



Article

Identification of Black Rot Resistance in a Wild *Brassica* Species and Its Potential Transferability to Cauliflower

Xiao-Guang Sheng ^{1,†}, Ferdinando Branca ^{2,†} , Zhen-Qing Zhao ¹, Jian-Sheng Wang ¹, Hui-Fang Yu ¹, Yu-Sen Shen ¹ and Hong-Hui Gu ^{1,*}

¹ Institute of Vegetables, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China; xguang@zaas.ac.cn (X.-G.S.); zhaozq@zaas.ac.cn (Z.-Q.Z.); wangjs@zaas.ac.cn (J.-S.W.); yuhf@zaas.ac.cn (H.-F.Y.); yusen612@163.com (Y.-S.S.)

² Department of Agriculture, Food and Environment, University of Catania, 95124 Catania, Italy; fbranca@unict.it

* Correspondence: guhh@zaas.ac.cn; Tel.: +86-571-86417316

† X.-G.S. and F.B. contribute equally to this work.

Received: 20 August 2020; Accepted: 7 September 2020; Published: 16 September 2020



Abstract: Black rot is a destructive disease that affects *B. oleracea* crops, causing significant losses to growers throughout the world. The purpose of this study was to screen out new sources resistant to *Xanthomonas campestris* pv. *campestris* race 4 (Xcc4) in 26 cauliflower and six related wild species, and to assess the inheritance of resistance. The results indicate that most of the tested accessions were susceptible or had intermediate resistance, except the Boc4601 (a cauliflower stable inbred line) and PI435896, UNICT5168, and UNICT5169 (wild accessions). Among them, UNICT5169 (*Brassica montana*) and PI435896 (*Brassica balearica*) showed the strongest resistance to Xcc4, with significantly lower disease index (DI), area of the infected part (AIP) and proportion of the infected part to the total leaf area (PTL) values. UNICT 5169 was selected as an Xcc4-resistant parent because of its relatively good cross seed-setting rate with cauliflower cultivars. F₁ hybrids were successfully produced between this wild resistant accession (UNICT 5169) and one susceptible cauliflower breeding line (Boc3202-4), indicating the potential transferability of this resistance to cauliflower. The results of the symptoms severity evaluation of the F₂ population indicate that Xcc4 resistance in UNICT5169 is a quantitative trait, which guides future resistance gene location and black rot resistance breeding.

Keywords: black rot; *Brassica oleracea*; resistance; cross-compatibility; genetics

1. Introduction

Cauliflower (*Brassica oleracea* var. *botrytis*) is an important vegetable, which is widely grown in China, India, Italy, and other countries located mainly in Asia and Europe [1–3]. Black rot is a destructive disease caused by *Xanthomonas campestris* pv. *campestris* (Xcc), which reduces the performance, yield, and quality of *B. oleracea* crops [4–8]. This pathogen invades host plants through hydathodes or wounds, with rapid multiplication that produces high amounts of extracellular polysaccharides and xanthan, clogging the vascular system and, thereby forming typical “V”-shaped chlorotic lesions along the edges of the leaves [9]. With the enlargement of the lesions, the veins also become black and eventually, the entire plant might wither and die. The pathogen has a strong ability to spread and is difficult to control using standard agronomic practices. Pesticide is ineffective because of the potential hazards of pesticide residue and environmental pollution; therefore, breeding Xcc-resistant varieties is undoubtedly the most effective way to control black rot disease.

The search for sources resistant to *Xcc* is complicated because of the existence of nine races of this pathogen, among which races one and four are predominant worldwide [10,11]. Previous studies have reported that *Brassica* B-genomic crops are resistant to race one, such as *Brassica nigra* (BB), *B. juncea* (AABB), and *B. carinata* (BBCC), while A-genome crops are resistant to race four, including *B. rapa* (AA), *B. juncea* (AABB) and *B. napus* (AACC) [12–15]. However, *B. oleracea* crops with the C genome lack specific resistance to races one and four, whereas some resistance to non-mainstream (two, three, six, and seven) and non-specific races has frequently been found [16–19]. It is difficult to transfer the genes resistant to race one and four that exist in the *Brassica* A and B genomes to C-genome crops due to interspecific hybridization obstacles, hybrid sterility, etc. [20,21]. Therefore, breeders still seek new resistant sources of *B. oleracea* to develop black rot-resistant varieties.

The seven cultivated varieties of *B. oleracea* crops have several wild relatives, such as *B. balearica*, *B. incana*, *B. insularis*, *B. macrocarpa*, *B. montana*, and *B. villosa*. These closely related wild species generally have the same 18 chromosomes as the C-genome crops and present a certain degree of sexual cross-compatibility with the *B. oleracea* cultivars [22]. Moreover, these wild species possess high resistance to various pests and diseases. *B. incana* and *B. villosa* are reported to be resistant to *Brevicoryne brassicae*, *Sclerotinia sclerotiorum* and *Verticillium longisporum* [23–25]. In addition, *B. macrocarpa* and *B. insularis* are reported to have high resistance to *Leptosphaeria maculans* and *Pyrenopeziza brassicae*, respectively [26–28]. In our study, disease resistance tests were carried out on different cauliflower accessions and wild relatives and a new *Xanthomonas campestris* pv. *campestris* race 4- (*Xcc*4)-resistant source (UNICT 5169, *B. montana*) was identified from the wild species. F₁ hybrids were also successfully produced between this wild resistant accession and one susceptible cauliflower breeding line (Boc3202-4), indicating the potential transferability of this resistance to cauliflower. The symptoms severity evaluation results of the F₂ population indicate that *Xcc*4 resistance in UNICT 5169 (*B. montana*) is a quantitative trait, which guides future resistance gene location and black rot resistance breeding. Therefore, the present study was framed to identify new black rot resistance source(s) and explore the genetics of resistance in a wild relative of *B. oleracea*.

2. Materials and Methods

2.1. Plant Materials

A total of 32 accessions were screened for black rot resistance (Table 1), including 26 cauliflower materials (eight were F₁ varieties and the other 18 were the stable breeding lines) and six related wild species (*B. balearica*, *B. incana*, *B. insularis*, *B. macrocarpa*, *B. montana*, and *B. villosa*). Cauliflower varieties were purchased from a Chinese market and the other stable breeding lines were created through microspore culture or self-breeding of the purchased varieties. For the wild species, PI435896 and PI662587 were initially collected from U.S. National Plant Germplasm System (NPGS) in 2017. UNICT4785, UNICT5168, UNICT3512 and UNICT5169 were collected from the University of Catania in Italy in 2015. They showed a relatively consistent phenotype in the year of introduction. After that, we selected the most vigorous plant for each accession to carry out artificial self-pollination. For the black rot resistance test, PI435896 and PI662587 were at F₃ generation and UNICT4785, UNICT5168, UNICT3512 and UNICT5169 were at F₅ generation.

A total of 20 seeds for each accession were sown in a 72-hole-tray with nursery substrate and then kept in an artificial climate chamber at 24/2 °C day/night and 75–80% humidity. The first three days consisted of dark conditions, followed by a 10/14 h cycle of light and darkness. The light source was a full spectrum of light emitting diode (LED) with an intensity of 12,000 lux. After 35 days, seedlings with 4–5 true leaves were used for inoculation. Two detached leaves per seedling were selected for inoculation. The evaluations were conducted on nine seedlings per accession, with three replicates.

Table 1. Investigation of *Xanthomonas campestris* pv. *campestris* race 4 (Xcc4) Resistance of Different *B. oleracea* Accessions.

No.	Name	Species	Type	DI Score	Resistance	AIP (cm ²)	PTL (%)
1	SH-80	Cauliflower	F ₁	33.8 ± 2.2 p	IR	1.4 ± 0.2 op	14.4 ± 2.5
2	SH-88	Cauliflower	F ₁	45.7 ± 2.8 klm	IR	1.8 ± 0.3 n	17.9 ± 2.2
3	QN-65	Cauliflower	F ₁	50.6 ± 2.5 hij	S	4.3 ± 0.3 def	40.2 ± 2.8
4	QN-80	Cauliflower	F ₁	43.8 ± 2.4 lm	IR	2.9 ± 0.2 k	28.0 ± 2.0
5	QN-90	Cauliflower	F ₁	37.8 ± 2.3 o	IR	1.6 ± 0.2 no	15.9 ± 2.1
6	ZS-50	Cauliflower	F ₁	56.1 ± 1.2 fg	S	4.7 ± 0.2 c	46.3 ± 2.1
7	ZS-60	Cauliflower	F ₁	45.7 ± 1.5 klm	IR	3.1 ± 0.1 j	32.7 ± 1.1
8	BY-80	Cauliflower	F ₁	33.9 ± 2.9 p	IR	1.4 ± 0.1 op	14.3 ± 1.4
9	Boc3202-4	Cauliflower	DH	83.2 ± 2.5 a	HS	5.8 ± 0.3 a	53.1 ± 2.8
10	Boc3226-4A	Cauliflower	DH	56.9 ± 2.3 fg	S	4.5 ± 0.3 cd	46.1 ± 3.0
11	Boc3206-1	Cauliflower	DH	41.7 ± 2.2 mn	IR	2.2 ± 0.3 m	21.2 ± 2.9
12	Boc3206-4	Cauliflower	DH	58.5 ± 3.1 ef	S	4.2 ± 0.4 efg	45.3 ± 4.4
13	Boc3225-21	Cauliflower	DH	39.3 ± 1.8 no	IR	1.7 ± 0.1 n	17.2 ± 1.1
14	Boc3201-1	Cauliflower	DH	47.2 ± 3.2 jkl	IR	3.4 ± 0.3 ij	24.9 ± 2.3
15	Boc3005-1	Cauliflower	DH	43.7 ± 2.4 lm	IR	2.6 ± 0.2 l	28.4 ± 2.2
16	Boc3228-4	Cauliflower	DH	76.4 ± 3.1 b	HS	5.3 ± 0.4 b	52.8 ± 4.1
17	Boc3203-4	Cauliflower	DH	54.3 ± 4.1 gh	S	3.9 ± 0.4 h	42.1 ± 4.4
18	Boc4259	Cauliflower	SI	65.5 ± 3.1 c	S	4.6 ± 0.3 cd	44.5 ± 2.9
19	Boc4258	Cauliflower	SI	64.3 ± 2.6 cd	S	4.5 ± 0.3 cde	42.2 ± 2.8
20	Boc4222-3	Cauliflower	SI	61.4 ± 3.3 de	S	5.0 ± 0.3 b	51.4 ± 2.9
21	Boc4229	Cauliflower	SI	56.8 ± 1.9 fg	S	4.4 ± 0.2 cdef	45.2 ± 2.1
22	Boc4710-1	Cauliflower	SI	53.8 ± 2.3 gh	S	4.3 ± 0.2 def	43.3 ± 1.9
23	Boc4601	Cauliflower	SI	22.9 ± 1.3 q	R	1.0 ± 0.1 q	12.2 ± 1.2
24	Boc4604	Cauliflower	SI	65.3 ± 1.3 cd	S	5.2 ± 0.2 b	51.1 ± 2.2
25	Boc4605	Cauliflower	SI	51.6 ± 3.0 hi	S	3.9 ± 0.4 gh	39.9 ± 3.0
26	Boc4251	Cauliflower	SI	48.8 ± 2.5 ijk	IR	3.5 ± 0.3 i	35.8 ± 2.7
27	PI435896	<i>Brassica balearica</i>	WD	11.2 ± 0.6 r	R	0.5 ± 0.08 r	4.6 ± 0.9
28	PI662587	<i>Brassica insularis</i>	WD	38.8 ± 2.6 no	IR	1.7 ± 0.2 n	18.6 ± 2.3
29	UNICT4785	<i>Brassica macrocarp</i>	WD	42.4 ± 1.8 mn	IR	4.2 ± 0.2 fg	42.9 ± 2.1
30	UNICT5168	<i>Brassica villosa</i>	WD	26.0 ± 1.7 q	R	1.2 ± 0.1 pq	11.3 ± 1.1
31	UNICT3512	<i>Brassica incana</i>	WD	63.7 ± 2.4 cd	S	5.2 ± 0.3 b	44.5 ± 2.6
32	UNICT5169	<i>Brassica montana</i>	WD	7.8 ± 0.5 r	HR	0.3 ± 0.06 r	2.9 ± 1.2

Note: Accessions 1–8 are cauliflower varieties that are popular in different regions of China. Accessions 9–26 are important pure breeding lines of cauliflower. Accessions 27–32 are *Brassica oleracea*-related wild species. F₁, F₁ variety; DH, doubled haploid; SI, stable inbred line; WD, wild species. HR, high resistance, 10 < DI ≤ 30; R, resistant, 10 < DI ≤ 30; IR, intermediate resistance, 30 < DI ≤ 50; S, susceptible, 50 < DI ≤ 70; HS, highly susceptible, 70 < DI ≤ 100. DI, disease index; AIP, the area of the infected part; PTL, the proportion of the infected part relative to the total leaf area. The values in the columns with the same letters are not statistically different with Duncan's multiple range test at $p \leq 0.05$.

2.2. Inoculation and Disease Resistance Assay

Xcc4, which was kindly provided by Professor Liu Fan (Beijing Academy of Agriculture and Forestry Sciences, China), was used for the inoculation of all tested accessions. Bacterial cultures were grown in potato dextrose agar (PDA) medium for 48–72 h at 30 °C and then the medium was carefully removed using sterile distilled water. The turbidity of the bacterial suspension was adjusted to an absorbance of 1.4 at 600 nm, corresponding to a concentration of $1.0\text{--}3.0 \times 10^9$ cfu/mL. Seedlings with 4–5 true leaves were used for inoculation. The second and third leaves of the seedlings were cut from the petioles with scissors and placed inside a transparent plastic box. The bottom of the box was covered with two layers of non-woven fabric and 15 mL of sterile water was added. All items used in the experiment, including scissors, non-woven fabrics and boxes, were autoclaved (110 °C, 20 min). Two small holes were poked near a secondary vein, approximately halfway between the mid-rib and the leaf margin, in the upper third of the leaf. A bacterial suspension (3 µL) was inoculated on each hole. All inoculated leaves were kept in an artificial climate chamber at 29/25 °C day/night and 85–95%

humidity under a 10/14 h light/dark cycle. The light source was a full spectrum of LED with an intensity of 12,000 lux. The symptoms severity of infected leaves was investigated 10 days after inoculation.

Two methods were used to evaluate the severity of symptoms. One was the traditional grading method with a six-point scale from 0 to 9 based on the relative lesion size and severity (0 = no symptoms; 1 = slight necrosis or chlorosis surrounding the inoculated points; 3 = small V-shaped lesion covering 3–10% of leaf area; 5 = spreading V-shaped lesions covering 10–30% of leaf area; 7 = large V-shaped lesions covering 30–50% of leaf area; 9 = severely infected lesions covering 50%–100% of entire leaf area) [12]. The disease index (DI) was calculated as follows: $\sum (si \times ni) \times 100/9 N$ (si , the incidence level; ni , the number of leaves with the corresponding incidence level; N , the total number of leaves investigated). The DI value was divided into five levels: high resistance (HR), $DI \leq 10$; resistance (R), $10 < DI \leq 30$; intermediate resistance (IR) $30 < DI \leq 50$; susceptibility (S), $50 < DI \leq 70$; high susceptibility (HS), $70 < DI \leq 100$ [12].

The other method used to evaluate the severity of symptoms was based on the plant phenotype measurement system (PPMS, PlantExplorer Spectral HS, Netherlands). Fv/Fm is an important chlorophyll fluorescence parameter and indicator of the photochemical efficiency of photosystem II, which was used to evaluate the degree of leaf disease after infection by the black rot pathogen. Ten days after inoculation, all tested leaves were photographed by the PPMS and the Fv/Fm value of each leaf was calculated by its own PhenoVation Analysis software. The Fv/Fm score of the outermost part of the chlorotic area was considered the critical value, and for most infected leaves, the value was approximately 0.4. Therefore, leaves with an Fv/Fm value less than 0.4 were considered to be infected, and those with an Fv/Fm value higher than 0.4 were considered to be normal. The area of the infected part (AIP) spreading from the inoculated site and its proportion to the total leaf area (PTL) were used to evaluate the severity of infected leaves.

2.3. Cross-Compatibility Survey and F_1 Production

F_1 hybrids were obtained by artificial pollination between one wild accession with high resistance to *Xcc* race 4 (UNICT5169, male parent) and a cauliflower breeding line that showed high susceptibility (Boc3202-4, female parent). In the flowering period of the maternal line, three plants with strong growth and high-quality inflorescences were selected. A total of three inflorescences were selected from each plant, and six to ten flower buds that were going to open were preserved for each inflorescence and the rest were removed. The stamens of Boc3202-4 were removed with pointed tweezers after wiping with 75% alcohol, and then the pollen of UNICT5169 was pollinated on the stigmas. The number of seeds harvested from each pod was recorded. The cross-affinity index (CI) was calculated by the following formula: $CI = \text{total number of hybrid seeds} / \text{total number of cross-pollinated buds}$.

2.4. F_1 Hybrid Identification

The morphology of the F_1 hybrids and their parental lines including growth vigor, crown width and leaf profile, was evaluated under greenhouse conditions. Total genomic DNA was isolated from leaf tissue collected in bulk from tested plants using the traditional cetyltrimethylammonium bromide (CTAB) method [29]. Two specific simple sequence repeat (SSR) primer combinations were selected for their clear distinct polymorphic banding patterns between the parents (Table S1) [30].

PCR amplification was performed following Zhao et al. [30]. The PCR products were electrophoresed in 6% polyacrylamide denatured gels, ran at 100 W for 2 h, and the banding patterns were visualized using silver staining as described by Panaud et al. [31]. The gel was photographed after being dried at room temperature.

2.5. Inoculation of Parents and F_1 , and F_2 Generations

The cultivation procedures and inoculation methods were followed as described above. The parental plants and F_1 hybrids (nine plants per accession) were tested together with F_2 plants. For 103 F_2 generation plants, the inoculation tests of the detached leaves were performed twice.

The first time was at the seedling stage with 4–5 true leaves, and two true leaves were detached for inoculation. The second time was 15 days later and two new true leaves from the same plant were cut and inoculated.

2.6. Data Analysis

Statistical analyses such as Pearson's simple correlation and analysis of variance (one-way ANOVA and Duncan's multiple range test) were performed using SPSS Statistics software, version 21.

3. Results

3.1. Investigation of Resistance to *Xcc* race 4 in Cauliflower and Related Wild Accessions

Three days after inoculation, chlorosis began to appear at the inoculated sites of leaves in vitro. As time progressed, typical V-shaped chlorosis extending inward from the inoculation site was observed accompanied by the browning of the leaf vein. Ten days after inoculation, the severity of symptoms among the tested accessions was significant, signaling that it was an appropriate time to perform the investigation.

3.1.1. Investigation Based on the Traditional Grading Method

We evaluated all inoculated leaves by a six-point scale from 0 to 9 based on the relative lesion size and severity (Figure 1). The results indicate that most of the tested accessions were susceptible or had intermediate resistance, whereas few of them showed resistance to *Xcc*4 (Table 1). Among the F_1 varieties, six ones had intermediate resistance, with DI scores from 33.8 to 45.7, whereas the other two varieties were susceptible, with DI scores of 50.6 (QN-65) and 56.1 (ZS-50). Among the cauliflower pure breeding lines, the average DI score reached 55.1 with a range from 22.9 (Boc4601) to 83.2 (Boc3202-4). Boc4601 is a late-maturing line with a growth period of more than 100 days and was defined as resistant as its DI value is less than 30. There were five lines with a DI score of 30–50, i.e., intermediate resistance. The majority of the cauliflower breeding lines (10/18) showed susceptibility, with a DI value from 51.6 to 65.5. The DI values of the remaining two lines were 76.4 (Boc3228-4) and 83.2 (Boc3202-4) and these lines were classified as highly susceptible. The wild accessions showed significant differences in *Xcc*4 resistance. UNICT3512 had the highest DI score of 63.7 and was considered susceptible. Two accessions were classified as intermediate resistance (PI662587 and UNICT4785) and resistant (PI435896 and UNICT5168) according to their DI scores. UNICT5169 had the lowest DI value (7.8) and was the only material identified as highly resistant. However, according to the statistical analysis, there was no significant difference ($p \leq 0.05$) in the DI value between UNICT5169 and PI435896, indicating that their resistance level was similar.



Figure 1. Grading evaluation of infected leaves according to a six-point scale from zero to nine based on the relative lesion size and severity. (Upper row, photos under ordinary brightfield lighting; bottom row, photos analyzed by the plant phenotype measurement system (PPMS).

3.1.2. Investigation Based on the PPMS

The definitions of the tested materials based on AIP or PTL values were generally consistent with the results of the above-mentioned DI survey (Table 1). The DI value was positively correlated with AIP and PTL at the 0.01 level, and the correlation coefficients were 0.93 and 0.91, respectively (Table 2). The accessions with high DI scores generally had high AIP and PTL values. HR and R accessions had AIP values less than 1.2 cm² and PTL scores lower than 12.2%. The AIP values of IR accessions ranged from 1.4 to 3.5 cm², and the PTL scores ranged from 14.3% to 35.8%. For S and HS accessions, the ranges of the AIP and PTL values were 3.9–5.8 cm² and 39.9–53.1%, respectively. In addition, the wild accession UNICT4785 had a DI score of 42.4 and was classified as IR level, but it had high AIP and PTL values of 4.2 cm² and 42.9%, respectively. Based on the Fv/Fm threshold of 0.4, parts of the UNICT4785 leaves that were slightly distal from inoculated points were infected and injured by *Xcc4*, but no visible symptoms of chlorosis or wilting were observed. However, these injured parts of the leaves could be identified by the PPMS, and their area was also calculated (Figure 2). For UNICT5169 and PI435896, there was no significant difference ($p \leq 0.05$) in their AIP values.

Table 2. Correlation Analysis of Important Data Sets based on the Pearson's Coefficient.

	DI	AIP	PTL	I2-DI	I2-AIP
DI	/	0.93 **	0.91 **	/	/
AIP	0.93 **	/	0.98 **	/	/
PTL	0.91 **	0.98 **	/	/	/
I1-DI	/	/	/	0.97 **	/
I1-AIP	/	/	/	/	0.98 **

Note: DI, disease index; AIP, area of the infected part; PTL, proportion of the infected part relative to the total leaf area; DI, AIP, and PTL, three sets of data obtained from the investigation of leaf disease severity of the 32 accessions. I1-DI and I2-DI, DI value of inoculation 1 and 2 on F₂ plants; I1-AIP and I2-AIP, AIP value of inoculation 1 and 2 on F₂ plants. **, significantly different at $p \leq 0.01$.

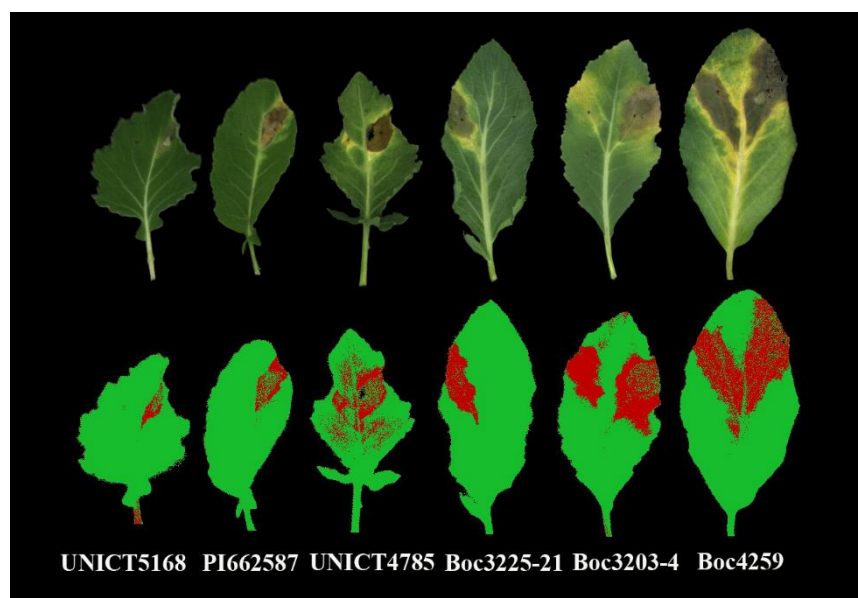


Figure 2. The severity of symptoms on leaves of different accessions 10 days after inoculation with the *Xcc4* bacterial suspension. (upper row, photos under ordinary brightfield lighting; bottom row, photos analyzed by the PPMS).

3.2. Cross-Compatibility Survey and F₁ production

UNICT5169 and PI435896 are wild relatives of *Brassica oleracea*, and these accessions showed high resistance to *Xcc4* among the tested materials. However, the self-compatibility of PI435896

and its cross seed-setting ability with cauliflower cultivars are both very poor, so it is difficult to obtain offspring seeds. To transfer the resistance to susceptible cauliflower breeding lines and to construct segregating populations, UNICT5169 (male parent) was selected to cross with Boc3202-4 (HS, cauliflower, female parent) to produce F_1 hybrids. A total of 78 buds of Boc3202-4 were emasculated artificially, pollinated with UNICT5169, and then bagged to prevent other pollen from touching the stigmas. After about 15 days of growth, the outer bag was removed to check the development of the seed pods. A total of 72 pollinated stigmas had successfully grown healthy seed pods and six ones died of yellowing, probably due to the damage caused to the stigmas during artificial pollination. Therefore, these yellowing and undeveloped stigmas were removed and not counted. The pods contained a maximum of three seeds, and most pods contained only one or two seeds. There were five empty pods, 24 and 34 pods with one and two seeds, respectively, and nine pods with three seeds. A total of 119 seeds were harvested, and the CI index was approximately 1.65 (Figure 3).

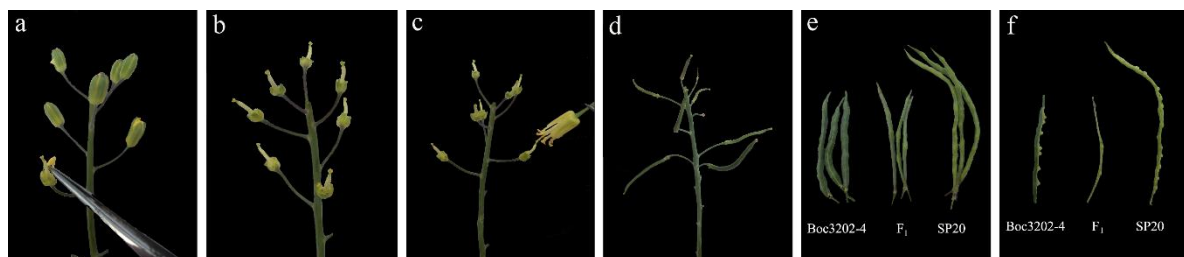


Figure 3. The process of artificial pollination between Boc3202-4 (HS, cauliflower, female parent) and UNICT5169 (HR, *B. montana*, male parent), and F_1 hybrid production. (a) Artificial stamens removal of Boc3202-4 buds. (b) Pistils of Boc3202-4 after the removal of stamens and petals. (c) UNICT5169 pollen was used for pollination of Boc3202-4 stigmas. (d) Developmental status of seed pods 15 days after cross-pollination. (e,f) Developmental status of seed pods 35 days after cross-pollination.

3.3. F_1 Hybrid Identification

F_1 hybrid authenticity was identified using phenotype and SSR molecular markers. The leaf morphology was intermediate between those of the parents, including leaf shape, color, and edge notch. However, F_1 plants did not have cauliflower-type curds. They were tall and had strong growth potential, and the overall phenotype tended to resemble that of the paternal wild species (Figure 4). Two pairs of SSR primers were screened out, because clear and stable polymorphic bands were amplified in the parents. The results show that all 119 F_1 plants contained specific bands of their parents, indicating that they were true hybrids (Figure 4).

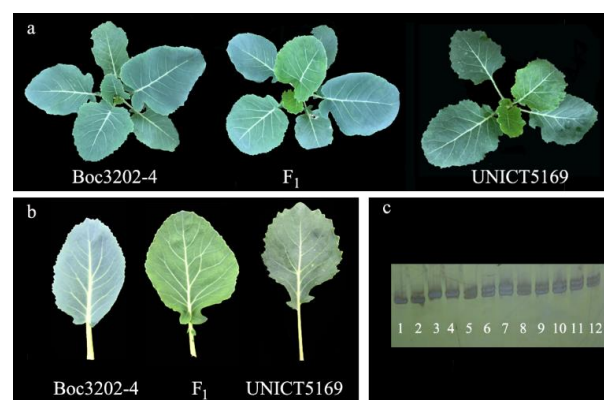


Figure 4. Identification of F_1 hybrids based on morphology and SSR molecular markers. (a) Plant morphology at the seedling stage. (b) Basal leaves of parental lines and the F_1 hybrid. (c) SSR analysis of parental lines and the F_1 hybrids: one and two, specific band for Boc3202-4; three and four, specific band for UNICT5169; 5–12, F_1 hybrids containing the bands from the parents.

3.4. Inoculation of Parents, F₁ and F₂ Generations

The average DI value of the F₁ hybrids was 39.4 and the AIP score was 1.9 cm², i.e., slightly lower than the mid-parent value of 44.4 and 2.9 cm², respectively, indicating that the *Xcc4* resistance of UNICT5169 is not completely dominant relative to the susceptible character (Table 3). Two F₁ plants were randomly selected, and an F₂ population was obtained by artificial self-pollination. The self-fertility of the F₁ hybrids was normal, and most pods contained more than five seeds. To better investigate the genetics of resistance, 103 F₂ plants were used to investigate their reaction to *Xcc4*. The inoculation tests of the detached leaves from F₂ plants were performed twice and there was a significant positive correlation ($p \leq 0.01$) between the two inoculations (Table 2).

Table 3. Investigation of *Xcc4* Resistance of Parents and F₁ Hybrids.

Accessions	Type	DI	AIP
Boc3202-4	female parent	80.8 a	5.6 a
UNICT5169	male parent	8.1 d	0.3 d
Mid-parent value	/	44.4 b	2.9 b
F ₁	F ₁ hybrids	39.4 c	1.9 c

Note: DI, disease index; AIP, the area of the infected part. The values in the columns with the same letters are not statistically different with Duncan's multiple range test at $p \leq 0.05$.

The results indicate that the DI and AIP scores of the F₂ plants were continuously distributed, and there were numerous variations. Moreover, a few F₂ plants showed transgressive inheritance and had higher resistance than UNICT5169. The distribution density of F₂ plants showed a slight right-skewed distribution with a main peak, indicating that the *Xcc4* resistance of UNICT5169 was a quantitative trait, and there might be a major gene control (Figure 5).

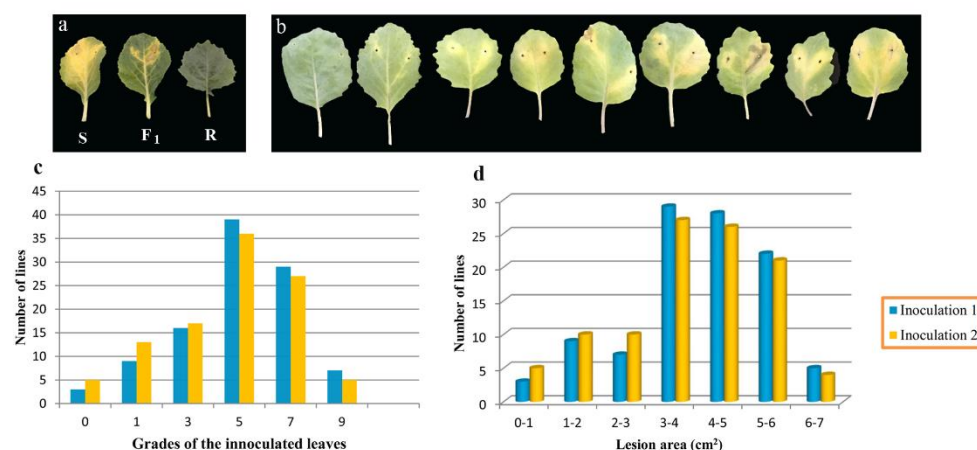


Figure 5. Investigation of symptoms severity to *Xcc4* in the F₂ population from Boc3202-4 (♀) and UNICT5169 (♂). (a) Resistance/sensitivity of F₁ hybrids and their parents to *Xcc4* (S, Boc3202-4. R, UNICT5169). (b) Resistance investigation of the F₂ plants. Frequency distribution of *Xcc4* resistance in the F₂ population based on artificial grading (c) and PPMS (d).

4. Discussion

Screening out materials resistant to black rot is required for the location and cloning of resistance genes, which is also the basis for resistance transfer and breeding new varieties of cauliflower resistant to black rot. The genes resistant to *Xcc4* mainly exist in *Brassica* A-genome crops, such as Chinese cabbage (*Brassica rapa*, $2n = 2x = 20$) [14,32] and rape (*Brassica napus*, AACC, $2n = 4x = 38$) [33]. However, due to the problems of interspecific hybridization and the sterility of hybrids, it is difficult to transfer the *Xcc4*-resistance genes from A-genome crops to *Brassica oleracea* crops. Although some genotypes

of *B. oleracea* have been identified as having *Xcc*-resistance [14,17,34], the breeding application of these resistant accessions has had limited success. In the present study, several accessions with good *Xcc4*-resistance were identified among the wild species, which could be crossed with cauliflower to obtain hybrid seeds without using costly methods such as embryo rescue or protoplast fusion. This discovery is very valuable because resistance to race four is very rare among C-genome crops. The identified wild accessions with high *Xcc4*-resistance can be directly utilized in sexual hybridization for the transformation of resistance to cauliflower and other *B. oleracea* crops.

The leaves infected with *Xcc* showed V-shaped lesions, but they were irregular, and it was difficult to calculate their area. The traditional method to evaluate the phenotype of infected leaves by a DI based on artificial grading is easily affected by sensory factors, which may lead to deviations in experimental results [19]. In this study, PPMS technology was used to assess the symptom severity of leaves inoculated with black rot pathogen. The diseased leaves with chlorotic or yellow lesions consistently exhibited a sharp decrease in the chlorophyll content and photosynthetic efficiency. Fv/Fm is an important chlorophyll fluorescence parameter that can reflect the photochemical efficiency of photosystem II. Therefore, the Fv/Fm value can be used to evaluate the degree of leaf disease after being infected by black rot pathogen. The results of this study show that the disease spot area calculated by the Fv/Fm value of 0.4 was consistent with the traditional DI evaluation, which can reflect the disease grade of leaves. In this way, it is possible to carry out high-throughput and accurate quantitative analysis of susceptible leaves using the PPMS technology. AIP and PTL are two important indexes to measure the disease severity of leaves infected with black rot pathogen. The present results indicate that the consistency of the two sets of data was quite high, so only one index needs to be used. In addition, PPMS technology can detect non-visible damage that occurs after *Xcc4* bacterial infection, thereby improving the accuracy of phenotypic investigation.

Previous research on the heredity laws of resistance to black rot in *B. oleracea* crops indicated that the different resistant accessions of inter- or intra-species had different inheritance patterns. One major recessive gene and two modifiers were reported to control resistance in the cabbage variety ‘Early Fuji’ [35]. With respect to the cauliflower accessions SN455 (no race information) and BR-161 (*Xcc* race one), a single dominant gene controls their resistance [36,37]. Vicente et al. [12] reported a single dominant locus governed resistance to *Xcc* race three in cabbage breeding lines BOH85c and PI436606. In this study, based on a combination of the F₁ and F₂ phenotypic values, the resistance to *Xcc4* in UNICT5169 is a quantitative trait that might be controlled by a major gene accompanied by multiple genetic modifications. However, a larger number of plants of the F₂ population and multiple types of segregation populations, such as BC₁, are needed to accurately assess the inheritance of *Xcc4* resistance.

5. Conclusions

In this study, *Xanthomonas campestris* pv. *campestris* race 4 resistance tests were carried out on different cauliflower accessions and wild relatives. Two methods of traditional DI classification and PPMS technology were used to evaluate the severity of symptoms. A new *Xcc4*-resistant source (UNICT 5169, *B. montana*) was identified and successfully hybridized with one susceptible cauliflower breeding line (Boc3202-4) to obtain F₁ hybrids, indicating the potential transferability of this resistance to cauliflower. The results of the symptoms severity evaluation of the F₂ population indicate that *Xcc4* resistance in UNICT5169 is a quantitative trait, which guides future resistance gene location and black rot resistance breeding.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/9/1400/s1>, Table S1: Two selected SSR primers with polymorphic parental bands.

Author Contributions: Conceptualization, X.-G.S. and H.-H.G.; methodology, Z.-Q.Z. and Y.-S.S.; software, X.-G.S. and Y.-S.S.; validation, J.-S.W. and H.-F.Y.; formal analysis, X.-G.S. and F.B.; investigation, J.-S.W.; resources, H.-H.G. and F.B.; data curation, X.-G.S.; writing—original draft preparation, X.-G.S.; writing—review and editing, H.-H.G. and F.B.; visualization, X.-G.S. and H.-F.Y.; funding acquisition, H.-H.G. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the National Key Research and Development Program of China (2017YFD0101805, 2017YFE0114500), Science and Technology Department of Zhejiang Province (LGN18C150010, 2016C02051-5-4), and Zhejiang Academy of Agricultural Sciences (A1).

Acknowledgments: We are grateful to Liu Fan of Beijing Academy of Agriculture and Forestry Sciences, China for providing the *Xcc* race 4.

Conflicts of Interest: The authors declared no conflict of interest.

References

1. Keck, A.; Finley, J. Cruciferous vegetables: Cancer protective mechanisms of glucosinolate hydrolysis products and selenium. *Integr. Cancer Ther.* **2004**, *3*, 5–12. [[CrossRef](#)] [[PubMed](#)]
2. Abdelkhalik, A.; Pascual, B.; Nájera, I.; Baixauli, C.; Pascual-Seva, N. Deficit irrigation as a sustainable practice in improving irrigation water use efficiency in cauliflower under mediterranean conditions. *Agronomy* **2019**, *9*, 732. [[CrossRef](#)]
3. He, F.; Thiele, B.; Santhiraraja-Abresch, S.; Watt, M.; Kraska, T.; Ulbrich, A.; Kuhn, A. Effects of Root Temperature on the Plant Growth and Food Quality of Chinese Broccoli (*Brassica oleracea* var. *alboglabra* Bailey). *Agronomy* **2020**, *10*, 702. [[CrossRef](#)]
4. Williams, P.H. Black rot: A continuing threat to world crucifers. *Plant Dis.* **1980**, *64*, 736–742. [[CrossRef](#)]
5. Vicente, J.G.; Holub, E.B. *Xanthomonas campestris* pv. *campestris* (cause of black rot of crucifers) in the genomic era is still a worldwide threat to *Brassica* crops. *Mol. Plant Pathol.* **2013**, *14*, 2–18. [[CrossRef](#)]
6. Bella, P.; Moretti, C.; Strano, C.P.; Zaccardelli, M.; Branca, F.; Buonauro, R.; Vincente, J.C.; Catara, V. Race typing and molecular characterization of *Xanthomonas campestris* pv. *campestris* strains occurring in Italy. *J. Plant Pathol.* **2015**, *97*, S17.
7. Bella, P.; Moretti, C.; Licciardello, G.; Branca, F.; Vincente, J.G.; Catara, V. Multilocus sequence typing analysis of Italian *Xanthomonas campestris* pv. *Campestris* strains suggested the evolution of local endemic populations of the pathogen and does not correlate with race distribution. *Plant Pathol.* **2019**, *68*, 278–287. [[CrossRef](#)]
8. Ragasová, L.; Penázová, E.; Gazdík, F.; Pecenka, J.; Cechová, J.; Pokluda, R.; Eichmeier, A. The Change of Bacterial Spectrum after Storage of *X. campestris* pv. *campestris* Inoculated Cabbage Heads (*Brassica oleracea* var. *capitata* L.). *Agronomy* **2020**, *10*, 443.
9. Tonguc, M.; Griffiths, P.D. Evaluation of *Brassica carinata* accessions for resistance to black rot (*Xanthomonas campestris* pv. *campestris*). *HortScience* **2004**, *39*, 952–954. [[CrossRef](#)]
10. Vicente, J.G.; Conway, J.; Roberts, S.J. Identification and origin of *Xanthomonas campestris* pv. *campestris* races and related pathovars. *Phytopathology* **2001**, *91*, 492–499. [[CrossRef](#)]
11. Fargier, E.; Manceau, C. Pathogenicity assays restrict the species *Xanthomonas campestris* into three pathovars and reveal nine races within *X. campestris* pv. *campestris*. *Plant Pathol.* **2007**, *56*, 805–818. [[CrossRef](#)]
12. Vicente, J.G.; Taylor, J.D.; Sharpe, A.G. Inheritance of race-specific resistance to *Xanthomonas campestris* pv. *campestris* in *Brassica* genomes. *Phytopathology* **2002**, *92*, 1134–1141. [[CrossRef](#)] [[PubMed](#)]
13. Westman, A.L.; Kresovich, S.; Dickson, M.H. Regional variation in *Brassica nigra* and other weedy crucifers for disease reaction to *Alternaria brassicicola* and *Xanthomonas campestris* pv. *campestris*. *Euphytica* **1999**, *106*, 253–259. [[CrossRef](#)]
14. Taylor, J.D.; Conway, J.; Roberts, S.J.; Astley, D.; Vicente, J.G. Sources and origin of resistance to *Xanthomonas campestris* pv. *campestris* in *Brassica* genomes. *Phytopathology* **2002**, *92*, 105–111. [[CrossRef](#)] [[PubMed](#)]
15. Sharma, B.B.; Kalia, P.; Yadava, D.K.; Singh, D.; Sharma, T.R. Genetics and molecular mapping of black rot resistance locus *Xca1bc* on chromosome B-7 in Ethiopian Mustard (*Brassica carinata* A. Braun). *PLoS ONE* **2016**, *11*, e0152290. [[CrossRef](#)]
16. Sharma, S.R.; Kapoor, K.S.; Gill, H.S. Screening against sclerotinia rot (*Sclerotinia sclerotiorum*), downy mildew (*Peronospora parasitica*) and black rot (*Xanthomonas campestris*) in cauliflower (*Brassica oleracea* var. *botrytis* subvar. *cauliflora*). *Indian J. Agri. Sci.* **1995**, *65*, 916–918.
17. Griesbach, E.; Loptien, H.; Miersch, U. Resistance to *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson in cabbage *Brassica oleracea* L. *J. Plant Dis. Prot.* **2003**, *110*, 461–475. [[CrossRef](#)]

18. Jensen, B.D.; Massomo, S.M.; Ignas, S.S.; Hockenhull, J.; Andersen, S.B. Field evaluation for resistance to the black rot pathogen *Xanthomonas campestris* pv. *campestris* in cabbage (*Brassica oleracea*). *Eur. J. Plant Pathol.* **2005**, *113*, 297–308. [[CrossRef](#)]
19. Saha, P.; Kalia, P.; Sharma, M.; Singh, D. New source of black rot disease resistance in *Brassica oleracea* and genetic analysis of resistance. *Euphytica* **2016**, *207*, 35–48. [[CrossRef](#)]
20. Mei, J.Q.; Liu, Y.; Wei, D.Y.; Wittkop, B.; Ding, Y.J.; Li, Q.F.; Li, J.N.; Wan, H.F.; Li, Z.Y.; Ge, X.H. Transfer of sclerotinia resistance from wild relative of *Brassica oleracea* into *Brassica napus* using a hexaploidy step. *Theor. Appl. Genet.* **2015**, *128*, 639–644. [[CrossRef](#)]
21. Wang, G.X.; Tang, Y.; Yan, H.; Sheng, X.G.; Hao, W.W.; Zhang, L.; Lu, K.; Liu, F. Production and characterization of interspecific somatic hybrids between *Brassica oleracea* var. *botrytis* and *B. nigra* and their progenies for the selection of advanced pre-breeding materials. *Plant Cell Rep.* **2011**, *30*, 1811–1821. [[CrossRef](#)] [[PubMed](#)]
22. Rvon, B.; Gustafsson, M.; Snogerup, S. Brassica sect. Brassica (Brassicaceae). II. Inter- and intraspecific crosses with cultivars of *B. oleracea*. *Genet. Resour. Crop Evol.* **1995**, *42*, 165–178. [[CrossRef](#)]
23. Singh, R.; Ellis, P.R.; Pink, A.C.; Phelps, K. An investigation of the resistance to cabbage aphid in *Brassica* species. *Ann. Appl. Biol.* **2010**, *125*, 457–465. [[CrossRef](#)]
24. Happstadius, I.; Ljungberg, A.; Kristiansson, B.; Dixelius, C. Identification of *Brassica oleracea* germplasm with improved resistance to Verticillium wilt. *Plant Breed.* **2010**, *122*, 30–34. [[CrossRef](#)]
25. Ding, Y.; Mei, J.; Liu, Y.; Wang, L.; Li, Y.; Wan, H.; Li, J.; Qian, W. Transfer of sclerotinia stem rot resistance from wild *Brassica oleracea* into *B. rapa*. *Mol. Breed.* **2015**, *35*, 225–229. [[CrossRef](#)]
26. Mithen, R.F.; Lewis, B.G.; Heaney, R.K.; Fenwick, G.R. Resistance of leaves of *Brassica* species to *Leptosphaeria maculans*. *Trans. Br. Mycol. Soc.* **1987**, *88*, 525–531. [[CrossRef](#)]
27. Mithen, R.F.; Lewis, B.G.; Heaney, R.K.; Fenwick, G.R. Glucosinolates of wild and cultivated *Brassica* species. *Phytochemistry* **1987**, *26*, 1969–1973.
28. Bradburne, R.; Majer, D.; Magrath, R.; Werner, C.P.; Lewis, B.; Mithen, R. Winter oilseed rape with high levels of resistance to *Pyrenopeziza brassicae* derived from wild *Brassica* species. *Plant Pathol.* **1999**, *48*, 550–558. [[CrossRef](#)]
29. Saghai-Marooof, M.A.; Soliman, K.M.; Jorgenson, R.A.; Allard, R.W. Ribosomal DNA spacer-length polymorphisms in barley. Mendelian inheritance, chromosomal location, and population dynamics. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 8014–8018. [[CrossRef](#)]
30. Zhao, Z.Q.; Gu, H.H.; Sheng, X.G.; Yu, H.F.; Wang, J.S.; Cao, J.S. Genetic diversity and relationships among loose-curd cauliflower and related varieties as revealed by microsatellite markers. *Sci. Hortic.* **2014**, *166*, 105–110. [[CrossRef](#)]
31. Panaud, O.; Chen, X.; McCouch, S.R. Development of microsatellite markers and characterization of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa* L.). *Mol. Gen. Genet.* **1996**, *252*, 597–607. [[CrossRef](#)] [[PubMed](#)]
32. Soengas, P.; Hand, P.; Vicente, J.G.; Pole, J.M.; Pink, A.C. Identification of quantitative trait loci for resistance to *Xanthomonas campestris* pv. *campestris* in *Brassica rapa*. *Theor. Appl. Genet.* **2007**, *114*, 637–645. [[CrossRef](#)] [[PubMed](#)]
33. Guo, H.; Dickson, M.H.; Hunter, J.E. *Brassica napus* sources of resistance to black rot of crucifers and inheritance of resistance. *HortScience* **1991**, *26*, 1545–1547. [[CrossRef](#)]
34. Hunter, J.E.; Dickson, M.H.; Ludwig, J.W. Source of resistance to black rot of cabbage expressed in seedlings and adult plants. *Plant Dis.* **1987**, *71*, 263–266. [[CrossRef](#)]
35. Bain, D. Reaction of *Brassica* seedlings to black rot. *Phytopathology* **1952**, *42*, 316–319.
36. Jamwal, R.S.; Sharma, P.P. Inheritance of resistance to black rot (*Xanthomonas campestris* pv. *campestris*) in cauliflower (*Brassica oleracea* var. *botrytis*). *Euphytica* **1986**, *35*, 941–943. [[CrossRef](#)]
37. Saha, P.; Kalia, P.; Sonah, H.; Sharma, T. Molecular mapping of black rot resistance locus *Xca1bo* on chromosome 3 in Indian cauliflower (*Brassica oleracea* var. *botrytis* L.). *Plant Breed.* **2014**, *133*, 268–274. [[CrossRef](#)]

