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Efficacy of virus elimination from *Rehmannia glutinosa* using simultaneous thermotherapy, chemotherapy, and meristem culture

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Abstract

Rehmannia glutinosa Libosch. is considered a valuable medicinal plant in Asia owing to its antianemic, antipyretic, anti-inflammatory, and antisenescence effects. Propagating the seeds of *R. glutinosa* is difficult; therefore, the plants are propagated vegetatively. However, this method increases the incidence of viral infection in commercial production. Experiments were conducted to determine the optimal micropropagation conditions for the production of healthy and virus-free *R. glutinosa* plants using thermotherapy, chemotherapy, and meristem tip culture. Thermotherapy (heat treatment at 37°C for 4 weeks), chemotherapy (addition of an antiviral agent into the growth medium), and meristem tip culture (0.3–0.5 mm) were separately performed. Reverse transcription-polymerase chain reaction tests were used to detect five viruses, namely, *Rehmannia* mosaic virus, Youcai mosaic virus, Broad bean wilt virus 2, Plantago asiatica *mosaic virus*, and *Rehmannia virus 1*. The virus elimination rate reached 55% with chemotherapy (60 mgL^{-1} ribavirin) and 10% with thermotherapy and 37% with meristem culture, respectively. The survival rates were high with thermotherapy (67.9%) and chemotherapy (55.2%) but low with meristem culture (29.4%). Complete inactivation of the viruses was challenging using these treatments; therefore, the treatments were combined. After the application of meristem culture followed by thermotherapy (37°C for 6 weeks) and chemotherapy (60 mgL^{-1} ribavirin), a high virus elimination rate (73%) and vigorous growth were observed. Furthermore, we obtained virus-free plants. Meristem culture followed by thermotherapy and chemotherapy may be effective for the elimination of major viruses from infected *R. glutinosa* plants.

Keywords: Apical meristem culture; Chemotherapy; *Rehmannia glutinosa*; RT-PCR; Thermotherapy.
Abbreviation: BAP -6-benzylaminopurine; BBWV2 - Broad Bean Wilt Virus 2; IAA - 3-Indole-Acetic Acid, MS - Murashige and Skoog; PlAMV - Plantago Asiatica Mosaic Virus; ReMV - Rehmannia Mosaic virus; ReV1 - Rehmannia Virus 1; *RNA* - Ribonucleic Acid; RT-PCR - Reverse Transcription-Polymerase Chain Reaction; YoMV - Youcai Mosaic Virus.

Introduction

Rehmannia glutinosa Libosch. is a perennial herb of the Scrophulariaceae family and is one of the most common medicinal crops in China and Korea (Cui et al., 2000). Roots of *R. glutinosa* are used in the treatment of hematologic disorders, insomnia, and diabetes (Xu, 1988). The main active components of *R. glutinosa* exert various beneficial effects and pharmacological actions on the blood, immune, cardiovascular, and nervous systems (Hasegawa et al., 1982; Bi et al., 2008; Liu et al., 2008; Zhang et al., 2008). Breeding of *R. glutinosa* involves the division of tubers or planting of seeds. However, propagation from seeds is challenging because of the low seed survival rate, poor reproduction, and slow harvesting. Therefore, these crops are generally propagated by dividing the roots (Park et al., 2009). *R. glutinosa* propagules generally exhibit poor seed germination, root viral infection, unpredictable yield, and slow plant growth (Park et al., 2009; Xu and Davey 1983; Zhao et al., 2007).

To address this breeding challenge, an effective propagation method using *in vitro* culture technology is required. Xu (1988) reported that tuber propagation caused severe plant damage owing to viral infection during propagation, resulting in decreased tuber yield. *R. glutinosa* generally gets infected by at least five viruses, namely, *Rehmannia mosaic virus* (ReMV), *Youcai mosaic virus* (YoMV), *Broad bean wilt virus 2* (BBWV2), *Plantago asiatica mosaic virus*, and *Rehmannia virus 1* (ReV1). Yellow spots, mosaics, or red veins on leaves are predominant symptoms of *R. glutinosa* viral infection, and depending on the type, these viruses are transmitted by sap and aphids.

The incidence of viral infection in *R. glutinosa* cultivation in Korea has not yet been investigated (Kwon et al., 2019). Viruses are particularly problematic in vegetative crops such as *R. glutinosa* because they are transmitted to vegetative bodies. The production of *R. glutinosa* plants without viruses involves high temperature treatment of *in vitro* and *ex vitro* plants (Ali et al., 2013; Feyissa et al., 2011; Lassois et al., 2013; Modarresi et al., 2016), apical meristem culture (Gong et al., 2019, Feyissa and Dugassa, 2011; Ramgareeb et al., 2010), and chemotherapy using antiviral agents, such as ribavirin (Khurana, 2004; Hu et al., 2015; Paunovic et al., 2007). Hu et al. (2015) reported that the efficiency of virus

elimination improved when high temperature and chemical treatments were simultaneously performed. They also indicated that the use of ribavirin (25 mgL^{-1}) and a high temperature treatment of 36°C resulted in a virus elimination efficiency of 95.0%. In this study, we compared the virus elimination efficiency of high temperature and chemical treatments and apical meristem culture to determine the optimum conditions for the production of virus-free *R. glutinosa* plants.

Result and Discussion

Virus detection measured by reverse transcription-polymerase chain reaction (RT-PCR) in* R. glutinosa *before virus elimination treatment

RT-PCR analysis of five viruses in *R. glutinosa* was accomplished before virus elimination treatment using PCR primer pairs (Table 1). Table 2 shows that the infection rates of ReMV, BBWV2, and YoMV were 100% and those of PIAMV and ReV1 were 23% and 42%, respectively. In this study, RT-PCR identified four types of viruses, ReMV, BBWV2, YoMV, and PIAMV, in different regions of Korea, which revealed that the infection rates of ReMV, BBWV2, and YoMV were 100% in Goesan-Chungcheongbukdo and 80% in Jeongeup-Jeollabukdo, but the PIAMV infection rates were different for each region, with 14%–16% in Eumseong, Goesan, and 100% in Jeongeup (Data not shown). According to Kwon et al., (2019), a total of 145 virus symptom samples were collected from major cultivation areas in Korea, and the results of the RT-PCR diagnosis showed that all collected leaf samples were infected with two or more viruses.

Virus elimination by thermotherapy, chemotherapy, and meristem culture

To produce virus-free plants from the virus-infected *R. glutinosa* through thermotherapy (37°C), chemotherapy (addition of 60 mgL^{-1} ribavirin into the medium), and apical meristem culture (0.3–0.5 mm), the virus elimination rate was compared using RT-PCR (Fig 1). After thermotherapy at 37°C for 4 weeks of *R. glutinosa*, the elimination rates of ReMV, BBWV2, and YoMV were 0%, whereas those of PIAMV and ReV1 were 10% and 40%, respectively (Table 3). Thermotherapy is a widely used virus elimination technique. Walkey (1980) revealed that when the apical meristem culture is directly exposed to high temperature, it affects the metabolic activity of meristem and inhibits virus replication. It has been reported that cassava is vegetatively propagated by the stem, and the cassava mosaic virus disappeared in treatment at 35°C–37°C for 3–4 weeks before plants culture (Garcia et al., 1993; Zok, 1993; Delgado and Rojas, 1993). Kidulile et al. (2018) obtained 50% virus-free plants when treated at 35°C as thermotherapy to remove African cassava mosaic virus; when treated at 40°C, the virus elimination rate as high as 69.5%, but the survival rate was low. As a result of treatment with ribavirin 60 mgL^{-1} during 4 weeks, 25%, 35%, and 30% elimination rates of ReMV, YoMV, and PIAMV were observed in the *in vitro* plants. It was confirmed that the elimination rates were different for each virus, 85% for BBWV2 and 100% for ReV1 (Table 3). Khurana (2004) found that virus elimination using antiviral chemicals is an important technique for virus-free plant production. Although antiviral agents are effective in removing viruses, it has been reported that they can cause weak damage such as phytotoxicity to plants at high concentrations but the effect disappears *in vitro* culture during 15 days. (El-Dougdoug et al., 2010). Hu et al. (2015) reported that when apple plants were treated with ribavirin at 15 mgL^{-1} and 25 mgL^{-1} *in vitro*, virus elimination rates were 74.4% and 75.0%. Modarresi et al. (2016) also reported that *Arabis mosaic virus* elimination when treated with ribavirin at a concentration of 30 mgL^{-1} for 4 weeks. Removal of *Prunus necrotic ringspot virus* from *in vitro* rose plants has been reported. Paunovic (2007) reported that when *in vitro* plants of plums were treated with ribavirin at 40 and 60 mgL^{-1}, 15.38% and 16.66% of virus-free plants, respectively, were obtained. Our findings were similar to those of previous studies. Table 3 shows that ReMV, BBWV2, and YoMV elimination rates after 4 weeks of apical meristem culture were low (0%–5%), but PIAMV and ReV1 elimination rates were extremely high (75%–100%). Chemotherapy showed that the ReMV, PIAMV, and YoMV elimination rates after 6 weeks of addition of 60 mgL^{-1} ribavirin into the medium were low (25%–35%), but BBWV2 and ReV1 elimination rates were extremely high (85%–100%). Thermotherapy showed that the ReMV, BBWV2, YoMV, and PIAMV elimination rates after 6 weeks of 37°C in the growth chamber were low (0%–10%), but ReV1 elimination rates were extremely high at 40%. In the virus elimination method for producing virus-free plants of crops, the apical meristem culture is affected by the excised meristem, crop variety, and size of the plant or virus species (Loebenstein, 2001). Wang et al. (2008) reported that exposing the mother plants to high temperature stress could inactivate the virus at the shoot tip in the apical tissue by inhibiting viral RNA synthesis. Sastry and Zitter (2014) also reported that apical meristem culture is generally used to remove viruses; an average size of 0.1–0.5 mm of apical culture with vigorous cell division or growth point in root tissue in the medium is recommended. It was found that it is effective to cut tissue that is not infected with the virus even at this apical division (Grout, 1990; Kane, 2005). On the other hand, Green and Lo (1989) cut and cultured 0.3 mm of the apical meristem, which they removed the Potato yellow dwarf virus but not the Raspberry bushy dwarf virus. Table 3 shows that thermotherapy and apical meristem culture exhibited low elimination rates of ReMV, BBWV2, and YoMV viruses, whereas PIAMV and ReV1 were removed. Virus-free plants of *R. glutinosa* could not be obtained if a single treatment method was used.

Comparison of shoot survival rate after virus elimination treatment

The growth characteristics of *in vitro* plants 4 weeks after administering the virus elimination treatment methods were compared (Table 4). After thermotherapy at 37°C for 4 weeks, the shoot height was 2.6 cm, the number of leaves was 16.2/explants, and the survival rate was 67.9%. Thermotherapy resulted in better growth characteristics than chemotherapy and apical meristem culture; however, thin stems and small leaves were observed. After treatment with ribavirin, the growth characteristics were found to be as follows: shoot height, 1.1 cm; 9.2 leaves/explants, and 55.2% survival rate. Apical meristem culture was considered to have a high mortality rate and a survival rate of 29.4% with a shoot length of 1.2 cm and 8.3 leaves/ explants (Table 4). Ali et al. (2013) showed that the survival rate of potatoes from apical meristem culture was 28.06%, the highest at 27 ± 1°C after incubation, which was higher than that at 30 ± 1°C and 35 ± 1°C. In comparison of the variety of potatoes, Diamant showed 17.52% survival rate, whereas Lalpakri and Heera showed 20.39% survival rate, showing different growth characteristics. Lee et al., (2013) reported that the survival rate of shoots during the *in vitro* plants introduction process

through thermotherapy and shoot apex culture to produce virus-free plants of 4 varieties, including apple 'Summer Dream', was as low as 25.0%–41.7% when thermotherapy at a low temperature of 35°C was also reported for a higher survival rate of shoots. However, the *in vitro* plants grown using apical meristem culture of sweet potato were treated at 37°C for 31 days at high temperature; the Awassa83 variety showed a 34.78% survival rate and the Awassa local variety showed a 54.55% survival rate (Feyissa and Dugassa, 2011). The results of this study were similar when thermotherapy was performed at 37°C for 6 weeks and the survival rate of common plants was 45.5%.

Comparison of virus elimination rate after thermotherapy and chemotherapy on* in vitro *plants derived from apical meristem culture

In Table 5, the results of comparing the elimination rates by virus type after the apical meristem culture followed by thermotherapy (37°C, 6 weeks) were obtained in a test in which ReMV 30%, BBWV2 35%, and YoMV 30%, and it showed higher virus elimination rates, i.e., 30%–35% than those shown in Table 3. In the case of PIAMV, 7 out of a total of 20 samples of plants showed 35% virus elimination rate, and in the case of ReV1, 19 out of a total of 20 subjects showed a 95% virus elimination rate. It has been reported that thermotherapy can be combined with the apical meristem culture applied to *in vitro* plants as a generally used virus elimination method (Lassois et al., 2013).

Kim et al. (2003) showed that for the elimination of GLRaV-3 from grapevines, thermotherapy for 6-8 hat 37 ± 2°C for 6–8 h and the 0.5–1.0 mm meristematic culture method were the most effective for virus elimination. It is reported that thermotherapy alone may not be effective. To obtain virus-free apple plants, the virus elimination efficiency of new cultivars that survived after thermotherapy and apical meristem culture was confirmed. Thus, in the case of ACLSV, 26 out of a total of 31 individuals were found to have an 84% elimination rate; in the case of ASGV, 21 out of 31 were found to have a virus elimination rate of 68%; and in ASSVd, 20 out of 24 showed 83% virus elimination (Lee et al., 2013). As a result of treatment with ribavirin 60 mgL^{-1} for 6 weeks, *in vitro* plants derived from *R glutinosa* of the apical meristem culture showed a virus elimination rate of 45% (BBWV2 80%, YoMV 45%, and PIAMV 50%). ReV1 showed a virus elimination rate of 100% (Table 5), and the virus elimination rate was on average 10% higher than when plants were treated with ribavirin 60 mgL^{-1} alone *in vitro*. When the apical meristem tissue of the potato was cut 0.1–1.0 mm, if the size of apical meristem was cut too small, there were a problem such as a somatic mutation in the plant (Wang et al., 2008). So, it has been shown that thermotherapy and chemotherapy method was more effective than apical meristem culture in obtain the virus-free potatoes (Cassells and Long, 1982; Ali et al., 2013; Aguilar-Camacho et al., 2016). The results of comparing the virus elimination rate after 6 weeks of *in vitro* plant culture at the apical meristem culture showed that ReMV 0%, BBWV2 15%, YoMV 10% were low, whereas the elimination rate of PIAMV 70% and ReV1 100% was higher than that of other viruses (Table 5). Cho et al. (2016) reported that various techniques such as thermotherapy are used for obtaining virus-free plants; however, in many cases, it is not possible to completely inactivate infected viruses by a single treatment.

In this test, the virus elimination rate was 37% in the case of the apical meristem culture; in the case of simultaneous treatment with thermotherapy, the virus elimination rate was 45%; and when the chemical treatment was administered at the same time, the virus elimination rate was considered to be the most effective at 64%. However, virus-free plants without five viruses of *R. glutinosa* were not obtained, and it is reported that virus elimination is more difficult when infection is with multiple types of viruses than when infection is with a single infection (Knapp et al., 1995; Paprstein et al., 2008).

Comparison of virus elimination rates after simultaneous treatment with thermotherapy and chemotherapy on* in vitro *and* ex vitro *plants derived from the apical meristem culture

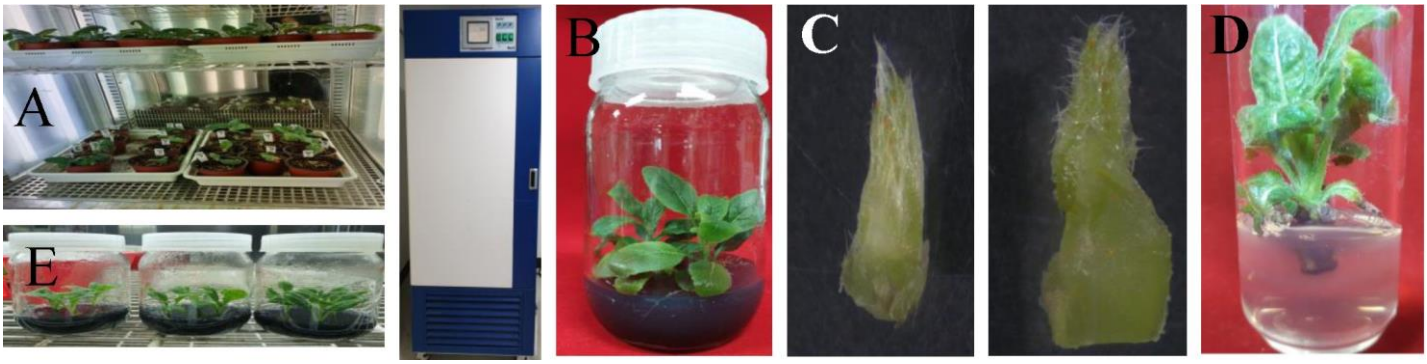
R. glutinosa virus was eliminated using single and combined treatments, but virus-free plants without the five types of *R. glutinosa* virus could not be obtained (Table 3 and Table 5). Therefore, to obtain virus-free plants, the apical meristem culture was simultaneously treated with ribavirin 60 and 80 mgL^{-1} in the medium and ribavirin sprayed 12 times with thermotherapy (37°C, 6 weeks) of *ex vitro* plants from apical meristem culture, and then virus elimination rates were compared using RT-PCR (Table 6). In Table 6, the results of comparing the elimination rates according to virus type after ribavirin 80 mgL^{-1} treatment and thermotherapy at 37°C for 6 weeks in the plant growth chamber were as follows: ReMV 55%, BBWV2 80%, and YoMV 60%, PIAMV 100% and ReV1 100%; PIAMV, and ReV1 viruses were completely eliminated. However, although ribavirin was administered at a high concentration of 80 mgL^{-1} and the virus elimination rate was high (an average of 79%), symptoms of phytotoxicity were observed as the leaves aged and withered, resulting in a high mortality rate (Paunovic et al., 2007). When *in vitro* plants grown at the apical meristem culture were treated with a ribavirin concentration of 60 mgL^{-1}, the growth characteristics were the best; the virus elimination rate was approximately 73% on average, and it was possible to obtain virus-free plants with the five types of *R. glutinosa* viruses removed. As a result of comparing the elimination rates by virus type, ReMV 40%, BBWV2 75%, and YoMV 50% were found; PIAMV 100% and ReV1 100% indicated the effect of completely removing the two viruses. Therefore, as a method for virus-free plants of *R. glutinosa*, the apical meristem culture followed by chemotherapy with 60 mg L^{-1} ribavirin, which is a concentration that minimizes its toxicity. When foliar sprayed with ribavirin concentration of 60 mgL^{-1} on *ex vitro* plants from apical meristem culture and then thermotherapy was treated for at 37℃ 6 weeks, the virus elimination rates were compared to 0% ReMV, 0% BBWV2, and 0% YoMV, it was not effective for the elimination of major viruses.

When *in vitro* plant from apical meristem culture plant growing in medium mixed with 60 mgL^{-1} of ribavirin were treated at 37°C for 6 weeks, virus free plants could be obtained (Fig. 2). According to Kim et al. (2003), when ribavirin was applied to infected plants via foliar application, the virus concentration was non-uniform even after treatment, making it impossible to determine the effect of the treatment. It was reported that it is only possible by combining the virus elimination methods. According to the results of this study, the apical meristem culture, thermotherapy and chemotherapy were treated simultaneously, it was more effective than the treatment in each for the elimination of major viruses from *R. glutinosa*. Furthermore, when *R. glutinosa* virus-free plants will be cultivated in the field, it is necessary to investigate the virus

Table 1. Primer pairs and expected size of RT- PCR products for detecting primers for *R. glutinosa* viruses.

List of PCR primers		Genome location	Size (bp)
Primers[a]	Sequence (5'→3')		
ReMV-F[b]	ACGCGTGGGGAGTGGTAGAAA	2,353-2,373	577
ReMV-R	GCGAAATGGGCTGGGTATGG	2,910-2,929	
YoMV-F[c]	GGGTCGCAAATTTTCCTTATCC	2,854-2,875	418
YoMV-R	CATCGTGTAGTGTGCCTTGTCA	3,250-3,271	
BBWV2-F[d]	AAACAAACAGCTTTCGTTCCG	R1 18-38	369
BBWV2-R	GCCATCTCATTAGCATGGA	R1 368-386	
PIAMV-F[e]	GCACTCCCCCACGGCGGCAGGTA	5,051-5,073	410
PIAMV-R	GGGGACGGAGGGGGAAGAGACTT	5,438-5,460	
ReV1-F[f]	TCATTGGCCCGACTTACAG	4,882-4,900	571
ReV1-R	AACGCGATGGCTTTAGATACA	5,432-5,452	

[a] The primers for each virus were designed to be species-specific; [b] ReMV - *Rehmannia mosaic virus*; [c] YoMV - *Youcai mosaic* virus; [d] *BBWV2* - Broad bean wilt virus *2;* [e] *PIAMV* - Plantago asiatica mosaic virus; [f] *ReV1*- Rehmannia virus *1*



Thermotherapy Chemotherapy Apical Meristem

Fig 1. Production process of virus-free *R. glutinosa* plants using thermotherapy, chemotherapy, and apical meristem.
A - Thermotherapy (37°C); B – Chemotherapy (ribavirin); C - Apical meristem; D - Apical meristem plants; E - Addition of ribavirin into the medium for culturing plants *in vitro.*

Table 2. Virus detection in *R. glutinosa* measured using RT-PCR before virus elimination treatment.

Types of virus	Number of tested	Number of Infected	Rate of Infection (%)
ReMV[a]	31	31	100
BBWV2	31	31	100
YoMV	31	31	100
PlAMV	31	7	23
ReV1	31	13	42

[a]ReMV - *Rehmannia mosaic virus;* BBWV2 - *Broad bean wilt virus 2;* YoMV - *Youcai mosaic virus;* PlAMV - *Plantago asiatica mosaic virus;* Rev1 - *Rehmannia virus 1*

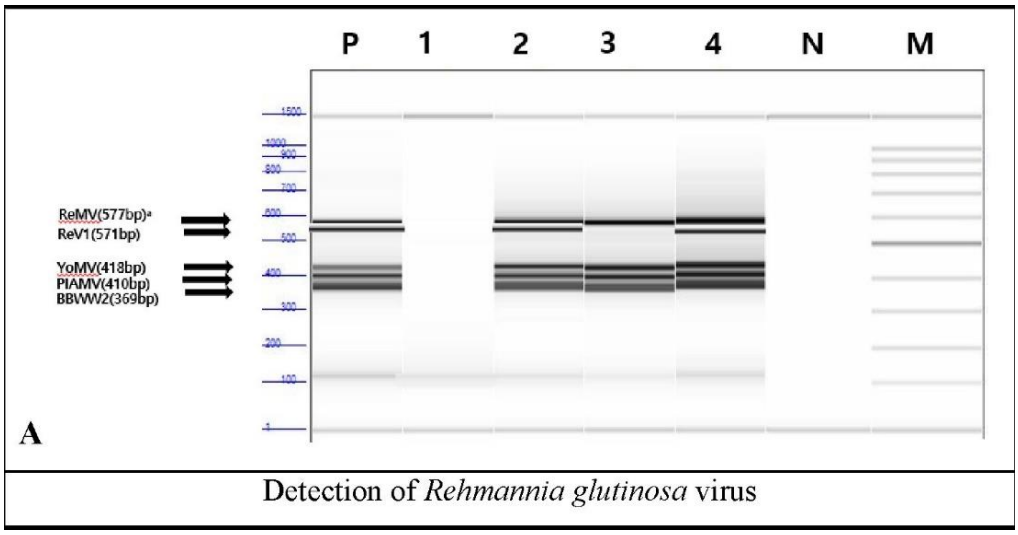


Figure 2. RT-PCR analysis of five viruses in *R. glutinosa* after virus elimination treatment. A-M: Marker; P: Positive; N- Negative control; 1 - Virus-free plants by virus elimination treatment; 2–4 – Infected samples. [a]ReMV - *Rehmannia mosaic virus*; BBWV2 - *Broad bean wilt virus* 2; YoMV - *Youcai mosaic virus*; PlAMV - *Plantago asiatica mosaic virus*; Rev1 - *Rehmannia virus 1*

Table 3. RT-PCR results of virus detection measurement performed at 4 weeks after virus elimination treatments.

Treatment	Number of tested	Rate of virus-free plant[a] (%)				
		ReMVb	BBWV2	YoMV	PlAMV	ReV1
Thermotherapy[c]	20	0	0	0	10	40
Chemotherapy[d]	20	25	85	35	30	100
Meristem culture[e]	20	0	5	5	75	100

[a]The numbers in parentheses indicate the number of virus-free plants analyzed; [b]ReMV - *Rehmannia mosaic virus,* BBWV2 - *Broad bean wilt virus,* YoMV - *Youcai mosaic virus,* PlAMV - *Plantago asiatica mosaic virus,* ReV1 - *Rehmannia virus* 1; [c] Treatment of thermotherapy: 37°C; [d] Treatment of chemotherapy: addition of ribavirin at 60 mg L^{-1} into the medium; [e] Meristem culture: *In vitro* plants

Table 4. The effect of virus elimination treatment method on *R. glutinosa* growth characteristics *in vitro* after 4 weeks of culture.

Treatment	Shoot length (cm)	No. of leaf (explant)	Leaf length (mm)	Survival rate (%)
Thermotherapy[b]	2.6 a[a]	16.2 a	20.5 a	67.9 a
Chemotherapy[c]	1.2 b	9.2 b	12.3 b	55.2 b
Meristem culture[d]	1.2 b	8.3 b	13.1 b	29.4 c

[a] Mean separation within columns using Duncan's multiple range test at P = 0.05; [b]Thermotherapy treatment –37°C; [c] Chemotherapy treatment - addition of ribavirin at 60 mg L^{-1} into the medium; [d] Meristem culture: *In vitro* plants

Table 5. RT-PCR results of virus detection measurement after meristem culture followed by thermotherapy or chemotherapy after 6 weeks

Treatment	Number of tested	Rate of virus-free plant[a] (%)				
		ReMV[b]	BBWV2	YoMV	PlAMV	ReV1
Meristem culture with thermotherapy[c]	20	30	35	30	35	95
Meristem culture with chemotherapy[d]	20	45	80	45	50	100
Meristem culture[e]	20	0	15	10	70	100

[a]The numbers in parentheses indicate the rate of virus-free plants analyzed; [b]*ReMV* - Rehmannia mosaic virus, *BBWV2* - Broad bean wilt virus, *YoMV* - Youcai mosaic virus, *PlAMV* - Plantago asiatica mosaic virus, *ReV1* - Rehmannia virus *1;* [c] Thermotherapy treatment - 37°C; [d] Chemotherapy treatment: addition of ribavirin at 60 mg L^{-1} into the medium [e] Meristem culture: *In vitro* plants.

Table 6. RT-PCR results of virus detection after meristem culture followed by thermotherapy and chemotherapy simultaneously after 6 weeks

Treatment	Number of tested	Rate of virus-free plant[a] (%)				
		ReMV[b]	BBWV2	YoMV	PlAMV	ReV1
I[c]	20	55	80	60	100	100
II[d]	20	40	75	50	100	100
III[e]	20	0	0	0	25	100

[a]The numbers in parentheses indicate the rate of virus-free plants analyzed; [b]ReMV- *Rehmannia mosaic virus,* BBWV2 - *Broad bean wilt virus,* YoMV - *Youcai mosaic virus,* PlAMV - *Plantago asiatica mosaic virus,* ReV1 - *Rehmannia virus* 1; [c]I - Meristem culture *(In vitro* Plants) + ribavirin 80 mg L^{-1} (addition into medium) + Thermotherapy (37°C); [d]II - Meristem culture *(In vitro* Plants) + ribavirin 60 mg L^{-1} (addition into medium) + Thermotherapy (37°C); [e]III: Meristem culture *(Ex vitro* Plants) + ribavirin 60 mg L^{-1} (spray 12) + Thermotherapy (37C)

morbidity rate, growth characteristics, and yield.

Materials and methods

Plant materials

The *R. glutinosa* variety "Dagang" grown at the Chungcheongbukdo Agriculture Research and Extension Services was used in this study. We conducted RT-PCR (reverse transcription-polymerase chain reaction) to detect ReMV, YoMV, BBWV2, PlAMV, and ReV1 in *R. glutinosa* plants before performing the virus elimination treatments. The RT-PCR results indicated that most plants had mixed viral infections of ReMV, YoMV, BBWV2, PlAMV, and ReV1.

In vitro cultures

Elongated shoots were cut at the node, thoroughly rinsed in running water for approximately30 min, immersed in 70% ethanol, surface-sterilized for approximately 30 s, and then washed once with sterile water. Next, we sterilized the surface again with 2% (v/v) sodium hypochlorite solution and 0.1% (v/v) Tween-20 for 12–15 min, followed by three rinses in sterile distilled water. *In vitro* plants were cultured on modified MS medium (Murashige and Skoog, 1962) containing 1 mgL^{-1} BAP [6-benzylaminopurine (MB Cell, Kisan Bio Co. Ltd., Korea)], 0.1 mgL^{-1} IAA (3-indole-acetic acid; MB Cell), 0.1 gL^{-1} ascorbic acid (MB Cell), 30 gL^{-1} sucrose (Junsei, Tokyo, Japan), 1 g charcoal (MB Cell), and 8.0 gL^{-1} agar (Junsei). Further, we adjusted the pH to 5.8 with 1 mol L^{-1} NaOH before autoclaving (AC-60, Daihan Scientific Co., Ltd., Korea) at 121°C for 20 min. Next, 80 mL of each medium was dispensed into 250 mL culture bottles. All cultures were maintained in a standard growth room at 24°C ± 1°C under a 16:8 h light/dark photoperiod with 40 pmol m^{-2} s^{-1} light intensity. At 30-day intervals, viable cultures were transferred to fresh MS medium, and the presence of ReMV, YoMV, BBWV2, PlAMV, and ReV1 in the cultures was reassessed by RT-PCR.

Isolation of RNA and RT-PCR

Total RNA was extracted by modifying the CTAB method (Gambino et al., 2008) using 20–50 mg plant samples for analysis. M-MLV reverse transcriptase was used to synthesize cDNA from the extracted RNA (Invitrogen, USA). Using the cDNA as a template, virus-specific primer sets were used for the PCR reaction (Table 1). For the PCR, 20 pL of the reaction solution was used, which included 1 pL of 0.2 pM primer, 1.6 pL of 2.5 mM dNTPs, and 0.1 pL 5 U TaKaRa Ex. Taq DNA Polymerase (TaKaRa Bio Inc., Japan), 2 pL 10× Taq Buffer, 1 pL cDNA, and 13.3 pL RNase-free water. The PCR conditions for initial denaturation were as follows: 45°C for 30 min and 94°C for 5 min, followed by DNA amplification (DNA denaturation at 94°C for 30 s, primer conjugation at 55–60°C for 30 s, and DNA extension at 72°C for 60 s) repeated 35 times. Finally, the reaction was completed at 72°C for 5 min. After the virus assay, the amplified DNA was examined using an automated capillary electrophoresis device (5200 Fragment Analyzer, Agilent, USA).

Virus elimination methods

Apical meristem culture

The meristem tissue (0.3–0.5 mm) was collected under a stereoscopic microscope and plated on an *in vitro* medium and cultured for 6 weeks. *In vitro* plants were cultured on modified MS medium (Murashige and Skoog, 1962) containing 1 mgL^{-1} BAP (6-benzylaminopurine; MB Cell), 0.1 mgL^{-1} IAA (1- naphthaleneacetic acid; MBCell), 0.1 gL^{-1} ascorbic acid (MB Cell), 30 gL^{-1} sucrose (Junsei), 1 gL^{-1} charcoal (MBCell), and 8.0 gL^{-1} agar (Junsei). Next, we adjusted the pH to 5.8 with 1 $molL^{-1}$ NaOH before autoclaving (AC-60, Daihan Scientific, Republic of Korea) at 121°C for 20 min. All cultures were maintained in a standard growth room at 24°C ± 1°C under a 16:8 h light/dark photoperiod (40 pmol m^{-2} s^{-1} light intensity). Viable cultures were transferred to fresh MS medium at 30-day intervals, and the presence of ReMV, YoMV, BBWV2, PIAMV, and ReV1 in the cultures was reassessed *using* RT-PCR.

Thermotherapy

Plants and *in vitro* plants were treated for 6 weeks in a plant growth chamber (VS-8407-1300, Vision Scientific, Republic of Korea) maintained at 37°C and 65% humidity (16:8 h light/dark photoperiod and 2000-lx light intensity) for thermotherapy. The temperature was gradually increased, and the thermotherapy periods were recorded when the temperature reached 37°C ± 0.5°C. The duration of all thermotherapy treatments was 6 weeks.

Chemotherapy

The antiviral compound used was ribavirin (MB Cell). Ribavirin was filter-sterilized using a Millipore filter (0.22 pm) and added into fresh MS media at final concentrations of 60 and 80 mgL^{-1}. Then, the shoots were transferred to ribavirin-containing culture media and cultured in a growth room under the conditions described above. The duration of all chemotherapy treatments was 6 weeks.

In vitro plants subjected to chemotherapy and thermotherapy simultaneously

In vitro plants derived from the apical meristem culture were transferred onto MS media containing ribavirin (prepared as described above) at concentrations of 60 and 80 mgL^{-1} and then subjected to the above-mentioned thermotherapy (37°C) treatments for 6 weeks. Each 250 mL glass flask contained 80 mL medium and four *R. glutinosa* shoots. In total, 30 shoots were utilized for each treatment. The growth of the *in vitro R. glutinosa* plants was periodically observed to assess the effects of the different treatments. After treatment, meristem tips ranging from 0.3 to 0.5 mm in size were dissected from shoot tips. All shoots dissected from treated plants were cultured on the same basic MS medium as described above. RT-PCR was used to detect viruses after two cycles of sub culturing, and the efficiency of virus elimination was calculated.

Data analysis

All analytical experiments were repeated twice. In each experiment a set of 20 plants were used for determination of each parameter. Data from each experiment were subjected to Duncan's multiple range test using SAS program (Version 6.21, SAS Institute Inc., Cary, NC, USA). Further, we conducted Fisher's LSD test to determine the mean separation. A probability level of 0.05 ($p < 0.05$) was considered for statistical significance in all data analyses.

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