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**INTERCROPPING WITH SUBTERRANEAN CLOVER ON  
GRAPEVINE NUTRITION AND DEVELOPMENT IN A  
VOLCANIC SOIL**

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**DOCTORAL THESIS IN FULFILLMENT OF  
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***“Intercropping with subterranean clover on grapevine nutrition and development in a volcanic soil”***

Esta tesis fue realizada bajo la supervisión de la Dra. MARIA DE LA LUZ MORA GIL perteneciente al Departamento de Ciencias Químicas y Recursos Naturales de la Universidad de La Frontera y es presentada para su revisión por los miembros de la comisión examinadora.

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*Le dedico esta tesis a mi Mamita*

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# Summary and thesis outline

Volcanic soils are characterized by limited availability of P (phosphorus) for plant uptake in agriculture. Most of P is fixed to Fe/Al oxides or constituting part of soil organic matter as organic P. For this reason, it is important to search management practices to increase the availability of P in the soil. Therefore, we propose the practice of intercropping as a management strategy for improving P use efficiency in volcanic soils. The main benefits of this practice are the improvement of crop yield, soil fertility, conservation of biodiversity, land-use efficiency, and carbon sequestration. Furthermore, intercropping contributes to the control of soil erosion, reduction of inputs application, and control of diseases and pests. Based on the above, intercropping appears as a useful tool in the current context of climate change and overpopulation.

As a result of climate change, fruticulture is moving from central Chile to the southern regions where traditionally are grown cereals and pastures. In this study, we assessed the feasibility of introducing viticulture in volcanic soils of southern Chile in combination with a traditional pasture. We proposed to grow subterranean clover (*Trifolium subterraneum* L.) with grapevine (*Vitis vinifera* L.) in intercropping for improving grapevine nutrition and development in a volcanic soil. In Chapter I, we present a General Introduction with the Hypothesis and Objectives of this research. We present the Theoretical Background in Chapter II describing the availability of P in volcanic soils and the use of intercropping as a tool for improving plant nutrition. We focused the review on the limitation of P in volcanic soils, the mechanisms of root exudation to mitigate P deficiency, and the use of intercropping as a management practice for improving P availability.

In the first experiment, (Chapter III) we characterized root exudates of grapevine and subterranean clover grown in monocropping and intercropping. Plants were cultivated in Hoagland hydroponic solution during two months under controlled conditions in the greenhouse. The results showed that the main chemical groups were organic acids, amino acids, and flavonoids in all treatments. Moreover, we identified different profiles in the root exudates of plants grown in monocropping and intercropping. We found kaempferol-3-glucuronide, glutathione, gluconic acid, and L-2-aminoadipic acid released only in intercropping treatment. These results indicate that the combination of different plant species modifies the composition of root exudates, suggesting a chemical communication between plants.

In the second experiment, (Chapter IV), we assessed the effect of intercropping between grapevine and subterranean clover on grapevine development in pots using an Andisol as a substrate. The experiment was performed under controlled conditions in greenhouse growing plants in pots in monocropping and intercropping for three months. The results showed that intercropping increases the accumulation of lignocellulosic compounds in the roots improving soil exploration. Besides, intercropping improves soil characteristics such as the concentration of soil available P, the increase of acid phosphatase activity, and the reduction of soil acidity. Furthermore, intercropping reduced the concentration of oxalic acid in the rhizospheric soil in comparison to monocropping. The differences in the concentration of oxalic acid were increasing over time, reaching the highest differences at the end of the experiment.

Finally, in Chapter V, we present the general discussion and conclusions of this research. We conclude that intercropping changes the chemical profile of root exudates particularly of

organic acids, amino acids, and flavonoids. The oxalic acid was the organic acid found in the highest concentration indicating differential stress conditions depending on the cultivation treatment. Intercropping causes competition with lower growth, and concentration of N and P in plant tissue. There are differences in lignocellulosic compounds depending on the nutritional status of grapevine plants. Intercropping enhances soil fertility caused by the increase of acid phosphatase improving the symbiotic relationships between plant and soil. This indicates that in intercropping treatment plants increase the release of organic compounds serving as attractants and energy source for soil microorganisms. Thus, intercropping appears as a promising tool to improve P use efficiency in a volcanic soil, aiming to establish a sustainable agricultural system.

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# **CHAPTER I**

## ***Introduction, Hypothesis, and Objectives***

## **1.1. General Introduction**

Phosphorus (P) is an essential element for all living plants with limited availability in volcanic soils (Mora et al., 2017; Ribera-Fonseca et al., 2018). The most important characteristics of volcanic soils are a high amount of organic matter, low pH, and high content of Fe/Al oxides/hydroxides (Borie and Rubio, 2003; Ribera et al., 2010). In these soils, most of P is fixed to Fe/Al oxides/hydroxides or constituting part of soil organic matter as organic P non-available for plants (Poblete-Grant et al., 2020; Redel et al., 2019). This limitation of P leads to an increase in the consumption of P fertilizers in agriculture raising the production costs. The P fertilizers are mostly obtained from phosphate rock which is a non-renewable resource with constantly increasing prices in the global markets (Karunanithi et al., 2015). Another concern is the eutrophication caused by the excessive use of P fertilizers disturbing the ecosystems particularly in aquatic environments (Gautier et al., 2018). In conditions of P deficiency plants develop different mechanisms for improving soil exploration including the release of root exudates to increase the availability of P in the soil (Carvalhais et al., 2011).

Among the most important mechanisms connecting plants and soil is the exudation of organic compounds by roots (Vives-Peris et al., 2020). Plants release organic compounds in the rhizospheric soil comprising the soil adjacent to the roots. Root exudates are classified according to their molecular weight in two main groups: i) High-molecular-weight compounds including polysaccharides, proteins, mucilage, and ectoenzymes; and ii) Low-molecular-weight compounds comprising organic acids, amino acids, sugars, and flavonoids (Pinton et al., 2007; Sasse et al., 2018). According to previous studies, the most important chemical groups involved in nutrient mobilization are organic acids, amino acids, and phenolics (Haichar et al., 2014; Proctor and He, 2017). The organic acids release phosphate

sorbed to Fe/Al oxides/hydroxides in volcanic soils competing with phosphate for the active sites (Darch et al., 2016; Delfim et al., 2018). The amino acids are a N source and enhance the hormonal system of plants functioning as a biostimulant (Ugolini et al., 2015). Moreover, the tyrosine-sulfated peptides are involved in root growth controlling root hair formation, nitrogen acquisition, and the differentiation of root cells (Oh et al., 2018). Flavonoids are released by roots as signaling compounds attracting beneficial microorganisms to the rhizosphere increasing P availability for plants (Tomasi et al., 2008). The molecules present in root exudates connect different plant species establishing a synergism to increase P use efficiency in the soil (Hallama et al., 2019). Furthermore, the activity of phosphatases enhances the mineralization rates of organic P present in the organic matter increasing P availability in the soil (Redel et al., 2019).

Farmers improve nutrient efficiency for plants performing practices such as intercropping, which consist of growing two or more crops at the same time in a single field. This is a traditional practice developed for many years particularly in regions without modern technology and with low input management (Altieri, 2002; Duchene et al., 2017). According to Morugán-Coronado et al. (2020); Sekaran et al. (2019), the most important benefits of intercropping are the improvement of nutrient retention, an increase of crop yield, conservation of soil fertility, minimizing the effects of pathogens and pests, and preventing soil erosion. Additionally, the improvement of physical properties, increasing water use efficiency in intercropping systems helps to control soil erosion (Ripoche et al., 2010). The most common type of intercropping is the combination of legumes with non-legumes companion plant species benefiting non-legumes with N-fixation supplied by legumes (Bargaz et al., 2017; Bukovsky-Reyes et al., 2019). The synergistic or antagonistic effect of

intercropping depends on the combination of plant species used and the soil where they are growing. However, the mechanisms involved in the interaction between plants and volcanic soil in intercropping systems remains poorly understood (Nassary et al., 2020; Ocimati et al., 2019).

Grapevine is one of the most important fruit crops in the world for producing fresh fruit and wine (Antolín et al., 2020; Bounab and Laiadi, 2019). In Chilean agriculture, grapevine cultivation covers approximately 198,000 ha (Aballay et al., 2020). In the cultivation of grapevine, one of the highest production costs is fertilization, making it necessary to increase nutrient use efficiency (Brunetto et al., 2020; Šarauskiš et al., 2019). For this reason, we searched the feasibility to practice intercropping for improving the efficiency and development of grapevine at a low cost (Morugán-Coronado et al., 2020). We included an herbaceous plant with a partial overlapping between roots to perform an interaction avoiding competition for water and nutrients (Celette et al., 2009). In southern Chile, one of the most important legume species are *Trifolium* spp. traditionally grown in farms and highly adapted to volcanic soils (Campillo et al., 2003). Subterranean clover (*Trifolium subterraneum* L.) is an annual self-reseeding plant without competition for water with grapevine during the summer (Abdi et al., 2020). Additionally, it covers the soil permanently during the year, providing a high release of root exudates able to mobilize P in volcanic soils (Smetham, 2003).

Previous studies performed by Garcia et al. (2018); Mercenaro et al. (2014), have found that the practice of intercropping using grapevine with herbaceous species such as *Dactylis glomerata* L., *Medicago polymorpha* L., and *Trifolium subterraneum* L. obtained beneficial effects in the nutrition and development of the grapevine. Particularly, the combination of

fruit trees with herbaceous plants helps to mitigate Fe deficiency because phytosiderophores released by herbaceous plants increase Fe acquisition for fruit trees under controlled conditions (Cesco et al., 2006). Considering the economical importance of grapevine production in Chilean agriculture, we propose the intercropping using subterranean clover as a useful tool to increase P use efficiency in volcanic soil. Based on the above, the focus of this research is to describe the effects of intercropping between grapevine and subterranean clover in the development of the grapevine.

## **1.2. Hypothesis and Research Objectives**

Based on the previous background, we addressed the following hypothesis:

### **1.2.1. Hypothesis**

Intercropping between subterranean clover and grapevine improve grapevine nutrition and development in a volcanic soil.

#### **1.2.2.1. General Objective**

To determine the effect of intercropping between subterranean clover and grapevine on grapevine nutrition and development in a volcanic soil.

#### **1.2.2.2. Specific Objectives**

1. To identify organic acids, amino acids, and flavonoids in root exudates of subterranean clover and grapevine grown in monocropping and intercropping.
2. To determine the effect of intercropping between subterranean clover and grapevine on soil fertility.

3. To estimate the effect of intercropping on the nutritional status and development of grapevine grown in a volcanic soil.

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## **CHAPTER II**

*Intercropping for improving phosphorus availability in volcanic soils: Current knowledge and gaps*

**Intercropping for improving phosphorus availability in volcanic soils: Current  
knowledge and gaps**

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**Abstract**

Phosphorus (P) is a limited nutrient in volcanic soils for plant uptake, highly fixed to Fe and Al oxides/hydroxides, or constituting soil organic matter. Phosphorus fertilizers produced from phosphate rock is a finite resource with an increasing demand for crop nutrition. Furthermore, the excessive application of P fertilizers pollutes the environment and increases production costs. In low P soils, one of the main responses of plants to mitigate P deficiency is the release of organic compounds by roots.

In soils, interactions between root exudates combining different plant species facilitate P acquisition in crops. Organic compounds present in root exudates may transform organic P into phosphate, or desorbing phosphate from soil minerals. The most important chemical groups involved in phosphate solubilization are organic acids, amino acids, and flavonoids. The main mechanisms observed are the substitution of phosphate, the mineralization of organic P, and the activation of soil microorganisms. These mechanisms explain the synergistic relationships between plants controlled by the release of root exudates into the soil.

In the search for improving P use efficiency in volcanic soils, intercropping is a useful practice performed for many years, able to enhance nutrient availability. This research summarizes the current knowledge regarding the capacity of root exudates to increase P availability for plants grown under intercropping. The combination of different plant species may permit access to unavailable P pools in soil increasing nutrient use efficiency in agriculture. In the present scenario of the scarcity of fertilizers, intercropping is presented as a useful tool to increase crop production in volcanic soils.

**Keywords:** Volcanic soils, soil phosphorus, intercropping, root exudates.

## 2.1. Introduction

Volcanic soils are characterized by low pH, high content of exchangeable Al, and a low amount of bases easily leached by rainfall (Mora et al., 2017; Ribera-Fonseca et al., 2018). In volcanic soils, P is highly fixed to Fe/Al oxides/hydroxides or constituting soil organic matter as organic P (Borie et al., 2019; Brucker and Spohn, 2019; Poblete-Grant et al., 2020). This limitation causes a low availability of phosphate ( $\text{PO}_4^{3-}$ ) where approximately 1% of total P is available for plant uptake (Borie and Rubio, 2003). Currently, phosphate rock, the major raw material for the manufacture of P fertilizers, is a finite resource, with an expected demand of 45,858,000 tons of  $\text{P}_2\text{O}_5$  in 2020 and limited availability for the next 50 years (Fertahi et al., 2020; Karunanithi et al., 2015; Wang et al., 2020).

In the rhizosphere, plant roots release a wide range of compounds such as organic acids, amino acids, and flavonoids improving P availability (Cesco et al., 2010; Preece and Peñuelas, 2020). Among the most important organic acids released by roots for solubilizing P in the rhizosphere are oxalic, and citric acids (Tawaraya et al., 2014). Previous studies have revealed that compounds such as organic acids and amino acids present in root exudates may improve the P use efficiency in soils (Ishikawa et al., 2002; Spohn et al., 2013). Additionally, the mycorrhiza fungi colonizing roots change the profile of root exudates and increase the availability of P in the soil (Ferrol et al., 2019). The interaction between root exudates of different species may establish synergism between plants increasing P use efficiency in the soil (Hallama et al., 2019).

In the search for alternatives to overcome P deficiency in volcanic soils, intercropping appears as a useful tool for mitigating P limitation (Dissanayaka et al., 2015). This practice has been performed for many years worldwide, particularly in regions under low input management, and remaining without modern technology (Wang et al., 2017). The main benefits of intercropping are the improvement of nutrient use efficiency, crop yield, soil fertility, conservation of biodiversity, C sequestration, prevention of soil erosion, and control of diseases and pests (Campanhola and Pandey, 2019; Mouradi et al., 2018; Raseduzzaman and Jensen, 2017). In intercropping, the partial overlapping between roots permits the exchange of compounds and the establishment of symbiotic relationships with soil microorganisms as was indicated by Wang et al. (2018). Furthermore, the colonization of roots by mycorrhizas allows the transfer and exchange of P by the external mycelia between different plants growing simultaneously (Ferrol et al., 2019). The right combination of plant species grown under intercropping enhance P acquisition, and different root types and architecture control the uptake of available phosphorus from different soil depths (Darch et al., 2018).

Based on the above, the focus of this review is to describe the effects of root exudates released to the rhizosphere of crops grown under intercropping on the improvement of P use efficiency. Furthermore, we described the limitation of P in volcanic soils, the mechanisms involved in the mitigation of P deficiency, and the use of intercropping as a tool for increase P availability in volcanic soils.

## **2.2. The limitation of P in volcanic soils**

In volcanic soils, the availability of P for plants is determined by properties such as pH, mineralogy, and content of organic matter (Delfim et al., 2018; Zúñiga et al., 2019).

Phosphate is mainly sorbed on the surface of clay minerals and bound to Fe and Al oxides. This binding capacity is higher in acidic soils decreasing as increasing soil pH (Marinari et al., 2020). Approximately 85-90% of P applied to soils remains unavailable for plant uptake, thus being a limiting factor for crop production (Mejías et al., 2013).

The major form of P is  $\text{H}_2\text{PO}_4^-$  with pK values in the soil solution of  $\text{pK}_1=2.15$ ,  $\text{pK}_2=7.20$ ,  $\text{pK}_3=12.33$ . Hence, a major proportion of phosphate is fixed to Fe and Al oxides by specific and electrostatic interactions constituting mononuclear (labile) and binuclear (recalcitrant) complexes (Fuentes et al., 2008). Silicate minerals or a high amount of  $\text{Ca}^{2+}$  reacts producing orthophosphate with  $\text{CaCO}_3$  sorbing phosphate to Fe and Al minerals (Fuentes et al., 2006). Conversely, P input in arable soils increases Fe deficiency because it improves Fe-P complexation decreasing phosphate availability for plants (Fujii et al., 2019).

Previous studies performed by Hanudin et al. (2014) have found that constituents of soil organic matter such as humic and fulvic acids increase the fixation of P. The accumulation in high-molecular-weight compounds as humate-Al-phosphate complexes reveal C/P ratios of 31 to 71 depending on the agricultural management (Borie et al., 2019; Borie and Rubio, 2003). The complexity of soil organic matter makes it difficult to determine the bioavailability of organic P in soil (Stock et al., 2019). The fractions of organic P in soils may contain approximately between 30 to 80% of the total soil phosphorus (Borie et al., 2019; Brucker and Spohn, 2019). Additionally, phytates cover approximately 50% of organic P pool constituting complexes of P-humic and fulvic acid associated with Fe and Al oxides/hydroxides (Borie et al., 1989; Takahashi and Dahlgren, 2016). However, organic matter bound to clays holds phosphate groups by ligand exchange with hydroxyl groups causing competition between P and clays for adsorption on active sites (Cartes et al., 2015).

The recalcitrance of organic P is highly variable in volcanic soils, with residual P as the most recalcitrant fraction of organic phosphorus. Residual P is composed between 21 and 42% of organic P as inositol hexakisphosphate stabilized in the organic matter, indicating that the recalcitrance of P is associated with mineral sorption and complexation of soil organic matter (Redel et al., 2016; Velásquez et al., 2016). As is showed in Table 2.1, organic P includes inositol phosphates as the major form constituting about 42 to 67% of organic P, and compounds such as myo-inositol, phosphate, hexakisphosphate, and nucleotides (Fuentes et al., 2006).

**Table 2.1.** Different forms of organic and inorganic P found in volcanic soils.

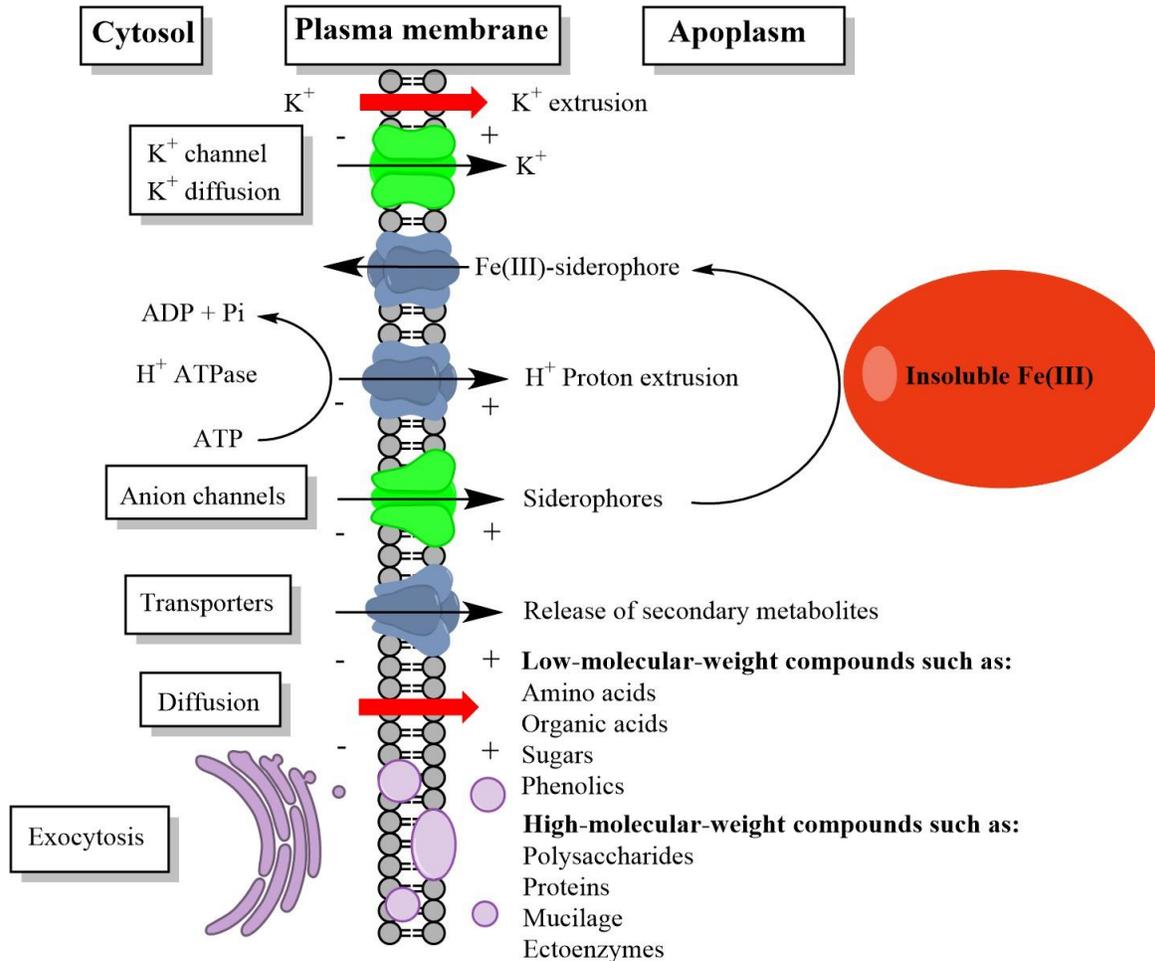
Forms of P	Name	Molecular formula	References
Inorganic	Aluminum phosphate	AlPO <sub>4</sub>	(Ishikawa et al., 2002)
	Apatite	Ca <sub>5</sub> OH(PO <sub>4</sub> ) <sub>3</sub>	
	Iron phosphate	FePO <sub>4</sub> ·H <sub>2</sub> O	
	Orthophosphate	H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> ·HPO <sub>4</sub> <sup>2-</sup>	(Fuentes et al., 2006)
	Pyrophosphate	PO <sub>4</sub> <sup>3-</sup> H <sub>4</sub> P <sub>2</sub> O <sub>7</sub>	
Organic	Fulvic-P	C <sub>14</sub> H <sub>9</sub> O <sub>12</sub> P	(Borie and Rubio, 2003; Borie and
	Humic-P	C <sub>9</sub> H <sub>6</sub> NO <sub>10</sub> P	Zunino, 1983)
	Myo-inositol hexakisphosphate	C <sub>6</sub> H <sub>18</sub> O <sub>24</sub> P <sub>6</sub>	(Borie et al., 1989; Redel et al., 2016; Velásquez et al., 2016)
	Scyllo-inositol hexakisphosphate	C <sub>6</sub> H <sub>18</sub> O <sub>24</sub> P <sub>6</sub>	
	D-chiro-inositol hexakisphosphate	C <sub>6</sub> H <sub>18</sub> O <sub>24</sub> P <sub>6</sub>	
	Neo-inositol hexakisphosphate	C <sub>6</sub> H <sub>18</sub> O <sub>24</sub> P <sub>6</sub>	
	Phosphodiester	C <sub>196</sub> H <sub>245</sub> N <sub>80</sub> O <sub>115</sub> P <sub>19</sub>	(Mise et al., 2018)
	Phytate	C <sub>6</sub> H <sub>18</sub> O <sub>24</sub> P <sub>6</sub>	

In volcanic soils with a high content of organic P and sorption to minerals, plants have developed two main mechanisms to deal with P deficiency: i) to improve P use efficiency in plant metabolism, and ii) increase soil exploration by roots for P scavenging and uptake (Mo et al., 2019; Zheng et al., 2019). Additionally, plants accumulate carbon compounds in roots increasing root-shoot biomass ratio and improving soil exploration by highly branched roots (Magalhaes et al., 2017). The improvement of soil exploration includes changes in the root growth, an increase in the amount of root P transporters, and the release of compounds into the rhizosphere interacting with soil microorganisms (Barra et al., 2019; Campos et al., 2018; Parra-Almuna et al., 2020). Under P deficiency, plants release organic compounds by roots to solubilize unavailable phosphorus in the soil (Carvalhais et al., 2011). It is noteworthy that exudates released from roots constitute a relevant strategy to mitigate the P deficiency in soils.

### **2.3. Root exudation as a mechanism to mitigate P deficiency in the soil**

According to Gargallo-Garriga et al. (2018); Neumann and Römheld, (2007) root exudates are composed by i) low-molecular-weight compounds such as organic acids, sugars, amino acids, ureides, and lipids as major groups released by diffusion; and ii) and high-molecular-weight compounds including polysaccharides, proteins, and mucilages among the most important released by transporters located in the plasma membrane. Figure 2.1 shows the transport of compounds from the root to the rhizosphere, where low-molecular-weight compounds are released by passive transport without energy consumption, and high-molecular-weight compounds are released by active transport with energy consumption. The effluxes of organic acids from roots to soil solution are driven by passive transport of organic

anions (due to a higher concentration of 1,000-fold in the cytosol) and active proton pumping by  $H^+$ -ATPase activity (Coskun et al., 2017; Sasse et al., 2018).



**Figure 2.1.** Mechanisms of root exudation in the plasma membrane of plant roots (adapted from Neumann and Römheld, 2007; Tsai and Schmidt, 2017).

Diverse mechanisms are carried out in the roots for increase P availability including proton release to maintain the balance of internal charge, the exudation of phosphatases, and low-molecular-weight organic acids for P mineralization (Preece and Peñuelas, 2020; Vives-Peris

et al., 2020). The release of protons ( $H^+$ ) to maintain internal charge balance may modify the solubility of organic P compounds affecting adsorption/desorption reactions and its availability for crops (Wei et al., 2019). Root exudation modify pH in soil solution changing the properties of the mineral surface, desorbing P by its competition with phosphate for active sites, and chelating cations mainly Al and Fe (Spohn et al., 2013). The deficiency of P increases the exudation rates, requiring negative charges provided by organic acids pumping for the release of organic acids (Pang et al., 2018). Under P deficiency, plants promote the accumulation of flavonoids in vegetative tissues for protection against adverse environmental conditions (Tomasi et al., 2008). The release of organic compounds depends on the distribution of roots influencing the efficiency in the release of root exudates to the rhizospheric soil. The deficiency of P induces a higher capacity of soil exploration modifying root growth, density, and architecture (Gérard et al., 2017). Herbaceous species have an architecture with widely spread roots in the topsoil making highly efficient in the release of organic compounds to the soil (Proctor and He, 2017; Saleem et al., 2018).

Root architecture influences the distribution of root exudates in the soil profile released mainly in the root tips (Magalhaes et al., 2017). Root growth is spread out to avoid competition for nutrients in the rhizosphere and the overlapping between lateral roots may indicate the effect of exudates in root architecture (Canarini et al., 2019; Tückmantel et al., 2017). The scarcity of P in soils modifies root architecture, promoting responses such as the growth of root hairs, lateral root formation, and mass of cluster roots to increasing nutrient use efficiency (Zheng et al., 2019). The geometry of roots changes P absorption rate especially under P starvation improving the formation of cluster roots and root branching (Campos, 2020). Similarly, plant growth regulators increase in adverse environmental

conditions enhancing P use efficiency (Karunanithi et al., 2015). The most important chemical groups from root exudation involved in the increase of P availability in volcanic soils are organic acids, amino acids, and flavonoids as was found by Contreras et al. (2019). In Table 2.2 it is observed the compounds released by plants growing under hydroponic solution focused on the mechanisms of root exudation without the interference of soil. The concentration of root exudates depends on the chemical mechanisms such as sorption/desorption to soil, and biological processes are referred to microbial consumption (Hallama et al., 2019).

Additionally, compounds present in root exudates include siderophores which mitigate Fe deficiency chelating iron for plants growing under Fe-limiting conditions (Kumar et al., 2019). The release of siderophores attracts microorganisms to the rhizosphere increasing the enzyme activity performed by soil bacteria (Cesco et al., 2006). The improvement of soil microbial activity enhances the availability of other nutrients such as N and P important for plants. In particular, N-fixing bacteria are highly influenced by the release of siderophores because they require large quantities of Fe for oxygen transfer in respiration and the nitrogenase enzyme complex (Crowley and Kraemer, 2007). Furthermore, the release of unavailable P by the dissolution of iron oxides releasing both Fe and phosphates available for both plant acquisition (Zhu et al., 2018).

**Table 2.2.** Organic acids, amino acids, and flavonoids found in root exudates of plants grown under intercropping in hydroponic solution.

Type of compound	Name	Molecular formula	Plant species involved	References
Organic acids	Acetic acid	C <sub>2</sub> H <sub>4</sub> O <sub>2</sub>	<i>Sedum alfredii</i> and <i>Zea mays</i> L.	(Jiang et al., 2013)
	L-2-Aminoadipic acid	C <sub>6</sub> H <sub>11</sub> NO <sub>4</sub>	<i>Trifolium subterraneum</i> L. and <i>Vitis vinifera</i> L.	(Contreras et al., 2019)
	Caffeic acid	C <sub>9</sub> H <sub>8</sub> O <sub>4</sub>	<i>Lactuca sativa</i> L. and <i>Cichorium intybus</i> L.	(Maucieri et al., 2017)
	Chicoric acid	C <sub>22</sub> H <sub>18</sub> O <sub>12</sub>	<i>Lactuca sativa</i> L. and <i>Cichorium intybus</i> L.	(Maucieri et al., 2017)
	Chlorogenic acid	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	<i>Lactuca sativa</i> L. and <i>Cichorium intybus</i> L.	(Maucieri et al., 2017)
	Cinnamic acid	C <sub>9</sub> H <sub>8</sub> O <sub>2</sub>	<i>Glycine max</i> L. and <i>Zea mays</i> L.	(Gao et al., 2014)
	Citric acid	C <sub>6</sub> H <sub>8</sub> O <sub>7</sub>	<i>Sedum alfredii</i> and <i>Zea mays</i> L.	(Jiang et al., 2013)
	<i>p</i> -Coumaric acid	C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>	<i>Glycine max</i> L. and <i>Zea mays</i> L.	(Gao et al., 2014)
	5-Dodecenoic acid	C <sub>12</sub> H <sub>22</sub> O <sub>2</sub>	<i>Vitis vinifera</i> L.	(Contreras et al., 2019)
	Ferulic acid	C <sub>10</sub> H <sub>10</sub> O <sub>4</sub>	<i>Glycine max</i> L. and <i>Zea mays</i> L.	(Gao et al., 2014)
	Formic acid	CH <sub>2</sub> O <sub>2</sub>	<i>Sedum alfredii</i> and <i>Zea mays</i> L.	(Jiang et al., 2013)
	N-Formylaspartic acid	C <sub>5</sub> H <sub>7</sub> NO <sub>5</sub>	<i>Trifolium subterraneum</i> L.	(Contreras et al., 2019)
	Gallic acid	C <sub>7</sub> H <sub>6</sub> O <sub>5</sub>	<i>Vitis vinifera</i> L. and <i>Avena sativa</i> L.	(Marastoni et al., 2019)
	4-Hydroxyphenylacetic acid	C <sub>8</sub> H <sub>8</sub> O <sub>3</sub>	<i>Vitis vinifera</i> L.	(Contreras et al., 2019)
	Malic acid	C <sub>4</sub> H <sub>6</sub> O <sub>5</sub>	<i>Vitis vinifera</i> L. and <i>Trifolium subterraneum</i> L.	(Contreras et al., 2019)
	Oxalic acid	C <sub>2</sub> H <sub>2</sub> O <sub>4</sub>	<i>Vitis vinifera</i> L. and <i>Trifolium subterraneum</i> L.	(Contreras et al., 2019)
	Salicylic acid	C <sub>7</sub> H <sub>6</sub> O <sub>3</sub>	<i>Glycine max</i> L. and <i>Zea mays</i> L.	(Gao et al., 2014)
	Succinic acid	C <sub>4</sub> H <sub>6</sub> O <sub>4</sub>	<i>Sedum alfredii</i> and <i>Zea mays</i> L.	(Jiang et al., 2013)
	Tartaric acid	C <sub>4</sub> H <sub>6</sub> O <sub>6</sub>	<i>Sedum alfredii</i> and <i>Zea mays</i> L.	(Jiang et al., 2013)
	Tartronic acid	C <sub>3</sub> H <sub>4</sub> O <sub>5</sub>	<i>Vitis vinifera</i> L.	(Contreras et al., 2019)
Amino acids	Glutathione	C <sub>10</sub> H <sub>17</sub> N <sub>3</sub> O <sub>6</sub> S	<i>Trifolium subterraneum</i> L. and <i>Vitis vinifera</i> L.	(Contreras et al., 2019)

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	Glycylglycine	$C_4H_8N_2O_3$	<i>Trifolium subterraneum</i> L. and <i>Vitis vinifera</i> L.	(Contreras et al., 2019)
	L-threonine	$C_4H_9NO_3$	<i>Trifolium subterraneum</i> L.	(Contreras et al., 2019)
Phenols	Cyanidin-3-glucoside	$C_{21}H_{21}ClO_{11}$	<i>Lactuca sativa</i> L. and <i>Cichorium intybus</i> L.	(Maucieri et al., 2017)
	Cyanidin-3-malonyl-glucoside	$C_{24}H_{23}O_{14}^+$	<i>Lactuca sativa</i> L. and <i>Cichorium intybus</i> L.	(Maucieri et al., 2017)
	Epicatechin	$C_{15}H_{14}O_6$	<i>Trifolium subterraneum</i> L.	(Contreras et al., 2019)
	Genistein	$C_{15}H_{10}O_5$	<i>Vicia faba</i> L. and <i>Triticum aestivum</i> L.	(Liu et al., 2019)
	Hesperetin	$C_{16}H_{14}O_6$	<i>Vicia faba</i> L. and <i>Triticum aestivum</i> L.	(Liu et al., 2019)
	Isorhamnetin	$C_{16}H_{12}O_7$	<i>Trifolium subterraneum</i> L.	(Contreras et al., 2019)
	Kaempferol-3-Glucuronide	$C_{21}H_{18}O_{12}$	<i>Vitis vinifera</i> L. and <i>Trifolium subterraneum</i> L.	(Contreras et al., 2019)
	Naringenin	$C_{15}H_{12}O_5$	<i>Vicia faba</i> L. and <i>Triticum aestivum</i> L.	(Liu et al., 2019)
	Rutin	$C_{27}H_{30}O_{16}$	<i>Vitis vinifera</i> L. and <i>Avena sativa</i> L.	(Marastoni et al., 2019)

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### **2.3.1. Organic acids to improve P availability in soil**

The organic acids released by plant roots are classified in two main groups: i) low-molecular-weight organic acids (LMWOA) such as oxalic, malic, citric, and succinic acids; and ii) high-molecular-weight organic acids (HMWOA) referred mainly to humic and fulvic acids (Bergelin et al., 2000; Magdziak et al., 2020). The LMWOAs are the most important in root exudates involved in soil phosphate solubilization (Carvalhais et al., 2011; Menezes-Blackburn et al., 2016). Particularly, LMWOA mobilize P from Fe-P and Al-P complexes as well as reducing the sorption of phosphorus fertilizers in soils. The chelation and ligand exchange are the main mechanisms by which organic acids solubilize phosphate (Hallama et al., 2019; Ishikawa et al., 2002). As is described in Table 2.3, most of the unavailable P is bound to Fe or Al oxides in volcanic soils, where plants release carboxylates that complexes Fe and Al solubilizing phosphate in the rhizosphere (Delgado et al., 2015). Additionally, organic acids block the active sites for phosphate adsorption in soil minerals increasing P availability in soil solution (Zúñiga-Feest et al., 2010). The role of organic acids in P availability is closely related to the complexation capacity of aluminosilicates such as allophane, releasing phosphate, and complexing Al simultaneously (Mejías et al., 2013; Takahashi and Dahlgren, 2016).

**Table 2.3.** Equilibrium dissolution reactions of different P forms in acidic soils.

Soil type	Equilibrium reaction	References
Acidic soils	$\text{Fe}(\text{OH})_3 + \text{H}_2\text{PO}_4^- \leftrightarrow \text{FePO}_4 + \text{OH}^- + 2\text{H}_2\text{O}$	(Zhu et al., 2018)
Acidic soils	$\text{FePO}_4 \cdot 2\text{H}_2\text{O}_{(s)} \leftrightarrow \text{Fe}^{3+} + \text{PO}_4^{3-} + 2\text{H}_2\text{O}$	(Zhu et al., 2018)
Acidic soils	$\text{Fe}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}_{(s)} \leftrightarrow 3\text{Fe}^{2+} + 2\text{PO}_4^{3-} + 8\text{H}_2\text{O}$	(Bolan et al., 2005)
Acidic soils	$\text{AlPO}_4 \cdot 2\text{H}_2\text{O}_{(s)} \leftrightarrow \text{Al}^{3+} + \text{PO}_4^{3-} + \text{H}_2\text{O}$	(Bolan et al., 2005)
Acidic soils	$\text{AlPO}_4 + 2\text{H}^+ \leftrightarrow \text{Al}^{3+} + \text{H}_3\text{PO}_4^-$	(Toor and Kim, 2019)
Slightly acidic soils (pH = 6)	$\text{Al}(\text{OH})_2\text{H}_2\text{PO}_4 + \text{OH}^- \leftrightarrow \text{Al}(\text{OH})_3 + \text{H}_2\text{PO}_4^-$	(Hanudin et al., 2014)
Acidic soils	$\text{MnPO}_4 \cdot 1.5\text{H}_2\text{O}_{(s)} \leftrightarrow \text{Mn}^{3+} + \text{PO}_4^{3-} + 1.5\text{H}_2\text{O}$	(Bolan et al., 2005)

The exudation of organic acids depends on different factors controlling the release rate to the surrounding soil. The release rate of organic compounds from roots depends on the environmental conditions and inner characteristics of the roots (Carvalhais et al., 2011). The root age is determinant in the release of organic acids to the rhizosphere, where mature root hairs reach the highest exudation peak in comparison with juvenile and senescent roots (Proctor and He, 2017). These exudates play an important role in the release of Fe to the rhizosphere from iron compounds contributing to the desorption of phosphate from mineral surfaces (Valentinuzzi et al., 2015). Additionally, the increase of organic acids under P deficiency releases phosphate and stimulates the development of microorganisms in the rhizosphere (Darch et al., 2016). The microorganisms contribute to the degradation of soil organic matter, where organic P is mineralized into phosphate available for plant acquisition (Vranova et al., 2013). The mineralization process performed by microorganisms evolves solubilizing the labile P until the most recalcitrant forms of P in the soil (Hallama et al., 2019).

### 2.3.2. The role of amino acids on P availability

In comparison to organic acids, amino acids play a minor role in complexing and mobilizing P for plants in soil solution (Delgado et al., 2015; Zúñiga-Feest et al., 2010). However, the release of amino acids is promoted under conditions of low salinity; therefore, in acidic soils the release of amino acids is considerable, involving passive and active transport (Neumann and Römheld, 2007). In legume plants, the most important amino acids present in root exudates are leucine, valine, lysine, and glycylglycine, easily degradable by soil microorganisms (Bobille et al., 2019; Contreras et al., 2019). These compounds participate in the chemical signaling between neighboring plants controlling the development of roots. The peptides present in root exudates modify the hormonal system of plants particularly auxins, gibberellins, cytokinin, and abscisic acid (Oh et al., 2018). These hormones control the development of roots and shoots including water and nutrients uptake (de Bang et al., 2017).

Amino acids are released in root exudates, but plant roots may also recapture amino acids from soil controlling the growth of microbial communities in the rhizosphere (Carvalhais et al., 2011). Amino acids such as alanine, promote phosphatase activity improving phosphate mineralization (Sasse et al., 2018; Spohn et al., 2013). These compounds play diverse roles such as the stimulation of plant growth-promoting rhizobacteria (PGPR) and colonization of root surfaces (Vives-Peris et al., 2018). The association of legumes with *Rhizobium* bacteria improves the availability of N and P, increasing P use efficiency by symbiotic nitrogen fixation (Nyoki and Ndakidemi, 2017; Tajini et al., 2012). The N-fixation is performed by *Rhizobium* bacteria transforming atmospheric N<sub>2</sub> into ammonium (NH<sub>4</sub><sup>+</sup>), then into nitrite (NO<sub>2</sub><sup>-</sup>), and finally into nitrate (NO<sub>3</sub><sup>-</sup>) all available for plant acquisition (Coskun et al., 2017).

### **2.3.3. Improvement of P availability by flavonoids**

According to Sasse et al. (2018); Vives-Peris et al. (2020), have been identified a wide range of flavonoids in vascular plants with an important amount in the root exudates of legumes. Flavonoids undergo important changes in soils such as the use by microorganisms as a nutrient source, fixation to clay minerals, sorption to soil organic matter, polymerization in organic compounds, chemical transformation, degradation, or chelation to Fe or Al (Hassan and Mathesius, 2012). Although in minor degree compared with LMWOA, flavonoids compete with phosphate ions for sorption sites, desorbing phosphates from soil-mineral surfaces, forming complexes with Fe and Al, increasing P availability in the rhizosphere (Cesco et al., 2012). Besides, flavonoids released by plant roots under P deficiency solubilize Fe-bound P, reduce microbial enzyme activity, and citrate mineralization of soil microorganisms competing with plants for P uptake (Tomasi et al., 2008). Legume roots may survive in conditions of P deficiency releasing flavonoids such as genistein, luteone, and wightone (Coskun et al., 2017; Vance et al., 2003). Plants release flavonoids before the exudation of organic acids, suggesting that flavonoids attract microorganisms to the rhizosphere (Cesari et al., 2019). Flavonoids in the rhizosphere play multifunctional roles such as the regulation of root growth and influence the cycling of P uptake in soil (Ray et al., 2018). The availability of flavonoids in soils depends on the fixation to clays and organic matter, or formation of protein complexes, using as C source, exudates by roots and water leaching (Mehmood et al., 2019). The release of flavonoids in the rhizosphere modifies the structure of bacterial communities changing the flux and pools of amino acids in the soil such as asparagine, glutamine, and glutamate (Czaban et al., 2018).

#### **2.4. Intercropping for improving P availability in volcanic soils**

The reports described above indicate that root exudates constitute an important strategy for plants to mitigate P deficiency (Carvalhais et al., 2011). Therefore, the increase of P availability by intercropping is an important tool for the action of root exudates and the direct uptake of P from the soil solution and the transfer of P by crop biomass (Hallama et al., 2019). Intercropping is closely related to root exudation and symbiotic relationships between plants in P uptake (Bargaz et al., 2017). Plants establish symbiotic relationships with mycorrhiza which enhance soil exploration and with bacteria involved in the production of phosphatases (Sekaran et al., 2019). Besides, the root exudation pattern, root depth, and arrangement influence directly in the partial overlapping of the roots (Proctor and He, 2017). The use of herbaceous species in intercropping helps to transform residual soil phosphorus into organic P available for soil microorganisms (Campanhola and Pandey, 2019). The carboxylates released by legume roots chelates Fe and Al increasing available P in the soil. Furthermore, intercropping improve the activity of phosphatase transforming organic P of soil organic matter into soluble phosphate available for both plant species (Wang et al., 2017). The content of organic matter in the soil is increased under intercropping management stimulating the microbial activity in the soil (Tumwebaze and Byakagaba, 2016). In Table 2.4 we present previous studies of intercropping in soils derived from volcanic ashes.

**Table 2.4.** Previous studies describing the effect of intercropping on plant nutrition in soils derived from volcanic ashes.

Experimental conditions	Species associated	Intercropping effect	References
Field	<i>Terminalia amazonia</i> , <i>Inga edulis</i> , and <i>Gliricidia sepium</i>	Intercropping increases plant growth and biomass accumulation	(Nichols et al., 2001)
Field	<i>Paraserianthes falcataria</i> , <i>Brassica oleracea</i> L. var. <i>Capitata</i> L., <i>Brassica chinensis</i> L., and <i>Zea mays</i> L.	Intercropping maintains yields and increases land-use efficiency especially in steeply sloping lands	(Nissen et al., 2001)
Field	<i>Manihot esculenta</i> Crantz with <i>Glycine max</i> L., and <i>Arachis hypogaea</i> L.	Intercropping increases soil fertility in different manners depending on soil conditions	(Pypers et al., 2011)
Field	<i>Coffea arabica</i> L., <i>Coffea canephora</i> L., and <i>Musa</i> × <i>paradisiaca</i>	Intercropping increases productivity, improving the efficiency of soil management	(van Asten et al., 2011)
Field	<i>Sorghum bicolor</i> L., <i>Crotalaria juncea</i> L., and <i>Helianthus annuus</i> L.	There were no clear effects of intercropping in nutrient uptake and stability	(Miyazawa et al., 2014)
Field	<i>Triticum durum</i> Desf. and <i>Trifolium subterraneum</i> L.	Intercropping increases wheat yield but it is important the arrangement for weed control	(Campiglia et al., 2014)
Pot	<i>Zea mays</i> L. and <i>Lupinus albus</i> L.	Intercropping mobilize sparingly soluble P forms for maize uptake	(Dissanayaka et al., 2015)
Field	<i>Coffea arabica</i> L., <i>Coffea canephora</i> L. <i>Artocarpus</i>	Agroforestry systems have a higher potential to sequester SOC than monocropping	(Tumwebaze and Byakagaba, 2016)

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	<i>heterophyllus</i> Lam., and <i>Mangifera indica</i> L.		
Field	<i>Musa × paradisiaca</i> , <i>Phaseolus vulgaris</i> L., and <i>Glycine max</i> L.	Intercropping improves banana yield, but it is necessary to prune the cover crop for light penetration	(Ocimati et al., 2019)
Field	<i>Zea mays</i> L. and <i>Phaseolus vulgaris</i> L.	Intercropping increases yields and total biomass of both plant species	(Nassary et al., 2020)

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Generally, the release of organic acids by legumes such as citrates mobilizes organic P making it available for cereal crops (Mouradi et al., 2018). Previous studies performed by Darch et al. (2018) found that intercropping combining cereals with legumes increases by 20% P uptake without the application of P fertilizers. An increase of the activity of acid phosphatase with carboxylates including malate, citrate, and succinate, finally solubilize organic P. The deficiency of P in soils promotes the release of carboxylates by legumes, specifically malate and citrate facilitating P uptake in cereals, indicating that intercropping is effective to improve nutrient management (Li et al., 2016; Zhou et al., 2019).

The practice of intercropping using cereals combined with legumes improves the yield of cereal plants but with a lower vegetative growth given by competition between plants (Nassary et al., 2020). The vegetative growth and plant metabolism is controlled by the availability of nutrients and also by light penetration for the plants growing below in the intercropping system (Ocimati et al., 2019). Therefore, the reports describe clear evidence about the important effects of the action of root exudates in P availability for plants grown in intercropping (Figure 2.2).

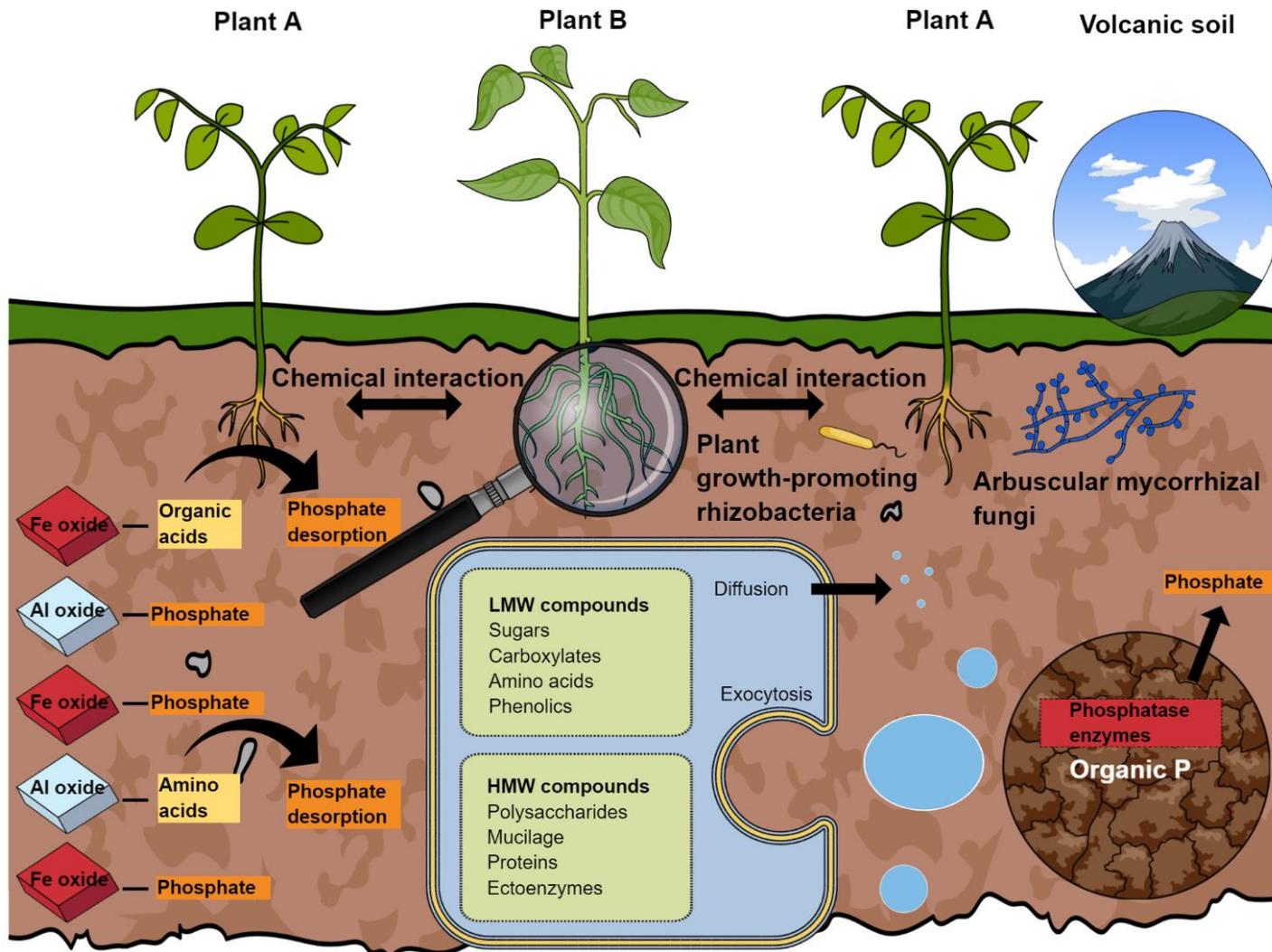


Figure 2.2. Hypothetical scheme for P solubilization by root exudates in volcanic soils with plants grown in intercropping.

## **2.5. Combination of herbaceous species with grapevine and the effects on grapevine nutrition**

The main benefits of using herbaceous plants with grapevine are the increase in the content of soil organic matter, erosion control, water retention, and improvement of symbiotic relationships with soil microorganisms (Garcia et al., 2018). Among the most important herbaceous species are from genera *Lolium*, *Dactylis*, *Trifolium*, *Medicago*, and *Festuca* spp. (Mercenaro et al., 2014). The main differences between grass and legumes are the N-fixation by the legumes increasing nutrient uptake while the grass species are mostly used for detoxifying grapevine from an excessive concentration of micronutrients such as Cu and Mn (Lessandro De Conti et al., 2019). However, previous studies state that a combination of grass with legume species obtain beneficial effects in grapevine development. Among the herbs used there are perennial and annual plants with different behavior depending on the life cycle. The use of annual plants is recommended to avoid the competition for water between clover and grapevine (Celette and Gary, 2013). This is an advantage for subterranean clover because it is an annual reseeding plant that dies during summer after burying the seed in the soil for the following year (Smetham, 2003).

Notwithstanding the belowground interactions are not fully understood, intercropping is an excellent manner to promote interactions between plants and soil microorganisms. The rhizodeposition of organic compounds by herbaceous plants attract soil mycorrhizas and bacteria increasing the mobilization of nutrients (Duchene et al., 2017). In Table 2.5 we present current experiments performed to improve the grapevine nutrition using herbaceous plants. However, the effect of intercropping between grapevine and herbaceous species on P availability in volcanic soils remains unclear.

**Table 2.5.** Previous experiments using herbaceous species in intercropping on grapevine nutrition and development.

Substrate	Species involved	Effect of intercropping	References
Calcaric Fluvisol	<i>Vitis vinifera</i> L., <i>Festuca arundinacea</i> Shreb., <i>Hordeum vulgare</i> L.	Competition for water and N between plants in unfertilized soil	(Celette et al., 2009)
Calcaric Fluvisol	<i>Vitis vinifera</i> L., <i>Festuca arundinacea</i> Shreb., <i>Hordeum vulgare</i> L.	Competition for water and N between plants along the grapevine cycle	(Celette and Gary, 2013)
Calcareous soil	<i>Vitis vinifera</i> L., <i>Festuca rubra</i> L.	Intercropping helps to prevent Fe chlorosis	(Covarrubias and Rombolà, 2014)
Calcareous alluvial soil	<i>Vitis vinifera</i> L., <i>Trifolium subterraneum</i> L., <i>Ornithopus compressus</i> L., <i>Medicago polymorpha</i> L., <i>Lolium perenne</i> L., <i>Dactylis glomerata</i> L.	Intercropping controls excessive vegetative growth and increases the concentration of total anthocyanins in the fruits	(Mercenaro et al., 2014)
Calcareous alluvial soil	<i>Vitis vinifera</i> L., <i>Bromus hordeaceus</i> L., <i>Avena sterilis</i> L., <i>Vulpia myuros</i> L., <i>Medicago polymorpha</i> L., <i>Dactylis glomerata</i> L., <i>Lolium rigidum</i> Gaud.	Intercropping controls excessive vigor of plants, increase yield and phenolic content in the berry	(Muscas et al., 2017)
Typic Argiudoll	<i>Vitis vinifera</i> L., <i>Festuca rubra</i> L.	Intercropping controls vegetative growth, and increase concentration of anthocyanins and free amino nitrogen in fruits	(Coniberti et al., 2018)

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Hoagland nutrient solution	<i>Vitis vinifera</i> L., and <i>Trifolium subterraneum</i> L.	Change of root exudates chemical profile	(Contreras et al., 2019)
Hapludalf soil	<i>Vitis vinifera</i> L., <i>Paspalum plicatulum</i> Michx., <i>Axonopus affinis</i> Sw.	Intercropping mitigates Cu and Mn toxicity improving P uptake	(Lessandro De Conti et al., 2019)
Nutrient solution	<i>Vitis vinifera</i> L., <i>Avena sativa</i> L.	Alleviation of Cu toxicity by phenolic compounds released by oat roots grown in combination with grapevine	(Marastoni et al., 2019)

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## **2.6. Concluding remarks and future perspectives**

The release of root exudates by plant roots play an important role in plant P nutrition mediating interactions between roots and microorganisms. Furthermore, elucidating the mechanisms between plants-soil-microorganisms is essential in the improvement of P nutrition. The interactions between grapevine plants grown under intercropping with subterranean clover in high P-fixing volcanic soils are not fully understood. Furthermore, the association between grasses and legumes including grapevine may be a feasible alternative for improving P use efficiency in the field.

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## CHAPTER III

### *Prospecting intercropping between subterranean clover and grapevine as potential strategy for improve grapevine performance*

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**Prospecting intercropping between subterranean clover and grapevine as potential strategy for improve grapevine performance**

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## Abstract

Intercropping is an agricultural practice commonly used to improve plant nutrition. In this study, we prospected the interaction between root exudates of subterranean clover (*Trifolium subterraneum* L.) and grapevine (*Vitis vinifera* L.). This experiment was focused on the detection of organic acids, amino acids, and flavonoids in root exudates released by grapevine and subterranean clover grown separately and together. Furthermore, we quantified low-molecular-weight organic acids (LMWOA) in root exudates. To test the effect of root exudates in plant-plant chemical signaling, both species were grown in Hoagland hydroponic solution. The experimental design contained three treatments: T1 (subterranean clover, monocropping); T2 (grapevine, monocropping) and T3 (subterranean clover + grapevine, intercropping). The exudate profile showed that the main compounds were amino acid, flavonoids and organic acids in all treatments. Specifically, amino acids exudates (~ 20%) were L-threonine by subterranean clover in monocropping (T1) and glutathione in intercropping with grapevine (T3). Glycylglycine was detected in exudates released by subterranean clover (T1) and both plants under intercropping (T3). Regarding flavonoids (~10%), epicatechin was detected only in exudates of subterranean clover (T1). Interestingly, we detected kaempferol-3-glucuronide, L-2-aminoadipic and gluconic acids were found only under intercropping. The LMWOA were oxalic, malic, citric, and succinic. Oxalic acid was released in higher concentration. We highlight that succinic acid reached the highest concentration under intercropping on day-30. These results strongly suggest that amino acids, flavonoids and organic acids act as signaling compounds between plant-plant interaction, can be utilized for improving grapevine plant performance.

**Keywords:** Intercropping, grapevine, subterranean clover, root exudates.

### **3.1. Introduction**

Intercropping is an agricultural practice, used by farmers with the purpose of increasing nutrients availability and crop productivity (Martin-Guay et al., 2018). In contrast with monocropping, which is characterized by growing a single crop species. Intercropping has been practiced for many years in different climatic regions and under low input management, generally remaining without modern agricultural technology (Altieri, 2002; Raseduzzaman and Jensen, 2017). The main benefits of intercropping are the increase of nutrient efficiency, improving diseases and pest control, water infiltration, weed management, soil erosion, and market risk reduction, making these agro-ecosystems very resilient to stress and continuously changing conditions (Duchene et al., 2017; Marastoni et al., 2019). The association of different plant species can benefit improving efficiency in plant nutrient uptake from soil or deleterious in cases of allelopathic relationships. For instance, intercropping is an effective practice, useful in the mitigation of iron deficiency in citrus, grapevine and olive plants, due to the mechanisms used by plants to improve iron availability into the rhizosphere by means of releasing phytosiderophores, organic acids and other nutrients such as N (Cañasveras et al., 2014; Cesco et al., 2006; Covarrubias and Rombolà, 2014). It is emphasized that intercropping improves nutrient uptake efficiency, in legume-grass systems through the influence of both root exudates interaction (Tajini et al., 2012).

Root exudates are defined as carbon-containing compounds released by plant roots into the rhizosphere (Sasse et al., 2018). Root exudates can be classified into two main groups: (i) high-molecular-weight compounds, constituted mainly by sugars (mucilages) and proteins, and (ii) low-molecular-weight compounds comprising organic acids, sugars, amino acids, among others (Bais et al., 2006). The ability of plants to release compounds into the

rhizosphere is one of the most remarkable traits of plant roots, where compounds play an important ecological role (Raghothama and Karthikeyan, 2005; Sasse et al., 2018).

The most important phosphate solubilizers identified in legume roots through P substitution by organic acids forming complexes with minerals containing  $\text{Fe}^{3+}$ ,  $\text{Al}^{3+}$  or  $\text{Ca}^{2+}$  are citric, malic and succinic acids (Ishikawa et al., 2002; Jia et al., 2018). In legume plants, the most important amino acids present in root exudates are leucine, valine, and lysine, which works as nutrient sources for plants (Bobille et al., 2016). It is noteworthy that root exudates of legume plants are characterized by having compounds such as amino acids with high relevance to increase nutrient efficiency (Coskun et al., 2017). Furthermore, flavonoids can undergo profound changes in soil, such as being used as a nutrient source, being adsorbed by clay minerals and organic matter, or being part of polymerization, chemical transformation, degradation or chelation of different ions (Ray et al., 2018). Flavonoids compete with phosphate ions ( $\text{PO}_4^{3-}$ ) for sorption sites, desorbing phosphates from soil-mineral surfaces, forming complexes with  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$ , and increasing P availability in the rhizosphere (Cesco et al., 2012; Li et al., 2016).

In Chilean agriculture, one of the most important economic crops is grapevine, reaching an area of 137,000 ha. Grapevines are exported in the global markets producing 732,000 tons of table grapes year 2017, and producing over 1,000 millions of liters of wine (Buzzetti-Horta and Banfi-Piazza, 2018). The main varieties grown in Chile are Cabernet Sauvignon, Carménère and Chardonnay produced in transversal valleys located in central Chile (Gennari, 2014; Montes et al., 2012). It is emphasized that volcanic soils are good substrates for grapevine and vineyard establishment providing the nutrients required by plants, whereby its cultivation under intercropping system can be a relevant strategy (Madruga et al., 2015).

Subterranean clover is an important legume comprising 1,000,000 ha of pastures grown in Andisols mainly in southern Chile due to its favorable conditions (Sudy-Bustamante and Guerrero-López, 2018). Therefore, both grapevine and subterranean clover grown in intercropping systems can improve the rhizospheric interactions among plant roots with positive effects (L. De Conti et al., 2019). From the agronomic point of view, subterranean clover is attractive because it is an annual, self-reseeding legume not competing with grapevine for water during the summertime (McLaren et al., 2017). Hence, the model root interaction between grapevine and subterranean clover is proposed to elucidate plant-plant chemical signaling via root exudates performing an experiment under hydroponic culture conditions to evaluate their intrinsic capacity to interact and modulate the release of radical exudates for prospecting its possible influence to increase nutrient availability. Under this context, the objectives of the present study were: (1) to determine the profile of root exudates of subterranean clover and grapevine under monocropping and intercropping conditions and (2) to determine the exudation rates of LMWOAs of subterranean clover and grapevine grown under both cropping conditions.

### **3.2. Materials and Methods**

As previously reported, we focused this study on two model plants: subterranean clover and grapevine, which have relevant economic importance and feasibility to grow in Andisols of southern Chile. The following methodology was established to determine root exudates profile focused on organic acids, amino acids, and flavonoids of both plant species grown under monocropping and intercropping.

### 3.2.1. Plant material and growth conditions

We used subterranean clover (*T. subterraneum* L.) cv. Seaton Park, a common cultivar resistant to root rot; 1-year old grapevine plants of cv. Chardonnay grafted on rootstock 1103 Paulsen (*V. berlandieri* x *V. rupestris*) were provided by the Guillaume nursery (San Fernando, Libertador General Bernardo O'Higgins region, Chile). This rootstock is characterized by their high resistance to drought, nematodes, and phylloxera (Serra et al., 2013; Tsegay et al., 2014). Both grapevine and subterranean clover are widely cultivated in commercial farms of La Araucanía region. The experiment was performed for two months (January and February 2017) under greenhouse conditions at Universidad de La Frontera, Temuco, Chile. The experiment consisted of three treatments: T1 = subterranean clover, T2 = grapevine, and T3 = subterranean clover x grapevine (intercropping), with three replicates arranged in a design with a completely random distribution. At the experiment establishment was used clover plants grown in germination plates for 2 weeks and 1-year-old grapevine plants from the nursery, using a density dose of 200 plants m<sup>-2</sup> for subterranean clover and 1 plant m<sup>-2</sup> for grapevine.

Subterranean clover and grapevine were cultivated in a hydroponic system using Hoagland hydroponic solution under monocropping (T1, T2) and intercropping (T3) (Hoagland and Arnon, 1950). The experiment was carried out using 2 L pots with a density of 16 subterranean clover plants per pot in T1, 1 grapevine plant per pot in T2 meanwhile, 1 grapevine plant with 8 clover plants were grown in T3 using a 3 L container. Bubbling pumps continuously aerated the hydroponic solution and adjusting pH at 5.0 every day. Growth conditions during the experiment were 20 °C; 60% relative humidity; light/dark cycle of 15/9 h and a photosynthetic photon flux density of 400-500 mmol m<sup>-2</sup> s<sup>-1</sup> during the daytime. Root

exudates samples of each treatment were collected 30 and 60 days after the establishment of the experiment. Root exudate collection was performed as described below.

### **3.2.2. Collection of root exudates**

Root exudates were collected taking plants from hydroponic solution into 50 mL of water for chromatography (LC-MS grade) for 2 hours to finally pour in 50 mL falcon vials. The collected root exudates were lyophilized. Afterward, samples were resuspended in 1 mL of water HPLC grade, filtered using pore size 0.22  $\mu\text{m}$  filters to clean up the samples, and finally kept under frozen conditions at  $-20\text{ }^{\circ}\text{C}$  for further analysis. Organic acids, amino acids, and flavonoids present in root exudates of subterranean clover and grapevine were analyzed on days 30 and 60 after the experiment establishment.

### **3.2.3. High-performance liquid chromatography (HPLC) coupled to mass spectrometry for root exudates profile characterization**

The identification of compounds present in root exudates was performed using HPLC/UV-ESI MS/MS according to Eldhuset et al. (2007); Marschner et al. (2011) and Haichar et al. (2014). Samples (10  $\mu\text{L}$ ) were injected in Shimadzu Prominence LC-20AD with detector UV/VIS coupled to a mass spectrometer (Biosystems/MDS Sciex 3200 Qtrap) equipped with ionization source by Electrospray Turbo VTM. HPLC was equipped with RP-C18 Inertsil ODS-3 column (2.1 x 250 mm, 3 $\mu\text{m}$ ) using a flux rate of 0.25 mL  $\text{min}^{-1}$  at 35  $^{\circ}\text{C}$ . Samples were eluted with a mobile phase composed by formic acid:water (1:9) as solvent A and formic acid:methanol (1:9) as solvent B. Samples were analyzed according to the following gradient profile: 0.1-3 min, 5% B; 3-15 min, 10% B, 30-35 min, 50%, 50-70 min, 100%, 80-90 min 5%. The detection wavelength was performed at 254 nm. The software used in the detection and HPLC control was Analyst 1.5.1. All compounds were identified by

comparison of fragmentation patterns referring to on-line database [www.massbank.jp/PeakSearch.html](http://www.massbank.jp/PeakSearch.html) and [www.spectra.psc.riken.jp/menta.cgi/respect/search/fragment](http://www.spectra.psc.riken.jp/menta.cgi/respect/search/fragment). HPLC analysis was focused on the detection of organic acids, amino acids, and flavonoids (profile content was expressed as a percentage relative to total).

#### 3.2.4. Quantification and release rate of LMWOAs

LMWOAs quantified in analytical HPLC were not detected in HPLC/UV-ESI MS/MS because having molar mass lower than  $150 \text{ g mol}^{-1}$  approximately, while compounds presented in Table 3.1 contain higher molecular weights detected by mass spectrometry. The identification and quantification of LMWOAs present in root exudates were performed using the HPLC method reported by Nicola Tomasi et al. (2008); Hassan and Mathesius (2012); Jia et al. (2018). Samples (20  $\mu\text{L}$ ) were injected into analytical HPLC (Prominence LC-20A, Shimadzu, Kyoto, Japan) equipped with a C-18 column (300 x 4.6 mm I.D; particle size 5  $\mu\text{m}$ ). Samples were eluted with a mobile phase composed by solvent A:  $\text{H}_3\text{PO}_4$  200 mM (pH 2.1); solvent B: methanol, solvent C: acetonitrile and solvent D: water, a flow rate of 1  $\text{mL min}^{-1}$  at 30  $^\circ\text{C}$ . Peaks data evaluation was processed by the HPLC software Primaide 1.0. The acid detection was performed at 210 nm. Sigma® provided the standard solutions of oxalic, malic, citric, and succinic acids. The identification of LMWOAs was based on peak retention time in comparison to the respective commercial standards. Respective standard curves were performed on organic acid quantifications. Root release rate of LMWOAs was expressed as  $\text{nmol g}^{-1} \text{ h}^{-1}$ .

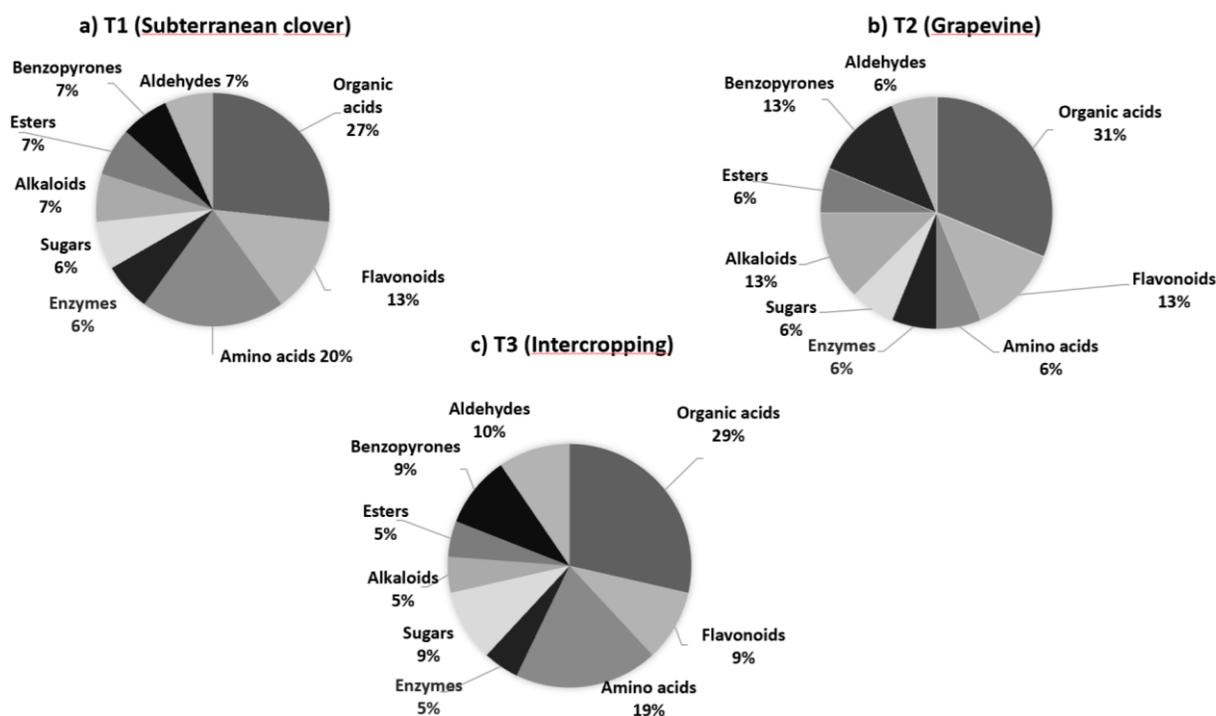
### **3.2.5. Statistical analysis**

Significant differences ( $p < 0.05$ ) were tested using one-way analysis of variance (ANOVA) and Tukey's test. All statistical tests were performed using the statistical package Statistix 10.0.

## **3.3. Results**

### **3.3.1. Identification of compounds present in root exudates of plant species under monocropping and intercropping**

Results indicate that root exudates of subterranean clover grown in monocropping are primarily composed of organic acids (27%), amino acids (20%) and flavonoids (13%). In contrast, in a lower proportion, we found alkaloids, esters, sugars, and benzopyrones (Figure 3.1a). Grapevine exudates grown in monocropping were constituted by organic acids (31%), amino acids (6%) and flavonoids (13%) whereas sugars and enzymes were found in lower proportion (Figure 3.1b). Figure 3.1c shows exudates released by subterranean clover and grapevine under intercropping are composed of organic acids (29%), amino acids (19%) and flavonoids (9%). This study was specifically focused on the identification of organic acids, amino acids and flavonoids, because according to previous studies they are the most important compounds involved in nutrient mobilization (Koo et al., 2005; Kostic et al., 2015).



**Figure 3.1.** Root exudates composition of subterranean clover and grapevine grown in hydroponic solution for 60 days separately and under intercropping system. T1= subterranean clover, T2 = grapevine and T3 = subterranean clover + grapevine (Intercropping).

### 3.3.2. Organic acids, amino acids, and flavonoids present in root exudates of plant species under monocropping and intercropping

Table 3.1 shows the detail of organic acids, amino acids and flavonoids found in root exudates released by grapevine and subterranean clover grown separately and under intercropping systems. It is emphasized that *trans*-cinnamic acid was detected in the root exudates of all treatments (Table 3.1).

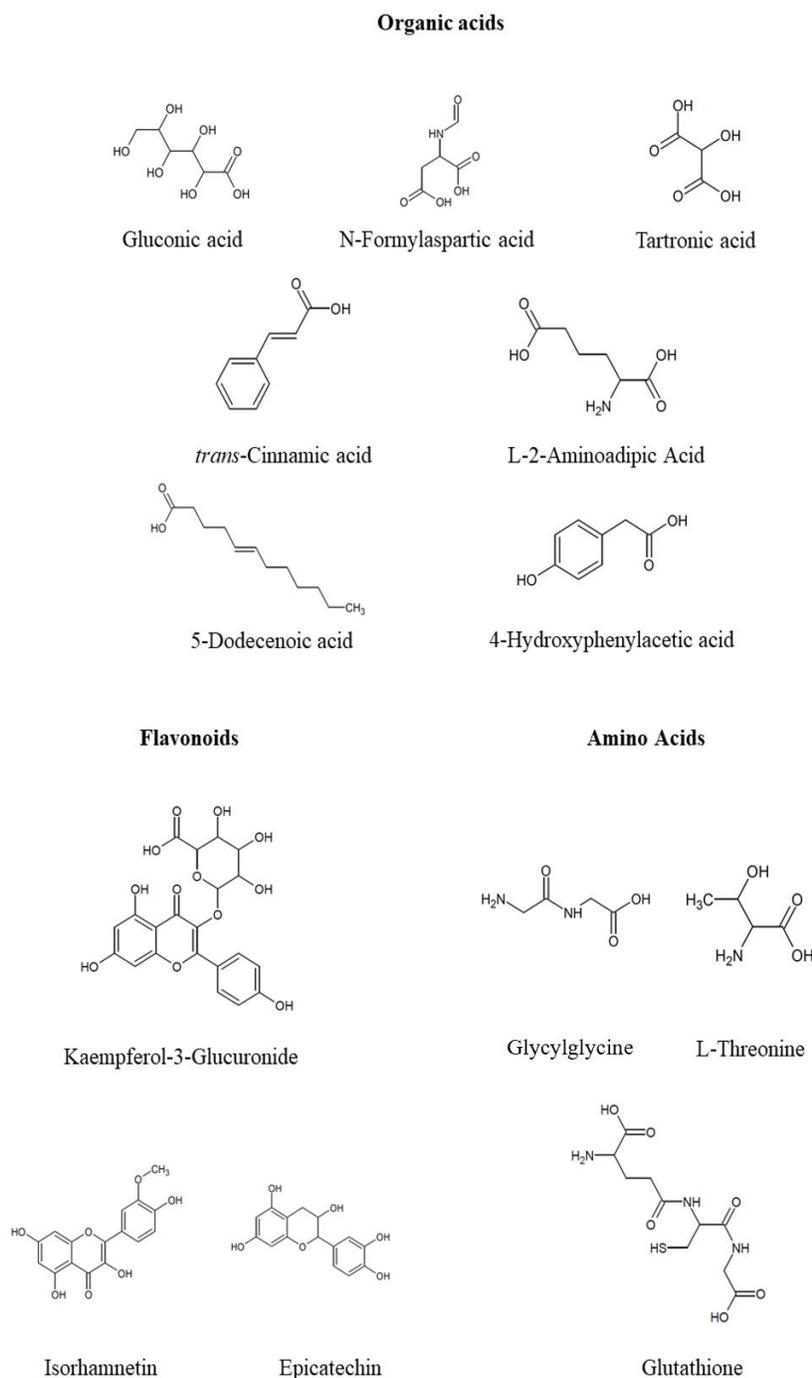
**Table 3.1.** Organic acids, amino acids, and flavonoids composition identified by HPLC in root exudates of different treatments at the end of the experiment (day-60) in hydroponic solution. T1 = subterranean clover, T2 = grapevine, and T3 = subterranean clover + grapevine. ND = not detected.

Treatment	Type of compound	Compound	Molecular formula	Retention time (min)	Centroid $m/z$ (Da)	Fragments (Da)		
T1 (subterranean clover) Monocropping	Organic acids	N-Formylaspartic acid	C <sub>5</sub> H <sub>7</sub> NO <sub>5</sub>	39.1	159.8	115.9	87.8	
		<i>trans</i> -Cinnamic acid	C <sub>9</sub> H <sub>8</sub> O <sub>2</sub>	39.1	146.9	118.9	103.0	147.0
	Amino acids	Glycylglycine	C <sub>4</sub> H <sub>8</sub> N <sub>2</sub> O <sub>3</sub>	2.9	133.0	75.0		
		L-threonine	C <sub>4</sub> H <sub>9</sub> NO <sub>3</sub>	32.6	119.9	72.8	55.7	
T2 (grapevine) Monocropping	Flavonoids	Epicatechin	C <sub>15</sub> H <sub>14</sub> O <sub>6</sub>	5.4	290.8	122.9	206.9	
		Isorhamnetin	C <sub>16</sub> H <sub>12</sub> O <sub>7</sub>	73.9	315.0	315.1	300.1	
	Organic acids	5-Dodecenoic acid	C <sub>12</sub> H <sub>22</sub> O <sub>2</sub>	77.8	196.8	178.9	196.9	
		4-Hydroxyphenylacetic acid	C <sub>8</sub> H <sub>8</sub> O <sub>3</sub>	22.8	150.9	136.0	104.9	
	Amino acids	<i>trans</i> -Cinnamic acid	C <sub>9</sub> H <sub>8</sub> O <sub>2</sub>	39.1	146.9	118.9	103.0	147.0
		Tartronic acid	C <sub>3</sub> H <sub>4</sub> O <sub>5</sub>	85.1	118.9	118.8	95.9	
		ND	ND	ND	ND	ND		
		Flavonoids	Isorhamnetin	C <sub>16</sub> H <sub>12</sub> O <sub>7</sub>	73.9	315.0	315.1	300.1
T3 Intercropping	Organic acids	L-2-Aminoadipic acid	C <sub>6</sub> H <sub>11</sub> NO <sub>4</sub>	37.2	160.0	115.9	160.0	
		<i>trans</i> -Cinnamic acid	C <sub>9</sub> H <sub>8</sub> O <sub>2</sub>	39.1	146.9	118.9	103.0	147.0
	Amino acids	Gluconic acid	C <sub>6</sub> H <sub>12</sub> O <sub>7</sub>	70.3	194.9	129.0	195.0	74.9
		4-Hydroxyphenylacetic acid	C <sub>8</sub> H <sub>8</sub> O <sub>3</sub>	22.8	150.9	136.0	104.9	

Amino acids	Glutathione	$C_{10}H_{17}N_3O_6S$	35.2	613.5	613.4
	Glycylglycine	$C_4H_8N_2O_3$	2.9	133.0	75.0
Flavonoids	Kaempferol-3- Glucuronide	$C_{21}H_{18}O_{12}$	39.1	463.1	287.2 463.0
	Isorhamnetin	$C_{16}H_{12}O_7$	73.9	315.0	315.1 300.1

In relation to organic acids, N-formylaspartic acid was found only in the root exudate of subterranean clover grown in monocropping (T1). 5-dodecenoic was found in grapevine monocropping (T2). Interestingly, gluconic acid was found only under intercropping (T3). It is noteworthy that 4-hydroxyphenylacetic acid was found under mono and intercropping of grapevine. Regarding amino acids, its noteworthy that glutathione is the tripeptide (glutamate, cysteine, and glycine) only released under intercropping. The results showed that subterranean clover released L-threonine under monocropping (T1). Meanwhile, amino acids were not detected in grapevine under monocropping (T2).

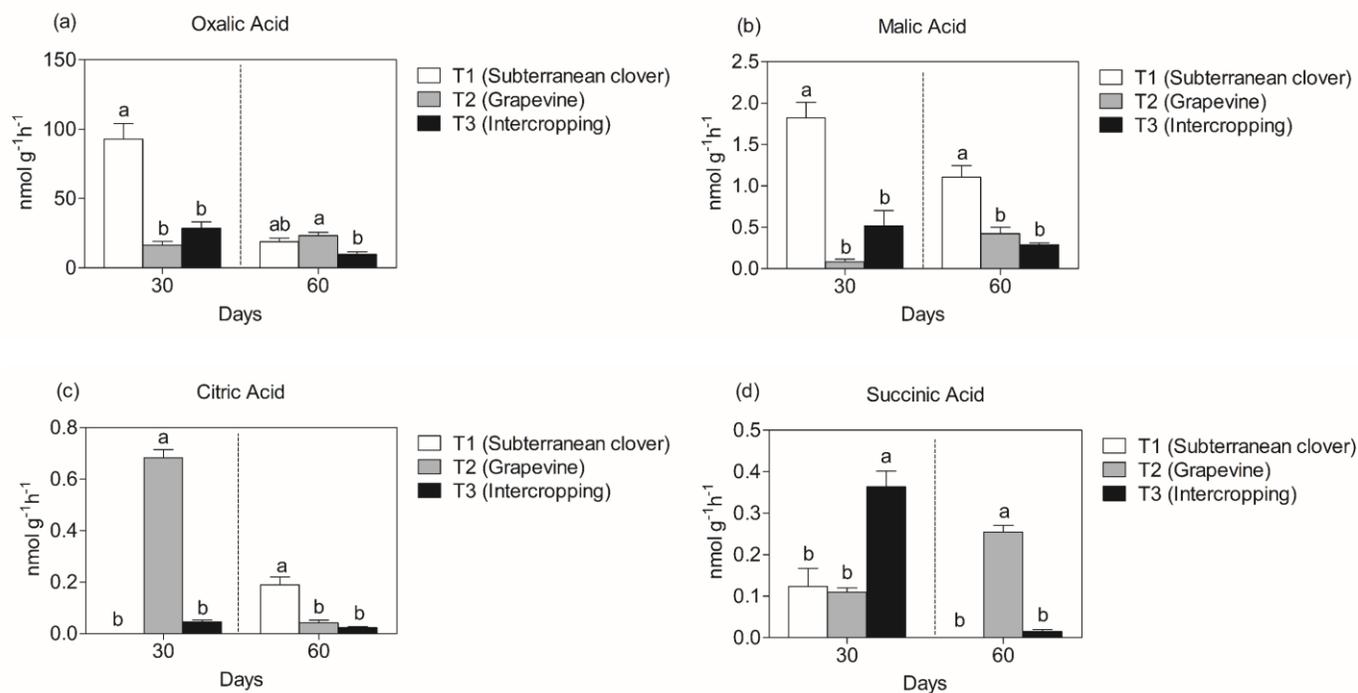
Respect to flavonoids, isorhamnetin was found in root exudates released by all treatments. Epicatechin was detected only in root exudates of subterranean clover under monocropping (T1). It highlights that kaempferol-3-glucuronide was found only under intercropping (T3). The detail of the chemical structures of compounds is found in Figure 3.2 and spectra of compounds found in root exudates detected by HPLC/UV-ESI MS/MS are presented in supplementary Table 1.



**Figure 3.2.** Chemical structure of identified compounds through HPLC/UV-ESI MS/MS present in root exudates released by subterranean clover and grapevine grown separately and together (intercropping) under hydroponic conditions on day-60.

### 3.3.3. Exudation rates of LMWOAs released by plant roots

Figure 3.3 shows the exudation rate associated with LMWOAs, which was analyzed 30 and 60 days after the beginning of the experiment. The release rate of oxalic acid is significantly higher than the other LMWOAs. Figure 3.3a indicates that oxalic acid has a higher exudation rate in subterranean clover (T1) ( $92.930 \pm 19.440 \text{ nmol g}^{-1}\text{h}^{-1}$ ) on day-30 but this rate decreases ( $28.590 \pm 13.760 \text{ nmol g}^{-1}\text{h}^{-1}$ ) when grown under intercropping with grapevine (T3). Figure 3.3b shows that malic acid has the highest exudation rate in subterranean clover (T1) ( $1.820 \pm 0.320 \text{ nmol g}^{-1}\text{h}^{-1}$ ) on day-30, showing significant differences with the other treatments. Figure 3.3c showed significant differences in citric acid with the highest release rate in grapevine (T2) ( $0.680 \pm 0.060 \text{ nmol g}^{-1}\text{h}^{-1}$ ) on day-30. Meanwhile, the highest release rate in subterranean clover (T1) ( $0.190 \pm 0.050 \text{ nmol g}^{-1}\text{h}^{-1}$ ) on day-60. Finally, Figure 3.3d showed significant differences in the exudation rate of succinic acid reaching the highest rate in intercropping treatment (T3) ( $0.360 \pm 0.063 \text{ nmol g}^{-1}\text{h}^{-1}$ ) on day-30. Whereas, on day-60 the highest release rate was in grapevine (T2) ( $0.260 \pm 0.025 \text{ nmol g}^{-1}\text{h}^{-1}$ ).



**Figure 3.3.** Root exudation rates of organic acids of subterranean clover and grapevine 30 and 60 days after the beginning of the experiment. (a), (b), (c), and (d) show quantification of oxalic, malic, citric, and succinic acids respectively. T1 = subterranean clover (Monocropping); T2 = grapevine (Monocropping) and T3 = subterranean clover + grapevine (Intercropping). Error bars indicate standard error of the mean. Letters indicate statistical significance according to ANOVA (Tukey's test) ( $p < 0.05$ ) ( $n = 3$ ). \*ns = no significant differences between treatments.

### 3.4. Discussion

Root exudates influence in chemical signaling in plant-plant interaction grown under intercropping, modifying compound profiles and its concentration. Subterranean clover is a legume characterized by release organic acids, amino acids, and flavonoids with ecological relevance to increase plant performance. In this study, plants grown in Hoagland solution

under sufficient nutrition condition; whereby, compounds present in root exudates were released as part of plant metabolism. Therefore, we evaluated the intrinsic ability of both grapevine and subterranean clover to interact at root exudates level for prospecting intercropping as a strategic tool to increase nutrient uptake.

Data highlight that both species subterranean clover and grapevine roots can release a wide range of compounds belonging to different types of chemical compounds depending on cropping system (monocropping and intercropping); for example, organic acids, flavonoids, amino acids, enzymes, sugars, alkaloids, esters, benzopyrones, and aldehydes (Brunetto et al., 2016). It is noted worthy that benzopyrones, alkaloids and flavonoids have an important role as mechanisms of defense against pathogens and benzopyrones as antifungal and antibacterial (Matsuura and Fett-Neto, 2015; Mhlongo et al., 2018).

Regarding amino acids, the results showed that subterranean clover grown in monocropping releases 20% of amino acids of the overall exudates sampled, which is higher compared to grapevine (6%). The data indicated that the percentage under intercropping (19%) is similar to obtained in subterranean monocropping. These results agree with Kusliene (2014), who found that legume plants had higher contents compared to non-legume plants, which favor N nutrition of grapevine. Furthermore, other experiments performed on root exudates using plants of *Trifolium repens* L. obtained similar results (Lesuffleur and Cliquet, 2010). In this study, we found that only L-threonine (essential amino acid) was found in root exudates of subterranean clover. Interestingly, glutathione was found only in exudates of plants grown under intercropping, which highlights as plant-plant signaling compounds between both plant species.

In relation to peptides, the results showed the presence of glycylglycine a dipeptide of glycine, which was only found in root exudates of subterranean clover (T1) and under intercropping (T3). It is emphasized that peptides have a relevant ecological role due to mediate the communication between plants, transmitting signals by receptors located in plant roots. Thus, these small signaling peptides improve morphological and physiological traits increasing plant nutrient uptake (de Bang et al., 2017). Although many signaling peptides and receptors have been identified, further knowledge is needed to clarify the mechanisms involved in this crosstalk among plants (Oh et al., 2018). Some amino acids such as L-tryptophan promote auxin activity in plants. However, interactions with other amino acids can inhibit plant growth, making necessary further studies about peptides role in soil (Rouphael et al., 2017). Therefore, the presence of both amino acids and peptides in root exudates can play a relevant role for growth regulation due to plant roots are capable of absorbing amino acids and peptides present in biostimulants translocating from soil inside the plant where regulate plant growth (Ugolini et al., 2015).

Additionally, flavonoids have a relevant role at root level due to the release of nutrients adsorbed to mineral surfaces into the soil solution, whereby its presence in root exudates have relevance to improving plant nutrition (Sasse et al., 2018). It noteworthy that kaempferol-3-glucuronide is present only in plants grown under intercropping; suggesting that the interaction of both plants promotes its release as signaling compound. Metabolic pathway of kaempferol-3-glucuronide production comes from *p*-coumaric acid inside the cells (Kallscheuer et al., 2017). Kaempferol-3-glucuronide is a flavonol with antioxidant properties found in root exudates of *Abelmoschus esculentus* according to the reported by (Ray et al., 2018). Furthermore, the data indicated that isorhamnetin is an O-methylated

flavonol present in both species grown separately and in intercropping. Isorhamnetin have been found in grapevine plant tissues and white clover according to Shi et al. (2018).

Particularly, organic acids found in root exudates contain both aromatic (*trans*-cinnamic acid, 4-hydroxyphenylacetic acid) and aliphatic groups (gluconic, N-formylaspartic, tartronic, L-2-aminoadipic, 5-dodecenoic), which is in correspondence to the study performed by Adeleke et al. (2016). The results indicated that *trans*-cinnamic acid was released in all evaluated treatments. *trans*-cinnamic acid is an allelochemical influencing metabolic processes such as seed germination and plant root growth, involved in lignin and flavonoids biosynthesis (Salvador et al., 2013). It highlights that L-2-aminoadipic acid was detected only in intercropping, suggesting its role between the plant-plant signaling.

LMWOAs determined by HPLC technique were oxalic, malic, citric, and succinic, which according to previous studies are the most common acids found in root exudates, specifically in legume species. Composition and concentration of LMWOAs are in correspondence with Sandra López-Rayó et al. (2015), who found that oxalic, tartaric, malic and ascorbic acids are present in root exudates of grapevine, with similar concentrations of oxalic acid found in this study ( $4,549 \text{ nmol g}^{-1} \text{ h}^{-1}$ ). Furthermore, similar results indicating a high concentration of oxalic acid were obtained by Eldhuset et al. (2007). Conversely, differences in oxalic acid tend to decrease over time which can be associated to a higher release rate in immature roots as is proposed by Proctor and He, (2017). In relation to the release rate of LMWOAs over time, there are different patterns depending on cropping system. These patterns can be due to a different release rate of these compounds in mature roots developed during the final period of the experiment. The data indicated that subterranean clover plants showed a higher concentration of LMWOAs compared to grapevine, confirming the hypothesis that it has a

higher capacity to release compounds in the rhizosphere (Lesuffleur and Cliquet, 2010; Ponce et al., 2004). Therefore, the results showed that subterranean clover constitutes a relevant source of LMWOAs in root exudates. Interestingly, the succinic acid was significantly higher in root exudates released under intercropping, indicating its importance during the interaction of both plants. In contrast, the other LMWOAs, where the malic and citric acid reached the highest concentration in subterranean clover (T1) and grapevine (T2), respectively.

The results showed that bioassays performed in hydroponic conditions allowed to determine the differential profile of root exudates of grapevine and subterranean clover cultivated under monocropping and intercropping, but the underlying mechanisms are still unknown. Furthermore, data showed the relevant importance that subterranean clover constitutes a substantial source of root exudates, playing a key role during root exudates interaction through intercropping.

### **3.5. Conclusions**

This study provides relevant evidence that subterranean clover growing together with grapevine plays a key role to modulate root exudates profile and plant-plant interaction. Besides, it was shown that root exudates had a wide range of chemical compounds with organic acids, amino acids, and flavonoids as the most important in both grown plants. Interestingly, intercropping has a significant effect differentiating root exudates profiles where kaempferol-3-glucuronide, glutathione, gluconic acid, and L-2-aminoadipic acid were released only under intercropping conditions. Data indicated that subterranean clover has a high potential for being selected as a promissory strategy to improve industrial grapevine plant performance.

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## CHAPTER IV

*Exploring the potential of intercropping between subterranean clover with grapevine on grapevine development and soil characteristics*

**Exploring the potential of intercropping between subterranean clover with grapevine on  
grapevine development and soil characteristics**

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## Abstract

Intercropping with subterranean clover (*Trifolium subterraneum* L.) may be a useful practice to increase phosphorus (P) acquisition in Andisols characterized by low P availability due to elevated sorption capacity. In this study, we aimed to evaluate the effect of intercropping with subterranean clover on the nutritional status of grapevine (*Vitis vinifera* L.). We performed a pot experiment using Andisol as the substrate in a greenhouse for three months with plants grown in monocropping and intercropping. The experimental design contained four fertilization treatments: Control (without fertilization); N (nitrogen applied); P (phosphorus applied); and NP (nitrogen and phosphorus applied). We sampled soil solution periodically, and at the end of the experiment, we collected soil samples and plant material for further physico-chemical analysis. Results revealed that intercropping with subterranean clover decreased the vegetative growth of grapevine reducing the concentrations of carbon (C), N, and P in plant tissue. Interestingly, intercropping with these species increased soil pH and acid phosphatase activity, thus mitigating acidity, and improving P availability. Characterization of root surfaces of grapevine by Confocal Laser Scanning Microscopy (CLSM) showed that lignin and cellulose increased significantly in intercropping with N fertilization compared to monocropping. Thermogravimetric Analysis (TGA) coupled to GC/MS released carboxylic acids, ketones, sugars, phenols, furans, and amines as major groups at high temperature from grapevine roots. We found significant differences in the relative abundance of ketones and phenols between monocropping and intercropping treatments, particularly with P applied. The concentration of oxalic acid was higher under monocropping in comparison to intercropping treatment, especially at the end of the experiment. Intercropping with subterranean clover may strengthen grapevine plants by increasing the roots' lignocellulosic compounds and mitigating environmental stress. Based

on the results we may suggest that the use of subterranean clover in intercropping is a promising tool for improving soil properties and nutrient use efficiency.

**Keywords:** Intercropping, subterranean clover, grapevine, Andisols.

#### **4.1. Introduction**

In the current scenario of global warming and overpopulation, sustainable intensification of agricultural production is crucial (Rockström et al., 2017). Intercropping with leguminous species, such as subterranean clover may not only improve plant nutrition but may also be useful for weed suppression especially in grapevine vineyards (Trigo-Córdoba et al., 2015).

Grapevine is one of the most frequently cultivated fruit trees, comprising approximately 7.4 million hectares in the world (Antolín et al., 2020). Grapevine products are exported to the global markets for example 732,000 tons of table grapes year 2017, producing over 1,000 million liters of wine (Cejudo-Bastante et al., 2018). In Chilean agriculture, the grapevine is one of the most important commercial fruit crops, occupying an area of approximately 198,000 ha (Aballay et al., 2020). It was emphasized that Andisols are suitable substrates for grapevine and vineyard establishment providing the nutrients required by plants (Madruga et al., 2015). However, Andisols are characterized by low amounts of available P caused mainly by the sorption of phosphate to Fe and Al hydroxides or organic P incorporated into organic matter (Borie and Rubio, 2003; Poblete-Grant et al., 2020). The availability of P in these soils is therefore closely related to the concentration of C and N (Panichini et al., 2017). Furthermore, climate change has moved the viticulture to the southern regions of Chile, making it necessary to study the suitability of Andisols for grapevine cultivation (Zambrano et al., 2018). Root exudates can release phosphate from insoluble  $\text{FePO}_4$  and  $\text{AlPO}_4$  and

increase phosphatase activity transforming organic P into available phosphate for plant uptake (Mise et al., 2020). Additionally, the effect of *Rhizobium* nodulation of legume roots may increase N and P availability in soil (Nyoki and Ndakidemi, 2017). Therefore, we propose the use of subterranean clover (*Trifolium subterraneum* L.) as model legume plants to grow in intercropping with grapevine. In southern Chile, subterranean clover is one of the most important species grown traditionally in pastures and highly adapted to Andisols (Campillo et al., 2003). Subterranean clover is a self-reseeding plant widely grown with a highly efficient release of organic acids, amino acids, and flavonoids (Contreras et al., 2019; Smetham, 2003). In legumes, the release of root exudates mediates plant-microorganisms relationships, improving colonization of symbiont bacteria in rhizospheric soil and roots (Ankati and Podile, 2019). Intercropping may thus enhance grapevine development through the improvement of plant nutritional status, particularly in Andisols with low nutrient availability.

Thus, in this study, we focused on subterranean clover as a facilitator for grapevine development in Andisols. We carried out a pot experiment under controlled conditions and determined: i) plant growth and elemental composition, ii) physicochemical soil parameters, iii) roots surface properties, iv) thermally labile compounds of grapevine roots, and v) concentration of oxalic acid in soil solution. The study aimed to determine the effect of intercropping with subterranean clover on grapevine nutrition and development.

## 4.2. Materials and Methods

### 4.2.1. Soil substrate and plant material

We used an Andisol obtained from an agricultural field located in La Araucanía region (at 38°47'07.6''S 72°16'36.3''W) belonging to the Vilcún soil series. The soil was sampled at 0-20 cm depth, air-dried and sieved to pass a 2-mm sieve. General characteristics of the soil are shown in Table 4.1. Before the beginning of the experiment, we applied a dose of 1 ton ha<sup>-1</sup> (1 g pot<sup>-1</sup>) of dolomite to the soil until reaching a sum of bases of 7.59 cmol+ kg<sup>-1</sup>. Furthermore, we applied basal fertilization of 100 kg ha<sup>-1</sup> of K by adding 0.16 g pot<sup>-1</sup> KCl to all treatments. We used subterranean clover (*T. subterraneum* L.) cv. Seaton Park, a widespread cultivar used in pastures and grapevine plants of 1-year old cv. Chardonnay grafted on rootstock 1103 Paulsen (*V. berlandieri* x *V. rupestris*), which were provided by Guillaume nursery located in San Fernando, Libertador General Bernardo O'Higgins Region, Chile.

**Table 4.1.** Chemical characteristics of soil used at the beginning of the experiment.

Parameter	Soil content
pH (H <sub>2</sub> O)	5.38
Organic matter (%)	13.00
Olsen P (mg kg <sup>-1</sup> )	3.34
K (mg kg <sup>-1</sup> )	176
Na (cmol+ kg <sup>-1</sup> )	0.11
Ca (cmol+ kg <sup>-1</sup> )	5.28
Mg (cmol+ kg <sup>-1</sup> )	1.75
Al (cmol+ kg <sup>-1</sup> )	0.23
Al saturation (%)	2.94
CEC (cmol+ kg <sup>-1</sup> )	7.82
Sum of bases (cmol+ kg <sup>-1</sup> )	7.59

#### 4.2.2. Experimental design and growth conditions

We performed the experiment for three months under greenhouse conditions at the University of La Frontera, Temuco, Chile, using two crop treatments: monocropping (grapevine) and intercropping (grapevine + subterranean clover) with four fertilization treatments. The experiment was performed under the following conditions: with an average temperature of 20 °C; relative humidity of 60%; a light intensity of 406 mmol photons m<sup>-2</sup>s<sup>-1</sup> and a light/dark cycle of 15/9 h. We used pots of a volume of 3 L containing 2.5 kg of dry sieved soil, plants were 1-year-old grapevines from the nursery. Four contrasting fertilizer doses were applied: (1) 0.21 g pot<sup>-1</sup> corresponding to 100 kg ha<sup>-1</sup> of urea fertilization (N); (2) 0.04 g triple

superphosphate  $\text{pot}^{-1}$  corresponding to  $20 \text{ kg ha}^{-1}$  of P (P); and (3) a combined treatment (NP) consisting of  $0.21 \text{ g urea}$  and  $0.04 \text{ g triple superphosphate pot}^{-1}$ . The fertilizers were applied after planting, dissolved in water used during the first irrigation. Furthermore, we established a control treatment without fertilizer addition.

In treatments using subterranean clover, we sowed 30 seeds per pot, and 1 week after germination we thinned to reach 20 plants of subterranean clover per pot using a density of  $600 \text{ plants m}^{-2}$ . In monocropping treatment, we used 1 plant of grapevine per pot, and in the intercropping treatment, we used 1 plant of grapevine with 20 plants of subterranean clover per pot. We irrigated plants daily using distilled water as needed during the experimental period. Weed control was performed manually in a similar manner for all treatments during the whole experiment. We arranged pots in a factorial design with four replicates and randomized every week during the experiment. After the first month of the experiment, we maintained two shoots per plant of grapevine with one lateral per shoot.

#### **4.2.3. Collection of soil solution from pots**

During the experimental period, we collected soil solution at months 1, 2, and 3 after the beginning of the experiment. We used Rhizon samplers with a diameter of 2.5 mm and a mean pore size of  $0.15 \mu\text{m}$ , which was introduced in one of the five holes located in the pot sides at 10 cm depth with a diameter of 3.0 mm. During each sampling, we introduced Rhizon samplers coupled to a syringe to extract soil solution under vacuum when pots were filled with 1 L of distilled water. We collected the extract of soil solution, which was filtered with a syringe filter of  $0.22 \mu\text{m}$  pore size. Afterward, samples were kept frozen at  $-80 \text{ }^\circ\text{C}$  in Falcon tubes. Subsequently, we lyophilized the soil solution collected for further HPLC analysis.

#### **4.2.4. Determination of plant growth and elemental composition**

At the end of the experiment, we measured shoot growth, root growth, and leaf chlorophyll index using a portable chlorophyll meter SPAD Minolta 502 (Konica Minolta, Inc., Osaka, Japan) performing five measurements per leaf blade in the three first leaves per each shoot. Then, we weighed plant material, dried it at 60 °C for 24 h to determine dry matter (DM). An aliquot was ground in mortar for further analysis. In the first instance, we determined the concentration of P in roots and leaves of grapevine following the methodology of molybdo-vanadate described by Sadzawka et al. (2007).

The concentration of P in leaves and roots was expressed as g kg<sup>-1</sup> of dry weight (DW), and P uptake was expressed as mg plant<sup>-1</sup>. We calculated the P uptake as the product of plant biomass and concentration of P in shoot and roots (P uptake = shoot DW \* shoot P concentration + roots DW \* roots P concentration) based on P determinations performed by Redel et al. (2019). The concentration of C, N, and C/N ratio of plant and soil samples were determined with a Eurovector Elemental Analyzer (Isoprime-Euro EA 3000) by the dry combustion method (up to 1,200 °C) and expressed as g kg<sup>-1</sup> of dry weight (DW).

#### **4.2.5. Characterization of roots surface under microscopy**

We observed the fluorescence of lignocellulosic compounds in the surface of roots using a Confocal Laser Scanning Microscope based on the methodology performed by Pontigo et al. (2018); Vega et al. (2019). To visualize lignin and cellulose present in the surface of roots, we stained the samples with 0.1% safranin O, and 1% Congo red simultaneously. Samples were stained with each dye for 20 min, rinsed with distilled water three times to remove dye excess, and then viewed in Confocal Laser Scanning Microscope (CLSM) Olympus Fluoview 1000. We measured auto-fluorescence at excitation/emission of 546/590, and

635/690 nm while images were processed using the software FV10-ASW version 2.0c (Olympus, Japan). We analyzed the fluorescence intensity of images using the software SigmaScan Pro version 5.0 (Systat, Inc., San Jose, CA), expressing fluorescence intensity as relative fluorescence unit (RFU).

#### **4.2.6. Thermogravimetric analysis (TGA) of grapevine roots**

After the drying and milling of samples, we performed the pyrolysis using a TGA coupled to a GC/MS. We placed approximately 4.5 mg of samples of grapevine roots into the equipment. The thermogravimetric analysis was performed using a TGA (STA 6000 Perkin Elmer, USA) under an inert atmosphere with helium as carrier gas at a flow rate of 70 mL min<sup>-1</sup>. The temperature program included heating from 50 to 600 °C at a heating rate of 50 °C min<sup>-1</sup>, then in 5.8 min (340 °C), we loaded the loop to start the volatiles transfer using helium at a flow rate of 49 mL min<sup>-1</sup> in a transfer line from the TGA to the GC/MS.

We carried out the pyrolysis using a GC/MS (Clarus 680 Perkin Elmer) with an ELITE-5MS II column. The equipment (length: 30 m; internal diameter: 250 µm; film thickness: 0.25 µm; maximum temperature: 350 °C). The initial temperature of GC oven was set at 50 °C during 2 min, then increased from 50 to 250 °C at a rate of 5 °C min<sup>-1</sup>, and finally kept at 250 °C for 5 min using a total time of 47 min. The flow rate of the carrier gas (helium) was 1 mL min<sup>-1</sup> at a temperature of 250 °C in the GC-MS transfer line.

We used a detector (Clarus SQ 8T Perkin Elmer) with an electron energy of 70 eV and the molecular weight of compounds analyzed ranged between 40 to 300 g mol<sup>-1</sup> with a temperature of 150 °C during 47 min. Besides, we classified the products of pyrolysis from root samples of grapevine in seven chemical groups: carboxylic acids, ketones, sugars,

phenols, furans, amines, and others. In supplementary Table 2 we listed the compounds found in different treatments of grapevine roots by GC/MS obtained at 340 °C.

#### **4.2.7. Collection, and analysis of soil**

At the end of the experiment, we collected the soil from pots, removed roots from surrounding soil, mixing the soil, and finally stored it at 4 °C for further enzyme analysis. We quantified acid phosphatase activity in soil using the spectrophotometric determination of *p*-nitrophenol (PNP) described by Tabatabai and Bremner (1969). Briefly, we homogenized soil samples, by sieving through a 2-mm sieve, and weighed 0.1 g of soil. Then, 0.1 mL of *p*-nitrophenylphosphate (PNP) 0.05 M and 1.0 mL of modified universal buffer (MUB) were added at pH 6.5 for the determination of acid phosphatase. Afterward, we incubated samples at 37 °C for 1 hour. Then, we added 0.1 mL of CaCl<sub>2</sub> 0.5 M; 0.4 mL NaOH 0.5 M and centrifuged for 7 minutes at 10,000 rpm. Finally, we read the enzyme hydrolytic activity in a microplate reader at 400 nm. It was expressed as μmoles of *p*-nitrophenylphosphate (PNP) g<sup>-1</sup> h<sup>-1</sup>. Conjointly with the quantification of acid phosphatase, soil samples were air-dried to determine soil pH and the concentration of Olsen P according to Sadzawka et al. (2007).

#### **4.2.8. Quantification of oxalic acid from soil solution by high-performance liquid chromatography (HPLC) technique**

Before HPLC injection, we resuspended soil solution samples in 5 mL of distilled water (LC-MS Grade) for HPLC analysis. We quantified oxalic acid present in soil solution according to López-Rayó et al. (2015) and Contreras et al. (2019). Sigma® provided the standard solution of oxalic acid and the identification was based on peak retention time in comparison to the respective commercial standard. We elaborated a calibration curve with values ranging

from 0 to 200 mg L<sup>-1</sup>. The retention time for oxalic acid was 2.7 min at 210 nm. Samples (20 µL) were injected into analytical HPLC (Prominence LC-20A, Shimadzu, Kyoto, Japan) equipped with a C-18 column (Merck) (300 x 4.6 mm I.D; particle size 5 µm). We eluted samples in a mobile phase composed of 80% of solvent A: H<sub>3</sub>PO<sub>4</sub> 200 mM (pH 2.1) and 20% of solvent B: methanol. The concentration of oxalic acid in soil solution was expressed as mmol L<sup>-1</sup>. Evaluation of peaks data was processed by the HPLC software Primaide 1.0.

#### **4.2.9. Statistical analysis**

All experiments were performed in quadruplicate, and values expressed as mean ± standard deviation (SD). Significant differences were tested using one-way analysis of variance (ANOVA) and comparisons of the mean were performed using Tukey's HSD test ( $p < 0.05$ ). All tests were performed using the statistical package Statistix 10.0. Finally, we clustered all data obtained at the end of the experiment in a principal component analysis (PCA) to establish groups and their relationships.

### **4.3. Results**

#### **4.3.1. Plant growth and elemental composition**

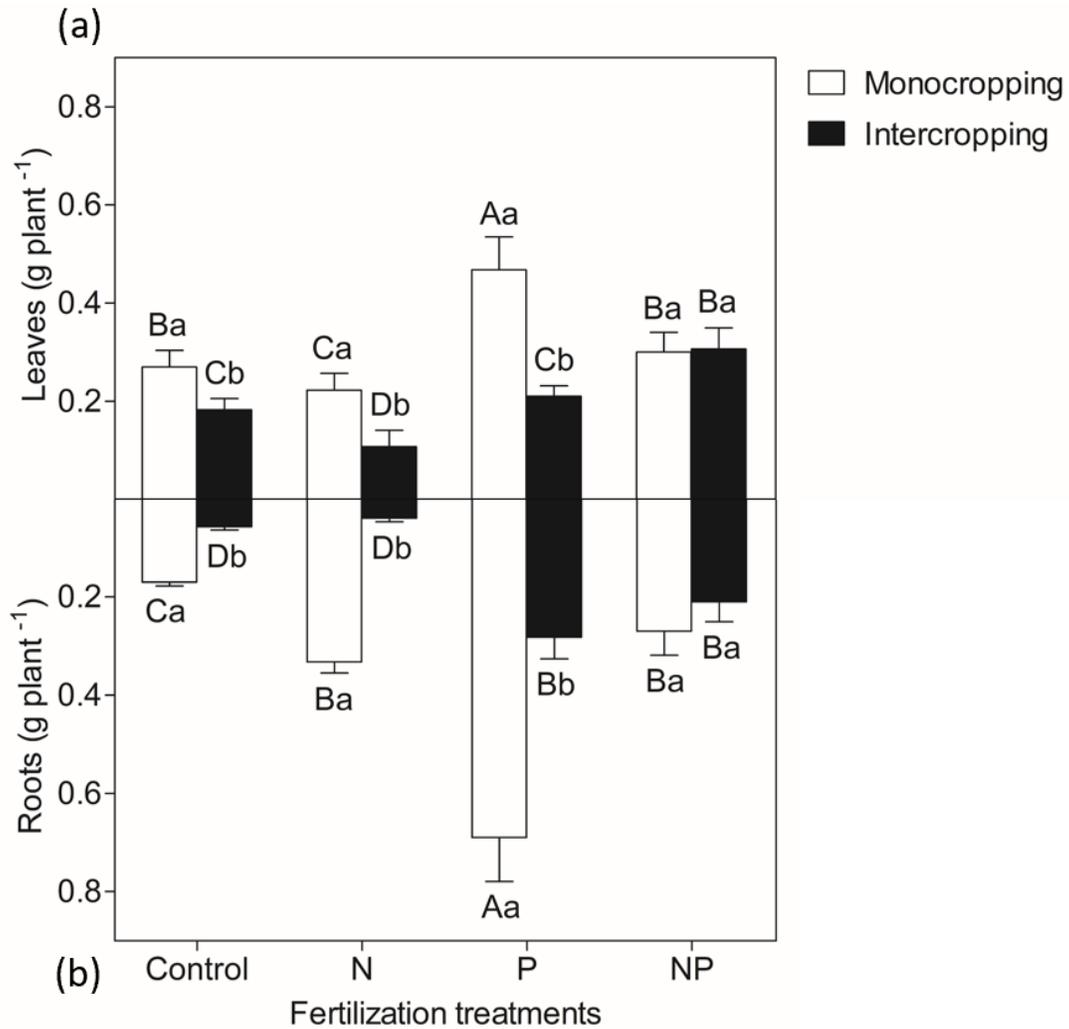
Table 4.2 shows the leaf chlorophyll index, number of leaves, and shoot length at the end of the experiment. The leaf chlorophyll index varied between  $17.07 \pm 1.96$  and  $14.60 \pm 0.78$ , with significantly higher value in intercropping in comparison to monocropping. The number of leaves ranged between  $5.03 \pm 0.86$  and  $21.33 \pm 1.70$  and was significantly higher under monocropping as compared to intercropping. Shoot length ranged between  $9.00 \pm 0.82$  cm and  $17.33 \pm 1.25$  cm and revealed significant differences among monocropping and intercropping in treatment control and treatments with combined N and P fertilization.

**Table 4.2.** Leaf chlorophyll index and grapevine growth in different treatments collected at the end of the experiment. Values are the mean of four replicates  $\pm$  standard deviation. Upper case letters indicate differences between fertilization treatments. Lower case letters indicate differences between crop treatments. Values of the probability of one-way ANOVA (Tukey's test) ( $p < 0.05$ ).

Treatment		Shoot			Roots		
		Leaf chlorophyll index (SPAD)	Number of leaves	Shoot length (cm)	Primary root length (cm)	Number of secondary roots	Root density per plant ( $\text{g L}^{-1}$ )
Control (without fertilization)	Monocropping	16.60 $\pm$ 0.36 <sup>Ba</sup>	18.33 $\pm$ 1.89 <sup>Aa</sup>	17.33 $\pm$ 1.25 <sup>Aa</sup>	20.44 $\pm$ 0.63 <sup>Ca</sup>	29.33 $\pm$ 1.53 <sup>Aa</sup>	0.68 $\pm$ 0.01 <sup>Eb</sup>
	Intercropping	16.73 $\pm$ 1.27 <sup>Aa</sup>	8.50 $\pm$ 1.47 <sup>Db</sup>	10.33 $\pm$ 1.25 <sup>Bb</sup>	18.00 $\pm$ 0.82 <sup>Cb</sup>	20.44 $\pm$ 1.23 <sup>Bb</sup>	0.86 $\pm$ 0.05 <sup>Da</sup>
N (nitrogen)	Monocropping	15.47 $\pm$ 0.12 <sup>Ba</sup>	14.00 $\pm$ 0.82 <sup>Ca</sup>	11.00 $\pm$ 0.82 <sup>Ba</sup>	24.44 $\pm$ 0.79 <sup>Aa</sup>	19.22 $\pm$ 1.37 <sup>Ca</sup>	1.33 $\pm$ 0.05 <sup>Ba</sup>
	Intercropping	17.07 $\pm$ 1.96 <sup>Aa</sup>	5.03 $\pm$ 0.86 <sup>Eb</sup>	10.33 $\pm$ 0.47 <sup>Ba</sup>	18.67 $\pm$ 1.70 <sup>Cb</sup>	12.38 $\pm$ 1.46 <sup>Db</sup>	1.49 $\pm$ 0.13 <sup>Aa</sup>
P (phosphorus)	Monocropping	16.53 $\pm$ 0.31 <sup>Aa</sup>	21.33 $\pm$ 1.70 <sup>Aa</sup>	11.00 $\pm$ 0.82 <sup>Ba</sup>	21.67 $\pm$ 1.25 <sup>Bb</sup>	28.25 $\pm$ 1.43 <sup>Aa</sup>	0.75 $\pm$ 0.03 <sup>Db</sup>
	Intercropping	15.97 $\pm$ 0.12 <sup>Bb</sup>	8.03 $\pm$ 0.78 <sup>Db</sup>	10.67 $\pm$ 0.94 <sup>Ba</sup>	24.33 $\pm$ 1.25 <sup>Aa</sup>	18.98 $\pm$ 1.88 <sup>Cb</sup>	1.40 $\pm$ 0.07 <sup>Aa</sup>
NP (nitrogen and phosphorus)	Monocropping	15.30 $\pm$ 0.70 <sup>Ba</sup>	16.70 $\pm$ 1.69 <sup>Ba</sup>	10.40 $\pm$ 0.57 <sup>Ba</sup>	23.67 $\pm$ 1.70 <sup>Aa</sup>	17.00 $\pm$ 0.82 <sup>Ca</sup>	1.42 $\pm$ 0.05 <sup>Aa</sup>
	Intercropping	14.60 $\pm$ 0.78 <sup>Ba</sup>	7.00 $\pm$ 1.63 <sup>Db</sup>	9.00 $\pm$ 0.82 <sup>Bb</sup>	20.00 $\pm$ 0.82 <sup>Cb</sup>	19.11 $\pm$ 1.80 <sup>Ca</sup>	1.01 $\pm$ 0.03 <sup>Cb</sup>

Additionally, Table 4.2 shows the root growth of grapevine under different treatments. The primary root length varied between  $18.00 \pm 0.82$  cm and  $24.44 \pm 0.79$  cm. It was significantly different between monocropping and intercropping in all fertilization treatments. The number of secondary roots ranged between  $12.38 \pm 1.46$  and  $29.33 \pm 1.53$  and was significantly different between monocropping and intercropping in all treatments except for NP. Root density per plant showed values between  $0.68 \pm 0.01$  g L<sup>-1</sup> and  $1.49 \pm 0.13$  g L<sup>-1</sup>. There were significant differences between monocropping and intercropping in all fertilization treatments except for N. The highest root densities were found in intercropping treatments with N fertilization, and the lowest values were recorded for the monocropping control.

The dry weight of grapevine leaves in different treatments is shown in Figure 4.1a and those of roots are shown in Figure 4.1b. The highest weights were observed in monocropping treatments with P fertilization ( $0.47 \pm 0.14$  g plant<sup>-1</sup>). In this treatment, we found significant differences between monocropping and intercropping. The lowest leaf weights were found in intercropping treatments with N fertilization ( $0.11 \pm 0.07$  g plant<sup>-1</sup>), while the other treatments revealed intermediate values. The highest values for root dry mass were observed in monocropping treatments with P fertilization ( $0.69 \pm 0.18$  g plant<sup>-1</sup>), whereas the lowest values were found in intercropping treatments with N fertilization ( $0.04 \pm 0.01$  g plant<sup>-1</sup>). Differences between monocropping and intercropping were observed in control treatments and fertilized treatments. In various treatments, roots of grapevine grown using intercropping exhibited lower weights than plants grown in monocropping. Our results indicate that monocropping led to an increased dry weight of leaves and roots.



**Figure 4.1.** Dry weight of leaves and roots of grapevine in different treatments collected at the end of the experiment. (a) dry weight of grapevine leaves, and (b) dry weight of grapevine roots. Abbreviations: Control: without fertilization; N: nitrogen applied; P: phosphorus applied; NP: nitrogen and phosphorus applied. Values are the mean of four replicates and bars indicate standard deviation. Upper case letters indicate differences between fertilization treatments. Lower case letters indicate differences between crop treatments. Values of the probability of one-way ANOVA (Tukey's test) ( $p < 0.05$ ).

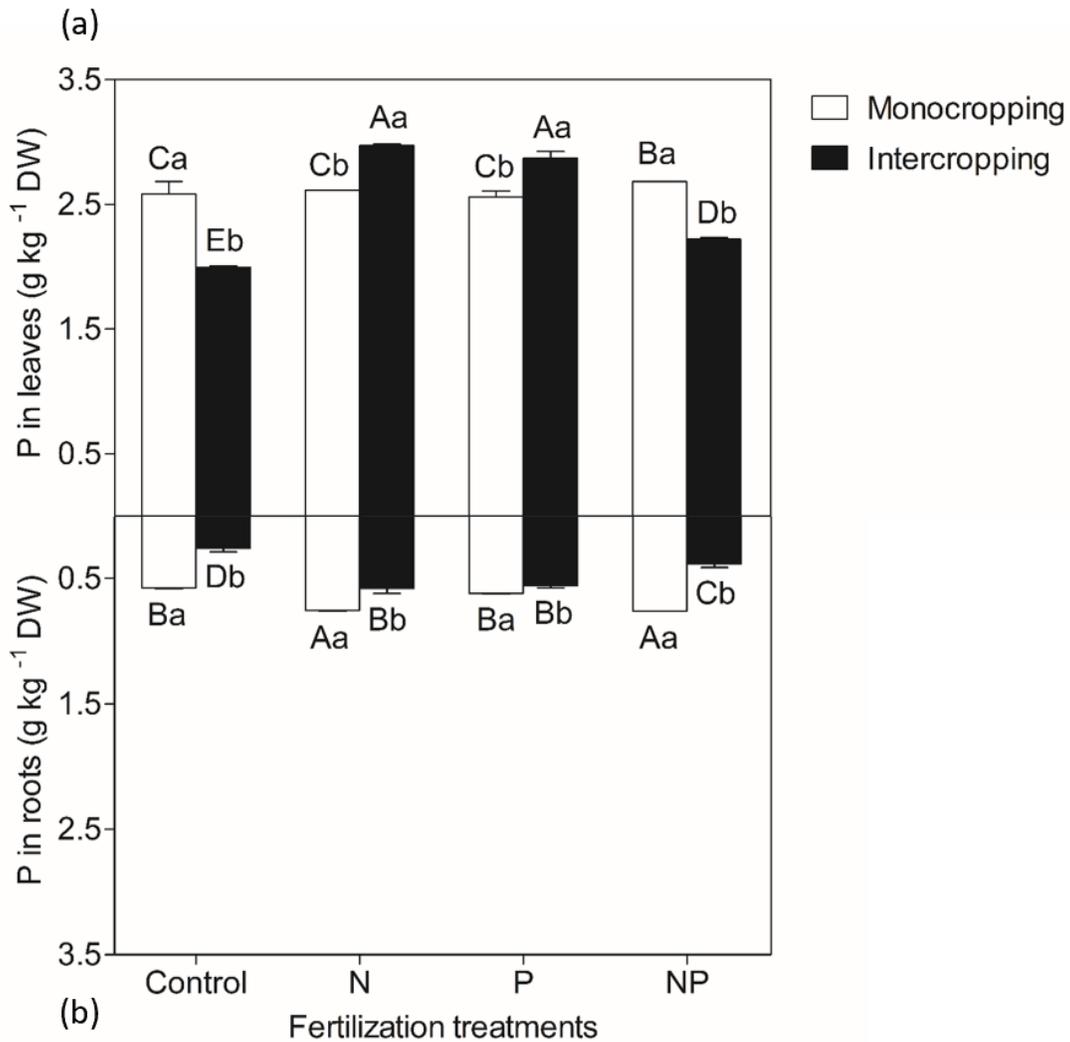
The concentration of C, of grapevine leaves collected at the end of the experiment, did not show significant differences among treatments (Table 4.3). In contrast, leaf N concentrations revealed significant differences between monocropping and intercropping in NP, P, and control treatments (Table 4.3). The highest values were observed in leaves from P fertilized monocropping ( $45.87 \pm 0.83 \text{ g kg}^{-1} \text{ DW}$ ), and the lowest values were noted for the intercropping control ( $30.97 \pm 2.92 \text{ g kg}^{-1} \text{ DW}$ ). Finally, the C/N ratio showed significant differences between monocropping and intercropping in all treatments except for N (Table 4.3). Monocropping with P fertilization showed the lowest values ( $9.16 \pm 0.24$ ), and the highest C/N ratio was recorded in intercropping control ( $13.29 \pm 1.19$ ).

**Table 4.3.** Concentration of carbon, nitrogen, and C/N ratio in leaf and root samples of grapevine in different treatments collected at the end of the experiment. Values are the mean of four replicates  $\pm$  standard deviation. Upper case letters indicate differences between fertilization treatments. Lower case letters indicate differences between crop treatments. Values of the probability of one-way ANOVA (Tukey's test) ( $p < 0.05$ ).

Treatment		Leaves			Roots		
		C (g kg <sup>-1</sup> DW)	N (g kg <sup>-1</sup> DW)	C/N ratio	C (g kg <sup>-1</sup> DW)	N (g kg <sup>-1</sup> DW)	C/N ratio
Control (without fertilization)	Monocropping	409.44 $\pm$ 19.32 <sup>Aa</sup>	38.06 $\pm$ 2.56 <sup>Ba</sup>	10.78 $\pm$ 0.51 <sup>Bb</sup>	432.17 $\pm$ 9.70 <sup>Ab</sup>	10.10 $\pm$ 0.24 <sup>Aa</sup>	42.80 $\pm$ 0.82 <sup>Cb</sup>
	Intercropping	410.95 $\pm$ 15.01 <sup>Aa</sup>	30.97 $\pm$ 2.92 <sup>Db</sup>	13.29 $\pm$ 1.19 <sup>Aa</sup>	455.72 $\pm$ 10.47 <sup>Aa</sup>	8.57 $\pm$ 0.64 <sup>Bb</sup>	53.39 $\pm$ 3.46 <sup>Aa</sup>
N (nitrogen)	Monocropping	414.14 $\pm$ 11.55 <sup>Aa</sup>	42.14 $\pm$ 1.50 <sup>Aa</sup>	9.83 $\pm$ 0.32 <sup>Ca</sup>	434.88 $\pm$ 7.03 <sup>Aa</sup>	10.99 $\pm$ 0.38 <sup>Aa</sup>	39.42 $\pm$ 1.45 <sup>Ca</sup>
	Intercropping	423.74 $\pm$ 11.76 <sup>Aa</sup>	44.34 $\pm$ 1.75 <sup>Aa</sup>	9.58 $\pm$ 0.56 <sup>Ca</sup>	442.62 $\pm$ 13.02 <sup>Aa</sup>	10.43 $\pm$ 0.80 <sup>Aa</sup>	41.37 $\pm$ 2.97 <sup>Ca</sup>
P (phosphorus)	Monocropping	419.86 $\pm$ 12.86 <sup>Aa</sup>	45.87 $\pm$ 0.83 <sup>Aa</sup>	9.16 $\pm$ 0.24 <sup>Db</sup>	431.30 $\pm$ 5.80 <sup>Ab</sup>	10.17 $\pm$ 1.48 <sup>Aa</sup>	40.70 $\pm$ 3.51 <sup>Cb</sup>
	Intercropping	417.84 $\pm$ 7.39 <sup>Aa</sup>	36.80 $\pm$ 1.70 <sup>Cb</sup>	11.37 $\pm$ 0.47 <sup>Ba</sup>	454.53 $\pm$ 15.59 <sup>Aa</sup>	9.51 $\pm$ 0.80 <sup>Ba</sup>	46.93 $\pm$ 3.06 <sup>Ba</sup>
NP (nitrogen and phosphorus)	Monocropping	413.62 $\pm$ 15.90 <sup>Aa</sup>	42.22 $\pm$ 1.88 <sup>Aa</sup>	9.80 $\pm$ 0.29 <sup>Cb</sup>	432.08 $\pm$ 13.90 <sup>Aa</sup>	10.34 $\pm$ 0.44 <sup>Aa</sup>	41.91 $\pm$ 3.38 <sup>Ca</sup>
	Intercropping	423.38 $\pm$ 19.76 <sup>Aa</sup>	37.04 $\pm$ 1.75 <sup>Cb</sup>	11.46 $\pm$ 0.84 <sup>Ba</sup>	442.05 $\pm$ 10.14 <sup>Aa</sup>	10.70 $\pm$ 0.96 <sup>Aa</sup>	42.30 $\pm$ 3.69 <sup>Ca</sup>

For root samples, we did not observe any significant differences in the C concentration except for monocropping control and monocropping fertilized with P, which showed lower values than the other treatments (Table 4.3). N concentrations were different only in control treatments (Table 4.3). We observed significant differences in C/N ratios between monocropping and intercropping for control, and P fertilized treatments. The lowest values were found in monocropping with N ( $39.42 \pm 1.45$ ) and the highest values were observed in intercropping treatments without fertilization ( $53.39 \pm 3.46$ ) (Table 4.3).

Figure 4.2 shows the P concentration of leaves and roots of grapevine in different treatments. The concentration of P in leaves grown under intercropping was significantly higher than for those grown under monocropping in treatments with N fertilization and for those with P fertilization. Conversely, in treatments with combined N and P fertilization and control, the concentration of P of grapevine grown under intercropping was significantly lower than those grown under monocropping. The concentration of P in grapevine leaves, was the highest under intercropping with N fertilization ( $2.97 \pm 0.02 \text{ g kg}^{-1} \text{ DW}$ ), while the lowest concentration was recorded in the treatment control ( $2.00 \pm 0.02 \text{ g kg}^{-1} \text{ DW}$ ) (Figure 4.2a).

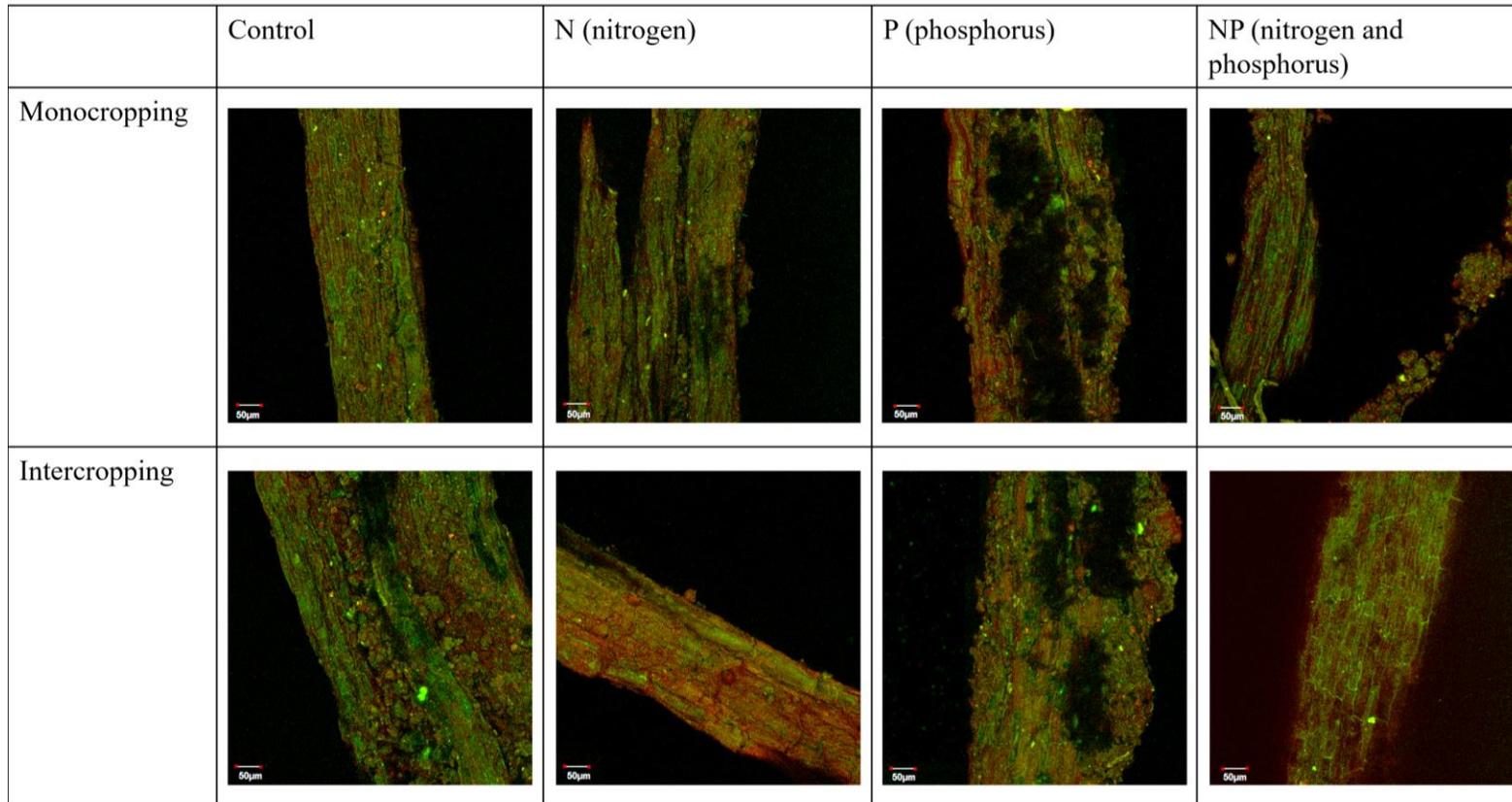


**Figure 4.2.** Concentration of P in leaves and roots of grapevine in different treatments collected at the end of the experiment. (a) the concentration of P in grapevine leaves, and (b) the concentration of P in grapevine roots. Abbreviations: Control: without fertilization; N: nitrogen applied; P: phosphorus applied; NP: nitrogen and phosphorus applied. Values are the mean of four replicates and bars indicate standard deviation. Upper case letters indicate differences between fertilization treatments. Lower case letters indicate differences between crop treatments. Values of the probability of one-way ANOVA (Tukey's test) ( $p < 0.05$ ).

Roots grown under monocropping showed higher concentrations of P than those grown under intercropping in the treatments: N, P, and NP (Figure 4.2b). The highest values were recorded under monocropping with N fertilization ( $0.76 \pm 0.01 \text{ g kg}^{-1} \text{ DW}$ ) and NP fertilization ( $0.76 \pm 0.01 \text{ g kg}^{-1} \text{ DW}$ ).

#### **4.3.2. Characterization of root surface under microscopy**

Initially, we characterized roots using Confocal Laser Scanning Microscopy (CLSM) to visualize the distribution of lignin and cellulose in the root surface. Besides, we observed the lignin and cellulose through fluorescence. We observed the highest value of fluorescence in grapevine grown under intercropping with N fertilization ( $1,240 \pm 235 \text{ RFU}$ ), and the lowest value under monocropping with P fertilization ( $640 \pm 96 \text{ RFU}$ ) with intermediate values in other treatments (Figure 4.3). These results may indicate the importance of P for the root development of the grapevine.



**Figure 4.3.** Characterization of surface polymers of grapevine roots in different treatments collected at the end of the experiment. Abbreviations: Control: without fertilization; N: nitrogen applied; P: phosphorus applied; NP: nitrogen and phosphorus applied. We observed lignin and cellulose fluorescence by excitation/emission wavelength 546/590 nm, and 635/690 nm by Confocal Laser Scanning Microscope.

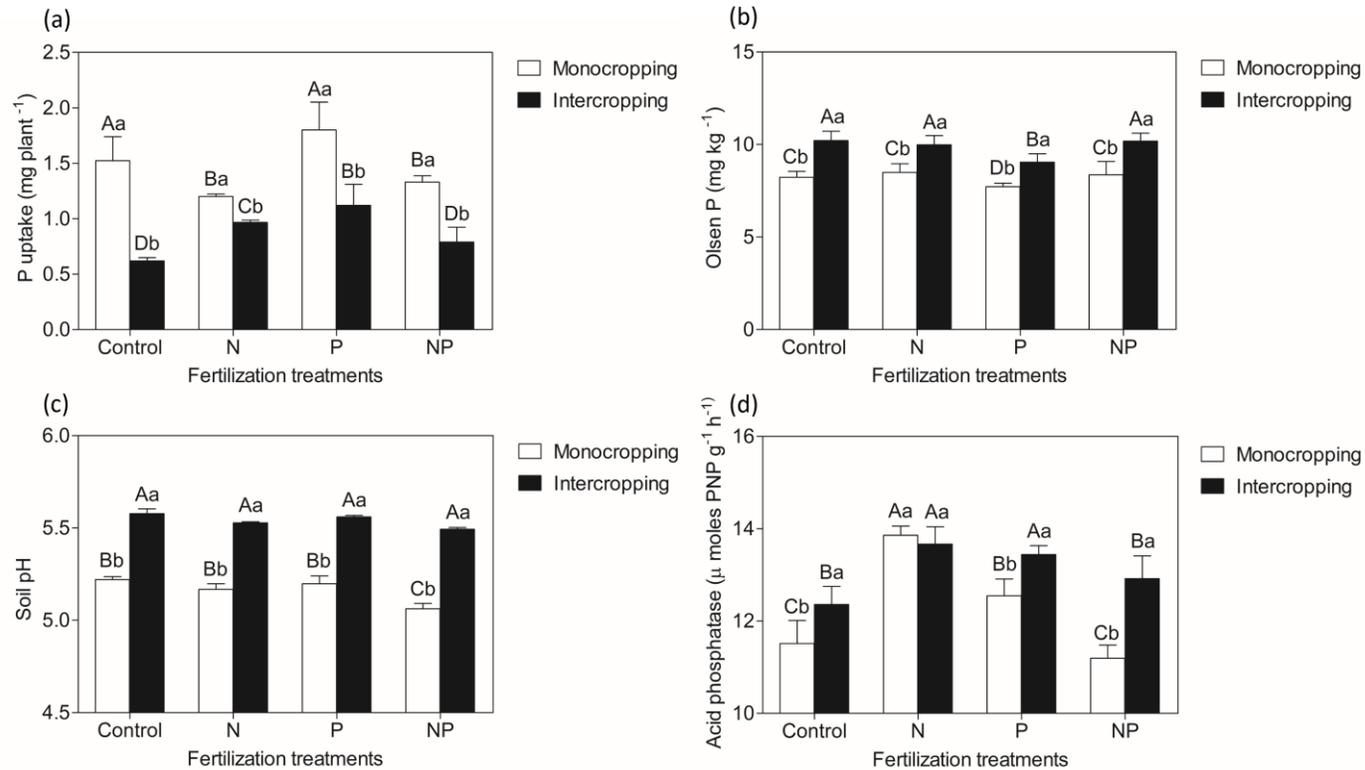
### 4.3.3. Thermogravimetric analysis (TGA) of grapevine roots

During the pyrolysis, lignin and hemicellulosic compounds were decomposed into polysaccharides of smaller molecular weight. Regarding carboxylic acids, the highest abundance was found in monocropping (N) (37.84%) and the lowest in intercropping (Control) (22.36%). The contribution of ketones to the pyrolysis products ranged from 24.76% to 32.43% and sugar contribution was low and close to the detection limit. Similarly, we observed low contributions of phenols and amines ranging between 0.5 and 3%. The polysaccharide-derived pyrolysis products furans showed a contribution between 6.09 and 8.94%. The contribution of unspecific compounds ranged between 29 and 37%.

In supplementary Table 2 we present the detected, and not detected (ND) compounds depending on fertilization treatments. For example, we observed that there were compounds not detected in treatments with complete fertilization (NP) and without fertilization (Control), identified only with N or P fertilization. These compounds are 2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)-; 2,4(3H,5H)-Furandione, 3-methyl-; Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-; Vanillin; Benzaldehyde, 4-hydroxy-3,5-dimethoxy-; and 5-tert-Butylpyrogallol. We also observed compounds present in all fertilization treatments except NP. These compounds are 5-Hydroxymethyldihydrofuran-2-one; Creosol; and Catechol. Finally, we identified compounds present in all treatments except in intercropping with N and P fertilization. These compounds are 2-Pentanone, 1-(2,4,6-trihydroxyphenyl); 1,4:3,6-Dianhydro- $\alpha$ -D-glucopyranose;  $\beta$ -D-Glucopyranose, 1,6-anhydro-; Phenol, 2-methoxy-4-(1propenyl)-; and (E)-2,6-Dimethoxy-4-(prop-1-en-1-yl) phenol.

#### **4.3.4. Soil analysis**

In soil, Olsen P values ranged between  $7.62 \pm 0.33 \text{ mg kg}^{-1}$  and  $10.55 \pm 0.76 \text{ mg kg}^{-1}$  (Figure 4.4b). All treatments showed significant differences with higher values for intercropping and the lowest values for monocropping. The concentrations of C, N, and C/N ratio of soil samples are shown in Table 4.4. The C concentration of soil was higher under monocropping than under intercropping, particularly in N and NP treatments. Comparing all fertilization treatments, the highest values were recorded under monocropping with N fertilization ( $80.54 \pm 2.70 \text{ g kg}^{-1}$ ) and the lowest values under monocropping control ( $70.72 \pm 3.13 \text{ g kg}^{-1}$ ).



**Figure 4.4.** Determination of (a) P uptake by grapevine plants, (b) soil Olsen P, (c) soil pH, and (d) activity of soil acid phosphatase at the end of the experiment. Abbreviations: Control: without fertilization; N: nitrogen applied; P: phosphorus applied; NP: nitrogen and phosphorus applied. Values are the mean of four replicates and bars indicate standard deviation. Upper case letters indicate differences between fertilization treatments. Lower case letters indicate differences between crop treatments. Values of the probability of one-way ANOVA (Tukey's test) ( $p < 0.05$ ).

The P uptake of grapevine plants in different treatments is shown in Figure 4.4a. Treatments under monocropping showed higher P uptake than those under intercropping in all fertilization treatments. We observed the highest content under monocropping with P fertilization ( $1.80 \pm 0.36$  mg plant<sup>-1</sup>). The lowest value of P uptake was determined under intercropping without fertilization ( $0.62 \pm 0.04$  mg plant<sup>-1</sup>) (Figure 4.4a). Soil pH was significantly higher under intercropping than in monocropping (Figure 4.4c). The highest value was  $5.58 \pm 0.05$  in intercropping without fertilization, and the lowest value was  $5.06 \pm 0.06$  in monocropping with NP fertilization. The highest activity of acid phosphatase was recorded in soil under N fertilization regardless of the cropping treatment (Figure 4.4d). We observed the lowest activity of phosphatase under monocropping treatments without fertilizers applied and with complete fertilization.

**Table 4.4.** Concentration of carbon, nitrogen, and C/N ratio in soil samples of grapevine pots in different treatments collected at the end of the experiment. Values are the mean of four replicates  $\pm$  standard deviation. Upper case letters indicate differences between fertilization treatments. Lower case letters indicate differences between crop treatments. Values of the probability of one-way ANOVA (Tukey's test) ( $p < 0.05$ ).

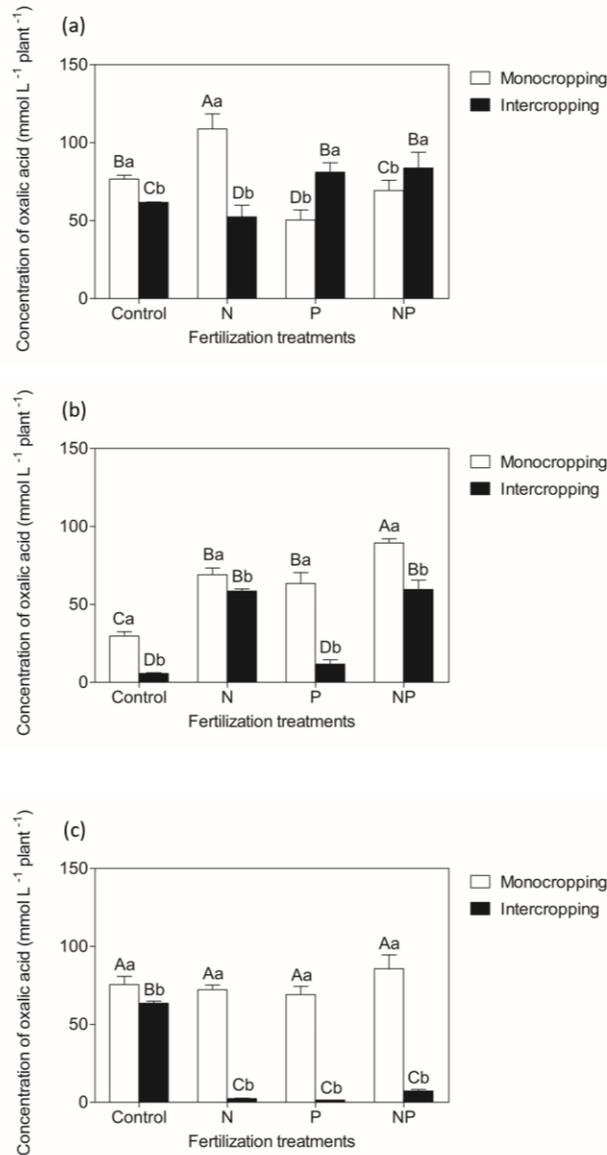
Treatment		C (g kg <sup>-1</sup> DW)	N (g kg <sup>-1</sup> DW)	C/N ratio
Soil samples				
Control (without fertilization)	Monocropping	70.72 $\pm$ 3.13 <sup>Ca</sup>	6.11 $\pm$ 0.36 <sup>Ca</sup>	11.59 $\pm$ 0.35 <sup>Aa</sup>
	Intercropping	73.49 $\pm$ 3.78 <sup>Ba</sup>	7.06 $\pm$ 0.75 <sup>Aa</sup>	10.98 $\pm$ 1.01 <sup>Aa</sup>
N (nitrogen)	Monocropping	80.54 $\pm$ 2.70 <sup>Aa</sup>	7.57 $\pm$ 0.09 <sup>Aa</sup>	11.73 $\pm$ 1.27 <sup>Aa</sup>
	Intercropping	72.01 $\pm$ 1.42 <sup>Cb</sup>	7.23 $\pm$ 0.01 <sup>Aa</sup>	10.70 $\pm$ 0.49 <sup>Aa</sup>
P (phosphorus)	Monocropping	76.55 $\pm$ 3.68 <sup>Ba</sup>	5.67 $\pm$ 0.14 <sup>Db</sup>	11.13 $\pm$ 1.38 <sup>Aa</sup>
	Intercropping	75.40 $\pm$ 3.74 <sup>Ba</sup>	6.38 $\pm$ 0.17 <sup>Ba</sup>	12.06 $\pm$ 1.08 <sup>Aa</sup>
NP (nitrogen and phosphorus)	Monocropping	78.84 $\pm$ 1.50 <sup>Aa</sup>	7.39 $\pm$ 0.46 <sup>Aa</sup>	10.20 $\pm$ 1.17 <sup>Aa</sup>
	Intercropping	73.04 $\pm$ 1.95 <sup>Bb</sup>	6.88 $\pm$ 0.27 <sup>Ba</sup>	11.21 $\pm$ 0.84 <sup>Aa</sup>

The N concentration of soil was higher under monocropping than intercropping especially in treatments with N+P fertilization. Although the N treatment under monocropping showed higher values than those under intercropping, in contrast, the intercropping treatment with P fertilization had higher N values than the monocropping treatment with P fertilization. Comparing all fertilization treatments, we found that the highest N concentrations were observed under monocropping with N fertilization (7.57  $\pm$  0.09 g kg<sup>-1</sup>), and the lowest values under monocropping with P fertilization (5.67  $\pm$  0.14 g kg<sup>-1</sup>). The C/N ratio of soil ranged

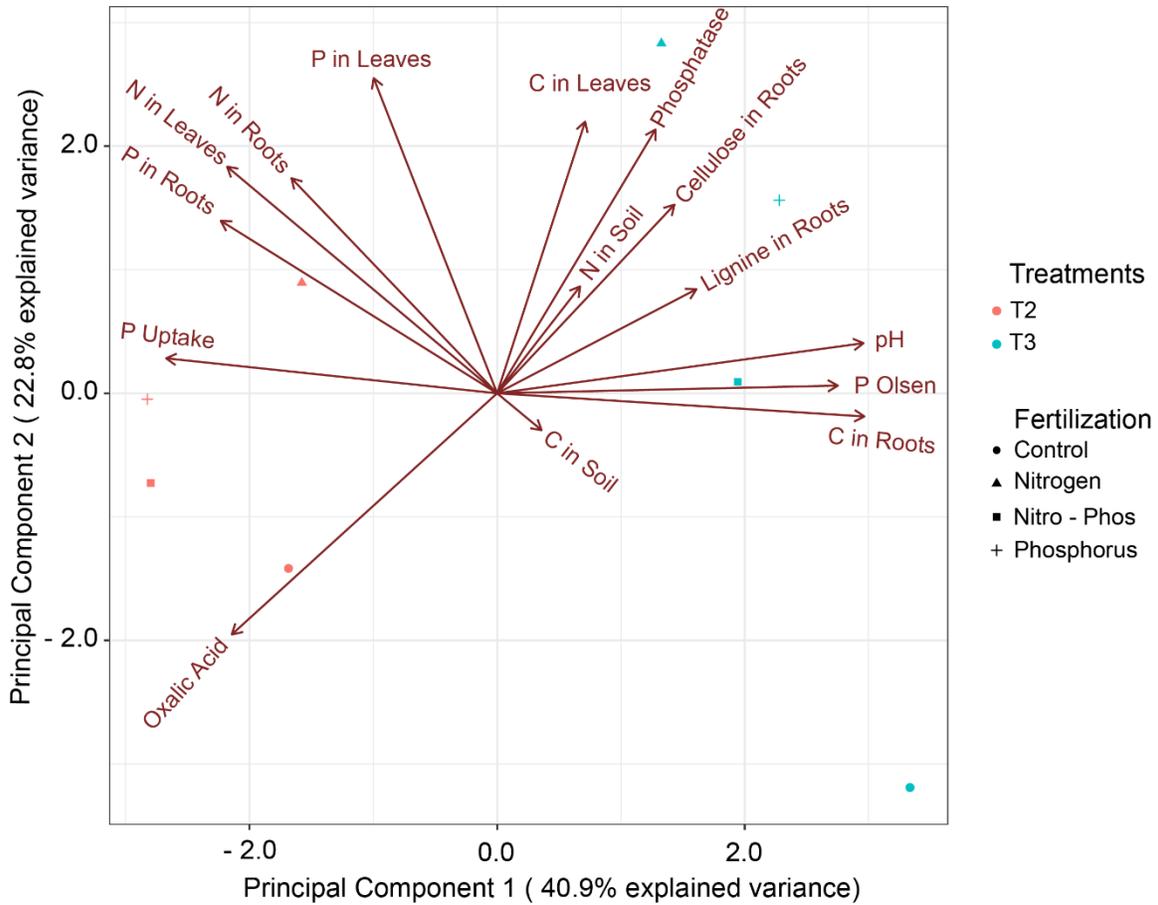
between  $10.20 \pm 1.17$  and  $12.06 \pm 1.08$ . Albeit there are significant differences between treatments in the concentration of C and N, we did not find significant differences in the soil C/N ratio (Table 4.4).

#### **4.3.5. Quantification of oxalic acid from soil solution by HPLC technique**

To determine the effect of root exudates on P availability in soil, we quantified the concentration of oxalic acid in soil solution in the first (Figure 4.5a), second (Figure 4.5b), and third (Figure 4.5c) months after the beginning of the experiment. Monocropping treatments showed a higher release of oxalic acid than intercropping treatments (Figures 4.5a, b, c). In the first month, the highest value was obtained for N fertilized monocropping treatment ( $108.82 \pm 19.32 \text{ mmol L}^{-1} \text{ plant}^{-1}$ ), while the lowest values were recorded under N fertilized intercropping treatment ( $52.39 \pm 15.14 \text{ mmol L}^{-1} \text{ plant}^{-1}$ ). In the second month, concentrations of oxalic acid decreased and ranged between  $5.71 \pm 0.82 \text{ mmol L}^{-1} \text{ plant}^{-1}$  and  $89.41 \pm 5.39 \text{ mmol L}^{-1} \text{ plant}^{-1}$ . After the third month, monocropping and intercropping treatments with N and P fertilization showed higher concentrations of oxalic acid than those under monocropping.



**Figure 4.5.** Concentration of oxalic acid in the first (a), second (b), and third (c) months after the beginning of the experiment determined by analytical HPLC technique. Abbreviations: Control: without fertilization; N: nitrogen applied; P: phosphorus applied; NP: nitrogen and phosphorus applied. Values are the mean of four replicates and bars indicate standard deviation. Upper case letters indicate differences between fertilization treatments. Lower case letters indicate differences between crop treatments. Values of the probability of one-way ANOVA (Tukey's test) ( $p < 0.05$ ).



**Figure 4.6.** Principal component analysis (PCA) of soil and plant variables and their relationships at the end of the experiment. Treatments T2: monocropping and T3: intercropping. Control: without fertilization; Nitrogen: nitrogen applied; Phosphorus: phosphorus applied; Nitro - Phos: nitrogen and phosphorus applied.

In PCA presented in Figure 4.6, we found a negative correlation between the concentration of oxalic acid with the activity of acid phosphatase, soil pH, Olsen P, and the concentration of lignocellulosic compounds in roots. Moreover, we observed a negative correlation between P uptake with soil pH, Olsen P, and C concentration in roots in PCA (Figure 4.6).

Furthermore, in monocropping, we observed a higher release of oxalic acid, P uptake, the concentration of P and N in roots, and leaves. Conversely, in intercropping, we found the highest values of C in leaves and roots, lignocellulosic compounds, activity of acid phosphatase, soil pH, and Olsen P.

#### **4.4. Discussion**

Regarding plant growth, intercropping mitigates the excessive vegetative growth of grapevine decreasing the uptake of N and N in leaves and roots (Mercenaro et al., 2014). We found a direct correlation between the concentration of P and N in leaves of grapevine suggesting that the deficiency of P in Andisols is related to phosphate and nitrogen availability.

We found a direct correlation between the concentration of cellulose and P in leaves of the grapevine, which could indicate a relationship between cellulose and plant nutrition. Indeed, the lignocellulosic compounds of grapevine roots were found to be strongly influenced by the nutritional status of plants, particularly by N, and P (Fernandes et al., 2013). These nutrients are directly involved in polysaccharides formation, which is a major constituent of the cell wall of roots (Lee et al., 2018; Moore et al., 2014). The accumulation of lignin and phenolic compounds in plants depends on management practices such as Si application as is described by Vega et al. (2019). Another management practices such as the application of manure increase the concentration of N and cellulose in plant tissue, which is reflected in higher growth and soil exploration by roots (Ramcharan and Richard, 2017). Regarding lignin concentration, we observed a direct relationship with N fertilization under intercropping management, similar to a study by Peres Soratto et al. (2019). The increase of lignocellulosic compounds in plants grown under intercropping may be caused by signal

peptides that are involved in the formation of Casparian strip, lateral roots, and nitrogen acquisition (de Bang et al., 2017; Oh et al., 2018).

Regarding C in soil, the absence of significant differences in C concentration may be caused by the short duration of this experiment of three months, because previous studies confirm the positive effect of intercropping in C concentration performed in the long-term (Bright et al., 2017; Latati et al., 2017; Nyawade et al., 2019). In agreement with Panichini et al. (2017), in the short-term, management practices have no significant effect on the organic matter of Andisols, which have a high capacity to store carbon rapidly mineralized by soil microorganisms. The concentration of C in plant tissue was higher for intercropping in comparison to monocropping, suggesting that intercropping may increase plant C uptake by a decrease in photosynthate levels (Tezza et al., 2019). In vineyards with herbaceous species growing between alleys, where C is captured by the grapevine and herbs, accumulating C in plant tissue of grapevine (Wolff et al., 2018).

Significantly higher pH values in soil under intercropping compared to monocropping were observed for all fertilization treatments. These differences are explained by the highest concentration of soil solution in plants grown under monocropping, revealing the highest stress in this treatment in comparison to intercropping. The practice of intercropping with subterranean clover thus helps to mitigate soil acidity in comparison to the grapevine grown alone (Wang et al., 2017). Furthermore, in Andisols the highest availability of P ranged between pH 4.5 to 5.5 where plants prefer  $\text{H}_2\text{PO}_4^-$  over  $\text{HPO}_4^{2-}$ , agreeing with the data obtained particularly for intercropping treatments (Vistoso et al., 2012).

Interestingly, intercropping increased enzyme activity in the soil, suggesting that the release of root exudates in the rhizosphere increases the activity of acid phosphatase (Gong et al., 2019; Mouradi et al., 2018). Phosphatase is the most sensitive soil enzyme to the composition of root exudates, stimulated by N depositions such as alanine and inhibited by others such as malate and citrate (Zhang et al., 2019). Previous studies have found that root exudates such as alanine and glucose stimulate the activity of phosphatase contributing to the mineralization of organic P in Andisols (Spohn et al., 2013). Moreover, the release of flavonoids in the rhizosphere stimulates phosphatase activity and function as microbial chemoattractants (Cesco et al., 2012; Tomasi et al., 2008).

In Andisols, P is strongly sorbed to Fe/Al minerals and thus low-molecular-weight organic acids (LMWOA) may release phosphate to the soil solution (Fujii et al., 2019). Under stressful conditions in the soil, plants release organic acids such as oxalic, malic, citric, and succinic acids as was found by Mora et al. (2009). However, in the experiment using grapevine plants the most abundant LMWOA quantified in root exudates was oxalic acid (Contreras et al., 2019). A higher concentration of oxalic acid in soil under monocropping may indicate the release of oxalic acid as a defense mechanism in response to plant stress caused by P deficiency (Tawaraya et al., 2014). This indicates that the release of oxalic acid in the rhizospheric soil is a response of plants grown under stressful environmental conditions (Li et al., 2019). Additionally, the reduction of oxalic acid release under intercropping may be caused by an accumulation of Ca-oxalate inside the root cells (López-Rayó et al., 2015). In the intercropping treatment, the concentration of oxalic acid in soil solution is inversely correlated to the concentration of N and P in plant tissue. This suggests that plants grown in monocropping released more oxalic acid to the soil to increase their N and P uptake (Ma et

al., 2020; Qian et al., 2019). Oxalic acid plays an important role in attracting beneficial bacteria for plants in the soil. Moreover, the release of oxalic acid promotes the synthesis of acid phosphatase in the soil increasing the activity of P solubilizer microorganisms (Darch et al., 2016). Regarding N, the release of oxalic acid in the soil solution also accelerates microbial N mineralization transforming organic N into plant-available forms (Yuan et al., 2018).

Summarizing, grapevine plants grown under monocropping are associated with a higher release of oxalic acid, P uptake, P in roots, and N in leaves and roots. Plants grown in monocropping released a higher concentration of oxalic acid as a survival mechanism under stressful conditions (Millaleo et al., 2018). Conversely, intercropping increases the lignocellulosic constitution of roots, reduces the release of oxalic acid by plant roots, improving soil properties such as acid phosphatase activity, soil pH, and concentration of P in soil. According to Palmieri et al. (2019), oxalic acid inside of plants regulates the content of calcium which is directly related to the lignocellulosic compounds in the roots. Based on the above, we may suggest that in monocropping oxalic acid was released to the soil, and under intercropping oxalic acid inside the plants promoted lignification of roots. On the other hand, the decrease in the concentration of oxalic acid under intercropping suggests that soil bacteria may consume oxalic acid as carbon source which is also supported by the increase of acid phosphatase activity under intercropping treatment.

#### **4.5. Conclusions**

Our results showed that intercropping with legumes induces a lower growth of grapevine, as revealed by the highest growth and concentration of C, N, and P in plants cultivated under

monocropping. Intercropping increases the accumulation of lignocellulosic compounds in the cell wall of grapevine roots, which strengthens roots improving soil exploration.

The practice of intercropping enhances soil characteristics, increasing soil pH, Olsen P, and the activity of acid phosphatase. Plants respond to this improvement of soil with a decrease in the concentration of oxalic acid released to the soil solution under conditions of intercropping. The reduction of the concentration of oxalic acid indicates that intercropping is an effective strategy to mitigate plant stress caused by P deficiency in comparison to monocropping treatment.

Based on the above, intercropping between subterranean clover and grapevine may be a useful tool to improve soil characteristics, mitigating plant stress, and strengthen the roots of grapevine plants.

#### **Conflict of interest**

The authors have no conflict of interest.

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## **CHAPTER V**

### ***General Discussion and Conclusions***

## 5. General Discussion and Conclusions

### 5.1. General Discussion

In the present study, intercropping using grapevine with subterranean clover changed the chemical profile of root exudates and improved grapevine performance. The compounds found only in intercropping treatment were kaempferol-3-glucuronide, glutathione, gluconic acid, and L-2-aminoadipic acid. Previous studies performed by Marastoni et al. (2019) have reported the variation in the composition of root exudates by intercropping practice using grapevine and oats, particularly in the release of gallic acid and rutin. The gluconic acid is synthesized by the oxidation of glucose for pyrroloquinoline quinone, and glucose dehydrogenase attracting P solubilizing Gram-negative bacteria (Ludueña et al., 2017). Rhizospheric bacteria use gluconic acid to lower root immunity suppressing oxidative burst, facilitating root colonization (Yu et al., 2019). This suggests that the exudation of gluconic acid by plants grown in intercropping may play the same function, suppressing the defense system of neighboring plants. Under field conditions, subterranean clover may prevail over other legume species by their annual life cycle, reseeding in each year, and they die during summertime avoiding competition for water with the grapevine (Abdi et al., 2020; Smetham, 2003). Furthermore, the most abundant LMWOA here quantified in root exudates was oxalic acid in the root exudates of subterranean clover grown under monocropping.

In the experiment growing plants in Andisol, the highest concentration of oxalic acid in root exudates in comparison to other LMWOA has been described by previous studies (López-Rayó et al., 2015; Mora et al., 2009). Previous reports have revealed that oxalic acid is an indicator of plant stress, released as a response under adverse environmental conditions promoting the symbiosis between plant and soil microorganisms such as mycorrhizas and

bacteria (Durán et al., 2016; Guo et al., 2018; Palmieri et al., 2019). The reduction of the concentration of oxalic acid observed in the soil solution of intercropping treatment suggests that the combination of both species decreases plant stress (Contreras et al., 2019). Oxalic acid helps to solubilize P compounds into available P forms for plant acquisition in the soil releasing fixed P and increasing the activity of soil microorganisms for P mineralization (Ma et al., 2020).

The practice of intercropping reduces the excessive vegetative growth of grapevine increasing the accumulation of phenolic compounds in plants (Mercenaro et al., 2014; Muscas et al., 2017). However, the control of excessive growth performed by herbaceous species grown in intercropping did not affect significantly the yield and development of grapevine (Giese et al., 2014). The highest growth of monocropping treatment in comparison to intercropping is associated with the highest concentration of N and P in grapevine tissue as was found by Latati et al. (2017). The increase of N in the tissue of grapevine grown under monocropping may be explained by the competition between plants of subterranean clover and grapevine, especially in the early stages of plant development. We found that regardless intercropping increases the activity of acid phosphatase and Olsen P in soil, plants of intercropping treatments showed a lower P uptake and concentration of P.

Regarding the concentration of C in grapevine tissue, the highest concentration of C in plants grown in intercropping may be explained by the lowest release of organic compounds to the soil solution. In monocropping, plants release higher amounts of organic compounds to the soil solution, causing losses of C from plant tissue to the soil (Celette and Gary, 2013; De Conti et al., 2020). In roots, the concentration of lignocellulosic compounds is strongly influenced by the availability of nutrients such as N and P (Peres Soratto et al., 2019;

Ramcharan and Richard, 2017). Furthermore, the lignocellulosic constitution of roots is highly influenced by signaling peptides increasing the cell elongation, N uptake, the formation of the Casparian strip, and development of root hairs (Oh et al., 2018; Roupheal et al., 2017). The concentration of N and P in roots increases the accumulation of compounds such as sugars and phenols constituting cell walls which strengthen the structure of roots of grapevine facilitating soil exploration (Fernandes et al., 2013; Moore et al., 2014; Vega et al., 2019). The accumulation of lignocellulosic compounds in the roots increase the resistance to drought provided by the accumulation of lignin monomers in the cell walls (Keller, 2020). Additionally, the absence of competition for water during the summer with annual subterranean clover may induce a higher resistance to drought in grapevine grown in intercropping (Smetham, 2003).

The augmentation of organic compounds in the rhizosphere as an energy source for microorganisms increases the activity of acid phosphatase under intercropping treatment (Bargaz et al., 2017). The enhancement of the activity of acid phosphatase in the soil increases the mineralization rates of organic P into phosphate available for plants (Redel et al., 2019; Zhou et al., 2019). According to Mouradi et al. (2018), the P in soil is also a source of energy for microorganisms involved in N-fixation such as *Rhizobium* bacteria. The deficiency of P causes stress in the legume plant increasing N-fixation, which finally mitigates P starvation in soil (Latati et al., 2017). The flavonoids present in root exudates improve the colonization of legume roots by N-fixing bacteria *Frankia* and *Rhizobium*. The most important flavonoids attracting these bacteria are genistein, daidzein, and isoliquiritigenin released mainly in the apical root zones (Cesco et al., 2012, 2010).

Additionally to the increase of N-fixation by subterranean clover, intercropping contributes to the ammonification and nitrification by soil microorganisms decreasing ammonia and N<sub>2</sub>O emission to the atmosphere (Chen et al., 2019; Du et al., 2020). The practice of intercropping using legumes increases the concentration of N and P in the soil, reducing leaching, runoff, and retaining nutrients in the soil (Mndzebele et al., 2020; Toselli et al., 2020). Thus, the improvement of grapevine nutrition by intercropping is revealed by the enhancement of soil fertility and symbiosis with soil microorganisms increasing the availability of N and P for plant uptake (Garcia et al., 2018). The supply of microorganisms provided by subterranean clover to the grapevine are N-fixing bacteria interacting with other organisms such as mycorrhizas. The N fixed by bacteria and soil P, are transferred directly by the mycorrhiza hyphae to the roots of plants. (Sekaran et al., 2019). Additionally, mycorrhizas improves the use of residual P associated with soil organic matter low available for plant acquisition (Borie et al., 2019). On the other hand, mycorrhiza helps to mitigate stress caused by Al toxicity, particularly in acidic soils increasing the tolerance to exchangeable Al (Seguel et al., 2017, 2016b, 2016a). Furthermore, the release of organic acids by plant roots is an additional mechanism to alleviate Al toxicity through Al exclusion from the roots (An et al., 2014; Palmieri et al., 2019). The reduction of the concentration of oxalic acid in the soil solution of plants grown in intercropping suggests that the practice of intercropping may reduce the saturation of Al in the soil.

Summarizing, the differences between monocropping and intercropping in the composition of root exudates, soil fertility, symbiotic relationship with soil microorganisms, drought resistance, and in the lignocellulosic constitution of roots can modify the translocation of photosynthates to the grape (Antolín et al., 2020; Mercenaro et al., 2014). Changes in the

concentration of anthocyanins, phenolics, phytoalexins, and flavonoids in the berry may change the quality of fruit and susceptibility to diseases (Burdziej et al., 2019; Donnini et al., 2016; Frioni et al., 2018). Exploring if the combination of grapevine with subterranean clover increases the accumulation of phenolic compounds in the grape which may improve the quality of fruit and wine. The future trends for this research are to evaluate the effect of intercropping in the nutrition and development of grapevine in the field. Finally, it is necessary to elucidate the implications of intercropping in the use efficiency of fertilizers and water for grapevine cultivation in the context of climate change.

## **5.2. General Conclusions**

Intercropping changes the chemical profile of root exudates, particularly of organic acids, amino acids, and flavonoids. The differences were regarding chemical groups and compounds found in subterranean clover and grapevine. We found kaempferol-3-glucuronide, glutathione, gluconic acid, and L-2-aminoadipic acid released only under intercropping. The potential of glutathione may be as a biostimulant for grapevine; however, further research is required to fully understand the effect of the other compounds on plant nutrition.

The release of organic acids is a response of plant roots facing stressful conditions with oxalic acid as the most abundant in root exudates and soil solution. Intercropping changes the concentration of low-molecular-weight organic acids (LMWOA) helping to release P fixed to the Fe/Al oxides/hydroxides increasing soil fertility for plant acquisition. The practice of intercropping mitigates drastically the stress of plants revealed by the lower concentration of oxalic acid in the soil solution in comparison to monocropping treatment.

Intercropping modifies the lignocellulosic composition of grapevine roots, increasing the accumulation of sugars and ketones which strengthens roots enhancing soil exploration. Furthermore, the practice of intercropping increases soil fertility, particularly the activity of acid phosphatase, soil pH, and P concentration in the soil.

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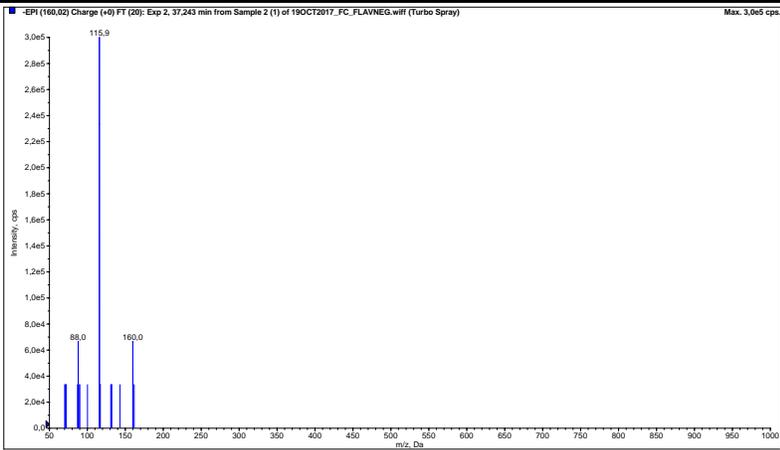
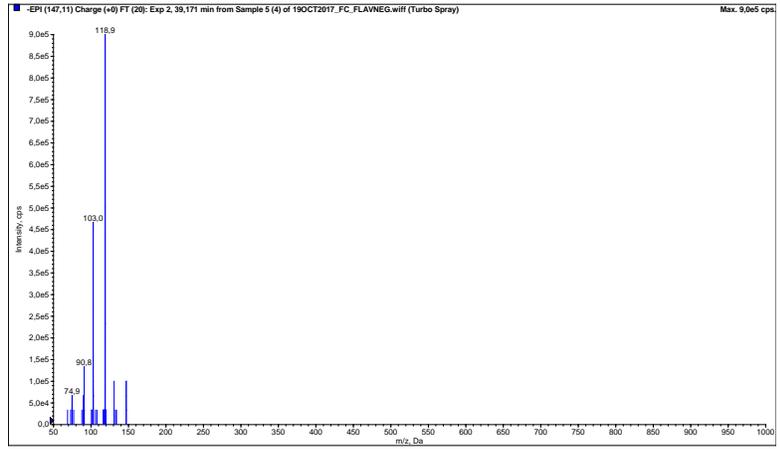
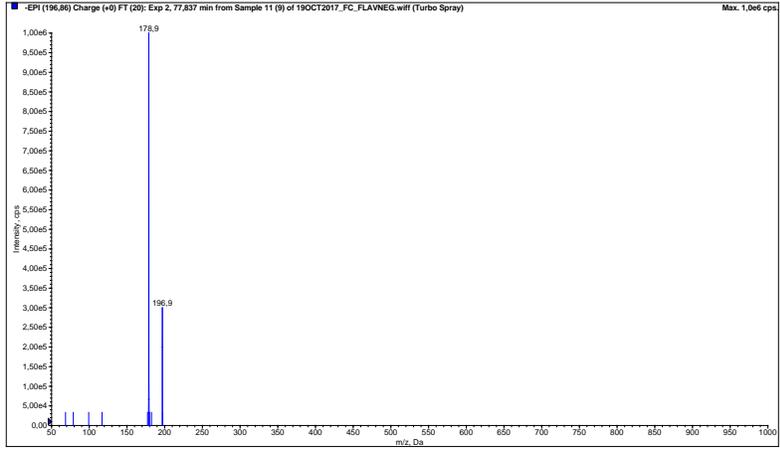
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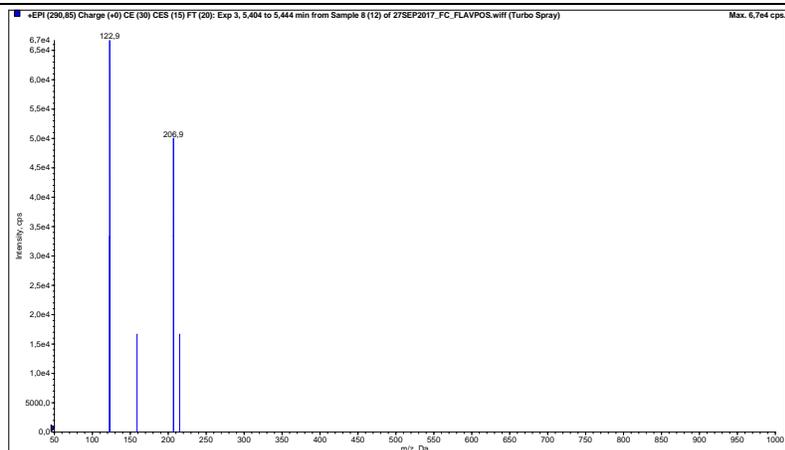
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# Appendix

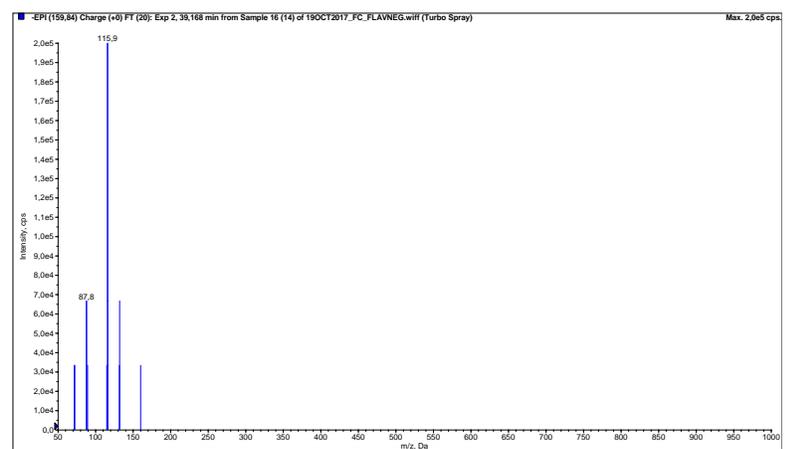
**Supplementary Table 1.** Spectra of compounds found in root exudates of plants grown in hydroponic solution detected by HPLC/UV-ESI MS/MS and described in Chapter III.

Compound	Spectra
L-2-Aminoadipic acid	 <p>■ -EPI (160.02) Charge (+0) FT (20): Exp 2, 37.243 min from Sample 2 (1) of 19OCT2017_FC_FLAVNEG.wiff (Turbo Spray) Max. 3.0e5 cps</p>
<i>trans</i> -Cinnamic acid	 <p>■ -EPI (147.11) Charge (+0) FT (20): Exp 2, 39.171 min from Sample 5 (4) of 19OCT2017_FC_FLAVNEG.wiff (Turbo Spray) Max. 9.0e5 cps</p>
5-Dodecenoic acid	 <p>■ -EPI (196.86) Charge (+0) FT (20): Exp 2, 77.837 min from Sample 11 (9) of 19OCT2017_FC_FLAVNEG.wiff (Turbo Spray) Max. 1.0e6 cps</p>

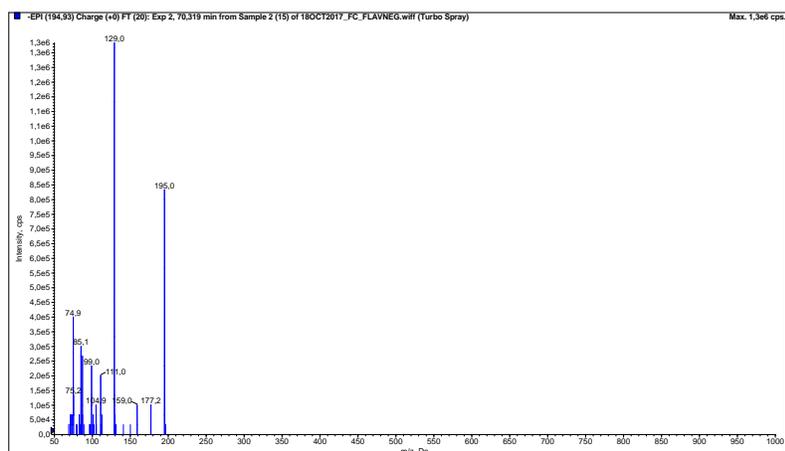
## Epicatechin



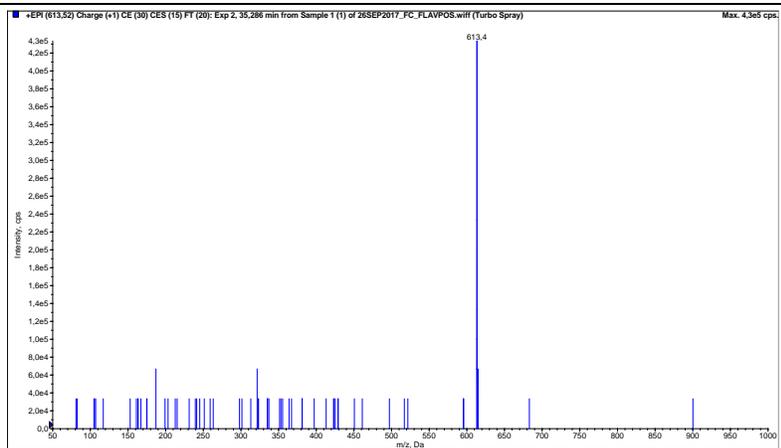
## N-Formylaspartic acid



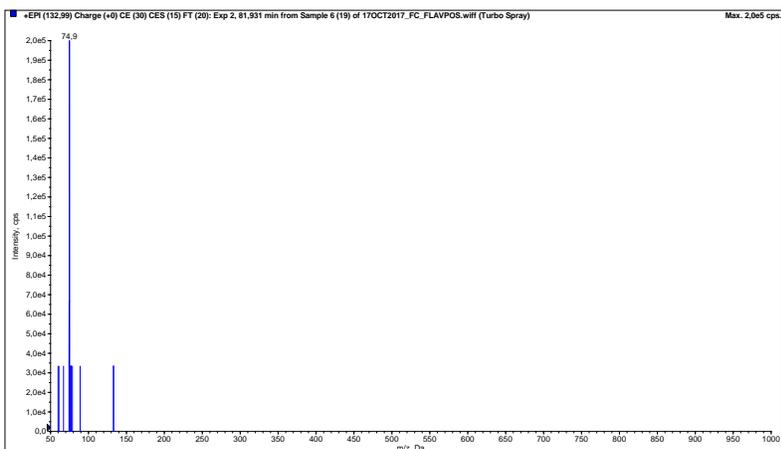
## Gluconic acid



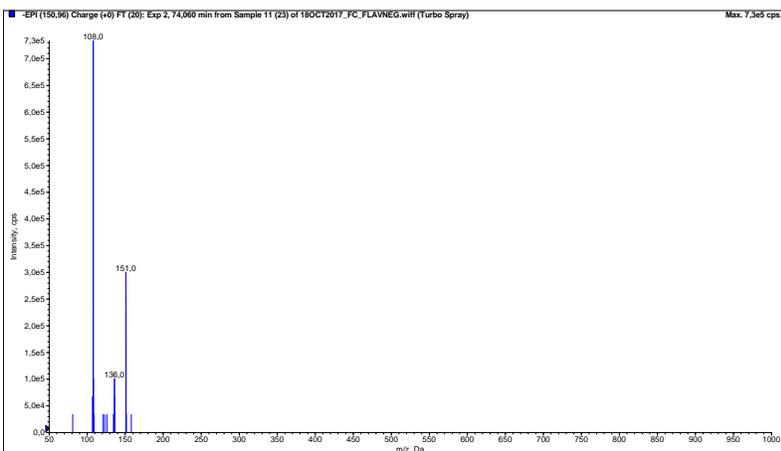
## Glutathione



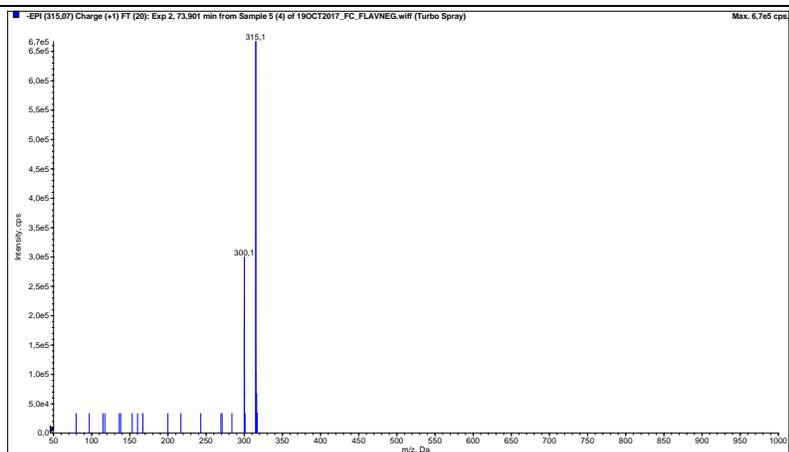
## Glycylglycine



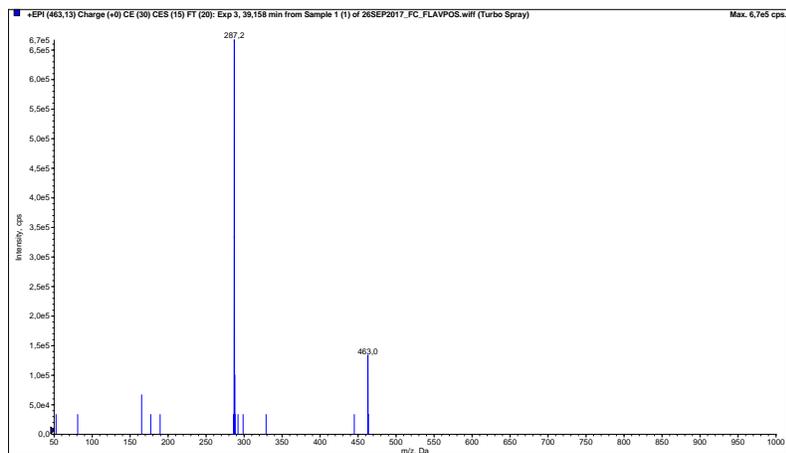
## 4-Hydroxyphenylacetic acid



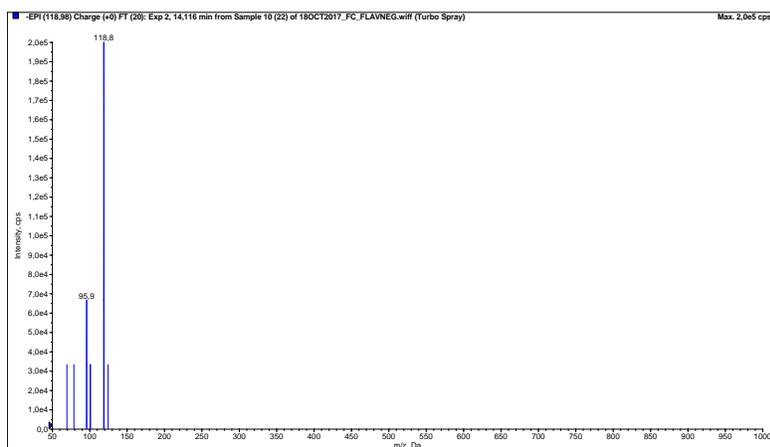
## Isorhamnetin



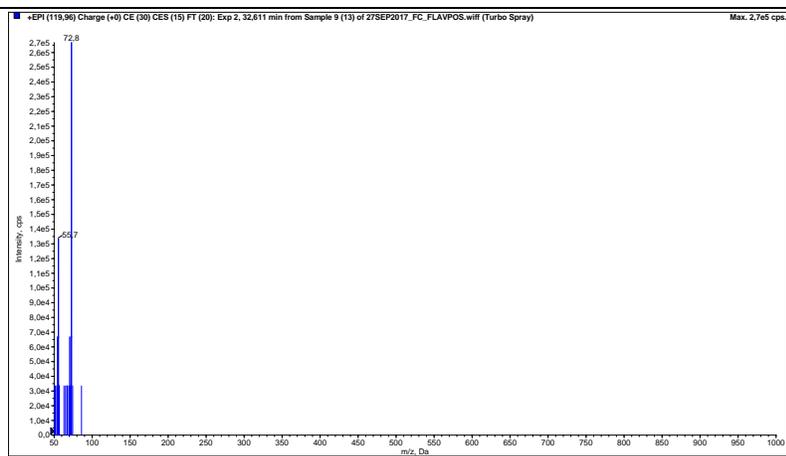
## Kaempferol-3-Glucuronide



## Tartronic acid



## L-threonine

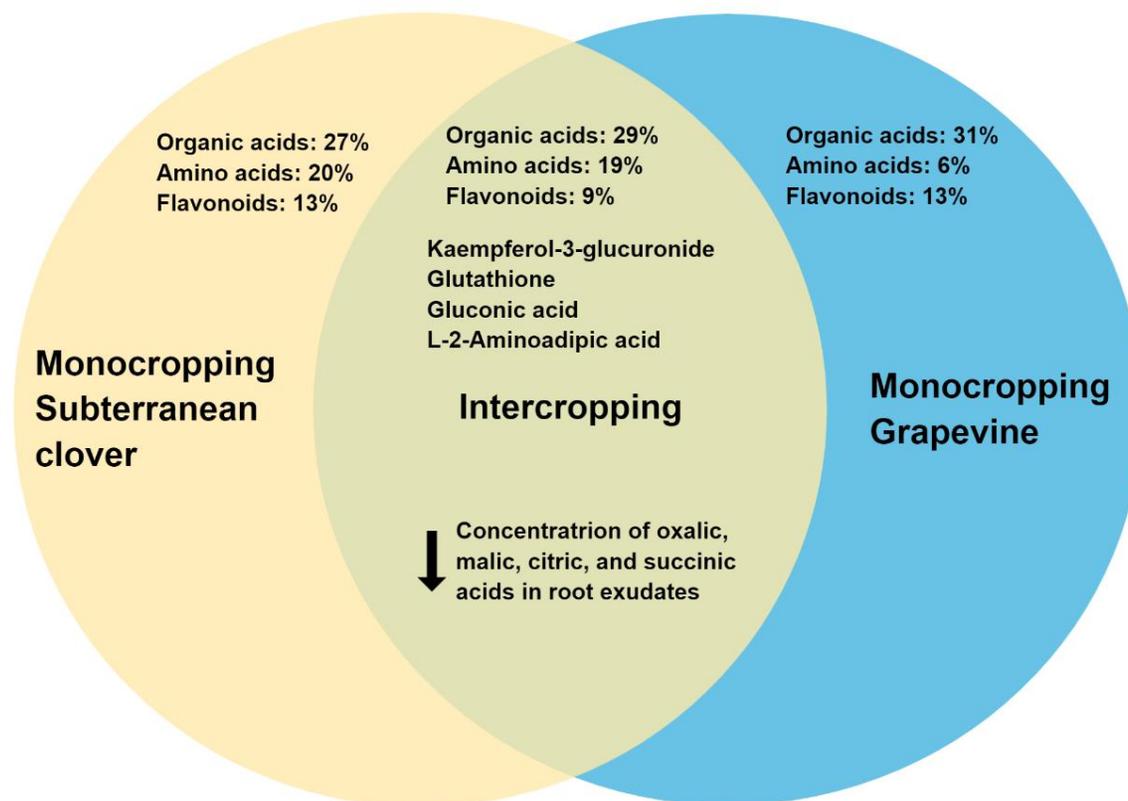


**Supplementary Table 2.** Compounds found after pyrolysis from different treatments obtained at 340 °C and described in Chapter IV.

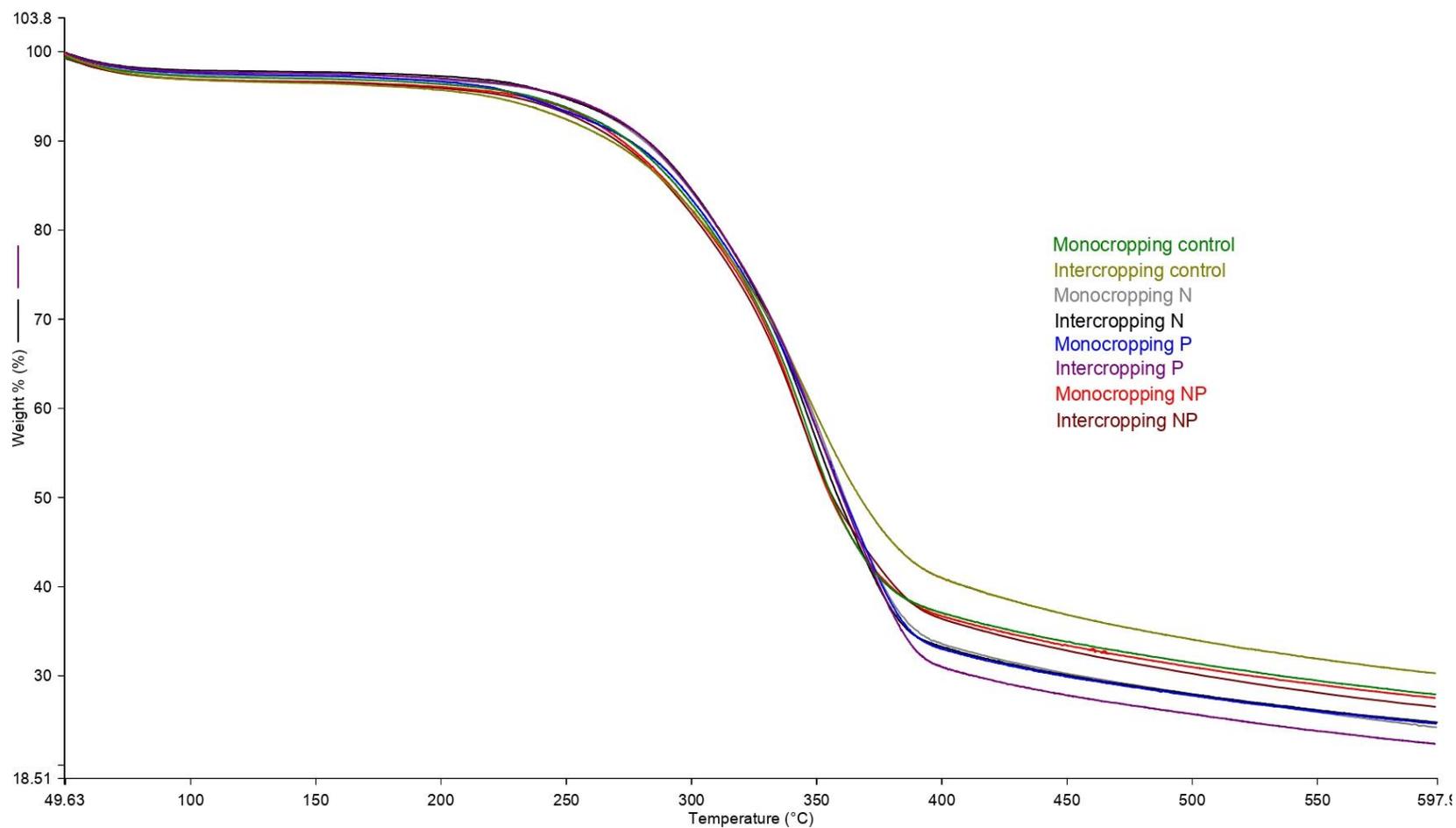
Abbreviations: Control: without fertilization; N: nitrogen applied; P: phosphorus applied; NP: nitrogen and phosphorus applied. Values are expressed as a percentage of relative abundance (%). ND: not detected.

Compound Name	Relative abundance (%)							
	Monocropping (Control)	Intercropping (Control)	Monocropping (N)	Intercropping (N)	Monocropping (P)	Intercropping (P)	Monocropping (NP)	Intercropping (NP)
<i>Carboxylic Acids</i>	28.86	22.36	37.84	31.49	32.40	32.37	26.88	27.48
Acetic acid	27.09	20.98	35.12	29.85	30.56	30.32	25.45	25.69
Propanoic acid, 2-oxo-, methyl ester	1.77	1.38	2.72	1.64	1.84	2.06	1.43	1.79
<i>Ketones</i>	28.05	24.76	32.43	30.08	28.72	30.03	26.95	26.14
2-Propanone, 1-hydroxy-	14.79	9.66	14.02	14.49	13.13	14.08	13.63	12.73
2-Cyclopenten-1-one, 2-methyl-	0.13	0.08	ND	0.10	0.11	0.07	0.15	0.11
Apocynin	ND	0.03	0.07	0.04	0.07	ND	ND	ND
2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)-	ND	ND	ND	0.05	0.07	0.08	ND	ND
1,2-Ethanediol, monoacetate	3.67	7.13	6.31	4.51	4.69	5.12	3.70	3.72
2-Propanone, 1-(acetyloxy)-	0.92	0.62	0.89	1.15	1.06	0.79	0.93	1.11
4-Cyclopentene-1,3-dione	0.18	0.16	0.26	0.33	0.28	0.20	0.18	0.24
2-Cyclopenten-1-one, 2-hydroxy-	1.11	0.92	1.29	1.30	1.22	1.28	1.15	1.13
2,4(3H,5H)-Furandione, 3-methyl-	ND	ND	ND	0.08	0.06	0.09	ND	ND
5-Hydroxymethyldihydrofuran-2-one	0.11	0.10	0.10	0.08	0.11	0.11	ND	ND
Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	ND	ND	ND	0.18	0.19	0.18	ND	ND
2-Pentanone, 1-(2,4,6-trihydroxyphenyl)	0.08	0.07	0.15	0.14	0.19	0.16	0.06	ND
Ethanone, 1-(2-furanyl)-	0.19	0.11	0.12	0.12	0.16	0.01	0.24	0.19
Methyl glyoxal	6.86	5.88	9.22	7.32	7.19	7.67	6.93	6.90
<i>Sugars</i>	0.32	0.37	0.34	0.36	0.35	0.35	0.27	ND
1,4:3,6-Dianhydro- $\alpha$ -D-glucopyranose	0.11	0.06	0.08	0.11	0.10	0.10	0.08	ND
$\beta$ -D-Glucopyranose, 1,6- anhydro-	0.21	0.31	0.26	0.25	0.25	0.25	0.19	ND
<i>Phenols</i>	2.18	1.94	2.29	2.63	2.67	2.83	1.59	1.51

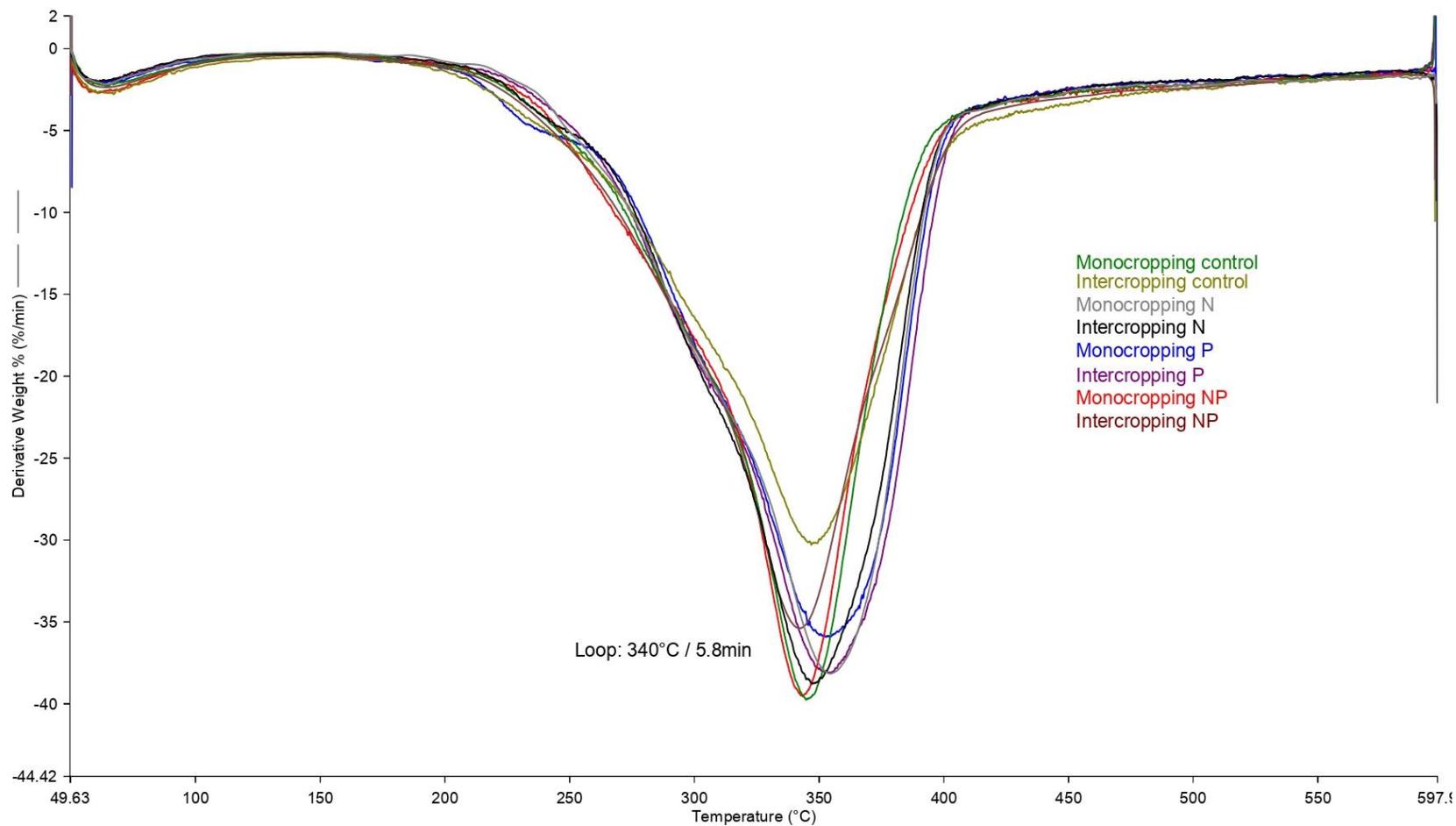
Phenol	0.41	0.73	0.26	0.31	0.23	0.15	0.34	0.46
Phenol, 2-methoxy-	0.44	0.34	0.50	0.43	0.49	0.39	0.45	0.48
2-Methoxy-4-vinylphenol	0.25	0.19	0.42	0.38	0.27	0.44	0.17	0.21
Vanillin	ND	ND	0.09	0.06	0.07	0.08	ND	ND
Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	ND	ND	ND	0.06	0.10	0.12	ND	ND
Phenol, 2,6-dimethoxy-	0.41	0.24	0.31	0.50	0.46	0.49	0.24	0.28
Phenol, 2-methoxy-4-(1propenyl)-	0.13	0.10	0.18	0.17	0.20	0.22	0.13	ND
(E)-2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	0.14	0.11	0.25	0.20	0.31	0.35	0.10	ND
Creosol	0.06	0.04	0.07	0.06	0.06	0.07	ND	ND
Hydroquinone	0.14	0.06	0.11	0.12	0.11	0.11	0.10	0.09
Catechol	0.07	0.07	0.09	0.08	0.07	0.09	ND	ND
1,2-Benzenediol, 3-methoxy-	0.04	ND	ND	ND	0.05	0.05	ND	ND
5-tert-Butylpyrogallol	ND	ND	ND	0.11	0.11	0.12	ND	ND
3,5-Dimethyl-4-hydroxytoluene	0.10	0.06	ND	0.14	0.14	0.16	0.07	ND
<i>Furans</i>	8.35	6.09	8.94	8.04	7.88	7.95	8.06	7.62
Furfural	3.09	2.26	3.14	3.11	2.86	2.82	2.87	2.79
2-Furanmethanol	2.14	1.36	1.94	2.09	1.89	1.91	2.04	1.88
Furan-2-carbonyl chloride, tetrahydro-	0.19	ND	ND	0.12	0.15	0.08	0.23	0.19
5-Hydroxymethylfurfural	0.07	0.10	0.13	0.12	ND	0.14	0.06	ND
2(5H)-Furanone	1.48	1.12	1.44	1.07	1.31	1.28	1.52	1.47
2-Formyl-9-[β-d-ribofuranosyl]hypoxanthine	0.06	0.07	0.20	0.10	0.15	0.20	0.10	ND
(S)-5-Hydroxymethyl-2[5H]-furanone	1.16	1.02	1.89	1.28	1.36	1.36	1.07	1.13
3-Furaldehyde	0.14	0.16	0.20	0.14	0.15	0.15	0.17	0.16
<i>Amines</i>	0.75	0.76	1.55	0.88	1.03	1.27	0.85	0.85
Pyrrolidine, 2-butyl-1-methyl-	0.39	0.38	0.46	0.41	0.37	0.41	0.41	0.36
Oxazolidine, 2,2-diethyl-3-methyl-	0.36	0.38	1.09	0.47	0.66	0.86	0.44	0.48
<i>Others</i>	33.63	29.60	33.40	31.63	29.15	29.95	35.33	36.43
Succindialdehyde	2.46	1.29	1.80	2.16	1.79	1.88	1.92	2.03
Cyclopropyl carbinol	0.62	0.38	0.55	0.73	0.58	0.71	0.50	0.47



**Supplementary Figure 1.** Venn diagram describing the results obtained in the experiment with plants grown in hydroponic solution explained in Chapter III.



**Supplementary Figure 2.** Diagram of TGA in different treatments of grapevine roots grown in pots described in Chapter IV. Abbreviations: Control: without fertilization; N: nitrogen applied; P: phosphorus applied; NP: nitrogen and phosphorus applied.



**Supplementary Figure 3.** Diagram of derivative TGA in different treatments of grapevine roots grown in pots mentioned in Chapter IV. Abbreviations: Control: without fertilization; N: nitrogen applied; P: phosphorus applied; NP: nitrogen and phosphorus applied.