

Preliminary investigation and detection based on loop-mediated isothermal amplification (LAMP) of phytoplasmas associated with diseases in *B. napus* L.



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ABSTRACT

In the last decade, some disease occurred on our experimental farms that had caused serious losses. They were not caused by fungi, bacteria or viruses. By loop-mediated isothermal amplification (LAMP) technique, the detection results pointed to the possible pathogen as phytoplasma. The investigation results implied that phytoplasmas could cause more than 13 kinds of symptoms in almost all parts of plants in *B. napus* L., including witches' broom, multi-stems, aggregate main inflorescences, and flat stems. The incidences of these phytoplasma-associated diseases in our experimental farms rose from 1.61% in 2010 to 6.00% in 2021. Some phytoplasma infected plants died without any growing points. These studies would be helpful for detecting phytoplasmas diseases, selecting disease resistant germplasm and improving varieties with disease resistances in *B. napus* L.

1. Introduction

Mycoplasma (also named mycoplasma-like organism, MLO), which is called phytoplasma in plants, is a self-replication smallest organism with the size (0.2–1.0 μm) between those of bacteria and virus. That is why some mycoplasmas could successfully go through sterilizing filter membrane (0.45 μm) (Marcone C and Ragozzino A, 1995). Mycoplasmas have not fixed shapes because they only have cell membranes without cell walls and they are difficult to be separated and cultured *in vitro*. They extensively exist in nature, as the main pathogens of human beings (Zheng et al., 2020; Zhang et al., 2021), livestock (Wu et al., 2017; Ji et al., 2018; Xu et al., 2020; Jiang et al., 2021), poultry (Chen et al., 2010) and plants. There were many reports of phytoplasma-related diseases in plants all over the world. In Europe, phytoplasma-related diseases mainly were reported in apple trees (Malandraki et al., 2015), cherry plums (Avramov et al., 2011), *B. napus* L. (Bertaccini et al., 1998), *B. rapa* L. (Kamińska et al., 2012) and other *B.* vegetables (Marcone and Ragozzino, 1995). In Asia, phytoplasmas-related diseases were found in many plant species, such as *Arundo donax* in India (Ajay et al., 2016), potato in South Korea (Jung et al., 2003), tomato in Saudi Arabia (Lhudaib and Azq, 2011), sugar cane in Vietnam (Hoat et al., 2013) and Thailand (Roddee et al., 2017), mango and so on in Pakistan (Fahmeed et al.,

2009). There were also reports of phytoplasma diseases in North America (Olivier et al., 2009; Chittem et al., 2015), South America (Banzato et al., 2021), Africa (Dickinson et al., 2007) and Australia (Streten et al., 2005). In China, phytoplasma-related diseases were reported in some crops, for example in rice, barley, wheat, soybean, sesame and sugarcane (Kuai et al., 2000). Additionally, phytoplasma-related diseases were reported in several forestry plants and caused serious damages and losses, such as grapevine phytoplasma yellows (Ge, 2006), witches' broom in jujube trees and paulownia (Kuai et al., 2000; Zhang et al., 1994). Cai (2007) investigated the hazards of phytoplasma-related diseases in Yunan Province in China. She found that phytoplasmas harmed more than 13 kinds of ornamental plants and caused several similar symptoms in them. In Cruciferae, phytoplasma-related diseases were reported in cabbage, broccoli, turnip, kale, *B. rapa* L. and *B. napus* L. in Europe and North America (Bertaccini et al., 1998; Kamińska et al., 2012; Chittem et al., 2015). In China, phytoplasma-related diseases were found in cabbage and broccoli in Yunnan Province (Mou et al., 2012; Cai et al., 2016). Zhang et al. (2020) found phytoplasma-related diseases in *B. juncea* L. in Inner Mongolia.

Since it was hard to isolate and culture phytoplasmas *in vitro*, Koch's law which was extensively used in the detection of pathogens is usually difficult in detection of phytoplasmas (Ge, 2006). However, there were

several other methods to distinguish among bacteria, virus and phytoplasmas. Firstly, the symptoms of bacteria and virus usually were local pathological changes (such as disease spots, rots or death in some parts of the plants) and those of phytoplasmas were only systematically developmental abnormalities (such as lack of stems, witches' broom, shortened internodes, dwarf, clustered main inflorescences or assembled branches and so on) (Liu et al., 2018). Secondly, the responses of virus and phytoplasmas to the treatments of penicillin and tetracycline were different. Phytoplasmas is sensitive to tetracycline but not to penicillin, which can be used as an auxiliary diagnosis of phytoplasmas-associated disease (Ishii et al., 1967; Ge, 2006). Thirdly, most of experts detected phytoplasmas with polymerase chain reaction (PCR) because phytoplasmas had conserved sequences in 16S rDNA and 23S rDNA, and they designed specific primers to amplify the DNA of phytoplasmas (Marcone C and Ragozzino A, 1995; Cai, 2007; Chittem et al., 2015). However, detection of phytoplasmas by PCR need extraction of total DNA of diseased plants, DNA amplification and gel electrophoresis in molecular technique laboratories with a series of expensive equipment (Yin, 2013). Fourthly, a novel method termed loop-mediated isothermal amplification (LAMP) that amplified DNA was developed and utilized in the detection of mycoplasmas in tissue culture and phytoplasmas (Notomi et al., 2000). Guatelli et al. (1990) created an isothermal amplification method of nucleic acids by a multi-enzyme reaction modeled after retroviral replication *in vitro*. According to this method, Notomi et al. (2000) developed LAMP which amplified DNA with high specificity, efficiency and rapidity under isothermal conditions. Obura et al. (2010) successfully used LAMP to detect phytoplasma diseases of Napier stunt in eastern Africa. By now, LAMP has been extensively utilized in detection of barley yellow dwarf viruses in China (Zhao et al., 2010) and in screening Napier grass accessions for resistance to Napier grass stunt disease (Deng et al., 2013; Wamalwa et al., 2017).

Ten years ago, we found diseased plants and serious losses in our experimental farm. The pathogens of these diseases excluded fungi, bacteria, and virus. In order to detect the pathogens, we tried to detect the diseased plants using LAMP. We also investigated symptoms of phytoplasma-related disease and the incidences of diseased plants in the past 10 years in our experimental farm. We expected these studies would benefit for detection of phytoplasma-associated diseases, for breeding cultivars with disease resistances, and for disease control in rapeseed production. According to previous publishing, we found it was the first report about phytoplasmas associated diseases in *B. napus* L. in China.

2. Materials and methods

2.1. Materials

The investigated population in this study included 1400 *B. napus* L. accessions, most of them were accessions bred in our laboratory and a few of them were introduced from abroad. The detected materials with LAMP were sampled leaves mixture from 11 symptomatic plants of accessions with stable and serious symptoms in the past several years, including DL130, Zhongyou 821, 84010, 384B, Xinyou 1, Line 2, Yuyou 1, (88024 × GN), Y25, (Shuangyou 9 × Ningyou 10) and (H155 × Qva) etc. Hybrid Shuangyouza 1008 cultivated in net sheds was used as control for its normal and healthy.

2.2. Field trials

All materials were sown in October at Tanghe experimental station (113.0648 E, 32.7384 N), which was located at Nanyang basin (in Henan Province) and up reaches of the Huai River. It belongs to temperate monsoon climate. During the rapeseed growth period, it was warm and with plenty of rainfalls. Cold injuries seldom occurred in winter there. All investigated materials were planted in two-row plots with 0.33 m row distance and 0.1 m plant distance. The controls of weeds, diseases and insects, fertilization and other agronomic treatments in the farm followed

traditional recommendations for winter oilseed rape production in local region. The symptom incidences of phytoplasma associated disease accessions were investigated at the flowering stages from 2010 to 2021. Shuangyouza 1008 was planted in net sheds as control.

2.3. Detection of phytoplasmas

We detected phytoplasmas using LAMP protocol kit (MycAway-Color One-step Mycoplasma Detection Kit UNG Plus, Shanghai Yeasen Biological Scientific Limited Company) according to the methods described by Obura et al. (2010). This protocol conducted LAMP to amplify 16S and 23S conserved regions of phytoplasmas DNA with two pair primers (BIP: 5'-TCAGCAACAAAACCTTTT GAAACTGAATTGGATTAGTTACTATAAG TGC-3'; FIP: 5'-GTGTTCAAGC AAAAGAACCTTCTTCTGCAGATACATT GGGAACT- 3'; B3: 5'-AAACACT CCAACATCATACG-3'; F3: 5'-CGCA-CATCCAAACGCATA- 3') (Obura et al., 2010). In order to increase amplification efficiency, the protocol kit added another pair of primers: BLoop: 5'-ATTTTAAAAAATAATAATGTAATCGG-3'; FLoop: 5'-GTAAT-GAAGATAATAAAATATCAAGAT-3' (Deng et al., 2013). This protocol could only detect if the pathogen is phytoplasma, but it does not determine the specific strain of phytophthora.

It was easy to identify symptoms of phytoplasma-associated diseases at flowering stage. We sampled 10 pieces of leaves with sterilized 1 mL pipette tips and put the leaf samples into 2 mL tubes. The leaf samples were sterilized with 5% sodium hypochlorite solution (84 disinfectant) for 5 min followed by rinsing with sterilized water for 3 times. Then, sterilized water (1 mL) was added to the tube with leaf samples and grinded the leaf samples into homogenate. The homogenate was centrifuged at 20 000 rounds per minute for 10 min. Added supernatant (1 μL), MycAway-Color A (24 μL) and MycAway-Color B (1 μL) into a tube (1 mL). Shook the tube for blending and finally added a drop of mineral oil for covering. Two specific reaction tubes were used as controls: added 1 μL MycAway-Color C to the reaction tube instead of 1 μL of the supernatant as positive control (CK1) and one reaction tube without the supernatant and MycAway-Color C as negative control (CK2). Bathed the reaction tube with the mixture in 63 °C water in thermostatic water bath. Took out the reaction tube and observed the color of mixture on a white fluorescent light box. Recorded and compared the colors of the mixtures with those of CK1 and CK2. The purplish blue of the mixture in the reaction tube (CK2) indicated negative result, which meant no phytoplasma in the leaf sample. If the color of mixture was sky blue (CK1), it showed positive result which indicating phytoplasma in the sample.

2.4. Investigation of symptoms caused by phytoplasmas

At flowering stage, we investigated the diseased accessions and recorded the symptoms in 1400 *B. napus* L. accessions in the experimental farm. We took leaf samples from 10 diseased plants of each kind of symptom and detected phytoplasmas using LAMP according to the methods described above. When the positive ratio of one kind of symptom detection result reached to 80%, we judged its pathogen was phytoplasmas, and with its symptom associated.

2.5. Investigation of incidences of diseased accessions

At flowering stage, we investigated 1400 *B. napus* L. accessions and recorded the accessions with phytoplasmas symptoms in the experimental farm. When the incidences of diseased plants in one accession population reached to more than 50%, the accession was recorded as be susceptible to phytoplasmas. The incidences of diseased accessions = Infected accessions Number/Total investigated accession number × 100%.

2.6. Data analysis

All data in this study were analyzed with Excel software.

3. Results and analysis

3.1. Phytoplasmas detection

According to the methods described above, we detected the diseased plants using LAMP. Results indicated that this method could successfully detect *B. napus* L. phytoplasmas. In Fig. 1, the colors in tube 1, 2, 3, 4, 7 and 8 were purplish blue in accordance with CK1, which means that they were negative without phytoplasmas. However, the colors in tube 5, 6, 9 and 10 were sky blue in accordance with CK2, which meant positive and had phytoplasmas in them. These results indicated successful detection of this method for phytoplasmas in *B. napus* L.

In order to investigate the reliability of phytoplasma detection, we compared the detection results with field symptoms between healthy Shuangyouza 1008 (CK) and diseased Zhongyou 821. All plants from Shuangyouza 1008 showed negative reaction in phytoplasma detection, which was in accordance with the symptoms in experimental field. In cultivar Zhongyou 821, 91.43% diseased plants showed positive in detection. These results indicated that this kind of phytoplasma detection method was reliable, although 8.57% diseased plants were not correctly detected. It suggested that right operations during detection was needed. Even in the asymptomatic plants from susceptible cultivar of Zhongyou 821, about 82.86% plants were detected to be positive. It was speculated that although these plants were infected by phytoplasma, the symptoms haven't shown up yet (Table 1).

According to this kind protocol of phytoplasmas detection, we only grinded the leaf samples instead of extracting DNA. Since DNA amplification reaction took place under isothermal condition (63°C), the reaction only needed to incubate in a 63°C water bath for an hour instead of a PCR cycler. The results were visible: sky blue of the mixture in the reaction tube meant positive while purplish blue meant negative. Therefore, gel electrophoresis was not needed. Compared with the detection by PCR, the protocol using LAMP in this study was simple, specific, sensitive, efficient and rapid. Of course, this protocol only detect if the pathogens were phytoplasmas or not without any strain information. If there are requirements to distinguish the strain, we need nested PCR, sequencing the products and comparing them.

3.2. Investigation of phytoplasma symptoms

At flowering stage, we investigated the susceptible accessions and recorded the symptoms in 1400 *B. napus* L. accessions in the experimental farm. After detecting the diseased plants with this protocol, we found the detecting results of 13 kinds of symptoms were positive and their positive ratio reached to 80%. So we speculated that their pathogens were phytoplasmas (Table 2, Figs. 2 and 3). Several symptoms of phytoplasma disease are shown in Fig. 2: plant with witches' broom from the base part (Fig. 2A and B); witches'broom from the upper stem (Fig. 2C); aggregate, flat and bending main inflorescences (Fig. 2D); aggregate branches (Fig. 2E); aggregate main inflorescences (F); double flat stems (Fig. 2G); plant without branches (Fig. 2H). In Fig. 3, other 6 kinds of symptoms of phytoplasmas were showed associating in leaves, buds and growth points: albino leaves (Fig. 3A); wrinkle leaves (Fig. 3B);

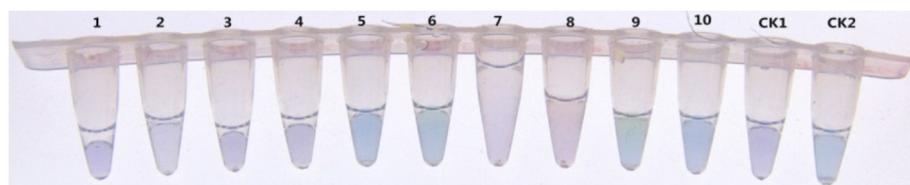


Fig. 1. Results of phytoplasmas detection using LAMP in *B. napus* accessions. Samples in tube 1, 2, 3, 4, 7 and 8 are taken from healthy normal accessions from the experimental farm, while those in tube 5, 6, 9 and 10 are taken from accessions with stable and serious symptoms DL130, 84010, 384B and Xinyou 1. CK1 is negative check Shuangyouza 1008. CK2 is positive check Zhongyou 821.

Table 1
Result reliable investigations of phytoplasmas detection using LAMP in *B. napus* accessions.

Material type	Total No.	Positive results		Negative results	
		Number	Rate (%)	Number	Rate (%)
Uninfected Shuangyouza 1008 (CK)	8	0	0.00	8	100
Asymptomatic plants from susceptible variety Zhongyou 821	35	29	82.86	6	17.14
Symptomatic plants from susceptible variety Zhongyou 821	35	32	91.43	3	8.57

Table 2
Detection results of different symptoms of phytoplasma-associated diseases with LAMP in *B. napus* L.

Line	Origins	Symptoms in experimental field	Ratio of positive plants detected by LAMP detection (%)	Coincidence between phytoplasmas detection with LAMP and field symptoms
H237	92B	witches'broom from the base part	91.33	+*√**
H303	146B	witches'broom at the upper stem	88.45	+√
H533	Zhongshuang 4	Aggregate, flat and bending main inflorescences	83.94	+√
G39	Zhongyou 821	aggregate main inflorescences	87.69	+√
G53	384B	Aggregate branches	89.21	+√
G357	Xingyouza 1	Double flat stems	84.65	+√
G789	Yuyou 9 × Ningyou 10	Plants without branches	90.12	+√
G795	Gujie × Gubai	Albino leaves	84.53	+√
G795	Gujie × Gubai	Albino buds	87.41	+√
G835	PSR-2 × 94-1	Wrinkle leaves	82.92	+√
G409	98009 × GN	Apetalous flowers	83.67	+√
G855	DL066 × (220 × 84004)	Plants without growth points	90.25	+√
G497	Y25	Stunting plant	92.00	+√
S25(CK)	Shuangyouza 1008	No symptom	0.00	-√

*: + Positive. -Negative. **:√ Coincidence.

apetalous flowers (Fig. 3C); albino buds (Fig. 3D); plants without growth points (Fig. 3E and F); stunting plants (Fig. 3G).



Fig. 2. Symptoms of disease associated with phytoplasmas in branches and main inflorescences. A and B: Plant with witches' broom at the base part; C: Witches' broom at the upper stem; D: Aggregate, flat and bending main inflorescences; E: Aggregate branches; F: Aggregate main inflorescences; G: Double flat stems; H: Plant without branches.

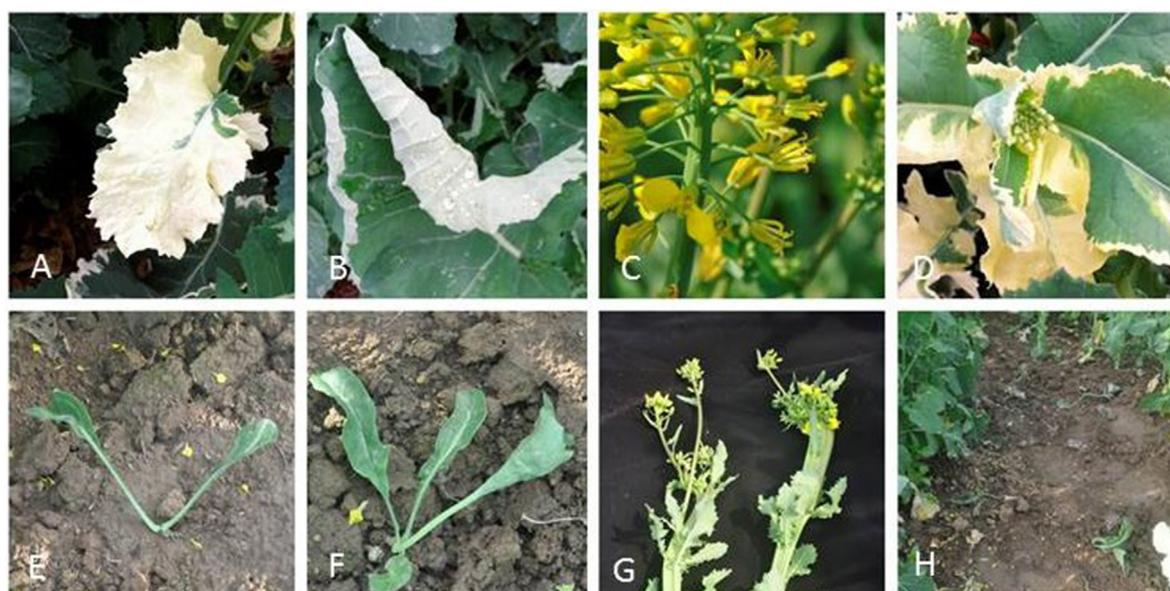


Fig. 3. Symptoms of disease associated with phytoplasma in leaves, buds and growth points. A: Albino leaves; B: Wrinkle leaves; C: Apetalous flowers; D: Albino buds; E and F are diseased plants without growth points; G: Stunting plant; H: Almost disappearance of an infected population.

3.3. Investigation of phytoplasma-associated diseases incidences

According to the detected symptoms of phytoplasma-associated diseases above, we investigated the incidences rate of 1440 accessions in Tanghe experimental farm from 2010 to 2021 (Fig. 4). In all investigated accessions, the phytoplasma-associated diseases incidences rose from 1.61% in 2010 to 6.00% in 2021, which rose 22.72% per year. The incidence of phytoplasma-associated diseases in 2021 was 2.7 times of that in 2010. The investigation results indicated that phytoplasma-associated diseases had become a problem in Tanghe experimental station. The reason perhaps was that experimental materials were sown and harvested every year, which led to phytoplasmas accumulation and transmission extensively among trial materials.

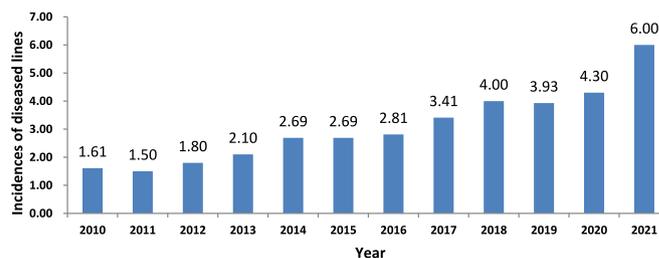


Fig. 4. Infected incidences of phytoplasmas diseases from 2010 to 2021 in experimental station.

4. Discussion

Mycoplasmata (also named phytoplasmas in plants) are main pathogens in human beings, animals and plants. Phytoplasma-associated diseases in rapeseed were firstly found in Europe. Bertaccini et al. (1998) found witches' broom disease caused by phytoplasmas in *B. napus* in Czech Republic and Italy. Calari et al. (2011) reported that phytoplasmas could be transmitted by seeds of winter rapeseed. Zwolińska et al. (2017) found that leafhopper was the transmission media of phytoplasma in *B. napus* in Poland. In northern America, there were phytoplasma-associated diseases in canola fields in many counties of North Dakota (Chittem et al., 2015). In China, phytoplasma-associated diseases were found in cauliflower in Yunnan Province (Cai et al., 2016), cabbage in Yunnan Province (Mou et al., 2012) and *B. juncea* in Inner Mongolia (Zhang et al., 2020). As for one of the most important oilseed crops in China, *B. napus* has not been reported to be infected by phytoplasmas. This study is the first report of phytoplasma-associated diseases in *B. napus* in China.

There were several methods to detect phytoplasmas. Firstly, Koch's law should be used to detect phytoplasmas because this law was classically and extensively used in the detection of pathogens, but it was difficult to isolate and artificially culture phytoplasmas *in vitro*. Therefore it is hard to use in detection of phytoplasmas (Ge, 2006). Secondly, since most of mycoplasma colonies have typical sunny side up egg-like shape under microscope, so Xu et al. (2020) primarily detected them by colonies shapes. However, this method also needs to isolate and culture phytoplasmas which was difficult to carry out. Thirdly, it was easy to distinguish virus from phytoplasmas according to the responses to penicillin and tetracycline. Virus was positive to the treatments of penicillin while phytoplasmas were negative. On the contrary, phytoplasmas were positive to the treatments of tetracycline while virus was negative (Ge, 2006). This kind of detection method also could not be used extensively because it needed to isolate and culture phytoplasmas. Fourthly, nest PCR were the most extensively used method to detect phytoplasmas because phytoplasmas had conserved sequences in 16S rDNA and specific primers could be designed (Marcone C and Ragozzino A, 1995; Cai, 2007; Chittem et al., 2015).

PCR detections need a set of expensive molecular instruments. LAMP was developed to amplify DNA with high specificity, efficiency and rapidly under isothermal conditions (Notomi et al., 2000). Obura et al. (2010) successfully used LAMP to detect Napier stunt phytoplasma in eastern Africa. The mechanism of MycAway-Color One-step Mycoplasma Detection Kit UNG Plus was the utilization of LAMP in detecting mycoplasma infection in cell culture. This protocol could detect samples under isothermal conditions without the need for DNA extraction and PCR recyclers. In this study, we tried to use them to detect phytoplasmas in *B. napus* L. However, since this kind of method detected the conserved sequence region, it only could detect the frequently occurred species of phytoplasmas (about 36 species) instead of distinguishing detailed strains of pathogen. Further differentiations need to detect with traditional nested PCR, sequence the PCR products and compare the sequences with the database (Marcone C and Ragozzino A, 1995; Cai, 2007; Chittem K and Mendoza L).

According to Cai (2007), Kuai et al. (2000), Bertaccini et al. (1998), Marcone C and Ragozzino A (1995), the symptoms of phytoplasma-related diseases mainly were as followings: (1) Lack of stems or witches' broom; (2) Shortened internodes, dwarf, clustered main inflorescences or assembled branches; (3) Flat stems; (4) Abnormal colored leaves or wrinkle leaves; (5) Apetalous flowers, albino buds, phyllody or sterile flowers; (6) Abnormal or decrescent siliques; (7) Stunting or lack of growth. Previous researches usually only focused on one symptom of phytoplasmas in one diseased plant. In this study, we investigated the symptoms of phytoplasmas for more than 10 years and found that there were 13 kinds of symptoms in *B. napus*, which almost included all kinds of symptoms of phytoplasmas in other plant species.

In this study, the incidences of phytoplasma-associated diseases in our

experimental station kept rising in past decade. Although these results did not obtain from production fields, we also should pay attention to them. In this study, we only regarded the accessions with incidence rate more than 50% as susceptible accessions. Actually, there were some accessions with incidence rate less than 50%. Marcone C and Ragozzino A (1995) found that the phytoplasma-associated diseases incidence rate ranged from 8% to 15% (some reaching 25%) in *B. crops* (such as cabbage, sprouting broccoli, turnip and kale) field in southern Italy. Lamey et al. (2001) reported that the state average incidence of aster yellow-symptomatic plants (one kind of phytoplasma related disease) in canola fields in 2000 was 4.5%, 8 out of the 16 counties represented some fields with incidences more than 5%. Chittem et al. (2015) found that a few canola fields were seriously affected by phytoplasma-related diseases in McLean and Cavalier counties of North Dakota in 2012 with incidences ranging between 10% and 20%.

In China, *B. napus* is one of the most important oilseed crops. It is necessary to identify, detect, prevent and control phytoplasma-associated diseases in production and cultivar breeding in *B. napus*. Firstly, it can help researchers and breeders to avoid interferences and misleads. Since phytoplasmas-associated diseases have spread extensively in *B. napus*, researchers regarded some diseased plants with phytoplasmas symptoms as mutants and paid attention to utilize them. Secondly, accurate identification and testing can help develop techniques to prevent and control phytoplasma-related diseases. For example, once phytoplasmas related diseases were identified, breeders could find resistant germplasm for improving resistant varieties. In addition, only when breeders find real pathogens of these diseases, they could select or resynthesize agents to control. Thirdly, it can help farmers to select correct management measures to decrease yield loss caused by phytoplasmas associated diseases. For example, farmers can eliminate diseased plants, spray insecticides to control the media insects. Fourthly, it can help customs to make quarantine policies for importing rapeseed. China is an important country of importing rapeseed in the world. The rapeseeds usually are imported from North American countries and European countries where phytoplasmas related diseases were reported (Roddee et al., 2017; Hoat et al., 2013; Fahmeed et al., 2009; Chittem et al., 2015).

However, this study is a primary investigation and detection of phytoplasmas-associated diseases in *B. napus* in China. Although the incidences and symptom investigation of the diseased accessions have lasted for 11 years, they were limited in the experimental farm instead of production fields. In addition, this study only identified and detected phytoplasmas, and could not distinguish their strains in details. In the future, further studies should investigate incidences of phytoplasmas associated diseases in *B. napus* in China and analyze biodiversity of their strains. Finally, we should try to find measurements to control the diseases.

Data availability statement

The datasets used and/or analyzed in this study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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