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Effect of manure application timing on roots, canopy and must quality in *Vitis vinifera* 'Merlot': a case study in Italy, North-East

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Summary

The maintenance and improvement of soil fertility are among the most important management practices in viticulture. The system efficiency fertilization (SEF) which is a new concept based on a maximum utilization of organic fertilizers (*i.e.*, manure) has become very important, especially within the organic viticulture sector, since other fertilizers are not allowed. The aim of this study was to determine the effect of different manure application timing on the root, shoot, and the grapevine yield, accumulation, and quality of biochemical compounds in the grape must since the timing effect was not previously investigated. The study was carried out on 'Merlot' variety organically cultivated, whose production aims at obtaining high-quality red wines. Three treatments were applied: NT (Non-Treated), T1 (Treated1- manure applied in late October) and T2 (Treated2 - manure applied in late February). After two study-years, the undertaken research has shown positive influences of soil manure application on the canopy features (T1), yield, and yield components (T2), along with a major accumulation of the primary metabolites (T2) (soluble solid, carbohydrates, chlorophyll). Yet, the secondary metabolites (polyphenols and anthocyanins) were promoted in the grape must at harvest time, especially when the manure was applied in late October (T2). Considering the benefits of manure application in the T2, after two study years, this timing is recommended in order to improve 'Merlot' grapes for high-quality red wine production.

Key words: grapevine; organic fertilization timing; radical system; shoot; grape must quality.

Introduction

In the whole agriculture sector, the maintenance and the performance of physiological functions rely on many factors, however, one of the most important factors is having the presence of a well-nourish cultivation substrate (*i.e.,* soil) (García-Orenes *et al*. 2016, Tomasi *et al.* 2021). Also, the nutrients available in the soil must necessarily be available for plants, especially in the rhizosphere. Thus, nutrients uptake by plants depend on the water flow as well as their presence in exchangeable form (Keller 2005). Plants require balanced amounts of macronutrients, such as calcium, magnesium, nitrogen, phosphorus, potassium and sulphur in high relative quantity (> 0.1 % of dry matter) and each of them is essential in order to complete the plant life cycle (Maathuis 2009, Silva *et al.* 2016, Mian *et al.* 2022). Hence, a balanced nutrition is essential for the vine growth, development, health and, therefore, to achieve high quality wine production (Sala *et al.* 2012, Tomasi *et al.* 2020; Salomé *et al.* 2016) especially in biodiversity hot spots such as in the Mediterranean. In Mediterranean areas, viticulture is an important land use. Vineyards are frequently found on inherently poor soils and are submitted to intensive management practices, which threaten soil functioning and associated ecosystem services. To encourage winegrowers and stakeholders to be reflective and adapt their vineyard practices, we evaluated the effects of three soil management practices (inter row plant cover duration, weeding and fertilization strategies. Soil fertilization is one of the most important viticultural techniques, consequently, hardly impact the yield and grape quality; however, unbalanced applications or excessive inputs can have negative effects on yield and quality (Lin *et al.* 2016). Traditional intensive agricultural practices have been negatively impacting soil quality, especially when organic fertilization is not applied (*i.e.* manure) (García-Orenes *et al.* 2016). Indeed, organic fertilization by using manure results in soil fertility improvements and quality indicators, also increasing the microbial diversity, and the crop performance (Wahab *et al.* 2018). Moreover, the manure applied in the soil is subjected to a number of physical and chemical modifications in order to release the macro and micro - nutrients, then becoming available for plant uptake (Gaiotti *et al.* 2017). This process is not immediate but takes a while, since it relies on many factors (e.g., microbial community effect on nutrient release, mineralization, etc.) (Lisek *et al.* 2016). The maintenance and improvement of soil fertility are important aims in viticulture, taking also into account that grapevines are usually cultivated in poor phytonutrient soils under organic management, and thus the system efficiency fertilization (SEF) based on an optimum utilization of manure has become of great importance as a managing tool. Generally, plants have a strong nutrient uptake in two growth stages: spring

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(*i.e.* after dormancy,) and before the winter dormancy (*i.e.* autumn), where the root system shows the maximum activity (Fregoni 2013). The objective of this work was to evaluate the effect of two bovine manure application timing on *Vitis vinifera* 'Merlot' organically cultivated, to understand how the manure application in different root activity stages can influence agronomic and enology (must) performances not previously investigated, also, to give a final general glance to winegrowers to how they can perform the fertilization.

Material and Methods

Plant material and experimental setup: The trial was conducted in the period 2019-2020 in Cormons (GO, 45°57′N 13°28′E), Friuli-Venezia Giulia region (Italy, NE), in an organic vineyard of *Vitis vinifera* cv. 'Merlot' grafted onto Kober 5BB rootstock. The vines were established in 2005 and trained to a free-cordon trellis set 1.50 m above ground with one catch wire. Vine spacing was 0.8 m × 2.3 m (intra and inter row) for a density of about 5,435 vines·ha^{-1}. The vine rows were approximately 90 m long and east-west oriented. Winter cane pruning was performed leaving the same bud number per each treatment and per vine (15). The canopy management in late spring and summer was limited to a light mechanical summer trimming of the vegetation at ± 40 cm above vineyard floor to optimize the machines operation and improve the grapes microclimate. Local standard practices were followed for pest and disease management in the organic sector, by applying only sulphur (S) and copper (Cu). The vines were irrigated by a drip subsoil irrigation system, according to the weather conditions to restore the field capacity. The treatments were applied in late October (T1- Treated1; BBCH: 95; 2019 and 2020) and in the last decenary of February (T2 - Treated2; BBCH: 01; 2020 and 2021) (BBCH: Biologische Bundesanstalt, Bundessortenamt and Chemical industry), in both tested years, with 1,500 kg·ha^{-1} of bovine manure, as generally applied in this vineyard according with reintegration of nutrient outputs (e.g., fruit, canes, etc – data not shown). No fertilization was performed in the NT (Non-Treated, check). Manure was incorporated in the soil by using a plough in the middle of the row, thus, not near the plants in order not to interfere with roots development at 20 cm below-ground. A randomized complete block design was adopted, where root, canopy, and grapes measures were carried out in three random blocks (each block consisting of 20 contiguous vine plants) along the internal row of each treatment (NT, T1 and T2). 'Merlot' variety was selected for this study, as it currently ranks in the top-ten among *Vitis vinifera* cultivars grown in Italy (Mian *et al.* 2021) the aetiological agent of grapevine downy mildew (DM. Finally, each measure was taken after two study years (in 2021) since we reputed that only after one growing season the manure application could not have induced any effect in the analysed parameters. Measures taken were the roots measurements, canopy features and grape must at harvest time (± 18-20 °Brix).

Pedological, climatic and manure characterization: The soil of the experimental vineyard and the manure applied were characterized at the beginning of the experiment. The samples were analysed by an external laboratory applying standard methods, as indicated by Italian Law (Decree n. 79/1992 and Decree n. 185/1999) and just adopted by Tomasi *et al.* (2020). Physical and chemical analysis of the samples confirmed the soil homogeneity within the selected plots for this experiment and the similarity between the two manures applied in both study years. Climatic data were taken from the ARPA meteorological station of Capriva del Friuli (GO, Italy), located near the experimental site.

Grapevine root studies: The profile wall method suggested by Boehm (1979) and recently used (Mian *et al.* 2022) seemed the most appropriate to determine root number. During the dormant period, three vines per treatment with similar scion diameter section were sampled within the measuring blocks. For each vine, a trench of approximately 1.20 m depth and 1.20 m with was dug in parallel to the vine row, at 0.50 m from the vine trunk. Fine roots were counted by using a 1.0 m high and 1.0 m wide grid system positioned against the profile wall considering the centre grapevine trunk as reference. Fine roots were chosen since they are the most metabolically active (Magalhães *et al.* 2011). Data are expressed as total vine roots·m^{-2}.

Furthermore, carbohydrates stored in the roots play a fundamental role in grapevines (winter survival and for plant activity in the following season) (Loescher *et al.* 1990). Thus, along with the root counting, root samples were collected, stored, and further analysed. The carbohydrate (*i.e.* starch, plus alcohol soluble sugars: glucose, sucrose, etc) concentration was determined in three replicates per organ and per treatment according to a colorimetric method (Loewus 1952) using anthrone reagent (Merck, Darmstadt, Germany). Absorbance readings at 620 nm were performed using a Shimadzu UV Mini-1240 spectrophotometer (Kyoto, Japan). Data are expressed in mg·g^{-1} dry weight (DW).

Leaf area, shoots number and chlorophyll content: The whole canopy development (typically mid-July and around veraision – BBCH: 79 - 83) was characterized. For each vine, the number of shoots and leaves were counted (n°). Leaves (all size) were collected and positioned on a 1.0 m × 1.0 m panel and photographed with a digital camera. Photos were processed with the Image program (National Institutes of Health, USA) and the following parameters were recorded: leaf size (cm^2) and total leaf area per vine (m^2). SPAD value was recorded by using a SPAD-502 - KONICA MINOLTA Europe, as this value is used to assessing the leaf chlorophyll content.

Yield, yield components and grape composition: Both treatments were harvested at technological maturity (18-20 °Brix), in different data. All the vines from each block per treatment were individually hand-picked. Yield·vine^{-1} (kg) and cluster·vine^{-1} (n°) were recorded, Cluster weight (g) was calculated as well. Fruit composition at harvest time (± 18-20 °Brix) was measured on a sample of 1.0 kg berries collected randomly from all vines of each block. Soluble Solids (SS; °Brix) were measured by using a refractometer (Atago PR32) at 20 °C, pH and titratable acidity (expressed as g·L^{-1} of tartaric acid - TA) were measured using an automatic titrator (Crison Micro TT 2022) by titration with 0.1N NaOH.

Anthocyanins and polyphenols in berry skins are of great importance for red wine sensory profile (Tomasi *et al.* 2021). Thus, following the method as described by De Rosso *et al.* (2016 such as Recioto, Amarone di Valpolicella and Raboso Passito). Changes of polyphenolic composition of the grapes as a consequence of withering were studied by ultra-high performance liquid chromatography-quadrupole time of flight mass spectrometry (UHPLC/QTOF), in 30-berry samples per treatment, the total polyphenols and anthocyanins were quantified in grape skin. Gas chromatography/mass spectrometry (GC/MS) analysis was performed using a 6850-gas chromatography system by Agilent Technologies (Santa Clara, CA, US), fitted with a fused silica HP-INNOWax polyethylene glycol capillary column (30 m × 0.25 mm, 0.25 µm inner diameter) (Agilent Technologies, Santa Clara, CA, U.S.A.), coupled with HP 5975C mass spectrometer and 7693A automatic liquid sampler injector (Agilent Technologies, Santa Clara, CA, U.S.A.) The values are expressed in $\mu g\ g^{-1}$ in the berry skin.

S t a t i s t i c a l   a n a l y s i s : One-way analysis of variance was performed using STATISTICA version 8 (StatSoft, Inc.). The determination of differences between treatments means was carried out using Tukey Test ($p \leq 0.05$, 0.01, 0.001%).

Results and Discussion

C h a r a c t e r i z a t i o n   o f   t h e   s t u d y   s i t e   a n d   m a n u r e   a p p l i e d : In Tab.1 the physical and chemical analyses for the study site are listed. Following the USDA (United States Department of Agriculture), the soil was physically classified as silt-loam. Chemically, the element content is on average for the soil in this study area. In Tab. 2 is possible to note the manure characterization used in both study-years. No great differences (chemical and physical) existed between the two manures applied in the two study-years, apart from the nitrogen (N) content, that was higher in 2020 with respect to 2019. Finally, Tab. 3 reports the meteorological data of growing seasons of both study years. As can be noted, both vintages showed fully comparable data for each measure taken into account (rainfalls, T°, humidity (%) and solar radiation (MJ m^{-2}).

T a b l e 1

Soil site characterization at the beginning of study

Parameter	Value
Sand (%)	3.4
Silt (%)	66.8
Clay (%)	29.8
Organic matter (%)	1.44
Total Nitrogen (N ‰)	7.61
pH (soil/water ratio = 1:2.5)	3.89
Total carbonates ($CaCO_3$ %)	15.63
Active carbonates ($CaCO_3$ %)	13.71
Available P_2O_5 (mg·kg^{-1})	178.6
Exchangeable K_2O (mg·kg^{-1})	115.36
Exchangeable MgO (mg·kg^{-1})	198.5
Exchangeable CaO (mg·kg^{-1})	201.52

T a b l e 2

Organic manure composition applied in both tested years (2019 and 2020)

Organic manure composition	2019	2020
Total nitrogen (N ‰)	7.2	5.5
P (P_2O_5) (%·g^{-1} DW)	0.44	0.37
K (K_2CO_3) (%·g^{-1} DW)	0.66	0.57
H_2O (v/v)	22	19
Organic Components (C) (%·g^{-1} DW)	16.8	15.6
C/N ratio	23	28
pH	6.9	7.2

R o o t   a n d   c a n o p y   s y s t e m s : In Tab. 4 the root system and canopy data are reported. Firstly, we investigated the number of fine roots per m^2. The treatments had no effect on total roots, however T1 trended towards having the highest number of roots, especially when compared to NT (100.6 and 74.6 roots m^{-2}, respectively). T2, which was responsible for 96 roots m^{-2}, showed a performance similar to T1. Considering the importance of the root system on nutrients and water uptake it would be of great interest having more study years to understand the manure effects on the radical system (i.e., root morphology, length, profile distribution, surface, volume, area). Secondly, regarding the number of leaves per vine (NL), both NT and T1 (1328 and 1342, respectively) showed a statistical lower NL in comparison to the T2 (1766). Thirdly, the leaf area (LA) was statistically higher in T2 (7.2 m^2), compared to NT (5.5 m^2), as expected by the highest LN. The results showed by T1 (6.2 m^2) did not differ from NT, being also similar to T2. Treatment T2 promoted higher NL and LA, that could lead to expected higher yield and quality. Nevertheless, as later on presented (Tab. 5), the T1 was the treatment responsible for higher yield and must quality. A plausible explanation for this can be related to the fact that higher NL associated to higher LA can alter the canopy layers (*i.e.* layers overlapping), yet an important issue for plant diseases (Mian *et al.* 2021). Moreover, a worse microclimate can also justify the results. Not only, higher NL and LA can increase the photosynthesis rate in the outer canopy, however, blocking the sun light reaching the inner canopy, thus reducing the whole plant system photosynthesis rate. This can result in reduction of carbohydrate accumulation and translocation from the leaves to the fruits, impacting grape yield and quality in T2 (as observed in this study) (Gaiotti *et al.* 2017, Martínez-Lüscher *et al.* 2021). Finally, this is also confirmed by what Botelho *et al.* (2021) observed.

Finally, the SPAD index was significantly affected by the treatments: T1 showed the highest value (54.3), followed by T2 (51.5), and NT (48.1). The SPAD index may be an important indicator of grape must composition. In fact, a number of studies related the SPAD value with N status in plants that can lead to a higher photosynthetic activity, thus to a major metabolites' accumulation in the reproductive (*i.e.* grapes) and storage organs (*i.e.* roots, trunk) (Esfahani *et al.* 2008, Jiang *et al.* 2017; Peng *et al.* 1995). In this sense, T1, responsible of the highest starch plus alcohol soluble sugar content, can well explain why SPAD index was taken

Table 3

Meteorological data of the study site during the growing season in 2019 and 2020 study-years

Year	Month	Rainfalls (mm)	Mean T°	Mean Humidity (%)	Radiation (MJ m^{-2})
2019	April	151.9	13.5	72.4	14.9
	May	236.5	14.6	79.3	16.1
	June	10.4	25.2	61.8	27.3
	July	95.0	24.7	64.6	25.1
	August	33.8	24.3	68.3	21.6
	September	103.6	19.2	73.0	15.8
		Total: 631.2	Mean: 20.3	Mean: 69.9	Total: 120.7
Year	Month	Rainfalls (mm)	Mean T°	Mean Humidity (%)	Radiation (MJ m^{-2})
2020	April	16.5	14.3	47.8	21.7
	May	67.6	17.8	60.8	21.9
	June	166.2	20.9	70.8	22.3
	July	66.3	23.8	62.3	25.4
	August	165.5	24.3	68.6	20.7
	September	186.4	20.1	68.5	16.7
		Total: 668.5	Mean: 20.2	Mean: 63.1	Total: 128.7

Table 4

Root and canopy characterization after two-study years in NT, T1 and T2. Within columns, values that are assigned by different letters are significantly different at $\alpha \leq 0.05$, 0.01, 0.001%, respectively. Statistical analysis was carried out using the Tukey Test. Data are the mean of three replicates

	Total vine roots m^{-2}	Leaves/vine (n°)	Leaf area (cm^2)	Shoots/vine (n°)	Leaf area/vine (m^2)	SPAD	Starch plus alcohol soluble sugars ($mg \cdot g^{-1}$ DW)
NT	74.6 a	1328 b	41.8 a	83 a	5.5 b	48.1 b	99.7 a
T1	100.6 a	1342 b	46.8 a	84 a	6.2 ab	54.3 a	173.0 a
T2	96 a	1766 a	41.1 a	83 a	7.2 a	51.5 ab	125.8 a

Table 5

Yield and Yield Composition after two-study years in NT, T1 and T2. Within columns, values that are assigned by different letters are significantly different at $\alpha \leq 0.05$, 0.01, 0.001%, respectively. Statistical analysis was carried out using the Tukey Test. Data are the mean of three replicates

	Yield/vine (kg)	Cluster/Vine (n°)	Cluster weight (g)
NT	1.8 b	9.0 a	200 a
T1	3.0 a	12.6 a	230 a
T2	2.4 ab	10.0 a	240 a

into account in this study. Finally, it also correlated with the highest °Brix found again in T1 (Tab. 6). The starch plus alcohol soluble sugar content in the roots wasn't statistically altered by the treatments. Although, T1 and T2 showed a trend towards increasing the averages observed (173.0 and 125.8 $mg \cdot g^{-1}$ dry weight, respectively). NT presented only 99.7 $mg \cdot g^{-1}$ DW. We can conclude by stating that the manure application in late February promoted canopy development, while in late October increased the chlorophyll content (SPAD index).

Yield and yield components study: In Tab. 5 the yield and yield components results are reported. T1 showed the highest yield production (3 $kg \cdot vine^{-1}$), this result was followed by T2 (2.4 kg) which showed an average statistically value differing from the yield presented in NT (1.8 kg). Additionally, T1 and T2 trended towards having a greater number of clusters per vine (12.6 and 10 $cluster \cdot vine^{-1}$, respectively), when compared to NT (9 $cluster \cdot vine^{-1}$), however, without statistical significance. As cluster weight, no statistical significance arose. Nevertheless, T2 tended to have the heaviest cluster weight with 240 g, whilst in T1 and NT were 230 and 200 g, respectively. The greater yield found in T1 may be explained considering the greater number of clusters per vine and cluster weight, compared to NT and T2 (even if the differences are only tendential). These conditions can concretely be translated into a greater yield/vine (Gaiotti *et al.* 2017). In fact, the difference in the yield was +1.2 kg in favour to T1 when compared to NT, hence statistically significant, as NT is the control with no manure application. When T1 is compared to T2, the difference in yield/vine is only significant (+ 0.6 kg). The same difference was found comparing NT and T2 (+ 0.6 kg). This led us to hypothesize that the little differences found in cluster weight, and cluster $vine^{-1}$ could have led to the higher yield in T1, with a less strong effect in T2, however, more marked for both treatments with respect to NT. As last consideration, as found out by Ruiz Diaz and Sawyer (2008), the manure application time is important

Table 6

Grape Must Composition at Harvest time after two study-years in NT, T1 and T2. Within columns, values that are assigned by different letters are significantly different at $\alpha \leq 0.05, 0.01, 0.001\%$, respectively. Statistical analysis was carried out using the Tukey Test. Data are the mean of three replicates

	Soluble solids (°Brix)	Titrable acidity (g tart. ac.·L^{-1})	pH	Total anthocyanins (µg·g^{-1} berry skin)	Total polyphenols (µg·g^{-1} berry skin)
NT	18.7 b	4.9 a	3.70 a	188.1 b	943.5 b
T1	20.9 a	3.7 b	3.71 a	229.1 a	1151.3 a
T2	19.3 b	4.4 ab	3.75 a	197.5 b	1080.5 a

also for the N availability with can influence the yield and yield related parameters.

Grape must composition at harvest time: Tab. 6 shows the grape must composition at harvest time. The SS showed a higher value in T1 (20.9 °Brix), while NT and T2 were statistically minor than T1, similar to each other (18.7 and 19.3 °Brix, respectively). The titrable acidity showed statistical differences as well: the least value was presented by T1 (3.7 g tart. ac. L^{-1}), and similar parameters were observed in NT and T2 (4.9 and 4.4 g tart. ac.·L^{-1}, respectively). Surprisingly, no differences arose for the pH (± 3.70). We might assume that in T1 the tartaric acid (*i.e.*, titrable acidity) was less conserved than the malic acid, which in turn might also explain the similarities found for the pH. As previously reported, the malic organic acid is more conserved and less subjected to precipitation, but strongly contributes to pH value (Tomasi *et al.* 2021). Furthermore, it is of great importance in 'Merlot' wines, because it guarantees a good-microbiological stability of wines when the malolactic fermentation is applied. Thus, it would have been of certain interest analysing the malic acid level.

Concerning the secondary metabolites, T1, once again showed the highest value of total anthocyanins (229.1 µg·g^{-1} berry skin), followed by T2 and NT, which showed no differences among their averages (197.54 and 188.1µg·g^{-1} berry skin, respectively). Regarding the total polyphenols, T1 and T2 recorded the highest and similar values (1151 and 1080.5 µg·g^{-1} berry skin, respectively) and NT was the least (943.5 µg·g^{-1} berry skin). The highest concentration of total anthocyanins and polyphenols in T1 and T2 (for anthocyanins, T2 without statistical support) could be explained by the higher content of SS especially in T1 since SS are the precursors of these metabolites. In fact, sugars and anthocyanins plus polyphenols follow a closely correlated parallel synthesis pathway and accumulation (Tomasi *et al.* 2021; Li *et al.* 2019).

Conclusion

The system efficiency fertilization (SEF) is an important new concept based on a maximum utilization of organic fertilizers, especially in the organic sector. In our study, concerning the parameters being analysed, we report that plants have promoted yield and grape must quality in T1, whilst a promotion of the canopy in T2 occurred. Yet, the control (NT) showed almost always less values for each type of data. In fact, the bovine manure applicated in late February (T2) induced the highest canopy development whilst the application in the last decenary of October (T1) promoted the yield and must quality. It might be assumed that the manure applied in late October released macro and micro – nutrients slower (Gaiotti *et al.* 2017), but the elements were available for plants for a longer period, thus increased the plant activity especially in terms of berry-must chemical composition, having led to a better physiological and photosynthetic activity in T2. Probably, by applying the manure in late February (T1), the N manure releasing is speeded up due to the further higher temperatures that faster mineralize the N, and this might have promoted only the canopy development (*i.e.* vegetative plant growth) (Gaiotti *et al.* 2017). Finally, the manure application in late October might be the best choice to increase both yield and must technological composition, of great importance in order to obtain high-quality organic 'Merlot' wines. Last but not least, an anticipated technological maturation, with improved quality, might also constitute an advantage in terms of grape health status, especially in climatically difficult environments.

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Conflicts of Interest

The authors declare to not have conflict of interest.

Author Contributions

All authors have contributed equally. All authors have read and agreed to the published version of the manuscript.

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