

Production of Apple Mosaic Virus–Free Pear and *Rosa damascena* Plants Through Tissue Culture

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ABSTRACT

Effect of chemical agents Virazole (Ribavirin) levels, on *Rosa damascena* and *Pyrus communis* production were investigated in this study. The results concluded that, 25% Chlorox reduced the contamination to a minimum value and gave highest survival percentage of pear and rose growth. The best results of the callus growth and shoot length of the rose cultivar were recorded with the addition of 3.0 or 4.0 mg/l BA and 2.0 mg/l IAA. The interaction effect revealed that growing the explant of pear explant in MS medium containing 1.0 mg/l BA+1 or 2 mg/l IAA gave the longest shoots (0.92 cm and 0.66 cm). Data also recorded that the concentration of ribavirin (20-100 mg/l) was effective in eliminating ApMV, while (10-100 mg/l) was not successful in eliminating ApMV from *Rosa* or pear.

INTRODUCTION

Pear is one of the oldest fruit crops widely grown in temperate and sub-tropical regions of the world ^[1].

Damask rose (*Rosa damascena* Mill.) from Rosaceae family is one of the most important medicinal plants. The rose plants are generally propagated by stem cuttings, such techniques used to produce disease free plants ^[2].

Apple mosaic virus (ApMV), one of the oldest known virus diseases of apple trees induced noticeable symptoms on leaves and fruits ^[3].

Tissue culture technique has been proved as a potential tool for quick and mass propagation of several plant species ^[4].

Shoot regeneration of two pears (*Pyrus communis* L.), namely 'bartlett' and 'dargazi' was successfully developed of genetic engineering research ^[5]. Pear (*Pyrus* species) shoot tips and nodal explants from three pear genotypes were cultured on Murashige and Skoog medium (MS) with five different plant growth regulator combinations ^[6]. Source of cytokinin utilized influences the induction and proliferation of axillary buds pear tree ^[7].

Saklani et al. ^[8] Found best multiplication of nodal rose explants occurred in the combination of BAP and KN (5.0 mg l⁻¹+2.5 mg l⁻¹). Silva et al. ^[9] on pear tree "Cascatense" found that 30 g/L sucrose was the most effective concentration growth and development of shoots ^[10].

Ribavirin is one of the well-known antiviral medicines which is highly effective against several DNA and RNA viral infections and their protein synthesis ^[11].

Papstein et al. [12] Studied that attempts to fully eliminate viruses by ribavirin in lower concentration 20 mg/l were not successful. However, the success of chemotherapy with ribavirin at 100 mg/l was 76% for ACLSV elimination after the second round.

Cieslinska [13] studied that chemotherapy and chemotherapy *in vitro* and found Ribavirin at concentration 25-100 mg/l was effective in eliminating ACLSV from PNRSV from "Empress" plum shoots. The higher concentrations of Virazole (50-100 mg/l) were phytotoxic for treated shoots, especially in case of "Empress" plum.

Hu et al. [14] *in vitro* plants of sand pear (*Pyrus pyrifolia* cv. *Jinshui*), showed that chemotherapy with ribavirin at 15–25 µg/ml for 5-30 days could enhance the growth and proliferation of *in vitro* pear plants.

Papstein et al. [12] Studied that attempts to fully eliminate viruses by ribavirin in lower concentration 20 mg/l were not successful.

Hu et al. [14] stated that Ribavirin treatments at 15 and 25 µg/ml resulted in virus elimination rates of 74.4% and 75.0%, respectively.

Mosaic is presumably the most commonly encountered viral disease in roses. Chemotherapy and chemotherapy methods were also applied separately and their antiviral effect compared with the chemo-chemotherapy [15].

MATERIALS AND METHODS

These experiments were carried out at Biotechnology department, faculty of science, Taif University during years 2015, 2016 and 2017. This study designed to investigate the Production of Apple Mosaic Virus –free pear and *Rosa damascena* plants through tissue culture, to select the best growth.

Shoots of pear and *Rosa damascena* (Taif Rose) plants were kindly obtained from Taif region, Saudi Arabia kingdom. Actively growing shoots were used as the explants source. The terminal shoots were collected from growing plants and were cut into 0.5-1.5 cm pieces.

Explants Sterilization

Shoot tip explants in size from 5 to 10 mm in length were excised and washed under running tap water. Explants were surface sterilized by immersing in an aseptic solution of different concentrations of Sodium hypochlorite solution (10, 20, 25 and 30%) for 10 min, then followed by Ethanol alcohol for one minute, finally rinsed several times distilled water.

Media Preparation

The basal medium MS which described by Murashige and Skoog was used to cultivate pear and rose *in vitro*. The medium was solidified with phytigel (2.5 g^l), and pH was adjusted to 5.7-5.8 with either 1N KOH or 1N HCl before phytigel addition and the media were autoclaved at 121°C at 1.1 kg.cm² for 20 min. Cultures were incubated in room chamber at 25 ± 2°C with 3000 lux under 16 h photoperiod and 8 h darkness.

Explants were cultured in test tubes containing MS medium, supplemented with sucrose (25 g/l) and different combinations of Indol acetic acid (IAA) and benzyl adenine (BA) were added as well. Combinations were as follows:

- 1) BA at 1 mg/l and IAA at 1.0 and 2 mg/l
- 2) BA at 2 mg/l and IAA at 1.0 and 2 mg/l
- 3) BA at 3 mg/l and IAA at 1.0 and 2 mg/l
- 4) BA at 4 mg/l and IAA at 1.0 and 2 mg/l

Callus formation was expressed as follows:

- + refers to: callus < 2 g/explant
- ++ refers to: 2 g < callus < 4 g/explant
- +++ refers to: callus > 4 g/explant.

Elimination of Apmv-free From Pear and *Rosa damascena* (Taif Rose) by Chemotherapy Treatment

Fruit and *Rosa damascena* (Taif Rose) trees infected with apple mosaic virus were used as a source of planting materials and healthy plants Control, trees were chosen after detecting the virus several times by ELISA for test. Samples of hard wood cutting from pear (*Pyrus communis*) and *Rosa damascena* (Taif Rose) plants were carried out to study the effect of virus elimination by chemotherapy (Virazole-Ribavirin).

The collected samples were sub-cultured on M.S medium supplemented with different Concentration of (Virazole-Ribavirin) 10 mg^l, 20 mg^l, 30 mg^l and control without Virazole-Ribavirin. Agar was used at 7 g^l and sucrose at 30 g^l. After 4 weeks from

culturing on medium, the following data were recorded then test by DAS-ELISA examination and an account of percentage of survival plants after one month.

Statistical Analysis

The obtained data were analyzed using completely randomized design (two factors). The Least Significant Difference (LSD) test was used for comparison among means according to the method of Steel and Torri^[16].

RESULTS

Sterilization

As shown in **Figure 1** regardless sterilizations levels of pear and rose plants after the preliminary experiment which aimed to investigate the effect of the concentration of commercial bleach (Chlorox 52.5 g/l NaOCl) and the duration of the sterilization process; among the concentrations and the durations tested, 25% Chlorox plus drops of a detergent (Tween 80) for 30 minutes after a pretreatment of soaking the shoots in 70% ethanol for one minute, reduced the contamination to a minimum and gave the highest survival percentage of pear and rose growth.

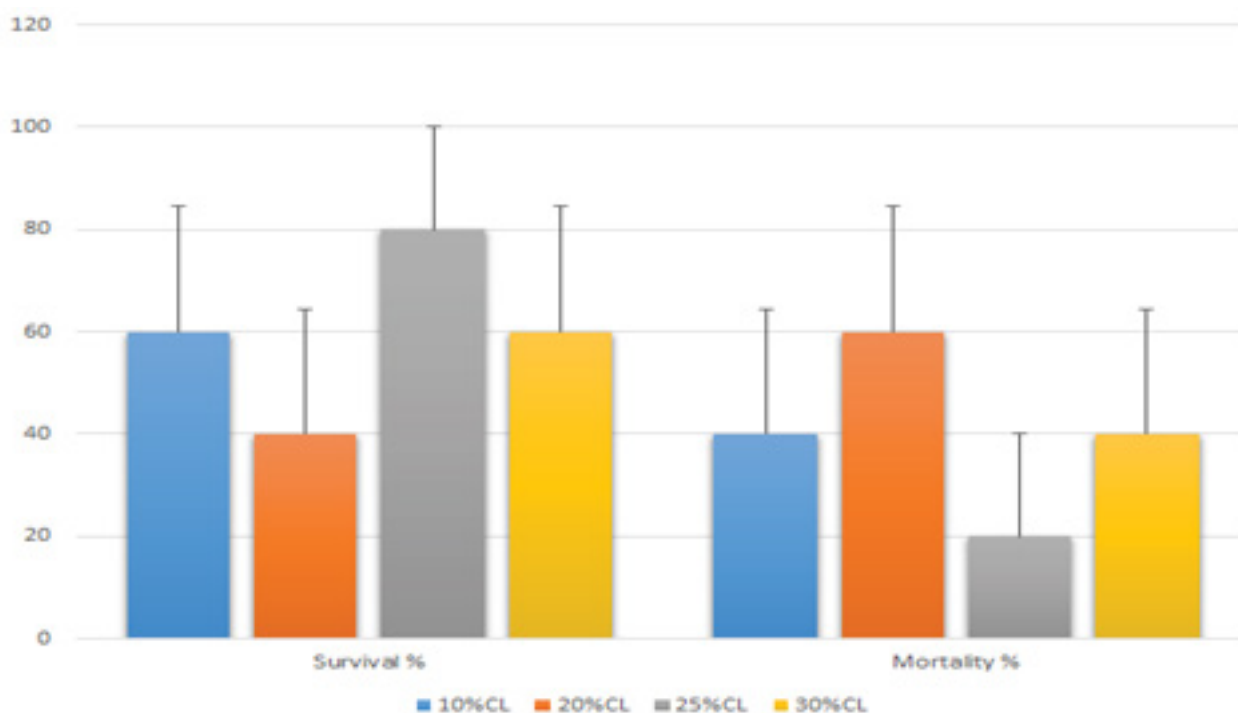


Figure 1. Effect of sterilization treatments on explant growth of pear and rose shoots.

Effect of BA and IAA on the Growth and Development of Rose Plant

Survival %

Concerning the response of survival percentage to media composition (**Table 1**). The results clearly indicated that the highest survival percentage (100%) was recorded regardless the media containing 2 or 3 mg/l BA supplemented with 1 mg/l IAA, also with BA at concentrate 4 mg/l supplemented with 2 mg/l IAA.

The addition of BA at 1.0, 2.0 or 3.0 mg/l+1 mg/l IAA as well as BA at 4.0 mg/l+2 mg/l IAA in the MS medium were the most effective treatments in increasing the survival percentage compared with control and the other treatments. Culturing the rose explants on control media reduced the survival percentage to the minimum values (60.00%).

Callus formation

As shown in **Table 1**, callus formation of the Media cultivar was best on the medium supplemented with 2 mg/l IAA and medium containing 4.0 mg/l BA+2.0 mg/l IAA. The best result of the callus growth of the rose cultivar was recorded with the addition of 3.0 or 4.0 mg/l BA and 2.0 mg/l IAA. No callus growth was obtained when the explant were cultured on MS medium without hormone. A great increase in the callus growth of the rose cultivar was recorded with the increase of NAA to 2 mg/l with 4 mg/l BA (**Figure 2**).

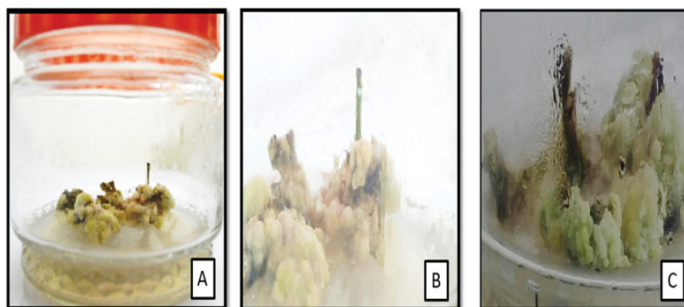


Figure 2. The best induction of *Rosa damascene* callus (A) 3 mg/l BA+2 mg/l IAA; (B & C) 4 mg/l BA+2 mg/l IAA.

Table 1. Effect of BA and IAA treatments on explant growth and development of *Rosa damascena* (Taif Rose) plant.

Mean ± SE*: Significant difference (p<0.05) between compared groups using student t-test.

Treatments	Survival %	Callus formation	Number of shoots/plant	Length of shoot (cm)	Number of leave/plant
0BA+0 IAA	60.00 ± 24.50	-	0.80 ± 0.20	0.80 ± 0.20	0.50 ± 0.16
1BA+IAA	80.00 ± 20.00	+	1.20 ± 0.38	1.32 ± 0.34	1.20 ± 0.38
1BA+2IAA	80.00 ± 20.00	++	1.80 ± 0.50	1.70 ± 0.46	2.40 ± 0.68*
2BA+1IAA	100.00 ± 0.00	+	3.40 ± 0.25*	3.88 ± 0.43*	4.00 ± 0.32*
2BA+2IAA	80.00 ± 20.00	++	2.00 ± 0.84	1.58 ± 0.71	2.00 ± 0.84
3BA+1IAA	100.00 ± 0.00	++	4.60 ± 0.60*	5.50 ± 0.60*	5.20 ± 0.38*
3BA+2IAA	60.00 ± 24.50	++	3.40 ± 1.40	3.84 ± 1.57	3.20 ± 1.36
4BA+1IAA	80.00 ± 20.00	++	4.60 ± 1.17*	5.70 ± 1.43*	4.20 ± 1.11*
4BA+2IAA	100.00 ± 0.00	+++	6.00 ± 0.32*	7.70 ± 0.38*	6.80 ± 0.20*

Shoot number

As shown in **Table 1**, regardless media composition, the addition of BA at different concentrations increased the shoot number compared with control and BA at 3.0 or 4.0 mg/l with 2 mg/l IAA produced the highest number of shoots/explant. Increasing level of NAA to 1 mg/l reduced the formation of shoots in most treatments (**Figure 3**).

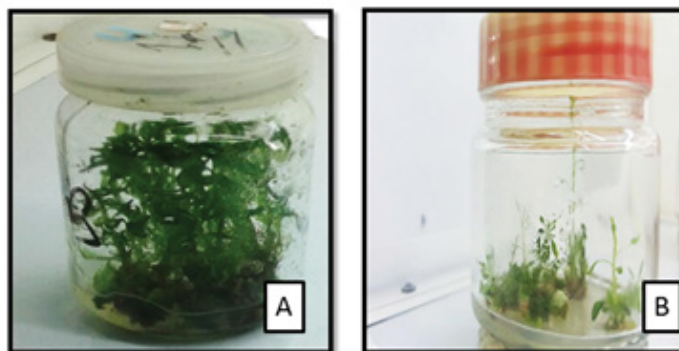


Figure 3. The best shoot tip of *Rosa damascene* (A) and Pear (B). (A) MS with 3 mg/l BA+2 mg/l IAA; (B) MS with 3 mg/l BA+1 mg/l IAA

Growing the explants of rose cultivar on MS medium containing 3 or 4mg/l BA gave the highest number of shoots/explant (4.60 and 6.0,respectively), whereas the lowest number of shoots of (0.80) was recorded with control MS medium without BA or IAA.

Shoot length

The results shown in **Table 1**, it indicated that there was a significant difference of the shoot length between hormone treatments. The MS culture Medea with 4 mg/l BA and 1 or 2 mg/l IAA formed significantly average of shoot length more than the other media, the shoot lengths were 5.70 and 7.70 cm, for the rose culture, respectively.

Growing the explant f in MS medium containing 0 mg/l BA+0 IAA gave the shortest shoots (0.80 cm).

Leaf shoots

As shown in **Table 1**, regardless media composition, the addition of BA at different concentrations increased the shoot number compared with control and BA at 3.0 or 4.0 mg/l with IAA produced the highest number of shoots/explant. The MS medium containing 4 mg/l BA gave the highest leaf number/explant (6.80), whereas the lowest number of leaves was recorded with MS medium without BA or IAA.

Effect of BA and IAA on the Growth and Development of Pear Plant

Survival %

Concerning the response of survival percentage to media composition (**Table 2**), the results clearly indicated that the highest survival percentage (100.00%) was recorded with untreated treatment.

The addition of BA at 3.0 mg/l+1 or 2 mg/l IAA as well as BA at 4.0 mg/l+1 or 2 mg/l IAA in the MS medium was the most effective treatments in increasing the survival percentage compared with the other BA treatments. Culturing the pear explants on media containing 2 mg/l IAA+1 mg/l BA reduced the survival percentage to the minimum values.

Callus formation

As shown in **Table 2**, callus formation of the pear explant was best on the medium supplemented with BA at 2 mg/l+1 or 2 mg/l IAA and medium containing 4 mg/l BA+2.0 mg/l IAA and produced great increase in the callus growth. Raising the level of 0 BA+0 IAA showed no callus formation. Decreasing callus growth was obtained when the explant were cultured on MS medium with the low level of BA.

Table 2. Effect of BA and IAA treatments on explant growth and development of pear plant. Mean \pm SE*: Significant difference ($p < 0.05$) between compared groups using student t-test.

Groups	Survival %	Callus formation	Number of shoots/plant	Length of shoot (cm)	Number of leave/plant
OBA+OIAA	100.00 \pm 0.00	-	2.80 \pm 0.40	1.36 \pm 0.10	2.60 \pm 0.40
1BA+1IAA	60.00 \pm 24.50	+	2.20 \pm 0.92	0.92 \pm 0.38	1.60 \pm 0.68
1BA+2IAA	40.00 \pm 24.50*	+	1.60 \pm 0.98	0.66 \pm 0.40	1.20 \pm 0.73
2BA+1IAA	60.00 \pm 24.50	++	2.60 \pm 1.08	0.96 \pm 0.40	1.60 \pm 0.68
2BA+2IAA	80.00 \pm 20.00	++	3.40 \pm 0.87	1.30 \pm 0.34	2.60 \pm 0.67
3BA+1IAA	80.00 \pm 20.00	+	3.60 \pm 0.93	1.24 \pm 0.32	2.80 \pm 0.73
4BA+1IAA	80.00 \pm 20.00	+	2.20 \pm 0.58	1.02 \pm 0.26	1.80 \pm 0.49
4BA+2IAA	80.00 \pm 20.00	++	2.20 \pm 0.58	1.06 \pm 0.26	2.90 \pm 0.58

Shoot number

As shown in **Table 2**, regardless media composition, the pear explant formed number of shoots more significantly with BA at 2 and 3 mg/l+IAA at 1 or 2 mg/l, which gave 3.40 and 3.60 shoots, respectively. The addition of BA at 1 mg/l+2 mg/l IAA decreased the shoot number compared with control or the other BA treatments and produced the lowest number of shoots/explant (**Figure 3**).

Shoot length

The results shown in **Table 2**, indicated that there was a significant difference of the shoot length between pear explants treatments. The untreated control pear explant formed significantly average of shoot length more than the other treatments, and produced 1.36 cm.

The addition of BA or IAA to MS medium had no significant effect on the shoot length (except for BA at 3 or 4 mg/l+IAA at 1 or 2 mg/l). The interaction effect revealed that growing the explant of pear explant in MS medium containing 1.0 mg/l BA+1 or 2mg/l IAA gave the longest shoots (0.92 cm and 0.66 cm).

Leaf shoots

As shown in **Table 2**, regardless pear explants on control MS medium formed number of leaves more significantly than 1 or 2 mg/l BA+1 or 2 mg/l IAA and produced 2.60. The addition of BA at 3 or 4 mg/l+1 or 2 mg/l IAA increased the leaf number compared with control and produced the highest number of leaves/explant.

Effect of BA and IAA treatments on explant growth and development of pear plant (**Table 2**).

Elimination of Apmv-free From Pear and Rosa damascena (Taif Rose) by Chemotherapy Treatment

The obtained results in **Table 3**, showed that, chemotherapy *in vitro* were applied to eliminate Apple Mosaic Virus. ELISA assays conducted after chemotherapy by virazole (ribavirin) for 40 days indicated that in at concentration (20-100 mg^l) was effective in eliminating ApMV. but concentration (10-100 mg^l) was not successful in eliminating ApMV from Rosa or pear while the higher concentrations of ribavirin (30-100 mg^l) exhibited shoot tip of pear and Rosa as possible sign of chemical toxicity (**Figures 4-8**).

Table 3. Influence of chemical substance Virazole (Ribavirin) on ApMV-free Rosa damascene (Taif Rose) and pear.

Treatments of Ribavirin (mg/L)	Name of plants	No. of:		Survival plantlets	No. of: plantlets tested			Virus free plantlets(%)
		Cultured plantlets	Survival plantlets	Total	Total	Healthy	Infected	
Control (Zero mg/L)	Rosa	10	10	100%	10	0	10	0%
10		10	10	100%	10	0	10	0%
20		10	3	30%	10	3	7	35%
30		10	6	65%	10	1	9	10%
Control (Zero mg/L)	Pear	10	10	100%	10	0	10	0%
10		10	10	100%	10	0	10	0%
20		10	3	35%	10	4	6	40%
30		10	4	54%	10	2	8	21%

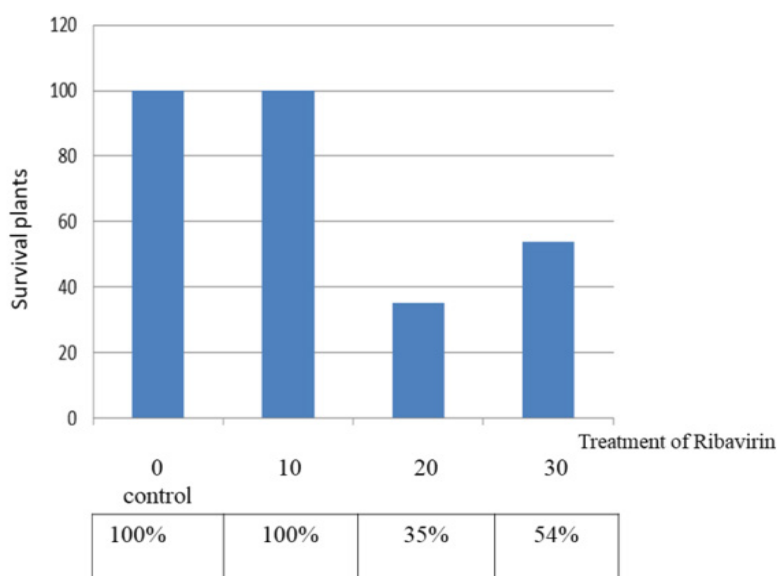


Figure 4. Influence of chemical substance Virazole (Ribavirin) survival in pear plants.

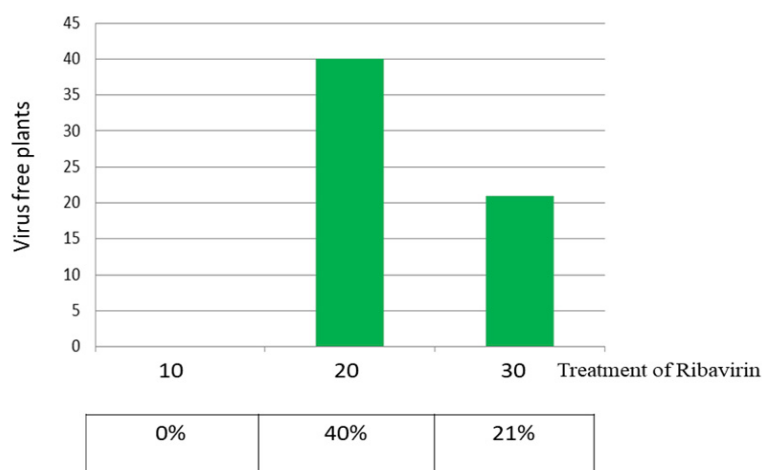


Figure 5. Influence of chemical substance Virazole (Ribavirin) on ApMV-free Pear plants.

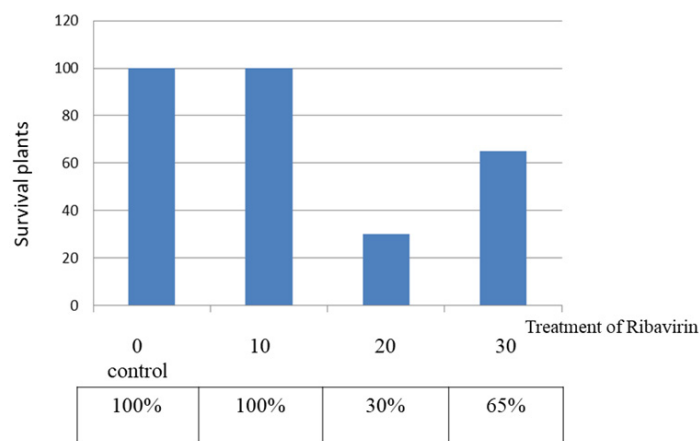


Figure 6. Influence of chemical substance Virazole (Ribavirin) survival in *Rosa damascena* (Taif Rose) plants.

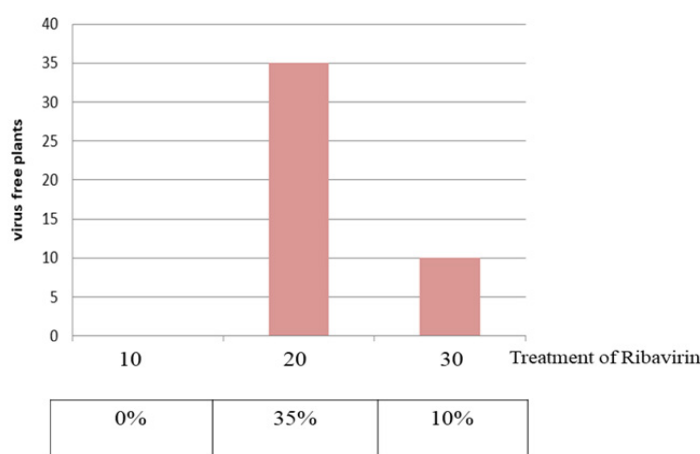


Figure 7. Influence of chemical substance Virazole (Ribavirin) on ApMV-free *Rosa damascena* (Taif Rose) plants.

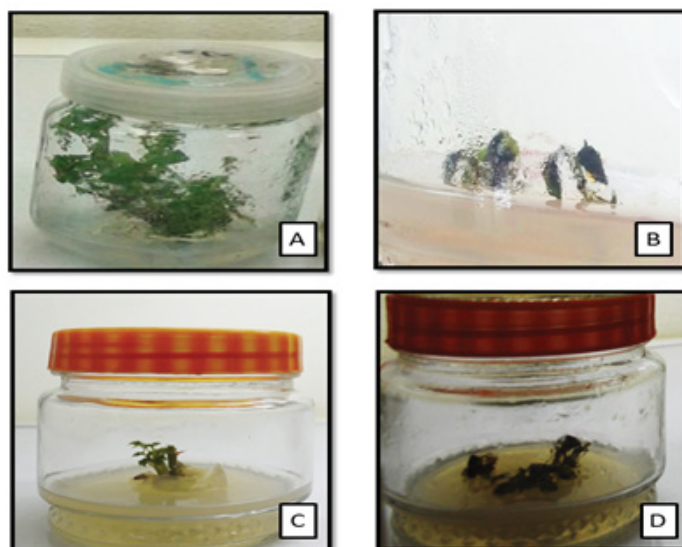


Figure 8. The higher concentrations of virazole-ribavirin were phytotoxic for treated shoots. (A) Pear control; (B) Pear with 30 mg/l; (C) Rosa control; (D) Rosa with 30 mg/l.

DISCUSSION

The aim of this study was to detect the Production of Apple Mosaic Virus-free pear and *Rosa damascena* plants through tissue culture, to select the best growth.

Apple Mosaic Virus which can cause disease of stone fruit and Rosa on susceptible rootstocks and greatly decreasing the growth and productivity of infected trees so need to prevent the productivity losses of fruit trees caused by viruses via tissue culture.

Data showed that callus formation of the pear explant was best on the medium supplemented with BA at 2 mg/l+1 or 2 mg/l IAA and medium containing 4 mg/l BA+2.0 mg/l IAA and produced great increase in the callus growth. The same results are obtained with Ilahi et al. and Sakr et al. [17,18].

Results of this study found that, regardless media composition, the pear explant formed number of shoots more significantly with BA at 2 and 3 mg/l+IAA at 1 or 2 mg/l, and this results are in a good agreement with results obtained by Ilahi et al. [17] obtained good shoot proliferation with 1, 2 and 3 mg/l BA. Tasy [19] on carnation cv Opale, mentioned that shoot production increased with increasing BA concentration (from 0.0 to 4.0 mg/l), but increased shoot vitrification El-Sawy and Bekheet, [20] on *Dieffenbachia picta* cv. Tropica. Found that BA at 1.0 or 2.0 mg/l was more effective for increasing the number of shoots. Also, ElSawy et al. [21] on *Dracaena* cv. Tricolour, obtained the largest number of shoots/explant with 4.0 mg/l of BA.

Ribavirin is one of the well-known antiviral medicines which are highly effective against several DNA and RNA viral infections and their protein synthesis [11].

Increasing ribavirin concentration in tissue culture led to malformation and unwanted proliferation of shoots and inhibition of the bud growth [15].

The higher concentration of Ribavirin was phytotoxic. Plants treated with (40, 80 mg.l⁻¹) Ribavirin had poor growth and apex necrosis. The highest concentration of Ribavirin (100 mg.l⁻¹) caused the death of the apple shoots [22,23].

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 [15,24].

CONCLUSION

Propagation and growth of *Rosa damascena* (Taif Rose) and *Pyrus communis* in *in vitro* cultures were significantly improved due to growth regulators treatment. Moreover, Available evidence suggests that the Virazole (Ribavirin) concentrations may act to explain the increasing in growth of plants under study and hence increase the plants protect the replication of the virus and thus inhibit it.

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