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Effect of Substrate Solarization for the Control of Fungi: The Case Study of *Fusarium circinatum*, the Quarantine Agent of Pine Pitch Canker

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Abstract. Pine pitch canker is one of the much concerning forest diseases in Europe, affecting both adult and young plant hosts. Substrates used in nurseries, especially the ones incorporating *Fusarium circinatum* host plant residues, such as pine bark, may represent a vehicle for the spread of the fungus. The present study tested the efficacy of solarization on the elimination of *F. circinatum* inoculum from an artificially inoculated substrate, after three and seven weeks of treatment application, compared with a non-solarized control treatment. The results show a reduction of the viable inoculum density of *F. circinatum* from the substrate after seven weeks, which suggests that substrate solarization may be a process to minimize biotic risks in a nursery.
Key words: damping-off, *Gibberella circinata*, integrated management, nurseries, soil disinfection

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Efeito da Solarização do Substrato no Controlo de Fungos: o Caso de Estudo de *Fusarium circinatum*, Agente de Quarentena do Cancro Resinoso do Pinheiro

Sumário. O cancro resinoso do pinheiro é uma das doenças florestais mais preocupantes na Europa, afetando plantas hospedeiras adultas e jovens. Os substratos usados em viveiros, principalmente aqueles que incorporam resíduos de plantas hospedeiras de *Fusarium circinatum*, como a casca de pinheiro, podem representar um veículo de disseminação do fungo. O presente estudo testou a eficácia da solarização na eliminação do inóculo de *F. circinatum* de um substrato artificialmente inoculado, após três e sete semanas, em comparação com um controlo não solarizado. Os resultados mostram uma redução da densidade do inóculo viável de *F. circinatum* no substrato após sete semanas, o que sugere que a solarização do substrato pode ser um processo de minimização de riscos bióticos em viveiro.

Palavras-chave: *damping-off*, *Gibberella circinata*, gestão integrada, viveiros, desinfeção do solo

Effet de la Solarisation du Substrat pour le Contrôle des Champignons: le Cas d'Étude du *Fusarium circinatum*, Agent de Quarantaine du Chancre Résineux du Pin

Résumé. Le chancre résineux du pin est l'une des maladies forestières les plus préoccupantes en Europe, affectant les adultes et les jeunes plantes hôtes. Les substrats utilisés dans les pépinières, en particulier ceux incorporant des résidus de plantes hôtes de *Fusarium circinatum*, telle que l'écorce de pin, peuvent représenter un véhicule pour la propagation du champignon. La présente étude a testé l'efficacité de la solarisation sur l'élimination de l'inoculum de *F. circinatum* d'un substrat inoculé artificiellement, après trois et sept semaines, par rapport à un témoin non solarisé. Les résultats montrent une réduction de la densité d'inoculum viable de *F. circinatum* à partir du substrat après sept semaines, ce qui suggère que la solarisation du substrat peut être un processus pour minimiser les risques biotiques dans une pépinière.

Mots-clés: fonte des semis, *Gibberella circinata*, gestion intégrée, pépinières, désinfection des sols

Introduction

Fusarium circinatum Nirenberg & O'Donnell (synonym *Gibberella circinata* Nirenberg & O'Donnell) is the causal agent of Pine Pitch Canker (PPC), one of the major pine diseases, affecting *Pinus* spp. and *Pseudotsuga menziesii* (Mirb.) Franco (NIRENBERG and O'DONNELL, 1998).

Pine Pitch Canker affects both adult trees and young seedlings. In adult trees, the main symptoms are pitch cankers on the trunk or in large lateral branches. Multiple branch infections may cause severe crown dieback and lead to tree mortality. PPC also causes reduced growth and lower timber quality. The fungus may also infect roots, shoots, flowers, cones and seeds. In forest nurseries, PPC is often responsible for damping-off and massive mortality of seedlings, causing large economic losses (DRENKHAN *et al.*, 2020).

Fusarium circinatum is natural from Central America (WIKLER and GORDON, 2000), but since it was first discovered in the USA in 1946, it has been introduced across the globe and currently has a worldwide distribution (BARNARD and BLAKESLEE, 1980; MURAMOTO and DWINELL, 1990; VILJOEN *et al.*, 1994; WINGFIELD *et al.*, 2002; DRENKHAN *et al.*, 2020). In Europe it was first reported in Spain in 2005 (LANDERAS *et al.*, 2005) and after that it spread across other European countries, including France (EPPO, 2006), Italy (CARLUCCI *et al.*, 2007) and Portugal (BRAGANÇA *et al.*, 2009). However, in France and Italy it is already eradicated. Due to the damages and the rapid spread of the fungus, this pathogen is currently included in the A2 list by the EPPO (European and Mediterranean Plant Protection Organization) and the Annex IIA of the Commission Implementing Regulation (EU) 2019/2072 as a quarantine organism present in EPPO countries area but not yet extensively distributed (VETTRAINO *et al.*, 2018).

The introduction of PPC into new areas, specially at great distances from the origin, seems to be related with the transport of infected plant material or seeds (STORER *et al.*, 1998; DRENKHAN *et al.*, 2020). *Fusarium circinatum* is a seedborne pathogen that can survive both superficially and internally in asymptomatic seeds (STORER *et al.*, 1998), which favors the spread of the disease. In forest stands, dispersal of *F. circinatum* spores occurs through wind, water and insect vectors, infecting trees through wounds caused by insect feeding or pruning (GORDON *et al.*, 2001; SCHWEIGKOFLER *et al.*, 2004; DVOŘÁK *et al.*, 2017). *Fusarium circinatum* spores may also survive in soil and wood debris for large periods of time (DWINELL and BARROWS-BROADDUS, 1978; GORDON *et al.*, 2001; SERRANO *et al.*, 2017).

In Portugal, as in most instances of introduction into new areas, the disease was first found in nurseries (BRAGANÇA *et al.*, 2009). Rigorous measures have been taken to prevent the infection to spread across the country, which also lead to great economical constraints for forest seedlings producers.

Currently, not many ways for the control of *F. circinatum* are available (AGUSTÍ-BRISACH *et al.*, 2012). In nurseries, the most important means to prevent new infections is to ensure the use of disease-free seed. Moreover, the use of pathogen-free irrigation water, sterile growth substrates and containers are of much relevance (WINGFIELD *et al.*, 2008).

Hence, it is important to find new integrated management strategies to fight against this fungus. The search for environmentally friendly solutions to fight against it is also of major relevance. Soil solarization is an example of such a technique used in agriculture for long time and against diverse pathogens (KATAN *et al.*, 1976; MORRA *et al.*, 2018). This process aims to decrease the level of inoculum of potential pathogens trough the increase of soil temperature using a plastic film, which retains temperature and humidity (KATAN *et al.*, 1976). It had been used for various species of fungi, including the genus *Fusarium* (PATRÍCIO *et al.*, 2007; BARAKAT and AL-MASRI, 2012; MORRA *et al.*, 2018). In the present study, we aimed to evaluate the potential effect of solarization on eliminating *F. circinatum* inoculum from a widely used substrate on nurseries.

Methodology

To assess the effect of solarization on *F. circinatum* inoculum viability in the substrate, a seven-weeks long period trial were carried out from August 9th to September 27th, 2019. Two treatments were compared: solarization (S), and non-solarization control (NS). Each treatment was applied in 70×40×15 cm boxes filled with substrate (Figure 1). A total of four boxes were used, two boxes per treatment. For each treatment, one box was used for *F. circinatum* viability testing and other for temperature monitoring.

Site of experiment

The experiments were carried out in Oeiras, Portugal (38°41'45.5"N 9°19'14.8"W).

A commercial substrate commonly used in nurseries for pines and other forest species seeding was used to perform the experiments. Substrate consisted of

humus based on forest residues, with predominance of maritime pine bark, and selected blond peat 0–40 mm and 2 kg of controlled release fertilizer with NPK 17-9-11 + 2 MgO 2 kg m^{-3} (conductivity: 80–120 µS cm^{-1}; pH 5.5–6.5; organic matter composition >70%; water content: 40%).

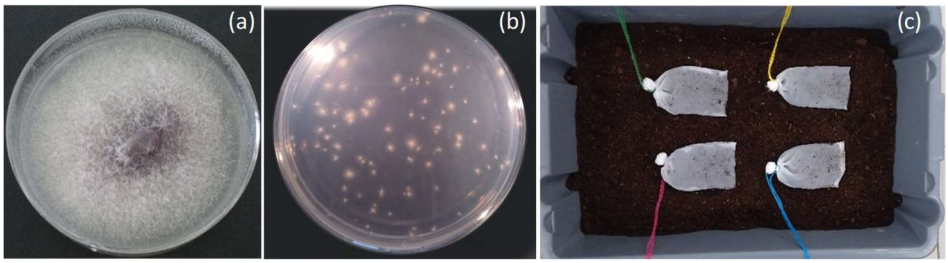

Figure 1 - *Fusarium circinatum* culture on PDA after 10 days incubation in dark, at 25±2°C (a) and CFU's on DCPA after 3 days incubation at room temperature, 23±2°C (b); nylon bags distribution, containing inoculated substrate, in the boxes (c), which were than completed with substrate and sealed with two layers of clear plastic film

Solarization method

Four boxes (70×40×14 cm) were fulfilled with the substrate and covered with two layers of clear plastic film (transparent polyethylene sheet of approximately 50 µm thickness). At 10 cm depth, four 30 g bags containing substrate artificially inoculated with *Fusarium circinatum* spore suspension were deposited in each of two boxes. The solarization treatment consisted in covering the box containing the substrate with the plastic film and exposing it to direct solar light. The control treatment (non-solarization) was applied to the second box containing artificially inoculated substrate and consisted of covering the box with the plastic film and keeping it apart from direct solar light exposure. The remaining two boxes were also exposed to each treatment for temperature recording as explained in subsection 2.3.

Soil temperature recording

To assess the thermal performance of the solarization and non-solarization treatments in the seven-weeks long period, one data-logger was placed at 10 cm depth in the substrate, inside each of two boxes. One was exposed to solarization

treatment and the other was exposed to the non-solarization control treatment. Each data-logger recorded the temperature with a record interval of 1 min. The sums of hours in which the temperatures feat in each temperature class (<25ºC; 25–30ºC; 30–35ºC; 35–40ºC; 40–45ºC; ≥45ºC) were calculated.

Substrate inoculation with Fusarium circinatum

A *F. circinatum* isolate (MEAN 1353) obtained from the culture collection of the INIAV institute (MEAN – "Micoteca da ex-Estação Agronómica Nacional" sited in Oeiras, Portugal) and previously isolated from *Pinus* sp. in Portugal was used to artificially inoculate the substrate. A spore suspension was prepared from cultures on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, EUA) grown at 25±1°C for 10 days in 90 mm Petri plates. Sterile deionized water was added to the colonies and spores were dislodged using a sterile glass rod. The spore suspensions were resuspended in sterile deionized water and concentration adjusted to 2×10^5 conidia mL^{-1} using a haemocytometer (American Optical Company, USA). The substrate which had previously been autoclaved for 30 min, was inoculated with 1 ml of the spore suspension per 1 g of substrate (initial fresh weight, water content 40%). The inoculated substrate was placed in little nylon bags, containing the equivalent of 30 g of substrate (initial fresh weight, water content 40%). A total of eight bags, four bags per treatment, were placed at 10 cm depth in each box and exposed to the respective treatment.

Viability of Fusarium circinatum

The viability of *F. circinatum* propagules was determined at the beginning of the experiment and 3 and 7 weeks after the application of solarization and non-solarization treatments. For assessing initial substrate inoculum concentration, after inoculation, two samples equivalent to 5 g of initial fresh weight (water content 40%) were weighed. For accessing 3 and 7 weeks substrate inoculum concentration, two bags (replicates) were removed from each treatment box at the respective time. One substrate sample, equivalent to 5 g of initial fresh weight (water content 40%), was weighed from each bag. For all, each 5 g substrate sample was diluted in 50 mL sterile distilled water with 0.01% Tween 20. Serial dilutions were made. For each dilution (10^{-2}, 10^{-3} and 10^{-4}), 500 ml of the suspension were placed in each of five Petri dishes (90 mm) containing dichloran-chloramphenicol peptone agar (DCPA; IOOS *et al.*, 2009), totalizing 15 plates per bag. The plates were then incubated at room temperature, approximately 23±2°C.

After that, the number of colony forming units (CFU's) per plate was counted. Colonies not immediately associated to *F. circinatum*, were sub-cultured on PDA and Spezieller-Nährstoffarmer Agar (SNA) to obtain an accurate identification. To each sample, the counting was performed with the 5 plates whose number of CFU's was between 20 to 100 or closer. The mean of each two replicates corresponded to the substrate inoculum concentration at each time and treatment. The viability of *F. circinatum* was calculated as the concentration of colony forming units (CFU's) per g of substrate (dry weight) (WONG *et al.*, 2011).

Results

Substrate temperature

Substrate temperature was greatly higher in solarized soil treatments compared with the control (Table 1 and Figure 2). The means of substrate temperatures (ºC) recorded during the solarization period were 32.8 and 23.3 during the first three weeks and 31.1 and 23.6 during the total seven weeks of the trial, under solarization and non-solarization control treatments, respectively. The substrate temperatures (ºC) vary between 22.5 and 25.5 in control and from 17.0 to 55.5 in solarization treatment during the seven weeks of the trial. Under solarization, the number of hours recorded for the temperature class (≥45ºC) were 49 after 3 weeks and 53 h after 7 weeks, revealing less extreme heat temperatures in September 2019, when compared with the first three weeks of the trial (August 2019).

Viability of Fusarium circinatum

The solarization treatment was able to decrease *F. circinatum* inoculum viability in the substrate from 2.05×10^5 to 2.47×10^3 CFU's per g of dry weight after seven weeks of treatment application. It was a greater decrease when compared with the control treatment, whose final *F. circinatum* viable inoculum density consisted of 4.57×10^4 CFU's per g of dry weight (Figure 3). Nonetheless, the contrary was observed after 3 weeks of treatment application. In this case, the viability of the inoculum was lower in the control (1.13×10^5 CFU's per g of dry weight) than in the substrate exposed to solarization (1.7×10^5 CFU's per g of dry weight).

Table 1 - Number of hours for different temperature classes recorded at 10 cm substrate depth under the treatments Solarization (S) and Non-Solarized Control (NS) during 3 weeks (August 9th-August 30th) and during 7 weeks (August 9th-September 27th)

Temperature class	Number of hours (3 weeks)		Number of hours (7 weeks)	
	NS	S	NS	S
<25ºC	503	113	1175	331
25 -30ºC	1	113	1	279
30 -35ºC	0	88	0	214
35 -40ºC	0	75	0	165
40 -45ºC	0	66	0	134
≥45ºC	0	49	0	53
Total (hours)	504	504	1176	1176
Max (ºC)	25.5	50.5	25.5	50.5
Min (ºC)	23	19	22.5	17
Average (ºC)	23.3	32.8	23.6	31.1
Sd (ºC)	0.3	8.2	0.4	7.7

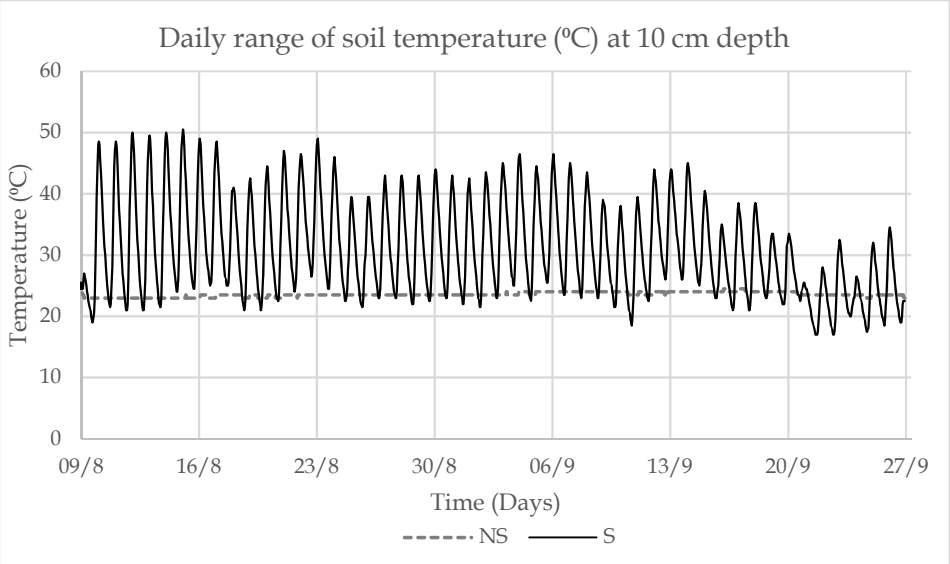

Figure 2 - Daily range of temperatures in substrate, at 10 cm depth, from 9th August to 27th September 2019, during Solarization (S) and Non-Solarized Control (NS) treatments

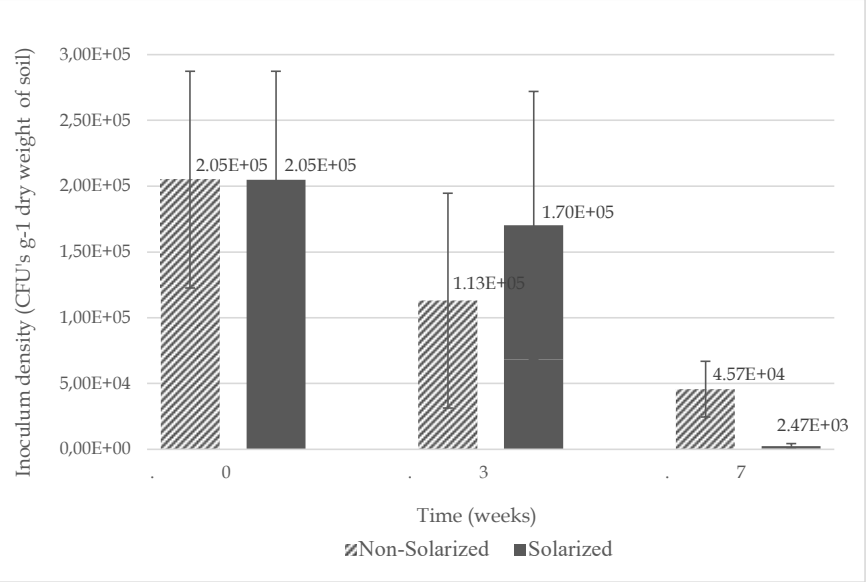

Figure 3 - Effect of solarization and non-solarization (control) treatments in the viability of *Fusarium circinatum* inoculum, in the substrate, after 3 and 7 weeks of treatment application (average density ± SD, CFU's g^{-1} dry weight of substrate). S – Solarized, NS – Non-Solarized Control

Discussion and conclusions

Substrates used in nurseries, especially the ones including *F. circinatum* host plant residues, such as pine bark, may represent a vehicle for the spread of the fungus (DWINELL and BARROWS-BROADDUS, 1978; GORDON *et al.*, 2001). Therefore, it is important to find ways to prevent infection from the use of these substrates. The present study tested the efficacy of solarization on the elimination of *F. circinatum* inoculum from an artificially inoculated substrate. The results suggest that substrate solarization is effective to reduce *F. circinatum* density from the substrate, even though this process takes time and may not eliminate the fungus completely.

Although not leading to complete elimination of *F. circinatum* inoculum in the substrate, solarization treatment was able to reduce *F. circinatum* viable inoculum density in the substrate from 2.05×10^5 to 2.47×10^3 CFU's per g (dry wight) after 7 weeks of application, which was a greater decrease when compared with the

control treatment whose final *F. circinatum* viable inoculum density consisted of 4.57×10^4 CFU's per g of dry weight. BARAKAT and AL-MASRI (2012) tested soil solarization treatments against *Fusarium oxysporum* f. sp. *lycopersici* also after 3 and 7 weeks of application and only after seven weeks of solarization, the population of *F. oxysporum* (CFU's) in soil was significantly reduced.

In the present study, the observed decrease in inoculum density might be related with the number of hours of exposition to higher temperatures in solarization treatment when compared with the control treatment (see Table 1 and Figure 2). Nevertheless, a decrease in the inoculum density was also observed in the control treatment. This might be related with the transference of the inoculum from optimal conditions (culture media and adequate humidity and temperature, approx. 25ºC) to the substrate.

Although the temperatures reached in the solarized substrate from the 4th until the 7th week (4 hours ≥45ºC) were less extreme than the temperatures from the 1st to the 3rd week of the trial (49 hours ≥45ºC), the decrease in viable inoculum density was higher in the last four weeks, than it was in the first three weeks. Yet, sublethal temperatures ≥35ºC exposition was 190 hours in the first three weeks and 162 hours in the last 4 weeks, completing a total of 352 hours during the seven weeks of the trial. These results outline the relevance of the long exposition time to sublethal temperatures in the reduction of inoculum density. FREEMAN and KATAN (1988) verified that the exposition of conidia and chlamydospores of *Fusarium oxysporum* f. sp. *niveum* to temperatures from 38 to 42ºC caused a reduction in propagule viability and resulted in a weakening effect in the surviving propagules. *Fusarium circinatum* propagules may also be affected by the exposition to these temperatures. In fact, QUESADA *et al.* (2019) tested growth of 15 isolates of *F. circinatum* isolates in culture at three temperatures: 25, 27, and 31°C and observed that all isolates showed a significant decrease in growth at 31°C.

Considering the amount of time needed for solarization treatment to be effective on the elimination of various soilborne fungi, various studies have been performed with enhanced technics of soil solarization to increase its effect on fungal inoculum reduction and decrease the amount of time required for its effectiveness against various fungal species, including *Fusarium* spp. (BARAKAT and AL-MASRI, 2012; WONG *et al.*, 2011; MORRA *et al.*, 2018). MORRA *et al.* (2018) tested the efficacy of a biodegradable black liquid consisting in a gel carbon black mixture when sprayed on the soil in order to work as a thermal solar panel, which led to a reduction in the application of the treatment, when compared with traditional solarization; BARAKAT and AL-MASRI (2012) tested the efficacy of

double layers of polyethylene sheets, and sheets containing ethylene vinyl alcohol, with the first leading to improvements when compared to regular soil solarization; other authors (WONG *et al.*, 2011) verified that the incorporation of decomposing vegetable organic matter in the soil, combined with solarization treatment application, may improve the efficacy of soil solarization against *Fusarium oxysporum* f. sp. *lycopersici*. The effect of soil solarization, followed by the application of biocontrol agents, such as *Trichoderma* spp., or fungicides has also been studied (PATRÍCIO *et al.*, 2007).

Although being presently difficult to currently apply this treatment in nurseries given the amount of time and space required to achieve the amount of substrate needed and given the uncomplete *F. circinatum* inoculum elimination, this study suggests that solarization may decrease viable inoculum density on a substrate and represents an interesting treatment in other contexts. In fact, the efficacy of soil solarization on decreasing fungal propagules have been studied and described by various authors on diverse soilborne pathogens, including the genus *Fusarium* (BARAKAT and AL-MASRI, 2012; MORRA *et al.*, 2018).

Various efforts have been developed concerning the fight against this fungus. The use of certified material is of much relevance to reduce the risk of infection trough this via. Moreover, some studies point out new alternative substrates from pine bark incorporating substrates (SILVA, 2018).

In conclusion, substrate solarization was able to decrease *F. circinatum* inoculum density in the substrate after seven weeks of treatment application, which gives good perspectives for future fungal control using this technique. Under the conditions tested, the fungus was not completely eliminated from the substrate, hence, an optimization of the solarization technic could be useful in order to achieve complete elimination of the fungus from the substrate, making solarization a viable solution to minimize biotic risks in a nursery. Given the relevance of pine pitch canker on nurseries around the world, it is extremely important that more and more effective fighting technics against this fungus are discovered and applied in an integrative management strategy.

In the present study, a tendency towards a decrease in the viability of *F. circinatum* was observed. However, further studies with larger essays should be performed to attest these results and assess the potential of substrate solarization for the control of pine pitch canker and also for other fungal diseases on nurseries and on other contexts.

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