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PHENOTYPIC VARIABILITY OF MYRTLE (*MYRTUS COMMUNIS* L.) AND SEASONAL ACCUMULATION OF PHENOLIC COMPOUNDS

Docente guida: Prof. Maurizio Mulas

Dottoranda: Miriam Catzeddu

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General abstract

This thesis investigates the variability of chemical composition in *Myrtus communis* L. under different biological and environmental contexts, with a particular focus on phenolic compounds in berries and mineral and bioactive constituents in leaves. By integrating post-harvest studies, phenotypic characterisation, and multi-environment field trials, the work aims to provide a comprehensive understanding of how genotype, developmental stage, and environmental conditions shape the accumulation and stability of key phytochemicals in myrtle.

Chapter 1 aimed to evaluate the effects of different post-harvest storage conditions on the quality of myrtle berries, with a specific focus on changes in phenolic compounds. In particular, the study investigated how three storage temperatures influenced weight loss and the evolution of total phenols, tannins, and anthocyanins in four cultivars with pigmented and unpigmented berries, in order to identify optimal storage conditions and to better understand the potential metabolic changes of phenolic compounds during shelf-life.

The second chapter focuses on the phenotypic characterization of two contrasting cultivars through the profiling of specialized metabolites, mainly flavonols and anthocyanins, across different ripening stages and growing seasons. Using chromatographic techniques, this section investigates how genotype, developmental stage, and interannual variability affect phenolic accumulation, highlighting the distinct nutritional and nutraceutical potential of pigmented and unpigmented berries over fruit development.

Chapter 3 aimed to investigate the expression of the main genes involved in flavonoid biosynthesis in pigmented and unpigmented myrtle berries, with the purpose of linking molecular data to the phenotypic and chemical differences observed between the two berry types. In particular, the study examined the relationship between gene expression and the accumulation of flavonoids and anthocyanins across sampling dates and years, in order to clarify the molecular mechanisms underlying berry pigmentation and the distinct biochemical profiles of the two cultivars.

The work presented in Chapter 4 examines the influence of cultivar, season, growing site, and thermal regime on the mineral and chemical composition of myrtle leaves. Based on multi-environment field trials, this analysis identifies seasonal and thermal drivers of variability in mineral nutrients, polyphenols, and chlorophyll content, while also considering the valorisation potential of leaf biomass as a sustainable source of bioactive compounds for industrial applications.

1. General introduction

1.1 Botany and ecology

The *Myrtus communis* L. is an evergreen shrub spontaneous of the Mediterranean maquis, belonging to the order of *Myrtales* and to family *Myrtaceae*, which includes 75 genera and approximately 3,000 species (Camarda and Valsecchi, 1983). *Myrtus communis* is the only species of the genus *Myrtus* found in the wild in Europe, with its range extending into North Africa and West Asia (Aleksic and Knezevic, 2014; Fiori, 1925; Picci and Atzei, 1996).

M. communis L. includes two subspecies, *communis* and *tarentina*, which differ in some morphological traits (Medda et al., 2022a; Medda and Mulas, 2021). Subspecies *communis*, can grow up to 5 meters tall and has long internodes, large undecussate leaves, long pedicellate flowers, calyx with acute lobes, larger corolla, and ellipsoidal or ovoid berries (Fiori, 1925; Parlatore, 1842). The subspecies *tarentina* is a shorter shrub that does not exceed 2 metres in height. It has short internodes and small leaves that are thickened and arranged in 4 rows. The flowers have a short pedicel, and the calyx has rounded, ciliated lobes. The corolla is small, and the berry is spherical or subglobose (Fiori, 1925).

Varieties within these subspecies are distinguished based on morphological features. In subspecies c*ommunis*, the *communis* var. *italica* is notable for its erect branches, ovate-lanceolate leaves measuring 10-15 mm in width, petals ranging from seven to eight in number, and prominent stamens. The *romana* variety exhibits large, light green leaves measuring 30-35 mm in width, often arranged in whorls. The *boetica* variety is characterized by short branches, ovate-lanceolate leaves approximately 20 mm wide, large petals, and long stamens and the *lusitanica* variety presents itself as a low shrub with narrow leaves and small flowers. For subspecies *tarentina*, the *tarentina* variety is distinguished by its shaggy branches and ovate-lanceolate leaves, while the *microphylla* variety features lanceolate leaves with a dark green coloration.

Myrtle is a plant native to the Mediterranean basin, thriving up to 800 meters of altitude in mild climates. It shows remarkable adaptability to harsh environmental conditions but remains sensitive to cold winds and frost. Conversely, it tolerates drought and prefers sandy, loose, permeable soils with a neutral or sub-acidic reaction. In Sardinia and Corsica, it is part of the low Mediterranean scrub (Usai et al., 2018). Plant height ranges between 0.5 and 3 m (Usai et al., 2020), with an assurgent habit. The bark is reddish then, upon aging, takes on a greyish colour with longitudinal fissures.

Leaves are evergreen, opposite, occasionally decussate in four rows, dark green on the upper surface, and light green and opaque on the lower page with the presence of aromatic glands. The leaf blade shapes varying from elliptical to lanceolate or ovate-lanceolate. Flowers are hermaphroditic, white or pink in colour, with a dialysepalous calyx of five sepals and a dialypetalous corolla of five petals. Numerous stamens with yellow anthers surround an inferior ovary with three lodges (Mulas et al., 2001, 1997). The fruit is a fleshy berry, which can be globose, ovoid, ellipsoidal, or pyriform. The unripe fruit has an acidulous taste, transitioning to a resinous flavour upon ripening. Their colour ranges from black to blue, or pinkish to white. Each berry contains one to eight seeds per locule.

Based on the coloration the fruit take on ripening, two varieties can be distinguished: *melanocarpa*, with pigmented, bluish-black or reddish berries, and *leucocarpa*, with unpigmented, yellowish or white berries.

The myrtle is a typical species of Mediterranean climate environment characterized by alternating cold, rainy winters and hot, dry summers. Its seasonal behaviour reflects climatic conditions.

Vegetative activity begins in spring, between late March and early April, which is when the air temperature begins to rise. The formation of new shoots occurs from the buds located in the terminal part of the branch, while the remaining mid-basal part retains the previous year's leaves. Once its cycle is over, the leaves fall off, leaving this part of the branch bare. The

average life span of myrtle leaves varies according to several factors, such as branch age, environmental conditions, and ecotype.

Vegetative growth continues until June with the elongation of shoots and the appearance of new leaves, this then stops in the summer period due to excessive aridity. A vegetative resumption may occur in the fall if early rains occur in favour of elongating the distal parts and shoots of the year.

When temperatures drop in November, myrtle plants halt their vegetative development, which will then restart in spring (Mulas, 2012a).

Around mid-May, flower bud formation occurs, from differentiated buds on the year's still-growing shoots. Around mid-May the emission of flower peduncles begins but, the actual flowering begins in June and ends in August. In some cases, flowering can also occur in September-October. Flower differentiation in myrtle affects the basal portion of the shoot, therefore, greater shoot length allows more fruit to develop.

Fruit will begin to attach in late July, early August; this occurs in a scalar fashion, both on the plant but also on the shoot itself.

The berries begin to turn colour in late August-September, then 4-5 months after flowering in November-December ripening is complete (Mulas, 2012a). The development cycle of the myrtle is influenced by water availability. Water stress causes old leaves to fall, thus decreasing photosynthetic activity, which impairs fruit growth, increases the risk of fruit drop and flower dripping in summer. Good water availability before flowering and fruit set, on the other hand, favours shoot development (Mulas, 2012a).

1.2 Myrtle plant uses

The myrtle plant (*Myrtus communis* L.) has been widely recognised for its diverse applications spanning various sectors, including food, cosmetics, pharmaceuticals, and traditional medicine (De Luca et al., 2022; Mulas, 2012b). This evergreen shrub has been used

since ancient times for its distinctive properties and valuable products derived from its various parts (Mulas, 2012b).

Myrtle has long been integrated into culinary traditions. The berries and leaves are used in the preparation of food to flavour meats and sauces, and the berries are specifically used to produce a typical liqueur from Sardinia (Italy) called "*Mirto di Sardegna*" (De Luca et al., 2022; Mulas, 2012b). This liqueur, obtained through hydro-alcoholic infusion, is an important economic resource for Sardinia, officially recognised by the European Commission in 2008, mandates exclusive use of Sardinian berries without added flavours or colourings. The leaves and berries of the myrtle plant have also been used as spices for the purpose of flavouring food and as a hop substitute in the production of beer.

The evergreen foliage of myrtle, in combination with its abundant flowering and adaptability, has led to its widespread popularity as an ornamental plant. Its high degree of branching and compact growth makes it suitable for cultivation in pots, gardens, and green urban spaces. The plant's capacity to re-flower in autumn or even winter serves to enhance its ornamental appeal. In addition, myrtle contributes to ecological restoration initiatives by facilitating the renaturalisation of degraded areas, a process requiring minimal water and maintenance, and being resistant to disease and pollution.

The myrtle plant has been identified as a versatile industrial resource. The leaves and wood of the plant yield furfural, a compound that is employed in the production of paints, lacquers, and plastics. The high tannin content of the leaves supports leather tanning processes. Myrtle wood, renowned for its durability, is used in the crafting of fishing creels, baskets, and other artisanal items (Mulas, 2012b). Furthermore, the essential oil obtained through distillation is employed in the dyeing of textiles and wool.

Myrtle is also used for animal feed, in perfume and cosmetic industries and pharmaceutical applications (De Luca et al., 2022; Usai et al., 2015).

Since ancient times different organs of the myrtle plants have been used as a natural medicine to treat many diseases, such as cough, skin disorders, gastrointestinal symptoms of peptic ulcers and haemorrhoids. Giampieri et al. (2020) reported many pharmacological activities of myrtle, including antioxidant, anticancer, antidiabetic, antiviral, antibacterial, antifungal, hepatoprotective, and neuroprotective properties.

The biological activities of myrtle are mainly associated with its. The biological activities of myrtle may be associated with its rich phytochemical composition, including phenolic compounds, flavonoids, tannins, anthocyanins, and volatile constituents of the essential oil such as myrtenyl acetate, α-pinene, 1,8-cineole, and limonene.

Pharmacological and phytochemical studies on the volatile oil of myrtle and its formulations have been also widely performed (Guzelmeric et al., 2022) and myrtle has also been intensively studied for its essential oil obtained from the whole aerial part of the plant, leaves, flowers, and berries (Usai et al., 2020).

Furthermore, myrtle extracts have shown potential in addressing sleep disorders, oesophageal reflux symptoms, and colitis (Jabri et al., 2016).

1.3 Phenotypic and genetic variability: cultivar selection

In recent decades, there has been an increased demand for myrtle biomass from the processing industry for liquor production. This has led to the domestication and selection process of the species, with the aim of protecting wild populations from indiscriminate harvesting. Furthermore, the biological and antioxidant properties of myrtle have led the scientific world to conduct numerous studies in various areas of application of the myrtle plant.

The domestication process was initiated in Sardinia in 1995 and mainly involved three phases of study (Medda and Mulas, 2021).

In the first phase, from 1995 to 1997, the selection of wild populations to be used as mother plants was conducted by observing and sampling plants in 36 localities in the region. A

total of 70 mother plants were studied, propagated agamically, and analysed for morpho-biometric characters using a descriptor list properly created for the myrtle species.

In the second step of domestication process (1997-2001), the selection of previously propagated plants was carried out according to the following characteristics:

- agamic propagation and nursery management aptitude;
- agronomic performance;
- apparent disease tolerance;
- fruit and leaf biochemical quality.

As a result of this selection, 40 wild parent lines were identified. Their behaviour was observed both at the site of origin and in the experimental fields keeping the same ecological and management conditions. The candidate cultivars were subjected to morphological and qualitative biomass analysis.

From 2001 to 2006, the third stage of the domestication process was carried out with the objective to explore the variability of new hybrids obtained by open pollination. Subsequently, these hybrids were compared for different characters such as aptitude for agamic propagation, nursery viability, agronomic performance, disease tolerance, and fruit and leaf quality. The work was performed crossing three myrtle cultivar selected in the previous second phase. A total of 387 seedlings were observed, and 20 clones were finally selected.

The 40 varietal lines selected from wild flora, in addition to the others 20 lines obtained from freely pollinated seedlings, were cultivated in the collection field of the “Azienda Didattico Sperimentale Antonio Milella” of the University of Sassari, situated in Fenosu, Oristano, in the central-western part of Sardinia. The planting pattern was configured to be 1 m in length per row, with a 3.15 m separation between rows, and the entire area was subjected to grassing.

1.4 Biochemical composition of myrtle

The growing interest of the scientific community in myrtle is mainly due to its richness in secondary metabolites, particularly phenolic compounds, which chemically include numerous molecules composed of one or more aromatic ring linked to one or more hydroxyl groups. Among these compounds, which also serve the function of plant defence against biotic and abiotic stresses, we can find phenolic acids, condensed tannins, flavonoids, and lignin (Haminiuk et al., 2012).

Phenolic compounds function as antioxidant agents by scavenging against certain radicals such as reactive oxygen and nitrogen species (ROS and RNS respectively) (Cruciani et al., 2020; Franco et al., 2019; Saxena et al., 2012). In fact, numerous studies report their beneficial effect against certain diseases such as Parkinson's (Dias et al., 2013), Alzheimer's (Emerit et al., 2003), cardiovascular disease (Ravarotto et al., 2018), and diabetes (Forbes et al., 2008). In vitro analyses have shown that phenolic compounds (including flavonoids) possess greater antioxidant power than carotenoids and vitamins C and E (Dai and Mumper, 2010). Polyphenolic profile can be used as a quality marker in cultivar selection of myrtle and other fruit species (Bertrams et al., 2013; González-de-Peredo et al., 2019; Milivojević et al., 2013; Sarais et al., 2016).

Genotype, growth site, climatic conditions, and maturation stage influence the quantitative and qualitative composition of phenolic compounds, their antioxidant activity, and biological properties (Cruciani et al., 2019; Hennia et al., 2018; Jabri et al., 2021). Therefore, it is important to identify the best time to harvest to maximize the antioxidant activity of myrtle berries and leaves (Babou et al., 2016; Medda et al., 2021a).

Numerous studies have aimed to identify phenolic compounds present in extracts of myrtle berries and leaves. From these studies, it was found that the main flavonoids contained in myrtle leaves are myricetin, quercetin, kaempferol, and catechin derivatives; in addition, the

leaves contain ellagitannins and phenolic acids, such as ellagic and gallic acid (D'urso et al., 2018; Mulas and Melis, 2008; Romani et al., 1999).

Myrtle berries, on the other hand, are rich in gallic and ellagic acid, flavonols such as myricetin-3-O galactoside, myricetin-3-O-ramnoside and myricetin-3-O-arabinoside, and flavanols such as (+)-catechin, (-)-epicatechin-3-O-gallate, (-)-epigallocatechin and (-)-epigallocatechin-3-O-gallate (Barboni et al., 2010). The genotypes with pigmented berries are rich in anthocyanins (mainly in the epicarp of the berry and to a reduced extent in the pulp), with the prevalence of delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside, malvidin 3-glucoside, cyanidin 3-arabinoside, delphinidin 3-arabinoside, petunidin 3-arabinoside, and malvidin 3-arabinoside (Sarais et al., 2016; Scorrano et al., 2017).

Studies comparing pigmented and unpigmented myrtle genotypes showed higher total phenolic and anthocyanin contents in pigmented berries than in unpigmented berries (Messaoud and Boussaid, 2011; Özcan et al., 2020). In addition, it has been observed that the unpigmented berry despite lacking anthocyanins has antioxidant power equal to that of the pigmented berry, this could be due to the high content of ellagitannins (Serreli et al., 2017).

In a recent study (Medda et al., 2020) a pigmented and an unpigmented cultivar were compared from the point of view of gene expression of the main structural genes involved in flavonoid biosynthesis. The study showed that the different pigmentation between the two genotypes could be due to the over-regulation of some genes, particularly the LDOX (Leucoanthocyanidin diogenase) gene and UFGT (UDP-Glucose Flavonoid 3-O-Glucosyl Transferase) gene.

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2. Chapter 1: Evolution of phenolic compounds in myrtle berries at different post-harvest conditions

2.1 Abstract

Myrtle berries are the raw material of liqueur, food, and cosmetic industries. Storage of this fruit allows a larger period of processing. However, there is a lack of knowledge about the optimal storage temperature and the effects on phenolic compounds in the berries. An experiment using berries of four cultivars (two with pigmented and two with unpigmented berries) stored at 2, 10, and 16 °C during 21 days plus a shelf-life period of 3 days at 20 °C was designed. Fruit weight loss at 2 and 16 °C was around 50%, while at 10 °C was around 30%. The highest value of weight loss was in the pigmented cultivar 'Ilaria' in berries stored at 16 °C (57.95%). Total phenols and tannins increased in all the cultivars, with the greatest increase of phenols at 2 °C for the 'Maria Antonietta' cultivar (from 10.94 to 27.79 mg GAE/g FW); while anthocyanins were detected only in the pigmented ones with the highest values in the cultivar 'Ilaria' (12.72 mg cyanidin (C3G)/g FW at 2 °C). The absence of significant correlation between the weight loss and the content of phenols leave to suppose that a new biosynthesis of those compounds occurred.

2.2 Introduction

Secondary metabolites of plants are compounds that are necessary for their survival and play a significant role in the interaction between the plant and the environment (Hartmann, 1996). Among these, a very important group is represented by phenolic compounds, consisting of one or more aromatic rings to which one or more hydroxyl groups are attached. Phenolic compounds can be classified based on the number of carbons in the skeleton, their solubility in water, or as flavonoid or non-flavonoid groups (Haminiuk et al., 2012).

The flavonoid group is particularly present in the outermost layers of fruit, as their synthesis is favoured by sunlight, and they are known to have antioxidant power and a defence

function against biotic and abiotic stress. This group also includes anthocyanins, natural pigments found in flowers and fruit that act as attractants for insects, thus promoting pollination and seed dispersal (Haminiuk et al., 2012).

Another group of polyphenols present in fruit are tannins, divided into two main subgroups: condensed and hydrolysable tannins. Tannins can precipitate proteins and confer the characteristics of astringency and bitterness to fruit.

In the diverse array of plant species that contain a high number of phenolic compounds, the *Myrtus communis* L. is considered a shrub belonging to the Mediterranean maquis that has applications in several industrial sectors, including medicinal, pharmaceutical, cosmetic, liqueur, and ornamental products (Mulas, 2012a). In the Mediterranean basin, myrtle is primarily employed in the production of the typical liqueur, which is obtained through the hydro-alcoholic infusion of the leaves and berries. The leaves are collected during the flowering period, which coincides with the balsamic stage, while the berries are harvested when they have reached full maturity, which occurs between November and December, depending on the variety.

A considerable number of studies (Bayir Yeğin et al., 2022; Medda et al., 2020; Özcan et al., 2020; Usai et al., 2015) have focused on the characteristics of fresh berries, leaves, extracts, and essential oils. However, few have examined the impact of a different method of storage in the post-harvest period on the quality of the fruit (Angioni et al., 2011; Angioni and Schirra, 2011; Fadda et al., 2017; Mulas et al., 2013).

Storing the berries after harvesting for a certain period could be advantageous to the liqueur industry, as the berries would be infused in stages, thus enhancing the quality of the liqueur marketed. This would be particularly beneficial given that the liqueur loses its organoleptic properties over time. Storage could also increase the content of certain phenolic compounds, which would be advantageous for use in the pharmacological and cosmetic industries (Connor et al., 2002).

The objective of this study was to examine the response of various myrtle cultivars to different post-harvest temperatures of storage by considering polyphenol, tannin and anthocyanin content.

2.3 Materials and Methods

2.3.1 Plant material and extracts preparation

Myrtle berries were obtained from the University of Sassari's collection field in Fenosu (Oristano, Central-Western Sardinia, Italy; 39°54′12″ N, 8°37′19″ E; 12 m a.s.l.). The four cultivars analysed were 'Maria Antonietta' and 'Ilaria', with pigmented berries, and 'Grazia' and 'Caterina', with unpigmented berries. The cultivars were previously characterized and evaluated for their horticultural and technological properties (Dessena et al., 2017; Mulas et al., 2002; Mulas and Cani, 1999) and are also known as CUG11, RUM13, RUM14, and V8, respectively.

Sampling was conducted when the berries of each cultivar reached full ripeness. Specifically, harvest dates were 25 November for ‘Grazia’, 28 November for ‘Maria Antonietta’ and ‘Ilaria’, and 5 December for ‘Caterina’. The berries were hand harvested, ensuring that the peduncle remained attached to the fruit to prevent injury.

In the laboratory, 1.8 kg samples of each cultivar were divided into plastic trays for a total of 18 sub-samples of 100 g each: 9 trays were used to monitor the weight loss during the storage period, the other 9 were employed for the collection of berries for subsequent chemical analysis. The berries designated for weight loss assessment were initially weighed and subsequently stored at three distinct temperatures (2 °C, 10 °C, and 16 °C) with a relative humidity of 90% until day 21. From day 21 until day 24, all berries were placed at room temperature (20 °C) and 65% of relative humidity. The weight was monitored on days 0, 7, 14, 21, and 24 post-harvest.

The berries selected for chemical analysis were stored in the same conditions as the others. For the chemical analyses, the berries were sampled at the time of harvest and on day

14, 21 and 24. Ten g of berries were taken, and seeds were removed. The remaining berry pulp was ground using a mortar and pestle with the aid of liquid nitrogen.

A 0.5 g portion of the pulverized sample was mixed with 10 ml of acidified ethanol (1% hydrochloric acid) then it was left to macerate in the dark at room temperature overnight. Subsequently, the samples were filtered, and chemical analyses were conducted immediately or after storage at -20 °C.

2.3.2 Reagents

All reagents used in this study were purchased from Carlo Erba reagents S.r.l. (Milan, Italy), except for catechin that were purchased from Merck (Darmstadt, Germany).

2.3.3 Total phenol, tannin, and anthocyanin content

The content of total polyphenols was determined using the Folin-Ciocalteu assay (Singleton and Rossi, 1965). A total of 0.25 ml of acidified ethanolic extract, pure or suitably diluted, was mixed with 15 ml deionised water and 1.25 ml Folin-Ciocalteu reagent and after 3 minutes, 2.5 ml of 20% sodium carbonate solution was added. The solution was made up to a volume of 25 ml with deionised water and left in the dark for 90 minutes. Absorbance readings were taken at 750 nm on a CARY 50 Scan UV-Vis VARIAN spectrophotometer (Amsterdam, The Netherlands). Results of total phenols were expressed as mg gallic acid equivalent (GAE)/g fresh weight (FW) based on a calibration curve (gallic acid 0.5-14 µg/ml, $R^2 = 0.9977$).

Total tannins content was determined as reported by Fadda and Mulas, (2010). A mixture with 4 ml of diluted extract, 2 ml of ethanol and 4 ml of vanillin solution (1% vanillin in 70% sulphuric acid) was incubated at room temperature for 30 minutes. Then, the solution absorbance was read at 500 nm using a spectrophotometer. A calibration curve was performed using catechin (CE) solution ranging from 0.25 to 12 µg/ml ($R^2 = 0.9985$). Tannins concentration was expressed as mg of CE/g FW.

Total anthocyanins were determined according to Lee et al., (2005). 0.5 ml of ethanolic extract were diluted with 2 ml of 0.025 M potassium chloride (pH 1) and 0.4 M sodium acetate

(pH 4.5), separately. The solutions were incubated for 30 minutes and then the absorbance was read at 520 and 700 nm. The anthocyanin content was calculated using the equation:

$$\text{Anthocyanin [mg/g FW]} = (A \times MW \times DF) / (\varepsilon \times 1 \times g\ FW)$$

where A (Absorbance) = (A520 nm – A700 nm) pH1.0 – (A520 nm – A700 nm) pH4.5; MW = molecular weight of cyanidin-3-glucoside; DF = dilution factor; 1 = pathlength in cm; ε = molar extinction coefficient; g FW = fresh weight of berries used for the extract preparation.

2.3.4 Statistical analysis

Statistical analyses were performed with the MSTAT-C programme (Michigan State University, East Lansing, MI, USA). Separation of the means was conducted using Duncan's multiple-range test with a p-value ≤ 0.01. The correlation coefficients (r) among parameters were calculated using Pearson's coefficient at $p \leq 0.001$ and $p \leq 0.05$.

2.4 Results

2.4.1 Weight loss

The weight loss of berries of all cultivars showed a comparable trend (Figure 1). The berries that maintained the greatest degree of hydration were those stored at 10 °C. The highest value of weight loss was observed in the pigmented cultivar 'Ilaria' in berries stored for 21 days at 16 °C after the shelf-life, with a value of 57.95% of the initial weight.

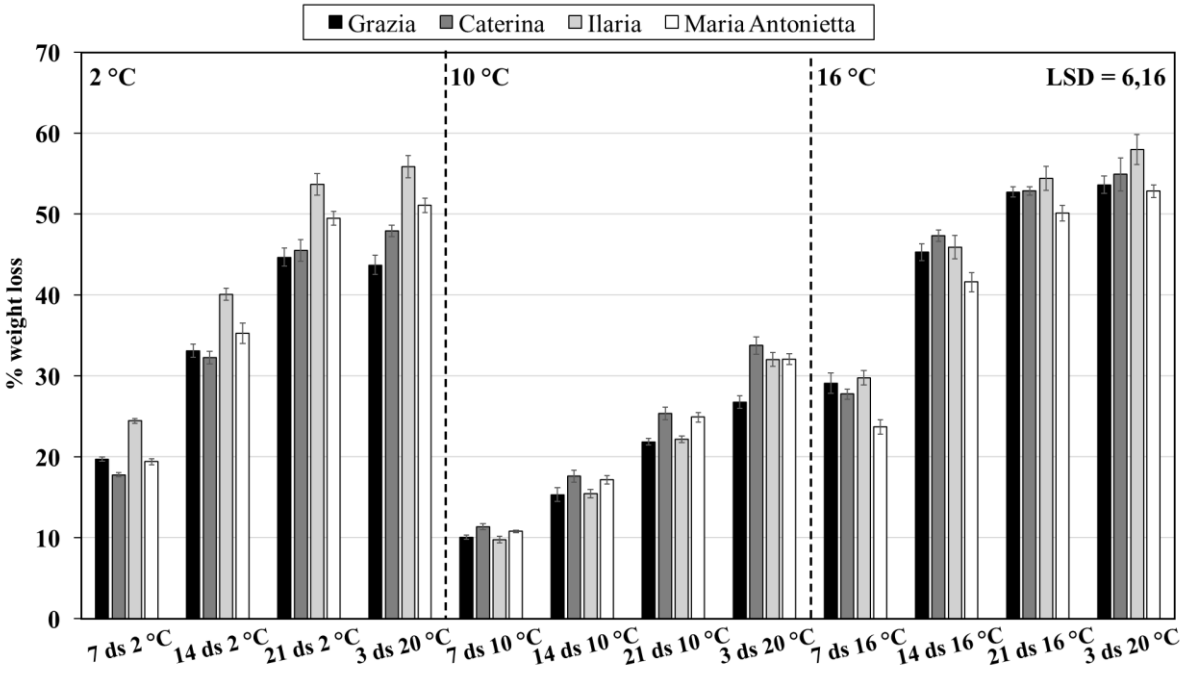

Figure 1. Weight loss trend in the berries of the cultivars 'Grazia', 'Caterina', 'Ilaria' and 'Maria Antonietta' for the storage time of 21 days at the 3 different storage temperatures (2 °C, 10 °C, 16 °C) plus 3 days of shelf life at 20 °C. The data represent the average of 3 replicates, and the error bar represents the standard error. The LSD value discriminate statistically significant differences according to Duncan test ($p \leq 0.01$).

2.4.2 Total phenol, tannin, and anthocyanin content

The polyphenol content of the berries at the time of harvest (Figure 2a) was highest in the pigmented cultivar 'Maria Antonietta', with a value of 12.36 mg gallic acid equivalent (GAE)/g fresh matter (FW). The cultivar that showed the lowest quantity of polyphenols at harvest was instead 'Caterina' with 2.86 mg GAE/g FW. 'Ilaria' and 'Maria Antonietta' showed a significant rise of total phenolic compounds at the end of shelf life (Figure 2b). The highest increase, from 10.94 to 27.79 mg GAE/g FW, was observed in berries of 'Maria Antonietta' stored at 2 °C. For the cultivar 'Grazia', storage at 2 °C and 10 °C resulted in a significant and progressive loss of phenolic compounds. In the 16 °C treatment, there was a decrease on day 14 and then the content increased slightly on subsequent dates. The berries of the 'Caterina' cultivar stored at 2 and 10 °C exhibited relatively stable polyphenol content compared to harvest day values, while an increase was observed in berries stored at 16 °C on the 24th day.

The cultivar with the lowest tannin content at the time of harvest was 'Caterina' (Figure 3a), with a value of 0.83 mg catechin equivalent (CE)/g FW. During the storage period (Figure 3b), the unpigmented berries exhibited low tannin content values, with no increase observed at the three different storage temperatures. In contrast, the pigmented cultivars showed a significant increase in all treatments, particularly in the berries stored at 2 and 16 °C.

The anthocyanin content of the unpigmented berries was found to be insignificant. The pigmented berries exhibited an increase in anthocyanin content on all sampling dates across all storage temperatures (Figure 4b). The highest values were observed in the cultivar 'Ilaria', with 12.72 mg cyanidin (C3G)/g FW in berries stored at 2 °C for 24 days.

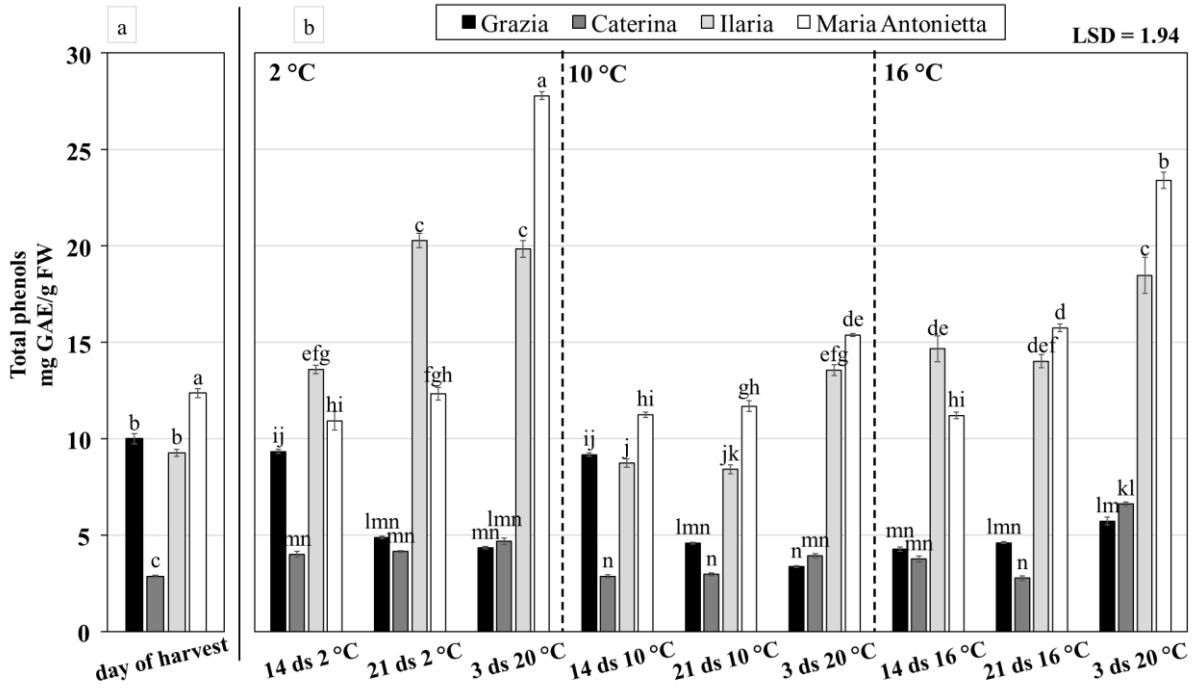

Figure 2. (a) Total polyphenol content in berries of the cultivars 'Grazia', 'Caterina', 'Ilaria' and 'Maria Antonietta' in mg gallic acid equivalent (GAE) per g fresh weight on the day of harvest. **(b)** Total polyphenol content in berries for the storage time of 21 days at the 3 different storage temperatures (2 °C, 10 °C, 16 °C) plus 3 days of shelf life at 20 °C. The data represent the average of 6 replicates, and the error bar represents the standard error. Different letters represent statistically significant differences with the Duncan test ($p \leq 0.01$).

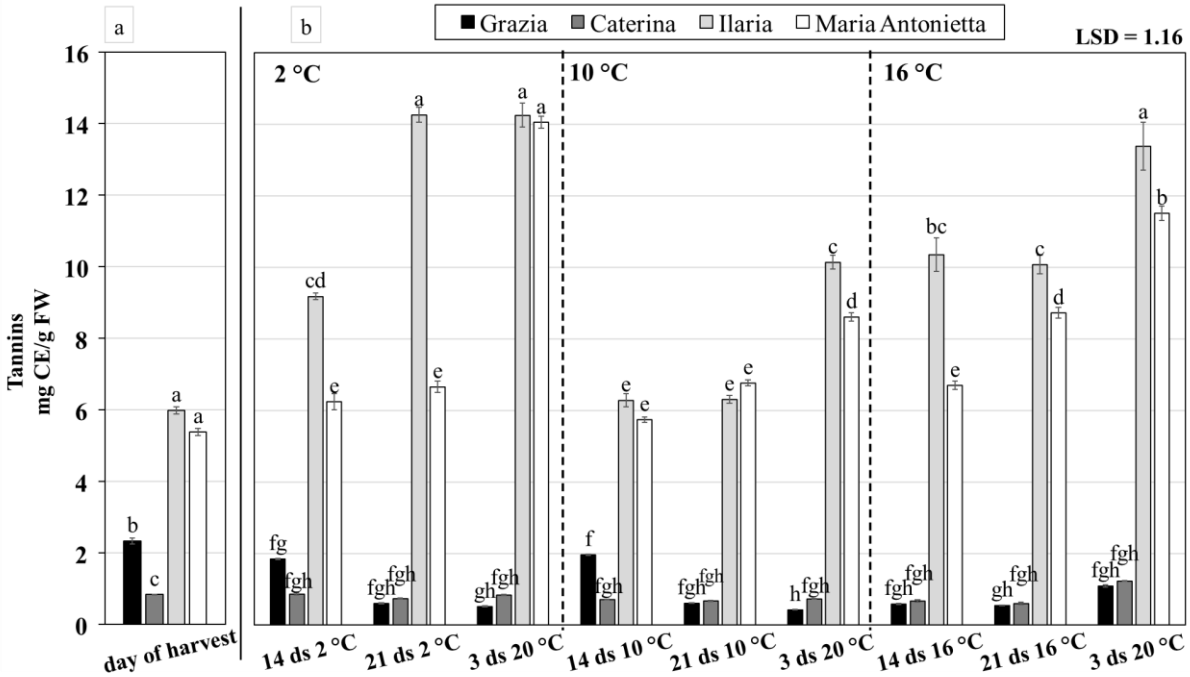

Figure 3. (a) Total tannin content in berries of the cultivars 'Grazia', 'Caterina', 'Ilaria' and 'Maria Antonietta' in mg catechin equivalent (CE) per g fresh weight on the day of harvest. **(b)** Total tannin content in berries for the storage time of 21 days at the 3 different storage temperatures (2 °C, 10 °C, 16 °C) plus 3 days of shelf life at 20 °C. Data represent the average of 6 replicates, and the error bar represents the standard error. Different letters represent statistically significant differences with the Duncan test ($p \leq 0.01$).

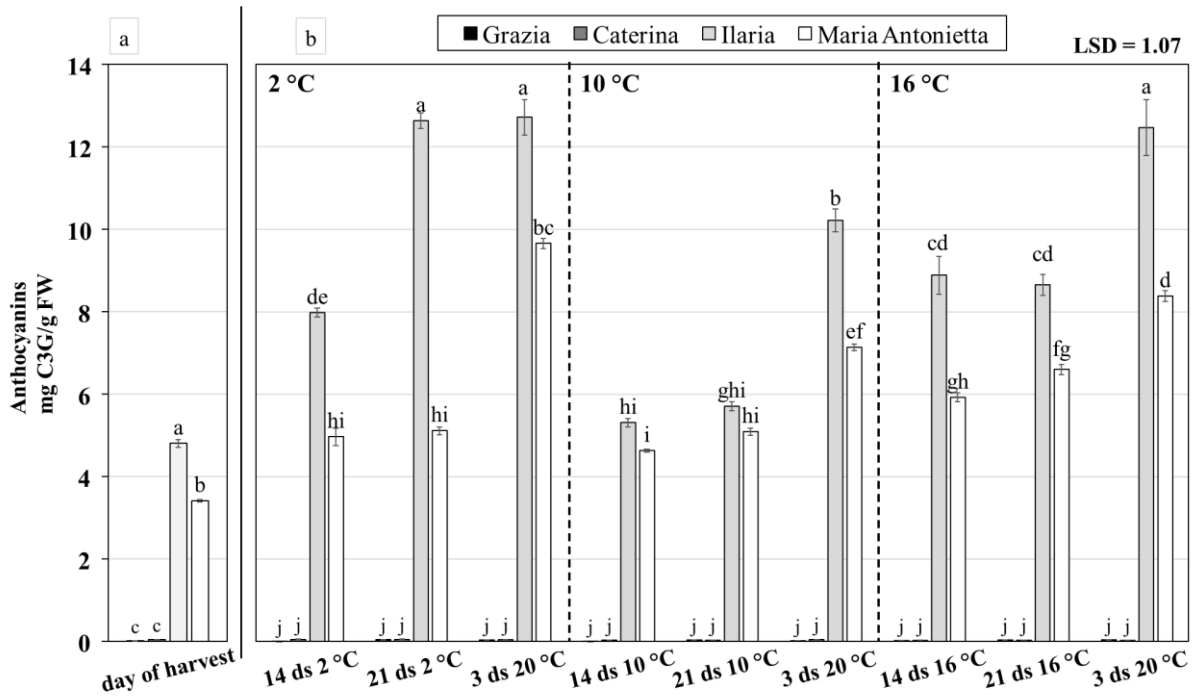

Figure 4. Total anthocyanin content in berries of the cultivars 'Grazia', 'Caterina', 'Ilaria' and 'Maria Antonietta' in mg cyanidin (C3G) per g fresh weight on the day of harvest. **(b)** Total anthocyanin content in berries for the storage time of 21 days at the 3 different storage temperatures (2 °C, 10 °C, 16 °C) plus 3 days of shelf life at 20 °C. The data represent the average of 6 replicates, and the error bar represents the standard error. Different letters represent statistically significant differences with the Duncan test ($p \leq 0.01$).

2.4.3 Correlation between phenolic compounds and weight loss

Correlation analysis, reported in Table 1, showed a highly positive correlation between the different phenolic compounds (r = 0.946 between polyphenols and tannins; r = 0.891 between polyphenols and anthocyanins; r = 0.988, between tannins and anthocyanins with $p <$ 0.001). No correlation was found between weight loss and the phenolic compounds analysed, except for a low correlation ($p < 0.05$) between weight loss and polyphenols.

Table 1. Correlation coefficients between phenolic compounds and berry weight loss.

	Polyphenols	**Tannins**	**Anthocyanins**
Tannins	**0.946*** **		
Anthocyanins	**0.891*** **	**0.988*** **	
Weight loss (%)	**0.334***	0.301	0.287

** = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.*

2.5 Discussion

All cultivars analysed showed an increasing trend in weight loss during storage. Weight loss increased significantly during storage (0-21 days), whereas a slight increase was observed after shelf-life. This trend was clear for berries stored at 2 and 16 °C, whereas for those stored at 10 °C a significant increase was observed even after shelf-life. This may be due to the higher residual hydration of berries treated at 10 °C, as they showed a lower rate of weight loss during storage than berries treated at 2 or 16 °C. A rise in weight loss was previously reported by Fadda et al., (2017), in berries stored at 2 °C for 30 days. Similarly, Angioni et al. (2011) demonstrated an increase in weight loss in berries stored at 2 °C for three months. Despite the similar increasing trend, the weight loss rates reported by these authors were lower than those found in this work. In the same way, 'Daniela' and 'Barbara' cultivars stored at 10 °C for 15 days at 90% relative humidity (Mulas et al., 2013), exhibited slightly less weight loss than the berries of the present study stored for 14 days at 10 °C. These differences may be due to a varietal factor, or to the storage conditions applied. This may be attributed to the fact that, in addition to temperature, relative humidity conditions were controlled, which may have reduced berry transpiration. A similar argument can be made for berries of the 'Daniela' and 'Barbara' cultivars stored at 10 °C for 15 days at 90% relative humidity in a previous experiment (Mulas et al., 2013), which exhibited slightly less weight loss than the berries of the present study stored for 14 days at 10 °C. Some varietal differences in weight loss were also observed in this work, possibly related to a different transpiration rate due to some differences in berry water content and thickness of the epicuticular wax layer.

The content of phenolic compounds in myrtle berries during storage exhibited a differentiated trend depending on the cultivar analysed and the storage conditions adopted. In pigmented berries, the phenolic compounds increased during storage and shelf life, whereas an opposite behaviour was observed in unpigmented cultivars. Fadda et al., (2017) reported for the pigmented cultivar 'Nadia', stored at a temperature of 2 °C, an increase in phenolic

compound content from the day of harvest until the 20th day of storage, followed by a decrease in the final stages of storage. A similar behaviour was observed for the pigmented cultivars 'Ilaria' and 'Maria Antonietta', which showed a significant increase in polyphenol content in berries stored at 2 °C. The other storage temperatures produced an increase in total phenols, but the rate of such increase was lower than that observed at 2 °C. The effect of temperatures on phenolic compounds was studied on other species as well (Koyama et al., 2022; Mallik and Hamilton, 2017). In three blueberry genotypes stored at 2 °C, Mallik and Hamilton (2017) showed an increase in total phenol content from the 14th day of storage. The synthesis of phenolic compounds during low temperature storage may be the fruit's response to cold stress through the activation of the phenylalanine ammonialyase pathway (Maghoumi et al., 2023).

In the work of Mulas et al., (2013), the storage of the berries at 10 °C did not significantly influence the polyphenol content. This result was also confirmed for the cultivar 'Caterina'. Conversely, for the other unpigmented cultivar 'Grazia' a decrease in phenolic content was observed during the storage period.

In the previously cited study, Mallik and Hamilton (2017) demonstrated that the polyphenol content in different blueberry cultivars stored at room temperature (21 °C) exhibited an increase, variably depending on the specific cultivar. This is comparable to the behaviour of myrtle berries of pigmented cultivars stored at 16 °C, whereas for the unpigmented cultivars, ‘Grazia’ exhibited a decline in polyphenol content during storage, and ‘Caterina’ showed an increase at the final date of monitoring.

The effect of storage conditions on tannin concentration depends on the cultivar. In the cultivar 'Caterina' no statistically significant differences were identified between the berries at harvest and those stored, regardless of the temperature analysed. By contrast, the cultivar 'Grazia', showed a decrease in tannin content under all storage conditions. Similarly, in myrtle berries of ‘Barbara’ cultivar stored at 10 °C for 15 days, a decrease in tannin content was observed in comparison to the harvest date when the berries were harvested 180 days after full

bloom (DAF) (Mulas et al., 2013). Conversely, no statistically significant differences were observed between berries harvested at 210 DAF and those stored for 15 days at 10 °C, similarly to the findings for the cultivar 'Daniela' in both sampling dates (Mulas et al., 2013).

The pigmented cultivars exhibited divergent behaviour, with an increase in tannin content observed, depending on the specific cultivar and storage temperature.

The anthocyanin content in the berries of the unpigmented cultivars was negligible, as previously reported (Maghoumi et al., 2023; Medda et al., 2020). In contrast, the pigmented berries of both cultivars exhibited an increase in anthocyanin content at all storage temperatures, with the cultivar 'Ilaria' showing the most significant increases. Fadda et al., (2017) reported that myrtle berries stored at 2 °C exhibited an increase in anthocyanin content after 20 and 30 days of storage. Conversely, Mulas et al., (2013) observed a decline in anthocyanin content in two myrtle cultivars harvested 180 days after flowering when stored at 10 °C for 15 days. However, berries harvested at earlier times exhibited an increase in anthocyanin content, suggesting that if the accumulation of these compounds is still at an early stage, their synthesis may continue even after harvest.

The correlation between the content of total polyphenols, tannins and anthocyanins were high ($r = 0.891–0.988$; $p < 0.001$). This finding aligns with previous studies that have reported a strong correlation between these compounds in different blueberry cultivars (Connor et al., 2002; Mallik and Hamilton, 2017). However, no significant correlation was observed between fruit weight loss during storage and the polyphenolic compounds analysed, except for a weak correlation between weight loss and total polyphenols ($r = 0.334$, $p < 0.05$). Similarly, Connor et al., (2002) found no correlation between blueberry weight loss and phenol and anthocyanin content in fruit stored at 5 °C for 7 weeks.

2.6 Conclusions

This study showed that storage at 10 °C was the most effective condition for limiting weight loss in myrtle berries, whereas storage at 2 °C and 16 °C resulted in greater dehydration, with the highest

values observed in the cultivar ‘Ilaria’. Phenolic compounds – including polyphenols, tannins, and anthocyanins – increased consistently during storage in the pigmented cultivars ‘Ilaria’ and ‘Maria Antonietta’, while unpigmented cultivars showed a more variable or declining trend, confirming that the biochemical response to post-harvest conditions is strongly genotype-dependent. The absence of a significant correlation between weight loss and phenolic accumulation indicates that the observed increases reflect an active de novo biosynthesis, likely associated with the activation of the phenylalanine ammonia-lyase pathway in response to cold stress, rather than a simple concentration effect. The strong intercorrelations among phenolic classes further support a coordinated regulation of the phenylpropanoid pathway during post-harvest storage.

From a practical standpoint, storage at 2 °C may represent a suitable strategy to maximise the accumulation of bioactive compounds in pigmented cultivars intended for pharmaceutical and cosmetic applications, whereas 10 °C appears to be the most suitable condition for preserving berry quality during storage for liqueur production. Future research should focus on the molecular mechanisms underlying cultivar-specific responses to post-harvest temperatures and on the potential of modified atmosphere packaging to further improve the post-harvest management and functional quality of myrtle berries.

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3. Chapter 2: Phenotypic characterisation of two myrtle cultivars through the assessment of peculiar specialized metabolites (flavonol and anthocyanin) content via high-performance liquid chromatography coupled with Diode Array detection (HPLC/UV-Vis-DAD).

3.1 Abstract

Bioactive compounds in food, particularly anthocyanins and flavonols in myrtle (Myrtus communis L.) berries, have gained scientific interest due to their health-promoting properties. While pigmented varieties are well-documented, the phenolic profile of unpigmented cultivars remains less explored. This study aimed to evaluate and compare the phenolic composition of two myrtle cultivars – one pigmented ('Maria Antonietta') and one unpigmented ('Caterina') – at different stages of ripening to assess their potential for food, pharmaceutical, and nutraceutical applications. Berries were sampled monthly from fruit set to full ripening over two consecutive seasons (2021 and 2022). Phenolic compounds were identified and quantified using HPLC-DAD and HPLC-ESI-MS. A three-way analysis of variance (ANOVA) was conducted to assess the effects of cultivar, harvest month, and year. Nineteen specialized metabolites, including nine anthocyanins, nine flavonols, and ellagic acid, were identified. Cultivar, month, and year significantly affected phenolic accumulation ($p < 0.001$). Anthocyanins, predominantly glucoside derivatives of malvidin, delphinidin, and petunidin, were detected almost exclusively in the pigmented cultivar, peaking at full maturity (up to 6682.80 μg/g FW in 2021). Conversely, total flavonols decreased progressively form July to December in both cultivars, though the unpigmented 'Caterina' exhibited a more pronounced reduction (up to 82.5%) compared to 'Maria Antonietta'. Phenolic accumulation is highly dependent on genotype, harvest time, and climatic variations. Pigmented myrtle berries are excellent sources of anthocyanins at maturity, whereas unpigmented cultivars are rich in flavonols early in their development, highlighting the distinct nutraceutical potential of both cultivars.

3.2 Introduction

In recent decades, there has been a notable increase in scientific interest in bioactive compounds in food, largely due to the substantial evidence of their beneficial effects on human health (Jimenez-Garcia et al., 2013; Morales, 2025).

While bioactive compounds such as simple phenols, flavonoids, anthocyanins, terpenes, and alkaloids are not directly involved in primary metabolic processes essential for plant growth and reproduction, they play crucial protective roles by conferring resistance against both environmental and biotic stressors (Zhuang et al., 2023). For humans, these compounds have been demonstrated to be beneficial in the prevention of several chronic diseases, including cardiovascular disease, type 2 diabetes, obesity and certain forms of cancer (Mattioli et al., 2020; Saxena et al., 2012). In particular, polyphenols found in fruit, vegetables and medicinal plants have been associated with powerful antioxidant activities (Stagos, 2019; Swallah et al., 2020), as they are capable of neutralising free radicals and reducing oxidative stress, which is a key mechanism underlying the development of ageing and degenerative disease processes (Rao and Zheng, 2025; Zagoskina et al., 2023).

Among polyphenols, particular interest has been directed towards flavonoids and anthocyanins, which are extensively found in dark-coloured berries and fruit. Flavonoids constitute a large family of plant secondary (specialized) metabolites, which include subfamilies such as flavanones, flavonols, flavones, catechins and anthocyanins (Santos et al., 2017). Each of these subfamilies plays a specific role in modulating inflammatory and immune responses. Anthocyanins, responsible for the red, blue and purple pigmentation of a wide range of fruits, are a significant source of dietary antioxidants (Mattioli et al., 2020).

Myrtle (*Myrtus communis* L.) is an evergreen plant typical of the Mediterranean region, with a history of use in both gastronomy and traditional medicine (Mulas, 2012a). In addition to being used to produce liqueurs and flavourings, the berries are a valuable source of bioactive compounds with numerous health-promoting properties. Among the main groups of compounds

identified in myrtle berries are polyphenols, particularly anthocyanins and flavonols, which have been demonstrated to possess antioxidant, anti-inflammatory and antimicrobial activity (Correddu et al., 2019; Cruciani et al., 2019; Tuberoso et al., 2010).

The anthocyanins are among the most extensively studied components in myrtle berries. They belong to the flavonoid group and include cyanidin-3-O-glucoside, delphinidin-3-O-glucoside and malvidin-3-O-glucoside, the beneficial effects of which have been widely documented (Mattioli et al., 2020; Scorrano et al., 2017). The pigmented varieties of myrtle, characterised by an intense purplish-red colour, are particularly rich in these anthocyanins, which contribute not only to the colour but also to the fruit's antioxidant capacity (Barboni et al., 2010; Messaoud and Boussaid, 2011).

In addition to anthocyanins, myrtle berries contain a wide range of flavonols (mainly quercetin and myricetin) and flavan-3-ols catechins. Previous studies have demonstrated that flavonols play a significant role in reducing the risk of inflammatory and degenerative diseases through their capacity to modulate biochemical pathways associated with oxidative stress (Babou et al., 2016; Roy et al., 2022). Other compounds have been identified, including tannins, phenolic acids (such as gallic acid) and essential oils that contribute to the aromatic profile of myrtle and may possess antimicrobial properties (Franco et al., 2019; Petretto et al., 2016).

Despite the rich chemical composition of myrtle berries, the less studied, unpigmented varieties display a distinct phenolic profile and lack of concentration of anthocyanins (Medda et al., 2021a). However, they can still contain notable amounts of flavonols and other polyphenols (Medda et al., 2021a; Messaoud and Boussaid, 2011). A comparison of pigmented and unpigmented varieties may therefore prove insightful in assessing their potential as sources of bioactive compounds.

In this context, high-performance liquid chromatography (HPLC) coupled with a second-generation spectrophotometric detector (DAD) is widely recognised as the technique of choice for the quantitative and qualitative analysis of anthocyanins and flavonols. The aim of

this study was to evaluate differences in phenolic composition and assess potential implications for food, pharmaceutical and nutraceutical applications by analysing the anthocyanin and flavonol content of two myrtle cultivars, one pigmented and one unpigmented, at different stages of berry ripening using HPLC/DAD.

3.3 Materials and Methods

3.3.1 Plant material

Myrtle berries were sampled from selected plants in the spontaneous flora and collected in the experimental field of the University of Sassari located in Fenosu (Central-West Sardinia, Italy: 39°53', 8°37'E, 12 m a.s.l.). The study focused on two cultivars: 'Maria Antonietta' (CUG11) with pigmented berries and ‘Caterina’ (V8) with unpigmented berries.

Sampling was conducted for two seasons (2021 and 2022) from fruit set to full ripening. Berries were hand-harvested monthly for a total of five sampling dates per year, transported to the laboratory, and stored in the freezer at -20 °C until subsequent analyses. Three replicates were taken for each sampling date.

3.3.2 Identification and quantification of phenolic compounds via HPLC/DAD

Berries were defrosted overnight in the refrigerator. The pulp, including the epicarp, was ground with a pestle and mortar. For each replicate, 1 g was weighed and placed in cylindrical glass vials with a screw cap and then 2 ml of 80% aqueous methanol acidified with formic acid (1%) was added. The solution was left in agitation overnight, and the resulting heterogeneous mixture was centrifuged at 4000 rpm for 20 minutes. The supernatant was filtered through a 0.45 μm PTFE filter and transferred into 1 ml HPLC amber vials and immediately analysed.

Chromatographic analyses were carried out on an Ultimate3000 UHPLC-focused instrument equipped with a binary high-pressure pump, a photodiode array detector, a thermostatic column compartment and an automated sample injector (Thermo Fisher Scientific, Inc., Milan, Italy). Collected data were processed through a Chromeleon Chromatography

Information Management System v. 6.80. Chromatographic runs were all carried out using a reverse-phase column (Gemini C18, 250 × 4.6 mm, 5 µm particle size, Phenomenex Italia s.r.l., Bologna, Italy) equipped with a guard column (Gemini C18 4 × 3.0 mm, 5 µm particle size, Phenomenex Italia s.r.l., Bologna, Italy). Myrtle extracts polyphenols were eluted with the following gradient of B (2.5 % formic acid in acetonitrile) in A (2.5 % formic acid in water): 0 m in. 10 % B; 20 m in. 35 % B; 25 min: 10 % B. The solvent flow rate was 1 ml/min, the temperature was kept at 25 °C, and the injector volume selected was 10 µl. DAD acquisitions were all performed according to Siracusa et al. (2019).

To unambiguously identify the chromatographic signals and/or to confirm peak assignments, a series of HPLC/ESI/MS analyses were performed on a selected number of representative samples. Chromatographic analyses were performed using the same conditions described above, while ESI mass spectra were acquired by a Thermo Scientific Exactive Plus Orbitra MS (Thermo Fisher Scientific, Inc., Milan, Italy) using a heated electrospray ionization (HESI II) interface. LC/ESI/MS settings and mass spectra acquisition were conducted according to the previous work of Siracusa et al. (2019).

All solvents and reagents used in this study were high purity laboratory solvents from VWR (Milan, Italy); HPLC grade water and acetonitrile were also obtained from VWR. High purity standars cyanidin 3-O-glucoside, rutin, chlorogenic acid, sinapic acid and p-coumaric acid were purchased from Sigma (VWR chemicals, Milan, Italy), whilst naringin, hesperidin and vitexin were from Extrasynthese (Lyoin, France).

All analyses were carried out in triplicate; the results are reported in micrograms (µg) of compound per gram of fresh weight (g FW).

3.3.3 Statistical Analysis

Statistical analyses were conducted using RStudio software. Descriptive statistics were first calculated, followed by a three-way analysis of variance (ANOVA) to assess the effects of cultivar, year, and month, as well as their interactions. When significant differences were

detected, mean separation was performed using Fisher's least significant difference (LSD) test at the 95% confidence level.

3.4 Results

This study represents the first investigation of the phenolic profile of myrtle berries at different stages of ripeness, comparing two cultivars characterised by different fruit colour ('Maria Antonietta', with pigmented berries, and 'Caterina', with unpigmented berries).

The analysis of variance revealed highly significant effects ($p < 0.001$) of year, month and cultivar on both anthocyanin and flavonol content (Table 1).

The study's results indicated that the pigmented cultivar 'Maria Antonietta' exhibited the highest anthocyanin concentration at full maturity in 2021, with a total anthocyanin content of 6680 µg/g FW (Table 2). On all sampling dates, the unpigmented cultivar exhibited anthocyanin levels that were either close to zero or undetectable.

Maximum flavonols concentrations were recorded in the 'Maria Antonietta' cultivar, with 5926 µg/g FW in berries sampled in October 2022, and in the 'Caterina' cultivar, with 5066 µg/g FW in berries sampled in August 2021.

Table 1. F values and significance levels (p-values) from the three-way analysis of variance (ANOVA), considering cultivar, year, month, and their interactions as fixed effects.

	Anthocyanins		Flavonols	
	F value		F value	
Year	51.64	***	26.23	***
Month	848.57	***	936.92	***
Cultivar	1282.32	***	320.96	***
Year:Month	32.5	***	214.56	***
Year:Cultivar	48.6	***	106.90	***
Month:Cultivar	826.82	***	71.86	***
Year:Month:Cultivar	29.98	***	71.12	***

*** = $p < 0.001$

Table 2. Content of anthocyanins and flavonols measured on berries of cultivar 'Maria Antonietta' (CUG11) and 'Caterina' (V8) in year 2021 and 2022 in five sample dates per year.

			Total anthocyanins	Total flavonols
Year	Sampling date	Cultivar	(μg/g FW)	(μg/g FW)
2021	jul	CUG11	26.63 ± 0.74 e	4214.97 ± 91.94 d
	aug		23.24 ± 0.31 e	4286.90 ± 132.15 d
	oct		33.35 ± 0.49 e	3118.94 ± 81.80 f
	nov		1301.99 ± 10.59 c	1633.24 ± 16.09 j
	dic		6682.80 ± 565.79 a	2712.34 ± 217.17 g
	jul	V8	2.36 ± 0.01 e	4203.07 ± 397.76 d
	aug		0.00 ± 0.00 e	5065.55 ± 348.67 b
	oct		0.00 ± 0.00 e	3463.47 ± 15.34 e
	nov		8.40 ± 0.53 e	1020.74 ± 148.31 k
	dic		58.70 ± 0.54 e	735.07 ± 109.64 l
2022	jul	CUG11	18.02 ± 0.16 e	4587.11 ± 1.34 c
	aug		17.21 ± 1.13 e	4160.34 ± 13.00 d
	oct		34.97 ± 3.21 e	5926.45 ± 114.52 a
	nov		829.16 ± 7.40 d	2324.18 ± 10.55 h
	dic		4521.66 ± 348.09 b	1985.63 ± 155.87 i
	jul	V8	0.00 ± 0.00 e	2217.18 ± 10.35 hi
	aug		0.00 ± 0.00 e	3487.49 ± 22.96 e
	oct		0.00 ± 0.00 e	4533.62 ± 50.38 c
	nov		14.31 ± 4.79 e	2687.70 ± 9.83 g
	dic		15.04 ± 2.30 e	543.64 ± 35.54 l
LSD			245.18	243.73

Different letters represent statistically significant differences with the Fisher's LSD test ($p \leq 0.05$).

Nineteen compounds, namely 9 anthocyanins, 9 flavonols and ellagic acid, were identified and quantified by HPLC. In detail, among the anthocyanins, three delphinidin derivatives, three petunidin derivatives, one cyanidin derivative, and two malvidin derivatives were detected (Table 3). The presence of anthocyanins was detected almost exclusively in the pigmented cultivar and, in particular in the more advanced stages of ripening, corresponding to the appearance of the characteristic dark-blue colouration of the fruit, for which these compounds are responsible.

The flavonols included five myricetin derivatives, three quercetin derivatives, and one kaempferol derivative (Table 3). The evolution during maturation of several metabolite of cultivar CUG11 and V8, in year 2021 and 2022, was reported in Table 2. During maturation, an

increase in anthocyanin content was recorded in the two years of analysis. The highest amount was observed in 2021 in the CUG11 cultivar (6682.80 µg/g) at the harvest time. Regarding the flavonols content, a decrement was detected in all cultivars, from July to December (2021 and 2022). It was noted that V8 showed a greater decrease in total flavonols content in 2021 and 2022, 82.5 and 75.5%, respectively. Instead, a decrease of 35.6 and 56.7% was observed in 2021 and 2022 in the fruits of the cultivar CUG11. Table 3 showed a comprehensive list of metabolites, with their relative quantitative ranges. Quantification was performed using calibration curves in the range 0.5–50 mg/L ($R^2 > 0.99$)

Table 3. Peak list obtained through HPLC/DAD and HPLC/ESI-MS analyses, for myrtle berries biochemical markers.

Compound code	Rt, min	Compound identification	MW
		Anthocyanins – 520 nm	
A1	5.25	delphinidin-3-O-galactoside	465.38
A2	5.69	delphinidin-3-O-glucoside	465.38
A3	6.11	petunidin-3-O-rutinoside	625.60
A4	6.52	cyanidin-3-O-glucoside	484.83
A5	7.03	petunidin-3-O-glucoside	479.40
A6	7.58	delphinidin-3-O-arabinoside	435.38
A7	8.51	malvidin-3-O-glucoside (oenin)	493.43
A8	9.38	petunidin-3-O-arabinoside	449.40
A9	10.94	malvidin-3-O-arabinoside	463.43
		Flavonols and ellagic acid – 350 nm	
F1	9.82	myricetin-3-O-rutinoside	626.38
F2	10.87	myricetin-3-O-glucoside	480.38
F3	11.59	myricetin-3-O-arabinoside	450.38
F4	12.53	myricetin-3-O-rhamnoside (myricitrin)	464.37
EA	12.93	ellagic acid	302.19
F5	13.20	quercetin-3-O-glucoside	464.32
F6	14.43	kaempferol-3-O-glucoside (astragalin)	448.38
F7	15.01	quercetin-3-O-rhamnoside	448.38
F8	15.89	myricetin	318.36
F9	19.89	quercetin	302.36

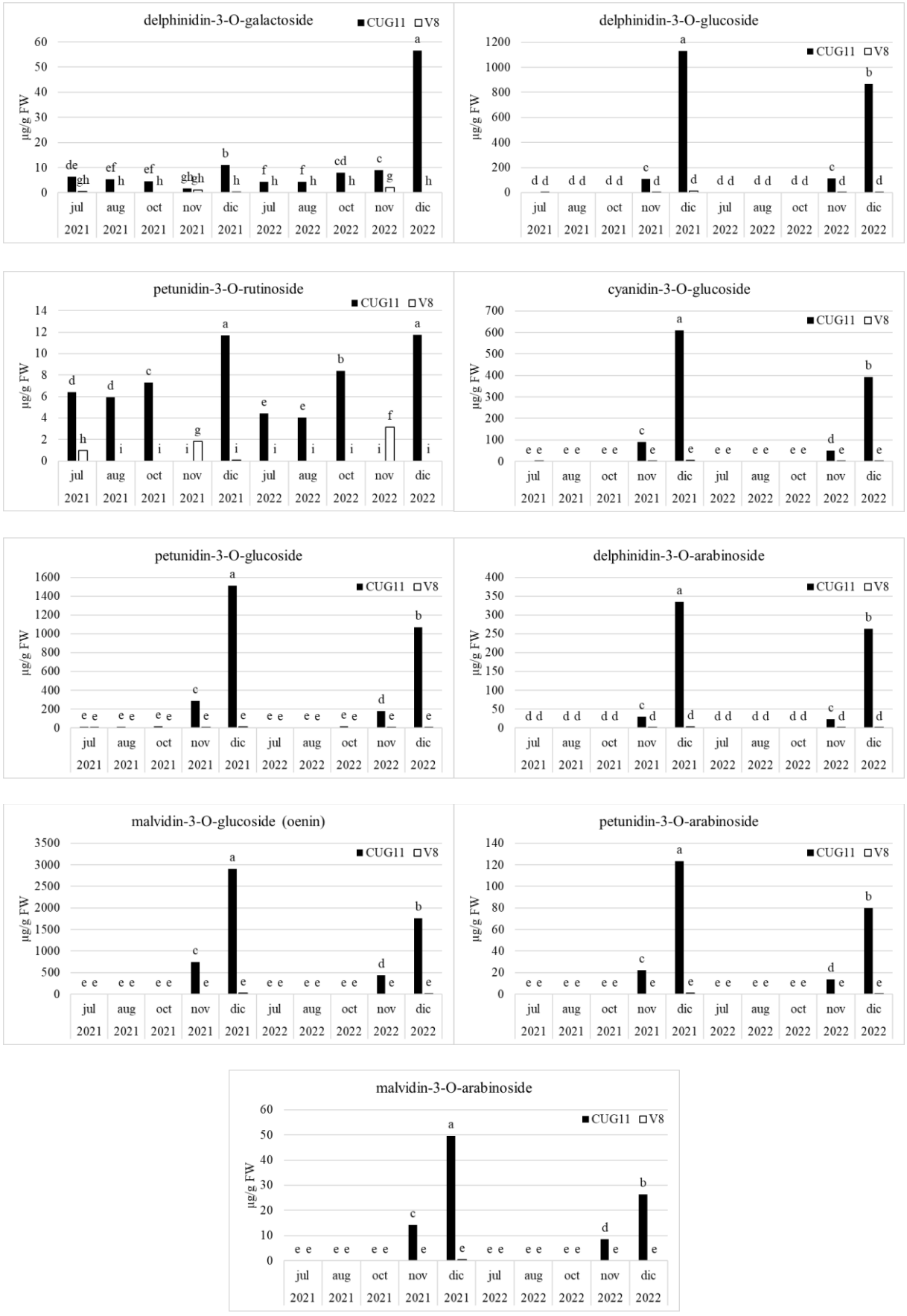

Figure 1. Evolution of anthocyanin accumulation in myrtle berries tissues for pigmented 'Maria Antonietta' cultivar (CUG11) and unpigmented 'Caterina' cultivar (V8). Data are the means of three replications and data labelled with the same letters are non-significantly different for $p \leq 0.05$.

Regarding the individual anthocyanins, a different trend was observed in the two years of analysis in all cultivars (Figure 1). It was found that the most abundant pigments were A7, A5 and A2 in both years at the harvest time. Indeed, it was noted that A7, A8 and A9 had been quantified only in November and December, while the other pigments had been detected already at the beginning of the experiment.

In all cultivars, it was observed that F2, F4 and EA were the most abundant compounds in both years at the harvest time (Figure 2). Overall, in December, the pigmented cultivar CUG11 had higher amounts of flavonols than the unpigmented cultivar V8. Generally, a reduction in individual flavonols was observed during maturation in all cultivars.

Regarding the cultivar CUG11, F7 and F3 compounds showed the largest reduction from July to December in the years 2021 and 2022 (Figure 2). Instead, F4 and F7 showed the highest decrement during maturation in cultivar V8 in both years.

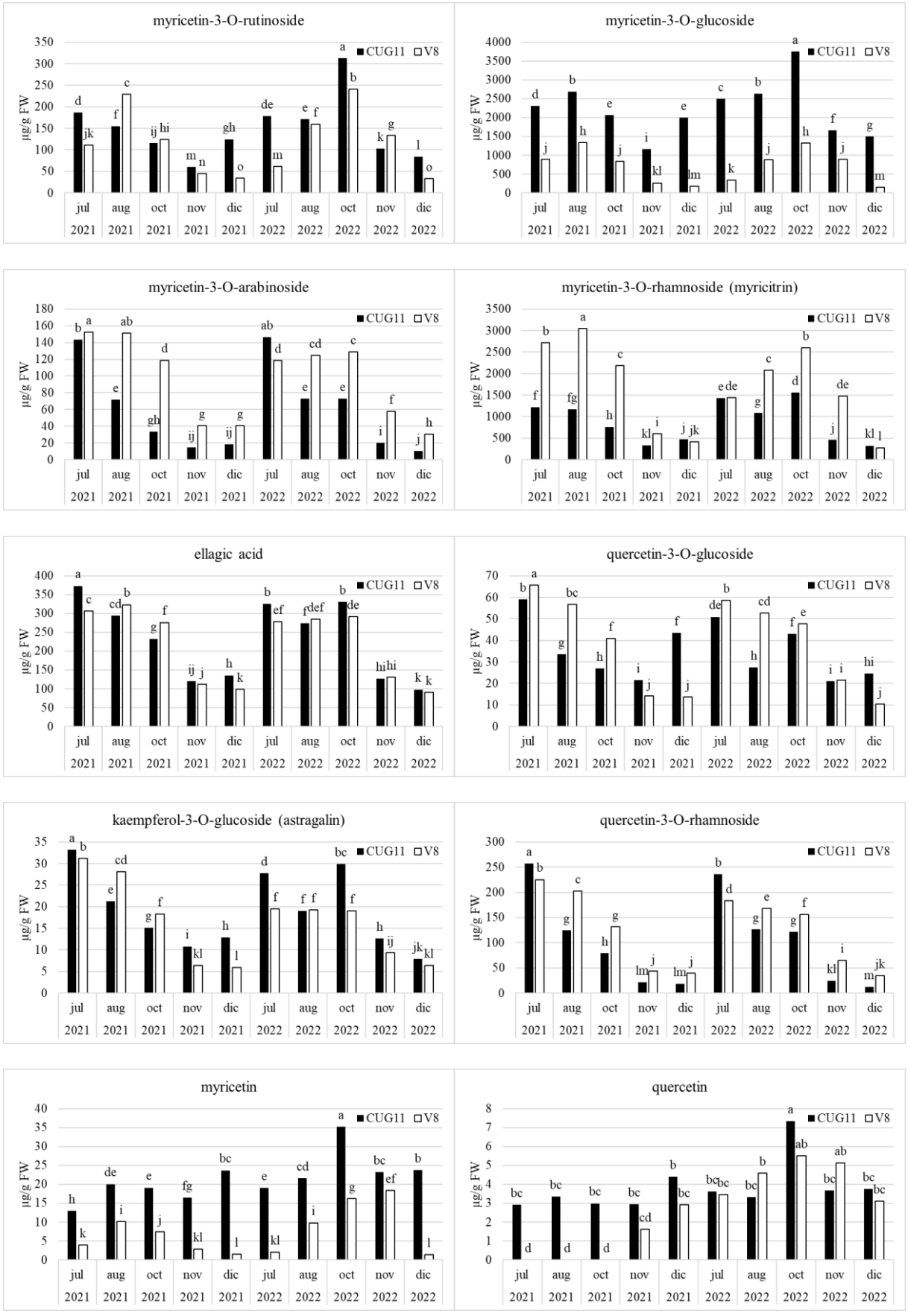

Figure 2. Trend of flavonols and ellagic acid accumulation in myrtle berries in pigmented 'Maria Antonietta' cultivar (CUG11) and unpigmented 'Caterina' cultivar (V8). Data are the means of three replications and data labelled with the same letters are non-significantly different for $p \leq 0.05$.

3.5 Discussion

The results of this study demonstrated that the phenolic composition of myrtle berries is strongly influenced by genotype, fruit developmental stage, and climatic conditions, with marked effects on anthocyanin and flavonol accumulation during ripening. Anthocyanins were detected almost exclusively in the pigmented cultivar CUG11 ('Maria Antonietta'), confirming the strong genetic control of pigment biosynthesis in myrtle berries. Glucoside derivatives represented the predominant anthocyanins in all samples, particularly malvidin-3-O-glucoside, delphinidin-3-O-glucoside, and petunidin-3-O-glucoside, in agreement with previous reports on *Myrtus communis* L. berries (Maldini et al., 2016; Scorrano et al., 2017). Anthocyanin accumulation progressively increased during ripening, especially during the final stages of maturation (November-December), coinciding with the development of the characteristic dark-blue berry pigmentation. This trend reflects the activation of the flavonoid biosynthetic pathway and the upregulation of structural genes involved in anthocyanin synthesis during fruit maturation.

Anthocyanin biosynthesis is highly dependent on climatic conditions, particularly temperature, solar radiation, and thermal fluctuations (Cheng et al., 2014; Medda et al., 2022b; Modica et al., 2025). Therefore, all pigments showed differences over the two years of analysis, and the highest anthocyanin concentration was observed in 2021 in cultivar CUG11 (6682.8 μg/g FW). Total anthocyanin content recorded in this study was higher than that previously reported for other myrtle cultivars (Fadda and Mulas, 2010; Medda et al., 2021b). Moderate temperatures and marked day–night thermal amplitudes are known to favour pigment accumulation, whereas heat stress may reduce anthocyanin biosynthesis and stability. Consequently, the higher anthocyanin levels observed in 2021 may reflect more favourable environmental conditions during the final ripening stages. In contrast, the unpigmented cultivar V8 showed negligible anthocyanin levels throughout fruit development, confirming the absence or strong repression of the anthocyanin biosynthetic pathway in this genotype.

The ANOVA analysis showed that all factors, including year, month, and cultivar, and their interactions with anthocyanins and flavonols, were highly significant. In particular, the highest F values were recorded for month and cultivar. This aspect confirmed that harvest time plays a key role in modulating phenolic metabolism during berry ripening and in the biosynthesis of several biochemical compounds. In myrtle berries, fruit maturation is characterized by important biochemical and physiological changes, including sugar accumulation, colour development, progressive anthocyanin accumulation, and modifications in the composition of other phenolic compounds (Aidi Wannes et al., 2009; Fadda and Mulas, 2010; Medda et al., 2022b). In contrast, flavonol content progressively decreased during ripening, confirming the metabolic shift occurring during the final stages of fruit development. Therefore, the identification of the optimal harvest stage is particularly important for maximizing the accumulation of specific bioactive compounds and improving the technological and nutraceutical value of the berries (Fadda and Mulas, 2010; Medda et al., 2022b).

Flavonol content measured during fruit development and ripening stages followed an opposite trend compared with anthocyanins, decreasing progressively from the early developmental stages to full ripening in both cultivars and years, in agreement with previous studies on myrtle and other berry species (Fadda and Mulas, 2010; Vvedenskaya and Vorsa, 2004). This pattern is consistent with the physiological role of flavonols in young fruits, where they act as protective compounds against UV radiation and oxidative stress. During maturation, the metabolic flux is progressively redirected toward anthocyanin biosynthesis, resulting in lower flavonol accumulation.

Among flavonols, myricetin and quercetin derivatives were the predominant compounds. In particular, myricetin-3-O-rhamnoside and quercetin-3-O-rhamnoside were the most abundant compounds in cultivar V8, confirming previous findings (Cvitković et al., 2022; Taamalli et al., 2014), whereas myricetin-3-O-arabinoside was also abundant in cultivar CUG11. Moreover, cultivar V8 exhibited a greater reduction in total flavonol content during

ripening (82.5% in 2021 and 75.5% in 2022) compared with CUG11 (35.6% and 56.7%, respectively), suggesting a more sustained flavonoid metabolism in the pigmented genotype during the later stages of fruit development.

Ellagic acid was consistently detected among the major phenolic compounds in both cultivars throughout berry development and ripening. Its persistence during maturation may be associated with the hydrolysis of ellagitannins, contributing to the accumulation of free ellagic acid in mature berries. Considering its well-known antioxidant and anti-inflammatory properties (Franco et al., 2019), the presence of ellagic acid further supports the nutraceutical relevance of both myrtle genotypes regardless of pigmentation.

3.6 Conclusions

The results of this study show that genotype, fruit ripening stage, and environmental conditions strongly influence the phenolic composition of myrtle berries. The pigmented cultivar CUG11 is characterized by high anthocyanin accumulation at full maturity, while the unpigmented cultivar V8 shows higher flavonol levels, particularly at early developmental stages.

Overall, anthocyanins increased during ripening, whereas flavonols decreased, indicating a clear shift in flavonoid metabolism during fruit development. These contrasting patterns highlight the different phytochemical profiles of the two cultivars and their complementary nutritional value.

Harvest timing is therefore crucial to optimize the recovery of specific phenolic compounds. Early harvest stages are more suitable for flavonol preservation, while late harvest maximizes anthocyanin content and antioxidant potential in pigmented berries. These findings may support the targeted use of myrtle berries in food and nutraceutical applications.

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4. Chapter 3: Expression analysis of flavonoid-related genes in pigmented and unpigmented myrtle berries.

4.1 Abstract

Flavonoids are key secondary metabolites involved in fruit pigmentation, antioxidant activity and stress protection. In myrtle (*Myrtus communis* L.), the molecular mechanisms underlying the difference between pigmented and unpigmented berries remain only partially understood. This study investigated the expression of eight structural genes involved in flavonoid biosynthesis, namely PAL, CHS, CHI, DFR, FLS, LDOX, UFGT and LAR, in two cultivars with contrasting berry pigmentation, 'Maria Antonietta' (pigmented) and 'Caterina' (unpigmented). Gene expression was analysed by RT-qPCR during fruit development through two complementary approaches: a comparative analysis of three ripening stages in both cultivars and a seasonal analysis of the pigmented cultivar across five developmental stages in two growing seasons. The results showed marked genotype-dependent differences, with the pigmented cultivar exhibiting higher expression of PAL and of the late anthocyanin biosynthetic genes DFR, LDOX and UFGT, particularly during early stages of berry development. In contrast, the unpigmented cultivar showed higher or delayed expression of some early-pathway genes, such as CHS and CHI. Across two seasons, most genes displayed a similar developmental trend, but transcript abundance was generally higher in 2021 than in 2022, suggesting a seasonal influence on pathway regulation. Overall, the data indicate that berry pigmentation in myrtle is associated with coordinated transcriptional regulation of the phenylpropanoid and anthocyanin branches and provide new insights into the developmental and genotypic control of flavonoid biosynthesis in this Mediterranean species.

4.2 Introduction

Flavonoids represent a substantial and significant class of secondary metabolites in plants. These compounds are distinguished by a carbon skeleton comprising a hexagonal ring

of six carbon atoms, linked to three additional carbon atoms, and terminating with a sixth carbon atom (Mao et al., 2025). Within this classification, several subclasses have been identified, including flavones, flavonols, flavanones, anthocyanins, and proanthocyanidins. These compounds play fundamental roles in plant physiology, contributing to tissue pigmentation, protection against UV radiation and oxidative stress, and defence against pathogens and herbivores (Falcone Ferreyra et al., 2012; Gutierrez et al., 2017; Liu et al., 2021). From a nutraceutical perspective, flavonoids are widely recognised for their antioxidant, anti-inflammatory, anticancer, antiviral, antibacterial, cardioprotective, and neuroprotective properties (Baldi et al., 2023; Falcone Ferreyra et al., 2012; Santos et al., 2017).

Myrtle (*Myrtus communis* L.) is an evergreen shrub that is characteristic of Mediterranean scrubland and belongs to the *Myrtaceae* family. It is distributed extensively along the Mediterranean coastline and is particularly representative of the Sardinian flora, where it plays an important ecological, cultural and economic role. The berries, which have a long history of use in the production of liqueurs and herbal preparations, are a natural source of phenolic compounds, including flavonoids and anthocyanins, which are responsible for pigmentation and antioxidant activity (Medda et al., 2020; Mulas, 2012a).

Several phytochemical studies have reported significant variability in the content and composition of secondary metabolites in myrtle, attributable to genetic differences, environmental conditions, and fruit ripening stage (Medda et al., 2021a; Petretto et al., 2016). It has been demonstrated that pigmented genotypes exhibit higher levels of phenolic compounds in comparison to unpigmented ones (Bakar et al., 2021; Özcan et al., 2020).

This phenotypic and metabolic diversity represents a valuable model for studying the molecular mechanisms underlying flavonoid biosynthesis, especially the transcriptional control regulating differential pigment accumulation between pigmented and unpigmented varieties.

Flavonoid biosynthesis originates from the shikimic acid pathway, which provides the aromatic precursors for the formation of phenolic acids and compounds of the phenylpropanoid

pathway. The key enzyme, phenylalanine ammonia-lyase (PAL), catalyses the conversion of phenylalanine into cinnamic acid, representing the initial step in the phenylpropanoid pathway. Subsequently, cinnamic acid is converted to p-coumaroyl-CoA through a series of reactions catalysed by cinnamate 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL). At this point, p-coumaroyl-CoA enters the flavonoid-specific pathway, where chalcone synthase (CHS) condenses three units of malonyl-CoA with one of p-coumaroyl-CoA to form naringenin chalcone. Subsequent isomerisation to naringenin, catalysed by chalcone isomerase (CHI), represents the branching point towards the different flavonoid subclasses, including flavones, flavonols, anthocyanins, and proanthocyanidins. Flavanone 3-hydroxylase (F3H) catalyses the conversion of naringenin into dihydroflavonols, which undergo further modification by flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H), determining the structural diversification of pigments. Subsequently, dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) are responsible for the formation of anthocyanidins, which are stabilized as coloured glycosides through the action of UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT) (Gutierrez et al., 2017; Liu et al., 2021; Zhuang et al., 2023; Zifkin et al., 2012). This process is a key step for anthocyanin accumulation within vacuoles.

Research conducted on various fruit species has demonstrated that there is tight regulation of the genes involved in anthocyanin biosynthesis during the development of fruit, with the expression of these genes also being modulated by environmental factors such as light intensity, temperature and water availability. In strawberries (*Fragaria* × *ananassa*), the application of red/blue (RB) and UVB light have been demonstrated to result in a substantial increase in the levels of flavonoids and anthocyanins. This response is associated with differential expression of structural genes and transcription factors that are implicated in the biosynthesis of anthocyanins (Chen et al., 2024). In apple trees (*Malus domestica*), Baldi et al. (2023) demonstrated organ-specific regulation of the enzymatic isoforms of the

phenylpropanoid/flavonoid pathway, highlighting differences in gene expression between different tissues during fruit development.

Transcriptomic studies on blueberry (*Vaccinium corymbosum*) have demonstrated that the biosynthesis of anthocyanins is subject to regulation by a coordinated transcriptional control of the flavonoid pathway in its entirety. This results in an increased activity of structural genes coinciding with the accumulation of anthocyanins during the process of ripening (Zifkin et al., 2012).

Within the *Rubus* genus, numerous studies have delineated the temporal and spatial regulation of key genes implicated in flavonoid biosynthesis. In particular, Chen et al. (2012) isolated and analysed the expression of genes involved in the anthocyanin and proanthocyanidin branches, such as RuCHS, RuDFR, RuANS, RuANR, RuLAR and a glucosyltransferase (RuGT), in addition to the transcriptional factor RuMYB10. The results show that anthocyanin accumulation during ripening is correlated with the up-regulation of RuANS and RuMYB10, while RuLAR and RuANR follow the proanthocyanidin profile. As demonstrated by Gutierrez et al. (2017), a metabolic flow of flavonoids from leaves to ripe fruits was also observed, in addition to the coordination of transcriptional regulation. Huang et al. (2023) confirmed that in blackberry fruits and blackberry-raspberry hybrids, the expression of the main genes involved in the flavonoid and anthocyanin pathways (RuPAL, RuFLS, RuANS, RuLAR, RuUFGT) varies during fruit development and between different cultivars, highlighting genotype-specific differences in expression patterns during ripening.

In the case of myrtle (*Myrtus communis* L.), however, knowledge of the molecular regulation of the flavonoid pathway appears to be limited. Medda et al. (2021b) analysed the expression of structural genes (PAL, CHS, CHI, DFR, LDOX, FLS, ANR, LAR, UFGT) in two cultivars with pigmented and unpigmented fruit, highlighting an increase in the expression of the PAL, CHI, DFR, LDOX and UFGT genes in the pigmented cultivar. In contrast, the unpigmented cultivar exhibited an increase in CHS gene expression during the final stage of

ripening, while DFR gene expression decreased in the final stages. In addition, other studies on myrtle have reported positive correlations between PAL enzyme activity and flavonoid and anthocyanin content in pigmented fruits (Medda et al., 2020).

In view of this, the present study aims to investigate the expression of genes involved in flavonoid biosynthesis in two myrtle cultivars, one pigmented (‘Maria Antonietta’) and one unpigmented (‘Caterina’), to understand the molecular basis of differences in pigment accumulation. The work involved two complementary approaches: a first comparative analysis of gene expression in three stages of fruit ripening in a single year between the two cultivars, and a second analysis aimed at evaluating the modulation of gene expression in the pigmented cultivar in five stages of ripening in two different sampling years. The results of this study facilitate a more comprehensive understanding of the temporal and genotypic regulatory mechanisms that govern flavonoid biosynthesis in myrtle, thereby providing a novel foundation for the genetic and functional enhancement of this Mediterranean species.

4.3 Materials and methods

4.3.1 Plant material

Myrtle berries were sampled from plants selected from the wild and cultivated at the experimental field of the University of Sassari, located in Fenosu (Oristano, Italy). The study involved two cultivars: ‘Maria Antonietta’ (CUG 11), with pigmented berries, and ‘Caterina’ (V8), with unpigmented berries.

Sampling was carried out for two consecutive seasons (2021 and 2022), from fruit set to full ripeness. The berries were harvested manually at monthly intervals, for a total of five sampling dates per year, then transported to the laboratory and stored at -80 °C until analysis.

4.3.2 Total RNA extraction and cDNA synthesis

For these analyses, the peel of the berries, removed with a scalpel without the pulp, was used. The peels were crushed in liquid nitrogen using a pestle and mortar. For RNA extraction,

the protocol reported by Medda et al. (2021) was followed with some modifications. To 0.7 g of ground tissue, 12 ml extraction buffer (1 M Tris-HCl pH 9, SDS 2.5%, PVP 2%, β-mercaptoethanol 5%) was added and the samples vortexed. Then 12 ml chloroform:isoamyl alcohol (24:1) was added and centrifuged at 6000 rpm for 45 minutes. The aqueous phase (approximately 10 ml) was removed, and 1.6 ml of 2 M potassium chloride was added. The samples were left on ice for one hour and then centrifuged at 6000 rpm for 45 minutes. The supernatant was removed, and 0.1 volume of 3 M sodium acetate and 0.8 volume of cold isopropanol were added, the samples were shaken thoroughly and centrifuged at 6000 rpm for 45 minutes. The supernatant was discarded, and the pellet was washed twice with 70% ethanol. After drying, the pellet was dissolved in 1.5 ml of DEPC water and transferred to 2 ml tubes. 0.1 volume of 2 M potassium acetate was added, and the samples were left on ice for one hour, followed by centrifugation at 14000 rpm for 20 minutes.

The supernatant was collected and transferred to two separate 1.5 ml tubes to which 1 volume of 4 M lithium chloride was added. The samples were left overnight at 4 °C and then centrifuged at 14000 rpm for 20 minutes. The supernatant was discarded, and the precipitated RNA was washed twice with 70% ethanol. The pellet was allowed to dry and then was resuspended in 30 µl of DEPC water. The qualities and the quantities were evaluated using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and by gel electrophoresis (agarose 1% in TBE 10x).

The total RNA was then diluted to 25 ng/µl and cDNA synthesis was performed using the SuperScript IV VILO Master Mix (Invitrogen) according to the manufacturer's protocol. Specifically, the reaction mixture contained 4 µl of SuperScript IV VILO No RT Control and 16 µl of RNA to obtain a final reaction volume of 20 µl. The thermal cycler conditions for cDNA synthesis were: 10 min at 25 °C, 50 °C for 10 min and 85 °C for 5 min.

Primers used for RT-PCR were those described by Medda et al. (2021b) and are listed in Table 1 of the Supplementary Materials.

4.3.3 Quantitative Real-Time RT-PCR (RT-qPCR)

The relative quantification qRT-PCR was carried out using the Rotor-Gene Q thermal cycler (Qiagen). The PCR mixture, with a final volume of 20 µl, was composed of: 50 mM MgCl2, 10x NH4, 5 µM dNTPs, 50 µM SYTO-9, 0.2 units of Taq polymerase, 10 µM of each gene-specific forward and reverse primer, and 100 ng of the cDNA sample. The thermal profile applied to all PCR amplification programmes consisted of an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes.

All genes were quantified in duplicate assays. Actin was employed as the reference gene to normalize the transcriptional data, and relative expression was determined by the normalized 2^−ΔΔCt method. Results are expressed as 'fold change' with respect to the calibrator sample. Standard deviation, standard error and Tukey test were calculated by using RStudio software (version 2024.12.1.563).

4.4 Results

4.4.1 Comparative expression analysis between pigmented and unpigmented cultivars

The expression profiles of eight flavonoid biosynthetic genes were evaluated during three stages of fruit development (July, September and October) in the pigmented cultivar 'Maria Antonietta' (CUG11) and the unpigmented cultivar 'Caterina' (V8) (Figure 1). Overall, marked differences in transcript accumulation were observed between the two cultivars, particularly for genes involved in the anthocyanin biosynthetic branch.

The PAL gene showed significantly higher transcript levels in the pigmented cultivar during fruit development, with expression progressively increasing from July to October and reaching its maximum value at the final sampling stage. In contrast, PAL expression remained comparatively lower in the unpigmented cultivar throughout the ripening period.

The expression pattern of CHS differed substantially between cultivars. In ‘Maria Antonietta’, CHS transcripts were abundant at the earliest developmental stage (July) but rapidly declined thereafter. Conversely, ‘Caterina’ exhibited the highest CHS expression in September, followed by a sharp decrease at full ripeness.

A similar cultivar-dependent pattern was observed for CHI. The pigmented cultivar showed very high transcript accumulation during July, whereas expression decreased markedly during the subsequent stages. In contrast, the unpigmented cultivar exhibited its highest CHI expression in September, with transcript levels exceeding those detected in CUG11 at the same stage.

Genes directly associated with anthocyanin biosynthesis, including DFR, LDOX, and UFGT, displayed higher expression in the pigmented cultivar, particularly during the early stages of fruit development. DFR expression reached its highest level in July in ‘Maria Antonietta’ and progressively declined during ripening. Similarly, LDOX and UFGT showed strong transcript accumulation in July in the pigmented berries, whereas expression remained substantially lower in ‘Caterina’.

The FLS gene exhibited a pronounced expression peak in July in the pigmented cultivar, followed by a strong reduction during fruit maturation. In the unpigmented cultivar, FLS transcripts were comparatively low in all sampling dates.

The expression of LAR, a key gene involved in proanthocyanidin biosynthesis, was highest during the earliest developmental stage in both cultivars and progressively decreased throughout ripening, with no statistically significant differences detected between ‘Maria Antonietta’ and ‘Caterina’ throughout fruit development.

Overall, the pigmented cultivar was characterized by enhanced expression of PAL, DFR, FLS, LDOX and UFGT, whereas the unpigmented cultivar showed relatively higher expression of CHS and CHI during intermediate developmental stages.

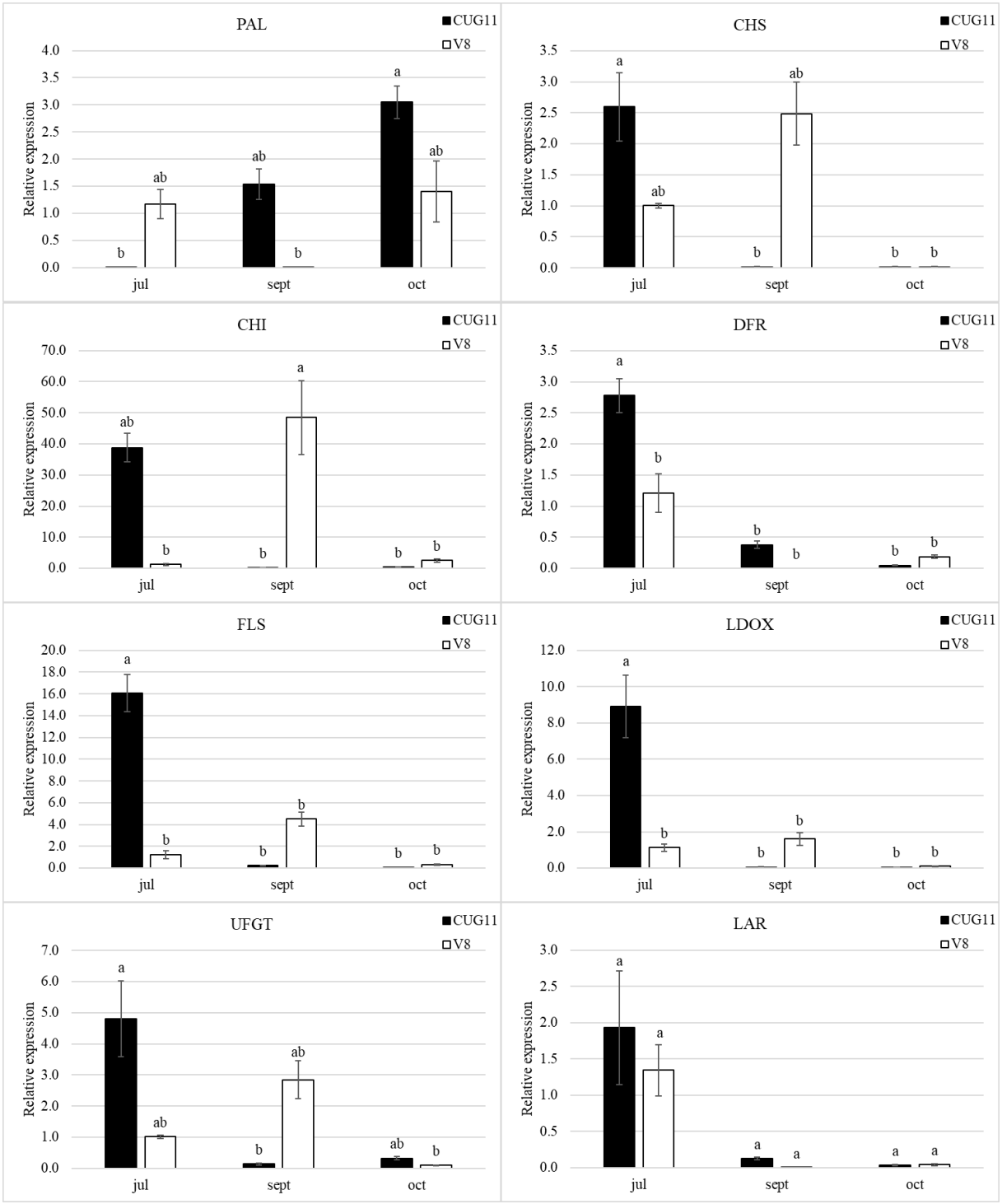

Figure 1. Expression analysis of PAL, CHS, CHI, DFR, FLS, LDOX, UFGT and, LAR during three stages of pigmented 'Maria Antonietta' (CUG11) and unpigmented 'Caterina' (V8) myrtle fruit development. Transcript levels of each gene were assessed by RT-qPCR and normalized using actin as reference gene. Results were calculated relative to a calibrator sample (jul 2021 from unpigmented samples) using the formula 2-ΔΔCt. Error bars represent standard error of three biological replicates. Different letters represent statistically significant differences with the Tukey B-test ($p < 0.05$). The Tukey B-test was carried out comparing all means data detectable at the different maturation stages for both the cultivars.

4.4.2 Gene expression dynamics during fruit development in the pigmented cultivar across two seasons

The expression profiles of the same flavonoid biosynthetic genes were further investigated in the pigmented cultivar ‘Maria Antonietta’ during five developmental stages over two consecutive growing seasons (2021 and 2022) (Figure 2).

The PAL gene showed a progressive increase in transcript abundance during fruit maturation in both years. Expression remained relatively low during the early developmental stages and increased markedly towards ripening, reaching maximum values in December. Although the overall pattern was similar in both seasons, transcript levels were generally higher in 2021 than in 2022.

In contrast, CHS expression was strongly concentrated in the earliest sampling stage. High transcript levels were detected in July 2021, whereas expression remained close to baseline values throughout the remainder of fruit development and during the entire 2022 season.

The genes CHI, DFR, FLS, LDOX, UFGT, and LAR exhibited a similar temporal trend. For all these genes, transcript accumulation was highest during the earliest developmental stage (July), followed by a rapid decline and maintenance of very low expression levels during the subsequent stages of fruit development.

Among these genes, UFGT displayed particularly elevated expression during July 2021, while considerably lower transcript levels were detected in the corresponding stage of 2022. Likewise, DFR, FLS, LDOX and LAR showed higher expression in 2021 compared with 2022, although both seasons shared the same general temporal pattern.

Overall, most flavonoid biosynthetic genes exhibited maximum expression during the initial stages of fruit development, whereas PAL represented the only gene showing a progressive increase during ripening. Furthermore, transcript abundance was generally higher in 2021 than in 2022, suggesting a seasonal effect on the regulation of flavonoid-related genes.

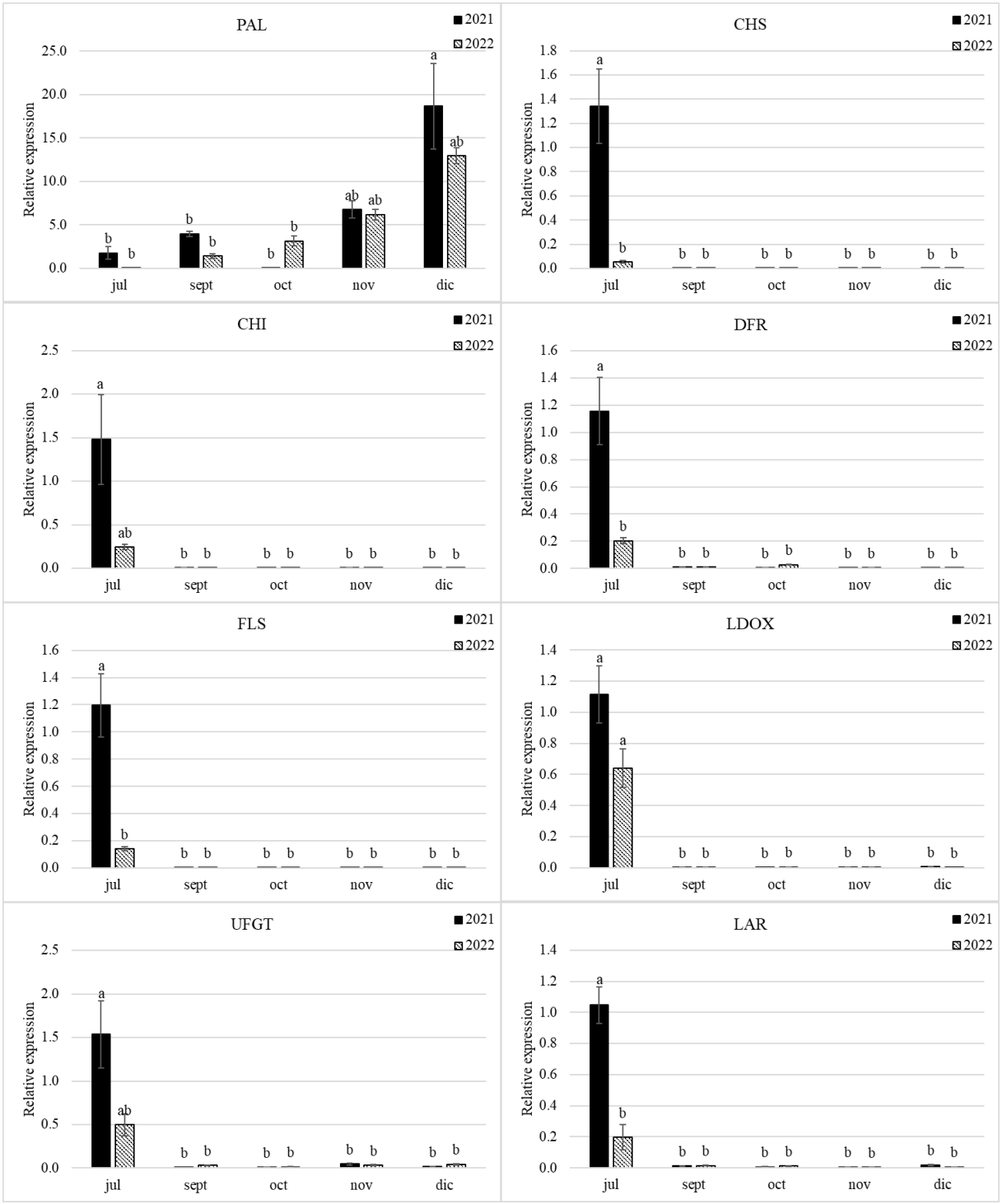

Figure 2. Expression analysis of PAL, CHS, CHI, DFR, FLS, LDOX, UFGT and, LAR during five stages of pigmented 'Maria Antonietta' myrtle fruit development in two years (2021 and 2022). Transcript levels of each gene were assessed by RT-qPCR and normalized using actin as reference gene. Results were calculated relative to a calibrator sample (jul 2021 from pigmented samples) using the formula 2-ΔΔCt. Error bars represent standard error of three biological replicates. Different letters represent statistically significant differences with the Tukey B-test ($p < 0.05$). The Tukey B-test was carried out comparing all means data detectable at the different maturation stages for both the years.

4.5 Discussion

The present study investigated the transcriptional behaviour of key structural genes involved in flavonoid biosynthesis in myrtle berries through two complementary approaches: a comparison between the pigmented cultivar ‘Maria Antonietta’ and the unpigmented cultivar ‘Caterina’, and a temporal analysis of the pigmented cultivar across two growing seasons. Overall, the results indicate that flavonoid accumulation in myrtle is strongly influenced by transcriptional regulation and that important differences exist between genotypes. This interpretation is consistent with current models of flavonoid biosynthesis, in which coordinated regulation of structural genes contributes to the relative accumulation of anthocyanins, flavonols and proanthocyanidins through the branching flavonoid pathway (Falcone Ferreyra et al., 2012; Liu et al., 2021; Mao et al., 2025).

The comparison between the pigmented cultivar ‘Maria Antonietta’ and the unpigmented cultivar ‘Caterina’ highlighted the strongest differences for PAL and for the late anthocyanin biosynthetic genes DFR, LDOX and UFGT. In the pigmented genotype, PAL showed a progressive increase during berry development, reaching the highest expression at the latest stage analysed, whereas its expression remained lower in the unpigmented cultivar. The higher PAL expression observed in the pigmented cultivar is consistent with the central role of this enzyme in catalysing the entry step into the phenylpropanoid pathway and providing precursors for downstream phenylpropanoid and flavonoid biosynthesis (Liu et al., 2021; Zhuang et al., 2023). Together with the enhanced expression of DFR, LDOX and UFGT, this pattern is consistent with the coordinated activation of genes involved in different steps of the flavonoid pathway during berry ripening. In myrtle, this interpretation is supported by previous reports showing that PAL activity correlates positively with anthocyanin and total polyphenol accumulation during maturation (Medda et al., 2020). The higher PAL expression observed in ‘Maria Antonietta’ is therefore consistent with enhanced phenolic metabolism in pigmented berries (Medda et al., 2020; Medda et al., 2021b).

The most informative cultivar-dependent differences concerned DFR, LDOX and UFGT. These genes are classically regarded as key components of anthocyanin formation because they mediate the conversion of dihydroflavonols into anthocyanidins and the subsequent glycosylation, a process that contributes to anthocyanin stability and accumulation in vacuoles (Liu et al., 2021; Zhuang et al., 2023). In the pigmented cultivar, their transcript abundance was markedly higher than in the unpigmented genotype, especially at the earliest stage analysed. This result agrees closely with the work of Medda et al. (2021b), who reported higher expression of PAL, CHI, DFR, LDOX and UFGT in the dark-blue myrtle cultivar compared with the white one, together with a positive association between gene expression and anthocyanin accumulation. The concordance between the two studies supports the hypothesis that pigmentation in myrtle is mainly associated with enhanced expression of late anthocyanin biosynthetic genes rather than with a generalized upregulation of the entire flavonoid pathway (Medda et al., 2021b).

The interpretation of CHS and CHI expression patterns is less straightforward. These genes encode early enzymes of the flavonoid pathway and therefore do not exclusively predict anthocyanin accumulation, since the intermediates generated downstream of their activity can contribute to multiple branches, including flavonols and proanthocyanidins (Falcone Ferreyra et al., 2012; Liu et al., 2021). In the present study, CHS showed higher expression in the pigmented cultivar at the earliest stage, whereas the unpigmented cultivar displayed a more evident peak at an intermediate stage; CHI also showed a cultivar-specific temporal pattern, with higher early expression in ‘Maria Antonietta’ and a later maximum in ‘Caterina’. Similar behaviour has been reported in blackberry, where early and shared-pathway genes may peak before the visible phase of pigmentation and then decline as fruit maturation progresses, whereas late anthocyanin biosynthetic genes show a stronger association with pigmented fruit (Chen et al., 2012; Zifkin et al., 2012). Accordingly, the elevated CHS and CHI transcripts detected in the unpigmented cultivar should not be interpreted alone as evidence of increased

anthocyanin synthesis, but rather as an indication that activation of upstream flavonoid genes alone is not sufficient to promote anthocyanin accumulation, which likely depends on the regulation of downstream branch-specific steps (Chen et al., 2012; Medda et al., 2021b).

This interpretation is further supported by the expression of FLS, which shares common dihydroflavonol substrates with DFR and may influence the balance between flavonol and anthocyanin biosynthesis (Falcone Ferreyra et al., 2012; Liu et al., 2021). In the pigmented myrtle cultivar, FLS was highly expressed at the earliest stage and then decreased sharply during maturation. A similar increase in flavonol-related gene expression during early fruit development has been observed in other berry species, where flavonols play important roles during early developmental stages and in light-exposed tissues, contributing to photoprotection and antioxidant functions (Gutierrez et al., 2017; Huang et al., 2023; Santos et al., 2017). In myrtle, this pattern may reflect a relevant contribution of flavonol biosynthesis during early berry development, before anthocyanin accumulation becomes predominant at later stages. This interpretation is also compatible with phytochemical evidence indicating that pigmented myrtle berries contain substantial levels of flavonols in addition to anthocyanins (Medda et al., 2021a; Medda et al., 2021b).

The expression pattern of LAR provides additional information on pathway regulation. LAR is associated with the biosynthesis of flavan-3-ols and proanthocyanidins, and its expression has often been reported to increase during early developmental stages or in tissues with active tannin accumulation (Chen et al., 2012; Liu et al., 2021). In both myrtle cultivars, LAR expression was highest at the earliest stage and then declined progressively, with no strong genotype-dependent differences. This suggests that proanthocyanidin-related metabolism is mainly associated with early fruit development in myrtle and is unlikely to represent a major factor explaining the contrasting pigmentation between the two cultivars. Comparable temporal patterns have been reported in berry fruits, where proanthocyanidin-related genes such as LAR are more closely associated with early developmental stages, whereas anthocyanin biosynthetic

genes become predominant during later ripening (Chen et al., 2012; Zifkin et al., 2012). The absence of a pronounced cultivar effect on LAR in the present study is consistent with the idea that the main transcriptional differences between pigmented and unpigmented myrtle berries involve the anthocyanin branch rather than the proanthocyanidin branch (Medda et al., 2021b).

The temporal analysis of the pigmented cultivar across two growing seasons provides a more complete view of the developmental dynamics of flavonoid biosynthesis. Most genes analysed, including CHS, CHI, DFR, FLS, LDOX, UFGT and LAR, showed maximum expression during the earliest sampling stage, followed by a strong decline during subsequent development. This pattern is consistent with the idea that flavonoid biosynthesis is particularly active during the early phases of berry growth, when fruit tissues are undergoing intense development and flavonoids may contribute to protection against environmental stresses and oxidative challenges (Falcone Ferreyra et al., 2012; Liu et al., 2021). In several fruit crops, early activation of flavonoid genes has been associated with developmental processes and the accumulation of compounds involved in antioxidant and protective functions before the ripening-associated increase in visible pigmentation (Chen et al., 2012; Zifkin et al., 2012).

Within this developmental framework, PAL behaved differently from the other genes, showing a progressive increase until the last sampling stage in both seasons. This result is particularly relevant because it indicates that the phenylpropanoid entry point remains active throughout ripening even when most downstream flavonoid genes are reduced. A similar pattern has been observed in myrtle and other fruit systems, where PAL remains associated with the maintenance of phenolic biosynthesis during later maturation stages (Medda et al., 2020; Baldi et al., 2023). In myrtle, this pattern may reflect the continued demand for phenylpropanoid precursors during late ripening, supporting the synthesis or maintenance of phenolic compounds beyond the major anthocyanin pool. The progressive increase in PAL therefore appears to represent a broader ripening-associated phenylpropanoid response rather than a pigment-specific event (Medda et al., 2020; Liu et al., 2021).

The seasonal comparison between 2021 and 2022 suggests that environmental conditions modulate the magnitude of gene expression without substantially altering the overall temporal pattern of the pathway. Transcript levels were generally higher in 2021 than in 2022 for most genes analysed, although the developmental profile remained highly similar between years. This is consistent with the well-established sensitivity of flavonoid biosynthesis to environmental factors, including light conditions, temperature and water availability (Liu et al., 2021; Chen et al., 2024; Zhuang et al., 2023). Studies in strawberry and other berry species have shown that environmental variation can influence the expression of structural genes involved in flavonoid metabolism and consequently affect anthocyanin accumulation and fruit colour development (Chen et al., 2024; Zifkin et al., 2012). In the present study, the seasonal differences are therefore plausibly attributable to variation in meteorological conditions between the two years rather than to a change in the general developmental regulation of the pathway, although climatic parameters were not directly considered in this study. The preservation of similar expression trends across seasons suggests that myrtle berries maintain a relatively stable developmental transcriptional pattern, with environmental conditions acting mainly as quantitative modulators (Chen et al., 2024; Medda et al., 2021b).

From a broader comparative perspective, the results obtained in myrtle align with studies in other berry species. In blueberry, transcriptome analyses have shown that anthocyanin accumulation during ripening is accompanied by coordinated activation of structural genes of the flavonoid pathway, particularly those involved in late anthocyanin biosynthesis (Zifkin et al., 2012). In blackberry, accumulation of coloured pigments also coincides with increased expression of genes such as DFR, ANS/LDOX and UFGT, whereas early biosynthetic genes may peak earlier or contribute to alternative flavonoid branches (Chen et al., 2012; Gutierrez et al., 2017; Huang et al., 2023). Studies in berry fruits further highlight the importance of stage-specific and branch-specific regulation, with proanthocyanidin-related genes being more associated with early developmental phases and anthocyanin-related genes becoming more

closely linked to ripening-associated pigmentation (Chen et al., 2012; Zifkin et al., 2012). The present results place myrtle within this broader biological framework and suggest that, despite species-specific differences, similar regulatory principles may underlie flavonoid accumulation across anthocyanin-rich berries (Liu et al., 2021; Zhuang et al., 2023).

A limitation of the cultivar comparison is that the analysis was restricted to the developmental stages shared by both genotypes, since late-season samples of ‘Caterina’ were not available. This limitation should be considered when interpreting genotype-specific differences, since late maturation can substantially modify flavonoid dynamics and may reveal additional regulatory differences that were not captured here. Nevertheless, the stages shared by both cultivars already show a coherent pattern: the pigmented genotype is characterised by stronger activation of the anthocyanin branch, whereas the unpigmented genotype displays comparatively higher or delayed expression of some early pathway genes. This pattern is consistent with differential regulation of flavonoid branches rather than a simple absence of flavonoid metabolism in unpigmented berries (Medda et al., 2021b; Bakar et al., 2021; Özcan et al., 2020).

Overall, the data support a model in which myrtle berry pigmentation is associated with coordinated transcriptional regulation of the phenylpropanoid and anthocyanin branches, with PAL contributing to precursor supply and DFR, LDOX and UFGT representing the main transcriptional features associated with the pigmented phenotype. The early developmental phase appears to be critical for activation of the flavonoid network, whereas later maturation is characterised by a marked reduction of most structural genes and a sustained increase in PAL. In the pigmented cultivar, this suggests a developmental shift from broad activation of flavonoid biosynthesis in young berries toward a more selective phenylpropanoid activity during ripening, potentially linked to the maintenance of phenolic metabolism and fruit quality. Taken together, these findings expand the current understanding of flavonoid regulation in myrtle and provide a basis for future studies integrating transcriptomic, metabolomic and regulatory approaches in

this Mediterranean species (Medda et al., 2020; Medda et al., 2021b; Zifkin et al., 2012; Chen et al., 2012).

The transcriptional patterns observed in the present study are also consistent with previous biochemical evidence in myrtle indicating that differences in berry pigmentation are mainly associated with anthocyanin accumulation, while flavonols and other phenolic classes contribute to the overall antioxidant profile and may vary according to developmental stage and environmental conditions (Medda et al., 2021a; Bakar et al., 2021).

4.6 Conclusions

This study provides evidence that flavonoid biosynthesis in myrtle berries is regulated in a developmentally and genotype-dependent manner. The comparison between pigmented and unpigmented cultivars indicates that berry colour is mainly associated with the stronger activation of PAL and of the late anthocyanin genes DFR, LDOX and UFGT. The pigmented cultivar ‘Maria Antonietta’ showed a transcriptional profile consistent with enhanced anthocyanin accumulation, whereas the unpigmented cultivar ‘Caterina’ displayed a different temporal pattern, especially for CHS and CHI.

The seasonal analysis of the pigmented cultivar further showed that most flavonoid-related genes are highly expressed in early fruit development and then decline during ripening, while PAL increases progressively toward the final stages. This suggests that the phenylpropanoid pathway remains active throughout maturation, possibly supporting broader phenolic metabolism beyond pigmentation alone.

Taken together, these findings confirm that pigmentation in myrtle berries is linked to the coordinated regulation of specific structural genes in the flavonoid pathway rather than to a uniform activation of the entire network. The observed seasonal variation also indicates that environmental conditions can modulate the intensity of transcriptional responses without altering the overall developmental pattern. Future studies integrating transcriptomic,

metabolomic and regulatory analyses will be essential to further clarify the mechanisms controlling flavonoid accumulation in myrtle fruits.

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5. Chapter 4: Effects of cultivar, season, growing site, and thermal regime on leaf mineral composition in *Myrtus communis* L.

5.1 Abstract

Myrtus communis L. leaves are a valuable source of bioactive compounds and mineral elements for food, nutraceutical, and pharmaceutical applications. This study investigated the influence of cultivar, harvest season, location, and thermal regime on the mineral and chemical composition of myrtle leaves. Five cultivars ('Nadia', 'Marta', 'Daniela', 'Tonina', and 'Grazia') were monitored over two years in two Sardinian locations, Alghero and Oristano.

Statistical analysis identified season as the main factor affecting most leaf parameters. Nitrogen content was significantly higher in spring than in autumn, while phosphorus and potassium also showed marked seasonal variations. Location significantly influenced ash content, with higher values recorded in Oristano, likely reflecting differences in soil composition. Cultivar-specific variability was particularly evident for total polyphenols and chlorophylls, with 'Marta' and 'Daniela' exhibiting distinct chemical profiles. Total polyphenol concentrations ranged from 7,880 to 14,017 mg/100 g DW among the analysed samples.

Correlation analysis revealed significant relationships between mineral accumulation and thermal parameters expressed as hour-degree sums. Potassium showed strong negative correlations with heat accumulation and positive correlations with chill-hour accumulation, while phosphorus exhibited moderate but significant relationships with thermal trends. Sodium, zinc, copper, and manganese were positively associated with increasing heat accumulation.

These findings demonstrate that genotype, environmental conditions, and seasonal thermal regimes strongly influence the chemical and mineral composition of myrtle leaves. The study also highlights the potential valorisation of autumn biomass as a sustainable source of bioactive compounds for industrial and nutraceutical applications.

5.2 Introduction

Myrtle (*Myrtus communis* L.) is a wild plant that grows as a shrub or small plant, belonging to the *Myrtaceae* family and the order *Myrtales* (Mulas, 2012a). Its area of distribution includes the coastal areas of the Mediterranean and West Asia (Yildirim et al., 2015).

In Sardinia (Italy) it grows at altitudes ranging from sea level to 800 m a.s.l., preferring neutral or sub-acidic soils that have moisture availability in the substrate. Despite its sensitivity to strong winds, it is a species that exhibits remarkable adaptability to extreme conditions of exposure, cold and aridity (Mulas, 2012a).

In traditional medicine, various parts of the plant were used as a remedy for numerous diseases due to the presence of bioactive compounds in the tissues. Currently, myrtle finds applications in the pharmaceutical, cosmetic and food industries (Giampieri et al., 2020). The leaves and fruits are used to extract essential oil, with the highest yield obtained from the leaves typically occurring during the flowering period, which corresponds to the balsamic period. (Hazrati et al., 2022). In addition, myrtle can also be used as a food supplement for grazing animals due to its high nutritional value in both the leaves and fruits (Medda and Mulas, 2021).

In Sardinia, the main industrial product derived from myrtle is *"Mirto di Sardegna"*, a traditional liqueur obtained by hydroalcoholic infusion of pigmented berries or leaves, producing respectively the red and white liqueur of myrtle.

Previous studies have demonstrated that the chemical composition of *Myrtus communis* leaves is strongly influenced by environmental factors, genotype, and phenological stage, leading to significant variability in both primary and secondary metabolites (Medda et al., 2021a; Petretto et al., 2016). The leaves are particularly rich in phenolic compounds, mainly flavonoids (such as myricetin and quercetin derivatives) and hydrolysable tannins, which have been associated with potent antioxidant, antimicrobial, and anti-inflammatory activities (D'Urso et al., 2018; Pereira et al., 2017; Romani et al., 1999). These bioactive constituents,

together with essential oils (particularly rich in α-pinene, limonene, 1,8-cineole, α-terpineol and linalool), are responsible for the pharmacological potential and aromatic profile of the species (Hazrati et al., 2022; Usai et al., 2015). The by-products of *M. communis* leaves resulting from essential oil extraction contain significant levels of phenolic substances with remarkable biological activity (Abdessemed et al., 2024).

Although most research has focused on the phytochemical composition of myrtle, few studies have investigated the mineral content of the leaves and its relationship with other chemical constituents (Nedjimi, 2024; Peñuelas et al., 2001; Yildirim et al., 2015).

According to Peñuelas et al. (2001), the most abundant mineral compound in myrtle leaves was C, followed by N, Ca, and K. Yildirim et al. (2015) observed that, in the three myrtle genotypes analysed, the most abundant minerals in the leaves were potassium (K), calcium (Ca), magnesium (Mg) and phosphorus (P). In a subsequent study, Nedjimi, (2024) reported that the descending sequence of the average content of essential elements in *M. communis* was as follows: K > Fe > P > Mn > Zn > Cu > V > Cr.

A better understanding of the relationships between the mineral and phenolic composition of leaves and their respective infusions would provide valuable insights for their valorisation as functional beverages or natural antioxidant sources.

Based on these considerations, the present study aimed to characterize the chemical and mineral composition of *Myrtus communis* leaves and their infusions from five cultivars grown in two Sardinian locations. Furthermore, correlations between leaf mineral content and thermal parameters (degree hours above and below specific temperature thresholds) were investigated to assess the influence of environmental factors on leaf composition. This research contributes to a deeper understanding of how genotype and growing conditions affect the quality of myrtle raw materials intended for industrial and nutraceutical applications.

The aim of this research was to establish the basic quality parameters of myrtle leaves for their use in liqueur production and other applications. To this end, the study examined the

influence of leaf mineral composition on the chemical characteristics of the corresponding infusions. A comparative analysis was carried out on leaves harvested in spring (balsamic period) and autumn to evaluate the potential use of autumn biomass — collected simultaneously with fruits — for the development of mechanized harvesting systems. The work focused on the variability in mineral composition of myrtle leaves as affected by cultivar, season, year, and collection site, and on correlations with temperature regimes expressed as degree hours above and below specific thermal thresholds.

5.3 Materials and methods

5.3.1 Plant material

The leaves were collected from plants growing in two experimental orchards located in Alghero (40° 39' north latitude, 8° 21' east longitude, 39 m above sea level) and Oristano (39° 53' north latitude, 8° 37' east longitude, 12 m above sea level). Five cultivars were analysed: 'Nadia', 'Marta', 'Daniela' and 'Tonina' with pigmented berries and 'Grazia' with unpigmented berries.

Leaf sampling was carried out by hand for two years in two seasons: May-June, before bloom during the balsam period, and November-December, when the berries ripened.

For each cultivar, three random samples of leaves were collected and stored in plastic bags at -22 °C until the laboratory analysis or their use for infusion in a hydroalcoholic solution. The leaves were washed three times with deionised water and allowed to dry before the analysis or before they were put in the hydroalcoholic solution.

5.3.2 Leaf infusions

The infusion was dressed on a laboratory scale in conformity with the industrial recipe. Leaves of each cultivar (20 g) were put in airtight closure jars containing 96% ethanol (120 ml) and placed in the dark (in a carton) for 3 months at room temperature. The infusions were then filtered and stored to be analysed.

5.3.3 Dry matter of leaves

Dry matter was determined by weight loss after oven-drying an amount of leaf sample at 105 °C for 24 hours. The percentage of dried matter was calculated in weight percentage of the initial fresh matter. All analyses were conducted in three replicates.

5.3.4 Ashes content of leaves

One g of dried matter was put in a porcelain capsule and then burnt with an open flame until it was incinerated. Then the capsule was placed in a muffle kiln at 550 °C for 16 hours. After the cooling of the capsules, the ashes content was determined by weight of the residuals in weight percentage of the initial dry matter. All analysis were conducted in three replicates.

5.3.5 Chemical analysis

Phenolic compounds extraction

Frozen leaves (10 g) were crushed and added to 100 ml of methanol containing 0.1% of HCl for 24 h at 4 °C. After filtration through filter paper, the solution was made up to a volume of 100 ml with acidified methanol and stored in glass bottles at 4 °C until analysis.

Total polyphenols analysis

Total polyphenols content was determined by the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965). An aliquot (0.5/1 ml) of pure or properly diluted extract (depending on the expected polyphenol concentration) or hydroalcoholic infusion was placed in a 50 ml flask and 35 ml deionised water and 2.5 ml Folin-Ciocalteu reagent were added. After 3 minutes, 5 ml of 20% sodium carbonate solution was added, and the solution was incubated at 70 °C for 20 minutes to allow it to become coloured. After cooling the solution under running water, it was made up to 50 ml with deionised water. The absorbance was read at a wavelength of 750 nm with a CARY 50 Scan UV-Vis VARIAN spectrophotometer (Amsterdam, The Netherlands). The results were expressed as milligrams of pure (+)catechin

per 100 g of leaf dry weight (mg /100 g DW). For the calibration curve, increasing concentrations of a pure catechin standard were used.

Tannins analysis

Four ml of diluted leaf extract or hydroalcoholic leaf infusion, 4 ml of vanillin reagent (1% vanillin in 70% sulphuric acid) and 2 ml of 96% ethanol were placed in glass tubes and stored in the dark for 20 minutes. The absorbance was measured at 500 nm with a spectrophotometer and compared with a reference of 6 ml of 96% ethanol and 4 ml vanillin reagent. Results were expressed as mg catechin equivalent per 100 g leaf dry weight according to the calibration curve.

Chlorophyll analysis

For each replicate of each sample, 20 leaf discs of 1 cm diameter were taken, weighed and placed in screw-top plastic flasks with 10 ml of 80% acetone.

The prepared flasks were stored in a refrigerator in the dark for 48 hours, after which the extract was homogenised using a model homogeniser: Heidolph Diax 600.

The homogenised extract was filtered through filter paper and then read on a spectrophotometer at wavelengths of 664.5 nm and 647 nm for chlorophyll a and chlorophyll b respectively. For the determination of chlorophyll in the hydroalcoholic infusions of the leaves, 2 ml of the infusion was added to 10 ml of 80% acetone and the same absorbance readings were taken as for the leaves.

The chlorophyll content was calculated using the following formulas:

$$Chlorophyll\ a\ (mg/l) = 12.63 * A_{664.5} - 2.52 * A_{647}$$

$$Chlorophyll\ b\ (mg/l) = 20.47 * A_{647} - 4.73 * A_{664.5}$$

Mineral elements analysis

Macro and micro-elements

One g of dried matter (or 25 ml of infuse) was reduced to ashes. The ashes were dissolved in 4 N HCl (5 ml), filtered and added of deionised water up to 100 ml to obtain a stock solution.

The macro-nutrients were determined directly at this dilution, while micro-nutrients were determined after an opportune dilution (usually 40 times) of the stock solution. Determinations were quantified with an atomic absorption spectrophotometer equipped with a hollow cathode lamp (Perkin Elmer mod A. Analist 100). All analyses were conducted in three replicates.

Phosphorus quantitative analyses

In a 100 ml volumetric flask, 10 ml of the stock solution were added of 7.5 N sulphuric acid (5 ml), ammonium molybdate (5 ml) and hydrazine sulphate (1 ml).

After 30 minutes in a thermostatic bath at 100 °C to develop a blue colour, the solution was cooled and added of deionised water up to 100 ml. The solution absorbance was measured at 650 nm. The concentration of phosphorus was deduced from a standard curve built with solutions containing increasing concentrations of phosphorus. All analyses were conducted in three replicates.

Total nitrogen analyses

One g of dried leaves (or 25 ml of hydroalcoholic leaf infusion) was put in a tube with selenic mixture and concentrated sulphuric acid (14 ml). After digestion at 100 °C, 40% sodium hydroxide was added, the solution was distilled to release ammonia and the ammonia solubilized in 25 ml of 0.1 N sulphuric acid. Then the excess of acid was titrated with 0.1 N sodium hydroxide and the total nitrogen calculated. All analyses were conducted in three replicates.

The percentage of nitrogen in the sample was thus determined:

$$\%N = [(25 - ml_{NaOH\ 0.1\ N}) * 14 * 0.1 * 100]/1000$$

where:

25 = ml sulphuric acid 0.1 N placed;

ml NaOH 0.1N = used for titration;

14 = molecular weight of nitrogen;

0.1 = titre of soda ash used for titration and sulphuric acid used to neutralise the ammonia;

1000 = weight of sample in mg.

5.3.6 Physical analysis

Colour evaluation of hydroalcoholic leaf infusion

For colour evaluation, 1 ml of the hydroalcoholic infusion was placed in a 5 cm diameter Petri dish with a transparent lid and placed on a sheet of white paper. Measurements were carried out using a MINOLTA CR-200 tristymulus colorimeter. The definition of the colour of the infusion was obtained by means of the three-dimensional coordinates L, a, and b.

5.3.7 Statistical analysis

Data relating to the composition of myrtle leaves was analysed to identify possible correlations with recorded weather conditions. During 2021, the number of hours which air temperature was below, or above defined threshold values was calculated separately for each semester (see Tables S3-S4). Hour-degrees (HD) sums were obtained by considering temperature thresholds between 0 and 35 °C at 2.5 °C intervals, corresponding to the minimum and maximum values observed during the study period. Based on the hourly temperature database, the hours below and above each threshold were counted to estimate the cumulative HD values for the analysed periods.

The data obtained were then subjected to analysis of variance (ANOVA) using RStudio software (version 2024.12.1.563) to evaluate the effect of the main experimental factors (cultivar, location and season) using a split-split-plot factorial model. The same software was used to perform a Duncan's test for mean separation and to calculate Pearson's linear correlation coefficients between mineral elements and their concentrations as a function of HD sums.

5.4 Results

5.4.1 Meteorological data

During the two years of observation, significant climatic variations were observed at the sites. The data indicate that Alghero registered higher rainfall and lower maximum and minimum temperature values than Oristano, which recorded the highest maximum temperatures, peaking at 42.6 °C in 2021.

The weather conditions in the two growing areas in the two years of sampling are reported in Figure 1. Soil analyses were performed for both locations and are reported in Table 1 and Table 2.

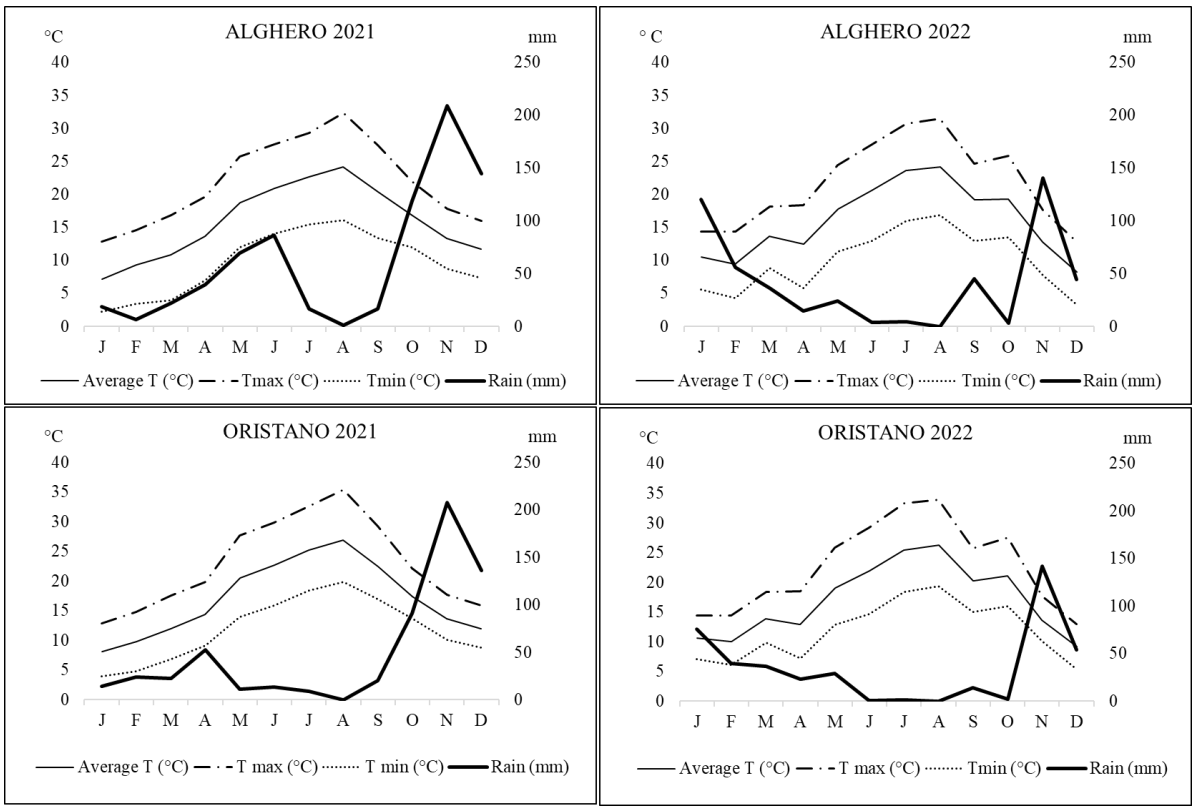

Figure 1. Monthly averages of maximum, minimum and average temperature and rainfall recorded in 2021 and 2022 at the two experimental sites.

Table 1. Summary of statistical analysis to assess the difference in soil composition between the two locations. [z]

Variables	skeleton >2 ‰	sand 2-1 ‰	sand 1-0.5 ‰	sand 0.5-0.2 ‰	coarse sand ‰	fine sand ‰	total sand ‰	silt ‰	clay ‰
Location	1.80	37.76 **	21.35 **	2.59	1.59	1.16	1.25	0.34	1.57
Depth	0.63	0.22	0.04	0.46	0.14	0.00	0.03	2.04	0.01
Location:Depth	0.96	0.83	1.58	0.33	0.01	0.02	0.00	4.13	0.16

	pH	SOM	C	N total	C/N	P_abs	K2O_abs	P2O5_abs
Location	39.67 **	1.09	1.28	14.44 **	8.51 **	8.58 **	57.21 **	9.09 **
Depth	1.47	0.00	0.00	0.06	0.59	1.43	0.66	1.45
Location:Depth	0.08	0.13	0.15	1.38	0.85	0.91	1.24	0.89

	Cu	Zn	Fe	Mn
Location	26.95 **	0.96	10.88 **	24.52 **
Depth	2.00	0.07	0.00	0.05
Location:Depth	0.94	0.03	0.31	3.16

	Ca	Mg	Na	K	∑ Exchange bases meq	Ca/Mg	Mg/K
Location	0.00	0.32	20.47 **	57.33 **	0.14	0.66	7.86 *
Depth	0.00	0.05	0.45	0.66	0.00	0.00	0.72
Location:Depth	0.00	0.27	2.93	1.25	0.04	0.34	0.33

[z]numerical values refer to the calculated F, while significance is indicated as: " " = not significant; "*" = $p \leq 0.05$; "**" = $p \leq 0.01$.

Table 2. Soil analysis in the two locations (Alghero and Oristano) sampled at two different depths (0-30 cm and 30-60 cm).[z]

Location	Depth cm	skeleton >2 ‰	sand 2-1 ‰	sand 1-0.5 ‰	sand 0.5-0.2 ‰	coarse sand ‰	fine sand ‰	total sand ‰	silt ‰	clay ‰
Alghero	0-30	34.9 a	19.3 a	35.4 a	132 a	187 a	423 a	609 a	122 a	269 a
Alghero	30-60	43.6 a	23.0 a	37.4 a	112 a	172 a	424 a	597 a	140 a	263 a
Oristano	0-30	49.0 a	66.2 b	79.1 b	87 a	232 a	462 a	684 a	129 a	187 a
Oristano	30-60	43.1 a	60.2 b	64.3 b	90 a	214 a	456 a	670 a	112 a	218 a

		pH	SOM g/kg	C g/kg	N total g/kg	C/N	P_abs ppm	K2O_abs ppm	P2O5_abs ppm
Alghero	0-30	8.09 b	18.3 a	10.6 a	0.89 a	12.3 b	17.0 b	237 a	38.8 b
Alghero	30-60	8.40 b	18.3 a	10.5 a	0.94 a	11.1 ab	19.4 b	187 a	44.2 b
Oristano	0-30	6.58 a	23.2 a	13.7 a	1.67 b	7.9 a	11.4 a	684 b	25.8 a
Oristano	30-60	6.79 a	20.8 a	12.1 a	1.38 ab	8.7 ab	11.2 a	541 b	25.5 a

		Ca ppm	Mg ppm	Na ppm	K ppm	∑ Exchange bases meq	Ca/Mg	Mg/K
Alghero	0-30	3214 a	286 a	161 a	199 a	19.6 a	7.45 a	4.70 ab
Alghero	30-60	3218 a	272 a	184 a	157 a	19.5 a	7.49 a	5.61 b
Oristano	0-30	3172 a	320 a	310 b	570 b	21.3 a	6.05 a	1.83 a
Oristano	30-60	3097 a	262 a	253 b	451 b	19.9 a	7.50 a	1.90 a

		Cu ppm	Zn ppm	Fe ppm	Mn ppm
Alghero	0-30	4.84 b	2.63 a	5.12 a	3.52 a
Alghero	30-60	3.88 b	2.35 a	4.76 a	5.32 a
Oristano	0-30	1.46 a	1.63 a	34.87 b	43.57 b
Oristano	30-60	1.39 a	1.09 a	27.37 ab	25.03 ab

[z]Values with different letters are significantly different ($p \leq 0.05$) according to Tukey's test.

5.4.2 Chemical composition of leaves and leaf infusions as influenced by cultivar, season, and locality

Statistical analysis reported in Table 3 revealed a significant influence of cultivar on the ashes, chlorophyll b and total polyphenol content ($p \leq 0.01$), while more limited effects were observed for dry weight and chlorophyll a ($p \leq 0.05$).

The season was found to be the most significant factor, with considerable differences observed for all variables examined, except for tannins and total polyphenols.

The location had a significant effect on dry weight, ashes, chlorophyll a and b, and dry weight, but did not affect tannins and total polyphenols.

The interactions between the factors demonstrated a range of responses, including an effect of cultivar x season on dry weight, ash, and total polyphenols; an effect of cultivar x location on dry weight, ash, total polyphenols, and tannins; and marked effects of season x location on ashes, chlorophylls, and tannins. Conversely, the three-way interaction (cultivar x season x location) was found to be highly significant for dry weight and total polyphenols ($p \leq 0.01$) and, to a lesser extent, for ash ($p \leq 0.05$).

Table 3. Summary of statistical analysis to assess the influence of cultivar, location, and season on the composition of myrtle leaves.[z]

Variables	Dry weight	Ashes	Chlorophyll a	Chlorophyll b	Tannins	Total polyphenols
Cultivar (A)	3.35 *	13.76 **	3.39 *	5.51 **	0.47	7.76 **
Season (B)	249.77 **	50.15 **	8.46 **	5.44 *	0.13	0.91
AxB	7.16 **	10.95 **	1.02	1.63	0.42	5.86 **
Location (C)	4.19 *	20.80 **	4.84 *	18.11 **	0.74	0.61
AxC	3.77 **	3.30 *	0.84	1.53	2.86 *	3.90 **
BxC	2.79	31.54 **	6.11 *	13.07 **	16.11 **	0.48
AxBxC	8.07 **	2.52 *	0.93	1.16	0.86	5.93 **

[z]numerical values refer to the calculated F, while significance is indicated as: " " = not significant; "*" = $p \leq 0.05$; "**" = $p \leq 0.01$.

The analysis of variance (ANOVA) presented in Table 4 was conducted to assess the influence of cultivar, season and location on the characteristics of myrtle leaf infusions. The cultivar significantly influenced the chlorophyll a content, the colorimetric parameters L and a, and the total tannin and polyphenol content. The season had a significant impact on chlorophylls, the colorimetric parameter b, and tannins, while it had no effect on L, a, and total polyphenols. The location of the cultivation had a significant effect on a ($p \leq 0.01$) and, to a lesser extent, on b and total polyphenols ($p \leq 0.05$). The interactions between the factors demonstrated a range of responses, including a highly significant effect of cultivar x season on tannins and total polyphenols, an influence of cultivar x location on all colorimetric parameters

and tannins, and an effect of season x location on b only ($p \leq 0.05$). In conclusion, the interaction between cultivar, season and location was found to be highly significant for L, tannins and total polyphenols ($p \leq 0.01$), and significant for a ($p \leq 0.05$).

Table 4. Summary of statistical analysis to assess the influence of cultivar, location, and season on the composition of myrtle leaf infusions. [z]

Variables	Chlorophyll a (mg/l)	Chlorophyll b (mg/l)	L	a	b	Tannins (mg/l)	Total polyphenols (mg/l)
Cultivar (A)	3.02 *	1.46	6.98 **	3.87 **	1.57	4.65 **	8.67 **
Season (B)	73.67 **	66.67 **	0.45	0.07	52.88 **	13.26 **	0.29
AxB	0.94	0.47	1.97	2.14	0.35	6.11 **	6.67 **
Location (C)	1.00	1.32	0.65	9.44 **	5.25 *	0.07	6.32 *
AxC	1.62	1.17	3.40 *	4.76 **	2.72 *	5.19 **	2.44
BxC	0.46	0.40	0.77	0.24	6.23 *	0.71	0.68
AxBxC	0.91	0.63	3.60 **	3.30 *	2.30	4.48 **	4.86 **

[z]numerical values refer to the calculated F, while significance is indicated as: " " = not significant; "*" = $p \leq 0.05$; "**" = $p \leq 0.01$.

The composition of myrtle leaves of the five cultivars, sampled in the two locations and during the two seasons is illustrated in Table 5. The data presented are the mean values obtained from two years of sampling.

The dry matter content demonstrates significant variation among cultivars, locations, and seasons, with values ranging from 33.64% in the 'Tonina' cultivar (spring, Alghero) to 47.02% in the 'Nadia' cultivar (winter, Oristano). In general, the leaves harvested in Oristano demonstrate higher values than those in Alghero.

The ash content exhibited a range from 3.75% to 5.67%, with the 'Nadia' cultivar harvested in Oristano during winter exhibiting the highest value.

Regarding chlorophyll content, the range of chlorophyll a was from 2.31 to 3.73 mg/gDW, while the range of chlorophyll b was from 0.79 to 2.24 mg/gDW. The highest concentrations of both pigments were observed in the spring samples from Oristano, particularly in the 'Grazia' cultivar, while the lowest values were found in the 'Daniela' cultivar.

The tannin content demonstrates significant variability between cultivars, with values ranging from 198.82 to 452.54 mg/100g DW. Among all the samples, the 'Marta' cultivar has both the highest value in the spring sampling in Alghero and the lowest in the winter sampling in the same location.

Finally, the total polyphenol range was found to be between 7880.91 mg/100g DW and 14017.80 mg/100g DW. The highest concentrations were found in the 'Marta' cultivar harvested in Alghero in spring, while the lowest values were recorded in the 'Tonina' cultivar in the same location and season.

Table 5. Average of chemical composition in leaves of *Myrtus communis* L. cultivars ('Nadia', 'Marta', 'Daniela', 'Tonina', 'Grazia') growing in two localities (AHO = Alghero, ORI = Oristano) in two seasons (S = spring, W = winter). The data reported represent the average of the two years of sampling, to obtain a representative seasonal average value for each cultivar and location. [z]

Location	Season	Cultivar	Dry weight (%)	Ashes (% DW)	Chlorophyll a (mg/gDW)	Chlorophyll b (mg/gDW)	Tannins (mg/100gDW)	Total polyphenols (mg/100gDW)
Alghero	Spring	Nadia	36.22 fg	4.59 d-g	2.92 abc	1.12 bc	315.35 ab	11848.63 ab
		Marta	37.55 def	4.21 e-h	2.45 bc	1.09 bc	452.54 a	14017.80 a
		Daniela	36.71 efg	4.74 c-g	2.41 bc	0.84 c	334.41 ab	8540.45 cd
		Tonina	33.64 g	4.66 c-g	3.12 abc	1.33 bc	294.58 ab	7880.91 d
		Grazia	35.54 fg	4.07 gh	2.43 bc	1.22 bc	329.87 ab	10490.79 bcd
	Winter	Nadia	35.93 fg	4.72 c-g	2.42 bc	1.11 bc	282.69 ab	9516.75 bcd
		Marta	36.54 efg	4.45 d-g	2.89 abc	1.15 bc	198.82 b	11309.97 bc
		Daniela	38.91 c-f	4.90 b-e	2.35 bc	0.79 c	311.65 ab	11794.51 ab
		Tonina	35.59 fg	4.10 gh	2.99 abc	1.24 bc	324.52 ab	8941.14 bcd
		Grazia	39.91 cde	3.75 h	2.84 abc	1.06 bc	306.56 ab	9085.16 bcd
Oristano	Spring	Nadia	41.23 bc	5.43 ab	3.08 abc	1.62 ab	283.99 ab	10138.57 bcd
		Marta	40.90 bcd	4.26 e-h	3.04 abc	1.68 ab	346.65 ab	10162.60 bcd
		Daniela	44.07 ab	4.14 fgh	3.00 abc	0.95 bc	247.32 b	9631.89 bcd
		Tonina	45.73 a	4.70 c-g	3.40 ab	1.40 bc	266.32 ab	9332.48 bcd
		Grazia	41.75 bc	4.23 e-h	3.73 a	2.24 a	232.00 b	11134.66 bc
	Winter	Nadia	47.02 a	5.67 a	2.72 abc	1.08 bc	373.81 ab	11865.54 ab
		Marta	40.80 bcd	5.31 abc	2.84 abc	0.94 bc	379.43 ab	9944.11 bcd
		Daniela	43.84 ab	4.82 b-f	2.31 c	0.81 c	350.07 ab	8646.27 cd
		Tonina	41.28 bc	5.09 a-d	2.86 abc	0.97 bc	373.90 ab	10287.10 bcd
		Grazia	41.47 bc	5.30 abc	3.00 abc	1.05 bc	366.58 ab	9524.21 bcd

[z]The results are expressed as means of six replicates. Values with different letters are significantly different ($p \leq 0.01$) according to Duncan's multiple range test.

Table 6 presents the composition of the infusions obtained from the myrtle leaves of the five cultivars, sampled in the two locations and during the two seasons. The data presented represent the mean values obtained from two years of sampling.

The highest values of chlorophyll a and chlorophyll b were observed in the infusions obtained from "Tonina" leaves harvested in winter in Alghero, at 29.67 and 8.35 mg/l respectively, while the lowest values were recorded in the infusions of "Daniela" with leaves harvested in spring in Oristano.

Concerning the colorimetric parameters, the highest L values were observed in the winter infusions of "Tonina" (45.69 – Oristano), those of a in "Nadia" infusions (-3.22 – spring – Oristano) and those of b in "Marta" infusions (17.42 – winter – Alghero).

Tannin content varied between 65.55 mg/l in infusions of “Tonina” and 422.83 mg/l in infusions of “Grazia,” with both sets of leaves collected in Oristano during the winter.

The highest polyphenol content in the infusions was recorded in the "Daniela" cultivar (19,141 mg/l – winter – Alghero), while the lowest was observed in "Tonina" (5,908 mg/l – winter – Oristano).

Statistical analysis reported in Table 7 revealed that the season has the strongest and most significant impact on most mineral macroelements of leaves. It significantly influences N, P, Na and K. The cultivar also influences almost all macroelements, particularly N, Ca and Mg. The location has a significant effect on N, P and Ca. However, it has no significant effect on Na, K, and Mg. The interaction between cultivar and season is significant for all the analysed elements, except for Ca, while the interaction between season and location is particularly significant for N, P and Mg, but not for other elements such as Na and Ca. The interaction between cultivar, season and location resulted in statistically significant variations in N, P, K, and Mg content.

Table 6. Average of chemical composition in leaf infusions of Myrtus communis L. cultivars ('Nadia', 'Marta', 'Daniela', 'Tonina', 'Grazia') growing in two localities (AHO = Alghero, ORI = Oristano) in two seasons (S = spring, W = winter). The data reported represent the average of the two years of sampling, to obtain a representative seasonal average value for each cultivar and location. [z]

Location	Season	Cultivar	Chlorophyll a (mg/l)	Chlorophyll b (mg/l)	L	a	b	Tannins (mg/l)	Total polyphenols (mg/l)
Alghero	Spring	Nadia	20.49 b-f	4.58 a-d	46.29 bc	-3.88 a-e	14.91 a-d	133.30 c	13851.92 ab
		Marta	16.72 ef	3.70 d	47.30 abc	-3.92 a-e	14.13 bcd	135.29 c	17845.08 a
		Daniela	16.57 ef	3.93 bcd	47.04 abc	-4.34 cde	14.01 bcd	112.54 c	13347.64 ab
		Tonina	19.44 c-f	4.73 a-d	47.96 ab	-4.22 a-e	13.36 cd	114.41 c	11815.83 ab
		Grazia	18.89 def	4.91 a-d	47.92 ab	-4.02 a-e	13.62 cd	101.22 c	11170.32 ab
	Winter	Nadia	27.72 ab	7.62 ab	45.69 c	-3.73 a-d	15.65 a-d	196.06 bc	16389.02 a
		Marta	22.95 a-f	7.24 a-d	48.93 a	-4.84 e	17.42 a	138.92 c	11856.43 ab
		Daniela	25.28 a-d	7.57 abc	46.80 bc	-4.10 a-e	15.95 abc	283.06 b	19140.61 a
		Tonina	29.67 a	8.35 a	45.99 bc	-3.88 a-e	16.38 ab	152.53 bc	11896.10 ab
		Grazia	23.35 a-f	6.31 a-d	47.42 abc	-4.19 a-e	16.49 ab	169.64 bc	12462.28 ab
Oristano	Spring	Nadia	20.89 b-f	5.59 a-d	46.54 bc	-3.22 a	13.29 d	176.49 bc	13889.87 ab
		Nadia	20.89 b-f	5.59 a-d	46.54 bc	-3.22 a	13.29 d	176.49 bc	13889.87 ab
		Daniela	15.73 f	3.56 d	47.84 ab	-3.81 a-d	13.21 d	126.99 c	12649.56 ab
		Tonina	18.55 def	3.99 bcd	47.88 ab	-4.27 b-e	14.22 bcd	116.83 c	12042.00 ab
		Grazia	16.85 ef	3.79 cd	47.22 abc	-4.17 a-e	15.32 a-d	128.50 c	11350.13 ab
	Winter	Nadia	27.31 abc	8.13 a	46.17 bc	-3.84 a-e	15.03 a-d	138.22 c	12732.77 ab
		Marta	24.37 a-e	6.23 a-d	47.19 abc	-3.55 abc	14.35 bcd	103.22 c	13820.66 ab
		Daniela	26.24 a-d	7.15 a-d	46.38 bc	-3.25 ab	15.05 a-d	166.51 bc	14314.05 a
		Tonina	21.97 a-f	5.88 a-d	49.02 a	-4.63 de	15.32 a-d	65.55 c	5907.61 b
		Grazia	22.41 a-f	6.61 a-d	47.91 ab	-3.73 a-d	16.32 ab	422.83 a	15870.28 a

[z]The results are expressed as means of six replicates. Values with different letters are significantly different ($p \leq 0.01$) according to Duncan's multiple range test.

Table 7. Summary of statistical analysis to assess the influence of cultivar, location, and season on the mineral composition (macroelements) of myrtle leaves in 2021. [z]

Variables	N	P	Na	K	Ca	Mg
Cultivar (A)	43.51 **	1.42	4.90 *	4.11 *	7.01 **	14.96 **
Season (B)	24.47 **	128.38 **	54.61 **	372.17 **	1.90	0.85
Location (C)	22.51 **	47.01 **	0.55	0.40	14.82 **	0.02
AxB	23.17 **	19.00 **	25.45 **	10.40 **	2.70	10.53 **
AxC	25.27 **	3.81 *	0.28	9.35 **	1.61	5.10 **
BxC	15.98 **	13.38 **	0.25	5.41 *	0.53	45.99 **
AxBxC	5.63 **	12.83 **	0.75	7.64 **	2.95 *	8.72 **

[z]numerical values refer to the calculated F, while significance is indicated as: " " = not significant; "*" = $p \leq 0.05$; "**" = $p \leq 0.01$.

Analysis of variance revealed a highly significant main effect of sampling season on the microelement composition of myrtle leaves (Table 8). Cultivar influenced only Fe content, whereas location showed no statistically significant effect. Among the microelements, Fe and Mn were the most responsive to both the main effects and the interactions between experimental factors.

Table 8. Summary of statistical analysis to assess the influence of cultivar, location, and season on the mineral composition (microelements) of myrtle leaves in 2021.[z]

Variables	Cu		Zn		Fe		Mn	
Cultivar (A)	1.71		1.22		11.46	**	3.87	*
Season (B)	20.25	**	108.63	**	59.97	**	52.70	**
AxB	3.23		1.43		3.64	*	10.16	**
Location (C)	0.01		1.49		0.87		2.51	
AxC	2.78		4.68	**	4.31	*	3.45	*
BxC	1.51		0.03		131.82	**	6.71	*
AxBxC	2.46		1.36		3.35	*	5.08	**

[z]numerical values refer to the calculated F, while significance is indicated as: " " = not significant; "*" = $p \leq 0.05$; "**" = $p \leq 0.01$.

The graphical representation (Figure 2 and 3) of the results relating to the mineral composition of the leaves for each cultivar made it possible to highlight, in greater detail, the influence of the variables considered on the elements analysed.

The leaves of the 'Daniela' cultivar, sampled in winter in Alghero, exhibited the lowest concentration of N (1.24%), while in the same sampling in Alghero, the 'Nadia' cultivar demonstrated the highest value (1.97%). The P content exhibited a range from 0.19% in 'Grazia' during the winter sampling in Alghero to 0.63% in 'Tonina' in the spring sampling in Oristano.

The 'Tonina' cultivar exhibited the highest Na values (0.10% in winter in both locations), whereas the lowest value (0.03%) was observed in 'Tonina' in the spring sampling in Alghero and in 'Daniela' in the winter sampling in Oristano.

Regarding K, the values ranged from 0.40% to 1.08% in 'Nadia', respectively in the winter sampling in Oristano and spring sampling in Alghero.

The analysis revealed Ca concentrations ranging from 0.83% in the ‘Grazia’ sample collected in spring in Alghero to 1.30% in the ‘Daniela’ sample obtained in winter in Oristano.

The Mg content was found to be at its lowest in the ‘Grazia’ sample (0.18%) in Oristano and at its highest in the ‘Nadia’ sample (0.37%) in Alghero, both of which were winter samples.

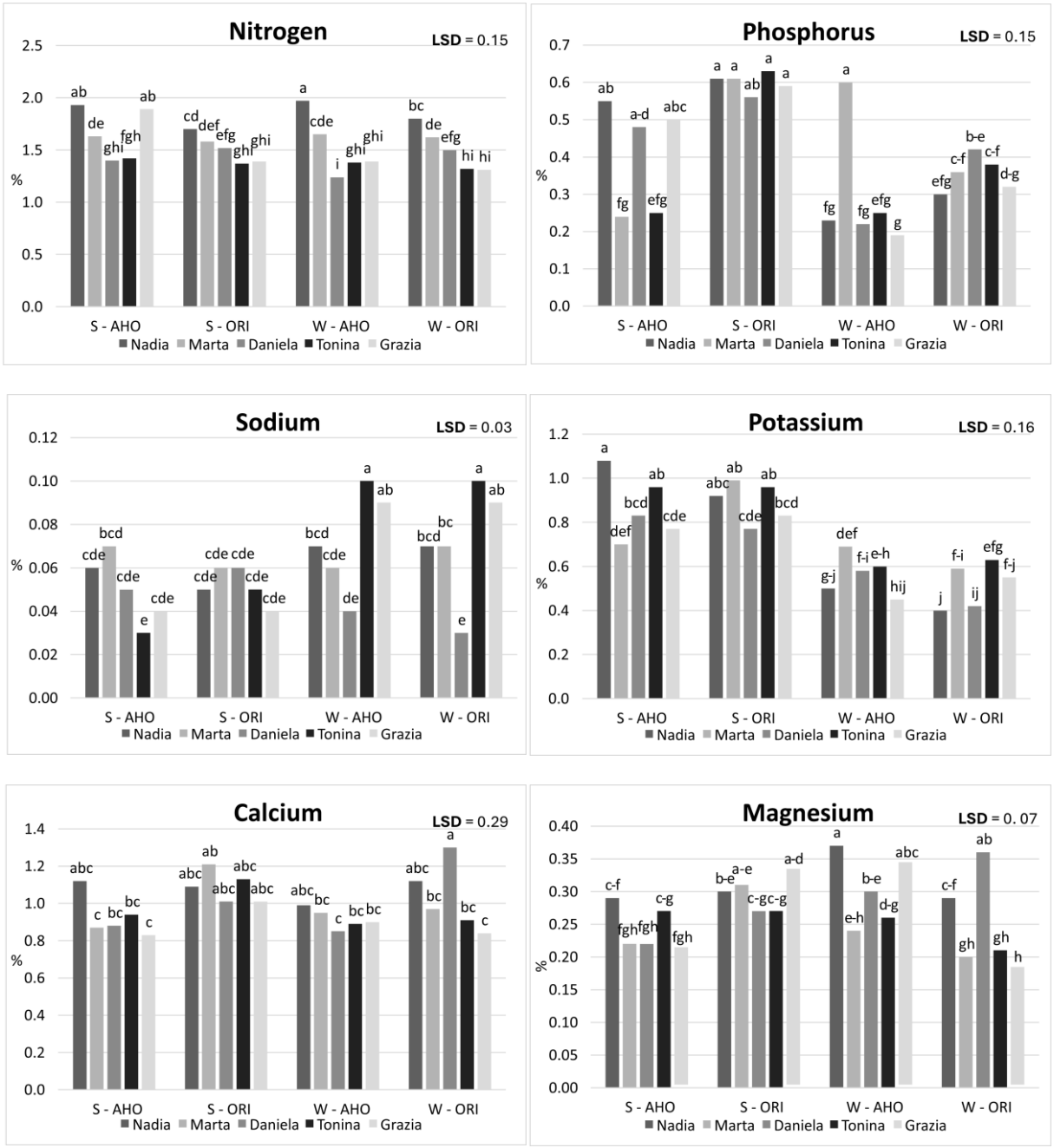

Figure 2. Average of macroelements concentration in leaves of Myrtus communis L. cultivars (‘Nadia’, ‘Marta’, ‘Daniela’, ‘Tonina’, ‘Grazia’) growing in two localities (AHO = Alghero, ORI = Oristano) in two seasons (S = spring, W = winter) in 2021. Data are the means of three replicates. Values with different letters are significantly different ($p \leq 0.01$) according to Duncan’s multiple range test.

Regarding microelements, the levels of Cu ranged from 5.83 ppm (‘Grazia’, spring, Oristano) to 15.00 ppm (‘Daniela’, winter, Alghero), while those of Zn ranged from 14.17 ppm in the leaves of ‘Marta’ harvested in spring in Alghero to 38.33 ppm in those of ‘Nadia’ harvested in winter in Alghero. The minimum value of Fe was recorded in ‘Daniela’ (29.17 ppm) during the winter months in Oristano, while the maximum value was observed in ‘Marta’ (196.67 ppm) in the spring. Finally, the concentration of Mn was found to be lowest in the winter sampling in Oristano in ‘Grazia’ (30.83 ppm) and highest in the winter sampling in Alghero in ‘Marta’ (110.83 ppm).

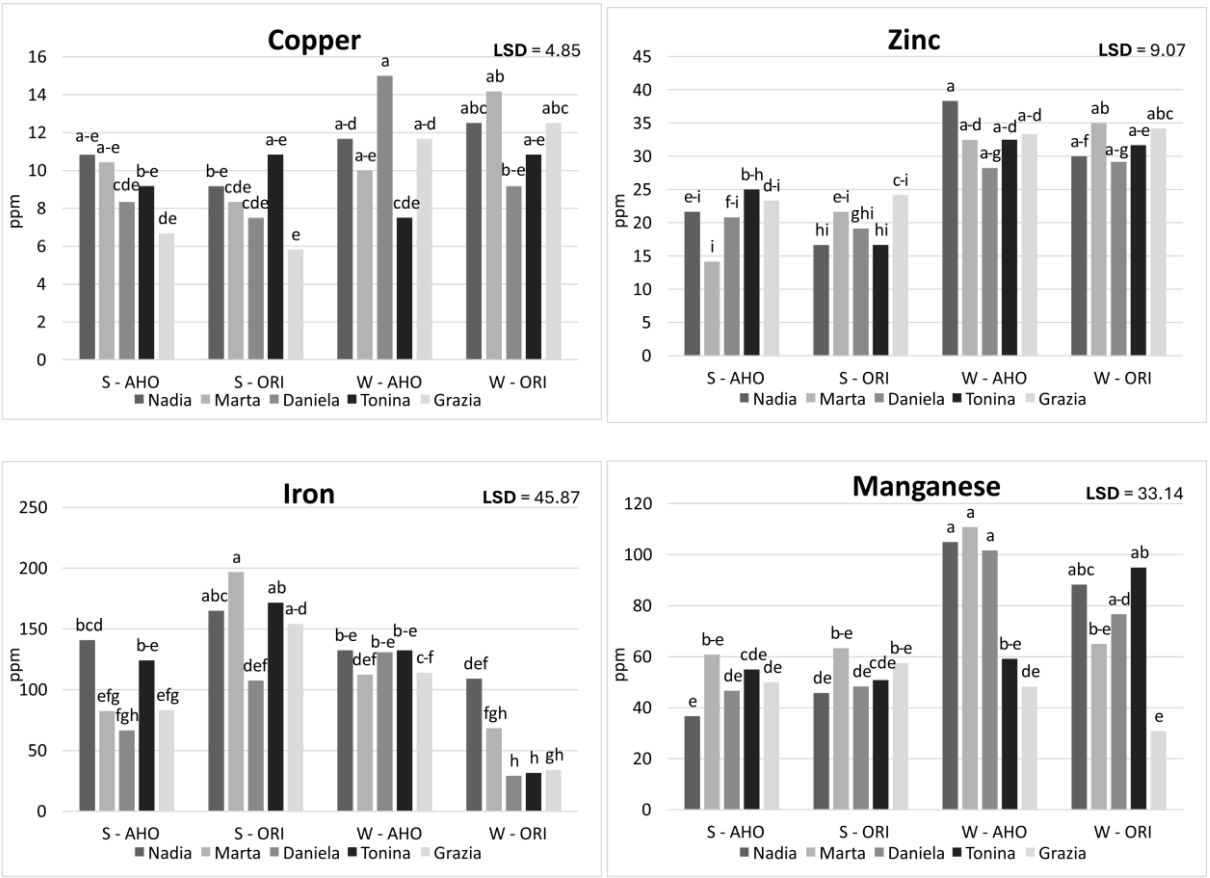

Figure 3. Average of microelements concentration in leaves of Myrtus communis L. cultivars (‘Nadia’, ‘Marta’, ‘Daniela’, ‘Tonina’, ‘Grazia’) growing in two localities (AHO = Alghero, ORI = Oristano) in two seasons (S = spring, W = winter) in 2021. Data are the means of three replicates. Values with different letters are significantly different ($p \leq 0.01$) according to Duncan’s multiple range test.

The analysis of mineral elements contained in myrtle leaves revealed significant positive relationships between P and K and Ca (Table 9); plants that accumulated more P in their leaves also accumulated K and Ca. Conversely, a negative correlation was observed between P and the microelements Cu and Zn; plants that accumulated more P accumulated less Cu and Zn in their leaves.

Sodium exhibited a positive correlation with Zn, while K demonstrated a positive correlation with Fe and a negative correlation with Zn and Mn. In addition, a positive correlation was identified between Mg and Ca, as well as between Mg and Fe, and between Zn and Mn. No significant correlations were identified for N, either with macroelements or with microelements.

Table 9. Coefficients (*r)* of linear correlation between the principal macroelements and microelements concentration of leaves, observed in the two experimental sites in spring and winter 2021 and 2022.

Variable	N	P	Na	K	Ca	Mg	Cu	Zn	Fe
P	0.163								
Na	-0.136	-0.354							
K	0.091	**0.652** **	-0.387						
Ca	0.267	**0.469** *	-0.333	0.172					
Mg	0.176	-0.035	-0.300	-0.122	**0.568** **				
Cu	-0.051	**-0.501** *	0.216	-0.431	-0.126	-0.081			
Zn	-0.029	**-0.524** *	**0.486** *	**-0.713** **	-0.210	0.086	0.430		
Fe	0.169	0.345	-0.234	**0.551** *	0.314	**0.471** *	-0.195	-0.354	
Mn	0.099	-0.237	0.001	**-0.469** *	0.025	0.243	0.342	**0.479** *	-0.058

*Bold data are significant at $p \leq 0.05$ level; **bold data are significant at $p \leq 0.01$.

A comprehensive analysis was conducted on the macroelement content (N, P, Na, K, Ca and Mg) in myrtle leaf infusions, with a focus on cultivar, seasonal and geographical variations (Table 10). The range of N values was from 26.45 ppm to 53.56 ppm, with the highest content

found in the ‘Tonina’ cultivar in winter in Alghero and the lowest in ‘Grazia’ in spring in the same location.

Phosphorus (P) exhibited a comparable trend, exhibiting variations between 19.40 ppm in ‘Grazia’ (winter, Alghero) and 53.43 ppm in ‘Nadia’ (winter, Alghero).

Sodium (Na) demonstrated the highest degree of variability among the elements analysed, with a maximum of 136.67 ppm in ‘Nadia’ '(winter, Alghero) and a minimum of 32.27 ppm in ‘Grazia’ (spring, Alghero).

The study revealed that K levels reached 310.27 ppm in the ‘Nadia’ cultivar during the spring months in Oristano, with the lowest observed value (110.88 ppm) recorded in the ‘Tonina’ cultivar during the winter months in the same location.

Calcium (Ca) exhibited a range of values from 14.38 ppm (‘Tonina’, winter, Oristano) to 64.00 ppm (‘Grazia’, winter, Oristano), with generally higher levels observed in the samples collected in Oristano.

Finally, Mg levels exhibited significant variation, ranging from 23.83 ppm in ‘Tonina’ (winter, Oristano) to 85.47 ppm in ‘Nadia’ (winter, Alghero).

Table 10. Average of macroelements concentration in leaf infusions of *Myrtus communis* L. cultivars ('Nadia', 'Marta', 'Daniela', 'Tonina', 'Grazia') growing in two localities (AHO = Alghero, ORI = Oristano) in two seasons (S = spring, W = winter). The data reported represent the average of the two years of sampling, to obtain a representative seasonal average value for each cultivar and location. [z]

Location	Season	Cultivar	N		P		Na		K		Ca		Mg	
			ppm		ppm		ppm		ppm		ppm		ppm	
Alghero	Spring	Nadia	33.56	bcd	36.93	a-d	40.27	c	246.13	a-f	31.47	ab	38.93	e-i
		Marta	30.60	cd	36.15	a-d	51.73	bc	261.83	a-e	27.20	ab	58.00	b-e
		Daniela	29.30	cd	35.95	a-d	33.33	c	252.70	a-e	26.13	ab	38.80	e-i
		Tonina	30.38	cd	36.50	a-d	43.20	c	205.63	d-i	20.30	b	28.80	hi
		Grazia	26.45	d	31.75	bcd	32.27	c	266.63	a-d	26.00	ab	54.73	c-g
	Winter	Nadia	42.56	b	53.43	a	136.67	a	285.60	abc	33.87	ab	85.47	a
		Marta	30.15	cd	25.10	cd	107.20	a	274.80	abc	48.67	ab	47.20	c-h
		Daniela	36.59	bc	25.67	cd	108.40	a	229.07	b-g	24.00	ab	75.23	ab
		Tonina	53.56	a	24.38	cd	54.73	bc	296.43	ab	22.98	ab	56.50	b-f
		Grazia	33.21	bcd	19.40	d	95.23	ab	169.08	g-k	25.28	ab	64.95	bc
Oristano	Spring	Nadia	34.01	bcd	31.63	bcd	38.53	c	310.27	a	20.40	b	61.47	bcd
		Marta	33.11	bcd	38.93	abc	32.93	c	286.30	abc	24.00	ab	66.40	bc
		Daniela	35.02	bcd	39.25	abc	40.13	c	222.93	c-h	16.93	b	48.80	c-h
		Tonina	42.41	b	28.62	bcd	42.27	c	197.73	e-i	26.80	ab	58.53	b-e
		Grazia	34.47	bcd	45.66	ab	41.87	c	239.53	b-f	43.87	ab	35.80	ghi
	Winter	Nadia	37.84	bc	40.67	abc	41.37	c	126.42	jk	17.35	b	42.18	d-i
		Marta	37.61	bc	45.83	ab	36.80	c	150.40	ijk	17.47	b	36.93	f-i
		Daniela	40.69	b	21.93	cd	51.20	bc	183.20	f-j	36.67	ab	64.00	bc
		Tonina	29.86	cd	28.20	bcd	38.47	c	110.88	k	14.38	b	23.83	i
		Grazia	34.72	bcd	28.33	bcd	59.13	bc	159.87	h-k	64.00	a	48.33	c-h

[z]The results are expressed as means of six replicates. Values with different letters are significantly different ($p \leq 0.01$) according to Duncan's multiple range test.

The contents of microelements (Cu, Zn, Fe and Mn) in myrtle leaf infusions are illustrated in Table 11. The concentration of Cu ranged from 0.10 to 0.74 parts per million (ppm), with the highest value recorded in the Daniela cultivar in spring in Oristano and the lowest in Grazia in the same season in Alghero.

Zinc (Zn) exhibited variations between 0.28 and 0.52 ppm, with the highest values recorded in Daniela during the winter months in Alghero and the lowest in Marta and Tonina, both in the winter months in Oristano.

Iron (Fe) demonstrated a range of concentrations between 0.22 and 0.83 ppm, with the maximum value observed in the Tonina cultivar in spring in Alghero and the minimum in Marta during winter in the same location.

Manganese (Mn) was identified as the most prevalent microelement across the range of analyses conducted, exhibiting concentrations ranging from 0.25 ppm in Marta (winter, Oristano) to 2.27 ppm in the same cultivar during the spring season in Alghero.

Table 11. Average of microelements concentration in leaf infusions of *Myrtus communis* L. cultivars ('Nadia', 'Marta', 'Daniela', 'Tonina', 'Grazia') growing in two localities (AHO = Alghero, ORI = Oristano) in two seasons (S = spring, W = winter). The data reported represent the average of the two years of sampling, to obtain a representative seasonal average value for each cultivar and location. [z]

Location	Season	Cultivar	Cu ppm		Zn ppm		Fe ppm		Mn ppm	
Alghero	Spring	Nadia	0.29	bc	0.43	abc	0.43	abc	1.66	ab
		Marta	0.32	bc	0.42	abc	0.48	abc	2.27	a
		Daniela	0.15	bc	0.42	abc	0.48	abc	1.66	ab
		Tonina	0.32	bc	0.45	abc	0.83	a	2.19	a
		Grazia	0.10	c	0.48	ab	0.69	ab	1.23	abc
	Winter	Nadia	0.24	bc	0.44	abc	0.40	bc	0.54	c
		Marta	0.15	bc	0.38	abc	0.22	c	0.66	bc
		Daniela	0.24	bc	0.52	a	0.49	abc	0.81	bc
		Tonina	0.21	bc	0.33	bc	0.27	bc	0.42	c
		Grazia	0.15	bc	0.40	abc	0.54	abc	0.58	bc
Oristano	Spring	Nadia	0.17	bc	0.38	abc	0.50	abc	1.38	abc
		Marta	0.21	bc	0.38	abc	0.37	bc	1.67	ab
		Daniela	0.74	a	0.40	abc	0.51	abc	0.78	bc
		Tonina	0.26	bc	0.30	c	0.37	bc	1.22	abc
		Grazia	0.37	bc	0.39	abc	0.36	bc	1.26	abc
	Winter	Nadia	0.46	b	0.32	bc	0.48	abc	0.39	c
		Marta	0.17	bc	0.28	c	0.27	bc	0.25	c
		Daniela	0.21	bc	0.35	abc	0.24	c	0.42	c
		Tonina	0.13	c	0.28	c	0.31	bc	0.27	c
		Grazia	0.15	bc	0.36	abc	0.24	c	0.30	c

[z]The results are expressed as means of six replicates. Values with different letters are significantly different ($p \leq 0.01$) according to Duncan's multiple range test.

5.4.3 Correlation between chemical composition of leaves and thermal regime

Heat hours accumulation

Pearson's correlation coefficients were determined between the macroelement and microelement content of myrtle leaves and the sum of hour degrees (HD) above the thermal threshold, revealing different trends among the various elements (Table 12).

The compounds Na, Cu, Zn and Mn demonstrated a positive correlation with rising thermal thresholds. Sodium (Na) exhibited a moderately significant correlation ($p \leq 0.05$) across most HD sums (except for 2.5 °C). The analysis revealed significant correlations ($p \leq 0.05$) for Cu across numerous HD sums ranging from 0 °C to 27.5 °C. The correlation became highly significant ($p \leq 0.01$) at HD sums equal to or greater than 30 °C. Zinc (Zn) exhibited the strongest and most consistent positive relationship with heat accumulation, demonstrating high and highly significant correlations at all thresholds analysed. Manganese (Mn) demonstrated significant correlations ($p \leq 0.05$) at thresholds ranging from 0 °C to 7.5 °C and at 35 °C. Additionally, within the intermediate-high range of 10–32.5 °C, the correlations were found to be highly significant ($p \leq 0.01$).

A negative correlation was observed between the levels of P and K compounds. For P, the correlations become significant at 12.5 °C and remain so for many thresholds; the most negative and highly significant value is observed at 32.5 °C ($r = -0.572$, $p \leq 0.01$). The results demonstrate a strong negative correlation of K across all HD sums tested ($p \leq 0.01$), indicating a consistent decrease in potassium content with rising degree days above a certain threshold. Iron (Fe) exhibited a highly significant negative correlation with the thermal threshold at 35 °C ($r = -0.592$, $p \leq 0.01$), while for all other thresholds, it showed insignificant correlations.

The data revealed a weak and insignificant correlation between nitrogen (N), calcium (Ca) and magnesium (Mg) and accumulated temperature.

Chill hours accumulation

The correlation between the macroelements and microelements in the leaves and the HD sums below the temperature thresholds was significant for all compounds except N, Ca and Mg (Table 13).

Phosphorus (P), potassium (K) and iron (Fe) exhibited positive correlations with HD sums. In particular, P demonstrated moderately significant correlations ($p \leq 0.05$) for specific temperature thresholds. For K, the correlations are significant even at low thresholds ($p \leq 0.05$)

and become highly significant ($p \leq 0.01$) from 7.5 °C to 32.5 °C, indicating an increase in leaf K content with an increase in accumulated cold hours. Iron exhibited significant positive correlations ($p \leq 0.05$) starting at 12.5 °C and highly significant correlations ($p \leq 0.01$) for HD sums equal to or greater than 30 °C.

Conversely, Na, Cu, Zn and Mn exhibited negative correlations with HD sums. Sodium (Na) showed significant correlations ($p \leq 0.05$) in thresholds between 5 °C and 30 °C. Copper (Cu) demonstrated significant correlations (both $p \leq 0.05$ and $p \leq 0.01$) within the HD sum range of 7.5 °C to 32.5 °C. Zinc (Zn) exhibited a significant negative correlation across a wide range of thresholds, with most intermediate thresholds showing highly significant correlations ($p \leq 0.01$). However, at 35 °C, the correlation weakens and becomes non-significant. Manganese showed significant negative correlations ($p \leq 0.05$) with HD sums between 5 °C and 30 °C.

Table 12. Coefficients (*r)* of linear correlation between the concentration of mineral macroelements and microelements of leaves, and hour-degree sums for temperatures above thresholds ranging from 0 to 35 °C as observed in the two experimental sites from January 1, 2021, to December 31, 2021.

	Temperature thresholds (°C) for above sums of hour-degrees calculation														
Variable	**0 °C**	**2.5 °C**	**5 °C**	**7.5 °C**	**10 °C**	**12.5 °C**	**15 °C**	**17.5 °C**	**20 °C**	**22.5 °C**	**25 °C**	**27.5 °C**	**30 °C**	**32.5 °C**	**35 °C**
N	-0.230	-0.266	-0.252	-0.237	-0.215	-0.180	-0.181	-0.188	-0.175	-0.177	-0.200	-0.183	-0.162	-0.153	-0.149
P	-0.355	-0.164	-0.253	-0.330	-0.408	**-0.502 ***	**-0.502 ***	**-0.493 ***	**-0.524 ***	**-0.517 ***	**-0.452 ***	**-0.506 ***	**-0.553 ***	**-0.572 ****	**-0.487 ***
Na	**0.498 ***	0.437	**0.470 ***	**0.488 ***	**0.498 ***	**0.499 ***	**0.504 ***	**0.512 ***	**0.510 ***	**0.508 ***	**0.501 ***	**0.512 ***	**0.513 ***	**0.513 ***	**0.487 ***
K	**-0.773 ****	**-0.638 ****	**-0.707 ****	**-0.748 ****	**-0.773 ****	**-0.791 ****	**-0.801 ****	**-0.818 ****	**-0.821 ****	**-0.814 ****	**-0.785 ****	**-0.820 ****	**-0.836 ****	**-0.846 ****	**-0.831 ****
Ca	0.039	0.169	0.113	0.045	-0.034	-0.136	-0.127	-0.098	-0.133	-0.132	-0.079	-0.111	-0.152	-0.163	-0.028
Mg	0.195	0.272	0.237	0.231	0.229	0.210	0.197	0.172	0.164	0.177	0.220	0.168	0.132	0.102	-0.067
Cu	**0.503 ***	0.389	**0.447 ***	**0.479 ***	**0.502 ***	**0.522 ***	**0.531 ***	**0.545 ***	**0.550 ***	**0.543 ***	**0.513 ***	**0.548 ***	**0.567 ****	**0.579 ****	**0.590 ****
Zn	**0.795 ****	**0.649 ****	**0.723 ****	**0.775 ****	**0.816 ****	**0.850 ****	**0.856 ****	**0.862 ****	**0.871 ****	**0.866 ****	**0.835 ****	**0.866 ****	**0.883 ****	**0.889 ****	**0.816 ****
Fe	-0.223	-0.048	-0.134	-0.162	-0.176	-0.204	-0.228	-0.272	-0.283	-0.262	-0.191	-0.278	-0.332	-0.375	**-0.592 ****
Mn	**0.556 ***	**0.485 ***	**0.521 ***	**0.557 ***	**0.590 ****	**0.615 ****	**0.612 ****	**0.602 ****	**0.608 ****	**0.610 ****	**0.603 ****	**0.605 ****	**0.604 ****	**0.598 ****	**0.469 ***

*Bold data are significant at $p \leq 0.05$ level; **bold data are significant at $p \leq 0.01$.

Table 13. Coefficients (*r)* of linear correlation between the concentration of mineral macroelements and microelements of leaves, and hour-degree sums for temperatures under thresholds ranging from 0 to 35 °C as observed in the two experimental sites from January 1, 2021, to December 31, 2021.

	Temperature thresholds (°C) for under sums of hour-degrees calculation														
Variable	**0 °C**	**2.5 °C**	**5 °C**	**7.5 °C**	**10 °C**	**12.5 °C**	**15 °C**	**17.5 °C**	**20 °C**	**22.5 °C**	**25 °C**	**27.5 °C**	**30 °C**	**32.5 °C**	**35 °C**
N	0.280	0.278	0.260	0.234	0.211	0.184	0.182	0.181	0.170	0.172	0.191	0.178	0.159	0.141	0.091
P	-0.035	0.024	0.206	0.314	0.388	**0.472 ***	**0.462 ***	0.437	**0.448 ***	0.438	0.385	0.424	**0.444 ***	0.395	0.160
Na	-0.344	-0.374	**-0.455 ***	**-0.490 ***	**-0.503 ***	**-0.513 ***	**-0.508 ***	**-0.498 ***	**-0.492 ***	**-0.490 ***	**-0.485 ***	**-0.490 ***	**-0.479 ***	-0.438	-0.262
K	**0.457 ***	**0.514 ***	**0.679 ****	**0.767 ****	**0.810 ****	**0.843 ****	**0.839 ****	**0.827 ****	**0.826 ****	**0.822 ****	**0.802 ****	**0.817 ****	**0.814 ****	**0.760 ****	**0.489 ***
Ca	-0.283	-0.251	-0.152	-0.098	-0.047	0.028	0.012	-0.017	-0.009	-0.018	-0.066	-0.031	-0.014	-0.055	-0.197
Mg	-0.325	-0.312	-0.238	-0.142	-0.086	-0.051	-0.028	0.00	0.031	0.031	0.005	0.021	0.068	0.141	0.287
Cu	-0.249	-0.293	-0.425	**-0.504 ***	**-0.546 ***	**-0.579 ****	**-0.579 ****	**-0.573 ****	**-0.578 ****	**-0.575 ****	**-0.554 ***	**-0.569 ****	**-0.576 ****	**-0.548 ***	-0.376
Zn	**-0.457 ***	**-0.518 ***	**-0.687 ****	**-0.767 ****	**-0.809 ****	**-0.850 ****	**-0.839 ****	**-0.815 ****	**-0.810 ****	**-0.804 ****	**-0.779 ****	**-0.799 ****	**-0.790 ****	**-0.714 ****	-0.390
Fe	-0.124	-0.073	0.117	0.291	0.387	**0.447 ***	**0.475 ***	**0.506 ***	**0.545 ***	**0.542 ***	**0.499 ***	**0.528 ***	**0.585 ****	**0.648 ****	**0.680 ****
Mn	-0.377	-0.412	**-0.495 ***	**-0.511 ***	**-0.519 ***	**-0.538 ***	**-0.520 ***	**-0.491 ***	**-0.476 ***	**-0.472 ***	**-0.462 ***	**-0.471 ***	**-0.449 ***	-0.369	-0.097

*Bold data are significant at $p \leq 0.05$ level; **bold data are significant at $p \leq 0.01$.

5.5 Discussion

The present study provides a comprehensive characterisation of the mineral composition of *Myrtus communis* L. leaves across multiple cultivars, sampling seasons, and growing locations in Sardinia (Italy). The results obtained confirm the dominant role of N as the most abundant macroelement in the leaf tissue, followed by Ca, K, P, Mg, and Na. This result is consistent with the findings of Peñuelas et al. (2001), who reported N as the second most abundant element in *M. communis* leaves after C. It diverges, however, from those of Yildirim et al. (2015) and Nedjimi (2024), who identified K as the dominant mineral in wild myrtle populations — a discrepancy that may reflect the different trophic status of the plants examined, since both of those studies were conducted on wild-growing shrubs in natural environments, whereas the present study was carried out on cultivated orchards established on soils with probably higher organic N content. Among microelements, Fe and Mn were the most prevalent, followed by Zn and Cu, consistent with the general pattern reported for *M. communis* by other studies (Nedjimi, 2024; Yildirim et al., 2015).

Season was identified as the most significant source of variation for most mineral elements in myrtle leaves, exerting highly significant effects on macroelements (K, P, Na, and N, Table 7) and all microelements (Table 8). This finding is consistent with previous studies on Mediterranean plant ecology and nutrient cycling, which have shown that phenological stage, nutrient remobilization, growth dynamics, and environmental conditions strongly influence plant mineral composition and stoichiometry (Milla et al., 2004; Millard and Grelet, 2010).

Cultivar was the second source of variability of several mineral elements in myrtle leaves, particularly N, Ca, and Mg for macroelements (Table 7) and, Fe and Mn for microelements. Genotype effects of similar magnitude have been documented for mineral leaf composition in olive cultivars (Dimassi et al., 1999), where N, P, K, Ca, and Mg concentrations varied significantly between genotypes even under identical soil and climatic conditions.

The collection site emerged as a critical determinant of the mineral composition of myrtle leaves, with highly significant location effects on N, P, and Ca (Table 7). The two sites, Alghero

and Oristano, differed markedly in their pedochemical characteristics, providing a natural experimental framework to investigate soil–plant mineral relationships. Alghero exhibited a distinctly alkaline soil reaction (pH 8.09–8.40), while Oristano presented a sub-acidic to neutral pH (6.58–6.79), a difference that was statistically highly significant (Table 1). The observed pH differences between the two sites likely contributed to the differential bioavailability of several essential elements, since soil pH is considered a master variable regulating nutrient solubility, mobility, and plant uptake (Barrow and Hartemink, 2023).

The soil analysis (Table 2) revealed that the Oristano site contained substantially higher levels of exchangeable K_2O compared to Alghero, as well as higher total N content, and dramatically elevated Fe and Mn. These differences in soil elemental pools, combined with the lower pH at Oristano, are expected to enhance the bio-availability of redox-sensitive elements (Rengel et al., 2023; Violante et al., 2010). The increased Fe and Mn concentrations in Oristano soils are consistent with the greater solubility and bioavailability of these elements under sub-neutral acidic conditions, where metal cations become more mobile and available for plant uptake (Rengel et al., 2023), potentially influencing the mineral profile of *M. communis* leaves.

Despite the large contrast in soil K_2O pools between the two locations, no statistically significant main effect of location was detected on leaf K concentrations (Table 7). This result suggests that *M. communis* may maintain relatively stable leaf K concentrations despite differences in soil K availability, indicating a degree of physiological regulation of K uptake and allocation. Potassium homeostasis in plants is maintained through the coordinated activity of multiple transport systems, including members of the HAK/KUP/KT transporter family, which regulate K uptake, translocation, and cellular distribution according to plant physiological demand (Véry et al., 2014). Comparable responses have been reported in olive (*Olea europaea* L.), where leaf K concentrations showed limited variation across contrasting soil types despite differences in soil properties affecting K availability (Chatzistathis et al., 2010).

In contrast, location exerted a strong and consistent effect on leaf P concentrations, with leaves from Oristano displaying higher P values in spring despite the lower absolute soil P content at this site. This pattern is likely explained by the strong pH dependence of phosphorus chemistry in soils. Under the alkaline conditions observed at Alghero (pH 8.09–8.40), phosphate availability is typically reduced due to precipitation and adsorption reactions with Ca compounds, which limit P solubility and plant uptake. Conversely, the slightly acidic to near-neutral pH of Oristano soils (6.58–6.79) likely favoured greater P bioavailability, as phosphorus fixation by both Ca at high pH and Fe/Al oxides at low pH is minimised within this intermediate pH range (Barrow and Hartemink, 2023; Tully and Ryals, 2017). This pedological framework may therefore explain the significantly higher leaf P concentrations observed at Oristano.

Micronutrient dynamics between locations showed a complex pattern, as no significant main effect of location was detected for leaf Cu, Zn, Fe, or Mn concentrations (Table 8), whereas significant Season × Location interactions emerged, particularly for Fe and Mn. The absence of a consistent location effect despite the marked differences in soil Fe and Mn concentrations between sites suggests that seasonal physiological regulation of nutrient uptake and internal redistribution may strongly influence leaf micronutrient accumulation. Nevertheless, spring sampling revealed substantially higher leaf Fe concentrations at Oristano (107.50–196.67 ppm) than at Alghero (66.67–140.83 ppm), while winter responses varied among cultivars, contributing to the significant interaction effect. The higher Fe concentrations observed at Oristano during spring are consistent with the greater Fe solubility and bioavailability typically associated with slightly acidic to near-neutral soils, together with the increased physiological demand for Fe during periods of active vegetative growth and photosynthetic activity (Barrow and Hartemink, 2023; Vélez-Bermúdez and Schmidt, 2022).

The Pearson correlation matrix (Table 9) revealed a coherent network of positive and negative associations among mineral elements in myrtle leaves, providing insight into the stoichiometric constraints governing mineral accumulation and into possible synergistic or

antagonistic interactions during nutrient uptake and translocation. However, correlation analysis alone does not demonstrate causal physiological interactions, and the observed relationships should therefore be interpreted as indicative rather than definitive evidence of nutrient synergism or antagonism.

The strong positive correlations between P and K ($r = 0.652$, $p \leq 0.01$) and between P and Ca ($r = 0.469$, $p \leq 0.05$) likely reflect the coordinated regulation of these nutrients during periods of active growth. Phosphorus and K typically reach maximum concentrations in spring, when cell division, membrane synthesis, and phloem transport are particularly intense. This co-variation is consistent with the ecological stoichiometry framework, in which P and K are recognised as growth-related elements closely associated with metabolic activity (Elser et al., 2010).

In contrast, the significant negative correlations of P with Cu ($r = -0.501$, $p \leq 0.05$) and Zn ($r = -0.524$, $p \leq 0.05$) may indicate antagonistic interactions during nutrient uptake and transport. High P availability in the rhizosphere can reduce Zn solubility through the formation of insoluble zinc phosphate compounds and may interfere with Zn uptake and translocation (Broadley et al., 2007). Similarly, elevated P concentrations may affect Cu acquisition through competitive effects on membrane transport systems involved in micronutrient absorption (Rengel et al., 2023).

The positive correlation between K and Fe ($r = 0.551$, $p \leq 0.05$) may reflect the simultaneous enhancement of K and Fe uptake during spring, particularly under the soil conditions observed at Oristano, where both K availability and Fe solubility are favoured. Conversely, the negative correlations between K and Zn ($r = -0.713$, $p \leq 0.01$) and between K and Mn ($r = -0.469$, $p \leq 0.05$) are consistent with competitive interactions among cations during root uptake. These antagonistic relationships may be mediated by partially shared transport systems and non-selective cation channels involved in mineral acquisition and translocation (Broadley et al., 2007; Rengel et al., 2023). The positive correlation between Zn and Mn ($r = 0.479$, $p \leq 0.05$) further supports the hypothesis of partially shared uptake and transport pathways for these divalent micronutrients.

Finally, the significant positive correlations between Mg and Ca ($r = 0.568$, $p \leq 0.01$) and between Mg and Fe ($r = 0.471$, $p \leq 0.05$) are coherent with the central physiological role of Mg in chlorophyll structure and with its close metabolic association with Fe in the photosynthetic apparatus. Magnesium is a key structural component of chlorophyll molecules, whereas Fe is essential for electron transport and chloroplast development (Briat et al., 2015; Cakmak and Yazici, 2010). Both elements are therefore required for the development and functioning of photosynthetic reaction centres, and their coordinated variation likely reflects the increased demand for chloroplast biogenesis and photosynthetic activity during periods of active growth.

The correlation analyses between leaf mineral element concentrations and hour-degree (HD) sums computed above and below temperature thresholds from 0 to 35 °C (Tables 12 and 13) yielded physiologically coherent patterns and highlighted potential relationships between thermal environment and mineral nutrition in *M. communis*.

Potassium exhibited significant positive correlations with HD sums above threshold values in the 12.5–27.5 °C range ($r > 0.672$), indicating that leaf K concentrations were higher in the warmer site (Oristano) and during the warmer spring season. This pattern is consistent with the well-known temperature dependence of K uptake and transport in plants, as moderate temperatures generally enhance root metabolic activity and nutrient absorption efficiency (Gierth and Mäser, 2007; Véry et al., 2014).

Phosphorus showed significant positive correlations with HD sums below thresholds in the 12.5–35 °C range ($r > 0.472$), as well as with HD sums above thresholds at similar temperature ranges. This pattern likely reflects the combined influence of moderate temperatures favourable for growth and the relatively high soil P availability observed at the Oristano site. Under these conditions, temperatures are sufficiently warm to sustain active growth and metabolic demand for P while maintaining favourable soil chemical conditions for P availability. Conversely, severe heat stress may reduce P uptake efficiency through alterations in root activity and membrane transport processes (Khan et al., 2023).

Sodium showed systematic and statistically significant negative correlations with HD sums above moderate temperature thresholds (5–30 °C), indicating that leaves from sites or periods characterised by a greater number of warm hours accumulated lower Na concentrations. This finding may indicate a tendency for Na accumulation under cooler conditions, potentially associated with osmotic adjustment or reduced ion dilution during periods of lower transpiration. As transpiration rates and xylem flux decrease under cool conditions, Na may accumulate more passively within leaf tissues. Similar trends have been reported in stress-tolerant Mediterranean species such as Atriplex halimus, where elevated leaf Na concentrations were associated with colder environments (Dessena and Mulas, 2021).

Zinc was the microelement most strongly correlated with thermal parameters, showing remarkably consistent positive correlations with HD sums below thresholds across the entire 0–35 °C range ($r = 0.649–0.889$). This pattern suggests that leaf Zn concentrations were favoured under cooler and less thermally extreme conditions. Such an association may be related to the established role of Zn in membrane stability, antioxidant protection, and cellular homeostasis under environmental stress conditions (Cakmak, 2000; Peck and McDonald, 2010). In Mediterranean evergreen species such as *Myrtus communis*, high temperature and irradiance are known to induce oxidative and physiological stress responses affecting mineral balance and leaf metabolism (Gratani et al., 2013; Tattini et al., 2006).

This result may also reflect the inverse relationship between Zn and K dynamics already observed in the dataset ($r = −0.713$), potentially associated with interactions among cation uptake and transport processes. Such antagonistic dynamics often emerge when plants prioritize osmotic adjustment through P accumulation at the expense of divalent cation uptake (Broadley et al., 2007; Rengel et al., 2023). Alterations in ion homeostasis and nutrient allocation under stress conditions have already been described in *M. communis*, particularly under salinity and high irradiance, where plants regulate ionic balance and osmotic adjustment mechanisms to cope with environmental constraints (Acosta-Motos et al., 2015; Tattini et al., 2006).

Additionally, high temperatures may negatively affect Zn absorption and translocation by altering root membrane functionality, transpiration rates, and water relations, contributing to the lower Zn concentrations observed under warmer conditions (Peck and McDonald, 2010). In *M. communis*, Mediterranean summer stress conditions have been associated with marked changes in stomatal regulation, water balance, and leaf physiological performance (Gratani et al., 2013; Mendes et al., 2001), processes that may indirectly influence micronutrient transport and accumulation.

Iron showed a significant negative correlation specifically with HD sums above the 35 °C threshold ($r = -0.592$), indicating that Fe accumulation was suppressed when the number of hours above 35 °C increased. Maximum temperatures at Oristano exceeded 42 °C in 2021 (Figure 1), and extreme heat is known to impair nutrient acquisition and Fe assimilation processes, potentially inducing functional Fe deficiency even under adequate soil Fe availability (Briat et al., 2015; Rengel et al., 2023).

5.6 Conclusions

This study demonstrated that the chemical and mineral composition of myrtle leaves is strongly influenced by season, cultivar, growing site, and thermal regime, confirming the complex interaction between genotype and environmental conditions in determining leaf quality. Among the experimental factors analysed, season had the greatest influence on most parameters, particularly on nitrogen, phosphorus, potassium, chlorophylls, and several microelements, highlighting the strong physiological response of myrtle leaves to seasonal metabolic changes. Leaves collected in spring generally exhibited higher nitrogen and chlorophyll contents, whereas autumn samples showed higher dry matter and ash accumulation, supporting the potential valorisation of autumn biomass collected during fruit harvesting.

The analysed cultivars exhibited marked differences in their chemical profiles, particularly for total polyphenols, tannins, and chlorophylls, confirming a strong genotype-dependent variability in the biosynthesis and accumulation of bioactive compounds. In particular, ‘Marta’ and

‘Daniela’ showed distinctive phenolic profiles, while significant differences between Alghero and Oristano reflected the influence of local soil composition and climatic conditions on mineral uptake and leaf metabolism.

Correlation analysis revealed coordinated relationships between mineral elements and thermal parameters expressed as hour-degree sums. Potassium and phosphorus were positively associated with chill-hour accumulation and negatively correlated with increasing heat accumulation, whereas sodium, zinc, copper, and manganese showed the opposite trend, suggesting that thermal regimes strongly affect nutrient allocation and mineral homeostasis in myrtle leaves. The significant correlations observed among several macro- and microelements further support the existence of coordinated physiological and metabolic regulation mechanisms linked to environmental adaptation.

From a practical perspective, these findings demonstrate that myrtle leaves represent a valuable source of minerals and phenolic compounds for industrial, nutraceutical, and pharmaceutical applications, and that seasonal and environmental conditions should be carefully considered to optimise raw material quality. In particular, autumn leaf biomass may represent a sustainable resource for the development of mechanised harvesting systems and to produce functional extracts and infusions. Future research should focus on the molecular and physiological mechanisms underlying genotype-specific responses to environmental and thermal conditions, as well as on the relationship between mineral composition, secondary metabolism, and the bioactive properties of leaf-derived products.

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6. General conclusions

The increasing interest in *Myrtus communis* L. as a source of bioactive compounds for the food, nutraceutical, cosmetic, and pharmaceutical industries has strengthened the need to better understand the factors regulating the accumulation of valuable metabolites in different plant organs. In recent decades, the domestication and selection programme carried out in Sardinia has led to the identification of numerous cultivars characterized by distinct agronomic, biochemical, and technological traits. Within this framework, the study of phenolic compounds and their regulation has become particularly important, considering their contribution to the biological properties of myrtle and their potential use in functional products.

The present doctoral thesis focused on the variability of chemical composition in myrtle, investigating how genotype, developmental stage, post-harvest conditions, seasonal dynamics, and environmental factors affect the accumulation of phenolic compounds in berries and the mineral and biochemical composition of leaves. Through the integration of post-harvest studies, metabolomic characterization, gene expression analyses, and multi-environment field trials, this work contributes to a deeper understanding of the physiological and biochemical mechanisms underlying myrtle quality.

The main results obtained during the PhD programme are summarized below.

The study of post-harvest storage conditions demonstrated that berry response is strongly genotype dependent. Storage at 10 °C was the most effective condition for limiting fruit weight loss, whereas storage at 2 °C and 16 °C induced greater dehydration. Pigmented cultivars showed a progressive increase in total phenols, tannins, and anthocyanins during storage, while unpigmented cultivars exhibited a more variable behaviour. The absence of a significant relationship between weight loss and phenolic accumulation suggests that the increase in bioactive compounds was not merely due to concentration effects associated with dehydration, but rather to an active metabolic response involving de novo biosynthesis of phenolic compounds. These findings indicate that low-temperature storage may represent a useful strategy to enhance the

functional value of pigmented berries intended for pharmaceutical and cosmetic applications, while intermediate temperatures are more suitable for preserving fruit quality for industrial processing.

The phenotypic characterization of pigmented and unpigmented cultivars through HPLC analyses highlighted the strong influence of genotype, fruit developmental stage, and growing season on phenolic composition. Nineteen specialized metabolites, including anthocyanins, flavonols, and ellagic acid, were identified and quantified during fruit development. Anthocyanins accumulated almost exclusively in the pigmented cultivar and reached their highest concentrations at full ripening, confirming the nutraceutical value of mature pigmented berries. Conversely, flavonols progressively decreased during fruit development in both cultivars and were particularly abundant during the earliest developmental stages. The unpigmented cultivar maintained appreciable levels of flavonols and ellagic acid throughout ripening, demonstrating that the absence of anthocyanins does not necessarily imply a lower phytochemical value. Overall, these results emphasize the importance of harvest timing and cultivar selection according to the desired phytochemical profile and final industrial application.

The investigation of flavonoid biosynthesis at the molecular level provided further insight into the mechanisms responsible for the contrasting pigmentation observed between cultivars. The pigmented cultivar showed higher expression levels of key structural genes involved in the phenylpropanoid and anthocyanin pathways, particularly PAL, DFR, LDOX, and UFGT. In contrast, the unpigmented cultivar exhibited lower expression of these genes and a different temporal regulation of early flavonoid pathway genes. Seasonal analyses revealed that gene expression patterns were generally consistent between years, although transcript abundance varied according to environmental conditions. The combined interpretation of metabolomic and molecular data indicates that berry pigmentation in myrtle is associated with coordinated transcriptional regulation of the anthocyanin biosynthetic pathway and confirms the central role of late biosynthetic genes in determining anthocyanin accumulation and fruit coloration.

The study of leaf composition demonstrated that season, cultivar, growing site, and thermal regime significantly affect both mineral nutrients and bioactive compounds. Seasonal variation emerged as the major source of variability for most parameters, while cultivar-specific differences were particularly evident for total polyphenols and chlorophyll content. The analysis of thermal parameters revealed significant relationships between temperature accumulation and nutrient dynamics, indicating that environmental conditions strongly influence leaf physiology and mineral balance. In particular, potassium was negatively associated with heat accumulation and positively associated with cooler conditions, whereas several microelements showed contrasting responses to thermal regimes. These findings highlight the complexity of genotype × environment interactions in myrtle and provide useful information for optimizing leaf harvest management and biomass valorisation.

Overall, the results obtained in this doctoral thesis confirm that the chemical composition of myrtle is highly dynamic and regulated by the interaction between genetic and environmental factors. Genotype plays a fundamental role in determining the accumulation of phenolic compounds and the expression of flavonoid biosynthetic genes, while developmental stage, storage conditions, season, and thermal environment modulate these responses. The integration of biochemical, molecular, and environmental approaches adopted in this work provides new knowledge on the mechanisms governing metabolite accumulation in myrtle and contributes to the identification of cultivars and management strategies aimed at maximizing the quality of berries and leaves.

From an applied perspective, the findings of this thesis support the use of pigmented cultivars as valuable sources of anthocyanins and other phenolic compounds for nutraceutical, pharmaceutical, and cosmetic applications, while also highlighting the potential of unpigmented cultivars and leaf biomass as alternative sources of bioactive molecules. Furthermore, the information generated on post-harvest behaviour, phenolic metabolism, gene expression, and mineral composition may contribute to future breeding programmes and to the development of

sustainable production systems capable of enhancing the economic and functional value of myrtle cultivation.

7. Supplementary materials

Chapter 2

Table S1. Sequence of primers used for RT-qPCR analysis.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Amplicones (bp)
ACT	AGATGACCCAGATTATGTTTGAGACCTTC	ACCATCACCAGAATCCAACACAATACC	122
PAL	CAACCCTGTGACCAACCATG	TTCTCTTCCAGGTGCCTCAG	174
CHS	AGTCTTCTGCTCCACCTCTG	GATCTCAGAGCAGACGACGA	199
CHI	GCCACAGATGATGCCTTCTT	CTCTTCCTCCTCCTCCTCGT	200
DFR	CGCGAATTTGCTCAGGAAGA	AGCCCTTTCTCTCTGCATGT	183
FLS	TACTGGTCCCGAACGATGTC	AACACTGCCCATGACATTCG	193
LDOX	AGGTTGGAGAAGGAAGTCGG	AGGATCTCAATGGTGTCCCC	245
UFGT	CCAGAAGAGGACATCGAGCT	GCCGAGAGTCTGCCTGATAT	237
LAR	TGACATCGGGAAGTTCACCA	TGATGATGACACGAGGGAGG	155

Table S1. Mineral composition of myrtle leaves (macroelements) in 2021.[z]

Cultivar	Season	Location	N (%)	P (%)	Na (%)	K (%)	Ca (%)	Mg (%)
Nadia	Spring	Alghero	1.93 ab	0.55 ab	0.06 cde	1.08 a	1.12 abc	0.29 c-f
		Oristano	1.70 cd	0.61 a	0.05 cde	0.92 abc	1.09 abc	0.30 b-e
	Winter	Alghero	1.97 a	0.23 fg	0.07 bcd	0.50 g-j	0.99 abc	0.37 a
		Oristano	1.80 bc	0.30 efg	0.07 bcd	0.40 j	1.12 abc	0.29 c-f
Marta	Spring	Alghero	1.63 de	0.24 fg	0.07 bcd	0.70 def	0.87 c	0.22 fgh
		Oristano	1.58 def	0.61 a	0.06 cde	0.99 ab	1.21 ab	0.31 a-e
	Winter	Alghero	1.65 cde	0.60 a	0.06 cde	0.69 def	0.95 bc	0.24 e-h
		Oristano	1.62 de	0.36 c-f	0.07 bc	0.59 f-i	0.97 bc	0.20 gh
Daniela	Spring	Alghero	1.40 ghi	0.48 a-d	0.05 cde	0.83 bcd	0.88 bc	0.22 fgh
		Oristano	1.52 efg	0.56 ab	0.06 cde	0.77 cde	1.01 abc	0.27 c-g
	Winter	Alghero	1.24 i	0.22 fg	0.04 de	0.58 f-i	0.85 c	0.30 b-e
		Oristano	1.50 efg	0.42 b-e	0.03 e	0.42 ij	1.30 a	0.36 ab
Tonina	Spring	Alghero	1.42 fgh	0.25 efg	0.03 e	0.96 ab	0.94 bc	0.27 c-g
		Oristano	1.37 ghi	0.63 a	0.05 cde	0.96 ab	1.13 abc	0.27 c-g
	Winter	Alghero	1.38 ghi	0.25 efg	0.10 a	0.60 e-h	0.89 bc	0.26 d-g
		Oristano	1.32 hi	0.38 c-f	0.10 a	0.63 efg	0.91 bc	0.21 gh
Grazia	Spring	Alghero	1.89 ab	0.50 abc	0.04 cde	0.77 cde	0.83 c	0.21 fgh
		Oristano	1.39 ghi	0.59 a	0.04 cde	0.83 bcd	1.01 abc	0.33 a-d
	Winter	Alghero	1.39 ghi	0.19 g	0.09 ab	0.45 hij	0.90 bc	0.34 abc
		Oristano	1.31 hi	0.32 d-g	0.09 ab	0.55 f-j	0.84 c	0.18 h

[z]The results are expressed as means of three replicates. Values with different letters are significantly different ($p \leq 0.01$) according to Duncan's multiple range test.

Table S2. Mineral composition of myrtle leaves (microelements) in 2021.[z]

Cultivar	Season	Location	Cu (ppm)		Zn (ppm)		Fe (ppm)		Mn (ppm)	
Nadia	Spring	Alghero	10.83	a-e	21.67	e-i	140.83	bcd	36.67	e
		Oristano	9.17	b-e	16.67	hi	165.00	abc	45.83	de
	Winter	Alghero	11.67	a-d	38.33	a	132.50	b-e	105.00	a
		Oristano	12.50	abc	30.00	a-f	109.17	def	88.33	abc
Marta	Spring	Alghero	10.43	a-e	14.17	i	82.50	efg	60.83	b-e
		Oristano	8.33	cde	21.67	e-i	196.67	a	63.33	b-e
	Winter	Alghero	10.00	a-e	32.50	a-d	112.50	def	110.83	a
		Oristano	14.17	ab	35.00	ab	68.33	fgh	65.00	b-e
Daniela	Spring	Alghero	8.33	cde	20.83	f-i	66.67	fgh	46.67	de
		Oristano	7.50	cde	19.17	ghi	107.50	def	48.33	de
	Winter	Alghero	15.00	a	28.27	a-g	130.83	b-e	101.67	a
		Oristano	9.17	b-e	29.17	a-g	29.17	h	76.67	a-d
Tonina	Spring	Alghero	9.17	b-e	25.00	b-h	124.17	b-e	55.00	cde
		Oristano	10.83	a-e	16.67	hi	171.67	ab	50.83	cde
	Winter	Alghero	7.50	cde	32.50	a-d	132.50	b-e	59.17	b-e
		Oristano	10.83	a-e	31.67	a-e	31.67	h	95.00	ab
Grazia	Spring	Alghero	6.67	de	23.33	d-i	83.33	efg	50.00	de
		Oristano	5.83	e	24.17	c-i	154.17	a-d	57.50	b-e
	Winter	Alghero	11.67	a-d	33.33	a-d	114.17	c-f	48.33	de
		Oristano	12.50	abc	34.17	abc	34.17	gh	30.83	e

[z]The results are expressed as means of three replicates. Values with different letters are significantly different ($p \leq 0.01$) according to Duncan's multiple range test.

Table S3. Calculated hour-degree sums for temperatures above thresholds ranging from 0 to 35 °C as recorded in the two experimental sites from 1st January 2021 to 31st December 2021 used for correlations with mineral leaves content.

Site	Temperature thresholds (°C) for above sums of hour-degrees calculation														
	0 °C	**2,5 °C**	**5 °C**	**7,5 °C**	**10 °C**	**12,5 °C**	**15 °C**	**17,5 °C**	**20 °C**	**22,5 °C**	**25 °C**	**27,5 °C**	**30 °C**	**32,5 °C**	**35 °C**
Alghero															
I sem.	4284	4070	3716	3274	2770	2219	1626	1110	794	557	302	128	17	0	0
II sem.	4416	4416	4383	4209	3864	3410	2726	2031	1499	1077	736	472	267	130	67
Total	8700	8486	8099	7483	6634	5629	4352	3141	2293	1634	1038	600	284	130	67
Oristano															
I sem.	4344	4321	4120	3730	3167	2449	1840	1308	900	643	427	193	37	3	0
II sem.	4416	4416	4415	4388	4148	3685	3083	2465	1896	1368	969	645	417	228	115
Total	8760	8737	8535	8118	7315	6134	4923	3773	2796	2011	1396	838	454	231	115

Table S4. Calculated hour-degree sums for temperatures under thresholds ranging from 0 to 35 °C as recorded in the two experimental sites from 1st January 2021 to 31st December 2021 used for correlations with mineral leaves content.

Site	Temperature thresholds (°C) for under sums of hour-degrees calculation														
	0 °C	**2,5 °C**	**5 °C**	**7,5 °C**	**10 °C**	**12,5 °C**	**15 °C**	**17,5 °C**	**20 °C**	**22,5 °C**	**25 °C**	**27,5 °C**	**30 °C**	**32,5 °C**	**35 °C**
Alghero															
I sem.	60	274	628	1070	1574	2125	2718	3234	3550	3787	4042	4216	4327	4344	4344
II sem.	0	0	33	207	552	1006	1690	2385	2917	3339	3680	3944	4149	4286	4349
Total	60	274	661	1277	2126	3131	4408	5619	6467	7126	7722	8160	8476	8630	8693
Oristano															
I sem.	0	23	224	614	1177	1895	2504	3036	3444	3701	3917	4151	4307	4341	4344
II sem.	0	0	1	28	268	731	1333	1951	2520	3048	3447	3771	3999	4188	4301
Total	0	23	225	642	1445	2626	3837	4987	5964	6749	7364	7922	8306	8529	8645

Table S5. Summary of statistical analysis to assess the influence of cultivar, year, location, and season on the mineral composition (macroelements) of myrtle leaf infusions in 2021 and 2022.[z]

Variables	N		P		Na		K		Ca		Mg	
Cultivar (A)	18.40	**	54.16	**	10.59	**	51.07	**	3.34	*	44.74	**
Year (B)	0.06		189.14	**	238.17	**	42.07	**	10.07	**	41.59	**
AxB	3.90	**	51.83	**	13.28	**	37.51	**	2.78	*	18.45	**
Season (C)	63.94	**	70.95	**	288.49	**	465.88	**	1.47		44.15	**
AxC	2.13		75.68	**	16.78	**	47.24	**	0.70		93.47	**
BxC	0.02		121.09	**	67.96	**	80.45	**	12.75	**	10.81	**
AxBxC	1.77		36.68	**	15.31	**	28.35	**	1.49		50.16	**
Location (D)	5.10	*	17.15	**	204.86	**	459.27	**	0.01		58.04	**
AxD	14.38	**	49.35	**	16.92	**	30.74	**	5.48	**	17.38	**
BxD	63.47	**	8.58	**	17.67	**	33.24	**	1.08		13.69	**
AxBxD	9.86	**	53.38	**	22.82	**	12.43	**	7.04	**	32.77	**
CxD	55.10	**	3.13		190.32	**	550.94	**	0.03		410.65	**
AxCxD	34.92	**	21.51	**	10.54	**	101.11	**	2.07		65.96	**
BxCxD	11.78	**	13.35	**	1.09		30.76	**	0.23		57.92	**
AxBxCxD	15.87	**	51.80	**	11.27	**	85.56	**	2.00		15.18	**

[z]numerical values refer to the calculated F, while significance is indicated as: " " = not significant; "*" = $p \leq 0.05$; "**" = $p \leq 0.01$.

Table S6. Summary of statistical analysis to assess the influence of cultivar, year, location, and season on the mineral composition (microelements) of myrtle leaf infusions in 2021 and 2022.[z]

Variables	Cu		Zn		Fe		Mn	
Cultivar (A)	32.11	**	13.80	**	4.71	**	106.99	**
Year (B)	110.30	**	10.12	**	43.63	**	3157.18	**
AxB	23.60	**	1.26		5.15	**	66.99	**
Season (C)	75.81	**	22.18	**	57.02	**	7915.07	**
AxC	35.46	**	5.03	**	5.27	**	194.04	**
BxC	65.56	**	264.88	**	202.25	**	3015.33	**
AxBxC	55.33	**	17.39	**	2.18		71.46	**
Location (D)	56.58	**	107.50	**	33.14	**	1143.70	**
AxD	47.91	**	0.94		11.29	**	69.72	**
BxD	102.89	**	2.60		25.56	**	961.96	**
AxBxD	40.51	**	8.13	**	9.16	**	72.84	**
CxD	22.63	**	2.21		4.09	*	121.10	**
AxCxD	80.70	**	7.40	**	9.53	**	61.77	**
BxCxD	23.67	**	10.38	**	8.91	**	328.97	**
AxBxCxD	86.87	**	5.51	**	4.21	**	175.82	**

[z]numerical values refer to the calculated F, while significance is indicated as: " " = not significant; "*" = $p \leq 0.05$; "**" = $p \leq 0.01$.

Table S7. Coefficients (*r)* of linear correlation between the principal macroelements and microelements concentration of leaf infusions, observed in the two experimental sites in spring and winter 2021 and 2022.

Variables	N	P	Na	K	Ca	Mg	Cu	Zn	Fe
P	0.099								
Na	0.035	0.255							
K	0.228	0.200	0.106						
Ca	-0.135	0.028	**0.406** **	-0.15					
Mg	0.294	0.034	**0.423** **	**0.321** *	0.242				
Cu	-0.051	0.137	-0.095	-0.269	-0.086	-0.080			
Zn	-0.165	0.156	**0.343** *	0.167	0.205	0.194	0.248		
Fe	-0.130	0.193	0.011	-0.011	-0.025	-0.055	0.211	**0.662** **	
Mn	-0.201	0.083	-0.302	0.256	-0.064	-0.130	0.056	**0.459** **	**0.641** **

*Bold data are significant at $p \leq 0.05$ level; **bold data are significant at $p \leq 0.01$.

Table S8. Coefficients (*r)* of linear correlation between the concentration of mineral macroelements and microelements of infusions leaves, and hour-degree sums for temperatures above thresholds ranging from 0 to 35 °C as observed in the two experimental sites from January 1, 2021, to December 31, 2022.

	Temperature thresholds (°C) for above sums of hour-degrees calculation														
Variable	**0 °C**	**2.5 °C**	**5 °C**	**7.5 °C**	**10 °C**	**12.5 °C**	**15 °C**	**17.5 °C**	**20 °C**	**22.5 °C**	**25 °C**	**27.5 °C**	**30 °C**	**32.5 °C**	**35 °C**
N	**0.384** *	**0.375** *	**0.367** *	**0.339** *	**0.337** *	**0.331** *	0.305	0.297	0.292	0.288	0.282	0.278	0.267	0.239	0.158
P	-0.187	-0.147	-0.172	-0.197	-0.206	-0.201	-0.159	-0.111	-0.092	-0.108	-0.149	-0.144	-0.146	-0.176	**-0.343** *
Na	0.203	0.107	0.117	0.141	0.188	0.280	**0.338** *	**0.335** *	**0.314** *	**0.324** *	0.307	0.310	0.259	0.090	-0.099
K	**-0.371** *	-0.251	-0.245	**-0.320** *	**-0.395** *	**-0.445** **	**-0.483** **	**-0.514** **	**-0.529** **	**-0.525** **	**-0.518** **	**-0.510** **	**-0.514** **	**-0.558** **	**-0.457** **
Ca	-0.005	-0.057	-0.065	-0.049	-0.010	0.048	0.106	0.147	0.158	0.149	0.107	0.108	0.089	0.020	-0.181
Mg	0.127	0.154	0.135	0.087	0.069	0.077	0.079	0.066	0.049	0.052	0.051	0.045	0.009	-0.077	-0.149
Cu	-0.108	-0.032	-0.087	-0.129	-0.151	-0.166	-0.145	-0.136	-0.148	-0.142	-0.119	-0.154	-0.189	-0.164	-0.103
Zn	**-0.408** **	**-0.508** **	**-0.578** **	**-0.587** **	**-0.517** **	**-0.361** *	-0.236	-0.201	-0.212	-0.206	-0.237	-0.274	**-0.363** *	**-0.406** **	**-0.476** **
Fe	**-0.507** **	**-0.606** **	**-0.643** **	**-0.630** **	**-0.555** **	**-0.428** **	**-0.333** *	-0.289	-0.282	-0.284	**-0.325** *	**-0.361** *	**-0.415** **	**-0.401** **	**-0.419** **
Mn	**-0.730** **	**-0.741** **	**-0.770** **	**-0.788** **	**-0.763** **	**-0.700** **	**-0.649** **	**-0.622** **	**-0.616** **	**-0.616** **	**-0.640** **	**-0.673** **	**-0.704** **	**-0.641** **	**-0.515** **

*Bold data are significant at $p \leq 0.05$ level; **bold data are significant at $p \leq 0.01$.

Table S9. Coefficients (*r)* of linear correlation between the concentration of mineral macroelements and microelements of infusions leaves, and hour-degree sums for temperatures under thresholds ranging from 0 to 35 °C as observed in the two experimental sites from January 1, 2021, to December 31, 2022.

	Temperature thresholds (°C) for under sums of hour-degrees calculation													
Variable	**0 °C**	**2.5 °C**	**5 °C**	**7.5 °C**	**10 °C**	**12.5 °C**	**15 °C**	**17.5 °C**	**20 °C**	**22.5 °C**	**25 °C**	**27.5 °C**	**30 °C**	**32.5 °C**	**35 °C**
N	-0.262	**-0.326 ***	**-0.349 ***	**-0.330 ***	**-0.334 ***	**-0.331 ***	-0.303	-0.294	-0.289	-0.282	-0.274	-0.266	-0.243	-0.154	0.198
P	0.082	0.103	0.157	0.192	0.205	0.201	0.157	0.106	0.084	0.099	0.142	0.133	0.129	0.145	0.238
Na	0.277	0.045	-0.048	-0.102	-0.164	-0.268	**-0.331 ***	**-0.327 ***	-0.303	-0.309	-0.285	-0.280	-0.198	0.144	**0.709 ****
K	0.152	0.154	0.202	0.303	**0.391 ***	**0.448 ****	**0.488 ****	**0.521 ****	**0.537 ****	**0.537 ****	**0.535 ****	**0.532 ****	**0.541 ****	**0.599 ****	0.152
Ca	0.176	0.112	0.093	0.066	0.021	-0.043	-0.106	-0.149	-0.161	-0.152	-0.106	-0.106	-0.081	0.038	**0.394 ***
Mg	-0.023	-0.127	-0.123	-0.078	-0.061	-0.072	-0.075	-0.061	-0.041	-0.041	-0.036	-0.023	0.033	0.211	**0.401 ****
Cu	-0.080	-0.037	0.062	0.118	0.146	0.164	0.141	0.133	0.144	0.136	0.107	0.145	0.184	0.126	-0.102
Zn	**0.528 ****	**0.542 ****	**0.610 ****	**0.616 ****	**0.539 ****	**0.373 ***	0.239	0.201	0.213	0.207	0.244	0.290	**0.407 ****	**0.495 ****	**0.431 ****
Fe	**0.525 ****	**0.609 ****	**0.658 ****	**0.647 ****	**0.569 ****	**0.435 ****	**0.334 ***	0.287	0.279	0.279	**0.324 ***	**0.367 ***	**0.435 ****	**0.406 ****	0.184
Mn	**0.480 ****	**0.647 ****	**0.741 ****	**0.781 ****	**0.764 ****	**0.703 ****	**0.649 ****	**0.620 ****	**0.612 ****	**0.611 ****	**0.636 ****	**0.675 ****	**0.709 ****	**0.566 ****	-0.090

*Bold data are significant at $p \leq 0.05$ level; **bold data are significant at $p \leq 0.01$.

Table S10. Calculated hour-degree sums for temperatures above thresholds ranging from 0 to 35 °C as recorded in the two experimental sites from 1st January 2021 to 31st December 2022 used for correlations with mineral leaf infusions components.

Site	Temperature thresholds (°C) for above sums of hour-degrees calculation														
	0 °C	**2,5 °C**	**5 °C**	**7,5 °C**	**10 °C**	**12,5 °C**	**15 °C**	**17,5 °C**	**20 °C**	**22,5 °C**	**25 °C**	**27,5 °C**	**30 °C**	**32,5 °C**	**35 °C**
Alghero															
I sem.	4284	4070	3716	3274	2770	2219	1626	1110	794	557	302	128	17	0	0
II sem.	4416	4416	4383	4209	3864	3410	2726	2031	1499	1077	736	472	267	130	67
III sem.	4319	4217	3990	3618	3038	2327	1634	1042	721	502	294	161	61	15	3
IV sem.	4365	4282	4135	3920	3581	3200	2776	2225	1664	1205	801	509	273	81	12
Total	17384	16985	16224	15021	13253	11156	8762	6408	4678	3341	2133	1270	618	226	82
Oristano															
I sem.	4344	4321	4120	3730	3167	2449	1840	1308	900	643	427	193	37	3	0
II sem.	4416	4416	4415	4388	4148	3685	3083	2465	1896	1368	969	645	417	228	115
III sem.	4341	4331	4261	4008	3461	2580	1722	1156	835	571	343	181	95	22	3
IV sem.	4414	4396	4313	4149	3871	3512	3088	2669	2142	1489	949	645	402	201	40
Total	17515	17464	17109	16275	14647	12226	9733	7598	5773	4071	2688	1664	951	454	158

Table S11. Calculated hour-degree sums for temperatures under thresholds ranging from 0 to 35 °C as recorded in the two experimental sites from 1st January 2021 to 31st December 2022 used for correlations with mineral leaf infusions components.

Site	Temperature thresholds (°C) for under sums of hour-degrees calculation														
	0 °C	**2,5 °C**	**5 °C**	**7,5 °C**	**10 °C**	**12,5 °C**	**15 °C**	**17,5 °C**	**20 °C**	**22,5 °C**	**25 °C**	**27,5 °C**	**30 °C**	**32,5 °C**	**35 °C**
Alghero															
I sem.	60	274	628	1070	1574	2125	2718	3234	3550	3787	4042	4216	4327	4344	4344
II sem.	0	0	33	207	552	1006	1690	2385	2917	3339	3680	3944	4149	4286	4349
III sem.	25	127	354	726	1306	2017	2710	3302	3623	3842	4050	4183	4283	4329	4341
IV sem.	51	134	281	496	835	1216	1640	2191	2752	3211	3615	3907	4143	4335	4404
Total	136	535	1296	2499	4267	6364	8758	11112	12842	14179	15387	16250	16902	17294	17438
Oristano															
I sem.	0	23	224	614	1177	1895	2504	3036	3444	3701	3917	4151	4307	4341	4344
II sem.	0	0	1	28	268	731	1333	1951	2520	3048	3447	3771	3999	4188	4301
III sem.	3	13	83	336	883	1764	2622	3188	3509	3773	4001	4163	4249	4322	4341
IV sem.	2	20	103	267	545	904	1328	1747	2274	2927	3467	3771	4014	4215	4376
Total	5	56	411	1245	2873	5294	7787	9922	11747	13449	14832	15856	16569	17066	17362