Appendices

Appendix 1: The List of Potential Quarantine of Sugarcane Harmful Organisms (Table 1)

Table 1 The list of potential quarantine of sugarcane harmful organisms

Insect	
1	*Diaprepes abbreviata* (L.)
2	*Diatraea saccharalis* (Fabricius)
3	*Graphognathus leucoioma* (Boheman); *Naupactus leucoloma* (Boheman)
4	*Opogona sacchari* (Bojer)
5	*Rhabdoscelus lineaticollis* (Heller)
6	*Rhabdoscelus obscurus* (Boisduval)
7	*Rhabdoscelus ferrugineus* (Olivier)
8	*Coptotermes* spp. (non-Chinese)
9	*Selenaspidus articulatus* Morgan
10	*Diatraea crambidoides* (Grote)
11	*Diatraea grandiosella* (Dyar)
12	*Diatraea centrella* Moschler
13	*Chilo partellus* (Swinhoe)
14	*Eldana saccharina* Walder
Fungus	
15	Sugarcane wilt disease (*Cephalosporium sacchari* E.J. Butler et Hafiz Khan)
16	Corn downy mildew (non-native species) (*Peronosclerospora* spp.)
17	Sugarcane leaf scorch (*Stagonospora sacchari* Lo et Ling)
18	Sugarcane downy mildew [*Peronosclerospora sacchari* (Miyake) shaw]
19	Sugarcane smut (*Ustilago scitaminea* Syd.)
Bacteria	
20	Sugarcane leaf scald [*Xanthomonas albilineans* (Ashby) Dowson]

(continued)

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Table 1 (continued)

21	Sugarcane gumming disease [*Xanthomonas axonopodis* pv. *vasculorum* (Cobb) Vauterin et al.]
22	Ratoon stunting disease (*Leifsonia xyli* subsp. *xyli*, Lxx)
Phytoplasma	
23	Sugarcane white leaf (*Sugarcane white leaf Phytoplasma*)
23	Sugarcane grassy shoot (*Sugarcane grassy shoot Phytoplasma*)
Nematode	
25	*Radopholus similis* (Cobb) Thorne
26	*Heterodera sacchari* Luc and merny
27	*Meloidogyne javanica* (Treub) Chitwood
Viruses and viroids	
28	*Sugarcane streak virus*, SSV
29	*Sugarcane Fiji disease virus*, SFDV
30	*Sugarcane mosaic virus*, SCMV
31	*Sorghum mosaic virus*, SrMV
32	*Sugarcane yellow leaf virus*, ScLV
33	*Sugarcane bacilliform virus*, SCBV
34	*Sugarcane streak mosaic virus*, SCSMV
Weeds	
35	*Sorghum halepense* (L.) Pers. (Johnson grass and its cross breeds)
36	*Sorghum almum* Parodi

Appendix 2: The List of Pesticides Prohibited and Restricted in China

In order to ensure the agricultural production safety, the agricultural product quality safety, and the ecological environment safety and safeguard the health and life safety of the people, according to the relevant provisions of the "Pesticide Management Regulations" of the Ministry of Agriculture in the Announcement of No. 194th, No. 199th, No. 274th, No. 322nd, No. 1157th, No. 1586th, No. 1745th, and No. 2032nd, they clearly stipulated the pesticide species which were forbidden to be produced, sold, and used in China and the pesticides that could not be used and were restricted in vegetables, fruit trees, tea, and Chinese herbal medicines. In order to facilitate the understanding of these relevant situations, the next list of pesticides prohibited and restricted in China is now summarized in the following table for reference.

If you want to understand and master more information, but in China Pesticide Information Network (http://www.chinapesticide.gov.cn) (Table 2).

Table 2 Pesticides prohibited and restricted in China

Number	Names of pesticide	Prohibited/restricted requirement	Ministry of Agriculture announcement document number
1.	HCH	Prohibited	No. 199th (2002.06.05)
2.	DDT	Prohibited	No. 199th (2002.06.05)
3.	Camphechlor	Prohibited	No. 199th (2002.06.05)
4.	Dibromochloropropane	Prohibited	No. 199th (2002.06.05)
5.	Chlordimeform	Prohibited	No. 199th (2002.06.05)
6.	EDB	Prohibited	No. 199th (2002.06.05)
7.	Nitrofen	Prohibited	No. 199th (2002.06.05)
8.	Aldrin	Prohibited	No. 199th (2002.06.05)
9.	Dieldrin	Prohibited	No. 199th (2002.06.05)
10.	Mercury compounds	Prohibited	No. 199th (2002.06.05)
11.	Arsena	Prohibited	No. 199th (2002.06.05)
12.	Acetate	Prohibited	No. 199th (2002.06.05)
13.	Bis-ADTA	Prohibited	No. 199th (2002.06.05)
14.	Fluoroacetamide	Prohibited	No. 199th (2002.06.05)
15.	Gliftor	Prohibited	No. 199th (2002.06.05)
16.	Tetramine	Prohibited	No. 199th (2002.06.05)
17.	Sodium fluoroacetate	Prohibited	No. 199th (2002.06.05)
18.	Silatrane	Prohibited	No. 199th (2002.06.05)
19.	Methamidophos	Prohibited	No. 322nd (2003.12.30)
20.	Parathion methyl	Prohibited	No. 322nd (2003.12.30)
21.	Parathion	Prohibited	No. 322nd (2003.12.30)
22.	Monocrotophos	Prohibited	No. 322nd (2003.12.30)

(continued)

Table 2 (continued)

Number	Names of pesticide	Prohibited/restricted requirement	Ministry of Agriculture announcement document number
23.	Phosphamidon	Prohibited	No. 322nd (2003.12.30)
24.	Fenamiphos	Prohibited	No. 1586th (2011.06.15)
25.	Fonofos	Prohibited	No. 1586th (2011.06.15)
26.	Phosfolan-methyl	Prohibited	No. 1586th (2011.06.15)
27.	Calcium phosphide	Prohibited	No. 1586th (2011.06.15)
28.	Magnesium phosphide	Prohibited	No. 1586th (2011.06.15)
29.	Zinc phosphide	Prohibited	No. 1586th (2011.06.15)
30.	Cadusafos	Prohibited	No. 1586th (2011.06.15)
31.	Coumaphos	Prohibited	No. 1586th (2011.06.15)
32.	Sulfotep	Prohibited	No. 1586th (2011.06.15)
33.	Terbufos	Prohibited	No. 1586th (2011.06.15)
34.	Paraquat	Prohibited	No. 1745th (2012.04.24)
35.	Chlorsulfuron	Prohibited	No. 2032nd (2013.12.09)
36.	Metsulfuron-methyl	Prohibited	No. 2032nd (2013.12.09)
37.	Ethametsulfuron-methyl	Prohibited	No. 2032nd (2013.12.09)
38.	Asomate	Prohibited	No. 2032nd (2013.12.09)
39.	Urbacide	Prohibited	No. 2032nd (2013.12.09)
40.	Omethoate	Cannot use in cabbage and citrus trees	No. 1586th (2011.06.15)
41.	Phorate	Cannot use in vegetables, fruit trees, tea, and Chinese herbal medicines	No. 199th (2002.06.05)
42.	Isofenphos-methyl	Cannot use in vegetables, fruit trees, tea, and Chinese herbal medicines	No. 199th (2002.06.05)
43.	Demeton	Cannot use in vegetables, fruit trees, tea, and Chinese herbal medicines	No. 199th (2002.06.05)

(continued)

Table 2 (continued)

Number	Names of pesticide	Prohibited/restricted requirement	Ministry of Agriculture announcement document number
44.	Carbofuran	Cannot use in vegetables, fruit trees, tea, and Chinese herbal medicines	No. 199th (2002.06.05)
45.	Aldicarb	Cannot use in vegetables, fruit trees, tea, and Chinese herbal medicines	No. 199th (2002.06.05)
46.	Ethoprophos	Cannot use in vegetables, fruit trees, tea, and Chinese herbal medicines	No. 199th (2002.06.05)
47.	Phosfolan	Cannot use in vegetables, fruit trees, tea, and Chinese herbal medicines	No. 199th (2002.06.05)
48.	Isazofos	Cannot use in vegetables, fruit trees, tea, and Chinese herbal medicines	No. 199th (2002.06.05)
49.	Dicofol	Cannot use in tea	No. 199th (2002.06.05)
50.	Fenvalerate	Cannot use in tea	No. 199th (2002.06.05)
51.	Daminozide	Cannot use in peanut	No. 274th (2003.04.30)
52.	Fipronil	Aside from maintaining health of corn and its other parts, as dry field seed coating agent, it is prohibited for use in other aspects	No. 1157th (2009.02.25)
53.	Isocarbophos	Cannot use on citrus trees	No. 1586th (2011.06.15)
54.	Methomyl	Cannot use on citrus trees, apple trees, tea trees, and cruciferous vegetables	No. 1586th (2011.06.15)
55.	Endosulfan	Cannot use on apple trees and tea trees	No. 1586th (2011.06.15)
56.	Methyl bromide	Cannot use on strawberries or cucumbers	No. 1586th (2011.06.15)
57.	Chlorpyrifos	Prohibited for use in vegetables	No. 2032nd (2013.12.09)
58.	Triazophos	Prohibited for use in vegetables	No. 2032nd (2013.12.09)
59.	Rodenticide	Stopped approval of its subpackage registration; approved subpackage registration will no longer be renewed	No. 274th (2003.04.30)
60.	High/highly toxic pesticide	Stopped approval of its subpackage registration	No. 194th (2002.04.22)

Appendix 3: Artificial Conservation Technology for Adult *Synonycha grandis* During the Overwintering Stage

1 Technical Background

1.1 *Ceratovacuna lanigera* Zehntner (Homoptera: Aphididae) is one of the main pests of sugarcane, and infestations can cover more than 80% of the total sugarcane-growing areas in China. Yield decreases resulting from C. *lanigera* damage are generally 18−35.3%, but can reach 45%, and the sucrose content can be decreased by 5.48−8.16%. Currently, the prevention and control of *C. lanigera* mainly relies on chemical pesticides, but the long-term continuous use of highly toxic broad-spectrum pesticides has killed large numbers of natural pest enemies, destroyed the ecological balance of ecosystems, triggered pesticide resistance, and polluted the environment. Furthermore, this has led to traces of pesticide residues in sugar that are harmful to human health. Therefore, research and development on the utilization of natural enemies for biological control of *C. lanigera* is urgently needed, hence the current drive for the production of pollution-free sucrose products and the development of the modern green sugar industry.

1.2 *Synonycha grandis* Thunberg (Coleoptera: Coccinellidae) is an important natural enemy of *C. lanigera* that is widely distributed in sugarcane-growing areas in China. Populations of this pest predator can be large, and during its lifetime, a single *S. grandi* individual may prey on 32,000 *C. lanigera*, even though this pest enemy tends to emerge slightly later than *C. lanigera* in the field under natural conditions. During the initial period of *C. lanigera* emergence, from May to June, the population of *S. grandi* in sugarcane fields is too small, and the reproduction rate is low, and during the main emergence period of *C. lanigera*, from July to August, *S. grandi* exerts little control over *C. lanigera*. This is because the overwintering survival rate of *S. grandi* is low under natural conditions, since natural overwintering sites are destroyed when sugarcane is harvested, and many *S. grandi* die when sugarcane leaves are burnt off. Therefore, exactly how to protect overwintering *S. grandis* individuals and improve the overwintering survival rate is an important topic for research. *S. grandis* emerge sporadically in May and June, and the release of large numbers into sugarcane fields could promote reproduction, thereby taking advantage of this natural pest enemy.

2 Technical Solutions

2.1 The natural overwintering environment of *S. grandis* adults is the sugarcane sheath (the middle and lower parts of the plant). Using an indoor simulation strategy,

before the arrival of cold conditions in the winter comes, large numbers of *S. grandis* adults can be collected from the field and laced in an indoor overwintering environment for artificial rearing during the winter months, to generate adequate numbers for sugarcane protection and ensure their availability at the most effective time.

2.2 Simulation overwintering conditions can be created in a glass jar of 18 cm in diameter and 30 cm in height, into which six to eight segments of 15−20 cm-long sugarcane withered sheath are placed vertically, and the jar is placed upside down onto a 5 cm diameter Petri dish with absorbent cotton soaked in clear water or sucrose water. Frozen *C. lanigera* are then scattered around the Petri dish, *S. grandis* adults are placed inside (50 adults per jar), and the jar brim is tightly covered with a sand net for indoor rearing at 15−20 °C. Food is replaced once every 3 days, and the rearing jar is kept clean.

3 Technical Advantages and Benefits

3.1 By creating a protective indoor overwintering environment and providing suitable artificial feed, the overwintering survival rate of *S. grandis* can be significantly improved, and these adults can quickly restore their reproductive capacity when feeding on fresh *C. lanigera*. Through this combined artificial reproduction and reintroduction, when *C. lanigera* appear during May and June, large numbers of *S. grandi* can be released into sugarcane fields, where they can quickly proliferate and establish a large of population that is able to effectively control *C. lanigera* numbers.

3.2 The artificial overwintering environment for *S. grandis* adults is simple, the raw materials can be obtained anywhere, and the apparatus can be reused; hence the cost is minimal.

3.3 The artificial feed used in this technology is composed of sucrose water and frozen *C. lanigera*. Sucrose water, consisting of 1:10 sucrose/tap water (w:w), is cheap and readily available, and *C. lanigera* are collected from the field, dispensed onto a 10 cm diameter Petri dish, sealed, and stored in a freezer at −18 °C. This nutritious artificial feed can be obtained anywhere and is simple to process, convenient, and affordable.

3.4 The optimum time for collecting *S. grandis* adults for artificial overwintering is late November and can be carried out alongside the stripping of old foot leaves before harvest, saving man-hours and effectively doubling the benefit with half the effort.

3.5 This overwintering preservation method is easy to understand and carry out, making it widely applicable in most if not all sugarcane areas.

4 Specific Implementation Steps

4.1 Feed Preparation

4.1.1 Frozen *C. lanigera* preparation: Every year from August to October, a large number of fresh *C. lanigera* adults can be collected from the field, dispensed on a 10 cm diameter Petri dish, sealed, and stored in a freezer at −18 °C for preservation and then used as needed.

4.1.2 Sucrose water preparation: One part white granulated sugar is mixed with ten parts tap water, stirred until completely dissolved, and used as needed.

4.2 Indoor Overwintering Preparation

In a glass jar of 18 cm diameter and 30 cm height, six to eight segments of 15−20 cm-long sugarcane withered sheath are placed vertically, and the jar is placed upside down on a 5 cm diameter Petri dish containing absorbent cotton soaked in sucrose water. Frozen *C. lanigera* are then scattered around the bottom of the jar.

4.3 Collection of Adults for Artificial Overwintering

In late November each year, *S. grandis* adults are collected from old leaf sheaths from the lower parts of sugarcane stalks in sugarcane fields.

4.4 Indoor Overwintering Breed Conservation

S. grandis adults collected from sugarcane fields placed into indoor overwintering sites at a density of 50 adults per jar, the jar brim is tightly covered with a sand net, and rearing is performed at 15−20 °C. Fresh feed is replaced once every 3 days, and the jar is kept clean.

Appendix 4: Molecular Detection Techniques of Sugarcane Important Diseases

Sugarcane diseases are one of the greatest threats to sugarcane production. Especially, the introduction of non-native or exotic species facilitates the spread of diseases in sugarcane in recent years. In addition, due to the diverse and complex ecological system in sugarcane planting area and pathogenicity differentiation,

various pathogens that can cause diseases to sugarcane have appeared, which has brought serious risks to sugarcane production in China (Xu et al. 2006; Huang et al. 2007a, b; Li et al. 2010a, b; Huang and Li 2011; Wang et al. 2015). Rapid and effective diagnosis and detection of the pathogens causing important diseases to sugarcane in different ecological areas are very important to identify the species/subspecies and genetic diversity of these pathogens and then provide theoretical references for the control of sugarcane diseases (Li and Huang 2012).

In recent years, the Sugarcane Research Institute of Yunnan Academy of Agricultural Sciences has conducted researches on the rapid detection of major diseases in sugarcane such as bacilliform virus disease, ratoon stunting, smut, and rust, which were financially supported by the Earmarked Fund for Modern Agro-industry Technology Research System of China and the Earmarked Fund for Modern Agro-industry Technology Research System of Yunnan Province. After years of effort, the molecular techniques for rapid detection of 13 pathogens that cause ten important diseases of sugarcane including smut, rust, leaf scald, ratoon stunting, red stripe, mosaic, Fiji, yellow leaf, white leaf, and bacilliform virus were established, which will provide scientific basis for effective diagnosis and control of sugarcane diseases, detection of virus-free seedling, and quarantine management of exotic species. And these PCR techniques developed by the Sugarcane Research Institute of Yunnan Academy of Agricultural Sciences are introduced in this article.

1 PCR Detection of Sugarcane Smut

1.1 DNA Extraction

0.5 g of the spores of *Ustilago scitaminea* Sydow (which causes sugarcane smut) or sugarcane tissue infected by the pathogen was weighed and ground in a mortar by adding liquid nitrogen. The total DNA of leaf was extracted using Plant DNA Extraction Kit, following the manufacturer's instruction. The quality of extracted DNA was identified using Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

1.2 Specific Primers for the Amplification of *U. scitaminea* Genome

The primers were designed according to the conserved nucleotide sequences of *bE* mating-type gene of *U.scitaminea* genome previously reported (Singh et al. 2004). The forward primer was bE4:5′-CGCTCTGGTTCATCAACG-3′, and the reverse primer was bE8:5′-TGCTGTCGATGGAAGGTGT-3′. The target fragment was 420 bp in size.

1.3 PCR Assay

The PCR amplification system contained 2.5 μL of 10 × PCR buffer, 2.0 μL of 25 mmol/Lol/L $MgCl_2$, 1.0 μL of 10 mmol/L dNTPs, 0.2 μL of *Taq* polymerase (5 U/μL), 2.0 μL of each primer (10 μmol/L), and 2 μL of DNA template, and sterile ultrapure water was added to the total volume of 25 μL. PCR was started with an initial denaturation step at 94 °C for 4 min, followed by 35 thermal cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, and terminated with a final extension at 72 °C 10 min. Three replicates were prepared for each sample.

1.4 Results Analysis

The PCR product (10 μL) was electrophoresed on a 1.0% agarose gel and visualized under UV light using Bio-Rad imaging system. The presence of a band at 420 bp indicated a positive test for *U. scitaminea*, and the absence of the band indicated a negative test (Fig. 1).

2 PCR Detection of Sugarcane Rust (Orange Rust and Brown Rust)

2.1 PCR Detection of Orange Rust Pathogen [*Puccinia kuehnii* (Kruger) Butler]

2.1.1 DNA Extraction

The total DNA of leaf sample (0.2 g) infected by *Puccinia kuehnii* (Kruger) Butler which causes orange rust in sugarcane was extracted using Plant DNA Extraction

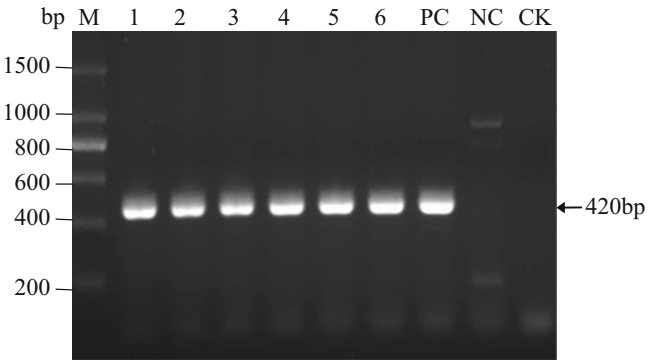

Fig. 1 PCR detection of sugarcane smut

Kit, following the manufacturer's instruction. The quality of extracted DNA was identified using Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

2.1.2 Specific Primers for the Amplification of *P. kuehnii* Genome

The primers were designed according to the conserved nucleotide sequences of the ITS region of *P. kuehnii* genome previously reported (Glynna et al. 2010). The forward primer was Pk1-F:5′-AAGAGTGCACTTAATTGTGGCTC-3′, and the reverse primer was Pk1-R:5′-CAGGTAACACCTTCCTTGATGTG-3′. The target fragment was 527 bp in size.

2.1.3 PCR Assay

The PCR amplification system with a total volume of 25 μL contained 10 μL of ddH_2O, 12.5 μL of 2 × PCR Taqmixture, 0.5 μL of DNA template, and 1.0 μL of each primer (10 μmol/L). PCR was started with an initial denaturation step at 94 °C for 5 min, followed by 35 thermal cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min, and extension at 72 °C for 30 s, and terminated with a final extension at 72 °C for 7 min. Three replicates were prepared for each sample.

2.1.4 Results Analysis

PCR product (10 μL) was electrophoresed on a 1.5% agarose gel and visualized under UV light using Bio-Rad imaging system. The presence of a band at 527 bp indicated a positive test for *P. kuehnii*, and the absence of the band indicated a negative test (Fig. 2).

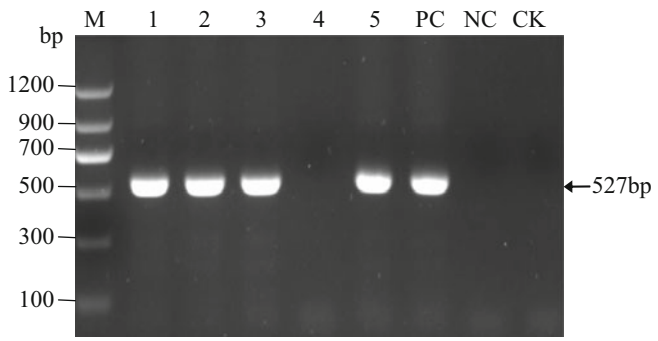

Fig. 2 PCR detection of sugarcane orange rust

2.2 PCR Detection of Brown Rust Pathogen (*Puccinia melanocephala* H. Sydow and P. Sydow)

2.2.1 DNA Extraction

The total DNA of leaf sample (0.2 g) infected by *Puccinia melanocephala* which causes brown rust in sugarcane was extracted using Plant DNA Extraction Kit, following the manufacturer's instruction. The quality of extracted DNA was identified using Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

2.2.2 Specific Primers for Amplification of *P. melanocephala* Genome

The primers were designed according to the conserved sequence of the ITS region of *P. melanocephala* genome (Wang et al. 1999). The forward primer was Pm1-F:5-′-AATTGTGGCTCGAACCATCTTC-3′, and the reverse primer was Pm1-R:5-′-TTGCTACTTTCCTTGATGCTC-3′. The target fragment was 480 bp in size.

2.2.3 PCR Assay

The PCR amplification system with a total volume of 25 μL contained 10 μL of ddH_2O, 12.5 μL of 2 × PCR Taqmixture, 0.5 μL of DNA template, and 1.0 μL of each primer (10 μmol/L). PCR was started with an initial denaturation step at 94 °C for 5 min, followed by 35 thermal cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min, and extension at 72 °C for 30 s, and terminated with a final extension of 7 min at 72 °C. Three replicates were prepared for each sample.

2.2.4 Results Analysis

PCR product (10 μL) was electrophoresed on a 1.5% agarose gel and visualized under UV light using Bio-Rad imaging system. The presence of a band at 480 bp indicated a positive test for *P. melanocephala*, and the absence of the band indicated a negative test (Fig. 3).

3 PCR Detection of Sugarcane Leaf Scald

3.1 Sample Collection and Pretreatment

Ten plants of each sugarcane sample were selected at maturity stage at five sampling sites. One node at the middle or lower part of stalk was collected from each plant, cut into a segment of 7 cm in length, and then cut in the vertical direction into four

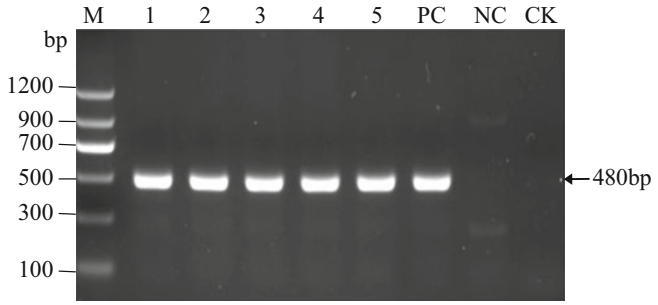

Fig. 3 PCR detection of sugarcane brown rust

copies. The samples were squeezed with pliers to collect about 25 mL of juice. The juice was mixed in a 50 mL centrifuge tube and preserved at −20 °C till analysis. The tools for sample collection and pretreatment were washed with water and then sterilized with 75% ethanol before use.

3.2 Sample Preparation

1.5 mL of the collected cane juice was transferred into a centrifuge tube and centrifuged at 13000 r/min for 3 min. After the supernatant was discarded, 1000 μL of sterile water was added to the precipitate. Then, centrifugation at 13000 r/min for 3 min was performed and repeated twice. Finally, 20–200 μL of sterile water was added to the precipitate and preserved at −20 °C till analysis.

3.3 Specific Primers for Amplification of *Xanthomonas albilineans* (Ashby) Dowson Genome

The primers were designed according to previously reported conserved sequence of *Xanthomonas albilineans* (Ashby) Dowson genome which causes leaf scald in sugarcane (Pan et al. 1998). The forward primer was XaF:5′-CCTGGTGATGACGCTGGGTT-3′; the reverse primer was XaR:5′-CGATCAGCGATGCACGCAGT-3′; and the target fragment was 600 bp.

3.4 PCR Detection

PCR amplification system (25 μL) contained 8.5 μL of ddH2O, 2×Easy Taq PCR SuperMix 12.5 μL, 2.0 μL of DNA template, and 1.0 μL of each primer (20 μg/μL). PCR was started with an initial denaturation step at 94 °C for 5 min, followed by ten

thermal cycles of denaturation at 94 °C for 45 s, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min; ten thermal cycles of denaturation at 94 °C for 45 s, annealing at 65 °C for 1 min, and extension at 72 °C for 2 min; and ten thermal cycles of denaturation at 94 °C for 45 s, annealing at 65 °C for 1 min, and extension at 72 °C for 3 min, and terminated with a final extension of 10 min at 72 °C. Three replicates were prepared for each sample.

3.5 Results Analysis

PCR reaction product (10 μL) was electrophoresed on a 1.0% agarose gel and visualized under UV light using Bio-Rad imaging system. The presence of a band at 600 bp indicated a positive test for *X. albilineans*, and the absence of the band indicated a negative test (Fig. 4).

4 PCR Detection of Sugarcane Ratoon Stunting Disease (RSD)

4.1 Sample Collection and Pretreatment

Sample collection and pretreatment for PCR detection of *Leifsonia xyli* subsp. xyli (Lxx) which causes ratoon stunting disease in sugarcane were the same as for PCR detection of *X. albilineans*.

4.2 DNA Extraction

For each sample, 2000 μL of sugarcane juice was collected in a centrifuge tube, centrifuged at 12000 r/min for 10 min, and the supernatant was discarded. The leaf total DNA was extracted using Plant DNA Extraction Kit, following the

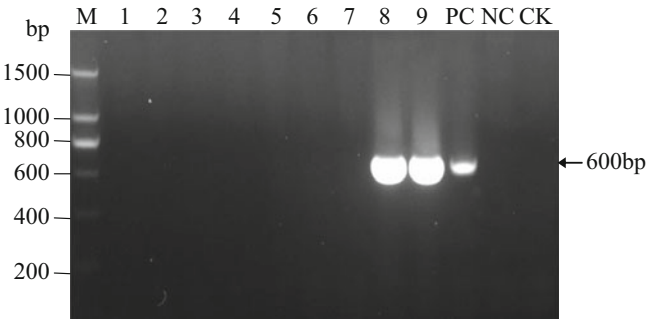

Fig. 4 PCR detection of sugarcane leaf scald

manufacturer's instruction. The quality of extracted DNA sample was identified by Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

4.3 Specific Primers for Amplification of *L. xyli* Genome

The primers were designed according to the conserved sequence of 16S-23SrDNA intergenic region of *L. xyli* (Fontana et al. 2013). The forward primer was Lxx1: 5′-CCGAAGTGAGCAGATTGACC-3′, and the reverse primer was Lxx2: 5′-ACCCTGTGTTGTTTTCAACG-3′. The target fragment was 438 bp.

4.4 PCR Assay

The PCR amplification system with a total volume of 20 μL contained 8.6 μL of ddH2O, 8 μL of 2 × PCR Taqmixture, 3 μL of DNA template, and 0.2 μL of each primer (20 μg/μL). The PCR conditions consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 thermal cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min, and were terminated with a final extension of 7 min at 72 °C. Three replicates were prepared for each sample.

4.5 Results Analysis

The PCR product (10 μL) was electrophoresed on a 1.0% agarose gel and visualized using Bio-Rad gel imaging system. The presence of a band at 438 bp indicated a positive test for *L. xyli*, and the absence of the band indicated a negative test (Fig. 5).

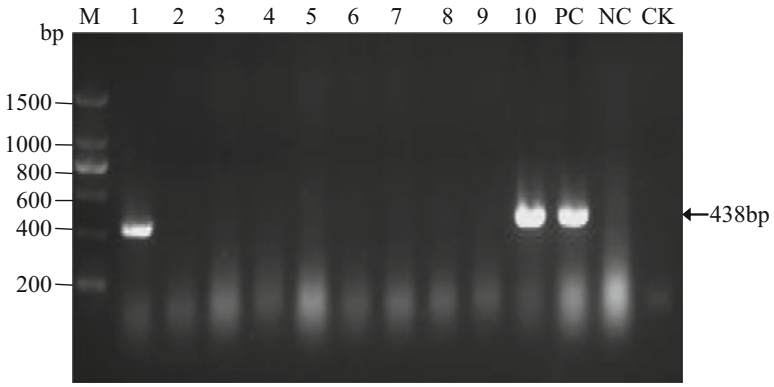

Fig. 5 PCR detection of RSD

5 PCR Detection of Sugarcane Red Stripe

5.1 DNA Extraction

0.2 g of sugarcane leaf was ground into powder in a mortar by adding liquid nitrogen. The total DNA was extracted using Plant DNA Extraction Kit, following the manufacturer's instruction. The quality of extracted DNA samples was identified by Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

5.2 Specific Primers for the Amplification of *Xanthomonas rubrilineans* (Lee et al.) Starr et Burkh

Specific primers were designed according to conserved sequences of 16S-23S rDNA intergenic region of *Xanthomonas rubrilineans* (Lee et al.) Starr et Burkh (Smith and Vandevelde 1994). The forward primer was P0f: 5′-GAGAGTTTGATCCTGGCTCAG-3′; the reverse primer was P6r: 5′-CTACGGCAACCTTGTTACGA-3′. And the target fragment was 1500 bp in length.

5.3 PCR Assay

The PCR amplification system with a total volume of 25 μL contained 11.0 μL of ddH_2O, 12.5 μL of 2 × PCR *Taq*mixture, 1.0 μL of DNA template, and 0.25 μL of each primer (10 μg/μL). The PCR conditions consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 thermal cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, and were terminated with a final extension of 7 min at 72 °C. Three replicates were prepared for each sample.

5.4 Results Analysis

10 μL of the PCR product was electrophoresed on a 1.5% agarose gel and visualized using the Bio-Rad gel imaging system. The presence of a band at 1500 bp indicated a positive test for *X. rubrilineans*, and the absence of the band indicated a negative test (Fig. 6).

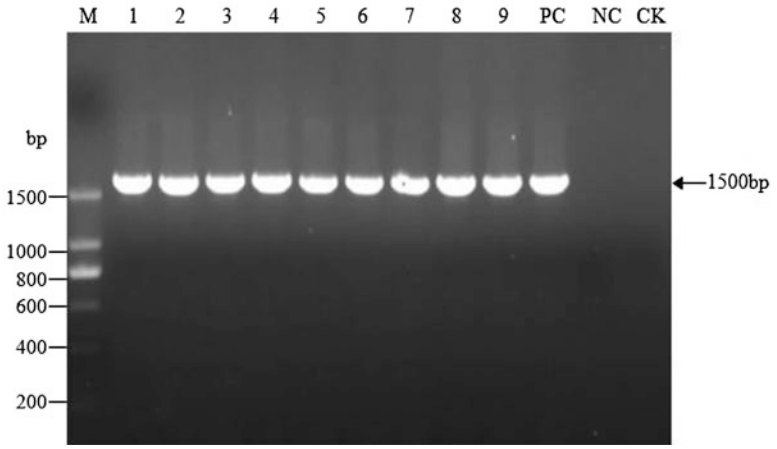

Fig. 6 PCR detection of sugarcane red stripe

6 RT-PCR Detection of Mosaic Viruses (SCMV, SrMV, and SCSMV)

6.1 RT-PCR Detection of *Sugarcane mosaic virus* (SCMV)

6.1.1 RNA Extraction

Fresh leaf (0.1 g) of sugarcane was ground into powder in a mortar, by adding liquid nitrogen. The total RNA was extracted by using Plant RNA Extraction Kit, following the manufacturer's instruction. The quality of extracted RNA was identified by Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

6.1.2 Specific Primers for Amplification of SCMV

The primers were designed according to the conserved sequences of SCMV coat protein (CP) encoding gene registered in GenBank. The forward primer was SCMV-F:5′-GATGCAGGVGCHCAAGGRGG -3′; the reverse primer was SCMV-R:5-′-GTGCTGCTGCACTCCCAACAG-3′. The target fragment was 924 bp in size.

6.1.3 RT-PCR Assay

Reverse transcription was performed using TransScript One-Step gDNA Removal and cDNA Synthesis Supermix Kit. The reverse transcription mixture (10 μL) contained 5 μL of 2 × TS reaction mix, 1.5 μL of DEPC water, 0.5 μL of 0.5 μg/μ

L Oligod $(T)_{18}$, 0.5 μL of RT/RI enzyme mix, 0.5 μL of gDNA remover, and 2.0 μL of RNA template. The reverse transcription conditions consisted of 30 min at 42 °C and 5 s at 85 °C. The PCR mixture (20 μL) contained 7.2 μL of ddH_2O, 10 μL of 2×Easy Taq PCR SuperMix (Beijing TransGen Biotech Co., Ltd), 2 μL of cDNA template, and 0.4 μL of each primer (20 μg/μL). The PCR conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 thermal cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, and were terminated with a final extension of 10 min at 72 °C. Three replicates were prepared for each sample.

6.1.4 Results Analysis

10 μL of the PCR product was electrophoresed on a 1.0% agarose gel and visualized using Bio-Rad gel imaging system. The presence of a band at 924 bp indicated a positive test for SCMV, and the absence of the band indicated a negative test (Fig. 7).

6.2 RT-PCR Detection of *Sorghum mosaic virus* (SrMV)

6.2.1 RNA Extraction

Fresh leaf (0.1 g) of sorghum was ground into powder in a mortar, by adding liquid nitrogen. The total RNA was extracted by using Plant RNA Extraction Kit, following the manufacturer's instruction. The quality of extracted RNA was identified by Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

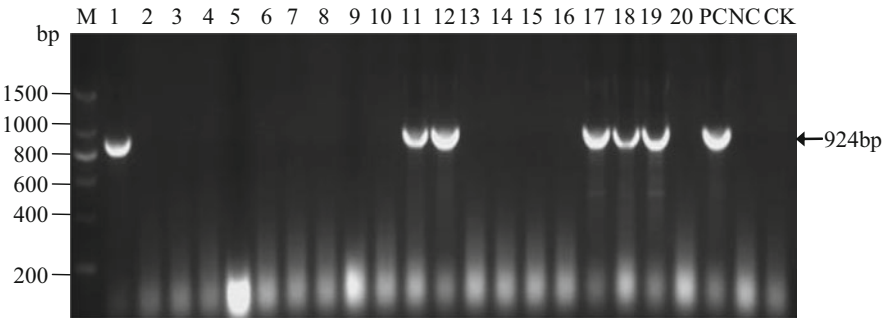

Fig. 7 RT-PCR detection of SCMV

6.2.2 Specific Primers for Amplification of SrMV Genome

The specific primers were designed according to the conserved sequences of SrMV coat protein (CP) encoding gene registered in GenBank. The forward primer was SrMV-F:5′-CATCARGCAGGRGGCGGYAC-3′; and the reverse primer was SrMV-R:5′-TTTCATCTGCATGTGGGCCTC-3′. The target fragment was 828 bp in size.

6.2.3 RT-PCR Assay

Reverse transcription was performed using TransScript One-Step gDNA Removal and cDNA Synthesis Supermix Kit. The reverse transcription mixture (10 μL) contained 5 μL of 2 × TS reaction mix, 1.5 μL of DEPC water, 0.5 μL of 0.5 μg/μL Oligod $(T)_{18}$, 0.5 μL of RT/RI enzyme mix, 0.5 μL of gDNA remover, and 2.0 μL of RNA template. The reverse transcription conditions consisted of 30 min at 42 °C and 5 s at 85 °C. The PCR mixture (20 μL) contained 7.2 μL of ddH_2O, 10 μL of 2×Easy Taq PCR SuperMix (Beijing TransGen Biotech Co., Ltd), 2 μL of cDNA template, and 0.4 μL of each primer (20 μg/μL). The PCR conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 thermal cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, and were terminated with a final extension of 10 min at 72 °C. Three replicates were prepared for each sample.

6.2.4 Results Analysis

10 μL of the PCR product was electrophoresed on a 1.0% agarose gel and visualized using Bio-Rad gel imaging system. The presence of a band at 828 bp indicated a positive test for SrMV, and the absence of the band indicated a negative test (Fig. 8).

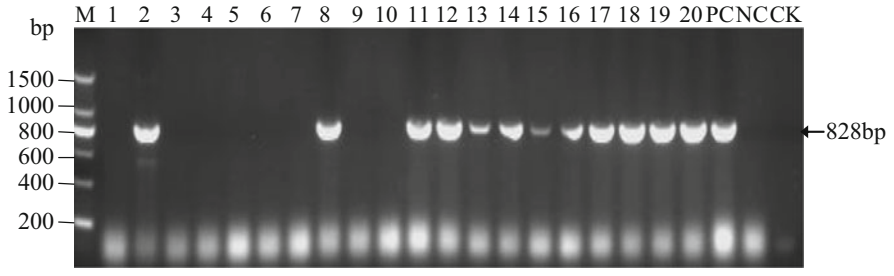

Fig. 8 RT-PCR detection of SrMV

6.3 RT-PCR Detection of *Sugarcane streak mosaic virus* (SCSMV)

6.3.1 RNA Extraction

Fresh leaf (0.1 g) of sugarcane was ground into powder in a mortar, by adding liquid nitrogen. The total RNA was extracted by using Plant RNA Extraction Kit, following the manufacturer's instruction. The quality of extracted RNA was identified by Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

6.3.2 Specific Primers for Amplification of SCSMV Genome

The primers were designed according to the conserved sequences of the coat protein (CP) encoding gene of SCSMV registered in GenBank. The forward primer was SCSMV-F: 5′-ACAAGGAACGCAGCCACCT-3′; the reverse primer was SCSMV-R: 5′-ACTAAGCGGTCAGGCAAC-3′. The target fragment was 939 bp in size.

6.3.3 RT-PCR Assay

Reverse transcription was performed using TransScript One-Step gDNA Removal and cDNA Synthesis Supermix Kit. The reverse transcription mixture (10 μL) contained 5 μL of 2 × TS reaction mix, 1.5 μL of DEPC water, 0.5 μL of 0.5 μg/μL Oligod $(T)_{18}$, 0.5 μL of RT/RI enzyme mix, 0.5 μL of gDNA remover, and 2.0 μL of RNA. The reverse transcription conditions consisted of 30 min at 42 °C and 5 s at 85 °C. The PCR mixture (25 μL) contained 9.5 μL of ddH_2O, 12.5 μL of 2×Easy Taq PCR SuperMix (Beijing TransGen Biotech Co., Ltd), 2 μL of cDNA template, and 0.5 μL of each primer (20 μg/μL). The PCR conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 thermal cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, and were terminated with a final extension of 10 min at 72 °C. Three replicates were prepared for each sample.

6.3.4 Results Analysis

10 μL of the PCR product was electrophoresed on a 1.0% agarose gel and visualized using Bio-Rad gel imaging system. The presence of a band at 939 bp indicated a positive test for SCSMV, and the absence of the band indicated a negative test (Fig. 9).

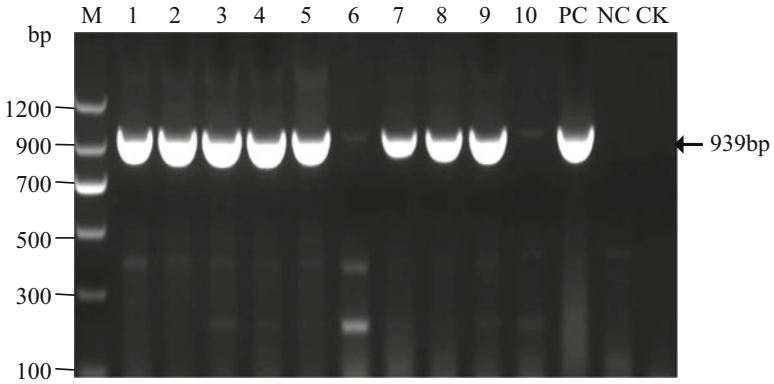

Fig. 9 RT-PCR detection of SCSMV

7 RT-PCR Detection of Sugarcane Fiji Disease

7.1 RNA Extraction

Fresh leaf (0.1 g) of sugarcane was ground into powder in a mortar, by adding liquid nitrogen. The total RNA was extracted by using Plant RNA Extraction Kit, following the manufacturer's instruction. The quality of extracted RNA was identified by Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

7.2 Specific Primers for Amplification of *Sugarcane Fiji disease virus* (SFDV)

The specific primers were designed according to the conserved sequences of the segment 9 (S9) region of *Sugarcane Fiji disease virus* genome previously reported (Cai et al. 2004). The forward primer was FDV7F:5′-CCGAGTTACGGTCAGACTGTTCTT-3′; the reverse primer was FDV7R:5′-CAGTGGTGACGAAAT GATGGCGA-3′. The target fragment was 450 bp in size.

7.3 RT-PCR Assay

One-step RT-PCR detection was performed using C. therm RT-PCR Kit. To a 0.5 mL PCR tube, 1.0 μL of RNA template, 0.25 μL of each primer (20 μg/μL), and 11.0 μL of ddH_2O were added and mixed. The mixture was denatured at 99 °C for 2 min and then immediately transferred on ice. Subsequently, 5 μL of 5 × buffer,

2.5 μL of 10% PVP, 1.25 μL of 100 mmol/L DTT, 1.25 μL of 100% DMSO, 1.0 μL of 5% BSA, 0.5 μL of 20 mmol/L dNTPs, and 1.0 μL of C. therm polymerase were sequentially added to the denatured mixture. The PCR conditions consisted of 30 min at 57 °C; 2 min at 95 °C; 35 cycles of 1 min at 95 °C, 1 min at 57 °C, and 1 min at 72 °C; and 8 min at 72 °C. Three replicates were prepared for each sample.

7.4 Results Analysis

10 μL of the PCR product was electrophoresed on a 1.0% agarose gel and visualized using Bio-Rad gel imaging system. The presence of a band at 450 bp indicated a positive test for SFDV, and the absence of the band indicated a negative test (Fig. 10).

8 RT-PCR Detection of Sugarcane yellow leaf virus *(ScYLV)*

8.1 RNA Extraction

Fresh leaf (0.1 g) of sugarcane was ground into powder in a mortar, by adding liquid nitrogen. The total RNA was extracted by using Plant RNA Extraction Kit, following the manufacturer's instruction. The quality of extracted RNA was identified by Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

8.2 Specific Primers for Amplification of ScYLV

The primers were designed according to the conserved sequences of the ScYLV coat protein (CP) encoding gene registered in GenBank. The forward primer was ScYLV-F:5′-AATCAGTGCACACATCCGAG-3′, and the reverse primer was ScYLV-R:5′-GGAGCGTCGCCTACCTATT-3′. The target fragment was 634 bp in size.

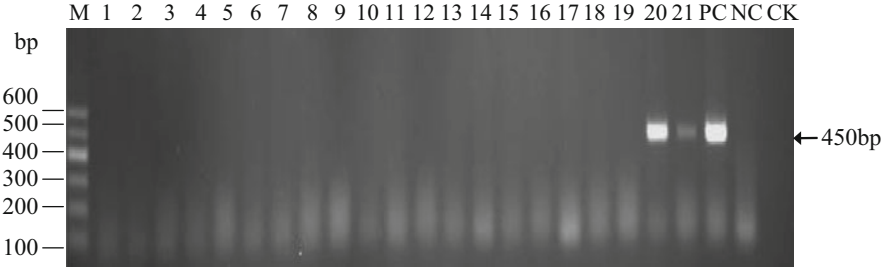

Fig. 10 RT-PCR detection of sugarcane Fiji virus

8.3 RT-PCR Assay

RT-PCR amplification of SCYLV CP gene was performed using One-Step RNA PCR Kit (TaKaRa, Dalian). In detail, 6.2 μL of ddH_2O, 0.8 μL of PrimeScript one-step enzyme mix, 10.0 μL of 2 × 1 step buffer, 0.5 μL of each primer (20 μmol/L), and 2.0 μL of RNA template were added sequentially into a PCR tube. After centrifugation at a low speed for a few seconds, the tube was loaded into PCR instrument to perform the PCR reaction under the conditions of 30 min at 45 °C; 2 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 52 °C, and 1 min at 70 °C; and 5 min at 70 °C. Three replicates were prepared for each sample.

8.4 Results Analysis

10 μL of the PCR product was electrophoresed on a 1.0% agarose gel and visualized using Bio-Rad gel imaging system. The presence of a band at 634 bp indicated a positive test for SCYLV, and the absence of the band indicated a negative test (Fig. 11).

9 PCR Detection of Sugarcane bacilliform virus *(SCBV)*

9.1 DNA Extraction

About 0.3–0.5 g of sugarcane leaf was ground into powder in a mortar, by adding liquid nitrogen, and then transferred into a 2 mL centrifuge tube. The total DNA was extracted by using Plant DNA Extraction Kit, following the manufacturer's instruction. The quality of extracted DNA was identified by Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

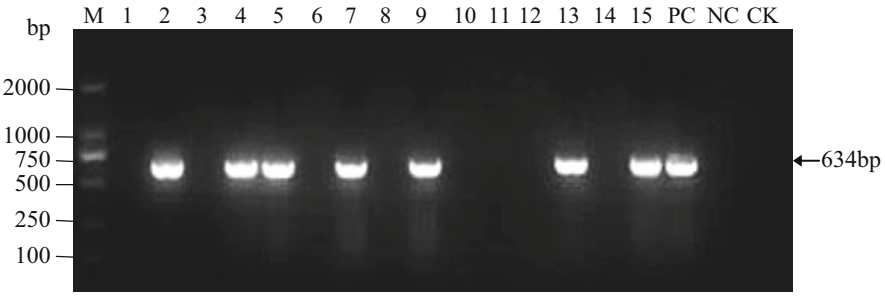

Fig. 11 RT-PCR detection of ScYLV

9.2 Specific Primers for Amplification of SCBV

The primers were designed according to the conserved region of SCBV genome previously reported[14]. The forward primer was PC1:5′-ACCAGATCCGAGATTACAGAAG-3′, and the reverse primer was PC2:5′-TCACCTTGCCAACCTTCATA -3′. The target fragment was 589 bp in size.

9.3 PCR Assay

In detail, 7.6 μL of ddH_2O, 10 μL of 2 × PCR *Taq*mix, 2 μL of DNA template, and 0.2 μL of each primer (20 μmol/L) were added into a PCR tube sequentially. After low-speed centrifugation for 10 s, PCR was started with an initial denaturation step at 94 °C for 2 min, followed by 30 thermal cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, and terminated with a final extension of 5 min at 72 °C. Three replicates were prepared for each sample.

9.4 Results Analysis

10 μL of the PCR product was electrophoresed on a 1.0% agarose gel and visualized under Bio-Rad gel imaging system. The presence of a band at 589 bp indicated a positive test for SCBV, and the absence of the band indicated a negative test (Fig. 12).

10 Nested PCR for Detection of Sugarcane White Leaf Phytoplasma

10.1 DNA Extraction

0.2 g of sugarcane leaf was ground into powder in a mortar, by adding liquid nitrogen. The total DNA was extracted by using Plant DNA Extraction Kit,

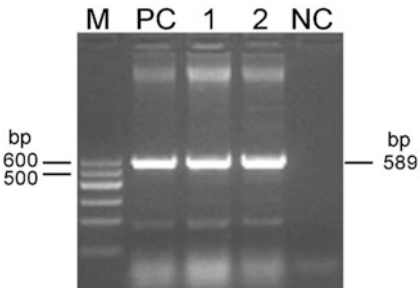

Fig. 12 PCR detection of SCBV

following the manufacturer's instruction. The quality of extracted DNA was identified by Eppendorf AG 22331 Protein/Nucleic Acid Analyzer.

10.2 Primers for Amplification of SCWL Phytoplasma

Universal primers P1/P7 and R16F2n/R16R2 for amplifying the 16S rDNA gene of phytoplasma previously reported were used for the amplification of SCWL phytoplasma in the present study (Gundersen and Lee 1996): P1:5′-AAGAGTTTGATCCTGGCTCAGGATT-3′,P7:5′-CGTCCTTCATCGGCTCTT-3′, and the target fragment was 1840 bp in size; R16F2n:5′-GAAACGACTGCTAAGACTGG-3′, R16R2:5′-TGACGGGCGGTGTGTACAAACCCCG-3′, and the target fragment was 1240 bp.

10.3 Detection of SCWL by Nested PCR

The first-round PCR was carried out using primers P1 and P7, in a total volume of 25 μL, containing 1 μL of total genomic DNA, 2.5 μL of 10 × PCR buffer, 1 μL of primer P1 (20 μmol/L), 1 μL of primer P7 (20 μmol/L), 2.0 μL of $MgCl_2$ (25 mmol/L), 2.0 μL of dNTPs (10 mmol/L), 0.2 μL of *Taq* polymerase (5 U/μL), and 15.3 μL of ddH_2O, under the conditions as follows: pre-denaturation at 94 °C for 3 min and 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. The second-round PCR was performed in a total volume of 25 μL, containing 1 μL of 30 times diluted first-round PCR product as the template, 1 μL of primer R16F2n (20 μmol/L), and 1 μL of primer R16R2 (20 μmol/L), and the volumes of other reagents were the same as the first-round PCR system, under the conditions as follows: pre-denaturation at 94 °C for 3 min and 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1 min. Three replicates were prepared for each sample.

10.4 Results Analysis

10 μL of the PCR product was electrophoresed on a 1.0% agarose gel and visualized under Bio-Rad gel imaging system. The presence of a band at 1240 bp indicated a positive test for SCWL, and the absence of the band indicated a negative test (Fig. 13a, b).

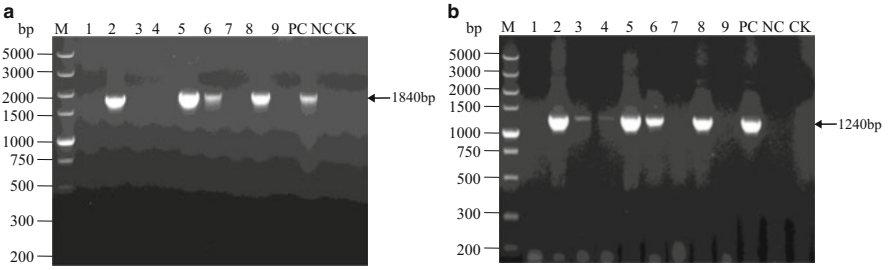

Fig. 13 (**a**) 1st PCR detection of SCWL. (**b**) 2nd PCR detection of SCWL

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Appendix 5: Molecular Detection Technique for Sugarcane Brown Rust Resistance Gene Bru1

1 Instrument and Equipment

PCR thermal cycler, gel imaging system, desk centrifuge, vortex mixer, constant temperature water bath, electrophoresis apparatus, electronic balance (balance sensitivity: 0.01–0.0001 g), microwave oven, −20 °C refrigerator, adjustable-volume pipettor, micropipette tip, centrifuge tube, PCR tube, and other related instruments and equipments.

2 Reagent

The Plant DNA Extraction Kit, 2×EasyTaq PCR SuperMix (with dye), the restriction enzyme RsaI (10000 U), DNA marker, 50×TAE buffer (diluted 50 times before use), ddH2O, agarose, and Goldview.

3 Materials

The resistant control cultivar R570 (known to contain Bru1) or ROC1 and ROC22 are used as positive control, the susceptible control variety Xuanzhe 3 or Yuetang 60 and Co290 that do not contain Bru1 are used as negative control, and sterilized deionized water is used as blank control.

4 Operation Procedure

1. Total DNA extraction

Total DNA is extracted from 0.1 g of the fully expanded first young leaf and ground into powder by liquid nitrogen. Use the Plant DNA Extraction Kit to extract the total DNA of the leaf, following the manufacturer's instruction. The quality of the extracted DNA is assessed using an AG 22331 Protein/Nucleic Acid Analyzer.

2. Primers

R12H16 is used to amplify a 570 bp fragment with the primer pair Fw: 5′-CTACGATGAAACTACACCCTTGTC-3′ and Rv: 5′-TTATGTTAGCGT GACCTATGGTC-3′, while 9O20-F4 is used to amplify a 200 bp fragment with the primer pair Fw: 5′-TACATAATTTTAGTGGCACTCAGC-3′ and Rv: 5′-ACCATAATTCAATTCTGCAGGTAC-3′.

3. PCR amplification

Amplification of the marker R12H16 is carried out in a 25 μL reaction mixture containing 9.5 μL ddH2O, 12.5 μL 2×EasyTaq PCR SuperMix, 2 μL total DNA template, and 0.5 μL each primer (20 μg/μL). The program is as follows: 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C; and a final extension for 5 min at 72 °C. Amplification of the marker 9O20-F4 is performed in a total volume of 25 μL containing 13 μL ddH2O, 10.2 μL 2×EasyTaq PCR SuperMix, 1 μL total DNA template, and 0.4 μL each primer (20 μg/μL). The program is as follows: 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C; and a final extension for 5 min at 72 °C. After amplification, 15 μL of 9O20-F4 PCR product is digested with 2.5 μL 10× digestion buffer and 1.0 μL Rsa I (10,000 U), and 6.5 μL ddH2O was added to a final volume of 25 μL. This digestion mix was incubated at 37 °C for 2 h and then 10 min at 65 °C.

4. Result and its identification

10 μL of R12H16 PCR product is analyzed by electrophoresis on a 1.5% agarose gel (add 0.005% of the Goldview in the gel before running), and 10 μL of 9O20-F4-Rsa I PCR product is analyzed by electrophoresis on a 2% agarose gel (add 0.005% of the Goldview in the gel before running). After electrophoresis, use the gel imaging system (Bio-Rad) to observe and identify. When a 570 bp target band is present in the R12H16 PCR product of the positive control, a 200 bp target band is present in the 9O20-F4-PCR-RsaI product of the positive control, and no band is present in the negative control and blank control, and we can identify that the detection result is valid. If a tested sample can simultaneously amplify the target bands of R12H16 and 9O20-F4 markers, it can be identified that this tested sample contains the brown rust resistance gene Bru1 (Fig. 14).

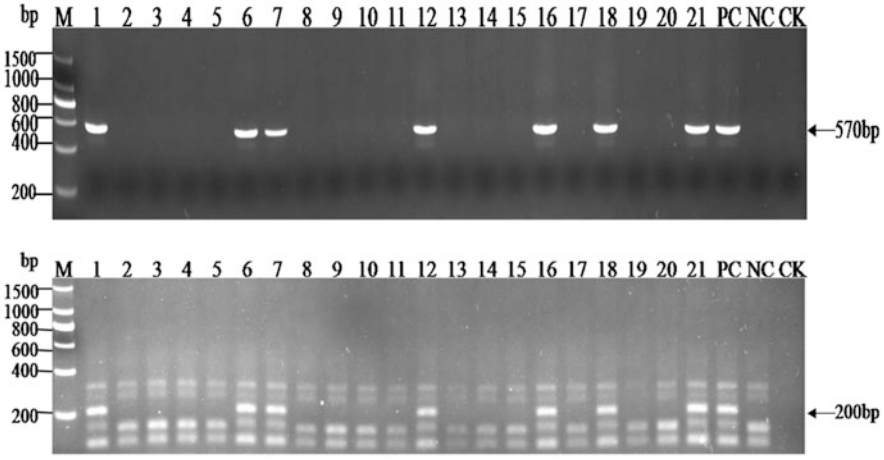

Fig. 14 PCR detection results of sugarcane brown rust resistance gene *Bru*1

Appendix 6: Production Technology Specification of Disease-Free Sugarcane Seedcane with Hot Water Treatment (DB 53/ T 370–2012) (Yunnan Provincial Standard)

1 Range

The standards specified herein cover the terms and definitions, main diseases removed, detoxification equipment and water temperature requirements, seedcane hot water treatment and propagation, seedcane quality requirements, test methods and rules, packaging, marking, storage, and transportation.

This standard applies to the production technology of disease-free sugarcane seedcane with hot water treatment.

2 Normative Files

The following documents are essential for the application of this document. For dated reference documents, only those dated references apply to this document. For undated references, the latest edition (including all the amendments) applies to this document.

NY/T 1796 sugarcane seedcane.

3 Terms and Definitions

The following terms and definitions apply to this document:

3.1 Disease-Free Sugarcane Seedcane with Hot Water Treatment

The seedcane has been treated by hot water in 50 ± 0.5 °C for 2 h, which does not contain the object to be detected in this standard.

3.2 The Percentage of Allowable Virus Infection

The allowable percentage of virus infection of disease-free sugarcane seedcane with hot water treatment.

3.3 The Rate of Virus Infection Detection

The percentage of plants detected as virus-infected in disease-free sugarcane seedcane with hot water treatment among the total number of tested plants.

4 Main Diseases Removed

4.1 Ratoon stunting disease (*Leifsonia xyli* subsp. *xyli*, Lxx)
4.2 Sugarcane mosaic virus, SCMV
4.3 Sorghum mosaic virus, SrMV

5 Detoxification Equipment and Water Temperature Requirements

5.1 Composition

The equipment for hot water treatment to produce disease-free sugarcane seedcane comprises a treatment box, automatic temperature control, and a temperature detection cabinet.

5.2 Volume

The recommended dimensions of the treatment box are 2.5 × 2.3 × 2.6 m, and the effective water body capacity should be 13,000 L, which can effectively treat 1200–1600 kg of seedcane.

5.3 Power

The recommended heating power is 120 kw. Water circulation is provided by a water pump with a power of 5.5 kw and a flow of 86.6 m^3 per hour. The water is circulated 6.7 times per hour.

5.4 Water Temperature

The automatic temperature control and temperature detection cabinet should keep the water at 50 ± 0.5 °C.

6 Seedcane Hot Water Treatment

6.1 Sample Collection

6.1.1 When the sugarcane matures, representative samples should be chosen randomly according to the varieties and the planting period.

6.1.2 Take six to ten sugarcane stems for each sample. First, cut off the young leaves with scissors, and put them in a sample collection bag for testing. Using a knife, cut each sugarcane stem at the middle and lower part of the stem internodes (about 7 cm long), squeeze 25 mL of sugarcane juice with forceps into a 50 mL centrifuge tube, and store at −20 °C. The sampling tools should be sterilized using 75% alcohol after each sample is taken.

6.2 Pathogen Detection

6.2.1 Electron microscopy (EM), serology (enzyme-linked immunosorbent assay (ELISA)), and PCR detection techniques are used to detect pathogens such as ratoon stunting disease and sugarcane mosaic disease.

6.2.2 According to the test results, sugarcane varieties with ratoon stunting disease, SCMV, and SrMV are treated with hot water.

6.3 Hot Water Treatment

6.3.1 Choose robust mature sugarcane stems, and remove the sugarcane leaves.

6.3.2 The sugarcane seedcane are cut into segments with three to five buds, placed in net bags stacked in a basket, and the baskets are soaked in flowing cold water for 48 h.

6.3.3 The water temperature is then heated to 51–52 °C, and the baskets with the seedcane are placed into the water such that the seedcanes are completely immersed.

6.3.4 Hot water treatment (50 ± 0.5 °C) continues for 2 h.

6.3.5 The seedcanes are removed from the treatment pond, sprayed with cold water, and soaked in 50% carbendazim WP 800 times liquid for 5–10 min. After disinfection, the seedcane can be planted and can also be stored for 1–2 days until the germ has hardened.

7 Seedcane Propagation

7.1 Nursery Site Selection

The nursery should be located conveniently for transportation and management. An ideal site should have conventional means of irrigation, flat terrain, good soil and land fertility, water conservation facilities, and access to roads. The primary

seedcane nursery should be located near the workshop producing disease-free sugarcane seedcane using hot water treatment.

7.2 Seedcane Propagation

7.2.1 The primary seedcane nursery is particularly used to breed disease-free seedcane using hot water treatment.

7.2.2 The disease-free seedcane is stored for 1–2 days before planting after the germ has hardened. Seedcane is planted at 120,000–150,000 buds per hectare, with double row top placement.

7.2.3 The primary seedcane nursery needs to be strictly protected from disease and pest infestation. All operations should be carried out by trained technicians. Once infected sugarcane is detected, it should be removed as soon as possible to prevent further infection.

7.2.4 In the following year, a large number of seedcanes are used for propagation that were harvested from the primary seedcane nursery. These seedcanes do not need to be treated with hot water, but secondary infection should be prevented.

7.2.5 In the secondary seedcane nursery, from planting seedcane until harvest, field management should be strengthened and strict protection observed; disease occurrence should be checked regularly.

7.2.6 In the 3rd year, the tertiary seedcane nursery is planted with seedcanes harvested from the secondary seedcane nursery. They do not need hot water treatment, but must be regularly inspected for sugarcane diseases.

7.2.7 The seedcane harvested from the tertiary nursery is disease-free seedcane, which can be provided to sugarcane farmers for planting.

7.3 Field Management

7.3.1 Field fertilization and cultivation management should be in line with the local production practice.

7.3.2 Attention should be paid to disease and pest monitoring, and seedcane purity should be tested. During the whole process of seedcane cutting and planting, the tools that come in contact with the sugarcane should be disinfected using 75% alcohol.

8 Seedcane Quality Requirements

Sugarcanes that tested positive with EM, ELISA, or PCR were virus-infected. The proportion of allowable virus-infected seedcane at all levels (the three test objects listed in the standard) and other quality requirements are shown in attached Table 3.

Table 3 Quality requirements of disease-free sugarcane seedcane with hot water treatment

Items	Requirements					
	Varietal purity %	Inclusion %	Stem diameter cm	Water content %	Germination rate %	Viruses detection rate %
Primary seedcane nursery	100.0	≤1.0	≥2.2	60–75	≥80.0	0.0
Secondary seedcane nursery	100.0	≤1.0	≥2.2	60–75	≥80.0	≤5.0
Tertiary seedcane nursery	100.0	≤1.0	≥2.2	60–75	≥80.0	≤10.0

9 Test Methods

Execute according to the provisions of NY/T 1796.

10 Test Rules

Execute according to the provisions of NY/T 1796.

11 Packaging, Marking, Storage, and Transportation

Execute according to the provisions of NY/T 1796.

Appendix 7: Technical Specification for Identification of Resistance to Sugarcane Rust Disease (DB 53/T 530–2013) (Yunnan Provincial Standard)

1 Range

This standard specifies herein cover terms and definitions, material preparation, pathogen collection, preparation of inoculum, inoculation, disease investigation, and disease resistance evaluation for detection of sugarcane rust disease resistance.

These standards are suitable for using the artificial inoculation method to identify and evaluate resistance to sugarcane rust disease in *Saccharum* L. and germplasm resources for related species and interspecies hybrids.

2 Terms and Definitions

The following terms and definitions apply:

2.1 Sugarcane Rust

Sugarcane brown rust of caused by *Puccinia melanocephala* H.Sydow and P. Sydow (synonym: *Puccinia erianthi* Padw. et Khan).

3 Material Preparation

The planting time is from March to May. A highly susceptible variety "Xuanzhe No. 3" was used as a susceptible control. A highly resistant variety "Mintang 70–611" was used as a disease-resistant control. Treated materials are planted in plastic buckets; soil and organic fertilizer (3:1) are mixed and placed into a plastic bucket (diameter 35 cm, height 30 cm) to 2/3 of the capacity of the bucket, normal management. There are four buckets for each material, and when inoculated, 20 plants with a consistent growth rate are selected from each bucket.

4 Pathogen Collection

The sugarcane rust occurrence period is from August to October; to collect disease sugarcane leaves with typical lesions from the diseased sugarcane areas, the samples were placed in a plastic sample collection bag and sprayed with water to moisturize, brought to the laboratory for spore extraction, and used directly for inoculation.

5 Preparation of Inoculum

The diseased sugarcane leaves are collected from the field and soaked for 1–2 h in plastic pots which contain 2/3 water, and then they were taken out with rubbing by hand or double-layer gauze filtration, and the filtrate served as spore suspension. After mixing well, the number of spores was counted with a blood cell count board. The inoculation concentration was 8×10^4 to 10×10^4 spores/mL.

6 Inoculation

6.1 Inoculation Period

In sugarcane elongation stage (seedling 4–6 months of age), inoculation should be carried out on a windless evening.

6.2 Inoculation Methods

Before inoculation, the bucket planting materials should be fully watered to increase the humidity, using spray inoculation method. The inoculum was evenly sprayed on the sugarcane leaves with a manual knapsack sprayer, and the inoculation amount was controlled that the spore suspension did not flow on the sugarcane leaves. After the first inoculation, do it one more time the next day.

6.3 Post-inoculation Management

After inoculation, the bucket planting materials were placed in the shading net to manage normally. They were sprayed daily two to three times with water and were not sprayed during the rainy days.

7 Disease Investigation

The incidence of test materials was investigated one by one after inoculation for 4–5 weeks. The disease is described and recorded according to the leaf's infected situation. And when investigated the incidence, visual estimation the percentage of leaves infected area of top visible hypertrophic zone.

8 Disease Resistance Evaluation

Evaluation of disease resistance is shown in attached Table 4.

Table 4 Evaluation standard for identification of resistance to sugarcane rust

Rating scale	Disease resistance	Leaf's infected situation
1	Highly resistant (HR)	No symptoms
2	Resistance (R)	There are necrotic spots, lesions accounted for leaf area below 10%
3	Moderately resistant (MR)	There are some uredinial on the plant, lesions accounted for leaf area of 11–25%
4	Moderately susceptible (MS)	The upper 1–3 leaves have some uredinial, while the lower leaves have a lot of uredinial, lesions accounted for leaf area of 26–35%
5	Susceptible 1 (S1)	The upper 1–3 leaves have a lot of uredinial, while the lower leaves have a slight necrosis, lesions accounted for leaf area of 36–50%
6	Susceptible 2 (S2)	The upper 1–3 leaves have extremely many uredinial, and the lower leaves have more necrosis than the rating 5, lesions accounted for leaf area of 51–60%
7	Susceptible 3 (S3)	The upper 1–3 leaves have extremely many uredinial, and the lower leaves have necrosis, lesions accounted for leaf area of 61–75%
8	Highly susceptible 1 (HS1)	The upper 1–3 leaves have some necrosis, lesions accounted for leaf area of 76–90%
9	Highly susceptible 2 (HS2)	Leaf necrosis, plant dying, lesions accounted for leaf area of 91–100%

Appendix 8: Technical Specification for the Comprehensive Control of Sugarcane Borers (DB 53/T 531–2013) (Yunnan Provincial Standard)

1 Range

The standard conditions outlined below describe the agricultural, physical, biological, and chemical methods for controlling sugarcane borer species. These standard conditions are applicable for the comprehensive prevention and control of *Sesamia inferens* Walker, *Chilo infuscatellus* Snellen, *Argyroploce schistaceana* Snellen, *Proceras venosatus* Walker, *Tryporyza intacta* Snellen, and *Chilo auricilia* Dudgeon.

2 Prevention and Control Objects

Sesamia inferens Walker
Chilo infuscatellus Snellen

Argyroploce schistaceana Snellen
Proceras venosatus Walker
Tryporyza intacta Snellen
Chilo auricilia Dudgeon

3 Control Principles

3.1 Adhere to the basic principle of "prevention first, comprehensive prevention and control."

3.2 For effective control, an early warning and monitoring system is essential for determining diseases in the first and second generations. Agricultural control should form the basis of this approach, and physical control is the main control method, supplemented by biological control, and chemical control should be utilized where needed and carefully coordinated to achieve comprehensive prevention and control measures.

4 Prevention and Control Methods

4.1 Agricultural Control

4.1.1 Sugarcane rotation with peanuts, soybeans, and rice or intercropping with vegetables and green manure crops can create favorable conditions for the survival and reproduction of natural enemies of sugarcane borers.

4.1.2 Choosing disease-free and healthy seedcane plants as seedcane is essential as is timely (early) seedcane planting and fertilization.

4.1.3 During the dormant phase in February and March, deadheart seedlings should be removed from the base, and all pests killed. Pest larvae which are deep in the center of seedlings can be lanced and killed with iron wire, and leaves should be stripped at the appropriate time.

4.1.4 Sugarcane buried 3−5 cm should be harvested using a hoe to cut through the soil surface, diseased leaves should be removed, and stumps and field weeds should be discarded after sugarcane harvest.

4.2 Physical Control

During the sugarcane borer adult peak period (March–July), a lamp is installed every 2−4 ha, each with a radiation radius of 100−120 m, a wavelength of 320−400 nm, and a power output of 30 W, at an installation height of 1−1.5 m (distance from the rim of the insect container to the earth). Lamps are then illuminated between 20:00 and 22:00.

4.3 Biological Control

4.3.1 Sex Pheromone Control

4.3.1.1 Sex Pheromone Trapping Method

Before each generation of pupae begins eclosion, *a* ~ 20 cm diameter trap pot is set in the sugarcane field, lures are placed across the surface of the pot every ~1 cm, and 30−45 trap pots are set per hectare, to kill the maximum number of male moths.

4.3.1.2 Mating Disruption Method

For this method, 3000 plastic tubes per hectare are combined with lures containing sex pheromone (spaced ~2.5 cm/a) which are evenly inserted into the midrib of sugarcane leaves (insertion area is 1.8 × 1.8 m), and lures are replaced every 15−20 days.

4.3.2 Release of *Trichogramma* As a Biological Control Agent

Trichogramma wasps are among the main natural enemies of various sugarcane borers in sugarcane fields, and they can benefit from artificial reproduction and field release. A total of 150,000 *Trichogramma* individuals per hectare are repeatedly released into sugarcane fields at the beginning of and during the peak breeding period, at 75−120 release points, five to seven times per year. Pesticide application should be delayed until well after the release of *Trichogramma* (~7 to 20 days).

4.3.3 Control Using *Trichogramma* Carrying Viruses

When using *Trichogramma* carrying a virus, they should be released during the peak ovipositing season of the targeted sugarcane borer species and in accordance with the density of the pest. Approximately 75−105 individuals per hectare should be released in an equidistant manner. *Trichogramma* egg cards containing the virus for controlling the sugarcane borer species are hung in the shade of sugarcane plants between early morning and 10:00 or after 15:00 as appropriate. Chemical pesticides cannot be used simultaneously.

4.3.4 Protecting Natural Pest Enemies

Apanteles flavipes Cameron (*Cotesia flavipes* Cameron), *Sturmiopsis inferens* Townsend, egg parasitoids, and other natural pest enemies can be used effectively against sugarcane borers. These species are widely distributed in sugarcane areas, and the parasitic rate is generally 15−35%. From early spring, an efficient, low-toxicity selective insecticide can also be applied to the root zone.

4.4 Chemical Control

4.4.1 At the end of February and in early March, ratoon planting and soil loosening should take place, and in May and June, when soil is "hilled up," each hectare should be treated with 3.6% broad-spectrum bisultap granules (GR), 3.6% common type bisultap GR, 5% carbosulfan GR, 5% monosultap•chlorpyrifos GR, 8% chlorpyrifos•phoxim GR (45−90 kg), 15% chlorpyrifos GR (15−18 kg), 30% chlorobenzamide•thiamethoxam suspension concentrate (SC; 600 mL), or 40% chlorobenzamide•thiamethoxam water-dispersible granules (WG; 600 g), mixed evenly with 600 kg of dry fine soil or chemical fertilizer. Treatments should be evenly applied to sugarcane ditches, sugarcane stumps, or sugarcane base and quickly covered with soil or plastic film.

4.4.2 In March and April, incubation of the first and second generation of sugarcane borer eggs reaches the peak stage, at which point each hectare should be subjected to treatment with a selection of 30% chlorantraniliprole•thiamethoxam SC (600 mL), 40% chlorantraniliprole•thiamethoxam WG (600 g), 98% cartap hydrochloride wettable powder (WP; 1000× concentrate), 25% bisultap water aqua (AP; 200× concentrate, 20% avermectin•fenitrothion emulsifiable concentrate (EC; 600× concentrate), and 95% monosultap dustable powder (DP; 1000× concentrate) by spraying onto leaves twice over 10−15 days. Pesticides should be applied in an alternating manner.

Appendix 9: Technical Specification for Identification of Resistance to Sugarcane Mosaic Disease (DB 53/T 637–2014) (Yunnan Provincial Standard)

1 Range

The standards specified herein cover terms and definitions, material selection and planting, inoculation of virus source, preparation of inoculum, inoculation method, disease investigation, and disease resistance evaluation for detection of sugarcane mosaic disease resistance.

These standards are suitable for using the artificial inoculation method to identify and evaluate resistance to sugarcane mosaic disease, which is caused by *Sugarcane mosaic virus* (SCMV), *Sorghum mosaic virus* (SrMV), and *Sugarcane streak mosaic virus* (SCSMV) in *Saccharum* L., germplasm resources for related species, and interspecies hybrids. These standards can also serve as a reference for the identification and evaluation of resistance to mosaic disease caused by the other viruses.

2 Terms and Definitions

The following terms and definitions apply:

2.1 Sugarcane Mosaic Disease

Mosaic virus diseases are caused by *Sugarcane mosaic virus* (SCMV), *Sorghum mosaic virus* (SrMV), and *Sugarcane streak mosaic virus* (SCSMV).

3 Material Selection and Planting

3.1 Material Selection

Healthy sugarcane plants without SCMV, SrMV, or SCSMV viruses should be chosen.

3.2 Control Materials

For identification materials, we used the "ROC 22" variety as a susceptible control and the "Mintang 70–611" variety as a disease-resistant control.

3.3 Material Handling

Plant material was cut into double-bud segments, soaked for 48 h in flowing cold water, heated at 50 ± 0.5 °C for 2 h, and then treated with 70% thiazoxine WG and 50% carbendazim WP (1:800 liquid soaking) for 5–10 min.

3.4 Soil Preparation

After sterilizing soil using high temperature, sterilized soil and organic fertilizer (3:1) are mixed and placed into a plastic bucket (diameter 35 cm, height 30 cm) to 2/3 of the capacity of the bucket.

3.5 Material Planting

Treated materials are planted in plastic buckets (six buckets for each material) and placed in a pest control greenhouse at 20−30 °C for cultivation. When inoculated,

5 plants with a consistent growth rate are selected from each bucket, giving a total of 30 plants.

4 Inoculation of Virus Source

Screened sugarcane plants exhibiting typical mosaic symptoms are infected with SCMV, SrMV, or SCSMV alone. SCMV, SrMV, and SCSMV viruses are then isolated and extracted from young diseased leaves to provide a virus source for inoculation and inoculated into the susceptible "ROC 22" sugarcane variety for breeding and preservation.

5 Preparation of Inoculum

Young diseased "ROC22" leaves harboring viruses are mixed with three volumes of 0.1 M phosphate buffer pH 7.2 (v:w) containing 0.2% Na_2SO_4, homogenized by grinding, and filtered through a double gauze to generate the filtrate as the virus inoculum, which should be freshly prepared just before each use.

6 Inoculation Methods

6.1 Inoculation Method Selection

Inoculation can be performed using the friction or cutting stem methods.

6.2 Cutting Stem Inoculation Method

Using test material of 4−5 months of age, the aboveground parts are rapidly removed along the soil surface, and 50 μL virus inoculum drops are injected into the sugarcane plant root incision. Each material is inoculated into 30 plants, and plants are then shaded for 24 h. After inoculation, plants are placed in a pest control greenhouse at 20−30 °C and observed.

6.3 Friction Inoculation Method

On a cool evening, 500−600 mesh quartz sand (10:1) is added to the virus inoculum, mixing well, and the thumb and index finger are used to gently scrape the sugarcane leaf epidermis at the seedling leaf base. When the test material has grown two to

Table 5 Evaluation standards for identification of resistance to sugarcane mosaic disease

Rating	Incidence (%)	Disease resistance
1	0	Highly resistant (HR)
2	0.1−10.0	Resistant (R)
3	10.1−33.0	Moderately resistant (MR)
4	33.1−66.0	Susceptible (S)
5	66.1−100	Highly susceptible (HS)

three pieces of complete leaves, the first inoculation is performed, and inoculation is repeated three times every 7 days, giving 30 plants for each material. After inoculation, plants are placed in a pest control greenhouse at 20−30 °C and observed.

7 Disease Investigation

At 20 days after cutting stem inoculation and 7 days after the first friction inoculation, observation was performed once every 15 days until no further changes were evident. The date of inoculation, inoculation plant number, date of appearance of first disease symptoms, and cumulative number of diseased plants should be documented, and the cumulative diseased plants rate (BP, %) is calculated using the formula (1)

$$\mathrm{BP} = (\mathrm{sn1}/\mathrm{sn2}) \times 100\% \tag{1}$$

where Sn1 = cumulative number of diseased plants and Sn2 = cumulative total number of plants.

8 Disease Resistance Evaluation

Evaluation of disease resistance according to grading standards is shown in attached Table 5.