	Miaoli	Jhuolan	+	+	+	+	-	-	-	-	AVR.
41	FOL-34-1	Tomato			+	+	+	+	-	+	+	-	2

Table 4. The genotyping result by using five groups of primers, ITS4/5, FOF1/FOR1, uni, sp13, and sp23 primers comparing with the bioassay result from Bonny Best, UC-82L, and MH-1 cultivars. Plus sign means there are band showing on the agarose gel and showing symptoms of Fusarium wilt, meanwhile minus sign means there are no band showing on the agarose gel and no symptoms of Fusarium wilt shown in the bioassay.

no.	Isolate no.	uni	sp13	sp 23	Putative Race	Bioassay Result	consistency
1	Fol-180	+	+	-	1	2	X
2	Fol-181	+	+	-	1	2	X
3	Fol-185	+	+	-	1	2	X
4	Fol-207	+	+	-	1	2	X
5	Fol-231	+	-	+	2	2	V
6	Fol-233	+	-	+	2	2	V
7	Fol-322	+	+	-	1	2	X
8	Fol-328	+	+	-	1	2	X
9	Fol-329	+	+	-	1	2	X
10	Fol-330	+	+	-	1	2	X
11	Fol-331	+	+	-	1	2	X
12	Fol-334	+	+	-	1	2	X
13	Fol-335	+	+	-	1	2	X
14	F.sp-224	+	+	-	1	2	X
15	F.sp-225	+	+	-	1	2	X
16	F.sp-227	+	+	-	1	2	X
17	F.sp-237	+	-	-	?	3	X
18	F.sp-238	+	-	+	2	3	X

Table 5. Showing the result of virulent FOL isolates from the PCR result with uni, sp13 and sp23 primers and comparing the putative race with our bioassay result, where we found only two isolates (Fol-231 and Fol-233) that are consistent with the putative race. Plus sign means there are band showing on the agarose gel, meanwhile minus sign means there are no band showing on the agarose gel.

Isolates	Species	Percent Iden.
Fol-32	*Fusarium solani*	100%
fol-196	*Fusarium solani*	100%
fol 291	*Fusarium proliferatum*	100%
fol 325	*Fusarium acutatum* (*F.fujikuroi* species complex)	99.53%
fol 337-1	*Fusarium croci* (*F. solani* species complex)	98.96%
fol 338-1	*Fusarium croci* (*F . solani* species complex)	98.96%
fol 339-1	*Fusarium croci* (*F . solani* species complex)	98.94%
fol 340-1	*Fusarium croci* (*F . solani* species complex)	98.96%

Table 6. Showing the result of other Fusarium species that we have collected by looking their DNA sequence in the NCBI BLAST GenBank, with 98~100% similarity.

Conclusion and Future Prospective

To conclude, from the genotyping result showed that race 2 FOL isolates are still predominant in Taiwan, with 16 isolates that are race 2 out of the 39 isolates, not including the control groups (FOL-008 and FOL 34-1). From the bioassay result, we have found the appearance of race 3

isolates in Taiwan (FOL 337 and FOL 338), but both isolates still need confirmation for the future study. As for the phenotyping result, unfortunately, we cannot use uni, sp13, and sp23 primers that were recommended from the previous study in Japan (Hirano and Arie, 2006), because of the incompatibility between the putative race and the bioassay result that we had collect. Therefore, in the future, we hope, we can identify or develop another useful marker that can help for the determination of FOL race that compatible with Taiwan's population. We also hope, the founding of race 3 in this study can be researched more in the future.

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