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CITRUS JAPONICA RESPONSE TO PLANT GROWTH REGULATORS THROUGH MICROPROPAGATION

A.Y. HANAWAT* and M.N. NAIF

Department of Horticulture and Landscape Gardening, College of Agriculture, Al-Qasim Green University, Al-Qasim, Iraq
*Corresponding author's email: abdulla.hinwat@gmail.com
Email address of co-author: makki@agre.uoqasim.edu.iq

SUMMARY

The study aimed to evaluate the effects of different concentrations of putrescine (Put: 0, 2.5, 5, and 7.5 mg L^{-1}) and forchlorfenuron (CPPU: 0, 0.4, and 0.8 mg L^{-1}) on the in vitro growth and multiplication of shoots in Japanese orange *(Citrus japonica*). Explants cultured on a Murashige and Skoog (MS) medium received supplements of 2 mg L^{-1} BA (benzyl adenine) and 0.1 mg L^{-1} NAA (1-naphthaleneacetic acid) for four weeks. Results revealed that increasing Put concentration in the multiplication medium significantly improved stem length, the number of leaves, fresh weight, and plant survival. The highest values were notable at Put (7.5 mg L^{-1}) for stem length (2.127 cm), leaves/explant (8.268), and 0.7944 mg and 85.6% survival, respectively, compared with the control. The latter recorded the lowest values (0.843 cm, 5.911, 0.7333 mg, and 67.8%, respectively). Similarly, increasing the CPPU concentration (0.8 mg^{-1}) revealed the highest values for shoots/explant (CPPU: 4.025), shoot length (2.090 cm), leaves/explant (8.687), fresh weight (0.8975 mg), dry weight (0.2192 mg), and survival (91.7%). The interaction between CPPU (0.8 mg L^{-1}) and Put (7.5 mg L^{-1}) concentrations recorded the topmost average stem length (2.750 cm), leaf number (9.533 leaves/explant), and survival percentage (100.0%).

Keywords: Kumquat **(***Citrus japonica*), plant tissue culture, putrescine (Put), CPPU, NAA, BA

Key findings: The interaction between CPPU (0.8 mg L^{-1}) and Put (7.5 mg L^{-1}) concentrations recorded the highest average stem length (2.750 cm), leaf number (9.533 leaves/explant), and survival percentage (100.0%) in the Japanese orange.

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INTRODUCTION

The Marumi kumquat, scientifically known as *Fortunella japonica* and *Citrus japonica*, belongs to the family Rutaceae and originates from Southeastern China. The fruit resembles a small orange, approximately the size of a large olive, and grows abundantly on small, slow-growing shrubs. Kumquats are valued both for their ornamental appeal due to their thornless nature and compact growth habit and their culinary uses, particularly in making jams, jellies, candied fruits, and various desserts, thanks to their distinctive sweet-sour flavor. Citrus species, including kumquats, hold significant nutritional, economic, and medicinal importance. Their fruits are rich in essential minerals, such as potassium, calcium, iron, magnesium, sodium, sulfur, and phosphorus, and are a notable source of vitamin C (Hasan *et al.,* 2016).

The propagation of citrus trees in general, and kumquats in particular, only employs traditional methods and depends on the availability of suitable explants. Tissue culture offers an opportunity to produce genetically homogeneous and reliable plants for the intensive propagation of many woody plants, including citrus (Abd, 2022). Among the key factors influencing in vitro plant propagation are plant growth regulators (PGRs), which are vital in regulating growth and developmental processes. CPPU (forchlorfenuron), a synthetic cytokinin, is approximately 10 times more potent than benzyl adenine (BA) in promoting lateral shoot development and overcoming apical dominance (Heedchim *et al.,* 2021).

Putrescine (Put), a low-molecular-weight polyamine ($H_{12}N_2{\cdot}2HCl{\cdot}C_4$), acts as a precursor for other polyamines such as spermidine and spermine. It stimulates cell division and promotes cell proliferation in the callus. Moreover, it improves shoot formation, works synergistically with cytokinins such as BAP (6-Benzylaminopurine or BA), promotes root formation, improves tissue survival rate, and affects the balance of auxins and cytokinins within the medium (Ahmadabadi *et al.,* 2023). This study sought to evaluate the individual and combined effects of putrescine and CPPU on the in vitro growth and multiplication of kumquat shoots.

MATERIALS AND METHODS

The study took place in the Plant Tissue Culture Laboratory of the Department of Horticulture and Landscape Engineering, College of Agriculture, Al-Qasim Green University, from November 2022 to June 2023. The purpose of the study was to evaluate the effects of the growth regulators forchlorfenuron, or CPPU, and putrescine (Put) on the in vitro growth and multiplication of Kumquat (*Fortunella japonica*) shoots.

Sterilization of explants

The removal of shoots from the plant used a sharp surgical blade before cutting these into 2-cm-long explants containing internodes. These parts entailed placement in a 250 ml conical flask, followed by adding a few drops of liquid detergent (bright), and thereafter, washing thoroughly and placing under running water for an hour to remove dirt and surface contaminants. For surface sterilization, these parts continued their transfer to a stratified airflow chamber, where their sterilization ensued by immersion in a mercuric chloride ($HgCl_2$) solution for 5 min with continuous shaking. After the specified sterilization period, the explant incurred thorough washing with sterile distilled water three times for 3 min each time to remove any residual effect of the sterilizing agent in preparation for the culture stage.

The culture medium used was Murashige and Skoog (MS, 1962), prepared according to the manufacturer's instructions (Callsion, USA) at a concentration of 4.43 g/L. Further promoting growth succeeded in the medium supplemented with 3% (w/v) sucrose and 100 mg/L myo-inositol. In addition, it contained 2 mg/L benzyladenine (BA) and 0.1 mg/L naphthalene acetic acid (NAA).

The BA preparation proceeded by dissolving 100 mg in a few drops of absolute ethanol and bringing the volume up to 100 mL

with distilled water, with 2 mL/L of this stock added to the medium. NAA preparation was similarly, adding 0.1 ml/L. The medium reached solidification with 7 g/L of agar (Agar-Agar type), with the pH adjusted to 5.7 ± 0.1 using 1 N HCl or 1 N NaOH.

The cultures' incubation in a growth chamber had a 25 °C ± 2 °C temperature under 1000 lux light intensity, with a 16-hour photoperiod for four weeks. At the end of the incubation period, recording the following growth parameters included shoot multiplication rate, shoot length, the number of leaves, fresh weight, dry weight, and percentage of shoot survival.

Multiplication rate (number of shoots/explant)

Ten culture tubes received selection from each treatment for each rootstock. The number of shoots resulting from the cultured nodal segments reached being counted for each replicate (one tube). The total number of shoots in each treatment underwent summation and division by the number of replicates to obtain the average number of shoots per treatment.

The calculation of the average length of shoots/explant (cm) occurred by placing each shoot on graph paper and measuring their lengths. Summing up the shoot lengths for each replicate entailed dividing by the number of shoots to give the average shoot length per replicate. The average number of leaves/shoots sustained calculation by the number of leaves for each of the formed shoots before extracting the average number of leaves for each shoot. Fresh weight computation of shoots (mg) used a sensitive balance at the end of the experiment. Measuring the dry weight of shoots (mg) continued after drying the shoots formed from the end of the incubation period (taking 10 replicates) in an electric oven at 65 °C until the weight was constant. The percentage of implant survival calculation was according to the percentage of viable transplants at the end of the exposure period (four weeks) to different concentrations of putrescine and CPPU.

Experiment design and statistical analysis

The conducted study had a factorial experiment using a completely randomized design, according to Al-Rawi and Khalafallah (2000), with two factors. These were three concentrations of CPPU × four concentrations of Put, and with 10 replicates per treatment. The ready-made statistical software Genstat, under the Windows operating system, served to perform statistical analyses. The treatment means, as compared, used the least significant difference ($LSD_{0.05}$) test to determine the significance of differences among the means.

RESULTS

Multiplication average (number of shoots/explant)

The results indicate that increasing the concentration of CPPU caused a significant rise in the number of shoots per explant (Table 1). The concentration treatment of 0.8 mg L^{-1} recorded the highest multiplication average of 4.025 shoots/explant, which remarkably surpassed other concentrations, compared with the control treatment, which gave the lowest average of 2.887 shoots/explant.

The same table shows statistically significant differences exist in the number of shoots among the varying concentrations of Put added to the nutrient medium. The concentration of 5 mg L^{-1} gave the highest multiplication average of 4.200 shoots/explant, compared with the lowest number of shoots, at 2.333 shoots/explant. Regarding the interaction between CPPU and Put, one notes from the same table that the interaction treatment (0.8 mg L^{-1} CPPU × 5 mg L^{-1} Put) excelled and gave the maximum average of 4.900 shoots/explant. In contrast, the interaction treatment (0 mg L^{-1} CPPU × 0 mg L^{-1} Put) recorded the lowest average number of shoots, reaching 1.750 shoots/explant.

Table 1. Effect of the growth regulator Put and CPPU and their interaction on the average number of shoots of Kumquat plants after four weeks of cultivar in vitro.

CPPU concentration (mg L^{-1})	Put concentration (mg L^{-1})				CPPU means
	0	2.5	5	7.5	
0	1.750	2.450	3.500	3.850	2.887
0.4	2.450	2.800	4.200	3.850	3.325
0.8	2.800	3.850	4.900	4.550	4.025
Put means	2.333	3.033	4.200	4.083	
$LSD_{0.05}$	= Put			0.1418 = CPPU0.1637	
$LSD_{0.05}$	Interaction = 0.2835				

Table 2. Effect of growth regulator (CPPU) and PUT and their interaction on the average stem length of Kumquat plants after four weeks of culture in vitro.

CPPU concentration (mg L^{-1})	Put concentration (mg L^{-1})				CPPU means
	0	2.5	5	7.5	
0	0.550	0.990	1.430	1.650	1.155
0.4	0.880	1.320	1.760	1.980	1.485
0.8	1.100	2.090	2.420	2.750	2.090
Put means	0.843	1.467	1.870	2.127	
$LSD_{0.05}$	Put = 0.1300, CPPU = 0.1501				
$LSD_{0.05}$	Interaction = 0.2601				

Average length of shoots (cm)

The study findings revealed that CPPU significantly excelled in increasing the average shoot length with the rise in its concentration in the nutrient medium (Table 2). The highest value of this trait resulted in the concentration of 0.8 mg L^{-1}, reaching 2.090 cm, versus the control treatment (0 mg L^{-1}), which recorded 1.155 cm.

The results indicated significant differences among the concentrations of Put used in studying the average shoot length when grown on the multiplication medium. The concentration of 7.5 mg L^{-1} substantially surpassed the other concentrations, giving the longest average shoot length of 2.127 cm. In contrast, the control concentration (0 mg L^{-1}) recorded the shortest value of 0.843 cm.

As for the interaction between CPPU and Put added to the nutrient medium, the table indicates that the interaction treatment (0.8 mg L^{-1} CPPU × 7.5 mg L^{-1} Put) significantly surpassed all other interaction treatments, giving an average shoot length of 2.750 cm. On the other hand, the interaction treatment (0 mg L^{-1} CPPU × 0 mg L^{-1} Put) gave the lowest average shoot length of 0.550 cm.

Average number of leaves/explant

The results showed adding CPPU to the growth medium notably affected the increase in the number of leaves formed on the shoots (Table 3). The treatment with 0.8 mg L^{-1} gave the optimum average of the most leaves, amounting to 8.268 leaves/explant, while the control treatment (0 mg L^{-1}) recorded the lowest average of 5.911 leaves/explant.

The results further indicated there emerged differences between the concentrations of Put used to study the average number of leaves formed on shoots growing in the multiplication medium. The concentration of 7.5 mg L^{-1} significantly exceeded the rest by giving the highest average of 8.268 leaves/explant, while the control concentration (0 mg L^{-1}) gave the lowest average number of leaves at 4.100 leaves/explant.

The outcomes of the interaction between Put and CPPU concentrations in the same table indicate considerable differences appeared between the interaction treatments. The interaction of 0.8 mg L^{-1} CPPU × 7.5 mg L^{-1} Put gave the ultimate average number of leaves, reaching 9.533 leaves/explant, while the interaction of 0 mg L^{-1} CPPU × 0 mg L^{-1}

Table 3. Effect of the growth regulator Put and CPPU and their interaction on the leaf number of Kumquat plants after four weeks of culture in vitro.

CPPU concentration (mg L^{-1})	Put concentration (mg L^{-1})				CPPU means
	0	2.5	5	7.5	
0	4.100	5.740	6.458	6.765	5.766
0.4	6.150	6.765	7.790	8.508	7.303
0.8	7.483	8.508	9.225	9.533	8.687
Put means	5.911	7.004	7.824	8.268	
$LSD_{0.05}$	Put = 0.1522, CPPU = 0.1757				
$LSD_{0.05}$	Interaction = 0.3044				

Table 4. Effect of the growth regulator Put and CPPU and their interaction on the average fresh weight of Kumquat plants after four weeks of culture in vitro.

CPPU concentration (mg L^{-1})	Put concentration (mg L^{-1})				CPPU means
	0	2.5	5	7.5	
0	0.4800	0.6267	0.5867	0.6300	0.5808
0.4	0.8033	0.8233	0.8900	0.8700	0.8467
0.8	0.9167	0.9067	0.8833	0.8833	0.8975
Put means	0.7333	0.7856	0.7867	0.7944	
$LSD_{0.05}$	Put = 0.03621, CPPU = 0.4182				
$LSD_{0.05}$	Interaction = 0.07243				

Put recorded the least value of 4.100 leaves/explant.

Average fresh weight of multiplying shoots (mg)

The addition of CPPU in different concentrations to the multiplication medium significantly increased the average fresh weight of multiplied shoots (Table 4). The highest value was evident at 0.8 mg L^{-1}, reaching 0.8975 mg. In contrast, the control treatment (0 mg L^{-1}) gave the lowest fresh weight, amounting to 0.5808 mg.

The results showed noteworthy differences between the concentrations of Put in the average fresh weight of shoots. The concentration of 7.5 mg L^{-1} significantly surpassed the other concentrations by giving the highest average fresh weight of 0.7944 mg. Meanwhile, the control concentration (0 mg L^{-1}) gave the lowest average of 0.7333 mg.

The outcomes of the interaction between the two studied factors indicate the occurrence of significant differences in the average fresh weight. The interaction treatment of 0.8 mg L^{-1} CPPU × 2.5 mg L^{-1} Put remarkably surpassed all other treatments, giving the maximum average of 0.9067 mg. In contrast, the interaction of 0 mg L^{-1} CPPU × 0 mg L^{-1} Put recorded the minimum value of 0.4800 mg.

Average dry weight of multiplying shoots (mg)

The results revealed significant differences existed among various concentrations of CPPU added to the multiplication medium for the average dry weight of shoots (Table 5). The average dry weight increased with the rise in CPPU concentration, reaching the highest value of 0.2192 mg at 0.8 mg L^{-1}. The control treatment (0 mg L^{-1}) recorded the lowest dry weight at 0.1688 mg. Similarly, the concentrations of Put considerably affected the average dry weight. The concentration of 5 mg L^{-1} gave the ultimate average of 0.2144 mg, while the control treatment (0 mg L^{-1}) gave the least value of 0.1894 mg. The interaction results indicate meaningful variations appeared among the combinations. The treatment of 0.4 mg L^{-1} CPPU × 5 mg L^{-1} Put gave the maximum average dry weight of 0.2300 mg, while the minimum value of 0.1283 mg was evident in the interaction of 0 mg L^{-1} CPPU × 0 mg L^{-1} Put.

Table 5. Effect of the growth regulator Put and CPPU and their interaction on the average dry weight of Kumquat plants after four weeks of culture in vitro.

CPPU concentration (mg L^{-1})	Put concentration (mg L^{-1})				CPPU means
	0	2.5	5	7.5	
0	0.1283	0.1800	0.1850	0.1817	0.1688
0.4	0.2000	0.1967	0.2300	0.2017	0.2071
0.8	0.2400	0.2067	0.2283	0.2017	0.2192
Put means	0.1894	0.1944	0.2144	0.1950	
$LSD_{0.05}$	Put = 0.01242, CPPU = O.1434				
$LSD_{0.05}$	Interaction =0.2484.				

Table 6. Effect of the growth regulator Put and CPPU and their interaction on the transplant survival (%) of Kumquat plants after four weeks of culture in vitro.

CPPU concentration (mg L^{-1})	Put concentration (mg L^{-1})				CPPU means
	0	2.5	5	7.5	
0	56.7	60.0	66.7	70.0	63.3
0.4	66.7	73.3	76.7	86.7	75.8
0.8	80.0	90.0	96.7	100.0	91.7
Put means	67.8	74.4	80.0	85.6	
$LSD_{0.05}$	Put = 5.06, CPPU = 5.85				
$LSD_{0.05}$	interaction10.13 =				

Percentage of implant survival (%)

The study findings state the concentrations of Put added to the nutrient medium significantly influenced the percentage of plant survival (Table 6). The concentration of 7.5 mg L^{-1} gave the highest survival rate of 85.6%, with the lowest percentage recorded by the control treatment at 67.8%. Regarding the effect of CPPU concentrations on survival percentage, the results showed a clear trend of increased survival with higher CPPU levels. The highest survival rate of 91.7% manifested at 0.8 mg L^{-1}, while the lowest rate of 63.3% was evident in the control treatment. The interaction between Put and CPPU also had a notable effect. The combination of 0.8 mg L^{-1} CPPU × 7.5 mg L^{-1} Put resulted in the maximum survival percentage of 100%. In contrast, the lowest percentage of survival (56.7%) appeared in the control interaction (0 mg L^{-1} CPPU × 0 mg L^{-1} Put).

DISCUSSION

The distinct role of putrescine in the process of cell division clearly increased the number of cells in biologically active regions (Cohen, 1998). The effect may also be due to the role of putrescine in enhancing cytokinin accumulation in plant tissues. The effect may also be due to the role of putrescine in enhancing cytokinin accumulation in plant tissues. as cytokinin contributes to reducing apical dominance and promoting the growth of lateral shoots (Table 1). Additionally, putrescine may increase potassium accumulation and enhance the concentrations of growth regulators. Potassium is essential for the activation of a wide range of enzymes, including kinases, which stimulate protein and nucleic acid synthesis. This, in turn, enhances cell division processes, particularly in meristematic tissues. These effects may collectively contribute to the emergence and proliferation of shoots (Mengel, 2007).

Putrescine also plays a role in increasing the levels of auxins and cytokinins, thereby positively affecting various growth parameters (both nutritional and vegetative). This results in an improved survival rate of cultured plants, as indicated in Table 6. Regarding the fresh weight of the living biomass, this may be due to putrescine's role in protecting cell membranes, thus reducing water loss (Yamaguchi and Blumwald, 2005; Minguet *et al.,* 2008).

The observed increase in most vegetative traits (Tables 1, 2, 3, 4, and 5) can refer to the positive effects of cytokinins. These effects include their efficiency in breaking apical dominance and stimulating lateral shoot development, which leads to an increase in the number of shoots (Werner *et* al., 2003 , Sharma *et al.,* 2009). Additionally, cytokinins promote cell division, differentiation, and the formation of a nutrient-attracting center in lateral shoots. This facilitates nutrient translocation, thereby accelerating growth and boosting shoot density (Taiz and Zeiger, 2010). The elongation of shoots observed (Table 2) after the addition of cytokinin (BAP) may be ascribable to its role in stimulating both cell division and elongation, as well as its influence on nucleic acid biosynthesis (Iqbal *et al.,* 2017). The increase in the average number of leaves (Table 3) and branch length (Table 2) is also due to the stimulating effect of cytokinins on cellular division and differentiation, leading to the development of more organized shoot structures.

Several researchers have confirmed the importance of cytokinin concentrations in tissue culture systems, including Hasan and Khasim (2015), Miilion *et al.* (2015), and Hossain *et al.* (2016). The findings of this study are in agreement with those of Salman and Khairy (2016), as well as Ahmed and Salem (2020), who reported that cytokinins significantly increased the number of leaves and branch lengths in Stromello rootstock compared with other concentrations. These results are also consistent with Hamza (2011), who observed a substantial increase in the number of leaves at low cytokinin concentrations.

The positive results in fresh weight (Table 4) and dry weight (Table 5) following CPPU application are due to the role of cytokinins in enhancing water movement within growing shoots and nutrient translocation. Cytokinins stimulate cell division and proliferation, which increases carbohydrate accumulation, contributing to an elevation in both fresh and dry shoot biomass (Tan *et al.,* 2007; Dabrowski *et al.,* 2015). These agreed with what Naif (2013) found, as the addition of cytokinin at different concentrations to the watertight medium resulted in a significant increase in the fresh and dry weights of the growths formed from five citrus roots, including *Citrumelo swingle*. Likewise, this study's results agreed with what Naji (2013) obtained. He found that adding cytokinin at different concentrations to the medium led to a notable rise in the fresh and dry weights of growths emerging from two-node microcultivation of some citrus roots**.**

Safana *et al.* (2022) investigated the effect of adding NAA auxin at concentrations of 1, 2, and 3 mg/L and chitosan at concentrations of 10, 15, 20, and 25 mg/L to the MS culture medium. This sought to determine the optimal concentration for rooting kumquat (*Citrus japonica*) shoots propagated from stem nodule biogenesis. The results showed culturing kumquat shoots in the MS medium supplemented with 2.0 mg/L NAA resulted in a statistically significant high rooting percentage (92.0%) and number of roots (3.20 roots per plant). Furthermore, outcomes showed adding 15 mg/L chitosan to the culture medium prepared with 2.0 mg/L NAA led to a notable rise in the same parameters (80% rooting percentage and 5.60 roots).

CONCLUSIONS

In *Citrus japonica*, the individual higher doses of Put and CPPU showed the best performance in terms of shoots/explant, shoot length, leaves/explant, fresh weight, dry weight, and survival. The interaction between CPPU (0.8 mg L^{-1}) and Put (7.5 mg L^{-1}) concentrations recorded the highest average stem length (2.750 cm), leaf number (9.533 leaves/explant), and survival percentage (100.0%) in *Citrus japonica*.

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