Study on Virus Elimination and Virus Detection Technology of Lily Filament

Virus Detection and Production of virus free plant materials from the Lily by slecting the basal of the filament flower as a explants

ABSTRACT

Lilium is a perennial bulbous flower of Lily family *Liliaceae* , with high ornamental and economic value. However, Lily is vulnerable to virus infection, which seriously affects the yield and quality of Lily, and poses a great threat to the production, sales, especially export of Lily, and has caused huge economic losses to the related industries. Therefore, the research on lily virus removal methods and virus detection technology has important practical significance to improve the ornamental value and economic value of lily.

In this study, the filaments of four susceptible lily varieties,' Valdisole' (A),'Adoration'(LA),' Ice Cube'(OT) and 'Zantriana' (O), were used as explants. The filaments of lily were divided into three parts, namely, top, middle , and base. In this paper, the virus detection of tissue culture seedlings induced by lily filaments was carried out by using DAS-ELISA and RT-PCR, and the removal effects of Cucumber mosaic virus,(CMV) and lily symptomless virus (LSV), two common viruses in lily, were explored, and the two detection technologies were compared.

In terms of virus-free effect, CMV virus can be basically removed from tissue culture seedlings induced by filament, but LSV virus removal effect is not good. From the two detection methods, RT-PCR is more sensitive than DAS-ELISA, but RT-PCR requires higher test conditions and technical requirements. Therefore, appropriate virus detection methods can be selected according to actual conditions and severity.

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Key words: lily filament, filaments culture, detoxification, virus detection

1.INTRODUCTION

Lilium, a perennial bulbous flower of *Liliaceae*, is one of the world's rare cut flowers, which is widely used in landscaping, food, and medicine, and has high ornamental and economic value[1]. However, lily is very susceptible to many ~~virus~~ viral infection because it mainly depends on nutrition. After virus infection, the growth ~~potential is~~of the plant become weakened, the plants are dwarfed, the flowers bloom early, the flowers are deformed and smaller, the colors are reduced, and the leaves appear mosaic, shrinkage, distortion, withered spots, and necrosis in early spring, which seriously affect the yield and quality of lily, bring great threat to the production and sales of lily, especially the export, and cause huge economic losses to related industries[2-3]. Therefore, it is of great practical significance to explore the virus removal method and virus detection technology for improving the ornamental ~~value~~ and economic value of lily.

In addition to fungal ~~diseases~~, ~~virus~~ viral disease has become the main disease next to lily. Because virus replication is closely related to plant metabolism and some viruses have strong

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stress resistance, there are still no effective control measures. Therefore, obtaining virus-free seedlings is the key to producing high-quality lily [4-11]. Phillips(1962) was the first to study the virus-free lily. After his successful virus-free lily stem tip culture, many foreign scholars succeeded in using the stem tip culture. In 1966, Mori and Hamaya obtained virus-free lily plants through stem tip culture [12]. At present, several countries in the world have successfully obtained lily virus-free vaccines by this method, which has been applied on a large scale in production. In addition, there are some new ways. Asano et al. (1978) used filaments of *Lilium longiflorum* for tissue culture and obtained virus-free bulbs. Through in vitro culture technology, Israel has also successfully established a virus-free procedure for lily [13]. Virus-free lily is mainly carried out through the combination of shoot tip culture, bead bud culture, chemical method, and heat treatment. Experiments show that the comprehensive method of shoot tip culture+heat treatment+chemical treatment makes the virus-free effect of lily ideal. However, there are few available shoot tips on lily bulbs, which are difficult to operate and have high technical requirements, which undoubtedly increase the cost of virus-free[14-17]. Filaments as explants, the disinfection and induction culture process is simple and easy to operate, which can provide abundant test materials after propagation. At the same time, filaments grow fast and have little possibility of a virus. Taking lily filaments as test materials is an ideal material for virus-free research, but there are few experiments on lily filaments, so this experiment is innovative.

In this study, four susceptible lily varieties,' Val di Sole',' Adoration',' Ice Cube' ,and 'Zantriana' were used as experimental materials, and the non-toxic seed seedlings of *Lilium pumilum DC*. were used as blank control. By exploring the detoxification effects of different varieties and different filament parts, And compared the differences between two different virus detection technologies, summed up a set of perfect virus-free cultivation technology and rapid and accurate virus detection technology system, which provided scientific guidance for the non-toxic production of this lily variety.

2. MATERIALS AND METHODS

2.1 Plant materials

The test materials ~~were~~ of the lily varieties imported from Holland through SINO Floriculture Co,Ltd, which were planted continuously in the garden plant practice base of Beijing Agricultural College for 2 years, and their flower buds were taken for testing. The test materials ~~were detected by RT-PCR to contain~~ were tested for CMV and LSV viruses RT-PCR using viral specific primers. Specific varieties, characteristics, and serial numbers are as follows:

Table1 The number and characteristics of the four kinds of lily

Coding No.	Part	Name	Bloom Color
1	A	'Val di Sole'	Yellow
2	LA	'Adoration'	White
3	OT	'Ice Cube'	White
4	O	'Zantriana'	Pink

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Provide the details of the primers and protocol used fro testing

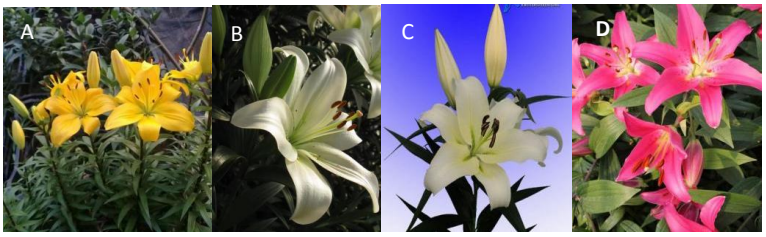

Fig1: A: A 'Val di Sole' B: LA 'Adoration' C: OT 'Ice Cube' D: O' Zantriana'

Enzyme-linked immunosorbent assay kits for lily symptomless virus (LSV) and cucumber mosaic virus (CMV) are products of American ADGEN company, TransZol PlantRNA extraction kit (TransGen Biotech), RNase-free Water, isopropanol, chloroform, absolute ethanol and RT-PCR reverse transcription kit (TransGen Biotech).

2.2 Materials treatments

Four diseased lily buds with consistent growth, immature and mature traits such as 'Val di Sole', 'Adoration', 'Icecube' ,and 'Zantriana' were selected from the experimental base, and three buds were selected from each variety as three replicates. The cut buds were washed ~~under~~ in running tap water for 30–60 min, and the surface water was sucked dry with filter paper before disinfection. On the ultra-clean bench, the buds were fully immersed in 75% alcohol solution for 10s, and then the alcohol on the buds was roasted with an alcohol lamp (about 40s). Selecting robust lily flower buds, nipping petals with tweezers, peeling filaments, cutting the peeled filaments into three sections, namely a base section, a middle section, and a top section, respectively inoculating the three sections on a culture medium, and recording the three sections with a marker pen; Each bottle was connected with 6 filament segments, and 9 bottles of one variety, totally 36 bottles. After inoculation, the formation of callus was observed, and the survival numbers of explants under each treatment were recorded. After about four weeks, statistical analysis was performed on the data, and the bulbar seedlings cultured after induction were proliferated[18-26]. The presence virus ~~of~~in the tissue culture seedlings was ~~detected~~ confirmed by DAS-ELISA and RT-PCR, and the virus-carrying status of the varieties was counted. In this study, the seedlings of *Lilium pumilum DC*. which were not infected with CMV and LSV viruses by RT-PCR were used as the blank control. Sucrose 50g/L, agar 5g/L, and PH6.0 were added to the above medium. The filaments were cultured at the temperature of (24± 2)℃and the light intensity of 1500～2000lx (light time of 10–12 h/d) in the room.

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2.3 Determination of lily virus by DAS-ELISA

~~The experimental materials were tissue culture seedlings induced from the early filament base.~~ Five offspring seedlings in tissue culture were randomly selected from each bud filament, with 15 plants in each variety and 60 samples in four varieties. An enzyme-linked immunosorbent assay (double antibody sandwich method) was used for confirm the viruses (CMV and LSV).

2.4 RT-PCR detection technology of lily virus

~~The test material was tissue culture seedling induced from the base of the floral filament in the early stage. Five~~The Five offspring seedlings (the same as the offspring seedlings in

DAS-ELISA method) derived from floral filament expansion of each bud were randomly selected, totaling 15 plants per variety and 60 samples for four varieties.

Total RNA ~~of plants~~ was extracted ~~with~~ using TransZol Plant RNA extraction kit produced by TransGen Biotech was subjected PCR using specific primers for LSV and CMV

~~(1)~~ (

~~2) PCR system was established based on the specific primers for LSV and CMV designed by Xu Rongxue and Ming Jun et al[27]. The sequences are~~ shown in Table 2.

Table 2 Priner sequences size and RT-PCR product size

Viruses	Upstream primer(5'- 3')	Upstream primer(5'- 3')	Product size (bp)
LSV	GAAGAAGCACGCTGGACTG	CGCCTGATGATCCCCTC	171
CMV	ATGGACAAATCTGAATCAACCAG	TCAGACTGGGAGCACTCCAG	657

(3) The concentration of each primer in PCR, the denaturation, annealing ,and extension conditions of PCR as well as the cycle times were optimized to determine the optimal reaction mode. Reverse transcription at 42~~℃~~℃ for 30 min and 94~~℃~~℃ for 5 min. The volume of PCR reaction is 20uL, including 2×One-Step Reaction Mix10ul, Easy script one-step enzyme mix 0.4ul, CMV and LSV upstream and downstream primers (10uM) 0.4uL, 0.4ul cDNA, RNase-free Water 8.4 uL. The optimized PCR program was as follows: pre-denaturation at 95℃ for 4min, followed by denaturation at 94℃for 30s, annealing at 52℃for 30s, extension at 72 ℃ for 1min, 35 cycles, extension at 72 ℃for 10min and finally the reaction at 4℃. Amplification was performed on DNA Engine®Thermal Cycler, agarose (1%) gel electrophoresis was performed, and the electrophoresis results were observed using a GelDoc1000 gel analyzer (Bio-Rad) and photographed.

(5) Samples with sizes close to the target fragments amplified by PCR were sent to our company for sequencing, and the sequencing results were compared with the color images and relevant sequences in NCBI.

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3. RESULTS AND DISCUSSION

3.1 The induction rate of the base of the filament was the best

Table3 Lily filament number of flower buds,the inoculation site,the survival rate and thinduction rate

Lily Varieties	Serial number	Bud number (piece)	Vaccination site	Inoculated filaments number(piece)	Survival number (piece)	Survival Rate(%)
'Val di Sole'	1	3	Top	18	0	0
			middle	18	0	0
			base	18	11	61.1
'Adoration	2		top	18	0	0

’		3	middle	18	1	5.6
			base	18	18	100
			top	18	0	0
‘Ice Cube’	3	3	middle	18	1	5.6
			base	18	18	100
			top	18	0	0
‘Zantriana’	4	3	middle	18	1	5.6
			base	18	18	100

The statistical analysis of the test data showed that the induction rates of the four lily varieties in different parts of the filament ~~were(:~~ base > middle > top)~~.,~~ ~~and~~ the induction rate of the base of the filament was the best as compared to middle and top. The induction rate of the base of the flower that 'Adoration', ‘Zantriana’ and 'Ice Cube' varieties was as high as 100%, while that of the 'Val di Sole' variety was slightly lower, 61.1%; The induction rate in the middle part of the filament of the cultivars of 'Adoration', 'Ice Cube', and ‘Zantriana’ was only 5.6%, while that in the middle part of the filament of the cultivar of 'Val di Sole' was 0%. No growth point was induced at the top of filament in four varieties. This ~~experiment~~ results showed that different lily varieties had different induction effects on the filament. The induction rates of different filament parts in the same variety were also different. This ~~result was~~ may be due to ~~probably because~~ the basal part of the filament had enough nutrients, and the tissue from the base to the top was tender ~~and tender, and the content of nutrients was small~~it contains very small amount nutrients. In addition, it was related to the length of filament disinfection time. The longer the time was, the lower the survival rate of filament would be. The buds with different development degrees might also affect the test result. The specific reason remains to be further discussed.

3.2 Detection of CMV and LSV virus by DAS-ELISA in lily

Table 4 The value of CMV detected by OD

OD	1	2	3	4	5	6	7	8	9	10	11	12
A	1.196	0.108	0.100	0.101	0.103	0.105	0.093	0.096	0.082	0.001	0.002	0.001
B	1.205	0.099	0.106	0.094	0.122	0.118	0.104	0.116	0.151	0.001	0.001	0.002
C	1.202	0.099	0.108	0.105	0.281	0.112	0.117	0.105	0.159	0.001	0.001	0.001
D	0.082	0.117	0.091	0.108	0.102	0.114	0.111	0.115	0.136	0.001	0.002	0.001
E	0.088	0.104	0.099	0.285	0.129	0.133	0.103	0.099	0.125	0.002	0.001	0.001
F	0.182	0.100	0.101	0.098	0.108	0.120	0.115	0.109	0.119	0.001	0.002	0.002
G	0.221	0.106	0.104	0.111	0.116	0.114	0.102	0.126	0.167	0.001	0.002	0.001
H	0.188	0.081	0.094	0.096	0.110	0.002	0.001	0.001	0.002	0.002	0.001	0.002

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Note: Well A1-C1 is the positive control, Well F1-H1 is the negative control, Well D1-E1 is the blank value, and the rest are test samples in the table. The OD value of each sample was detected by ELISA reader, and the average OD value of the positive control was calculated to be 1.201. The mean OD value of that negative control was 0.197; The blank mean OD value was 0.085.

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It can be concluded from the table that the detoxification rate of 'Val di Sole' is 100%; The virus-free rate of 'Adoration' was 93.3%. The virus-free rate of ' Ice Cube' was 93.3%. The

virus-free rate of ‘Zantriana’ was 100%. The experimental results showed that 'Val di Sole' and ‘Zantriana’ could completely remove CMV ~~virus~~, while 'Adoration' and 'Ice Cube' could not completely remove CMV ~~virus~~.

Table5 The value of LSV detected by OD

OD	1	2	3	4	5	6	7	8	9	10	11	12
A	0.747	0.079	0.093	0.098	0.082	0.163	0.083	0.101	0.230	0.001	0.000	0.001
B	0.715	0.102	0.099	0.085	0.090	0.088	0.103	0.090	0.165	0.001	0.001	0.002
C	0.726	0.089	0.088	0.094	0.104	0.127	0.101	0.098	0.138	0.001	0.001	0.001
D	0.062	0.085	0.130	0.093	0.186	0.109	0.078	0.136	0.093	0.001	0.000	0.001
E	0.052	0.086	0.085	0.095	0.094	0.086	0.163	0.107	0.143	0.000	0.001	0.001
F	0.099	0.088	0.156	0.099	0.097	0.099	0.102	0.111	0.119	0.001	0.000	0.000
G	0.085	0.089	0.094	0.082	0.099	0.084	0.096	0.096	0.170	0.001	0.000	0.001
H	0.076	0.069	0.073	0.100	0.092	0.000	0.001	0.001	0.000	0.000	0.001	0.000

Note: Well A1-C1 was the positive control, Well F1-H1was the negative control, Well D1-E1 was the blank value, and the rest were test samples in the table. The OD value of each sample was detected by ELISA reader, and the average OD value of the positive control was calculated to be 0.729. The mean OD value of that negative control was 0.087; The blank average OD value was 0.057, and the relevant data statistics were shown in Table 5

The detoxification rate of 'Val di Sole' was 26.7%. The detoxification rate of ‘Adoration' was 13.3%; The detoxification rate of 'Ice Cube' was 20%; The detoxification rate of ‘Zantriana’ was 26.7%. The results showed that the four lilies could not completely remove LSV and the detoxification rate was low.

3.3 The detectable rate of LSV ~~virus~~ was higher than that of CMV ~~virus~~ by DAS-ELISA detection

Table6 The statistics of DAS-ELISA detection

Variety Name	CMV Virus-free rate（%）	The quantity detection（piece）	Average relevance ration（%）	LSV Virus-free rate（%）	The quantity detection（piece）	Average relevance ration（%）
‘Val di Sole’	100.0	0		26.67	11	
‘Adoration’	93.33	1	3.33	13.33	13	78.33
‘Ice Cube’	93.33	1		20.0	12	
‘Zantriana’	100.0	0		26.67	11	

As can be seen intuitively from the table, samples harbored up to 78.33% LSV and a very low 3.33% CMV. It indicated that the removal rate of LSV ~~virus~~ from the four varieties of 'Val di Sole', 'Adoration', 'Ice Cube', and ‘Zantriana’ was very low, while the removal rate of CMV ~~virus~~ was very high or basically removed.

The results of this study showed that the detectable rate of LSV ~~virus~~ was higher than that of

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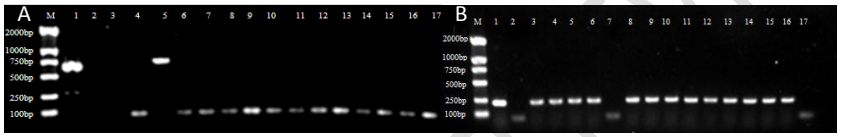
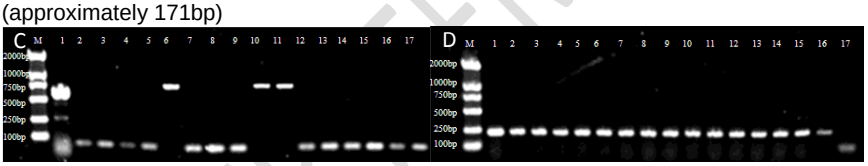
CMV virus, and the detectable rate of CMV ~~virus~~ was extremely small, only 3.33%, which might be related to the extent to which plant material was infected by virus and the characteristics of the virus itself. Liu Fen also pointed out in ~~the~~ that ~~research that the~~ infection rate of LSV of *Lilium davidii* was greater than that of CMV[28], and LSV ~~virus~~ was difficult to remove from the *Lilium davidii* . From the detoxification effect of varieties, the detoxification effects of CMV and LSV from high to low were ‘Zantriana’, 'Val di Sole', 'Ice Cube', 'Adoration'; The detoxification effect of Lily 'Val di Sole' and ‘Zantriana’ varieties were better than that of 'Adoration' and' Ice Cube' varieties. Plants ~~with~~ having CMV ~~virus~~ infection was also ~~carry~~ infected with LSV ~~virus~~. This result might be related to the characteristics of 'Adoration' and 'Ice Cube'. The of 'Adoration' and 'Ice Cube' was more robust than the 'Val di Sole' and ‘Zantriana’ in growth and not suitable for degradation. Once infected, the virus might also be difficult to remove.

3.4 Detection of CMV in lily ~~virus~~ by RT-PCR

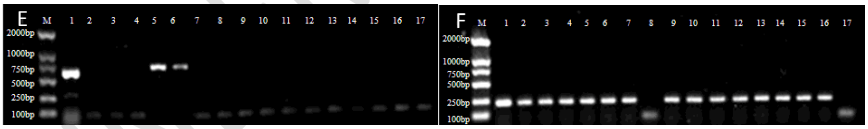
Test results of CMV: A (about 657bp) and LSV: B (about 171bp) of 'val di Sole'



Worship the results of the 'Adoration' cultivars CMV: C (approximately 657bp) and LSV:D (approximately 171bp)



Test Results for CMV:E (approximately 657bp) and LSV:F (approximately 171bp) ' Icecube'.



Results of CMV:G (about 657bp) and LSV:H (about 171bp) Test for ‘Zantriana’.

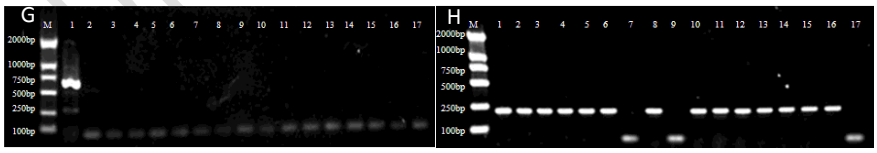


Fig. 2: Well 1 was the original plant, and the LSV virus band was clear and bright. Well 2–16 were the filament tissue culture seedlings of three buds of this variety, 15 plants in total, and Well 17 was the blank control.

3.5 The detoxification rate of CMV ~~virus~~ was higher than that of LSV ~~virus~~ by RT-PCR virus detection

A PCR assay was performed on 15 samples from each lily variety of 'Val di Sole', 'Adoration', 'Ice Cube', and ‘Zantriana’. The detoxification rates of CMV and LSV were counted as follows:

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Table 7 The statistics of RT-PCR detection

Serial number	Variety Name	CMV Virus-free Rate（%）	LSV Virus-free Rate（%）
1(A)	‘Val di Sole’	93.3	13.3
2(LA)	‘Adoration’	80.0	0.0
3(OT)	‘Ice Cube’	86.7	6.7
4(O)	‘Zantriana’	100.0	13.3

As shown in Table 6, the virus-free rates of CMV ~~virus~~ on the base of flower filament of 'Val di Sole', 'Adoration', 'Ice Cube' and ‘Zantriana’ were 93.3%, 80.0%, 86.7% ,and 100.0%, respectively. The detoxification rates of LSV were 13.3%, 0.0%, 6.7% and 13.3%, respectively. It was obvious that the detoxification rate of CMV ~~virus~~ was higher than that of LSV ~~virus~~. From the detoxification effect of varieties, the detoxification effects of CMV and LSV were ‘Zantriana’, ' Val di Sole', 'Ice Cube', 'Adoration' from high to low; The detoxification effect of 'Val di Sole' and ‘Zantriana’ varieties was better than that of 'Adoration' and 'Ice Cube' varieties. Plants with CMV virus also carry LSV ~~virus~~.

4.CONCLUSION

The experiment by Yao et al. showed that the differentiation rate of filament was significantly higher than that in other parts and the differentiation required a shorter time [29]. In this study, the color of the silk gradually changed from pale yellow or white to green, with both ends of the incision tilted, 5–7 d after inoculation. After about 20d, the incision site was obviously enlarged, indicating that callus had been produced in the tissue. After 25–30 d of culture, an exophytic callus was formed around the incision at the lower end of the explant. The protuberant callus varied in size, and the surface was uneven. About 40 d to 45 d, bud differentiation appeared on the callus, and then the filaments were further elongated and thickened, and the base was further expanded. The buds came together to form a bud cluster and gradually extended out of the green leaves from about 56 d to 60 d. In this process, the top and middle parts were almost free from swelling. The above test results are similar to the studies by Jiang Chunhua [30], Zhou Zufu [31], Zhao Daping [32] and others.

In this experiment, the growth point was easy to grow from the base (lower end of morphology) of the filament, with a high induction rate, and a low induction rate in the middle part, with all the tops dead, which was consistent with the study by Liu et al. that only the part close to the base of the receptacle could induce buds or callus, while other parts could not be induced [33]. This experiment showed that different lily varieties had different induction effects on filament. The induction rate of the same variety also differed in different the filament parts. This result was probably because the basal part of the filament had enough nutrients, and the tissue from the base to the top was tender and tender, and the content of nutrients was small. In addition, it was related to the length of filament disinfection time. The longer the time was, the lower the survival rate of filament would be. The buds with different development degrees might also affect the test result. The specific reason remains to be further discussed

The virus-free rates of CMV virus on the base of flower filament of 'Val di Sole', 'Adoration', 'Ice Cube' and ‘Zantriana’ were 93.3%, 80.0%, 86.7%, and 100.0%, respectively. The

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detoxification rates of LSV were 13.3%, 0.0%, 6.7% and 13.3%, respectively. For virus elimination rate, CMV virus is higher than LSV virus, and LSV virus elimination rate is low, the highest 13.3%, the lowest is 0%. From the detoxification effect of varieties, the detoxification effects of CMV and LSV were 'Val di Sole', 'Ice Cube', 'Adoration' from high to low; The detoxification effect of 'Val di Sole' and ‘Zantriana’ varieties was better than that of 'Adoration' and 'Ice Cube' varieties. Plants harboring CMV virus also harbored LSV virus, which was consistent with the trend in detection conclusions by DAS-ELISA. The numerical values of the two detection methods were compared, and it was found that the detection accuracy of RT-PCR technology was more accurate than that of enzyme-linked immunosorbent assay (DAS-ELISA), indicating that the sensitivity of RT-PCR technology was high.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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