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Doctoral School of Biology and Sportbiology

Ph.D. Thesis

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The impact of TiO_2 nanoparticles treatment, developmental, and environmental factors on phenolic content and antioxidant capacity of grapevine (*Vitis vinifera* L.) leaves

Ph.D. Thesis

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List of symbols and abbreviations

Symbols and abbreviations	Meaning
$^{\bullet}OH$	Hydroxyl Radical
$^{1}O_2$	Singlet Oxygen
AOC	Antioxidant Capacity
d	day
DW	Dry Weight
FCR	Folin Ciocalteu Reaction
FLGs	Flavonol glycosides
FRAP	Ferric Reducing Antioxidant Power
H_2O_2	Hydrogen Peroxide
HCA	Hydroxycinnamic Acids
HPLC	High-Performance Liquid Chromatography
K-glc	Kaempferol-3-*O*-glucoside
K-glr	Kaempferol-3-*O*-glucuronide
MO	Methyl Orange
$O_2^{\bullet-}$	Superoxide Radical Anion
Q-gal	Quercetin-3-*O*-galactoside
Q-glc	Quercetin-3-*O*-glucoside
Q-glr	Quercetin-3-*O*-glucuronide
Q-rut	Quercetin-3-*O*-rutinoside
ROS	Reactive Oxygen Species

TEAC	Trolox Equivalent Antioxidant Capacity
TF	Total Flavonol
TiO_2 NPs	Titanium Dioxide Nanoparticles
TPC	Total Phenolic Content
UV	Ultraviolet
λv	The Radiation Energy

1. Literature overview

1. 1. General introduction

TiO_2 is attracting a great deal of research due to its unique physiochemical features, such as crystal phase, size, and morphology (Nakata and Fujishima, 2012). An estimated 7.25 million tonnes of TiO_2 are produced each year across the world. In nature, TiO_2 has four polymorphs, namely anatase, rutile, brookite, and TiO_2 (B) (Reghunath *et al*., 2021). TiO_2 is utilized in a broad range of applications such as paints, inks, sunscreens, cosmetics, toothpaste, paper, plastics, textile, and air/water purification (Lyu *et al*., 2017). Furthermore, TiO_2 NPs are used for plant growth and protection. Applying TiO_2 NPs on plants improves root length, plant height, fresh biomass, chlorophyll content, photosynthesis rate, nutrient uptake, polyphenol content, and antioxidant capacity (Rafique *et al*., 2018). However, not all studies showed a similar pattern of positive effects of TiO_2 NPs on plants. TiO_2 NPs can also show toxic effects on plants depending on several factors, such as crystal phase, size, morphology, and concentration of nanoparticles, as well as plant species and application method (Cox *et al*., 2017). In addition, application of TiO_2 NPs is one of the new strategies to improve growth and plant performance under biotic and abiotic stress (Gohari *et al*., 2020).

Due to their high photocatalytic activity, TiO_2 NPs play a role similar to natural stress in increasing ROS (such as $^{\bullet}OH$, $^1O_2$, $O_2^{\bullet-}$, and H_2O_2). Therefore, plants develop numerous mechanisms to resist these stresses, including enzymatic and non-enzymatic responses (Sharma *et al*., 2012). Phenolic compounds are a large family of secondary metabolites. They play a crucial role as antioxidants. They can be classified into two main groups: non-flavonoids and flavonoids. Non-flavonoids include phenolic acids, stilbenes, and lignans. Flavonoids are classified into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins (Cheynier *et al*., 2012). Flavonols in plants have physiological functions ranging from microbial interactions to pollen fertility and free radical scavenging. However, their most widespread roles appear to be as UV protectants and as co-pigments in flowers and fruit. Quercetin and kaempferol derivatives are the main flavonols found in *Vitis vinifera* species. Of these flavonols, mainly quercetin-3-*O*-glucoside and -3-*O*-glucuronide are found in grapevine leaves (Bouderias *et al*., 2020; Kőrösi *et al*., 2019a; Downey *et al*., 2003).

Besides TiO_2 NPs, many other factors influence the biosynthesis of phenolic compounds, among which the most important is the genotype (variety). Additionally, other factors are related to developmental and environmental factors (Li *et al*., 2020). The metabolism and production of phenolic compounds are affected by seasonal factors, such as precipitation, temperature, and solar radiation intensity. Abiotic and biotic stresses have been extensively studied as single stresses. However, plants are constantly exposed to different combinations of stresses that exceed the damage caused by single stresses. For example, it was shown that combining drought and heat stress had a considerably greater negative impact on the development and production of plants than each stress administered alone. In contrast, heat and salt combination provided a higher degree of tolerance compared with salt stress alone (Rizhsky *et al*., 2002; Rivero *et al*., 2014).

In plants, developmental age is a strong determinant of stress responses. The juvenile-to-adult transition is a holistic process triggered by both endogenous (metabolic, hormonal, and genetic) and exogenous (environmental) factors (Rankenberg *et al*., 2021). Plants are influenced by environmental factors, both biotic (e.g., fungi, viruses, bacteria, insects, and herbivores) and abiotic (e.g., light, heat, cold, salt, and drought), which accelerate ROS accumulation. Production of polyphenol metabolites is one strategy for protection against environmental stress, and the amount of these compounds depends on leaf age. Young leaves contain more flavonoids than older leaves (Masa *et al*., 2016). The larger concentrations of these chemicals in young leaves may allow for greater resistance to stress than in mature leaves by keeping the antioxidant machinery functioning and ROS production at tolerable levels (Fini *et al*., 2011; Loreto *et al*., 2004).

1. 2. Titanium dioxide nanoparticles (TiO_2 NPs) and their effects on plants

1. 2. 1. TiO_2 NPs and their properties

Nanotechnology is a broad field in the twenty-first century, as in the past decades this technology has opened up new applications in many areas such as biotechnology and agro-industry (Gruère *et al*., 2011; Scott *et al*., 2018; Lyu *et al*., 2017), due to the unique physical and chemical properties of nanoparticles, such as crystal phase, size, and particle morphology. TiO_2 NPs are one of the most widely used metal oxide nanoparticles, with an annual production capacity of 7.25 million tonnes (Szymanska *et al*., 2016; Khan *et al*., 2019).

TiO_2 is a white powder, odorless, insoluble, and non-combustible. It is bright and has a high refractive index. It has a molecular weight of 79.9 g mol^{-1}, a relative density of 4.26 g cm^{-3} at 25 °C, and a melting point of 1843 °C (Shi *et al*., 2013). Due to their high catalytic activity and usage in a variety of applications, TiO_2 NPs are mass-produced all over the world. TiO_2 is frequently used as a white pigment, with around four million tons consumed annually as a white color in liquids, pastes, or as a coating on solids. It accounts for 70% of the total amount of dyes produced globally. TiO_2 is also found in plastics, paper, ink, medicines, electronics, and food. It may even be used to whiten skim milk as a color. They are also utilized in cosmetics such as sunscreens because of their UV-protective properties (Shi *et al*., 2013). TiO_2 NPs have antimicrobial activities when exposed to UV light. Furthermore, TiO_2 NPs are employed to filter air and water via catalytic activity under UV radiation (Wang *et al*., 2016).

There are a number of TiO_2 polymorphs including synthetic ones. TiO_2 NPs exist in four main crystalline structures in nature: anatase and rutile comes with a tetragonal structure, brookite with an orthorhombic structure, and TiO_2 (B) (monoclinic) (Fig. 1) (Tanvir *et al*., 2015; Reghunath *et al*., 2021). Anatase and rutile, while occurring naturally, can be synthesized in the laboratory without difficulty, and these forms are those which have been employed most in studies of photocatalysis. Brookite is a naturally occurring phase and is extremely difficult to synthesize. TiO_2 (B) occurs in nature and can also be obtained synthetically in the laboratory. Rutile is considered the most stable phase, while anatase and brookite are transition forms of rutile (Reyes-Coronado *et al*., 2008). Brookite has a larger cell volume (eight TiO_2 groups), which makes it less

stable than the anatase and rutile (two TiO_2 groups) phases. However, TiO_2 (B) exhibits good thermal stability and could be converted to anatase at 800 °C (Reghunath *et al*., 2021).

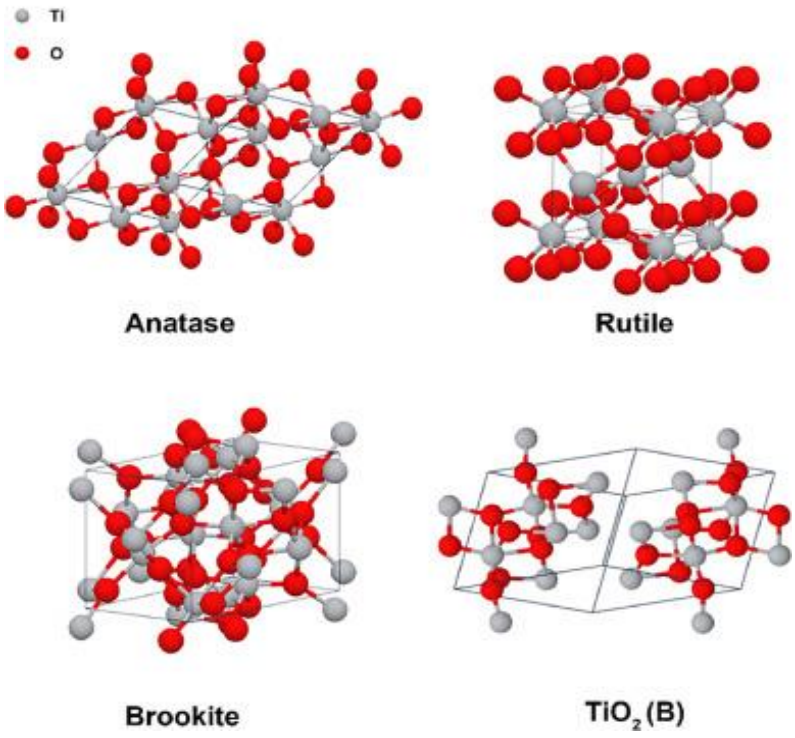


Fig. 1. TiO_2 crystal structures (Reghunath *et al*., 2021).

The band gap energy of a TiO_2 NPs describes the energy needed to excite an electron from the valence band to the conduction band. Anatase has a band gap of 3.2 eV, corresponding to a UV wavelength of 387 nm. In contrast, rutile has a smaller band gap of 3.0 eV with excitation wavelengths that extend into the visible at 410 nm (Kubacka *et al*., 2012). Much of the research is applied to improving the photocatalytic activity by reducing the electron-hole pair recombination and extending absorption to longer wavelengths (to harvest a greater proportion of solar radiation). It is reported that the combination of the anatase and rutile phases of TiO_2 exhibits greater photocatalytic activity than that of pure anatase or rutile phase. Since, the anatase/rutile dual phase in the samples suppresses electron/hole recombination by blocking photoelectrons at the anatase/rutile interface, leading to more efficient separation of the photogenerated electron–hole pairs and increase photocatalytic activity (Scanlon *et al*., 2013). The controversy is over the energetic alignment of the band edges of the rutile and anatase (Fig. 2). Type II (rutile) the transfer

of photogenerated electrons from anatase to rutile, and the transfer of holes from rutile to anatase at a clean interface (Kawahara *et al*., 2002). Type II (anatase) is discussed by Scanlon *et al*. (2013), in the mixed rutile and anatase samples, the electrons move from the rutile to the anatase phase, while the holes move in the opposite direction. Type I level alignment, both electron and hole transfer to the rutile phase (Fig. 2) (Ko *et al*., 2017). Between these three proposed valence and conduction band alignment mechanisms for the anatase/rutile interface, most researchers agree with the second case, i.e., Type II (anatase). The functional properties of TiO_2 NPs are strongly dependent on their size and morphology. The average sizes of anatase and rutile nanoparticles in Degussa P25 have been reported to be 85 nm and 25 nm, respectively (Ohno *et al*., 2001). According to Ko *et al*. (2017), the level of alignment between anatase and rutile varies with size: (i) type II (anatase) for bulk and larger nanoparticles; (ii) type I when at least one nanoparticle type has a diameter of less than 15 nm; and (iii) type II (rutile) when at least one nanoparticle type has a diameter of less than 2.5 nm (Fig. 2).

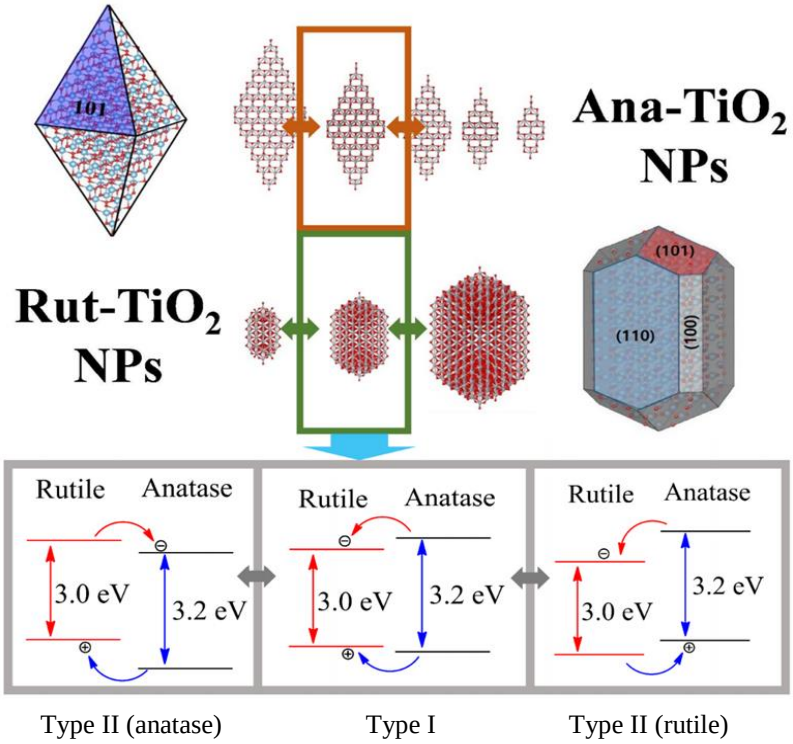


Fig. 2. Schematic diagram showing three proposed valence and conduction band alignment mechanisms for the anatase/rutile size-dependent. Type-II (anatase), Type-II (rutile), and Type-I, with arrows indicating associated charge transfer possibilities (Ko *et al*., 2017).

Degussa P25 is a commercial TiO_2 often used as a benchmark model photocatalyst because of its excellent photocatalytic activity. It contains more than 70% anatase with a minor amount of rutile and a small amount of amorphous phase (Jiang *et al*., 2018). According to Ohtani *et al*. (2010), P25 is 78% anatase, 14% rutile, and 8% amorphous. On the other hand, Datye *et al.* (1995) reported that P25 has no identifiable amorphous phase. The inhomogeneity of P25's crystalline composition is responsible for this variance. This disparity can be explained by the inhomogeneity of the crystalline composition of P25 inside the same package and among various batches of manufacture (Ohtani *et al*., 2010). Several reports show different ratios of anatase and rutile in the samples. The typical crystalline composition of P25 was evaluated to be 70/30 (Bacsa and Kiwi, 1998), 75/25 (Han *et al*., 2018), 77/23 (Fu *et al*., 2018), 80/20 (Bickley *et al*., 1991), 81/19 (Han *et al*., 2018), or 87/13 (He *et al*., 2019) of anatase and rutile, respectively. However, whether the microstructures of the two phases (anatase and rutile) are interwoven or exist separately remains a point of contention. For instance, Ohno *et al.* (2001) found that the rutile phase exists separately from anatase particles. In contrast, Bickley *et al.* (1991) reported that anatase and rutile phases in the P25 powder often appeared in close proximity. As calculated, around 15% rutile nanoparticles more likely exist on the surface of anatase with the formation of a heterojunction structure. In addition, transmission electron microscopy (TEM) measurement shows that some anatase particles are covered with rutile clusters or thin overlayers. Although isolated nanoparticles with sole rutile phase coexist (Jiang *et al*., 2018).

In general, in the inorganic material field, particles show two main types of morphology. The first is the shape of the primary particle, which can be regarded as a single crystal; the second refers to the shape of the secondary particle, which is an agglomeration of primary particles, including hollow, porous, and solid spheres. The primary particles show four types of structures: zero-, one-, two-, and three-dimensional. Many TiO_2 nanostructural materials, such as spheres, nanorods, fibers, tubes, sheets, and interconnected architectures, have been fabricated (Nakata and Fujishima, 2012). The structural dimensionality (or morphology) of TiO_2 NPs can significantly alter their properties and affect their efficiency in photocatalytic reactions (Fig. 3) (Mamaghani *et al*., 2020).

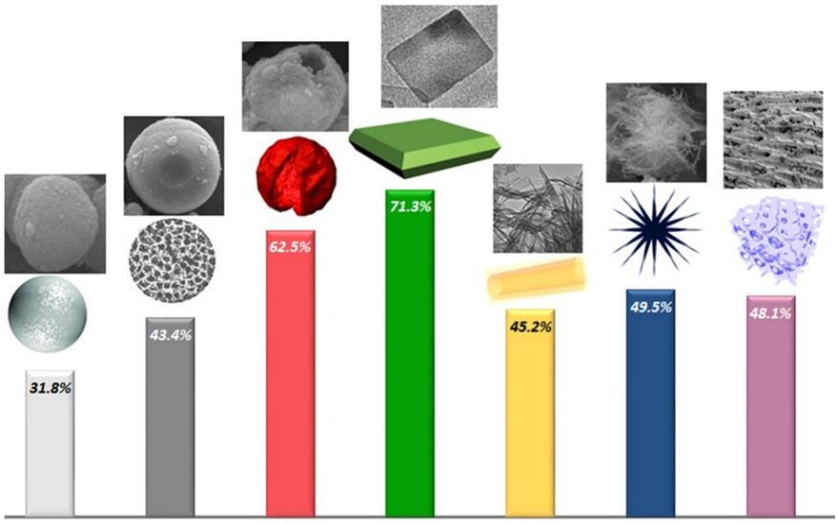


Fig. 3. Displays the comparison efficiency of photocatalytic activity over different morphologies (Mamaghani *et al*., 2020).

For evaluating the morphological control of a primary particle, it is important to know which of its facets are exposed, this is because each crystal facet exhibits different properties. The equilibrium shape of anatase has been reported as a truncated bipyramidal shape constructed by eight {101} and two {001}. A tetragonal prism bounded by {110} and terminated by a pair of tetragonal pyramids bounded by {011} was the equilibrium shape of rutile (Fig. 4) (Wang *et al*., 2019). The most stable plane among the rutile crystal facets was {110}. Theoretical studies demonstrated that anatase {100} facets are more active and accordingly exhibit higher catalytic activity than {001} and {101} facets (Li and Xu, 2010). In other study by Xu *et al.* (2013), {111} facet exhibited higher photocatalytic activity in comparison to {001}, {101}, and {010} facets.

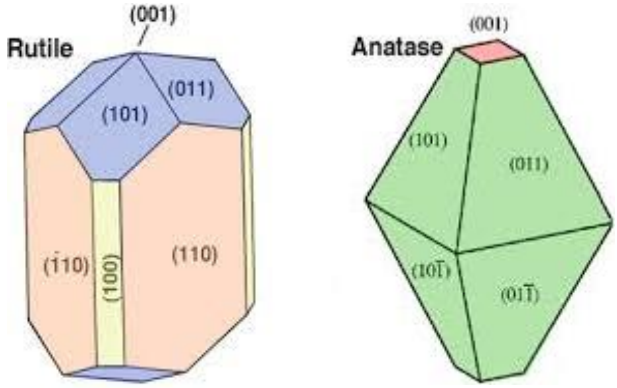


Fig. 4. The equilibrium shape of rutile and anatase (Wang *et al*., 2019).

1. 2. 2. Photocatalytic property

Of a great number of oxide semiconductors, TiO_2 is the most widely used as a promising photocatalyst because of its high photocatalytic efficiency. Photocatalysis is based on the activation of TiO_2 NPs by light. When a TiO_2 NPs material is irradiated with photons whose energy is higher or equal to its band gap energy, a promotion of an electron from the valence band (VB) to the conduction band (CB) occurs with the concomitant generation of a hole in the valence band VB as shown in Fig. 5. The photo-generated charge carriers (electrons and holes) react with the donor (e.g., H_2O) or acceptor (e.g., O_2) molecules adsorbed on the surface of TiO_2 NPs, thus producing ROS ($^{\bullet}OH$ and $O_2^{\bullet-}$, respectively). The overall process can be described by the following reactions:

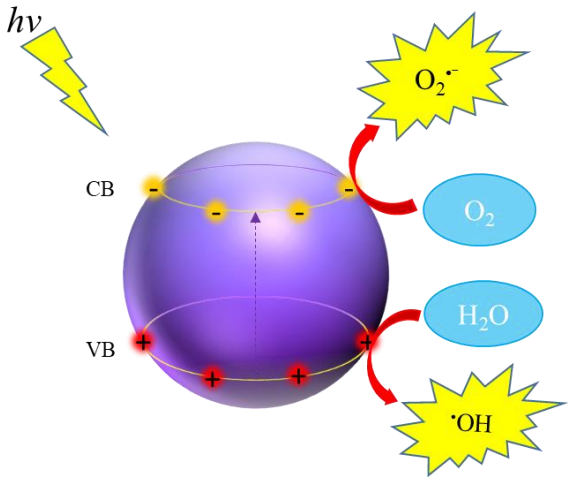


Fig. 5. Schematic formation of $O_2^{\bullet-}$ and $^{\bullet}OH$ radicals on TiO_2 nanoparticles (Kőrösi *et al*., 2019b).

- $TiO_2 + h\nu \rightarrow e^-_{CB} + h^+_{VB}$ (1)
- $e^-_{CB} + O_2 \rightarrow O_2^{\bullet-}$ (2)
- $h^+_{VB} + H_2O \rightarrow {}^{\bullet}OH + H^+$ (3)

In the presence of a contaminant organic molecule, which is adsorbed on the catalyst surface, the $^{\bullet}OH$ radical reacts to produce adducts, followed by the fragmentation of the molecular structure into several intermediate species until the total mineralization of the contaminant is completed with the formation of CO_2 and H_2O. The success of the photocatalytic process is therefore strictly dependent on the competition between the reaction of the electron with water on the TiO_2 NPs surface and the electron-hole recombination process that releases heat or radiation (Kőrösi *et al*., 2016; 2019b).

1. 2. 3. Effects of TiO_2 NPs on plants

It is largely accepted that TiO_2 NPs exposure results in the cellular generation of ROS, leading to both positive and negative impacts on plant growth. Numerous studies have clarified the benefits of TiO_2 NPs for plants, which may directly or indirectly improve their growth. Plants grown under a wide range of TiO_2 NPs resulted in plants with greater root length, plant height, and fresh biomass. In addition, chlorophyll content, photosynthesis rate, and antioxidant system were improved (Li *et al*., 2015). TiO_2 NPs' interactions with plants create a variety of biochemical changes. For instance, the external application of TiO_2 NPs to maize increased anthocyanins content in comparison with control (Morteza *et al*., 2013). Szymanska *et al.* (2016) showed that TiO_2 NPs can elevate the antioxidant level of *Arabidopsis thaliana*.

In contrast, some evidence shows that TiO_2 NPs have a negative impact on plants. TiO_2 NPs toxicity depends on plant species, concentration and particle size of NPs, and exposure conditions. Not all species exhibit the ability to resist the effects of NPs. There have been several studies investigated the genotoxic potential of TiO_2 NPs on different species. For instance, *V. narbonesis* L. the dose at which genotoxicity was found to be significant began at 2%, while significant toxic dosage for *Zea mays* L. began at the lowest concentration of 0.2% (Cox *et al*., 2017). Several reports have described the negative and toxic effects of high concentrations. High concentrations of TiO_2 NPs mainly result in the elevated production of ROS, followed by chlorophyll degradation and cellular toxicity. The toxic effects of TiO_2 NPs have been reported in barley (Feizi *et al*., 2012), tobacco (Hou *et al*., 2019), onion (Filho *et al*., 2019), wheat (Silva *et al*., 2019), and spinach (Fenoglio *et al*., 2009) plants. Furthermore, the surface area of NPs, their reactive nature and tendency to aggregate are other possible reasons for their toxicological effects. In addition, the NPs application method, via soil, hydroponics, or foliar delivery, has been shown to have different efficiencies (Zhao *et al*., 2020).

TiO_2 NPs are used as elicitors to protect plants from a variety of biotic and abiotic challenges. They enhanced different defense mechanisms involved in plant tolerance. Pretreatment with TiO_2 NPs significantly alleviated the stress of UV-B radiation on *Arabidopsis thaliana.* In addition, TiO_2 NPs activated the antioxidant system of plants, improved the activity of antioxidant enzymes, and promoted the synthesis of flavonoids (Wang *et al*., 2021). Moreover, it has been shown that the foliar application of TiO_2 NPs could also improve salinity and drought stresses

tolerance of wheat and cotton plants (Mustafa *et al*., 2021; Shallan *et al*., 2016). TiO_2 NPs have also shown significant antimicrobial and antibacterial activity. Satti *et al.* (2021) used TiO_2 NPs with a size of 40–65 nm and a spherical shape on wheat affected by a fungal disease. It was demonstrated that TiO_2 NPs reduced the disease severity, thus ultimately improving the quality and yield of wheat plants.

1. 3. Reactive oxygen species (ROS) and their role in plants

When oxygen is incompletely reduced, ROS are formed (Sharma *et al*., 2012). $O_2^{\bullet -}$, H_2O_2, $^1O_2$, and $^{\bullet}OH$ make up the majority of it. They are prevalent in a variety of subcellular compartments such as chloroplasts, mitochondria, and peroxisomes, because of the intense metabolic activity that normally occurs in these compartments (Gill and Tuteja, 2010). These chemical species can operate as intracellular signaling molecules, which are crucial for controlling a wide range of physiological processes of living organisms (Das and Roychoudhury, 2014). The fundamental biochemical features of ROS underpin the mechanisms necessary for living organisms' development. However, their overproduction induces oxidative stress, which can damage lipids, proteins, and deoxyribonucleic acid (DNA) (Sharma *et al*., 2012; 2019; Gill and Tuteja, 2010).

A number of biotic and abiotic stimuli that enhance the intracellular levels of ROS disrupt the balance between the generation and scavenging of ROS. When the level of ROS is increased and exceeds the defense mechanisms, the cell is in a state of oxidative stress. As a result, during times of stress, defense systems against oxidative damage are triggered in order to control dangerous levels of ROS (Sharma *et al*., 2012; Das and Roychoudhury, 2014). The intracellular balance between generation and removal must be precisely controlled and/or effectively processed. This is necessary to minimize possible harm caused by ROS to cellular components as well as sustain plant growth, metabolism, development, and overall productivity. Antioxidants, both enzymatic and non-enzymatic, keep the balance between ROS generation and detoxification (Sharma *et al*., 2012; Huang *et al*., 2019).

Understanding the oxidative mechanisms in plants may aid in developing plants more adapted to the environment. Plant stress tolerance has been linked to the preservation of strong antioxidant capacity to eliminate hazardous amounts of ROS. Several studies have shown that keeping a high amount of antioxidants in a plant's cells can help it defend itself from oxidative

damage by rapidly scavenging harmful levels of ROS and restoring redox homeostasis (Carvalho *et al*., 2015).

1. 4. The effect of developmental and environmental factors on phenolic content in plants

Biosynthesis of phenolic compounds starts from the shikimic acid pathway and subsequently diversifies, largely depending on cell type, developmental stage, and environmental cues (Patra *et al*., 2013). Phenolic compounds are widely distributed in different plant cells, tissues, and organs, which may possess different phenolic compounds at different developmental stages (Bartwal *et al*., 2013). Phenolic compounds are mainly genetically influenced. However, environmental factors such as nutrition, temperature, and lighting conditions can also affect their synthesis and accumulation (Brouillard and Dangles, 1994; Mol *et al*., 1998; Harborne, 1980) because the phenolic compound pathways and their regulation are particularly sensitive to environmental changes.

1. 4. 1. Effect of developmental factors on phenolic compounds

Age of a plant is a powerful determinant of its responses to stress. Differential vulnerability to diverse environmental stresses is widely observed at both the organ and whole-plant levels. Both endogenous (ROS, hormones, and genetic) and exogenous (environmental) factors impact the holistic process of transition from juvenile to adult (Huang *et al*., 2019). Any morphological, physiological, or biochemical change that occurs as a result of differential regulation of developmental processes can be considered an Age-related changes (ARCs), even though not all ARCs are apparent. Because ARCs are irreversible, they can be viewed as accumulating rather than transient. Therefore, ARCs are observable events that cumulatively describe the aging process (Rankenberg *et al*., 2021). For instance, the onset of leaf senescence is age-dependent and includes a complex interaction of internal and environmental factors that influence its timing, progression, and completion (Lee and Masclaux-Daubresse, 2021).

The changes that occur in a plant during its growth are strongly influenced by the way it interacts with the environment (Juvany *et al*., 2013). When plants are exposed to abiotic stress, ROS levels typically rise. Reducing these ROS is an important aspect of dealing with abiotic stresses. A complex antioxidant network evolves in plant cells to scavenge ROS and so regulate their levels according to the requirements of cell signalling (Xia *et al*., 2015). Because of

differences in the capacity to generate signaling cascades and acclimative responses, old and young leaves have varied acclimatization capabilities (Huang *et al*., 2019). Transitions from the juvenile to adult phase are marked by gradual changes in leaf polyphenols. Phenolic contents protect plants from biotic and abiotic stress, and their density generally decreases in developing leaves. For instance, the youngest leaves appear red due to the accumulation of anthocyanins, which are gradually lost during greening (Barker *et al*., 1997; Liakopoulos *et al*., 2006). Stages of leaf development influence the phytochemical and antioxidant properties of the leaf. Therefore, phenolic compounds are generally different for each position (age) of leaves (Anwar *et al*., 2017). For example, Campa *et al.* (2017) found that hydroxycinnamic acids and flavonoids were marketably different in young, mature, and old leaves. Schoedl *et al.* (2012) examined the concentrations of sixteen polyphenols for three specific age groups selected to carry out leaf developmental stages. They showed that there is a difference in the levels of polyphenol concentrations between the positions of the leaves in the shoot. Phenylalanine ammonia-lyase and shikimate dehydrogenase levels were highest during the earliest stages of leaf development and decreased progressively as the leaves matured (Blume and McClure, 1979). High levels of anthocyanins in young leaves are correlated with a low risk of photoinhibition (Manetas *et al*., 2002). While the mature leaves contained more phenolics and flavonoids than the young and old *Aquilaria beccariana* leaves (Anwar *et al*., 2017). In contrast, Liu *et al.* (2020) reported that after maturation, flavonols increased, whereas flavanols and phenolic acids decreased. The total catechin content was reported to be higher in young leaves than in old ones. FLGs were analyzed in the leaves of six currant cultivars (*Ribes* spp.). The results suggest that the genetic background of the cultivars is quite important. Furthermore, the time of collection and leaf position had a greater impact on the composition than the year of harvest or the growing latitude (Yang *et al*., 2015).

1. 4. 2. Effect of environmental factors on phenolic compounds

Plants can continually modify phenolic compounds to adapt to the demands of an ever-changing environment (Mannino and Micheli, 2020). These compounds are primarily synthesized to combat abiotic stress such as high and low temperatures, drought, salinity, and UV stress (Imran *et al*., 2021; Isah, 2019), and biotic stress as bacteria, fungi, insects, and nematodes (Gimenez *et al*., 2018). Phenolic compounds are continually produced in plants, while other or more recent

amounts are formed in response to stress signals (Pollastri and Tattini, 2011; Ramakrishna and Ravishankar, 2011). Table 1 shows the effect of environmental factors on phenolic compounds, TPC, and AOC in plants.

Seasons: are characterized by a number of environmental factors that influence plant development throughout the year, including soil composition, temperature, drought, rainfall, sunshine intensity, and day length. Phenolic levels are changed throughout plant development in response to these changes to avoid any harm. Hydrolysable tannins were detected in large quantities during wet seasons, but flavonoids were predominantly produced during dry seasons. These findings show that climate change may be one of the factors influencing plant phenolic compound levels (Santos *et al*., 2011). In the leaves of *C. paliurus*, there was a considerable seasonal fluctuation in phenolic compounds, with the maximum level appearing in May, July, and November (Cao *et al*., 2019). Furthermore, total phenols, flavonoids, flavonols, and stilbenes were highest in leaves of six grape varieties gathered in September compared to other seasons (Katalinic *et al*., 2013; 2009). FLGs levels in black currant (*Ribes nigrum* L.) leaves rose from July to August, peaking in early October in one variety and late August in other varieties (Liu *et al*., 2014). The FLGs, on the other hand, are found to be at their peak levels in late July to mid-August, followed by a drop in currant leaves (Yang *et al*., 2015).

Drought: drought frequently causes a rise in oxidative stress and, as a result, an increase in the amount of polyphenol content. For example, polyphenol content increased in grapevine leaves treated by drought stress (Griesser *et al*., 2015; Cui *et al*., 2017). In addition, the duration and severity of stress can influence the phenolic content of plants differently. Short-term drought stress enhances the polyphenol content in leaves (Nakabayashi *et al*., 2014; Ma *et al*., 2014). Long-term drought stress, on the other hand, was shown to lower the overall concentration of phenols, phenolic acids, and antioxidant activity in grape leaves and roots (Król *et al*., 2014). Plant tissues containing anthocyanins are usually drought-resistant. For example, a study showed that the drought resistance of purple cultivars is better than that of green cultivars (Bahler *et al*., 1991). Nakabayashi *et al.* (2014) showed that over-accumulation of anthocyanins enhanced tolerance against oxidative stress and dehydration. Flavonoids have protective functions during drought stress. Differential accumulation of flavonoids and hydroxycinnamate in the leaves of *Ligustrum vulgare* under excessive light and drought stress (Tattini *et al*., 2004).

Temperature: temperature strongly influences phenolic compounds. Low temperatures decrease total phenolic concentrations in grapevine (*Vitis vinifera*, Himrod cultivar), but the phenolic acids are increased. While total antioxidant activity was lower after low temperature (+10 °C daytime and +7 °C at night) than in unstressed leaves (Król *et al*., 2015; Amarowicz *et al*., 2010). Temperature variations have multiple effects on the polyphenols in plants. Several studies have examined the effects of increased temperatures on phenolic production in plants (Shamloo *et al*., 2017).

Light and UV irradiation: many experts have agreed that light has a substantial impact on phenolic compounds. According to several research studies, there is a positive relationship between increased light intensity and the amounts of polyphenols in plants. For example, exposure of grape leaves to sunlight activated biosynthesis of light-responsive phenols in order to avoid the harmful effects of light stress (Kocsis *et al*., 2015). It was also observed that there was an effect of the type and duration of radiation on the accumulation of polyphenols (Blancquaert *et al*., 2019; Del-Castillo-Alonso *et al*., 2015; Morales *et al*., 2010; Tegelberg *et al*., 2004). The synthesis of hydroxycinnamic acids (HCA) was stimulated by strong visible light, but flavonoid production was particularly enhanced by UV radiation (Kolb *et al*., 2001). Under low UV conditions, higher amounts of HCA were found when compared to flavonoids. Conversely, when solar UV radiation was increased, a decrease in the amount of HCA was observed in conjunction with an intense accumulation of flavonoids (Bidel *et al*., 2007; Burchard *et al*., 2000), especially the increased concentration of flavonols when UV-B is enhanced (Ryan *et al*., 1998; Gregan *et al*., 2012). In addition, prolonged exposure to UV rays or high doses has been observed to increase the concentration of flavonols in both exposed and unexposed tissues (Bidel *et al*., 2015).

Other environmental factors, including salinity, soil type and composition, wounding, metal ions, circadian rhythm, and geography, clearly impact the synthesis and accumulation of phenolic compounds (Verma and Shukla, 2015). Salt stress often creates both ionic as well as osmotic stress in plants, resulting in the accumulation or decrease of polyphenols in plants. Phenolic contents are reported to increase in response to salt stress in grapevine leaves (Mohammadkhani, 2018). Furthermore, an increase in polyphenol content with increasing salinity has been observed in a number of plants (Parida *et al*., 2002; Mane *et al*., 2010; Ksouri *et al*.,

2007). Sarker and Oba (2020) showed augmentation in polyphenol yield with high antioxidant capacity in both sensitive and tolerant varieties.

In nature, plants are unlikely to be exposed to biotic or abiotic stressors in isolation. Multiple stressors such as heat, drought, salinity, and pathogen attack are more likely to occur. Because stress reactions are often antagonistic, predicting molecular responses to many stressors based on single stress data is difficult, if not impossible. Researchers have just lately begun to investigate multiple-stress interactions, discovering, for example, that plant responses to a combination of heat and drought differ from those to each single stress (Rizhsky *et al*., 2002; Rivero *et al*., 2014).

Plants exposed to a combination of two or more stimuli exhibited a distinct physiological and biochemical stress response that could not be predicted based on exposure to the individual stresses. These responses are affected by interactions between the different stresses, which can act either antagonistically or synergistically. For instance, an antagonistic interaction resulting from a combination of heat and drought stress was demonstrated in tobacco. However, the heat and salt combination showed a synergistic effect, which provided a higher degree of tolerance compared with salt stress alone. Therefore, the results gained from the limited number of studies on stress combinations emphasize the gap between the information acquired by most single stress studies and the knowledge needed to develop crops that are tolerant to suboptimal field conditions (Rizhsky *et al*., 2002; Rivero *et al*., 2014).

Table 1. The phenolic compounds, TPC, and AOC of many plants are affected by environmental factors.

Phenolic compounds, TPC, and AOC	Environmental factor	Significant change in concentration	Plant species	Plant parts	References
Polyphenol	Severe drought stress	Increased	*Vitis vinifera* L.	Leaves	Griesser *et al*., 2015
Phenolic acids	Long-term drought stress	Decreased	*Vitis vinifera* L.	Leaves and roots	Król *et al*., 2014
Anthocyanin	Drought stress	Increased	*Vitis vinifera* L.	Leaves	Cui *et al*., 2017
TPC	Drought stress	Increased	*Vitis vinifera* L.	Roots	Weidner *et al*., 2009
Flavonoids	Water deficit	Increased	*Arabidopsis*	Leaves	Nakabayashi *et al*., 2014;
Flavonols	Water deficit	Increased	*thaliana* L.	Shoots and roots	Shojaie *et al*., 2016
Flavonoids and HCA	Excess light and drought	Increased	*Ligustrum vulgare*	Leaves	Tattini *et al*., 2004
Phenolics	Cold stress	Decreased	*Vitis vinifera L.*	Leaves	Król *et al*., 2015
TPC and AOC	Low-temperature stress	Decreased	*Vitis vinifera L.*	Leaves	Amarowicz *et al*., 2010
Phenolic acids	Low-temperature stress	Increased	*Vitis vinifera L.*	Leaves	Amarowicz *et al*., 2010
Total phenolic acids and total flavonoids	Increased temperature	Increased	*Triticum spp.*	Whole	Shamloo *et al*., 2017
Anthocyanin	Low temperature and light	Increased	*Vitis vinifera L.*	Berries	Azuma *et al*., 2012
Anthocyanin	Hight temperature and dark	Decreased	*Vitis vinifera L.*	Berries	Azuma *et al*., 2012
Phenolic content	Light	Increased	*Vitis vinifera L.*	Leaves	Kocsis *et al*., 2015

p-caffeoyl-tartaric acid and myricetin-3-*O*-glucoside	UV	Increased	*Vitis vinifera L.*	Leaves	Del-Castillo-Alonso *et al*., 2015
Flavonols, flavanols, and stilbenes.	UV	Unchanged	*Vitis vinifera L.*	Leaves	Del-Castillo-Alonso *et al*., 2015
TPC and flavonol	UV-B	Increased	*Vitis vinifera L.*	Berries	Gregan *et al*., 2012
HCA	Visible light	Increased	*Vitis vinifera L.*	Leaves	Kolb *et al*., 2001
Flavonoids	UV light	Increased	*Vitis vinifera L.*	Leaves	Kolb *et al*., 2001
Phenolic content	UV radiation	Increased	*Betula pendula*	Leaves	Morales *et al*., 2010; Tegelberg *et al*., 2004
Flavonol	UV-B	Increased	*Centella asiatica*	Leaves	Bidel *et al*., 2015
Quercetin and kaempferol	UV-B	Increased	*Petunia*	Leaves	Ryan *et al*., 1998
HCA and flavonoids	UV-A and UV-B	Increased	Rye and three woody species	Leaves	Burchard *et al*., 2000; Bidel *et al*., 2007
Phenolic content	Salinity stress	Increased	*Vitis vinifera L.*	Leaves and roots	Mohammadkhani, 2018
Polyphenol content	Salinity stress	Increased	*Cakile maritima*	Leaves	Ksouri *et al*., 2007

1. 5. Phenolic compounds

Phenolic compounds are a chemically diverse group of approximately 10,000 distinct chemicals, some of which are soluble only in organic solvents, others are water-soluble carboxylic acids and glycosides, yet others are insoluble macropolymers (Taiz and Zeiger, 2012). Because of their chemical diversity, phenolics play a number of roles in plants. Many of them are anti-herbivore and anti-pathogen chemicals. Mechanical support, attracting pollinators and seed dispersers, absorbing harmful UV radiation, and inhibiting the development of neighboring competitive plants are some of the other roles (Mierziak *et al*., 2014; Ferreyra *et al*., 2012; Samanta *et al*., 2011).

The shikimic acid pathway and the malonic acid pathway are the two primary pathways through which plant phenolics are biosynthesized (Taiz and Zeiger, 2012) (Fig. 6). Most phenolics are biosynthesized through the shikimic acid pathway. Erythrose-4-phosphate is coupled with phosphoenolpyruvate (PEP) to create phenylalanine. Then, by the removal of an ammonia molecule, Phenylalanine ammonia-lyase (PAL) catalyzes the conversion of phenylalanine to trans-cinnamic acid. Many phenolic compounds, such as flavonoids, undergo this reaction, which is a crucial regulatory step in their production (Sharma *et al*., 2019). Polyphenol end products are transported to a variety of intracellular or extracellular locations, with the majority of them ending up in the vacuole (Braidot *et al*., 2008).

Polyphenols can be classified into two main groups: non-flavonoids and flavonoids. Non-flavonoids include phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), stilbene, and lignan. Flavonoids are classified into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins (Cheynier *et al*., 2012). Flavonoids are the most common and widespread group of phenolic compounds. The primary carbon structure of flavonoids contains 15 carbons. The chemical structures of this class of compounds are based on a C6-C3-C6 skeleton. They are arranged in two aromatic rings (Rings A and B) linked to an oxygenated homo-cyclic ring (Ring C) (Teixeira *et al*., 2013) (Fig. 7). The heterocyclic ring is closed in most flavonoids, but remains open in chalcones and dihydroclons. The difference in the oxidation and substitution state of the C ring determines the different classes of flavonoids (Panche *et al*., 2016). The primary carbon flavonoid structure may contain many substituents. Hydroxyl groups are usually found at positions 4, 5, and 7, but they can also be found at other positions. The majority of flavonoids are

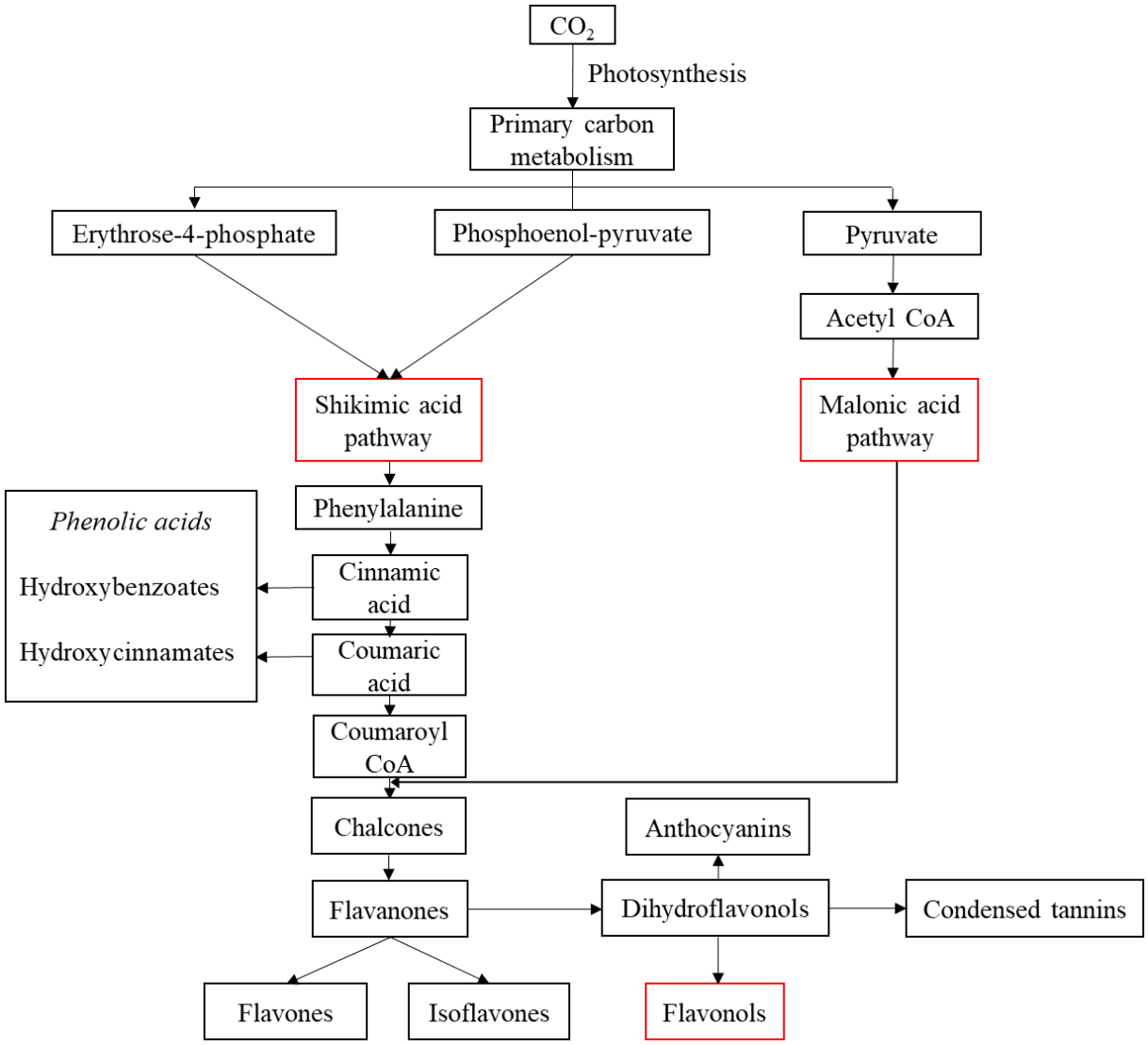


Fig. 6. A simplified view of the major pathways of phenolic compounds biosynthesis (Taiz and Zeiger, 2012).

naturally found in the form of glycosides (Hichri *et al.*, 2011). While both hydroxyl groups and sugars increase the water solubility of flavonoids, other substitutes, such as methyl ether or modified units, make flavonoids lipophilic (hydrophobic), and flavonoids are classified based on the degree of oxidation of the three-carbon bridge.

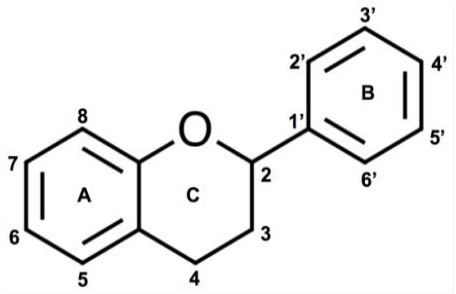


Fig. 7. Flavonoid ring structure and numbering (Teixeira *et al.*, 2013).

Flavonols are products of the flavonoid biosynthetic pathway, which also gives rise to both anthocyanins and condensed tannins in plants. Flavonols or 3-hydroxyflavones differ from many other flavonoids in that they have a double bond between positions 2 and 3 and an oxygen (ketone group) at position 4 of the C ring, like flavones. However, they differ from them by having a hydroxyl group at position 3. Most flavonols are found in the form of *O*-glycosides and rarely in the form of *C*-glycosides, and their conjugated derivatives (glycones) are primarily related to sugars and hydroxycinnamic acids, or organic acids (Iriti and Faoro, 2009). Flavonols in plants have physiological functions ranging from microbial interactions to pollen fertility and free radical scavenging. However, their most widespread roles appear to be as UV protectants and as copigments in flowers and fruit. Copigmentation is an association between flavonols and anthocyanin pigments that confers stability on the coloured form of the anthocyanin molecule, resulting in increased colour or altered hue. Flavonols are considered to act as UV- and photo-protectors because they absorb strongly at both UV-A and UV-B wavelengths (Taiz and Zeiger, 2012).

1. 6. Grapevines

The grape plant (*Vitis vinifera* L.) is one of the oldest and most important crops in the world. The total area harvested from grapes in the world is estimated at about 7.5 million hectares. Moreover, it has great economic importance and is the most widely cultivated species, which is used in many products (OIV, 2019). The grapevine (*Vitis vinifera*) belongs to the family Vitaceae (Fig. 8), which comprises about 60 inter-fertile wild *Vitis* species distributed in Asia, North America, and Europe under subtropical, Mediterranean, and continental–temperate climatic conditions. *Vitis vinifera* is the single *Vitis* species that has acquired significant economic interest over time because of its traits, such as early ripening, berry size, yield, musky flavor, fertility, sugar content, and acidity. This species reproduces well because it has hermaphrodite (sometimes female) flowers, but is highly susceptible to different diseases, such as phylloxera, powdery mildew, and downy mildew, that can limit plant productivity and cause a severe yield reduction. Some other *Vitis* species are used as rootstocks due to their resistance to phylloxera, cryptogamic diseases, lime, drought, and salinity tolerance. Therefore, many grape breeders have crossed *Vitis vinifera* with other resistant species or interspecific complex hybrids (*V. amurensis*, French-American hybrids, etc.) to obtain high-quality and disease-resistant grapes. For example, the North

American *V. rupestris*, *V. riparia*, or *V. berlandieri* are used as breeding rootstock due to their resistance against grapevine pathogens, such as phylloxera, oidium, and mildews. *V. amurensis* is not very sweet and acidic, but this species is characterized by greater resistance to cold (-40 °C), as well as greater resistance to powdery mildew and downy mildew (Keller, 2015; Reynier, 2012). Currently, there are about 5,000 to 10,000 varieties of *Vitis vinifera* grapes, varying in appearance and fruit quality, distributed throughout Europe and most of Asia.

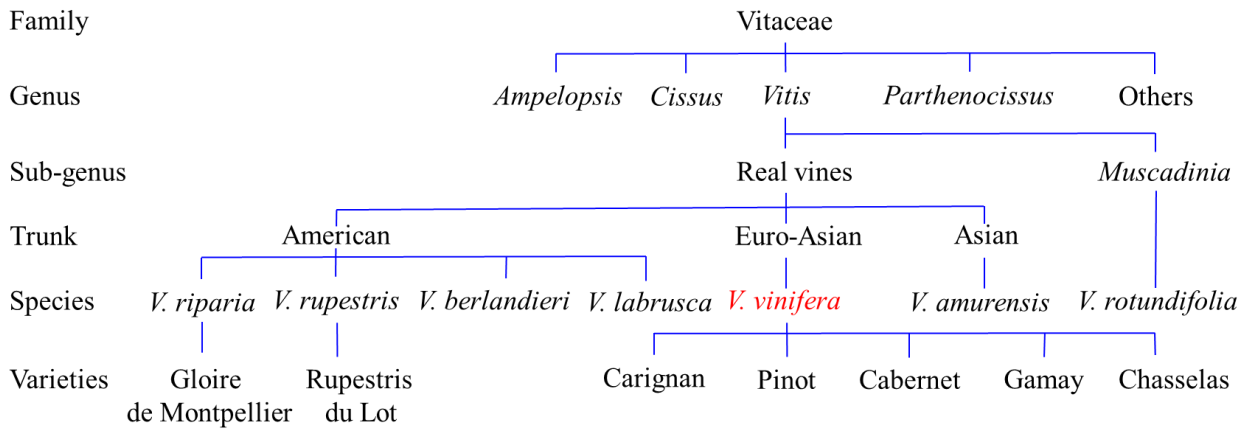


Fig. 8. Vitaceae family (Reynier, 2012).

1. 6. 1. Phenolic compounds in grapevines leaves

In addition to studies of berries, the phenolic composition of other parts of grapes, such as roots, stems, and leaves have also been studied. There are at least 183 phenolic compounds identified, which are distributed differently along the length of the grape. Grape leaf petioles and blades have two to three times the antioxidant capacity of berries, according to studies on antioxidant activity (Hmamouchi *et al*., 1996; Doshi *et al*., 2006). The leaves contain 132 different phenolic compounds, the majority of which are phenolic acids, flavonoids, and coumarin (Goufo *et al*., 2020). Flavonols are the most abundant flavonoids in grape leaves, accounting for around 83% of total phenol levels. In the literature, at least 35 compounds derived from four glycones have been identified: myricetin, quercetin, kaempferol, and isorhamantin (Goufo *et al*., 2020; Goufo *et al*., 2019; Hmamouchi *et al*., 1996; Teszlák *et al*., 2018; Kőrösi *et al*., 2019a; Bouderias *et al*., 2020). Fig. 9 shows some of the chemical structures of FLGs. Grape leaves are employed in the pharmaceutical, cosmetic, and food sectors because they are abundant in phenolic compounds

and have high antioxidant potential. They are used to treat various diseases, such as hypertension, diarrhea, and varicose veins (Dani *et al*., 2010). Furthermore, grape leaf extract has anti-inflammatory, anti-pain, and antipyretic properties (Aouey *et al*., 2016).

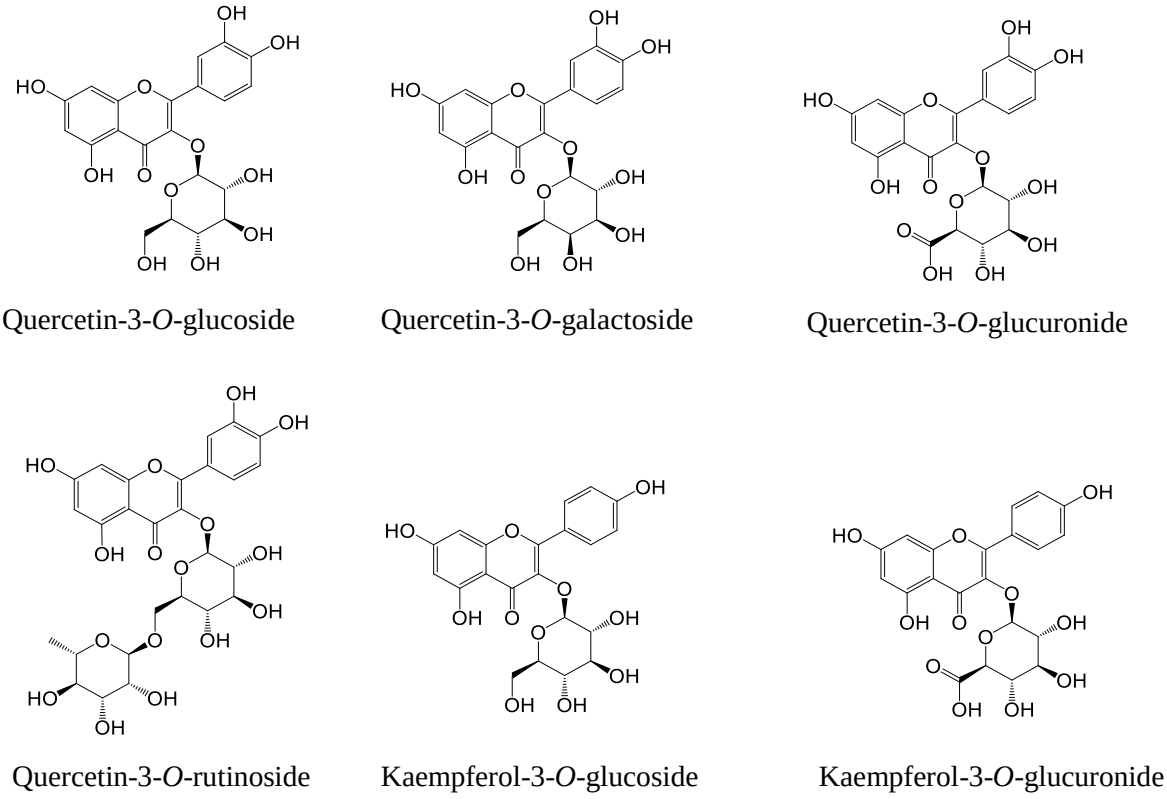


Fig. 9. Chemical structures of flavonol glycosides (Taiz and Zeiger, 2012).

2. Aims of the thesis

Phenolic compounds are produced primarily in plants for their growth, development, and protection. However, its accumulation in plants is affected by many environmental and developmental factors.

The main aims of this study are to investigate the effects of TiO_2 NPs on phenolic content and antioxidant capacity in the leaves of five grapevine varieties. In addition, the effects of leaf position (age) and seasons on flavonol glycosides distribution in Cabernet Sauvignon leaves are presented.

To address these points, we studied the following:

1. Impact of TiO_2 NPs treatment on phenolic compounds in the leaves of five grapevine varieties

1. 1. To what extent can the TiO_2 NPs treatment affect the polyphenol levels and antioxidant capacity in the leaves of different grapevine varieties?1. 2. Is there a relationship between phenolic compounds and antioxidant capacities, and can this relation be affected by TiO_2 NPs treatment?
1. 3. Can phenolic profiles and antioxidant capabilities be utilized as chemotaxonomic characteristics to distinguish grapevine varieties?

2. The age and season dependent changes in the distribution of flavonol glucoside in Cabernet Sauvignon leaves.

2. 1. How are FLGs distributed depend on the leaf position?
2. 2. Does the FLGs content of the leaves depend on the season?
2. 3. Is there a relationship between different FLGs, and can this relation be affected by seasons?

3. Materials and Methods

3. 1. Experimental site

A field experiment was performed on the southfacing slopes of the Mecsek Hills in Hungary at the Research Institute for Viticulture and Oenology's central station (University of Pécs). The soil was a Ramann-type brown forest soil mixed with clay formed on red sandstone covered by Pannonian sediment. Meteorological data such as natural broadband UV radiation, precipitation, temperature, and relative humidity were monitored using the WS600 automatic weather station (Lufft GmbH, Germany) equipped with a CUV5 radiometer (Kipp & Zonen, Deft, the Netherlands). The automatic weather station was set up 100 m close to the experimental site in both cases. According to our meteorological information between 1950 and 2010, the location receives 782 mm of precipitation per year, 2021 hours of yearly sunlight, and an annual mean temperature of 11.6 °C. During the experimental period, the microclimate was ideal for the vines' development (Teszlák *et al*., 2013; Kőrösi *et al*., 2019a; Bouderias *et al*., 2020).

3. 2. Experimental design

3. 2. 1. Treatment of leaves with TiO_2 NPs (Degussa P25 TiO_2 NPs)

Twenty-three-year-old vines of *Vitis vinifera* L. varieties Cabernet Franc, Cabernet Sauvignon, Kadarka, Kékfrankos, and Merlot were investigated under non-irrigated conditions. Each variety was grafted on a generally used rootstock (Teleki 5C, *Vitis berlandieri* x *Vitis riparia* hybrid). Vines were grown with 2 × 1 m vine spacing with north-south row orientation on mid-high cordon trellis system under standard management practice of grape gene bank in our institute. The five varieties studied have different origins and taxonomic positions according to ampelographic classifcations: Cabernet Franc, Cabernet Sauvignon, Kadarka (convarietas pontica), Kékfrankos (convarietas orientalis), and Merlot (convarietas occidentalis). The five varieties have the same exposure, they are close to each other (on the same tarrace) and growing in similar soil and microclimatic conditions, because of typical set up of gene bank collection (10 vine stock per cultivars). During the TiO_2 experiment 8 bud per m^2 (2 x 1 m row and vine spacing) crop load was used in the mid-high cordon trellis system.

Mature and healthy sun-adapted leaves from the 3^{rd}–5^{th} nodes were used for the measurements. TiO_2 NPs treatment was performed on 23 May 2017 using 1 mg ml^{-1} Degussa P25 TiO_2 dispersion in high purity deionized water without any additives. The leaves were treated by using simple manual sprayer. Some milliliters of dispersion were sprayed onto the adaxial surface of leaves until the dispersion covered them homogenously and then allowed to dry (Fig. 10). Control leaves were treated solely with deionized water. After two weeks of treatment, five control and five treated leaves per vine stock were collected from each variety. Three individual vines were chosen for the treatments. The collected grapevine leaves were dried at 35 °C in dark for 24 hours and then grounded in a porcelain mortar.

Fig. 10. Grapevine leaf treated with Degussa P25 TiO_2 NPs.

3. 2. 2. The collection of leaf samples, taking into account their position (age)

On the south-facing slopes of the Mecsek Hills, thirteen-year-old vines of *Vitis vinifera* L. cultivar 'Cabernet Sauvignon' were studied in non-irrigated open-field conditions. The vines were grafted using a T5C rootstock (*Vitis berlandieri* x *Vitis riparia*). In a vertical shoot positioned umbrella training method, vines were grown with 3.5 x 1.2 m vine spacing and an East-West row orientation. The grape growing technology was convencional in case of plant protection and the canopy management, but of course we did not use toping and leaf removal during the leaf position experiment. After the pruning, the normal canopy management was the shoot positioning and shoot selection with focusing to the optimal canopy structure of the given training system. During the leaf position experiment the normal crop load was using with 20-24 buds per vine.

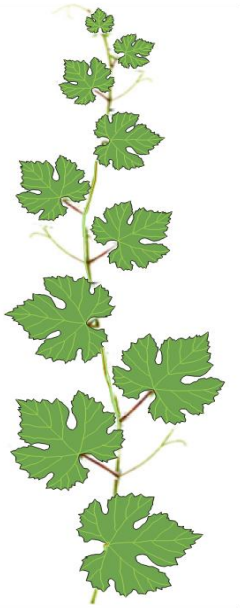


Fig. 11. Drawing of Cabernet Sauvignon shoot, from base (old leaf) to apex (young leaf).

On June 14th and September 10th, 2018, leaf samples were gathered from randomly selected shoots of nine individual vines (Fig. 11). Three shoots from different vines were merged, and leaves from the same leaf positions were pooled. Because the developmental stage of the shoots differed significantly between the two seasons, the shoots in June and September had 28 and 42 leaf levels (one leaf per node), respectively. We established a correlation system between leaf position and matching leaf age according to the BBCH scale based on our phenological monitoring of the variety Cabernet Sauvignon during the vegetative season (Lorenz *et al*., 1995; Coombe, 1995; Duchene *et al*., 2010). The grapevine leaves were harvested and lyophilized. The lyophilized leaves were ground to a soft powder using a mortar and pestle.

3. 3. Sample preparation and extraction

25 mg of each powder sample was extracted with 1.0 ml of 60% (v/v) aqueous methanol solution acidified with formic acid (1% (v/v)), and subsequently sonicated in water bath for 30 min. The resulting suspensions were centrifuged at 20,660 × g and the supernatants were filtered through 0.22 μm PTFE syringe filters (Labex Ltd., Hungary). The obtained supernatants were

analyzed by using HPLC and different assays for the determination of total phenolics and antioxidant capacity.

3. 4. Methods

3. 4. 1. Photocatalytic test of Degussa TiO_2 NPs

The photocatalytic activity of commercial Degussa P25 TiO_2 NPs was tested by the degradation of methyl orange (MO) as a model compound. For the photocatalytic test, 50 ml of 0.05 w/v% aqueous dispersion containing 10 mg L^{-1} MO was used. The photocatalytic test was performed in an acidic aqueous dispersion (pH = 5.1 ± 0.1). Prior to the light exposure, the dispersion was stirred in the dark for 30 min. The dispersion was irradiated at room temperature for different times (for one hour; 2 ml of the sample was collected every ten minutes) by using a 15-W UV-A light source (F15 W T8 BL368, Sylvania). The distance between the dispersion and the lamp was 10 cm (Fig. 12). Before analysis, the dispersion was centrifuged at 22,660 × g for 10 min. The concentration of MO was determined by UV-vis spectroscopy (Shimadzu UV-1800).

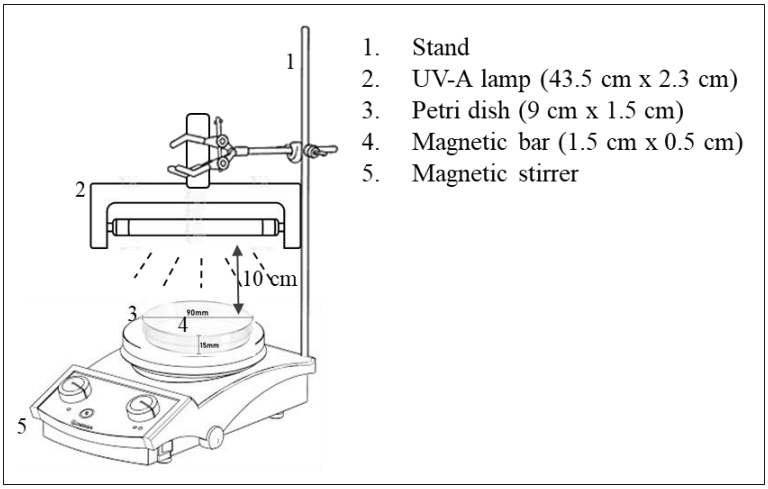


Fig. 12. Schematic draw describes the photocatalytic test

3. 4. 2. Measurement of scavenging of H_2O_2

25 ml of leaf extract (Cabernet Sauvignon) was mixed with 200 µl H_2O_2 in beaker of 100 ml. Prior to the light exposure, the dispersion was stirred in the dark for 30 min. The dispersion was irradiated at room temperature for one hour (1 ml of the sample was collected every ten minute) by using a 15-W UV-A light source (F15 W T8 BL368, Sylvania). The distance between the dispersion and the lamp was 5 cm. Before analysis, the dispersion was centrifuged at 22,660 × g for 10 min and the supernatants were filtered through 0.22 µm PES syringe filters (Labex Ltd., Hungary). The obtained supernatants were analyzed by using HPLC.

3. 4. 3. High-performance liquid chromatography analysis (HPLC-DAD)

Chromatographic analysis was performed using a Perkin Elmer Series 200 HPLC system and a Phenomenex Kinetex® 2.6 µm XB-C18 100 Å, 100 × 4.6 mm column. The column temperature was kept constant at 25 °C. In the mobile phase, (A) 0.1% formic acid and (B) a mixture of 0.2% formic acid and acetonitrile (1:1) were used. The flow rate was 1 ml min^{-1}. The elution program (Table 2) was comprised of isocratic and both linear and nonlinear gradient steps for the separation. A diode array detector (DAD) at 330 nm (for caftaric acid) and 350 nm (for flavonols) measured the absorbance of 5 µl of methanolic extract injected into the HPLC system. Analytical standards with known concentrations were used to create quantification calibration curves.

Table 2. Elution profile for HPLC analysis.

Step	time, min	A, %	B, %	Curve
0	15	100	0	0
1	5	100	0	0
2	3	90	10	0
3	5	80	20	-9
4	8	60	40	-2
5	4	30	70	1
6	3	0	100	0

3. 4. 4. Total phenolic content (TPC): Folin-Ciocalteu Reaction (FCR) assay

FCR assay was used to quantify total phenolic content (Folin and Ciocalteu, 1927). In a cuvette, 20 µl of 10x diluted leaf extracts were mixed with 500 µl of diluted Folin-Ciocalteu reagent (1:10). After 5 min of incubation, 500 µl of Na_2CO_3 (6% w/v) solution was added. A spectrophotometer was used to measure the absorbance at 760 nm after 90 min of incubation at room temperature. Total phenolics in leaf samples were expressed as µmol of gallic acid equivalent per mg of DW using a gallic acid calibration curve.

3. 4. 5. Ferric-reducing antioxidant power (FRAP)

FRAP provide the Fe^{3+} reducing capacity of the leaf extracts measuring the absorbance change of ferrous TPTZ complex (Szőllősi and Szőllősi Istvánné Varga, 2002). FRAP reagent was prepared by mixing 12.5 ml of acetate buffer (300 mM, pH 3.6), 1.25 ml of TPTZ solution (10

mM TPTZ in distilled water) and 1.25 ml of 20 mM $FeCl_3$ solution (in 40 mM HCl). 10 µl of diluted leaf extracts were added to 190 µl of FRAP reagent in microplate wells. After 30 min incubation at room temperature, absorbance was measured at 620 nm in plate reader. Calibration curve was made with ascorbic acid, and FRAP data of leaf samples were expressed as µmol ascorbic acid equivalent per mg DW.

3. 4. 6. Trolox equivalent antioxidant capacity (TEAC)

TEAC measurements were based on the method reported by Re *et al.* (1999) adapted for plant materials as described earlier (Majer and Hideg, 2012). The main reaction is the reduction of $ABTS^{\bullet+}$, which was prepared by mixing 9.7 ml of phosphate buffer (50 mM, pH 6.0), 100 µl of ABTS (0.1 mM), 100 µl of horse radish peroxidase (0.0125 mM) and 100 µl of H_2O_2 (1 mM). After 15 min incubation at room temperatures, 10 µl of leaf sample extract was added to 190 µl of $ABTS^{\bullet+}$ solution, and then the conversion of the cation radical into colorless ABTS was followed by measuring the absorbance at 651 nm. Calibration curve was made with trolox and TEAC data of leaf samples were expressed as µmol trolox acid equivalent per mg DW.

3. 5. Chemicals and reagents

For liquid chromatography, acetonitrile and methanol (LiChrosolv® Reag. Ph Eur, Merck, Germany) were used as gradient grade. Molar Chemicals Ltd., Hungary, provided formic acid (Alfa Aeasar, 97%). Extrasynthese (Genay, France) provided reference substances for Q-rut, Q-glc, Q-glr, K-glc, and K-glr. Dimethyl sulfoxide, DMSO, (≥99.9%, Sigma-Aldrich), 2,4,6- tris (2-pyridyl)-s-triazine, TPTZ, (98%, Sigma-Aldrich), 2,2′-azino-bis (3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt, ABTS, (≥98%, Sigma-Aldrich), 4-oxo-2,2,6,6-tetramethylpiperidine, TMPO, (99.4%, Molar Chemicals Ltd., Hungary) were used as received. 5,5-dimethyl-1-pyrroline N-oxide, DMPO, was synthesized as previously described, and it was freshly distilled before usage. To get highly pure water, an ultra-pure water system (LaboStar™ 7 TWF-UV, Germany) was employed.

3. 6. Statistical analysis

Statistical analyses were carried out using IBM SPSS 24.0 (IBM SPSS Inc., Chicago, IL, USA). Standard deviation and paired t-test were calculated on all data sets. Results were considered statistically significant at $P < 0.05$. Pearson correlation coefficients were calculated to

determine the degree of correlation of CA, FLGs, FCR, FRAP, and TEAC, for both control and treated leaves. The greater absolute value of Pearson coefficient (r) indicates stronger correlation at 0.05 and 0.01 level of signifcance. Multivariate analyses were conducted by means of canonical discriminant functions to evaluate the similarity of grapevine varieties using CA, FLGs, FCR, FRAP, and TEAC of control leaves.

4. Results and discussion

4. 1. Impacts of TiO_2 NPs treatment on phenolic compounds in grapevine leaves

4. 1. 1. Photocatalytic activity of P25 TiO_2 NPs

Commercial TiO_2 (Degussa P25) is widely accepted as the benchmark because of its excellent photocatalytic activity. MO is a popular anionic azo dye that is harmful to both the environment and organisms. The photocatalytic performance of P25 TiO_2 NPs was examined by measuring the decomposition of MO under UV irradiation. For the photocatalytic assay, 50 mL of 0.05% w/v P25 aqueous dispersion containing 10 mg L^{-1} MO was used. Before exposure to light, the dispersion was stirred in the dark for 30 min. Then, it was exposed to UV-A light for an hour. The photocatalytic degradation of MO results is shown in Fig. 13a. Irradiation of an aqueous solution of MO in the presence of P25 TiO_2 NPs leads to its decomposition. Without the presence of P25 TiO_2 NPs, for all considered times, any change in the MO concentration was not observed. In Fig. 13b, the color change of MO was examined in the absence and presence of P25 TiO_2 NPs**.** The color of MO solution did not change in the absence of P25 TiO_2 NPs, but the color of dispersion varied progressively over irradiation duration in the presence of P25 TiO_2 NPs (Fig. 13b). Furthermore, Fig. 13c shows the typical UV-vis spectrum of MO (10 mg L^{-1}) in the presence and absence of the P25 catalyst at different irradiation times. As it can be seen, the intensity of the peak at 464 nm progressively decreases within 60 min of irradiation time. In fact, after 60 min, there was a degradation of more than 76% MO, whereas there was no change in the absence of P25 (Fig. 13c). Thus, the degradation of more than 76% of MO during UV light in a short time (60 min) confirms the high photocatalytic activity of P25 TiO_2 NPs. Different experiment conditions can influence the photocatalytic activity of P25 TiO_2 NPs, such as concentration of P25 and MO, and irradiation time. Guettai and Ait Amar (2005) performed experiments with P25 TiO_2 (0.8 g L^{-1}) and a different concentration of MO under UV irradiation and in the dark. In the case

of (≤50 mg/L), complete degradation of MO was achieved (97.4%) after 5 hours of UV irradiation. However, no degradation of the MO was seen in the dark and in the presence of TiO_2.

These findings may be explained by the large band gap value, which delays the electron-hole recombination process and hence boosts photocatalytic activity (Fig. 13d). Mixed-phase TiO_2 (anatase/rutile) has been shown to have better photocatalytic activity than pure anatase. Because electron transit from the rutile to the anatase phase minimizes anatase recombination, more effective electron-hole separation and better catalytic activity are achieved (Scanlon *et al*., 2013; Hurum *et al*., 2003). The controversy is over the energetic alignment of the band edges of the rutile and anatase. Ko *et al.* (2017) suggest three alignment types that are size-dependent: Type I, Type II (rutile), and Type II (anatase). However, most research agrees with Type II (anatase) (Scanlon *et al*., 2013; Tobaldi *et al*., 2019). Our transmission electron microscopy (TEM) measurement shows that the average size of the P25 TiO_2 NPs in our sample is about 28 nm (Kőrösi *et al*., 2019a), which also agrees with type II (anatase). Besides size, TiO_2 morphology and crystal phase also have an influence on photocatalytic activity. TiO_2 photocatalytic activity tested by using different toxic organic pollutants in soil, air, or water. TiO_2NPs have been shown to degrade organic pesticides and herbicides in soils (Mir *et al*., 2014; Li *et al*., 2016), and also for decontaminating toxic organic pollutants in air and water treatment (Mamaghani *et al*., 2020; Lazar *et al*., 2012). Seven TiO_2 morphologies were synthesized and evaluated for the photocatalytic oxidation (PCO) of methyl ethyl ketone (MEK) in air (Mamaghani *et al*., 2020). TiO_2 nanosheets outperformed other morphologies with a removal efficiency of 71.3%, which was roughly two times higher than commercial P25 (Mamaghani *et al*., 2020). A synergistic effect between two phases, two facets, or other conditions such as H_2O_2 may impact TiO_2 photocatalytic activity. Titania nanosheets' excellent performance was attributed to the high percentage of exposed {001} facets and the synergistic effect of {001} and {101} facets (Mamaghani *et al*., 2020). As shown in this study, the synergistic was between two phases (anatase and rutile) in P25. In addition, it has been demonstrated that rutile is highly effective in the photodegradation of MO in the presence of H_2O_2. By using 4.4 mM H_2O_2, ~90% of total decolorization was achieved after 2 min of UV-A irradiation. However, MO deterioration with Degussa P25 TiO_2 was only 7% under the same testing conditions (Kőrösi *et al*., 2019b).

Furthermore, the photocatalytic activity of TiO_2 polymorphs has been frequently debated in the literature. It is generally accepted that anatase has higher photocatalytic activity than the

rutile phase. A study has been carried out to examine the photocatalytic activity of anatase and rutile TiO_2 NPs by demonstrating their efficient photocatalytic degradation of various organic dyes under both short and long irradiations. Rutile was shown to be just as efficient as anatase in degrading indigo carmine (IC) dye. But anatase was shown to be substantially more effective against methylene blue (MB), methyl orange (MO), rhodamine B (RB), and eriochrome black T (EBT) under short UV irradiation. Under long UV irradiation, the photodegradation study of these organic dyes also showed similar trends to both anatase and rutile. However, for EBT photodegradation, the results revealed that both phases had identical photocatalytic activity. The maximal degradation efficiency employing anatase and rutile titania photocatalysts was reported to be 88% and 77% for MB under short UV irradiation, respectively, and roughly 65% for EBT under long UV irradiation. Even after five cycles, the photocatalyst retained a degradation efficiency of 83% for anatase and 71% for rutile when tested against MB (Gautam *et al*., 2016).

Regarding the mechanism of the photodegradation of MO by TiO_2, the path begins with the excitation of TiO_2 with UV light to produce the electron-hole pair, which reacts with water and oxygen to generate ROS. Finally, these types of ROS lead to the degradation of the MO, as shown in the next equation and Fig. 13d.

$$\text{ROS } (O_2^{\bullet-},\ {}^{\bullet}OH,\ {}^1O_2) + MO \longrightarrow CO_2 + H_2O$$

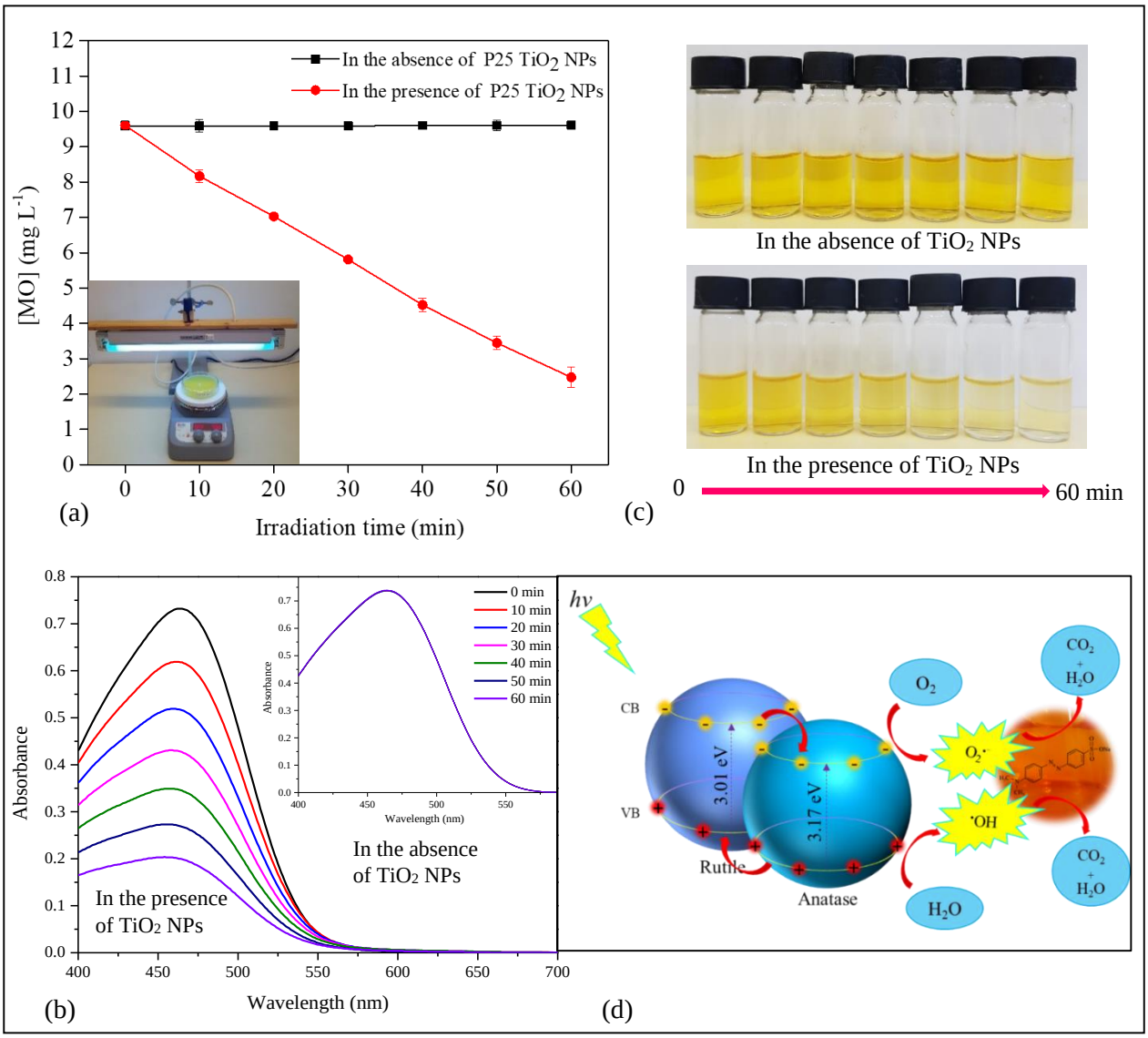


Fig. 13. Photocatalytic degradation of MO using Degussa P25 TiO_2 NPs. The concentration of TiO_2 NPs was 0.5 mg ml^{-1} in dispersion and the MO concentration was 10 mg L^{-1}. (a) Photocatalytic activity of MO, (b) Dispersion color change over 60 min with and without P25, (c) UV-VIS spectrum of MO in the presence and absence of P25 catalyst at various irradiation times, (d) Schematic of the valence and conduction band alignment mechanisms for anatase and rutile of P25 TiO_2 NPs.

4. 1. 2. Phenolic compounds in grapevine leaves of five varieties

Typical HPLC-DAD chromatograms of methanolic leaf extract of five varieties are presented in Fig. 14. Seven phenolic compounds have been identified, with the highest level recorded for Q-glr, followed by CA, Q-glc, K-glr, Q-rut, K-glc, and Q-gal. All the varieties showed the same sorts of phenolic compounds. However, they possess minor differences in the individual compound concentrations. Among the varieties examined in this research, the highest amount of CA was found in Merlot (4568 mg kg^{-1} DW), followed by Kékfrankos (4086 mg kg^{-1} DW) and Cabernet Franc (4065 mg kg^{-1} DW), while the lowest content of CA was determined in Cabernet Sauvignon and Kadarka 1356 mg kg^{-1} DW and 1320 mg kg^{-1} DW, respectively. Considering the FLGs concentration levels per variety, the leaves of Merlot tend to contain the highest levels of Q-glr and K-glr than the other varieties. However, Cabernet Franc is characterized by the highest content of Q-glc and Q-gal (2610 mg kg^{-1} DW and 630 mg kg^{-1} DW, respectively), as well as K-glc (996 mg kg^{-1} DW). When compared to the other four varieties, Kékfrankos has a higher concentration of Q-rut (Fig. 14).

Phenolic acids and flavonols play multiple functions in plants, such as UV protection, pigmentation, and growth (Agati and Tattini, 2010). Their distribution varies according to the plant organ, seasons, plant age, and geographically (Doshi *et al*., 2006; Park and Cha, 2003; Bouderias *et al*., 2020; Pantelic *et al*., 2017). For example, Doshi *et al.* (2006) showed that phenolic compounds are accumulated in different concentrations in berries, leaves, and shoots. Also, Park and Cha (2003) reported that the concentration of flavonols contained in the grape leaves was higher than that obtained from the skin. Furthermore, we previously demonstrated that FLGs in Cabernet Sauvignon grapevine leaves were age and season sensitive (Bouderias *et al*., 2020). The phenolic content of 22 grapevine leaves from various varietal sources in Serbia was examined. Leaf extracts were primarily characterized by phenolic acids, flavonols, and flavan-3-ols, albeit quantities varied depending on geographical origin (Pantelic *et al*., 2017). In this study, we found that both CA and FLGs accumulate in various amounts in grapevine leaves from different varieties. We suggest that such differences are likely due to the genetic characteristics of the grape. Thus, each variety of grape has an effect on phenolic content and profiles. As a result, the capability of each variety to confront the stress differs from one variety to another.

The high concentration of CA and Q-glr indicates their importance in grapevine leaves. These compounds may have an important role in leaf defense against UV damage. Several studies agree that these two compounds are produced in high concentrations in the plant due to their UV-protective benefits (Bouderias *et al*., 2020; Kőrösi *et al*., 2019a).

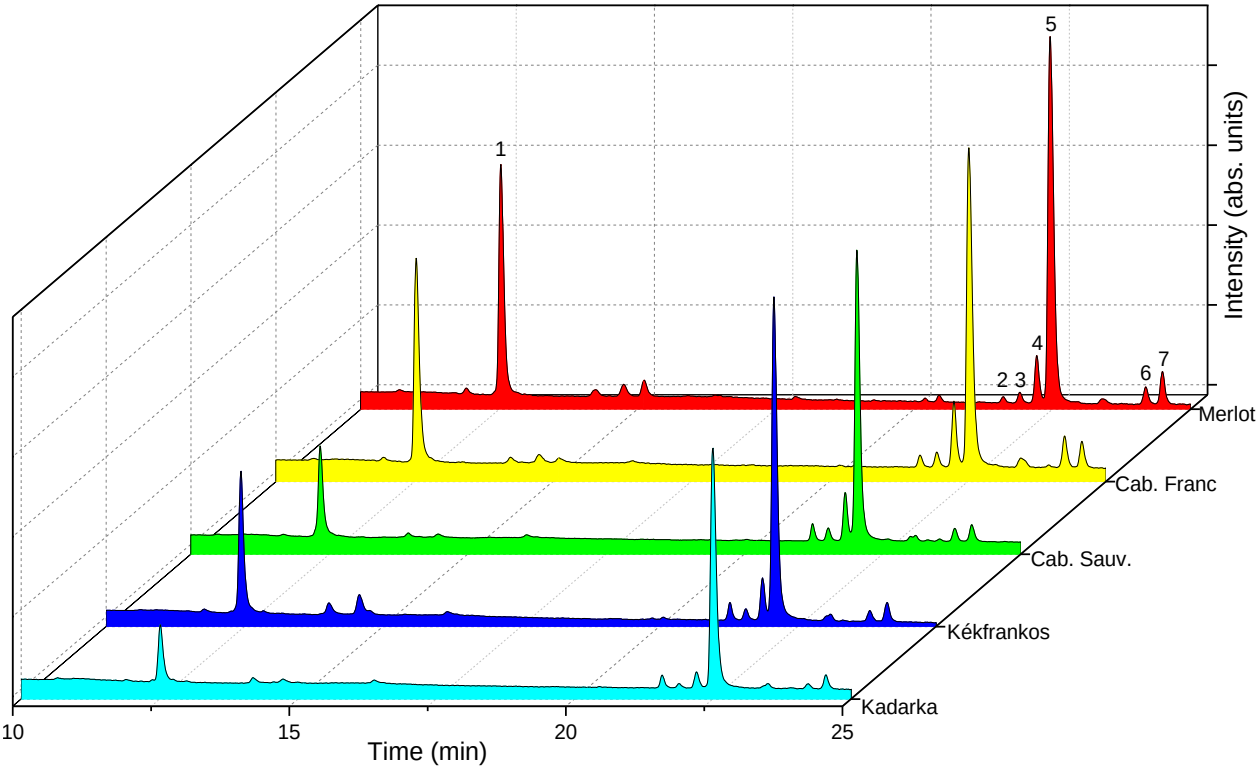


Fig. 14. Typical HPLC-DAD chromatograms of different grape leaf extracts recorded at 350 nm. (1) CA, (2) Q-rut, (3) Q-gal, (4) Q-glc, (5) Q-glr, (6) K-glc, and (7) K-glr.

4. 1. 3. Impacts of TiO_2 NPs on caftaric acid and flavonol glycosides

Several studies have shown that ROS plays an important role in plant abiotic stress responses by activating stress-response and defense pathways (Mittler, 2017; You and Chan, 2015). Due to their high photocatalytic activity, TiO_2 NPs play a role similar to natural stress in increasing ROS (such as $^{\bullet}OH$, $^1O_2$, $O_2^{\bullet-}$, and H_2O_2). Therefore, plants develop numerous mechanisms to resist these stresses, including enzymatic and non-enzymatic responses (Fig. 15). Secondary metabolites are one of the stress response mechanisms. Phenolic compounds, a family of specialized metabolites that include phenolic acids and polyphenols such as flavonols and

anthocyanins and have significant radical scavenging action, help to reduce oxidative stress. In our study, we evaluated CA and FLGs to investigate the response of the non-enzymatic defense mechanism of grapevine leaves treated with TiO_2 NPs. Figs. 16 and 17 show that CA and FLGs are significantly influenced by TiO_2 NPs treatment. CA level was found to be high in all the treated leaves, with the exception of the Kékfrankos variety, which did not show change after treatment (Fig. 16). In addition, TiO_2 NPs also boosted the biosynthesis of FLGs, with the exception of Q-glr which decreased in treated leaves, especially in Kékfrankos (Fig. 17).

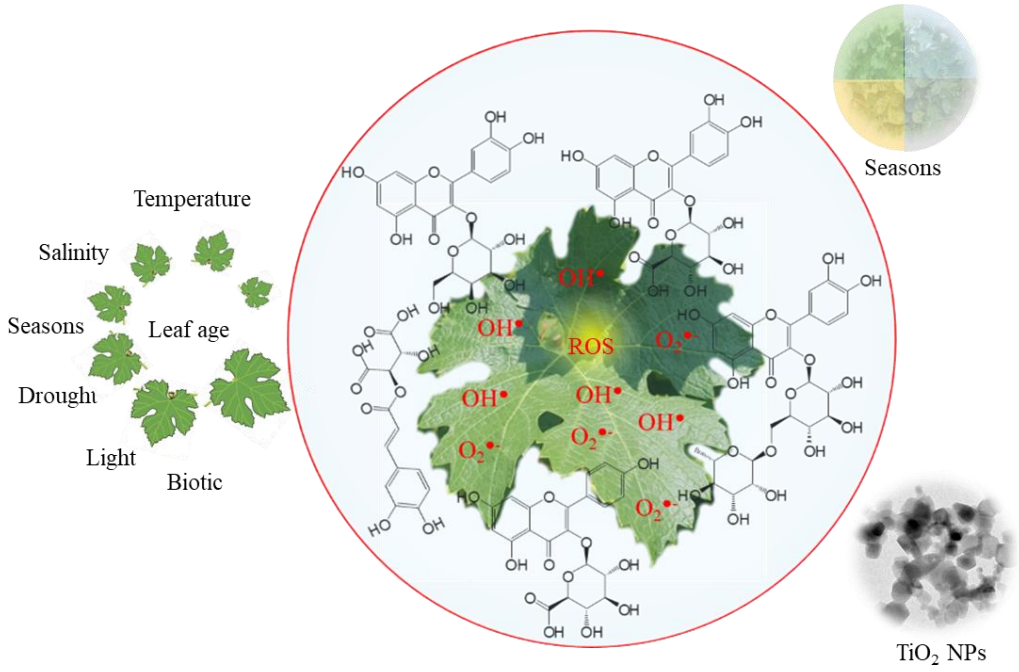


Fig. 15. Schematic summary of TiO_2 NPs, the environmental and developmental factors that influence the concentration of polyphenols in grapevine leaves.

Hydroxycinnamic acids and flavonols are known as the most stress-sensitive phenolic compounds in grapevine leaves. They are strong antioxidants, and therefore they have the ability to mitigate the adverse effects of stress-induced ROS (Latouche *et al*., 2012; Csepregi *et al*., 2016). It has been demonstrated that continuous or high doses of UV light increase the concentration of flavonols in leaves (Bidel *et al*., 2015). Furthermore, Nakabayashi *et al*. (2014) indicated that flavonols and anthocyanins can mitigate drought stress. However, the levels of all phenolic acids in leaves and roots decreased significantly under long-term drought stress (Król *et al*., 2014).

Amarowicz *et al.* (2010) examined the effect of low-temperature stress on phenolic compounds in grapevine leaves and discovered an increase in phenolic acids. In addition, grapevine (*Vitis vinifera* L.) leaves infected with Bois noir produced more phenolic and flavonoid compounds, whereas lignin concentration decreased (Negro *et al*., 2020). In the comparison of these biotic and abiotic stress with our study, we find that there is a similar trend or behavior of an increase or decrease in phenolic compounds after TiO_2 NPs treatment. On the other hand, TiO_2 NPs and other nanoparticles, such as silver (Ag), gold (Au), and Zinc oxide (ZnO) nanoparticles, also show several effects on plants (Siddiqui *et al*., 2015). For example, the application of TiO_2 NPs to maize increased anthocyanins (Morteza *et al*., 2013). The spherical Ag NPs induced the anthocyanin accumulation in *Arabidopsis* seedlings (Syu *et al*., 2014).

ROS in plants behaves like a double-edged sword; it is beneficial at low concentrations, but damaging at higher concentrations in the cell. The decreased Q-glr in treated leaves proves that this compound acts as a first line of defense in plant leaves during stress. It has also been proven that this compound accumulates in high concentration in young leaves and then decreases in old leaves that have suffered more from environmental stress during their life cycle (Bouderias *et al*., 2020). Esca infection, on the other hand, increases Q-glr by 35% (Goufo *et al*., 2019).

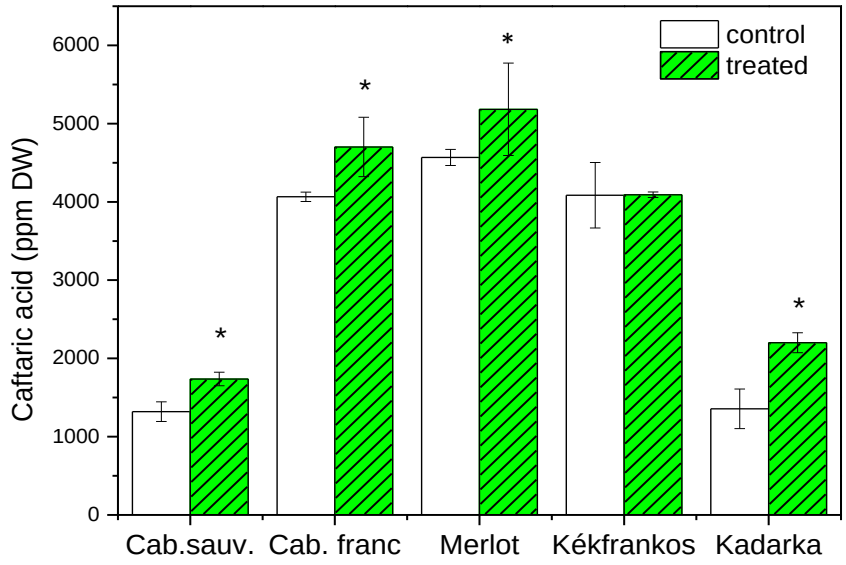


Fig. 16. Illustrates the effect of Degussa P25 TiO_2 NPs treatment on the caftaric acid content in the leaves of five red grapevine varieties. *significant at $P < 0.05$ level.

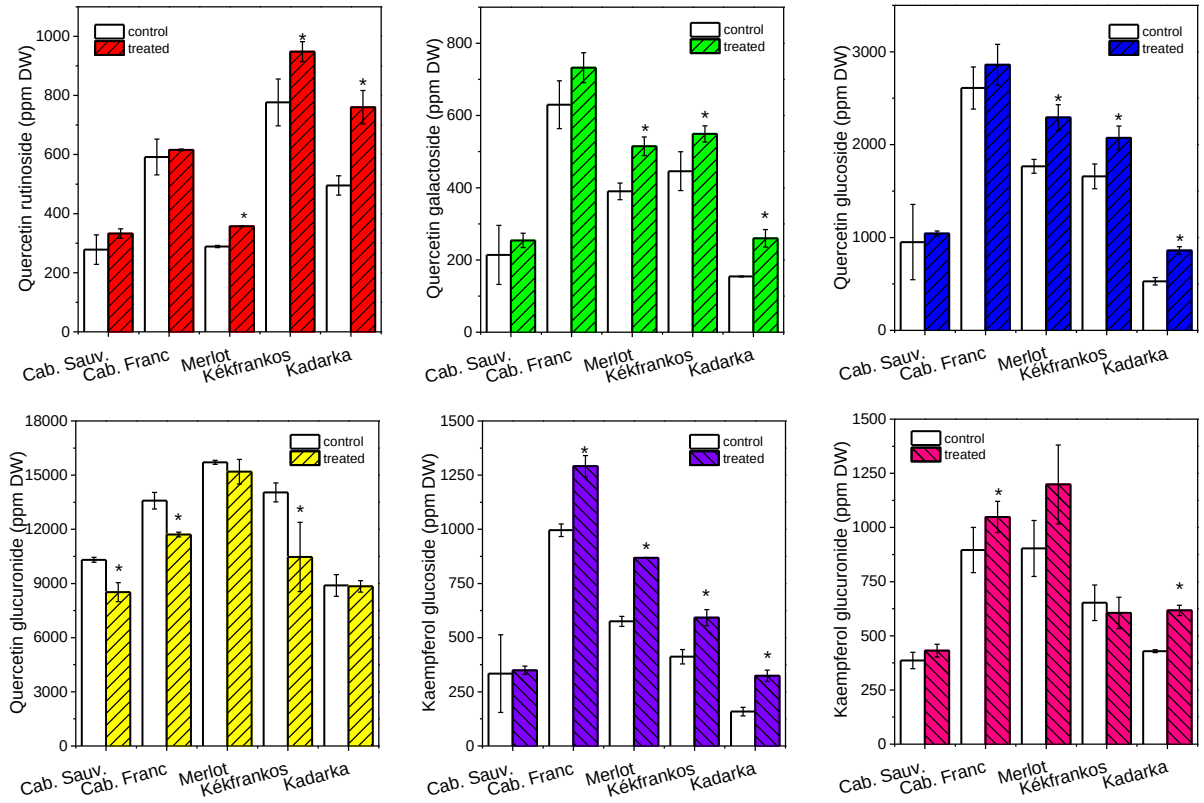


Fig. 17. Shows the effect of Degussa P25 TiO_2 NPs treatment on the flavonol glycosides in the leaves of five red grapevine varieties. *significant at $P < 0.05$ level.

4. 1. 4. Effects of TiO_2 NPs on the total phenolic contents and antioxidant capacities

Plants have a lot of different types of antioxidants, mostly polyphenols and flavonoids, which exhibit antioxidant activity (Rice-Evans *et al*., 1996; Szőllősi and Szőllősi Istvánné Varga, 2002). Numerous assays have been used to measure the antioxidant capacity of samples (Re *et al*., 1999; Pietta, 2000; Huang *et al*., 2005). Although current antioxidant assays have many advantages, such as simple procedure, quick analysis time, cheap reagents, and simple instrumentation being used, however, these strengths are insufficient to support the efficacy and reliability of these assays. In general, the antioxidant pathways are nonspecific and insensitive. At present, there is no universal and improved protocol for determining antioxidant capabilities. There is no standardized and strictly validated assay that can give a comprehensive picture of the antioxidant capacity that a test sample possesses. Therefore, a combination of several (at least three) assays must be performed to obtain a realistic assessment of the antioxidant capacity

exhibited by the sample (Sadeer *et al*., 2020). We employed colorimetric assays (FCR, FRAP, and TEAC) to quantify total phenolic content and antioxidant capacity in this work since they are the most accessible and extensively used techniques for assessing the antioxidant activities of biological materials.

TPC and AOC of control and treated leaves are shown in Fig. 18. The control leaves showed a noticeable difference in the TPC and AOC among grapevine leaf varieties. TPC was highest in Kékfrankos. This variety also showed the most effective FRAP and TEAC radical scavenger activity. The lowest values for TPC, FRAP, and TEAC were obtained in the samples of Kadarka and Cabernet Sauvignon. After TiO_2 NPs treatment, the general level of TPC, together with FRAP and TEAC values, were significantly influenced. The overall TPC and AOC of all varieties increased, however they declined dramatically in Kékfrankos.

Several studies used different methods to measure total phenolic content and antioxidant activity, such as FCR, FRAP, and TEAC. The redox potential of Fe (III) salt (~ 0.70 V) is similar to that of $ABTS^{\bullet-}$ salt (0.68V). Thus, the TEAC and FRAP assays are virtually identical, with the exception that TEAC is performed at neutral pH and the FRAP test is performed at acidic (pH 3.6) conditions. In addition, a strong linear correlation between TPC and antioxidant activity has been found (Huang *et al*., 2005). This finding is in line with our results. In both control and treated samples, all of the varieties showed the same trend in FCR, FRAP, and TEAC.

The TiO_2 NPs promoted the TPC and AOC in all the varieties, with the exception of Kékfrankos. It has been shown that TiO_2 NPs activate the antioxidant system of plants, improve the activity of antioxidant enzymes, and promote the synthesis of flavonoids (Wang *et al*., 2021). In addition, Szymanska *et al.* (2016) showed that a higher concentration of TiO_2 NPs can elevate the antioxidant level of *Arabidopsis thaliana*. On the other hand, biotic and abiotic stress also showed an increase in the AOC in the plants. Microbially damaged grape berries showed higher AOC compared to healthy grape berries (Balík *et al*., 2009). Drought and light also change in the phenolic content and augmented AOC in barly (Kowalczewski *et al*., 2020). In addition, TPC decreased in plants grown in amended soil by TiO_2 NPs, while AOC increased (Soran *et al*., 2021). The low content of two important phenolic compounds (CA and Q-glr) (Figs. 16 and 17), which play a key role in leaf defense, may be explain the drop in TPC and AOC in Kékfrankos. These findings are in agreement with the abiotic stresses, which also show an influence on polyphenol concentrations and AOC. Similarly, to the Kékfrankos situation under photocatalytic stress, the

long-term water deficit condition resulted in the depletion of TPC both in grapevine leaves and roots (Król *et al*., 2014). Cold stress also resulted in a decrease in the phenolic levels, leading to a lower radical scavenging capacity in the grapevine leaves (Król *et al*., 2015). In addition, *in vitro*, Li *et al.* (2022) reported that the TPC of tea decreased significantly after the addition of 0.5 % (w/w) TiO_2 NPs.

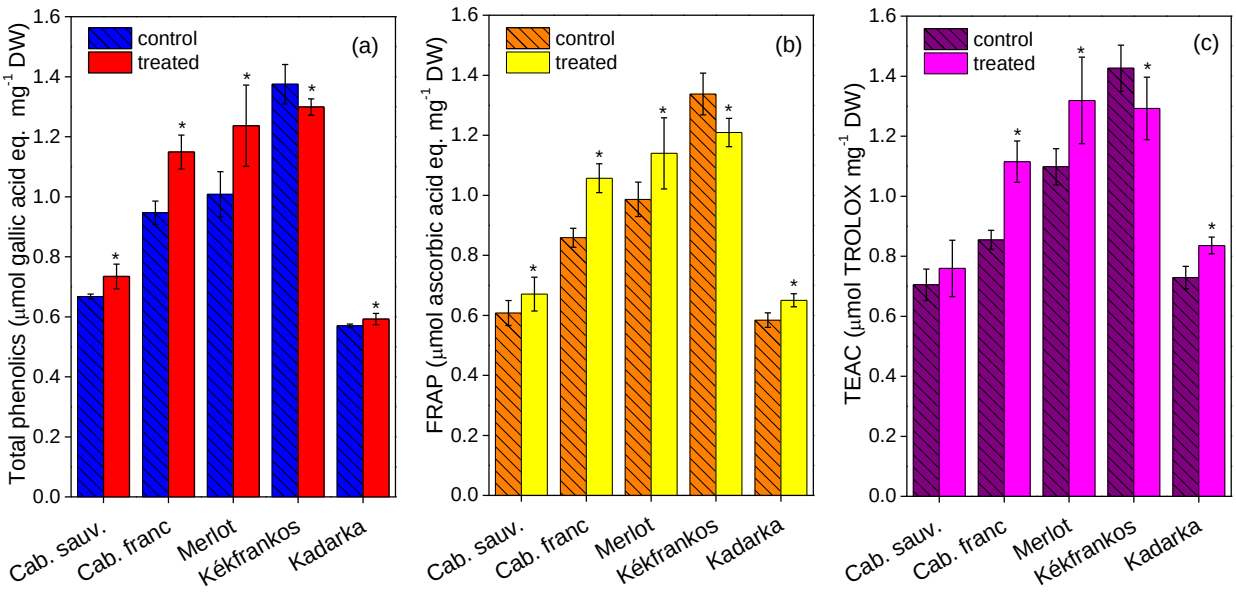


Fig. 18. Shows the effect of Degussa P25 TiO_2 NPs treatment on total phenolic content and antioxidant capacity by using three different assays: FCR (a), FRAP (b), and TEAC (c) in the leaves of five red grapevine varieties. *significant at $P < 0.05$ level.

4. 1. 5. The relationship between phenolic compounds and antioxidant capacity, and how TiO_2 NPs treatment affects it

Also in this work, the relationship between individual FLGs, CA, TPC, and AOC values was investigated. Correlation matrixes were produced using Pearson correlation coefficients. Data for control and treated leaves is presented in Tables 3 and 4, respectively.

Correlation analysis on the data set of grapevine leaves revealed that there are significant and positive correlations between individual FLGs with each other and also with CA, with the exception of Q-rut. This compound was neither correlated with other FLGs nor CA.

A significant correlation was found between FCR, TEAC, FRAP, and FLGs, with the exception of K-glc. Both CA and Q-glr are considered the main phenolic compounds in the leaf

extracts. Strong positive correlations were found between these compounds and FCR, TEAC, and FRAP. The high antioxidant activity of these compounds may explain their presence in high concentration in the leaves. The correlation coefficient between FCR, TEAC, and FRAP is 0.986^{**}–0.947^{**}–0.976^{**}. A similar correlation between TPC and AOC has been found by Balík *et al.* (2009). They showed that antioxidant activity was most closely correlated with the content of total polyphenols in infected grape berries (correlation coefficient = 0.8336–0.9952).

The TiO_2 NPs treatment did not change the correlations between CA, FLGs, FCR, FRAP, and TEAC. This indicates that TiO_2 NPs have the same effect on all phenolic compounds (CA and FLGs), TPC and AOC, which led to the stability of the correlations between them after treatment.

Table 3. Correlation analysis of CA, FLGs, FCR, FRAP, and TEAC values in control grapevine leaves (five varieties in three replications, N= 15).

		CA	Q-rut	Q-gal	Q-glc	Q-glr	K-glc	K-glr	FCR	FRAP	TEAC
CA	Pearson Correlation	1	0.303	0.781**	0.791**	0.955**	0.655**	0.887**	0.868**	0.841**	0.769**
	Sig. (2-tailed)		0.272	0.001	0.000	0.000	0.008	0.000	0.000	0.000	0.001
Q-rut	Pearson Correlation	0.303	1	0.496	0.345	0.147	0.174	0.136	0.522*	0.569*	0.515*
	Sig. (2-tailed)	0.272		0.060	0.208	0.601	0.535	0.628	0.046	0.027	0.050
Q-gal	Pearson Correlation	0.781**	0.496	1	0.984**	0.715**	0.907**	0.788**	0.647**	0.585*	0.421
	Sig. (2-tailed)	0.001	0.060		0.000	0.003	0.000	0.000	0.009	0.022	0.118
Q-glc	Pearson Correlation	0.791**	0.345	0.984**	1	0.755**	0.953**	0.834**	0.607*	0.533*	0.367
	Sig. (2-tailed)	0.000	0.208	0.000		0.001	0.000	0.000	0.016	0.041	0.179
Q-glr	Pearson Correlation	0.955**	0.147	0.715**	0.755**	1	0.615*	0.858**	0.855**	0.816**	0.751**
	Sig. (2-tailed)	0.000	0.601	0.003	0.001		0.015	0.000	0.000	0.000	0.001
K-glc	Pearson Correlation	0.655**	0.174	0.907**	0.953**	0.615*	1	0.802**	0.377	0.290	0.114
	Sig. (2-tailed)	0.008	0.535	0.000	0.000	0.015		0.000	0.166	0.294	0.687
K-glr	Pearson Correlation	0.887**	0.136	0.788**	0.834**	0.858**	0.802**	1	0.601*	0.567*	0.475
	Sig. (2-tailed)	0.000	0.628	0.000	0.000	0.000	0.000		0.018	0.027	0.074
FCR	Pearson Correlation	0.868**	0.522*	0.647**	0.607*	0.855**	0.377	0.601*	1	0.986**	0.947**
	Sig. (2-tailed)	0.000	0.046	0.009	0.016	0.000	0.166	0.018		0.000	0.000
FRAP	Pearson Correlation	0.841**	0.569*	0.585*	0.533*	0.816**	0.290	0.567*	0.986**	1	0.976**
	Sig. (2-tailed)	0.000	0.027	0.022	0.041	0.000	0.294	0.027	0.000		0.000
TEAC	Pearson Correlation	0.769**	0.515*	0.421	0.367	0.751**	0.114	0.475	0.947**	0.976**	1
	Sig. (2-tailed)	0.001	0.050	0.118	0.179	0.001	0.687	0.074	0.000	0.000	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4. Correlation analysis of CA, FLGs, FCR, FRAP, and TEAC values in TiO_2 NPs-treated grapevine leaves (for five varieties in three replications, N= 15).

		CA	Q-rut	Q-gal	Q-glc	Q-glr	K-glc	K-glr	FCR	FRAP	TEAC
CA	Pearson Correlation	1	0.045	0.866**	0.918**	0.828**	0.813**	0.789**	0.822**	0.845**	0.891**
	Sig. (2-tailed)		0.873	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Q-rut	Pearson Correlation	0.045	1	0.209	0.047	-0.307	-0.081	-0.255	0.213	0.292	0.204
	Sig. (2-tailed)	0.873		0.455	0.868	0.266	0.774	0.358	0.446	0.292	0.465
Q-gal	Pearson Correlation	0.866**	0.209	1	0.981**	0.574*	0.931**	0.653**	0.844**	0.856**	0.795**
	Sig. (2-tailed)	0.000	0.455		0.000	0.025	0.000	0.008	0.000	0.000	0.000
Q-glc	Pearson Correlation	0.918**	0.047	0.981**	1	0.689**	0.943**	0.725**	0.859**	0.861**	0.827**
	Sig. (2-tailed)	0.000	0.868	0.000		0.005	0.000	0.002	0.000	0.000	0.000
Q-glr	Pearson Correlation	0.828**	-0.307	0.574*	0.689**	1	0.634*	0.895**	0.573*	0.602*	0.715**
	Sig. (2-tailed)	0.000	0.266	0.025	0.005		0.011	0.000	0.026	0.018	0.003
K-glc	Pearson Correlation	0.813**	-0.081	0.931**	0.943**	0.634*	1	0.790**	0.665**	0.667**	0.641*
	Sig. (2-tailed)	0.000	0.774	0.000	0.000	0.011		0.000	0.007	0.007	0.010
K-glr	Pearson Correlation	0.789**	-0.255	0.653**	0.725**	0.895**	0.790**	1	0.476	0.516*	0.610*
	Sig. (2-tailed)	0.000	0.358	0.008	0.002	0.000	0.000		0.073	0.049	0.016
FCR	Pearson Correlation	0.822**	0.213	0.844**	0.859**	0.573*	0.665**	0.476	1	0.985**	0.933**
	Sig. (2-tailed)	0.000	0.446	0.000	0.000	0.026	0.007	0.073		0.000	0.000
FRAP	Pearson Correlation	0.845**	0.292	0.856**	0.861**	0.602*	0.667**	0.516*	0.985**	1	0.959**
	Sig. (2-tailed)	0.000	0.292	0.000	0.000	0.018	0.007	0.049	0.000		0.000
TEAC	Pearson Correlation	0.891**	0.204	0.795**	0.827**	0.715**	0.641*	0.610*	0.933**	0.959**	1
	Sig. (2-tailed)	0.000	0.465	0.000	0.000	0.003	0.010	0.016	0.000	0.000	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4. 2. The five grapevine varieties' characteristics

Discriminant analyses were used to characterize the five grapevine varieties using caftaric acid and flavonol levels (Fig. 19a), FCR, FRAP, and TEAC data sets (Fig. 19b). The phenolic profiles of Cabernet Franc and Kadarka were close to each other, while other varieties showed significant differences (Fig. 19a). At the same time, Cabernet Sauvignon and Kadarka, with significantly different genotypes, belonged to the same group based on their FCR, FRAP, and TEAC values (Fig. 19b). Kékfrankos, with its high antioxidant capacity (Fig. 18), was highly different from the other varieties. Cabernet Sauvignon and Cabernet Franc varieties have differing compositions even though they have comparable genetics. Cabernet Sauvignon is a hybrid grape formed by crossing Cabernet Franc (a red grape) with Sauvignon blanc (a white grape) (Bowers and Meredith, 1997).

In order to classify grapevine varieties based on the flavonol profile, Mattivi and his co-authors (2006) investigated the presence of flavonols in the berry skins of 91 grape varieties (*Vitis vinifera* L.). They found that red and white varieties have different flavonol profiles. In red varieties, the main flavonol was quercetin, followed by myricetin, kaempferol, laricitrin, isorhamnetin, and syringetin. In white varieties, the main flavonol was quercetin, followed by kaempferol, and isorhamnetin. On the other hand, three varieties: Cabernet Sauvignon, Cabernet Franc, and Sauvignon blanc, show different concentrations of individual and total flavonols. This may agree with our finding that even if they belong to a similar genotype, they have different characteristics. Furthermore, our findings are quite similar to those of previous studies. It turns out that the chemical composition of grape leaves may be used to discriminate between their different origins (Banjanin *et al*., 2020; Gülcü *et al*., 2020; Pantelic *et al*., 2017). The phenolic content, radical scavenging activity, and mineral composition of 22 grapevine leaves of diverse varietals in Serbia have been determined. A variation in the chemical composition was shown to be a convenient way to differentiate among the grape leaves of diverse varietal origins (Pantelic *et al*., 2017). The utilization of percentages also allows a better verification of similarities between varieties belonging to the same family, often very different from one another as far as the absolute quantities of anthocyanins are concerned, but with similar profiles (Mattivi *et al*., 1990). The anthocyanin profile has been used to classify red *Vitis vinifera* varieties. Therefore, analyzing anthocyanins proved useful in grapevine classification and chemotaxonomy (Castellarin *et al*.,

2006). It is expected that, in a similar way to anthocyanins, it can be possible to use flavonols for taxonomical classification and metabolite profiling (Bogs *et al*., 2006; Jeong *et al*., 2006), thus providing new information on the metabolism of flavonoids in both red and white grape varieties (Mattivi *et al*., 2006). As a result, phenolic profiles and antioxidant capacities may be used as chemotaxonomic parameters to distinguish grapevine varieties.

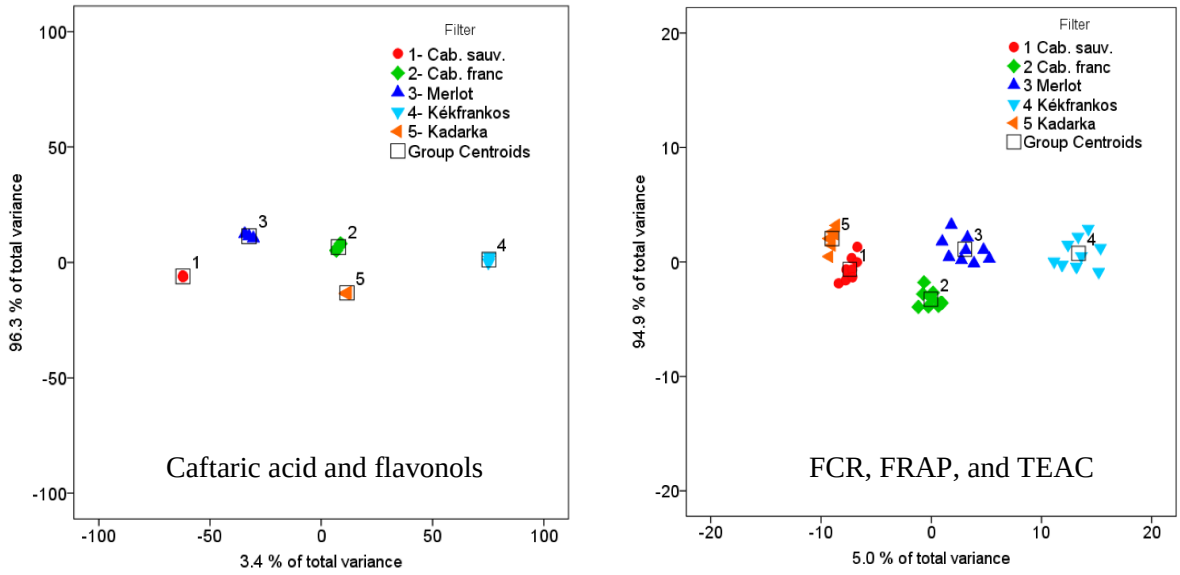


Fig. 19. Canonical discriminant function based on (a) caftaric acid and flavonols, (b) FCR, FRAP, and TEAC in control leaves of five grapevine varieties. Group centroids indicate the similarities or distance between varieties.

4. 3. Influence of the leaf position (age) and seasons on the flavonol glycosides distribution in grapevine leaves

Although the phenolic compounds' biosynthesis and accumulation research are progressing, reports on the influences of developmental and environmental factors on the synthesis and accumulation of these compounds in plants are still rare. Environmental factors like soil composition, temperature, rainfall, and UV radiation incidence can all have an impact on phenolic compound concentrations. According to our meteorological information for the past 49 years, the location received 11.6 °C of temperature, 2021 hours of sunlight, and 782 mm of precipitation. In 2018, the temperatures and hours of sunshine increased, while precipitation decreased.

Temperatures, sunshine hours, and precipitation totaled 14.1 °C, 2186 hours, and 717 mm, respectively (Appendix 4). During the experimental period, the microclimate was ideal for the vines' development. When comparing climate data (average temperatures, hours of sunshine, and precipitation) in June and September for 49 years and in 2018, the results show that these measurements increased in both months in 2018, except for precipitation in September 2018, which decreased. In June 2018, all these measurements were higher than in September (Appendix 5 and 6). Among phenolic compounds, flavonols can be influenced by the development of the plant and by environmental changes. Thus, flavonols represent a chemical interface between plants and the environment. In this work, we investigated how leaf age and season (June and September) influence FLGs' distribution and accumulation in grapevine leaves.

4. 3. 1. The main flavonol glycosides in grapevine leaves

Flavonols are important constituents of plant cells and are known for their multiple physiological roles, including screening of incoming solar radiation and scavenging various free radicals. They not only protect different components of the cell from damage, but also play a vital role in plant growth and development (Pollastri and Tattini, 2011). Different environmental stresses can change their levels in plant organs (Blancquaert *et al*., 2019). Besides these factors, we examined the influence of leaf age and season on FLGs accumulation in the grapevine leaves.

In order to identify the main phenolics present in Cabernet Sauvignon leaves, HPLC-DAD analyses were carried out. A representative chromatogram is depicted in Fig. 20. The results showed that quercetin- and kaempferol glucosides are the main FLGs in the leaves. Six FLGs, namely, Q-rut, Q-gal, Q-glc, Q-glr, K-glc, and K-glr were identified. Q-glr was the predominant derivative with a high concentration (~12,650-16,520 ppm DW), while the second most abundant flavonol glycoside was Q-glc (~1,125-4,380 ppm DW). Q-rut, Q-gal, and kaempferol glycosides were detected in a lower concentration range, typically below ~1,000 ppm DW (Bouderias *et al*., 2020).

Detailed studies of the flavonoid chemistry of *Vitis* spp. varieties have revealed that the majority of samples produce common flavonols. Quercetin and kaempferol are produced in considerable quantities. In addition, several studies have shown that the most *Vitis* spp. produce glucuronide of quercetin in considerable amounts (Dresch *et al*., 2014; Topalovic *et al*., 2012),

while Q-glc and Q-rut were reported in other studies as main compounds (Hmamouchi *et al*., 1996; Park and Cha, 2003; Farhadi *et al*., 2015; Katalinić *et al*., 2009).

FLGs have been shown important role in ROS scavenging in both *in vitro* and *in planta*. Our measurements *in vitro* proved that FLGs play an excellent role as ROS scavenging (Fig. 21). FLGs in grapevine leaf extract eliminated the $^{\bullet}OH$ radicals, which were generated by UV-photolysis of H_2O_2, from the medium (Bouderias *et al*., 2020). *In planta*, ROS altered significantly the FLGs content in response to TiO_2 NPs stress was detected in five different varieties of *Vitis vinifera* L leaves (Kőrösi *et al*., 2019a).

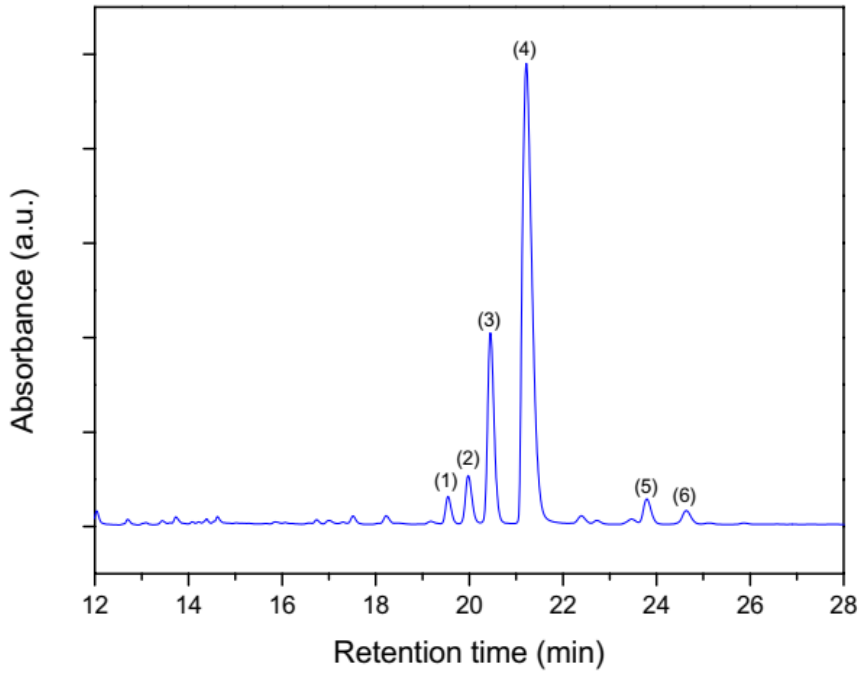


Fig. 20. Typical HPLC-DAD chromatogram of methanolic leaf extract of Cabernet Sauvignon: (1) Q-rut, (2) Q-gal, (3) Q-glc, (4) Q-glr, (5) K-glc, and (6) K-glr.

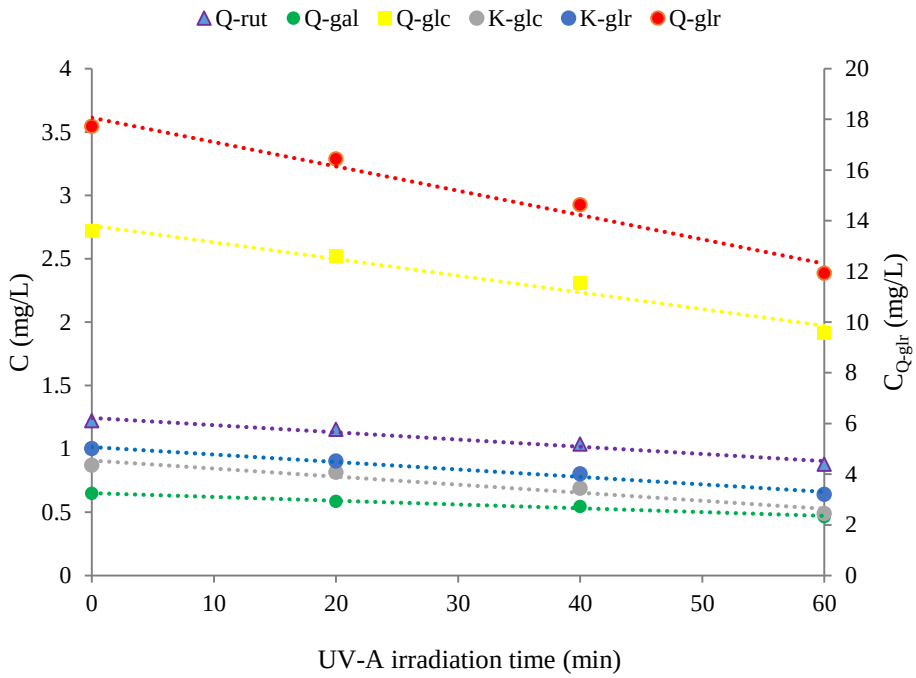


Fig. 21. Monitoring of flavonol composition of an aqueous grapevine leaf extract containing 140 mmol L^{-1} H_2O_2 under UV-A irradiation as a function of time. Based on the following equation, ˙OH radicals were generated by UV-photolysis of H_2O_2: $H_2O_2 + hv \rightarrow$ ˙OH + ˙OH Due to the scavenging of ˙OH radicals, the level of flavonol glycosides decreased gradually as a function of time. The concentration of Q-rut, Q-gal, Q-glc, K-glc, K-glr are depicted on the left axis of the diagram while the concentration of Q-glr is presented on the right axis.

4. 3. 2. Impact the leaf position (age) on the distribution of flavonol glycosides

Each leaf has a different level of exposure to the environmental conditions, which may have an effect on the distribution of phytochemicals and antioxidant capacity in *Vitis vinifera* L. The aims of this study were to compare individual FLGs of Cabernet Sauvignon leaves at different stages of maturity based on their position on a shoot. Leaves at different positions from base to apex in a shoot represented gradient decrease in the leaf age (from old to young) (Fig. 22).

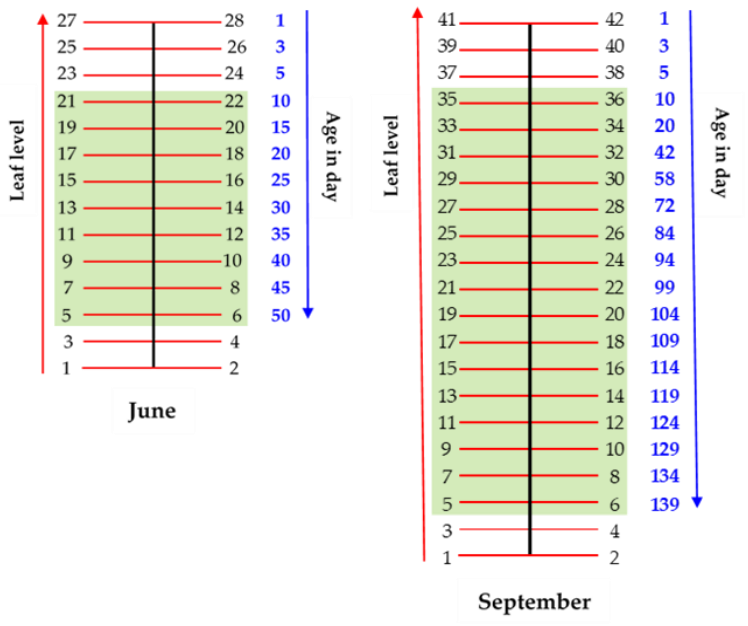


Fig. 22. Schematic draw of Cabernet Sauvignon shoots with 28 and 42 leaf levels harvested in June and September, respectively. Black numbers demonstrate the leaf levels while blue numbers on the right side show the corresponding leaf age. The green marks indicate which leaves were analysed by HPLC-DAD.

The results in Fig. 23 show that the concentrations of Q-rut and Q-glr are present in high concentrations in the young and old grapevine leaves. However, the constant increase of Q-glc, Q-gal, K-glc, and K-glr concentrations towards the shoot base led to their accumulation in old leaves at high concentrations. The changes in the flavonol levels are well indicated by the slope of the lines fitted to the data points. May be this difference in the concentrations of FLGs due to their distribution and accumulation on two different leaf sides (adaxial and abaxial). Therefore, we suggest that Q-glr and Q-rut accumulate faster in the adaxial as a first line of defense, due to the exposure of this leaf part to UV (sun). The high levels of these compounds prove their strong antioxidant capacity in the defense processes of grapevine from the early stage of leaf development, while the abaxial side of the leaf that was not directly exposed to the sun, accumulates Q-glc, Q-gal, K-glc, and K-glr more slowly than the first side as a second line of defense. Bidel *et al.* (2015) showed that low UV-B irradiance induced the accumulation of flavonols in the exposed epidermis without any parallel increase in the mesophyll or opposite lamina surface. At higher UV-B irradiances, the epidermis that was not directly exposed to UV-B

also began to accumulate flavonols, albeit in lower amounts than the exposed epidermis. But with multiple and successive daily exposures, flavonols accumulate progressively throughout all leaf tissues.

The study of Bhakta and Ganjewala (2009) revealed that leaf position influences the type and level of secondary metabolites and thereby their antioxidant properties. For instance, principal component analysis illustrated distinct differences in overall phenolic profiles between old and young tea leaves. After maturation, flavanols and phenolic acids decreased, whereas flavonols increased (Liu *et al*., 2020). i.e., flavanols and phenolic acids are more active or special accumulate in young and mature leaves, whereas, flavonols are more efficient in old leaves. This may be a good explanation for our study why all individual flavonol glycosides are high in old leaves.

Tables 5 and 6 support the results which have been shown in Fig. 23. In addition, Q-glr level was significantly decreased in old leaves, in September (Table 6). Its decrease in the last developmental stage (old leaf) may be explained by overstress in this period of the leaf life cycle. As it is known, oxidative stress plays a key role at both ends of the leaf life cycle. Maintaining an adequate cellular ROS/antioxidant (redox) balance that allows growth and prevents oxidative damage in young emerging leaves, while later on photo-oxidative stress induces cell death in senescing leaves (Juvany *et al*., 2013). For example, the UV-B-induced Q-glr in *C. asiatica*, suggests that flavonoid-mediated UV-B protection may be conferred both by UV-B screening and the quenching of ROS. At the highest UV-B irradiance, mesophyll tissue preferentially accumulated Q-glr, and in a smaller amounts K-glr (Agati and Tattini, 2010; Agati *et al*., 2012; Bidel *et al*., 2015). In addition, our previous study proves that strong photocatalytic oxidative stress can induce a decrease in Q-glr content in grapevine leaves (Kőrösi *et al*., 2019a).

Phenolic content can be influenced by developmental and environmental factors as well (Li *et al*., 2020; Rankenberg *et al*., 2021). Flavonoids are mainly genetically influenced. In addition, environmental factors such as nutrients, temperature, and lighting conditions can have an effect on flavonoid composition (Brouillard and Dangles, 1994; Mol *et al*., 1998; Harborne, 1980). Besides environmental influence, changes in flavonols can also be explained by grape development changes (Downey *et al*., 2003).

Flavonol levels in Shiraz and Chardonnay grapes were measured throughout the berry development process. The results indicated that two distinct periods of flavonol synthesis occur in

grapes, the first around flowering and the second during the ripening of the developing berries. The expression of FLS genes (*VvFLS1*) was found in leaves, tendrils, pedicels, buds, and inflorescences as well as in developing grapes. Expression was highest between flowering and fruit set, then decreased, increasing again during ripening, coincident with a rise in flavonols per berry. In contrast, *VvFLS2* expression was much lower than *VvFLS1* expression and did not change during berry development (Downey *et al*., 2003).

To investigate the transcription patterns of five flavonol synthases (FLSs), the messenger RNA (mRNA) levels of those genes were determined in the leaves, flower buds, flowers, and berry skins of Cabernet Sauvignon. The transcription patterns of the five FLSs varied with the organ and stage. In the leaves, the mRNAs of *FLS2*, *FLS3*, and *FLS5* were detected in small and medium-sized leaves, and the accumulation decreased as they grew, while the mRNA of *FLS4* was detected in all of the leaves tested. In the flower buds and flowers, the mRNAs of all five FLSs were detected. In the berry skins, the mRNAs of *FLS2*, *FLS4*, and *FLS5* were detected at an early stage of development (June), but this accumulation decreased afterward and was hardly detected at the pre-veraison stage (July). From the veraison stage (August) to the harvest stage (September), the mRNAs of *FLS4* and *FLS5* accumulated in the skins again (Fujita *et al*., 2006).

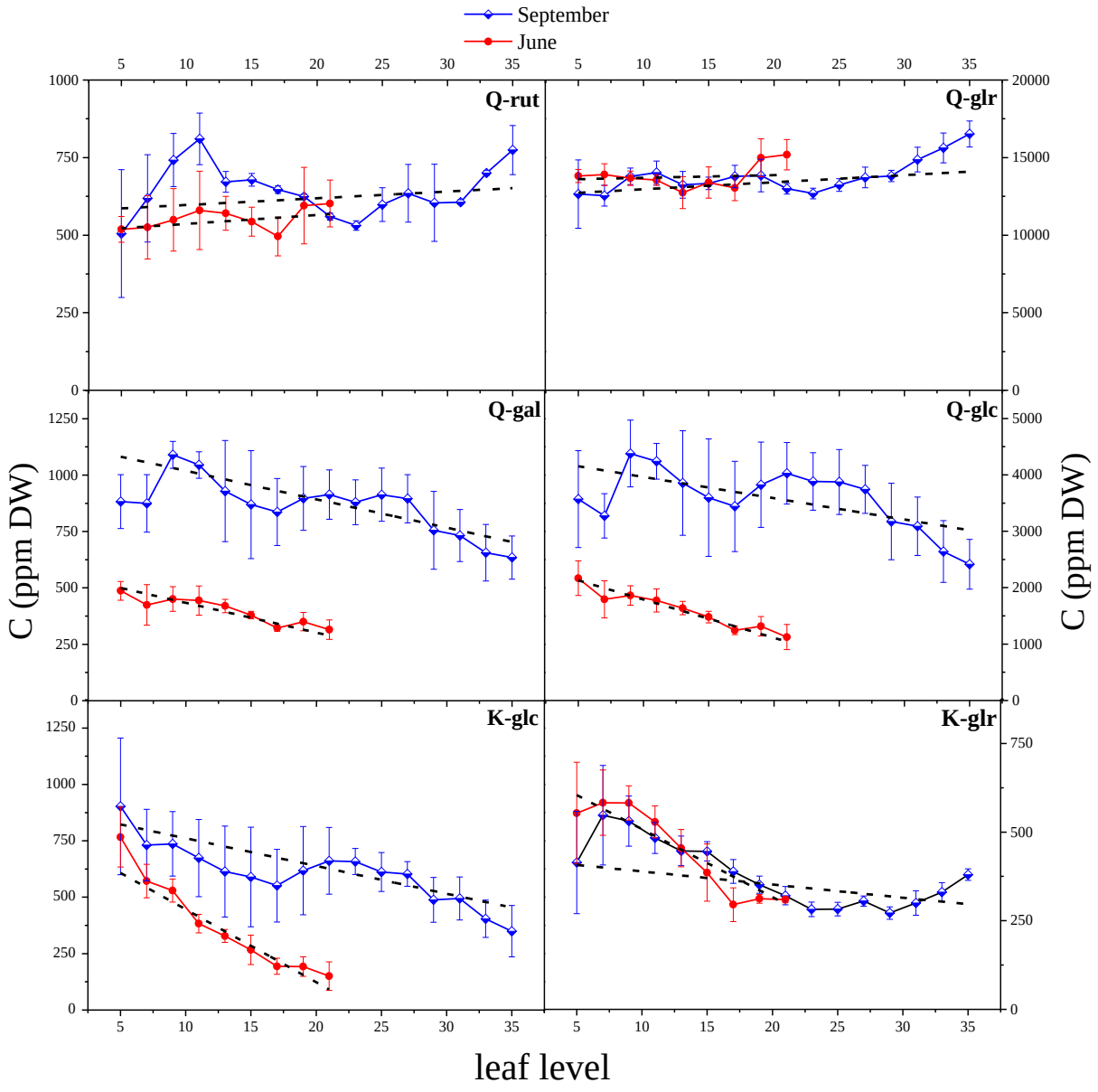


Fig. 23. Shows how the concentrations of flavonol glycosides change in Cabernet Sauvignon leaves based on leaf position and season. Concentrations are expresses in µg g^{-1} dry weight. Linear fitting marked with dashed lines was performed by Origin 9 software.

Table 5. Flavonol levels and total flavonol content of the leaves harvested from 5-10 and 17-22 leaf levels in June. Data are expressed in μg g^{-1} DW. Results clearly demonstrate that the amounts of Q-gal, Q-glc, K-glc, and K-glr were significantly lower at higher leaf levels (i.e. younger leaves). Q-rut, Q-glr, and total flavonol concentrations did not differ significantly between the two age-groups.

			Q-rut	Q-gal	Q-glc	Q-glr	K-glc	K-glr	Total flavonol
June	Leaves 17-22	Shoot#1	616±65	305±35	1066±171	14831±2088	128±35	285±35	17231±2046
		Shoot#2	610±86	360±32	1349±141	14814±828	193±37	311±13	17637±1035
		Shoot#3	466±47	322±11	1269±23	13565±844	216±3	321±19	16159±908
		mean±sd[a]	564±85	329±28	1228±146	14403±726	179±46	306±18	17009±764
	Leaves 5-10	Shoot#1	613±64	493±47	2014±176	13702±525	586±102	603±71	18011±779
		Shoot#2	522±20	443±63	1884±342	13510±525	693±203	621±30	17672±1165
		Shoot#3	458±21	426±77	1926±434	14180±150	589±106	494±110	18074±429
		mean±sd[a]	531±78	454±35	1941±67	13797±345	623±61	573±69	17919±216
		p (t-test)[b]	0.6437	0.0083	0.0015	0.2615	0.0005	0.0029	0.1181

[a]Mean values ± standard deviation for the individual shoots (1-3) with 5-10 or 17-22 leaf levels.
[b]P is the results of t-test between the 5-10 and 17-22 leaves.

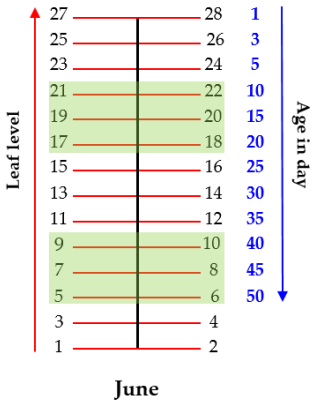
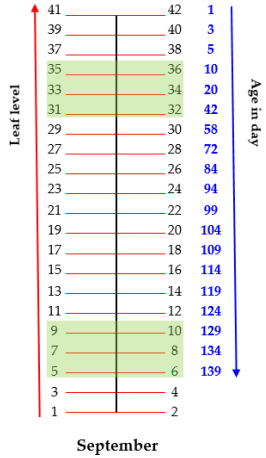


Table 6. Flavonol levels and total flavonol content of the leaves harvested from 5-10th and 31-36 th leaf levels in September. Data are expressed in μg g^{-1} DW. Q-gal, Q-glc, K-glc, and K-glr significantly lower for younger leaves (at 31-36th leaf levels). Q-rut and total flavonol concentrations did not differ significantly between the two age-groups. Q-glr concentration significantly increased in the younger leaves.

			Q-rut	Q-gal	Q-glc	Q-glr	K-glc	K-glr	Total flavonol
September	Leaves 31-36	Shoot#1	687±85	782±59	3194±392	14830±652	524±62	323±46	20340±412
		Shoot#2	723±126	562±53	2205±343	16480±668	362±108	367±30	20699±408
		Shoot#3	671±49	679±58	2746±325	15706±1186	362±55	321±45	20484±846
		mean±sd[a]	694±27	674±110	2715±496	15672±826	416±94	337±26	20508±180
	Leaves 5-10	Shoot#1	472±168	884±237	3528±1309	12091±1817	665±191	425±55	18015±3747
		Shoot#2	752±38	999±50	3976±409	13681±1013	857±273	572±54	20836±1614
		Shoot#3	641±150	964±98	3717±433	13217±810	848±142	547±121	19934±1390
		mean±sd[a]	622±141	949±59	3741±225	12996±818	790±108	514±79	19595±1441
		p (t-test)[b]	0.4339	0.0188	0.0310	0.0163	0.0106	0.0205	0.3396



4. 3. 3. Influence the seasons on the flavonol glycosides content

Leaf development, from early leaf growth to senescence, is tightly controlled by plant development and the environment (Juvany *et al*., 2013). Besides checking how FLGs are affected by leaf age, the effect of seasons was also revealed. From Fig. 23, it is also possible to distinguish two groups of the individual FLGs according to the change of seasons. The first group includes Q-glc, Q-gal, and K-glc. These FLGs doubled in September compared to June. However, the trend in their concentration changes towards the shoot tip was similar to June. The second group includes Q-glr, Q-rut, and K-glr. This group showed no significant difference between the two seasons, despite the passage of 90 days. Moreover, Student's t-tests confirmed that the leaves harvested in September contained significantly higher levels of Q-glc, Q-gal, K-glc, and K-glr (Table 7). Even though the last leaf position has the same age (10 days), Q-glc and Q-gal levels were multiplied in September leaves (Table 8). Fig. 25, and Tables 7 and 8 also proved that the level of TF is higher in September, independently of the leaf position. Even though the total flavonol level showed a significant difference between two seasons, there was no difference between the total flavonol content of the young and old leaves in one season (Tables 5 and 6).

Several studies have reported that UV radiation increases the biosynthesis of flavonols in plants (Kolb and Pfündel, 2005). Our study showed that FLGs increase in September. Therefore, besides UV radiation, other factors may also have the possibility of increasing flavonol production, such as low temperature and rainfall (Bhatia *et al*., 2018; Nenadis *et al*., 2015). He *et al.* (2010) outlined that low temperatures, such as 25 ºC, favored the anthocyanin biosynthesis. Furthermore, it was shown that light and low temperatures have a synergistic effect on the expression of genes within the flavonoid biosynthesis pathway (Azuma *et al*., 2012).

Environmental variables are key factors that determine the final amounts of FLGs in leaves. The doubling of Q-glc, Q-gal, and K-glc concentrations (Fig. 24) and TF (Fig. 25) in September indicates that, besides their protective functions, these FLGs are likely linked to other roles such as growth. Mierziak *et al.* (2014) showed that flavonoids may be responsible for mediating ROS-induced signaling cascades vital to cell growth and differentiation. In addition, flavonoids can regulate auxin efflux to recognize the extracellular environment. Therefore, control of auxin transport by flavonoids can be important in the stress-induced morphogenetic response of plants.

Fig. 24 demonstrates the flavonol profile of the youngest and oldest leaves in June and September. In both seasons, old leaves showed a decrease in Q-glr and an increase in Q-glc and K-glc. In June, the Q-glr ratio for the youngest leaves (21-22th levels) was 86%, and it was decreased to 75% in the oldest ones (5-6th levels). While Q-glc and K-glc ratios increased from 6 to 12% and 1 to 4%, respectively (the difference between the two leaf ages is 40 days). By comparing the leaves at the 35-36th and 5-6th nodes in September, we found that the Q-glr ratio decreased from 78 to 67%, whereas Q-glc and K-glc fractions increased from 11 to 19% and 2 to 5%, respectively (the difference in their ages is 130 d).

Despite having the same leaf age (10 d) in both seasons, the leaves harvested in September contained c.a. two times higher Q-glc level. Interestingly, although the difference in age between the youngest leaf in September (node 35-36th) and the oldest leaf (node 5-6th) in June is 40 d, the ratios of Q-glr, Q-glc, and K-glr are similar (Fig. 24). In the case of the oldest leaf in both seasons, where the difference in age is 90 d, FLGs such as Q-gal, Q-glc, and K-glr were found to be significantly higher in September (Table 8). With regard to the youngest leaf in June (10 d) and the oldest one in September (130 d), the differences between Q-glr, Q-glc, and K-glr ratios were significantly different, but the trend decreasing of Q-glr and increasing Q-glc and K-glr was the same as in each season. Their ratios in June were 86%, 6%, and 1%, respectively, while in September they were 67%, 19%, and 5%.

By comparing the leaf positions in June and September, even though 90 d had elapsed between the two sampling dates (the oldest leaf in both seasons) and 130 d between the youngest and oldest leaf in June and September, respectively, the trend observed in June was not changed, but with different ratios. 130 d is the longest period between two seasons. During this period, Q-glr was decreased, and Q-glc was increased to its maximum in September. This explain that flavonols influence daily depends on the stress which exposed to plants. In addition, the similarity between the oldest leaf in June and the youngest leaf in September can explain the effect of environmental conditions on flavonol accumulation and plant growth.

In most cases, several environmental stresses jointly cause drastic changes in phenolic compound levels. In recent years, there have been many reports on the effects of drought stress (Griesser *et al*., 2015; Król *et al*., 2014; Cui *et al*., 2017; Weidner *et al*., 2009), salinity (Mohammadkhani, 2018), light and UV (Kocsis *et al*., 2015; Del-Castillo-Alonso *et al*., 2015; Del-

Castillo-Alonso *et al*., 2015; Gregan *et al*., 2012; Kolb *et al*., 2001), and temperature (Król *et al*., 2015; Amarowicz *et al*., 2010; Azuma *et al*., 2012) on phenolic compounds in grapevine. However, the response of plants to multiple abiotic stresses is unique and cannot be directly predicted by examining each stress individually. The differences in plant phenolic content are often difficult to interpret, as many abiotic conditions usually interfere with complex internal factors. The environmental conditions in which plants grow are very complex. This study may have an important role in showing the effect of multiple stresses on the defense system of plants, taking into account the age of the leaves and the time of picking. Therefore, there is a need for a broader study on the extent to which the defense system of leaves of different ages is affected by individual stress and multiple stresses.

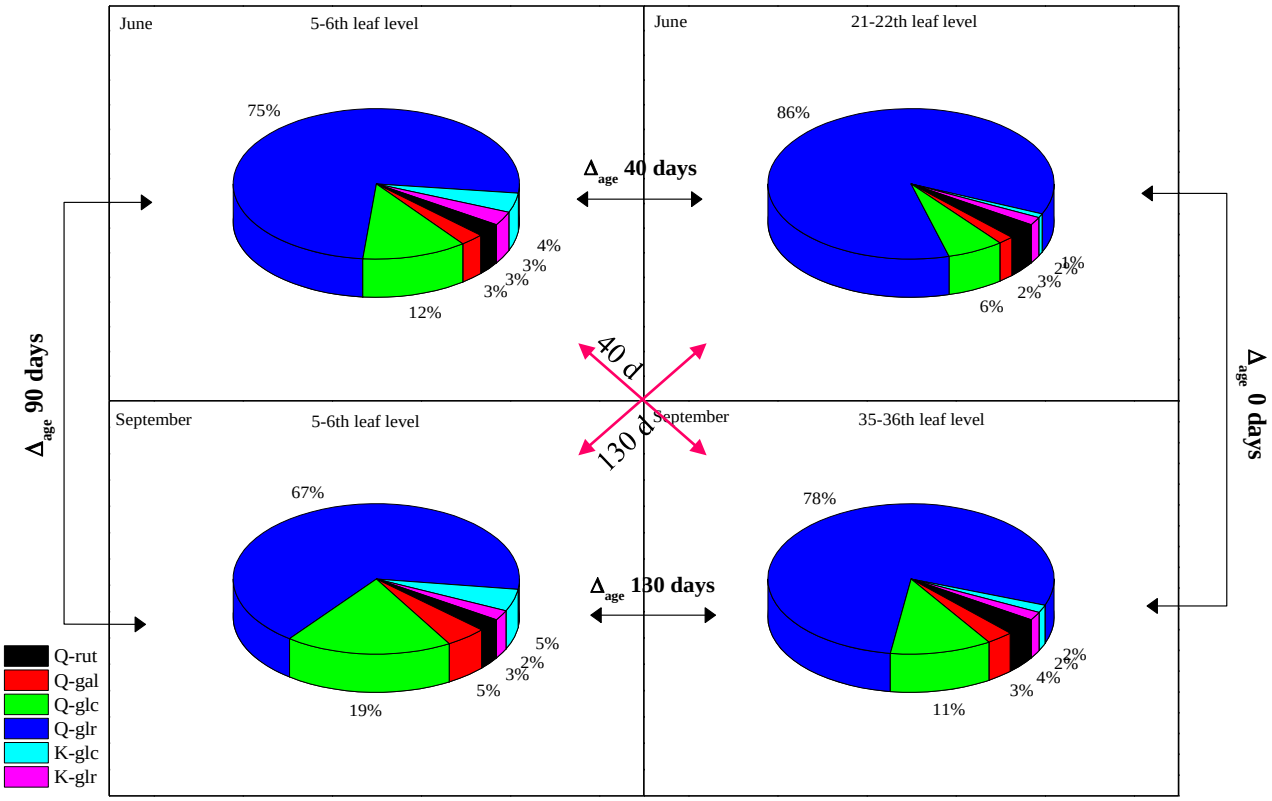


Fig. 24. Flavonol profiles of the oldest and the youngest Cabernet Sauvignon leaves harvested in June and September.

Table 7. Flavonol levels and total flavonol content of the leaves harvested in June and September. Leaves were collected from 5-22th and 5-36th leaf levels in June and September, respectively.

	Leaves 5-36	Q-rut	Q-gal	Q-glc	Q-glr	K-glc	K-glr	Total flavonol
September	Shoot#1	616±109	942±141	3949±733	13711±1228	678±125	369±84	20265±1899
	Shoot#2	660±119	832±182	3433±780	13860±1428	611±203	389±106	19785±1274
	Shoot#3	656±92	815±146	3310±623	13810±1240	528±193	383±114	19501±1387
	mean±sd[a]	644±24	863±69	3564±339	13793±76	605±75	380±10	19850±386
	Leaves 5-22							
June	Shoot#1	626±60	413±97	1591±459	14149±1224	333±213	434±153	17547±1152
	Shoot#2	557±61	396±51	1584±301	13679±1126	402±248	456±139	17075±1241
	Shoot#3	477±35	388±64	1623±366	13601±755	392±173	445±113	16927±1135
	mean±sd[a]	553±74	399±13	1600±21	13810±297	376±37	445±11	17183±324
	p (t-test)[b]	0.11475	0.0003	0.0104	0.9303	0.0090	0.0018	0.0008

[a]Mean values of flavonols for all the leaves of the individual shoots (#1-#3). Data are expressed in µg g^{-1} dry weight.
[b]P values show the results of t-test between 5-22th and 5-36th leaf levels.

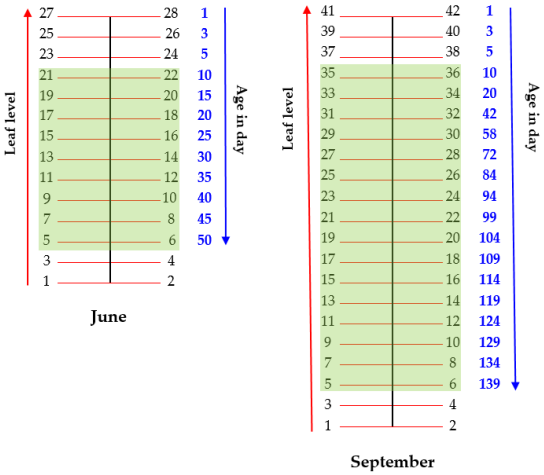
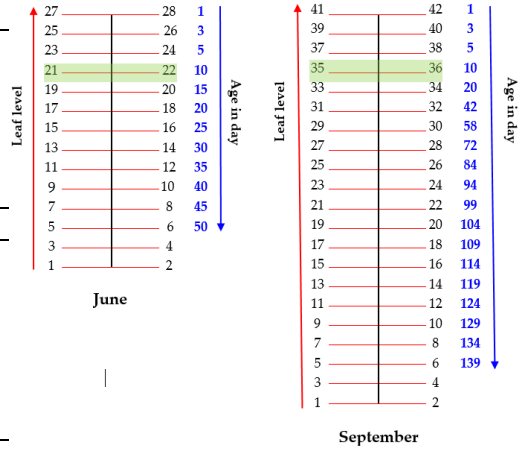


Table 8. Comparison the leaves with same age but in different seasons. 10-days-leaves leaves harvested from 21-22th and 35-36 th leaf levels in June and September, respectively. Flavonol levels and total flavonol content are expressed in µg g^{-1} DW. Q-gal, Q-glc, and K-glr and total flavonol levels were significantly (P<0.05) higher in September. Q-rut, Q-glr and K-glc concentrations did not differ significantly between the two seasons.

			Q-rut	Q-gal	Q-glc	Q-glr	K-glc	K-glr	Total flavonol
September	Leaves 35-36	Shoot#1	769	739	2865	15561	480	372	20786
		Shoot#2	856	551	1982	17111	268	399	21168
		Shoot#3	698	614	2397	16894	300	369	21272
		mean±sd[a]	774±79	635±96	2415±442	16522±839	349±114	380±17	21075±256
June	Leaves 21-22	Shoot#1	622	265	868	16285	88	303	18431
		Shoot#2	666	345	1226	14874	151	307	17567
		Shoot#3	518	333	1280	14401	214	320	17066
		mean±sd[a]	602±76	315±43	1125±224	15187±980	151±63	310±9	17688±691
		p (t-test)[b]	0.0529	0.0062	0.0107	0.1475	0.0573	0.0029	0.0013



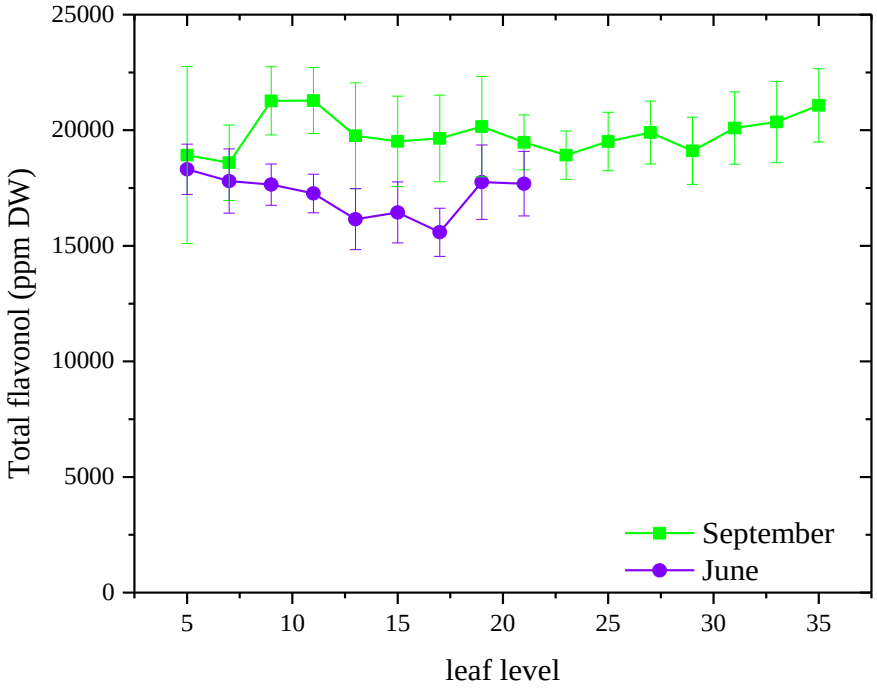


Fig. 25. Total flavonol level of Cabernet Sauvignon leaves harvested in June and September as a function of leaf levels.

4. 3. 4. The relationship between flavonol glycosides and how seasons affect them

Pearson's correlation analysis confirms the correlation between FLGs in both seasons (Table 9). In June, K-glr showed strong positive correlations with the Q-glc, Q-gal, and K-glc levels, while Q-rut content correlated positively with Q-glr and K-glr content in September. In addition, both seasons showed a strong positive correlation between Q-glc, Q-gal, and K-glc.

In addition, plotting analysis measurements assert the correlation between Q-gal, K-glc, and K-glr in June. For these FLGs, the slopes of the fitted lines differ in both June and September, indicating their different proportions in the two seasons. A positive correlation was observed between Q-rut and Q-glr levels in September. However, the slope of the fitted lines was very similar, showing the same Q-rut: Q-glr ratio in both seasons (Fig. 26).

Q-glc and Q-gal have a linear correlation value of 0.961 (Fig. 27). This shows that they have a strong, positive, and linear relationship. Despite the fact that their ultimate concentrations differed, they both demonstrated an upward trend in leaf age and sampling season. This strong

correlation between Q-glc and Q-gal is most likely due to the two compounds' glycon natures being similar. Both glucose and galactose are isomers, and their structures are quite similar. As a result, they may have similar or the same functions in the plant. The molecular structure of phenolics has been shown to impact their antioxidant capacity (Cao *et al*., 2019; Ono *et al*., 2010).

Table 9. Pearson's correlation analysis between individual FLGs of Cabernet Sauvignon leaves collected in June and September. Pearson's correlation coefficients were calculated pair wise for n n=27 and n=48 data sets in June and September, respectively.

**Correlation is significant at the 0.01 level.

*Correlation in significant at the 0.05 level.

		Q-rut	Q-gal	Q-glc	Q-glr	K-glc	K-glr
June	Q-rut	1	0.248	0.002	0.406*	-0.249	-0.099
	Q-gal		1	0.961**	-0.178	0.751**	0.719**
	Q-glc			1	-0.207	0.864**	0.741**
	Q-glr				1	-0.195	-0.246
	K-glc					1	0.843**
	K-glr						1
September	Q-rut	1	0.215	0.122	0.614**	-0.067	0.535**
	Q-gal		1	0.964**	-0.253	0.797**	0.417**
	Q-glc			1	-0.23	0.800**	0.262
	Q-glr				1	-0.304*	0.064
	K-glc					1	0.449**
	K-glr						1

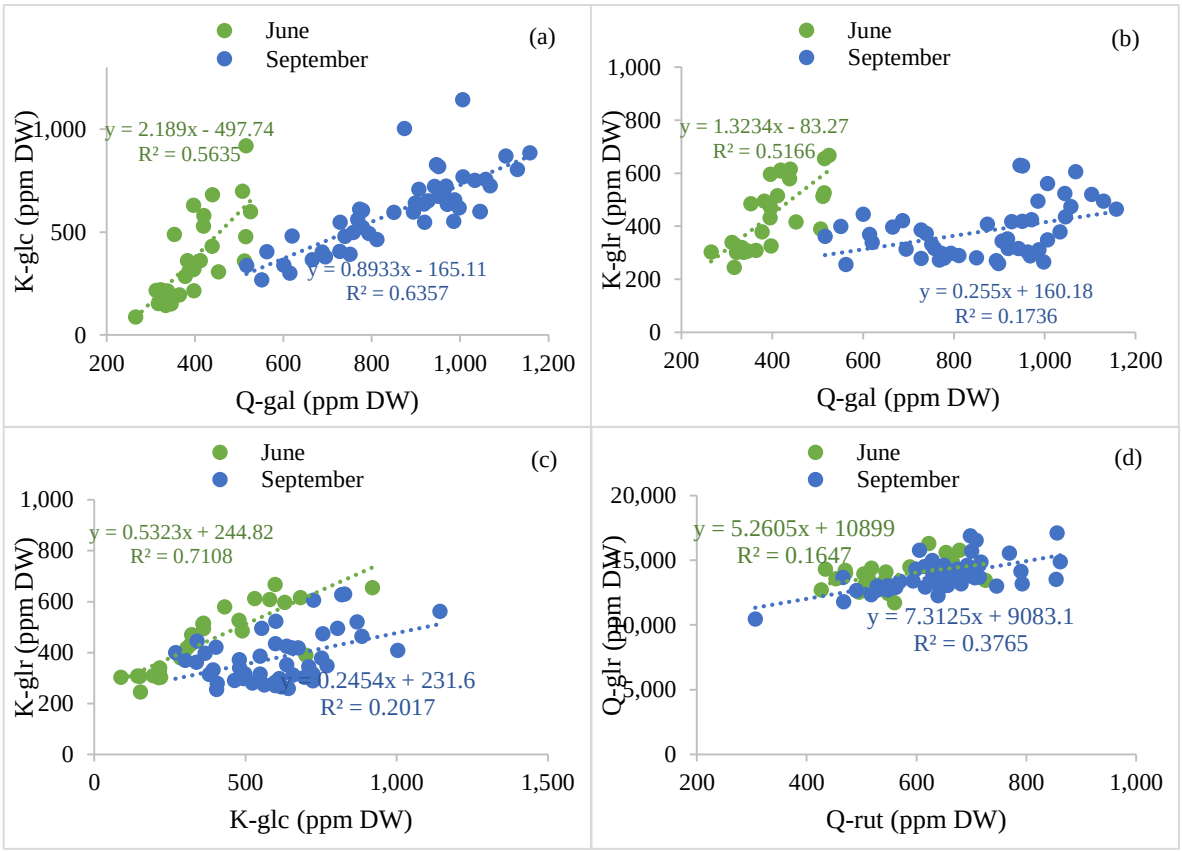


Fig. 26. The main correlation between individual flavonol glycosides in June (green) and September (blue) (a) K-glc vs. Q-gal, (b) K-glr vs. Q-gal, (c) K-glr vs. K-glc, and (d) Q-glr vs. Q-rut.

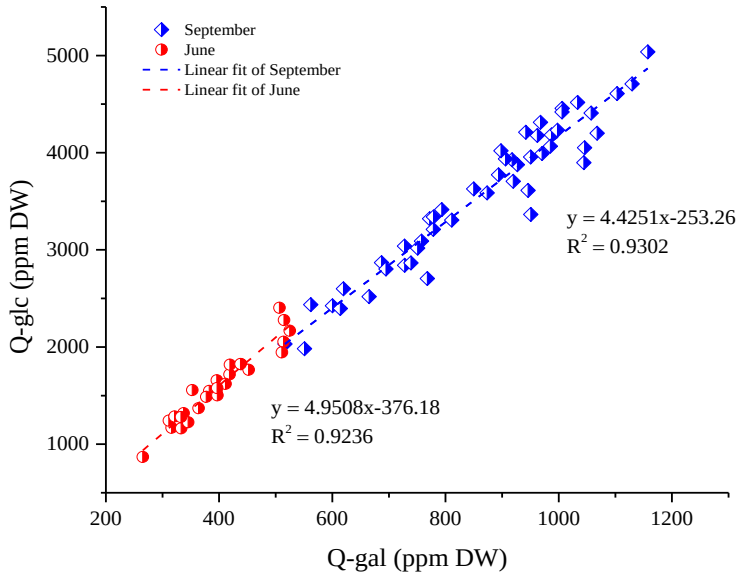


Fig. 27. The correlation between Q-glc and Q-gal in June (red) and September (blue). Fitted lines are marked with dashed line. The results of linear regression are displayed below the corresponding data.

5. Conclusions

Phenolic compounds are widely distributed secondary metabolites with different metabolic functions in plants. The main phenolic compounds in the grapevine leaves of five red varieties of *Vitis vinifera* were investigated by using HPLC-DAD. Leaf extract showed the same content of caftaric acid and six flavonol glycosides (Q-glc, Q-gal, Q-glr, Q-gal, K-glc, and K-glr) in all varieties but with different concentrations. Caftaric acid and Q-glr are shown to be the dominant compounds. These compounds can be influenced by several environmental and developmental factors. Therefore, the influences of TiO_2 NPs treatment, age, and seasons on these phenolic compounds were investigated.

In vitro experiments confirmed the photocatalytic activity of Degussa P25 TiO_2 NPs and their capacity to generate reactive oxygen species (ROS) by testing the degradation of methyl orange (MO). In order to investigate the effects of these ROS on plant responses, aqueous dispersion of P25 TiO_2 NPs was used for the foliar exposure of five grapevine varieties in field conditions. The photocatalytically produced ROS significantly boosted the production of phenolic compounds with the exception of Q-glr, which did not change with Merlot and Kadarka, or decreased significantly in the case of Cabernet Franc, Cabernet Sauvignon, and Kékfrankos. In addition, the treatment altered the total phenolic content and antioxidant capacity of grapevine leaves. With the exception of Kékfrankos, TiO_2 NPs increased FCR, FRAP, and TEAC values of the varieties investigated. In this case, it is possible to distinguish tolerant genotypes that possess high efficiency in their anti-oxidative system and can tolerate stress better than sensitive ones. Furthermore, significant and positive correlations were detected between phenolic compounds and antioxidant capacity, while the TiO_2 NPs treatment did not change these correlations.

Our results indicate that the five vine varieties each possess individual characteristics. However, TiO_2 NPs treatment seems to result in considerable changes in phenolic content and antioxidant capacity regardless of the genotype. A good choice for suitable concentration of TiO_2 NPs may boost the leaves' antioxidant system in response to the photogenerated ROS, but excessive concentrations may lead to damage. Further studies are necessary to unfold the correlation between physicochemical properties and phytotoxicity of TiO_2 NPs.

Furthermore, the results showed that flavonol glycosides are differently distributed between young and old leaves of Cabernet Sauvignon. It was observed that the concentrations of Q-rut and Q-glr were high since the beginning of leaf life and remained stable in older leaves. In contrast, Q-gal, Q-glc, K-glc, and K-glr showed low concentration in young leaves and then increased over time, allowing their accumulation in old leaves. Therefore, all flavonols were found in high amounts in old leaves, only Q-glr showed a significant decrease in old leaves at September.

Seasons, in addition to leaf age, had an effect on flavonol glycoside accumulation. Eventhough the trend in both seasons (Summer and Autumn) was the same, the concentrations of Q-gal, Q-glc, and K-glc increased considerably in September. While Q-glr, Q-rut, and K-glr did not show any significant difference between the two seasons.

Interesting correlations were found between the flavonol glycosides in grapevine leaves. Strong positive correlations were detected between the Q-glr and Q-rut levels, while Q-glc content was positively correlated with Q-gal and K-glc levels. Furthermore, the strongest correlation ($r = 0.961$) was found between Q-glc and Q-gal in both June and September.

Our findings revealed that leaf position and season are critical sample criteria that should be taken into account when studying flavonol levels.

6. New scientific achievements

The main aims of this study were to investigate the effects of TiO_2 NPs treatment, leaf position (age), and seasons on phenolic content and antioxidant capacity in the grapevine leaves.

Based on our results, we conclude that:

1. TiO_2 NPs treatment boosted the production of phenolic compounds in grapevine leaves.
2. TiO_2 NPs treatment increased leaf TPC and AOC of the varieties investigated (Cabernet Franc, Cabernet Sauvignon, Kadarka, and Merlot), with the exception of Kékfrankos.
3. Significant and positive correlations are found between individual FLGs with each other and also with CA and AOC. The TiO_2 NPs treatment did not change this correlation between phenolic compounds and AOC either.
4. Based on chemotaxonomic, the five grapevine varieties showed different characteristics.
5. Depending on the leaf position (age), FLGs in grapevine leaves can be divided into two groups:

    5. 1. The first group: includes Q-glc, Q-gal, K-glc, and K-glr. The concentrations of these flavonols were influenced by leaf age.

    5. 2. The second group: includes Q-glr and Q-rut. The concentrations of these flavonols were not influenced by leaf age.

6. In grapevine leaves level of FLGs were higher in September than that of in June.

7. Positive correlations were found between FLGs, while the strongest positive correlation was found between Q-glc and Q-gal in both June and September.

7. Future perspective

1. **TiO_2 NPs treatment**

Plants are subjected to many biotic and abiotic stresses that limit crop productivity and cause devastating economic and social impacts. To deal with these conditions, plants develop various avoidance or tolerance mechanisms. Plant responses and acclimation processes vary depending on the duration and severity of the stress, as well as on the plant's age and stage of growth. Phenolic compounds, vitamins, and proteins play a crucial role in plant growth and protection against various biotic and abiotic stresses. TiO_2 NPs can be used as an elicitor to enhance the biosynthesis of these compounds in plants. However, the benefits and toxicity of TiO_2 NPs depend on several factors, such as the plant species, concentration and particle size of the NPs, and exposure conditions. Thus, it is necessary to extend the study on the effect of TiO_2 NPs on plants. Several studies under different conditions can be performed to investigate the positive and negative effects of TiO_2 NPs on plants.

1.1. Some studies have found that the properties of nanoparticles, such as size, shape, and type, have diverse impacts on plants. As a result, expanding the study to evaluate how TiO_2 NPs with diverse properties affect grapevine leaves is required. In addition, it should be testing TiO_2 NPs at different concentrations to detect what is the optimal concentration for the highest antioxidant capacity and what is the limit concentration that can induce toxicity.

1. 2. In this study, Kékfrankos showed a different response after TiO_2 NPs treatment than the other studied varieties. Therefore, the study can be extended to treat more varieties through which resistant and susceptible varieties can be known.

1. 3. The research may be expanded to look at the effects of TiO_2 NPs on other compounds that are important in plant defense and growth, such as phenolic compounds (anthocyanins and resveratrol) and vitamins.

1. 4. In this study, we focused on the effect of TiO_2 NPs on phenolic compounds and the antioxidant capacity of grapevine leaves. The effect of TiO_2 NPs on other grapevine parts/organs, such as seeds and berries, can also be investigated. The treatment of grapevine seeds with TiO_2 NPs under irradiation before sowing may affect the germination rate. TiO_2 NPs may also be used to protect grape berries from various pathogens. Thus, it can be tested for their effects at different stages of berry development.

1. 5. Keep track of the experiment's time to identify the best time for treatment and harvesting.

2. **Leaf position (age)**

In nature, environmental stresses often occur together and exhibit unparalleled compound effects on plants, causing changes in the cellular, metabolic, and physiological activities of the plant. Phenolic compounds are one of the most important antioxidants that can play a crucial role as a defense line in plants, and therefore it is necessary to conduct further experiments to find out how multiple stresses may affect the distribution of flavonol glycosides. Thus, taking into account leaf position (age), the effects of individual and combined stress on the distribution of flavonol glycosides in leaves can be compared *in vitro*.

8. Summary

***Background and aims*.** During their development stages, plants are exposed to stress due to several environmental factors (Juvany *et al*., 2013). To protect themselves from this stress, they biosynthesize phenolic compounds, which have the property of antioxidants. Flavonols are one of the main polyphenolic subclasses. They are mainly accumulated in the epidermal cells of plant tissues in response to solar radiation. UV-B-induced increase in the quercetin to kaempferol ratio may offer protection against UV-B stress. Flavonols have an antioxidant function during plant response to different environmental stresses (Sharma *et al*., 2012). Grapevine leaves are rich in polyphenols, especially flavonol derivatives such as quercetin and kaempferol glycosides (Hmamouchi *et al*., 1996).

Diverse types of NPs are now being employed as innovative and efficient elicitors of phenolic compounds for various plant species. One of the most prevalent types of "nano-elicitors" is titanium dioxide nanoparticles (TiO_2 NPs) (Anjum *et al*., 2019; Khan *et al*., 2021; Lala, 2021). TiO_2 NPs' ability to form reactive oxygen species (ROS) when excited by ultra-violet (UV) light makes them useful for successfully inactivating a variety of pathogens. In living organisms, ROS also has a signaling role. As a result, ROS produced by TiO_2 NPs can affect both enzymatic and non-enzymatic defense mechanisms. Szőlősi *et al.* (2020) found that these NPs had both beneficial and harmful impacts on plants. Furthermore, it has been reported that the combination of TiO_2 NPs with another stress can play an important role in reducing the damage to a plant (Singh *et al*., 2016).

The primary goals of this thesis were to investigate the effects of TiO_2 NPs on phenolic compounds in grapevine *Vitis vinifera* leaves, as well as how age and seasons influence flavonol glycosides distribution.

Materials and methods. The grapevine leaves of five varieties; Cabertnet Franc, Cabernet Sauvignon, Kadarka, Kékfrankos, and Merlot were treated with 1 mg ml^{-1} Degussa P25 TiO_2, where the plants are exposed to natural sunlight with relatively high UV radiation (with a maximum of ~ 45 W m^{-2}). After two weeks of exposure, the polyphenol profile was determined in the leaves by HPLC-DAD. The total phenolic content and antioxidant capacity were detected by FCR, TEAC, and FRAP assays.

Leaf samples of Cabernet Sauvignon were collected from randomly chosen shoots of individual vines in both June and September. Three shoots of different vines were combined and leaves were pooled at the same leaf positions. The shoots were different in the two seasons, they possessed 28 and 42 leaf levels in June and September, respectively. After sample preparation, flavonol glycosides were analysed by using the HPLC–DAD system.

Results. TiO_2 NPs have been used to test their influence on phenolic compounds and antioxidant capacity in grapevine leaves. Degussa P25 TiO_2 NPs were used in our study. Degussa P25 is one of the most active commercial photocatalysts. P25 TiO_2 NPs showed the ability to absorb light at wavelengths below 410 nm which enables it to produce ROS. These ROS showed significant influences on the chemical composition and antioxidant capacity of grapevine leaves. Phenolic compounds, including CA and FLGs (Q-glc, Q-gal, Q-rut, K-glc, and K-glr) were increased, while Q-glr decreased. Increasing the antioxidant capacity was compatible with an increase of phenolic compounds in all varieties, with the exception of Kékfrankos, where it decreased significantly. In addition, significant and positive correlations were detected between phenolic compounds and antioxidant capacity, and they did not change after TiO_2 NPs treatment. Regardless of TiO_2 NPs treatment, the phenolic compounds and antioxidant capacity analyzes showed that the five red varieties have different characteristics. Therefore, this confirmed that they belong to different genotype groups.

Leaf age and seasons are also two other main factors that can control polyphenols in the plant. Our analysis of phenolic compounds in grapevine leaves of Cabernet Sauvignon showed interesting results. In the case of leaf age, two flavonol glycoside groups have been distinguished. The first group showed that Q-glr and Q-rut were high in both young and old leaves. The second group includes Q-glc, Q-gal, K-glc, and K-glr that are low in young leaves, then increase gradually to reach the high concentration in old leaves. We also noted that grapevine increased Q-glc, Q-gal, and K-glc biosynthesis more in September than in June. In addition, interesting correlations were observed between flavonol glycosides. The strongest correlation was found between Q-glc and Q-gal in June and September, also Q-glc showed a positive correlation with K-glc. Strong positive correlations were found between Q-glr and Q-rut.

Conclusions. Our study showed that caftaric acid and six flavonol glycosides (Q-glr, Q-glc, Q-gal, Q-rut, K-glr, and K-glc) are the main phenolic compounds of grapevine leaves of five red

varieties. TiO_2 NPs treatment, leaf age, and seasons seem to have an effect on these compounds. TiO_2 NPs treatement has been shown effect on concentrations of caftaric acid, flavonol glycosides, and antioxidant capacity. In general, caftaric acid and flavonol glycosides increased, with the exception of Q-glr, which decreased significantly. In addition to increasing polyphenol content, antioxidant capacity also increased with all varieties, with the excepetion of Kékfrankos.

Furthermore, our results showed that the six individual flavonol glycosides are distributed differently between young and old leaves. Where two groups were observed, Q-glr and Q-rut were high in all leaf ages, while Q-glc, Q-gal, K-glc, and K-glr were lower in young leaves and high in old leaves. As well, Q-glc, Q-gal, and K-glc were significantly accumulated in September than June.

In conclusion, the amount of phenolic compounds in vine leaves is changing depending on TiO_2 treatment, variety, developmental stages, and seasons.

***Keywords**:* Grapevine *Vitis vinifera* leaves, polyphenols, caftaric acid, flavonol glycosides, antioxidant capacity, TiO_2 NPs, ROS, leaf age, seasons.

9. Összefoglalás

Előzmények és célok. A növények fejlődési szakaszaik során számos környezeti tényező hatására stressznek vannak kitéve (Juvany *et al*., 2013). Hogy megvédjék magukat ettől a stressztől, fenolos vegyületeket szintetizálnak, amelyek antioxidáns tulajdonságokkal rendelkeznek. Ezek közé tartoznak a flavonolok, a polifenolok egyik fő alosztálya, melyek főleg a növények epidermális sejtjeiben halmozódnak fel a napsugárzás hatására. A kvercetin/kempferol arány UV-B sugárzás által kiváltott növekedése védelmet nyújthat az UV-B stressz ellen. Emelletta a flavonolok antioxidáns funkciót töltenek be a növények különböző környezeti stresszre adott válaszai során (Sharma *et al*., 2012). A szőlőlevelek gazdagok polifenolokban, különösen flavonolszármazékokban, például kvercetinben és kaempferol-glikozidokban (Hmamouchi *et al*., 1996).

A különféle típusú nanopartikulumok (NP) számos növényfajban a fenolos vegyületek termelésének új és hatékony gerjesztői. A titán-dioxid nanorészecskék (TiO_2 NP-k) ezen 'nano-elicitorok' egyik leggyakrabban használt típusa (Anjum *et al*., 2019; Khan *et al*., 2021; Lala, 2021). A TiO_2 NP-k azon képessége, hogy ultraibolya (UV) fénnyel gerjesztve reaktív oxigénfajtákat (ROS) termelnek, hasznossá teszi őket különféle kórokozók hatékony inaktiválására. Ismert, hogy a ROS-nak élő szervezetekben jelátviteli szerepe is van, ezért a TiO_2 NP-k által indukált ROS hatással lehet az enzimes és nem enzimatikus védekezési rendszerekre is. Így a NP-k kettős természetet mutatnak, jótékony és káros hatásuk is lehet a növényekre (Szőllősi *et al*., 2020). Emiatt a TiO_2 NP kezelés más stresszekkel való kombinációja fontos szerepet játszhat a növények károsodásának csökkentésében (Singh *et al*., 2016).

Dolgozatom fő célja az volt, hogy megvizsgálja a TiO_2 NP kezelés hatását a szőlő (*Vitis vinifera*) leveleinek fenolos vegyületeire, valamint az, hogy az életkor és az évszakok hogyan befolyásolják a flavonol-glükozidok mennyiségi eloszlását.

Anyagok és módszerek. A Cabernet Sauvignon levélmintákat egyedi szőlőtőkék véletlenszerűen kiválasztott hajtásairól gyűjtöttem júniusban és szeptemberben. A hajtások a két évszakban eltérő hosszúságúak voltak, júniusban 28, szeptemberben 42 levélszinttel rendelkeztek. Három különböző szőlőtőke ugyanazon levélemeletről gyűjtött mintáit egyesítettük. A mintákat

liofilizáltuk, porítottuk és extraháltuk hangyasavas metanollal. A flavonol-glükozidokat HPLC-DAD rendszerrel elemeztük.

Öt kékszőlő-fajta (Cabertnet Franc, Cabernet Sauvignon, Kadarka, Kékfrankos és Merlot) leveleit 1mg ml^{-1} Degussa P25 TiO_2-dal kezeltük az ültetvényben, ahol a növények természetes napfénynek vannak kitéve viszonylag magas UV sugárzással (maximum ~ 45 W m^{-2}). Két hét expozíció után a polifenol profilt a levelekben HPLC-DAD módszerrel határoztuk meg. A teljes fenoltartalmat és az antioxidáns kapacitást FCR, TEAC és FRAP módszerekkel mutattuk ki.

Eredmények. A Cabernet Sauvignon szőlőlevelekben található fenolos vegyületek elemzése érdekes eredményeket mutatott. A levelek kora alapján két flavonol-glikozid csoportot tudtunk elkülöníteni. Az első csoportba tartozó Q-glr és Q-rut koncentrációja magas mind a fiatal mind az öreg levelekben. A második csoportba tartozó Q-glc, Q-gal, K-glc és K-glr koncentrációja a fiatal levelekben alacsony, majd fokozatosan növekszik míg eléri az idősebb levelekre jellemző legmagasabb koncentrációt. Arra is rámutattunk, hogy a Q-glc, Q-gal és K-glc szintézise a levelekben szeptemberben erőteljesebb, mint júniusban. Emellett összefüggéseket figyeltünk meg a különböző flavonol-glikozidok között. A legszorosabb korrelációt a Q-glc és a Q-gal között találtuk, valamint a Q-glc pozitív korrelációt mutatott a K-glc-vel. Szintén erős pozitív korrelációt találtunk a Q-glr és a Q-rut között is.

A TiO_2 NP kezelés hatását is vizsgáltuk a szőlőlevelek fenolos összetételére. Vizsgálatunk során TiO_2 NP-ként Degussa P25-öt használtunk, amely az egyik legaktívabb kereskedelmi fotokatalizátor. Különböző koncentrációjú anatáz és rutil keverékéből áll, melyek 410 nm alatti hullámhosszú fényre ROS-t generálnak. Ezek a ROS molekulák szignifikáns hatással voltak a szőlőlevelek kémiai összetételére és antioxidáns kapacitására. A fenolos vegyületek, köztük a CA és az FG-k (Q-glc, Q-gal, Q-rut, K-glc és K-glr) mennyisége növekedett, míg a Q-glr csökkent. Az antioxidáns kapacitás növekedése minden szőlőfajta esetében összeegyeztethető volt a fenolvegyületek mennyiségének növekedésével, a Kékfrankos kivételével, ahol jelentősen csökkent. Emellett szignifikáns pozitív korrelációt találtunk a fenolos vegyületek és az antioxidáns kapacitás között, ami megmaradt a TiO_2 NP-k kezeléseket követően is. A TiO_2 NP-k kezelésétől függetlenül a fenolos vegyületek és az antioxidáns kapacitás elemzései azt mutatták, hogy az öt kékszőlő-fajta eltérő tulajdonságokkal rendelkezik, igazolva a genotípusbeli különbségeket.

***Következtetéseink*.** Vizsgálataink kimutatták, hogy a kaftársav és hat flavonol-glikozid (Q-glr, Q-glc, Q-gal, Q-rut, K-glr és K-glc) a vizsgált öt kékszőlő-fajta leveleinek fő fenolos vegyületei. Ugyanakkor a levelek kora, az évszakok és a TiO_2 NP-k kezelés hatással vannak ezen vegyületek mennyiségére. Eredményeink azt mutatták, hogy a hat egyedi flavonol-glikozid eltérő koncentrációban oszlik meg a fiatal és az öreg levelek között. Az eloszlás alapján két csoportot figyeltünk meg, a Q-glr és a Q-rut koncentrációja minden levélkorban magas volt, míg a Q-glc, Q-gal, K-glc és K-glr alacsonyabb volt a fiatal levelekben és magasabb az öregebbekben. Valamint több Q-glc, Q-gal és K-glc halmozódott fel a levelekben szeptemberben, mint júniusban.

A TiO_2 NP kezelések hatására a kaftársav és a flavonol-glikozidok koncentrációja általában növekedett, kivéve a Q-glr-t, amely jelentősen csökkent. A polifenoltartalom növelésével párhuzamosan az antioxidáns kapacitás is nőtt minden fajtánál a Kékfrankos kivételével.

Összefoglalva, a szőlőlevélben lévő fenolos vegyületek mennyisége a fajtától, a fejlődési szakaszoktól, az évszakoktól és a TiO_2 kezeléstől függően változik.

***Kulcsszavak*:** szőlő (*Vitis vinifera)* levél, polifenolok, kaftársav, flavonol-glükozidok, antioxidáns kapacitás, TiO_2 NP, ROS, levélkor (levélszint), évszakok

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11. Scientific publications and conference abstracts

11. 1. Thesis publications

Kőrösi L., **Bouderias S**., Csepregi K., Bognár B., Teszlák P., Scarpellini A., Castelli A., Hideg É., Jakab G. Nanostructured TiO_2-induced photocatalytic stress enhances the antioxidant capacity and phenolic content in the leaves of *Vitis vinifera* on a genotype-dependent manner. *J. Photochem. Photobiol. B.* (2019). 190, 137−145; https://doi.org/10.1016/j.jphotobiol.2018.11.010 **(IF: 3.261)**

Bouderias S., Teszlák P., Jakab G., Kőrösi L. Age- and season-dependent pattern of flavonol glycosides in Cabernet Sauvignon grapevine leaves. *Sci. Rep*. (2020). 10, 14241. https://doi.org/10.1038/s41598-020-70706-7 **(IF: 4.379)**

11. 2. Other publications

Kőrösi L., Bognár B., **Bouderias S**., Castelli A., Scarpellini A., Pasquale L., Prato M. Highly-efficient photocatalytic generation of superoxide radicals by phase-pure rutile TiO_2 nanoparticles for azo dye removal. *Appl. Surf. Sci*. (2019). 493, 719−728; https://doi.org/10.1016/j.apsusc.2019.06.259 **(IF: 6.386)**

Bouderias S., Teszlák P., Bognár B., Csepregi K., Hideg É., Jakab G., Kőrösi L. Fotoreaktív nanorészecskék hatása vörösborszőlő-fajták levelének polifenol összetételére és tápelemtartalmára. *Kertgazdaság* (1998). 51: 3 pp. 31−42, 12 p. (2019).

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11. 3. Thesis conference abstracts

Bouderias S., Teszlák P., Jakab G., Kőrösi L. Nanostructured titanium dioxide as an antimicrobial agent on grapevine (*Vitis vinifera* L.) leaves: A phytotoxicological study. In: Tamás, László; Zelenyánszki, Helga (eds.) Fiatal Biotechnológusok Országos Konferenciája "FIBOK 2018": Abstract Book. Budapest, Hungary: JATEPress Kiadó, (2018). pp. 113−114., 1 p.

Bouderias S., Teszlák P., Balázs B., Csepregi K., Hideg É., Jakab G., Kőrösi L. Boosting the antioxidant capacity, phenolic content, and macro- and microelements in *Vitis vinifera* leaves by photoreactive TiO_2 nanoparticles. 8th interdisciplinary doctoral conference book of abstracts. Pécs, (2019). P. 13.

Bouderias S., Teszlák P., Czégény G., Jakab G., Kőrösi L. Application of anatase and rutile titanium dioxide nanoparticles in vineyard: response of enzymatic and non-enzymatic defense system of grapevines. 4th national conference of young biotechnologists. "FIBOK 2020": Abstract Book. Debrecen, Hungary, (2020). pp. 38., 1 p.

11. 4. Other conference abstracts

Bouderias S., Teszlák P., Jakab G., Kőrösi L. Drought stress-induced changes of flavonol profile in grapevine leaves (*Vitis vinifera* L.) In: David, Edwards; Rodomiro, Ortiz - 3rd Agriculture and Climate Change Conference. (2019). p. 1.

Teszlák P., **Bouderias S**., Jakab G., Kőrösi L. Szárazság stressz indukálta változások a szőlőlevél polifenolos összetételében in: Puskás, János (eds.) 11. Szőlő és klíma konferencia: Program és az előadások összefoglalói. (2019). p. 29.

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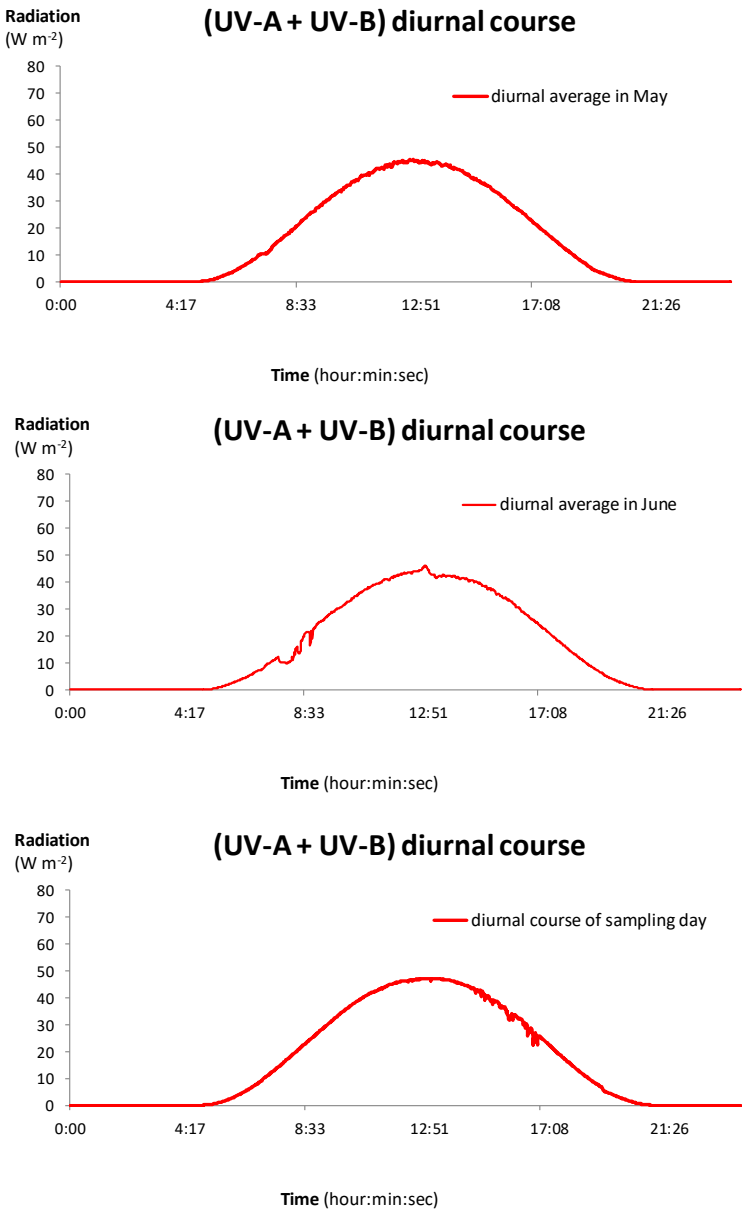
Sakina Bouderias

13. Appendix



A. 1. Representative diurnal course of natural broadband (UV-A + UV-B) radiation in May, June and sampling day, 2017. Analysis of the meteorological data set showed that grapevine leaves received high level of UV-A+UV-B radiation during the growing season (close to daily maximal value of ~45 Watt m^{-2}).

A. 2. Meteorological conditions measured during the experimental period. Values represented mean temperature (T_{mean}), amount of precipitation and relative humidity of air (RH_{mean}).

*= values of 60 years average between years 1950 and 2010 according to the meteorological database of Research Institute for Viticulture and Oenology, Pécs, Hungary.

	T_{mean} (°C)		Precipitation (mm)		RH_{mean} (%)
	Long-term average*	2017	Long-term average*	2017	2017
May	16.9	18.2	75	48	59.70
June	20.1	23.9	96	60	60.30

A. 3. Shows the location of the five grapevine varieties that were treated by TiO_2 NPs. The five varieties have the same exposure; they are close to each other (on the same terrace), and they were grown in similar soil and microclimatic conditions.

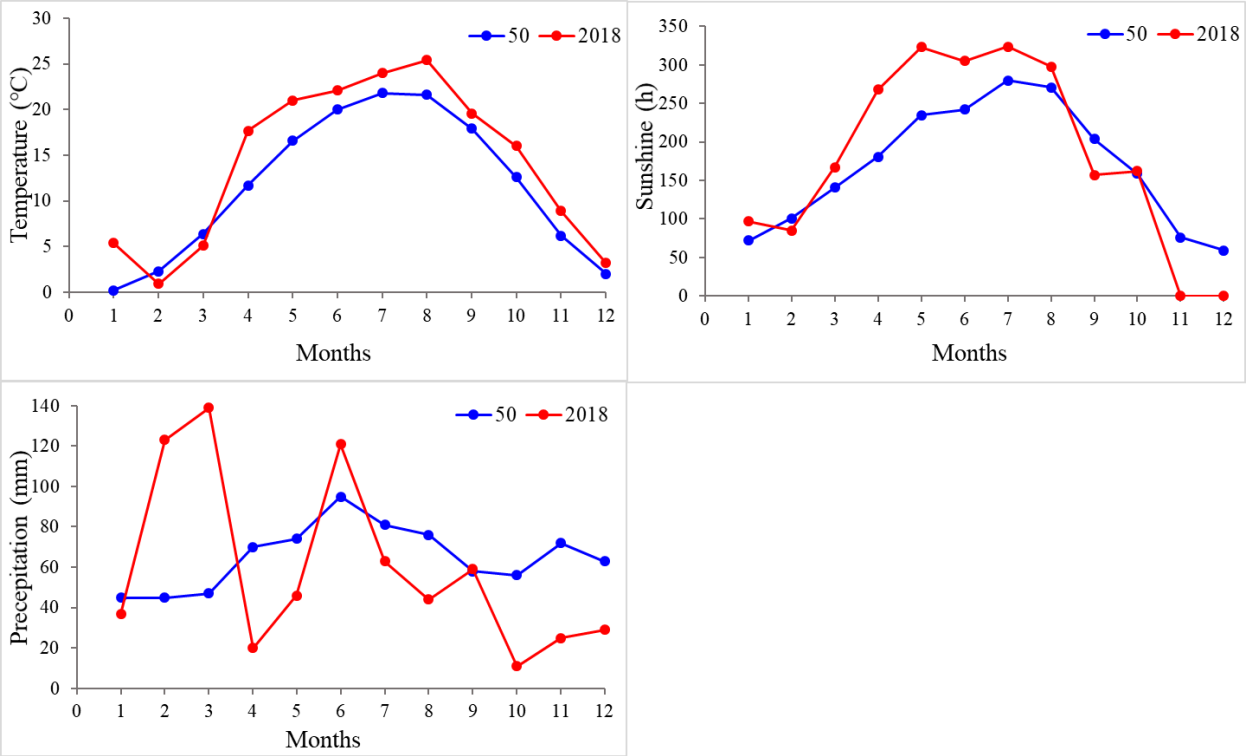
Vineyard of Mária
Table 33

Plantation:	1994-1996
Area:	2800 m^2
Area:	0,28 ha
Spacing area:	2,0 x 1,0 m 2m^2

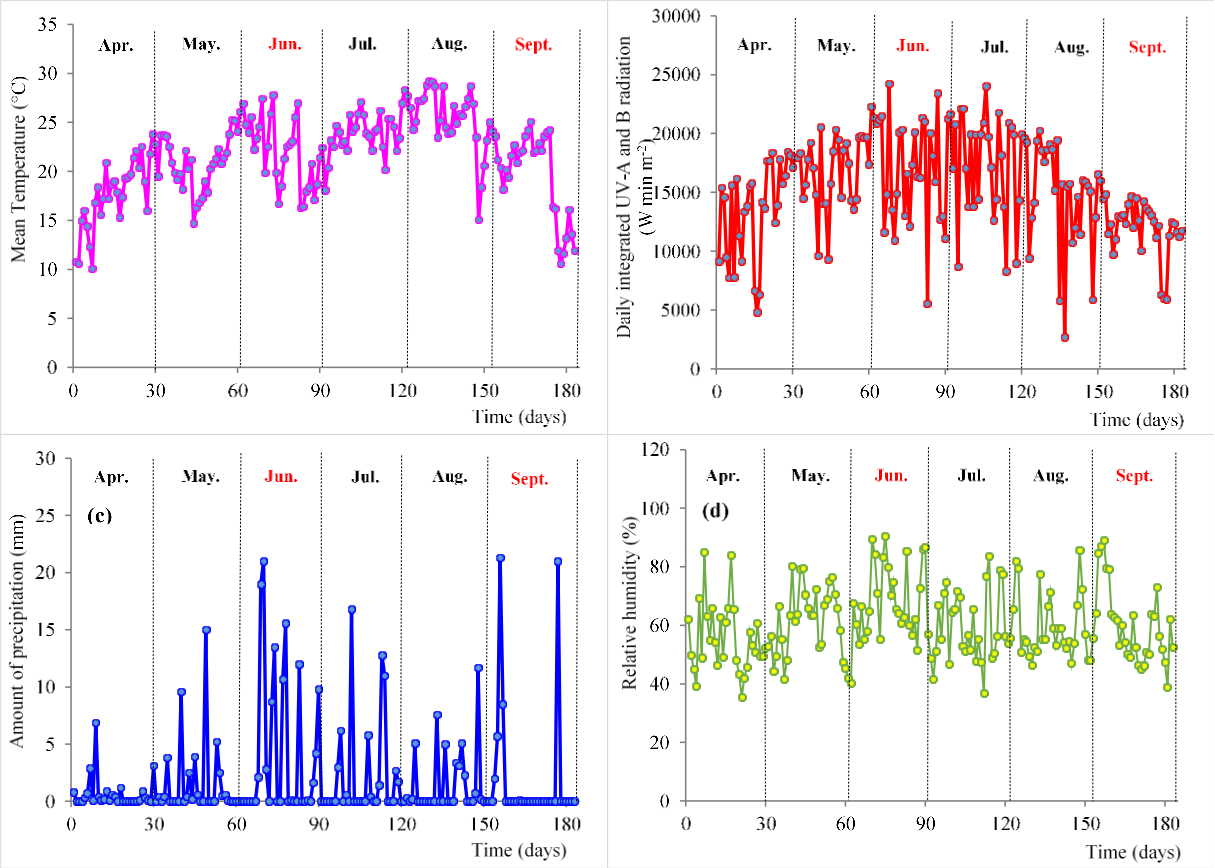
Terrace:	Number of varieties:	Number of plants:
a:	30	309
b:	31	295
c:	27	275
Total:	**88**	**879**

N W

Titán	Kármin	Teinturier femelle	Petit Bouschet	Syrah noir	Szent Lőrinc	Zweigelt	Merlot noir	Kékoportó (Portugieser)		
Alicante Bouschet	Bíbor kadarka	Gamay Fréaux	Muscat Bouschet	Barbera	Gamay noir	Blauburger	Cabernet franc	Kadarka P.9	c	
Rubintos	Grand noir	Gamay teinturier	Carignan noir	Molnárszőlő	Vranac	Pinot noir	Cabernet sauvignon	Kékfrankos Kt.1		
Fresno seedless	Lubik piros / Boucherau	Olimpia testvére	Muscat de Terracina	Gizella emléke	Izbégi Katinka	Boglárka (KM.159)	Olimpia	Pölöskei muskotály	Kossuth szőlő	
Demir kapija	Izsáki nagyszemű	Madeleine Royal	Olivette blanche	Berki 4	Mócsai Mariska	KM.8 (Italia x Szőlősk. kir.)	Rekord	KM.183 (Csilla)	Csaba gyöngye	b
Sicilien	Muscat candia ?	Madeleine angevine	Rizamat	Krasznay Erzsébet	Pécsi áldás	Téli muskotály	Favorit	Narancsízű	Pannónia kincse	
Kozma Pálné muskotály	RF.5 (Reflex)	Göcseji zamatos	Julius Caesar	Angyal Dezső muskotály	Horthy Miklósné	Thallóczy Lajos muskotály	Damjanich tábornok	Ezeréves Magyarország emléke	Szauter Gusztáv	
Judit	F.24/1	Zala gyöngye	Csaba gyöngye, piros	Kocsis Irma	Benkő Julianna	Bem tábornok	Darányi Ignác	Mathiász Pipiske	Szauter Gusztávné	a
Anita	Magnélküli nagybogyójú gömbölyű	Suzy	Irsai Olivér	Gloria Hungariae	Attila	Tompa Mihály	Cegléd szépe	Mathiász Jánosné muskotály	Szőlőskertek királynője muskotály	



A. 4. Compare the meteorological data for 49 years (blue) and in 2018 (red): (a) mean temperature, (b) hours of sunshine, and (c) amount of precipitation. For 49 years, the average annual temperature was 11.6 °C, the site receives 2021 hours of sunshine and 782 mm annual precipitation. In 2018, the average annual temperature was 14.1 °C, the site receives 2186 hours of sunshine and 717 mm annual precipitation.



A. 5. Meteorological data between April and September in 2018: (a) mean temperature, (b) daily integrated UV-A and B radiation, (c) amount of precipitation, and (d) relative humidity of air. In June, the average temperature was 22.1 °C, the site receives 121 mm precipitation, and 305 hours of sunshine. In September, the average temperature was 19.6 °C, the site receives 59 mm precipitation, and 157 hours of sunshine.

A. 6. Meteorological conditions measured during the experimental period. Values represented mean temperature, hours of sunshine, and amount of precipitation.

*= values of 49 years average between years 1951 and 2000 according to the meteorological database of Research Institute for Viticulture and Oenology, Pécs, Hungary.

	Temperature (°C)		Hours of sunshine (h)		Precipitation (mm)	
	Long-term average*	In 2018	Long-term average*	In 2018	Long-term average*	In 2018
June	20.0	22.1	242	305	95	121
September	17.9	19.6	204	157	58	59