UNIVERSITÀ DEGLI STUDI DI TORINO

SCUOLA DI DOTTORATO

DOTTORATO IN
SCIENZE AGRARIE, FORESTALI E ALIMENTARI

CICLO: XXXVI

Strategies for improving fruit and grapevine production and for increasing resilience in new ecological scenarios: early detection of graft incompatibility in *Castanea* spp. and *Vitis* spp. through physiological and chemical approaches.

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ANNI
2021; 2022; 2023

Index

Introduction

Castanea sativa Mill.

The European chestnut (*Castanea sativa*), also known as Sweet chestnut, belongs to the *Fagaceae* family, genus *Castanea*. This includes 12 or 13 species, according to the classification, and it is widespread in the boreal hemisphere(Beccaro, Alma et al. 2019). Large species diversity characterises the wide distribution of chestnut in Asia, North America, and Europe, hence reflecting not only the adaptation of the genus *Castanea* to diverse environmental conditions, but also to different management strategies encompassing orchards for fruit production, coppices for timber production, and naturalised populations providing several ecosystem services(Martín, Mattioni et al. 2017). *C. sativa* trees are found on very different pedoclimates, though they prefer soft and deep acidic soils with a pH ranging from 4 to 6.5; rainfall needs to range from 700 to 1500 mm/year, according to the soil type.

European chestnut is more adapted to shade and north-oriented slopes, and can grow well from sea level (Caucasus) up to over 1500 m a.s.l. (North Spain, South Italy). Indeed, natural and planted forests of sweet chestnut cover the species' ecological limits, spreading from the Caucasus to Portugal, reaching the southern United Kingdom, the Canary Islands, and the Azores archipelago. It is also locally present in Lebanon and Syria(Bounous 2002).

Sweet chestnut is one of the oldest domesticated species, widespread throughout the Roman Empire and commonly cultivated during the Medieval period, becoming so indispensable for the survival of mountain populations that these cultures were identified as "chestnut civilizations"(Conedera, Krebs et al. 2004). Therefore, sweet chestnut represents an important resource in Europe for its great ecological (large ecosystem biodiversity and landscape value), economic (fruit, wood, honey, and tannin production), and cultural relevance.

However, since the beginning of the 20th century, the growing areas of sweet chestnuts have dramatically decreased because of several social, cultural, and environmental changes. Such challenges include the progressive depopulation of

mountain areas, diet changes, climate change, and the establishment and spread of diseases and pests(Gullino, Larcher et al. 2009; Spathelf, van Der Maaten et al. 2014). The latter encompass ink disease caused by the oomycetes *Phytophthora cambivora* (Petri) Buisman and *P. cinnamomi* Rands, chestnut blight associated with the ascomycete *Cryphonectria parasitica* (Murrill) M.E. Barr, the emerging nut rot due to *Gnomoniopsis castaneae* G. Tamietti, and the infestation and control of the Asian gall wasp *Dryocosmus kuriphilus* Yasumatsu(Rigling and Prospero 2018; Lione, Danti et al. 2019).

This negative trend is now reversing, as chestnut cultivation represents an interesting alternative to traditional fruit crops which are currently experiencing a challenging time, due to many factors such as rising prices for raw materials, climate change, and the spread of new pests and diseases. This is the case especially in north-western and central parts of Italy.

Intensive orchards of hybrid and European chestnuts are rising also in lowland areas, where agricultural practices typical of major fruit crops are adopted.

In conclusion, the health benefits deriving from the consumption of chestnut-based products, especially from *C. sativa*, are considered to be among the driving forces behind this renewed interest(Beccaro, Donno et al. 2020). Consequently, an intense breeding activity has been leading to the selection of many promising cultivars and rootstocks in recent years(Gamba, Cisse et al. 2021).

Modern rootstocks and graft incompatibility

The climate change has dramatic consequences on agriculture because of the spread of new pests and diseases and the resulting abiotic stresses to which plants are subjected(Corwin 2021). The development of rootstock genotypes able to adapt to these changing scenarios is among the most promising cultivation strategies. In the last decades, research in chestnut breeding resulted in a high number of registered rootstocks and cultivars. The most sought-after traits by breeders are resistance or low susceptibility to diseases, adaptability to changing climatic conditions, agronomic traits, ease of propagation, and graft compatibility. This latter represents the main issue connected to their diffusion;

since the introduction of new interspecific hybrid chestnut cultivars and rootstocks, low graft success became problematic(Beccaro, Alma et al. 2019).
At present, few works are available on chestnut graft incompatibility. Given the renewed interest for this cultivation, graft incompatibility is therefore a topical issue limiting the adoption of new rootstocks and cultivars and further work is needed to explore the relationships of historical local cultivars grafted on these new genotypes. As grafting is the most used propagation technique on *Castanea* spp., it is essential to find techniques able to early predict this disaffinity, to drive breeding programs and therefore the development of the chestnut cultivation.

Table grapevine (*Vitis* spp.), a potential alternative to traditional fruit crops in Northern Italy

Over the past decade, the damages caused by the bacterial canker of kiwifruit led farmers to find other valuable and suitable crops in Italy. Together with high-density chestnut groves, new plantations of table grapevine started to be implemented re-adapting the growing structures used for kiwifruit. Normally grown in the Centre and South Italy, this cultivation is showing encouraging results at higher latitudes. The high thermal excursion determines an intense fruit coloration; the sugar/acidity ratio is favoured by the geographical characteristics of the area and the late ripening period compared to southern grapevines increases the market value of this crop.
The propagation through grafting is widely used in grapevine cultivation, as it allows to overcome many problems, among which phylloxera represents the main issue. In recent years, the University of Milan has been focusing on selecting new rootstocks for *Vitis* spp. able to mitigate the effects of climate change. The series M-rootstocks were released in 2014, chosen for their tolerance to iron-limited conditions, resistance to salinity, and good tolerance to drought(Bianchi, Caramanico et al. 2020). These new genotypes could represent a valuable crop strategy to face the current viticulture challenges for wine and table grapes. However, doubts regarding the compatibility level between these newly selected rootstocks especially with table grape cultivars remains.

State of the art on graft incompatibility

Since the appearance of symptoms of anatomical abnormalities can take years, it is then necessary to identify experimental procedures that address the question of whether a certain breed will be compatible or not, to facilitate the selection of rootstocks suitable for the modern fruit-growing sector. In the last decades, investigations on graft incompatibility have significantly increased, actively facing this challenge through different approaches(Vahdati, Sarikhani et al. 2021). Interesting results have been found studying secondary compounds, physiological parameters, morphological and histological aspects(Azimi, Ozkaya et al. 2016; Skočajić, Gašić et al. 2021). In particular, research has focused on phenolic compounds and their putative role as markers of either compatible or incompatible grafts. Quali-quantitative differences in the phenolic patterns between scion/rootstock can imply disfunctions at the graft union, leading to consider them as potential markers of graft incompatibility(Errea, Garay et al. 2001; Hudina, Orazem et al. 2014; Loupit and Cookson 2020). Chemical and physiological techniques have been tested in this regard with effective results on different fruit species.

Beside chemical traits, rootstocks may affect considerably many physiological processes occurring in the scion. Stomatal opening, usually expressed as stomatal conductance, and leaf chlorophyll content are useful parameters evaluating early diagnosis of incompatibility before visual symptoms occur(Losciale, Zibordi et al. 2008; Gamba, Donno et al. 2022). These quick and non-destructive parameters were found to be effective in the detection of differences among grafting combinations, standing out as supporting tools for chemical analysis.

Aim of the Ph.D. project

The project of the present thesis titled “Strategies for improving fruit and grapevine production and for increasing resilience in new ecological scenarios: early detection of graft incompatibility in *Castanea* spp. and *Vitis* spp. through physiological and chemical approaches” aimed at investigating multidisciplinary

approaches for the study of graft incompatibility in chestnut (*Castanea* spp.) and table grapevine (*Vitis* spp.). As symptoms of anatomical abnormalities and finally the graft failure can take years, the final goal was to find one or more techniques suitable to early predict scion/rootstock incompatibility. Grafting is a complex mechanism with a range of different physiological, chemical, and anatomical interactions. Thus far, very few studies have been carried out exploring these dynamics occurring during grafting formation in chestnut and table grape propagation. In view of the growing importance that these species are having, promoting the breeding of new cultivars and rootstocks, the combined study of these parameters can contribute to acquiring greater insight into the graft incompatibility matter.

Within the project, the implications of phenolic compounds during the grafting development of compatible and incompatible table grape and chestnut combinations were reviewed. Several phenolic compounds chosen among the most involved in the grafting process, based on preliminary investigations and literature, were considered. The identification and quantification of these potential markers were performed via HPLC, preceded by their extraction via green techniques. Chemical dynamics were explored at the graft union, above and below it in the outer and inner woody tissues, to find out whether a certain pattern in the phenolic production could be predictive of the graft incompatibility. In parallel, physiological traits such as stomatal conductance, fluorescence rate, and leaf chlorophyll content were assessed during the growing season as support tools. Finally, preliminary morphological observations were carried out at the graft interface of the experimental combinations.

The chapters of the present thesis

In **chapter 1 "From the nursery to the vineyard: a low-impact approach to secondary metabolite extraction during grafting"** a methodological research to define a sustainable and effective extraction method for the evaluation of phenol compounds in table grape woody tissues is presented. Though much research has been conducted on grapevine analysing the expression and

accumulation of phenolics during grafting, no literature is available for table grape. In the light of this, a blended approach for the extraction of phenol compounds from woody tissues was implemented, based on positive previous results on chestnut tissues. A preliminary overnight maceration through methanol solvent was combined with a green extraction via an ultrasound-assisted extractor (UAE). Three key extraction variables were tested in different combinations, namely the amount of plant material, the ultrasound-assisted extraction time, and the volume of extraction solvent. Four classes of polyphenols were investigated and chosen for their established role in grafting dynamics. The results provided a promising and eco-friendly methodology for the study of phenol compounds involved in the grafting process, which can be useful in exploring the interactions between new rootstock genotypes and table grape cultivars. This article has been submitted to Horticulturae, MDPI (Gamba et al., submitted).

In **Chapter 2 "Quali-Quantitative Study on Phenol Compounds as Early Predictive Markers of Graft Incompatibility: A Case Study on Chestnut (*Castanea* spp.)"** an experimental work on the implications of specific phenolic compounds during grafting development of compatible and incompatible chestnut combinations was reported. The separation, identification and quantification of the phenolic markers via HPLC was preceded by an ultrasonic green extraction. The phytochemical fingerprint was performed on the inner and outer tissues at the graft union, above and below it at two crucial stages (callusing and end of vegetative cycle) for each grafting combination. Two chromatographic methods were tested for a total of 15 phenol compounds. The aim of the research was to assess the potential application of these secondary metabolites as markers for the early prediction of graft incompatibility in chestnut propagation. The paper has been published on Horticulturae, MDPI (Gamba et al., 2021).

In **chapter 3 "Graft incompatibility in chestnut: a multidisciplinary approach for early identification in *Castanea* spp."** the chemical mechanisms

and the physiological response of several grafting combinations of *Castanea* spp. cultivars and rootstocks were investigated. The analysis on phenol compounds involved inner and outer tissues of chestnut grafts and focused on specific compounds selected based on previous research. Phenol compounds were firstly extracted using green extraction technology (ultrasounds). Separation, identification, and quantification were performed via HPLC. Analysis of physiological aspects was carried out using porometer, chlorophyll content meter, and Arborcheck® instrumentation. For the first time the study of the physiological response to grafting was assessed on chestnut. The results seem to validate the effectiveness of this multipurpose approach for the prediction of graft incompatibility in chestnut, supporting previous chemical findings.
This work has been presented at the XXXI International Horticultural Congress (IHC 2022, Angers, France) and published on Acta Horticulturae, ISHS (Gamba et al., 2023).

In **chapter 4 "Greater insights into chestnut (*Castanea* spp.) graft incompatibility through the monitoring of chemical and physiological parameters"** a multidisciplinary study on chestnut graft incompatibility dynamics is presented, based on the previous experience gained during the Ph.D. program. The work explored the physiological and chemical dynamics at the graft union, above and below it in different intraspecific and interspecific chestnut grafts. In particular, the implications of phenolic compounds during grafting development of compatible and incompatible combinations were reviewed at two phenological times. Stomatal conductance and leaf chlorophyll content were assessed during the growing season as support tools, being non-destructive useful indicators of plant water status. The aim of the study was to identify an effective approach for an early detection of incompatibility in chestnut propagation, to accelerate rootstock breeding and the development of resilient materials.
This article has been submitted to Scientia Horticulturae, Elsevier (Gamba et al., submitted).

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1. From the nursery to the vineyard: a low-impact approach to secondary metabolite extraction during grafting

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1.2. Abstract

The detrimental impact of climate change on crop yields, biodiversity, soil health, and water use necessitate the generation of innovative cultivation strategies. The development of crops able to tolerate stresses can be highly effective in adapting to the ever-changing ecological scenarios caused by climate change. For example, using rootstocks that are selected for their lower susceptibility to altered abiotic and biotic conditions can help mitigate the negative effects of climate change on crop productivity, soil health, and water use. The phenomenon of graft incompatibility is a significant limitation to the spread of new rootstock genotypes. Research has focused on the issue undertaking different approaches. Studies on the concentrations of phenols have highlighted the role of these molecules as markers of incompatibility in several horticultural species, although no specific research has been reported for table grape. In this study, an eco-friendly preliminary method to extract polyphenols from table grape tissues is proposed, coupling a traditional maceration using a solvent with a green technique with an ultrasound-assisted extractor. The following parameters were

compared: i) sample weight (0.1 g, 0.5 g, and 1 g); ii) time of ultrasound-assisted extraction (10 min, 20 min, and 30 min); iii) solvent volume for maceration (10 mL, 15 mL, and 20 mL). Four phenol classes were considered based on previous works on *Vitis* spp.: cinnamic acids, flavonols, benzoic acids, and catechins. The characterization of polyphenolic biomarkers was carried out via HPLC. 1 g of plant material, 30 minutes of sonification, and 20 mL of organic solvent was the combination of factors that resulted in the most efficient fingerprint, both quantitatively (267.68 ± 3.91 mg/100 g Fresh Weight [FW]) and qualitatively, with the four classes analysed significantly represented. This is the first work that proposed an extraction protocol for phenol compounds in table grape woody tissue based on both ecological and routine techniques. The definition of a protocol is a crucial first step for further analysis of secondary metabolites involved in graft incompatibility and ultimately for the adoption of new rootstocks for table grape cultivation.

Keywords: polyphenols; green techniques; graft incompatibility; hplc; rootstocks; mitigation strategies

1.3. Introduction

Climate change has dramatic consequences on agriculture due to the spread of new pests and diseases and the resulting abiotic stresses to which plants are subjected [1]. Mitigation strategies have been identified and they can be highly effective in facing the negative effects of climate change [2]. Among these strategies, one pivotal direction is to develop rootstock genotypes selected for their adaptability to changing environmental conditions. Although investigations on rootstocks have been very limited throughout the 20th century, in the last decades the interest has significantly increased. Rootstock choice can impart resistance to abiotic conditions related to weather (water scarcity, thermal stress) and soil (salinity, toxicity of heavy metals, stress related to soil pH, nutrient deficiency). Rootstocks can provide several benefits, including the ability to

create a well-developed and dense root system, which increases the plant's capacity to extract water from the soil. The vigor of the plant can also be positively influenced by rootstocks, as it affects light interception and carbon assimilation[3]. Moreover, rootstocks can confer plant resistance to soil-borne pests and pathogens, as demonstrated by the phylloxera-resistant American rootstocks[4]. Many horticultural species belonging to *Solanaceae* family are now grafted using rootstocks resistant to soil pathogens (*Fusarium* spp., *Verticillium* spp.), nematodes, bacteria, and extreme temperatures[5]. In the last decades, the University of Milan has been focusing on the selection of new rootstocks for grapevine cultivation, able to mitigate the effects of climate change. The series M-rootstocks were released in 2014, chosen for their tolerance to iron-limited conditions, resistance to salinity, and good tolerance to drought[6]. These new genotypes could represent a valuable crop strategy to face the current viticulture challenges for wine and table grapes.

Currently, selecting plant materials that can ensure high yield and quality standards while being able to withstand the impact of climate change is one of the most promising crop adaptation strategies. Research is actively addressing these challenges to guide genetic improvement and the breeding of new resilient rootstocks. However, the main challenge with using and spreading newly selected rootstock genotypes is the issue of incompatibility, which can occur during grafting propagation[7].

During the past decades, extensive research has focused on the issue of graft incompatibility on different species such as grapevine (*Vitis* spp.), olive (*Olea europea*), chestnut (*Castanea* spp.), peach (*Prunus* spp.), melon (*Cucumis melo*), and litchi (*Litchi chinensis*)[8-13] undertaking different approaches. Though, mechanisms underlying graft incompatibility are not yet fully understood, and only a few and/or outdated works are available for minor species. Many factors may interfere with the results interpretation, such as the technique employed and the degree of graft incompatibility. The identification of valuable transcript or metabolite markers of compatibility/incompatibility is challenging because of the high number of control samples and grafted individuals to be tested[14].

Moreover, in woody plants, delayed graft incompatibility can occur, which makes interpretations more complicated and the experiment time-consuming[15]. One promising avenue of research in addressing graft incompatibility has been the investigation of secondary metabolites, with a particular focus on phenol compounds. Studies have shown that these compounds may play a crucial role in the formation of graft unions, due to their involvement in cell division, development, and differentiation[16]. Research has demonstrated a relationship between the accumulation of polyphenols and the degree of compatibility in many fruit species, suggesting that manipulating phenol levels could be a potential strategy for improving graft success rates[17-21]. The identification and quantification of phenol molecules from vegetal samples are performed through high-performance liquid chromatography (HPLC), preceded by solvent extraction. Because of the complexity in the composition of the different phenol classes, there are no universal extraction procedures, and the extract composition depends on the technique and the solvent used[22]. Usually, solvents employed can be either organic or aqueous. Among them, the most commonly used ones are water, acidified water, methanol, propanol, ethanol, acetone, and their mixtures[23]. Usenik et al. proposed an extraction protocol on apricot (*Prunus armeniaca*) consisting of a 10 days maceration via acetone:water (80:20 v/v)[20]. The same protocol, slightly modifying the days of maceration and volume of solvent was performed on grapevine[8]. On eucalyptus (*Eucalyptus gunnii*), phenol compounds were extracted via methanol:ethanol (1:1 v/v) solvent[24]. Many parameters may influence the extraction yield of phenol compounds, such as the type of solvent, extraction time and temperature, liquid/solid ratio, sample matrix and size of the particles, and the plant species analysed[25]. Although conventional techniques are widely used for phenolic compound extraction, they suffer some setbacks that cannot be overlooked, namely the consumption of large amounts of hazardous organic solvents, the accumulation of residues, and the use of high-energy inputs[23].

In recent years, new extraction techniques for secondary metabolites were developed to improve extract quality and efficiency and to reduce solvent

consumption and extraction time. These techniques use different mechanisms such as ultrasound (ultrasound-assisted extraction, UAE), microwave energy (microwave-assisted extraction, MAE), supercritical fluids (supercritical fluid extraction, SFE), and elevated pressures (accelerated solvent extraction, ASE)[26].

The UAE method is a sustainable alternative to traditional extraction methods as it requires low solvent and energy inputs. This technique simplifies manipulation and reproducibility, as it can be performed at atmospheric pressure and ambient temperature and gives higher purity to final products. Finally, it can be employed in small and large-scale settings[27]. The UAE exploits the acoustic cavitation that damages the cell walls of the vegetal samples and favors the release of bioactive compounds[26]. In the last decade, this green technique has been successfully employed for the extraction of many compounds from fruits, vegetables, and plant materials[28, 29]. A recent study on *Punica granatum*[30] compared the extraction of antioxidants from the peel using the UAE method and conventional solvent maceration. Results reported higher antioxidant yields (+24%) and reduced extraction time (-90%) in the case of the UAE method. Another work carried out on tomatoes (*Solanum lycopersicum*) demonstrated that the use of the UAE technique allowed to increase the extraction yield of carotenoids by 143%, without causing any degradation of these compounds[31]. Finally, this environmentally friendly technique was used to extract biologically active molecules, such as polyphenols and other secondary metabolites, from *Castanea* spp. by-products. Specifically, this work analysed the nutraceutical composition of chestnut wastes deriving from bud-derivates, highlighting their ecological value and suggesting their circular reuse in alternative to composting or incineration[32].

The aim of the present work is to define a sustainable and effective extraction method for the evaluation of phenol compounds in table grape woody tissues. Though much research has been conducted on grapevine analyzing the expression and accumulation of phenolics during grafting, no literature is available for table grapes. Could phenol compounds be suitable markers of graft incompatibility on

table grapes, as observed on other tree crops? If so, could previous extraction protocols be efficiently adapted for detecting phenol compounds? Based on these premises, methodological research was conducted combining the conventional extraction method with the UAE technique, to reduce the use of organic solvents, extraction time, and increase the extraction yield of phenol compounds. The implementation of an extraction protocol for secondary metabolites in table grape woody tissues, based on both ecological and economical techniques, is an essential initial step towards researching graft incompatibility and ultimately enabling the use of new, sustainable rootstocks.

1.4. Materials and methods

1.4.1. Plant material

The plant material used in this experiment is grapevines of the table grape cultivar "Regal", one of the most commonly grown cultivars in the Piemonte region, grafted on the "420A" rootstock. While field experience suggests compatibility between these genotypes, no formal studies have been conducted yet to confirm this. For our experiment, the scion materials were provided by Cooperativa Monvisofruit soc. Agricola, while Vignevolute s.s.a. supplied the 420A rootstock. In April 2022, plants were grafted at Vignevolute nursery, located in Montà d'Alba (Cuneo province). This nursery belongs to Vignevolute s.s.a. and has extensive experience in *Vitis* propagation, with an annual production of about half a million grafted vines. Scions and rootstocks were subjected to thermotherapy, which consisted of a pre-treatment at 30°C for 20 minutes, followed by a treatment of woody materials at 50°C for 45 minutes to prevent future infections and contaminations, especially of phytoplasmas[33]. Then, vines were grafted using a machine able to perform the whip and tongue graft. Next, table grape vines were dipped into a solution made of *Trichoderma* spp., zeolite, and copper and then into paraffin to prevent dehydration at the grafting union. Finally, grafted vines were placed in forcing boxes filled with wet sawdust and stored for about four weeks at 30°C and 80-90% relative humidity. These conditions are essential to induce callusing formation. In May, table grape grafts were removed from

forcing boxes, dipped again into paraffin, and finally planted into 1.5 L pots. At this stage, known as callusing, plant material was sampled for the further experimental extraction of phenol compounds. The grafting union was removed from each plant cutting 1 cm above and 1 cm below. Samples were then stored refrigerated at -80°C until laboratory analysis. The extraction of secondary metabolites was performed on inner and outer tissues separately. For this reason, the cortex and barks were separated from internal tissues; then, they were ground in a mortar using liquid nitrogen to maintain the cold chain. Finally, the tissue powder was mixed, weighed, and stored at -80°C until the following extraction, as reported in previous similar studies [8, 10, 34, 35].

1.4.2. Extraction setup

In this study, different parameters were evaluated to define a protocol for the extraction of polyphenolic compounds produced by the plant tissues during grafting formation in table grape. Starting from previous studies[8, 10] a blended approach was implemented, based on a preliminary overnight maceration through methanol solvent followed by an ultrasound-assisted extraction (UAE). In particular, the amount of plant material resulting from the grafting union (0.1 g, 0.5 g, and 1 g), the time of ultrasonic extraction (10 min, 20 min, and 30 min), and the volume of solvent used for the extraction (10 mL, 15 mL, and 20 mL) were evaluated. The experimental work started by considering the amount of plant material (step 1 in Table 1). Phenol extraction was performed by testing three different quantities of plant material composed of internal and external table grape milled tissues, leaving the extraction time and the solvent volume fixed at 30 minutes and 20 mL, respectively, as previously reported for chestnut[10]. Once defined the proper plant tissue quantity, in step 2 three extraction times via ultrasound-assisted extractor were tested (10, 20, and 30 min), leaving unchanged the amount of plant material previously selected (1 g) and the solvent volume (20 mL). Step 3 concluded by evaluating the extraction of phenol compounds using different volumes of extraction solvent based on methanol, water, and HCl (10,

15, and 20 mL), keeping fixed the plant material (1 g) and the extraction time (30 min), as resumed in Table 1.

Table 1 *Experimental steps for the definition of the extraction protocol.*

Step	ID code	Plant material (g)	Extraction time (min)	Extraction solvent (mL)
1	**0.1 g**_30 min_20 mL	**0.1**	30	20
	0.5 g_30 min_20 mL	**0.5**	30	20
	1.0 g_30 min_20 mL	**1.0**	30	20
2	1.0 g_**10 min**_20 mL	1.0	**10**	20
	1.0 g_**20 min**_20 mL	1.0	**20**	20
	1.0 g_**30 min**_20 mL	1.0	**30**	20
3	1.0 g_30 min_**10 mL**	1.0	30	**10**
	1.0 g_30 min_**15 mL**	1.0	30	**15**
	1.0 g_30 min_**20 mL**	1.0	30	**20**

Many phenolics have been considered as markers based on previous studies on *Vitis* spp.[8, 18, 35]: 2 catechins, 5 flavonols, 2 benzoic acids, and 4 cinnamic acids. For each combination of parameters, selected amounts of the ground material were added to a specific quantity of extraction solvent based on methanol, water, and HCl (95:4.5:0.5, v/v/v) overnight. Then, polyphenolic compounds were extracted by an ultrasonic bath for 10, 20, and 30 minutes at 23 kHz (Reus sarl, Drap – France). Finally, all the extracts were centrifuged at 4000 rpm for 10 minutes and filtered through a 0,45 μm filter (polytetrafluoroethylene membrane - PTFE) before the HPLC-DAD analysis [28].

1.4.3. Chromatographic analysis

The characterization of polyphenolic biomarkers was carried out by a 1200 Agilent HPLC - UV-Vis Diode Array Detector (Agilent Technologies, Santa Clara, CA, USA). The molecules were separated on a Kinetex C18 column (4.6x150 mm, 5 μm, Phenomenex, Torrance, CA, USA) [36]. The different HPLC methods and their specific parameters are reported and described in the Supplementary Materials (Table S1).

1.4.4. Statistycal Analysis

One-factor ANOVA test (SPSS 22.0) was performed on the data, and then a Tukey's HSD post hoc comparison test ($n = 3$; $P < 0.05$) was used to compare the mean values. Results were reported as mean value ± standard deviation (SD). Different letters were used to highlight significant statistical differences concerning the Tukey test ($P < 0.05$).

1.5. Results and discussion

Some scientific papers have proposed a protocol for the extraction of phenol compounds from *Vitis* spp. woody tissues. The procedures available were adapted starting from works on other fruit species with similar tissue composition[37], and the final protocol was set up for wine grapes[8]. Nevertheless, no studies have been carried out on the phenol expression in table grape woody tissues. In this study, a suitable and sustainable method to extract polyphenols was proposed evaluating different combinations of sample weight, time of extraction, and solvent volume.

1.5.1. Step 1: amount of plant material

The experimental study focused on the weight of the plant samples as the primary variation factor. Phenol extraction is normally performed on tissues previously milled using liquid nitrogen. The process is time, energy, and sample-consuming for the operator, especially if tissues have more than one year or belong to hardwood species. Indeed, this operation cannot be mechanized, as polyphenols are considered heat-labile compounds and could undergo thermal degradation with the use of homogenizers[38]. It is essential therefore to find the minimum amount that allows for an efficient phenol extraction. Moreover, reduced quantities imply a limited consumption of solvent, which leads to lower waste production and minor environmental and economic costs[39].

The extraction by maceration coupled with an ultrasound system and HPLC identification of phenolics from plant material at minimum levels may be an excellent analytical strategy to obtain a phytochemical profile in accordance with

sustainable international protocols. Since no studies addressed the issue for table grape tissues, the so-called liquid/solid ratio (expressed in mL of solvent per g of solid) must be investigated to define a protocol that maximizes the extraction yield and minimizes the solvent consumption and the sample preparation time.

Most of the extraction methods reported in the literature for phenol compounds used a liquid/solid ratio in the range of 5–50 mL/g[40]. On other woody crops, the amount of plant material used in literature for phenol characterization varies depending on the species investigated and on the extraction technique, ranging from 0.01 g for apricot[20], 0.1 g for pear[19], 0.5 g for Siberian elm[41], and up to 10 g for pear-quince[21].

The current study examined three different weights of plant samples: 0.1 g, 0.5 g, and 1 g, and the extraction efficiency was evaluated as the sum of the phenolic compounds extracted. Figure 1 presents the results of the extraction efficiency based on the quantity of plant material used for the extraction.

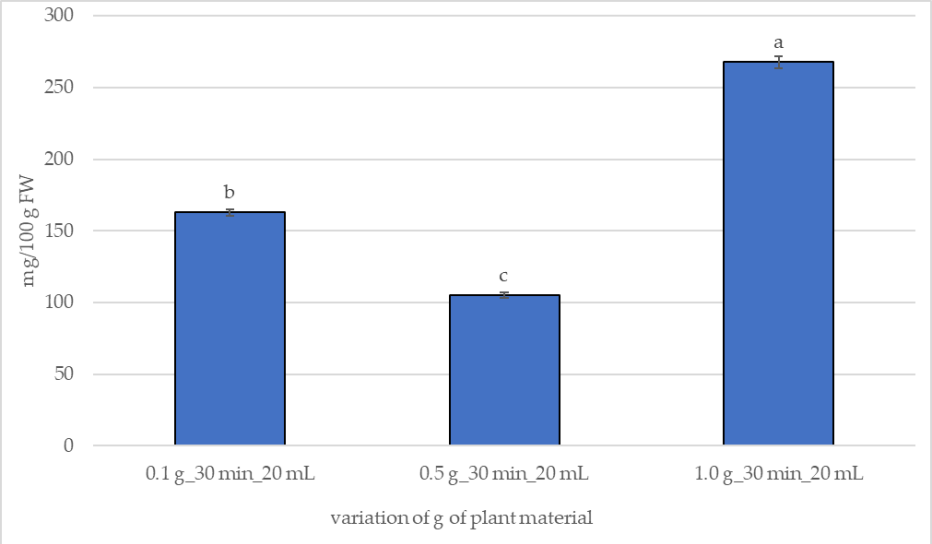

Figure 1. Influence of the different amounts of plant material on the extraction efficiency, expressed as the sum of the phenol classes considered for the analysis obtained by HPLC fingerprint. The results are reported as mg/100 g FW (FW = fresh weight). Mean value and standard

deviation are given for each sample (n = 3). Different letters for all the considered groups indicate significant statistical differences ($p < 0.05$).

According to Figure 1, 1 g of plant material was found to be the best amount (267.68 ± 3.91 mg/100 g FW). Reduced quantities of the sample were not enough to detect all the phenol classes investigated (Figure 2). Cinnamic acids, which are almost one-third of the total phenolic compounds identified with 1 g, were not found with 0.1 and 0.5 g of plant material. The most represented was the class of flavonols (34.99%), followed by the catechins (31.06%).

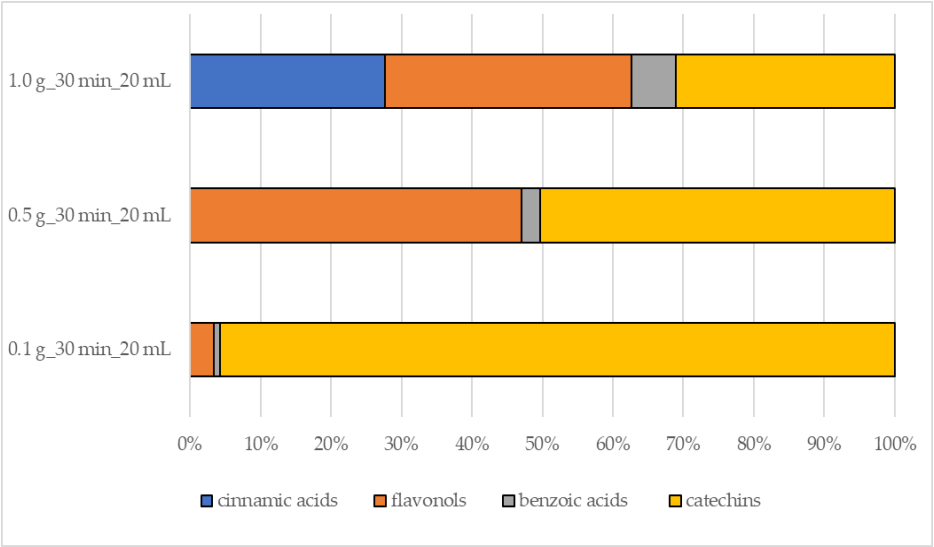

Figure 2. Phytochemical fingerprint according to the amount of plant material used for the extraction of phenol compounds.

In recent years cinnamic acids have been proven to be related to the grafting process in *Vitis* spp. In particular, the concentration of sinapic acid increases in incompatible combinations, highlighting its role as a potentially suitable marker of graft incompatibility[8, 18]. Rather different is the behaviour of another cinnamic acid, the chlorogenic acid, that was found to be more accumulated at the grafting union of compatible combinations, especially during callusing and rooting stages[18]. As its chemical structure is very similar to those of some lignin

intermediates, chlorogenic acid could be considered an intermediate of the lignin biosynthetic pathway[42]. The employment of this compound to produce lignin monomers is linked to physiological or environmental signals. The grafting process can be considered as an intense stress for plants, therefore the re-routing of the chlorogenic acid towards lignin during grafting formation can be expected, especially with compatible unions. The concentration of this molecule was not detected using 0.1 and 0.5 g of plant material, while 1 g allowed to obtain a significant value of 34.76 ± 1.55 mg/100 g FW. Two other cinnamic acids were linked to graft incompatibility in recent works. Ferulic and coumaric acids have been found in higher concentrations in incompatible unions of *Uapaca kirkiana*[43], while ferulic acid was more abundant in *Vitis* spp.[8] incompatible grafts. Finally, research on graft incompatibility between Sato-zakura cherry cultivars and *Prunus avium* rootstock pointed out the role of coumaric and ferulic acids as biomarkers for possible delayed incompatibility[44]. In the present work, no traces of ferulic acid were detected for all the amounts of plant material tested, while coumaric acid was found in low concentration only with 1 g.

Based on the lack of previous studies on table grapes and on the findings on other fruit species, this preliminary research focused on the expression of the main classes of phenol compounds involved in the grafting process, rather than delving into the dynamics of individual markers. In light of this, the amount of plant material that yielded the broadest and most comprehensive phenol fingerprint across the four investigated classes was determined to be 1 g.

1.5.2. Step 2: time of extraction using UAE

Among the conventional extraction techniques of phenol compounds, maceration has been one of the most used. It consists in blending pulverized plant material into the appropriate solvent, which can extract valuable compounds by a proper selection of solvent polarity[45]. The main limitations of maceration are the large amounts of time and solvent required. Nowadays, thanks to the availability of more practicable and eco-friendly techniques this method has been gradually abandoned[23]. In this study, two techniques were combined to increase the

sustainability of phenol extraction; a traditional maceration using solvent was coupled with a green method using an ultrasound-assisted extractor. In this step, the amount of sample material and the solvent volume were left constant, 1 g and 20 mL. Then, three extraction times were tested for the UAE (10, 20, and 30 min). Considering the ultrasound-assisted extractor used in this work, the reduction of the extraction time would allow an energy saving of 0.034 kWh (20 min) and 0.066 kWh (10 min). Moreover, the operator would be less exposed to noise, which is a physical hazard in the occupational setting. These benefits add to the environmental advantages of ultrasounds if compared to other techniques, represented by lower use of non-renewable resources and lower energy consumption[46].

Figure 3 reports the sum of phenol compounds extracted with different UAE times. Reducing the time from 30 to 20 and 10 minutes statistically lowered the extraction yield, respectively of 36.28% and 23.21%.

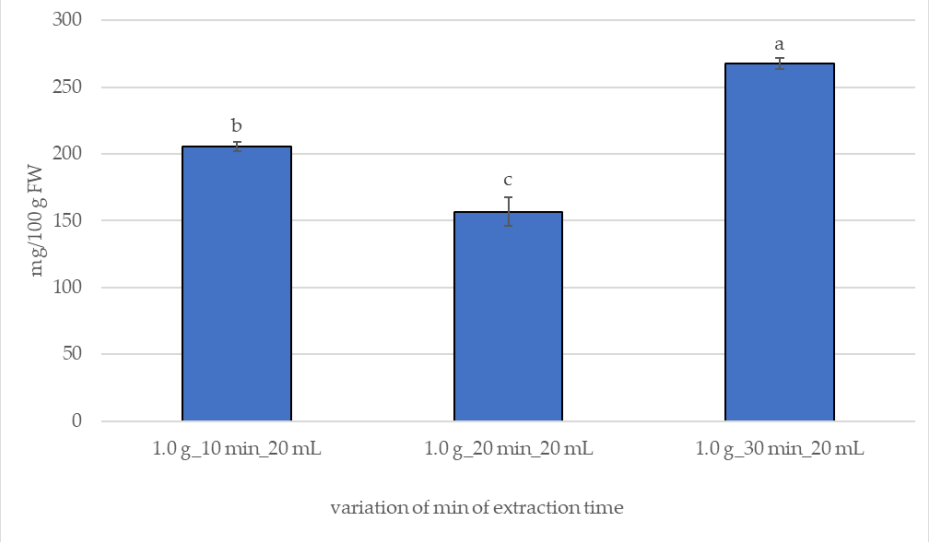

Figure 3. Influence of the different extraction times on the extraction efficiency, expressed as the sum of the phenol classes considered for the analysis obtained by HPLC fingerprint. The results are reported as mg/100 g FW (FW = fresh weight). Mean value and standard deviation are given for each sample (n = 3). Different letters for all the considered groups indicate significant statistical differences ($p < 0.05$).

The lower extraction time (10 min) did not allow to detect the phenol class of the cinnamic acids (Figure 4), showing a pattern already observed using the lower quantity of sample material during the first step (0.1 g). Decreasing from 30 to 20 minutes resulted in an extraction loss of 78.78% for cinnamic acids (74.09±1.18 vs 18.69±1.12 mg/100 g FW), and 52.02% for flavonols (93.65±0.96 vs 44.93±1.40 mg/100 g FW).

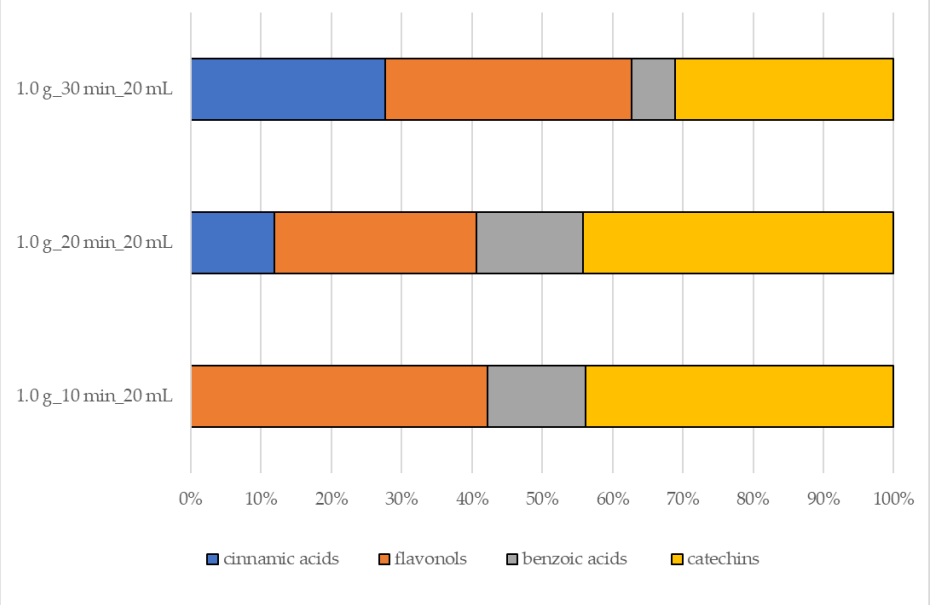

Figure 4. Phytochemical fingerprint according to the extraction times tested for the extraction of phenol compounds.

Gallic acid, catechins, sinapic and ferulic acids, and epicatechins are all compounds that were found in higher concentrations in tissues of incompatible grafts of different species[8, 19, 20]. Accordingly, low concentrations of these molecules may be interpreted as a sign of affinity. Among the benzoic acids, gallic and ellagic acids were investigated because of their role in grafting dynamics. In particular, gallic acid turned out to be a good marker of graft incompatibility for several species, such as *Prunus armeniaca*, *Vitis* spp., and *Castanea* spp.[10, 20, 35]. The concentration of this compound was found to be higher at the graft union

at early stages of development, such as callusing, and decreasing during the growing season. Grafting propagation represents a stress for plants, especially if two incompatible genotypes are combined. Gallic acid is normally biosynthesized under stress conditions since it is active in protecting plant cells from oxidative stress[47]. At present, little research has been done on its antioxidant effects in the reaction to stress. However, an increased production of this molecule could be related to incompatibility between partners, as an attempt to overcome the grafting stress. Ellagic acid has a similar behavior, decreasing during grafting establishment, though differences between compatible and incompatible combinations were significant only on *E. gunnii*[24].

Though on one hand, phenol markers increase in the case of incompatible grafts, for at least one compound it was observed a different behavior. In fact, the biosynthesis of chlorogenic acid seems to be stimulated in the case of compatible unions, as it was reported in *Vitis*[18]. The extraction time of 30 minutes enabled the extraction of the most abundant amount of cinnamic acids. Among them, the most expressed was chlorogenic acid (46.92%), supporting the compatibility of the experimental combination tested. The remaining part of this class of compounds was represented by caffeic acid (40.69%), and coumaric acid (12.39%). In previous research on *Vitis*, caffeic acid was found in very low quantities only at the callusing stage of both compatible and incompatible grafts, so its biosynthesis may not be related to disaffinity[18]. As regards coumaric acid, its involvement in graft dynamics has still not been clarified on grapevine. Considering the low quantity measured on table grape tissues during this study, it was not considered as a proper predictive marker.

The most effective extraction time according to the results was 30 minutes, as it was possible to obtain a thorough screening of the four classes of phenol compounds considered.

1.5.3. Step 3: volume of extraction solvent

Conventional solid-liquid extraction or conventional methods are two terms defining any process that requires simple maceration in a solvent, normally at

room temperature, and at atmospheric pressure[40]. Despite its simplicity, this technique has been questioned because of its drawbacks in terms of environmental impacts which are not justified by higher extraction yield efficiency. Based on these considerations, the present work coupled a green extraction technique (UAE) to the traditional maceration. The extraction solvent used was methanol, water, and HCL (95:4.5:0.5, v/v/v). Methanol is an organic solvent among the most used for polyphenol extraction, especially from plant matrices[48, 49]. The combination of these two techniques was evaluated on table grape aimed at reducing the volume of methanol-based solvent making the extraction process more sustainable[50]. A recent work on *Crocus sativus* has shown the effectiveness of this combined method on petal extracts for the assessment of total phenolic content[51]. In this study on saffron, this hybrid approach was compared to a traditional maceration at 45°C under magnetic stirring, using two different extraction solvents. The combined method via sonification allowed to obtain an extract with a statistically higher total phenol content if compared to the method via maceration.

In the present study, 1 g of sample material was macerated overnight at room temperature testing different solvent volumes and further placed in an ultrasonic bath filled with distilled water. The sonification process took 20 minutes, which was the best extraction time as emerged by the second step. The phenol extraction efficiency was evaluated by comparing 10, 15, and 20 mL of organic solvent (Figure 5).

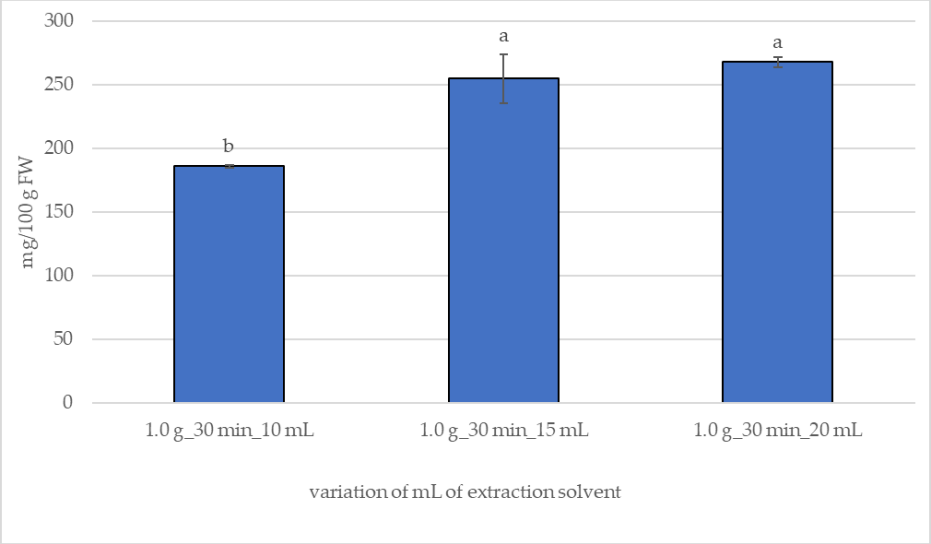

Figure 5. Influence of the different volumes of solvent on the extraction efficiency, expressed as the sum of the phenol classes considered for the analysis obtained by HPLC fingerprint. The results are reported as mg/100 g FW (FW = fresh weight). Mean value and standard deviation are given for each sample (n = 3). Different letters for all the considered groups indicate significant statistical differences ($p < 0.05$).

The significantly lower extraction yield was obtained using 10 mL of organic solvent (185.79 ± 1.30 mg/100 g FW). No statistical differences in the amount of total phenol compounds were found using 15 mL (254.67 ± 19.25 mg/100 g FW) or 20 mL (267.68 ± 3.91 mg/100 g FW).

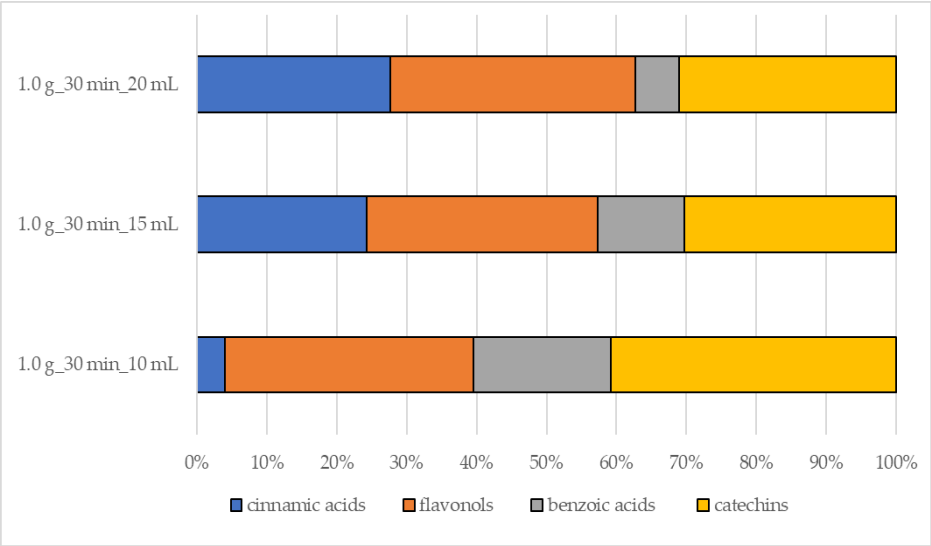

Figure 6. Phytochemical fingerprint according to the volume of solvent tested for the extraction of phenol compounds.

The use of 20 mL of extraction solvent provided the most effective and comprehensive results in terms of characterizing cinnamic acids, flavonols, and catechins. Though, there were no statistically significant differences with the results of using 15 mL. The low yield of benzoic acids could further support the compatibility between the genotypes analysed in this study, as no traces of gallic acid were detected at any solvent volumes. Due to the lack of works on table grape that focused on the expression of phenol compounds related to graft incompatibility, the main phenol classes involved in graft incompatibility according to earlier works on other fruit crops have been taken into account[8, 18, 35]. Considering the focus on multiple markers rather than on single/few specific compounds, 20 mL as the proper volume of organic solvent was proposed, since it furnished the broader bands for the four phenolic classes analysed.

This work focused on three key parameters influencing the extraction of phenol compounds. Therefore, further studies are planned to validate and verify these findings, testing additional extraction variables and new grafting combinations

with the aim of developing an optimized extraction protocol of polyphenols from table grape woody tissues. Investigations on the level of extraction solvent should be deepened, since the results of this study suggest that it could be slightly reduced between 10 and 15 mL for certain phenol compounds without compromising the extraction potential. Lessening the quantity of organic solvent would be a great enhancement in terms of extraction process sustainability.

The identification of potential markers for graft compatibility, such as benzoic acids, catechins, and cinnamic acids, could help to identify new genotypes with high compatibility rates and resilience to environmental stressors such as climate change. The development of new, resilient rootstocks could lead to increased yields, improved quality, and reduced environmental impact, making viticulture more sustainable and profitable.

1.6. Conclusions

The present study is the first that addressed the matter of graft incompatibility in table grape from a biochemical perspective. In particular, the work focused on the development of a preliminary extraction method for phenol compounds in woody tissues. Four classes of polyphenols were investigated, chosen for their established role in grafting dynamics. Three key extraction variables were tested in different combinations, namely the amount of plant material, the ultrasound-assisted extraction time, and the volume of extraction solvent. 1 g of plant material, 30 minutes of sonification, and 20 mL of organic solvent resulted in the most efficient fingerprint, both quantitatively and qualitatively, with the four phenol classes significantly represented. The results provided a promising and sustainable methodology for the study of phenol compounds involved in the grafting process, which can be useful in exploring the interactions between new rootstock genotypes and table grape cultivars.

This research supports further investigation into graft incompatibility in table grape and enhance the development of innovative rootstocks for the future of viticulture.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Chromatographic conditions of the used methods; Figure S1: Flow chart representing the extraction setup.

Author Contributions: Conceptualization, Giovanni Gamba, Dario Donno and Gabriele Beccaro; Data curation, Giovanni Gamba and Dario Donno; Formal analysis, Giovanni Gamba and Dario Donno; Investigation, Giovanni Gamba and Dario Donno; Methodology, Giovanni Gamba and Dario Donno; Resources, Giovanni Gamba; Supervision, Gabriele Beccaro; Validation, Paolo Sabbatini and Gabriele Beccaro; Writing – original draft, Giovanni Gamba; Writing – review & editing, Dario Donno, Zoarilala Razafindrakoto, Paolo Sabbatini and Gabriele Beccaro. All authors have read and agreed to the published version of the manuscript.

Funding: this research was funded by Next Generation EU - National Recovery and Resilience Plan (PNRR) – National Research Centre for Agricultural Technologies -AGRITECH, Task 4.1.2 (SPOKE 4), identification code: CN00000022.

Acknowledgments: the authors thank Vignevolute s.s.a. and Società Cooperativa Agricola Monvisofruit that made facilities and plant material available for this experimental work. We would like to express our sincere gratitude to Giovanni Marchisio, who contributed greatly to the research with his availability and expertise.

Conflicts of Interest: The authors declare no conflict of interest.

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2. Quali-Quantitative Study on Phenol Compounds as Early Predictive Markers of Graft Incompatibility: A Case Study on Chestnut (*Castanea* spp.)

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Quali-Quantitative Study on Phenol Compounds as Early Predictive Markers of Graft Incompatibility: A Case Study on Chestnut (*Castanea* spp.)

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2.2. Abstract

In recent years, research has focused on phenolic compounds and their putative role as markers of graft incompatibility. Thus far, no studies have been conducted on the role of phenolic compounds in chestnut (*Castanea* spp.). The present study investigated the content of phenolic compounds in different combinations of *Castanea* spp. cultivars and rootstocks. Analyses were performed on the inner and outer tissues of chestnut grafts at two phenological sampling stages. The separation, identification and quantification of the phenolic markers via HPLC were preceded by an ultrasonic green extraction. Two chromatographic methods were tested for a total of 15 phenol compounds. Flavonol compounds were not detected, while cinnamic acids were found in low concentrations. The amount of gallic acid turned out to be higher at the graft union of the incompatible combination (20.11 ± 1.47 mg/100 gFW vs 8.94 ± 1.08 mg/100 gFW). The same pattern was observed for catechin (15.79 ± 1.83 mg/100 gFW vs 9.63 ± 1.98 mg/100 gFW). Differences in tannin concentrations seemed to be species-specific, and were apparently not related to graft incompatibility. The present

work underlines the potential application of certain phenol compounds for the early prediction of graft incompatibility in *Castanea* spp.

Keywords: compatibility; polyphenols; grafting; propagation; clonal rootstocks; HPLC; green extraction; biochemical compounds

2.3. Introduction

In recent years, the comprehension of the grafting process has made great progress; however, graft incompatibility remains one of the major obstacles in woody plant breeding, and for the nursery industry. Successful grafting is a complex biochemical and structural process that begins with an initial wound response, followed by callus formation, leading to the creation of a continuous cambium and plasmodesmata, and the establishment of a functional vascular system between the two grafting partners [1]. The critical points where incompatibility can occur are many, primarily when the meristematic tissues of both the scion and rootstock fail to merge and build a new cambium connection [2]. Furthermore, the fact that the new vascular connections could not be well differentiated, or were weakly established, has been postulated as the main reason for incompatibility in woody plants [3,4]. In fact, graft incompatibility can induce the undergrowth or overgrowth of the scion, which can provide dysfunctions in water and nutrients' flow through the graft union, and can cause the wilting of the plant. Usually, it occurs at early stages, when vascular connections are forming, but it can also appear at the fruiting stage, when the plant has a high demand for water and nutrients [5].

Because this incompatibility can occur either at an early phase or after years, with the tree breaking at the union point, it causes severe economic damage. In order to avoid incompatibility, the graft partners often belong to the same genus, although the grafting of different species is also common in order to induce resistance to soil-borne pests and diseases, and to enhance the tolerance to abiotic stresses [6]. It is therefore necessary to identify experimental procedures that address whether a certain breed will be compatible or not. In recent years,

investigation has focused on phenolic compounds and their putative role as markers of either compatible or incompatible grafts.

Phenolic compounds, the biosynthesis of which is triggered by wounding and infections, are produced and accumulated during the callusing phase. It seems that the quantitative and qualitative differences in the phenolic patterns between the scion and the rootstock can imply dysfunctions at the graft union in different fruit trees, which leads us to consider them as potential markers of graft compatibility or incompatibility [7–10]. As a matter of fact, these compounds can be used as markers of graft incompatibility at an early stage: it is known that in *Vitis* spp., gallic acid increases in the case of incompatible grafts, while ferulic acid decreases [10]. In *Prunus armeniaca* (L.) grafted on incompatible *Prunus* rootstocks, the amount of phenolic compounds is higher, and is generally associated with the formation of small cells in incompatible scion–rootstock combinations that did not form successful unions in vitro [7]. Moreover, in less compatible apricot scion–rootstock combinations, a higher level of flavanols like catechin and epicatechin were detected [7,11,12]. High concentrations of catechin and epicatechin were also measured in pear–quince incompatible grafts before the appearance of visible incompatibility symptoms [13]. While the role of phenolic compounds has been tested in *Vitis* spp., *Prunus* spp. and *Pyrus communis* (L.), no studies have been conducted on *Castanea* spp.

In the last few years, the cultivation of chestnut has been experiencing a revival phase, affirming itself as an important fruit crop, especially in the European area. This renewed interest in its cultivation has caused the shifting of its production from being extensive to intensive. In chestnut farming, grafting has proven to be a very successful and reliable method of asexual propagation. However, incompatibility can occur, especially between European species and hybrid rootstocks, which are the most utilized in the orchard industry [6,14]. Graft incompatibility has several causes, above all of which are some anatomical issues: it is known that the unusual fluted stem structure existing in several species can cause the misalignment of tissues between the scion and the rootstock, as well as a necrotic layer at the grafting point which will act as a

barrier between the two partners. In incompatible grafts, a mass of parenchymatous tissue interrupts the normal vascular connection between the rootstock and scion; the interruption of cambial continuity was also observed, probably due to the proliferation of phloem fiber tissue. Eventually, the lack of winter hardiness, infection by chestnut blight at the union point, poor grafting techniques, and scion–rootstock genetic distance can also provoke graft failure [14]. As described above, a successful grafting also depends on biochemical and metabolic reactions between the two partners. It seems that peroxidases play a potential role in grafting, as these enzymes are involved in lignin formation and lignin–carbohydrate bonding [15]. Graft incompatibility in chestnut can be classified into 'early', if it is seen in the first two years, and 'late', if it occurs after at least 5–7 years [6].

The aim of this research was to investigate an innovative biochemical approach for the early prediction of graft incompatibility between different chestnut genotypes. In order to do this, it focused on identifying and measuring phenolic compounds at the graft union, below it and above it to find out whether a certain pattern in the phenolic production could be predictive of graft incompatibility. It is a procedure that requires routine HPLC analysis, and it can be performed in the first year of grafting, thus allowing us to determine the success a priori before visual symptoms occur.

2.4. Materials and Methods

2.4.1. Plant Material

Scions of *Castanea sativa* (Mill.) and *Castanea mollissima* (Bl.) were grafted onto different seed and clonal rootstocks of *C. sativa, Castanea crenata* × *Castanea sativa* and *Castanea pumila* (L.) Mill. Tissues from the grafting point, 5 cm above and 5 cm below it were collected at two phenological sampling stages: at the callusing time (CAL), 60 days after grafting, and at the end of the vegetative cycle (EVC), 255 days after grafting. Callus formation is an essential step for the graft development, but it is not enough to ensure its success. During

this step, an intense production and accumulation of secondary metabolites occurs. Many studies have reported differences in phenol expression at this stage. The plant material was collected at the Chestnut R&D Center, Piemonte (Chiusa Pesio, Cuneo Province, Italy), and from Castellino Riccardo Vivai. The cultivars used for the experimentation were 'Marrone della Val Susa' (*C. sativa*), a valuable marrone type variety historically cultivated in the Piemonte Region, and 'Chushuhong', a Chinese cultivar (*C. mollissima*). Four grafting combinations were tested, having been chosen based on their known compatibility level, as summarized in Table 1 and described below:

i. Chinese cultivar 'Chushuhong' × Euro-Japanese hybrid seedling rootstock (CH × s_EUJAP);

ii. European cultivar 'Marrone della Val Susa' × Euro–Japanese hybrid seedling rootstock obtained from a progeny field (MSUS × sp_EUJAP);

iii. European cultivar 'Marrone della Val Susa' × Euro–Japanese hybrid clonal rootstock 'Marsol CA07' (MSUS × c_EUJAP);

iv. European cultivar 'Marrone della Val Susa' × *C. pumila* seedling rootstock (MSUS × s_PUM).

Table 1. Grafting combinations tested and the known level of compatibility.

Combination	Scion	Rootstock (Species)	Known Compatibility Level
CH × s_EUJAP	'Chushuhong' (*Castanea mollissima*)	hybrid seedling (*C. crenata* × *C. sativa*)	Incompatible
MSUS × sp_EUJAP	'Marrone della Val Susa' (*Castanea sativa*)	seedling progeny 'MB221′ (*C. crenata* × *C. sativa*)	compatible
MSUS × c_EUJAP	'Marrone della Val Susa' (*Castanea sativa*)	clonal 'Marsol CA07′ (*C. crenata* × *C. sativa*)	compatible
MSUS × s_PUM	'Marrone della Val Susa' (*Castanea sativa*)	Seedling (*C. pumila*)	unknown

Trees were grafted in 2021 with the whip graft technique, which is widely used on chestnut because it guarantees a structural resistance at the graft union point.

Mastic was applied on the upper cut of the scion to avoid dehydration, and then a specific biodegradable grafting tape was used to bond the union point tightly.

For each graft combination and at each phenological stage, five plants were sampled and stored at −80 °C until the analytical analysis. Three small sections of 5 cm each were then obtained from every plant: one section above the graft (corresponding to the scion), one at the graft union, and one below the graft (corresponding to the rootstock).

Then, each section was cut longitudinally, and the bark and cortex were separated from the inner tissues. The outer and inner tissues were immediately frozen in liquid nitrogen and separately ground in a mortar. This step had to be performed very rapidly in order to avoid tissue oxidation and the hydrolysis process, which may modify the phenolic composition of the samples. For each section, the ground tissues derived from the five plants of each graft combination were then mixed, weighed and stored at −80 °C until extraction, as described in a previous study on *Vitis* spp. [16].

2.4.2. Extraction of the Polyphenolic Markers

In this study, several phenolic compounds were considered, having been chosen from among those that were found to be more involved in the grafting process: 4 cinnamic acids (caffeic, chlorogenic, coumaric and ferulic acids), 5 flavonols (hyperoside, isoquercitrin, quercetin, quercitrin, rutin and ellagic acid), 2 benzoic acids (ellagic and gallic acids), 2 catechins (catechin, epicatechin) and 2 tannins (castalagin, vescalagin). A preliminary solvent extraction was performed: 1 g of the ground material was added to 20 mL of a mix of methanol, water and HCL (95:4.5:0.5, v:v:v) overnight. Then, the secondary metabolites were extracted by an ultrasonic bath for 30 min (Hielscher Ultrasonics UP200 St, Teltow, Germany). Later, each sample was centrifuged at 4000 rpm for 10 min, and then the liquid phase was filtered through a 0.45 μm filter (polytetrafluoroethylene membrane—PTFE) and finally analysed by HPLC-DAD [17].

2.4.3. Chromatographic Analysis

The separation, identification, and quantification of the phenolic markers were performed by an Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA, USA) with manual injection (20 µL sample loop) coupled to an UV-Vis Diode Array Detector.

The chromatographic separation was performed on a Kinetex C18 column (4.6 × 150 mm, 5 µm, Phenomenex, Torrance, CA, USA) [18].

The different procedures utilised in the analysis are described in the Supplementary Materials (including Table S1).

2.4.4. Statistical Analysis

Data were subjected to a one-factor ANOVA test (SPSS 22.0), and the mean values were compared with Tukey's HSD post-hoc comparison test at $p < 0.05$ ($n = 3$). The data are expressed as the mean value ± standard deviation (SD). The significant statistical differences ($p < 0.05$) are highlighted by different letters according to the Tukey test.

2.5. Results and Discussion

The aim of the present work was to verify whether phenol compounds could be used as markers of graft compatibility in chestnut propagation, in order to forecast at a very early stage the grafting success rate. In this study, five classes of phenols—for a total of 15 bioactive compounds—were analysed: benzoic acids (ellagic and gallic acids), cinnamic acids (caffeic, chlorogenic, coumaric and ferulic acids), catechins (catechin and epicatechin), tannins (castalagin and vescalagin) and flavonols (hyperoside, isoquercitrin, quercetin, quercitrin and rutin). From all the sections analysed, the graft union allowed us to better discriminate the differences in terms of the expression of phenol compounds. In light of this consideration, the reported results focus on the graft union tissues.

The most represented bioactive class was tannins (75.19% at CAL and 81.10% at EVC), followed by catechins (21.21% and 16.20%), benzoic acids (3.32% and

2.45%) and cinnamic acids (0.27% and 0.25%). Compounds belonging to the flavonol class were not detected.

Although the discussion of the results deals with all of the phenols cited above, only a few compounds showed statistically significant differences between the compatible MSUS × c_EUJAP ('Marrone della Val Susa' grafted on clonal 'Marsol CA07') and incompatible CH × s_EUJAP ('Chushuhong' × hybrid seedling) graft combinations, confirming what was found on fruit species such as *Pyrus, Vitis* and *Prunus* spp. Moreover, this is the first work that focused on the role of phenolic compounds in chestnut grafting, contributing to the improvement of the knowledge of this under-studied crop.

2.5.1. Benzoic Acids

Table 2 reports the concentration of benzoic acids found at the graft area for all of the combinations tested, as measured at CAL and EVC stages.

Table 2. Benzoic acid fingerprint of the tissues at the graft union. The results are reported as mg of the bioactive compound per 100 g fresh weight (FW).

Phenological Stage	Bioactive Class	Compound	CH × s_EUJAP		MSUS × s_EUJAP		MSUS × c_EUJAP		MSUS × s_PUM	
			Mean Value (mg/100 g_{FW})	SD	Mean Value (mg/100 g_{FW})	SD	Mean Value (mg/100 g_{FW})	SD	Mean Value (mg/100 g_{FW})	SD
Callusing	Benzoic acids	ellagic acid	31.10 a	2.84	36.02 a	3.36	32.41 a	2.64	35.23 a	4.75
		gallic acid	20.11 c	1.47	11.40 ab	1.87	8.94 a	1.08	14.09 b	1.19
End of vegetative cycle	Benzoic acids	ellagic acid	24.63 a	3.71	28.62 a	4.64	26.10 a	2.18	27.17 a	2.56
		gallic acid	15.71 c	1.70	6.77 ab	1.55	5.58 a	1.57	11.55 bc	1.05

The mean value and standard deviation are given for each sample (n = 3). Different letters for each compound indicate the significant differences at $p < 0.05$.

According to the results, the concentration of gallic acid had a general pattern in all the tested combinations, decreasing from CAL to the EVC. From the analysis of the amounts detected at CAL on the graft union tissues, clear and statistically significant differences emerged. The incompatible combination CH × s_EUJAP recorded the statistically higher accumulation of this compound (20.11 ± 1.47

mg/100 gFW), followed by MSUS × s_PUM (14.09 ± 1.19 mg/100 gFW), the compatibility level of which is still unknown. The smallest quantities of gallic acid were found in MSUS × c_EUJAP (8.94 ± 1.08 mg/100 gFW), which were over two times lower than in the incompatible combination, and in MSUS × s_EUJAP (11.40 ± 1.87 mg/100 gFW), with both having a well-known affinity.

These data highlight a variability among the compatible combinations, depending on the origin of the rootstock: the concentration of gallic acid lightly increased in the seedling rootstock combination MSUS × s_EUJAP with respect to clonal rootstock MSUS × c_EUJAP, both at the CAL and EVC stages.

At the EVC stage, a decrease of the gallic acid amounts was recorded in all the tested combinations. The higher quantity in the graft union tissues, as for the CAL stage, was found in CH × s_EUJAP (15.71 ± 1.70 mg/100 gFW), followed by MSUS × s_PUM (11.55 ± 1.05 mg/100 gFW), which did not differ statistically from the incompatible combination. The compatible combinations showed the largest decrease of gallic acid, −40.61% for MSUS × s_EUJAP and −37.58% for MSUS × c_EUJAP. The letter grafting combination had the statistically significant lowest value (5.58 ± 1.57 mg/100 gFW).

These results are in accordance with previous work on apricot (*Prunus armeniaca*) establishing that gallic acid production is higher in less-compatible graft combinations, especially at early stages [9]. In *Vitis* spp., this acid proved to be a good marker of compatibility, as it was more abundant in less-compatible graft combinations [10,16,19]. As well as *Vitis* spp., a study on *Eucalyptus gunni* (Hook.f.) phenol composition showed a gallic acid peak in incompatible grafts [20]. In this study, in vitro plantlets of *E. gunni* were sampled at the grafting union, and gallic acid was quantified 20 and 40 days after grafting. The amount of this acid decreased severely from 20 to 40 days in both the compatible and incompatible combinations, with the highest concentration being recorded at 20 days in the incompatible grafts. As suggested by many authors [12,19,21], the higher amount of gallic acid at the graft union may be explained by its antioxidant power, which enhances the reductive state of the cells and confers tolerance to the oxidative stress that exists mainly at the graft union. Gallic acid is a benzoic

acid normally produced under stress conditions, so it may be the mechanism through which the plant attempts to overcome the stress caused by the graft.

Ellagic acid and gallic acid had a similar pattern, with both decreasing from CAL to EVC, but in the case of ellagic acid, this decrease was less pronounced. Indeed, its concentration in all of the combinations shrank by about 20%, following a pattern already observed on *E. gunni,* where this compound showed statistically significant differences among compatible and incompatible combinations [20]. This compound did not show any deep differences between compatible and incompatible combinations, leading us to think that it may not be a suitable marker of graft compatibility in chestnut.

Among the benzoic acids investigated, only gallic acid furnished positive results, supporting previous outcomes on fruit crops' graft incompatibility.

2.5.2. Cinnamic Acids

Based on previous research findings, the content of caffeic, chlorogenic, coumaric and ferulic acids was measured. Indeed, previous works demonstrated that cinnamic acids could be involved in graft compatibility in *Vitis* spp. and *P. communis* [10,19,22]. Moreover, the effect of these compounds during the differentiation of vascular tissues on *Olea europaea* (L.) was investigated, and the results highlighted that ferulic acid had higher concentrations in grafting combinations that did not differentiate an intact and well-functioning vascular system [23].

The chestnut tissues analysed at the graft union for all the combinations had very low amounts of cinnamic acids, in the range of 2 mg/100 gFW, which seems to suggest that they may not be reliable markers of graft compatibility (Table 3).

Table 3. Cinnamic acid fingerprint of the tissues at the graft union. The results are reported as mg of the bioactive compound per 100 g fresh weight (FW).

Phenological Stage	Bioactive Class	Compound	CH × s_EUJAP		MSUS × s_EUJAP		MSUS × c_EUJAP		MSUS × s_PUM	
			Mean Value (mg/100 g_{FW})	SD	Mean Value (mg/100 g_{FW})	SD	Mean Value (mg/100 g_{FW})	SD	Mean Value (mg/100 g_{FW})	SD
Callusing	Cinnamic acids	caffeic acid	1.04 a	0.38	1.27 a	0.34	1.43 a	0.30	1.15 a	0.23
		chlorogenic acid	1.02 a	0.15	1.49 a	0.10	1.80 a	0.27	1.08 a	0.08
		coumaric acid	n.d.	/	n.d.	/	n.d.	/	n.d.	/
		ferulic acid	1.02 a	0.29	1.07 a	0.23	1.95 a	0.39	1.35 a	0.27
End of vegetative cycle	Cinnamic acids	caffeic acid	0.62 a	0.28	1.05 a	0.16	1.28 a	0.18	0.96 a	0.15
		chlorogenic acid	1.22 a	0.14	2.28 a	0.26	2.37 a	0.25	1.25 a	0.19
		coumaric acid	n.d.	/	n.d.	/	n.d.	/	n.d.	/
		ferulic acid	0.46 a	0.06	1.00 a	0.10	1.64 a	0.18	0.74 a	0.14

The mean value and standard deviation are given for each sample (n = 3). Different letters for each compound indicate significant differences at $p < 0.05$.

n.d.= not detected

Caffeic and ferulic acids had a very similar trend, decreasing from CAL to EVC and showing a higher amount in the compatible combination MSUS × c_EUJAP (at CAL, the caffeic acid concentration was 1.43 ± 0.30 mg/100 gFW, while the ferulic acid quantity was 1.95 ± 0.39 mg/100 gFW). However, these slight differences did not differ from a statistical point of view. The similar trend and quantity which showed in the data may be due to the metabolic connection between these two phenol compounds, as ferulic acid derives from caffeic acid through the COMT enzyme [24].

In contrast to the ferulic and caffeic acid patterns, chlorogenic acid increased from CAL to EVC, and its concentration was slightly higher compared to the two previous compounds. At both phenological sampling stages, the chlorogenic acid level was higher in the compatible grafts, particularly in the ones grafted on clonal rootstock. At EVC, MSUS × c_EUJAP reached the maximum amount of 2.37 ± 0.25 mg/100 gFW, while a lower value of chlorogenic acid was detected in the incompatible combination CH × s_EUJAP (1.22 ± 0.14 mg/100 gFW), followed by MSUS × s_PUM (1.25 ± 0.19 mg/100 gFW). However, as for caffeic and ferulic acids, these differences did not have statistical relevance.

Coumaric acid was not detected by the HPLC in any of the chestnut samples, even though many studies reported an accumulation of this acid in incompatible grafting combinations of other tree species [8,9,20].

The concentration of these three cinnamic acids at the graft point may be justified by the fact that these compounds are involved in the biosynthesis of lignin, and for that reason they are required in huge amounts at the union point, where the scion and rootstock must synthesize new lignin to complete the grafting process. In fact, ferulic and caffeic acids stabilize the cell wall and contribute to the improvement of the mechanical stability of the graft union and thereby the graft success [25]. Some authors have also suggested that under particularly stressful conditions, there could be crosstalk between the phenol pathway and the lignin pathway, and hence chlorogenic acid could be converged to the lignin biosynthesis process [24].

These experimental data establish that cinnamic acids are found in very poor concentration (around 2 mg/100 gFW), and that it is not possible to evidence a clear and divergent pattern between compatible and less-compatible graft combinations. Therefore, these phenols might participate in the determination of graft incompatibility, but they cannot be considered reliable markers in the forecasting of graft success in *Castanea* spp. Although ferulic acid proved to be a reliable marker of compatibility in *Vitis* [10], it seems that it is not the same scenario in *Castanea* spp. These findings corroborate the importance of species relativity, and therefore the need to identify one or more markers which are specific for a certain genus or species.

2.5.3. Catechins

Among the phenols analysed in the literature as potential markers of graft incompatibility, catechin and epicatechin seem to be suitable indicators in several woody species. It seems that these compounds could have a role in hindering cambial connections between the cells of the scion and rootstock, affecting the callus formation and lignin biosynthesis [16].

In particular, with regard to catechin, an accumulation of this compound was found in incompatible grafting combinations at the graft union in *Eucalyptus gunnii, Pyrus communis* and *Vitis* spp. [13,19,20], above the graft interface in *Prunus armeniaca* and *Vitis* spp. [3,9,16], and below it in *Pyrus communis* grafted on different pear rootstocks [22].

The results of the present study on catechin and epicatechin accumulation showed a clear trend common to most of the other phenol compounds analysed, such as ellagic, gallic, ferulic and caffeic acids. Indeed, their concentration slightly decreased from CAL to EVC (Table 4). This pattern can be explained because of the higher oxidative environment that prevails in the first stages of graft formation. As these compounds have a well-known antioxidant function, their accumulation at callusing contributes to confer tolerance to oxidative stress to cells [12]. However, only the catechin concentration differed significantly between the compatible and incompatible combinations, recording the higher concentration at CAL in CH × s_EUJAP (15.79 ± 1.83 mg/100 gFW) and the statistically lower concentration in the compatible combination MSUS × c_EUJAP (9.63 ± 1.98 mg/100 gFW).

Table 4. Catechin fingerprint of the tissues at the graft union. The results are reported as mg of bioactive compound per 100 g fresh weight (FW).

Phenological Stage	Bioactive Class	Compound	CH × s_EUJAP		MSUS × s_EUJAP		MSUS × c_EUJAP		MSUS × s_PUM	
			Mean Value ($mg/100\ g_{FW}$)	SD	Mean Value ($mg/100\ g_{FW}$)	SD	Mean Value ($mg/100\ g_{FW}$)	SD	Mean Value ($mg/100\ g_{FW}$)	SD
Callusing	Catechins	catechin	15.79 b	1.83	11.05 ab	1.39	9.63 a	1.98	12.98 ab	1.92
		epicatechin	290.22 a	3.69	287.14 a	7.59	301.59 a	13.43	281.16 a	9.19
End of vegetative cycle	Catechins	catechin	13.33 b	2.10	9.30 ab	1.25	6.31 a	1.20	10.44 ab	1.14
		epicatechin	235.22 a	8.04	220.14 a	11.03	249.59 a	6.57	222.16 a	9.18

The mean value and standard deviation are given for each sample ($n = 3$). Different letters for each compound indicate the significant differences at $p < 0.05$.

Regarding the epicatechin expression at the graft union, the concentration of this compound was much higher if compared to catechin. However, no differences were detected for all of the combinations at the two sampling stages. Similar results were obtained by Assunção et al., 2016, on four grafting combinations of

Vitis spp. [10]. In this study, the compatibility level among combinations did not influence the expression of epicatechin. On the contrary, statistically significant differences were detected among the grafting sections. Based on these findings, they concluded that epicatechin could be useful only as an auxiliary compound in grafting incompatibility evaluation.

2.5.4. Tannins

This class of secondary metabolites can be found in many species, although few studies have investigated their role in the grafting process. Tannins are involved in the response to wounding, increasing in content as a part of the plant's defense system when mechanical injuries occur through an upregulation of genes involved in their synthesis [26]. Moreover, previous research on sorghum [27] has demonstrated that an accumulation of tannins has negative effects on the proliferation of callus, limiting the formation of vascular tissues. Therefore, it seems that tannins could negatively impact healing processes on the long term, as in the case of grafting propagation.

As further confirmation of these findings, studies on the anatomical properties of *Pinus radiata* (D. Don.) grafts found an excessive tannin accumulation in incompatible unions, as indicated by bark tissues with abnormal dark stains [28]. The present study focused particularly on two compounds, castalagin and vescalagin, as the most representative molecules in chestnut tissues [29]. Indeed, a 2014 work on different bark samples of *C. sativa* pointed to vescalagin and castalagin as the most abundant compounds [30].

The results show a common trend for both the compounds analysed (Table 5). In fact, the castalagin and vescalagin concentrations increased from the CAL to EVC stage. The higher increase in concentration was recorded in the incompatible combination CH × s_EUJAP (+14.3% castalagin, +13.4% vescalagin), followed by the compatible MSUS × c_EUJAP (+13.78% castalagin, 13.16% vescalagin). These results appear to contradict what was stated on *Carya illinoinensis* (Wangenh.) by Su et al., 2021 [26], where grafted plants revealed a decreasing trend of tannin content during graft union development.

Table 5. Tannin fingerprint of the tissues at the graft union. The results are reported as mg of bioactive compound per 100 g fresh weight (FW).

Phenological Stage	Bioactive Class	Compound	CH × s_EUJAP		MSUS × s_EUJAP		MSUS × c_EUJAP		MSUS × s_PUM	
			Mean Value ($mg/100\ g_{FW}$)	SD	Mean Value ($mg/100\ g_{FW}$)	SD	Mean Value ($mg/100\ g_{FW}$)	SD	Mean Value ($mg/100\ g_{FW}$)	SD
Callusing	Tannins	castalagin	380.43 a	13.95	468.06 bc	10.50	505.38 c	21.79	435.56 b	17.72
		vescalagin	517.14 a	21.22	673.69 c	17.06	682.57 c	29.15	624.08 b	19.43
End of vegetative cycle	Tannins	castalagin	434.83 a	11.80	529.62 bc	9.89	575.00 c	23.01	485.53 b	12.30
		vescalagin	586.57 a	18.76	749.72 c	15.96	772.42 c	22.44	705.23 b	14.13

The mean value and standard deviation are given for each sample ($n = 3$). Different letters for each compound indicate the significant differences at $p < 0.05$.

It is known that the content of tannins is highly species-dependent. A study on Chinese (*C. mollissima*) and American (*Castanea dentata* (Marsh.) Borkh.) chestnuts investigated the constitutive levels of the two major groups of tannins, hydrolysable tannins and proanthocyanidins, revealing a higher concentration of these compounds in the stems of *C. dentata* [31].

The findings of the present study highlighted a great variability in the quantity of tannins among the combinations. These differences seem to be species-specific and are not apparently related to graft incompatibility. In fact, the amount of castalagin and vescalagin increased during the vegetative cycle in all of the graft combinations, with almost the same growth rate.

Although tannins have great importance in many industrial sectors—such as animal feed, leather and food, and especially in wine production—the reason of their abundance in *Castanea* species is still unclear. This study highlighted a common trend for all of the grafting combinations tested. This concentration increase during the vegetative cycle may suggest that tannin production could be related to the biosynthesis of structural tissues such as lignin.

2.6. Conclusions

Chestnut cultivation is experiencing a revival phase, affirming itself as an important fruit crop worldwide. In consideration of the growing importance of

this species, the breeding of new cultivars and rootstocks is very active. Grafting is the main technique used to propagate chestnut, though incompatibility represents a critical issue. Therefore, one or more techniques which are able to predict graft incompatibility early are needed, as for other valuable fruit crops.

The present work is the first that addresses the question on *Castanea* spp. from a biochemical point of view, focusing on the quali-quantitative expression of phenol compounds. In particular, the aim of the work was to find one or more phenol compounds that could work as markers for an early identification of incompatibility, before visual symptoms occur. In total, 15 bioactive compounds belonging to five phenol classes were chosen based on the literature, and their expression at different phenological periods and on different graft sections was analysed. The results are encouraging, supporting the hypothesis that some molecules are more suitable than others for the prediction of incompatibility phenomena. More specifically, the amount of gallic acid was over two times higher at the grafting union of incompatible combinations. The same trend was observed for catechin, with higher statistically significant values detected in incompatible grafts. The gallic acid at the graft union of incompatible combinations recorded an increase of +125% at callusing and +181% at the end of the vegetative cycle compared to the concentration of compatible ones. As for catechin, it increased by +64% at the first sampling period, and by +111% at the end of the vegetative season in incompatible grafts.

Some compounds did not seem to be suitable markers of incompatibility, such as cinnamic acids, which are present in very low quantities in chestnut tissues. Other molecules like ellagic acid and epicatechin, which turned out to be good markers on other fruit species, need further research. The same goes for castalagin and vescalagin, the concentrations of which largely differed among the tested combinations. These compounds increased in *Castanea* tissues throughout the vegetative cycle, showing a percentage increase of approximately 14% for both compatible and incompatible grafting combinations. This common increasing trend indicates that the differences in tannin concentrations could be species-specific, and apparently not related to graft incompatibility.

Future works addressing the issue will have to enlarge the number of rootstocks and cultivars tested in order to strengthen and validate the present findings.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1—Table S1: Conditions of the methods used for the chromatographic analysis.

Author Contributions: Conceptualization, G.G., D.D. and G.L.B.; Data curation, G.G. and D.D.; Formal analysis, G.G. and D.D.; Investigation, G.G. and V.C.; Methodology, G.G. and D.D.; Re-sources, G.G. and D.D.; Supervision, G.L.B.; Validation, D.D., Z.R.R. and G.L.B.; Writing—original draft, G.G. and V.C.; Writing—review and editing, Z.R.R. and G.L.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research has not received external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to thank Mauro Caviglione and Marco D'Oria for the helpfulness and professionalism with which they grafted all of the chestnut combinations.

Conflicts of Interest: The authors declare no conflict of interest.

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3. Graft incompatibility in chestnut: a multidisciplinary approach for early identification in *Castanea* spp.

ISHS Acta Horticulturae 1366: XXXI International Horticultural Congress (IHC2022): International Symposium on Innovative Perennial Crops Management

Graft incompatibility in chestnut: a multidisciplinary approach for early identification in *Castanea* spp.

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Keywords: propagation, clonal rootstocks, HPLC, phenolic compounds, chlorophyll content
DOI: 10.17660/ActaHortic.2023.1366.53

3.2. Abstract

Among the key factors aimed at improving fruit production, grafting plays a pivotal role. The rootstock genotype influences crop efficiency and fruit quality, resilience in new ecological scenarios and susceptibility to pests and diseases. In recent years, research on rootstock/scion relationships has been investigated, but graft incompatibility still represents a limit to nursery activities. Research has been concentrating on the role of polyphenols as markers of either compatible or incompatible grafts. Promising results have been achieved on several fruit species such as *Vitis* spp., *Prunus* spp. and *Pyrus* spp., analysing the qualitative and quantitative expression of phenolic compounds in scion and rootstock. Indeed, different patterns can determine dysfunctions at the graft union, highlighting them as potential markers for graft incompatibility. Moreover, several physiological parameters such as chlorophyll content, fluorescence rate, and stomatal conductance proved to be effective tools in the early detection of physiological stresses related to graft incompatibility. Thus far, very few studies have been carried out on phenolic compounds and physiological responses in chestnut (*Castanea* spp.) grafting. The present work investigated the biochemical mechanisms and the morpho-physiological status of several combinations of *Castanea* spp. cultivars and rootstocks. The analysis involved the inner and outer tissues of chestnut grafts at two phenological stages. Phenol compounds were firstly extracted using green extraction technology (ultrasounds). Separation, identification, and quantification were performed via HPLC. Analysis of

physiological aspects was carried out using a porometer, chlorophyll content meter, and Arborcheck® instrumentation. The results seem to validate the effectiveness of this multipurpose approach for the prediction of graft incompatibility in chestnut.

Keywords: propagation, clonal rootstocks, HPLC, phenolic compounds, chlorophyll content

3.3. Introduction

Grafting is one of the most used propagation techniques in fruit tree production. It involves joining tissues of two plants so that they continue their growth together. Many are the reasons why plants are grafted: i) to propagate recalcitrant species difficult to propagate by other means; ii) to induce early entry into production; iii) to benefit from the properties of selected rootstocks (Garner, 2013). Indeed, the rootstock genotype strongly influences crop efficiency and fruit quality, resilience in new ecological scenarios and susceptibility to pests and diseases. Though, the main issue connected to grafting is the phenomenon of incompatibility, which may compromise its success. In recent years, research on rootstock/scion relationships has been investigated, but graft incompatibility still represents a limit to the breeding sector. In literature, studies have been conducted on several fruit species such as pear, grapevine, and apricot (Hudina *et al.*, 2014; Canas *et al.,* 2015; Usenik *et al.*, 2006). The aim of these works was the study of phenol compounds accumulated at the graft interface of compatible and incompatible scion/rootstock combinations. Beside polyphenols, other predictive markers seem to be peroxidase and phenylalanine ammonia-lyase enzymes, as they are involved in the biosynthesis of polyphenols and lignin formation. Together with biochemical and genetic techniques, morpho-physiological investigations have been shown to be effective in detecting stresses related to graft incompatibility. Parameters such as chlorophyll content, fluorescence rate, stomatal conductance and internal anatomy of the graft union allowed

differentiation between compatible and incompatible grafts (Calatayud *et al.*, 2013; Chen *et al.*, 2016).

Chestnut is among the numerous species propagated through grafting and it stands out as an interesting alternative to traditional fruit crops in our region. The potential health benefits derived from the consumption of fresh and processed chestnut products have been the focus of several studies, increasing the interest in the European chestnut (*Castanea sativa* Mill.) in the food industry. The large intraspecific diversity among sweet chestnut cultivars makes them very appreciated for their organoleptic and nutritional properties, with particular regard to "Marrone-type" cultivars (Beccaro *et al.*, 2020). However, the lack of knowledge on graft compatibility among valuable Italian cultivars and new innovative rootstocks limits cultivation. The aim of this study was to apply one or more of the above-mentioned predictive methods to chestnut.

3.4. Materials and Methods

3.4.1. Plant materials

Experimental activities foresaw the production of grafted trees comparing different combinations, chosen based on their known compatibility level (Table 1). Chestnuts were grafted with the whip and tongue technique in the early spring of 2021 and grown in an open field at the Chestnut R&D Center Piemonte (Chiusa Pesio, NW Italy - 620 m asl). One-year-old clonal rootstocks and stored refrigerated scions were used. The use of clonal material is very important as it minimises the genetic variability factor, that characterises seedling rootstocks. The clonal rootstock tested was 'Marsol CA07' (*Castanea crenata* x *C. sativa*) obtained through cuttings. A third combination using a seedling rootstock of *C. pumila*, a chestnut species native to North America, was also tested. Scions belong to the 'Marrone della Val Susa' cultivar, among the most valuable Italian *C. sativa* cultivars, and 'Chushuhong', a *C. mollissima* cultivar used to set the incompatible combination.

Table 2 Grafting combinations tested and corresponding compatibility level.

Rootstock	Cultivar	Code	Compatibility
Marsol CA07	Chushuhong	Ch x CA07	-
Marsol CA07	Marrone della Val di Susa	MS x CA07	+
Castanea pumila	Marrone della Val di Susa	MS x Cpum	?

3.4.2. Sampling time

Two phenological periods were set at callusing (CAL, around 60 DAG) and at the end of the vegetative cycle (EVC, around 270 DAG). The choice was driven by preliminary tests performed on chestnut, and by previous studies on other fruit species. Five samples for each combination and each period were collected. Samples consisted of the graft section and tissues above and below it (Figure 1), that were cut off and stored in a -80°C refrigerator until the analysis.

Figure 1 Chestnut grafting sample collected at callusing stage (CAL).

3.4.3. Physiological observations

Morpho-physiological traits were measured throughout the vegetative cycle on five trees per combination. Arborcheck® instrumentation was used to investigate foliar chlorophyll and fluorescence. Chlorophyll content was determined in SPAD units. Arborcheck® automatically coupled fluorescence values with chlorophyll content to determine the physiological status of each combination, expressed in terms of vitality and stress. Vitality represents the overall physiological health of the tree which affects the ability of its own photosynthetic system to function and adapt to different conditions. It is the sum of: i) photosynthesis efficiency, which is the ability of the photosynthetic systems within the leaf to convert light energy from the sun to the complex carbohydrates required for sustained tree growth, and ii) chlorophyll content, limited by the amount of carbohydrate available for growth which reduces nutrient uptake resulting in leaf chlorosis and necrosis. Stress is the current physiological state of the tree in response to any ongoing biotic or abiotic stress factor. It consists of consists of 4 Stress Indicator (Si) parameters calculated from the chlorophyll fluorescence measurements: Si1, the maximum quantum yield of PSII photochemistry (F_v/F_0); Si2, ground fluorescence in dark-adapted state (F_0); Si3, variable fluorescence in dark-adapted state ($F_v = F_m - F_0$); Si4, from the fluorescence parameter Area above the fluorescence curve. Provides an estimation of the size of the reduced plastoquinone pool. Stomatal conductance was measured with an LI-600 porometer, clipping 20 leaves for each combination.

3.4.4. Plant material preparation

Biochemical analysis was performed on the inner and outer tissues of the tested combinations at the CAL and EVC stages, the bark and cortex were separated from the inner tissues of each section. Using liquid nitrogen, outer and inner tissues were frozen and then ground in a mortar. The ground tissues were weighed and stored at −80 °C until further extraction.

3.4.5. Phenols extraction, separation, identification, and quantification

Two methods were tested for biochemical fingerprinting: method A (cinnamic acids and flavonols) and method B (benzoic acids, catechins, and tannins) for a total of 16 phenolic compounds belonging to five classes. Primary solvent extraction (maceration) was followed by a second extraction by ultrasound-assisted green technologies. 1 g of ground material was left overnight in 20 mL of a mix of methanol, water and HCL (95:4.5:0.5, *v*:*v*:*v*). Samples were then plunged into an ultrasonic bath for 30 min (UP200 ST, Hielscher Ultrasound Technology, Teltow, Germany). Finally, each sample was centrifuged at 4000 rpm for 10 min, and the liquid phase was filtered through a 0.45 µm filter (polytetrafluoroethylene membrane—PTFE). An Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA, USA) coupled to an UV-Vis Diode Array Detector was used to separate, identify, and quantify the phenolic markers (Donno *et al.*, 2021).

3.4.6. Statistical analysis

The variance analysis (ANOVA) for the comparison of means, followed by Tukey's honest significant difference (HSD) multiple range test ($p < 0.05$), was performed on the data obtained. Analysis was performed with SPSS Statistics 22.0 (IBM, Armonk, NY, USA, 2013).

3.5. Results and discussion

3.5.1. Physiological observations

Preliminary results showed discrimination between compatible and incompatible combinations for all the physiological traits analysed. The chlorophyll content of the leaves was measured twice during the vegetative cycle and recorded significantly lower values in the incompatible combination (Ch x CA07) compared to the compatible one (MS x CA07) for both sampling periods. A

similar pattern was observed for stomatal conductance, with reduced stomatal activity in Ch x CA07 (Table 2). These findings are in accordance with the results of Calatayud *et al*. (2013) on melon.

Table 3 Chlorophyll content and stomatal conductance measured on the grafting combinations tested during the vegetative cycle.

Grafting combination	**Chlorophyll content (SPAD units)**				**Stomatal conductance (mol m-2 s-1)**	
	period 1 (28/07/21)		**period 2 (02/09/2021)**			
	SPAD	SD	SPAD	SD	G_{sw}	SD
Ch x CA07	41.02 b	±3.08	34.97 c	±4.65	0.019 b	±0.013
MS x CA07	47.19 a	±3.75	48.00 a	±3.51	0.303 a	±0.112
MS x Cpum	40.94 b	±3.14	41.38 b	±1.59	0.047 b	±0.032

Confirmations arrived from the Arborcheck®, that recorded considerable differences in terms of overall vitality (the sum of photosynthesis efficiency and chlorophyll content) and stress (stress consists of four stress indicator parameters calculated from the chlorophyll fluorescence measurements) at all measurement periods (Figure 2).

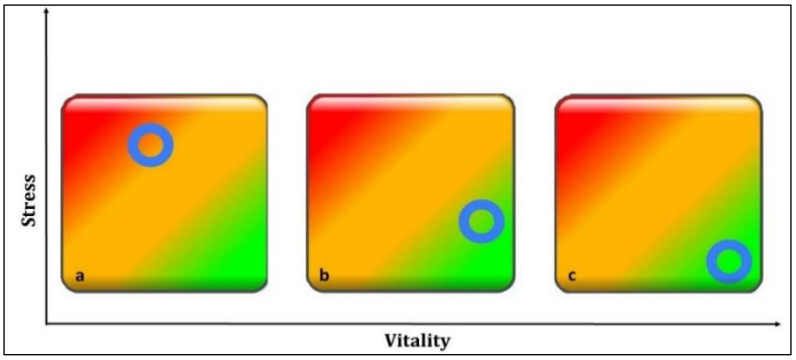

Figure 2 Overall vitality and level of stress of the grafting combinations measured with Arborcheck® instrumentation on 28 July 2021; Ch x CA07 (a), MS x CA07 (b), MS x Cpum (c).

3.5.2. Biochemical observations

The biochemical fingerprinting provided interesting results, in line with what was obtained on other fruit species. In particular, the incompatible combination Ch x CA07 showed higher values of benzoic acids and catechins at CAL sampling time (Figure 3), as previously observed in other fruit species (Musacchi *et al.*, 2000; Assunção *et al.*, 2019).

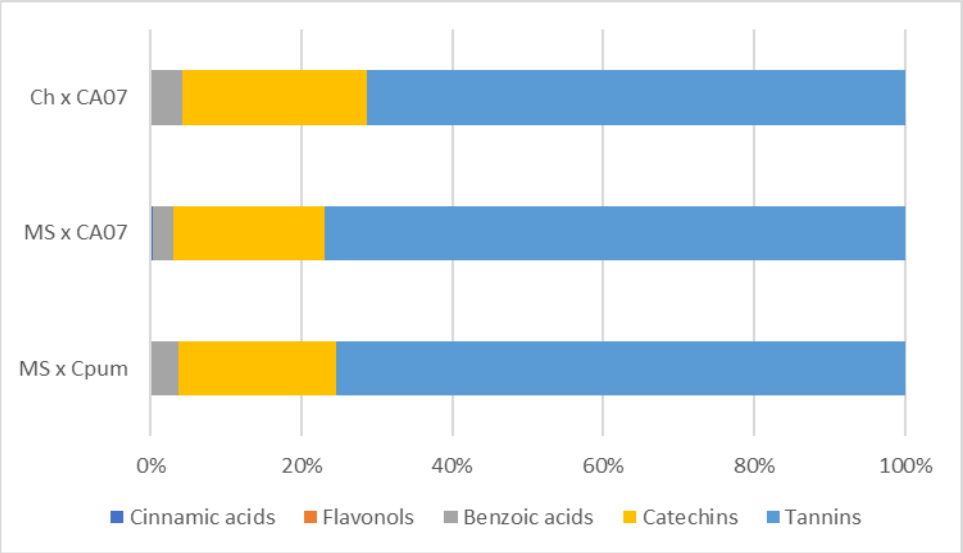

Figure 3. Phenolic fingerprint at graft union in the grafting combinations at callusing sampling time.

The same pattern was observed at the EVC stage (Figure 4). At both stages, cinnamic acids were detected only at trace concentrations, while flavonols were not found. Tannins were the compounds present in larger quantities for both the phenological stages and in all the tested grafting combinations. *Castanea* species are normally very rich in tannins, and no patterns related to incompatibility emerged.

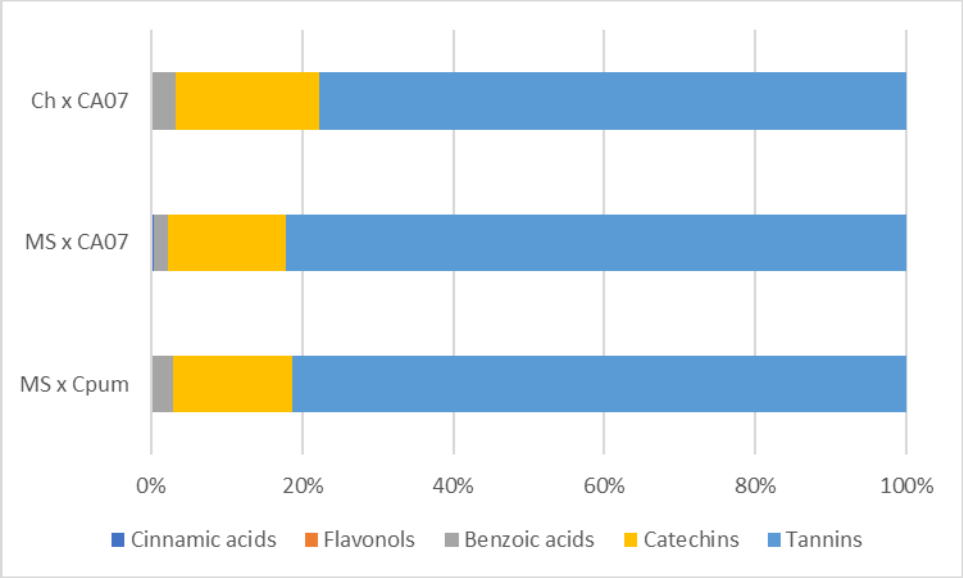

Figure 4. Phenolic fingerprint at graft union in the grafting combinations at the end of the vegetative cycle sampling time.

3.6. Conclusions

The present study analysed the phenomenon of graft incompatibility on chestnut under a multidisciplinary approach. From a physiological point of view, the content of chlorophyll, the overall vitality expressed as a function of chlorophyll content and fluorescence rate, and the stomatal conductance allowed to discriminate between compatible and incompatible grafting combinations. Analysis was found to be handy, instant, and non-destructive. The study on phenol compounds highlighted a pattern already observed in other fruit species. Indeed, the concentration of catechins and benzoic acids turned out to be higher

in incompatible grafts. The combined use of biochemical and physiological parameters seems to be a reliable tool to early predict graft incompatibility in chestnut. Further research should be implemented to validate these findings, enlarging the number of combinations tested.

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4. Greater insights into chestnut (*Castanea* spp.) graft incompatibility through the monitoring of chemical and physiological parameters

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4.2. Abstract

In recent years considerable effort has been done to study the mechanisms underlying grafting formation, with the aim of identifying potential predictive techniques of graft incompatibility to be employed in breeding programs. Research has been focusing on the principal cash crops, while few works are available in the literature for minor species such as chestnut. Given the renewed interest for this cultivation, graft incompatibility is therefore a topical issue limiting the adoption of new rootstocks and cultivars. The present study explored the physiological and chemical dynamics occurring in different intraspecific and interspecific chestnut grafts with the aim of identifying an effective approach for the early detection of incompatibility in chestnut propagation, to accelerate rootstock breeding and the development of resilient materials. The total phenolic content (TPC) and the identification and quantification of specific phenol classes were reviewed at two phenological times via spectrophotometric and

chromatographic analysis. Stomatal conductance (Gsw) and leaf chlorophyll content were assessed during the growing season as support tools, being non-destructive useful indicators of plant water status. Significant differences in the physiological traits among compatible and incompatible grafting combinations were recorded and remained consistent during the season. Leaf chlorophyll content assessed in SPAD units ranged from 55.50±2.78 - 48.04±1.61 in compatible combinations to 43.95±2.45 - 31.30±2.88 in incompatible ones. Stomatal conductance showed an average value of 0.542±0.021 mol m-2 s-1 for compatible combinations, significantly higher than that observed in incompatible unions (0.072±0.007 mol m-2 s-1). TPC increased statistically from CAL to EVC in all the sections of all the combinations, with a greater accumulation of phenol compounds in the graft area of the incompatible unions, especially in the inner woody layers. The phytochemical fingerprint calls into question the role of benzoic acids, primarily gallic acid, and catechins as markers of graft incompatibility on chestnut. The multidisciplinary approach undertaken contributed to acquiring greater insight into the graft incompatibility matter.

Keywords: clonal rootstocks; phenolic compounds; stomatal conductance; chlorophyll content; propagation

4.3. Introduction

In nature, grafting is a phenomenon in which two plant segments connect and grow as a single entity thanks to the establishment of a vascular continuity between them. It is reasonable to think that artificial grafting was first performed by observing the natural process, as many techniques known as “approach grafting” took similar forms[1]. Historical sources testify how this technique has been practiced for millennia on perennial crops, since ancient Chinese and Greeks[2]. In the last century, grafting took hold also on horticultural species, mainly belonging to the *Solanaceae* and *Cucurbitaceae* families[3].

The importance of grating using selected genotypes as rootstocks is well recognized. They impact significantly on crop production and quality, influencing

many morpho-physiologic traits such as canopy architecture, nutritional uptake, vigour, tolerance or resistance to pests and diseases, flowering time, cold hardiness, and resistance to replant disease[4, 5]. Moreover, rootstocks can impart resistance or increased tolerance to abiotic stresses like salinity, hypoxia, toxicity of heavy metals, and stress connected to soil pH, which are becoming more frequent due to climate change[6, 7]. Successful grafts pass through a series of morphological, physiological, and molecular events consisting in hormonal signaling, protein turnover, gene expression, phenol metabolism, and ion uptake and transport ending with the establishment of the vascular connection. Incompatible grafts result from the failure of one or more of these events[8]. Disaffinity among two genotypes can be due to various factors: taxonomic distance, pathogens, environmental conditions, and poor craftsmanship above all[9]. In the last decades, considerable effort has been made to study the mechanisms underlying grafting formation, to understand the causes of graft incompatibility and to identify potential metabolic markers to be employed in breeding programs. Research has been focusing on the principal cash crops, while few works are available in the literature for minor species. Many studies focused on the accumulation of phenolic compounds at the graft union, as these secondary metabolites play a primary role in many metabolic processes such as cell division, development and differentiation[9]. Moreover, being involved in defense responses, their increased biosynthesis could be a mechanism with which plants try to hinder the oxidative stress related to graft incompatibility[10]. However, the concentration of certain phenolic molecules could limit the proliferation and differentiation of callus, thereby hindering the formation of new vascular tissues[11]. Qualitative and quantitative analysis of phenol concentration highlighted the role of certain molecules as promising markers of graft incompatibility on several species, especially with new cultivar/rootstock combinations [12-14].

Beside chemical traits, rootstocks may affect considerably many physiological processes occurring in the upper part of the grafted trees, namely the scions. Effects on the physiology could be linked to the root system, especially in the

case of dwarfed trees, but also to the graft incompatibility among genotypes. Indeed, according to the grafting combination, rootstocks can interfere with stomatal opening and closure, net CO_2 assimilation, and intracellular CO_2 through hydraulic and hormonal signalling[8, 15]. Stomatal opening is usually expressed as stomatal conductance (g_s), a useful parameter evaluating the rate of water vapour exiting or CO_2 entering through stomata, which is a measure of leaf transpiration. As the incomplete wound healing in incompatible unions causes vascular discontinuity affecting the plant water status, these physiological parameters could help in the early diagnosis of incompatibility before visual symptoms occur[16-18]. At the early grafting process, incompatible combinations may show a complete or partial vascular discontinuity associated with phloem degeneration at the graft union. This condition could affect the activity of the new phloem and xylem, leading to detrimental effects on the ascendent water flow and on the descendent photo-assimilates[19]. Beside gas exchange measurements, analysis of the leaf chlorophyll content proved to be an efficient tool to discriminate among different degrees of compatibility[20]. A practical way to assess this parameter is via a hand-held SPAD meter, a device widely used for the rapid and non-destructive assessment of the chlorophyll content. It gives relative SPAD values proportional to the amount of leaf chlorophyll. Many studies on different horticultural species confirmed the effectiveness of this parameter in corroborating analytical evidence of graft incompatibility[21-24].

At present, few works are available on chestnut graft incompatibility. Beside the historical, ecological, and cultural relevance that this cultivation has represented for centuries, it is a considerable economic resource (fruit, wood, tannin production, and honey) for mountainous and hilly areas[25]. Despite having gone through a gradual decline during the 20th century, in the last decades the interest for this cultivation has been renewed. Intensive orchards are rising also in lowland areas, adopting agricultural practices typical of major fruit crops. Among them, the use of clonal rootstocks is of great interest. Consequently, breeding activity is enhancing, and new resilient genotypes are recently being released.

Graft incompatibility is therefore a topical issue limiting the adoption of new rootstocks and cultivars, crucial materials for the revitalization of the chestnut cultivation.

The present study explored the physiological and chemical dynamics at the graft union, above and below it in different intraspecific and interspecific chestnut grafts. In particular, the implications of phenolic compounds during grafting development of compatible and incompatible combinations were reviewed at two phenological times. Stomatal conductance and leaf chlorophyll content were assessed during the growing season as support tools, as non-destructive indicators of plant water status. The aim of the study was to identify an effective approach for an early detection of incompatibility in chestnut propagation, to accelerate rootstock breeding and the development of resilient materials.

4.4. Materials and methods

4.4.1. Plant materials

Plant materials consisting in grafted trees were grown at the Chestnut R&D Center Piemonte, in Chiusa Pesio (44°18'21"N 7°40'50"E, 620 m above mean sea level). Scions were collected in February 2022 from the Castanetum germplasm collection field located at the Chestnut R&D Center and dark-stored at 4°C until grafting. The mother plants were guaranteed in terms of varietal trueness-to-type and phytosanitary requirements, to avoid the influence of diseases during graft development. Grafts were performed in March 2022 using the whip and tongue technique, which ensures a high success rate and good stability thanks to the tongue that holds rootstock and scion together. The trial foreseen the use of two-years-old clonal rootstocks cultivar “Marsol CA07” self-produced via cutting at the Chestnut R&D Center and two seedling rootstocks of *C. mollissima and C. crenata* grown in the Castanetum. Four experimental combinations were selected based on their compatibility level (Table 1) and grown in the open field.

Table 4 Experimental grafting combinations chosen for the study, with their known compatibility level.

CODE	ROOTSTOCK	SCION	COMPATIBILITY LEVEL
BBxCA07	Marsol CA07 (*C. crenata x C. sativa*, clonal)	Bouche de Bétizac (*C. crenata x C. sativa*)	compatible
MSxCA07	Marsol CA07 (*C. crenata x C. sativa*, clonal)	Marrone Val Susa (*C. sativa*)	compatible
MSxCren	*C. crenata* (seedling)	Marrone Val Susa (*C. sativa*)	incompatible
MSxMoll	*C. mollissima* (seedling)	Marrone Val Susa (*C. sativa*)	incompatible

Graft samples for the chemical analysis were collected at two phenological stages: callusing (CAL) and end of vegetative cycle (EVC), according to previous studies on chestnut and other major fruit species[18, 26, 27]. The first sampling time corresponds to approximately 60 days after grafting (DAG), while EVC is during the dormancy period, at the end of the year. Experimental graft combinations were cut 5 cm above and below graft union, to include scion and rootstock tissues. Ungrafted plant materials were also sampled, so as to have information about the content in phenol compounds in the starting genotypes. Samples were stored at -80°C refrigerator until further analysis on woody tissues.

4.4.2. Sample preparation

The extraction of secondary metabolites was carried out on external and internal tissues, independently. Bark, cambium, and phloem were separated from the inner tissues; afterwards, they were ground to powder in a mortar. Liquid nitrogen was used to facilitate grinding operations and to maintain the cold chain, essential to limit phenol degradation. Finally, milled tissues were weighed and stored at -80°C until further analysis[26, 28].

4.4.3. Extraction of secondary metabolites from woody tissues

For the extraction of phenol compounds, a blended approach was employed, consisting in a conventional extraction method via solvent followed by an ultrasound-assisted extraction. 1 g of sample was placed into 20 mL of a mix of methanol, water and HCl (95:4.5:0.5, v:v:v) overnight. Then, tubes containing

sample and extraction solvent were moved into an ultrasonic bath for 30 minutes at 23 kHz (Reus sarl, Drap – France). Later, each sample was centrifuged at 4000 rpm for 10 minutes and finally stored at 4 °C and 95% relative humidity until further analysis.

4.4.4. Spectrophotometric analysis - total phenolic content (TPC) assessment

The total phenolic content was assessed using the Folin-Cioâlteu method[29], partially modified[30]. The absorbance was read with a single-beam UV–Vis spectrophotometer (1600-PC, VWR International, Milan, Italy) at a wavelength of 760 nm. Results were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (FW).

4.4.5. HPLC fingerprint: bioactive compounds characterization

The following phenolic classes have been considered for the phytochemical fingerprint, according to previous studies[26, 27, 31, 32]: catechins (catechin, epicatechin), benzoic acids (ellagic and gallic acids), and tannins (castalagin, vescalagin). The liquid phase of the samples previously prepared was filtered through a 0.45 μm filter (polytetrafluoroethylene membrane—PTFE) and finally analysed by HPLC-DAD[33]. For the analysis, an Agilent 1200 High-Performance Liquid Chromatograph - UV-Vis Diode Array Detector (Agilent Technologies, Santa Clara - California, USA) was used. The molecules were separated on a Kinetex C18 column (4.6x150 mm, 5 μm, Phenomenex, Torrance - California, USA)[34]. The chromatographic conditions of the method used for the characterization of the benzoic acids, catechins and tannins are reported in Table 2.

Table 5 chromatographic conditions of the method used for the identification and quantification of benzoic acids, catechins, and tannins.

Class of interest	Stationary phase	Mobile phase	Wavelenght
Benzioc acids, catechins and tannins	KINETEX – C18 column (4.6 × 150 mm, 5 µm)	A: H_2O/CH_3OH/HCOOH (5:95:0.1 v/v/v), pH = 2.5; B: CH_3OH/HCOOH (100:0.1 v/v)	280

Elution conditions
Gradient analysis: 3%B to 85%B in 22 min + 85%B in 1 min (2 min conditioning time); flow: 0.6 mL min^{-1}

4.4.6. Physiological observations

Physiological responses of chestnut to graft incompatibility were assessed by measuring the chlorophyll content and the stomatal conductance to water (g_{sw}). The first parameter was recorded with Arborcheck® ArbCm 01 and expressed as SPAD units, which are estimates of chlorophyll content in the leaf. A steady-state handheld porometer (Licor LI-600) was used to measure leaf-level stomatal conductance, expressed in mol m^{-2} s^{-1}. In particular, LI-600 evaluates stomatal conductance to water vapour (g_{sw}), which gives a rate of water vapour exiting through the stomata. Measurements were taken each 15 days from August to September, from 1100 to 1300 hours. Monitoring was carried out on 30 fully developed sun-facing leaves belonging to five trees per combination, choosing days with comparable environmental conditions. For each monitoring day, both parameters were evaluated on the same leaves, avoiding midrib or any other large vein, chlorotic areas, and holes. Instruments were placed in similar positions in the bottom-right section, choosing a suitable point mid-way between the outer edge of the leaf and the midrib.

4.4.7. Statistical analysis

Chemical and physiological results were evaluated by analysis of variance (ANOVA) for comparison of means (RStudio, version 2022.02.2+485). Significant statistical differences among biological repetitions were investigated using Tukey's HSD multiple range test ($P < 0.05$).

4.5. Results and discussion

4.5.1. Physiological traits

4.5.1.1. Leaf chlorophyll content

The measurements on the chlorophyll content underlined statistically significant differences, that have remained consistent throughout the vegetative cycle (Table 2). In particular, the BBxCA07 combination showed the highest leaf chlorophyll content, followed by the MSxCA07. Among the incompatible combinations, MSxCren recorded intermediate values, while MSxMoll had the lowest chlorophyll content.

Table 6 Leaf chlorophyll content of the experimental grafting combinations tested, expressed as SPAD units. Different letters for all the considered groups indicate significant statistical differences ($p < 0.05$).

	Compatibility level	16/08/2022		30/08/2022		14/09/2022		28/09/2022	
		Mean value (SPAD units)	SD	Mean value (SPAD units)	SD	Mean value (SPAD units)	SD	Mean value (SPAD units)	SD
BBxCA07	Compatible	55.50 a	2.78	55.15 a	2.68	55.27 a	2.04	54.24 a	1.56
MSxCA07	Compatible	49.16 b	2.16	48.11 b	2.19	50.04 b	0.77	48.04 b	1.61
MSxCren	Incompatible	43.95 c	2.45	43.45 c	3.05	43.53 c	1.44	43.65 c	2.17
MSxMoll	Incompatible	37.50 d	3.59	35.70 d	3.87	32.61 d	1.56	31.30 d	2.88

During the growing season, the SPAD index had a physiological decrease (Graphic 1), to a greater extent in the case of MSxMoll (-16.53%). The compatible combinations had a similar downward trend (-2.27% for BBxCA07 and -2.28% for MSxCA07), while MSxCren had the lowest reduction (-0.68%). These differences in the leaf chlorophyll content seem to indicate distinct levels of graft compatibility. The highest values were observed in the BBxCA07, which is the genetically closest combination ((*C. sativa* x *C. crenata*) x (*C. crenata* x *C. sativa*)). The values of the other compatible combination MSxCA07 were very similar to those of a previous study[18]. Few studies in literature report SPAD values for *Castanea sativa* species, mainly related to water stress trials such as

water deficiency or water logging, as in the case of a study on 2-year-old European chestnut seedlings[35]. The well-watered control had SPAD values slightly lower if compared to the present findings on compatible graft combinations. This could be related to the different growing conditions of the study of Camisón et al., 2019, with chestnut seedlings grown in 2-litre pots.

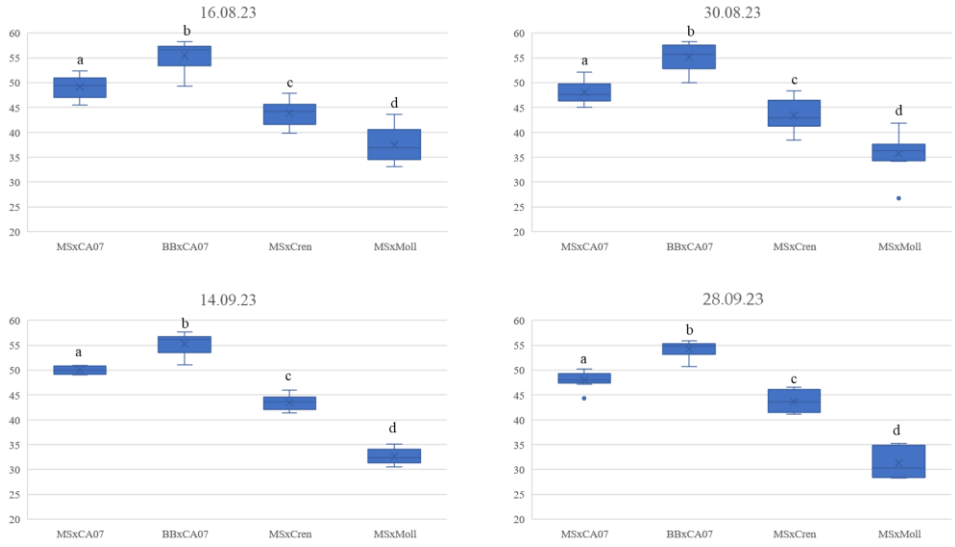

Figure 3 Leaf chlorophyll content of the experimental graft combinations measured during the vegetative cycle and expressed in SPAD units. Bouche de Bétizac x Marsol CA07 (BBxCA07), Marrone Val Susa x Marsol CA07 (MSxCA07), Marrone Val Susa x C. crenata *(MSxCren), Marrone Val Susa x* C. mollissima *(MSxMoll). Mean value and standard deviation are given for each sample (n = 3). Different letters for all the considered groups indicate significant statistical differences (p < 0.05).*

On the contrary, focusing on other genus such as *Citrus* or *Prunus*, many studies dealt with the leaf chlorophyll content related to graft incompatibility. A recent work investigated the changes in the leaf chlorophyll content of *Citrus maxima* cultivars grafted on different rootstocks (*Poncirus trifoliata* and *Citrus junos*). The incompatible combination exhibited the lowest SPAD values, severely decreasing from 182 DAG to 203 DAG[24]. Another work on *Citrus* spp. by Wang et al., (2022) validates the use of SPAD reading as a preselection index for evaluating graft compatibility of stock and scion[21]. Similar results were obtained on "Summergrand" nectarine cultivar (*P. persica*) grafted onto two

different plum rootstocks, as reported by Amri et al., 2021[36]. The comparison between graft combinations and ungrafted rootstocks highlighted lower SPAD values in the case of the incompatible union, accompanied by typical symptoms of the “translocated” incompatibility (yellowing and curling of the leaves, vigour reduction and unhealthy appearance of shoots). A reduction in the rate of shoot growth due to graft incompatibility may lead to a decline in leaf carbon export from scion to rootstock, with consequently limited nitrogen assimilation[37]. As a result, SPAD values could drop due to the blockage of carbohydrate allocation[38].

Another work on graft incompatibility in *Prunus* spp. evaluated the leaf chlorophyll content of three peach cultivars grafted on different clonal rootstocks. Tree death caused by graft incompatibility was preceded by a drastic reduction in SPAD index values five months after field planting[22].

According to the findings of the present study, to previous experience[18], and excluding any disorders due to pests, diseases, or nutrient deficiencies, chlorophyll contents lower than 40 SPAD units could therefore be reliable indicators of stress related to graft incompatibility in chestnut.

4.5.1.2. Stomatal conductance

Stomatal conductance was assessed via a porometer on the same leaves used to measure the chlorophyll content. The monitoring has been carried out during days with similar weather conditions and in a specific time interval. The values of stomatal conductance recorded during the vegetative season, expressed as the rate of water vapor exiting through stomata, are reported in Figure 1.

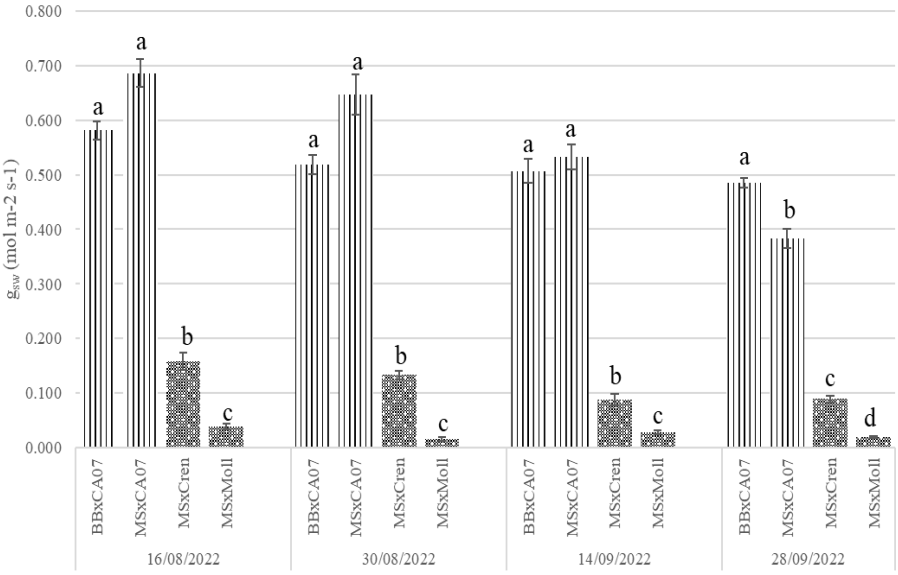

Figure 4 Stomatal conductance to water vapor (g_{sw}) for the experimental grafting combinations, expressed as mol m^{-2} s^{-1}. Bouche de Bétizac x Marsol CA07 (BBxCA07), Marrone Val Susa x Marsol CA07 (MSxCA07), Marrone Val Susa x C. crenata *(MSxCren), Marrone Val Susa x* C. mollissima *(MSxMoll). Means (n = 30) followed by the same letter are not significantly different at P < 0.05 (Duncan's test).*

Significant differences can be clearly observed among compatible (striped bars) and incompatible (dotted bars) grafting combinations. Differences remained consistent throughout the growing season, with greater values for Bouche de Bétizac and Marrone Val Susa grafted onto clonal hybrid rootstock Marsol CA07. Only the last measurement highlighted differences among these two combinations, with a higher average value for BBxCA07. The pattern that emerged from the monitoring of g_{sw} is similar to the one observed with the leaf chlorophyll content. For both the parameters, the incompatible combinations performed the lowest values, particularly in the case of MSxMoll. These values are in accordance with a previous preliminary study on chestnut graft incompatibility[18]. Apart from this work, no authors have investigated the changes of stomatal conductance related to chestnut graft incompatibility. However, there are some studies on other fruit species that focused on the issue.

A paper by Losciale et al., (2008) reports a study on pear 'Bosc' grafted on pear seedlings and quince EMC, analysing the effects of rootstocks on the photosynthetic efficiency under similar conditions of light and temperature[17]. Trees grafted on the incompatible rootstock EMC recorded lower stomatal conductance as a consequence of the limited hydraulic conductivity, which led to reduced transpiration and net photosynthesis. These results confirm previous research on two pear cultivars grafted onto rootstocks with different degrees of graft compatibility; leaf transpiration, stomatal conductance, and net photosynthesis were reduced in the case of grafts with quince rootstock EM[16]. Graft incompatibility among some pear cultivars and quince rootstock leads to a poor synthesis of vascular bundles at the graft union, inhibiting water and nutrient transport[39].

4.5.2. Chemical dynamics

4.5.2.1. Total phenolic content

The total phenolic content was measured in the inner and outer tissues of the three sections composing the graft. Table 2 reports the values obtained for each section and in each woody layer of the experimental grafting combinations tested.

Table 7 Total phenolic content measured in the three sections composing the grafts and in the inner and outer woody layers. Bouche de Bétizac x Marsol CA07 (BBxCA07), Marrone Val Susa x Marsol CA07 (MSxCA07), Marrone Val Susa x C. crenata *(MSxCren), Marrone Val Susa x* C. mollissima *(MSxMoll). The mean value and standard deviation are given for each sample (n = 3). Different letters for each compound indicate the significant differences at p < 0.05.*

SAMPLING TIME	SECTION	WOODY LAYER	BBxCA07 compatible		MSxCA07 compatible		MSxCren incompatible		MSxMoll incompatible	
			mean	SD	mean	SD	mean	SD	mean	SD
			(mg GAE/100 g FW)		(mg GAE/100 g FW)		(mg GAE/100 g FW)		(mg GAE/100 g FW)	
callusing	scion	external	2923.44 b	21.04	3109.00 a	61.10	3138.25 a	45.59	3182.84 a	8.53
		internal	391.63 b	2.81	303.17 c	13.40	463.25 a	9.42	485.72 a	15.73
	graft union	external	2800.90 a	20.25	2813.47 a	21.70	2863.00 a	61.22	2212.07 b	85.39
		internal	529.55 b	22.22	403.17 c	19.16	739.30 a	33.40	669.19 a	51.16
	rootstock	external	2892.50 c	79.49	3028.88 bc	15.62	3186.45 a	28.87	3036.59 b	63.46
		internal	291.65 b	21.35	270.86 c	11.44	364.50 a	14.90	432.31 a	20.44
end of vegetative cycle	scion	external	3168.34 c	38.82	3307.99 a	12.64	3208.11bc	7.22	3276.50 ab	38.71
		internal	593.39 d	12.29	1232.25 c	27.41	1547.67 b	97.80	1749.89 a	23.47
	graft union	external	3283.67 ab	5.21	3329.86 a	80.90	3210.60 b	20.42	3236.21 ab	16.74
		internal	791.26 c	9.12	1293.67 b	17.52	3110.13 a	24.18	3149.67 a	5.97
	rootstock	external	3166.30 b	6.73	3244.98 a	29.51	3215.55 ab	14.40	2959.03 c	29.81
		internal	465.43 d	7.27	814.21 b	3.64	694.70 c	36.17	945.22 a	27.60

TPC was quantified also on the inner and outer tissues of the ungrafted genotypes, namely on Bouche de Bétizac, Marrone Val Susa, Marsol CA07, *C. mollissima*, *C. crenata*. Knowing the initial quantities of phenol compounds in each genotype was useful for the interpretation of the results.

Based on previous works, it seems that the major chemical changes related to graft incompatibility occur at the graft union, though examples of phenol translocation towards scion/rootstock have been reported. On chestnut, only Karadeniz et al., (1993) focused on the study of TPC dynamics during grafting establishment[40]. In this preliminary experiment, different grafting techniques were compared at different times, assessing the success rate and the total phenolic content in phloem tissues of the graft union. Results show that the amount of these secondary metabolites has been increasing all along the vegetative cycle. A more recent study by Amri et al., (2021) found that incompatible peach/plum unions led to a statistically significant increase of TPC and PAL enzymatic activity in the scion tissues during the leaf fall period. Authors found a positive correlation between PAL activity and TPC, which leads them to believe that the change in the PAL activity might have a primary role in the accumulation of phenolic compounds[36]. These data confirm an earlier chemical study on peach (*P. persica*) and Japanese apricot (*P. mume*) incompatible grafts[10].

In the present study, the amount of TPC increased statistically from CAL to EVC in all the sections of all the combinations, as expected, except for the outer tissues of scion and rootstock of MSxCren and MSxMoll. The external tissues, composed of bark, cambium, and phloem, had a significantly higher content of phenol compounds compared to the internal ones for all the sections analysed (scion, graft union, and rootstock). These differences remained consistent during the growing season. Though, no patterns emerged analysing the TPC in the external layers. A rather different behaviour was observed in the inner tissues, where the amount of phenol compounds turned out to be higher in the incompatible combinations for all the sections. In particular, the amount of TPC at the graft union was over three times higher at EVC in the case of incompatible combinations (Figure 3).

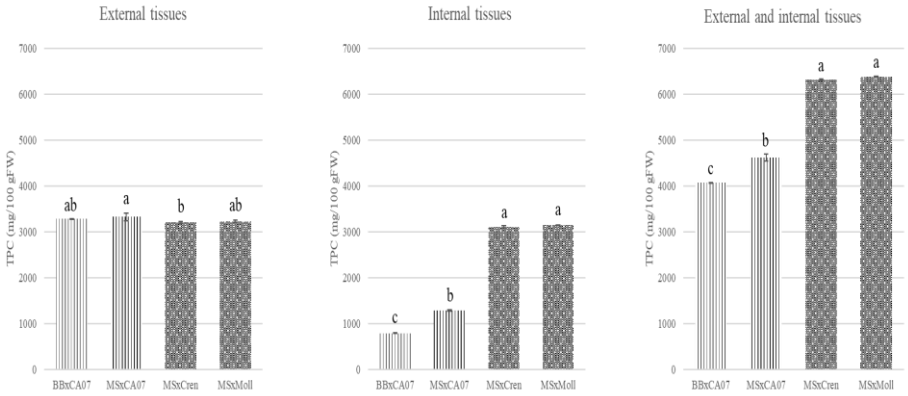

Figure 5 Total phenolic content in the external and internal woody layers of graft union, values measured at the end of the vegetative cycle. Bouche de Bétizac x Marsol CA07 (BBxCA07), Marrone Val Susa x Marsol CA07 (MSxCA07), Marrone Val Susa x C. crenata *(MSxCren), Marrone Val Susa x* C. mollissima *(MSxMoll). The mean value and standard deviation are given for each sample (n = 3). Different letters for each compound indicate the significant differences at $p < 0.05$.*

As shown in Figure 3, a greater accumulation of phenol compounds was observed in the graft area of the incompatible unions (dotted bars), especially in the inner woody layers. Between the compatible combinations, MSxCA07 recorded a statistically higher amount of TPC (1293.67 vs 791.26 mg GAE/100 g FW). These differences could indicate, as observed with the leaf chlorophyll content, distinct levels of graft compatibility.

As grafting represents a source of stress for plants, several chemical pathways are involved during scion-rootstock establishment. Among these, the metabolism of phenols is enhanced, as they are implicated in the processes of stress and wounding. These secondary metabolites play a primary role especially in the early growth stages of connections, being related to lignin formation and protein bounding[41, 42].

In the present work, at CAL the content of phenol compounds in the external tissues seemed to be influenced primarily by the physiological enhancement of phenol metabolism and by the genotype. On the contrary, minor differences found in the inner tissues of the scion and graft union could be related to graft incompatibility. At EVC, differences in terms of phenol accumulation were

significantly more pronounced, especially at the graft union and in the internal tissues. The greater amount of phenols in the inner tissues of MSxCren and MSxMoll scions are in accordance with previous research and seems to indicate a translocation of these secondary metabolites towards the upper part of the graft[10, 36].

The assessment of the total phenolic content seems to represent a useful preliminary tool to discriminate among compatible/incompatible combinations. Several studies reported an accumulation of TPC in the case of incompatible combinations, supporting the present findings[43, 44]. However, a thorough identification and quantification of the main classes and molecules responsible for this accumulation is needed.

4.5.2.2. Polyphenolic markers identification and quantification

The analysis of phenol compounds in the different sections composing chestnut grafts indicated tannins as the most represented class. In particular, castalagin was the predominant compound found, as it has been already observed on chestnut, and was mostly located in the external woody tissues[26, 45]. Asiatic species (*C. mollissima* and *C. crenata*) showed a lower concentration of tannins if compared to *C. sativa*, as revealed by the study of the ungrafted genotypes.

At the graft union, where the biosynthesis of polyphenols seems to be higher during grafting development, the concentration of benzoic acids, catechins, and tannins increased significantly during the growing season, showing higher amounts at the EVC stage. However, the accumulation of phenol compounds appeared to be driven by the phenomenon of incompatibility only partially (Figure 4). In particular, the concentration of catechins (catechin and epicatechin) in compatible and incompatible combinations did not allow to define a clear trend, being in contrast to previous studies[26, 31, 46].

Figure 6 Phytochemical fingerprint of the outer and inner woody tissues composing the graft union. Bouche de Bétizac x Marsol CA07 (BBxCA07), Marrone Val Susa x Marsol CA07 (MSxCA07), Marrone Val Susa x C. crenata *(MSxCren), Marrone Val Susa x* C. mollissima *(MSxMoll).*

The concentration of the benzoic acids, and primarily of gallic acid, calls into question the role of these compounds as markers of graft incompatibility on chestnut, supporting recent preliminary findings on the same species[47]. The genotype of the starting materials (scions and rootstocks) could also influence the phenolic levels at the graft interface, modifying the amounts of the selected incompatibility/compatibility markers. For this reason, further studies are necessary to better define the potential ranges of these molecules.

Polyphenol compounds have been investigated on many fruit species because of their involvement in grafting formation. Though, studying the behaviour of these biomolecules is challenging because of multiple reasons: influence of the environmental conditions, difficulties in finding homogenous nursery materials, and establishment of fully incompatible combinations are among the main limitations. Regarding this latter aspect, experiences in the field seem to suggest

that chestnut incompatibility may fall into the so-called localized incompatibility. Because of morpho-physiological alteration at the graft union, this type of disaffinity causes malformations which eventually leads to impaired union formation; after some years grafted trees can break at the junction, with considerable economic losses[48, 49]. The above-listed aspects make the study of graft incompatibility very complex, thereby justifying the analysis of phenol compounds in terms of classes rather than single compounds.

4.6. Conclusions

The present study investigated incompatibility in interspecific chestnut grafting under a multidisciplinary approach. Stomatal conductance and leaf chlorophyll content were confirmed to be effective and non-destructive validation tools; the monitoring of these physiological parameters during grafting formation allowed to clearly discriminate between compatible and incompatible grafting combinations.

The analysis of the total phenol content confirmed a pattern already observed in previous research on other fruit species. The biosynthesis of phenol compounds increased during the vegetative cycle and became higher at the graft union. Comparing the TPC in the graft tissues at the end of the vegetative cycle, incompatible combinations recorded the statistically highest values. The accumulation of polyphenols was concentrated in the inner tissues. Finally, the phytochemical fingerprint suggests how the phenol compounds investigated could be driven by the phenomenon of incompatibility only partially, in contrast to earlier works.

The study of chestnut graft incompatibility remains a challenge for multiple reasons and the identification of techniques able to explore the interactions between new rootstock genotypes and chestnut cultivars is of great interest. The current scientific findings support the development of an integrated multidisciplinary approach for the early prediction of graft incompatibility, rather than single methods.

The combined study of chemical and physiological parameters contributed to acquiring greater insight into the graft incompatibility matter. Further research will be implemented to validate these findings, enlarging the number of genotypes and broadening the spectrum of phenol classes and compounds, with the final goal of supporting the development of new and resilient rootstocks.

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General findings and discussion

In recent years, traditional fruit crops are experiencing a challenging time, due to many factors (rising prices for raw materials, climate change, and the spread of new pests and diseases…). By way of example, the kiwifruit cultivation is strongly decreasing because of the damages caused by the bacterial canker (Psa) and by the kiwifruit vine decline, forcing farmers to find other valuable and suitable crops in Italy. Chestnut and table grape cultivations represent interesting alternatives to major fruit crops, especially in northwestern and central parts of Italy. As evidence of this, an intense breeding activity has been leading to the selection of many promising genotypes for *Castanea* and *Vitis* spp. However, still scarce information is available regarding the interactions between new rootstocks and cultivars.

The Ph.D. project explored the physiological, chemical, and anatomical dynamics occurring during grafting formation in chestnut and table grape propagation to find an early predictive approach of graft incompatibility.

The role of phenol compounds as potential incompatibility markers was investigated on chestnut and table grape. The study of the implications of phenolic compounds during grafting development was carried out starting from previous works on similar fruit species, since few works have addressed the issue on *Castanea* and *Vitis* spp. In particular, no works were reported in the literature for table grape. Because of this lack of knowledge, most of the activities focused on developing an extraction method for phenols from woody tissues (Chapter 1). Four classes of polyphenols were investigated and chosen for their established role in grafting dynamics. The results provided a promising and sustainable methodology for extracting phenol compounds involved in the grafting process. Further studies are planned to validate and verify these findings, testing additional extraction variables and new grafting combinations to optimize the extraction protocol. This preliminary methodological research lays the foundations for future studies on graft incompatibility in table grapevine.

On chestnut, phenol compounds involved in grafting development were investigated on the tree sections composing graft (upper and lower parts, graft

union) and in several interspecific experimental combinations. Phenol classes were selected according to literature and to previous preliminary works. Experimental activities allowed to further select the most involved phenol compounds; then, analyses were repeated enlarging the number of genotypes under test. At first, results were found to be in accordance with previous studies on other fruit species (Chapter 2). Though, improving the extraction technique and broadening the genotypes, results seemed to be contradictory (Chapter 4). For this reason, the analysis of the total phenol content (TPC) was implemented, as a further validation tool. This latter parameter furnished interesting results, highlighting statistically higher concentrations of phenol compounds in the inner tissues at the graft interface of incompatible combinations (Chapter 4). The assessment of the TPC seems to represent a useful preliminary tool to discriminate among compatible/incompatible combinations, especially if coupled with physiological monitoring. The biosynthesis of polyphenols seems to increase during grafting development, showing higher amounts at the end of the vegetative cycle. However, the accumulation of phenol compounds appeared to be driven by the phenomenon of incompatibility only partially. In particular, the concentration of catechins and benzoic acids in compatible and incompatible combinations did not allow to define a clear trend, being in contrast to previous studies. Probably, the genotype of the starting materials (scions and rootstocks) could also influence the phenolic levels at the graft interface, modifying the amounts of the selected incompatibility/compatibility markers. For this reason, further studies are necessary to better define potential ranges of these molecules. To validate chemical outputs, physiological observations were carried out starting from the second Ph.D. year. Physiological traits such as chlorophyll content and stomatal conductance are handy, rapid, and non-destructive to acquire, and turned out to be very useful corroborating tools of graft incompatibility on several horticultural species. Within the Ph.D. project leaf chlorophyll content, fluorescence rate, and stomatal conductance have been investigated during multiple vegetative cycles on chestnut grafting combinations. The chlorophyll content in the leaves of incompatible unions recorded statistically lower values

during all the growing seasons. According to the results and excluding any disorders due to pests, diseases, or nutrient deficiencies, chlorophyll contents lower than 40 SPAD units could therefore be reliable indicators of stress related to graft incompatibility in chestnut. Significant differences were observed also measuring the stomatal conductance, emphasising the pattern that already emerged from the monitoring of the leaf chlorophyll content (Chapter 3 and 4). The incomplete wound healing due to incompatibility brings to vascular discontinuity, negatively influencing plant water status.

On table grape physiological observations did not show any clear pattern. Future trials will be implemented testing new rootstock selections, whose distribution is still limited. As *Vitis* rootstocks are normally meant for grape production, it will be interesting to observe their behaviour with table grape cultivars.

During the Ph.D. period abroad at Empresa de Transformación Agraria S.A., S.M.E., M.P. (TRAGSA) coordinated by Dr. Beatriz Cuenca, different aspects related to propagation and nursery management were addressed. Many genotypes clonally produced were grafted for experimental purposes. Morphological, physiological, and chemical aspects were investigated on innovative hybrid rootstocks grafted with traditional cultivars. Preliminary histological observations were carried out on several chestnut grafting combinations and furnished promising results, allowing to observe early symptoms of disaffinity at the graft interface.

Conclusions

The development of innovative rootstocks and cultivars able to adapt to the negative impacts of climate change and to the spread of new pests and diseases is one of the most promising mitigation strategies for horticulture. Research can support the breeding sector by finding tools to early predict the compatibility of these nursery materials.

The Ph.D. experimental activities focused primarily on the implications of phenolic compounds during grafting development in chestnut and table grape

propagation, coupling chemical aspects with morpho-physiological observations. Results suggest that some classes of polyphenols could work as incompatibility markers on *Castanea* and *Vitis* spp., confirming previous works on other horticultural species. Though, the analysis of the phytochemical fingerprint questioned the role of certain well-known incompatibility markers for chestnut, such as benzoic acids and catechins.

Studying the behaviour of these biomolecules is challenging because of multiple reasons: the influence of the environmental conditions, difficulties in finding homogenous nursery materials, and establishment of fully incompatible combinations are among the main limitations. These aspects make the study of graft incompatibility very complex. Therefore, the current scientific findings support the development of an integrated multidisciplinary approach for the early prediction of graft incompatibility, rather than single methods.

The monitoring of the physiological parameters during the vegetative cycle was a useful complementary tool to discriminate among compatible and incompatible genotypes, as well as the analysis of the total phenolic content.

To conclude, the combined study of chemical and morpho-physiological parameters contributed to acquiring greater insight into the graft incompatibility matter in chestnut and table grapevine. Further research will be implemented to validate these findings, enlarging the number of genotypes and broadening the spectrum of phenol classes and compounds, with the final goal of supporting the development and the subsequent spread of new and resilient rootstocks and cultivars.

Ringraziamenti