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Development of *in vitro* mutagenesis and gene silencing protocols in *Vitis* spp. to promote resistance against fungal and oomycete pathogens

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2,4-D – 2,4-dichlorophenoxyacetic acid

B5 – Gamborg culture medium (Gamborg, 1968)

BAP – 6-Benzyl-aminopurine

bp – Base pairs

CIM – Callus induction medium

DNA, RNA – Deoxyribonucleic acid, ribonucleic acid

dNTPs – Term referring to the 4 deoxyribonucleotides: dATP, dCTP, dGTP and dTTP

dsRNA – Double-stranded RNA

EC(s) – Embryogenic callus (es)

GA3 – Gibberellic acid

hRNA – Hairpin ribonucleic acid

IBA – Indole-3-butyric acid

IM – Initiation medium

LB – Luria broth medium

MBs – Meristematic bulks

mRNA – Messenger ribonucleic acid

MS – Murashige and Skoog culture medium (Murashige and Skoog, 1962)

MS ½ – Murashige and Skoog culture medium (Murashige and Skoog, 1962) with half concentration of major salts.

NAA – 1-Naphthalene acetic acid

NEC – Non-embryogenic callus

NN – Nitsch and Nitsch culture medium (Nitsch and Nitsch, 1969)

PEMs – Proembryogenic masses

PCR -– Polymerase chain reaction

PGR(s) – Plant growth regulator(s)

PTGS – post-transcriptional gene silencing

qPCR – Quantitative-Real-time PCR

RNAi – RNA interference

SEs – Somatic embryos

SE – Standard error

siRNA – small interfering RNA

T-DNA – Transfer DNA

Taq polymerase - Enzyme originally isolated from the bacteria *Thermus aquaticus*

TDZ – Thidiazuron

X6 – Embryo development and maintenance medium

Abstract

Downy mildew, powdery mildew, and grey mold are the phytopathological diseases primarily responsible for economic losses in grapevine culture worldwide. These are polycyclic diseases of the aerial plant system. Starting from a few primary infection centres, under favourable environmental conditions, secondary cycles can lead to exponential epidemic developments which are difficult to contain, forcing the use of numerous preventive treatments, like phytochemicals. In this context, viticulture plays a fundamental role; in fact, the 65% of all fungicides used in agriculture are applied to vineyards, compared to the 3% of agricultural area occupied in Europe. This awareness has led to a new impulse towards the research and the development of alternative strategies to chemical means of protection, like the exploitation of genetic approaches, aimed at increasing the resistance of the host. In this scenario, one of the main purposes of this thesis has been to broaden the panorama of genetic variability, through techniques already known and used in the genetic improvement programs, such as chemical mutagenesis and somaclonal variability, or through the application of RNA interference (RNAi) approaches applied to the viticultural context, by introducing new host resistances, while maintaining the varietal identity. Furthermore, the importance of *in vitro* morphogenesis, in terms of de novo shoot organogenesis and somatic embryogenesis, has been emphasized as essential tool for the application of biotechnological approaches to be used on different grapevine cultivars and rootstock. Several lines of research have shown that the success of *in vitro* plant regeneration and the competence of genetic transformation greatly depends on the genotype of the species of interest. Therefore, both *in vitro* regeneration and *Agrobacterium*-mediated transformation systems have been efficiently endorsed for the induction of a post-transcriptional gene silencing mechanism mediated by double-stranded RNA (dsRNA) molecules, by inserting RNAi-based gene constructs in grapevine (Host Induced gene silencing – HIGS), which target genes of *Botrytis cinerea* and *Plasmopara viticola* that act as virulence effectors. Parallelly, the complex system of the pathogen-host interaction can be altered in favour of the latter through the optimization of a new RNAi approach, which is based on the exogenous application on the plant surface of the same dsRNA molecules to reduce the virulence of fungal and oomycetes pathogens. The efficacy of the exogenous application of dsRNA molecules targeting genes of *Botrytis cinerea* has been tested and considered suitable to obstruct and prevent the disease and delay its appearance on grapevine leaves and berries.

1 - Chapter 1- LITERATURE REVIEW: Biotechnological approaches: gene overexpression, gene silencing, and genome editing to control fungal and oomycete diseases in grapevine[1]

1. Introduction

Grapevine is one of the world's most commonly produced fruit crops, with a yield of about 79 million tons of grapes produced only in 2018 (Faostat Database, 2018). High-quality grapes for table consumption and wine production are derived from varieties of only one vine species, *Vitis vinifera L. subsp. sativa*, whereas other species are exploited as rootstocks (Fournier-Level et al., 2010), or are used in breeding programs solely for introducing new important traits in selected cultivars (Vezzulli et al., 2019). However, this kind of application is somewhat controversial due to international rules, particularly in Europe, where a limitation is imposed on the use of cultivars derived only from *Vitis vinifera* within breeding programs (De la Fuente Lloreda, 2018).

Considering their high pedo-climatic adaptation capacity, the cultivation of *Vitis vinifera* cultivars become possible between 30° to 50°N and S latitude (Real et al., 2015). In the presence of favourable weather conditions (generally mild temperatures and high humidity), during the crop cycle, almost every organ of the plant is susceptible to attack by the main fungal and oomycetes diseases, such as downy mildew, powdery mildew, and grey mould, caused by *Plasmopara viticola* (Berk. and Curtis) Berl. & De Toni, *Erysiphe necator* Schwein., and *Botrytis cinerea* Pers., respectively (Armijo et al., 2016). According to recent global surveys, these diseases in regards to the main winegrowing regions in the world were considered by researchers and production professionals as the most harmful for grape production (Bois et al., 2017; Boso et al., 2019; Martínez-Bracero et al., 2019; Cortiñas Rodríguez et al., 2020)

Grapevine breeding programs are mainly focused on inducing resistance against biotic agents, especially those that have a history of attacking European *Vitis* since the late nineteenth century such as grape phylloxera and mildews. Mildews, originated from North America, and they were introduced in most European *Vitis vinifera* varieties that proved to be highly susceptible due to the absence of co-evolutionary processes between pathogens and plants (Toffolatti et al., 2018). Research work on resistant (R) genes and their introduction in selected cultivars for genetic improvement by classical breeding is generally challenging and requires several generations of backcrosses, during which a strict selection must be carried out, trying to synchronously preserve either important agronomic/oenological characteristics or traits of interest. The varietal rigidity imposed by registered designations of origin, long juvenile phase, and high heterozygosity leads to costly and longer breeding technical times when classical breeding is applied on *Vitis* species (Dalla Costa et al., 2017). Furthermore, although classical breeding and agrochemical approaches were considered effective at first, in the long run, they could lead to the emergence of resistant pathogen strains, since they mainly confer a qualitative type of resistance, which is prevalently monogenic (Guimier et al., 2019). To cope with these threats, farmers have massively used pesticides, arousing conflicting opinions regarding the environmental sustainability and the quality of viticulture and wine production processes (Hussain et al., 2009).

Studies in the field of plant molecular biology and biotechnology may support plant defence strategies allowing researchers to select traits that could undermine pathogen's aggression (Parmar et al., 2017). Genetic

transformation remains generally the most commonly exploited strategy compared to several others biotechnological approaches, as it allows researchers to stably insert specific gene sequences in a host plant. Genetic transformation also permits the importation of more than one R gene, this creates the condition to have potential additive or synergistic effects. The validation of R genes in *Vitis* species is possible, but it requires the development of efficient regeneration and transformation protocols in order to genetically transform these plants, which often lead researchers to opt for model plants like *Nicotiana tabacum* or *Arabidopsis thaliana*, as host to implement these studies (Xing et al., 2006). Classical genetic engineering techniques, mainly based on standard genetic transformation methods through the insertions and consequent expression of heterologous genes of interest such as resistance (R) genes or other defence genes represents the basis on which the new generation of biotechnologies are founded (Dalla Costa et al., 2017).

Overexpression of defence genes against crop fungal pathogens symbolizes one of the main biotechnological tools exploited to counterbalance pathogen aggressiveness, and consequent yield losses (Wally and Punja, 2010; Parmar et al., 2017). Pathogenesis-related proteins (PR-proteins), antimicrobial peptides, secondary metabolites, and specific compounds can be overexpressed in host cells with a direct effect at the target level. Alternatively it is also possible to stimulate host defence biosynthetic pathways (e.g. through the overexpression of transcription factors that enhance the plant defence-related genes)(Ali et al., 2018). In addition to these biotechnological strategies, new breeding techniques (NBTs) such as genome editing mediated by CRISPR/Cas9 technology, a high precision tool capable of strategically introduce targeted mutations in the host genome (Gentile and La Malfa, 2019), or cisgenesis/intragenesis which allow the inclusion of gene sequences from sexually compatible plants (Holme et al., 2013), have been developed and optimized during the past decades. RNA interference (RNAi) mechanism, where double-strand RNAs (dsRNAs) molecules trigger the mRNA degradation or translational repression, is another powerful tool to subvert pathogenic attacks while the downregulation of gene expression occurs (Limera et al., 2017; Taning et al., 2020).

This literature review aims to provide an overview of target genes discovered in *Vitis* species and assessed through the above-mentioned biotechnological strategies to increase tolerance to the most severe pathogens, such as grey mould, powdery and downy mildews.

2. Plants response mechanisms to pathogenic attacks

Plants have a series of biochemical or physical barriers, that belong to the general constitutive defences which can prevent fungi from entering the plant cells. Plant cell walls and related compounds such as trichomes, wax layers, cuticle, cellulose, and pectin lamellae are the main physical impediments to fungal ingression. For example, the presence of numerous trichomes on the lower leaf epidermis reduces downy mildew primary infection due to the increased exposure of zoospore to dehydration (Kortekamp and Zyprian, 1999). After infection, several histological responses help plant cell by curbing pathogen invasion. Callose deposition forming, cell wall thickenings commonly known as papillae, lignin, and other phenolic compounds production nearby fungal penetration site, have shown an active defence role during the early stages of plant invasion (Voigt, 2014). For instance, the expression of stress-induced callose synthase PMR4 (Powdery Mildew

Resistance 4) has proven to provide complete penetration resistance against *Arabidopsis* powdery mildew (Ellinger et al., 2013).

During evolutionary-conserved plant defence processes, two effective and subsequential mechanisms occur to actively respond to pathogen and pest infections; firstly, the activation of the response machinery takes place following the perception of non-specific molecules produced by the pathogen; secondly, there occurs a specific recognition of pathogens virulence factors, through the products encoded by R genes (Jones and Dangl, 2006). Plants are able to generally recognise bacteria, fungi, oomycetes, and viruses thanks to either the presence or the production of specific conserved molecules, known as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) that act as elicitors of plant defence responses (Newman et al., 2013). In the case of pathogenic attack, the presence of some receptors and co-receptors known as pattern recognition receptors (PRRs) on the plant surface at the membrane level, can efficiently recognise PAMPs thus, allowing the establishment of PAMP-triggered immunity (PTI) defence response, leading to the impediment of early-stage fungal growth, without killing the pathogen cells (Boutrot and Zipfel, 2017).

In order to suppress these barriers, pathogens can secrete a plethora of effectors which, in some cases can be identified by the plant cell thanks to the presence of Resistance proteins (R proteins), which lead to the Effector-triggered Immunity (ETI) (Poltronieri et al., 2020). This plant immunity response is stronger than PTI, as it is able to elicit the activation of additional defence signalling mechanisms including PR-genes expression induction, local hypersensitive responses (HR), and consequently programmed cell death (PCD) (Gong et al., 2019). Unfortunately, due to the lack of specific R genes, the most important *Vitis vinifera* cultivars have proved to have inadequate defence responses to limit the invasion of both biotrophic and necrotrophic Fungi and Chromista pathogens (Armijo et al., 2016).

3. Genetic engineering for the expression of candidate genes involved in fungal-oomycete resistance

Prior to genetic transformation processes, a fundamental part is the identification of candidate genes that exert in the host an active role in the enhancement of plant defences, such as pathogenesis-related proteins, antimicrobial peptides, transcriptional factors, products of the secondary metabolism, and defence-related genes. The expression/overexpression through genetic engineering techniques is still one of the most common biotechnological tools used to validate cisgenic and transgenic sequences that induce/improve resistance against specific pathogens in *Vitis* spp. (Table 1).

Table 1. Representative attempts of genetic transformation applied in *Vitis* species to enhance resistance against the most harmful fungal and oomycetes pathogens.

Gene name	Gene source	Description	Biological effect	Host	References
Pathogenesis-related proteins					
***Non-expressor of Pathogenesis Related 1* (*VvNPR1.1*)**	*Vitis vinifera*	Key signal in salicylic acid pathway and local basal resistance to biotrophs	Enhanced resistance to *Erysiphe necator*	*Vitis vinifera* cv. Chardonnay	(Le Henanff et al., 2011)
***Rice chitinase* (*RCC2*)**	*Oryza sativa*	PR protein Class I Chitinase	Major resistance to *Erysiphe necator* and slight resistance to *Elsinoe ampelina*	*Vitis vinifera* cv. Neo Muscat	(Yamamoto et al., 2000)
***Chitinase and ribosome-inactivating protein* (*RIP*)**	*Hordeum vulgare*	These genes encode for two antifungal proteins	Susceptibility to *Erysiphe necator* and *Plasmopara viticola* infection equal to that of the control	*Vitis vinifera* cv. Seyval blanc	(Bornhoff et al., 2005)
***Rice Chitinase* (*Chi 11*)**	*Oryza sativa*	Pathogenesis-related protein	Late and reduced manifestation of *Erysiphe necator* symptoms	*Vitis vinifer* cv. Pusa Seedless	(Nirala et al., 2010)
Chitinase* and *ß-1,3-glucanase	Scab-infected Sumai 3 wheat	Pathogenesis-related proteins	Chitinase was more effective than glucanase in conferring tolerance to *Plasmopara viticola*	*Vitis vinifera* cv. Crimson Seedless	(Nookaraju and Agrawal, 2012)
VpPR4-1	*Vitis pseudoreticulata*	PR4 protein are considered chitin-binding proteins	Improved tolerance to *Erysiphe necator*	*Vitis vinifera* cv. Red Globe	(Dai et al., 2016)
***Thaumatin-like protein* (*Vvtl-1*)**	*Vitis vinifera* cv. Chardonnay	Pathogenesis-related protein 5	Increased resistance to *Erysiphe necator* and *Elsinoe ampelina*	*Vitis vinifera* cv. Thompson Seedless	(Dhekney et al., 2011)
***Thaumatin-like protein* (*VqTLP29*)**	*Vitis quinquangularis* cv. Shang-24	Pathogenesis-related protein 5	Increased resistance to powdery mildew, but decreased resistance to *Botrytis cinerea*	*Arabidopsis thaliana*	(Yan et al., 2017)
***Thaumatin-like protein* (*VaTLP*)**	*Vitis amurensis* Rupr. "Zuoshan-1"	PR-5 proteins have endo-β-1,3-glucanase activity; binding β-1,3-glucan	Reinforced resistance to *Plasmopara viticola*	*Vitis vinifera* cv. Thompson Seedless	(He et al., 2017)
VpPR10.1	*Vitis pseudoreticulata*	PR10 have *in vitro* ribonuclease activity	Increased tolerance to *Plasmopara viticola*	*Vitis vinifera* cv. Thompson Seedless	(Su et al., 2018)
Antimicrobial Peptides (AMPs)					
***Natural Magainin-2* (*Mag2*) /*synthetic derivate* (*MS199*)**	Magainin extracts from the skin of *Xenopus laevis* frog	Magainins with broad-spectrum *in vitro* antimicrobial activity against bacteria and fungi	Resistance to bacterial diseases such as crown gall diseases, minor susceptibility against *Erysiphe necator*	*Vitis vinifera* cv. Chardonnay	(Vidal et al., 2006)

Magainin-2* (*mag2*) + *PGL	Magainin extracts from the skin of *Xenopus laevis* frog	AMP belonging to the Magainins family	PGL protein seems to inhibit *Botrytis cinerea* spore germination	*Vitis vinifera* cv. Chardonnay	(Rosenfield et al., 2010)
Transcription factors					
VvWRKY2	*Vitis vinifera* cv. Cabernet Sauvignon	WRKY protein isolated after *Plasmopara viticola* infection	Increased tolerance to *Botrytis cinerea* and broad-spectrum fungal resistance	*Nicotiana tabacum* cv. Xanthi	(Mzid et al., 2007)
VvWRKY33	*Vitis vinifera*	WRKY protein	Enhanced resistance to *Plasmopara viticola*	*Vitis vinifera* cv. Shiraz	(Merz et al., 2015)
VpWRKY3	*Vitis pseudoreticulata* Accession "Baihe-35-1"	WRKY protein isolated after *Erysiphe necator* infection	Improved tolerance to *Ralstonia solanacearum*	*Nicotiana tabacum* cv. NC89	(Zhu et al., 2012)
***Ethylene response factors* (*VpERF2* and *VpERF3*)**	*Vitis pseudoreticulata*	Transcription factor isolated after *Erysiphe necator* infection	Enhanced resistance to *Ralstonia solanacearum* and *Phytophtora parasitica var. nicotianae* Tucker	*Nicotiana tabacum* cv. NC89	(Zhu et al., 2013)
***C-repeat-binding factor dehydration-responsive element-binding factor 1C* (*MrCBF2/DREB1C*)**	*Muscadinia rotundifolia* "Noble"	Transcription factor isolated after *Plasmopara viticola* inoculation	Enhanced resistance to *Peronospora parasitica*	*Arabidopsis thaliana* "COL0"	(Wu et al., 2017)
***bZIP transcription factor* (*VvbZIP60*)**	*Vitis vinifera* cv. Jing Xiu	Transcription factor that activates the accumulation of salicylic acid and the expression of PR1 protein	Enhanced resistance to powdery mildew	*Arabidopsis thaliana*	(Yu et al., 2019c)
***DOF protein* (*VvDOF3*)**	*Vitis vinifera*	Protein involved in plant growth, development and plant defence	Enhanced resistance to powdery mildew	*Arabidopsis thaliana*	(Yu et al., 2019b)
***Tify protein* (*VvTIFY9*)**	*Vitis vinifera*	Protein highly expressed in leaves. Play an active role in SA pathway	Increased resistance to powdery mildew	*Arabidopsis thaliana*	(Yu et al., 2019d)
***C2H2-type zinc finger protein* (*VvZFP11*)**	*Vitis vinifera*	This protein expression is regulated by salicylic acid and methyl jasmonate	Enhanced resistance to powdery mildew	*Arabidopsis thaliana*	(Yu et al., 2016)
Secondary stress-related metabolites					
***PR10 promotor- Stilbene synthase* (*Vst1*)**	*Vitis vinifera* cv. Optima	Stilbenes production	Decreased susceptibility to *Botrytis cinerea*	41B rootstock (*Vitis vinifera* cv. Chasselas x *Vitis berlandieri*)	(Coutos-Thévenot et al., 2001)

***Stilbene synthase* (*Vst1*)**	*Vitis vinifera*	Stilbenes production	Reinforced resistant against *Botrytis cinerea*	*Vitis vinifera* cv. Sugraone	(Dabauza et al., 2015)
***Stilbene synthase* (*STS*)**	*Vitis pseudoreticulata*	Stilbenes synthesis	Transgenic plants with high resveratrol content	*Vitis vinifera* cv. Thompson Seedless	(Fan et al., 2008)
Calcium-dependent protein kinase (CDPK) (*VaCPK20*)	*Vitis amurensis*	Regulator of the biosynthetic pathways of resveratrol	Increased expression of *STS7* gene, enhanced resveratrol production	Cell cultures of *Vitis amurensis* rupr.	(Aleynova-Shumakova et al., 2014)
***Stilbene synthase* (*VpSTSgDNA2*)**	*Vitis pseudoreticulata*	Stilbenes production	Improved tolerance against *Erysiphe necator*	*Vitis vinifera* cv. Chardonnay	(Dai et al., 2015)
***Stilbene synthase* (*VpSTS*)**	*Vitis pseudoreticulata*	Stilbenes production	Improved resistance to powdery mildew	*Arabidopsis thaliana*	(Jiao et al., 2016)
***Stilbene synthase* (*VaSTS19*)**	*Vitis amurensis*	Stilbenes production	Improved resistance to *Botrytis cinerea* and powdery mildew	*Arabidopsis thaliana*	(Wang et al., 2017b)
***Stilbene synthase* (*VqSTS6*)**	*Vitis quinquangularis*	Stilbenoids accumulation	Improved resistance to *Erysiphe necator*	*Vitis vinifera* cv. Thompson Seedless	(Liu et al., 2019)
		Defence-related genes			
***Jasmonate-ZIM domain protein* (*VqJAZ4*)**	*Vitis quinquangularis* clone Shang-24	This gene is upregulated after *Erisiphe necator* inoculation	Improved resistance to powdery mildew and enhanced susceptibility to *Botrytis cinerea*.	*Arabidopsis thaliana*	(Zhang et al., 2019)
***Polygalacturonase-inhibiting proteins* (*pPGIPs*)**	Pear fruit	PGIPs are plant cell-wall proteins that specifically inhibit fungal endo-polygalacturonases (PGs).	Increased resistance to *Botrytis cinerea* and slight tolerance to *Xylella fastidiosa*	*Vitis vinifera* cv. Thompson Seedless and Chardonnay	(Agüero et al., 2005)
***Two endochitinase* (*ech*42 and *ech*33) and one *N-acetyl-β-d-hexosaminidase* (*nag*70)**	*Trichoderma harzianum, Trichoderma virens*	Extracellular endochitinase of biocontrol agents and chitonylitic genes	Enhanced resistance to *Botrytis cinerea.* Tolerance to *Erysiphe necator* in *ech*42-*nag*70 expressing transgenic plants	*Vitis vinifera* cv. Thompson Seedless	(Rubio et al., 2015)
E3 ubiquitin ligase Erysiphe necator-induced RING finger protein 1 (VpEIRP1)	*Vitis pseudoreticulata* Baihe 31-1 accession	This protein activates plant defence response through the proteolysis of VpWRKY11 transcription factor	Enhanced resistance to powdery mildew	*Arabidopsis thaliana*	(Yu et al., 2013)
***F-box/Kelch-repeat protein* (*VpEIFP1*)**	*Vitis pseudoreticulata*	Transcription of EIFP protein is induced after powdery mildew infection and activation of PR genes	Enhanced tolerance to *Erysiphe necator*	*Vitis vinifera* cv. Red Globe and *Arabidopsis thaliana*	(Wang et al., 2017a)

***Metacaspases* (*VrMC2* and *VrMC5*)**	*Vitis rupestris*	Executors of hypersensitive response (HR), isolated after *Plasmopara viticola* infection	Programmed cell death (PCD) activation	*Nicotiana tabacum* cv. Bright Yellow 2 and *Vitis vinifera* cell cultures	(Gong et al., 2019)
***Serotonin N-acetyltransferase* (*VvSNAT2*)**	*Vitis vinifera*	Protein essential for melatonin production and for SA and JA signalling pathways activation	Improved resistance to powdery mildew	*Arabidopsis thaliana*	(Yu et al., 2019a)
***Resistance to Powdery Mildew 8 locus* (*RPW8.2*)**	*Arabidopsis thaliana*	Protein that encodes for small basic protein, with weak homology with NB-LRR protein	*Erysiphe necator* hyphal growth and sporulation were significantly restricted	*Vitis vinifera* cv. Thompson Seedless	(Hu et al., 2018)
***Ubiquitin ligase* (*VpPUB23*)**	*Vitis pseudoreticulata*	Type E3 ubiquitin ligase is involved in many immune regulation responses	Decreased resistance to *Erysiphe necator*	*Vitis vinifera* cv. Thompson Seedless	(Zhou et al., 2014)
***Ubiquitin ligas* (*VaPUB*)**	*Vitis amurensis*	U-box protein E3 ligase causes downregulation of PR10	Transgenic plants were susceptible as control to *Plasmopara viticola*	*Vitis vinifera* cv. Thompson Seedless	(Jiao et al., 2017)
VaHAESA	*Vitis amurensis* cv. Shuanghong	Pattern recognition receptor (PRR) that belongs to leucine-rich repeat receptor-like protein kinase	Induce H_2O_2, NO, and callose accumulation. Leaves showed less spores and *Plasmopara viticola* infected areas than control	*Vitis vinifera* cv. Thompson Seedless	(Liu et al., 2018)

3.1 Overexpression of pathogenesis-related proteins

Once elicitors have been recognized by the plant, the contact with the pathogen induces different defence mechanisms in host cells, such as the reinforcement of structural barriers, the synthesis of secondary stress-related metabolites such as phytoalexins and the provision of PR proteins (Pieterse et al., 2014) as depicted in Figure 1.

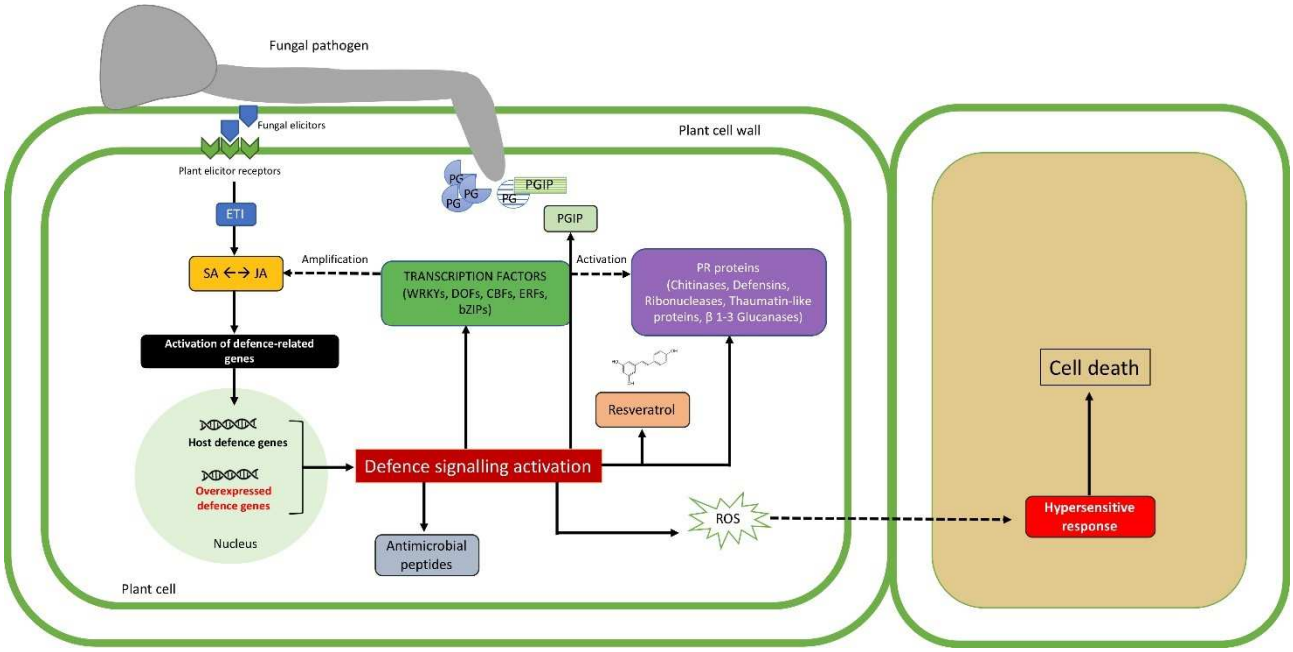


Figure 1: Description of transgenic plant-pathogen arms race during pathogenesis. The first impediment to fungal invasion is represented by chemical and physical barriers already existing before the infection. The trophic activity begins with lytic enzyme production (e.g. polygalacturonase, PG), that can be suppressed by the production of specific inhibitors (e.g. polygalacturonase inhibitors, PGIP), which can be expressed also by the use of genetic engineering techniques. A specific recognition takes place when elicitors coded by *avirulence* (*Avr*) genes of the fungal cell are recognized by host receptors, driving effector-triggered immunity (ETI). ETI results in the activation of defence gene expression (i.e defence molecules, antimicrobial peptides, phytoalexins), through the salicylic/jasmonic acid (SA/JA) signalling pathways (Poltronieri et al., 2020). The aforementioned defence molecules together with pathogenesis-related proteins (PRs) and Transcription factors can be overexpressed in the host cell, imparting a harmful effect against the pathogens. Intriguingly, transcription factors are responsible for activating plant defence response, and their overexpression leads to the stimulation of SA/JA signalling pathways.

The extreme results of ETI is the hypersensitivity response that, thanks to reactive oxygen species (ROS) accumulation, leads to the programmed cell death (PCD), isolating and detaining the propagation of the pathogen in other plant cells (Gong et al., 2019).

PR proteins (PRs) are a class of soluble proteins that was isolated for the first time from tobacco tissues after infection with the tobacco mosaic virus (TMV) (Van Loon and Van Kammen, 1970). In general, all the proteins expressed in response to both abiotic and biotic stresses are included in this category (Ali et al., 2018). The expression of some of these proteins can also be triggered by the accumulation of plant hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), which are related to plant defence (Ali et al., 2018). PRs are relevant in resistant responses against fungal attack as they are generally involved in the formation of necrotic lesions that limit pathogen invasion and growth. Furthermore, they are activated in different ways depending on the pathogen trophic behaviour. Biotrophs turn on the SA pathway in the plant, that triggers *NPR1* gene (*non-expressor of PR gene 1*) expression, which in turn induces the transcription and production of the SA-mediated gene proteins (*PR1*, *PR2*, *PR5*) that circulates in the sap, giving rise to Systemic Acquired Resistance (SAR) (Ali et al., 2017, 2018). The overexpression of *Vitis vinifera NPR1.1* gene increased resistance to powdery mildew in this species through the constitutive activation of *PR1* and *PR2* genes expression also in uninfected plants (Le Henanff et al., 2011). Necrotrophs induce the activation of JA pathway in the plant, which induces the local accumulation of JA-mediated proteins (*PR4, PR5, PR12*), leading to Local Acquired Resistance (LAR) (Ali et al., 2018). At present, 17 PR families were classified from different plant species and some of them have shown evident antifungal activity such as β 1-3 glucanases (PR2) (Antoniw et al., 1980), chitinases (PR3, PR4, PR8, PR11) (Van Loon, 1982; Métraux et al., 1988; Melchers et al., 1994), thaumatin-like proteins (PR5) (Van Loon, 1982), proteinase inhibitors (PR6) (Green and Ryan, 1972), peroxidases (PR9) (Lagrimini et al., 1987), ribonuclease like-proteins (PR10) (Somssich et al., 1986), defensins (PR12) (Terras et al., 1995), thionins (PR13) (Epple et al., 1995).

Chitinases and β 1-3 glucanases show antifungal activity thanks to their direct attack on fungi cell wall, causing its fragmentation and disaggregation (Nookaraju and Agrawal, 2012). Two *Vitis vinifera* transgenic lines, expressing the *rice chitinase* gene (*RCC2*), exhibited HR and a significant reduction in powdery mildew symptoms (suppression of both conidial germination and mycelial growth) caused by *Erysiphe necator* and slight resistance to anthracnose when compared with the non-transformed control (Yamamoto et al., 2000).

Bornhoff and collaborators showed the ineffectiveness of protection against powdery and downy mildew in transgenic *V. vinifera* plants expressing chitinase and RIP (Ribosome-inactivating protein) isolated from barley (Bornhoff et al., 2005), disapproving the expectation of having synergistic effect due to the co-expression of these two enzymes. On the contrary, Nookaraju and co-authors observed a low susceptibility to *Plasmopara viticola*, characterised by a 15 to 35% reduction in hyphal growth in plants expressing both *chitinase* and *β 1-3 glucanase* genes (Nookaraju and Agrawal, 2012). By using the gene construct codifying for the *rice chitinase* gene *chi 11* researchers obtained two grapevine transgenic lines characterized by a high chitinase activity, which displayed smaller lesions and delayed manifestation of powdery mildew symptoms (Nirala et al., 2010). Many chitin-binding proteins belong to PR4 family, described mainly as wound-inducible proteins, and triggered by fungi infection in several plants. The isolation from *V. pseudoreticulata* of a PR-4 protein and its overexpression in the susceptible *V. vinifera* genotype Redglobe led to an increased resistance to powdery mildew, inhibiting hyphal growth (Dai et al., 2015).

Thaumatin proteins belong to the PR5 family and are characterized by a thaumatin domain and a PR5-like protein kinase receptor (Yan et al., 2017). Their anti-oomycete mechanism relies on their β 1-3 glucan binding and endoglucanase activities (He et al., 2017). Dhekney and colleagues obtained two cis-genic grapevine lines showing broad-spectrum antifungal resistance, by expressing a gene construct codifying for the *Vitis vinifera* thaumatin-like protein 1 (VVTL-1), that conferred a 10 days delay in symptoms manifestation, compared to the non-transformed control after powdery mildew infection, and a significant resistance to black rot, a fungal disease caused by *Guignardia bidwellii* (Dhekney et al., 2011). Transgenic *Vitis vinifera* expressing a thaumatin-like protein gene isolated from *Vitis amurensis* showed decreased susceptibility to downy mildew, reducing infected area, and the number of sporangia (He et al., 2017). Further studies in this field suggested that some *thaumatin-like protein* (*TLP*) genes, perform better against biotrophs rather than necrotrophs, like *TLP29* gene of *V. quinquangularis* (*VqTLP29*) expressed in *A. thaliana*, where an increased susceptibility to *Botrytis cinerea* was detected (Yan et al., 2017). PR 10 proteins are highly expressed after pathogen invasion, and their anti-fungal capability seems to be also associated to their RNase/DNase activity, and to their role in the control of flavonoid biosynthesis (Casañal et al., 2013; Wang et al., 2014). *VpPR10.1* gene inserted through *Agrobacterium*-mediated transformation in the Thompson Seedless cultivar led to reduced hyphal growth of *Plasmopara viticola*, through callose deposition around hyphae and haustoria, and hydrogen peroxide accumulation compared to non-transgenic lines (Su et al., 2018).

3.2 Gene expression of antimicrobial peptides

In addition to PR proteins, scientists revealed that some antimicrobial peptides (AMP) were proved to have antifungal activity (Jenssen et al., 2006). However, overexpression of AMP does not always result in an enhanced resistance against fungi, maybe due to the activity of endogenous proteases which can inactivate peptides, neutralizing their antimicrobial properties (Hightower et al., 1994). Magainins are a class of antimicrobial peptides that interfere with fungal-membranes function, altering their polarity and inducing cell mortality, but they do not interfere with the membrane of host cells. Grapevine plants overexpressing either natural or synthetic magainins have shown enhanced resistance to *Agrobacterium vitis* and *Erysiphe necator* (Vidal et al., 2006). In another study, the ectopic expression of *Magainin-2* (*mag 2)* and *PGL* genes generated plants slightly resistant to powdery mildew (Rosenfield et al., 2010).

3.3 Overexpression of transcriptional factors

The role of transcriptional factors is strictly correlated with signalling pathways, and they play the role of regulating the expression of *PR* genes or enzymes implicated in defence responses (Eulgem, 2005). The three more relevant transcription factor families that are unique in plants are WRKY proteins, ethylene-responsive-element-binding factors (ERFs), and basic-domain leucine-zipper (bZIP) (Singh et al., 2002).

WRKY proteins family includes 74 *Arabidopsis* members having the aminoacidic sequence WRKYGQK and a zinc-finger-like-motif in common (Merz et al., 2015). Following *Plasmopara viticola* infection in grapes, *VvWRKY2* gene upregulation took place before the increased expression of *PR* genes, suggesting its involvement in phyto-pathogenesis (Mzid et al., 2007). In tobacco, the ectopic expression of *VvWRKY2* gene

led to a broad-spectrum resistance against fungi and oomycetes (Mzid et al., 2007). A reduction of up to 70% sporulation of *Plasmopara viticola* infecting grapevine leaves was also recorded in cisgenic plants expressing the *VvWRKY33* gene (Merz et al., 2015), and a 40% decreased susceptibility was recorded when 41B rootstock overexpressed *VvWRKY1* gene (Marchive et al., 2013).

In *Arabidopsis,* almost 124 ERF transcription factors are known to be involved in cold and drought tolerance as well as pathogen resistance (Singh et al., 2002). The transcription factor *VpWRKY11*, that is a negative regulator of basal resistance in *Arabidopsis*, undergoes a proteolytic degradation operated by *Erysiphe necator*-induced RING finger protein 1 (EIRP1), thanks to its E3 ligase activity. Overexpression of *VpEIRP1* gene in *Arabidopsis* showed enhanced resistance to powdery mildew and bacterial diseases (Yu et al., 2013). The overexpression of three *ERF* genes isolated from *Vitis pseudoreticulata* showed a different level of tolerance against pathogenic fungi in tobacco and *Arabidopsis* plants, suggesting that more research is required for these transcription factors (Zhu et al., 2013). *Muscadinia* is another genus of *Vitaceae* family, characterized by plants naturally immune to different pathogens, including downy mildew. The overexpression of CBF2 transcription activator isolated from *M. rotundifolia* in *Arabidopsis* gave decreased susceptibility to downy mildew (*Peronospora parasitica*), and enhanced cold and drought tolerance, although causing morphological changes and flowering delay (Wu et al., 2017).

Almost 75 transcription factors with a basic region/leucine zipper motif (bZIP) have been identified in *Arabidopsis*, which are able to regulate different mechanisms from plant defence signalling pathways to seed and flower development (Jakoby et al., 2002). The ectopic expression of *bZIP60* isolated from *Vitis vinifera*, decreased the severity of the symptoms of powdery mildew on *Arabidopsis* (Yu et al., 2019c). DOF transcription factors derived by the expression of *DOF* gene family, which includes 25 members in *Vitis vinifera*, are involved in plant development and gene expression regulation (Costenaro da Silva et al., 2016). *VvDOF3* gene overexpressed in *Arabidopsis* acted as a transcription factor and increased the resistance to powdery mildew (Yu et al., 2019b).

3.4 Overexpression of secondary metabolites generally induced by biotic stress

Grapevine cultivation became also appealing from a nutraceutical point of view, due to the abundance of molecules with high antioxidant capacity contained in grapes. Stilbene, flavonols, and anthocyanins represent the major phenolic compounds that are responsible for generating beneficial effects also to plants, and they assist plant-pathogens arms races (Hasan and Bae, 2017). One of the most famous compounds, known for its anti-cancer property is resveratrol, a stilbene polyphenol, constitutively present at low concentration in all grapevine organs. Resveratrol tends to accumulate in areas close to *Erysiphe necator*, *Plasmopara viticola,* and *Botrytis cinerea* infection sites, limiting pathogen spread and diffusion since these fungi are unable to metabolize this component. The antimicrobial and antifungal activity of resveratrol has been highlighted by some studies showing that this compound displays an inhibitory effect on *Botrytis* conidia germination and mycelium growth, leading to ultra-structural changes on conidia (e.g. granulation of cytoplasm and disorganization of cell content) (Adrian and Jeandet, 2012).

The biosynthetic pathway common to all the major phytoalexins in grapevine requires the activity of stilbene synthase (STS), which, by condensing three Malonyl CoA molecules with one molecule of Coumaryl CoA, leads to resveratrol production (Coutos-Thévenot et al., 2001). *In vitro* infection tests with *Botrytis cinerea* were carried out on grapevine micro cuttings transformed with *V. vinifera Vst1* gene under the control of a promoter inducible by fungal infection (Pr10 promoter isolated from alfalfa); the results obtained confirmed an enhanced resistance against the pathogen in the transgenic lines that exhibited the highest resveratrol synthesis level (Coutos-Thévenot et al., 2001). In another study, the expression of *Vst1* gene under the cauliflower mosaic virus 35S promoter resulted in smaller botrytis necrotic lesion size in transgenic grapevine in comparison with control plants, demonstrating the existence of a positive correlation among resveratrol content and resistance to *Botrytis cinerea* (Dabauza et al., 2015). In transgenic *Arabidopsis* plants, the expression of the *Vitis pseudoreticulata STS* gene (*VpSTS*), encoding a novel stilbene synthase, led to greater resistance to powdery mildew, since its expression seemed to be stimulated by SA, as happens with the expression of its homologous allele from susceptible *Vitis vinifera* (Jiao et al., 2016). The same *STS* gene was successfully introduced in Thompson Seedless somatic embryos, showing an increased concentration of resveratrol in the transgenic lines; however, no studies on increased pathogen resistance were carried out in this work (Fan et al., 2008).

On Chardonnay transformed plants, the same target genes led to an increased production of hydrogen peroxide and consequently reduced mycelium growth of *Erysiphe necator* (Dai et al., 2015). *STS19* gene from Chinese wild grape was able to reduce powdery mildew and grey mould susceptibility when expressed in transgenic *Arabidopsis* plants, through SA/JA signalling pathways enhancement (Wang et al., 2017b). Although the genetic transformation aimed at improving the phytoalexin content seems to be a good strategy to reinforce plant immunity, at the same time, it can cause physiological and morphological alterations of the vine, especially during flowering and berry ripening (Coutos-Thévenot et al., 2001).

Other molecular pathways are related to phytoalexin synthesis. It has been demonstrated that a positive correlation exists between calcium-mediated signalling and a high amount of resveratrol, corroborated by the fact that treatments with calcium channels blockers reduced resveratrol synthesis in transgenic *Vitis amurensis* cells (Aleynova-Shumakova et al., 2014). The overexpression of the calcium sensor protein CDPK20 stimulated resveratrol production in *Vitis amurensis* cells, although only an increased *VaSTS7* gene expression was elicited, while the expression of other *STS* genes remained unaltered (Aleynova-Shumakova et al., 2014).

3.5 Overexpression or gene expression of other defence-related genes

Jasmonate Zim domain (JAZ) proteins are transcriptional repressors of JA signalling pathways, and participate in secondary metabolites biosynthesis, in addition to their involvement in response to biotic and abiotic stresses (Zhang et al., 2019). *VqJAZ4* gene (a jasmonate-ZIM gene from *Vitis quinquangularis*) transcription was induced after SA and MeJA application and by *Erysiphe necator* infection, evidencing its role in defence mechanisms (Zhang et al., 2019). Transgenic *Arabidopsis* plants expressing this gene showed enhanced resistance to powdery mildew and increased ROS accumulation and callose production, compared to non-transgenic control. However, the same *VqJAZ4* expressing lines appeared more susceptible to grey mould,

possibly because *VqJAZ4* gene suppresses the expression of JA-related genes, and therefore impeding JA signalling pathways activation, making the control of necrotrophs by the plant more thwarted (Zhang et al., 2019).

One essential event for fungal and oomycetes pathogenesis is the production of polygalacturonases (PG) which acts in the impairment of host cell walls. The introduction of polygalacturonases inhibitors (PGIP) in the plant through genetic engineering can limit the degradation of host cells and also as a result of enzymatic activity of cell walls compounds make available some glucosidic fragments that can react as effectors thus, eliciting host defence (Agüero et al., 2005). Twelve of the eighteen transgenic lines of *Vitis vinifera* expressing the pear *PGIP* gene showed a significant reduction in necrotic lesion size after inoculation with *Botrytis cinerea* (Agüero et al., 2005). In this study, the movement of PGIP through the xylem and the graft union was also demonstrated. This is an important result that shows the obtainment of genetically improved non-transgenic scion grafted on transgenic rootstock.

Trichoderma spp. are generally introduced in the field, notably in organic farming, as biocontrol agents for fungal disease management. The isolation of endochitinases and hexosaminidases from these biocontrol fungi and their gene transfer in grapevine cultivars allowed the obtainment of plants with enhanced tolerance to *Botrytis cinerea* and *Erysiphe necator* (Gray et al., 2014). Ubiquitination in plants plays different strategic roles, especially regarding the selective degradation of proteins. The overexpression of the E3 ubiquitin ligases from *Vitis pseudoreticulata* led to an increased susceptibility in transgenic plants to powdery mildew (Yu et al., 2013).

Stimulation of HR represents a useful strategy, particularly effective for its rapid mode of action. It has been demonstrated that the metacaspases MC2 and MC5 identified in *V. rupestris* upon *Plasmopara viticola* inoculation, were involved in the execution of HR, activating the most efficient ETI in this genotype (Gong et al., 2019). The ectopic expression of caspase-like regulators in plants related to cell apoptosis could increase resistance to broad-spectrum diseases, which mediates defence-related programmed cell death (Gong et al., 2019).

Melatonin is a molecule firstly discovered in mammals, where it acts as a fundamental regulator of circadian rhythms, and its presence was also confirmed in higher plants. Considering its innate antioxidant activity, this molecule can be used in agriculture both as a plant growth regulator and as a biostimulator to cope with stress conditions (Arnao and Hernández-Ruiz, 2014). The melatonin biosynthetic pathway includes modifications of L-tryptophan and the acetylation of serotonin, operated by serotonin-N-acetyl transferase (SNAT). Expressing the *SNAT2* gene cloned from *V. vinifera* in *Arabidopsis* plants led to a greater accumulation of melatonin and reduced susceptibility to powdery mildew (Yu et al., 2019a).

In the *Arabidopsis* genome, a locus that confers resistance to different mildews, *RPW8*, has been characterized and it is composed of two genes *RPW8.1* and *RPW8.2* (Xiao et al., 2001); the latter is located in the extrahaustorial membrane, that covers the fungal haustorium and promotes accumulation of hydrogen peroxide. The expression of this gene (characterized by an efficient inhibition of fungal growth and

sporulation) in transgenic *Vitis vinifera* plants demonstrated that this resistance can be transferred in this species as well (Hu et al., 2018).

PTI in plants could be promoted by the expression of PRRs dedicated to PAMPs recognition. Transient expression of *VaHAESA*, a *V. amurensis* leucine-rich repeat receptor-like protein kinase, in grapevine leaves determined significant reduction in an infected area by downy mildew, assisted by callose deposition, H_2O_2 and NO accumulation on the nearby infection site (Liu et al., 2018).

4. RNAi: Host- or Spray- induced gene silencing against Fungi and Oomycetes

In addition to owning genetic heritage, nucleic acid molecules can be managed as tools that follow specific recognition by the vegetal cell and can be processed in various ways, giving a start to a set of signals that may stimulate the induction of different defence responses up to the block of specific mRNA translation (Bhat and Ryu, 2016).

Post-transcriptional gene silencing is arbitrated by the activity of small RNAs (sRNAs) (Huang et al., 2019). In the plant kingdom, the class of sRNAs is represented by microRNAs (miRNAs) originating from endogenous *MIR* loci, and small interfering RNAs (siRNAs) derived from long double-stranded RNA (dsRNA) molecules (Hou et al., 2019). Using RNA templates, RNA dependent RNA polymerases (RDRP) are responsible for de novo synthesis of secondary siRNAs, that exert a stronger role in gene silencing than primary siRNAs (Dalakouras et al., 2020). This eukaryotic conserved silencing mechanism allows transcripts degradation or protein production restraint, through sRNAs production having full or partial complementary sequences with the target mRNA. sRNAs are loaded into argonaute (AGO) protein, part of RISC complex (RNA-induced silencing complex), where they become probes for binding with complementary RNA targets thus, exerting their silencing ability (Limera et al., 2017).

Host defence mechanisms and pathogen virulence strategies are linked through cross-kingdom mechanisms (Huang et al., 2019). In the same way the plant sends siRNAs to silence target genes of the pathogens, the pathogen uses the same mechanism to increase its virulence by impairing host immunity genes (Cai et al., 2018b; Hou et al., 2019). Bidirectional cross-kingdom RNAi can be exploited for generating silencing effects through the introduction of RNA molecules in transgenic plants that can counteract fungal and oomycetes virulence genes (Wang et al., 2016). Nowara and collaborators showed that the accumulation of dsRNAs in barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) targeting the fungal glucanosyl transferase, *Avrk1* and *Avra10* effectors caused a reduction in haustorium formation of the causative agent of powdery mildew *Blumeria graminis* (Nowara et al., 2010). Gene silencing of other effector proteins led to similar results against powdery mildew in *Hordeum vulgare* (Pliego et al., 2013; Whigham et al., 2015).

Once one or more genes to be silenced have been identified by studying plant-pathogen interaction processes (Figure 2a), several approaches can be used to deliver dsRNAs into plants. The potential of Host-induced gene silencing (HIGS) approach in crop disease management can be explored through the expression of RNAi constructs against various target genes in the host, as shown in Figure 2b. Resistance to *Fusarium graminaerum* was obtained by HIGS approach in *Arabidopsis* and barley plants, by targeting the fungal sterol 14α

demethylase (CYP51) (Koch et al., 2013). Others *Fusarium spp*. related diseases were successfully controlled by HIGS strategy in embryogenic cells of banana targeting two FOC proteins, whose roles are strongly related to fungal growth, development, and pathogenesis (Ghag et al., 2014) or *chitinase* genes in *Triticum aestivum* (Cheng et al., 2015). *Barley-stripe mosaic virus-induced* RNAi worked efficiently also against *Puccinia tritici* hitting three pathogenicity genes, and reducing leaf wheat rust (Panwar et al., 2013). Downregulation of *Verticillium dahliae hygrophobins 1* (*VdH1*) gene important for microsclerotia production, led to reduced wilt symptoms in cotton transformed plants (Zhang et al., 2016).

RNAi machinery has been demonstrated to be also functional against oomycetes pathogens. Significant reduction in *Phytophtora spp.* load and disease progression was recorded in HIGS potato plants targeting the *Avr3a* effector, and *G protein-β-subunit 1* (*PiGPB1*) gene of this pathogen species (Vega-Arreguín et al., 2014; Jahan et al., 2015; Sanju et al., 2015).

HIGS strategy has also been applied in lettuce plants to express silencing constructs targeting *Highly Abundant Message* (*HAM34)* or *Cellulose Synthase* (*CES1*) genes of *Bremia lactucae*, making these plants resistant to downy mildew (Govindarajulu et al., 2015).

All these studies have demonstrated the efficacy of this technique (HIGS) in specific genes downregulation, highlighting their high potential that can be managed in future grapevine genetic improvement programs aimed at increasing resistance to biotic stresses.

In addition to gene silencing against an external pathogen, is it also possible to exploit the RNAi mechanism to target endogenous genes, that have a negative influence in the pathosystem. An example is the case of *MLO* (*Mildews Locus O)* genes with transmembrane domain, considered as susceptibility genes (S-genes), that alter vesicle-associated and actin-dependent defence pathways (Panstruga, 2005). Knockdown of *VvMLO7* gene through constitutive expression of long non-coding dsRNA led to a significant reduction of powdery mildew disease severity in the transgenic grapevine cultivar Brachetto (Pessina et al., 2016).

However, the application of HIGS approach is limited by poor public acceptance and strict legislative rules applied to GMO cultivation, and also by the lack of efficient *in vitro* regeneration and genetic transformation protocols for several crops, and more often for all the genotypes within the same species (Sabbadini et al., 2019b).

The fact that simple exogenous application of polynucleotides can affect mRNA levels of important virulence-related genes of pathogens/plants without modifying the host genome, opens new opportunities for the development of new scientific techniques and crop improvement strategies (Dubrovina et al., 2019). Extracellular-self DNA and RNA could be also applied to the plants in order to stimulate their immune response (Hou et al., 2019). The attack of siRNA production machinery in the pathogen, through RNAi, has the potential to inhibit the pathogen virulence itself. Wang and collaborators demonstrated that *Botrytis cinerea DCL1/2*- long dsRNAs, targeting expression of dicer proteins essential for sRNA production, exogenously applied on the surface of detached leaves and fruits of different plant species, including grapes, can be efficiently taken up by the necrotrophic fungus, providing a relevant protection against grey mold (Wang et al., 2016). Similar observations were made when spray applications of long non-coding dsRNA molecules,

which target three genes required for the biosynthesis of *Fusarium graminearum* ergosterol, efficiently inhibited the fungal growth at the sprayed (local) as well as the non-sprayed (distal) parts of detached leaves, probably due to the basipetal and acropetal transportation along the vascular system of the silencing signal (Koch et al., 2016). In many cases, researchers preferred to adopt a multitarget approach, by silencing simultaneously two or more target genes entailing in pathogenesis. White mould and grey mould symptoms can be significantly decreased in *Arabidopsis* and *Brassica napus* leaves, respectively through foliar application of 20 different dsRNAs targeting various genes, evidencing the possibility to counteract closely related fungi while applying the same dsRNAs molecules on various crops (McLoughlin et al., 2018). The same conserved target gene among various fungi, such as β2-tubulin gene of *Fusarium asiaticum,* could be selected for RNAi, altering damaging effects afforded by *Fusarium* spp., *Botrytis cinerea*, *Magnaporthe oryzae* and *Colletotrichum truncatum* (Gu et al., 2019).

In addition to HIGS, the exogenous application of long dsRNAs, small dsRNAs and hairpin RNAs has been recently studied and proposed as a new environmental-friendly crop protection tool (Cagliari et al., 2019). Spray induced gene silencing (SIGS) allows the adsorption of dsRNA by either plant cells and tissues, where it can be processed from host RNAi machinery and/or then progressively conveyed on pathogen cells, or directly adsorbed and processed by the fungal cell driving gene silencing through their own RNAi machinery (Figure 2c) (Koch et al., 2016; Sang and Kim, 2020). However, the exact mechanisms behind the uptake of exogenous dsRNAs and their use to activate RNAi machinery in the plant and/or pathogen cells is still unclear, and they seem to be affected by the method of exogenous application used combined with the absorption capacity of different plant organs (Dalakouras et al., 2018; Dubrovina and Kiselev, 2019; Das and Sherif, 2020; Wang and Dean, 2020).

The appearance of pathogens resistant strains to fungicide can be counteracted using fungicides with different modes of action or with a combined application of dsRNAs. A reduction of *Fusarium asiaticum* pathogenicity and resistance to phenamacril, caused by a mutation in the *myosin-5-gene* (*Myo-5*) was recorded with the continuous application of the phenamacril and dsRNA-*Myo-5* as treatments on wheat speaklets (Song et al., 2018). Long or small dsRNAs could be supplied to plants via low-pressure or high-pressure spray, petiole adsorption, or trunk injection (Koch et al., 2016; Wang et al., 2016; McLoughlin et al., 2018; Dalakouras et al., 2020). Through petiole adsorption and trunk injection methods, dsRNAs were shown to be limited to the apoplast and transported only along the xylem, without penetrating the plant cell. These results were demonstrated by Dalakouras and co-authors that applied both these techniques into *Vitis vinifera* observing that the delivered hairpin-RNAs (hpRNAs) were systemically transported and detectable in leaves, distant from the treated area, from one up to 10 days post-application, but no siRNAs deriving from DCL-processed hpRNAs were found (Dalakouras et al., 2018). Furthermore, when siRNAs were applied by petiole absorption also into GFP-expressing *N. bethamiana* no silencing effect on GFP transcripts was observed (Dalakouras et al., 2018). Nevertheless, it seems possible by using these exogenous application techniques to directly reach fungal or oomycetes that normally colonize the apoplast and xylem tissue, where, after undergoing internal processing, dsRNAs can exert their biological activity (Dalakouras et al., 2020). On the other hand, high-

pressure spray of siRNAs had the potential to ensure both local and systemic gene silencing on tobacco plants (Dalakouras et al., 2016).

Selecting fungicide sites of action, Nerva and colleagues, constructed a single long dsRNA molecule that exerted protection against grey mould *in vitro*, on grapevine detached leaves, and grapes at post-harvest, applied through the high-pressure spray and petiole adsorption. Despite different levels of protection have been recorded among the dsRNAs delivery methods, interestingly all the techniques that facilitate the provision of intact dsRNA to the fungus were assumed as effective (Nerva et al., 2020).

Some authors have also reported the possibility to use SIGS to target endogenous genes in plants and downregulate their mRNA levels both locally and systemically (Dalakouras et al., 2016; Dubrovina et al., 2019; Bennett et al., 2020). To our knowledge, the data present in the literature are limited to the foliar application of dsRNA molecules to silence transgenes expressed in model plant species, like *Arabidopsis* and *N. benthamiana*; however, these results open new scenario for the use of SIGS also to target endogenous gene sequences, like susceptibility genes in grapevine and other crops, to enhance plant defence responses.

5. Genome editing

Genome editing is a powerful technique that facilitates the generation of multiple types of genome modification, like insertion, deletion, or mutation, having various implications in genetic studies of animal and vegetal cells (Limera et al., 2017).

Among the three typologies of engineered nucleases that are at the base of genome editing techniques, Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) (CRISPR/Cas9) is the lower-cost, simpler and faster system compared with the other enzymes such as zinc finger nuclease (ZFN) or transcription activator-like effector nuclease (TALEN) (Demirci et al., 2018). Specific modifications in DNA sequence could be driven by the CRISPR/Cas9 reprogrammed system, which needs the insertion of a well-designed single guide RNA (sgRNA) molecule into cells through the different types of vectors (Figure 2 d) (Demirci et al., 2018). SgRNAs are constituted by a single molecule of RNA composed of specific crRNA-trascrRNA (transactivating RNA) chimera sequence (Limera et al., 2017). Mutation efficiency could be notably increased by designing multiple target sgRNA for one target gene (Wang et al., 2018). Ren and collaborators introduced a single plasmid containing specific sgRNA through *Agrobacterium*-mediated transformation into Chardonnay suspension cells to alter the biosynthetic pathway of tartaric acid (Ren et al., 2016). *L-idonate dehydrogenase* (*idnDH*) gene was successfully mutated using CRISPR/Cas9 system, without recording off-target events and highlighting the importance of high GC content in sgRNA sequence in order to obtain high efficiency in genome modifications (Ren et al., 2016). In fact, also previous researches, carried out on other plant species, showed that sgRNAs designed to have a GC content above 50% led to an higher editing efficiency probably due to the final binding capability of these molecules to their targets, which, in some species genomes have high GC contents in specific regions (Ma et al., 2015; Pan et al., 2016). *Vitis vinifera Phytoene Desaturases* (*VvPDS*) was efficiently knockout in cell masses of Neo Muscat (Nakajima et al., 2017), Chardonnay, and 41B rootstock by CRISPR/Cas9 binary vectors expressing a sgRNA with 65% GC content (Ren et al., 2019). Notably, the first application of target genome editing (TGE)

for increasing resistance against *Botrytis cinerea* in grapevine was reported by Wang and colleagues (Wang et al., 2018). The mutations of *VvWRKY52* gene, induced by TGE in Thompson Seedless transgenic plants led to a significant reduction in *Botrytis cinerea* colonies, especially in biallelic grapevine mutant lines (Wang et al., 2018). Mutation efficiency driven by CRISPR/Cas9 system is widely dependent on different factors (technical methods, plant genotype, gene target, *in vitro* regeneration and selective conditions) as already known for genetic transformation techniques, and others specifically for this approach such as the choice of the Cas 9 promoter and sgRNA sequence (Ma et al., 2016). As an alternative to classical genetic transformation, a plasmid-mediated procedure that can lead to the generation of transgenic-free new varieties, which is based on direct delivery of CRISPR/Cas9 ribonucleoproteins (RNPs) generally into protoplasts, is available (Osakabe et al., 2018). Although *in vitro* plant regeneration of protoplast can be applied to some herbaceous species, in recalcitrant woody fruit plants species, the development of this technique is hampered by many factors, and attempts on grapevine are relatively recent and need further studies (Osakabe et al., 2018; Bertini et al., 2019). A plasmid-free method to obtain genome-edited plants was elaborated by Malnoy and collaborators, in which CRISPR/Cas9 RNPs were directly inserted in protoplasts of grapevine cultivar Chardonnay and apple cultivar Golden delicious. Grapevine protoplasts were obtained from embryogenic calli, and the induction of site-directed mutation of the *Mildew Locus O-7* (*MLO7)* gene was demonstrated, however, the regeneration of new genome-edited plants was not reported (Malnoy et al., 2016). Direct delivery of CRISPR/Cas9 RNPs was also described by Osakabe and colleagues, who detailed the advantages and critical steps in the obtainment of mutated *IdnDH* grapevine plants regenerating from protoplast or directly regenerated after classical *Agrobacterium-mediated* transformation (Osakabe et al., 2018). In whichever manner, genome-editing technology could be effectively applied for grapevine susceptibility gene knockout, which would be a beneficial plant defence strategy. This is the case of some *Vitis vinifera* cultivars, where susceptibility against downy and powdery mildews was decreased through the exploitation of the CRISPR/Cas9 system, by transforming embryogenic calluses in order to induce target mutagenesis of specific susceptibility genes (Giacomelli et al., 2018). In the future, targeted genome editing can be exploited to insert new genes or modify genes regulating plant-pathogen interaction at the expense of pathogens. Different to the others NBTs, that are based on the introduction of foreign DNA sequences in the host genome, TGE represents an innovative method that can induce specific modification in the existing genome limited to the introduction of single-point mutations (Zimny et al., 2019).

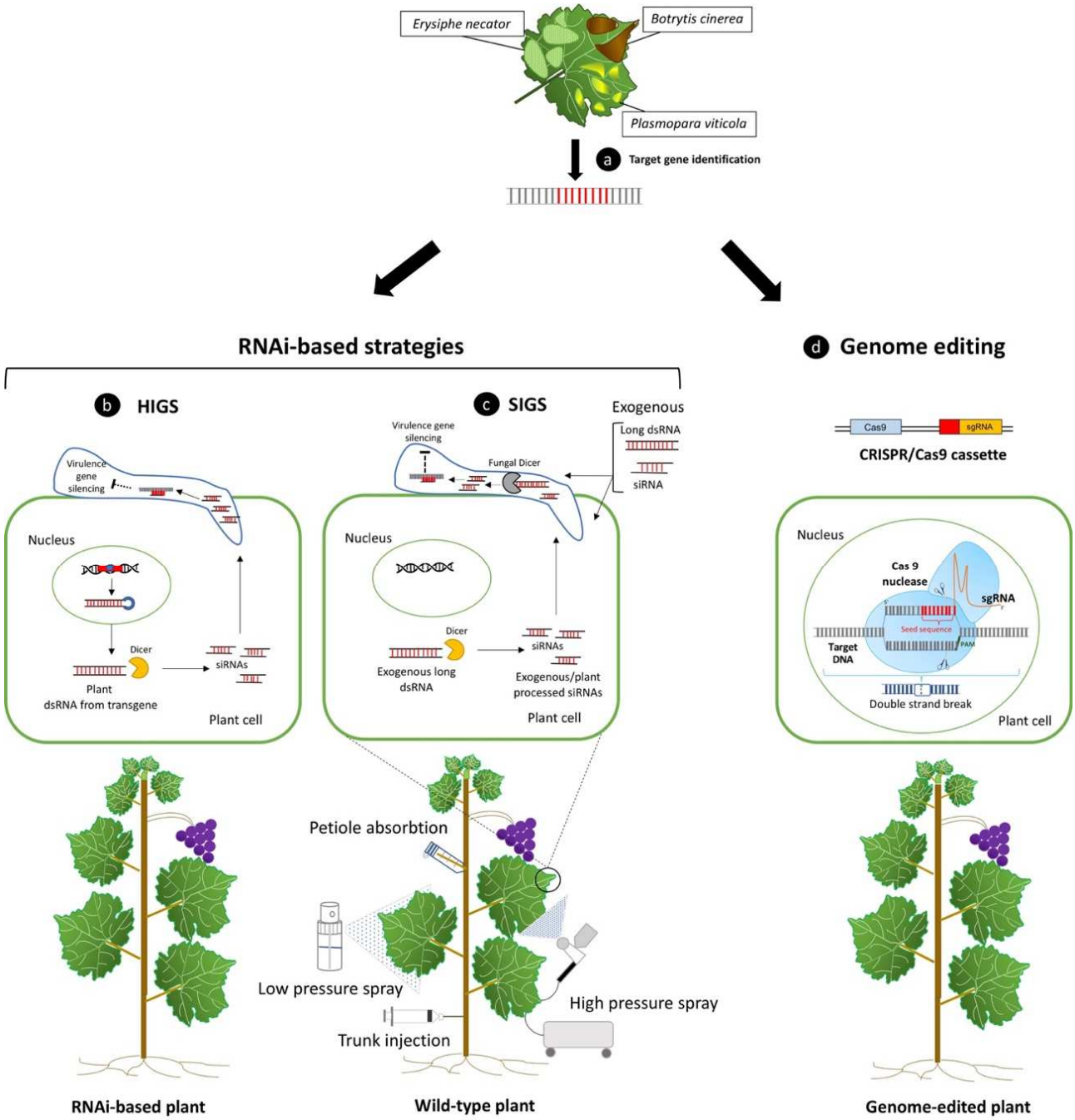


Figure 2: In addition to trans/cisgenesis methods, the expression of RNAi gene constructs in the plant, the exogenous applications of double strand (dsRNA) molecules targeting host/pathogen genes, or plant genome editing, represent valid alternatives to enhance plant immunity during pathogenesis. a) Candidate genes capable of limiting pathogen aggression or improve plant defence responses can be identified during the infection processes caused by the fungal and oomycetes causal agents of the most impactful diseases for grapevine production. RNAi-based strategies can be exploited to improve plant defence by providing dsRNAs to the plant cell through the expression of an introgressed hairpin-based gene construct in the plant genome, or through their delivery by exogenous application. b) In Host-Induce Gene Silencing (HIGS), as a result of transcription of an RNAi sequence, a long dsRNA molecule is formed. When this molecule is recognized by Dicer-like protein, it is cleaved into siRNAs, which can knockdown related target gene expression (Wang et al., 2016). c) A transgenic-free procedure in which dsRNAs are directly sprayed on the surface of plants and pathogens is known as Spray-Induced Gene Silencing (SIGS). These molecules can be absorbed by both types of cells, and,

depending on the delivery method used, dsRNAs can be processed by either the fungal/oomycetes and host RNAi machinery, leading to virulence gene knockdown and the reduction of pathogen detrimental effects. Low-pressure spray, high-pressure spray, petiole adsorption, and trunk injection of dsRNAs represent different available exogenous dsRNA delivery methods to confer plant protection against different plant pathogens, included fungi (Dalakouras et al., 2020). d) CRISPR/Cas9 system can be used for inducing targeted genome editing in plants, included the inactivation of specific plant susceptibility genes expression, which can help to regulate plant-pathogen interaction processes and disease resistance enhancement. Cas 9 protein complex is guided by artificially designed single guide RNA molecule (sgRNA) and leads to double-strand breaks (DSBs) of targeted DNA. SgRNA contains a seed sequence (around 8-12 bp) complementary to target DNA that guides the binding of the Cas 9 protein to the target genomic sequence. The site of cleavage takes place 3 nucleotides upstream to the protospacer adjacent motif (PAM) (Limera et al., 2017).

6. Biosafety considerations and overview of breeding technologies applied to enhance resistance against fungal and oomycete diseases in grapevine

Nowadays, various methods for grapevine genetic improvement are available, including both traditional breeding methods and new biotechnological approaches. The development and application of each of these strategies are often linked to several technical advantages and disadvantages; furthermore, they often give rise to new biosafety issues and public concerns (Table 2). A brief description of the new breeding techniques, compared with traditional breeding system, referring to the possibility of increasing in grapevine resistance to fungal and oomycetes diseases is reported below.

Traditional breeding: With the application of this technology it must be accepted that new cultivars will be similar to the original clones but not the same, evidencing the importance of assisted tools such as Marker Assisted Selection (MAS) that, thanks to the possibility to use the molecular markers, can be employed for the detection of genes of interest (Yang et al., 2016). Cultivars suitability for the wine market must be tested and classified as a new type of wine. Generally, this is a long-term program, that requires deep knowledge of genetic resources, and the new clones corresponding to the original clones recognized internationally for particular wine brands can be identified with difficulty (Riaz et al., 2009; Myles, 2013). Moreover, if resistance to diseases is provided by the insertion of R genes from less susceptible genotypes, it will be easily overcome by the onset of new pathogen strains (Myles, 2013).

Transgenesis: this technique allows the overexpression of both homologous and heterologous genes, including antifungal proteins. Whenever regeneration/transformation protocols are available, this technology can confer stable resistance to diseases to any grapevine cultivars, mostly preserving the agronomic characteristics of the original clone. Great potential is given by the high availability of gene vectors and selectable marker genes. The presence of transgenes in the plant genome and its release into the environment make risk assessment and public acceptance more difficult (Hartung and Schiemann, 2014).

Cisgenesis/Intragenesis: these technologies make it possible to introduce genes originally present in the same species or in sexually compatible ones into one genotype, through genetic transformation (Schouten et al., 2006; Rommens et al., 2007; Joshi et al., 2011). Compared to transgenesis, despite the availability of several grapevine resistance genes, it is more difficult to create full cisgenic gene constructs for the lack of efficient

cisgenic promoters and selectable markers thus, making the selection of stably transformed plants more complicated (Limera et al., 2017) (Table 2).

Gene silencing – HIGS - RNAi technology: HIGS uses the same transgenesis approach but the inserted RNAi gene construct can be designed with high specificity and minimizing off-target effects (Limera et al., 2017). It is applicable to downregulate/modulate the expression of plant endogenous genes and to target genes of grapevine pests and diseases. The expression of a new short RNAi sequence instead of new proteins facilitate risk assessment (Zotti et al., 2018). With the increasing knowledge of pests, fungi, and virus genomes this technology offers an effective and flexible tool for introducing stable resistances in grapevine cultivars.

Gene silencing – SIGS - RNAi technology: It is not considered a transgenic approach because it is not based on recombinant DNA technology, and it involves the application of small RNA molecules, with a much higher target effect. The new products are regulated as new natural molecules and not as GMO. To ensure better delivery of dsRNAs new formulates and production systems that will reduce production costs are under validation (Taning et al., 2020). It has been demonstrated that the SIGS pathway is greatly independent of the canonical defence pathways, hence conferring a "less expensive" and efficient immunity to cells, in comparison to an active pattern-or effector-triggered immunity (PTI/ETI) in progress which is expensive in terms of cellular energy (Koch et al., 2016).

Genome editing – CRISP/Cas9: this is the most recent technology for inducing target mutations in grapevine. The potential of this technology applied to grapevine depends on the identification of specific susceptibility gene sequences to be modified in the grapevine genome. Some important results have been already identified; however, the results can be affected by the type of target gene (Malnoy et al., 2016; Pessina et al., 2016; Ren et al., 2016; Osakabe et al., 2018). Different studies have demonstrated the risk of this technology in inducing off-target effects, even though it has reduced risk on the environment and on the consumer (Zischewski et al., 2017). The efficiency of this technology also depends on the methods used for the insertion of CRISPR/Cas9 protein needed for genome editing. In the case of genome editing induced by genetic transformation the new plants are definitely GMO and regulated as such. The limited availability of efficient regeneration protocols from somatic tissue or protoplasts remains the main limiting factor in applying this technology for targeting mutation in different grape cultivars.

Table 2: Description of different biotechnological approaches highlighting biosafety concerns and consumer acceptance

Technology	*Type of modification*	*Target origin and description*	*Time needed*	*Classification according to EU-Legislation*	*Side effects*	*Biosafety concerns*	*Consumer acceptance (proposed)*
Traditional breeding	Breeding and several backcrosses generation, introgression breeding, induced mutagenesis and somatic hybridization	Genes found in crossable, sexually compatible organisms	At least 10-15 years	Non-GMO	Altered clone identity, partial resistance to biotic stresses	No biosafety concerns and basic regulation needed (Rommens et al., 2007)	High (Lusk and Rozan, 2006)
Transgenesis	Genetic transformation	Overexpression of genes also from non-sexually compatible organisms, presence of gene sequences (i.e. promoter, selectable marker gene) from non-compatible organisms	Around 1-2 years	GMO	Release in the environment of genes of different origins; expression of new protein products with possible allergen/toxic effects	Expression of unknown protein/enzyme; use of antibiotic/herbicide resistance markers, lack of coexistence with non-GM, organic cultivations	Low (Hartung and Schiemann, 2014; Delwaide et al., 2015; Lucht, 2015; Malyska et al., 2016; Frewer, 2017)
Cisgenesis/ Intragenesis	Genetic transformation	Expression/overexpression of a gene originating from the recipient plant itself (cisgenesis), or expression of full/partial coding sequence originating from a sexually compatible plant (intragenesis)	Around 1-2 years	GMO	Scarce availability of efficient cisgenic selectable marker genes	cisgenic/intragenic plants solves the current biosafety problems regarding the presence of foreign genes in the plant host genome (Telem et al., 2013)	Medium/High (Schouten et al., 2006; Rommens et al., 2007; Jacobsen and Schouten, 2008)
Gene silencing-HIGS	Genetic transformation	Overexpression of non-coding dsRNAs downregulating exogenous or endogenous gene expression	Around 1-2 years	GMO	Efficacy of gene silencing varies with the genes and target organisms. Possible off-target effects in non-target organisms (NTOs)	Reduced off-target effects by designing RNAi sequences with high specificity and verified with bioinformatic studies. Minimal biosafety concerns when HIGS is applied only to rootstocks by trans-grafting technique (Arpaia et al., 2020)	Medium (Mezzetti et al.; Casacuberta et al., 2015; Papadopoulou et al., 2020)
Gene silencing-SIGS	No-genetic modification	External application of non-coding dsRNAs downregulating exogenous or endogenous gene expression	Few months	No-GMO	Efficacy of gene silencing depends on the efficiency and specificity of the RNAi sequence and on the degree of adsorption	RNAi sequence should be selected in order to avoid off-target effects (Dalakouras et al., 2020). The absence of negative effects, that can be caused by the nanotechnology-based delivery method used, on the	Medium/High (Shew et al., 2017; Taning et al., 2020)

					showed by plants and pathogens cells	environment and human health needs to be demonstrated	
Genome editing	Genetic transformation/plasmid-free protoplast transformation	Artificially gene modification/ target random mutation	About 1 2 years. More time necessary if transgene segregation is required from T0 plants, or if protoplast in vitro regeneration is required	No-GMO /GMO in Europe (ECJ-2018)	Possible appearance of off-target mutations; difficulties in plant regeneration from protoplasts	Transgene integration, effect of the expression of Cas 9 proteins, specificity and fidelity of Cas 9 protein (El-Mounadi et al., 2020)	Medium/High (Voytas and Gao, 2014; Ishii and Araki, 2016)

7. Conclusions

Plants and pathogenic fungi/oomycetes are living organism and their interaction give rise to a series of interlocking events culminating in plants immunity deficiency or vulnerability. The establishment of a plant disease is a complex mechanism whose resolution can be achieved by the application of integrating defence strategies. Gene overexpression, gene silencing, and genome editing are mainly used for studying gene functions and can be efficiently exploited to control pathogenic diseases caused by Fungi and Chromista kingdom. All these biotechnological approaches could be exploited for decreasing both pathogen virulence and plant susceptibility to diseases. In contrast to traditional breeding methods, the application of biotechnological techniques allows the breeder to act specifically at the gene level, avoiding the introduction of undesirable genes in the new improved grapevine cultivar. A detailed study of candidate genes involved during the infection process is required in order to select the best target for protecting plants or counteracting pathogenicity and virulence gene expression.

Regeneration and transformation of recalcitrant *Vitis vinifera* cultivars remains to be the biggest challenge for the application of genetic engineering-related biotechnologies (Ren et al., 2016). Once engineered plants have been obtained and before their commercialization, they must be subjected to strict regulation in order to guarantee the safety of their products towards the environment and on humans thereof. For this reason, researchers started to develop alternative strategies to classical biotechnological tools such as SIGS or Cisgenesis and intragenesis, avoiding the introgression in the host genome of foreign genes and the use of antibiotic resistance genes as selectable markers (Sabbadini et al., 2019c).

All together these approaches have the opportunity to offer preservation of plant health during the pathogenic challenge by providing a broad spectrum of defence mechanisms ranging from an overproduction of various compounds to RNA-mediated silencing, passing through specific gene inactivation.

1 This literature review has been published as follows:

Capriotti, L., Baraldi, E., Mezzetti, B., Limera, C., & Sabbadini, S. (2020). Biotechnological Approaches: Gene Overexpression, Gene Silencing, and Genome Editing to Control Fungal and Oomycete Diseases in Grapevine. International Journal of Molecular Sciences, 21(16), 5701.

THESIS PROBLEM STATEMENT

The most employed strategy in protecting vineyards from cryptogamic diseases such as grey mold, powdery mildew and downy mildew are the massive use of phytochemicals, which appear to have an impact on human health and ecosystems in general. During a single growing season, a total of 15 treatments may be necessary only for the control of such biotic adversities, which should provide for an alternation of active substances to avoid the occurrence of selective pressure in pathogens (Delaunois et al., 2014). In the current scenario of increasing global warming the management of fungal diseases has become a challenge, and the repeated and continuous use of fungicides is considered a practice no longer sustainable. The knowledge acquired on candidate resistant (R) genes, having an active role in plant defence mechanisms, has allowed numerous breeding programs to integrate these traits into selected cultivars, even though, with some limits in the conservation of the proper qualitative characteristics of the original clones. Given their gene-specific mode of action, biotechnological techniques come to the aid of breeders, allowing them to generate simple and fast modifications in the host, without introducing other undesired genes (Capriotti et al., 2020). One of the objectives of those who work with these new technologies is to make them widely applicable to all existing varieties, and to encourage their widespread application, especially to typical products of a territory such as Prosecco in Veneto region. Biotechnological tools can be applied for the protection of plants from chronic fungal and oomycetes diseases or for the development of new biodiversity in agriculture, aware of the potential of these technologies to lead towards new horizons. Plant cell regeneration competence is a key factor in determining the success of a biotechnological application, and the development of an efficient regeneration protocol is of primary importance (Dalla Costa et al., 2017). Different biological and technical barriers hinder the widespread applicability of efficient *in vitro* regeneration systems that together with a multi-year cycle and the presence of numerous polyphenolic exudates, make such processes not always achievable in all the genotypes.

The induction of random point mutations induced by chemical mutagens, or that can potentially occur during the *in vitro* regeneration processes, a phenomenon known as somaclonal variation, opens up new perspectives in the treatment of fungal and oomycete diseases or in obtaining new plants with useful variation, including diseases resistance, to be selected in large populations of mutagenized plants. New clones derived from this morphogenic ways have the high advantage to be classified as derived clones of the original cultivars so easier to register and transferred to the market, without any impediment related to any additional rules. Moreover, resistant clones of determined grape cultivars can be produced with the more precise biotechnology based on the downregulation of pathogen genes, which act as virulence effectors, by exploiting the RNAi mechanism, representing another biotechnological tool that can increase plant defence. RNAi processes can be induced by the overexpression of a hairpin gene construct codifying for a dsRNA molecule or alternatively by the exogenous application of the same dsRNA molecule on plants and pathogens surface, exploiting the capacity of pathogen cells to take up environmental dsRNA and processing them in the executor molecules of gene silencing (Qiao et al., 2021).

Activities

With the final aim to develop different strategies to stem the damage caused by the etiological agents *Plasmopara viticola* and *Botrytis cinerea* in grapevine, this research program covered some aspects ranging from the implementation of intravarietal genetic variability through somatic embryogenesis and chemical mutagenesis, to the application of host-induced gene silencing (HIGS) and spray-induced gene silencing (SIGS), as post-transcriptional gene silencing techniques. More specifically the objectives and aims addressed were as follows:

1. To review the existing literature on new biotechnological tools and their application in *Vitis* spp. for increasing their resistance to the main fungal and oomycete pathogens.
2. To optimize *in vitro* regeneration protocols both through the induction of somatic embryogenesis in different grapevine genotypes using different starting explants, and by the regeneration via organogenesis through the meristematic bulk (MB) system, and from cotyledons and hypocotyls derived from somatic embryos.
3. To employ MB regeneration system to apply chemical mutagens on plants to increase the genetic variability in grapevine cultivars, in order to select plant more tolerant to powdery mildew and other fungal diseases.
4. To analyse a new population derived from the germination of somatic embryos, towards the behaviour to downy mildew infection, and at the same time assess the possible variability between the cultivar Ancellotta and Lambrusco Salamino.
5. To develop genetic transformation protocols in various grapevine cultivars and rootstocks through *Agrobacterium*-mediated method using meristematic bulk slices as explants, for selecting the most efficient in terms of transgenic shoot production.
6. To apply HIGS in *Vitis vinifera* cultivars against *Plasmopara viticola* and *Botrytis cinerea* targeting their *Dicer-like 1* and *2* genes by both inducing shoot production from MB slices and cotyledons-hypocotyls derived from somatic embryos, or through the induction of secondary embryogenesis starting from somatic embryos.
7. To develop a low-pressure spray strategy for topical application of RNAi molecules against *Botrytis cinerea* in grapevine leaves and fruits as a preventive or curative treatment.

Research section

2 - CHAPTER TWO: *In vitro* morphogenesis protocols in grapevine cultivars and rootstocks

1. Introduction

The development of an efficient *in vitro* culture technique is the basis for the application of every biotechnological approach and can be used to speed up genetic improvement programs. Micropropagation, based on the *in vitro* axillary shoot proliferation, is considered one of the most employed technique to obtain a great number of healthy plants in a relatively short period (Kumsa, 2020), for all crops including grape. Cellular totipotency represents the ability to produce all the differentiated cells in an organism and is the biological principle on which is based plant morphogenesis from somatic and germline tissues (Karami et al., 2009). Fragmented shoot apices, auxiliary buds, inter-node segments, leaves and petioles could be cultured for the obtainment of adventitious shoots organogenesis according to the application of exogenous phytohormones (Xie et al., 2016).

Explants of different origin react to growth regulators in distinct ways, in which small changes in plant growth regulators (PGRs) concentrations can vary gene activation pattern, leading to disparate morphogenic responses (Khan et al., 2015). This process, which is based on the intrinsic plasticity of plant tissues, depends not only on the application of hormones (mainly auxins and cytokinins), but also and above all on the ability of the tissue to respond to hormones with a development program (Gaspar et al., 2003; Hurný et al., 2020). Organogenesis process may be described in three phases, considering the cell attitude towards the balancing of phytohormones. In the first phase, cells acquire the competence to regenerate new organs through cell de-differentiation and an undifferentiated growth due to the high mitotic activity. In the next phase, competent cells are stimulated towards a development program determined for a specific organ, based on the added hormones (high concentration ratio of cytokines/auxins stimulate the growth of the apical part, lower ratio promote root development). Morphogenesis proceeds autonomously and independently of the added hormones during the last step (Sugiyama, 2015). Unfortunately, *in vitro* regeneration of most important crop plants does not strictly follow these stages; hence, the development of predictable and routine *de novo* shoot organogenesis programs remains a challenge (Duclercq et al., 2011). An innovative and efficient regeneration protocol was fine-tuned by Mezzetti and co-authors (Mezzetti et al., 2002), consisting on the production of a complex vegetative structure full of meristematic cells, so-called meristematic bulk (MB). The entire regeneration process starts from *in vitro* proliferating shoots, with a high competence for plant regeneration. This innovative regeneration platform allowed to conduct significant researches from genetic transformation trials to the study of chimerism in grapevine plants that benefit from this (Mezzetti et al., 2002; Bertsch et al., 2005; Xie et al., 2016). In the current study, the meristematic bulk, after being processed and cut into slices were used in genetic transformation and mutagenesis experiments in order to collect putative transgenic or mutagenized new lines. In addition to organogenesis, somatic embryogenesis has the potential to provide an efficient model system in functional studies as well as being used practically for large-scale plant propagation (Von Arnold et al., 2002; Correia et al., 2019). Research in somatic embryogenesis is largely described in terms of culture improvement

or protocol adaptation to various genotypes, while molecular pattern and physiological aspects remains commonly not much addressed (Marsoni et al., 2008). Woody plant species, including grapevine, are commonly recalcitrant to genetic transformation, and somatic embryogenesis represents the most applied regeneration strategy for this purpose (Prieto et al., 2019; Song et al., 2019). Stable genetic transformation is hindered by the development of chimaeras organs, minimal in somatic embryogenesis in which the entire regeneration pattern starts from a single cell (Gambino et al., 2007; Dai et al., 2015). The non-zygotic *in vitro* embryogenesis regeneration process also termed somatic embryogenesis consists in the formation of somatic embryos with a structure very similar to a zygotic embryo but derived from the morphogenesis of mature somatic tissues (Martinelli and Gribaudo, 2009). Although somatic embryogenesis was proved to be a successful tool for grapevine *in vitro* regeneration optimized for many *Vitis* species, this technique is strongly genotype-dependent, and its efficiency varies according to the starting explant chosen. Different patterns of gene expression are observed during the acquisition of embryogenic competence, which makes induction and maintenance of this process particularly difficult (Schellenbaum et al., 2008; Yang and Zhang, 2010). The entire morphogenetic process relies on a unique developmental pathway characterized by various phases, that can be resumed as follows (Martinelli and Gribaudo, 2009):

1. Induction of callogenesis and acquisition of embryogenic competence
2. Culture and development of embryogenic calluses and characterization of embryo potential
3. Long term culture of embryogenic calluses and embryos proliferation
4. Somatic embryos conversion in whole plantlets

Culture initiation is a determining factor being dependent on the genotype, type of starting explant, phenological phase and composition of culture medium in terms of basal salts and plant growth regulators (PGRs). During the first step, somatic cells must undergo to an internal cell reprogramming of gene expression pattern leading to a polarized cell division (Corredoira et al., 2019). The obtainment of somatic embryos is possible through direct or indirect developmental way, often not well distinguishable. The indirect somatic embryogenesis starts with the development of an embryogenic callus followed by the emergence of pro embryogenic masses (PEMs), from which cell cluster or single cells originate new somatic embryos (Horstman et al., 2017). The direct somatic embryogenesis occurs directly from the explant, without the callus development phase, through the proliferation and development of already formed pre-embryogenic determined cells. In many woody plant species, direct somatic embryogenesis is preferred for clonal mass propagation rather than indirect somatic embryogenesis, which is characterised by a high incidence of somaclonal variation (Ghadirzadeh-Khorzoghi et al., 2019). Excluding immature zygotic embryos, their somatic counterpart are widely used in breeding programs, and the most employed initial explants in grapevine are anthers, ovaries, leaves, petioles, tendrils and nodal sections (Vidal et al., 2009). Although the mineral compositions of the culture medium is noteworthy, the type and concentrations of PGRs have been demonstrated to be crucial in the embryogenic callus induction phase (Corredoira et al., 2019). Organs *in vitro* regeneration is generally controlled by auxins and cytokinins, with the first ones that exert an important role in both early-stage embryo patterning and somatic embryos induction (Nic-Can and Loyola-Vargas, 2016). Other factors can influence

embryogenic responses, such as pH and photoperiod conditions, carbonic sources, gelling agents, and the presence of antioxidant compounds (Martinelli and Gribaudo, 2009). Depending on the degree of regeneration recalcitrance of each genotype, somatic embryos can arise from the starting explants following a one-step process in which induction and expression of embryogenic competence occurred in the same medium, or a two-step process, in which, after being cultured on an induction medium, calluses are transferred to an expression medium (normally containing lower cytokinins-auxins concentration) (Corredoira et al., 2019). The maintenance of embryogenic activity is commonly achieved by the frequent subculture of PEMs that are continuously produced through their culture on PGRs free medium, or by inducing secondary embryogenesis in a recurrent cycle (Vannini et al., 2005). The somatic embryos mainly developed asynchronously, because not all embryogenic calluses differentiate at the same time. Secondary embryogenesis allows the reduction of the physiological differences in embryo developments, increasing the number of somatic embryos produced (Maillot et al., 2009).

The study of embryo defected mutants in model plants allowed to identify few genes specifically related to embryogenesis program, but in most cases the inability to regenerate embryos was caused by events occurring during cell division and differentiation or by an altered perception of hormones (Correia et al., 2019). The downregulation via post transcriptional gene silencing of specific genes related to non-embryogenic callus formation can be used as useful strategy to increase regeneration/transformation potential of recalcitrant woody plant species (Correia et al., 2019).

The response in term of primary and secondary embryogenesis is strictly related to the genotype and starting type of tissue. For this reason, in particular for grape, it is important to study the response of the different genotypes and somatic tissue in order to develop the most efficient protocol for inducing high production of primary and/or secondary somatic embryos to be used for the application of the New Breeding Techniques (cisgenesis, gene editing and RNAi), or in particular in the case of grape rootstocks also for the development of new techniques for the efficient and high-quality plant propagation.

However, one of the major limitations of this regeneration technique regards embryo quiescence and endodormancy, with consequent low germination rate and plant development (Gray and Purohit, 1991). There are two critical steps: one during somatic embryos germination, consisting in the conversion of embryo structures into plantlets, and the other in the acclimatization phase. Variable values of germination rate were reported in somatic embryogenesis studies, often lower than 50% (Martinelli et al., 2001b; Carimi et al., 2005). Jayasankar and co-authors demonstrated in their research that somatic embryos obtained on solid culture exhibited physiological dormancy and a different morphology compared to their liquid-medium-derived counterparts, enhancing the germination rate by more than 40% (Jayasankar et al., 2003). The most efficient dormancy-breaking pre-treatment applicable to somatic embryos is cold (generally 2 months) alone or in combination to the addition of GA_3 (gibberellic acid) in the culture medium (Larrouy et al., 2017). In the view of increasing regeneration potential, overcoming the risky embryo-plantlet conversion stage, we described in this study the adventitious bud formation on cotyledons and hypocotyls, exploiting their juvenile physiological status (Vilaplana and Mullins, 1989). Cotyledons and hypocotyls of somatic embryos were considerate suitable

explants on a par with their zygotic counterpart, usually employed in recalcitrant species such as *Prunus* spp. (Mante et al., 1989). De novo shoot organogenesis was possible to achieve starting from different tissues due to the cellular pluripotency that characterized plant cells. During the last year of research, we developed and optimized an innovative regeneration protocol, using cotyledons and hypocotyls of somatic embryos as starting material. This work aimed to test different regeneration protocols (organogenesis and somatic embryogenesis) on various grapevine cultivars and rootstocks, in order to identify the genotype response to the different morphogenesis process and develop the most efficient regeneration protocol for the selected cultivars and rootstocks.

2. Material and methods

2.1 Organogenesis from meristematic tissues in grapevine cultivars and rootstocks

2.1.1 Plant material

The research work included different grape cultivars and rootstocks, well known for the economic importance for the grapevine industry (Table 1). Albana, Ancellotta, Chardonnay, Glera, Lambrusco Salamino, Merlot, Pinot Grigio, Sangiovese, Thompson Seedless, Vermentino, 110 Richter, 1103 Paulsen, and Kober 5BB meristematic bulk were provided by Vitroplant Italia S.r.l., Cesena, Italy, and used in genetic transformation and chemical mutagenesis experiments. Ancellotta, Chardonnay, Lambrusco Salamino, 110 Richter, Kober 5BB, Thompson Seedless have been used in somatic embryogenesis studies.

Table 1: *Vitis vinifera* cultivars and *Vitis* hybrid rootstocks employed for meristematic bulk production in order to visualize their regeneration occurred during *in vitro* culture for the application of genetic transformation. To each genotype is associated a brief description regarding the main destination, the cultivated area in Italy, the main characteristics, and whether somatic embryogenesis has been applied during this research programme. All the information have been acquired on the Italian national vine variety register, and for rootstocks from the work conducted by Warmund and co-authors in 2013 (Warmund et al., 2013).

Genotypes	Purpose/Destination	Cultivation (Ha) (ISTAT, 2010)/vine shoots certified material production ISTAT 2018-2019)	Note	Somatic embryogenesis
Albana	White grape variety, exclusively for wine production	1.523 cultivated on Emilia Romagna (North Italy)	High vegetative vigour	☒
Ancellotta	Red grape variety, belonging to Lambrusco group, exclusively for wine production	4.343 cultivated on Emilia Romagna (Emilia –North-Western Italy)	Constant and abundant production	☑
Ciliegiolo	Red grape variety, for wine production and direct consumption	1.830, Toscana region and central Italy	Derive from Sangiovese	☒
Chardonnay	White grape variety, exclusively for wine production	19.709, originally from Burgundy (central-eastern France), from Trentino it spreads throughout Italy	Used for sparkling wine production	☑
Glera	White grape variety, exclusively for wine production	18.255 cultivated on Veneto region (North-eastern Italy)	Prosecco production	☒
Lambrusco Salamino	Red grape variety, exclusively for wine production	5.003 cultivated on Emilia Romagna and Lombardia regions (North western Italy)	Cylindrical bunch having the shape of salami	☑
Merlot	Red grape variety, exclusively for wine production	28.042 cultivated in all Italy, mainly Friuli Venezia Giulia and Veneto (North eastern Italy)	Gives intense red colour to wines	☒
Pinot grigio	Grey grape variety, exclusively for wine production	17.281 mainly cultivated in North Italy	It is often vinified in white	☒
Sangiovese	Red grape variety, exclusively for wine production	71.558 cultivated in all Italy, excluding North-eastern regions	It is one of the classic cv. that enters on the Chianti wine formula and other important wines	☒
Thompson Seedless	White grape variety, exclusively for fresh or dried consumption	209.077 vine shoots produced in 2019	Small, oval berries without seeds	☑
Vermentino	White grape variety, exclusively for wine production	4.562 cultivated in central Italy, mainly Liguria, Toscana and Sardegna region	Produces high quality grapes in the coastal areas	☒
110 Richter (*Vitis berlandieri* Rességuier n. 2 x *Vitis rupestris* Martin)	Rootstock	306.409 units produced in 2018; on average widespread rootstock throughout Italy	Appreciated for its good vigour, its constant production over time and for its moderate resistance to drought and calcareous soils	☑
Kober 5BB (*Vitis berlandieri* x *Vitis riparia* Teleki sel. 5A)	Rootstock	168.758 units produced in 2018; on average widespread rootstock throughout North Italy	Induces great vegetative vigour, excessive in fertile soils. It is moderately resistant to calcareous soils	☑

1103 Paulsen (*Vitis berlandieri* Rességuier n. 2 x *Vitis rupestris* Du Lot)	Rootstock	2.702.503 units produced in 2018; on average widespread rootstock throughout Italy, especially in Sicily	Vigorous and elastic rootstock, it has a high affinity with all varieties. It resists to drought and adapts well to almost all soils, even clayey-calcareous ones. Tolerant to magnesium deficiency (Livigni et al., 2019)	☒

In vitro proliferating shoots of two selected *Vitis vinifera* cultivars Ciliegiolo, Thompson Seedless and the two rootstocks, 110 Richter (*V. berlandieri* x *V. rupestris*) and Kober 5BB (*V. berlandieri* x *V. riparia*), were used as starting plant material for the induction of meristematic bulks and the study of the regeneration potential. The vegetative material was subcultured every 30 days on a medium containing MS salt and vitamins (Murashige and Skoog, 1962), 30 g L^{-1} sucrose, 4.4 µM of 6-benzylaminopurine (BAP) and 7 g L^{-1} plant agar. The explants were kept in a growth chamber at 24 ± 1°C under a photoperiod of 16-h light (70 µmol m^{-2} s^{-1}) provided by white fluorescent tubes.

2.1.2 MB induction and maintenance

The MB induction and maintenance was obtained following the protocol established by Mezzetti and co-workers on 2002 (Mezzetti et al., 2002). Briefly, shoot tips derived from proliferating cultures were processed and placed on the initiation medium (IM) described previously (Mezzetti et al., 2002), with the addition of 0.01 µM 1-Naphthaleneacetic acid (NAA). At each monthly subculture (a total of 3), the apical dome was eliminated and the concentration of BAP in the IM medium was increased (from 4.4 µM (IM1), 8.8 µM (IM2), up to 13.2 µM (IM3)). The obtained MBs (Fig. 1 a) were maintained and proliferated by slicing them (Fig. 1 b) on IM3 culture medium and used as starting materials for the subsequent regeneration and transformation experiments. The explants were kept in a growth chamber at 24°C under a photoperiod of 16-h light (70 µmol m^{-2} s^{-1}) provided by white fluorescent tubes.

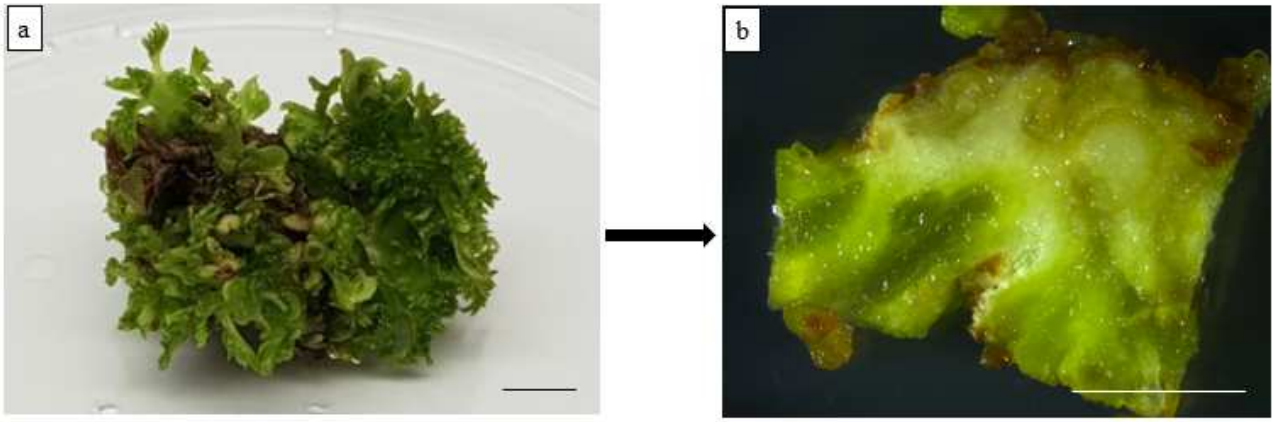


Figure 1: Organogenesis-based regeneration method applied on grapevine: a) Meristematic bulks (MBs) obtained after 3 weeks on regeneration medium (IM3); b) Thompson Seedless meristematic bulk slices; (*bars* = 5 mm).

2.1.3 MB regeneration efficiencies

A total of 20 MB slices (1 cm^2, 2 mm thick), divided in four replicates, were placed on the IM3 regeneration medium. The explants were transferred on fresh media every 3 weeks for a total of three subcultures, cutting off the new regenerating shoots at each subculture. The experiment was repeated twice. Regeneration response of MB slices of Ciliegiolo, Thompson Seedless, 110 Richter, and Kober 5BB was monitored recording the following data: percentage of regenerating MB slices [(number of MB slices regenerating shoots/total MB

slices treated) × 100], and mean number of green shoots regenerated from each explant at each culture condition. All the data were recorded after each sub-culture (at 3, 6 and 9 weeks).

2.2 Primary and secondary somatic embryogenesis and embryo maintenance of grapevine cultivars and rootstocks

2.2.1 Initiation of embryogenic process

Canes of *Vitis vinifera* cv. Ancellotta, Lambrusco Salamino, and rootstock 110 Richter (*Vitis berlandieri* x *Vitis riparia*) were collected from vineyard (Reggio-Emilia, Emilia Romagna region, North-Western Italy) during the winter season, and then stored at 4°C for at least one month, preserving the dehydration of vegetal material. At the appropriate time, *Vitis* cuttings were surfaced sterilized with a solution containing 50 % of a commercial bleach (NaClO 1%) for five minutes and then rinsed several times using tap water. The branches were cut at their distal ends (only some centimetres), and transferred into a vessel filled with sterile distilled water up to half, respecting their original geotropism, and maintained in a growth chamber with white fluorescent lights (65 µmol m^{-2} s^{-1} and 16 hours photoperiod) at 25°C ± 1. From three to five weeks, depending on the genotypes, the buds have sprouted and developed the inflorescences, that before the full bloom stage were used as starting explant for the induction of somatic embryo.

2.2.2 Initiation of somatic embryos from whole flowers, stamens, and pistils

Flowers were excised and observed under a stereomicroscope to identify the right developmental stage of whole flowers, stamens, and pistils. Based on the appearance of explants, stamens and pistils have been selected at stages II and III (Dhekney et al., 2009), and whole flowers at stages IV and V (Gribaudo et al., 2004) considered as the most suitable for somatic embryogenesis induction. Dissected inflorescences (Fig. 2 a) were manipulated previous their immersion for 1 minute in ethanol (70% v/v) and surfaces sterilization in 25% NaClO solution with few drops of Tween 20 in continuous agitation for 10 minutes, followed by three 5-minutes washes in sterile distilled water. Stamens (Fig. 2 b) were collected removing flower calyptra and were placed, including their filaments and anthers, in the center of the Petri dishes, while the pistils were arranged in their entirety at the perimeter of each Petri dish. Whole unopened flowers (Fig. 2 c) were dissected from the inflorescence, retaining their pedicels, placing them horizontally on induction medium. All these explants were cultured from 5 up to 7 weeks in dark condition at 25°C and checked regularly after being transferred to mid light conditions (30 µmol m^{-2} s^{-1} and 16 hours photoperiod) at 25°C ± 1.

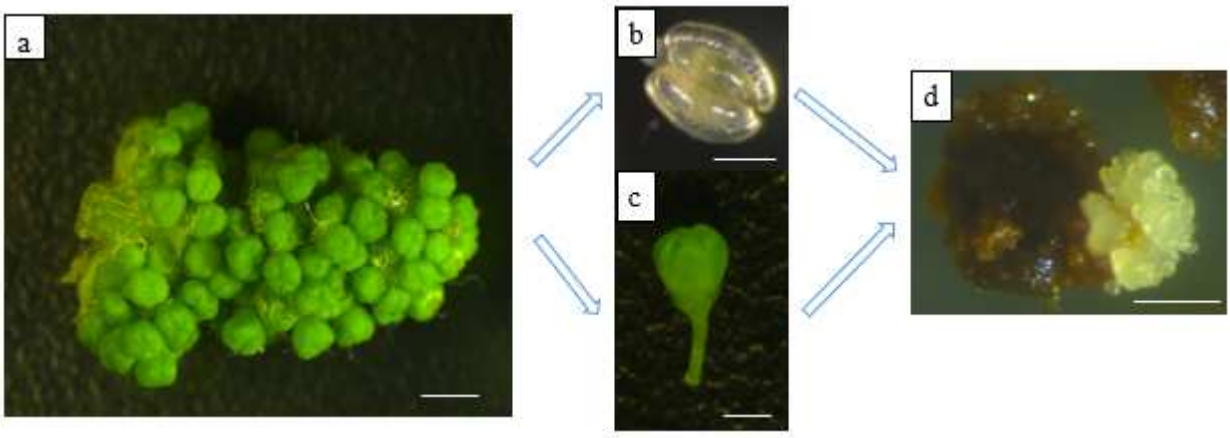


Figure 2: Grapevine inflorescence (a), anther (b), and whole flower (c) at the right stage of development of Ancellotta cultivars, embryogenic calluses (d) developed from the explants after 10 weeks of culture on induction medium (whole flowers on MS1). (*bars* = 2 mm on a and d; 1 mm on b-c).

A total of 20 whole flowers, or stamens and pistils coming from the disruption of five flowers, were cultured in Petri dishes containing two different callus induction media, MS1 (Dhekney et al., 2009) and PIV (Franks et al., 1998), and subjected to two different cytokinins/auxin ratio. MS1 contained Murashige and Skoog basal salts and vitamins (Murashige and Skoog, 1962), 7 g L^{-1} plant agar (Duchefa Biochemie), 20 g L^{-1} sucrose, 4,5 µM N6-benzylaminopurine (BAP), and 5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) (Duchefa Biochemie). PIV was composed of Nitsch and Nitsch basal salt (Nitsch and Nitsch, 1969) and B5 vitamins, 7 g L^{-1} plant agar, 60 g L^{-1} sucrose, 9 µM BAP, and 5 µM 2,4-D. In all media used the pH was adjusted to 5,8 with KOH 1M before autoclaving at 121°C for 20 minutes. Embryogenic calluses (EC) (Fig. 2 d) that gradually emerged from flowers, stamens or pistils were transferred to hormone-free X6 medium (X6) (Li et al., 2001; Dhekney et al., 2009, 2016), consisting of a modified MS medium (Murashige and Skoog, 1962) lacking glycine and supplemented with 3.033 g L^{-1} KNO_3 and 0.364 g L^{-1} NH_4Cl as the sole nitrogen sources, 60.0 g L^{-1} sucrose, 1.0 g L^{-1} *Myo*-inositol, 7.0 g L^{-1} plant agar (Duchefa), and 0.5 g L^{-1} activated charcoal. Pro-embryogenic masses (PEMs) and somatic embryos (SE) were sub-cultured every 2 weeks on fresh medium, in low light condition (15 µmol m^{-2} s^{-1} and 16 hours photoperiod), to maintain high embryogenic competence. Elongated and partially rooted SEs at advanced cotyledonary stage were transferred to glass pots containing a culture medium composed of MS basal salts and vitamins supplemented with 30 g L^{-1} sucrose and 1.1 µM BAP. Plantlets with rootlets were cleaned in water and transferred to small pots filled with soil potting mix. Plant acclimatization was carried out in three-four weeks in the growth chamber, decreasing gradually air-saturation level.

2.2.3 Induction of somatic embryos from stem nodal segments

Somatic embryogenesis experiments were performed using nodal segments (composed by one auxiliary bud and a short stem segment) from 4-weeks-old *in vitro* elongated plantlets of Thompson Seedless, Chardonnay,

110 Richter, and Kober 5BB, provided by Vitroplant Italia S.r.l., Cesena, Italy. Nodal segments were placed on callus induction medium (CIM) (Maillot et al., 2016) containing MS with half-strength major salts, 7 g L^{-1} plant agar, 25 g L^{-1} sucrose, and supplemented with two different concentrations of PGRs, 9 µM 2, 4-D plus 4.5 µM BA (CIM1), or 18 µM 2, 4-D plus 9 µM BA (CIM2). Stem nodal segments, fifty for each genotype, were placed horizontally on each of the two media (CIM1 and CIM2) and kept for one month in dark condition at 24°C. Subsequently, to stimulate the emergence of pro-embryogenic cell clusters and embryos all the explants were transferred to solid Medium A containing 60 g L^{-1} sucrose, 2.5 g L^{-1} activated charcoal, 20 µM indole-3-acetic acid (IAA), 10 µM 2-naphtoxyacetic acid (NOA), and 1 µM BAP in mid-light condition (30 µmol m^{-2} s^{-1} and 16 hours photoperiod) at 24°C. Only embryogenic calluses were transferred to X6 embryo development and maintenance medium, for the continuous culture of PEMs.

2.2.4 Induction of secondary embryogenesis

The embryogenic ability was maintained for each genotype tested through the induction of secondary somatic embryogenesis. Cluster of SEs, or types II single somatic embryo as described by Zhou and colleagues (Zhou et al., 2014), in turn, were stimulated to induce secondary embryogenesis, providing higher and synchronous embryo production, in accordance to what was reported by Maillot and co-authors (Maillot et al., 2006). These explants were selected between the other embryos on X6 medium and were cultured for one month in dark condition on medium termed E96 (Maillot et al., 2006) containing MS salts and vitamins, with half concentration of major salts, 60 g L^{-1} sucrose, 7 g L^{-1} plant agar, 9 µM 2,4-D and 4.5 µM BAP. All the explants were transferred to Medium A (Maillot et al., 2006) for embryogenic callus growth and development, incubated under light (15 µmole m^{-2} s^{-1} and 16 hours photoperiod), and sub-cultured to fresh medium every four weeks. At every subculture on fresh Medium A, well-developed embryogenic calluses were transferred to fresh X6 medium for embryos development. The obtained somatic embryos were maintained and proliferated by transferring them on X6 culture medium and used as starting materials for the subsequent regeneration and transformation experiments.

2.2.5 Embryogenic efficiencies

Using whole flowers, stamens, and pistils, the number of embryogenic calluses arose from the explants were counted and reported at 5 and 10 weeks after culture initiation. Total embryogenic efficiency (%) was noted as the total number of embryogenic calluses (adding those obtained at 5 and 10 weeks) produced by the total number of explants. Using stem nodal segments, embryogenic efficiency (%) was calculated 10 weeks after culture initiation as following described: [(number of nodal segments regenerating embryogenic calluses /total nodal segments treated) × 100]. This was applied to each genotype and culture medium with different PGRs tested as described above. The percentage value of callogenesis was also reported as the number of explants that produced dedifferentiated callus to total segments placed in culture.

2.3 De novo shoot organogenesis from cotyledons and hypocotyls of grapevine cultivar somatic embryos

2.3.1 Culture conditions

Well-developed whitish somatic embryos of Ancellotta, Lambrusco Salamino, and Thompson Seedless were continuously produced on X6 medium (Fig. 3 a). These explants were obtained following the different protocols previously described and have been cut separating hypocotyls from cotyledons (Fig. 3 b-c). These explants were placed on Petri dishes filled with two different novel regeneration media consisting of NN basal salts and vitamins (Nitsch and Nitsch, 1969) supplemented with 15 g L^{-1} of sucrose, 7 g L^{-1} of plant agar, and two specific hormone combination: one including 4.4 µM of BAP and 0.49 µM of IBA (3-Indol-butirryc acid) (culture medium 1), and the other with 13.2 µM of BAP (culture medium 2).

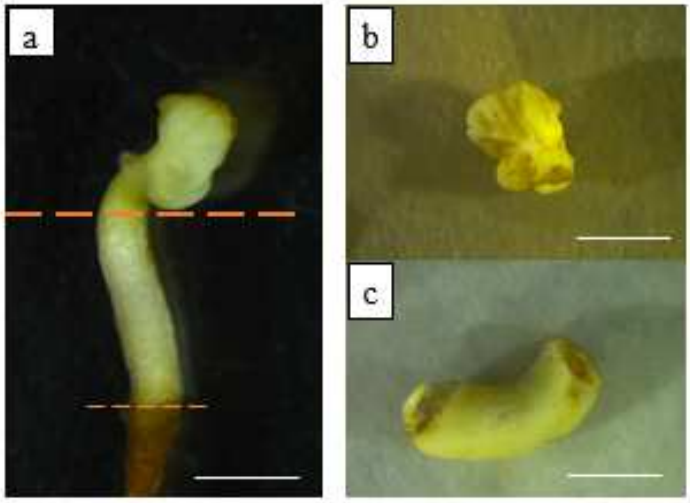


Figure 3: Well developed whitish somatic embryos (a) were dissected separating cotyledons (b) and hypocotyls with their distal ends cut off (c) (*bars* = 5 mm).

A total of 50 cotyledons and 50 hypocotyls for each genotype and for each culture medium were stimulated to differentiate new organs, and placed in the growth chamber at 24 ± 1°C under a photoperiod of 16-h light (70 µmol m^{-2} s^{-1}), provided by white fluorescent tubes. Explants were controlled for more than 12 weeks, transferring them to the same fresh culture medium every three weeks. 9 weeks after culture, the regeneration rate (%) was recorded as the number of explants able to regenerate shoots compared with the total number of explants relative to each type of explant on each substrate. The regeneration experiment was replicated three times. At the third subculture, explants were moved to microboxes (Micropoli, IT) in which PGR content in the media was halved at each step to stimulate internodes elongation, then, elongated shoots were transferred to PGR-free MS substrate for rooting. Elongated and rooted plantlets were acclimatized, transferring them into pots containing a commercial soil peat.

2.3.2 Cotyledons and hypocotyls regeneration efficiencies

A total of 50 cotyledons and 50 hypocotyls of Ancellotta, Lambrusco Salamino, and Thompson Seedless, divided in five replicates (ten explants for each petri plate), were placed on the two-regeneration media each (50 explants for each medium). The explants were transferred on fresh media every 3 weeks for a total of three subcultures. The experiment was repeated three times. Regeneration responses of cotyledons and hypocotyls respectively on each medium and for each genotype were monitored recording the percentage of regenerating explants [(number of explants regenerating shoots/total explant treated) × 100]. Considering the high regeneration potential which made it difficult to distinguish the individual shoots, it was not possible to evaluate the mean number of green shoots regenerated from each explant at each culture condition. All the data were recorded after each sub-culture (at 3, 6, and 9 weeks), but only those obtained at 9 weeks were noted.

2.4 Statistical analysis

The regeneration experiment on cotyledons and hypocotyls was replicated three times. Regeneration data were subjected to analysis of variance (ANOVA), with cultivar and regeneration medium tested as sources of variation. The mean comparisons between cultivar and regeneration medium were determined using Student-Newman-Keuls t-test at $p < 0.05$. All analyses were performed with the Statistica 7 software (Statsoft Tulsa,CA, USA). Data on percent regeneration were transformed by the arcsine square root transformation, ARSIN (SQRT (X)) before analysis.

3 Results

3.1 Organogenesis from meristematic tissues in grapevine cultivars and rootstocks

3.1.1 Regeneration capacity of MBs

A regeneration test was conducted for the variety Ciliegiolo, and for the two rootstocks, 110 Richter and Kober 5BB. Thompson Seedless has been used as the reference genotype for the protocol described by (Mezzetti et al., 2002). MB slices obtained from the four genotypes were placed on IM3 medium, and data on the regeneration efficiency (percentage of slices producing at least one shoot, data not shown) and the mean number of regenerating shoots per explant were collected at 3, 6, and 9 weeks of cultivation (Fig. 4 a). All the genotypes showed a comparable regeneration efficiency with values close to 100% of regenerating slices (data not shown), starting from 3 weeks of culture and maintained for the entire culture period (Fig. 4 b). The rootstock Kober 5BB showed the highest mean number of regenerated shoots per explant with values comparable to those exhibited by Thompson Seedless (Fig. 4 a). 110 Richter's MB slices exhibited the lowest regeneration efficiency in terms of the mean number of shoots per explant throughout the data acquisition periods (Fig. 4 a). Ciliegiolo showed the same trend as Thompson Seedless and Kober 5BB until 6 weeks of cultivation, after which the number of regenerated shoots declined (Fig. 4 a).

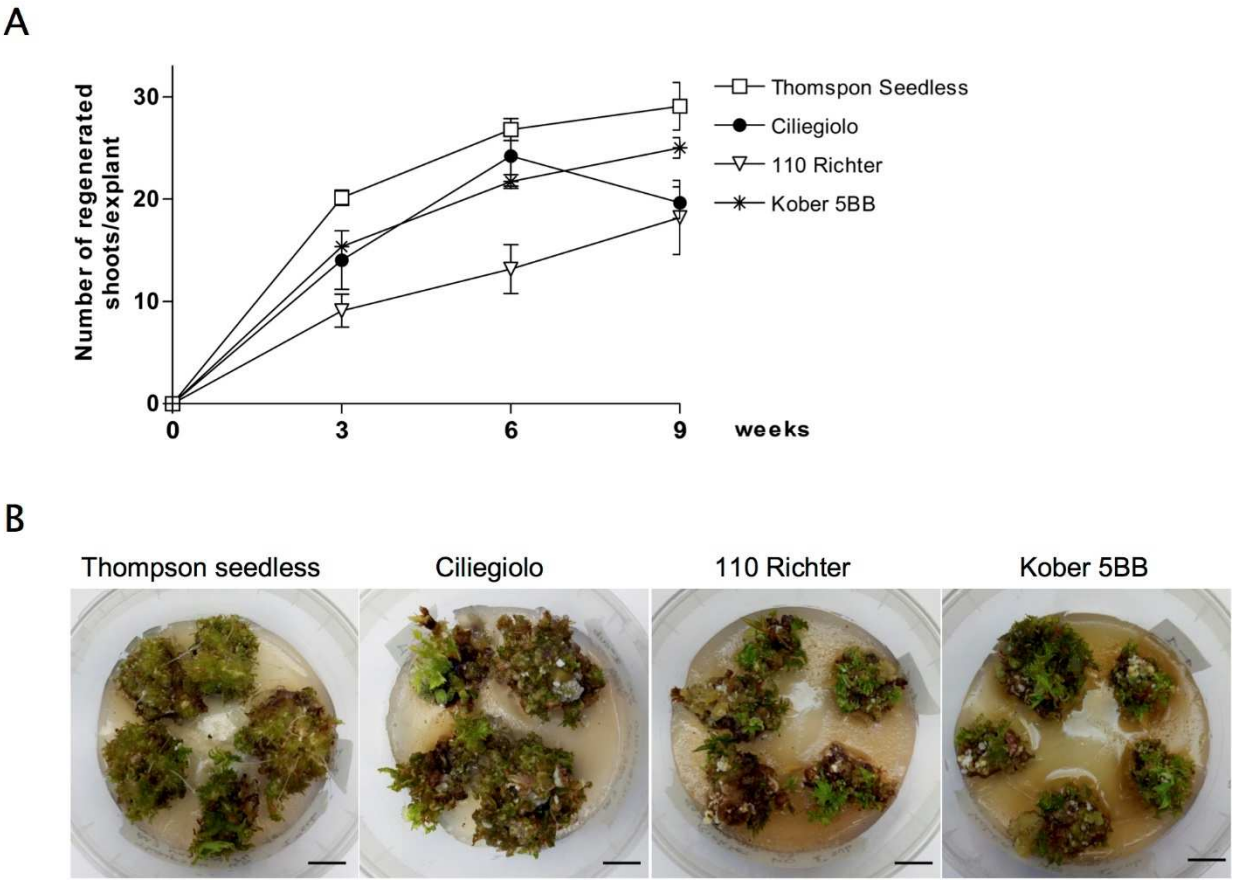


Figure 4: Regeneration efficiency obtained from MB slices of Thompson Seedless, Ciliegiolo, 110 Richter and Kober 5BB: (a) Mean number of shoots per explant. Data were acquired at 3, 6 and 9 weeks of cultures. Data reported are means of ± SE (n = 20). (b) A representative image of the MB slices regeneration of the four genotypes on IM3 medium, after three weeks of culture (*bar* = 1 cm).

After this preliminary study, the same system with slight modifications precisely in PGR composition of IM3 have been adopted and optimized thanks to the collaboration of Vitroplant for the regeneration of the following genotypes: Albana, Ancellotta, Chardonnay, Ciliegiolo, Glera, Lambrusco Salamino, Merlot, Pinot Grigio, Sangiovese, Thompson Seedless, Vermentino, 110 Richter, 1103 Paulsen, and Kober 5BB. Among these genotypes Glera, Pinot Grigio, and 1103 Paulsen had some problem during the *in vitro* regeneration, with the production of exudates that were released on the culture medium with consequent tissue browning and scarce shoot production, also when were cultured on media enriched to TDZ until a concentration of 2 µM.

3.2 Primary and secondary somatic embryogenesis and embryo maintenance of grapevine cultivars and rootstocks

3.2.1 Embryogenic efficiencies using floral-derived starting explants

This study aimed to individuate the best floral-derived starting explants for the induction of primary somatic embryogenesis on Ancellotta, Lambrusco Salamino, and 110 Richter genotypes, using MS1 and PIV as induction culture media. From 225 up to 275 stamens, 25 up to 55 pistils, and 160 up to 400 whole flowers

were placed in culture for each genotype and each culture medium, based on the availability of branches capable of differentiating inflorescences. The number of embryogenic calluses were recorded at 5 and 10 weeks after culture initiation, and the related embryogenic efficiency was calculated as the total number of embryogenic calluses induced from the total number of explants. The results of the experiment (Table 2) evidenced that for all the genotypes tested, PIV seems to be the most suitable induction medium when whole flowers are used as starting explant, as well as for MS1, which induced a good embryogenic response in stamens.

The highest embryogenic efficiency was recorded when whole flowers were used as starting explants, for almost all the genotypes and the culture media tested (Table 2). The highest percentage of embryogenic calluses (ECs) formation was observed for 110 Richter whole flowers cultured on PIV medium. Although first ECs can be seen from the first months of culture, the emergence of new embryogenic tissues was reported also sporadically after six months of culture, especially from Lambrusco Salamino whole flowers. Regardless of the genotype and the culture medium, the whole flowers provided a greater number of embryogenic calluses than the stamens and lastly the pistils.

Ancellotta and Lambrusco Salamino somatic embryos germinated into two-three weeks of culture on MS medium supplemented with 1.1 μM of BAP. 110 Richter somatic embryos germination will be evaluated as soon as the embryos have reached the cotyledonary stage.

Table 2: Number of embryogenic calluses formed from various starting explants after 5 and 10 weeks of culture on PIV and MS1 induction medium. Efficiency of somatic embryogenesis using stamens, pistils, and whole flowers of Ancellotta, Lambrusco Salamino, and 110 Richter.

Genotype	Culture media	Type of Explant	Number of explants	Number of new embryogenic calluses after		*Embryogenic efficiency
				5 weeks	10 weeks	
Ancellotta	MS1	Stamens	275	4	7	4%
		Pistils	55	-	1	1,81%
		Whole flowers	180	-	12	6,67%
	PIV	Stamens	125	1	2	2,4%
		Pistils	25	-	-	0%
		Whole flowers	180	1	13	7,78%
Lambrusco Salamino	MS1	Stamens	250	5	6	4,4%
		Pistils	50	1	-	2%
		Whole flowers	400	3	19	5,5%
	PIV	Stamens	250	4	3	2,8%

		Pistils	50	1	0	2%
		Whole flowers	200	6	8	7%
110 Richter	MS1	Stamens	225	-	4	1,78%
		Pistils	45	1	-	2,22%
		Whole flowers	180	2	5	3,89%
	PIV	Stamens	225	-	-	0%
		Pistils	45	1	-	2,22%
		Whole flowers	160	8	5	8,13%

*Embryogenic efficiency was reported as the number of embryogenic calluses arose during 10 weeks from the total number of explants (%)

3.2.2 Embryogenic efficiencies using *in vitro* nodal segments

One hundred *in vitro* growing stem nodal segments each for Thompson Seedless, Chardonnay, 110 Richter, and Kober 5BB were dissected from proliferating *in vitro* shoot cultures and used as starting explants to induce somatic embryogenesis (Fig. 5 a). 100% of callogenesis was reported for all the genotypes in both culture media, but the majority of the calluses were non-embryogenic. Chardonnay and 110 Richter nodal segments failed to produce somatic embryos, while only one explant for both Thompson Seedless and Kober 5BB originated embryogenic calluses or a somatic embryo, respectively (Fig. 5 b, c).

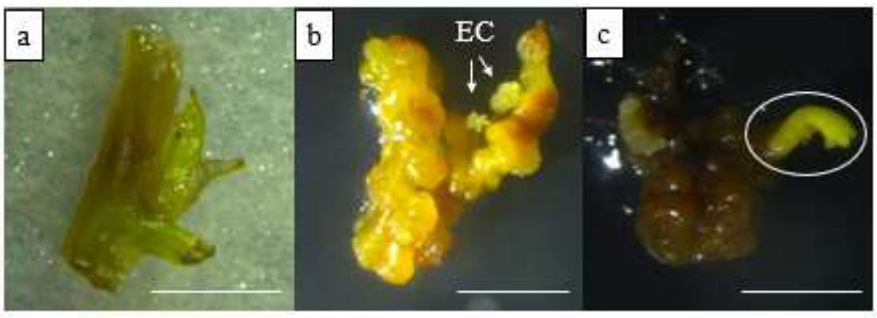


Figure 5: Stem nodal segments selected and dissected from *in vitro* growing plantlets (a); Thompson Seedless stem nodal segments after two months from culture initiation, embryogenic calluses (EC) were produced nearby the lateral bud (b). Kober 5BB stem nodal segments produced one single embryo (circled in white) through direct somatic embryogenesis after two months from culture initiation (c) (*bars* = 5 mm).

More in detail, one explant of Thompson Seedless started to produce two embryogenic calluses situated near the lateral bud after two months of culture on CIM2 (2% embryogenic efficiency). In the meantime, one explant of Kober 5BB induced direct somatic embryogenesis from nodal segments cultured on CIM1, through the formation of one somatic embryo. Embryogenic calluses of Thompson seedless were proliferated on X6 medium, stimulating the production of new somatic embryos, establishing an embryo line for this cultivar. The

single embryo of Kober 5BB was cultured on the same proliferation medium, but it started to elongate, inhibiting the possibility to regenerate new somatic embryos for this rootstock.

3.2.3 Induction of secondary embryogenesis

Embryogenic cultures were previously obtained from the cultivars Ancellotta, Lambrusco Salamino, Thompson Seedless starting from various type of explants. In turn, Cluster of SEs or type II SE at cotyledonary stage have been cultured on E96 medium to induce secondary embryogenesis, increasing the availability of embryos to be used in genetic transformation or to induce somaclonal variability as a source of new genetic variability. After four weeks of culture, all the explants developed different type of callus, including compact and yellow calluses (Fig. 6 a). After their transfer onto the medium A, non-embryogenic calluses stopped to grow and progressively turned brown. Clusters of secondary embryos appeared gradually on some calluses within an additional culture period of 1–4 months on the medium A, sub culturing explant every 4 weeks on fresh medium. Embryogenic structures, composed by several translucent embryos (Fig. 6 b) at different developmental stage (mainly at globular stages), were selected and cultured on X6 medium, on which they formed new embryogenic cultures (Fig. 6 c). Both type of explants has been successfully used, in all cultivars, for the establishment of new cultures of proliferating somatic embryos.

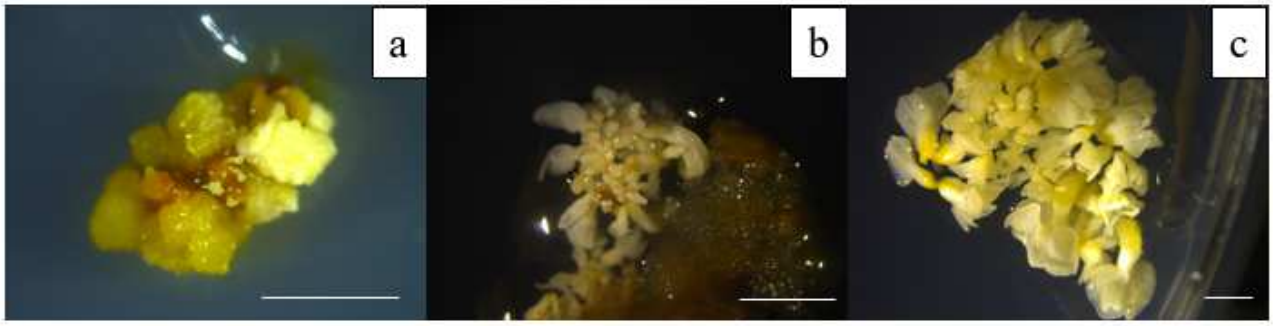


Figure 6: Initiation of secondary embryogenesis from embryo clusters: development of embryogenic calluses and different type of calluses on E96 medium after 1 month of culture (a), embryogenic callus growth and development of embryogenic aggregates starting from 1 months of culture on culture medium A (b), synchronous production of SE proliferated on PGR-free X6 medium (c) (*bars* = 2 mm).

3.3 De novo shoot organogenesis from cotyledons and hypocotyls of grapevine cultivar somatic embryos

After culture on NN media supplemented with BAP alone or in combination with IBA for 3 weeks, adventitious shoot regeneration occurred in all the cultivar tested on cotyledons less in the region close to the hypocotyl and most on their adaxial surface, especially along the central vein (Fig. 7 a, b, c). For the hypocotyls most adventitious shoot regeneration occurred from the distal end opposite to the radicles, mainly in correspondence of the wounded surface area of hypocotyls (Fig. 7 e, d, f).

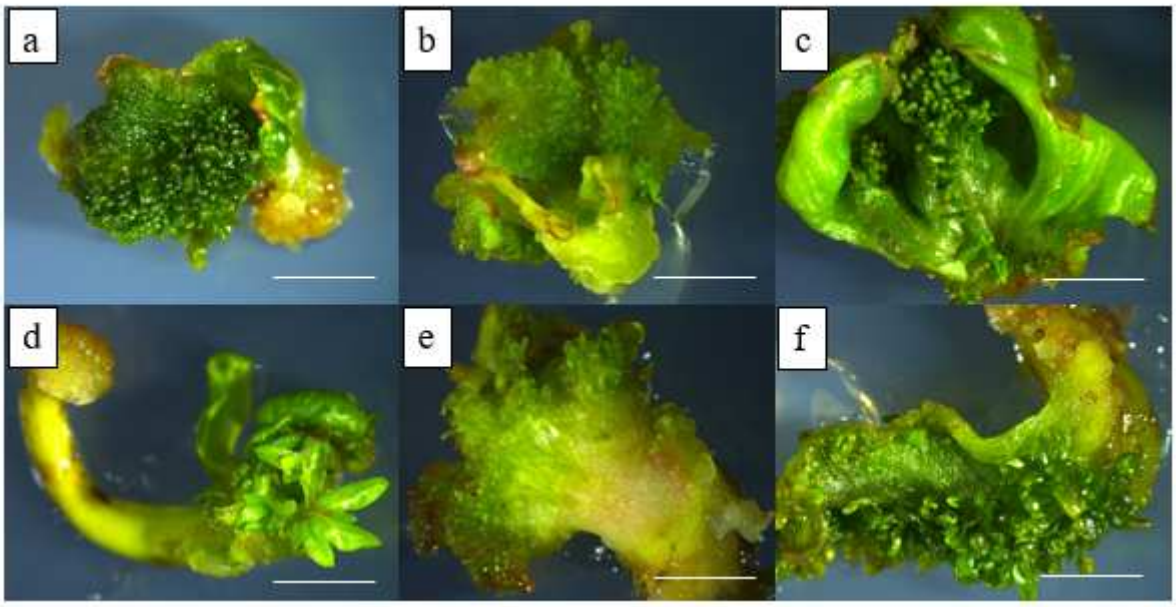


Figure 7: De novo shoot organogenesis from cotyledons (a, b, c) and hypocotyls (d, e, f) of Lambrusco Salamino (a, d), Thompson Seedless (b, e), and Ancellotta (c, f) after three weeks of culture. (*bars* = 5 mm).

The regeneration efficiencies in terms of explants that were able to produce at least one shoot have been recorded after 9 weeks of culture, sub culturing explant onto fresh media at 3 weeks intervals (Figure 8).
Among the different type of tissue derived from the somatic embryos, cotyledons were the most regenerative starting explants, reaching a value higher than 65% for each genotype cultured on both culture media (Figure 8). Comparing shoot regeneration (%) from cotyledons of the various genotypes cultured on the same culture medium, the most responsive were cotyledons of Thompson Seedless cultured on medium 1 (90%), and on medium 2, where Ancellotta cotyledons reached the highest regeneration rate (92%) (Figure 8). Comparing the cotyledons regeneration rates of the same genotype placed in diverse culture substrates we found significant differences in two of the three genotypes tested. In Thompson Seedless, culture medium 1 allowed to record an enhancement of 18% in regeneration compared to culture medium 2. In Ancellotta the best regeneration medium was the second, which differs from the first by 28 percentage points. In Lambrusco Salamino, culture medium 2 allowed a slight and negligible improvement in explants regeneration compared to culture medium 1.
On both substrates, Ancellotta and Thompson Seedless hypocotyls reached a regeneration response up to 30-40 %, while explants of Lambrusco Salamino showed a high incidence of necrosis, with a reduced regeneration response (20%). Comparing the regeneration rate reached by the same genotype on different substrates hypocotyls of the three cultivars showed the higher regeneration response only when were cultured on medium 1, although their percentage values of shoot regeneration were statistically similar.

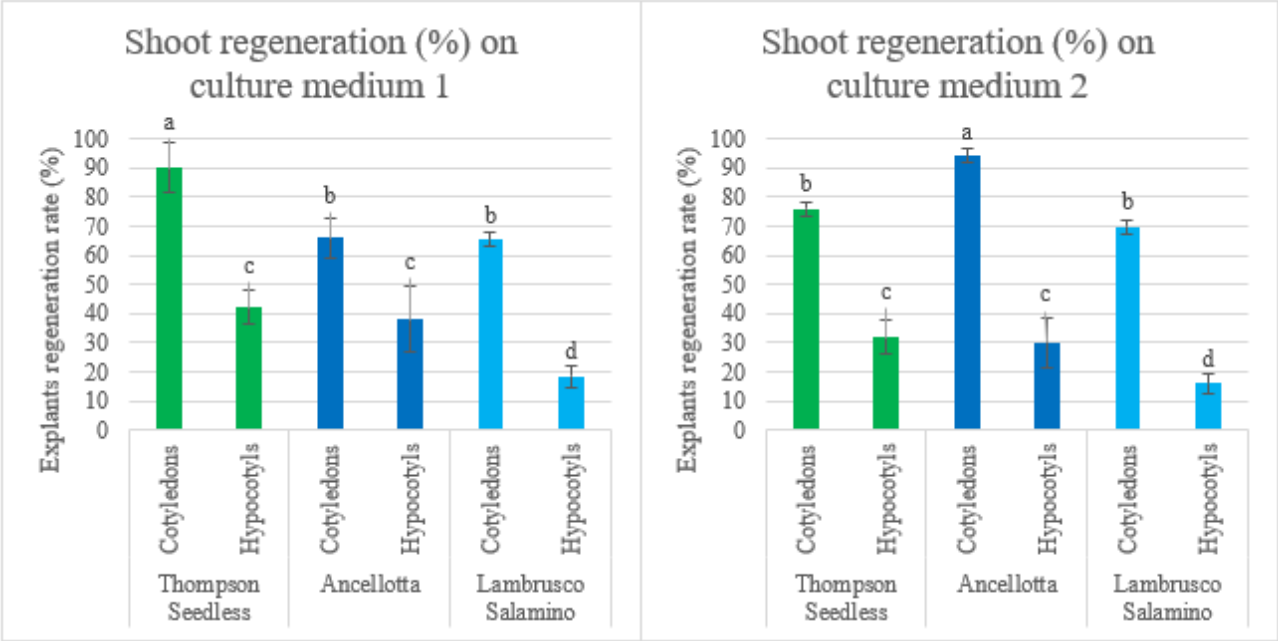


Figure 8: Regeneration rate (%) of Ancellotta, Lambrusco Salamino, Thompson Seedless cotyledons and hypocotyls cultured on regeneration medium 1 and 2, acquired after 9 weeks of culture. Means with different letters are significantly different according to the Student-Newman-Keuls ($p < 0.05$) ± SE ($n = 50$). Error bars represent the standard errors of three replications.

After nine weeks of culture, only proliferated explants were maintained in fresh regeneration medium containing a halved concentration of cytokinins compared to the initial substrate composition, keeping the two-substrate separated. At 12 weeks, cotyledons and hypocotyls were not more distinguishable. These resulting structures had a high regeneration capacity, characterized by a continuous shoot proliferation (Figure 9). Elongated shoots were isolated in MS medium without PGRs to stimulate root production, that occurred after two or three weeks.

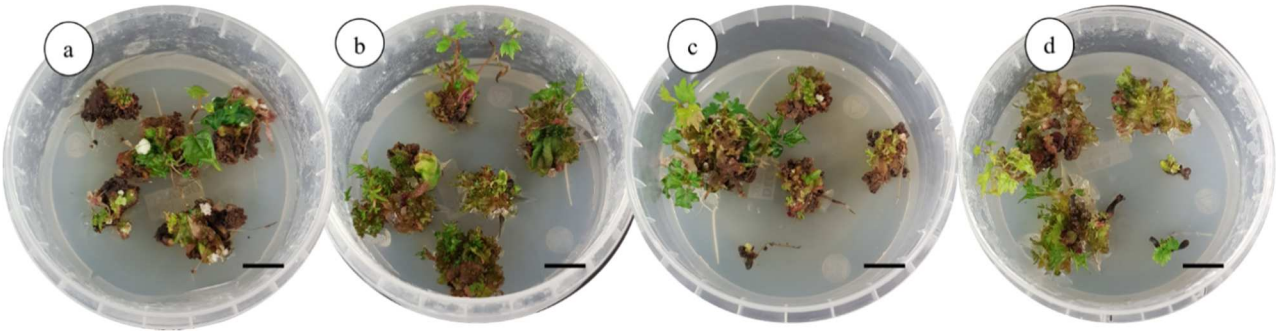


Figure 9: A representative image of Lambrusco Salamino shoot proliferating calluses derived from the culture of cotyledons (a, b), and hypocotyls (c, d), on culture medium 1 (a, c), and culture medium 2 (b, d) with halved cytokinin concentration, after twelve weeks of culture (*bar* = 1 cm).

In the following sub-cultures, elongated shoots which have been produced by regenerative structures, were isolated in MS PGRs-free medium and one month after were acclimatized, giving rise to plants with a typical appearance and conformation of the cultivar of reference (Figure 10).

Figure 10: A representative image of Lambrusco Salamino, Ancellotta and Thompson Seedless plantlets, after 1 months of acclimatization in greenhouse. These plants derived from the induction of shoot organogenesis from cotyledons and hypocotyls.

4. Discussion

4.1 Organogenesis from meristematic tissues in grapevine cultivars and rootstocks

Nowadays, most of the research on grapevine biotechnology programs is mainly focused on somatic embryogenesis (Prieto et al., 2019). Meristematic bulk production enable us to save time and hours of work compared to the time-consuming regeneration pathway of somatic embryogenesis (Xie et al., 2016). In addition, MBs regeneration system has the advantage of starting from micro propagated material, therefore it is independent from the seasonality to which is linked the availability of flowers and induction times provided for somatic embryogenesis. Among other assets, this technique is highly adaptable as it can be easily used in the propagation of different grapevine cultivars and rootstocks without requiring any modification in the methods employed. However, the response in MBs production and maintenance seems to be influenced by the genotypes, also into the same species, and usually require a different combination of PGRs. The regeneration protocol via organogenesis, developed as previously described, was applied for the first time to the local wine grape cultivar Ciliegiolo, and two rootstocks, 110 Richter and Kober 5BB, and was compared with the table grape cultivar Thompson Seedless. To our knowledge, there are no reports on the *in vitro* regeneration of explants from Ciliegiolo, and few studies exist on the *in vitro* regeneration by organogenesis of explants of the two rootstocks (Clog et al., 1990; Martinelli et al., 1996; Torregrosa and Bouquet, 1996; Bayir et al., 2007), for which somatic embryogenesis is the preferred regeneration system (Torregrosa et al., 2000; Ben Amar et al., 2007; Gambino et al., 2007). At 9 weeks, the average number of shoots regenerated per explants was constantly higher to 15 in all genotype tested, demonstrating that MS-based medium, combined with 13.2 µM of BAP and 0.01 µM of NAA was a suitable regeneration substrate for high shoot production for all genotypes.

We prove that MB regeneration system can be effectively used to induce efficient regeneration of various grapevine cultivars as Albana, Ancellotta, Chardonnay, Ciliegiolo, Glera, Lambrusco Salamino, Merlot, Pinot Grigio, Sangiovese, Thompson Seedless, Vermentino, and *Vitis* hybrids rootstocks including 110 Richter, 1103 Paulsen and Kober 5BB.

4.2 Primary and secondary somatic embryogenesis and embryo maintenance of grapevine cultivars and rootstocks

Somatic embryogenesis is a unique developmental process that practically expressed cellular totipotency, confirming that somatic cells contain all the genetic information to execute plant development, not necessarily requiring sexual fertilization (Correia et al., 2016). Proteomics studies associated with somatic embryogenesis have highlighted differences between embryogenic and non-embryogenic calluses, allowing to refine the culture conditions to increase the production of embryogenic tissues (Marsoni et al., 2008; Correia et al., 2016).

One of the main aims of this study was to determine the most suitable culture medium combination and floriferous explants type for the induction of somatic embryogenesis on Ancellotta, Lambrusco Salamino, and 110 Richter. Whole flowers, stamens, and pistils had similar efficiency in SE induction for almost all genotypes tested in the different culture media. However, whole flowers were the favorite explants, especially when were cultured on PIV culture medium. To our knowledge, Ancellotta and Lambrusco Salamino somatic embryogenesis induction has not been previously reported in scientific literature, therefore we developed the first efficient regeneration protocol via somatic embryogenesis for both these cultivars.

In *Vitis* spp. the immature anthers are the most widely used starting explants which led to the obtainment of high level of embryogenesis induction in other studies (Dhekney et al., 2009; Gambino et al., 2020). According to what was reported by other researchers, anthers seemed to be the most responsive starting explant compared to pistils (Dhekney et al., 2009; Acanda et al., 2014). However, the genotype play an important role to determine the best explant reactivity in SE induction (Malenica et al., 2020). Our results showed that whole flowers are recommendable explants for establishing grapevine embryogenic cultures, to be preferred to stamens and pistils for their ease of collection and excision, allowing to start the *in vitro* culture of a high number of explants in a relatively short time (Gambino et al., 2007).

Although the maintenance of grapevine cuttings is possible, the availability of these type of explants is generally limited. The physiological status, especially for anthers and ovaries had a great influence on somatic embryogenesis responses, and the selection of the right stage of their development requires an expert eye (Gribaudo et al., 2004; Dhekney et al., 2009). Studies regarding the use of alternative explants have been generally restricted to leaves (Martinelli et al., 1993, 2001b; Zhu et al., 1997; Das et al., 2002; Dhekney et al., 2009), tendrils (Salunkhe et al., 1997) and meristematic tissues (Maillot et al., 2006, 2016), for few genotypes. The use of nodal segments excised from *in vitro* grown plantlets opened the possibility to have potential explants available at each time of the year, ensuring long-term production of somatic embryos (Maillot et al., 2016). The induction process consisted of two phases distinguished by two different culture media (CIM and

Medium A). In our experiment, contrary to what was reported by Maillot and co-authors in 2016, we did not observe the production of any embryogenic calluses on Chardonnay, although an improved clone of the cosmopolitan cultivar Chardonnay has been used. However, Thompson Seedless and Kober 5BB stem sections regenerated somatic embryos via indirect and direct somatic embryogenesis, respectively, even if for Kober 5BB only one embryo has been produced, that did not proliferate. The regeneration events took place mainly nearby bud region area, emphasizing the results obtained by the aforementioned researchers. Although the present strategy has proven not to be an efficient regeneration system when compared to the use of flower as starting explant, the induction of somatic embryogenesis from nodal segments can potentially induce embryogenic calluses all year round, requiring only the availability of *in vitro* proliferating cultures. In addition to flower explants widely used to induce somatic embryogenesis, it has been shown that these meristematic tissues can induce an efficient regeneration of somatic embryos for the cultivar Thompson Seedless. We demonstrated that two possibilities are available for having somatic embryos ready for the application of the new breeding techniques: i) the new induction of embryogenic calluses starting from floriferous or nodal segments explants or ii) the somatic embryos proliferation through the induction of secondary somatic embryogenesis using cluster or single SEs.

4.3 De novo shoot organogenesis from cotyledons and hypocotyls of somatic embryos

Embryonic tissues are characterized by a great regeneration competence, which can be exploited to maintain morphogenic potential intact for several years (Martinelli et al., 2001a). In other woody fruit plants such as apple and pear, sections of cotyledons and/or hypocotyls derived from zygotic embryos were used for adventitious bud induction (Korban and Skirvin, 1985; Browning et al., 1987). In grapevine, only a few research publications highlighted the usefulness of this regeneration strategy applied on whole somatic embryos or their portions (Vilaplana and Mullins, 1989; Martinelli et al., 2001b). Our study determined that both cotyledons and hypocotyls were proper starting explants for stimulating the production of adventitious buds, in contrast with the findings from Vilaplana *et al*., 1989, whereby they did not observe shoot regeneration from buds formed on cotyledons. Mature somatic embryos of two Italian cultivars were obtained by the methods of Dhekney et al., 2009, while those of Thompson Seedless were induced from the culture of nodal segments previously described (Maillot et al., 2016). Two regeneration media have been selected according to the research work conducted on 1989 by Vilaplana and colleagues having different cytokinin/auxin ratio. The results obtained in the present study confirmed the high organogenetic competence of cotyledonary leaves also reported by Martinelli and co-authors in 2001, plus the valorization of hypocotyl explants that regenerated shoots to a variable percentage from 18 to 40% in all cultivars tested. This efficient regeneration system could be exploited for massive plant propagation and in genetic transformation trials (Martinelli et al., 2001b). As reported by Vilaplana *et al*., (1989), we observed the continuous induction of adventitious buds from the explants, that appeared as round green bumps developing the first plantlets after only 40 days from culture initiation (Fig. 11 a). Even the apical meristems of somatic embryos had undergone cell proliferation, leading to the evolution of vegetative structures (enriched in auxiliary well-formed buds), not always clearly distinguishable from those developed by the new adventitious buds formed (Fig. 11 b). *De novo* shoot

organogenesis frequently occurred in cotyledons nearby the midvein, and in hypocotyls in an area close to their cut distal ends.

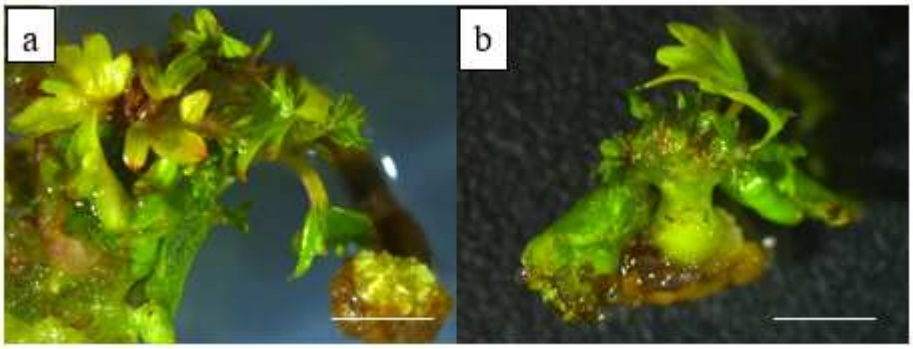


Figure 11: shoot regeneration from hypocotyls (a) and cotyledons (b) of Lambrusco Salamino after 40 days of culture on culture medium 1. *De novo* shoot organogenesis occurred in the distal ends, generally in the opposite to where the primary root was (a). Suspected proliferation of apical meristems which prevailed over the adventitious regeneration of buds was observed between the two cotyledonary leaves.

In the three *Vitis vinifera* cultivars tested, promising results have been obtained in terms of plantlets production, especially using cotyledons, suggesting that they have similar competence to regeneration.

At the time of data recording in this study, our assays showed that the couple of culture media considered were adequate for cotyledon related shoot organogenesis, although the most suitable was culture medium 1 and medium 2 for Thompson Seedless and Ancellotta, respectively.

Embryo dormancy and low plantlets conversion rate, negative aspects related with the culture of somatic embryos, could be bypassed applying this innovative strategy, increasing regeneration potential, and avoiding plant losses.

5. Conclusion

De novo shoot organogenesis and somatic embryogenesis are the two main plant regeneration ways to apply new breeding techniques (Limera et al., 2017). Regeneration process commonly start with cell division and dedifferentiation allowing the acquisition of organogenetic competence, thanks to the cell perception of exogenous phytohormones, and continued with organ initiation and development (López-Ruiz et al., 2019). Shoot regeneration are based on cell pluripotency, in which stem meristematic cells were stimulated to produce unipolar adventitious structures.

In this study we applied an efficient propagation and maintenance of MB, as well as the use of MB in the regeneration of several *Vitis vinifera* cultivars and three hybrid rootstocks. MB slices have been considered as our model regeneration unit, from which we promote shoot proliferation, enhancing the inherent regeneration potential in the presence of high content of cytokinins in the culture medium. Successful regeneration has been achieved for the following genotypes: Albana, Ancellotta, Chardonnay, Ciliegiolo, Glera, Lambrusco Salamino, Merlot, Pinot Grigio, Sangiovese, Thompson Seedless, Vermentino, and *Vitis* hybrids rootstocks including 110 Richter, 1103 Paulsen and Kober 5BB (Table 3). However, the regeneration of Glera, Pinot

Grigio and 1103 Paulsen require further adjustments of the protocols in order to reduce the incidence of tissue oxidation and necrosis.

On the other hand, somatic embryogenesis exploits the properties of totipotent cells, which have the maximum level of freedom, leading to the simultaneous regeneration of a complete adult organism (somatic embryo) (Verdeil et al., 2007). The regeneration procedures for the obtainment of actively proliferating somatic embryos starting from floral explants have been set up on Ancellotta, Lambrusco Salamino and 110 Richter (Table 3). Thompson Seedless somatic embryos have been obtained by the culture of stem segments of *in vitro* plantlets, extending the period for obtaining these types of explants to the whole year. Embryogenic cultures have been maintained for long period of time through the induction of secondary embryogenesis, starting from cluster of SE or single embryos at cotyledonary stage. Generation and continuous proliferation of somatic embryos of two Italian cultivars (Lambrusco Salamino and Ancellotta) have been achieved for the first time, and different floriferous explants sources were used for embryogenic callus induction.

In vitro culture is a fundamental prerequisite to obtain competent tissues that could be easily manoeuvred in genetic improvement programs by mutagenesis or genetic engineering (Torregrosa et al., 2001). In this study somatic embryogenesis and *de novo* shoot organogenesis have been also combined inducing adventitious bud formation from cotyledons and hypocotyls derived from somatic embryos, leading to precocious high shoot production (less than 60 days). A conspicuous shoot production has been observed following the culture of cotyledons and hypocotyls of Ancellotta, Lambrusco Salamino, and Thompson Seedless (Table 3).

All those techniques have been applied in dept to broaden the possibilities and avenues to increase regeneration efficiency from an initial explant. All the regeneration strategies described above have been efficiently implemented for Ancellotta, Lambrusco Salamino and Thompson Seedless (Table 3), which resulted more reactive to these *in vitro* morphogenesis processes in comparison with the other grape cultivars tested.

Genotypes	**Organogenesis from meristematic tissues**	**Somatic embryogenesis**		**Organogenesis from cotyledons and hypocotyls**
		From floral-derived explants	From stem nodal sections	
Cultivars (*Vitis vinifera*)				
Albana	↑↑↑			
Ancellotta	↑↑	↑↑↑		↑↑↑
Ciliegiolo	↑↑↑			
Chardonnay	↑↑		↓	
Glera	↔			
Lambrusco S.	↑↑↑	↑↑↑		↑↑↑
Merlot	↑↑↑			
Pinot grigio	↑			
Sangiovese	↑↑			
Thompson S.	↑↑↑		↑↑	↑↑↑
Vermentino	↑↑↑			
Rootstocks (*Vitis* hybrids)				
110 Richter	↑↑	↑↑↑	↓	
Kober 5 BB	↑↑		↑	
1103 Paulsen	↑			

Table 3: representative table in which a resume of the regeneration strategy adopted for each cultivar and rootstock has been made. The arrows indicate the level of efficiency in the applicability of each regenerative technique to individual

genotypes, under the peculiar conditions followed. Legend = ↑↑↑: high efficiency; ↑↑: high-intermediate efficiency; ↑: low-intermediate efficiency; ↔ low efficiency; ↓: no results

Despite of many researchers reported efficient plant regeneration protocols via organogenesis or via somatic embryogenesis, the regeneration ability, especially in terms of embryonic cell production, is strongly genotype dependent. Further studies are needed to clarify how morphogenesis is affected by different agents, such as type and physiological status of starting explant, the basal salt and PGRs composition in the culture medium, and the culture conditions.

3 - CHAPTER THREE: Somaclonal variation and induced mutagenesis in grapevine cultivars

1. Introduction

Biotic stresses represent serious agronomic problems throughout the crop cycle. Genetic variation is the basis of genetic improvement programs that mainly aim of extending it. This could be enlarged by exploiting numerous techniques, starting with classical genetic recombination until arriving at genetic manipulation to induced targeted variability. As the words suggest, recombination allows the creation of new genes combination of homologous DNA belonging to diverse parental, not acting on gene structures (Mutation Breeding: Theory and Practical Applications - A. M. van Harten - Google Libri). Till now, one of the most applied techniques allowing the increase of genetic variability aimed at improving important agronomic traits in staple and fruit crops is the application of commercial breeding (Sabbadini et al., 2021). Through the hybridization, performing selected crossing with wild species or accession that had an evolutionary history with a specific pathogenic causal agent, it is possible to obtain new individuals having traits that confer resistance to the related biotic stress, after an accurate selection of the progenies that may require several years, or even decades (Karanjalker and Begane, 2016). Molecular tools, as the exploitation of Marker-assisted selection (MAS), come to aid conventional breeders to considerably reduce the time required to complete a genetic improvement program (Sabbadini et al., 2021). In the course of evolution, several new species have emerged as a result of heritable changes that appeared in genetic pre-existing material throughout a process attributable to the terms of genetic mutations. Although natural spontaneous mutation occurs at very low frequencies, they represent the primary source of genetic variability, and their interaction with the environment has resulted in phenotypes that were selected by growers or first breeders since they had improved characteristics (Oladosu et al., 2016). This evolutionary process can be exploited in plant breeding programs due to the possible utility in functional gene studies and to provide new opportunities in the constitution of a new cultivar (Chaudhary et al., 2019). Mutation breeding arose thanks to the possibilities to induce mutation in plants, thanks to the administration of physical, chemical, or biological agents, constituting a middle way between modern plant breeding and new breeding techniques (NBTs) (Oladosu et al., 2016). This approach remains an integrative approach to the limitations that the new technologies could be considered as a primary path of precise mutation through genetic transformation and genome editing still have (Chaudhary et al., 2019; Sabbadini et al., 2021).

Markedly, traditional mutagenesis has the limitation of creating an uncontrollable variation, but it can be new and unexpected, and the efficiency of the result depends on the amount of material treated and the selection procedure adopted. Traditional randomly induced mutagenesis also has the important advantage that it is not based on the use of the DNA recombinant technology therefore not classified as a genetically modified organism in all international legislations, including EU legislation, regarding the creation of new plant genetic materials (Eriksson et al., 2019).

Although there are no substantial differences in the type of mutation induced by chemical and physical agents, the only chemical treatment allows breeders to achieve a high rate of alterations, without affecting the fertility

and vitality of mutagenized plants (Shu et al., 2012). Precisely for this reason, it is essential to evaluate well, through specific tests, the dose of mutagen that is optimal for obtaining the greatest number of useful mutations while keeping the number of those low mutations that are lethal or lead to plant infertility. Compared to the physical one, chemical mutagenesis not required complicated equipment and facilities, and is characterized by some other advantages such as the broader spectrum of active molecules available than ionizing and non-ionizing radiations; the possibility to obtain more specific mutation with the choice of a certain compounds, and the ability to generate mainly gene mutations (Mba, 2013). It is noteworthy that chemical agents contribute point mutations, potentially acting at the gene level, knock-down or varying correlated activity of the corresponding protein (Oladosu et al., 2016).

According to their mechanisms of action, three main classes of chemical mutagens are distinguished:

1. Base analogs: these compounds have a structure similar to that of purine or pyrimidine bases and which can therefore be incorporated into DNA during replication causing mismatches that give rise to replacements of bases. 5-bromouracil and 2-aminopurine belongs to this category.
2. Intercalating agents: these compounds can bind to the DNA double helix distorting it and causing the addition or loss of one or a few base pairs, i.e. insertions or deletions, involving one slippage of the reading module of triplets ("frameshift mutation"). Therefore, the intercalating agents, such as acridines or the notorious ethidium bromide cause more relevant effects compared to other chemical mutagens that commonly induce substitutions of bases.
3. Alkylating agents: these compounds could react with nucleic acids, causing modifications by nucleotides alkylation, with consequent substitutions or, more rarely, deletions of bases.

The most popular chemical agents used in mutation breeding are alkylating agents, which promote alkylation of nucleobases and phosphate groups, mainly causing transitions, transversions, deletions, insertions, inversions, DNA single- and double-strand breaks (Shu et al., 2012).

EMS-induced mutation has proved effective in almost 15 plant crops, using mainly seeds as a target plant material (Jankowicz-Cieslak and Till, 2016). In its most frequent event, the action of the monofunctional EMS alkylating agent takes place through the formation of alkylated guanine in the N-7 position. Alkylated guanine may react differently than the normal guanine, and in such a way that guanine can pair with thiamine, thus leading to base-pair errors (Kodym and Afza, 2003). In an aqueous solution, the hydrolyzation or degradation of half of the initial EMS active compound is reached after 93 hours at 20°C and after 26 hours at 30°C (Mba et al., 2010).

Multifunctional compounds belonging to inorganic azide groups are characterized by herbicidal, nematocidal, and fungicidal properties as well as being applied as mutagens; they are also employed for industrial purposes (i.e. as a source of nitrogen in the airbag). Sodium azide is a chemical mutagen that exerts its mutagenic potential itself or through the formation of intermediates such as hydrazoic acid (HN_3) (Kleinhofs et al., 1978) or azido-alanine (Rosichan et al., 1983) which have a negative influence on metabolic process such as cellular respiration and oxidative phosphorylation, indispensable for energy generation (Kleinhofs et al., 1978). Even though there is no evidence that this substance causes chromosome aberrations, may have an influence during

meiotic cellular activity, leading to chromosome translocation (Shin and Jeung, 2011). Sodium azide is generally more active while the replication of the genetic material takes place on a large amount in the treated organism (Pearson et al., 1975)

Olsen and co-authors reported in their research the growth of plants devoid of pro-anthocyanidin or anthocyanin as a consequence of high incidence in transition mutations (more frequently in G/C-A/T direction) rather than transversions in a barley sodium azide-mutagenized population (Olsen et al., 1993).

The efficiency of chemical mutagenesis is strongly dependent on the nature of the active compound selected, the concentration of the mutagen, temperature, pH, and length of the treatment (Mba et al., 2010; Oladosu et al., 2016). The optimal dose of mutagen is the one that induced lethality from 25 to 50% among the putative mutated plants after the treatment with the active compound in a defined time interval (Kour et al., 2017).

Especially for perennial fruit crops, seeds are not the best starting material, in which these species are mainly propagated agamically for retaining their genetic integrity (Predieri, 2001). Mutagenesis could be methodically combined with tissue culture, ensuring high phytosanitary conditions, and enlarging the possibility to identify the mutation in the correspondent phenotype of new regenerated plants (Predieri, 2001; Bednarek et al., 2007; Jain et al., 2010).

The combination of tissue culture and chemical mutagenesis allows to enhance the creation of genetic variation of selected plants, in particular for vegetatively propagated plants (Jain et al., 2010).

An event unavoidable resulting from the application of *in vitro* plant regeneration is the somaclonal variation, which in the perspective of genetic improvement could represent a further source of genetic variability. Côte and co-workers in 2001 coined the term somaclonal variation to identify genetic and phenotypic variations that arose from *in vitro* multiplication, which can be manifested as either somatically or meiotically stable events (Wang and Wang, 2012).

The genetic alteration mainly reflected in somaclonal variants are epigenetic changes and gene mutations, which may be stable and heritable (Krishna et al., 2016). Genetic somaclonal variation includes several modifications: from ploidy changes and chromosome rearrangements to specific genetic sequence changes (Wang and Wang, 2012). Therefore, these related new characteristics may be stably transmitted to the progeny. Epigenetic aspects as the regulation of gene expression they may or may not have a genetic origin with consequent evidence regarding the heritability of these variations (Kaeppler et al., 2000). Although different studies revealed the occurrence of somaclonal variability, the main causes are prevalently unknown, nonetheless variation could be induced with tissue culture or could be pre-existing in the tissues that undergo morphogenic processes (Bairu et al., 2011). On somaclonal variants, three classes of alterations have been generally reported: i) heritable changes consisting in the modification of the genome at the gene or chromosome level; ii) epigenetic changes as results of the development on an artificial culture environment, and iii) heritable but reversible changes due to the variation of gene expression pattern (Karp, 1995).

Plant tissue culture enhances the frequency of the appearance of mutations due to the presence of numerous stress factors and the addition of several chemical compounds, including PGRs (Dalla Costa et al., 2017).

In vitro regeneration of vegetatively propagated crops allows to test a large number of homogeneous individuals in limited space. Organogenesis and somatic embryogenesis morphogenic ways have the potential to give rise to somaclonal variants in a higher frequency compared to those that normally occur in a natural environment (Wang and Wang, 2012). The advent of a mutation mainly affects a single cell and the regeneration of a new organism from this cell leads to the expression of this mutation in the future individual (Suprasanna and Nakagawa, 2012). Taking into account the above it might be inferred that techniques such as somatic embryogenesis that induce regeneration from single cells are more effective in the production of mutated individuals than those in which the regenerative process takes place from a group of cells as in organogenesis (Subban et al., 2021). It has been demonstrated that the propagation technique that involves direct or indirect shoot regeneration has the risk of leading to the formation of chimeric plants with non-homogeneous characteristics (Datta and Chakrabarty, 2008). Phillips and co-authors in 1994 highlighted a greater mutagenic potential in those regenerative processes that involve the production of callus composed by undifferentiated cells (Phillips et al., 1994). Hence, indirect organogenesis and indirect somatic embryogenesis are probably the preferred regeneration pathways for the application of tissue culture somaclonal variant approach. Tissue culture response is generally genotype-dependent and somaclonal variation emerged to vary among different species mainly due to the genetic stability of the starting tissues. It has been postulated that chromosome instability and variation could happen in higher frequency in polyploids rather than haploids or diploids (Karp, 1995). The level of exposure of the "unprotected" genetic material to different type and concentrations of PGRs and other gaseous hormones (i.e. ethylene), the length of the entire morphogenic process which may include more than ten sub-cultures are just some of the many factors that can be associated with the onset of mutations (Sun et al., 2013; Vitamvas et al., 2019). Also, the chosen organ may have a role in the occurrence of somaclonal variation: the oldest or well-specialized tissues are able to generate regenerants having more various phenotypes rather than young and not-well specialized counterparts (Leva et al., 2012).
Information about somaclonal variation is rather scarce in both grapevine and other woody plants, and only a few examples have been reported, almost exclusively regarding the application of somatic embryogenesis. An attempt of chimeral breeding through the organogenesis conjunction of meristematic bulk tissues of two *Vitis vinifera* cultivars (Cabernet sauvignon and Babic) has been conducted, resulting in some level of tissue heterogeneity, and seven chimeric plants have been obtained after accurate phenotyping and genotyping analyses (Preiner et al., 2019). On *Vitis vinifera*, some studies have reported various type of somaclonal variants, thanks to the application of flow cytometry, or specific molecular techniques such as the analysis of DNA marker (as microsatellites), amplified fragment length polymorphism (AFLPs), and random amplification of polymorphic DNA (RAPD) (Prado et al., 2010; Dalla Costa et al., 2017). Although differences have been found in plants regenerated by somatic embryogenesis, to date no new grapevine lines have been isolated and commercialized using this technology (Dalla Costa et al., 2017). One of the most reported changes in grapevines is the ploidy variation (Acanda et al., 2013), such as the appearance of tetraploid plants regenerated from somatic embryos in higher frequency as the result of somatic embryogenesis related variability compared to colchicine treatment (Kuksova et al., 1997) or as a consequence of the solely colchicine

application on *Vitis vinifera* auxiliary buds (Notsuka et al., 2000). Nevertheless, the majority of the modifications are epigenetic like DNA methylation, and the relatively important markers for the recognition of the phenomenon were described through the analysis of different Pinot Noir clones (Ocaña et al., 2013).

With the aim to create a large genetic variability in grapevine (*Vitis vinifera*) cultivars large populations of plants were regenerated *in vitro* through shoot organogenesis and somatic embryogenesis. Meristematic bulk regeneration system (Mezzetti et al., 2002) has been applied for Ancellotta, Chardonnay, Lambrusco Salamino, Merlot, Pinot Grigio cultivars, whereas floriferous explants of Ancellotta and Lambrusco Salamino cultivars have been regenerated inducing the formation of somatic embryos using protocols described in chapter two (Dhekney et al., 2016). In the experiments with the meristematic bulks were also applied chemical mutagens (EMS and Sodium azide), at different concentrations and times of application. For both regeneration morphogenic ways (organogenesis and somatic embryogenesis) were isolated and developed a large population of new putative mutagenized plants. A preliminary selection for identifying mutants with increased tolerance/resistance to major grape diseases (powdery mildew and downy mildew) has been performed in the first year of plant development in greenhouse. In almost all the different populations, plants expressing reduced symptoms have been identified. All plants have been transferred to the field to be assessed at reduced severe disease infection in order to identify clones with stable increased tolerance/resistance to the diseases but maintaining the standard characteristics of the cultivar of origin.

2. Material and methods

2.1. Induced mutagenesis through the applications of chemical mutagens

2.1.1 Initiation and maintenance of meristematic bulk

Lateral buds of *Vitis vinifera* cv. Ancellotta, Chardonnay, Lambrusco Salamino, Merlot, and Pinot Grigio, were initially provided by Ampelos, the Italian viticulture nursery consortium, and then introduced to *in vitro* conditions and processed by Vitroplant Italia s.r.l. for the creation of the meristematic bulks (Mezzetti et al., 2002). As reported in chapter two, the constitution of the meristematic bulk (MB) resulted after consecutive subculture of vegetative structure deprived of their apex, in MS basal salt and vitamins, supplemented with 30 g L^{-1} of sucrose and 7 g L^{-1} of plant agar, increasing by one unit the BAP content in the culture medium at each transfer.

Meristematic bulk slices were used as starting material for the development of protocols for the application of *in vitro* chemical mutagenesis. Vegetal materials were sub-cultured monthly in low auxin to cytokinin ratio condition, on an MS basal medium (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} of sucrose, 7 g L^{-1} of plant agar, varying type, and phytohormone content depending on the genotype considered. Shoot organogenesis occurred in a growth chamber under a photoperiod of 16-h light (70 µmol m^{-2} s^{-1}) provided by white fluorescent tubes.

On Chardonnay, Merlot, and Pinot Grigio MBs proliferation occurred in the presence of 8.8 µM of N^6-benzylamino-purine (BAP) and 0.225 µM of thidiazuron (TDZ) on an MS medium having half major salt

concentrations (MS 1/2), while MBs of Ancellotta and Lambrusco Salamino were cultured in the same culture condition but on culture medium containing full strength MS with 8.8 µM of BAP and 1 µM of TDZ.

2.1.2 Preparation of EMS and sodium azide solutions

Preliminary studies regarding the toxicity of ethyl methanesulfonate (EMS, SIGMA ALDRICH) and sodium azide (NaN_3, SIGMA ALDRICH) on MB slices have been conducted to define the magnitude of the EMS or NaN_3-work solution concentrations and times of application. All the solutions were prepared fresh just before the application of chemical treatments, following two steps. In the first step, distilled autoclaved water has been poured on 50 mL Falcon tubes as the solvent of the solution. After the filtration using a 0,22 µm filter, Dimethyl sulfoxide (DMSO) 2% v/v was added to the solution as the carrier agents. The volume of the final solution was fixed at 40 mL. 10 % of EMS and 5% of sodium azide stock solutions have been prepared with autoclaved distilled water and sterilized by filtration, using specific personal protective equipment (PPE) (full-face gas mask in carbon filters, gloves, lab coat). The second step occurred a few minutes before explants treatment in which EMS or sodium azide has been added at the calculated volume in the working solution composed by water-DMSO mixture in Falcon tubes. The solutions have been mixed to obtain a homogeneous emulsion. In EMS application, working solution were concentrated 4.85 mM, 24.28 mM and 48.56 mM (0,05%, 0,25%, 0,5%), while 4.61 mM and 6.15 mM (0,03%, 0,04%) have been selected for sodium azide treatments. As negative control, the same explants were treated with the same solution without the addition of chemical mutagenic agents. Ancellotta and Lambrusco Salamino cultivars have been treated only using EMS (0,05%, 0,20%, 0,25%), while the remaining cultivars were subjected to both active agents.

2.1.3 Chemical mutagenic treatments, and isolation of putative mutagenized shoots.

MBs were processed in sterile conditions, preparing several slices (around 1 cm^2 and 2 mm thick) from each MB, using a scalpel blade, following by slices placement in Petri dishes filled with sterile distilled water, ensuring a good imbibition for at least 1 hour. After previous experimental observation, the length of treatment with mutagenic compounds has been fixed at three hours for EMS and four hours for sodium azide-based treatments. During the treatments, MB slices were continuously stirred in a rotary shaker (110 rpm) at the growth chamber conditions. Negative control without the chemical agents has been planned at the execution of each experimental trial. A total of 320, 420, and 200 MB slices of Chardonnay, Merlot, and Pinot Grigio respectively underwent mutagenic treatment using both active agents, whereas 330 MB slices of Ancellotta and Lambrusco Salamino each were subjected to increasing concentrations of EMS, as reported in the previous subchapter.

After the treatment, MB slices were washed three times in sterile distilled water in single Falcon tubes (50 mL), vigorously mixing the washing solution at each step. The working solution has been disposed of according to specific rules for special waste management. MB slices were collected from Falcon tubes using forceps and placed in plastic microboxes (micropoli Italia) containing the regeneration medium having the same composition of proliferation medium from which they derive. The entire regeneration process (from an

ex vivo plant to a new putative mutagenized individual) may require less than one year. In order to evaluate the regeneration potential of MB slices, after 28 days with the different treatments, the putative regenerated shoots were visualized under sterile conditions, carefully counting the number of well-formed shoots (at least 1 cm high), and then calculating the percentage of the explants that produce at least one shoot. Subsequently, MBs slices were transferred in a fresh regeneration medium, reducing by half cytokinins concentration following a scheme reported below (Fig. 1).

Figure 3: Regeneration process after chemical mutagenesis treatment. At each subculture, explants were transferred on culture media with reduced concentration of cytokinins, until elongated and rooted putative mutagenized shoots in the presence of a small amount of BA were acclimatized in the greenhouse.

After two sub-cultures, well-developed shoots which extended their internodes were selected and transferred in a medium containing a low amount of BAP. Elongated shoots were acclimatized and elongated by Vitroplant Italia S.r.l. Plants were grown in the glasshouse until they reached the right stage for transplanting in a larger greenhouse, where agronomical and resistance characteristics concerning the main grapevine pathogens have been evaluated. These putative mutagenized plants of Ancellotta and Lambrusco Salamino are currently *in vitro* waiting the adequate environmental condition for the acclimatization phase.

2.2. Somatic embryogenesis associated to somaclonal variation

2.2.1 Plant material

Adult field-grown plants of *V. vinifera* L. cv. Ancellotta and Lambrusco Salamino were selected from the grapevine field collection of mother plants of the phytosanitary centre of Reggio Emilia and dormant vine shoots have been collected during January 2019 and stored at 4°C for at least one month. Vine branches were surfaced sterilized and manipulated in pieces having two-four buds and were placed in a pot filled with tap water in the growth chamber to stimulate shoot and inflorescences development. Inflorescences at stage H of the Baggiolini (1952) phenological scale, corresponding to separated clusters, were dissected viewing the microspores stage using an MZ10F fluorescence stereomicroscope (Leica Microsystems GmbH, Wetzlar,

Germany). The collected flower clusters were washed with 70% of ethanol for 1 minute and then sterilized as described by Dhekney et al., 2009. After the washing step, the dissection of whole flowers, immature stamens (anther plus filament), and ovaries took place for culture initiation.

2.2.2 induction of somatic embryogenesis.

For the initiation of the embryogenic cultures, basal medium containing Nitsch and Nitsch (Nitsch and Nitsch, 1969) salts and Murashige and Skoog (Murashige and Skoog, 1962) were employed with 60 g L^{-1} and 20 g L^{-1} of sucrose respectively. Two media were tested: PIV based on NN basal salt with B5 vitamins supplemented with 9 µM of BAP, and 5 µM of 2,4-D; and MS1 containing Murashige and Skoog basal salts and vitamins plus 4.5 µM of BAP, and 5 µM of 2,4-D. The pH of each medium was adjusted to 5.8 prior to autoclaving at 98 kPa and 121°C, and the media were solidified using 7 g L^{-1} of Plant Agar (Duchefa Biochemie, Haarlem, Netherlands). Twenty-five stamens and five ovaries, or twenty-five whole flowers were placed on 90-mm-diameter polystyrene Petri plates containing 25 mL of medium. The cultures were maintained at 24±1°C in continuous darkness and sub-cultured onto fresh medium after five weeks.

2.2.3 Embryo differentiation and conversion into plantlets

Embryogenic calluses at the percentages reported in chapter two were selected and isolated onto X6 medium, for development and embryo differentiation, while the remaining explants were cultured on the same fresh induction medium. Well-developed somatic embryos, with their hypocotyls, cotyledons, and an apical root axis (Fig. 2 a), were collected from X6 medium and cultured for four weeks on a medium consisting of MS-based differentiation medium supplemented with 30 g L^{-1} sucrose and 1.1 µM of BAP in glass pots.

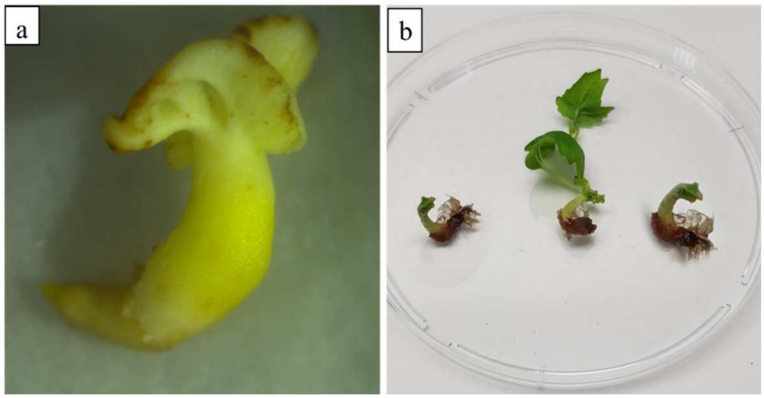


Figure 2: well-developed somatic embryos isolated from X6 somatic embryos proliferation medium (a). Somatic embryos germination and rooting in an MS PGR free medium after 4 weeks of culture (b).

Plant conversion occurred through embryo germination and root development on the same medium (Fig. 2 b). Glass pots were taken over by Vitroplant company that made the work of plant acclimatization and development on the greenhouse.

2.3 Greenhouse evaluation of mutagenized/somaclonal varied populations

2.3.1 Plant development and cultivation

Putative mutagenized populations of Chardonnay, Merlot, and Pinot Grigio after the acclimatization step which occurred in the equipped greenhouse of Vitroplant, have been carried out in April to Dalmonte nursery (Brisighella, Ravenna, Emilia Romagna), where have been transplanted in 18 cm diameter plastic pots. As the growing season progresses the main shoots have been developed at greenhouse cultivation conditions in an upright position thanks to the use of supports of reeds. After the active growth phase about six months later, the plants had a main well-developed shoot about 150 cm high, with expanded leaves. Ancellotta and Lambrusco Salamino somatic-embryo derived plants after acclimatization were transferred in a greenhouse of the Phytosanitary Consortium of Reggio Emilia. These plants, that were transplanted in larger pots (18 cm diameter) showed higher vigor, probably due to a juvenile effect induced by somatic embryogenesis regeneration process.

2.3.2 In vivo screening for tolerance to powdery mildew on the mutagenized population

Chardonnay, Merlot, Pinot Grigio grapevine plants were followed during the active growing season in a dedicated space in the greenhouse at Dal Monte nursery located in Faenza. These plants were intentionally non-treated with pesticides in order to observe any different changes also regarding pathogen tolerance. In the autumn season, a severe infection of powdery mildew, caused by *Erysiphe necator*, homogeneously affected the plant population. The response of the plant populations, of the different cultivars, to this natural infection was evaluated by assigning a disease severity index to each plant. For powdery mildew six classes have been identified, based on plant disease symptoms distribution (0 = 0% infected leaf surface; 1 = 0,1-5%; 2 = 5,1-15%; 3 = 15,1-40 %; 4 = 40,1-70%; 5 > 70%). The powdery mildew severity value (%) at the first observation was calculated using the following formula: Disease severity (%) = ((Σ (the number of diseased plants × disease severity index)) / (maximum class value (5) × the number of plants rated)) × 100 (Choi et al., 2007). Unfortunately, these plants at that controlled conditions, were not infected by downy mildew so it was not possible to perform an early screening also for this disease.

2.3.3 In vivo screening for tolerance to downy mildew on somaclonal varied population

Ancellotta and Lambrusco Salamino grapevine acclimatized somatic embryos-derived plants were placed in a dedicated greenhouse belonged to provincial phytosanitary consortium of Modena and Reggio Emilia and monitored during the active growing season. These plants were intentionally non-treated in order to observe any different changes also regarding pathogen tolerance. In early September, plants were moved outside the greenhouse and as the first test, all the plants were inoculated with *Plasmopara viticola* through low spray treatment of a solution containing the sporangia of the pathogen. The oomycetes propagules were collected from infected leaves selected in a vineyard located in the province of Reggio Emilia. Exploiting the artificial inoculum in the open greenhouse we evaluated the presumed mutated population, by the disease severity evaluation, that allowed us to associate at each plant a disease severity class index. Disease development was

examined at 7 days post-inoculation (dpi), by calculating the disease severity index shown by each plant. Relying on the percentage ratio among infected leaf area and total leaf area, each plant was rated in a class, following this class division: 1 = 0,1-5%; 2 = 5,1-15%; 3 = 15,1-40 %; 4 = 30,1-70%; 5 > 70%

The downy mildew severity value (%) at the first observation was calculated using the following formula: Disease severity (%) = ((Σ (the number of diseased plants × disease severity index)) / (maximum class value (5) × the number of plants rated)) × 100 (Choi et al., 2007).

2.4 Data analysis

The definition of the optimal dose of mutagen was possible through the estimation of the lethal dose 50, defined as the concentration that reduces the number of regenerated shoots by 50%. A total of 30 MB slices were considered for each cultivar and each treatment; the experiment was replicated three times. The mean number of green shoots regenerated from each explant and the percentage of regenerating MB slices [(number of MB slices regenerating shoots/total MB slices treated) × 100], which has undergone a specific mutagenic treatment in the current culture condition has been recorded at 28 days after treatment application for all the cultivars.

In the somaclonal variation experiment, the number of Ancellotta and Lambrusco Salamino plants originating from the induction, development, and conversion of somatic embryos into plants was recorded counting the final number of plants in pots cultivated in the greenhouse, after the acclimatization step.

Towards the behaviour of plants in response to powdery mildew that occurred naturally in the greenhouse and separately to the tolerance against artificial inoculated downy mildew, the disease severity index was assigned at each plants of the presumed-varied populations of the different cultivars (Ancellotta, Chardonnay, Lambrusco Salamino, Merlot, Pinot Grigio). The percentage of plants that expressed a certain class value compared to the total plant population of a cultivar was detected. The average of disease severity index and the total disease severity (%) associated with each variety were also reported.

Data for each variable were analysed by using analysis of variance to detect any significant differences. Post hoc comparison (Student-Newman-Keuls) was used to compare the level of resistance of each variety. Principal components analysis was performed for the results of all variables. All calculations were performed by using the Statistica 7 software (Statsoft Tulsa, CA, USA).

3. Results

3.1 induced mutagenesis through the applications of chemical mutagens

3.1.1 Regeneration capacity of MBs after the application of chemical mutagens

The high regeneration potential of MB slices had allowed the emergence of shoots, putatively mutagenized, arose from different points of the meristematic slice explants. Each chemical treatment induced a strong browning and necrosis of the slices tissues in the first two weeks of subculture on regeneration medium, followed by the appearance of new green areas, that started to regenerate shoots thanks to the cell multiplication stimulation induced by the phytohormones added to the medium. The exposure time and

mutagenic compound concentration of treatment that allows 50 % reduction of shoots regenerated in 28 days (LD 50), in comparison with control treatment (without active agents), has been considered appropriate.

EMS treatments on Ancellotta and Lambrusco Salamino

For each genotype and each treatment condition 90 meristematic MBs, divided into three biological replicates, have been taking into consideration for the evaluation of regeneration potential. After the detection of explant regeneration rate (explants that produce shoots/total number of explant) and the average number of shoots that regenerated 28 days after the mutagenic treatment, on Ancellotta and Lambrusco Salamino it was observed a sustained decline trend in shoot production as the concentration used increases (Figure 3). The regeneration response that followed the application of the alkylating agent was statistically equal in the cultivar tested. Considering the percentage of explants that regenerate at least one shoot, only on MBs of Lambrusco Salamino treated with the highest concentration of EMS we observed a significant reduction, compared to the control.

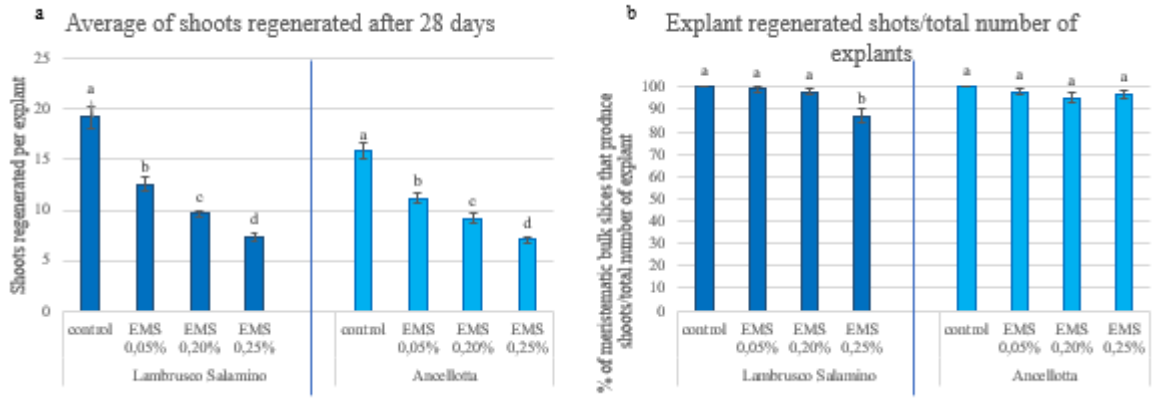


Figure 3: Regenerative responses 28 days after the application of EMS 0,05%, 0,20%, 0,25% for 3 hours on Ancellotta and Lambrusco Salamino MB slices. The average of shoots regenerated by each explant (a) and the explant that regenerate at least one shoots by the total number of treated explants were reported (b). Small letters represent differences in regeneration between the different treatments within a single cultivar. Means with different letters are significantly different according to the Newman-Keuls test ($p < 0.05$) ± SE.

The calculation of the lethal dose of EMS (Table 1) showed a reduction of shoot production variable from about 30% at the lowest concentration of EMS to around 60% at the highest EMS concentration. Each EMS employed concentration had a detrimental effect on shoot production in both Italian cultivars.

Table 1: EMS dose associated with the reduction of shoot regenerated on average by MB slices, compared to the non-treated control.

Genotype	EMS 0,05%	EMS 0,20%	EMS 0,25%
Ancellotta	34,41	50	61,77
Lambrusco Salamino	29,81	42,38	55,64

During the propagation and the following selection of putative mutagenized Ancellotta and Lambrusco Salamino lines, we observed some differences at the phenotype level that could be an indication of the modification of the clone. Strong vitrification of the plant material and color chimeric breaks in the leaves probably caused by chlorophyll deficiency (Figure 4) are among the phenotypic variations found in the mutagenized material compared to the untreated control.

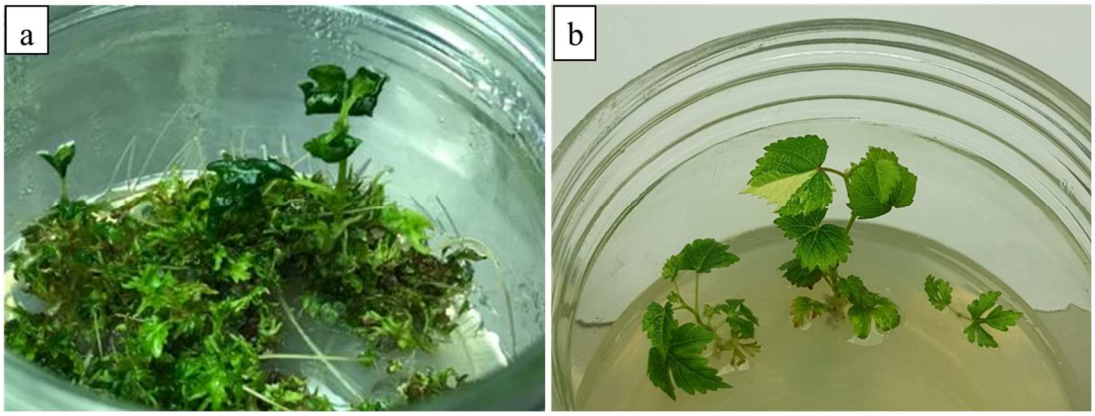


Figure 4: severe vitrification of vegetal material was found during the proliferation of Ancellotta MB slices (a); leaves color breaks were reported in a Lambrusco Salamino plantlet at the last *in vitro* subculture (b).

EMS and sodium azide treatments on Chardonnay, Merlot, Pinot Grigio

For each genotype and each treatment condition, 90 meristematic bulk slices have been taking in consideration (divided in three biological replicates) for the evaluation of regeneration potential, expressed as average number of shoots regenerated after 28 days in regeneration medium and the percentage of regenerating MB slices.

At normal culture conditions after the "no treatment" (negative control), MB slices of all the three cultivars had a range of regeneration that varied between 15 shoots/explant for Merlot and 19 shoots/explant for Chardonnay. Both EMS and sodium azide had a strong effect in reducing shoot regenerations, with an increasing trend at increasing concentrations of mutagenic compound (Fig. 5). MB slices of Chardonnay cultivar showed a strong susceptibility to chemical mutagens, also at the lowest concentrations, which makes their use interesting for the application of this technique. EMS resulted particularly effective in reducing shoot regeneration, especially in dose equal or higher than 0,25%. In Merlot cultivar, the application of 0,05% of EMS did not have an effect in terms of reduction in shoot regeneration, contrary to what happened when MB slices were treated with a higher concentration of EMS. EMS and sodium azide at all concentrations were strong efficient in the contraction of regeneration when were applied on Pinot Grigio MB slices. In this case, it was observed a more energetic activity showed by sodium azide then EMS-based treatment at comparable concentrations. Concerning the regenerating explants, EMS at the highest concentration strongly affected the number of explant able to produce at least one shoot in almost all the cultivars. On Chardonnay and Merlot this detrimental effect it is also maintained at concentrations of 0,25%, while in Pinot Grigio did not produce

any significant effect compared to the untreated control. Sodium azide at the highest concentration inhibited shoot production in MB slices of Chardonnay and Merlot, while in Pinot Grigio this effect was not relevant.

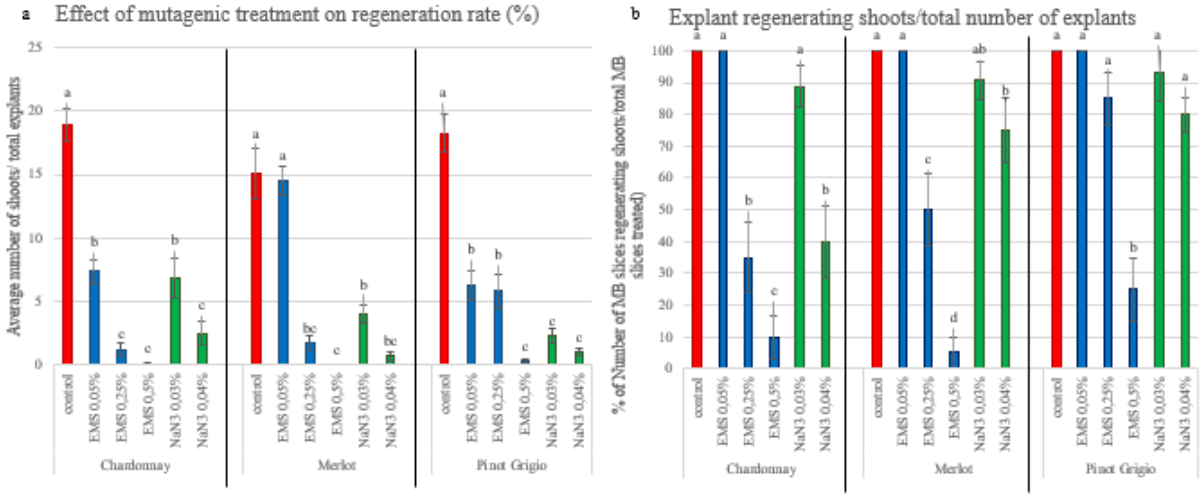


Figure 5: Chardonnay, Merlot, Pinot Grigio MB slices regenerative responses 28 days after the application of EMS 0,05%, 0,25%, 0,50% for 3 hours or 0,03%, 0,04% of sodium azide for 4 hours. The average of shoots regenerated by each explants (a) and the explant that regenerate at least one shoots by the total number of treated explants were reported (b). Small letters represent differences in regeneration between the different treatments within a single cultivar. Means with different letters are significantly different according to the Newman-Keuls test ($p < 0.05$) ± SE.

The average number of shoots regenerated by each explant dataset was used to quantify the degree of regeneration reduction caused by each treatment at a specific concentration (Table 2).

Table 2: Percentage value of shoot regeneration reduction in comparison with control treatment observed after the application of specific concentration of EMS and sodium azide on Chardonnay, Merlot, and Pinot Grigio.

Genotype	EMS 0,05%	EMS 0,25%	EMS 0,5%	NaN3 0,03%	NaN3 0,04%
Chardonnay	61,21	93,67	99,21	63,85	86,81
Merlot	3,97	88,41	99,67	73,18	95,36
Pinot Grigio	68,22	65,48	98,36	87,40	94,52

One month after mutagenic treatment, a strong reduction in new shoots emergence has been recorded for all the active agents and the concentration employed, with the exclusion of the lowest concentration of EMS when was applied in Merlot, that results mainly ineffective. At the concentrations used in the trial, EMS treatment exerted a heavy and durable effects, also demonstrated by the high incidence of necrosis in MBs. All these data suggest that concentration and type of mutagens used during this experimentation may increase the probability to have new individuals having diverse genome identity, due to the appearance of point mutations. EMS 0,05%, EMS 0,25%, $NaN_3$0,03%, and $NaN_3$0,04%, were employed frequently in extra mutagenic treatments as a compromise between mutagenicity power and regeneration reduction, excluding procedures that affect new shoots emergence too weakly or too strongly. After regeneration and proliferation, elongated

shoots were isolated in a PGR-free MS medium, and subsequently, they were acclimatized in the glasshouse by Vitroplant.

3.2 Inducing somaclonal variation by somatic embryogenesis

3.2.1 Embryo differentiation and conversion into plantlets

A highly efficient protocol for the somatic embryogenesis of grapevine cv. Ancellotta and Lambrusco Salamino was successfully applied following the method described by Dhekney and colleagues in 2009 (Dhekney et al., 2009), based on the use of BAP as the cytokinin in combination with 2,4-D, using MS and NN basal medium. The embryogenic response occurred mainly in whole flower explants, at the base of the stamen filaments and in ovaries, although the culture of whole flowers saves time (Gambino et al., 2007). Embryogenic calluses were sub-cultured in somatic embryos development medium (X6), showing each stage of somatic embryo differentiation process (globular, heart, torpedo, cotyledonary) (Fig. 6) (Sharma et al., 2004).

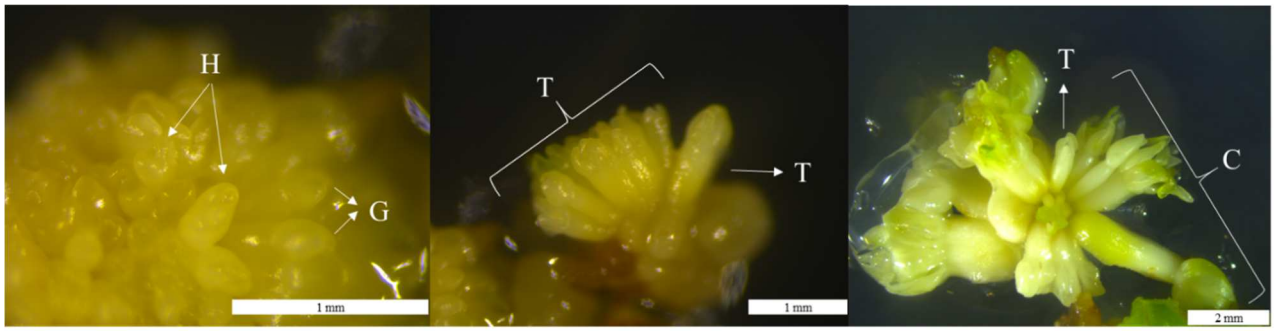


Figure 6: Lambrusco Salamino somatic embryos in subsequential differentiation stages. G: globular; H: heart; T: torpedo; C: cotyledonary.

Grapevine somatic embryos germinated and converted to plants normally at intermediate rates (data not shown) without adding growth regulators in the medium. Over a year and a half, a total of 2499 plants were originated from somatic embryos conversion into plantlets, of which 1132 of Ancellotta and 1167 of Lambrusco Salamino. During the growing season plants were transplanted in single pots (Fig. 9 a) and kept under the greenhouse environment (Fig. 9 b) where they expressed their vegetative growth, until the vegetative rest occurred around the month of November.

3.3 Greenhouse evaluation of mutagenized populations

3.3.1 Screening of chemical mutagenized populations for tolerance to powdery mildew

A total of 1418 plants were grown in the greenhouse, distinguished as follow: 619 of Chardonnay, 517 of Merlot, and 282 of Pinot Grigio. The plants have been intentionally not treated with pesticide applications and an intense powdery mildew infection occurred. This natural infection was used to conduct the first screening to detect plants tolerance/resistance to powdery mildew. Relying on powdery mildew symptoms distribution,

each plant was classified for the diffusion of the symptoms induced by the natural infection, so to identify at this preliminary screening some potential variants with increased tolerance/resistance to the disease.

As a result of this first early screening, Pinot Grigio, Chardonnay, and Merlot populations showed a variation in plant susceptibility to *Erysiphe necator* (Fig. 7). The plants were distributed following a bell-shaped curve, and significant differences were recorded between the genotypes towards disease tolerance.

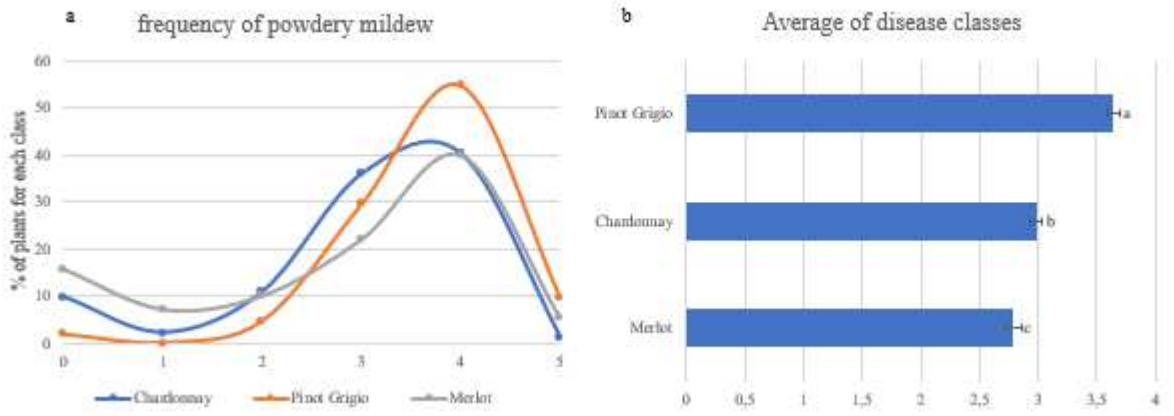


Figure 7: Powdery mildew severity index collected during the first survey, on natural inoculated Chardonnay, Merlot, Pinot Grigio plants derived from the mutagenic treatments of meristematic bulk slices. In all the cultivars, plants distribution among the six classes (a) and average of disease severity index (b) were reported. Means with different letters are statistically diverse according to Newman-Keuls test ($p < 0.05$) ± SE.

The three populations showed a high frequency of plants having a well diffused infection, this confirming the efficacy of the natural infection and the normal tolerance known for these grapevine cultivars. However, each population also showed a group of variants showing low and very low level of infection (some even 0). Notably, Pinot Grigio was the most susceptible cultivars compared to Merlot, in which the highest number of symptomless plants were identified. Considering the other cultivars, Chardonnay population showed an intermediate level of susceptibility, distinguishable from the others. In the overall, populations of the three cultivars differed for an average of disease severity, corresponding to 55,78%, 59,67%, and 72,77% for Merlot, Chardonnay, and Pinot Grigio, respectively.

Over the total numbers of 287, 517, and 609 plants of Pinot Grigio, Merlot, and Chardonnay populations respectively, a total of 5, 81, and 59 plants of Pinot Grigio, Merlot, and Chardonnay, respectively, were identified, from random locations in the greenhouse, without showing any disease symptoms (Fig. 8 a). In addition to the variation of the level of disease symptoms (Fig. 8 b, c, d, e), in Merlot population plants showing a phenotype with strong red color in leaves and petioles have been also identified (Figure 8 f-g). This result can be considered of interest to confirm the potential of traditional mutagenesis in inducing variation that can be promising for creating a wider genetic variability in grapevine cultivars. Clearly, this is an early screening and all data observed needs to be confirmed in further assessments in subsequent filed cultivation cycles.

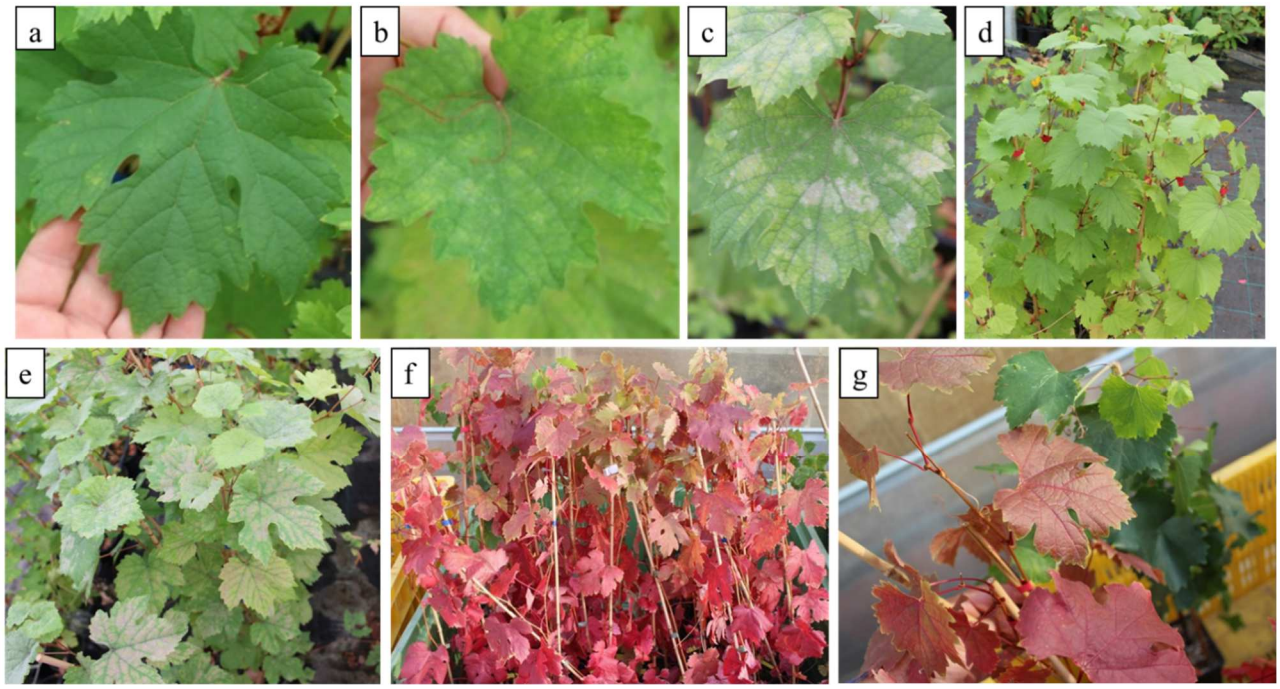


Figure 8: Putative mutagenized plants on greenhouse naturally infected with powdery mildew: healthy leaves (a), low powdery mildew leaf infection (b), severe powdery mildew leaf infection (c). Symptomless plants (d) were marked and isolated from the other infected plants (e). Phenotype completely red were detected in some Merlot plants (f-g); putatively modified merlot plant with completely red leaves and petioles placed next to a plant with a green phenotype (g).

3.3.2 Screening of somatic embryo-derived population for tolerance to downy mildew

Early in the season, a fungicidal application was necessary in order to not compromise the initial stages of growth. In September, plants have been trimmed to reduce vegetative vigor. A few weeks later the plants were placed outside the greenhouse (Fig. 9 c) and 14 days later were inoculated with *Plasmopara viticola.*

Figure 9: Ancellotta and Lambrusco Salamino plants were acclimatized in the greenhouse (a) transplanted in single pots and monitored during the spring (b). Plants were moved outside the greenhouse where were challenged with *Plasmopara viticola* (c). Downy mildew symptoms were evenly distributed throughout the plants (d).

To assess the variability of the two populations in response to *Plasmopara viticola* infection, 7 days after artificial inoculation (Fig. 9 d), each plant was assessed for the extension of the diseases. Each plant was evaluated through the classification in five disease classes (from 1 to 5), relying on plant disease severity index. According to the results of the first survey, plants were distributed following a bell-shaped curve (Fig. 10).

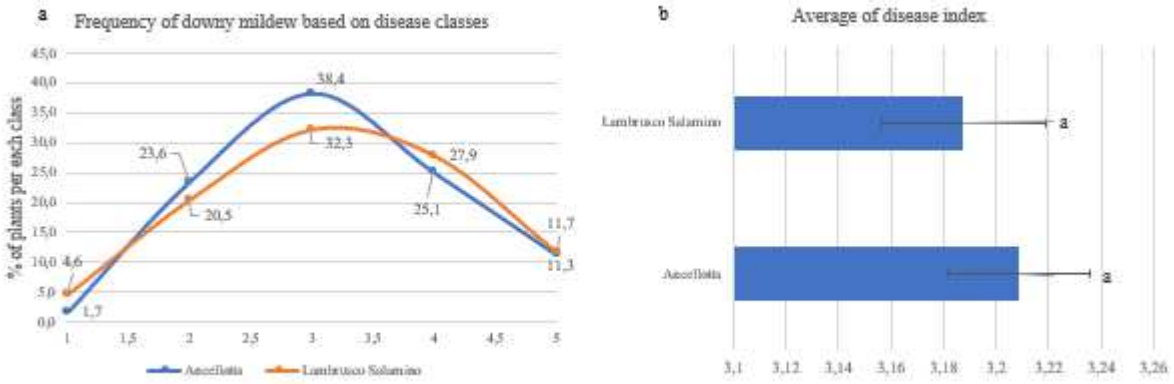


Figure 10: downy mildew severity index collected during the first survey, on artificial-inoculated Ancellotta and Lambrusco Salamino plants derived from somatic embryos. In both cultivars, plants distribution among the five classes (a) and average of disease severity index (b) were reported. Means with identical letters are statistically equal according to Newman-Keuls test ($p < 0.05$) ± SE.

In Lambrusco Salamino 4,6% of plants showed less or no disease symptoms, while the same behaviour was reported on 1,7% Ancellotta plants, corresponding to 54 and 22 plants respectively. Although the disease severity index curves were slightly different between the two cultivars, we did not observe substantial differences in the average value of downy mildew severity index (Fig. 10 b). During this observation, Ancellotta plants resulted on average more susceptible to downy mildew compared to Lambrusco Salamino ones. In Ancellotta the downy mildew severity value was 64,17% overall, while for Lambrusco Salamino was 63,75%. Here, the results of a preliminary survey have been reported. However, additional tests are needed to confirm the tolerance against downy mildew while observing any phenotypical variations.

4. Discussion

4.1 induced mutagenesis through the applications of chemical mutagens

The combination of tissue culture and chemical mutagenesis allow to increase genetic variability in grapevine cultivars, this combining the exploitation of existing cell variability of somatic tissue and the new genetic variability induced by chemical mutagens. The use of somatic embryogenesis as cell differentiation process is of high interest for this type of application reducing the risk to obtain chimeric plants (Kour et al., 2017). Despite the beneficial potential related to the consolidation of these techniques, only rare applications were efficiently developed on fruit crops, i.e. two banana cultivars were released after the application of *in vitro* induced mutation (Miri et al., 2014). One of the main reasons could be the scarce availability of efficient regeneration protocols for these species which are not very easy to cultivate *in vitro* (Sabbadini et al., 2019c).

Chemical mutagens were applied in fruit crops to investigate disease tolerance (i.e. on *Musa spp*. against *Fusarium oxysporum*), tolerance to salinity, heavy metal and toxins; morphological changes (i.e. dwarfism), and the improvements of specific agronomical traits (i.e. earliness) (Predieri, 2001). To our knowledge, this is the first time that EMS and sodium azide mutagenesis has been performed using Meristematic bulk of various grapevine cultivars. The common and international important cultivars Chardonnay, Merlot, and Pinot Grigio have been identified by the members of the research convention as the most suitable subjects for the administration of the mutagenic treatment. Being so widespread it is important to find resistant clones to reduce the environmental impact of their cultivation. The experience gained with these cultivars has been extended to other cultivars of national importance (Ancellotta and Lambrusco Salamino) for wine production. The optimal dose of mutagens should be the concentration that guarantees a 50% reduction in regenerated shoots per treated unit. Even though the individuals belonged to the same species, it is interesting to note that for some treatments a cultivar-specific response has been recorded. Whether in Chardonnay and Pinot Grigio the treatment with EMS at the lowest concentration led to a conspicuous reduction of the regenerated shoots, the same concentration on Merlot did not affect regenerative rate. Generally, in the massive application of mutagenesis, the concentrations considered suitable were those that led to a regeneration reduction which averages at 60 and 75%. Intriguingly, on Chardonnay, sodium azide at both concentrations was preferred to EMS administration, although some tests were performed at the lowest EMS concentration. For the remaining varieties, treatments with a higher sodium azide concentration and those with an EMS concentration of 0.25% were preferred. For Ancellotta and Lambrusco Salamino, LD 50 was clearly defined subjecting meristematic bulk slices to a solution containing 0,20% of EMS for 3 hours of treatment. In fact, the same LD 50 intended as a reduction in the vitality of Petunia seeds was achieved applying 0,1% of EMS (Berenschot et al., 2008). Variable LD 50 values were selected in different studies in which induced mutation applied on banana shoot tips led to individuate as suitable an exposure at around 1,55% of EMS for 30-60 minutes, supported by RAPD analysis (Shirani Bidabadi et al., 2012) or an exposure of 2.63% and 1.6% for 2 h and 3 h (Dandeniya Arachchige and Abeysinghe, 2016). EMS treatments at 0,25% strongly affected the number of explants able to regenerate shoots considering the total number of MB slices treated on Chardonnay and Merlot (35-50%), while Lambrusco Salamino, Ancellotta, and Pinot Grigio maintained high value of regeneration (higher than 85%) at the same concentration used. A comprehensive operating protocol should entail *in vitro* regeneration protocol and selection, *in vivo* (e.g. greenhouse) verification of modified traits, accurate field evaluation of existence, stability, and usefulness of improved features (Predieri, 2001). The behaviour toward the tolerance to powdery mildew of the putative mutagenized population composed by Chardonnay, Merlot, and Pinot Grigio EMS-sodium azide treated plants were documented during the first s under greenhouse cultivation system. In almost all cultivars tested, a high incidence of severe disease index was recorded. 10%, 16%, and 2% of Chardonnay, Merlot, and Pinot Grigio plants respectively were symptomless and were individuated in different locations into the greenhouse, that were surrounded by heavily infected plants belonging to the same cultivar. This preliminary screening confirmed the possibility to exploit a genetic variability induced by the *in vitro* regeneration process, itself and in combination with specific mutagenic treatments (e.g. EMS, sodium

azide), including the possibility to have mutations of interest for identifying new clones with increased resistance to specific diseases.

Furthermore, the presence of red individuals detected on cv. Merlot confirmed the possibility to generate new genetic variation also related to other important traits (Fig. 8 f-g).

Powdery mildews tolerant plants were marked and isolated from the others, for testing and conducting further investigations.

As soon as they are ready, Ancellotta and Lambrusco Salamino plants deriving from chemical mutagenesis will undergo the same evaluations made on mutagenized population of Chardonnay, Merlot, and Pinot Grigio.

The results showed the suspected variability among lines of the three international cultivars isolated through tissue culture and EMS-sodium azide mutagenesis, that necessitate to be monitored and evaluated in future field observations, through the execution of specific infection trials under different conditions to confirm the acquired genetic resistance.

4.2 Somaclonal variation on somatic embryos

Embryogenic capability exploit cellular totipotency, and all the process started mainly from one cell that acquire the competence to regenerate an adult organism, limiting the regeneration of chimeric organisms (Faure et al., 1996). The passage from a natural context to the aseptic artificial *in vitro* environment require specific physiological adjustments which inevitably lead to molecular changes the greater the more intense is the degree of cellular undifferentiation that occurred during regeneration. Stress related to the manipulation of the material with the addition of numerous chemicals which are not considered dangerous, together with a molecular-physiological status that stimulate cell division can encourage the occurrence of modification in the resultant regenerated individuals. Each regeneration event is potentially capable of giving rise to somaclonal variants that are mostly un-predictable, not reproducible, and aleatory and could be pre-existing in the starting explants or induced during the culture process (Neelakandan and Wang, 2012). Analogous studies on grapevine using somatic embryogenesis regeneration system revealed the polyploidy as the most frequent modification in the somaclonal variant population (Prado et al., 2010; Acanda et al., 2013). The increase of ploidy level is generally achieved through colchicine treatment that act by preventing the segregation of chromosomes during meiosis originating organisms containing double the chromosome number than usual (Manzoor et al., 2019). On *Vitis vinifera* two studies highlighted the potential of somaclonal variation associated with the selection made with culture filtrate. The first allowed to isolates individuals after protoplast regeneration from embryogenic calluses selected in the presence of increasing concentration of *Botrytis cinerea* filtrate, reporting the production of tetraploid lines (Reustle and Matt, 2000). The second study involved the exposure of pro embryonic clusters on *Elsinoe ampelina* culture filtrate, leading to the establishment of resistant plants in greenhouse and open field tests, associated with an enhanced chitinase production (Jayasankar et al., 2000). *In vitro* conditions are related to high relative humidity, a fixed temperature and photoperiod, the presence of susceptible host and the abundance of nutrients in the regeneration media, which inevitably favor the development of pathogens that often grow uncontrolled, therefore they are difficult to manage (Rao and Sandhya, 2016). Although some researches enhanced the

existence of a positive correlation between the use of either fungal-related toxins and culture filtrate and the obtainment of plant lines resistant to the related pathogen, we decided to did not select the material *in vitro*, in order to explore and visualize any possible variation arose from somatic embryo-derived plants. Our preliminary results highlighted the presence of genetic variability, which made it possible to detect in the early selection, plants tolerant to the oomycete *Plasmopara viticola*

During the first open greenhouse observation, an artificial inoculation of *Plasmopara viticola* was performed in order to screen and classify each plant for its response towards the pathogen. Interestingly, 54 Lambrusco Salamino and 22 Ancellotta plants showed reduced symptoms or no symptoms at all. These plants were marked and jointly with the others were monitored for further observations to visualize valuable variations also in response to test inoculation of the most devastating fungal and oomycetes agents. It was no possible to observe any phenotypical changes probably due to the high plant vigor related to the transient juvenile status, that is characteristics in plants developed from zygotic and/or somatic embryos. Our preliminary results seem to confirm the possibility of detecting resistant individuals in populations generated by this *in vitro* regeneration system.

3.3 Conclusion

In contrast to classical breeding, mutational breeding results simpler and faster, allowing to vary one or two characteristics of the entire genome, keeping the original clone characteristics unaltered, although is in effect a GMO-free approach (Khan et al.). This kind of induced mutation was commonly applied for genetic improvement of the major cereal crop, and others such as cotton, cowpea, peanut, sesame, and soybean almost exclusively propagated by seed (Roychowdhury and Tah, 2013). Efficient protocols for somatic embryogenesis and the direct application of chemical mutagens exploiting meristematic bulk regeneration system have been established and demonstrated in various *Vitis vinifera* cultivars (Tab. 3). Indirect somatic embryogenesis applied on Ancellotta and Lambrusco Salamino has allowed to obtain a population of 2500 plants on which to evaluate the presence of useful modifications through somaclonal variation phenomenon especially in promoting resistance to downy mildew (Tab. 3). The fact that somaclonal variation occurs at higher frequency in plants regenerated by the application if *in vitro* culture, increases the possibilities that some changes could involve important agronomical traits in a positive novel way which may not be achieved by conventional breeding (Mohan Jain, 2001). At the first survey, after the inoculation test, some somatic embryo-derived plants showed enhanced resistance to downy mildew (Tab. 3). However, somaclonal variation was also reported stimulating de novo shoot organogenesis. The combination of somaclonal variation and the direct application of chemical mutagens as alkylating agents (EMS) or sodium azide, theoretically enhanced the possibilities to have mutations in the resultant population. EMS and sodium azide treatments of Chardonnay, Merlot, Pinot Grigio MB slices led to the production of a population of 1413 individuals that were affected by an intended strong infection of powdery mildew in the greenhouse. Symptomless plants for almost all the genotypes have been identified, few in Pinot Grigio, more in Chardonnay, and a high number in Merlot. For this cultivar were also individuate plants with red leaves and petioles, highlighting the possibility to induce natural variability for other interesting traits for grape cultivars.

Table 3: Summary table of the first survey carried out on cultivars that have been tested, specifying *in vitro* regeneration method, mutagenic chemical agents used, assessment of resistance to a certain type of pathogen and number of plants with low symptoms

Cultivars	**Regeneration method**	**Mutagenic treatment**	**Tested pathogen**	**Total number of plants in greenhouse**	**Number of plants with low levels of infection**
Ancellotta	Somatic embryogenesis	-	*P. viticola*	1132	22
Chardonnay	Meristematic bulk	EMS/Sodium azide	*E. necator*	619	59
Lambrusco Salamino	Somatic embryogenesis	-	*P. viticola*	1167	54
Merlot	Meristematic bulk	EMS/Sodium azide	*E. necator*	517	81
Pinot Grigio	Meristematic bulk	EMS/Sodium azide	*E. necator*	282	5

EMS Mutagenic treatment was also applied on Ancellotta and Lambrusco Salamino cultivars, where future comparison with the somatic-embryo derived population could lead to promising results. If the regeneration from somatic embryos proves effective in the production of somaclonal variants it could be exploited to create new genetic variability, without having to use chemicals that are also dangerous for human health when used for the mutagenic treatment.

Plants of all populations will be transferred to the field for continuing monitoring their behaviour under open field cultivation conditions, at reduced pest management regime, so to be better identify and confirm the presence of clones with increased tolerance/resistance to diseases and also to identify other genetic variants of interest for the improvement of the different cultivars tested. The most promising clones selected in the preliminary screening have been also grafted on standard rootstocks (Fig. 11) so to anticipate their more precise assessment not only on one plant but on a plot of at least ten clonally propagated plants.

Figure 11: Grafting of Chardonnay, Merlot, and Pinot Grigio promising tolerant clones to powdery mildew

4 - CHAPTER FOUR: Genetic transformation efficiency on various grapevine cultivars and *Vitis* rootstocks and application of HIGS against *Botrytis cinerea* and *Plasmopara viticola*

1. Introduction

Agriculture and the selection of interesting phenotypes for productive and qualitative characteristics has been possible for some centuries only via targeted crosses between improved parents or through the identification and consequent propagation of mutated individuals. The advent of genetic engineering made it possible to accelerate modern breeding especially in fruit plants characterized by a long juvenile phase and to study the function of genes unknown until then. Genetic engineering is a powerful technique that allows to act in a precise way, modifying one or few traits of the original clone without affecting the clone identity and its characteristics (Gentile and La Malfa, 2019).

However, genetic engineering approach is often hampered by technical and biosafety issues. Concerning the methodological aspects, a crucial pre-requisite in genetic transformation studies is the availability of an efficient robust regeneration method, which is often difficult to develop, especially for fruit tree species (Sabbadini et al., 2019c). In addition, transformation and regeneration competences, which are intrinsic features strongly influenced by the genotype, must coexist in the plant cells to allow whole plants to be regenerated from the genetically modified cells (Delporte et al., 2014). Genetic manipulation of plant genome can be carried out in either of the two methods: direct or indirect DNA transfer, through particle bombardment (biolistic) or *Agrobacterium*-mediated transformation, respectively. In transgenic plants modified using different *Agrobacterium* strains the copy number of the transgene introgressed into the plant genome are usually low (Gray et al., 2014).

Typically, transformation efficiency, especially during the first phases after *A. tumefaciens* infection, involves few plant cells, depending on the genotype recalcitrance. Therefore, an effective selection strategy is essential to identify the few transgenic cells among those that have not been introgressed the gene of interest, and to obtain transgenic plants in the later steps. The selection of transformed events is normally achieved by the use of selectable marker genes, expressed in the host together with the gene of interest, that provide growth advantages only to the modified cells, i.e. *neomycin phosphotransferase II* (*NPTII*) gene that confers resistance to the antibiotic kanamycin (Gray et al., 2014). The presence of reporter gene in a transformation system, such as the enhanced green fluorescent protein (eGFP), or the cis-gene *VvMybA1* involved in anthocyanin production, can be an additional tool for the real-time visualization of transformed cells, with the possibility of identifying untransformed cells and/or shoots that would otherwise not be identifiable (Dutt et al., 2018).

Plant genetic engineering applications can be expanded through the use of various molecular tools and diverse gene transfer technologies (Delporte et al., 2014). The sequencing of the grapevine genome has certainly increased and clarified some fundamental aspects (Jaillon et al., 2007), allowing the identification of gene sequences related to the control of important agronomical characteristics, including those involved in resistance to biotic and abiotic stresses, although some of them need to be deepened (Sabbadini et al., 2019c).

Candidate genes can be identified by molecular tools, and their function can be efficiently unveiled by gene overexpression or gene silencing (Prieto et al., 2019). Precision breeding or new breeding techniques (NBTs) which are gaining ground more and more, especially in the last decade, have the potential to modify gene expression such as RNA interference (RNAi), and RNA-guided editing of genomes (Prieto et al., 2019). RNA interference is a rapid and sequence-specific mechanism that can lead to post-transcriptional gene silencing through the presence of double-stranded RNAs (dsRNAs) molecules. These molecules can be designed to have sequence homology to target specific genes of a certain pathogen (Agrawal et al., 2003). The RNAi machinery consists mainly of two phases. During the first phase called initiation, long dsRNAs molecules are cleaved into small interfering RNAs (siRNAs) of 21 to 25 nt, through the action of Dicer-like proteins (DCL), that are a family of RNAse enzymes. In the effector phase siRNA are loaded into a RNA induced silencing complex (RISC), which, by reducing siRNAs into single strands, works through the pairing and consequent silencing of homologous mRNAs (Xu et al., 2019). This evolutionary conserved mechanism has been discovered for the first time in plants, during an experiment performed on Petunia, and later, RNAi has been found in many other eukaryotic organisms (Eamens et al., 2008). Agriculture can benefit from the RNAi approach through the production of plants that stably express long or short dsRNAs-encoding gene constructs able to modulate target gene expression, according to the technique called Host Induced Gene Silencing (HIGS) (Das and Sherif, 2020). One of the main application of HIGS system consists in the induction of resistance against abiotic and biotic stresses, and successful experiments were documented, especially regarding insect/pest management (Taning et al., 2020). In woody plant species, some viruses as PRSV and PPV, have been efficiently controlled through the expression of hairpin-based gene constructs in Papaya (Jia et al., 2017) and Plum species (Sabbadini et al., 2019a; Sidorova et al., 2019), respectively.

The major phytopathological problems in grapevine cultivation, especially in Europe, are represented by the attacks of the pathogenic fungi *Botrytis cinerea*, *Erysiphe necator* and the oomycete *Plasmopara viticola.* Prof. Hailing Jin from the University of California, Riverside discovered that during the pathogenesis, the ubiquitous fungus *Botrytis cinerea* sends siRNAs as effector molecules to silence plant immunity genes in the host (Wang et al., 2016). DCL1/2 double mutant of *Botrytis cinerea* strain led to a loss of virulence demonstrating that the production of these molecules depends on the activity of both DCL 1 and DCL 2 of *Botrytis cinerea* (Huang et al., 2019). According to the discovery made by Cai et al., also plants, as parts of their immune response, send siRNAs molecules to fungal cells mainly through an extracellular vesicles trafficking among the cells belonging to different kingdoms (Cai et al., 2018a). RNAi *Bc-DCL 1/2* gene construct have been validated by the same research group in transgenic Arabidospis plants, confirming the susceptibility reduction to the pathogen compared to wild type plants (Wang et al., 2016). DCL genes are responsible for the production of siRNAs effectors in other pathogenic fungi such as *Verticillium* spp. (Wang et al., 2016). Genomic studies on *Plasmopara viticola* performed by Brilli et al., have revealed the presence of *dicer like* genes (not well classified) also in this pathogen (Brilli et al., 2018). To investigate if these genes may have a role in the production of siRNAs in *Plasmopara viticola*, and if their silencing can lead to a reduction in the pathogenicity of this oomycete, two new hairpin gene constructs targeting *Pv-DCL 1* or *Pv-DCL1/2* were designed and

produced in collaboration with Prof. Elena Baraldi of University of Bologna. Both these hairpin gene constructs also express the *eGFP/NPTII* gene cassettes, harbored by the binary silencing vector pK7GWIWG2, introduced into EHA101 *Agrobacterium* strain.

In this study a gene construct expressing only the *NPTII* and *eGFP* genes, was used in genetic transformation trials to screen diverse grapevine cultivars (Ancellotta, Albana, Chardonnay, Ciliegiolo, Glera, Lambrusco Salamino, Merlot, Pinot Grigio, Sangiovese, Thompson Seedless, Vermentino), and hybrids rootstocks (110 Richter, 1103 Paulsen, Kober 5BB) for their transformation competence, exploiting the meristematic bulk regeneration system. The results obtained from these first experiment led to the identification of Thompson Seedless as the most efficient genotype in terms of regeneration and transformation efficiency; therefore, this genotype was used later also to carry out HIGS experiments: The hairpin gene construct targeting *Bc DCL1/2* (Wang et al., 2016) have been introgressed through *Agrobacterium*-mediated transformation trials on Thompson Seedless meristematic bulk slices, to obtain RNAi plant lines capable to actively produce long *Bc DCL1/2* dsRNAs. Both hairpin gene constructs targeting *Pv-DCL 1* or *Pv-DCL1/2* were introduced via *Agrobacterium*-mediated transformation in Thompson Seedless meristematic bulk slices. In addition, targeting the *Dicer-like* genes of *Plasmopara viticola,* Ancellotta, Lambrusco Salamino, and Thompson Seedless somatic embryos (SEs) have been also used as starting material for the induction of secondary somatic embryogenesis post-Agro-infection, although the adopted protocol needs to be revised. A novel use of mature somatic embryos was also deepened in this study, allowing to derive cotyledons and hypocotyls from which organogenesis was induced following the *Agrobacterium*-mediated transformation, leading to the production of several putative transgenic lines that have to be molecularly characterized. Lastly, a comparison of genetic transformation efficiency of MB slices, cotyledons and hypocotyls employed as starting explants was evaluated.

2. Material and methods

Vectors and *Agrobacterium tumefaciens* strains

The gene constructs used for the transformation experiments are reported below:

1. *A. tumefaciens* strain EHA105 harbouring pK7WG2 binary vector which has in its T-DNA the *eGFP* reporter gene and the *NPTII* genetic cassette as a selectable marker, that confers resistance to kanamycin (Sabbadini et al., 2019c). The bacterium cells were selected on YEB medium containing 75 mg L^{-1} spectinomycin, and 50 mg L^{-1} rifampicin.
2. *A. tumefaciens* strain GV3101 harbouring pHELLSGATE 8 binary vector which has in its T-DNA an RNAi construct targeting *DCL 1-2* of *Botrytis cinerea* as well as the *NPTII* genetic cassette as selectable marker (Wang et al., 2016). The selective antibiotics are 50 mg L^{-1} spectinomycin, 50 mg L^{-1} rifampicin, and 50 mg L^{-1} gentamicin on LB medium.
3. *A. tumefaciens* strain EHA101 harbouring pK7GWIWG2(II) binary vector has in its T-DNA an RNAi construct targeting *DCL 1-2* (chimaera construct), or *DCL 1* (441) of *Plasmopara viticola*, as well as

the *eGFP* and the *NPTII* genetic cassettes. The selective antibiotics are 50 mg L^{-1} spectinomycin, 50 mg L^{-1} rifampicin on LB medium.

The first gene construct has been kindly provided by Prof. Tiziana Pandolfini, and PhD Barbara Molesini from the University of Verona, while the second construct was provided by Prof. Hailing Jin from the University of California, Riverside. The gene construct targeting *Dicer-like genes 1* and *2* of *Plasmopara viticola* was designed and prepared in collaboration with Prof. Elena Baraldi from the University of Bologna. Among the *Dicer-like genes 1* (441) and *2* (331) that were identified in *Plasmopara viticola* cDNA, fragments of 258 and 257 nt respectively have been selected. These fragments were both inserted by overlapping PCR, or individually, in pGEM plasmids and verified by sequencing. Using the GATEWAY® with clonase II® conversion technology, that is based on a site-specific recombination reaction, it was possible to transfer the two target gene fragments the chimaera ones (515 nt) and 441 (258 nt) to the final plasmid pK7GWIWG2(II) which is specific for the application of post-transcriptional gene silencing (Karimi et al., 2002).

Gene construct and regeneration method used for each genotype

Genetic transformation trials have been organized based on the availability of the related starting explant. Albana, Ancellotta, Chardonnay, Ciliegiolo, Glera, Lambrusco Salamino, Merlot, Pinot Grigio, Sangiovese, Vermentino, Thompson Seedless, 110 Richter, 1103 Paulsen, Kober 5BB meristematic bulk slices were subjected to *Agrobacterium*-mediated transformation using EHA105 *35S-eGFP-NPTII* gene construct. Thompson Seedless MBs have been employed in HIGS experiments, to obtain putative transgenic lines which express EHA101 *Pv DCL 1/2* RNAi, EHA101 *Pv DCL 1* RNAi, GV3101 *Bc DCL 1/2* RNAi hairpin-based gene constructs. Ancellotta, Lambrusco Salamino, and Thompson Seedless somatic embryos, cotyledons and hypocotyls were employed as starting explant (somatic embryos via somatic embryogenesis, cotyledons and hypocotyls via organogenesis) in genetic transformation experiments using EHA101 *Pv DCL 1* or *Pv DCL 1/2* RNAi gene construct, as reported in Table 1.

Table 1: Summary table of all genetic transformation trails performed on *Vitis* species which are reported by distinguishing genotype, type of explant, method of regeneration and gene construct used

Genotype	Starting explant	Regeneration method	Gene construct
Albana, Chardonnay, Ciliegiolo, Glera, Merlot, Pinot Grigio, Sangiovese, Vermentino, 110 Richter, 1103 Paulsen, Kober 5BB	MBs	Organogenesis	EHA105 *35S-eGFP-NPTII*
Ancellotta, Lambrusco Salamino	MBs	Organogenesis	EHA105 *35S-eGFP-NPTII*
	SE	Somatic embryogenesis	EHA101 *Pv DCL 1/2* RNAi
	Cotyledons and hypocotyls	Organogenesis	EHA101 *Pv DCL 1/2* RNAi
			EHA101 *Pv DCL 1* RNAi
Thompson Seedless	MBs	Organogenesis	EHA105 *35S-eGFP-NPTII*
			EHA101 *Pv DCL 1* RNAi
			EHA101 *Pv DCL 1/2* RNAi
			GV3101 *Bc DCL 1/2* RNAi
	SE	Somatic embryogenesis	EHA101 *Pv DCL 1/2* RNAi
	Cotyledons and hypocotyls	Organogenesis	EHA101 *Pv DCL 1/2* RNAi
			EHA101 *Pv DCL 1* RNAi

2.1 *Agrobacterium*-mediated transformation of grapevine meristematic bulk slices

2.1.1 Plant material

Meristematic bulks (MBs) of the *Vitis vinifera* cultivars Albana, Ancellotta, Chardonnay, Ciliegiolo, Glera, Lambrusco Salamino, Merlot, Pinot Grigio, Sangiovese, Thompson Seedless and Vermentino, as well as the *Vitis* hybrid rootstocks 110 Richter, 1103 Paulsen, and Kober 5BB have been introduced to *in vitro* condition and micro propagated by Vitroplant Italia s.r.l. MBs maintenance was carried out through their transfer into fresh IM3 medium (Mezzetti et al., 2002) every 4 weeks. The IM3 PGRs compositions was adjusted for each genotype (Figure 1) and TDZ was also tested in combination with BAP.

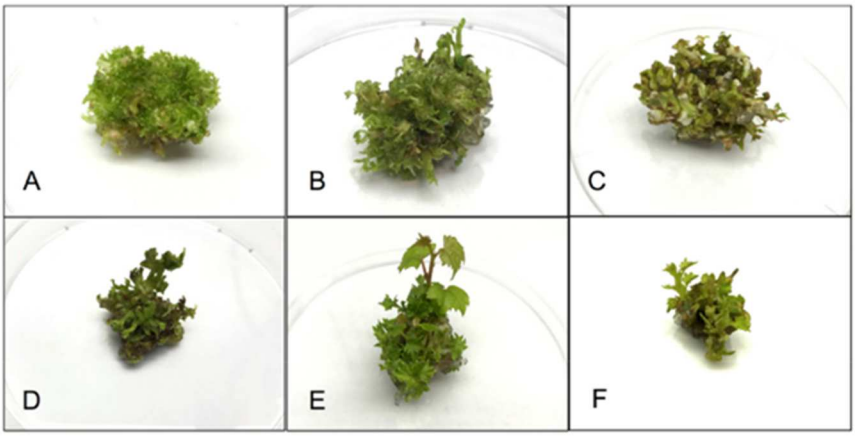


Figure 1: Meristematic bulks of grapevine cultivars and rootstocks: a) Thomspon Seedless; b) Sangiovese; c) Glera; d) Vermentino; e) 110 Richter; f) 1103 Paulsen.

Culture medium was optimized for each genotype utilized for meristematic bulks maintenance and proliferation. MS basal medium supplemented with 13.2 µM of BAP and 0.1 µM of NAA was used for Thompson Seedless and Ciliegiolo cultivars MBs maintenance, the other genotypes were cultured in the presence of 8.8 µM of BAP and 0.23 µM of TDZ.

A variable number of slices of about 2 mm thick were obtained from each meristematic agglomerate, which underwent the removal of any necrotic portions, roots, or shoots. This material cultured in an optimal regeneration medium in a growth chamber, maintained at 24 ° C, with 16 hours of light (70 µmol m^{-2} s^{-1}), thus obtained acquires a high regenerative competence and becomes the optimal starting material both for micro-propagation and for genetic transformation.

2.1.2 Chemical reagents and media preparation

- Culture medium for *A. tumefaciens*: YEB medium (5 g L^{-1} beef extract, 1 g L^{-1} yeast extract, 5 g L^{-1} for each of tryptone and sucrose, and 480 mg L^{-1} $MgSO_4$) for the growth of EHA105 *A. tumefaciens* strain, or LB medium (10 g L^{-1} of tryptone, 5 g L^{-1} of yeast extract, 10 g L^{-1} of NaCl) for

the growth of GV3101 or EHA101 *A. tumefaciens* strains; liquid media were eventually solidified with 7 g L^{-1} of bacto-Agar (SIGMA). Before autoclaving, the pH was adjusted to 7.2 using 1N NaOH.

- Meristematic bulk infection solution (MS20) is a liquid medium based on MS basal salt and vitamins supplemented with 20 g L^{-1} of sucrose. Before autoclaving, the pH was adjusted to 5.2 using 1N KOH.
- Co-culture medium (MSH0) is based on MS basal salt and vitamins supplemented with 30 g L^{-1} sucrose and solidified with 7 g L^{-1} plant Agar (Duchefa). Before autoclaving, the pH was adjusted to 5.7 using 1N KOH.
- Regeneration and selection medium for Thompson Seedless and Ciliegiolo cultivars is based on MS basal salt and vitamins supplemented with 30 g L^{-1} sucrose, 13.2 µM BAP, 0.1 µM NAA, 70 mg L^{-1} kanamycin, 200 mg L^{-1} cefotaxime, 200 mg L^{-1} carbenicillin, and solidified with 7 g L^{-1} plant agar (Duchefa). Before autoclaving, the pH was adjusted to 5,7 using 1N KOH.
- Regeneration and selection medium for Albana, Ancellotta, Chardonnay, Glera, Lambrusco Salamino, Merlot, Pinot Grigio, Sangiovese, Vermentino, 110 Richter, Kober 5BB, 1103 Paulsen is based on MS basal salt and vitamins supplemented with 30 g L^{-1} of sucrose, 13.2 µM BAP, 0.23 µM TDZ, 70 mg L^{-1} kanamycin, 200 mg L^{-1} cefotaxime, 200 mg L^{-1} carbenicillin, and solidified with 7 g L^{-1} of plant agar (Duchefa). Before autoclaving, the pH was adjusted to 5,7 using 1N KOH.

2.1.3 Grapevine MBs transformation conditions.

The transformation protocol adopted has been the same as it was described by Sabbadini and co-authors with slight modifications (Sabbadini et al., 2019c). Either LB or YEB solid medium containing the specific selective antibiotics was used for plating *A. tumefaciens* which was incubated at 28°C for 48 hours. *A. tumefaciens* single colonies were picked from the plate and transferred to falcon tubes where were left to grow in liquid YEB/LB medium at a temperature of 28°C, on a shaker at 200 rpm until they reach an OD_{600} = 0,7–1,0 (using a spectrophotometer). Bacterial cells were pelleted, centrifuging at 4°C for 15 minutes at 2500 rpm and re-suspended in 40 mL of MS20 liquid medium supplemented with 100 µM acetosyringone. The solution was vigorously mixed and placed on a rotary shaker for 2 h at 24°C. Slices obtained from the meristematic bulks, fifteen at a time, were separately dipped in the bacterial suspension for 15 min. After which they were blotted onto sterile filter papers and placed on MSH0 medium in which a sterile filter paper soaked in a solution containing MS20 plus 100 µM acetosyringone was set on the top of the medium. Plates containing explants were co-cultured for 48 h at 24°C in completely dark conditions.

2.1.4 Transformation competence of the *Agrobacterium*-infected MBs of various grapevine cultivars and rootstocks screened via *eGFP* combined to kanamycin selection

A total of 50 MB slices for each genotype were utilized for each transformation trial, for a total of 100 MB treated, therefore each genotype has been employed in two distinct transformation trials. The genetic transformation efficiency was detected in the following cultivars: Albana, Ancellotta, Chardonnay, Ciliegiolo, Glera, Lambrusco Salamino, Merlot, Pinot Grigio, Sangiovese Thompson Seedless Vermentino, and rootstocks (110 Richter, 1103 Paulsen, and Kober 5BB); After the co-culture with *Agrobacterium*, all the

explants for each genotype were placed in microboxes containing fresh IM3 medium supplemented with the appropriate combination of PGRs, 200 mg L^{-1} of cefotaxime and carbenicillin each to contain *Agrobacterium* survival, and 70 mg L^{-1} of kanamycin. The explants were transferred on fresh culture media every three weeks and observed under the stereomicroscope Leica MZ10F to detect calli sectors and shoots emitting green fluorescence (λEX = 480 nm and λEM = 510 nm). Images were acquired by the Leica DFC 450 C camera and processed through the Leica Application Suite V.4.5. At each subculture (3, 6, and 9 weeks), new regenerating shoots and calli areas not fluorescent were cut and discarded, and only putative transgenic zones were transferred to fresh media. The number of MB slices expressing *eGFP* was counted and expressed as a percentage of the total explants infected. The experiment was repeated twice. When new shoots were visible, generally after 9 weeks from the agro-infection, they were transferred on IM medium supplemented with the same antibiotic composition, but with 4.4 μM of BAP stimulating shoot elongation. Subcultures that followed occurred halving the cytokinin content at each step, maintaining the same antibiotic regime.

2.1.5 Genomic DNA extraction and Southern blot analysis of Thompson Seedless transgenic plants

Genomic DNA of Thompson Seedless *eGFP* putative transgenic lines were isolated from 0.5–1 g of frozen leaves using the "Illustra DNA extraction kit PHYTOPURE" (GE Healthcare) according to the manufacturer's instructions. Then, genomic DNA was cleaned up using NucleoSpin Plant II (Macherey-Nagel). The DNA (20 μg) was digested with 40 U of HindIII in the presence of spermidine (5 mM) and BSA (0.1 mg/mL), then electrophoresed on a 0.8% agarose gel at 4.5 V cm−1, and transferred on positively charged Hybond N+ membrane (GE Healthcare). DNA probe for the analysis, spanning a 341 bp-long portion of the 35 S promoter used to drive eGFP expression, was obtained by PCR with the following forward (F) and reverse (R) primers: F 5′-CTTCGTCAACATGGTGGAGCACGACA-3′ and R 5′-TGGAGATATCACATCAATCCACTTG-3′. The probe was labelled with dCTP [α 32P] (Perkin Elmer) by random priming using "Prime-It II Random Primer Labeling Kit" (Stratagene). Unincorporated nucleotides were removed with the "Illustra AutoSeq G-50 Dye Terminator Removal Kit" (GE Healthcare). After overnight hybridization at 42°C in ULTRAhyb buffer (Ambion) in the presence of 106 cpm ml−1 of labelled probe, the blot was washed twice in 2X SSC containing 0.1% SDS at 42°C for 5 min, and twice in 0.1XSSC containing 0.1% SDS at 42°C for 15 min. Autoradiography was then performed using CL-XPosure film (Thermo Scientific).

2.1.6 Genetic transformation for the obtainment of plants expressing RNAi gene constructs against *Plasmopara viticola*

Agrobacterium tumefaciens EHA101 strain harbouring two different RNAi fragments that target *DCL 1* (441 gene construct) and *DCL 1/2* (chimaera gene construct) of *Plasmopara viticola* have been alternatively employed for genetic transformation trials on Thompson Seedless cultivar (Fig. 2).

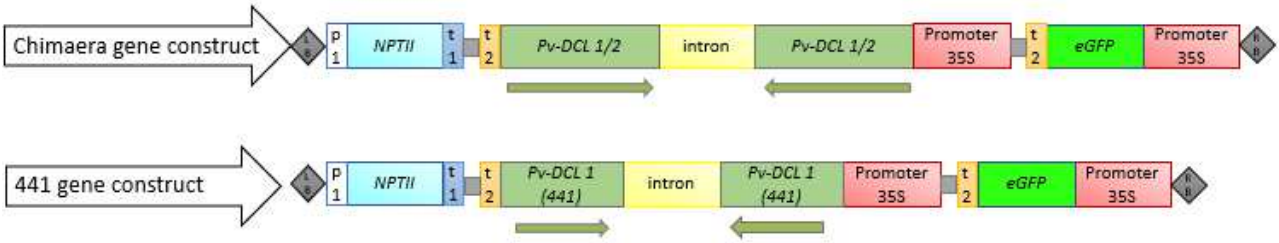


Figure 2: Schematic presentation of the two RNAi gene constructs targeting *Dicer-like 1 and 2* (chimaera, 515 nt) or *Dicer-like 1* (441, 258 nt) of *Plasmopara viticola*. LB, left border sequence; p1, promoter of the *nopaline synthase* gene (in white); *NPTII*, *neomycin phosphotransferase II* gene conferring resistance to kanamycin; t1, terminator of the nopaline synthase gene (in blue); t2, terminator of the Cauliflower Mosaic Virus 35S (in orange); *Pv-DCL* 1/2 or *Pv-DCL* 1 fragment in reverse orientation; intron; *Pv-DCL* 1/2 or *Pv-DCL* 1 fragment in sense orientation; p35S, promoter of the Cauliflower Mosaic Virus 35S; RB, right border sequence.

Agrobacterium-mediated infection was carried out on 720 MB slices of Thompson Seedless using chimaera construct (targeting *DCL1/2*) and 480 MB slices of the same cultivars using 441 gene constructs (targeting *DCL1*) following the transformation protocol described above, for a total of ten genetic transformation trials, processing about 120 MB slices at each time. Putative transgenic regenerated shoots have been isolated and cultured separately in glass pots, filled with the selection media having the same antibiotic regime and high cytokinin concentration (BAP 13.2 µM) to stimulate proliferation of the putative transformed shoots.
Genetic transformation efficiency was assessed after 9 weeks of selection and was calculated as the percentage of MB regenerating eGFP shoots / total MB slices treated.

2.1.7 Genetic transformation for the obtainment of plants expressing an RNAi gene construct against *Botrytis cinerea*

For the transformation trials, we used the *Agrobacterium tumefaciens* strain GV3101 harbouring the plasmid pHellsgate-*Bc-DCL1/2* provided by Prof. Hailing Jin from the University of California, Riverside (Figure 3) (Wang et al., 2016). This hairpin gene construct was used for the application of host induced gene silencing against *Botrytis cinerea* in the grapevine cultivar Thompson Seedless.

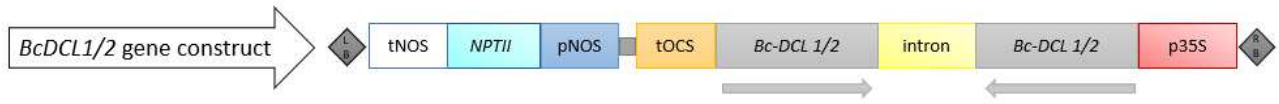


Figure 3: Schematic presentation of the *Bc-DCL1/2* hairpin gene construct used for transformation. The construct is based on the plasmid vector pHELLSGATE 8 (Helliwell and Waterhouse, 2003). LB, left border sequence; tNOS, terminator of the *nopaline synthase* gene; *NPTII*, *neomycin phosphotransferase II* gene conferring resistance to kanamycin; pNOS, promoter of the nopaline synthase gene; tOCS, terminator of the octopine synthase gene; *Bc-DCL* 1/2 fragment in reverse orientation (490 nt); intron; *Bc-DCL* 1/2 fragment in sense orientation (490 nt); p35S, promoter of the Cauliflower Mosaic Virus 35S; RB, right border sequence.

Following the same method described in the previous sub-chapters, a total of 600 MB slices of Thompson Seedless were agro-infected, in five separate genetic transformation trials. Since there are no reporter genes, the selection of the putatively transformed material was only possible through the action of kanamycin added in the selection medium. The genetic transformation efficiency was reported as the percentage of MB regenerating green shoots able to root in a kanamycin enriched medium/ total MB slices treated.

2.1.8 Acclimatization of putative transgenic lines

Cytokinin contents have been decreased at each subculture until 1 µM and elongated shoots have been selected. Root emergence was favored in glass tubes or alternatively in glass pots in PGR and antibiotic free MS medium where they stayed for a month. After rooting, the acclimatization occurred through the transfer of explants in alveolar seedbeds (50 x 30 x 5,5 cm) filled with commercial peat and placed in a dedicate growth chamber, at high humidity for at least three weeks.

2.1.9 Molecular characterization of Thompson Seedless *Bc-DCL1/2* and *Pv-DCL1/2* transgenic plants

Thompson Seedless *Bc-DCL1/2* and *Pv-DCL1/2* putative transgenic lines have been analyzed by using Thermo Scientific Phire Plant Direct PCR Kit (Fisher Scientific). A 0,5 mm diameter leaf sample was collected by each plant line into a tube containing 20 µL of the dilution buffer. After processing samples by grinding and vortexing, 1,25 µL have been used as a template in 50 µL PCR. The PCR program consisted of an initial denaturation at 98°C for 5 min, followed by 40 cycles of denaturation at 98°C for 5 s, annealing at 63,6°C for 5 s (for the amplification of *35S*) or at 60°C for 5 s (for the amplification of *Pv-DCL1/2* sequence), and extension at 72°C for 20 s, and a final extension at 72°C for 1 min. PCR products were analysed by electrophoresis on 1% agarose gels stained with Invitrogen SYBR Safe (Fisher Scientific). DNA bands were visualized using a GelDoc EZ Imaging System (Bio-Rad). DNA amplicon for the analysis, spanning a 515 bp-long portion of the *Pv-DCL1/2*, was obtained by PCR with the following forward (F) and reverse (R) primers: F 5′- ATGATGGACACCTCGTTGTG -3′ and R 5′- GCGTACAATCTCGGGTGTG -3′. The detection of the transgenic status of *Pv-DCL1* and *Bc-DCL1/2* plant lines has been performed through the amplification of promotor *35S* gene sequence, following the same PCR program, using an annealing temperature of 63,6°C.

2.2 *Agrobacterium*-mediated transformation of grapevine somatic embryos

2.2.1 Plant material

Ancellotta, Lambrusco Salamino somatic embryos were induced from the culture of floral explant (whole flowers, stamens, and pistils) as reported by Dhekney and co-authors (Dhekney et al., 2016), while Thompson Seedless SEs were initiated by culturing stem segments (containing one lateral bud) of *in vitro* growing plants (Maillot et al., 2016). These somatic embryos were constantly proliferated separating and selecting pro-embryonic masses (PEM) on X6 medium (Li et al., 2001), and transferred to fresh medium every two weeks. Somatic embryos at mid-cotyledonary stage, corresponding to the SE Type II (Dai et al., 2015) were selected as starting explant in *Agrobacterium*-mediated transformation experiments, for the induction of secondary embryogenesis post Agro-infection.

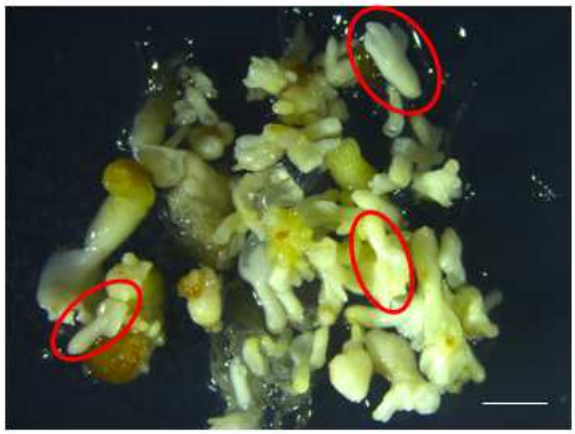


Figure 4: Somatic embryos were selected at mid-cotyledonary stage in Agrobacterium-mediated transformation trials. The right stage (SE type II) has been circled in red (bar = 2 mm).

2.2.2 Grapevine somatic embryos genetic transformation conditions

The transformation protocol adopted has been the same as described by Dhekney and co-authors with slight modifications (Dhekney et al., 2016). *A. tumefaciens* EHA101 strain harbouring *Pv DCL 1/2* gene construct (Fig. 2) was employed in genetic transformation experiments. The bacteria were left to grow in Petri dishes containing LB solid medium supplemented with 50 mg L^{-1} of spectinomycin and 50 mg L^{-1} of rifampicin for 48 hours at 28°C. Single colonies were introduced in falcon tubes filled with LB liquid medium and the selective antibiotics, and were incubated overnight at 28°C on a rotary shaker (180 rpm). The overnight cultures (OD_{600} = 0,7-1) were spin at 4200 g for 8 minutes at room temperature. The supernatant was discarded, and the pellet was resuspended adding 20 mL of X2 medium (having the same composition of X6 medium, adding 20 g L^{-1} of sucrose instead of 60 g L^{-1}), and mixed for 3 hours at the same overnight culture conditions. At the same time, cotyledonary somatic embryos (SEs) were carefully selected, avoiding tissue wounding, and placed in groups of 20 in sterile Petri plates. 5 mL of *Agrobacterium* culture were incubated for 7-10 minutes with SEs in constant agitation. After the removal of the bacterial solution using a micropipette, SE were settled in Petri dishes, where two layers of sterile filter paper have been soaked previously in liquid DM medium (DKW basal salts (DRIVER and KUNIYUKI, 1984); 0,3 g L^{-1} KNO_3; 1 g L^{-1} myo-inositol; 2

mg L^{-1} each of thiamine –HCl and glycine; 1 mg L^{-1} nicotinic acid; 30 g L^{-1} sucrose; 5.0 µM BAP; 2.5 µM each NOA and 2,4-D; pH was adjusted at 5.7). Following co-cultivation for 72 hours at 24°C in darkness, SEs were observed under a UV stereomicroscope for the evaluation of transient GFP expression. Explants were washed for 3 days at 110 rpm in conical flask filled with 100 mL DM medium with 200 mg L^{-1} each of carbenicillin and cefotaxime, and 15 mg L^{-1} kanamycin to inhibit remnant bacterial growth. Washed cultures were transferred to each 100 × 15 mm Petri dishes containing 25 mL solid DM medium (same composition of liquid DM medium, plus 7 g L^{-1} plant agar) with 200 mg L^{-1} each of carbenicillin and cefotaxime and 100 mg L^{-1} kanamycin. Callus development and proliferation occurred in dark conditions at 24°C for 4 weeks. After this period, callus cultures were transferred to Petri dishes containing 30 mL of X6 medium with 200 mg L^{-1} each of carbenicillin and cefotaxime, and 70 mg L^{-1} kanamycin for the regeneration of secondary transformed embryo. The remaining explant that did not show any callogenesis or developed non embryogenic calluses were left on the same DM medium or moved to a fresh DM with the same antibiotic concentration. The presence of modified SE lines was screen at weekly intervals, and the cultures were maintained in dark. Independent SE lines are identified by bright GFP fluorescence and kanamycin resistance. Callus culture were transferred every 4 weeks on fresh X6 medium.

2.2.3 Identification of transient GFP expression

Five *Agrobacterium*-mediated transformation trials have been performed on a total of 101, 97, and 107 SEs of Thompson Seedless, Ancellotta, and Lambrusco Salamino, respectively. Transient GFP expression was assessed immediately after co-culture (three days after Agro-infection), and transient GFP expression efficiency was evaluated for each cultivar as the number of SEs that express GFP fluorescence/total SEs treated.

2.3 *Agrobacterium*-mediated transformation of somatic embryo-derived cotyledons and hypocotyls, via organogenesis

2.3.1 Plant material

Ancellotta, Lambrusco Salamino and Thompson Seedless mature somatic embryos at advanced cotyledonary stage have been selected as starting explant for promoting de novo shoot organogenesis, dividing cotyledons from hypocotyls (Figure 5).

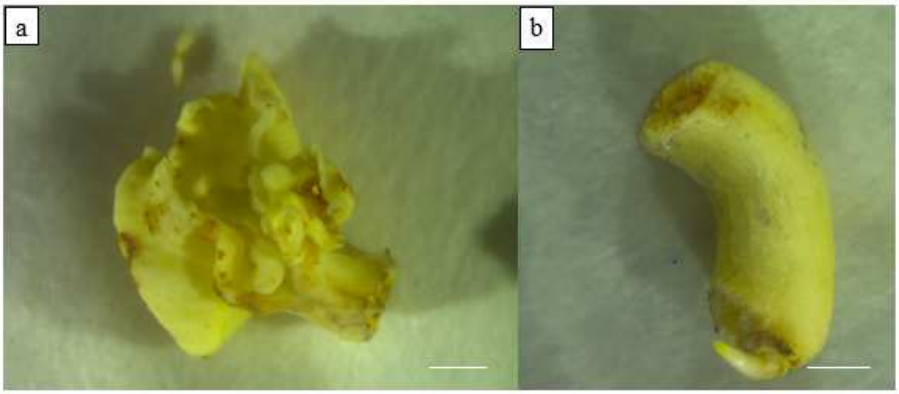


Figure 5: Cotyledons (a) and hypocotyls (b) of Thompson Seedless 3 days after dissection (bar = 2 mm).

2.3.2 Grapevine cotyledons and hypocotyls genetic transformation conditions

The genetic transformation protocol was optimized as the result of the combination of the two transformation protocols used for MBs (Mezzetti et al., 2002) and for SEs (Dhekney et al., 2016) to which we refer. Whitish mature SEs selected from three-to four-week-old SE culture on X6 medium, were cut, separating cotyledons and hypocotyls that were placed on fresh X6 medium. Overnight liquid culture of *A. tumefaciens* EHA101 carrying the hairpin gene construct *Pv-DCL 1/2* (OD_{600} = 0,7-1) in LB liquid medium with selective antibiotics, were spin at 4200 g x 8 minutes at room temperature. The bacterial pellet was resuspended in 20 mL of X2 medium and 100 µM of acetosyringone and mixed in a rotary shaker at 180 rpm at 24°C for 3 hours. Afterwards, cotyledons and hypocotyls were introduced in falcon tubes and treated for 15 minutes at 24°C, covering the falcons with aluminum foil. Explants were blotted dry and co-cultured in Petri dishes containing X2 solid medium covered with filter paper previously soaked with a solution of X2 and 100 µM of acetosyringone for 48 hours at 24°C in dark condition. The washing step occurs at the end of co-culture, arranging the embryos in sterile Petri dishes where a solution of deionized sterile water supplemented with 200 mg L^{-1} each of cefotaxime and carbenicillin was poured and mixed for five minutes. Samples were blotted dry and cotyledons were placed with abaxial face in contact with the culture media, while hypocotyls were cultured horizontally in microboxes filled with two different regeneration and selective media containing NN medium (Nitsch and Nitsch, 1969) including 30 g L^{-1} of sucrose, 7 g L^{-1} of plant agar, 70 mg L^{-1} kanamycin, and 200 mg L^{-1} each of carbenicillin and cefotaxime. Two phytohormones combination were used: one with 13.2 µM BAP (culture medium 2), and the other that includes a combination of 4.4 µM BAP and 0.49 µM IBA (culture medium 1).

De novo shoot organogenesis occurred under light provided by white fluorescent tubes (16 hours of light at an intensity of 70 µmol m^{-2} s^{-1}).

2.3.3 Genetic transformation efficiency determination in cotyledons and hypocotyls cultured on two regeneration/selection media

50 cotyledons and 50 hypocotyls of each Thompson Seedless, Ancellotta, and Lambrusco Salamino cultivars for each culture medium were agro-infected. The expression of green fluorescent protein in inoculated

cotyledons/hypocotyls and developing plantlets was observed every 3 weeks under the stereomicroscope for GFP-fluorescence visualization. Genetic transformation efficiency was reported for each type of explant at 9 weeks, as the number of explants and/or shoots expressing green fluorescence on the total number of explants treated.

2.3.4 Comparison of genetic transformation efficiency using MB slices, cotyledons and hypocotyls as starting explants in genetic transformation trials

100 meristematic bulk slices, 100 cotyledons and 100 hypocotyls belonging to Thompson Seedless table grape cultivar have been inoculated with *Agrobacterium tumefaciens* strain EHA101 which contain *Pv DCL 1* RNAi fragments and *NPTII* and *eGFP* coding regions, following the specific procedures reported in the previous sub-chapters. After co-culture, the explants were left to grow in the regeneration media composed by MS basal salt and vitamins (Murashige and Skoog, 1962) including 30 g L^{-1} of sucrose, 7 g L^{-1} of plant agar, 13.2 µM BAP, 0.1 µM NAA, 70 mg L^{-1} kanamycin, and 200 mg L^{-1} each of carbenicillin and cefotaxime.
Expression of green fluorescent protein in inoculated MB, cotyledons, and hypocotyls, and developing plantlets was observed every 3 weeks under the stereomicroscope for GFP-fluorescence visualization. Genetic transformation efficiency was reported for each type of explant at 9 weeks, as the number of explants and/or shoots expressing green fluorescence on the total number of explants treated.

2.4 Statistical analysis

All acquired data from each trial were analysed by one-way ANOVA, and Student Newman-Keuls test ($p < 0.05$) is the pot-hoc test that was used to identify significant differences. The results obtained from transformation trials were analysed.

3. Results

3.1 *Agrobacterium*-mediated transformation of grapevine meristematic bulk slices

3.1.1 Transformation competence of the *Agrobacterium*-infected MBs of various grapevine cultivars and rootstocks screened via *eGFP* combined to kanamycin selection

Putative transgenic events from the ten cultivars and the three rootstocks tested (Table 7) were regenerated from slices of meristematic bulks inoculated with *A. tumefaciens* EHA105 strain expressing the *eGFP* and *NPTII* coding regions. After co-culture with *Agrobacterium*, MB slices were placed in microboxes that contained the selection medium and transferred onto fresh media every three weeks. Visual screening of transformed cells using eGFP was conducted at each subculture to detect and dissect the putative transformed areas and/or proliferating shoots. Transformation efficiency, intended as the percentage of MB slices expressing eGFP on the total infected explants, was assessed at three, six and nine weeks after agro-infection. The percentages of MB slices showing eGFP fluorescence are detailed in Fig. 6.

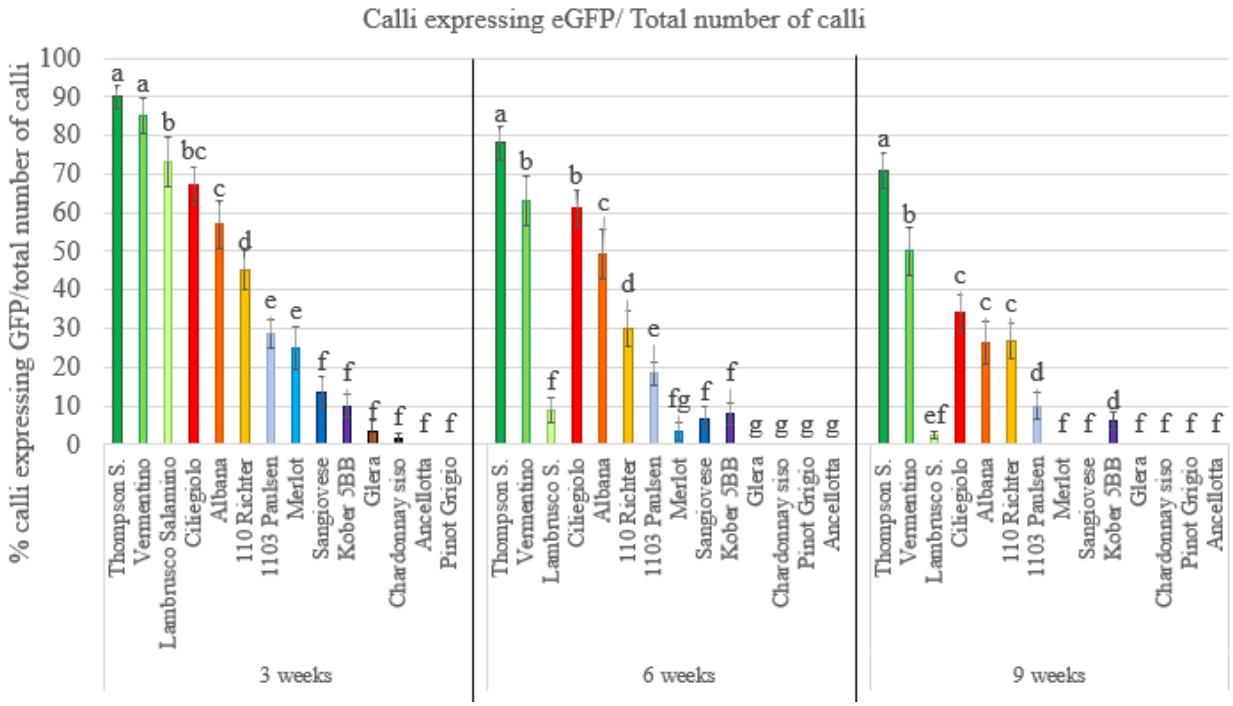


Figure 6: Percentage of MB slices showing eGFP fluorescence, selected by the combined use of kanamycin and eGFP screening, after 3, 6, and 9 weeks from selection initiation. Means with different letters are significantly different according to Newman-Keuls test ($p < 0.05$) ± SE ($n = 50$), and compare results obtained from the different genotypes at the same acquisition period. Two independent experiments were performed with similar results.

The sets of transformation trials were performed in order to screen the cultivars and the hybrid rootstock concerning their competence for transformation, exploiting the regeneration ability of MB slices. Compared to the other genotypes, Thompson Seedless exhibited the highest percentage of transformed MB areas at almost all data acquisition periods, with values of 90%, 78%, and 71% of eGFP-fluorescent explants after three, six or nine weeks after genetic transformation, respectively. Among the other cultivars, we were not able to produce transgenic calli in Ancellotta, Chardonnay, Glera, Merlot, Pinot Grigio, and Sangiovese, while Vermentino, Albana, and Ciliegiolo showed an intermediate level of genetic transformation efficiency up to 9 weeks. Lambrusco Salamino MBs have been one of the most performant cultivar in terms of MB that express at least one fluorescent area on the total inoculated explants at three weeks. This competence was not maintained in the subsequent subcultures during which the transformation efficiency was reduced to very low levels, which could suggest that the selection antibiotic concentration was excessive enough to break down the regenerative capacity of the explants. The transformation efficiency of hybrid rootstocks decreased with subculturing and were generally lower than those obtained by Thompson Seedless, Vermentino, Ciliegiolo, and Albana. Among all the rootstocks, 110 Richter reached the highest transformation efficiency values of about 45%, 30%, and 26,73% in the three consequent subcultures, respectively. A scarce number of calli expressing GFP were counted at 9 weeks for the rootstocks 1103 Paulsen and Kober 5BB, while no transgenic events were detected on Ancellotta, Chardonnay, Glera, Merlot, Pinot Grigio, and Sangiovese cultivars at the same period. This method led to the isolation of transgenic calli for almost all the genotypes (Figure 7 a, b, c),

while five transgenic shoots arose in the solely Thompson Seedless cultivar which expressed eGFP (Fig. 7 d), and were isolated and characterized through Southern blot analysis.

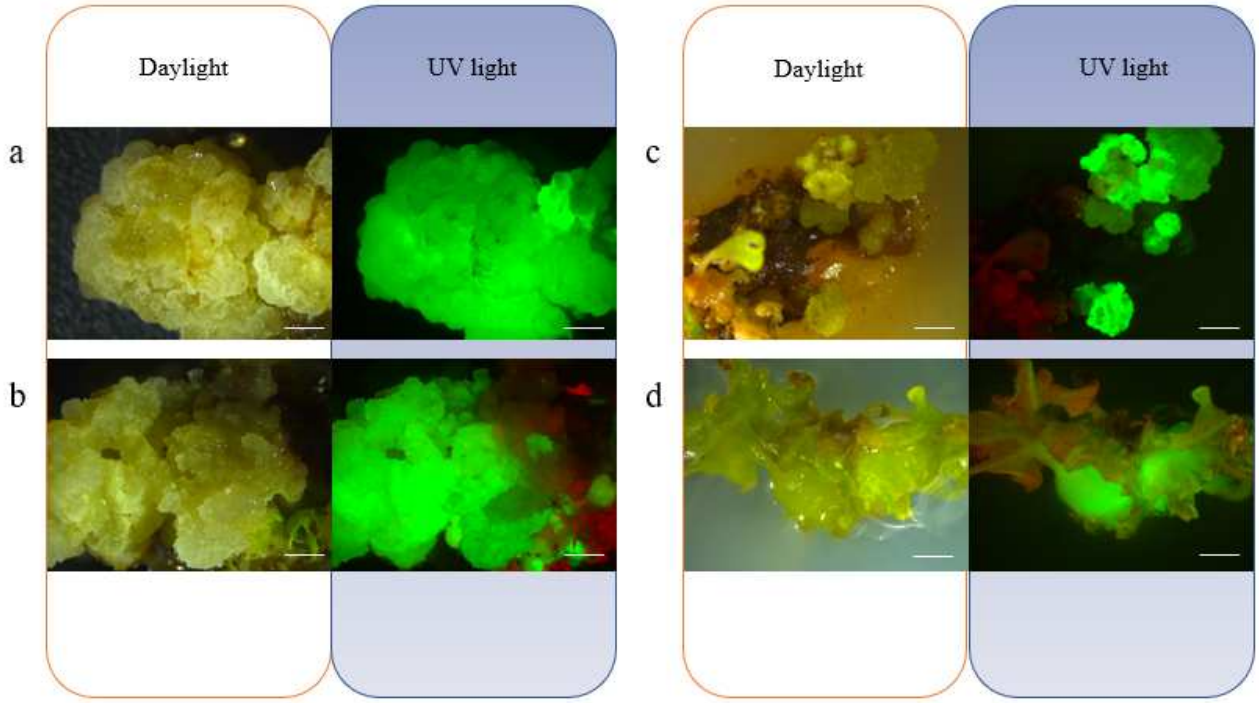


Figure 7: eGFP-fluorescing calli of Albana (a), Vermentino (b), 110 Richter (c) and shoots of Thompson Seedless (d) after 9 weeks of culture on media containing 70 mg L^{-1} of kanamycin. (bar = 2 mm).

Southern blot analysis

Thompson Seedless was the only genotype that regenerated transgenic shoots (Fig. 8 a, b): five lines from MB slices selected on 70 m g L^{-1} kanamycin were recovered, some of which were isolated after the first 3 weeks of selection (Fig. 8 a), and several transgenic shoot regenerated after 9 weeks of culture on medium. The putative transformed shoots were isolated and transferred on rooting medium. Five putative transgenic lines (i.e #1, #2, #3, #4, and #5) obtained through the combined use of eGFP and kanamycin selection were able to produce roots *in vitro*. Southern blot analysis revealed that line #3 contained one copy of the transgene; lines #1 and #2, had five and four copies respectively (Fig. 8 c). The remaining two lines, # 4 and #5, displayed the same pattern of hybridization signals (i.e. 3 copies of the transgene), thus representing a single transformation event (Fig. 8 c).

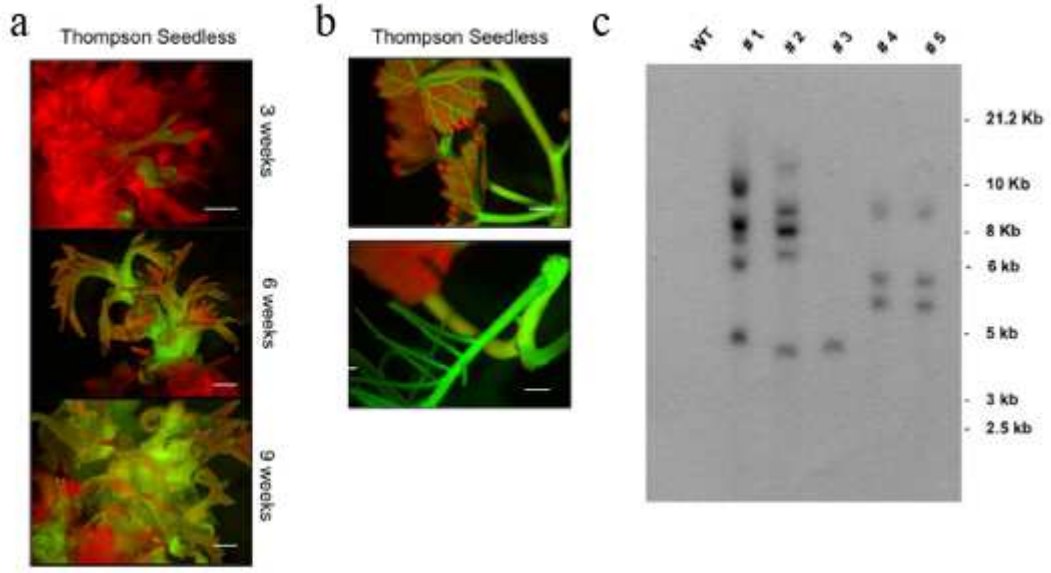


Figure 8: eGFP-detection and transgenic state evaluation in different grapevine genotypes: (a) Thompson Seedless transgenic shoot regeneration at 3, 6, 9 weeks on kanamycin 70 mg L−1; (b) Thompson Seedless elongated and rooted transgenic shoot. Uniform fluorescence with bright green color was observed in transformed tissues under UV light (bar = 2 mm); (c) Transgenic state of T0 35 S::eGFP grapevine transgenic plants evaluated by Southern blot. 20 µg of genomic DNA from control non-transformed plant (lane 1) and transgenic lines #1, #2, #3, #4, and #5 (lanes 2–6, respectively) was digested with HindIII. The image was obtained after overnight exposure.

3.1.2 Transformation efficiency of the *Agrobacterium*-infected MBs with hairpin gene construct against *Plasmopara viticola*

A. tumefaciens strain (EHA101) carrying two different gene constructs were used to transform MB slices of Thompson Seedless. Only regenerants expressing eGFP picked up from individual MB cultured on a kanamycin-enriched medium were counted as putative primary transgenic clones. Using the chimaera gene construct (*Pv-DCL1/2*), 5 shoots expressing diffuse fluorescence (Fig. 9 e-f) were produced during the regeneration/selection phase, and one shoot exhibited a chimeric behaviour. When the *Pv-DCL1* gene construct was used, the number of shoots expressing diffuse fluorescence were three (Figure 9 a-b) plus one chimeric line (Figure 9 c-d). All these independent lines were isolated and proliferated, increasing the number of shoots for each line. For the chimeric plant management, a prolonged culture in kanamycin and a strong mechanical treatment were applied in order to eliminate the calli areas that did not show fluorescence, encouraging the development of only fluorescent-putatively transformed cells. Overall, 0,69% and 0,63% of MB slices were able to regenerate at least one putative transgenic shoot expressing diffuse fluorescence under UV light using *PvDCL1/2* and *Pv DCL1*, respectively.

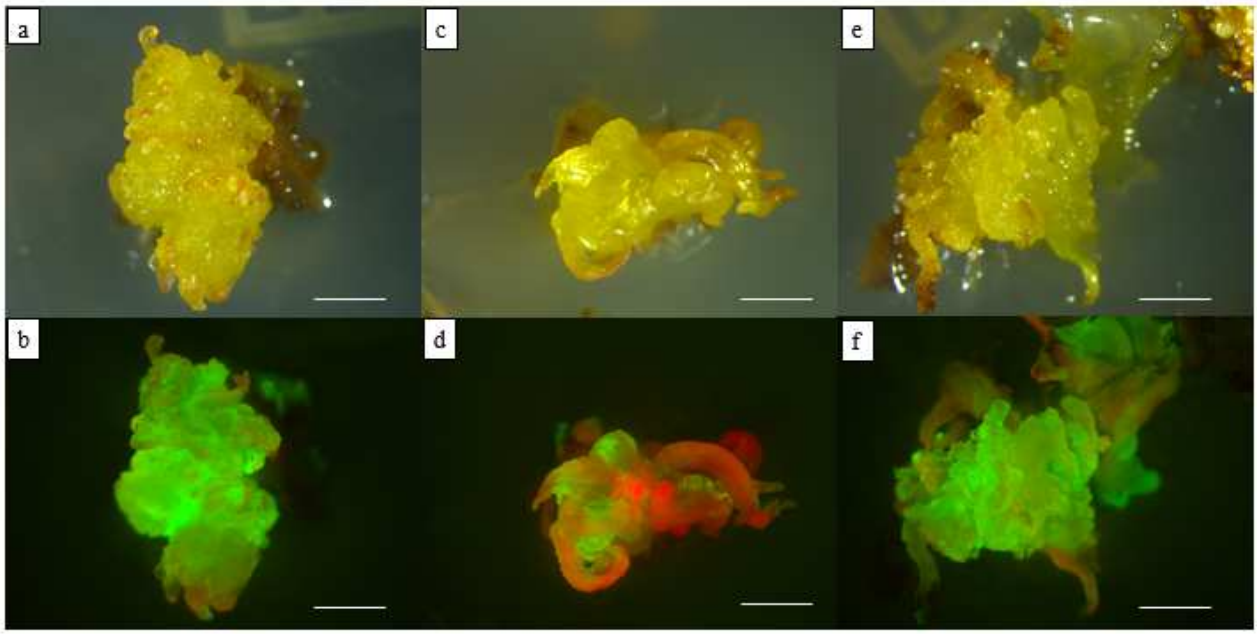


Figure 9: eGFP-fluorescing shoots regenerated from eGFP calli of Thompson Seedless after 12 weeks of culture transformed with *Pv-DCL 1* gene construct (a, b, c, d), and *Pv-DCL 1/2* gene construct (e, f). Chimeric explants are composed by fluorescent cell clusters dispersed in red areas. (bar = 2 mm).

Putative transgenic explants appeared strongly affected by vitrescence (fig 9 a-d), with consequent problems during the elongation of the plant material. Most of the elongated shoots isolated from a medium containing 1 µM of BAP and 25 mg L^{-1} of kanamycin have not produced roots on PGR-free MS medium. After five more additional months, thanks to the expertise built up by Vitroplant, we were able to root five putative transgenic lines that emitted strong fluorescence when shoots or roots were visualized under UV light (three transformed with chimaera gene construct and two with *Pv DCL1* RNAi gene construct), that were acclimatized in alveolar seedbeds (Figure 10).

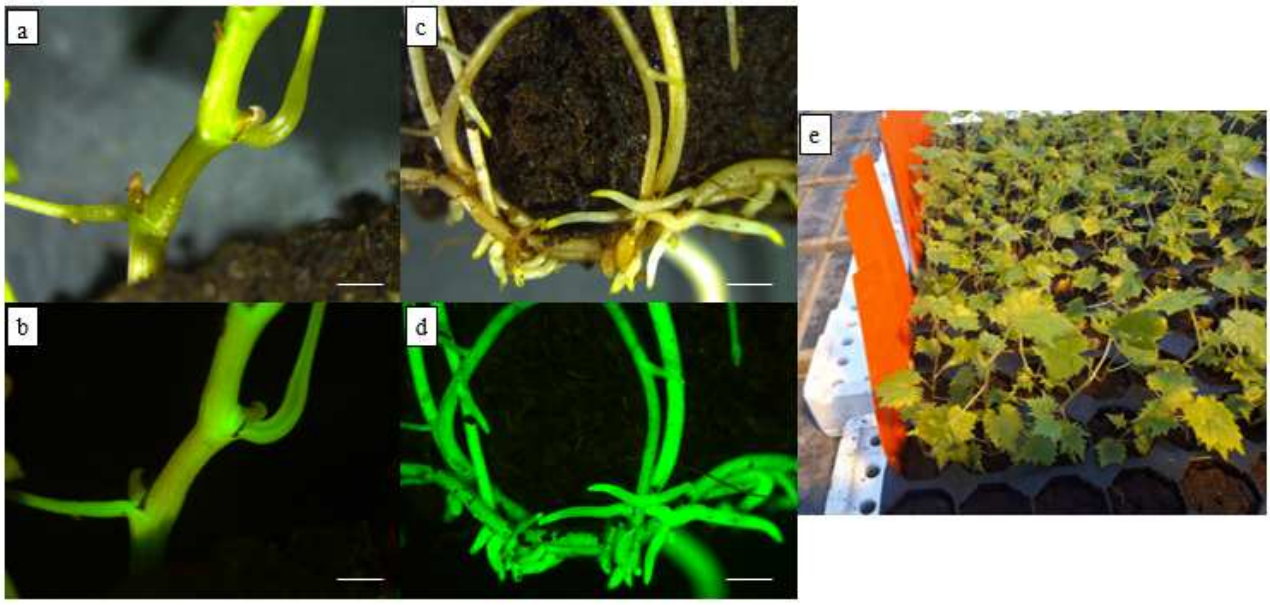


Figure 10: Transgenic *Pv DCL1/2* Thompson Seedless plants displayed in white light (a, c) or under UV light (b, d). Putative transgenic plants after the acclimatization phase in alveolar seedbeds placed on the greenhouse e).

Molecular analysis of transgene integration

PCR analysis have been conducted on the five *Pv DCL1/2* and on the three *Pv DCL1* Thompson Seedless independent lines that expressed fluorescence signal in order to confirm the integration of transgenes into the grape genome. Considering the *Pv DCL1/2* lines, the dilution protocol was applied starting from *in vivo* leaf samples, for #2, #3 lines, whereas *in vitro* material was used for the lines #0, #1, #4. The results indicated that a 515 bp DNA fragment corresponding to the *Pv DCL1/2* gene sequence was present in all the five lines analysed, but it was absent in the wild type line and in the negative control sample containing water (Fig. 11). All transgenic plants analysed produced the *Pv DCL1/2* gene-specific 515 bp fragment after PCR amplification.

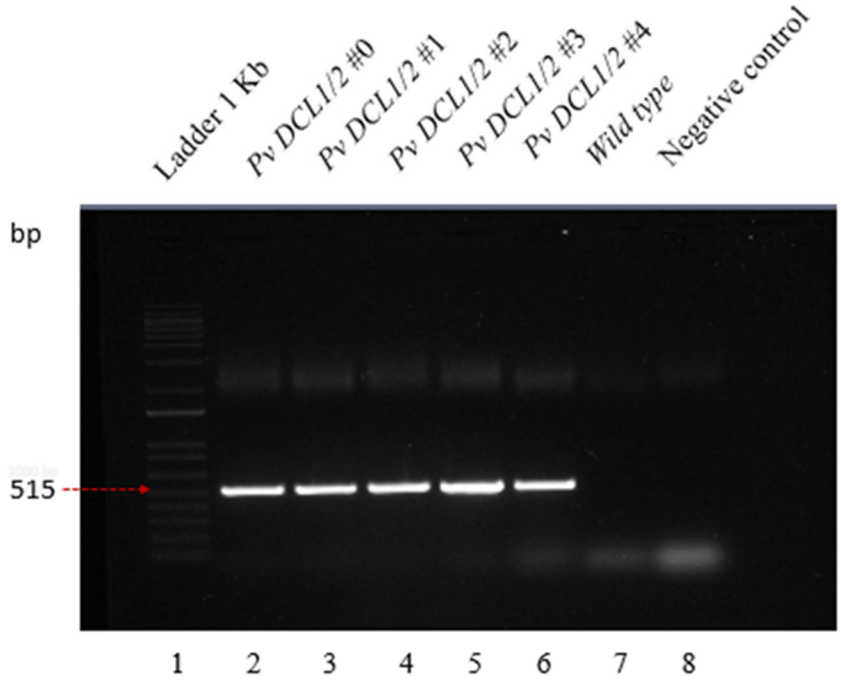


Figure 11: Molecular analysis of *Pv DCL1/2 V. vinifera* cv. Thompson Seedless grapevine lines. PCR analysis of DNA isolated from leaf tissue of non-transformed and transgenic Thompson Seedless plants. A 515 bp DNA fragment corresponding to *Pv DCL1/2*-specific hairpin gene sequence is shown. Negative control, deionized sterile water; *Wild type*, non-transformed Thompson Seedless; *Pv DCL1/2* #0, #1, #2, #3, #4, transgenic Thompson Seedless plants transformed with EHA101 *Pv DCL1/2* RNAi gene construct.

Regarding the *Pv DCL1* lines the dilution protocol was applied, starting from *in vitro* leaf samples, for the lines #9, #10, #11. The results indicated that a 340 bp DNA fragment corresponding to the *35S promoter* gene sequence was present in all the three lines and in the positive control analysed, but it was absent in the wild

type line and in the negative control sample containing water (Fig. 12). All transgenic plants analysed produced the *35S promoter* gene-specific 340 bp amplicon after PCR amplification.

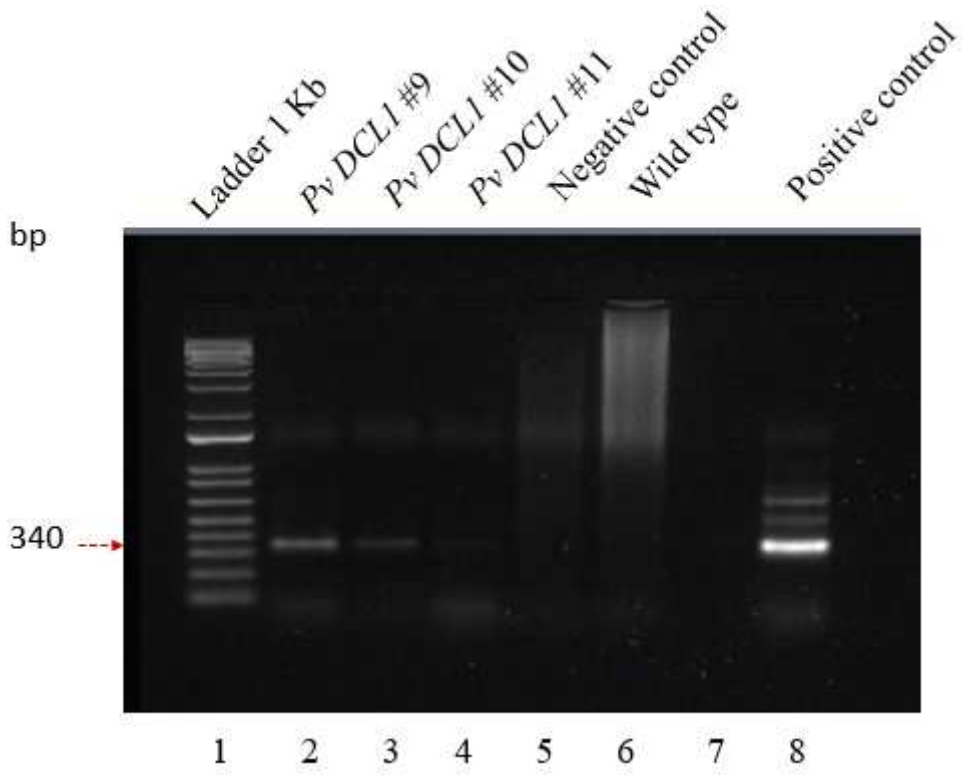


Figure 12: Molecular analysis of *Pv DCL1 V. vinifera* cv. Thompson Seedless grapevine lines. PCR analysis of DNA isolated from *in vitro* leaf tissue of non-transformed and transgenic Thompson Seedless plants. A 340 bp DNA fragment amplified from the *35S*-specific gene sequence is shown. Positive control, plasmidic DNA extracted from *A. tumefaciens* EHA101 carrying *Pv DCL1* RNAi gene construct; Negative control, deionized sterile water; *Wild type*, non-transformed Thompson Seedless; *Pv DCL1* #9, #10, #11, transgenic Thompson Seedless plants transformed with EHA101 *Pv DCL1* RNAi gene construct.

3.1.3 Transformation efficiency of the *Agrobacterium*-infected MBs with hairpin gene construct against *Botrytis cinerea*

In this study, 600 MB slices of Thompson Seedless were inoculated with *Agrobacterium tumefaciens* GV3101 harbouring *Bc-DCL1/2* hairpin gene construct. As observed in the other transformation trials, tissues became brown due to antibiotic selection but in this case the identification of putative transgenic cells was more difficult due to the absence of the reporter gene *eGFP*. Only some callus area maintained a high regenerative capacity while the other parts of the meristematic tissues became progressively necrotic and were discarded. When new shoots were visible (Figure 13 a), they were selected and transferred on selection medium enriched with the same high cytokinin content (13,2 µM), maintaining 70 mg L^{-1} of kanamycin for the whole process. After several subculture characterised by the increase of the auxin/cytokinin ratio (Figure 13 b) a total of four putative transgenic lines, corresponding to a transformation efficiency equal to 0,67%, were isolated and were

able to root in a kanamycin-enriched PGR free MS medium (Figure 13 c). three putative transgenic line was successfully acclimatized in plastic pots (Figure 13 d), and the other one is still rooting *in vitro.*

Figure 13: Selection process of putative transgenic lines regenerating on kanamycin-enriched medium. Green shoots that gradually regenerates in kanamycin enriched medium were identified (a). These lines were proliferated separately from the other explants in glass pots having the same antibiotic composition, but a lower content of PGR (BAP 4.4 µM) (b). After several sub-cultures, elongated shoots were left to grow in a PGR-free medium, maintaining the same kanamycin concentration (c). Plants were acclimatized in plastic pots and then placed in the greenhouse (d) (bar: 5 mm).

Molecular analysis of transgene integration

PCR analysis have been conducted on the four *Bc DCL1/2* Thompson Seedless independent lines that in order to confirm the integration of transgenes into the grape genome. Regarding the *Bc DCL1/2* lines, the dilution protocol was applied starting from *in vivo* leaf samples, for #5, #7, #8 lines, whereas, *in vitro* material was used for the lines #6. The results (Fig. 14) indicated that a 340 bp DNA fragment corresponding to the *35S promoter* gene sequence was present in all the four transgenic lines analysed and in the positive control represented by *Pv DCL1/2* #3 transgenic line, but absent in the wild type line and in the negative control sample containing water. All transgenic plants analysed produced the *35S* gene-specific 340 bp fragment after PCR amplification.

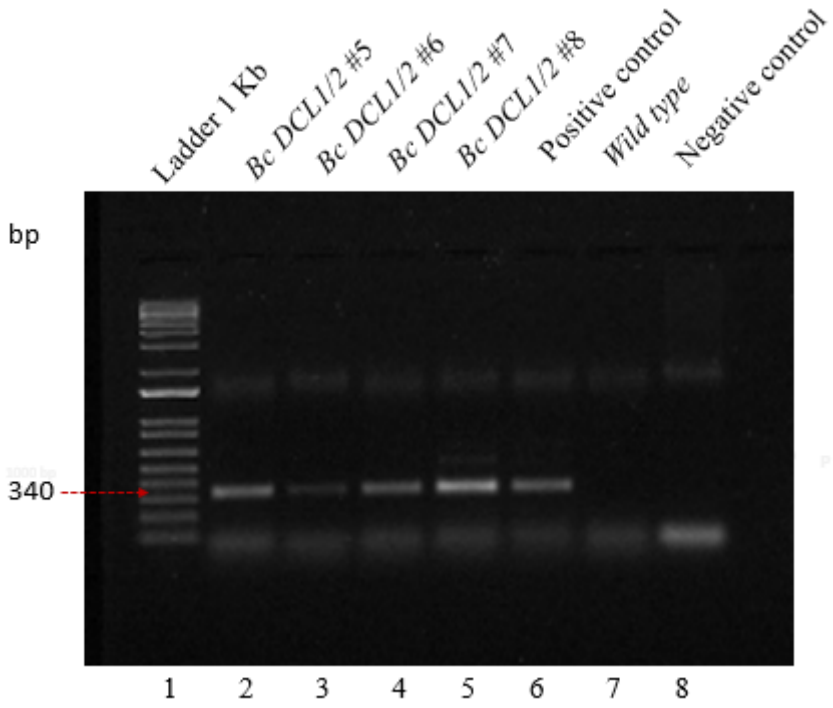


Figure 14: Molecular analysis of *Bc DCL1/2 V. vinifera* cv. Thompson Seedless grapevine lines. PCR analysis of DNA isolated from leaf tissue of non-transformed and transgenic Thompson Seedless plants. A 340 bp DNA fragment amplified from the *35S promoter*-specific gene sequence is shown. Negative control, deionized sterile water; Positive control, *Pv DCL1/2* #3 Thompson Seedless transgenic line; *Wild type*, non-transformed Thompson Seedless; *Bc DCL1/2* #5, #6, #7, #8 transgenic Thompson Seedless plants transformed with GV3101 *Bc DCL1/2* RNAi gene construct.

3.2 *Agrobacterium*-mediated transformation of grapevine somatic embryos

Embryogenic calli were successfully initiated and developed in somatic embryos for each of the cultivar (data are reported in chapter two). The somatic embryos were initiated in culture in DM medium (Dhekney et al., 2016) with the selective antibiotic kanamycin and the antibiotics capable to curb *A. tumefaciens* growth, to stimulate secondary embryogenesis after *Agrobacterium*-mediated transformation using a hairpin RNAi gene construct harboured by EHA101 strain. Agro-infected explants were co-cultured with *A. tumefaciens* for 72 hours in dark condition. After co-culture, somatic embryos have been observed under a UV stereomicroscope for GFP expression determination. Therefore, their receptivity to genetic transformation was analysed (Table 2). Thompson Seedless appeared highly efficient, showing a diffuse fluorescence on a numerous number of somatic embryos (Fig. 15 e-f and Table 2). About 90% of somatic embryos of Ancellotta exhibited transient transformation visible by fluorescence emission (Fig. 15 a-b). Lambrusco Salamino (Fig. 15 c-d) reached an efficiency in GFP expression that averaged around 50%.

Table 2: Transient genetic transformation efficiency of Thompson Seedless, Ancellotta, and Lambrusco Salamino after co-culture with *A. tumefaciens*. The results are related to one transformation trial.

Cultivars	Total explants agro-infected	Total explants expressing GFP	Transient genetic transformation (%) (3 days after infection)
Thompson Seedless	101	101	100%
Ancellotta	97	92	89,24%
Lambrusco Salamino	107	46	49,22%

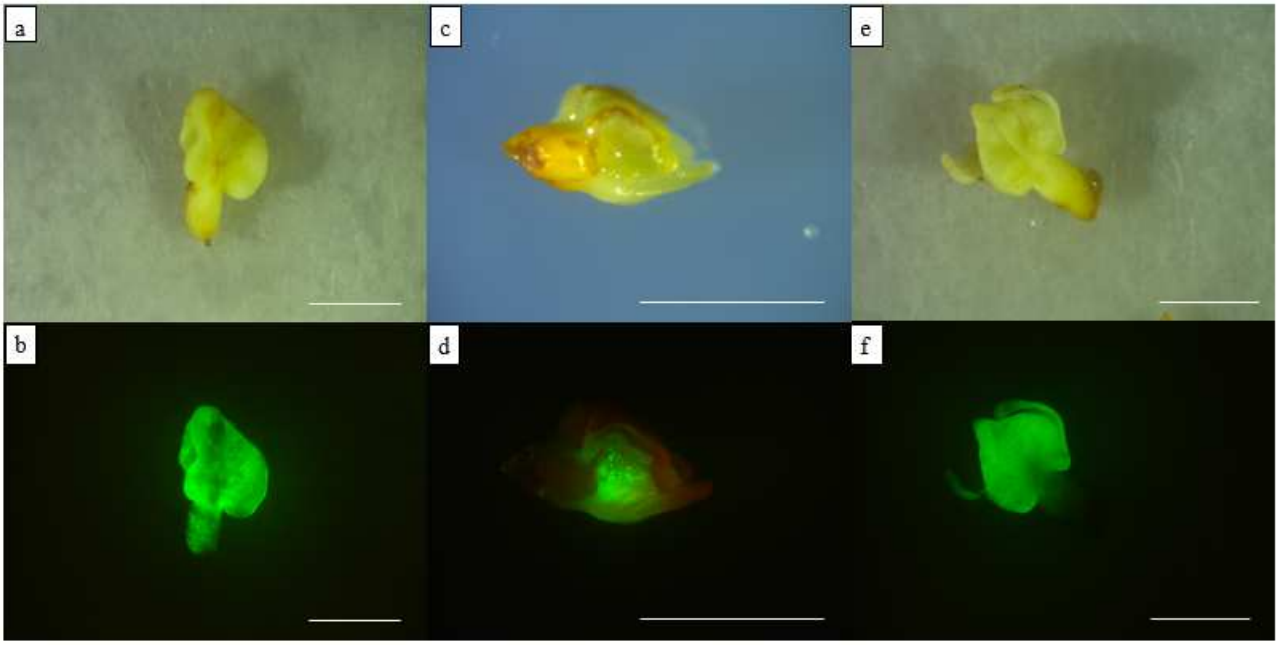


Figure 15: Representative images collected after *Agrobacterium*-mediated transformation of Ancellotta (a-b), Lambrusco Salamino (c-d), and Thompson Seedless (e-f) somatic embryos selected at the advanced cotyledonary stage, illuminated by white light (a, c, e) and UV light (b, d, f) (bar: 2 mm).

After *Agrobacterium* inoculation and more precisely after the washing step, most somatic embryos became brown, producing exudates which darkened the washing solution. Following 4 weeks in completely dark

condition at 24°C, some explants of Ancellotta (Fig. 16 a-d) and Thompson Seedless (Fig.16 e-h) started to develop calluses, which had a conformation very similar to embryogenic calluses.

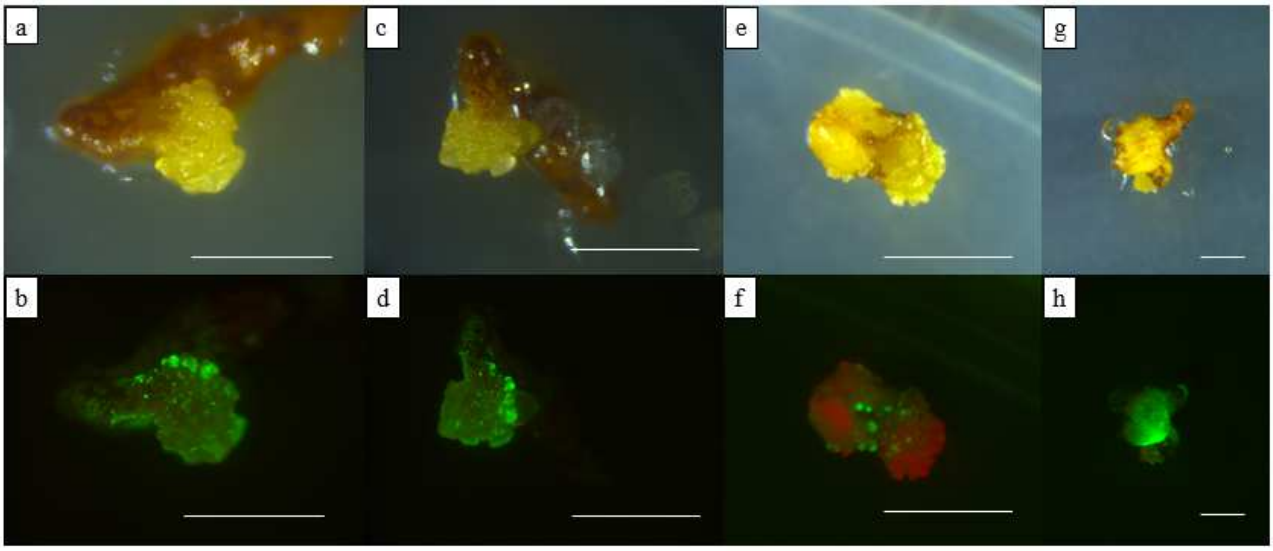


Figure 16: Representative images of calluses developed after 4 weeks of culture on DM medium with 200 mg L^{-1} each of carbenicillin and cefotaxime, and 100 mg L^{-1} of kanamycin of Ancellotta (a-d), and Thompson Seedless (e-h) somatic embryos, illuminated by white light (a, c, e, g) and UV light (b, d, f, h) (bar: 2 mm).

Ancellotta and Thompson Seedless developed promising calli after only four weeks of culture, while in Lambrusco Salamino the development of the calli was almost nil. Only explants that showed calli were transferred on X6 medium, supplemented with 70 mg L^{-1} of kanamycin, 200 mg L^{-1} of carbenicillin and cefotaxime. Unfortunately, all somatic embryo-derived explants did not continue to develop the presumed embryogenic calluses or even to produce transgenic somatic embryo lines during around six months of selection occurred by monthly transfer on fresh X6 medium. Somatic embryo-derived explants have stopped growing and they turned brown, progressively losing their fluorescence, up to necrosis during selection, as we observed in the previous transformation tests conducted on Ancellotta and Lambrusco Salamino somatic embryos. Moreover, maintaining the explants on DM medium for one or two additional months (transferring them to fresh medium at 4 weeks interval), all the explants failed to develop well-shaped embryogenic calluses, compromising the effectiveness of the whole experiment.

3.3 *Agrobacterium*-mediated transformation of somatic embryo-derived cotyledons and hypocotyls, via organogenesis

3.3.1 Genetic transformation efficiency in cotyledons and hypocotyls cultured on two regeneration/selection media

For transformation studies Ancellotta, Lambrusco Salamino, and Thompson Seedless cotyledons and hypocotyls were employed to stimulate the induction of adventitious bud formation, trying to preferentially stimulate new regeneration from transformed cells. Two PGR combinations were tested, the first was characterized by a higher auxin/cytokinin ratio (culture medium 1) and the second with the exclusive presence

of BAP. Transformation efficiency was determined after 9 weeks of selection by the visualization of the expression of GFP. Cotyledons and hypocotyls started to form adventitious buds that were visible already after 3 weeks in Thompson Seedless cotyledons. Transformation experiments for Thompson Seedless cotyledons with kanamycin selection produced an overall transformation efficiency higher than 50% in both culture media used (Fig. 17)

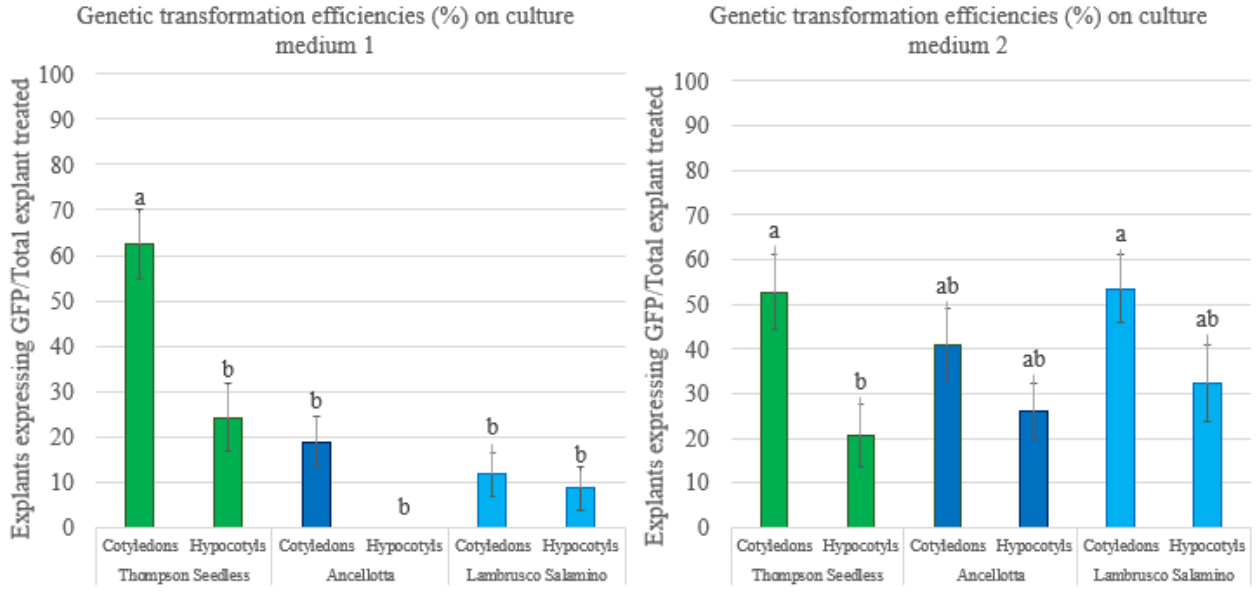


Figure 17: Genetic transformation efficiency (%) at 9 weeks of Ancellotta, Lambrusco Salamino, Thompson Seedless cotyledons and hypocotyls cultured on regeneration/selection media 1 and 2. Means with different letters are significantly different according to the Student-Newman-Keuls ($p < 0.05$) ± SE (n=50).

Cotyledons of Thompson Seedless had the highest transformation efficiency on culture medium 1 while sharing high levels of genetic transformation efficiency with Lambrusco Salamino cotyledons when were cultured on medium 2. As we observed during the regeneration experiments, cotyledons exerted a great regeneration competence rather than hypocotyls. Differences in the fluorescent expression rate have been found when the same type of explant was cultured on different media, especially regarding cotyledons: they ranged from less than 10% for Thompson Seedless (switching from medium 1 to medium 2) to more than 40% for Lambrusco Salamino (switching from medium 2 to medium 1). The culture medium containing the highest cytokinin concentration allowed to increase the number of explants showing green fluorescent spots in a significant manner on Ancellotta and Lambrusco Salamino cotyledons, comparing the same explants in the two different culture media. Hypocotyls had a reduced incidence of fluorescence on the total number of treated explants compared to cotyledons counterpart, and no significant differences have been reported culturing them in the two-culture media. In Thompson Seedless an average of 10 % of green fluorescent spots developed fluorescent shoots, corresponding to 6 and 3 putatively transformed lines on culture medium 1 from cotyledons (Fig. 18 a-b) and hypocotyls (Fig. 18 j-k), respectively, and 7 and 6 fluorescent lines regenerated on culture medium 2 from cotyledons (Fig. 18 c-f) and hypocotyls (Fig. 18 l-m), respectively. These shoots were characterized as independent putative transgenic lines to be confirmed with molecular analysis. Roots emitting

fluorescence were also reported in Thompson Seedless cotyledons cultured on medium 1 (Fig. 18 a-b). For the first time, we observed the production of a fluorescent shoot in Ancellotta cultivar, although chimeric, from an agro-infected cotyledon (Fig. 18 g-h) cultured on culture medium 2, a fact that was never been observed during genetic transformation of MBs of the same cultivar. We also isolated a green fluorescent callus of Lambrusco Salamino, that being non-morphogenic, failed to produce transgenic shoots.

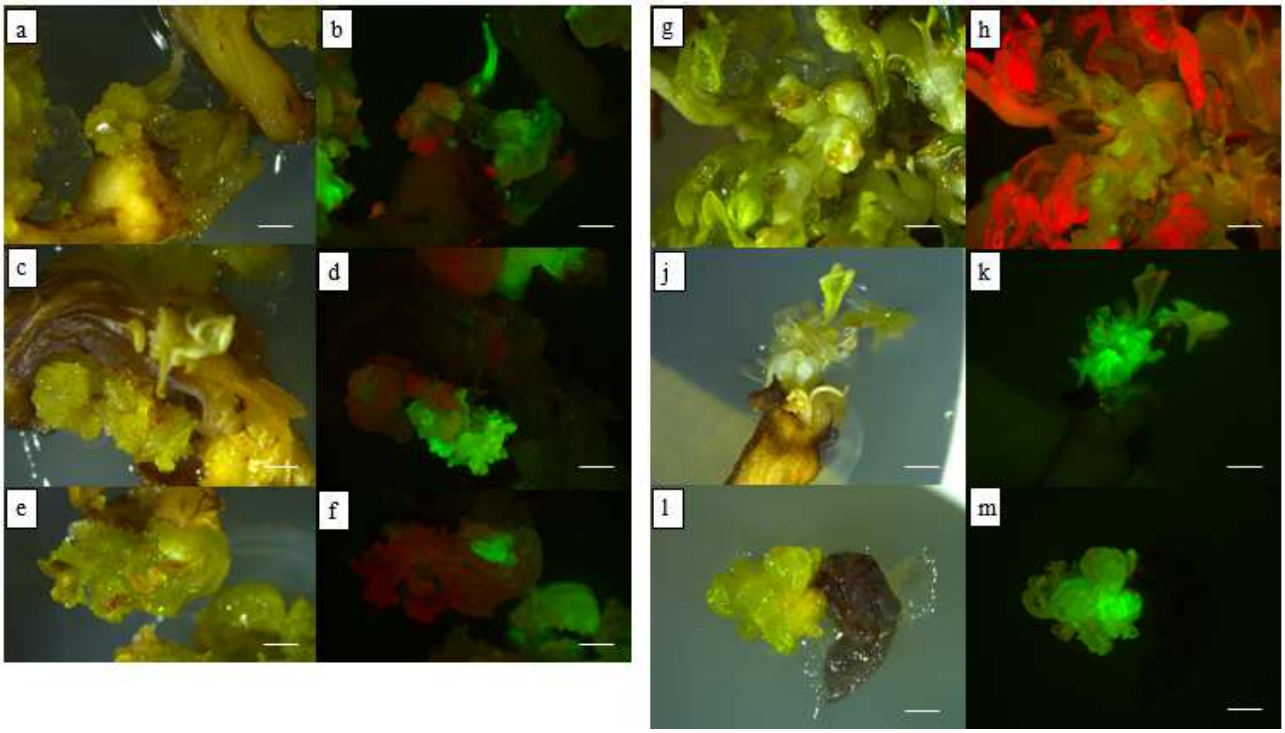


Figure 18: Representative images of shoots or shoots-derived structures developed after 9 weeks from agro-infection on Thompson Seedless (a-f) and Ancellotta (g-h) cotyledons, and Thompson Seedless hypocotyls (j-m), illuminated by white light (a, c, e, g, j, l) and UV light (b, d, f, h, k, m). (a-b) Thompson Seedless cotyledons cultured on culture medium 1; (c-f) Thompson Seedless cotyledons cultured on culture medium 2; (g-h) Ancellotta cotyledons cultured on medium 2; (j-k) Thompson Seedless hypocotyls cultured on medium; l-m) Thompson Seedless hypocotyls cultured on medium 2 (bar: 2 mm).

3.3.2 Comparison of genetic transformation efficiency using MB slices, cotyledons and hypocotyls as starting explants in genetic transformation trials

GFP reporter gene allowed real-time monitoring of transgene expression, immediately after agro-inoculation. Genetic transformation efficiency was assessed at 9 weeks of selection on the same number of MB slices, cotyledons and hypocotyls that were inoculated with the same gene construct (targeting *Pv-DCL 1*). The experiment was carried out using the model cultivar Thompson Seedless, the only one capable of regenerate transgenic shoots form all the explants tested, in order to individuate the most efficient starting explant in terms of putative transgenic events (calli or shoots) arose from a fixed number of samples, set at 100. The selection occurred in the same regeneration/selection medium optimized for MB by Sabbadini and co-authors (Sabbadini, et al. 2019b).

In line to this experiment, it was possible to associate at each starting explant one class of genetic transformation rate (%): about 20% of hypocotyls, 40% of MBs, and 60% of cotyledons explants expressed GFP considering the total number of treated explants (Figure 19).

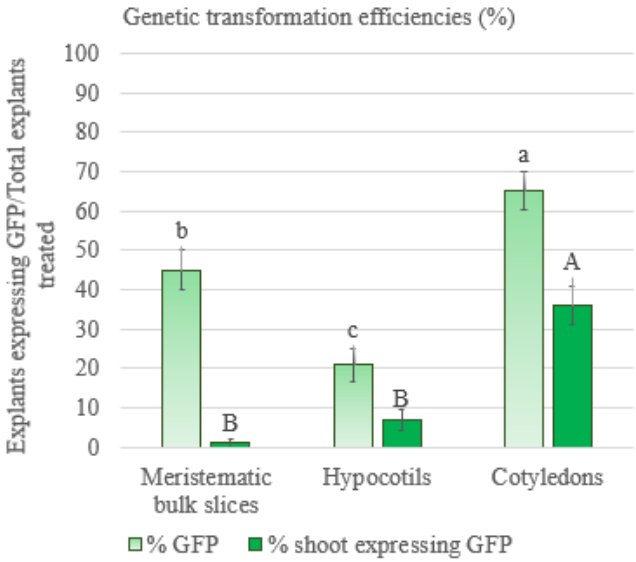


Figure 19: Percentage value of transformation efficiency reported both as the explants that expressed at least one fluorescent calli area/total treated explants and the explants that regenerate at least one fluorescent shoot/total explants treated. Transformation efficiency was recorded after 9 weeks of genetic transformation experiments conducted on MB slices, hypocotyls and cotyledons of Thompson Seedless cultivar transformed with *Pv-DCL1* RNAi gene constructs. Small letters represent differences in GFP expression between the different explants in terms of calli that showed at least one fluorescent dot on its surface; capital letters represent differences in GFP expression between the various explants in terms of shoots expressing GFP. Means with different letters are significantly different according to the Student-Newman-Keuls ($p < 0.05$) ± SE (n=100).

Differences have been found on the emergence of transgenic shoots and their development. Cotyledons showed the highest significant transformation rate in terms of explants expressing GFP and fluorescent shoots, compared to meristematic bulk slices and hypocotyls explants. Although MB slices had in average a significantly higher number of explants expressing GFP than hypocotyls, this difference resulted no more significant when we compared the number of shoots expressing GFP between the two types of explants. A total of 1, 7 and 36 shoots showed GFP on MB, hypocotyls, and cotyledons, respectively. Cotyledons and hypocotyls were able to regenerate more than one putative transgenic shoot from the same explant (Figure 20 e-f).

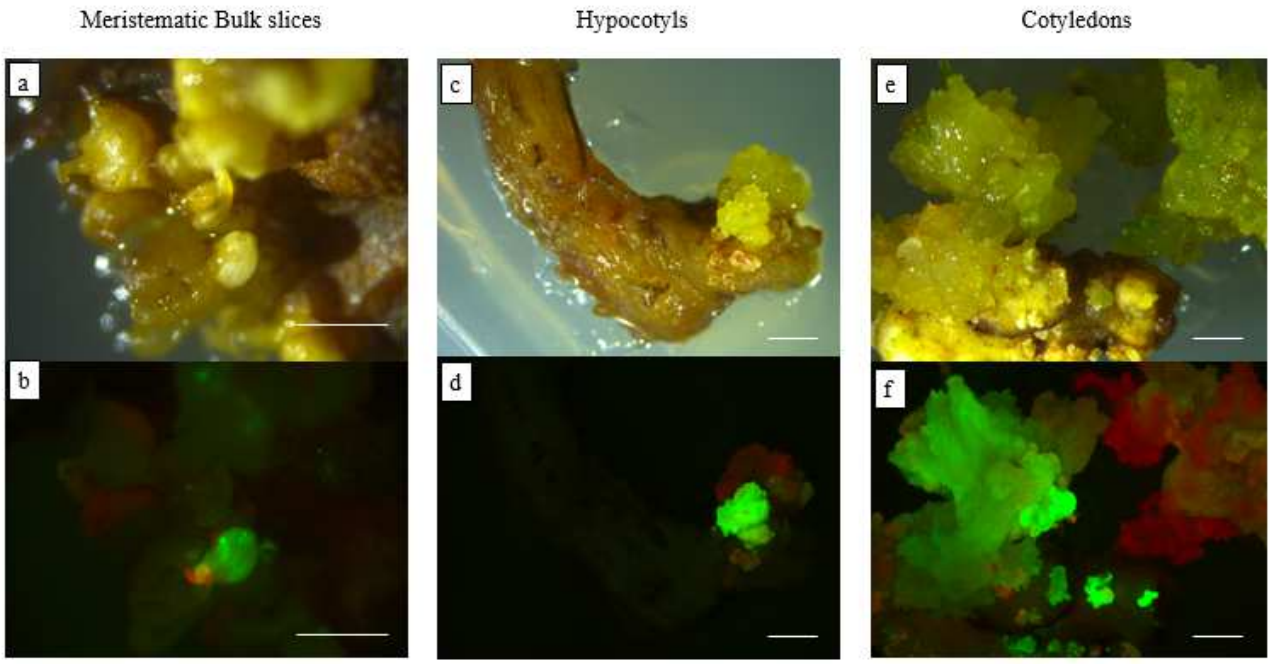


Figure 20: Representative images of shoots or shoots-derived structures developed after 9 weeks of culture on MS medium with 200 mg L^{-1} each of carbenicillin and cefotaxime, and 70 mg L^{-1} of kanamycin using Thompson Seedless MB slices (a-b), hypocotyls (c-d), and cotyledons (e-f), illuminated by white light (a, c, e) and UV light (b, d, f) (bar: 2 mm).

4. Discussion

4.1 *Agrobacterium*-mediated transformation of grapevine meristematic bulk slices

The regeneration and transformation protocol via organogenesis described here, that was designed by Mezzetti and co-authors in 2002 (Mezzetti et al., 2002) was applied in various grapevine cultivars (Albana, Ancelotta, Chardonnay, Ciliegiolo, Glera, Lambrusco Salamino, Merlot, Pinot Grigio, Sangiovese, Vermentino, Thompson Seedless) and *Vitis* spp. rootstocks (110 Richter, Kober 5BB, 1103 Paulsen), in order to detect their transformation competence after the *Agrobacterium*-mediated transformation with *35S-eGFP-NPTII* gene construct. The main aim was to discover a genotype that is capable of producing putatively transgenic shoots, to be used for the expression of RNAi gene constructs designated to silence target genes of pathogens. Although organogenesis from MBs was applied in other analogous researches (Mezzetti et al. 2002; Sabbadini et al. 2019b; Xie et al. 2016), the most exploited regeneration method for transgenic plant production in *Vitis* species remain somatic embryogenesis (Torregrosa et al., 2000; Gambino et al., 2007; Prieto et al., 2019). The method reported here is faster and easy to apply comparing the time-consuming somatic embryogenesis, that may require special equipment and expertise (Iocco et al., 2001; Martinelli et al., 2001b; Dhekney et al., 2009), anticipating the regeneration of transgenic plants by at least two months (Xie et al., 2016). Before genetic transformation experiments, 70 mg L^{-1} of kanamycin was assessed in a toxicity trial on non-transformed MB, as the most performant concentration that inhibits shoot regeneration, balancing the regeneration competence at a level where the regeneration of so-called escape shoots is disadvantaged (Sabbadini et al., 2019c).

Except for Ancellotta, Glera, Merlot, Sangiovese, Pinot Grigio, 1103 Paulsen, the application of MB organogenesis system after agro-infection led to the production of transgenic calli from the other genotypes, and only on Thompson Seedless also of transgenic shoots. The genotype and the selection procedures are shown to play a crucial role in successful grapevine transformation (Dhekney et al., 2012). Etiolated meristematic buds (Dutt et al., 2007) and meristematic bulks (Xie et al., 2016) of Thompson Seedless produced respectively 18 and 111 independent lines, using different concentration of kanamycin during selection. Contrary to what was reported by other researches, we did not observe transgenic shoot production on cv. Chardonnay (Xie et al., 2016).

The identification of putative transgenic lines, the isolation of transformed cell from the rest, the removal of necrotic or non-transformed areas of the calli and the individuation of chimeric individuals was made easier combining the presence of the selective agents (kanamycin at 70 mg L^{-1}) and the recurring visualization of fluorescence under UV stereomicroscope emitted by transformed cells in the MB (Sabbadini et al., 2019b). The usefulness of the green fluorescent protein reporter gene has been recognized in transgenic studies that involved different species, allowing to easily monitor and discriminate escapes shoots from the transformed ones (Rakosy-Tican et al., 2007).

From the results obtained, as mentioned by Sabbadini and co-authors a high regeneration competence does not correspond to a high transformation efficiency, which seems to be a genetic predisposition with the mutual interaction with PGRs added during selection (Sabbadini et al., 2019c). Not only interspecific but also intraspecific variability was reported in this study. Apart from Thompson Seedless and Vermentino among the cultivars, and 110 Richter among the rootstocks tested, exhibited the highest percentage of calli expressing GFP on the total number of inoculated MB, although they have led to the exclusive production of transgenic calli expressing eGFP. Thompson Seedless transgenic shoots regenerated from meristematic green cell clusters, differently from what happened in the other genotypes tested where the GFP expressing areas appeared in non-organogenic tissues, mainly organized in a whitish callus.

The screening of the various genotypes has allowed us to select Thompson Seedless as the candidate table cultivar for the application of Host induced gene silencing. Genetic transformation of Thompson Seedless using *eGFP-NPTII* gene construct was 5% while using hairpin gene constructs targeting *DCL 1/2* of *Plasmopara viticola* and *Botrytis cinerea*, genetic transformation efficiencies were quite lower. This reduction in transformation efficiency may be related to the diverse transformation efficiencies exerted by different *Agrobacterium* strains. The highest genetic transformation efficiency was recorded using EHA105 strain, which was considered a super-virulent competent strain especially on grapevine (Torregrosa et al., 2002), rather than EHA101 and GV3101 that were the biological vectors of *Pv-DCL 1/2* or *Pv-DCL 1,* and *Bc-DCL 1/2* gene constructs respectively. However, all these strains were employed in successful genetic transformation trials (Bouquet et al., 2006; Fan et al., 2008; VIDAL et al., 2010). The use of RNAi gene construct could be attractive as the hairpin-shaped sequences do not provide transgene derived protein accumulation in the plant cells, as it normally occurs using a standard gene construct (Sidorova et al., 2019). The recovery of HIGS Thompson Seedless lines required a longer time compared to the traditional genetic

transformation applying the same protocol. However, a total of 5, 3, and 4 independent putative lines have been obtained applying this protocol using *Pv-DCL 1/2*, *Pv-DCL 1,* and *Bc-DCL 1/2* RNAi constructs respectively, and two additional chimeric lines (expressing *Pv-DCL 1/2*, *Pv-DCL 1* hairpin gene construct) are now under selection, in order to stimulate the development of calli area that express a bright fluorescence.

4.2 *Agrobacterium*-mediated transformation of grapevine somatic embryos

According to comparable study (Li et al., 2001; Dai et al., 2015; Dhekney et al., 2016) whether embryonic competence induction require high auxin concentration, the morphogenesis expression and therefore SE emergence occur usually lowering or removing auxin in the culture media (T. H. Franco et al., 2006). The transition from medium DM to X6 was performed precisely to stimulate the emergence of the embryos, as reported in the original protocol.

Unfortunately, Transient GFP expression gradually decreased in both intensity and frequency over time as cultures were subjected to kanamycin selection. Li and co-authors in 2006 highlighted the influence of a 7 days preculture of Thompson Seedless SEs in fresh X6 medium in increasing stable transformation efficiency compared to SEs agro-infected without preculture (Li et al., 2006), although they obtain transgenic SE lines also without pre-culturing the starting material. The selection of the right stage of development is important to determine the applicability of genetic transformation, commonly type II somatic embryos have been considered the most suitable starting explant involving genetic transformation tests of SEs (Franks et al., 1998; Zhou et al., 2014). In accordance to what was reported by Dai and collaborators, the *Agrobacterium*-mediated transformation using SEs did not produce transgenic SE lines, contrary to what happened by transforming PEM, and the same patterns of ceased development and constant increase of necrosis during selection was observed (Dai et al., 2015). However, other researchers, including those who elaborated the protocol that we followed in the course of this study, successfully obtained transgenic independent lines from the secondary embryogenesis induction of SEs (Martinelli and Mandolino, 1994; Franks et al., 1998; Li et al., 2006; Zhou et al., 2014; Dhekney et al., 2016). In genetic transformation attempts oriented in introducing transgenes that can enhance resistance to particular or a group of fungal or viral pathogens, the preferred starting explant used were embryogenic calluses (Le Gall et al., 1994; Xue et al., 1999; Legrand et al., 2003; Gambino et al., 2005; Agüero et al., 2006). Embryogenic calluses are composed by whitish globular masses, similar to PEM, therefore are already specialized for SE production, but they generally require an additional culture on an expression medium to permit the complete histodifferentiation of SEs (Corredoira et al., 2019). The induction of secondary somatic embryogenesis probably requires a further effort of de-differentiation and cellular reprogramming which, together with the stress induced by kanamycin, makes it difficult to sustain the entire process (Pais, 2019). All this could explain the fact that without the application of genetic transformation, secondary somatic embryogenesis was successfully achieved on Ancellotta, Lambrusco Salamino, and Thompson Seedless (described in chapter two) but using different media (Maillot et al., 2006). Further investigations will be organized in new attempts through the selection of somatic embryos type I (Franks et al., 1998) the execution of pre-culture treatment (Li et al., 2006) and the addition of antioxidant compounds (Perl et al., 1996).

4.3 *Agrobacterium*-mediated transformation of somatic embryo-derived cotyledons and hypocotyls, via organogenesis

The embryonic tissues are characterized by a high degree of regenerative competence. This regenerative competence could be exploited to draw up an efficient regeneration protocol that can be applied to different agricultural crops. Mature zygotic embryos of different cereal crops have been widely employed for biotechnological purposes (Ji et al., 2013). However similar experiments on woody plants are scarce, probably due to the high heterozygosity level and the need to cultivate clones with characteristics identical to those typical of the cultivated selected variety. The use of zygotic embryos for transgenic plant production have been reported in species such as *Eucalyptus* (Prakash and Gurumurthi, 2009) and *Salix* (Yang et al., 2013), but there were also selected as efficient starting explant for inducing regeneration via organogenesis or somatic embryogenesis in other related hardwood species (Polito et al., 1989; Tang and Guo, 2001; Du and Pijut, 2008) Although somatic embryos are the final results of a specific developmental pathway that can be induced asexually *in vitro*, their morphology is practically identical to the zygotic embryos (Correia et al., 2016). For this reason, in place of zygotic embryos, we used somatic embryos of grapevine cultivars Ancellotta, Lambrusco Salamino, and Thompson Seedless starting from the indications provided by the only two similar researches conducted on *Vitis* species (Vilaplana and Mullins, 1989; Martinelli et al., 2001b)

The protocols fine-tuned in this study demonstrated a direct regeneration from cotyledons and hypocotyls obtained from somatic embryos and an effective transformation response in terms of shoots expressing green fluorescence, especially in Thompson Seedless cultivar. In addition to meristematic bulk, the combined *eGFP/NPTII* selection system which involves the addition of 70 mg L^{-1} of kanamycin and careful visualization of transformation events (Sabbadini et al., 2019c), was efficiently applied on cotyledons and hypocotyls. The two regeneration media tested, similar to those adopted for the regeneration of mature dormant somatic embryos of Sultana, Grenache, and Gloryvine (Vilaplana and Mullins, 1989), resulted efficient in almost all the cultivars tested, although when more cytokinins were added to the culture medium, an increased fluorescence incidence on cotyledons explants of Ancellotta and Lambrusco Salamino at 9 weeks of selection was observed. This innovative system allowed to increase the number of fluorescent shoots produced in the model cultivar, and to bring to light the regeneration of a chimeric Ancellotta shoot, that was left in *in vitro* proliferation to select only transformed meristematic cells. The greater transformation efficiency of this type of explant was confirmed through the execution of an experiment aimed at defining the best starting explants among MB slices, cotyledons, and hypocotyls for the *Agrobacterium*-mediated transformation on Thompson Seedless cultivar. Applying the cultural conditions previously described, cotyledons explants reached the highest regeneration and transformation efficiency, leading to the production of several independent putative transgenic lines, which are currently in proliferation *in vitro*. However, all the explants including MB slices and hypocotyls played an active role in the regeneration of putatively transformed calluses and shoots.

Noteworthy is the receptivity to genetic transformation of the Ancellotta cultivar when cotyledons and hypocotyls or slices of meristematic bulk are chosen as starting explants. Using MB slices, the transformation efficiency of Ancellotta was nil since the first subculture at three weeks of selection. When cotyledons or

hypocotyls were subjected to Agro-infection, genetic transformation efficiency was about 40% for the first and 25% for the second type of explant, leading to the isolation of one chimeric line. In accordance to Sabbadini and co-workers on 2019, genotype has an influence on determining the transformation competence, but this fact suggest that the regeneration protocol adopted could have a powerful impact in the isolation and selection of new transgenic organs.

5. Conclusion

This study has confirmed the efficiency and adaptability of the *in vitro* regeneration protocol, developed by Mezzetti et al., 2002, for the obtainment of MBs of different cultivar and rootstock of *Vitis*, with a high shoot regeneration capability. The results obtained from the transformation trials, using the gene construct *35S-eGFP-NPTII*, were useful as they helped to identify, among the genotype tested, Thompson Seedless and 110 Richter as the best grapevine cultivar and rootstock. Being Thompson Seedless the only genotype capable of differentiating transgenic shoots, meristematic bulk were used to obtain lines expressing RNAi constructs, targeting particular genes important for the pathogenesis of *Plasmopara viticola* and *Botrytis cinerea*, that have been confirmed by molecular analyses, using *in vitro* or *in vivo* leaf samples. In HIGS experiments against *Plasmopara Viticola*, five lines and three lines expressing *Pv DCL 1/2* and *Pv DCL 1*, respectively, are available for gene construct validation, the same concept is valid for the four HIGS lines expressing *DCL 1/2* specific for *Botrytis cinerea* (Table 3). As the subcultures progressed, we focused on the obtainment of somatic embryos, trying to apply the protocol described by Dhekney and collaborators in 2016 to induce secondary somatic embryogenesis after inoculation with *Agrobacterium tumefaciens* EHA101 carrying the RNAi gene chimaera construct against *Plasmopara viticola*. Despite having performed six transformation assays on Ancellotta and Lambrusco Salamino, no somatic embryos arose from fluorescent putative embryogenic calluses that grew in DM medium in the presence of kanamycin. In the future, other experiments will be run, applying some adjustments to the protocol in order to optimize the growth and selection of fluorescent embryogenic calluses and consequent SEs development. Alternatively, mature somatic embryos at advanced cotyledonary stage were used as the source of somatic structures (cotyledons and hypocotyls), which during *in vitro* cultivation conditions were able to form adventitious buds and therefore to regenerate new shoots. The combined use of this regeneration platform with the *Agrobacterium*-mediated transformation, led to a significant increase production of putative transgenic Thompson Seedless HIGS-*Pv* lines, in the same time period, and the obtainment of one *Pv DCL 1/2* Ancellotta line (Table 3). In this study, different regeneration strategies have been efficiently employed to obtain a suitable number of transgenic lines, which will allow to validate the gene constructs against *Plasmopara viticola* and to confirm the validity of those already inserted in *Arabidopsis* plants for the control of *Botrytis cinerea* (Wang et al., 2016).

Table 3: Summary table of all transgenic lines obtained on Thompson Seedless and Ancellotta which are reported by distinguishing genotype, type of explant, gene construct used and the regeneration method.

Genotype	Starting explant	Gene construct	Number of transgenic lines	Regeneration method
Thompson Seedless	MBs	EHA105 *35S-eGFP-NPTII*	5 independent transgenic lines starting from 200 MBs	Organogenesis
		EHA101 *Pv DCL 1* RNAi	3 transgenic lines starting from 480 MBs	
		EHA101 *Pv DCL 1/2* RNAi	5 transgenic lines starting from 720 MBs	
		GV3101 *Bc DCL 1/2* RNAi	4 transgenic lines starting from 600 MBs	
	SE	EHA101 *Pv DCL 1/2* RNAi	0	Somatic embryogenesis
	Cotyledons	EHA101 *Pv DCL 1/2* RNAi	About 3 transgenic lines from 50 cotyledons*	Organogenesis
		EHA101 *Pv DCL 1* RNAi	About 5 transgenic lines from 100 cotyledons*	
	Hypocotyls	EHA101 *Pv DCL 1/2* RNAi	About 2 transgenic lines from 50 cotyledons*	
		EHA101 *Pv DCL 1* RNAi	About 4 transgenic lines from 100 cotyledons*	
Ancellotta	Cotyledons	EHA101 *Pv DCL 1/2* RNAi	About 1 transgenic line from 50 cotyledons*	

* These transgenic lines expressed fluorescence signal under UV but are currently proliferating *in vitro*

5 - CHAPTER FIVE: Spray-induced gene silencing against *Botrytis cinerea* on grapevine leaves and berries

1. Introduction

The constant increase of the world's human population and the negative impact of climate change on agricultural production risk putting a strain on the primary sector (Nishimoto, 2019). Plant production can be strongly limited by abiotic adversities such as stress or water stagnation, salinity, exceptional weather events, cold or heat, and biotic adversities, such as pathogens, insects, and weeds. To defend plants against the abiotic adversity, farmers commonly adopt a preventive approach, choosing the genotype that best suits the environmental conditions of the place; for biotic stresses, both preventive and curative approaches are required. Plants defence against pathogens and pests often results in the application of pesticides with more or less specific action during the course of the crop cycle. Pesticides used for crop protection are globally increasing each year, and the average use of pesticides per area of cropland has gone from almost 1,55 kg/ha in 1990, to 2,63 kg/ha in 2018 (FAOSTAT, 2018). China, USA, and Argentina are the countries with the greatest use of pesticides, and Italy holds the European record among the countries that use most pesticides per cultivated area (WorldAtlas, 2018). These molecules are commonly liquid and applied by spray treatment which can give rise to point-like contamination, for example following an accidental spill or water washing of the spraying machines, or widespread contamination due to volatilization and consequent relapse, drift during treatments, surface flow, and leaching (Carter, 1999). Although they undergo processes of dispersion in the environment and degradation by biotic and abiotic factors, these substances are generally first tested *in vitro* for the evaluation of possible acute toxicity, given the poor selectivity of some active compounds (Van Der Werf, 1996). Scientist have demonstrated that the spatial distribution in latitude from the equator of various pathogens and pests around the world has been promoted by global warming (Bebber et al., 2013). Fungi and oomycetes are greatly affecting food production, negatively affecting both pre-and post-harvest management, and they represent one of the main targets of pesticides application (Bebber and Gurr, 2015). In addition to environmental and health issues, the frequent and continuous application of fungicides can also lead to the selection of resistant pathogen strains, in which their action may only refer to one or few target site (Fisher et al., 2012). In this scenario, it is essential to broaden the knowledge of alternative solutions to protect plants and their products, and to develop more compatible and respectful strategies for the ecosystem as a whole.

Recent studies have discovered a novel mechanism of communication between plants and pathogens, termed cross-kingdom RNA interference (RNAi), a coordinated phenomenon in which fungi send small interfering RNAs (siRNAs) into the host plant to silence host immune response genes, and plants send sRNAs packaged in extracellular vesicles into fungal pathogens to silence virulence-related genes (Weiberg et al., 2013; Cai et al., 2018b; Huang et al., 2019; Zeng et al., 2019). The production of siRNA effectors can be hindered in the pathogen through the direct application of specific dsRNA molecules, following the spray-induced gene silencing approach (SIGS), or through the genetic modification of the plant that expresses the dsRNA molecules inducing the RNAi mechanism (host-induced gene silencing (HIGS) strategy), exploiting the

bidirectional dsRNAs exchange between host and pathogen (Capriotti et al., 2020). Researchers have demonstrated that some pathogenic fungi, such as *Botrytis cinerea* and *Fusarium graminearum*, are able to adsorb dsRNAs from the environment or from HIGS *Arabidopsis* lines; these molecules are processed by pathogen RNAi machinery into siRNA that give rise to the silencing of complementary target genes (Koch et al., 2016; Wang et al., 2016). A fundamental pre-requisite in the induction of RNAi-mediated silencing of targeted fungal pathogen genes is the capability of the fungus to efficiently take up environmental dsRNA (Kettles et al., 2019). When this capacity has been determined, different dsRNA delivery methods could be applied based on the biology of the target organism and the characteristics of the host. High-pressure spray, low-pressure spray, trunk injection, petiole adsorption and root imbibition are the available methods for the direct application of the principal actors of RNAi mediated Post-Transcriptional Gene Silencing (PTGS) (Dalakouras et al., 2020). In the *Fusarium graminaerum*-barley pathosystem, the spray treatment of long dsRNA targeting three genes related to ergosterol biosynthesis led to a reduced pathogenicity of the fungus compared to the administration of siRNA (Koch et al. 2016). Koch and co-authors discovered that long dsRNAs were preferentially translocated in their entirety through the vascular system, due to the low presence of processed siRNAs in barley plants. These and other researches highlighted that both exogenously applied long dsRNA and siRNA are capable to stimulate fungal RNAi machinery to silence specific genes. Unfortunately, both long dsRNA and siRNA molecules are susceptible to enzymatic degradation by RNAse and other abiotic factors such as UV light and water rinse. Both these kind of RNA molecules can be protected and efficiently conveyed by several chemical compounds, and clay-nanosheets represents one of the most promising vectors, also taking into consideration the low cost of production (Taning et al., 2020).

The present study aimed to evaluate the window of protection against *Botrytis cinerea* provided by a unique dsRNA-based treatment applied through a low spray approach on grapevine tissues. During this trial, the capability to reduce or prevent grey mold disease of long *Bc-DCL 1/2* dsRNA naked or complexed with LDH Nanoparticles (Bioclay) applied on the surface of grapevine leaves attached to the plants, and on detached fruits, has been observed at 1, 7, 14, 21, 28 days after a single application followed by an artificial inoculum of *Botrytis cinerea*. Naked or Bioclay *Bc-DCL 1/2* dsRNAs were also evaluated as curative treatments, that have been performed one day after pathogen inoculation for their capacity to block grey mould disease progression. SIGS approach have been successfully applied on grapevine leaves and berries to control grey mould both in pre-and post-harvest in a protected environment.

2. Material and methods

2.1 In vitro synthesis and bacterial-derived dsRNA

The synthesis of the dsRNA started from the individuation of the target genes in *Botrytis cinerea*. The 490 nucleotides (nt) DNA fragment, obtained by overlapping PCR, was the same used in previous research works published by Wang and co-authors on 2016 that targeted *Dicer-like* genes *1* and *2* of *Botrytis cinerea* (Wang et al., 2016). The T7 promoter sequence was introduced into both 5' and 3' ends of the DNA fragment by PCR, respectively.

A PCR reaction have been performed in a final volume of 50 µL (adding 2 µL forward primer; 2 µL reverse primer; 4 µL dNTP 2,5mM; 0,5 µL Taq Polymerase enzyme;10 µL 5XHF BUFFER; 0,5 µL of DNA template; and 31 µL of sterile water).

Afterwards, a 0,1% of agarose gel have been run, testing the presence of dsRNA molecules and the purification of PCR product was conducted on a total volume of 400 µL through the addition of 1000 µL of pure ethanol and 40 µL of sodium acetate 3M for 2 hours , followed by a centrifugation step, for 10 minutes at 13000 g. The resultant pellet was washed again adding 700 µL of ethanol (75%), followed by a centrifugation for 5 minutes, and the re-suspension in water.

For RNA transcription, the MEGAscript® RNAi Kit instructions have been followed (Life Technologies, Carlsbad, CA), where cleaned PCR product serves as a template for the following reactions conducted on a total volume of 20 µL, also reported on the manufacturer instruction: 8 µL of template; 2 µL 10x-T7 reaction buffer; 2 µL ATP; 2 µL CTP; 2 µL UTP; 2 µL GTP; 2 µL T7 enzyme mix, at a temperature of 37°C for 4 hours. After the transcription reaction, 30 µL of DEPC water were added to dilute the sample and then the RNA concentration was checked at the nanodrop.

Afterwards, 1000 µL of pure ethanol and 40 µL of sodium acetate 3M were added to precipitate the sample, at 4°C overnight. After being quantified at the nanodrop, the reaction product was stored at -80°C.

In vitro transcribed RNA, at a concentration of 20 ng μL^{-1} was employed for low-pressure spray experiments on grape berries.

Bacterially expressed *Bc-DCL 1/2* dsRNA originating from *Escherichia coli* H115 was provided by Prof. Neena Mitter research group, at the University of Queensland, who prepared the molecules as naked dsRNA alone or loaded in layered double hydroxides (LDH) (Mitter et al., 2017).

2.2 Empty Nanoclay and Bioclay (Nanoclay loaded with dsRNA)

Layered double hydroxides or Nanoclay (Mitter et al., 2017) in the form of single sheet like-clay nanoparticles were used as dsRNAs carrier during low-pressure spray treatments for the control of grey mold on grapevine leaves and berries. LDH is composed by a series of hydroxides of divalent or trivalent metal ions which make the structure positively charged to be able to interact with negatively charged dsRNA, forming a complex called Bioclay, stabilized by electrostatic bonds. In this research collaboration, Prof. Neena Mitter provided the molecules for the realization of the experiments such as LDH (empty Nanoclay), naked dsRNAs molecules targeting *Bc-DCL 1/2*, and Bioclay consisting of *Bc-DCL 1/2* dsRNAs loaded into clay nanosheets at the mass ratio of 1:4 (dsRNA: LDH). The stock solutions were stored at -20°C in falcon tubes wrapped by parafilm. All the Bioclay experiments were conducted using a final concentration of both naked and formulated dsRNA equal at 50 ng μL^{-1}.

2.3 Fungal culture and infection conditions

Botrytis cinerea B05.10 strain taken from -80°C stock was routinely cultured on fresh Maltose Extract Agar medium (MEA) consisting of 20 g L^{-1} malt extract, 10 g L^{-1} bacto-protease peptone, and 15 g L^{-1} agar. Ten days after the culture, sporulating Petri dishes were used to collect conidia. 5 mL of distilled sterile water were

spread on the surface of the medium, and with the aid of a sterile inoculating loop, mycelium was removed and filtered using a cell strainer 70 µm Nylon mesh (Fisherbrand), specific for conidia selection. Using a hemocytometer, the conidia have been counted, to reach a final concentration of 10^5conidia/mL in distilled deionized water, supplemented with 10 g L^{-1} of Difco Sabouraud Maltose Broth (MB) (fisher scientific).

2.4 External application of dsRNAs on the surface of grapevine leaves

Cultivar Cabernet Sauvignon grapevine cuttings were left to root in a sandy seedbed during the winter period and were transplanted to single plastic pots containing commercial soil mix (Figure 1 a). Grapevine shoots developed on the greenhouse environment were irrigated manually avoiding wetting of the foliar surface. In around four-five weeks plants had at least one well-developed shoot in active growth and around 10 expanded leaves for each plant (Fig. 1 b). At this stage each plant was subjected to dsRNA treatments. The dsRNA topical application experiment included four treatments: the negative control consisting of RNAse-free water, bacterial-derived naked *Bc-DCL 1/2* dsRNA at a concentration of 50 ng μL^{-1}, Nanoclay empty through the application of empty LDH, and Bioclay (Nanoclay + bacterial-derived *Bc-DCL 1/2* dsRNA molecules at a concentration of 50 ng μL^{-1}). Plants were inoculated with *Botrytis cinerea* conidia at either 1, 7, 14, 21, and 28 days post-treatment and relative biomass and necrotic lesions were counted and analysed at 4 days post-inoculation (dpi). For each treatment and each time observation, three plants were sprayed covering all the leaf surface until draining and they were left to grow in greenhouse-controlled conditions (20–30°C, 16–18-h photoperiod, and a high light intensity until 1,400 µmol m^{-2} s^{-1}) (Fig. 1 c). Just before the treatment, a sign made with tape was affixed to the shoots to identify the only leaves that have been sprayed. At the designed time, five grapevine leaves for each treatment and each time-shift were detached and placed up to a filter paper imbibed with DEPC water adherent to the bottom of a transparent plastic box. Detached leaves were challenged with *Botrytis cinerea* conidia through a low-pressure spray that was applied evenly wetting the entire foliar surface. The box was close with the proper lid creating an environment with high humidity content. During disease development, the boxes were placed at 20°C in dark conditions. The whole experiment was replicated two times, starting from new grapevine plants of the same cultivar provided by Prof. Philippe Rolshausen, from the University of California, Riverside.

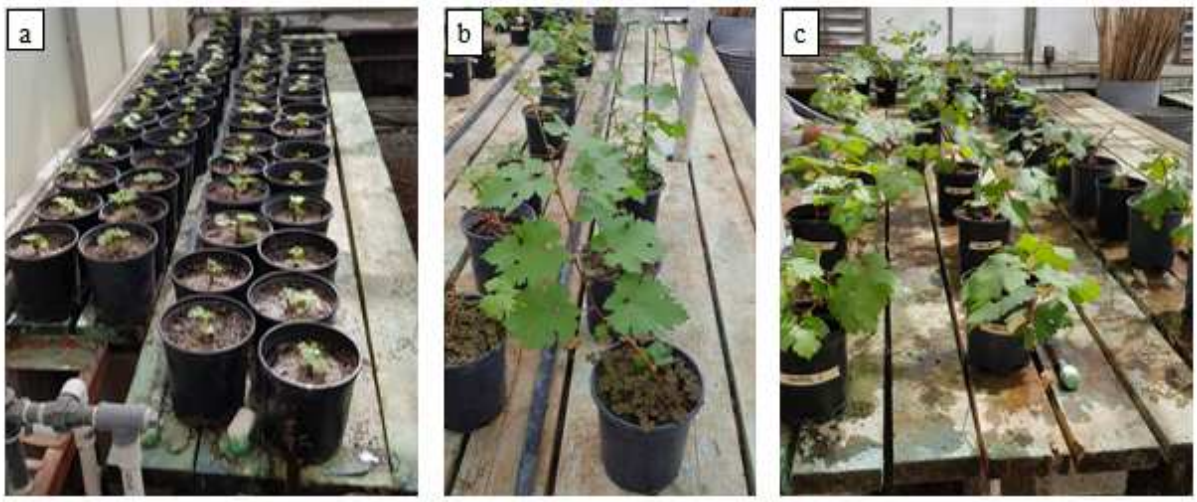


Figure 1: Development of grapevine plants in greenhouse-controlled environment. Rooted cuttings were transplanted in single plastic pots filled with commercial soil mix (a). Only plants with well-developed shoots and expanded leaves were selected for treatment applications (b). Grapevine plants were left to grow after treatment application before *Botrytis cinerea* inoculation (c).

Leaves detached from the original plants were also employed to test the efficacy of treatments when performed 1 day after pathogen inoculation (curative approach). The leaves were settled in an analogous way to previous experiments. 1 day after pathogen inoculation, the treatments were applied using the spray bottle on detached leaves in a unique box, using a divider panel avoiding spray contamination during the treatments.

2.5 External application of dsRNAs on the surface of grape berries

Grape berries of *Vitis labrusca* "concord" were purchase fresh at a local supermarket and selected as experimental hosts plant material. Berries were positioned in plastic boxes up to a filter paper imbibed with RNAse-free water before treatment application. Four treatments including the negative control (DEPC water), *in vitro* synthetized *Bc-DCL 1/2* naked dsRNA (20 ng μL^{-1}), Nanoclay empty, and Bioclay (Nanoclay + bacterial-derived *Bc-DCL 1/2* dsRNA molecules at a concentration of 50 ng μL^{-1}) were deposited in the form of a drop of 20 µL on the surface of each fruit using a micropipette. For each treatment ten fruits have been treated. 1, 3, and 7 days after, each fruit was challenged with 10 µL of *Botrytis cinerea* infection solution and incubated in the dark at room temperature. The experiment was replicated three times.

2.6 DNA extraction and data analyses

Six *Vitis vinifera* leaf discs (7 mm in diameter) picked out in the same area for all the samples were pooled in a 2 mL tubes 96 h after *Botrytis* inoculation, frozen in liquid nitrogen and were pulverized by the mechanical action exerted by four chrome steel balls previously entered in the Eppendorf tubes. 400 µL of pre-heated 2 x CTAB extraction buffer were added to the samples, and after mixing and removing the chrome steel balls, the solution was incubated in a water bath at 65°C for 1 hour. 600 µL of chloroform were introduced in the tubes mixing the samples by hand and centrifuging at 13.000 g for 10 minutes, collecting about 400 µL of supernatant. The extraction step with chloroform was repeated at least two times, until the upper aqueous phase

became clear. 500 µL of ethanol (1:1,2 V:V) and 30 µL of sodium acetate (3 M) were added, mixed by hand and incubated for 20 minutes. Samples were centrifuged at 13.000 g for 20 minutes; the supernatant was discarded and 400 µL of 75% ethanol were used to wash DNA pellet. The last centrifugation at 13.000 g for 5 minutes was necessary to discard ethanol, and evaporation under a chemical hood for at least 30 minutes was carried out as last step. Samples were resuspended in 50 µL of deionized sterile water and quantified using nanodrop.

The relative quantification of plant and fungal DNA in infected tissues was done by performing two real-time PCR reaction using specific primer that identified fungal and plant housekeeping genes (Gachon and Saindrenan, 2004). Grapevine actin (primers forward 5'-CCATGATGTGGGTGGAATGATAGTC and reverse 5'-AGCAGATGAGATTGCTACCAAATGAAG) and a specific *Internal Transcribed Spacer* (*ITS*) of *Botrytis cinerea* (primers forward 5'-TCGAATCTTTGAACGCACATTGCGC and reverse 5'-TGGCAGAAGCACACCGAGAACCTG) have been selected as genes to detect.

The real-time PCR assays were conducted using a BIO RAD CFX96. All DNA samples at a concentration of 200 ng μL^{-1} were analysed by real-time PCR assays using the specific primers and SYBR Green 1 (SigmaAldrich). Real-time PCR was performed in a final volume of 20 µL, containing 2 µL forward primer, 2 µL reverse primer, 10 µL 10x buffer, 1,6 µL dNTP (2,5 mM); 0,5 µL × SYBR Green 1 and 0,5 µL Taq Polymerase. Thermal cycling conditions were as follows: 1 min at 94°C, followed by 40 cycles each consisting of 15 s at 94°C, and then 30 s at 58°C and 30 s at 72°C. Melting curve analysis was carried out using the following program: 95°C for 15 s, followed by a constant increase in temperature from 60°C to 95°C at a rate of 0,5°C per 30 s. For relative quantitative real-time PCR (qPCR) we used the comparative threshold cycle (Ct), that is the cycle number in which fluorescence can be detected from the background fluorescence. The differences in Ct found between the various targets, allowed to identify the DNA of *Botrytis cinerea* relative to its host DNA using ΔΔCt method (Gao et al., 2004). In addition to relative biomass quantification of *Botrytis cinerea*, necrotic lesion sizes were measured at 4 dpi through image acquisition and relative area was calculated using ImageJ software.

2.7 statistical analyses

To analyse the data, STATISTICA 7 software was used to compute hypothesis testing. To determine whether dsRNA-based treatments on the surface of the leaf differed from the controls, data were subjected to a one-way analysis of variance (ANOVA with $p < 0.05$), followed by a comparison of means using a Student-Newman-keuls post hoc test with significance levels set at $p < 0.05$.

3. Results

3.1 Foliar spray applications of dsRNA reduce *Botrytis cinerea* infection in grapevine

To take an additional step towards a likely future large-scale application, around 400 µL (50 ng μL^{-1}) of long *Bc-DCL 1/2* dsRNA molecules and their controls were sprayed as naked dsRNA, or formulated with clay nanosheets (BioClay) on the surface of each grapevine leaf on plants growing in the greenhouse. 1, 7, 14, 21,

28 days after the treatments, leaves were detached and challenged with *Botrytis cinerea*. The severity of the symptoms was assessed 4 days post-inoculation in a plastic box with high humidity content, through image acquisition, calculation of necrotic lesion area and relative biomass quantification of the pathogen compared to the host's DNA. At 4 dpi, dsRNA-treated leaves sprayed 1 and 7 days before inoculation developed less frequently brownish lesions, that were substantially sporadic than those on water or empty Nanoclay-sprayed leaves, used as controls in this experiment (Fig. 2 a).

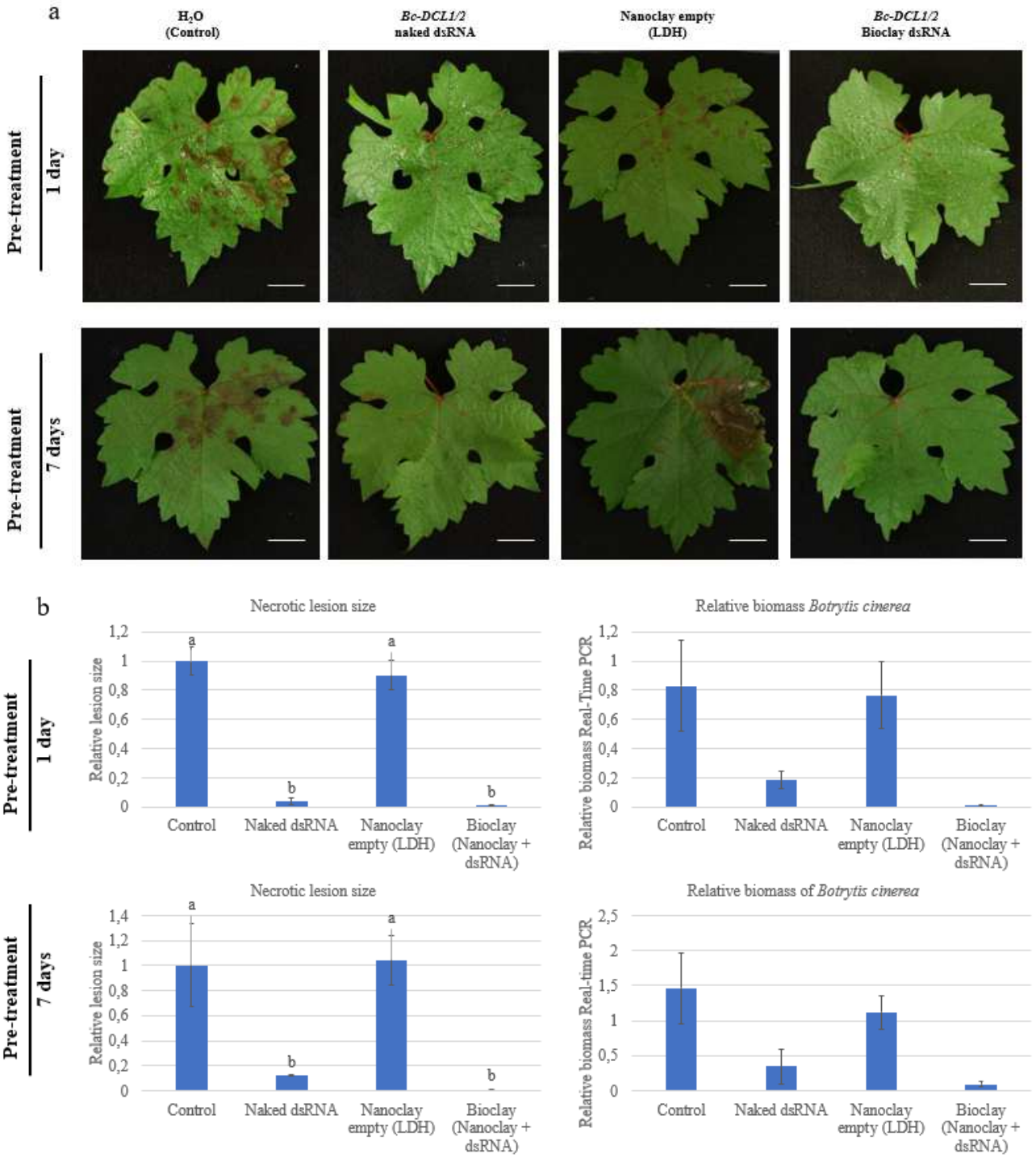
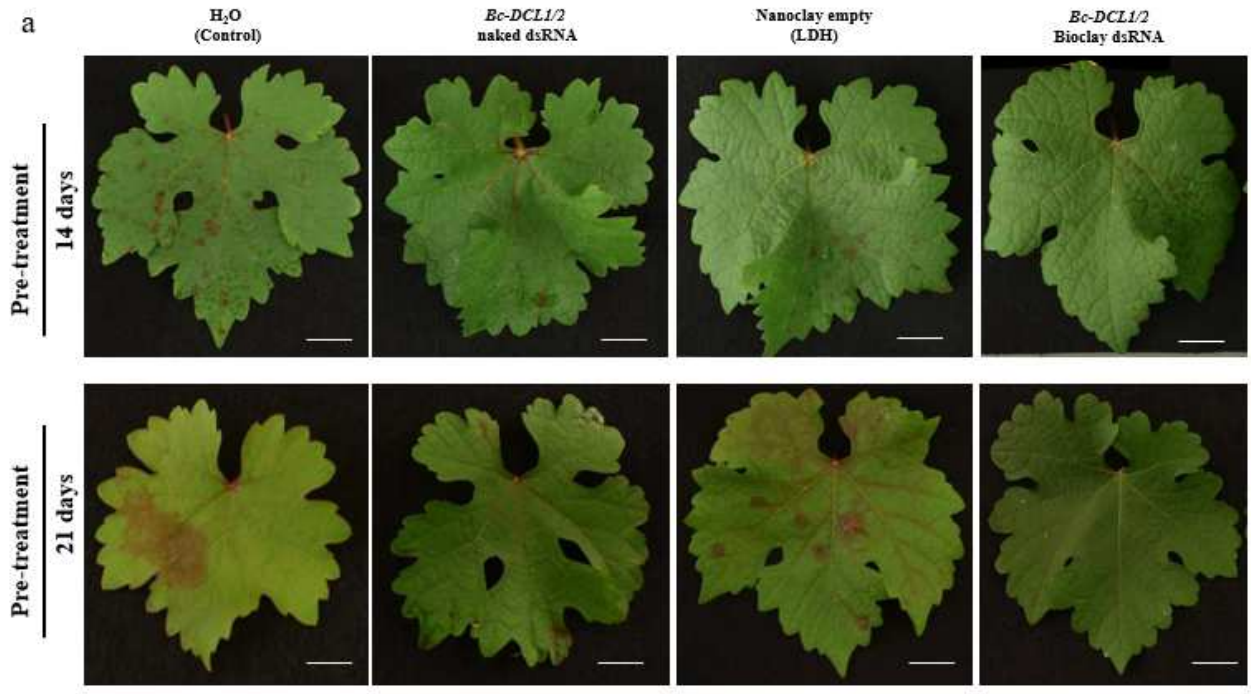


Figure 2: Treatment with *Bc-DCL1/2* - dsRNAs protected grapevine leaves from *Botrytis cinerea* up to 7 days. The grapevine leaves were pre-treated with Naked *Bc-DCL1/2*-dsRNAs, Bioclay *Bc-DCL1/2*-dsRNAs, Nanoclay empty and DEPC water for 1 and 7 days, respectively, and then infected with *Botrytis cinerea*. The pictures were taken at 4 dpi (up to 5 and 11 days after RNA pre-treatment) (bar: 2 cm) (a). The relative lesion sizes were measured using software ImageJ, and error bars indicate the standard error of ten plant leaves (five for each replicate). The relative biomass was measured by quantitative PCR, and error bars represent standard error of three technical replicates. Means with different letters indicate statistically significant differences ($p < 0,05$) (b).

qPCR analysis of fungal DNA levels, based on the ratio between fungal ITS and grapevine actin, confirmed reduced fungal growth on dsRNA-treated leaves (Figure 2 b). Similar results have been obtained applying the same set of treatment but performing *Botrytis* inoculation 14 and 21 days after spray treatment. Interestingly, dsRNA-based treatments continue to confer an excellent protection efficiency of the foliar surface compared to control treatments (Fig. 3 a), which were appreciable both through the visualization and characterization of the symptoms, and the molecular quantification of the relative biomass of the pathogen (Fig. 3 b). The relative biomass of the pathogen in grapevine leaves treated with naked dsRNA and infected at 21 days after treatment application was increased compared to the same value for the leaves treated and inoculated after 14 days, representing a timid sign of loss of effectiveness. Up to now the treatment with naked dsRNA and Bioclay have effectively controlled the grey mold, although it was not possible to identify the most performing treatment in terms of effectiveness.



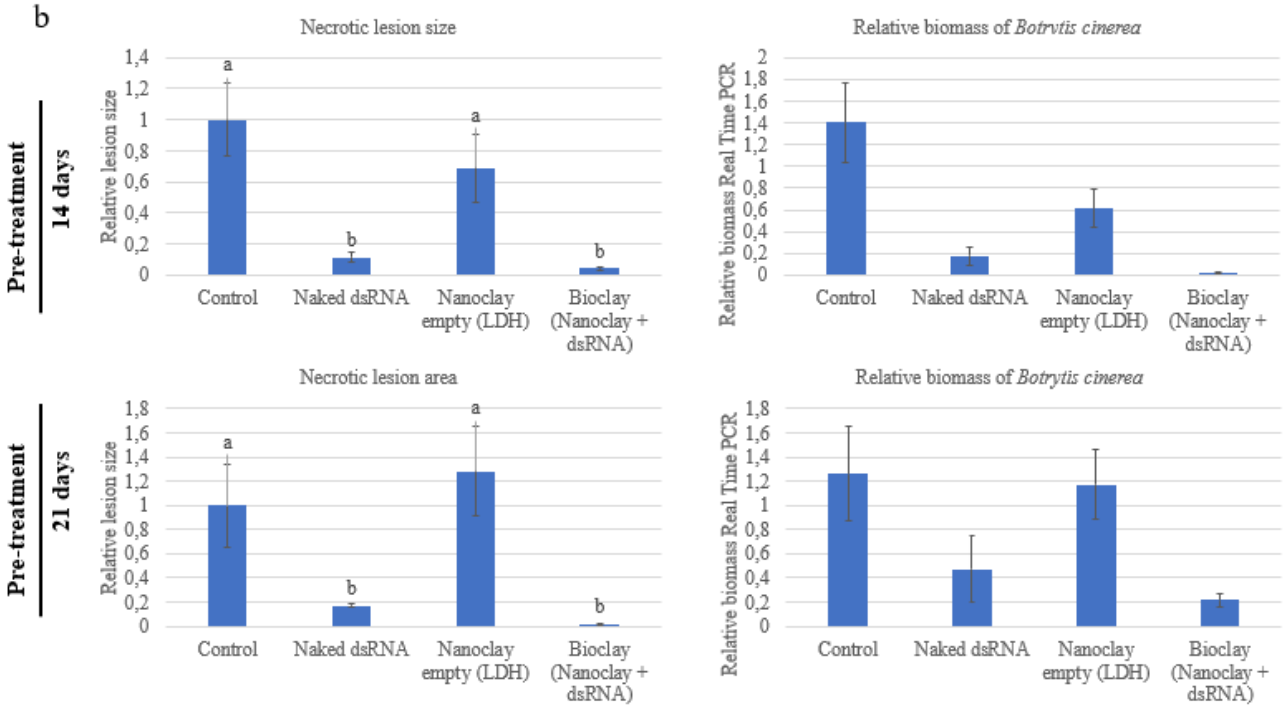


Figure 3: Treatment with *Bc-DCL1/2* -dsRNAs protected grapevine leaves from *Botrytis cinerea* up to 21 days. The grapevine leaves were pre-treated with Naked *Bc-DCL1/2*-dsRNAs, Bioclay *Bc-DCL1/2*-dsRNAs, Nanoclay empty and DEPC water for 14 and 21 days, respectively, and then infected with *Botrytis cinerea*. The pictures were taken at 4 dpi (up to 18 and 25 days after RNA pre-treatment) (bar: 2 cm) (a). The relative lesion sizes were measured using software ImageJ, and error bars indicate the standard error of ten plant leaves (five for each replicate). The relative biomass was measured by quantitative PCR, and error bars represent standard error of three technical replicates. Means with different letters indicate statistically significant differences ($p < 0,05$) (b).

To further explore the potential of SIGS, we assessed the silencing efficiency of dsRNA-based treatments exerted after 28 days from the last application and alternatively applied as spray curative treatment on detached grapevine leaves, 1 day after pathogen inoculation. Necrotic lesions developed in almost all the leaves, excluding those treated with Bioclay, which continue to remain unaffected as observed in the previous experiments (Fig. 4 a). The curative ds-RNA based treatment was as effective as when the pre-treatment was administered at the same time-shift, compared to water and Nanoclay empty controls. These findings were supported by relative biomass quantification of *Botrytis cinerea* (Fig. 4 b).

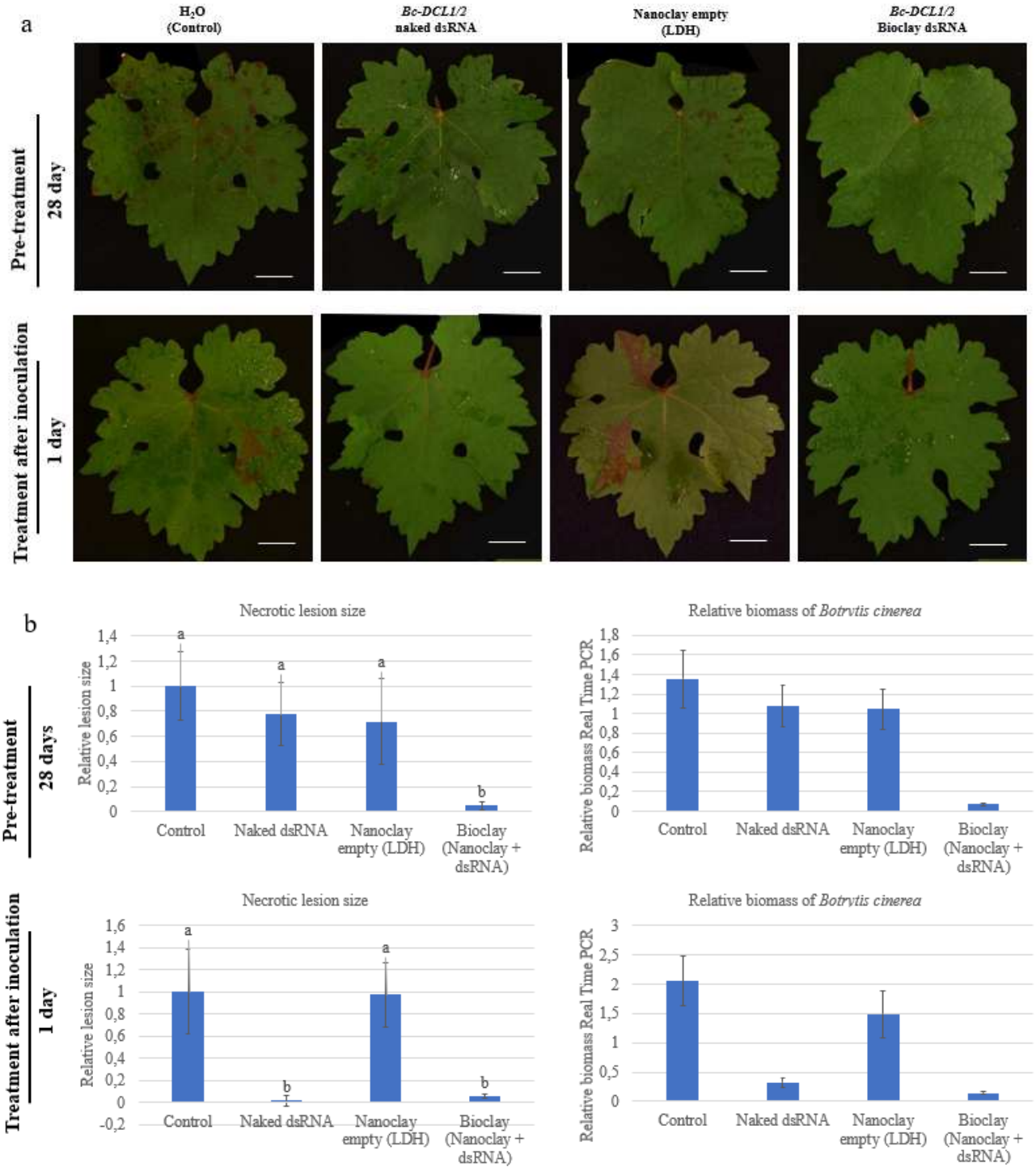


Figure 4: Treatment with *Bc-DCL1/2* -dsRNAs protected grapevine leaves from *Botrytis cinerea* when were applied 1 day after inoculation and up to 28 days as a pre-treatment only in the form of Bioclay. The grapevine leaves were pre-treated with Naked *Bc-DCL1/2*-dsRNAs, Bioclay *Bc-DCL1/2*-dsRNAs, Nanoclay empty and DEPC water for 28 days respectively, and then infected with *Botrytis cinerea*. The same treatments have been also applied 1 day after pathogen inoculation. The pictures were taken at 4 dpi (up to 32 days after RNA pre-treatment and up to 3 days as post-treatment) (bar: 2 cm) (A). The relative lesion sizes were measured using software ImageJ, and error bars indicate the standard error of ten plant leaves (five for each replicate). The relative biomass was measured by quantitative PCR, and error bars represent standard error of three technical replicates. Means with different letters indicate statistically significant differences ($p < 0,05$) (B).

3.2 Drop applications of dsRNA reduce *Botrytis cinerea* infection in grape berries

RNAse-free water, *in vitro*-transcribed naked *Bc-DCL 1/2* dsRNA concentrated at 20 ng μL^{-1}, empty Nanoclay, and Bioclay containing bacterial derived *Bc-DCL 1/2* dsRNA at 50 ng μL^{-1} were applied in the form of drops onto the surface of grape berries, for the evaluation of protection provided by the molecules when inoculation was performed 1, 3, and 7 days after the treatment. At the defined time, *Botrytis* conidia were introduced in the form of 10 µL drops which have been placed in the same area as the administration of each treatment.

At each time, the lesion size was assessed at 4 dpi through image acquisition, and lesion area measurement using ImageJ software. After four days post-inoculation, grapes treated with DEPC water and empty Nanoclay resulted covered by a thick blanket of sporulated hyphae, giving the characteristic grey colouration to the infected tissues (Fig. 5 a). Naked dsRNA treatment conferred protection at both 1 and 3 days, while at 7 days the pathogen has been able to infect the treated fruits more markedly. Bioclay treatment is the only one that managed to contain the advancement of grey mould in a significant way also 7 days after its application, as it can be seen from the measurements of the infected areas carried out on each fruit (Fig. 5 b).

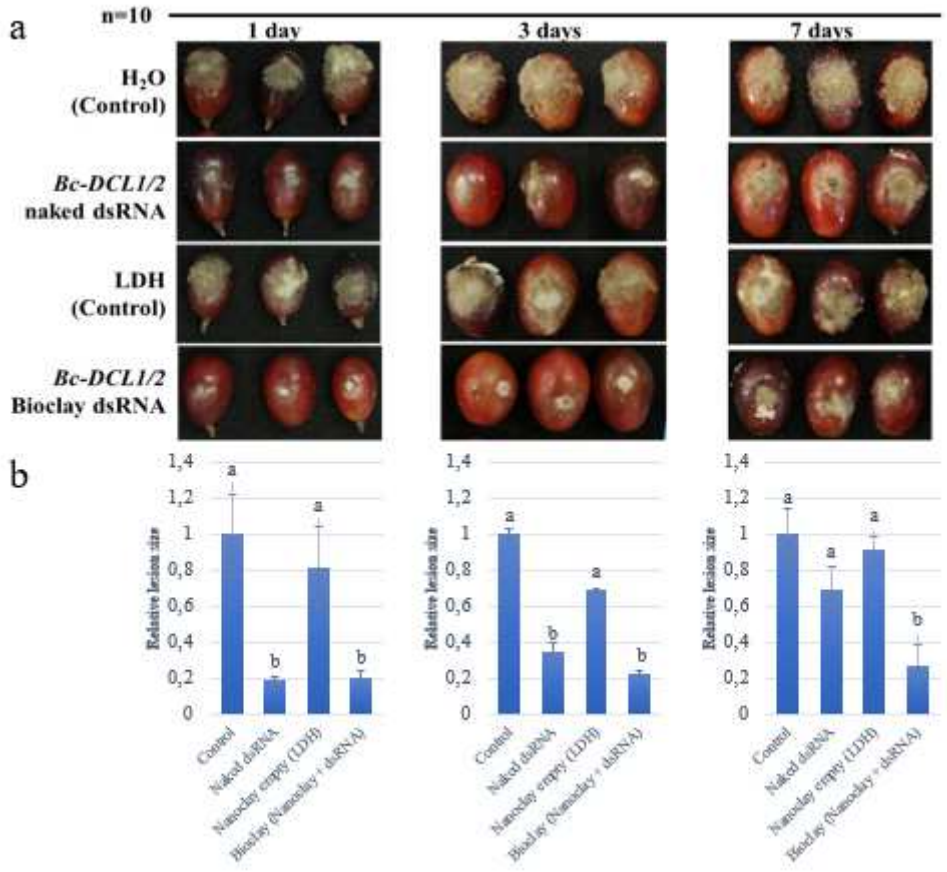


Figure 5: Treatment with *Bc-DCL1/2* -dsRNAs protected grapes from *Botrytis cinerea* up to 3 days and up to 7 days in the only form of Bioclay. The grape berries were pre-treated with Naked *Bc-DCL1/2*-dsRNAs, Bioclay *Bc-DCL1/2*-dsRNAs, empty Nanoclay and DEPC water for 1, 3, and 7 days, respectively, and then infected with *Botrytis cinerea*. The pictures were taken at 4 dpi (up to 4, 7, and 11 days after RNA pre-treatment) (a). The relative lesion sizes were

measured using software ImageJ, and error bars indicate the standard error of thirty grapes (ten for each replicate). Means with different letters indicate statistically significant differences ($p < 0,05$) (b).

4. Discussion

The silencing of *Botrytis cinerea Dicer-like genes 1* and *2* has been amply demonstrated to play a crucial role in reducing fungal growth and pathogenicity when both long non-coding *Bc-DCL 1/2* dsRNA and siRNAs were sprayed on the surface of fruits and leaves of various plant species (Wang et al., 2016). The current study supports this discovery by offering new aspects regarding the persistence and effectiveness of the application of RNA-based molecules that silence *DCL 1/2* genes crucial to the pathogenesis of *Botrytis cinerea*. The potential of SIGS for crop protection was explored through the direct application of naked dsRNA or conveyed by Nanoclay, constituting the Bioclay (Mitter et al., 2017) into grapevine plants. To test the duration of the effectiveness of antifungal dsRNA-based treatments with the corresponding controls, the inoculation with fungal pathogen was performed at intervals of time spaced from each other about 7 days. Naked *Bc-DCL 1/2* dsRNAs were effective up to 21 days from the last and unique administration on *in planta* grapevine leaves, whereas Bioclay *Bc-DCL 1/2* dsRNA resulted operative for the whole period considered, also after 28 days from the treatment. We showed that LDH nanocarrier system for dsRNA can be employed in SIGS approach for increasing dsRNA stability through a gradual releasing of the molecules and the firm adhesion to the leaf surface, allowing to prolong the window of protection against *Botrytis cinerea* (Mitter et al., 2017).

It has been discovered that processed siRNAs can move from cell to cell through plasmodesmata, although also systemic transport of RNA molecules has been documented to confer gene silencing in vegetal organs not directly involved in the treatment (Dalakouras et al. 2020; Koch et al. 2016; Mitter et al. 2017; Nerva et al. 2020).

Naked or formulated dsRNA were tested on grapes because are usually the main target organs of the fungus that consequently led to direct economic losses and vinification-related problems such as the production of extracellular enzymes and glucans that falls under indirect economic losses. Furthermore, few pesticides can be used for the post-harvest defence of grapes as many have long shortage times, with consequent residuality in the final products. Therefore the development of this fungus is generally limited by applying various physical treatments to fruits and vegetables (Usall et al., 2016). Our results suggest that dsRNA molecules in the form of drop treatments can protect grapes up to 3 days from the attack of the necrotrophic fungus *Botrytis cinerea*. *Bc-DCL 1/2* dsRNA complexed with Nanoclay conferred a though resistance also at 7 days after treatment, while the treatment with unprotected dsRNA was no longer effective. However, it should be taken into account that the two treatments were distinguished by different dsRNA production method. Also Nerva and colleagues (2020), who adopted the high-pressure spray delivery method, reported a significant reduction in *Botrytis cinerea* growth on leaves and bunches treated with dsRNAs targeting three fungicide site of action, followed by an artificial inoculation with the pathogen (Nerva et al., 2020). The application of long dsRNAs instead of siRNA has been preferred because they can be processed by the RNAi mechanism of the fungus originating a plethora of siRNA, with the possibility of having multiple intervention sites, very useful to avoid the evolution of resistant pathogen strains (Koch et al. 2016). SIGS approach is an alternative GMO-free

strategy that allows to induce specific gene silencing, that have the potential to be directly involved in pathogen and pest management. The use of carrier molecules such as cationic nanoparticle, layered double hydroxides (LDH), and carrier peptide has the potential to enhance spray induce gene silencing effectiveness both preventing dsRNA degradation and increasing the permeability and the cellular uptake of this compounds (Jiang et al., 2014; Numata et al., 2014; Mitter et al., 2017). Even though the synthesis cost of dsRNA molecules was high until recently, some emergent Biotech companies i.e. GreenLight Biosciences, AgroRNA (Genolution) developed innovative methods which allow to reduce production costs from the current 1000 dollars per gram for *in vitro* transcription up to values of less than one dollar per gram (Taning et al., 2020).

5. Conclusion

SIGS represents an environmentally friendly alternative to pesticide application, acting in a specific manner through the silencing of genes important for virulence or the biosynthesis of fundamental components. *DCL 1/2* genes proved to be suitable target genes to knockdown through complementary mRNA degradation in which they are involved in fungal RNAi machinery through the cleavage of long dsRNAs into siRNAs (21-24 nt), that act as pathogen effectors that enhance the aggressiveness of *Botrytis cinerea. Vitis vinifera* cv. Cabernet sauvignon leaves growing in the greenhouse and detached American fox grapes (*Vitis labrusca* 'Concord') have been selected as appropriate starting material to test the efficacy and the applicability of this technique for plant/fruit protection purposes. The development of grey mold on leaf explants was vigorously restrained by the application of naked *Bc-DCL 1/2* dsRNA up to 21 days, whereas Bioclay *Bc-DCL 1/2* dsRNA allowed to extend the protection window over 28 days. Grapes during the post-harvest period at room temperature resulted more susceptible to *Botrytis cinerea* necrotrophic attack. However, Bioclay *Bc-DCL 1/2* dsRNA treatment was able to confer a visible protection also seven days after the last application, while the window of protection was reduced to three days if the dsRNA treatment was performed as such (naked dsRNA). During these experiments, *Bc-DCL 1/2* were confirmed as valid candidate target genes for defending grapevine organs from the advent of grey mold, and the loading of dsRNA molecules into appropriate carriers such as Nanoclay can extend their persistence in the plant surface, consequently prolonging their silencing efficacy.

Although further studies are needed, especially regarding the mechanisms of RNA uptake and movements into the organisms, RNAi-mediated silencing offers a great opportunity both in endogenous gene regulation and modulation of gene expression particularly useful in pathogen-pest management of crops.

General Conclusions

In Europe, around 3.160.718 hectares have been cultivated with grapevine in 2019, of which 697.910 hectares in Italy (FAOSTAT, 2019), which despite being the third country at European level for the vineyard area invested (at first place there is Spain followed by France) is the first in the world for the quantity of wine produced (49.5 million hectolitres), ahead of France and Spain. Globally wine production reached 274.4 million hectolitres (OIV, 2016). Grapevine represents one of the most important fruit crops in the world for both wine production and fresh grapes consumption. The evolution of the use of the fruit has passed through the three stages: direct use of the fruit (seasonal event), use of the dry fruit (conservable food) and use of the fermented juice to make it a foodstuff that can be preserved until the next harvest. The use of grapes and their related products is well documented in Greek, but above all Roman history. The recent past of viticulture has gone through dark times, especially following the introduction of the American *Vitis* species in the middle of the 19th century which allowed the spread of fungal and oomycetes pathogens, and newly introduced insects for which there has been no co-evolution between pathogens and hosts. *Vitis vinifera* is highly susceptible to powdery mildew, downy mildew and phylloxera, and although different techniques were applied to protect plants, this species is considered until now highly prone to those fungal diseases, that together with grey mold represented the most impactful fungal and oomycetes diseases that affect grape production. Although conventional breeding is easily applicable to short-growing herbaceous species, its applicability is more limited to fruit-bearing woody plants due to their multiannual crop cycle, the long juvenile phase, and problems of high heterozygosity and self-incompatibility during fertilization. The crossing and/or hybridisation techniques are highly disadvantaged especially in vines as they often lead to the loss of the high-quality characteristics typical of each grape variety, and very frequently a vine is associated with a wine product with well-defined characteristics that must remain constant over time. Compared to the implementation of these traditional genetic improvement techniques, the application of *in vitro* techniques allowed us to preserve the clone identity and it was one of the principal steps that we followed for the employment of plant biotechnologies. In addition to mass propagation, during the *in vitro* techniques, different regenerative responses have been collected by mainly manipulating the phytohormones content and combination in the culture medium. Having had the opportunity to work with different *Vitis vinifera* cultivars and hybrid *Vitis* rootstocks, several regeneration techniques have been optimized, including organogenesis from meristematic bulks, somatic embryogenesis and organogenesis from cotyledons and hypocotyls derived from mature somatic embryos. Meristematic bulk regeneration system has been taken as a reference model for the implementation of techniques such as genetic transformation and chemical mutagenesis. The development of a suitable, easy-to-apply regeneration protocol is an essential prerequisite for increasing the possibility of developing new transgenic or mutated individuals, and it is from the optimisation of these protocols that we started with the research. Somatic embryos have been obtained in Ancellotta, Lambrusco Salamino and 110 Richter from the culture of floral explants as stamens, pistils, and whole flowers, while Thompson Seedless ones have been obtained from the culture of nodal segments on different induction and expression media. We optimize the adventitious bud formation using Ancellotta, Lambrusco Salamino and Thompson Seedless cotyledons and hypocotyls as starting explants,

reaching high regenerative responses in terms of new shoots production. All regeneration strategies allowed to produce plants having a phenotype correspondent to the original clone, although during somatic embryos germination some of them failed to develop well-shape plantlets, probably due to the somatic embryos endodormancy, which has been easily overcome by the induction of shoot regeneration from dissected cotyledons and hypocotyls. Once the regenerative response has been tested and the meristematic bulk regeneration method has been selected, chemical mutagenesis approach has been considerate as the preferred method indicated to induce random mutations and therefore genetic variability within clones of Ancellotta, Chardonnay, Lambrusco Salamino, Merlot, Pinot Grigio. Following the application of different concentration of EMS and sodium azide we have produced a putative mutagenized population composed by 609, 517, and 287 plants of Chardonnay, Merlot, and Pinot Grigio, respectively, that were evaluated for their susceptibility to a spontaneous severe powdery mildew infection occurred during the vegetative season in the greenhouse in a first assay. 59, 81 and 5 symptomless plants of Chardonnay, Merlot, and Pinot Grigio respectively were individuated in random locations of the greenhouse; in Merlot, some plants appeared completely red enhancing the possibility of obtaining tangible variations for some individuals. On Ancellotta and Lambrusco Salamino cultivars the application of EMS on MBs resulted in the development of chimeric individual, that need to be tested in greenhouse/field. During some *in vitro* regeneration techniques, especially those that require a high degree of cellular de-differentiation, the onset of somaclonal variations limits the clonal propagation, but at the same time represents an opportunity to induce genetic variability. The somaclonal variability have been evaluated through the conversion of numerous somatic embryos into plantlets of Ancellotta and Lambrusco Salamino such as to produce a new putatively varied population formed by 1332 and 1167 plants of Ancellotta and Lambrusco Salamino. Acclimatized plantlets were left to grow in the greenhouse for an entire growing season, at the end of which the plants were brought outdoors and an artificial inoculation of *Plasmopara viticola* sporangia has been carried out. 7 days after, the disease severity has been assessed for each plant, and about 22 Ancellotta and 54 Lambrusco Salamino showed reduced downy mildew symptoms compared to the other plants of the same cultivar. Further observations and characterization are needed in the putative varied populations to confirm the variation observed and to consider new traits preferentially related to pathogen resistance. If these lines were confirmed resistant/tolerant it would be interesting to understand which genes were involved and which modification occurred.

In addition to chemical mutagenesis and somaclonal variability, that generally act by randomly varying the plant genome, we have optimized a genetic transformation protocol, by introducing a construct that induces kanamycin resistance to the plant and expresses the eGFP protein, useful for visual selection of transformed cells. Meristematic bulk slices of various grapevine cultivars (Albana, Ancellotta, Chardonnay, Ciliegiolo, Glera, Lambrusco Salamino, Merlot, Pinot Grigio, Sangiovese, Vermentino, Thompson Seedless) and *Vitis* spp. rootstocks (110 Richter, Kober 5BB, 1103 Paulsen) have been screened towards their receptivity to *Agrobacterium*-mediated transformation (EHA105 strain) thanks to the counting of the MB that expressed green fluorescence on the total explant treated. These experiments allowed to identify Thompson Seedless as the only *Vitis* genotype among those tested that led to transgenic shoot regeneration.

The discovery of the RNAi silencing mechanism opened the possibility to silence specific target genes involved in the pathogenesis as the enhancer of virulence or in the constitution of pathogen biological structures or function (Agrawal et al., 2003). *Botrytis cinerea* pathogenic fungus has developed an innovative virulence strategy that allowed the production of small interfering RNA (siRNA) acting through the silencing of host immunity genes (Wang et al., 2016). *Dicer-like genes 1* and *2* of *Botrytis cinerea* are necessary for siRNA production, and their silencing guided by long and short dsRNA molecules leads to a significant reduction in disease development on leaves and fruit of different plant species (Wang et al., 2016). This approach has been explored both by the application of host-induced gene silencing (HIGS) in which *Bc-DCL 1/2* dsRNA fragments were constitutively expressed in transgenic Thompson Seedless lines, and the GMO-free application that involved the topical administration of dsRNA molecules on the plant and fungal surface. Genetic transformation of 600 MB slices of Thompson Seedless with GV3101 *Bc-DCL1/2* RNAi gene construct led to the obtainment of four independent putative transgenic lines that have been molecularly characterized. Alongside, a single low spray application of naked *Bc-DCL 1/2* dsRNA on leaves and grapes was able to attenuate fungal disease development up to 21 days and 3 days on leaves and grape berries, respectively. The formulation of *Bc-DCL 1/2* dsRNA with layered double hydroxides Nanoclay formed a complex termed Bioclay that was able to prolong the window of protection, allowing to observe healthier leaves compared to the control 28 days after the single application, and healthier grapes 7 days after the unique treatment.

Genome analyses allowed to identify the presence of the same target genes (*DCL 1/2*) that have been selected as potential genes to silence the impactful Oomycete *Plasmopara viticola*, to reduce its virulence (Brilli et al., 2018). With this aim, thanks to the collaboration established with Prof. Elena Baraldi from the University of Bologna, we designed two RNAi gene construct targeting *DCL 1* (441 construct), and *DCL 1/2* (chimaera construct) of *Plasmopara viticola*, harboured by EHA101 *A. tumefaciens* strain. Various starting explants of Ancellotta, Lambrusco Salamino and Thompson Seedless have been employed using the aforementioned gene constructs following the MB system and somatic embryogenesis regeneration methods. Five and three fully fluorescent shoots were generated agro-infecting MB slices with chimaera and 441 gene constructs, respectively. The number of transgenic shoots recovered using organogenesis from cotyledons as starting explants was significantly higher of those obtained agro-infecting MB slices or hypocotyls. The obtained transgenic lines have been acclimatized, and molecularly characterized, and in the near future they will be tested to evaluate their resistance to *Plasmopara viticola*.

Some aspects need to be elucidated such as the genotype-dependent genetic transformation efficiency, the characterization of putative varied/mutagenized individuals, and the validation of *Pv-DCL 1* and *Pv-DCL 1/2* gene construct in Thompson Seedless transgenic HIGS lines.

All these techniques have been explored to extend the application of different biotechnologies including or not the production of genetically modified individuals with a view to making modern viticulture more sustainable, especially as regards the management of pathogens during the crop cycle and at post-harvest.

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