Micropropagation of Grapevine and Strawberry from South Russia: Rapid Production and Genetic Uniformity

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Abstract: Grapes and strawberries are major fruit crops with a dynamic, fast-growing global market. In addition to conventional techniques, biotechnological tools allow the preservation of valuable genotypes of both crops and the large-scale propagation of high-quality material. We have developed new protocols for expeditious and reliable micropropagation of grapes and strawberries cultivated in south Russia. In vitro cultivation on semisolid media was combined with rapid propagation in bioreactors. A six-week cycle of propagation in a bioreactor resulted in a 300-fold increase in fresh mass, with a propagation rate of ~5 in grapes and ~20 in strawberries. Genetic analysis using the inter simple sequence repeat (ISSR) DNA fingerprinting technique confirmed the full uniformity of the regenerants. The results of this research will support the local horticultural industry with fast and efficient propagation systems of new breeding lines, introduced varieties, and elite cultivars.

Keywords: *Vitis*; *Fragaria*; ISSR; in vitro culture

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1. Introduction

Grapes and strawberries are major fruit crops with a dynamic, fast-growing global market. Numerous grapevine varieties are used to produce fresh fruits, wines, juices, raisins, and other products [1]. The whole fruit, skin, leaves, and seeds of the grape plant are used in cosmetics and the pharmaceutical industry [2]. In addition to being tasty, strawberries are prevalent in the human diet because of their high content of essential nutrients and beneficial phytochemicals [3]. Therefore, crop improvement and timely monitoring of product quality and propagation of the highest quality plant material are critical for grape and strawberry production.

Improving grapes by breeding for desired characteristics is slow and restricted by their high heterozygosity and relatively long life cycles [4]. Therefore, the usual horticultural practice is to propagate grapes vegetatively using grafting and cuttings [5]. Similarly, vegetative propagation in strawberries from plugs or bare-rooted units ensures the retention of additive and non-additive genetic traits [6]. In addition to conventional techniques, plant biotechnology has been used to preserve valuable genotypes of both crops, propagate high-quality material on a large scale, perform physiological studies, and obtain genetically transformed types. The application of tissue culture of strawberries and grapes provides technical support for the industrial production of these important crops [7–10].

Although laboratory protocols have been developed for the micropropagation of several grape [8] and strawberry [11–13] varieties, mass propagation in vitro is a costly and laborious process requiring specialized and expensive equipment, limiting the use of biotechnology in the propagation. The cost of labour represents between 40 and 60% of the total price of the harvested plants [14,15]. The search for more efficient micropropagation

technologies and reduced labour costs has led to the cultivation of plants in vitro in a liquid medium. During the adaptation of microbiological bioreactor systems for the specific needs of plant micropropagation [16], temporary immersion bioreactors (TIBs) proved successful for this purpose [17]. Bioreactor application provides optimal growth conditions by regulating chemical and physical parameters, which results in maximum yields of high-quality regenerants. It also keeps the production costs as low as possible by integrating automated facilities and simple, low-cost devices [17,18].

Optimal plant production in bioreactors is cultivar-specific and requires precise control of the plant morphogenesis in liquid culture systems. In addition, in vitro micropropagation can lead to genetic changes, mutations, and somaclonal variation [19]. Genetic and epigenetic changes can quickly cause the loss of characteristics essential to the parental forms. Somaclonal variation in berry plants might lead to morphological variants, sporadic occurrences of abnormal fruits and a hyper-flowering habit [20]. Therefore, molecular strategies have been developed to analyze the results of propagation and to reduce clonal variation to manageable levels. Randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) marker analyses have been developed to identify genetic diversity in micropropagated plants to verify trueness-to-type. ISSR primers targeting microsatellites are abundant throughout the plant genome and are more reproducible than RAPD markers [19].

In this study, we have developed protocols for expeditious and reliable micropropagation of grapes and strawberries cultivated in south Russia. We combined in vitro cultivation on solid media with immersion systems in TIBs and employed molecular markers to assess genetic uniformity among regenerants. The results of this research will support the local horticultural industry with fast and efficient propagation of new breeding lines, introduced varieties, and elite cultivars.

2. Materials and Methods

2.1. Plant Material Preparation

Grapevine (*Vitis vinifera* cv. Traminer Pink) and strawberry plants (*Fragaria × ananassa* cv. Murano) were collected from large farms on the Black Sea coast in May 2021. The green shoots were placed in plastic bags and taken to the laboratory in a mobile cooler. In the laboratory, the shoots were cleaned of leaves and petioles and thoroughly washed with running water for 10 min. The initial explants of grapes included two-node microcuttings with apical meristems, 2–3 leaf primordia, and axillary meristem(s). Strawberry explants were taken as 3–5 mm crown sections, with one or two meristems. The explants were disinfected by immersion in 70% ethanol for 10 s, soaked for 15 min in a 2% sodium hypochlorite solution with two drops of Tween –20 (0.1%), and rinsed three times for 5 min in sterilized distilled water.

2.2. Cultivation Conditions and Propagation

For the initial stage, all explants of both crops were grown on a basal Murashige and Skoog (MS) medium [21] with the addition of 30 g/L sucrose, 0.2 mg/L indolylbutyric acid (IBA) and 2 mg/L 6-benzylaminopurine (6-BAP). The pH was adjusted to 5.7 ± 0.1 using 0.1 N NaOH or HCl before adding 8% of agar (Sigma-Aldrich, Saint Louis, MO, USA). The medium was autoclaved at 121 °C at a pressure of 1.2 kg/cm² for 40 min. Following sterile filtration, 6-BAP and IBA were added to the autoclaved medium. Twenty-five millilitres of the medium was poured into sterile 240 mL glass tissue culture vessels with a vented polypropylene lid.

Ten explants were introduced into each vessel. The explants were incubated at 24 ± 1 °C under a 16-h photoperiod and illumination with 40-watt cold-white fluorescent lamps at an intensity of 105–115 µmol PPFD/m²/s (PPFD = photosynthetic photon flux density). Growing shoots were separated and subcultured in fresh medium every four weeks under similar conditions. After three cycles of four weeks (twelve weeks in total) of

the initial cultivation, the explants of grapes and strawberries were separated into three groups of 120 shots each and cultivated for six weeks under different conditions:

1. Solid medium. The explants were subcultured in 240 mL glass vessels on solid MS medium (agar 8%) smended with 30 g/L sucrose, 2 mg/L 6-BAP at pH 5.7 ± 0.1 , 12 plants per vessel.
2. TIB-1, ten bioreactors containing 12 explants each. Explants were cultivated in temporary immersion bioreactors (TIB) RITA (VITROPIC, Saint-Mathieu-de-Tre'Viers, France; <http://www.vitropic.fr/rita>, accessed on 2 January 2022) containing 200 mL liquid medium MS with 30 g/L sucrose, 2 mg/L 6-BAP (pH 5.7 ± 0.1) and immersed for 1 min every hour. The medium was autoclaved at 121 °C at a pressure of 1.2 kg/cm² for 40 min.
3. TIB-2, ten bioreactors containing 12 explants each. TIB-2 used the same conditions as for TIB-1, but the medium was prepared using specialized automatic media preparatory Mediawel 10 (Alliance Bio Expertise, France; <https://www.alliance-bio-expertise.com/en/mediawel10>, accessed on 2 January 2022) with filling temperature 43 °C, sterilization temperature 121 °C, sterilization time 40 min, and a temperature control accuracy of ± 0.5 °C.

Vegetative mass accumulation and the number of shoots per explant were recorded after six weeks of culture. The rates of shoot growth and propagation were calculated at the end of the propagation cycle.

2.3. Rooting, Hardening, and Acclimation

Well-developed shoots were transferred to fresh 1/2 MS medium smended with 1 mg/L IBA. The rest of the cultivation parameters remained unchanged. After four weeks of rooting, regenerants were carefully transferred to 5 cm pots filled with a peat–perlite mixture (3:1) and moved to growth cabinets with a relative humidity that gradually decreased from 100 to 60%, a temperature of 22 ± 1 °C, and a photoperiod of 16/8 (day/night) hours. After four weeks, plants were transferred to a greenhouse.

2.4. Genetic Analysis

Ten samples were randomly selected from each propagation treatment at the plantlet hardening stage, collected in tubes (1.5 mL), cooled to a temperature of -20 °C, and stored for subsequent DNA extraction. The total genomic DNA from 50–100 mg plant tissue of each sample was isolated using the DNA extraction procedure described by Lodhi et al. [22] with a minor modification: 1 mM dithiothreitol (DTT) was used instead of 2% β -mercaptoethanol. The amount of DNA was quantified using a fluorometric method with an Allsheng Fluorometer, Fluo-100 (China) and a QuantiFluor dsDNA System reagent kit (Promega).

Twenty-nine ISSR primers (UBC, Vancouver, BC, Canada) were tested, using genomic DNA from three randomly sampled individuals. Two primers, UBC807 and UNC810, presented the best amplification standards (unmistakable, reproducible loci with good resolution quality) (Table 1).

Table 1. ISSR primers with their respective sequences of nucleotides, a total number of amplified loci and size of bands in grape and strawberry plantlets propagated in vitro.

ISSR Primer	Sequence on Nucleotides, 5'-3'	Number of Loci in Grape	Number of Loci in Strawberry
UBC807	AGA GAG AGA GAG AGA GT	5	7
UBC810	GAG AGA GAG AGA GAG AT	7	5

These were selected to amplify the genomic DNA of 30 samples from each crop 10 from each propagation treatment. The PCR reaction was carried out in 20 μ L of a reaction

mixture consisting of 10 µL LightCycler 480 SYBR Green I Master Mix (LifeScience, Roche, Penzberg, Germany), 40 ng total DNA (5 µL), 1 µL water, and 4 µL of the corresponding primer (0.33 µM). PCR was performed using an automatic analyzer LightCycler96 (Roche Life Science), as follows: initial denaturation at 95 °C for 5 min, then 45 denaturation cycles at 95 °C for 10 s, annealing at 54 °C (grapes) or 56 °C (strawberry) for 25 s, and elongation at 72 °C for 25 s. The resulting amplicons were separated using horizontal electrophoresis in 2% agarose gel and TBE buffer in the presence of ethidium bromide (0.5 µg/mL) [23]. The resulting bands were visualized using a SERVA BlueCube 300 gel documentation system (SERVA Electrophoresis GmbH, Heidelberg, Germany). A DNA length marker 100+ bp DNA Ladder (Evrogen, Moscow, Russia) was used as a size standard.

Statistical analysis was performed using the Statistica 13.3.0 software (TIBCO Statistica, 2017, Palo Alto, CA, USA) with default parameters. The significance of differences was tested by one-way ANOVA, followed by Tukey's HSD.

3. Results

3.1. Micropropagation on Solid Medium and in Bioreactors

Microcuttings were taken at the growth stage and, following sterilization, introduced into the solid medium for 12 weeks. During this period, the grape explants enlarged, and their mass increased from 20–25 mg to 40–50 mg (Figure 1A,B). Following the initial culture on solid medium, the regenerants were separated into three groups and subcultured under different conditions on solid medium or in TIBs.

Figure 1. Growth and propagation in vitro of grapevine *Vitis vinifera* cv. Traminer Pink under different conditions. All media were smented with 2 mg/L 6-BAP. (A), initial explant—apical point with two young leaves (scale bar = 2 mm). (B), plant regeneration after two weeks growing on solid medium smented with 2 mg/L 6-BAP (scale bar = 2 mm). (C), rooting of regenerants after four weeks culture on solid medium smented with 0.5 mg/L IBA (scale bar = 5 mm). (D), a cluster of new shoots after six weeks of cultivation in TIB-1 (scale bar = 5 mm). (E), a cluster of new shoots after six weeks of cultivation in TIB-2 (scale bar = 5 mm).

On solid medium, only a slight increase in the shoot mass from ca. 40 to 60 mg was recorded during the subsequent six weeks of cultivation (Figure 2). Shoot proliferation was not observed. At the same time, the dramatic difference in mass accumulation and new shoot formation in grapes was already observed after 2–3 weeks of cultivation in the bioreactors. The development of plants in both reactors (TIB-1 and TIB-2) was slightly reduced in the first week, but during the second week of cultivation, their mass increased to 388 and 1038 mg, respectively, and after a six-week cycle, massive shoot proliferation was observed in plants in both TIB-1 and TIB-2: 12 initial shoots produced 55.8 ± 5.3 and 60.6 ± 7.5 shoots, respectively. After six weeks of propagation and cultivation, the fresh mass plants in TIB-1 increased from 30 to 1000 mg, while those in TIB-2 was significantly higher and increased to 1600 mg (Figure 2). However, in a few vessels, we recorded early necrosis of the older regenerants (Figure 1E).

Figure 2. Fig replaced Accumulation of fresh mass in regenerants of grapevine *Vitis vinifera* cv. Traminer Pink cultivated in vitro for six weeks under different conditions. The significance of differences (asterisks) was calculated using Tukey's pairwise comparison following ANOVA ($p \leq 0.05$; $n = 12$). Vertical lines indicate standard errors.

The propagation dynamics of strawberries was even more dramatic than that of grapes. When regenerants continued their development on solid medium, they propagated slowly (Figures 3 and 4), while the accumulation of fresh mass in TIBs was rapid and had increased 20-fold after only three weeks of cultivation.

Figure 3. Growth and propagation in vitro of strawberry (*Fragaria × ananassa* cv. Murano). (A), plant regeneration after 2 weeks growing on solid medium smented with 2 mg/L 6-BAP and 0.2 mg/L IBA (scale bar = 10 mm). (B), rooting of regenerants after four weeks culture on solid medium smented with 0.5 mg/L IBA (scale bar = 10 mm). (C), a cluster of new shoots after five weeks of cultivation in TIB-1 (scale bar = 10 mm). (D), a cluster of rooted shoots after six weeks of cultivation in TIB-2; medium smented with 1 mg/L IBA (scale bar = 10 mm).

A six-week cycle of propagation in TIB-2 resulted in an almost 300-fold increase in fresh mass, from 60 to 35,000 mg (Figure 4). TIB-1 propagation was slower but still very efficient. After a six-week cycle, massive shoot proliferation was observed in plants in both TIB-1 and TIB-2; 12 plants produced 63.7 ± 14.2 and 244.0 ± 24.3 , respectively.

Figure 4. Fig replaced Accumulation of fresh mass in regenerants of grapevine strawberry *Fragaria × ananassa* cv. Murano cultivated in vitro for six weeks under different conditions. The significance of differences (asterisks) was calculated using Tukey's pairwise comparison following ANOVA ($p \leq 0.05$; $n = 12$). Vertical lines indicate standard errors.

3.2. Rooting and Acclimation

Following shoot proliferation, the regenerants were transplanted to the media smented with IBA. On solid media, 100% of the grape plantlets formed roots after 2–4 weeks of cultivation. In TIBs, however, media smented with 1 mg/L IBA for 4 weeks resulted in excessive root formation and suppression of shoot development (Figure 5A–C). When the IBA concentration was reduced to 0.5 mg/L, after 1–2 weeks, 100% of plantlets with root system suitable for hardening (Figure 5D) was obtained. Rooted plantlets were transferred to climatic chambers and then to a greenhouse (Figure 5E–G). The survival rate after hardening was 92% for the plantlets rooted on solid medium and 87% for the plantlets that originated from TIBs.

Figure 5. Rooting and hardening of grapevine cv. Traminer Pink and strawberry cv. Murano cultivated in vitro. Scale bar = 1 cm. (A), grapes: rooting after two weeks of cultivation in TIB with 1 mg/L IBA. (B), grapes: rooting after two weeks of cultivation in TIB with 1 mg/L IBA. (C), grapes: rooting after four weeks of cultivation in TIB with 1 mg/L IBA. (D), grapes: rooting after one-week cultivation in TIB with 0.5 mg/L IBA. (E), grapes: rooting after four weeks culture on solid medium with 1 mg/L IBA. (F), grape plantlet after hardening for four weeks replanted in a greenhouse. (G), bare-rooted strawberry plant after hardening for four weeks and acclimation in a greenhouse.

All strawberry regenerants developed root systems within 3–4 weeks, both on solid medium and in TIBs. The plantlets were then transferred to climatic chambers for hardening. A very small number of plantlets died during hardening and acclimation. The percentage of healthy acclimated regenerants was 97% and 95% for solid medium and TIBs, respectively.

3.3. Genetic Uniformity

Genetic analysis using ISSR primers was performed just prior to plant hardening. No polymorphism was detected, thus verifying the genetic uniformity of the micropropagated plants. All the sampled plants were determined to be genetically identical (Figure 6).

Figure 6. Representative image of ISSR analysis of regenerants of grapevine cv. Traminer Pink (A,B) and strawberry cv. Murano (C,D) cultivated in vitro. In each of the three propagation treatments, the propagated plantlets are identical. Five plants (1–5) are presented for each treatment. TIB-1, temporary immersion bioreactors; TIB-2, temporary immersion bioreactors with automatically prepared media. M—marker, 100 bp DNA Ladder.

4. Discussion

Grapes and strawberries are important and desirable crops in south Russia, but local horticulture still depends on imported propagation material. In order to secure the production of healthy elite varieties, rapid and reliable systems of propagation are essential. In addition to conventional techniques, the use of biotechnological tools allows for the preservation of valuable genotypes of both crops and the large-scale propagation of superior material.

Although protocols for micropropagation on solid media have been developed for numerous varieties of grapes and strawberries, they are relatively expensive and labour intensive. On the other hand, the use of bioreactors not only secures higher multiplication rates but also reduces labour costs [24]. TIBs were reported as an efficient technique for the propagation of pear [25], berries [9,16,19], carnations [26] and other horticultural crops. However, micropropagation on solid media still prevails in both laboratory and commercial practice.

In this research, two techniques were combined: in vitro cultivation was initiated on solid media and then advanced to rapid propagation and rooting in bioreactors (Figure 7). The full cycle took ca. six months, similar to the results of Mhatre et al. [8] on solid media, but with a higher propagation rate and mass accumulation. Thus, a six-week cycle of propagation in TIBs resulted in an increase of fresh mass of up to 300-fold, with a propagation rate of ~5 for grapes and ~20 for strawberries (Figures 2 and 4). Our studies also confirm previous reports on successful regeneration of berry crops on semisolid medium, followed by culturing in a bioreactor system [9]. Similarly, in carnation, TIB culture resulted in more than ten times shoot production [26]. This is probably due to better contact of the

plant with the culture medium, optimal nutrient and growth regulator supply, and aeration and medium circulation [27].

Figure 7. Scheme of the proposed protocol for grape and strawberry propagation, based on initial cultivation on solid medium, followed by fast propagation and rooting in a bioreactor.

The proposed protocol will allow semi-automation of the micropropagation that is not possible on a solid medium. In this context, using specialized automatic media preparatory Mediawel 10 provided an additional advantage to automated plant propagation. Surprisingly, in both studied crops, automatic media preparation resulted in a nearly 2-fold increase in shoot mass, while with strawberries, the use of this media also resulted in a 4-fold increase in the propagation rate (Figures 2 and 4). Although the media in our systems TIB-1 and TIB-2 had the same chemical composition, the advantages of fully automatic preparation are more precise temperature (± 0.5 °C), pressure control and continuous stirring of the medium during preparation and dispensing. Necrosis of the older regenerants, recorded in a few TIB vessels, was probably caused by the insufficient ventilation of bioreactors [28].

Following shoot proliferation, rooting was initiated by cultivation on media smented with IBA. Interestingly, however, a lower concentration of IBA was more efficient in TIBs. This fact can be explained by a more efficient accumulation of phytohormones in the immersion systems and better cultivation conditions, as was shown in *Olea* [29].

The percentage of acclimatized strawberry and grape plants after rooting on a solid medium was slightly higher in comparison with plants propagated in TIBs. The reason for this difference might be root morphology in the plantlets. Although the difference in the hardening rates between the solid medium and TIBs was not critical (only 5–7%), further improvement in technology will be needed to optimize the production of a well-developed root system in TIBs.

Finally, genetic assessment using ISSR markers confirmed the complete uniformity of the regenerants. Therefore, the developed protocol is eminently adaptable to elite varieties of both crops. In addition, it will enable micropropagation of disease- and virus-free materials, such as those recently obtained in our laboratory. The results of this research will support the local horticultural industry with efficient propagation systems of new breeding lines, introduced varieties, and elite cultivars.

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