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ORIGINAL ARTICLE

# Regeneration of plants from embryogenic callus-derived protoplasts of Garganega and Sangiovese grapevine (*Vitis vinifera* L.) cultivars

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## Abstract

Protoplasts are useful research tools for basic and applied plant science, but the regeneration of whole plants from protoplasts is challenging in most of agronomically important crops, including grapevine (*Vitis vinifera* L.). Here we describe an efficient protocol for the induction of embryogenic callus, the isolation of protoplasts, and the regeneration of whole grapevine plants in two Italian grapevine cultivars. Embryogenic callus was induced successfully from stamens collected from immature flowers. Isolated protoplasts were tested to confirm their viability and then cultivated using the disc-culture method, at a density of $1 \times 10^5$ protoplasts/mL in solid Nitsch's medium supplemented with 2 mg/L 1-naphthaleneacetic acid and 0.5 mg/L 6-benzylaminopurine. After 3–4 months, the protoplasts of both cultivars regenerated with similar efficiency into cotyledonal-stage somatic embryos. The somatic embryos were transferred to solid Nitsch's medium supplemented with 30 g/L sucrose and 2 g/L gellan gum, and were maintained in the dark for 4 weeks. This step was necessary for the embryo to complete germination, allowing subsequent shoot elongation in response to light on a medium with 4 µM 6-benzylaminopurine. Then root elongation occurred after transferring on a medium with 0.5 µM 1-naphthaleneacetic. After ~ 6 months from the isolation of protoplasts, normal plants were regenerated, which were moved to the greenhouse. The protoplasts could also be transfected using the polyethylene glycol method, as confirmed using a plasmid carrying the yellow florescent protein marker gene. The new method is therefore compatible with biotechnological applications such as gene transfer and genome editing.

## Key message

This study reports an improved protocol for embryogenic callus induction, protoplast isolation andwhole plant regeneration of two *Vitis vinifera* cultivars. Protoplasts showed high transfectionefficiency.

**Keywords** *Vitis vinifera* · Embryogenic callus · Protoplast isolation · Plant regeneration · Protoplast transfection

## Abbreviations

| | |
|---|---|
| PEG | Poly-ethylene glycol |
| YFP | Yellow fluorescence protein |
| CRISPR/Cas | Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein |
| FDA | Fluorescein diacetate |
| NAA | 1-Naphthaleneacetic acid |
| 6-BAP | 6-Benzylaminopurine |
| RNPs | Ribonucleoproteins |
| MES | 2-(N-morpholino)ethanesulfonic acid |

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## Introduction

Grapevine (*Vitis vinifera* L.) is an economically important fruit crop whose ripe berries are rich in sugars and secondary metabolites, including anthocyanins, tannins, carotenoids, norisoprenoids, terpenes and other volatile organic compounds. The development of grapevine plants and especially the berries has been studied in great detail, and the molecular basis of development has been well characterized due to the availability of a high-quality draft genome sequence (Jaillon et al. 2007) and high-throughput analytical methods to study the transcriptome, proteome and

metabolome (Zamboni et al. 2010; Fasoli et al. 2012, 2018). During the last 10 years, many candidate genes that directly control biotic and abiotic stress responses or berry ripening have been identified using such methods (Kuhn et al. 2014; Serrano et al. 2017; Fasoli et al. 2018). However, grapevine is generally recalcitrant to transformation and regeneration, and there are no comprehensive mutant libraries, so only a few studies have been published in which the functions of such candidate genes have been tested directly by gene transfer or targeted mutation (Rinaldo et al. 2015; He et al. 2018; Dal Bosco et al. 2018).

Protoplasts are plant cells lacking the typical polysaccharide wall. They are prepared by mechanical and/or enzymatic treatments that leave the cell contents bound by an intact plasma membrane. Protoplasts remain viable and are useful tools for both basic and applied scientific research because they facilitate techniques that are not applicable to walled plant cells, including gene transfer by chemical transfection with polyethylene glycol (PEG) or by electroporation, cell sorting and isolation by flow cytometry, the analysis of membrane biology, the induction of somaclonal variation, and plant breeding by protoplast fusion (Davey et al. 2005). Like walled plant cells, protoplasts also remain amenable to alternative gene transfer methods such as particle bombardment and transformation by *Agrobacterium tumefaciens*. Recently, protoplasts have also been used for genome editing in many plant species (Xie and Yang 2013; Subburaj et al. 2016; Woo et al. 2015). This is a form of targeted mutagenesis based on sequence-specific nucleases and is considered the new frontier in plant breeding. The most widely used genome editing platform is based on clustered regularly interspaced short palindromic repeats (CRISPR) targeted by an RNA-guided nuclease known as CRISPR-associated protein 9 (Cas9). Although the combination of protoplasts and CRISPR/Cas9 has made targeted mutagenesis possible in almost any plant species, the bottleneck is the ability to regenerate fertile plants from protoplasts. This step is necessary for the analysis of gene function in whole plants, but is still very challenging in most agronomically important crops. Plant cells are generally totipotent, which means that fully differentiated and even non-dividing cells can be persuaded to dedifferentiate and re-enter the cell cycle, producing a mass of undifferentiated cells called a callus. In the appropriate medium, callus can regenerate into whole plants via one of two routes: organogenesis, which involves the direct development of shoots and roots, or somatic embryogenesis, in which plant development is completely recapitulated, including the embryonic stage. In some species, callus formation can be triggered from a protoplast culture, allowing whole plants to be regenerated from protoplasts that have been genetically transformed or modified by genome editing. Regeneration from protoplasts can be divided into four main phases: formation of a new cell wall, cell elongation and initial divisions, proliferation to form a micro-callus and then a macro-callus, and finally regeneration by organogenesis or somatic embryogenesis (Papadakis et al. 2009).

In grapevine, protoplasts can be prepared from cells isolated from leaves, roots or berry mesocarp (Papadakis and Roubelakis-Angelakis 1999; Fontes et al. 2010). Recently, protoplasts were also prepared from the embryogenic callus of a Chardonnay cultivar and genome editing was achieved by transformation with the traditional guide RNA/Cas9 plasmid DNA or by the direct introduction of guide RNA/Cas9 ribonucleoproteins (Malnoy et al. 2016; Osakabe et al. 2018). However, it was not possible to regenerate whole plants from these genome-edited protoplasts. Indeed, only two previous reports have described the successful regeneration of plants from grapevine protoplasts, one representing the interspecific hybrid Seyval Blanc, which comprises 50% *V. vinifera*, 37% *V. rupestris* and 13% *V. licencumii* (Reustle et al. 1995), and one for the *V. vinifera* cultivar Koshusanjaku (Zhu et al. 1997).

To overcome this barrier, here we report a stepwise protocol for the regeneration of whole plants from embryogenic callus-derived protoplasts of two Italian grapevine cultivars: the white-skinned cultivar Garganega, and the red-skinned cultivar Sangiovese. Although regeneration was achieved in both cases, the formation of somatic embryos and the subsequent regeneration process were more efficient in Garganega. We also achieved the PEG-mediated transfection of protoplasts representing both varieties, as shown by the expression of a yellow fluorescent protein (YFP) marker gene. These accomplishments therefore provide the basis for functional analysis, biotechnological applications and precision crop breeding in grapevine.

## Materials and methods

### Plant material and the induction of embryogenic cultures

Embryogenic Garganega and Sangiovese callus was initiated from immature stamen cultures. Briefly, inflorescences were collected from plants of both cultivars growing in an experimental vineyard in the province of Verona, Italy. The flowers were surface sterilized by immersing them in 100 mL of 7% $Ca(ClO)_2$ containing one/two drops of Tween-20 for 50 s with constant agitation, followed by three 5-min washes in sterile distilled water. Stamens (anthers with intact filaments) were carefully separated from the calyptra and pistil before placing 50 stamens on plates containing PIV medium (Franks et al. 1998). After 2–3 months, embryogenic callus was transferred to $C1^P$ medium and subcultured in the same medium every 4 weeks (Iocco et al. 2001).

### Protoplast isolation

Protoplasts were prepared as described by Zhu et al. (1997). Briefly, protoplasts were isolated from embryogenic callus after 7–10 days of subculture in $C1^P$ medium by incubation for 6 h on a gyratory shaker in 10 mL filter-sterilized digestion solution per 1 g of embryogenic material. The digestion solution comprised 2% w/v Cellulase Onozuka, 1% w/v Macerozyme R-10, 0.05% w/v Pectolyase Y-23, 10 mM $CaCl_2$, 5 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) and 0.5 M mannitol (pH 5.7). The mixture was filtered through a nylon sieve (60 µm) and the protoplasts were washed twice with washing solution (10 mM $CaCl_2$ and 0.5 M mannitol). The viability of the protoplasts was tested under UV light after staining with 0.5 mg/mL fluorescein diacetate (FDA).

### Protoplast culture for somatic embryogenesis

Isolated protoplasts were cultivated at a density of $1 \times 10^5$ protoplasts/mL using the disc-culture method (Zhu et al. 1997). Briefly, 800-µL droplets containing protoplasts in solid Nitsch's medium supplemented with 2 mg/L 1–naphthaleneacetic acid (NAA), 0.5 mg/L 6-benzylaminopurine (6-BAP), 0.3 M glucose, 0.09 M sucrose and 2 g/L gellan gum (pH 5.7) were poured into Petri dishes. After solidification, liquid Nitsch's medium with the same composition but supplemented with 0.3% activated charcoal was added as a reservoir. The liquid medium was replaced every 2 weeks with fresh medium as described above but without glucose. Cultured protoplasts were maintained at 28 °C. After 3–4 months of culture, protoplast-derived cotyledonal somatic embryos were transferred to solid Nitsch's medium supplemented with 30 g/L sucrose and 2 g/L gellan gum (pH 5.7) and maintained in the dark for 4 weeks to allow complete germination.

### Embryo development and regeneration of whole plants

Embryo development and plant recovery were carried out as described by Li et al. (2014). Germinated somatic embryos were transferred to C2D4B medium (C2D medium supplemented with 30 g/L sucrose, 4 µM 6-BAP and 7 g/L TC agar, pH 5.8) and maintained under light (65 µE, 16-h photoperiod) at 25 °C for 3–4 weeks. Plantlets were transferred to MSN medium (Murashige and Skoog (MS) medium containing 30 g/L sucrose, 0.5 µM NAA and 7 g/L TC agar, pH 5.8) to promote elongation of roots and development of the whole plant. Vigorous plants were transferred to potting soil and acclimated in a growth room for ~ 2 weeks before transfer to the greenhouse.

### PEG-mediated protoplast transfection and analysis of fluorescence

Protoplasts were transfected as described by Woo et al. (2015). We resuspended $5 \times 10^5$ protoplasts of each cultivar in 200 µL of MMG solution (0.4 M mannitol, 15 mM MgCl2, 4 mM MES [pH 5.7]) and gently mixed with 50 µg of the pEGB3Ω1-35S::*YFP*::Tnos vector carrying the *yfp* marker gene (Sarrion-Perdigones et al. 2013) and 210 µL freshly prepared PEG solution (40% (w/v) PEG 4000, 0.2 M mannitol and 0.1 M $CaCl_2$). The mixture was incubated for 20 min at 25 °C in darkness before slowly adding 950 µL W5 solution (2 mM MES pH 5.7, 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl). The resulting solution was mixed well by pipetting. Protoplasts were pelleted by centrifugation at 100×*g* for 3 min and resuspended gently in 1 mL WI solution (0.5 M mannitol, 20 mM KCl and 4 mM MES pH 5.7). Finally, the protoplasts were transferred to multi-well plates and cultured in the dark at 25 °C. YFP expression in transfected protoplasts was monitored 24, 48 and 72 h post-transfection using a Leica MZ 16 F stereomicroscope equipped with a Leica CLS 150 X light source and YFP filter set comprising an excitation filter (500/20 nm) and a barrier filter (535/30 nm).

## Results

### Induction of embryogenic callus and protoplast isolation

Garganega and Sangiovese anthers were collected from immature flowers and cultured in PIV medium to induce embryogenic callus development. The callus appeared after about 3 months in both cultivars, with no differences in callus induction efficiency. Protoplasts of Garganega and Sangiovese were isolated from the callus after 7–10 days of subculture in $C1^P$ medium (Fig. 1a). During subculture, many of the Sangiovese embryogenic callus pieces turned brown, and these were discarded. The quantity of embryogenic material used for protoplast isolation was 0.2 g for both cultivars and the yield in both cases was ~ $2 \times 10^6$ protoplasts (Fig. 1b). The isolated protoplasts were tested for viability by FDA staining under UV light (Fig. 1c). Comparison of the epifluorescence and white light images (Fig. 1b, c) showed that most protoplasts remained viable immediately after isolation, as confirmed by their intense green fluorescence. The integrity of protoplasts in both cultivars exceeded 80%.

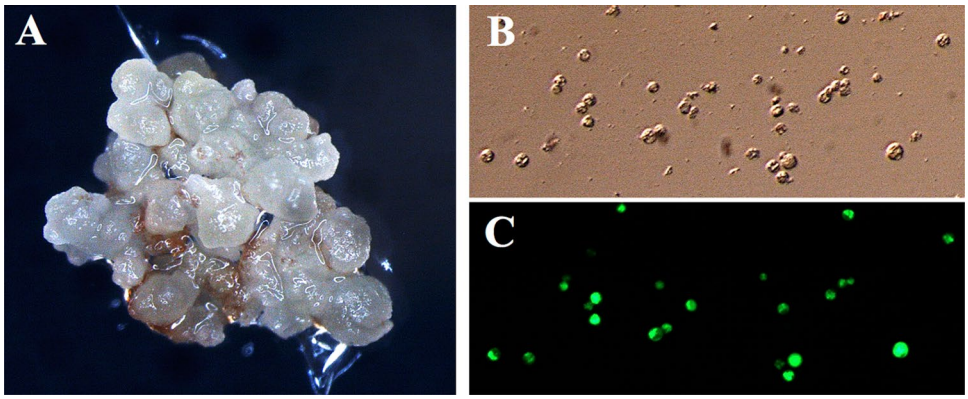


**Fig. 1** Protoplast isolation from embryogenic callus. **a** Garganega embryogenic callus after 7–10 days of subculture. **b** Protoplasts isolated from the callus—white light. **c** Protoplasts isolated from the callus and labeled with FDA—UV light

## Cell division, microcolony formation and somatic embryogenesis

Isolated protoplasts of both cultivars were cultivated at a density of $1 \times 10^5$ protoplast/mL using the disc-culture method. In this method, the droplets of solid culture medium containing isolated protoplasts are surrounded by liquid culture medium supplemented with activated charcoal, which prevents the browning of the culture and thus promotes cell division and colony formation (Zhu et al. 1997). The first protoplast cell division occurred after 10 days in both cultivars (Fig. 2a, b). Further cellular divisions occurred after ~ 30 days (Fig. 2c, d) and microcolonies of both cultivars were observed after ~ 40 days from protoplast isolation (Fig. 2e, f), suggesting that embryogenesis was not induced directly from protoplasts but rather from the protoplast-derived callus. After ~ 2 months, somatic embryos recovered on the liquid Nitsch's medium initiated germination and the typical globular and heart stages of embryo development were visible in both cultivars (Fig. 2g–j). Mature cotyledonary embryos were formed starting 3 months after the protoplast culture (Fig. 2k–m). After 4 months, starting from the same number of isolated protoplasts, we recovered 87 Garganega and 78 Sangiovese cotyledonary embryos, suggesting that Garganega has a slightly higher embryo regeneration efficiency.

## Regeneration of whole plants

We observed that the direct transfer of cotyledonary embryos to solid Nitsh's medium under light has caused browning and was ultimately lethal. For this reason, cotyledonary somatic embryos were maintained in the dark in Nitsh's medium supplemented with sucrose for about 1 month to allow complete embryo germination. Only germinated somatic embryos were then transferred to C2D4B medium under light for shoot elongation (Fig. 3a). The number of germinated embryos obtained was 55 of 87 (63%) for Garganega and 33 of 78 (42%) for Sangiovese. The remaining somatic embryos were either immature or abnormal, and these were discarded.

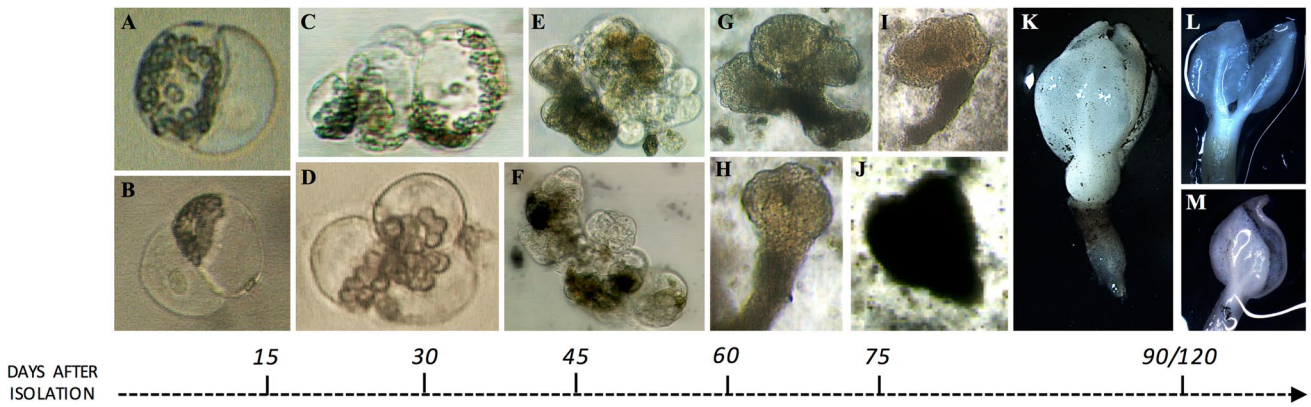


**Fig. 2** Protoplast development to mature embryo. **a**, **b** First cellular divisions, appearing ~ 10 days after protoplast isolation. **c**, **d** Further cellular divisions appearing ~ 30 days after protoplast isolation. **e**, **f** Microcolony formation ~ 40 days after protoplast isolation. **g–i** Globular stage of embryo development. **j** Heart stage of embryo development, appearing 2/3 months after protoplast isolation. **k–m** Cotyledonal stage mature embryos appearing ~ 3 months after protoplast isolation

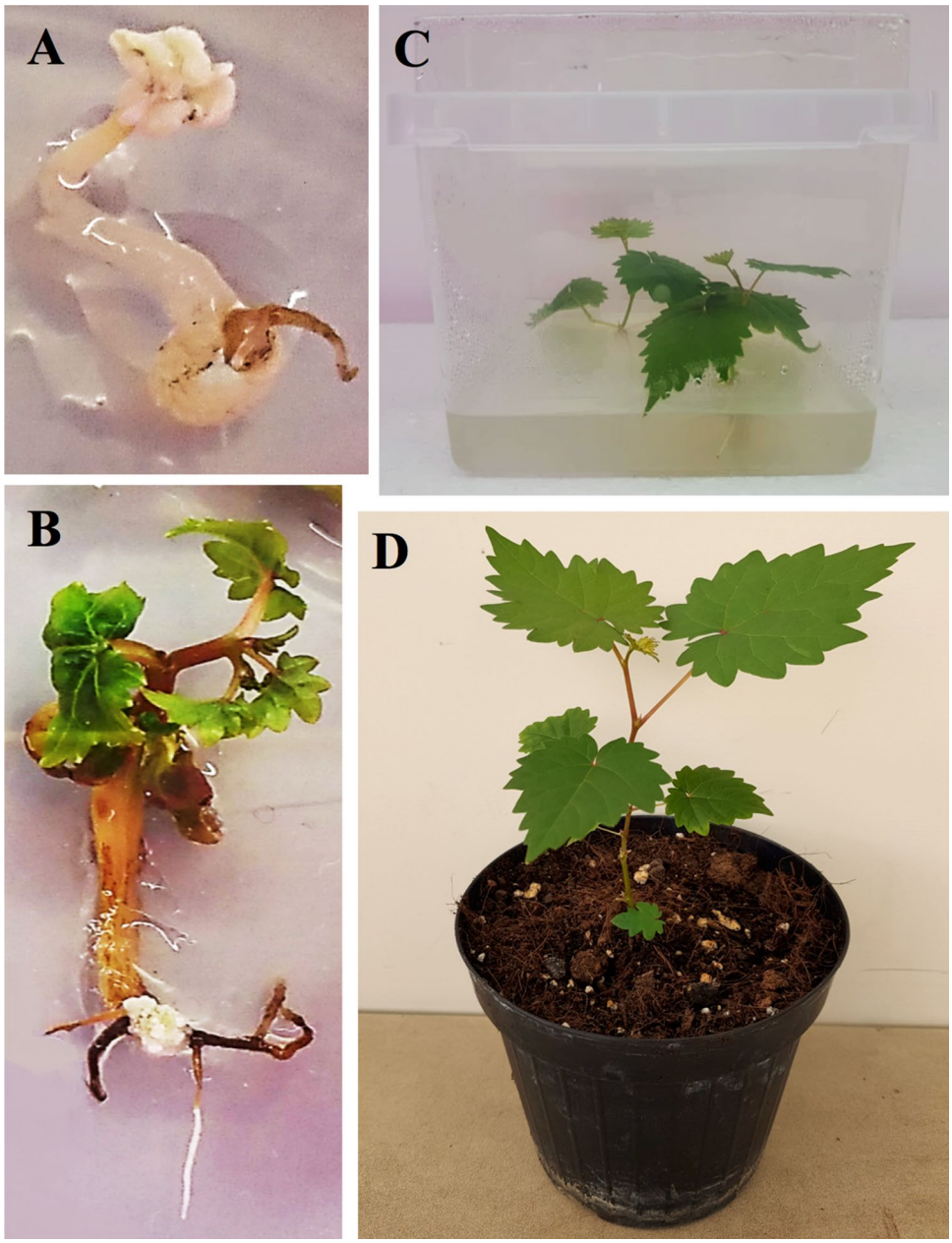


**Fig. 3** Regeneration of whole plants from somatic embryos. **a** Germinated somatic embryo. **b** Plantlet from somatic embryo. **c** Two in vitro regenerated plants. **d** Regenerated protoplast-derived whole plant

Shoot elongation from germinated embryos generally occurred within 5 weeks after transfer to light, but in some cases the process took as long as 10 weeks (Fig. 3b). We recovered 21 Garganega but only nine Sangiovese plantlets from germinated embryos. These were transferred to MSN medium to allow root elongation and further plant development. The germinated embryos developed into whole plants with expanded leaves and roots after 4 weeks (Fig. 3c). All the 21 Garganega plantlets developed into whole plants, but we recovered only seven whole plants from the Sangiovese plantlets. After acclimation, the regenerated plants were transferred to the greenhouse. The plants of both cultivars showed normal growth and morphology (Fig. 3d).

## PEG-mediated protoplast transfection

PEG-mediated transfection was carried out using protoplasts of both varieties to evaluate their transformation efficiency, with *yfp* as a visible marker gene. We transfected $1 \times 10^5$ protoplasts in medium containing 40% PEG 4000 and 50 µg of the plasmid pEGB3Ω1-35S::*YFP*::Tnos, carrying a cassette for YFP overexpression. We monitored YFP fluorescence 24, 48 and 72 h post-transfection, and detected a signal at all three time points (Fig. 4a). Transfected protoplasts showed homogeneous YFP expression and there was no apparent increase in fluorescence from 24 to 72 h post-transfection. The absence of a signal in the negative control (protoplasts

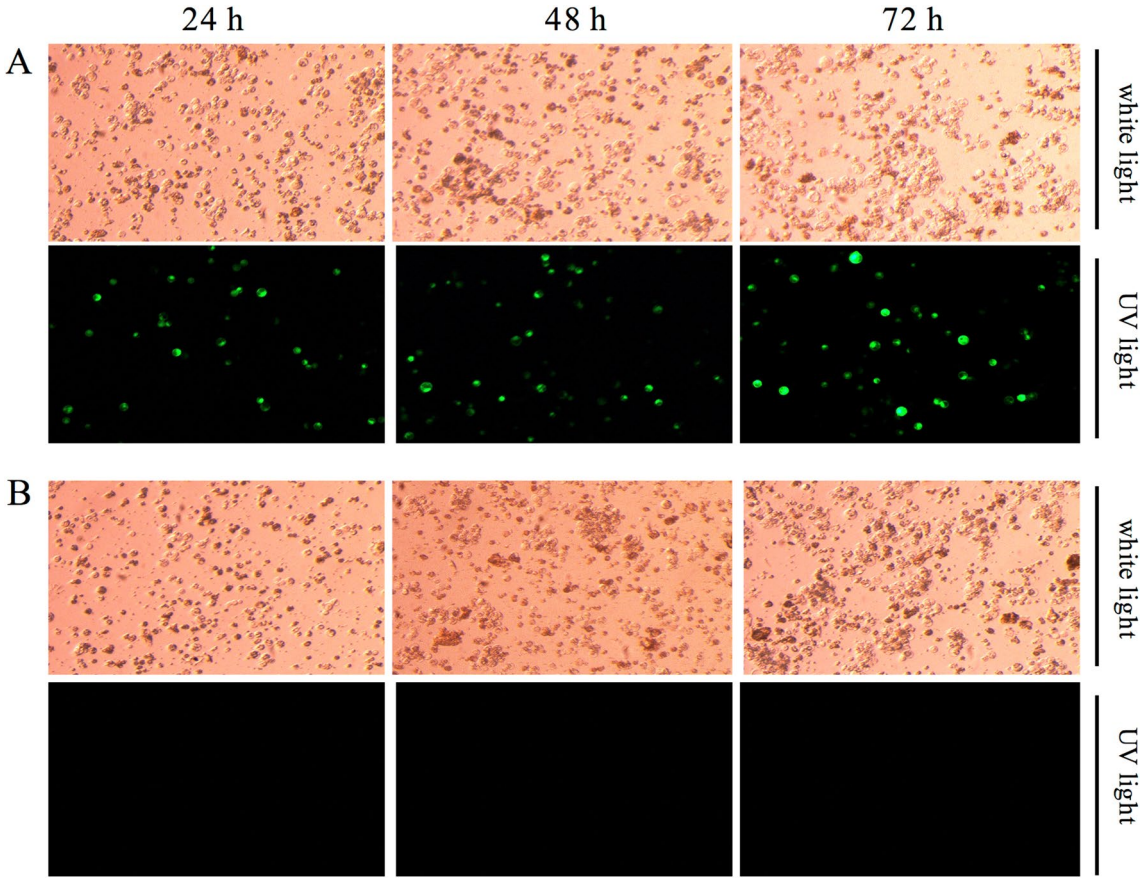


**Fig. 4** PEG-mediated transfection of protoplasts. **a** Protoplasts transfected with plasmid containing the YFP expression cassette. **b** Protoplasts transfected with an empty vector, as negative control. The YFP signal was detected by fluorescence microscopy 24, 48 and 72 h post-transfection

transfected with the empty vector) confirmed the success of the transfection (Fig. 4b).

## Discussion

Plant protoplasts are useful for basic and applied research, particularly for the functional analysis of genes and the modification of plants by gene transfer or genome editing (Fontes et al. 2010; Malnoy et al. 2016). However, the protoplasts themselves are only useful for the analysis of cellular functions, and the regeneration of whole plants is necessary to determine how genes affect plant development or physiology. This is a major bottleneck in many plant species, including grapevine, which is highly recalcitrant to regeneration.

Grapevine protoplasts have been isolated from various organs, but the regeneration of whole plants has been achieved in only a handful of cases and only when the protoplasts were isolated from embryogenic tissue (Reustle et al. 1995; Zhu et al. 1997). These regeneration protocols have not been widely adopted because they tend to be inefficient and highly genotype dependent, which is challenging in a species renowned for its huge range of cultivars.

To address this challenge, we have developed an efficient protocol for the regeneration of whole grapevine plants from protoplasts isolated from embryogenic callus, and have demonstrated its feasibility in two diverse cultivars (Garganega and Sangiovese) representing the north-east and center viticultural regions of Italy, respectively. Protoplasts of both cultivars were isolated from embryogenic callus derived from anthers. The protoplasts were cultivated as previously described by Zhu et al. (1997), including the key step of disc-culture cultivation in solid medium in the presence of activated charcoal to adsorb contaminants released from protoplasts, which would otherwise promote browning and ultimately cell death. This approach was similarly effective in both cultivars, leading to the efficient recovery of a large number of cotyledonary somatic embryos.

The direct transfer of these cotyledonary somatic embryos to the light did not result in further development. We therefore modified the original protocol described by Zhu et al. (1997) by introducing a dark adaption period lasting ~ 1 month, which was necessary for the cotyledonary

somatic embryos to complete germination and become competent for full regeneration. We found that the dark-adaption strategy was more effective in Garaganega embryos, producing a larger number of germinated embryos, but nevertheless we also recovered a significant number of Sangiovese embryos suitable for regeneration.

The regeneration of whole plants from germinated embryos was achieved by strictly following the method described by Li et al. (2014). This included cultivation on C2D4B medium supplemented with 6-BAP to promote shoot elongation, followed by transfer to MSN medium supplemented with NAA to induce root elongation and plant development. The overall regeneration efficiency from cotyledonal embryos to whole plants was three times higher in Garganega (24%) than Sangiovese (9%). These values are similar to the ~ 12% efficiency originally reported for the cultivar Koshusanjaku (Zhu et al. 1997).

Although the two cultivars showed differences in regeneration efficiency, the ability of this new protocol to support the regeneration of diverse genotypes indicates that it should be applicable to many other cultivars used for wine production. Some of the Sangiovese callus clones showed evidence of browning, which indicated their lack of competence for further development. This behavior appears to be related to the recalcitrance of this and other cultivars to stable transformation mediated by *A. tumefaciens* (our unpublished data). However, we were able to achieve the transformation of Sangiovese protoplasts by PEG-mediated transfection, and this could represent an alternative strategy to introduce new traits into recalcitrant cultivars.

Our new regeneration protocol encourages the application of biotechnological approaches to grapevine protoplasts, including genome editing for the introduction of targeted genetic changes with unprecedented control and accuracy. Genome editing has been successfully applied in grapevine by the transfection of protoplasts with standard guide RNA/Cas9 vectors, by the direct introduction of guide RNA/Cas9 ribonucleoproteins (Malnoy et al. 2016; Osakabe et al. 2018) and by the transformation of embryogenic callus with *A. tumefaciens* (Ren et al. 2016). These studies have shown that the protoplast transfection (with standard vectors or ribonucleoproteins) is likely to be the most effective genome editing approach in grapevine because regenerating plants from a single transformed or edited cell avoids the formation of chimeric regenerants, which is a common problem when the target is a multicellular tissue such as callus. However, the regeneration of genome-edited plants from transformed protoplasts has yet to be reported. Moreover, the direct introduction of guide RNA/Cas9 ribonucleoproteins was demonstrated to be able to overcome off-targets effects and unwanted integration of plasmid vector into the genome (Malnoy et al. 2016). Although we did not directly test our isolated protoplasts by genome editing, we confirmed that the protoplasts of both species were amenable to PEG-mediated transfection, as shown by the uniformly intense YFP signal first observed 24 h post-transfection and lasting until at least 72 h post-transfection. Therefore, our improved protocol for the regeneration of grapevine plants from protoplasts through embryogenesis may address the limitations encountered in previous attempts to generate genome-edited plants from protoplasts.

## Conclusion

We have developed an efficient protocol for the regeneration of whole plants, representing two Italian wine grapevine cultivars, from protoplasts isolated from embryogenic callus. The protoplasts were cultivated using the disc-culture method at a density of $1 \times 10^5$ cells/mL, and were regenerated by first encouraging them to form somatic embryos. The first cell division occurred ~ 10 days after protoplast isolation, microcolonies appeared after ~ 1 month, and cotyledonal somatic embryos were observed after ~ 3 months. A critical step was the maintenance of cotyledonary embryos in the dark for 1 month before transfer to shoot elongation medium because this allowed the embryos to complete germination and thus to become competent for further development. Germinated somatic embryos were transferred to the light for shoot elongation followed by root elongation and growth, resulting in the recovery of whole plants ~ 6 months after protoplast isolation. The protoplasts were amenable to PEG-mediated transfection, indicating that the combination of transfection and our new regeneration procedure could be used for the application of biotechnological approaches such as genome editing in a wider range of grapevine cultivars than previously envisaged.

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**Author contributions** EB performed the protoplast isolation, the regeneration of whole plants from somatic embryos and the PEG-mediated transfection of protoplasts; MP conceived the study; SZ and GBT supervised the study and wrote the manuscript. All the authors contributed to the discussion of the results, reviewed the manuscript and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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