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Research Article
Production of Virus-Free Rose Plants using Meristem-Tip Culture and *in vitro* Thermotherapy

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Abstract

Background and Objective: Tissue culture and thermotherapy were proved to be suitable in eliminating viruses of many plants. This study was designed in an attempt to produce virus-free Al-Taif rose plants (*Rosa damascena* Trigintipetala Dieck) through the practical application of the tissue culture approach and thermotherapy. **Materials and Methods:** Double Antibody Sandwich-Enzyme-Linked Immunosorbent Assay (DAS-ELISA) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) techniques were used to detect the presence of *Apple mosaic virus* (ApMV) and *Strawberry latent ringspot virus* (SLRV) in rose plant materials collected from Taif, KSA. RT-PCR was more sensitive than DAS-ELISA in detecting the 2 viruses. **Results:** Three different meristem-tip sterilization methods were compared and results revealed that treatment 3 (T_3: 70% Ethanol for 1.0 min and 15% Clorox (Sodium hypochlorite 5.25%) for 10 min) was the most suitable as 97.78% of cleaned meristem tips survived. Meristem tips with different lengths were thermotherapy-treated for different durations. It was indicated that meristem tips of 0.5 or 1.0 cm and heat-treated at 37°C for four weeks gave the highest percentage of meristems that were able to differentiate into micro-shoots. **Conclusion:** RT-PCR detection of ApMV and SLRV revealed that using thermotherapy-treatment, for 4 weeks, of 0.5 cm long meristem tips was successfully applied to eliminate the 2 viruses in 92 and 96% of regenerated plantlets, respectively.

Key words: Rose, ApMV, SLRV, virus-free, tissue culture, thermotherapy, DAS-ELISA, meristem

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mosaic Disease (MD) is considered to be one of the most important economic viral diseases infecting plants and a very common disease of all types of cultivated roses throughout the world. Several different viruses have been recorded as causal agents of MD i.e., *Apple mosaic virus* (ApMV)[1-3], *Arabis mosaic virus* (ArMV)[4], *Prunus necrotic ringspot virus* (PNRSV)[5,6] and *Strawberry latent ringspot virus* (SLRV)[7].

Shoot tip culture is one of the options to control diseases, especially viruses[8-10]. Several studies showed that tissue culture is a successful technique to eliminate plant viruses for producing virus-free plant materials[11-13]. Conci *et al.*[14] confirmed that the efficiency of this process could be increased by treating plant materials with heat (Thermotherapy treatment) before shoot tip excision. Spanò *et al.*[15] used meristem-tip culture and *in vitro* thermotherapy for eliminating 3 viruses infecting artichoke (*Artichoke latent virus* (ArLV), *Tomato infectious chlorosis virus* (TICV) and *Artichoke italian latent virus* (AILV)).

Vivek and Modgil[3] conducted a successful trial to produce virus-free apple plants cv. Oregon Spur-II were infected with 4 viruses (*Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV)) by applying a combination of thermotherapy at 37-40°C for 4 weeks and meristems of different sizes.

Torres *et al.*[16] used shoot tip culture and dry heat thermotherapy (37°C for 35 days) to recover virus-free cv. Amarante garlic plants. Vivek and Modgil[3] stated that high efficiency of virus elimination from horticultural plants could be reached using the suitable size of meristem tip, therefore, they detected ApMV in meristems $\geq$0.2 mm^3.

As a result of the seriousness of viral diseases on plants, several studies have been conducted to rapidly detect viruses and produce virus-free plants using several different approaches including tissue cultures, thermotherapy treatment, chemical treatment and others. Therefore, this study was designed to attempt to produce virus-free rose plants by using a combination of tissue culture and thermotherapy treatments.

MATERIALS AND METHODS

Study area: It is worth noting that the experimental part of this study was carried out at the Scientific Research Center at Taif University, KSA during the period from January, 2019 up to May, 2021.

Collection of rose plant samples: About 60 samples of *Rosa damascena* Trigintipetala Dieck (Al-Taif rose) were collected from Taif, KSA during the period from January-February, 2019. These plants were divided into 2 groups, the 1st contained 48 samples exhibiting virus-like symptoms and the 2nd contained 12 phenotypically healthy rose plants. Samples were collected in paper bags (30×50 cm) and then stored at 4°C until use. These samples included branches of rose plants loaded with shoot tips containing axillary buds from which apical meristems could be extracted.

Serological and molecular detection of rose viruses in collected samples: Antisera specific to both ApMV and SLRV were used in the Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA) technique for detecting the presence of the 2 viruses in rose plant materials by following the protocol of Rubio *et al.*[17] and Boonham *et al.*[18].

Two primer pairs (Table 1) specific to ApMV[1,2] and SLRV[7] were synthesized *via* an Applied Biosystem DNA/RNA Synthesizer, AGERI, ARC, Giza, Egypt and used for RT-PCR[19] detection of both viruses. All RT-PCR products were analyzed on 1.0% agarose gel in TBE buffer containing aliquots of ethidium bromide (0.5%) and electrophoresis was applied at 65 V for 1.5 hrs.

Extraction and preparation of meristem tips: Healthy and virus-infected rose plants were used as sources of meristem tips with lengths of 0.3, 0.5 and 1.0 cm under stereo-microscope. An average of 21 meristem tips per rose plant was obtained.

Sterilization treatment of meristem tips: Several 540 meristem tips were excised from the healthy rose plants and divided into 3 groups (180 meristems each) to compare the efficiency of 3 different sterilization treatments and detect the most suitable one. The 1st meristem tip group was exposed to 70% ethanol for 30 sec and 20% Clorox (Sodium hypochlorite 5.25%) for 10 min (T_1). The 2nd meristem tip group was exposed to 70% ethanol for 1.0 min and 15% Clorox for 10 min (T_2). The 3rd meristems group was exposed to 70% ethanol for 1.0 min and 20% Clorox for 10 min (T_3). The treated meristems were then rinsed in sterile distilled water 3 times.

Thermotherapy treatments: To eliminate the 2 viruses from virus-infected rose meristem tips, a total number of 270 meristems, divided into 9 replicates (10 meristems each), with different lengths (0.3, 0.5 and 1.0 cm) were

Table 1: Primer pairs used for RT-PCR detection of ApMV and SLRV

Viruses	Primers	Sequence	Expected PCR products (bp)	References
ApMV	Forward	5'-Cgtgaggaagtttaggttg-3'	425	1,2
	Reverse	5'-Gcctcctaatcggggcatcaa-3'		
SLRV	Forward	5'-Cctctccaacctgctagact-3'	370	7
	Reverse	5'-Aagcgcatgaaggtgtaact-3'		

Table 2: Full strength MS medium containing 3.0% sucrose, 0.7% phytoagar supplemented with different additions was used for *in vitro* propagation of rose meristems

Stages	Active charcoal (%)	Benzyl aminopurine (BAP) (mg L^{-1})	Kinetin (Kn) (mg L^{-1})	Indole-3-butyric acid (IBA) (mg L^{-1})
Shoot initiation	0.3	2.0	1.0	nil
Shoots multiplication	nil	3.0	1.0	nil
Elongation	nil	1.0	1.0	nil
Rooting	nil	nil	nil	2.0

thermotherapy-treated at 37°C for 4-6 weeks (30 meristems for each length). Meristem tips that produced callus were discarded, while those produced micro-shoots were tested for the presence of the 2 viruses by RT-PCR as previously described.

Cultivation media and *in vitro* tissue culture conditions: All sterilized meristems were separately cultivated on full strength MS medium for shoot initiation (Table 2) for 3 weeks. The thermotherapy-treated meristems which showed RT-PCR negative results (virus-free) were subjected to *in vitro* regeneration (Shoot initiation, shoot multiplication, shoot elongation and rooting) as reported by Attia *et al.*[20] (Table 2). As a control, MS medium without any growth regulators was used for all the *in vitro* cultures treatments. In a growth chamber, the *in vitro* cultures were incubated at 26±2°C on a 16/8 hrs light/dark and 3,000 lux light intensity provided by cool-white fluorescent light for 3 weeks for each stage. In the case of rooting, to remove adhered agar and traces of medium, the rooted plantlets containing about 4 or 5 roots with lengths ranging from 3-5 cm were carefully rinsed with warm H_2O. Plantlets were then transplanted to plastic pots (10 cm in diameter) containing sterile peat moss.

Statistical analyses: The experiments of this study were conducted in 3 replicates and data were subjected to statistical analysis using Analysis of variance (ANOVA) and Duncan's multiple range tests with a significance of $p<0.01$ level. Data of the study were analyzed using ASSISTAT version 7.7 beta (2016) computer package.

RESULTS

Presence of ApMV and SLRV in collected samples: Rose is one of the important flowering shrubs whose name derives from the Latin word Rosa. Due to its exposure to viral infections that could affect its economics, this research is one

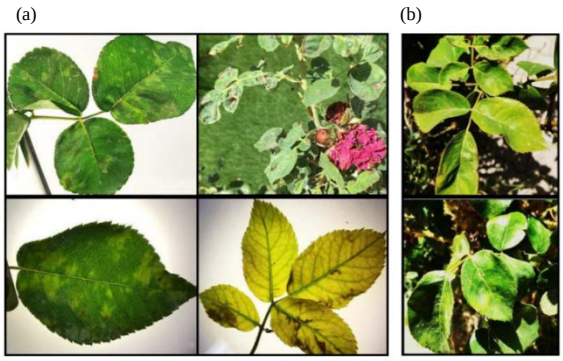

Fig. 1 (a-b): Taify rose plant samples were collected from the Taif region, (a) Samples exhibiting virus-like symptoms and (b) Symptomless plant samples

of the attempts conducted to eliminate some rose viruses (ApMV and SLRV) and produce virus-free rose plants, using a combination of tissue culture (meristem tips with different lengths) and thermotherapy treatment. To achieve such a goal, 60 rose plants exhibiting virus-like (Fig. 1a) or healthy-like symptoms (Fig. 1b) were initially collected from Taif city, KSA and screened for the presence of the 2 rose viruses of interest.

DAS-ELISA and RT-PCR were used to detect the presence of ApMV and SLRV in collected rose plant samples. As shown in Table 3, ApMV and SLRV were detected using DAS-ELISA in 18 and 9 rose plant samples, respectively. Whereas RT-PCR detection revealed the presence of ApMV and SLRV in 12 and 09 rose plant samples, respectively. Virus-free rose plants were used as a source for meristems that were excised under a stereo-microscope and used to evaluate the efficiency of 3 meristem-tip sterilization treatments. On the other hand, meristems excised from virus-infected rose plant samples were used in thermotherapy treatment experiments.

Effect of sterilization treatments on the survival of meristem tips: As shown in Table 4, the highest survival rate

Table 3: Summary of ELISA and RT-PCR detection of 2 rose viruses in collected rose samples

Detection tools		Virus in rose plant samples			
		ApMV		SLRV	
ELISA	RT-PCR	Number	Percentage	Number	Percentage
-ve	-ve	30	50	42	70
-ve	+ve	12	20	9	15
+ve	+ve	18	30	9	15
Total	60	100	60	100	

Table 4: Effect of 3 sterilization treatments on the number of cleaned-survived meristem tips (NC-SMT) extracted from a virus-infected rose plant

Sterilization treatments	ANMT	ANC-SMT	ANC-SMT (%)
T_1: 70% Ethanol for 30 sec and 20% Clorox (Sodium hypochlorite 5.25%) for 10 min	60	18.3^{c}	30.6^{c}
T_2: 70% Ethanol for 1.0 min and 15% Clorox (Sodium hypochlorite 5.25%) for 10 min	60	58.7^{a}	97.8^{a}
T_3: 70% Ethanol for 1.0 min and 20% Clorox (Sodium hypochlorite 5.25%) for 10 min	60	34.3^{b}	57.2^{b}

ANMT: Average number of meristem tips and ANC-SMT: Average number of cleaned-survived meristem tips. The Duncan's test at a level of 5% of probability was applied, the averages followed by the same letter do not differ statistically. Every treatment had 3 replicates and every replicate had 60 meristem tips

Table 5: Differentiation frequency of thermotherapy-treated meristem tips incubated for different periods

Meristem-tip length (cm)	Period (weeks)	ANMT	Type of meristem differentiation: Callus* ANC	Callus* ANC (%)	Micro-shoots ANMS	Micro-shoots ANMS (%)
0.3	4	10	9.3^{a}	93.3^{a}	00^{f}	00^{f}
	5	10	5.3^{d}	53.3^{d}	00^{f}	00^{f}
	6	10	00^{f}	00^{f}	00^{f}	00^{f}
0.5	4	10	00^{f}	00^{f}	8.3^{b}	83.3^{b}
	5	10	00^{f}	00^{f}	4.3^{e}	43.3^{e}
	6	10	00^{f}	00^{f}	00^{f}	00^{f}
1.0	4	10	00^{f}	00^{f}	7.3^{c}	73.3^{c}
	5	10	00^{f}	00^{f}	3.7^{e}	36.7^{e}
	6	10	00^{f}	00^{f}	00^{f}	00^{f}

*: Meristems differentiated into callus were discarded, ANMT: Average number of meristem tips, ANC: Average number of callus and ANMS: Average number of micro-shoots. The Duncan's test at a level of 5% of probability was applied and the averages followed by the same letter are not statistically different. Every treatment had 3 replicates and every replicate had 30 meristem tips

of cleaned meristem tips was obtained from the 2nd treatment (T_2) in which the percentage of Clorox was reduced from 20% (in the 1st and 3rd treatments) to 15%. The average numbers of cleaned survived meristem tips were 18.3, 58.7 and 34.3 in the 1st, 2nd and 3rd treatments, respectively.

Effect of size of meristem tips and duration of thermotherapy on their differentiation: Meristem tips of 3 different lengths were excised and thermotherapy-treated by incubation at 37°C for 3 different periods. Results in Table 5 showed that meristems of all lengths that were thermotherapy-treated for 6 weeks and incubated on shoot initiation medium failed to differentiate into callus or micro-shoots. While the other groups of meristem tips, thermotherapy-treated for 4 and 5 weeks, differed in their differentiation response. Meristem tips of 0.3 cm in length and incubated for 4 and 5 weeks were differentiated into callus and therefore discarded. Meristem tips of 0.5 and 1.0 cm and incubated for 4 and 5 weeks, were differentiated into micro-shoots but in different proportions. It has been proven that the micro-shoot regeneration frequency obtained from meristem tips of 0.5 and 1.0 cm and incubated for 4 weeks was higher (83.3 and 73.3%) than that obtained from those incubated for 5 weeks (43.3 and 36.7%).

Evaluation of meristem tip culture and thermotherapy in eliminating ApMV and SLRV: RT-PCR was successfully applied to detect the presence of ApMV and SLRV in the meristem tips that were successfully regenerated and differentiated into shoots. These could be subjected to micropropagation, once confirmed to be virus-free, to produce virus-free plants. Table 6 showed that the percentage of virus-free regenerated plants produced from meristem tips incubated for 4 weeks was higher than that produced from meristem tips incubated

for 5 weeks, regardless of the length of the meristems. The percentage of ApMV-free regenerated plants obtained from 0.5 cm meristem tips were 92.0% (Fig. 2a), 76.9% (Fig. 2b) and from 1.0 cm of meristem tips were 62.0% (Fig. 2c), (50.0%) (Fig. 2d) incubated for 4 and 5 weeks, respectively. Regarding the SLRV, the percentages of virus-free regenerated plants obtained from 0.5 cm meristem tips were 96.0% (Fig. 3a), 84.6% (Fig. 3b) and from 1.0 cm of meristem tips were 79.2% (Fig. 3c), (50.0%) (Fig. 3d) incubated for 4 and 5 weeks, respectively.

Table 6: RT-PCR detection of ApMV, and SLRV in thermotherapy-treated meristems incubated for different durations

			ApMV				SLRV			
			Virus-infected		Virus-free		Virus-infected		Virus-free	
Meristems lengths (cm)	Incubation periods (weeks)	Total tested meristems	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
0.5	4	25	2	08.0	23	92.0	1	4.00	24	96.0
	5	13	3	23.1	10	76.9	2	15.4	11	84.6
1.0	4	24	9	37.5	15	62.5	5	20.8	19	79.2
	5	12	6	50.0	06	50.0	6	50.0	06	50.0

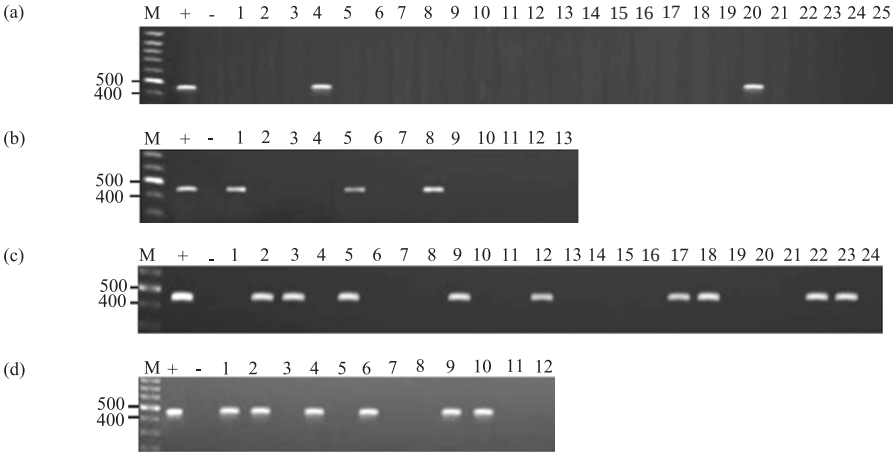

Fig. 2(a-d): RT-PCR detection of ApMV in thermotherapy-treated meristems with lengths of (a-b) 0.5 and (c-d) 1.0 cm incubated for 4 and 5 weeks, respectively
M: 100 pb DNA ladder

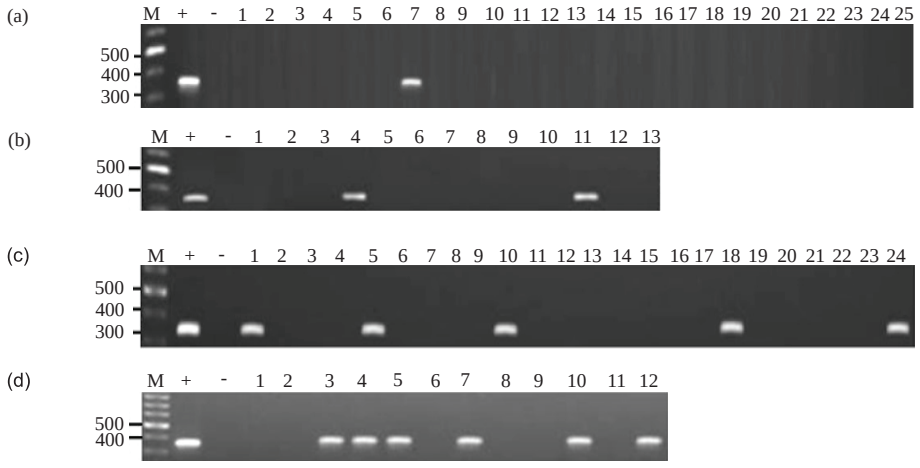

Fig. 3(a-d): RT-PCR detection of SLRV in thermotherapy-treated meristems with lengths of (a-b) 0.5 and (c-d) 1.0 cm incubated for 4 and 5 weeks, respectively
M: 100 pb DNA ladder

Fig. 4(a-e): Stages of *in vitro* micropropagation of virus-free Taif rose meristem, (a) Shoot initiation, (b) Shoot multiplication, (c) Shoot elongation, (d) Rooting and (e) Virus-free plantlet

Several 43/74 treated meristem tip samples were tested negative, by RT-PCR, for both ApMV and SLRV and therefore were subjected to micropropagation steps (Shoot initiation, shoot multiplication, shoot elongation, rooting and virus-free plantlet) as shown in (Fig. 4a-e). A total number of 195 virus-free plantlets were obtained and became ready for acclimatization with the assumption that the number is stable in all micropropagation stages.

DISCUSSION

In this study, Taify rose plants free from ApMV and SLRV were produced using a combination of plant tissue culture and thermotherapy. Rose is the most popular cut flower in the world and for this, it must be produced in high quality and also protected, by biotechnology methods, from diseases[21]. Among several types and forms of roses worldwide, Al-Taif rose of Taif, KSA (the king of roses) is considered to be the best. It has more than 3000 species with different colours, which are used in gifts, medicines and expensive perfume extractions. Also, it is used in washing the honourable Kaabah every year after being mixed with Zamzam water. Rose Mosaic Disease (RMD) is caused by different viruses is the most common disease of rose in different countries[6,19-21]. Some investigators revealed that RMD affect roses flower earlier than healthy plants and produces a significant reduction in the number and diameter of the rose flowers as well as a reduction in number, diameter and length of the rose shoots and finally reduction number of flower petals[6,22]. The experimental results showed that RT-PCR was more sensitive and accurate for detecting both viruses which are supported by other investigators[23-25] who reported that PCR was more sensitive than DAS-ELISA in detecting plant viruses, particularly those that are found in low concentrations or symptomless plant leaves, phloem-limited viruses and viruses present in the apical meristems.

The survival of cultured meristem tips is suggested to depend on their rate of microbial contamination[26] and hence it was important to use a suitable sterilization method to minimize bacterial and fungal contamination. Among the 3 different sterilization treatments tested in this study, using 15% Clorox (T_2 treatment) was the most suitable in obtaining the highest percentage of the average survived and cleaned meristem explants. This is in harmony with the findings reported by Attia *et al.*[20].

Several studies applied tissue culture techniques on meristem tips, in different lengths, infected with viruses to try to eliminate them. Success in eliminating viruses using this method may be due to the absence of protoplasmic strands, faster cell division rate than virus replication and high hormonal concentrations and activities in meristem tips[3,8-10]. Moreover, several reports indicated that elevated temperatures could be used as well, to eliminate virus infection and about 50% of viruses could be eliminated from cultivated crops using heat treatment[27]. Results reported in this study showed that the highest percentage of virus-free

explants was obtained from meristem tips of 0.5 cm long. These results are in agreement with that previously reported in which the tissue culture technique was used alone or in combination with thermotherapy to eliminate viruses in the virus-affected plants[28,29]. In this study, the survival rate increased with increasing the length of meristem tips and shorter meristem tips were unable to regenerate into micro-shoots which is supported by Babaei *et al.*[30] Elimination of viruses from virus-infected plant materials was previously studied by several investigators[2,11,13,16].

CONCLUSION

Thermotherapy treatment of 0.5 cm long meristem tips for 4 weeks was successfully applied to produce a large number of Al-Taif rose plantlets free of ApMV and SLRV, the causal agents of rose mosaic disease. RT-PCR detection of ApMV and SLRV revealed that using thermotherapy-treatment, for 4 weeks, of 0.5 cm long meristem tips was successfully applied to eliminate the 2 viruses in 92 and 96% of regenerated plantlets, respectively.

SIGNIFICANCE STATEMENT

It has been proven in several studies that the combination of meristem tip culture and thermotherapy is highly beneficial in producing virus-free plants as one of the most important diseases that affect plants. Thermal may reach 30-40°C in a thermal room for varying periods according to the plant used. The importance of using the meristem tip of the plant could be summarized in getting rid of viruses from infected plants because the meristem tips are full of meristematic tissues that have a tremendous ability to divide and the damaged cells are replaced quickly, which facilitates the possibility of obtaining virus-free plants.

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