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Control of oak root disease by fosetyl-Al

Trunk injection of fosetyl-aluminium controls the root disease caused by *Phytophthora cinnamomi* on *Quercus ilex* woodlands

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Summary

In Spain, *Quercus* open woodlands are animal ranching systems of organic production seriously threatened by the exotic pathogen *Phytophthora cinnamomi.* The root disease it causes kills thousands of oaks annually. Effective disease management needs to integrate different techniques, and the use of a resistance inducer such as fosetyl-Al can play a key role, since the use of potassium phosphite is prohibited in Spain. In a woodland where the pathogen recently arrived, 60 holm oaks in three different defoliation classes (asymptomatic, slight, and moderate defoliation) were selected for trunk injection with pressurized capsules containing 4% of commercial fosetyl-Al or water (controls). Holm oaks were checked periodically for defoliation and presence of the pathogen in roots and rhizosphere soil. Three years after treatments, defoliation was significantly lower in oaks treated with fosetyl-Al, which even increased canopy cover, in comparison with control oaks, independently of the initial defoliation class considered. Chlamydospore density in rhizosphere soil, as well as the presence of the pathogen into the roots, were not significantly influenced by fosetyl-Al treatments, although a trend to a lower presence of *P. cinnamomi* in roots was observed in treated oaks at every soil inoculum density detected. This study has shown that fosetyl-al, a phosphonate registered as a fungicide in the European Union, provides protection to holm oaks against *P. cinnamomi*, even exhibiting a therapeutic effect on preexisting infections. Consequently, this effective measure should be considered as part of the integrated approach to control this highly destructive pathogen in holm oak woodlands.

Keywords: *Dehesa*; fungicide; holm oak; oak decline; phosphite; phosphonate; resistance inducer.

Introduction

Dehesa is an open woodland ecosystem created and maintained by humans and their livestock (Scarascia-Mugnozza *et al*., 2000). The *dehesa* ecosystem spans an area of approximately 10^6 ha in Spain (Carevic *et al*., 2010), with monospecific holm oak (*Quercus ilex* subsp. *ballota*) or cork oak (*Q. suber*) the most frequent tree species. In Spain, *dehesas* are located mainly in the south, covering 27% of the total surface in the Andalusia region. The ecological, economic and social importance of *dehesa* systems justify its special protection (Habitats and Natura 2000 EU Directives). Despite preservation efforts, *dehesa* is threatened by poor or even non-existent tree regeneration and the dramatic effects of decline caused by *Phytophthora cinnamomi* (Sánchez *et al*., 2006). Similar agroecosystems also occur in Portugal (*montado*), Sardinia (Italy), Crete and northern Greece, North Africa and California (USA) (savanna-like ecosystems) and, in some cases, they are also threatened by *P. cinnamomi* root disease (Moreira & Martins, 2005; Garbelotto *et al*., 2006; Scanu *et al*., 2013). In Spain, Phytophthora root disease is killing thousands of trees every year (Romero *et al*., 2007a), with an estimated mortality of approximately 500,000 oak trees killed by this pathogen between 2006-2016 in Huelva, Andalusia, the most affected province in Spain (personal communication, ASAJA-Huelva). Different control techniques are available for use in an integrated control system which need to be implemented woodland to woodland for effective disease management. This include avoidance of high livestock loads and soil movements, encouraging soil drainage (Fernández *et al*., 2008), avoidance of highly susceptible herbaceous crops (Serrano *et al*., 2012b), application of calcium fertilizers to the soil (Serrano *et al*., 2012a) or biofumigant crops (Ríos *et al*., 2016). Together with integrated control, the use of resistance inducers is another suitable option to control the high rates of oak mortality caused by this pathogen. Potassium phosphite (K_2HPO_3) is the most frequently used product against Phytophthora diseases (McDonald *et al*., 2001). Phosphite treatment mainly reduces disease by stimulation of plant defense mechanisms, rather than through direct inhibition of pathogen growth (Guest & Grant, 1991). However, phosphite may also be directly inhibitory to *P. cinnamomi* mycelial growth (González *et al*., 2017). Equally, potassium phosphite is the main product used to control Phytophthora diseases of forest trees worldwide (Fernández-Escobar *et al*., 1999; Hardy *et al*., 2001; Gentile *et al*., 2009; Schmidt & Garbelotto, 2010), but its use was prohibited in Spain because the product was marketed as phosphoric fertilizer (RD506/2013, www.juntadeandalucia.es/agriculturaypesca/raif/novedades/2014/novedad_14050702.html). The potential antifungal activity of phosphite means that its commercialization requires toxicity analyses and residue persistence tests to enable registration as a fungicide. Before prohibition, phosphite was applied as trunk injections to control *P. cinnamomi* infections on oak trees in Spain (Fernández-Escobar *et al*., 1999). Recently, other systemic products registered as fungicides were tested on potted *Quercus* seedlings for use in the management of *Quercus* forest health (González *et al*., 2017). Fosetyl-aluminium (aluminium tris-O-ethyl phosphonate, fos-al), an alternative to phosphite, has been widely used in the management of diseases caused by Peronosporales, including some Phytophthora diseases of forest trees (Silva *et al*., 2016). Applied at doses recommended by the manufacturers in pot experiments, fos-Al reduced the disease symptoms in holm and cork oaks exposed to *P. cinnamomi* more effectively than phosphite (González *et al*., 2017). Therefore, the purpose of this work was to i) obtain statistical evidence to endorse the effectiveness of fos-Al to prevent *P. cinnamomi* root disease in mature holm oak trees, and ii) test the evolution of crucial events for disease outbreak (soil inoculum density and root infection) in treated and untreated oaks.

Material and Methods

Site characteristics and disease history

Dehesa Los Bueyes (Huelva, Spain, 37º 36.38' N; 7º 18.21' W) is an agroforestry system of Mediterranean climate composed of pure stands of *Q. ilex* subsp. *ballota* with 30-40 trees per ha of semi-natural pasture. This *dehesa* is primarily an acorn-Iberian pig and sheep ranching system. *Phytophthora cinnamomi* disease was first diagnosed in Dehesa Los Bueyes in 2001, after its isolation from rootlets of two symptomatic trees (M.E. Sánchez, unpublished data). Starting from this first detected focus, the presence of the root disease in this *dehesa* was reported for 2003-2005 (Romero *et al*., 2007b), killing a 45% of the trees across nearly 19 ha (295 killed oaks from a total of 643) until 2011. The disease has spreading downhill until it reached an asphalt pathway that divides Dehesa Los Bueyes in two. In 2010-11, very heavy autumn rains across the site caused runoff water to drag infested soil over the pathway, reaching the area previously free of disease. Three years later, foliar yellowing and wilting were evident on the former healthy half, just near the pathway.

Field surveys and samplings

In early autumn 2014, a field survey was carried out in Dehesa Los Bueyes to evaluate oak defoliation on the side of the pathway where the pathogen recently arrived. Each oak in an area of 12 ha (400 × 300 m) was visually classified in a 0-5 defoliation class (DC) scale (adapted from Lakatos & Mirtchev, 2014), being 0 = 0-10% defoliation (asymptomatic oaks), 1 = 11-25% defoliation (slight), 2 = 26-50% (moderate), 3 = 51-75% (severe), 4 = defoliation > 75% (very severe), 5 = dead oak. Diameter at breast height (DBH) of every tree was also measured. Twenty mature *Q. ilex* trees (DBH > 20 cm) per each main defoliation class found (DC 0, DC 1 and DC 2) were randomly selected. Then, 20 oaks initially belonging to DC 0 (asymptomatic oaks), DC 1 (slight defoliation) and

DC 2 (moderate defoliation) were selected, making a total of 60 oaks. For each DC, 10 oaks were treated with fos-Al and the other 10 with only water to act as controls.

Defoliation assessment was repeated twice a year on every selected oak for 3 years, in spring (May) and autumn (November), evaluating just after rainfall events. After 3 years of evaluations, the relative area under the disease progress curve (rAUDPC) was calculated as a percentage regarding the potential maximum value (Campbell & Madden, 1990), as follows:

$$\mathrm{rAUDPC} = \frac{100}{(s_{max} \times t_e)} \times \sum_{i=1}^{n} \frac{(s_i \times s_{i+1})}{2} \times (t_{i+1} \times t_i)$$

where s_i = defoliation value for observation number i, s_{max} = maximum value of defoliation (5), t_i = number of months between treatment and evaluation i, t_e = total evaluation period, and n = number of evaluations.

Together with the initial evaluation and autumn evaluations, soil and root samples were obtained from each selected oak. Root excavations (two holes per tree) were carried out 1 m from the base of the trunk at a depth of 10-50 cm. A total of approximately 200 cm^3 of symptomatic feeder roots and around 1 Kg of rhizosphere soil were collected and mixed to provide one single sample per tree. The samples (roots and soil) were placed into plastic bags, kept in a cool box and carried to the laboratory for further isolation.

The initial sampling was carried out in autumn 2014 and repeated in the autumn of 2015, 2016 and 2017.

Fosetyl-aluminium treatments

In autumn 2014, after the first evaluation and sampling, 10 oaks per DC were randomly selected to be treated with fos-al. Treatment was performed by trunk injection, applying pressurized capsules (Inyect©, Fertinyect SL, Córdoba) each one containing 200 ml of

4% of commercial product (ALIETTE©, Bayer) in water solution, as recommended by the company. One capsule was applied to each 20-25 cm of trunk perimeter, at 50 cm from the ground line. Depending on its DBH, each oak received 3-4 capsules. Control trees (10 per initial DC) were equally treated, but only water filled pressurized capsules applied. Treatments were applied in the middle of a sunny day, with a temperature of 20-25ºC. Trunk absorption of the capsules' contents took 20-30 min approximately.

***Phytophthora* isolations**

Rotten feeder roots obtained in each field sampling were washed under running tap water, air dried, cut in 4 mm-long segments and directly plated on NARPH medium (Hüberli *et al*., 2000). Six Petri dishes, each containing six root segments were plated per sample and dishes were incubated at 22º C in the dark for 5 days. Colonies growing from necrotic root segments were identified as *P. cinnamomi* based on hyphal morphology (characteristic coralloid hyphae with abundant grapelike clustered chlamydospores and rounded hyphal swellings, Romero *et al*., 2007b) observed under the inverted microscope (Olympus IMT-2, ×40) and data expressed as percentage of positive isolation for each oak tree: (number of root segments yielding one *P. cinnamomi* colony / total number of root segments plated in NARPH) × 100.

Soil samples (around 1 Kg each) obtained in each field sampling were air dried at room temperature until constant weight and sieved (2 mm). Then, 10 g of homogenized dry soil (ρ = 1.1) was suspended in 100 ml of sterilized water agar (0.2%) and shaken. One-milliliter aliquots were taken from the soil-water agar mix continuously handly stirred and plated on Petri dishes containing 20 ml of NARPH medium, using a sterile glass spreader to distribute the material over the agar surface. This dilution was previously shown to produce a countable number of colonies from soil samples of declining oaks (Romero *et al*., 2007b). For each soil sample, a total of 20 Petri dishes were prepared.

Dishes were incubated at 24º C in the dark for 24 h, then the agar surface of each dish was washed with sterile water, removing the soil-water agar mix. Dishes were re-incubated at 24º C in darkness for another 72 h. Colonies obtained were identified as *P. cinnamomi* based on hyphal morphology observed under the inverted microscope (characteristic coralloid hyphae with abundant grapelike clustered chlamydospores and rounded hyphal swellings, Romero *et al*., 2007b) and counted. Inoculum densities were expressed as colony-forming units per g dry soil ($CFUg^{-1}$).

Additionally, to establish the relationship between the presence of the pathogen in roots and soil, linear regressions were performed with *P. cinnamomi* positive isolation from roots (%) and data of soil inoculum density ($CFUg^{-1}$) as the best fit of adjusted R-squared. Data were grouped by the initial DC (30 trees per grouped data).

Statistical analyses

Data of defoliation, percentage of *P. cinnamomi* isolation from root samples, and soil inoculum density ($CFUg^{-1}$) along the evaluations, were checked for homoscedasticity by the Levene's test, and two-way Repeated Measures AOV tests were performed with oak treatment (fos-Al or water), initial DC, and the interaction treatment×initial DC as factors. To fit continuity, data of soil inoculum density were transformed to $(CFUg^{-1})^{0.5}$ before AOV analysis. A two-way General AOV was used for rAUDPCs considering the same factors (treatment, initial DC, and treatment×initial DC). For every analysis, mean values were compared by the Fisher's LSD test for $\alpha = 0.05$ (Statistix software 9.0).

Results

The most frequent DCs at the initial evaluation were 0, 1 and 2; and only a few trees exhibited severe defoliation (DC 3). Very severe defoliation (DC 4) or dead oaks (DC 5) were not detected at the experimental plot.

For defoliation considered along the evaluation period, the interaction treatment×initial DC was not significant ($F = 0.67$; $P = 0.5196$), but significance was achieved depending on treatment ($F = 7.97$; $P = 0.0154$), with a significantly lower defoliation for fos-Al treated oaks in comparison with water-treated (control) ones, independently of the initial defoliation class considered (Figure 1a). Moreover, treated oaks even increased their canopy cover.

Average values of rAUDPCs obtained for disease progression (defoliation) are in Figure 1b. ANOVA showed significant differences for rAUDPCs depending on oak treatment ($F = 13.08$, $P = 0.0007$) and initial DC ($F = 157.89$, $P < 0.0001$), but not for the interaction between both variables ($F = 0.69$, $P = 0.5056$). Comparison of means showed significantly lower values for rAUDPCs in holm oaks treated with fos-Al in comparison with control oaks (Figure 1b).

Mean values of inoculum density in oak rhizosphere soils are in Table 1. No significant differences were found depending on oak treatment ($F = 0.01$, $P = 0.9184$) nor initial DC ($F = 0.77$; $P = 0.4834$). For presence of *P. cinnamomi* in roots, average data of positive isolations are shown in Table 2. Significant differences were only found depending on initial DC ($F = 5.57$, $P = 0.0195$). The comparison of means showed a significantly higher presence of the pathogen in roots as the initial DC increases, but differences were not significant when treatment was considered.

The best fit for the relationship between chlamydospore soil density ($CFUg^{-1}$) (x) and positive isolation from roots (%) (y) was a linear model: $y = -0.1132x + 5.8913$ ($R^2 = 0.75$) for fos-Al treated oaks, and $y = -0.1121x + 6.645$ ($R^2 = 0.83$) for controls (Figure 2). An inverse relationship between variables was found for both groups of data (treated or control oaks), which did not differ significantly in variances ($P = 0.4611$), slopes ($P = 0.8551$) nor elevations ($P = 0.4216$).

Discussion

This study showed that the application of fos-Al by trunk injection was effective protecting holm oaks against *P. cinnamomi* root disease. Our results agree with those obtained with potassium phosphite against the same disease affecting oaks (Fernández-Escobar *et al*., 1999; Sánchez *et al*., 2006), *Eucalyptus* and other Australian native species (Hardy *et al*., 2001; Shearer *et al*., 2006), chestnuts and walnuts (Gentile *et al*., 2009) and another Phytophthora root or stem diseases affecting forest trees (Garbelotto & Smith, 2009; Dalio *et al*., 2014). One single treatment of 4% fos-Al was able to stop holm oak defoliation in the field, and even increasing crown density, even though the benefit of fos-Al became significant 2 years after treatment, like chestnut, walnut or declining Mediterranean *Quercus* treated with potassium phosphite (Fernández-Escobar *et al*., 1999; Gentile *et al*., 2009).

Gentile *et al*. (2009) pointed that phosphonate effectiveness is inversely proportional to initial disease severity, and it is generally assumed that phosphonate injections only provide protection against new *Phytophthora* infections when trees are healthy or slightly diseased. However, in this work, protection afforded by fos-Al resulted independent of the initial defoliation exhibited by treated holm oaks, from asymptomatic to moderately defoliated oaks. It remains unknown whether severely defoliated holm oaks (more than 50% of crown transparency) could also be protected by fos-Al injections.

The monoethyl phosphonate fos-al, or its breakdown product (phosphite), has a mixed mode of action in plant tissues: a direct action by direct inhibition of pathogen growth (González *et al*., 2017) or critical stages in the life cycle of *P. cinnamomi* (King *et al*., 2010), but protection against Phytophthora diseases appears specially based on its indirect action by implementing the natural defense mechanisms of the host plant to arrest pathogen development (Berkowitz *et al*., 2013). Considering that in the present work, injected holm oaks, including some asymptomatic ones, were already root-infected by *P. cinnamomi* when treatments were applied (as shown by *P. cinnamomi* isolation from root samples in early autumn 2014, before treatments), fos-Al possibly gave protection against new infections (preventive action), but some therapeutic effect is also feasible, since pre-existing infections did not continue in treated oaks and a recovery of canopy cover was observed. In this way, we expected that this likely therapeutic effect of fos-Al would result in a lower root colonization in treated holm oaks in comparison with controls for similar chlamydospore densities in soil, which in turn did not depend on fos-Al treatment either. However, when data of percentage of *P. cinnamomi* isolation from roots along the experiment were analyzed, this presumed difference between treated or untreated oaks was not found. Nevertheless, when correlations between chlamydospore density in the rhizosphere soil and root isolations were established, trend curves showed a lower presence of the pathogen in roots of treated trees in comparison with control roots at all soil chlamydospore densities found.

For quite some time, there are reports on the effectiveness of fos-Al against Phytophthora diseases on agricultural tree crops (i.e. El-Hamalawi *et al*., 1995), but little information is available on fos-Al application on natural or semi natural ecosystems, commonly attributed to its higher cost in comparison with potassium phosphite. However, costs are not so different. As an example, in Spain the cost for a single fos-Al injection is around 2.50 Euro, that made 7.50-10.00 Euro per tree, considering each tree needs an average of 3-4 injectors; while the same treatment injecting potassium phosphite has a cost of 2.30 Euro per injector (6.90-9.20 Euro per tree) (data from Fertinyect SL, Córdoba, Spain). Nevertheless, the effectiveness of fos-Al in forest trees was already demonstrated against *Phytophthora austrocedri* on Austral cypress (*Austrocedrus chilensis*) (Silva *et al*., 2016) or against *P. cinnamomi* on walnuts (Belisario *et al*., 2009).

In the present work, it was experimentally proved that fos-Al applied by trunk injection give protection against the root disease caused by *P. cinnamomi* in holm oaks growing in open woodlands (*dehesas*). This active ingredient is included as fungicide at the EU Pesticide Database and marketed in Spain as fungicide for use in agriculture and some forestry crops (i.e. *Cuppresus* spp.). Nevertheless, more research is needed to know the potential effectiveness of fos-Al treatment in heavily diseased oaks (more than 50% defoliation), the minimum effective concentration of fos-Al applied to holm oaks, or the length of protection afforded. We found at least 3 years of protection, but this period could be longer, may be comparable to the frequency of potassium phosphite injections (4 years) to protect Australian forest species from *P. cinnamomi* root disease (Shearer & Fairman, 2007).

As conclusion, while more research is needed, fos-Al trunk injection was evidenced as an effective measure which should be counted in the integrated management to fight against the highly destructive disease that *P. cinnamomi* causes in *dehesa* ecosystems.

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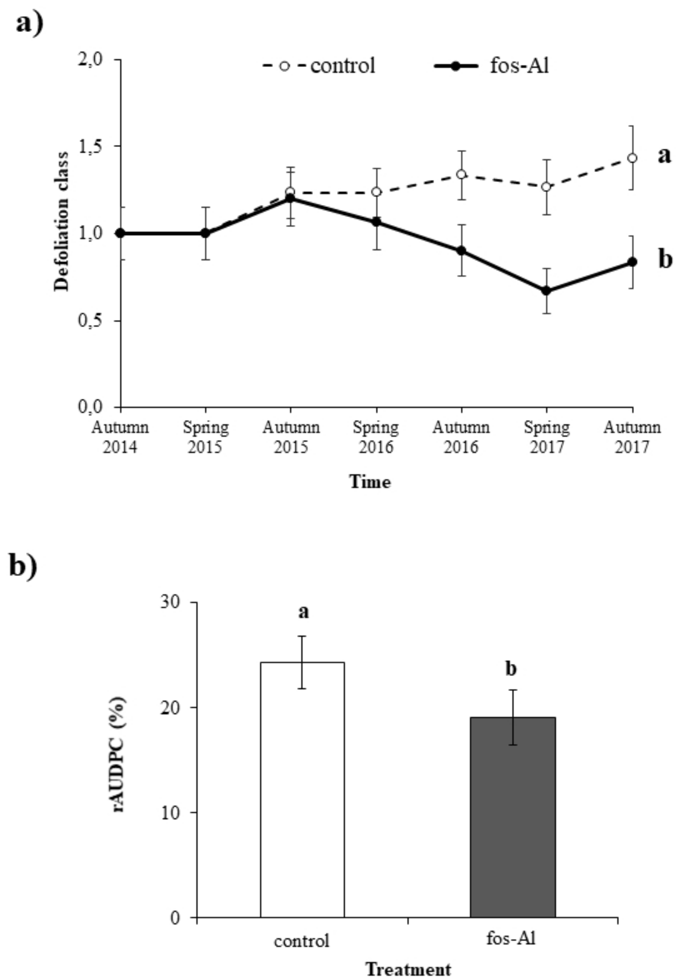
Table 1 Inoculum density (average ± SE of viable *Phytophthora cinnamomi* propagules per gram of dry soil, $CFUg^{-1}$) detected in samples of rhizosphere soil from oaks treated with water (control) or fosetyl-aluminium (fos-Al) trunk injections. Data are grouped by initial oak defoliation class (DC), being 0 = 0-10% defoliation (asymptomatic oaks), 1 = 11-25% (slight defoliation), 2 = 26-50% (moderate defoliation)

Table 2 Percentage (average ± SE) of positive *Phytophthora cinnamomi* isolation from roots of holm oaks treated with water (control) or fosetyl-aluminium (fos-Al) trunk injections. Data are grouped by initial oak defoliation class (DC), being 0 = 0-10% defoliation (asymptomatic oaks), 1 = 11-25% (slight defoliation), 2 = 26-50% (moderate defoliation)

Figure 1 a) Average values and SE of defoliation recorded in holm oaks treated with water (control) or fosetyl-aluminium (fos-Al) trunk injections along the period autumn 2014-autumn 2017. b) Average values and SE obtained for the relative area under the disease progress curve (rAUDPC) for defoliation recorded over time in holm oaks treated with water (control) or fosetyl-aluminium (fos-Al) trunk injections. Bars marked with different letters significantly differ according with the Fisher's LSD test for $P < 0.05$

Figure 2 Relationship between percentage of positive isolation of *Phytophthora cinnamomi* from roots [(number of root segments yielding one *P. cinnamomi* colony / total number of root segments plated in NARPH) × 100] and inoculum density (viable propagules per gram of dry soil, $CFUg^{-1}$) detected in the rhizosphere along the period autumn 2014-autumn 2017 for holm oaks treated with water (control) or fosetyl-aluminium (fos-Al) trunk injections. Dots are the average values obtained (white dots = controls; black dots = fos-Al treated oaks), and the lines are the adjusted linear regressions



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190x254mm (96 x 96 DPI)

Table 1 Inoculum density (average ± SE of viable *Phytophthora cinnamomi* propagules per gram of dry soil, CFUg^{-1}) detected in samples of rhizosphere soil from oaks treated with water (control) or fosetyl-aluminium (fos-Al) trunk injections. Data are grouped by initial oak defoliation class (DC), being 0 = 0-10% defoliation (asymptomatic oaks), 1 = 11-25% (slight defoliation), 2 = 26-50% (moderate defoliation)

Treatment	Initial DC	Soil inoculum density (CFUg^{-1})			
		autumn 2014	autumn 2015	autumn 2016	autumn 2017
control	0	52.6 ± 10.4	37.7 ± 11.0	28.1 ± 5.4	12.4 ± 3.2
	1	78.2 ± 32.7	60.5 ± 19.6	21.6 ± 9.6	6.8 ± 3.3
	2	63.1 ± 11.3	33.6 ± 8.0	29.3 ± 5.4	11.6 ± 2.5
fos-Al	0	35.8 ± 9.1	28.7 ± 8.3	28.0 ± 7.2	15.4 ± 4.3
	1	67.3 ± 13.1	40.4 ± 10.9	23.5 ± 6.0	13.7 ± 4.4
	2	62.5 ± 14.9	45.7 ± 11.3	16.4 ± 4.0	24.9 ± 13.3

Table 2 Percentage (average ± SE) of positive *Phytophthora cinnamomi* isolation from roots of holm oaks treated with water (control) or fosetyl-aluminium (fos-Al) trunk injections. Data are grouped by initial oak defoliation class (DC), being 0 = 0-10% defoliation (asymptomatic oaks), 1 = 11-25% (slight defoliation), 2 = 26-50% (moderate defoliation)

Treatment	Initial DC	Positive isolation from roots (%)			
		autumn 2014	autumn 2015	autumn 2016	autumn 2017
control	0	0.3 ± 0.3	0.0 ± 0.0	2.9 ± 1.0	4.8 ± 1.6
	1	0.6 ± 0.4	0.3 ± 0.3	2.8 ± 1.2	6.4 ± 2.3
	2	0.6 ± 0.6	0.3 ± 0.3	4.3 ± 1.4	7.8 ± 1.5
fos-Al	0	0.3 ± 0.3	0.3 ± 0.3	0.6 ± 0.6	4.0 ± 1.7
	1	0.6 ± 0.4	0.0 ± 0.0	2.0 ± 0.8	6.4 ± 2.0
	2	0.8 ± 0.4	0.0 ± 0.0	5.8 ± 2.1	10.2 ± 3.4

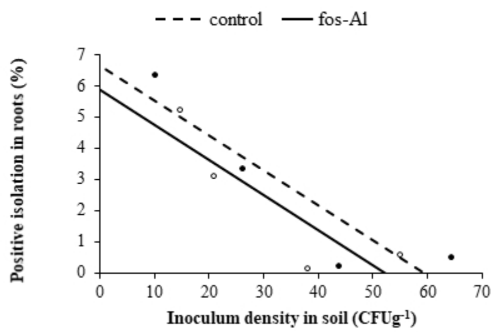

Figure 2 Relationship between percentage of positive isolation of Phytophthora cinnamomi from roots [(number of root segments yielding one P. cinnamomi colony / total number of root segments plated in NARPH) × 100] and inoculum density (viable propagules per gram of dry soil, CFUg-1) detected in the rhizosphere along the period autumn 2014-autumn 2017 for holm oaks treated with water (control) or fosetyl-aluminium (fos-Al) trunk injections. Dots are the average values obtained (white dots = controls; black dots = fos-Al treated oaks), and the lines are the adjusted linear regressions

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