Genetic improvement of Oenological Interesting Lucanian Grapevine Varieties through CRISPR/Cas9-Mediated Mutagenesis: somatic embryogenesis and protoplast regeneration

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Abstract. Cultivated grapevine varieties, propagated vegetatively, exhibit intra-varietal genetic variability due to somatic mutations. While this variability has been exploited in the past for clonal selection, current environmental challenges and market demands necessitate more robust methods for genetic improvement. This study investigates somatic embryogenesis and protoplast culture as essential steps for the application of CRISPR/Cas9 genome editing in grapevine. We optimized somatic embryogenesis protocols for six grapevine varieties (*Aglianico*, *Aleatico*, *Greco bianco*, *Merlot*, *Primitivo*, *Crimson*). Optimal hormone ratios were identified for each genotype, leading to efficient induction of embryogenic calli and subsequent development of somatic embryos. Viable protoplasts were isolated from three varieties (*Crimson*, *Greco bianco*, *Primitivo*) and cultured in three different nutrient solutions. Protoplast multiplication was observed, with *Crimson* and *Primitivo* exhibiting higher multiplication rates compared to *Greco bianco*. Efficient and reproducible somatic embryogenesis and protoplast culture protocols are crucial for enabling targeted genetic modifications via CRISPR/Cas9 in grape, thought regeneration of whole plants from protoplast culture requires further investigation.

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1 Introduction

Before the introduction of *PIWI* or disease-resistant genotypes [1], grapevine germplasm was primarily the result of continuous selection of varietal clones with spontaneous somatic mutations. These mutations generally did not substantially alter the typical characteristics of the original grapevine variety, allowing it to be cultivated for the production of specific wines [2]. The limited genetic diversity within existing varieties hinders the selection of new clones exhibiting enhanced tolerance to water stress, reduced susceptibility to pathogens, and shorter vegetative and reproductive cycles, emphasizing the need for new methods for inducing genetic variability in grape [3]. The recently developed CRISPR/Cas9 genome-editing system offers a promising approach to generate small, precise genetic variations in existing genotypes while preserving their overall genetic background [4,5]. In grapevine, CRISPR/Cas9 has the potential to generate mutations that confer improved pathogen tolerance, stress resistance, or modified fruit and must characteristics, without disrupting the established varietal traits. However, the application of CRISPR/Cas9 technology requires efficient in vitro plant regeneration systems like somatic embryogenesis and protoplast regeneration that need to be optimized for each specific variety [6-8]. Somatic embryogenesis involves the development of embryos from non-zygotic cells and provides the starting material for editing as well as multiplication of edited plants. Protoplast regeneration, on the other hand, involves the isolation of individual plant cells (protoplasts) lacking a cell wall from embryogenic callus, followed by their in vitro culture and differentiation into somatic embryos. Introducing the CRISPR/Cas9 machinery into protoplasts before regeneration can result in plants bearing the desired genetic modifications.

This study investigates the optimization of somatic embryogenesis in five wine and one table grape varieties (*Aglianico*, *Aleatico*, *Greco bianco*, *Merlot*, *Primitivo*, and *Crimson seedless*) and the establishment of a protoplast culture system for *Crimson*, *Grecobianco* and *Primitivo* varieties.

2 Materials and methods

2.1 Somatic embryogenesis

A somatic embryogenesis protocol was developed using floral buds as explants, rather than stamens. Buds were collected from inflorescences at microsporogenesis stages IV (tetrads) or VI (pollen grains) [9]. To induce embryogenic callus formation, buds, after removing the base of the calyptra, were aseptically cultured in the dark on Murashige and Skoog (MS) solid medium supplemented with equimolar concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP)[10]. Over variable durations depending on the variety, compact calli derived from cell proliferation evolved into proembryogenic masses (PEM), which produced somatic embryos following a modification of the medium's hormonal composition. Somatic embryos were germinated using a specific substrate and subsequently transferred to light conditions to enable plantlet development.

2.2 Protoplast isolation, cultivation and multiplication assessment

Protoplasts were isolated from embryogenic calli of *Crimson seedless*, *Greco bianco*, and *Primitivo* by enzymatic digestion (0.22% macerozyme-R10 SERVA and 0.21% cellulase Worthington) at 25 °C for approximately 15 hours. Following two filtration and washing steps in a MMG solution (0.5M Mannitol, 4mM MES pH 5.7, 15mM $MgCl_2$), protoplasts were re suspended at a concentration of 2×10^5 cells per mL in MMG. Protoplast suspension was then mixed with an equal volume of a 3.2% sodium alginate solution. The resulting mixture was dispensed in drops (beads) of 100µL onto a solid medium containing 20mM CaCl2. The solidified beads were transferred to Petri dishes containing the chosen nutrient solution. The dishes were maintained in the dark at 25 °C with gentle agitation, and the nutrient solutions were refreshed bi-weekly.

The multiplication capacity of protoplasts from *Crimson seedless*, *Greco bianco*, and *Primitivo* genotypes was assessed over 60 days of culture in three different nutrient solutions: a Nitsch and Nitsch Basal (NN) medium[6] and two variants of the NN medium with reduced cytokinin (1.7µM6-BAP, 0.7µM Kinetine) (NN1) or increased auxin (1.67µM IAA, 1.67 µM NOA) (NN2) contents. Three replicate Petri dishes for each genotype received the same nutrient solution treatment. The multiplication capacity of protoplasts for each of the three genotypes in each of the three treatments was assessed over 60 days. The number of microcolonies within 5 beads of each Petri dish was counted weekly by inspecting five random fields of view per bead using a 40x objective on an inverted microscope (Zeiss Axiovert).

3 Results and discussion

Cell proliferation was evident on the distal part of the buds after 30 days of culture. Compact calli appeared within the following two months depending on the variety. Almost all calli evolved into proembryogenic masses (PEM) over varying timeframes. Subsequently, the hormonal composition of the medium was modified to induce differentiation of somatic embryos. Specifically, 2,4-D was removed, while the 6-BAP concentration was significantly reduced. These reductions were tailored in a genotype-specific manner across the six investigated genotypes, reflecting variations in their hormone sensitivity. Germinating somatic embryos were transferred to light conditions for plantlet development. Optimal hormone combinations for the induction, differentiation,

andgermination of somatic embryos were identified for all six genotypes under study (Fig. 1).

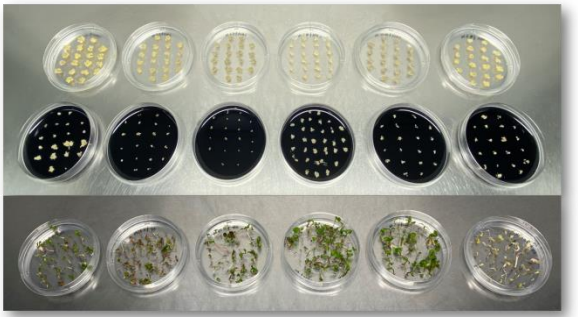

Fig. 1. Somatic embryogenesis in five wine and one table grape varieties. From left to right: Ag. *Aglianico*, Al. *Aleatico*, Gb. *Greco bianco*, Me. *Merlot*, Pr. *Primitivo*, Cr. Crimson seedless.

The study demonstrates the efficacy of somatic embryogenesis for grapevine propagation, emphasizing the critical role of balanced hormone manipulations in achieving embryogenic competence of calli, as well as embryo induction and differentiation, and plantlet germination.

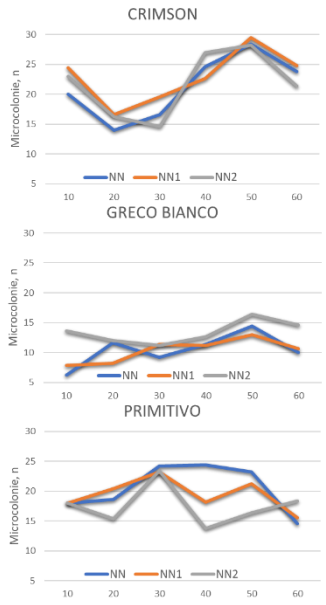

Fig. 2. Growth of *Crimson*, *Greco bianco* and *Primitivo* protoplasts embedded in sodium alginate beads with three liquid nutrition solutions (NN, NN1, NN2) over 60 days of culture. Growth was measured as the number of micro colonies.

The observation of genotype-specific hormonal requirements underscores the need for optimized protocols and customized media for somatic embryogenesis in grapevine. Viable protoplasts were successfully isolated from embryogenic calli of *Primitivo*, *Greco bianco* and *Crimson*. Protoplasts embedded in 1.6% sodium alginate beads were cultured in Petri dishes with three liquid nutrient solutions (NN, NN1, and NN2) for over three months. The multiplication capacity of protoplasts of each genotype in the three nutrient solutions was assessed over 60 days by weekly counts of the number of microcolonies within 5 beads of each of the three replicate Petri dishes. The *Crimson seedless* and *Primitivo* genotypes exhibited higher multiplication capacity compared to *Greco Bianco* (Fig. 2).However, no significant effect of the nutrient solution type on the cell multiplication rate was observed across the three genotypes(Fig. 3). After 4-5 months of cultivation, microcolonies showed a decrease in growth rate and often underwent necrosis.

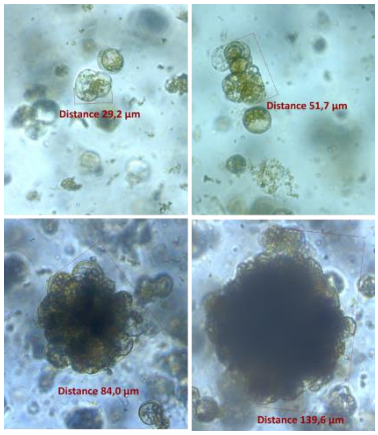

Fig. 3. *Primitivo* protoplasts. Size of protoplast microcolonies at 10, 20, 80 and 95 days from the start of cultivation.

4 Conclusions

This study provides essential groundwork for developing more robust and reliable tissue culture systems for genetic engineering and breeding of important grapevine varieties, particularly in the Lucanian region. The study has successfully defined efficient protocols for somatic embryogenesis in *Aglianico*,*Aleatico*, *Grecobianco*, *Merlot*, *Primitivo* and *Crimson seedless* grape varieties. Efficient isolation and cultivation protocols for *Greco bianco*, *Primitivo* and *Crimson seedless* protoplasts have also been developed. The genotype-specific requirements for somatic embryogenesis underscore the importance of customized optimization. While protoplast culture showed promising results for cell multiplication, the long-term viability and regeneration of plants from protoplasts requires further investigation. Only the availability of efficient and reliable transfection and regeneration systems will allow the routine use of CRISPR/Cas9 enzymatic mutagenesis in grapevine genetic improvement.

The research activities described in this study were partially conducted within the framework of Project RESO (REsilienza e SOstenibilità delle filiere ortofrutticole e cerealicole per valorizzare i territori) funded by Italian National Operative Programs for Research and Innovation (PON) 2014-2020.

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