



北部地區辣椒炭疽病病原鑑別及 抗病種原篩選

作物改良課
曾盟群





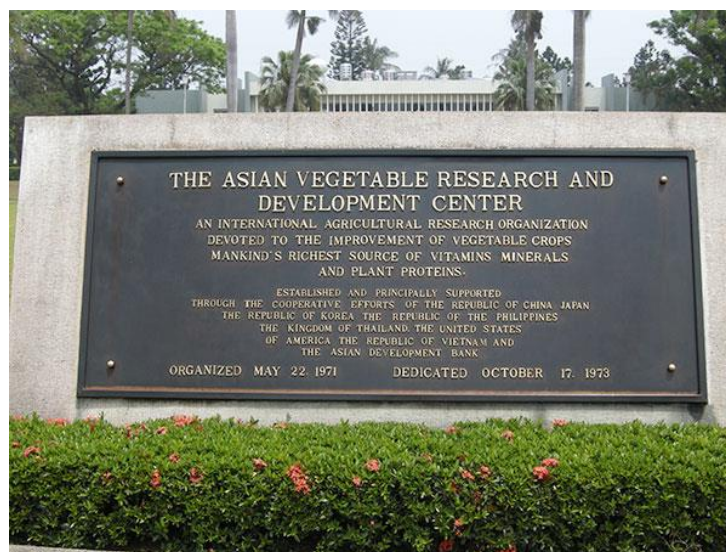
計畫目的

- 農委會科技計畫「強化亞蔬中心與臺灣研究機構蔬菜科技合作研發」
- 為強化本會所屬試驗研究機關研究人員與亞蔬中心之合作研發，並培養國內農業人員國際化視野及英語能力，前業函請貴機關派員赴亞蔬中心進行3個月之合作研究。



亞蔬-世界蔬菜中心

- 總部位於台灣臺南市善化區，於1971年5月22日設立，前身為亞洲蔬菜研究發展中心（the Asian Vegetable Research and Development Center），由中華民國、美國、日本、韓國、泰國、菲律賓、越南等七國政府和亞洲開發銀行共同簽約成立





Introduction - chili anthracnose

From Greek *anthrax* coal, carbuncle + *nosos* disease





Anthracnose - origin

- Causing agent: *Colletotrichum* spp.
- Wide host range ~3,200 species of monocot and dicot plants





Strategies to control chili anthracnose

Chemical control

- Fungicide application

Cultural practices

- Lower planting density
- Reduce nitrogen input (farmer recommendation)

Breeding resistant cultivars

- Understanding the pathogen
- Host plant
- Interaction
- Selection (phenotyping)





Identification of *Colletotrichum* spp.

Morphological

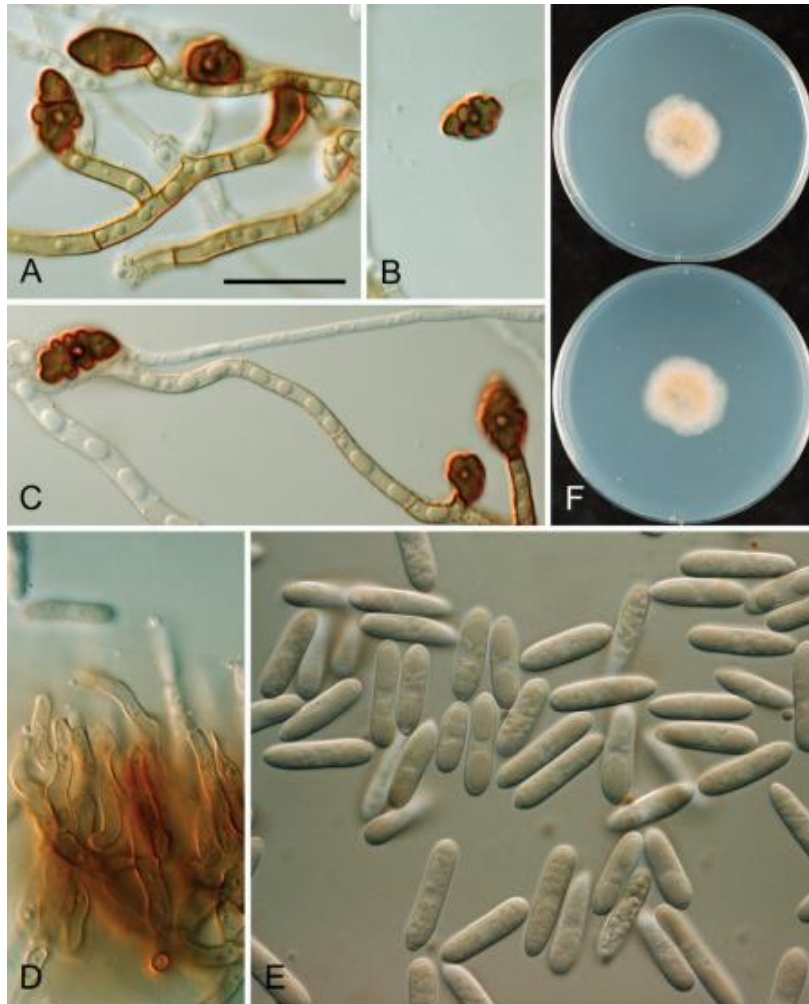
- Conidia shape
- Colony color
- Colony growth
- Appressoria

Molecular

- ITS sequence
- Multi-loci analyses:
 - Actin (ACT)
 - Calmodulin (CAL)
 - Chitin synthase I (CHS-1)
 - DNA lyase (APN2)
 - manganese superoxide dismutase (SOD2)

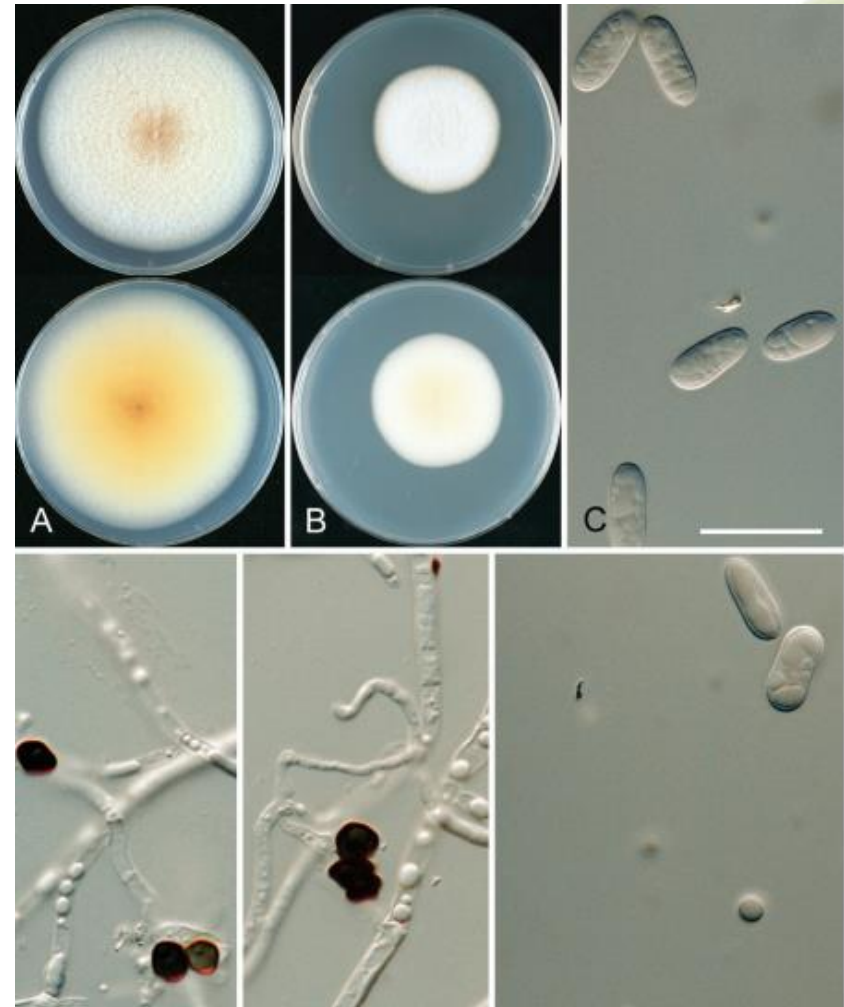


Identification by morphology- example



Colletotrichum aeschynomenes

8



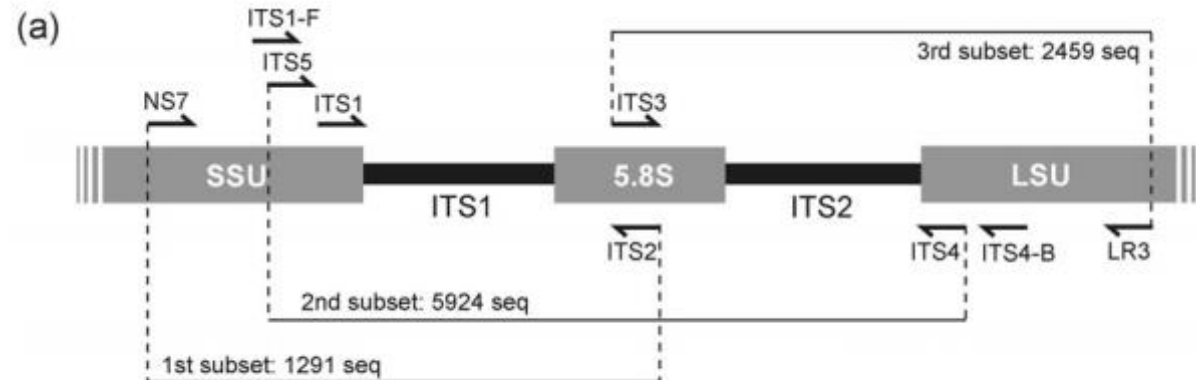
Colletotrichum aenigma.

Weir et al., 2012



ITS – Nuclear ribosomal internal transcribed spacer

- Widely used for fungus diversity research.
- Can be amplified with single PCR.
- All primers sequence are available.



(b)

Primer	Author	Primer sequence	Position
<i>Forward primers</i>			
NS7	[19]	GAGGCAATAACAGGTCTGTGATGC	1403-1426
ITS1-F	[18]	CTTGTCATTTAGAGGAAGTAA	1723-1744
ITS5	[19]	GGAAGTAAAAGTCGTAACAAGG	1737-1758
ITS1	[19]	TCCGTAGGTGAACCTGCGG	1761-1779
ITS3	[19]	GCATCGATGAAGAACGCAGC	2024-2045
<i>Reverse primers</i>			
ITS2	[19]	GCTGCGTTCATCGATGC	2024-2043
ITS4	[19]	TCCTCCGCTTATTGATATGC	2390-2409
ITS4-B	[18]	CAGGAGACTTGTACACGGTCCAG	2526-2548
LR3	[13]	CCGTGTTTCAAGACGGG	3029-3045



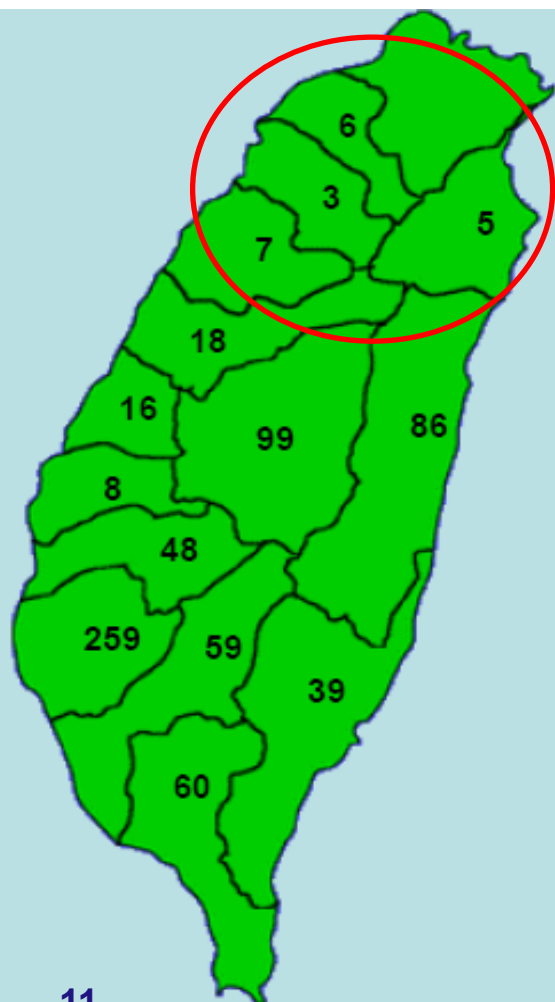
Colletotrichum species associated with pepper anthracnose in Taiwan

Table 3. Comparisons of putative *Colletotrichum* species associated with pepper anthracnose.

Characteristics	<i>C. acutatum</i>	<i>C. boninense</i>	<i>C. truncatum</i>	<i>C. gloeosporioides</i>
Mycelium color	White to gray	White	White to gray	White to dark gray
Reverse colony color	Orange-pink or dark olive	Cream, yellow, or orange	Dark brown	Black, gray, white or pink
Presence of acervuli	Nil	Sometimes present	Often present	Sometimes present
Conidia shape	Fusiform or cylindrical, most with acute end	Cylindrical with obtuse end, often with a hilum-like conidial base	Falcate with acute apex	Cylindrical with obtuse end
Conidia size(μm)	15.1(12.8–16.9) × 4.8(4.0–5.7)	14.9(14.2–16.0) × 6.5(6.0–7.1)	23.9(21.8–28.4) × 4.8(4.4–5.4)	15.6(14.9–16.8) × 4.7(4.3–4.9)
L/B ratio	3.1	2.3	5	3.3
Appressorium	round to ovate	ovate, sometimes with shallow lobe	Nt.	ovate to irregular in shape with lobes
Growth rate at 28°C (mm/day)	5.3 (4.0–6.0)	9.0 (8.0–11.0)	7.3 (5.8–8.6)	13.3 (10.4–15.0)
Protease activity	Most with strong	None	None	None, with one exception
ITS1 length	181 bp	190 bp	177 bp	171 bp
Specific primers	CaINT2/ITS4	Cg2f/ITS4	CcINT2/ITS4	CgINT/ITS4
Remark		Designated as <i>Cg</i> type 2 before		



Colletotrichum spp. associated with pepper anthracnose in Taiwan



Colletotrichum isolates collected during 1992-2009 from Taiwan

Species	No.
<i>C. acutatum</i> (Ca)	468
<i>C. boninese</i> (Cb)	34
<i>C. capsici</i> (Cc) = <i>C. truncatum</i>	90
<i>C. gloeosporioides</i> (Cg)	115
<i>Colletotrichum</i> sp.	6
Total	713

C. gloeosporioides species complex

- Diversified: 22 species plus one subspecies.
- First identified on *Citrus*, with other species morphologically similar fungi from other hosts.
- One of the causal agents of chili anthracnose in Taiwan



C. acutatum

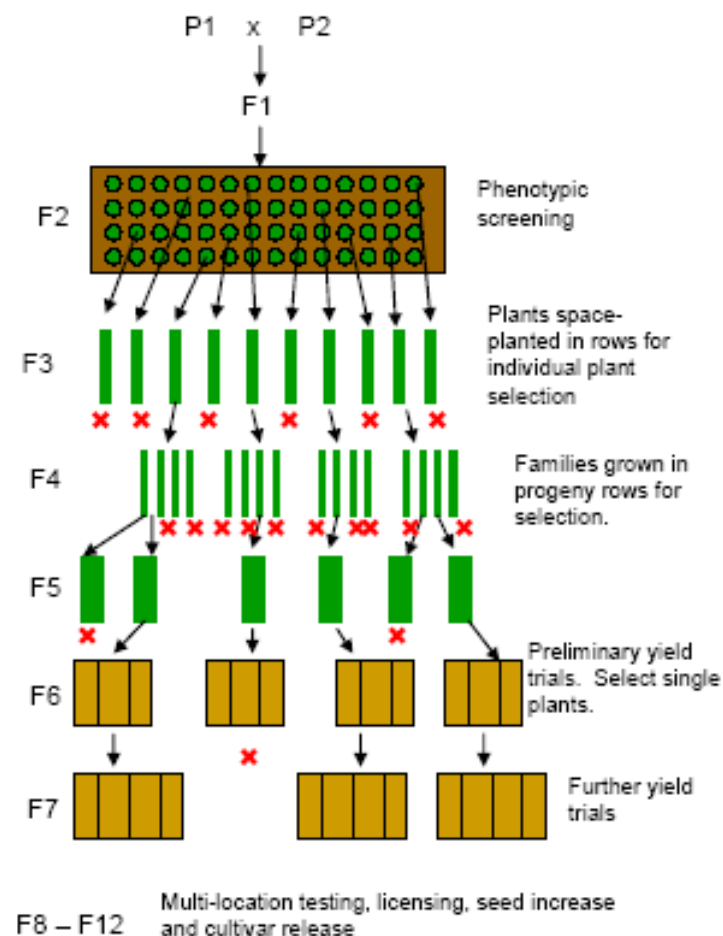


C. gloeosporioides

Breeding for resistance – General

- Resistant source: genetic material
- Cross/Hybridization
- Selection
 - Resistant progeny
 - Other favorable traits

PEDIGREE METHOD



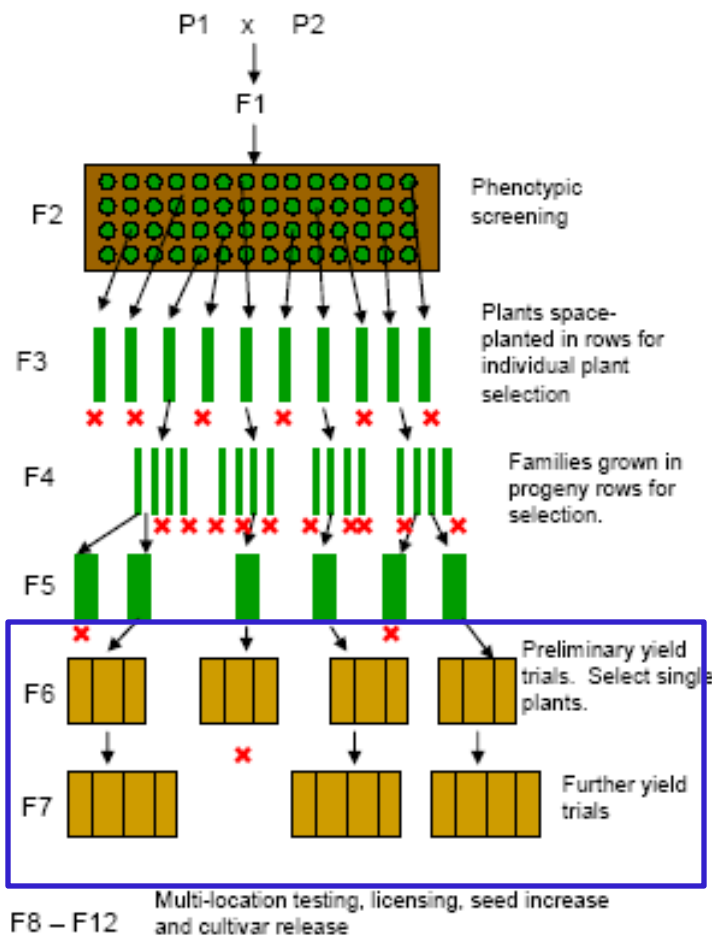


Methods for screening for chili anthracnose resistance

- Field Screening



PEDIGREE METHOD





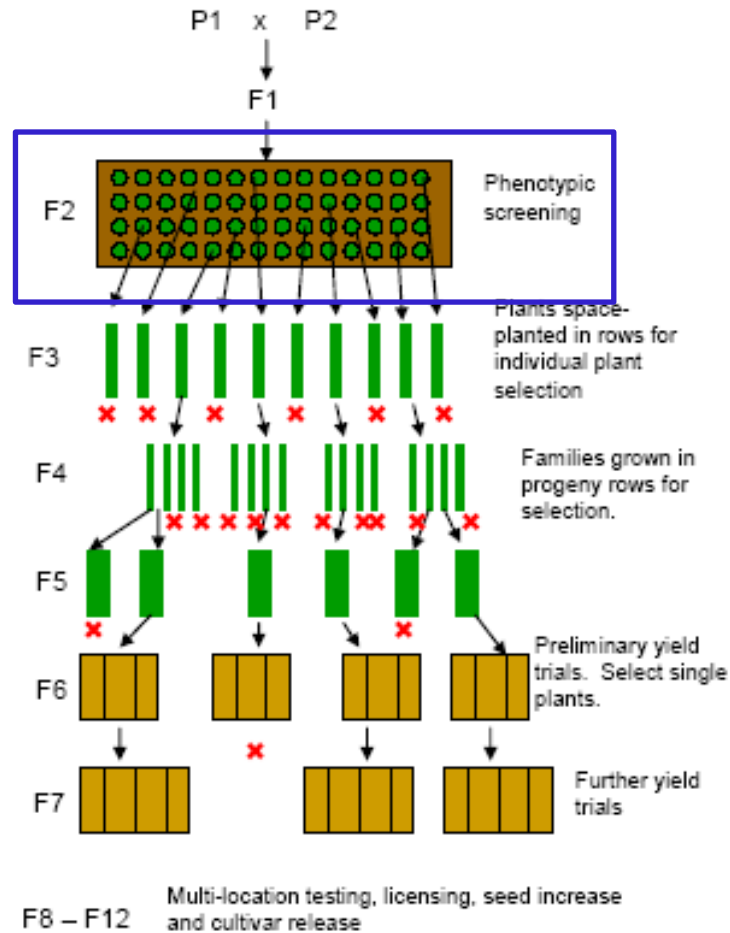
Artificial inoculation methods developed by AVRDC

Microinjection

Spray



PEDIGREE METHOD

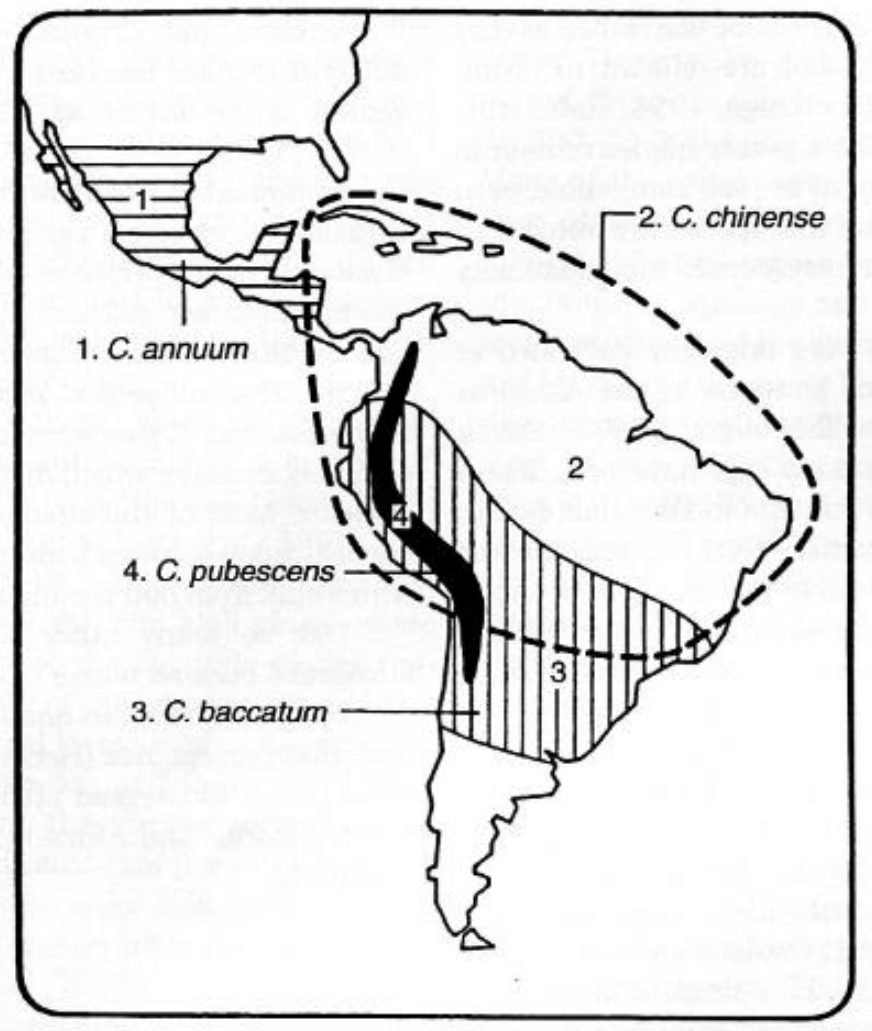




The chili germplasm

- **5 cultivated species**

- *Capsicum annuum*
- *Capsicum chinense*
- *Capsicum frutescens*
- *Capsicum baccatum* var. *pendulum*
- *Capsicum pubescens*





Chili anthracnose resistance breeding at AVRDC

- Primary resistant source: PBC932 (*C. chinense*)
- Resistance against *C. acutatum* has been successfully introgressed into *C. annuum* breeding program.



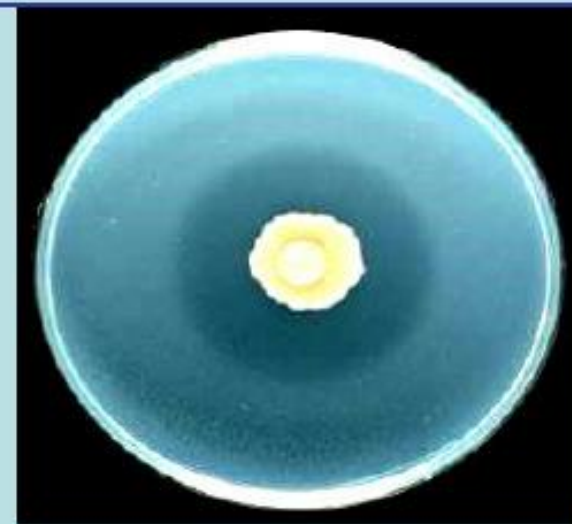


Anthracnose observed on breeding lines derived from PBC932 at AVRDC in 2005

Field symptom



C. acutatum was identified



M a b c d e f g h i j k l m





Justification

- Breeding for anthracnose resistance is a long-term goal which requires further understanding of several aspects.
- There are less isolates and limited information of *Colletotrichum* species in Northern Taiwan.
- Although the most predominant *Colletotrichum* species in Taiwan is *C. acutatum*, chili anthracnose may also be caused by several *Colletotrichum* species, screening for multi-resistance is necessary.



Objectives

- I. Identify the *Colletotrichum* isolates from **Northern Taiwan** using morphological and molecular characterization.

- II. Identify resistant sources against *C. gloeosporioides* on 20 chili pepper genotypes.



I. Identification of *Colletotrichum* isolates associated with chili anthracnose from Northern Taiwan

- To identify the causal agents of anthracnose in Northern Taiwan
- Field collection: April to July 2015
- Chili or sweet pepper fields





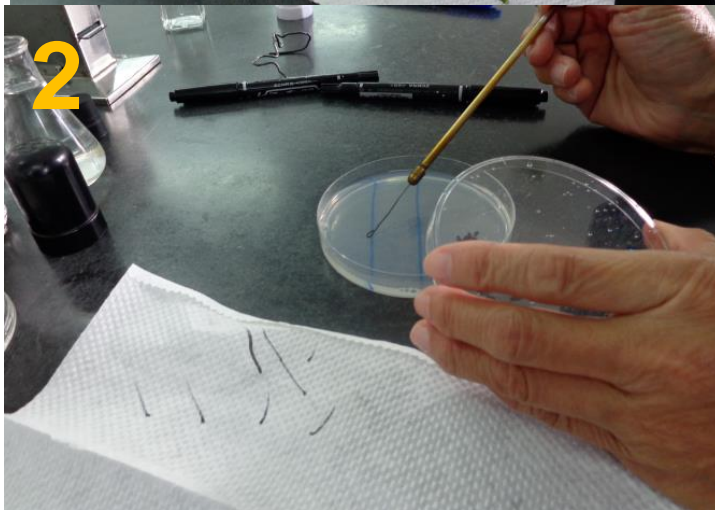
Locations of isolate collections



- | |
|-------------|
| 1. Tucheng |
| 2. Shulin |
| 3. Pingjhen |
| 4. Hukou |
| 5. Cyonglin |
| 6. Wufong |



Isolation of *Colletotrichum* spp. from chili fruits





Isolates collected from anthracnose infected fruits

Isolate no.	Genus	Date collected	Host plant	Sampled from	County	Location
Coll-1316	<i>Colletotrichum</i>	2015. 04. 22	Sweet pepper	Mature Fruit	Hsinchu	Cyonglin
Coll-1317	<i>Colletotrichum</i>	2015. 04. 22	Sweet pepper	Mature Fruit	Hsinchu	Cyonglin
Coll-1318	<i>Colletotrichum</i>	2015. 04. 22	Sweet pepper	Mature Fruit	Hsinchu	Cyonglin
Coll-1320	<i>Colletotrichum</i>	2015. 06. 17	Chili pepper	Mature Fruit	Taoyuan	Pingjhen
Coll-1321	<i>Colletotrichum</i>	2015. 06. 17	Chili pepper	Green Fruit	Taoyuan	Pingjhen
Coll-1322	<i>Colletotrichum</i>	2015. 05. 22	Chili pepper	Fruit	Taipei	Tucheng
Coll-1324	<i>Colletotrichum</i>	2015 .07 .08	Sweet pepper	Green Fruit	Hsinchu	Wufong
Coll-1325	<i>Colletotrichum</i>	2015 .07 .09	Chili pepper	Mature Fruit	Hsinchu	Hukou
Coll-1326	<i>Colletotrichum</i>	2015 .07. 21	Chili pepper	Mature Fruit	Taipei	Shulin
Coll-1327	<i>Colletotrichum</i>	2015 .07. 21	Chili pepper	Mature Fruit	Taipei	Shulin



Morphological and molecular characterization

- **10 uncharacterized isolates from Northern Taiwan**
- **5 reference isolates previously characterized by AVRDC**
 - Coll-153 (*C. acutatum*)
 - Coll-302 (*C. bonienense*)
 - Coll-524 (*C. acutatum*)
 - Coll-491 (*C. gloeosporioides*)
 - Coll-930 (*C. truncatum*)



Morphological Characterization

- **Observations**
 - Growth rate
 - Protease activity
 - Conidia shape
 - Colony morphology





Morphological characterization

Growth rate (mm/day)



A 4.5 mm² colony was transferred to a PDA plate.

28°C 12H light/Dark photoperiod.

3 replications

The colony size was measured 6 days after subculture.

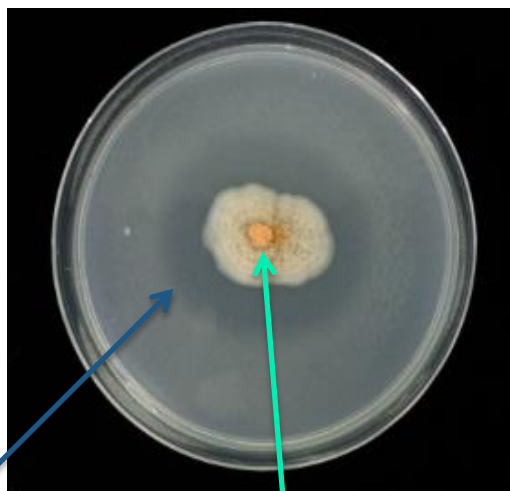


Morphological characterization

Protease activity



Clear Zone

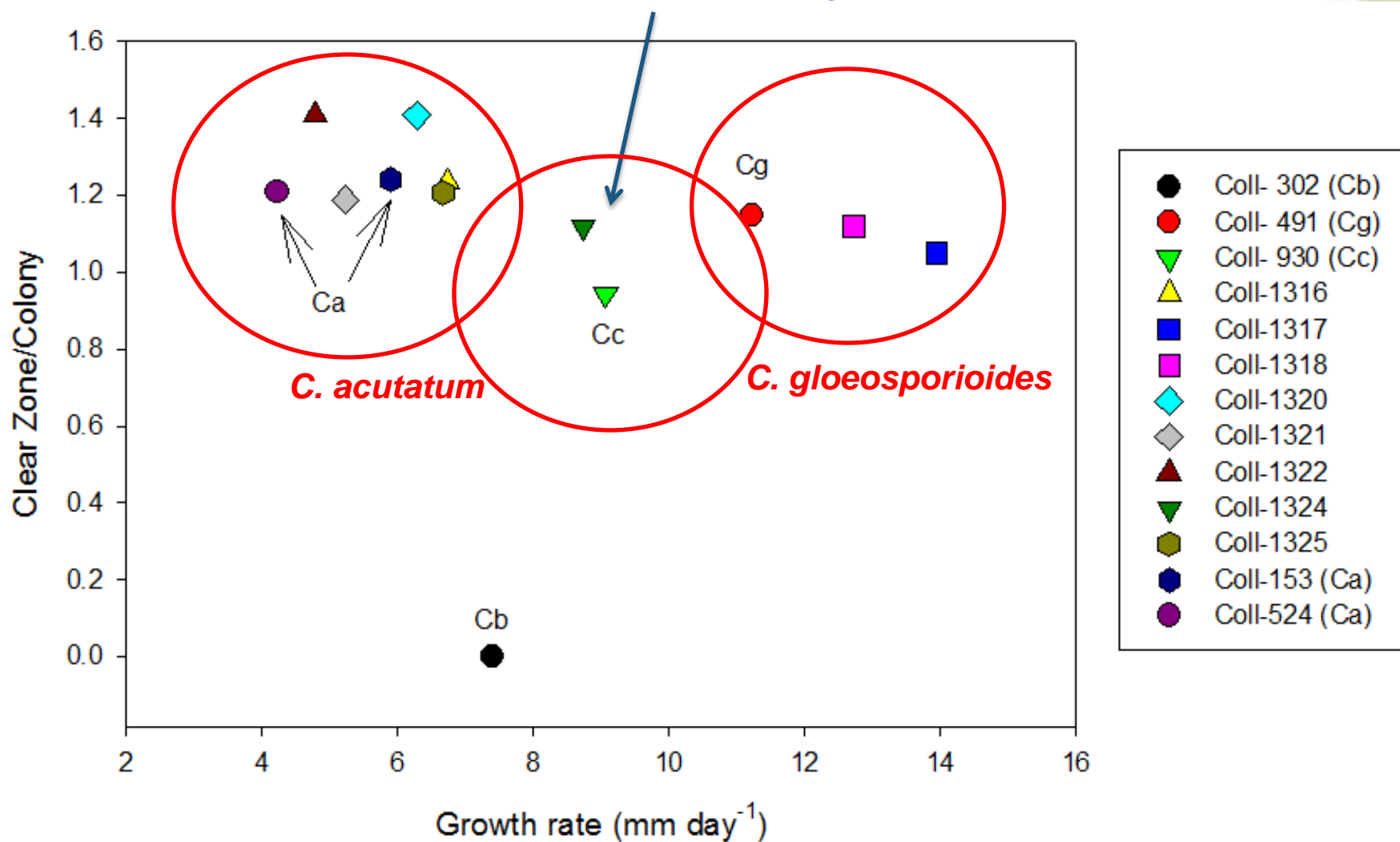


Colony

- Culture on CHM medium for 3 days with 28°C 12H light/Dark photoperiod.
- The ability of digesting Casein
- Determined by the diameter of clear zone divided by diameter of colony.
- *C. acutatum* has the strongest protease activity



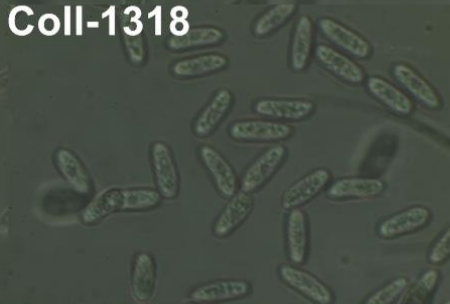
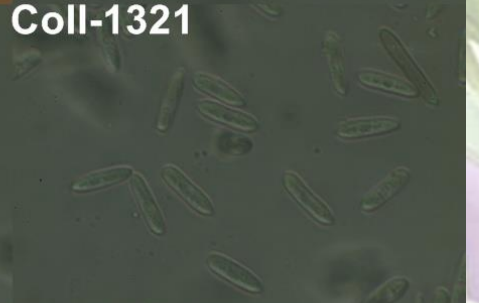
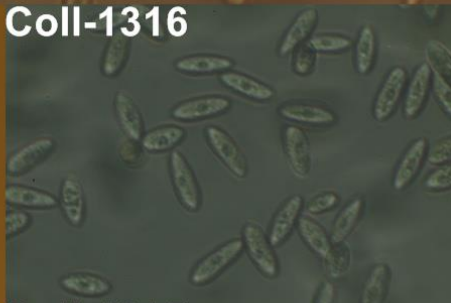
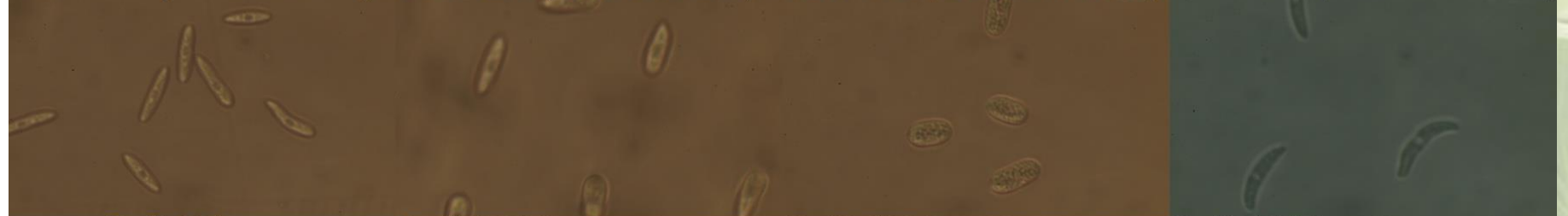
C. acutatum, *C. truncatum* or *C. gloeosporioides*?





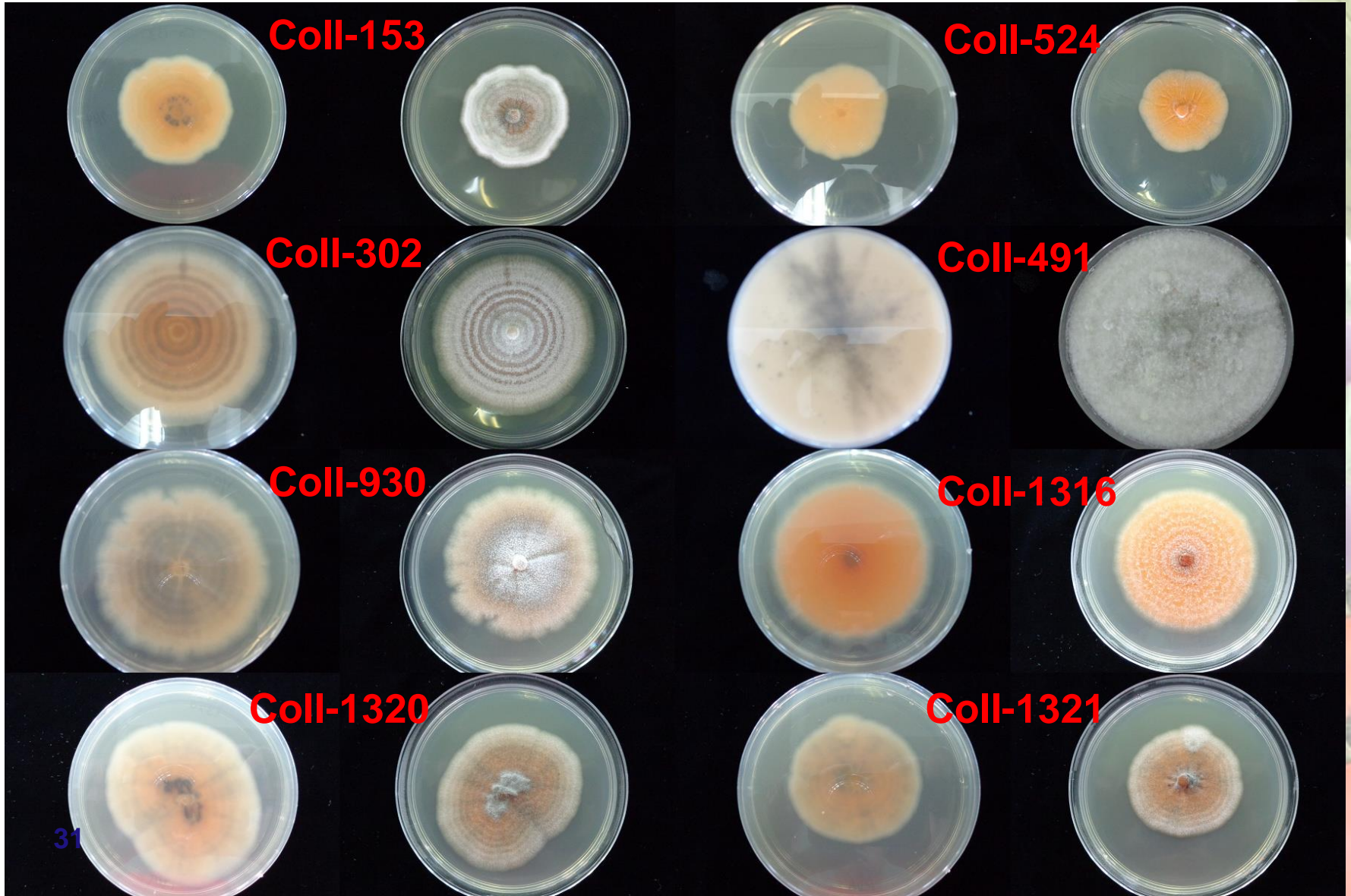
Conidia shape of isolates

Coll-153 (*C.acutatum*) Coll-524 (*C.acutatum*) Coll-302 (*C.boninense*) Coll-930 (*C. truncatum*)





Colony morphology of isolates



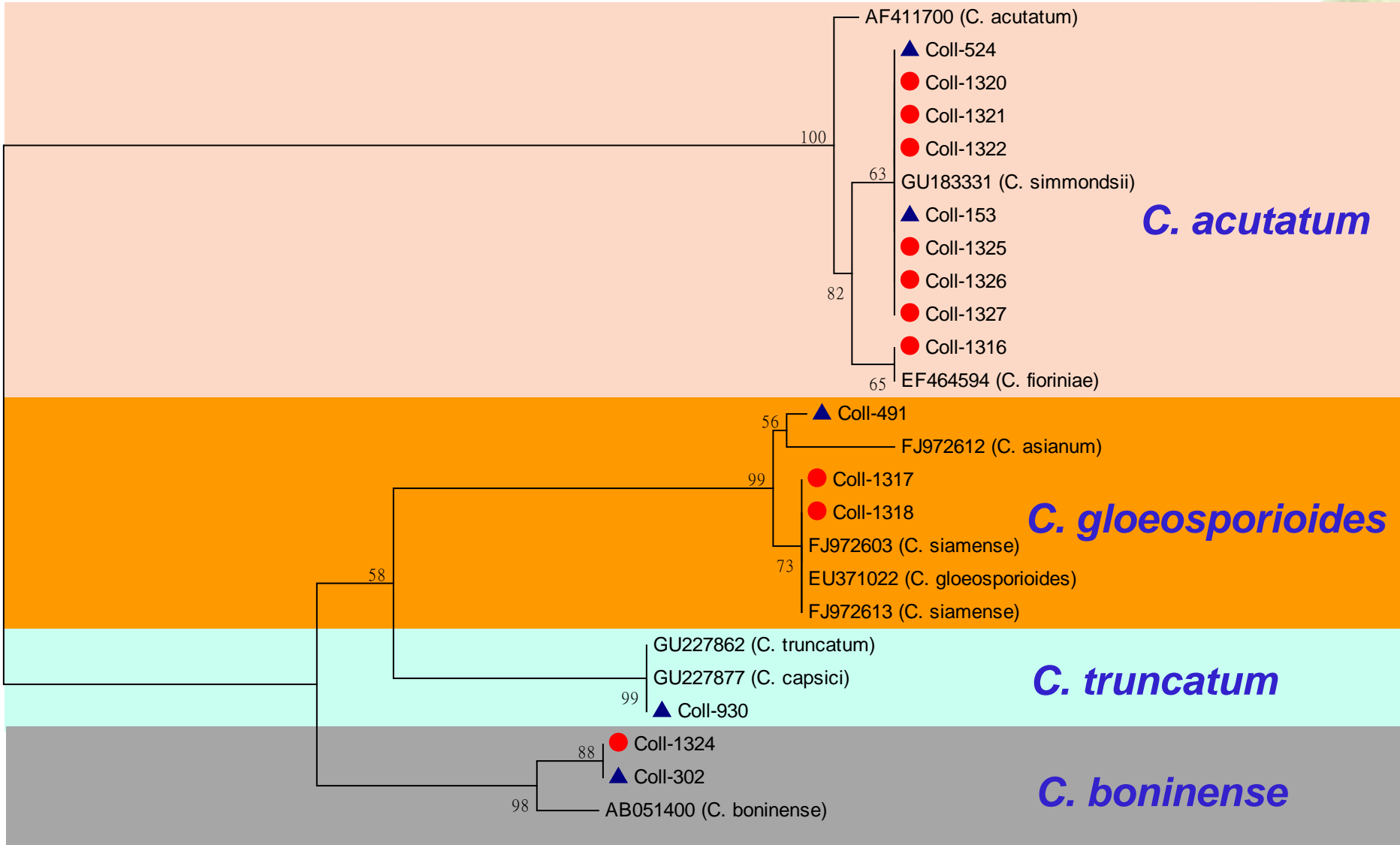


Molecular characterization – ITS sequence

- The region between ITS4 to ITS5 was sequenced.
- The phylogenetic tree was constructed by MEGA software using neighbor-joining method with 1000 bootstrap test.
- 10 reference sequences for *Colletotrichum* spp. (NCBI GeneBank) were included as references for species identification.



Molecular characterization - phylogenetic analysis



0.01



Results of morphological and molecular identification

<u>Isolate no.</u>	<u>Genus</u>	<u>Species morphology</u>	<u>ITS sequence</u>
Coll-1316	<i>Colletotrichum</i>	<i>acutatum</i>	<i>acutatum (fioriniae)</i>
Coll-1317	<i>Colletotrichum</i>	<i>gloeosporioides</i>	<i>gloeosporioides</i>
Coll-1318	<i>Colletotrichum</i>	<i>gloeosporioides</i>	<i>gloeosporioides</i>
Coll-1320	<i>Colletotrichum</i>	<i>acutatum</i>	<i>acutatum (simmondsii)</i>
Coll-1321	<i>Colletotrichum</i>	<i>acutatum</i>	<i>acutatum (simmondsii)</i>
Coll-1322	<i>Colletotrichum</i>	<i>acutatum</i>	<i>acutatum (simmondsii)</i>
Coll-1324	<i>Colletotrichum</i>	<i>gloeosporioides</i>	<i>boninense</i>
Coll-1325	<i>Colletotrichum</i>	<i>acutatum</i>	<i>acutatum (simmondsii)</i>
Coll-1326	<i>Colletotrichum</i>	<i>acutatum</i>	<i>acutatum (simmondsii)</i>
Coll-1327	<i>Colletotrichum</i>	<i>acutatum</i>	<i>acutatum (simmondsii)</i>



Summary of *Colletotrichum* identification

- 1 *C. boninense*, 2 *C. gloeosporioides*, 7 *C. acutatum* were identified.
- 7/10 isolates were identified as *C. acutatum* which accords with previous survey result by AVRDC.
- Coll-1324 is a *C. boninense* complex with protease activity.

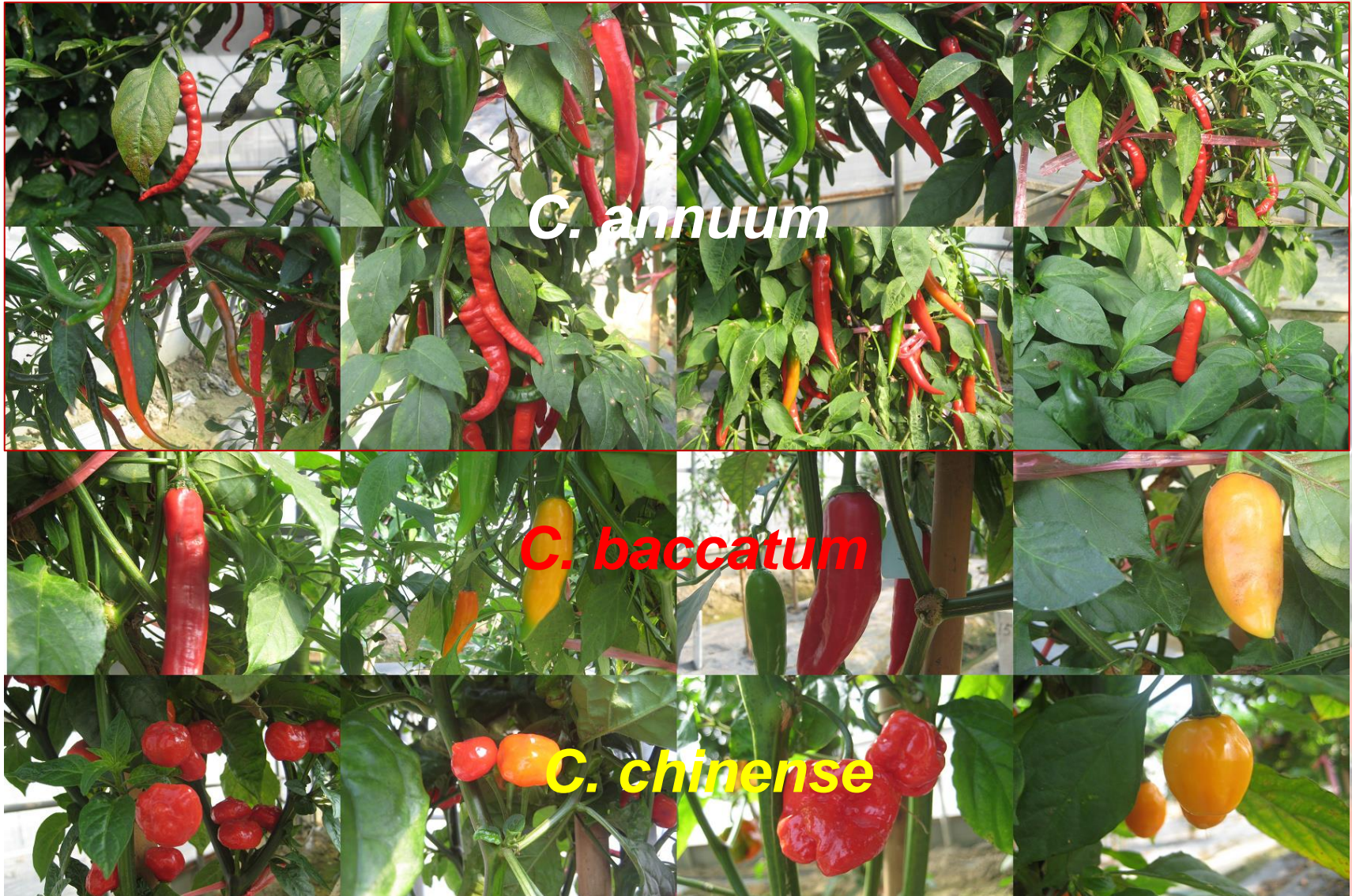


II. Evaluation of 20 chili genotypes against *C. gloeosporioides*

- *C. gloeosporioides* (isolate: Coll-1318)
- Plant material: 12 *C. annuum*, 5 *C. baccatum*, 3 *C. chinense* accessions at the green and red fruit stage.
- Microinjection inoculation: 5×10^6 conidia/ml (10x higher).
- Incubation at 100% RH for 24 hr, then kept at 98% RH at 28°C in the dark.
- Fruit lesion diameter was measured at 10 DAI.



20 chili genotypes for international trial



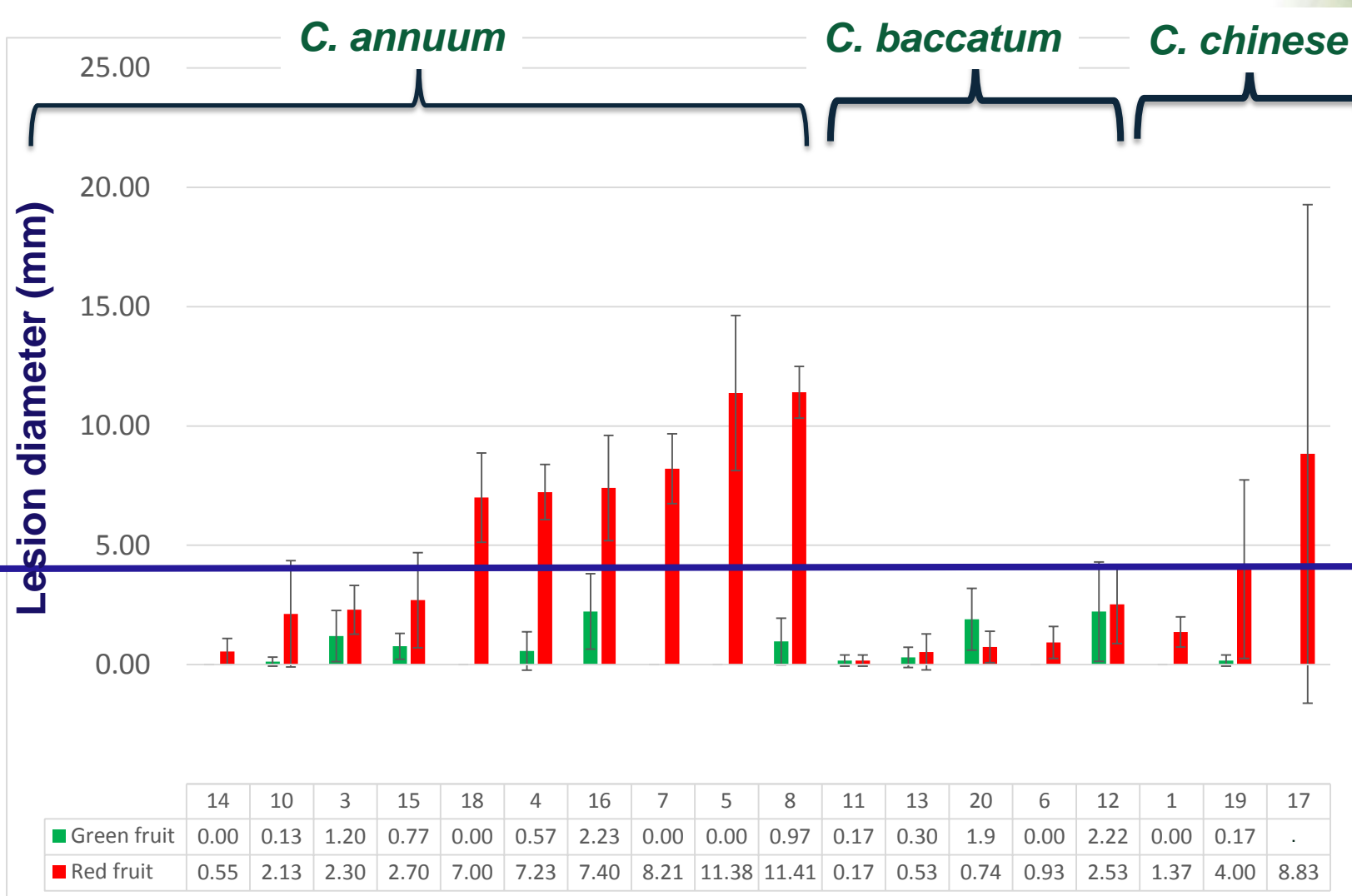


Microinjection inoculation





Summary of microinjection using *C. gloeosporioides*













S

R



Resistant genotypes at red fruit stage

AVPP9609 (PBC932)	AVPP0519	AVPP0514	AVPP0105	AVPP9905
				
VI04432	VI046805 (PBC81)	VI063172	AVPP9608	V1046804 (PBC80)
				



Discussion

- All genotypes showed resistance to *C. gloeosporioides* at the green fruit stage.
- All accessions of *C. baccatum* were resistant to *C. gloeosporioides* at the green and red fruit stage.
- Susan's Joy (*C. annuum*) is normally susceptible to *C. acutatum* and *C. truncatum* but was highly resistant to *C. gloeosporioides* at the green and red fruit stage.



Recommendation

- The continuing collection of isolates helps us to understand the population dynamics of *Colletotrichum* spp.
- Susan's Joy may be a good research material to study the possible defense mechanisms regarding on different *Colletotrichum* species.
- Identifying resistant chili genotypes against *C. gloeosporioides* will contribute to chili pepper varieties with broad resistance against different species of *Colletotrichum*.



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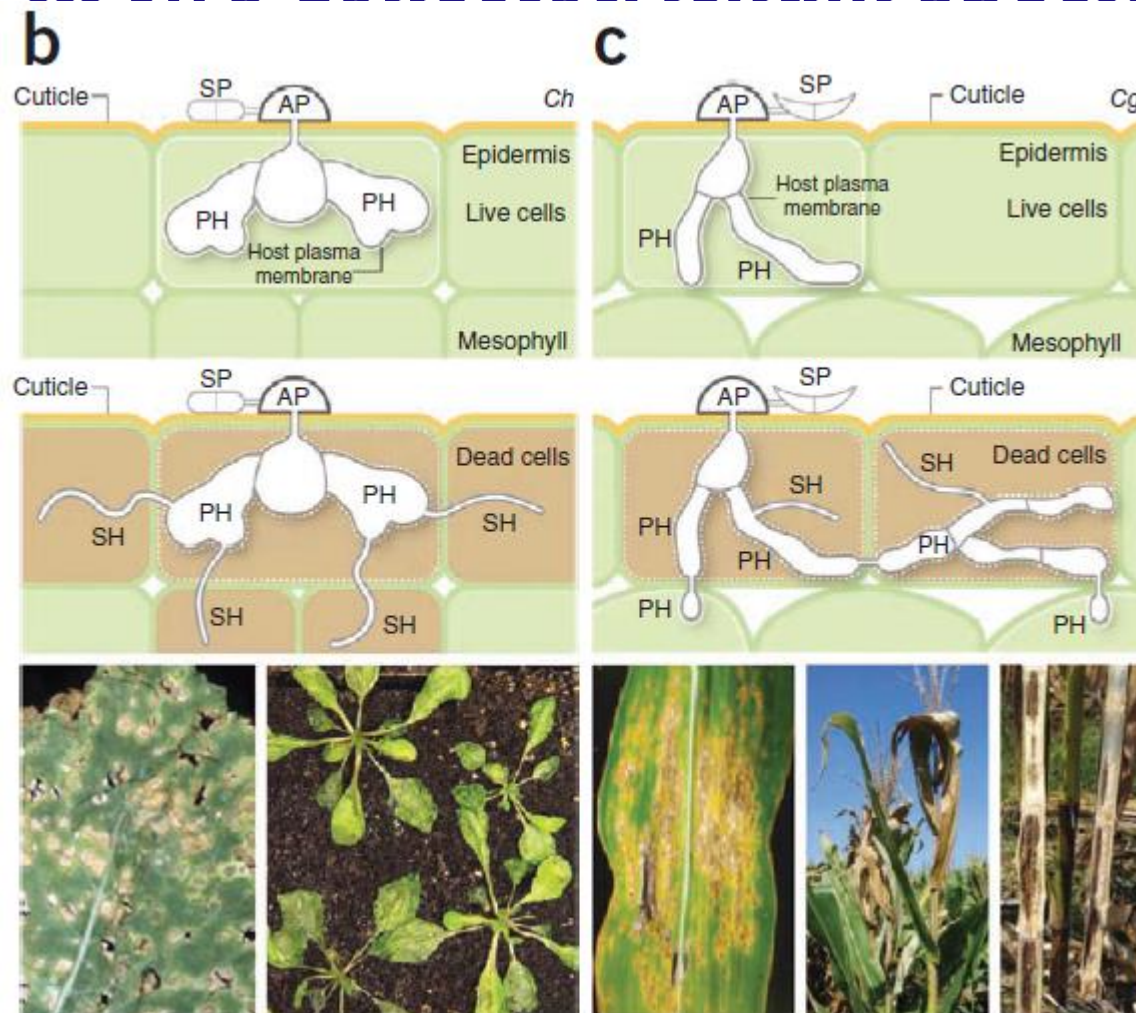




High lesion size variations within the same *Capsicum* genotypes on *C. gloeosporioides* inoculation



Biotrophic verses Necrotrophic stage in two *Collectotrichum* species





Colletotrichum species associated with pepper anthracnose in Taiwan

Table 3. Comparisons of putative *Colletotrichum* species associated with pepper anthracnose.

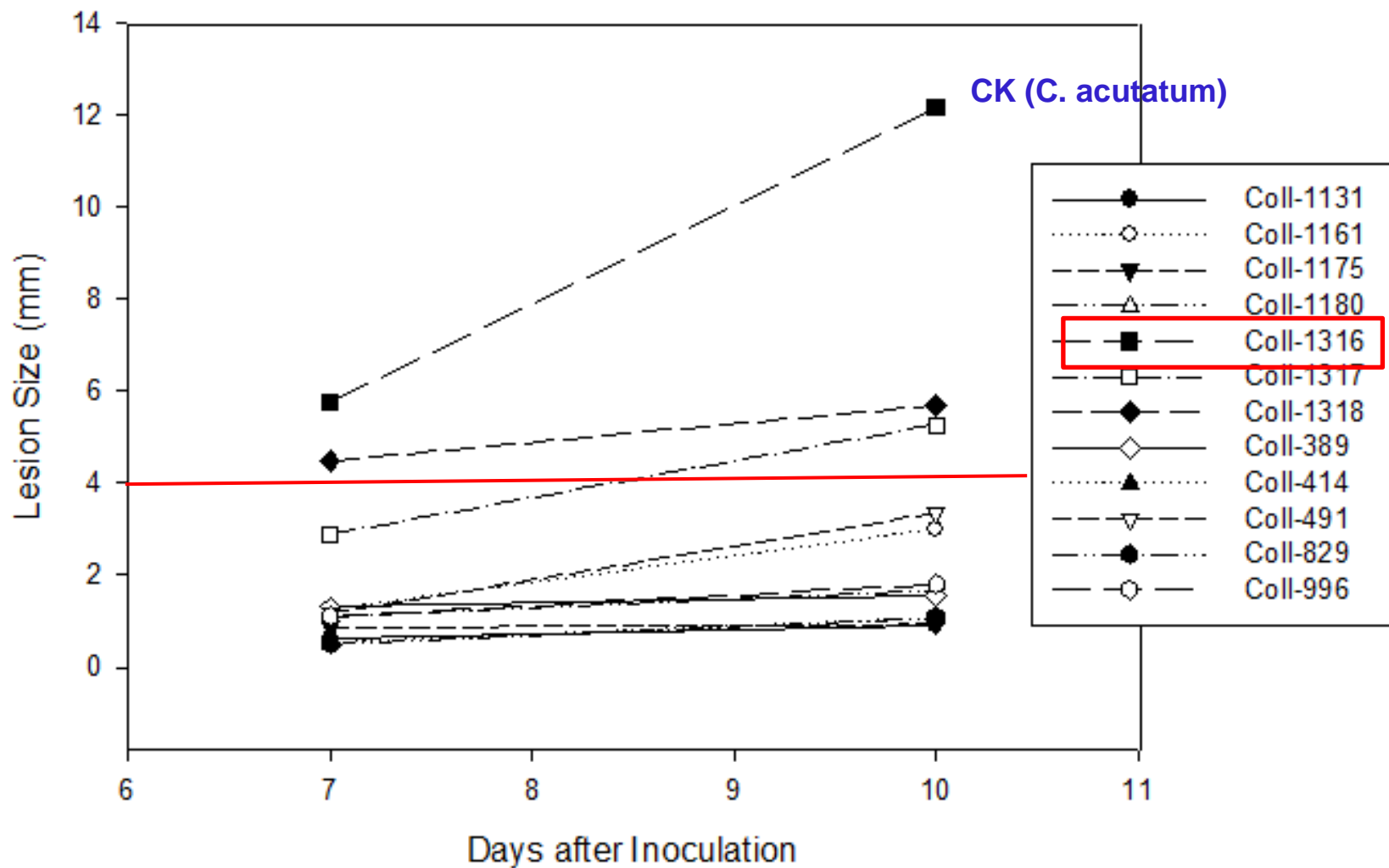
Characteristics	<i>C. acutatum</i>	<i>C. boninense</i>	<i>C. capsici</i>	<i>C. gloeosporioides</i>
Mycelium color	White to gray	White	White to gray	White to dark gray
Reverse colony color	Orange-pink or dark olive	Cream, yellow, or orange	Dark brown	Black, gray, white or pink
Presence of acervuli	Nil	Sometimes present	Often present	Sometimes present
Conidia shape	Fusiform or cylindrical, most with acute end	Cylindrical with obtuse end, often with a hilum-like conidial base	Falcate with acute apex	Cylindrical with obtuse end
Conidia size(μm)	15.1(12.8–16.9) × 4.8(4.0–5.7)	14.9(14.2–16.0) × 6.5(6.0–7.1)	23.9(21.8–28.4) × 4.8(4.4–5.4)	15.6(14.9–16.8) × 4.7(4.3–4.9)
L/B ratio	3.1	2.3	5	3.3
Appressorium	round to ovate	ovate, sometimes with shallow lobe	Nt.	ovate to irregular in shape with lobes
Growth rate at 28°C (mm/day)	5.3 (4.0–6.0)	9.0 (8.0–11.0)	7.3 (5.8–8.6)	13.3 (10.4–15.0)
Protease activity	Most with strong	None	None	None, with one exception
ITS1 length	181 bp	190 bp	177 bp	171 bp
Specific primers	CaINT2/ITS4	Cg2f/ITS4	CcINT2/ITS4	CgINT/ITS4
Remark		Designated as <i>Cg</i> type 2 before		



Colletotrichum isolates –summary of results?

Isolate no.	Genus	Species	Date collected	Host plant	Sampled from	County	Location
Coll-389	<i>Colletotrichum</i>	<i>gloeosporioides</i>	2002. 08. 13	Sweet pepper	fruit(R)-1	Nantou	Jhushan/延山里
Coll-414	<i>Colletotrichum</i>	<i>gloeosporioides</i>	2003. 07. 21	Sweet pepper	fruit(R)-2	Nantou	Sinyi/Chiu mei
Coll-491	<i>Colletotrichum</i>	<i>gloeosporioides</i>	2004. 09. 20	Chili pepper	branch	Tainan	Shanhua/AVRDC
Coll-829	<i>Colletotrichum</i>	<i>gloeosporioides</i>	2008. 07. 23	Chili pepper	fruit (R)-2	Pingtung	Hengchun
Coll-996	<i>Colletotrichum</i>	<i>gloeosporioides</i>	2009. 07. 29	Sweet pepper	fruit (R)-4	Nantou	Puli/Da ping ting
Coll-1131	<i>Colletotrichum</i>	<i>gloeosporioides</i>	2010. 09.03	Sweet pepper	fruit (yellow)	Nantou	Sinyi
Coll-1161	<i>Colletotrichum</i>	<i>gloeosporioides</i>	2011. 06.13	Chili pepper	fruit (R)-2	Kaohsiung	Meinong
Coll-1175	<i>Colletotrichum</i>	<i>gloeosporioides</i>	2011. 06.22	Chili pepper	fruit (R)-1	Changhua	Fangyuan
Coll-1180	<i>Colletotrichum</i>	<i>gloeosporioides</i>	2011. 07.05	Sweet pepper	fruit (G)	Hualien	Shoufong
Coll-1316	<i>Colletotrichum</i>	<i>acutatum</i>	2015. 04. 22	Sweet pepper	fruit (R)	Hsinchu	Cyonglin
Coll-1317	<i>Colletotrichum</i>	<i>gloeosporioides</i>	2015. 04. 22	Sweet pepper	fruit (R)	Hsinchu	Cyonglin
Coll-1318	<i>Colletotrichum</i>	<i>gloeosporioides</i>	2015. 04. 22	Sweet pepper	fruit (R)	Hsinchu	Cyonglin

Screening for high virulent *C. gloeosporioides* isol.





Summary of microinjection using *C. gloeosporioides* and *C. acutatum*

AVPP/VI Code	Other Name	Species	Avg. lesion diameter (mm)			
			Cg-1318G	Cg-1318R	Ca-153G	Ca-153R
1. AVPP9609	PBC 932-6-2, TC07619	<i>C. chinense</i>	0.00	1.37	4.67	6.70
3. AVPP0514	0537-7559	<i>C. annuum</i>	1.20	2.30	9.30	7.97
4. AVPP0520	PBC1713, TC06842	<i>C. annuum</i>	0.57	7.23	14.27	10.50
5. AVPP9813	9852-173	<i>C. annuum</i>	0.00	11.38	14.93	17.37
6. AVPP9608	PBC 880	<i>C. baccatum</i>	0.00	0.93	4.88	6.23
7. AVPP0518	0537-7507	<i>C. annuum</i>	0.00	8.21	17.83	16.77
9. VI059328	PBC142, C05573	<i>C. annuum</i>	0.97	11.41	18.20	15.13
10. AVPP9905	9955-15; Susan' s Joy	<i>C. annuum</i>	0.13	2.13	17.67	19.91
11. VI044352	Tc6498	<i>C. baccatum</i>	0.17	0.17	3.03	4.73
12. VI046804	PBC 80,TC06643	<i>C. baccatum</i>	2.22	2.53	-	0.71
13. VI046805	PBC 81,TC06644	<i>C. baccatum</i>	0.30	0.53	-	0.33
14. AVPP0519	0538-8525	<i>C. annuum</i>	0.00	0.55	10.47	18.40
15. AVPP0105	PP9950-5197,PP0107-7058	<i>C. annuum</i>	0.77	2.70	12.47	18.40
16. AVPP0508	PP0537-7516	<i>C. annuum</i>	2.23	7.40	18.20	-
17. VI60881	Bhut Jolokia	<i>C. chinense</i>	-	8.83	-	-
18. AVPP0022	PP0030-772-09-1	<i>C. annuum</i>	0.00	7.00	1.63	8.73
19. AVPP1240	PP1235-5715BK	<i>C. chinense</i>	0.17	4.00	11.87	11.89
20. VI063172	PBC1752, PI594137 01 SD	<i>C. baccatum</i>	1.90	0.74	2.00	6.63



Plant material- 20 chili genotypes

AVPP/VI Code	Other Name	Species	Origin
1.AVPP9609	PBC 932-6-2, TC07619	<i>C. chinense</i>	USA
2.AVPP9610	PBC 550a, C05669	<i>C. annuum</i>	Indonesia
3.AVPP0514	0537-7559	<i>C. annuum</i>	AVRDC
4.AVPP0520	PBC1713, TC06842	<i>C. annuum</i>	Korea
5.AVPP9813	9852-173	<i>C. annuum</i>	AVRDC
6.AVPP9608	PBC 880	<i>C. baccatum</i>	Australia
7.AVPP0518	0537-7507	<i>C. annuum</i>	AVRDC
8.AVPP0207	0038-9155-5-1	<i>C. annuum</i>	AVRDC
9.VI059328	PBC142, C05573	<i>C. annuum</i>	India
10.AVPP9905	9955-15; Susan's Joy	<i>C. annuum</i>	AVRDC
11.VI044352	Tc6498	<i>C. baccatum</i>	unknown
12.VI046804	PBC 80,TC06643	<i>C. baccatum</i>	Peru
13.VI046805	PBC 81,TC06644	<i>C. baccatum</i>	Peru
14.AVPP0519	0538-8525	<i>C. annuum</i>	AVRDC
15.AVPP0105	PP9950-5197,PP0107-7058	<i>C. annuum</i>	AVRDC
16.AVPP0508	PP0537-7516	<i>C. annuum</i>	AVRDC
17.VI60881	Bhut Jolokia	<i>C. chinense</i>	USA
18.AVPP0022	PP0030-772-09-1	<i>C. annuum</i>	AVRDC
19.AVPP1240	PP1235-5715BK	<i>C. chinense</i>	AVRDC
20.VI063172	PBC1752, PI594137 01 SD	<i>C. baccatum</i>	USA



Difficulties in identifying anthracnose resistance

Inconsistency in genetic inheritance:

- Depends on inoculation method used
- Pathogen species and pathotype used
- Different resistance depending on green or red fruit stage

Other issues:

- Lengthy maturity time of chili peppers
- Lack of resistance in cultivated *C. annuum* species
- Hybridization problems between *C. annuum* and *C. baccatum*

