Agrochemical characterization of *Vitis labrusca* grape pomace and its effect on a soil-plant system

Caracterización agroquímica del orujo de uva de *Vitis labrusca* y su efecto sobre el sistema suelo-planta

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Abstract

This study characterized the agrochemical properties of *V. labrusca* grape pomace (GP) and evaluated the effect on the rhizobacteria *Azospirillum brasiliense* and horticultural crops, determining safety as fertilizer and/or mulching. Two first bioassays were performed with the GP at different concentrations evaluating toxicity on *A. brasiliense*, and on tomato and lettuce seeds. A third bioassay evaluated GP mulching effects on tomato and lettuce plants growing with amounts varying between 20 and 80 t ha^{-1}. Agrochemical characterization showed that GP is rich in potassium and phosphorus, with a low content of Na^+ salts (SAR < 15). The GP at 2.5% (w v^{-1}) significantly increased survival rates of N_2-fixing rhizobacteria. Results on seed germination revealed lettuce was more susceptible to increasing GP concentrations. The application of 20 t ha^{-1} of GP in greenhouse experiments increased lettuce and tomato root biomass. Furthermore, the aerial part of tomato showed no toxicity symptoms. These results open the possibility of considering *V. labrusca* GP as mulching without prior treatment in tomato crops.

Keywords
agrowaste • revalorization • macronutrients • *Azospirillum brasiliense* • tomato • lettuce

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Resumen

Este estudio tuvo como objetivo caracterizar las propiedades agroquímicas del orujo de uva (GP) de *V. labrusca* y evaluar su efecto sobre *Azospirillum brasiliense* y cultivos hortícolas para determinar su uso seguro como fertilizante y/o mulching. Los bioensayos se realizaron con el GP a diferentes concentraciones para evaluar su toxicidad sobre *A. brasiliense* y semillas de tomate y lechuga. Para evaluar el efecto de GP sobre el crecimiento de plantas de tomate y lechuga se aplicaron diferentes dosis como mulching en el rango de 20-80 t ha^{-1}. La caracterización agroquímica mostró que GP es rico en potasio y fósforo; y tiene bajo contenido de sales de Na^+ (SAR < 15). El GP al 2,5% (p v^{-1}) fue responsable de un aumento significativo en la tasa de supervivencia de las rizobacterias fijadoras de N_2. Los resultados de la germinación de las semillas revelaron que la lechuga fue más susceptible al aumento de las concentraciones de GP. La aplicación de 20 t ha^{-1} de GP en los experimentos de invernadero incrementó la biomasa radicular de lechuga y tomate. Además, la parte aérea del tomate no presentó síntomas de toxicidad. Estos resultados abren la posibilidad de considerar el aprovechamiento de *V. labrusca* GP como mulching, sin utilizar ninguna tecnología de tratamiento antes de su aplicación al suelo en cultivos de tomate.

Palabras claves
residuo agronómico • revalorización • macronutrientes • *Azospirillum brasiliense* • tomate • lechuga

Introduction

The wine industry is one of the oldest and most important in the world. In 2019, wine production was estimated at around 258 Mhl (27). Grapes for winemaking belong to the Genus *Vitis* sp, being *V. vinifera* and *V. labrusca* the two main producing species (20). *V. vinifera* is cultivated in arid soils, poor in organic matter, while the hybrid *V. labrusca* originated in eastern USA, grows in different edaphoclimatic regions and is highly resistant to diseases (18, 24). In South America, mainly in Argentina and Brazil, *V. labrusca* cultivated areas have increased in the last decade (24, 40).

Annually, the wine industry produces 13 x 10^6 t of solid waste or by-products worldwide, with grape pomace (GP) being the most abundant (10-13 Mt per year). In terms of circular economy, GP could generate new products of different values (7, 21), closing the cycle as mulching or organic fertilizer due to its lignocellulosic nature (8). Mulching improves soil structure, keeps moisture, moderates temperature, and increases organic matter (OM), returning soil nutrient cycling environments to pristine soil patterns (25). On the other hand, when the agronomic purpose is to increase crop yield, organic fertilizers of plant origin constitute an interesting agroecological alternative for intensive crops like vegetables, flowers, and orchards (22). Nevertheless, a proper agronomic management program incorporating lignocellulosic by-products, such as GP, needs to characterize the product and establish an appropriate use. Unlike the well-known physicochemical characteristics of *V. vinifera* GP (6, 16, 34), the relevant agrochemical properties of *V. labrusca* GP have not been studied yet. Direct incorporation of *V. vinifera* GP into the soil could negatively affect plant growth due to inhibitory compounds or given competition between soil microorganisms for essential nutrients such as nitrogen (6). However, this study only considers GP physicochemical properties, leaving aside other sensitive parameters in the soil-plant systems.

Biological indicators are living organisms, such as plants, animals, and/or microorganisms exploited to detect toxic substances in terrestrial and aquatic ecosystems (45). The effect of agrowastes on the soil-plant system can be evaluated by lettuce (*Lactuca sativa*) and tomato (*Solanum lycopersicum*) as biological indicators, as well as by the free-soil nitrogen-fixing bacterium *Azospirillum brasiliense* (14, 36, 37, 41). The combination of agrochemical characterization together with biological indicators enables the development of appropriate agronomic management programs (22).

This study characterized the agrochemical properties of *V. labrusca* var. *Isabella* GP and evaluated toxicity effects on *A. brasiliense*, tomato and lettuce determining GP safety as fertilizer or/and mulching.

MATERIAL AND METHODS

Study area and GP sampling

The study considered twenty-four ha of *V. labrusca* var *Isabella* at "Cooperativa de la Costa" wine-cellar located in Berisso, Buenos Aires, Argentina (34°53'22.79" S; 57°49'21.11" W (28). The soil is Rendzic Leptosol (11) with an A-horizon of 20-40 cm and a high content of well-humified organic matter, under which there is an AC or C-horizon constituted by layers of small shells (1). In 2013, the winery produced 350 hL of wine and a total of 12 t of GP, representing 25% of grape total weight (29).

Samplings were carried out in 2015, 2016 and 2017 seasons. Each year, 10 random GP samples were collected immediately after grape pressing and blended to obtain a compound sample. One part of the sample was separated to determine moisture content. The rest was oven-dried at 60°C to constant weight and then divided into sub-samples. These were stored in hermetically sealed in containers until GP physicochemical characterization. Part of fresh material from the 2017 campaign was separated and stored at -20°C for subsequent bioassays.

Agrochemical GP characterization

Ashes and OM were determined by calcination (6). Percentage contents of organic carbon (C) and total nitrogen (N) were determined after Walkley and Black (1934) and micro-Kjeldahl methods (5) respectively, and the C/N ratio was calculated. Total phosphorus (P) was determined by the yellow vanadate-molybdate method (19). Extractable and exchange cations were obtained by the saturated paste extraction method and the Ammonium acetate method 1N pH7, respectively (35). Calcium (Ca^{+2}) and Magnesium (Mg^{+2}) were estimated by complexometry with 0.02 N EDTA; Sodium (Na^{+}) and Potassium (K^{+}) by flame photometry. Sodium adsorption ratio (SAR) was calculated using the following equation (31) (eq. 1).

$$\mathbf{SAR = (Na^{+}\ ext) / [\ (Ca^{+2}\ ext + Mg^{+2}\ ext /2]^{1/2}} \qquad (1)$$

The Mg^{+2}/K^{+}, Ca^{+2}/Mg^{+2}, and Ca^{+2}/K^{+} ratios were calculated using the obtained values for individual exchangeable cations (17). Cation exchange capacity (CEC) was determined by steam distillation and percentage base saturation (S) was calculated by eq. 2 (33).

$$\mathbf{S\ (\%) = (\Sigma\ exchangeable\ cations/CEC)\ {*}100} \qquad (2)$$

Electric conductivity (EC) and pH values were measured in the aqueous soluble fraction (1:5 ratio) by conductometry and potentiometry, respectively (19). Moisture content (%) was determined by oven-drying samples at 60°C until constant weight and calculating the difference between wet and dry weight. Water-soluble phenols (WSP) were determined as described by Osono and Takeda (2001). Three technical replicates were used to determine each physicochemical parameter. Values were averaged to obtain a unique value for each year. Mean, range and t variance were estimated with three annual values. The classification criterion proposed by Fernández Linares *et al.* (2006) and Havlin *et al.* (1999) allowed interpretation.

Bioassays with GP application

Three bioassays determined GP safety as fertilizer or/and mulching: a) GP toxicity evaluation on a N fixing microorganism (*A. brasiliense*), b) GP toxicity evaluation on germination of tomato and lettuce seeds; and c) GP effect on growth parameters of tomato and lettuce plants.

a)- GP toxicity evaluation on a N fixing microorganisms (*A. brasiliense*)

GP effects on survival of *A. brasiliense* CECT 590 T were evaluated according to

Saparrat *et al.* (2010). A bacterial suspension (100 µl) was inoculated in 900 µl of the GP sterilized by filtration (0.22 µm millipore membrane) at different concentrations: 2.5, 3.5, 5, and 10% (w v^{-1}). Controls were made using sterile distilled water and 4 replicates per treatment were utilized. Cultures were grown at 28°C and 150 rpm for 24 h. Colony-forming units (CFU mL^{-1}) were estimated by the dilution and plating method using the selective medium Congo red. The data were transformed to logarithms and analyzed by ANOVA and a Tukey test ($p < 0.05$).

b)- GP toxicity evaluation on germination of tomato and lettuce seeds

GP phytotoxicity was evaluated after the effects observed on germination and root growth of lettuce and tomato according to Tiquia *et al.* (1996) with modifications. Seeds of each species were placed on filter paper inside Petri dishes (9 mm diameter) in contact with 3 ml of the GP at different concentrations: 2.5, 5, 10, and 20% w v^{-1}. Negative and positive controls consisted of paper soaked with sterile distilled water and 1 M of a $CuSO_4$ solution, respectively. Four replicates per treatment were incubated at 25°C for 7 days in the dark.

Number of germinated seeds and root length (mm) were determined. Relative germination percentage (G) (eq. 3), relative root length (RL) (eq. 4), and germination index (GI) (eq. 5) were calculated using the following equations:

$$\text{G (\%)} = \text{(number of germinated seeds at X GP concentration/ number of seeds germinated in negative control)} * 100 \quad (3)$$

$$\text{RL (\%)} = \text{(mean root length at X GP concentration/ mean root length in negative control)} * 100 \quad (4)$$

$$\text{IG} = (\text{G} * \text{RL}) / 100 \quad (5)$$

A 2 mm primary root defined seed germination. The data obtained were analyzed by ANOVA and Tukey test ($p < 0.05$). Percentual data were transformed using arc sen $\sqrt{p}$, before statistical analysis.

c) GP effect on growth of tomato and lettuce plants

The effect of GP on lettuce and tomato plants was determined using ten plants per treatment and according to Sampedro *et al.* (2004) with modifications. Seedlings with three expanded leaves were placed in 5 L pots filled with a mixture of soil and sand in a 1:1 ratio (v v^{-1}). GP mulching was added to the pots at different doses: 20, 40, and 80 t h^{-1} (wet weight equivalent to 60, 120 and 240 g of GP per pot). The plants were grown for 1 month in a greenhouse and periodically watered. Pots without GP were set up as control. The number of expanded leaves per plant was estimated by direct counting. Leaf greenness (GrI) was measured using a portable chlorophyll meter (SPAD-502, Minolta Corp. Japan), randomly selecting three expanded leaves per plant. Total leaf area per plant was captured by photography and analyzed using J image software (38). Plants were harvested and soil adhered to roots was washed with running water. The aerial part of the plants and roots were oven-dried at 60°C to constant weight determining aerial and root dry biomass. The data were analyzed for each plant species comparing dose effects. A Kruskal-Wallis test and a non-parametric multiple contrast as *post hoc* test ($p < 0.05$) analyzed the number of expanded leaves. The remaining data were analyzed by ANOVA and Tukey test ($p < 0.05$).

Results

Agrochemical GP characterization

The C/N ratio < 25 suggested an equilibrate decomposition of OM. Among extractable cations, GP showed high levels of K^+ and low Na^+, high CEC (> 40), and a varied macronutrient supply (exchangeable cations, high content of K^+, followed by Ca^{+2}, and low Mg^{+2}). The GP exhibited a high base saturation index (S > 40), high EC (> 4 dS m^{-1}), extremely acidic pH, and high hygroscopicityy (> 70% humidity, table 1, page XXX).

Table 1. Physicochemical characteristics of *V. labrusca* var. *isabella* GP.

Tabla 1. Características fisicoquímicas del OU de *V. labrusca* var. *isabella*.

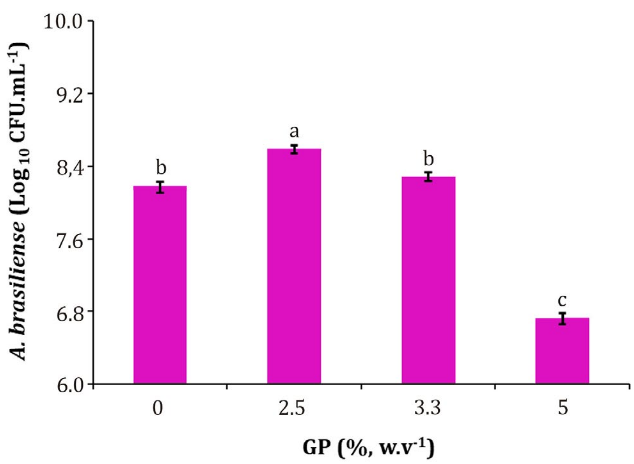
Parameter	Mean	Range	VC[1]
OM (g kg^{-1})	9.3	9.1-9.4	0.2
Ash (%)	7.5	6.4-9.1	18.9
TOC (g kg^{-1})	4.71	3.42-5.51	2.39
TN (g kg^{-1})	0.21	0.2-0.22	0.58
C/N	22.4	16.6- 26.3	26.5
TP (g kg^{-1})	3.1	2.9-3.4	8.6
Ext K^+ (g kg^{-1})	37.6	27.7-51.7	33.3
Ext Na^+ (mg kg^{-1})	64	57-75	15.1
Ext Ca^{+2} (g kg^{-1})	4.4	3.7-5.0	15.0
Ext Mg^{+2} (g kg^{-1})	0.7	0.5-0.8	23.2
SAR[2]	1.3^{-3}	1.1^{-3}- 1.51^{-3}	14.7
Exch K^+ ($cmol_c$ Kg^{-1})	49.3	43.9-54.1	10.4
Exch Ca^{+2} ($cmol_c$ Kg^{-1})	7.2	6.6-7.6	7.2
Exch Mg^{+2} ($cmol_c$ Kg^{-1})	0.9	0.4-1.7	72.9
Exch Na^+ ($cmol_c$ Kg^{-1})	0.4	0.4-0.5	21.0
Exch Ca^{+2}/ Exch Mg^{+2}	10.47	4.5-16.5	57.8
Exch K^+/ Exch Ca^{+2}	6.9	5.8-7.6	14.2
Exch K^+/ Exch Mg^{+2}	75.9	25.8-124.5	65.0
CEC ($cmol_c$ Kg^{-1})	43.7	41.5-46.7	6.2
S[3] (%)	132.4	125.7-137.7	4.64
EC (dS m^{-1})	5.8	5.4-6.1	6.1
pH	3.7	3.6-3.7	0.7
Moisture (%)	79.2	78.1-80.1	1.3
WSP[4] (mg g^{-1})	74.0	73.4-75.4	1.5

1, Variance coefficient. 2, Sodium adsorption ratio. 3, Base saturation index. 4, Water-soluble phenols. Extractable (Ext) and interchange (Exch) cations. n= 3 annual samples.

1, Coeficiente de variación. 2, Radio de adsorción de sodio. 3, Índice de saturación de bases. 4, Fenoles hidrosolubles. Cationes extractables (Ext) e intercambiables (Exch). n= 3 muestras anuales.

GP toxicity evaluation on a N fixing microorganism (*A. brasiliense*)

GP concentrations showed significant differences in *A. brasiliense* growth ($F= 684.50$; $p < 0.001$). No CFU of the diazotrophic bacteria was recovered at 10% GP concentration (figure 1).



Means followed by the same letter are not significantly different (Tukey test, $p < 0.05$).

Los datos son medias de cuatro réplicas ± D.E. Medias seguidas por la misma letra no presentan diferencias significativas (Test de Tukey, $p < 0,05$).

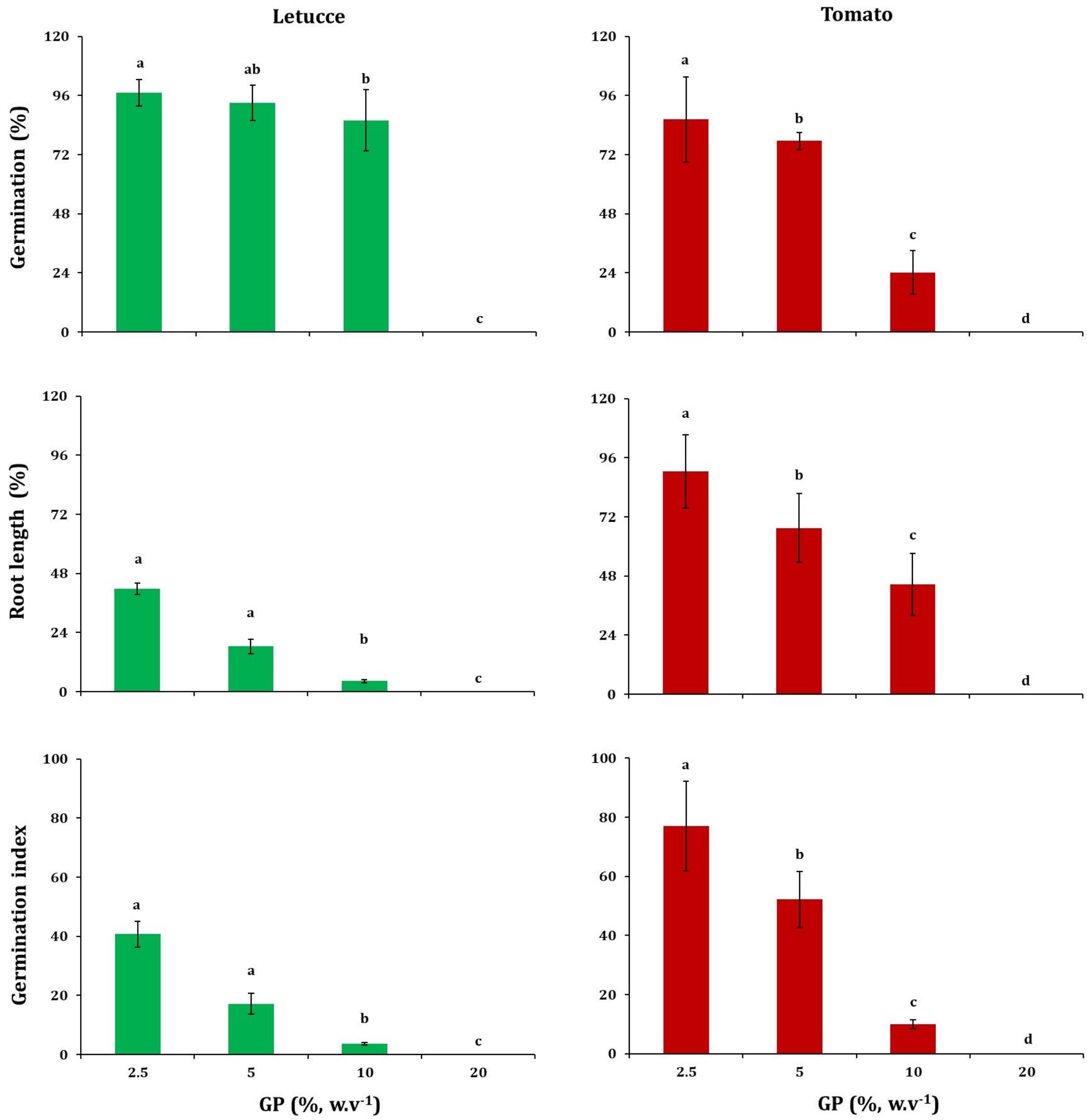
Figure 1. *A. brasiliense* growth after 24 h of incubation in several concentrations of GP (%, w v^{-1}). The data are means of four replicates ± S.D.

Figura 1. Crecimiento de *A. brasiliense* después de 24 h de incubación en distintas concentraciones de OU (%, p v^{-1}).

GP toxicity evaluation on germination of tomato and lettuce seeds

Significant differences were found in relative germination (G) ($F= 86.04$; $p <0.001$), relative root length (RL) ($F= 583.56$; $p <0.001$) and germination index (GI) ($F= 348.69$; $p <0.001$) when lettuce seeds were incubated at different GP concentrations. The G values at 2.5, 5 and 10% GP concentration equalled or exceeded 90%. The RL and GI, registered values below 50% indicating a toxic effect of GP at all concentrations evaluated (figure 2a, c, e).

For tomato, significant differences were found in G ($F= 368.32$; $p <0.001$), RL ($F= 145.96$; $p <0.001$) and GI ($F= 278.02$; $p <0.001$). The toxic effect in G, RL and GI increased with GP concentration (figure 2b, d, f).



Mean ± S.D. of four replicates followed by the same letter are not significantly different (Tukey test, $p < 0.05$).

Los datos son medias de cuatro réplicas ± D.E. Medias seguidas por la misma letra no presentan diferencias significativas (Test de Tukey, $p < 0,05$).

Figure 2. Effect of GP addition on germination, root length and germination index of lettuce and tomato plants.

Figura 2. Efecto de la adición del OU sobre la germinación, longitud radicular e índice de germinación de plantas de lechuga y tomate.

GP effect on growth of tomato and lettuce plants.

Significant differences were found in expanded leaf number (EL) (H= 8.54; p= 0.0259), greenness index (GrI) (F= 14.21; p < 0.0001), total leaf area (TLA) (F= 95.71; p < 0.0001), aerial dry weight (ADW) (F= 77.05; p < 0.0001) and root dry weight (RDW) (F= 40.54; p < 0.0001) of lettuce. The TLA, ADW and RDW significantly decreased with GP doses over 40%. The addition of 20 t ha^{-1} of GP as mulch showed an increase in RDW.

For tomato plants, significant differences were found in GrI (F= 6.72; p= 0.0038), TLA (F= 46.47; p < 0.0001), ADW (F= 21.00; p < 0.0001) and RDW (F= 30.54; p < 0.0001). The GP presented the highest toxic effect on ADW and TLA at doses of 40-80 t ha^{-1}. In contrast, GrI significantly increased at the maximum dose. Also, a non-toxic effect of GP was observed on RDW at 20 t ha^{-1} (table 2).

Table 2. Expanded leaf number (EL), greenness index (GrI), total leaf area (TLA), aerial dry weight (ADW) and root dry weight (RDW) of lettuce and tomato plants grown with increasing GP doses.

Tabla 2. Número de hojas expandidas (EL), Índice de verdor (GrI), Área foliar total (TLA), Biomasa seca área (ADW) y biomasa seca radicular (RDW) de plantas de lechuga y tomate en respuesta a diferentes dosis crecientes de OU.

Doses (t ha^{-1})	EL (n° $plant^{-1}$)		GrI (SPAD units)		TLA (cm^2 $plant^{-1}$)		ADW (g $plant^{-1}$)		RDW (g $plant^{-1}$)	
Lettuce										
0	11	a	28.2 ± 1.8	a	656.9 ± 85.3	a	3.6 ± 0.4	a	0.6 ± 0.1	b
20	9	b	19.8 ± 2.9	b	295.7 ± 18.9	b	2.3 ± 0.3	b	0.8 ± 0.1	a
40	9	b	23.3 ± 1.9	b	180.0 ± 30.7	c	1.0 ± 0.2	c	0.3 ± 0.0	c
80	9	b	22.1 ± 1.7	b	195.9 ± 41.7	c	1.0 ± 0.3	c	0.3 ± 0.0	c
Tomato										
0	11	A	31.2 ± 4.0	B	552.1 ± 57.2	A	9.1 ± 2.2	A	0.6 ± 0.1	B
20	11	A	31.2 ± 1.9	B	548.7 ± 43.9	A	9.5 ± 0.6	A	0.8 ± 0.1	A
40	10	A	36.3 ± 4.8	AB	275.7 ± 56.5	B	3.7 ± 1.1	B	0.4 ± 0.0	C
80	11	A	38.1 ± 3.5	A	295.6 ± 41.2	B	4.0 ± 1.8	B	0.4 ± 0.1	C

Data means ± S.D. of 10 replicates. For each row and vegetal species, different letters indicate significant differences (p < 0.05; Tukey test or Kruskal-Wallis test).

Los datos son medias de 10 réplicas ± D.E. Para cada fila y especie vegetal, diferentes letras indican diferencias significativas (p < 0,05; Test de Tukey o Kruskal-Wallis).

Discussion

This study found high OM contents in *V. labrusca* GP, as reported for *V. vinifera* and other agroindustrial by-products like solid olive mill “alperujo” (*Olea europaea* L.) and coffee pulp (2, 3, 4, 6). Unlike oil and coffee by-products, grape pomaces have high NT content and a C/N ratio nearly under the recommended limits for organic fertilizing (< 25) (6, 22).

Depending on the origin, commercial organic fertilizers have diverse amounts of essential nutrients (23). Bustamante *et al.* (2008) found 1.15 g kg^{-1} total P for *V. vinifera*, while in this study *V. labrusca* showed a higher total P content, resulting in a more attractive by-product for the agronomic industry.

While most organic fertilizers usually require EC values under 4 dS m^{-1}, the value obtained in this study was higher and considered detrimental to plant growth (22). However, the main salts found in *V. labrusca* GP are Ca^{+2} and/or Mg^{+2} chlorides and sulfates (SAR < 15). In this sense, the pomace could be considered a good alternative to animal manure causing soil disintegration due to concentrated Na^{+} salts (9, 10). Furthermore, *V. labrusca* pomace exhibited a CEC value similar to that of highly productive soils (>45), probably given by the available functional groups negatively charged (phenols, carboxylic acids, etc.) found in OM (15). Hence, soil addition of GP might reduce leaching, thus increasing essential cations availability (39).

Soils with a saturation base close to 100% exhibit alkaline pH (14). In this study, the pomace showed an elevated base saturation while being an acidic by-product, probably given to the presence of organic acids like malic and tartaric, common in grapes, that together with K+ and its effect over the diminished free tartaric acid, define by-product acidity (3). On the other hand, the high moisture content in this by-product (79.2%) is within the range reported for *V. vinifera* pomace and other agroindustrial wastes such as alperujo and coffee pulp ranging between 64-80% (3, 4).

Introducing OM into the system could differentially affect soil microorganisms and plants, depending on application dose and tolerance ranges (26, 41). *A. brasiliense* is a soil-free nitrogen-fixing bacteria producing several plant signalling molecules like phytohormones (auxins and gibberellins) (13, 32). The positive effect of GP on this diazotrophic bacteria has not been previously reported using other by-products (37).

When seeds were exposed to 10% GP concentration, primary roots showed a brownish colour in both plant species, probably given by cell necrosis caused by toxic compounds in GP or polymerization of compounds (chromophores) in root exudates as a defence response. A similar symptom was reported in tomato roots as an allelochemical effect of *Sicyos deppei* (Cucurbitaceae) (33). Some phenols act as allelochemical compounds related to polar narcosis (structural and functional alteration of cell membrane), oxidation uncoupling, alterations in electrophilicity, hydrophobicity, and dissociation and union of H in biomolecules (30, 44). Since *V. labrusca* pomace is rich in soluble phenolic compounds, these substances could be related to the mentioned root symptoms.

In the plant experiments, the stimulant effect of GP on root systems at the lowest dose could be due to greater nutrient availability (9). In intensive crops, such as lettuce and tomato, the recommended dose of an organic fertilizer aimed at maintaining soil productive capacity is 40 t ha^{-1} (42). The results obtained in this study would limit GP utilization as an organic fertilizer, but applications at the lowest dose could be used as mulching in tomatoes and possibly in other crops with similar tolerance ranges.

Conclusions

V. labrusca GP presents physicochemical characteristics associated with soil health. Its high content of phosphorus and potassium, as well as low sodium and low SAR values differentiate this winery by-product from the one derived from *V. vinifera*. Low concentrations of *V. labrusca* GP promotes *A. brasiliensis* and tomato root system without altering aerial biomass, an effect known as hormesis. Toxicity symptoms of *V. labrusca* GP on plant growth at highest doses restricts its usage as an organic fertilizer. However, further ongoing field experiments will evaluate the effect of GP as mulching on tomato growth and other microorganisms indicating soil biological quality.

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