TẠP CHÍ KHOA HỌC
TRƯỜNG ĐẠI HỌC QUY NHƠN

Xác định chủng virus khảm thuốc lá và virus khảm dưa chuột trên giống Địa hoàng 19 (*Rehmannia glutinosa* varieties 19) bằng kỹ thuật Enzyme Linked Immunosorbent Assay

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TÓM TẮT

Virus khảm thuốc lá (Tobacco Mosaic Virus - TMV) và virus khảm dưa chuột (Cucumber Mosaic Virus - CMV) thường gây bệnh hại trên cây Địa hoàng. Nghiên cứu xác định chủng virus TMV và CMV bằng kỹ thuật Enzyme-Linked Immunosorbent Assay (ELISA) trên các cây mẹ được lấy mẫu để nhân giống, nhằm loại bỏ tác nhân gây bệnh hại ngay từ giai đoạn vào mẫu ban đầu. Kết quả cho thấy các mẫu thân, rễ củ, chồi cấp một và cây *in vitro* giống Địa hoàng 19 (*Rehmannia glutinosa* variety 19) đều không bị nhiễm hai chủng virus TMV và CMV. Đây là nguồn vật liệu khởi đầu phục vụ nhân giống cây sạch bệnh tạo nguồn giống cây Địa hoàng 19 *in vitro* đảm bảo chất lượng tốt.

Từ khóa: *Địa hoàng 19, kỹ thuật ELISA, virus khảm dưa chuột, virus khảm thuốc lá.*

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Detection of tobacco mosaic virus and cucumber mosaic virus on *Rehmannia glutinosa* variety 19 by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

Tobacco Mosaic Virus **(**TMV) and Cucumber Mosaic Virus (CMV) are common pathogens of *Rehmannia glutinosa* Libosch. The study conducted experiments to detect TMV and CMV viruses by Enzyme-Linked Immunosorbent Assay (ELISA) on the mother plants sampled for propagation to eliminate pathogens from the initial sampling stage. The detection results of the stem, tuberous roots, primary shoots, and *in vitro* seedlings of *Rehmannia glutinosa* variety 19 (RG19) showed that the samples were not infected with two types of TMV and CMV viruses. This is the starting material for propagating disease-free seedlings to create a highly qualified source of ***in vitro*** RG19 for crops.

Keywords: *Rehmannia glutinosa variety 19, ELISA, cucumber mosaic virus, tobacco mosaic virus.*

1. INTRODUCTION

Rehmannia glutinosa Libosch (RG) root contains iridoid glucoside compounds, phenylpropanoids, polysaccharides, and phenolic acids,[1] which are widely used in traditional Chinese medicines possessing anti-anemia, antipyretic, anti-inflammatory activities and the effect on lowering blood sugar, anti-aging,[2] and strengthening the immune system.[3] During the RG cultivation using tuberous roots, asexual propagation materials have been practiced through many successive propagation seasons; however, there was a problem of decreasing crop yield and productivity, which led to the degradation of the variety, causing smaller tuber diameter and more extended tuber length output and quality decline. Wang *et al*.[4] identified the Tobacco Mosaic Virus **(**TMV) and the Cucumber Mosaic Virus (CMV) that are common pathogens on RG grown in Henan province, Shandong province, China, and confirmed that they are the cause of the decline in RG varieties. According to Teng *et al*.,[5] TMV and CMV have caused severe damage to RG crops due to their effects on sprout degradation, leading to yield and quality decline. Some other authors have determined the cause of diseases on RG from TMV and CMV viruses. Matsumoto *et al*.[6] detected 27% of RG plants infected with TMV in the first year, 31% in the second year, and up to 63% in the third year, grown in Fukuoka, Japan by ELISA. The damage also reduced the verbascoside content in the tubers each year (verbascoside content reached 0.021% in the first year and only 0.016%

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in the third year). Zhang *et al*.[7] detected the virus that infected RG in Henan province, China by ELISA and gene sequencing. The results showed that TMV is the primary virus infecting RG. Sequencing Gene CP of TMV isolated from RG showed that samples' nucleotide and amino acid sequences were 86.5% and 94.3% with strain TMV U1, respectively, and up to 79.3% and 95.0% against other TMV strains. It was named the Rehmannia Mosaic Virus strain (ReMV) and belongs to the genus Tobamovirus. Liao *et al*.[8] isolated a ReMV strain from the plants grown in Taiwan and Henan province by Chen *et al*.,[9] China. Wardani *et al*.[10] also isolated the harmful ReMV strain on Tobacco plants in Yogyakarta, Indonesia.

In order to control harmful viruses on RG, Teng *et al*.[5] created two varieties of RG, including LBA1 and LBA2, that are resistant to infection by TMV and CMV viruses by genetic engineering. In addition, plants propagated by *in vitro* seedlings can also control diseases caused by viruses by using growth tips as explants or strictly controlling mother plants, using disease-free mother plants as a source of breeding materials. Therefore, we conducted the study to detect TMV and CMV viruses on *Rehmannia glutinosa* variety 19 (RG19) to select disease-free plants as a source of *in vitro* propagation.

2. RESEARCH METHODS

2.1. Research materials

RG19 was provided by Hung Vuong University and accepted for circulation according to notice No. 909/TB-TT-CLT dated July 31, 2020, of the Department of Crop Production, Ministry of Agriculture and Rural Development, Vietnam. Samples of five-month-old RG19 were collected at three locations: Dan Quyen commune, Tam Nong district, Phu Tho province, Vietnam; Bach Luu commune, Song Lo district, Vinh Phuc province, Vietnam; and Trung Yen commune, Son Duong district, Tuyen Quang province, Vietnam. Primary *in vitro* shoot samples and *in vitro* seedlings were provided by the Center for Environmental Biotechnology, Hung Vuong University - Phu Tho, Vietnam.

Laboratory chemicals: PathoScreen® Kit to detect TMV and CMV viruses purchased from Agdia-USA. Laboratory equipment: Biotek ELISA system, sample crusher Tissue Lyser II, thermal printer, sample storage refrigerator, micropipettes.

2.2. Research duration and location

The study was conducted from February 2021 to December 2022 at the Department of Molecular Pathology, Vietnam Institute of Agricultural Genetics and Center for Biotechnology - Environment, Hung Vuong University.

2.3. Research methods

2.3.1. Sampling method

Selection of mother plants: Only well-grown plants were selected: five plants/collection site × three collection sites (one collection site/province × three provinces). Each plant was tested for viruses both from stem and tuberous roots. Thirty samples were collected due to each sampled plant was separated into stem and tuberous root parts for virus testing.

Selection of primary shoots: Using the tuberous roots of the selected mother plants, proceeded to *in vitro* samples. After twenty days, the shoots from the tuber roots were identified as primary shoots. Thirty primary shoots were selected from thirty tuber slices for virus testing.

Selection of *in vitro* samples for rooting: Randomly selected one plant pot-1 from thirty pots of rooting plants in the laboratory for virus testing.

2.3.2. ELISA test method

The ELISA test was conducted according to the instructions of the PathoScreen® Kit as follows:

Sample fixation: Crush the test samples in GEB-coated buffer at a ratio of 1:10 and centrifuge at 10,000 rpm for five minutes to collect the supernatant, which was then added to three microplates (100 µl/well) for each

test sample. The positive and negative control samples were added into separate wells with the same volume in three replicates.

Sample incubation: Pack the sample tray tightly into the box and incubate at 2°C - 8°C for 12 hours.

The first sample washing: Samples were washed with PBST buffer seven times, 100 µl/time after incubation.

Immobilization of antibody-binding enzyme: Add 100 µl/well of alkaline phosphatase enzyme into each test sample tube and incubate for 2 hours at room temperature.

The second sample wash: After enzyme incubation, the samples were washed with PBST buffer eight times, 100 µl/time.

Addition of PNP substrate: PNP substrate concentration 1 mg/ml was added to the wells, 100 µl/well, then incubated for sixty minutes in the dark at room temperature. PNP will be hydrolyzed to nitrophenol phosphate under the catalysis of alkaline phosphate enzyme. Nitrophenol phosphate is a yellow substance that can be detected by the eye or by an ELISA reader. Reactive coloration and colorimetric test results were evaluated using an ELISA Biotek machine, determining the OD value at 450 nm.

Statistical data analysis: The collected data were analyzed using the IRRISTAT 5.0 program. The treatments' means were compared using the Least Significant Difference (LSD) test at the 0.05 level.

3. RESULTS AND DISCUSSION

3.1. Testing of TMV and CMV virus on stem and root samples of RG19

Stem and tuberous root test samples from healthy and well-developed RG19 seedlings were used for TMV and CMV assays, and the results can be seen in Table 1. Table 1 data showed that thirty samples of stem and roots in the experimental wells were colorless, which indicates the negative test results. In the TMV test, the OD values for all thirty samples ranged from 0.047 to 0.074, while the negative control value was 0.067. In the CMV test, the OD values for all thirty samples ranged from 0.050 to 0.061, while the negative control value was 0.056. The positive control is stable, and the colorimetric results of the three wells are all yellow for positive test results. The OD value for TMV was 1.820, which is significantly twenty-four times higher than the negative reactions. Similarly, the OD value for CMV was 2.908, which is significantly forty-seven times higher than that of negative reactions. Thus, all thirty samples of stem and root tubers of RG19 were not infected with two types of TMV and CMV. The tuberous roots of these plants are used as material in the samples for *in vitro* propagation.

Table 1. Detection of TMV and CMV virus on the stem and root of RG19.

No.	Sample ID	TMV Virus			CMV Virus		
		Color	OD value	Result	Color	OD value	Result
1	PT - R1	Nil	$0.049^{a} \pm 0.002$	-	Nil	$0.050^{a} \pm 0.005$	-
2	PT - R2	Nil	$0.061^{a} \pm 0.003$	-	Nil	$0.053^{a} \pm 0.006$	-
3	PT - R3	Nil	$0.050^{a} \pm 0.004$	-	Nil	$0.052^{a} \pm 0.000$	-
4	PT - R4	Nil	$0.047^{a} \pm 0.002$	-	Nil	$0.050^{a} \pm 0.004$	-
5	PT - R5	Nil	$0.051^{a} \pm 0.004$	-	Nil	$0.056^{a} \pm 0.002$	-
6	PT - T1	Nil	$0.059^{a} \pm 0.009$	-	Nil	$0.055^{a} \pm 0.003$	-
7	PT - T2	Nil	$0.054^{a} \pm 0.004$	-	Nil	$0.056^{a} \pm 0.002$	-
8	PT - T3	Nil	$0.059^{a} \pm 0.002$	-	Nil	$0.057^{a} \pm 0.005$	-
9	PT - T4	Nil	$0.052^{a} \pm 0.005$	-	Nil	$0.053^{a} \pm 0.002$	-

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10	PT - T5	Nil	$0.057^{a} \pm 0.008$	-	Nil	$0.050^{a} \pm 0.002$	-
11	VP - R1	Nil	$0.059^{a} \pm 0.001$	-	Nil	$0.061^{a} \pm 0.001$	-
12	VP - R2	Nil	$0.068^{a} \pm 0.004$	-	Nil	$0.059^{a} \pm 0.002$	-
13	VP - R3	Nil	$0.059^{a} \pm 0.001$	-	Nil	$0.060^{a} \pm 0.007$	-
14	VP - R4	Nil	$0.055^{a} \pm 0.004$	-	Nil	$0.053^{a} \pm 0.001$	-
15	VP - R5	Nil	$0.073^{a} \pm 0.003$	-	Nil	$0.059^{a} \pm 0.008$	-
16	VP - T1	Nil	$0.058^{a} \pm 0.006$	-	Nil	$0.060^{a} \pm 0.002$	-
17	VP - T2	Nil	$0.066^{a} \pm 0.003$	-	Nil	$0.060^{a} \pm 0.003$	-
18	VP - T3	Nil	$0.064^{a} \pm 0.003$	-	Nil	$0.061^{a} \pm 0.005$	-
19	VP - T4	Nil	$0.050^{a} \pm 0.004$	-	Nil	$0.054^{a} \pm 0.003$	-
20	VP - T5	Nil	$0.063^{a} \pm 0.005$	-	Nil	$0.056^{a} \pm 0.006$	-
21	TQ - R1	Nil	$0.059^{a} \pm 0.007$	-	Nil	$0.056^{a} \pm 0.006$	-
22	TQ - R2	Nil	$0.056^{a} \pm 0.006$	-	Nil	$0.058^{a} \pm 0.007$	-
23	TQ - R3	Nil	$0.057^{a} \pm 0.003$	-	Nil	$0.054^{a} \pm 0.002$	-
24	TQ - R4	Nil	$0.064^{a} \pm 0.004$	-	Nil	$0.060^{a} \pm 0.005$	-
25	TQ - R5	Nil	$0.066^{a} \pm 0.004$	-	Nil	$0.060^{a} \pm 0.002$	-
26	TQ - T1	Nil	$0.066^{a} \pm 0.007$	-	Nil	$0.058^{a} \pm 0.002$	-
27	TQ - T2	Nil	$0.063^{a} \pm 0.008$	-	Nil	$0.059^{a} \pm 0.009$	-
28	TQ - T3	Nil	$0.063^{a} \pm 0.008$	-	Nil	$0.056^{a} \pm 0.004$	-
29	TQ - T4	Nil	$0.074^{a} \pm 0.004$	-	Nil	$0.060^{a} \pm 0.004$	-
30	TQ - T5	Nil	$0.069^{a} \pm 0.004$	-	Nil	$0.053^{a} \pm 0.007$	-
31	PC (+)	Yellow	$1.820^{b} \pm 0.128$	+	Yellow	$2.908^{b} \pm 0.033$	+
32	NC (-)	Nil	$0.067^{a} \pm 0.014$	-	Nil	$0.056^{a} \pm 0.008$	-
$LSD_{0.5}$			*0.038*			*0.012*	

Note: Positive (+), Negative (-), Phu Tho (PT), Vinh Phuc (VP), Tuyen Quang (TQ), negative control (NC-), positive control (PC+), Root (R), Trunk (T)

Different letters (a, b) in the same column represent significant differences $p < 0.05$.

3.2. Detection of TMV and CMV virus on primary shoots of RG19

After twenty days in the root sample of RG19, the tuber root slices began to sprout to produce primary shoots. For TMV and CMV virus testing, thirty primary shoots were selected from tuberous root samples. The test results are summarized in Table 2.

Table 2. Detection of TMV and CMV virus on primary shoots of RG19.

No.	Sample ID	TMV Virus			CMV Virus		
		Color	OD value	Result	Color	OD value	Result
1	PS 1	Nil	$0.050^{a} \pm 0.001$	-	Nil	$0.049^{a} \pm 0.002$	-
2	PS 2	Nil	$0.054^{a} \pm 0.005$	-	Nil	$0.053^{a} \pm 0.004$	-
3	PS 3	Nil	$0.052^{a} \pm 0.002$	-	Nil	$0.051^{a} \pm 0.003$	-
4	PS 4	Nil	$0.048^{a} \pm 0.002$	-	Nil	$0.048^{a} \pm 0.001$	-
5	PS 5	Nil	$0.054^{a} \pm 0.005$	-	Nil	$0.052^{a} \pm 0.004$	-
6	PS 6	Nil	$0.060^{a} \pm 0.007$	-	Nil	$0.059^{a} \pm 0.007$	-
7	PS 7	Nil	$0.060^{a} \pm 0.006$	-	Nil	$0.059^{a} \pm 0.007$	-
8	PS 8	Nil	$0.060^{a} \pm 0.002$	-	Nil	$0.060^{a} \pm 0.003$	-

9	PS 9	Nil	$0.055^{a} \pm 0.003$	-	Nil	$0.054^{a} \pm 0.002$	-
10	PS 10	Nil	$0.062^{a} \pm 0.005$	-	Nil	$0.059^{a} \pm 0.002$	-
11	PS 11	Nil	$0.060^{a} \pm 0.005$	-	Nil	$0.057^{a} \pm 0.002$	-
12	PS 12	Nil	$0.055^{a} \pm 0.005$	-	Nil	$0.050^{a} \pm 0.005$	-
13	PS 13	Nil	$0.061^{a} \pm 0.014$	-	Nil	$0.055^{a} \pm 0.007$	-
14	PS 14	Nil	$0.072^{a} \pm 0.012$	-	Nil	$0.063^{a} \pm 0.009$	-
15	PS 15	Nil	$0.071^{a} \pm 0.011$	-	Nil	$0.063^{a} \pm 0.012$	-
16	PS 16	Nil	$0.070^{a} \pm 0.010$	-	Nil	$0.064^{a} \pm 0.006$	-
17	PS 17	Nil	$0.058^{a} \pm 0.001$	-	Nil	$0.058^{a} \pm 0.001$	-
18	PS 18	Nil	$0.063^{a} \pm 0.005$	-	Nil	$0.060^{a} \pm 0.005$	-
19	PS 19	Nil	$0.056^{a} \pm 0.002$	-	Nil	$0.055^{a} \pm 0.001$	-
20	PS 20	Nil	$0.055^{a} \pm 0.002$	-	Nil	$0.053^{a} \pm 0.003$	-
21	PS 21	Nil	$0.070^{a} \pm 0.003$	-	Nil	$0.065^{a} \pm 0.005$	-
22	PS 22	Nil	$0.062^{a} \pm 0.002$	-	Nil	$0.072^{a} \pm 0.010$	-
23	PS 23	Nil	$0.062^{a} \pm 0.002$	-	Nil	$0.064^{a} \pm 0.003$	-
24	PS 24	Nil	$0.057^{a} \pm 0.001$	-	Nil	$0.056^{a} \pm 0.002$	-
25	PS 25	Nil	$0.065^{a} \pm 0.005$	-	Nil	$0.064^{a} \pm 0.004$	-
26	PS 26	Nil	$0.061^{a} \pm 0.002$	-	Nil	$0.060^{a} \pm 0.002$	-
27	PS 27	Nil	$0.058^{a} \pm 0.005$	-	Nil	$0.055^{a} \pm 0.003$	-
28	PS 28	Nil	$0.060^{a} \pm 0.005$	-	Nil	$0.060^{a} \pm 0.004$	-
29	PS 29	Nil	$0.065^{a} \pm 0.004$	-	Nil	$0.065^{a} \pm 0.002$	-
30	PS 30	Nil	$0.065^{a} \pm 0.004$	-	Nil	$0.063^{a} \pm 0.004$	-
31	PC (+)	Yellow	$1.755^{b} \pm 0.124$	+	Yellow	$1.849^{b} \pm 0.128$	+
32	NC (-)	Nil	$0.082^{a} \pm 0.019$	-	Nil	$0.068^{a} \pm 0.009$	-
$LSD_{0.5}$			*0.037*			*0.038*	

Note: positive (+), negative (-), negative control (NC-), positive control (PC+), primary shoot (PS). Different letters (a, b) in the same column represent significant differences $p < 0.05$.

Table 2 shows that all thirty primary shoot samples were negative for two types of TMV and CMV viruses. The experimental wells and the negative control samples showed colorless results. However, the positive control samples showed positive results in yellow. In TMV virus detection, the OD value of thirty samples ranged from 0.048 to 0.072. The negative and positive control values were 0.082 and 1.755, respectively. Similarly, in CMV virus tests, the OD value of thirty samples ranged from 0.048 - 0.072, while the positive control value was high at 1.849, which is significantly twenty-five times higher than negative reactions (0.068). Thus, the *in vitro* primary shoot samples from the roots of RG19 were determined to be uncontaminated by TMV and CMV viruses. As a result, these samples were used in the next steps for bulk multiplication.

3.3. Detection of TMV and CMV virus on *in vitro* seedlings RG19

The primary shoots of RG19 continued to be transplanted to the bud propagation medium to multiply the number of shoots rapidly. After five times multiplication, selected shoots that met the standards for transplanting were transferred to the rooting medium to create complete *in vitro* seedlings with complete roots, stems, and leaves. Thirty *in vitro* seedlings from different culture flasks were selected to detect TMV and CMV virus. The detection results are summarized in Table 3.

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Table 3. Detection of TMV and CMV viruses on RG19 in *in vitro* seedlings.

No.	Sample ID	TMV virus			CMV virus		
		Color	OD value	Result	Color	OD value	Result
1	IS 1	Nil	$0.051^{a} \pm 0.002$	-	Nil	$0.051^{a} \pm 0.006$	-
2	IS 2	Nil	$0.049^{a} \pm 0.004$	-	Nil	$0.054^{a} \pm 0.003$	-
3	IS 3	Nil	$0.045^{a} \pm 0.002$	-	Nil	$0.049^{a} \pm 0.003$	-
4	IS 4	Nil	$0.048^{a} \pm 0.002$	-	Nil	$0.051^{a} \pm 0.001$	-
5	IS 5	Nil	$0.051^{a} \pm 0.006$	-	Nil	$0.056^{a} \pm 0.004$	-
6	IS 6	Nil	$0.052^{a} \pm 0.002$	-	Nil	$0.059^{a} \pm 0.003$	-
7	IS 7	Nil	$0.050^{a} \pm 0.002$	-	Nil	$0.057^{a} \pm 0.003$	-
8	IS 8	Nil	$0.053^{a} \pm 0.003$	-	Nil	$0.056^{a} \pm 0.002$	-
9	IS 9	Nil	$0.058^{a} \pm 0.003$	-	Nil	$0.059^{a} \pm 0.004$	-
10	IS 10	Nil	$0.049^{a} \pm 0.004$	-	Nil	$0.050^{a} \pm 0.019$	-
11	IS 11	Nil	$0.056^{a} \pm 0.008$	-	Nil	$0.058^{a} \pm 0.011$	-
12	IS 12	Nil	$0.054^{a} \pm 0.007$	-	Nil	$0.060^{a} \pm 0.009$	-
13	IS 13	Nil	$0.060^{a} \pm 0.014$	-	Nil	$0.056^{a} \pm 0.003$	-
14	IS14	Nil	$0.054^{a} \pm 0.004$	-	Nil	$0.062^{a} \pm 0.006$	-
15	IS 15	Nil	$0.055^{a} \pm 0.005$	-	Nil	$0.055^{a} \pm 0.003$	-
16	IS 16	Nil	$0.054^{a} \pm 0.002$	-	Nil	$0.058^{a} \pm 0.002$	-
17	IS 17	Nil	$0.055^{a} \pm 0.006$	-	Nil	$0.056^{a} \pm 0.005$	-
18	IS 18	Nil	$0.052^{a} \pm 0.005$	-	Nil	$0.049^{a} \pm 0.004$	-
19	IS 19	Nil	$0.057^{a} \pm 0.010$	-	Nil	$0.054^{a} \pm 0.005$	-
20	IS 20	Nil	$0.056^{a} \pm 0.009$	-	Nil	$0.057^{a} \pm 0.007$	-
21	IS 21	Nil	$0.063^{a} \pm 0.014$	-	Nil	$0.060^{a} \pm 0.005$	-
22	IS 22	Nil	$0.055^{a} \pm 0.001$	-	Nil	$0.061^{a} \pm 0.001$	-
23	IS 23	Nil	$0.056^{a} \pm 0.002$	-	Nil	$0.056^{a} \pm 0.004$	-
24	IS 24	Nil	$0.053^{a} \pm 0.003$	-	Nil	$0.061^{a} \pm 0.002$	-
25	IS 25	Nil	$0.059^{a} \pm 0.002$	-	Nil	$0.061^{a} \pm 0.002$	-
26	IS 26	Nil	$0.050^{a} \pm 0.003$	-	Nil	$0.055^{a} \pm 0.004$	-
27	IS 27	Nil	$0.056^{a} \pm 0.002$	-	Nil	$0.058^{a} \pm 0.005$	-
28	IS 28	Nil	$0.054^{a} \pm 0.002$	-	Nil	$0.060^{a} \pm 0.004$	-
29	IS 29	Nil	$0.054^{a} \pm 0.003$	-	Nil	$0.059^{a} \pm 0.005$	-
30	IS 30	Nil	$0.054^{a} \pm 0.005$	-	Nil	$0.058^{a} \pm 0.001$	-
31	PC (+)	Yellow	$2.020^{b} \pm 0.085$	+	Yellow	$1.901^{b} \pm 0.036$	+
32	NC (-)	Nil	$0.051^{a} \pm 0.002$	-	Nil	$0.053^{a} \pm 0.002$	-
$LSD_{0.5}$			*0.026*			*0.014*	

Note: positive (+), negative h (-), negative control (NC-), positive control (PC+), In vitro seedlings (IS)
Different letters (a, b) in the same column represent significant differences $p < 0.05$.

Table 3 data shows that all thirty *in vitro* seedlings of RG19 and NC samples were negative for two types of TMV and CMV viruses, while PC samples tested with yellow color. In the TMV test, the OD value of thirty samples ranges from 0.045 - 0.063; the negative control was 0.051; the positive control was 2.020, which is significantly thirty-two times higher than the negative reactions. For the CMV test, the OD value of thirty samples ranges from 0.049 - 0.062. With the same pattern, the negative control was 0.053; the positive control was 1.901, which is significantly thirty times higher than the negative reactions. Thus, none of the thirty *in vitro* seedling samples were infected with TMV or CMV viruses.

3.4. Discussion

According to Wang *et al*.[4] and Teng *et al*.,[5] RG plants infected with TMV and CMV often lead to sprouting degradation, causing smaller tuber diameter and reducing the yield and quality of tubers. Similarly, Teng *et al*.,[5] Matsumoto *et al*.,[6] and Zhang *et al*.[7] found some strains of TMV and CMV viruses in RG when cultivated in China and Japan. Of which, the research by Matsumoto *et al*.[6] showed that the TMV virus appeared on RG cultivars with a high rate of plants infected in the first year (27%). This infection rate increased to 31% and 63% in the second and third years. These infected plants also caused a decrease in verbascoside content, which was only recorded at 0.021% and 0.016% in the first year and the 3rd year, respectively. Therefore, it is necessary to select a variety of RG that are not infected with TMV or CMV for cultivation. Teng *et al*.[5] used genetic engineering to create two varieties of RG, LBA1, and LBA2, that were resistant to infection by TMV and CMV viruses.

R. glutinosa plants infected with the TMV and the CMV often have symptoms of leaf mosaic (mosaic pattern of light and dark green), yellow spots on the leaves, stunning symptoms, and necrotic spots.[4,7,8,10] When choosing mother plants during breeding, these are easy-to-identify characteristics to eliminate plants infected with TMV and CMV. In addition, breeding the plant micropropagation system is an ideal solution to the problems caused by diseases. Tissue culture helps eliminate diseases caused by viruses. Plant cell and tissue culture are important in manipulating plants for improved crop varieties. The plant regeneration system is essential to micropropagation approaches leading to plant improvement in *R. glutinosa***.** Plant tissues of *R. glutinosa* will provide a source of disease-free seedlings.

Viruses can also be eliminated by *in vitro* propagation. Before propagation, testing for TMV and CMV viruses on the mother plants, which provide propagation material, is essential to eliminate pathogens from the initial sampling stage. The test results of the stem, tuberous roots, and *in vitro* RG19 seedlings collected from Phu Tho, Vinh Phuc, and Tuyen Quang showed that the samples were not infected with two types of TMV and CMV viruses. These *in vitro* seedlings are the starting material for the propagation of disease-free plants, which contributes to providing a source of good-quality seedlings for cultivation.

4. CONCLUSION

Samples from stems and roots of RG19 grown in the three provinces of Phu Tho, Vinh Phuc, and Tuyen Quang used as materials for *in vitro* culture were not infected with TMV and CMV viruses. Thirty *in vitro* primary shoot samples and thirty complete *in vitro* seedling samples were not infected with two types of TMV and CMV viruses. The test results of the ELISA technique were all negative.

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