Research Status of Clubroot Disease of Cruciferous Crops in China

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Contents

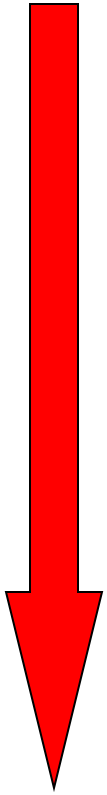
Clubroot disease in China

- Found in Jiangxi, Hunan, Taiwan provinces in 1950s
- Spread out quickly at the end of 1990s
- Distributed over 1 million ha - covering most areas of China and most severely in the southwest, northeast, and middle regions
- Damage found on Chinese cabbage, cabbage, canola, mustards, etc.

Fast spread-out

Clubroot disease incidence(%) on canola in Anhui

Year	Field	Plant	Highest plant-diseased
2003	0.11	1.06	5.67
2004	1.20	1.11	5.89
2005	1.82	2.03	7.80
2006	5.89	2.89	12.00
2007	9.06	7.12	21.07
2008	10.33	40.08	66.67
2009	28.96	10.33	45.78
2010	41.70	8.80	52.34
2011	71.70	19.49	54.40
2012	71.90	24.47	92.20



China Team of clubroot disease

In 2010, the Ministry of Agriculture, China set up a nationwide program titled “Research and Demonstration of Control Technologies for Clubroot Disease of Cruciferous Crops (201029030) ”, including:

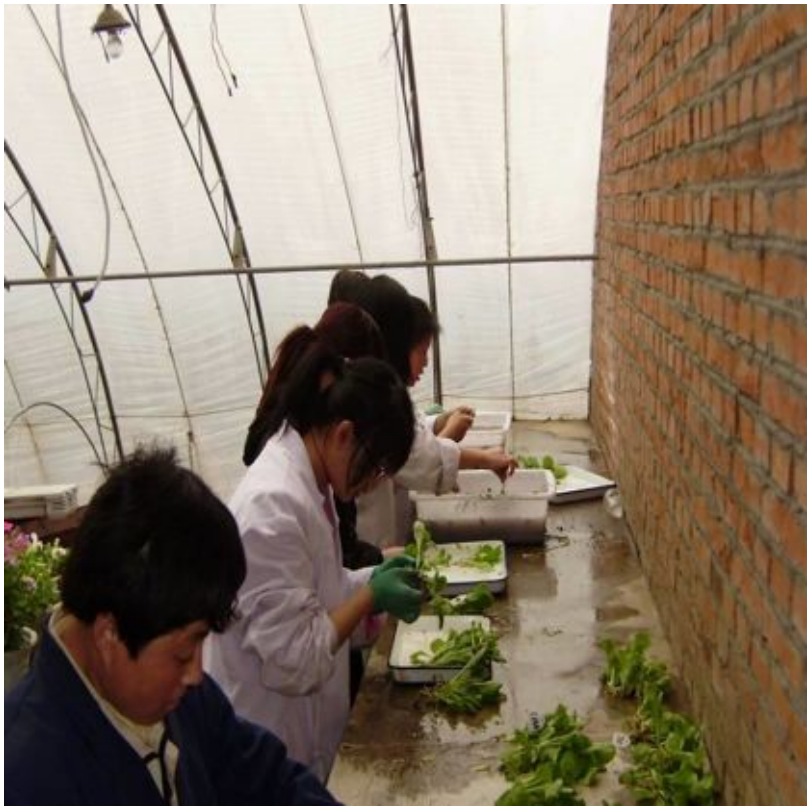
16 public institutions, such as the agricultural universities of Yunnan, Sichuan, Huazhong, Hunnan, China, Shenyang, Anhui and Tibet, comprehensive universities of Southwest, Zhejiang, and Science and Technology of East China and the agricultural academies of Yunnan, Jiangxi, China (CAAS), Beijing and Liaoning. A total of 25 principal scientists are involved into the program under the leadership of Yunnan Agricultural University.

Other members' research

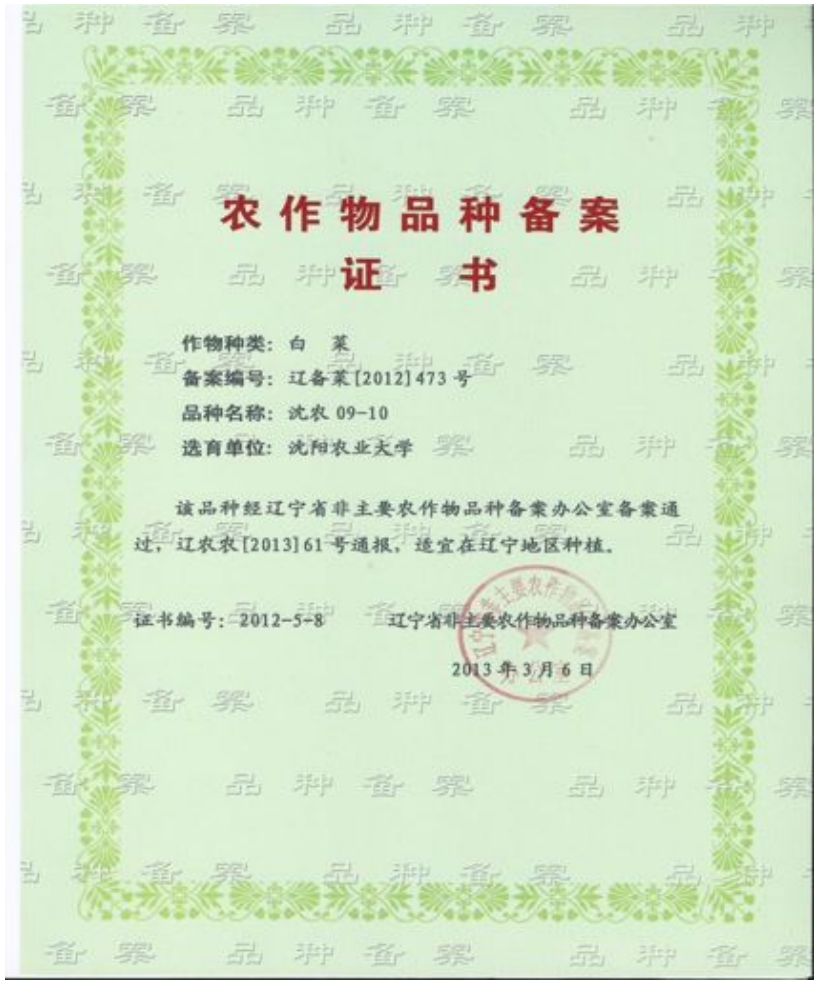
1. Resistance breeding: Chinese cabbage, cabbage, and cauliflower

Liaoning team's achievements:

- resistance breeding of Chinese cabbage
- >100 lines including male sterile and restorer lines
- 4 combinations released commercially



Shennong 09-10



农作物品种备案证书

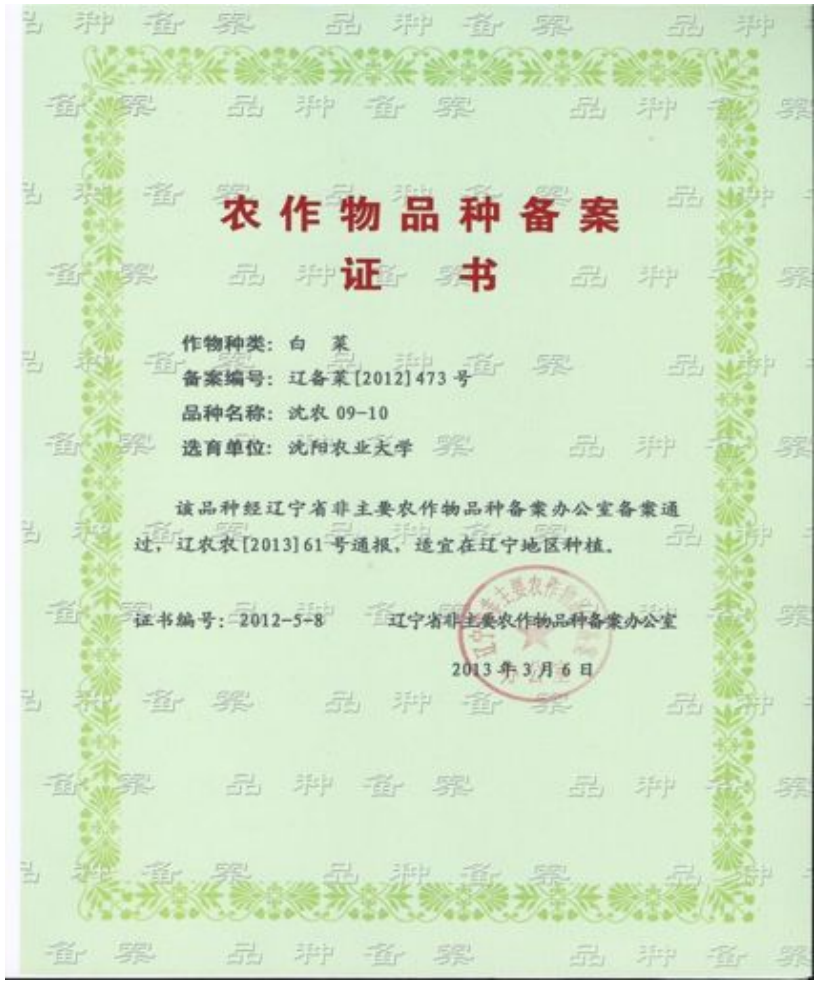
作物种类：白　菜
备案编号：辽备菜[2012]473号
品种名称：沈农09-10
选育单位：沈阳农业大学

该品种经辽宁省非主要农作物品种备案办公室备案通过，辽农农[2013]61号通报，适宜在辽宁地区种植。

证书编号：2012-5-8　　辽宁省非主要农作物品种备案办公室

2013年3月6日

Shengnong 09-10F1



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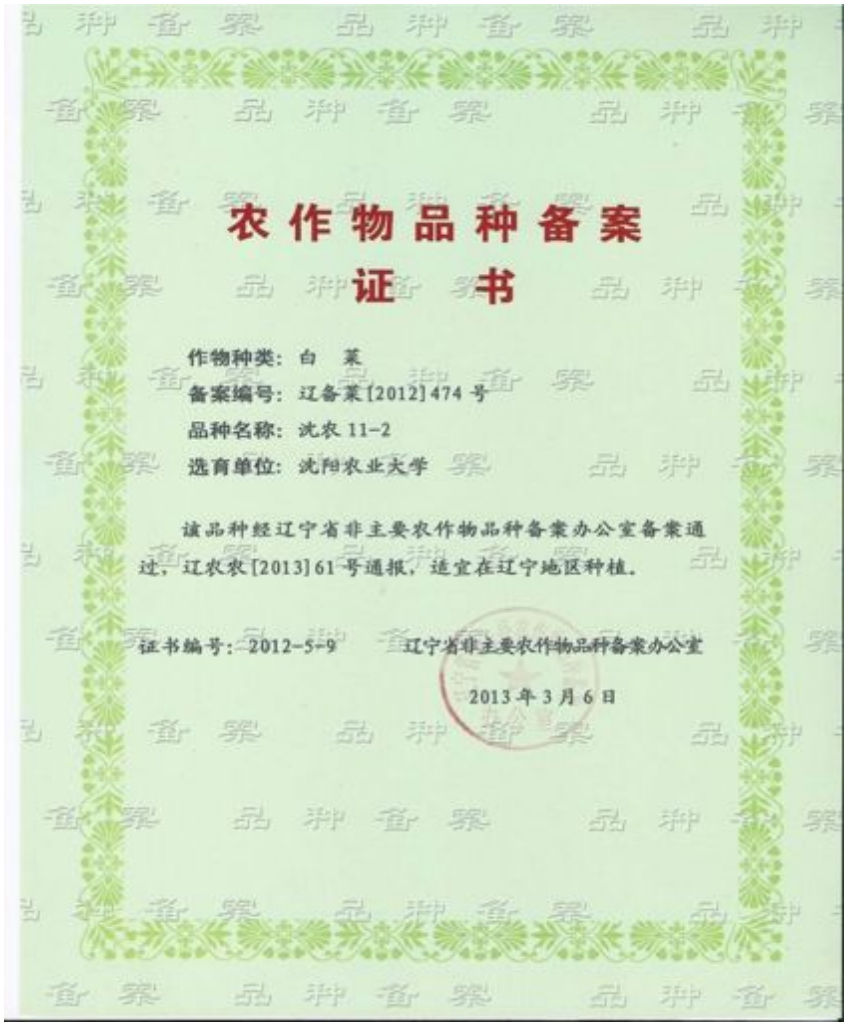
证书编号：2012-5-8　　辽宁省非主要农作物品种备案办公室

2013年3月6日

Shengnong 11-2F1



农作物品种备案证书

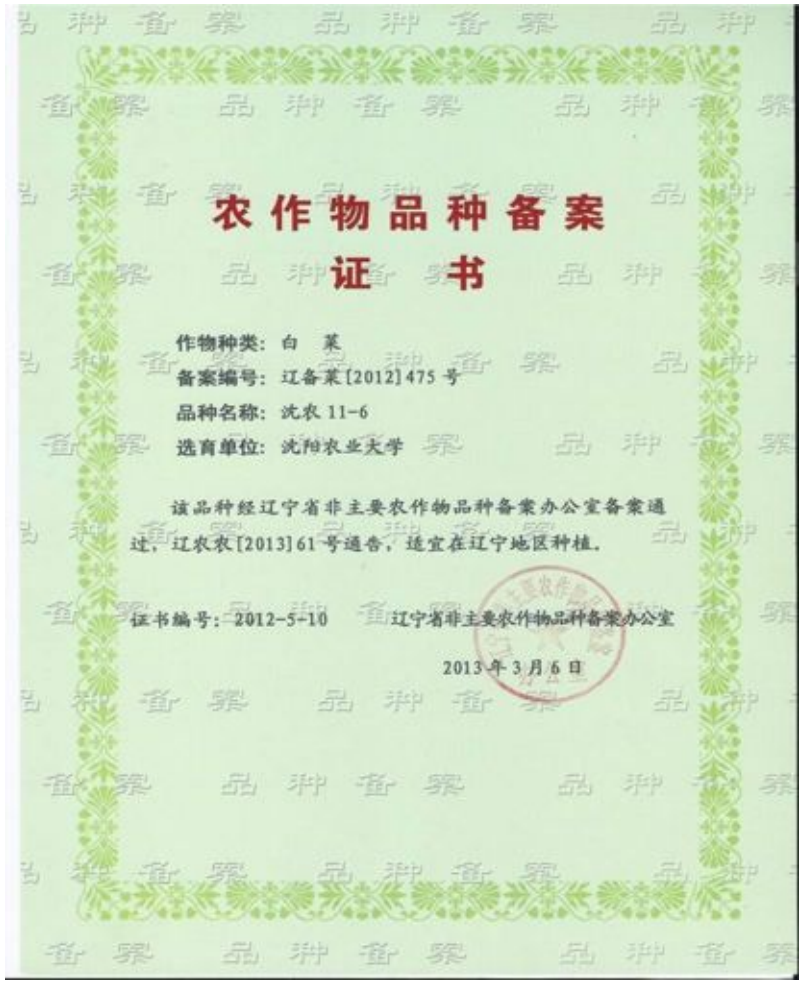
作物种类：白　菜
备案编号：辽备菜[2012]474号
品种名称：沈农 11-2
选育单位：沈阳农业大学

该品种经辽宁省非主要农作物品种备案办公室备案通过，辽农农[2013]61号通报，适宜在辽宁地区种植。

证书编号：2012-5-9　　辽宁省非主要农作物品种备案办公室

2013年3月6日

Shennong 11-6F1



农作物品种备案
证书

作物种类：白　菜
备案编号：辽备菜[2012]475号
品种名称：沈农 11-6
选育单位：沈阳农业大学

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证书编号：2012-5-10　　辽宁省非主要农作物品种备案办公室

2013年3月6日

Jiangming HE's team

YAU team

Zhianbai 1

Resistant Cauliflowers from Beijing Team

Cf9, DI 3.2%

Cf6 , DI 19.7%

Cf11 , DI 5.3%

Cf12 DI 9.8%

Cf5 , DI 12.9%

Cf24 , DI 11.6%

Cf26 , DI 20.1%

Other members' research

2. Identification of physiological races

Identification system

Inoculum: 2.0×10^{6}-^{7} spores/ g soil

Williams system including:

2 cabbages: Jersey queen(JQ), Badger Shipper(BS)

2 rutabagas: Laurentiana(LT), Wilhelmsburger(WB)

Race distribution in China

Williams race model

Note: + means S; - means R.

Variety	Race Model															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
JQ	+	+	+	+	−	+	+	−	−	+	−	+	−	−	−	−
BS	−	+	−	+	−	−	+	−	−	+	+	−	+	+	+	−
LT	+	+	+	+	−	−	−	+	+	−	+	−	+	−	−	−
WB	+	−	−	+	−	−	−	−	+	+	+	+	−	+	−	+

Races distribute in China

There are 10 races, such as Races 2, 3, 4, 5, 7, 8, 9, 10, 11, 13 and 15 in China, but Races 4, 7 are dominant and distributed in all the clubroot disease regions.

Other members' research

3. Control methodology

- More than 20 fungicides were tested for evaluating their control effects, the most useful ones are fluazinam and cyazofamid.
- Before seeding, spray cyazofamid (a.i. 1000g/ha), then drench fluazinam (a.i. 2000g/ha) after seed germination.
- This combination can control the disease by about 70% - 80%.

Canola seed dressing

- **Fluazinam with 1% sodium alginate dressed canola seed;**
- **Cyazofamid with 1% sodium alginate dressed conola seed;**
- **75% control effect.**

Canola seed dressing with chemicals

Treatment	Location	I	II	Mean	Control effect%
		Incidence%			
Cya.	Xiuning	55.00	28.57	37.10	35.21
	Zhijiang	30.19	14.75	22.47	49.75
Flua.	Xiuning	5.33	0	2.70	95.28
	Zhijiang	13.73	8.33	11.03	75.34
CK	Xiuning	60.78	53.73	57.26	-
	Zhijiang	57.14	32.29	44.72	-

Dressed canola seeds

Control effect with fluazinam

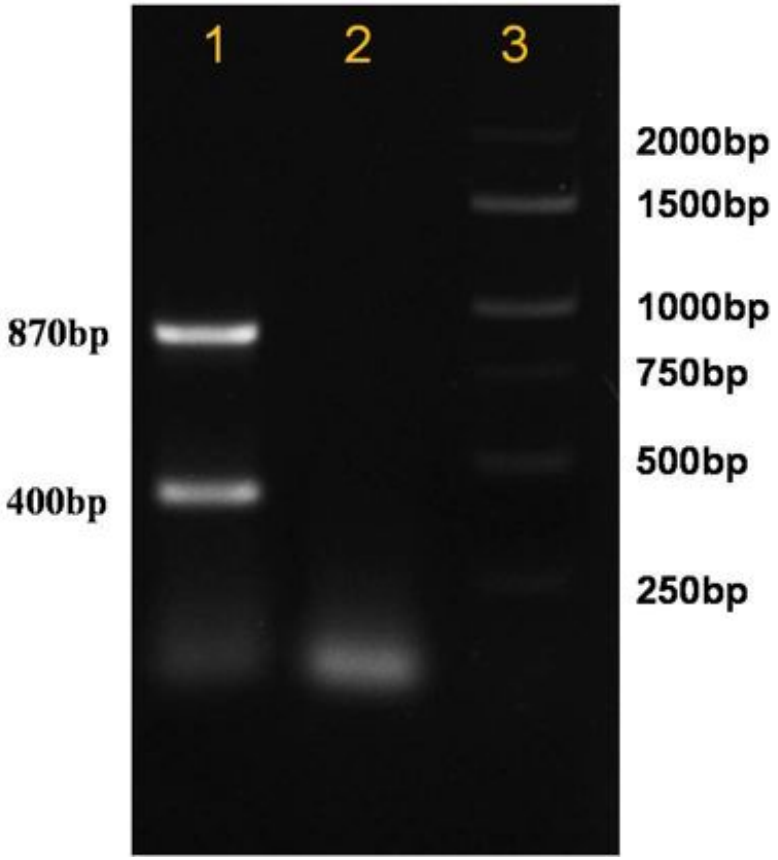
Control effect with cyazofamid

Negative control

Other members' research

4. Diagnostic technology

Development of rapid detection kit



Please contact Prof. Liqun ZHANG, China Agri. Uni. by his email: zhanglq@cau.edu.cn, if you need more details.

Other members' research

5. Biocontrol

- Five groups, Yunnan Agri. Uni., Sichuan Agri. Uni., Hunan Agri. Uni., Zhejiang Uni. Southwest Uni., are involved in screening bioagents and some strains of *Streptomyces*, *Bacillus* spp. and *Lysobacter* sp. are effective.

YAU team's research

- 1. The floating system (establishing brassica seedlings in water) spreads clubroot disease:
  - the disease area growing as fast as this system in Yunnan
  - the experiment confirmed the hypothesis.

Yunnan team's research

Table 1 Design for confirmation of water carrying the pathogen

处理 Tretment	作物 Crop	基质 Substrate	栽培方式 Culture style	浇灌 Wateri
1	大白菜 Chinese cabbage	腐殖土 Humus	传统苗床 TSB	无根钟菌水
2	大白菜 Chinese cabbage	灭菌腐殖土 SH	传统苗床 TSB	无根钟菌水
3	大白菜 Chinese cabbage	灭菌腐殖土 SH	传统苗床 TSB	晋山城关育苗
4	大白菜 Chinese cabbage	灭菌腐殖土带菌量 10^3 个/g SH with 10^3 spores/g	传统苗床 TSB	无根钟菌水
5	甘蓝 Cabbage	腐殖土 Humus	传统苗床 TSB	无根钟菌水
6	甘蓝 Cabbage	灭菌腐殖土 SH	传统苗床 TSB	无根钟菌水
7	甘蓝 Cabbage	灭菌腐殖土 SH	传统苗床 TSB	晋山城关育苗
8	甘蓝 Cabbage	灭菌腐殖土带菌量 10^3 个/g S H with 10^3 spores/g	传统苗床 TSB	无根钟菌水
9	大白菜 Chinese cabbage	灭菌腐殖土 SH	漂浮接种带菌量 10^3 个/g Floating on SW with 10^3 spores/g	无根钟菌水
10	大白菜 Chinese cabbage	灭菌腐殖土 SH	昆明沙朗乡育苗池水漂浮 Floating on KSW	无根钟菌水
11	大白菜 Chinese cabbage	灭菌腐殖土 SH	晋山城关育苗池水漂浮 Floating on YCW	无根钟菌水
12	大白菜 Chinese cabbage	灭菌腐殖土 SH	无根钟菌水漂浮 Floating on SW	无根钟菌水

1)1～8:传统育苗 Traditional nursing; 9～12:漂浮育苗 Floatation nursing; SH: Sterilized humus; TSB: Traditional seedling bed; SW: Sterilized water; KSW: Water collected from a floatation culturing system of Shalang Township, Kunming; YCW: Water collected from a floatation culturing system of Yanshan Township; CW: Clean water without *Plasmodiophoru brassicae* .

Table 2 Disease incidence and disease inde of cabbage clubroot disease after different treatment

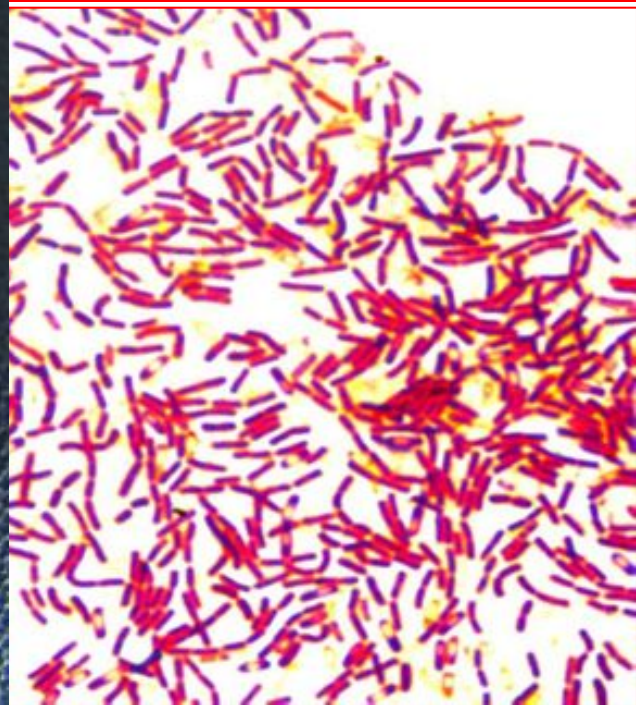
处理 Treatment	发病率/% Disease incidence	病情指数 Disease inde
1	0.00	0.00
2	0.00	0.00
3	47.34 b	12.22 b
4	94.52 a	23.35 a
5	0.00	0.00
6	0.00	0.00
7	12.52 d	2.13 ef
8	20.94 cd	4.30 de
9	43.75 b	8.81 c
10	29.57 c	6.30 cd
11	29.64 c	6.05 cd
12	0.00	0.00

YAU team's research

2. Biocontrol with *Bacillus subtilis* XF-1

Based on ecological balance, XF-1 was isolated from *Plasmodiophora brassicae*-infected soil in 2004 and patented (ZL200810058919.0) with sequence information in 2008 after we tested more than 1000 strains.

I. Morphology



II. Control effect

Application methods

- Dress seeds with 10^7 CFU/ml before seeding
- Drench the soil after seeding
- Drench the rhizospheric soil of seedlings three times, at 7, 14 and 21 days after germination
- Pellet-dress seeds.

Control effect on Chinese cabbage clubroot in the greenhouse, 2007

Days after germination	Disease index	Control effect(%)
12	0	100
17	0	100
22	0	100
27	0.60	98.70
60	1.19	98.33
85	14.29	85.44

The field trial in Songming County at 85th day after seeding in 2007

	CK	I	II	III	Average
Total plant	219	174	176	156	169
Diseased plant	106	10	8	3	7.00
Incidence(%)	0.48	0.06	0.05	0.02	0.04
Control effect (%)	-	88.12	90.60	96.03	91.58

Chinese cabbage

Chinese cabbage

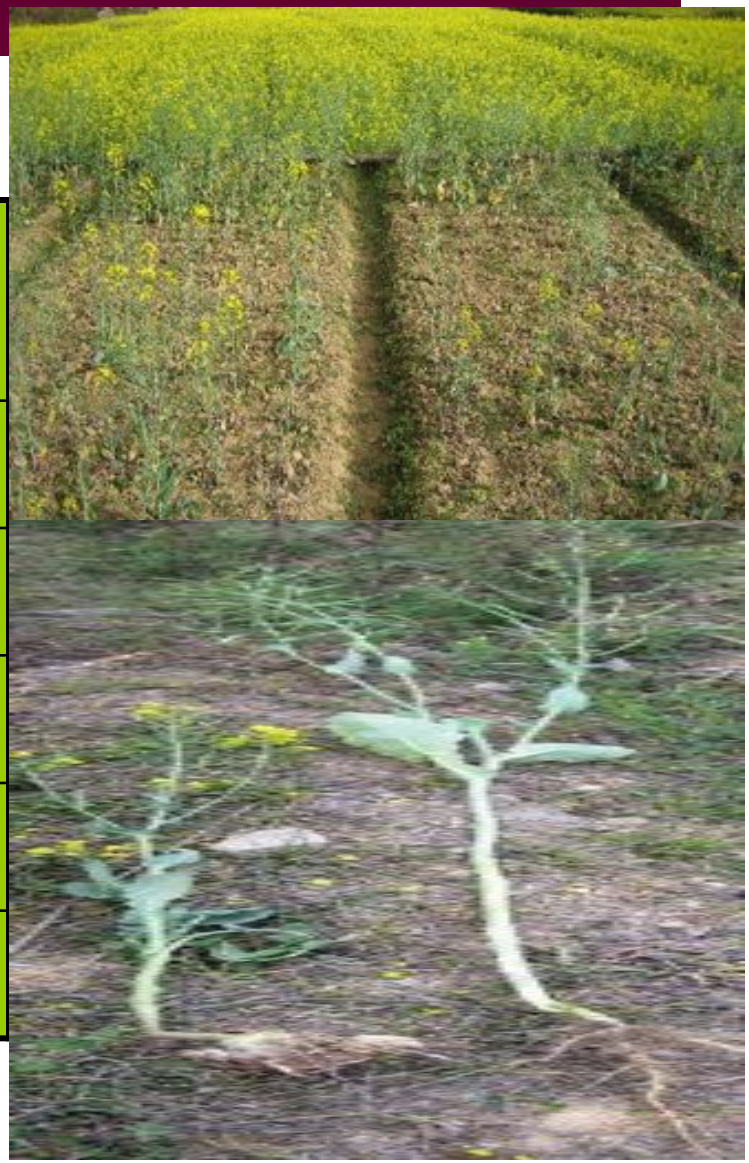
Treatment with XF-1

Application in Yunnan in 2011

Application in Liaoning in 2012

Control of clubroot in canola in 2008

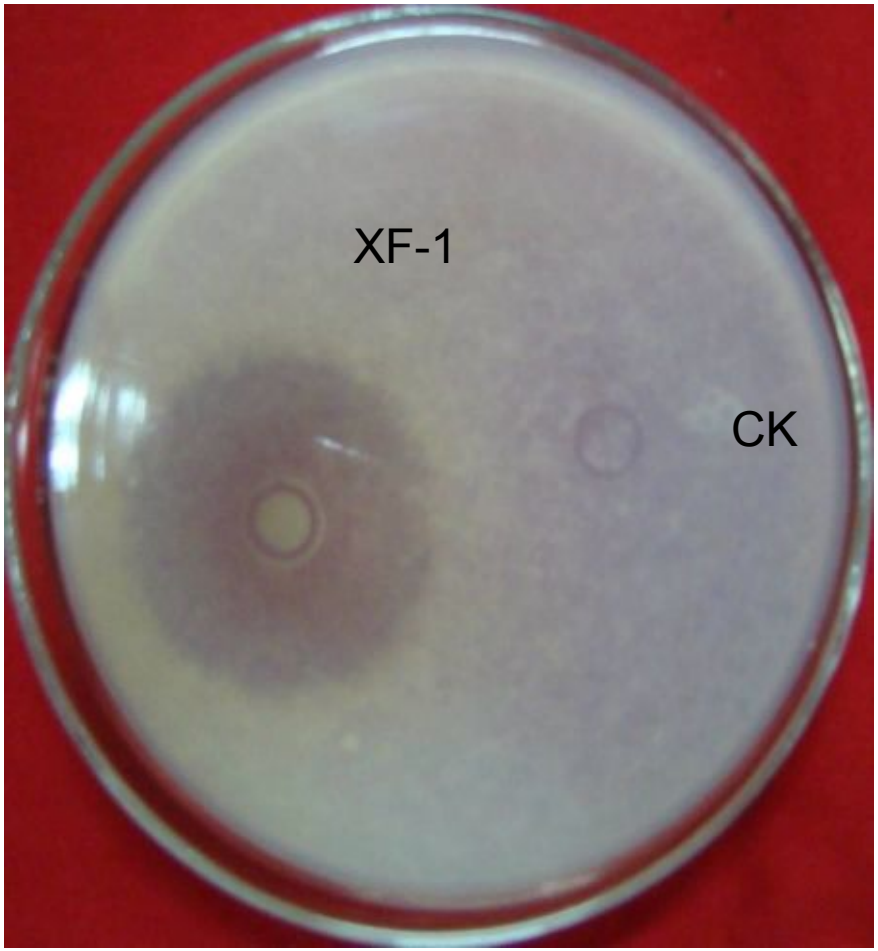
County	Tr.	Area (ha)	Control (%)	Yield increase (%)
Longyang	XF-1	37	94.6	25.1
	Hymexazol	20	88.0	19.3
Longling	XF-1	6	79.8	24.8
Changnin	XF-1	6	93.1	28.7
	Chlorothalonil	1	69.4	13.4



III. Why?

1. Chitosanase gene *csn*, 834bp long and encodes a 30 kD protein consisting of 277aa, was found to be involved in the disease control of XF-1
2. *PBR1* gene, 753bp long, encodes a 25kD protein composed of 251 aa, which is patented

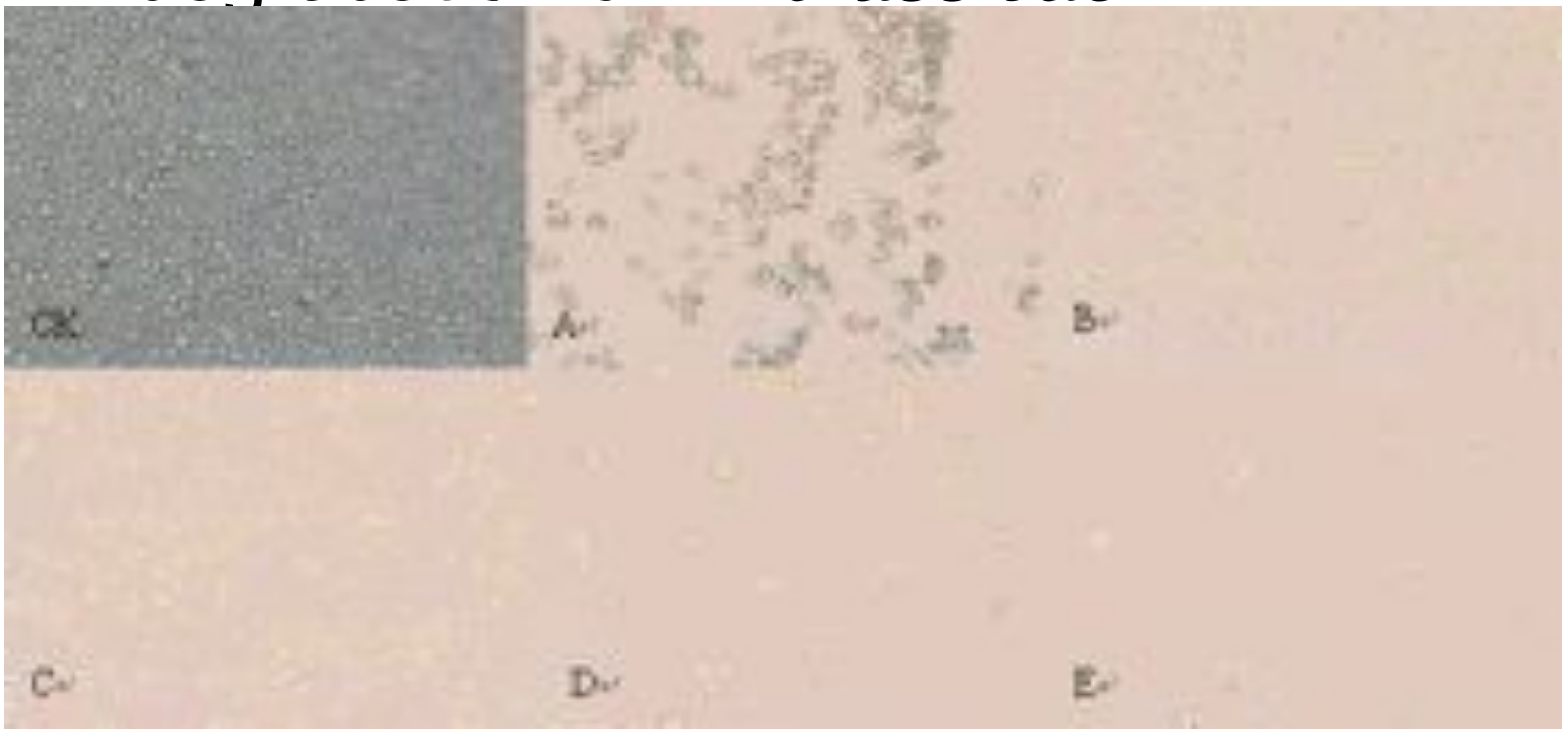
The proteins were precipitated by ammonium sulfate.



Fusarium solani

1) 1st protein:

degradation of *P. brassicae*



CK：untreated；A：immediately treated；
B：30 min later；C：1 hour later；D：2 hours later；E：3 hours later

The temperature-endurable protein sequence

1 MKISMQKADF WKKAAISLLV FTMFFTLMMS ETVFAAGLNK DQKRRAEQLT

51 SIFENGTTEI QYGYVERLDD GRGYTCGRAG FTTATGDALE VVEVYTKAVP

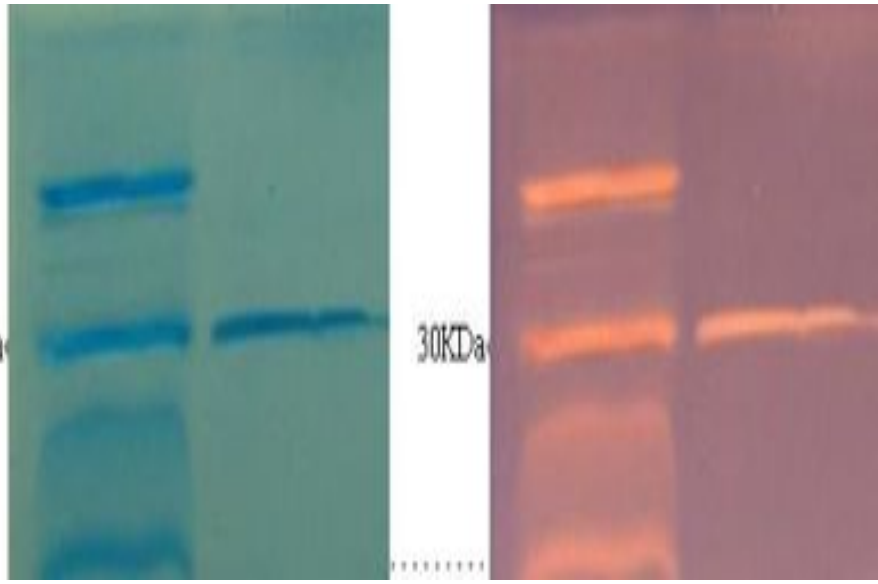
101 NNKLKKYLPE LRRLAKEESD DTSNLKGFAS AWKSLANDKE FRAAQDKVND

151 HLYYQPAWRR SDNAGLKTAL ARAVWYDTVI QHGDGDDPDS FYALIKRTNK

201 KAGGSPKDGI DEKKMLNKFL DVRYDDLMNP ANIDTRDEWR ESVARVDVLR

251 SIAKENNYNL NGPIIMRSNE YGNFVIK

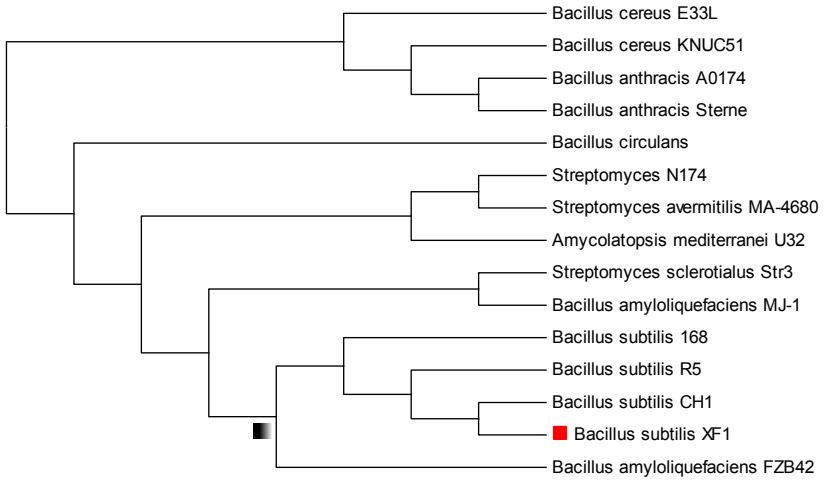
Matched peptides shown in Bold Red



At 121℃, sterilized for 20min, then separated on SDS-PAGE

Analogue to Chitosanase

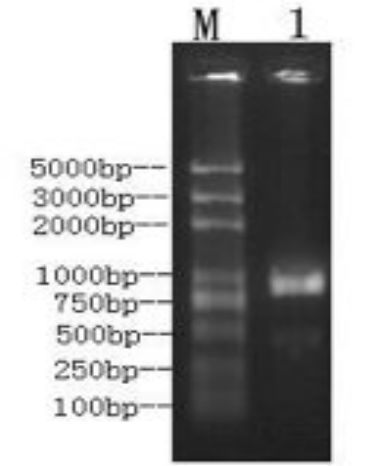
	XF-1	B168 and R5
Gene length(bp)	834	834
Similarity(%) in DNA sequence	99	99
Nucleic acid difference	69, 140, 141, 174, 179, 765, 768, 774, 789	69, 140, 141, 174, 179, 765, 768, 774, 789
Similarity(%) in aa sequence	98	98
aa difference	19, 47, 60, 256, 257	19, 47, 60, 256, 257
Amino acid	Ile, Val, Thr, Asp, Lys	Met, Glu, Ile, Glu, Asp



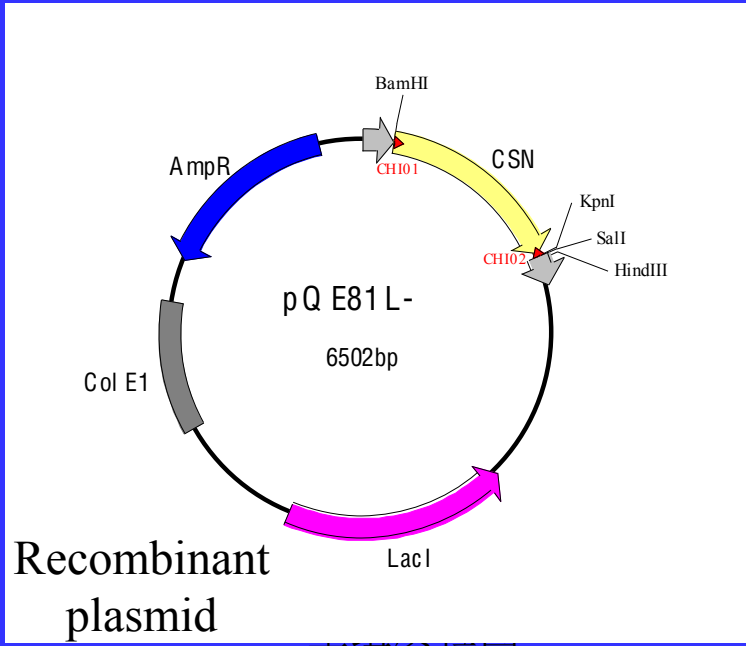
csn amplification of XF-1 and B168 from their genomes

- Specific primers

CHI01 (*Bam*HI) :
5’-GCGGATCCCATGAAAATCAGTATGCAAACAG-3’
CHI02 (*Kpn*I) :
5’-GAGGTACCTGTCTCTTGTCTTTTCCGCATC-3’

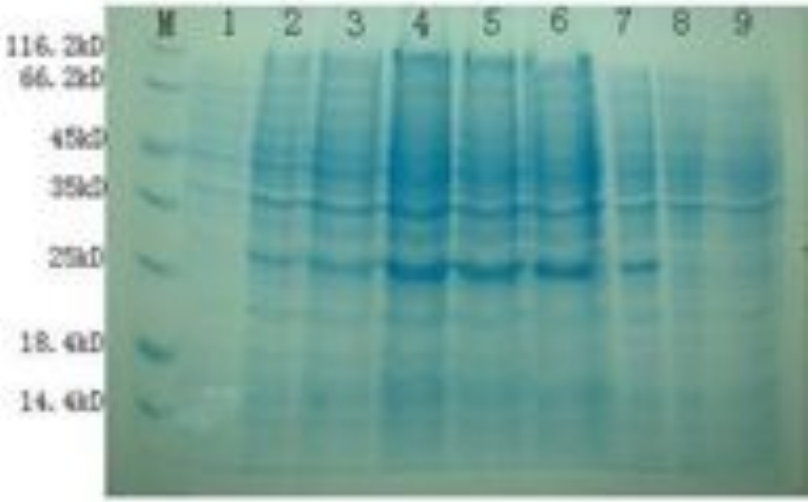


Profile of ***csn*** **gene by** PCR amplification

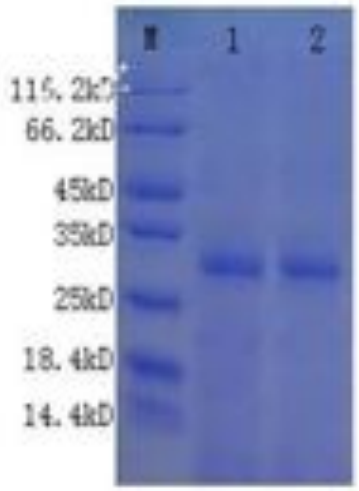


Recombinant plasmid

csn genes of XF-1 and B168 expressing in *E. coli* B21

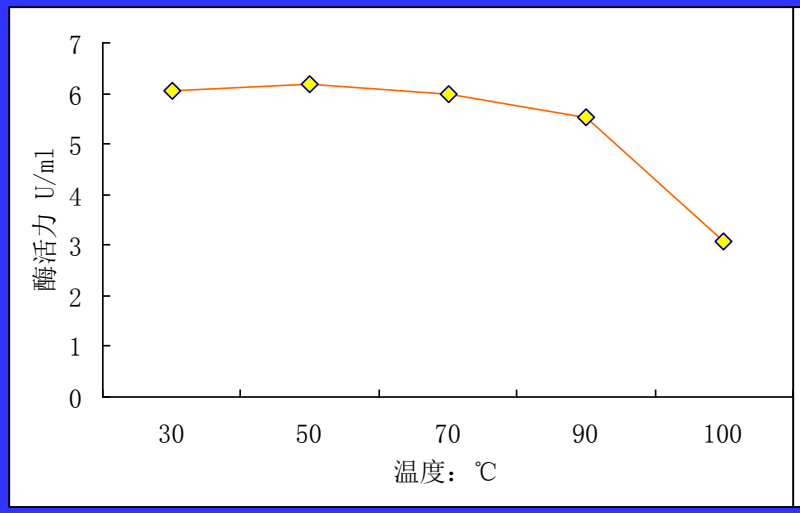


SDS-PAGE profile of induced *csn* gene expression of XF-1 and B168

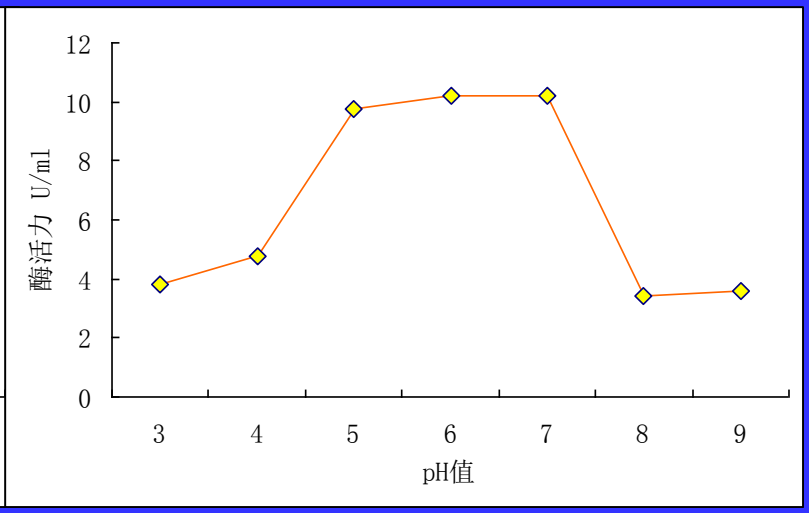


Purified chitosanase by Ni-NTA

Activity of the Chitosanase expressing in *E. coli* B21 by testing Ethyl amide glucose with spectrophotometer 174 (UV 2100)



Temperature

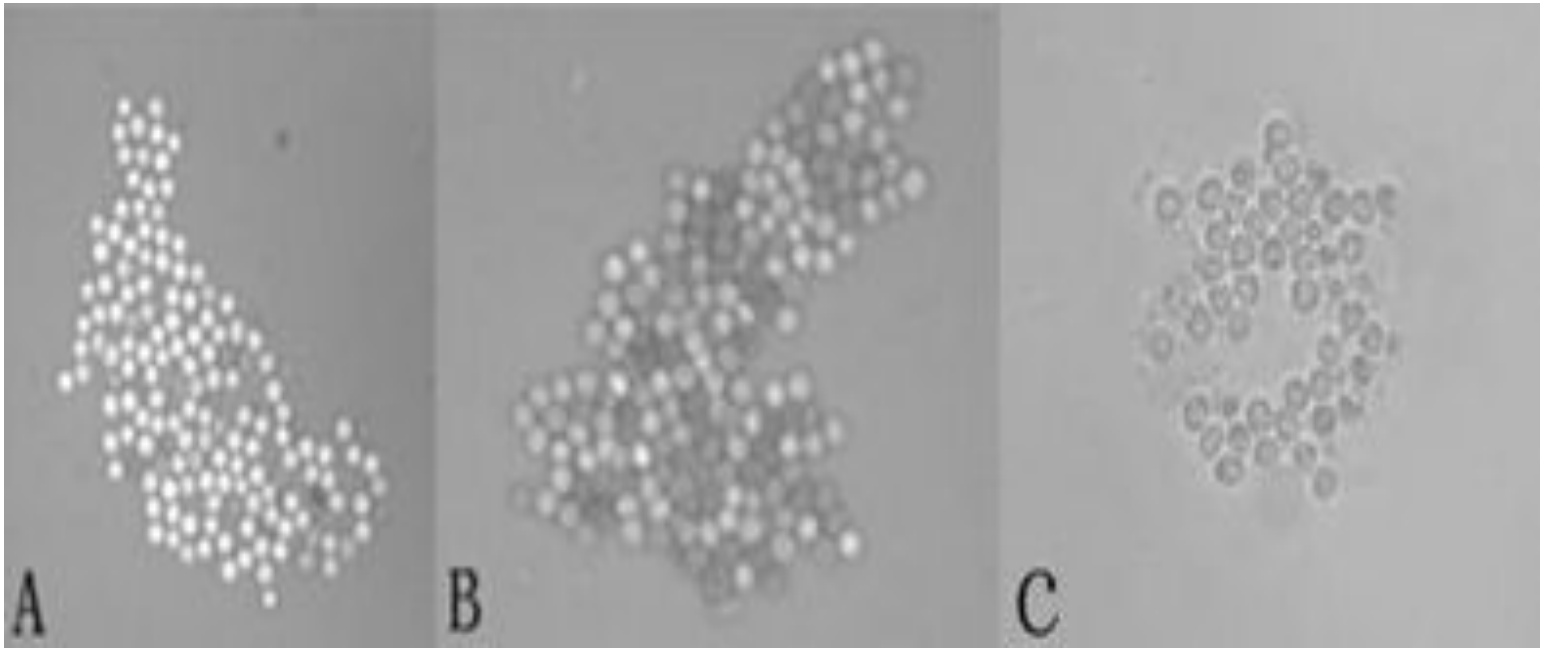


pH value

Suppression effect of chitosanase from XF-1 and B168

Fungus	Suppression effect			
	Exp. Protein of XF-1	XF-1 strain	Raw protein of XF-1	Exp. Protein of B168
M. grisea. rice blast	++	+++	++++	-
C. lunata. leaf spot of maize,	++	+++	+++	-
F. oxysporum f. sp. *Dianthi*, carnation wilt	++	++++	+++++	-
F. solani, root rot of pseudogengseng,	+++	++++	+++++	-

Notice： -： no inhibition; ++: ≥0.2cm; +++： ≥0.5cm;++++: ≥0.8cm; +++++: ≥1.0cm for inhibitory zone.



The resting spores treated with **Chitosanase expressed by *E. coli***

A. Ck；B. Treated for 24h；C. Treated for 36h

2) The 2nd protein

- By the same procedure, *PBR* gene was cloned.

Suppression effect of the purified PBR1 proteins from XF-1 and B168

Fungus	Protein source	
	XF-1	B168
M. oryzae. rice blast	+++	_
C. lunata. leaf spot of maize,	++++	_
F. oxysporum f. sp. *Dianthi*, carnation wilt	++++	_
F. solani, root rot of pseudogengseng,	++	_

注： Notice： ++: ≥0.2cm; +++：≥0.5cm;++++: ≥0.8cm.

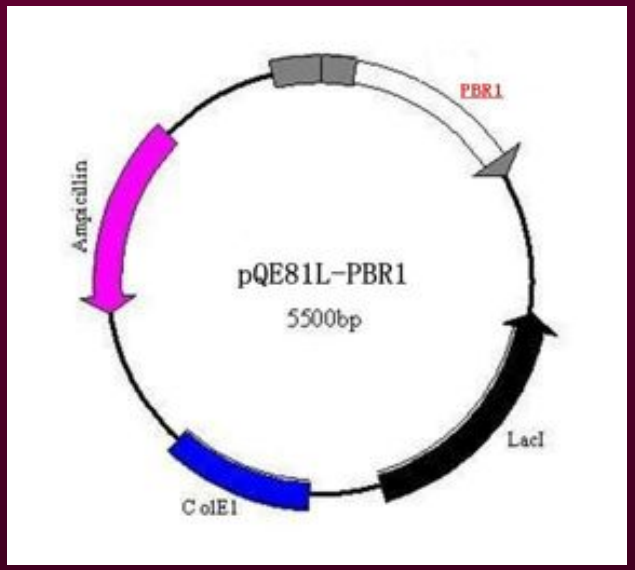
PBR1 gene amplified from XF-1 genome

- Primers designed based on the aa sequence: PBR01 and PBR02

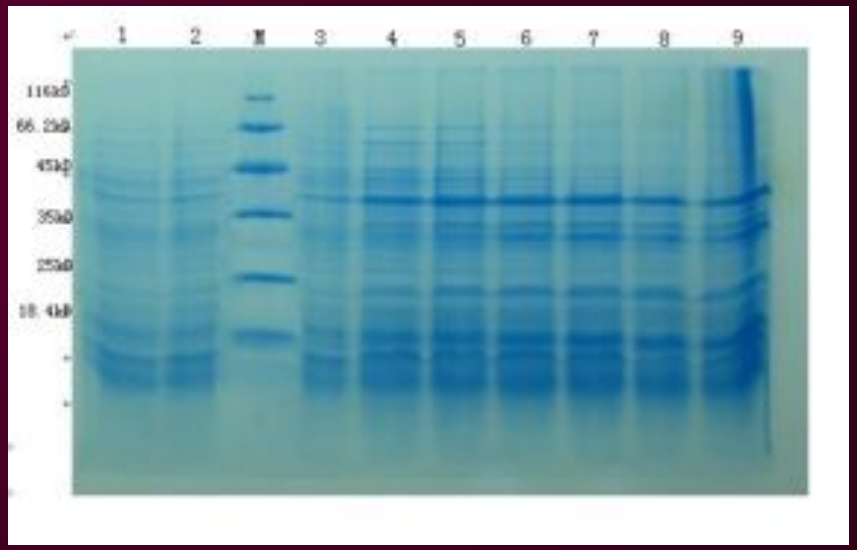
PBR1 gene amplified by PCR

1: B168; 2 and 3: XF-1

PBR1 gene expressing in *E. coli* B21



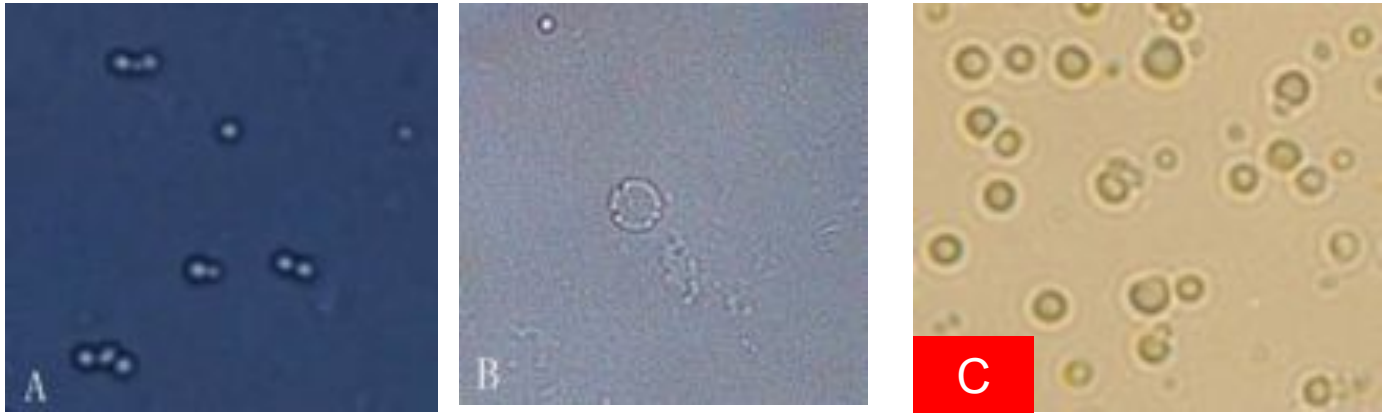
Recombinant pQE-81L



Profile of the induced *PBR1* gene on SDS-PAGE

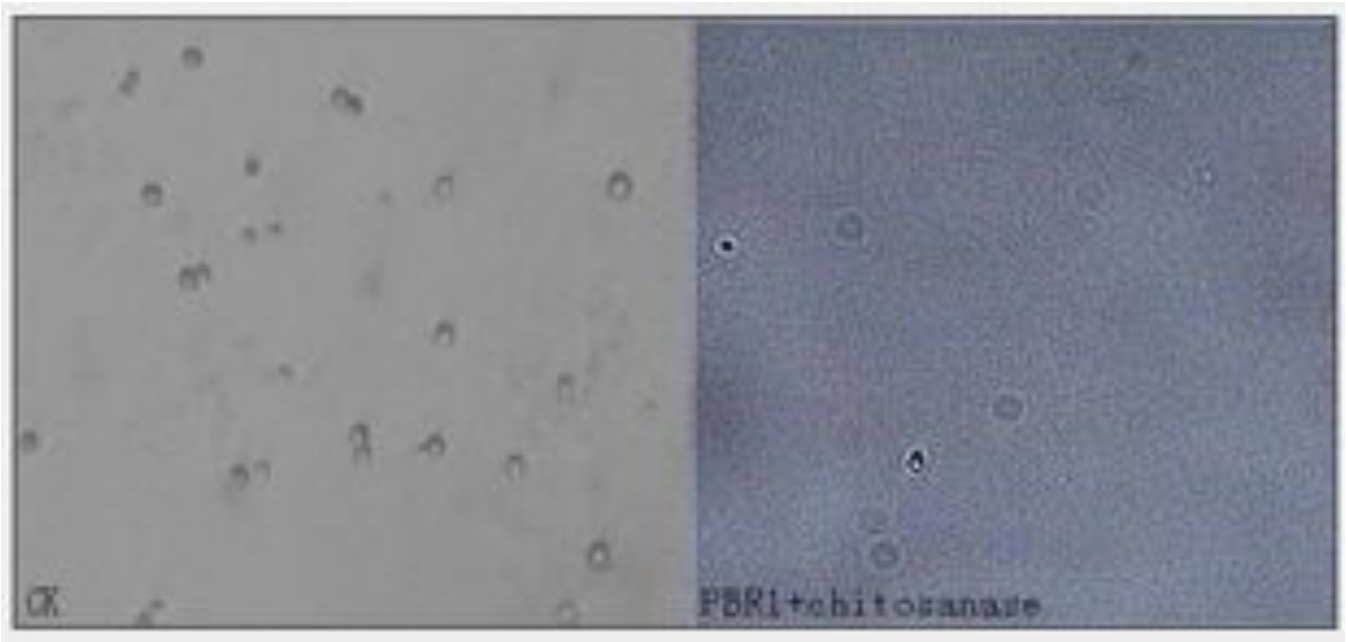
Inhibition of PBR1 protein expressed in *E. coli* B21

Effect of the PBR1 fusion proteins from XF-1(left) and B168 (right) on *Fusurium solani*



Inhibition effect of PBR1 proteins on *P. brassicae*
（A：untreated；B：treated for 24h with the protein from XF-1; C: treated for 24h with the protein from B168）

Combination of chitosanase with PBR1 protein

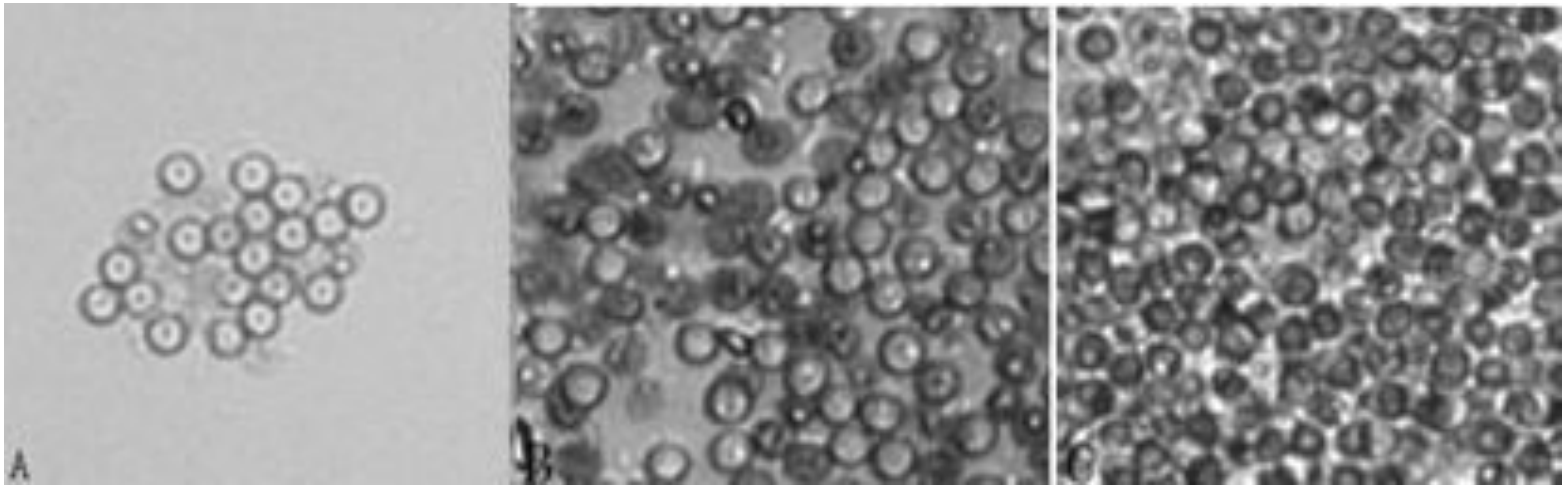


PBR1 application: Development of a tool to screen potential strains for clubroot control

- Designed a pair of primers based on *PBR1* sequence: PBR1-01 and PBR1-02 (the sequences would be released after our patent)
- 14 strains have the specific bands, about 750bp long from 55 Bacillus strains, but only 4 strains have the complete sequence of *PBR1*, 753bp bands, the other ten have 752bp bands
- The sequence of 2 strains, 41-1 and 6-11, have the exactly same sequences as XF-1 and can suppress the resting spores of *P. brassicae*

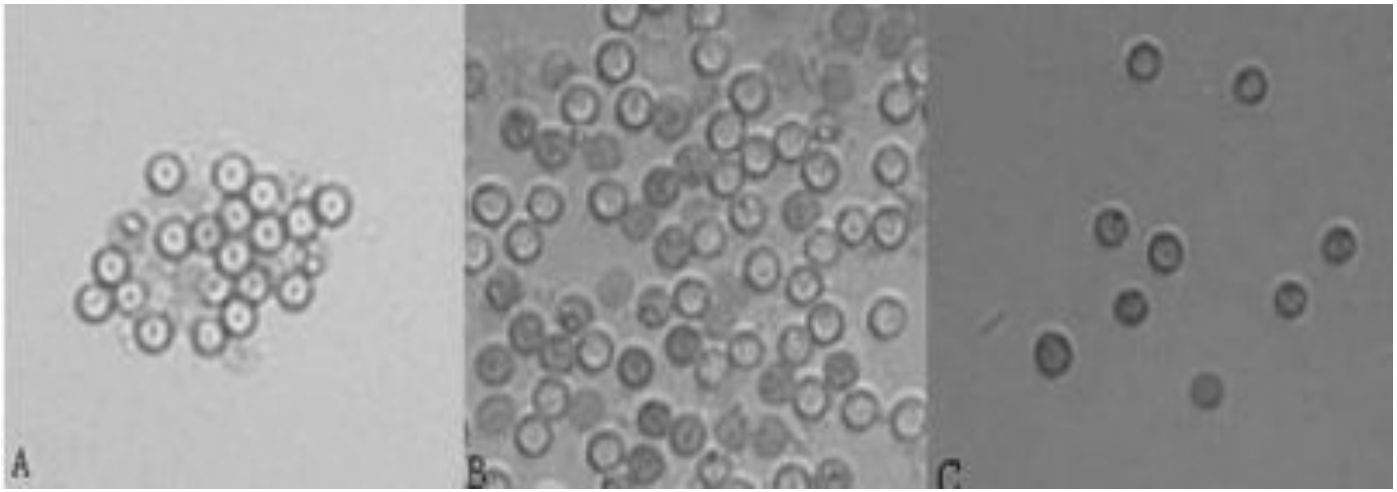
Strain 6-11 could kill the resting spores of *P. brassicae*

The supernatant from Strain 6-11 culture could killed the spores with 90% and 100% in 24h and 48h based on staining with Evans Blue.

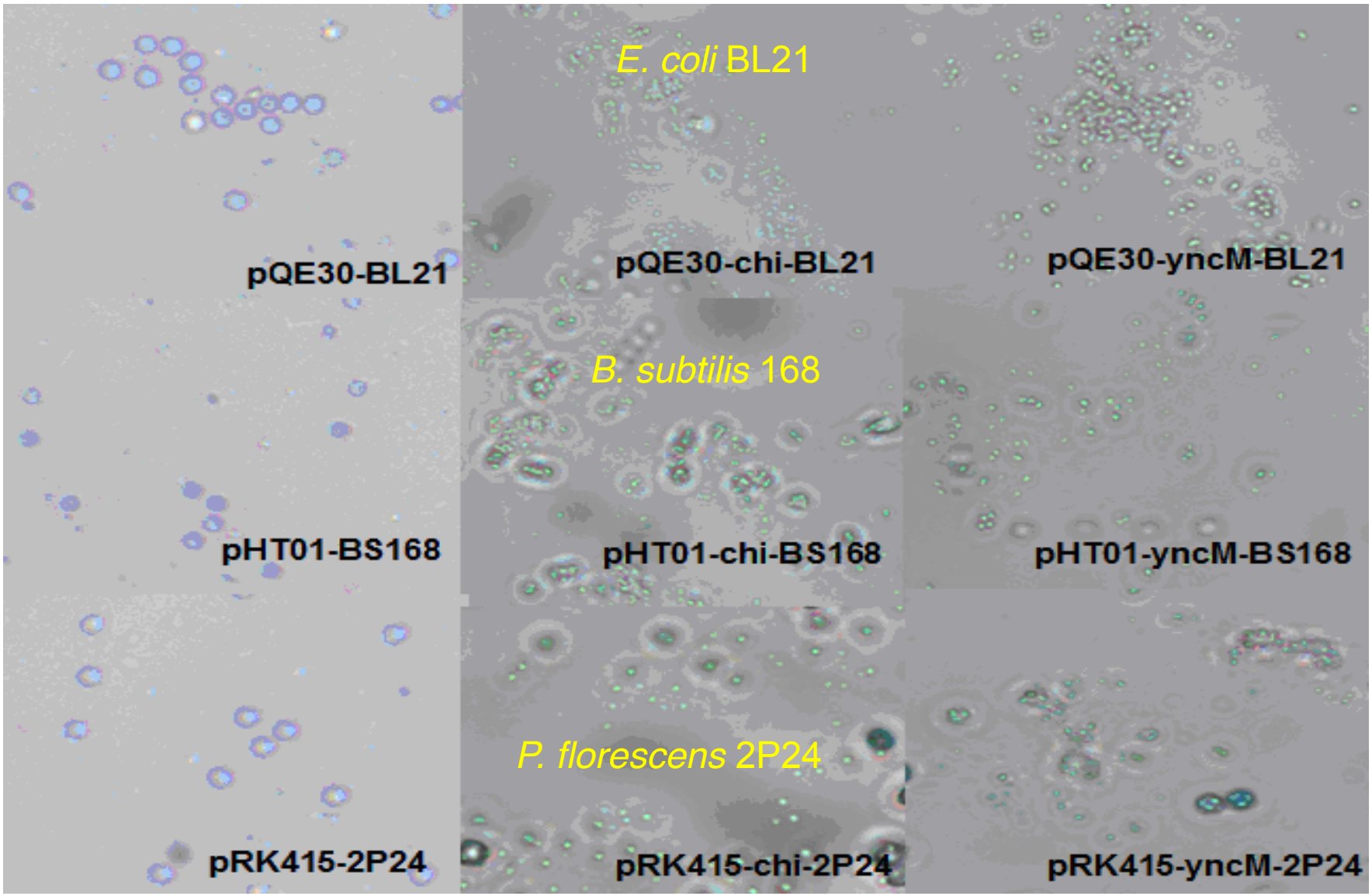


Strain 41-1 could kill the resting spores of *P. brassicae*

The supernatant from Strain 6-11 culture could killed the spores with 50% and 80% in 24h and 48h based on staining with Evans Blue.



PBR1 and *csn* genes' application by gene transformation



Conclusions

- **Clubroot disease is very severe in China**
- **A nationwide team cooperates on the disease control**
- **The team focuses on pathogenic variation, resistance breeding, biological and chemical controls, agricultural management, and biocontrol agent formulation**
- ***Bacillus subtilis* XF-1 is an effective biocontrol agent, which can control the disease by two proteins, chitosanase and PBR, a new protein.**

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