Detection of some virus strains on *Rehmannia glutinosa* variety 19 by Enzyme Linked Immunosorbent Assay

ABSTRACT

Tobacco Mosaic Virus (TMV) and Cucumber Mosaic Virus (CMV) are common pathogens of *Rehmannia glutinosa*. The study carried out experiments to detect of TMV, CMV virus by Enzyme Linked Immunosorbent Assay (ELISA) on the mother plants that will be sampled for propagation, in order to eliminate pathogens from the initial sampling stage. The dectection results of stem, tuberous roots, primary shoots, and *in vitro* seedlings of *Rehmannia glutinosa* variety 19 (RG19) showed that the samples were not infected with 2 types of TMV and CMV viruses. This is the starting material for propagation of disease-free seedlings to create a highly qualified source of *in vitro* RG19 for crops.

Keywords: *Rehmannia glutinosa variety 19, Enzyme Linked Immunosorbent Assay, Cucumber Mosaic Virus, Tobacco Mosaic Virus.*

1. INTRODUCTION

Rehmannia glutinosa (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[2,3], and strengthening the immune system[4]. 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 longer tuber length output and quality decline. Wang et al.[5] 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.[6] , TMV and CMV have caused serious 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, CMV viruses. Matsumoto et al.[7] detected 27% 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, only 0.016% in the third year). Zhang et al.[8] detected the virus that infected RG in Henan province, China by ELISA and gene sequencing. The results showed that TMV is the main virus infecting RG. Sequencing Gene CP of TMV isolated from RG showed that the nucleotide and amino acid sequences of samples were 86.5% and 94.3% with strain TMV U1, respectively, and up to 79.3% and 95.0% against other TMV strains, respectively. It was named as Rehmannia Mosaic Virus strain (ReMV), belongs to the genus Tobamovirus. Liao et al.[9] isolated a ReMV strain from the plants grown in Taiwan and in Henan province by Chen et al.[10], China. Wardani et al.[11] also isolated the harmful ReMV strain on Tobacco plants grown in Yogyakarta, Indonesia.

In order to control harmful viruses on RG, Teng et al.[6] 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 source of breeding materials. Therefore, we carried out the study to detect of 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 the notice No. 909/TB-TT-CLT dated July 31, 2020 of the Department of Crop Production, Ministry of Agriculture and Rural Development[12]. Samples of 5-month-old RG19 were collected at 3 locations: Dan Quyen commune, Tam Nong district, Phu Tho province; Bach Luu commune, Song Lo district, Vinh Phuc province; and Trung Yen commune, Son Duong district, Tuyen Quang province. Primary *in vitro* shoot samples and *in vitro* seedlings were

provided by the Center for Environmental Biotechnology, Hung Vuong University - Phu Tho.

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

2.2. Research time and place

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 trees were selected as following: 5 trees/site × 3 points (1 point/province × 3 provinces); Each plant was tested for viruses both from stem and tuberous roots. A total of 30 samples were collected.

Selection of primary shoots: Using the tuberous roots of the selected mother plants, proceeded to *in vitro* samples. After 20 days, the shoots that emerged from the tuber roots were identified as primary shoots. 30 primary shoots then were selected from 30 different tuber slices for virus testing.

Selection of *in vitro* samples for rooting: Randomly selected 1 plant/pot from 30 pots of rooting plants in the laboratory for virus testing.

2.3.2. E LISA test method

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

Sample fixation: Crush the test samples in GEB-coated buffer at a ratio of 1:10, centrifuge at 10,000 rpm for 5 minutes to collect the supernatant which was then add to three microplates (100 µl/well) for each test sample. The positive control and negative control samples were also added into seperated wells with the same volume in three replicates.

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

The first sample washing: After incubation, samples were washed with PBST buffer 7 times, 100 µl/time.

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: The samples after incubation with enzyme was washed with PBST buffer 8 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 60 min 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, so it can be detected by the eye or by an ELISA reader. The test results were evaluated by reactive coloration and colorimetric using an ELISA Biotek machine, determining the OD value at 450 nm.

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 are summarized in Table 1. Table 1 data showed that 30 samples of stem and roots in the experimental wells showed colorless. That means the test results were negative. In the TMV test, the OD values for all 30 samples ranged from 0.047 to 0.089 while the negative control value was 0.067. In the CMV test, the OD values for all 30 samples ranged from 0.050 to 0.084 while this value of the negative control was 0.056. The positive control is stable, its colorimetric results of 03 wells are all yellow for positive test results. The OD value for TMV was 1,820 which is 20 times higher than that of the negative reactions. Similarly, The OD value for CMV was 2,908 which is 34 times higher than that of negative reactions. Thus, all 30 samples of stem and root tubers of RG19 were not infected with 2 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.

N0	Sample ID	TMV Virus			CMV Virus		
		Color	OD value	Result	Color	OD value	Result
1	PT - R1	Nil	0.049 ± 0.002	-	Nil	0.050 ± 0.005	-
2	PT - R2	Nil	0.061 ± 0.003	-	Nil	0.053 ± 0.006	-
3	PT - R3	Nil	0.050 ± 0.004	-	Nil	0.052 ± 0.000	-
4	PT - R4	Nil	0.047 ± 0.002	-	Nil	0.050 ± 0.004	-
5	PT - R5	Nil	0.051 ± 0.004	-	Nil	0.056 ± 0.002	-
6	PT - T1	Nil	0.059 ± 0.009	-	Nil	0.055 ± 0.003	-
7	PT - T2	Nil	0.054 ± 0.004	-	Nil	0.056 ± 0.002	-
8	PT - T3	Nil	0.059 ± 0.002	-	Nil	0.057 ± 0.005	-
9	PT - T4	Nil	0.052 ± 0.005	-	Nil	0.053 ± 0.002	-
10	PT - T5	Nil	0.057 ± 0.008	-	Nil	0.050 ± 0.002	-
11	VP - R1	Nil	0.059 ± 0.001	-	Nil	0.061 ± 0.001	-
12	VP - R2	Nil	0.068 ± 0.004	-	Nil	0.066 ± 0.010	-
13	VP - R3	Nil	0.059 ± 0.001	-	Nil	0.060 ± 0.007	-
14	VP - R4	Nil	0.055 ± 0.004	-	Nil	0.053 ± 0.001	-
15	VP - R5	Nil	0.073 ± 0.003	-	Nil	0.069 ± 0.002	-
16	VP - T1	Nil	0.058 ± 0.006	-	Nil	0.060 ± 0.002	-
17	VP - T2	Nil	0.066 ± 0.003	-	Nil	0.064 ± 0.005	-
18	VP - T3	Nil	0.064 ± 0.003	-	Nil	0.061 ± 0.005	-
19	VP - T4	Nil	0.050 ± 0.004	-	Nil	0.054 ± 0.003	-
20	VP - T5	Nil	0.063 ± 0.005	-	Nil	0.056 ± 0.006	-
21	TQ - R1	Nil	0.059 ± 0.007	-	Nil	0.056 ± 0.006	-
22	TQ - R2	Nil	0.056 ± 0.006	-	Nil	0.058 ± 0.007	-
23	TQ - R3	Nil	0.057 ± 0.003	-	Nil	0.061 ± 0.012	-
24	TQ - R4	Nil	0.084 ± 0.010	-	Nil	0.081 ± 0.013	-
25	TQ - R5	Nil	0.089 ± 0.012	-	Nil	0.084 ± 0.007	-
26	TQ - T1	Nil	0.066 ± 0.007	-	Nil	0.062 ± 0.007	-
27	TQ - T2	Nil	0.063 ± 0.008	-	Nil	0.059 ± 0.009	-
28	TQ - T3	Nil	0.063 ± 0.008	-	Nil	0.062 ± 0.011	-
29	TQ - T4	Nil	0.074 ± 0.004	-	Nil	0.067 ± 0.006	-
30	TQ - T5	Nil	0.069 ± 0.004	-	Nil	0.053± 0.007	-
31	PC (+)	Yellow	1.820 ± 0.128	+	Yellow	2.908 ± 0.033	+
32	NC (-)	Nil	0.067 ± 0.014	-	Nil	0.056 ± 0.008	-

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

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

After 20 days in the root sample of RG19, the tuber root slices began to sprout to produce primary shoots. 30 primary shoots were selected from 30 different tuberous root samples for TMV and CMV virus testing. The test results are summarized in Table 2.

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

N0	Sample ID	TMV Virus			CMV Virus		
		Color	OD value	Result	Color	OD value	Result
1	PS 1	Nil	0.005 ± 0.001	-	Nil	0.049 ± 0.002	-
2	PS 2	Nil	0.054 ± 0.005	-	Nil	0.053 ± 0.004	-
3	PS 3	Nil	0.052 ± 0.002	-	Nil	0.051 ± 0.003	-
4	PS 4	Nil	0.048 ± 0.002	-	Nil	0.048 ± 0.001	-
5	PS 5	Nil	0.054 ± 0.005	-	Nil	0.052 ± 0.004	-
6	PS 6	Nil	0.06 0 ± 0.007	-	Nil	0.059 ± 0.007	-
7	PS 7	Nil	0.060 ± 0.006	-	Nil	0.059 ± 0.007	-
8	PS 8	Nil	0.060 ± 0.002	-	Nil	0.06 0 ± 0.003	-
9	PS 9	Nil	0.055 ± 0.003	-	Nil	0.054 ± 0.002	-
10	PS 10	Nil	0.062 ± 0.005	-	Nil	0.059 ± 0.002	-
11	PS 11	Nil	0.060 ± 0.005	-	Nil	0.057 ± 0.002	-
12	PS 12	Nil	0.055 ± 0.005	-	Nil	0.050 ± 0.005	-
13	PS 13	Nil	0.061 ± 0.014	-	Nil	0.055 ± 0.007	-
14	PS 14	Nil	0.072 ± 0.012	-	Nil	0.063 ± 0.009	-
15	PS 15	Nil	0.071 ± 0.011	-	Nil	0.063 ± 0.012	-
16	PS 16	Nil	0.07 0 ± 0.010	-	Nil	0.064 ± 0.006	-
17	PS 17	Nil	0.058 ± 0.001	-	Nil	0.058 ± 0.001	-
18	PS 18	Nil	0.063 ± 0.005	-	Nil	0.060 ± 0.005	-
19	PS 19	Nil	0.056 ± 0.0 02	-	Nil	0.055 ± 0.001	-
20	PS 20	Nil	0.055 ± 0.002	-	Nil	0.053 ± 0.003	-
21	PS 21	Nil	0.07 0 ± 0.003	-	Nil	0.065 ± 0.005	-
22	PS 22	Nil	0.086 ± 0.007	-	Nil	0.072 ± 0.010	-
23	PS 23	Nil	0.086 ± 0.007	-	Nil	0.081 ± 0.010	-
24	PS 24	Nil	0.057 ± 0.001	-	Nil	0.056 ± 0.002	-
25	PS 25	Nil	0.065 ± 0.005	-	Nil	0.064 ± 0.004	-
26	PS 26	Nil	0.061 ± 0.002	-	Nil	0.060 ± 0.002	-
27	PS 27	Nil	0.058 ± 0.005	-	Nil	0.055 ± 0.003	-
28	PS 28	Nil	0.06 0 ± 0.005	-	Nil	0.06 0 ± 0.004	-
29	PS 29	Nil	0.065 ± 0.004	-	Nil	0.065± 0.002	-
30	PS 30	Nil	0.065 ± 0.004	-	Nil	0.063 ± 0.004	-
31	PC (+)	Yellow	1.755 ± 0.124	+	Yellow	1.849 ± 0.128	+
32	NC (-)	Nil	0.082 ± 0.019	-	Nil	0.068 ± 0.009	-

Note: Positive (+) ; Negative (-), negative control (NC-), positive control (PC+), primary shoot (PS).

Table 2 data shows that, all 30 primary shoot samples were negative for 2 types of TMV and CMV viruses. The experimental wells and the negative control samples showed colorless result, however the positive control samples showed positive results in yellow. In TMV virus detection, the OD value of 30 samples ranged from 0.048 to 0.086. This value recorded at negative and positive control were 0.082 and 1,755, respectively. Similarly, CMV virus test the OD value of 30 samples ranged from 0.048 - 0.081, whilw the positive control value was high at 1,849 which is 23 times higher than negative reactions (0.068). Thus, the *in vitro* primary shoot samples from the roots of RG19 were determined as uncomtaminated from TMV and CMV viruses.

As the 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 were continued to be transplanted to the bud propagation medium to rapidly multiply the number of shoots. After 5 times of multiplication, selected shoots that met the standards for transplanting were transferred to the rooting medium to create complete *in vitro* seedlings with with complete roots, stems and leaves. Selection of 30 *in vitro* seedlings from 30 different culture flasks were used to detect TMV and CMV virus. The detection results are summarized in Table 3.

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

N0	Sample ID	TMV virus			CMV virus		
		Color	OD value	Result	Color	OD value	Result
1	IS 1	Nil	0.044 ± 0.003	-	Nil	0.051 ± 0.006	-
2	IS 2	Nil	0.049 ± 0.004	-	Nil	0.054 ± 0.003	-
3	IS 3	Nil	0.045 ± 0.002	-	Nil	0.049 ± 0.003	-
4	IS 4	Nil	0.048 ± 0.002	-	Nil	0.051 ± 0.001	-
5	IS 5	Nil	0.051 ± 0.006	-	Nil	0.056 ± 0.004	-
6	IS 6	Nil	0.052 ± 0.002	-	Nil	0.059 ± 0.003	-
7	IS 7	Nil	0.050 ± 0.002	-	Nil	0.057 ± 0.003	-
8	IS 8	Nil	0.053 ± 0.003	-	Nil	0.056 ± 0.002	-
9	IS 9	Nil	0.058 ± 0.003	-	Nil	0.059 ± 0.004	-
10	IS 10	Nil	0.049 ± 0.004	-	Nil	0.050 ± 0.019	-
11	IS 11	Nil	0.056 ± 0.008	-	Nil	0.058 ± 0.011	-
12	IS 12	Nil	0.054 ± 0.007	-	Nil	0.060 ± 0.009	-
13	IS 13	Nil	0.060 ± 0.014	-	Nil	0.066 ± 0.016	-
14	IS14	Nil	0.054 ± 0.004	-	Nil	0.062 ± 0.006	-
15	IS 15	Nil	0.055 ± 0.005	-	Nil	0.055 ± 0.003	-
16	IS 16	Nil	0.054 ± 0.002	-	Nil	0.058 ± 0.002	-
17	IS 17	Nil	0.055 ± 0.006	-	Nil	0.056 ± 0.005	-
18	IS 18	Nil	0.052 ± 0.005	-	Nil	0.049 ± 0.004	-
19	IS 19	Nil	0.057 ± 0.010	-	Nil	0.054 ± 0.005	-
20	IS 20	Nil	0.056 ± 0.009	-	Nil	0.057 ± 0.007	-
21	IS 21	Nil	0.063 ± 0.014	-	Nil	0.064 ± 0.011	-
22	IS 22	Nil	0.055 ± 0.001	-	Nil	0.061 ± 0.001	-
23	IS 23	Nil	0.056 ± 0.002	-	Nil	0.056 ± 0.004	-
24	IS 24	Nil	0.053 ± 0.003	-	Nil	0.061 ± 0.002	-
25	IS 25	Nil	0.088 ± 0.049	-	Nil	0.091 ± 0.051	-
26	IS 26	Nil	0.050 ± 0.003	-	Nil	0.055 ± 0.004	-
27	IS 27	Nil	0.056 ± 0.002	-	Nil	0.058 ± 0.005	-
28	IS 28	Nil	0.054± 0.002	-	Nil	0.063 ± 0.002	-
29	IS 29	Nil	0.054 ± 0.003	-	Nil	0.059± 0.005	-
30	IS 30	Nil	0.084 ± 0.005	-	Nil	0.088 ± 0.004	-
31	PC (+)	Yellow	2.02 0 ± 0.085	+	Yellow	1.901 ± 0.036	+
32	NC (-)	Nil	0.051 ± 0.002	-	Nil	0.053 ± 0.002	-

Note: Positive (+) ; Negative h (-), negative control (NC-), positive control (PC+), In vitro seedlings (IS)

Table 3 data shows that, all 30 samples of *in vitro* seedlings of RG19 and NC samples were negative for 2 types of TMV and CMV viruses while PC samples tested with yellow color. In TMV test, the OD value of 30 samples ranges from 0.044 - 0.088; the negative control was 0.051; the positive control was 2.020 which was 23 times higher than that of the negative reactions. For CMV test the OD value of 30 samples ranges from 0.049 - 0.091. With the same pattern, the negative control was 0.053; the positive control was 1,901 which was 21 times higher than that of the negative reactions. Thus, all 30 *in vitro* seedling samples were not infected with 2 types of TMV virus and CMV virus.

3.4. Discussion

According to Wang et al. [5] and Teng et al.[6] RG plants infected with TMV, CMV often lead to sprout degradation, causing smaller tuber diameter, reducing yield and quality of tubers. Similarly, Matsumoto et al.[7], Zhang et al.[8] and Teng et al.[6], some strains of TMV and CMV viruses were found in RG when cultivated in China and Japan. Of which, the Research by Matsumoto et al.[7] showed that the TMV virus appeared on RG cultivar with a high rate of plants infected in the first year (27%). This infected rate was increased to 31% and 63% in the second year and the third year respectively. These infected plant also caused an decrease in verbascosid content which were 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 is not infected with TMV, CMV for cultivation. Teng et al.[6] used genetic engineering to create two varieties of RG named LBA1 and LBA2 that were resistant to infection by TMV and CMV viruses. Viruses can also be eliminated by *in vitro* propagation. Before conducting propagation, it is very important to test for TMV and CMV viruses on the mother plants which provide material for propagation in order to eliminate pathogens from the initial sampling stage . The test results of 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 2 types of TMV and CMV viruses. These *in vitro* seedlings are the starting material for propagation of disease-free plants which contributes to the provision of a source of good quality seedlings for cultivation.

4. CONCLUSION

Samples from stems and roots of RG19 grown in 3 provinces of Phu Tho, Vinh Phuc and Tuyen Quang used as materials for *in vitro* culture were not infected with TMV and CMV viruses. 30 *in vitro* primary shoot samples and 30 complete *in vitro* seedling samples were not infected with 2 types of TMV and CMV viruses. The test results by ELISA technique were all negative.

REFERENCES

1. I. M. Chung, J. J. Kim, J. D . Lim, C. Y. Yu, S. H. Kim, S. J . Hahn. Comparison of resveratrol, SOD activity, phenolic compounds and freeamino acid in *Rehmannia glutinosa* under temperature and water stress, *Environ. Exp. Bots.*, **2006**, *56*, 44-53.
2. I. Kitagawa, Y. Fukuda, T. Taniyama, M. Yoshikawa. Chemical studies on crude drug processing. VII. On the constituents of *Rehmanniae* radix: absolute stereostructures of rehmaglutins A, B and D isolated from Chinese *Rehmanniae* Radix, the dried root of *R. glutinosa* Libosch., *Chem. Pharm. Bull.*, **1991**, *39*, 1171-1176.
3. R. X. Zhang, M. X. Li, Z. P. Jia. *Rehmannia glutinosa* : review of botanicals, chemistry and pharmacology, *J. Ethnopharm.*, **2008**, *117*, 199-214.
4. H. Zhao, J. Tan, C. H. Qi. Photosynthesis of *R. glutinosa* subjected to drought stress is enhanced by choline chloride through alleviating lipid peroxidation and increasing proline accumulation, *Plant Growth Reg.*, **2007**, *51*, 255-262.
5. M. Wang, I. F. Lin, L. Q. Huang, Y. F. Chen, Z. G. Wu, G. F. Li, E. S. Weim, R. X. Fang. TMV and CMV widely infect cultivated *Rehmannia glutinosa* Libosch. *Acta Phytopathological Sinica*, **2006**, *36(2)*, 189-192.
6. Z. Teng, Y. Shen, J. Li, Z. Lin, M. Chen, M. Wang, M. Li, H. Dong, L . Huang . Construction and quality a nalysis of t ransgenic *Rehmannia glutinosa* c ontaining TMV and CMV coat p rotein, *Molecules*, **2016**, *21*, 1134-1145.
7. M. Matsumoto, Y. Shoyama, I. Nishioka, H. Iwai, S. Wakimoto. Identification of viruses infected in *Rehmannia glutinosa* Libosch. var *purpurea* Makino and effect of virus infection on root yield and iridoid glycoside contents . *Plant Cell Reports*, **1989**, *7*, 636-638.
8. Z. Zhang, L. Zhang, Q. Qiao, Y. Wang, X. Jin. Identification of viral pathogens of *Rehmannia glutinosa* disease in Henan province, *Acta Phytopathologica Sinica*, **2004**, *34(5)*, 395-399 .

9. J. Liao, C. Hu, J. Kao, T. Deng. Identification of Tobacco mosaic virus infecting *Rehmannia glutinosa*, *Plant Pathol. Bull.*, **2007**, *16*, 61-69.

10. J Chen, C. Feng, X. Guo, Y. Zhou, T. Gu, X. Zhuang, L. Cheng, K. Zhang. Development of polyclonal antibodies based serological methods for detection of the R ehmannia mosaic virus in field plants. *Frontiers in Sustainable Food System*, **2022**, *6*, doi.org/10.3389/fsufs.2022.1013470 .

11. T. P. Wardani, S. Hartono, S. Sulandari, S. Somowiyarjo. Double i nfections of Rehmannia mosaic virus and Potato virus Y on Tobacco p lants in c entral Java and s pecial r egion of Yogyakarta, *Jurnal Perlindungan Tanaman Indonesia*, **2021** , *25(2)*, 133-140 .

12. Department of Crop Production, Ministry of Agriculture & Rural Development. Notice No. 909/TB-TT-CLT dated July 31, 2020 about the announcement of circulation of the variety Diva 19. **2020** .