Scientia Horticulturae 215 (2017) 38–48

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

# Anaerobic soil disinfestation reduces survival and infectivity of *Phytophthora nicotianae* chlamydospores in pepper

Paula Serrano-Pérez[a,*], Erin Rosskopf[b], Ana De Santiago[a], María del Carmen Rodríguez-Molina[a]

[a] CICYTEX, Instituto de Investigaciones Agrarias Finca La Orden-Valdesequera, A-5 km 372, 06187, Guadajira, Badajoz, Spain
[b] USDA, ARS, United States Horticultural Research Laboratory, 2001 S. Rock Rd., Fort Pierce, FL 34945, USA

ARTICLE INFO

*Article history:*
Received 24 May 2016
Received in revised form 2 December 2016
Accepted 5 December 2016
Available online 15 December 2016

*Keywords:*
Biofumigation
Biodisinfestation
Biological soil disinfestation
Reductive soil disinfestation

ABSTRACT

*Phytophthora nicotianae* is the principal causal agent of root and crown rot disease of pepper plants in Extremadura (western Spain), a spring-summer crop in this region. Preplant soil treatment by anaerobic soil disinfestation (ASD) may effectively control plant pathogens in many crop production systems, but field conditions and availability of C sources can limit its practical application. A laboratory experiment was conducted to study *P. nicotianae* control by ASD with low temperatures and several carbon (C) sources: rice bran, rapeseed cake, grape pomace and brewer's spent grain. Survival and infectivity of pepper by *P. nicotianae* chlamydospores were reduced with all C sources assayed and redox potential in all ASD treatments indicated that reductive soil conditions were achieved. Rice bran (20 tons $ha^{-1}$); rapeseed cake (20 tons $ha^{-1}$), and grape pomace (40 tons $ha^{-1}$) were also assayed in a field experiment in early spring. Survival and infectivity of *P. nicotianae* were also reduced with all C sources. An increase of dehydrogenase and urease activities and a strong pH decline were observed with rice bran and rapeseed cake, probably related to an increase of anaerobic bacterial populations in soil. Application of ASD with high C source rates may be effective in control of *P. nicotianae* under low temperature conditions.


## 1. Introduction

The ban on methyl bromide as a soil fumigant and the limitations on the use of other chemical products (1,3-dichloropropene and chloropicrin are under review, European Directive 91/414/EEC Regulation 1107/2009 on Plant Protection Products) have created a need for the evaluation of other forms of soilborne pathogen control (Colla et al., 2012).

Anaerobic soil disinfestation (ASD) (also referred as biological soil disinfestation, reductive soil disinfestation) is a biologically-based preplant soil treatment method developed to control plant disease and manage yield decline in many crop production systems (Blok et al., 2000; Shinmura, 2000). ASD involves incorporating an easily decomposable carbon-rich organic amendment into the soil, creating anaerobic soil conditions through soil saturation and covering with plastic film for a period of several weeks (Rosskopf et al., 2015). Soil saturation is critical for achieving anaerobic conditions. The use of clear gas-impermeable film, besides preventing the resupply of oxygen through the soil surface, elevates soil temperature and maintains soil moisture (Fennimore et al., 2013).

The efficacy of ASD is likely to be affected by field soil temperatures, consistency and degree of the anaerobic conditions, soil type, nature of the indigenous soil microorganisms, duration of the treatment, and susceptibility of the target plant pathogen (Koike and Gordon, 2015).

The degradation of organic matter is associated with microbial changes in the soil environment related to pathogen suppression (Hong et al., 2014; Huang et al., 2015; Mazzola, 2011; Mazzola et al., 2012; Mazzola and Manici, 2012; Momma et al., 2011; Mowlick et al., 2012, 2013a,b,c). Moreover, the application of soil amendments into satured soil leads to stronger reductions in redox potential (Eh) because oxygen is being consumed by the stimulated soil microbiota and reduced products are being released into the soil solution (Momma, 2008).

Incorporation of organic matter with ASD can enhance microbial biomass and enzyme activities as a result of increased organic C content in soil (Haynes, 1999). The dehydrogenase enzyme activ-

* Corresponding author.
*E-mail addresses:* paula.serranop@gobex.es (P. Serrano-Pérez), Erin.Rosskopf@ARS.USDA.GOV (E. Rosskopf), anarosario.santiago@gobex.es (A. De Santiago), carmen.rodriguez@gobex.es (M.d.C. Rodríguez-Molina).

http://dx.doi.org/10.1016/j.scienta.2016.12.003
0304-4238/

ity serves as indicator of the microbiological redox systems in soils, since most of this enzyme is produced by anaerobic microorganism (Tabatabai, 1982; Trevors, 1984) and it is often used as a direct measure of soil microbial activity (Trevors, 1984; García and Hernández, 1997). Pathogen suppression has been related to significantly higher values of dehydrogenase, β-glucosidase, urease and phosphatase activities (Núñez-Zofío et al., 2011). The reduction processes could increase phosphorus (P) solubility in acidic soils (Scalenghe et al., 2002) and phosphatase enzyme plays a critical role in P-cycle (Speir and Ross, 1978). Urease enzyme is responsible for the hydrolysis of organic material applied to the soil into $NH_3$ and $CO_2$, so it is related with the N-cycle. Generally, the ASD process has been related, among other factors, with changes in the redox potential, with the accumulation of $NH_4^+$ in the soil, increases of the organic matter content, and reductions of soil pH (which can affect to P solubility) (Butler et al., 2014b; Núñez-Zofío et al., 2011; Runia et al., 2014).

Solid materials, such as cereal bran, are easily incorporated, but it is often difficult to achieve sufficient disinfestation deep in the soil profile because their effect is limited to the depth of carbon source incorporation, which is typically approximately 15–20 cm (Strauss and Kluepfel, 2015). However, ASD using rice and wheat bran is being used in commercial strawberry fields in California (Shennan et al., 2014) and tomato and melon crops in Japan (Momma et al., 2013).

Although ASD does not require either high temperature (Goud et al., 2004; McCarty et al., 2014; Momma et al., 2006, 2013; van Overbeek et al., 2014; Runia et al., 2014) or long-term incubation (Butler et al., 2012b,c; Momma et al., 2010), soil temperature is, in addition to carbon source, a factor affecting the efficacy of ASD treatment on disease control (Shennan et al., 2013). ASD treatments where C-source rates were low (less than 1 mg $g^{-1}$), did not consistently decrease viability of *Sclerotinia sclerotiourum* or incidence of Fusarium root rot (Butler et al., 2014a). Thereby, these authors suggested that C source rate higher than 4 mg $g^{-1}$ of soil is required when soil temperatures during ASD treatment are low (15–25 °C).

*Phytophthora nicotianae* Breda de Haan (=*P. parasitica* Dastur) is the principal causal agent of root and crown rot in open field paprika pepper crops of Extremadura (western Spain), where the pathogen survives mainly as chlamydospores (Rodríguez-Molina et al., 2010). Solarization in summer effectively inactivated chlamydospores of *P. nicotianae* in field trials in this region (Rodríguez-Molina et al., 2016) and there have been reported decreases in densities of this pathogen after soil solarization (Butler et al., 2012b; Coelho et al., 1999; Juarez-Palacios et al., 1991; Lacasa et al., 2015). However, pepper for paprika is a spring-summer crop in Extremadura and non-chemical soil treatments to implement in spring, before the transplant of pepper plants, are necessary. Biofumigation with the application of *Brassicaceae* species as green manure in combination or not with seed meal of *Brassica carinata* (Biofence) was assayed in spring field conditions with no significant impact on survival and infectivity of *P. nicotianae* chlamydospores (Lacasa et al., 2015; Rodríguez-Molina et al., 2016).

The objective of the present study was to evaluate in laboratory and field conditions the effect of different C-sources for ASD with moderate soil temperatures on survival and infectivity of *P. nicotianae*. The effect of treatments on soil properties related with ASD process was assessed.

## 2. Materials and methods

### 2.1. Soil and organic amendments

A field experiment was established in a sandy loam soil (pH = 6.5; organic matter = 0.55%) at the Agricultural Research Institute Finca La Orden-Valdesequera (Extremadura, western Spain) and this soil was also collected for laboratory experiments. No soil fumigants had ever been previously applied to this area.

**Table 1**
Field application rates of different C sources calculated based on total carbon content.

| C source[z] | C (%) | C/N | Field dose[y] (kg $m^{-2}$) |
|---|---|---|---|
| Rice bran | 47 | 21 | 2 |
| Rapeseed cake | 50 | 11 | 2 |
| Grape pomace | 52 | 27 | 4 |
| Brewer's spent grain | 44 | 15 | 3.5 |

[z] Humidity: 51% in grape pomace; 40.2% in brewer's spent grain and insignificant in rice bran and rapeseed cake.
[y] Equivalent to 4 mg C $g^{-1}$ of soil (Butler et al., 2012c).

The C-sources used for ASD in this work were: rice bran powder, rapeseed cake pellet (var. *Tocatta*, a glucosinolate-free variety), grape pomace, and brewer's spent grain. All amendments were analyzed with a C/H/N determinator (CHN628 model, LECO Corporation, MI, USA) to determine carbon (C) content and carbon:nitrogen (C/N) ratio in order to calculate the field dose required according to the recommendations of Butler et al. (2012c) for soils with low temperatures (15–25 °C) during ASD (Table 1). The brewer's spent grain was mixed with chicken poultry pellet to decrease the C/N ratio. The brewer's spent grain treatment was not included in the field experiment. With the exception of rapeseed cake pellet (from Neiker-Tecnalia, Vitoria-Gasteiz, Spain) and chicken poultry pellet (from Fertinagro, Teruel, Spain), all amendments were obtained from local industries.

### 2.2. Inoculum production

The isolate P-23 of *P. nicotianae* used in the experiments was obtained from a pepper plant grown in Extremadura that showed disease symptoms (wilting and root and crown rot) and its pathogenicity was confirmed previously (Rodríguez-Molina et al., 2010).

Chlamydospores were produced using the procedure proposed by Rodríguez-Molina et al. (2016) that modifies the methodology described by Tsao (1971) and Mitchell et al. (1992). Briefly, six 15-mm diameter V8 juice agar plugs of the *P. nicotianae* isolate were transferred to a 20-cm Petri plate containing 150 ml of clarified V8 juice broth. After incubation at 25 °C in the dark for 10 days, V8 juice broth was removed and 150 ml of sterile distilled water was added to submerge the mycelial mat and the plate was further incubated at 18 °C in the dark for a minimun of 7 days. Once the chlamydospores were formed, the mycelial mats were rinsed in sterile distilled water on a cheesecloth and blended for 7 min in a homogenizer (MICCRA D-1, ART-moderne Labortechnik, Germany) with 50 ml of sterile distilled water and then subjected to a 60 s cycle (active interval: 0.9 s, passive interval: 0.1 s) of sonication (HD 2070, Sonoplus, Bandelin, Germany). The resulting suspension was centrifugated (2 min; 1760 rpm) and the pellet was resuspended in sterile distilled water. The total number of chlamydospores in the suspension was estimated with the aid of a Neubauer hemocytometer and viability of chlamydospores was determined by staining with rose bengal solution (Tsao 1971). Fresh chlamydospores were applied to soil [50 chlamydospores $g^{-1}$ dry soil] in a bioassay with 10 pepper plants to check the quality of the inoculum (data not shown).

Inoculum bags were prepared with agryl cloth containing sieved (2 mm sieve) and disinfected (autoclaved 1 h at 121 °C twice in two consecutive days) field soil inoculated with chlamydospores of *P. nicotianae* (5 g of soil and 2500 chlamydospores for the laboratory

experiments and 100 g of soil and 5000 chlamydospores for the field experiments) and the bag was closed by tying with string.

### 2.3. Laboratory experiments

In order to evaluate the effectiveness of different organic amendments as C sources for ASD, a closed controlled-temperature system was set up to emulate the physical, chemical, and microbial changes that take place in the field during ASD. In this system, chlamydospores of *P. nicotianae* were exposed to (i) all the compounds released into the soil solution during the ASD process or only to (ii) the volatile compounds generated during the process. The controlled system consisted of hermetic 1-l glass containers (14 cm height and 10 cm diam) that were filled with 600 g of soil that were mixed with the organic amendment and inoculated with a suspension of chlamydospores to obtain a final concentration of 50 chlamydospores $g^{-1}$ dry soil. Small inoculum bags, with 5 g of soil and 2500 chlamydospores, were also prepared and hung in the headspace of the container, avoiding contact with the soil placed in the bottom of the container. The soil in the bottom was saturated with 90 ml of tap water and the containers were hermetically sealed, placed in a complete randomized design with four replicates per treatment inside a programmable incubator and incubated for 4 weeks. The temperature regime in the incubator was that prevailing in soil at a depth of 20 cm during solarization in spring in western Spain [17.5 °C for 5 h/day; 22.5 °C for 5 h/day; 27.5 °C for 4 h/day; 32.5 °C for 2 h/day; 27.5 °C for 3 h/day; 22.5 °C for 5 h/day], as recorded in previous field experiments (Rodríguez-Molina et al., 2016).

Three different rates were assayed for each C source: half field dose (D1); field dose (D2) and one higher than field dose (D3). The treatments were: rice bran 1 kg $m^{-1}$ (RB1) (the conversion between weight and area units was based on the soil bulk density of 1.163 g $cm^{-3}$ and soil incorporation of 20-cm depth); rice bran 2 kg $m^{-1}$ (RB2); rice bran 3 kg $m^{-1}$ (RB3); rapeseed cake 1 kg $m^{-1}$ (RC1); rapeseed cake 2 kg $m^{-1}$ (RC2); rapeseed cake 4 kg $m^{-1}$ (RC3); grape pomace 2 kg $m^{-1}$ (GP1); grape pomace 4 kg $m^{-1}$ (GP2); grape pomace 8 kg $m^{-1}$ (GP3); brewer's spent grain 1.75 kg $m^{-1}$ + chicken poultry pellet 1.15 kg $m^{-1}$ (BC1); brewer's spent grain 3.5 kg $m^{-1}$ + chicken poultry pellet 2.3 kg $m^{-1}$ (BC2); brewer's spent grain 7 kg $m^{-1}$ + chicken poultry pellet 4.6 kg $m^{-1}$ (BC3). Control treatments, non-amended and inoculated (CPhy + ) and non-amended and non-inoculated (CPhy-) were included in the experiment. In addition, to verify the importance of soil microbiota in ASD, treatments with autoclaved soil (1 h at 120 °C twice in two consecutive days) mixed with the field dose were included for each C source: (RB2-AS); (RC2-AS); (GP2-AS) and (BC2-AS). Autoclaving was done before organic material application. Moreover, only as control for redox potential (Eh) data, a non-amended, non-inoculated and non-wetted treatment was included.

At the end of the incubation period, the containers were opened and the soil from the inoculum bags was analyzed to estimate the number of chlamydospores of *P. nicotianae* surviving after the volatile exposure. Briefly, the 5 g of soil from the bags were added to 45 ml of 0.25% water-agar (1:10, v:v) and stirred for 2 min; five 1-ml aliquots from this slurry were spread evenly over each of five Petri plates containing 12 ml of NARPH medium (Romero et al., 2007). After 48 h of incubation in the dark at 25 °C, the soil overlay was removed by gently washing the agar surface with tap water. Macroscopically visible colonies of *P. nicotianae* were counted and reported as CFU $g^{-1}$ dry soil.

The treated soil in the bottom of the containers was recovered and used to determine inoculum survival and infectivity.

After the incubation period, the redox potential of the soil (Eh; mV) was measured directly with a handheld ORP meter (HI36203, Hanna Instruments Inc., Woonsocket, RI, USA) while the pH was measured in slurry (1:1, v:v, with deionized water) with a pH electrode (HI12963, Hanna Instruments Inc., Woonsocket, RI, USA).

### 2.4. Field experiment

A randomized complete block design experiment with four replicates and a plot size of 1.5 × 1.5 m was established including three C sources for ASD treatment and two controls. There was a separation of 2 m between plots to prevent the cross-contamination between treatments. The treatments evaluated were: rice bran 20 tons $ha^{-1}$ (RB); rapeseed cake 20 tons $ha^{-1}$ (RC); grape pomace 40 tons $ha^{-1}$ (GP); control non-amendment (CP) and control non-amended and without plastic cover (C). On 13 April 2015, the C sources were added to the soil at 20-cm depth using a motorized plow and four inoculum bags, with 100 g of soil and 50 chlamydospores per gram of dry soil, were buried in each plot at 20-cm depth. Transparent polyethylene film (0.05 mm thick) was used to cover the plots (except treatment C), and the plastic was tucked in under the soil at the edges when it was applied. The soil was irrigated with 50 mm of water through a localized irrigation system under the plastic cover at the beginning of the experiment and, 14 days after, it was irrigated again with the same dose to keep it near to field capacity (VWC at 0.21 $m^3$ $m^{-3}$) in the first 24-cm depth. Following the 4-week ASD treatment, on 11 May 2015, plastic covers were removed, inoculum bags were recovered for inoculum survival and infectivity determination and one composite soil sample was collected at 0–25-cm depth from each plot to analyze physicochemical soil properties and microbial activity. In plots where weeds had grown, all vegetation was cut within the quadrant of the plot, weighed, and then oven-dried (102 °C) to calculate dried weight $m^{-2}$.

Soil temperature was continuously monitored at 20-cm depth, using soil probes and an automatic data logging system (HOBO Weather Logger, Pocasset, MA, USA) as well as moisture (volumetric water content, VWC) using 10HS sensors ($ECH_2O$, Decagon Devices Inc., Pullman, WA, USA), during the 4-week period. Soil samples were collected for soil pH analysis before initiation of treatments and every 7 days during the ASD treatment. A pH electrode (HI12963, Hanna Instruments Inc., Woonsocket, RI, USA), in a 1:1 v/v slurry of soil and deionized water, was used for pH determination.

### 2.5. Survival and infectivity of inoculum

For inoculum survival determination, 2 g of soil was flooded with distilled water in individual 9-cm Petri plates, and immature carnation petals were floated over the soil suspension to bait *P. nicotianae* (Tello et al., 1991). After 2–7 days, carnation petals were microscopically observed for the presence of *P. nicotianae*. In laboratory experiments, 10 subsamples from the soil in the bottom of each container were analyzed as described above, and in the field trial, three subsamples were prepared from each inoculum bag. Results of survival are expressed as the percentage of soil samples where *P. nicotianae* was detected (Lacasa et al., 2015).

The remainder of the soil from the bottom of containers in laboratory experiments and from each field inoculum bag was used to perform bioassays in order to determine chlamydospores infectivity on pepper plants. The soil was transferred to 250 $cm^3$ plastic pots (5 pots per container from laboratory experiments and 1 pot per inoculum bag from field experiments) with a disinfested (autoclaved for 1 h at 121 °C) vermiculite:peat (1:3, v:v) mixture. One pepper seedling (*Capsicum annuum* L. cv. *Jaranda*) at the 2–4-true-leaf stage was transplanted into each pot, with its roots placed in contact with the treated soil. Plants were grown in a growth chamber with a 16 h light at 28 °C/8 h dark at 24 °C cycle, and disease symptoms were recorded every week over a period of 2 months.

As plants died, roots were washed with tap water and fragments of roots and crown were placed on potato dextrose agar and on NARPH medium to isolate the pathogen. At the end of the bioassay, the root systems of all plants were examined for the presence of disease symptoms. Results of infectivity are expressed as the percentage of diseased plants (Coelho et al., 2000; Lacasa et al., 2015) and, because the blocks were kept separate, there were four repetitions per treatment.

### 2.6. Physicochemical soil properties

Soil samples were collected from each plot at two times: (i) before treatment application (0 weeks) and (ii) at the end of the experiment (4 weeks). Prior to analysis, soils were air-dried and ground to pass through a 2-mm sieve. Organic matter (OM) was determined by dichromate oxidation (Walkley and Black, 1934). The electrical conductivity (EC) was measured in water at a soil:extractant ratio of 1:5. Ammonium ($NH_4^+$) content was determined after extraction with 2 M KCl according to (Mulvaney, 1996). Available phosphorus (P) was measured using the molybdate reactive method (Murphy and Riley, 1962) after bicarbonate extraction (Olsen, 1954).

### 2.7. Soil enzyme activity

Enzyme activities were determined on the same soil samples collected for physicochemical characterization. β-glucosidase activity was determined by using 25 mM *p*-nitrophenyl-β-D-glucopyranoside (EC 219.661.3) as substrate according to Eivazi and Tabatabai (1988) and it was determined as the amount of p–nitrophenol (PNP) formed from p–nitrophenyl-β-D-glucoside (PGN). Acid phosphatase activity was determined at pH 6.3, using 16 mM *p*-nitrophenyl phosphate (EC 206.353.9) as substrate according to Tabatabai and Bremner (1969). For both enzyme activities, the concentration of PNP was determined photometrically at 400 nm. Dehydrogenase activity was determined by measuring the amount of triphenylformazan (TPF) released after incubating the soil with 2,3,5-triphenyl-tetrazolium chloride. TPF was extracted with methanol (Trevors et al., 1982) and determined by reading at 490 nm. Urease activity was assayed by the modified method of Nannipieri et al. (1980). Briefly, 4 ml of 0.1 M phosphate buffer (pH 7.1) and 1 ml of 1.067 M urea were added to 1-g soil samples. The samples were shaken at 25 °C for 1 h, and thereafter, 5 ml of 2 M KCl were added to terminate the reaction. The N-$NH_4^+$ was measured by colorimetric analysis according to Mulvaney (1996) at 667 nm.

### 2.8. Data analysis

Data were analyzed by one-way analysis of variance (ANOVA), comparing means of the treatments by Tukey's multiple range test, in the case of percentage of survival and infectivity of inoculum, and by a Dunnett's T3 test, that does not assume equal variances, in the case of weed data. Data of *P. nicotianae* chlamydospores survival exposed to the volatile compounds generated during ASD, expressed as CFU $g^{-1}$ dry soil, were subjected to one-way ANOVA for each C-source on log-transformed data ($\log(x+1)$) followed by Tukey's multiple range test. Field pH was analyzed with two-way analysis of variance for repeated measures (rmANOVA), including statistical significance for the effects of treatment (between-subjects factor) and sampling time (within-subjects factor), as well as the interactions between them. When assumption of sphericity was violated, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. Data on soil enzyme activity and soil properties were also subjected to two-way rmANOVA, studying the effects of treatments (between-subjects factor) and sampling time (within-subjects factor) and their interactions. Post-rmANOVA means comparisons by profile contrast were carried out with Bonferroni's correction. Unless otherwise specified, effects and differences were considered significant at $P < 0.05$. All analyses were performed with the software package SPSS version 20.0 (SPSS Inc., Chicago, Illinois, USA).

## 3. Results

### 3.1. Laboratory experiments

All doses of the amendments assayed, except GP, significantly decreased the infectivity of the pathogen with respect to the control without amendment, but differences between doses among the same C source were not detected in any case (Fig. 1). For RB, RC, and BC treatments the doses D2 and D3 decreased the survival bellow 40%, significantly lower than in the control treatments (above 85%), and the dose D1 was not statistically different from the control (Fig. 1). With RB and BC amendments, decreases in survival with doses D2 and D3 were significantly higher than with dose D1, but differences were not significant with RC amendment. With GP treatment, neither infectivity nor survival decreased significantly with respect to the control, regardless of the dose assayed. This lack of significance may be due largely to low levels of infectivity and survival detected in the control treatment. Conversely, treatments with the field dose (D2) with autoclaved soil (RB2-AS, RC2-AS, BC2-AS and GP2-AS) did not show reduction either in survival or in infectivity compared with the control, except in BC2-AS where a decline of survival was observed (Fig. 1).

The volatiles released by the amendments in the ASD laboratory experiment did not affect the viability of chlamydospores with any of the doses tested, except the treatments RB1 and RB2 that caused significant decreases in viability compared to RB3 (Table 2). Since there were no significant differences between the controls CPhy+ and CPhy- for each amendment, the data of these treatments were pooled for statistical analyses. Significant decreases in viability of chlamydospores were found in the control treatments.

Soil Eh was kept around 237 mV in non-amended and non-wetted soil (Cnw) (Table 3); however, it dropped sharply below −124 mV in all wetted soils. The lowest Eh was observed in the soils amended with brewer's spent grain and poultry manure, in which Eh decreased near to −200 mV, even with autoclaved soil, but it was not significantly lower than with those soils amended with the other C-sources. No differences were found in pH values among treatments at the end of the experiment (data not shown).

### 3.2. Field experiment

Soil temperatures at 20-cm in plastic covered treatments ranged from 15 to 35 °C and there was a similar trend of soil temperatures in all covered treatments. In covered plots, the greatest number of hours registered (∼200 h) were between 20 °C and 25 °C and approximately 150 h above 25 °C (Fig. 2). Soil temperatures in the non-covered treatment ranged from 12 to 27.5 °C, accumulating the highest number of hours (217 h) around 17.5 °C and only 15 h above 25 °C.

Soil moisture in covered plots was maintained around field capacity (20–22 $m^3\ m^{-3}$ of VWC) throughout the experiment, while in plots without plastic cover, soil moisture decreased inconstantly due to isolated rains to values of 12 $m^3\ m^{-3}$ of VWC.

Soil pH did not significantly change over time in the control treatments (C and CP) or in the GP treatment (Fig. 3). However, a strong decline of soil pH was observed for RB and RC treatments during the first week of the field experiment, with pH values below 6. After the first week, pH values gradually increased until the end of

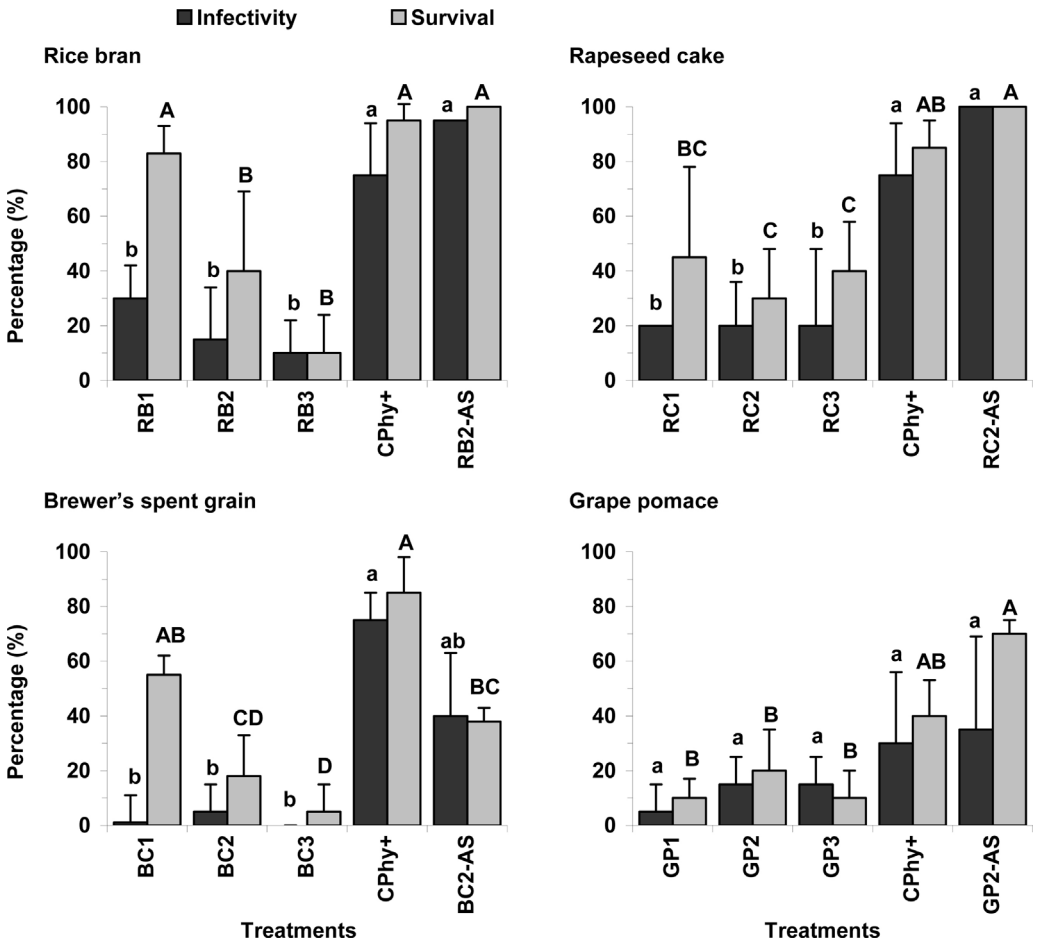


**Fig. 1.** Survival and infectivity of *Phytophthora nicotianae* after ASD laboratory experiments. Rice bran 1 kg m$^{-2}$ (RB1); rice bran 2 kg m$^{-2}$ (RB2); rice bran 3 kg m$^{-2}$ (RB3); rapeseed cake 1 kg m$^{-2}$ (RC1); rapeseed cake 2 kg m$^{-2}$ (RC2); rapeseed cake 4 kg m$^{-2}$ (RC3), grape pomace 2 kg m$^{-2}$ (GP1); grape pomace 4 kg m$^{-2}$ (GP2); grape pomace 8 kg m$^{-2}$ (GP3); brewer's spent grain 1.75 kg m$^{-2}$ + chicken poultry pellet 1.15 kg m$^{-2}$ (BC1); brewer's spent grain 3.5 kg m$^{-2}$ + chicken poultry pellet 2.3 kg m$^{-2}$ (BC2); brewer's spent grain 7 kg m$^{-2}$ + chicken poultry pellet 4.6 kg m$^{-2}$ (BC3) and non-amended and inoculated control (CPhy+). Treatments followed by AS were prepared with autoclaved soil (1 h, 121 °C twice in two consecutive days). Survival was determined by detecting *P. nicotianae* with carnation petals as baits and expressed as% of positive baits. Infectivity was determined by bioassays with pepper plants and expressed as% of diseased plants. Values are means ± SD (n = 4). For each C-source and parameter, bars with different letter are significantly different (ANOVA ($P < 0.05$) followed by Tukey's multiple range test ($P < 0.05$)).

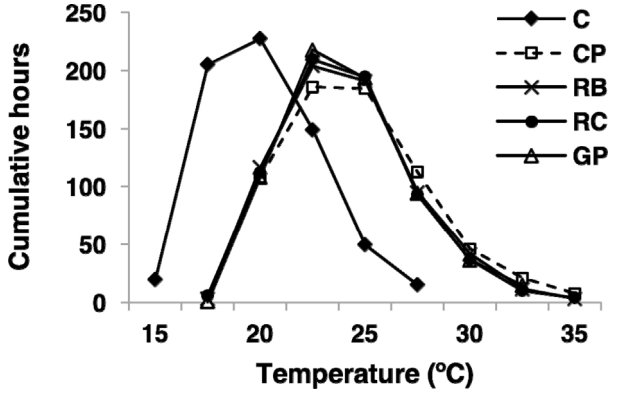


**Fig. 2.** Mean soil temperature at 20-cm depth during the ASD field experiment, expressed as cumulative time between different temperature ranks. Non-amended control without plastic cover (C); non-amended control (CP); rice bran 20 tons ha$^{-1}$ (RB); rapeseed cake 20 tons ha$^{-1}$ (RC); grape pomace 40 tons ha$^{-1}$ (GP). Values represent means of data collected from two sensors.

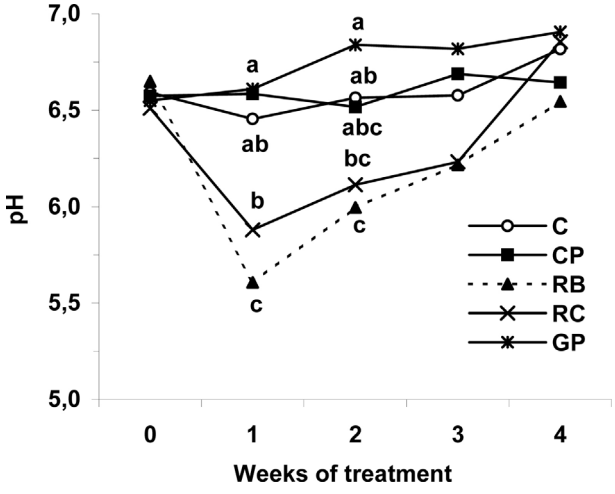


**Fig. 3.** Evolution of soil pH during the ASD field experiment. Non-amended control without plastic cover (C); non-amended control (CP); rice bran 20 tons ha$^{-1}$ (RB); rapeseed cake 20 tons ha$^{-1}$ (RC); grape pomace 40 tons ha$^{-1}$ (GP). Values are means (n = 4). Means for each week of treatment followed by a different letter are statistically different (rmANOVA ($P < 0.05$) followed by multiple comparison test with Bonferroni's corrections ($P < 0.05$)).

the 4-week experiment, when no differences were found between treatments (Fig. 3).

There was a significant effect of treatment on survival of *P. nicotianae* from recovered inoculum bags (Fig. 4). The estimated

**Table 2**
Number of chlamydospores of *P. nicotianae* surviving after exposure to the volatile compounds generated during the ASD process.

| C source | Treatments [z] | CFU $g^{-1}$ dry-soil [y] |
|---|---|---|
| Rice bran | RB1 | 315 ± 108 b |
| | RB2 | 366 ± 103 b |
| | RB3 | 608 ± 140 a |
| | RB2-AS[x] | 294 ± 124 b |
| | C | 196 ± 90 c |
| Rapeseed cake | RC1 | 378 ± 44 a |
| | RC2 | 536 ± 67 a |
| | RC3 | 482 ± 254 a |
| | RC2-AS[x] | 355 ± 97 a |
| | C | 230 ± 140 b |
| Grape pomace | GP1 | 324 ± 157 ab |
| | GP2 | 419 ± 52 a |
| | GP3 | 430 ± 82 a |
| | GP2-AS[x] | 480 ± 74 a |
| | C | 308 ± 127 b |
| Brewer's spent grain | BC1 | 129 ± 44 ab |
| | BC2 | 267 ± 83 a |
| | BC3 | 210 ± 179 ab |
| | BC2-AS[x] | 187 ± 117 ab |
| | C | 102 ± 84 b |

[z] Rice bran 1 kg m-2 (RB1); rice bran 2 kg m-2 (RB2); rice bran 3 kg m-2 (RB3); rapeseed cake 1 kg m-2 (RC1); rapeseed cake 2 kg m-2 (RC2); rapeseed cake 4 kg m-2 (RC3), grape pomace 2 kg m-2 (GP1); grape pomace 4 kg m-2 (GP2); grape pomace 8 kg m-2 (GP3); brewer's spent grain 1.75 kg m-2 + chicken poultry pellet 1.15 kg m-2 (BC1); brewer's spent grain 3.5 kg m-2 + chicken poultry pellet 2.3 kg m-2 (BC2); brewer's spent grain 7 kg m-2 + chicken poultry pellet 4.6 kg m-2 (BC3). Data of non-amended and inoculated control and non-amended and non-inoculated control were combined (C).

[y] Initial inoculum density was 500 chlamydospores g-1 dry soil. Values are means ± SD (n=4) of CFU g-1 dry soil. Means for each C-source followed by a different letter are statistically different (ANOVA ($P < 0.05$) followed by Tukey's multiple range test ($P < 0.05$)).

[x] Autoclaved 1 h, 121 °C twice in two consecutive days.

**Table 3**
Soil redox potential (Eh) measured directly in the ASD containers at the end of laboratory experiments.

| C source | Treatments [z] | Eh [y] |
|---|---|---|
| Rice bran | RB1 | −192 ± 10 cd |
| | RB2 | −182 ± 01 bcd |
| | RB3 | −202 ± 06 cd |
| | RB2-AS[x] | −147 ± 33 bc |
| Rapeseed cake | RC1 | −182 ± 04 cd |
| | RC2 | −198 ± 13 cd |
| | RC3 | −203 ± 13 cd |
| | RC2-AS[x] | −175 ± 13 bcd |
| Grape pomace | GP1 | −173 ± 35 bcd |
| | GP2 | −189 ± 10 cd |
| | GP3 | −181 ± 14 bcd |
| | GP2-AS[x] | −159 ± 03 bcd |
| Brewer's spent grain | BC1 | −211 ± 22 d |
| | BC2 | −210 ± 14 d |
| | BC3 | −195 ± 29 cd |
| | BC2-AS[x] | −212 ± 25 d |
| Controls | C | −124 ± 15 b |
| | Cnw | 237 ± 27 a |

[z] Rice bran 1 kg $m^{-2}$ (RB1); rice bran 2 kg $m^{-2}$ (RB2); rice bran 3 kg $m^{-2}$ (RB3); rapeseed cake 1 kg $m^{-2}$ (RC1); rapeseed cake 2 kg $m^{-2}$ (RC2); rapeseed cake 4 kg $m^{-2}$ (RC3), grape pomace 2 kg $m^{-2}$ (GP1); grape pomace 4 kg $m^{-2}$ (GP2); grape pomace 8 kg $m^{-2}$ (GP3); brewer's spent grain 1.75 kg $m^{-2}$ + chicken poultry pellet 1.15 kg $m^{-2}$ (BC1); brewer's spent grain 3.5 kg $m^{-2}$ + chicken poultry pellet 2.3 kg $m^{-2}$ (BC2); brewer's spent grain 7 kg $m^{-2}$ + chicken poultry pellet 4.6 kg $m^{-2}$ (BC3). Data of non-amended and inoculated controls and non-amended and non-inoculated controls were combined (C). Data of non-amended, non-inoculated and non-wetted treatments of each amendment were pooled (Cnw).

[y] Values are means ± SD (n = 4). Means followed by a different letter are statistically different (ANOVA ($P < 0.05$) followed by Tukey's multiple range test ($P < 0.05$)).

[x] Autoclaved 1 h, 121 °C twice in two consecutive days.

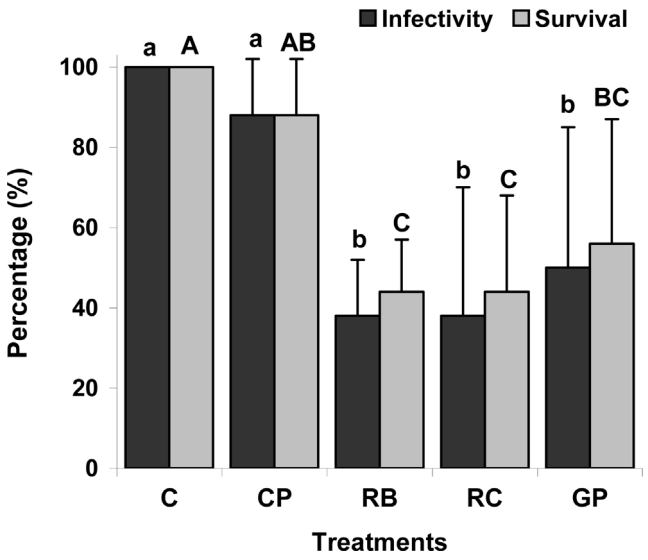


**Fig. 4.** Survival and infectivity of *Phytophthora nicotianae* in inoculum bags buried at 20-cm depth in the soil during the ASD field experiment. Non-amended control without plastic cover (C); non-amended control (CP); rice bran 20 tons $ha^{-1}$ (RB); rapeseed cake 20 tons $ha^{-1}$ (RC); grape pomace 40 tons $ha^{-1}$ (GP). Survival was determined by detecting *P. nicotianae* with carnation petals as baits and expressed as% of positive baits. Infectivity was determined by bioassays with pepper plants and expressed as% of diseased plants. Values are means ± SD (n = 4). For each parameter, bars with different letter are significantly different (ANOVA ($P < 0.05$) followed by Tukey's multiple range test ($P < 0.05$)).

survival was high (above 88%) in both control treatments without C sources (C and CP), achieving 100% of survival when plastic cover was not used, while percentages of survival were significantly reduced (below 56%) in all ASD treatments compared to the control C, as well as compared to the control CP in RB and RC treatments. No statistical differences were found between ASD treatments. The use of a plant disease bioassay confirmed the pathogenicity of the surviving population of *P. nicotianae*. There was a significant effect of treatment on infectivity, with trends very similar to that observed for survival. All ASD treatments (RB, RC and GP) resulted in significant decreases in infectivity compared to the controls (C and CP), but there were no differences in infectivity among ASD treatments (Fig. 4).

Weed biomass was affected by treatment (Fig. 5). Control with plastic cover had the highest level of weeds, with *Cyperus rotundus* and *Setellaria media* being the predominant species. However, weed populations were low in the treatments RC and RB, as well as in the uncovered control. GP treatment resulted in inconsistent control due to differences between blocks.

### 3.3. Physicochemical soil properties

Physicochemical soil properties before and after ASD field experiment are presented in Table 4. The soil analysis before C-source incorporation (0 weeks) did not show differences between plots for any parameter studied, indicating homogeneity of the plots before establishment of trial. At the end of the field experiment, there were significant increases in EC (except for GP treatment), although without significant differences between treatments. All ASD treatments significantly increased the values of OM and were different from controls. The increases in $NH_4^+$ were also significant in all cases, as well as differences between treatments at the end of the experiment, with the quantity of $NH_4^+$ in treatment RC being significantly greater than in C, CP, and GP treatments. The amount of $NH_4^+$ in RB was also high, although it was not significantly different from CP and GP. The increases in available P were significant only in RB and RC treatments, although

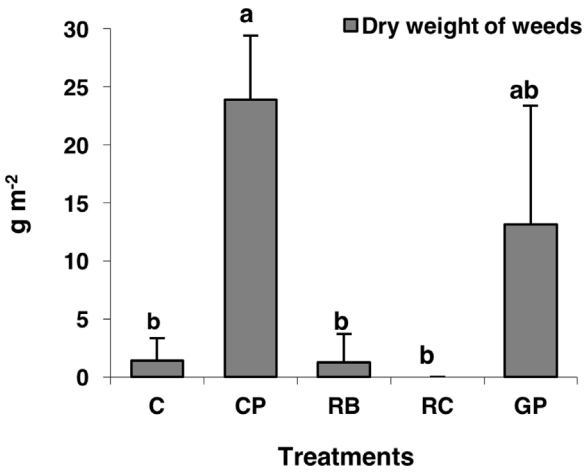


**Fig. 5.** Weeds grown in plots after ASD field experiment, calculated as dried weight $m^{-2}$ at the end of the field experiment. Non-amended control without plastic cover (C); non-amended control (CP); rice bran 20 tons $ha^{-1}$ (RB); rapeseed cake 20 tons $ha^{-1}$ (RC); grape pomace 40 tons $ha^{-1}$ (GP). Values are means ± SD (n = 4). Bars with a different letter are statistically different (ANOVA (P < 0.05) followed by Dunnet's T3 test (P < 0.05)).

**Table 4**
Soil properties before C-sources incorporation (0 weeks) and at the end of the treatments in the field experiment (4 weeks).

| | EC dS/m | OM % | $NH_4^+$ mg/kg soil | P mg/kg soil |
|---|---|---|---|---|
| *0 weeks* [z] | | | | |
| | 0.042 ± 0.002 | 0.54 ± 0.02 | 0.2 ± 0.03 | 31.83 ± 1.23 |
| *4 weeks* [x] | | | | |
| C | 0.069 ± 0.005 a* | 0.53 ± 0.07 c | 7.72 ± 0.42 c* | 33.61 ± 1.90 a |
| CP | 0.059 ± 0.005 a* | 0.52 ± 0.08 c | 8.19 ± 0.76 bc* | 34.93 ± 5.15 a |
| RB | 0.058 ± 0.005 a* | 1.24 ± 0.26 a* | 14.91 ± 4.56 ab* | 37.91 ± 3.14 a* |
| RC | 0.076 ± 0.004 a* | 0.76 ± 0.04 b* | 34.99 ± 33.20 a* | 34.81 ± 3.26 a* |
| GP | 0.048 ± 0.005 a | 0.8 ± 0.09 b* | 8.46 ± 0.50 bc* | 34.85 ± 4.23 a |

Non-amended control without plastic cover (C); non-amended control (CP); rice bran 20 tons $ha^{-1}$ (RB); rapeseed cake 20 tons $ha^{-1}$ (RC); grape pomace 40 tons $ha^{-1}$ (GP).

[z] No significant differences between plots. Values for each parameter are means of all plots ± SD (n = 20).

[x] Means ± SD (n = 4). For each parameter, means with different letter are statistically different (rmANOVA (P < 0.05) followed by multiple comparison test with Bonferroni's corrections (P < 0.05)).

*Means are statistically different from the mean at 0 weeks for each parameter.

no differences were found between treatments at the end of the experiment.

### 3.4. Soil enzyme activity

Soil enzymatic activities before and after ASD treatments are presented in Table 5. Similar to the physicochemical properties, there were no differences among plots at the beginning of experiments for any enzymatic activity studied. RB and RC treatments significantly increased the activity of dehydrogenase in the soil, although only RB treatment was significantly different from C, CP, and GP at the end of the experiment. Urease activity increased significantly in RC treatment and it was significantly different from C, CP, and GP treatments. The levels of acid phosphatase activity did not change significantly after the experiment in any case, although RC was the treatment with the highest value. Significant increases in β-glucosidase activity were found in C, RB, and GP treatments, but no differences were found between treatments at the end of the experiment.

## 4. Discussion

In this study, ASD using rice bran powder (RB), rapeseed cake pellets (RC), brewer's spent grain (BC), and grape pomace (GP) as C-sources under spring soil temperature conditions was effective in reducing infectivity and survival of *P. nicotianae* compared to the controls, both in field and laboratory experiments. Under laboratory conditions addition of GP did not result in significant reduction, possibly due to the low level of infectivity and survival detected in the control.

The direct thermal disinfestation caused by increased soil temperatures (solarization) is disregarded in our study, given the relatively low temperatures registered during the field experiment and the incubation temperature regime in laboratory experiments. Coelho et al. (2000) demostrated that under a thermal exposure of 35 °C for 480 h *P. nicotianae* can survive for a long period, requiring a minimum of 38 °C, in excess of 288 h to cause a population decline below 1 ppg of soil.

The infectivity of *P. nicotianae* chlamydospores of pepper plants was lower than 50% for all C-sources assayed in the field experiment while, in laboratory experiments, it was below 20% in all ASD treatments. This difference may be related to the controlled conditions in the laboratory in contrast to the field. Moreover, homogeneous distribution of the amendments in the soil, and the way in which the inoculum was introduced into the soil in the field experiment, may also explain in part the higher levels of infectivity, since the inoculated soil was wrapped in hydrophilic agril cloth and the chlamydospores were not in direct contact with the treated soil. Butler et al. (2012a) also used buried inoculum packets in assessing control of *F. oxysporum* and *S. rolfsii* and their results suggested that pathogen control in field conditions would be more consistent than the observed in the soil isolated inside the inoculum bag, due to greater exposure of natural inoculum to sites of residue decomposition within the soil.

For each C-source, the three doses assayed under laboratory conditions resulted in similar and significant reductions of infectivity with respect to the control treatment, and BC was the most effective C-source. However, survival with the lowest dose (D1) for each C-source was not different from the control treatments, indicating that half field rate was not effective for suppression of the inoculum, although it is possible that the treatments caused a weakening of inoculum, which lowered its infectivity (Rodríguez-Molina et al., 2016; Termorshuizen and Jeger, 2014). Results of the field experiment, with percentages of survival similar to those of infectivity for all treatments, confirm the results found under laboratory conditions, indicating that the dose of 4 mg of C per g of soil recommended by Butler et al. (2012c) for ASD treatment with low soil temperatures is effective to control the disease under regional spring field conditions. Moreover, increasing the dose is unnecessary with regard to pathogen suppression and the application of the highest dose (D3) may be infeasible under field conditions.

None of the ASD treatments assayed eliminated the pathogen inoculum, neither in laboratory nor in field experiments. Probably, a reduction of 60% in chlamydospores survival is not sufficient to control the disease in field conditions, but it is necessary indicate that the inoculum density used in our experiments (50 chlamydospores $g^{-1}$ dry soil) was extremely high compared to inoculum densities found in naturally infested soils (Shew, 1983).

Application of rice bran or rapeseed at 2 kg $m^{-2}$ as C-sources in ASD reduced effectively *P. nicotianae* inoculum in the field conditions of the present study. The same rate of rice bran was evaluated in California strawberry fields and reduced the number of *V. dahliae* microsclerotia by 85–100% with soil temperatures above 17 °C (Shennan et al., 2013). Previously, Yossen et al. (2008) had reported effective control with wheat bran at 1 kg $m^{-2}$ and soil temperatures over 30 °C, in a carnation greenhouse naturally infested with

**Table 5**
Soil enzyme activities before C-sources incorporation (0 weeks) and at the end of the treatments in the field experiment (4 weeks).

| | Dehydrogenase mg TPF $kg^{-1}$ soil h | Phosphatase mg PNP $kg^{-1}$ soil h | Urease mg N-$NH_4^+$ $kg^{-1}$ soil h | β-Glucosidase mg PNP $kg^{-1}$ soil h |
|---|---|---|---|---|
| *0 weeks* [z] | | | | |
| | 1.02 ± 0.11 | 148.38 ± 8.02 | 2.57 ± 0.21 | 55.69 ± 3.92 |
| *4 weeks* [x] | | | | |
| C | 0.99 ± 0.29 b | 165.77 ± 15.80 a | 1.93 ± 0.26 b | 84.43 ± 6.11 a* |
| CP | 0.77 ± 0.16 b | 148.01 ± 9.24 a | 1.28 ± 0.24 b | 52.67 ± 18.83 a |
| RB | 4.82 ± 1.06 a* | 157.33 ± 15.82 a | 3.32 ± 0.88 ab | 89.51 ± 19.87 a* |
| RC | 3.27 ± 2.01 ab* | 183.16 ± 73.23 a | 6.3 ± 4.09 a* | 78.78 ± 7.48 a |
| GP | 1.63 ± 0.52 b | 154.04 ± 23.27 a | 1.7 ± 0.07 b | 82.71 ± 29.10 a* |

Non-amended control without plastic cover (C); non-amended control (CP); rice bran 20 tons $ha^{-1}$ (RB); rapeseed cake 20 tons $ha^{-1}$ (RC); grape pomace 40 tons $ha^{-1}$ (GP).
[z] No significant differences between plots. Values for each parameter are means of all plots ± SD (n = 20).
[x] Means ± SD (n = 4). For each parameter, means with different letter are statistically different (rmANOVA ($P < 0.05$) followed by multiple comparison test with Bonferroni's corrections ($P < 0.05$)).
*Means are statistically different from the mean at 0 weeks for each parameter.

*F. oxysporum*. On other hand, Zavatta et al. (2014) obtained similar strawberry yields using dry ground grape pomace at 2 kg $m^{-2}$ (with a pre-plant fertilizer), rice bran or methyl bromide in a California trial.

The effectiveness of *Brassicaceae* species as a C-source in ASD has also been evaluated with positive results in suppression of several pathogens (Blok et al., 2000; Hewavitharana et al., 2014; McCarty et al., 2014; Mowlick et al., 2013c, 2014). The rapeseed cake pellets used in our study were produced with a glucosinolate-free variety used for livestock feeding, so that the toxic effect of isothiocyanates released from *Brassicaceae* tissues to the soil atmosphere is not the mechanism involved in the control under ASD process, which is consistent with previous studies (Blok et al., 2000; Goud et al., 2004; Mazzola et al., 2001). Biofumigation with *Brassicaceae* cover crops, or *Brassica* pellets or both amendments, under spring soil temperature conditions similar to those in field experiments reported here, was not effective in suppression of *P. nicotianae*, although reduction of infectivity was found related to the application of plastic cover, suggesting the involvement of anaerobiosis in this reduction (Lacasa et al., 2015; Rodríguez-Molina et al., 2016).

The application of plastic without amendment (CP treatment) did not suppress *P. nicotianae* in contrast to ASD treatments, although anaerobic conditions were achieved in both cases, suggesting that anaerobiosis alone is not sufficient for pathogen suppression. Momma et al. (2013) reported that survival of *F. oxysporum* placed in hydrogen gas-treated water was not affected, which also indicates that reductive condition is not sufficient for pathogens suppressions. Blok et al. (2000) also found anaerobic conditions in covered plots regardless of incorporation of organic amendment, but application of plastic alone did not result in significant reduction of pathogen populations. Although suppression of soilborne pathogens under anaerobic conditions generated during flooding is well-known (Menzies, 1963; Pullman et al., 1981; Stover, 1955), in all of these studies, a long period of flooding was necessary for pathogen suppression. A recent study showed that the survival period of a pathogen under anaerobic conditions became shorter as the incubation temperature became higher. Thereby, *P. cactorum* did not survive after an incubation period of 147 days at 25 °C or 43 days at 30 °C under anaerobic atmosphere (Ebihara and Uematsu, 2014). These conditions of time-temperature under anaerobiosis did not occur in our trials.

In our laboratory experiments, adding C-sources to the non-autoclaved soil induced stronger reductions in redox potential (Eh ≈ −200 mV) than in the control treatment without amendment (Eh ≈ −124 mV). In treatments with autoclaved soil, reduction would be induced by microbes immigrated with organic amendment, this being more noticeable in BC2-AS treatment. Similar results were observed by Wen et al. (2015) using rice or maize straw at 30 °C during a 30-day controlled experiment, in which soil Eh decreased to approximately −200 mV in ASD treatment, while in the flood alone treatment Eh was maintained around −100 mV. During the ASD process, the speed and magnitude of the Eh decrease varied with the organic matter added (Kobara et al., 2007; Uematsu et al., 2007). In a 7-week field experiment of ASD for nut-tree nurseries, rice bran applications at 15.7 and 20.2 tons $ha^{-1}$ suppressed *Agrobacterium tumefaciens* and *Pythium* spp. and low redox potentials (Eh ≈ −300 mV) were observed (Strauss and Kluepfel, 2015). In California, Shennan et al. (2013) reported that a cummulative anaerobicity (average redox potentials below a critical value indicative of anaerobic conditions) of 50,000 mV $h^{-1}$ at 25 °C of soil temperature was necessary for control of *V. dahliae*. This anaerobic threshold may be used as an indicator that the ASD process is being performed successfully.

Under the reductive soil conditions of the ASD, metal ions such as $Mn^{2+}$ and $Fe^{2+}$ formed in soil solution might be one of the mechanism of pest suppression (Momma et al., 2011; van Bruggen and Blok, 2014). Changes in color in the ASD treated soils were observed in our laboratory experiments, showing grayish-blueish colors, indicating the accumulation of toxic $Fe^{2+}$ and $Mn^{2+}$ ions in the soil solution (FAO, 2010) although in this work, these elements were not directly determined. In the same way, gray patches predominated on the soil surface in RB and RC plots at the end of the field experiment, indicating reduced soil conditions (FAO, 2010).

The decline in soil pH in RB and RC treatments, as well as an unpleasant odor, suggested that toxic organic acids were released to the soil solution, through the fermentation of the C-sources by anaerobic bacteria, as some authors have previously observed (Butler et al., 2012a,b; Huang et al., 2015; Momma et al., 2006; Runia et al., 2014). After the first phase of pH decline, a gradual increase of pH was observed by the decomposition of the organic acids (Rosskopf et al., 2015) and by the ammonification process (Ladd and Jackson, 1982). In contrast, soil pH was minimally affected by GP treatment suggesting that organic acids from the anaerobic breakdown of this C-source were not present in sufficient quantities to lower soil pH (Butler et al., 2014a), perhaps due to the less easily decomposable components of this amendment. Several studies have shown that soil pH response is highly influenced by the type of amendment incorporated into soil (Rosskopf et al., 2015). The enzyme β-glucosidase is very sensitive to changes in pH, and soil management practices (Acosta-Martínez and Tabatabai, 2000; Madejón et al., 2001), however, no significant differences in its activity were detected between treatments. This may be due to the recovery of initial values of soil pH when this enzymatic activity was analyzed at the end of the field experiment.

High accumulation of $NH_4^+$ and significant increases in urease activity were observed with RB or RC treatments according to previous ASD studies (Butler et al., 2014b; Núñez-Zofío et al., 2011; Runia et al., 2014). This accumulation of $NH_4^+$ suggests inhibition of nitri-

fication due to lack of oxygen (Bodelier et al., 1996), although the process known as dissimilatory nitrate reduction could also play a role (Broadbent and Stojanovic, 1952). In a recent study, Cao et al. (2014) suggested that a high concentration of $NH_4^+$ was associated with suppression of mycelial growth and zoospore germination of *P. capsici* in an anaerobically digested pig slurry. In contrast, the higher C/N ratio of GP amendment (27:1) could lead to immobilization of N into soil microbial biomass (Rosskopf et al., 2015).

A slightly impact of RB and RC treatments on soil P status was observed probably due to the higher solubility of the P in these acidified soils (Scalenghe et al., 2002), although that did not affect to the acid phosphatase activity.

Before treatments, dehydrogenase levels in soil were similar to those reported in other studies (Leirós et al., 2000; Quilchano and Marañón, 2002; Trasar-Cepeda et al., 2000). RB and RC treatments increased the enzyme production to levels four times higher than control values. Dehydrogenase activity increases under anaerobic conditions because it is produced by anaerobic microorganisms (Wolińska and Stępniewska, 2012) and it is influenced more by the ease of the decomposition of the organic matter than by the quantity of organic matter incorporated into the soil (Mandal et al., 2007). Presumably, the success of the RB and RC treatments in the suppression of *P. nicotianae* was related to an increase of anaerobic bacteria, such as *Clostridium* and *Bacillus* spp., as some authors have studied extensively (Hong et al., 2014; Momma et al., 2010; Mowlick et al., 2012, 2013a,b, 2014; Rosskopf et al., 2014; Wen et al., 2016) and related to the more easily decomposable components of these C-sources. This is also supported by the higher urease activity and liberation of $NH_4^+$ to soil shown with RB and RC.

In autoclaved soil, no C-source assayed was effective in reducing infectivity or survival of *P. nicotianae*, in agreement with the results by Momma et al. (2010) with *F. oxysporum* f.sp. *lycopersici*, indicating that pathogen control has a biological component.

The weed population before ASD was low due to the dry conditions of soil and the lack of optimal soil temperatures for germination. This weed status was similar to the weeds grown in the uncovered control after ASD. However, in the covered treatments, the plastic cover promoted optimal conditions for weed germination. In this study, the effect of ASD on weed biomass was measured immediately after plastic was removed so we did not determine if there was long-term effective control of weeds with RB and RC treatments or simply showed delayed emergence.

Decrease in viability of *P. nicotianae* was found during the laboratory experiments when chlamydospores of the control treatments were exposed to volatile compounds released from wetted soil. These results may be explained by the research of van Agtmaal et al. (2015) who found 15 potential suppressive volatile compounds (VOCs) generated after subjecting untreated soil to anaerobic stress by watering. These antifungal compounds may be released by the natural microflora, but they were absent in the ASD soils owing to the temporal changes in the soil microbial community. Obviously, more research is needed to investigate this hypothesis.

Previous work that combined plastic cover with organic amendments during spring did not show the expected results to replace chemical treatments for the control of *P. nicotianae* in Extremadura (Lacasa et al., 2015; Rodríguez-Molina et al., 2016). However, from the results of the present study, ASD treatment utilizing RB, RC, BC or GP as C-sources at the dose of 4 mg of C $g^{-1}$ of soil and at moderate soil temperatures typical of spring in this region are effective in reducing infectivity and survival of *P. nicotianae*. This paper could be considered as a preliminary study on ASD to reduce survival of *P. nicotianae* chlamydospores with different organic C-sources available. Application of ASD in spring is a promising alternative to chemical treatments and to summer solarization, compatible with warm-season vegetable production, such as is needed for open-field paprika pepper crop production. In future works, we have intention of testing ASD in commercial fields with naturally infested soil and evaluate the viability of the treatments in economic terms.

## Acknowledgments

The authors wish to thank to EJ Palo Núñez, C Palo Osorio and M Esteban by their technical assistance. To the anonymous referees for their constructive criticism. They are also grateful to Cerveza Cerex, Viñaoliva Soc Coop. and Arrocerías Dorado S.A. by supply the C sources. This research was supported by INIA [proyect RTA2011-00005-C03-02] and FEDER funds. Paula Serrano-Pérez is the recipient of a predoctoral fellowship from INIA.

## References

Acosta-Martínez, V., Tabatabai, M.A., 2000. Enzyme activities in a limed agricultural soil. Biol. Fertil. Soils 31 (1), 85–91.
Blok, W.J., Lamers, J.G., Termorshuizen, A.J., Bollen, G.J., 2000. Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. Phytopathology 90, 253–259.
Bodelier, P., Libochant, J.A., Blom, C., Laanbroek, H.J., 1996. Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptation of ammonia-oxidizing bacteria to low-oxygen or anoxic habitats. Appl. Environ. Microbiol. 62, 4100–4107.
Broadbent, F. lE., Stojanovic, B.F., 1952. The effect of partial pressure of oxygen on some soil nitrogen transformations. Soil Sci. Soc. Am. J. 16, 359–363.
Butler, D.M., Rosskopf, E.N., Kokalis-Burelle, N., Albano, J.P., Muramoto, J., Shennan, C., 2012a. Exploring warm-season cover crops as carbon sources for anaerobic soil disinfestation (ASD). Plant Soil 355, 149–165.
Butler, D.M., Kokalis-Burelle, N., Muramoto, J., Shennan, C., McCollum, T.G., Rosskopf, E.N., 2012b. Impact of anaerobic soil disinfestation combined with soil solarization on plant-parasitic nematodes and introduced inoculum of soilborne plant pathogens in raised-bed vegetable production. Crop Prot. 39, 33–40.
Butler, D.M., Eichler Inwood, S.E., McCarty, D.G., Sams, C.E., Wszelaki, A.L., Dee, M.E., et al., 2012c. Optimizing anaerobic soil disinfestation for Tennessee. In: Proceedings of Annual International Research Conference of Methyl Bromide Alternatives and Emissions Reductions, Orlando, FL, pp. 11–13.
Butler, D.M., Ownley, B.H., Dee, M.E., Eichler Inwood, S.E., McCarty, D.G., Shrestha, U., et al., 2014a. Low carbon amendment rates during anaerobic soil disinfestation (ASD) at moderate soil temperatures do not decrease viability of *Sclerotinia sclerotiorum* sclerotia or Fusarium root rot of common bean. Acta Hortic. 1044, 203–208.
Butler, D.M., Kokalis-Burelle, N., Albano, J.P., McCollum, T.G., Muramoto, J., Shennan, C., et al., 2014b. Anaerobic soil disinfestation (ASD) combined with soil solarization as a methyl bromide alternative: vegetable crop performance and soil nutrient dynamics. Plant Soil 378, 365–381.
Cao, Y., Chang, Z., Wang, J., Ma, Y., Yang, H., Fu, G., 2014. Potential use of anaerobically digested manure slurry to suppress Phytophthora root rot of chilli pepper. Sci. Hortic. (Amsterdam) 168, 124–131.
Coelho, L., Chellemi, D.O., Mitchell, D.J., 1999. Efficacy of solarization and cabbage amendment for the control of *Phytophthora* spp. in north Florida. Plant Dis. 83, 293–299.
Coelho, L., Mitchell, D.J., Chellemi, D.O., 2000. Thermal inactivation of *Phytophthora nicotianae*. Phytopathology 90, 1089–1097.
Colla, P., Gilardi, G., Gullino, M.L., 2012. A review and critical analysis of the European situation of soilborne disease management in the vegetable sector. Phytoparasitica 40, 515–523.
Ebihara, Y., Uematsu, S., 2014. Survival of strawberry-pathogenic fungi *Fusarium oxysporum* f. sp. *fragariae*, *Phytophthora cactorum* and *Verticillium dahliae* under anaerobic conditions. J. Gen. Plant Pathol. 80, 50–58.
Eivazi, F., Tabatabai, M.A., 1988. Glucosidases and galactosidases in soils. Soil Biol. Biochem. 20 (5), 601–606.
FAO, 2010. Visual Soil Assessment (VSA) Field Guides, Online: http://www.fao.org.
Fennimore, S.A., Serohijos, R., Samtani, J.B., Ajwa, H.A., Subbarao, K.V., Martin, F.N., et al., 2013. Tif film, substrates and nonfumigant soil disinfestation maintain fruit yields. Calif. Agric. 67, 139–146.
García, C., Hernández, T., 1997. Biological and biochemical indicators in derelict soils subject to erosion. Soil Biol. Biochem. 29 (2), 171–177.
Goud, J.-K.C., Termorshuizen, A.J., Blok, W.J., van Bruggen, A.H.C., 2004. Long-term effect of biological soil disinfestation on verticillium wilt. Plant Dis. 88, 688–694.
Haynes, R.J., 1999. Labile organic matter fractions and aggregate stability under short-term grass-based leys. Soil Biol. Biochem. 31, 1821–1830.
Hewavitharana, S.S., Ruddell, D., Mazzola, M., 2014. Carbon source-dependent antifungal and nematicidal volatiles derived during anaerobic soil disinfestation. Eur. J. Plant Pathol. 140, 39–52.
Hong, J., Butler, D.M., Noling, J.W., He, Z., Rosskopf, E.N., Burelle, N., et al., 2014. Comparison of anaerobic soil disinfestation and drip-applied organic acids for

raised-bed specialty crop production in florida. VIII International Symposium on Chemical and Non-Chemical Soil and Substrate Disinfestation, 221–228, 1044.
Huang, X., Wen, T., Zhang, J., Meng, L., Zhu, T., Cai, Z., 2015. Toxic organic acids produced in biological soil disinfestation mainly caused the suppression of *Fusarium oxysporum* f sp. *cubense*. BioControl 60, 113–124.
Juarez-Palacios, C., Felix-Gastelum, R., Wakeman, R.J., Paplomatas, E.J., DeVay, J.E., 1991. Thermal sensitivity of three species of *Phytophthora* and the effect of soil solarization on their survival. Plant Dis. 75, 160–1164.
Kobara, Y., Uematsu, S., Tanaka-Miwa, C., Sato, R., Sato, M., 2007. Possibility of the new soil fumigation technique with ethanol solution. Proceedings of 2007 Annual Research Conference on Methyl Bromide Alternatives and Emissions Reduction, 74.
Koike, S.T., Gordon, T.R., 2015. Management of Fusarium wilt of strawberry. Crop Prot. 73, 67–72.
Lacasa, C.M., Martínez, V., Hernández, A., Ros, C., Lacasa, A., del Mar Guerrero, M., et al., 2015. Survival reduction of *Phytophthora capsici* oospores and *P. nicotianae* chlamydospores with Brassica green manures combined with solarization. Sci. Hortic. (Amsterdam) 197, 607–618.
Ladd, J.N., Jackson, R.B., 1982. Biochemistry of ammonification. Nitrogen in Agricultural soils, 173–228.
Leirós, M.C., Trasar-Cepeda, C., Seoane, S., Gil-Sotres, F., 2000. Biochemical properties of acid soils under climax vegetation (Atlantic oakwood) in an area of the European temperate–humid zone (Galicia, NW Spain): general parameters. Soil Biol. Biochem. 32, 733–745.
Madejón, E., Burgos, P., López, R., Cabrera, F., 2001. Soil enzymatic response to addition of heavy metals with organic residues. Biol. Fertil. Soils 34 (3), 144–150.
Mandal, A., Patra, A.K., Singh, D., Swarup, A., Masto, R.E., 2007. Effect of long-term application of manure and fertilizer on biological and biochemical activities in soil during crop development stages. Bioresour. Technol. 98, 3585–3592.
Mazzola, M., Manici, L.M., 2012. Apple replant disease: role of microbial ecology in cause and control. Annu. Rev. Phytopathol. 50, 45–65.
Mazzola, M., Granatstein, D.M., Elfving, D.C., Mullinix, K., 2001. Suppression of specific apple root pathogens by *Brassica napus* seed meal amendment regardless of glucosinolate content. Phytopathology 91, 673–679.
Mazzola, M., Shennan, C., Muramoto, J., 2012. Application sequence and soil biology influence anaerobic soil disinfestation induced disease suppressio. Proc. 2012 Annu. Int. Res. Conf. Methyl Bromide Alternatives and Emissions Reduction http://mbao.org /2012/57Mazzola.pdf.
Mazzola, M., 2011. Potential of biofumigation of soilborne pest control in strawberry. In: 2011 Proceedings of Annual International Research Conference on Methyl Bromide Alternatives and Emissions Reductions, San Diego (CA), pp. 41–47.
McCarty, D.G., Inwood, S.E.E., Ownley, B.H., Sams, C.E., Wszelaki, A.L., Butler, D.M., 2014. Field evaluation of carbon sources for anaerobic soil disinfestation in tomato and bell pepper production in Tennessee. HortScience 49, 272–280.
Menzies, J.D., 1963. Survival of microbial plant pathogens in soil. Bot. Rev. 29, 79–122.
Mitchell, D.J., Kannwischer-Mitchell, M.E., Singleton, L.L., Mihail, J.D., Rush, C.M., 1992. Methods for Research on Soilborne Phytopathogenic Fungi. APS Press, St. Paul, MN, pp. 31–38.
Momma, N., Yamamoto, K., Simandi, P., Shishido, M., 2006. Role of organic acids in the mechanisms of biological soil disinfestation (BSD). J. Gen. Plant Pathol. 72, 247–252.
Momma, N., Momma, M., Kobara, Y., 2010. Biological soil disinfestation using ethanol: effect on *Fusarium oxysporum* f. sp. *lycopersici* and soil microorganisms. J. Gen. Plant Pathol. 76, 336–344.
Momma, N., Kobara, Y., Momma, M., 2011. $Fe^{2+}$ and $Mn^{2+}$, potential agents to induce suppression of *Fusarium oxysporum* for biological soil disinfestation. J. Gen. Plant Pathol. 77, 331–335.
Momma, N., Kobara, Y., Uematsu, S., Kita, N., Shinmura, A., 2013. Development of biological soil disinfestations in Japan. Appl. Microbiol. Biotechnol. 97, 3801–3809.
Momma, N., 2008. Biological soil disinfestation (BSD) of soilborne pathogens and its possible mechanisms. JARQ 42, 7–12.
Mowlick, S., Hirota, K., Takehara, T., Kaku, N., Ueki, K., Ueki, A., 2012. Development of anaerobic bacterial community consisted of diverse clostridial species during biological soil disinfestation amended with plant biomass. Soil Sci. Plant Nutr. 58, 273–287.
Mowlick, S., Inoue, T., Takehara, T., Kaku, N., Ueki, K., Ueki, A., 2013a. Changes and recovery of soil bacterial communities influenced by biological soil disinfestation as compared with chloropicrin-treatment. AMB Express 3, 46.
Mowlick, S., Takehara, T., Kaku, N., Ueki, K., Ueki, A., 2013b. Proliferation of diversified clostridial species during biological soil disinfestation incorporated with plant biomass under various conditions. Appl. Microbiol. Biotechnol. 97, 8365–8379.
Mowlick, S., Yasukawa, H., Inoue, T., Takehara, T., Kaku, N., Ueki, K., et al., 2013c. Suppression of spinach wilt disease by biological soil disinfestation incorporated with *Brassica juncea* plants in association with changes in soil bacterial communities. Crop Prot. 54, 185–193.
Mowlick, S., Inoue, T., Takehara, T., Tonouchi, A., Kaku, N., Ueki, K., et al., 2014. Usefulness of Japanese-radish residue in biological soil disinfestation to suppress spinach wilt disease accompanying with proliferation of soil bacteria in the *Firmicutes*. Crop Prot. 61, 64–73.
Mulvaney, R.L., 1996. Methods of soil analysis. Part 3. Chemical methods. Soil Sci. Soc. Am. B Ser. 5, 1123–1184.
Murphy, J., Riley, J., 1962. A modified single solution method for the determination of phosphate in natural waters. Anal. Chim. Acta 27, 31–36.
Núñez-Zofío, M., Larregla, S., Garbisu, C., 2011. Application of organic amendments followed by soil plastic mulching reduces the incidence of *Phytophthora capsici* in pepper crops under temperate climate. Crop Prot. 30, 1563–1572.
Nannipieri, P., Ceccanti, B., Cervelli, S., Matarese, E., 1980. Extraction of phosphatase, urease, proteases, organic carbon, and nitrogen from soil. Soil Sci. Soc. Am. J. 44, 1011–1016.
Olsen, S. R., 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate.
Pullman, G.S., DeVay, J.E., Garber, R.H., Weinhold, A.R., 1981. Soil solarization: effects on verticillium wilt of cotton and soilborne populations of *Verticillium dahliae Pythium* spp., *Rhizoctonia solani*, and *Thielaviopsis basicola*. Phytopathology 71, 954–959.
Quilchano, C., Marañón, T., 2002. Dehydrogenase activity in Mediterranean forest soils. Biol. Fertil. Soils 35, 102–107.
Rodríguez-Molina, M.C., Morales-Rodríguez, M.C., Osorio, C.P., Núñez, E.P., Alonso, E.V., Maya, M.S.D., et al., 2010. Short communication. *Phytophthora nicotianae*, the causal agent of root and crown rot (Tristeza disease) of red pepper in La Vera region (Cáceres Spain). Spanish J. Agric. Res. 8, 770–774.
Rodríguez-Molina, M.C., Serrano-Pérez, P., Palo, C., 2016. Effect of biofumigation with *brassica* pellets combined with Brassicaceae cover crops and plastic cover on the survival and infectivity of inoculum of *Phytophthora nicotianae* Breda de Haan. Pest Manag. Sci. 72, 1295–1301.
Romero, M.A., Sánchez, J.E., Jiménez, J.J., Belbahri, L., Trapero, A., Lefort, F., et al., 2007. New *Pythium* taxa causing root rot on mediterranean *Quercus* species in south-west Spain and Portugal. J. Phytopathol. 155, 289–295.
Rosskopf, E., Hong, J., Kokalis-Burelle, N., Butler, D., 2014. Eliminating obstacles for the adoption of anaerobic soil disinfestation in florida tomato. Tomato Institute Proceedings.
Rosskopf, E.N., Serrano-Pérez, P., Hong, J., Shrestha, U., Rodríguez-Molina, M. del C., Martin, K., et al., 2015. Anaerobic soil disinfestation and soilborne pest management. In: Meghvansi, M.K., Varma, A. (Eds.), Organic Amendments and Soil Suppressiveness in Plant Disease Management. Springer, pp. 277–305.
Runia, W.T., Thoden, T.C., Molendijk, L.P.G., van den Berg, W., Termorshuizen, A.J., Streminska, M.A., et al., 2014. Unravelling the mechanism of pathogen inactivation during anaerobic soil disinfestation. VIII International Symposium on Chemical and Non-Chemical Soil and Substrate Disinfestation, 177–193 (1044).
Scalenghe, R., Edwards, A.C., Ajmone Marsan, F., Barberis, E., 2002. The effect of reducing conditions on the solubility of phosphorus in a diverse range of European agricultural soils. Eur. J. Soil Sci. 53 (3), 439–447.
Shennan, C., Muramoto, J., Baird, G., Koike, S., Bolda, M., Mazzola, M., 2013. Optimizing anaerobic soil disinfestation for soilborne disease control. In: 2013 Proceedings of Annual International Research Conference on Methyl Bromide Alternatives and Emissions Reductions, San Diego (CA), pp. 11–13.
Shennan, C., Mazzola, M., Rosskopf, E.N., Kokalis-Burelle, N., Momma, N., Butler, D.M., et al., 2014. Anaerobic soil disinfestation for soil borne disease control in strawberry and vegetable systems: current knowledge and future directions. VIII International Symposium on Chemical and Non-Chemical Soil and Substrate Disinfestation, 165–175, 1044.
Shew, H.D., 1983. Effects of soil matric potential on infection of tobacco by *Phytophthora parasitica* var *nicotianae*. Phytopathology 73, 1160–1163.
Shinmura, A., 2000. Causal agent and control of root rot of welsh onion. PSJ Soilborne Disease Workshop Report, 133–143.
Speir, T.W., Ross, D.J., 1978. Soil phosphatase and sulphatase. Soil Enzymes 203, 197–250.
Stover, R.H., 1955. Flood-fallowing for eradication of *Fusarium oxysporum* f.sp *cubense:* III. Effect of oxygen on fungus survival. Soil Sci. 80, 397–412.
Strauss, S.L., Kluepfel, D.A., 2015. Anaerobic soil disinfestation: a chemical-independent approach to pre-plant control of plant pathogens. J. Integr. Agric. 14, 2309–2318.
Tabatabai, M.A., Bremner, J.M., 1969. Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1, 301–307.
Tabatabai, M.A., 1982. Soil enzymes. In methods of soil analysis. Part 2. Chem. Microbiol. Prop. 9 (2082), 903–947.
Tello J., Varés, F., Lacasa, A., 1991. Manual de laboratorio. Diagnóstico de hongos, bacterias y nematodos fitopatógenos. MAPA. Dirección General de Sanidad de la Producción Agraria. Madrid. 485 pp.
Termorshuizen, A.J., Jeger, M.J., 2014. Assessing inoculum of soilborne plant pathogens: theory and practice in decision-making for soil disinfestation. VIII International Symposium on Chemical and Non-Chemical Soil and Substrate Disinfestation, 75–80, 1044.
Trasar-Cepeda, C., Leirós, M.C., Gil-Sotres, F., 2000. Biochemical properties of acid soils under climax vegetation (Atlantic oakwood) in an area of the European temperate–humid zone (Galicia, NW Spain): general parameters. Soil Biol. Biochem. 32, 733–745.
Trevors, J.T., Mayfield, C.I., Inniss, W.E., 1982. Measurement of electron transport system (ETS) activity in soil. Microb. Ecol. 8, 163–168.
Trevors, J.T., 1984. Dehydrogenase activity in soil: a comparison between the INT and TTC assay. Soil Biol. Biochem. 16 (6), 673–674.
Tsao, P.H., 1971. Chlamydospore formation in sporangium-free liquid cultures of Phytophthora parasitica. Phytopathology 61, 1412–1413.

Uematsu, S., Tanaka-Miwa, C., Sato, R., Kobara, Y., Sato, M., 2007. Ethyl alcohol as a promising material of reductive soil disinfestation for controlling root knot nematode and soilborne plant diseases. In: Proceedings of 2007 Annual International Research Conference on Methyl Bromide Alternatives and Emissions Reductions, MBAO, USEPA and USDA, San Diego, pp. 71–75.

Walkley, A., Black, I.A., 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci. 37, 29–38.

Wen, T., Huang, X., Zhang, J., Zhu, T., Meng, L., Cai, Z., 2015. Effects of water regime crop residues, and application rates on control of *Fusarium oxysporum* f. sp. *cubense*. J. Environ. Sci. 31, 30–37.

Wen, T., et al., 2016. Effects of biological soil disinfestation and water regime on suppressing *Artemisa selengensis* root rot pathogens. J. Soils Sediments 16, 215–225.

Wolińska, A., Stępniewska, Z., 2012. Dehydrogenase Activity in the Soil Environment. INTECH Open Access Publisher.

Yossen, V., Zumelzu, G., Gasoni, L., Kobayashi, K., 2008. Effect of soil reductive sterilisation on Fusarium wilt in greenhouse carnation in Córdoba, Argentina. Australas. Plant Pathol. 37, 520–522.

Zavatta, M., Shennan, C., Muramoto, J., Mazzola, M., 2014. Evaluating C-sources for anaerobic soil disinfestation. In: 2014 Proceedings of Annual International Research Conference on Methyl Bromide Alternatives and Emissions Reductions, Orlando, pp. 11–12.

van Agtmaal, M., van Os, G.J., Hol, W.H.G., Hundscheid, M.P.J., Runia, W.T., Hordijk, C.A., et al., 2015. Legacy effects of anaerobic soil disinfestation on soil bacterial community composition and production of pathogen-suppressing volatiles. Front. Microbiol. 6, 701.

van Bruggen, A., Blok, W., 2014. Soil disinfestation and food safety issues. VIII International Symposium on Chemical and Non-Chemical Soil and Substrate Disinfestation, 437–445, 1044.

van Overbeek, L., Runia, W., Kastelein, P., Molendijk, L., 2014. Anaerobic disinfestation of tare soils contaminated with *Ralstonia solanacearum* biovar 2 and *Globodera pallida*. Eur. J. Plant Pathol. 138, 323–330.