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Improvement of *in vitro* production of mandarin microscions and enhancement success of *in vivo* micrografting

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ABSTRACT

Traditional propagation method of citrus is a main cause of virus transportation from the infected trees through scions. So, the aim of this work is improvement the in vitro production and hardening of micro scions of mandarin. As well as, employment of the micro-scions to be successfully grafted on rootstock grown in greenhouse, as a new commercial method, through examine the effect of growth retardant and auxin on grafting successful percent of in vitro produced micro-scions in greenhouse. In this study, micro-scions of chinese mandarin were resulted from multiplication stage after cultured it on MS medium supplemented with different growth retardant types and concentrations as paclopetrazol (PP333) at different concentrations 0.5, 0.6, and 0.7mg/l and absacic acid (ABA) at different concentrations 2.5, 5.0 and 7.5µg/l for one month before be used as scions in greenhouse. After harvesting of micro-scions, it transferred to greenhouse and dipped in 10 mg/l IBA for different duration (0, 5, 10 and 15 min) prior to grafting process in greenhouse. After 60 days from grafting, scions derived from MS medium supplemented with 0.5 mg/l PP333 combined with dipping in IBA for 15 min, scions derived from MS medium supplemented with 0.7 mg/l PP333 combined with dipping in IBA for10 min, scions derived from MS medium supplemented with 5.0 µg/l ABA combined with dipping in IBA for 10 min, scions derived from MS medium supplemented with 7.5 µg/l ABA without dipping in IBA and scions derived from MS medium supplemented with 7.5 µg/l ABA combined with dipping in IBA for 10 min) maximized successful grafts percentage (100 %) compared with control (40%). Growth parameters were superior after 50 and 80 days of grafting as a result of the most treatments compared with control.

Keywords: Micrografting, PP333, ABA, IAA, mandarin, citrus.

1. Introduction

Citrus is one of the most important fruit crops in the world which are well grown at latitude 35°N-35°S (Ruiz *et al.,* 2000). Citrus is considered as the major fruit crops in Egypt, due to cultivated area reached to (204095 Hectare) representing about 29% of the total fruit area (700854 ha), the total fruitful area of Citrus reached about (175734 ha) approximately, which produce about 4272886 metric tons, from which around 1.34 million tons are exported, Egypt ranking as the sixth biggest producer of orange throughout the world after Brazil, China, US, EU, and Mexico (Abobatta, 2019).

In vitro micrografting is an important technique supporting the micropropagation of a range of plant species, particularly woody plant species. Over the past several decades, in vitro micrografting has become a strategy to facilitate shoot recovery and acclimatization of in vitro-grown horticultural species (Wang *et al.,* 2022).

Following the advent of in vitro plant tissue culturing in the early 1900s a grafting system using tissue culture (micrografting) in pathogen management to facilitate the eradication, indexing and transmission of pathogens, as well as the assessments of graft incompatibility induced by pathogen infection (Chilukamarri *et al.,* 2021). Using micro shoots witch production from tissue culture in citrus grafting in greenhouse is very important to production Citrus seedlings virus-free. Pathogen-free citrus

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selections; including virus-free plants, have been obtained by nucellar embryos culture, nucellar tissue culture, thermotherapy, clonal selection, indexing, and by shoot-tip grafting (STG) (Bitters *et al.,* 1972, Roistacher *et al.,* 1976, Singh *et al.,* 2008 and Hamza *et al.,* 2013). Micropropagation of shoot tips has been successfully employed to produce virus-free plants in citrus (Carvalho *et al.,* 2002). While, (Panattoni *et al.,* 2013) reported that tissue culture regenerate plants in breeding programs, without guarantee for eliminating viruses from plants.

At the present there is a worldwide shortage in the supply of planting material due to new strict regulations to produce and commercialize citrus plants; these regulations include upgraded infrastructure to prevent pest arrivals and test to confirm negatively diagnose for pathogens such as CTV (Citrus tristeza virus), CEVd (Citrus Exocortis Viroid) and HLB (Huanlongbing) (Vashisth *et al.,* 2020; ICA, 2019) in both rootstock and grafts.

Grafting techniques are determined as joining and unite pieces of living tissues from different plants. So, they will fuse to form and function as one plant. One of the most important keys to successful budding and grafting is properly positioning scion on the rootstock. It is essential to have good contact of cambium or growing layer of the scion and rootstock as the success of graft depends on cambium which is located just below the bark and is a layer of active dividing cells responsible for the production of the conducting vascular system of the stem and for the production of wood and bark. Grafting methods include, wedge grafting or cleft grafting, saddle grafting, side veneer or side cleft grafting, epicotyl grafting, splice grafting and whip or tongue grafting (Elam, 1997).

Micrografting is a technique that potentially can combine the advantages of rapid *in vitro* multiplication with the increased productivity that results from grafting superior rootstock and scion combinations (Gebhardt and Goldbach, 1988). Micrografting success ranged between 26.7% to 51.88% in kinnow mandarin (Naz *et al.,* 2007; Singh *et al.,* 2008). Production of *Gardenia jasminoides* scions *in vitro* and use it for grafting on *G. thunbergia* rootstock in greenhouse is established with high successive percent (100%). This method decreases production coast because it does not need to rooting stage and the percent of successive grafting in greenhouse is higher than *in vitro* micrografting (Nower and Hamza, 2013).

Citrus can be micropropagated via tissue culture techniques, but it has not been a commercial method. The main goal of micropropagation is production of virus-free plantlets through micrografting technique, but, percentage of micrografting success is so low and the successive plantlets need further to be acclimatized with another loss percent (Hamza *et al.,* 2013).

The development and differentiation of vascular tissues during plant growth is finely regulated by almost every known phytohormone. Nevertheless, auxin appears to be the primary regulator of vascular cell differentiation and patterning with other hormones interacting with the auxin biosynthesis, transport and signalling pathways to fine-tune this process (Nanda and Melnyk, 2018).

Micrografting success of 28% for Valencia orange and 14% for Tahiti lime are consistent with reported studies for recovery of disease-free plants; however, to increase success level for massive plant propagation, it is recommended for further studies to consider evaluation of in vitro conditions such as increased sucrose concentration, in vitro adaptation of shoot tip meristems and auxin supply, among others (Isidro *et al.,* 2021).

Success of micrografting is associated with genotype, shoot tip source, rootstock culture and age, nutrient medium composition, sucrose concentration, graft technique and PGR (Plant Growth Regulator) supply in the medium (Miceli *et al.,* 2019)

So, the aim of this work is improvement the in vitro production and hardening of micro shoots of mandarin. As well as, employment of the micro-scions to be successfully grafted on rootstock grown in greenhouse, as a commercial method, through examine the effect of growth retardant and auxin on grafting successful percent of in vitro produced micro-scions in greenhouse.

2. Materials and Methods

This study was carried out in laboratories of Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), Sadat City University, Egypt, and south El-Tahrir Research Station, Horticultural Research Institute, Agriculture Research Center, Ministry of Agriculture, Egypt, during the period from 2017 to 2021.

2.1. Stages of in vitro shoot multiplication

Citrus reticulate Blanco fruits were harvested in 1st November and sterilized by 3.5% NaOCl for 15 min. followed by wash for three times with autoclaved distilled water. Then fruits were rinsed in 70% Ethanol for 5 second and they were burned in the laminar air flow and opened with scalpel for seeds separation. Subsequently, seeds were germinated on MS basal medium in 210 ml culture jars contained 50 ml basal medium. These jars were incubated in darkness until nucellar embryos germinated. Then, nucellar embryos shoot tips were cultured on MS multiplication media contained 2mg BA/l. Two successive subcultures were carried out fifteen days interval (Hamza *et al.,* 2013).

2.2. Shoots pre-hardening

2.2.1. Effect of different growth retardant types and concentrations on *in vitro* shoots pre-hardening

Multiplied shoots were cultured on MS medium supplemented with various concentrations of paclobutrazol (PP333) (0.0 as control, 0.5, 0.6, and 0.7 mg/l PP333) or different concentrations of Abscisic Acid (ABA) (2.5, 5.0 and 7.5µg /l) for 30 days. The cultures were incubated in high light intensity (3000lux). Leaves number/shoot, shoot length (cm) and shoot diameter (mm) were recorded also growth vigor were estimated after 30days.

2.2.2. Scions preparation

After 30 days from shoots culture on MS medium supplemented with growth retardant, the resulted shoots have been separately harvested from each in vitro treatment. Then, they were transferred to greenhouse. The collected shoots were carefully washed to remove medium. Leaves were removed and re-washed with a fungal disinfectant Rizolex T-50.

2.3. Effect of growth retardance and indole butyric acid (IBA) on improvement the formation of graft union

Shoots resulted from each in vitro treatment were divided into four groups in greenhouse. The four groups were rinsing in IBA solution 10 mg/l for different durations (0 as control, 5, 10 and 15 min) before grafting.

2.4. Rootstock preparation

The seeds of the vigorous rootstock Volkamariana were collected from full ripening fruits. Subsequently, seeds were planted in soil mixture container (sand and peat moss 3:1) in a greenhouse until germination then individually transplanted to black polyethylene bags (15cm) for three months before grafting (Fig.1).

Fig. 1: Rootstock cultivation and preparation in greenhouse.

2.5. Steps of tip pen grafting method

1- Rootstock was cut at 20 cm height from soil surface and apical incision in the rootstock Fig2 (A).
2- Then, grafts were prepared with a length of 1.5 to 2 cm the grafts were fixed by Plastic Fig2 (B and C).
3- Scion rootstock and union region was covered with a plastic bag for save suitable humidity around scion and grafting union. Polyethylene bags were gradually removed Fig2 (D).

4- Each treatment consisted of three replicates and each replicate contained ten plants. Successful grafts number (beginning growth of new leaves) were recorded after 15, 30, 45 and 60 days from grafting, leaves number and scion length (cm) were recorded after 50 and 80 after grafting.

Fig. 2: Steps of tip pen grafting method of micro scions on rootstock grown in greenhouse

2.6. Data analysis

Statistical analysis: Data of results were statistically analyzed by factorial randomized complete design using SAS (1988) package using computer software MSTAT-C (MSTAT Development Team, 1988). The least significant difference among levels of each treatment was compared using LSD. Test at 5% level according to (Steel *et al.,* 1997).

3. Results and Discussion

3.1. Effect of different growth retardant types and concentrations on shoots *in vitro* pre-acclimatization

Data in Table 1 and Fig.3 show that the highest shoot length (5cm) was recorded by MS medium-free hormone and MS supplemented with ABA (2.5 µg/l), while, the shortest values were recorded by MS supplemented with PP333 (0.7 mg/l).

Table 1: Effect of different growth retardant types and concentrations on *in vitro* shoots pre-hardening

Growth retardant types	Growth retardant Concentrations	Shoot length (cm)	Shoot diameter (mm)	Leaves No./shoot	*Growth vigor
Control	0.0	5.00	1.10	6.00	2.00
PP_{333}	0.5 mg/l	4.40	1.50	4.10	4.00
	0.6 mg/l	3.70	1.70	4.00	5.00
	0.7 mg/l	3.30	1.80	3.00	5.00
ABA	2.5 µg/l	5.00	1.10	6.00	3.00
	5.0 µg/l	4.50	1.35	4.00	4.00
	7.5 µg/l	4.50	1.34	5.11	5.00
LSD at level 5%		0.60	0.20	0.81	0.92

*Growth vigor was measured according to Pottino, (1981)

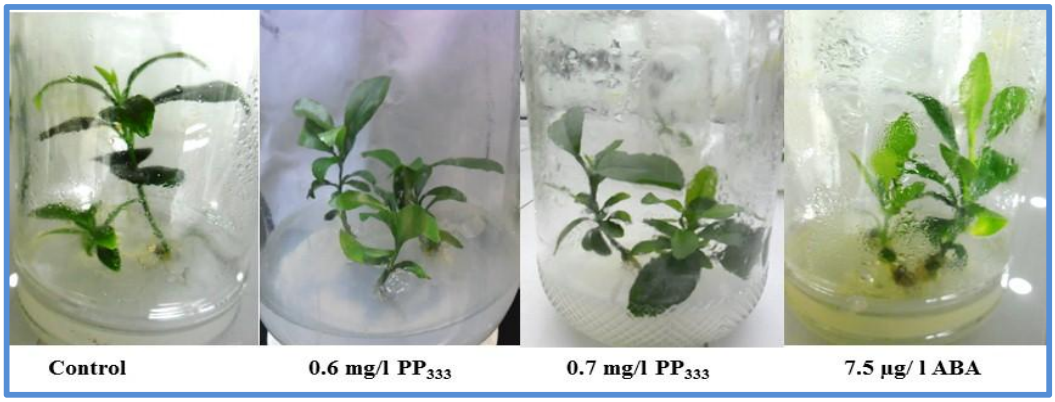

Fig. 3: Effect of different growth retardant types and concentrations on *in vitro* shoots pre-hardening

Concerning the shoot diameter, the highest value (1.8mm) was recorded by PP333 (0.7 mg/l), whereas the least value (1.1mm) was recorded by MS medium-free hormone. The highest leaves number (6 leaves/shoot) was recorded by MS medium-free hormone as well as MS supplemented with 2.5µg/l ABA. Finally, growth vigor clears the same response of the other parameters, where the highest values (5) were recorded by PP333 (0.6 and 0.7 mg/l) and ABA (7.5 µg/l) and the least value (2) was recorded by basal-MS.

3.2. Effect of different growth retardant types and indole butyric acid on micro grafting success of *Citrus reticulate Blanco* scions

Data in Table (2) and Fig (4) presented the micrografting success number and percentage as affected by pre-hardening agents (*in vitro*) and soaking for various periods time in IBA (*in vivo*).

Table 2: Effect of different growth retardant types and indole butyric acid on micro grafting success of *Citrus reticulate Blanco* scions.

Growth retardant types and concentrations (A)		Soaking period in IBA (min) (B) 0	5	10	15	Mean (A)
		Micro grafting success number (%) after 15 days				
Control		4.0 (40%)	6.0 (60%)	3.0 (30%)	5.0 (50%)	7.0 (70%)
PP_{333} (mg/l)	0.5	4.0 (40%)	7.0 (70%)	6.0 (60%)	8.0 (80%)	8.0 (80%)
	0.6	3.0 (30%)	4.0 (40%)	3.0 (30%)	3.0 (30%)	5.7 (57%)
	0.7	8.0 (80%)	6.0 (60%)	7.0 (70%)	4.0 (40%)	7.7 (77%)
ABA (µg/l)	2.5	4.0 (40%)	3.0 (30%)	4.0 (40%)	4.0 (40%)	7.0 (70%)
	5.0	4.0 (40%)	6.0 (60%)	6.0 (60%)	5.0 (50%)	7.7 (77%)
	7.5	6.0 (60%)	4.0 (40%)	4.0 (40%)	3.0 (30%)	8.0 (80%)
Mean (B)		4.71 (47%)	5.143 (51%)	4.7 (47%)	4.5 (45%)	
LSD at 5%		A: 0.008	B: 0.006	A&B: 0.02		
		Micro grafting success number (%) after 30 days				Mean (A)
Control		4.0 (40%)	8.0 (80%)	7.0 (70%)	9.0 (90%)	7.0 (70%)
PP_{333} (mg/l)	0.5	6.0 (60%)	8.0 (80%)	8.0 (80%)	10.0 (100%)	8.0 (80%)
	0.6	6.0 (60%)	5.0 (50%)	6.0 (60%)	6.0 (60%)	5.7 (57%)
	0.7	8.0 (80%)	8.0 (80%)	9.0 (90%)	6.0 (60%)	7.7 (77%)
ABA (µg/l)	2.5	7.0 (70%)	6.0 (60%)	8.0 (80%)	7.0 (70%)	7.0 (70%)
	5.0	8.0 (80%)	8.0 (80%)	9.0 (90%)	6.0 (60%)	7.7 (75%)
	7.5	10.0 (100%)	6.0 (60%)	8.0 (80%)	8.0 (80%)	8.0 (80%)
Mean (B)		7.00 (70%)	7.00 (70%)	7.8 (78%)	7.42 (74%)	
LSD at 5%		A: 0.003	B: 0.006	A&B: 0.02		
		Micro grafting success number (%) after 45 days				Mean (A)
Control		4.0 (40%)	8.0 (80%)	7.0 (70%)	9.0 (90%)	7.0 (70%)
(PP_{333}) (mg/l)	0.5	6.0 (60%)	8.0 (80%)	8.0 (80%)	10.0 (100%)	8.0 (80%)
	0.6	6.0 (60%)	6.0 (60%)	6.0 (60%)	6.0 (60%)	6.0 (60%)
	0.7	8.0 (80%)	8.0 (80%)	10.0 (100%)	7.0 (70%)	8.0 (80%)
ABA (µg/l)	2.5	7.0 (70%)	7.0 (70%)	8.0 (80%)	8.0 (80%)	7.5 (70%)
	5.0	8.0 (80%)	8.0 (80%)	10.0 (100%)	6.0 (60%)	8.0 (80%)
	7.5	10.0 (100%)	6.0 (60%)	9.0 (90%)	8.0 (80%)	8.2 (82%)
Mean (B)		7.0 (70%)	7.2 (72%)	8.2 (82%)	7.7 (77%)	
LSD at 5%		A: 0.008	B: 0.006	A&B: 0.02		
		Micro grafting success number (%) after 60 days				Mean (A)
Control		4.0 (40%)	8.0 (80%)	7.0 (70%)	9.0 (90%)	7.0 (70%)
PP_{333} (mg/l)	0.5	6.0 (60%)	8.0 (80%)	8.0 (80%)	10.0 (100%)	8.0 (80%)
	0.6	6.0 (60%)	6.0 (60%)	6.0 (60%)	6.0 (60%)	6.0 (60%)
	0.7	8.0 (80%)	8.0 (80%)	10.0 (100%)	7.0 (70%)	8.2 (82%)
ABA (µg/l)	2.5	7.0 (70%)	7.0 (70%)	8.0 (80%)	8.0 (80%)	7.5 (75%)
	5.0	8.0 (80%)	8.0 (80%)	10.0 (100%)	6.0 (60%)	8.0 (80%)
	7.5	10.0 (100%)	6.0 (60%)	10.0 (100%)	9.0 (90%)	8.7 (87%)
Mean (B)		7.0 (70%)	7.2 (72%)	8.4 (84%)	7.8 (78%)	
LSD at 5%		A: 0.15	B: 0.12	AxB: 0.31		

Fig. 4: Effect of different growth retardant types and indole butyric acid on micro grafting success of *Citrus reticulate Blanco* scions

Results revealed that, after 15 days of grafting, *Citrus reticulate* scions (micro scions) derived from MS medium supplemented with 0.5 mg/l PP333 and soaking in IBA for 15 minutes as well as scions derived from MS medium supplemented with 0.7 mg/l PP333 without treating with IBA clear maximum successful grafts percentage (80%) compared with control (40%). After 30 days of grafting, *Citrus reticulate* micro scions derived from MS medium supplemented with 0.5 mg/l PP333 and soaking in IBA for 15 minutes, and scions derived from MS medium supplemented with 7.5 µg/l ABA without soaking in IBA, enhanced successful grafts percentage to 100 % compared with other treatments and control (40%). After 45 days of grafting, micro scions resulted from MS medium supplemented with 0.5 mg/l PP333 and soaking in IBA for 15 minutes, and scions derived from MS medium supplemented with 0.7 mg/l PP333 and soaking in IBA for 10 minutes, scions derived from MS medium supplemented with 5.0 µg/l ABA and soaking in IBA for 10 minutes and scions derived from MS medium supplemented with 7.5 µg/l ABA without soaking in IBA augmented the success percentage of grafting (100 %) compared with control. After 60 days of grafting, *Citrus reticulate* micro scions derived from MS medium supplemented with 0.5 mg/l PP333 and soaking in IBA for 15 minutes, scions derived from MS medium supplemented with 0.7 mg/l PP333 and soaking in IBA for 10 minutes, scions derived from MS medium supplemented with 5.0 µg/l ABA and soaking in IBA for 10 minutes, and scions derived from MS medium supplemented with 7.5 µg/l ABA without dipping in IBA increased the success percentage of grafting to 100 %.

3.3. Effect of different growth retardant types and indole butyric acid on retard the response of micro grafting scions of *Citrus reticulate*

Results in Table (3) revealed that, after 15 days micro scions which were treated with 7.5 µg/l ABA in vitro and soaked in IBA for 15 minutes in vivo, gave the highest number and percentage of unresponsive grafts (70%). After 30 days, micro scion which were resulted from the same in vitro medium (MS+ 7.5 µg/l ABA) and soaked in IBA for five minutes gave the highest number (3 scions) and percentage (30%) of unresponsive grafts. After 45 days, micro Micro-scion were cultured on MS medium supplemented with 7.5 µg/l ABA with soaking in IBA for five minutes gave the highest number and percentage of un response grafts (3scion and 30%, respectively). After 60 days, micro scions derived from MS medium supplemented with 0.6 mg/l PP333 with 15 minutes soaking in IBA as well as scions resulted from 2.5 µg/l ABA with soaking in IBA for 15 minutes maximized the number and percentage of unresponsive grafts (30%). Generally, it could conclude that the effect of growth retardance can be vanished by the using of IBA and by the time. After 30 days, the soaking in IBA for five or ten min

vanished the effect of growth retardance in retarding the response of scions, which performed the same results of untreated scions (control). After 45 and 60 days, the most scions which treated with IBA for five minutes cleared 100% response scions.

Table 3: Effect of different growth retardant types and indole butyric acid on retard the response of micro grafting scions of *Citrus reticulate*

Growth retardant types and concentrations (A)		Soaking period in IBA (min) (B)				Mean (A)
		0	5	10	15	
		Number of un response micro scions (%) after 15 days				
Control (0.0)		3.0 (30%)	3.0 (30%)	5.0 (50%)	5.0 (50%)	4.0 (40%)
PP_{333} (mg/l)	0.5	4.0 (40%)	2.0 (20%)	3.0 (30%)	2.0 (20%)	2.7 (27%)
	0.6	4.0 (40%)	4.0 (40%)	5.0 (50%)	5.0 (50%)	4.5 (45%)
	0.7	2.0 (20%)	3.0 (30%)	3.0 (30%)	2.0 (20%)	2.5 (25%)
ABA (µg/l)	2.5	4.0 (40%)	6.0 (60%)	4.0 (40%)	5.0 (50%)	4.7 (47%)
	5.0	6.0 (60%)	4.0 (40%)	4.0 (40%)	5.0 (50%)	4.7 (47%)
	7.5	4.0 (40%)	6.0 (60%)	6.0 (60%)	7.0 (70%)	5.7 (57%)
Mean (B)		3.8 (38%)	4.0 (40%)	4.3 (43%)	4.4 (44%)	
LSD at 5%			A: 0.01	B: 0.01	A&B: 0.02	
		Number of un response micro scions (%) after 30 days				Mean (A)
Control (0.0)		0.0 (0.0%)	0.0 (0.0%)	1.0 (10%)	0.0 (0.0%)	0.3 (3%)
PP_{333} (mg/l)	0.5	2.0 (20%)	1.0 (10%)	1.0 (10%)	0.0 (0.0%)	1.0 (10%)
	0.6	1.0 (10%)	1.0 (10%)	2.0 (20%)	2.0 (20%)	1.5 (15%)
	0.7	1.0 (10%)	0.0 (0.0%)	1.0 (10%)	1.0 (10%)	0.7 (7%)
ABA (µg/l)	2.5	1.0 (10%)	2.0 (20%)	0.0 (0.0%)	2.0 (20%)	1.2 (12%)
	5.0	2.0 (20%)	2.0 (20%)	1.0 (10%)	2.0 (20%)	1.7 (17%)
	7.5	0.0 (0.0%)	3.0 (30%)	2.0 (20%)	2.0 (20%)	1.7 (17%)
Mean (B)		1.0 (10%)	1.3 (13%)	1.1 (11%)	1.3 (13%)	
LSD at 5%			A: 0.41	B: 0.31	A&B: 0.81	
		Number of un response micro scions (%) after 45 days				Mean (A)
Control (0.0)		0.0 (0.0%)	0.0 (0.0%)	1.0 (10%)	0.0 (0.0%)	0.3 (3.0%)
PP_{333} (mg/l)	0.5	1.0 (10%)	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	0.3 (3.0%)
	0.6	1.0 (10%)	0.0 (0.0%)	2.0 (20%)	2.0 (20%)	1.2 (12.0%)
	0.7	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	1.0 (10%)	0.3 (3.0%)
ABA (µg/l)	2.5	1.0 (10%)	0.0 (0.0%)	0.0 (0.0%)	2.0 (20%)	0.8 (8.0%)
	5.0	2.0 (20%)	2.0 (20%)	0.0 (0.0%)	1.0 (10%)	1.2 (12%)
	7.5	0.0 (0.0%)	3.0 (30%)	1.0 (10%)	1.0 (10%)	1.2(12%)
Mean (B)		0.7 (7.0%)	0.7 (7.0%)	0.6 (6.0%)	1.0 (10.0%)	
LSD at 5%		A: 0.34	B: 0.26	A&B: 0.68		
		Number of un response micro scions (%) after 60 days				Mean (A)
Control (0.0)		0.0 (0.0%)	0.0 (0.0%)	1.0 (10%)	0.0 (0.0%)	0.3 (3.0%)
PP_{333} (mg/l)	0.5	1.0 (10%)	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	0.3 (3.0%)
	0.6	1.0 (10%)	0.0 (0.0%)	2.0 (20%)	2.0 (20%)	1.2 (12.0%)
	0.7	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	1.0 (10%)	0.3 (3.0%)
ABA (µg/l)	2.5	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	2.0 (20%)	0.8 (8.0%)
	5.0	0.0 (0.0%)	2.0 (20%)	0.0 (0.0%)	0.0 (0.0%)	0.5 (5.0%)
	7.5	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)
Mean (B)		0.3(3.0%)	0.3 (3.0%)	0.5 (5.0%)	0.7 (7.0%)	
LSD at 5%			A: 0.29	B: 0.22	A&B: 0.58	

3.4. Effect of different growth retardant types and indole butyric acid on drying of micro-scions of *Citrus reticulate* after grafting

Date in Table 4 revealed that after 15 days of grafting, the highest number and percentage of dry grafts were observed in *Citrus reticulate* micro-scions derived from MS medium supplemented with 0.7 mg/l PP333 and *in vivo* dipping in IBA for 15 minutes followed by control (40% and 30% respectively), while, the most treatments minimized the number and percentage of dry grafts. After 30 days, the in vitro and the in vivo treatments accelerated the formation of graft unions and prevent the drying of

grafts. Anyway, the highest number and percentage of dry grafts were observed in control (60%). The same trend was observed after 45 and 60 days.

Table 4: Effect of different growth retardant types and indole butyric acid on drying of micro-scions of *Citrus reticulate* after grafting

Growth retardant types and concentrations (A)		Soaking period in IBA (min) (B) 0	5	10	15	Mean (A)
		Number (%) of drying of micro-scion after 15days				
Control		3.0 (30%)	1.0 (10%)	2.0 (20%)	0.0 (0.0%)	1.5 (15%)
PP_{333} mg/l	**0.5**	2.0 (20%)	1.0 (10%)	1.0 (10%)	0.0 (0.0%)	1.0 (10%)
	0.6	3.0 (30%)	2.0 (20%)	2.0 (20%)	2.0 (20%)	2.2 (22.0%)
	0.7	0.0 (0.0%)	1.0 (10%)	0.0 (0.0%)	4.0 (40%)	1.3 (13%)
ABA mg/l	**2.5**	2.0 (20%)	1.0 (10%)	2.0 (20%)	1.0 (10%)	1.5 (15%)
	5.0	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)
	7.5	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)
Mean (B)		1.4 (14.0%)	0.8 (8.0%)	1.0 (10.0%)	1.0 (10.0%)	
LSD at 5%			A 0.3962	B0.2995	A&B0.7924	
		Number (%) of drying of micro-scion after 30days				**Mean (A)**
Control		6.0 (60%)	2.0 (20%)	2.0 (20%)	1.0 (10%)	2.7 (27%)
PP_{333} mg/l	**0.5**	2.0 (20%)	1.0 (10%)	1.0 (10%)	0.0 (0.0%)	1.0 (10%)
	0.6	3.0 (30%)	4.0 (40%)	2.0 (20%)	2.0 (20%)	2.7 (27%)
	0.7	1.0 (10%)	2.0 (20%)	0.0 (0.0%)	3.0 (30%)	1.5 (15%)
ABA µg/l	**2.5**	2.0 (20%)	2.0 (20%)	2.0 (20%)	1.0 (10%)	1.7 (17%)
	5.0	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	2.0 (20%)	0.5 (5%)
	7.5	0.0 (0.0%)	1.0 (10%)	0.0 (0.0%)	0.0 (0.0%)	0.3 (3%)
Mean (B)		2.0 (20%)	1.7 (17%)	1.04 (10%)	1.31 (13%)	
LSD at 5%			A 0.4133	B 0.3124	A&B 0.8266	
		Number (%) of drying of micro-scion after 45days				**Mean (A)**
Control		6.0 (60%)	2.0 (20%)	2.0 (20%)	1.0 (10%)	2.7 (27.0%)
PP_{333} (mg/l)	**0.5**	3.0 (30%)	2.0 (20%)	2.0 (20%)	0.0 (0.0%)	1.7 (17.0%)
	0.6	3.0 (30%)	4.0 (40%)	2.0 (20%)	2.0 (20%)	2.7(27.0%)
	0.7	2.0 (20%)	2.0 (20%)	0.0 0.0%)	2.0 (20%)	1.5 (15.0%)
ABA (µg/l)	**2.5**	2.0 (20%)	3.0 (30%)	2.0 (20%)	0.0 (0.0%)	1.7(17.0%)
	5.0	0.0 (0.0%)	0.0 (0.0%)	0.0 (0.0%)	3.0 (30%)	0.8(8.0%)
	7.5	0.0 (0.0%)	1.0 E (10%)	0.0 (0.0%)	0.0 (0.0%)	0.3(30.0%)
Mean (B)		2.314 A	2.014 A	1.186 B	1.186 B	
LSD at 5%			A 0.4036	B 0.3051	A&B 0.8073	
		Number (%) of drying of micro-scion after 60days				**Mean (A)**
Control		6.0 (60%)	2.0 (20%)	2.0 (20%)	1.0 (10%)	2.7 (27.0%)
PP_{333} (mg/l)	**0.5**	3.0 (30%)	2.0 (20%)	2.0 (20%)	0.0 (0.0%)	1.7(17.0%)
	0.6	3.0 (30%)	4.0 (40%)	2.0 (20%)	2.0 (20%)	2.7(27.0%)
	0.7	2.0 (20%)	2.0 (20%)	0.0 (0.0%)	2.0 (20%)	1.5 (15.0%)
ABA (µg/l)	**2.5**	3.0 (30%)	3.0 (30%)	2.0 (20%)	0.0 (0.0%)	1.7 (17.0%)
	5.0	2.0 (20%)	0.0 (0.0%)	0.0 (0.0%)	4.0 (40%)	1.5 (15.0%)
	7.5	0.0 (0.0%)	4.0 (40%)	0.0 (0.0%)	1.0 (10%)	1.3 (13.0%)
Mean (B)		2.7 (27%)	2.4(24.0%)	1.1(11.0%)	1.4 (14.0%)	
LSD at 5%			**A 0.3860**	**B 0.2918**	**A&B 0.7720**	

3.5. Effect of different growth retardant types and concentrations combined with in vivo dipping in indole butyric acid on growth parameters of micro-scions of *Citrus reticulate* after grafting in greenhouse

After 50 days, results were recorded in greenhouse. Data presented in Table (5) and Fig. (5) showed that *Citrus reticulate* scions derived from MS medium supplemented with 0.5 or 0.7 mg/l PP333 combined with in vivo dipping in IBA for 15 minutes gave the highest scions length (5.0 and 4.6 cm, respectively).

Scions which dipping in IBA for 15 minutes without any growth retardant significantly maximized leaves number (9.0 leaves/scion), followed by micro-scions derived from 0.6mg/l PP333 individual or combined with in vivo dipping in IBA for 10 or 15min (8.0, 8.33 and 8,33 leaves/scion, respectively),

without significant differences between them. Also, scions derived from 2.5 µg/l ABA significantly enhanced the leaves number (8.66 leaves/scion). Finally, it could conclusion that after 50days, the negative effects of growth retardance vanished.

Table 5: Effect of different growth retardant types and concentrations combined with in vivo dipping in indole butyric acid on growth parameters of micro-scions of *Citrus reticulate* after grafting in greenhouse

Growth retardant types and concentrations (A)		**Soaking period in IBA (min) (B)**				**Mean (A)**
		0	**5**	**10**	**15**	
		Shoot length of micro-scion after 50days of grafting (cm)				
Control		3.00	4.00	3.75	3.80	3.638
PP_{333} (mg/l)	**0.5**	3.00	3.80	3.00	5.00	3.720
	0.6	3.50	2.60	3.60	3.00	3.175
	0.7	4.33	3.50	4.50	4.63	4.242
ABA **(µg/l)**	**2.5**	4.00	2.20	3.75	3.80	3.438
	5.0	3.10	4.00	3.76	3.70	3.642
	7.5	3.80	3.10	3.60	2.96	3.367
Mean (B)		3.54	3.31	3.71	3.84	
LSD at level 5%		A:0.17	B: 0.13	AxB:0.34		
		Leaves No./scion after 50 days of grafting				**Mean (A)**
Control		5.00	7.33	7.33	9.00	7.16
PP_{333} mg/l	**0.5**	5.00	7.33	6.00	7.33	6.41
	0.6	8.00	6.66	8.33	8.33	7.83
	0.7	5.66	6.66	7.00	4.00	5.83
ABA µg/l	**2.5**	8.66	5.66	8.00	5.66	7.00
	5.0	5.33	5.33	5.66	5.00	5.33
	7.5	5.33	5.00	4.83	4.00	4.79
Mean (B)		6.14	6.28	6.73	6.19	
LSD at 5%		A:0.83	B: 0.63	AxB: 1.67		

Fig. 5: Effect of different growth retardant types and concentrations combined with in vivo dipping in indole butyric acid on growth parameters of micro-scions of *Citrus reticulate* after 50 days of grafting in greenhouse

Growth parameters of scions grafting after 80days in greenhouse were recorded and presented in Table (6) and Figure (6). Results showed that *Citrus reticulate* scions derived from MS medium supplemented with 0.5 mg/l PP333 combined with in vivo dipping in IBA for 15 min maximized the scion's length (11.33 cm) followed by scions derived from the same in vitro treatment and 5min dipping in IBA (10.17cm). After 80 days from grafting, the scions without any growth retardant treatment witch dipping in IBA for 15 minutes, and scions derived from in vitro with 2.5 µg/l ABA combined with dipping in IBA for 10min significantly maximized leaves number (14 leaves/scion for each).the most growth retardance treatments cleared high growth response after 80 days of grafting.

Table 6: Effect of different growth retardant types and concentrations combined with in vivo dipping in indole butyric acid on growth parameters of micro-scions of *Citrus reticulate* after 80 days of grafting in greenhouse

Growth retardant types and concentrations (A)		Soaking period in IBA (min) (B)				Mean (A)
		0	5	10	15	
		Shoot length of micro-scion after 80days of grafting (cm)				
Control		8.00	8.33	10.00	10.17	9.13
PP_{333} (Mg/l)	0.5	9.00	10.17	9.17	11.33	9.92
	0.6	7.83	8.00	8.50	8.00	8.08
	0.7	8.00	7.67	9.67	9.50	8.71
ABA (µg\l)	2.5	8.00	6.00	7.33	7.50	7.21
	5.0	6.63	8.00	7.33	7.33	7.32
	7.5	8.17	8.00	8.33	5.33	7.46
Mean (B)		7.95	8.02	8.62	8.45	
LSD at level 5%		A:0.27	B: 0.21	AxB: 0.54		
		Leaves No./scion after 80 days of grafting				Mean (A)
Control		8.00	10.67	11.00	14.00	10.92
PP_{333} (mg/l)	0.5	7.33	10.33	8.33	10.67	9.16
	0.6	11.17	10.00	12.00	11.67	11.21
	0.7	9.00	9.33	10.33	7.66	9.08
ABA (µg/l)	2.5	11.00	12.33	14.00	10.00	11.83
	5.0	7.33	7.67	9.33	8.33	8.16
	7.5	8.33	7.00	7.66	6.33	7.33
Mean (B)		8.81	9.61	10.38	9.81	
LSD at 5%		A:0.33	B :0.25	AxB: 0.66		

Fig. 6: Effect of different growth retardant types and concentrations combined with in vivo dipping in indole butyric acid on growth parameters of micro-scions of *Citrus reticulate* after 80 days of grafting in greenhouse in greenhouse

4. Discussion

Pre-hardening treatments through addition different concentrations of PP333 or ABA to MS medium in vitro prior the harvesting of mandarin micro-scions maximized the success number and percentage of micrografting in greenhouse. Also, dipping the micro-scions in IBA for different periods prior the grafting process individual or in combination with in vitro treatments of PP333 or ABA enhanced the micrografting success and improved the formation of grafting union. As well as, treatments increased the values of growth parameters of micro-scions through acceleration the cell division which resulted in well formation of union grafting area. These results may reflect the PP333 and ABA effects on inducing storage substances in cells which may accelerate the cell division and formation of graft union. Also, auxin may affect the success of grafting through inducing the formation of graft union as well as accelerate the cell division and vascular formation.

Also, Results may be due to the ability of PP333, in low concentrations, as anti-gibberellin to induce storage which may save substances which may be needed to form grafts union. So, PP333 may be useful in enhancement grafts successful. Also, PP333 at the low concentration may be maximizing leaves number and scion length because it is so diluted and could be degradation quickly and have no negative effects on growth parameters. Results came in line with (Chronopoulou-Sereli, 1999) who reported that Paclobutrazol affects the content of plant growth regulators by inhibiting gibberellin synthesis, reducing ethylene evolution, and increasing cytokinin level. grafting successful). This may be due to the good vascular system formation in the case of tip pen grafting. Also, results came in line with the finding of Hamza *et al.,* 2013, who stated that 0.6 mg/l PP333 gave the highest percentage of successful grafts 88.77%.

Results are supporting the finding of (Asahina *et al.,* 2011; Yin *et al.,* 2012; Wang *et al.,* 2014; Melnyk *et al.,* 2015; Matsuoka *et al.,* 2016 and Chen *et al.,* 2017) who stated that consistent with auxin's pivotal role in vascular formation and wound healing, it is perhaps unsurprising that auxin may play an important role in graft union formation. Grafting induces the expression of auxin biosynthesis and signaling genes and exogenous auxin application is often necessary for in vitro grafting.

Also, results agree with (Elam., 1997) who stated that, It is essential to have good contact of cambium or growing layer of the scion and rootstock as the success of graft depends on cambium which is located just below the bark and is a layer of active dividing cells responsible for the production of the conducting vascular system.(Elfving and Visser, 2006) add that auxin is very important for vascular tissue proliferation and reconnection across the graft junction. Better shoot growth due to NAA may be due to the fact that auxin influence the production and function of cytokinin.

DiDonato *et al.* (2004); Sugimoto *et al.* (2010); and Melnyk *et al.* (2015) suggesting that how auxin is perceived rather than absolute auxin levels or efficiency of auxin transport may be a determining factor in grafting success. One of the genes important for graft formation, aberrant lateral root formation 4 (ALF4), acts downstream of auxin and regulates xylem pole pericycle cell division and lateral root formation. Also, meristematic cells in the vascular tissue, might be a key driver of vascular connection during grafting.

The current study was conducted to determine the potential of using auxin–cytokinin combination to improve the grafting process. Growth regulator like auxin released from the vascular bundle of stock and scion induces compatible unions through differentiation of vascular tissues, act as morphogenic substances as a result accelerate grafting success (Kondo *et al.,* 2014). Nanda and (Melnyk, 2018) explained that Gibberellins (GAs) are diterpene Phyto hormones with an important role in plant development, particularly in regulating plant growth, as GAs promote cell expansion, cell differentiation and cell proliferation. Micrografting of Kashi mandarin resulted in maximum (56.8%) response when the rootstocks were cultured in semisolid MS medium fortified with 0.5 mg L-1BAP and 0.1 mg L-1 indole-3-acetic acid (IAA) along with 5% sucrose (Singh, 2018).

5. Conclusion

Culturing of chinese mandarin micro scions which produced from multiplication stage on MS medium supplemented with suitable concentration of growth retardant; *i.e.,* 0.5 mg/l PP333 or 5.0 µg/l ABA for 30 days in vitro. Subsequently, transferred to the greenhouse and dipping it in IBA for 10 or 15 minutes before grafting gave the highest percentage of success grafts (100%) in greenhouse. This research considers a promising step in commercially production of in vitro micro scions and improve

the success of grafting in vivo. Also, this research insures to maintenance of the production of virus-free scions and eliminate viruses spread via grafting manipulation.

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