



RESEARCH ARTICLE

Morpho-physiology of sour orange seedling treated with melatonin under in vitro water stress

Suha Mohamed Naser^{1*}, Abrar M Almousawi¹, Dhurgham B Naji² & Wael Adnan Sahib Aljefry¹

¹Department of Horticulture and Landscape, University of Kufa, Najaf 964, Iraq

²Administrator of Kerbala Education, Occupational Instruction, Kerbala 964, Iraq

*Correspondence email - suham.alasede@uokufa.edu.iq

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Abstract

The study was conducted in the Micropropagation laboratory, Faculty of Agriculture, University of Kufa, from November 2023 to March 2024 to study the *in vitro* effects of Polyethylene glycol (PEG) in sour orange (*Citrus aurantium*) seed germination and seedlings growth using growth chamber seed culture. Mature sour orange seeds were cultured on Murashige and Skoog medium supplemented PEG 6000 at concentrations of 0, 0.5, or 1 % and 3 concentrations of melatonin (0, 0.5 or 1 mg L⁻¹) for 45 days to study water stress effect on seed germination percentage, seedling length, root length, fresh weight and dry weight. Results showed that water stress adversely affected these traits: seed germination percentage, seedling height, fresh and dry weight and root length, alongside the increase in PEG concentration compared to PEG 6000 free medium after 45 days of the seed culture. The nutrient medium supplemented with melatonin reduced the negative effect of water stress as it improved seed germination rate, seedling height, shoot fresh and dry weight and root length, as the concentration exceeded 1 mg L⁻¹. All the study indicators were significantly affected by the interaction treatments of PEG and melatonin, especially in the absence of PEG. Particularly, the interaction of 0 % PEG plus 1 mg L⁻¹ melatonin produced the highest values in all plant growth traits studied, while the interaction treatment at 1 % PEG with 1 mg L⁻¹ melatonin gave lower values in the traits abovementioned.

Keywords: botanical; citrus; *in vitro*; plant hormone; sapling; water stress

Introduction

Citrus cultivation in Iraq faces many problems, especially in the central and southern regions, due to the irrigation water shortage. Water shortage is a real issue encountered by many countries of the world that face agricultural expansion and the impact of water stress on the plants is negatively reflected on the production (1, 2). Sour orange (*Citrus aurantium* L.) is one of the most important assets that can be grafted on various types of citrus, due to the availability of its seeds in large quantities and because of its compatibility with the commercial varieties of citrus (3). It is also a significant and suitable asset in lands with medium and heavy soil texture (4). Such varieties can also tolerate high soil moisture and inappropriate environmental conditions and resist gummosis disease caused by high groundwater and some fungal pathogen infection (5). The grafted trees are characterized by being of high yield and fruits of excellent quality (6).

Scientific studies have shown that citrus species and varieties vary in their tolerance to different environmental stresses, including drought and water deficiency (7). Hence, many environmental stress issues can be solved by selecting tolerant plant varieties and using stress relievers to overcome the problem of lack of irrigation water prevailing in dry countries of the world, including Iraq (8-10).

One of these strategies is using tissue culture techniques as an important tool for provision amid homogeneous growth in terms of nutritional content, controlled environmental conditions and not restricted to a large space or specific time used in the study of tolerance to various environmental stresses, including water stress (11).

It has been found in earlier studies that PEG is an effective material for inducing water stress in plants *in vivo* (12). PEG can be added to the growth media at an appropriate concentration to reduce water hydration activities important to seed absorption and germination (13). Thus, the nutrient medium treated with PEG can weaken the ability of plants to absorb water to a level that represents natural drought conditions (14).

In the past few years, the attention of researchers has been directed to studying the role of the hormone melatonin in reducing water stress in many plants (15). In view of the limited studies related to evaluating the germination of sour orange (*Citrus aurantium*) seeds in Iraqi drought, this issue can be studied using the tissue culture technique by enhancing the nutrient medium with melatonin. Therefore, this research was conducted to study the physiological effects of water stress induced by PEG in the presence of melatonin on (*Citrus aurantium*) seed germination and seedling growth.

Materials and Methods

The research was conducted in the laboratories of the Date Propagation Unit in tissue culture at the Faculty of Agriculture, University of Kufa and included studying the effect of water stress and enhancing the nutrient medium with melatonin on seedling growth resulting from the cultivation of Sour orange seeds *in vitro*, as they were obtained from local markets. Ripe seeds were taken from the ripe fruits directly and sterilized after planting in planting containers according to the following steps:

Preparing seeds

The seeds were taken and washed with running water and then sterilized in the Laminar air flow cabinet by dipping in 96 % ethanol for 1 min and then immersed in a 2.5 % sodium hypochlorite solution (NaOCl) for 15 min with continuous stirring. Then, the seeds were washed three times with sterile distilled water for 3-5 min each time to remove the residual effect of the sterile material.

Preparing the nutrient medium (MS)

The Murashige and Skoog (MS) medium, ready (made by the American company Caisson), was used at the rate of 4.4 g L⁻¹, adding 30 g L⁻¹ sucrose. Afterward, PEG 6000 was added at concentrations of 0.0, 0.5, or 1.0 % in combination with melatonin at concentrations of 0.0, 0.5, or 1.0 mg L⁻¹. The acidity of the medium was adjusted to 5.7 using HCl₁ OR NaOH₁ standard solution. Agar was used for medium hardening at 7 g L⁻¹ and then the medium was distributed in the cultivation vials by 10 mL per bottle. The empty vials were closed and autoclaved (120 °C and a pressure of 1.04 kg/cm² for 20 min) for sterilization, then cooled to room temperature to be ready for the treatment combinations prepared.

Planting seeds in vials

A single seed was placed in each vial and 10 replicates (10 vials) were conducted on each treatment. The prepared vials were placed in the growing room for 45 days at 25±°C and a luminous intensity of 1000 lux using standard fluorescent lamps for 16 hrs day⁻¹ positioned above the culture containers, typically on shelving units, to ensure uniform light distribution across all vials.

The studied indicators

At the end of the incubation period, three replications (plants) were included in the study due to resource constraints (e.g., cost, time, or labor associated with biochemical assays), while ensuring these samples were representative of the overall population. Some traits were determined likewise; seed germination rate, seedling height (cm) using a ruler, from the surface of the nutrient medium to the top of the seedling, number of leaves, main root length (cm), seedling fresh and dry weight (mg) after being dried in electric oven at 65 °C and until weight was fixed.

Experimental design and data analysis

The experimental treatments included all possible interactions and were distributed for the two factors to nine treatments with three replications in a Randomized Complete Block Design (RCBD), six seedlings per experimental unit (16). The experiment data were collected and subjected to data analysis, where analysis of variance ANOVA was performed using the computing statistical program GenStat 12th (17). Differences among the treatments' means were compared according to the least significant difference (LSD) at a probability level of 0.05.

Results

Seed germination rate

The results indicate a significant difference between the PEG concentrations added to MS medium in seed germination ratio (Table 1). The germination percentage decreased with the increasing PEG in the medium, where the highest concentration (1 %) resulted in the least germination rate (53.33 %) after 45 days post-planting DPP, compared to the highest germination rate (70 %) in the control. However, an increase in melatonin concentration in the medium resulted in a higher seed germination rate, as the concentration exceeded 1 mg L⁻¹, resulting in a higher rate of seed germination, 83.33 %. The same Table 1 showed that interaction of 0 % or 0.5 % PEG and 1 mg L⁻¹ melatonin resulted in the highest germination of 90 % compared to a 40 % germination rate when using 0.5 or 1 % PEG in the absence of melatonin.

Table 1. Effect of PEG 6000 and melatonin concentrations and their interactions on sour orange seed germination rate (%) after 45 days of *in vitro* cultivation

PEG %	Melatonin mg L ⁻¹			Average
	0	0.5	1.0	
0	50	70	90	70.00
0.5	40	60	90	63.33
1.0	40	50	70	53.33
Average	43.33	60.00	83.33	
	PEG=15.01			
LSD <i>p</i> (0.05)	Melatonin = 15.01			
	Interaction=30.13			

*Values are means of three replications

Seedling height (cm)

In case of seedlings length (Table 2), the findings showed that PEG had also negative effect on seedlings height, as the lowest seedlings height, 1.78 cm, was recorded at 1 % concentration of PEG in comparison with treatment 0 % which resulted in 4.32 cm (Fig. 1,a). Unlike the PEG effect, melatonin increased concentration, especially up to 1 mg L⁻¹, gave the highest seedlings height of 4.52 cm compared to 2.01 cm in the (melatonin absence) control (Fig. 1 b). It was also shown that interaction treatment of 0 % and 0.5 %

Table 2. Effect of PEG 6000 and melatonin concentrations and their interactions on sour orange seedlings height (cm) after 45 days of *in vitro* cultivation

PEG %	Melatonin mg. L ⁻¹			Average
	0	0.5	1.0	
0	3.17	4.47	5.53	4.32
0.5	2.40	3.05	5.03	3.49
1.0	0.45	1.90	3.00	1.78
Average	2.01	3.14	4.52	
	PEG= 0.121			
LSD at <i>p</i> (0.05)	Melatonin = 0.121			
	Interaction=0.209			

*Values are means of three replications

PEG and 1 mg L⁻¹ melatonin resulted in seedling length of 5.53 and 5.03 cm, respectively, compared to the lowest seedling height (0.45 cm) when using 1 % PEG with 0 mg L⁻¹ melatonin (Fig. 1c).

Number of leaves

Findings (Table 3) showed that medium treated with PEG has significantly reduced the number of leaves, which decreased as the PEG concentration increased. The lowest number of leaves (3) was in the medium treated with 1 % PEG, compared to 6.03

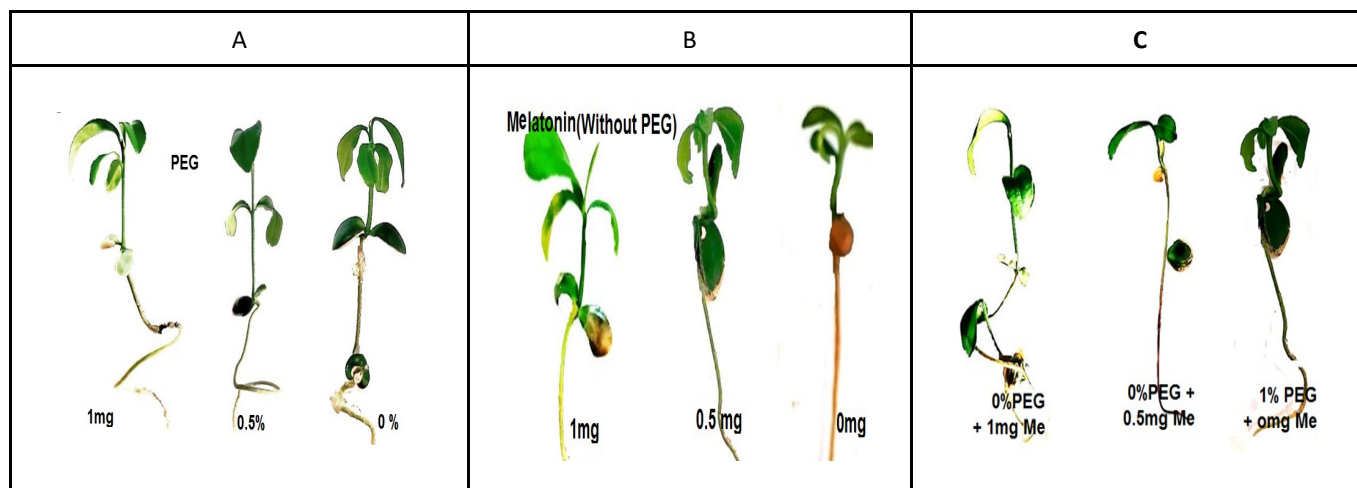


Fig. 1. Effect of the experimental factors PEG (A), melatonin (B) and interaction (C) on sour orange seedling growth parameters.

Table 3. Effect of PEG 6000 and melatonin concentrations and their interactions on sour orange leaf number after 45 days *in vitro*

PEG %	Melatonin mg L ⁻¹			Average
	0	0.5	1.0	
0	4.33	6.77	7.00	6.03
0.5	3.00	5.00	5.33	4.44
1.0	2.00	2.50	4.50	3.00
Average	3.11	4.76	5.61	
LSD at <i>p</i> (0.05)				
PEG= 1.32 Melatonin = 1.32 Interaction=2.67				

*Values are means of three replications

leaves seedling¹ in the untreated control on the initiation stage. On the other hand, melatonin increased the number of leaves significantly as concentrations increased compared to the untreated control. The highest number of leaves was recorded in the interaction treatment of 1 mg L⁻¹ melatonin and 0 % PEG, which gave 7 leaves, while the lowest value was when using 1 % PEG in the absence of melatonin (2 leaf plant⁻¹).

Root length

It is noted from Table 4 results that seedlings' root length was also negatively affected by increased PEG concentrations added to the nutrient medium. The 1 % PEG at 45 DPP resulted in a root length of 2.89 cm compared to the longest root achieved in the comparison treatment (0 % PEG), reaching 4.99 cm. Similarly, the results indicate an increase in root length in seedlings grown on melatonin-treated medium. The dose of 1 mg L⁻¹ melatonin had significantly higher root length up to 4.99 cm, while untreated medium gave the lowest root length of 3.22 cm. At 45 DPP, the highest seedling's root length of 6.83 cm was recorded in medium treated with 0 % PEG and 1 mg L⁻¹ melatonin, with

Table 4. Effect of PEG 6000 and melatonin concentrations and their interactions on sour orange root length (cm) after 45 days of *in vitro* cultivation

PEG %	Melatonin mg L ⁻¹			Average
	0	0.5	1.0	
0	3.66	4.49	6.83	4.99
0.5	3.33	4.00	4.28	3.87
1.0	2.67	2.16	3.85	2.89
Average	3.22	3.55	4.99	
LSD at <i>p</i> (0.05)				
PEG= 0.421 Melatonin= 0.421 Interaction=0.839				

*Values are means of three replications

significant difference from the lowest root length 2.16 cm in the interaction treatment of 1 % PEG and 0.5 mg L⁻¹ melatonin.

Fresh weight

The results in Table 5 showed that applying PEG to the nutrient medium significantly reduced the average fresh weight after 45 days of planting seeds and the fresh weight decreased with increasing the concentrations of PEG where the lowest value was at concentration of 1 % PEG which gave 243 mg, while the highest fresh weight 402 mg was in the untreated control. However, melatonin applied to the medium increased plant fresh weight as the concentration increased. The 1 mg L⁻¹ significantly gave the highest average fresh weight of 384 mg compared with the lowest

Table 5. Effect of PEG 6000 and melatonin concentrations and their interactions on sour orange seedling fresh weight (mg) after 45 days of *in vitro* cultivation

PEG %	Melatonin mg. L ⁻¹			Average
	0	0.5	1.0	
0	349	408	449	402
0.5	274	334	387	332
1.0	157	228	317	243
Average	260	323	384	
LSD at <i>p</i> (0.05)				
PEG= 121.01 Melatonin = 121.01 Interaction=239.11				

*Values are means of three replications

rate of fresh weight of 260 mg for the concentration of 1 mg L⁻¹. The interaction of PEG and melatonin (Table 5) indicates significant differences among treatments for the plant fresh weight.

Dry weight

The results in Table 6 indicate a significant difference between the different concentrations of PEG added to the MS medium in

Table 6. Effect of PEG 6000 and melatonin concentrations and their interactions on sour orange seedling dry weight (mg) after 45 days of *in vitro* cultivation

PEG %	Melatonin mg L ⁻¹			Average
	0	0.5	1.0	
0	58.16	66.00	74.33	66.16
0.5	45.67	61.49	60.83	56.00
1.0	30.67	56.16	58.85	48.65
Average	44.8	61.22	64.67	
LSD at <i>p</i> (0.05)				
PEG= 8.12 Melatonin= 8.12 Interaction=16.23				

*Values are means of three replications

the average dry weight. The table indicates that the average dry weight of the seedling decreased with the increase in the concentration of PEG added to the nutrient medium until it reached the lowest rate at the highest concentration of PEG 1 % to yield 48.65 mg. The highest dry weight rate was recorded when the comparison was treated, which gave 66.16 mg. The same table shows that there is a significant difference between the concentrations of melatonin in the average dry weight of the seedlings, as the concentration exceeds mg L^{-1} significantly by giving it the highest dry weight rate of 64.67 mg against (44.8 mg) for the concentration of 0 mg L^{-1} . The results of the overlap between PEG concentrations and melatonin concentrations contained in Table 6 indicate significant differences in the rate of dry weight, as the concentration overlap treatment of 0 % PEG with 1 mg L^{-1} melatonin significantly exceeded 74.33 mg while the lowest rate for dry weight at interference treatment 0 mg L^{-1} melatonin with a concentration of 1 % PEG of 30.67 mg.

Discussion

Water stress disrupts both physiological and biochemical processes in plants, resulting in decreased plant growth (18). In the current research, water stress significantly reduced the parameters of seed germination and seedling development in orange. Any reduction in water absorption, seeds or plants, possibly impairs the vital bioactivities of the plant represented by the essential metabolic processes during germination, which crucially require water for enzymatic reactions, nutrient balance and cellular elongation. Water deficit stress likely compromises vital activities associated with seeds or seedlings' growth, which negatively reflects early plant development, as evidenced by the observed reduction in sour orange indicators (19). Furthermore, water deficit stimulates shifting in the cellular hormonal balance towards the synthesis and accumulation of growth inhibitors such as abscisic acid (ABA) and phenols. Besides, the levels of hormonal growth stimuli such as auxins and gibberellins, responsible for cell division and elongation, tend to increase. In contrast, under stress conditions, imbalanced cellular hormones trigger inhibited shoot elongation and reduced overall seedling vigor, which are well-established physiological indicators of drought stress in plants (20).

The observed decrease in shoot dry weight of seedlings under water stress situations revealed the negative effect of water shortage on vital physiochemical activities in plants. Consequently, water stress disrupts cell homeostasis, impedes water uptake and confines cellular turgor pressure, all of which are critical for cell expansion and biomass accumulation and subsequently decline stored metabolites (21). Nevertheless, the decrease in dry weight may be ascribed to the degradation of carbohydrate synthesis, protein accumulation and other essential storage compounds essential for plant growth when the plant is exposed to water stress (22). In addition, water scarcity hinders the mobility, solubility and absorption of nutrients from the root zone, thereby further limiting plant growth and development (23). This complex disturbance emphasizes how vulnerable early seedling growth stages are to drought and how crucial it is to reduce water stress to maintain biomass production.

The response to passive root growth under water stress is mostly due to carbohydrate transfer to the roots under acute water stress, where root growth indicates the extent of

adaptability to the lack of water in the plant (24). However, the incorporation of melatonin into the nutrient medium remarkably improved seedling tolerance to water stress, especially at sufficient concentrations, by mitigating the severity of drought-induced growth inhibition. Sour orange plants grown in a melatonin-supplemented medium presented substantial increments in crucial growth parameters, including higher plant height, leaf count and fresh and dry weight. These improvements may be attributed to melatonin's function in boosting plant growth by modulating a wide array of biophysiological processes, including hormonal signaling pathways, osmotic regulation and antioxidant defense (25). Additionally, it mitigates stress by altering the internal nutritional balance in plant tissues, thus supporting better growth performance in situations where water supply is limited (23).

Future studies could benefit from comparison analyses involving exogenous bioactive chemicals such as GABA, potassium phosphite, flavonoids and phenolics, which are also known to influence seed germination and early seedling development under stress circumstances, even though this study focused solely on melatonin effects.

Conclusion

We conclude that water stress conditions in the micropropagation medium can be successfully performed using PEG, which has negatively affected citrus seed germination and growth of resulting seedlings. On the other hand, the addition of melatonin to the growing medium, in the presence of PEG, reduced plant stress mediated by salinity and had a positive effect on seed germination rate and seedling growth. Based on the findings of this study, planting citrus seeds through tissue culture with a melatonin-treated medium could be an effective technique to alleviate water stress damage.

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Authors' contributions

SMN established the research idea, selected the experimental factors and interpreted the results. AMA carried out the laboratory tests and wrote the research in general. DBN participated in experimental design and performing data analysis. WASA, carried out the laboratory work related to data collection and revising paper references. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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