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Somatic Embryogenesis in *Vitis* for Genome Editing: Optimization of Protocols for Recalcitrant Genotypes

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Abstract: New Plant Breeding Techniques (NPBTs) protocols have been developed to produce new grape varieties with improved quantitative and qualitative characteristics. Reliable transformation protocols for grapes are based on the generation/induction of embryogenic callus cells that are then transformed. Varieties such as Italia have proven to be very recalcitrant to regeneration via somatic embryogenesis. In this work, the development of a protocol for improved production of embryogenic calluses is described. Two sterilization protocols were tested: (a) a lower active chlorine concentration for a longer time (LS); and (b) a higher chlorine concentration for a shorter time (HS), in combination with the absence or presence of citric acid in the growing substrate in the first growth media. The embryogenic calluses formation in Chardonnay, a cv. with a high embryogenic response, was significantly higher in presence of citric acid in the initial growing substrate regardless of the sterilization protocol. In Aglianico, a cv. with a lower embryogenic response, no significant differences were observed. Instead, in a recalcitrant cv. as Italia, we obtained a 13-fold increase in embryogenic calluses formation performing sterilization of flowers with the HS protocol compared to LS.

Keywords: table grapes; genetic improvement; new breeding techniques; *Vitis vinifera* L.; embryogenic callus

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1. Introduction

Italy is among the major grape producers (13.5% table grape, 86.5% wine grape), with a large vineyard-dedicated surface area [1]. Italian table grape production has long been dominated by the "Italia" variety, which has large white aromatic but seeded berries.

The on-growing demand for seedless grapes has boosted the search for novel seedless varieties. As a result, in the last decade, numerous new seedless varieties have been registered in the Italian National Register of Vine Varieties [2]. Most of these varieties have a non-EU origin, which might result in adaptation difficulties in European cultivation areas.

This research started in 2008 in the CREA center of Viticulture and Enology of Turi (Italy), intending to obtain novel seedless varieties appreciated by consumers but able to perform well in the Italian context using traditional breeding techniques together with *embryo rescue* [3–5]. In general, the main objectives for the genetic improvement of table grape breeding programs are large seedless berries with appreciated organoleptic characteristics, an extension of the harvest calendar, simplified cultivation management of the vineyard, good productivity, enhancement of nutraceutical composition, improved aptitude for cold storage, suitability for transport, and long shelf-life. Moreover, an improved tolerance or

resistance to fungal diseases (gray mold, powdery mildew, and downy mildew) is a goal in common with both table and wine grape breeding programs.

Genetic improvement toward these goals has been performed exclusively by traditional cross-breeding, with the most important drawback being that the meiotic crossing-over will transfer entire chromosomal tracts without control and will result in the acquisition of unwanted characters together with the desired ones.

In recent years, thanks to the introduction of New Plant Breeding Techniques (NPBTs), genetic improvement could be performed with cis-genesis and genomic editing [6,7]. Unlike traditional cross-breeding, the NPBTs allow modifying a chosen character, keeping intact the other typical characteristics of the variety by adding or modifying only target genes. The NPBTs, strictly speaking, are indeed genetic engineering techniques but do not lead to genetically modified organisms (GMOs) according to the most influential scientific opinions [8]. Contrary to GMOs, NPBTs will not transfer exogenous DNA sequences from unrelated, genetically distant organisms, often containing selectable markers such as antibiotic resistances. NPB techniques simulate sexual crossings (cis-genesis) and mutagenesis (genome editing). Briefly, cis-genesis consists of the transfer of intact genes and their regulatory regions in the same orientation. These genes must originate from the same or closely related sexually compatible species. Genome-editing instead consists of a target mutagenic approach, using the abilities of the CRISPR/CAS system to introduce single or double cuts in precise locations of the genome and exploiting the endogen DNA repair mechanism by introducing random mutations. A more detailed review of NPBTs and their applications on crop protection can be found in del Giudice et al. (2021) [9].

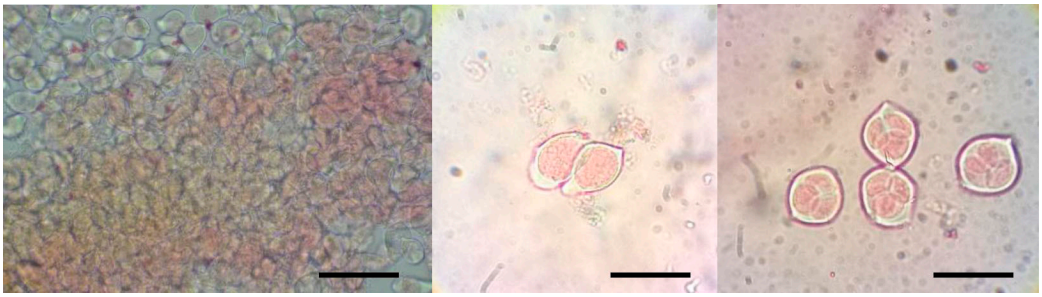
In perennial woody plant breeding, the main advantage of these techniques is the significant costs and time reduction and especially the possibility of introducing desired characters in historical, well-known, appreciated, and valuable varieties. The application of these new technologies will often require a phase in which an embryogenic callus is used. Embryogenic calluses are special proliferating cells, in vitro cultivated from somatic tissues that, given the appropriate hormonal conditions or stimuli, acquire or bear the ability to form embryos without going through meiosis, crossing over, and development in seeds. Therefore, embryos derived from these calluses will have the same gene heritage as the tissue donor plant, which is a critical aspect to keep peculiar varietal characteristics. In NPBTs, genetic engineering is performed on cells of embryogenic calluses and somatic embryos are formed. These embryos can regenerate the whole edited or cisgenic plant. A wide application of these techniques is hindered by the null or very low aptitude to generate embryogenic calluses of many important varieties. Therefore, the search for the optimization of methods to improve the embryogenesis ability of recalcitrant genotypes is of paramount importance for the application of NPBTs [10–12].

In the present work, we will illustrate the CREA's research activity that allowed to obtain somatic embryos of a highly recalcitrant variety such as Italia, laying the foundations for the application of NPBTs in *Vitis vinifera* L.

2. Materials and Methods

2.1. Plant Materials

Immature inflorescences of 13 *Vitis vinifera* L. varieties—Aglianico N., Autumn Royal N., Chardonnay B., Crimson Rs., Glera B., Italia B., Negromaro N., Palieri N., Primitivo N., Red Globe Rs., Sultanina B., Summer Royal N., and Victoria B.—were harvested from 15-year-old plants grown in the experimental field of the CREA (Azienda Lamarossa, Rutigliano, Southern Italy; 40°57′26″ N; 17°00′26″ E) between April to June 2019 and 23 April to 26 May 2020. The inflorescences were collected for the first year in the stages of early flower development I to VI and in the second year in stages I and II, according to Gribaudo et al. [13], when the pollen mother cells (PMC) were in the late premeiotic phase and early formation of the tetrad [13,14]. The development phase of the explants was determined by the examination of the microsporogenesis stage (Figure 1) with an optical microscope (Nikon Labophotmod. Y) after acetocarmine staining of crushed anthers [15,16].



(**A**) (**B**) (**C**)

Figure 1. Flower development stages: (**A**) PMC in late premeiotic phase (stage II); (**B**) PMC with callose wall (stage III); (**C**) Tetrads (stage IV). Bars: (**A**) 40 µm; (**B**) and (**C**) 20 µm.

The freshly collected explants were immersed in ethanol (70% *v/v*) for 30 s, then two different sterilization protocols with sodium hypochlorite were applied: a) sodium hypochlorite (0.8% active chlorine) for 10 min (LS); and b) sodium hypochlorite (2% active chlorine) for 2 min (HS). In the first year of experiments, only the LS protocol was used.

In both the LS and HS protocols, a few drops of Tween 20 were added under mild stirring. After rinsing in sterile water 3 times (one minute each), for each cultivar half of the explants were plated and a half was kept at 4 °C and plated at the latest within seven days to avoid possible contamination problems. In the first year, whole flowers of all 13 varieties were cultured (Table 1) while in the second year we focused on three varieties with different levels of recalcitrance; therefore, whole flowers of only Aglianico, Chardonnay, and Italia were cultured (Supplementary Materials Table S1). Whole flowers were used as explants, as already proposed by other authors for *Vitis* and *Pistacia vera* L. [17,18], instead of the stamens and ovaries. Using whole flowers is not only time-saving but allows greater ease of cultivation, results in less damage of stamens and ovaries, and allows a greater number of explants to be plated.

Table 1. Embryogenic callus formation on several varieties employed in the first year of experimentation.

Variety Name	Explants Number	Embryogenic Callus (%)
Aglianico N.	2030	0.34
Autumn Royal N.	560	0.89
Chardonnay B.	1050	4.1
Crimson Rs.	560	0.71
Glera B.	2100	0.05
Italia B.	5670	0.09
Negromaro N.	1120	0.09
Palieri N.	1260	0.16
Primitivo N.	1680	0
Red Globe Rs.	1750	0.06
Sultanina B.	1120	3.7
Summer Royal N.	1190	1.3
Victoria B.	1540	0.06

2.2. Media and Culture Conditions

Explants have been initially cultivated in the following media for callus induction (PIV) [13,17,19,20]: Nitsch and Nitsch mineral salt [21], Murashige and Skoog vitamins [22], 6% sucrose, and 0.3% Gellan Gum. The basal medium was supplemented with 4.5 µM 2,4-D and 8.9 µM BA. In the second year of experimentation citric acid was added to the media as an antioxidant in the dose of 0.15 g L^{-1} only to half of the samples [23]. The pH was adjusted to 5.8 with KOH 1 M before autoclaving (121 °C for 20 min). Flowers were cultured in 90 mm Petri plates containing 25 mL of medium (70 explants/plate) sealed with PARAFILM ®. The cultures were kept at 26 °C in the dark.

After the formation of the calluses (about 3–4 months) for their maintenance in the long term, two different substrates (GS1CA and C1) were sequentially used. These substrates have a composition similar to PIV except for the growth regulators. For GS1CA: 10 µM NOA, 1 µM BA, 20 µM IAA (the latter was filter-sterilized and added after autoclaving), and 0.25% activated charcoal [13,17–19]. For C1: 1 µM BA, 5 µM 2,4-D [24,25], and 0.15 g L^{-1} of citric acid. The pH was adjusted to 5.8 with KOH 1 M or HCl 0.5 M. The cultures were maintained under the conditions described above. The transfer of calluses on fresh substrate occurred monthly [26], alternating 1 month on GS1CA and 2 months on C1.

The Nitsch and Nitsch mineral salt, Murashige and Skoog vitamins, and plant growth regulators were purchased from Duchefa, Haarlem, The Netherlands. The Gellan Gum was purchased from Alfa Aesar by Thermo Fisher Scientific, Bond Street Ward Hill, MA, USA. Acetocarmine was purchased from Chem Cruz, Santa Cruz Biotechnology, Inc., Finnel, Dallas. The number of explants lost due to infection was recorded after one month in culture. Total calluses production was recorded after 8 months. Callus quality was evaluated differentiating calluses according to their morphology and embryogenic competence in agreement with previous literature data [9,10].

2.3. Statistical Analysis

The statistical procedures described in detail in the following paragraphs, including the principal component analysis (PCA) and ANOVA with interaction, were performed using the open-source R statistical software (R version 3.6.3, 29 February 2020) [27]. The R packages used are listed in alphabetical order: Cars [28] and FactoMineR [29]. Angular transformation was applied to the data before statistical analysis.

3. Results

In order to set up an optimal protocol for induction of embryogenic callus from *Vitis vinifera* varieties, we gathered several culture media from different works [19,25]. In the first round of experimentation in 2019, we assayed a large number of varieties (Table 1), testing our experimental condition and sampling times. We obtained embryogenic calluses from almost all tested varieties, except for Primitivo. The efficiency was in line with the literature data or, in some cases, even higher [12,13]. For recalcitrant varieties of particular interest, such as Italia, unfortunately, a success rate of less than one per thousand was observed (Table 1). Concerning the sampling time of the inflorescences, the explants from Italia (n = 5670) were qualified following the stage flowers reported in the literature [13]. It was observed the formation of rare (n = 4) embryogenic calluses only in stages I and II, corresponding to the late premeiotic phase of PMCs. Based on these evaluations, we have defined this harvesting period as optimal for a recalcitrant variety such as Italia.

The optimal sampling time and a tested protocol defined in the first year of experimentation were replicated in the second year on three selected varieties (Italia, Aglianico, and Chardonnay), aiming to improve their efficiencies by acting on some parameters, namely, antioxidants in media and conditions of sterilization. Regarding the latter, contrasting effects on the ratios of clean culture were found between LS and HS conditions. Indeed, in Italia HS was fully effective (100% i.e., no loss of samples due to infections) while LS was less effective (86%); in Chardonnay, both HS and LS achieved 100% efficiency; on the contrary, in Aglianico HS was less effective compared to LS (70% vs. 84%). The callus morphology and embryogenic competence were correlated as observed by others [11,12]. The friable (non-embryogenic) calluses (FC) appear pale yellow or white, the cells are weakly adhered to each other, with high water content. In the hard calluses (HC), the cells are strongly cohesive, this type of callus also was not embryogenic. The embryogenic ones (EC) appear granular with a light yellow or white color (Figure 2); however, it has been observed that even browned granular calluses have developed embryos in the long run. The friable calluses showed a faster growth rate than the embryogenic ones, confirming previous observations [12].

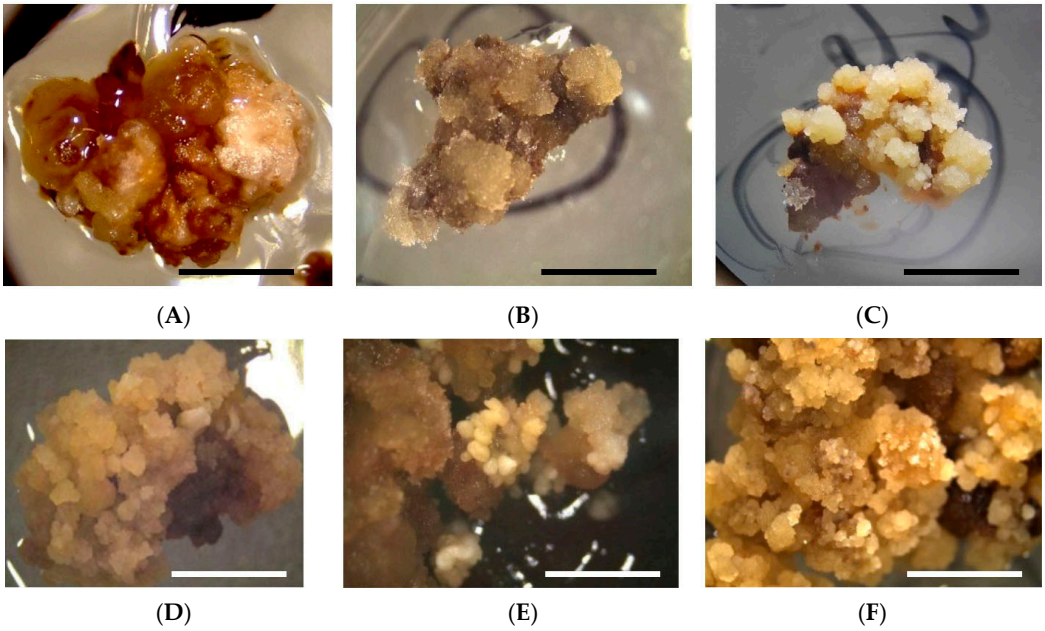


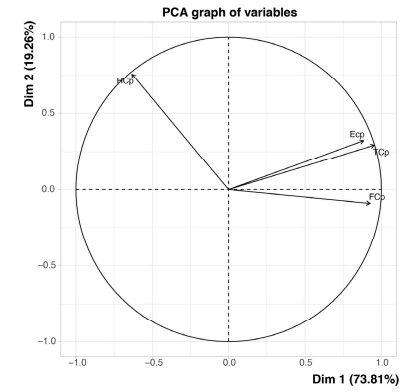
Figure 2. Type of callus at 8 months of culture: (**A**) hard callus Italia; (**B**) friable callus Italia. Embryogenic callus: (**C**,**D**) Italia, (**E**) Aglianico, and (**F**) Chardonnay. Bars: 5 mm.

The different types of calluses were recorded and expressed as percentages in relation to total explants in culture. A PCA was performed on the continuous variables: total (TC), friable (FC), embryogenic (EC), and hard (HC) calluses. The presence/absence of citric acid and the sterilization were used as supplementary categorical variables. In Figures 3C, 4C and 5C, each of the categories of the supplementary variables has been reported with a confidence ellipse. The sum of the first two dimensions describes 93.07%, 92.19%, and 78.6%, of the total variance of the dataset for Chardonnay, Aglianico, and Italia, respectively.

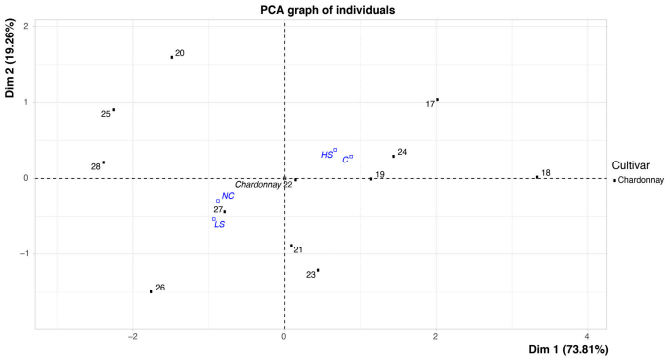
The influence of an early presence of citric acid (C) together with higher chlorine concentration (HS) on Chardonnay was evident for both embryogenic calluses and total calluses formation (EC and TC).

For Aglianico samples, it was found that shorter sterilization with a higher chlorine content (HS) and the presence of citric acid in the early stages in the culture media (C) favor the formation of hard calluses (HC). The influence of the investigated factors was not straightforward for other types of callus formation.

For Italia, the influence of both factors on embryogenic calluses formation was clear (Figure 5A,B). The initial absence of citric acid (NC) coupled with shorter sterilization with a higher chlorine content (HS) were favorable factors for the formation of EC. On the other hand, the initial presence of citric acid (C) and a lower chlorine content (LS) were favorable conditions for HC and TC formation.



(**A**)



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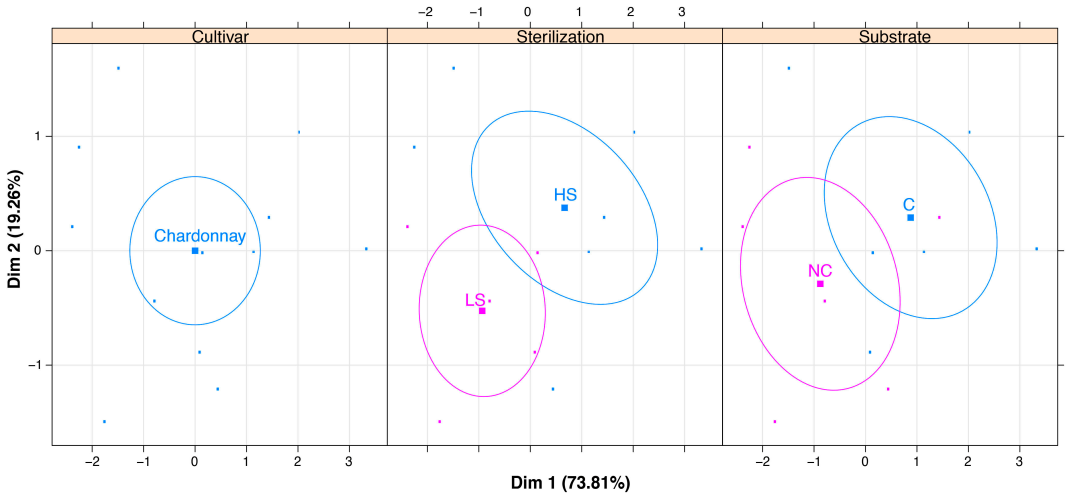
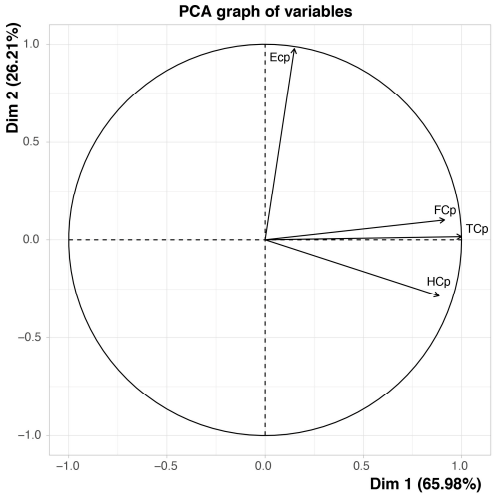
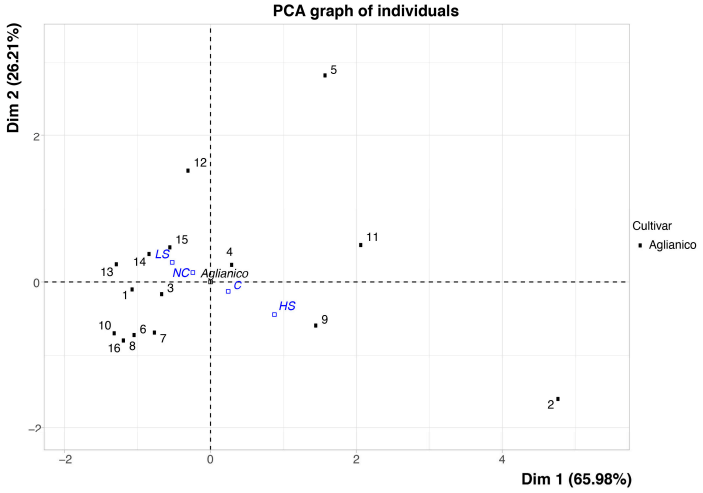


(**C**)

Figure 3. PCA plot for Chardonnay: (**A**) loading plot, (**B**) score plot, and (**C**) categories of the supplementary variables.

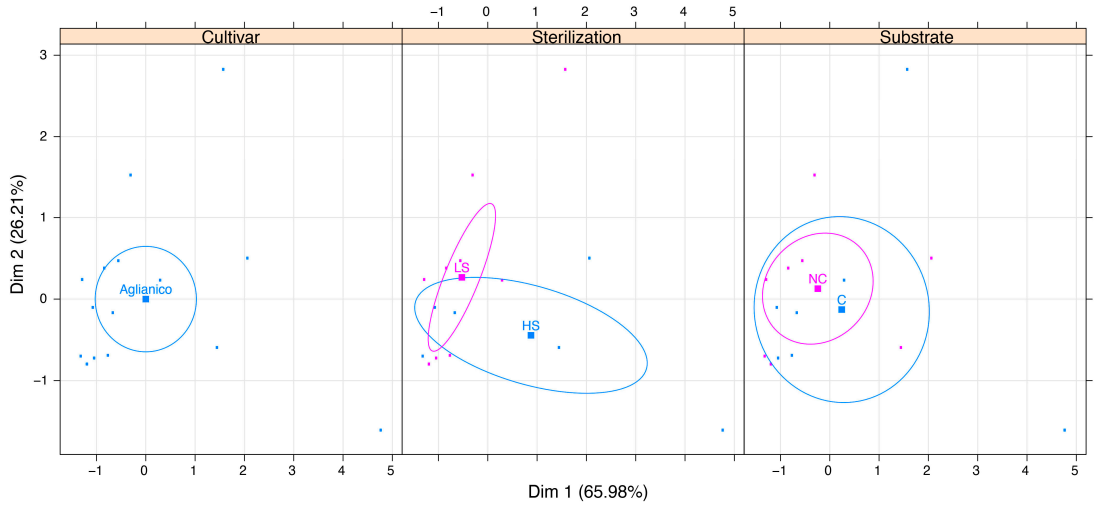


(**A**)



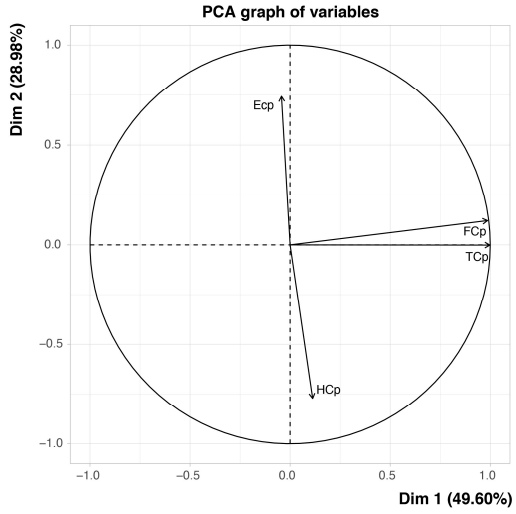
(**B**)

Figure 4. *Cont.*

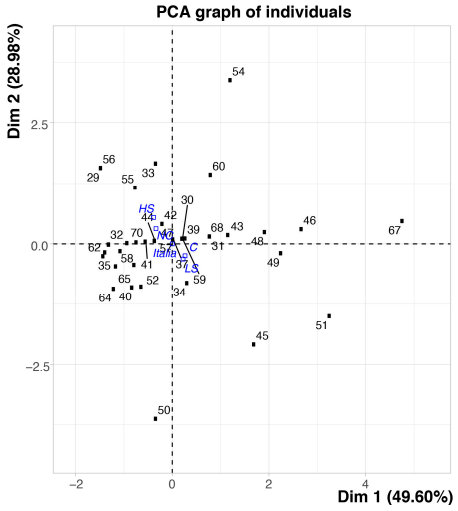


(**C**)

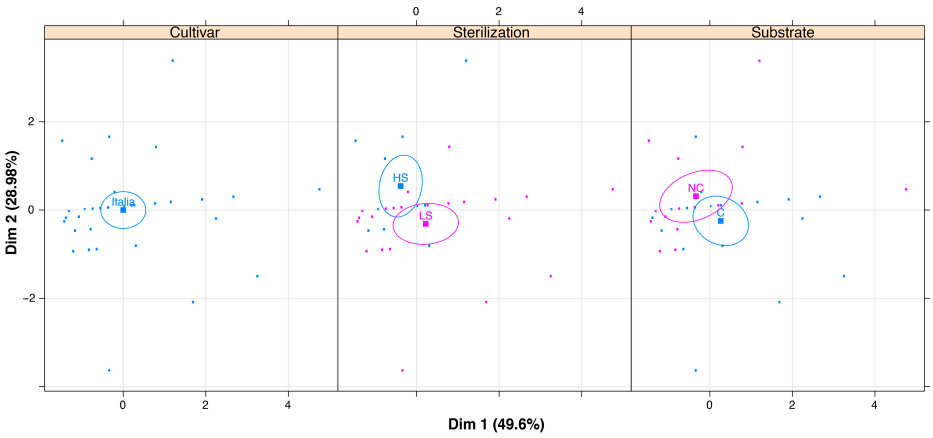
Figure 4. PCA plot for Aglianico: (**A**) loading plot, (**B**) score plot, and (**C**) categories of the supplementary variables.



(**A**)



(**B**)



(**C**)

Figure 5. PCA plot for the Italia (**A**) loading plot, (**B**) score plot, and (**C**) categories of the supplementary variables.

These results show the cv. Italia embryogenic calluses formation is favored by the absence of citric acid in the early stage of culture. On the contrary, embryogenic calluses production for Chardonnay is favored by citric acid added early to the culture media. For both Italia and Chardonnay shorter sterilization with higher chlorine content favored the embryogenic calluses formation. For Aglianico, it is not clear the influence of different factors in the formation of embryogenic calluses.

Following the PCA, a three-way ANOVA with interaction was performed to evaluate the combined effect of the three categorical experimental factors, namely, Variety, Sterilization, and Substrate, on the continuous variables TC, FC, HC, and EC. The interactions between Sterilization x Variety and Substrate x Variety for EC were found significant. Therefore, the effect of the Substrate and Sterilization factors changes depending on the level of the Variety factor (Supplementary Materials Table S2). Due to the influence of the Variety, each of the three cultivars was analyzed separately (Supplementary Materials Table S3). Regarding EC, for the cultivar Italia, it was significantly influenced by the factor Sterilization ($p \leq 0.01$), while for Chardonnay it was influenced by the factor Substrate ($p \leq 0.01$). Furthermore, for Chardonnay, the factors Substrate and Sterilization separately affect the total TC ($p \leq 0.05$).

In particular, in Italia EC increased approximately 13-fold, and in Chardonnay, TC increased 2-fold by sterilizing the flowers with HS procedure, compared to LS. Otherwise, in Chardonnay, the presence of acid citric in the substrate increased the TC and EC by 2- and 3-fold, respectively, while high sterilization (HS) increased the TC approximately by 2-fold (Table 2). Concerning Aglianico, no treatment had significant effects.

Table 2. Effect of the substrate (with citric acid, C, and without, NC) and sterilization procedure on the percentage of different callus types.

Cultivar	Callus Types (%)	Substrate			Sterilization		
		C	NC		LS	HS	
Italia	Total (TC)	14.73	9.18	n.s.	13.36	10.71	n.s.
	Friable (FC)	12.30	8.25	n.s.	11.74	8.66	n.s.
	Hard (HC)	2.04	0.54	n.s.	1.54	1.16	n.s.
	Embryogenic (EC)	0.39	0.38	n.s.	0.07	0.89	**
Aglianico	Total (TC)	20.90	15.80	n.s.	12.93	27.38	n.s.
	Friable (FC)	11.25	9.82	n.s.	7.43	15.71	n.s.
	Hard (HC)	8.04	4.02	n.s.	3.21	10.71	n.s.
	Embryogenic (EC)	1.61	1.96	n.s.	2.29	0.95	n.s.
Chardonnay	Total (TC)	50.71	26.43	*	22.86	49.80	*
	Friable (FC)	20.71	13.10	n.s.	9.14	22.45	n.s.
	Hard (HC)	4.05	5.48	n.s.	4.00	5.31	n.s.
	Embryogenic (EC)	25.95	7.86	**	9.71	22.04	n.s.

Citric acid: C always present, NC added after 3–4 months. Flower sterilization: HS: with 2% of active chlorine for 2′; LS: with 0.8% of active chlorine for 10′. n.s., not significantly different; *, significantly different according to Duncan's test at $p \leq 0.05$; **, significantly different at $p \leq 0.01$.

These results confirm the PCA outcome concerning the effect of the sterilization on Italia and the substrate on Chardonnay. On the other hand, for Aglianico, the PCA results were not confirmed. Overall, the analyses performed indicate that the Sterilization and Substrate factors had a significant effect on callus production with a strong difference based on the Variety factor.

4. Discussion

The present work tackled one of the most critical aspects of the application of NPBTs to vines and in general in all plant species: handling recalcitrant varieties. To regenerate a new plant with genetic engineering, it is necessary to have tissues with embryogenic competence. These tissues in vines can be obtained from cultures of flowers, ovaries, or anthers collected in a specific and narrow window of time. Unfortunately, the response to culture conditions

with embryogenic callus production was generally low. The influence of the genotype in callus induction and plant regeneration is well known [30]. Due to the aforementioned difficulties, the majority of the published works employed genotypes that respond very well to the conditions of culture, can produce large quantities of calluses with embryogenic competence, and regenerate with high efficiency [19,31]. Unfortunately, many valuable grape varieties are not included among the few well-responding genotypes. In this work, we have selected a highly appreciated table grape variety, the Italia, which is considered recalcitrant. The only reported study on this genotype showed a very low production of calluses that often show no embryogenic competence [12]. In our experimental design, to evaluate the efficacy of the conditions tested, in addition to Italia we have also worked on other varieties that have a different ability to produce embryogenic calluses. As a variety with a low to medium embryogenic efficiency, we used Aglianico, a black grape variety typical of southern Italy. As a control, we used a variety with high embryogenic efficiency, namely, Chardonnay blanc [17,31,32].

Our preliminary results have shown how culturing whole flowers instead of only the filaments of anthers or ovaries was an efficient strategy that allows processing in a short time and more explants with good results. This was very important especially for varieties with very low rates of embryogenesis.

The protocol adopted in the first year of experimentation in this work allows obtaining embryogenic calluses from almost any of the tested genotypes.

The results of this work support the findings of previous authors also on other varieties concerning the influence of genotype on calluses formation [13,17,26,32]. Besides the strong genotype influence, the formation of total and embryogenic calluses was influenced by the type of sterilization and the cultivation substrate. Of particular importance for the Italia variety was the finding that a significant variation in the frequency of formation of the embryogenic callus was found when the flowers were treated with more stringent sterilization conditions. This behavior, also observed in Chardonnay blanc, can be interpreted as a result of necrosis of the tissues of the calyptra due to the higher concentration of sodium hypochlorite. Since the production of embryogenic calluses derives from the anthers and ovaries, there would be a reduced impact on the total proliferation of cells from the tissues of the calyptra, responsible for the production of non-embryogenic friable calluses with a higher growth rate [12]. Non-competent calluses grow fast and cause the few and slow-growing embryogenic ones to disperse whilst competing for nutrients and space available in the culture media. The beneficial effects of stressful conditions have been also observed in other species, such as the formation of embryogenic calluses in the culture substrate of *Cattleya maxim*, which was determined by saline stress due to 0.3 M NaCl [33].

Another improvement in the culture conditions concerns the addition of citric acid, which can keep the calluses vital for a longer period [23]. Improving the vitality of calluses in the long term was necessary to apply NPBTs. Since it was necessary to obtain the relevant quantities of calluses, a long time was required to multiply the calluses characterized by slow growth. Furthermore, it has been observed that in the long run even some browned calluses, believed to have lost vitality and embryogenic competence, eventually did produce embryos. Citric acid has been shown, at least in Chardonnay, which was the genotype with the highest embryogenic efficiency, to improve the vitality of calluses in the long term, thus increasing the efficiency of the procedure. On the other hand, no consistent conclusions could be drawn for the effect of citric acid in the more recalcitrant varieties such as Italia and Aglianico.

The results of this work describe how our modified protocol significantly increases the production of embryogenic calluses, which was a fundamental aspect for the applicability of NPBTs such as cisgenesis and genome editing. The positive results obtained for the highly recalcitrant variety chosen in this work are promising, although the applicability to other valuable recalcitrant *Vitis* cultivars needs further investigation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7110511/s1, Table S1: Numbers of different callus types in each Substrate

Sterilization combination for each variety, scored after eight months of culture, losses due to infection were excluded, Table S2: Analysis of Variance for callus types with three factors, Table S3: Analysis of Variance of embryogenic calluses (EC) on each Cultivar.

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Abbreviations

CRISPR/CAS	clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR-associated proteins);
2,4-D	2,4-dichlorophenoxyacetic acid;
BA	6-benzylaminopurine;
KOH	potassium hydroxide;
NOA	2-naphthoxyacetic acid;
IAA	indole-3-acetic acid;
HCl	hydrochloric acid;
ANOVA	analysis of variance;
NaCl	sodium chloride.

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