

ASSESSMENT OF THE SUCCESS OF MICRO GRAFTING CLEMENTINE TIMING ON SOUR ORANGE

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ABSTRACT

This study was carried out at three locations (Laboratory of Plant Tissue Culture, Fields of the College of Agriculture, University of Diyala and one of the private nursery at the city of Baqubah. The applied experiments included two separate experiments on the scion Mandarin Clementine micro grafted on Sour orange rootstock. The first micro grafting experiment aimed to develop a method of vegetative propagation of Clementine and transferring the micro grafts from a laboratory to the field and comparing grafting dates (spring, early autumn, late autumn) with the laboratory grafting after treating grafting region with different concentrations of gibberellin (0, 0.3 or 0.4 mg. L⁻¹). The effect of grafting dates on the percentages of success of grafted plants after one month of acclimatization. The experiment was carried out according to completely randomized design (CRD) as a factorial experiment with two factors and three replications for the first one, and as a single factor experiment with three replicates for the second experiment. Results showed a decline in the percentage of success of the grafting and a slight success correlated with increased gibberellin concentration in the aforementioned experiment at early fall grafting. A second field experiment, was conducted with the aim of studying the success of maintained micro propagated plants during summer season and the effect of foliar spray with salicylic acid (0, 200 or 400 mg. L⁻¹) and marine algae extract (0, 5 or 10 g. L⁻¹) on some characteristics of vegetative growth and some chemical characteristics. The experiment was carried out according to the Randomized Complete Block Design (RCBD), as a factorial experiment with two factors and three replications. No significant effect for both factors was recorded in most vegetative characteristics, while chemical composition was significantly affected caused by the two factors.

Key words: Grafting, *In vitro*, Citrus, Marine algae extract, Salicylic acid.

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تقييم نجاح تركيب لالانكي كلمنتاين الدقيق ووقت التركيب على أصل النارنج

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المستخلص

نفذت الدراسة الحالية في مختبر زراعة الانسجة النباتية وحقول كلية الزراعة جامعة ديالى واحد المشاتل الخاصة في مدينة بعقوبة، إذ تضمنت تجربتين منفصلتين على طعم اللالانكي كلمنتاين واصل النارنج. تضمنت التجربة الاولى التركيب الدقيق بهدف تطوير طرائق الإكثار الخضري للالانكي كلمنتاين ونقل تقانة التركيب الدقيق للحمضيات من العمل المختبري الى الحقل ودراسة تأثير مواعيد التركيب (التركيب الربيعي، التركيب الخريفي المبكر، التركيب الخريفي المتأخر مقارنة بالتركيب المختبري) وازافة تراكيز مختلفة من حامض الجبرلين GA3 (0، 0.3 أو 0.4 ملغم. لتر⁻¹) في النسبة المئوية لنجاح التركيب وتضمنت ايضا دراسة تأثير مواعيد التركيب في النسبة المئوية لنجاح النباتات المركبة بعد شهر على الاقل. نفذت التجربة وفق تصميم C.R.D كتجربة عاملية بعاملين وثلاثة مكررات للصفة الاولى، وكتجربة بسيطة بثلاثة مكررات للصفة الثانية، أظهرت النتائج تراجع التركيب الخريفي المبكر في النسبة المئوية لنجاح التركيب وتأثير طفيف لزيادة تراكيز حامض الجبرلين في الصفة المذكورة، فيما ظهر تفوق التركيب الخريفي بموعديه في النسبة المئوية لنجاح النباتات المركبة بعد شهر على الاقل، كما تضمنت الدراسة الحالية تجربة ثانية وهي التجربة الحقلية تأثير الرش الورقي بكل من حامض السالسليك (0، 200 أو 400 ملغم. لتر⁻¹) ومستخلص الطحالب البحرية (0، 5 أو 10 غم. لتر⁻¹) في بعض صفات النمو الخضري والكيميائية وأظهرت النتائج عدم وجود تأثير معنوي لكلا العاملين في اغلب الصفات الخضرية، فيما تبين وجود تأثيرات معنوية لزيادة التراكيز لكلا العاملين في الصفات الكيميائية.

الكلمات المفتاحية: التركيب، خارج الجسم الحي، الحمضيات، مستخلص الطحالب البحرية، حامض السالسليك.

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INTRODUCTION

Sour orange (*Citrus aurantium* L.) is an excellent rootstock for most citrus species and is suitable for heavy and medium soil texture, having a deep root system, large vegetative growth and tolerates high ground moisture, cold and salinity to some extent. One of the main defects of this rootstock is its easy infection with Tristeza (4). Clementine is a popular variety of Mandarin (*Citrus reticulata* Blanco.), it is an early ripening variety with desirable qualities (6). The most common method of propagating clementine is vegetative propagation by grafting on citrus root stocks. T-grafting is the most common grafting method (8). However, it is time consuming since the production of a grafted citrus seedlings requires two years to be ready for cultivation in the field (one year to grow the rootstock will be ready for grafting and a second year for the growth of the grafts). Micro grafting which is one of plant tissue culture application, is a promising method for propagating citrus species. The grafting process generally requires that the plant stem used as a scion contains a number of buds then dissected and placed on a rootstock in a professional manner. The later can be a seedling or a small vegetatively produced plant. Because of the continuous demand on budded citrus, large numbers at a short time, under controlled conditions, free of diseases and insects, year round production, such technology must be developed and automated. Incompatibility between grafts and rootstocks has been recorded in interspecific than interspecific grafts which may cause failure in the grafting region (5), and it may be due to physiological, metabolic and molecular mechanisms, although the mechanism is still unclear (7). Micro grafting technique is commonly used on woody species, especially fruit trees, as it was performed on different types of citrus species so scions confer virus resistance. The technology was developed by Murashige et al (17) in which they obtained small numbers of grafted citrus plants after fitting the growing apex of the scion on seedling rootstocks ex vivo. This technology was developed later by Navarro et al (19) who laid down the basic rules for micro grafting, with a success ranged between 30 - 50%. The

basis of the technology is the placing the top of a branches consisting of the apical meristem (meristematic dome) with 3 - 2 leaf primordia (0.1-0.5 mm in length) on a chosen rootstock grown in vitro for a period of two weeks. The technology is utilized for producing virus free micro grafted plants based on the fact that the meristematic apices are devoid of viruses due to the absence of direct vascular contact between the meristematic cells of the developing apex and the stem tissues (11, 18) achieved micro grafting success in Mandarin Clementine reached 42% when they micro grafted the apical meristem with three leaf principles (0.11 - 0.16 mm long), while Keita, and Noguchi (13) indicated a lower success rate when he used the same technique. (12) studied the degree of compatibility between five types of citrus roots with the clementine which was used as scion. They reported differences in the percentage of success when clementine was micro grafted on orange variety Valencia, Troyer. Strange, Citrumelo, and Carrizo citrange, giving a success rate of 14, 20, 31, 12, and 18% respectively. Pasquale et al (20) reported a difficulty in micro grafting Clementine on other types of citrus. Salicylic acid is a growth regulator of phenolic compounds that has regulatory roles in plant growth and improving its tolerance to stress, as it works to regulate ion absorption, hormonal balance, stomatal movement and flowering. It also accelerates the formation of chlorophyll and carotene pigments. In addition to its role in increasing photosynthesis the enzymes activity as well as contributes to increasing plant tolerance to osmotic stress, heat stress and salt stress (10), It also has a positive effect on the vegetative growth (2) and on the fruiting characteristics (3). Seaweed extract is among the important sources that have been employed in agricultural production when supplemented to chemical fertilizers. It stimulates plant growth at appropriate concentrations since it contains macro and micro nutrients and even growth-promoting substances such as auxins, auxin-like substances, vitamins, amino acids, organics, as well as the amino acid Glycine betaine, which is an osmoticum and thus attributes to the role of these extracts in increasing the plant's tolerance to salinity and drought. Seaweed

extract releases nutrients slowly compared to mineral fertilizers (15) It also has a positive effect on the vegetative growth (22). Other researchers also showed that seaweed extract improves growth and increases production in many fruit trees (1). Based on the big demand on citrus trees by farmers, and the clear shortage in market supply and because of the high-cost production by conventional budding and grafting methods and certainly its limitation to be conducted in certain months, thus the present study was designed to produce Clementine trees grafted on Sour orange rootstock, using the technique of the micro grafting after treatment with salicylic and seaweed extract.

MATERIALS AND METHODS

Two experiments, a micro grafting experiment, and a field experiment were conducted, the micro grafting experiment which included micro grafting in vitro and micro grafting in vivo (field). The later was carried out at three dates (spring, early fall, and late fall) using Cleft grafting. Gibberellic acid (GA3) at three concentrations (0.0, 0.3 or 0.4 mg. L-1) was examined by immersing the grafts in the solution for two minutes before micro grafting. The experiment was carried out according to CRD as a factorial experiment with two factors and three replications for the first trait, and one factor experiment with three replicates for the second trait. Results were analyzed using the analysis of variance using the statistical program SAS, and the differences between the means were compared according to the Duncan's Multiple Range Test at a probability level of 0.05. Mandarin clementine seedlings of 2 years old grafted on sour orange rootstock were used as a source of grafts, leaves were removed manually and branches (3 cm long) were washed with running water for 20-30 minutes, then put in plastic bags. In vitro grafting: The Explants prepared for surface cultivation were sterilized to get rid of contaminants by immersing them in a solution of sodium hypochlorite (NaOCl) at a concentration of 0.6% for 15 minutes and the volume was completed to 100 ml with sterile distilled water for each concentration with the addition of two drops of liquid soap to reduce leaf surface tension. The plant parts were

immersed in the sterilization solution and washed with sterile distilled water three times, with constant stirring.

Preparation and sterilization of the medium Murashige and Tucker medium (MT) (16) supplemented with 30 g. L-1 sucrose and 7 g. L-1 agar was used. The pH was adjusted to 5.7 -5.8 (21). The medium was distributed into 200 ml glass vials of 20 ml each, then sterilized with an autoclave at 121 ° C and 1.04 kg cm-2 for a period of 15 minutes.

Tools of Sterilization

All tools used in vitro work, such as tweezers, scalpels, petri dishes, as well as distilled water used for washing plant parts were sterilized with autoclave at a temperature of 120 ° C and under pressure of 1.04 kg.cm2 for 15 minutes. The rootstock seedlings grown on MS medium were used when they reached 3-5 cm and a diameter of 1-2 mm, thus seedlings were ready for micro grafting. Grafted seedlings were lifted from the culture bottles and transferred to sterile petri dishes under sterile conditions. The tops of seedlings were chopped to 2-5 cm to be grafted on, then the roots were shortened to 4-6 cm and the axillary buds were removed. After that, an incision was made in the middle of the upper section of the rootstock at a length of 3 mm and the operation was performed with small forceps and a scalpel No.11 which was carried on holder No. 3.

Scion preparation

After performing a superficial sterilization on the plant parts, their lower ends that may have been damaged by the sterile material were cut off and the leaves were removed except for the apical leaves as they were shortened and the goal of keeping them was to facilitate holding the plant part during the grafting. After that, a cut was made in the lower two sides in the form Wedge, and the branches were then treated with gibberellic acid solution.

Grafting implementation

Using small forceps, the branch was transferred from the solution and inserted in the cut area inside the incision and then the area was wrapped with a cotton thread to ensure that the bark of the rootstock was matching to the bark of the graft then the grafted seedling was transferred inside the vial. Vials were covered with aluminum foil and then incubated in the growth chamber at a

temperature of 24 ° C. under a light intensity of 2000 Lux for 16 hours of lighting, day-1, and the data were recorded 15 days after grafting.

Field grafting

The rootstock seedlings resulting from the seed germination experiments were used in the grafting. After germination, seedlings were lifted from the vials and the acclimatization process was performed by washing them from the medium residue with running water and soaking them with fungicide, then transferred into 5 cm diameter pots filled with a previously sterilized peat moss with a heat sterilizer and the seedlings were placed in the growth room for a period of 7 days. Thereafter, they were transferred to the greenhouse to be ready for field grafting. Ends were cut, leaving about 1.5-2 cm of the apical tops, and the axillary buds were removed. An incision was made at the middle of the upper section of the rootstock with a length of 3 mm and the operation was performed with small forceps and a scalpel No. 11 which carried out on a holder No. 3.

Scion preparation

Leaves were removed from the branches except apical ones which were shortened in order to facilitate holding the explant during grafting. After that, the branches were then cut from both lower sides in the shape of a wedge, branches were then immersed with gibberellin acid solution.

Grafting execution of

Using small forceps, branches were transferred from the solution and inserted in the cut area inside the incision, tied with a cotton thread, and pots containing the grafted plants were covered with plastic transparent bags to preserve moisture. Data were recorded 15 days after the grafting (Fig.1).

Grafting dates

- 1- Spring grafting was conducted during the period 12-15 April, 2020.
- 2- An early fall grafting was carried out during the period 15-17, October, 2020.
- 3- A late fall grafting was carried out during the period 15-17, November, 2020.

Studied traits

1-The percentage of micro grafting success (%) = (number of successful grafted plants / number of total grafted plants) *100

The percentage success rate of grafted plants was calculated after one month of acclimatization.

2-The effect of gibberellic acid was not considered, so only the effect of grafting dates was calculated, as the plants that exhibited success in the micro grafting were taken into account in each replicate and for each season which were acclimatized. The percentage of succeeded grafted plants after one month of acclimatization were calculated = (number of plant showed successful grafting after one month of acclimatization / number of plants that succeeded in grafting before acclimatization) *100.

Field experiment

The experiment was carried out in a nursery in Baqubah city / Diyala governorate on grafted plants resulting from the spring grafting after transferring plants from 5 cm diameter pots to 20 cm diameter pots filled with a mixture of peat moss and soil mixture at a ratio 4: 1 (v/v). Grafted plants were sprayed with three concentrations of salicylic acid (0, 200, 400 mg.l-1), and three concentrations of marine algae extract (0, 5, 10 g.l-1). The treatments were carried out after all the plants formed four or more leaves. The experiment was carried out according to the Randomized Complete Block design (RCBD), as a factorial experiment with two factors and three replications. The results were analyzed using the analysis of variance by the statistical program SAS, and the differences between the means were compared according to the Duncan's Multiple Range Test at a probability 0.05 level.

Salicylic acid: Salicylic acid powder was dissolved in distilled water and sprayed on leaves at three concentrations (0, 200, 400 mg. L-1), using six replicate plants (starting from 19/7/2020), at 15-day intervals.

Seaweed extract: The seaweed was extracted with water and sprayed on the leaves at three concentrations (0, 5, 10 g. L-1), and using three plant replicates (starting from 22/8/2020) at 15 days intervals.

Studied traits

Initial measurements were taken on 15/7/2020, and final measurements were taken on 15/12/2020 which included:

1-Effect of gibberellin concentration and the date of grafting and their Interaction on the percentage of micro grafting success.

2- The mean increase in plant height (cm) which was calculated using a ruler, the height

was recorded before the start of the experiment and after the completion of the treatments, and the percentage increase in the plant height was calculated.

3- Mean number of branches. plant⁻¹.

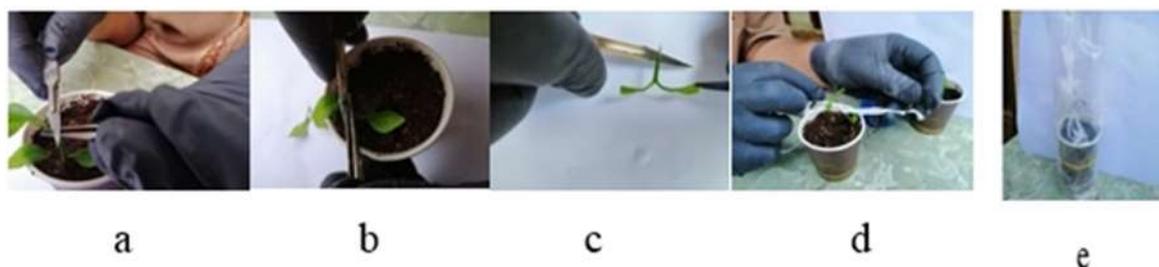


Figure 1. Steps for field grafting (a) Earring top of rootstock seedling (b) A slit in the rootstock seedling was done (c) The bottom of the scion in the form of a wedge was done (d) The scion was inserted to the rootstock (e) The grafted plant was covered with polyethylene to retain moisture

RESULTS AND DISCUSSION

Micro grafting experiment

Percentage of success of the micro grafted plants.

Results in Table 1 show a steady proportional increases in the percentages of grafting success with increasing gibberellin concentration in which the branches were immersed before grafting, despite of the non-significant differences between mean values. Results revealed clearly that the time of year in which micro grafting is carried out is so critical, since the that the late autumn grafting date significantly exceeded other treatments (96.30%), while the early autumn grafting date gave the lowest percentage (11.11%). The interaction between 0 mg.L⁻¹ gibberellin concentration at the late autumn grafting, interaction between the 0.3 mg.L⁻¹ and both the spring date and the late autumn date and the interaction between gibberellin at 0.4 mg.L⁻¹ and the laboratory micro grafting displayed

the highest percentage of success in grafting reached 100% as shown in Figure 2. While the interaction between the concentration of gibberellin at 0 mg. L⁻¹ and the early autumn grafting and the interaction between the concentration of gibberellin at 0.3 mg.L⁻¹ and the early autumn grafting, gave the lowest percentage of success (0%). Grafting success in vitro (at the laboratory scale) has enormously been increases with increasing gibberellin concentration. Although gibberellin role in promoting cell division and elongation has been documented elsewhere in the literature, it has improved the percentage of grafting success. Results revealed that late autumn micro grafting date is influential towards rapid integration between scion and rootstock. The drop in atmospheric temperature at this time of year in the region where the experiment was conducted could be the influential factor raised the percentage of grafting success.

Table 1. Effect of gibberellin concentration, the date of grafting and the interaction between them on the percentage of micro grafting success

The concentration of gibberellin mg. L ⁻¹	Grafting date			Laboratory	Mean effect of gibberellin concentration
	Spring	Early autumn	Late autumn		
0	88.89 a	0 c	100 a	33.33 Bc	55.55 A
0.3	100 a	0 c	100 a	77.77 A	69.44 A
0.4	66.66 ab	33.33 bc	88.89 a	100 A	72.22 A
Mean effect of grafting date	85.18 AB	11.11 C	96.30 A	70.37 B	

* Means with similar letters do not differ significantly at a probability level of 0.05 according to Duncan polynomial test

Percentage of grafted plants success after one month of acclimatization

Significant differences in the percentages of survival of grafted plants after a month of acclimatization are shown in Table 2, as the late autumn grafting gave the highest percentage of success in grafted plants after one month of acclimatization, reached 85%, while the laboratory grafting gave the lowest rate recording 16.10%.

Table 2. Effect of grafting date on the percentage of success in grafted plants after one month of acclimatization.

Grafting date	Success of grafted plants after one month of acclimatization (%)
Spring	43.67 AB
Early autumn	66.67 AB
Late autumn	85 A
Laboratory	16.1 B

* Means with similar letters do not differ significantly at a probability level of 0.05 according to Duncan polynomial test.

The results in Tables 1 and 2 shows that the late fall grafting gave the highest mean values in both characteristics, and the low success rates in the early autumnal grafting may be because of the high temperature during the period of the grafting procedure, as it reached in the period from 15-20/10/2020 to 40 oC and this may led to the death of the tender tissues of the scion. Table 1 shows an increase in the percentage of success of the grafted plants with increasing the concentration of gibberellin, as the effect of internal gibberellin leads to an increase in the speed of healing in the grafting region by stimulating cell division and expansion (9). Data in Table 2 shows the superiority of the date of the fall grafting over the spring and the laboratory micro grafting. The low percentage of plant success resulted from the laboratory grafting may due to the delicacy of plants resulting from in vitro propagation and their endurance to field conditions.

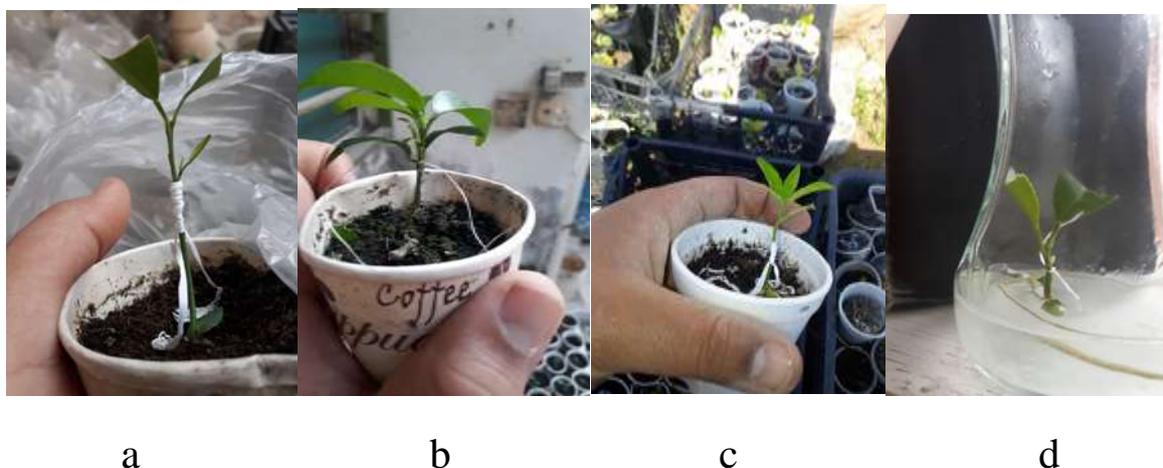


Figure 2. Successfully grafted plants a) Grafted plant in the laboratory after 15 days. b) Grafted plant in the field after 12 days .c) A plant produced from the spring grafting, after a month of acclimatization. d) A plant produced from a late autumn grafting, after one month of acclimatization

Field experiment

Results displayed in Table 3 show a negative effect of increasing salicylic acid on the mean increase in plant height, as there was no significant difference between the two treatments 0 and 200 mg. L⁻¹ (35.65 and 32.44%, respectively) while the treatment of 400 mg. L⁻¹ recorded 28.15%). The results of the same Table show the presence of

significant effects after treatment with seaweed extract, as treatment with 10 g. L⁻¹ recorded 42.35% which outperformed compared to treatment with 5 g. L⁻¹ (29.38%), which again outperformed at a concentration of 0 g.L⁻¹ (24.51%). Results of the same Table also show that there were significant differences when the two factors were interacted, as the interaction between the

concentration of salicylic acid 0 mg. L⁻¹ and the concentration of seaweed extract 10 g. L⁻¹ achieved the highest % increase in plant height, which reached 55.18%, The interaction

between the concentration of salicylic acid 200 mg. L⁻¹ and the concentration of seaweed extract 0 g. L⁻¹ recorded the lowest increase in plant height (8.19%).

Table 3. Effect of salicylic acid and seaweed extract and their interaction on the mean increase in plant height (%).

Salicylic acid mg. L ⁻¹	Seaweed extract g.L ⁻¹			Mean effect of salicylic acid
	0	5	10	
0	21.60 e	30.16 d	55.18 a	35.65 A
200	8.19 f	48.53 b	40.62 c	32.44 A
400	43.74 bc	9.45 f	31.25 d	28.15 B
Mean effect of seaweed extract	24.51 C	29.38 B	42.35 A	

* Means with similar letters do not differ significantly at a probability level of 0.05 according to Duncan polynomial test

Mean number of branches. plant⁻¹

The mean number of branches was not significantly affected after treatment with

salicylic acid and seaweed extract and even at the interaction between them (Table 4).

Table 4. Effect of spraying salicylic acid and seaweed extracts and their interaction on the mean number of branches

Salicylic acid L ⁻¹	Seaweed extract g.L ⁻¹			Mean effect of salicylic acid
	0	5	10	
0	1.333 a	1 a	2 a	1.44 A
200	2 a	2 a	2 a	2 A
400	2.333 a	1 a	2 a	1.78 A
Mean effect of seaweed extract	1.889 A	1.333 A	2 A	

* Means with similar letters do not differ significantly at a probability level of 0.05 according to Duncan polynomial test.

The results in Table 4 show a significant effect in the treatment with salicylic acid and seaweed extract, and their interaction on the mean increase in plant height, as a positive effect was recorded after seaweed extract treatment. Meanwhile, there were no significant differences due to the effect of treatment with salicylic acid and seaweed extract on the mean number of plant branches. The reason for the increase in plant height may be due to that seaweed extract containing nutrients that lead to increase the plant's metabolic activities, including potassium, which is necessary for activating enzymes necessary for the synthesis of amino acids and proteins, as well as help to synthesize chlorophyll which is important in the process of photosynthesis and the formation of sugars, proteins and ATP, and thus lead to increase plant growth and development, which

ultimately lead to an increase in vegetative growth characteristics (14).

CONCLUSION

Results of study was showed that the micro grafting of citrus species can be adopted in which rootstocks are neither very tiny (in vitro) nor very large (conventional grafting). Choosing the right timing for micro grafting alongside the immersion of grafts with the proper concentration of gibberellin, seem to be significant factors in improving the alignment between grafts and rootstocks. Foliar spraying with natural extracts such as seaweed accompanied with plant derived hormones such as salicylic acid improves rootstock-graft union.

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