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RESEARCH ARTICLE

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# Winter biodisinfestation with Brassica green manure is a promising management strategy for *Phytophthora capsici* control of protected pepper crops in humid temperate climate regions of northern Spain

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## Abstract

*Phytophthora capsici* causes root and crown rot of protected pepper (*Capsicum annuum* L.) crops in the Basque Country (northern Spain), a humid temperate climate area. The objective was to determine the effect of winter biofumigation and plastic cover (biodisinfestation) with *Sinapis alba* L. (cultivar 'Ludique') fresh green manure on the survival of introduced *P. capsici* inoculum (oospores) in a greenhouse soil. After autumn-winter Brassica cover crop soil incorporation in February, oospores remained at 15 and 30 cm depth for four weeks´ time exposure in two consecutive years, 2009 and 2010. Oospores viability was estimated with a plasmolysis method and infectivity with a potted pepper bioassay. Viability was significantly higher in biodisinfestated oospores than in the non-treated control in both years (81% and 21% relative increase in 2009 and 2010, respectively) and significantly higher at 30 cm depth than at 15 cm in 2009 (24% relative increase). Conversely, biodisinfestation significantly delayed disease incidence progression until the first half of infectivity bioassays in both years compared to the non-treated soils (83% and 75% relative decrease of dead plants in 2009 and 2010 respectively). The low soil temperatures could explain the low oospore survival reduction and suggest that the suppressiveness expressed in the infectivity bioassay was related with an increase of microbial activity in the biodisinfestation treatment. We conclude that winter biodisinfestation with fresh Brassica green manure is a promising management strategy for *Phytophthora* root rot control of protected peppers crops in regions of humid temperate climate such us northern Spain.

**Additional keywords:** oospores; *Capsicum annuum*; biosolarization; biofumigation; *Sinapis alba*.

**Abbreviations used:** AUDPC (area under the disease progress curve); DP (diseased plant); RAUDPC (relative area under the disease progress curve).

**Authors' contributions:** MNZ and SL participated in the conception and design of the research. MG, MO, EO, AOB and SM participated in the other stages of the work, including the performing of experiments, data analyzing, revision of the intellectual content and the drafting of the paper.

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## Introduction

*Phytophthora capsici* is one of the main soil-borne pathogens that causes root and crown rot of protected pepper (*Capsicum annuum* L.) crops worldwide (Ristaino & Johnston, 1999) and in the Basque Country (northern Spain) (Riga *et al*., 2000; Larregla *et al*., 2015), an area characterized by a humid temperate climate. This pathogen species produces survival spores (oospores) that are capable to persist in the soil and act as the main initial inoculum that causes primary infections in the next crop cycle (Erwin & Ribeiro, 1996). The presence of *P. capsici* is also reported in the southeast of Spain with a warm dried climate causing root and crown rot disease, not only in solanaceous plants (pepper, eggplant and tomato) but also in curcubitaceous like

zucchini, watermelon and cucumber (De-Cara-García *et al*., 2018). Optimal, minimum and maximum temperatures for mycelia growth of *P. capsici* have been reported in the range of 25-32ºC, 5-7ºC and 38-42°C (Erwin & Ribeiro, 1996). Oospores survival was scarcely affected at constant temperatures ≤ 35ºC in controlled laboratory conditions in moistened soil. Required exposure times for thermal inactivation of *P. capsici* oospores were 199-22-6.6-4.7-1.0 h at 40-45-47.5-50-53ºC of constant temperatures, respectively, in these constant temperature regimes. Regarding the cycling temperature regimes, 4 h-40ºC regime killed 100% of oospores after 28 days, while the 5 h-35ºC regime after 70 days killed only 75% (Etxeberria *et al*., 2011b).

In greenhouses of the Basque Country, pre-plant soil disinfestation with chemical fumigants has been traditionally used to control pepper root and crown rot, but social changes and legal limitations on the use of chemical disinfectants boosted the evaluation of environmentally friendly and healthy alternatives of soil-borne pathogen control in the last years (Colla *et al*., 2012). One of these alternatives was the implementation of fresh green manure into the soil, which was reported to have a significant effect on soil-borne pathogen diseases (Larkin, 2013; Lacasa *et al*., 2015).

In some cases, the pathogen can be established in soils but the disease becomes progressively less severe even though the pathogen persists in soil, which is known as suppressiveness (Cook & Baker, 1983; Chandrashekara *et al*., 2014). The suppression of soilborne pests by naturally occurring biocides released in soil when tissues of Brassicaceous plants or fresh green manure crops are decompose in soil is known as biofumigation (Kirkegaard *et al*., 1993; Angus *et al*., 1994). Biofumigation using Brassicas residues as fresh green manure (Angus *et al*., 1994) and as rotation crops fitted into farming systems (Rudolph *et al*., 2015), is an effective practice for the control of soil-borne pathogens while responding to environmental and health concerns as well as legal limitations (OJ, 2009) of soil chemical disinfestation. Among Brassicas, *Sinapis alba* (white mustard) was reported to be effective in soil-borne pathogen control in different crops like root rot in common Mediterranean crops (De-Cara-García *et al*., 2018), root and crown rot in pepper and tomato (Morales-Rodriguez *et al*., 2014) and powdery scab in potato crops (Larkin, 2013).

Under certain conditions, soils can develop a suppressive effect which means that pathogens hardly establish in these soils or develop disease in the crop (Shurtleff & Averre, 1997). The main mechanisms that explain the suppressive effects of the use of Brassica species for soil-borne pest control are the biocidal compounds released into the soil when Brassica tissues are decomposed (Kirkegaard & Sarwar, 1998; Sarwar & Kirkegaard, 1998; Bending & Lincoln, 1999; Manici *et al*., 2000; Matthiessen & Kirkegaard, 2006;) and the increased populations of antagonistic organisms after the incorporation of organic matter in the soil (Cohen *et al*., 2005; Mazzola *et al*., 2001).

The effectiveness of biofumigation combined with solarization, solar heating by covering a soil with a transparent plastic film (Katan & Gamliel, 2014), has been described in Mediterranean areas with soil-borne pathogens problems (Lacasa *et al*., 2015; De-Cara-García *et al*., 2018). In the case of areas with humid temperate climate, in which temperatures are insufficient for direct or indirect effects on pathogen control, the application of biosolarization in winter could contribute to control soil-borne pathogen. In this case in which temperature would probably not be high enough to produce solarization effects, it is more proper to use the term biodisinfestation.

In the Basque Country, pepper crops are grown in unheated greenhouses from early-spring to early-autumn. Thus, the proper time to practice biodisinfestation in this area can be before (winter season) or after (fall season) the period in which the greenhouse is occupied by the pepper crop cycle (Arriaga *et al*., 2011). However, biodisinfestation at the end of winter can be considered a more viable option than in autumn, because of its greater proximity in time with the crop cycle, the progressive increase of solar radiation and consequently in temperature and microbial activity in the plastic-sealed amended soil. Brassica green manures can influence soil suppressiveness by several mechanisms different to the glucosinolates and isothiocyanates pathway, such as indirect effects on the pathogen associated with changes in the populations of antagonistic organisms, as well as the more direct suppressive effects of compounds released from the tissues that may not be related to glucosinolates or isothiocyanates. In addition, other mechanisms include beneficial impacts on organic matter, nutrition, soil structure and erosion control which are common to most green manures (Mcguire, 2003; Matthiessen & Kirkegaard, 2006; Prasad *et al*., 2015). Thus, the establishment of the crop after an autumn-winter cover crop of Brassica regardless of its glucosinolate content, could be regarded as an adequate control method for *Phytophthora* root and crown rot.

The objective of this study was to evaluate the effectiveness of winter biodisinfestation as green manure with an autumn-winter cover crop cycle of *S. alba* (white mustard) on the survival of *P. capsici* oospores in the agroclimatic greenhouse conditions of

the Basque Country, a humid temperate area with lower temperatures than the Mediterranean region.

## Material and methods

### Field trial characteristics

Trials were performed during two consecutive years (2009 and 2010) in an unheated tunnel type greenhouse, located at NEIKER Research Station (Derio, Basque Country, northern Spain) with a size of 42 × 9.4 $m^2$. Soil type was a clay-loam, with 5.5% of organic matter content, a C/N ratio of 15.1, pH=6.4 and an electrical conductivity of 2.15 dS $m^{-1}$.

### Treatments and experimental design

In this study, the effect of soil biodisinfestation with *S. alba* fresh green manure implementation and transparent polyethylene plastic film cover (0.05 mm thick) was assessed on viability and infectivity of *P. capsici* soil inoculum (oospores) at two different soil depths (15 cm and 30 cm) after four weeks of treatment exposure.

The experiment was factorial with a treatment structure of two crossed factors: i) soil treatment with two levels (Sa_P: *S. alba* fresh green manure amendment plus soil plastic tarping; Na_NP: non-amended and non-plastic control) and ii) depth of buried *P. capsici* inoculum with two levels (15 cm; 30 cm).

Non-amended and non-tarped soil was considered as the non-disinfested control treatment. Soil treatments were arranged in a randomized complete block design with three replicates and a plot size of 6 × 4.7 $m^2$. Treatments were applied in two consecutive years (2009 and 2010) in the same replicate plots in order to observe reiteration effects.

### Brassica crops and application of Brassica fresh green manure

Seeds (15 kg $ha^{-1}$) of *S. alba* cv. 'Ludique' were sown the 14$^{th}$ of November 2008 and the 1$^{st}$ of October 2009 in the experimental greenhouse in the same three replicate plots. *Sinapis alba* plants were watered through sprinkling irrigation as needed throughout the growing period. At blooming (17 weeks after treatments), *S. alba* plants were harvested, manually chopped and subsequently incorporated in the soil using a rotavator on the 19$^{th}$ of Febuary 2009 and the 18$^{th}$ of Febuary 2010 at a rate of 4.5 and 7.2 kg $m^{-2}$ fresh weight in each year respectively.

Oospores of *P. capsici* (soil bags for infectivity and meshes for viability) were buried at 15 and 30 cm soil depth in each experimental plot. Brassica amended plots were watered by sprinkling irrigation for one hour to ensure that soil was moistened to 25-30 cm and covered with transparent polyethylene plastic film (0.05 mm thick) for four weeks. Experimental plots from the non-treated soil control treatment were also irrigated in order to maintain same conditions of soil moisture.

### Production of oospores

*Phytophthora capsici* oospores were produced *in vitro* by pairing isolates of different mating types on soft pea agar (7.5 g $L^{-1}$ agar) in order to assist oospore extraction according to Pittis & Shattock (1994). Agar was supplemented with 0.1 g $L^{-1}$ β-sitosterol to increase oospore formation. A1 Spanish isolates from pepper (00/004, 02/206 and 06-13-03) were mated with A2 isolates (CBS 554.88 and CBS 370.72) from the Dutch Type Culture Collection (Centraalbureau voor Schimmelcultures, CBS). After four weeks of incubation (in darkness at 20ºC), oospores were extracted from agar by blending in sterile distilled water (10 mL $plate^{-1}$).

### Effect of treatments on oospores viability

Oospores viability was evaluated through a plasmolysis method. The oospore suspension was filtered through a 100 µm nylon mesh. The concentration of oospores in the filtered suspension was determined with a Fuchs-Rosenthal counting chamber and an optical microscope at 200X magnification in order to ensure the required number of oospores. After determining suspension concentration, oospores were then placed in 1 × 1 cm 25 µm nylon meshes (Sefar Nitex 03 25/19, SEFAR, Switzerland) by vacuum filtration. Embedded oospores in 25 µm nylon meshes (at least 1000 oospores per mesh piece) were placed within 5 × 5 cm envelops made from permeable 1,550 µm nylon mesh (Sefar Nitex 06-1550/60). A total of 12 envelops were buried in each greenhouse experimental plot in 3 different locations (3 locations × 2 depths × 2 exposure times). One envelop from each location and depth was removed after two and four weeks from the treatment onset. For each envelop, 100 oospores were observed microscopically to estimate percentage viability using the plasmolysis method (Jiang & Erwin, 1990), effective on *P. capsici* oospores according to Etxeberria *et al*. (2011a).

### Effect of treatments on oospores infectivity

In the case of infectivity assessment, oospore suspension used for the soil inoculation of the

infectivity bioassay was pretreated with Glucanex (β-glucanase, cellulase, protease, and chitinase activities) (SIGMA, USA) at a concentration of 20 mg $mL^{-1}$ for 48 h at 20°C in order to remove sporangia and mycelial debris. Five aliquots of 100 µL of Glucanex-treated oospore suspensions were poured onto selective PARPH (pimaracin-ampicillin-rifampicin-pentachloronitrobenzene-hymexazol) agar medium (Jeffers & Martin, 1986) to ensure the absence of viable hyphal fragments or sporangia. Soil samples were previously air-dried, sieved through a 5 mm mesh screen and sterilized (121°C for one hour on two successive days). The infested-soil-layer technique (Bowers & Mitchell, 1991) was used for the infectivity bioassay, and oospores of *P. capsici* were added to moist sterilized soil (10,000 oospores in 100 g soil) of each treatment plot and then wrapped in a polypropylene fabric material made from a frost protection blanket (RAISA, Spain) to form a small bag which was buried at 15 and 30 cm soil depth in four locations of each greenhouse experimental plot (eight soil bags per plot). The soil bags remained buried for four weeks. Upon treatment completion, the bags of infested soil were unearthed from each replicate plot. The infested soil from each bag (100 g) was deposited in a 12-cm-diameter pot on top of a layer of sterilized perlite substrate (2 g) and covered with another layer of sterilized peat (10 g). A pepper plant in the two-true-leaf stage (about 30 days of age) was transplanted into the uninfested top layer of peat in each pot. The pepper cultivar used was 'Derio', which is susceptible to *P. capsici*. The potted plants were maintained for 90 days in a growth chamber at 25ºC with a 14:10 h photoperiod. Plants were observed twice a week and percentage of diseased plants was registered. The area under the disease progress curve (AUDPC) was calculated to quantify disease progress according to Madden *et al*. (2007):

$$AUDPC = \sum_{j=1}^{n_j-1} \left(\frac{y_j + y_{j+1}}{2}\right)(t_{j+1} - t_j)$$

where $Y_j$ is an assessment of disease (percentage) at the $j_{th}$ observation, $t_j$ is time (in days) at the $j_{th}$ observation, and $n_j$ is the total number of observations.

In order to compare bioassays of different duration trials, AUDPC was relativized and divided by the duration of each trial in days (RAUDPC), and expressed as %-days per day units. The percentage of diseased plants (DP) and the progression of disease (RAUDPC) in the infectivity bioassay were both calculated till mid-point (47 days in 2009 and 46 days in 2010) and till end-point of bioassay (90 days in 2009 and 88 days in 2010) respectively in order to analyze the different progression of disease among the different treatments (see Table 2 below).

## Temperature registration

Temperature was registered with probes connected to a Hobo datalogger (Weather Station, OCC, US, H8-4 32K). Air temperature in the middle of the greenhouse at 2 m height and soil temperature in each soil treatment experimental plot (central block) was recorded at 15 and 30 cm depth every 30 min.

## Statistical analysis

The mixed linear model used for parametric analysis of the factorial experiment of two crossed factors (Soil treatment, Depth) contained both fixed- and random-effects. Soil treatment, depth and their interaction were considered fixed factors, and the block a random factor. Analyses were performed with a two-way factor (soil treatment, depth) analysis of variance (ANOVA). Means + standard errors (n=3 replicates of 4 plants per treatment for oospores infectivity, and n=9 envelops per treatment for oospores viability) were calculated and, when the *F* ratio was significant, least significant differences between means of significant main factors were analysed with the Tukey *HSD* test and between means of significant interactions were studied with least squares means adjusted for multiple comparisons with Tukey-Kramer. The significance level for *F* tests and mean comparisons was fixed at $p$=0.05. The statistical program used was SAS (Statistical Analysis Software, SAS Inst., Cary, NC, USA; release 9.3).

Mean values across all the subsamples within each experimental unit (replicate plot per treatment in each block) were determined for each dependent variable in the analysis. The experiment was a randomized complete block design with three replicate plots per treatment. For the variable oospores viability, each experimental unit was composed by three subsamples (each subsample was a count of 100 oospores) (Table 1). For the variable oospores infectivity, each experimental unit was composed by four subsamples (each subsample was an inoculated soil bag) (Table 2).

Viability percentage values of *P. capsici* oospores relativized to the initial viability (Viabr) were transformed with "arcsine ($\sqrt{(Viabr/100)}$)" (Table 1). In the oospores infectivity plant bioassays, DP was transformed with "arcsine ($\sqrt{(DP/100)}$)" (Table 2).

Data on variables percentage of oospores viability and diseased plants from the oospores infectivity bioassays were transformed with the arcsin square-root transformation to stabilize the variances. Values of significance probability ($p$-values), parameter

**Table 1.** Effect on *Phytophthora capsici* oospores viability within each exposure time (2 and 4 weeks) after field disinfestation treatments in the 2009 and 2010 year greenhouse experiments. Three replicate plots with three locations per plot and one count of 100 oospores per location were used per treatment. Mean values (n=9) ± standard errors.

| Soil treatment[a] | Depth (cm)[a] | Viabr0 (%)[b] | Viabr2 (%)[b] | Viabr4 (%)[b] |
|---|---|---|---|---|
| | | **Year 2009** | | |
| Sa_P | 15 | 100.0±6.8 | 46.7±10.8 | 55.7±6.1 |
| Na_NP | 15 | 100.0±6.8 | 30.3±2.6 | 25.3±2.1 |
| Sa_P | 30 | 100.0±6.8 | 43.1±5.1 | 61.3±6.5 |
| Na_NP | 30 | 100.0±6.8 | 37.9±1.7 | 39.6±4.9 |
| Mean Sa_P | – | 100.0±6.8 | 44.9±5.8 | 58.5±3.9 (I)[c] |
| Mean Na_NP | – | 100.0±6.8 | 34.1±1.7 | 32.4±3.1 (II) |
| – | Mean 15 | 100.0±6.8 | 38.5±5.8 | 40.5±4.5 (2)[d] |
| – | Mean 30 | 100.0±6.8 | 40.5±2.7 | 50.4±4.7(1) |
| **Factor** | | **Two way ANOVA [e]** | | |
| Treatment | $F_{1, 32}$ [f] | – | 2.390 | 25.82 |
| | *p*-value | – | 0.1323 | <0.0001 |
| Depth | $F_{1, 32}$ | – | 0.070 | 4.540 |
| | *p*-value | – | 0.792 | 0.042 |
| Treatment × Depth | $F_{1, 32}$ | – | 0.760 | 0.320 |
| | *p*-value | – | 0.389 | 0.576 |
| Residual | CPE[g] | – | 0.047 | 0.029 |
| | | **Year 2010** | | |
| Sa_P | 15 | 100.0±1.3 | 61.1±1.2 (ab)[h] | 52.5±2.3 |
| Na_NP | 15 | 100.0±1.3 | 58.9±2.4 (b) | 48.3±2.6 |
| Sa_P | 30 | 100.0±1.3 | 67.3±1.5 (a) | 62.2±3.3 |
| Na_NP | 30 | 100.0±1.3 | 56.0±2.8 (b) | 46.3±4.6 |
| Mean Sa_P | – | 100.0±1.3 | 64.2±1.2 (I)[i] | 57.3±2.3 (I)[i] |
| Mean Na_NP | – | 100.0±1.3 | 57.4±1.8 (II) | 47.3±2.6 (II) |
| – | Mean 15 | 100.0±1.3 | 60.0±1.3 | 50.4±1.7 |
| – | Mean 30 | 100.0±1.3 | 61.7±2.1 | 54.3±3.4 |
| **Factor** | | **Two way ANOVA [e]** | | |
| Treatment | $F_{1, 30}$ [f] | – | 10.860 | 10.550 |
| | *p*-value | – | 0.003 | 0.003 |
| Depth | $F_{1, 30}$ | – | 0.760 | 1.520 |
| | *p*-value | – | 0.391 | 0.228 |
| Treatment × Depth | $F_{1, 30}$ | – | 5.100 | 3.760 |
| | *p*-value | – | 0.031 | 0.062 |
| Residual | CPE[g] | – | 0.004 | 0.009 |

[a]Factorial experiment with treatments structure of two crossed factors: Soil treatment with two levels (Sa_P: *Sinapis alba* fresh green manure amendment plus soil plastic tarping; Na_NP: non-amended and non-plastic control). Depth of buried *P. capsici* oospores with two levels (15 cm; 30 cm). [b]Viabr2 (%), Viabr4 (%): oospores viability percentage values at 2 and 4 weeks exposure time respectively relativized to the initial viability Viab0 (%). In year 2009, Viab0 (%) = 57.2 ± 3.9%, which was relativized to Viabr0 = 100 + 6.8%. In year 2010, Viab0 (%) = 65.5 ± 0.9%, which was relativized to Viabr0 = 100 ± 1.3. [c]In 2009, values in the same column within each exposure time followed by the same Roman numeral in brackets indicate no significant differences between levels of the main factor Soil treatment based on the Tukey *HSD* test ($p<0.05$). [d]In 2009, values in the same column within each exposure time followed by the same Arabic numeral in brackets indicate no significant differences between levels of the main factor Depth based on the Tukey *HSD* test ($p<0.05$). [e]Values of significance probability (*p*-values), parameter estimates ($F_{1,32}$, CPE) and residual in two-way ANOVA correspond to transformed variables, as indicated in the Statistics section. [f]*F* statistic with numerator and denominator degrees of freedom used in its calculation. [g]CPE: Covariance parameter estimate of the residuals of the statistical model. [h]In 2010, values in the same column within each exposure time followed by the same lower-case letter in brackets indicate no significant differences between levels of the interaction of Soil treatment and Depth factors based on the Tukey test ($p<0.05$). [i]In 2010, values in the same column within each exposure time followed by the same Roman numeral in brackets indicate no significant differences between levels of the main factor Soil treatment based on the Tukey *HSD* test ($p<0.05$). When differences were non-significant, letters were omitted.

**Table 2.** Infectivity of *Phytophthora capsici* oospores in bioassays with pepper plants after field disinfestation treatments in the 2009 and 2010 year greenhouse experiments. Three replicates of four plants each per treatment. Mean values (n=3) ± standard errors.

| Soil Treatment[a] | Depth (cm)[a] | $DP_{47}$ (%)[b] | $RAUDPC_{47}$ (% days/day)[c] | DP (%)[b] | RAUDPC (% days/day)[c] |
|---|---|---|---|---|---|
| | | | **Year 2009** | | |
| Sa_P | 15 | 8.3±8.3 | 0.07±0.07 | 83.3±8.3 | 1.18±0.19 |
| Na_NP | 15 | 91.7±8.3 | 1.09±0.21 | 100.0±0.0 | 2.43±0.15 |
| Sa_P | 30 | 16.7±8.3 | 0.22±0.18 | 91.7±8.3 | 1.43±0.22 |
| Na_NP | 30 | 58.3±22.0 | 0.78±0.38 | 91.7±8.3 | 1.91±0.47 |
| Mean Sa_P | _ | 12.5±5.6(II)[d] | 0.14±0.09(II) | 87.5±5.6 | 1.31±0.14 |
| Mean Na_NP | _ | 75.0±12.9(I) | 0.93±0.21(I) | 95.8±4.2 | 2.17±0.25 |
| _ | Mean 15 | 50.0±19.4 | 0.58±0.25 | 91.7±5.3 | 1.81±0.30 |
| _ | Mean 30 | 37.5±14.1 | 0.50±0.23 | 91.7±5.3 | 1.67±0.26 |
| **Factor** | | | **Two way ANOVA[e]** | | |
| Treatment | $F_{1,8}$[f] | 17.640 | 11.080 | 1.500 | 9.060 |
| | *p*-value | 0.003 | 0.010 | 0.267 | 0.017 |
| Depth | $F_{1,8}$ | 0.360 | 0.110 | 0.000 | 0.230 |
| | *p*-value | 0.565 | 0.746 | 1.000 | 0.644 |
| Treatment × Depth | $F_{1,8}$ | 1.960 | 0.920 | 1.500 | 1.770 |
| | *p*-value | 0.199 | 0.364 | 0.267 | 0.220 |
| Residual | CPE[g] | 0.143 | 0.170 | 0.061 | 0.247 |
| | | | **Year 2010** | | |
| Sa_P | 15 | 16.7±8.3 | 0.11±0.07 | 100.0±0.0 | 1.77±0.08 |
| Na_NP | 15 | 66.7±8.3 | 0.47±0.15 | 100.0±0.0 | 1.95±0.06 |
| Sa_P | 30 | 16.7±16.7 | 0.12±0.12 | 91.7±8.3 | 1.73±0.23 |
| Na_NP | 30 | 66.7±22.0 | 0.49±0.17 | 100.0±0.0 | 2.06±0.19 |
| Mean Sa_P | _ | 16.7±8.3(II)[d] | 0.11±0.06(II) | 95.8±4.2 | 1.75±0.11 |
| Mean Na_NP | _ | 66.7±10.5(I) | 0.48±0.10(I) | 100.0±0.0 | 2.01±0.09 |
| _ | Mean 15 | 41.7±12.4 | 0.29±0.11 | 100.0±0.0 | 1.86±0.06 |
| _ | Mean 30 | 41.7±16.7 | 0.30±0.12 | 95.8±4.2 | 1.89±0.15 |
| **Factor** | | | **Two way ANOVA[e]** | | |
| Treatment | $F_{1,8}$[f] | 9.850 | 0.023 | 1.000 | 2.650 |
| | *p*-value | 0.014 | 0.023 | 0.347 | 0.142 |
| Depth | $F_{1,8}$ | 0.000 | 0.894 | 1.000 | 0.030 |
| | *p*-value | 1.000 | 0.894 | 0.347 | 0.860 |
| Treatment × Depth | $F_{1,8}$ | 0.150 | 0.958 | 1.000 | 0.220 |
| | *p*-value | 0.705 | 0.958 | 0.347 | 0.649 |
| Residual | CPE[g] | 0.149 | 0.052 | 0.023 | 0.073 |

[a]Factorial experiment with treatments structure of two crossed factors: Soil treatment with two levels (Sa_P: *Sinapis alba* fresh green manure amendment plus soil plastic tarping; Na_NP: non-amended and non-plastic control). Depth of buried *P. capsici* oospores with two levels (15 cm; 30 cm). [b]$DP_{47}$ (%), DP (%): Percentage of diseased pepper plants by *P. capsici* oospores in bioassay after 47 days (mid-point of bioassay) and after 90 days (end-point of bioassay) respectively. In 2010, the variable was $DP_{46}$ (%). [c]$RAUDPC_{47}$ (%-days per day), RAUDPC (%-days per day): Relative area under the disease progress curve in bioassay with pepper plants expressed in percentage-days per day after 47 days (mid-point of bioassay) and after 90 days (end-point of bioassay) respectively. In 2010, the variable was $RAUDPC_{46}$ (%-days per day). [d]Values in the same column followed by the same Roman numeral in brackets indicate no significant differences between levels of the main factor soil treatment based on the Tukey *HSD* test ($p<0.05$). When differences were non-significant, letters were omitted. [e]Values of significance probability (*p*-values), parameter estimates ($F_{1,8}$, CPE) and residual in two-way ANOVA correspond to transformed variables, as indicated in the Statistics section. [f]*F* statistic with numerator and denominator degrees of freedom used in its calculation. [g]CPE: Covariance parameter estimate of the residuals of the statistical model.

estimates and residual in two-way ANOVA correspond to transformed variables (see footnotes in Tables 1 and 2).

# Results

### Soil temperatures

Daily temperatures were similar over two years. In the 2009 greenhouse experiment, daily maximum air temperatures varied from 19.6 to 38.3°C with an average value of 27.5°C and daily minimum air temperatures varied from 1.2 to 7.8°C with an average value of 3.8°C. In the amended-plastic tarped soil, the average of maximum and minimum daily temperatures were 13.9 and 13.1°C at 15 cm depth and 16.7 and 12.7ºC at 30 cm depth respectively. In the non-amended and non-tarped soil, the average of maximum and minimum daily temperatures were 15.1 and 11.1°C at 15 cm depth and 13.3 and 12.0ºC at 30 cm depth respectively.

### Effect of treatments on oospores viability

In the 2009 year experiment, oospore viability for four weeks exposure time was significantly affected by the main factors soil treatment ($p$<0.0001) and depth ($p$=0.042) (Table 1). In the 2010 year experiment, viability was significantly affected by the main factor soil treatment ($p$=0.003 and $p$=0.003 for two and four weeks exposure times, respectively) and the soil treatment × depth interaction which resulted significant for the first exposure time ($p$=0.031) and almost significant for the final exposure time ($p$=0.062) (Table 1). The significant soil treatment × depth interaction in year 2010 indicated that oospore viability only was affected by soil treatment at 30 cm depth but not at 15 cm, showing a higher survival in the biodisinfestated treatment than in the non-treated control. Winter biodisinfestation with *S. alba* did not reduce oospores viability, with final values significantly greater than in the non-treated control in both years (81% and 21% relative increase to the non-treated control in 2009 and 2010 respectively) (Table 1). Viability was significantly higher at 30 cm depth than at 15 cm in 2009 (24% relative increase) (Table 1).

### Effect of treatments on oospores infectivity

In most of the variables studied to characterize oospores infectivity in the bioassay with pepper plants, no significant effects were found for the main factors soil treatment, depth or their interaction in any of the two years (Table 2). Neither DP nor RAUDPC showed significant differences at the end of bioassay (end-point of bioassay; 90 days in 2009 and 88 days in 2010). Nevertheless, a significant delay in disease progression (Fig. 1) was observed till mid-point of bioassay (47 days in 2009 and 46 days in 2010) in the biodesinfestated treatment in relation to the non-treated control, as shown by the significantly lower values of the variables $DP_{47}$ ($p$=0.003) and $RAUDPC_{47}$ ($p$=0.01) in 2009 (Table 2) and the variables $DP_{46}$ ($p$=0.014) and $RAUDPC_{46}$ ($p$=0.023) in 2010 (Table 2). Mean values of these variables in the biodesinfestated treatment showed a relative decrease to the non-treated control of 83% and 85% in 2009 and 75% and 77% in 2010 respectively.

# Discussion

Soil temperature remained below certain levels (40-53°C) that are considered critical to the survival of *P. capsici* oospores (Etxeberria *et al*., 2011b). An absence of thermal inactivation was observed in this greenhouse experiment, located in the humid temperate Atlantic agroclimatic conditions of northern Spain. The low soil temperatures registered in our experiment (averages of 13.5 and 14.7ºC at 15 and 30 cm depth respectively) would explain the limited production of biocidal compounds by decomposition from Brassica tissues and the reduced efficacy against *P. capsici* buried inoculum, in line with the observations of Gamliel & Stapleton (1993), Wang *et al*. (2009) and Prasad *et al*. (2015) in other pathosystems. These authors observed that some biocidal compounds, such as methyl sulfide and dimethyl disulphide, were only detected in heated Brassica amended soils and not in the unheated amended soils.

Our results are in line with the experiments performed by Lacasa *et al*. (2015) in paprika crops in open field conditions in Western Spain (Extremadura), in which biodisinfestation in spring with *S. alba* fresh green manure at low soil temperatures (below 25 °C for more than 80% of exposure time) did not improve infectivity reduction of *Phytophthora nicotianae* chlamydospores introduced inoculum. Similar results were obtained by Rodriguez-Molina *et al*. (2016) in the same region after spring biodisinfestation with defatted seed meal *Brassica* pellets. In contrast, these authors observed that spring biofumigation with plastic cover with *Brassica* pellets plus *S. alba* fresh green manure had no significant effect on the survival of *P. nicotianae* but caused reduction in chlamydospores infectivity.

Conversely, survival of *P. capsici* oospores in Mediterranean agroclimatic conditions was much

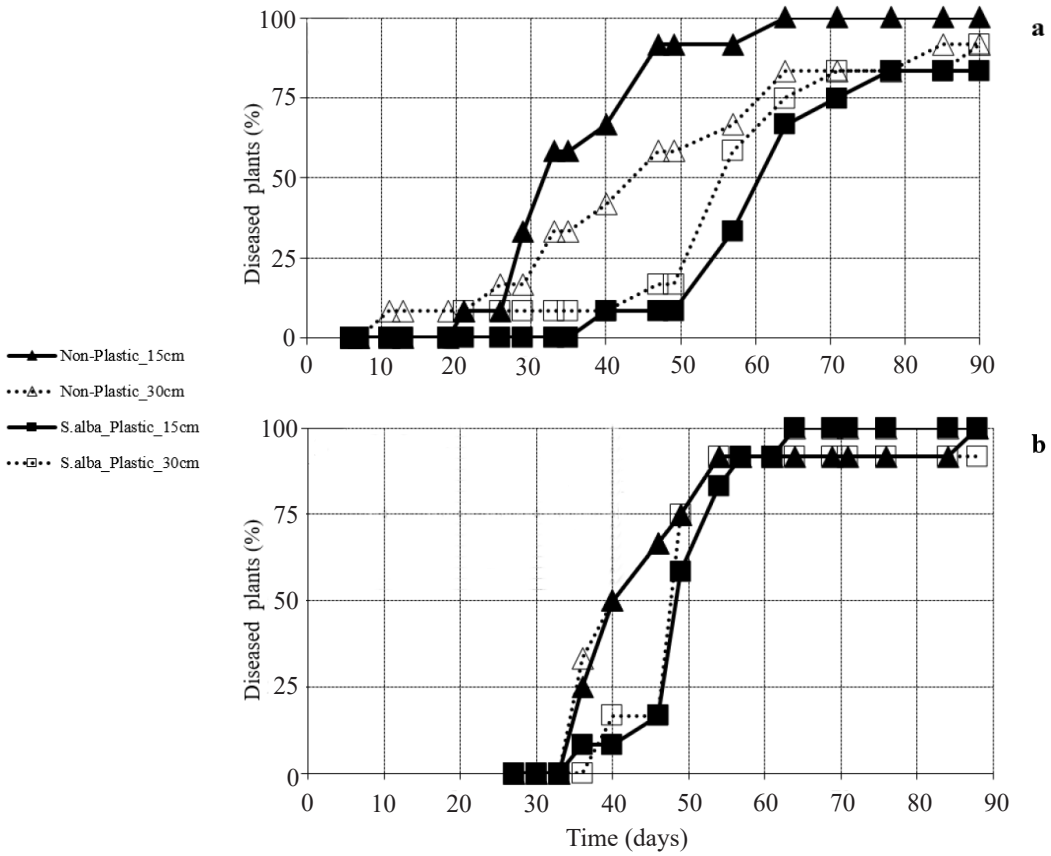


**Figure 1.** Temporal progression in the percentage of diseased plants in bioassays with potted pepper plants cv. 'Derio' (susceptible to *Phytophthora capsici*) and with soil previously inoculated with oospores for the determination of *P. capsici* oospores infectivity after four weeks exposure time to soil disinfestation treatments in the greenhouse experiments. Each point represents the mean of three replicates and each replicate is composed by four plants. Mean values (n=3). Years: 2009 (1a) and 2010 (1b).

reduced or almost extinct when soil temperatures were high during the biodisinfestation treatment. This was the case of greenhouses located in southeastern Spain that practice biosolarization in August-September with exposure times of six weeks and that apply fresh sheep manure mixed with other types of organic amendments such as fresh green manure of several Brassicaceae (Lacasa *et al*., 2015), defatted *Brassica carinata* seed meal dehydrated pellets (Guerrero *et al*., 2010) or several agro-industrial byproducts such as sugar-beet molasses (Lacasa *et al*., 2010) or Brewers' spent grain (Guerrero *et al*., 2014). In all these studies, depending on amendment treatments, the amended and plastic-tarped soil at 15 cm depth accumulated between 370 and 545 h at temperatures above 40ºC. These high temperatures enhance the effectiveness of the treatment (Katan & Gamliel, 2014) and the release of a greater variety of biocidal compounds (Matthiessen & Kirkegaard, 2006; Prasad *et al*., 2015, 2016). Thus, a synergic effect between temperature and the release of Brassica biocidal compounds might account for the greater survival reduction observed in *Phytophthora* inoculum.

However, the oospores infectivity results (Table 2) suggest that the application of *S. alba* as a green manure can increase the suppressive capacity of the soil since disease development was significantly delayed in the biodisinfestated treatments in both years when compared to the non-treated control treatments at both soil depths. This suppressiveness seems to be mediated by the increase in the soil's microbial activity linked to the organic matter decomposition of fresh green manure and changes in the soil microbioma composition which are involved in the suppression of several soil-borne pathogens (Larkin *et al*., 2007; Mazzola *et al*., 2017). The infectivity results of our experiment could be partially explained by the same mechanism observed by Mazzola *et al*. (2001) in the suppression of *R. solani* apple root rot by proliferation of soil microbes after *Brassica napus* seed meal amendment regardless of its glucosinolate content.

The low temperatures recorded in our experiment could explain the low reduction of *P. capsici* oospore survival and suggest that biofumigation mechanism pathway glucosinolates-myrosinase-isothiocyanates has had little effect on the low suppressiveness expressed in our infectivity bioassay. The generation of toxic compounds (mainly isothiocyanates) from the glucosinolates present in the species of Brassicas can inhibit the germination of oospores, as demonstrated by Manici *et al*. (2000) for oospores of *Pythium* spp., preventing or delaying the establishment of disease. On the other hand, the culture and subsequent biodisinfestation with *S. alba* significantly increased the dehydrogenase and glucosidase activities with respect to the non-treated control (percentages increases of 66% and 17%) in the first year (2009) and the dehydrogenase, glucosidase, urease and phosphatase activities (percentages increases of 90%, 31%, 52% and 37%) in the second year of reiteration (2010). The increase of microbial activity can influence the control of soil pathogens through antagonistic mechanisms such as parasitism, competition and antibiosis (Janvier *et al*., 2007), which could be linked to the initial observed delay in oospores infectivity in the biodisinfestation treatment with *S. alba* (Fig. 1; Table 2) and in agreement with other studies conclusions (Mazzola *et al*., 2001, 2018; Kirkegaard & Matthiessen, 2004; Cohen *et al*., 2005; Núñez-Zofío *et al*., 2012; Hewavitharana & Mazzola, 2016).

Suppressive effects of *P. capsici* were observed on the subsequent greenhouse pepper crop after applying a fresh green manure of *S. alba* as opposed to the utilization of *B. carinata* pellets which did not reduce the disease in previous experiments with greenhouse pepper crops in northern Spain (Núñez-Zofío *et al*., 2011a,b). The rhizosphere effect derived from the presence of *S. alba* plants during winter might be responsible for the observed higher soil biodiversity, possibly resulting in higher suppressiveness and lower disease incidence in the subsequent pepper crop cycles grown in years 2009 (2.8% in *S. alba* + plastic *vs* 42.6% in non-treated control) and 2010 (14.0% in *S. alba* + plastic *vs* 29.6% in non-treated control) (Núñez-Zofío *et al*., 2011b, 2012). This could be more related with the long-term (several weeks) suppressive effects of the use of Brassica linked to mechanisms of increase of microbial activity (Mazzola *et al*., 2001; Cohen *et al*., 2005) than with the short-term effect (a few days) linked to mechanisms of generation of biocidal isothiocyanates derived from glucosinolates hydrolysis through the myrosinase enzymatic system (Kirkegaard & Sarwar, 1998; Sarwar & Kirkegaard, 1998; Matthiessen & Kirkegaard, 2006; Prasad *et al*., 2015, 2016).

According to Lacasa *et al*. (2015), in the Mediterranean climate conditions of Southeastern Spain, the thermal effect of summer biosolarization had more impact than the biofumigant effect of compounds generated by Brassicas on *P. capsici* oospores viability. In contrast, in winter biodisinfestation with Brassicas in the humid temperate climate of northern Spain, thermal and biofumigant effects were insufficient for *P. capsici* oospores short-term inactivation, in agreement with reduced efficacy of soil solarization by cabbage amendment in climates such as in the Southeastern United States, where cloud cover and rainfall interrupted the heating process of the soil to eliminate *P. capsici* oospores at a depth of 25 cm (Coelho *et al*., 1999). However, long-term suppressive effects were achieved and final crop disease incidence was reduced. Reiteration of *S. alba* crops as fresh green manure could contribute to achieve long-term suppressive effects with the increase in soil's microbial activity and mobilization of specific antagonistic soil bacterial populations that reduced crop disease incidence in the subsequent pepper crop cycles (Mazzola *et al*., 2001; Cohen *et al*., 2005; Núñez-Zofío *et al*., 2011b, 2012).

In view of the obtained results, it can be concluded that winter biodisinfestation with fresh Brassica green manure is a promising management strategy for *Phytophthora* root rot control of protected peppers crops in regions of humid temperate climate such us northern Spain. Nevertheless, further research will be necessary to guarantee an effective *Phytophthora* biodisinfestation by fitting green manure species in combination with other fresh organic amendments.

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