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ORIGINAL ARTICLE

Lignin nanoparticles containing essential oils for controlling *Phytophthora cactorum* diseases

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Abstract

Phytophthora cactorum is a plant pathogen affecting a wide range of hosts, causing economically damaging diseases, such as damping off and root rot, in fields and nurseries. Current plant protection strategies are often inadequate to control *Phytophthora* diseases. Hence, an attempt was made to evaluate the potential of a novel control method using lignin nanoparticles loaded with essential oil of *Thymus serpyllum* (EO-LNPs) for controlling *P. cactorum* infections on *Pinus nigra* in vitro and greenhouse conditions. Nanoparticles were characterized by Py-GCMS and the drug efficiency, and drug loading capacity were determined using HPLC. *T. serpyllum* essential oils (EOs) were characterized by gas chromatography (GC-FID and GC-MS). Under in vitro conditions, the median effective concentration (EC_{50}) values were 20.453 and 88.711 μg/ml, for EOs and EO-LNPs, respectively. Furthermore, in vivo tests revealed that thyme EOs and EO-LNPs were very effective in reducing the mortality of inoculated pine seedlings, with an inhibition rate of 93% and 100%, respectively. Results reported in this study open the possibility of using EO-LNPs for improving plant health in greenhouse settings. The design of new protection strategies relying on lignin nanoparticles conforms to the principles of the circular economy.

KEYWORDS
lignin, nanoparticles, *Phytophthora cactorum*, *Pinus nigra*, *Thymus serpyllum*; essential oils

1 | INTRODUCTION

Phytophthora is a genus containing more than 150 species (Yang et al., 2017) that are mainly plant pathogenic organisms. *Phytophthora* spp. are frequently associated with ornamental plants, especially in nurseries where *Phytophthora cactorum* is one of the most common species isolated (Jung et al., 2016). *P. cactorum* (Lebert & Cohn) (Erwin & Ribeiro, 1996) can infect an extremely large number of hosts, causing root rot, wilting and mortality as well as foliar and fruit (Erwin & Ribeiro, 1996; Jung et al., 2016).

There is a very high risk related to the introduction of *Phytophthora* species into new habitats via the movement of plants (Frankel et al., 2020; Santini et al., 2013). For instance, *P. tentaculata* was inadvertently introduced by restoration planting into new areas in California (Rooney-Latham & Blomquist, 2014; Rooney-Latham et al., 2015) while *P. ramorum* entered Europe through the trade of infested ornamental plants (Ivors et al., 2006; Mascheretti et al., 2008). Once established in a new country or region, these pathogens are very difficult to eradicate or manage. They can spread easily causing wilting and death of hosts, with further consequences of economic losses and damage to ecosystems and landscapes.

For instance, the presence of *Pinus nigra* (European black pine) in Europe could be compromised by *Phytophthora* diseases. Black pine is a fast-growing conifer with a wide but fragmented distribution across Europe and Asia Minor, mainly found in mountain areas. It is characterized by slow regeneration after forest fires and therefore gets replaced by faster growing species This process results in reduction of the black pine-dominated habitat in southern Europe. Thus, production of healthy seedlings in nurseries is key to preserving forest biodiversity. Unfortunately, nursery production of black pine is seriously affected by *P. cactorum* (Peterson, 1962).

The use of conventional synthetic fungicides for controlling diseases caused by *Phytophthora* spp. leads to development of resistant pathogens and has negative impacts on the environment and human health. Therefore, it is desirable to identify more eco-friendly control systems against *Phytophthora* spp.

Bio-based agrochemicals are becoming a "hot topic" regarding pest control in agriculture. However, currently the utilization of bio-based products in this field is still negligible compared to synthetic products. From this point of view, there are some substances which are considered promising, especially if delivered in the form of nanoparticles. Among the bioactive compounds which could be used as natural biocides are essential oils (EOs), which are "products obtained from a natural raw material of plant origin, by steam distillation, by mechanical processes from the epicarp of citrus fruits, or by dry distillation, after separation of the aqueous phase if any by physical processes" (ISO 9235:2013). The antimicrobial activity of these natural products has been widely studied and several biological effects (e.g., cytotoxicity, phototoxicity, mitochondrial damage) are well known (Bakkali et al., 2008; Kejlova et al., 2010). Due to the great number of constituents, EOs seem to have no specific cellular targets and could be considered as biocides with broad-spectrum activities (Antonelli et al., 2020; Bakkali et al., 2008; Isman, 2000).

In the literature, a small number of reports highlight the biocontrol activities of EOs are on *Phytophthora* spp. such as *P. colocasiae* (Sameza et al., 2014), *P. capsici* (Liu et al., 2017) and *P. cactorum* (Lee et al., 2008.), to name a few. EOs of *Eucalyptus globulus* inhibited growth of *P. colocasiae* (Sameza et al., 2014). Similarly, oils extracted from foliage of *Eupatorium adenophorum* significantly controlled *P. capsici* blight of pepper, through multiple mode of actions including disruption of the cell membrane system (Liu et al., 2017). A concentration of 28×10^{-3} mg/ml of several EOs, including citronellol, neral and geranial, led to approximately 100% inhibition of *P. cactorum* (Lee et al., 2008.). One of the major concerns with the use of EOs in the field is the duration of action. Apart from EOs themselves, the delivery system has a crucial role as it can enhance the biocidal properties of an active principle while also regulating its release time. In recent years, lignin applied in a nanoparticle form has gained increasing interest as a drug delivery system (Alqahtani et al., 2019; Zikeli & Vinciguerra, 2020; Zikeli et al., 2018). Lignin nanoparticles are known to stabilize EOs and promote growth inhibition activity against microbes, but their efficacy has been demonstrated mainly against different bacteria (Chen et al., 2020; Freitas et al., 2020). Lignin possesses many interesting properties for industrial applications because of its biodegradability, along with antioxidant, antimicrobial and stabilizer properties (Glasser, 2019; Hodásová et al., 2015; Zikeli et al., 2019; Zikeli & Vinciguerra, 2020). It is, therefore, a material with great potential for use as a carrier matrix in biocide activities. The consequences of using lignin in a drug delivery system are variable due to the role of hydrogen bond interactions, which depends on the quantity of aromatic and aliphatic hydroxyl groups in the specific type of lignin (Glasser, 2019). Indeed, results in this type of work are strictly correlated to the source of the lignin (hardwood, softwood, grass) and the process of extraction which determines the overall physicochemical properties of the lignin, and its cross-linking characteristics, which is necessary in biopolymer production (Glasser, 2019). Using lignin nanoparticles as a delivery system is also of great benefit in that the residues are abundant in agricultural and forest harvesting wastes. Furthermore, finding applications for lignin wastes is green chemistry, and also applies directly to circular economy principles avoiding single use in bioenergy (Colantoni et al., 2013; Delfanti et al., 2014; Paletto et al., 2019; Pieratti et al., 2019). Certainly, the potential for use of lignin wastes for high-value-added products is great and can be much more favourable than solely thermal use via combustion in terms of lower environmental impact (Lettner et al., 2020; Wang et al., 2018).

In the present study, lignin nanoparticles combined with EOs of wild thyme were evaluated for their ability to control mortality caused by *P. cactorum* in black pine (*P. nigra* J.F. Arnold, 1785) seedlings.

2 | MATERIAL AND METHODS

2.1 | Culture media and organisms

Strains of *P. cactorum* were isolated in 2019, from bleeding lesions on the collar of beech trees in Monti Cimini (Viterbo, Italy), as described by Vettraino et al. (2008), with some modifications. Briefly, tissues collected from the margin of necrotic areas were sterilized (ethanol 70%, 3 s, rinsed 3 times in distilled water, dried in a laminar flow hood) and transferred to PARBhy agar (Vettraino et al., 2005). *Phytophthora* isolates were identified initially based on colony growth patterns and morphological traits of sporangia, oogonia and antheridia. For two representative *P. cactorum* isolates, PF10 and PF11, identification was confirmed by sequencing the ITS rDNA and cox I regions, followed by a BLAST search at GenBank (Kroon et al., 2004; Vettraino et al., 2011).

2.2 | Essential oils from *Thymus serpyllum*

EOs of *T. serpyllum* were kindly provided by Flora srl, Florence, Italy. Flowering plants of *T. serpyllum*, common name wild thyme, were collected by hand on an organic farm in Turkey. EOs were extracted by hydrodistillation using a Clevenger-type apparatus and analysed by GC–MS (Shimadzu QP 2010 Plus) fitted with FID and Japan capillary column (0.32 mm i.d., length: 30 m, film thickness 0.25 μm). The injector temperature was maintained at 280°C while the ion source

temperature was set at 230°C. A total of 0.2 μl of oil sample was injected into the column with a split ratio of 80:1. The temperature program was 60°C for 2 min, 250°C for 5 min at 10°C/min and 280°C for 15 min at 10°C/min. The peak normalization method was used to estimate the oil composition (%). Peaks were identified by comparing individual mass spectra with the NIST (National Institute of Standards and Technology) database and Wiley 229 mass spectrometry libraries. The analysis was carried out by Flora srl.

2.3 | Preparation and characterization of nanoparticle dispersion

Lignin nanoparticles loaded with essential oil (EO-LNPs) were prepared using a modified protocol from Zikeli and Vinciguerra (2020). Acidolysis lignin (AL) from beech wood was dissolved in DMSO together with EOs from wild thyme and subjected to dialysis against water. The ratio of AL to EOs in this work was highly in favour of EOs (AL:EOs = 1:3), in order to prepare a biocide delivery system (BDS) of high efficacy in the subsequent in vitro and in vivo experiments. Thus, 100 mg of AL and 300 μl of EOs were dissolved in 10 ml DMSO and, after centrifuging, filled into a SpectraPor® dialysis tube (SpectraPor 1 Dialysis Membrane Standard RC Tubing, 6–8 kDa, Spectrum Labs, USA) which was immersed in an excess of distilled water (4 L) for 1 h. The resulting EO-LNPs dispersions were stored at 4°C until further use for analysis and biocide experiments. Aliquots of the samples were freeze-dried for further analysis.

EO-LNPs were characterized by Py-GCMS after freeze-drying as described in Zikeli and Vinciguerra (2020). Drug loading efficiency and drug loading capacity were determined using HPLC and external calibration with pure EOs following formulas (1) and (2) below. Drug release was investigated using in vitro release experiments via a dialysis method as described in Zikeli and Vinciguerra (2020).

$$\text{DLE}\,(\%) = (\text{weight of EOs in EO} - \text{LNPs/weight of EO added initially}) \times 100 \quad (1)$$

$$\text{DLC}\,(\text{wt}\,\%) = (\text{weight of EOs in EO} - \text{LNPs/weight of EO} - \text{LNPs}) \times 100 \quad (2)$$

2.4 | Determining antifungal activity in vitro

Wild thyme EOs were evaluated for the ability to control *P. cactorum* PF10 and PF11 growth using the technique described by Tian et al. (2011). PDA (10 ml/Petri dish) was amended with various concentrations of EOs (24, 48, 96, 144, 192 and 240 μg/ml) via loaded nanoparticles (EO-LNPs) or unformulated EOs, dissolved in 0.1% DMSO. PDA supplemented with the same amount of DMSO or blank formulation (i.e., NPs without oils) used in treated cultures was used as control. Cultural media and DMSO were purchased from Merck (Milan-Italy). As control, a fungicide (Infinito®, Bayer) containing fluopicolide (62.5 g/L) + propamocarb hydrochloride (625 g/L) was used to a final concentration of 1.2 μl/ml. Five replicate cultures per treatment were used. The experiment was conducted twice. Petri dishes were inoculated with an agar plug (8 mm diameter) of each isolate obtained from active margins of colonies grown on PDA. Cultures were incubated at 25 ± 2°C, in the dark.

After 7 days, the colony diameter was measured, and the mycelial growth inhibition (MGI) was calculated as follows:

$$\text{MGI} = \left[(\text{dc} - \text{dt})/\text{dc} \times 100\right]:$$

where "dc" is mean colony diameter of control cultures and "dt" is mean colony diameter of treatment (Mohammadi et al., 2015).

Subsequently, median effective inhibitory concentration (EC_{50}) values for EOs and the fungicide "Infinito®" (expressed as ppm) against *Phytophthora* isolates were calculated. To test whether treatments were fungistatic or fungicidal, a disc plus pathogens was subcultured from treated cultures to fresh PDA. The effect was considered fungistatic if pathogen growth occurred at 25 ± 2°C after 10 days. The minimum inhibitory concentration values of NPs, oil and fungicide were determined as the lowest concentration of compound that inhibited *P. cactorum* growth 7 days after subculture and were expressed in μg/ml.

2.5 | Effect of the thyme essential oils on *Phytophthora cactorum* diseases in greenhouse conditions

Seeds of black pine (*P. nigra*) were disinfected (10% sodium hypochlorite for 15 min, rinsed three times in sterile distilled water and air-dried in a laminar flow cabinet) and sown in pots (2 × 2 × 3 cm), containing steam-sterilized soil (2:1 perlite/clay soil). One week after germination, *Pinus* seedlings were transplanted to new pots (15 cm diameter and 16 cm height) with different treatments. EOs (135 μg/ml) and EO-LNPs (135 μg/ml) were included in the test as well as the reference fungicide (1.2 μl/ml). A total of 12 treatments were prepared (A = untreated, B = *P. cactorum* PF10; C = *P. cactorum* PF11, D = LNPs; E = EOs; F = EO-LNPs; G = *P. cactorum* PF10 + EO-LNPs; H = *P. cactorum* PF11 + EO-LNPs; I = *P. cactorum* PF11 + EOs; L = *P. cactorum* PF10 + EOs; M = *P. cactorum* PF10 + Fungicide; N = *P. cactorum* PF11 + Fungicide). *Phytophthora* inoculum was prepared by inoculating sterilized millet seeds (Vettraino et al., 2001). Infested seeds were added to potting soil at a rate of 3% (wt/vol). All pots were maintained in a greenhouse at 23–27°C, 60%–70% relative humidity, 16 h light and 8 h darkness. Plants were flooded after 1 day for 48 h to promote sporulation.

Mortality of plants was recorded weekly throughout the duration of the experiment (3 weeks). The pathogen was reisolated from soils by baiting and confirmed morphologically.

2.6 | 6 Statistical analyses

Experiments were carried out in a completely randomized design. Measurements of mycelial inhibition were subjected to one-way ANOVA, and means comparisons were made using Duncan's multiple range tests. The effective dose for 50% (ED50) inhibition was calculated using probit analysis. Mortality data were compared using

chi-square tests. Differences at $p \leq 0.05$ were considered to be significant. All statistical analysis was made using R software (R Core team, 2021).

3 | RESULTS

3.1 | Chemical composition of the essential oils

Based on the GC-MS analysis, 19 primary compounds were identified in the EOs of wild thyme. Most compounds, except for carvacrol, thymol, linalool, γ-terpinene and *p*-cymene, were present at concentrations below 1.5%. Carvacrol was the most abundant component (68.49%, Figure 1).

3.2 | Lignin nanoparticles dispersions loaded with essential oils

3.2.1 | Qualitative analysis of freeze-dried EO-LNPs using Py-GCMS

The main components of EOs from wild thyme were detected by Py-GCMS of the freeze-dried EO-LNPs dispersion. They included carvacrol and lignin pyrolysis products such as syringol, guaiacol, coniferyl alcohol or 4-vinylsyringol (Figure 2). The relative sizes of the peaks changed compared to the composition of pure EOs determined by GC-MS. In EO-LNPs, carvacrol was detected in higher amounts (74.8%) together with thymol (4.1%), while *p*-cymene, γ-terpinene and linalool were detected only in trace quantities (Figure 2, Table 1).

3.2.2 | Quantification of EO-LNPs dispersion using HPLC

EO-LNPs (6.9 mg/ml of lignin) loaded with 15.8 mg/ml of EOs from wild thyme were prepared via simultaneous entrapment that resulted in highly loaded LNPs dispersions. The DLE and DLC values for EO-LNPs were approximately 61.9% and 69.6%, respectively.

3.2.3 | Release behaviour in vitro of EO-LNPs in vitro using dialysis and HPLC

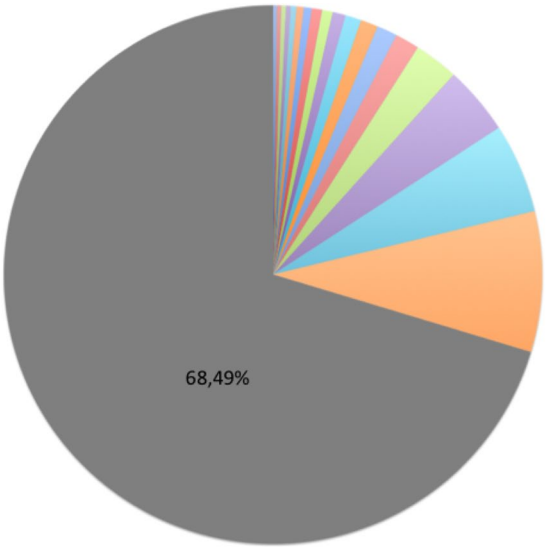
Under the experimental conditions used here, release behaviour in vitro showed a strong retaining effect against EOs release from the EO-LNPs dispersions. In the first 24 h, much lower quantities of wild thyme EOs were released from EO-LNPs compared to the pure EOs dispersion. After 24 h, whereas almost 100% of EOs were released from the pure EOs dispersion, EO-LNPs dispersion still contained approximately 20% of EOs initially present (Figure 3, Table 2).

3.2.4 | Scanning electron microscopy (SEM)

SEM photos showed distinct particles at the nano-scale, but also chain-like agglomerates with a length of up to several micrometres (Figure 4a). At high magnification (Figure 4b–c), EO-LNPS with diameters of approximately 100 nm can be identified. However, these particles were present in irregular rounded shapes and a number of patches may be detected.

3.3 | Anti-oomycete Assay

No significant differences in virulence were detected between the *P. cactorum* PF10 and PF11 (ANOVA, $p < 0.05$). Thus, data for the two isolates were combined. The antifungal activity of thyme EOs was tested against *P. cactorum* in vitro. Both *P. cactorum* PF10 and PF11 were sensitive to the fungicide Infinito®, since neither isolate grew on PDA amended with the compounds at 1.2 μg/ml. Thyme EOs caused either partial or complete inhibition of *P. cactorum* growth A dose-dependent increase in inhibition rate was observed



Main components	Content (%)
β-fellandrene	0,23
Limonene	0,25
Aromadendrene	0,27
Sabinene	0,28
1.8 cineole	0,33
Borneol	0,42
c-sabinene idrato	0,46
α-terpineol	0,61
terpinen-4-ol	0,62
α-pinene	0,76
β-caryophyllene	0,94
α-thujene	1,02
α-terpinene	1,19
β-myrcene	1,42
Thymol	2,59
γ-terpinene	4,02
p-cymene	5,24
Linalool	8,22
Carvacrol	68,49

FIGURE 1 Chemical composition of *T. serpyllum* EOs obtained from gas chromatography-mass spectrometry (GC-MS) analysis

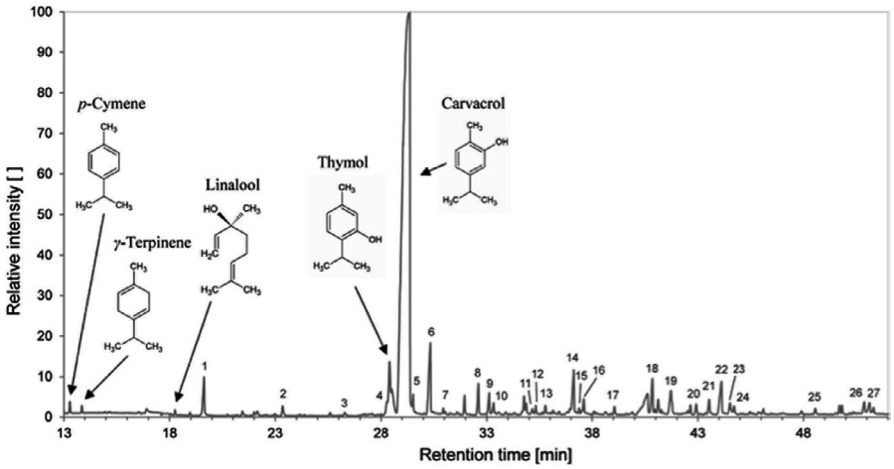


FIGURE 2 Py-GCMS chromatogram of freeze-dried lignin nanoparticles loaded with *T. serpyllum* EOs

with *P. cactorum* strains treated with EOs or EO-LNPs, with EC_{50} values of 120.453 and 88.711 μg/ml, respectively (Figure 5).

EO-LNPs inhibited pathogen growth at a minimum concentration of 48 μg/ml, lower than for EOs (120 μg/ml). There was a total (100%) inhibitory effect at 240 μg/mL for EO-LNPs with double value for EOs (480 μg/ml). Thyme EOs caused the death of *Phytophthora* isolates in 7 days, when used at concentrations over 480 μg/ml.

The fungicides and plant EOs applied as a soil drench on *Pinus* seedlings significantly (Chi-square; $p < 0.05$) reduced mortality of the plants, compared to the nontreated plants in greenhouse assays. The fungicide Infinito® had the most significant effect against the pathogen with 100% healthy seedlings despite inoculation. In contrast, approximately 27% of inoculated plants died. Mortality of treated pine seedlings was first observed by 10 days after inoculation. In the presence of EOs, 7% of seedlings died, within 3 weeks of inoculation. In contrast, no mortality was observed in the EO-LNP treatments.

4 | DISCUSSION

In this work, we explored thyme EOs, free and encapsulated in lignin nanoparticles, as new agents for the treatment and control of *P. cactorum* in *P. nigra* seedlings. According to our understanding, and the information obtained from the reviewed literature, this is the first study to elaborate a strategy for controlling *Phytophthora* growth within the framework of EU bioeconomy strategy, 2012. The use of biomass residues to develop eco-friendly antimicrobial substances, in fact, promotes good practices to operate the bioeconomy within safe ecological limits.

Thyme EOs have proved to have high efficacy in constraining several microorganisms including *Chaetomium*, *Fusarium*, *Aspergillus* and *Stachybotrys* species (Antonelli et al., 2020) and *Phytophthora* spp. (Lu et al., 2013; Mohammadi et al., 2015; Quintanilla et al., 2002). However, to date, *T. serpyllum* has not yet been studied in relation to its biological control effect against *Phytophthora* species.

EOs of *T. serpyllum* contain several molecules, including borneol, isobutyl acetate, caryophyllene, 1,8-cineole, citral, citronellal, citronellol, *p*-cymene, geraniol, linalool, α-pinene, γ-terpinene, α-terpineol, terpinyl acetate and thymol in relatively high concentrations, but carvacrol is the major active compound (PDR for Herbal Medicines).

Carvacrol is already known to have bactericidal and fungicidal activities against Gram-positive and Gram-negative bacteria, fungi and oomycetes (Antonelli et al., 2020; Ayres Cacciatore et al., 2020; Hyldgaard et al., 2012; Martín et al., 2010; Szczepanski & Lipski, 2014).

It is a volatile secondary metabolite insoluble in water with a single hydroxyl group (–OH) adjacent to the methyl group in the aromatic ring. The hydrophilic nature of the -OH group and the ability of carvacrol to exchange its protons have important roles in its antimicrobial activities. It alters the structure and function of cell membranes causing an increase in permeability. Its antifungal activity, together with that of thymol, is linked to the formation of lesions in the cell membranes and a reduction in ergosterol content in fungi. Carvacrol affects physiological processes inside the cell, influences virulence factors, affects gene expression and thus reduces virulence of pathogens (Liu et al., 2019). It can alter hyphal morphology of fungi and *Phytophthora* spp., increasing cytoplasmic coagulation, vacuolations, hyphal shrivelling and protoplasm leakage (Soylu et al., 2006). Another mechanism that could be involved in the interaction between carvacrol and plant resistance is associated with phenols, known to act as activators of plant defences as highlighted on elms (Martín, Solla, Witzell, et al., 2010; 2010; Martín et al., 2012).

Data reported here confirmed that EOs of *T. serpyllum* were effective in management of *P. cactorum* diseases of *P. nigra* seedlings. We might hypothesize that the antimicrobial activities could be linked to high concentrations of carvacrol. However, a synergism with other compounds characterizing the plant extract cannot be excluded (Heghes et al., 2019). For instance, *T. serpyllum* EOs analysed in this study contained considerable amounts of *p*-cymene (5.4%) and linalool (8.22%), which cause increases in intracellular cAMP (Antonelli et al., 2020), and γ-terpinene, which is, together with *p*-cymene, a biochemical precursor of thymol and carvacrol in the phenol biosynthetic pathway (Mahboubi & Ghazian Bidgoli, 2010; Saei-Dehkordi et al., 2010).

TABLE 1 Relative abundances of pyrolysis products from freeze-dried lignin nanoparticles dispersions loaded with essential oil from wild thyme (EO-LNPs)

#	Pyrolysis product	RT [min]	EO-LNPs [%]
-	*p*-Cymene	13.23	0.3
-	*γ*-Terpinene	13.78	0.1
-	Linalool	18.32	0.2
1	Guaiacol	19.59	1.3
2	4-Methylguaiacol	23.32	0.4
3	4-Ethylguaiacol	26.25	0.1
4	4-Vinylguaiacol	28.24	0.6
-	Thymol	28.41	4.1
-	Carvacrol	29.15	74.8
5	Eugenol	29.29	0.6
6	Syringol	30.14	3.5
7	Isoeugenol (cis)	30.82	0.2
8	Isoeugenol (trans)	32.51	1.0
9	4-Methylsyringol	33.02	0.7
10	Vanillin	33.22	0.4
11	Homovanillin	35.11	0.2
12	4-Ethylsyringol	35.27	0.3
13	Acetoguaiacone	35.72	0.3
14	4-Vinylsyringol	37.07	1.9
15	Guaiacylacetone	37.34	0.2
16	4-Allylsyringol	37.55	0.4
17	4-Propenylsyringol (cis)	39.01	0.3
18	4-Propenylsyringol (trans)	40.76	1.4
19	Syringaldehyde	41.63	1.3
20	Homosyringaldehyde	42.87	0.4
21	Acetosyringone	43.47	0.7
22	Coniferyl alcohol (trans)	44.06	1.9
23	Coniferaldehyde	44.47	0.7
24	Syringylacetone	44.68	0.3
25	Sinapylalcohol (cis)	48.52	0.2
26	Sinapylalcohol (trans)	50.86	0.7

Note: The symbol "–" refers to compounds present as traces.

The data presented here confirmed analytical pyrolysis as potent technique for the analysis of polymeric constituents in addition to volatile compounds (Ferraz et al., 1997; Romagnoli et al., 2018; Vinciguerra et al., 2011). Data from Py-GCMS showed that, when considering the ratio of EOs to lignin, a much higher value (79.5%) in favour of EOs was evident compared to the 54% reported by Zikeli and Vinciguerra (2020). However, Zikeli and Vinciguerra (2020) already indicated that a proportion of the EOs might not be fully entrapped but present as loosely bound or free pure oil micelles around the EO-LNPs in the dispersion. Pure EO micelles might still be retained and hindered from evaporation by the EO-LNPs present in the suspension.

Based on the DLE and DLC values, EOs from *T. serpyllum* were considered suitable to be entrapped into lignin nanoparticles. The DLE values for the EO-LNPs prepared in this work were over 60% and comparable with values reported in other publications (Dai et al., 2017; Zikeli & Vinciguerra, 2020). Conversely, the DLC attained higher values than data reported in the literature (Dai et al., 2017; Zikeli & Vinciguerra, 2020), probably for an overestimated quantification due to the presence of pure EO micelles in the EO-LNPs dispersion.

EO-LNPs delayed the release of EOs into the surrounding medium within the first 48 h of the experiment. The ratio of lignin to EOs was highly in favour of EOs in the present work, but even a low quantity of lignin matrix material compared to the loaded EOs was sufficient to successfully retard the release of EOs from the aqueous dispersion of EO-LNPs. It is worth noting that the efficiency of the process is led not only by the amount but also the structure of lignin, which is a variable to be considered in further investigations (Ferraz et al., 1997). The higher concentration of EOs, strongly in favour of EOs in the presented work, resulted in unified drop-like spots of EOs visible under SEM, but probably caused by the heating effect of the SEM electron beam.

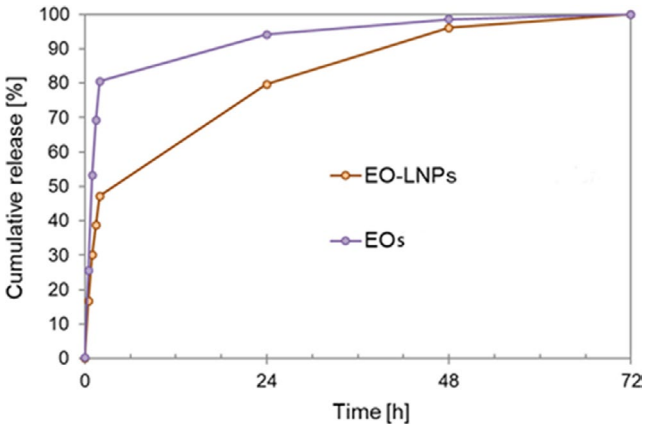


FIGURE 3 Release profile of *T. serpyllum* EOs incorporated into EO-LNPs compared with the release profile of pure *T. serpyllum* EOs dispersed in water

TABLE 2 Essential oil cumulative release of the lignin nanoparticles essential oil dispersions (EO-LNPs) and the pure essential oil dispersions (EOs) over time

Essential oil cumulative release [%]		
Time [h]	EO-LNPs	EOs
0.5	16.6	25.5
1	30.1	53.2
1.5	38.6	69.1
2	47.2	80.5
24	79.7	96.8
48	96.0	98.5
72	98.7	100.0

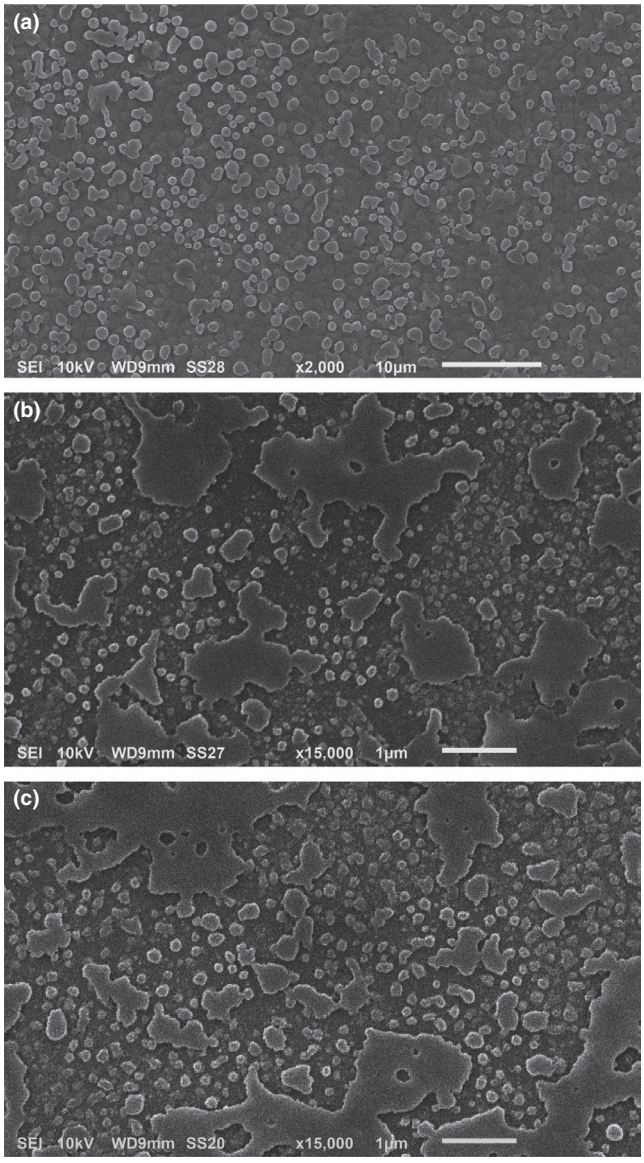


FIGURE 4 Scanning electron microscopy photos of EO-LNPs. (a): ×2.000 magnification; (b) and (c): ×15.000 magnification

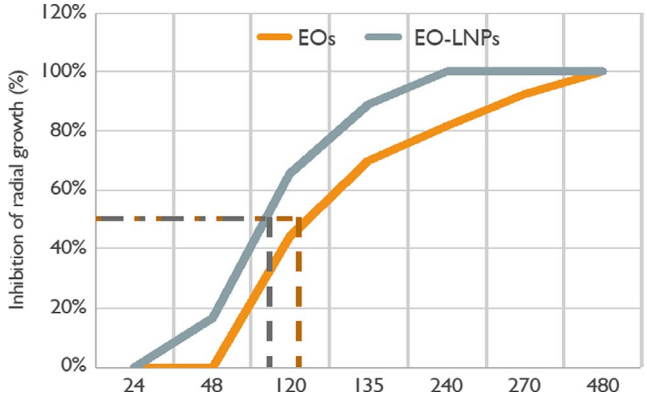


FIGURE 5 Percentage of inhibition of the radial growth of *P. cactorum* on PDA amended with EOs and EO-LNPs

Consequently, the prepared EO-LNPs with diameters around 100 nm, which can be clearly seen in Figure 4 at high magnification (Figure 4b–c), show more irregular shapes compared to those presented in Zikeli and Vinciguerra (2020). This irregularity is probably derived from the high concentration of EOs in the structure of the nanoparticles, particularly the high load of carvacrol molecules. Thus, the mass ratio of EOs and lignin was probably the main driving force influencing the form and shapes of EOs loaded lignin nanoparticles, when comparing the present work with that of Zikeli and Vinciguerra (2020).

This study showed that encapsulated EOs of wild thyme were more effective than pure EOs in controlling *P. cactorum* both in vitro. The encapsulation slows evaporation of volatile EOs, allowing a delayed and continuous release of the compounds that could affect *P. cactorum* growth. Treatments with EOs and EO-LNPs had no impact on growth of pine seedlings in our study. Nevertheless, phytotoxicity can be of concern when certain EOs are applied to plants or soil. Bi et al. (2012), for example, reported that oregano EOs inhibited seedling emergence of common bean (*Phaseolus vulgaris*), beet (*Beta vulgaris*), carrot (*Daucus carota*) and pepper (*Capsicum annuum*). Therefore, specific EOs should be selected for different hosts to avoid phytotoxic consequences.

In conclusion, our results indicated that the application of *T. serpyllum* EOs might be an alternative method to synthetic chemicals for integrated management strategies against diseases caused by *P. cactorum*. The formulation of biologically derived active compounds as nanoparticles increased the efficacy of the control strategy.

In addition to their biocompatibility and excellent stability, innovative lignin nanoparticles exhibited a strong capacity to carry thyme EOs with sustained release. The use of lignin in agriculture provides an example of the circular economy concept and will increase the added value chain towards sustainability instead of a simpler use for lignins in the bioenergy sector (Vinardell & Mitjans, 2017; Wang et al., 2018).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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