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Research Article

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Recovery from Viral Infections through in Vitro Techniques in the Local Grapevine Cultivar "Shesh I Zi"

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ABSTRACT

Local grapevine cultivars in Albania are of great significance, representing the country's rich genetic diversity, cultural heritage, and potential to produce distinctive, high-quality wines for competitive international markets. The establishment of scientific protocols for determining the genetic identity and phytosanitary status of propagating material is essential for ensuring the production of healthy grapevine seedlings. Between 2021 and 2023, the phytosanitary status of the local cultivar Shesh i Zi was evaluated. DAS-ELISA assays were performed using Agritest (Italy) kits to detect Grapevine fanleaf virus (GFLV), Grapevine fleck virus (GFkV), Grapevine leafroll-associated viruses 1 and 3 (GLRaV-1, GLRaV-3), and Grapevine virus A (GVA). GLRaV-3 was detected in 6 of the 18 tested samples. Virus elimination was performed using meristem culture on MS medium. Thirty-five meristems were excised from apical shoots; after 40 days in a vegetative growth chamber, 27 explants were obtained and micro propagated. In vitro thermotherapy was applied for two months, starting at 30 °C, followed by one week at 38 °C. After acclimatization and growth in a screenhouse, 83% of plants tested negative for GLRaV-3, confirming the effectiveness of the protocol. The recovery of virus-free Shesh i Zi plants is a critical step towards implementing a rigorous certification program for valuable local cultivars, supporting their preservation, enhancing opportunities for domestic nurseries, and aligning with the requirements for Albania's future EU accession.

Keywords: Local Grapevine, Meristem-Tip Culture, Thermotherapy, GLRaV-3, DAS-ELISA

Introduction

The preservation of local grapevine germplasm from phytosanitary degradation, as well as from genetic erosion, requires the implementation of scientific protocols for the production of high-quality plant propagation material. Local grapevine cultivars are characterized by their adaptation to phytoclimatic and soil conditions, high productivity for both fresh consumption and industrial processing, relative resistance to diseases and pests, and a diversity of forms and genotypes derived from bud mutations. These characteristics highlight their production potential and suitability for clonal selection. Notable examples include Shesh i Bardhë, Shesh i Zi, Kallmet, and Vlosh, along with several minor local varieties, all of which possess valuable traits and significant genetic potential for the selection and breeding of new cultivars with desirable qualities. Local cultivars occupy around 60% of the total vineyard area in Albania, with Shesh i Bardhë and Shesh i Zi representing the most dominant share of this surface. The vineyard collection at the Experimental Base comprises 153 cultivars, including 55 local ones: 19 white wine cultivars, 18 red wine cultivars, 10 white table grape cultivars, and 8 black table grape cultivars.

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This collection aims to explore, identify, and expand the local germplasm pool for varietal improvement through sexual hybridization, thereby supporting the development of Albanian viticulture and agrotourism.

The cultivar Shesh i Zi originates from Central Albania and is widely cultivated throughout the country. It ripens in September, produces high-quality wine, and is also suitable for consumption as a table grape.

Sanitary inspections in vineyards have revealed a high incidence of viral and viroid infections in plants showing characteristic symptoms [1,2]. Such infections have significant economic impact, as they reduce both agronomic productivity and oenological quality.

Plant tissue culture plays a key role in phytopathology by advancing techniques to combat pathogens responsible for viral infections in plants [3,4].

Accurate identification of viral agents and the species targeted for sanitation is essential for selecting the most effective eradication methods, such as in vitro and in vivo thermotherapy and in vitro meristem-tip culture [5,6]. Implementing clonal and sanitary selection programs for local varieties of national importance is vital, as these strategies remain the most effective means of preserving and recovering indigenous germplasm in fruit and viticulture.

In Albania, the legal framework for the certification of plant propagation material has been updated to align with European Union regulations. This includes D.C.M. No. 86 of 2022 ("Law on Viticulture and Wine"), harmonized with EU Regulation No. 1308/2013 of December 11, 2017, as well as Law No. 10 416, dated April 7, 2011, "On Planting and Propagating Material," as amended by Laws No. 67/2013 (February 14, 2013), No. 105/2015 (October 1, 2015), No. 12/2019 (March 5, 2019), No. 99/2024 (September 12, 2024), and No. 21/2025 (March 13, 2025) (updated version). Although the legislation is almost harmonized with EU requirements, its implementation in practice does not yet meet all regulatory standards.

Material and Methods

Plant Material

Plant material of the local grapevine cultivar Shesh i Zi was collected during the dormant season, at the time of vineyard pruning (January–February) in the years 2021–2023, from the collection of local grapevine cultivars at the Shamogjin Experimental Base, Vlora Agricultural Technology Transfer Centre. Dormant cuttings were selected for subsequent diagnostic testing to determine the presence of viruses, viroids, or other similar pathogens.

Phytosanitary Assessment

The phytosanitary status of Shesh i Zi was evaluated using serological diagnostic assays for the detection of major grapevine viruses, following established protocols [7-9,5,1]. The viruses tested included Grapevine fanleaf virus (GFLV), Grapevine fleck virus (GFkV), Grapevine leafroll-associated virus 1 (GLRaV-1), Grapevine leafroll-associated virus 3 (GLRaV-3), and Grapevine virus A (GVA). Based on the results of these assays, plants found to be infected with viruses were selected for sanitation treatments.

DAS-ELISA Diagnostics

Virus detection was performed using the Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA) method, as described by Clark and Adams, with commercial kits from Agritest, Italy (Set Grapevine CERT 5 ELISA, 1000 × 5 tests), which included one positive and one negative control reagent. For each sample, a plate layout was prepared, and plant tissue was ground in extraction buffer at a 1:10 ratio, with the extracts stored at 4 °C [10]. ELISA plates were sensitized by adding 100 µL of IgG (coating buffer) to each well, covered with cling film, placed in a sealed plastic box, and incubated for 2 h at 37 °C. Plates were then washed three times for 3 min with washing buffer, and 100 µL of clarified plant extract was added to each well, followed by overnight incubation at 4 °C. After a second washing step (three washes of 3 min each), 100 µL of alkaline phosphatase-conjugated IgG diluted in conjugate buffer was added, and plates were incubated for 2 h at 37 °C. Plates were again washed three times and 100 µL of p-nitrophenyl phosphate substrate (1 mg/mL in substrate buffer) was added to each well. Color development (yellow) was allowed to proceed at room temperature for 30 min to 2 h, and absorbance was measured at 405 nm at 30 min intervals using an ELISA plate reader.

Following diagnosis, plants of the cultivar Shesh i Zi found to be infected with Grapevine leafroll-associated virus 3 (GLRaV-3) were subjected to in vitro sanitation treatment. The treatment, conducted between May and September, employed a combination of in vitro techniques as described by Savino et al., Barba et al., and Bottalico et al. [11-13].

Sanitation Techniques

Meristem-tip Culture

Shoots (4–5 cm) of the Shesh i Zi cultivar were excised from GLRaV 3 infected plants and prepared for in vitro culture under aseptic conditions in a laminar flow cabinet. Explants were surface sterilized in 10% sodium hypochlorite (NaOCl) containing one to two drops of Tween 20 for 20 min, followed by thorough rinsing with sterile distilled water. Meristem-tip culture was performed following Gautheret and Alpi, isolating the apical dome with one or two leaf primordia—a region generally free from viruses [14-20]. Using a stereomicroscope, 35 meristem tips (0.4–0.6 mm) were excised and placed onto nutrient medium with macro- and micronutrients, vitamins, 0.2 mg/L BAP, 0.1 mg/L NAA, 30 g/L sucrose, and 7 g/L agar. Cultures were maintained at 24 °C under a 16 h light/8 h dark photoperiod with ~3500 lux fluorescent illumination.

In vitro thermotherapy

Following propagation, in vitro-grown shoots underwent thermotherapy in a controlled growth chamber—first at 30 °C

for one week (pre-treatment), then for 60 days at 38 °C under 16 h light/8 h dark and ~5000 lux illumination. Sterilized shoots (1.5–2 cm), cultured for eight weeks, were exposed to two treatment durations: 20 or 40 days at 38 °C. Culture vessels were placed in water baths to maintain lower substrate temperature and reduce heat stress. Post-treatment, meristems (0.4–0.6 mm) were excised and transferred to nutrient medium. After 40-day acclimatization, plants were tested using DAS ELISA to confirm virus elimination.

In Silico Analysis

To complement the serological diagnosis, an "in silico" analysis of GLRaV-3 genomic sequences was performed. Reference sequences were retrieved from the NCBI GenBank database, including complete and partial genomes representing different GLRaV-3 phylogenetic groups [17,18]. Sequence alignment was carried out using MUSCLE v3. and phylogenetic trees were constructed with MEGA X (Kumar et al., 2018) using the Maximum Likelihood method with 1,000 bootstrap replicates [19]. The genomic regions corresponding to the epitopes recognized by DAS-ELISA antibodies were identified and compared across isolates to assess potential sequence variability affecting antibody binding [20]. Protein domain prediction was carried out using InterProScan to support the structural interpretation of diagnostic targets [21].

Results and Discussions

Based on the diagnostic results (Table 1) for the detection of GFLV, GFkV, GLRaV-1, GLRaV-3 and GVA, obtained using DAS-ELISA serological tests, infected plants were identified for sanitation through in vitro techniques as recommended by the literature [22].

Table 1: Phytosanitary status of Shesh i Zi grapevine cultivar as determined by DAS-ELISA.

Cultivars	Virus GFLV	Virus GFkV	Virus GVA	Virus GLRaV-1	Virus GLRaV-3
P-Sheshi Zi 1	-	-	-	-	-
P-Shesh i Zi 2	-	-	-	-	-
P- Shesh i Zi 3	-	-	-	-	-
P- Shesh i Zi 4	-	-	+	-	+
P- Shesh i Zi 5	-	-	-	-	-
P- Shesh i Zi 6	-	-	-	-	-
P- Shesh i Zi 7	-	-	-	-	+
P- Shesh i Zi 8	-	-	-	-	-
P- Shesh i Zi 9	-	-	-	-	-
P- Shesh i Zi 10	-	-	+	-	+
P- Shesh i Zi 11	-	-	-	-	-
P- Shesh i Zi 12	-	-	-	-	+
P- Shesh i Zi 13	-	-	-	-	-
P- Shesh i Zi 14	-	-	-	-	-
P- Shesh i Zi 15	-	-	+	-	+
P- Shesh i Zi 16	-	-	-	-	-
P- Shesh i Zi 17	-	-	-	-	+
P- Shesh i Zi 18	-	-	-	-	-

Phytosanitary testing indicated that the Shesh i Zi plants were free from GFLV, GFkV, and GLRaV-1; however, 6 out of 18 plants tested positive for Grapevine leafroll-associated virus 3 (GLRaV-3) [22,23].

To eliminate the phloem-limited virus GLRaV-3, in vitro sanitation techniques were applied, namely meristem-tip culture and in vitro thermotherapy following all procedural phases: inoculation, propagation, rooting, and acclimatization [24,25]. Thirty-five meristems were excised from apical buds, and after 40 days in the growth chamber, 27 explants (77%) were successfully established.

Following the growth and elongation phase, new shoots developed from meristematic tissue via organogenesis on a standard MS medium (Figure 1A, B). These shoots were maintained in the growth chamber under controlled conditions. After propagation, plantlets were transferred to a rooting medium supplemented with 0.1 mg/L indole-3-butyric acid (IBA) to stimulate root system development. Rooted plantlets were subsequently acclimatized under in vivo conditions with controlled temperature and light parameters (Figure 1C–F).

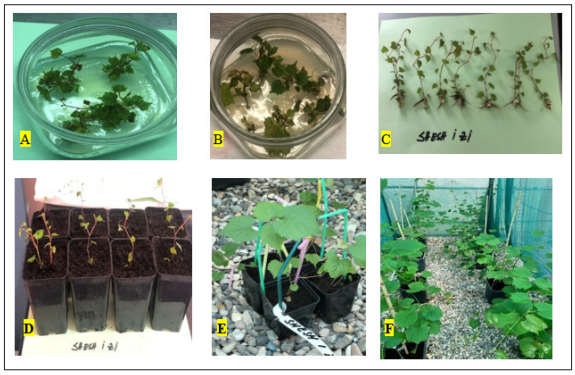

Figure 1: Stages of in vitro culture and sanitation process for the production of healthy plants of Vitis vinifera L. cv. Shesh i Zi infected with Grapevine leafroll-associated virus 3 (GLRaV-3). Virus elimination was achieved through a combination of meristem-tip culture and in vitro thermotherapy. (A) Plantlets during subculture. (B) Plantlets in the propagation phase. (C) Rooting stage of plantlets. (D) Acclimatization of plantlets under controlled in vivo conditions. (E) Acclimatized plants grown in screenhouse conditions. (F) Healthy, virus-free plants ready for field planting.

The plant material, sanitized using a combination of meristem-tip culture and in vitro thermotherapy, was tested by DAS-ELISA to verify its final phytosanitary status. Leaf samples were collected from both the apical and basal portions of each shoot. Of the six plants initially infected with GLRaV-3, five tested negatives after treatment, corresponding to an 83% sanitation success rate (Table 2).

Table 2: Sanitation results for Vitis vinifera L. cv. Shesh i Zi infected with GLRaV-3, treated with a combination of meristem-tip culture and in vitro thermotherapy. Virus detection was carried out using DAS-ELISA two months after thermotherapy at 38 °C, showing 83% of plants free from GLRaV-3

Cultivar	Virus	Treatment duration	Diagnostic method	Result	Sanitized plants (%)
Shesh i Zi	GLRaV-3	2 months at 38 °C	DAS-ELISA	Negative: 5 / Positive: 1	83%

The observed health improvement confirms the effectiveness of meristem-tip culture—an aseptic tissue culture method that targets virus-free regions of the plant—when combined with in vitro thermotherapy, a heat-based treatment that inhibits viral replication [26]. This combined approach proved effective in eliminating Grapevine leafroll-associated virus 3 (GLRaV-3) from the Shesh i Zi cultivar [27].

Conclusions

The present study demonstrates that the combined application of meristem-tip culture and in vitro thermotherapy is an effective strategy for eliminating Grapevine leafroll-associated virus 3 (GLRaV-3) from the autochthonous Vitis vinifera L. cultivar Shesh i Zi, achieving an 83% sanitation success rate. This integrated approach exploits the natural virus-free status of meristematic tissue and the inhibitory effect of elevated temperatures on viral replication, resulting in the recovery of healthy, virus-free planting material.

The practical applications of in vitro sanitation techniques extend beyond the rehabilitation of individual infected vines. They represent a valuable tool for the recovery, conservation, and sustainable use of old vineyards, particularly those containing minor or local cultivars. These cultivars, which form a diverse and heterogeneous viticultural heritage in Albania and across the wider Balkan and Mediterranean regions, are of high cultural and genetic significance. Their preservation is crucial not only for maintaining biodiversity but also for safeguarding unique enological traits that contribute to the identity and competitiveness of regional wines.

In the context of growing consumer interest in authenticity, traceability, and the rediscovery of local grape varieties, such sanitized plant material offers winemakers an opportunity to diversify and enhance the quality of their production. Implementing rigorous phytosanitary programs based on advanced in vitro techniques will be fundamental to supporting both the commercial value and the long-term sustainability of viticulture in Albania, in line with EU phytosanitary standards and certification requirements.

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