INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–1106/2021/25–4–805–812
DOI: 10.17957/IJAB/15.1732
http://www.fspublishers.org

Full Length Article

Plasmodiophora brassicae in Yunnan and its Resistant Sources in Chinese Cabbage

Xueyu Han[1†], Junlong Yin[1,2†], Ikram Ullah[1], Enzhu Luo[1] and Yanling Yue[1*]
[1]*College of Landscape and Horticulture, Yunnan Agricultural University, Kunming 650201, China*
[2]*Chinese Academy of Tropical Agricultural Sciences, Proving Ground, Danzhou 571737, China*
[*]For correspondence: yanling-yue@126.com; 2217714953@qq.com
[†]Contributed equally to this work and are co-first authors
Received 01 August 2020; Accepted 02 January 2021; Published 25 March 2021

Abstract

The Williams differential system was employed for pathotype identification of 34 *Plasmodiophora brassicae* root samples collected from Yunnan Province and pathotypes 1, 2, 4, 10 and 14 were detected. Pathotype 4 was dominant with 70.59% of all the samples in Yunnan. The distribution of the *P. brassicae* pathotypes was mapped. Resistance to *P. brassicae* (clubroot disease) was investigated in 22 Chinese cabbage cultivars and it was found that the cultivar Shangpin had multiple resistances and was immune while Shangpin CR527 and Shangpin CR523 were resistant to *P. brassicae*. These cultivars can be used by farmers as sources of resistance to *P. brassicae*, to aid them in reducing disease in their crops. Seven known clubroot-resistant genes were detected in the 22 Chinese cabbage cultivars. *CRa* and *CRb* were found to be the most resistant to *P. brassicae* pathotype 4. Beisheng CR12 was resistant to pathotypes 1, 4, 10 and 14, but did not carry any known resistance genes, which indicated that unknown resistant genes were present. This study will lay the foundation for the control of clubroot disease and promote disease-resistant breeding of Chinese cabbage.

Keywords: Clubroot; Differential hosts; *Plasmodiophora brassicae*; Resistance identification

Introduction

Clubroot, caused by *Plasmodiophora brassicae* (*P. brassicae*), is an obligate parasitic soil-borne disease of plants causing 25–60% of yield losses in crucifer crops annually (Faggian *et al.* 1999; Howard *et al.* 2010; Suo *et al.* 2015). *P. brassicae* infects the roots, causing swelling or formation of galls on the roots. The distorted root reduces water and nutrient uptake and leaf heads fail to develop (Faggian *et al.* 1999). Clubroot was first reported in 1737 on the west coast of the Mediterranean Sea and southern Europe (Karling 1942). In Asia, it was first reported in Taiwan, China (1936) and then spread to Japan and Korea. At present, there are reports of clubroot occurrence in the Northeast, Southwest, Shandong and the upper and middle reaches of the Yangtze River in China (Suo *et al.* 2015). The resting spores of *P. brassicae* can survive for more than a decade within the soil (Karling 1942), even if the cruciferous crops were no longer cultivated. Once a suitable host is available for infection, the resting spores activate and invade the roots, and additional resting spores are released after root decomposition, thus threatening the sustainable production of cruciferous vegetables (Fei *et al.* 2015). Some measures have been taken to control this pathogen in infested fields. Crop rotation is commonly used to prevent the spread of clubroot disease (Howard *et al.* 2010), however, this method is not a practical way to eradicate spores (Peng *et al.* 2015). Soil amendments with lime have also been considered as an effective management strategy to reduce clubroot infestation, by increasing soil pH (Lv *et al.* 2018). However, the repetitive use of lime destroys the soil structure, ultimately affecting sustainable production (Webster and Dixon 1991). Fluazinam, a synthetic fungicide, has been shown to control *P. brassicae* (Howard *et al.* 2010; Yuan *et al.* 2016), however, improper use of the soil and plant residues greatly affects human health and environmental safety (Lee *et al.* 2012). Endophytic actinomycetes isolated from the roots of Chinese cabbage can effectively control clubroot (Lee *et al.* 2008), but the activity against *P. brassicae* is susceptible to soil environmental conditions, and their field efficacy is inconsistent and often ineffective (Saravanan *et al.* 2003; Mcgrann *et al.* 2017). Wang *et al.* (2016) found that the *Streptomyces albospinus* CT205, proved to be effective in preventing clubroot disease, although environmental and climatic factors severely affect its performance. Biocontrol agents are expensive and, if not applied properly, can lead to an increased production cost. Therefore, the application of

resistant varieties is considered to be one of the most effective and environmentally friendly approaches for treating clubroot disease.

Clubroot has been present in Yunnan for approximately 30 years. It first appeared in the Chenggong area of Kunming and has been identified in Chuxiong, Yuxi, Qujing, Dali, and other areas. Yunnan is the main vegetable producing area in China, and Chinese cabbage is an important export vegetable of Yunnan Province, cultivated in an area of approximately 100 000 hm^2 with a yield of 4.06 million. The soil contaminated by *P. brassicae* in the main production regions accounts for approximately 20% of the total area and the loss of crops is generally 20–40%, but can be up to 100%. Several resistant Chinese cabbage varieties, such as CR Huimin, Kanggen 51 and Tiejia No.1, have been developed and introduced (Tan and Yue 2013; Yin *et al*. 2018), although these studies are mostly directed at a single *P. brassicae* pathotype and a single sampling site, lacking comprehensive evaluation of pathotype differentiation, variety resistance and a suitable applied range in the province. In order to fully understand the distribution of clubroot in Yunnan Province, *P. brassicae* samples were collected from 34 different clubroot growing areas in 2017–2019 and their pathotypes and resistant cultivars of Chinese cabbage were identified, which would provide useful information for farmers to prevent and control clubroot.

Materials and Methods

Plant pathogen materials

A total of 34 *P. brassicae* samples were collected from the main cruciferous vegetable growing areas in Yunnan Province. The samples, along with collection sites and hosts, are shown in Table 1. For pathotype identification of the 34 *P. brassicae* samples, the hosts proposed by Williams, including two cabbage cultivars Jersey Queen (JQ), Badger Shipper (BS) and two turnip cultivars Laurentian (LT), Wilhelmsburger (WB), were obtained from the Liaoning Academy of Agricultural Sciences, China. Twenty-one Chinese cabbage-resistant varieties and the susceptible control variety '83-1'were purchased from markets.

Preparation of the pathogen, plant inoculation and disease assessment

Resting spores were extracted from samples of *P. brassicae* using the method described by Zheng *et al.* (2019). The peat and resting spores were mixed evenly, with 1×10^7 spores per gram of pathogen soil. Peat, perlite, and vermiculite with a volume ratio of 2:1:1 were mixed and packed in seedling plugs with 72 holes, and 1.0 grams of pathogen soil was added to each hole. Germinated seeds of Williams' identified hosts, and 22 Chinese cabbage varieties were sowed on pathogen soil, one seed per hole, and one seedling plug per variety. They were fertilised with Hoagland nutrient solution in the greenhouse (average night temperature 18°C and average day temperature 25°C). After 40 days of inoculation, pathotypes of 34 *P. brassicae* were identified according to the resistant and susceptible responses by Williams' hosts (Williams 1996). The severity of the club development on each plant was rated using a scale of 0, 1, 3, 5, 7 and 9, according to The Grading Standard for Clubroot Resistance of *Brassicaceae* Crop Seedling Period, where 0 = no clubbing, 1 = one or a few small clubs on the lateral roots, 3 = clubs on the main roots with diameters less than 2 times that of the stem base, 5 = clubs on the main roots with diameters 2–3 times that of the stem base, 7 = clubs on the main roots, with diameters 3–4 times that of the stem base, and 9 = clubs on the main roots with diameters more than four times that of the stem base, or the swollen root is blackening. The index of disease (ID) was calculated using the following formula:

$$ID\ (\%) = \sum (n\times0+n\times1+n\times3+n\times5+n\times7+n\times9)/(N\times9)\times100$$

where n is the number of plants in each class, N is the total number of plants, and 0, 1, 3, 5, 7 and 9 are the symptom severity classes.

DNA extraction and primer design

The Plant DNA Extraction Kit (http://www.bioteke.com/) was used to extract DNA from the leaves of 22 Chinese cabbage varieties and 4 Williams' hosts, according to the manufacturer's instructions. Primers for disease-resistant genes were designed based on published molecular marker sequences of CR loci: *Crr1* (Suwabe *et al.* 2003), *Crr2* (Suwabe *et al.* 2003), *Crr3* (Hirai *et al.* 2004), *CRa* (Ueno *et al.* 2012), *CRb* (Hatakeyama *et al.* 2013), *CRc* (Ueno *et al.* 2012) and *CRk* (Ueno *et al.* 2012). The list of primers used is listed in Table 2.

PCR reaction procedure and gel electrophoresis

Each locus was amplified with 2 ng of template DNA in a 25 μL reaction volume containing 1 μL of each primer and 12.5 μL of 2× PCR master mix (Takara, Japan). Thermal cycling conditions comprised denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing temperature of each primer at 61°C, 55.5°C, 55.5°C, 54.5°C, 57°C, 52.2°C, 49°C, 47°C for 30s and extension at 72°C for 30s followed by extension at 72°C for 5 min in a BioTeke GeneAmp PCR system. Amplified PCR products were separated by electrophoresis in a 1.5% agarose gel in Tris-acetate-EDTA buffer, stained with EB (ethidium bromide) and photographed.

Results

Identification of pathotypes

The total collection of 34 *P. brassicae* isolates was assessed. The susceptible or resistant responses of Williams' hosts to *P. brassicae* are shown in Table 3. The 34 *P. brassicae*

Table 1: Sampling codes, site and hosts of 34 *Plasmodiophora brassicae* samples

Code	Site	Hosts	Code	Site	Hosts	Code	Site	Hosts	Code	Site	Hosts
P1	Songming	Chinese cabbage	P2	Songming	Chinese cabbage	P3	Yingjiang	Chinese cabbage	P4	Yuxi	Chinese cabbage
P5	Panlong	Chinese cabbage	P6	Lufeng	Chinese cabbage	P7	Yaoan	Chinese cabbage	P8	Nanhua	Chinese cabbage
P9	Chenggong	Chinese cabbage	P10	Yingjiang	Chinese cabbage	P11	Jianshui	Chinese cabbage	P12	Jianshui	Chinese cabbage
P13	Ludian	Chinese cabbage	P14	Yiliang	Brassica oleracea	P15	Yiliang	Chinese cabbage	P16	Luliang	Chinese cabbage
P17	Luliang	Chinese cabbage	P18	Qujing	Chinese cabbage	P19	Dali	Chinese cabbage	P20	Luquan	Chinese cabbage
P21	Ludian	Chinese cabbage	P22	Ludian	Chinese cabbage	P23	Xundian	Cauliflower	P24	Luliang	*Brassica oleracea*
P25	Anning	Cauliflower	P26	Wuding	Chinese cabbage	P27	Shilin	Chinese cabbage	P28	Jinning	Chinese cabbage
P29	Fumin	Chinese cabbage	P30	Xishan	Chinese cabbage	P31	Weishan	Chinese cabbage	P32	Tonghai	Chinese cabbage
P33	Xuanwei	Chinese cabbage	P34	Qiubei	Chinese cabbage						

Table 2: Primers used for detection of clubroot resistant genes

Locus	Primer name	Primer sequences	Product size/bp	References
Crr1	BRMS-088-FW	TATCGGTACTGATTCGCTCTTCAAC	R263/S233	(Suwabe *et al*. 2003).
	BRM-088-RV	ATCGGTTGTTATTTGAGAGCAGATT		
Crr2	BRMS-096-FW	AGTCGAGATCTCGTTCGTGTCTCCC	R220/S189	(Suwabe *et al*. 2003).
	BRMS-096-RV	TGAAGAAGGATTGAAGCTGTTGTTG		
Crr3	OPC11-2F	GTAACTTGGTACAGAACAGCATAG	R1300/S1000	(Hirai *et al*. 2004).
	OPC11-2R	ACTTGTCTAATGAATGATCATGG		
CRa	SC2930-T-FW	TAGACCTTTTTTTTGTCTTTTTTTTTAC	R800	(Ueno *et al*. 2012).
	SC2930-Q-FW	CAGACTAGACTTTTTGTCATTTAGA	S800	
	SC2930-RV	CTAAGGCCATAGAAATCAGGTC		
CRb	KBrH129J18R-FW	AGAGCAGAGTGAAACCAGAACT	R254/S194	(Hatakeyama *et al.* 2013).
	KBrH129J18R-RV	GTTTCAGTTCAGTCAGGTTTTTGCAG		
CRc	B50-C9-FW	GATTCAATGCATTTCTCTCGAT	R800	(Ueno *et al.* 2012).
	B50-6R-FW	AATGC ATTTTCGCTC AACC	S800	
	B50-RV	CGTATT ATATC TCTTT CTCCA TCCC		
CRk	HC688-4-FW	TCTCTG TATTGCGTTGACTG	R1000	(Ueno *et al.* 2012).
	HC688-6-RV	ATATGTTGAAGCCTATGTCT	S1000	
	HC688-7-RV	AAATATATGTGAAGTCTTATG ATC		

Table 3: Resistant and susceptible response (severity based on a disease index, DI) of Williams' differential hosts to collections of *Plasmodiophora brassicae* from Yunnan Province, China

Code	Williams hosts and disease index								Pathotypes	Code	Williams hosts and disease index								Pathotypes
	JQ	DI	BS	DI	LT	DI	WB	DI			JQ	DI	BS	DI	LT	DI	WB	DI	
P1	+	44	+	16	+	50	+	11	4	P18	+	13	+	26	+	7	-	0	2
P2	+	21	+	33	+	28	+	30	4	P19	+	36	+	15	+	20	+	11	4
P3	+	85	+	44	+	41	+	54	4	P20	-	0	+	11	-	0	+	11	14
P4	+	26	+	61	+	11	+	35	4	P21	+	12	+	11	+	16	+	11	4
P5	+	56	+	74	+	59	+	42	4	P22	+	32	+	17	+	41	+	39	4
P6	+	19	+	31	+	40	-	0	2	P23	+	17	+	11	-	0	+	35	10
P7	+	26	+	44	+	47	+	22	4	P24	+	23	+	21	+	22	+	42	4
P8	+	31	+	34	+	46	+	19	4	P25	+	20	+	20	+	13	+	13	4
P9	+	31	+	11	+	19	+	35	4	P26	+	44	+	24	+	48	+	42	4
P10	+	40	+	35	+	27	+	17	4	P27	+	40	+	11	-	0	+	11	10
P11	+	26	+	32	+	26	+	42	4	P28	+	43	+	17	+	11	+	18	4
P12	+	20	+	22	+	12	+	19	4	P29	+	28	+	46	+	34	+	38	4
P13	+	7	+	11	-	0	+	35	10	P30	+	20	+	20	+	15	+	23	4
P14	+	21	-	0	+	21	+	21	1	P31	+	38	+	19	-	0	+	27	10
P15	+	15	+	15	+	22	+	24	4	P32	+	30	+	50	+	13	+	18	4
P16	-	0	+	11	-	0	+	11	14	P33	+	19	+	22	+	12	+	16	4
P17	+	25	+	24	-	0	+	4	10	P34	+	21	+	18	+	14	+	17	4

Note: "+" represents susceptible reaction, "-" represents resistant reaction
JQ, BS, LT, and WB are Williams hosts (Jersey Queen, Badger Shipper, Laurentian, Wilhelmsburger). P1-P34 are the 34 *P. brassicae* samples. "Pathotypes": the identified result of Pathotypes

could be classified into five pathotypes: pathotypes 1, 2, 4, 10 and 14. Among them, 24 samples were pathotype 4, accounting for 70.59% of the total samples (Table 3). The results indicated that pathotype 4 was the predominant pathotype in Yunnan Province. All the Williams' hosts (JQ, BS, LT and WS) were susceptible to *P. brassicae* of pathotype 4, which confirmed that this variety had the strongest pathogenicity of those studied.

The disease index of hosts JQ, BS, LT and WS differed after inoculation with pathotype 4. The disease index of JQ was 12 in P21 but reached 85 in P3, whereas the disease index of BS was 11 in P9 and P21, but 74 in P5. Similarly, the results for LT ranged from 11 to 59 and the disease index of WS ranged from 11 to 54 for pathotype 4. This shows pathogenic diversity among *P. brassicae* of pathotype 4 in Yunnan Province. The disease index of hosts

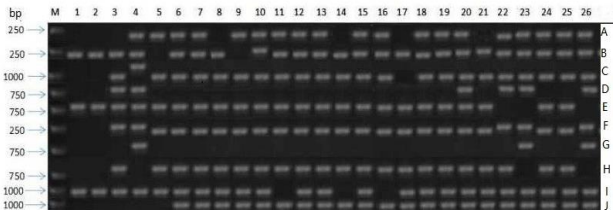

Fig. 1: Amplification results of different resistant genes
Note: M: Marker DL2000; 1–4: Williams Identification Hosts Badger Shipper, Jersey Queen, Wilhelmsburger, Laurentian; 5: CK cultivars 83-1; 6–26: cultivars Chunqiushengen, CR65, Tianci, Jinfu baby cabbage, Guizu, Degaorongyao, Kanggen 911, Taineng CR119, Beisheng CR12, Chinese cabbage King, CR mogen, Xinkanggen, CR gaokangwang, Kangbingwang CR117, Guoshen CR167, Shenggen No.1, Shangpin, Shangpin CR527, Degao CR117, Tiejia No.1, Shangpin CR523. A, B, C, (D, E), F, (G, H), (I, J): amplification results of *Crr1* (BRMS-088), *Crr2* (BRMS-096), *Crr3* (OPC11-2), *CRa* (SC2930-T, SC2930-Q), *CRb* (KBrH129J18R), *CRc* (B50-C9, B50-6R), *CRk* (HC688-6, HC688-7) respectively. The product size of resistant and susceptible is shown in Table 3

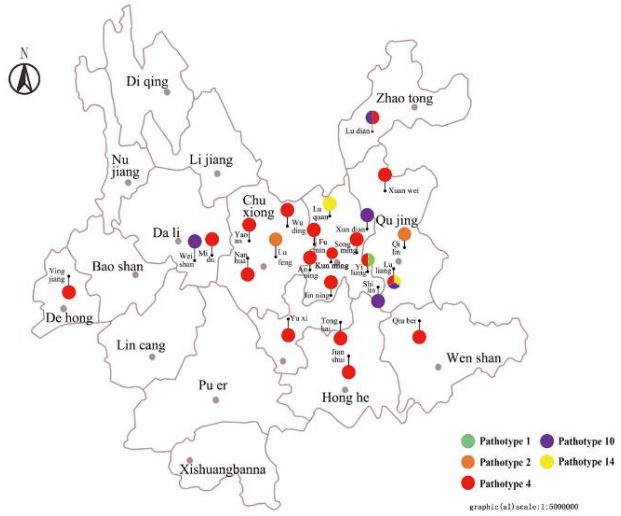

Fig. 2: Distribution of pathotypes of *Plasmodiophora brassicae* in Yunnan

was higher in P3 and P5 than in others, indicating that the pathogenicity of P3 and P5 was stronger than that of other *P. brassicae* of pathotype 4.

Diseases reaction of Chinese cabbage cultivars

We assessed the disease responses of 22 Chinese cabbage cultivars to 34 *P. brassicae* isolates (Table 4). The control variety (83-1) showed the typical clubroot symptoms with all *P. brassicae* isolates, which indicates that the method of inoculation was suitable. The other 21 Chinese cabbage varieties exhibited different resistance responses to the 34 *P. brassicae* isolates. Shangpin was immune to each of the 34 collections, whereas the disease index of Shangpin CR527 and Shangpin CR523 were less than 10, indicating strong resistance. From the resistance expression of cultivars to pathotype 4, there were only three cultivars namely Shangpin, Shangpin CR527 and Shangpin CR523 that were immune to P5, less than the other varieties included in pathotype 4, which shows that P5 was a stronger version of *P. brassicae.* There were few sources which showed resistance to P5.

As shown in Table 4, CR8, CR9, CR10, CR17, CR18 and CR21 expressed immunity to P14 (pathotype 1). The disease indices of CR2, CR3, CR5, CR13, CR14 and CR16 were lower than 10, indicating that they were resistant to P14. CR17, CR18 and CR21 exhibited immunity, while CR1 and CR10 were resistant to P6 and P18 (pathotype 2). CR9, CR17, CR18 and CR21 were immune, and CR12 and CR16 were resistant to P13, P17, P23, P27 and P31 (pathotype 10). CR9, CR13, CR14, CR16, CR17, CR18, CR19 and CR21 showed immunity, and CR1 and CR8 were resistant to P16 and P20 (pathotype 14).

Molecular markers for resistance

To investigate the relationship between the clubroot-resistant varieties and known CR resistance loci, PCR amplification of the seven known molecular markers was applied for genotyping Williams' hosts and the 22 cultivars.

The results (Fig. 1, Table 5) indicated that LT contained the most disease-resistant genes: *Crr3*, *CRa*, *CRb*, *CRc* and *CRk*. WB contained three disease-resistant genes: *CRa*, *CRb* and *CRk*. Only the resistant gene *CRk* was detected in the BS and JQ. Among the 21 tested varieties, CR18 and CR21 contained four disease-resistant genes, heterozygous *CRa* and *CRk*, homozygous *CRb* and *CRc*, which accounted for 9.52% of the total tested varieties. CR17 was checked for three disease-resistant genes, homozygous *CRa*, *CRb* and heterozygous *CRk*, which accounted for 4.76%. CR5 was examined for two resistant genes, homozygous *Crr2* and heterozygous *CRk*, accounting for 4.76%. CR15 contained two resistant genes, heterozygous *CRa* and *CRk*, which accounted for 4.76%, and CR7, CR9 and CR11 did not test for any disease-resistant genes, accounting for 14.29% of the total tested cultivars. The other cultivars contained only one heterozygous *CRk* gene (61.9%).

Discussion

There are few reports related to the study of pathotype differentiation of *P. brassicae* in China, and only a few pathotypes have been detected, such as pathotype 4 in areas of Shandong and Jilin Province and pathotypes 2, 4 and 11 in the Liaoning area (Shen *et al.* 2009). Pathotypes 7 and 11 have been reported in the Sichuan area (Chen *et al.* 2016), and pathotypes 1, 4, 9 and 13 in the Hunan area (Peng *et al.* 2013). The clubroot in the Shandong, Jilin, Liaoning, Sichuan, and Hunan areas could be explained by the cultivation of resistant varieties. In this study, 5 pathotypes, pathotype 1, 2, 4, 10 and 14, were investigated in Yunnan Province and only Shangpin was determined as immune to all pathotypes, indicating that *P. brassicae* in Yunnan is more complex and pathogenic than in other areas in China.

Table 4: Resistant and susceptible response (severity based on a disease index, DI) of 22 differential varieties to collections of *Plasmodiophora brassicae* from Yunnan Province, China

Varieties		83-1	CR1	CR2	CR3	CR4	CR5	CR6	CR7	CR8	CR9	CR10	CR11	CR12	CR13	CR14	CR15	CR16	CR17	CR18	CR19	CR20	CR21
P1	DI	61	0	4	0	43	16	10	0	0	0	0	3	0	0	0	17	2	0	0	0	12	1
	R	S	I	R	I	S	S	S	I	I	I	I	R	I	I	I	S	R	I	I	I	S	R
P2	DI	57	31	4	5	70	5	15	11	0	0	0	52	0	2	0	57	11	0	0	2	23	5
	R	S	S	R	R	S	R	S	S	I	I	I	S	I	R	I	S	S	I	I	R	S	R
P3	DI	85	19	57	9	43	33	41	0	0	0	1	43	0	19	5	49	32	0	0	13	0	0
	R	S	S	S	R	S	S	S	I	I	I	R	S	I	S	R	S	S	I	I	S	I	I
P4	DI	72	25	42	52	58	15	35	17	0	0	0	30	11	7	10	56	17	0	0	38	14	0
	R	S	S	S	S	S	S	S	S	I	I	I	S	S	R	S	S	S	I	I	S	S	I
P5	DI	86	26	41	31	37	79	19	43	31	31	33	50	30	31	33	48	50	0	0	46	54	0
	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	I	S	S	I
P6	DI	65	3	20	11	65	14	28	32	38	48	0	11	17	10	27	80	27	0	0	19	25	0
	R	S	R	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S	I	I	S	S	I
P7	DI	80	33	78	19	62	56	19	39	20	4	0	10	0	1	27	48	46	0	0	11	15	0
	R	S	S	S	S	S	S	S	S	S	R	I	S	I	R	S	S	S	I	I	S	S	I
P8	DI	67	17	26	0	41	48	22	0	0	8	2	53	23	0	0	27	27	0	0	13	12	0
	R	S	S	S	I	S	S	S	I	I	R	R	S	S	I	I	S	S	I	I	S	S	I
P9	DI	56	11	26	17	59	33	19	56	19	16	20	4	0	0	36	11	23	0	0	35	28	2
	R	S	S	S	S	S	S	S	S	S	S	S	R	I	I	S	S	S	I	I	S	S	R
P10	DI	72	4	9	2	46	16	16	32	0	38	0	29	33	6	7	41	26	0	0	31	13	0
	R	S	R	R	R	S	S	S	S	I	S	I	S	S	R	R	S	S	I	I	S	S	I
P11	DI	65	1	19	0	47	28	23	42	17	22	1	15	19	0	0	70	7	0	0	7	7	0
	R	S	R	S	I	S	S	S	S	S	S	R	S	S	I	I	S	R	I	I	R	R	I
P12	DI	76	9	13	0	31	29	0	21	0	4	0	29	37	4	11	51	4	0	0	0	36	0
	R	S	R	S	I	S	S	I	S	I	R	I	S	S	R	S	S	R	I	I	I	S	I
P13	DI	37	5	22	7	69	6	8	8	0	0	2	17	0	0	9	32	6	0	0	0	13	0
	R	S	R	S	R	S	R	R	R	I	I	R	S	I	I	R	S	R	I	I	I	S	I
P14	DI	46	19	7	2	63	8	17	25	0	0	0	37	11	1	5	50	2	0	0	17	15	0
	R	S	S	R	R	S	R	S	S	I	I	I	S	S	R	R	S	R	I	I	S	S	I
P15	DI	57	19	7	2	63	8	17	25	19	0	0	37	11	1	5	50	2	0	0	17	6	0
	R	S	S	R	R	S	R	S	S	S	I	I	S	S	R	R	S	R	I	I	S	R	I
P16	DI	67	8	19	0	72	11	14	22	2	0	26	26	0	0	0	40	0	0	0	0	25	0
	R	S	R	S	I	S	S	S	S	R	I	S	S	I	I	I	S	I	I	I	I	S	I
P17	DI	70	39	65	21	78	44	69	36	37	0	10	44	3	19	20	46	5	0	0	13	15	0
	R	S	S	S	S	S	S	S	S	S	I	S	S	R	S	S	S	R	I	I	S	S	I
P18	DI	44	7	63	5	12	12	4	0	0	1	7	20	2	2	0	45	0	0	0	7	16	0
	R	S	R	S	R	S	S	R	I	I	R	R	S	R	R	I	S	I	I	I	R	S	I
P19	DI	65	22	13	4	54	18	1	4	0	0	0	26	3	0	6	20	1	0	0	3	0	0
	R	S	S	S	R	S	S	R	R	I	I	I	S	R	I	R	S	R	I	I	R	I	I
P20	DI	67	8	19	11	72	0	8	22	2	0	26	26	11	0	0	40	0	0	0	0	25	0
	R	S	R	S	S	S	I	R	S	R	I	S	S	S	I	I	S	I	I	I	I	S	I
P21	DI	22	39	70	17	61	14	23	0	6	31	0	21	9	0	3	61	34	0	0	19	33	0
	R	S	S	S	S	S	S	S	I	R	S	I	S	R	I	R	S	S	I	I	S	S	I
P22	DI	72	2	27	28	67	44	14	36	20	27	0	42	0	0	0	61	15	0	0	50	17	0
	R	S	R	S	S	S	S	S	S	S	S	I	S	I	I	I	S	S	I	I	S	S	I
P23	DI	37	5	22	7	69	6	8	8	0	0	2	17	0	0	9	32	6	0	0	0	13	0
	R	S	R	S	R	S	R	R	R	R	I	R	S	I	I	R	S	R	I	I	I	S	I
P24	DI	67	24	37	29	33	5	13	7	0	8	0	0	12	6	0	57	19	0	3	32	61	0
	R	S	S	S	S	S	R	S	R	I	R	I	I	S	R	I	S	S	I	R	S	S	I
P25	DI	33	28	0	1	22	17	2	11	0	0	0	19	0	0	0	28	0	0	0	11	6	0
	R	S	S	I	R	S	S	R	S	I	I	I	S	I	I	I	S	I	I	I	S	R	I
P26	DI	65	11	14	15	52	9	32	0	0	21	3	2	0	14	0	48	19	0	0	11	32	0
	R	S	S	S	S	S	R	S	I	I	S	R	R	I	S	I	S	S	I	I	S	S	I
P27	DI	57	31	24	4	56	12	1	4	0	0	3	26	0	0	4	24	1	0	0	0	3	0
	R	S	S	S	R	S	S	R	R	I	I	R	S	I	I	R	S	R	I	I	I	R	I
P28	DI	56	39	46	21	78	44	16	36	37	1	10	44	3	19	20	46	5	0	5	15	13	0
	R	S	S	S	S	S	S	S	S	S	R	S	S	R	S	S	S	R	I	R	S	S	I
P29	DI	63	3	20	17	37	21	17	16	39	32	3	15	5	15	10	59	23	0	1	36	36	0
	R	S	R	S	S	S	S	S	S	S	S	R	S	R	S	S	S	S	I	R	S	S	I
P30	DI	52	6	42	5	12	12	16	0	0	1	7	20	2	2	0	45	0	0	0	16	17	0
	R	S	R	S	R	S	S	S	I	I	R	R	S	R	R	I	S	I	I	I	S	S	I
P31	DI	46	16	34	5	58	14	8	7	0	0	7	29	0	0	6	44	3	0	0	0	16	0
	R	S	S	S	R	S	S	R	R	I	I	R	S	I	I	R	S	R	I	I	I	S	I
P32	DI	73	16	63	2	62	24	9	7	0	0	0	23	12	1	34	58	5	0	0	7	9	0
	R	S	S	S	R	S	S	R	R	I	I	I	S	S	R	S	S	R	I	I	R	R	I
P33	DI	35	22	8	3	18	13	5	14	2	0	3	25	0	0	0	36	0	0	0	15	4	0
	R	S	S	R	R	S	S	R	S	R	I	R	S	I	I	I	S	I	I	I	S	R	I
P34	DI	38	26	4	7	24	19	3	12	1	0	5	17	0	0	0	27	0	0	0	12	9	0
	R	S	S	R	R	S	S	R	S	R	I	R	S	I	I	I	S	I	I	I	S	R	I

Note: Disease index<10 meaned Resistant (R), Disease index≥10 meaned susceptible (S), Disease index=0 meaned immune (I). From CR1 to CR21: Chunqiushenggen, CR65, Tianci, Jinfu baby cabbage, Guizu, Degaorongyao, Kanggen 911, Taineng CR119, Beisheng CR12, Chinese cabbage King, CR Mogen, Xinkanggen, CR Gaokangwang, Kangbingwang CR117, Guoshen CR167, Shenggen No.1, Shangpin, Shangpin CR527, Degao CR117, Tiejia No.1, Shangpin CR523

Pathotypes of P. brassicae and their distribution in Yunnan are shown in Fig. 2.

Clubroot disease was detrimental in Kunming and its surrounding areas, Fumin, Anning, Jinning, Songming and Yiliang. Kunming became a disease centre, spreading outward to some areas of Chuxiong, Dali, Dehong, Lijiang,

Table 5: Identification results of molecular markers

Code	Cultivars	Resistant site						
		Crr1	*Crr2*	*Crr3*	*CRa*	*CRb*	*CRc*	*CRk*
1	BS	\	-	\	-	-	\	+
2	JQ	\	-	\	-	-	\	+
3	WB	\	-	-	±	+	-	+
4	LT	-	-	+	±	+	+	+
5	83-1（CK）	-	\	-	-	-	-	+
6	CR1	-	-	-	-	-	-	±
7	CR2	-	-	-	-	-	-	±
8	CR3	\	-	-	-	-	-	±
9	CR4	-	\	-	-	-	-	±
10	CR5	-	+	-	-	-	-	±
11	CR6	-	-	-	-	-	-	±
12	CR7	-	-	-	-	-	-	-
13	CR8	-	-	-	-	-	-	±
14	CR9	\	-	-	-	-	-	-
15	CR10	-	-	-	-	-	-	±
16	CR 11	-	-	-	-	-	-	-
17	CR12	\	-	-	-	-	-	±
18	CR13	-	-	-	-	-	-	±
19	CR14	-	-	-	-	-	-	±
20	CR15	-	-	-	±	-	-	±
21	CR16	\	-	-	-	-	-	±
22	CR17	-	-	-	+	+	-	±
23	CR18	-	-	-	+	+	+	±
24	CR19	-	-	-	-	-	-	±
25	CR20	-	-	-	-	-	-	±
26	CR21	-	-	-	+	+	+	±

Note: "+" Homozygous persistent site; "-" Homozygous susceptible site; "±" Heterozygous resistant site; "\" no site. From CR1 to CR21: Chunqiushenggen, CR65, Tianci, Jinfu baby cabbage, Guizu, Degaorongyao, Kanggen 911, Taineng CR119, Beisheng CR12, Chinese cabbage King, CR Mogen, Xinkanggen, CR Gaokangwang, Kangbingwang CR117, Guoshen CR167, Shenggen No.1, Shangpin, Shangpin CR527, Degao CR117, Tiejia No.1, Shangpin CR523

Table 6: Resistance varieties of different Pathotypes

Race	Cultivars
Race 1	Taineng CR119, Beisheng CR12, Chinese cabbage King, Shangpin, Shangpin CR527, Degao CR117, ShangpinCR523
Race 2	Shangpin, Shangpin CR527, Shangpin CR523
Race 4	Shangpin
Race 10	Beisheng CR12, Shangpin, Shangpin CR527, Shangpin CR523
Race 14	Beisheng CR12, CR Gaokangwang, Kangbingwang CR117, Shenggen NO.1, Shangpin, Shangpin CR527, Degao CR117, Shangpin CR523

Honghe and Qujing, and to counter this expansion, we propose prevention and control strategies for clubroot disease in Yunnan Province. Although planting disease-resistant Chinese cabbage varieties or non-cruciferous crops in clubroot disease areas can reduce the number of *P. brassicae* spores in the soil and avoid the occurrence of clubroot, we need to invoke quarantine and disinfection of the seeds and seedlings in the adjacent areas of the disease area, avoiding soil pollution caused by *P. brassicae* from clubroot disease areas and preventing the expansion of the disease.

The pathogenicity of *P. brassicae* in Yunnan Province has changed over the past decade. The *P. brassicae* found in Lufeng was pathotype 12 in 2013 (Liu *et al.* 2013), but is now pathotype 2. Both of the 2 *P. brassicae* samples from Songming were pathotype 4 in our study, but pathotype 7 was identified in 2016 (Chen *et al.* 2016). Pathotype 2 had higher pathogenicity than 12, and pathotype 4 was higher than pathotype 7. These results indicate that clubroot disease in Lufeng and Songming had become more robust over time. Pathogenicity of pathotype 4 was the highest among all pathotypes. However, only 3 cultivars: Shangpin, Shangpin CR523 and Shangpin CR527, were resistant to all types of pathotype 4 in Yunnan; the other varieties expressed either immune, resistant or susceptible responses depending on the type of pathogen 4. The responses of the same cultivar to 24 samples of pathotype 4 were different, which indicated its pathogenicity differences and complexity. It was reported by Li *et al.* (2012) that they had successfully isolated several pathotypes (2, 4, 8 and 11) from pathotype 4 samples in Shenyang Province by separating single resting spores. Their results led to the assumption that pathotype 4 samples in Yunnan may be composed of different types of *P. brassicae.* At present, these types cannot be clearly distinguished using the Williams system. Therefore, a more accurate identification method is crucial for the prevention and control of clubroot, and for clubroot-resistant breeding.

The application of resistant varieties is the most economical, effective and sustainable measure for the control of clubroot. In this study, Shangpin, Shangpin CR527 and Shangpin CR523 were immune to pathotypes 1,

2, 10 and 14 of *P. brassicae*, however, only Shangpin was immune to all of the types of pathotype 4 of *P. brassicae* (Table 6). These results provide a reference for the selection of Chinese cabbage cultivars in the clubroot disease area of Yunnan Province. They could also provide germplasm for the breeding of clubroot-resistant Chinese cabbage.

Some molecular markers of clubroot resistance have been identified. *CRa* and *CRb* are alleles or closely linked disease resistance loci on the A03 chromosome (Kato *et al.* 2012; Hatakeyama *et al.* 2017). Chinese cabbage with *CRa* and *CRb* could be resistant to pathotype 4 of *P. brassicae* (Saravanan *et al*. 2003) *CRa* and *CRb* loci were detected in Shangpin, Shangpin CR527 and Shangpin CR523 in this study. Shangpin, was only detected in three resistant markers. These three markers were also detected in Shangpin CR523 and Shangpin CR527, but their resistance was lower, indicating that there are still undiscovered resistance sites in Shangpin, and need further research. According to Sakamoto *et al.* (2008), *Crr3* and *CRk* are two independent resistance loci on chromosome A03, with a genetic distance of 7 cM. However, in this experiment, the two resistance loci were quite different. The *Crr3* locus was not detected in the 21 tested varieties, but *CRk* loci were widely detected, even in the susceptible control '83-1'. It is suspected that *CRk* loci may not be closely related to disease resistance genes.

Beisheng CR12 (CR9) was immune to P1-P4, P12-P17, P22, P23, P25, P27 and P31-P34, relating to pathotypes 1, 4, 10 and 14 (Table 3), but did not carry any known resistance genes (Table 5), indicating that unknown resistant genes were present. Beisheng CR12 would be a potential genetic resource for developing resistant markers or genes to prevent clubroot.

Conclusion

P. brassicae was more complex and pathogenic in Yunnan than in other areas of China. The two different strategies and three cultivars were suggested to prevent and control clubroot. Further studies are needed to find the potential genes resistant-clubroot in cultivars Beisheng CR12 and Shangpin.

Acknowledgements

This work was supported financially by the Key Research and Development Project of Yunnan Province (No. 2018BB021). We are thankful to the Vegetable Industry Technology System Experimental Stations in Jianshui, Dehong, Tonghai, Luliang, Kunming and Ludian for collection of *P. brassicae* root samples.

Author Contributions

Designed the experiments: YLY. Performed the experiments: JLY, XYH and EZL.Analyzed the data: XYH and JLY. Contributed reagents/materials/analysis tools: IU and EZL. Wrote the paper: XYH and YLY.

Conflict of Interest

We, the authors, declare no conflict of interest of any kind among ourselves of the institutions where the work was done

Data Availability Declaration

All data reported in this article are available with the corresponding author and will be produced on demand

References

Chen J, XS Ren, HY Song, CQ Li, TC Yuan, J Si (2016). Identification of races of *Plasmodiophora brassicae* from four different regions and resistance identification of new cabbage cross combinations. *J Southwest Univ* 38:67–72

Faggian R, SR Bulman, AC Lawrie, IJ Porter (1999). Specific polymerase chain reaction primers for the detection of *Plasmodiophora brassicae* in soil and water. *Phytopathology* 89:392–397

Fei WX, SF Wang, BC Hu, QS Li, SM Hou, SB Rong, XY Wu (2015). Control effect of different fungicides on clubroot and yield loss caused by *Plasmodiophora brassicae* in *Brassica napus*. *Plant Prot* 41:201–204

Hatakeyama K, T Niwa, T Kato, T Ohara, T Kakizaki, S Matsumoto (2017). The tandem repeated organization of NB–LRR genes in the clubroot-resistant *CRb* locus in *Brassica rapa L. Mol Genet Genomics* 292:397–405

Hatakeyama K, K Suwabe, RN Tomita, T Kato, T Nunome, H Fukuoka, S Matsumoto (2013). Identification and characterization of *Crr1a*, a gene for resistance to clubroot disease (*Plasmodiophora brassicae* Woronin) in *Brassica rapa* L. *PLoS One* 8; Article e54745

Hirai M, T Harada, N Kubo, M Tsukada, K Suwabe, S Matsumoto (2004). A novel locus for clubroot resistance in *brassica rapa* and its linkage markers. *Theor Appl Genet* 108:639–643

Howard RJ, SE Strelkov, MW Harding (2010). Clubroot of cruciferous crops – new perspectives on an old disease. *Can J Plant Pathol* 32:43–57

Karling JS (1942). *The Plasmodiophorales*, 2nd edn. Springer, New York, USA

Kato T, K Hatakeyama, N Fukino, S Matsumoto (2012). Identificaiton of a clubroot resistance locus conferring resistance to a *Plasmodiophora brassicae* classified into pathotype group 3 in Chinese cabbage (*Brassica rapa* L). *Breed Sci* 62:282–287

Lee JE, JS Kang, YW Ki, JH Park, IC Shin, HC Koh (2012). Fluazinam targets mitochondrial complex I to induce reactive oxygen species-dependent cytotoxicity in SH-SY5Y cells. *Neurochem Intl* 60:773–781

Lee SO, GJ Choi, YH Choi, KS Jang, JC Kim (2008). Isolation and characterization of endophytic actinomycetes from Chinese cabbage roots as antagonists to *Plasmodiophora brassicae*. *Microbiol Biotechnol* 18:1741–1746

Li Q, XQ Shen, XC Geng, L Li (2012). Separate inoculation of single resting spores and identification of *Plasmodiophora brassicae* races. *Plant Prot* 38:95–101

Liu F, LH Zhang, GH Ji (2013). Identification of physiological race of *Plasmodiophora brassicae* in Yunnan and Tibet. *Chin Veg* 20:77–81

Lv B, Y Wang, H Xai, Z Yao, C Jiang (2018). Differences in effects of different amendments on growth and soil fertility of Chinese cabbage in yellow brown soil and red soil. *Sci Agric Sin* 51:4306–4315

Mcgrann GRD, T Yoxall, LJ Paterson, JMG Taylor, IG Birmpilis, DR Walters, ND Havis (2017). Control of light leaf spot and clubroot in brassica crops using defence elicitors. *Eur J Plant Pathol* 148:447–461

Peng G, D Pageau, SE Strelkov, BD Gossen, S Hwang, R Lahlali (2015). A >2-year crop rotation reduces resting spores of *Plasmodiophora brassicae* in soil and the impact of clubroot on canola. *Eur J Agron* 70:78–84

Peng SS, ZH Ren, XL Huang, MJ Liu, LF Sun, EM Liu (2013). Physiological race identification on clubroot of cruciferous crops caused by *Plasmodiophora brassicae* in Hunan. *J Changj Veg* 6:46–49

Sakamoto K, A Saito, N Hayashida, G Taguchi, E Matsumoto (2008). Mapping of isolate-specific QTLs for clubroot resistance in Chinese cabbage (*Brassica rapa* L. sspp. *pekinensis*). *Theor Appl Genet* 117:759–767

Saravanan T, M Muthusamy, T Marimuthu (2003). Development of integrated approach to manage the fusarial wilt of banana. *Crop Prot* 22:1117–1123

Shen XQ, K Nie, Q Wu, YG Zhang, XH Meng (2009). Initial research report on differentiation identification of Chinese cabbage clubroot main physiological races. *Chin Veg* 1:59–62

Suo H, LZ Chen, H Xu, B Song, XX Fan, XH Yuan, CZ He (2015). Research progress in clubroot of crucifers. *J Anhui Agric Sci* 126:115–117

Suwabe K, H Tsukazaki, H Iketani, K Hatakeyama, M Fujimura, T Nunome, H Fukuoka, S Matsumoto, M Hirai (2003). Identification of two loci for resistance to clubroot (*Plasmodiophora brassicae woronin*) in *Brassica rapa L. Theor Appl Genet* 107:997–1002

Tan C, YL Yue (2013). Identification of different Chinese cabbage [*Brassica campestris* L. sspp. *pekinensis* (Lour) *Olsson*] varieties resistance to clubroot (*Plasmodiophora brassicae*). *Chin Veg* 8:91–94

Ueno H, E Matsumoto, D Aruga, S Kitagawa, H Matsumura, N Hayashida (2012). Molecular characterization of the *CRa* gene conferring clubroot resistance in *Brassica rapa*. *Plant Mol Biol* 80:621–629

Wang SM, L Yin, T Shen, H Yang, B Shen (2016). Biological characteristics of *Streptomyces albospinus* CT205 and its biocontrol potential against cucumber fusarium wilt. *Biocont Sci Technol* 26:951–963

Webster MA, GR Dixon (1991). Calcium, pH and inoculum concentration influencing colonization by *Plasmodiophora brassicae*. *Mycol Res* 95:64–73

Williams PH (1966). A system for the determination of races of *Plasmodiophora brassicae* that infect cabbage and rutabaga. *Phytopathology* 56:624–626

Yin JL, ZY Qian, YF Zheng, CX Zhao, YL Yue (2018). Physiological race identification of *Plasmodiophora brassicae* and screening of resistant source in Chinese cabbage at Yingjiang County. *Chin Veg* 8:55–57

Yuan Q, IS Druzhinina, XY Pan, ZL Yuan (2016). Microbially mediated plant salt tolerance and microbiome-based solutions for saline agriculture. *Biotechnol Adv* 34:1245–1259

Zheng J, XL Wang, Xiao Y, SQ Wei, D Wang, Y Huang, WM Wang, H Yang (2019). Specificgenes identified in pathotype 4 of the clubroot pathogen *Plasmodiophora brassicae*. *Plant Dis* 103:495–503