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Chemical composition and antioxidant activity of *Fomitopsis pinicola* growing on coniferous and deciduous substrates

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Abstract

Xylotrophic fungi are widespread in all types of forests. The composition of their primary and secondary metabolites including biologically active compounds depends on species and growth substrate; therefore, fungi can become a valuable raw material for biotechnology, pharmaceuticals, and food industry. *Fomitopsis pinicola* is one of the common species in Russia and Europe, growing predominantly on *Pinus sylvestris* L., but in mixed forests of the Urals it is also found on *Betula pendula* Roth. As metabolism of angiosperms and gymnosperms, and their timber properties are different, growth substrate could affect the chemical composition of fungi fruit bodies; therefore our study aimed at metabolite composition of extracts, obtained from *F. pinicola* collected from birch and pine, and their antioxidant activity. Qualitative analysis revealed alkaloids, phenolics, and anthraquinones. Saponins were found only in the samples obtained from pine. Thin layer chromatography of extracts revealed the same qualitative composition of phenolics, but their amount was higher on birch – 4.2 mg g^{-1} then on pine – 3.1 mg·g^{-1}. In ABTS test extracts showed the same antiradical activity. The metabolomics profile obtained by UHPLC-MS totally revealed 116 compounds, and each fungi sample contained more than 70 of them. Thus, the type of substrate influenced on the profile of metabolites and quantitative composition in *F. pinicola* fruit bodies.

Key words: *Betula pendula*, fungal methanol extracts, *Pinus sylvestris*, TLC, UHPLC-MS.

Introduction

Forests occupy 46.6 % of the Russian Federation territory, which is about 20 % of all forests on the planet. They are not only a source of wood, but also a powerful sequester of carbon, regulating the climate both in local and global scales. Forests provide us with medicinal and food plants, mushrooms, etc. Sverdlovsk Region is one of the ten regions in Russia in terms of area (68 %) covered by forests. The largest city in the region – Yekaterinburg – is located on the eastern slope of

the Ural Mountains, in the southern taiga subzone and is surrounded by mixed forests, represented by *Pinus sylvestris* L. with an admixture of *Betula pendula* Roth. (FAO 2012).

Common forest pests in the Urals are presented by parasitic and saprotrophic wood-destroying fungi. Some of them being parasites control the number of host trees, others being saprotrophic decay dead wood and return CO_2 to the atmosphere. One of the most typical species in the Urals is *Fomitopsis pinicola* (Sw.) P. Karst., which mainly inhabits pine, but also occurs on birch (Gründemanna et al. 2020). Large fruit bodies, wide distribution, and specific form and colour make it easily recognizable. This species damages timber, causing brown wood rot. At the same time it is used in traditional medicine in Siberia, China and Korea (Bishop 2020, Gründemanna et al. 2020).

In recent decades, there has been an increasing interest to new natural raw materials, including fungi that contain biologically active compounds for medical purposes. The search for biologically active substances (BAS) as bioprotectors and plant growth regulators for agriculture is also relevant (Ermoshin et al. 2021b). Large stocks and ease identification make *F. pinicola* promising for study as a raw material for the obtaining BAS for pharmaceuticals and biotechnology. *In vitro* experiments have shown that the chloroform extract of *F. pinicola* fruit bodies negatively affects the growth of some cancer cell lines (Gao et al. 2017). For alkaline extracts, the antidiabetic effect has been demonstrated in rats (Lee et al. 2008), as well as an immunomodulating, anti-inflammatory and antioxidant effects of its polysaccharides. It has been shown that fruit bodies contain ergosterol, sesquiterpenes, lanostane triterpenes and their glycosides (Bishop 2020, Janardhanan et al. 2020). Moreover, fungi fruit bodies are rich in phenolics which are known as the powerful reducing agents, and other biologically active substances – anthroquinones, carbohydrates, amino acids (Ermoshin et al. 2021a). However, this species has been studied to a much lesser extent than Reishi or Chaga used by the official medicine (Gründemanna et al. 2020, Janardhanan et al. 2020).

Most of the works do not indicate the type of growth substrate for studied species, though it is known that the composition of the fruit body is influenced by the growth substrate, and partially the fruit bodies are made of fragments of wood on which the fungus grows. Thus, the purpose of our work was to compare the chemical composition of ethanol and methanol extracts and the antioxidant activity of ethanol extracts from tinder fungus *F. pinicola* collected from the most typical growth substrates – pine and birch.

Materials and Methods

Biological material was collected in a mixed forest in the vicinity of the biological station of the Ural Federal University, Sverdlovsk region, Sysertsky district (56°36'4" N 61°3'25" E). Fruit bodies were collected by route method from all tree trunks on the transect. The number and the mass of the collected fruit bodies were assessed in the laboratory (Ermoshin et al. 2021a).

The chemical composition and *in vitro* antioxidant activity of fruit bodies were studied in ethanol (95%) extracts. Ethanol (3 mL) was added to a dry biomass of fruit bodies (150 mg). The mixture was treated with ultrasound for 15 min (570 W) at 50 °C. Then the mixture was centrifuged.

The supernatant was poured into a Falcon tube. Extraction was repeated 3 more times, supernatants were pooled together, and the extract volume was adjusted to 15 mL. The qualitative composition of the main groups of BAS was assessed using standard pharmacopeial tests (Zhu et al. 2017, Shaikh and Patil 2020, Ermoshin et al. 2021a). The amount of the total phenolics was determined spectrophotometrically in the reaction with Folin-Checolteu reagent and expressed in terms of gallic acid (Sulkowska-Ziaja et al. 2012). The antioxidant activity *in vitro* was determined in three tests and expressed as the total reduction potential, as the antiradical activity in ABTS-test, and antioxidant activity in membrane lipid peroxidation model (Ermoshin et al. 2021a, b).

Ethanol extracts were also studied for the qualitative characteristics of the birch and pine bark metabolites by thin layer chromatography (TLC) in a solvent system toluene: ethyl acetate: formic acid (30:18:2), and phenolic compounds were visualised with ferric chloride, flavonoids with aluminum chloride and terpenes with phosphoric-tungstic acid (Bertrams et al. 2013, Hamad et al. 2019, Blondeau et al. 2020, Ermoshin et al. 2021a).

The identification of individual phenolics and terpenoid compounds was carried out in extracts prepared from 50 mg of dry material in 1 mL of methanol. The mixture was treated with ultrasound at 40 °C for 30 min, and then extracts were separated by centrifugation, purified by passing through a reverse-phase column (C-18) and a microfilter with pore size of 0.02 μm. The identification of compounds was done using UHPLC-DAD-MS method. UHPLC-DAD was performed with an Agilent 1290 UHPLC coupled with a 6430 triple quadrupole mass spectrometer (Agilent Technologies, Singapore). Separations were performed on an Agilent ZORBAX RRHD Eclipse Plus C18 column (50×2.1 mm, i.d. 1.8 μm). UHPLC mobile phase was composed of 0.1 % formic acid as A and methanol as B. The A:B ratio was varied to the following program: 0 min, 85:15; 2 min, 73:27; 4 min, 65:35; 7 min, 50:50; 10 min, 40:60, 12 min, 25:75, 17 min, 0:100. The follow rate was 0.3 $mL \cdot min^{-1}$, the injection volume was 2 μL, and the column temperature was 30 ºC. The list of standards was compiled based on the literature data (Kim et al. 2008, Gao et al. 2013, Ermoshin et al. 2022).

Statistical data processing was done using Statistica 8 (StatSoft). The significance of differences in the abundance of fruit bodies on different substrates was determined by Fisher's criterion. The assessment of chemical composition similarity was carried out according to Jaccard coefficient. Differences in quantitative indicators are shown by the nonparametric U-Mann-Whitney test. Analyzes were carried out in 3–4 analytical and 2–3 biological replicates.

Results and Discussion

The studied species was found mainly on pine. However, the species is also known to inhabit deciduous species, including birch, but with a significantly lower abundance (Bishop 2020, Gründemanna et al. 2020). The biomass and the number of collected fruit bodies are presented in Table 1. Most of the basidiocarps were collected from pine trunks – 4 times more often than on birch. By mass 90 % of all fruit bodies were collected from pine. Basidiocarps on birch were twice smaller in mass than on pine.

Table 1. Frequency and total weight of basidocarps on different substrates.

Species	Number of trees and percent of trees	Total dry mass of the collected fruit bodies, g/%	Average dry mass of fruit bodies per tree, g
Pine	19 (79.2 %)	665/89.9	35
Birch	5 (20.8 %)*	75/10.1*	15
Total	24 (100 %)	740/100	

Note: * – the difference is significant at $p < 0.05$ (Fisher's test).

The bark of trees is the outer layer of the trunks, and its chemical composition directly depends on the composition of the wood. Despite the difference in composition, at least a quarter of the wood components are found in the bark (Ghavidel et al. 2021), therefore, we have studied the chemical composition of the bark.

The extracts from pine and birch bark contained the main groups of BAS (Table 2) as phenolics, flavonoids, triterpenes in both species; tannins and alkaloids were not detected, which corresponds to literature data (Bertrams et al. 2013, Hamad et al. 2019). Anthraquinones were found in the pine bark and saponins were presented only in the birch bark.

A quantitative analysis was performed for phenolics and flavonoids. They are known as important BAS with multiple health effects and activities (Selamoglu 2017). There was no significant difference in the content of phenolic compounds between species; however, the content of flavonoids in the pine bark was more than 5.5 times higher than in the birch bark.

Table 2. The main groups of BAS in the bark of birch and pine.

Sample (extract)	Phenolics, $mg \cdot g^{-1}$	Flavonoids, $mg \cdot g^{-1}$	Tannins	Anthraquinones	Alkaloids	Terpenes	Saponins
Pine bark	32.3 ±1.0	13.2 ±0.8	-	+	-	+	-
Birch bark	30.6 ±0.6	2.3 ±0.3**	-	-	-	+	+
Basidiocarps from pine	3.1 ±0.1	n.d.	-	+	+	+	+
Basidiocarps from birch	4.2 ±0.2*	n.d.	-	+	+	+	-

Note: (+) – component detected; (-) – qualitative reactions are negative; n.d. – not determined, below the limit of detection, in a quantitative method; * – differences between pine and birch are significant at $p < 0.05$ (U-criterion); ** – differences between pine and birch are significant at $p < 0.01$ (U-criterion).

The analysis of individual compounds was carried out by the method of TLC (Table 3). In birch bark 8 spots were found (presumably 5 terpenes, 2 phenols and 1 flavonoid), and 10 spots – in pine (presumably 2 terpenes, 2 phenols, 2 flavonoids and 4 four spots were not identified to class of chemicals). Tree spots were common to both substrates. Two of them were terpenes, and one corresponded to the quercetin standard. Salicylic acid was found in the birch bark, according to the standard. The index of similarity for the chemical composition of substrates was 0.2 according to Jaccard criterion. Thus, pine as a substrate had a greater variety of metabolites than birch. Not only were a greater number of individual compounds observed in the pine bark, but also a higher content of phenolics and flavonoids.

The results obtained do not contradict the literature data (Hamad et al. 2019, Ramadhani et al. 2021). We supposed that *F. pinicola* basidiocarps harvested from pine would also have a wider variety of metabolites.

Table 3. TLC analyses of pine and birch bark.

No	Spot colour				Rf	Distribution coefficient relative to			Sample		Group of compounds
	Without chemical processing	With $AlCl_3$	With phosphoric-tungstic acid	With $FeCl_3$		Quercetin	Gallic acid	Salicylic acid	Birch bark	Pine bark	
1	-	-	grey	-	0.06	0.14	0.22			+	n.d.
2	brown	brown	-	-	0.07	0.19	0.33	0.12		+	n.d.
3	-	yellow	-	grey	0.12	0.28	0.46	0.18		+	flavonoid
4	rose	brown	rose	-	0.14	0.34	0.57	0.22	+		terpene
5	-	-	-	grey	0.19	0.42	0.68	0.29	+		phenolic
6	grey	grey	-	grey	0.26	0.64	1.09	0.43		+	phenolic
7	violet	grey	-	violet	0.35	0.83	1.42	0.55		+	phenolic
8	yellow	green	yellow	grey	0.45	1.00	1.69	0.67	+	+	quercetin
9	-	-	violet	-	0.54	1.20	1.83		+	+	terpene
10	grey	grey	-	blue	0.63	1.52	2.60	1.00	+		salicylic acid
11	-	-	violet	-	0.71	1.57	2.39		+		terpene
12	-	-	violet	-	0.77	1.71	2.61		+	+	terpene
13	-	-	violet	-	0.81	1.80	2.74		+		terpene
13	-	-	grey	-	0.88	1.97	3.00			+	n.d.
15	-	-	grey	-	0.95	2.11	3.22			+	n.d.

Note: Rf – retention factor, n.d. – not determined, below the limit of detection in a quantitative method.

Comparison of basidiocarp extracts showed that their chemical composition differed from the bark composition. For example, alkaloids were found only in the fruit bodies. Anthraquinones were detected in basidiocarps growing on both substrates, while in the bark they were found only in pine. The presence of saponins depended on the growth substrate. The amount of phenolics in the fruit bodies was significantly less than in the growth substrate, and flavonoids were found in trace amounts. Even though pine bark was richer in phenolics than birch bark, fruit bodies collected from pine contained 25 % less phenolics than basidiocarps from birch.

Our results on the antioxidants such as phenolic compounds and anthraquinones, found in *F. pinicola* basidiocarps, correspond to literature data. (Yen et al. 2000, Sulkowska-Ziaja et al. 2012). We compared *in vitro* antioxidant activity of extracts from basidiocarps growing at the same territory and having the same age but differing in the growth substrate (Table 4).

Table 4. *In vitro* antioxidant activity of extracts.

Substrate	ABTS-test, %	Total reduction potential, %	Inhibition of lipid peroxidation, %
Pine	92.9 ±0,1	553 ±12	44.9 ±11,7
Birch	92.8 ±0,2	795 ±24*	32.3 ±19.0*

Note: * – differences between pine and birch are significant at $p < 0.05$ (U-criterion).

Basidiocarps growing on birch had a higher level of phenolics and reduction potential than those growing on pine. In the model of membrane lipid peroxidation the opposite pattern was observed – the extract from fruit bodies growing on birch inhibited the formation of malondialdehyde by a quarter weaker than the extract of fruit bodies from pine. Probably, the mechanism of LPO inhibition was not directly related to the antioxidant effect of phenolics since they are hydrophilic molecules and do not dissolve in the lipid phase of membranes. Regardless of the growing substrate, the fungi extracts had the same high antiradical activity in the ABTS test. Thus, *F. pinicola* extracts from both substrates had a high antioxidant activity, which corresponds to literature data (Sulkowska-Ziaja et al. 2012, Nowacka et al. 2015, Onar et al. 2016, Sevindik et.al. 2017) and makes it interesting for biotechnology, pharmaceutical and nutraceutical usage as the possible sources of antioxidants.

TLC revealed only three metabolites in basidiocarp extracts, which could not be reliably determined to class. The method turned out to be uninformative for distinguishing the composition of the fruit bodies extracts for one species of fungi growing on different substrates, therefore, to compare the composition of the fruit bodies, HPLC was done in combination with mass spectrometry. We focused on the search for the most common phenolic compounds with pronounced antioxidant activity and biological effects, such as anti-cancer, antiviral, antibacterial, hypoglycemic, and others (Sulkowska-Ziaja et al. 2012, 2018). Except phenolic compounds, we have paid attention to terpenoids known for their anticancer activity (Gao et al. 2013, Cör et al. 2018, Dasgupta and Acharya 2019). The results are presented in Table 5.

Table 5. Content of individual phenols and terpenes in fruit bodies.

Compound	Precursor ion / Production, $m \cdot z^{-1}$	Substrate	
		Pine, $\mu g \cdot g^{-1}$	Birch, $\mu g \cdot g^{-1}$
	Phenolics		
Gallic acid	169/125	8.04±0.40	1.32±0.05*
Chlorogenic acid	353/191.1	0.02±0.00	0.03±0.00
Caffeic acid	179/135	0.81±0.04	1.21±0.05
P-coumaric acid	163/119	0.33±0.00	0.33±0.02
Cinnamic acid	147/103.6	n.d.	3.61±0.20*
Protocatechuic acid	153/109	42.67±2.10	56.87±2.80
Catechin	289/109	n.d.	2.65±0.10*
Epicatechin	289/245	0.05±0.00	n.d.
Rutin	610/300	0.21±0.01	0.44±0.02*
Luteolin	285/133	0.21±0.01	0.36±0.02

Compound	Precursor ion / Production, m·z^{-1}	Substrate	
		Pine, μg·g^{-1}	Birch, μg·g^{-1}
Kaempferol	285/133	0.56±0.03	0.64±0.03
Terpenes			
20-OH lucidenic acid A	455/149	n.d.	0.23±0.01
20-OH lucidenic acid N	457/none	0.53 ±0.03	0.10±0.00*
Ganodermanotriol	473/437.3	n.d.	36.76±1.50*
Ganoderiol A	475/457.3	8.60±0.4	15.16±0.70*
Ganoderiol D	489/471.2	33.97±1.40	6.25±0.37*
Ganoderiol F	477/none	1.15±0.05	0.89±0.04
Lucidumol A	473/259.1	0.47±0.02	1.01±0.05
Ergosterol peroxide	429/none	263.49±13.00	167.68±8.30*

Note: * – differences between pine and birch are significant at $p < 0.05$ (U-criterion).

Protocatechuic acid was the predominant phenolics, which is known as a powerful antioxidant. Hydroxybenzoic acid which is often presented in fungi (Kim et al. 2008) was not found in the studied species. The sum of identified phenolic compounds was only 1.5–2 % of the amount of phenolics determined spectrophotometrically. Flavonoids were not detected by qualitative reactions, and spectrophotometrically their content was determined in trace amounts, thus, flavonoids did not play a significant role in the antioxidant activity of *F. pinicola* fruit bodies extracts. However, they were determined by the method of UPHLC-MS. A lot of the compounds in studied extracts were not identified, but most likely they belonged to polyphenols. In terms of the concentration and diversity of identified compounds, birch samples were richer in BAS. This makes the study of basidiocarps collected from birch rather interesting compared to pine.

UHPLC revealed small concentration of terpenes, characteristic to *Ganoderma* (Reishi). They were also found in trace amounts in other fungal species, that are not related to *Ganoderma* genus (Gao et al. 2013). These compounds are known to perform an anticancer activity, and even in small concentration they can inhibit tumor cells (Gao et al. 2013). Ergosterol peroxide which is known as pronounced anticancer compound (Dasgupta and Acharya 2019) dominated among individual terpenes and phenols. Its concentration (0.16–0.26 mg·g^{-1}) was higher in extracts from fruit bodies grown on pines then on birches.

A detailed study of chromatograms (Fig. 1) showed at least 74 compounds (molecular weight individual peaks) in fruit bodies from birch, and 72 in basidiocarps from pine. Thirty of them were common for both fungi extracts (they have the same retention time and ion mass). Jaccard similarity coefficient was 0.26, which was close to the value of the coefficient for chemical composition of bark from TLC analysis.

The search for new antioxidants is relevant and promising, since oxidative stress is a common response to the pathogenesis of many diseases. Reactive oxygen species disrupt the structure of proteins; increase the frequency of mutations, which lead to the tumor risks, the heart diseases, and so on. Therefore, the

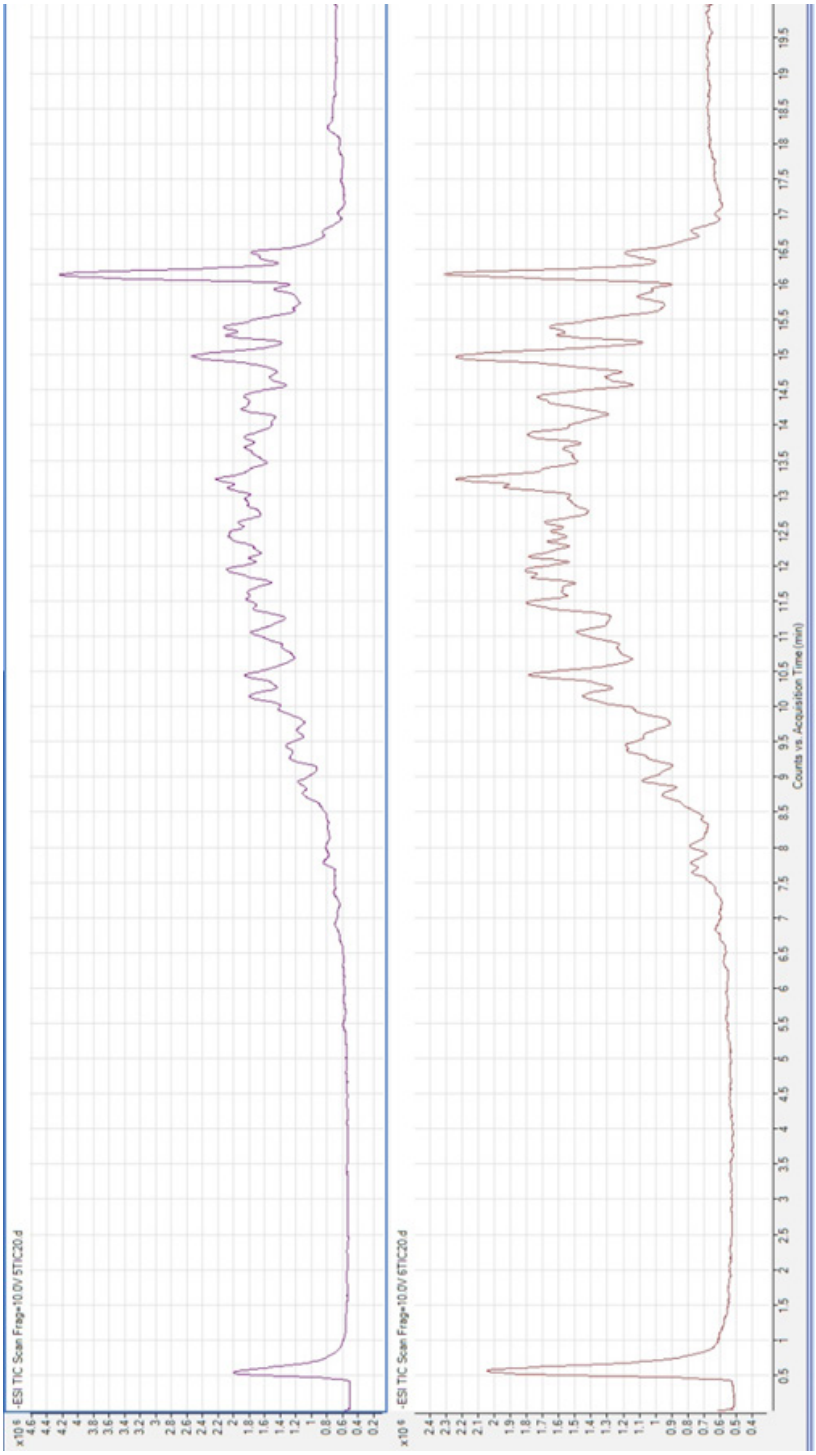

Fig. 1. HPLC-MS analyses: total ionic current, positive ionization (+ TIC).
Note: above – extract from basidiocarps growing on birch, below – on a pine tree.

search for new natural antioxidants is an important task. At the same time, it matters to know from which substrate the fruit bodies of fungi should be collected. In our study raw materials collected from birches had a higher content of phenolic compounds and greater antioxidant activity.

Conclusion

The chemical composition of extracts from *F. pinicola* fruit bodies collected on the same territory at the same time, growing on pine or birch revealed the same groups of BAS, except for saponins, which were found only in basidiocarps from pine. The analyses of the metabolic profiles by finger-printing method revealed the influence of the substrate on the chemical composition of fungi extracts – more than 70 compounds were found in each sample, and only 30 were common. The total amount of phenolics and antioxidant activity were higher in the fruit bodies, collected from birch. Protocatechuic acid, a powerful antioxidant, prevailed among phenolics in both fungi extracts. Both samples contained terpenes, characteristic for *Ganoderma* (Reishi), but in much smaller quantities. Ganoderiol D predominated among others. High concentration of anti-cancer compound ergosterol peroxide found in both fungi extracts.

Thus, the growing substrate affects the qualitative and quantitative composition of *F. pinicola* fruit bodies. The type of fungal growth substrate can change the profile of the metabolites in them. The data obtained gives the prospects for further study and use of *F. pinicola* in pharmaceuticals. This species is typical for pines, but could be also find on birch trunks in mixed forests in much smaller quantities. More detailed study of *F. pinicola* fruit bodies, growing on a birch is necessary.

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Influence of essential oil of *Thymus pulegioides* L. harvested in forest area on *Myzus persicae* S. in tobacco and *Apis mellifera* L.

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Abstract

In laboratory conditions, the effect of Essential oil (EO) vapours from *Thymus pulegioides* L. against *Myzus persicae* S. on tobacco and *Apis mellifera* L. bees were tested. Aphids' mortality increased when concentration increased. A low mortality of *M. persicae* of 9.7 % was reported at 0.5 μL·L^{-1} air, at 1 μL·L^{-1} air mortality was 31.6 %, and at 2 and 3 μL·L^{-1} air mortality was 100 %. Concentration of 2 μL·L^{-1} air was found to be the minimum which has maximum efficacy of 100 %. Differences between efficacy of various concentrations were found to be statistically significant. EO vapours tested at concentration of 2 μL·L^{-1} air applied under the same conditions to *A. mellifera* showed 100 % toxicity to the bees as well. No mortality was reported in the control.

Key words: green peach aphid, honey bees, mountain thyme.

Introduction

The control of economically important agricultural pests with biological substances is gaining popularity. Green peach aphid *Myzus persicae* S. is such an economic pest, rapidly multiplying and attacking huge variety of plants (Blackman and Eastop 2000). Green peach aphid has been found to exhibit resistance to neonicotinoid insecticides (Devine et al. 1996), moreover the application of some is highly restricted in Europe (Cressey 2017). Insecticides contain substances dangerous to human health (Meena et al. 2018). EO as biopesticides are one of the alternatives of chemical insecticides (Pavela et al. 2010) and can be applied against *M. persicae* as shown by previous research (Radev 2022). EO are extracted from a number of plants including mountain thyme *Thymus pulegioides* L. The species is distributed in forest clearings and pastures. Thyme EO is characterised by antimicrobial (Elfellah et al. 1984) and antioxidant (Youdim et al. 2002) activity.

The object of present study is to test the insecticidal effect of *T. pulegioides* essential oil on *Myzus persicae* S. and *Apis mellifera* L. (non-target organism).

Materials and Methods

To extract EO from *T. pulegioides*, stems and flowers were collected from forest pastures in the Rhodopes mountain during flowering phase, June 2021. The amount of 1.5 kg was distilled for 3 h in a micro-distiller. The EO was stored in a glass container.

Insecticidal efficacy of *T. pulegioides* EO vapours were tested in laboratory conditions against *M. persicae* on tobacco using a similar to Digilio et al. (2008) method. Tobacco planted plants in pots were infested with aphids. After 5 days, plants with a known number of aphids were placed in 10 L air-tight cylinders. Concentrations of 0.5, 1, 2 and 3 $\mu L \cdot L^{-1}$ air and one control were tested in three replicates for each concentration. The oil was pipetted on filter paper and placed at the bottom of the cylinder. After 24 h, the efficiency was calculated according to Abbott (1925). Aphids that did not react when touched were considered as dead.

The minimum concentration that had highest efficacy was tested on 3 days old *A. mellifera* bees. Bees were newly emerged, caged and fed on 50 % sugar syrup. Three replicates and a control were used for the experiment; bees were treated with EO for 24 h under the same conditions described for aphids. Each replicate contained twenty bees.

The results were statistically processed by using one-way Anova in Excel.

Results and Discussion

The test results showed that *T. pulegioides* EO vapours have insecticidal effect against *M. persicae*. Aphids' mortality increased when concentration increased. A low mortality of aphids of 9.7 % found at 0.5 $\mu L \cdot L^{-1}$ air, at 1 $\mu L \cdot L^{-1}$ air mortality was 31.6 %, and at 2 and 3 $\mu L \cdot L^{-1}$ air it was 100 %. Concentration of 2 $\mu L \cdot L^{-1}$ air was found to be the minimum concentration which has maximum efficacy. A significant statistical differences were found between the efficacy of the various concentrations ($F > F_{crit}$, Anova: Single factor, p = 0.000) (Fig. 1). Mortality results for

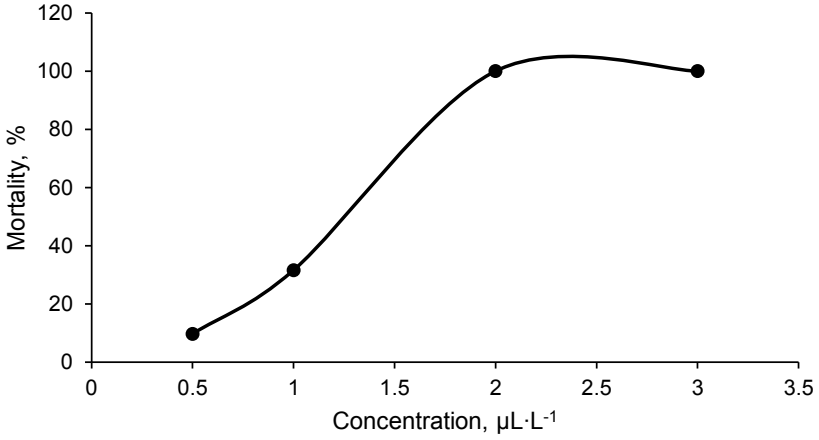

Fig. 1. Insecticidal efficiency of *T. pulegioides* EO against *M. persicae* at different applied doses.

the concentrations 0.5 μL·L^{-1} air and 1 μL·L^{-1} air show higher efficacy compared to those of Digilio et al. (2008). Khaled et al. (2017) showed toxic effect of *Thymus capitatus* L. EO by fumigation as well as by spraying against *M. persicae*.

The concentration of 2 μL·L^{-1} air was tested for toxicity on *A. mellifera.* The data shows very high toxicity of 100 % (means ±SD 100 ±0.0) of *T. pulegioides* EO vapours on treated bees while no mortality was recorded in the control. High statistical difference was found between the tested and control groups ($F > F_{crit}$, Anova: Single factor, $p = 0.000$).

Conclusions

EO vapours of *T. pulegioides* showed an insecticidal effect against *M. persicae* on tobacco and *A. mellifera*. The study information can be used in the production of tobacco seedlings, and in bees for pest control. More in-depth studies are needed in this field of study.

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Impact of essential oil from *Satureja kitaibelii* Wierzb. ex Heuff. harvested from mountain area on *Myzus persicae* Sulzer in tobacco and *Apis mellifera* L.

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Abstract

Essential oil (EO) vapours from *Satureja kitaibelii* Wierzb. ex Heuff. were tested in laboratory conditions against *Myzus persicae* Sulzer in tobacco and *Apis melifera* L. Efficacy of 100 % against *M. persicae* at 3 μL·L^{-1} air was established. Low efficacy of 17.6 % was found at 0.5 μL·L^{-1} air, increasing to 71.3 % at concentration of 1 μL·L^{-1} air, and reaching 95.3 % at 2 μL·L^{-1} air. The concentration of 3 μL·L^{-1} air applied under the same conditions to worker bees of *A. mellifera* rendered toxicity of almost one hundred per cent – 99.5 %, correspondingly. In the control group of bees, no mortality was established. The lifespan of bees depends on multiple factors and further studies are needed, also in field conditions.

Key words: green peach aphid, honey bees, mountain savory.

Introduction

The application of biopesticides in agriculture is a relevant issue today. *Myzus persicae* Sulzer is an important crop pest which reproduces fast and forms large colonies; it's widely distributed and causes significant economic damages (Bass et al. 2014). The aphid is known to develop resistance to insecticides (Contreras et al. 2013). The use of pesticides creates environmental risk of pollution and has unfavourable effects on non-target organisms (Tilman et al. 2011). Essential oils (EO) are an alternative, but more studies are needed for their further application (Radev 2022). EO could be obtained from various plants and such a plant is *Satureja kitaibelii* Wierzb. Ex Heuff. *Satureja* is also called mountain savory (Lawless 2002), naturally distributed in mountain areas (Centeno 2003). *Satureja* sp. EO has fungicidal (Soares et al. 2016), insecticidal (Tepe 2015) and nematicidal (Faria et al. 2015) activity.

The aim of this research is to study the potential insecticidal effect of *S. kitaibelii* EO, on *M. persicae*, and *A. mellifera* L. as a non-target organism.

Materials and Methods

The plant material of *S. kitaibelii* for obtaining EO was collected in flowering stage from a mountain area of the Eastern Rhodopes in July 2021. The collected sample of 1.5 kg was distilled for 3 h in a micro EO distillery with volume of 8 L. EO was stored in glass container.

The insecticidal efficacy of EO vapours of *S. kitaibelii* against *M. persicae* in tobacco was tested in laboratory conditions using a method similar to Digilio et al. (2008). Potted tobacco plants were infested with aphids. After 5 days the plants with counted aphids were placed in airtight cylinders with volume 10 L. Concentrations of 0.5, 1, 2 and 3 $\mu L \cdot L^{-1}$ air and one control without EO were tested with three repetitions for each variant. EO was placed onto filter paper on the cylinder floor. The mortality rate was taken after 24 h. The aphids which did not react to push were counted dead. EO efficacy was according to Abbott (1925).

The concentration with the highest efficacy was tested on 3 days old *A. mellifera* bees. In each repetition and control group 20 bees were placed for 24 h under the same conditions. The bees were newly emerged, separated in a cage and fed on 50 % sugar syrup.

The results were statistically processed by using one-way Anova in Excel.

Results and Discussion

According to the laboratory tests EO from *S. kitaibelii* exhibits insecticidal activity against *M. persicae*. When the concentration is increased aphid mortality also increases. According to Abbott (1925) the curve shows 100 % mortality at dose of 3 $\mu L \cdot L^{-1}$ air. Low efficacy of 17.7 % was reported at 0.5 $\mu L \cdot L^{-1}$ air, increasing to 71.3 % at 1 $\mu L \cdot L^{-1}$ air dose, and at 2 $\mu L \cdot L^{-1}$ air reaching efficacy of 95.3 % ($F > F_{crit}$, $p = 0.000$, $p \leq 0.05$) (Fig. 1).

The concentration of 3 $\mu L \cdot L^{-1}$ air exhib-

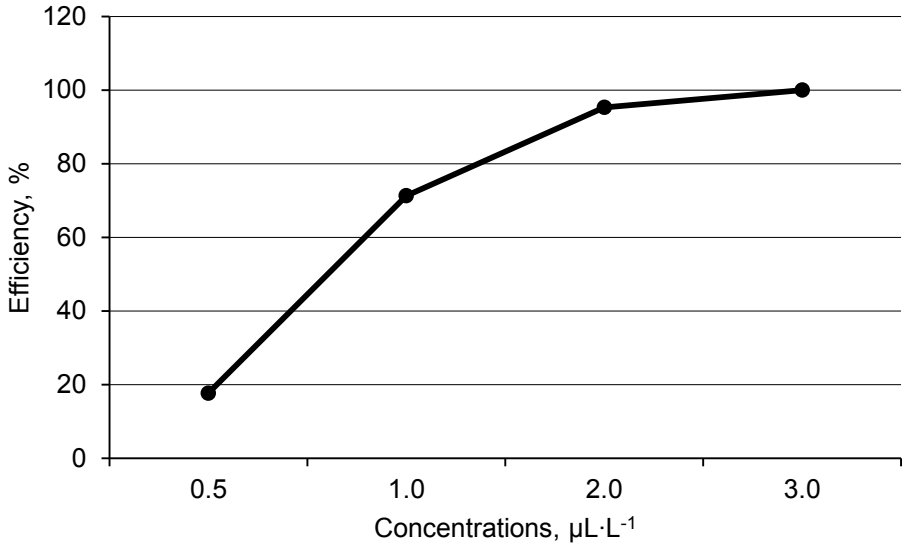

Fig. 1. Insecticidal efficacy of *S. kitaibelii* EO against *M. persicaea* in different doses.

iting the highest efficacy was tested for toxicity to *A. mellifera* in order to establish its effect on bees. The results show toxicity of EO vapours of *S. kitaibelii* to bees as well (means ±SD 99.5 ±0.87), while no mortality was reported for the control group of bees ($F > F_{crit}$, $p = 0.000$, $p \leq 0.05$). According to Regnault-Roger (1997), the way in which EOs are applied matters. The lifespan of bees depends on multiple factors and further studies are needed.

Conclusions

It has been established that EO vapours of *S. kitaibelii* exhibit insecticidal effect *against M. persicae* in tobacco and *A. mellifera*. The results can find practical application in the production of tobacco seedlings, and in bees for pest control. Field studies are required.

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Vegetation characteristic and carbon stock variation of mangrove forests in Sambas Regency, West Kalimantan province, Indonesia

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Abstract

Mangrove area is a transitional area with abundant natural resources, providing environmental services for coastal communities in regulating water systems, preventing seawater intrusion, as well as providing oxygen and carbon stock (C-stock). Comprehensive data of mangrove forests in Sambas Regency, West Kalimantan province is very limited. This study aims to monitor the condition of mangrove forests and estimate their potential carbon stocks in Sambas Regency. There are six dominant mangrove species in Sambas Regency, namely *Avicennia alba*, *A. marina*, *Bruguiera cylindrica*, *B. gymnorrhiza*, *Lumnitzera littorea*, and *Rhizophora apiculata*. Vegetation density varies from sparse to dense, ranging from 800–4700 trees per ha with a canopy cover of 55.00–86.63 %. The range of mangrove species diameter values is from 4.39 ±0.69 – 27.45 ±21.14 cm. Potential C-stock ranged from 7.17 ±0.32 – 546.67 ±70.83 Mg C·ha^{-1} with an average of 130.62 ±166.34 Mg C·ha^{-1}. The highest potential of mangrove C-stock was found at the Sebubus location. Mangrove vegetation in this study has a high potential of carbon stock, the potential value close to Kubu Raya Regency, West Kalimantan province, and higher than several mangrove ecosystems in Indonesia. In general, the condition of mangrove vegetation in this area is still good and has great potential for carbon stock. However, protection and sustainable use are needed in conserving the mangrove ecosystem in this area.

Key words: canopy cover, mangrove density, mangrove ecosystems, structure and composition.

Introduction

Mangrove forest is a unique ecosystem as a constituent of coastal ecosystems in tropical and subtropical regions (Giri et al. 2010), and has the ability to adapt to high salinity and tidal conditions of seawater (Kandasamy and Bingham 2001). The role of mangrove ecosystems is important in coastal areas, roots that stick to the ground as a support for plant defense can function as fish habitat. In addition, due to its physical characteristics, mangroves are able to act as a barrier to seawater intrusion, prevent coastal erosion and abrasion, breaking waves, and protect the

mainland from sea storms if the mangrove conditions are still well maintained (Saifullah 1985).

Indonesia is an archipelagic country with the second-longest coastline in the world, located in the tropics area, and has a high potential for mangrove forests. According to data from the IMEF (2021), the area of mangroves in Indonesia reaches 3.12 million ha, with 92 % of them in good condition (dense canopy). Furthermore, data on the area of mangroves in West Kalimantan province is estimated at 161,967 ha or the eighth widest of 34 provinces in Indonesia.

Coastal ecosystems consisting of mangrove forests, tidal salt marshes, and seagrass have a high potential for the carbon stored in living biomass (above and below ground), non-living biomass and soil, known as blue carbon (Howard et al. 2014). According to Murdiyarso et al. (2015) and Donato et al. (2011), mangrove forests can store 3–5 times higher carbon than lowland tropical forests. Based on the estimation of Alongi et al. (2015), mangrove and seagrass areas in Indonesia have carbon potential as much as 3.4 Pg or contribute about 17 % of the total blue carbon potential in the world.

The coastal area of West Kalimantan province according to the Zoning Plan for Coastal Areas and Small Islands of 2014–2034 (West Kalimantan Provincial Regulation Number 7 of 2014) consists of two regions, namely the northern coastal area (Pontianak Regency, Bengkayang Regency, Singkawang City, and Sambas Regency) and southern coastal areas (Kubu Raya Regency, North Kayong Regency, and Ketapang Regency). Carbon stock estimation in West Kalimantan has been widely carried out in Kubu Raya Regency, but not with 6 other regencies, including Sambas Regency. This study aims to estimate the potential of carbon stocks in the northern coastal area of West Kalimantan province, especially Sambas Regency, based on several characteristics of the mangrove vegetation found. The results of this study are expected to be authentic information regarding the characteristics and potential of mangrove vegetation carbon stocks in Sambas Regency.

Material and Methods

Study area

The study area was determined by detecting the presence of mangroves using Landsat 8 satellite imagery (data acquisition in January 2019 – September 2021 to minimize cloud cover), with a false-colour RGB (NIR-SWIR-Red) band composite. Areas that appear red-brown in coastal areas are detected as mangrove areas (Fig. 1). Surveys and research data collection was carried out from September to October 2021. A total of 4 out of 6 districts on the coast of Sambas Regency were selected as study areas, with a total of 10 observation plots (Selakau District: 1 plot, Pemangkat District: 1 plot, Jawai District: 2 plots, and Paloh District: 6 plots).

Analysis of the structure and composition of mangrove forests was carried out by making an observation plot of 10×10 m^2 perpendicular to the coastline representing the stratification of mangrove forests based on guidelines from the IIS (2020). Measurements in each plot were carried out on each trees with stem diameter (DBH) 4 cm, species identification was carried out based on the reference of Rusila Noor et al. (2012) and Giesen et al. (2006). The density and basal area of mangrove vegetation were calculated in each research plot and then

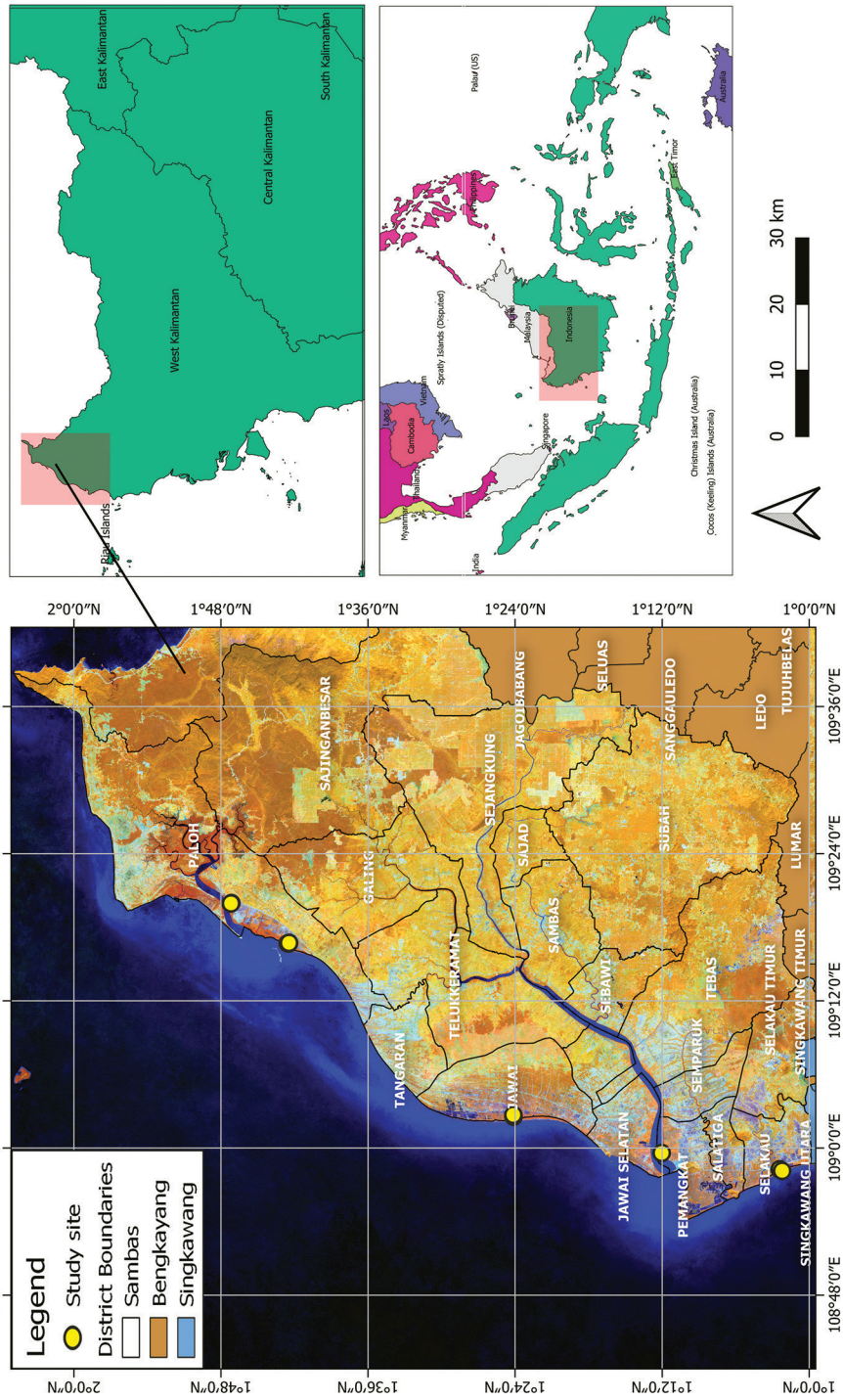
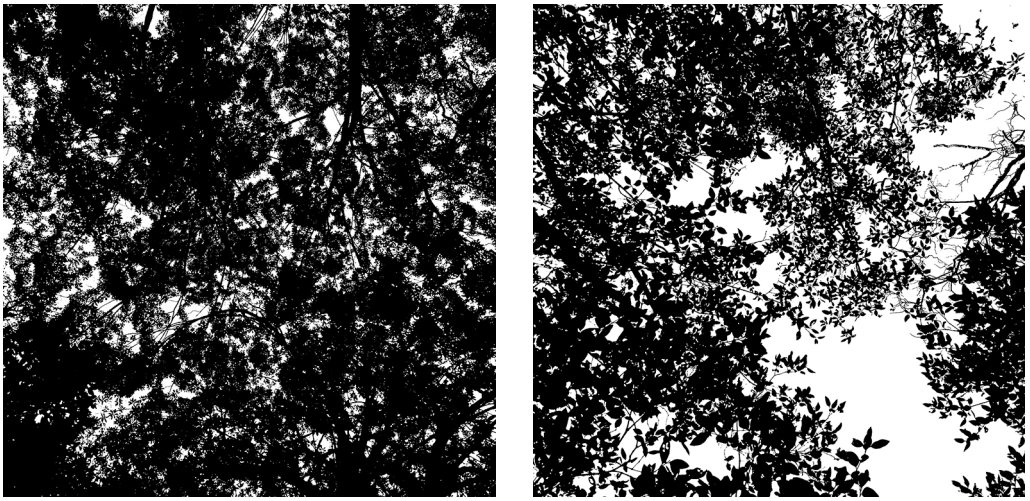


Fig. 1. Map of study area showing mangrove vegetation distribution (Landsat 8 RGB NIR-SWIR-Red Band Combination).

converted per reference unit. Vegetation canopy density (Fig. 2) was measured using the hemispherical photography method based on the IIS (2020) reference. The photograph was taken vertically to the canopy, four times in each plot, consisting of one picture in the center of each side of the 5×5 m^2 sub-plot. The hemispherical photo was analyzed by ImageJ software to measure the dark pixel (as a canopy) and white pixel (as the sky).

Biomass and C-stock estimates were carried out in each vertical stratification of mangrove forests, consisting of estimates of above-ground biomass (AGB) and carbon stocks (AGC), belowground vegetation biomass (BGB) and carbon stocks (BGC). The estimation of biomass and carbon stocks was carried out by non-destructive methods using observation plots and based on available species-specific allometric or common allometric formulations (Table 1). The estimation of belowground biomass and C-stock is carried out using AGB:BGB ratio approach in mangrove forests (Kauffman and Donato 2012).



a **b**

Fig. 2. Hemispherical photography method for measuring canopy cover: a – Dense canopy cover (≥ 75 %), b – Medium canopy cover (50–75 %).

Table 1. Allometric equations to determine biomass and C-stock in this study.

No	Species	Allometric	ρ, g·cm^3	Sources
1	*Avicennia alba* Blume	$AGB = 0.251 \cdot \rho \cdot D^{2.46}$ $BGB = 0.199 \cdot \rho^{0.899} \cdot D^{2.22}$	0.506	Komiyama et al. (2005)
2	*Bruguiera cylindrica* (L.) Blume		0.749	Komiyama et al. (2005)
3	*B. gymnorrhiza* (L.) Lam.		0.699	Komiyama et al. (2005)
4	*Lumnitzera littorea* (Jack) Voigt		0.670	Komiyama et al. (2005) and Zanne et al. (2009)
5	*Rhizophora apiculata* Blume		0.770	Komiyama et al. (2005)

No	Species	Allometric	ρ, g·cm^3	Sources
6	*Avicennia marina* (Forssk.) Vierh.	AGB = 0.1848·$D^{2.3524}$ BGB = 0.1682·$D^{1.7939}$	-	Dharmawan and Siregar (2008)
		Other allometrics		
1	Dead tree – Decay status 1	B = 0.975·AGB		Kauffman and Donato (2012)
2	Dead tree – Decay status 2	B = 0.8·AGB		Kauffman and Donato (2012)
3	Aboveground C-stock	C-stock = 0.47·AGB		Kauffman and Donato (2012)
4	Belowground vegetation C-stock	C-stock = 0.39·AGB		Kauffman and Donato (2012)

Note: ρ is wood density, D is diameter at breast height, B is biomass.

Results and Discussion

Structure and composition of mangrove forest

Field data collection from the observation plots was carried out to describe the structure and composition of mangrove forests in Sambas Regency. Based on observations, it was found that there were 6 dominant mangrove species in Sambas Regency, namely *Avicennia alba, A. marina, Bruguiera cylindrica, B. gymnorrhiza, Lumnitzera littorea*, and *Rhizophora apiculata*. Tree density about 800–4700 individuals per ha, vegetation height about 3–18 m, and canopy density about 55–86.63 %. The number of natural tillers of each type of mangrove is quite varied in the observation plot, there are several locations with the potential for a large number of mangrove tillers, such as in the locations of Malek, Sebubus and Sarang Burung Usrat Village. The availability of sufficient natural seedlings is one of the important factors in maintaining the sustainability and regeneration of mangrove vegetation in the future.

The range of mangrove species diameter values in the observation plot is about 4.39 ±0.69 – 27.45 ±21.14 cm, where the Selakau location has the smallest average individual diameter and the Sebubus location has the largest average individual diameter (Table 2). The range of diameter values found at the location is smaller than that of mangrove forest vegetation in Kubu Raya Regency (CFCRRD-FORDA and CIFOR 2011) and Mempawah Regency (Rafdinal et al. 2019), West Kalimantan province, with individual mangrove diameters found to reach more than 55 cm and 80 cm. However, from the value of individual density per ha, mangrove vegetation in Sambas Regency was found to be higher than the report from Rafdinal et al. (2019) in Mempawah Regency. The large diameter of mangrove tree can describe in general the age of mangrove vegetation at the site, in addition to the suitability of ecological factors that support the life of the mangrove vegetation. Sebubus location has a fairly high range of individual mangrove diameter values, this is presumably because this location is not directly facing the sea, so the received wave intensity is minimal compared to several other locations in Sambas Regency.

Table 2. Structure and composition of mangrove forest in Sambas Regency.

Location (District, Village)	Code	Description	Major species	*N* (DBH ≥4 cm)	DBH Average ±SD, cm	Total seedling
Selakau	Sel1	Tree density 1500 ha^{-1}, Canopy density 55 %, Vegetation height about 3–4 m	*Avicennia marina*	15	4.83 ±0.72	32
Pemangkat	Pem1	Tree density 800 ha^{-1}, Canopy density 58 %, Vegetation height about 5–8 m	*Avicennia marina*	8	15.00 ±3.66	8
Paloh, Malek	Mal1	Tree density 1700 ha^{-1}, Canopy density 81.91 %, Vegetation height about 3–4 m	*Avicennia marina*	7	11.92 ±2.66	0
			Bruguiera gymnorrhiza	10	7.58 ±3.49	125
Paloh, Malek	Mal2	Tree density 4700 ha^{-1}, Canopy density 76.89 %, Vegetation height about 3–4 m	*Avicennia marina*	30	7.08 ±2.80	45
			Bruguiera gymnorrhiza	17	6.15 ±3.14	8
Paloh, Malek	Mal3	Tree density 3400 ha^{-1}, Canopy density 71.04 %, Vegetation height about 5–6 m	*Avicennia marina*	12	7.30 ±3.82	4
			Bruguiera gymnorrhiza	22	4.39 ±0.69	529
Paloh, Sebubus	Seb1	Tree density 2200 ha^{-1}, Canopy density 86.63 %, Vegetation height about 8–13 m	*Rhizophora apiculata*	22	12.55 ±4.82	9
Paloh, Sebubus	Seb2	Tree density 2400 ha^{-1}, Canopy density 81.70 %, Vegetation height about 8–13 m	*Rhizophora apiculata*	16	7.15 ±2.96	4
			Bruguiera cylindrica	8	19.51 ±13.09	3
Paloh, Sebubus	Seb3	Tree density 2000 ha^{-1}, Canopy density 83.98 %, Vegetation height about 12–18 m	*Avicennia marina*	4	26.75 ±6.87	0
			Bruguiera cylindrica	11	12.91 ±8.13	133
			Lumnitzera littorea	5	27.45 ±21.14	47
Jawai, Sarang Burung Usrat	USR1	Tree density 1700 ha^{-1}, Canopy density 72.36 %, Vegetation height about 4–6 m	*Avicennia alba*	17	9.46 ±3.85	65
			Rhizophora apiculata	0	0	6
Jawai, Sarang Burung Usrat	USR2	Tree density 1900 ha^{-1}, Canopy density 81.92 %, Vegetation height about 8–12 m	*Avicennia alba*	19	10.60 ±4.68	25
			Rhizophora apiculata	0	0	4

Carbon stock

Total C-stock estimation of mangrove vegetation using non-destructive methods in Sambas Regency is about 7.17 ±0.32 – 546.67 ±70.83 Mg C·ha^{-1} with an average of 130.62 ±166.34 Mg C·ha^{-1} (Table 3). The highest C-stock value from all locations was found at Sebubus village, Paloh District (546.67 ±70.83 Mg C·ha^{-1}), while the lowest was found at Selakau site, Selakau District (7.17 ±0.32 Mg C·ha^{-1}). The highest value of biomass and C-stock at the Sebubus location was due to the composition of a high average tree diameter (12.91 ±8.13 – 27.45 ±21.14 cm) and a fairly high tree density (2000 trees ha^{-1}) compared to several other observation locations.

Table 3. Total vegetation C-stock of mangrove forest in Sambas Regency.

Location code	*N* (DBH≥4 cm)	Aboveground vegetation C-stock, Mg C·ha^{-1}	Belowground vegetation C-stock, Mg C·ha^{-1}	Total vegetation C-stock, Mg C·ha^{-1}
Sel1	15	5.48 ±0.28	1.69 ±0.078	7.17 ±0.32
Pem1	8	43.91 ±5.82	7.01 ±0.90	50.92 ±6.32
Mal1	17	38.32 ±3.63	10.40 ±1.18	48.72 ±3.12
Mal2	47	49.23 ±2.59	14.42 ±0.98	63.66 ±2.08
Mal3	34	23.09 ±2.30	6.67 ±0.43	29.76 ±1.71
Seb1	22	127.08 ±11.81	44.26 ±4.46	171.33 ±9.53
Seb2	24	209.38 ±46.19	63.71 ±15.32	273.10 ±34.56
Seb3	20	437.37 ±94.32	109.30 ±29.07	546.67 ±70.83
USR1	17	32.50 ±3.26	12.68 ±1.46	45.17 ±2.64
USR2	19	50.80 ±6.79	18.93 ±2.69	69.73 ±5.32
Average	22.3	101.72 ±132.43	28.91 ±34.22	130.62 ±166.34

The potential for vegetation carbon stocks in Sambas Regency is greater than some mangrove forest locations, such as study from Harishma et al. (2020) in mangrove ecosystems of Kerala, India (AGB and BGB; 35.14 and 23.42 Mg C·ha^{-1}), study from Savari et al. (2020) in Gowatr mangrove forest, Gulf of Oman (AGB and BGB; 28.51 and 66.69 Mg C·ha^{-1}), and study from Wanthongchai and Pongruktham (2019) in mangroves of the Lower Andaman Coast, Thailand (AGB and BGB; 2.25 and 1.5 Mg C·ha^{-1}). The potential C-stock in Sambas Regency was close to the value of the largest mangrove forest in West Kalimantan, namely in Kubu Raya Regency (AGB and BGB; 134.8 and 14.3 Mg C·ha^{-1}) (Murdiyarso et al. 2015) (Fig. 3). The amount of vegetation carbon stock value is influenced by the structure and composition of mangrove vegetation in each location, especially the density and average diameter of individual mangrove trunks found.

Mangrove forests in Sambas Regency have great carbon potential and can support climate change mitigation efforts. Based on the contribution of carbon stocks of each species, the three species with the highest contribution were *Bruguiera cylindrica* with a value of 35.86 Mg C·ha^{-1}, *Lumnitzera littorea* with a value of 33.28 Mg C·ha^{-1} and *Avicennia marina* with a value of 23.40 Mg C·ha^{-1}. These three species have the highest contribution to carbon stocks because they have a high density (*Avicennia marina* are quite dominant) and have a high average tree

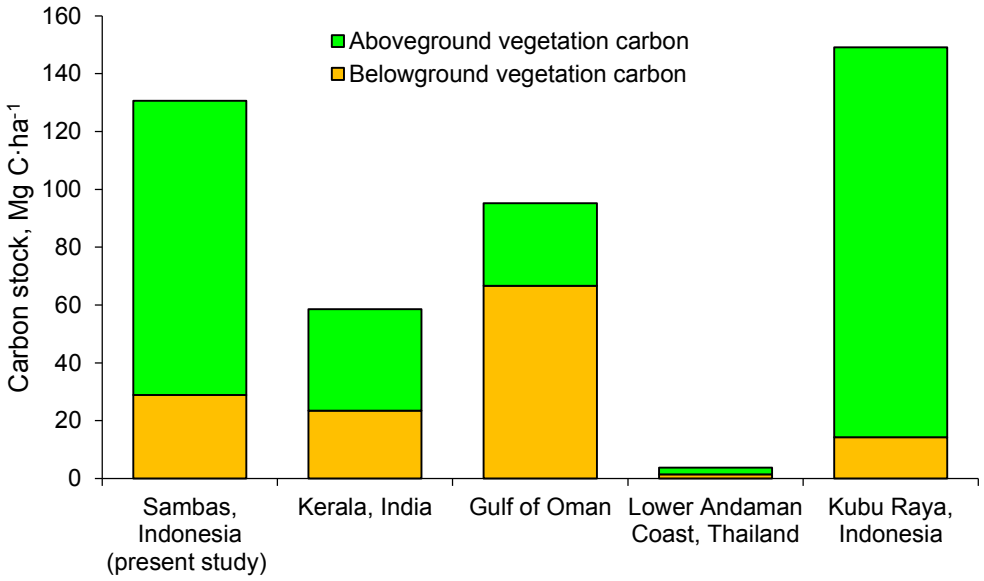


Fig. 3. Comparation of several mangrove forest carbon stock.

diameter (*Bruguiera cylindrica* and *Lumnitzera littorea*). According to Komiyama et al. (2005), tree density and tree diameter affect the carbon potential value of a species in a mangrove forest ecosystem. Based on the structure and composition, also potential carbon stock, Sambas Regency has a high potential for mangrove forests compared to several mangrove forests in Indonesia and other countries. Conservation efforts from both the community and the local government are needed to maintain the potential of existing mangrove forests and sustainable use for the preservation of mangrove forests in the Sambas Regency.

Conclusions

An assesment of mangrove vegetation in Sambas Regency in this study found six dominant mangrove species, namely A*vicennia alba, A. marina, Bruguiera cylindrica, B. gymnorrhiza, Lumnitzera littorea* and *Rhizophora apiculata*. Vegetation density varies from sparse to dense, ranging from 800–4700 trees·ha^{-1} with a canopy cover of 55.00–86.63 %. The range of mangrove species diameter values ranged from 4.39 ±0.69 – 27.45 ±21.14 cm. Potential vegetation C-stock is about 7.17 ±0.32 – 546.67 ±70.83 Mg C·ha^{-1} with an average of 130.62 ±166.34 Mg C·ha^{-1}, the highest potential was found at the Sebubus Village.

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Age structure, growth rate and condition of *Barbus petenyi* (Cyprinidae) in the middle and upper stream of the Ogosta River

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Abstract

This is a study of length and weight growth of Romanian barbel from the middle and upper stream of the Ogosta River. Population parameters estimates were based on scale annual interpretation. The estimates were compared with growth data obtained in previous periods from other Bulgarian water courses. Methodologically, 100 individuals were collected by electrofishing in 2020. Specimens' age ranged from 1 to 5 years. More than half of the sample was represented by one-year-old fish. Older fish were relatively few. The lengths of all sampled individuals ranged from 39 mm to 202 mm. A relationship between standard length (*SL*) and scale radius (*S*) was described by an equation: $SL = 2.7077 \cdot S$, $r^2 = 0.9485$, $p < 0.005$. A relationship between fish gutted weight (*W*) and *SL* was represented by an equation: $W = 0.00002 \cdot SL^{2.8936}$; $r^2 = 0.9956$, $p<0.005$.

Key words: growth of weight, linear growth, Romanian barbel, size and age composition.

Introduction

Romanian barbel (*Barbus petenyi* Heckel, 1852) is a rheophilic fish typically encountered in the middle and upper reaches (Michaylova 1960, 1970; Marinov 1986) of Danube River tributaries in Romania and Bulgaria (Kottelat and Freihof 2007). The species is also found in the Kamchia River of the Black Sea watershed in Bulgaria (Drenski 1951, Kottelat and Freihof 2007). This is one of the 'meridionalis group' barbels, which are medium sized and adapted to moderately cold water (Bianco 2012). Five-year-old specimens reach over 20 cm in length (Michaylova 1960). However, some authors indicate 28 cm (Marinov 1986) and even 30 cm as the maximum species length (Peshev et al. 2003, Karapetkova and Zhivkov 1995). The Romanian barbel is one of the most numerous species of the middle zone of the North Bulgarian Rivers (Karapetkova and Dikov 1986; Dikov et al. 1988, 1994). This species is of interest for recreational fishing.

The aim of this study was to establish the growth parameters of the Romanian barbel from the Ogosta River and fill the knowledge gap that exist for this species.

Study Area

The Ogosta River (Fig. 1) is the largest watercourse in the north western part of Bulgaria (Fig. 2). With a length of 144.1 km, the river ranks 7th in the ranking of the 'big' rivers of Bulgaria (Hristova 2012). The Ogosta River springs below the Vrazha Glava peak (1935 m) in the Balkan Mountains. After collecting waters from many tributaries, the river flows into the Danube River, not far from the town of Oryahovo. Built in 1986 near the city of Montana, the Ogosta Dam, divides the river into two parts. This study was conducted in the middle zone of the river, located above the dam (Table 1).

Ecological characteristics of the studied section of the river were established as follows: the river meanders through a hilly area and so distinct changes of the depth of its flow were observed and recorded. The river bottom was covered with larger and smaller stones, as well as gravel. The terrain was inclined, which enabled rapid water flow. The banks were overgrown with black alder and white willow; their branches covered the riverbed. Fish found refuge from the strong current in hidden holes in the roots of these sur-

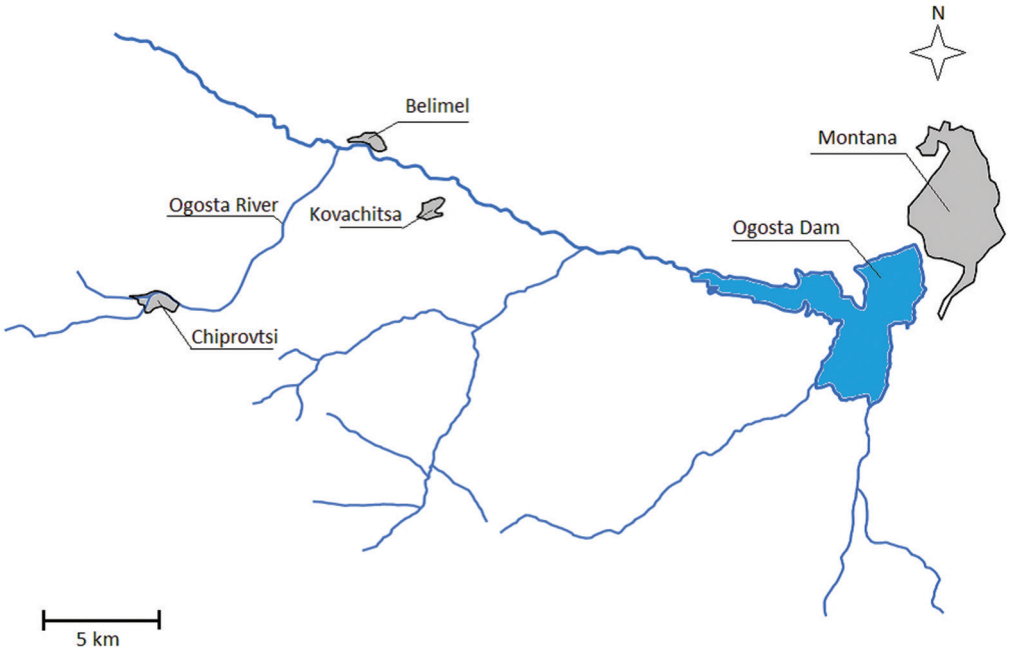

Fig. 1. Schematic map of Ogosta River middle zone, where the Romanian barbel was found.

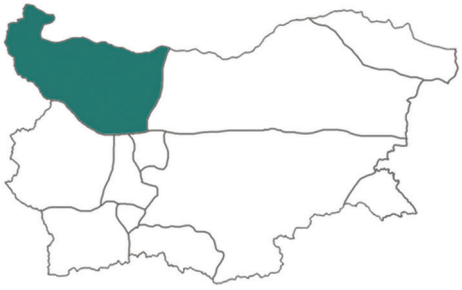

Fig. 2. Location of the studied region of Bulgaria.

rounding trees. The studied part of the riverbed lied between 190 and 380 m above the sea level. The study took place in the autumn, when the measured daily water temperature reached 8–12 °C while dissolved oxygen values were between 4.8 and 7.6 mg·m^{-3}.

Materials and Methods

The study was based on a sample of 100 individuals of Romanian barbel. Sampling took place at five sampling areas in the middle zone of the Ogosta River (Table 1). Sampling material was collected in the autumn of 2020 by electrofishing. A SAMUS 725G converter was used, providing up to 640 V direct current (DC), frequency 50 Hz and output power reaching up to 200 W. Catch was performed according to the EN 14011:2004 instruction (Water quality Sampling of fish with electricity). Measurements were conducted of total fish length (*TL*) and standard fish length (*SL*) with a precision of 1 mm and gutted weight (*W*) with a precision of 1 g. Age was determined by measuring fish scales, using an Olympus CX 31 microscope at a 40× magnification.

Table 1. Sampling areas in the Ogosta River.

No	Location	Geographic coordinates		Altitude, m a.s.l.	Date of sampling
		N	E		
1	Near the confluence of the Ogosta River and the Ogosta Dam.	43°23'49"	23°03'58"	195	23.10.2020
2	Near the strawberry farm.	43°24'20"	23°03'16"	221	22.10.2020
3	Near the village of Gorna Kovachitsa.	43°25'20"	23°00'40"	250	23.10.2020
4	Near the village of Belimel.	43°25'33"	22°57'30"	305	22.10.2020
5	Near the town of Chiprovtsi.	43°23'42"	22°55'26"	380	22.10.2020

In addition to Romanian barbel, 10 fishes from 2 families were recorded in the middle part of the Ogosta River. Species identification was made according to Kottelat and Freyhof (2007).

A fish's linear growth was determined via a back-calculation of length (*SL)* from the diagonal caudal radius of a scale (*S*) (Zhivkov 1981). This relation is described by a linear equation (1):

$$SL = a + b \cdot S, \quad (1)$$

where: *SL* – standard length of fish, mm; *S* – diagonal caudal radius of a fish scale (eyepiece micrometer scales divisions); *a*, *b* – equation coefficients.

Absolute annual linear incensement was accepted as a characteristic of the growth (Zhivkov 1972), calculated by formula (2):

$$t = S \cdot L_n - S \cdot L_{n-1}, \quad (2)$$

where: *t* – absolute annual linear incensement, mm; *Ln*; L_{n-1} – average standard length of fish for two consecutive years, mm.

Gutted weight (*BW*) values were estimated by equation (3) Ricker (1979), used by many authors (Zhivkov 1981, 1999; Prodanov 1982; Raikova-Petrova and Zhivkov 1993; Kukushkin 1997; Belomacheva et al. 2005):

$$BW = a \cdot SL^b, \text{ g} \quad (3)$$

where: S*L* – standard length of fish, mm; *a, b* – equation coefficients.

A comparison of the length growth of different populations of Romanian barbels is made by ranking them according to their average length at the same age (Zhivkov 1972).

Condition of a population is studied in three ways:

1) By calculating a classical coefficient (K_f) of the Fulton equation (4):

$$K_f = (BW \cdot SL^{-3}) \cdot 10^2 \quad (4)$$

where: *SL* – weighted average standard body length, cm; *BW* – weighted average gutted body weight, g.

2) By calculating a coefficient K_b, using the Fulton equation, but instead of exponent 3, exponent *b* from the population weight-length relationship from equation (3) is used.

3) By comparison of weight growth (*TW*) of fish from different populations, a method proposed by many authors (Goldspind 1979; Zhivkov 1981, 1999; Basami and Grove 1985; De Silva 1985; Raikova-Petrova 1992). A relationship is expressed by the equation (5):

$$TW_L = a \cdot SL^b, \text{ g}, \quad (5)$$

where: *L* – length of fish, mm; *TW* – total weight of fish g; *a*, *b* – equation coefficients.

In order to obtain comparable values of *W* in equation (3), pre-selected rounded values of *L* (50, 100, 150, 200 and 250 mm) are successively substituted in place of *L* (*L* = 50, *L* = 100, *L* = 150, …). Using equation (5) with the listed values of *L* (mm), allows obtaining the corresponding values of mass *TW*: $TW_{L=50}$, $TW_{L=100}$, $TW_{L=150}$, $TW_{L=200}$, $TW_{L=250}$. The so-obtained mass values ($TW_{L=50}$, $TW_{L=100}$, $TW_{L=150}$, $TW_{L=200}$, $TW_{L=250}$) for each of the studied populations are then compared (Zhivkov 1981, 1999; Raikova-Petrova and Zhivkov 1993).

Results and Discussion

Age and size composition

The age of Romanian barbel from the Ogosta River ranges from 1 to 5 years. The first age group is the most abundant and accounts for more than half of the catch (56 %) (Fig. 3). The number of fish of other age groups decreases progressively with age. Only one specimen was found in the fifth age group.

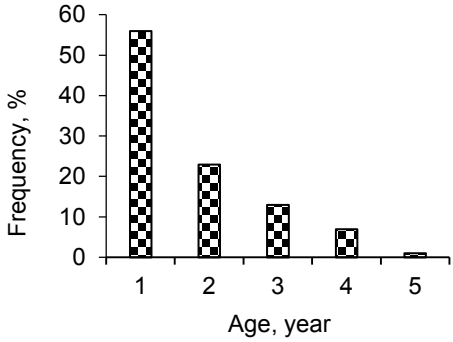

Fig. 3. Age structure of Romanian barbel from the Ogosta River.

Similar age structure reported Michaylova (1960) for the Vedena River, where first and second age groups represented 85 % from the sample. In this river, however, the most numerous were two-year-old fish (49 %). The available data show that only two five-year-old individuals (1 %) were caught in the Vedena River. A lack older groups' specimen from both studied rivers, Vedena and Ogosta, is likely a result of increased natural mortality or fishing elimination (Pravdin 1966).

In the sample from the Ogosta River, the largest fish is a five-year-old individual with body length 172 mm (*TL* 202 mm) and body weight 71 mm (*TW* 80.6 g). Mean length is 83 ±26 mm and mean weight 10

±12 g. The following size groups are the most numerous: 51–60 mm; 61–70 mm. Specimens longer than 100 mm are relatively few (Fig. 4).

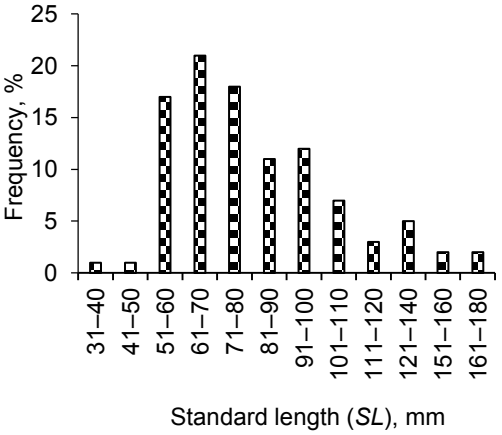

Fig. 4. Size classes of Romanian barbel from the Ogosta River.

Growth rate

A relationship between standard length and scale radius is well expressed by a linear function (Fig. 5). Length growth of different age groups is relatively constant (Table 2). Due to the onset of sexual maturity (Nikolsky 1965) two-year-old fish have a slight delay of linear growth. In subsequent years, length growth remained close to that, observed during the first year. Exceptions are five-year-old fish, which exhibit the fastest growth. Older and larger fish are likely to be more successful in finding food in this watercourse.

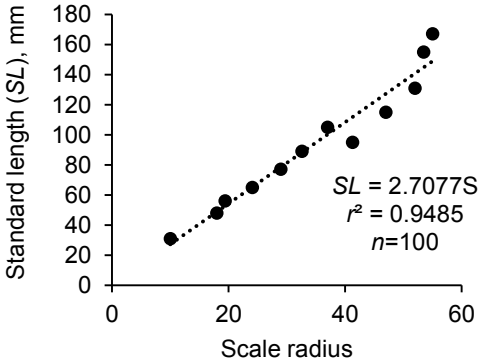

Fig. 5. Growth in length of Romanian barbel from the Ogosta River.

Table 2. Back-calculated standard body length (*SL*) of Romanian barbel of the Ogosta River.

Year	Age group	N	Mean back calculated *SL* (mm) at age				
			SL_1	SL_2	SL_3	SL_4	SL_5
2020	I	56	65				
2019	II	23	35	87			
2018	III	13	30	54	108		
2017	IV	7	30	54	79	138	
2016	V	1	32	54	84	100	149
Mean calculated *SL*, mm		100	38	62	90	119	149
Mean observed *SL*, mm		100	65	89	111	135	172
Annual increment, mm			38	30	36	38	49

A well-defined, almost functional relationship between body length and weight is characteristic of Romanian barbel (Fig. 6). Weight growth is characterized by a gradual increase up to the fifth year (Table 3). Two-year-old fish exhibit some growth retardation both in terms of length and weight. Probably, this is a result of in-

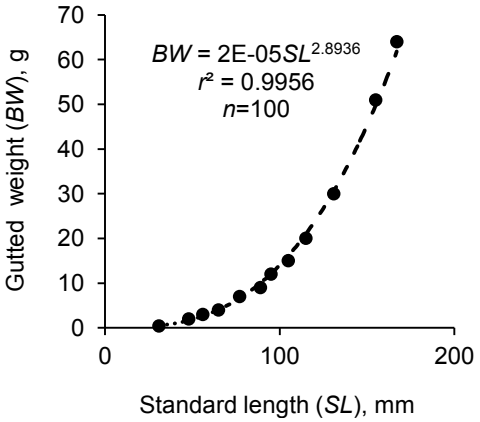

Fig. 6. Growth in weight of Romanian barbel from the Ogosta River.

creased sexual function; for this species, it takes place in their second year (Michaylova 1960). Five-year-old fish have the highest weigh growth.

Available data for this species allows a comparison of linear growth, using observed total body length (*TL*) (Table 4). The highest linear increment was associated with fish of the Rositsa River, and the lowest one – with fish of the Osam and Lom rivers (Danube watershed) (Marinov 1986). Observed total body length of Ogosta River fish is very close to that reported by Michaylova (1960) for the Vedena River. Although the Vedena River is located in southern Bulgaria and the Ogosta in northern Bulgaria, both rivers are part of the Danube basin. Growing conditions in the two rivers are obviously similar, as colder climate in Northern Bulgaria is compensated by the lower altitude of the Ogosta riverbed.

Table 3. Back-calculated gutted body weight (*BW*) of the Romanian barbel in the Ogosta River.

Year	Age group	N	Mean back calculated *BW* (g) at age				
			BW_1	BW_2	BW_3	BW_4	BW_5
2020	I	56	3				
2019	II	23	4	8			
2018	III	13	1	2	15		
2017	IV	7	1	2	6	31	
2016	V	1	1	2	7	12	39
Mean calculated *BW*, g		100	2	4	10	22	39
Mean observed *BW*, g		100	4	10	19	36	71
Annual increment, g			2	2	8	15	27

Table 4. Comparison of mean observed total body length (*TL*) of Romanian barbel of the same age.

Source	River	Total body length (*TL*), mm					
		N	TL_1	TL_2	TL_3	TL_4	TL_5
Marinov 1986	Rositsa	20	76	142	163		
Marinov 1986	Nishava	15	77	125			
Michaylova 1960	Vedena	168	75	108	158	170	202
Our data 2020	Ogosta	100	78	108	131	160	202
Marinov 1986	Dryanovska	20	70	101	133	152	
Marinov 1986	Vit	32	71	120	127		
Marinov 1986	Archar	20	43	100	117		
Marinov 1986	Dryanovska	32	48	86	109	142	

Source	River	Total body length (*TL*), mm					
		N	TL_1	TL_2	TL_3	TL_4	TL_5
Marinov 1986	Lom	15	42	86			
Marinov 1986	Osam	25	42	86			

However, use of calculated standard body length (*SL*) for comparative analysis is recommended by many authors (Prodanov 1982; Raikova-Petrova 1992; Zhivkov 1981, 1999; Raikova-Petrova and Zhivkov 1993; Kukushkin 1997; Belomacheva et al. 2005). This method would give more accurate results. In this way, individual deviations are reduced and a general trend in length growth is better expressed. Our data show that calculated *SL* for the species is lower than observed (Table 2). Applying standard length (*SL*) instead of total length (*TL)* prevents potential errors, due to caudal fin damage.

Condition factor

Condition factor is calculated by the Fulton equation – K_f. The calculated K_f for barbel from the Ogosta River, K_f =1.4, is higher than the average reported for specimen from the Vedena River – K_f = 0.6–2.56 (Mihailova 1960). The author points out that K_f is higher only for four- and five-year-old fish from the Vedena River, which are few in the sample (about 5 %).

Many authors prefer the use of K_b to Fulton's coefficient K_f because it gives more accurate results. In this study, the coefficient K_b =1.75 is much larger than K_f.

By substituting rounded values for the body length (50 to 250 mm) into the equation expressing *BW*/*SL* ratio – BW_L=0.00002$L^{2.8396}$, the weight at a corresponding length is obtained. The results are as follow: BW_{50} = 2 g, BW_{100} = 12 g, BW_{150} = 40 g, BW_{200} = 92 g, BW_{250} = 176 g. Those data allow the comparison of the growth of the Romanian barbel from different populations.

Conclusions

The population of Romanian barbel of the Ogosta River is predominated by one-year-old fish. Adult and larger fish are very few. Replenishment dominates over residue.

Species' length growth is closer to that of fish of the Vedena River (the Iskar River watershed). The condition factor and Fulton's coefficient of Romanian barbel of the Ogosta River is higher than the highest reported for barbel of the Vedena River.

Acknowledgments

I am grateful to my colleague G. Gruychev for his help in collecting the material of this study. This study is financially supported by UOGS 'Petrohan' at LTU-Sofia (project number: 8/2020).

Authors' statement

The present study was conducted in accordance with the national legislation. The electrofishing was conducted with a permit 31/26.08.2020, issued by the Minister of the Ministry of Agriculture, Food and Forestry of Bulgaria. Most of the fish caught were released back into the water. Only the quantity indicated in the electro-

fishing permit was detained.

Conflict of interest

The author declares no conflict of interest.

Data availability statement

The data that support the findings of this study are available on request from the author.

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Dendroclimatic investigation of Caucasian spruce (*Picea orientalis* (L.) Link) in Moscow botanical gardens

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Abstract

Caucasian spruce (*Picea orientalis* (L.) Link) has a native natural area at Caucasus and Pontic Mountains around the eastern Black Sea in Russia, Georgia, Armenia and Turkey. This species forms pure stands or mixed with other conifers and hardwoods. In Moscow it is cultivated at the Arboretum of Main Botanical Garden of Russian Academy of Sciences N. V. Tsitsin and at the Arboretum of Botanical Garden of Moscow State University M. V. Lomonosov. It has been established that the following meteorological parameters have the main adverse effect on the growth of this spruce species: temperature in February, precipitation in February, March, April, May and July, temperatures of April and July. In the conditions of global climate change, this species may become important for forest crops and urban landscaping.

Key words: dendrochronology, dendroclimatology, dendroecology, tree-ring analysis.

Introduction

Caucasian spruce (*Picea orientalis* (L.) Link) has a native natural area at Caucasus and Pontic Mountains around the eastern Black Sea in Russia, Georgia, Armenia and Turkey. This species forms pure stands or mixed with other conifers and hardwoods, often as a dominant on moist, shaded slopes or in ravines, at altitude 1000–2000 m a.s.l. (Eckenwalder 2009).

In Moscow this species is cultivated and introduced at the arboretums of Main Botanical Garden of Russian Academy of Sciences N. V. Tsitsin and Botanical Garden of Moscow State University M. V. Lomonosov. According to the results of long-term observations in the Botanical Garden of the Russian Academy of Sciences, this species is characterized by low winter hardiness (Demidov 2005). Caucasian spruce has stable growth only in the southern and western regions of Russia and is damaged by frosts already at the latitude of Kiev (Kolesnikov 1974).

The high sensitivity of Caucasian spruce to moisture regime is well-known in the Russian forest science. But at the same time, it is relatively frost-resistant species, which has been formed the tree line high in the mountains. Also, this species is characterized by great shade tolerance and its undergrowth does not toler-

ate direct exposure to sunlight (Tkachenko 1955).

Despite the fact that the species sometimes demonstrates high frost resistance in its natural range, it is impossible to predict its winter hardiness when introduced in other geographical conditions. Winter hardiness is a complex indicator that depends not only on specific temperature values in certain periods of winter, but also on the combination and order of alternation of such values, as well as on the combination of temperature values with the values of other meteorological parameters. These provisions can be illustrated by the example of Siberian fir (*Abies sibirica* Ledeb.). In its natural range, this species is able to tolerate severe winter frosts up to -50 °C and below, but when cultivated in Western Europe, it is damaged by frost at relatively small low temperatures below zero (Bulygin 1991). This makes it necessary to conduct experimental studies of the growth of the success of the species in the conditions of introduction in each individual region. Dendroclimatic method provides such an opportunity, as it allows to retrospectively analyze a series of experiments set by nature, the results of which are recorded in the variability of annual rings.

The fossil remains of the Caucasian spruce indicate that its range has repeatedly changed due to the impact of climate change (Kapper 1954). Taking into account the scenario of climate warming in the European part of Russia, the Eastern spruce may become important as a valuable forest-forming breed: in native area it forms stands with wood volume 800–1000–1500 $m^3 \cdot ha^{-1}$ cubic meters per hectare (Tkachenko 1955). It may have perspective for urban tree planting too.

Dendroclimatic analysis makes it possible to better understand the factors critical to the good condition of this species and to predict its response to climate change (Fritts 1976). Some numbers of works are devoted to the dendroclimatic analysis of the growth of the eastern spruce in its natural range. So, the study of Özkan (1999) attempted to describe the variations in the annual growth of Caucasian spruce and to give a mean annual ring curve using annual rings.

The effect of climate change for Caucasian spruce forest was investigated by group of Turkish scientists according to regional climate model (RegCM3) (Tüfekçioğlu et al. 2008). Climate change could significantly influence distribution, diversity, structure and stability of the oriental spruce ecosystems. According to RegCM3 regional climate model, the temperatures will increase 2–4 °C in the region in the next century. Future climate scenarios predict 200–300 mm increase in precipitation in the eastern part of the region, while the western part won't have any increase in precipitation in the next century. Temperature increases in the western part of the region can cause more stress on spruce trees and would probably increase bark beetle attacks. Also, fire could become an important threat in the western part of the region. It is possible to observe 400–800 m upward shift in the spruce belt in the western part. Tree line of spruce stands would probably move upwards both in western and eastern part of the North-eastern Black Sea Region.

Also, Caucasian spruce was investigated by bioclimatic modelling methods (Örücü 2021). This study seeks to identify the change that will occur in future in the current habitat of *Picea orientalis* L.

under climate change scenarios. To this end, a model was developed in the MaxEnt 3.4.1 software programme using 19 bioclimatic variables obtained from the Worldclim database. The study made use of the Community Climate System Model (CSSM) version 4 and the Representative Concentration Pathway (RCP) 4.5 and RCP 8.5 scenarios for 2050–2070 developed in accordance with the fifth report of the Intergovernmental Panel on Climate Change (IPCC). The results of the Jackknife test, which was used to identify the bioclimatic variables that affect the geographical distribution of the species, mostly showed that isothermality was the variable that generated the greatest gain. In conclusion, the total extent of the 'suitable' and 'highly suitable' areas within the study area, constituting the current distribution area of the oriental spruce, is found to be 45,323 km^2 by 2070, according to the model, this figure rises to 65,042 km^2 in the RCP 4.5 scenario and 90,861 km^2 in the RCP 8.5 scenario. In view of the results of the study, it is anticipated that the oriental spruce will start to occur in those areas that will come in future to display climatic characteristics, similar to those in its natural distribution area.

The works of Dutch scientists are of particular interest in the context of our research, too (Song et al. 2021). Conifers are key components of many temperate and boreal forests and are important for forestry, but species differences in stem growth responses to climate are still poorly understood and may hinder effective management of these forests in a warmer and drier future. They studied 19 Northern Hemisphere conifer species planted in a 50-year-old common garden experiment in the Netherlands to (1) assess the effect of temporal dynamics in climate on stem growth, (2) test for a possible positive relationship between the growth potential and climatic growth sensitivity across species, and (3) evaluate the extent, to which stem growth is controlled by phylogeny. Eighty-nine percent of the species showed a significant reduction in stem growth to summer drought, 37 % responded negatively to spring frost and 32 % responded positively to higher winter temperatures. Species differed largely in their growth sensitivity to climatic variation and showed, for example, a four-fold difference in growth reduction to summer drought. Both growth sensitivity to climate and growth potential were partly phylogenetically controlled. Conclusions: A warmer and drier future climate is likely to reduce the productivity of most conifer species. Scientists did not find a correlation between growth potential and growth sensitivity to climate; instead, some species combined high growth potential with low sensitivity to summer drought. The results of this investigation may help to select productive species that are able to cope with a warmer and drier future.

Dendroclimatic studies of Caucasian spruce in the conditions of introduction on the European territory of the Russian Plain have not been carried out until now. Such kind of research allows us to identify specific climatic factors limiting the success of the growth of the species in the conditions of introduction. Knowledge about them makes it possible to predict the success of the growth of the species in the region under different scenarios of global climate changes. The most goal of the investigation was to establish climatic factors affecting tree ring width forming, where ring width considered as an indicator of good or bad health condition of trees.

Materials and Methods

The coring of wood samples in the eastern spruce plantings on the territory of Main Botanical Garden of Russian Academy of Sciences N. V. Tsitsin and Botanical Garden of Moscow State University M. V. Lomonosov was carried out during the period 2009–2010. The coring was made by a Pressler drill. The characteristics of sample trees are given in tables 1 and 2. A comparative characteristic of tress can be seen from photos on figures 1 and 2. The number of samples could not be larger, as the number of collection of specimen in arboreta was limited.

Table 1. Characteristics of the sample trees of the Caucasian spruce in November 2009 in Botanical Garden of Russian Academy Sciences.

Number	DBH, cm	Height, m	Height of coring, m
1	10	10	1
2	10	9	0.70
3	12	11	1
4	12	11	1
Average	11	10	0.9

Table 2. Characteristics of the sample trees of the Caucasian spruce in September 2014 in Botanical Garden of Moscow State University.

Number	DBH, cm	Height, m	Height of coring, m
1	5	3.0	0.8
2	4	3.2	0.8
3	4	2.8	0.8
4	4	4.0	0.8
5	5	2.5	0.8
Average	4.4	3.1	0.8

Despite the comparable age, the trees are differed in biometric characteristics. It relates with condition of growth in plantings. In the Arboretum of Main Botanical

Fig. 1. Caucasian spruce curtain in the Arboretum of the Russian Academy of Sciences (photo July 2010).

Fig. 2. Caucasian spruce in the Arboretum of Moscow State University (photo September 2014).

Garden they grew in an open landscape, in sparse planting. In the Arboretum of Moscow State University, they grew under the canopy of a multi-species stand. That is why the sizes of trees differ more than two times.

Caucasian spruce has annual rings with a clearly distinguishable structure, a clear boundary between early and late wood. This enables reliable measuring of the width of annual ring even with its small size. The general view of the annual rings of the Caucasian spruce is shown on Figure 3.

Fig. 3. Structure of annual rings at the accounting tree of the Caucasian spruce.

Measurements of the width of the annual rings were carried out using a binocular microscope MBS-10 with accuracy 0.05 mm or more. Before measuring the core, surface was cut with a scalpel and after that rubbed with chalk powder. Cross-dating was carried out by visual analysis of graphs in Microsoft Excel. All the chronologies were cross-dated before they were averaged in chronologies for plots.

For dendroclimatic analysis the climagramm method was used (Lovelius 1997, Rumyantsev 2010). The essence of this method is to compare the average values of meteorological parameters for different groups of years. Such meteorological parameters as the average monthly air temperature and the monthly precipitation amount were compared.

Correlation method was used for correlation analysis, too. Correlation method uses as simple and reliable instrument for estimation connections between annual rings width and meteoparameters fluctuations. Traditionally raw ring data series expose to indexation procedure in order to remove the trend line before correlation analysis (Fritts 1976, Cook et al. 1992). But in our case, we observe untypical trend of ring width time variability expressed in a stable increase in the width of the annual ring, which may relate with the trend of meteoparameters, especially important that it may relate with positive trends of winter temperature dynamics, which are clearly observed for Moscow climatic data. That is the reason for work with absolutely ring width data during correlation coefficients calculation. In our case, for the 27-year-long time series, there are 25 degrees of freedom for a confidence level of 0.95 (0.05) and the significant are correlation coefficients with values 0.38 or more.

Results of correlation analysis sometimes give possibilities for radial growth modelling according meteoparameters dynamic. For investigating chronology, such kind modelling has been completed by linear regression equation with using of standard Microsoft Excel functions.

Results and Discussion

Individual chronologies by the ring width for accounting trees were averaged and the results for two objects are shown at Figure 4.

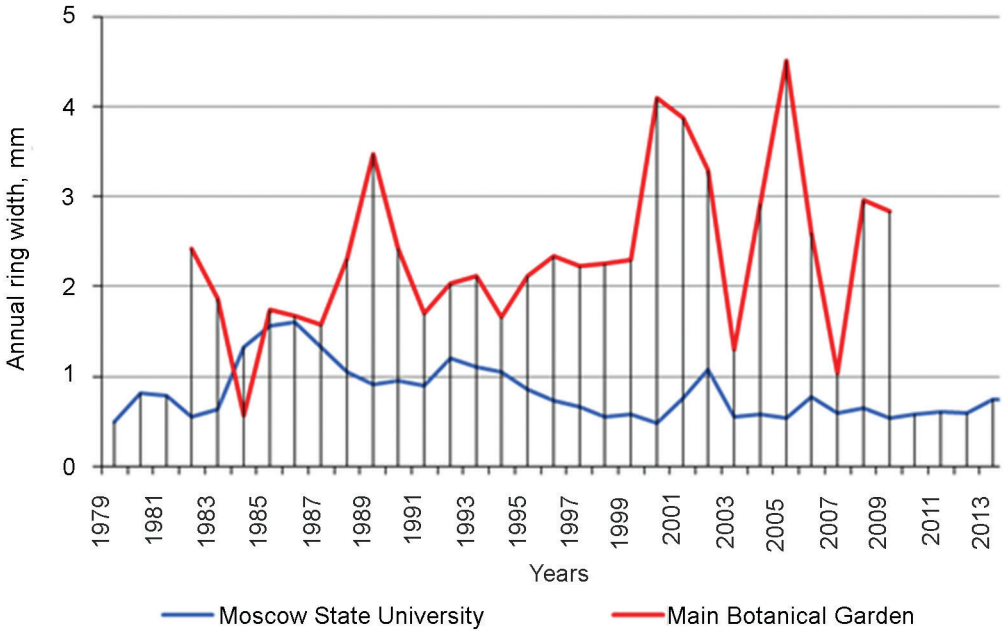

Fig. 4. Average annual ring width by the years for two arboretums.

As it can be seen from graphs at Figure 4, both plantings have a comparable age: about 45 years in 2022. The more accurate determination of the age is impossible due to core sampling at a height of more than 0 m, different growth rates in height at objects, as well as the uncertainty of age of used planting material. It is quite clear that the radial growth of spruce in conditions of Main Botanical Garden of Russian Academy of Sciences exceeds the radial growth in the conditions of Botanical Garden of Moscow State University by several times (the specific amount of excess varies for different years). This should be explained by the factor of inhibition of spruce growth under the influence of interspecific competition at the second object. Also, interannual fluctuations in radial growth are more pronounced on the first object than on the second. So, for significant dendroclimatic analysis is suitable for the chronology from main Botanical Garden only. Trees in the Main Botanical Garden of Russian Academy of Sciences have a trend of increasing radial increment. It is differed from normal age trend in radial growth time series, which is negative. It can be explained by steady decrease in the level of competition with other plants related or may be related with the trends in time series some meteoparameters.

For trees from MSU Arboretum, a high level of oppression on the part of the dominant tree canopy provides high asynchrony in the fluctuations of growth in individual chronologies and, as a result, when they averaged, gives a chronology with unexpressed weather changes. This chronology is characterized by low sensitivity and is unsuitable for dendroclimatic analysis.

Analyzing the dynamics of radial

growth, it is possible to distinguish by the years favourable and unfavourable for the growth of Caucasian spruce (years of local maximums and local minimums of the average width of the annual ring). The group of years with favourable environmental conditions for growth includes: 1982, 1989, 2000, 2005 and 2008. The group of years with unfavourable environmental conditions for growth includes: 1984, 1991, 1994, 2003 and 2007. More complete results for the years of low and high growth would be obtained if the key years in each sample were considered separately. It could then be possible to show what percentage of trees have decreased and increased growth in the given years. But due to the limitations in dendrochronological material, this technique cannot be implemented. The results of the dendroclimatic analysis by climagramm method are shown in figures 5 and 6.

Thus, based on the analysis of the graphs in figures 5 and 6, it

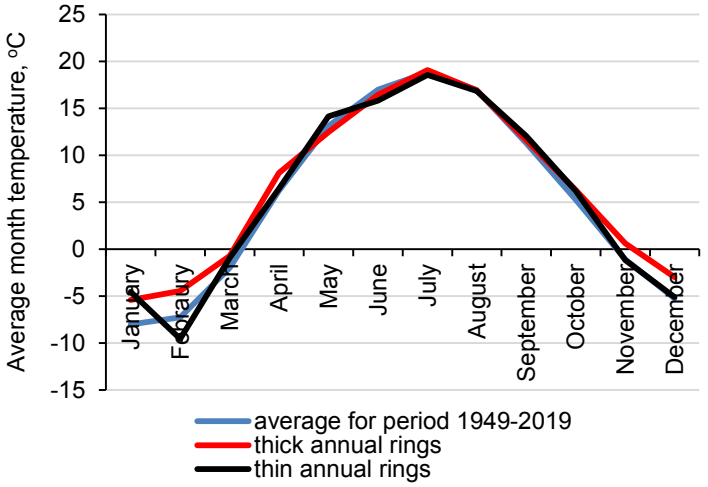

Fig. 5. The comparison of average month air temperatures in the years favourable (thick annual rings) and unfavourable (thin annual rings).

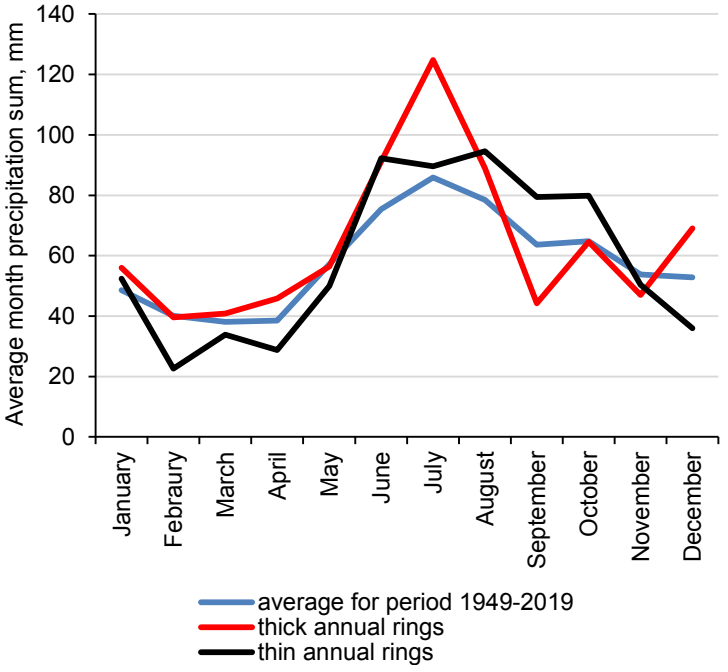

Fig. 6. The comparison of average month precipitation sums in the years favourable (thick annual rings) and unfavourable (thin annual rings).

should be concluded that high temperatures in February, as well as increased precipitation in July, are favourable for the growth of Caucasian spruce. The lack of precipitation (below average values) in February, March, April and May has negative effect on the tree ring forming and trees health. During May (and other dry periods in July) the trees may need watering. In the conditions of the onset of climate warming, Caucasian spruce may turn out to be a promising species for creating forest crops and urban landscaping, but its planting will require regular watering. Currently, it is advisable to lay test forest plantings of Caucasian spruce ecotypes in the north-west regions (as a region with great number of precipitation) of the European part of Russia, in Smolensk region for example. This species may be perspective for forest plantations in Belorussia too.

The temperature of February is a critical factor for the survival of Caucasian spruce in Moscow. Probably this is effect of winter drought. It is well-known that winter drought can be of great importance for the wintering condition of conifers (Roll-Hansen and Roll-Hansen 1998). It takes place in sunny, windy and frosty weather. In this case, the plant cannot get water from the soil and the needles can lose so much water that it will cause it to die off. But February temperature changes with a pronounced trend towards climate warming (Fig. 7).

As it can be seen from the trend of temperature dynamics in February by 2045, its average values in Moscow will reach 0 C by 2045. The conditions of existence in the Moscow region will be favourable for the Caucasian spruce.

Graphs at Figure 8 show the dynamics of radial growth in the Caucasian spruce and the linear trend for this time series is demonstrated. The growth trend has a clear similarity with the trend of temperature changes in February (Fig. 7). For estimation correlations between this series, also as for estimation the influence of other meteoparameters on growth, the correlation analysis was used. Calculations were made both for the parameters in the year of the formation of the annual ring, and for the parameters of the previous year. The calculation results are shown in Table 3.

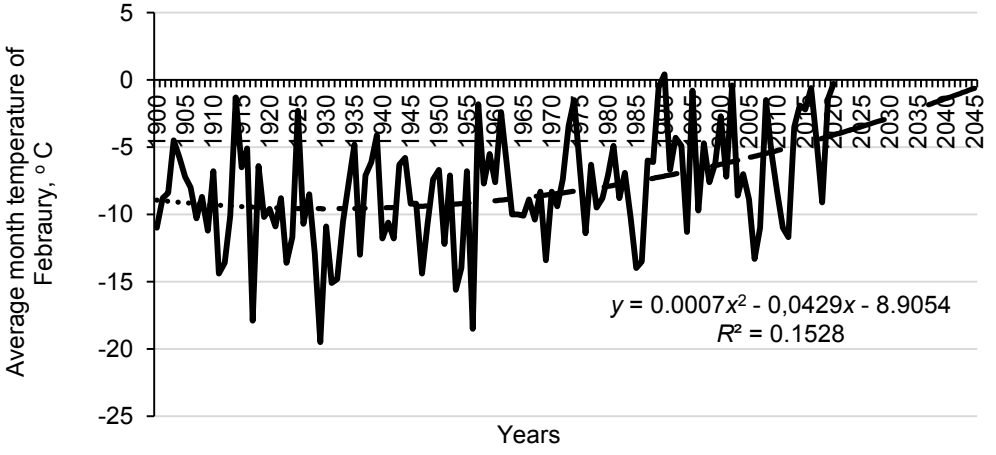

Fig. 7. Dynamics of February temperature in Moscow and forecast of its changes.

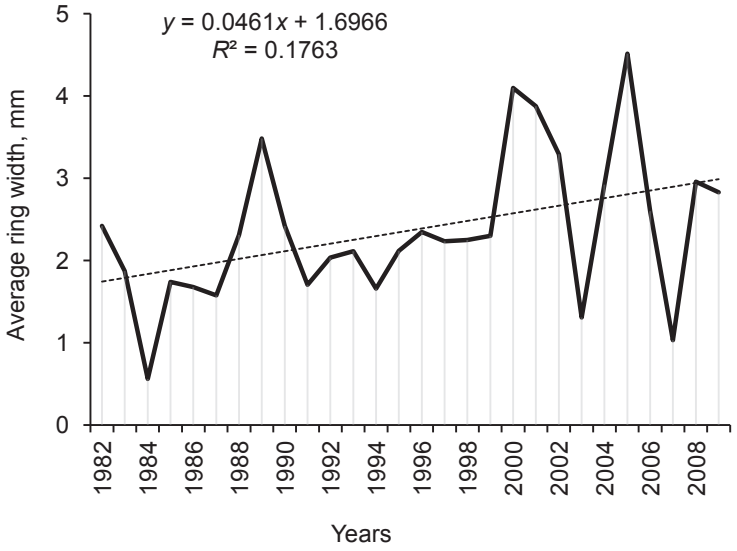

Fig. 8. Radial growth dynamics of Caucasian spruce and trend line.

Table 3. Correlation analysis results.

Month	Month average air temperature		Month precipitation sum	
	Previous year	Current year	Previous year	Current year
January	0.12	0.18	0.05	0.05
February	0.16	0.40*	0.34	0.54*
March	0.15	-0.02	0.18	0.35
April	0.14	0.40*	-0.27	0.00
May	-0.35	-0.34	0.25	0.24
June	0.21	0.11	-0.14	-0.02
July	0.48*	0.46*	0.26	0.11
August	0.23	0.07	0.09	-0.11
September	0.07	0.16	-0.24	-0.47*
October	0.27	0.02	-0.24	0.01
November	-0.05	0.27	-0.05	-0.05
December	0.14	0.02	0.34	0.48*

Note: * Significant value of correlation coefficient for $p = 0.05$.

As a result, the significant and biologically explained correlations are observed for February precipitation sum of current year, for February, April and July temperatures of current year and for July temperatures of current year. Thus, the correlation analysis method confirmed the positive influence of mild, slightly frosty and snowy conditions in February on the successful growth of Caucasian spruce in the Main Botanical Garden of the Russian Academy of Sciences. Warm April and July are favourable for Caucasian spruce growth, too. The regression analysis was used

for modelling the spruce growth dynamic depending of meteo parameters dynamic. As a result, the growth dynamic was described by equation (1):

$$R=-3.77337-0.01677\cdot T_2+0.042831\cdot T_4+0.026253\cdot P_2+0.27725\cdot T_{7\text{-}1}-0.03226\cdot T_7, \quad (1)$$

where:

R – the mean of average tree ring width value in the calendar year;

T_2 – the mean of average month air temperature in February in the current calendar year;

T_4 – the mean of average month air temperature in April in the current calendar year;

P_2 – the mean of precipitation sum in February in the current calendar year;

$T_{7\text{-}1}$ – the mean of average month air temperature in July in the previous calendar year;

T_7 – the mean of average month air temperature in July in the current calendar year.

Recent model describes 53 % of real growth variability, and has correlation coefficient 0.73 and synchroneity coefficient 81 % with real growth time series (Fig. 9).

This result is expected taking into account the data of dendroclimatic analysis by the method of climagramms. The temperature of February is associated with a radial growth in a curvilinear correlation: its fluctuations within certain limits do not affect fluctuations in growth, while strong deviations from average values have a strong effect on growth.

Conclusions

Thus, the study identified the main specific climatic factors that cause poor growth of Caucasian spruce in Moscow. The lack of precipitation during the cultivation of spruce can be compensated by watering. It is important that watering is timed to the specific identified months. February frosts are an almost unavoidable factor limiting the growth of Caucasian spruce in Mos-

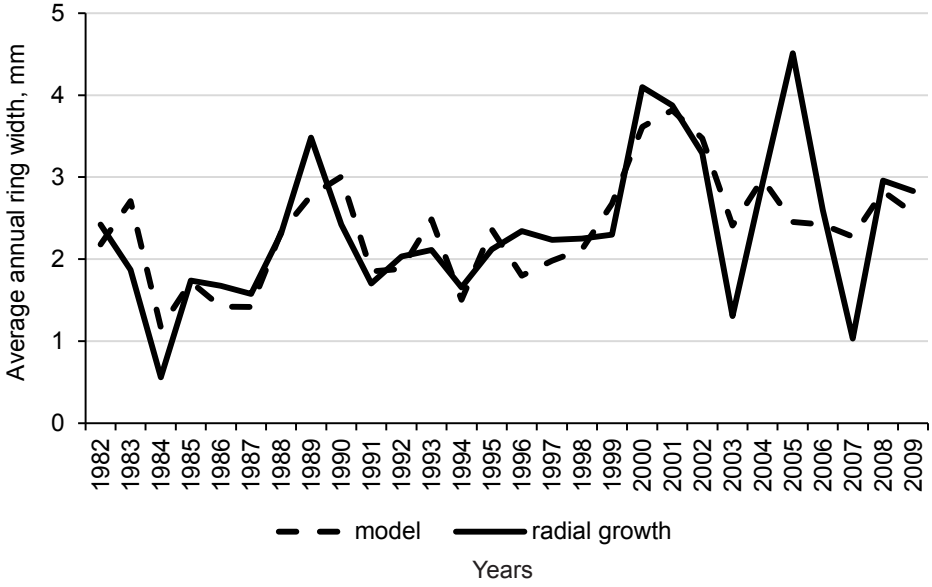

Fig. 9. Radial growth dynamics of Caucasian spruce and results of its modelling.

cow. However, the dynamics of the average monthly temperature in February is such that by 2045 the conditions for the growth of the Caucasian spruce in Moscow should become favourable. This species may become promising for plantation breeding in the Moscow region.

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Post-fire restoration of broad-leaved forests in the foothills of Dagestan

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Abstract

The article presents the results of the analysis of woody vegetation renewal peculiarities after a crown fire, which broke out in 2010 in a beech-yew forest in Termenlik district (965 m a.s.l.) on the south-eastern slope of Gimrinskiy Ridge in the Foothill of East Caucasus in Dagestan. For the description of the changes, which occurred on the post-fire spot in 2019, eight sample plots with areas 100 m^2 each were arranged. A geobotanical description of the sample plots was conducted according to the generally accepted method, which included the definition of the microrelief, tree species composition, their distribution in the layers and biometric parameters. For each tree species, projective cover in percentage was revealed, as well as abundance, sequence of penetration and distribution, dynamics of the development of the above-ground part and the results of their competitive interaction were evaluated. Owing to the appearance of new species of seed origin with accelerated growth in the composition of the forest stand, such as *Populus tremula* L., *Salix caprea* L., *Acer campestre* L., *Fraxinus excelsior* L., a suggestion was made about the change of the succession trend and the formation of a newly reformed coenosis without participation of the basic dominant species of the primary forest, namely *Fagus orientalis* Lipsky, *Carpinus caucasica* L., *Taxus baccata* L., *Acer platanoides* L. A method of mechanical removal of the dominant species *Populus tremula* L. was offered, leading to the enhancement of the competitive advantages of pioneer species characteristics of this locality.

Key words: broad-leaved forest, competition, competitive advantages, crown fire, dominance, pioneer species, species richness.

Introduction

In Dagestan, due to the relative dryness of the climate, fires are a serious negative factor of destruction of established arboreal planting. Fires break out here in predominantly foothill woods in summer during prolonged drought and in intramountain and high-mountain forests in winter and early spring.

On the whole, forests occupy a relatively insignificant territory in Dagestan – 10.6 % (561,800 ha). According to the composition of the dominant species, they are represented by low-lying and mountain bottomland poplar and willow forests, foothill oak and beech-hornbeam forests, intramountain pine forests, high-mountain

pine-birch and beech forests.

The above-mentioned plantations vary based on different susceptibility and resistance to fires and possess different demutation potential. It is widely accepted that coniferous forests are more susceptible to fires than broad-leaved ones (Proença et al. 2010).

At the same time, depending on ecological conditions of growth, conditions of origin, intensity of distribution, repetitiveness and duration of interfire intervals, the renewal of coenoses in the broad-leaved, as well as in coniferous forests, are subject to certain general patterns. Thus, out of the herbaceous plants the most widespread pioneer post-fire species, regardless of the type of forest, are *Chamaenerion angustifolium* (L.) Scop., *Impatiens noli-tangere* L.*, Urtica dioica* L.*, Calamagrostis langsdorffii* L. (Krasnoschyokov et al. 2010; Sabirov and Sabirova 2011, 2017; Sabirov 2019, Sabirov et al. 2019). Out of the arboreal species in place of burnt coniferous forests, due to its biological and ecological features, the first to appear is the pine, and the possibilities of its fixation in the burned-out forest grow despite the relative poverty of conditions of soil nutrition. Out of post-fire pioneer arboreal plants the most frequently mentioned species are *Populus tremula* L., *Salix caprea* L., *Quercus petraea* (Matt.) Liebl., *Acer campestre* L. (Komarova 1986, 1992; Komarova et al. 2007) and especially aspen species (Kuleshova et al. 1996, Kay 1997, Tereshkin and Tereshkina 2006, Komarova et al. 2007, Kalinin 2008, Ivanova and Golubtsova 2010, Khapugin et al. 2013, Krasnow and Stephens 2015).

This paper presents the results of studying the sequence of penetration and distribution of tree species in the post-fire area of a broad-leaved forest, the dynamics of development of their aerial parts, the competitive advantages of some species, and changes in the succession trend by mechanical removal of dominants.

The state of knowledge of the issue

The role of fires in the formation of forests has been summarized in many works (Melekhov 1981; Sannikov 1981; Komarova 1986; Furyaev 1988, 1996; Sabaeva 2005a). They deal with the conditions of their appearance, intensity and peculiarities of their distribution, repetitiveness linked to the ecological regimen of natural complexes, morphological structure and the character of post-fire coenoses, as well as the duration of interfire intervals.

It is universally recognized that fires have a strong impact on the condition of afforestation and are one of the main factors of their attenuation and death. The resistance of forests to fires is influenced by many factors: climate, structure of coenoses, trees size (Keyser et al. 2017). While comparing climatic and typological conditions in order to explain the patterns of renewal of forests after fire, even the local variety of geological layers turned out to be important (Torres et al. 2016).

Meanwhile, many researchers consider fire an important factor stimulating forest formation in nature. The resulting change of layers and the following post-fire successions are considered to be the most widespread form of forest dynamics on the territory of North Siberia (Rozanov 1999), in the south of Siberia (Buryak et al. 2007), in the basin of the Amur river (Sokolova and Verkhoturov 2019), in the Central Yakutia (Utkin 1965, Lytkina and Protopopova 2006, Nikolaev 2010), in Crimea. The forest is formed after the fire depending on its periodicity, ecotope con-

ditions, intensity of combustion providing the so-called 'pyroecological' conditions (Druzhinin 2013).

Special significance during the assessment of vegetation in the burned-out forest is attributed to the initial number of newly settled species, which are responsible for the speed and direction of the succession process (Sabaeva and Nikitina 2004; Sabaeva 2005b, 2006; Kalinenko et al. 2006). In the very first years the number of species can reach 'from 17 to 30' thousand examples per hectare and even 235 thousand examples per hectare (Dancheva et al. 2015). Later the increase in number of each species can vary depending on their biological peculiarities, provision with moisture and moesorelief. According to the universally accepted opinion, the absence of competition in the first years after fire leads to the spread of the species, mainly anemochores, and the bloom of their seeds is possible tens of kilometres away. In some cases burned-out forests are covered with grassy vegetation from main-rooted and rhizomatous species (Asadulaev and Omarova 2016), which preserve leader buds in the soil.

In the conditions of sufficient humidification of the soil after fires, the most frequent species to spread, out of the arboreal species, are the species of aspen and willow, which can lead to the change in the development of the stand of trees in the direction of enhancement of their role (Kuleshova et al. 1996, Tereshkin and Tereshkina 2006, Kalinin 2008, Khapugin et al. 2013). The role of aspen as pioneer species of the post-fire renewal of forests, contributing to the biodiversity of forest landscapes, is indicated in reference to many regions of the Holarctic (Kay 1997, Komarova et al. 2007, Ivanova and Golubtsova 2010, Krasnow and Stephens 2015). For example, in the western part of the USA, aspen, having the ecological amplitude, grows in wermuth steppes, in orohylile forests and sometimes reaches the tree line (Mitton and Grant 1996). It has been noted that, compared to coniferous forests, aspen stands provide enhanced afflux of water and resistance of ecosystems to fires (Shepperd et al. 2006). For this and other reasons many researchers consider aspen forests the most important type of post-fire deciduous forests (Long and Mock 2012). Aspen is widespread not only after fires, but also on the territories deserted after the extraction of the minerals, landslides, timber cutting and other breach of forest systems (Shepperd et al. 2006).

The question of longevity of the existing and newly emerged aspen forests is being discussed in the literature. The majority of the specialists hold the opinion that the longevity of the existing aspen forestry is achieved through clonal reproduction, and the capture of new violated territories occurs due to the multiple seedlings (Einspahr and Winton 1977, Turner et al. 2003, Landhäusser et al. 2010, Krasnow et al. 2012, Fairweather et al. 2014, Krasnow and Stephens 2015). The latter opinion about the spread of aspen trees on burned-out areas as a result of drifting of seeds from the outskirts has also been proven on the post-fire spot examined by us in the locality Termenik in the Foothill Dagestan (Asadulaev and Omarova 2016). As there are no big forests of this species here, there are only sporadic examples among beech and hornbeam forests. In some works on preservation of longevity of aspen forests, it is recommended to conduct rejuvenation of existing stands, removal of coniferous trees, controlled setting fire and protection from herbivores (Shepperd et al. 2001, Jones et al. 2005, Bates et al. 2006).

Among other post-fire pioneer arboreal species in the warmest habitats with periodically dry and poor soils, *Quercus mongolica* Fisch. ex Ledeb., *Acer mono* Maxim. ex Rupr., *Pinus koraiensis* Siebold & Zucc. (Southern Sikhote-Alin) have been cited (Komarova 1986, 1992; Komarova et al. 2007).

The most widespread post-fire pioneer species out of the herbaceous plants are *Chamaenerion angustifolium* (in rhododendron and clusterberry pine forests) (Krasnoschyokov et al. 2010), *Corydalis* (in pseudo-taiga larch forests of the Central Khangai) (Dorzhsuren and Krasnoschyokov 2007), *Impatiens noli-tangere* – in the areas with greater soil humidity, *Urtica dioica* – on rich and humid soils in oak forests of the Mordovia State Nature Reserve.

At the first stages of post-fire renewal in the course of the pyrogenic succession the number of species of herbaceous plants initially grows, while at the later stages it decreases. Only by the fourth year perennial plants slowly replace annual ruderal species (Sabirov and Sabirova 2011, 2017; Sabirov et al. 2019). On the spots with low fire intensity the richness of annual herbaceous plants is higher, while on the spots with high intensity there has been observed the opposite situation. The number of indicator species decreases as fire intensity grows. There has been detected a higher level of carbon, microbal biomass and nitrogen on spots without fire compared to post-fire spots (Heydari et al. 2016). The grass canopy is thought to fix the surface faster than trees and it protects it from erosion, which is especially important in mountain conditions.

The majority of researchers noted that in the first years after the ground fires conditions are formed, which are close to the optimal for the emergence, survival and growing of plantlets of trees owing to the removal of competition on the part of herbaceous-shrub layer, improvement of water and physical peculiarities of soils and enhancement of biological cycling of substances (Sannikov 1981, Maslakov 1984, Dylis 1985, Evdokimenko 2008). In other cases, a significant impact on the reconstruction of vegetation after fire is made by the so-called ‘nanny-plants’, i.e. secondary succession can be launched in the state of pioneer coenosis by means of increase in the frequency of emergence of seedlings of tall shrubs, aiphyllous and deciduous trees under the small shrubs (Siles et al. 2008).

The most extensive information in literature related to post-fire succession processes and the consecutive stages of changing of dominant species has been presented in reference to the Russian coniferous forests. It has been noted that their reconstruction varies in different regions and it depends on certain physiographic and geological-geomorphological conditions. The first issue to be addressed is the fact that the radical change of light conditions after the fires leads to disappearance of the majority of typically hylile photophygous species from the coenosis. Newly created ecological niches are immediately inhabited by heliophilous plants the most widespread of which from the herbaceous species is *Chamaenerion angustifolium*.

The first species, out of the arboreal ones, which appears on the burned-out spot during the burning of coniferous forests due to its biological and ecological peculiarities, is *Pinus koraiensis* but the possibilities of its settling for a long period are limited, except for the ‘extreme’ landscapes. Under the crown layers of the pine, as well as under the birch, which accompanies it, fir begins to grow, which

slowly occupies the second layer of the forest stand and later penetrates the upper layer. It is impossible for the pine undergrowth as a heliophilous plant to grow here till the next fire passes. Such a regimen of existence of pine forests is called pyrogenic stability. In the majority of cases autochthonous coniferous forests are replaced after the fire by the derivative secondary parvifoliate (birch and aspen) or light coniferous forests (Sannikov and Sannikova 1985).

Besides, in reference to *Pinus koraiensis* there has been substantiated a hypothesis of 'impulse pyrogenic induction' of its renewal. An idea was proposed of the fire as 'information matrix' providing the continuity of horizontal structure of phytocoenoses. The modern ideas of the diverse role of cyclically repeating fires in the transformation of the main components of biogeocoenoses in the forests of light coniferous formation of the taiga zone of the northern hemisphere are considered to be proven (Sannikov 1981, Sannikov and Sannikova 1985). In the pine and fir forests (for example, on the territory of the Kola Peninsula) existing at different stages of post-fire successions there have been demonstrated similar common patterns of formation of age-related, size and vitality structure of coenopopulations. Such data are the evidence of existence of unified mechanisms in the core of which there lie laws of 'intrapopulation competitive interaction of the specimens and ecological and coenotic regulation of renewal processes' (Stavrova et al. 2016).

The most detailed study of the content, structure, formation and development of arboreal ecosystems after fires has been conducted by Sabirov (Sabirov and Sabirova 2011, 2017; Sabirov et al. 2019). On the island of Sakhalin in the light coniferous forests formed by *Larix cajanderi* Mayr, dark coniferous forests formed from *Picea ajanensis* Fisch. ex Carrière. and *Abies sachalinensis* (F. Schmidt) and in the dark coniferous forests with admixture of broad-leaved breeds *Phellodendron sachalinense* (F. Schmidt) Sarg., *Quercus mongolica* Fisch. ex Ledeb*, Kalopanax septemlobus* (Thunb.ex A. Murr.) Koidz*.*, *Acer mono* ssp. *mayrii* (Schwer.) Kitam. Single plants of initial species appear in the second year after the fire. By the third year *Chamaenerion angustifolium*, *Copaifera langsdorffii* Desf., *Vaccinium vitis-idaea* L. actively settle. By the fifth year, *Carex van-heurckii* Müll. Arg., *Scorzonera radiata* Fisch. ex Ledeb., as well as *Artemisia arctica* Less., *Tilingia ajanensis* Regel & Tiling. begin to settle successfully. In the tenth year after fire the occurrence of *Copaifera langsdorffii* Desf., *Chamaenerion angustifolium* decreases significantly and the occurrence of *Carex van-heurckii* Müll. Arg. reaches 95–97 %. At the same time there appear larch, fir, aspen, Siberian dwarf pine (*Pinus pumila* (Pall.) Regel), *Betula middendorffii* Trautv. & C.A. Mey., *Duschekia maximowiczii* (Callier) Pouzar, *Spiraea betulifolia* Pall. In place of burned-out larch forests, sometimes develop pioneer species of mosses, lichens and the Kurile bamboo (*Sasa kurilensis* (Rupr.) Makino & Shibata), completely covering the soil surface, which poses a serious obstacle to the successful dissemination and growth of the indigenous arboreal coenoses.

On the whole, the duration of the renewal of the fir forest in the southern taiga is estimated to be 120–150 years. If we consider a multiple-aged spruce forest as the final phase of the succession, then 150 more years are required for its formation.

There is less information on the evaluation of post-fire successions in the

broad-leaved forests in the literature. The sequence of renewal of such forests in the east of the USA and the south-east of Canada can be cited as an example (Krasnow et al. 2012, Krasnow and Stephens 2015). Here the dominant species of pioneer coenoses in the first year is *Digitaria*, in the second year it is *Rumex*. After the second year, there appear perennial herbs, among which woundwort (*Solidago*) prevails. By the 15–20-th year the bluegrass (*Andropogon*) and other graminoids prevail. By the 30–35-th year the poic stage can be followed by the bush stage where at first low bushes (*Vaccinium, Gaylussacia*) dominate, then tall bushes do (*Quercus ilicifolia* Wangenh.). By the 50-th year the forest stand is completely restored. The first dominant species is the pitch pine (*Pinus rigida* Mill.), later the scarlet and white oaks (*Quercus coccinea* Muenchh., *Quercus alba* L.) appear. These species form young pine and oak forests with well-developed bush cover. Only by the 200-th year, if there are no recurring forests and other breach, an oak forest is formed with insignificant participation (or complete absence) of pine and bushes.

Materials and Methods

The research was carried out in a section of beech-yew forest burned down in 2010 in the Foothills of Dagestan, located at an altitude 965 m above sea level on a slope with north-eastern exposure (Fig. 1).

Study of the patterns of post-fire renewal of the forests in Dagestan has not been conducted before. We initiated this research in 2011–2013 in the area of

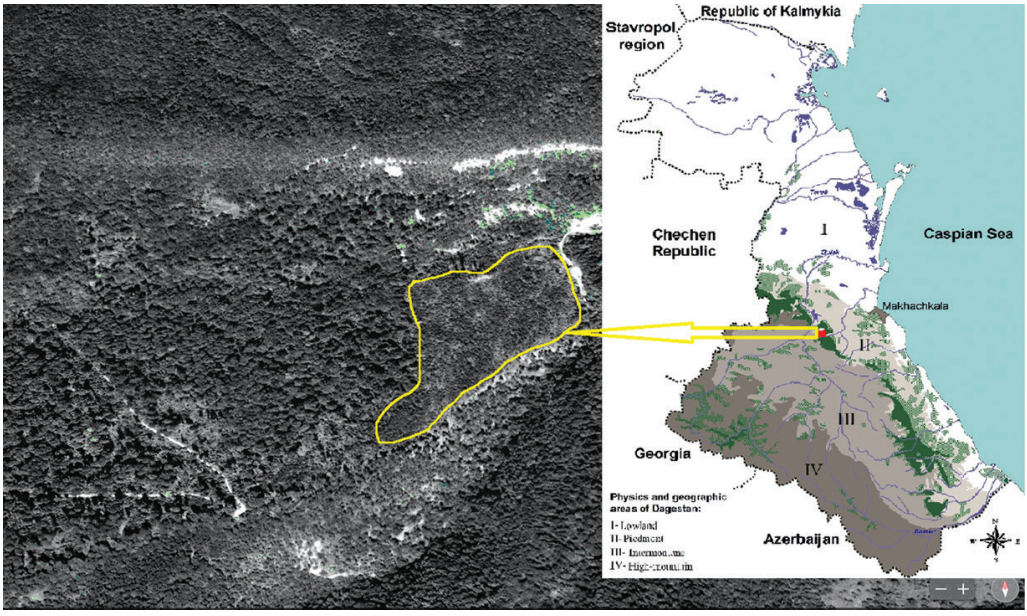

Fig. 1. The area of the burnt forest in the locality Termenlik on the map of East Dagestan.

Note: Caucasus (red dot) and a satellite picture on the Google map (yellow outline). On the map of Dagestan under number II a territory occupied by foothill broad-leaved forests is designated.

beech and yew forest burnt in a crown fire in the locality Termenlik in Foothill Dagestan (Asadulaev and Omarova 2016).

Six years later (in 2019) we continued to study the above-mentioned area. Tasks were set of revealing the sequence of penetration and distribution of arboreal species in the area, their species richness and abundance, dynamics of development of the above-ground part and evaluating the results of their interaction and competitive advantages. There were simultaneously arranged events aimed at changing the trajectory of post-fire renewal of the species composition of the forest using the method of mechanical removal of the dominant species (Krasnow and Stephens 2015), in our case it was *Populus tremula*.

According to the classification offered by Melekhov (1981), the post-fire forestland in the locality Termenlik in the Foothill Dagestan, where we conducted our study, has been assessed by us as the forestland with the forest stand destroyed in the crown fire (the year of fire being 2010).

For the description of further successive changes, which have occurred here since 2011, in 2019 we arranged eight sample areas (SA), 100 m^2 each (10×10 m). The geobotanical description of the SA was conducted following the generally accepted methods (Methods … 2002) and included the definition of the microrelief, revealing the tree species composition, their distribution in layers and biometric parameters of each species. For each tree species, a projective cover in percentage and the number of specimens were revealed. The development of arboreal plants was defined based on the height and diameter of the crown.

The assessment of renewal of arboreal vegetation after the crown fire was conducted in 2019 using the indices of species richness (alpha-diversity) and abundance. The first index was defined as a number of species per area unit, the second one is considered to be more substantial and denotes the number of specimens in each species (its functional weight) (Gasanov 2006). For the evaluation of the species richness Menhinick's index by equation (1) was calculated (Gasanov 2008).

$$D_{Mn} = \frac{S}{\sqrt{N}}, \quad (1)$$

where: D_{Mn} is Menhinick's index; S is number of revealed species; N is total number of specimens.

The basis of the latter index consists of different combinations between the number of revealed species (S) and the total number of specimens of all species (N). The great value of the index, according to the calculations, corresponds to a greater diversity and is related to, first of all, the measure of achievement by the coenosis of ecological balance with the environmental conditions.

While comparing the numerical indices of the species abundance, a question of the main dominant species and the succession trend of development of the post-fire broad-leaved forest in the Foothill Dagestan arises. To our mind, one can get the answer to this question by comparing two factors, namely numerical superiority of the species at the initial stage and the growth rates (biometric factors) of the trees.

The first factor is based on the relative abundance of species and can be calculated using the Shannon's index (P_j), which is the ratio of the number of specimens of given species to their total number in the sample, i.e. $P_j = \frac{n}{N}$.

Mathematical treatment was conducted by methods accepted in biology using the programme Statistica for Windows 6.0.

Results and Discussion

Evaluation of post-fire renewal of arboreal vegetation

On the examined spot after a year after the fire (in 2011) sprouts were discovered of seeds, ratoons and an undergrowth of ten arboreal species (*Acer platanoides* L., *Populus tremula*, *Fagus orientalis* Lipsky, *Carpinus betulus* L. *Tilia cordata* Mill., *Rubus caucasicus* Focke, *Euonymus europaeus* L., *Euonymus latifolius* (L.) Mill., *Euonymus verrucosus* Scop., *Sambucus nigra* L.). In the unburned forest adjacent to the post-fire area, in addition to the above-mentioned species, there are also: *Fraxinus excelsior* L., *Populus tremula*, *Rubus caucasica* Focke, *Quercus petraea*, *Ligustrum vulgare* L., *Mespilus germanica* L., *Rhamnus cathartica* L., *Ulmus glabra* Huds., *Acer campestre*, *Cerasus avium* L., *Crataegus monogyna* Jacq., *Crataegus* sp., *Pyrus caucasica* Fed., *Prunus divaricata* Ledeb., *Quercus macranthera* Fisch. & C.A. Mey. ex Hohen., *Rosa* sp., *Rosa canina* L., *Rubus idaeus* L., *Sorbus aucuparia* L., *Salix caprea*, *Sambucus nigra*, *Swida australis* (C.A. Mey.) Pojark. ex Grossh., *Betula litwinowii* Doluch., *Cornus mas* L., *Lonicera caprifolium* L., *Malus orientalis* Uglitzk., *Tilia cordata*, *Rubus caucasicus*, *Taxus baccata* L., *Ulmus minor* Mill., *Viburnum lantana* L., *Pinus kochiana* Klotzsch ex K. Koch, *Robinia pseudoacacia* L.

The number of pioneer species (due to the abundance of soil nutrition resources, the absence of competitors) in the next two years increased to 23 species (Table1). At the same time, the values of the species richness index remained very low. In subsequent years (in our case, from 2013 to 2019), most of the species growing in the vicinity (some earlier, others later) penetrated into the post-fire territory, including *Acer platanoides* L. *Carpinus betulus, Fagus orientalis*.

At the same time the number of species per area unit has increased significantly (in 2011 there were 10 species, in 2019 – 23), and the index 'the total number of specimens of all species' has changed slightly, or even decreased because of the increase in the size of specimens.

Table 1. Participation of tree species in the post-fire restoration of broad-leaved forest (year of fire – 2010, year of accounting – 2019).

Species	The number of specimens on the spot (100 m²)								Species richness	Specimens density of species per 100 m²	Occurrence, %
	1	2	3	4	5	6	7	8			
Acer platanoides	46	45	35	27	28	34	44	34	293	36.6	100
Populus tremula	9	51	12	7	26	40	22	6	173	22.6	100
Carpinus caucasica	22	0	36	52	2	2	18	22	154	19.3	87.5
Fagus orientalis	4	0	24	28	1	2	3	2	64	8.0	87.5
Salix caprea	8	7	4	0	7	6	18	5	55	6.9	87.5
Fraxinus excelsior	0	0	1	4	0	0	4	0	9	1.1	37.5
Tilia cordata	0	2	0	2	2	0	0	0	6	0.8	37.5
Betula litwinowii	0	0	0	0	4	1	1	0	6	0.8	37.5
Quercus iberica	1	1	0	0	0	0	0	0	2	0.3	25.0
Ulmus glabra	0	0	0	0	0	0	0	1	1	0.1	12.5
Malus orientalis	0	0	0	0	0	0	1	0	1	0.1	12.5

Crataegus monogina	1	0	0	0	0	0	0	0	1	0.1	12.5
Rubus caucasicus	1	1	1	0	1	1	0	0	5	0.6	62.5
Euonymus europaeus	1	1	0	1	0	0	1	1	5	0.6	62.5
Euonymus verrucosus	0	1	1	1	0	0	0	1	4	0.5	50.0
Sambucus nigra	0	0	1	0	0	0	1	1	3	0.4	37.5
Rosa canina	0	0	1	1	0	0	1	0	3	0.4	37.5
Euonymus latifolius	0	1	1	0	0	0	0	0	2	0.3	25.0
Rhamnus cathartica	0	1	0	0	0	0	0	0	1	0.1	12.5
Cornus mas	1	0	0	0	0	0	0	0	1	0.1	12.5
Ligustrum vulgare	0	0	0	1	0	0	0	0	1	0.1	12.5
Mespilus germanica	0	0	0	0	0	0	1	0	1	0.1	12.5
Lonicera caprifolium	0	0	0	0	0	0	0	1	1	0.1	12.5
Specimen density	94	111	117	124	71	86	115	74	792	99	
Species density	10	10	11	10	8	7	12	10	9.8		
Menhinick's index D_{Mn}	1.03	0.95	1.01	0.90	0.95	0.75	1.12	1.16	0.82		
The Shannon index									1.757		
Pielou's evenness index									0.845		

Based on the results of calculations made on the spot, Menhinick's index for the estimation of which there were used factors *S* (the number of discovered taxa) and N (the total number of specimens) has changed from 0.75 (on the spot with 7 species and 86 specimens) up to 1.16 (on the spot with 10 species and 74 specimens), the maximum number of specimens being 124.

Of the total number of species (39) growing in the unburned part of the forest, 16 species have not yet been found in the post-fire area during the period of ten years.

The missing species are the habitual species for the lower layers of broad-leaved forests of the Foothill Dagestan, namely *Cerasus avium*, *Crataegus* sp., *Pyrus caucasica*, *Prunus divaricata*, *Quercus macranthera*, *Rosa* sp., *Rubus idaeus*, *Sorbus aucuparia, Taxus baccata*, *Ulmus minor*, *Viburnum lantana*, except for *Pinus kochiana* and *Robinia pseudoacacia*, which are not characteristic of such forests in Dagestan. The last species is invasive and occurs on the lightened spots along the roads, and the pine is solitary here and occurs on pebble outcrops along the river downcuts.

In the next years, given the preservation of the species abundance on the same level (around 800 specimens per 800 m^2) and increase in the number of species up to 37 (without the last two species), Menhinick's index will acquire a value equal to 1.3. Such an increase is to be expected in the next 10 years. Simultaneously with size enlargement of the trees growing here there will enhance a competition which will contribute to the withdrawal of a significant number of specimens (with the preservation of the total number of species, the number of each species can significantly decrease). The index factor will increase accordingly. On the whole, based on Menhinick's index, the optimum factors of species richness for the sample

areas had not been reached by the year of 2019 and this process will be enhanced intensively.

Additional information on the condition of arboreal vegetation on the postfire spot is provided by the factor of species density, which is the number of species per sample area unit. This parameter in the sample area fluctuates between 7 and 12 species per 100 m^2.

In the first and the following years in all sample areas without exception two species were discovered (occurrence): *Acer platanoides* and *Populus tremula*. The difference between these two dominant species lies in the fact that the first species prevails based on the number and biomass gain (crown height and diameter), which defines its competitive advantages. A little more rarely (87.5) occur *Salix caprea*, *Fagus orientalis*, *Carpinus betulus*, whose biomorphological parameters are also much lower than those of *Populus tremula*.

The species abundance and the species density in the ecological studies are used for the evaluation of the prevalence of the species and its functional weight in the coenosis. The notion of species domination is derived from the species abundance (Gasanov 2008). In our case the highest indices of domination have been observed in the following species: *Acer platanoides* (293), *Populus tremula* (173), *Carpinus betulus* (154), *Fagus orientalis* (64), *Salix caprea* (55), the functional weight of which is 93.3 %. The share of Norway maple alone is 40 %. Practical experience shows that Shannon's diversity index (P_j) lies in the interval between 1.5 and 3.5 and rarely exceeds 4.5. In our case P_j = 1.757. As the number of species in the coenosis grows, so do the factors of the index. That is why in order to obtain the characteristics independent from the number of species in the sample, one apply to the calculation of the Pielou's evenness index (the ratio of the observed diversity to the maximum one).

The evenness index is rather high here, which makes it 0.845. As this factor takes the value from 0 to 1, one can deduce that the analyzed sample includes the majority of the species of the studied arboreal coenosis, and the high value of evenness corresponds to the situation of equal abundance of all species.

Biometric indicators of the development of arboreal species

Crown height and diameter are the most important biomorphological indicators reflecting the development of trees and their competitive advantages in woodlands. A high positive correlation was revealed between these indicators in the trees that grew on the site after fire (N = 754, r = 0.899). This dependence gave us the basis for comparing the data obtained for different types of species and using them to interpret post-fire processes.

In addition, according to the results of a two-way analysis of variance, differences in biomorphological indicators between the trees of the same species growing on different sites werenot proven (Table 2). This indicates the representativeness of the trial site data, i.e. the size of the plots of 100 m^2 fully reflects the overall morphological diversity of the arboreal species present here. At the same time, differences in height and diameter between species are significant; the effect of species differences (h^2, %) in height reaches 57.2 %, which is slightly higher than in crown diameter – 49.3 %. Based on statistical indicators for SA and for species, a further comparative analysis of arboreal species was carried out by the height of their crowns.

Table 2. The results of analysis of variance by crown indicators species.

Factors	Statistical factors						
	Df Effect	MS Effect	Df Error	MS Error	*F*	*p*	h^2, %
Crown height							
Sample area	7	22.04	13.7	13.20	1.66	0.19	3.4
Species	12	68.56	746	1.07	63.84	0.0000	57.2
Crown diameter							
Sample area	7	11.22	14.6	12.98	0.86	0.55	0
Species	12	65.33	746	1.51	43.09	0.0000	49.3

Note: *F* – Fischer's criterion; Confidence level: *$p < 0.05$, ** – $p < 0.01$, *** $p < 0{,}001$; h^2 – force of influence of a factor.

The correlation of the group of trees by crown height, irrespective of their belonging to a certain species, is represented in Figure 2. There are only ten groups with sizes from 0.5 to 9 m. The proportion of 'taller' trees assigned to four groups from 5 to 9 m on all the sites is insignificant and amounts to a maximum of 5 %, except for the sample area No.6, where the proportion of trees of these groups is about 26 %. The presence of bigger trees in sample area No.6 can be possibly explained by the environmental conditions, as this sample area is characterized by a more inclined microrelief, which contributes to a slightly bigger accumulation of moisture and elements of mineral nutrition. The majority of trees (from the total number of trees) can be attributed to plants from 1 to 2 m. *Populus tremula* and *Salix caprea* are practically not represented in this group. The group of the smallest plants with height of 0.5 m (the minimal size) is sufficiently representative (in some sample areas their share is

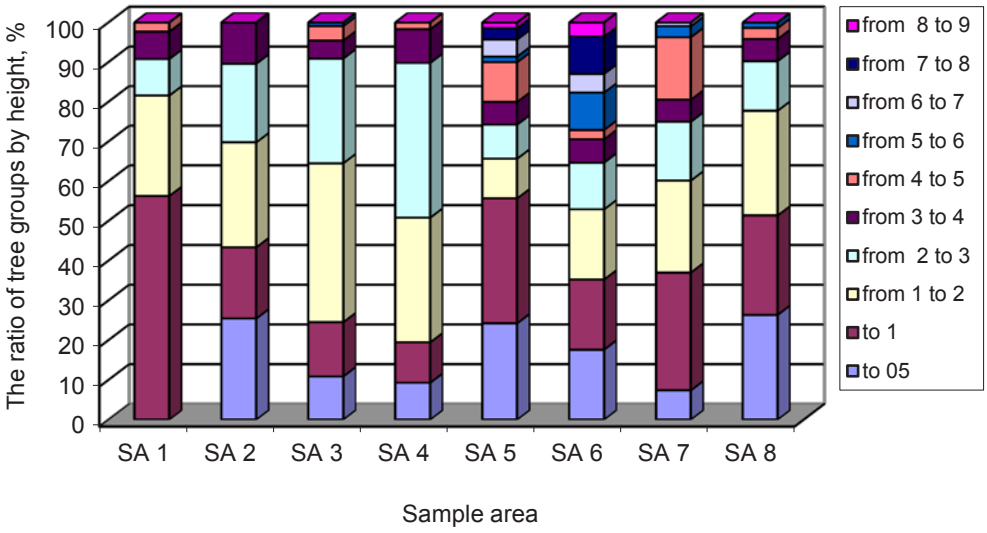

Fig. 2. Quantitative ratio of the trees according to their height in the sample area irrespective of their belonging to the species.

up to 20 %).

The significant number of plants of the latter group is associated with two factors. First of all, it points to the presence of species, which grow very slowly at the young age. For example, 5-year-old maple trees reach the height of 0.5 m, which is indicator of insignificant annual gain. Secondly, a high share of 0.5 m trees in the sample area points to the fact that every year seeds of plants of different species reach the sample area and the seeds of plants have been germinating for 10 years already (since 2011).

The picture that shows the distribution of specimens of different species by their sizes turned out to be more informative (Fig. 3). As shown in the figure, the whole of the left side (groups with specimens sized 4 m and above) is occupied by two species, namely *Populus tremula* and *Salix caprea*, while the right side is occupied by the 5 remaining species, i.e. *Fraxinus excelsior*, *Tilia cordata*, *Carpinus betulus*, *Fagus orientalis*, *Acer platanoides*. A special place is occupied by *Acer platanoides*, which has the highest share of plants with insignificant growth (293 specimens), (P_j = 0.369), where 95 % of specimens have the height less than 0.5 m.

The dispersion of the crown height index *CV* (%) in the sample area is significant in the species, while the lime-tree has the lowest one (5.6–31.7), which proves the identical growth rate of the specimens of this species. Populus trees have the highest dispersion (24.0–74.0) (Table 3). In this species in all sample areas there prevail middle-sized plants – up to 3 m (mode – 2.9), at the same time some specimens reach significant maximum sizes – up to 8.5 m. There also occur plants with the amount of growth less than 0.5 m. Hornbeam, ash-tree and lime-tree have approximately identical maximum size of the trees; the share of their height from the total height of all the trees (M_j, %) is approximately the same – 10–11 %. The maximum share from the total height of all trees is reserved for the aspen, which secures absolute competitive advantage for this species.

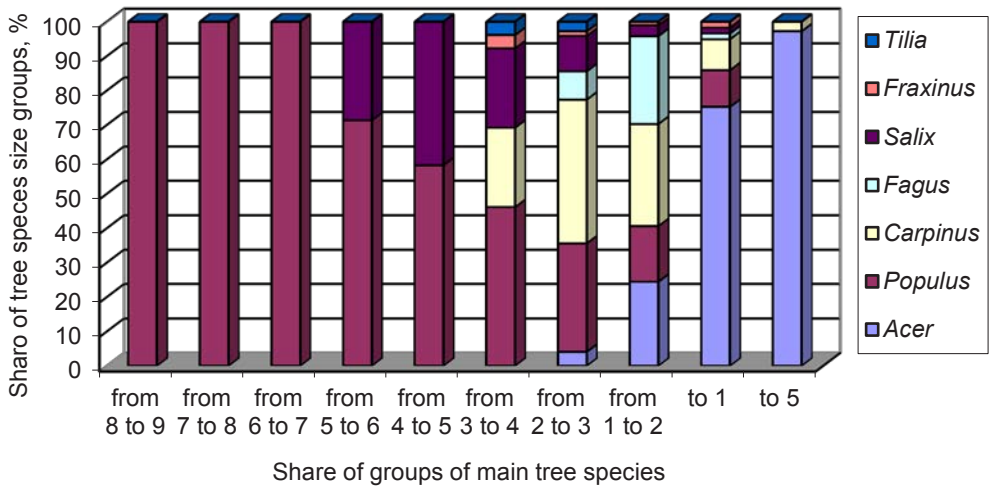

Fig. 3. The share of size groups of seven arboreal species on the post-fire forest spot.

Table 3. Statistical characteristics of the data based on the crown height of the main arboreal species in the post-fire forest area.

Indices	Tree species by genera							
	Populus	***Salix***	***Carpinus***	***Fraxinus***	***Tilia***	***Fagus***	***Acer***	***Betula***
CV, %	24.0–74.0	13.6–60.9	15.7–53.1	22.9–50.3	5.6–31.7	6.7–52.1	42.1–79.3	21.0
Mode, m	2.9	3.0	1.3	0.4	1.0	0.9	0.3	0.2
Maximum height, m	8.5	5.5	3.5	3.5	3.3	2.5	2.5	2.0
M_i, %	27	18	11	11	10	8	8	6

Note: *CV*, % is dispersion of the crown height index; M_i, % is the share of the index of the maximum height of each species in the total height index of the trees of all species.

At the same time, aspen trees have demonstrated significant drops in the amount of growth depending on the year; in 2014 it was reduced by two and a half compared to 2013 (up to 0.5 m), remaining on the same level for four more years (Fig. 4). The dynamics of growth of two other dominant species (in terms of number) of the second layer (maple and beech) has been homotypic and stable for years and it does not reflect the dependence on the ambient conditions.

The fluctuation of the annual amounts of growth shows that aspen is more sensitive to the conditions of moisture-providing of the soil and it, apparently, reflects the drops in the amount of precipitation in the same period (Table 4). According to the data provided by Buynaksk weather station in 2014, the amount of precipitation from April to July dropped by 136.7 mm (from 391 to 254.3 mm).

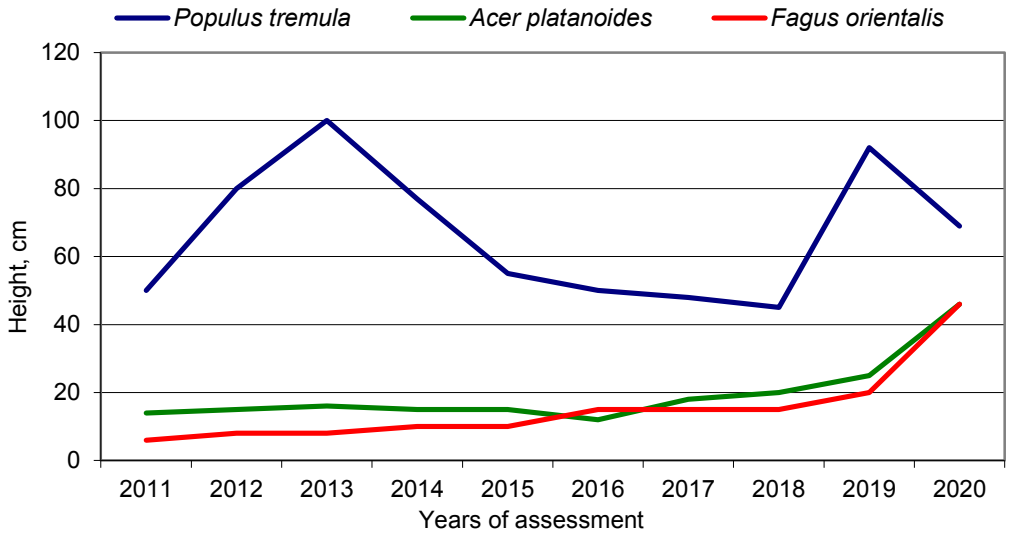

Fig. 4. Shoot growth dynamics of the dominant species in the sample area after the fire in the location Termenlik.

Table 4. The data reflecting the changes in the average late-spring and early-summer temperature and precipitation (from April to July) by year for the area Termenlik.

Indices	Years									
	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
Average temperature, °C	18.4	20.8	18.9	19.9	19.0	19.7	18.8	19.8	20.0	19.4
The sum of precipitation, mm	382	265	391	254	222	248	299	277	171	208

August 2014 was especially arid – only 7.8 mm. Radical fluctuations in the length of growth of the aspen can be explained by a greater dependence of the plants of this species on climatic conditions of the location, especially on the humidity of the soil (Tereshkin and Tereshkina 2006, Khapugin et al. 2013, Krasnow and Stephens 2015). In the following years the amount of precipitation was low, as well.

In two other species the lines representing the annual dynamics show gradual increase in the amount of growth. The growth curve (especially at the beginning of ontogeny) is considered to be species-specific and is less dependent on the ambient conditions than in the next years. The annual amounts of growth of the maple and beech in the first eight years after fire were insignificant, and in 2019 and 2020 the burgeon growth intensified – 22 and 24 cm, respectively. Such growth dynamics is associated with a more pronounced internal genetic control of the escape of these species (under relatively stable environmental conditions), and also means less dependence on precipitation fluctuations.

More intense annual burgeon growth naturally provides bigger total crown height of the trees. The average height of aspen trees in the sample area is 6.8 m, the maple – around 2.0 m, the beech – 1.5 m, which secures significant competitive advantages for the first species.

Conclusions

The study of the mechanisms and stages of the forest renewal after fire is an important task requiring long-term observations, the knowledge of natural pre-fire flora, methods of geobotanical studies, physiological peculiarities and the types of adaptive strategies of plants participating in the succession process. The obtained data, as a result of years of study, will hold importance for the explanation of natural processes of the renewal of broad-leaved forests in the Foothill Dagestan after fires, and at the same time they will be relevant for organizing events aimed at their artificial reconstruction.

By the 10-th year after fire the introduction of the main arboreal species from the part of the forest untouched by fire had practically finished (Pielou's evenness index = 0.845). Out of 39 potential species, 23 were discovered. The missing species are habitual for the lower layers of broad-leaved forests of the Foothill Dagestan, namely: *Cerasus avium*, *Crataegus* sp., *Pyrus caucasica*, *Prunus divaricata*, *Quercus macranthera*, *Rosa* sp., *Rubus idaeus*, *Sorbus aucuparia*, *Taxus baccata*, *Ulmus minor*, *Viburnum lantana, Pinus kochiana* and *Robinia pseudoacacia*, which are not dominant forest-making species and cannot define the trajectory of development of the demutation forest.

All sample areas without exception in

the first and the following years were dominated by two species – *Acer platanoides* (293 specimens per 800 m^2) and *Populus tremula* (173). High indices of abundance were registered in reference to the species *C. caucasica* (154), *Fagus orientalis* (64), *Salix caprea* (55). The difference between the indicated species lies in the fact that *Populus tremula* is only represented by a greater number, but also surpasses other species in terms of height and crown diameter (the average height of aspen trees is 6.8 m, maple – 2.0 m and beech – 1.5 m).

The numerical superiority and high growth rates (biometric factors) of the trees at the initial stage secure absolute competitive advantages for *Populus tremula* defining the trajectory of development of the post-fire demutation forest. Taking into account the resource and nature conservation value of the beech and yew forest for the locality Termenlik, the removal of the species dominating at the first stage of the forest renewal is important. This action is considered to lead to the enhancement of competitive advantages of other species with predictable consequences. In the studied post-fire coenosis the development will change in the direction of the beech and yew forest initial for this locality, and not the coenosis with the dominant aspen.

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Mycorrhizal status of *Diospyros celebica* Bakh. (Ebenaceae), an endangered endemic species from Sulawesi Island, Indonesia

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Abstract

Arbuscular mycorrhizal fungi (AMF) play an important role in the composition and growth of plants in forest ecosystems. Research on mycorrhizal status in endemic plant species is still lacking. This study aims to determine the AMF status of ebony (*Diospyros celebica* Bakh.), which is one of the endemic plant species that are threatened with extinction on the island of Sulawesi, Indonesia. The collection of rhizosphere soil samples and ebony roots was carried out in three ebony stands on Sulawesi Island, namely Maleali village and Tovalo village, Parigi Motong district, Central Sulawesi province and in Ako village, Pasangkayu district, West Sulawesi province, Indonesia. Identification of AMF species was carried out at the Forest Biotechnology Laboratory, Bogor Agricultural Institute. We found 19 AMF species from two genera (*Glomus* and *Acaulospora*) associated with ebony, with the distribution of 7 AMF species in Maleali village, 5 and 7 AMF species in Tovalo village and Ako village, respectively. Furthermore, AMF colonisation on ebony roots was in the low category, between 0.33 % and 2.47 %, with a spore density of 38–162 per 50 g of soil. The results of this study indicate that *Diospyros celebica* is associated with AMF. This information can be used as a guide in the use of AMF to optimize the cultivation, conservation and restoration programs of this species in the future.

Key words: *Acaulospora,* colonisation, ebony, *Glomus*, spore density.

Introduction

Currently, there are very few data regarding the status of mycorrhizae in endangered plants in the world (Evelin et al. 2019). Bothe et al. (2010) reported that based on the International Union for Conservation of Nature (IUCN 2022a) database, only 139 rare plant species belonging to 44 families have been screened for mycorrhizal status (Wang and Oiu 2006). The 54 plant species are classified as endangered, 13 species as Near Threatened and 72 are included in the Least Concern category. And among the 54 species that are endangered, 11 plant species are categorised as critically endangered, 6 endangered and 30 vulnerable.

Tree species are threatened with extinction in Indonesia, which have been studied in symbiosis with arbuscular mycorrhizal fungi, among others; Kuku wood (*Pericopsis mooniana* Thw.) (Husna et al. 2015), Kalapi (*Kalappia celebica* Kosterm) (Asrianti et al. 2016), Bisbul (*Diospyros blancoi* A.DC.) (Ningsih et al. 2013) and Jenang (*Daemonorops draco* Blume) (Purwati et al. 2019). Meanwhile, there are still many other forest plant species that are threatened with extinction and it is not yet known whether they are in symbiosis with mycorrhizae or not. One example is *Diospyros celebica* Bakh. which by the people in Sulawesi island is known as ebony. This species has been categorised as an endangered (vulnerable A1cd) since 1998 in the Red List of IUCN (2022b).

Ebony (*D. celebica*) is a genus of Ebenacea family, one of the endemic plant species on the island of Sulawesi, where natural populations can only be found in Central Sulawesi (the districts of Poso, Parigi Moutong and Donggala), South Sulawesi (the district of Gowa, Barru, Maros, Sidrap and Mamuju) and Gorontalo (Hendromono 2008). Ebony produces luxury wood that has high economic value and has been ratified as the mascot of the province of Central Sulawesi through the Decree of the Governor of Central Sulawesi No. 660/78/1995 dated 27 February 1995 (Wulandari et al. 2017). Ebony has a very beautiful wood pattern arranged in black and brownish red strips, because of its distinctive wood pattern, very strong and durable. This species has a high commercial value and is one of the trade timbers classified as a luxurious type of wood that is in great demand by the public, both in the domestic and interntional markets and is one of the causes of its limited existence in nature.

Ebony is a type of wood that grows very slowly, with a life cycle of about 100 years and even up to 200 years. The slow growth will be an obstacle in its development, besides there is no much data about its growth criteria (Rahman and Abdullah 2002). Many replanting efforts have been carried out in logged-over areas, but the success rate for planting is very low, presumably due to a lack of knowledge about the ecology of where ebony species grow (Allo 2002).

Arbuscular mycorrhizal fungi (AMF) have the ability to colonisa various types of plants found in various habitats in the tropics (Zhi-Wei et al. 2001). They are found abundantly in almost all natural terrestrial communities and are in an obligate symbiosis with the roots of about 80 % of terrestrial plants. This form of symbiosis is the oldest, the origins of which can be traced back to 400 million years ago (Harley and Smith 1983, Smith and Read 2008). Arbuscular mycorrhizal fungi including Glomeromycota are beneficial soil microorganisms that have a key function in a complex network of biotic interactions below and above the soil (Turrini and Giovannetti 2012). However, the effectiveness of AMF in infecting host plants varies, because AMF has certain specifications for selecting host plants. Host plants and conditions of the surrounding environment will indicate the level of presence of AMF (Goltapeh et al. 2008).

One of the efforts to increase the growth of ebony seedlings in the context of their conservation efforts is the use of AMF. Utilisation of natural AMF from local plants will be more effective than the use of isolates from outside where the plants are grown. This is because AMF is a living microorganism with adaptability to a specific host and environment. Differences in location and rhizosphere will result in differences in the diversity of species and

populations of AMF (Widiastuti and Kramadibrata 1992). Therefore, a study was conducted on the status of AMF in ebony rhizosphere which is the first step in the exploration and utilisation of local superior AMF to increase the growth of ebony plants and before this species becomes extinct on the island of Sulawesi.

The objectives of this research were to study the colonisation and density of AMF spores in *Diospyros celebica* stands in Sulawesi Island, Indonesia.

Material and Methods

Research sites

This research was carried out for 8 months starting from April to December 2020. The locations of soil and root sampling of ebony plants were carried out in three locations (Fig. 1 and Table 1). Identification of the type, colonisation and spore density of AMF was carried out at the Forest Biotechnology Laboratory, Bogor Agricultural University (IPB), Bogor, West Java. Analysis of soil properties was carried out at the Soil Science Laboratory, Faculty of Agriculture, Tadulako University, Indonesia.

Diospyros celebica is a medium to large tree with a straight trunk that can reach a height of 40 m with a trunk diameter up to 120 cm and often large buttresses. The outer bark (Pepagan) is grooved and peeled off into small pieces, brown-black in colour. Single leaf alternating, oblong-elongated, measuring 10–35 cm × 2–7 cm, the underside is downy and gray-

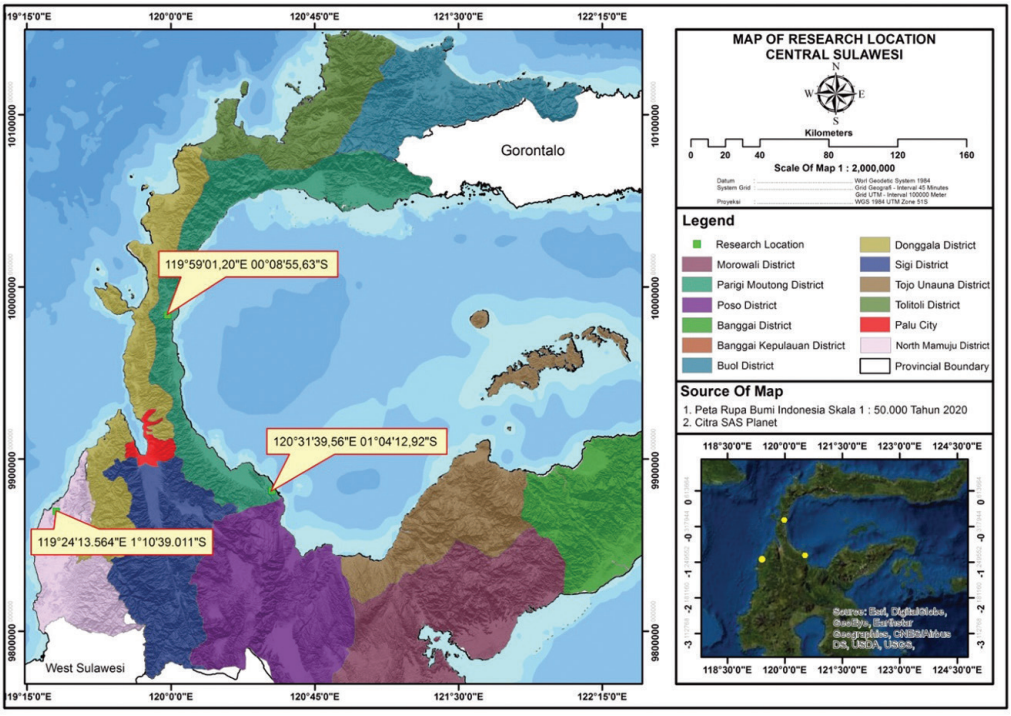


Fig. 1. Map of soil and root sampling locations in three ebony stands as a source of ebony seeds on the island of Sulawesi.

Table 1. Characteristics of ebony stands at the study site.

Location	Coordinates	Altitude, m a.s.l.	Stand age, years
Tovalo village, Kasimbar District, Parigi Moutong	00°08'55.63'' S 119°59'01.20'' E	56	30
Maleali village, Sausu District, Parigi Moutong Regency	01°04'12.92'' S 120°31'39.56'' E	36	40
Ako village, Pasangkayu District, Pasangkayu	1°10'39,011'' S 119°24'13,564'' E	73	25

green. The upper surface of the leaves is glossy and dark green. The flowers are clustered in the axils of the leaves, white. The fruit is ovate, hairy, young fruit is green and ripe fruit is yellow to brown (Gunawan et al. 2019, Fig. 2).

Soil and root sampling

Soil samples were carried out from the surface to a depth of 20 cm. They were taken randomly from ebony stands, where 10 trees with relatively the same growth

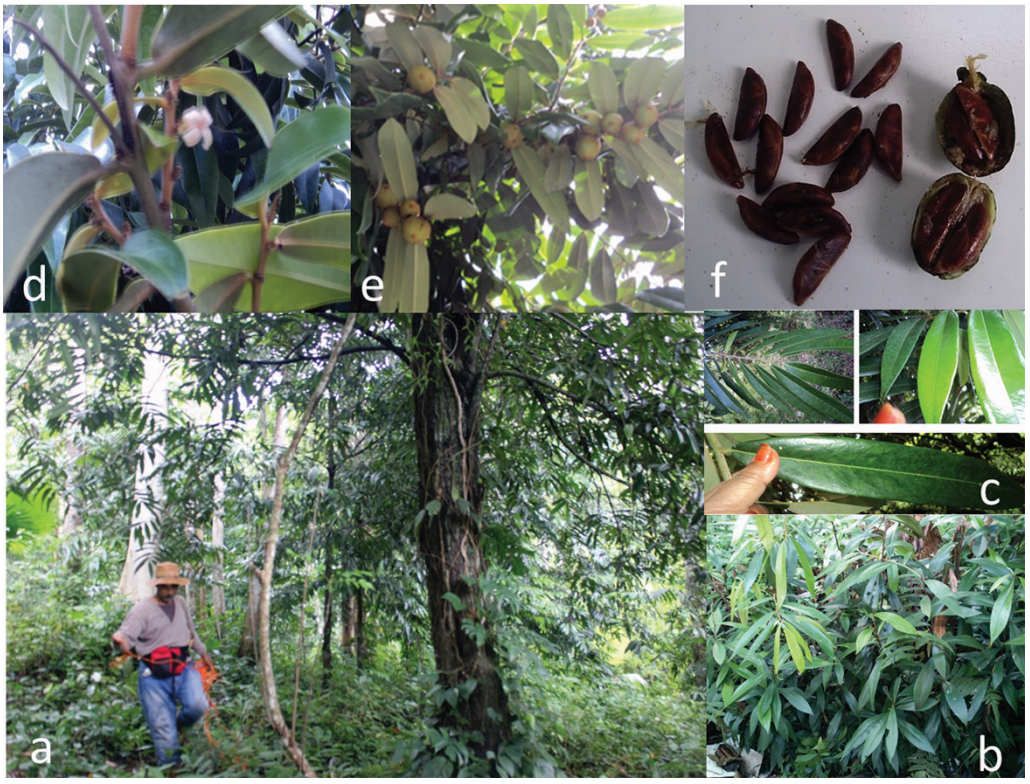


Fig. 2. Morphological features of *Diospyros celebica*: a) trees, b) seedlings, c) leaves, d) flowers, e) fruits, e) seeds (photos: Rukmi Rukmi).

vigor were selected at each study site. Furthermore, for each ebony tree, 4 soil sampling points were set with a distance of 1–2 m from the tree trunk, namely on the west, east, north and south sides. At each point 250 g of soil samples were taken and then composited, so that a total of 1000 g was obtained from each tree. Each soil sample was put in a plastic bag and labeled accordingly. All soil samples were air-dried in the laboratory for the purpose of isolation and identification of arbuscular mycorrhizal fungi spores and for analysis of soil properties.

Fine root samples from ebony trees were also collected at random from the tree from which the rhizosphere soil was taken. These roots were ensured to be taken from at least three lateral roots towards the smooth roots of the ebony tree. After harvesting, these were put in a plastic box, labeled according to the research location and taken to the laboratory. Root samples were rinsed under running tap water to remove soil and adhering dirt. After cleaning, they were cut into 1 cm size and stored in 70% alcohol solution until further analysis.

Isolation, identification of AMF and analysis of physico-chemical properties of soil

The technique used for the isolation of AMF spores was the wet pour-filter technique (Pacioni 1992). The spore extraction was then followed by a centrifugation technique (Brundrett et al. 1996). The spore preparations used Melzer's dye and polyvinyl lacto glycerol (PVLG) preservative placed separately on a glass slide. AMF spores obtained from the extraction after counting were placed in Melzer's and PVLG solutions and their types present in these two solutions were the same. Then the spores were carefully broken down by pressing the cover slip. Healthy spores were selected and stored on a slide that had been given a solution of PVLG and Melzer then covered with a cover glass. The colour change of spores in Melzer's solution is one of the indicators to determine the type of spores. The identification of was carried out by observing the morphological colour, shape, size, hyphae, spore ornamentation, and the reaction to the fusing solution (Schenck and Pérez 1988, Schüßler and Walker 2010). After that, the AMF spores were identified manually based on the description of the current AMF species (International Culture Collection of Vesicular-Arbuscular Endomycorrhizal Fungi in INVAM 2020). The spores were observed under a stereo microscope and were identified using an Axio Imager microscope with a magnification of 200×.

The spore density (per 50 g of soil) was calculated by directly counting their number present in each sample. AMF species richness was defined as the number of AMF species present per soil sample (Koske 1987).

The physico-chemical properties of the selected soil were analysed using a standard protocol. Organic C was quantified by Walkley-Black method (Walkley and Black 1934). Total N was analysed using Kjeldahl method (Kjeldahl 1883). P availability was analysed using the method by Bray and Kurtz (1945). Soil pH was determined using a digital pH meter and soil texture using a pipette method.

Arbuscular mycorrhizal fungi colonisation

The roots were put into a 10% KOH solution and stored for 2 weeks in the room. After that the KOH solution was discard-

ed and the root samples were washed with clean water. Furthermore, they were soaked in alkaline H_2O_2 and then heated for 45–60 min at a 90 °C. Next, the roots were cleaned and soaked in 1% HCL solution for 24 h, then were immersed in a dye solution (Trypan blue 0.05% + Glycerin 70% + 30% aquadest) for 24 h. The roots were then washed again and put into a 50% glycerin solution.

AMF colonisation counts used the infected root length method (Giovannetti and Mosse 1980). From the roots that have been treated with trypan blue, root samples with a length of ±1 cm were taken and arranged in the glass microscopy slides, and were carefully observed, the field of view showing colonisation was marked with (+), and if there was no colonisation it was marked with (-). In general, colonisation was characterised by the presence of AMF strucutures within the root such as extraradical and intraradical hyphae, vesicles, arbuscles or spores (Brundrett et al. 1984). To calculate the percentage of AMF infection in roots, formula (1) (Brundrett et al. 1996) was used:

$$\frac{A}{B} \cdot 100 \text{ , \%} \qquad (1)$$

where: *A* is the number of field of view colonised, *B* is total observed field of view.

The percentage level of AMF colonisation was classified according to the colonisation standard of O'Connor et al. (2001), namely: a) Not colonised: 0 % colonisation value; b) Low: colonisation value <10 %; c) Medium: colonisation value 10–30 %; d) High: colonisation value >30 %.

Data analysis

Levels for the treatment were calculated by analysis of variance The mean density and number of spores and the percentage of colonisation of arbuscular mycorrhizal fungi were compared using the test Tukey's Honestly Significant (LSD 0.05% confidence). Significant (ANOVA) using the SPSS software package (IBM SPSS Statistics 25, Armonk, NY, USA).

Results and Discussion

Arbuscular mycorrhizal fungi associated with ebony plants

The results showed that there were 7 types of AMF associated with ebony plants in Maleali village, Sausu district, Parigi Moutong Regency, Central Sulawesi, 5 species associated in Tovalo village, Kasimbar district, Central Sulawesi. Parigi Moutong, Central Sulawesi and 7 in Ako village, Pasangkayu District, Pasangkayu Regency, West Sulawesi. The description of each type can be seen in Table 2.

Table 2. Description of Arbuscular mycorrhizal fungi spores associated with ebony plants.

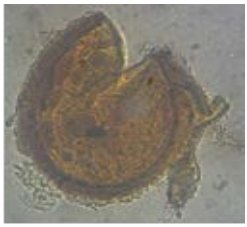
Species	Description				Location
	Shape	Size	Colour	Other ornament	
Glomus sp. 1	globalus	100×90 µm	brownish yellow	pigmented hyphae and layered spore walls	A
Glomus sp. 2	globalus	100×90 µm	yellow	pigmented hyphae and spore walls	A
Glomus sp. 3	globalus	130×120 µm	blackish brown	one spore wall, primidium and has not germinal wall	A

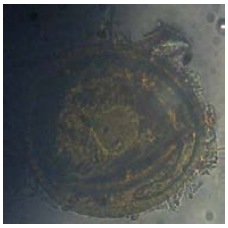
Species	Description				Location
	Shape	Size	Colour	Other ornament	
Glomus sp. 4	globalus	110×110 µm	blackish brown	one spore wall, does not have a germinal wall, there are non-figmental hyphae	A
Glomus sp. 5	globalus	180×180 µm	yellowish	hyphae and spore walls	A
Glomus sp. 6	ellipsoid	100×100 µm	yellow	one spore wall, do not have a germinal wall, have hyphae	B
Glomus sp. 7	globalus	90×90 µm	yellow	spore walls and primidiums	B
Glomus sp. 8	globalus	120×130 µm	brown	spore walls and primidiums	B
Glomus sp. 9	ovoid	90×90 µm	yellow	spore walls and non-figmental hyphae	B
Glomus sp. 10	globalus	150×150 µm	blackish brown	non-figmental hyphae	C
Glomus sp. 11	globalus	120×120 µm	reddish brown	spore walls, do not have a germinal wall	C
Glomus sp. 12	ovoid	110×110 µm	yellowish	hyphae, spore walls, do not have germinal walls	C
Glomus sp. 13	globalus	120×120 µm	brownish black	clear hyphae and a spore wall	C
Glomus sp. 14	globolus	110×110 µm	yellowish	non-figmental hyphae and a spore wall	C
Acaulospora sp. 1	globalus	250×350 µm	brown	spore wall and germinal wall, also non-figmental hyphae	A
Acaulospora sp. 2	globalus	290×320 µm	brown	one spore wall and germinal wall, there are cicatrix and saccule	A
Acaulospora sp. 3	globalus	180×180 µm	yellowish brown	a germinal wall, there are cicatrix	B
Acaulospora sp. 4	globalus	360×310 µm	brownish	one spore wall, germinal wall, there are cicatrix	C
Acaulospora sp. 5	globalus	270×270 µm	brownish	one spore wall and germinal wall	C

Note: A – Maleali village, Sausu District, Parigi Moutong Regency; B – Tovalo village, Kasimbar District, Parigi Moutong Regency; C – Ako village, Pasangkayu District, Pasangkayu Regency.

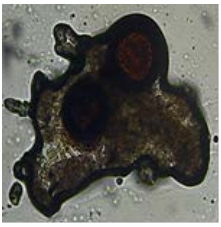
The types of each AMF associated with ebony at the three research sites can be seen in Figure 3.

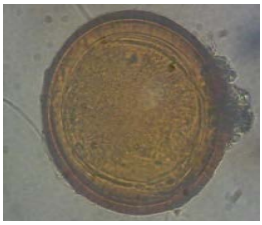
Based on the results of spore identification from the three research locations, it was found that only 2 AMF genera associated with ebony plants, namely *Glomus* and *Acaulospora*, where both genera had yellow to brownish black colour on average and were globalus shaped, but could be distinguished based on the characteristics and structure of each genus.

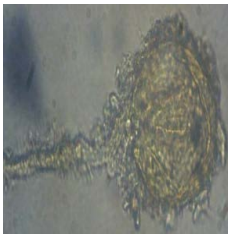

Glomus sp. 1

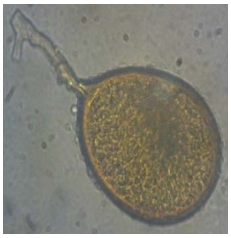

Glomus sp. 2

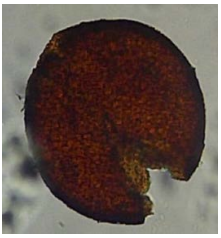

Glomus sp. 3

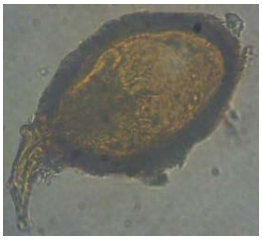

Glomus sp. 4

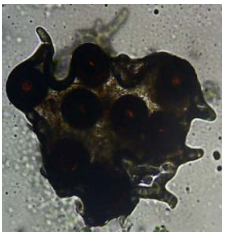

Glomus sp. 5


Glomus sp. 6

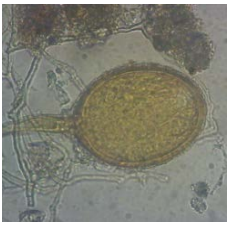

Glomus sp. 7

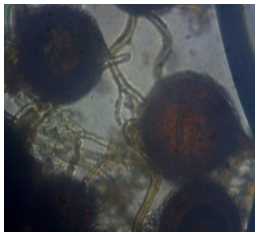

Glomus sp. 8

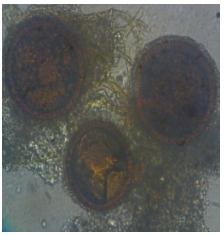

Glomus sp. 9

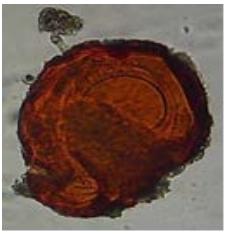

Glomus sp. 10

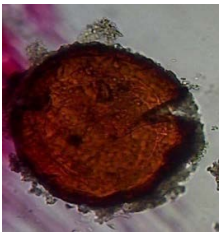

Glomus sp. 11


Glomus sp. 12

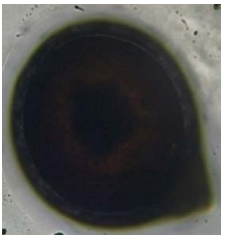

Glomus sp. 13


Glomus sp. 14


Acaulospora sp. 1


Acaulospora sp .2


Acaulospora sp. 3


Acaulospora sp. 4

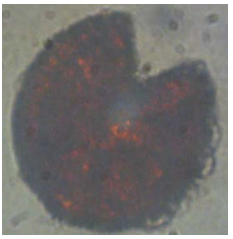

Acaulospora sp. 5

Fig. 3. Micromorphology of AMF associated with ebony in three locations.

The dominant genus *Glomus* was found at three locations (71.3–80 %) compared to the genus *Acaulospora* (Fig. 4). Some of the characteristics of genus *Glomus* were a distinctive hypal attachment that was not found in other genera. It was globose-sub globose, ovoid and obovoid in shape, yellow, brownish red, brown, and black in colour, wall layers 1–2. This genus can thrive at a pH of less than 5 to neutral. Furthermore, the genus *Acaulospora* had several characteristics, including globolus to elliptical, clear, yellow, or yellowish red in colour, 2–3 spore walls, there were substending hyphae (INVAM 2020). This genus was also more adapted to acidic soil conditions with a pH of less than 5 to neutral. The characteristic found was that the spores develop on the edge of the sporiferous saccule, so that in adult spores there would be one saccule which if released will leave a hole which is also called cycatric.

In this study, it was found that Glomeraceae family was abundant, where only two dominant genera were found, namely *Glomus* and *Acaulospora* which were found in the rhizosphere soil of ebony plants. Previous studies have also reported that they were also dominant in the forest which may be complementary to their host plant functions (Zhao et al. 2003, Jamiołkowska et al. 2018, Wang et al. 2019, Furrazola et al. 2020). The dominance of *Glomus* and *Acaulospora* in plantations may be related to the smaller spore size which allows them to reproduce faster in a shorter time (Wang et al. 2019). Confirmed by Husband et al. (2002), Li et al. (2007), Jefwa et al. (2012) and Pereira et al. (2014) is that *Glomus* and *Acaulospora* are the most common and most common arbuscular mycorrhizal fungi genera and species found infecting terrestrial plants

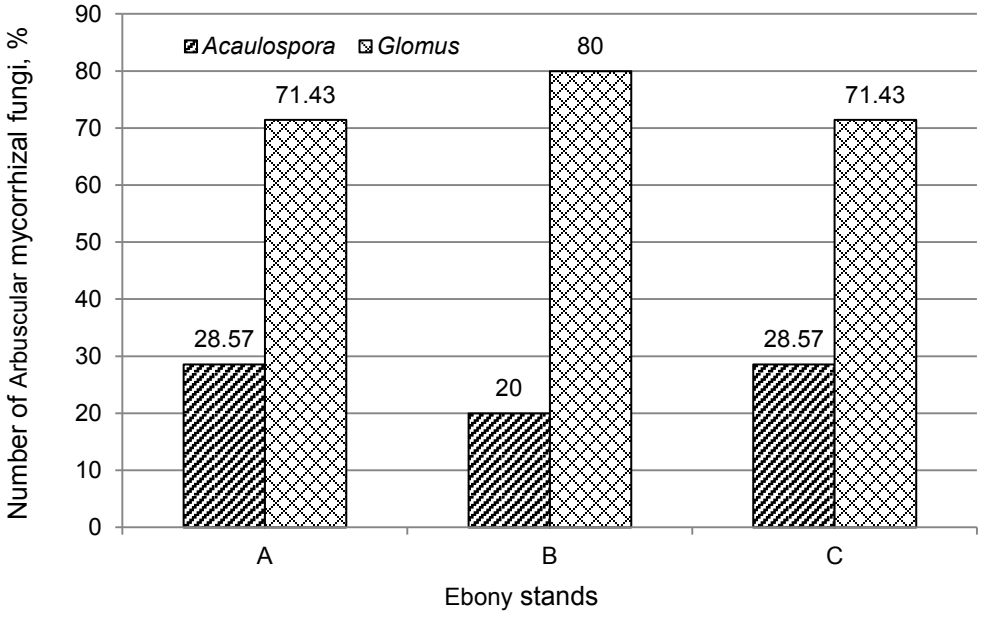


Fig. 4. Percentage of the number of arbuscular mycorrhizal fungi.

Note: A – Maleali village, Sausu District, Parigi Moutong Regency; B – Tovalo village, Kasimbar District, Parigi Moutong Regency; C – Ako village, Pasangkayu District, Pasangkayu Regency.

throughout the world, both in natural environments and those that have been modified by humans. Both genera were able to adapt to a very wide range of environmental conditions, including the different physico-chemical soil conditions in the three research sites (Table 3). The absence of other AMF genera in this study maybe due to the size of their spores. As explained by Hepper (1984) that large spores of AMF species require a longer period to develop compared to AMF species that have small spore sizes, and also because rhizosphere soil sampling is only done once.

The different soil physico-chemical conditions between the three research sites (Table 3), was one of the factors that greatly affected the level of spore density and the diversity of AMF species found. Based on observations, the shape and number of spores, type and genus of AMF found in each research location varied. This situation shows the diversity of AMF found in each research location. The diversity of species/genus and the number of spores found in this study can be caused by differences in rhizosphere conditions and research locations (Widiastuti and Kramadibrata 1993, Diop et al. 2021, Alrajhei et al. 2022).

The number of AMF species found in this study was less than the results of research by Nobre et al. (2018), where they found 16 AMF species associated with Babassu palm (*Attalea speciose* Mart.) in the eastern suburbs, Amazonia, Brazil, with the dominance of *Acaulospora* (6 species) followed by *Glomus* (3 species). More results were also found by Songachan et al. (2015), where they found 20 types of AMF in the rhizosphere of *Clerodenrum* species in Meghalaya, India.

According to Nurhalimah et al. (2014) the distribution of AMF is influenced by many factors, including soil type and structure, N and P nutrients in the soil, water, pH and soil temperature. Environmental factors affect the level of mycorrhizal infection in plants. Each AMF genus has a different character. *Glomus* with a wide distribution pattern is a genus that is able to survive and tolerate extreme soils and also has symbiotic ability and high adaptability to local conditions (Sianturi et al. 2005). According to Clark (1997), *Glomus* has the shortest dormancy period, has fairly good germination and the fastest germination time among other mycorrhizal genera (±6 weeks), while the narrowest distribution of AMF genus is *Acaulospora*.

Table 3. Soil characteristics.

Parameters	Soil Sampling Location		
	A	B	C
C-organic, %	1.87 ±0.01	2.41 ±0.02	1.72 ±0.01
N-total, %	0.18 ±0.02	0.25 ±0.02	0.18 ±0.02
pH (H_2O)	5.43 ±0.01	5.56 ±0.04	5.39 ±0.02
P_2O_5 Bray, ppm	6.18 ±0.03	7.80 ±0.17	5.96 ±0.02
Soil texture	Clay	Clay	Clay
Sand, %	39.02 ±0.03	42.74 ±0.02	5.52 ±0.04
Clay, %	43.34 ±0.65	40.90 ±0.57	70.84 ±0.60
Silt, %	17.64 ±0.35	16.36 ±0.13	23.94 ±0.10

Note: A – Maleali village, Sausu District, Parigi Moutong Regency; B – Tovalo village, Kasimbar District, Parigi Moutong Regency; C – Ako village, Pasangkayu District, Pasangkayu Regency. Each value is mean of three replications, ± Standard Deviation.

Spore density and arbuscular mycorrhizal fungi colonisation

The spore density of arbuscular mycorrhizal fungi (AMF) per 50 g of soil, the average number of spores, the percentage of colonisation of arbuscular mycorrhizal fungi and their

categories at three locations of ebony stands can be seen in Table 4.

Table 4. Density, average number of spores and colonisation.

Location study	Spore density per 50 g of soil	Colonisation, %	Category	Forms of colonisation		
				Mycelium	Vesicle	Arbuscular
A	72 ±5 b	0.73 ±0.15 b	Low	+	+	-
B	38 ±3.05 b	2.47 ±0.44 a	Low	+	+	-
C	162 ±12 a	0.33 ±0.26 b	Low	+	+	-

Note: A – Maleali village, Sausu District, Parigi Moutong Regency; B – Tovalo village, Kasimbar District, Parigi Moutong Regency; C – Ako village, Pasangkayu District, Pasangkayu Regency. Each value is the mean of three replications, ± Standard Deviation, + present, - absent. Means followed by same letter within a column are not significant at $p \leq 0.05$ level (Tukey's Honestly Significant test).

The results showed that the spore density varied between the three locations. The density found in the ebony rhizosphere soil in Ako village was higher (162 spores per 50 g soil) and was significantly different from the ebony stands in Maleali village and Tovalo village, which were only 72 and 38 spores per 50 g soil, respectively. This is lower than that of *C. crinite* rhizosphere soil, which is an average of 756 spores per 100 g of soil (Furrazola et al. 2020). Much higher results were reported that the AMF spore density associated with tree species in Eastern China plantations was between 4.38 to 76.38 spores per gram of soil (Wang et al. 2019) and 100 to 302 spores per gram of soil in the rhizosphere of *Attalea speciosa* (Nobre et al. 2018, Wang et al. 2019).

Liu et al. (2021) reported that soil pH is a major factor and negatively affects the mycorrhizal status of the rhizosphere and endosphere. The effect of soil pH on the results of this study also confirmed by Guo and Gong (2014) who observed that soil pH is the main factor in the formation of AMF composition in ecosystems that are stressed by salt levels, because soil pH affects spore germination and AMF hyphae growth (Gai et al. 2004). Soil pH at three locations of ebony stands tends to be acidic, namely 5.39–5.56. Low or very acidic soil pH has a direct relationship with an increase in the number and presence of AMF spores in the soil (Saputra et al. 2015). Generally, mycorrhizae are resistant to changes in soil pH so that in alkaline or very acidic soils AMF can still be found. However, the number of AMF depends on the adaptability of each type to be able to develop properly (Samsi et al. 2017). Soti et al. (2014) reported that the AMF colonisation rate was high in soils with low acidity (5.5–6.0) compared to soils with high acidity (4.0–4.5). Differences in season, temperature and humidity can also affect AMF sporulation (Koske 1987), and this causes the highest number of spores found in the rhizosphere of ebony plants in Ako village, Pasangkayu district, West Sulawesi, compared to the other two locations.

According to Suharno et al. (2016) the duration of the interaction between fungi and hosts is influenced by variations in the dependence of mycorrhizae on the host plant and the influence of land conditions on the ability of mycorrhizae to grow. The diversity of AMF is highly dependent on natural conditions. Differences in the location of growth and rhizosphere cause differences in the diversity of species

and populations of AMF (Cui et al. 2018, Velázquez et al. 2020, Husein et al. 2022, Islam et al. 2022). The diversity of AMF spores is due to, among other things, differences in soil properties (Alimi et al. 2021), management practice and elevation level (Marizal et al. 2016), organic matter content in the soils (Bedini et al. 2013, Rini et al. 2017), plant nutrition, light intensity and temperature change (Sun et al. 2013). The next environmental factor that affects the number of spores is C-organic (Samsi et al. 2017).

C-organic in ebony rhizosphere soil samples in Maleali village, Tovalo village and Ako village was 1.87 %, 2.41 %, 1.72 % respectively. The stand with high organic C content (Tovalo village) had fewer spores than the other rhizosphere soil samples. This is in line with the results of Sianturi et al. (2005) who also reported that a small number of AMF spores were found in soils with the highest organic C content. However, the highest number was found in soils with a very low C-organic content compared to soil samples with a high content (Samsi et al. 2017).

The results in Table 4 show that the samples of ebony roots from Tovalo village, Kasimbar District had the highest percentage of colonisation by AMF (2.47 %) which was significantly different from the percentage of colonisation in ebony root samples from Maleali village, Sausu District (0.73 %) and the lowest was in the sample of ebony roots from Ako village, Pasangkayu District (0.33 %). Ebony root colonisation was categorised as low. The same results were obtained by Turjaman et al. (2019), where they found a low percentage of AMF colonisation on several plant roots in the peat swamp forest of Jambi, Sumatra, Indonesia, namely *Alstonia scholaris* (L.) R.Br. (7 %) and *Macaranga pruinosa* (Miq.) Müll.Arg. (9 %).

The low AMF colonisation in ebony roots may be influenced by many factors, for example growth conditions, interactions with host plants, light conditions, temperature, competition of AMF with other soil microbes (Turjaman et al. 2019). The age factor of the old ebony plants (25–40 years) (Table 1) has relatively hard roots so that it has difficulty in the process of infection. AMF infection is more common in young roots (Hermawan et al. 2015). Edaphic and climatic factors, compatibility between fungi and their hosts, root properties and the presence of other soil microorganisms can affect AMF sporulation and its symbiosis with certain trees (Ferdousee et al. 2012).

Furthermore, soil P is also an important factor affecting the intensity of colonisation and growth of AMF spores. When it is abundant, plants will obtain a lot of P nutrients through their roots so they can inhibit growth, extension rate and activity of AMF extraradical hyphae (Zhang et al. 2014). This condition causes a reduction in the level of root colonisation, resulting in reduced P uptake and plant growth (Kiers et al. 2011, Li et al. 2013). Similar to available P and organic C, in this study, the highest total soil N was also obtained from ebony stands in Tovalo village, 0.25 %, followed by ebony stands in Maleali village and Ako village 0.18 %. N is a limiting nutrient in many terrestrial ecosystems (Vitousek and Howarth 1991), including influencing plant associations with AMF. Their development, both in terms of hyphal proliferation and spore formation is influenced and stimulated by addition of organic matter containing high N (Gryndler et al. 2003, Bukovská et al. 2016).

Based on the data obtained, the high density and average number of spores in Ako village was not followed by a high percentage of root colonisation by AMF. In

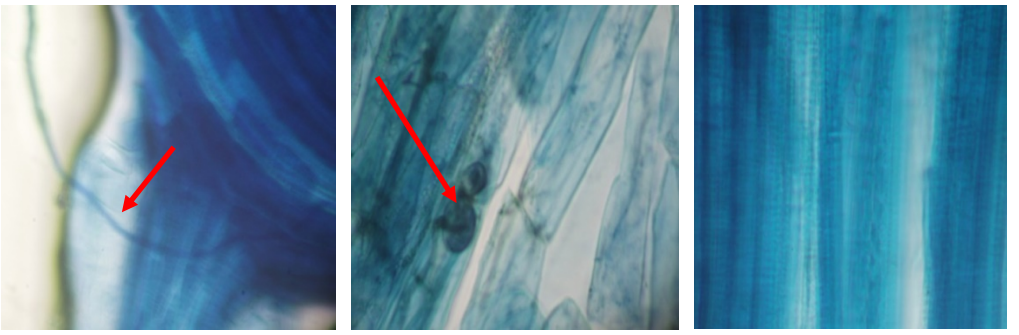
accordance with the explanation by Smith and Read (1997) it is not certain that plants with a high percentage of AMF colonisation will have high spore densities. This is due to spore formation factor which is influenced by many factors including edhapic ones and seasonal variation (Sivakumar 2013), the host plant (Sun et al. 2022), growth medium, fertilizer, temperature (Kilpeläinen et al. 2020) and light (Menge 1984). Zhang et al. (2014) stated that the level of root colonisation by AMF and spore density were closely related to the soil factor.

In addition, soil texture is also closely related to the development of plant roots, and indirectly affects the percentage of AMF in plant rhizosphere (Saputra et al. 2015). According to Foth (1994) soil that contains a lot of dust is stronger at storing water because it has small pores and has water absorption slowly so that water is stored in the soil for a long time. In this condition, the available water content is more so that mycorrhizal colonisation on the roots is very low. The results of observations of AMF infection in ebony roots can be seen in Figure 5.

Based on the results of observations, the percentage of AMF infection was characterised by the presence of structures such as AMF vesicles and hyphae in the ebony root tissue, whereas in roots that did not show any infection, these structures were not found. In the observation of root colonisation, the presence of arbuscular was not found in all the observed root samples. According to Smith and Read (1997) the existence of the arbuscular in the root is relatively short, ranging from 1–3 days. However, with the presence of one or more AMF structures, it can be said that there has been an association by AMF to its host plant.

Conclusions

Arbuscular mycorrhizal fungi associated with ebony plants from the three research sites consisted of 19 species belonging to two genera, namely the genus *Glomus* and *Acaulospora*. The spore density found in Ako village was higher (162 spores per 50 g soil). AMF colonization on ebony roots also varied the highest percentage of AMF infection was in ebony roots in Tovalo village (2.47 %). The results of this study provide basic information about the association of AMF with ebony plants under natural conditions. From the research results, it is recommended that further research is needed, especially the identification of AMF species in natural ebony for-



a b c

Fig. 5. Ebony roots infected by AMF: a – hyphae, b – vesicles, c – uninfected roots.

ests in various distribution areas, so that data on the diversity of AMF species associated with ebony is more complete and can be used as a reference in future conservation efforts, especially the application of these species. AMF has been found to support the cultivation, conservation and restoration of this species in the future.

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Fungal inhibition of *Pinus merkusii* cone extracts against *Phanerochaete chrysosporium*

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Abstract

This study aims to identify the extractives from *Pinus merkusii* Jungh. & de Vriese cone together with their evaluation for antifungal agent. The pine cone was obtained from Temanggung, central Java, Indonesia. The pine cone was grinded to size of 40 mesh and was extracted with *n*-hexane, ethyl acetate, and methanol in a Soxhlet apparatus. The antifungal test was performed through growth inhibition of cone extracts against *Phanerochaete chrysosporium* Burds. (white-rot) and the extractive responsible for antifungal was elucidated by GC-MS. The antifungal activity measurement showed that *n*-hexane extract as the highest inhibition with IC_{50} of 637.74 μg·ml^{-1}. The analysis of *n*-hexane extract through GC-MS exhibited diterpenoid as dominant compound and they were characterized as pimaric acid and abietic acid TMS. Therefore, the presence of terpenoids in *P. merkusii* cone extracts are potential for natural preservative.

Key words: lipophilic, natural preservative, pine, resin acids, terpenoids, white-rot.

Introduction

White-rot (*Phanerochaete chrysosporium* Burds.) is one of the organisms that caused detrimental effect on wood industry. This type of fungal disease caused depolymerisation of hemicellulose and lignin components, leaving undegraded crystalline cellulose hence its white colouration at the advance stages of decay (Goodell et al. 2020). The utilisation of wood as material for furniture and other uses proposed the application of wood preservative to protect from the organisms. In another hand, the uses of synthetic preservative triggers side effect on human and environment. Therefore, the alternative way includes researching natural preservatives from plants. The potential of natural preservatives from plants has been reported from previous studies; phenolics (Zabka and Pavela 2013, Pizzolitto et al. 2015) and lipophilic such as essential oils (Srivastava et al. 2008) as antifungal against genus

Aspergillus. In the case of white-rot, plant extracts also were applicated as preservative agent (Da Silveira et al. 2017, Xie et al. 2017). Due to the white-rot attacks affects in decreasing the mechanical, physical, and chemical properties of wood, the use of white-rot such as *Ph. chrysosporium* for model laboratory test also has been recorded (Oliveira et al. 2010).

Pine cone is a reproductive organ produced by *Pinus merkusii* Jungh. & de Vriese and other species from Pinophyta division. It is composed by lignin, cellu lose, resins, and other organic compounds. The woody female cone is a common biomass waste in pine plantations. In previous works, pine cone was reported to contain hydrophilics and lipophilics extractives such as tannins, flavonoids, monoterpenes, as well as diterpenes (Kilic et al. 2011, Xu et al. 2012, Zhao et al. 2019). Several bioactivities such as antibacterial, antitumor, immunoregulatory, and antifungal have been reported in pine cone from various species (Ulukanli et al. 2014, Yi et al. 2017).

In Indonesia, especially in Java Island, pine forest (*Pinus merkusii*) sited of 476,126 ha and thus the material of pine cone presence is in huge amounts (Masendra et al. 2018). However, information on the fungal inhibition effect of *P. merkusii* cone extract is still limited. Therefore, the purpose of this study is to identify *P. merkusii* cone extractives responsible to inhibit the growth of white-rot for future development as natural preservative against fungi.

Material and Methods

Cone collection and extraction

Air dried of *P. merkusii* cone (tree age: 30-year-old) was collected from Temanggung, Central Java, Indonesia (7°14' S, 110°2' E). The site elevation is 500 m a.s.l., precipitation of 3000–3500 mm and annual temperature of 24–30 °C. The pine cones were sieved in size of 40 mesh and 53.5 g of dry sample was successively extracted with *n*-hexane, ethyl acetate, and methanol for 6 h using Soxhlet equipment. To obtain dried extract the solution sample was evaporated by evaporator machine and then was weighed as extractive content (percentage of initial sample).

Fungal inhibition test

Ph. chrysosporium was chosen for antifungal activity. Its strain IPBCC 930259 was collected and purchased from Institute Pertanian Bogor Centre Collection, West Java, Indonesia. The fungus was pre-cultured on PDA (potato dextrose agar; Aldrich, Germany) in Petri dish of 90 mm diameter for 7 days at 25 °C. In this experiment the medium was autoclaved at 120 °C for 20 min. The performance of antifungal extracts against *Ph. chrysosporium* referred to method described by Lukmandaru (2013). 300 µL extract sample was spread on the surface of 20 mL PDA medium in Petri dish. After the solvent drying, *Ph. chrysosporium* from pre-cultured PDA was inoculated. The fungus was incubated at 25 °C and 70 % relative humidity for 5–7 days. The blank sample was conducted in the same method with replacing the extract by solvent only. The incubation was stopped when the growth of fungus in blank sample was 100 %. In this experiment, a standard of biocide/fungicide (Bioindustries, Yogyakarta) was compared for control positive. It consists of methylene bis thiocyanate and 2-thyocyanomethyl thiobenzothiazole. The growth inhibition (*GI*) of sample was cal-

culated through equation (1) as follows:

$$GI = \frac{A_1}{A_0} \cdot 100,\ \% \qquad (1)$$

where: $A_1 = \frac{\pi \cdot d_1^2}{4}$ is area of sample in cm^2 and d_1 is diameter of sample growth in cm; $A_0 = \frac{\pi \cdot d_0^2}{4}$ is area of blank in cm^2 and d_0 is diameter of blank growth in cm.

The fungal inhibition was performed in three replications and three concentrations to calculate IC_{50} (the concentration that inhibit 50% activity). The concentration of 500, 1000, and 2000 $\mu g \cdot mL^{-1}$ were used to performed IC_{50}. In addition, the performance of IC_{50} calibration from *n*-hexane, ethyl acetate, and methanol extract, were $y = 0.037x + 25.8$, $y = 0.0191x - 5$, and $y = 0.0449x + 12$, respectively. All equations have $R^2 > 0.9$, where x is IC_{50} in $\mu g \cdot mL^{-1}$ and y is 50 $\mu g \cdot mL^{-1}$.

Gas chromatography mass spectra (GC-MS study)

The highest value of *Ph. chrysosporium* inhibition from the pine cone extracts was analysed with GC-MS. The *n*-hexane extract was injected for identification of extractive. It was silylated according to method described by Preciado et al. (2016). Briefly, 1 mg *n*-hexane extract was dissolved in 50 µL pyridine. Into solution sample, 100 µL solution consisted of N,O-bis (trimethylsilyl) acetamide 99 µL and trimethylchlorosilane 1 µL were added. The sample was put in oven with 100 °C for 30 min. After cooling process, 1 mL *n*-hexane was added to the sample solution. For analysis, 1 µL sample was injected to the GC-MS machine using a GCMS-QP 2010 (Shimadzu, Japan). Gas chromatography condition was Rtx-5MS capillary column (30 m × 0.25 mm I.D. and 0.25 µm; GL Sciences, Tokyo, Japan). The sample detection temperature was 280 °C and column temperature was set from 170 °C (1 min) to 280 °C at 5 $°C \cdot min^{-1}$. Injection temperature of 200 °C was set and acquisition mass range was made 50–500 atomic mass units with helium as gas carrier. In addition, the split ratio of GC-MS machine was made of 20. MS of *n*-hexane extractives was compared with NIST11 (2011) library and literature (Azemard et al. 2016, Masendra et al. 2018). Furthermore, the calculation of each compound was done using peak relative method (Masendra et al. 2021b).

Statistical analysis

One-way ANOVA was used to observe the significancy value of solvent. ANOVA was performed using SPSS software (IBM, USA) with 95% confidence level. Tukey-HSD was applicated to analyse the data with significant difference.

Results and Discussion

Extractive content

Figure 1 shows cone of *P. merkusii* abundance in hydrophilic extracts and lipophilic extract. The methanol extractive gave the highest value of 6.35 %, while ethyl acetate gave the lowest percentage of 1.95 %. The lipophilic extraction or *n*-hexane extraction yielded 3.11 % extract. The extractive content of *P. merkusii* cone was reported in a previous work (Masendra et al. 2021a). In comparison, the methanol extractive of *P. merkusii* cone was higher than acetone, toluene-ethanol, and water of wood (Wijayanto et al. 2015). Further, *n*-hexane extractive content of *P. merksii* cone was higher than *n*-hexane extract of bark (Masendra et al. 2018), but was low-

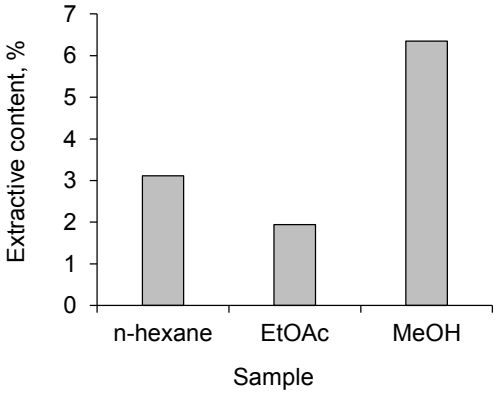

Fig. 1. Yield extractive of *P. merkusii* cone.
Note: EtOAc is ethyl acetate, MeOH is methanol (Masendra et al. 2021b).

er than *P. merkusii* wood dichloromethane extract (Wijayanto et al. 2015).

Antifungal assay and elucidation of extractive compounds

IC_{50} of pine cone extracts is displayed in Figure 2. The *n*-hexane extract exhibited highest inhibition 637.74 μg·mL^{-1}, followed by methanol (851.22 μg·mL^{-1}) and ethyl acetate (3446.01 μg·mL^{-1}). Further comparison with positive control of biocide (IC_{50} of 260 μg·mL^{-1}), the *n*-hexane extract was two times lower. The higher value of inhibition in *n*-hexane and methanol extracts than ethyl acetate extract indicates the lipophilic and hydrophilic compound involve in stopping the white-rot growth.

Due to higher inhibition of fungal in *n*-hexane extractive, thus, this lipophilic extractive was further discussed. The detection of *n*-hexane extract constituent found out four compounds (Fig. 3). The compound 1 was detected as sesquiterpene and compound 2–4 were identified as diterpenes through NIST11 library comparison (Table 1).

In Table 1 the compound with higher proportion in *n*-hexane extract was diterpene rather than sesquiterpene. Therefore, the characterisation of diterpene (compound 2–4) was further elucidated. The compound 2 showed fragment of mass-to-charge ratio (m/z) 374, 359, 309, 257, 241, 121, 73. The compound 3 proposed fragment of m/z 374, 359, 256, 241, 213, 185, 121, 73, and the last compound (4) had fragment of m/z 372, 359, 257, 239, 141, 105, 91, 73. The other fragmentations of compound 2–4 are displayed in Figure 4, while their comparation of mass spectra with previous works (Azemard et al. 2016, Masendra et al. 2018) is shown in Table 2.

Mass spectra interpretation of compound 2–4 were further discussed in this section. The appearance of m/z 73 in compound 2–4 suggests these compounds have TMS (-C_3H_9Si) in their structures (Fig. 5 for

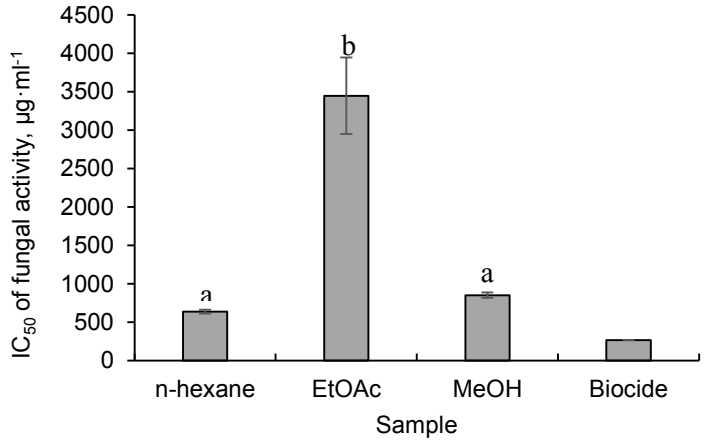

Fig. 2. Fungal inhibition from *P. merkusii* cone extracts.
Note: Different letters are statistically different at $p < 0.05$.

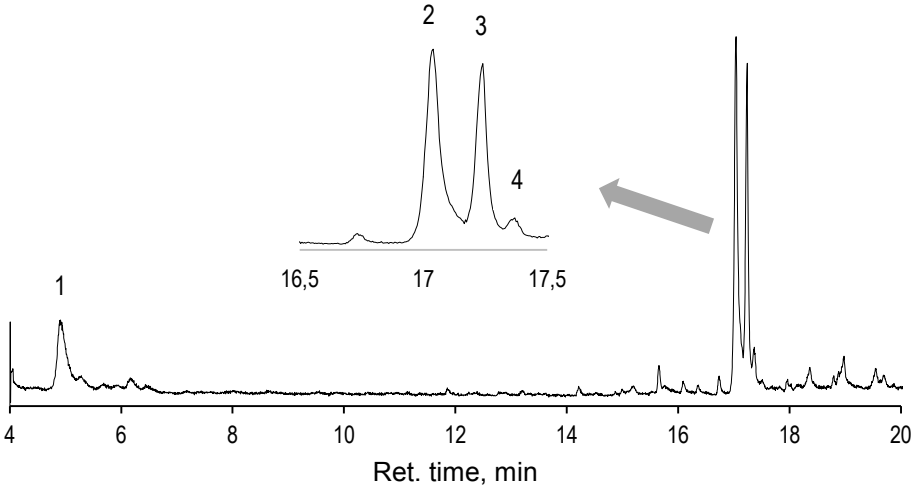

Fig. 3. GC-MS chromatogram of *P. merkusii* cone.
Note: compound 1 sesquiterpene, compound 2–4 diterpenes.

Table 1. Cone extract of *P. merkusii* constituents.

Compound	Constituents	Concentration, %	Group	Similarity index, %
1	Trans-longipinocarveol	12.91	Sesquiterpene	80
2	Pimaric acid TMS	49.12	Diterpene	80
3	Abietic acid TMS	34.43	Diterpene	80
4	Dehydroabietic acid TMS	3.54	Diterpene	88

Note: TMS is trimethylsilyl.

chemical structure of terpenoid detected in *P. merkusii* cone extract). Compound 2–4 showed total molecular peak (M^+) at m/z 374 (compound 2 and 3) and m/z 372 (compound 4). The presence mass spectra of m/z 359 [M^+ - m/z 15] in compound 2 and 3 indicated the loss of m/z 15 molecules and thus the compound 2 and 3 contain methyl (-CH_3) in their structure. While in compound 4, the m/z 372 to m/z 359 is indicated to the loss of m/z 13 molecules or methine (=CH-) in its structure. The next mass spectrum that appears in compound 2–4 is m/z 257 ($C_{19}H_{29}$) and m/z 256 ($C_{19}H_{28}$). Both m/z 257 and m/z 256 appear due to the loss of m/z 117 or 115 molecules ($C_4H_9O_2Si$) from m/z 374 or m/z 372. Moreover, the spectra in compound 2 and 3 have m/z 241 that estimated from the reduction of m/z 256 by m/z 15, while m/z 239 is made from the reduction of m/z 257 by m/z 18. The m/z 15 and m/z 18 is associated to the loss of methyl in compound 2 and 3, and water in compound 4. The other detected fragment in compound 2 and 3 is m/z 213. This fragment is made from reduction of m/z 241 by m/z 28, while m/z 211 in compound 4 is made from reduction of m/z 239 by m/z 28. The m/z 28 is associated to the loss

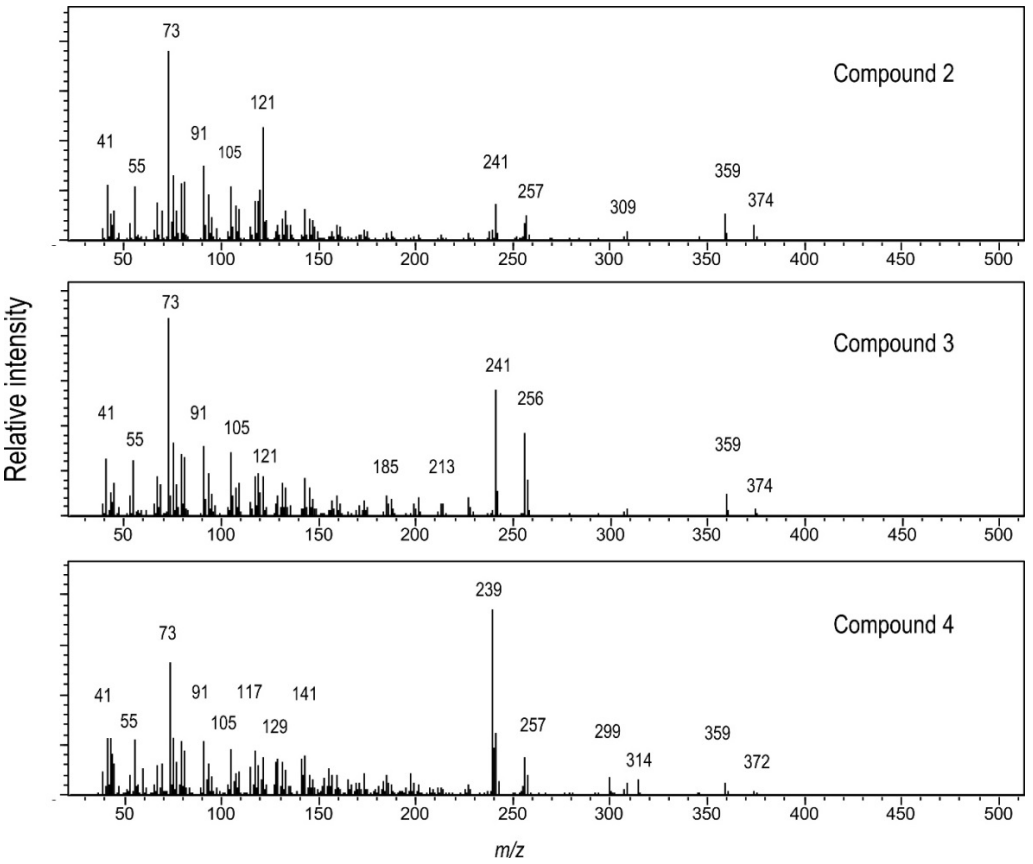

Fig. 4. Mass spectrum of compound 2–4.

Table. 2. Mass spectra comparison of compound 2–4 with literature.

Constituent	Mass spectra fragmentations
Pimaric acid TMS[a]	374 $[M]^+$ (10), 359 (27), 309 (3), 257 (19), 241 (19), 121 (100), 105 (14), 91 (15.1), 73 (95), 55 (22), 41 (5)
Compound 2	374 $[M]^+$ (8), 359 (13.7), 309 (3.3), 257 (13.8), 241 (16.9), 121 (55.5), 105 (22.3), 91 (33.3), 73 (100), 55 (24.2), 41 (21.1)
Abietic acid TMS[b]	374 $[M]^+$ (9), 359 (8), 256 (100), 241 (86), 213 (35), 185 (64), 157 (9), 143 (15), 129 (9), 105 (9), 91 (9), 73 (5)
Compound 3	374 $[M]^+$ (2.9), 359 (9.6), 256 (47.6), 241 (69.3), 213 (6.3), 185 (10.5), 157 (8.6), 143 (18.4), 129 (9.8), 105 (29.3), 91 (32), 73 (100)
Dehydroabietic acid TMS[a]	372 $[M]^+$ (15), 359 (11), 314 (2), 299 (2), 257 (11), 239 (100), 141 (10), 105 (3), 91 (4), 73 (27), 55 (5)
Compound 4	372 $[M]^+$ (2), 359 (6), 314 (7.5), 299 (9.6), 257 (11), 239 (100), 141 (15), 105 (20), 91 (25), 73 (65), 55 (25.3)

Note: [a] – Masendra et al. (2018), [b] – Azemard et al. (2016).

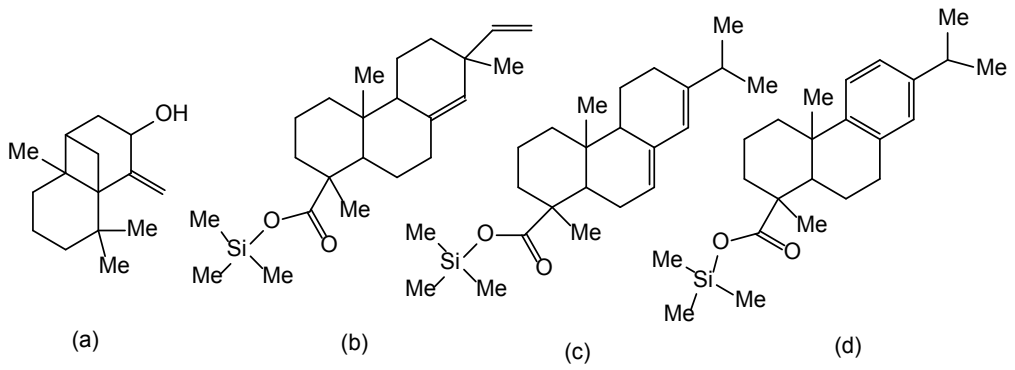

Fig. 5. Chemical structure of sesquiterpene and diterpenes.

Note: longipinocarveol (a), pimaric acid (b), abietic acid (c), and dehydroabietic acid (d) in TMS derivative.

of C_2H_4 (ethene). Hence, m/z 374 is predicted to be $C_{23}H_{38}O_2Si$ while m/z 372 is $C_{23}H_{36}O_2Si$. Therefore, these compounds were categorised as diterpenic compounds that contain four isoprenes (C_5H_8). Furthermore, the fragments of compound 2–4 were compared to previous work (Azemard et al. 2016, Masendra et al. 2018) and they were identified as diterpenic TMS derivative compounds, namely, pimaric acid for compound 2, abietic acid for compound 3 and dehydroabietic acid for compound 4 (Table 2). In addition, the comparison compound 2–4 with another genus *Pinus* was made. It was shown that the terpenic compound dominant in the *P. merkusii* cone is pimaric acid and abietic acid. The presence of those diterpenoids also was detected in the cone of *P. armandii* (Yang et al. 2010), *P. halepensis*, *P. pinea*, *P. sylvestris*, *P. nigra*, and *P. brutia* (Kilic et al. 2011).

The detection of the extractive in *P. merkusii* cone such as lipophilic and hydrophilic compound could be responsible as plant protector against microorganisms. In addition, in correlation with constituent of *n*-hexane and methanol extracts, the high fungal inhibition may due to the presence of diterpenoids and phenolics. The terpenoid especially diterpenic acids from *P. merkusii* cone such as pimaric acid TMS and abietic acid TMS were amounted in high concentration. Previously, it was reported that terpenic compounds from essential oil inhibited white-rot fungus (Xie et al. 2017). Related to the hydrophilic compounds, a previous work (Masendra et al. 2021a) reported that methanol extract of *P. merkusii* cone contained total phenols (54.5 %) and total flavonoid (3.4 %) as well as the detection of phloroglucinol and catechin. The phenolics in the *P. merkusii* cone might also act as fungal inhibitor (*Ph. chrysosporium*).

The mechanism of extractives in inhibiting of fungus growth could be defined by their lipophilicity and hydrophilicity. A previous work reported that lipophilic compound of eugenol, thymol, isoeugenol, and hydrophilic compound of vanillin act as inhibitor for *Fusarium verticillioides* (Sacc.) Nirenberg mycelia (Dambolena et al. 2012). The appearance of the mentioned properties is known by the presence of hydroxyl (-OH) for hydrophilicity and methyl for hydrophobicity in the compounds. In the phenolics and terpenes, it

was described that the presence of -OH and methyl group effected the fungal and bacterial activity (Sekine et al. 2009, Zengin and Baysal 2014, Pizzolitto et al. 2015, Masendra et al. 2021c). Therefore, in the current study, the presence of methyl group in the compound 2–4 as well as the presence of -OH group in trans-longipinocarveol, phloroglucinol, and catechin attribute in inhibiting the growth of mycelia from *Ph. crysosporium*. The presence of those compounds in *n*-hexane and methanol extracts suggests the pine cone of *P. merkusii* is potent for natural preservatives compound.

Conclusions

The fungal test of *P. merkusii* cone extracts against *Ph. chrysosporium* exhibited inhibition activity especially in *n*-hexane and methanol extracts. The investigation of *n*-hexane extractive detected terpenic compounds in the cone of *P. merkusii* and they were identified as trans-longipinocarveol, pimaric acid, abietic acid, and dehydroabietic acid. The higher presence of pimaric acid and abietic acid in *P. merkusii* cone indicated their role were dominant in inhibiting the growth of *Ph. crysosporium*. The presence of methyl group in both pimaric and abietic acid together with their lipophilicity suggested to have responsibility in stopping the fungus growth. The research concludes that *P. merkusii* cone is potential for future natural preservative development against white-rot, especially to *Ph. crysosporium*.

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Allometric equation for the biomass and carbon stock of cajuput (*Melaleuca cajuputi* Powell) stand in Wanagama Forest, Yogyakarta, Indonesia

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Abstract

Indonesia forests are ecosystems with mega biodiversity. Although many allometric equations have been developed for either forest types or tree species, a species–specific allometric equations are still important for precise estimation. The cajuput tree is a promising species that can provide non-timber forest products and mitigate climate change. In Indonesia, cajuput trees have been utilized as a source aromatic oil in forest plantations and natural forests. For climate change mitigation, cajuput could be useful as carbon stock. This research aimed to develop an allometric equation of naturally growing cajuput from Wanagama Forest using destructive sampling with 30 samples of cajuput trees to develop and 20 samples to validate the allometric equation. Power model law was used to develop the allometric equation. The requirement of the selected allometric equation was high coefficient determination (R^2_{adj}), low value of mean absolute bias, and low value of root mean square error. Results showed that and were the best allometric equations to estimate dry-weight biomass and carbon content, respectively. The predictor of diameter at breast height was robust in estimating the biomass and carbon of cajuput. Adding a predictor of total height did not significantly affect the allometric equation.

Key words: carbon accounting, climate change mitigation, non-timber forest products, teaching forest.

Introduction

Melaleuca forest is distributed in Australia and Southeast Asia, and is cultivated in other regions of the World, like Southern United States, and the Caribbean (Tran et al. 2013a). In Australia, the carbon stock of *Melaleuca* has not been reported adequately (Tran et al. 2013b). From an ecological perspective, the *Melaleuca* forest has potential for climate change mitigation (Tran et al. 2013a). However, less attention has been paid to the *Melaleuca* forest as carbon stock in Southeast Asia, such as Vietnam and Indonesia (Tran et al. 2015, Mulyana et al. 2021).

Melaleuca forest in Indonesia has been distributed in Maluku, Papua, and Java Island. Cajuput (*Melaleuca cajuputi* Powell) oil is well known in Indonesia as aromatic oil and is used in the traditional medicine. In Java Island, National Forestry Enterprise (Perhutani) and Forest Management Unit of Yogyakarta (KPH Yogyakarta) are the major producers of cajuput oil (Utomo et al. 2012; Mulyana et al. 2018, 2021) and own cajuput plantation and cajuput oil factory. In Maluku and Papua Island, cajuput grows naturally and is processed traditionally by the community around the cajuput forest (Parera et al. 2006; Widiyanto and Siarudin 2013, 2014; Siarudin and Widiyanto 2014). The species resources, both from natural stands and plantations has contributed significantly to the community economy (Parera et al. 2006, Indrajaya et al. 2013).

Cajuput trees play an essential role in the socio-economy and should be considered in climate change mitigation due to their ability to remove CO_2 from the atmosphere. Cajuput forest has a capacity to store carbon at around 169 ± 26 t C·ha^{-1} (aboveground) and 104 ±16 t C·ha^{-1} in soil and roots (Tran and Dargusch 2016). Specifically in Indonesia, the carbon stock of cajuput leaves/twigs in KPH Yogyakarta is 0.871 t C·ha^{-1} (Mulyana and Purwanto 2020). It is of particular interest to estimate the carbon stock of cajuput in natural forests because the characteristics of the species there differ from those in the plantations. For instance, the cajuput trees in Perhutani and KPH Yogyakarta have a similar height for all ages (around 1.5 m height), and the leaves/twigs are harvested periodically (approximately 9 months) (Utomo et al. 2012; Mulyana et al. 2019, 2021). The trees growing naturally in natural forests have complex diameter distribution and total height (Widiyanto and Siarudin 2013, 2014; Siarudin and Widiyanto 2014).

The cajuput plantation is one of the important source of products from KPH Yogyakarta. Meanwhile, naturally grown cajuput can be found in Forest with Special Purpose (Kawasan Hutan Dengan Tujuan Khusus/KHDTK) Wanagama in compartment 7. Mulyana et al. (2021) elaborated the use of allometric equation to estimate leaf/twig biomass in KPH Yogyakarta using three predictors, namely, height of the canopy, canopy diameter, and stem diameter. However, the accuracy is not satisfactory because of the low coefficient determination (0.428) and >1% aggregate deviation. Understanding cajuput allometry is crucial in estimating its potential as carbon stock or biomass energy source. The objective of the present study was to develop an allometric equation for estimating the biomass and carbon content of naturally grown cajuput stand in Wanagama Forest.

Material and Methods

Research site

Fieldwork was conducted in May 2021 at compartment 7 of Forest with Special Purpose (Kawasan Hutan Dengan Tujuan Khusus/KHDTK*)* of Wanagama (hereinafter called as Wanagama Forest, Yogyakarta). Wanagama Forest is located in Gunungkidul District, Province of Special Purpose Yogyakarta (Fig. 1). Compartment 7 is dominated by cajuput (*Melaleuca cajuputi*), gliricidia (*Gliricidia sepium* (Jacq.) Kunth ex Walp.), mixed forest, and shrub. The land use of compartment 7 consists of cajuput (23.4 ha), gliricidia (17.0 ha), shrub (14.2 ha), and mixed forest (9.0 ha).

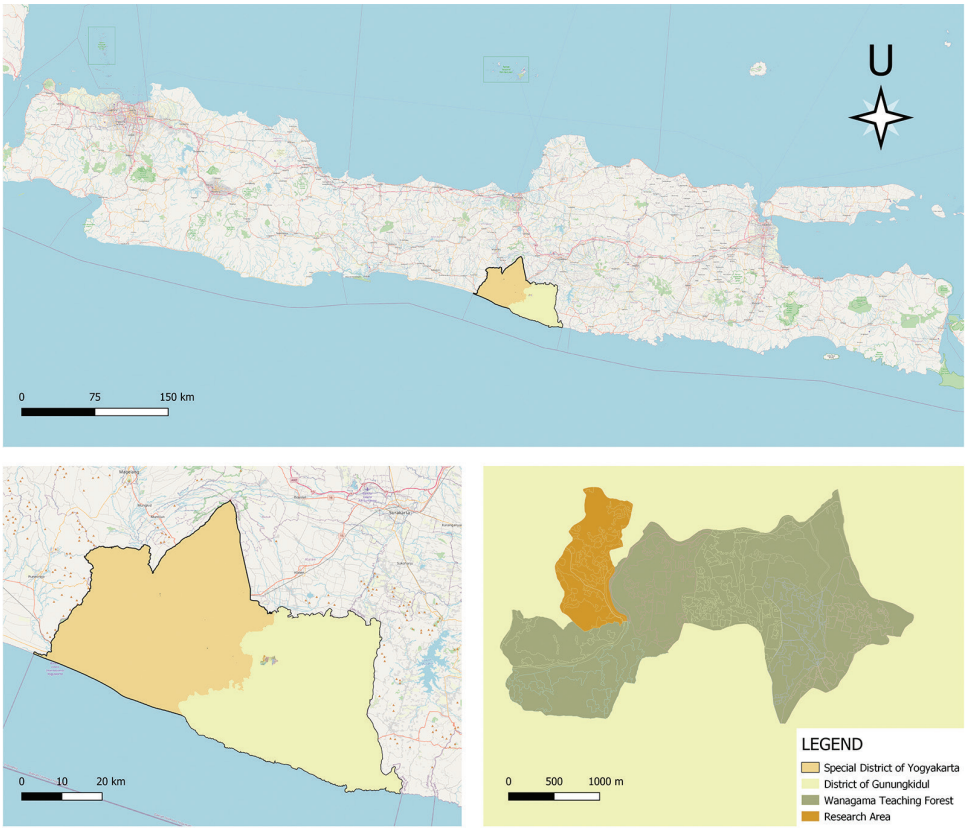

Fig. 1. Research site.

The cajuput stands in compartment 7 of Wanagama Forest were planted and grown naturally without specific treatments. This condition differed for the cajuput stands in Perhutani and KPH Yogyakarta, which were harvested at the height of around 1.5 m to utilize the leaves/twigs (Utomo et al. 2012, Mulyana et al. 2019). Utomo et al. (2012) and Mulyana et al. (2019) explained that the leaves and twigs of cajuput are harvested every year because of cajuput's ability to regenerate.

Data collection

Fifty cajuput trees were divided into two groups, namely, a sample set to develop an allometric equation (30 samples) and a sample set to validate the allometric equation (20 samples). Purposive sampling was used for the determining of selected samples. The selection was based on diameter distribution, accessibility of tree locations, and healthy conditions of the trees.

The selected samples were harvested at the height of 1.5 m, and the cut trees were divided into stems, branches, and leaves/twigs. In the future, the remaining cajuput stump can still produce new leaves/twigs that can be utilized for cajuput oil production (Utomo et al. 2012, Mulyana et al. 2019). Utomo et al. (2012) and Mulyana et al. (2019) described that the cajuput can produce leaves/twigs for

40 years.

The fallen sample trees were measured for their total height. Each section was weighed for the fresh-cut biomass using 0.001 kg precision digital scales. For the total stem biomass, the weight of fallen stem biomass and the biomass of the remaining stump were summed up. The remaining stumps were measured for the diameter at the base (D_b), diameter at the tip (D_t), and height of the stump (h). Volume (v) was then calculated (Fig. 2). The stem biomass was estimated using the equations (1–3) as follows:

$$V = \frac{D_b + D_t}{2} \cdot h, \quad (1)$$

$$B = V \cdot W_d, \quad (2)$$

$$H = h + h', \quad (3)$$

where: V is the volume, cm^3; D_b is the diameter at base, cm; D_t is the diameter at tip, cm; B is the biomass, g; W_d is the wood density, g/cm^3; h is the height of stump, cm; h' is the height of fallen cajuput sample, cm; and H is the total height of cajuput tree, cm.

The fresh-cut samples of wood, branches, and leaves were dried in the oven. According to Indonesia National Standard (SNI) No. 7724 about the measurement and calculation of carbon stock, the samples of stems and branches for oven drying must have at least 2 cm width and a disk shape. The minimum amount of leaves for drying was 100 g. The samples were dried in the oven at 70–85 °C for 72 hours until they reached a stable weight (Badan Standarisasi Nasional 2011).

Data analysis

The allometric models of dry-weight biomass and carbon stock of cajuput stand were developed from destructive sampling data and estimated using a single predictor (diameter or height) and two predictors (diameter and height). The power model is an allometric equation commonly used to estimate biomass and carbon and has been widely applied in mangrove forests (Ong et al. 2004, Kusmana et al. 2018), forest plantations (For-

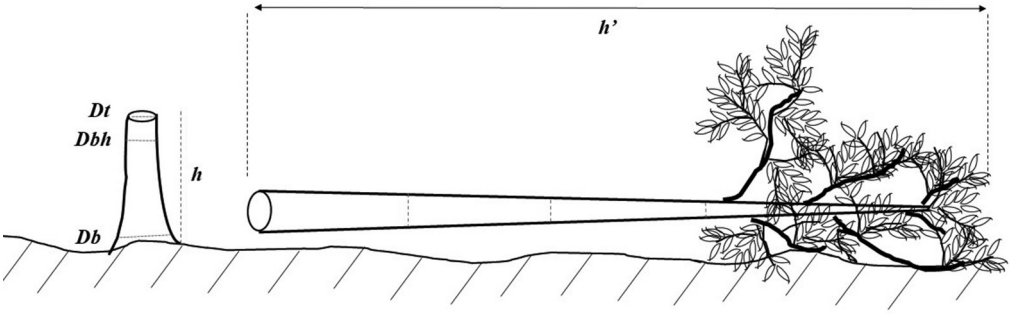

Fig. 2. Data collection of cajuput biomass.

tier et al. 2017, Mulyana et al. 2020). We evaluated the extent to which general or site-specific allometric equations, using diameter at breast height (DBH, dryland forests (Almulqu et al. 2019), and community forest (Wirabuana et al. 2021). Following the methods of Fortier et al. (2017) using diameter at breast height (DBH and Wirabuana et al. (2021), the simple power equations (4–6) to estimate biomass and carbon were as follows:

$$Y = \beta_0 \cdot D^{\beta_1}, \quad (4)$$

$$Y = \beta_0 \cdot H^{\beta_1}, \quad (5)$$

$$Y = \beta_0 \left(D^2 \cdot H\right)^{\beta_1}, \quad (6)$$

where: Y is the dry-weight biomass or total carbon, kg; D is the diameter, cm; H is the total height, m; and β_0 and β_1 are intercepts.

According to Lukito (2011), the average percentage carbon content (*PCC*) of cajuput was 67.53% in stems, 66.40% in branches, and 59.41 % in leaves. The dry-weight biomass was converted to carbon content using the calculated total carbon content in equation (7).

$$TC = \left(0.675 \cdot DW_s\right) + \left(0.664 \cdot DW_b\right) + \\ + (0.594 \cdot DW_l), \quad (7)$$

where: TC is the total carbon, kg; DW_s is the stem dry-weight, kg; DW_b is the branches dry-weight, kg; DW_l is the leave dry-weight, kg.

Biomass (dependent variable) was estimated using the predictors (independent variables) of diameter at breast height (D), total height (H), and the combination of diameter at breast height with total height ($D^2 \cdot H$). The best model was chosen based on the highest value of adjusted coefficient determination (R^2_{adj}) and the lowest values of root mean square error (RMSE) and mean absolute bias (MAB). These parameters can describe the bias between the observed and predicted values (Wirabuana et al. 2021). The equations (8–9) for MAB and RMSE were as follows:

$$MAB = \frac{\sum_{i=1}^{n} \left| Y - \overline{Y} \right|}{n}, \quad (8)$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n} \left(\left| Y - \overline{Y} \right| \right)^2}{n}} \quad (9)$$

where: *MAB* is the mean absolute bias, *RMSE* is the root mean square error, Y is the observed value, and is the predicted value.

The validation of the allometric equation is important in assessing the ability of the model to predict the biomass or carbon based on the selected predictor. This step must be conducted using a dataset differing from the one used in the development of allometric equations (Mulyana et al. 2020). In a small sample size, leave-one-out cross-validation can be applied to process the validation test (Wirabuana et al. 2021). The bias between the observed and predicted values can be analyzed using MAB and RMSE.

Results and Discussion

Statistic summary of cajuput samples

The total aboveground biomass of naturally grown cajuput in Wanagama Forest was allocated in the stems, branches, and leaves/twigs. The dominant biomass allocation was in the stem section, followed by the branches and leaves/twigs (Fig. 3). A similar biomass allocation was also found in the cajuput plantation at Perhutani, in which the aboveground biomass was distributed in the stems (69.44 %), branches (10.27 %), and leaves/twigs (20.29 %) (Lukito 2011).

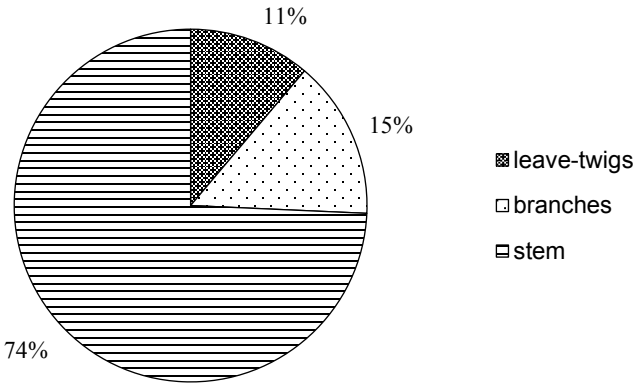

Fig. 3. Biomass allocation in cajuput tree.

A comprehensive research also found dominant stem biomass in the cajuput stand in a peat swamp forest in Indonesia, where the biomass was distributed in the stems (57.69%), roots (22.21 %), branches (17.94 %), leaves (1.35 %), flowers (0.16 %), and fruits (0.66 %) (Alpian et al. 2013). Therefore, the wide range of habitat of cajuput and the site quality could affect its growth and biomass distribution.

The cajuput samples used in developing an allometric equation (Table 1) had similar characteristics to those used in validating the allometric equation (Table 2). In general, the samples represented the real condition of the cajuput stand in Wanagama Forest. The average diameter at breast height of all cajuput samples (data set for development and validation) was 7.4 cm, and the total height average was 7.42 m.

Biomass (dependent variable) was estimated using the predictors (independent variables) of diameter at breast height (*D*), total height (*H*), and the combination of diameter at breast height with total height ($D^2 \cdot H$). The relationship of the total dry-weight of cajuput with the stem diameter, total height, and combination of diameter and total height exhibited a non-linear regression pattern (Fig. 4). This trend was similar to that in *Gliricidia sepium*

Table 1. Statistic summary of the measurements of cajuput samples to develop the allometric equation.

Variables	Minimum	Maximum	Average	Standard deviation
Diameter at breast height, cm	3.90	10.50	7.28	1.55
Total height, m	4.69	13.00	7.47	2.06
Fresh-cut weight of stem, kg per tree	3.20	54.05	20.60	11.01
Fresh-cut weight of branch, kg per tree	0.56	6.90	2.96	1.71
Fresh-cut weight of leave, kg per tree	0.64	9.70	3.07	1.87

Table 2. Statistic summary of the measurements of cajuput samples to validate the allometric equation.

Variables	Minimum	Maximum	Average	Standard deviation
Diameter at breast height, cm	3.60	12.40	6.90	1.99
Total height, m	3.60	9.20	7.17	1.15
Fresh-cut weight of stem, kg per tree	2.28	28.0	25.12	11.04
Fresh-cut weight of branch, kg per tree	0.25	7.98	1.88	1.77
Fresh-cut weight of leave, kg per tree	0.40	6.05	2.06	1.54

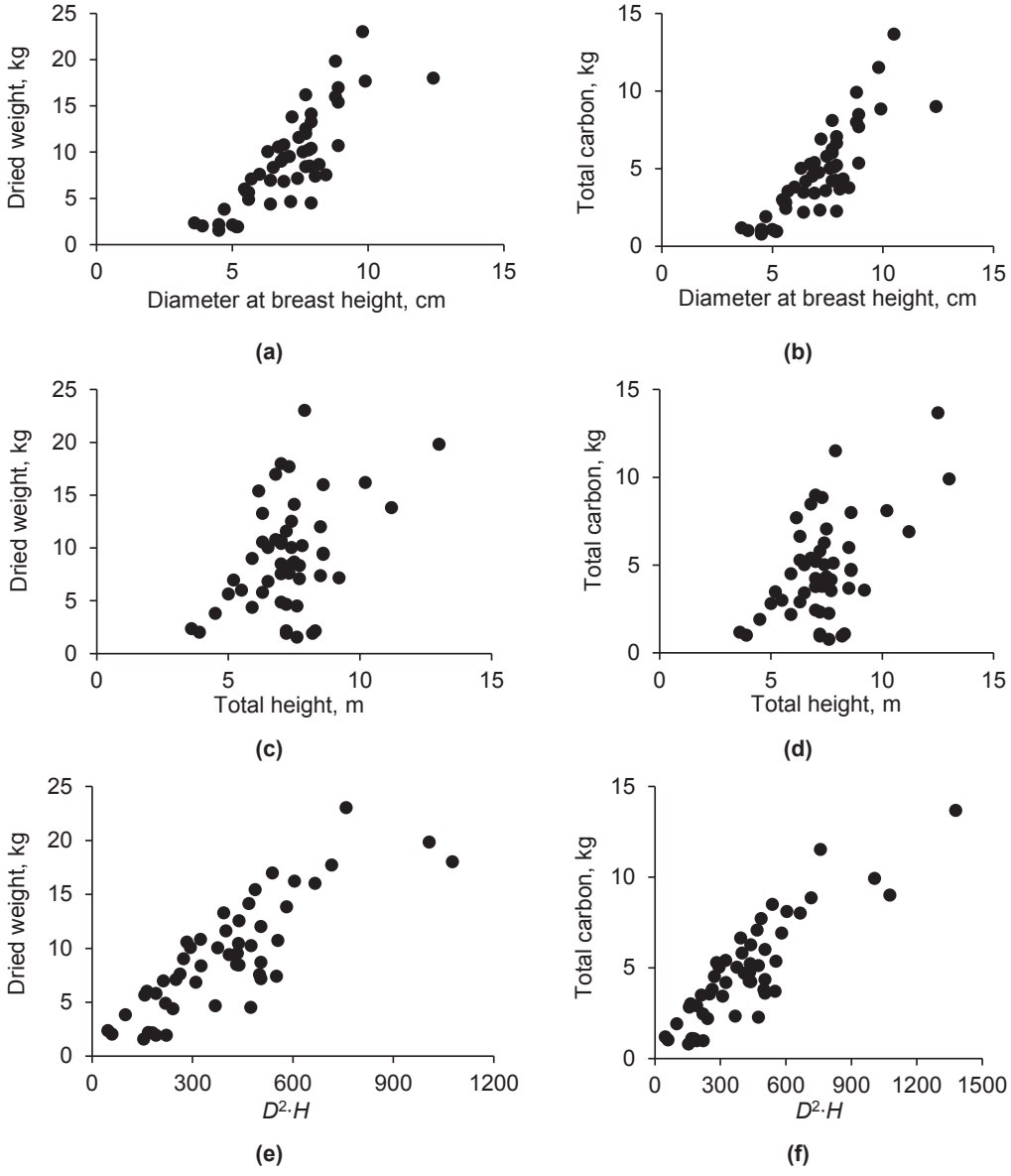

Fig. 4. Relationship among dried biomass (a, c, e) and total carbon (b, d, f) as dependent variables and diameter at breast height (*D*), total height (*H*), and combination of the two ($D^2 \cdot H$).

(Mulyana et al. 2020), *Ziziphus mauritiana* Lam. (Kurniawan and Pujiono 2019) but differed from that in cajuput trees in KPH Yogyakarta (Mulyana et al. 2021). Thus, the allometric equation of naturally grown cajuput in Wanagama Forest

can be compared with other species due to their similar characteristics. However, the allometric equation is not suitable for comparing the naturally growing cajuput in Wanagama Forest with the utilized cajuput in forest plantation.

Specific allometric equation for estimating dry-weight biomass in Indonesia

Allometric equations for estimating biomass and carbon have been applied in many forest types in Indonesia. For instance, Yamakura et al. (1986) developed an allometric equation in the tropical rain forest, Basuki et al. (2009)so far the equations for Dipterocarp forests have not been developed in sufficient detail. In this research, allometric equations are presented based on the genera of commercial species and mixed species. Separate equations are developed for the Dipterocarpus, Hopea, Palaquium and Shorea genera, and an equation of a mix of these genera represents commercial species. The mixed species is constructed from commercial and non-commercial species. The data were collected in lowland mixed Dipterocarp forests in East Kalimantan, Indonesia. The number of trees sampled in this research was 122, with diameters (1.30 m or above buttresses in tropical lowland dipterocarp forest at East Kalimantan, Maulana (2014) in Papua tropical forest, Almulqu et al. (2019) in dryland forest at East Nusa Tenggara, and Wirabuana et al. (2021) in community forest at Java. In addition, Kusmana et al. (2018) developed species–specific allometric equations for *Sonneratia* sp. in the mangrove forest, Mulyana et al. (2020) for *Gliricidia sepium* in bioenergy plantation, and Akbar (2012) for *Shorea teysmanniana* Dyer ex Brandis in peat swamp forest. Indonesia's forest biodiversity, either forest type or species, is enormous. Nevertheless, the allometric equations for specific forest types or sites and species must be further developed to eliminate under- or overestimation (Manuri et al. 2017) biogeographical zones and various succession and degradation levels of natural lowland forests in the Indo-Malay region. The only additional variable that significantly and largely contributed to explaining AGB variation is grouping based on wood-density classes. Context: There is a need for an AGB equation at tree level for the lowland tropical forests of the Indo-Malay region. In this respect, the influence of geographical, climatic and ecological gradients needs to be assessed. Aims: The overall aim of this research is to provide a regional-scale analysis of allometric models for tree AGB of lowland tropical forests in the Indo-Malay region. Methods: A dataset of 1300 harvested trees (5 cm ≤ trunk diameter ≤ 172 cm.

The power model in this research showed an excellent performance in estimating biomass and carbon using the predictors of diameter at breast height and combination of diameter and height. The coefficient of determination (R^2_{adj}) was similar for the allometric equations, but the values of intercept (β_0 and β_1) differed (Table 3). The intercept of β_0 in the allometric equation for estimating total carbon was lower than that in the allometric equation for estimating dry-weight biomass. This finding can be explained by the PCC of cajuput at around 59%–67% from dry-weight biomass.

According to Table 3, the predictors of diameter and combination of diameter and height can be considered as the best allometric equations. The allometric equations for estimating the dry-weight biomass and carbon content of cajuput were

Table 3. Allometric equations for estimating dry-weight biomass and carbon content.

Variables	Equation	β_0	β_1	R^2_{adj}	SEE	$p <$
Dry-weight biomass	$DW = \beta_0 \cdot D^{\beta_1}$	0.136	2.190	0.935	0.181	0.001
	$DW = \beta_0 \cdot H^{\beta_1}$	0.260	1.872	0.559	0.472	0.001
	$DW = \beta_0 \left(D^2H\right)^{\beta_1}$	0.084	0.813	0.942	0.171	0.001
Total carbon	$C = \beta_0 \cdot D^{\beta_1}$	0.142	1.951	0.912	0.211	0.001
	$C = \beta_0 \cdot H^{\beta_1}$	0.258	1.641	0.609	0.369	0.001
	$C = \beta_0 \left(D^2H\right)^{\beta_1}$	0.054	0.817	0.945	0.161	0.001

Note: *DW* is dry-weight biomass, kg; *C* is total carbon, kg; *D* is the diameter at breast height, cm; and *H* is the total height, m.

$DW = 0.136D^{2.190}$ and $C = 0.142D^{1.951}$, respectively. Both allometric equations showed a coefficient of determination above 0.900. A strong correlation was found between dry aboveground biomass or total carbon and the predictor of diameter at breast height.

The findings also confirmed previous works on existing allometric equations in Indonesia stating that biomass and carbon can be estimated using the diameter as predictor in the power model (Table 4). For the cajuput stand, which lives in the peat swamp forest in Indonesia, the power model can be used to estimate dry biomass using the predictor of diameter at breast height. However, the allometric equation for mixed species showed a lower coefficient of correlation than that for the single species.

Table 4. Summary of the existing allometric equations in Indonesia.

Species	Forest type	Equation	R^2_{adj}	References
Melaleuca cajuputi	Peat swamp	$DW = 0.128 \cdot DBH^{2.410}$	0.994	Alpian et al. (2013)
Sonneratia sp.	Mangrove	$DW = 0.299 \cdot DBH^{2.300}$	0.950	Kusmana et al. (2018)
Gliricidia sepium	Forest plantation	$C = 0.019 \cdot D_{30}{}^{2.719}$	0.958	Mulyana et al. (2020)
Shorea teysmanniana	Peat swamp	$DW = 0.09 \cdot DBH^{2.58}$	0.990	Akbar (2012)
Acacia nilotica L.	Savanna	$DW = 0.340 \cdot DBH^{1.970}$	0.984	Istomo and Farida (2017)
Ziziphus mauritiana	Savanna	$DW = 50.75 \cdot DBH^{2.350}$	0.959	Kurniawan and Pujiono (2019)
Mixed stands	Lowland dipterocarp forest	$DW = 0.125 \cdot DBH^{2.533}$	0.821	Manuri et al. (2016)

Note: *DW* is dry-weight biomass, kg; *C* is total carbon, kg; DBH is the diameter at breast height, cm; and D_{30} in cm is the diameter at 30 cm from the base.

Meanwhile, the predictor of total height (*H*) showed a weak correlation with the dry-weight (R^2_{adj} = 0.559, $p < 0.001$) or total carbon (R^2_{adj} = 0.559, $p < 0.001$) of cajuput. Statistical analysis also showed poorer, although significant correlation between diameter at breast height and total height (R^2_{adj} = 0.678, $p < 0.001$). This finding differed from the studies on Poplar species, in which the diameter at breast height had a strong correlation with total height (R^2 = 0.820, $p < 0.001$) (Fortier et al. 2017)using diameter at breast height (DBH. Owing to the unique characteristics of each species, the allometric equation must be species–specific.

The cajuput tree in Yogyakarta has unique characteristics. This research showed that the diameter at breast height posesses a low correlation with the total height. In addition, the crown dimension of cajuput could not accurately predict its biomass (Mulyana et al. 2021). Elaborating the allometric equation is still important in estimating cajuput biomass.

Validation of cajuput allometric equation

The allometric equation with predictor diameter and combination diameter and height produced high coefficients of determination (R^2_{adj}) and the predictor diameter showed the lowest bias (Table 5). By contrast, the total height predictor did not precisely estimate the biomass or carbon.

The use of a single predictor of diameter is highly efficient for fieldwork or rapid assessment. Total height is relatively difficult to estimate, and diameter can be measured easily and precisely (Tiryana 2016). Wirabuana et al. (2020) also recommended using the diameter as a predictor of tree biomass in community forests because of its lower variance than total tree height in the same area. Adding the predictor height did not contribute significantly to the allometric equation, either for dry-weight or carbon content estimation.

Table 5. The allometric equation to estimate carbon stock.

Variables	Equation	MAB	RMSE
Dry-weight biomass	$DW = \beta_0 D^{\beta_1}$	2.974	3.358
	$DW = \beta_0 H^{\beta_1}$	3.180	3.542
	$DW = \beta_0 \left(D^2 H\right)^{\beta}$	3.561	3.981
Total carbon	$C = \beta_0 D^{\beta_1}$	2.793	2.995
	$C = \beta_0 H^{\beta_1}$	2.785	2.929
	$C = \beta_0 \left(D^2 H\right)^{\beta_1}$	3.158	3.418

Note: *DW* is dry-weight biomass, kg; *C* is total carbon, kg; *D* is the diameter at breast height, cm; *H* is the total height, m; MAB is mean absolute bias; and RMSE is the root mean square error.

Conclusion

Diameter at breast height is the best predictor to estimate the dry-weight and carbon content of cajuput in Wanagama Forest. The allometric equations for estimating the dry-weight and carbon content of cajuput were $DW = 0.136 \cdot D^{2.190}$ and $C = 0.142 \cdot D^{1.951}$.

Recommendation

This research was conducted in marginal land, in which the soil formation was dominated by limestone and low water input. Further research on the allometric equation of cajuput can be performed in other types of forest ecosystems, such as dryland, savanna, and tropical rain forest in Indonesia. It will allow development of a comprehensive cajuput allometric equation, which can be applied to a wide range of forest ecosystems in Indonesia.

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Soil properties of the most productive *Tuber aestivum* habitats from midpart of Western Bulgaria

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Abstract

Only recently, truffle hunting in Bulgaria has established itself as an important source of income and due to the specific socio-economic aspects, there is a tendency for continuous increase of the truffle hunters' number. *Tuber aestivum* is the most important commercial truffle in Bulgaria. Because truffles are ectomycorrhizal fungi, at least part of their life cycle is related to plant partner, which in most cases is a tree species. Among those factors that affect growth and distribution of vegetation and of *Tuber* host plants, are pedoclimatic conditions. They are determined from a large extent on geographical and orographic characteristics, creating specific microclimatic conditions suitable for *Tuber* fruitbodies development. In this study we focus on various soil properties of the most productive *T. aestivum* habitats from midpart of Western Bulgaria. We have used Principal component and classification analysis (PCA) to determine the most important soil factors for fruitbody formation. The common feature of the most productive *T. aestivum* habitats from midpart of Western Bulgaria are high cation exchange capacity values (CEC) and low $CaCO_3$ content. Moreover, our research confirms that Ca^{2+}, total organic carbon and total nitrogen are among the most important factors for *T. aestivum* production. The results extend our knowledge on *T. aestivum* ecology and can be used to select the best areas for establishing truffle plantations in Bulgaria.

Key words: PCA, soil environment, summer truffle.

Introduction

Although it was found back in 1965 (Hinkova 1965, Dimitrova and Gyosheva 2008), it became clear that Burgundy truffle – *Tuber aestivum* Vittad. (syn. *Tuber uncinatum* Chatin) occurs frequently in a variety of habitats in Bulgaria. The annual truffle production for all commercial true truffle species (*Tuber* sp.) from natural sites during the last few years in Bulgaria according to unofficial and official sources exceed 500 t in average year but in good one can reach over double. For instance, according to the National Statistical Institute, the 2020-year export is 299 294 kg total of all *Tuber* sp., 210 720 kg of them are exported to Italy. More than two thirds of these truffles are represented by *T. aestivum* and this makes it the most economically important in Bulgaria. Unlike other commercial truffle species like *T. magna-*

tum Picco, which occurrence is restricted to small areas characterised by particularly suitable environmental parameters (Mello et al. 2004), *T. aestivum* occupies a wider ecological niche.

There is sufficient evidence, that most of the truffles have very wide range of plant species as ectomycorrhizal partners and in the past, this was subject to various studies (Bonito et al., 2010; Stobbe et al. 2013b, García-Montero et al. 2014).

Because truffles grow underground or are partially buried, the soil is their environment. For this reason, its physical, granulometric, hydrological and chemical properties separately and as a combination as well and the soil biota, i.e., soil complex environment are of utmost importance (Gryndler et al. 2017).

The moisture content in soils and particularly hydrological regime affects to the physiological activity of vegetation, which in turn affects the truffle harvest. Increase and inhibition of moisture occurs in plane relief and along rivers due to the rising groundwater levels. In thermophilic vegetation zone of Western Bulgaria, under the influence of continental climate with warm summers and rather cold winters, the soil moisture is mostly result of precipitation and therefore these soils are usually very dry in the second half of the vegetation period.

During the last two decades numerous studies of pedological conditions were done for *T*. *aestivum* productive sites in Europe, which spread from Spain and Sweden to Transylvanian Subcarpathian Hill in Romania (Wedén et al. 2004, García-Montero et al. 2009, Păcurar et al. 2019). However, there is no available information for Bulgaria until now.

The aim of this study is to fill this gap – to describe and analyse the soil properties of the most productive *T. aestivum* habitats from midpart of Western Bulgaria. Present data can be used to select the best areas for establishing truffle plantations in Bulgaria.

Material and Methods

Study area

Ten representative productive *T. aestivum* habitats were chosen from more than a hundred known to the authors in a maximum distance of 120 km from Sofia as a mean point of Central part of Western Bulgaria. The criteria were the higher ratio – productivity/area ($kg \cdot m^{-2}$) of truffle habitats, the duration of fruitbody formation and the heterogeneity in soil type and bedrock/vegetation/climate patterns (altitude) compared to the other appropriative sites within the region. Most of the habitats are visited on a weekly or biweekly bases from the end of May till the end of November or till the end of fruitbodies formation. The main topographical characteristics of studied truffle area and productivity are listed in the Table 1, but exact geographical coordinates are not provided due to the lack of legal framework regarding the collection of truffles.

The climate is moderate continental with average annual precipitation for the study region from 550 to 860 mm, with a maximum from May to June. The average annual temperature is from 11.2 ºC (S5 at 290 m a.s.l.) to 7.8 ºC (S4 at 1020 m a.s.l.), average maximum – from 28.4 ºC to 22.6 ºC (August and July) and average minimum from –5.3 ºC to -6.5 ºC (January) (Kyutchukova 1979, Koleva and Peneva 1990).

Table 1. Topographic, vegetation and productivity characteristics of studied area.

Sample	Elevation, m a.s.l.	Exposition	Slope, %	Relief	Microrelief	Host trees	Truffle productivity habitats area*	Annual productivity**
S1	550	NW	2	plane	plane	Qc	M	2
S2	580	E	4	plane	mound	Qc	S	3
S3	570	SW	2	plane	plane	Qc, Tt	M	2
S4	1020	NE	17	slope lower part	plane	Tt, Cs	M	1
S5	290	SW	2	plane	depressed	Cb, Qc, Qd	S	2
S6	720	NE	2	plane	depressed	Tt	M	1
S7	530	S	3	plane	plane	Tt	L	1
S8	890	NE	24	slope lower part	excavation	Cb, Fs	M	2
S9	640	W	3	plane	plane	Tt	S	1
S10	810	SW	14	slope upper part	plane	Ps, Pn	L	1

Note: * S ≤ 25 m^2, 25 m^2 < M ≤ 50 m^2, L > 50 m^2; ** 1 > 5 kg, 2 = 2.5÷5 kg, 3 < 2.5 kg; Qc – *Quercus cerris* L., Qd – *Quercus dalechampii* Ten., Tt – *Tilia tomentosa* Moench., Cs – *Castanea sativa* Mill., Cb – *Carpinus betulus* L., Fs – *Fagus sylvatica* L., Ps – *Pinus sylvestris* L., Pn – *Pinus nigra* Arn.

Soil sampling

A total 40 soil samples (30 plus 10) are collected at approximately 7 cm below the surface after removing litter and accompanying vegetation. Three soil cylinder cores are taken up to 10 cm from three *T. aestivum* fruitbodies found at each truffle habitat. Additionally, approximately 1 kg of soil is sampled for each place from not more than 15 cm distance of *T. aestivum* fruitbody. All soils analyses were performed in specialised laboratory at Forestry University, Sofia.

Soil analyses

To determine soil pH H_2O ISO 10390:2005 was used. Bulk density was measured according to ISO 11272:1998 and particle density by pycnometer method (ISO 787-10:1993). Soil porosity was determined by formula (1).

$$SP = \left(1 - \frac{BD}{PD}\right) \cdot 100, \quad (1)$$

where: *SP* is soil porosity in %, *BD* is bulk density in g·cm^{-3}, *PD* is particle density.

Soil organic matter was determined by modified Turin's method – 120 ºC, 45 min and $CuSO_4$ as a catalyst (Kononova 1963, Filcheva and Tsadilas 2002). Total nitrogen – Kjeldal's method (Kjeltec Auto 1030 Analyser). Available phosphorus (P_2O_5) and available potassium (K_2O) – acetate-lactate method (Ivanov 1984). Soil texture was measured as clay – <2 μm; silt – 2–63 μm and sand – 63–2000 μm (ISO 11277:2009). For exchangeable cations (Ca^{2+}, Mg^{2+}, K^+, Na^+) determina-

tion was used ISO 11260:2018. Content of calcium carbonates (percent) – Scheibler's method – ISO 10693:1995. Saturated water content, field capacity, wilting point and hygroscopic moisture content – Kachinskii method (Kachinskii 1963).

Statistical analysis

Collected data of all productive truffle habitats were analysed using R 4.1.2 (R Core Team, 2022) and main descriptive statistics of soil parameters were presented. Soil texture triangle diagram was built using R package soil texture 1.1.5 (Moeys 2018). For C:N ratio, ANOVA was performed, followed of a Tukey's HSD post hoc test, preceded by Shapiro-Wilk normality test and Levene's test of homogeneity of variance. Principal component and classification analysis (PCA) was done with the aid of package FactoMineR 2.4 (Husson et al. 2017) for 18 selected soil parameters. For graphic visualisation package ggplot2 3.3.5 (Wickham 2016) was used.

Results and Discussion

Geographical characteristics

Studied soils of most productive *T. aestivum* habitats from midpart of Western Bulgaria are located from 300 to 1000 m a.s.l. with all expositions included (Table 1). According to Stobbe et al. (2013a) southern habitats with a warm climate reach higher altitudes (Morocco, ~1,600 m a.s.l.), whereas northern habitats are often located near sea level (Sweden, <50 m a.s.l.), but it seems that even in Bulgaria, Burgundy truffle can reach very high altitudes close to the upper limit in southern parts of a country. The highest known elevation on which fruitbodies of *T. aestivum* are found in Bulgaria is ~1430 m a.s.l. and the lowest 15–30 m a.s.l. near Danube and the Black Sea (T. Nedelin unpublished data). The slopes are from gently to moderately steep. At higher altitudes the Burgundy truffle fruitbodies prefers growing from strongly sloping to steep terrains. One of the reasons might be the greater solar energy coming perpendicular to the surface when the crop began at the end of summer and/or the beginning of autumn at these places combined to the favorable soil moisture. The relief in most cases is plane or lower part of a slope, where the water drains away. The microrelief is plane to more or less depressed, but it can be also mound and is related to the particular climate conditions. Both last characteristics are very important in the respective season and host ability to form *T. aestivum* ECM. For instance, plane relief is preferred for artificial plantations in Hungary at low elevation (Gógán et al. 2012).

Host plants

Host plants of the studied areas are presented in Table 1. The most common (S1-S5, S8) are Fagaceae and with Pinaceae (S10), they play an important ecological role as ectomycorrhizal partners (Bonito and Smith 2016). Lindens are also one of the main Burgundy truffle hosts and it was found in the most productive sites (S3, S4, S6, S7, S9) An interesting observation is that *Corylus avellana*, although is widespread is not among the most productive *T. aestivum* host plants in the region. The possible reason is that it occurs mostly on acidic soils near Sofia.

Our results show that not all plant species have the same significance. They differ from the seasonal dynamics in the

formation of fruitbodies and their quantity. For instance, in Bulgaria, *Pinus sylvestris* and *Pinus nigra*, are most productive from the mid-summer, while *Tilia* sp. from the end of the spring (data not shown). However, it is difficult to separate the exact influence of pedoclimatic conditions and those of a host species.

Physical and granulometric soil properties

The soil granulometric diagram (Fig. 1) reveals broad range in soil particle size distribution. The most appropriative soils of productive areas are clay, silty clay loam, clay loam, silt loam, loam, sandy clay loam and sandy loam.

Unlike Carpathian Basin, where more than two-thirds of Burgundy truffle habitats have heavy clay soils (Bratek et al. 2010) only few in our studied area are with higher content of clay, but not too heavy (for example S5). In such, truffles are usually developing on the surface or up to 5–7 cm depth and can be seen with naked eyes. The crop in these cases sometimes can be remarkable (more than 1 kg per once visitation at ≈10 m^2 or less area. The fruitbodies are often larger and from our observation a single truffle can reach 200 g and more but the fruiting period is relatively short and production is very dependable from climate conditions. Compared to the other studies (Wedén et al. 2004, Raglione and Owczarek 2005, Bruhn and Hall 2011, Gógán et al. 2012, Robin et al. 2013, Stobbe et al. 2013a, Jamali 2017, Hilszczańska et al. 2019), truffle representative productive habitats from midpart of Western Bulgaria resembles the granulometric structure of most *T. aestivum* productive soils from Poland, Sweden, Italy, France, Belgium, Romania, Hungary and Iran as well as those on which artificial plantations have been established in Canada and in Israel.

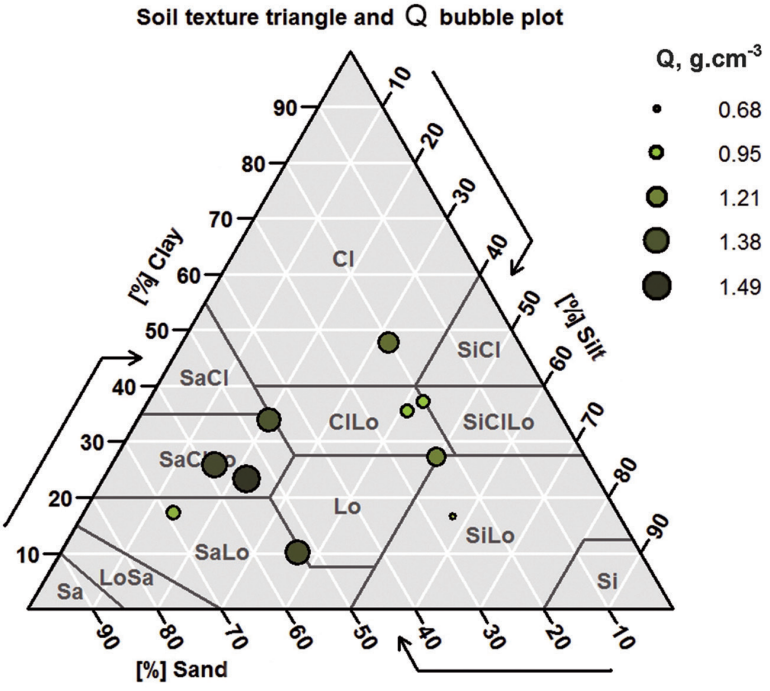

Fig. 1. Soil texture triangle and bulk density plot of studied truffle habitats.

Studied soils are Vertisols, Cambisols, Luvisols, Fluvisols and Rendzinas. The last one is from the

most favourable type in Western and Central Europe (Chevalier and Sourzat 2012, Stobbe et al. 2013a).

The bedrock is represented by sedimentary rocks, andesite, limestone, marlstone and conglomerate (Nishava KiT 2019; Silva 2003 2019). To our knowledge, andesite is not preferred bedrock for Burgundy truffle habitats and is still not reported in the literature.

In recent studies, bulk density (*BD*) of *T. aestivum* productive soils is reported around 1 (Bragato et al. 2021a, b). Our results show that *BD* varies from 0.68 to 1.49. The lowest value is observed in *P. nigra* and *P. sylvestris* plantation developed on Rendzinas with limestone bedrock (S10).

In Table 2 are presented main descriptive statistics parameters of studied soils.

Hydrological and chemical soil properties

Soil parameters such as water availability could influence truffle plantation success (Todesco et al. 2019). Three (S7, S9 and S10) of most productive habitats have similar hydrological properties. In them, soils are well drained, with higher val-

Table 2. Descriptive statistics of some of the most important properties of soil of studied truffle habitats.

Parameters	Mean	St.Dev.	min	max
HMC, Hygroscopic moisture content, %	10.73	±3.51	5.35	17.29
SWC, Saturated water content, %	46.89	±17.45	30.48	85.70
FC, Field capacity, %	31.76	±14.31	20.14	64.47
PWP, Permanent wilting point, %	14.38	±4.70	7.16	23.17
PD, Particle density, $g \cdot cm^{-3}$	2.53	±0.06	2.46	2.67
P, Porosity, %	53.90	±10.92	40.00	73.00
Q, Bulk density, $g \cdot cm^{-3}$	1.16	±0.27	0.68	1.49
pH, H_2O	7.41	±0.56	6.80	8.10
Sand, %	39.28	±18.62	20.16	68.89
Silt, %	33.27	±14.87	13.87	57.50
Clay, %	27.45	±11.30	10.04	47.66
SOM, Soil organic matter, %	5.58	±2.17	1.28	8.18
TOC, Total organic carbon, %	3.24	±1.26	0.75	4.74
TN, Total nitrogen, %	0.30	±0.12	0.09	0.52
C:N	10.74	±1.58	8.29	13.06
P_2O_5, $mg \cdot 100\ g^{-1}$	12.20	±7.64	2.24	24.72
K_2O $mg \cdot 100\ g^{-1}$	45.84	±17.31	22.61	76.69
$CaCO_3$, %	2.32	±3.40	0.60	11.90
Ca^{2+}, Exchangeable calcium, $cmol(+) \cdot kg^{-1}$	44.36	±28.55	17.14	92.29
K^+, Exchangeable potassium, $cmol(+) \cdot kg^{-1}$	0.91	±0.44	0.40	1.95
Mg^{2+}, Exchangeable magnesium, $cmol(+) \cdot kg^{-1}$	3.83	±1.80	2.17	7.53
Na^+, Exchangeable sodium, $cmol(+) \cdot kg^{-1}$	0.10	±0.12	0.03	0.40
EA, Exchangeable acidity, $cmol(+) \cdot kg^{-1}$	0.02	±0.00	0.01	0.02
CEC, Cation exchange capacity, $cmol(+) \cdot kg^{-1}$	49.13	±29.26	20.33	100.00

ues of HMC and both SWC and FC. The higher values of FC suggest that even at higher summer temperatures and southern expositions these soils have enough moisture to ensure fruitbodies development.

The mean value (7.4) and the range of pH (H_2O) – 6.8–8.1 of studied soils (Table 2) corresponds to the value pointed out to Burgundy truffle in Europe (Stobbe et al. 2013a, Păcurar et al. 2019). It refers also to the most soils in Bulgaria, spreading up to 1000–1200 m a.s.l.

SOM, TOC and TN vary in a wide range. For example, S7 soil is very poorly stocked according to the parameters above, but is one of the most productive truffle sites near Sofia with a lot of large and long-distance spreading truffle patches. This can be explained due to the suitable plant host (*T. tomentosa*), its age and plantation structure, available herbaceous plants (*Geum urbanum* L., *Ranunculus ficaria* L. and *Anemone nemorosa* L.), some of them are also known to support ECM (Gryndler et al. 2014) and can stored a plenty of carbohydrates respectively. It should be noted that the area is surrounded from two sites by water.

In most studied soils, C:N ratio is around 10, which corresponds to a number of studies, not only for *T. aestivum* but for some other truffles with commerce values (Hilszczańska et al. 2008, Chevalier and Sourzat 2012). This ratio indicates a preference of *T. aestivum* for soils that are poor in the readily degradable nitrogen. Compared to some observations from Italy (Piacenza), Sweden and France (Weden et al. 2004, Innangi et al. 2020), studied soils near Sofia shows slightly lower values resembling those from the region of Parma with a mean value of 10.8 (Gregori 2010). Our results show also, that in soils with loose and optimal structure, according to their bulk density, C:N ratio is ~10, while in those with compact and very compact structure, C:N ratio is ~12 (p = 0.0393, Tukey HSD = -1.974).

Calcium (Ca^{2+}) is essential element for plant growth, but also numerous of studies point out that it is a key factor for *Tuber* sp. occurrence and fruitbody formation (García-Montero et al. 2009, Gryndler et al. 2017, Hilszczańska et al. 2019). It is provided mostly from soil solids. Through ion exchange, elements such as Ca^{2+} and K^+ are released and escape into the soil solution where they can be readily absorbed by ECM. Its concentration in soil has a seasonal fluctuation, especially in broadleaves forests, and after its leaves fall to the ground, they decompose rapidly and release large amounts of Ca^{2+} ions that become adsorbed as exchangeable Ca^{2+} on humus and clay. Compared to the other studies (Hilszczańska et al. 2019), exchangeable calcium in most productive truffle habitats in midpart of Western Bulgaria varies in broader range – from 17.14 to 92.29 cmol(+)·kg^{-1}.

According to the total $CaCO_3$ content, with the exception of S10, studied soils are poor or little stocked. The cation exchange capacity (CEC) is an important variable, dependent on the amount of humus in the soil, and the amount and type of clays (Weil and Brady 2017). Low CEC soils need quick but frequent watering, while high CEC soils need slow water applied less often. In most natural or artificial forest sites, where *T. aestivum* occurs, CEC ranges from 5 to 45 cmol(+)·kg^{-1} (García-Montero et al. 2008, Robin et al. 2016). However, our result shows a range from 20 to 100 cmol(+)·kg^{-1} and mean of our study productive truffle habitats is around 50 (Table 2).

PCA – BIPLOT (Fig. 2) are built to present soils variables interaction and

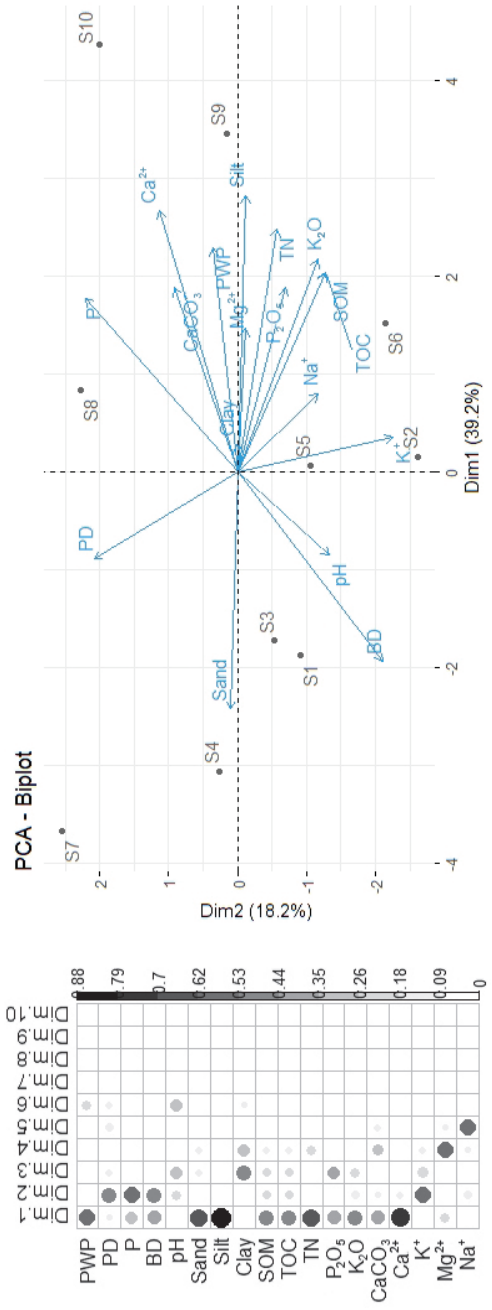

Fig. 2. PCA – BIPLOT and bubble plot (factor coordinates of dimensions), according to the most productive *T. aestivum* habitats in midpart of Western Bulgaria.

each soil sample impact (individual contribution) on the most productive *T. aestivum* habitats in midpart of Western Bulgaria.

Principal component analysis (PCA)

First two principal components (dimensions) express 57.4 % of the total inertia (Fig. 3), and first 5 of them fit more than 91 % of it.

The first principal component summarizes 39.2 % of the overall variance. The main structure elements of Dim 1 are presented from the variables (factor coordinates of PCA variables are presented in S-1): Silt (0.939), Ca^{2+} (0.887), TN (0.825), Sand (-0.807), i.e. its presence have negative effect on fruitbody formation of *T. aestivum* if we assume that first three components are favourable and PWP (0.759). Another possible hypothesis for the above, can be the fact that the Burgundy truffle ECM is of short distance exploration type (Zambonelli et. al. 1995) and in a higher sand content, the resistance in the soil is less, and would be conducive to the development of competitive long distances type ECM fungi.

The second principal component summarizes 18.2 % of the overall variance. The main structure elements of Dim 2 are constructed from the variables: K^+ (-0.751), P (0.735) and *BD* (-0.700). According to PCA – BIPLOT diagram, bulk soil density is negatively correlated with porosity and in other hand, K+ are negatively correlated to particle density. The third principal component focuses mainly on Clay and pH (15.1 %).

Individual contribution to PCA is presented in Figure 4 according to their bulk density. The variation of physical, granulometric, hydrological and chemical soil properties in optimal-lose soils from midpart of Western Bulgaria is much great-

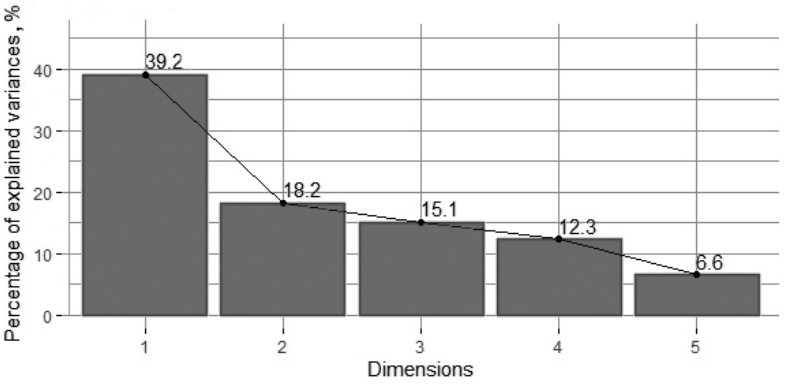

Fig. 3. Scree plot of % of total variance according to principal components (dimensions).

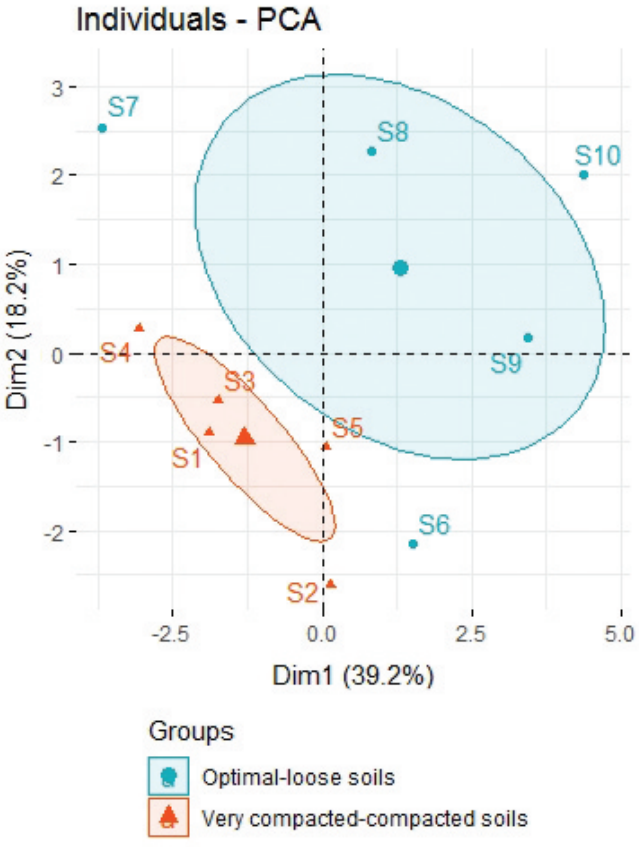

Fig. 4. Productive truffle habitats, according to their bulk density.

er than the very compacted-compacted soil group. According to our observations, in first, *T. aestivum* crop is more dependable on external (out of soil) parameters such as climate and especially precipitation. According to our observations, Rendzinas are among the most suitable soils and we suggest that *T. aestivum* ectomycorrhiza is not only element but also function of the forest ecosystems spreading in such soils. This is because most plants do not have simple, plant-only roots – they have mycorrhizae (Begon and Townsend 2021).

Conclusions

It has been found that productivity of *T. aestivum* habitats from midpart of Western Bulgaria varies between years in extremely wide borders and even all pedological conditions are perfect, there is no guaranty that the formation of fruitbodies will occur. The reason for this is complex, but the temperature and precipitation are of the most importance in combination with the soil mechanical and chemical properties as well as the hydrologi-

cal regime in the soil, which determines the spatial distribution of the ECM roots. Most productive *T. aestivum* habitats from midpart of Western Bulgaria are characterising with great diversity of physical, hydrological and chemical soil parameters. Their common feature is very high Cation exchange capacity values (CEC) and very low and low $CaCO_3$ content.

Compared to other *T. aestivum* PCA studies (and to other commercial truffles also like *T. melanosporum* Vittad.) our research confirms that Ca^{2+}, TOC and TN are among the most important factors for productive sites. As this is already well known, future efforts should focus on the factors affecting to the formation of ECM and fruitbodies in the context of interaction between the competing ectomycorrhizal fungal species.

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Habitat preferences by Western capercaillie (*Tetrao urogallus* L.) on the Lička Plješivica massif (Dinaric Alps)

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Abstract

The Western capercaillie is a large galliform bird whose populations are declining in Europe. By the middle of the 20th century it was present on six massifs of the Dinaric Alps in Croatia. In the meantime, the capercaillie has disappeared from two massifs and lek numbers have fallen by 77 %. The only area where lek numbers have increased is the Lička Plješivica, a massif on the border with Bosnia and Herzegovina. In this study we applied ENFA analysis to evaluate the habitat suitability for capercaillie on the Lička Plješivica massif. The habitat analysis showed that the capercaillie prefers steeper terrain and ridges, forest roads as well as forest associations with higher basal areas, and it avoids grasslands. The capercaillie in general prefers areas with a cold and humid climate. However, compared to the boreal region, the Alps and the Carpathian Mountains, the capercaillie shows a certain habitat preference for the Dinaric Alps. In conclusion, the high suitability of the habitat and its connection to the Bosnia-and-Herzegovinian capercaillie population are probably the causes underlying the increase in the capercaillie population on the Lička Plješivica massif.

Key words: avian habitat suitability, Balkan forest dwelling bird species, Croatia, leks, range decline.

Introduction

Since the middle of the 20th century a population decline has been observed of various species of forest grouse all over the Eurasian area. Currently, two main Eurasian metapopulations of Western capercaillie (*Tetrao urogallus* Linnaeus, 1758) are: boreal (inhabiting the area of northern Europe and Siberia, from Scandinavia to the River Yenisei) and alpine (inhabiting the Central European area) (Segelbacher et al. 2003). Boreal area is non-fragmented habitat with estimated

capercaillie population of about 1,000,000 birds. Differently, alpine population is estimated at only 30,000 birds that live in a highly fragmented area, frequently lacking corridors that should connect different populations. Alpine population is therefore divided into small, sometimes isolated, local populations (subpopulations) ranging from 100 to 1000 birds. Beside these areas, capercaillie can also be found on smaller areas of northern Scotland, Cantabria, Ardennes, Jura Mountains, Dinaric Alps, Rhodope Mountains, Rila Mountain, Pirin Mountain and Carpathian area. Though species distribution and still preserved large populations in Scandinavia and Russia prevents it to be declared as highly endangered, its populations in other (out boreal) European habitats are rapidly decreasing (Klaus et al. 1997, Storch 2007).

A similar trend was also observed in the Dinaric Alps. In the middle of the 20th century, the Western capercaillie (*Tetrao urogallus*) was present in the six main massifs in Croatia (Risnjak, Velika Kapela, Mala Kapela, Velebit, Ličko sredogorje and the Lička Plješivica) and almost all the leks were found above 1000 m altitude. The majority of leks were on Risnjak massif (112), while only one was found on Ličko sredogorje massif (Table 1, Fig. 1, Franić 1910, Rucner 1954, Turkalj 1956, Car 1970, Lukač 2004, Frković 2012) mostly in silver fir-beech or sub-mountain beech forests. On most Croatian part of Dinaric Alps populations of the species are composed of boreal lineage and belong to subspecies *Tetrao urogallus major* (Bajc et al. 2011), but some areas are not included in the analysis.

One of the factors behind the population decline is habitat changes. According to Sachot (2002), the decline or even local disappearance of this species has been observed in the following habitat types: a) highly cultivated areas of Western and Central Europe where capercaillie have only survived in smaller, protected areas such as National Parks, b) areas with intensive coniferous production in the Scandinavian region; c) highly-managed forest areas between the River Volga and the Ural Mountains; and d) well-preserved mountain forests on the southern border of its distribution (i.e. the Balkan Peninsula and some parts of the Alps).

In general, the capercaillie prefers habitats that include original spruce-beech-fir stands and a spruce vegetation area (Storch 1993, Saniga 2004). However, the relatively large and dispersed area inhabited by capercaillie has led to the development of certain peculiarities in its habitat preferences. For example, in the Scandinavian area the capercaillie inhabits mostly coniferous forests, while in the Cantabrian region (Spain) it can also be found in predominantly beech (*Fagus sylvatica* L.) forests with few conifers (Rodriguez and Obeso 2000). This suggests that the traditional understanding of the capercaillie's habitat is frequently influenced by the bird's habits, the season, food availability and the bird's sex (Storch 1993, 1994; Miettinen et al. 2008, 2010).

The aim of this study was to give balance of former and recent number of capercaillie leks and to analyse habitat quality from the model of Lička Plješivica, the only massif with an increasing capercaillie population in Croatia, as a prerequisite for the creation of guidelines for preservation of the Dinaric population, and its connection with the population in Bosnia and Herzegovina.

Material and Methods

Capercaillie monitoring

The presence of the capercaillie all over the massif was recorded by direct monitoring and by identified signs of their activities (tracks, faeces) tracking during the mating season (March – May), and searching for feathers during the moulting period (summer). Since these traditional methods can have negative impacts on capercaillie, expressed as disturbance during the mating season, or lead to consumption of energy in harsh environmental conditions (flight), we also used 45 camera traps as a non-invasive form of monitoring. The animals were monitored from April 2009 to May 2018. The localities where the presence of capercaillie was confirmed (observed birds, faeces, footprints, feathers, images on cameras) were recorded by GPS (Fujitsu Siemens PDA 560 S coupled with a Navman B-10 antenna magnifier).

Habitat evaluation on the Lička Plješivica massif

The Lička Plješivica massif is the second largest massif in Croatia, partly located in the neighbouring Bosnia and Herzegovina. The area above 1000 m altitude covers a surface of 42,700 m^2. Due to its fragmentation, we have only analysed habitat characteristics in the uninterrupted part that covers a total of 24,205 m^2 (Fig. 1). On this part of the massif the capercaillie is a resident species. According to Köppen climate classification (Šegota and Filipčić 2003), the area belongs to the *Df* climate type (boreal climate). The mean annual temperature varies from 2 to 8 °C, and mean July temperature from 10 to 17 °C. Annual precipitation is 1377–2458 mm (Zaninović 2008). The forests are managed. According to stand form the most dominant are European beech (64 %) and mixed silver fir *(Abies alba* Mill.*)* and European beech (33 %) forest stands. The rest of the stands (3 %) belong to management class of oaks (*Quercus petraea* (Matt.) Liebl., *Quercus pubescens* Will. and *Quercus cerris* L.), black pine (*Pinus nigra* J.F. Arnold) and Norvegian spruce (*Picea abies* (L.) H. Karst.). Mixed silver fir-European beech forests are managed in way of selective stand form, while other management class stands are managed as uneven-aged stand forma resident species.

Until now, several habitat suitability models (HSM) frameworks are developed and they can be divided in different techniques (Hirzel and Le Lay 2008), but for wildlife are relatively often in use Habitat Suitability/Selection Models (Guisan and Zimmermann 2000, Ottaviani et al. 2004, Graf et al. 2006, Braunisch and Suchant 2007, Bollmann et al. 2011). The models can use presence/absence or only presence data of focal species. However, when working with rare species, data are often scarce and choosing a wrong absence could significantly reduce the quality of a model. Namely, absence data in particular are often difficult to obtain accurately. A given location may be classified in the 'absence' set because the species could not be detected even though it was present. This problem can exist is situations where absence data are not available (many data banks), when are unreliable (most cryptic or rare species), or they are meaningless (invaders). That's why, in the paper a method was used based only on presence data (Braunisch et al. 2008) – Ecological niche factor analysis (ENFA, Hirzel et al. 2002).

ENFA computes suitability functions by comparing the species distribution in the

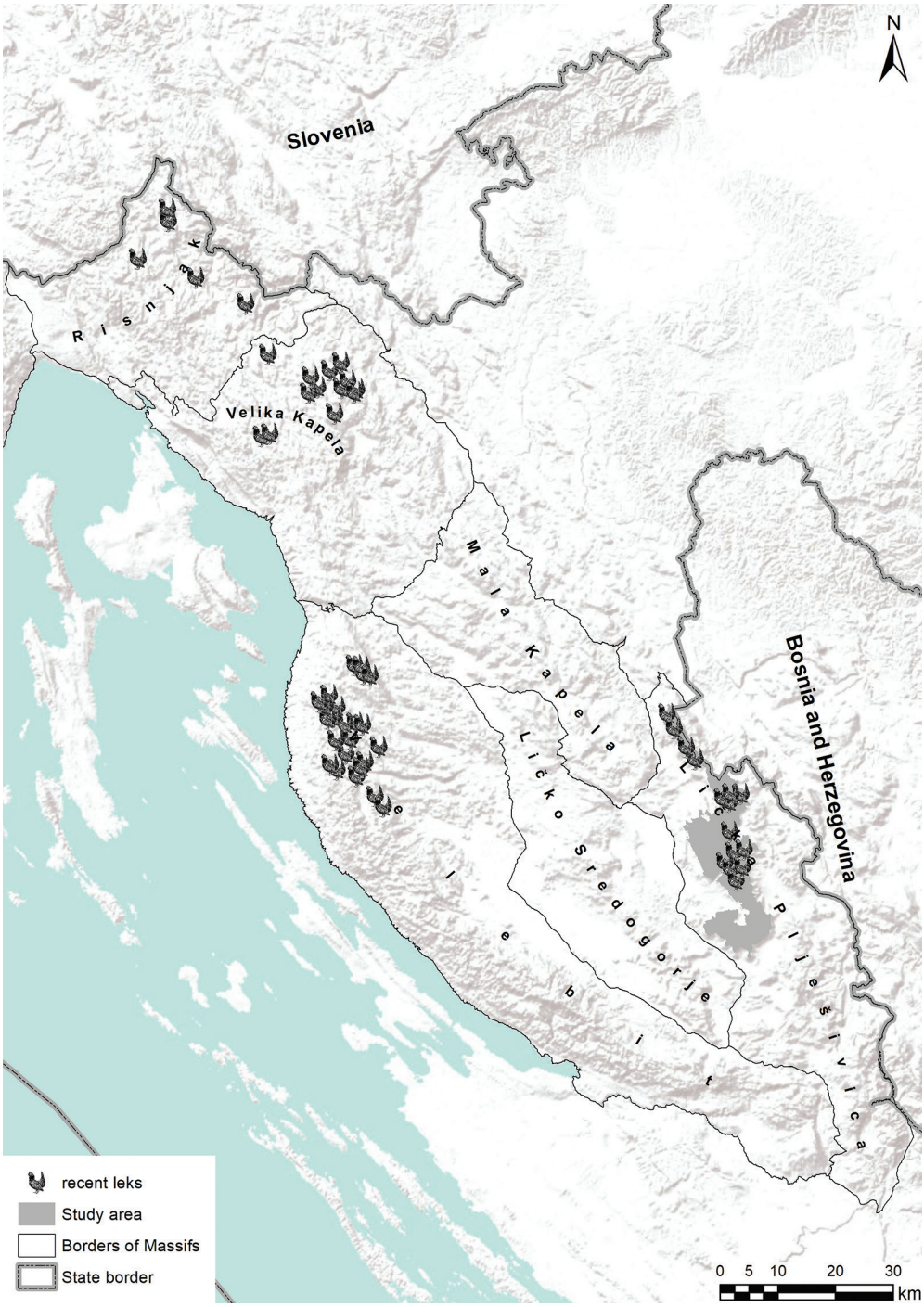

Fig. 1. Current distribution of capercaillie leks with respect to elevations.

ecogeographical space with that of the whole set of cells. The study area is divided in a lattice of adjacent cells (quadrats), each being characterized by a series of N ecogeographical variables (EGV). Thus, EGVs and species distribution are represented as set of raster maps.

Hirzel et al. (2002) created raster maps in 1×1 km (100 ha) lattice of cells. However, the Dinaric massifs in Croatia with altitude of 1000 m a.s.l. are so narrow that usually, neighbouring cells of 100 ha do not enter the researched area. For these reasons, the analysed area was divided into a network of smaller squares, measuring 100×100 m (1 ha). The capercaillie presence map was made in a way that capercaillie observation was recorded by GPS, which represented the centre of their presence (life range). Additionally, the quadrants located within a radius of 500 m from the GPS point were also included in the area. If the quadrants were further from the border of the 1000 m a.s.l. area, they were not considered. With this, the raster network proposed by Hirzel et al. (2002) was partly adjusted. EGVs were calculated for each square. In total, we used 22 EGVs of three categories of data:

a) Topographic (3 EGVs): elevation (01_tu_elev), inclination (02_tu_slope), topographic index (03_tu_topo).

b) Climate (15 EGVs): mean annual number of days with precipitation of at least 1 mm (04_tu_mnd_pr1mm), mean annual number of days with snowfalls above 1 cm (05_tu_mnd_sn1), mean annual number of cold days – t_{min}< 0 °C (06_tu_mancd), mean annual precipitation (07_tu_maprec), mean precipitation in spring (08_tu_manprec_spr), summer (09_tu_meanprec_summ), autumn (10_tu_manprec_aut) and winter (11_tu_meeanprec_wint), mean precipitation in vegetation period – 1st May to 31st October (12_tu_meanprec_veget), mean annual air temperature (13_tu_mean_annairtemp), mean air temperature in April (14_tu_meantemp_apr) and July (15_tu_meantemp_july), mean sun exposition per year (16_tu_mean_sunexpos), mean annual humidity (17_tu_mean_humid); and seasonal precipitation amounts, in percentages of multiannual average (in percentiles) in summer (18_tu_percprecip_summ).

c) Landscape variables (4 EGVs): percent of forest area (19_tu_perc_forest), distance of cell to roads (20_tu_dist_road, only forest roads are present in the study area), distance of cell to grasslands (21_tu_dist_grass) and basal area (22_tu_basal_area), Basal area is the common term used to describe the average amount of an area occupied by tree stems (m^2). It is defined as the total cross-sectional area of all stems in a stand measured at breast height (1.3 m), and expressed as per unit of land area – $m^2 \cdot ha^{-1}$ (Sgerm and Pranjić 1987).

Topographic variables were obtained from digitalized topographic maps (1:25,000) and transferred into a digital elevation model (DEM). A topographic index is a variable described by Guisan et al. (1999) and Weiss (2001). The Topographic Position Index (TPI) compares the elevation of each cell in a DEM to the mean elevation of a specified neighbourhood around that cell. Positive TPI values represent locations that are higher than the average of their surroundings, as defined by the neighbourhood (ridges). Negative TPI values represent locations that are lower than their surroundings (valleys). TPI values near zero are either flat areas (where the slope is near zero) or areas of constant slope (where the slope of the point is significantly greater than zero). According to TPI cells were classified in

six categories: valleys (1), lower slopes (2), gentle slopes (3), steep slopes (4), upper slopes (5) and ridges.

For TPI we used 'Land Facet Corridor Designer' program for ArcGIS 9.3 (Jenness et al. 2013).

Climate variables were derived by scanning and geocoding meteorological maps (raster maps in 100 ha resolution) (Zaninović 2008). Percentile discrepancies were obtained from the Croatian Meteorological and Hydrological Service, and evaluated using a modified form of Conrad-Chapman's method. Land usage was taken from a map of habitat types in Croatia (Bioportal 2019) and the digitalized forest database (CROATIAN FORESTS 2022) of Croatian Forests Ltd.

Maps were prepared using the ArcGIS 9.3 and Idrisi Selva 17.00 software packages. Prior to the analysis, data were standardized due to the different measuring units.

Data and aerial analysis and preparation of the habitat suitability map were made in the Biomapper 4.0 program (Hirzel et al. 2004), according to following procedure:

1. Testing on discrepancies of EGVs maps.

2. Box-Cox transformation (Box and Cox 1982) and standardisation of EGVs by retrieving means and dividing by standard deviations.

3. Calculation of correlations, making of global correlation tree and calculation of Standardised Levins' niche breadth index (B_{sta}, Krebs 1989). As Standardised Levins' niche breadth index is lower, the focal species are closer to habitat specialist. From this point of view HSM analysis was performed on EGVs with the lowest breadth index in each of three types of data – in total on the basis of 11 EGVs.

4. Calculation of marginality and specialisation. Both of them can be particular (for each EGV) and global (for all EGVs). Marginality represents the absolute difference between global mean and species mean, divided by 1.96 standard deviations of the global distribution. A large value (close to one) means that the species lives in a very particular habitat relative to the reference set. Specialisation represents the ratio of the standard deviation of the global distribution to that of the focal species. Specialization coefficients range from -1 to +1, but only their absolute value is meaningful. A high value indicates a narrow niche breadth in comparison with the available conditions. Global specialization coefficients range from 1 to infinity, but it is important for calculating global tolerance. For ease of interpretation, the global tolerance coefficient is defined as the inverse of the global specialisation and it ranges from 0 to 1. A high tolerance value indicates that within a given study area, the species occupies a relatively wide niche, and vice versa.

5. Calculation of habitat suitability (HS) map and it validation. Validation is performed by comparing the habitat suitability value of a validation subset (cross-validation procedure, FAQ 2005). The model quality was quantified using the continuous Boyce index ($B_{cont(W)}$, Hirzel et al. 2006), a threshold independent modification of the Boyce index (Boyce et al. 2002), which quantifies the relationship between the observed and expected number of validation points for different values of the habitat suitability index (HIS). This index varies from -1 to 1. Positive values indicate a model which predictions are consistent with the presences distribution in the evaluation dataset, values close to zero mean that the model is not different from a chance model, negative values indicate an incorrect model, which predicts

poor quality areas where presences are more frequent.

According to ENFA habitat suitability map was developed in a way that area is divided into five categories: a) 0 % of area in net cell is suitable for capercaillie, b) 1–25 % suitability, c) 26–50 % suitability, d) 51–75 %, and e) 76–100 % suitability.

Results

In contrast to other massifs in Croatia, the trend in the capercaillie population on the Lička Plješivica massif shows a 36 % increase in lek numbers. From the previously recorded 11 leks, in this study we found 4 new leks, which gives a total of 15 leks (Table 1). Data for lek numbers of all six massifs in the middle of the 20th century were obtained from literature (Franić 1910, Rucner 1954, Turkalj 1956, Car 1970, Lukač 2004, Frković 2012), game management plans and forest management plans and our direct observations.

The habitat suitability map based on 11 EGV explains 60 % of total variance with the two first factors (marginality and first factor of specialisation, Table 2, Fig. 2). Overall marginality is relatively high, very close to 1 (0.904). It means that capercaillie lives in a very particular habitat relative to the reference set. Positive scores in bold indicate capercaillie preference for microlocalities with higher value than average, while negative scores indicate capercaillie preference for microlocalities with lower value than average in the area.

According to the scores habitat suitability (HS) for capercaillie is most influenced by climatic EGVs. More precisely, HS largely increases with seasonal precipitation in summer (score 0.453), low mean air temperature in July (score -0.391), and during the year (score -0.388), relative air humidity (score 0.374), mean air temperature in April (score -0.367) and mean annual number of days with snowfalls above 1 cm (score 0.031). It means that, capercaillie prefers areas with above average rainfall during summer in regards to long term mean and in general colder microlocalities with more snow days.

The most important land-use variables are distance to roads (score -0.232) and slopes (score 0.189), distance to pasture (score 0.129) and basal area (score 0.121). Negative impact of road distance to capercaillie presence, in fact means that capercaillie prefers microlocalities closer to roads and other EGVs indicate preference of steeper terrains and ridges,

Table 1. Number of capercaillie leks according to the massifs in Croatia.

Massif	Area above 1000 m a.s.l., ha	Area above 900 to 1000 m a.s.l., ha	Numbers of leks in the middle of the 20th century	Current number of leks	Percentage of recent leks in relation to the middle of the 20th century, %
Risnjak	23,232	11,269	112	5	4
Velika Kapela	27,719	19,376	54	14	2
Mala Kapela	6243	13,016	9	0	0
Velebit	65,347	19,038	60	24	40
Ličko sredogorje	6237	11,976	1	0	0
Lička Plješivica	42,697	17,111	11	15	136
Total	171,475	91,787	247	58	23

Table 2. Values of the first four ENFA factors for capercaillie on the Lička Plješivica massif.

Parameter	Marginality factor	Specialization factors		
		Spec. 1	Spec. 2	Spec. 3
	25 %	35 %	9 %	6 %
05_tu_mnd_sn1	**0.310**	-0.045	**0.453**	**-0.656**
14_tu_meantemp_apr	**-0.367**	-0.047	**0.276**	**-0.557**
15_tu_meantemp_july	**-0.391**	**-0.724**	**-0.441**	**0.119**
22_tu_basal_area	**0.121**	-0.008	**0.164**	**0.133**
21_tu_dist_past	**0.129**	-0.018	**0.188**	**0.200**
20_tu_dist_road	**-0.232**	-0.006	**-0.107**	**-0.155**
13_tu_mean_annairtemp	**-0.388**	**0.685**	**0.153**	**0.143**
17_tu_mean_humid	**0.374**	-0.032	**-0.645**	**0.310**
18_tu_percprecip_summ	**0.453**	-0.017	**0.076**	**-0.208**
02_tu_slope	**0.189**	0.002	**-0.072**	0.013
03_tu_topo	-0.016	0.001	-0.005	**0.056**

Note: Numbers in bold indicate statistical significance the level of $p<0.05$. Percentages refer to explained marginality and specialization.

microlocalities more distant to grasslands and, finally, forest stands of thicker trunks of trees. Topographic index doesn't have significant impact on capercaillie presence.

Eigenvalue attributed to this first factor is relatively low (26.854). It means that randomly chosen cells in Lička Plješivica are about 30 times more dispersed on this axis than are the cells were capercaillie was recorded. In other words, at the Lička Plješivica locality Capercaillie are not very much sensitive to shifts from their optimal conditions on this axis. In addition to that is specialization factor score.

Specialization factor is moderate 1.562 (global tolerance is 0.64). According to the values, capercaillie shows slight tendency toward habitat generalist. It is normally, because we performed analysis of relatively specific area (part of the Lička Plješivica Mountain over 1000 m a.s.l.) which, in *sensu lato*, belong to 'capercaillie areas'. Specialisation scores in bold (Table 2) mean the capercaillie was found occupying a narrower range of values than available. First factor of specialisation explains 35 % of total variance, but only two EGVs significant explain – mean air temperature in July (score 0.724) and mean annual air temperature (score 0.685). Scores of specialisation should be interpreted as unsigned values.

If the suitable area (HS) is divided into the five qualitative classes, then based on the analysis and calibration, the final map of habitat suitability for the capercaillie (Fig. 2) has a relatively high reliability index ($B_{cont(W)} = 0.95 \pm 0.092$).

Based on the habitat suitability map, Lička Plješivica can be divided into two main zones: northern and middle part, and southern part of the massif. Unsuitable areas (0 %, Table 3) are mostly situated in the southern part of the Lička Plješivica, with relatively low portion (2.49 %). Low suitable areas (1 to 25 %) are situated also in the southern part of the massif and they comprise more than one third of the mountain (37.68 %). Moderate suitable ar-

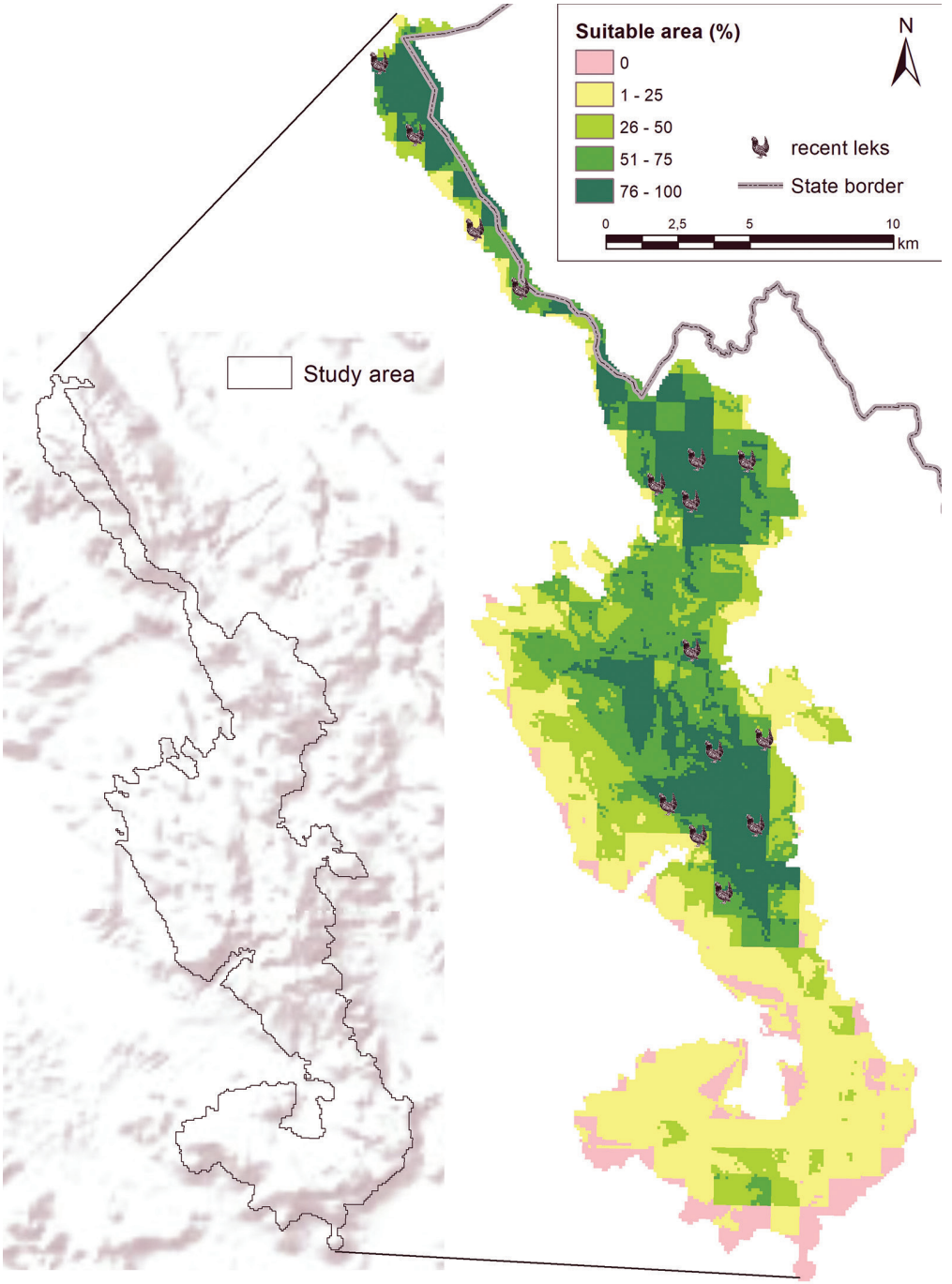

Fig. 2. Relief (left) and habitat suitability map for capercaillie on the Lička Plješivica massif (right).

Table 3. Classes of suitable areas for capercaillie at studied area of the Lička Plješivica massif over 1000 m a.s.l.

Classes of suitable areas	Description	Area, ha	Share in study area, %
0 %	Unsuitable	603	2
1 to 25 %	Low suitable	9121	38
26 to 50 %	Moderate suitable	4125	17
51 to 75 %	Suitable	5246	22
76 to 100 %	Very suitable	5110	21
Total		24,205	100

eas occupied middle part of the mountain with portion of 17.04 %. They surround better capercaillie habitats but some leks are located on this part of the Lička Plješivica. Moderate suitable areas are also placed in the middle part of the study area with some lower share (21.67 %). In this area are also present some leks. Finally, very suitable areas have share of 21.11 %. High quality habitats are located on the ridge of the Plješivica Mountain (Fig. 2), mostly in the northern part of the massif, and less in the midle part. Generally, quality of capercaillie habitats shows some decreasing gradient from north to the south of study area.

Discussion

As capercaillie out from boreal area inhabits high mountains with harsh terrain and is a rather shy species, near-accurate estimation of its population size is related to many difficulties, especially if using classic monitoring methods. Our results support the theses, because capercaillie was present in colder microlocalites, steeper terrain and on the ridges of Lička Plješivica.

Due to the habitat preferences already mentioned, the easiest way of assessing population size is to monitor lek activity. This however complicates habitat evaluation, since males and females have different habitat preferences. For example, while during summer and autumn females use old tree stands and forest clearings (Storch 1993), males only use old tree stands. Saniga (2004) reports that in the Carpathian Mountains males can be found on spruce-beech-fir stands (54 %) after the mating season, while during the summer and autumn they inhabit spruce forests (45 % and 60 % respectively). Similar habitat types, mostly dominated by conifers, prefers the Balkan capercaillie (*Tetrao urogallus rudolfi* Dombrowski, 1912) in Bulgaria (Plachiyski et al. 2018, 2019). This is understandable if known that capercaillie prefers stands where blueberry dominates on the ground floor (Storch 1995), which are strongly related to conifer forests. In our study area patches with blueberry are very rare, but nevertheless capercaillie prefers forest patches with relatively high basal area. More precisely, the patches are presented by groups of old trees in common beech forest stands. Except beech stands, some leks are placed in beech-fir stands. These stands officially have selective structure, but during visiting of leks it seems the stand structure is more uneven-age then selective, which will be more deeply studied in the future.

In our research, capercaillie leks were not strictly associated with coniferous forest stands but mixed silver fir and beech or pure sub-mountain beech stands. This indicates the adaptation of the capercaillie to the forest stand composition. Similar findings were recorded in Cantabria (Spain) where the capercaillie inhabits beech forests with a very small ratio of conifers (Rodriguez and Obeso 2000). Furthermore, forest stands in the analysed part of the Dinaric Alps are of uneven age (sub-mountain beech forests), or are selective (beech-fir forests). This makes it difficult to find a relationship between capercaillie presence and forest characteristics on the Dinaric Alps in comparison to the boreal region (Miettinen 2009). ENFA analysis did not confirm any relationship between capercaillie presence and the ratio of coniferous trees, but did confirm a correlation with the size of the trees. This finding confirms previous statements that capercaillie prefer older forests. Another habitat peculiarity is represented by the special type of forest zonality of the Western and Central Dinaric Alps. There the vegetation zones on hilly and mountain areas are initially sessile oak forests and continue with hilly beech forests – beech-fir forests, and end with sub-mountain beech forests (Horvat 1949). This represents the so-called 'Dinaric type of vertical distribution of tree community structure', in which zones of spruce forests do not follow the zones of beech-fir forests as in Central Europe. Therefore, the capercaillie on Dinaric Alps mainly inhabits mixed-aged sub-mountain beech forests. Another important factor in lek establishment is topography. Using the example of the Western Carpathians, Saniga (2002) reported that 27 out of 43 leks were located in the upper half of convex, lateral ridges, in a range of 30 to 300 m from the main ridge. It was hypothesized that these locations are easier to find in conditions of dust and foggy weather. Our results indicate that the capercaillie chooses steeper slopes and ridges (if other habitat requirements are met). Braunisch and Suchant (2007) hypothesize that the capercaillie prefers areas with snow deeper than 10 cm. According to them these areas: i) are more susceptible to the creation of clearances, with the consequent creation of a variety of living conditions, ii) have fewer predators and iii) allow birds to rest in the snow and reduce their energy loss. In our research, marginality analysis has shown similar results, with very high marginality score between capercaillie presence and number of days with snow cover over 1 cm. However, if the analysis goes slightly deeper, it is clear that the capercaillie avoids temperature extremes, both between seasons and years. In general, the capercaillie prefers a colder and humid climate.

Results of analysing capercaillie habitats in Switzerland (Sachot 2002) showed most potential disturbance factors didn't have negative impact on the capercaillie presence. For example, wild boar hunting has a very low influence. But, in Dinaric Alps all three big carnivore species (Brown bear – *Ursus arctos* Linnaeus, 1758, Grey wolf – *Canis lupus* Linnaeus, 1758, Eurasian lynx – *Lynx lynx* Linnaeus, 1758) were present and that's why big game species (Northern chamois – *Rupicapra rupicapra* Linnaeus, 1758; Red deer – *Cervus elaphus* Linnaeus, 1758; Roe deer – *Capreolus capreolus* Linnaeus, 1758; and Wild boar – *Sus scrofa* Linnaeus, 1758) are not abundant. Small game hunting is not present in the study area. Thus, hunting activity is present, but at low level. Moreover, capercaillie tend to be closer to narrow forest roads with soft

cover and nordic ski trails (Sachot 2002). In our study, capercaillie presence is more likely close to the roads. Possible explanation lays in the fact that the massif is very depopulated, relatively isolated and human visiting is very rare (foresters and, occassionaly, a few hunters or mountaineers). The situation is the same in most of Croatian part of Dinaric Alps. But, the road network size of study area in the paper is 14.26 $m \cdot ha^{-1}$. Road network density in out boreal part of the capercaillie distribution area varies from 4 to 5 $m \cdot ha^{-1}$ (Alps respective Jura, Switzerland, Brändli et al. 2016), 6.6 to 6.7 $m \cdot ha^{-1}$ (West Carpathian, Poland, Kroczak et al. 2016), 26.53 $m \cdot ha^{-1}$ (Rhodope and Rila mountains, Bulgaria, Krumov 2019) to 48.6 $m \cdot ha^{-1}$ (Alps, Austria, Toscani et al. 2020). According to these data, it seems forest road network in our study area is moderate and still doesn't show detrimental effect for capercaillie.

The reasons for the population decline in Croatia are still debated, but it seems that the geomorphology of the Dinaric Alps is one of the potential factors. The mountain line from Risnjak massif continues without interruptions via Velika and Mala Kapela massifs to Lička Plješivica, and further to Bosnia, Serbia and Montenegro. This relief enables the spread of capercaillie from Bosnia, where the population is still quite stable (Adamič et al. 2006), to Lička Plješivica, but not further to Mala Kapela massif. This lack of communication with other populations leads to isolation and the consequent local decline. Previous research stated that on Mala Kapela massif the capercaillie inhabits areas from 800 to 1400 m a.s.l. (Turkalj 1934). However, loss of connection with other populations and potential climate and habitat change have resulted in the rapid decline of capercaillie populations on this massif. On the other hand, to the south of the Velebit massif the altitudes are lower, and there are uninterrupted mountain habitats. Müller (1974) claims that the leks are positioned on higher ground as they are usually used as flying corridors for females during the mating season. New leks can be established in areas with a high density of females, or areas where subadult males are visiting females. If they manage to keep them until the mating season, a new lek is established (Gjerde et al. 2000).

From all we observed it is clear that on the Lička Plješivica massif only 6 % of the area above 1000 m a.s.l. is unsuitable for capercaillie, while 31 % is characterized as highly suitable. This, in combination with the connection to the Bosnian capercaillie population, has probably resulted in the gradual population increase.

Conclusions

Over the last 60 years, capercaillie populations in Croatia have exhibited a 77 % decline. The highest decline was observed on Risnjak massif, were lek numbers dropped from 112 in the middle of the 20th century to the present five. Interestingly, a slight increase in lek numbers was observed on the Lička Plješivica massif (from 11 to 15). The ENFA analysis applied showed that the capercaillie prefers colder areas, steeper terrains and ridges and areas closer to roads where forest stands are comprised with groups of older trees. On the other hand, the capercaillie avoids grasslands (open areas). Compared to boreal areas, the Alps and the Carpathian Mountains, the capercaillie shows certain habitat preferences in the Dinaric Alps. In other words, the ENFA analysis did not confirm a relationship between the presence of capercaillie and the ratio of conif-

erous trees. This is in accordance with the different zonality of forest vegetation in the Western and Middle Dinaric Alps. The so-called Dinaric type of vertical distribution of tree community structure, in which zones of beech-fir forests are followed again by beech forests, results in the fact that the capercaillie in the Dinaric Alps inhabits mainly uneven-age sub-mountain beech forests. In conclusion, the high suitability of Lička Plješivica as the capercaillie's habitat and its connection to the Bosnian capercaillie population are probably the reasons for the population increase observed on Lička Plješivica.

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Genetic analysis of selected high-yielding oleoresin trees of Aleppo pine (*Pinus halepensis* Mill.)

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Abstract

Oleoresin, a quantitative trait with great variability, is the most important non-wood product of Aleppo pine (*Pinus halepensis* Mill.). That being so, and the fact that oleoresin's added value has risen lately, the primary objective of this research was the selection of high-yielding (plus) and low-yielding oleoresin (control) Aleppo pine trees from two Greek populations (Chalkidiki and Euboea) and their genetic analysis with isozymes. The hypothesis tested was whether certain genetic parameters such as heterozygosity and fixation index *F*, are different between plus and control trees in the two populations. Horizontal starch gel electrophoresis was used in order to analyze the endosperms of the selected trees. Eleven enzyme systems were applied which resulted in 17 loci with 33 alleles. The mean expected heterozygosity was high for all groups of trees. No differences were found among mean fixation indices *F* across all groups of trees but a tendency was detected at certain loci between the two populations. Furthermore, a first analysis for the discrimination and the fingerprinting of certain selected trees was performed with molecular markers (RAPDs). In total, six primers were applied which resulted in 68 loci. The discrimination and fingerprinting of the selected trees were successful using just 9 loci. The results imply that since there is great variability in oleoresin yield and high expected heterozygosity among the plus trees of the Greek Aleppo pine populations, a breeding program in order to increase the oleoresin yield should be conducted.

Key words: high-yielding trees, isozymes, Mediterranean pine, RAPDs, resin production.

Introduction

Aleppo pine (*Pinus halepensis* Mill.) is a tree widely distributed alongside the Mediterranean basin. In Greece, its range extends from Chalkidiki, in the north, to Peloponnesus, in the south and it can also be found in certain islands of Aegean and Ionian Sea. It is of great ecological importance since it is a very drought tolerant species, very well adapted to the semiarid sites of Mediterranean and probably will be of special value in the future due to climate change and temperature increase. In many countries it is used for recreational purposes and its wood is also useful as firewood. However, the most important non-wood forest product of Aleppo

pine is oleoresin. In Greece, oleoresin is being tapped from the bole of the tree after wounding the trunk.

Oleoresin plays an important ecological role since it protects the wounded trees from invading insects and associated fungal pathogens (Croteau and Johnson 1985). In addition, its components (Karanikas et al. 2010) are very important feedstocks for industrial use. The volatile fraction, turpentine, is commonly used as paint thinner. Monoterpene and sesquiterpene flavor and fragrance agents are added to foods, beverages, perfumes, soaps, toothpaste, tobacco and other products (Verlet 1993). Recent studies have revealed the great importance of oleoresin in the improvement of diesel fuel (oil) as a scavenger of moisture (Tsanaktsidis et al. 2014a,b) or its use in advanced biofuels (Harvey et al. 2010), while more recently, the effect of oleoresin in pharmacology and on climate change is being investigated (Scaltsoyiannes et al. 2015, 2018). In Greece, many people residing in villages neighboring Aleppo pine forests make their living by oleoresin tapping which though is a difficult job with small profit, attracts more and more people due to the recent economic crisis. Due to the above-mentioned reasons, oleoresin production is of great importance.

Great variability in oleoresin flow and in subsequent annual yield has been observed among the individuals of a pine population. The environment contributes in some degree to this variability (Papaioannou and Megalophonos 1966, Coppen and Hone 1995, Westbrook et al. 2013), but of great importance is the genetic heritability. Many studies have revealed that oleoresin yield is a trait with medium to high heritability (Squillace and Bengston 1961, Hanover 1966, Squillace 1971, McReynolds et al. 1989). In Greece, the annual average oleoresin yield from a tree is about 3 kg, but many individuals yielding more than 10 kg·yr^{-1} have been reported (Tsoumis 1995, Karanikas et al. 2011).

Due to its importance in the Mediterranean basin, Aleppo pine has been the objective of many genetic analyses (e.g. Panetsos 1975, 1981, Schiller and Grunwald 1987). Isozyme studies have been conducted by Korol et al. (1995), Teisseire et al. (1995), Korol and Schiller (1996) and Agúndez et al. (1997). A couple of Greek populations have been studied by Loukas et al. (1983), Schiller et al. (1986) and Korol et al. (2002).

The calculation of mean expected heterozygosity ($\overline{H}_e$) or gene diversity of a certain species contributes to its protection since the allelic variation is critical to the survival of a species and allows organisms to adapt to changing environments. Furthermore, measuring genetic variation of a population is a first step in evaluating the tree improvement potential of the species (White et al. 2007). Aleppo pine's oleoresin yield is a quantitative trait and thus high gene diversity is necessary in order to proceed to a breeding program. A breeding program which uses selected high yielding oleoresin pines will result in progenies that yield larger quantities of oleoresin. Even if these selected trees are vegetatively propagated and then established in artificial plantations, the annual oleoresin yield would be larger and this would lead to a more profitable profession for the oleoresin tapping workers (Susaeta et al. 2014). In this frame Westbrook et al. (2013) associated SNPs with oleoresin flow in loblolly pine and developed a genomic prediction model to accelerate breeding for enhanced oleoresin flow.

Thus, the first objective of this research was to confirm the large phenotypic variation of oleoresin yield in the Greek popula-

tions of Aleppo pine and more specifically to locate and select individuals that yield large (plus trees) and small (control trees) quantities of oleoresin. The second objective was to acquire a detailed knowledge of allelic variation and heterozygosity of the 'populations' under consideration in order to evaluate the potential of a breeding program. A biochemical (isozyme) analysis of the selected trees was performed in order to calculate the gene diversity. In addition, heterozygosity and fixation indices were compared between high yielding and low yielding oleoresin trees aiming to unveil any differences that could lead to an association with oleoresin flow. The final objective was the fingerprinting of some selected genotypes using molecular markers and more specifically the technique of Random Amplified Polymorphic DNA (RAPD). These high-yielding trees, given a high diversity in their populations, can be vegetatively propagated through grafting to establish a seed orchard.

Materials and Methods

The two Aleppo pine populations included in the study were selected based on the following two criteria: (a) to represent different site and climate conditions and (b) to contain trees that are currently being tapped for commercial purposes. The two Aleppo pine populations that met these two criteria, were in Chalkidiki (40°01' N, 23°25' E) and in Euboea (38°49' N, 23°26' E), Greece (Fig. 1). The two areas

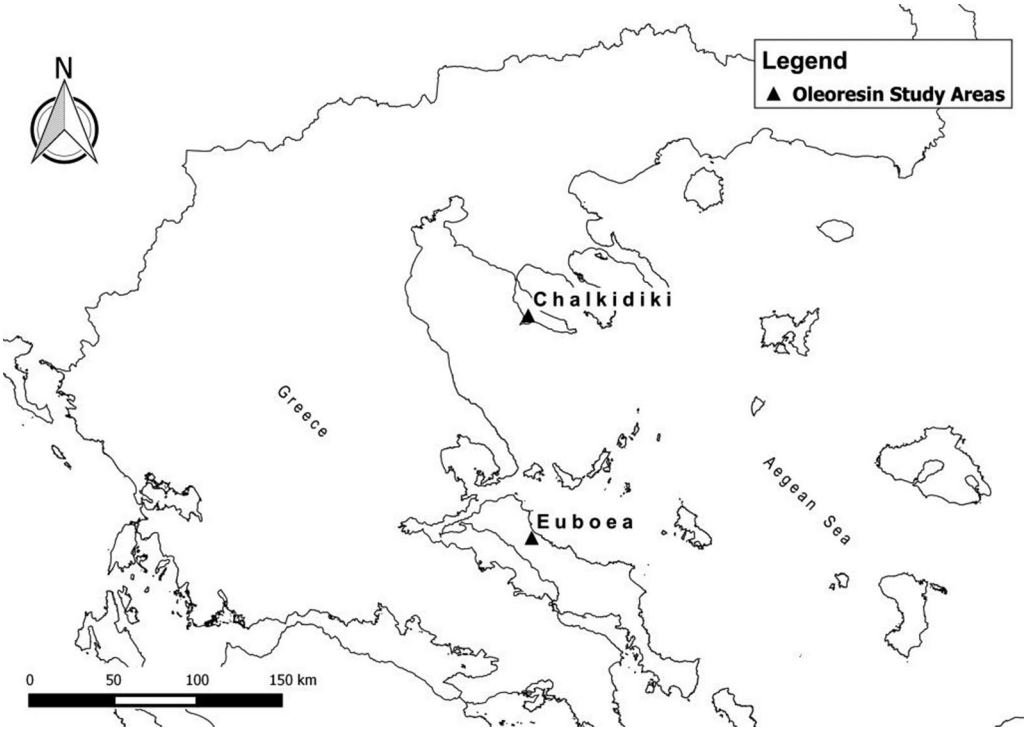

Fig. 1. Two Aleppo pine populations located in Chalkidiki (Northern Greece) and Euboea (Central Greece) respectively, were selected for this study.

are approximately 130 km apart. In these two selected areas, after consulting with the oleoresin tapping workers, we located trees that yield more than 10 kg·yr^{-1} (plus trees) and trees that yield less than 2 kg·yr^{-1} (control trees). The oleoresin yield of these individuals was measured for two successive years in order to confirm their annual yield. From the first northern population we selected 19 plus and 11 control trees, while from the second southern population we selected 11 plus and 9 control trees. In total, 50 individuals, 30 plus and 20 control trees, were selected. Female cones were collected from all the selected trees and were transferred to the lab. Then, the cones were put in the oven at 40 °C until they opened (about 10 days). The seeds were put in wet Petri dishes and when germinated, the endosperms were extracted and used for further laboratory analyses.

The plant tissue was ground and homogenized according to Conkle et al. (1982) and the isozyme analyses were conducted on horizontal starch gel electrophoresis. In total, three buffer systems were used; *Lithium-Borate-Tris-Citrate* (LBTC) and *Histidine-HCl* (H) according to Cheliak and Pitel (1985) and *Morpholine-Citrate* (D) according to Conkle et al. (1982), while the following eleven enzyme systems were applied: 6-phosphogluconate dehydrogenase, 6PGD (EC 1.1.1.44); menadione reductase, MNR (EC 1.6.99.2); isocitrate dehydrogenase, IDH (EC 1.1.1.42); leucine aminopeptidase, LAP (EC 3.4.11.1); phosphoglucose isomerase, PGI (EC 5.3.1.9); Acid phosphatase, ACP (EC 3.1.3.2); β-esterase, β-EST (EC 3.1.1.3); malate dehydrogenase, MDH (EC 1.1.1.37); phosphoglucomutase, PGM (EC 5.4.2.2); glucose 6-phosphate dehydrogenase, G6PD (EC 1.1.1.49); *g*lutamate dehydrogenase GDH (EC 1.4.1.2). Since the gymnosperms' endosperm is haploid tissue, seven (7) endosperms per tree were used in order to detect all the alleles of each locus (Morris and Spieth 1978). The probability of missing an allele when analyzing haploid seed tissue is calculated as 0.5^{n-1}, where n is the number of endosperms analyzed, and therefore, using seven seeds reduced this probability to 1.6 %. Frequency-based genetic analyses were applied using the cross-platform package GenAlex 6.41 (Peakall and Smouse 2006). More specifically, the following population genetic characteristics were calculated: the allele frequency per locus (A), the observed heterozygosity (H_o), the expected heterozygosity (H_e) and the fixation index F.

For additional statistical analysis, four groups of trees were denoted as conditional populations, 1 and 3 consisting of plus trees in Chalkidiki and Euboea, respectively, and 2 and 4 – of control trees. The significance of differentiation among the population pairs based on the genotypes (genotypic differentiation) was tested by exact G-test on contingency tables (Goudet et al. 1996). Additionally, allelic differentiation was tested for comparison. Unbiased estimates were obtained as described by Raymond and Rousset (1995a). The analysis was done in two steps – first the test for significant genotypic differentiation was performed for all population pairs, and the second step was done on pooled data – populations 1 and 3 (plus trees) were pooled and so were the populations 2 and 4 (control trees). Software Genepop v. 4.7 (Raymond and Rousset 1995b) was used for the analysis.

Furthermore, the fingerprinting of 4 high-yielding oleoresin pine trees was performed through the Random Amplified Polymorphic DNA (RAPDs) markers.

To avoid dominance, DNA was extracted from haploid tissue which in the case of conifers is the seed megagametophyte. Two of the fingerprinted trees were from Chalkidiki and the other two from Euboea. Seven megagametophytes from each tree were isolated by the same method, used in the case of isozymes. DNA extractions were performed by using the CTAB protocol (Doyle and Doyle 1987). DNA amplifications by Polymerase Chain Reaction (PCR) were carried out in a Mastercycler Eppendorf ep Gradient S Thermocycler using the profile proposed by Gómez et al. (2001). The volume of the reaction mixture was 25 μl and contained: 2.5 μl 10× Buffer, 2 mM Mg^{2+}, 200 μM dNTPs, 200 nM primer, 0.75 U Taq polymerase, 10 ng DNA. Total 30 oligonucleotide primers (Operon kits OPA01-10, OPE01-10, OPH01-10) were tested in amplification, polymorphisms and Mendelian inheritance. The chosen 6 (OPA01, OPA07, OPE02, OPH05, OPH07, OPH09) were used in the amplifications of DNAs from the four selected trees. The reactions were visualized under UV light, after running in 1.5% agarose gels in 0.5× TBE buffer and ethidium bromide staining. The GenAlex 6.41 (Peakall and Smouse 2006) software was used for the fingerprinting of the selected genotypes.

Results

The genetic analyses of 11 enzyme systems resulted in a total of 17 loci with 33 alleles (Table 1). Among them, 4 loci (Idh-A, Acp-A, Pgm, Gdh) were found monomorphic and the rest 13 were polymorphic. At the polymorphic loci, the detected number of alleles per locus was two, with the exception of three alleles at β-Est and four

Table 1. Loci and allele frequencies for each group of trees.

Locus	Allele	Chalkidiki		Euboea	
		CH_P	CH_C	E_P	E_C
6pgd-A	1	0.711	0.818	0.818	0.722
	2	0.289	0.182	0.182	0.278
6pgd-B	1	0.763	0.773	0.909	0.611
	2	0.237	0.227	0.091	0.389
Mnr	1	0.737	0.818	0.682	0.556
	2	0.263	0.182	0.318	0.444
Idh-A	1	1	1	1	1
Idh-B	1	0.289	0.227	0.045	0
	2	0.711	0.773	0.955	1
Lap	1	0.447	0.318	0.682	0.778
	2	0.553	0.682	0.318	0.222
β-Est	1	0.395	0.136	0	0
	2	0.368	0.455	0.864	0.813
	3	0.237	0.409	0.136	0.187
Pgi	1	0.105	0.136	0.409	0.056
	2	0.895	0.864	0.591	0.944
Mdh-A	1	0	0.091	0	0
	2	1	0.909	1	1
Mdh-B	1	1	0.955	0.909	0.944
	2	0	0.045	0.091	0.056
Mdh-C	1	0	0	0.045	0.056
	2	0.079	0	0.045	0
	3	0.105	0.091	0	0.056
	4	0.816	0.909	0.909	0.889
Mdh-D	1	0.868	0.636	0.591	0.722
	2	0.132	0.364	0.409	0.278
Acp-A	1	1	1	1	1
Acp-B	1	0.053	0	0.091	0.222
	2	0.947	1	0.909	0.778
Pgm	1	1	1	1	1
G6pd	1	0.579	0.636	0.682	0.667
	2	0.421	0.364	0.318	0.333
Gdh	1	1	1	1	1

Note: CH_P = Chalkidiki plus trees, CH_C = Chalkidiki control trees, E_P = Euboea plus trees and E_C = Euboea control trees.

alleles at Mdh-C. The frequency of the most common allele was always higher than 0.5 (minor polymorphism, *sensu* Lewontin 1985), with two exceptions concerning the locus β-Est-1. The trees from Chalkidiki exhibited a slightly larger polymorphism P_X = 76.47 % than the trees from Euboea P_E = 70.59 %. In total, the mean polymorphism was P = 73.53 %. Unique or private alleles were detected at β-Est-1 and Mdh-A-1 loci for the Chalkidiki individuals and at Mdh-C-1 locus for the Euboea individuals.

The allele frequencies per locus were similar across all the groups with certain exceptions (Table 1). The frequency of Idh-B-1 was 0.289 and 0.227 for the plus and control trees of Chalkidiki, respectively, while for the plus and the control trees of Euboea was just 0.045 and zero (0), respectively. The same pattern was found for the β-Est-1 locus where the frequency of Chalkidiki plus trees was 0.395, whereas the corresponding frequencies for the Euboea were zero (0). Thus, the frequency of these specific alleles could be used as a tool for the differentiation of the two populations, since high frequencies would indicate a sample from Chalkidiki and low (or even zero) frequencies would indicate a sample from Euboea. In contrast, the allele Mdh-C-1 was not detected at all among the selected trees of Chalkidiki while it was detected with small frequencies among the trees of Euboea. An interesting pattern can be found at the LAP enzyme system. The first allele Lap-1 was more frequent among the plus and control trees of Euboea than the trees o Chalkidiki where the second allele Lap-2 was more frequent. Furthermore, the allele Mdh-C-2 was detected (in small frequencies) only among the plus trees of both Chalkidiki and Euboea. This allele shows a tendency for differentiation between plus and control trees.

For both populations (Chalkidiki and Euboea), the mean observed heterozygosity was $\overline{H_o}$ =0.255 and the mean expected heterozygosity was $\overline{H_e}$ =0.244. As for the Chalkidiki trees, both plus and control ones, the largest observed heterozygosity (H_o) was detected at the G6pd locus (0.660) and the largest expected heterozygosity (H_e) at the β-Est locus (0.660). The maximum value of fixation index F was calculated for the Pgi locus (0.515) and its minimum value for the G6pd locus (–0.250) indicating large differences between H_o and H_e in both cases. The largest H_o for the Euboea trees was detected in the same locus as for the Chalkidiki trees, that is G6pd (0.650) but the largest H_e was detected on a different locus, namely, the Mnr locus (0.469). For Euboea, the F was maximum at the β-Est locus (0.650) revealing an excess homozygosity and on the other hand the minimum value was calculated at the G6pd locus (-0.481) revealing an excess of heterozygosity.

The comparison of plus and control trees at the two selected areas based on H_o, H_e and F index is presented at Table 2.

The mean H_o across all four groups was quite similar ranging from 0.250 to 0.267. That was also the case for the mean H_e which ranges from 0.224 to 0.254. Between the plus trees of the two populations, the highest H_o and H_e were detected at different loci. In the population of Chalkidiki, the highest H_o and H_e were observed at the β-Est locus while in the Euboea population the highest H_o and H_e were found at the Pgi locus. The mean fixation index F for each of the four groups was close to zero, ranging from –0.066 to +0.022, but with great variability within each group, since the corresponding SE was quite large. The comparison between

Table 2. Observed (H_o) and expected (H_e) heterozygosities and fixation index *F* for plus and control trees from Chalkidiki and Euboea.

Locus	Plus trees						Control trees					
	Chalkidiki			Euboea			Chalkidiki			Euboea		
	H_o	H_e	*F*	H_o	H_e	*F*	H_o	H_e	*F*	H_o	H_e	*F*
6pgd-A	0.579	0.411	–0.407	0.182	0.298	0.389	0.364	0.298	–0.222	0.333	0.401	0.169
6pgd-B	0.263	0.361	0.272	0.182	0.165	–0.100	0.455	0.351	–0.294	0.556	0.475	–0.169
Mnr	0.316	0.388	0.186	0.455	0.434	–0.048	0.364	0.298	–0.222	0.667	0.494	–0.350
Idh-A	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND
Idh-B	0.474	0.411	–0.152	0.091	0.087	–0.048	0.455	0.351	–0.294	0.000	0.000	ND
Lap	0.474	0.494	0.042	0.455	0.434	–0.048	0.636	0.434	–0.467	0.444	0.346	–0.286
B-Est	0.632	0.671	0.059	0.09	0.254	0.64	0.364	0.650	0.440	0.125	0.339	0.631
Pgi	0.105	0.188	0.441	0.818	0.483	–0.692	0.091	0.236	0.614	0.111	0.105	–0.059
Acp-A	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND
Acp-B	0.105	0.102	–0.029	0.182	0.173	–0.053	0.000	0.000	ND	0.222	0.375	0.407
Mdh-A	0.000	0.000	ND	0.000	0.000	ND	0.182	0.165	–0.100	0.000	0.000	ND
Mdh-B	0.000	0.000	ND	0.182	0.165	–0.100	0.091	0.087	–0.048	0.111	0.105	–0.059
Mdh-C	0.263	0.317	0.170	0.182	0.169	–0.073	0.182	0.165	–0.100	0.222	0.204	–0.091
Mdh-D	0.263	0.229	–0.152	0.636	0.483	–0.316	0.364	0.463	0.214	0.556	0.401	–0.385
Pgm	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND
G6pd	0.526	0.488	–0.080	0.636	0.434	–0.467	0.727	0.463	–0.571	0.667	0.444	–0.500
Gdh	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000	ND
Mean	0.250	0.254	0.022	0.256	0.224	–0.057	0.267	0.247	–0.066	0.251	0.231	–0.043
SE	0.058	0.055	0.048	0.065	0.046	0.075	0.059	0.050	0.076	0.063	0.049	0.071

Note: SE = standard error and ND = not defined.

the two plus groups, at 6pgd-A locus, demonstrates that the Chalkidiki trees have their minimum *F* value (–0.407) and the Euboea trees have their maximum *F* value (0.389) while at Pgi locus the Chalkidiki trees have their maximum *F* value (0.441) and the Euboea trees have their minimum *F* value (–0.692). A similar pattern at the two aforementioned loci can be found between the control trees from the two provenances. Thus, at two loci we find the maximum and minimum value alternately between the two groups of plus trees.

The exact tests of population differentiation at the different allozyme loci revealed that there were 12 significant p-values out of 75 possible (Table 3). Three significant cases of differentiation were detected between population pairs CHp and Ec, between CHp and Ep, and between CHc and Ec. Two significant p-values were found between the pairs CHc-Ep and one – between the populations Ep and Ec. The differentiation between Ep and Ec at the locus 6pgd-B was at the threshold level of significance ($p = 0.051$). When all loci were included in the analysis, there was significant differentiation between three population pairs – CHp-Ep, CHp-Ec, and CHc-Ec. No significant differentiation was found when the plus and control trees from the two localities were pooled (CHp+Ep vs. CHc+Ec).

The results of the genic differentiation (i.e., differentiation calculated based on allelic structure, Table 4) revealed practically the same picture: 12 significant

Table 3. *P*-values (unbiased estimate) of the genotype differentiation among populations tested by Fisher's exact test.

Population pairs	6Pgd-A	6pgd-B	Mnr	Idh-B	Lap	Est	Pgi	Acp-B	Mdh-A	Mdh-B	Mdh-C	Mdh-D	G6pd	All loci
CHp-CHc	0.288	1.000	0.566	0.742	0.398	0.149	1.000	0.520	0.126	0.367	0.568	0.068	1.000	0.586
CHp-Ep	0.349	0.245	0.784	**0.021**	0.125	**0.0003**	**0.006**	0.612	**–***	0.127	0.212	**0.015**	0.571	**0.00004**
CHp-Ec	1.000	0.381	0.236	**0.009**	**0.029**	**0.003**	0.743	0.234	–	0.321	0.288	0.210	0.760	**0.006**
CHc-Ep	1.000	0.362	0.478	0.148	**0.024**	0.051	0.065	0.476	0.477	1.000	0.477	1.000	1.000	0.245
CHc-Ec	0.719	0.276	0.064	**0.038**	**0.002**	0.161	0.738	0.075	0.478	1.000	0.770	0.742	1.000	**0.040**
Ep-Ec	0.746	**0.051**	0.492	1.000	0.716	**1.000**	**0.003**	0.460	**–**	1.000	0.850	0.440	1.000	0.462
CHp+Ep vs CHc+Ec	0.809	0.241	1.000	0.411	1.000	0.145	0.196	0.744	0.156	1.000	0.459	0.353	1.000	0.786

Legend: The meaning of CHp, CHc, Ep and Ec is explained in Table 1. *P*-values indicating significant differentiation are in bold.
Note: * Test was not possible because there was only one genotype.

Table 4. *P*-values (unbiased estimate) of the allelic differentiation among populations tested by Fisher's exact test.

Population pairs	6Pgd-A	6Pgd-B	Mnr	Idh-B	Lap	Est	Pgi	AcpB	Mdh-A	Mdh-B	Mdh-C	Mdh-D	G6pd	All loci
CHp-CHc	0.381	1.000	0.542	0.764	0.419	0.094	1.000	0.527	0.130	0.367	0.367	0.053	1.000	0.494
CHp-Ep	0.383	0.189	0.768	**0.023**	0.107	**0.0001**	**0.009**	0.619	**–***	0.130	0.150	**0.025**	0.560	**0.00003**
CHp-Ec	1.000	0.345	0.224	**0.011**	**0.024**	**0.0008**	0.663	0.162	–	0.322	0.209	0.264	0.771	**0.002**
CHc-Ep	1.000	0.413	0.485	0.184	**0.034**	**0.009**	0.089	0.488	0.489	1.000	0.488	1.000	1.000	0.198
CHc-Ec	0.705	0.312	0.093	0.053	**0.005**	0.055	0.613	**0.033**	0.491	1.000	0.773	0.739	1.000	**0.036**
Ep-Ec	0.705	0.054	0.517	1.000	0.724	1.000	**0.013**	0.381	**–**	1.000	0.848	0.508	1.000	0.647
CHp+Ep vs CHc+Ec	0.816	0.227	1.000	0.418	1.000	0.067	0.175	0.708	0.158	1.000	0.361	0.363	1.000	0.664

Legend: The meaning of CHp, CHc, Ep and Ec is explained in Table 1. *P*-values indicating significant differentiation are in bold.
Note: * Test was not possible because there was only one genotype.

cases of differentiation: four for the pair CHp-Ep (loci Idh-B, Est, Pgi and Mdh-D), three – CHp-Ec (loci Idh-B, Lap and Est), two for the pairs CHc-Ep (Lap, Est) and CHc-Ec (Lap and Acp-B) and one for the pair Ep-Ec (Pgi). At overall level, when all loci were considered, significant differentiation was detected between the pairs CHp-Ep, CHp-Ec, and CHc-Ec.

The genetic profiles of the individuals from Chalkidiki and Euboea that took place in this experiment are presented in Table 5. According to the 68 loci, we have done a check for the possible existence of identical genotypes among the selected trees. Based on the analyses all four individuals that were studied posed a different genotype.

Table 6 shows the number of individuals which exhibited the same genotype according to the number of loci that are included in the analysis.

Table 5. Genotypes at RAPD loci of 4 plus trees.

LOCUS	E3	CH5	E5	CH6	LOCUS	E3	CH5	E5	CH6	LOCUS	E3	CH5	E5	CH6
OPA01-250	11	00	11	00	OPA07-1720	11	00	00	00	OPH07-620	11	11	11	00
OPA01-400	11	11	11	11	OPA07-2000+	11	00	00	00	OPH07-700	00	00	11	11
OPA01-490	11	11	11	00	OPE02-600	11	00	11	00	OPH07-750	00	11	11	11
OPA01-500	00	11	00	11	OPE02-850	00	00	00	10	OPH07-850	11	11	11	11
OPA01-550	11	00	11	00	OPE02-900	00	11	00	00	OPH07-880	00	00	11	00
OPA01-620	00	11	00	00	OPE02-980	00	11	10	11	OPH07-980	11	11	11	11
OPA01-700	11	11	11	00	OPE02-1100	11	11	11	11	OPH07-1100	00	11	11	11
OPA01-750	00	11	00	11	OPE02-1230	11	11	11	11	OPH07-1470	00	11	11	00
OPA01-900	11	00	00	11	OPE02-1350	00	00	10	10	OPH07-1600	00	11	00	11
OPA01-1050	00	11	11	00	OPE02-1470	11	11	11	11	OPH07-1720	11	11	11	00
OPA01-1170	00	00	00	11	OPE02-1850	11	11	11	11	OPH07-2000	11	11	11	11
OPA01-1300	00	00	00	11	OPH05-300	11	11	11	11	OPH09-500	11	00	11	11
OPA01-1400	00	00	11	00	OPH05-550	11	11	00	11	OPH09-580	00	11	00	00
OPA07-400	11	00	00	00	OPH05-650	11	11	11	11	OPH09-620	00	11	00	00
OPA07-490	00	11	00	00	OPH05-700	00	11	11	00	OPH09-650	00	11	00	00
OPA07-750	00	00	11	11	OPH05-750	00	11	00	00	OPH09-700	00	11	00	11
OPA07-850	11	11	00	00	OPH05-860	00	00	11	11	OPH09-850	11	11	11	11
OPA07-980	00	11	11	00	OPH05-980	00	11	00	11	OPH09-980	00	11	00	11
OPA07-1050	00	00	00	11	OPH05-1100	00	00	00	11	OPH09-1100	11	00	11	00
OPA07-1230	00	11	11	11	OPH05-1350	11	11	11	11	OPH09-1230	00	11	00	11
OPA07-1350	11	00	00	00	OPH05-2000	11	11	11	11	OPH09-1600	11	11	11	00
OPA07-1400	00	00	00	11	OPH05-2000+	11	11	11	11	OPH09-2000+	11	11	11	11
OPA07-1600	00	00	11	00	OPH07-550	00	00	00	11					

Table 6. Matching and unique genotypes for the loci combination of 4 trees.

Number of loci	# with matching genotype	# with unique genotype	# matching except 1 locus	# matching except 2 loci	# matching except 3 loci
1	4	0	0	0	0
1+2	4	0	4	0	0
1+2+3	2	2	4	3	0
1+2+3+4	2	2	2	3	3
1+2+3+4+5	2	2	2	0	3
1+2+3+4+5+6	2	2	0	2	0
1+2+3+4+5+6+7	2	2	0	0	2
1+2+3+4+5+6+7+8	2	2	0	0	2
1+2+3+4+5+6+7+8+9	0	4	2	0	0
1+2+3+4+5+6+7+8+9+10	0	4	0	2	0
…	…	…	…	…	…
1+2+3+4+...+67+68	0	4	0	0	0

Note: # = Number of trees.

Complete separation of the selected individuals can be achieved if we take under consideration 9 loci. The possibility of finding two individuals with identical genotype is $7.3 \cdot 10^{-4}$, whereas for the total amount of the 68 loci the possibility is $2.7 \cdot 10^{-20}$.

Discussion

The isozyme literature for *P. halepensis* concerning the presence or absence of a locus and its corresponding alleles presents great variability. This becomes evident with the following discussion. Loukas et al. (1983) detected two polymorphic 6pgd loci for a Greek population in Attica which is in accordance with the current study but that wasn't the case for the rest of the studies reviewed. While in most of the studies there were detected two loci, the first of them (6pgd-A) was monomorphic (Schiller et al. 1986, Teisseire et al. 1995, Korol and Schiller 1996, Agúndez et al. 1997). Korol et al. (1995) detected just one monomorphic locus, whereas Schiller et al. (1986) and Korol et al. (2002) detected three loci with the first of them monomorphic. A similar difference among the current study and the previews studies can be found for the Mnr locus where we report two alleles in contrast with the other studies which detected two loci either monomorphic or polymorphic (Schiller et al. 1986, Korol and Schiller 1996, Korol et al. 2002).

In the case of β-Est the findings of the current study deserve a special mention. To the best of our knowledge this is the first time that this particular locus is reported for Aleppo pine. Three alleles were detected for the Chalkidiki population and two alleles for the Euboea population. The β-Est enzyme system was reported in other pines (*Pinus lawsonii* Roz. and *Pinus montezumae* Lamb.) where three loci were detected with 3, 2 and 2 alleles, respectively (Vargas et al. 2002).

Furthermore, we report for the first time the detection of four alleles at the Mdh-C locus in *P. halepensis*. Most stud-

ies detected four loci, mostly monomorphic (Schiller et al. 1986, Korol et al. 1995, Teisseire et al. 1995, Agúndez et al. 1997). Korol et al. (2002) reported four polymorphic loci with two alleles at Mdh-C locus while we detected four alleles at Mdh-C for the first time.

Studies with isozyme analyses of *P. halepensis* populations from all around the Mediterranean basin reported mean expected heterozygosity ($\overline{H}_e$) ranging from 0.020 to 0.143, smaller than the current study (Teisseire et al. 1995, Korol and Schiller 1996, Agúndez et al. 1997). Loukas et al. (1983) analyzed the Greek population of Attica and found high $\overline{H}_e$ = 0.179. On the other hand, Schiller et al. (1986) reported low levels of $\overline{H}_e$ for two Greek populations, the first in Peloponnesus ($\overline{H}_e$ = 0.056) and the second in Euboea ($\overline{H}_e$ = 0.065). More recently, Korol et al. (2002) analyzed one population from Chalkidiki and another from Euboea. The mean expected heterozygosity was $\overline{H}_e$ = 0.177 and $\overline{H}_e$ = 0.188, respectively. In the last two studies, the two Greek populations had the highest values among 17 and 20 Mediterranean populations, respectively. In the current study we detected even higher $\overline{H}_e$ values with $\overline{H}_e$ = 0.232 for Euboea and $\overline{H}_e$ = 0.255 for Chalkidiki. These high $\overline{H}_e$ values of the Greek populations demonstrate their high gene diversity and the level of adaptation they have acquired through the years. Probably, the environmental factors favour the presence of the species in the north-east Mediterranean. Moreover, the species adaptation will be tested in the case of Euboea where almost the entire Aleppo pine population was burned during the wildfires of August 2021. The level of natural regeneration and the subsequent survival of the species will be an indicator of the population's high gene diversity. It is also worth mentioning that the Chalkidiki population has very high gene diversity although it is situated close to the northern limits of the species.

As for the mean fixation indices *F*, we could say that for the three out of four groups, there is an excess of heterozygotes. One possible explanation for these excesses in heterozygotes is the fact that the trees that comprise these groups are selected for their oleoresin yield and therefore, are not selected randomly (White et al. 2007).

Significant differentiation between the population pairs was detected in 9 cases out of 63 possible (Table 3). They were in four loci out of the eleven studied and concerned different combinations of population pairs. Only in one case (Ep-Ec, locus Pgi) there was significant differentiation between plus- and control trees within the same locality. When all loci were included in the analysis, there was significant differentiation between three population pairs – CHp-Ep, CHp-Ec, and CHc-Ec. No significant differentiation was found when the plus and control trees from the two localities were pooled (CHp+Ep vs. CHc+Ec). Evidently, more advanced markers and more advanced statistical methods (Zou et al. 2016) would be necessary to reveal the genetic background of the oleoresin production and to proceed further to successful marker-assisted selection.

The application of six primers of RAPD markers was a simple and safe procedure for the identification and discrimination of four selected individuals. This specific technique can be used for the fingerprinting and separation of a plurality of further individuals, as demonstrated by the very low possibility of identical genotype calculated to specific loci on the analyzed individuals.

In conclusion, we could say that there

is great variability in oleoresin yield and high heterozygosity among the selected plus trees. These trees can form the basic material, through vegetative propagation, for the establishment of either seed orchards or artificial plantations aiming to multiple oleoresin yield and income of landowners. However, new, more informative markers and approaches could be put into use in order to identify the prospective individuals for oleoresin production. Besides the selection of plus trees and establishment of seed orchards, development of genome-wide association studies could help to identify candidate genes or genome regions contributing to the traits of interest. The seed orchards could provide opportunities for performing of controlled crosses necessary for the genetic association studies. Experience and analogies with other pine species could also facilitate such studies (Lauer et al. 2022).

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Artificial intelligence-based image processing for hazard identification in forestry operations – a sawmill case study

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Abstract

Monitoring forestry workers' safety through early warning and detection systems has potential to improve conditions and reduce costs incurred from preventable accidents and injuries (savings up to 0.5 million USD per annum) during timber harvesting and processing operations. This project has a primary goal of understanding the needs, challenges, and opportunities of using artificial intelligence image processing for hazard monitoring and developing a workwear embedded with such technology for sawmilling operations in order to ensure the wellbeing of the workers. The underlying algorithm can be further developed to be implemented with forklift mounted cameras and other wearable safety devices. The positive research findings can also be applied in other forestry environments such as harvesting where safety management for human and vehicle interactions are required.

Key words: hazard identification, image processing, safety management, vehicle human interaction, wearable device.

Introduction

Forestry industry is considered as one of the dangerous based on its health and safety record (Gejdoš et al. 2019). Some of these incidents are fatal. Forestry operations are considered to be among the top in terms of fatality rates, especially harvesting (Melemez 2015). They pose high risk of accidents due to factors such as: work environment involving difficult terrain and weather conditions; use of heavy machinery and power tools; harmful work conditions such as exposure to noise, dust, vibration, exhaust fume, and body posture; worker fatigue (Gejdoš et al. 2019). Personal and work characteristics combined with the work environment are believed to influence the creation of a hazardous environment that could be triggered by different mechanisms that cause an accident. To prevent accidents in forestry, a number of initiatives have been taken over the years, which include: stricter workplace health and safety regulations; mechanization of forestry operations; safety awareness and training among workers; enhanced commitment

of the higher management; improved safety behaviour of workers; to name a few (Tremblay and Badri 2018). In recent years, researchers have attempted to develop early warning systems to alert workers on the impending hazards (Newman et al. 2018). Among them, real-time alerts using sensor-based technology is becoming very popular (Bowen et al. 2019).

Despite significant progress within the monitoring device industry, the widespread integration of this technology into workplace health and safety portfolios remains limited. Although it has undoubtedly played a major role in the improvement of forest management and processing activities, its application for personalised safety monitoring has not been fully explored. The forestry sector lags some other industries such as manufacturing, mining and construction in the trialling and introduction of these technologies. Due to the hazardous working environments in the forestry sector, workers frequently face safety and health risks throughout the supply chain. Automated hazard monitoring systems based on sensor and short-range communication technologies are considered one of the most promising methods to help manage these risks. Automated monitoring systems can acquire data, convert it into structured information, and immediately deliver these to the worker as an early warning for corrective action. While industries such as healthcare, sports and fitness, manufacturing, mining, construction etc., have started using the above technologies, the forestry sector lags in trialling and using personalised safety monitoring systems in its workplaces. For examples, there is an increase use of wearable sensor system for worker safety with real-time monitoring and warning in many industries. The adoption of wearable technology has the potential for a result-oriented data collection and analysis approach to providing real-time information and early warning of potential hazards to forestry workers. A review of the literature indicates that the existing wearable technologies applied in other industrial sectors can be used within the forestry industry.

The most popular development in the construction and mining sectors is proximity sensors used to monitor and alert the worker amidst a plethora of large moving equipment on sites (Teizer and Cheng 2015). These technologies are capable of monitoring and providing real-time geo-referenced steaming of the site environment to safety managers (Mayton et al. 2012). They could monitor gases, dust, noise, light quality, altitude, etc., and help safety managers to continuously assess risks to workers and localize their prevalence. The resultant knowledge accumulated on the frequent cross-overs between workers and heavy equipment could lead to better site layout designs (Teizer and Cheng 2015). The ability for early warning of workers on potential collusion is another benefit of these proximity sensors which uses a personal tag on the worker and a reader on the equipment with antenna and other communication technology to trigger alerts when the there is an overlap of the radio frequency fields that defines each units' proximity warning space (Teizer et at. 2010, Marks and Teizer 2013). The other application is to create a virtual fencing of danger zones, locate 3D coordinates of workers using ultra-wide band (Giretti et al. 2009, Carbonari et al. 2011) or mobile infrared/ultrasonic sensors (Lee et al. 2009) to alert when they are within or closer to such zones. Park et al. (2016) found Bluetooth technology is better than magnetic and RFID based technologies for proximity sensors in avoiding worker

and heavy machinery collusion in construction sites.

Benefits of individual wearable sensors or systems can be integrated based on their attributes for multi-parameter monitoring of ergonomic and safety performance. While the existing sensor technology is being trialled in many industries, their application in forestry is limited due to both systemic issues and environmental conditions. The sensor-based techniques suffer from software instability, interruptions with very high electrical voltage in timber mills, the need for multiple tags and receivers limit practical deployment, and some technologies will not be suitable for internal environments (such as GPS). From an environmental perspective, traditional sensor-based technologies cannot deal with the dynamic nature of forestry operations, particularly radio frequency-based technologies need direct line of sight, and environmental heat can cause errors in sensors (such as pressure ones). Compared to above, vision-based systems have the advantage of not requiring the installation of multiple tags, they incur lower costs and are easier to maintain.

This paper reports a vision-based collusion prevention development trialled at timber mills in Mount Gambier South Australia to investigate their applicability in forestry industry surroundings. This study aims to bring development and implementation costs to a minimum by carefully selecting low cost and easy to maintain components, and with shared cost arrangement as part of the existing personal protective devices. Results of this study are expected to provide a new approach for automating remote monitoring of forestry workers' safety behaviour by fusing data on their location, physical strain, and proximity to hazardous conditions. This proposed workwear can potentially contribute to accident prevention (avoidance) and reduction and ensure worker welfare from plantation harvesting to sawmill.

Research Design and Methodology

The approach used in this study includes:

– Identifying and assessing safety hazards of harvesting/sawmilling operations based on literature, accident data obtained from SafeWork South Australia, interviews with field staff, and observation/work-studies carried out in the field;

– Identifying parameter requirements for hazard monitoring in above operations against available sensors;

– Development of a prototype of the or a vision-based wearable device, calibration, and validation trials in actual forestry settings.

Case study selection

Case studies are selected as these provide the most relevant and realistic industry requirements. In particular, they also serve as testing grounds for the proposed detection method, especially for the accuracy measures. In result, two large sawmills in Mount Gambier, Limestone Coast, South Australia, participated in this study. Initial consultation identified mobile plant and pedestrian interactions would be the key focus. Forklifts confined spaces having to manoeuvre between production lines and aisles with ground workers involved in manual operations (such as stacking, strapping, wrapping, packing, quality inspections, segregation, etc.) in proximity pose severe hazards of potential collusion. The 15-ton loaders operating outside the mill could come in contact with forklifts and ground workers there. The visibility of loaders is poor due to stacks of finished timber piled on the

ground. In addition, the mobile plant operator (both forklift and loader) could be in lack of visibility situation due to blind spots caused by the size of the load carried (very long timber). When the loader-forklift and ground worker presented in these confined spaces, with a large and heavy timber load in front of the plant, risk of contact collision would be or is high. It must be noted that the stacks of products can block both people and drivers' views entirely.

Therefore, mobile plant – worker interaction was the most critical area and selected as the case study for this development. Four scenarios were selected:

i. Human to vehicle (forklift and truck) – indoor;

ii. Human to vehicle (forklift and truck) – outdoor;

iii. Vehicle to vehicle (less common) – indoor;

iv. Vehicle to vehicle (forklifts and loaders) – outdoor.

With respect to the future embeddable requirements, an Android mobile application based on the standard on-device cameras was selected to develop this proof-of-concept system. The mobile devices could be mounted on the vest, helmet and other personal wear. In addition, the application can also be easily modified for other embedded devices in the market.

The core of this solution is a machine learning model which is built based on AI-based image processing algorithms, detecting both vehicles and human movement. This proposed solution does not rely on any network, nor additional cloud hardware to run so it can be rapidly deployed in any environment. The project team has designed and implemented a proof-of-concept (POC) AI algorithm to test the suitability of this approach in a multivehicle factory setting. This algorithm is expected to detect several objects: cars, trucks, forklifts and human in an accurate and efficient manner.

Trial procedures and Data Collection

Videos were captured in two occasions - pilot and testing. In total, approximately 8 video footage were taken to represent different scenarios. Therefore, the testing was able to cover all scenarios including both in-house and outdoor, ideal *vs* sub-optimal lighting condition, people to vehicle and vehicle to vehicle.

The system was developed as an Android application which took the video feeds directly from the front and rear cameras on the mobile devices. It was later modified to take video feeds from pre-captured mp4 files. Since the underlying algorithm stayed same, the result was applicable in real world scenarios.

Due to Covid restrictions, all researcher site visits have been prohibited. Therefore, for the trail in the plant, a purpose made helmet mount (Fig. 1) was used on the helmet of the plant supervisor during his daily routine work to capture real video footage for the system development and testing purposes.

Evaluation criteria

Several measures were employed to evaluate the success of this development as follows:

– Accuracy: Can the technology detect the potential risks in the above scenarios real-time?

– Reliability: Can the technology run stably in all environment (e.g. rain, dust, low light) with minimum human intervention for a sustained period?

– Cost: Does the adoption of technology (individual or vehicle based) result in

Fig. 1. DIY helmet mounted system (smart phone in use) – camera focusing set to 1 to 50 m.

huge cost?

– Adaptability: Can the technology be used in less ideal environment (e.g. no network)?

AI image classifier/Object detection

In addition to the traditional proximity sensor-based technologies, researchers such as Torbaghan et al. (2022) find that the computer vision technology and AI algorithms has long focused on the manufacturing production (e.g. fault detection); however, the recent emerging area is the safety management. Especially, by using the standard mobile devices such as smartphones and harvesting the machine learning capability built-into the modern phone processors, Zhang (2021) suggests that this approach does not only reduce the overall implementation cost but also improve the overall accuracy as that the phone sensors are often more advanced than the ones found inside the surveillance cameras.

The primary AI algorithm in safety management is object detection. Unlike traditional AI-base image classification algorithms, the proposed approach requires a model to detect objects real-time. This can be achieved in developing an AI Convolutional Neural Network (CNN) image classifier model. Jawale et al. (2020) suggests that the CNN algorithm can take in an input image, assign importance (learnable weights and biases) to various aspects/objects in the image to differentiate one from the other (Fig. 2).

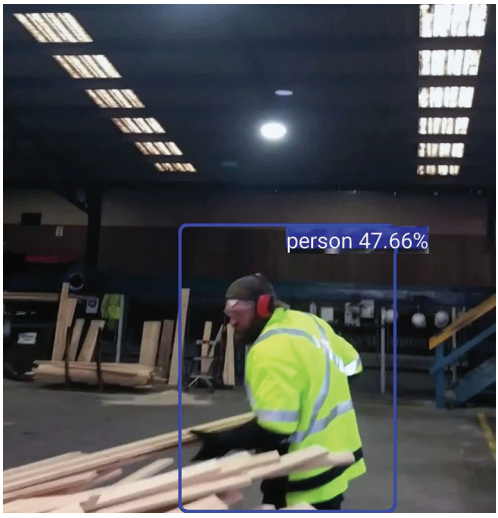

Fig. 2. Example of human detection.

CNN Image/object classification models usually have lots of parameters. Building an accurate model requires lots of labelled training data and computing power. In this project, the researcher team has decided to retrain an existing image classifier – Google TensorFlow image classifier, to fit with the proposed scenarios. This

approach is often referred as 'transfer learning'. It takes a piece of a model that has already been trained on a related task and reusing it in a new model. The existing image classifiers recognises a wide range of objects. However, there are only a few types that are of primary interest in the proposed scenarios such as people and vehicles (forklifts and trucks). Thus, during the retraining process, labelled vehicles' images have been fed onto the base model in order to deliver a more suitable scenario-fit CNN classifier model for this project.

It is noted that the retraining CNN image classification model is only considered as one component in the proposed application. Figure 3 shows the overall process.

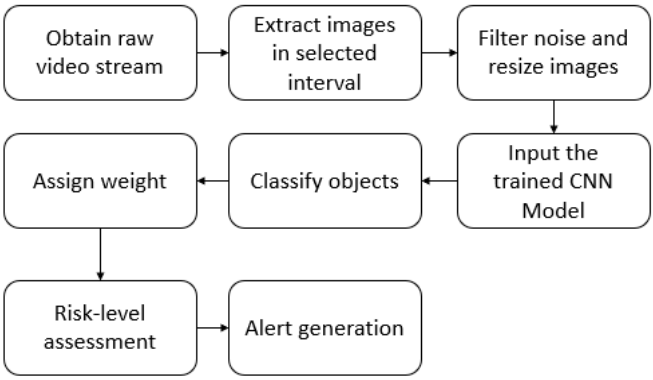

Fig. 3. Safety application flowchart.

Video stream to image: CNN classifier works at the image-level, thus, there is a need to take snapshots from the camera video stream periodically (1 image per second in this project).

Filter noise and resize images: Image denoising has a significant impact on CNN model accuracy. As reviewed by Ilesanmi and Ilesanmi (2021), there are several well-known methods such as linear and non-linear filters that can be applied to the input images. These methods are used in this project. In addition, by reducing the size of raw input images, the overall processing time can be reduced as well as extending device battery life. Thus, the raw image has been resized to 1024×768 before processing.

Assign weight to the classified results: it must be noted that the CNN classification does not give a binary result. Instead, it provides a threshold value. The higher values suggest that the detected objects are more likely to be a suggested type. While the retrained CNN model in this project does increase the threshold value of the detection of vehicles, it still presents mis-classified objects with lower threshold values. Especially, affected by real-time captures (e.g. blurred images due to movement), the overall threshold values are often lower than expected. Therefore, it requires a fine-tuning process to assign weight (cut-off threshold value) to the classified results. Once the classifier result is above this weighted value, then it is considered as a trustable detection.

Risk-level assessment and alert generation: in this project, the researcher team has primary focused on the object detection. In practice, the distance measure and vehicle speed are critical in the overall risk assessment. The actual risk assessment also needs to take other variables such as plant layout and organisation policy. More importantly, alert generation needs to consider human fatigue and other factors. Therefore, these two stages are considered as future research only.

Results and Discussion

Identify industry requirements: workplace health and safety concerns of the South Australian forestry industry

SafeWork SA collates worker compensation claims data obtained into a database for policy analysis. During 1st July 2002 – 31st June 2013, a total of 18,778 lost days were reported by the 860 forestry industry accident victims noted in the SafeWork SA database which translates into an average of 22 lost days per accident. Victims claimed AUD 12.1 million as compensation for medical treatment (an average of AUD 14,033 per accident). Given the number of accidents occurring in the forestry industry, harvesters, transporters and processors constantly seek novel strategies that promote safety. Innovation in implementing safer work methods and the use of technology has eliminated some of the traditional hazards, as indicated in the gradual reduction of accidents in South Australia. Forestry operations are typically characterised as physically demanding tasks that are often performed in harsh environment. Because of the continuous and repetitive exposure to physically demanding work, strains and sprains are the most common type of work-related, non-fatal injuries, which constitute 18 % and 26 % of harvesting and sawmilling injuries reported in South Australia. Furthermore, the continuous exposure to an excessive level of physical strain can lead to physical fatigue, which may result in decreased productivity and motivation, inattentiveness, poor judgment, poor quality work, job dissatisfaction, and increase in the risk of developing worker-related musculoskeletal injuries (MSIs) or cardiovascular disorders.

The data analysis found that MSIs are the largest injury types that accounted for 35 % of total injuries followed by incidence of struck by or trap between moving objects (26 %). It revealed that lower back injuries are among the most common MSIs among those reported (43 %). These occur when the demand of work exceeds the capacity of a worker's body or the worker repetitively performing heavy activities. Musculoskeletal injuries can also be found in other parts of the body, such as the shoulders (21 %), chests (11 %), and wrists (7 %). They are usually caused by overexertion, which is a leading cause of time-loss injury for the reported accidents. The analysis showed that half of the injuries, more than 100 lost days are attributable to overexertion. Overexertion is not only the most common event category, but also the most expensive, resulting in AUD 6.2 million in direct medical expenses to the industry during the review period.

Hazard identification and current mitigation measures

Most of these risks are handled through administrative control. When it fails, a reliable real-time proximity detection and alert system is needed on this site to provide ground workers with another layer of safety protection. There is a 3 m exclusion zone between the forklift and ground worker within enclosed spaces demarcated by a blue static colour arc light from the forklift. The blue light is intended to alert workers of their proximity to the forklift. However, there are confined spaces which do not provide a 3 m safe zone, particularly when the forklift is turning around with timber on it. Workers would normally step inside the production area as soon as they see a forklift. However, when they are heavily concentrating on a job

or get used to the constant movement of forklifts, complacency can creep in which poses a hazard to the worker. In addition, when someone steps inside the production area, they could hit by stationary or moving objects or caught in between objects (machine). The stacks of products and the physical layout of the factory floor may not give the workers safe zones to move to in an emergency.

During summer, the temperature inside dry mills could exceed 35°C and the fans could increase the noise levels which combined with worker fatigue during long shifts could very easily distract the concentration of a worker. The repetitive nature of tasks can cause workers to experience a decrease in awareness as well as limited visibility for forklift operator. The other factor that could lead to accidents is 'domestic blindness' and the fact that workers getting used to the movement of forklifts and the demarcation blue lights. Change of colour, when tested, did not work. A flashlight, something that is dynamic, with an irregular pattern could help get the attention of workers. The other major issue unearthed during the observations was the change of vision of the forklift operator between open areas and enclosed areas of the timber mill. The constant movement between open areas with sunshine to shed lighting could affect the operator's reflexes and impact the ability to negotiate the plant's movement when a pedestrian is the transition zone. This sudden blindness in the transit zone between in and outside of the timber mill poses serious hazards the pedestrians.

Initial desktop assessment of technologies

The literature and technology market scan suggest that the current sensor technology cannot address the above issues satisfactory. The current commercial state of art solutions has been developed based on proximity sensors which use radio signals such as Bluetooth and Wi-Fi network to detect approaching vehicles and human operators. However, each device costs about USD 5000–8000, which is too costly for day-to-day scalable adoption. These solutions also rely on expensive underlying network coverage which may not be available in all operation sites.

Some of the deployment and support challenges identified include:

– Ultrasound/IR/laser: directional, multiple sensors are required to cover 360 degrees resulting in data processing issues and increasing hardware cost and time to fit to equipment;

– Radio-based systems such as Bluetooth, etc.: easily interfered by the plant setup;

– Location awareness, e.g. GPS: existing technologies may fit well in one set of scenarios (e.g. GPS location tracking for outdoor), they do not fit well indoors where signals can be distorted or not available;

– Commercial solutions: require whole site data and high cost, limited accuracy;

– Administrative control: this solution involves separation of workers and machines, but this is not always feasible.

Therefore, with respects to the above-mentioned success measures, the study further examined the emerging technologies and determined that a risk Identification approach through AI-based image processing (object detection) could be a viable and justifiable approach.

Image recognition trial

With the on-site supervisors' assistance, a number of video clips were recorded to

cover both indoor and outdoor scenarios. Given that the indoor lighting is constant, special attentions were made to the outdoor lighting conditions to cover both day light and low light (cloudy late afternoon) conditions. It must be noted that the raw detection algorithm threshold values cannot be used as an accuracy measure. These thresholds are calculated results based on two factors:

1. Whether or not there is a potential recognisable object within the identified area;

2. Whether or not the correct object type is assigned.

Therefore, in this project, based on a number of trials, we have set an internal cut-off point value of 40 % (assign weight). In other words, POC algorithm believes that any detection with an internal threshold value above 40 % is a correct object-detection event relating to safety monitoring (human and vehicle).

In order to provide an accurate measurement of the real-world application, human manual assessment is essential. Thus, in total, 58 indoor, 61 outdoor daylight and 53 outdoor lowlight images were randomly extracted from the 5 recorded video clips (total duration 1 h) for human verification. The tagging person has been given the raw images and the result spreadsheet (exampled in Table 1). After checking the original image, the tagging person manually assigns value 1 for each correct detection, 0 for each incorrect detection in the 'detection success' column.

Based on the human tagging results, the overall detection accuracy is shown in Table 2.

Table 1. Human tagging.

Image	Time	Type	Area	Detection success
22	00:25.6	vehicle	RectF (-15.923696, 658.1464, 812.1965, 1560.0878)	1
57	01:06.5	person	RectF (812.2905, 1268.6532, 958.5147, 1741.5225)	1

Table 2. Accuracy measure.

Lighting condition	Success - people	Success - vehicle	Errors - people	Errors - vehicle	Accuracy, %
Outdoor lowlight	10	23	5	15	62.26
Outdoor daylight	26	29	1	5	**90.16**
Indoor constant light	27	27	2	2	**93.10**
Total	63	79	8	22	82.56

Note: Above 90 % accuracy (in bold) is considered as desirable target outcomes.

In the case that false alarms and misses were identified, reasons were also found. For example, some of the mobile trays (e.g. Fig. 4) were detected as vehicles as they shared similar characteristics as real vehicles by having 4 wheels as well.

Overall, the image processing algorithm is found highly accurate, especially under good lighting conditions. For example, Figure 5 has shown examples of successful detection of human and vehicles in both indoor and outdoor settings. Figure 5 also shows that the recognition process works in obstructed views successfully.

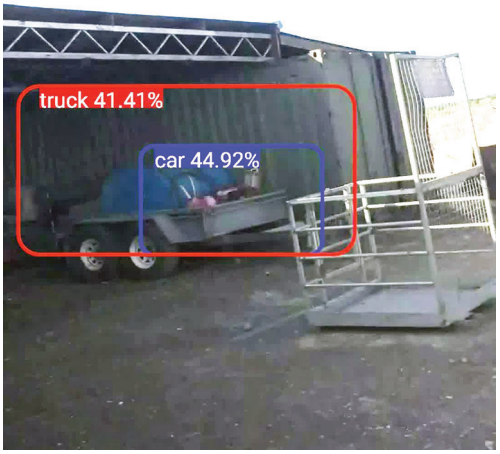

Fig. 4. Error – a mobile trailer is recognised as vehicles.

within a short period (e.g. ±2 s), the detection accuracy could be improved significantly. Also, a new detection model can be developed (trained) for lowlight conditions specifically.

In addition, from the development and testing, it was found that existing off-of-the-shelf algorithms to identify the proximity and direction of movement of the vehicle based on image processing techniques could be used in the future commercial development. When the camera has been mounted in a relatively fixed position (e.g. on top of the forklifts or factory walls), it is possible to derive distances between two tracked objects through triangulation by calculating the angle of the ground surface. However, this can be complex as

Fig. 5. Success – indoor and outdoor detection examples.

Future work

In summary, the end user participants of the study were highly satisfied with the proof-of-concept system and the highly accurate detection rate (90%+) under good lighting conditions that the system produced. However, the researchers still believe that the underlying AI object algorithm can be further enhanced to improve overall accuracy. For example, by combining detection results from multiple images both objects may be moving and this information needs to be calculated rapidly, multiple times per second, and then linked to the potential impact algorithm to identify and alert of possible impacts.

This study has focused on safety management in the processing environment (sawmills). However, the research findings are also applicable in other forestry environments. For example, in the harvesting environment, heavy machines

such as forestry skidders and log loaders are often used. Consequently, the safety management requirements for human and vehicle interactions are also significant. Therefore, it is expected to conduct research in the relevant settings in near future.

Conclusions

The estimated direct cost of accidents to the South Australian forestry industry is USD 1,050,000 per annum (accounting only for lost days and medical cost). This study has successfully identified and assessed health and safety hazards of forestry operations based on literature, past accident data, interviews and observation/work-studies carried out in the field/ identify the parameter requirements for hazard monitoring in forestry operations against readily available sensors. Success measures and coverage scenarios were emerged as the testing base for the proof-of-concept system.

The developed AI-based image processing system is expected to prevent work-related injuries in forest operations in addition to help maintain the health and wellbeing of workers. It could reduce work-related injury expenses such as insurance claims, lost days and lost productivity. As an indirect impact, it could improve the productivity of workers as they are provided with real-time safety situation-awareness that help them feel safer. In addition to those who are active, it could also help workers recovering from accidents and who have returned to work and new workers to become proficient and safe in their jobs with confidence.

There are many existing studies of the underlying AI technologies such as CNN for image processing in the field of computer science. However, turning these technologies into practical solutions indeed requires domain knowledge and practical experience. This applied research tried to explore the feasibilities of applying AI technologies in the forestry environment. Such a process is considered valuable in the forestry AI adoption projects.

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Physiological aspects of natural regeneration in coppiced forest dominated by *Quercus frainetto* Ten. and *Quercus cerris* L.

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Abstract

Comparison study of some physiological parameters of natural generative regeneration of *Quercus frainetto* Ten. (QF) and *Quercus cerris* L. (QC) was conducted *in situ* in an oak-dominated old coppiced forest. Gas exchange levels and sapling leaf pigment content were assessed simultaneously under the canopy (C-site) and in a gap (G-site) open in the stand two years before measurement. Differences in maximum photosynthetic capacity (A_{max}) and dark respiration rate (R_d), determined by light-response regressions were compared to evaluate leaves' carbon balance. Transpiration rate (E) and water use efficiency (WUE) were analyzed to compare changes in water regimes. Total chlorophyll content (TCC) and normalized difference vegetation index (NDVI) were monitored on the same plants. Species-specific responses to changed micro-condition after a canopy opening were observed. Both elements of the carbon balance (A_{max} and R_d) change much more substantially in QF leaves compared to QC in G-site. At the same time, the QF has a greater difference of E in the G-site compared to the C-site than in the QC. Such leads to a flattening of the WUE between two species at the G-site, in a background of a higher WUE of QF in the C-site. However, QF has similar, although not higher, TCC levels as in QC, as well as NDVI in G-site, compared to C-site.

Key words: natural regeneration, photosynthetic light response, physiological adaptation, water use efficiency.

Introduction

In recent years a broad and complex study of oak-dominated coppiced forests in Bulgaria has started. The process was driven by the goals set by the national meeting for managing of oak coppiced forests in Bulgaria (Kostov 2016). *Quercus frainetto* Ten. (QF) and *Q. cerris* L. (QC) have a determinant role in the Bulgarian oak coppiced forests (Kostov et al. 2017). In West Bulgaria, they often take part in mixed coppiced forests with the dominance of one or the other (Kostov et al. 2019). Although from different subgenera and sections (subg. *Quercus,* sect. *Quercus* and subg. *Cerris*, sect. *Cerris* respectively) of the genus *Quercus* (Hipp et al. 2019), according to the European atlas of forest tree species (de Rigo et al. 2016,

Mauri et al. 2016), *Quercus frainetto* Ten. and *Q. cerris* L. prefer similar ecological conditions with wider range for the second one.

As Kimmins (1997) says, 'Foresters will have to become much more familiar with autecology and ecophysiology of the plant communities they are managing if natural regeneration is to be accomplished successfully'. The main goals of the plant physiological studies in forest science may differ from an explanation of seasonal or age-related differences in some of the internal processes in the tree plants (Wolkerstorfer et al. 2011) to exploring the mechanisms for overcoming stress periods (Pietras et al. 2016; Stojanović et al. 2016 2017) or disturbances (Anev and Marinova 2021).

The present study aimed to compare Hungarian oak (*Quercus frainetto*) and Turkey oak (*Quercus cerris*) seedlings in adaptation to increased light intensity by comparing light curves of photosynthesis, transpiration rate and water-use efficiency (WUE) and total chlorophyll content. We also studied normalized difference vegetation index (NDVI) in relation to different microclimatic conditions, as a result of anthropogenic activity (gaps-opening phase during implementation of shelterwood with gaps regeneration system).

Materials and Methods

Site description

This *in situ* experiment was conducted in a mixed *Q. frainetto* (QF) and *Q. cerris* (QC) coppiced stand, which is a part of State Forestry Enterprise Elin Pelin and situated about 30 km east of Sofia in West Bulgaria. The site and stand characteristics are given in tables 1 and 2, respectively.

Table 1. Site description.

Characteristic	Value
Altitude, m a.s.l.	700
Latitude	42°35'38.99″
Longitude	23°36'34.60″
Slope, °	5
Aspect	North
Mean annual air temperature, °C	8.0
Total precipitation, mm·yr^{-1}	574

Table 2. Stand description.

Characteristic	*Q. frainetto*	*Q. cerris*
Age, years	60	60
Basal area, m²·ha^{-1}	9.2	13.1
Density, N·ha^{-1}	216	208
Mean diameter (DBH), cm	24	28
Mean height, m	15	18
V, m³·ha^{-1}	68	108

The regeneration on the site was abundant with more than 196,000 ha^{-1} (~29 %) seedlings for QF and more than 473,000 ha^{-1} (~71 %) for QC, mostly about 0.30 m up to 0.50 m of height and mostly about 5–6 up to 12–15-years-old, regularly distributed all over the area of the stand.

During the winter of 2017, a gap was opened with an area of 1252 m^2 within the experimental stand. This study was conducted in June 2019 when the saplings in the gap had developed leaf buds laid under the new light conditions.

Plant material

Three different natural regenerated sample seedlings from QF and QC, both in the gap and under the canopy, or total of 12 seedlings were used in this study. For the gas-exchange and pigment concentration measurements, a third fully developed leaf from the top of the experimental sap-

Fig. 1. Experimental leaves, taken from saplings: a) in the gap, and b) under the canopy.

lings was chosen (Fig. 1).

Gas-exchange and pigment concentration

Gas-exchange measurements were conducted with a portable photosynthesis system Li-6400 (Li-COR Inc., Lincoln, NE, USA), equipped with shielded thermocouples, a red light-emitting diode light source (6400-02), and a quantum PAR sensor (LI-190) in the chamber. Before measurements, the Li6400 was calibrated according to the manufacturer's recommendations (LiCor 2012). Following standard protocol (Evans and Santiago 2014), light-response curves for each leaf were recorded on a typical sunny day, between 09:00–12:00 at ambient levels of environmental factors: with CO_2-concentration at about 395 μmol-CO_2·mol^{-1}, leaf temperature 23.5 °C, and 43.3 % relative humidity and using eight levels of photosynthetic photon flux density (2000, 1000, 500, 250, 100, 50, 25 and 0 μmol·m^{-2}·s^{-1}). Photosynthetic light responses were individually analysed with the non-rectangular hyperbolic model (Prioul and Chartier 1977, Leverenz and Jarvis 1979, Marshall and Biscoe 1980) by formula (1):

$$An = \frac{\alpha \cdot \mathrm{PPFD} + A_{\max} - \sqrt{\left(\alpha \cdot \mathrm{PPFD} + A_{\max}\right)^2 - 4 \cdot \theta \cdot \alpha \cdot \mathrm{PPFD} \cdot A_{\max}}}{2 \cdot \theta} - R_d, \qquad (1)$$

where: An is the photosynthetic rate, PPFD is the incident photosynthetic photon flux density, α is the maximum apparent quantum yield (the initial slope of the curve), θ is the convexity of the light response curve, and A_{max} is the light-saturated gross photosynthetic rate, R_d is the dark respiration rate.

All regression lines were fitted to the data by applying the least-squares approach of the Microsoft Excel Solver routine using the Newton algorithm (Office 2016, Microsoft, Redmond, WA, USA).

Total chlorophyll content (TCC, g·m^{-2}) and normalized difference vegetation index (NDVI) were measured with a portable chlorophyll meter atLEAF+ (FT Green LLC, Wilmington, DE, USA) and PlantPen NDVI-300 (PSI Corporation, Czech Republic) respectively, using two readings per leaf (one on each of the left and right parts of the leaf). The atLEAF+ and

Plant-Pen NDVI-300 meters measure leaf absorptance or reflectance difference between red and near infra-red lights, and their measurements strongly correlated to the total chlorophyll and water content of leaf (Muraoka et al. 2012, Zhu et al. 2012, Novichonok et al. 2016).

Two-way ANOVA (Factor A: Species and Factor B: Conditions) with Tukey's multiple comparison test was used for statistical evaluation of experimental variants.

Results

Light curves of photosynthesis and CO_2-balance

Under the canopy, QF is more strongly suppressed, and its photosynthesis quickly reaches saturation. QC takes advantage of direct incident light more adequately and, at PPFD above 750 μmol(γ)·m^{-2}·s^{-1}, realizes higher photosynthesis than QF (Fig. 2a).

In the gap, the light curve of photosynthesis of QC leaves does not differ in comparison to such under the canopy. However, the photosynthetic light curve of QF was changing significantly, and already in a much more comprehensive range of PPFD levels, QF is competitive with QC (Fig. 2b).

In confirmation of the above, the parameters of the light curve regressions referring to the balance of CO_2 change specifically in the two species, depending on the condition – under the canopy or in the gap (Fig. 3).

In the gap, A_{max} is only 1.18 times higher for QC, while for QF, this difference is significantly higher (1.58 times). A larger increase in A_{max} may signal a faster adaptation to the illumination of QF than QC. At the same time, the dark respiration rate (R_d) is significantly higher within the gap only in QF (2.34 times), while QC varies

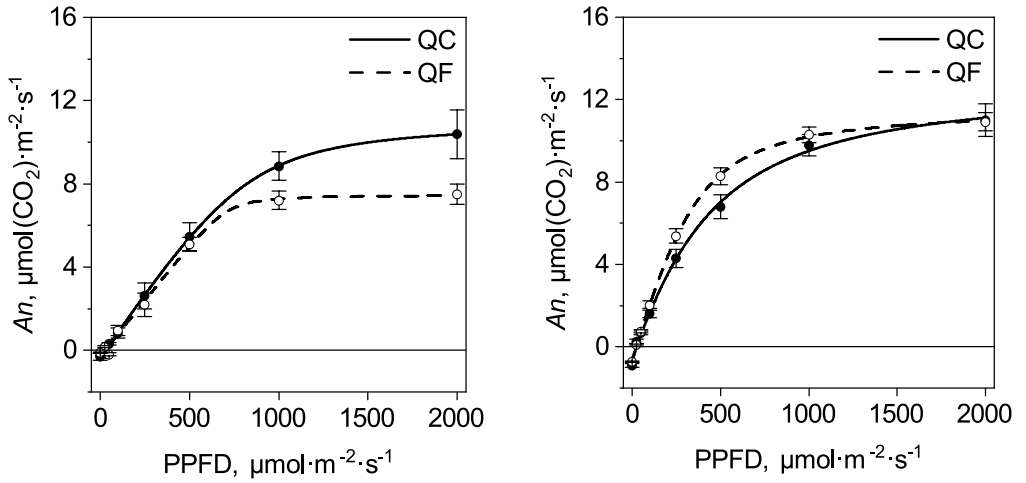

Fig. 2. Photosynthetic light-responses of *Quercus cerris* (QC) and *Quercus frainetto* (QF) under the canopy (a) and within the gap (b).

Note: Arithmetic mean values (n = 3) of the measured photosynthesis at different PPFD levels are shown with dots and their standard errors – with whiskers. The non-linear regressions according to eq. (1) are shown with lines.

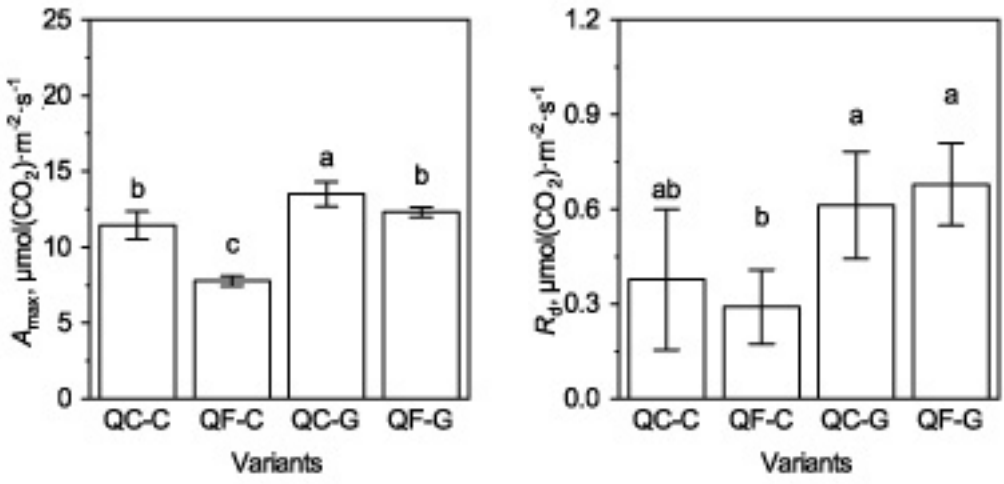

Fig. 3. Maximum photosynthetic rate (A_{max}) (left) and dark respiration rate (R_d) (right) of QC and QF within the gap (G) and under the canopy (C).

Note: Data represent the regression coefficient ±SE. Two-way ANOVA (Factor A: Species and Factor B: Conditions) (Tukey test) is applied to estimate the difference between all the variants. Different letters denote statistically significant differences (p-value < 0.05).

insignificantly between the canopy and gap, which gives an advantage in the carbon balance of QC compared to QF.

Water regime

Under the canopy, QF transpires with lower (1.66 times) intensity than QC, but within the gap, increased transpiration rate (2.7 times) much more than QC (1.51 times) and thus gained its water consumption with that of the QC (Fig. 4).

Thus, despite the more efficient use of water under the canopy of QF, the WUE in the gap is equalized between species and significantly lower than under the canopy.

Total chlorophyll content and normalized difference vegetation index (NDVI)

TCC was higher only in the leaves of QC in gap compared to the canopy, which can be interpreted as the formation of sun ecotype leaves characterized by thicker leaf lamella and, therefore, more chlorophyll per unit leaf area. However, for the QF leaves did not observe a similar difference in TCC under the canopy and in the gap (Fig. 5).

The leaves of both species retain high levels of NDVI (Gamon et al. 1995), despite specific variations in their chlorophyll content and water regime. The lack of significant change in NDVI can be interpreted as evidence of successful adaptation to gap conditions in both species.

Discussion

Turkey oak is a fast-growing tree with good adaptability (Savill 2013). It dominates in the regeneration and even at the same age and with equal participation in the stand density, its mean diameter and

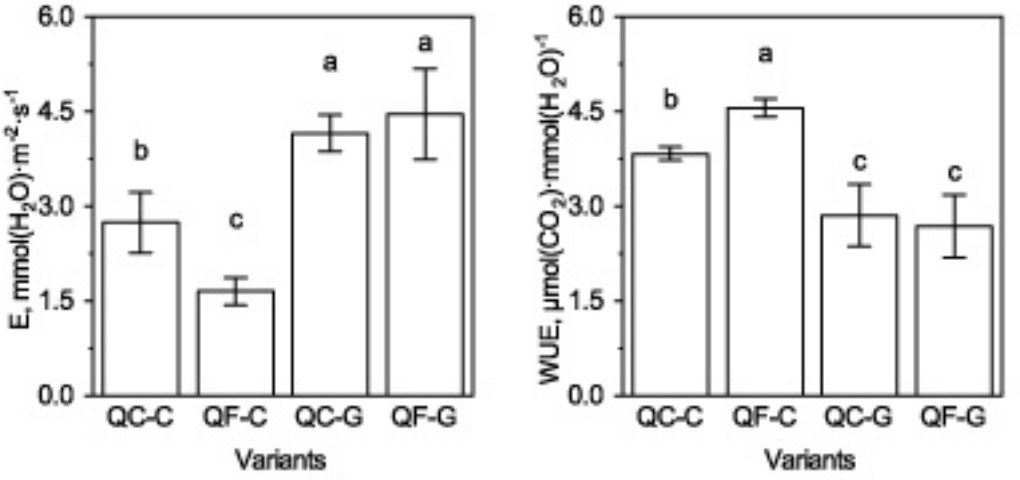

Fig. 4. Transpiration rate (E) (left) and water-use efficiency (WUE) (right) of QC and QF within the gap (G) and under the canopy (C).

Note: Data represent the mean values (n = 3) ±SE. Two-way ANOVA (Factor A: Species and Factor B: Conditions) (Tukey test) is applied to estimate the difference between all the variants. Different letters denote statistically significant differences (p-value < 0.05).

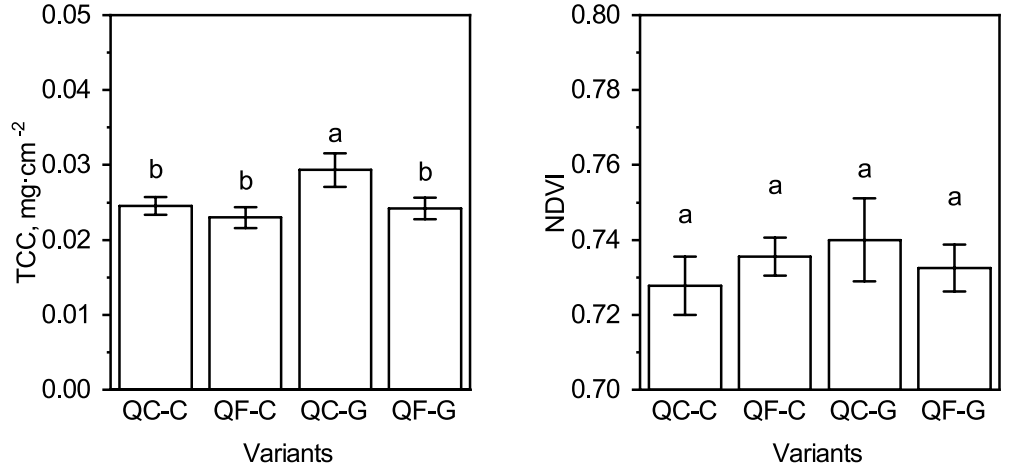

Fig. 5. Total chlorophyll content (TCC) (left) and normalized difference vegetation index (NDVI) (right).

Note: Data represent the mean values (n = 6) ±SE. Two-way ANOVA (Factor A: Species and Factor B: Conditions) (Tukey test) is applied to estimate the difference between all the variants. Different letters denote statistically significant differences (p-value < 0.05).

height and the basal area and stock volume per ha are greater. Since the site characteristics are favourable for both species, we can suggest this dominance of QC is a result from the physiological processes within the trees.

Condition-derived forms (Kimmins 1997) of light-response curves were

concluded for both species, considered light-dependent, showing suppression in their seedlings under the canopy with greater scale for QF. Yet, species-specific features were observed in their responses within the opening. Both groups in the gap reach high levels of net photosynthesis but also high levels of dark respiration and the saplings under the canopy stand contrary to them. The results of net photosynthesis within the gap are similar to those of older trees from the same species during the same season described by Wolkerstorfer et al. (2011), but those under the canopy show again an advantage of QC. Clearly, under the canopy, both species develop lower productive leaves with a lower response to the increasing light intensity, but this is more pronounced in QF. The higher light response under canopy conditions shows better light-dependent competitiveness of *Q. cerris* and higher light-related stress in *Q. frainetto* under the canopy.

CO_2 exchange shows higher capabilities of QC to include carbon into organic compounds under the canopy. It reaches relatively high values under both conditions, showing almost no sign of suppression under the canopy. QF manages to fix CO_2 at a slightly higher rate than QC in the gap, but under the canopy, it falls behind, showing the lowest maximal rate of photosynthesis. With the small amount of CO_2 released with respiration, we can say that CO_2 exchange in QF-C is highly suppressed.

Both species showed higher levels of transpiration than older trees during the same season (Wolkerstorfer et al. 2011), which can be referred to the higher rate of most physiological processes in younger individuals. Within the gap, QF increases its rate of transpiration on a greater scale than QC and decreases water use efficiency dramatically close to the QC WUE. The similar E and WUE in the gap show no signs of water stress. This could be another sign of light-dependent stress in QF under the canopy. QC remains more active under the canopy, so its transpiration rate remains relatively high.

All studied physiological aspects of the regeneration in this study show suppression of QF under the canopy and the ability of QC to remain vital under some level of shading, in conscience to Popovic et al. (1997). In the gap the species are with comparable performance with slightly earlier response in QF.

The NDVI index for all the groups is relatively high, showing that the plants chosen for the study are healthy, without a sign of drought-related stress. They also have close chlorophyll concentration levels, although QC-G has the greatest. The latest probably shows the mechanism for better light-dependent performance in this case, and it is higher chlorophyll concentration.

The uses of the two species differ, with the preference of the Hungarian oak for timber (Mauri 2016) and the Turkey oak primarily for fuel wood (Savill 2013). There should be chosen a primary aim in front of any mixed natural stand or plantation from these species that is going to be managed. The intensity and repetition of thinning during early stages of stand development can be corrected by this aim.

Conclusions

The study confirmed that both species are shade-intolerant, more pronounced for QF. The closed canopy suppresses both species but QF more. The opening of gaps in the canopy reflects in greater scale the QF but favours both species. The better

performance for QC under some levels of shading makes it more competitive from both, so that we can expect its dominance during the following stages of stand development for mixed *Q. frainetto – Q. cerris* stands without further selective thinning. The site conditions are favourable for both species, so the main question when managing naturally regenerated stands or establishing plantations on such conditions from these species should be the aim of the stand or do we want to produce fuel wood or timber.

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