OPEN ACCESS
Freely Available Online

JOURNAL OF AGRONOMY RESEARCH
ISSN NO: 2639-3166

Research Article

DOI : 10.14302/issn.2639-3166.jar-18-2084

NIRS Footprint of Bio-Fertilizers from Hay Litter-Bags

Giorgio Masoero[1,2,*], Marco Delmastro[3], Alberto Cugnetto[1], Giusto Giovannetti[4], Marco Nuti[5]

[1]Accademia di Agricoltura di Torino (TO).

[2]Dipartimento di Scienze Agrarie, Forestale e Alimentari, Università di Torino (TO).

[3]IMAMOTER, CNR, Albugnano (AT).

[4]Centro Colture Sperimentali, CCS-Aosta s.r.l. (AO).

[5]Università di Pisa (PI).

Abstract

The biofertilization of crops using microbial biota in the soil (MBS) is a modern practice that is used to sustain fertility. MBS agents can promote the yield and health of crops, by luxuriating in the shoot as well as in the root systems. Farmers devoted to systematic MBS fertilization are creating a "*Symbiotic*" (S) form of agriculture, which offers a greater advantage of resilience than Conventional (C) or organic farming. Since MBS is involved in organic matter degradation, hay-litter-bag probes can be used to reflect a global functionality of the active soil, in the short-medium term. It is here shown that the NIRS hay-litter-bag technique, intended not as mass decay but as a quality evolution of the hay probes, can be modelled as a valid footprint of S *vs.* C soils. A patented MBS was used in eight experiments in which litter-bags from an S treated thesis were compared with equivalent litter-bags from a non-inoculated C thesis. The chemical signature of the S *vs.* C in the litter-bag composition was a percentage decrease of sugars and fibres. A smart NIRS device was used to discriminate the origin of the S *vs.* C litter-bags and a sensitivity of 71% ($P<0.0001$) was obtained. External validations on 37 S farms showed that three NIRS models discriminated the true positive S spectra, with a sensitivity of 90% as single and 98% as compound probabilities The NIRS radiation of the hay-litter-bags confirmed the results of the S *vs.* C agriculture soil footprint. Moreover, the SCIO-NIR devices also made it possible to connect the S farms in a smart network.

Corresponding author: Giorgio Masoero, Accademia di Agricoltura di Torino, Via A. Doria 10, 10123, Torino, Italy.

Running Title: Smart NIRS network for bio-fertilizers

Citation: Giorgio Masoero, Marco Delmastro, Alberto Cugnetto, Giusto Giovannetti, Marco Nuti (2018) NIRS Footprint of Bio-Fertilizers from Hay Litter-Bags . Journal of Agronomy Research - 1(1):22-33. https://doi.org/10.14302/issn.2639-3166.jar-18-2084

Keywords: NIRS, biofertilizer footprint, hay-litter-bag quality, smart NIR-SCIO, rapid analyses.

Received: Apr 13, 2018 **Accepted:** May 07, 2018 **Published:** May 30, 2018

Editor: Paramanandham Joothi Pillai, Assistant Professor, Department of Zoology and Wildlife Biology, AVC College (Autonomous), Mannampandal - 609 305. Tamil Nadu. India.

Introduction

Biofertilizer arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) are prominent protagonists in the sustainability search for global agriculture [1], also concerning the horizons of the BioAg Alliance [2] and Engineering [3]. Their potential can be spread to the different agricultural systems described by Narain [4], thanks to the properties of resilience inductors [5]. A meta-analysis of field studies on the responses of wheat to AMF [6] has highlighted that field AMF inoculation can be proposed as an effective agronomic practice for wheat production, with aboveground biomass increases of around 20%, as assessed under Indian [7] and in high [8, 9] or low [10] Italian input conditions. AMF phenotypes are expressed in accord to the Law of the Minimum [11]. Phosphorus acquisition efficiency is the key feature [12, 13], but Thirkell et al. [14] managed to resolve the paradox of nitrogen: while N-mineral fertilization has been shown to elicit luxuriating and strong mutualism, similar responses have been found to be lacking following the addition of N-organic substances; the Authors, have shown that allowing hyphae access to an organic material can improve the total N and P content, with a simultaneous and substantial increase in the plant biomass (+66% for both the hypogeal and epigeal). The use of fertilizer microbial biotas of the soil (MBS), even at a minimal density of 14 AMF spores per maize seed [8], has multiple effects: acidification of the roots and stem [15]; greater resistance to disease [16]; fortifications of the functional properties, such as the antioxidant potential [17, 18]. Several beneficial effects have been observed along the forage-milk-meat chain [19, 20, 21].

The agricultural market crisis in Italy and Europe has led to a diversification of the supply of products, and also of the methods adopted to obtain different sustainable productions. For some time, several both conventional and organic farmers have engaged in a so called "*Symbiotic Agriculture*" (S) [22], in which a systematic use of MBS biofertilizers is adopted.

Considering the chemical parameters of the multifaceted soil fertility that could be rapidly predicted by means of an NIRS examination of the soil bulk sample [23], lacking of objective rapid measurements able to assess the microbial status of agrarian soils in the present work we aim to demonstrate that a biofertilizing change is real in biochemical functioning mechanisms, and that such a change can easily be testified.

The use of Litter-bags is a technique that has long been adopted in soil studies on microfauna evolution [24], as well as on mass and / or CHN decay driven by fungi [25]. The idea of coupling a litter-bag to a smart-NIRS technique has sprung from the availability of a new instrument that has been tested successfully with iced milk [26] on live rabbits [27] and for meat discrimination [21].

Experimental Procedure

The method presented in this study excludes weighting operation and is based on a footprint of a summary microbial transformation of a standard hay litter-bag evaluated according the percentage variation of the composition on a short-medium term. The work of Santoni [28] has indicated how the more numerous recalcitrant compounds (hemicellulose, ash, ether extract, crude protein and lignin) showed a parabolic upward trend that pointed out an attenuation of the accumulation percentage and a decrease after a maximum at around 60 d. In parallel, the labile substances (cellulose in the NDF and crude fibre) showed a less pronounced downward trend.

Litter-Bag

The easiest and most repeatable substrate for field-scale purposes was identified as being a hay for small animals ("*Vita Verde Small Animal Alpine Hay*", by Vitakraft pet care GmbH & Co. KG, Bremen, DE). The hay was ground in a 3 mm grid forage mill (Retsch GmbH, Haan, DE). About 5 g of hay was packed into half empty 10x10cm square polypropylene nets (1.5 mm mesh), which were then resealed using 4 staples, and a plastic label was added for identification and for easiness of finding purposes. The probes were inserted vertically near the roots and remained underground for about 60 days. They were then dried at mild temperature, gently cleaned and preserved at room temperature.

Litter-Bag Composition

The chemical composition of the hay and litter-bag residues was predicted, using a Perkin Elmer

IdentiCheck TM instrument (714-3333 nm), and the used equations were established on twelve species of crops, analysed at four stages, as reported by Tassone et al (2014) [29].

NIRS Discrimination of the Litter-Bag Origin

The extracted litter-bags were opened, and the surfaces of both sides were examined using a smart new miniaturized NIR web-based wireless spectrophotometer (SCIO v. 1.2, Consumer Physics, Tel Aviv) with a 740-1070 nm range. Three spectra were acquired on the two sides of the litter-bags. Chemometrics of the 331-point spectra was performed using a categorical discrimination procedure, integrated within the SCIO Lab proprietary software named AKA (Also Known As), and the confusion matrix, after normalization and 1th derivation of the spectra. The reclassification capacities in the Symbiotic (S) and Conventional (C) classes within each calibration experiment, where S litterbags were compared with C litterbags, or in validation experiments, with only S litterbags, were considered as the reference of the performances.

Materials and Methods

Eight experiments were set up under different conditions for calibration purposes in order to observe the NIR spectra and decomposition of the litter-bags, as well as the S *vs.* C discrimination ability. The involved crops were : *Lolium*, Wheat, Coffea, Grapevine, Pear, Quercus and Olive (Tab.1), and the litter-bag experiment concerned 106 C-type litter-bags, which were compared with 143 S-type litter-bags in two complex of 249 FT-Perkin Elmer and of 698 NIRS-SCIO spectra. The common denominator of the trials was the fertilisation of the soil with a patented MB, Micosat F ® (www.micosat.it), a consortium based on: AMF from finely ground cultivated sorghum roots, containing spores and *ifae* of *Funneliformis coronatus* GO01 and GU53, *F. caledonium* GM24, *F. intraradices* GB67 and GG32, *F. mosseae* GP11 and GC11, *F. viscosum* GC41; saprotrophic fungi: *Streptomyces spp*. ST60, *Streptomyces spp*. SB14, *Streptomyces spp*. SA51, *Beauveria spp*. BB48, *Trichoderma viride*, *T. harzianum, Trichoderma harzianum* TH01, *Trichoderma atroviride* TA28, *Trichoderma spp*.; rhizosphere bacteria: *Bacillus subtilis* BA41, *Pseudomonas fluorescens* PN53, *Pseudomonas spp*. PT65 and *Pochonia chlamidosporia*, in the relative percentage of 40% crude inoculum (AM fungi) and 21.6% bacteria and saprotrophic fungi.

In order to validate the litter-bag-NIR-SCIO technique, 37 farms belonging to the "*La Granda quality food consortium*" (Fossano, It), which started to use a systematic biofertilization of their fields five-six years ago in order to develop a Symbiotic production chain, introduced 89 litter-bags into S-type fields and meadows. The validation experiment lasted two years (A, B) and 318 spectra were obtained. The eight models that predicted the S *vs*. C type from the calibration experiments were applied to the validation data-set spectra. The classification percentage of the S-type spectra correctly predicted as S-type (sensitivity) was calculated for each model. In order to formalize an "*NIRS biofertilizer footprint*", the best three models were then considered for single and for compound probabilities of false negative, by applying a "symbiotic" score predicted from the three independent models to each spectrum: a value of 1 was scored for the S grade and a value of 0 for the C grade. The total symbiotic score of a litter-bag thus varied from 0 (Conventional, nine C=0 from the three models applied to the three spectra) to 3 (fully Symbiotic, when the three models all predicted S=1). The compound probability of the non-S outcome, that is, the false-negative cases, was then fitted from the 318 S spectra.

The composition of the litter-bags was analysed by means of a mixed one-way model considering the soil type (S *vs.* C) fixed and the effect of the experiments random [30].

Results

NIRS Discrimination of the Litter-Bag Origin

The calibration of the SCIO spectra from experiments 1-8 is reported in Tab. 2. The average AKA reclassifications were 71% for S (P<0.0001) and 62% for C (P<0.0001) , with variation coefficients of about 25% between experiments. The results were confirmed from the validation sets in experiments 9 and 10 (Tab. 2), where overall classification ratings of 78±4% for year A and 71±5% for year B were obtained. Among the eight models, numbers 2, 3 and 5 were the best ranking ones for the A and also for the B years : in fact, their average classification ability was 89.9±3.1% and 90.1±3.6%, respectively. Compounding information

Table 1. Setup of the calibration (#1-8) and validation (#9-10) experiments.

Experiment No.	Colture	Year	Site	No. Litter-bags	Microbial Biota Soil[1] Treatments
1	Lolium	16	Meadows	14	10 kg ha^{-1}, in 2016
2	Wheat	17	Field	25	3 kg ha^{-1} tanning
3	Coffea	16	Pot, GH	27	5 g pot^{-1} granular, in 2016
4	Grapevine	16	Pot	12	5 g pot^{-1}in 2014
5	Grapevine	17	Pot	12	5 g pot^{-1}in 2016
6	Pear	16	Orchard	34	10 kg ha^{-1} in 2016
7	Quercus - Truffle	16	Orchard	54	10 kg ha^{-1}, in 2015 and in 2016
8	Olive	17	Orchard	71	20 kg ha^{-1}, in 2016
9-A	Crops / Meadows	16	Fields	43	Symbiotic for five years
10-B	Crops / Meadows	17	Fields	46	Symbiotic for six years

[1]Microbial Biota Soil, MBS: Micosat F ®

Table 2. Calibration of NIR-SCIO spectra in eight experiments for the Conventional © and Symbiotic (S) footprint of litter-bags and validation on 37 Symbiotic farms of single and the best three chained models. Values in classification percentages (C->C and S->S = Sensitivity).

Exp	Crop	Year	Calibration				Validations					
			No. Spectra		Classification%		Set-A			Set-B		
			C	S	C->C	S->S	S->S	±	SEM	S->S	±	SEM
1	Lolium	16	26	12	90%	54%	57%	±	5.10%	43%	±	6.20%
2	Wheat	17	24	53	45%	91%	**90%**	±	3.10%	**94%**	±	3.00%
3	Coffea	16	24	59	50%	72%	**91%**	±	2.90%	**85%**	±	4.50%
4	Grapevine-1	16	26	28	51%	89%	60%	±	5.00%	41%	±	6.10%
5	Grapevine-2	17	18	19	52%	65%	**88%**	±	3.30%	**92%**	±	3.40%
6	Pear	16	62	64	73%	51%	84%	±	3.70%	81%	±	4.90%
7	Quercus-Truffle	16	59	58	73%	52%	77%	±	4.30%	57%	±	6.20%
8	Olive	17	55	111	60%	93%	79%	±	4.20%	72%	±	5.60%
	Total / Means		294	404	62%	71%						
	Prob. >50%				<0.0001	<0.0001						
9-A	Various	16		129			78%	±	4%			
10-B	Various	17		189						71%	±	5%
	Total / Means		0	318								
Means of the best three chained Models in bold (2, 3, 5)							89.90%	±	3.10%	90.10%	±	3.60%

from the best three chained models (Tab. 3) raised the probability of not obtaining one false negatives in a correct classification for true S membership to 98.4±0.12%.

The overall average validation grade was 2.73±0.25 (data not shown in the table), a sure sign of effective modifications in the litter-bag composition after the BMS treatments.

Litter-Bag Composition

As far as the evolution trend of the litter-bags (Tab. 4), compared to the original hay, is concerned, the overall result of the degradative processes increased the value of the multivariate crop maturity index towards a more mature type of forage, by 81% in C and 64% in S. The percentage of recalcitrant components increased in the litter-bags: hemicellulose (88% C and 98% S), ether extract (55 and 60%), ash (45 and 47%), indigestible NDF (26 and 9%) and lignine (ADL 17 and 19%). On the other hand, the labile components underwent an average relative decrease: crude fibre (-22 and -36%) and acid detergent fibre (ADF -19 and -22%). As for the MBS inoculation (Tab. 4), five significant variables distinguished the S litter-bags from the C ones: the nitrogen-free (NFE -3%, P=0.007) and the wall components decreased (NDF -10%, P=0.0006; indigestible NDF -13%, P=0.08 ; crude fibre -17%, P=0.0541), but the crude protein increased (+13%, P=0.0025), and was thus apparently more protected from the added BM. Moreover, the lipids increased (+6%, P= 0.0247).

Discussion

Litter-Bag Composition

According to Tassone et al. (2014) [29] concerning the algebraic formula of the crop maturity index for growing plants, the regression sign of the percentage on the days from sowing was positive for NDF, ADF and indigestible NDF, while it was negative for ash, crude protein, NDF digestibility and digestible-NDF. After haymaking, in the underground environment the grasses composing the litter-bags started an ontogeny involution, as a result of biotic and abiotic factors, but also because of BMS action. The observed rise in protein may be a sign of increased MBS growth [25, 31] , and the net result was that the fibrolytic communities elicited the attacks of the carbohydrates. In terms of crop maturity index, the BMS increased the evolution of the litter-bags towards a more mature type of residue, as can be observed in Fig. 1, where the lines of the S and C trends cross. Our results suggest that, as expected, BMS promotes the mechanisms that are favourable for an early maturation of the residual organic matter in the root horizon, and multi-annual observations are necessary [32]. BMS management is based on the inoculation of aerobic microbes, but, because of a luxuriating rhizosphere, and in spite of respiration-fermentation processes, the net long term result could improve the carbon footprint of the whole plant-soil system, and thus raise its sustainability. Several soil management practices, inspired by a conservative agriculture design for the improvement of the accumulation of soil organic matter, are largely

Table 3. Classification probability of the Symbiotic grade 3, 2 and the compound classification (3 or 2) >1, or false negative cases, from the three best equations in the validation of the 318 Symbiotic spectra.

Symbiotic Grade result /3	Prob.	Compound Classification (3 or 2) >1	±	SEM
3	71.7%			
2	26.7%	98.4%	±	0.12%

Table 4. Composition of the hay and of the litter-bags in the Conventional (C) and Symbiotic (S) fields.

Dry matter composition	Unit	C Conventional	S Symbiotic	S C^{-1} %	Prob.	Hay H	C H^{-1} %	S H^{-1} %
Crop maturity index	n	1.09	0.99	-20%	0.4306	0.6	81%	64%
Crude fibre	g kg^{-1}	136	112	-17%	0.0541	174	-22%	-36%
Indigestible NDF	g kg^{-1}	182	158	-13%	0.0823	145	26%	9%
Neutral detergent fibre – NDF	g kg^{-1}	427	385	-10%	0.0006	426	0%	-10%
Acid detergent fibre – ADF	g kg^{-1}	267	249	-6%	0.1828	329	-19%	-24%
Predicted dry matter at harvest	g kg^{-1}	153	146	-5%	0.2932	129	18%	13%
Nitrogen free extract – NFE	g kg^{-1}	529	518	-3%	0.007	475	11%	9%
Cellulose	g kg^{-1}	223	219	-2%	0.8151	206	8%	7%
Digestible NDF	g kg^{-1}	328	325	-1%	0.721	306	7%	6%
Gross energy	MJ kg-1	16.38	16.4	0%	0.9721	16.54	-1%	-1%
Lignine – ADL	g kg^{-1}	88	89	1%	0.8454	75	17%	19%
Ash	g kg^{-1}	200	202	1%	0.6953	137	45%	47%
In vitro total digestibility –IVTD	g kg^{-1}	822	841	2%	0.1686	855	-4%	-2%
NDF digestibility	g kg^{-1}	634	668	5%	*0.1649*	680	-7%	-2%
Hemicellulose	g kg^{-1}	148	156	5%	*0.3041*	79	88%	98%
Ether extract	g kg^{-1}	36	39	6%	*0.0247*	23	55%	65%
Crude protein	g kg^{-1}	123	141	15%	*0.0025*	127	-3%	11%

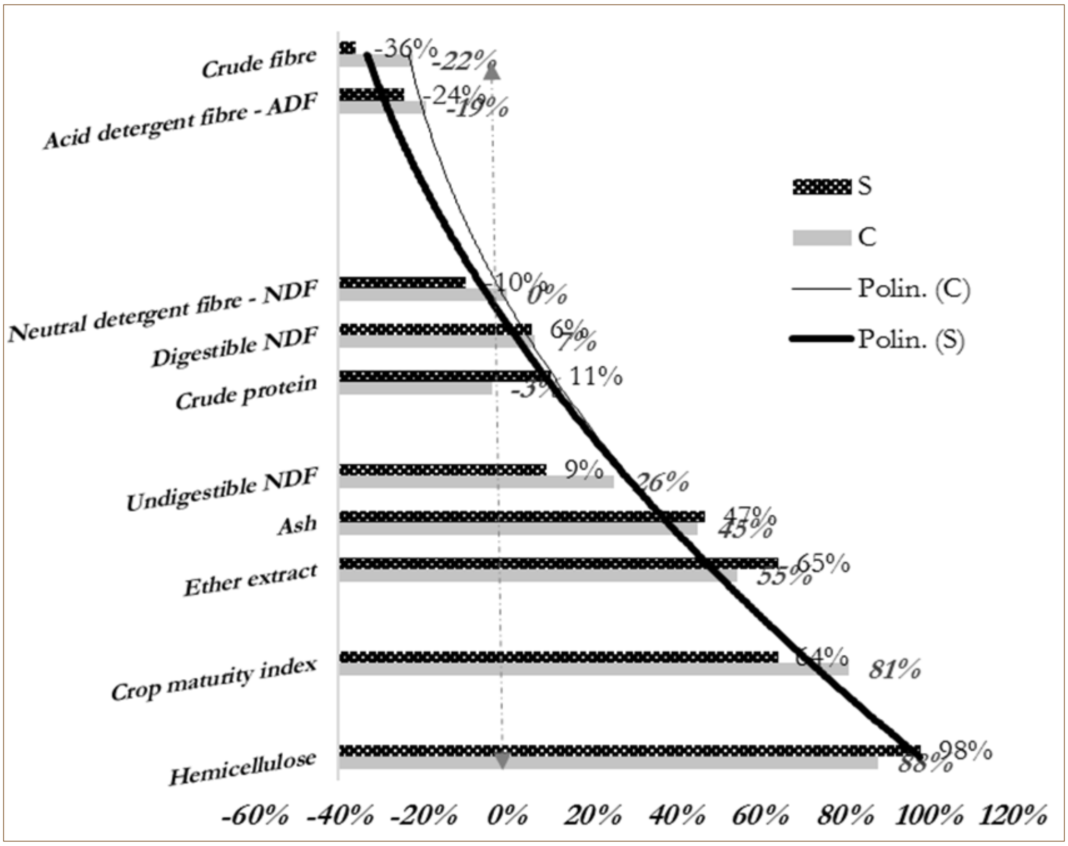

Figure 1. Relative deviation of the litter-bag residues from the hay composition after 60 d of landfilling for the Symbiotic and Conventional groups and litter maturity tendency enhanced in the S vs C conditions.

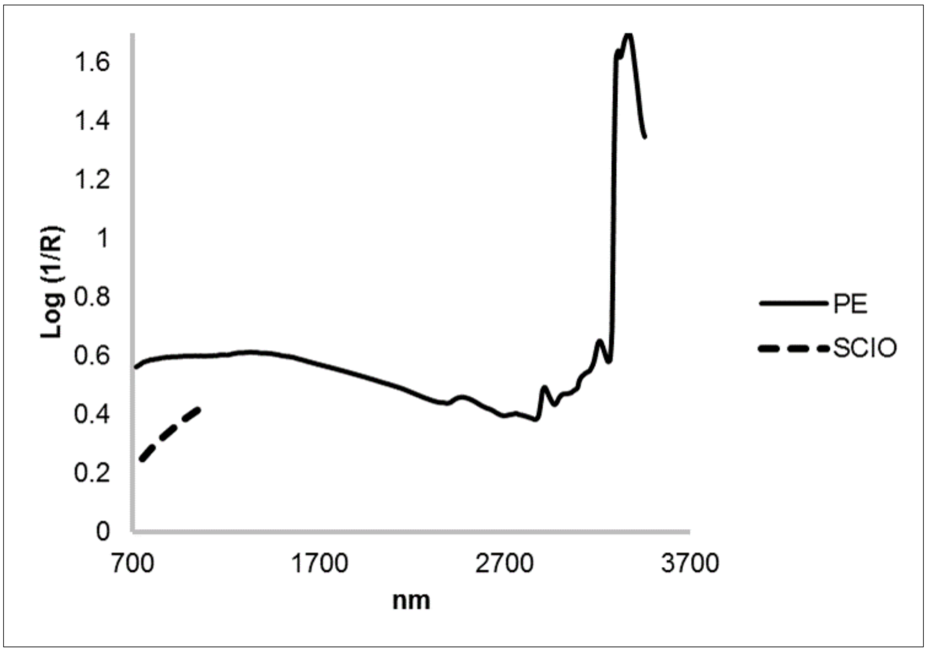

Figure 2. Average NIR spectra of the Litter-bags measured by the two instruments. It is possible to consider how short but rich the SCIO range is.

supported by EU agricultural policies, while the pro-MBS route has been totally neglected, in spite of the phosphate crisis that is expected from 2030 onwards [33].

The results of the present work are in agreement with Leolini's [34] results (ibid Table 12) obtained from a litter-bag quality study conducted on six natural sites in Spain, in which four litter types were considered. In fact, as a result of some mild differentiated activities, a significant linear increase was observed in the Spanish study for the ash and ether extract percentages, but the digestible NDF also increased, while no labile constituent was reduced. In the agrarian and well cultivated soils of the present work, the litter quality appeared to be modified to a great extent, and the MBS attacked the labile components, which arithmetically enhanced the percentages of the counterparts. In the MOLTE long-term organic experiment [26](ibid Fig. 72), only a mild footprint of the Organic vs. Conventional soil was found by means of a multivariate analysis of the predicted constituents (R^2 0.18±0.04) or, rather, in the direct NIR spectra discrimination (R^2 0.32±0.06).

Litter-bag decomposability, intended as mass decay, can be related to NIR spectra [35], and these correlations were also present in the data from Florence University: in [34] R^2 0.46 for the residual mass; in [28] 0.74 for the residual mass and 0.90 for the k decay term (recalculated). In the present work, the quantitative aspect of litter-bags was omitted, because of operational difficulties at a large scale, but also after results from the MOLTE experiment which elicited a more meaningful structural and functional relationship from the variability of the litter-bag quality than from considering the total lost mass or the exponential decay. MBS activities in litter-bag matrices mainly depend on the unexplored vast communities in the foreign soil, and to a lesser extent on the microbiome of the hay. The outcomes of litter-bag modifications are also modulated by abiotic factors, such as the pabulum conditions, i.e. thermic, water and mainly the redox-oxygen availability. According to [36], an incubation of litter-bags for two months could allow the net N mineralization to be estimated, and in the present BMS framework, an N preservation appeared. The effect of the MBS treatment on the symbiotic fingerprint appeared to be quite consistent and repeatable for the five significantly varied constituents.

NIRS Discrimination of the Litter-Bag Origin

As far as the discrimination problem is concerned, can these five signs be considered a valid support to obtain an univocal response that could help to testify the use of MBS as biofertilizers? The chemical composition of litter-bags needs a chemometric deconvolution of a broad NIR-IR spectra (714-3333 nm), which could be obtained from high-quality scientific instruments. These devices represent a valid tool to help understand some mechanisms, but are less portable for a large-scale dimension. Thus, thanks to the overtones and combinations of the organic molecules in the electromagnetic spectrum, originating in the IR region, a surprisingly small but rich NIR spectra 740-1070 nm (Fig. 2) can be capitalized on by means of vibrational spectroscopy. For field sampling and analyses operations on a smart-farm basis, the S footprint should be directly searched for in the electromagnetic spectrum. Considering the immensity of the biotas in different farms and crops, a rational choice among local models could protect against gross biases. The between-farm validation adopted in the present work is similar to a local vs. global chemometric procedure, utilized to manage large NIR datasets of soils in a better way [23]. The false negative litter-bags, with an S grade of 1, were mostly concentrated on two farms. This may have been the result of a real inefficacy of the BMS for those particular management conditions.

The outlooks on the use of NIRS regard both plant tissues and canopies [37, 38] as well as soil quality for precision agriculture purposes [39], all of which require new approaches to acquire soil data on landscapes [40, 23]. Direct NIR scanning of the soil horizons has also been proposed as a valid and practical tool to monitor the ontogeny and heterogeneity of detritus in soil, which is useful for the assessment of the carbon and nitrogen budget of the soil [41], but even for the macro components of soil biota: in fact, Zormoza et al. [42] found very high r-squares for AMF (0.91), Fungi (0.80), Protozoa (0.73), Actinomycetes (0.92) and Bacteria Gram+ (0.91), and also for enzymatic activities, while direct NIR scanning was not so reliable for exchangeable P (0.46).

Conclusions

Obtaining knowledge about functional soil biota is expensive, as well as long and hard to achieve. Moreover, problems in use may arise. Smart sensors that match offline solutions in performance while enabling size reductions, low power consumption, low unit costs, low maintenance costs and data fusion [43] are currently being investigated, however far from practical solutions. The proposed rapid comparative method of over 90% success is limited to some Italian farmers organized to monitor their progressive results from fields with probative results of litter-bags over the years. A relevant feature is that it would be possible to testify a future yield, even before harvesting. Above all, this form of indirect certification of the production process, based on the microbial soil footprint instead of a direct NIRS discrimination of the products, would eradicate difficult searches for specific markers of the S footprint in the final product. The natural increase in functional compounds in symbiotic farming products, and mainly in antioxidants, is a scientifically proven fact. However, as we are moving in a context of biological variability, it is unlikely that there will be no overlapping of one or a few characteristic substances between symbiotic and conventional products that are statistically evident at the individual level (and not only of averages detected in experimental trials with several replicates).

A diffuse web network could capitalise on this diffusive system of harmonized sampling and smart NIRS analyses, as suggested by Klakegg [44] referring to its potential use in future everyday cases.

Acknowledgements

The authors wish to thank: S. Capaldo and M. Seminara (Consorzio La Granda Quality, Fossano, CN); R. Bodrero (Commerciale Agricola, Villafalletto, CN); G.L. Malvicini and G.L. Turello (illycaffè S.p.A., Green Coffee Procurement Dept., Trieste); A. Bevilacqua (AGRION, Manta, CN); G. and M. Bergese (La Corte, Monasterolo di Savigliano, CN); R. Del Negro (Aix en Provence); S. Ravaglia (SIS Foraggere, BO); F. and R. Polo (ATS. Bio contrasto al CoDIRO, Ugento, LE) for their kind support in the setting up of the experiments and in the litter-bag management; thanks are also due to the Fondazione CRT, Torino for the financial support to the scientific activities of the Accademia di Agricoltura di Torino and special thanks to L. Leolini and M. Santoni for their pioneering work.

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