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Differences in morphological, biochemical, and genetic characteristics of grape explants grafted using various micrografting techniques

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Abstract

The study aimed to improve the micrografting technique for rapid *in vitro* grapevine plantlet propagation using Flame Seedless scion and Freedom as rootstock. Explants obtained *in vitro* were cultured in MS media containing 0.4 mg L^{-1} BAP. For micrografting, six methods were used: sterile wire ties, sterile metal needle, wax, aluminum foil, gel, and callus. Data was collected on morphological and biochemical parameters in micrografts produced using various procedures. With various degrees of success, all *in vitro* micrografting procedures successfully produced grafts. The best results of morphological parameters were recorded in micrografting by wax method with the highest survival (83.88%), number of shoots/explants (1.77), shoot length (1.66 cm), leaves/shoot (4.55), rooting (58.20 %), root length (1.27 cm). In contrast, the callus method had the least value for these parameters. Wax and aluminum foil methods were found to be a promising alternative to traditional grapevine propagation methods in the study.

Key words: Grapevines, tissue culture, micro grafting, Flame seedless, Freedom, solid stacking wax and alginate matrix

Introduction

Grapevine (*Vitis vinifera* L.) is a widespread and highly valuable horticultural crop belonging to *Vitaceae*. Around 6.931 million hectares are grown worldwide. Italy, France, the United States, Spain and China are the main producers, accounting for half the total world production of 74.276 million Mt (FAO, 2017).

In Egypt, grape occupies the second-ranked fruit after citrus and it has a great importance and plays an important role in the agricultural economy (Abido *et al.*, 2013). The necessity to modernize the planting material production technologies of fruit crops has been stimulated by many considerations, such as the trends towards increasing the planting densities in field-grown trees and the transition to intensive growing systems, which includes the selection of new parents for breeding programmers, the creation and introduction of new cultivars and the modernization of tree habit and pruning. All of these changes have created a demand for more and more quantities of quality planting material. The conventional system of propagation is time-consuming and the material raised is neither uniform nor healthy. Applying *in vitro* techniques in fruit growing can be a viable alternative to circumvent these problems.

Micrografting technique appears to be helpful in early screening of graft compatibility in fruit crops. Micrografting has applications for the physiological analysis of the rejuvenation of mature phase plants. It is possible to graft at any time of the year and to transplant the grafted plants when desired (Ali and Mirza, 2006). Grafting under *in vitro* conditions has several advantages both for production and research. *In vitro* shoot tip grafting has often been applied for the (i) improvement and rejuvenation of several tree species **(**Bandeira *et al.*, 2006); (ii) virus elimination (Bisognin *et al.*, 2008; Riberio *et al.*, 2008); (iii) study of physiological connections between rootstocks and scions such as (in) compatibility, root to shoot communication or transport and (iv) use in quarantine as this method has a minimum risk for importing plants**.** Due to the multiple uses and advantages of shoot tip grafting, this technology may be of interest or potential practical value to technicians, researchers and nursery operators. Professionals in each of these areas would benefit from introducing a more straightforward and efficient micrografting procedure that is less dependent on mastery of complex techniques and thus will contribute to the practical utility of micrografting as a tool in fruit tree biotechnology.

The present study was designed to optimize the micro nodes and micrografting technique for rapid propagation of grapevines plantlets *in vitro* to establish a suitable protocol of micrografting, including some methods of grafting such as sterile wire tie, sterile metal needle, wax, aluminum foil, gel and callus for grape plants of Flame scion on Freedom rootstock.

Materials and methods

Plant material: Flame Seedless as a scion and Freedom as a rootstock were used as micro nodes. Plants were purchased from the Horticultural Research Institute A.R.C.'s Viticulture Research Department. During 2017-2020, the research was carried out at the Tissue Culture and Germplasm Conservation Research Laboratory–Horticulture Research Institute–Agriculture Research Center–Giza, Egypt, in collaboration with the Agriculture Faculty–Cairo University.

Shootlet production: Explants of scion and rootstock were cut, ranging from 1.0 -2.0 cm in length. The microcuttings were divided into groups, each containing 2-3 nodes. The explants

of micro cutting (piece of 0.5 x 2 cm^2 carried 1-2 lateral buds) were taken from March to April. The explants were washed three times with sterile distilled water and immersed in 70% ethanol solution (v/v) for 30 sec, then washed in Clorox (commercial bleach containing 2.25% sodium hypochlorite) at 15% for 15 min, plus tween 20 (2 drops /100 mL) with agitation under aseptic conditions. Finally, the explants were washed three times with distilled water. The treated explants cultured on MS medium supplemented with BAP 0.2 mgL^{-1} for shootlet proliferation and 2,4 D mgL^{-1} for callus production and incubated at a photoperiod of 16/8 illumination, day and night at 27℃ for 1, 3 and 7days.

Micro graft method

Solid stacking wax: The explants were divided into 2-3 cm pieces for each scion and rootstock. They were put in a straight line and used a candle to drop wax on the union area between scion and rootstock. After cooling the explants and wax complete solidifying, the grafted explants were cultured vertically in MS Media.

Micro metallic needle: The metallic needle with a diameter of 0.45 mm and a length of 13 mm was cut and planted in the centre of the rootstock about 6 mm and the other side was planted in the scion. The grafted explant was then placed in culture media.

Aluminum foil: They were thin strips of aluminum foil used to bind the grafted plants. They have many disadvantages: its sharp ends causing the cutting of plant tissues, wrapping micro-grafted region need skilled labor and time-consuming procedure to apply, and the binding with these aluminum strips is not tight.

Sterile wire tie: The rootstock was cut V shape in the top and scion was cut to the opposite shape of V. The end of scion and top of rootstock was connected and tied by wool wire.

Alginate matrix gel: Sodium alginate gel was used to produce gel solid matrix with $CaCl_2$. This gel was used as a connection between scion and rootstock.

Regeneration of grafted explants: The micrografted explants were cultured on MS medium containing various concentrations of salts at 1000, 3000, 5000 and 7000 ppm of combination of $CaCl_2$: NaCl. After the incubation period, each treatment was used for growth measurement and chemical composition was tested according to A.O.A.C. (2006).

Morphological characteristics of shootlets for multiplication stage: The culture in this stage was incubated for one month and the parameters were determined:

Survival percentage= [No. of survived explants/total number of cultured explants] x 100.

Number of shoots was calculated as the number of developed shoots per explants.

Callusing %= [No. explants formed callus/ total number of cultured explants] x 100.

Rooting %= [No. of rooted explants/ total number of cultured explants] x 100.

Total soluble phenols changes: Extraction of phenolic compounds was carried out according to the method described by (Farhadi *et al.*, 2016).

Flavonoids: The flavonoids were determined by Farhadi *et al.* (2016) method using apeginin as a reference compound.

Oxidized enzymes: The plant materials used for the estimation of catalase (CAT), peroxidase (POX) and polyphenol oxidase (PPO) enzymes were the terminal buds in addition to the first and second young leaves**.** Catalase CAT activity was assayed. One unit of POD enzyme activity was defined as the amount of the enzyme that catalyzed the conversion of one micromole of H_2O_2 per minute at 25 ºC (Del-Toro-Sánchez *et al.*, 2013). The PPO enzyme activity was expressed as v the changes in the optical density/gram fresh weight/h (Bian and Jiang, 2009).

DNA extraction: Gene JET Plant Genomic DNA Purification Mini Kit (Thermo Scientific).

PCR reaction: MyTaq™ Red Mix (BIOLINE).

PCR conditions: 1.5 min at 94°C for initial denaturizing, 35 cycles of 45s at 94°C (denaturation), 45s at 45°C (annealing) and 1.5 min at 72°C (extension), then 45s at 94°C, 45s at 44°C, followed by 5 min at 72°C for final extension, and finally stored at 4°C (Hu *et al.*, 2006).

List of ISSR primers

S.No.	Name	Sequence
1	HB 10	GAG AGA GAG AGA CC (GA)6CC
2	UBC-807	AGA GAG AGA GAG AGA GT (AG)8T
3	UBC-809	AGA GAG AGA GAG AGA GG (AG)8G
4	UBC-811	GAG AGA GAG AGA GAG AC (GA)8C
5	UBC-812	GAG AGA GAG AGA GAG AA (GA)8A
6	UBC-817	CAC ACA CAC ACA CAC AA (CA)8A

Statistical analysis: Data obtained were recorded and exposed to the proper statistical analysis of complete randomized design in three replicates. Means obtained were differentiated using Duncan new multiple range test as described by (Demirhan *et al.,* 2010).

Results and discussion

Changes obtained by micrografting

Morphological changes: As shown in Table 2, morphological characters such as percentage of survival, number of shoots per plant, shoot length, number of leaves/shoot, percentage of rooting, root length and percentage of callus were significantly affected by different treatments. Concerning the effect of micrografting methods, data in Table 1 showed that the highest values with a significant effect on survival (83.86%) were recorded by the method of wax followed by aluminum foil (83.86%) followed by wire tie (54.99%) and then sterile metal needle (41.66%). In contrast, the lowest survival was recorded in the callus and gel method. Regarding the effect of micrografting methods, data in Table 2 showed that wax method significantly recorded the highest number of shoots/plant (1.77). At the same time, the lowest number of shoots/plant (0.00) was in callus micrografting method. Data in Table 2 showed that the highest value with a significant effect on the number of leaves per shoot (4.55)

was recorded by wax method and the lowest (0.00) in callus micrografting method. These findings are consistent with the results in apple (Aazami and Bagher, 2010), peach, plum and apricot (Hussain *et al.*, 2014). One of the main aims of micrografting is to provide a solution for some physiological and anatomical problems between related species and cultivars. This has been answered in citrus fruits, almond (Conejero *et al.*, 2013) and plum. Another common problem is the low successful graft union percentage. In most cases and for the majority of plants this is due to the small size of shoot-tip organ, making the excision, handling, grafting and subsequent maintenance of grafted assembly difficult and leading to the drying of shoot-tips and low graft integration. Despite that, *in vivo* derived shoot-tips are larger in size than *in vitro*-derived ones, making handling of them easier (Ali and Mirza, 2006). However, they contain more phenolic compounds and hormonal content resulting in higher polyphenol oxidases and peroxidase activity and hence higher browning and drying of fresh tissue occur just before and after scion and rootstock integration (Pahnekolayi *et al.*, 2019).

In vitro culture condition has the advantage that it is not dependent upon growing season. Furthermore, it is possible to perform micrografting at any desired time. Another principal benefit of *in vitro* culture condition over *in vivo* grown plants is attaining the high number of shoot-tip explants owing to possible frequent subcultures of individual shoots under controlled conditions. Unlike other asexual propagation methods, micrografting produces disease-free, especially virus-free plants, with possible benefits of scion rootstock combinations (Youssef *et al.*, 2009). In grapevine, micro-grafting is a high-tech method for producing disease-free material for cultivation and breeding programs for detecting virus infections (Youssef *et al.*, 2009). This method of propagation yields a homogenous clonal disease free population of plants, capable of high establishment and performance potential in field conditions. The success of micrografting primarily depends on the grafting technique. In our study cleft grafting, where the joined parts were legated with aseptic wax or aluminum foil, proved to be the most successful and efficient method for *in vitro* micrografting of grapevines. Grafting success was determined by callus formation and rooting. Callus tended to be formed at the junction of a graft union, arising from the living cells of both, scion and rootstock. In *in vitro* grafting of grape with phylloxera-resistant rootstock cultivars, the grafting success was determined by callus formation and rooting. In this study, the rootstock Freedom was successful in rooting, callus formation, and growth with Flame seedless scion. In our study, the micrografting survival was about 83.88% in wax method and 83.86% in aluminum foil in Freedom rootstock × Flame seedless scion. Eventually, micrografting is an alternative suitable propagation method leading to the higher growth potential of grafted populations.

Biochemical changes

Total soluble phenols: The method of grafting gave different changes in chemical contents in the grafting area, especially after grafting. The main chemical variation obtained in explants was oxidized enzymes activity, phenols, flavonoids, and total antioxidants compared with each rootstock and scion. The data in Table 3 showed a significant interaction effect between grafting methods and scion and rootstock. The data showed increase in total soluble phenols in the grafting area by using wax for Flame x Freedom (0.0162 %) compared with total phenols in Freedom (0.0112 %), Flame (0.0067 %).

Total antioxidants: The data in Table 3 showed both of grafting areas scored the lowest value of antioxidants (45.88 %) for F/F, indicating increased phenolic compounds but the scion and rootstock gave the highest level of total antioxidants 65.12 % for Freedom, 66.28 % for Flame. In addition, the oxidized enzyme activity increased in presence of phenolic compounds, as the phenolic compound in grafting area were high that increased CAT, PPO and POD enzyme activity. The data in Table 3 explain the peroxidase activity that gave the same results like catalase; where it increased with grafting methods (0.11 and 0.12 U/mL, respectively) for wax and alginate.

Total flavonoids: In the case of using wax and alginate in micrografting methods increase phenolic compound refer to decrease all compound scavenging with phenolic compound included flavonoids. The data in Table 3 showed that the percentage of total flavonoids decreased with micrografting method wax (solid stacking) and Flame (scion) (0.821 and 0.914%, respectively) compared with flavonoids in Freedom rootstock. On the other hand, the polyphenol oxidase activity increased to the highest level (0.008 U/mL) for grafting by wax method (solid stacking).

Plant growth regulators: According to the obtained data in Table 4, ABA content was the highest in grafting flame on Freedom (0.02981 µg/100 g FW). Moreover, GA_3 in the grafting area of Flame on FFreedom gave the highest value (0.3082 µg/100 g FW) compared with other methods (0.2322 µg/100 g FW) followed by IAA content which gave 0.1358 µg/100 g FW for grafting Flame on Freedom compare with the other method 0.0936 µg/100 g FW.

Genetical studies

ISSR assay: The ISSR are ideal as markers for genetic mapping and population studies because of their abundance and the high degree of polymorphism between individuals within a population of closely related genotypes. These properties indicate their potential role as good supplements for RAPD based genome

Table 2. Effect of various micro grafting methods in grape (Flame x Freedom) on morphological characters

Methods	Treatments	Survival %	No. of shoots	Shoot length	No. of leaves/ shoot	Rooting %	Root length	Callus %
Physical methods	Solid stacking wax	83.88a	1.77a	1.66a	4.55a	58.20a	1.27a	55.55a
	Alummonium foil	83.86a	1.44ab	1.55a	2.44b	55.53a	1.04a	49.99a
	Wire tie	54.99a	1.33ab	1.52a	1.66bc	23.14b	0.38ab	23.14b
	Sterile metal needles	41.66a	1.33ab	0.90b	1.00c	23.14b	0.43bc	0.00c
Chemical methods	Alginate matrix gel	37.03b	0.66bc	0.46bc	1.00c	8.33b	0.00c	0.00c
Biological methods	Callus binding cells	27.03b	0.00c	0.00c	0.00d	0.00c	0.00c	0.00c
	Metal sheet	23.14b	0.00c	0.00c	0.00d	0.00c	0.00c	0.00c
	LSD (5 %)	43.28	1.12	0.89	2.21	26.12	0.32	25.18

Table 3. Effect of various micro grafting methods in grape (Flame x Freedom) on antioxidant profile

Treatments	Total phenol (%)	Total antioxidants (%)	Total flavonoids (%)	CAT (U/mL)	POD (U/mL)	PPO (U/mL)
Freedom (Rootstock)	0.0112	65.12	1.826	0.002	0.005	0.004
Flame (Scion)	0.0067	66.28	0.914	0.003	0.006	0.003
Solid stacking wax (F/F)	0.0162	45.88	0.821	0.012	0.011	0.008
L.S.D 5%	0.0126	1.21	0.232	0.001	0.003	0.002

Table 4. Effect of various micro grafting methods in grape (Flame x Freedom) on PGRs contents by HPLC

PGRs	ABA		GA_3		IAA	
Retention time	RT	u/100 g	RT	u/100 g	RT	u/100 g
Solid stacking wax (F/F)	5.893	0.02981	3.462	0.23221	2.927	0.09366
Alginate matrix (F/F)	6.268	0.00911	3.435	0.3082	2.777	0.13589
L.S.D 5%		0.00231		0.00211		0.00182

analysis (Verma and Rana, 2013). Tested ISSR primers that resulted in polymorphic ISSR profiles, including six polies (GA) or (AG) or (CA); dinucleotide primers. The number of bands produced by the used primers ranged between 9 for the primer 6 (UBC-817) and 36 for the primer 5 (UBC-812). Primers 5 (HBC-812) $(GA)_8A$, 4 (UBC-811) $(AG)_8C$, (Table 1) produced higher number of total alleles compared to other primers. The total number of alleles produced by the primers is 145 including 124 polymorphic markers and only 21 monomorphic markers. Polymorphisms of 97.22 and 90.6% were found in two primers, UBC-812 and UBC-811 (Table 5). Meanwhile, primers 6 and 1 (UBC-817 and HB-10) had a low polymorphism of 33.33%. The polymorphism produced by the other two primers ranged from 85.71% for primer 3 (UBC-809) to 89.65% for primer 2 (UBC-907). The total percentage of polymorphic markers for all primers in the examined 145 accessions is 85.51%, which indicated high level of genetic variation among the examined scion, rootstock and micrografted explants. Photograph illustrating the ISSR fingerprinting of scion; rootstock and micrografted explant by selected primer are shown in Fig. 1. This include ISSR fingerprinting revealed in 3 phenotypes by primer (UBC-811). The approximate size of the largest fragment produced was found in Flame seedless scion followed by Flame seedless x Freedom.

It has been suggested to use more than one marker for a better analysis of genetic stability and variation and the possibility of somaclonal variants (Singh *et al.,* 2013; Rohela *et al.*, 2018). Among different molecular markers, SCoT markers are gaining much attention due to their excellent quality and better feasibility than other molecular markers in genetic diversity and homogeneity studies (Collard and Mackill 2009; Cabo *et al.,* 2014; Thakur *et al.*, 2016). Genetic stability of *in vitro* regenerated plants, analyzed by SCoT markers, reported in several plant species such as *Cleome gynandra* (Rathore *et al.*, 2014), *Citrullus lanatus* (Vasudevan *et al.*, 2017), *Sesamum indicum* (Elayaraja *et al.* 2019), and *Pisum sativum* (Ajithan *et al.*, 2019). Similarly, ISSR primer-based assessment of *in vitro* propagated plants was reported in Morus spp. (Rohela *et al.*, 2018), *Eleusine coracana* (Babu *et al.*, 2018), *Solanum trilobatum* (Pendli *et al.*, 2019),

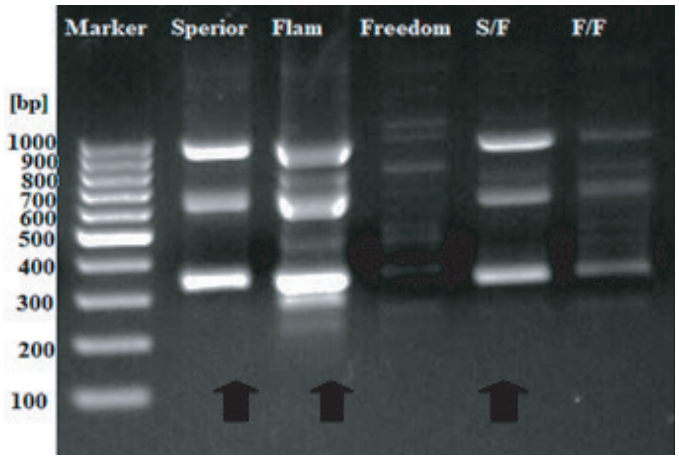

Fig. 1 ISSR-PCR (primer UBC-811) product of genomic DNA of the seven grafted grape (rootstock-scions) [M: molecular marker, S: Superior scion, F: Flame, Fr: Freedom, S/F: Superior x Freedom, F/F: Flame x Freedom]

Corallocarpus epigaeus (Vemula *et al.*, 2020), and *Albizia lebbeck* (Saeed *et al.*, 2019).

The pair-wise genetic similarity estimates, based on Nei's similarity coefficient, of the 27 landraces used in this study are given in Table 5.

Findings from the present study revealed the successful application of various *in vitro* micrografting techniques on compatible rootstock with the scion. The methods, ranked in descending order of efficacy, were as follows: wax, aluminum foil, sterile wire tie, sterile metal needle, gel, and callus. Notably, the wax micrografting method exhibited the most favorable outcomes in terms of morphological criteria, displaying the highest survival percentage among all tested techniques.

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Table 5. ISSR-PCR amplification products of DNA extracted from scion, rootstock and grafted explants of grape for six primers

Name	Sequence	CG %	TB	PB	MB	PPB %
HB 10	GAG AGA GAG AGA CC /$(GA)_6$CC	57.14	11	7	3	63.64
UBC-807	AGA GAG AGA GAG AGA GT/ $AG)_8$T	47.05	29	26	3	89.65
UBC-809	AGA GAG AGA GAG AGA/ GA_8 GG	52.9	28	24	4	85.71
UBC-811	GAG AGA GAG AGA GAG AC$(GA)_8$C	47.05	32	29	3	90.6
UBC-812	GAG AGA GAG AGA GAG AA$(GA)_8$A	47.05	36	35	1	97.22
UBC-817	CAC ACA CAC ACA CAC AA$(CA)_8$A	47.05	9	3	6	33.33

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