Hygienization and control of *Diplodia seriata* fungus in vine pruning waste composting and its seasonal variability in open and closed systems

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Abstract

After the ban on sodium arsenite, waste management alternatives to the prevalent burning method, such as the hygienization and biodegradation in solid phase by composting, are required for the pruned material from grapevines affected by various fungi. In this work the dynamics of a fungus associated with vine decay (*Diplodia seriata*) during the composting process of a mixture of laying hen manure and vine pruning waste (2:1 w/w) have been investigated in an open pile and a discontinuous

closed biodigester. Through the optimization of the various physical–chemical parameters, hygienization of the infected waste materials was attained, yielding class-A organo-mineral fertilizers. Nevertheless, important differences in the efficiency of each system were observed: whereas in the open pile it took 10 days to control *D. seriata* and 35 additional composting days to achieve full inactivation, in the discontinuous biodigester the fungus was entirely inactivated within the first 3-7 days. Finally, the impact of seasonal variability was assessed and summer temperatures shown to have greater significance in the open pile.

Keywords: biodigester; composting; grapevine disease; laying hen manure; vine pruning waste.

1. Introduction

Fighting grapevine decline diseases, both in young and adult plants, is of crucial importance due to their direct impact on the production volume and on the quality of grapes, making their eradication a priority for the wine sector. In particular, the increase in diseases in young plants is a matter of great concern for nursery operators and viticulturists. *Petri disease* or *Eutipiosis* is increasingly spreading in young plants and several publications claim that plants from nurseries are also affected (Aroca et al., 2010; Edwards et al., 2007).

The Esca (Black Measles) is one of the best known vine diseases which appears in the adult plant. Diseases of the vine wood are caused by pathogenic xylophagous fungi, such as *Diplodia seriata, Phaemoniella chlamidospora, Phaeoacremonium aleophilum, Eutypa lata, Fomitiporia mediterranea, Cylindrocarpon* spp., with symptoms that are multiple, complex and often confusing. *Diplodia seriata* is a fungus associated with

vine wood decay in Europe, USA, Australia and South Africa, which belongs to the family of *Botryosphaeriaceae*, that has been associated with *Black Dead Arm* and *Diplodia Cane Dieback* (Savocchia et al., 2015) and which is particularly virulent. Surico et al. (2006) argued that there should be concurrent abiotic and biotic factors to eventually produce external symptoms of the disease. Prophylactic measures with hot water and fungicides for the treatment of plants in the nursery multiplication process, targeted at controlling *P. chlamydospora, P. aleophilum* and *Cilindrocarpon* spp., are available (Waite & Morton, 2007).

Research conducted at different laboratories has also been aimed at evaluating the effectiveness of different biological products "*in vitro*" based on *Trichoderma harzianum, Trichoderma atroviride, Gliocladium roseum, Cephalosporium verticillium* and *Fusarium lateritium* to protect injured areas, but aforementioned treatments have not been effective in the control of Esca in the vineyard. After the ban on sodium arsenite, there have also been studies on the effectiveness of chitosan oligomers combined with *Trichoderma harzianum* (Chittenden & Singh, 2009) and chitosan-nanometals composites against *Diplodia seriata* (Matei et al., 2015). However, the best form of control is still the adoption of preventive measures such as the elimination of prunings of affected stocks, the protection of pruning wounds with fungicides and performing the pruning late so as to reduce the susceptibility of wounds (Larignon et al., 2009).

A way to achieve a simultaneous removal of the reservoir of pathogenic grapevine decline fungi (illustrated with *Diplodia seriata* fungus) in vine pruning waste and of those of other microorganisms present in manure is the use of composting processes. These processes, in addition, add value to the resulting product as an organo-mineral fertilizer.

An important aspect when composting manure is the addition of a bulking agent to optimize substrate properties (such as air space, moisture content, C/N ratio, particle density, pH and mechanical structure), thus positively affecting the decomposition rate (Bernal et al., 2009; Hao et al., 2004). Lignocellulosic agricultural and forestry by-products are commonly used as bulking agents in co-composting of nitrogen-rich wastes, such as animal manures (Guerra-Rodríguez et al., 2001; Huang et al., 2006). Consequently, and in order to conduct the composting process, laying hen manure has been incorporated as a livestock waste rich in nitrogen and the best poultry manure/vine pruning waste ratio has been determined so as to achieve the hygienization of the fungus in the vine pruning waste and to produce a good-quality compost. This pruning waste management approach is a suitable alternative to burning and to abandonment in the vineyard, which would contribute to the propagation of the diseases.

The aim of this work has been to compare the fungus removal efficiency in pruned vine shoots attained in an open compost pile against that of a discontinuous biodigester in different seasons. In an open pile it is essential to achieve a high temperature throughout the mass by periodic turning, while a discontinuous biodigester facilitates the establishment of the thermophilic temperatures and the aeration needed to achieve the hygienization of vine pruning waste, thus taking the temperature reached inside the mass as the process control parameter. Vine shoot prunings are lignocellulosic residues, whose structure is not altered in the composting process at temperatures below 50 °C, so mixing with laying hen manure greatly contributes toward reaching temperatures up to 70 °C. To attain the fungus hygienization, the US Environmental Protection Agency regulations (EPA, 2002), recommends maintaining a minimum temperature of the composting mass of 55 °C for 3 days (aerated static pile or in-vessel) or 15 days with 5 turns (windrow) to meet the regulatory requirements of class A fertilizers, and a

minimum of 40 °C for 5 days -during which temperature should exceed 55 ºC for at least 4 hours- to meet class B fertilizer requirements.

Finally, to ensure the compost quality according to the European Regulation (EC) No. 2003/2003 for fertilizers, the levels of nutrients and heavy metals at the beginning and at the end of the composting process were monitored, for both the open pile and the discontinuous biodigester.

2. Materials and methods

2.1. *Raw materials*

The pruned vine shoots came from *Tempranillo* variety vines from vineyards located in Cubillas de Santa Marta (Valladolid, Spain). The shoots were crushed into chips of a size not exceeding 2-4 cm and were stacked/piled until they were used in the Centro de Formación Agraria in Viñalta (Palencia, Spain) owned by Junta de Castilla y León.

Diplodia seriata, a fungus associated with grapevine decline in Castilla y León (Spain), was supplied by ITACyL (Valladolid, Spain) laboratories, after its isolation in MEA (malt extract agar) and PDA (potato dextrose agar) culture media (Martin & Cobos, 2007).

Laying hen manure, consisting of a mixture of the original bedding material (sawdust) and the poultry solid waste, was used for the composting of the vine pruning waste to ensure a final compost rich in nitrogen.

2.2. *Artificial inoculation assay*

In order to analyze aspects such as the disinfection capacity or the percentage of disappearance of the fungus, it is necessary to have infected vine shoots, in a controlled way, both in the piles and in the biodigester.

For this purpose, 800 inoculated vine shoots using *Diplodia seriata* (400 for the open pile and 400 for the biodigester) were prepared. The inoculation of the pruned shoots of *Tempranillo* grapevines was conducted on 15-20 cm long cane-segments, in which 2 or 3 leaf buds had been kept, and that had been previously sterilized by immersion in a 1.5% sodium hypochlorite solution for 2-3 min and washed thoroughly with sterile distilled water. Cane-segments were then dried for 30 min in a laminar flow chamber under ultraviolet light (using a UV sterilizer cabinet (Kowell, Gyeonggi-do, South Korea)), according to Martín and Martín (2013). After that, 5 cane-segments were used to confirm sterilization by using microbiology analysis, as explained in subsection 2.4.

To inoculate *D. seriata*, a basal cut was made in the sterilized cane-segments and an 8-mm mycelium agar plug from a growing culture was placed in the wound. The inoculated pieces were placed in test tubes and stored at 25 °C in the dark for two months (Martín & Martín, 2013). To confirm infection, 5 cane-inoculated segments were used to establish the initial percentage of infection before the composting process. Values were different in the different seasons (spring 65.3%, summer 100%, winter 66.6%) Figure 5.

2.3. *Composting devices: open pile and discontinuous biodigester*

Composting tests were performed at the Centro de Formación Agraria in Viñalta, Palencia (Spain), and took place from 3rd May to 19th July for the composters corresponding to the spring season, from 26th July to 12th October for those

146 corresponding to the summer season, and from 12th November to 2nd February for the
147 winter season.

148 A requirement for the optimization of the composting process in both the open (pile)
149 and the closed (discontinuous biodigester) systems is the determination of the most
150 appropriate laying hen manure:vine pruning waste ratio (the C/N ratio of the starting
151 mixture needs to be ca. 30). The C/N ratios of the starting materials were measured
152 (after drying at 100 °C for 48 hours and grinding in a ZM-100 ultracentrifugal mill
153 (Retsch, Haan, Germany) to a particle size <0.08 mm) with a CHN2000 apparatus
154 (LECO, Saint Joseph, MI, USA). Experimental C/N for laying hen manure (A) and vine
155 shoot prunings (B) were 10.11 and 54.71, respectively (Table 1), so the mixture ratios
156 for 100 kg were readily determined by using equation [1]:

157 $$\mathrm{A} \cdot (\mathrm{C/N})_{\mathrm{A}} + \mathrm{B} \cdot (\mathrm{C/N})_{B} = 100 \cdot (\mathrm{C/N})_{mixture} \quad [1]$$

158 These calculations indicate that the theoretical manure to vine pruning waste ratio
159 should be 55/45, i.e., 1.22. Nevertheless, in order to attain a good hygienization and
160 quality of the compost, it is also necessary to operate with a mixture that can reach high
161 composting temperatures. Preliminary tests with laying hen manure/vine pruning waste
162 ratios of 1.22:1, 1.5:1 and 2:1 were conducted, concluding that the best results for the
163 final hygienization and quality of compost were achieved with a 2:1 w/w manure:vine
164 pruning waste ratio (Matei et al., 2014).

165

166 **Table 1.** Composition of the starting materials.

Starting material	C (%)	H (%)	N (%)	C/N ratio	pH
Hen manure	31.8	4.27	3.14	10.11	7.35
Vine shoots pruning waste	41.03	5.48	0.75	54.71	6.30

167

For the preparation of the open pile (approx. 1200 kg in total), an initial layer (5-10 cm, 60 kg) of straw was placed directly on the ground, so as to absorb leachates, followed by a first 300 kg layer of laying hen manure, a second 150 kg layer of grapevine pruning waste, a third 300 kg layer of manure, a fourth 150 kg grapevine prunings layer, and finally a covering layer of manure (300 kg). In the discontinuous biodigester, 33 layers were prepared, with a bottom layer of straw (20 kg) to adsorb leachates and alternate layers of manure (30-35 kg) and crushed pruning waste (15-17.5 kg), covered by a final layer of manure.

The inoculated pruning waste was placed in layers 2 and 4 in the open pile (200 inoculated cane-segments per layer) and in layers 15, 17, 19 and 23 in the discontinuous biodigester (100 inoculated pruned vine shoots per each layer). In order to distinguish them from other cane-segments, they were marked with green paint, and to enable subsequent extraction, white bridles and nylon thread were used (Figure 1).

Water was added to each layer of crushed vine shoots to provide a moisture value of 50%, and moisture and temperature were monitored as indicated below. Aeration of the open pile was assured with the turnover process (after 28 days).

The discontinuous biodigester consisted of a Box-Compost (UVa, Valladolid, Spain) container and a Compostronic (UVa, Valladolid, Spain) device. Dimensions of the Box-Compost container were 2370×1080×1420 mm, and it was constructed with panels of polyester and polyurethane foam, with an external stainless skeletal frame (Figure 1*c*). Inside the Box-Compost container, temperature, moisture and oxygen contents of the composting mass were conditioned with a 7.5 KW air heater, water sprinklers and a 3 HP high pressure centrifugal ventilator, which were automatically controlled with a Compostronic device equipped with a HOBO U12 Temp/RH/Light/External data logger (ONSET, Bourne, MA, USA) with 4 external channels (Sánchez et al., 2008).

193

194 [FIGURE 1]

195 **Figure 1**. (*a*) inoculated cane-segment with white bridle; (*b*) extraction of cane-segments from the open
196 pile using nylon threads; (*c*) discontinuous biodigester; (*d*) detail of cane-segment with bridle and nylon
197 thread; (*e*) nylon threads for layer 23 in the discontinuous biodigester; (*f*) extraction of inoculated cane-
198 segments from the discontinuous biodigester.

199

200 **2.4. *Microorganisms analysis***

201 Hygienization analysis was conducted by extracting five of the pruned vine shoots
202 inoculated with *D. seriata* at a time from aforementioned layers of the two systems
203 under study (open pile and discontinuous biodigester), on a daily basis during the first
204 week and then on days 10, 20, 30, 40, 50 and 60 of the experiment. Each vine shoot was
205 cut into 6 chips that were placed in a Petri dish, (30 chips/sample) containing malt
206 extract agar (MEA) to which 0.5 g/l of chloramphenicol had been previously added.
207 Dieldrin acaricide (0.2 g/L) was also added to prevent the appearance of mites.
208 Subsequently, they were held in an incubator at 25 ºC for 15 days to morphologically
209 determine the growth of *Diplodia seriata* and other fungi (*Aspergillus, Acremonium,*
210 *Alternaria, Fusarium*) and bacteria (*Actinomycetes, Bacillus, Thiobacillus and*
211 *Enterobacter spp*) which grew from each chip. The resulting fungal colonies were
212 isolated in potato dextrose agar (PDA) and were grown at 25 ºC in alternating cycles of
213 12 hours of darkness and 12 hours of near ultraviolet light to induce sporulation (Martín
214 & Martín, 2013). Reading of the Petri plates containing chips started after 10 days and
215 was repeated every two weeks for two months.

216 The inactivation percentage of *Diplodia seriata* fungus was determined by counting
217 the number of infected chips (0-6) for each cane-segment of composted samples (30).
218 Initial percentage of infected chips before composting are represented by time 0 (Figure

219 5). *D. seriata* isolates were identified by macroscopic characteristic features, such as the
220 texture of the colonies, the color of the mycelium in the MEA medium, the shape of the
221 margin of the fungus in the Petri dish or spore morphology and conidiogenous cells,
222 according to Phillips (2007), and by resorting to PCR (SureTect Real-Time PCR System;
223 Thermo Scientific, Waltham, MA, USA) for confirmation when necessary.
224
225 **2.5. *Chemical, physical and phytotoxicity germination analyses***
226 Samples were analyzed for carbon, hydrogen and nitrogen concentrations with a
227 CHN2000 elementary analyzer (LECO, Saint Joseph, MI, USA); temperature and
228 moisture were tracked (3 repetitions) with a 638 Pt portable thermometer (Crison, Hach
229 Lange, Düsseldorf, Germany) and a HH1 moisture meter (Theta Meter, Moscow,
230 Russia), respectively; electrical conductivity (EC) and pH were measured with a
231 microCM 2200 conductivity meter (Crison, Hach Lange, Düsseldorf, Germany) and a
232 Basic 20 pH meter (Crison, Hach Lange, Düsseldorf, Germany), respectively.
233 In addition, a Medilow incubator (J.P. Selecta, Barcelona, Spain), a Heraeus T 6030
234 heating and drying oven (Thermo Scientific, Waltham, MA, USA), a J.P. Selecta
235 Mediclave autoclave, an ETHOS 900 microwave (Milestone Srl, Sorisole, BG, Italy)
236 and a Varian AA240 FS atomic absorption spectrophotometer (Agilent Technologies,
237 Santa Clara, CA, USA) with flame and graphite furnace for metals (UNE-EN
238 13650:2001 compliant) were also used to process the samples and further characterize
239 them.
240 For the phytotoxicity germination test, ten *Lepidium sativa* seeds were incubated
241 with 1 mL of compost extract at two different concentrations, either 1.7 g or 5 g of
242 compost/100 mL of distilled water, at 25 ºC in the dark for 72 hours on sterilized
243 cellulose filter paper (Whatman No. 1), according to Zucconi et al. (1981). Four

repetitions were prepared; the root length of germinated seeds was measured and compared with the growth of the dH_2O control that represents the 100%. The germination index (GI%) was calculated using the following formula [Equation 2]:

$$GI\% = \frac{\text{seed germination} \times \text{root length of treatment} \times 100}{\text{seed germination} \times \text{root length of control}} \quad [2]$$

The GI has been proven a very sensitive index (Zucconi et al., 1981), indicating non-phytotoxicity of the compost when values are higher than 60%.

2.6. *Statistical analysis*

The statistical analysis of the data was conducted using OriginPro 2016 SR1 (OriginLab, Northampton, MA, USA) ANOVA tools. Unless specifically stated otherwise, all statistical differences represent $p<0.05$.

3. Results and discussion

3.1. *Physicochemical parameters*

3.1.1. *Temperature, moisture and time*

When operating on an open pile system (Figure 2*a* and Figure 2*b*) or on a discontinuous biodigester (Figure 2*c* and Figure 2*d*), there is not a clear delimitation between the mesophilic (10-40 °C) and the thermophilic (40-75 ºC) phases, since they occur in a sequential manner (Niu et al., 2015; Schloss et al., 2003), but whereas the open pile required ten days to reach the thermophilic phase with a continuous moisture loss, the discontinuous biodigester reached the thermophilic phase in five days with a temperature of 75 ºC and a volumetric soil moisture of ca. 40-50%. Franke-Whittle et al. (2014) also observed water evaporation in an open pile during the thermophilic phases, due to the elevated temperatures (up to 73 °C), which contributed to the loss of

wet mass. High temperatures can also produce, especially in the uncontrolled windrow system (open pile), chemical reactions that can result in the formation of unwanted substances. In a closed system, the temperature can be controlled in every phase of the process, even during the hygienization, and these problems do not occur (Grüneklee, 1998).

To reactivate the composting process in the open pile, a turnover and water addition were required after 28 days, which appeared in the time evolution as a temperature increase and a gradual decrease in the volumetric soil moisture. In the discontinuous biodigester, external conditions had no effect as aeration was optimized to 5 minutes aerating every 24 hours. Therefore, in the biodigester it was the aeration the one that favored the hygienization of the vine pruning waste, due to the high temperature that was reached inside it (75 ºC).With regard to self-heating period, it was long enough (60 days) in order to ensure efficient hygienization of the input materials used (Franke-Whittle et al., 2014).

[FIGURE 2]

Figure 2. Evolution of temperature (ºC) and moisture (%) of the samples collected on a daily basis for piles (*a*,*b*) and discontinuous biodigesters (*c*,*d*). All measurements were conducted in triplicate: the reported values correspond to the average and the estimated standard deviation (e.s.d.) was <4.20% in all cases.

For the open pile in the spring and summer seasons, the thermophilic phase lasted up to 50 days with a volumetric moisture content of 41-42%, while in winter the moisture was 47% (see Figure 2*b*). These fluctuations can be ascribed to the influence of ambient conditions, according to Zhu (2006). Out of the three seasons under consideration, correlation between moisture and temperature and an appreciable significance were only

observed for the summer season (Table 2), both for the pile ($p=0.004$, $R^2=0.767$) and the digester ($p=0.016$; $R^2=0.643$). The fact that the significance was higher for the pile ($p<0.01$) than for the digester ($p<0.05$) was probably due to the higher moisture content in the former in comparison to the later (47% *vs*. 41%).

Table 2. Coefficients of determination and p-values for the correlations between the rate of disappearance of *Diplodia seriata* fungus and the different physicochemical parameters for the open piles (*top*) and the discontinuous biodigesters (*bottom*). (+)/(-) indicate positive/negative correlation, respectively.

Open Pile		*Diplodia seriata* (%)	Temperature (ºC)	Humidity (%)
Temperature (ºC)	spring	$R^2=0.348$ (-); p=0.026		
	summer	$R^2=0.832$ (-); p=0.000		
	winter	$R^2=0.326$ (-); p=0.032		
Humidity (%)	spring	$R^2=0.491$ (+); p=0.052	$R^2=0.004$ (+): p=0.873	
	summer	$R^2=0.552$ (+); p=0.034	$R^2=0.767$ (+); p=0.004	
	winter	$R^2=0.455$ (+); p=0.066	$R^2=0.027$ (+); p=0.695	
pH	spring	$R^2=0.141$ (-); p=0.185	$R^2=0.124$ (+); p=0.215	$R^2=0.000$ (+); p=0.975
	summer	$R^2=0.518$ (-); p=0.003	$R^2=0.614$ (+); p=0.000	$R^2=0.749$ (+); p=0.005
	winter	$R^2=0.382$ (-); p=0.018	$R^2=0.590$ (+); p=0.001	$R^2=0.258$ (+); p=0.198
EC (dS/m)	spring	$R^2=0.529$ (+); p=0.003	$R^2=0.607$ (-); p=0.001	$R^2=0.098$ (+); p=0.448
	summer	$R^2=0.428$ (+); p=0.011	$R^2=0.414$ (-); p=0.012	$R^2=0.711$ (+); p=0.008
	winter	$R^2=0.442$ (+); p=0.009	$R^2=0.481$ (-); p=0.005	$R^2=0.361$ (+); p=0.115

Closed biodigester		*Diplodia seriata* (%)	Temperature (ºC)	Humidity (%)
Temperature (ºC)	spring	$R^2=0.595$ (-); p=0.001		

	summer	R^2=0.285 (-); p=0.048		
	winter	R^2=0.496 (-); p=0.004		
Humidity (%)	spring	R^2=0.018 (+); p=0.295	R^2=0.057 (+); p=0.566	
	summer	R^2=0.086 (+); p=0.479	R^2=0.643 (+); p=0.016	
	winter	R^2=0.618 (+); p=0.020	R^2=0.080 (+); p=0.494	
pH	spring	R^2=0.313 (-); p=0.037	R^2=0.565 (+); p=0.001	R^2=0.129 (+); p=0.380
	summer	R^2=0.610 (-); p=0.000	R^2=0.065 (+); p=0.377	R^2=0.375 (+); p=0.106
	winter	R^2=0.470 (-); p=0.006	R^2=0.022 (+); p=0.607	R^2=0.608 (+); p=0.022
EC (dS/m)	spring	R^2=0.729 (+); p=0.000	R^2=0.461 (-); p=0.007	R^2=0.005 (+); p=0.864
	summer	R^2=0.265 (+); p=0.059	R^2=0.117 (-); p=0.230	R^2=0.002 (+); p=0.913
	winter	R^2=0.642 (+); p=0.000	R^2=0.044 (-); p=0.470	R^2=0.720 (+), p=0.007

The significant differences in the summer, also reported by Benito et al. (2006) when operating with pruned vine shoots mixed with foliage in open composting piles in different seasons, could be attributed to differential leaching of soluble salts as a result of seasonal precipitations.

In comparison to the work of Bustamante et al. (2012), whereas temperatures higher than 50 ºC could not be attained when cattle slurry was mixed with vine shoots pruning as bulking agent in a pile, laying hen manure allowed temperatures to reach ca. 70 ºC and produced much more effective composting and better hygienization, for the open piles and the biodigester.

3.1.2. *pH and electrical conductivity*

According to Sánchez-Monedero et al. (2001), the pH increases during the composting process both for open and closed systems due to the decomposition of the organic acids and the formation and release of ammonia by volatilization. The pH values for the open and closed devices in this study ranged from 7 to 9.5 and the EC was in the 1-3.5 dS/m range (Figure 3). The highest pH values were attained for piles and biodigesters operating in winter (pH 9.2), with a very high EC/pH significance ($p<0.001$) for that period.

The final values of pH and EC were similar in the different seasons: pH values (8-8.5) and EC values (1.8-1.3 dS/m) were typical of mature compost and could be regarded as indicative of good quality products. Correlations between temperature and EC in the open pile (Table 2) were: R^2 =0.607 (p=0.001) in spring, R^2=0.414 (p=0.012) in summer and R^2=0.481 (p=0.005) in winter. Similar pH values were observed by Benito et al. (2006) working with vine shoots pruning waste compost. These authors obtained significant differences ($p < 0.01$) between means from piles formed in different seasons, probably due to differential leaching of soluble salts as a result of seasonal precipitations.

[FIGURE 3]

Figure 3. pH and electrical conductivity (EC) variation in the samples taken from open piles (*a*,*b*) and discontinuous biodigesters (*c*,*d*). The reported values are the average of three measurements, and the e.s.d. was <1% in all cases.

3.1.3. *C/N ratio*

When operating with pruning waste, a compost can be considered stable or mature if the C/N ratio is around 15-20, while for values above 25 nitrogen would be tightly bound to organic matter and not available to be used by plants (Huang et al., 2004). For a (dry) laying hen manure/vine shoots 2:1 w/w ratio, the operating ranges in terms of C/N ratios for the open pile (14-22) and for the biodigester (12-20) (Figure 4) were within aforementioned interval for optimal ready-to-use compost, according to Rosen et al. (1993).

The seasonal analysis of the compost for the open pile showed that the highest values of the C/N ratio occurred in spring, ten days after the start of the process (C/N ratio=22), while the lowest ratio corresponded to the summer pile after 20 days (C/N ratio=14). The subsequent turnover allowed an increase in the C/N ratio and promoted compost maturation by the end of the process (with C/N ratio=20 in spring, C/N ratio=18 in winter and C/N ratio=16 in summer).

[FIGURE 4]

Figure 4. C/N ratio variation in the samples taken from (*a*) open piles and (*b*) closed batch biodigesters. All reported values correspond to the average of the three repetitions, and e.s.d. was <1.9% in all cases.

3.2. *Biological parameters and compost quality control*

3.2.1. *Diplodia seriata hygienization.*

The time scale for inactivation of *Diplodia seriata* fungus in the open pile and the discontinuous biodigester (Figure 5) showed 10 days were required for the partial elimination of the fungus and 35 days of composting were needed for its complete inactivation for the open piles. In the discontinuous biodigester (Figure 5*b*) the fungus

were inactivated in 3-7 days. Consequently, the compost hygienization was far quicker and more effective in the discontinuous biodigester than in the open pile, regardless of seasonal variability.

[FIGURE 5]

Figure 5. *Diplodia seriata* fungus inactivation (%) in the samples taken from (*a*) open piles and (*b*) discontinuous biodigesters.

A study of the inactivation of *Diplodia seriata* fungus as affected by different physicochemical parameters and in different seasons (spring, summer and winter) was also conducted. When operating in an open pile, there was an inverse correlation between the percentage of inactivation of *Diplodia seriata* and the temperature increase in all the three seasons (Table 2). The associated significance was particularly high in the summer, due to the higher temperatures reached in this season (R^2=0.832, p<0.001). The significance was lower for the fungus inactivation *vs*. moisture (R^2=0.552, p=0.034), *vs*. pH (R^2=0.518, p=0.003), *vs*. electrical conductivity (R^2=0.428, p=0.011) and *vs*. the percentage of other fungi (R^2=0.508, p=0.004). Dunkley et al. (2011) also demonstrated the effectiveness of the open pile composting process during the summer to destroy any pathogenic microorganisms that may be present. Rodríguez et al. (2012) confirmed the effectiveness of a discontinuous biodigester (a rotary drum reactor) using pruning waste and powdered sawdust as bulking agents, attaining a complete removal of pathogens after 3 days of composting.

3.2.2. *Other fungi and bacteria*

Survival of other fungi and bacteria (Figure 6) was generally lower in the discontinuous biodigester than in the open pile, regardless of the season. For example,

388 in the summer, other fungi and bacteria occurred in about 85% of chip samples from the
389 open pile , while in the discontinuous biodigester the percentage of positive samples
390 (40%) was less than half that of the open pile (40%). Franke-Whittle et al. (2014) also
391 observed that microbial communities changed over the 63 days of composting, which is
392 typical in composting systems.
393
394 [FIGURE 6]
395 **Figure 6**. Other fungi and bacteria time evolution for open piles (*a*,*b*) and discontinuous biodigesters (*c*,*d*)
396
397 3.2.3. *Germination index and compost quality*
398 The results of the *in vitro* germination tests in the aqueous extracts of mixtures of
399 vine pruning waste and laying hen manure at two dilutions (D1: 5 g compost/100 mL
400 water; D2: 1.7 g compost/100 mL water), conducted with cress (*Lepidium sativum*)
401 seeds, are summarized in Table 3. The germination rate was higher in compost extracts
402 from the open pile than from the discontinuous biodigester, and was slightly higher for
403 summer compost (D2: 210%). Germination indices at the end of the composting process
404 (D1: 113%; D2: 141%) were higher than at the start of the composting process (D1:
405 109%; D2: 117%). In both cases the compost would be considered mature since the GI
406 is above 60%, which indicates that it contains no phytotoxic substances that can
407 negatively affect the development of seeds (Zucconi et al., 1981).
408
409 **Table 3**. Germination index (GI) for *Lepidium sativum*, using aqueous extracts of the initial and final
410 compost, for the open pile and the discontinuous biodigester in the different seasons. Reported values are
411 the average of the four repetitions.

Treatment	GI (%)											
	Spring				Summer				Winter			
	Pile	σ	Biodigester	σ	Pile	σ	Biodigester	σ	Pile	σ	Biodigester	σ
D1 (initial)	105	12	63	7	105	6	61	8	162	23	150	16
D2 (initial)	123	13	68	4	114	7	125	19	174	20	147	17
D1 (final)	111	11	60	10	125	13	123	8	133	23	120	14
D2 (final)	126	14	90	10	210	13	135	14	191	22	164	15

412 * The germination index for the control (dH_2O) was 100. D1 and D2 stand for first and second dilution (5 g/100 mL
413 of water and 1.7 g/100 mL of water, respectively).

414

415 3.2.4. *Nutrients and heavy metals contents*

416 The application of the composted waste to the soil involves the provision of
417 substantial quantities of nutrients (Table 4). However, during the composting process a
418 relative increase in the concentration of metals took place, due to organic matter loss
419 and transformation (Canarutto et al., 1991; García et al., 1991). By comparison of the
420 contents of heavy metals in the open pile and in the discontinuous biodigester at the
421 beginning *vs*. at the end of the composting process (Table 4), slight increases in the
422 contents of Mg (0.64% *vs*. 0.78%) and Cr (16 ppm *vs*. 18.3 ppm) were observed for the
423 discontinuous biodigester, while for the open pile there were increases in the Ca (4.13%
424 *vs*. 4.47%), Cr (15.95 ppm *vs*. 20.80 ppm), Ni (8.26 ppm *vs*. 12.18 ppm) and Pb (11.10
425 ppm *vs*. 11.56 ppm) contents. No changes were observed for Na, K and Fe. However,
426 more studies and statistical analyses are needed in order to confirm these data.

427 The resulting fertilizing products could be classified as class A according to the
428 Annex V of the Spanish Royal Decree 506/2013 of 28th June (which transposes
429 Regulation (EC) No. 2003/2003 for fertilizers and its subsequent amendments), and the
430 quality of the obtained compost allows its application to agricultural soils since no item

exceeded the maximum limits for heavy metals in solids (mg/kg of dry matter). Nonetheless, the contents of Cu and Zn in the compost were close to the upper limit value for class A fertilizer (Cu: 70 ppm and Zn: 200 ppm) due to the manure used as a starting product.

Table 4. Nutrients and heavy metals contents at the beginning (P_0 and B_0) and at the end (P and B) of the composting process for the open pile (P) and the discontinuous biodigester (B).

Sample	%K	%Na	%Ca	%Mg	%Fe	Cr (ppm)	Cu (ppm)	Mn (ppm)	Zn (ppm)	Ni (ppm)	Pb (ppm)	Cd (ppb)
P_0, B_0 (initial)	1.27	0.24	4.13	0.64	0.27	16.0	96.6	650	448	8.3	11.1	217
P (final)	1.27	0.20	4.47	0.64	0.38	20.8	79.5	449	291	12.2	11.6	147
B (final)	1.30	0.25	3.93	0.78	0.22	18.3	96.1	579	395	8.6	9.9	160

4. Conclusions

The discontinuous biodigester was far more efficient than the open pile for the hygienization of the grapevine pruning waste inoculated with *Diplodia seriata*. High summer temperature was a significant factor in the inactivation of *D. seriata* in open compost piles (R^2=0.832, $p<0.001$). The chosen 2:1 w/w ratio for the laying hen manure/vine prunings waste mixture allowed the compost to reach the required high temperatures, and yielded an excellent fertilizer, free of pathogens, with a C/N ratio lower than 20, that favors the mineralization of the compost, and a germination rate well above 60%.

Regarding circular economy, the obtained compost will have a high agronomic and economic value and is particularly suitable for the soils of the vineyards, which have

very low organic matter content. The compost should be reintroduced into the wine production system in order to close the residual material cycle.

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565

Fig. 1. (a) Inoculated cane-segment with white bridle; (b) extraction of cane-segments from the open pile using nylon threads; (c) discontinuous biodigester; (d) detail of cane-segment with bridle and nylon thread; (e) nylon threads for layer 23 in the discontinuous biodigester; (f) extraction of inoculated cane-segments from the discontinuous biodigester.

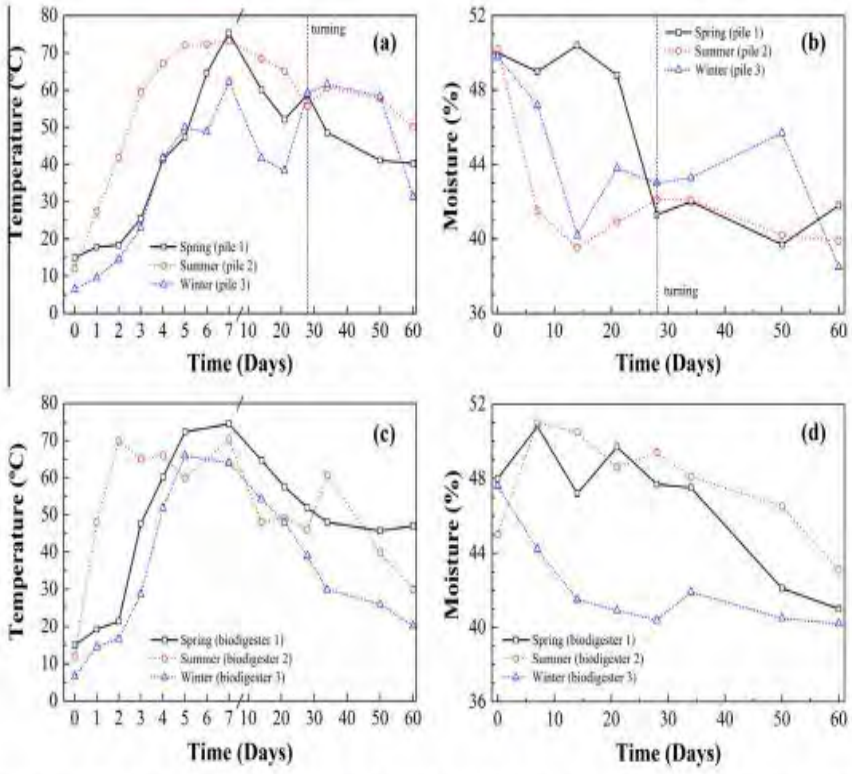

Fig. 2. Evolution of temperature (°C) and moisture (%) of the samples collected on a daily basis for piles (a and b) and discontinuous biodigesters (c and d). All measurements were conducted in triplicate: the reported values correspond to the average and the estimated standard deviation (e.s.d.) was <4.20% in all cases.

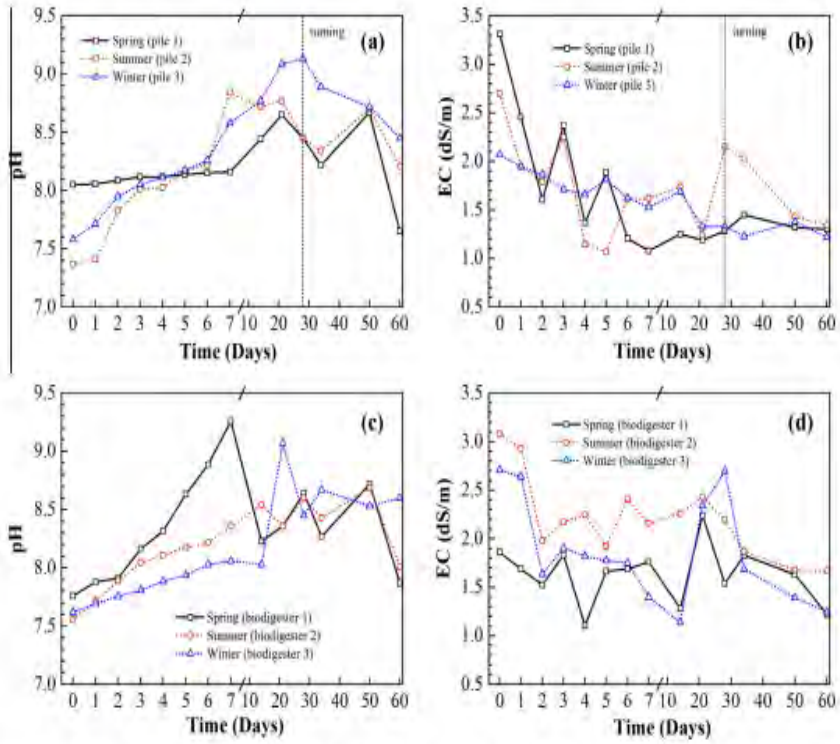

Fig. 3. pH and electrical conductivity (EC) variation in the samples taken from open piles (a and b) and discontinuous biodigesters (c and d). The reported values are the average of three measurements and the e.s.d. was <1% in all cases.

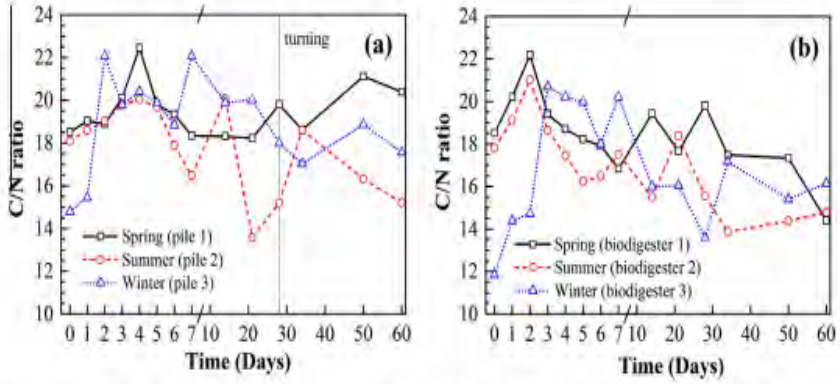

Fig. 4. C/N ratio variation in the samples taken from (a) open piles and (b) closed batch biodigesters. All reported values correspond to the average of the three repetitions and e.s.d. was <1.9% in all cases.

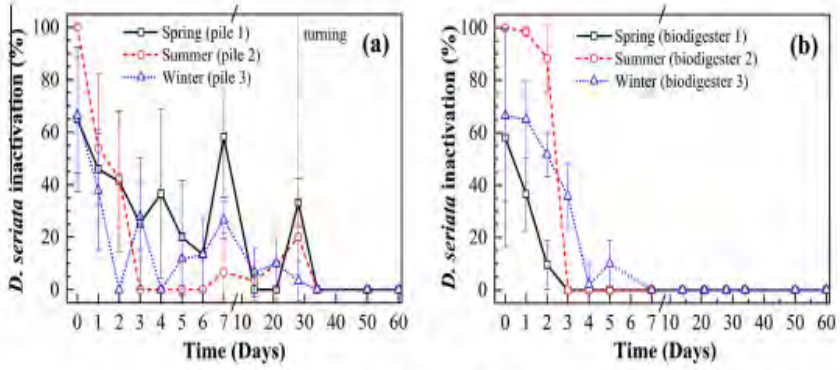

Fig. 5. *Diplodia seriata* fungus inactivation (%) in the samples taken from (a) open piles and (b) discontinuous biodigesters.

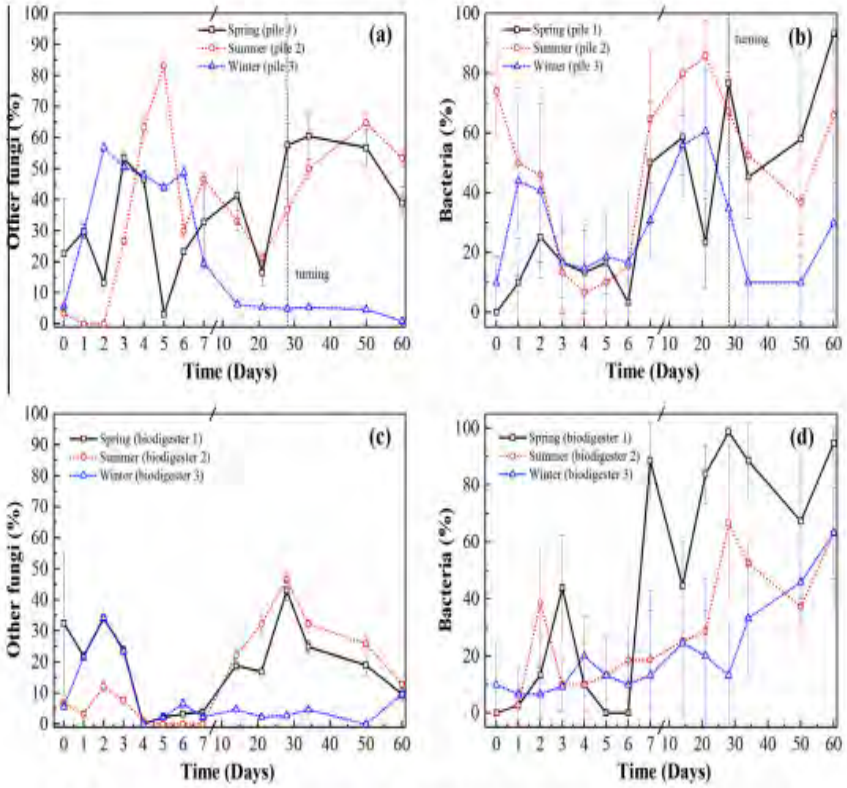

Fig. 6. Other fungi and bacteria time evolution for open piles (a and b) and discontinuous biodigesters (c and d).