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IMPACT OF PINE (*Pinus sylvestris* L.) AND SPRUCE (*Picea abies* (L.) Karst.) BARK EXTRACTS ON IMPORTANT STRAWBERRY PATHOGENS

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Phytopathogenic fungi induced considerable economic losses in strawberry production industry; therefore, more attention should be paid to development and implementation of preventative treatment that is environmentally friendly. Coniferous trees produce a wide variety of compounds, such as terpenoids and phenolics. Several studies are known on fungicidal activity of different components of coniferous tree bark. The aim of this study was to evaluate in vitro *pine (*Pinus sylvestris *L.) and spruce (*Picea abies *(L.) Karst.) bark ethanol extracts impact on pathogenous fungi causing diseases of strawberries. Products of processed pine (*Pinus sylvestris*) and spruce (Picea abies) bark were tested. During 2011 to 2013, several* in vitro *experiments were carried out to test the effectiveness of pine and spruce bark extracts against various phytopathogenic fungi isolated from strawberries:* Botrytis cinerea*,* Colletotrichum acutatum*,* Phytophthora cactorum *and* Mycosphaerella fragariae*. Radial growth tests showed that coniferous bark extracts inhibit mycelial growth of* B. cinerea, C. acutatum, P. cactorum *and* M. fragariae*. Extracts had the highest antifungal effect on* B. cinerea *two and five days after inoculation ($p < 0.05$). Bark extracts can reduce the sporulation of* B. cinerea, C. acutatum *and* P. cactorum*.*

Key words: Botrytis cinerea, Colletotrichum acutatum, Phytophthora cactorum, Mycosphaerella fragariae*, inhibition of mycelial growth, inhibition of sporulation.*

INTRODUCTION

Strawberry (*Fragaria* × *ananassa* Duch.) plants are susceptible to various pests and diseases, including those of fungal origin with significance worldwide. Fungal diseases occur in all parts of plants (flowers, fruits, leaves, crowns and roots). They reduce yields and quality of fruits and cause considerable economic losses (Paulus, 1990). Common strawberry phytopathogenic fungi are *Botrytis cinerea* Pers.*, Colletotrichum acutatum* Simmonds*, Phytophthora cactorum* (Lebert & Cohn) J. Schröt*, Mycosphaerella fragariae* (Tul.) Lindau*, Verticillium dahlia* Kleb. and *Rhizopus sp*. (De los Santos *et al.*, 2003). The most serious and widespread strawberry phytopathogens are *B. cinerea* and *C. acutatum*, which cause grey mould and anthracnose, respectively. *B. cinerea* is a phytopathogen that infects a wide range of hosts, although small fruit crops are most severely affected (Williamson *et al.*, 2007). In optimal conditions for the development of disease (humidity > 90% and temperature 20 °C), it can cause important economic losses of yield (Paulus, 1990). Fernández-Acero *et al.* (2007) quantified losses caused by the phytopathogenic fungi *B. cinerea* and *C. acutatum* to be between 10 and 100 million euro yearly in Europe. Thus, it is important to protect strawberries from harmful activity of pathogenic fungi, to achieve maximum production in the strawberry growing industry.

Fungal diseases conventionally may be controlled by cultural methods or systemic fungicides. Although chemical control by systemic fungicides is an inexpensive way to control strawberry pathogens, fungal ability to develop resistant strains leads to pathogen adaptation (Russel, 1995; Rosslenbroich and Stubler, 2000). Also, long-term use of fungicides has led to accumulation of fungicidal residues in the environment (Koul *et al.*, 2008). Therefore, in recent years there has been interest in alternative strategies, including use of natural biocontrol mechanisms or substances that protect plants (Miclea and Puia, 2010). Thus, more attention should be paid to development of environmentally friendly products that control plant diseases.

Plants contain a high number of organic compounds with enormous chemical diversity (Gottstein and Gross, 1992) and many of them possess antifungal properties. Coniferous trees produce a wide variety of compounds with antifungal properties, such as terpenoids and phenolics (Krauze-Baranowska *et al.*, 2002). Several studies have been made on antifungal effect of different components of coniferous trees (Hong *et al*., 2004; Ludley *et al*., 2008; Laugale and Daugavietis, 2009; Zarins *et al*., 2009). Coniferous bark is one of forest exploitation byproducts that also can be used for producing of plant protection products. Pan and Lundgren (1995) isolated 28 phenolic compounds from root bark of spruce and reported that some of them have antifungal and antibacterial activity. The studies on chemical composition of coniferous bark from trees grown in Latvia had been done by Verovkins *et al*. (2008). Reports are available on antifungal activity of pine (*Pinus sylvestris* L.) and spruce (*Picea abies* L.) extracts and essential oils against a wide range of fungi: *Fusarium culmorum*, *F. poae*, *F. solani* (Krauze-Baranowska *et al.*, 2002), *Heterobasidion parviporum, H. annosum, Fomitopsis pinicola, Ophiostoma piceae, Ceratocystis polonica, Phascidium coniferarum* (Alfredsen *et al.*, 2008), *Penicillium funiculum, Ulocladium oidemansii* (Motiejunaite and Peciulyte, 2004), and *Lophodermium seditiosum* (Klavina *et al.*, 2012). Although the mechanism of extract compound action against fungi is unknown, two possible ways have been identified: initiation of a defence mechanism in plants leading to systemic induced resistance (Albouvette *et al.*, 2006) or the ability of extract compounds to disrupt the integrity of fungal cell walls and membranes (Koul *et al.*, 2008). Natural substances of plant origin, like plant extracts and essential oils may be promising, because plant extracts are effective against phytopathogens (Alabouvette *et al.*, 2006, Koul *et al.*, 2008), their extraction is not complicated and time-consuming and extracts do not pollute the environment (Zarins *et al.*, 2009). In spite of considerable research effort on fungicidal properties of essential oils known as green pesticides, a few antifungal products based on plant essential oils have appeared in the market (Koul *et al.*, 2008).

It is important to understand the interactions among potential plant protective extract and pathogen to obtain valuable information, necessary for the development of biofungicide. The aim of this study was to evaluate pine *P. sylvestris* and spruce *P. abies* bark ethanol extract impact on pathogenous fungi causing diseases of strawberries *in vitro*.

MATERIALS AND METHODS

Pine and spruce bark ethanol extracts. Spruce bark extract (dry mass 30%) and pine bark extract (dry mass 26%) was prepared in the Latvian State Forest Research Institute "Silava". Bark was crushed with extrusion-type grinder M-1. The resulting mass was fractionated using sieves, and a fraction with particles size 0.5–1.0 mm was used for further production. Extraction was made with a "Büchi" Universal Extraction System B-811 in Soxhlet regime. Ethanol 96% (vol.) was used as a solvent.

The phenol concentration in the extract was determined. Phenols determination was based on optical density measurement of coloured oxidation products, obtained using Folin-Ciocalteu reagent (tungstic acid in alkaline medium results in blue colour). Gallic acid was used as a reference substance (Yermakov, 1987; Pasqualini *et al*., 2003; Mechnikova *et al*., 2007). Density of blue substances and reference substance (gallic acid) was measured at 765 nm. Concentration of total flavonoids was measured by differential spectroscopy method (Ngo and Zhohova, 2007). Optical densities of coloured substances after reaction with aluminium chloride were measured at 410 nm. A scanning spectrophotometer Genesys 10 UV was used for optical density measurements. The content of flavonoids in dry mass of both extracts was 1.16%, the content of phenols was 32.3% and 20.9%, in dry mass of spruce and pine extracts, respectively.

Fungal isolates. *Botrytis cinerea* isolate number LUBI-Z1 (2011) and *Mycosphaerella fragariae* isolate LUBI-M1 (2012) were obtained from naturally infected strawberry collected from the Pūre Horticultural Research Centre. *Colletotrichum acutatum* and *Phytophthora cactorum* were supplied from the Fungal Biodiversity Centre of the Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW) in September 2012 — strain numbers CBS 130239 and CBS 112275, respectively. *B. cinerea*, *M. fragariae* were maintained on Potato Dextrose Agar (PDA) medium (SIFIN, Berlin), *C. acutatum* and *P. cactorum* on Corn meal agar (CMA) and stored at 4 ± 2 °C. Isolates were passaged every six months. *B. cinerea* was grown on PDA for five days, *C. acutatum* and *P. cactorum* isolates — for ten days before being used in the study.

***In vitro* mycelial growth inhibition essay.** Antifungal activity of pine and spruce extracts on mycelial growth of *C. acutatum, P. cactorum* and *B. cinerea* were tested. Sterile molten PDA were cooled to 60 °C and pine or spruce trees bark extracts were added to obtain final dose of 0.1 g L^{-1}, 1 g L^{-1}, 10 g L^{-1}, 20 g L^{-1}. Agar plugs (5 × 5 mm) from pure cultures of the four tested fungi were placed in the centre of 85 mm Petri dish with pine or spruce trees bark extracts at various doses. Negative control contained only PDA medium, and positive control contained PDA with 1 g L^{-1} fungicide Signum® (piraclostrobin 6.7% + boscalid 26.7%, produced by BASF, Germany). Each treatment was replicated five times. Inoculated Petri dishes were incubated at 25 ± 2 °C in the dark. Radial growth of the mycelium in two directions was measured daily for ten days or till the fungus had reached the edge of the plate. Antifungal activity was expressed in terms of percentage of mycelial growth inhibition at each dose and calculated according to the following formula: $P\ (\%) = [DC - DT] / DC] \times 100$, where P = mycelial growth inhibition (%); DC and DT are the average diameters of fungal colony of control and treatment, respectively (Pandey *et al*., 1982; Zambonelli *et al*., 1996). The statistical analysis was made using the data, obtained on the second and fifth days for *B. cinerea* and on the third and eighth days for *C. acutatum, P. cactorum*, and *M. fragariae.*

Sporulation inhibition essay. The level of sporulation of the fungi *B. cinerea, C. acutatum* and *P. cactorum* were measured 14 days after incubation (25 ± 2 °C) on PDA with added different doses of bark extracts. Mycelial plugs with 10 mm diameter were taken from each Petri dish at two different places 10–15 mm from edge, produced spores (conidia or zoospore) were harvested by scrapping them off from plugs with the scalpel, and washed with 1 ml distilled sterile water. The mycelium and the spore mixture were filtered through cheesecloth. The number of spores per ml of filtrate was determined using haemocytometer and the result was transformed into spores/mm^2. Petri dishes, where mycelial diameter has not grown enough to take two samples with the diameter 10 mm, were not observed.

Statistical analysis. Sporulation inhibition assay data were log transformed prior to statistical analysis. Data from *in vitro* mycelial growth and sporulation inhibition essay were analysed using one-way ANOVA analysis at significance of differences $p < 0.05$. To determine which treatments were significantly different, the Tukey's honestly significant difference (HSD) multiple comparison test was used. Statistical analyses were conducted using the software R 2.14.1.

RESULTS

Impact of pine and spruce bark extract on mycelial growth. The antifungal activity of pine and spruce bark extracts was observed against all tested fungi: *Botrytis cinerea, Colletotrichum acutatum, Phytophthora cactorum* and *Mycosphaerella fragariae*. Bark extracts showed very high antifungal activity against *B. cinerea*, mycelial growth inhibition 65–100% on the second day of measurements; however, obtained data indicated that inhibition decreased during incubation period of fungi (Fig. 1). Mycelial growth of all investigated fungi were inhibited by pine and spruce bark extracts at various doses, except *P. cactorum* at spruce bark extract dose of 0.1 g L^{-1}, where extract even stimulated the growth of fungi (Figs. 1, 2). On the second and fifth days for *B. cinerea* and third day for *C. acutatum* and *P. cactorum*, no significant differences between spruce and pine ethanol extract at dose 20 g L^{-1} and conventional fungicide Signum® were found using Tukey's HSD test. Moreover, pine and spruce extracts at 20 g L^{-1} dose were even more effective against mycelial growth of *M. fragariae* than Signum® and showed statistically significant difference (Fig. 2A, B).

Impact of pine and spruce bark extract on sporulation. Pine and spruce bark extracts reduced sporulation of *B. cinerea, C. acutatum* and *P. cactorum* at different extract doses. At the lowest dose no sporulation reduction was observed, however, sporulation was inhibited at higher doses (Table 1). Pine bark extract reduced sporulation of *C. acutatum* more effective then spruce bark extract according to Tukey's honestly significant difference test, showing important decrease at dose of 1 g L^{-1} comparing to spruce bark extract, which showed significant difference only at the dose of 20 g L^{-1}.

DISCUSSION

Pine and spruce bark extracts at a higher dose — 20 g L^{-1} caused mycelial growth inhibition 100% of fungi *B. cinerea*, *C. acutatum* and *P. cactorum* and did not differ from conventional fungicide Signum®. Moreover, the extracts inhibited mycelial growth of *M. fragariae* more effectively than Signum®, although *M. fragariae* was more resistant to the impact of fungicide Signum®. The sporulation of *B. cinerea*, *C. acutatum*, and *P. cactorum* was also reduced by the extracts at higher doses. These results suggest that pine and spruce bark extracts can be used for environmentally friendly plant protection product development. Although knowing that different strains of *B. cinerea* may have different susceptibility against fungicides (Rosslenbroich and Stubler, 2000), it is important to investigate the impact of extracts on different *B. cinerea* strains.

Research data show that inhibition effect of extracts on mycelial growth decreases during incubation period of the tested fungi. Slow growth of fungi during the first days of incubation and the increase of growth speed afterwards may be explained by the ability of some fungi to produce certain enzymes that can catalyse the oxidation of the host metabolites (Vio-Michaelis *et al*., 2012). Phenolic compounds are the main antifungal agents in spruce and pine extracts

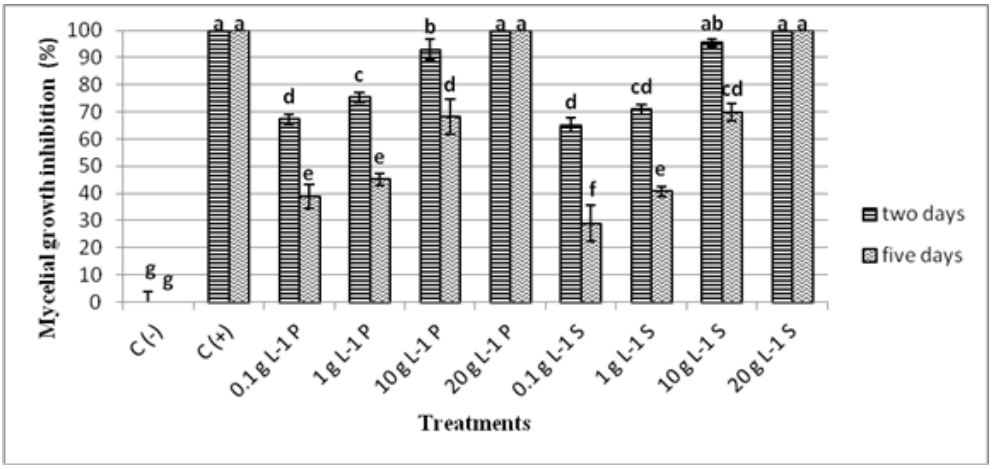

Fig. 1. Mycelial growth inhibition of *Botrytis cinerea* in the second and fifth days of incubation caused by pine (P) and spruce (S) bark extracts at different doses obtained by radial growth test. Data shown are mean values of 5 replicates with standard deviation. C (-) PDA without additives; C (+) PDA with fungicide Signum® 1 g L^{-1}. Bars with different letters indicating significant ($p < 0.05$) difference according to Tukey's honestly significant difference (HSD) multiple comparison.

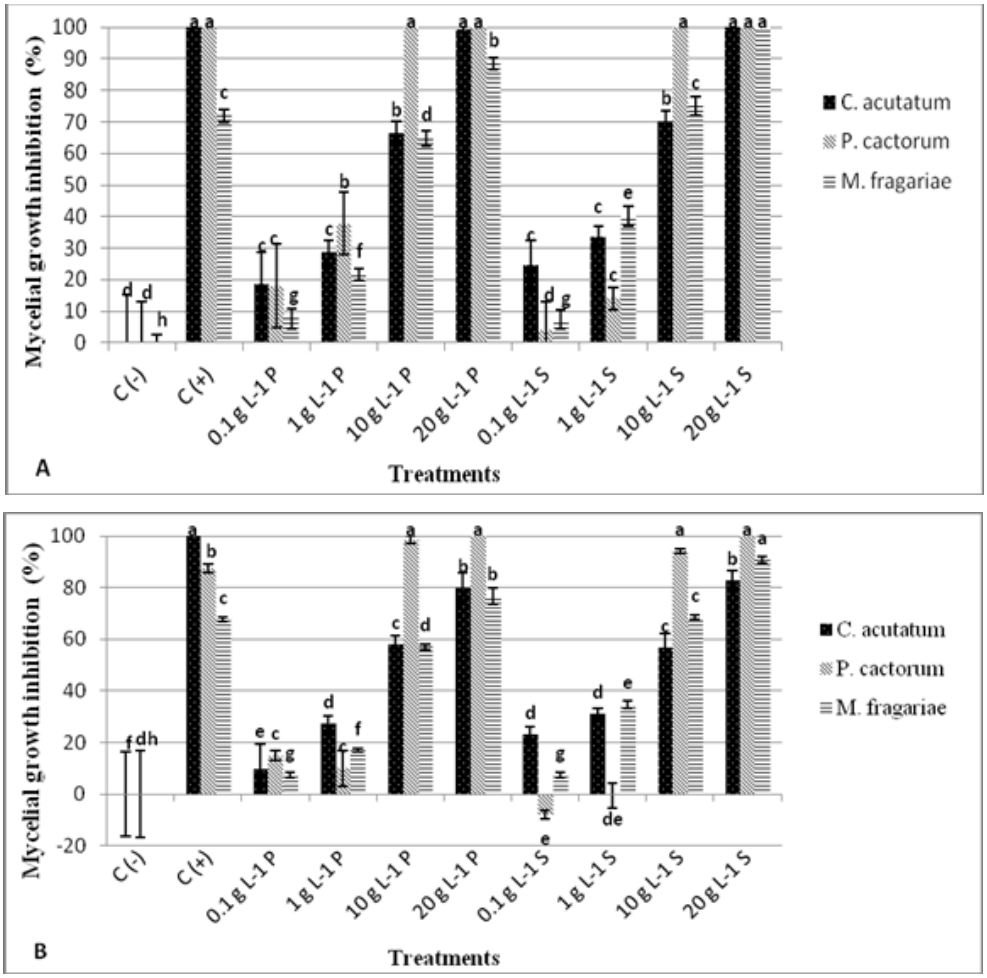

Fig. 2. Mycelial growth inhibition of *Colletotrichum acutatum, Phytophthora cactorum*, *Mycosphaerella fragariae* caused by pine (P) and spruce (S) bark extracts at different doses obtained by radial growth test. Data shown are mean values of 5 replicates with standard deviation. **A**, third day of incubation; **B**, eighth day of incubation. Controls: C (-) PDA without additives; C (+) PDA with fungicide Signum® 1 g L^{-1}. Bars with different letters compare growth inhibition of one fungus at various extract doses, indicating significant (p 0.05) difference according to Tukey's honestly significant difference (HSD) multiple comparison.

Table 1

NUMBER OF CONIDIA PER mm^2 PRODUCED BY FUNGI *Botrytis cinerea, Colletotrichum acutatum* AND *Phytophthora cactorum* GROWN ON POTATO DEXTROSE AGAR MEDIA

Treatments	Dose in PDA media, $g\ L^{-1}$	*Botrytis cinerea*		*Phytophthora cactorum*		*Colletotrichum acutatum*	
		Average amount of produced spore on 1 mm^2 media*					
Control		6.33×10^5	c	1.01×10^6	b	1.07×10^5	c
Pine ethanol extract	0.1	4.93×10^5	ac	7.01×10^5	b	9.66×10^4	c
	1	4.18×10^5	ac	6.76×10^5	a	6.01×10^3	a
	10	4.09×10^5	ab	N		4.78×10^3	ab
	20	2.96×10^5	a	N		1.52×10^3	a
Spruce ethanol extract	0.1	5.11×10^5	c	9.99×10^5	b	5.04×10^4	c
	1	5.81×10^5	ca	5.35×10^5	a	5.34×10^4	c
	10	3.32×10^5	b	N		3.03×10^4	c
	20	N		N		9.77×10^3	b

* Means with different letters in the same column indicate significant differences between values, according to Tukey's honestly significant difference test ($p < 0.05$). N – sporulation was not tested.

(Krauze-Baranowska *et al*., 2002). There are synergistic and antagonistic interactions known between different phenolic compounds, and the changes in the composition of these compounds could cause the changes of antimicrobial effect (Deba *et al*., 2008). This may explain the decrease of inhibitory effect after longer incubation period, as fungi may catalyse extract components not only by making them non-toxic, but also by changing phenolic composition.

The spores are a major vehicle for the dissemination of fungal diseases (Dahlberg and Van Etten, 1982), and thus sporulation is an important parameter that needs to be examined to evaluate possible usage of the extract in plant protection. At lowest dose of pine and spruce bark extracts sporulation reduction was not observed, however sporulation was inhibited at higher extracts doses. The effect may be associated not only with direct effect of extract or its

components on fungal sporulation, but also with the ability of the extract to disrupt or dissolve fungal cell walls and membranes (Koul *et al.*, 2008), preventing the development of mycelium. In this case it is possible that at a higher extract dose the mycelia of fungi are not fully grown and do not reach the age, required for sporulation, unlike the fungi with smaller extract doses. Presently the ways of action of different plant extracts and their components against fungi are still unknown, and only some information about possible mechanisms is available. Therefore, it is important to continue investigations of the mechanisms of coniferous extracts against fungi.

Gottstein and Gross (1992) reported that coniferous trees have an extremely high number of organic compounds of enormous chemical diversity, therefore evaluation of the effect of different extract fractions on fungi is necessary. Although this study presents promising results on antifungal properties of pine and spruce bark extracts, further investigations on the practical applicability and the way of action of pine and spruce extracts and separated extract compounds are required to evaluate the potential use of pine and spruce bark extracts against important diseases of berries and vegetables.

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PRIEDES (*Pinus sylvestris* L.) UN EGLES (*Picea abies* (L.) Karst.) MIZAS EKSTRAKTU IETEKME UZ NOZĪMĪGIEM ZEMEŅU PATOGĒNIEM

Zemeņu ražu būtiski samazina kaitēkļi un patogēno sēņu izraisītas slimības, tādēļ aizvien vairāk pētījumu tiek veltīti videi draudzīgu augu aizsardzības produktu izveidei. Skuju koki producē dažādus aktīvus savienojumus, piemēram, terpenoīdus un fenolus. Pētījuma mērķis bija novērtēt parastās priedes (*Pinus sylvestris* L.) un parastās egles (*Picea abies* (L.) Karst.) ekstraktu antifungālo iedarbību uz zemeņu slimības izraisošām sēnēm *in vitro*. Laikā no 2011. līdz 2013. gadam veiktas vairākas eksperimentu sērijas, lai pārbaudītu ekstraktu efektivitāti pret zemeņu slimības izraisošām fitopatogēnām sēnēm — *Botrytis cinerea, Colletotrichum acutatum*, *Phytophthora cactorum* un *Mycosphaerella fragariae*. Novērtējām ekstraktu ietekmi uz micēlija augšanu un sporulēšanas intensitāti. Sēņu radiālās augšanas tests parādīja, ka priežu un egļu mizas etanola ekstrakti inhibē *B. cinerea, C. acutatum, P. cactorum* micēlija augšanu. Pievienojot barotnei ekstraktu devu 20 g L^{-1}, *B. cinerea, C. acutatum* un *P. cactorum* novēro 100% micēlija augšanas inhibēšanu, kas būtiski neatšķiras no fungicīda Signum® efektivitātes ($p < 0,05$). Mūsu rezultāti liecina, ka sēne *M. fragariae* ir vairāk izturīga pret ekstraktu ietekmi.