

Identification of fungicidal components in poplar bark from short rotation plantations

Daniela Einer, Martina Bremer, Javane Oktaee, Steffen Fischer, André Wagenführ

Growth of mould fungi on packaging material reduces the product quality. Eco-fungicidal substances ensure an environmentally friendly approach to prevent this problem during the service life of the product. The bark of poplar trees, as a by-product of short rotation plantations, can be a new source for obtaining bio-fungicidal substances. The types and amount of the eco-fungicidal substances in different poplar clones were investigated with GC/MS and extracted from the raw material with different methods and adapted solvent series. After applying the eco-fungicidal substances to packaging material exposed to a mixture of mould fungi, the resistance against mould growth was monitored for several weeks. The bark extracts have shown promising results in reducing the growth speed of the mould and testing of separate fungicidal substances in the bark showed that substances such as phenol, chatecol and salicylic alcohol can prevent the mould growth.

Keywords: bark, short rotation coppices, poplar, mould fungi, bio-fungicides, fungicidal substances

Introduction

Moulded fibre products are used for different purposes, from the packaging of high-quality electronics to the cultivation of plants in greenhouses. All applications have a limited life span, from a few weeks to several months, including their production, storage, transport, and use. During the service life, intact structure and pleasant appearance are crucial to guarantee their unrestricted application.

Mould growth on the packaging material was reported by the producer during the storage or transport. The occurrence of mould, caused by unfavourable environmental conditions, affects product quality. It should be noted that mould fungi grow mostly on the surface and they do not attack the structure of the fibres, but their growth is an indicator of the fact that the condition is available for other wood (i.e. plant fibre) destroying fungi. It is desired to prevent the mould growth and at the same time keep the high biodegradability of the packaging products. So the material can go back to nature after its relatively short service life. Thus, utilization of bio-based fungicides to decelerate the mould growth might be the answer to meet both of the mentioned requirements.

Nowadays, many industries are focusing on the application of plantations as their primary source of fibre material. These plantations produce a huge quantity of by-products which are mostly in the form of bark chips.

One of the possible applications of bark can be in the production of bio-fungicidal substances. Bark in trees is responsible for protection against environmental threats such as wood-destroying microorganisms. There are around 10,000 chemical components in the tree that are presumed to be associated with natural durability (Yang and Jaakkola, 2011) and many of them have been reported to be present in the tree bark. A rough evaluation is that the phenolic components together with terpenes and terpenoids are the components that protect against fungi in wood (Nascimento et al., 2013; Yang and Jaakkola, 2011; Hart, 1989; Dix and Webster, 1995).

There have been some studies on identification of various potential fungicidal substances in the bark of different trees, for further application as bio-fungicides. Where some researchers reported an inhibiting effect of these materials the others could not find an exact correlation between the extracted content and the fungal growth (Özgenç et al., 2017; González-Laredo et al., 2015). This topic requires an in-depth analysis of any interesting species.

The aim of this research was to identify potential bio-fungicidal substances in the bark of poplar trees from short rotation plantations for extraction and application as biologically based fungicides for fibre moulded packaging materials. Therefore, the fungicidal compounds in poplar bark from different clones

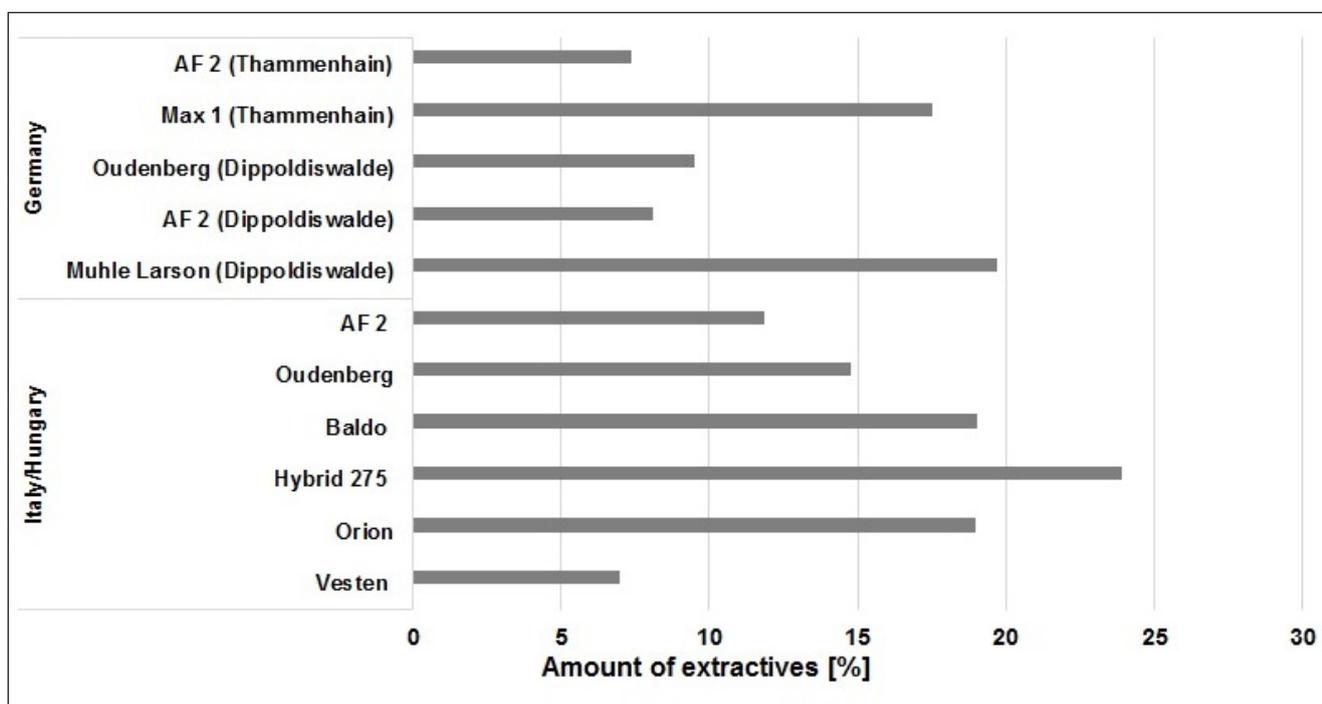


Fig. 1: Influence of clone type and growing site on the amount of extractives of poplar bark from short rotation plantations

Abb. 1: Einfluss von Klontyp und Anbauort auf die Extraktmenge aus Pappelrinde von Kurzumtriebsplantagen

as well as the level of fungicidal substances were studied to develop a method to transfer the natural resistance of the bark to these packaging materials.

Material and Methods

Poplar bark

The amount and type of the substances in the bark depends on the clones' characteristics and the environmental conditions (e.g. temperature, precipitation, soil composition, etc.). In this study, poplar bark of different hybrids was collected from different locations in Hungary, Germany, and Italy.

The clones were namely Muhle Larsen, Baldo, AF 2, Oudenberg, Vesten, Orion, Hybride 275, and Max 1. The selection of the examined poplar clones was based on the resources available in the project frame. Tree stems were manually debarked and bark was dried at below 45 °C in an oven for 72 h for further investigations.

Bark extraction

Prior to the extraction, bark chips were milled, using a cross beater mill, and passed through a 4 mm sieve. Extraction of bark was carried out with different methods. To identify the potential fungicidal substances, Soxhlet extraction was executed with solvents of different polarities (i.e. hexane, toluene, and ethanol). For the Soxhlet extraction, 2 g of milled bark were filled into a cellulose thimble and placed into a 30 ml Soxhlet apparatus. Extraction took place with 60 ml of solvent for 6 h. Afterward the extracted solutions were concentrated with a rotary evaporator and then analysed, using a GC-MS device. For separation of fungicides batch extractions at varying tem-

peratures (25 and 85 °C) and Soxhlet extraction were used. For batch extractions, 80 g of milled bark was stirred with 2 l solvent in a beaker for 1 h. The extraction was carried out three times in each solvent. Between the extraction steps, the solid was separated by filtration from the solution. Between 55 and 90 g of milled bark were used for Soxhlet extraction in a 500 ml

Tab. 1: Performed extractions

Tab. 1: Durchgeführte Extraktionen

sample	aim	method	solvent series
AF 2	Identification of fungicides	Soxhlet extraction	Hexane Toluene Ethanol
Max 1	Identification of fungicides	Soxhlet extraction	Hexane Toluene Ethanol
AF 2	Isolation of fungicides	Batch (25, 50, 85 °C)	Ethanol (99 and 70 %)
AF 2	Isolation of fungicides	Soxhlet extraction	Water Ethanol
AF 2	Isolation of fungicides	Soxhlet extraction	Water Hexane Ethanol
Max 1	Isolation of fungicides	Soxhlet extraction	Water Ethanol
Max 1	Isolation of fungicides	Soxhlet extraction	Water Hexane Ethanol

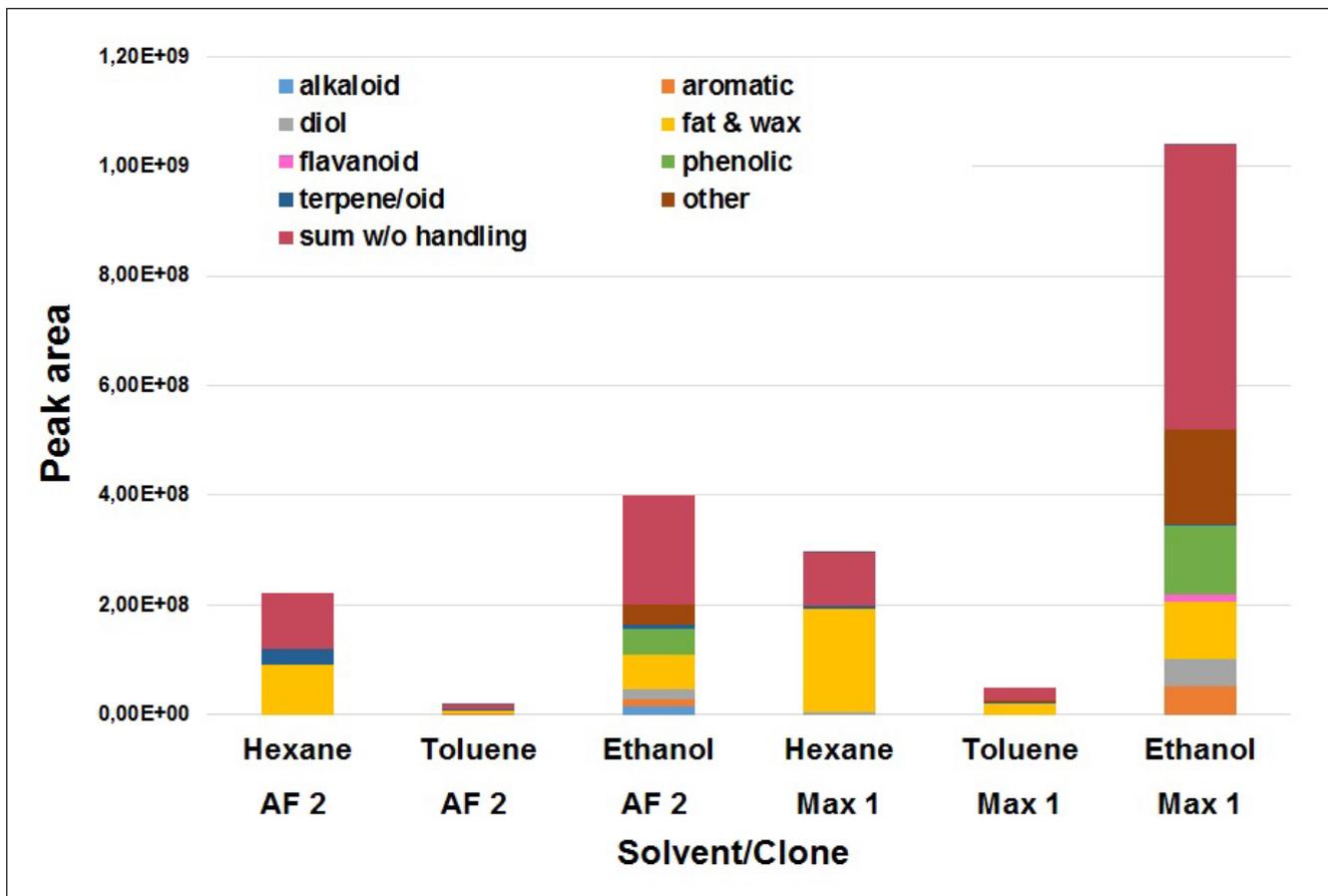


Fig. 2: Influence of solvent on extracted substances from AF 2 and Max 1 clones

Abb. 2: Einfluss des Lösungsmittels auf die aus AF 2 und Max 1 extrahierten Substanzen

Soxhlet apparatus with 1.2 l solvent. The extract solutions were concentrated with a rotary evaporator and air dried. Dry extracts were milled by ball mill. Tab. 1 gives an overview of the performed extractions.

The GC-MS analysis was carried out with GC 7890B (Agilent) coupled with MSD 5977 (SIM). The following conditions were used: column DB-1 from J&W Scientific (30 m * 0,25 mm, 0,25 µm film thickness), constant helium flow rate of 1 ml min⁻¹, oven program: from 80 °C to 300 °C with 5 °C per min hold time 10 min, injection temperature 250 °C and split was set between splitless and 15:1 depending on solvent and extraction method. All measurements were done in scan mode of the MS. A typical mass scan range from 35-500 m/z and ionization voltage from 70 eV were used. The identification of the components in the extract was assigned by comparison of their retention indices and mass spectra fragmentation pattern with those stored in the computer library and published literature. By comparison of peak areas, a semi-quantitative estimation of the substances was realized. Single substances were calibrated in ethanol and toluene for quantification.

Bark fibre production

Fibres were obtained from the bark using thermo-mechanical pulping (TMP). In the TMP method, the raw poplar bark is heated up with saturated steam. The chips are discharged to the fast rotating ribbon feeder, which rapidly injects the chips into

the refiner. The refining part contains a stationary and a rotating disc. These discs have grooves and dams on their surface which become finer from their centre to their periphery. Due to centrifugal force, the material is derived across the radius of the disc and gradually brakes into fibres. Fibres are mostly separated at the softened middle lamella. Fibres were then dried in an oven at 50 °C and stored for production of laboratory sheets.

Laboratory sheets

In producing moulded fibre products, different fibre materials and manufacturing processes are used. Among the most common raw materials are fresh cellulose, residual material from paper and cardboard, and fibres from perennials (e.g. *Miscanthus x giganteus*). Various additives, such as wet strength agents are added during different stages of production to adjust the desired properties in the final product. The intended use affects the material composition, for example, a higher proportion of fresh cellulose is used for the production of packaging for high-quality consumer goods.

The different manufacturing processes share the following production steps: dissolving the bulk material in water to produce the pulp; inserting of a preforming mould in the pulp, suction of water followed by accumulation of the fibres on the surface of the pre-forming mould; removal of the moulded fibre product for drying or transfer to post-forming.

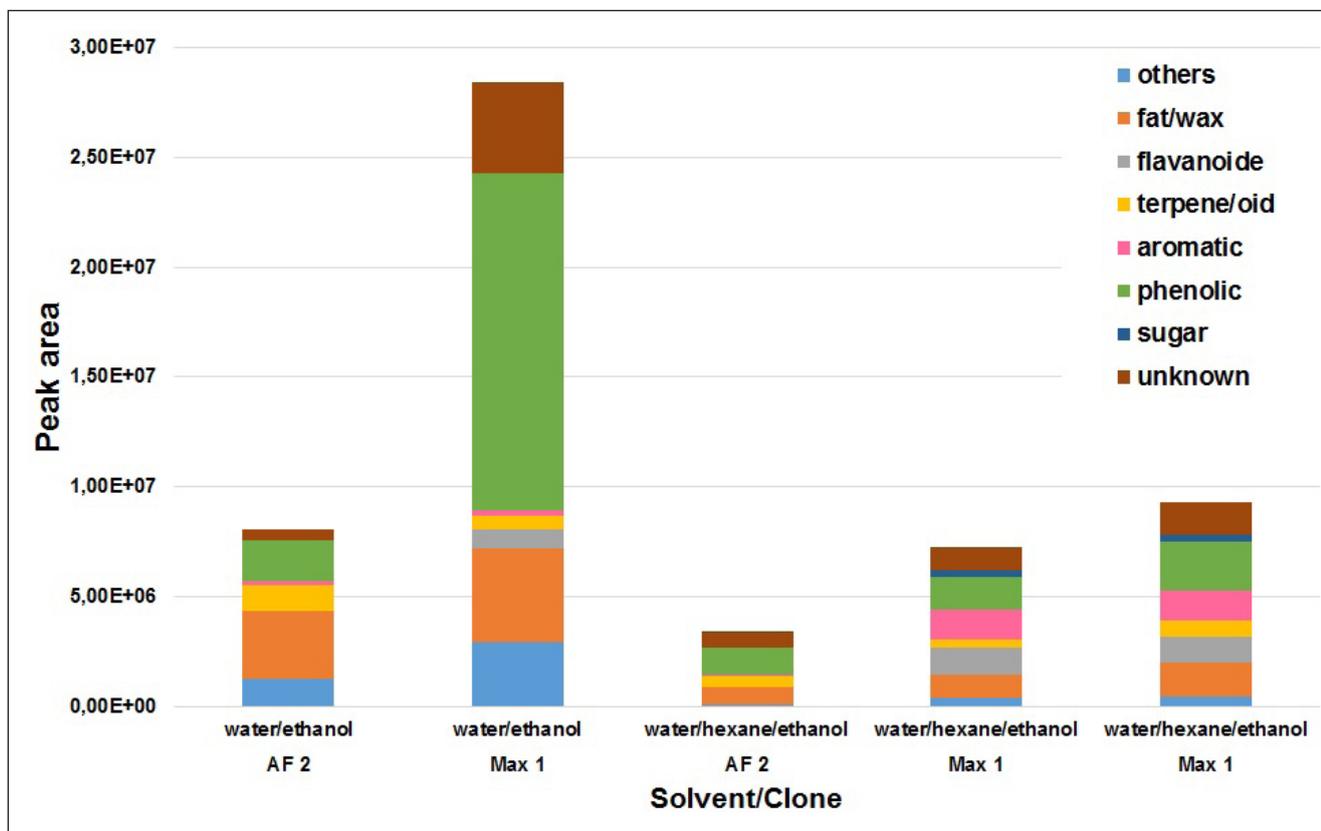


Fig. 3: Extracted substance classes in Max 1 and AF 2 clones with ethanol, with pre- extraction with water and hexane

Abb. 3: Substanzklassen, mit Ethanol extrahiert aus Max 1 und AF 2, nach Vorextraktion mit Wasser und Hexan

Plain laboratory sheets, with production processes similar to the industrial method, were produced with different percentage of bark fibres (30, 40, 50, and 60 % wt.) and cardboard. A series of laboratory sheets from combinations of cardboard and Miscanthus (30:70 % wt.) was made as control samples since it was the preferred combination of the project's partner company, which produces fibre moulded packaging. The additives that were applied were Giluton WS12 (5 % wt.) and Perglutan K2 (8 % wt.) from Kurita Europe GmbH.

Mould fungi

In designing of the test for determination of the fungicidal effects of the extracted substances, five different types of mould fungi (*Aspergillus niger*, *Paecilomyces variotii*, *Chaetomium globosum*, *Trichoderma virens*, *Penicillium pinophilum*) were selected, based on previous experience and suggestions of other researchers. A mixture of spore suspensions with a concentration of $5 \cdot 10^5$ spore/ml was used in preparation of mould tests (Oktae, 2019).

Two series of tests were performed. In the first tests, the aim was to determine the possible antifungal properties of the bark fibres as well as bark extracts, produced with methods described in section "Bark extraction" For that, Petri-dishes with malt-extract-agar-medium were prepared and the mixed spore suspension of the five mould fungi was spread on their surface. After an incubation time of three days at 27 ± 1 °C, the dishes were covered with mould fungi.

Solid extracts of bark were dispersed in distilled water using an ultrasound bath to 0.05 g/ml concentration. Laboratory sheets made of cardboard and Miscanthus were cut and soaked very shortly in the liquid and left overnight to dry.

Laboratory sheets, containing bark fibres or prepared with eco-fungicidal solution, were placed on the Petri-dishes. Then the samples were stored in an incubator for a period of four weeks. To assess the mould growth on the surface of the samples a visual observation based on Tab. 2 was carried out weekly.

In the second test setting, four different potential substances that were identified in the poplar bark were purchased

Tab. 2: Evaluation of fungal growth intensity

Tab. 2: Bewertung der Intensität des Wachstums der Schimmelpilze

Intensity of Growth	Evaluation criterion
0	No growth apparent under the microscope
1	No growth visible with the naked eye, but clearly visible under the microscope
2	Growth visible with the naked eye, covering up to 25 % of the sample surface
3	Growth visible with the naked eye, covering up to 50 % of the sample surface
4	Considerable growth, covering more than 50 % of the sample surface
5	Intensive growth, covering the entire sample surface

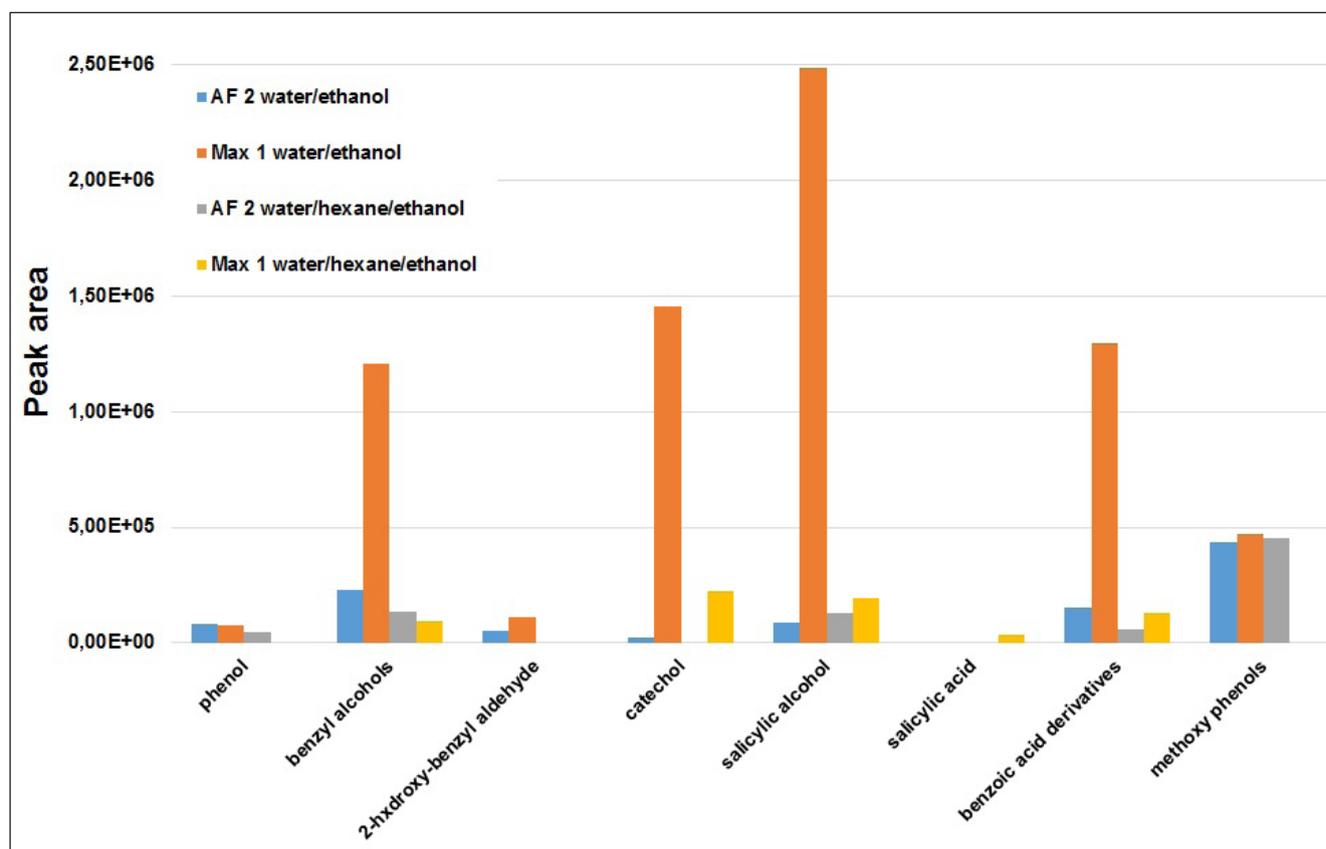


Fig. 4: Type and appearance of potential fungicidal aromatics and phenols in the bark extracts of AF 2 and Max 1 clones

Abb. 4: Art und Auftreten potentieller fungizider Aromaten und Phenole in Rindenextrakten der Klontypen AF 2 und Max 1.

and used to determine the ability of these substances in preventing the mould growth. These chemicals were namely: catechol, 4-Benzyloxyphenol, phenol, and salicylic alcohol and were chosen on the basis of the results of section "Fungicidal components in poplar bark". Different concentrations of 1.5, 1, 0.5, 0.3, and 0.1 % of these substances in distilled water were prepared and pipetted in the twelve well plates. As a nutrient for the fungi 2 % malt extract solution was used. The ratio of the malt extract to the fungicidal solution was kept 1:2. The wells on the twelve well plates have a capacity of 3 ml which was filled first with this solution and then 10 μ l spores were added to each well. The plates were sealed with Parafilm and placed on a shaker for two weeks at 150 rpm. The visual observation was carried out after this period.

Results and discussion

Fungicidal components in poplar bark

Extractions of different poplar clones from different locations were investigated for their amount of extractives. These investigations were carried out with Soxhlet extraction and a mixture of ethanol and toluene (1:1 ratio). In Fig. 1 the percentage of extractives of various studied clones, based on dried mass, is illustrated. As expected, the location of the plantations and the clone type showed to be influencing the amount of extractives. The amount of extractives in the clones AF 2 and Oudenberg is higher in more southern locations, which can be related to the

climate conditions. But, there is also a strong dependence on the clone. Muhle Larson and Max 1 both showed relatively high contents of extractives and were found in different locations.

Potential fungicidal substances, their isolation, and effect will be discussed with two clones from Germany, namely clone Max 1, with a high content of extracts, and AF 2 with relatively low content of extracts.

Two groups, aromatic substances and phenols, as potential antifungal substances, were in the focus of interest. However, fats and waxes can have a fungicidal effect through the hydrophobization of the material. These mentioned substances differ in polarity; therefore, solvents of different polarities were used to determine the optimal way for the extraction of fungicides.

It was possible to separate the different classes of extractive substances as shown in Fig. 2 for the poplar clones AF 2 and Max 1. Mainly fats and waxes are extracted with hexane, whereas aromatic substances and phenols are extracted with ethanol beside many other substance classes. Toluene extraction results in very small yields. These investigations confirmed that the content of extracts is much higher in clone Max 1. Especially, the substances of interest (aromatics and phenols) are in higher concentrations. In addition, the hydrophobing fats and waxes show higher quantities in hexane solvent as well as in ethanol. Moreover, many other substances were present which could not be identified, in ethanol extract.

In general, five substance groups were found as potential fungicides, which are listed in Tab. 3. In the same table, the identified

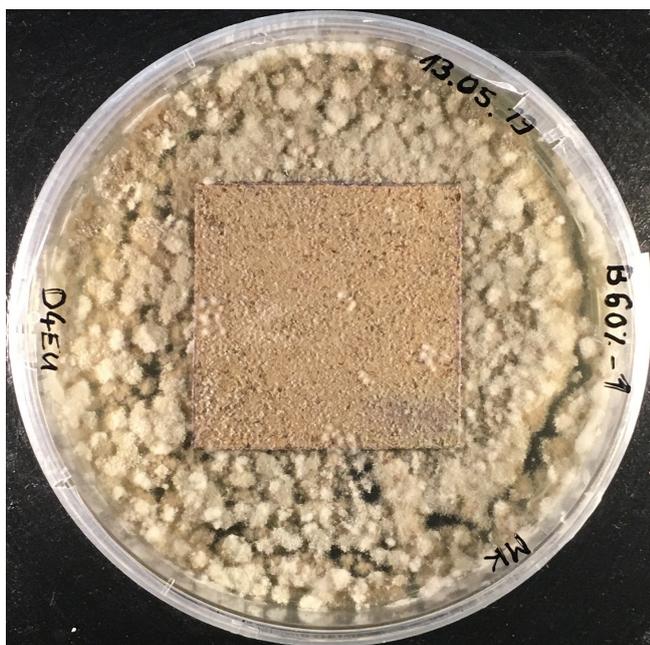


Fig. 5: Growth of mould fungi on the surface of fibre moulded packaging materials with 60 % wt. bark fibre substitution

Abb. 5: Wachstum von Schimmelpilzen auf der Oberfläche von fasergeformten Verpackungsmaterialien mit 60 % Massenanteil an Rindenfasern

substances for various clones are marked. Several phenols were found in all clones, whereas lignin-based substances (benzoic acid and benzaldehydes, cinnamonic acids and aldehydes and Vanillin) were very different in the extracts of clones. Most of the clone extracts contain salicylic acid and/or alcohol.

In the next step, the separation of potential fungicides was carried out. Batch extractions in ethanol were investigated. About 7.5 % wt. of the used bark could be separated as ethanol extract at room temperature for AF 2 (Germany). However, some of the solutions began to mould after a few days. This can be explained by the high proportion of oligomeric sugars. Therefore, a pre-extraction with water was subsequently carried out. As a result, the proportion of ethanol extracts was reduced to 4.5 %. Without pre-extraction in water, at higher temperatures, a slightly lower extract content in ethanol is gained.

In comparison, Soxhlet extractions of AF 2 and Max 1 were executed. At first, a pre-extraction in water was carried out for at least 24 hours. The water extracts of both clones were dark in colour and had a similar amount of solid content. In the subsequent ethanol extraction, the separated masses were very different, as expected. In Tab. 4 the masses of separated extracts at the diverse extraction steps are listed.

As mentioned before, hydrophobization can also inhibit fungi growth. For a separate investigation of these effects, an extra step of extraction with hexane was added between the water and the ethanol extractions. It was found that the amount of separated extracts was significantly reduced by the hexane extraction for both clones. For this purpose, further investigations are required.

As expected, mainly fats, waxes, aromatic and phenolic com-

pounds were identified in hexane extracts. The ethanol extracts contain much more substance classes. Interesting substance classes for the fungicidal effect are strongly reduced in the ethanol extract of hexane containing sequence as shown in Fig. 3. This reduction mainly affects benzyl alcohols, catechol, salicylic alcohol and benzoic acid derivatives within the phenolic compounds (Fig. 4).

Evaluating the fungicidal properties

The growth of mould on the surface of the fibre moulded packaging material with bark fibre and with bark extracts were recorded, with the method and test setting that was explained before. The results of this part showed that substituting Miscanthus fibres with bark fibres led to similar mould growth on the samples and higher amount of bark fibres (> 30 %) did not show any meaningful influence on the mould resistance property (Fig. 5).

Comparing different applied extracts showed that the best results were achieved when samples were coated with hexane extracted components of Max 1 hybrid. Results of the identification of the substances in extracts (Fig. 2) show that the highest amount of fat and waxes were identified in hexane extracts of

Tab. 3: Groups of identified potential fungicides

Tab. 3: Gruppen identifizierter potentieller Fungizide

Group	Substance	AF 2	Max 1	Mühle Larson	Vesten	Oudenberg	Baldo
Phenols	Phenol					x	
	Catechol	x	x	x	x	x	x
	4-propyl-resocinol	x		x	x	x	x
	Guaiacol	x	x	x	x	x	
	Benzoyloxyphenol	x		x		x	
Benzoic acid / aldehyde / derivatives	4-hydroxy-benzoic acid	x			x	x	
	2,5-dihydroxy-benzeneacetic acid	x				x	
	3-Hydroxy-4-methoxybenzoic acid	x					x
	2-hydroxy-4-methyl-benzaldehyde	x	x	x	x		x
	4-hydroxy-3,5-dimethoxy-benzoic acid	x					x
Cinnamon acid / aldehyde / derivatives	p-Hydroxycinnamic acid	x	x	x			
	3-Hydroxy-4-methoxycinnamic acid	x			x	x	x
	3,5-Dimethoxy-4-hydroxycinnamaldehyde	x				x	
Vanillin derivatives	Vanillin	x					
	Vanillic acid hydrazide	x					
Salicylic derivatives	Salicylic acid		x	x	x	x	x
	Salicyl alcohol	x	x	x	x		

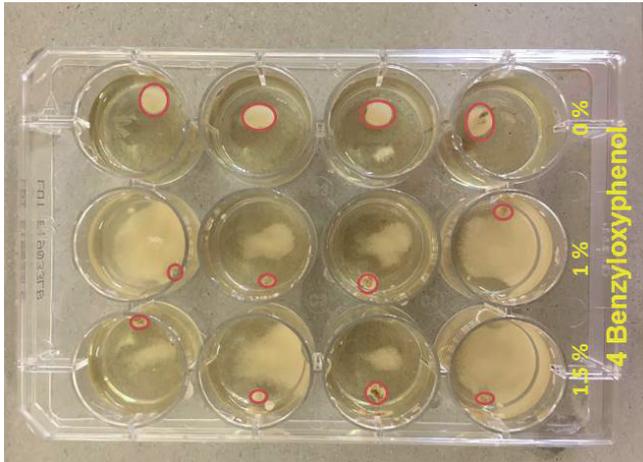


Fig. 6: Growth of mould fungi in liquid medium with fungicides identified in poplar bark

Abb. 6: Wachstum von Schimmelpilzen in Flüssigmedium mit in Pappelrinde identifizierten fungiziden Wirkstoffen

Max1, therefore, the higher resistance of these samples can be related to hydrophobization of the samples and not the direct anti-fungal properties of the components.

There were no differences detected between ethanol extracted solutions of two studied hybrids. In Tab. 5 the growth level of mould fungi on different samples is reported. Darker colours on the table present the more severe mould growth.

In testing of the neat substances, it was proved that application of phenol (> 0.1 %), chatecol (> 0.3 %), and salicylic alcohol (> 0.5 %) could stop the mould growth in the reported test setting. Where a reduction in growth was observed with the addition of 4-Benzyloxyphenol to the liquid the growth was not entirely prevented. On Fig. 6 the mould growth was marked with red for better visibility.

Outlook

As explained in the article, different factors such as location, age and harvesting time can affect the chemical composition of the bark of poplar clones. Therefore, it is of high interest to study these factors in the next steps of this research.

Tab. 4: Masses of extracts separated with Soxhlet

Tab. 4: Menge an mittels Soxhlet separierten Extrakten

Extraction sequence	step	Mass of extract [%] AF 2	Mass of extract [%] Max 1
Water/ethanol	Water	19.73	20.52
	Ethanol	2.54	10.38
Water/hexane/ethanol	Hexane	0.51	1.47
	Ethanol	0.63	4.03

The variation in the fungicidal substances in the inner and outer bark is another relevant topic to investigate, especially in older trees. Although the tests in the laboratory are developed for a worst-case scenario, it is interesting to investigate the durability of the material in the desired service life of 6 months.

The scope of this research was focused on stopping the growth of mould fungi on the packaging material as a current problem of the partner in the project. The effectiveness of the extracted fungicidal substances should be studied in exposure to wood destroying fungi as well (i.e. white rot, brown rot, and soft rot).

Acknowledgement

This project has received funding from the Bio Based Industries Joint Undertaking under the European Union's Horizon 2020 research and innovation programme under grant agreement No 745874.

The project "Dendromass4Europe – Securing Sustainable Dendromass Production with Poplar Plantations in European Rural Areas" aims at establishing sustainable, regional cropping systems for agricultural dendromass production on marginal land. The dendromass (lignous biomass, bark and wood) produced in SRC will be supplied to dedicated bio-based value chains. Four new bio-based materials will be created. A functionally adapted lightweight board for furniture production is planned as well as three bark based materials: eco-fungicidal moulded fibre pulp products, bark enriched wood-plastic composite profiles and bark enriched wood-plastic composite granulates.

Nine partners from seven EU countries as well as other experts are working on the project under the direction of Technische Universität Dresden, Department of Forest Policy and Forest Resource Economics. The partners include research institutions, companies and non-governmental organizations. For further information, check www.dendromass4europe.eu.

Tab. 5: Level of mould growth on fibre moulded samples

Tab. 5: Grad des Schimmelpilzwachstums auf fasergeformten Proben

Intensity of mould growth	1st	2nd	3rd	4th
Cardboard + Bark fibre 30 %	1	1	4	4
Cardboard + Bark fibre 40 %	0	1	4	5
Cardboard + Bark fibre 50 %	1	2	4	5
Cardboard + Bark fibre 60 %	0	0	4	4
Cardboard + Miscanthus (control)	1	2	3	4
Max 1, Ethanol extract	1	1	1	2
Max 1, Ethanol extract after Hexane	1	2	2	2
Max 1, n-Hexane	0	0	0	1
AF 2, Ethanol extract	1	1	2	2
Max 1, n-Hexane	0	0	0	1

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Dr. Martina Bremer studied chemistry at TU Dresden from 1985 to 1990 and finished her doctorate there in 1995. From 1995 to 2006 she worked at the Institute of Inorganic Chemistry at TU Bergakademie Freiberg. After that, she worked as researcher and secretary at the VECUR e.V. in Freiberg. Since 2008 she is working at the professorship of Wood and Plant Chemistry at TU Dresden. Among other topics, she is working on the material use of wood and plant components like extracts or lignin.

ABSTRACT

Identifizierung fungizider Bestandteile in Pappelrinde von Kurzumtriebsplantagen

Ein Wachstum von Schimmelpilzen auf Verpackungsmaterialien reduziert deren Produktqualität. Biofungizide Substanzen stellen eine umweltfreundliche Variante zur Vermeidung dieses Problems während der Nutzungsdauer des Produktes dar. Pappelrinde als Nebenprodukt von Kurzumtriebsplantagen ist neue Quelle für die Gewinnung fungizider Substanzen. Art und Menge der biofungiziden Substanzen wurden mittels GC/MS untersucht, die geeigneten Substanzen wurden mittels verschiedener Methoden und angepasster Lösemittelreihen aus dem Rohmaterial gewonnen. Verpackungsmaterialien mit applizierten biofungiziden Substanzen wurden einer Mischung aus verschiedenen Schimmelpilzen ausgesetzt. Die Resistenz gegenüber dem Wachstum der Schimmelpilze wurde über mehrere Wochen beobachtet. Die Rindenextrakte zeigten vielversprechende Ergebnisse bei der Reduzierung der Geschwindigkeit des Wachstums der Schimmelpilze. Die Prüfung ausgewählter, in der Rinde vorhandener Substanzen hat gezeigt, dass beispielsweise Phenol, Brenzcatechin und Salizylalkohol das Wachstum von Schimmelpilzen einschränken kann.

Schlüsselwörter: Rinde, Kurzumtriebsplantagen, Pappel, Schimmelpilze, Biofungizide, fungizide Inhaltsstoffe

M.Sc. Javane Oktaee studied Wood Science and Technology at University of Tehran in Iran. Since 2013 she is working at the professorship of Wood and Fibre Materials Technology at TU Dresden. She recently has completed her PhD in the field of natural fibre polymer composites. Her focus is on application of by-products of short rotation plantations, in particular bark.

Prof. Dr. rer. nat. Steffen Fischer studied Chemistry at TU Dresden from 1983 to 1988 and finished his doctorate there in 1992. From 1992 to 2003 he worked at the Institute of Inorganic Chemistry at TU Bergakademie Freiberg and finished his habilitation. After that, he worked as head of the department “Polysaccharide Chemistry” at Fraunhofer Institut for Applied Polymer Research in Potsdam. Since 2006 he is Professor of Wood and Plant Chemistry at TU Dresden and head of the Institute of Plant and Wood Chemistry.

Prof. Dr.-Ing. André Wagenführ studied Wood Technology and Fibre Material Technology at TU Dresden from 1980 to 1984 and finished his doctorate there in 1988. After some years in the industry, from 1992 he worked as the head of the Biology and Conservation Department at Institut für Holztechnologie Dresden (IHD). Since 1999 he is a Professor of TU Dresden and head of the Chair for Wood Technology and Fibre Materials Technology, in Institute of Natural Materials Technology of TUD.