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Research Article

Efficiency of a chemo-thermotherapy technique for eliminating *Arabis mosaic virus* (ArMV) and *Prunus necrotic ringspot virus* (PNRSV) from *in vitro* rose plantlets

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Abstract: Mosaic is presumably the most commonly encountered viral disease in roses. We have developed chemo-thermotherapy for eliminating *Arabis mosaic virus* (ArMV) and *Prunus necrotic ringspot virus* (PNRSV) from rose plants. Chemotherapy and thermotherapy methods were also applied separately and their antiviral effect compared with the chemo-thermotherapy. In this procedure, infected explants were regenerated on MS medium containing ribavirin at concentrations of 10, 20 and 30 mg/l for 20 and 40 days, followed by a thermotherapy treatment for 30 days at 38 °C for 16 hours and 22 °C for 8 hours per day. The complex of rose viruses (ArMV and PNRSV) were effectively eradicated from regenerated rose plantlets as verified by double-antibody sandwich enzyme- linked immunosorbent assay (DAS-ELISA). Thermotherapy alongside with chemotherapy (containing 30 mg/l per one month) during the period of four weeks was the best treatment for plantlet regeneration and virus elimination. The virus elimination efficiency from ArMV, PNRSV and ArMV + PNRSV infected plants were determined as 63.33%, 90.09% and 85.18%, respectively. A detailed procedure for elimination of mixed viruses is described.

Keywords: Rose mosaic, virus elimination, chemotherapy, thermotherapy, ribavirin

Introduction

Rose *Rosa hybrida* is an economically important ornamental plant (more than 5100 ha including other ornamentals) in Iran (Ministry of Agriculture Jihad, 2012) and it is among the most popular flowering plants in the world. Roses are developed as garden plants for the cut-blossom industry and as a wellspring of natural scents. The Netherlands, with rose cultivation area of about 8000 hectares is considered the global leader in rose production. Ecuador with 5000 hectares and Zambia, in Africa, with 80 percent of its cultivated land under roses (Flament *et al*., 1993; Weiss, 1997) are among the other major rose growing countries. Viruses which infect roses belong mainly to the *Ilarvirus* and *Nepovirus* genera (Horst, 2007). *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV) from *Ilarvirus* (Converse and Bartlett, 1979; Fulton, 1970; Moury *et al*., 2001), and *Arabis mosaic virus* (ArMV) and *Strawberry latent ring spot virus* (SLRSV) from *Nepovirus* alone or in combination with each other can cause serious damages to both garden and green house roses around the world (Johnston *et al*.,

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1995). Therefore, production of virus-free rose seedlings is considered to be a crucial step for production of healthy and marketable rose plants. Different techniques have been employed by scientists for obtaining virus-free plant material such as chemotherapy (Wambugu, 1985), thermotherapy (Pazhouhandeh, 2002) and meristem culture or a combination of these techniques. Efficiency of virus elimination depends on the efficiency of technique, type of virus or virus combination and host plant (Knapp *et al*., 1995). Thermotherapy has been used for eradication of viruses from many crop plants including potato, carnation, geranium, strawberry and citrus. By the use of this technique, plants are treated at high temperatures in growth chambers (Griffiths *et al*., 1990) and meristems are then excised and cultured on MS medium for regeneration of virus-free new shoots. Chemotherapy has also proven to be a good method in obtaining virus free biological materials from some viral infected plants. Treatment of plants with different chemical substances can inhibit virus replication, cell to cell movement or even long distance movement of some viruses in plant (Griffiths *et al*., 1990; Faccioli, 2001). These chemicals can also affect meristem growth in the plant (Matthews *et al*., 1992). However, it should not be ignored that in none of the cases complete elimination of treated viruses was recorded and that such techniques are able to only suppress the viral replication and their genome expression in the treated tissues.

Ribavirin is one of the well known antiviral medicines which is highly effective against several DNA and RNA viral infections and their protein synthesis (Meier *et al*., 2003). It is a well- known member of the nucleoside anti metabolite drugs that hinder duplication of viral hereditary material. Despite the fact that ribavirin is not effective against all viruses, its application has received considerable attention during last 15 years due to its wide range of activities and simplicity of use (Dhital *et al*., 2008; Mahmoud *et al*., 2009; Gută *et al*., 2010). Like other virazole`s carboxamide group, ribavirin create the indigene nucleoside drug that looks like adenosine or guanosine, depending on its rotation (Ortega-Prieto *et al*., 2013). In this way, when ribavirin is joined into RNA as an adenine or guanine, it combines similarly well with either uracil or cytosine and makes changes in RNA dependent replication in RNA virus infections. Such extreme changes can be deadly to RNA viruses (Elia *et al*., 2008). Previous studies have shown a reduction in PNRSV and *Prune dwarf virus* (PDV) titer in shoots of *Prunus avium* plants grown in hydroponics using thermotherapy and chemotherapy techniques (Howell *et al*., 2001). Here we report the chemo-thermotherapy technique developed for successful production of virus-free rose plant *in vitro*. A combination of chemotherapy using ribavirin and thermotherapy techniques were employed in this study to evaluate their antiviral efficiency against the PNRSV and ArMV in *Rosa hybrida* infected plant tissues.

Materials and Methods

Plant culture

Rose plants *Rosa hybrida* singly infected with ArMV or PNRSV and mixed infected with ArMV+PNRSV were used in this study. Their infections were confirmed by double- antibody sandwich enzyme- linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977; Rakhshandehroo *et al*., 2009) using the specific antisera (Bioreba, Reinach, Switzerland) according to manufacturer`s instructions. ELISA plates were coated with 100 µL of IgG of each virus diluted 1:1,000 in carbonated coating buffer (0.6 M Na_2CO_3 and 0.14 M $NaHCO_3$, pH 9.6) for each. Plant sap was extracted from approximately 0.5 g fresh tissue of each plantlet and tested in duplicated wells with appropriate positive and negative controls. Optical density (OD) was measured at nm using ELISA reader for each well (Stat Fax 2100, AWARNESS Tech. Inc. USA). Virus infected and healthy plants were distinguished based on the formula routinely used in seed certification systems (Hill and Jackson, 1984): R= X + 3SD

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where R = limit of infection to virus, X = mean of absorbance and S. D. = Standard deviation.

The younger green stems were cut into uninodal portions (0.5-1.0 cm in length) and surface sterilized in 30% ethanol for 30 seconds followed by 30% commercial bleach (Sodium hypochlorite) for 15 min and then washed four times (5 min each) with sterile distilled water and cultured on MS medium (Murashige and Skoog, 1962) containing 0.4 mg/l NAA and 0.4 mg/l BAP (pH 5.8). For rooting, after 20 and 40 days, the individual shoots were transferred on the half-strength MS medium supplemented with 6 mg/l IAA. The rooted plants were potted and kept under ideal greenhouse conditions at 22-25 °C and 60-70% relative humidity with 12-14h natural light. *In vitro* grown healthy rose plantlets under *in vitro* condition were used as negative control while infected rose plantlets were used as positive control both of which were tested by serology.

Chemotherapy

Three different concentrations of ribavirin also known as virazole (1-β D-ribofuranosyl-1, 2, 4, -triazole-3-carboxamide) were used for virus eradication from shoots. Nodal cuts of single or mixed infection with both ArMV and PNRSV were cultured *in vitro* on MS media containing 10, 20 and 30 mg/l of ribavirin (Pazhouhandeh, 2002) for a period of 20 and 40 days. The experiment was replicated three times (10 nodal cuts in each replication) using positive and negative controls.

To establish root for regenerated excised shoots, they were transferred to half-strength MS medium. Then the rooted rose plantlets were examined with DAS-ELISA for the presence of ArMV and PNRSV.

Thermotherapy

In vitro regenerated plantlets were placed in a growth chamber for 30 days (16 hours of light, 38 °C; 8 hours of dark, 22 °C). Untreated plantlets were kept at the same condition and used as control plants. Four weeks later, for shooting, the survived rose plantlets were moved to a fresh MS medium and kept at 24/21 °C day/night temperatures in a growth chamber.

Simultaneous application of chemotherapy and thermotherapy

Shoots were regenerated on MS media containing the above mentioned concentrations of ribavirin (10, 20 and 30mg/l) and then subjected to thermotherapy (as described above) for a period of 30 days. Plantlets were then rooted on the MS medium and tested for viruses as described earlier.

Explants were assayed for the presence of ArMV and PNRVS by DAS-ELISA method. Numbers of virus-free plants tested with DAS-ELISA were recorded for each infection type and virus treatment. Analysis of variance was done by the process described before (Gomez and Gomez, 1984) and Duncan`s multiple variety analysis including Dunnett`s test were carried out to evaluate the means. Percentage of regenerated explants [(Number of regenerated explants/ Number of explants treated) × 100] and virus-free plants [(Number of virus-free plantlets/ Number of regenerated plantlets) × 100)] were calculated and used for comparing virus therapy treatments (Lozoya *et al*, 1996; Falah *et al*., 2009).

Results

The antiviral effects of chemotherapy, thermotherapy and the combination of these two techniques on ArMV and PNRSV infection in rose plants were studied. Results indicated that increasing ribavirin concentrations to 30 mg/l for a period of 20 days significantly ($p < 0.001$) increased both percentage of regenerated explants and percentage of virus-free plantlets compared to the lower (10 and 20 mg/l) concentrations. In contrast, increasing treatment period from 20 to 40 days negatively impacted the regeneration of explants and production of virus-free plants (Fig. 1). The antiviral effect of chemotherapy on elimination of PNRSV was significantly higher ($p < 0.001$) than ArMV or the combined infection of viruses using different concentrations of ribavirin (Fig. 1A).

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On the other hand, the rate of shoot regeneration for PNRSV infected rose plants treated with the 30 mg/l of ribavirin for 40 days was significantly higher ($p < 0.001$) than other applied concentrations. The rate of viral elimination was comparatively less ($p < 0.001$) when lower concentrations were used (10 and 20 mg/l for 20 and 40 days) (Fig. 1B). The antiviral effect of 30 mg/l ribavirin for 20 days against mixed infections of ArMV + PNRSV was similar to that of 10 mg/l ribavirin for 40 days, while the rate of shoot regeneration was still remarkably higher ($p < 0.001$), when 30 mg/l of ribavirin was used (Fig. 1C).

Ribavirin treatment at the concentration of 20 mg/l for 40 days had no deleterious effects on explants. Multiple shoots with no sign of chemical toxicity were regenerated, while explants treated with 30 mg/l of ribavirin for 40 days exhibited corky nodal characteristics as possible sign of chemical toxicity (Fig. 2D).

Chemotherapy application *in vitro* for four weeks resulted in the production of lowest explant for elimination of regeneration and virus elimination (Table 1). The chemotherapy with 30 mg/l of ribavirin concentration showed significantly ($p < 0.001$) very effective in production of virus-free rose plants and induced the growth of shoots compared to untreated (no ribavirin).

The alternating high and low temperatures of 38 °C for 16 h in light and 22 °C for 8 h in dark (thermotherapy without using chemotherapy), produced virus-free plants at the rates of 55.55, 66.66 and 37.50% for ArMV, PNRSV and ArMV + PNRSV, respectively (Table 2).

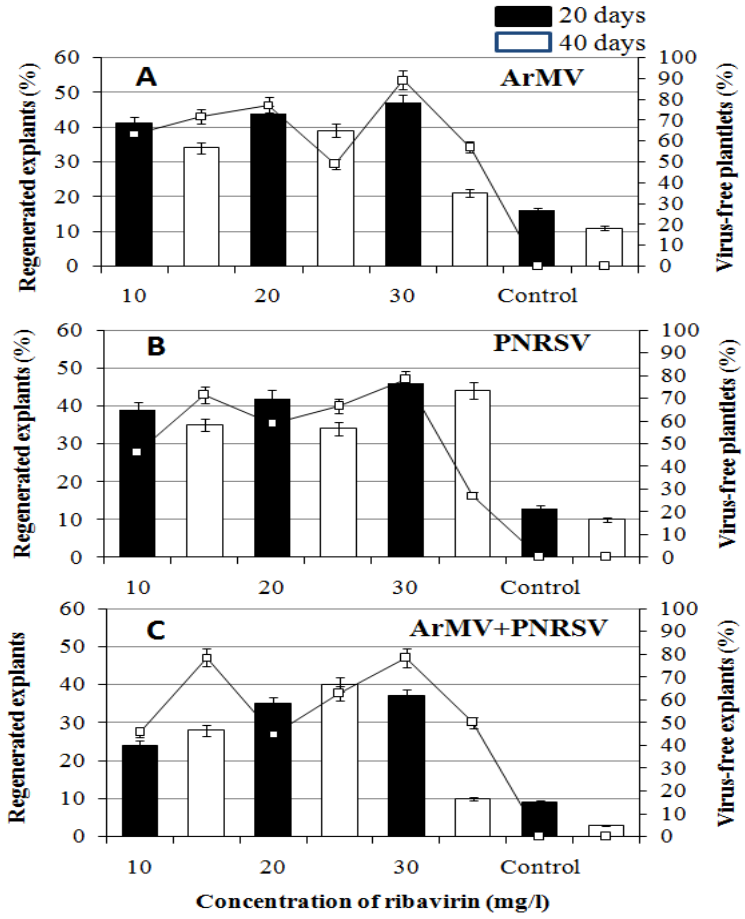

Figure 1 Effect of ribavirin concentrations on percentages of regenerated explants (bar) and virus-free plantlets (line) in chemotherapy. ArMV (A); PNRSV (B) and ArMV + PNRSV (C).

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Figure 2 Effects of ribavirin treatment with different concentrations for a period of 40 days on the survival of rose explants grown *in vitro*: untreated control (A); treated with 10 mg/l (B); 20 mg/l (C); and 30 mg/l (D) of ribavirin.

Table 1 Effects of ribavirin on the growth and regeneration of explants grown on MS media for different periods of time.

Ribavirin concentration (mg/l)	Infection type											
	ArMV				PNRSV				ArMV + PNRSV			
	Regenerated explants*%		Shoots height (mm)		Regenerated explants (%)[1]		Shoots height (mm)		Regenerated explants (%)[1]		Shoots height (mm)	
	20 days	40 days	20 days	40 days	20 days	40 days	20 days	40 days	20 days	40 days	20 days	40 days
0	34	21	18.7	19.1	39	22	16.7	23.5	18	13	17.3	21.8
10	82***	64***	21.2	22.6	78***	70***	25.6*	35.1**	48***	56***	19.8	35.0**
20	88***	78***	27.8	85.1***	84***	68***	32.1***	64.2***	70***	80***	22.8*	74.7***
30	94***	42***	91.5***	14.4	92***	88***	124.1***	11.0	74***	20*	79.2***	10.3

[1] Regenerated explants from 50 treated explants.
Values are expressed as Mean ± S.D. *** Data are significantly different ($p < 0.001$) from control (ribavirin at concentration 0 mg/l), ** Data are significantly different ($p < 0.01$) and * Data are significantly different ($p < 0.05$); using Dunnett`s test.
Rose plants treated with just the MS medium and free of the ribavirin are considered as negative control.

Table 2 The survival percentage of rose explants and virus-free plants using chemotherapy followed by a thermotherapy for 30 days.

Ribavirin concentration (mg/l)	Infection type					
	ArMV		PNRSV		ArMV + PNRSV	
	Regenerated explants (%)	Virus-free plantlets (%)[1]	Regenerated explants (%)	Virus-free plantlets (%)[1]	Regenerated explants (%)	Virus-free plantlets (%)[1]
0	33.33	55.55[ab]	40	66.66[b]	26.66	37.50[c]
10	43.33	61.53[a]	60	72.22[b]	36.66	36.36c
20	50.00	40.0[c]	60	64.28[b]	26.66	62.50[b]
30	100	63.33[a]	100	90.09[a]	90.00	85.18[a]
Control	0	0	0	0	0	0

[1] Virus-free plantlets: [(Number of virus-free plantlets/ Number of regenerated plantlets) × 100]
Means in a column followed by different letters are significantly different according to Duncan`s multiple range test (p □ 0.05).

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Combination of chemotherapy and thermotherapy referred to as chemo-thermotherapy had the highest curative effect on single infections of ArMV or PNRSV. However, ribavirin treatment of 30 mg/l followed by the heat treatment for four weeks resulted in regeneration of highest percentages of ArMV, PNRSV or ArMV + PNRSV virus-free plantlets (Table 2, Fig. 3).

Callus formation in the nodal parts of the shoots was observed with no effect on the shoot growth (Fig. 3A, B and C). In chemo-thermotherapy PNRSV, ArMV and ArMV + PNRSV were eliminated successfully and virus-free plants were produced at the rates of 90.09%, 63.33% and 85.18% respectively (Table 2).

Discussion

Application of chemotherapy technique, rendered rose plants virus-free. Effective eradication appeared to rely on the duration of treatments. In this study, total inactivation of the viral virulence was achieved at dose of 30 mg/l in a period of 20 days ($p < 0.001$).

In this research, DAS-ELISA method was applied to evaluate the antiviral effects of chemotherapy and thermotherapy *in vitro* against ArMV and PNRSV infecting rose plants. It has been reported that the temperature and the time of exposure are limited by the heat tolerance of the host plant, depending on species and variety (Gella and Errea, 1998), contrary to our results; high temperature and the period of time to which the rose explants were exposed did not effect their growth even in the highest concentration (30mg/l).

Ribavirin or Virazole has been utilized as a viricide against *Potato virus X*, *Potato virus Y* and *Potato virus S* (Cassells and Long, 1982; Klein and Livingston, 1983), and PNRSV on herbaceous *Begonia* spp. plants (Verma *et al*., 2005). Cieslinska (2007) reported that *Apple chlorotic leafspot virus* (ACLSV) from Myrobalan and PNRSV from "Empress" plum shoots, were eliminated successfully by using ribavirin at concentration of 10-100 mg/l. This was achieved only when chemotherapy and thermotherapy were combined, compared to the sole application of the ribavirin.

Phytotoxic impact of ribavirin has been reported previously (Elia *et al*., 2008) and its effects on regenerated plants even three years after treatment were found (Salazar, 1996). In the wake of previous studies in this research we tried to find the lowest concentration of ribavirin having the highest effects on virus eradication. Results of this study also showed that increasing ribavirin concentration in tissue culture led to malformation and unwanted proliferation of shoots and inhibition of the bud growth (Table 1, Fig. 2). Similar to what was found by Danci *et al*. (2009) that ribavirin increased significantly virus-free percentage, whereas regeneration volume dramatically decreased. This may be clarified by the reality that ribavirin can inhibit and reduce cellular activities presumably by blocking cellular RNAs (Loebenstein, 2001). Also increasing concentration of ribavirin resulted in phytotoxicity to plant tissues; probably due to the toxic impact to plants brought about by the chemotherapeutic agents (Bittner *et al*., 1987; Deogratias *et al*., 1989). Hence, Hansen (1984) found the incapability of increasing concentration of ribavirin to eradicate PNRSV from peach using foliar treatment of 500 ppm. However, using chemo-thermotherapy at the highest concentration (30mg/l) for 30 days showed optimum growth and high regenerated explants (Fig 3C). Using both chemotherapy and thermotherapy in this study was effective to eradicate ArMV and PNRSV from rose plants which is similar to results of eliminating PNRSV from *Prunus* sp. achieved by Cieślińska (2007). The obtained results have been better than using chemotherapy alone.

Another recommended technique–mircoshoot tips culture-was tested by Golino *et al*. (2007) regarding elimination of virus (es) infection in some species of roses infected with ApMV and PNRSV. This method could eradicate virus in about 72% of tested plants. However, Previati *et al*. (2008) used *in vitro* thermotherapy and shoot-tip culture to propagate and produce virus-free roses.

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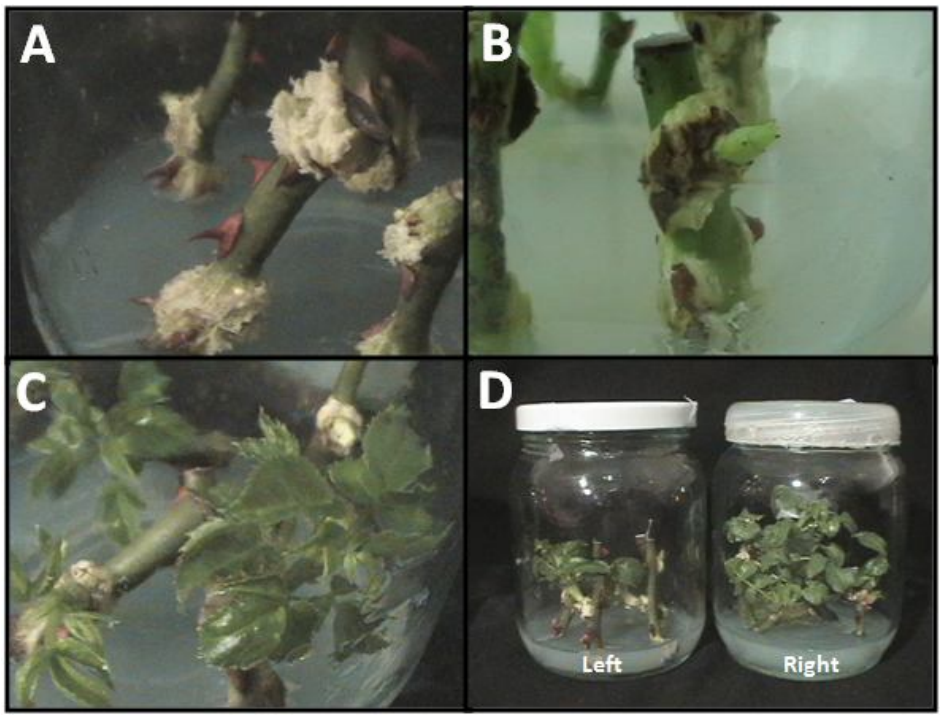

Figure 3 Effect of chemotherapy and thermotherapy with concentrations of ribavirin on the growth and survival of explants. Phytotoxic effects of ribavirin at the concentration of 10 mg/l (A); and 20 mg/l (B); optimum growth of shoots at the concentration of 30 mg/l (C); Untreated infected control rose plants (D Left) and the rose plant treated with 30 mg/l of ribavirin in chemo-thermotherapy (D Right).

Ribavirin may be active in its triphosphate form, which inhibits the 5' capping of viral RNAs (Learch, 1987) and/or inhibition of systemic virus movement (Cassells and Long, 1982). Virus-freed rose plants showed a higher yield and quality based on regenerated explants observed in this research (more produced leaves in greenish color) than the untreated explants. In practice, treating infected explants cultures with ribavirin appears to be a simple and effective way for eliminating complex of viruses such as PNRSV and ArMV from infected roses. Use of chemotherapy at concentration 30 mg/l during 20 days or using chemo-thermotherapy at concentration of 30 mg/l ribavirin could be the methods of choice for producing virus-free rose explants from infected roses, specially at the economical scale. However, chemo-thermotherapy technique saves time and also space and overcome issues of contamination from other sources.

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بررسی اثرات روش شیمی-گرمادرمانی در شرایط درون شیشه‌ای برای حذف ویروس موزاییک آرابیس و لکه حلقه‌ای بافت مرده‌ی هسته‌دارها در نهال گیاه رز

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چکیده: بیماری موزاییک احتمالاً مهم‌ترین بیماری ویروسی در گل رز است. در این مطالعه، با به‌کارگیری روش شیمی-گرمادرمانی سعی بر عاری‌سازی رز از ویروس موزاییک آرابیس (ArMV) و ویروس لکه حلقه‌ای بافت مرده‌ی هسته‌دارها (PNRSV) در مقایسه با کاربرد مجزای هر یک از دو روش یاد شده گردید. بدین‌منظور گیاهچه‌های آلوده به ویروس در محیط کشت MS محتوی ریباویرین به غلظت‌های ۱۰، ۲۰ و ۳۰ میلی‌گرم بر میلی‌لیتر به‌مدت ۲۰ و ۴۰ روز به‌علاوه روش دمادرمانی به‌مدت ۳۰ روز در دمای ۳۸ درجه سلسیوس به‌مدت ۱۶ ساعت و دمای ۲۲ درجه سلسیوس به‌مدت ۸ ساعت استفاده گردید. مجموع ویروس‌های رز (ArMV + PNRSV) به روش‌های ذکر شده به‌طور مؤثری حذف گردیدند که توسط آزمون داس الیزا تأیید شد. گرمادرمانی به‌همراه شیمی‌درمانی (محتوی ریباویرین در غلظت ۳۰ میلی‌گرم بر میلی‌لیتر در مدت زمان ۳۰ روز) مؤثرترین نوع درمان و حذف ویروس از گیاهچه‌ها در طول دوره ۴ هفته بود به‌طوری‌که باعث ویروس‌زدایی از گیاهان رز تک آلوده به ArMV وPNRSV و دوگانه آلوده به ArMV + PNRSV با کارآیی به‌ترتیب ۶۳/۳۳٪، ۹۰/۰۹٪ و ۸۵/۱۸٪ گردیدند. جزییات کامل روش مورد آزمون به‌منظور حذف ویروس‌های مختلف ذکر گردیده است.

واژگان کلیدی: بیماری ویروسی موزاییک رز، حذف ویروس، شیمی‌درمانی، گرما رمانی، ریباویرین

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