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In vitro Propagation of *Citrus aurantifolia* and its Conservation in Pakistan

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Abstract

Citrus varieties are propagated by both asexual and sexual methods. Generally, rootstocks are propagated sexually through seeds, while most of the commercial varieties are propagated by various asexual methods such as grafting and budding. The technology called plant tissue culture is used for commercial production of plants that are microbe and virus free and to save germplasm of infrequent or threatened species of plants. Fast reproduction techniques have the potential to produce plants free of viruses. Likewise, traditional propagation methods are old, time consuming and are difficult. Tissue culture techniques are assumed for reliable commercial production of economically important plants. In present study shoot tips of Citrus aurantifolia were collected from the field of BCI, NARC. Different disinfectant (Ethanol and NaOCl) with the concentration ranging from 10 to 20 percent were used for culture establishment. Maximum survival percentage was observed in 10% NaOCl. After 5 weeks, established cultures were shifted to hormonal growth media (BAP, GA3 and NAA) at the concentration ranging from 0.1 to 0.8 mg/L. Our finding results that maximum phenotypic characters (plant height, number of leaves and number of shoots were observed on BAP at the concentration of 0.1 to 0.3 mg/L, while maximum number of roots were observed on NAA at the concentration of 0.5mg/L. In present study, for in vitro conservation different conservants like sorbitol and mannitol (10, 20 and 30 g/L) were used. Incubation of cultures was maintained at 21°C under the light intensity of 1000 lux. Old cultures of Citrus aurantifolia were shifted in conservant media. Overall, remarkable difference was observed. In present study mannitol at the concentration of (10, 20 and 30 g/L) showed slow growth after 35 days. In future the plants in conservation media will be used for further propagation and for growing them in fields.

Keywords: *Citrus aurantifolia*, in vitro propagation, tissue culture, conservation, growth regulators, Pakistan

Introduction

The genus citrus belongs to family called Rutaceae and it includes 1300 species and 140 genera. There are few important fruits of genus citrus that includes *Citrus Paradis*, *Citrus Sinensis*, *Citrus Limon*, *Citrus Grandis*, *Citrus Aurantium*, *Citrus Medica*, *Citrus Reticulate* And *Citrus aurantifolia* [1,2]. *Citrus aurantifolia* (Christm) grows in hot subtropical or tropical region areas such as Southern Florida, Mexico, India, Egypt and the West Indies [3]. The plant has its place with Kingdom Plantae, Phylum: Magnoliophyta, Class: Mangoliopsida, Order: Sapindales, Family: Rutaceae, Genus: Citrus, Specie: *Citrus aurantifolia* [4].

Citrus aurantifolia is little shrubby tree, and its height is about 5m in length. The flower size is about one inch in diameter and color of flowers are white, yellow with a light purple tip on the margin. The fruits of *Citrus aurantifolia* are globular to ovate berry that is about 3 to 6cm in diameter and often have apical papilla [5]. Citrus fruits are cultivated in Pakistan on an land ranging from 192831 hectares with a annual production of 2395560 tones. Possible generation of citrus is 18-20 tons per hectare with an average production of 12.78 tons per hectare [6].

Citrus varieties are propagated by both asexual and sexual methods. Mostly, rootstocks are propagated sexually through seeds, many commercial types are grown via asexual techniques, and one significant asexual technique is micropropagation, which is utilized to create rootstock plants that are virus-free [7]. The root system of citrus is shallow, they are planted in teachers or into arranged and cracked rocky soil and give the roots a well support and

improve the trees to stand in high wind [8]. Over the traditional methods of propagation through seeds, cutting grafting, air layering, etc., commercial plant production using the micropropagation approach has many advantages. The generation of plants free of viruses can be achieved by rapid plant propagation [9,10].

Studying the impact of chemical disinfectants, growth regulators, and conservants (sorbitol and mannitol) on in vitro *Citrus aurantifolia* propagation was the goal of this research.

Material and Methods

The following procedures were followed during the in vitro propagation of the *C. aurantifolia* plant at National Agricultural Research Centre Pakistan. Explant of *C. aurantifolia* was collected from the field of NARC. The shoot tips of plant were cut 4-5 cm each shoots size. Explant preparation was done by removing all the leaves present on shoots, remaining part of the excised shoot was further cut into different segments, 2 cm each. The explants segments were then surface sterilized via adding a pinch of detergent and two drops of tween-20 (1 drop for 20 ml) and then shake for 2 to 4 minutes. After that explants segment was put under running tap water for 30-45 minutes.

The common principle media used in tissue culture lab is the MS Media (Murashige and Skoog). Four treatments were used, To was control of MS media (without any hormonal concentration), while T1, T2 and T3 were hormones used in propagation as shown in table 1 and it was further mixed in MS media before adding agar.

Table 1: Hormones and chemicals concentration

To	T1	T2	T3
MS	**BAP**	**GA3**	**NAA**
MS	0.1mg/L	0.1mg/L	0.5mg/L
MS	0.2mg/L	0.3mg/L	1mg /L
MS	0.4mg/L	0.5mg/L	1.5mg/L
MS	0.6mg/L	1mg /L	
MS	0.8mg/L		

The experiment of conservation was conducted to conserve the explants of C.aurantifolia in conservation media for the purpose of slow growth and longtime storage. Three treatments were used, To was control of MS media (without any conserving chemical), while T1 (Sorbitol) and T2 (Mannitol) were chemicals used for slow growth conservation as shown in (Table 2). The experiment of conservation was conducted on lowest temperature 21°C under the light intensity of 1000 lux.

The explants prepared from the shoot tips of *C. aurantifolia* were again aseptically cut before placing in the culture medium (MS media). Test tubes containing 10ml of solid MS media were used for this purpose. Explants segments were cultured into the medium by using forceps under aseptic conditions. The experiment was performed under optimized conditions of light and temperature. The culture was incubated at 25°C under 16 hour's photoperiod (2,000 lux) with white florescent tube for 3-4 weeks. Established plants after 4 weeks were further sub cultured on MS media with different hormonal treatments. After 2 to 5 weeks morphological data were recorded.

Table 2: Conservation media and their chemical concentration

To	T1	T2
MS	Sorbitol	Mannitol
MS	10g/L	10g/L
MS	20g/L	20g/L
MS	30g/L	30g/L

Results

In vitro culture establishment was accompanied by using chemical disinfectants (Ethanol and NaOCl) with variant concentrations, i.e., 10% ethanol and 90% distilled water for 15 mintues, 15% ethanol and 85% distilled water for 15 minutes, 20% ethanol and 80% distilled water and 10% NaOCl and 90% distilled water, 20% NaOCl and 80% distilled water were applied to the explants for 15 minutes respectively. Results showed that maximum survival rate (100%) was obtained with 10% Clorox NaOCl and (98%) survival rate was obtained from 10% Ethanol. Lowest survival rate was given by 20% NaOCl survival rate. Observations showed that increasing concentration of Ethanol and NaOCl may adversely affect the plant (Table 3).

Table 3: Optimization for Ethanol and NaOCl concentration for in vitro culture establishment of *C. aurantifolia*

Disinfectant Concentration	Total No. of Plants	No. of Plants Survived	Survival%	Fungal Contamination	Bacterial Contamination
10% Ethanol	50	49	98	0	1
15% Ethanol	30	24	80	2	4
20% Ethanol	20	12	60	6	2
10% NaOCl	50	50	100	0	0
20% NaOCl	20	10	50	8	2

Following morphological parameters (Plant Height, Number of leaves, shoots and roots) were studied. The explants were cultured under sterile conditions in MS media with different hormonal concentrations separately. With the help of measuring scale, the height of each plant was recorded with weekly intervals of time. Our results show that *C. aurantifolia* shown maximum plant height of (3.9 cm and 2.5 cm) with BAP and GA3 at the concentration of (0.1mg/L to 0.2mg/L.) as compared to NAA (0.5 cm) at the concentration of 0.5ml/L and MS control media. Our finding results that lower concentration of hormones BAP and GA3 (0.1 to 0.2mg/L), have great effect on plant height as compared to the higher concentration of these hormones (0.4 mg to 0.8mg/L). The MS control media does not show any effect on plant height. Five weeks old cultures were shifted in

conservants with different treatments (Figure 1).

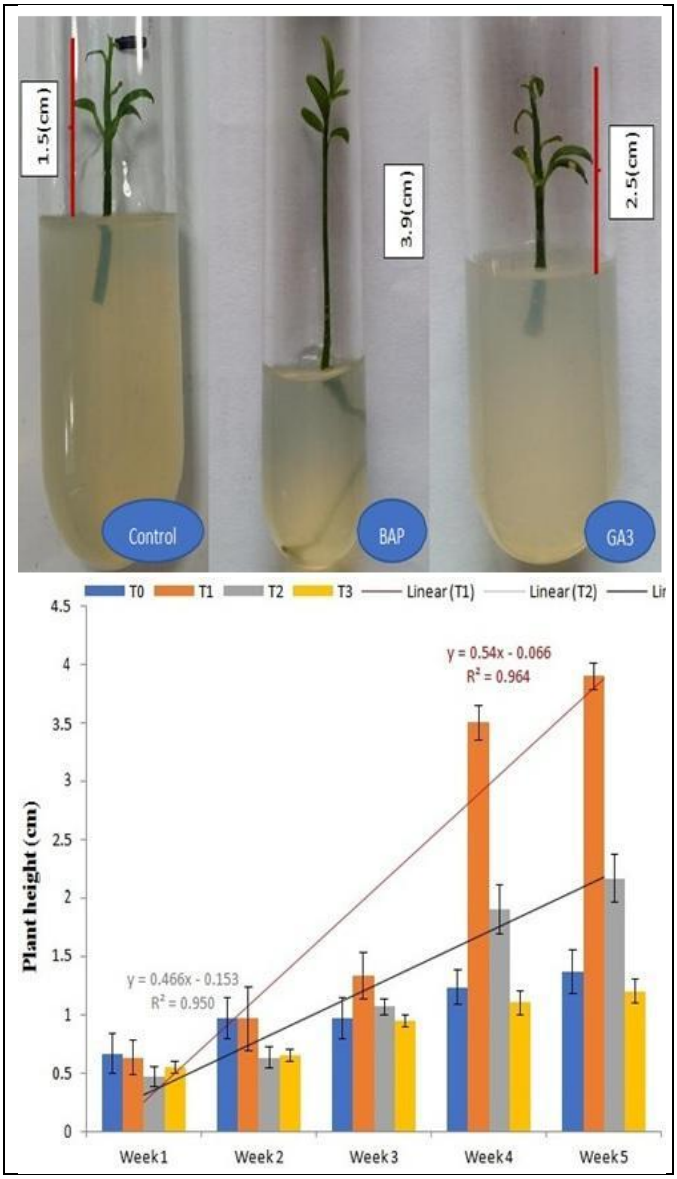


Figure 1: Effect of growth regulators on plant height of *C. aurantifolia* after regular intervals of 5 weeks.

The number of leaves of each plant was recorded with weekly intervals of time. Our result showed that maximum number of leaves (6 to 4) were observed in BAP and GA3 at the concentration of (0.1mg/L) as compared to NAA (0.5mg/L) having (3) number of leaves and MS control media with (2) number of leaves. Our finding results that lower concentration of hormones BAP and GA3 (0.1mg/L) and NAA (0.5mg/L) have great effect on plant height as compared to the higher concentration of these hormones (0.2 to 1.5mg/L). Whereas the MS control media does not show any effect on the number of leaves of *C. aurantifolia* (Figure 2).

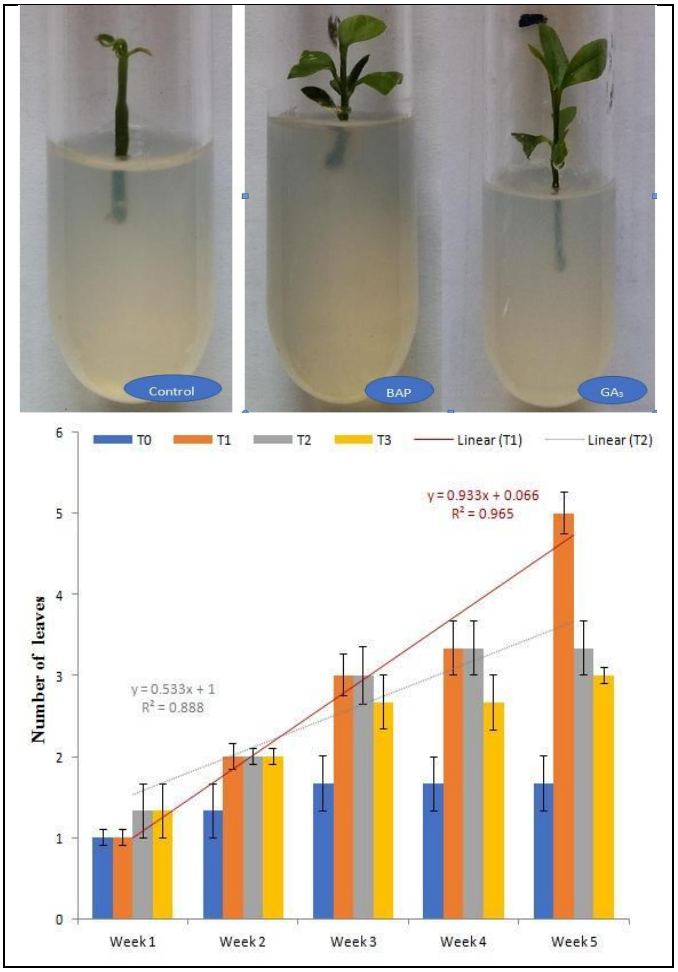


Figure 2: Effect of growth regulators on number of leaves of *C. aurantifolia* after regular intervals of 5 weeks.

The number of shoots of each plant was recorded with weekly intervals of time. Our result showed that highest number of shoots (2 to 4) was found with BAP at the concentration of (0.1mg/L) and NAA at the concentration of (1mg/L). While GA3 and MS control media does not show any effect on shoots number and remain with only one number of shoot which was cultured in them. Our finding results that lower concentration of hormones BAP (0.1mg/L) and NAA 1mg/L have great effect on shoots numbers as compared to the higher concentration of these hormones (0.4 to 1.5mg/L) (Figure 3).

The number of roots of each plant was recorded with weekly intervals of time. Our result showed that highest number of roots (2 to 4) was found with NAA at the concentration of (0.5mg/L)). While BAP, GA3 and MS control media does not show any effect on roots number. Our finding results that lower concentration of NAA (0.5mg/L) have great effect on roots numbers as compared to the higher concentration of NAA (1.5mg/L) (Figure 4).

Five weeks old cultures were used for conservation studies and different concentration of sorbitol (10, 20 and 30 g/L) and mannitol (10, 20 and 30 g/L) were used. Incubation of cultures was maintained at 21°C under the

light intensity of 1000 lux. All the phenotypic characters recorded (number of shoots, roots, leaves and plant height) found that lowest conservation (slow growth) was observed with mannitol. While sorbitol and MS media does not prove good for conserving the plants of *Citrus aurantifolia* and maintained their fast growth which is not best for conserving plants (Figure 5 and Figure 6).

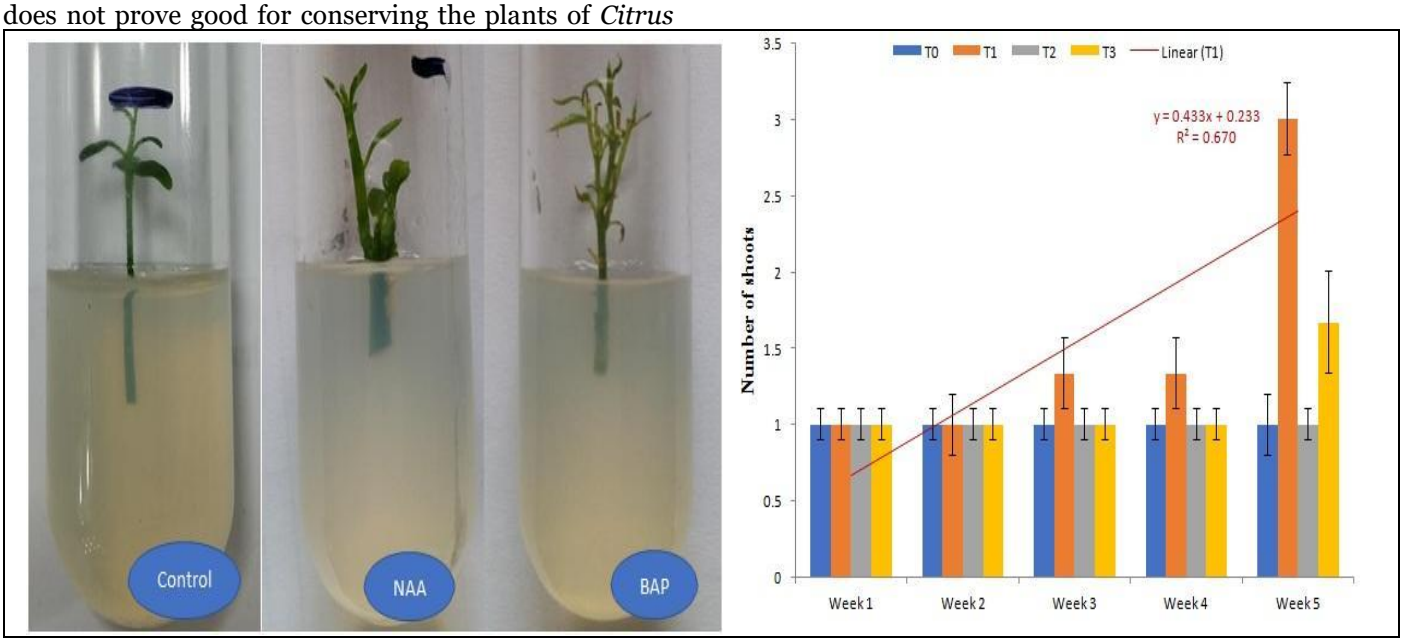


Figure 3: Effect of growth regulators on number of shoots of *C. aurantifolia* after regular intervals of 5 weeks.

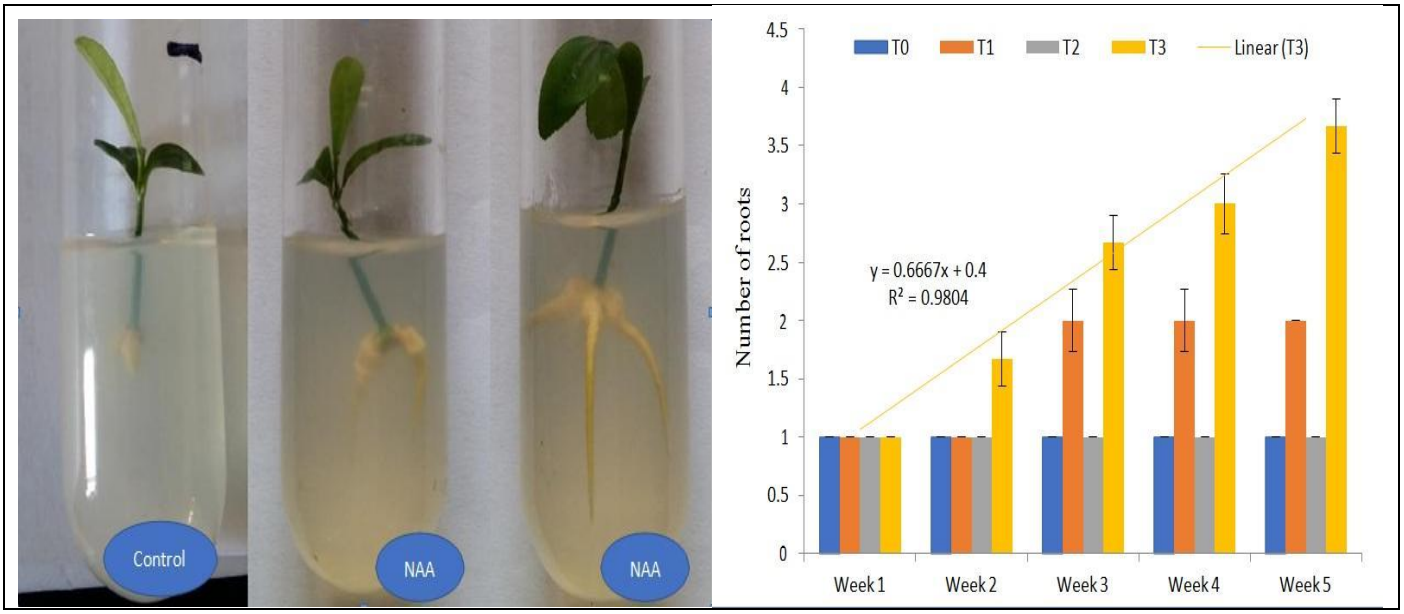
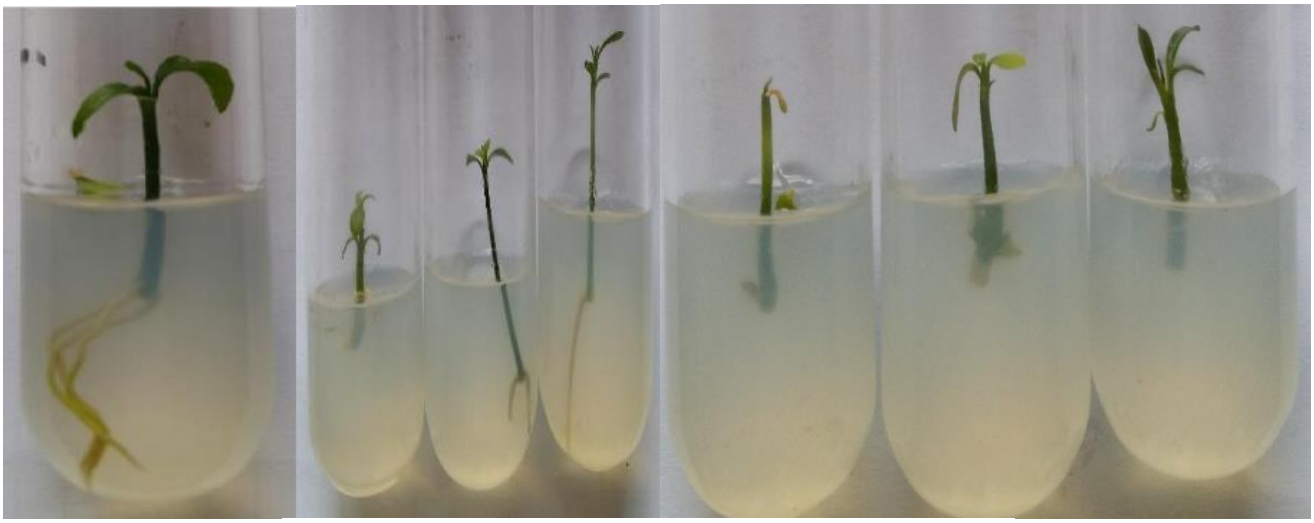


Figure 4: Effect of growth regulators on number of roots of *Citrus aurantifolia*.



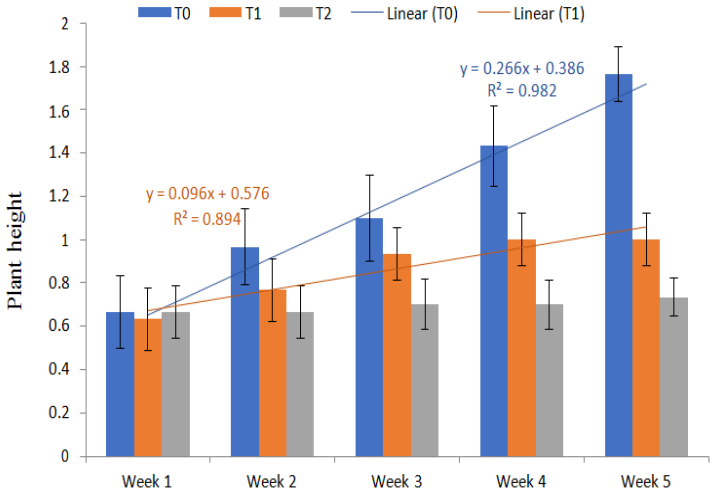
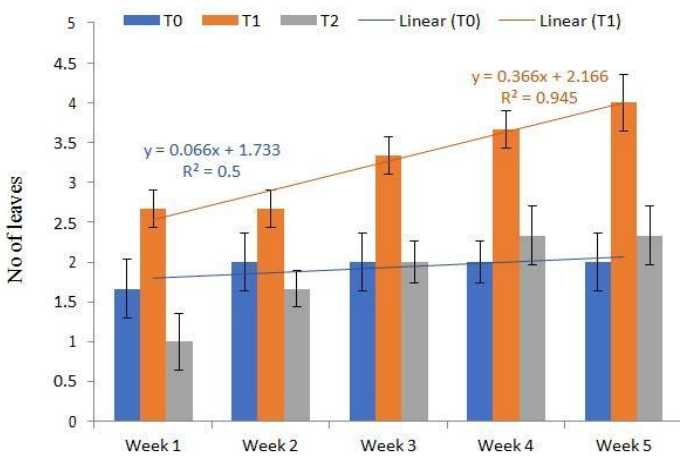


Figure 5: Effect of conservants after 35 days.



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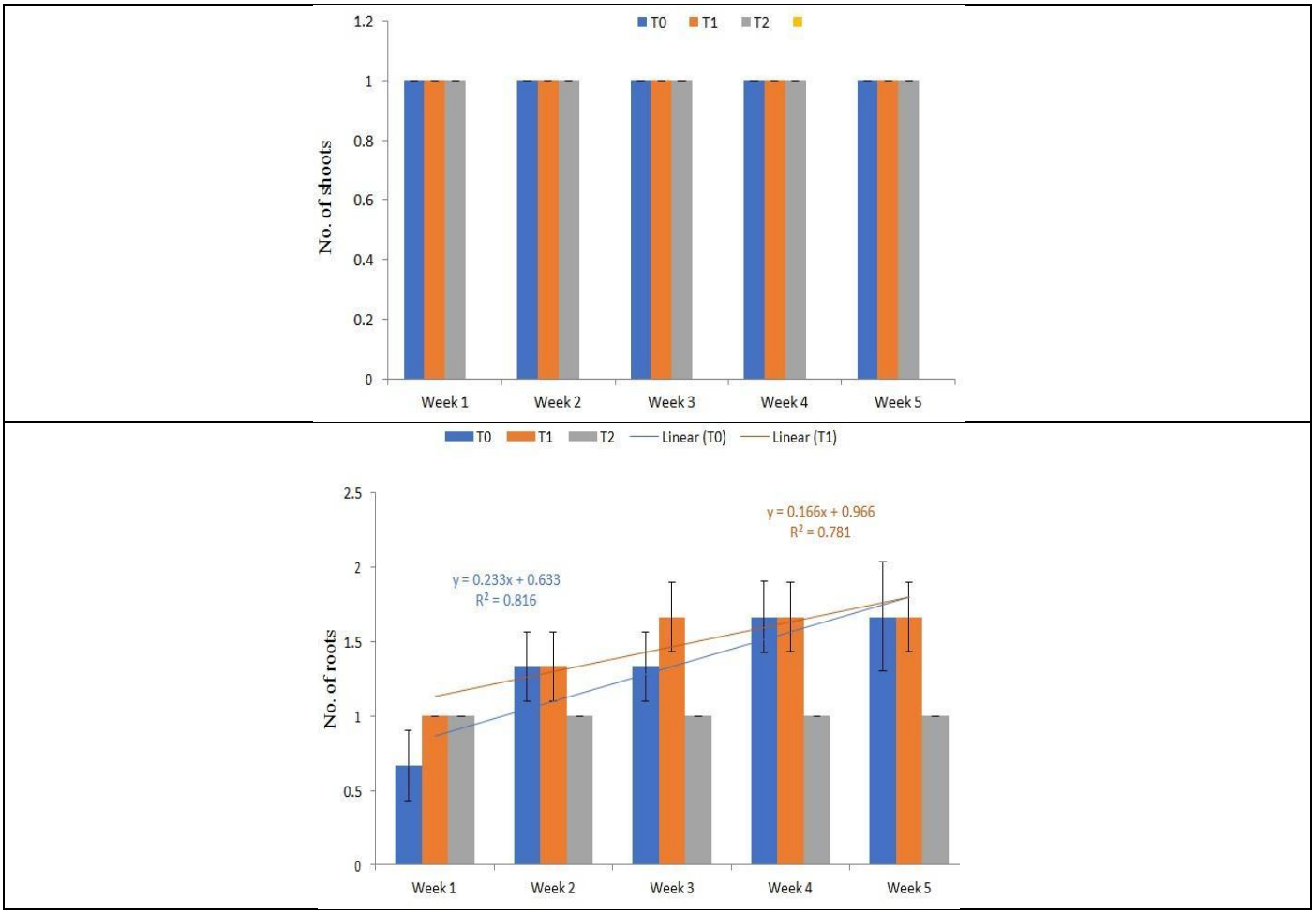

Figure 6: Effect of conservants on plant height, leaves, shoots and roots of *Citrus aurantifolia*

Discussion

A very important technique in tissue culture which was used for conserving the in vitro plant growth is using the chemicals for obtaining slow growth [11]. Therefore, in present study an effort was made for In vitro conservation of *Citrus aurantifolia* and its conservation to investigate phenotypical behaviour of citrus cultivar within the short period (5 months) propagation and conservation. The *C. aurantifolia* plant was propagated in vitro at the National Agricultural Research Centre of Pakistan using an explant that was harvested, according to the following protocols. Control of MS media and three hormones (BAP, GA3 and NAA) were used to study their effect on propagation of *Citrus aurantifolia*. Selected plants show significant phenotypic variations by using these hormones regarding to the morphological data.

Our results asupport the results of Sarker et al., [12] because in their finding the highest number of leaves shoots, roots were observed in growth hormones as compared to MS control media. They used BAP and GA3 at the concentration of (1.5 mg/L and 0.5mg/L). Similarities found between both finding, which might be due to the functions of these hormones and their effect on plant growth. BAP is a synthetic cytokinin that stimulates plant growth and development [13]. GA3 has the ability to arouse quick root and stem growth [14], GA3 generally used in the concentration ranging from (0.01 to 10 mg/L) respectively [15]. Auxin family hormone NAA is used in a variety of commercial plant rooting products [16].

In present study two conserving chemicals sorbitol and mannitol were used for observing plant responses to these conserving chemicals. The plants show positive response on these chemicals as compared to the MS control media. Our finding also agreed with the results of Shibli et al., [17], they reported that metabolic process of plants was interrupt by the nutrients supplies in low amount, due to low amount of nutrients the metabolic process disturbs which might be reduced the protein synthesis, due to lack of numbers of cofactors the enzymatic activity also disturbs. The low amount of nutrients also disturbs the phosphorus compounds like (ATP) synthesis and thus it conquered the activity of hormones in the segments of plants.

Conclusion

Based on present results it was concluded that low concentration (0.1 to 0.5mg/L) of growth regulators (BAP, GA3 and NAA) show best results in propagation of Citrus autrantifolia and produce rapid growth of its shoots, roots leaves and height as compared to control of MS. It was concluded that 21°C is optimum temperature under the light intensity of 1000 lux for conserving the plants of *Citrus aurantifolia* in storage room and conserving chemicals mannitol and sorbitol at the concentration of (10 to 30g/L show positive response and maintained slow growth after 5-6 weeks. In future the plants in conservation media will be used for further

propagation and for growing them in fields.

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