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Propagation of citrus rootstocks by stem cutting using auxin pretreatments: the case of citrumelo (*Citrus paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.)

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Abstract

In order to develop effective methods for rapid clonal multiplication of citrus rootstocks, we propose herein to investigate the effect of exogenously applied auxins on rooting and establishment of spring cuttings of two citrumelo accessions (*Citrus paradisi* Macf. x *Poncirus trifoliata* L. Raf), *i.e.* Swingle and Sacaton. Cuttings were taken from young greenhouse seedlings and exposed for 24 hours to different treatments including three auxin types (indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA)) and two concentrations of each one (100 and 200 ppm). Treated cuttings were then raised along with control cuttings under greenhouse conditions for six weeks. The results taken at the end of this period have shown significant variations in response to auxin treatments regarding survival, rooting and sprouting parameters. These followed similar patterns for the two accessions and were found to be dependant on the nature of the auxin rather than its concentration, the genotype or the interaction of these factors. Among the different combinations tested, IBA at 100 ppm level gave the highest survival (100%), the highest rooting percentage (100%) and was selected as the most suitable for promoting sprouting of citrumelo cuttings. By contrast, the application of NAA gave poor results and exerted a particular inhibitory effect on initiation of Swingle citrumelo roots and sprouts.

1. Introduction

Cutting is often the preferred method of propagation in horticulture since it is cheap, fast, simple and does not require the special techniques necessary in grafting, budding or micro-propagation. For citrus, - as many fruit crops - this method is necessary not only to maintain desirable characteristics of the mother plant [1] , but also to provide them with desirable quantities of true-to-type rootstocks. This fact may be of great importance since rootstocks are known to affect many scion characteristics such as vigor, yield, fruit quality and disease resistance, conditioning therefore the success of citrus industries [2-3-4-5-6] . As opposed to seedling, the products of cutting are uniform plants that do not exhibit the differences resulting from genetic variations [7] . Furthermore, it is reported that cutting has the tendency to decrease the juvenile stage of plants and reduce the time of nursery development [8]. The study of Verma et al. [9], for example, have shown a higher sprout length in cuttings of kagzi lime as compared to budded plants and nucellar seedlings.
A review of literature reveals that numerous studies were carried out to estimate the success of cutting in citrus species and have divided them into two groups: (i) Easy-to-root species such as lemons (*Citrus limon*), acid

limes (*Citrus aurantifolia*) and citrons (*Citrus medica*), and (ii) difficult-to-root species including sweet orange (*Citrus sinensis*), mandarins (*Citrus reticulate*) and trifoliate orange (*Poncirus trifoliata*) [10]. This ranking was confirmed later by Villas Boas et al. [11] who studied the rooting of stem cuttings of 16 citrus cultivars and reported low rooting rates in sweet orange and tangerines and higher rates in lemon 'Siciliano' and the citrons 'Etrog' and 'Diamante'. However, little is still known about the rooting ability of hybrid cultivars. Swingle Citrumelo, for example, which resulted from a cross between grapefruit (*Citrus paradisi*) and trifoliate orange in 1907 did not become widespread until the mid to late 1980s when it started to be used in Florida orchards [12]. This delay was mainly due to a shortage in supply of seeds and nursery trees because of quarantines related to citrus bacterial spot disease. It appeared also that some seeds of other citrumelos were used for propagation and mistakenly identified as Swingle during that period which may explain the unexpectedly poor performance of groves planted on Swingle [12] . Nowadays, although citrumelo proved to be tolerant to salinity [13-14] , alkalinity [15] , CTV (Citrus Tristeza Virus), nematodes and Phytophthora diseases [16], its behavior is known to be accession-dependant [17] . Moreover, its use is still limited to some countries such as Brazil and the U.S. It is therefore important to establish sound and accurate methods for mass production of this rootstock before considering a clonal selection or undertaking a breeding program.

Besides genotypic influence, the rooting potential of plant cuttings depends on growth regulators, especially auxins. Indeed, it has been established that endogenous and exogenous levels of auxins plays a key role in promoting root initiation in plants [18]. The indole-3-acetic acid (IAA) was the first plant hormone to be applied exogenously for rooting purposes [19]. In the same year, Zimmerman and Wilcoxon [20] discovered that several new synthetic auxins, among them indole-3-butyric acid (IBA) also promoted rooting. Later, various workers demonstrated that IBA is very effective in promoting rooting of many difficult-to-root species [21-22-23-9]. In citrus, Bhusal et al. [24] reported that cuttings of different species treated with IBA displayed a higher number and length of roots relative to non-treated. Similarly, Rossal and Kersten [25] showed that a quick dipping of 'Valencia' sweet orange cuttings before planting increased significantly the percentage of formed callus in the basal part of the roots. However, many inconsistencies can be noticed among these experiments due to differences in concentrations, formulas, additives or treatment durations. As a consequence, one can still find varieties and cultivars - in almost every species - that do not root even after treatment with auxins.

Thus, the objective of the present investigation was to evaluate the performance of spring cuttings of two citrumelo accessions, i.e. Swingle and Sacaton, in response to different auxin treatments in order to determinate an efficient method to produce uniform and true-to-type cuttings of this rootstock with predictable characteristics.

2. Experimental details

2.1. Plant material and growth conditions

In order to investigate the effect of auxin treatments on rooting and establishment of citrumelo cuttings (*Citrus paradisi* Macf. x *Poncirus trifoliata* L. Raf.), a greenhouse experiment was conducted at the Regional Center for Agricultural Research in Kenitra (Morocco) in late April 2015. The choice of this period was based on the fact that cuttings collected in late spring root most readily [26]. Uniform stem cuttings, with an average length of 12 cm and containing 3 to 4 buds were collected in the greenhouse from two-year-old healthy seedlings belonging to two citrumelo accessions: Swingle citrumelo and Sacaton citrumelo. The use of small, spindly and angular wood was avoided when possible, as this type of wood wasreported to be difficult to root [27].

2.2. Application of auxin treatments

After taking all the leaves off, the basal ends of the cuttings were soaked for 24 hours in water solutions containing Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and Naphtalene acetic acid (NAA) at 100 and 200 ppm concentrations. Control lots were treated with tap water for the same period. All cuttings were then planted in 60x40x12 cm sized plastic trays filled with sterilized peat moss and kept in mini tunnels to maintain a suitable environment for vegetative growth: 25-35°C temperature and 40-75% relative humidity. The irrigation was applied immediately after planting and every two days thereafter with tap water.

The experiment was laid out in a Split-Split-Plot Design with three replications and 10 cuttings per replication. Main plots consisted of two genotypes (Swingle and Sacaton), subplots consisted of the 3 treatments (IAA, IBA and NAA) and sub-subplots consisted of three auxin concentrations (0, 100 and 200 ppm).

2.3. Data collection and Analysis

After six weeks of growth, the cuttings were uprooted and relevant data were recorded. These included the number of roots per cutting, the number of sprouts per cutting, root length, sprout length and sprout diameter. Survival rate and rooting/sprouting percentages were also calculated based on the observations. Collected data were analyzed statistically using SAS package (SAS 9.0). Data were first subjected to analysis of variance at p=0.05 and square root techniques were applied wherever necessary (percentages). The means were compared using Duncan's multiple range test at 5% significance level.

3. Results

3.1. Effect of auxin treatments on cutting survival

ANOVA results indicated that survival rate was significantly affected by auxin type and concentration after six weeks of growth ($P<0.05$). However, no significant differences were found between treatments for Swingle citrumelo cuttings, although their survival rates ranged between 87% and 100% (Figure 1). By contrast, the response of Sacaton citrumelo cuttings varied among auxin treatments with maximum rates at 100 ppm IAA and 100 ppm IBA (100%) and minimum rates at 200 ppm IAA (84%).

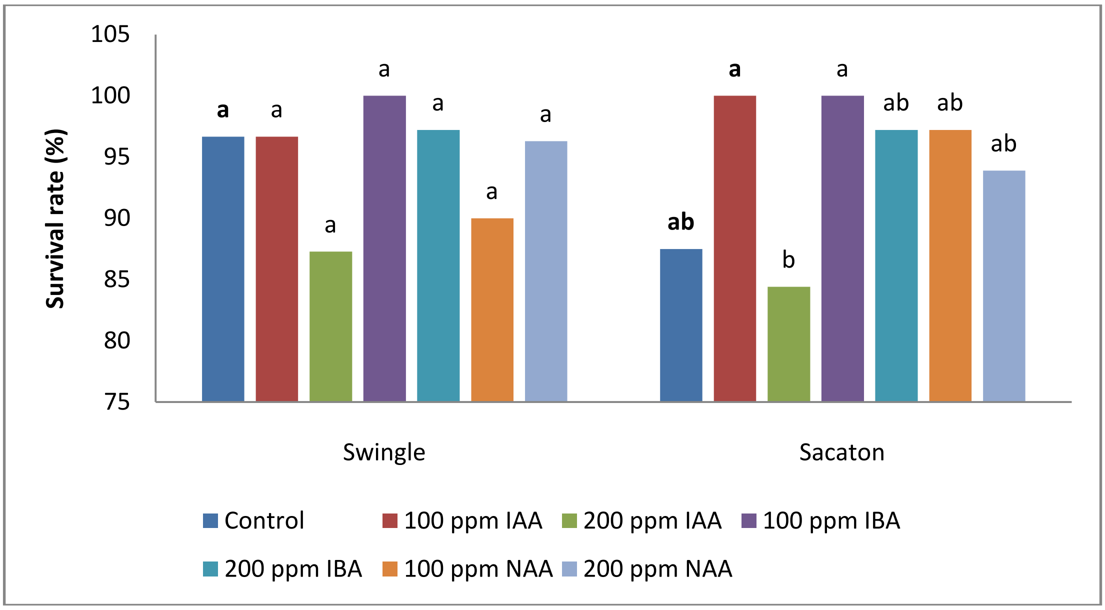

Figure 1: Effect of Auxin treatments on survival of citrumelo cuttings

3.2. Effect of auxin treatments on rooting

Six weeks after planting, cuttings of both citrumelo accessions formed roots to varying degrees. In instances where roots were not induced, some calluses were often developed at the basal end of cutting in response to auxin treatments. The basal end was swollen and the color was changed from green to whitish green.

The results recorded from the tested cuttings, including the percentage of rooting, the number of roots and root length are presented in the table 1. The analysis of variance showed a highly significant effect ($P<0.001$) of auxin type on all these parameters. By contrast, the effects of genotype and auxin concentration were only significant on the number of roots. In both accessions, maximum rooting percentage of 100% was achieved under the 100 ppm IBA treatment. However, cuttings treated with IAA at 200 ppm exhibited a higher number of primary roots reaching averages of 15.74 and 16.67 roots per cutting, respectively for Swingle and Sacaton accessions. The lowest values were recorded under control and NAA treatments with a rooting percentage of 68 to 81% and an average number of roots ranging from 2.89 to 4.39 roots per cutting.

Data recorded in respect to root length showed also a variation depending on the treatment applied. In Sacaton citrumelo cuttings, the longest roots were obtained under 100 ppm IAA (7.01 cm), 100 ppm IBA (6.78 cm) and 200 ppm IBA (6.47 cm), whereas it was the treatment 200 ppm IAA which stimulated the best elongation of Swingle citrumelo roots with an average length of 8.07 cm. The shortest roots were recorded under NAA and control treatments regardless of the accession studied. The corresponding values for these treatments varied from 4.12 and 4.79 cm.

Table 1: Effect of auxin treatments on rooting of citrumelo cuttings

Genotype	Treatment	Rooting[1] (%)		Number of roots[2]		Root lenght[2] (cm)	
C. Swingle	Control	74.35	bc[3]	2.89	c	4.70	b
	100 ppm IAA	96.67	ab	8.72	b	6.65	ab
	200 ppm IAA	93.94	ab	15.74	a	8.07	a
	100 ppm IBA	100.00	a	9.31	b	7.44	ab
	200 ppm IBA	94.19	ab	9.03	b	6.75	ab
	100 ppm NAA	73.33	bc	2.33	c	4.79	b
	200 ppm NAA	68.15	c	2.62	c	4.48	b
C. Sacaton	Control	75.00	b	3.12	d	4.12	b
	100 ppm IAA	96.67	ab	11.52	bc	7.01	a
	200 ppm IAA	94.10	ab	16.67	a	5.13	b
	100 ppm IBA	100.00	a	9.47	c	6.78	a
	200 ppm IBA	97.22	ab	12.74	b	6.47	a
	100 ppm NAA	81.48	ab	3.78	d	4.72	b
	200 ppm NAA	75.56	b	4.39	d	4.65	b
Analysis of variance[4]							
Genotype (G)		NS		**		NS	
Type of auxin (T)		***		***		***	
Concentration (C)		NS		***		NS	
G x T		NS		NS		NS	
G x C		NS		NS		NS	
T x C		NS		***		NS	
G x T x C		NS		NS		NS	

[1] Mean of 3 replications.
[2] Mean of 30 replications.
[3] For each accession, superscripts within columns indicate mean separation between treatments by Duncan's multiple range test at 0.05 level.
[4] Significant effects are indicated by asterisks: * = $P < 0.05$, ** = $P < 0.01$ et *** = $P < 0.001$, while non significant effects are indicated by NS.

3.3. Effect of auxin treatments on sprouting

ANOVA results revealed a significant effect of auxin type on all sprouting parameters studied at 0.01 level. The effects of genotype, auxin concentration and the interaction Auxin type x Auxin concentration were also significant with respect to the number of sprouts, sprout length and the number of leaves per cutting.

Among treatments, IBA at 100 ppm resulted in maximum number of sprouted cuttings after 45 days of growth with percentages of 94.44 and 100% respectively for Swingle and Sacaton cultivars, whereas control and NAA treatments resulted in the lowest values, ranging from 73 to 78% (Table 2).

Similarly, data pertaining to the number of sprouts per cutting, the length of sprouts and the number of leaves per sprout were the highest under the concentration 100 ppm of IBA with means of 2.06, 7.94 cm, 13.34 respectively in Swingle and 2.16, 7.34 cm, 13.22 in Sacaton (Table 2). This treatment was closely followed by the 100 ppm IAA treatment and contrasted to control and NAA treatments which resulted in minimum values. We should particularly note that NAA treatments resulted in lower values than control in the case of Swingle citrumelo cuttings suggesting a specific toxic effect.

In contrast to the aforementioned parameters, the results showed no or few differences among treatments in regards to sprout diameter.

Table 2: Effect of auxin treatments on sprouting of citrumelo cuttings

Genotype	Treatment	Sprouting[1] (%)	Number of sprouts[2]	Sprout lenght[2] (cm)	Sprout diameter[2] (mm)	Number of leaves[2]
C. Swingle	Control	78.06 b[3]	1.59 bc	3.65 cd	1.03 a	7.22 c
	100 ppm IAA	92.50 ab	1.83 ab	5.57 b	1.21 a	10.72 b
	200 ppm IAA	90.61 ab	1.52 bc	4.61 bcd	1.03 a	8.61 bc
	100 ppm IBA	94.44 a	2.06 a	7.94 a	1.36 a	13.34 a
	200 ppm IBA	85.10 ab	1.21 c	5.24 bc	1.06 a	7.73 c
	100 ppm NAA	73.33 b	1.43 bc	2.81 d	0.98 a	6.33 c
	200 ppm NAA	75.56 b	1.45 bc	3.05 d	1.33 a	6.21 c
C. Sacaton	Control	75.00 c	1.54 cd	3.25 c	0.98 b	6.37 c
	100 ppm IAA	96.67 ab	2.04 ab	6.89 ab	1.38 a	12.74 ab
	200 ppm IAA	90.77 ab	1.39 d	4.91 bc	1.09 b	8.61 bc
	100 ppm IBA	100.00 a	2.16 a	7.34 a	1.50 a	13.22 a
	200 ppm IBA	97.22 ab	1.88 abc	6.86 ab	1.39 a	11.94 ab
	100 ppm NAA	88.89 bc	1.50 cd	3.88 c	1.15 b	7.34 c
	200 ppm NAA	84.44 bc	1.68 bcd	3.89 c	1.14 b	7.97 c
Analysis of variance[4]						
Genotype (G)		NS	*	*	NS	**
Type of auxin (T)		**	**	***	**	***
Concentration (C)		NS	***	**	NS	***
G x T		NS	NS	NS	NS	NS
G x C		NS	NS	NS	NS	NS
T x C		NS	**	NS	*	**
G x T x C		NS	NS	NS	NS	*

[1] Mean of 3 replications.
[2] Mean of 30 replications.
[3] For each accession, superscripts within columns indicate mean separation between treatments by Duncan's multiple range test at 0.05.
[4] Significant effects are indicated by asterisks: * = P < 0.05, ** = P < 0.01 et *** = P < 0.001, while non significant effects are indicated by NS.

4. Discussion

Most of tested cuttings survived after six weeks of growth even in the non-treated control. This may be due to the suitable substrate and/or environmental conditions (temperature, humidity). Indeed, the effect of growth medium and seasonal variations on cutting establishment is well documented and peat medium was particularly recommended for growth of citrus cutting [28-29]. Furthermore, both accessions rooted successfully under water treatment, which implies that these can be ranked as easy-to-root cultivars. According to Habermann et al. [30] and Alam et al. [31] , rooting and survival of cuttings are correlated since the initiation of roots can promote rehydration of tissue and prevent their death from water stress.

All rooting parameters tended to increase to a similar extent in the two accessions in response of exogenous auxin application. We showed particularly in this study a high effectiveness of the combinations 100 ppm x IBA and 200 ppm x IAA. These findings are in line with previous works that have reported a higher activity of IBA and IAA in stimulating rooting of citrus species [24-32]. Some authors also stated that auxin concentrations may affect rooting response of cuttings [32-29]. In experiments where 100, 200 and 400 ppm concentrations of IAA and IBA were tested on cuttings of Eureka lemon, Homosassa sweet orange, sweet orange seedlings, sour orange, Calamindin, Cleopatra mandarin and Thornton tangelo [27], it was found (i) that 100 ppm concentration was no more effective than tap water (ii) a 200 ppm concentration greatly increased the number of roots induced on all varieties as compared with that obtained with tap water and (iii) a 400 ppm concentration caused some injury to the base of cuttings of some varieties and caused no apparent injury to cuttings of other varieties.

Figure 2: Appearance of Swingle citrumelo cuttings after six weeks of growth. A: Control ; B: 100 ppm IAA ; C: 200 ppm IAA ; D: 100 ppm IBA ; E: 200 ppm IBA ; F: 100 ppm NAA ; : 200 ppm NAA.

In contrast to IAA and IBA, cuttings treated with NAA had more difficulty to root. This might be due to differences in absorption and transport of these hormones. Indeed, although NAA proved to stimulate rooting of many plant species [33-34-35], it is thought that low levels of this hormone can be absorbed by treated tissues [36]. On the other hand, differences in uptake between IAA and IBA are unlikely according to Ludwig-Müller et al. [37] who followed the uptake of these two auxins for 24h and 48h and reported no significant differences during these time periods.

The literature reports also several cellular and biochemical explanations which may illustrate the differences observed in our study among auxins. Some authors attributed the effectiveness of IAA and IBA to their higher stability [1-38]. Others [39-40] studied the metabolism of IAA and IBA by mung cuttings and attributed the better activity of IBA to the rapid formation of IBAsp, an IBA conjugate, which promoted rooting better than IAA.. In the same line, Pythoud and Buchala [41] could not find any oxidation products of IBA in cuttings of

Populus tremula and assumed that this fact might be the cause of the better activity of IBA in rooting. It was also extensively reported that IAA and IBA have the particularity to increase the endogenous levels of free IAA and free IBA, which seem to play a key role in root initiation (42-43-44-45-46). In citrus, experiments on Rangpur lime have shown an increase in endogenous level of IAA in the bark of cuttings which was more than three times higher at the day 19 after exogenous treatment than at day 0 and correlated well with ease of rooting [47]. Similarly, Epstein et al. [48] observed a concomitant increase in free IBA with the rooting of easy- and difficult-to-root cultivars of sweet cherry (*Prunus avium* L.). They postulated that easy-to-root, as opposed to difficult-to-root cultivars, have the ability to hydrolyze the ester conjugate at the appropriate time to release free IBA, which may promote root initiation. This may be a plausible explanation in our case if we consider that citrumelos are easy-to-root cultivars.

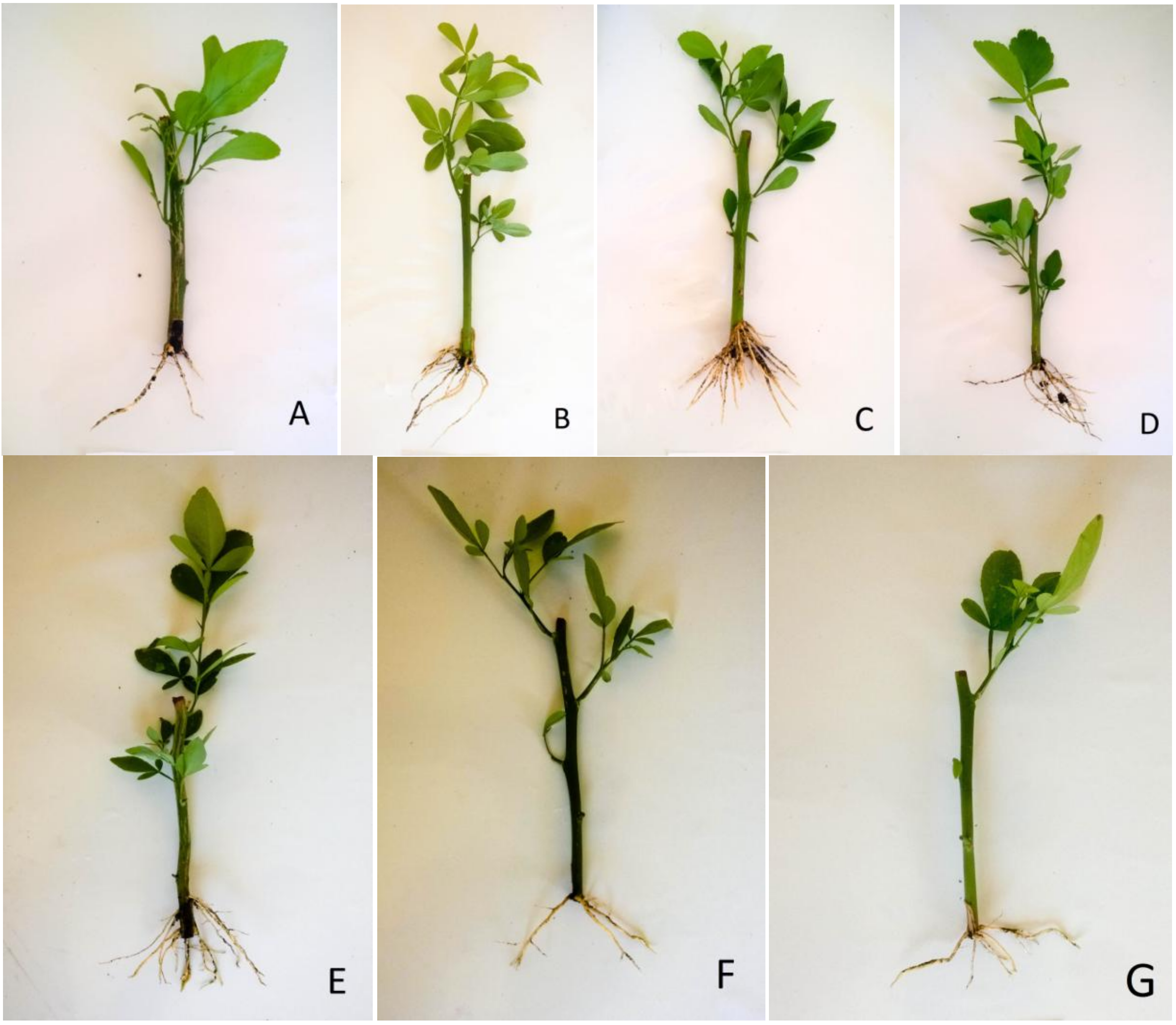

Figure 3: Appearance of Sacaton citrumelo cuttings after six weeks of growth. A: Control ; B: 100 ppm IAA ; C: 200 ppm IAA ; D: 100 ppm IBA ; E: 200 ppm IBA ; F: 100 ppm NAA ; : 200 ppm NAA.

Besides rooting, there were considerable variations in sprouting of citrumelo cuttings depending on the nature and the concentration of auxins applied. In general, NAA treatments did not differ from control, while IBA and IAA gave satisfactory results, particularly at 100 ppm level. Previous workers have also noticed an increasing effect of IBA on bud sprouting of sweet lime [49] and Satsuma orange [50] compared to untreated cuttings. Bhatt and Tomar [22] obtained maximum sprouting percentage (68.50%), maximum sprout length (6.59 cm) and maximum sprout diameter (0.32 cm) under the concentration 500 ppm after dipping *Citrus aurantifolia*

Swingle cuttings in concentrated solutions of IBA. According to the literature, the increase in sprouting may be due to more number of leaves and vigorous system, which may enhance the absorption of mineral and water and promote the production and assimilation of carbohydrates that are necessary for growth [31]. It is also thought that easy-to-root species can absorb more auxin and transport more of it to the aerial parts [18] where it can increase linear growth of cutting tissues through stimulation of cell elongation [51]. In this case, the differences in effectiveness among auxins may have resulted from differences in transport velocity as suggested by Epstein and Sagee [52] who found that IBA was transported in midribs of *Citrus* leaves at a somewhat lower (approximately 60%) rate than IAA.

In conclusion, the results of the present investigation clearly indicate that cuttings of citrumelo root easily and develop within a short time after treatment with low auxin concentrations for 24 h before planting. We particularly recommend the use of IBA and IAA at the 100 ppm concentration for this purpose. We should also note that this technique gave better results than those reported by early workers on citrumelo [53] and various other citrus species using the quick dip method [54-32-55-47]. Thus, it should be generalized in order to be used in large scale for mass production of improved material and effective establishment of citrus nursery stocks.

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