

Soluble Biobased Substances in soil or Salicylic Acid on leaves affect the foliar pH and soil biovariability of Grapes-as explained by the NIR Spectroscopy of Litterbags and Teabags

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Short title: Treatments in vineyards modify soil bioactivity and Biovariability

Running title: Acidic treatments in vineyard

Abstract

The purpose of the trial was to check the effects of two grapevine treatments on the plant activity and on the bioactivity and biovariability of the soil. An alkaline complex of Soluble Biobased Substances (SBS) was used in soil at 30 g per plant in a single solution. Salicylic Acid (SA) was used on leaves at 150 mg l⁻¹ every two weeks at 50 ml plant. The plants were examined for their foliar pH and NIR spectra. The soil bioactivity was monitored by means of hay-Litterbag-NIRS (LBN) in combination with the Teabag Index (TBI), using rooibos and green tea that had been buried for 60 days. The evolution of the TBI presented here concerns the TBI-NIRS spectroscopic method used for discriminant analysis. A new algorithm was used to estimate the soil microbiome from the green Teabag spectra. The obtained results showed that the plants and the soil responded to the treatments. In fact, SBS, but not SA, lowered the leaf pH by 5%, an unexpected and original result. Both treatments increased the variability of the leaf composition, with a lower discrimination, based on the NIR spectra, from the Control (75%) to 44% (SA) and 38% (SBS). The TBI method, which is based on weights, was less efficient (67%) than the TBI-NIRS of rooibos (96%) or the LBN of hay (80%), but it was like the TBI-NIRS of green tea (74%). The LBN analyses indicated that the mycorrhizal index had increased by 8% in SA but had reduced by 7% in SBS, while both treatments reduced the activity of the microbes, which did not affect the soil respiration rate. The mineral N in the soil was substantially raised by about 11÷69%. The Taxa profiles showed marked deviations from the

Control. Moreover, the SBS treatment reduced the *Glomeromycota* by 35%, which matched to the reduction in the mycorrhizal index. The most favored Bacteria from the treatments were *Proteobacteria* and *Actinobacteria*, with *Mortierellomycota* being the most penalized. Neither treatment affected the production, but both delayed the technological maturity by 9-11%, while the SBS retarded the phenolic maturity by about 18%. It has been concluded that a simple treatment of vines can affect the bioactivity in the leaves and berries as well as the biovariability of the soil.

Introduction

Healthy soils form the basis of any sustainable viticulture, and a central task is that of making the living medium that contains micro and macro-organisms, which perform many ecological functions and provide ecosystem services, more efficient¹. The resistance of cultivated plants to climate change can be summarized as the fight against thermal, water and saline stresses. The vine can be considered resilient to irrigation, so much so that it can be considered the norm. Such management practices as green pruning, colored nets, anti-hail nets and grassing are more and more frequently used to counteract thermal and solar excesses. The administration of humic acid and fulvic acids (HA) on the ground or even on the leaves can be included in the virtuous practices that can be evaluated. According to Mohamadineia et al.², the use of HA as a soil method is better than as a spray method in vineyards. However, as far as biostimulants are concerned, the foliar route is the preferred one. Popescu and Popescu³ confirmed that foliar application with HA can improve the growth, yield, and berry quality attributes of grapevines, while according to Irani et al.⁴, biostimulants have a high potential for promoting fruit yield and the quality of grapevines in drought-prone regions. However, the burial of HA in the soil has shown other positive effects, for example, a nematocidal activity⁵⁻⁶, while other authors fear negative consequences concerning the microbial biodiversity of the soil⁷⁻⁸, as well as concerning the stability of the enzymatic system of the soil⁹, although positive aspects have been highlighted for a potassium humate application and for soil properties, microbial activity, and Jerusalem artichoke yield components¹⁰, and have been confirmed by Kumar et al.¹¹. The combination of HA and biostimulants has been experimented by various authors. Eman et al.¹² minimized the use of nitrogenous fertilizers on grapevines by using HA and yeasts. Martinez et al.¹³ showed that the joint application of compost, mineral fertilizer, and microbial inoculant can improve root development in table grapes (*Vitis vinifera* L.) and soil quality in integrated nutrient management programs.

Ibrhaim et al.¹⁴, through the foliar application of HA and salicylic acid (SA), showed that SA was more effective than HA and recommended the use of SA as a foliar spray to enhance the productivity and nutritional quality of red sweet peppers. SA is a natural growth regulator of vascular plants that influences several physiological and metabolic processes. The foliar application of SA may increase the yield of vegetable species by reducing stress-induced growth inhibition¹⁵. In general, HA and SA positively affect the growth and development of plants by acting as an organic-mineral fertilizer and a phytohormone, respectively. However, the responses of vegetable species and cultivars can differ significantly. In grapevines according to El-kenawy¹⁶, the best results were obtained with SA (500 ppm) + fulvic acid (500 ppm) without the addition of chitosan. In salt-stressed grapes the optima growth and yield were realized for a SA 1 mM dose¹⁷. SA treatments of 6 and 9 mM have been found to be more effective in reducing salt stress over the plants of 41 B and 1103 P American grapevine rootstocks¹⁸. In heat stress, an SA pretreatment maintained a higher Rubisco activation state¹⁹. Moreover, exogenous SA can promote the growth and Se uptake of grapevines under Se stress, with 250 ppm having been shown to be the most effective concentration²⁰. Using SA at 400 ppm three times was highly effective in palm²¹ and in *Citrus sinensis*²². SBS-Humic and/or SA foliar treatments can affect fruit

quality. Patel et al.²³ showed that among the considered treatments SBS @ 1.5 % was found to be more effective on Mango fruit than SA @ 1000-1550 and 2000 ppm, and significantly affected the maximum shelf life, TSS, total sugar, reducing sugar, non-reducing sugar and ascorbic acid, whereas the acidity, physiological loss in weight and spoilage were minimum.

Experimental procedure

As highlighted in the introduction, the studies on treatments with HA on the soil are not numerous and there are even fewer that concern soil biodiversity, while none are available about leaf treatments with SA. In this experiment, we have studied an alternative to HA, namely a non-commercial alkaline product derived from municipal biowastes, which is extracted from non-renewable sources. The alternative simple and non-joint effects of a soil treatment with SBS and an SA leaf treatment have been compared in this completely randomized experiment. The phenotypic evaluation on the plants took into consideration the leaf composition, as disclosed by the NIR spectra, and especially the leaf pH, the grape production, and the quality of the grapes pertaining to their technological and phenolic maturity. Indirect methods, based on the detection of the bioactivity of hay or of red and green Tea litters, coupled with NIR spectroscopy, were applied to evaluate the soil microorganisms. An attempt was also made to predict the bacterial and fungal biota from the NIR spectra of green Tea to evaluate the soil biodiversity.

Material and Methods

Description and management

The trial was conducted in the western Po valley (45.08180636477463, 7.958740094755527) on an adult vineyard of Malvasia red wine, located on a steep South oriented slope. A conventional management of the vineyard, following an integrated pest management protocol, is adopted, and it had not been fertilized in the three years before the research. The vines are pruned using the mono lateral guyot system with espalier training and at a density of 3.460 plants per hectare. During the vegetative period, the soil

Table 1. Soil composition.

Component	Content
Sand (%)	29.25
Silt (%)	50.65
Clay (%)	19.6
pH soil	7.9
Ca carbonate total (%)	6.4
CaCO ₃ active %	2.6
Organic matter %	1.355
N Total %	0.085
C/N ratio	9.3
Cation_exchange_capacity (meq 100 g ⁻¹)	17.8
Ca exchangeable (meq 100 g ⁻¹)	19.14
Mg exchangeable (meq 100 g ⁻¹)	1.65
K exchangeable (meq 100 g ⁻¹)	0.21
Mg to K ratio	7.9
P assimilable (ppm)	5
Fe assimilable DTPA (ppm)	29
Fe assimilable ETPA (ppm)	0
Mn assimilable ETPA (ppm)	10
Cu assimilable (ppm)	3

Table 2. Elemental analysis of the Soluble Biobased Substances (SBS).

Constituent	% (res. 60°C) ⁻¹	g 30 g(fluid) ⁻¹
Dry matter	86.58	3.90
Solid volatile	36.82	1.66
N	2.30	0.10
C	27.88	1.25
H	1.14	0.05
S	0.48	0.02
C/N ratio	12.1	
pH 8.40		Dry matter 7.27%

management involves three grass mulchings between the rows and three mechanical processings under the rows. The topsoil composition is reported in Tab. 1.

Treatments

An experimental alkaline (pH 8.40) complex of Soluble Biobased Substances (SBS) was used for the soil treatment to provide some humic substances, derived from biogas residues (Tab. 2). A total of 30 ml of the complex, diluted in 750 ml of water, was distributed over each six plants in a single solution on July 6, 2022.

The humic acid extraction process was performed using compost obtained from the anaerobic digestion of municipal green biowastes. The process²⁴ involves hydrolyzing the compost in an k-alkaline solution and producing value added (SBS). The suspended solids were separated and the liquid solution, containing around 7% of dry matter, was used for the above-mentioned treatments. A typical solution of SBS has 4% of humic extract: humic acids + fulvic acids in a 3:2 ratio (which means 2.4% w/w humic and 1.6% w/w fulvic acids).

The other treatment considered in the experiment was based on Salicylic Acid (SA), a slightly soluble substance (2000 ppm) that was used at a concentration of 150 ppm. The solution reached pH 7.12 (-4% vs. water at pH 7.44) and was sprayed onto the leaves of six plants every two weeks at a dose of 50 ml plant.

Foliar measurements and NIRS analyses.

Two leaves per plant were sampled seven times from July 6 till September 6. The leaves were analyzed for the petiole pH using a Hamilton Peek Double-Pore F, / Knick combined 35 x 6 (LxØ) glass-plastic electrode, at two decimals. The NIR spectroscopy of the leaf blade was conducted in single scan mode using an NIR-SCiO™ smart device, as described in Masoero et al.²⁵. The foliar pH data were analyzed, by means of the XLSTAT 2019.4.1²⁶ software, according to a bifactorial ANOVA, with three treatments and seven times replicates. The chemical components of the leaves were predicted, by means of NIRS, through the application of previously published PLS models²⁷⁻²⁸, calculated using WinISI-III (FOSS NIRSystem/Tecator, Infrasoft International, LLC) software.

Quality measurements

A total of 30 berries were collected randomly from each plant at two different times near the harvest and subsequently taken to the laboratory. Three subsets of 10 berries were composed and weighed, then the

pulp, skins and seeds were divided and each berry was examined for pH using the Hamilton Double-Pore F electrode and by a bench refractometer for the degree brix, while a triplicate NIRS scan was applied to the pooled skins and seeds. The technological index was calculated as $\text{pH}^2 \times \text{brix}$, whereas the phenolic index was expressed as the ratio of the non-extractable to the extractable phenols and was predicted from the NIR spectra according Cugnetto et al.²⁹.

Set up, properties and spectra elaboration of the Litterbags and Teabags.

The litterbag-NIRS method was developed by Masoero et al.³⁰ and has been applied in numerous trials with biofertilizers³¹⁻³⁴. It consists of examining the NIR spectrum of ground alpine hay placed in a package of permeable sachets that have remained buried for 60 days. The chemometric elaboration of the spectra allows a discrimination of the various groups under study to be carried out. The method is that of the Partial Least Squares Discriminant (PLSD), which consists of first preparing the N*G incidence matrix, composed of N observations and G groups, in whose cells a value of 1 is assigned if an observation is present and 0 if an observation is absent. The PLS-Regression method is then used, and G equations fitted to the values 1 and 0 are calculated. The predictions obtained from the equations generate a matrix of values whose G columns are submitted to a linear discriminant analysis to ascertain the percentage of correct classifications (%Correct), which is the diagonal of the G² confusion matrix. In our case, we used the WinISI-III software (Infrasoft) to obtain the PLS equations from the NIR spectra and the XLSTAT software²⁶ to perform the discriminant analysis with cross-validation of the predictions.

Based on previous equations³⁵, the NIR spectra allowed us to predict 23 Litterbag-NIRS variables. These predictions were the final bromatological composition parameters of the hay residuals in the litter in relation to a feeding value for ruminants. Furthermore, some soil properties previously related to spectra ($\text{NO}_3^- \text{N}$, $\text{NH}_4^+ \text{N}$, Soil Induced Respiration) and the mycorrhizal index were estimated. Finally, in agreement with the proposal of Fontaine et al.³⁶ and Blagodatskaya et al.³⁷, it was possible to obtain an indirect evaluation of the microbial activity by comparing the initial composition of the hay and the final one of the litters. This scheme considers two conventional types of microbes: the so-called “r-” (r-labile-strategist) microbe, which refers to a negative allometric variation of substances that decrease during the decay, as opposed to a category of slow “k+” (k+ resistant-strategist) microbes, which refer to a positive allometry of the substances that increase in percentage in the litter. In the works of Masoero et al.³³ and Hett et al.³⁴, it was highlighted that the components that formed last in the herbs (acid detergent fiber, acid detergent lignin, crude fiber, cellulose) are the first to degrade, while the substances that formed first (ash, hemicellulose, crude protein) are of type “k”, whose relative presence in litters increase during the decay process.

The Teabag Index (TBI) method of Keuskamp et al.³⁸ involves burying two bags of Lipton green tea (EAN 87 22700 05552 5) and rooibos tea (EAN 87 22700 18843 8) for 90 days. At the end of the test, the two sachets are removed and weighed. In the TBI, two parameters are determined by means of two formulas: the stabilization factor TBI_S and the decomposition rate TBI_K. These parameters were elaborated as univariates by means of the ANCOVA model. In this work, for the first time, we have improved the TBI method by applying NIR spectroscopy to green and red tea residues. The spectra were elaborated by means of a PLS-D type procedure, exactly as was done for the hay Litterbags. At present, we cannot consider the allometry of the decomposition of the two types of tea with respect to the original ones, because there is a lack of chemical data to calibrate the procedure. Instead, starting from the NIR spectra of green tea, which were taken from the study of Staffolani et al.³⁹ in vineyards, it was possible to

obtain a set that was highly correlated with the bacterial and fungal biomass. In that study, which was carried out on nine clusters of Nebbiolo vines, the triplicate samples of soil, kept in a refrigerated environment between 1 and 6°C, reached the Bioma Makers company laboratory within 72 hours of sampling. The analysis performed by the laboratory was based on the sequencing and characterization of the 16SrRNA gene and ITS markers of bacteria, fungi and Archaea using Illumina MiSeq technology. The results reported the presence of taxa expressed as a percentage of the total identified taxa. In the present work, the PLS equations, recalculated after the cited study (unpublished), resulted an R^2 coefficient of 0.68 ± 0.13 for both *Bacteria* and *Fungi*, and these models were applied to the green tea spectra of the present study.

Results

Foliar pH and NIR spectra.

The SBS lowered the leaf pH by 5% (Tab. 3).

This did not happen at the beginning of the test but after 10 days, and it continued until the end (Fig. 1). It is worth noting that the pH trend during the summer reached its maximum around August 12, and then decreased .

Based on the analyses of the NIR spectra (Tab. 3), it was observed that the treatments had a strong effect on the leaves, as demonstrated by the 1-VR coefficient, which reached values of 0.47 in the comparison Control vs. Salicylic and of 0.66 in the comparison Control vs. SBS-Humic. The treatments affected the confusion matrix and the correct classifications. In fact, when the Control was set at a 75% level, the classification dropped to 44% for Salicylic and 38% for SBS-Humic, although the latter was not significant ($P = 0.34$).

This result originated from modifications in the components of the leaf blade (Tab. 4), with several decreases in hemicellulose (-17% and -15% for SA and SBS, respectively), crude protein (-5% and -1%) and dry matter (-1% and -2%). An increase was instead observed for the cellulose, ADF and not digestible NDF, so that the overall net effect was a rise in the Crop Maturity Index (+3% and +1%).

Litterbags and Teabags.

The average classification rate (Tab. 5) was 80% from the NIRS of the Litterbags, 96% from the NIRS of

Table 3. Effects of the treatments on the leaf pH and % Classification based on the NIR spectra of the leaves.

Treatment	No.	pH		NIRS 1-VR	% Class	P (%Class)
Control	89	3.75	a		75%	a 0.0001
SA-Salicylic	105	3.72	a	0.47	44%	b 0.0165
SBS-Humic	79	3.58	b	0.66	38%	b 0.3446
Total	273				52%	0.0001
ln (SA/C)%		-1%			-54%	
ln (SBS/C)%		-5%			-68%	

%Class = %Classification; 1-VR \equiv R-squares in cross-validation; a>b $P < 0.05$.

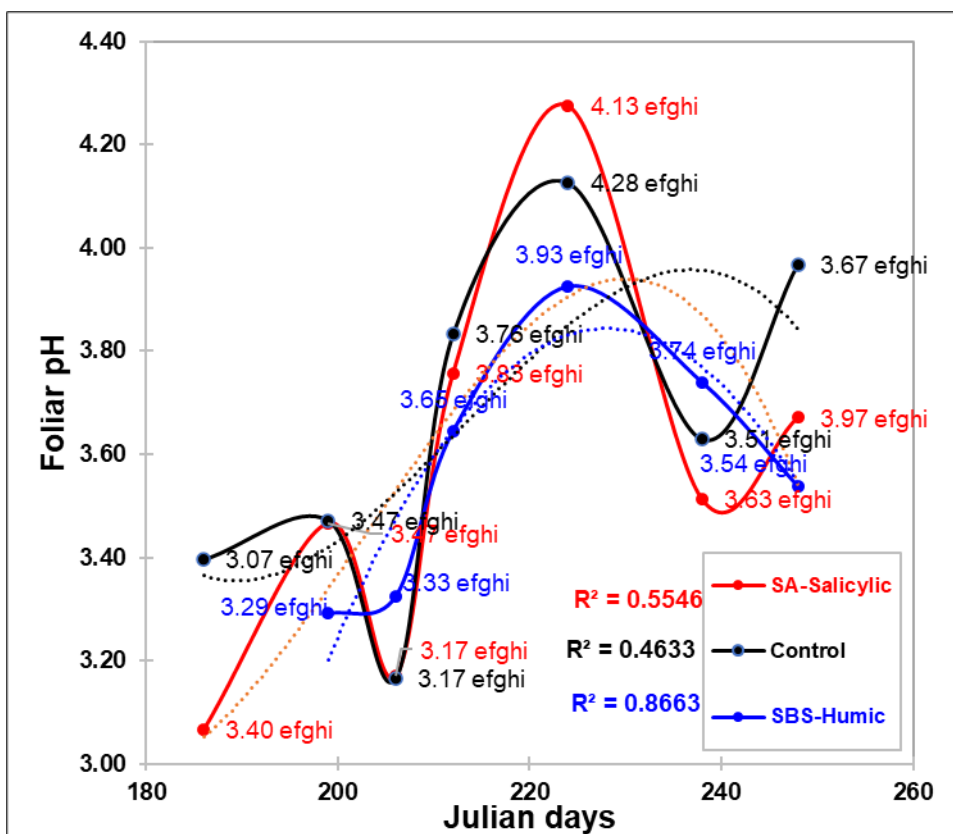


Figure 1. Trend of the leaf pH at seven moments of the trial in the three treatments. (a>b...>I, P<0.05)

Table 4. Effects of the treatments on the leaf composition.

Leaf component	Mean Control	ln (SA/C)		ln (SBS/C)	
Hemicellulose (%DM)	6.29	-17%	*	-15%	*
Crude Protein (%DM)	7.36	-5%	*	-1%	
Dry Matter (%)	32.03	-1%		-2%	*
NDF (%DM)	50.41	0%		-2%	*
Total Digestibility (%)	69.56	-1%	*	0%	
Cellulose (%DM)	35.74	2%	*	-1%	*
ADF (%DM)	44.12	2%	*	0%	
NDF not digestible (%DM)	30.83	2%	*	1%	
Crop Maturity Index	2.68	3%	*	1%	

* P<0.05.

the rooibos and 74% from the NIRS of the green tea. The calculations based on the weight loss were explanatory of the experiment, with an average classification of 67% (statistically significant different from the random value of 33%), but inferior to the level obtained from the NIR spectra. Therefore, it could be convenient to use rooibos as a marker of soil bioactivity as it can enhance the resolution power of the experiment.

The two parameters of the TBI showed different features in relation to the acidic treatments. In fact, the regression of TBI_K on TBI_S, which was negative in the Control, became positive in SA and strongly positive in SBS (Fig. 2), while at same time, TBI_S increased by 80% in SA, and the TBI_K factor increased by 116% in the SBS treatment (Tab.6).

The Litterbag-NIRS appeared preferable as it allowed the mechanics of the results to be interpreted on the basis of well-established equations. The results of the litterbag decomposition (Tab. 7) have been very instructive. The microbial k⁺ resistant-strategists decreased to a great extent (-13 and -28% in SA and SBS, respectively), whereas the r-labile-strategists decreased less (-8 and -4%) and their r-k difference therefore rose, especially in SBS (+19 and +115%). Another sign of a lower microbial activity in

Table 5. Discrimination analysis: effects of the Treatments on the % Classification (%Class) as obtained from the NIR Spectra of Litterbags and NIR spectra of Teabags.

Treatment	No. of Tea-bags	Weight of two Tea-bags (TBI_S, TBI_K)	Litterbags		Rooibos		Green tea	
			%Class	NS	% Class	NS	%Class	NS
C-Control	4	60%	36	72%	18	89%	18	67%
SA-Salicylic	3	80%	36	86%	21	100%	22	82%
SBS-Humic	5	60%	36	83%	18	100%	18	72%
Total-Average	12	67% c	108	80% b	57	96% a	58	74% bc
ln (SA/C)		28%		18%		12%		20%
ln (SBS/C)		0%		14%		12%		8%

NS = No. of NIR Spectra; a>b>c P 0.05.

Table 6. ANCOVA of the TBI_S (Stabilization) and TBI_K (Decomposition) parameters from the Teabag Index (TBI).

Treatment	TBI_S Stabilization		TBI_K Decomposition		b (S/K)	
C-Control	11.2%	b	0.59%	b	-0.1163	c
S-Salicylic	25.0%	a	1.17%	b	0.0212	b
SBS-Humic	12.7%	b	1.88%	a	0.0927	a
Average	16.3%		1.2%			
Ln(SA/C)	80%		68%			
Ln(SBS/C)	12%		116%			

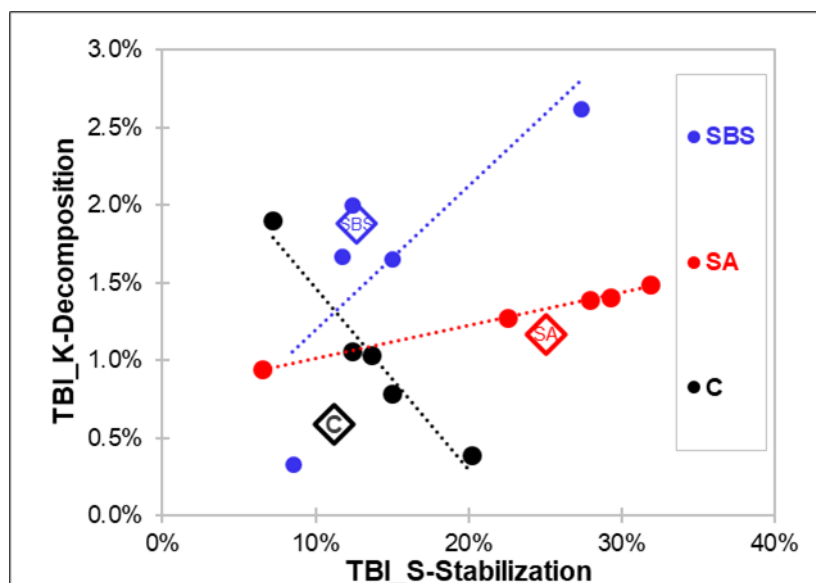


Figure 2. Regression of the TBI_K (Decomposition) on the TBI_S (Stabilization) parameters from the Teabag-Index in the three treatments (SBS-Humic, SA-Salicylic, C-Control).

the SBS-Humic treatment was the crop maturity index, which decreased by 11%. However, the soil respiration capacity increased (SIR +9 and +5%), albeit not significantly. The most penalized variables of the litter were the type "k+ resistant" ones (protein, ash, hemicellulose), which are linked to the k-strategists, while the "r-labile" variables, which represent the main components of the wall (crude fiber, ADL, NDF not-digestible, ADF, cellulose), were tendentially increased by the treatments. Interestingly, the Mycorrhizal index increased in S (+8%) but clearly decreased in SBS (-7%). A further result was a strong increase in the predicted N content of the soil, that is, of about 17% in S and 46% in SBS, with a prevalence in the NH_4 form.

The high-degree relationships between the NIR spectra and TBI parameters were assessed through a calibration-validation process (Tab. 8). In fact, it was possible to predict the two TBI-parameters from all the NIR spectra, but especially from the rooibos.

The abundance of *Bacteria* and *Fungi* predicted from the NIR spectra of the green tea (Tab. 9) was not different, but the *Taxa* profiles showed marked deviations from the Control. In particular, the SBS treatment altered the *Fungi* composition and showed a drop of 35% in *Glomeromycota*, which was correlated

with the reduction in the mycorrhizal index. Among the *Bacteria*, the most favored were *Proteobacteria* and *Actinobacteria*, while *Mortierellomycota* appeared to be the most penalized.

As far as the mineral soil N contents (NO_3^- -N as well as NH_4^+ -N) regressed on the *Taxa* are concerned, the results (not shown in the table) enhanced the negative coefficients more than the positive ones for *Fungi*, with a minimum for *Ascomycota* (-0.045). Similarly, the minima negative taxa from *Bacteria* were *Planctomycetota* (-0.06) and *Chloroflexi* (-0.04), as opposed to the most positive one (*Mucoromycota*, +0.05).

Table 7. Effects of the treatments on the Litterbag-NIRS variables.

Litterbag-NIRS variables	Type	Hay	Control	SA-Salicylic	SBS-Humic	P	Ln(SA/C)	Ln(SBS/C)
Microbes k+ "resistant-			7.45 a	6.48 b	5.38 c	<0.0001	-13%	-28%
Protein (%DM)	k+	7.88	12.72 a	12.23 a	10.59 b	<0.0001	-4%	-17%
Ash (%DM)	k+	6.77	19.54 a	18.23 b	17.02 c	<0.0001	-7%	-13%
Hemicellulose (%DM)	k+	9.48	16.77 a	15.02 b	15.75 b	0.001	-10%	-6%
NDF digestible (%DM)	r-	28.3	23.91 a	22.96 b	21.49 c	<0.0001	-4%	-10%
NDF Digestibility (%)	k+	46.7	53.22 a	51.22 b	47.87 c	<0.0001	-4%	-10%
Microbes r- "labile-			8.96 a	8.27 b	8.62 ab	0.001	-8%	-4%
Crop maturity Index			1.02 a	1.05 a	0.90 b	<0.0001	3%	-11%
Total Digestibility (%)	k+	61.8	78.99 a	78.13 b	76.40 c	<0.0001	-1%	-3%
N-free extracts (%DM)	r-	46.5	49.39 a	48.95 b	48.61 c	<0.0001	-1%	-2%
NDF (%DM)	r-	60	44.91 a	44.82 a	44.89 a	0.193	0%	0%
Mycorrhizal index			1.10 ab	1.20 a	1.02 b	<0.0001	8%	-7%
Energy (%DM)	r-	16.95	15.69 c	15.78 b	15.91 a	<0.0001	1%	1%
Lipids (%DM)	r-	2.87	2.85 b	2.88 a	2.89 a	0	1%	2%
Cellulose (%DM)	r-	38.35	22.05 b	22.95 a	22.20 b	0.024	4%	1%
ADF (%DM)	r-	21.65	28.12 b	29.80 a	29.136 b	0.001	6%	4%
Substrate Induced Respiration ($\mu\text{g Cmic g}^{-1}\text{ FW}$)			119.3 a	129.85 a	125.34 a	0.129	9%	5%
NDF Non digestible (%DM)	r-	32.3	21.00 c	21.86 b	23.39 a	<0.0001	4%	11%
ADL-Lignin (%DM)	r-	12.17	6.07 b	6.85 a	6.93 a	<0.0001	13%	14%
NO_3^- -N in soil ($\text{mg kg}^{-1}\text{ DM}$)			72.52 c	80.55 b	88.95 a	<0.0001	11%	23%
Crude fiber (%DM)	r-	12.98	8.67 b	11.49 a	9.48 b	0	33%	9%
NH_4^+ -N in soil ($\text{mg kg}^{-1}\text{ DM}$)			3.15 b	3.85 b	5.32 a	<0.0001	22%	69%
Difference r-k (%)			1.50 b	1.79 b	3.23 a	<0.0001	19%	115%

a>b>c; P<0.05.

Table 8. Predictability of the TBI_S and TBI_K parameters from the NIR spectra of the two types of Tea and the Hay.

NIR spectra	Teabag Index (TBI) Parameters	N	Mean	SD	RSQ	SECV	1-VR
Green Tea	S-Stabilization	47	16.1%	6.2%	0.64	4.2%	0.53
Green Tea	K-Decomposition	47	1.1%	0.5%	0.53	0.3%	0.47
Rooibos Tea	S-Stabilization	41	18.5%	6.4%	0.79	3.6%	0.69
Rooibos Tea	K-Decomposition	39	1.3%	0.4%	0.75	0.2%	0.72
Hay	S-Stabilization	92	15.5%	5.9%	0.69	3.7%	0.60
Hay	K-Decomposition	88	1.2%	0.5%	0.76	0.3%	0.70

RSQ = r-square in calibration mode; SECV = standard error in cross-validation mode; 1-VR = r-squares in cross-validation mode

Table 9. Effects of the SA-Salicylic / Control and SBS-Humic / Control treatments on the abundance of the *Taxa* in *Phyla Bacteria* and *Fungi*

<i>Taxa / Phyla</i>	Abundance of the Control	ln (SA/C)%	P	ln (SBS/C)%	P
<i>Bacteria</i>					
<i>Proteobacteria</i>	96.9	3.2	*	3.2	*
<i>Actinobacteriota</i>	87.9	4.1	*	3.9	*
<i>Firmicutes</i>	67.8	2.8		1.3	
<i>Bacteroidota</i>	25.7	4.2	*	3.9	*
<i>Planctomycetota</i>	8.3	-6.4	*	-7.0	*
<i>Chloroflexi</i>	12.0	-5.1	*	-4.3	*
<i>Verrucomicrobiota</i>	11.0	9.8	*	5.7	*
<i>Myxococcota</i>	11.1	-0.7		-0.3	
<i>Acidobacteriota</i>	6.0	-5.4		-0.1	
<i>Bdellovibrionota</i>	2.2	13.0	*	13.1	*
<i>Cyanobacteria</i>	0.7	2.4		13.3	*
<i>Planctomycetes</i>	1.8	17.9	*	11.0	*
<i>Spirochaetota</i>	0.3	68.7	*	64.2	*
<i>Chlamydiae</i>	0.1	19.2		-3.5	
<i>Fungi</i>					
<i>Ascomycota</i>	260.6	3.9		-6.3	*
<i>Basidiomycota</i>	82.0	4.6		-4.0	
<i>Mortierellomycota</i>	14.0	-8.3	*	-5.5	*
<i>Glomeromycota</i>	6.0	10.0	*	-35.0	*
<i>Mucoromycota</i>	3.9	6.0		4.0	

* P<0.05.

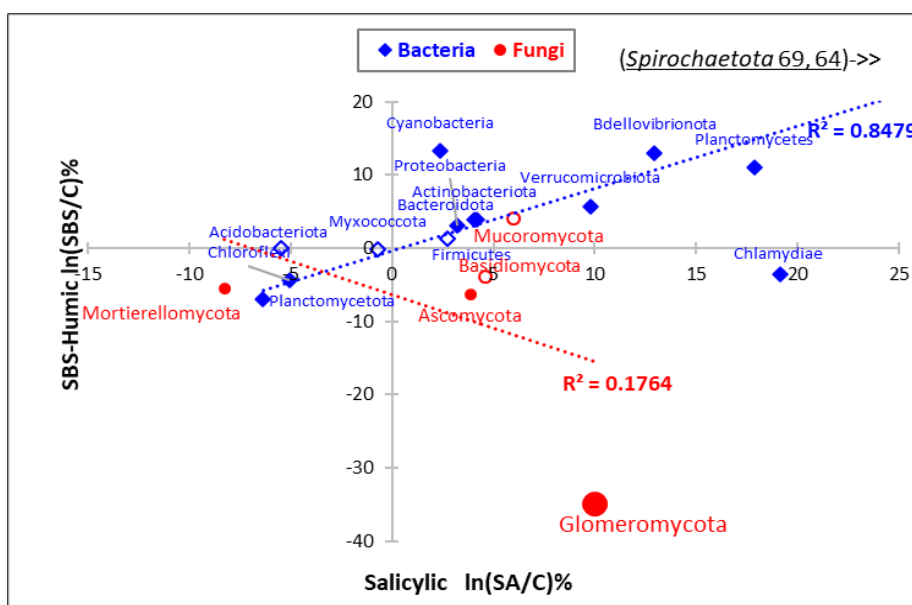


Figure 3. Regression of the Salicylic effect (SA, X axis) on the alkaline SBS-Humic effect (SBS, Y axis) for the *Taxa of Phyla Bacteria* (blue) and *Taxa of Phyla Fungi* (red).

Table 10. Effects of the treatments on production

Treatment	No. of bounces per plant	Cluster weight (Kg plant)	Bounce weight (g)
C-Control	27.5	5.2	186
SA-Salicylic	36.3	5.4	150
SBS-Humic	28.5	5.0	184
ln(SA/C)	28%	4%	-22%
ln(SBS/C)	4%	-4%	-1%

Production.

The treatments did not modify the productions (Tab. 10).

Quality.

The berries sampled from the treated groups (Table 11) resulted to be smaller than the Control (SA -9% and SBS -4%). In addition to the reduction in the mass of the berries, it has been observed that the treatments delayed the technological maturity by around 10%. This was due to a state of greater acidity, with a -4% reduction in pH for both treatments, albeit for the same amount of sugar. As far as the NIR spectra of the skin is concerned, a higher variability was observed in the SBS group, which was classified at 35%, a non-significant value, and was significantly lower than the other more homogeneous Control (67%) and SA (61%) groups.

The seeds of the three groups were highly and equally characterized by the NIR spectra (about 87%) (Tab. 12). As for the components of the seeds with similar total phenols, the non-extractables were 6%

Table 11. Effects of the treatments on the technological maturity (TM) and on the Classification from the NIRS of skins.

Treatment	No.	W.10 berries		Brix°		pH		Index TM		% Class. NIRS Skin	
C-Control	18	21.0	a	24.21	a	3.20	a	248	a	67%	a
SA-Salicylic	20	19.2	b	23.65	a	3.09	b	226	b	61%	a
SBS-Humic	18	20.1	ab	23.59	a	3.07	b	223	b	35%	b
P	56	0.018		0.083		0.000		0.001		0.000	
ln (SA/C)		-9%		-2%		-4%		-9%		-8%	
ln (SBS/C)		-4%		-3%		-4%		-11%		-63%	

a>b P<0.05.

Table 12. Effects of the treatments on the quality and phenolic maturity (PM) of the grape seeds.

Treatment	No.	NIRS % Class	Phenolic										
			Total		Extractable		Non extractable		Phenolic Maturity		Proanthocyanidines		
C-Control	60	88%	1.51	a	0.35	a	b	1.04	ab	2.29	a	0.47	b
SA-Salicylic	60	85%	1.60	a	0.39	a		1.12	a	2.32	a	0.48	b
SBS-Humic	60	87%	1.49	a	0.32	b		0.98	b	1.91	b	0.51	a
P	180		0.084		0.002			0.027		0.000		0.000	
ln (SA/C)		-4%	6%		11%			7%		1%		3%	
ln (SBS/C)		-2%	-1%		-7%			-6%		-18%		9%	

a>b; P<0.05.

lower for SBS. This corresponds to a lower phenolic maturity of around 18%, with a prevalence of proanthocyanidines (+9%).

Therefore, the treatment with humic acids generated a maturation delay of 18%.

Discussion

Soil traits.

How is it possible to imagine that a minimal treatment with acids could affect the bioactivity of the soil so much? All soil investigation systems show differences between treatments. In our case, the couple of TBI parameters derived from weighing the residues indicated that the TBI_S (Stabilization) factor, which depended on the green tea degradation, increased by 80% in SA, while the TBI_K (Decomposition) factor, linked to the rooibos degradation, increased by 116% in SBS. According to Mori⁴⁰, the two parameters are related in the positive mode, and greater emphasis should be placed on the TBI_S parameter, linked to green tea. Therefore, in the framework of the regression features exposed in Fig. 2, the Control group with the strong negative trend should be considered an outlier, thus making the comparison results invalid. As far as the chemical composition and quality degradation of the two teas are concerned, the study by Duddigan et al.⁴¹ was based on Nuclear Magnetic Resonance Spectroscopy, where the variability depended on the relative abundance of the components that are traditionally considered labile (O-alkyl C and carbohydrate C) against more recalcitrant components (alkyl C and methoxyl C of lignin), and the tea was disregarded as a feed decomposed in a rumen, which is the approach adopted in the LBN method. The mass decay of the green tea was biphasic, with a rapid decline to 14 d, and the loss of soluble tannins, followed by a slower decline rate to 91 d, whereas the mass of rooibos tea declined at a slow rate over the whole incubation period. The percentages of decomposition at 91 d were 80% and 52% for green and rooibos tea, respectively.

Since the NIR spectra and residual weight were well correlated (Tab. 8), the conclusions obtained from the weight tend to converge, but the spectra, as they also reflected finer quality variations, showed higher power of discrimination. TBI only has agroecological purposes, and is only rarely used for agronomic purposes

In a framework of a multifaceted experiment, Tóth et al.⁴² compared set-aside areas and cultivated areas in Hungary, which were cultivated using only rooibos buried for one month. The decomposition rate in the cultivated fields was 18.7%, whereas it was 25.83 (+32%) in the set-aside fields. However, in that study, the type of habitat did not affect the bacterial alpha diversity or community composition to any great extent ($P = 0.122$).

The Litterbag-NIRS variables predicted from the hay NIR spectra clearly indicate that SBS decreased the components of the k⁺ type (protein, ash, crop maturity index, NDF digestibility, hemicellulose) by about 28% and the r-type traits by about 4%. The SA treatment showed a similar but lower reduction of the k-variables (-13%), which was stronger in the r-variables (-8%). In short, the bioactivity detected from the quality of the hay litters was limited by both the SBS and SA. Our results are in agreement with those of Allison⁹ about the strong HA inhibition of enzyme activity, but are not in agreement with the results of a study on HA in pots of chicory⁸, in which the increase in bacterial biomass was related to the availability of carbonaceous substrates for heterotrophic growth: in fact their dose threshold resulted to be 2 g kg⁻¹ of soil, a value that is not comparable with the amendment used for our vines, which was instead around 0.125 g per kg of vineyard soil, that is, 16 times lower.

Contrary to expectations, no significant differences were found between the cultivated and set-aside soils in the Hungarian experiment Tóth et al.⁴², regarding bacterial alpha diversity and community composition. In the present trial, we have been able to profile the reduction in bioactivity in the litters through a biodiversity pattern in the bacterial and fungal taxa, a result that was beyond any expectation.

Our data confirm the rise in mineral N levels observed by Jin et al.⁷ in *Panax ginseng*, which amounted to 90-125% after dosing humic and fulvic acid at 0.8 g·Kg⁻¹ soil, an 8 times higher level than that applied in the present work. Moreover, their finding indicated that the microbial diversity of the rhizosphere soil and bulk soil was reduced after the humic acidic treatments. The results of the present work confirm the reduction in *Chloroflexi*, *Actinobacteria* and *Acidobacteria*.

Leaf traits.

The treatments affected the foliar composition, regardless of whether they were administered per leaf or in the soil. The most surprising result was the lowering of the leaf pH following the treatment with SBS. This physiological measure is not usual in agronomy. A first comparative analysis on forty-nine Mediterranean species in different environmental conditions⁴³ was followed by observations on tree species located along 3300 km of a water gradient⁴⁴ and on desert species in China⁴⁵. From a physiological point of view, a lowering of pH can be determined by a stress condition, be it water or heat⁴³, such as an excess of UV-A, B²⁵ or by a reduction of the solar cycle over the years⁴⁶. A negative relationship between artificial mycorrhization and leaf pH has been observed on corn⁴⁷, sorghum²⁷, and on seven crops⁴³, in the sense that a biofertilization with mycorrhizae corresponded to a lowering of pH.

Product quality.

The injection of SA into grapevine berries is an elicitor of phenolics and hydrolytic enzymes that delay, or even inhibit the ripening of the berries⁴⁸. Ranjbaran et al.⁴⁹, who sprayed SA on harvested berries, enhanced the increase of the total phenolic content of the berry skins after the shelf-life period. Similarly, in our trial, SA delayed the commercial maturity of the grape by 9%, but preserved the phenolic maturity of the seeds, and both these results could be interesting for winemakers to counteract climatic warming.

However, we cannot comment on the benefic effects obtained from massive SBS spraying on mango pointed out by Patel et al.²³ (SBS @ 1.5% or SA @ 1500 ppm) or on the advantages in grape quality

pointed out by Popescu and Popescu ³.

Conclusion

The systematic lowering of the leaf pH (-5%) by the SBS product containing Humic Acid is the first milestone that has been achieved in the present trial, and this result is in conflict with the expectations of a worse (-7%) predicted mycorrhization index of the vines. However, the better mycorrhization index obtained from the use of Salicylic Acid (+8%) left the pH unchanged (-1%). Both results need confirmation, possibly by resorting to artificial mycorrhization and verifying the effectiveness of the desired symbiosis in a batch experiment. However, the deviation of the leaf pH after a mild SBS soil treatment could be interesting to progress against the phytoplasmosis *flavescence dorée*.

The second milestone has been the confirmation of an always latent antagonism between chemical treatments and soil bioactivity. In the ongoing challenge of progress toward soil knowledge, the third milestone seems to us to be the demonstration of the efficacy of Litterbags-NIRS and TBI-NIRS in untangling the relationship between microbial bioactivity and biovariability quickly and indirectly.

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