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Prospecting intercropping between subterranean clover and grapevine as potential strategy for improving 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 subterranean clover exudates (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 acts as signaling compounds between plant-plant interaction, can be utilized for improving grapevine plant performance.

1. Introduction

Intercropping is an agricultural practice, used by farmers with the purpose of increasing nutrients availability and crop productivity [1]. 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 [2,3]. 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 [4,5]. 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 [6–8]. It is emphasized that intercropping improves nutrient uptake efficiency, in legume-grass systems through the influence of both root exudates interaction [9].

Root exudates are defined as carbon-containing compounds released by plant roots into the rhizosphere [10]. 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 [11]. 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 [10,12].

The most important phosphate solubilizers identified in legume roots through P substitution by organic acids forming complexes with

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minerals containing Fe^{3+}, Al^{3+} or Ca^{2+} are citric, malic and succinic acids [13,14]. In legume plants, the most important amino acids present in root exudates are leucine, valine, and lysine, which works as nutrient sources for plants [15]. 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 [16]. 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 [17]. Flavonoids compete with phosphate ions (PO_4^{3-}) for sorption sites, desorbing phosphates from soil-mineral surfaces, forming complexes with Fe^{3+} and Al^{3+}, and increasing P availability in the rhizosphere [18,19].

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 ton of table grapes year 2017, and producing over 1,000 millions of liters of wine [20]. The main varieties grown in Chile are Cabernet Sauvignon, Carménère and Chardonnay produced in transversal valleys located in central Chile [21,22]. It is emphasized that volcanic soils are good substrates for grapevine and vineyard establishment providing the essential nutrients required by plants, whereby its cultivation under intercropping system can be a relevant strategy [23]. Subterranean clover is an important legume-grass species comprising 1,000,000 ha of pastures grown in Andisols mainly in southern Chile due to its favorable conditions [24]. Therefore, both grapevine and subterranean clover grown in intercropping systems can improve the rhizospheric interactions among plant roots with positive effects [25]. From the agronomic point of view, subterranean clover is attractive because is an annual, self-reseeding legume not competing with grapevine for water during the summertime [26]. 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.

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 conditions.

2.1. Plant material and growth conditions

We used subterranean clover (*T. subterraneum* L.) var. Seaton Park, a common variety 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 [27,28]. 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 during 2 weeks and 1-year-old grapevine plants from the nursery, using a density dose of 200 plants m^{-2} for subterranean clover and 1 plant m^{-2} for grapevine.

Subterranean clover and grapevine plants were cultivated in a hydroponic system using Hoagland hydroponic solution under monocropping (T1, T2) and intercropping conditions (T3) [29]. 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 aereated 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^{-2} s^{-1} 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.

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) during 2 h 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 μm filters to clean up the samples, and finally kept under frozen conditions at -20 °C for further analysis. Organic acids, amino acids, and flavonoids present in root exudates of subterranean clover and grapevine were analyzed on day 30 and 60 after the experiment establishment.

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 [30–32]. Samples (10 μL) were injected in Shimadzu Prominence LC-20AD with detector UV/VIS coupled to 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 μm) using a flux rate of 0.25 ml/min at 35 °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 in on-line database www.massbank.jp/PeakSearch.html and 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).

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 g mol^{-1} approximately, while compounds presented in Table 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 [33–35]. Samples (20 μL) 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 μm). Samples were eluted with a mobile phase composed by solvent A: H_3PO_4 200 mM (pH 2.1); solvent B: methanol, solvent C: acetonitrile and solvent D: water, a flow rate of 1 ml/min at 30 °C. Peaks data evaluation was processed by the HPLC software Primaide 1.0. The acid

Table 1

Organic acids, amino acids and flavonoids composition identified 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	$C_5H_7NO_5$	39.1	159.8	115.9 87.8
		trans-Cinnamic acid	$C_9H_8O_2$	39.1	146.9	118.9 103.0 147.0
	Amino acids	Glycylglycine	$C_4H_8N_2O_3$	2.9	133.0	75.0
		L-threonine	$C_4H_9NO_3$	32.6	119.9	72.8 55.7
	Flavonoids	Epicatechin	$C_{15}H_{14}O_6$	5.4	290.8	122.9 206.9
		Isorhamnetin	$C_{16}H_{12}O_7$	73.9	315.0	315.1 300.1
T2 (grapevine) Monocropping	Organic acids	5-Dodecenoic acid	$C_{12}H_{22}O_2$	77.8	196.8	178.9 196.9
		4-Hydroxyphenylacetic acid	$C_8H_8O_3$	22.8	150.9	136.0 104.9
		trans-Cinnamic acid	$C_9H_8O_2$	39.1	146.9	118.9 103.0 147.0
		Tartronic acid	$C_3H_4O_5$	85.1	118.9	118.8 95.9
	Amino acids	ND	ND	ND	ND	ND
	Flavonoids	Isorhamnetin	$C_{16}H_{12}O_7$	73.9	315.0	315.1 300.1
T3 Intercropping	Organic acids	L-2-Aminoadipic acid	$C_6H_{11}NO_4$	37.2	160.0	115.9 160.0
		trans-Cinnamic acid	$C_9H_8O_2$	39.1	146.9	118.9 103.0 147.0
		Gluconic acid	$C_6H_{12}O_7$	70.3	194.9	129.0 195.0 74.9
		4-Hydroxyphenylacetic acid	$C_8H_8O_3$	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

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 nmol g^{-1} h^{-1}.

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. Results

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 (Fig. 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 (Fig. 1b). Fig. 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 [36,37].

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

Table 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 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 Fig. 2 and spectra of compounds found in root exudates detected by HPLC/UV-ESI MS/MS are presented in supplementary Table 1.

3.3. Exudation rates of LMWOAs released by plant roots

Fig. 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. Fig. 3a indicates that oxalic acid has a higher exudation rate in subterranean clover (T1) (92.930 ± 19.440 nmol g^{-1} h^{-1}) on day-30 but this rate decreases (28.590 ± 13.760 nmol g^{-1} h^{-1}) when grown under intercropping with grapevine (T3). Fig. 3b shows that malic acid has the highest exudation rate in subterranean clover (T1) (1.820 ± 0.320 nmol g^{-1} h^{-1}) on day-30, showing significant differences with the other treatments. Fig. 3c showed significant differences in citric acid with the highest release rate in grapevine (T2) (0.680 ± 0.060 nmol g^{-1} h^{-1}) on day-30. Meanwhile, the highest release rate in subterranean clover (T1) (0.190 ± 0.050 nmol g^{-1} h^{-1}) on day-60. Finally, Fig. 3d showed significant differences in the exudation rate of succinic acid reaching the highest rate in intercropping treatment (T3) (0.360 ± 0.063 nmol g^{-1} h^{-1}) on day-30. Whereas, on day-60 the highest release rate was in grapevine (T2) (0.260 ± 0.025 nmol g^{-1} h^{-1}).

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

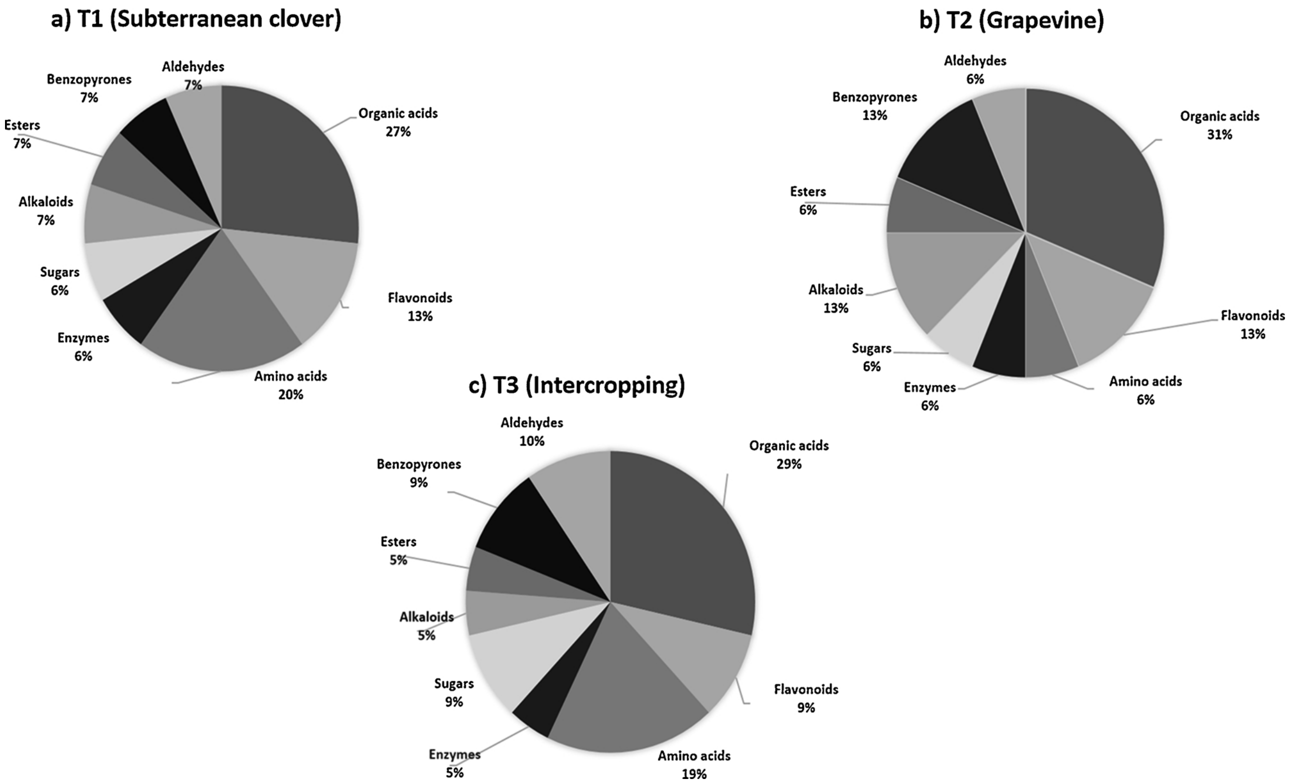

Fig. 1. Root exudates composition of subterranean clover and grapevine grown in hydroponic solution during 60 days separately and under intercropping system. T1 = subterranean clover, T2 = grapevine and T3 = subterranean clover + grapevine (Intercropping).

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 plants 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 [38]. 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 [39,40].

Regarding to 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 are in agreement to [41], 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 [42]. 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 [43]. Although many signaling peptides and receptors have been identified, further knowledge is needed to clarify the mechanisms involved in this crosstalk among plants [44]. 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 [45]. 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 [46].

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 [10]. 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 [47]. Kaempferol-3-glucuronide is a flavonol with antioxidant properties found in root exudates of *Abelmoschus esculentus* according to the reported by [48]. 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 [49].

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 [50]. 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 [51]. It highlights that L-2-aminoapidic acid was detected only in intercropping, suggesting its role between the plant-plant signaling.

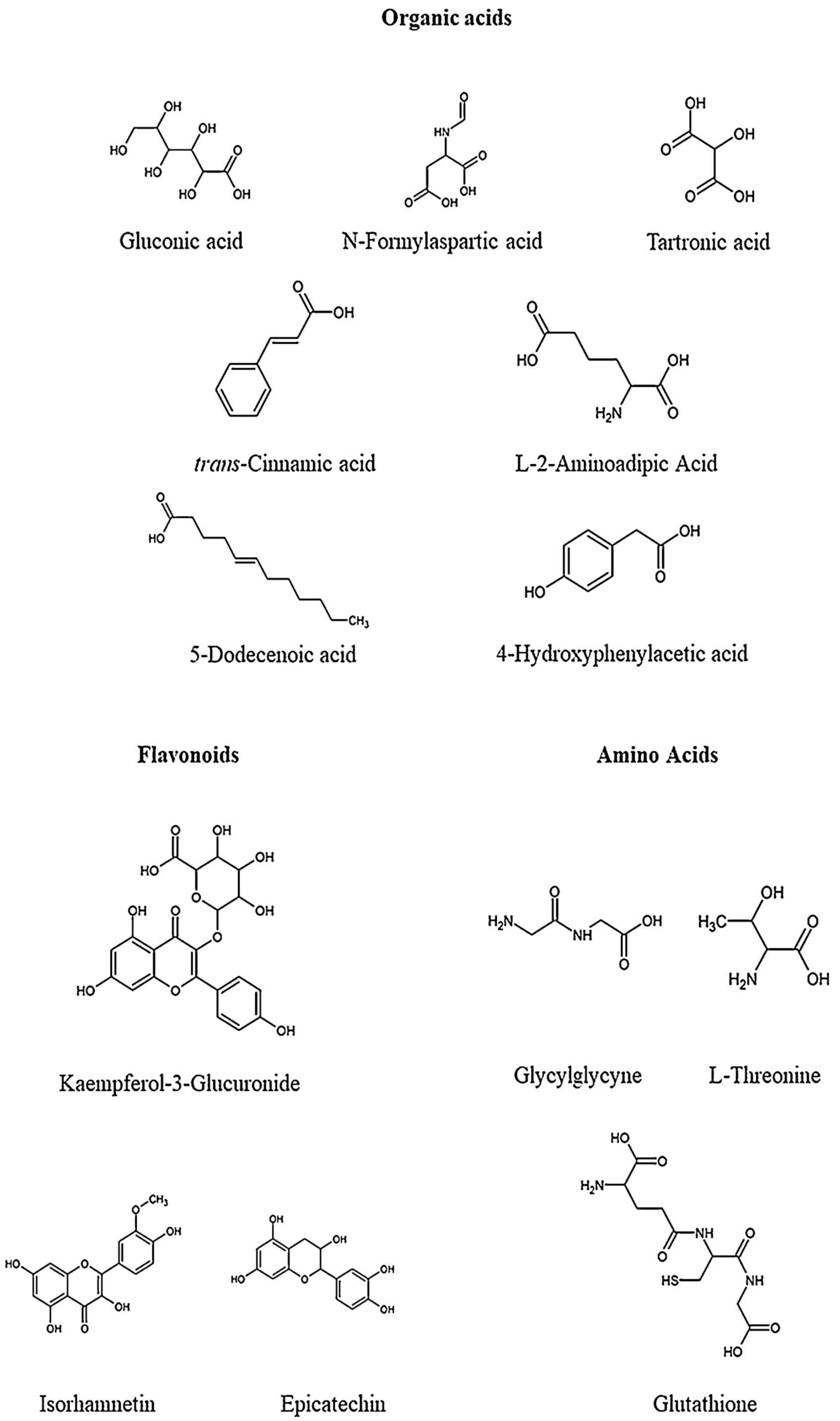

Fig. 2. Chemical structure of identified compounds through HPLC/UV- ESI MS/MS present in root exudates released by subterranean clover and grapevine grown separately and together (intercropping) under hydroponic conditions on day-60.

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 [52], 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 (4549 nmol $g^{-1} h^{-1}$). Furthermore, similar results indicating a high concentration of oxalic acid were obtained by [30]. 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 [53]. 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 [42,54]. 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

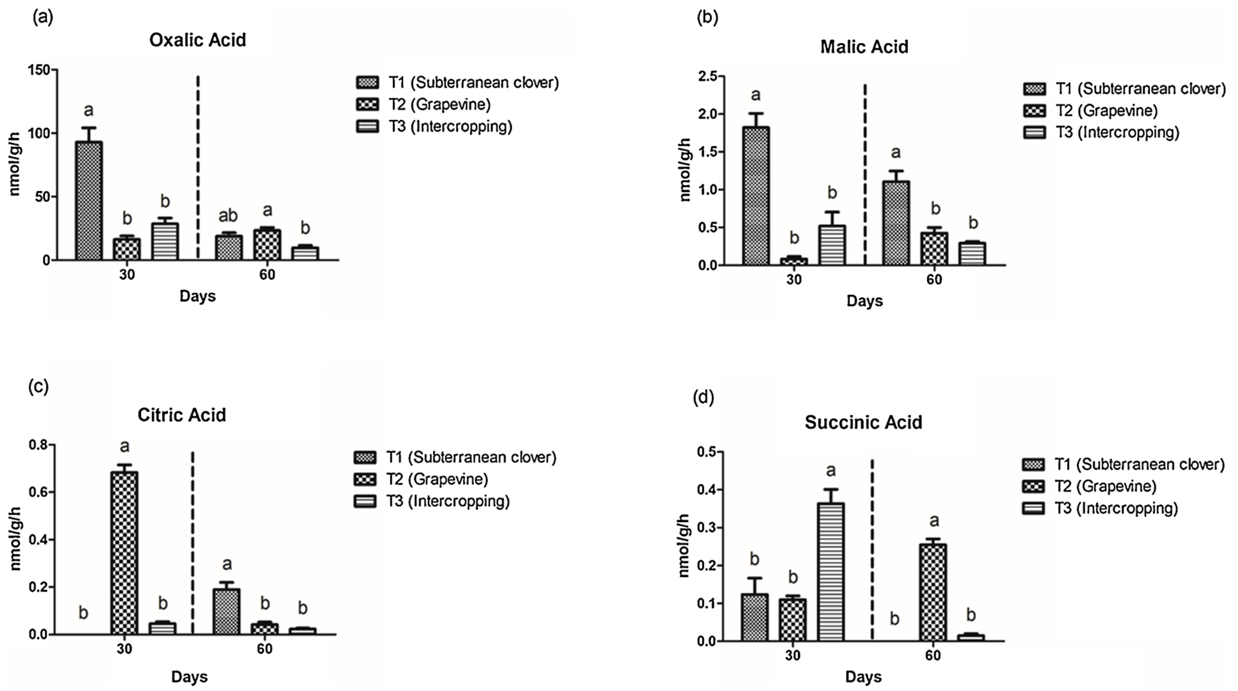

Fig. 3. Root exudation rates of organic acids of subterranean clover and grapevine 30 and 60 days after the beginning of the experiment. a, b, c, and d show quantification of oxalic, malic, citric and succinic acids respectively. T1 = subterranean clover (Monocropping); T2 = grapevine (Monocropping)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

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.

5. Conclusions

This study provides relevant evidence that subterranean clover grown under intercropping with grapevine play 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 be selected as a promissory strategy to improve industrial grapevine plant performance.

Conflict of interest

Authors has no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cpb.2019.100110.

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