Radosław Mirski[1], Jakub Kawalerczyk[1], Dorota Dziurka[1],
Kinga Stuper-Szablewska[2], Joanna Walkiewicz[1]

Selected Chemical and Physical Properties of Pine Wood Chips Inoculated with *Aspergillus* and *Penicillium* Mold Fungi

Odabrana kemijska i fizička svojstva drvne sječke od borovine inokulirane plijesnima *Aspergillus* i *Penicillium*

ORIGINAL SCIENTIFIC PAPER
Izvorni znanstveni rad
Received – prispjelo: 21. 11. 2022.
Accepted – prihvaćeno: 14. 7. 2023.
UDK: 674.8
https://doi.org/10.5552/drvind.2023.0076

ABSTRACT • *Mold fungi representing genera of Penicillium and Aspergillus commonly develop on the surface of freshly harvested wood chips during storage. As a result, they are often considered as low-quality material and intended for incineration. Thus, the aim of the present study was to investigate the effect of wood chips infestation with mold fungi representing genera of Aspergillus and Penicillium on their basic chemical and physical properties, such as: chemical structure (evaluated with FTIR spectroscopy), mass loss and hygroscopicity, after an incubation of 3, 6 and 9 weeks. Based on the visual assessment and ergosterol content analysis, it was found that inoculation of wood chips with molds led to the intense fungal development on their surface. However, as observed in FTIR spectra, the presence of molds caused no changes in wood chemical structure. Furthermore, no mass loss and no significant increase in the hygroscopicity of wood were observed. Therefore, pine wood chips overgrown by studied genera of fungi seem to be a valuable material for various applications.*

KEYWORDS: *mold fungi, wood chips, FTIR spectroscopy, mass loss, hygroscopicity*

SAŽETAK • *Plijesni rodova Aspergillus i Penicillium najčešće se razvijaju na površini svježe pripremljene drvne sječke tijekom skladištenja, zbog čega se takva sječka često smatra nekvalitetnim materijalom i spaljuje se. Stoga je cilj ovoga istraživanja bio istražiti utjecaj zaraženosti drvne sječke plijesnima rodova Aspergillus i Penicillium na njezina osnovna kemijska i fizička svojstva kao što su kemijska struktura (ispitano FTIR spektroskopijom), gubitak mase i higroskopnost nakon ikubacije od tri, šest i devet tjedana. Na temelju vizualne procjene i analize sadržaja ergosterola, utvrđeno je da je inokulacija drvne sječke plijesnima prouzročila intenzivan razvoj plijesni na površini*

[1] Authors are researchers at Poznań University of Life Sciences, Faculty of Forestry and Wood Technology, Department of Mechanical Wood Technology, Poznan, Poland. https://orcid.org/0000-0002-4881-579X; https://orcid.org/0000-0002-5539-1841; https://orcid.org/0000-0001-6197-7825; https://orcid.org/0000-0002-8365-3714
[2] Author is researcher at Poznań University of Life Sciences, Faculty of Forestry and Wood Technology, Department of Chemistry, Poznan, Poland. https://orcid.org/0000-0002-9011-8592

drvne sječke. Međutim, na FTIR spektrima je uočeno da plijesni nisu uzrokovale promjene u kemijskoj strukturi drva. Nadalje, nije uočen gubitak mase niti znatno povećanje higroskopnosti drva. Stoga se može zaključiti da je drvna sječka od borovine zaražena promatranim rodovima plijesni upotrebljiv materijal za različite primjene.

KLJUČNE RIJEČI: *plijesan, drvna sječka, FTIR spektroskopija, gubitak mase, higroskopnost*

1 INTRODUCTION
1. UVOD

Mold fungi are the example of microorganisms originating from different taxonomic groups, resulting in various physiological reactions. Due to this diversity, they have the ability to develop on many different types of surfaces such as e.g. wood, wood-based materials, gypsum boards, ceramics, etc. (Nielsen *et al*., 2004). Factors influencing the development of molds include the availability of nutrients, humidity, air flow, sunlight, temperature and pH (Schmidt, 2006). The temperature ranging from 20 to 35 °C is considered to be the optimum for the development of mold fungi (Viitanen and Bjurman, 1995). Moreover, relative air humidity of approx. 80 % seems to be a minimum required for their growth (Viitanen *et al*., 2011).

In terms of nutrients, mold fungi use starch, sugars and proteins causing the parenchyma cells to grow in the wood surface layer. According to Ahmed *et al*., (2013), the depth of penetration is usually around 1 mm. They do not cause a degradation of structural constituents of wood and, therefore, they are significantly less destructive comparing to brown-rot and white-rot fungi (Broda, 2020; Woźniak, 2022). Although it is believed that they only reduce the aesthetic value of wood without reducing its mechanical properties, there are indications in literature that mold fungi can also cause some changes in the wood characteristics. Darwish *et al*. (2013) observed that the development of molds can reduce crystallinity of cellulose and cause a change in FTIR (Fourier transform infrared spectroscopy) spectra indicating changes in the chemical structure of lignin and carbohydrates. Furthermore, Salem *et al*. (2016) stated that the colonization of mold fungi can contribute to the increase in the content of copper, chlorine and aluminium and a decrease in the content of carbon. Hamed and Mansour (2018) observed that the advanced development of molds can lead to a degradation of wood ultrastructure.

Wood chips are the example of wooden materials susceptible to mold fungi, especially when they are freshly harvested (Idler *et al*., 2019). According to Mirski *et al*. (2019), the annual production of wood chips during the softwood processing in Polish sawmills can reach millions of cubic meters. They are usually considered as a by-product occurring during roundwood cutting process and they are intended for the storage after the processing is completed. Usually, the storage process takes place outdoors, which means that the wood can be exposed to increased humidity. Moreover, the elevated temperature inside the chip pile also favors the growth of microorganisms (Krigstin *et al*., 2019). Studies performed by Alakoski *et al*. (2016) and Lieskovský *et al*. (2017) showed that mold fungi (including genera of *Aspergillus* and *Penicillium*) are commonly found in landfills. Although, as mentioned above, it is believed that molds do not deteriorate the basic properties of wood, a raw material visibly infested by them is considered to be of poor quality and is usually intended for incineration (Knoll *et al*., 1993). However, due to the progressive shortages of raw material (Taghiyari *et al*., 2020), the diversity of available energy biomass (Mat Aron *et al*., 2020) and the assumptions of sustainable economy (Kawamura and Wang, 2020), the alternative possibilities of using wood degraded by fungi are constantly sought. The example of potential applications for such material may be a preparation of termite attractants (Esenther *et al*., 1961; Su, 2005), synthesis of adhesives (Heritage, 1954; Kawamura and Wang, 2020; Li and Geng, 2005) and manufacturing of wood-based composites (Ayrilmis *et al*., 2015; Ge *et al*., 2018; Nemli *et al*., 2018). However, in order to look for new ways of application of pine wood chips covered with mold fungi, their properties should be thoroughly investigated.

Therefore, taking into account that the knowledge about the effect of mold fungi development on the properties of wood chips is scarce and the issue is expected to gain even more importance due to the increased processing of hardwood in the years to come in Poland, hardwood usually being even less resistant to biotic factors (Gendek *et al*., 2018; Zajączkowski *et al*., 2013), it was decided to carry out the experiments aimed at determining the changes in the basic properties of wood chips, such as: chemical structure, hygroscopicity and mass loss caused by the inoculation. Moreover, due to the intensive search for the new applications for degraded wood, the present research can be a valuable reference and contribute to finding new ways of use of raw material overgrown by molds, other than for energy purposes.

2 MATERIALS AND METHODS
2. MATERIJALI I METODE

Pine (*Pinus sylvestris* L.) wood chips produced during roundwood processing were supplied by KPPD

Szczecinek S.A. (Kalisz Pomorski, Poland). In order to get rid of larger pieces, wood chips were sieved with flat sieves using a mesh with square perforations of 50 mm × 50 mm. The individual fragments of the bark remaining in the mixture after sorting were removed manually. Due to the fact that both wood moisture content and chip size can potentially influence the growth of microorganisms (Hukka and Viitanen, 1999; Idler *et al*., 2019), each batch of wood chips was characterized before being placed in the chamber and before the application of inoculum. Moisture content (*MC*) of wood was determined immediately before the inoculation. 20 pieces of wood chips were collected from each batch of material and their *MC* was investigated using a dry oven method according to PN-77/D-04100 (1978). In addition, 500 chips were collected from each batch to determine their length, width and thickness using a calliper with an accuracy of 0.01 mm. Moreover, in order to characterize their shape, the degree of slenderness (λ), flatness (Ψ) and width coefficient (*m*) were calculated according to the equations presented in the study of Dukarska *et al*. (2021). Bulk density of wood chips was determined by investigating the mass of wood chips loosely poured in a vessel with a volume of 5000 cm^3.

For the production of inoculum, the species of molds representing genera of *Aspergillus* and *Penicillium,* identified with the use of genetic methods in previous studies involving the analysis of the biodegradation of wood chips during short-term storage, were used. A specific list of species can be found in a recently published article (Mirski *et al*., 2022). It is a favorable solution due to the fact that the composition of the inoculum reflects a diversity of mold fungi actually occurring in landfills. The spore suspension was prepared according to the method previously applied by Góral *et al*. (2015) and Buśko *et al*. (2014). Isolates were incubated on plates filled with agar medium for 4 weeks. The plates were washed with saline to prepare spore suspensions. The concentration of spore suspension was determined using the haematocrit at approx. 5 × 10^5 spores/ml. The proportion of *Aspergillus* spores to *Penicillium* spores was 1:1. The material was sprayed with the suspension in the amount of 100 ml/1 kg of wood chips. The inoculated material was stored for 3, 6 or 9 weeks in the chamber. For the incubation, the temperature of 25°C and the relative humidity (RH) of 95 % were applied. The assumed RH was obtained by keeping the material above supersaturated solution of potassium sulphate. Wood chips incubated for 3, 6 and 9 weeks were labeled as Z-3, Z-6, Z-9, respectively. Moreover, the reference variant which was not subjected to the inoculation was labeled as Z-0.

In order to assess the progress in the development of fungi on wood chips during the assumed incubation periods, the method previously used by Kwaśniewska-Sip *et al*. (2018) was applied. 50 pieces of wood were collected from each variant after the end of incubation and evaluated visually with the help of loupe with the magnification of 10×. Wood chips were classified with the five-class assessment system used by Imken *et al*. (2020): class 0 = no fungal growth, class 1 = 1-25 % of the surface infested, class 2 = 26-50 % of the surface infested, class 3 = 51-75 % of the surface infested, class 4 = 76-100 % of the surface infested.

One of the commonly used methods of chemical analysis allowing for the determination of fungal development is the determination of ergosterol (ERG) content. For this purpose, the method previously used to analyze its content in wood dust (Pędzik *et al*., 2021; Stuper-Szablewska *et al*., 2017; Szwajkowska-Michałek *et al*., 2020) and bark (Szwajkowska-Michalek *et al*., 2019) was applied. A detailed description of the determination using UPLC can be found in the above-mentioned papers.

Commonly used Fourier transform infrared spectroscopy was used to determine the effect of mold growth on the chemical structure of wood chips. The advantage of this technique is that it requires little time and material to perform the analysis (Chen *et al*., 2010; Traoré *et al*., 2016). It is used for example to assess the structure of wood components (Siuda *et al*., 2019), the effect of various degrading factors (Pandey and Pitman, 2003) and protective measures (Woźniak *et al*., 2020). (5 ± 0.01) g of wood chips were ground in laboratory mill and sieved to collect the fraction of 0.125 mm. The wood powder was mixed with KBr at a 1/200 mg ratio. The analysis was performed with the use of Bruker FTIR IFS 66/s spectrometer (Bruker, Ettlingen, Germany) with the Fourier transform range (500–4000 cm^{-1}), registering 32 scans at the resolution of 4 cm^{-1}.

Before the inoculation, 20 wood chips from each variant were dried to a constant weight at (102±1) °C. Then their weight was determined with an accuracy of 0.001 g. Chips prepared in this way were seasoned for 24 hours with the rest of the mixture to equalize the MC. Then, they were placed in the chamber together with the remaining material. After the incubation process, their weight was determined again with an accuracy of 0.001 g after drying to a constant weight at (102±1) °C. The mass loss (*ML*) was calculated according to Eq. 1.

$$ML = \frac{m_1 - m_2}{m_1} \cdot 100\ \% \qquad (1)$$

Where: m_1 – mass of wood chip before inoculation (g), m_2 – mass of wood chip after incubation (g).

The methodology applied by Siuda (2019) to assess the hygroscopicity of oak wood modified with trialkoxysilanes was used to determine the effect of fungal inoculation on wood chips hygroscopicity. For this pur-

pose, (10±0.05) g of wood chips from each variant were collected and ground in a laboratory mill to obtain the fraction of 0.2 mm. 10 pellets were formed from the obtained material with the use of a hand press. The diameter of each pellet made from (1±0.01) g of wood powder was 12 mm. Produced pellets were dried in a laboratory oven to a constant weight at (102±1) °C, and then placed in sealed containers over a supersaturated ammonium phosphate solution. The use of chosen salt ensured the conditions of RH at the level of 90-95 %. The weight gain of pellets was measured after 15 min; 30 min; 1 h; 2 h; 4 h; 8 h; 24 h; 48 h; 72 h and 120 h.

The obtained results were analyzed with the use of Statistica 13.3 software. The empirical distributions of the features were presented as histograms and their interpretation was carried out using the Shapiro-Wilk test. This test is commonly used to check the normality of the distribution of random variables. In order to investigate the influence of independent variables on the dependent variable, a multivariate analysis of variance (ANOVA) was used. During the analysis, the following tests were used: Fisher test, *t*-Student test, HSD Tukey test and Kruskal-Wallis test depending on the type of data, at the significance level of $\alpha = 0.05$. The use of the above tests allowed for the assessment of the significance of differences by identification of homogeneous groups.

3 RESULTS AND DISCUSSION
3. REZULTATI I RASPRAVA

Based on the Shapiro-Wilk test results, it was found that only in the case of MC of chips labeled as Z-9 there was a normal distribution at the assumed significance level. For each variant, the chip length values were mostly concentrated around the average value (approx. 60 % of the results were in the range between 30 and 35 mm). A similar tendency was also observed in the case of width and thickness where approx. 80 % of the observations were in the range of 12-14 mm and approx. 70 % in the range of 4.0 – 4.5 mm, respectively.

Sieving the chips before the inoculation may have resulted in less variable dimensions due to the reduction in the occurrence of chips larger than 50 mm. When analyzing the MC distributions, it was found that, depending on the type of raw material, 40-50 % of the chips in the mixture were characterized by a *MC* of 20.0-20.5 %. Despite the fact that no normal distribution was found, it was decided that, as in the case of Mirski (2013) research, the power of statistical tests was sufficient enough to perform and evaluate the results of analysis of variance. The results of the analysis of the significance of differences between the variants are presented in Table 1.

Based on the analysis of homogeneous groups determined in the HSD Tukey test, it was found that regardless of the variant, there were no statistically significant differences in the dimensions, shape and *MC* of the materials. Therefore, when interpreting the results of further analysis, the influence of the size of chips and their initial MC on the development of microorganisms can be omitted.

After removing the wood chips from the chamber, different infestation levels were identified depending on the incubation time. The Shapiro-Wilk test showed that no normal distribution was found for infestation classes for either inoculated variant. Despite the fact that no normal distribution was found at the assumed significance level, the histograms showed that the vast majority of observations represented the mean value. Moreover, as can be seen from the data presented in Figure 1, the longer the assumed incubation time, the higher infestation class of the chips. Homogeneous groups determined in the Kruskal-Wallis test indicate that the differences between the variants were statistically significant. The greatest increase in the fungal growth was observed between the third and sixth week of the experiment, when the infested area increased from 1-25 % of the chip surface to 51-75 %. Further extension in the incubation time to 9 weeks caused a complete infestation of the wood surface. The progressive development of mold fungi on the surface of wood chips resulted from the optimal conditions for their growth (temperature of 25 °C and RH of 95 %) provided during the incubation (Sedlbauer, 2001; Viitanen

Table 1 Dimensions, moisture content and shape of the chips intended for inoculation
Tablica 1. Dimenzije, sadržaj vode i oblik sječke pripremljene za inokulaciju

Parameter *Parametar*	**Unit** / *Jedinica*	**Variant label** / *Oznaka varijante*		
		Z-3	Z-6	Z-9
l	mm	30.32 ± 3.21 *a*	30.38 ± 3.24 *a*	30.49 ± 3.35 *a*
b		12.84 ± 0.89 *a*	12.92 ± 1.02 *a*	13.05 ± 1.15 *a*
a		4.11 ± 0.29 *a*	4.14 ± 0.31 *a*	4.19 ± 0.37 *a*
MC	%	19.98 ± 0.99 *a*	20.00 ± 0.84 *a*	20.36 ± 0.59 *a*
Ψ	-	3.13 ± 0.19 a	3.14 ± 0.18 *a*	3.11 ± 0.31 *a*
λ		7.37 ± 0.57 *a*	7.35 ± 0.64 *a*	7.30 ± 0.76 *a*
m		2.36 ± 0.16 *a*	2.35 ± 0.21 *a*	2.34 ± 0.23 *a*
Bulk density	kg/m^3	193.67 ± 1.53 *a*	194.00 ± 1.73 *a*	193.33 ± 2.08 *a*

Mean value ± standard deviation; letters a, b mark homogeneous groups in HSD Tukey test / *srednja vrijednost ± standardna devijacija; slovima a, b označene su homogene skupine u HSD Tukey testu*

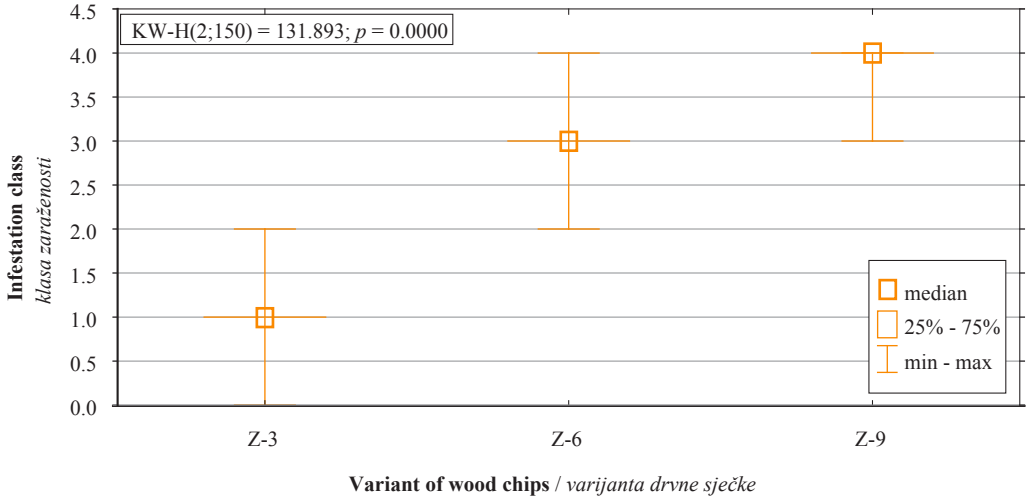

Figure 1 Infestation class of wood chips depending on incubation time
Slika 1. Klasa zaraženosti drvne sječke s obzirom na vrijeme inkubacije

and Bjurman, 1995). The effect of the incubation time on the infestation class tested in laboratory conditions may differ from the experiment carried out in a natural environment. In the case of wood stored in climatic chambers, constant conditions (RH, temperature) were ensured. On the other hand, in a natural environment characterized by variable weather conditions, which often differ significantly from the optimum, the so-called stress factors may occur. They result mainly from too low air RH or inadequate temperature (Zak and Wildman, 2004). Moreover, according to Johansson *et al*. (2013), the conditions of variable RH and ambient temperature during the incubation of wood slow down the growth of molds. This effect is particularly noticeable when the changes are rapid (Viitanen and Bjurman, 1995).

The results of ergosterol content determinations in inoculated wood chips differing in incubation times are presented in Figure 2. Homogeneous groups determined in the HSD Tukey test indicate that the content of ERG differed in a statistically significant way depending on the length of the incubation period and it increased over time. Between the third and sixth week of incubation, the ERG concentration increased by 14.50 mg/kg. Furthermore, in the case of the nine-week incubation, the ERG content increased by 57.33 mg/kg and 71.83 mg/kg when compared to the concentration determined in the sixth and third weeks, respectively. Therefore, the results of the analysis of ERG content correspond to the assessment of the infestation classes, which stays in agreement with the results presented by Pasanen *et al*. (1999). Thus, the results confirmed that, as the incubation time of wood chips is extended under favorable conditions, the area overgrown by molds is also extended.

The spectra of both sound and inoculated wood chips are presented in Figure 3. A wide peak corresponding to stretching vibrations of OH bonds of hydroxyl groups was observed in the range of 3700-3050 cm^{-1} (Ghahri *et al*., 2018). Moreover, in the range of

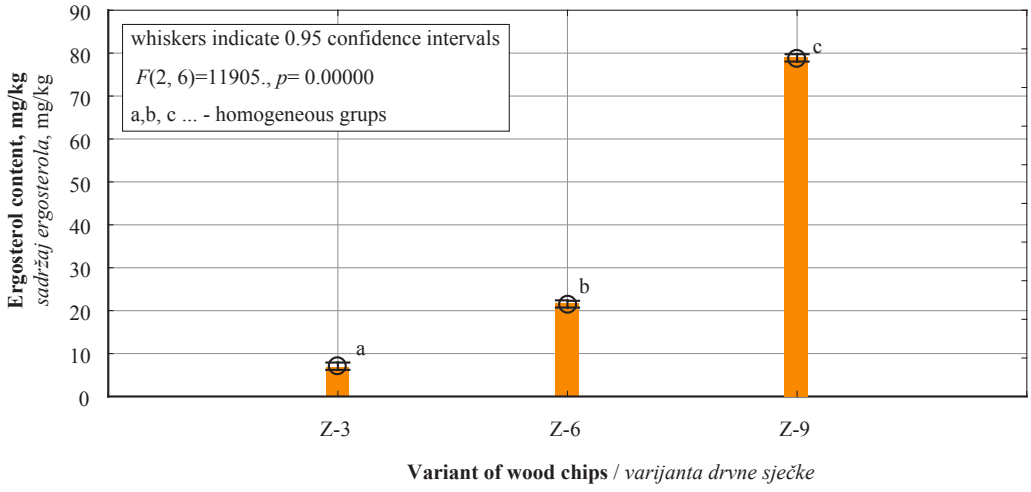

Figure 2 Ergosterol content in wood chips depending on incubation time
Slika 2. Sadržaj ergosterola u drvnoj sječki ovisno o vremenu inkubacije

2980-2830 cm^{-1}, stretching vibrations of C-H were identified and it was overlapped by the C-O stretching vibrations generated by fatty acids (Gupta *et al*., 2011). At about 1737 cm^{-1}, a peak from a vibration of C=O bond of hemicelluloses was recorded (Li *et al.*, 2011). In the fingerprint region (1800-700 cm^{-1}), several more band characteristics of wood were noted. The peak occurring at 1510 cm^{-1} corresponded to the vibrations of the aromatic ring of lignin. The peaks at 1372 cm^{-1} and 1161 cm^{-1} originated from deformation vibrations of C-H bonds and vibrations of C-O-C bonds, respectively. These bands were derived from the polysaccharides such as cellulose and hemicelluloses (Pandey and Pitman, 2003). At 1234 cm^{-1} there were also signals from carbon-oxygen stretching bonds occurring in lignin. Furthermore, the peak observed at 898 cm^{-1} was assigned to C-H deformation bond. The comparison between reference wood sample and those inoculated showed no significant changes in the course of FTIR spectra, regardless of the incubation period. This observation indicates that inoculation of wood chips with mold fungi did not have a significant effect on their chemical structure. Jelle and Hovde (2012) investigated the possibility of using attenuated total reflection infrared spectroscopy (ATR-FTIR) to detect mold fungi on the surface of various materials, including wood. Moreover, the authors found that the application of this method has a great application potential and that it may be helpful in determining the presence of mold on the surface of wood and gypsum boards. However, as they emphasized, this method requires further research consisting of determinations of possible limitations, reliability in the case of various species of both fungi and wood and possible directions of application.

The average values of the wood chips mass before inoculation and after the incubation process were compared. Based on the results of t-Student test, it was found that there were no statistically significant differences between the average mass before inoculation and average mass after incubation (p-value > 0.05). The incubation time had no effect on the obtained results. The percentages of mass loss are shown in Figure 4. Based on the presented average percentage values, it cannot be concluded that the growth of mold fungi caused a mass loss of wood chips. The ML of approx. 0.05 % most likely resulted from an error in the measurement method. Moreover, based on the Fisher test, it was found that there were no statistically significant differences between the variants (p-value > 0.05). Thus, the incubation time had no effect on the percentage mass loss. Mass loss in wood degraded by decaying fungi results from the decomposition of cell wall, which is caused by the decomposition of its structural components – carbohydrate substances such as cellulose or hemicelluloses and lignin. However, FTIR spectra did not indicate any significant changes in the content of these substances. Idler *et al.* (2019) confirmed that during the storage of fragmented wood, mold fungi are not a direct cause of mass loss due to the non-structural substances used for development. However, according to the authors, they may contribute indirectly to the activity of decaying microorganisms through a synergistic effect.

Figure 5 shows changes in moisture content during the exposure of homogenized wood to increased RH (90-95 %).

The course of changes in *MC* was very similar regardless of the wood chips variant. After 120 hours of exposure to high RH, wood reached *MC* of approx. 28 %, which is close to the fiber saturation point (FSP). According to Krzysik (1978), FSP for pine wood is 29 %. A very similar level of *MC* after 120 h was observed for homogenized, unmodified oak wood (Siuda 2019). In order to confirm that the time of incubation had no effect on the changes in *MC*, ANOVA was carried out. Based on the analysis of homogenous groups deter-

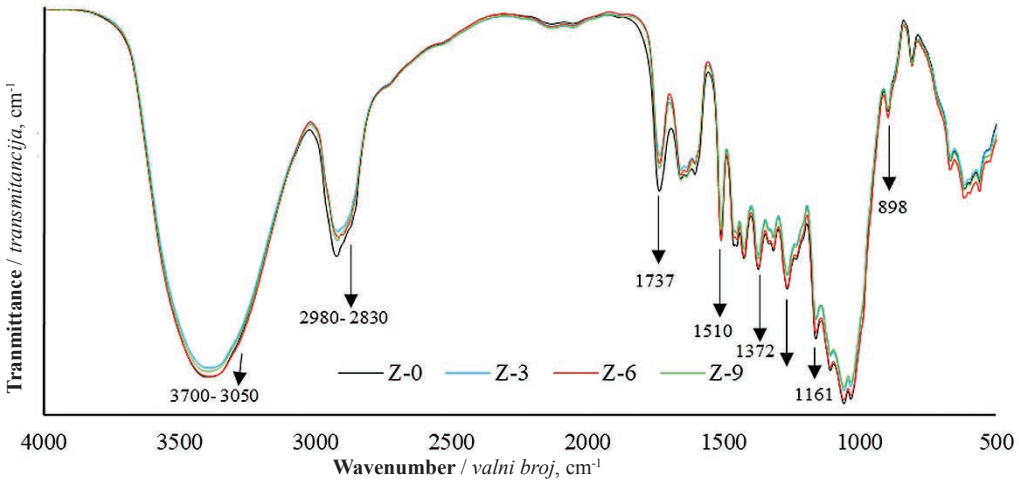

Figure 3 Course of FTIR spectra of wood chips depending on variant
Slika 3. FTIR spektri drvne sječke ovisno o njezinoj varijanti

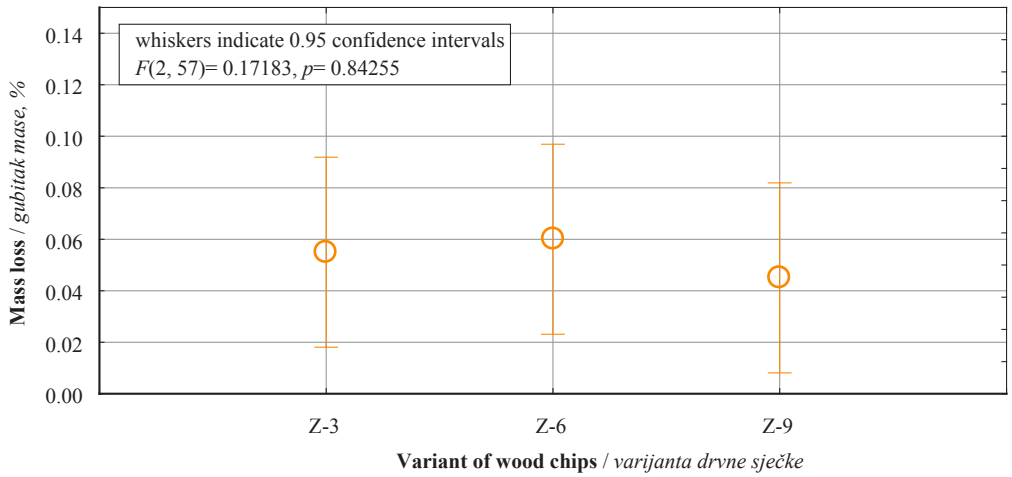

Figure 4 Percentage mass loss depending on the variant of wood chips
Slika 4. Postotak gubitka mase ovisno o varijanti drvne sječke

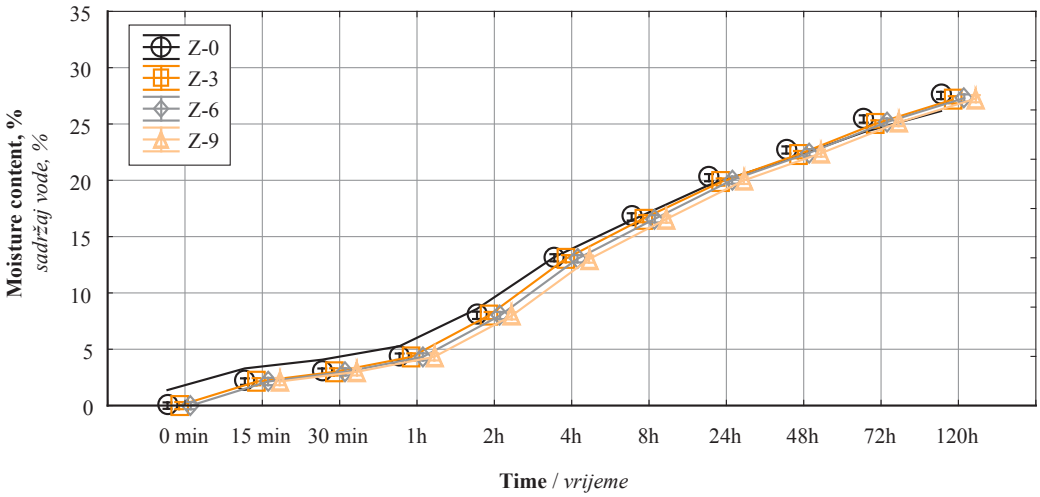

Figure 5 Changes in wood moisture content depending on wood chips variant
Slika 5. Promjene sadržaja vode u drvu ovisno o varijanti drvne sječke

mined in the HSD Tukey test, it was found that there were no statistically significant differences in *MC* between the variants for all measurement times. Thus, the incubation time and the development of molds had no effect on the hygroscopicity of wood. Therefore, the effect described by Rowell (2005) and Clausen (2010), who found that wood covered with mold fungi may be characterized by increased water absorption, was not observed. However, their development is not accompanied by deep penetration into the anatomical structures of wood. Taking into account that, in the case of advanced development of mold fungi, the hyphae may move through the pits degrading their membrane, the increase in absorbency may only occur in the surface layer of the infested material (Nilsson, 2009).

4 CONCLUSIONS
4. ZAKLJUČAK

The present research investigated the effect of wood chips inoculation with *Aspergillus* and *Penicillium* fungi on their selected properties. The results of visual assessment and ergosterol content have shown differentiation in infestation due to the length of the incubation period. The results of FTIR analysis, mass loss and hygroscopicity determinations show no changes caused by a surface infestation with molds. Therefore, pine wood chips, which are commonly overgrown by molds, especially during storage when they are freshly harvested, can be considered as a valuable material that can potentially find alternative applications, other than for energy purposes. In the future, research concerning the use of this type of material will be conducted in various ways.

Acknowledgements – Zahvala

This research was funded by the National Centre for Research and Development, BIOSTRATEG3/344303/14/NCBR/2018. The study was also supported by funding for statutory R&D activities as research task number 506.224.02.00 of the Faculty of Forestry and Wood Technology, Poznan University of Life Sciences. The research was performed within the framework of the doctoral thesis: "Possibility of using wood

chips exposed to mold growth in a chipboard manufacturing process", and the paper contains the same data.

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Corresponding address:

Dr. JAKUB KAWALERCZYK
Department of Mechanical Wood Technology, Faculty of Forestry and Wood Technology, Wojska Polskiego 38/42, 60-637 Poznan, POLAND, e-mail: kawalerczyk@up.poznan.pl