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Detection and identification method of Cucumber green mottle mosaic virus

黄瓜绿斑驳花叶病毒检疫检测与鉴定方法

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Detection and identification method of Cucumber green mottle mosaic virus

1 Scope

This document specifies the sampling methods, quarantine detection, result determination and reporting, sample processing, file preservation of cucumber mottle mosaic virus.

This document is applicable to the quarantine detection and identification of cucumber mottle mosaic virus, in agricultural plant quarantine.

2 Normative references

The provisions in following documents become the provisions of this Standard through reference in this Standard. For the dated documents, only the versions with the dates indicated are applicable to this document; for the undated documents, only the latest version (including all the amendments) is applicable to this standard.

GB 15569 Quarantine protocol for the movement of agricultural plants and plant products

GB/T 28071 Detection and identification of cucumber green mottle mosaic virus

3 Terms and definitions

There are no terms and definitions, that need to be defined in this document.

4 Principles

Cucumber green mottle mosaic virus (CGMMV) belongs to the genus Tobacco mosaic virus, which spreads with seeds, scions, rootstocks. The important basis for the virus quarantine detection and identification is typical symptoms and other rapid diagnosis. It is combined with the detection results of virus double antibody sandwich enzyme-linked immunosorbent (ELISA) or the results of reverse transcription polymerase chain reaction (RT-PCR), for comprehensive judgement.

5 Reagents and materials

Sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), anhydrous disodium hydrogen phosphate (Na_2HPO_4), anhydrous potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), anhydrous sodium sulfite (Na_2SO_3), sodium hydroxide (NaOH), sodium azide (NaN_3), ethanol (CH_3CH_2OH), disodium p-nitrophenylphosphonate (PNPP), diethyl pyrocarbonate (DEPC), deionized water, commercially available antibodies, commercially available enzyme-labeled antibodies, commercially available PCR reagents, commercially available Trizol reagents, etc. The ratio of reagents, which are required in the testing, is as shown in Appendix A.

All reagents are of analytical pure.

6 Instruments and utensils

PCR instrument, desktop high-speed refrigerated centrifuge, spectrophotometer, electronic balance, enzyme-linked plate, enzyme-linked reader, enzyme-free plate machine, micropipette, water bath, electrophoresis instrument and horizontal electrophoresis tank, gel imaging system, incubators, refrigerators, etc.

7 Sampling

7.1 Seeds

The sampling quantity and method shall be carried out, according to the provisions of GB 15569. The sampling shall be sufficient and representative.

7.2 Leaves and fruits

Randomly select the diseased or suspected diseased leaves or fruits, mainly young leaves and fruits. Each fruit sample shall be not less than 200 g; the leaves shall be not less than 10 g. They shall be sealed in a Ziplock bag and stored at 4 °C, indicating the information, such as the name of the plant, collection time, place, collector.

8 Testing

8.1 Symptom inspection

Check the leaves, stems, fruits, for comparison with typical symptoms of cucumber green mottle mosaic virus disease (see Appendix B).

8.2 Rapid diagnosis

See Appendix C, for rapid diagnostic test strips or diagnostic kits. If using commercially available test strips or kits, follow the instructions to operate.

8.3 Enzyme linked immunosorbent assay (ELISA)

See Appendix D, for enzyme linked immunosorbent assay of cucumber green mottle mosaic virus. If a commercially available kit is used, follow the instructions to operate.

8.4 Reverse transcription polymerase chain reaction detection (RT-PCR)

Refer to Appendix E, for the reverse transcription polymerase chain reaction detection (RT-PCR) of cucumber green mottle mosaic virus. If using a commercially available kit, follow the instructions to operate.

9 Results judgement and reporting

9.1 Result judgment

9.1.1 Typical symptoms

If the fruit is diseased and has typical symptoms, it can be initially judged.

9.1.2 Rapid diagnostic results

If the test result of rapid diagnostic test strip or diagnostic kit is positive, it can be judged as highly suspected.

9.1.3 Enzyme linked immunosorbent assay (ELISA)

Under the premise that the OD value of the negative control optical density value is ≤ 0.1, AND positive control's OD value is greater than 2 times the negative control's OD value, if the sample hole's OD value is greater than 2 times the negative control's OD value, it is determined as a positive reaction, that is, the sample has cucumber green mottle mosaic virus.

9.1.4 Reverse transcription polymerase chain reaction detection (RT-PCR)

Observe the electrophoresis results. On the premise that the positive control has a band at 654 bp, whilst the blank and negative controls have no band, if the sample to be tested has a band at 654 bp, THEN, it is judged as a positive reaction, that is, the sample has cucumber green mottle mosaic virus.

9.1.5 Result report

Fill in the identification results, in the plant pest sample identification report, in Appendix F.

Appendix A

(Informative)

Reagent preparation method

A.1 Coating buffer

Take 1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, 0.2 g of NaN_3. Use deionized water to dilute it to 1000 mL. Adjust the pH to 9.6. Store it at 4 °C.

A.2 Washing buffer

Take 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, 8.0 g of NaCl, 0.2 g of KCl, 0.5 g of Tween-20. Use deionized water to dilute it to 1000 mL. Adjust pH to 7.4. Store it at room temperature.

A.3 Extraction buffer

Take 2.0 g of egg white powder, 20.0 g of polyvinylpyrrolidone (PVP, K30) (molecular weight 44000 ~ 54000), 1.3 g of Na_2SO_3, 0.2 g of NaN_3, 20.0 g of Tween-20. Dissolve it in 1000 mL of 1XPBST. Adjust pH to 7.4. Store it at 4 °C.

A.4 Enzyme-labeled antibody buffer

Take 2.0 g of bovine serum albumin, 10.0 g of polyvinylpyrrolidone (PVP, K30), 0.2 g of NaN_3. Dissolve it in 1000 mL of 1XPBST. Adjust to pH 7.4. Store it at 4 °C.

A.5 Substrate buffer

Take 97.0 mL of diethanolamine, 0.1 g of $MgCl_2$, 0.2 g of NaN_3. Use deionized water to dilute it to 800 mL. Adjust pH to 9.8. Store it at 4 °C.

Appendix B

(Informative)

Basic information of cucumber green mottle mosaic virus

B.1 Host range

Watermelon, gourd, cucumber, pumpkin, gourd and other melon crops.

B.2 Biological characteristics

B.2.1 Lethal temperature

90 °C ~ 100 °C.

B.2.2 Dilution limit

10^{-7} mL/L ~ 10^{-6} mL/L.

B.2.3 In vitro survival period

Up to 119 d or longer. It can be kept for several years at 0 °C. The virus can survive in the seeds for 240 d ~ 540 d. After the diseased residue is buried in the soil for 420 d, the virus still has infection activity.

B.2.4 Morphology of virions

The virions are rod-shaped by electron microscope, about 300 nm long and 15 nm wide.

B.3 Typical symptoms

B.3.1 Symptoms of watermelon infection

The leaves have irregular chlorotic, in mottled mosaic; the green part is uplifted; the leaf margins are rolled up; the leaves are slightly narrower and thinner. Fruiting pedicels sometimes show brown necrotic streaks. There are filamentous yellow fibers in the pulp; the pulp around the seeds becomes dark red and water-stained. In severe cases, the pulp becomes dark red, with a lot of cavities in it; it becomes softening, rotten, smelly, which is inedible (see Figure B.1 and Figure B.2).

Appendix D

(Informative)

Double antibody sandwich enzyme-linked immunosorbent assay for cucumber green mottle mosaic virus

D.1 Preparation of sample

D.1.1 Seeds

Take a few seeds. Peel them. Add sample extraction buffer (W:V = 1:8). Grind and homogenize it. Centrifuge it, at 5000 r/min at 4 °C for 5 min. Store it for later use.

D.1.2 Leaves

Take leaves. Add extraction buffer (W:V = 1:8). Grind and homogenize it. Centrifuge at 5000 r/min for 5 min at 4 °C. Store for later use.

D.2 Coating

Use coating antibody buffer solution, to dilute the coating antibody of CGMMV, according to the dilution ratio specified by the kit. Mix well. Add it to the enzyme-linked plate. Add 100 μL to each hole. Put it in a humidor. Incubate at 37 °C, for 4 h.

D.3 Washing

After the incubation, pour out the reagents in the reaction holes. Use PBST washing solution, to rinse it 3 ~ 4 times, for 15 s each time. Then invert the microplate on absorbent paper, to naturally dry the residual liquid in the holes.

D.4 Add antigen (sample to be tested)

Add the antigen (sample to be tested) to the enzyme-linked plate. At the same time, add 100 μL of positive control, negative control, blank control (sample extraction buffer). Repeat 3 times for the sample to be tested. Incubate for 2 h, in an incubator or at room temperature (25 °C) h. Store the remaining samples, in a -20 °C refrigerator, for future reference.

D.5 Washing

It is carried out, according to D.3.

D.6 Add enzyme-labeled antibody

Use enzyme-labeled antibody buffer, to dilute the enzyme-labeled antibody of CGMMV,

Appendix E

(Informative)

Reverse transcription polymerase chain reaction (RT-PCR) assay of cucumber green mottle mosaic virus

E.1 Pretreatment of utensils

To prevent RNA degradation by RNA enzyme, the vessels are treated by DEPC water (V:V=1:1000) before the test. Place the mortar, gun tip, centrifuge tube, etc. in DEPC water at 37 °C. Soak it for 24 h. Take out. Sterilize it at 121 °C for 30 min. Bake it dry for later use.

E.2 Extraction of total RNA

Take symptomatic leaves. Grind them fully in liquid nitrogen. Transfer them into a 1.5 mL centrifuge tube. Add 1 mL of Trizol reagent. Let it stand for 5 min, at room temperature. Then add 200 μL of chloroform. Vortex-shake it for 15 s. Place at room temperature for 5 min. Centrifuge it at 12000 r/min, at 4 °C for 15 min. Transfer the supernatant to a new centrifuge tube. Add 600 μL of isopropanol. Mix well. Let it stand at room temperature for 10 min. Centrifuge it at 12000 r/min, at 4 °C for 15 min. Discard the supernatant. Add 1 mL of 75% ethanol into the precipitate, to wash it once. Absorb the supernatant. Dry it at room temperature for 3 min ~ 5 min. Add 30 μL of DEPC ddH_2O, to dissolve the total RNA.

E.3 RT-PCR reaction system

E.3.1 Primer sequence (CGMMV-654F/R)

Upstream primer (Primer F): 5'-CGTGGTAAGCGGCATTCTAAACCTC-3';

Downstream primer (Primer R): 5'-CCGCAAACCAATGAGCAAACCG-3'.

E.3.2 RT-PCR experimental steps

E.3.2.1 cDNA synthesis

Prepare the RT reaction system (20 μL), according to the following components. The reaction is performed, by adding ddH_2O without RNA enzyme as a negative control. The reaction conditions are 42 °C for 1 h, to synthesize cDNA, inactivate reverse transcriptase at 70 °C for 10 min. The reaction system is as shown in Table E.1.

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