Woodland reserves within an urban agglomeration as important refuges for small mammals

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Abstract

The aim of the study was to determine the species richness (S, Chao-1 index) and diversity (Shannon–Wiener H′ index, diversity profiles) of small mammal assemblages in woodland reserves in an urban agglomeration and to compare the similarity of assemblages (with the use of Ward’s method) in terms of proportions of small mammals connected to the habitats of different level of naturalness. The work was conducted from 2004–2015 at 9 woodland reserves in Warsaw (Poland). On the basis of the analysis of pellets of tawny owls Strix aluco, 2792 individuals were identified (24 species). Reserves supported from 7 to 16 of the small mammal species, the highest overall number of species estimated (Chao-1) was 19. Species present in every reserve were Apodemus flavicollis, A. agrarius, Rattus norvegicus, Sorex araneus and Talpa europaea. Least frequent were Microtus agrestis and M. subterraneus. Seven species of bats were detected. Species diversity was lower in the biggest forest complexes, where forest rodents dominated small mammal assemblage. The heterogeneity of habitats within reserve and in the surroundings, in combination with limited human-interference, resulted in an increase in the species diversity. Overall, the reserves under study were an important refuge for small mammals within the Warsaw agglomeration. However, safeguarding of adjacent areas against excessive anthropogenic change is needed and ecological corridors that link different areas need to be retained.

Key words

rodents, soricomorphs, bats, urbanisation, habitat fragmentation, tawny owl pellets

Introduction

Urbanisation leads to the destruction of woodlands adjacent to towns and cities. The process is happening most rapidly near large metropolexes with well-developed industries and growing human populations (Faulkner 2004; review in Marzluff et al. 2008). Woodlands or forests close to large cities are subject to broad-scale changes, typically the construction of building plots, the development of features, such as wastewater treat-
ment plants, landfills for the disposal of waste, roads or cemeteries. In extreme cases, especially near city centres, widespread deforestation and the removal of tree cover can be very common. Often, the only means of restricting the influence of urbanisation to areas of woodland located within city limits is to protect areas of woodland by making them nature reserves (Łaszek and Sendzielska 1989; Wojtatowicz 2005).

Highly-urbanised areas, and in particular the areas located in the centres of large metroplexes, are invariably characterised by impoverished mammal faunas, especially small mammal faunas (Goszczyński et al. 1993; McKinney 2008). In urban environments, factors that reduce the richness and diversity of small mammal assemblages (especially those characteristic of woodland or forest) include the fragmentation of forest and woodland, as well as the isolation that increasingly characterises remaining patches (Dickman and Doncaster 1987; Baker et al. 2003; Gomes et al. 2011; Łopucki et al. 2013). Despite being protected, woodland reserves established within metroplexes tend to be characterised by an impoverished mammal fauna. For example, the Australian reserves of this type in cities have a third fewer native species of small mammals than those located outside of cities (Buckmaster et al. 2010). Nevertheless, the establishment of reserves may still increase the suitability of the habitat after several years of protection (Lesiński and Gryz 2012). Even within city limits, the composition of small-mammal assemblages differs markedly across an urbanisation gradient, with lower species richness and dominance of species connected to markedly transformed habitats typical for central zone (Gryz et al. 2008).

The aim of this work was to characterise the small mammal fauna of woodland reserves established within a large urban area. The number of species and species diversity was compared between different sites. In addition, the quality of habitat present at different sites and the role that reserves played in protecting small mammals were assessed.

**Material and Methods**

**Study sites**

This work was conducted in Warsaw, Poland’s capital and largest city, with almost 2 million inhabitants. The pellets of tawny owls *Strix aluco* were searched within 12 of the city’s woodland reserves. Finally, data from 9 of areas (Fig. 1) were used because in the three other reserves, either tawny owls were not recorded (Gryz and Krauze-Gryz 2013) or a sufficient number of pellets was not found. A characterisation of the sites is presented below. The data from literature (Łaszek and Sendzielska 1989; Czerwiński et al. 2003; Wojtatowicz 2005; Luniak 2010; Stolarz 2011) and the author’s own observations were used.

**Las im. Jana III Sobieskiego (the King Jan III Sobieski Wood)**

This woodland reserve has been protected since 1934 and covers 115 ha. The central and southern parts retain oak *Quercus* spp. stands over 170 years old, while other areas have stands dominated by oaks and Scots pines *Pinus sylvestris* with a mix of various broadleaved species. The reserve is part of a larger forest complex covering approximately 1000 ha. This area, which lacks natural watercourses or bodies of water, is connected to a limited degree with extensive forest areas located beyond the Warsaw city limits. Pellets were collected here during the years from 2009–2015 from three tawny owl territories.
Olszynka Grochowska
This 57 ha reserve was established in 1983. The entire woodland complex is protected. It is surrounded by single-family housing and railway infrastructure. Scots pine, birch *Betula pendula* and oak are present in the tree stands, and wet areas support black alder *Alnus glutinosa*. A drainage channel is present in the southern part of the reserve. Pellets were collected from a single tawny owl territory during the years from 2011–2014.

Zakole Wawerskie (Wawer Meander)
This reserve is part of the old floodplain terrace of the River Vistula, which was established in 2002. Ninety percent of this wet 56 ha area is covered by natural stands of alder between 40 to 60 years in age. The western part also has meadowlands, reed beds and scrub habitats. The northern limits of this protected area are delimited by a canalised watercourse emptying into the Vistula. An area of more than 150 ha to the south is made up of meadow, pasture, arable land and wasteland. In contrast, land to the east and north of the Wawer Meander has become highly urbanised. Pellets were collected from a single tawny owl territory during the years 2011–2013.

Kawęczyn
This woodland reserve is 70 ha and was established in 1998. The reserve is bounded by two roads with heavy traffic and a rail line. However, there are adjacent woodlands that make the entire area of contiguous woods approximately 150 ha. This area is barely linked to the more extensive Olszynka Grochowska area and the Sobieski Wood. The stands in this area are primarily composed of Scots pine, oak and birch, though most of this site was only afforested in the 1950s. Pellets from a single tawny owl territory were collected here during the years 2010–2013.

Bagno Jacka (Jacek Marsh)
This area of nearly 20 ha was established in 1981 to protect a raised bog and transitional mire. Ninety percent of this reserve is occupied by forest up to 70 years old. Tree species primarily consist of Scots pine and birch. The peatland here is surrounded by pine stands that grow on mineral soils. To the south of the reserve, there are urbanised areas. On the other sides of the reserve, there are military training grounds that cover approximately 8000 ha. Pellets were obtained from a single tawny owl territory during the 2011–2014 period.

Las Bielański (Bielany Wood)
Since 1978, this area of 130 ha has been protected as a multi-species broadleaved woodland. Habitats in this reserve include oak-lime-hornbeam forest, riparian forest and alder carr. The protection of woodland extends to a rather large area of approximately 170 ha. The oldest oak stands here exceed 250 years in age. The reserve area is crossed by two small watercourses. On the eastern side, the reserve is partly separated from the River Vistula by a 3-lane expressway, while the northernmost limits are connected to the large Kampinos (Kampinoski) National Park, which extends up to the city limits of Warsaw. In contrast, urban development is present to the west and south. Pellets from the three tawny owl territories were collected here during the years from 2007–2014.

Skarpa Ursynowska (Ursynów Scarp)
This reserve protects the scarp rising above what was once the proglacial valley that carried meltwaters from the ice sheets. This valley is now considered to be the Vistula Valley. Protection was provided to a 23 ha area here in 1996, and the reserve includes a multi-species stand of broadleaved woodland. However, the woodland and scrub habitat only accounts for 30–40% of the protected area. The remainder of the reserve is composed of meadowland and ruderal plant associations. The scarp runs to the south of the reserve and is connected to woods present at the southern edge of the Warsaw agglomeration. To the southeast, the reserve borders have more extensive open areas, although these areas are destined for more urban development. Pellets from two tawny owl territories were collected here during the 2006–2012 period.

Las Natoliński (Natolin Wood)
A 105 ha area is protected since 1991. Although this land was formerly an area of parkland that extended around the residences of Polish magnates, spontaneous regenerations of woodland took place during the post-War period. The oldest stands of oak are more than 250 years old. Ash *Fraxinus excelsior*, elm *Ulmus* spp. and black alder are also well-represented in these woods. The reserve area also includes heritage buildings and
open areas. To the west of the reserve are developed areas, while on the other sides, there are extensive areas of farmland, although farmland is diminishing because more and more plots are being sold to urban developers. Pellets from three tawny owl territories were collected during the years 2004–2011.

Las Kabacki (Kabaty Wood)
This is Warsaw’s largest protected area, which covers more than 900 ha. Kabaty Wood was established in 1980. The multi-species tree stands here are dominated by Scots pines and oaks, which grow in fertile habitats. Some stands are more than 120 years old. The forest complex is surrounded by developed areas, except in the east, where the reserve is adjacent to arable land and an urban park. This reserve is contiguous with other tracts of land via the Vistula Scarp. Pellets from five tawny owl territories were collected here during the years from 2004–2010.

Methods
Inventory of the mammal fauna was conducted with the standard, non-invasive procedure of examining tawny owl pellets (Heisler et al. 2015). Alternative inventory methods, such as trapping (using a variety of different traps), invariably limit the number of species detected below the number actually present (Gortat et al. 2014). In addition, trapping is difficult to execute in areas that are heavily travelled by people. Trapping also often leads to unintended deaths of the animals under study (Gryz and Krauze 2007; Gryz et al. 2008). Analysis of the dietary composition of the tawny owls can offer a robust quantitative and qualitative reflection of the small vertebrate assemblage present in an area (Mikkola 1983; Gryz and Krauze 2007; Lesiński et al. 2008; Zmihorski et al. 2011; Heisler et al. 2015). The tawny owl is a rather sedentary species, which stays in a single territory throughout the year. This permits a high degree of certainty that the prey hunted by the owls was taken in areas close to where the pellets were collected. Depending on habitat type, the home ranges of tawny owls are known to cover between 10+ and several tens of hectares (Redpath 1995; Sunde and Bølstad 2004). Tawny owls are also known to be opportunistic feeders, thus, the study of their pellets may provide a robust estimate of the diversity of mammal species present, at least for the species of the following genera: Rodentia, Soricomorpha, Chiroptera and Carnivora. However, in Europe, the adults of only two carnivorous species can be caught by tawny owl, that is least weasel Mustela nivalis and stoat Mustela erminea. In other cases, the juveniles are preyed upon. The tawny owl can consume prey as small as Poland’s smallest bat or shrew species (weighing just a few grams), as well as large rodents whose weights may exceed 250 g, such as the water vole Arvicola amphibius and brown rat Rattus norvegicus (Lesiński et al. 2009).

Pellets were collected on a regular (monthly, quarterly or half-yearly) basis to ensure that the diet of the tawny owls in all seasons of the year was represented. Subsequent laboratory analysis was based on standard procedures (Raczyński and Ruprecht 1974; Yalden and Morris 1990). Fragments of bone were identified using the key of Pucek (1984) or other detailed works (Ruprecht 1979, 1987). The material collected in the years 2004–2015 originated either within the protected areas under study or close to the reserve boundaries. Pellets were obtained beneath trees in places where tawny owls were considered to be commonly present. Some pellets were also taken from nest boxes or tree holes. Some of the data documenting the mammal fauna of the Bielany, Natolin and Kabaty Woods and Jacek Marsh reserves were also taken from the literature (Gryz and Krauze 2007; Lesiński and Gryz 2012; Stolarz and Lesiński 2012).

The results are presented in terms of the percentage of all the mammal individuals reported, that were accounted for by individuals of any given taxon. Species richness (S) was determined, as well as an estimate of the overall species richness of small and medium-sized mammals at a given site on the basis of the Chao-1 index. This index accounts for the number of species represented just once or twice in a given sample (Chao 1984). Species diversity was estimated using the ln based Shannon-Wiener H’ index, but also by generating diversity profiles of Tothmeresz (1995). The latter takes species richness into account, as well as the Shannon-Wiener and Simpson diversity indices. To compare the diversities characteristic of different sites, a t-test was used to compare the diversities calculated using the Shannon formula as was proposed by Hutcheson (1970). The similarity of the species assemblages was compared using the Ward’s method by calculating Euclidean distances. Statistical analyses were performed using the Past3 program (http://folk.uio.no/ohammer/past/).
Results

Overall, the sites yielded the remains of 2792 individuals representing 24 species (Tab. 1). Species present at all sites were the yellow-necked mouse *Apodemus flavicollis*, striped field mouse *A. agrarius*, brown rat *Rattus norvegicus*, common shrew *Sorex araneus* and mole *Talpa europaea*. Also, widespread was the common vole *Microtus arvalis*, house mouse *Mus musculus* and bank vole *Myodes glareolus*, each of which was recorded in 8 of the 9 sites. The least widespread of the rodents reported was the field vole, *Microtus agrestis*, which was recorded at just two sites, and the pine vole *M. subterraneus*, root vole *M. oeconomus* and the wood mouse *Apodemus sylvaticus*, each of which was present at 4 sites.

The highest proportions of woodland rodents (i.e., *A. flavicollis* and *M. glareolus*) among prey items were 77% observed in the Kawęczyn reserve, as well as 69% and 64% in the Kabaty Wood and Olszynka Grochowska sites, respectively. The voles associated with open areas were present at the highest frequencies at the Jacek Marsh (where they accounted for 11% of the overall assemblage), at Natolin Wood (8%), and at the Wawer Meander, Bielany Wood, Ursynów Scarp and Kabaty Wood reserves. The rodents associated with the presence of human beings (*Mus musculus, A. agrarius* and *R. norvegicus*) accounted for the greatest proportions of the assemblages reported at the Ursynów Scarp and Wawer Meander sites (50% and 29%, respectively), the Olszynka Grochowska site, and the Sobieski and Natolin Woods (Fig. 2).

The Ward’s method suggested that there was a faunal similarity between the Bielany reserve and the Kabaty Woods. However, the Jacek Marsh and Sobieski Wood sites were also included in the same group. A second group included the Wawer Meander and the Ursynów Scarp sites, as well as Kawęczyn and the Natolin Wood, with the latter forming two separate clusters alongside the separated Olszynka Grochowska (Fig. 3).

The numbers of species reported for different sites ranged from 7 (at the Kawęczyn reserve) to 16 (at the Jacek Marsh and the Bielany and Natolin Woods). There was no relationship between these numbers and either the reserve area (r = –0.06, P > 0.05) or the sample size.
Table 1. The percentage, species richness (reported – S or estimated – Chao-1) and diversity (Shannon-Wiener index – H’)
of small mammals detected in Warsaw woodland reserves based on the analysis of tawny owl pellets. Woodland reserves: Sobieski Wood (1), Olszynka Grochowska (2), Wawer Meander (3), Kawęczyn (4), Jacek Marsh (5), Bielany Wood (6), Ursynów Scarp (7), Natolin Wood (8), Kabaty Wood (9)

<table>
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<tr>
<th>Prey</th>
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<td>Microtus arvalis</td>
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<td>M. oeconomus</td>
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<td>M. agrestis</td>
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<td>Microtus spp.</td>
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<td>Σ Microtus</td>
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<td>2.7 2.1 20.3</td>
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<td>Myodes glareolus</td>
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<td>33.4 41.8 1.6</td>
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<tr>
<td>Apodemus flavicollis</td>
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<td>A. sylvaticus</td>
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<td>A. agrarius</td>
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<td>Apodemus spp.</td>
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<td>Micromys minutus</td>
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<td>Neovison vison</td>
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<tr>
<td>Mustela nivalis</td>
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<td>Number of species – S*</td>
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<td>Chao-1*</td>
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<td>1.53 1.40 1.90</td>
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Woodland reserves within an urban agglomeration as important refuges for small mammals

The estimated overall species richness of small mammal species (Chao-1) was greatest at the Jacek Marsh reserve and smallest at the Ursynów Scarp reserve. The values for Chao-1 index were comparable with the numbers of species actually obtained for the Ursynów Scarp, Natolin Wood and Sobieski Wood reserves. In Olszynka Grochowska site, Jacek Marsh and Bielany Wood, the estimated numbers of species were greater by 3 than those actually reported, while in the case of Kabaty Wood, the difference between the estimated and observed values was highest and greater by 6 species (Tab. 1).

In turn, the diversity of species of small mammals was highest at the Natolin Wood site ($H' = 2.21$), followed by the Jacek Marsh ($1.89$). However, these two sites differed significantly in this regard ($t = 2.28, df = 189.96, p < 0.05$). The lowest estimated value for diversity was for Kawęczyn reserve ($H' = 1.13$). This value differed significantly from the values estimated for the Olszynka Grochowska site ($H' = 1.40$) ($t = -2.41, df = 186.37, p < 0.05$) and the Kabaty Wood ($H' = 1.39$) ($t = 2.30, df = 199.34, p < 0.05$). The species diversities of small mammal assemblages of the Kabaty Wood and Olszynka Grochowska were not found to differ significantly ($t = -0.07, df = 464.61, p > 0.05$).

The analyses of diversity profiles (Fig. 4) revealed that the highest diversity was at the Natolin Wood, however it was comparable to Jacek Marsh. Jacek Marsh had a similar profile to the Bielany Wood and the Wawer Meander reserves. A rather similar profile characterised the Sobieski and Kabaty Wood reserves, the Olszynka Grochowska site and the Ursynów Scarp, while the Kawęczyn reserve had the lowest diversity of all sites surveyed.

**Discussion**

Our study showed that the number of species recorded (as well as a total estimated number of species – Chao-1) differed greatly between woodland reserves in Warsaw. Also, the analyses of species diversity (Shannon-Wiener $H'$ index) indicated important discrepancies between areas. Nevertheless, when diversity profiles, based on species richness and the Shannon-Wiener $H'$ and Simpson diversity indices (Tothmeresz 1995), were taken into account, it could be seen that only one site (Kawęczyn) was markedly different from all the others. Possible explanations for these results are discussed below.
The greatest species diversity (reflected both by Shannon-Wiener H’ index and diversity profiles), and relatively high levels of species richness, were observed in the Natolin Wood. This site also had the most even spread of species associated with forests (accounting for less than 50%), open areas and human development. This high diversity reflects the heterogeneity of the habitat (i.e., the presence of extensive grasslands and water bodies) within the reserve (Krojerová-Prokešová et al. 2016), as well as the close proximity of both open and developed areas. In addition, this area of woodland only experiences limited human interference, indeed, the structure of this reserve is actually reminiscent of a primitive forest. In this case, a moderate level of urbanisation has promoted higher levels of species diversity, in a manner consistent with the ‘intermediate disturbance hypothesis’ (McKinney 2008). Similar number of small mammals was recorded in SW suburban zone of Warsaw, in a moderately transformed habitat with high percentage of vegetation cover (Romanowski et al. 2016). On the contrary, Kawęczyn was a reserve with the highest share of typical forest rodents (Myodes glareolus and Apodemus flavicollis). At the same time, the smallest number of species was reported from this reserve, as well as the smallest level of species diversity (both H’ index and diversity profile). Nevertheless, the species characteristic of areas highly modified by human activity (Mus musculus, A. agrarius and R. norvegicus) here accounted for less than 5% of the overall small mammal assemblage. The results of this kind are analogous to those obtained from Białowieża National Park (Jędrzejewski et al. 1994; Gryz et al. 2012), an extensive forest with a low human-interference. Similar proportions for the frequency of occurrence of these two groups of rodents (i.e., connected to forests and human-transformed areas) were also obtained for the Kabaty Wood reserve. Here, a relatively small number of species was also detected, although the difference between the actually-recorded and the estimated overall species richness (Chao-I) was greatest, which suggests that with a bigger sample size a higher number of species would be probably recorded. In the case of the two aforementioned reserves, they either protect large complex of woodland or are a part of the bigger wood, so the presence of species connected to open/human-transformed areas is limited to forest edges. On the contrary, in the case of small woods, the higher ‘edge effect’ ensures the penetration by species associated with human-transformed areas (i.e., Apodemus agrarius) or with open areas (i.e., voles), and their considerable share in total small-mammal assemblage.

Previous studies from Warsaw showed that urbanization influenced the species composition of rodents, with striped-field mouse being a dominant species (and reaching extreme densities) in considerably human-transformed habitats (i.e., parks, cemeteries, etc.) (Babińska-Werka et al. 1979; Gortat et al. 2014). So, this is the species whose share in the small-mammal assemblage can be an index of the level of naturalness of the woodland area. In this view, the Ursynów Scarp reserve could be defined as the least pristine among all the reserves studied. The only woodland species present at this site was yellow-necked mouse, but it only accounted for a small portion of the overall assemblage. In fact, the species most abundant here were those characteristic of areas extensively disturbed by humans and they accounted for more than half of all of the specimens recorded, and a proportion of A. agrarius was the highest (as compared to other reserves). This site also had low species richness and relatively low species diversity. The Ursynów Scarp is a small area located well within the city, with neighbourhood areas that are partially built-up. The findings here are thus consistent with those from other studies, where urbanisation has resulted in a decrease in species diversity and richness. These studies have also found that urbanisation also decreases the representation of native species (Cavia et al. 2009) and the proximity to developed areas generally reduces the diversity of mammal species (Dickman 1987).

The areas with a high level of species richness recorded were the Jacek Marsh and Bielany Wood. The first of these is a fragment of suburban woodland that is connected to larger areas of forest beyond the city. Thus, the species that were rare in the metroplex, such as the Microtus agrestis, were present in this reserve. Three species of bats have also been reported there. The other reserve, the Bielany Wood has the greatest number of bat species (4), along with three Microtus species. At the same time, woodland rodents made up over 50% of the small mammals detected at this site. In this woodland, changes in small-mammal community were recorded over last decades as a result of legal
protection and restoration of stands, with an increase of *A. flavicollis* and soricomorphs, and a decrease of *A. agrarius, Rattus norvegicus* and *M. musculus* (Lesiński et al. 2012).

Although voles are typical for open area, they are absent (on the contrary to field-stripped mouse), from the central quarters of Warsaw (Gryz et al. 2008), and their share in rodent community decreases with an urbanization gradient (Andrzejewski et al. 1978; Gortat et al. 2014). Therefore, in our study they were present in all but one (Kawęczyn) woodland reserves, but their share was highest in sites that were surrounded by or connected to open areas such as meadows or arable lands (i.e., Wawer Meander, Ursynów Scarp, Natolin Wood). In the case of the Jacek Marsh voles (i.e. *Microtus arvalis*) were probably caught at the road/railway embankment, covered with low-cut grass and regularly mown, which marked the edge of the reserve. It can be assumed that small-mammal assemblage of agricultural lands surrounding the woodland reserves under this study is relatively high. Due to changes in land use within city borders, they are often represented by fallow or set-aside land, old orchards. Such habitats are known to support higher biodiversity of small mammals than intensively cultivated fields (Janova and Heroldová 2016).

The group of small mammals that are very sensitive to habitat fragmentation are shrews (Vergnes et al. 2013). In Warsaw, they are absent from green areas located in the central quarters (Gryz et al. 2008; Krauze-Gryz et al. 2016) and areas of high anthropopressure degree (Gortat et al. 2014). Nevertheless, they were present in all the studied woodland reserves. It may be assumed that the woodland reserves under study were big enough to maintain viable population of shrews.

Bats can be regarded as prey species caught most often by chance. In all of the areas studied, it may be possible to record more bat species as the list of bat species of the Warsaw agglomeration counts 15 (Lesiński et al. 2001; Popczyk et al. 2008).

None of the representatives of the dormouse family Gliridae were recorded in this study. The closest known localities for the hazel dormouse *Muscardinus avellanarius* are in the Kampinos Forest, which is mostly National Park land (Lesiński et al. 2013) or a part of the Mazowiecki Landscape Park (Lesiński and Gryz 2008). Another species rarely recorded in areas developed by humans is the northern birch mouse *Sicista betulina*, which was recorded relatively close to the Jacek Marsh reserve (Lesiński et al. 1998), but has never been reported from the Warsaw agglomeration.

Woodland reserves under this study offered variable conditions for small mammals. This was due to their history, habitat structure within the reserve and in the surroundings, as well as human interference. However, according to Ward’s method, similarities were found. For example, the Wawer Meander and the Ursynów Scarp reserves, which formed one group, are both strictly connected to Vistula floodplain, both are long-narrow woods, surrounded by open, at least partially wet, areas. In the other example, the Bielany and Kabaty Woods are characterised by similar habitat types, with a considerable share of old stand, and they are both located in a similar distance from the city centre.

According to the work done by Dickman (1987), the richness of small mammal species within a city can be maintained through a network of woodland areas over 0.65 ha in size. All of the areas examined in the present study are far larger than this recommended size, hence, the reserves in Warsaw likely ensure the effective protection of mammals associated with woodlands. However, in case of the smaller sites studied – especially where these are surrounded by areas subject to extensive anthropogenic influence – changes in species composition were apparent and the penetration by species associated with open areas or the presence of people was also observed. Nevertheless, these results suggest that the system of protected areas in Warsaw provide a safeguard to populations of most small mammal species. However, a further key element to the protection of these small mammals is the prevention of additional damage to areas adjacent to reserves, as well as the retention and maintenance of the ecological corridors that connect the land between different sites.

**References**


Babińska-Werka J., Gliwicz J., Goszczyński J. 1979. Synurbization processes in population of *Apode-


Is cut-stump and girdling an efficient method of black cherry Prunus serotina Ehrh. eradication?

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ABSTRACT

Efforts to prevent the invasion of black cherry Prunus serotina Ehrh. have a long history in Western Europe. However, effective methods of eliminating it that do not bear negative side effects for ecosystems have not yet been developed. Mechanical methods are the first choice in environmentally sensitive areas. In this study, we aimed to find answers to the questions: does the application of cutting at a height of 1 m from the ground limit the sprouting capacities of black cherry? And, is stem girdling an effective method of eliminating black cherry? The study was carried out in the Kampinos National Park, on two mixed pine forest plots with undergrowth of black cherry. Three mechanical methods of elimination were applied: cut-stump at the base, cutting at a height of 1 m above the ground and girdling of the stem at a height of ca 1 m above the ground. In both locations, 225 trees were treated, at three different dates corresponding with three different phenological phases of black cherry development. The evaluation of effectiveness of treatments was based on the sprouting capacity of the tree afterwards, which included: the number of generated sprouts, the length of three longest sprouts, dry mass of sprouts, and the assessment of tree survival rate. It was discovered that girdling is a significantly more effective method of control than ground-level cut-stump or cutting at a height of 1 m above the ground in the conditions of central Poland. However, in the season of treatment, even though recurring sprouts were removed, only a part of the girdled trees died (24% to 54%). There is a slight difference between the sprouting response of cutting at a height of 1 m above the ground (4% to 24% of dead trees) and the basal cut-stump method (0% of dead trees).

KEY WORDS

Mechanical methods of elimination, mixed pine forests, invasive plants, protected natural areas, sprouts

INTRODUCTION

The prevention of biological invasions is an important task faced by the environmental protection services, following the implementation of Convention on Biological Diversity (CBD 1992) and Regulation (EU) No 1143/2014 of the European Parliament and Council on 22 October 2014. Such work requires a thorough knowledge of expansion of the invasive species, its biology and ecology, and the effectiveness of methods of elimination. Black cherry Prunus serotina Ehrh., originating in North America, is one of the 100 most
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Expansive organisms in Europe, where in many countries (e.g., the Netherlands, Germany, Denmark) it is referred to as a ‘wood weed’ (DAISE; Starfinger 1997; Vanhellemont 2009). It occurs in the whole area of Poland (Tokarska-Guzik 2005). It has been inventoried in an area of ca 100,000 ha of forests managed by the State Forests National Forest Holding (PGL LP), which constitutes 1.4% of their total area (Bijak et al. 2014). The invasion of black cherry has also expanded in protected areas. It occurs in 10 out of 23 Polish national parks, and it is the most frequently combatted species there (Najberek and Solarz 2011; Bomanowska et al. 2014).

Even though the elimination of black cherry has a long history in Western Europe, efficient methods that would not bear negative side effects for ecosystems have not been developed (Muys et al. 1992; Starfinger et al. 2003). It is well known that elimination is costly and time consuming, and its results can be lost through discontinuation of procedures, which leads to a reinvasion of the species. High effectiveness is shown by chemical controls, including, for example, the application of substances containing glyphosate (Van den Meersschaut and Lust 1997; Csiszár and Korda 2015). However, the application of chemical substances negatively impacts many components of the environment, and they should not be applied in protected regions (Tokarska-Guzik et al. 2012). On terrain that is especially environmentally sensitive, it is necessary to limit to mechanical methods, among which the most commonly applied is basal cut-stump. This is the least labour-intensive method, but it is also ineffective, as many sprouts shoot off from the remaining stump (Starfinger et al. 2003; Halarewicz 2012; Namura-Ochalska 2012; Annighöfer et al. 2012). This occurs because black cherry displays a high regeneration capacity after damage, which is exploited in its natural habitats for the sake of forest regeneration (Husch 1954; Marquis 1990). For this reason, other methods are sought for practical uses, such as cutting the stump at a higher level, girdling and the introduced underplanting of the cultivated seedlings (e.g., Otręba et al. 2014; Krzysztofiak and Krzysztofiak 2015; Namura-Ochalska and Borowa 2015; Demeter and Lesku 2015). Experiments with application of various methods of elimination, including mechanical methods, have been carried out so far in Belgium and Italy (Van den Meersschaut and Lust 1997; Annighöfer et al. 2012). The experiments indicate a high effectiveness of girdling, falling just below that of chemical methods of control.

The aim of this study is to ascertain whether, in the habitat conditions of central Poland, cutting stumps at the height of ca 1 m from the ground limits the sprouting capacity of black cherry, and if girdling of the stem is an effective method of eliminating black cherry.

The field experiment was carried out in the Kampinos National Park, where black cherry is one of the most frequent alien tree species, and where the scope of work aimed at its limitation spans more than 400 ha per year (Otręba et al. 2014). Due to its high environmental value, the Kampinos forest is protected as a national park and as a part of the Natura 2000 and Biosphere Reserve programmes. The obtained results will enable development of a more effective and efficient method of mechanical elimination of black cherry – a species alien among the European flora.

Material and methods

Study sites location and characteristics

The Kampinos National Park, located in the central Mazovian Lowland, is nearly entirely situated in the Warsaw Valley, with only its southern and eastern fragments reaching the Łowicz-Blonie Plain and the Warsaw Plain, respectively. The main characteristic of the Park’s landscape is the presence of belts of dunes and swamps formed in the proto-valley of the Vistula River. Forests cover 73% of the Park’s 385 km² area. The coniferous pine forests of various levels of soil fertility, moisture and anthropogenic transformation dominate in the Park (Andrzejewska et al. 2010). The climate is temperate, with a prevalence of continental characteristics over Atlantic ones. The mean yearly temperature is 7.7°C, the vegetation period lasts 185 days, and the mean yearly precipitation level is 547 mm (Andrzejewska 2003). The year 2015, when the experiment was carried out, was exceptionally dry and warm (Andrzejewska, unpubl.).

Plot design and characteristics

The study area was located in the eastern part of the Kampinos National Park, in the protection district of Laski, on two plots: at Sieraków (N – 52,30290, E – 20,84810), forest division number 345, and Lipków (N – 52,2834, E – 20,81810), division no. 185. The criteria of uniform-
ity of habitat conditions, species composition, and vertical canopy structure within each location were used for the selection of study plots. The similarity of locations was not taken into account. Plots were characterised by the presence of abundant undergrowth forming a lower shrub layer of black cherry that was not yet eliminated. Both plots are located at the outskirts of the forest complex. In Sieraków, the plot borders private woods and lands belonging to the Laski village; in Lipków it borders a built-up area of the Hornówek village. Both locations are situated at the edge of the Warsaw Plain.

The environmental profile of the study plots was based on appraisal descriptions (Protection Plan of the Kampinos National Park, KPN 2002) that were then verified in the field. The Sieraków canopy is characterised by a three-layer structure and two-generational nature. The higher stratum of the canopy is dominated by pedunculate oak, silver birch and Scots pine, the last reaches the age of 175 or 94 years. The lower layer is composed mainly of black cherry, reaching a cover of ca 30%. The floor is dominated by a neophyte, Impatiens parviflora DC., accompanied by species of nitrophilous ecotones: Moehringia trinervia (L.) Clairv., Geranium robertianum L., Alliaria petiolata (M.Bieb) Cavara et Grand and scarce coniferous species (diagnostic species, in the syntaxonomy after Matuszkiewicz 2001). According to the Protection Plan (2002), the forest type is described as a fresh mixed coniferous forest formed on Brunów Arenosol Albic on sand, with traces of post-agrarian character.

The plot in Lipków is covered by the Scots pine canopy aged 79 years, with an admixture of silver birch and sparse pedunculate oaks. Its abundantly formed (70% of cover) lower layer consists mostly of two species: black cherry and Norway maple. The floor, reaching on a significant area only up to 20% of cover, is dominated by Rubus caesius L., accompanied by Vaccinium myrtillus L., Dryopteris carthusiana (Vill) H.P. Fuchs and Moehringia trinervia (L.) Clairv. According to the Protection Plan (2002), the forest type is described as fresh mixed forest formed on Haplic Podzol on sand.

**Premises of the experiment**

The study to assess the effectiveness of given methods of mechanical elimination of black cherry was carried out during the vegetation season of 2015, in the form of an experiment in natural conditions. Overall, 450 trees were selected, 225 in each of the two locations: Sieraków and Lipków. The trees occupied ca 0.6 ha in each case.

Three types of procedures of mechanical elimination of black cherry were applied: 1) cut-stump at the base level with a chain saw (this commonly used method is a kind of reference probe); 2) cutting the tree at a height of ca 1 m above the ground level with a chain saw; and 3) girdling by removal of the bark, phloem and cambium at a width of ca 20 cm around the whole circumference of the stem at a height of ca 1 m above ground level with a draw knife.

The procedures were carried out three different times, each time on 25 trees in each location. The dates when the cuts were performed corresponded to different phenological phases of the life cycle of black cherry and to different vegetation conditions. The cuts were performed on April 8th (which reflects early spring and the beginning of the vegetation period), then on June 2nd (which corresponds to late spring and peak of black cherry’s blooming season), and on July 29th (which corresponds to summer and the formation of black cherry’s fruits).

The trees subjected to procedures of mechanical elimination were directly adjacent to one another in the terrain, which balanced the impacts of their microhabitat on the results of the experiment. Specimens with large crowns and at least 4 cm DBH were selected for the experiment. For each tree, the following measurements were taken: diameter at breast height (accurate to 0.1 cm), height (accurate to 50 cm, using a Suunto hypsometer), and length of the crown (accurate to 50 cm, using a Suunto hypsometer). The trees were individually numbered in the field to enable their identification.

Drawing on an experiment carried out in Belgium (Van den Meeresschaut and Lust 1997) for the sake of increasing the effectiveness of the procedures, an additional treatment was performed: it consisted of tearing off new sprouts and recreating girdles in the cases where stem tissues were regenerated; the removal of sprouting shoots was repeated every 8 weeks. For the trees treated first in spring, it was done three times (that is, on June 1st, July 28th and September 20th); for the trees treated in late spring, twice (on July 28th and September 20th); and for those treated in the summer, once (on September 20th). The evaluation of effective-
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The efficiency of each procedure of elimination of black cherry was based on an analysis of the following parameters reflecting the sprouting capacity after the procedure: the number of generated sprouts, length of three longest sprouts and dry mass of generated sprout shoots. The dry mass was weighed after 48 hours of drying at a temperature of 105°C. The sums of measurements obtained in each control were used for analysis: that is, for the early spring procedures, from three controls; for the late spring ones, from two controls; and only for the summer cuts, from a single control.

Each time, the crown-regenerating capacity of the girdled trees was assessed using a two-point scale (dead or alive). Capacity for regeneration – reflected in the percentage of dead trees in the total number of those treated by each procedure – was used for assessment of the effectiveness of each tested procedure. The stems that did not generate any sprouts by the end of the season (on September 20th) were considered dead. In the case of the girdled trees, the crowns were also dead.

**Statistical Analysis**

Biometric parameters and variables reflecting the sprouting capacity did not generally demonstrate a normal distribution (Shapiro and Wilk’s W test) and did not meet the criterion of variation homogeneity (Levene’s test). Due to this fact, the Kruskal–Wallis nonparametric multiple comparison of mean ranks test was used for all samples (Stanisz 2006), followed by pairwise comparison (post-hoc test) using Mann-Whitney test with Bonferroni corrected p values. The significance level was set to $p = 0.05$. The two populations of trees from two plots were considered as independent blocks. Calculations and charts were made with the applications Past 3 (Hammer et al. 2001) and Microsoft Office Excel.

**Results**

**Biometric parameters of the trees used in the experiment**

Stem diameter at DBH, the height of trees and the length of crown are presented using a mean for all trees treated with the same procedure, regardless of the date of procedure (Tab. 1). The girth of trees at DBH ranged between 4.3 cm and 21.2 cm, the height ranged from 3 m to 17 m, and the crown length ranged between 0.5 m and 9 m. The trees on Lipków plot were thicker and had more developed crowns than those in Sieraków, and the differences are statistically significant (Mann-Whitney’s U test: DBH: $z = -4,1236$, $p = 3,73E-05$; height: $z = -1,8691$, $p = 0,0616$; crown length: $z = -3,5742$, $p = 0,0004$). On the other hand, the girth, height and length of crown of the treated trees within each plot did not display statistically significant differences (Kruskal–Wallis’ test: DBH: Sieraków $H = 2.989$, $p = 0.2242$; Lipków $H = 1.69$, $p = 0.4295$; height: Sieraków $H = 3.246$, $p = 0.1943$; Lipków $H = 3.808$, $p = 0.1465$; crown length: Sieraków $H = 1.45$, $p = 0.4787$; H = 0.4206, $p = 0.8068$).

<table>
<thead>
<tr>
<th>Location</th>
<th>Treatment</th>
<th>No of trees</th>
<th>DBH (cm)</th>
<th>Height (m)</th>
<th>Crown length (m)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>min.</td>
<td>max.</td>
<td>median</td>
</tr>
<tr>
<td>Sieraków</td>
<td>Cut-stump</td>
<td>75</td>
<td>4.8</td>
<td>21.2</td>
<td>8.1*</td>
</tr>
<tr>
<td></td>
<td>Cut at 1 m</td>
<td>75</td>
<td>6.1</td>
<td>18.5</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>Girdling</td>
<td>75</td>
<td>6.0</td>
<td>18.1</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>Total</td>
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<td>4.8</td>
<td>21.2</td>
<td>8.3*</td>
</tr>
<tr>
<td>Lipków</td>
<td>Cut-stump</td>
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<td>16.2</td>
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<tr>
<td></td>
<td>Girdling</td>
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<td>21.2</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>225</td>
<td>4.3</td>
<td>21.2</td>
<td>9.5**</td>
</tr>
</tbody>
</table>

* – ** – pairs statistically significant at $p = 0.05$. 
Sprouting response of black cherry to mechanical elimination procedures

Sprouting capacity as assessed by the number of sprout shoots generated after the procedure differed significantly with regard to the type of procedure applied (early spring treatment $H = 86.07$, $p = 4.45 \times 10^{-17}$, late spring treatment $H = 82.91$, $p = 1.696 \times 10^{-16}$, summer treatment $H = 89.19$, $p = 7.872 \times 10^{-19}$) (Fig. 1). Noticeably less sprouts were recorded in the case of girdled trees, and the difference with regard to the other treatments was statistically significant in each date of application at $p < 0.01$. Within the same treatment, no differences between plots were significant. The trees girdled in early spring (Fig. 1a) sprouted four times fewer shoots (25 shoots on average in both Sieraków and Lipków) than those to which cut-stump was applied (Sieraków, 103 shoots on average; Lipków, 99 shoots on average). The maximum number of sprouts recorded among the trees cut at the base was 120 (in the Sieraków plot), while it was 73 among the girdled ones (in the Lipków plot). As the date of girdling grew later, the percentage of trees generating sprouts dropped. Practically all trees

![Graph 1](image1.png)  
**Figure 1.** Total number of black cherry sprout shoots collected in the 2015 vegetation season – quartile method. Sprout generation response of cut or girdled trees: a – in early spring, with three-time removal of sprouts; b – in late spring, with two-time removal of sprouts; c – in the summer, with a one-time removal of sprouts. A = basal cut-stump; B = stem cut at a height of 1m above the ground, C = stem girdling; S = Sieraków plot, L = Lipków plot

![Graph 2](image2.png)  
**Figure 2.** Total length of the 3 longest sprout shoots of black cherry collected in the 2015 vegetation season – quartile method. Sprout generation response of trees cut or girdled: a – in early spring, with three-time removal of sprouts; b – in late spring, with two-time removal of sprouts; c – in the summer, with a one-time removal of sprouts. A = basal cut-stump, B = stem cut at the height of 1m above the ground, C = stem girdling; S = Sieraków plot, L = Lipków plot
girdled in the summer failed to generate sprouts (100% for Sieraków, 96% for Lipków) in the given vegetation season. High cuts usually (in 5 per 6 cases) resulted in generation of a smaller number of sprouts in comparison to the control basal cut-stump; however, the differences in mean values ranging between 15% and 38% were not statistically significant (Fig. 1).

Also, regarding the total length of three longest sprout shoots, girdling proved to most effectively limit the generative capacities of black cherry at all given dates of application of the procedure, on both study plots (Fig. 2) (early spring treatment $H = 107.5, p = 1.366E-21$, late spring treatment $H = 102.9, p = 1.025E-20$, summer treatment $H = 103.6, p = 5.024E-22$). The difference with the two other types of procedures was in all cases statistically significant at $p < 0.01$. Cutting stems at a height of 1 m limited the lengthwise growth of the shoots in comparison to the basal cut-stump procedure, and this result was strengthened by delaying the procedure. Differences were statistically significant in four out of six variants of the procedure (i.e., for early spring cuts in Lipków, for late spring cuts in Sieraków, and for the summer cuts on both study plots).

Girdling proved to be the procedure that most significantly limited the biomass of generated sprouts, regardless of the date of procedure (Fig. 3). Its effect differed statistically from that of the other two methods at $p < 0.01$. The dry mass of sprout shoots after girdling in early spring (Fig. 3a) was 13 times smaller than after basal cut-stump in the study plot in Sieraków (82.7 g on average after basal cut-stump and 6.5 g after girdling) and 25 times smaller in the study plot in Lipków (98.8 g on average after basal cut-stump and 3.9 g after girdling). The effectiveness of the basal cut-stump procedure and stem cutting at the height of 1 m above ground in early spring was similar to the analysed measure. However, as the application of procedure was delayed, the effectiveness of cutting at a height of 1 m rose. It gave a statistically significantly different result from the one obtained from basal cut-stump after the summer application of the procedures (Fig. 3c). For each date of the procedure and study plot, the mean values of the dry mass of sprouts after cutting at the height of 1 m were lower by 2% to 67% than the mean values obtained from the application of basal cut-stump.

The block effect did not occur in most analyses; that is, the differences in obtained values did not vary with relation to the given plot. A statistically significant difference between the results from Sieraków and Lipków was recorded in only 2 out of 27 cases (Fig. 2a, A and B treatment).

![Figure 3. Total dry mass of sprout shoots of black cherry collected in the 2015 vegetation season – quartile method. Sprout generation response of trees cut or girdled: a – in early spring, with three-time removal of sprouts; b – in late spring, with two-time removal of sprouts; c – in the summer, with a one-time removal of sprouts. A = basal cut-stump, B = stem cut at the height of 1 m above the ground, C = stem girdling; S = Sieraków plot, L = Lipków plot.](image-url)

**Dying of trees after the procedure of mechanical elimination**

The application of basal cut-stump and of a cut at the height of 1 m in early spring combined with triple removal of sprouts did not contribute to killing any speci-
mens of black cherry in the season when the procedure was carried out (Tab. 2). In the case of girdling, the symptoms of dying out (dead crown and lack of sprouts in the autumn control) were recorded in the case of every other tree in the Sieraków study plot and every fourth one in the Lipków study plot. A similar result was observed in the case of trees girdled in late spring, after double removal of sprouts – although in this case, there were more dead trees in the Lipków study plot than in the Sieraków one. In the case of the procedure carried out in summer, all trees treated with basal cut-stump and girdling survived in the first year of treatment, and only in the case of cutting at a height of 1 m did a part of the trees not sprout new shoots.

Table 2. Effectiveness of mechanical elimination of black cherry in the Kampinos National Park represented by the percentage of trees dying in the year of application of a treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Location</th>
<th>Dead trees* (%)</th>
<th>Date of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Early spring</td>
</tr>
<tr>
<td>Basal cut-stump</td>
<td>Sieraków</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Lipków</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cut at a height of 1m</td>
<td>Sieraków</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Lipków</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Girdling</td>
<td>Sieraków</td>
<td>52</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Lipków</td>
<td>24</td>
<td>44</td>
</tr>
</tbody>
</table>

* The trees that did not generate sprouts by the date of last removal (i.e., September 20th) in the 2015 vegetation season; in the case of girdled trees, their crowns were also dead.

**Discussion**

Is girdling an effective method of eliminating black cherry in the conditions of central Poland?

Each of the mechanical procedures of eliminating black cherry tested in this study stimulated the awakening of dormant buds and growth of sprout shoots in trees. The degree of the response depended on the type of activity and was noticeably and statistically significantly lowest in the case of girdled trees. The trees girdled in early spring generated about 4 times less sprout shoots than the trees cut at the same date. Reduction of sprouting capacity assessed by measurement of the three longest shoots and of the dry mass of shoots was significantly higher in the case of girdled trees. However, sprout shoots were present among all trees, and when they remained, the possibility that dead crown will be substituted by young shoots needed to be taken into consideration.

The trees girdled in early spring produced living leaves, even though cambium and phloem were cut in the phase when buds break (at the beginning of vegetation season). The first symptoms of the crowns’ dying were recorded in summer, and in autumn, the crowns of 62% of the trees were dead (mean from two study plots), and 20% suffered from decreased vitality. The high vitality of tree crowns throughout most of the vegetation season probably had a limiting impact on the sprouting capacity of stems below the girdle. The crowns, remaining after girdling, limited the penetration of sun to the damaged stem, while on the contrary, the cutting procedures created a clearing that allowed much more light into the bottom of the forest. Several studies show that black cherry reacts with intense growth to light supply (among other, Husch 1954; Starfinger 1997; Closset-Kopp et al. 2007). Therefore, a possibility of delayed sprouting response due to the awakening of dormant buds in the next vegetation season needs to be taken into consideration. Therefore, the experiment requires continuation, and the final conclusions will be possible only after another vegetation season. The need for caution in assessing the effectiveness of procedures in the first year was pointed out by Van den Meerschaut and Lust (1997).

In autumn of first year, the degree of dying out of trees girdled in early and late spring reached 24–52% depending on the study plot and date of procedure (Tab. 2). In response to the question stated in the objectives of this study, it must be said that based on the results obtained, girdling is not a completely effective method of eliminating black cherry; however, it is much more effective than basal cut-stump or cutting at a height of 1 m above the ground.

A high effectiveness of stem girdling the black cherry in comparison to felling and to a procedure that consisted of bending the stem combined with snapping the stem to cause an incomplete break of the cambium at a height of ca 80 cm was recorded in an experiment carried out in northern Italy (Annighöfer et al. 2012). After the vegetation season, the girdled trees
is cut-stump and girdling an efficient method of black cherry Prunus serotina Ehrh. eradication?

did not differ from the control trees (not subjected to any procedure) with regard to the size and mass weight of the sprout shoots, but the number of sprouts was similar (between ten and twenty on average) to that of trees subjected to other procedures. Also, a comparison of effectiveness of chemical, biological and mechanical methods in an experiment carried out in Belgium showed that girdling brings the best results. With regard to the dates and number of additional procedures consisting of removal of sprouts, the percentage of dead trees in the second year of the experiment was between 35% and 90% (Van den Meersschaut and Lust 1997). These authors recommended girdling for application in environmentally sensitive areas (in nature reserves) where it is forbidden to use more effective chemical or biological methods. Some practitioners have expressed different opinions: based on active protection activities carried out in the Roztocze National Park, they assessed the girdling procedure as possessing low effectiveness, due to abundant sprouting below the girdle, high labour intensiveness and high requirements regarding the execution of girdle (Tittenbrun and Radliński 2015).

Does application of cutting at a height of 1 m above the ground limit sprouting capacity of black cherry?

To limit the regeneration of black cherry, cutting stems at a height of 0.5–1 m above the ground has been tested in practical activities (Krzyztofiak and Krzyztofiak 2015; Tittenbrun and Radliński 2015). Cutting at a height of 1 m followed by systematic removal of sprouting shoots every couple of years is also practiced in the Kampinos National Park, on ca 1,000 ha of forest of the Rózin division (Namura-Ochalska and Borowa 2015). Activities consisting of systematically disturbing the black cherry to inhibit its growth, result in dying out of a part of the trees and radically reduce its generative breeding (Namura-Ochalska and Borowa 2015; Tittenbrun and Radliński 2015).

In this experiment, we raised the question of whether the application of cutting at a height of 1 m above the ground significantly reduces the sprouting response of affected trees. The obtained results show this procedure to demonstrate a slight reducing impact in comparison to basal cut-stump. A lack of statistically significant differences was seen for the number of sprout shoots at all three dates of procedures and for both study plots (Fig. 1). The reduction of dry mass of shoots was significant only for the summer cut (Fig. 2). High cutting had a reducing impact on the index of total length of three longest shoots; the difference was significant for four out of six combinations of the experiment (Fig. 3). The highest effectiveness of cutting at a height of 1 m – expressed both by the three parameters of sprouting capacity as well as by percentage participation of dead trees – was obtained only for the summer procedure. Further study will show whether this result will be maintained in the next vegetation season.

A slightly higher effectiveness of cutting at the height of 0.5–1 m than ours was obtained in Belgium. As a result of the procedure carried out in autumn with single or two-time removal of sprouts, a mortality of 22% and 31% was obtained, which then grew to 48% and 55% in the second year of experiment (Van den Meersschaut and Lust 1997). A different result from ours was obtained in a comparison of sprouting capacity of the three species: Acer spicatum Lam. (mountain maple), Betula papyrifera Marsh. (paper birch) and Prunus pensylvanica L. (pin cherry), cut at four heights: 0, 15, 45, and 75 cm (Jobidon 1997). After 2 years, the higher-cut stems had significantly more sprouts; however, these differences decreased with the following season of sprout generation and after 10 years, they ceased to be significant. That experiment, in contrast to our study, focused on much younger trees aged only three years.

Practitioners point out the convenience of distribution of sprouts in the higher part of damaged stem, from the perspective of enabling subsequent cuts. The study carried out here supports that opinion. Although sprouts were most frequently distributed along the whole of stem, they were most numerous and reached the greatest sizes in the higher parts of stems (field observation).

Impact of cutting and sprout removal on stimulating the regeneration of black cherry

The results obtained confirm known studies showing a strong sprouting response by black cherry to cutting. This undermines the reasons for its application as a method of limiting this species’ invasion (among others, Marquis 1990; Starfinger et al. 2003; Halare-
The repeated (three times per season) removal of sprouts was aimed at causing the trees to die out, as had taken place in the experiment carried out in Belgium (Van den Meerschaut and Lust 1997). The application of 2–3 additional procedures for two following years also resulted in high mortality of cut trees in studies carried out by Van der Kruis (1990 after Van den Meerschaut and Lust 1997). However, in the first vegetation season of our experiment, no specimen cut in early spring at the base or at a height of 1 m died. Sprout removal contributed, on the other hand, to the awakening of dormant buds and growth of sprouts. The highest number of sprout shoots was recorded during the first control, while the number dropped with the repetition of procedure. The method of carrying out the experiment made it possible to take into account the measurements of all sprouts, while leaving sprouts without stripping leads to natural elimination of some of them (Annighöfer et al. 2012). In Italy, black cherries similar to those in our experiment, growing under the canopy and characterised by a similar girth, produced on average 36 sprouts (+- SD 21) after one year from cutting (Annighöfer et al. 2012), while we obtained a total of on average 103 sprouts (+- SD 52) from three controls in the Sieraków study plot and on average 99 sprouts (+- SD 34) in the Lipków study plot (Fig. 1a).

Due to a very strong sprouting response by black cherry to mechanical damage, it is necessary to consider extraction and removal of the root. This procedure brings noticeably better results, but it is much more time-consuming and difficult to carry out in vast areas. The introduction of indigenous tree species as competition for black cherry is also an effective method of limiting the occurrence of this neophyte (Starfinger et al. 2003; Otręba et al. 2014; Namura-Ochalska and Borowa 2015).

Conclusions

In the habitat conditions of central Poland, stem-girdling of black cherry has proven to be a significantly more effective method of elimination than basal cut-stump or cutting at a height of 1 m above the ground. Nonetheless, in the season of applying the procedure, even with additional procedures of sprout removal, only a part of the girdled trees died out (24–52%). Thus, even the procedure of girdling was not effective in the first year of experiment. The procedure of stem cutting at a height of 1 m from the ground only partially limited the sprouting response of the trees. The number of sprouts on the trees cut at the base and at a height of 1 m did not differ statistically significantly. High cutting, on the other hand, did limit the total length of the three strongest shoots. Despite application of additional procedures of removal of sprouts, all trees cut in early spring maintained vitality in autumn of the first year of experiment. For all tested procedures, it is possible to observe an increase in their effectiveness in limiting sprouting capacity (to 100% in the case of girdling) with a later date of applying the procedure.

The number of repetitions of the sprout-shoot removal procedure needed to cause complete death (and in consequence, elimination) of a tree is an important piece of information for use in practical elimination of the black cherry. However, observations carried out in one vegetation season cannot be treated as conclusive. The continuation of experiment in the following year is necessary for a complete and reliable assessment of the effectiveness of both the tested methods, as well as the dates of their application and the number of repetitions. Only then will it be possible to evaluate the actual effectiveness of applied procedures of mechanical elimination of black cherry in the Kampinos National Park.

Due to the high sprouting response by black cherry to mechanical damage, in areas of environmental value it seems worthwhile to consider application of more time-consuming and costly methods, such as extraction and root removal, as well as the planting of competitive indigenous species with the goal of eliminating black cherry.

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REFERENCES


Najberek K., Solarz W. 2011. Biological invasions in the Polish national and landscape parks (in Polish


Stanisza A. 2006. An accessible course in statistics with the application of STATISTICA PL with examples in medicine (in Polish). Statsoft, Poland.


Starfinger U., Kowarik I., Rode M., Schepker H. 2003. From desirable ornamental plant to pest to accepted additional to the flora? — the perception of alien tree species through the centuries. Biological Invasions, 5, 323–335.


Tokarska-Guzik B. 2005. The establishment and spread of alien plant species (kenophytes) in the flora of Poland. Wydawnictwo Uniwersytetu Śląskiego, Katowice, Poland.


Response of the callus cells of fir (Abies nordmanniana) to in vitro heavy metal stress

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ABSTRACT

The aim of the presented research was to investigate the effect of three heavy metals – lead, cadmium and copper – on the callus cells of Abies nordmanniana. The toxicity degree and toxicity effect of the selected heavy metals was determined on the embryonic level. On the basis of the spectrometric analyses as well as macroscopic and microscopic observations, this research referred to the accumulation of heavy metals in tissues, assuming that this mechanism is related to the acquisition of tolerance by cells exposed to this type of abiotic stress. Moreover, the effect of the genotype of fir on the cell defence, that is, the induction of tolerance, was analysed. Understanding of the issues related to the heavy metal resistance of plant genotypes in future may contribute to the selection of genotypes of individuals that are more resistant to stress factors, particularly in the multi-directional and rational forest management. The results showed that lead (20 mg l⁻¹), which proved to be the most toxic amongst the three examined heavy metals, has the most severe negative effects on the tissue of fir trees. Copper (20 mg l⁻¹) was accumulated for a long time in the cells of fir trees, and it was not degraded or excreted outside the tissues even after three weeks of in vitro culture. Of the three tested genotypes, G14 had the greatest tendency to accumulate each of the examined metals, that is, it appeared to be the least tolerant genotype.

KEY WORDS

Abies, abiotic stress, callus, embryogenic suspension culture (ESC), metals, somatic embryogenesis

INTRODUCTION

Environmental pollution with heavy metals has increased greatly because of the rapid worldwide industrial development. Studies carried out on this subject include the effect of metals on humans, animals, and plants. Analyses of the effect of heavy metals on plants, including forest trees, were conducted by Lovrenecic et al. (2008), Szymura (2009) and Zacchini et al. (2009) and the problem was discussed by Maksymiec (2007). Some studies also include the research on callus tissues carried out on the cellular level. Those studies allowed to understand the in vitro effect of various heavy metals on plant tissues including cell tolerance and the possibility of further cell growth (Israr et al. 2006; Nehnevajova et al. 2007; Iori et al. 2012). This issue was investigated in details in the presented study, which analysed the accumulation of copper, lead and cadmium in the cells of three genotypes of embryogenic callus of Abies nordmanniana (Steven) Spach. The in vitro culture and...
spectrometry allowed to solve three problems: the toxicity effect of the examined heavy metals on fir tissues, induction of stress tolerance in different genotypes and the effect of the time of metal accumulation on further organogenetic growth and development.

Caucasian fir is one of the most important species grown mostly in Christmas tree plantations, but its in vitro cultured embryogenic callus has become, because of this work, a point of reference for studies on the effect of heavy metals on gymnosperm trees, mainly from the genus *Abies*. The analyses included three heavy metals from two different groups, that is, metals necessary for proper growth and development of trees (Cu) and toxic metals (Cd and Pb). It needs to be pointed that only some heavy metals (i.e., those whose specific gravity of 1 cm³ is lower than 5 g) present in the environment are necessary for proper functioning of plant cells. These metals include copper (Cu), iron (Fe), zinc (Zn), manganese (Mn), cobalt (Co) and nickel (Ni). The remaining heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg) and aluminium (Al) are not used in cellular metabolism and can be toxic for plants even in very small quantities (Page et al. 2006).

Despite many years of studies on the reactions of plant cells to the impact of heavy metals, still several issues remain unexplained, mainly due to the fact that so far there is no detailed study on the biochemical changes caused by heavy metals in plant cells. There are both extensively studied issues, such as metal transport through membranes, heavy metal detoxification, oxidative stress and its neutralisation and those for which the available literature is scarce, such as the impact of metals on nucleic acids (Nowakowska and Oliver 2013; Woźni and Przybył 2004; Williams et al. 2000).

**Material and Methods**

**Plant material**

Mature open pollinated seeds of Caucasian fir (*A. nordmanniana* (Steven) Spach) acquired in the seed year 2009 were derived from Vallø region on the east coast of the island of Zealand in eastern Denmark (E: 55°24'; N: 12°15'), from seed plantations marked with numbers 13 and 19. Seeds of *A. nordmanniana* were subjected to a two-stage, 24-h disinfection process with the use of ethyl alcohol (30 s), 10% sodium hypochlorite (NaOCl) for 15 min, thorough rinsing in deionised water and polyvinylpyrrolidone water solution (PVP) (Sigma-Aldrich) for 24 h at 7 ± 1°C, before again rinsing in five changes of sterile deionised water in laminar flow cabinet (Nawrot-Chorabik 2016).

Embryogenic callus (ESC, embryogenic suspension culture) was initiated by somatic embryogenesis on zygotic embryos isolated from mature seeds of Caucasian fir.

The analyses of the impact of heavy metal were conducted on three selected genotypes of embryogenic callus (cell lines) characterised by a high proliferation rate, which were marked as G2, G14 and G21. About 300 mg of callus tissue of each genotype was placed in triplicate on Petri dishes with modified solid Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt 1972) containing 3.2 mM benzyladenine, 4.6 mM kinetin, 4.5 mM 2,4-dichlorophenoxyacetic acid and 2.0% sucrose. Heavy metals were added to the medium, composed as described above, and the final concentration of each heavy metal was 20 mg l⁻¹. Copper was used in the form of CuSO₄ × 5H₂O, lead in the form of the standard solution of Pb(NO₃)₂ in 2% HNO₃ and cadmium in the form of the standard solution of Cd(NO₃)₂ in 2% HNO₃. The standard solutions were purchased from ULTRA Scientific. Heavy metals were not added into the media in control combinations. pH of the medium was set at 5.7. All reagents used for *in vitro* culture and spectrometric analyses were used from Sigma-Aldrich company.

The *in vitro* culture was conducted in the dark in a phytotron chamber at 24°C and 50% humidity.

**Spectrometric analysis**

After one, two and three weeks of culture, the calluses of Caucasian fir were collected from the medium and covered with 10 cm³ of concentrated nitric acid (HNO₃).
The obtained partially digested suspension–solution was digested for 60 min in Microvela mineraliser in a microwave field until complete digestion. The wet-digested solution was transferred to a 50-cm³ volumetric flask, and the content of individual heavy metals in subsequent samples was determined in the inductively coupled argon plasma emission spectrometer iCAP 6000 series (Thermo Scientific, MA, USA). The control callus tissue (cultured in vitro on a medium without heavy metals) was also subjected to spectrometric analysis. The measurements were carried out by using a two-dimensional CID (charge injection semiconductor detector), which allowed for carrying out emission measurements of all elements present in the samples simultaneously with the synchronous recording of all necessary emission lines.

**Statistical analysis**
Statistical analysis of data was conducted using two tests: the Tukey multiple comparison test preceded by the F-test – one-way analysis of variance (ANOVA) – as well as the Tukey multiple comparison test preceded by the Friedman test for repeated measure systems (Tab. 1–3). Because significant results of the F-test (analysis of variance) do not inform us between which of the examined metals there are differences (the test shows only which groups differ from each other); therefore, further multiple ‘post-hoc’ comparisons were conducted in order to determine between which metals there are statistically significant differences at the 0.05 significance level. These tests allowed to draw the objective conclusions from the resulting numerical data. All statistical analyses were conducted using STATISTICA version 7.1 (StatSoft Inc., Tulsa, OK, USA).

**RESULTS**
Answers to the following questions were obtained: which of the selected heavy metals (Cu, Pb and Cd) is the most harmful to the cells of fir, whether there is the effect of the plant tissue genotype on the tolerance level to stress caused by heavy metals and whether the time of metal accumulation in plant cells (one, two and three weeks) induces their tolerance to this stress factor.

The results of this research indicated that lead has the strongest negative effects on the tissue of fir. The concentration of lead in cells is greater than that of the remaining metals (copper and cadmium). The difference between the mean content of copper and cadmium is statistically insignificant, but it is statistically significant in the case of lead (Tab. 1).

**Table 1. Statistical analysis of data – multiple comparisons test (Tukey) preceded by the F-test of one-way ANOVA**

<table>
<thead>
<tr>
<th>Metal</th>
<th>Average metal content</th>
<th>Result of ANOVA test</th>
<th>Probability p in multiple comparisons test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>0.123146</td>
<td>F = 5.574</td>
<td>0.0055*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.0055*</td>
<td></td>
</tr>
<tr>
<td>Cadmium</td>
<td>0.124294</td>
<td>0.9976</td>
<td>0.01506*</td>
</tr>
<tr>
<td>Lead</td>
<td>0.173593</td>
<td>0.0125*</td>
<td>0.01506*</td>
</tr>
</tbody>
</table>

* Statistically significant difference at the level of α = 0.05.

On the basis of the results of spectrometric analyses, the statistical analysis was performed for each of the elements separately and showed that the genotypes 2 and 21 are statistically different from the genotype 14. This genotype had the greatest tendency to accumulation of each of the metals, which means that it is the weakest genotype. Genotypes 2 and 21 were resistant to all tested metals (Tab. 2). The values of accumulation in their cells had a downward trend (Fig. 1).

**Table 2. The effect of the genotype of A. nordmanniana callus on the accumulation of copper, lead and cadmium confirmed by the statistical analysis of data**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average metal content</th>
<th>Friedman’s test result</th>
<th>Probability p in multiple comparisons test</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2</td>
<td>0.1116</td>
<td>X² = 12.667</td>
<td>0.0053*</td>
</tr>
<tr>
<td>G14</td>
<td>0.16537</td>
<td>p = 0.0018*</td>
<td>0.0005*</td>
</tr>
<tr>
<td>G21</td>
<td>0.09240</td>
<td></td>
<td>0.4003</td>
</tr>
</tbody>
</table>
It was shown that only copper (CuSO₄ × 5H₂O) showed an increase in the accumulation from one to three weeks. Statistical significance of differences between week 1 and 3 was proved by the Tukey test (Tab. 3). The presence of other examined heavy metals induced tolerance to this stress factor.

Table 3. The effect of time of accumulation of copper, lead and cadmium on the tolerance induction in the cells of A. nordmanniana confirmed by the statistical analysis of data

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Time (week)</th>
<th>Average metal content</th>
<th>Friedman's test result</th>
<th>Probability p in multiple comparisons test</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2</td>
<td>1</td>
<td>0.09684</td>
<td>( \chi^2 = 9.556 ) ( p = 0.0048^* )</td>
<td>0.1732, 0.0094*</td>
</tr>
<tr>
<td>G14</td>
<td>2</td>
<td>0.12496</td>
<td></td>
<td>0.1732, 0.3059</td>
</tr>
<tr>
<td>G21</td>
<td>3</td>
<td>0.14764</td>
<td></td>
<td>0.0094*, 0.3059</td>
</tr>
<tr>
<td>G2</td>
<td>1</td>
<td>0.10791</td>
<td>( \chi^2 = 9.556 ) ( p = 0.0062^* )</td>
<td>No statistically significant differences</td>
</tr>
<tr>
<td>G14</td>
<td>2</td>
<td>0.12790</td>
<td></td>
<td>No statistically significant differences</td>
</tr>
<tr>
<td>G21</td>
<td>3</td>
<td>0.13707</td>
<td></td>
<td>No statistically significant differences</td>
</tr>
<tr>
<td>G2</td>
<td>1</td>
<td>0.14888</td>
<td>( \chi^2 = 0.697 ) ( p = 0.7165 )</td>
<td>No statistically significant differences</td>
</tr>
<tr>
<td>G14</td>
<td>2</td>
<td>0.17217</td>
<td></td>
<td>No statistically significant differences</td>
</tr>
<tr>
<td>G21</td>
<td>3</td>
<td>0.19973</td>
<td></td>
<td>No statistically significant differences</td>
</tr>
</tbody>
</table>

* Statistically significant difference at the level of \( \alpha = 0.05 \).

In conclusion, the results of spectrophotometric analysis and statistical analysis of data indicated the effect of heavy metals – copper, cadmium and lead – which is reflected in the morphological changes of the callus tissue. The final effect was evidenced by the transformations in the phenotype of embryogenic callus mass as a wound tissue, expressed as the gradual die-
Response of the callus cells of fir (Abies nordmanniana) to in vitro heavy metal stress

back of its cells. These changes resulted from negative, organogenetic transformations of cells, whose metabolism was disturbed by the introduction of heavy metals into the growth medium (Fig. 2). On the same type of medium in the case of cadmium (Fig. 2C) and lead (Fig. 2D), the tissue was dehydrated, was compact and did not undergo further morphogenesis. There were no green fragments, that is, those containing chlorophyll, which indicated a deficit of new cells, whilst in the existing cells, the synthesis of this pigment was inhibited.

Figure 2. Organogenetic changes in the callus of Abies nordmanniana caused by heavy metals after three weeks of in vitro culture on SH medium: A – control embryogenic callus (heavy metal-free medium); B – callus with somatic embryos on medium containing 20 mg l−1 copper; C – darkening callus with necrotic changes, not producing embryos on medium containing 20 mg l−1 cadmium; D – dead, dehydrated callus with characteristic necrotic changes on medium containing 20 mg l−1 lead; bars indicate 5.0 mm
After a few days, visible necroses and leakage of mucilaginous substance were recorded, which caused the death of the callus (Fig. 1C, D). Only in the case of copper (Fig. 2B), the necroses were fragmentary and only a small percentage of somatic embryos died. Although the callus tissue was slightly dehydrated compared to the control callus, it remained alive (Fig. 2A).

**Discussion**

This study presents the results and conclusions from three areas of research on heavy metals on the embryonic level. The numerical data obtained from the spectrometric analysis, illustrated in the graphs, showed the relationships between different metals and genotypes (Fig. 2). Macroscopic and microscopic observations of phenotypic changes in the callus tissue of *A. nordmanniana* and finally the statistical analysis of the obtained data confirmed the final conclusion: lead (20 mg l\(^{-1}\)) has the most toxic effects on the tissue of fir (Fig. 2D; Tab. 1–3). The results obtained in this study indicate that lead proved to have the strongest negative impact on the tissue of fir. Its concentration in plant cells exceeded the concentration of the other tested heavy metals (copper and cadmium). What is more, the difference between the mean content of copper and cadmium is statistically insignificant, whilst it is statistically significant between lead and the remaining metals (Tab. 1). Maksymiec (2007) emphasises that the effect of toxic lead influence on plants is strong and fast inhibition of growth processes of the above- and underground parts, as well as the decrease in the activity of the photosynthetic apparatus, often correlated with progressing senescence processes. The *in vitro* studies on the effect of lead were conducted by numerous authors, amongst others, Agrawal and Sharma (2006) on the shoots of *Holarrhena antidysenterica* flowering plant of the genus *Wrightia* based on the concomitant changes in protein. They concluded that the rate of inhibition in morphogenesis was in the order of Cd > Pb > Cu > Zn. Lead, being the second most inhibitive metal, was responsible for phytotoxic effects of *H. antidysenterica* on *in vitro* regeneration. As shown in the literature, lead deposits occupy a significant part of vacuoles and, in extreme cases, fill it almost entirely. Lead causes disturbances in cellular metabolism, which is manifested in the form of intensive vacuolisation, concentric endoplasmic reticulum, lobed nucleus and numerous multivesicular bodies present in the cell (Woźni and Przybył 2004). The presented studies on this metal emphasise the significant role of lead in the cellular morphogenesis based on the example of callus of *A. nordmanniana*. Callus treated with lead died after 30 days, which was particularly evident in the genotype 14. It was shown that the addition of 20 mg l\(^{-1}\) of this metal to the medium was toxic to cells of fir. Strong necrotic changes, rapid dehydration of cells and no induction of somatic embryos on the callus were observed microscopically (Fig. 2D).

Toxic effects of metal ions can be observed not only in the ultrastructure of cells but also on a molecular level, that is, in the expression or inactivation of certain genes. Changes in the DNA have further consequences, such as robust transcription or biosynthesis of some proteins, and cause induced imbalances in activated oxygen metabolism as well as antioxidant enzymes (Schröder et al. 2003). Analysing of those mechanisms is legitimate but not always necessary to obtain answers to some questions.

In *Brassica juncea*, studies were conducted on the accumulation of lead, cadmium and zinc in *in vitro* cultures, with regard to somaclonal variation. In this research, the emphasis was put on shoot regeneration with callus. It appeared that whilst more than 90% of hypocotyls formed callus on the control medium, only 50% of all hypocotyl explants formed callus in the presence of 40 µM Cd. The best regenerants were chosen under hydroponic conditions, which, after selection, could be used for the purposes of phytoremediation (Nehnevajova et al. 2007).

This shows that research on heavy metals is multi-level and multi-directional; however, there are few studies conducted on trees in which the effect of genotype on the tolerance to heavy metals was verified. Therefore, this problem was addressed in the presented study, and it has become undoubtedly one of the most important aspects of this research. Solution to the issue related to the genotypes resistant to heavy metals in future could contribute to the selection of genotypes of individuals more or less resistant to stress factors, especially with the assumption of multi-directional and rational forest management.

The statistical analysis, based on the results of spectrometric measurements, indicated that the genotypes 2 and 21 are statistically different from the genotype 14.
This genotype accumulated the greatest amounts of each of the analysed metals, indicating that this is the weakest genotype. In contrast, genotypes 2 and 21 were resistant to all of the examined metals (Tab. 2). The values of accumulation in their cells had a downward trend (Fig. 1). Probably those genotypes formed defence strategies against stress factors allowing either to avoid stress or to tolerate it. The first strategy involves the development and launching of the cell mechanisms that prevent or hinder the metal ion transgression of a barrier formed by the cellular membrane, that is, to prevent their entry into the protoplast. The second – and more likely – strategy is based on the development and launching of mechanisms inside the cell that neutralise the metal ions and the corrective mechanisms that remove the damage caused by the heavy metals. Therefore, the second strategy allows for the growth and development of a cell after the penetration of metal into the protoplast, which was noticed in the case of the genotypes 2 and 21 (Fig. 2).

Another aspect addressed in this study concerned the time of accumulation of copper, lead and cadmium in the cells of A. nordmanniana. It was demonstrated that amongst the tested metals, only the accumulation of copper increased from one to three weeks. The Tukey test confirmed the statistical significance of differences in the concentrations between week 1 and 3 (Tab. 3). The tolerance to this stressor was generated by the presence of other examined heavy metals. Copper is treated as an essential microelement for living organisms, and it is a relatively low toxic metal. Unlike other heavy metals, it is accumulated in chloroplasts and vesicles of various origin (endoplasmic reticulum (ER) and Golgi apparatus (GA)). This metal, together with iron, is one of the elements that participate in the redox processes. Spectrometric analyses indicate that too high concentrations of copper (20 mg l\(^{-1}\)) can cause negative effects in plant cells. This can possibly result from the generation of a large excess of reactive oxygen species (ROS) and free radicals (FR), which ultimately leads to oxidative stress in a cell, that is, a situation in which more ROS and FR are produced than metabolised. Copper in low concentrations is well metabolised by plants and its high concentrations result in long-term accumulation of this element in plant tissues and thus the inhibition of growth and development and adverse changes in organogenetic metabolism. The reason for long-term accumulation of copper can be either the mentioned oxidative stress or the fact that copper is transported not to vacuoles but to ER and GA, and the mechanism of disposal of copper from the cells has not been fully elucidated yet. This may also be caused by the transport proteins, but this hypothesis requires further advanced research leading to the identification of such proteins. Similar to the presented study, Agrawal and Sharma (2006), in their studies conducted on medicinal herbaceous species H. antidysenterica, analysed the toxicity effect of different metals, including copper. The conclusions were drawn based on the protein analysis carried out by sodium dodecyl sulphate polyacrylamide gel electrophoresis. These authors showed that two new peptides were synthesised (29 and 20 kDA) in response to both copper and zinc. In their research, low concentration of copper (1 mg l\(^{-1}\)) resulted in good organogenesis in shoots of H. antidysenterica. On the other hand, in higher concentrations of copper, the induction of multiple shoots buds was successful but the growth was limited. Whilst zinc in vivo was the only metal with concentrations significantly higher in the leaves than that in the root and stem (Milić et al. 2012). Patterson III and Olson (1983) studied the effect of heavy metals on variety of substrates. Copper, nickel and cobalt solutions were added to a filter paper, mineral soil and organic soil substrates to test the effects of these metals on the germination and radicle elongation of woody species to eastern North America. For all species, toxicity followed the pattern Ni > Cu > Co for filter paper and Ni > Co > Cu for mineral and organic soils. Deciduous species were more readily damaged by these metals than the coniferous species. This study carried out on the medium as a substrate broaden the knowledge of, amongst others, copper and cadmium in the species of gymnosperms. It turns out that these metals more adversely affect the tissue of trees.

To summarise, it needs to be emphasised that in vitro culture is considered to be a valuable tool in micropropagation of trees (Pospišilová et al. 1999; Nawrot-Chorabik 2008), genetic studies of, for example, somaclonal variation (Nawrot-Chorabik 2009) and cryopreservation (Charne et al. 1988), as well as in biotechnology in, for example, studies on pathogenicity on embryonic level in dual cultures (Nawrot-Chorabik 2014; Nawrot-Chorabik et al. 2016; Sieber et al. 1990; Vookova et al. 2006). Moreover, as evidenced by the presented paper,
the in vitro culture can also be used in the selection of plant material being tolerant to stress factors, such as toxic concentration of heavy metals. The latter application is significant especially in the case of woody species characterised by long reproductive cycles.

**Conclusion**

The toxicity degree and toxicity effect of the selected heavy metals were determined on the embryonic level – lead (20 mg l⁻¹) has the most toxic effects on the fir tissue.

There are genotypes of trees that are resistant to the effects of heavy metals, and there are also those genotypes that do not show any resistance.

The in vitro culture can be used in the selection of plant material being tolerant to stress factors, such as toxic concentration of heavy metals.

It should be cultured in vitro the callus genotypes that are resistant to heavy metals in future that could contribute to the selection of genotypes of individuals more or less resistant to stress factors, especially with the assumption of multi-directional and rational forest management.

The results of these studies can be useful to solve the problems of tolerance to stress, the effect of heavy metals on plants at the cellular level, a point genotype plants in the immune response, remediation and so on.

**Acknowledgement**

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**References**


Nowakowska J., Oliver J.D. 2013. Resistance to environmental stresses by *Vibrio vulnificus* in the viable
but nonculturable state. *FEMS Microbiology ecology*, 84, 213–222.


Accumulation of heavy metals in needles and bark of *Pinus* species

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**Abstract**

During the research, cumulative properties of conifer needles *P. armandii*, *P. banksiana*, *P. mugo*, *P. nigra*, *P. sylvestris* and *P. wallichiana* in reference to Cu, Ni, Mn, Fe, Zn and Cd were analysed, and the factors which have an impact on the chemical composition of the bark of those species were identified. During the study, the age of needles and the content of the examined components in soil was taken into consideration. The content of metals in the needles varied, depending on a species and the age of the coniferous needles. In most cases, a higher level of content of those metals was determined in 2 years old needles, except for Cu and Zn (*P. banksiana*) as well as Zn (*P. nigra*), in which case, higher concentration of metals in 1 year old needles was determined. The obtained results indicate that the heavy metals’ concentration in the samples of needles was relatively low, except for Ni (*P. armandii*, *P. sylvestris*, *P. wallichiana*) and Cd (*P. armandii*, *P. banksiana*), which showed higher levels. Among the examined elements, Mn was accumulated in the largest volume in the needles of *P. banksiana*, Fe and Cu in the needles of *P. wallchiana*, Ni in the needles of *P. sylvestris*, Zn in the needles of *P. nigra* and Cd in the needles of *P. armandii*. The bark samples represented a clearly acidic reaction, with pH levels from 3.7 (*P. sylvestris*) to 4.9 (*P. armandii*). The highest quantities of Mn, Fe, Cu and Cd were accumulated by the bark of *P. armandii*, Ni in the bark of *P. sylvestris* and Cu in the bark of *P. mugo*.

**Key words**

heavy metals, needles, bark, *Pinus* species

**Introduction**

The genus *Pinus* is one of the most widely spread in the northern hemisphere. Its species appear both in natural forests and in many botanical gardens. There are 115 known species of trees and bushes from the *Pinus* genus all over the world. For many years, these species have been the object of scientific research as to morphological differences (Christensen and Dar 1997), survival rate of the plovers during the forest fires (Reyes
and Casal 2012), nutritional requirements (Parzych and Sobisz 2012), production and use of timber (Espelta et al. 2003), quantity and quality of the organic litterfall as a source of nutritional components (Astel et al. 2009), impact of the tree crowns on the properties of soil (Poláková et al. 2015), as well as monitoring research. In Poland and in many European countries, 1 year old needles and 2 years old needles of Pinus sylvestris and P. nigra as well as their bark are used for evaluation of the level of air pollution (Lamppu and Hutunen 2002; Yilmaz and Zengin 2003; Świercz 2006; Chrzan 2013; Parzych and Jonczak 2013, 2014). Tree needles absorb a series of chemical components that are necessary for supporting the vital processes, from soil and air (Migaszewski 1997). The intake of nutritional components by trees is regulated by metabolic processes. For adequate growth and development, in addition to micro-components, trees need adequate quantity of micro-components; this role is played to a large extent by heavy metals. However, excessive concentration of these elements in the environment is undesirable and harmful for most trees. As per Augusto et al. (2002), the species of coniferous trees absorb (about 35%) more components than leafy trees (25%) from the air due to a larger surface of the needles. The chemical composition of the needles is characterized by a specific variability resulting from the age of needles, a natural fertility of the habitat and the factors conditioning the intake of soil components. The bark of trees depending on the structure and porosity connected therewith, stops dusts and aerosols in the quantity proportional to its surface (Chrzan 2013). Pinus nigra, in comparison to other species of coniferous trees, shows high tolerance for changes of pH of the soil (Arsova 1999), while Pinus sylvestris to diversified abundance and moisture of the soil (Parzych and Jonczak 2013). Additionally, the artificial planting of some species of Pinus is practiced more often in order to limit erosion of the soil (Pusz et al. 2015), reconstruction of forests after fire (Espelta et al. 2003) and in order to improve the quality of degraded soils (Wójcik and Krzaklewski 2009).

The aim of this research was to compare the accumulative properties of needles of Pinus armandii Franch., Pinus banksiana Lamb., Pinus mugo Turra, Pinus nigra J.F. Arn., Pinus sylvestris L. and Pinus wallichiana A.B. Jacks. in relation to Cu, Ni, Mn, Fe, Zn and Cd and to identify the factors shaping the chemical composition of the bark of such species. In the research, the age of needles and the content of examined components in the soil were taken into account.

**Materials and methods**

**Research area**

The research was conducted within the area of the forest at the University botanic garden (48°45’ N, 21°19’ E) in Košice (Slovakia) in the summer season in 2015. The garden was established in 1950 and at present its area is 30 ha. It is situated at an altitude of 218 to 370 m a.s.l. From the northern side, it is surrounded by natural forests. The vital part of the Park area (24 ha) is covered by forest communities with a participation of the species of the genera such as: Pinus: P. armandii, P. banksiana, P. mugo, P. nigra, P. sylvestris and P. wallichiana. These species were introduced in order to increase the stabilization of the bottom. The tree stands selected for the research are 40 to 45 years old now. Their average height is from 6.0 m (P. mugo) to 19.9 m (P. sylvestris), and the average breast height is from 34.0 cm (P. mugo) to 105.0 cm (P. sylvestris) (Tab. 1). The tree stands grow in the shallow soils of cambisol type (Mochnacký 2001; Kebel and Koštálik 2011). The average annual air temperature over the examined area remains at the level of +8.4 °C, and the lowest temperatures are in January (−3.4 °C). The average annual volume of precipitation is 643 mm and comes in June.

**Table 1. Characteristic of examined stands**

<table>
<thead>
<tr>
<th>Species Code</th>
<th>Species Name</th>
<th>Average height [m]</th>
<th>Average dbh [cm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. arm</td>
<td>Pinus armandii</td>
<td>12.5</td>
<td>64.5</td>
</tr>
<tr>
<td>P. ban</td>
<td>Pinus banksiana</td>
<td>11.0</td>
<td>48.5</td>
</tr>
<tr>
<td>P. mag</td>
<td>Pinus mugo</td>
<td>6.0</td>
<td>34.0</td>
</tr>
<tr>
<td>P. nig</td>
<td>Pinus nigra</td>
<td>18.0</td>
<td>72.5</td>
</tr>
<tr>
<td>P. syl</td>
<td>Pinus sylvestris</td>
<td>19.9</td>
<td>105.0</td>
</tr>
<tr>
<td>P. wal</td>
<td>Pinus wallichiana</td>
<td>18.0</td>
<td>64.0</td>
</tr>
</tbody>
</table>
Sampling and analysis

5 trees, each of 6 species of Pinus (P. armandii, P. banksiana, P. mugo, P. nigra, P. sylvestris i P. wallichiana), were earmarked for the study (Tab. 1). Directly under the crowns of the trees, soil samples were taken for physical and chemical analysis from a depth of 0–20 cm, 20–40 cm and 40–60 cm. The samples were dried at a temperature of 65°C, ground in the mortar and sieved through the sieve (1 mm). In the soil, the organic matter content was marked by using the method of roasting in a muffle furnace at a temperature of 550°C and pH in a water solution in a weight proportion (1:2.5) by a potentiometer method. From each tree, samples of needles from the seventh whorl of weight 10–20 g were taken for the tests, separating the 1 year old needles from the 2 years old needles, as per the recommendations of ICP Manual Forest (Rautio et al. 2010). After their transport to the laboratory, the needles were carefully washed in deionized water to remove any particulate matter. From the trunks of the trees, at the breast height (1.3 m), samples of the external layer of bark were taken (about 10 g). The bark and the needles were dried in paper bags at a temperature of 65°C and were homogenized in a laboratory grinder (A11 IKA, Germany). The samples had been kept in tightly closed polyethylene bags till the time of analyses. In the samples of bark and needles, the pH was determined in a water solution in proportion 1:10 by means of the potentiometer method. In order to determine the metallic elements, the soil samples (1.0 g) and the samples of needles (0.5 g) and bark (0.5 g) were mineralized in the solutions of 65% HNO₃ and 30% H₂O₂ in order to obtain clear and colourless solution. The samples with soil were drained. Then all the samples were supplemented with deionized water (Hydrolab, HLP 10, Poland) to a volume of 50 ml.

Table 2. The median physicochemical properties of the soil samples (±standard deviation) taken under trees of Pinus species with Kruskal–Wallis test results

<table>
<thead>
<tr>
<th></th>
<th>Depth [m]</th>
<th>Pinus armandii</th>
<th>Pinus banksiana</th>
<th>Pinus mugo</th>
<th>Pinus nigra</th>
<th>Pinus sylvestris</th>
<th>Pinus wallichiana</th>
<th>Kruskal–Wallis test</th>
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<td></td>
<td></td>
<td>pH</td>
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<td>0.0–0.2</td>
<td>0.2–0.4</td>
<td>0.4–0.6</td>
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<td></td>
<td>6.4 ± 0.2</td>
<td>6.2 ± 0.2</td>
<td>6.0 ± 0.3</td>
<td>6.1 ± 0.1</td>
<td>6.2 ± 0.1</td>
<td>6.0 ± 0.3</td>
<td>6.4 ± 0.1</td>
<td>12.6291</td>
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<td>0.6 ± 0.4</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.4</td>
<td>0.5 ± 0.1</td>
<td>15.3873</td>
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<td></td>
<td>0.0–0.2</td>
<td>0.2–0.4</td>
<td>0.4–0.6</td>
<td>0.1 ± 1.2</td>
<td>0.2 ± 1.2</td>
<td>0.1 ± 1.2</td>
<td>0.0 ± 1.0</td>
<td>15.0151</td>
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<td>OM %</td>
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<td></td>
<td>554.6 ± 12.3</td>
<td>549.5 ± 10.5</td>
<td>571.0 ± 9.2</td>
<td>564.9 ± 19.6</td>
<td>561.4 ± 9.9</td>
<td>500.1 ± 36.9</td>
<td>640.3 ± 15.3</td>
<td>16.4966</td>
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<td>Mn</td>
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<td></td>
<td>350.30 ± 1037</td>
<td>352.25 ± 842</td>
<td>369.40 ± 916</td>
<td>364.0 ± 24.3</td>
<td>360.4 ± 16.3</td>
<td>374.5 ± 14.39</td>
<td>360.4 ± 16.82</td>
<td>13.9537</td>
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<td>Fe</td>
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<td></td>
<td>32.3 ± 0.9</td>
<td>31.0 ± 1.0</td>
<td>32.6 ± 0.8</td>
<td>32.3 ± 0.8</td>
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<td>35.8 ± 1.0</td>
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<td>Cu mg/kg</td>
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<td></td>
<td>35.2 ± 4.5</td>
<td>29.2 ± 4.3</td>
<td>35.7 ± 3.2</td>
<td>42.7 ± 4.4</td>
<td>52.7 ± 2.4</td>
<td>76.8 ± 5.0</td>
<td>73.6 ± 6.7</td>
<td>15.7368</td>
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<td>Ni</td>
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<td></td>
<td>84.8 ± 6.4</td>
<td>71.4 ± 5.3</td>
<td>72.7 ± 2.7</td>
<td>74.5 ± 7.2</td>
<td>67.3 ± 4.2</td>
<td>84.5 ± 4.5</td>
<td>68.1 ± 7.1</td>
<td>15.2151</td>
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<td>Zn</td>
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<tr>
<td></td>
<td>1.7 ± 0.7</td>
<td>1.5 ± 0.3</td>
<td>1.7 ± 0.2</td>
<td>0.7 ± 0.4</td>
<td>0.9 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.6</td>
<td>9.7185</td>
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<td>Cd</td>
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</table>

Note: the bold p-values are statistically significant.
The Zn, Cu, Ni, Mn, Fe and Cd content was determined by the method of absorption atomic spectrometry using the Aanalyst 300 instrument (Perkin Elmer, USA). The tests were carried out following the original standards of Merck (KGaA, 1g/1000ml). The metals were determined with the following wave lengths: 213.9 mm Zn, 324.8 Cu, 232.0 Ni, 279.5 Mn, 248.3 Fe and 228.8 Cd. All the analyses were performed in three replicates.

**Statistical analysis**

Distribution of physical and chemical data related to the soil, needles and bark was measured by means of application Shapiro Wilk test. The statistical validity between pH and the heavy metals content in the soil, needles and bark of the tested species of *Pinus* was established on the basis of a non-parametric Kruskal Wallis test (Tab. 2, 4; Fig. 1). To identify the factors that

![Figure 1](image_url)

**Figure 1.** The contents of Cu, Ni, Zn and Cd in the 1-year and 2-years needles of *Pinus* species with the Kruskal–Wallis test results (triangle – median values, moustache – minimum and maximum values)
have an impact on the chemical composition of the bark of the species of *Pinus*, the method of Principal Components Analysis was applied (PCA). All calculations and charts were prepared in Statistica 7.1 software. In order to provide quality control of the obtained results, an analysis of the certified reference material of the plants was made (CRM 060). The obtained results were within the limits of the error ± 3%.

**Results**

**Physicochemical properties of soil**

The soils under the examined tree stand represented the slightly acidic and neutral reaction, showing little diversity depending on the depth (Tab. 2). An increased soil acidity along with the depth was observed under the tree stand of *P. banksiana*, which had an impact on the increase of bioavailability of manganese and iron for the root system and higher accumulation of Mn and Fe in the needles of that species (Fig. 1).

The organic matter content was diversified depending on the species from under which the soil samples were taken, as well as on the depth (Tab. 2). The highest content of the organic matter was discovered in the surface layers of the soil (0.0–0.2 m), from 8.5% under the tree stand of *P. wallichiana* to 12.7% under the crowns of *P. armandii*. In all the research stations, a decrease of organic matter along with the depth was discovered. The lowest content in the layer 0.4–0.6 m was found in the research stations of *P. mugo* (5.4%), and the highest under the tree stands of *P. nigra* (9.7%).

The content of heavy metals in the soil under the tree stands of *Pinus* was diverse depending on the species and depth of the layer. In the surface layer (0.0–0.2 m), the concentration of manganese remained at the level from 500.1 mg/kg (*P. sylvestris*) to 640.3 mg/kg (*P. wallichiana*), and in the layer of 0.4–0.6 m, from 508.3 mg/kg (*P. sylvestris*) to 716.4 mg/kg (*P. banksiana*) (Tab. 2). In the tested samples of soils, the lowest content of Fe was discovered under the tree stand of *P. mugo*, and the highest at the tree stands of *P. banksiana*. Under the tree stand of *P. banksiana*, an increase of Mn and Fe was observed in the deeper layers of the soil. In case of copper, the highest concentration was discovered in stations under the tree stand of *P. wallichiana* (from 69.7 mg/kg to 81.9 mg/kg), and the lowest in soil under *P. nigra* (30.1–32.8 mg/kg). The content of zinc in the surface layers of the soil remained at 73.6 mg/kg under the tree stand of *P. sylvestris* to 88.3 mg/kg at the tree stands of *P. wallichiana*. In the layer of 0.4–0.6 m, the concentration of Zn remained at a similar level as in the layer 0.0–0.2 m and was from 63.2 mg/kg (*P. sylvestris*) to 88.3 mg/kg (*P. nigra*). The lowest concentrations of Cd were discovered under the tree stand *P. nigra* (0.3–0.5 mg/kg), and the highest concentration was discovered in the case of *P. armandii* (1.5–1.7 mg/kg).

The Kruskal–Wallis test proved statistically significant differences in physical and chemical properties of the soils under the examined species of *Pinus* except for concentration of cadmium in the layers of the soil 0.0–0.2 and 0.2–0.4 m (Tab. 2).

**Physicochemical properties of Pinus species**

The needles of the examined species *Pinus* were characterized by diversity of pH, but both 1-year old and 2-years old needles represented a strongly acidic reaction. The highest level of acidity was characteristic in the needles of *P. banksiana* (pH: 4.00–4.18) and *P. armandii* (pH: 4.00–4.32), and the lowest in the needles of *P. sylvestris* (pH: 5.25–5.32) (Fig. 2). In majority of the examined species, a slightly higher acidity was represented by 1 year old needles and 2 years old needles except for *P. wallichiana*; in this case, a reverse relation was observed.

![Figure 2. pH of needles of Pinus species depending on the age of needles with the Kruskal–Wallis test results (triangle-median values, moustache – minimum and maximum values)](image-url)

The content of metals in the needles was diversified depending on a species and the age of the needles.
In most cases, a higher metal content was found in the 2 years old needles, except for Cu and Zn (P. banksiana), and Zn (P. nigra), where higher concentration was discovered in 1 year old needles. The highest concentration of Fe was characteristic for the needles of P. wallchiana (1-year old = 263.1 mg/kg, 2-years old = 298.6 mg/kg), and the lowest content was found in the case of P. sylvestris (respectively 114.1 mg/kg and 140.9 mg/kg). The highest concentration of manganese was found in the needles of P. banksiana (respectively 182.4 mg/kg and 217.7 mg/kg), and the lowest in the needles of P. wallchiana (17.8 mg/kg and 21.3 mg/kg) (Fig. 1, Tab. 3). The content of zinc in the examined samples were characteristic of the species and remained at the level 18.6–26.3 mg/kg in P. wallchiana, 48.7–55.0 mg/kg in P. armandii, 51.1–56.9 mg/kg in P. mugo, 47.4–54.2 mg/kg in P. sylvestris, 45.8–59.2 mg/kg in P. nigra and 53.9–57.1 mg/kg in P. banksiana (Fig. 1, Tab. 3). The highest concentration of copper was discovered in the needles of P. wallchiana (10.9–11.2 mg/kg), and the lowest in P. nigra (3.7–5.5 mg/kg). Nickel was accumulated in the highest volume in the needles of P. wallchiana (15.8–16.6 mg/kg), and the lowest in the needles of P. banksiana (3.7–5.5 mg/kg). In the case of cadmium, the highest concentration was discovered in the needles of P. armandii (2.5 mg/kg), and the lowest in the needles of P. mugo (0.1 mg/kg) (Fig. 1, Tab. 3). The examined needles accumulated heavy metals in the volume changing in the decreasing sequences:

- P. armandii: Fe > Mn > Zn > Ni > Cu > Cd, P. banksiana: Fe > Mn > Zn > Cu > Ni > Cd,
- P. mugo: Fe > Zn > Mn > Ni > Cu > Cd, P. nigra: Fe > Zn > Mn > Ni > Cu > Cd,
- P. sylvestris: Fe > Mn > Zn > Ni > Cu > Cd i P. wallchiana: Fe > Zn > Mn > Ni > Cu > Cd.

The Pinus species under consideration were grouped by the application of Ward method on the basis of similarity in accumulation of heavy metals in needles and two main groups were separated. In group I – P. armandii, P. banksiana and P. sylvestris were found, and in the second group – P. mugo, P. nigra and P. wallchiana were found (Fig. 3).

**Physicochemical properties of bark**

All samples of the examined bark had a strongly acidic reaction, having values of pH from 3.7 (P. sylvestris) to 4.9 (P. armandii) (Tab. 4). The heavy content of metals in the bark of the species Pinus was strictly dependent on the surface of the bark under consideration. Concentrations of Mn remained at the level from 13.0 mg/kg (P. nigra) to 67.3 mg/kg (P. armandii), and the iron content had the values from 253.8 mg/kg (P. wallchiana) to 1122.7 mg/kg (P. armandii). Zinc constituted substantially lower quantities in the bark having the values from 42.7 mg/kg (P. sylvestris) to 118.7 mg/kg (P. mugo). Nickel concentrations remained at the level from 41.4 mg/kg (P. wallchiana) to 90.6 mg/kg (P. sylvestris), and copper from 6.9 mg/kg (P. mugo and P. nigr-

<table>
<thead>
<tr>
<th>Species</th>
<th>Age of needles</th>
<th>Mn</th>
<th>Fe</th>
<th>Cu</th>
<th>Ni</th>
<th>Zn</th>
<th>Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinus armandii</td>
<td>1-year of needles</td>
<td>136.1 ± 1.5</td>
<td>152.3 ± 6.2</td>
<td>4.7 ± 0.3</td>
<td>15.6 ± 1.2</td>
<td>48.7 ± 1.5</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>2-years of needles</td>
<td>158.4 ± 2.0</td>
<td>254.2 ± 8.1</td>
<td>6.1 ± 1.1</td>
<td>13.0 ± 2.3</td>
<td>55.0 ± 0.7</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Pinus banksiana</td>
<td>1-year of needles</td>
<td>182.4 ± 1.2</td>
<td>168.5 ± 6.6</td>
<td>10.3 ± 0.5</td>
<td>3.5 ± 1.9</td>
<td>57.1 ± 0.9</td>
<td>1.4 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>2-years of needles</td>
<td>217.5 ± 3.0</td>
<td>270.5 ± 2.9</td>
<td>9.4 ± 0.3</td>
<td>8.0 ± 1.5</td>
<td>53.9 ± 1.8</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>Pinus mugo</td>
<td>1-year of needles</td>
<td>41.4 ± 1.8</td>
<td>229.9 ± 8.6</td>
<td>7.9 ± 0.5</td>
<td>8.2 ± 1.3</td>
<td>51.1 ± 1.4</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>2-years of needles</td>
<td>40.1 ± 0.5</td>
<td>250.8 ± 7.1</td>
<td>7.8 ± 0.9</td>
<td>10.6 ± 1.8</td>
<td>56.9 ± 0.6</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Pinus nigra</td>
<td>1-year of needles</td>
<td>19.8 ± 0.7</td>
<td>210.6 ± 7.8</td>
<td>3.7 ± 0.2</td>
<td>6.8 ± 3.2</td>
<td>59.2 ± 0.4</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>2-years of needles</td>
<td>17.8 ± 2.2</td>
<td>220.7 ± 4.7</td>
<td>5.5 ± 0.3</td>
<td>7.5 ± 0.7</td>
<td>45.8 ± 0.5</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Pinus sylvestris</td>
<td>1-year of needles</td>
<td>97.6 ± 0.5</td>
<td>114.1 ± 6.9</td>
<td>5.2 ± 1.1</td>
<td>7.3 ± 3.2</td>
<td>54.2 ± 2.2</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>2-years of needles</td>
<td>119.1 ± 1.0</td>
<td>140.9 ± 5.6</td>
<td>5.2 ± 0.6</td>
<td>17.1 ± 2.8</td>
<td>47.4 ± 0.8</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Pinus wallchiana</td>
<td>1-year of needles</td>
<td>17.9 ± 1.5</td>
<td>263.1 ± 8.4</td>
<td>10.9 ± 1.0</td>
<td>15.8 ± 3.1</td>
<td>18.6 ± 1.5</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>2-years of needles</td>
<td>21.3 ± 0.3</td>
<td>298.6 ± 3.6</td>
<td>11.2 ± 0.7</td>
<td>16.6 ± 1.5</td>
<td>26.3 ± 1.0</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

Table 3. The average ± standard deviation of heavy metals concentration (mg/kg) in the 1-year and 2-years needles of Pinus species
gra) to 10.1 mg/kg (P. armandii). From among the examined metals, cadmium was found in the lowest quantities, having the values from 0.8 mg/kg (P. banksiana) to 2.5 mg/kg (P. armandii). The results obtained from the research showed the statistically significant differences in the concentration of Mn, Fe, Cu, Ni, Zn and Cd in the bark of the species Pinus (Tab. 4).

In order to identify factors determining the chemical composition of the bark, Principal Components Analysis (PCA) was applied. pH and concentrations of Mn, Fe, Cu, Ni, Zn and Cd in the bark were used for calculation, depending on the species. 2 independent factors explaining 73% of variance of chemical composition of the examined bark samples were used as the main components (Tab. 5). For interpretation of data, only such values of the factor loadings were used,

![Figure 3. Similarity of the investigated Pinus species in relation to the contents of Mn, Fe, Cu, Ni, Zn and Cd in needles (Euclidean distance, Ward’s clustering method).](image)

**Table 4.** The median of pH and heavy metals concentration (mg/kg) in bark of Pinus species with Kruskal–Wallis test results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pinus armandii</th>
<th>Pinus banksiana</th>
<th>Pinus mugo</th>
<th>Pinus nigra</th>
<th>Pinus sylvestris</th>
<th>Pinus wallichiana</th>
<th>Kruskal–Wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>4.9 ± 0.2</td>
<td>3.9 ± 0.5</td>
<td>4.4 ± 0.6</td>
<td>4.5 ± 0.2</td>
<td>3.7 ± 0.3</td>
<td>4.2 ± 0.3</td>
<td>16.875 0.0047</td>
</tr>
<tr>
<td>Mn</td>
<td>67.3 ± 6</td>
<td>27.0 ± 2</td>
<td>35.3 ± 13</td>
<td>13.0 ± 2</td>
<td>31.7 ± 6</td>
<td>20.3 ± 2</td>
<td>14.4978 0.0127</td>
</tr>
<tr>
<td>Fe</td>
<td>1122.7 ± 6</td>
<td>613.2 ± 2</td>
<td>792.3 ± 5</td>
<td>629.2 ± 2</td>
<td>655.8 ± 5</td>
<td>253.8 ± 4</td>
<td>16.5789 0.0054</td>
</tr>
<tr>
<td>Cu</td>
<td>10.1 ± 1</td>
<td>8.6 ± 0.5</td>
<td>6.9 ± 0.7</td>
<td>6.9 ± 0.5</td>
<td>7.3 ± 0.2</td>
<td>7.7 ± 1.4</td>
<td>11.3569 0.0447</td>
</tr>
<tr>
<td>Ni</td>
<td>48.2 ± 1</td>
<td>42.7 ± 6</td>
<td>43.6 ± 7</td>
<td>80.5 ± 2</td>
<td>90.6 ± 2</td>
<td>41.4 ± 4</td>
<td>15.8304 0.0073</td>
</tr>
<tr>
<td>Zn</td>
<td>66.5 ± 2</td>
<td>57.4 ± 1</td>
<td>118.7 ± 1</td>
<td>76.0 ± 2</td>
<td>42.7 ± 1</td>
<td>59.3 ± 1</td>
<td>16.5789 0.0054</td>
</tr>
<tr>
<td>Cd</td>
<td>2.5 ± 0.9</td>
<td>0.8 ± 0.5</td>
<td>2.4 ± 0.8</td>
<td>1.2 ± 0.7</td>
<td>2.1 ± 1.6</td>
<td>1.9 ± 0.9</td>
<td>6.9527 0.2242</td>
</tr>
</tbody>
</table>

Note: the gray p-values are statistically significant.

**Table 5.** Results of principal component analysis (PCA) the heavy metals content in bark of Pinus species, n = 90

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>−0.78</td>
<td>0.02</td>
</tr>
<tr>
<td>Mn</td>
<td>−0.88</td>
<td>−0.22</td>
</tr>
<tr>
<td>Fe</td>
<td>−0.89</td>
<td>−0.01</td>
</tr>
<tr>
<td>Cu</td>
<td>−0.63</td>
<td>−0.64</td>
</tr>
<tr>
<td>Ni</td>
<td>−0.35</td>
<td>0.94</td>
</tr>
<tr>
<td>Zn</td>
<td>−0.35</td>
<td>0.89</td>
</tr>
<tr>
<td>Cd</td>
<td>−0.48</td>
<td>0.16</td>
</tr>
<tr>
<td>Eigenvalues</td>
<td>2.97</td>
<td>2.16</td>
</tr>
<tr>
<td>Explained variance [%]</td>
<td>42</td>
<td>31</td>
</tr>
</tbody>
</table>

Note: in gray factor loading higher than 0.7 are highlighted.
which were higher than 0.7. Factor 1 explained 42% of the chemical composition and grouped pH, Mn and Fe characterized by high, negative factor loadings. Factor 2 explained 31% changeability of chemical composition of the bark and was indirectly proportional to the content of Ni and Zn.

**Discussion**

**Physicochemical properties of soil**

The main deciding factor for availability of heavy metals for plants is the reaction of soil. The solubility of heavy metals is low in the neutral and alkaline reactions, and increases with the lowering of pH value (Gworek 2006), thereby increasing the bioaccumulation factors in plants (Gębski 1998). Increase of mobility of Zn, Mn and Cd is most effective with pH = 6.0, Fe at pH = 4.0, while Ni and Cu at pH = 5.5. Manganese, however, is characterized by increased solubility in alkaline environment as well (Alloway 1995).

Dead plant remains which constitute an important link in the circulation of matter and energy flow constitute the source of organic matter. Its quantity and quality influences the characteristics of the soil and nutrition for the plants (Astel et al. 2009; Polláková et al. 2015). Increased organic matter content in the surface layers of soil is a result of systematic influx of organic deposition that takes place throughout the year (Astel et al. 2009); its distribution depends on the species and qualitative properties of the tree stand (Jonczak et al. 2015). The largest quantities of organic matter were found in the soil under the tree stands of *P. armandii* and *P. nigra*, which result most probably from a slower speed of decomposition of their organic remains.

According to Kabata-Pendias and Pendias (1999), the occurrence of trace elements in the soil depends on their content in the parent rock and on the character of the soil creation processes. Atmospheric dust deposition, which is a carrier of many heavy metals, especially at the areas under influence of anthropogenic factors, had vital impact on concentration of trace elements in the surface layer of the soil (Tainio et al. 2010; Parzych and Jonczak 2014; Rapport 2016). Metals deposited in the surface layer of soil are bound by the constant phase of the soil, absorbed by organisms and undergo migration along with water filtering in. The quantity of bio-available forms of heavy metals is strictly dependent on the soil reaction and organic matter content (Parzych and Jonczak 2013). Increase of Mn and Fe content in the soil along with the depth under the three stand of *P. banksiana*, is an effect of increase of acidity of the soil in the layers 0.2–0.4 m and 0.4–0.6 m in relation to the layer 0.0–0.2 m. Acidification causes washing out of soluble forms of manganese and iron to the deeper layers of the soil. Similar phenomenon was observed under the tree stand of *P. sylvestris* at the area of Słowiński National Park (northern Poland), (Parzych and Jonczak 2013).

**Physicochemical properties of Pinus species**

Strong acidic reaction of the needles of the species of *Pinus* is characteristic for the coniferous tree stands, and in the case of *P. sylvestris*, it reaches the values of pH = 3.0–3.2 (Świercz 2006). The influence of external factors, such as alkaline emission, substantially lowers the acidity of needles of *P. sylvestris* (pH = 4.8–5.3) (Świercz 2003).

Concentrations of trace elements in the needles of the examined species of *Pinus* were relatively low, except for nickel (*P. armandii, P. sylvestris, P. wallchiana*) and cadmium, (*P. armandii, P. banksiana*), which showed increased levels of these metals. Small content of metals in the needles was a result of limited availability of particular metals from the soil due to pH. From among the examined metals, iron dominated in all the samples (Fig. 1), not exceeding however, the acceptable level (<375 mg/kg) (Kabata-Pendias and Pendias 1999). Iron content in the samples of needles *P. nigra* from Kościele were slightly higher than the ones which were found at the territory of Poland at the Słowiński National Park (SPN) (Parzych and Sobisz 2012), which reflects the impact of anthropogenic factors. Low content of Mn in the needles of *P. wallchiana* resulted from the negative reaction of the soil (Tab. 1), where manganese was available for plants to a small extent. Physiological demand of most plants for Mn is usually from 10 to 25 mg/kg. Toxic levels were found in none of the examined samples of the needles (>500 mg/kg) (Kabata-Pendias and Pendias 1999). Substantially higher Mn content was found in the needles of *P. sylvestris* and *P. nigra* at the area of SPN due to strongly acidic reaction of soils increasing the availability of manganese compounds for the root system. Concentrations of zinc in the needles
of the examined species of Pinus were close to the results obtained in the case of P. sylvestris and P. nigra at the area of SPN (Parzych and Sobisz 2012). The average zinc content in the over-ground parts of the plants, which were not under the impact of pollution, usually remains at the level of 10–70 mg/kg. To cover the physiological needs of most plants, sufficient concentration of zinc in the leaves is within 15–30 mg/kg, and copper at the level of 4–5 mg/kg. However, the average Cu content in over ground parts of the plants is usually from 5 to 20 mg/kg (Kabata-Pendias and Pendias 1999) and is highly diversified depending on the part of the plant, species and genus. However, Cadmium as a toxic metal, is accumulated by plants in a passive way (Baran and Jasiewicz 2009). Concentrations of Cd in the needles of the species Pinus represent little contamination of soils under the examined tree stands. The accumulative properties of the needles in relation to Mn, Fe, Cu, Ni, Zn and Cd indicate mutual similarities between some species of Pinus. In group I, P. armandii, P. banksiana and P. sylvestris are found, which have needles characterized by a relatively high iron, manganese and zinc content (Fe > Mn > Zn) and P. mugo, P. nigra and P. wallichiana, characterized by a high concentration of iron, zinc and manganese (Fe>Zn>Mn). Differences in accumulation of Zn and Mn in the needles of P. sylvestris and P. nigra was also found during the research done by Parzych and Sobisz (2012).

Physicochemical properties of bark

The tissue covering the plants is a very important and exceptionally sensitive bio-indicator of pollution of the natural environment. The test results confirmed that pH of the bark of trees is characteristic for the species and has the values of pH from 3.0 to 5.5 (Marmor andRandlane 2007). The reaction of the bark depends also on the age, health status of the trees and the properties of the substratum on which it grows (Chrzan 2013). The test results in the literature indicate that the bark of P. sylvestris shows a strongly acidic reaction with pH within 3.1–3.3 (Marko-Worłowska et al. 2010) and 3.1–3.9 (Chrzan 2013). Moreover, the bark of coniferous trees is characterized by higher acidity than the bark of leafy trees (Chrzan et al. 2010). The bark of the trees, depending on the structure and porosity, arrests the dust and aerosols in the quantities proportional to their surface (Chrzan 2013), sometimes showing even higher concentrations of a given metal than in the soil (Kuang et al. 2007). The obtained results of the research indicate that the highest quantities of Mn, Fe, Cu and Cd were accumulated by the bark of P. armandii, nickel by the bark of P. sylvestris, and Cu by the bark of P. mugo. The research done by Rykowska and Wasiak (2009) proves that the accumulation of heavy metals in the bark of the trees strongly depends on the species and on the impact of anthropogenic factors, which is also confirmed by the research concerning P. armandii, P. banksiana, P. mugo, P. nigra, P. sylvestris and P. wallichiana from Košice. As per Chrzan et al. (2010), the coniferous trees accumulate metals in higher quantities than the leafy species. The bark of Pinus sylvestris can accumulate from 32.4 to 143.3 mg/kg Zn (Chrzan 2013), and the bark of Robinia pseudoacacia from 1.4 mg/kg to 26.9 mg/kg Zn (Kraszkiewicz 2010).

The results of the factor analysis clearly diversify the sources of origin of heavy metals in the bark of the examined species Pinus. Factor 1 indicates the origin of iron and manganese for the same local sources of pollution, such as iron processing, machine and textile industry functioning in the area of Košice. Factor 2 comprises nickel and zinc, which are most often sourced from the atmospheric precipitation originating from industry, communication and transport (Rapport 2016).

Conclusions

The examined soils were abundant at an average level in the basic micro components, contained small quantities of cadmium, and their availability for trees depended on soil reaction. From among the tested species, the needles of P. banksiana were characterized by the highest level of acidity that had an impact on the soil reaction under the tree stand and on the higher accumulation of Fe and Mn in the needles.

In most cases, higher levels of content of those metals was determined in the 2 years old needles, except for Cu and Zn (P. banksiana) as well as Zn (P. nigra), in which case, higher concentration of metals in 1 year old needles was determined. This indicates a greater sensitivity of the needles of these species to Cu and Zn. From among the examined elements, Mn was accumulated in the largest volume in the needles of P. banksiana, Fe and Cu in the needles of P. wallichiana, Ni in the needles.
Accumulation of heavy metals in needles and bark of Pinus species

of P. sylvestris, Zn in the needles of P. nigra, and Cd in the needles of P. armandii. Chemical analyses of the needles of P. armandii, P. sylvestris and P. wallchiana indicate a small pollution of the tested area with nickel and the needles of P. armandii and P. banksiana show increased content of cadmium. The examined bark samples indicated strongly acidic reaction. The highest quantities of Mn, Fe, Cu and Cd were accumulated by the bark of P. armandii, Ni by the bark of P. sylvestris, and Cu by the bark of P. mugo.

REFERENCES


Genetic variation of silver fir progeny from Tisovik Reserve population determined via microsatellite and isozyme markers

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ABSTRACT

Progeny from 19 family lines of silver fir (Abies alba Mill.) from a small, native and isolated population from the Tisovik Reserve (Belarusian part of Białowieża Primeval Forest) growing in an experimental plot near Hajnówka (Polish part of Białowieża Primeval Forest) were analysed in terms of 4 nuclear microsatellite DNA loci and 9 isozyme systems with 14 loci. The aim of this study was to determine the genetic variation within and between progeny lines. Analysis of isozyme loci showed that all progeny lines, except the progeny lines T6 and T16, were characterised by an excess of heterozygotes and 20% of the detected variation occurred between progeny. Progeny formed two groups. Microsatellite loci showed that 6 progeny lines demonstrated an excess of heterozygotes and 12 an excess of homozygotes. On an average, the population was in the Hardy–Weinberg equilibrium. Analysis of molecular variance (AMOVA) showed that 14% of the detected variation occurred between offspring and the remaining 86% within progeny lines. The most distinct progeny line was T1, where the highest number of alleles per locus was detected. Generally, progeny of Tisovik is characterised by high level of differentiation as the offspring of isolated population that have limited number of individuals to crossing (only 20). In some progeny line, the private alleles that are detected may be the result of pollination from Polish part of Białowieża Forest where in 1920s and 1930s of XX century had planted the seedling of silver fir of unknown origin. The substructuring of population is observed, and the detected deficiency of heterozygotes may be ostensible as a result of the Wahlund effect. Such pattern of genetic structure could also be an effect of harsh environmental conditions exerting selection pressure and modifying the genetic composition of this population.

KEY WORDS

european silver fir, genetic diversity, isolated population, isozyme analysis, nuclear microsatellite DNA, progeny

INTRODUCTION

In terms of its economic and ecological value, silver fir (Abies alba Mill.) is one of the most important forest trees growing in the mountains of Central Europe. In the northern part of it species distribution, this tree occurs on lowlands. In these regions, fir is characterised by relatively small variation and lower adaptability to stress in comparison to other Pinaceae species, especially Scots pine and Norway spruce. The reason for
this seems to be the history of fir (the small number of glacial refugia) and populational genetic processes such as mutation, drift and selection during and after postglacial migration to the northern part of its natural distribution (Larsen 1986; Konnert and Bergman 1995; Breitenbach-Dorfer et al. 1997; Vendramin et al. 1999; Longauer et al. 2003; Liepelt et al. 2009). These populational processes were induced inter alia by climatic fluctuations. The reduced adaptability because of insufficient genetic variation is assumed and is, thus, the main predisposing factor exerting a permanent stress on silver fir and thereby making it sensitive to other factors such as frost, drought, acidification and biotic pests (Longauer et al. 2003). Another reason for reduced variation is the dieback of fir caused by anthropogenic factors such as inappropriate management systems (mainly cultivating fast-growing needle trees such as Scots pine and Norway spruce), air pollution, insect pests and others. Dieback of this species is especially evident in the northern part of fir distribution (Longauer 1994).

The Białowieża Primeval Forest is the only remaining natural lowland forest in Europe, where a small, natural and isolated population of fir can be found in the Tisovik Reserve. This population is located in Belarus – 120 km north of the natural range limit of this species and is a remnant of the post-glacial migration and contraction of the silver fir distribution. The Tisovik Reserve is the last site in the Białowieża Forest where fir occurs naturally and should be recognised as the fir locus classicus (Korczyk et al. 1997). A detailed description of the Tisovik Reserve is given in papers by Mejnartowicz (1996a, 1996b), Korczyk et al. (1997), Korczyk (1999, 2015a, 2015b), Goncharenko and Savitski (2000), and Pawlaczyk et al. (2005). The number of firs in Tisovik has never been abundant. According to the above authors, the number of fir trees ranged from 300 to 100 (between 1829, when this site was discovered by Górski (1829), and 1939, the beginning World War II). Currently, there are only 20 mature fir trees. This site is limited not only by the very small area where fir is growing (a mineral forest islet that is surrounded by the vast ‘Dziki Nikor’ peat-land) but also by animals that browse and graze the trees and seedlings and by local people who cut out trees. Decisive in the survival of this fir population, despite these many unfavourable factors, has been its capacity for intensive seeding and natural recovery. However, research conducted in 1995 showed that seeds germinated less and 60% was parasitized by monofagous entomofauna (mainly Megastigmus suspectus Borr. from Hymenoptera and Resseliella piceae Seitt. from Dip- tera) (Korczyk et al. 1997). These two species occur in Poland and Belarus only on European silver fir and came to Białowieża Forest together with fir in the Holocene climate optimum period and stayed with fir as a relict (Korczyk et al. 1997). Such a high level of seed parasitisation is the result of the very small number of fir trees. These facts suggest that Tisovik is probably a declining population, which could go through a ‘bottle neck’ and may completely disappear (Mejnartowicz 1996a, 1996b). In response to this threat, the preservation of its gene resource and the description of its diversity are an urgent priority. Therefore, in 1995, a plantation with Tisovik fir offspring was established in Hajnówka Forest District (Polish part of Białowieża Primeval Forest).

Earlier studies carried out on isozyme markers showed that Tisovik population were characterised by an excess of heterozygosity (Mejnarońicz 1996a, 1996b; Goncharenko and Savitski 2000), which may be the result of a long selection for adaptability to the rapidly changing environmental factors in the Białowieża Primeval Forest after the last glacial period. Heterozygous trees are expected to be more adaptable because natural selection reduces the frequencies of homozygotes in natural forest populations (Stern and Roche 1974).

The genetic analysis may provide essential information that is important to understand the mechanisms that allow a population to exist beyond the species natural distribution in a small and isolated site, where there are often extreme conditions (in the case of fir, e.g. low soil humidity, lower than 4°C of average temperature in February and destructive human activities over many years). The genetic variability may be a source of information for institutions dealing with conservation and provide information about the source of genetic variation and plasticity of silver fir in protected areas such as nature reserves, which bring together some of the most valuable and unique populations. Understanding the variability of the offspring will help trace trends and microevolutionary processes that are occurring in small and isolated populations.
The aim of the investigations was to characterise the genetic variation within and between the progeny of the Tisovik population on the basis of isozyme and nuclear microsatellite DNA loci and to compare the results obtained from these two markers. The general purpose will be to check whether a high level of heterozygosity discovered by Mejnartowicz (1996a, 1996b) and Goncharenko and Savitsky (2000) will be maintained in progeny.

**Material and Methods**

**Material**

In order to protect the gene pool and detect genetic variation in silver fir from the Tisovik Reserve, the provenance—progeny experiment plot was established by Prof. Adolf F. Korczyk from the Forest Research Institute in Białowieża and the Białystok University of Technology. In 1995, seeds from 20 silver fir trees were collected and sowed in a forest nursery in Nawojowa (Beskid Sądecki). Next, in 1998, three-year-old seedlings were moved to an experimental plot in Hajnówka Forest District in Poland (Leśna, Wilczy Jar, section 416Ag/416Cf). The area of silviculture is about 0.225 ha. Detailed description of seed collection and seedlings cultivation is given in Korczyk papers (1999, 2015b).

Plant material was collected from the progeny of 19 maternal trees from Tisovik Reserve. Each progeny line was represented by 8–15 trees. In sum, 274 progeny trees were studied. For analysis, 1-year-old needles and vegetative buds were collected from 15-year-old progeny in 2010. The plant material was stored in a freezer at a temperature of −20°C until the beginning of analysis.

**Methods**

**Isozyme separation**

Isozyme separation was conducted according to the procedure described by Cheliak and Pitel (1984) and HusSENDÖRFER et al. (1995). Crude cell extract was prepared by homogenisation of dormant buds in 80 ml of 0.2M Tris-HCl extraction buffer (1% ethylenediaminetetraacetic acid (EDTA) II, polyvidone (PVP) 40, PVP 80, TWEEN, polyethylene glycol, sodium ascorbate, 2-mercaptoethanol and 0.01% dithiothreitol, pH 7.2) and then paper wicks (Watmann 3MM) were soaked. Isozymes were separated in 10% starch gel in two buffer systems: (a) Tris-citrate (pH 8.2), lithium-borate (pH 8.3) and (b) Tris-citrate (pH 8.0) in a dilution of electrode buffer 1:15. The enzymes stained and buffer systems used for their resolution are listed in Table 1. Lithium-borate gels were separated at a constant voltage (240 V by 5 h), and Tris-citrate gels were separated at a constant current (75 mA by 4 h). After separation, the isozymes were detected on the gel slabs by using the staining methods of Cheliak and Pitel (1984) and HusSENDÖRFER et al. (1995).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abbr.</th>
<th>E.C.</th>
<th>Buffer system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate dehydrogenase</td>
<td>GDH</td>
<td>1.4.1.2</td>
<td>A</td>
</tr>
<tr>
<td>Glutamate oxaloacetate transaminase</td>
<td>GGT</td>
<td>2.6.1.1</td>
<td>A</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>LAP</td>
<td>3.4.11.1</td>
<td>A</td>
</tr>
<tr>
<td>Glucose phosphate isomerase</td>
<td>PGI</td>
<td>5.3.1.9.9</td>
<td>A</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>IDH</td>
<td>1.1.1.41</td>
<td>B</td>
</tr>
<tr>
<td>Shikimate dehydrogenase</td>
<td>SDH</td>
<td>1.1.1.25</td>
<td>B</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>PGM</td>
<td>5.4.2.2</td>
<td>B</td>
</tr>
<tr>
<td>Phosphogluconate dehydrogenase</td>
<td>PGD</td>
<td>1.1.1.44</td>
<td>B</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>MDH</td>
<td>1.1.1.37</td>
<td>B</td>
</tr>
</tbody>
</table>

**DNA extraction**

Genomic DNA from fir needles and buds tissue was extracted. Frozen needles and buds (100 mg of tissue) were powdered in liquid nitrogen, and the total genomic DNA was extracted using a modified ATMAB method (Doyle and Doyle, 1990) and then dissolved in 0.1 × TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) for subsequent use. The quality and quantity of extracted DNA were measured on a Nanodrop™ ND-1000 (ThermoScientific) spectrophotometer and diluted to a final concentration of 20 ng/μl.

**SSR amplification**

Four nrSSR markers that gave satisfactory amplification products in the analysed fir species were selected from the published literature (Cremer et al. 2006). However, the amplification and polymorphism potential for eight primer pairs of nuclear microsatellite loci were checked: SF 1, SF 2, SF 8, SF 83, SF 324, SF 333, SF b5
and SF g36. Some of these markers were monomorphic or did not give an amplification of products or showed unstable amplification. Four markers – SF 333, SF 1, SF 324 and SF b5 – were classified as variable and stably amplified and were then used in this study (Tab. 2).

Table 2. Characteristics of the used primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Motive</th>
<th>Allele size (bp)</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF 333 (DQ218463)</td>
<td>(CA)₁₂ (TA)₄</td>
<td>166–178</td>
<td>5'-ATTTGTTCATTTTTGGTCTTG-3' 5'-ACACAGGAAAAAGTCGGTAA-3'</td>
</tr>
<tr>
<td>SF 1 (DQ218453)</td>
<td>(CCG)₆</td>
<td>209–221</td>
<td>5'-TTGACGTGATTACAAATCCA-3' 5'-AAGAACGACACCATTCTCACC-3'</td>
</tr>
<tr>
<td>SF 324 (DQ218461)</td>
<td>(CCG)₆</td>
<td>106–112</td>
<td>5'-TTTGAACGGAAATCAAATTC-3' 5'-AAGAACGACACCATTCTCACC-3'</td>
</tr>
<tr>
<td>SF b5 (DQ218455)</td>
<td>(CT)₁₅</td>
<td>130–148</td>
<td>5'-AAAAAGCATCACTTTTCCTG-3' 5'-AAGAGGAGGGGATTACAAG-3'</td>
</tr>
</tbody>
</table>

Primers are from Cremer et al. (2006).

Polymerase chain reaction (PCR) was performed in a 2720 Thermal Cycler (Applied Biosystems© Waltham, Massachusetts, USA). Each amplification reaction contained 1× reaction buffer (Novazym), 0.2 mM of each deoxynucleotide, 0.25 µM of forward and reverse primer and 0.5U of HiFiTaq DNA polymerase (Novazym), approximately 50 ng of genomic DNA and de-ionised water to a total volume of 10 µl. The forward primer of each primer pair was fluorescent labelled with 6FAM (SF 1), VIC (SF 324), NED (SF b5) and PET (SF 333) dye (Applied Biosystems© Waltham, Massachusetts, USA) at its 5' end to avoid the mistakes when reading the similar variants lengths of different amplified loci.

The PCRs were completed using the following touchdown protocol: 5 min at 94°C followed by 10 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C; followed by 25 cycles of 30 s at 94°C, 50 s at 50°C and 40 s at 72°C; followed by a 7 min extension at 72°C and an indefinite hold at 4°C.

PCR products were separated with a 3130xl Genetic Analyzer (Applied Biosystems©) capillary electrophoresis system with GeneScan™600LIZ™ as an internal size standard. Individuals were analysed and genotyped using GeneMapper version 3.7 software (Applied Biosystems©).

Data analysis and statistical methods

For each isozyme (using PopGene32 by Yeh et al. (2000)) and microsatellite locus (using GenAIEx by Peakall and Smouse (2006)), the number of alleles (A), level of inbreeding amongst individuals within in each progeny (Fᵢₛ), overall level of inbreeding in the studied population (Fᵢₚ), measure of genetic differentiation amongst progeny lines (Fₛₚ), gene flow (Nm), expected (Hₑ) and observed heterozygosity (Hₒ) were estimated. The Hardy–Weinberg exact test of heterozygote deficiency (HWE) was computed using Genepop (Raymond and Rousset 1995) program. Gene flow (Nm) was estimated using Wright's (1978) formula: Nm = 0.25(1 − Fₛₚ)/Fₛₚ. For microsatellite loci, the number of null alleles was calculated using Micro-Checker 2.2.3 (van Oosterhout et al. 2004; 2006), and for isozyme markers, the Ewens–Watterson test for neutrality (Watterson 1977) was performed using PopGene32.

For both markers in each progeny line, the number of private alleles (Aᵢₚ) and the mean number of alleles per locus (Aᵢₚ) were computed using GenAIEx. In addition, the percentage of polymorphic loci (P%) for isozymes and allelic richness (AR) for microsatellite were calculated using PopGene32 and FSTAT v. 2.9.3.2, respectively (Goudet 1995; 2001).

The UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrograms for both markers based on the Nei (Nei 1972) genetic distance were constructed using MEGA software (Tamura et al. 2013). To calculate the Nei genetic distance (Nei 1972) between each pair of progeny lines, the PopGene32 (Yeh et al. 2000) was used.

In addition, to estimate the genetic differentiation amongst progeny lines, hierarchical AMOVA was conducted using GenAIEx v.6.4 software (Peakall and Smouse 2006) for both markers. AMOVA was used to describe the percentage share of genetic diversity within and amongst progeny lines in terms of the total genetic diversity. The level of genetic differentiation between populations was estimated using the Φ statistic (an analogue to F). Statistical significance was determined by random permutation, with the number of permutations set to 999.
### Results

Analysis of isozyme markers in the 9 enzyme systems, 14 isozyme loci (Gdh, Got-A, Got-B, Idh, Lap, Mdh-A, Mdh-B, Pgd-B, Pgi-A, Pgi-B, Pgm-A, Pgm-B, Sdh-A and Sdh-B) were detected. The parameters for isozyme loci were given in Table 3. Of the 14 loci examined, two loci (Sdh-A and Pgm-A) were monomorphic in all the studied progeny lines and displayed a homozygous pattern, so they were removed from further analyses. The remaining loci were polymorphic, with two to four alleles (A). Most polymorphic loci have two or three alleles, with an average of 2.6; only in Pgi-B and Gdh, four alleles were found.

The mean value of the inbreeding coefficient ($F_{IT}$) indicates an excess of heterozygotes. Six loci showed a deficit and six an excess of heterozygosity, in comparison with the expected levels. The genetic differentiation ($F_{ST}$) calculated over polymorphic loci was at a mid level and the level of gene flow (Nm) between the studied

### Table 3a. Genetic parameters for polymorphic isozyme loci: A – number of alleles; $F_{IS}$ – level of inbreeding amongst individuals within each progeny; $F_{IT}$ – overall level of inbreeding in studied populations; $F_{ST}$ – measure of genetic differentiation amongst progeny lines; Nm – gene flow; Ho and He – observed and expected heterozygosity, respectively; HWE – the Hardy–Weinberg exact test of heterozygote deficiency, *** – $p < 0.001$, ** – $p < 0.01$, ns – $p > 0.05$

<table>
<thead>
<tr>
<th>Locus</th>
<th>A</th>
<th>$F_{IS}$</th>
<th>$F_{IT}$</th>
<th>$F_{ST}$</th>
<th>Nm</th>
<th>Ho</th>
<th>He</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gdh</td>
<td>4</td>
<td>-0.053</td>
<td>0.169</td>
<td>0.211</td>
<td>0.936</td>
<td>0.168</td>
<td>0.203</td>
<td>***</td>
</tr>
<tr>
<td>Got-A</td>
<td>2</td>
<td>-0.287</td>
<td>-0.073</td>
<td>0.166</td>
<td>1.256</td>
<td>0.137</td>
<td>0.128</td>
<td>ns</td>
</tr>
<tr>
<td>Got-B</td>
<td>2</td>
<td>-0.163</td>
<td>-0.027</td>
<td>0.117</td>
<td>1.891</td>
<td>0.053</td>
<td>0.051</td>
<td>ns</td>
</tr>
<tr>
<td>Idh</td>
<td>3</td>
<td>-0.045</td>
<td>0.104</td>
<td>0.143</td>
<td>1.499</td>
<td>0.242</td>
<td>0.270</td>
<td>**</td>
</tr>
<tr>
<td>Lap</td>
<td>2</td>
<td>-0.094</td>
<td>0.045</td>
<td>0.126</td>
<td>1.727</td>
<td>0.147</td>
<td>0.154</td>
<td>ns</td>
</tr>
<tr>
<td>Mdh-A</td>
<td>2</td>
<td>-0.594</td>
<td>-0.476</td>
<td>0.074</td>
<td>3.145</td>
<td>0.726</td>
<td>0.492</td>
<td>***</td>
</tr>
<tr>
<td>Mdh-B</td>
<td>3</td>
<td>-0.519</td>
<td>-0.289</td>
<td>0.152</td>
<td>1.400</td>
<td>0.811</td>
<td>0.629</td>
<td>**</td>
</tr>
<tr>
<td>Pgi-A</td>
<td>3</td>
<td>0.200</td>
<td>0.481</td>
<td>0.351</td>
<td>0.463</td>
<td>0.042</td>
<td>0.081</td>
<td>***</td>
</tr>
<tr>
<td>Pgi-B</td>
<td>4</td>
<td>-0.349</td>
<td>-0.159</td>
<td>0.140</td>
<td>1.531</td>
<td>0.737</td>
<td>0.636</td>
<td>ns</td>
</tr>
<tr>
<td>Pgm-B</td>
<td>2</td>
<td>-0.026</td>
<td>0.156</td>
<td>0.177</td>
<td>1.165</td>
<td>0.084</td>
<td>0.099</td>
<td>ns</td>
</tr>
<tr>
<td>Pgd-B</td>
<td>2</td>
<td>-0.601</td>
<td>-0.485</td>
<td>0.072</td>
<td>3.199</td>
<td>0.726</td>
<td>0.489</td>
<td>***</td>
</tr>
<tr>
<td>Sdh-B</td>
<td>2</td>
<td>0.004</td>
<td>0.272</td>
<td>0.269</td>
<td>0.679</td>
<td>0.263</td>
<td>0.362</td>
<td>**</td>
</tr>
<tr>
<td>Mean</td>
<td>2.6</td>
<td>-0.210</td>
<td>-0.024</td>
<td>0.166</td>
<td>1.349</td>
<td>0.345</td>
<td>0.299</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>0.229</td>
<td>0.069</td>
<td>0.078</td>
<td>0.021</td>
<td>0.259</td>
<td>0.089</td>
<td>0.062</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3b. Genetic parameters for microsatellite loci: A – number of alleles; $F_{IS}$ – level of inbreeding amongst individuals within each progeny; $F_{IT}$ – overall level of inbreeding in studied populations; $F_{ST}$ – measure of genetic differentiation amongst progeny lines; Nm – gene flow; Ho and He – observed and expected heterozygosity, respectively; HWE – the Hardy–Weinberg exact test of heterozygote deficiency, *** – $p < 0.001$, ** – $p < 0.01$, ns – $p > 0.05$

<table>
<thead>
<tr>
<th>Locus</th>
<th>A</th>
<th>$F_{IS}$</th>
<th>$F_{IT}$</th>
<th>$F_{ST}$</th>
<th>Nm</th>
<th>Ho</th>
<th>He</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF 333</td>
<td>6</td>
<td>0.152</td>
<td>0.224</td>
<td>0.115</td>
<td>1.930</td>
<td>0.552</td>
<td>0.658</td>
<td>*** 0.105</td>
</tr>
<tr>
<td>SF 1</td>
<td>4</td>
<td>-0.276</td>
<td>-0.151</td>
<td>0.114</td>
<td>1.946</td>
<td>0.386</td>
<td>0.303</td>
<td>ns -0.092</td>
</tr>
<tr>
<td>SF 324</td>
<td>4</td>
<td>0.150</td>
<td>0.193</td>
<td>0.085</td>
<td>2.708</td>
<td>0.137</td>
<td>0.161</td>
<td>ns 0.067</td>
</tr>
<tr>
<td>SF b5</td>
<td>6</td>
<td>0.443</td>
<td>0.480</td>
<td>0.106</td>
<td>2.105</td>
<td>0.062</td>
<td>0.110</td>
<td>*** 0.132</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>0.073</td>
<td>0.149</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>Mean</td>
<td>5.0</td>
<td>0.1171</td>
<td>0.1865</td>
<td>0.105</td>
<td>2.172</td>
<td>0.2840</td>
<td>0.3080</td>
<td>0.0530</td>
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<tr>
<td>SE</td>
<td>0.148</td>
<td>0.130</td>
<td>0.007</td>
<td>0.183</td>
<td>0.113</td>
<td>0.124</td>
<td>0.050</td>
<td></td>
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</tbody>
</table>

progeny lines was 1.349 migrants per generation. In loci Gdh, Mdh-A, Mdh-B and Pgd-B, an exact test for the Hardy–Weinberg equilibrium indicated a significant excess of heterozygotes, but in the loci Pgi-A and Sdh-B, this test showed a significant excess of homozygotes (FIS). Observed heterozygosity (H_0) across the loci for all samples was higher than the mean expected heterozygosity (H_0) under the Hardy–Weinberg expectations. The Ewens–Watterson test for neutrality for each locus showed that the allele frequencies at all loci were selectively neutral in the studied population.

In the whole population of Tisovik, 31 alleles were detected, 4 of them had a frequency of less than 0.05 and 1 was very rare (<0.01). The parameters of genetic variation for each progeny line were given in Table 4. Two private alleles (A_p) were detected – in progeny line T9 (Pgi-A allele 1) and in T13 (Pgi-B allele 3). The percentage of polymorphic loci (P%) within the studied progeny lines ranged from 42.86 (T1) to 71.43 (T13), with a mean of 53.76; however, within the whole population, this percentage was 85.71%. The lowest value of mean number of alleles per locus (A_L) was noted in progeny lines T1 and T8, and the highest in T29. The mean number of alleles per locus (A_L) for the whole population was 2.36.

The highest values of the observed (H_0) and the expected (H_E) heterozygosity were in progeny line T5, and this was even higher than that in the population. Fixation indices FIS showed that all progeny lines except two (T6 and T16) exhibited an excess of heterozygotes.

Table 4. Parameters of genetic variation for each silver fir progeny line for isozyme and microsatellite loci: N – size of sample; P% – percentage of polymorphic loci; A – number of alleles; A_L – mean number of alleles per locus; A_R – mean allelic richness; A_p – private alleles; Ho and He – observed and expected heterozygosity, respectively; FIS – inbreeding coefficient

<table>
<thead>
<tr>
<th>Progeny line</th>
<th>N</th>
<th>Isozyme</th>
<th>SSR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P%</td>
<td>A</td>
</tr>
<tr>
<td>T1</td>
<td>14</td>
<td>42.86</td>
<td>20</td>
</tr>
<tr>
<td>T2</td>
<td>15</td>
<td>50.00</td>
<td>23</td>
</tr>
<tr>
<td>T3</td>
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<td>T4</td>
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</tr>
<tr>
<td>T9</td>
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<td>64.29</td>
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<tr>
<td>T11</td>
<td>15</td>
<td>35.71</td>
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<tr>
<td>T12</td>
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<td>24</td>
</tr>
<tr>
<td>T13</td>
<td>15</td>
<td>71.43</td>
<td>25</td>
</tr>
<tr>
<td>T15</td>
<td>15</td>
<td>57.14</td>
<td>25</td>
</tr>
<tr>
<td>T16</td>
<td>15</td>
<td>50.00</td>
<td>24</td>
</tr>
<tr>
<td>T17</td>
<td>15</td>
<td>57.14</td>
<td>25</td>
</tr>
<tr>
<td>T18</td>
<td>15</td>
<td>57.14</td>
<td>25</td>
</tr>
<tr>
<td>T21</td>
<td>13</td>
<td>57.14</td>
<td>24</td>
</tr>
<tr>
<td>T22</td>
<td>15</td>
<td>50.00</td>
<td>22</td>
</tr>
<tr>
<td>T23</td>
<td>8</td>
<td>57.14</td>
<td>24</td>
</tr>
<tr>
<td>T29</td>
<td>15</td>
<td>57.14</td>
<td>26</td>
</tr>
<tr>
<td>Mean</td>
<td>14.421</td>
<td>53.759</td>
<td>23.5</td>
</tr>
<tr>
<td>SE</td>
<td>0.185</td>
<td>2.341</td>
<td>0.421</td>
</tr>
<tr>
<td>Total</td>
<td>274</td>
<td>85.710</td>
<td>31</td>
</tr>
</tbody>
</table>
The highest excess of heterozygotes occurred in progeny line T22.

Hierarchical AMOVA showed that the coefficient of gene differentiation ($\Phi_{PT}$) between progeny lines was 0.198. Most genetic diversity (80%) resulted from the variation within progeny lines, whereas the variation amongst progeny lines was 20% (Tab. 5).

Pairwise comparison of $F_{ST}$ showed the highest genetic differentiation between progeny lines T8 and T23 ($F_{ST} = 0.234$, significant at $p < 0.01$) and the lowest between progeny lines T2 and T17 ($F_{ST} = 0.001$) (data in documentation of work, does not attached). Nei’s (1972) genetic distances between 19 progeny lines ranged from 0.018 between progeny lines T17 and T18 to 0.114 between T3 and T29 (data in documentation of work, does not attached). On dendrogram (Fig. 1a), the progeny formed two groups. The first groups is divided into two subgroups: the first subgroup with progeny lines T17, T18, T13, T1, T5 and T12 and the second with T8, T2, T6, T4 and T21. The progeny lines T9, T3 and T11 join these two subgroups. The second group included progeny lines T22, T23, T29, T15 and T16.

**Table 5.** Analysis of molecular variance (AMOVA): df – number of degrees of freedom; SSD – sum of squared deviation; MSD – mean squared deviation; $\Phi_{PT}$ – the population genetic differentiation; $p$ – testing probability; $Nm$ – gene flow

<table>
<thead>
<tr>
<th>Marker</th>
<th>Source of variance</th>
<th>df</th>
<th>SSD</th>
<th>MSD</th>
<th>Variance component</th>
<th>Percentage of total variance (%)</th>
<th>$\Phi_{PT}$</th>
<th>$p$</th>
<th>$Nm$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>amongst progeny</td>
<td>18</td>
<td>100.2</td>
<td>5.6</td>
<td>0.615</td>
<td>20</td>
<td>0.198</td>
<td>0.01</td>
<td>1.015</td>
</tr>
<tr>
<td>Isozyme</td>
<td>within progeny</td>
<td>255</td>
<td>636.2</td>
<td>2.5</td>
<td>2.495</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>273</td>
<td>736.4</td>
<td>3.1</td>
<td>3.109</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>amongst progeny</td>
<td>18</td>
<td>81.2</td>
<td>4.5</td>
<td>0.221</td>
<td>14</td>
<td>0.143</td>
<td>0.01</td>
<td>1.501</td>
</tr>
<tr>
<td>SSR</td>
<td>within progeny</td>
<td>255</td>
<td>338.3</td>
<td>1.3</td>
<td>1.327</td>
<td>86</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>273</td>
<td>419.5</td>
<td>1.5</td>
<td>1.548</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** UPGMA dendrograms for progeny lines based on Nei genetic distances calculated via (A) isozyme and (B) nrSSR markers

[Diagram A and B showing UPGMA dendrograms for progeny lines]
Analysis of nrSSR

From four to six alleles (A) were identified at each locus (Tab. 3b). At loci SF 333 and SF b5, an exact test for the Hardy–Weinberg equilibrium indicated a significant deficit of heterozygotes (HWE). Locus SF 1 expressed an excess of heterozygotes (F_{IS}, F_{IT}). Loci SF 324 and SF 1 were in the Hardy–Weinberg equilibrium (HWE). The mean value of the inbreeding coefficient of an individual relative to the progeny line (F_{IS}) was 0.117 (p ≤ 0.001), but relative to the population as a whole (F_{IT}) was 0.186 (p ≤ 0.001), indicating a deficit of heterozygotes. The genetic differentiation (F_{ST}) was 0.105, and the level of gene flow (Nm) between the studied progeny lines was 2.172 migrants per generation.

Mean observed heterozygosity (H_{O} = 0.284) across the loci for all samples was lower than the mean expected heterozygosity (H_{E} = 0.308) under the Hardy–Weinberg expectations, but, totally, the progeny exhibited the Hardy–Weinberg equilibrium. The highest proportion of null alleles was discovered in locus SF b5, and across the loci for all samples, this was low and amounted to 0.053.

The parameters of genetic variation for each progeny line were given in Table 4. The highest number of alleles (A), the highest mean number of alleles per locus (A_{L}) and allelic richness (A_{R}) for all loci were detected in progeny line T1, and the lowest in T15. In sum, of the 20 alleles detected, 5 were unique to particular progeny lines: two private alleles (A_{P}) in progeny lines T1 and T9 and one in progeny line T29. The highest value of observed heterozygosity (H_{O}) was detected in progeny line T3, and the lowest in T2. Fixation indices (F_{IS}) showed that 6 progeny lines (T1, T3, T4, T5, T6 and T22) exhibited an excess of heterozygotes and 13 a deficit of heterozygotes. The highest deficit of heterozygotes occurred in progeny line T2.

AMOVA showed that 14% of the total variability was present amongst progeny lines and the remaining (86%) was attributable to differences within progeny lines (Tab. 5). These differences are statistically significant (Φ_{PT} = 0.143; p < 0.01). The overall gene flow (Nm) amongst progeny lines was low and amounted to 1.5, which gives an estimate of the average number of migrants between all the studied progeny per generation.

F_{ST} showed the highest genetic differentiation between progeny lines T1 and T13 (F_{ST} = 0.341, significant at p < 0.0001) and the lowest between progeny lines T3 and T6 and also T6 and T21 (F_{ST} = 0.001), and the mean value was equal to 0.11. F_{ST} averaged across the loci also indicated that progeny lines T1, T4, T5 and T9 differed most significantly from the others (data in documentation of work, does not attached).

On the basis of the calculated Nei genetic distances, a UPGMA dendrogram was constructed (Fig. 1b). The progeny formed three groups: the first with the progeny of T2, T23, T15, T22, T21, T12, T18, T29 and T16; the second with the progeny of T11, T17 and T13. To these two groups joined the progeny T9. An additional third group included progeny of T3, T6, T8 and T5. To these progeny, offspring T4 is linked and T1 is the most differentiated progeny. Nei’s (1972) genetic distances between 19 progeny lines ranged from 0.004 between progeny lines T2 and T23 to 0.245 between T1 and T13 (data in documentation of work, does not attached).

Discussion

Genetic research into the maternal silver fir from the Tisovik Reserve has been relatively intensive over the past two decades, since the dieback of this population and the rapidly decreasing number of fir trees was first noticed. On isozyme markers, this stand was analysed by Mejnartowicz (1996a, 1996b) on 11 maternal trees (from 20 which still growing there) using 11 enzyme systems (17 loci) and by Goncharenko and Savitsky (2000) on 15 enzyme systems (22 loci).

Our results obtained from isozyme markers (12 loci, nine enzyme systems) showed that the value of polymorphic loci (P%) ranged from 28.57% (in the T8 progeny line) to 71.43% (in the T13 progeny line) with an average value of 53.76%, whereas in maternal trees, (Mejnartowicz 1996a, 1996b) 21.49% of polymorphic loci was detected. We discovered that the mean number of alleles per locus (A_{L}) is 1.677, slightly more than that in maternal trees, A_{L} = 1.353 (Mejnartowicz 1996a, 1996b) and 1.409 (Goncharenko and Savitsky 2000). The mean value of expected heterozygosity (H_{E}) was 0.245 and observed heterozygosity (H_{O}) 0.297; these values were higher than that in maternal trees: H_{E} = 0.079 and H_{O} = 0.123 (Mejnartowicz 1996a, 1996b) and, respectively, 0.111 and 0.127 (Goncharenko and Savitsky 2000). So progeny, similar to maternal trees, demonstrated the excess of heterozygotes, what confirm the inbreeding indices F_{IS} = −0.210 and F_{IT} = −0.024 for progeny and less for maternal trees (−0.035;
The largest excess of heterozygotes was noted in progeny lines T22 (−0.629) and T1 (−0.516) and the smallest in T18 (−0.011). Only two progeny lines – T6 and T16 – demonstrated a slight excess of homozygotes (0.013 and 0.073, respectively). Measurement of genetic differentiation between progeny lines (F_ST = 0.166) showed a greater level of variation than maternal population (0.038) (Goncharenko and Savitsky 2000). This conclusion confirmed the result of AMOVA, where ΦPT was 0.198.

Mejnartowicz (1996a, 1996b) compared Tisovik to eight other stands from Poland (Tomaszów Lubelski, Skarżysko, Komańcza, Międzygórze and four man-made stands from Białowieża Forest). He discovered that Tisovik is characterised by the lowest mean number of alleles per locus, percentage of polymorphic loci and expected heterozygosity with comparison to other populations (A_L ranged from 1.412 to 1.580, P% from 23.529 to 41.176 and H_E from 0.123 to 0.158) and showed excess of heterozygosity similar to that in case of Skarżysko and three stands from Białowieża. A dendrogram constructed based on the Nei genetic distances showed that Tisovik fir is the most different from other populations.

Goncharenko and Savitsky (2000) also compared the Tisovik population to six other populations from Ukraine (Drohobycz, Delyatyn and Worochta) and Poland (Jata, Roztocze and Łabowiec) and thus to populations from the north-eastern border of the species range. These populations were characterised by a higher number of alleles per locus (from 1.500 to 1.818) and a higher expected heterozygosity – from 0.113 to 0.147 (except Jata 0.100) – than was the case in Tisovik. Similar to Tisovik, four populations displayed an excess of heterozygotes and two – Jata and Drohobycz – an excess of homozygotes. Jata is a population that is growing on the border of the range, but Drohobycz is growing in dense distribution. Nei’s genetic distances and a dendrogram constructed based on the Nei genetic distances were as follows: SF 1 (Ho = 0.333, He = 0.598, A = 3), SF 333 (Ho = 0.391, He = 0.792, A = 6), SF 324 (Ho = 0.333, He = 0.348, A = 3) and SF b5 (Ho = 0.682, He = 0.883, A = 5). Comparing these values with our results, we found lower values, particularly for locus SF b5 (Ho = 0.062, He = 0.110), but higher numbers of alleles from 4 to 6 (only in locus SF 333 detected the same number of alleles). Each loci studied by Cremer et al. (2006) showed excess of homozygotes, similar to that in Tisovik progeny, except locus SF 1, which exhibited their excess.

Another Cremer et al.’s (2012) study was conducted in the Black Forest, a low mountain range in the south-western part of Germany. He compared mother trees to seeds on five SSR loci (three the same like in our work) and reported that values of expected and observed heterozygosity and the number of detected allelic richness were as follows: SF 1 (Ho = 0.534, He = 0.539, A_R = 5), SF 333 (Ho = 0.489, He = 0.720, A_R = 6), and SF b5 (Ho = 0.467, He = 0.471, A_R = 7) for forest stands and for seeds: SF 1 (Ho = 0.441, He = 0.509, A_R = 7), SF 333 (Ho = 0.557, He = 0.693, A_R = 7) and SF b5 (Ho = 0.457, He = 0.529, A_R = 8). So in seeds, more alleles were found in every loci, and both mother trees and seeds were characterised by excess of homozygotes. However, this excess was much larger for seeds in loci SF 1.
and SF b5 and for mother trees in locus SF 333. The values of \( H_O \) and \( H_E \) for seeds were similar to our results especially in loci SF 1 and SF 333, but much higher in locus SF b5. We found less number of alleles in comparison especially to seeds and, to a lesser extent, mature trees. We analysed seedlings (so middle phase of trees growth) but only in one small and isolated population.

Lately, Cvrčková et al. (2015) had studied eight populations of silver fir from Czech Republic using eight microsatellites markers, but only one locus was the same like in our research (SF 1). Cvrčková et al. (2015) detected five alleles in this locus and the expected and observed heterozygosity are as follows: \( H_E = 0.451 \) and \( H_O = 0.473 \). In Tisovik progeny, four alleles were detected and the expected and observed heterozygosity are 0.303 and 0.386, respectively, so locus SF 1 in these two cases showed excess of heterozygotes. Three other loci studied by Cvrčková et al. (2015) also showed similar pattern of homozygote deficiency.

Comparing the results obtained from 4 SSR and 12 isozyme loci, it may be concluded that the mean value of expected heterozygosity (\( H_E \)) was at a similar level as in isozymes (0.308 and 0.299), but that of observed heterozygosity (\( H_O \)) was lower (0.284 and 0.345). Four progeny lines (T5, T9, T11 and T21) had similar values of observed heterozygosity detected from isozyme and SSR markers, six (T1, T3, T4, T6, T8, T16) had higher values of observed heterozygosity for SSR markers and the remaining nine for isozymes (Fig. 2a). The parameters of inbreeding (\( F_{IS} \)) exhibited the fact that offspring were characterised by a slight excess of homozygotes (the largest was noted in progeny lines T2 and T18, and the smallest in T21). Six progeny lines (T1, T3, T4, T5, T6 and T22) demonstrated slight excesses of heterozygotes.

![Figure 2. Comparison between isozyme and SSR markers for (A) observed heterozygosity and (B) inbreeding index](image-url)
Only progeny line T6 showed an excess of homozygotes in isozymes and a deficiency in microsatellite markers (Fig. 2b). Measures of genetic differentiation amongst progeny lines ($F_{ST} = 0.105$; $\Phi_{PT} = 0.143$) showed smaller values than that detected by isozyme markers. The gene flow ($N_m$) amongst progeny lines was low (1.015 for isozyme and 1.501 for DNA markers), what may give evidence about internal division in this population on subgroups. It may be a result of more frequent pollination by some paternal trees (e.g. trees 6, 7, 8, 9, 13 or 18, which are growing on the middle of this stand) than others that may have less chance on pollination because growing on the border of this population (Pawlaczyk et al. 2005). The presence of the private alleles both in SSR and isozyme markers may be effected by pollination of foreign pollen from Polish part of Bialowieza Forest where in 1920s and 1930s of XX century had planted the seedling of silver fir of unknown origin (Mejnartowicz 1996a, 1996b, Korczyk et al. 1997).

Describing diversity of the offspring of Tisovik Reserve using SSR markers and comparing to other Polish populations from dense range (Jata Reserve – near Lublin, Kamienna Góra Reserve – Roztocze National Park and Łabowiec Reserve – Beskid Sądecki) (Pawlaczyk et al. 2010), it may be concluded that Tisovik progeny are characterised by a lower observed and expected heterozygosity ($H_o$ and $H_e$). In Pawlaczyk et al.’s (2010) paper, two loci were analysed (SF 1 and SF 333), and they detected three to five alleles per locus SF 1 and eight alleles per locus SF 333. Values of observed heterozygosity ($H_o$) for SF 1 ranged from 0.428 for Jata to 0.467 for Kamienna Góra and those of expected heterozygosity ($H_e$) ranged from 0.507 for Kamienna Góra to 0.517 for Jata. Respectively, these values for Tisovik were 0.386 for $H_o$ and 0.303 for $H_e$. Similarly, values of SF 333 ranged from 0.607 for Kamienna Góra to 0.627 for Łabowiec and those of $H_e$ ranged from 0.743 for Kamienna Góra to 0.755 for Łabowiec. Respectively, these values for Tisovik were 0.552 for $H_o$ and 0.658 for $H_e$. So Tisovik progeny are characterised by a lower observed and expected heterozygosity. Progeny of populations from dense range showed excess of homozygotes in these two loci, whilst Tisovik progeny in locus SF 333, but in locus SF 1 excess of heterozygotes. However, when four SSR loci are taken into account, the deficiency of heterozygotes was detected.

Differences in the levels of heterozygosity in Tisovik offspring between used markers may be the result of inbreeding and genetic drift, which is very likely in a small and isolated population. A similar situation has been described in Pinaceae by many authors, such as Potter et al. (2008) in Fraser fir and Głowacki et al. (2005) in dwarf mountain pine. Potter et al. (2008) detected a bigger deficit of heterozygosity in microsatellite markers than in enzyme markers. The consistently significant deficiency of heterozygotes across loci and populations was explained by Potter et al. (2008) on the basis of inbreeding, the Wahlund effect caused by spatial substructuring of intra-population demes and out of Hardy–Weinberg equilibrium than any overriding effect of null alleles.

The grade of variability may also be a result of long selection and adaptation to rapidly changing environmental factors in the Bialowieza Primeval Forest after the last glaciation. Fir is especially sensitive to the low temperatures present in February and the impact of the continental climate in this region (Klisz et al. 2016). The differences in heterozygosity found between the SSR and isozyme markers may result from the fact that proteins are more sensitive to selection. So homozygotes are removed from a population and heterozygotes are maintained as they are more suited to the environmental conditions.

Tisovik Reserve is a small population that counted only 20 mature trees and had been damaged by anthropogenic changes to sites. The impact of population size and human activity on the level of diversity of silver fir has been observed in the Sudeten Mts., where this site is considerably smaller than the population in the Carpathian Mts. Lewandowski et al. (2001) on 9 enzyme systems with 13 loci detected low number of alleles per locus (from 1.23 to 1.54) in the Sudeten Mts. and a low grade of variation. A comparison between the Sudeten and Carpathian Mts. silver fir in Poland was carried out by Mejnartowicz (2004) on 14 enzyme systems with 28 loci. He described the great Nei genetic distance (from 0.173 to 0.252) that separated the Sudeten and Carpathian populations and was the result of restricted gene flow between populations and their isolation. The Sudeten populations were characterised by a lower level of observed and expected heterozygosity (0.130 and 0.129, respectively) than the Carpathian ones (0.275 and 0.269, respectively) and lower genetic diversity. Another example of the impact of population
size on diversity was given by Mejnartowicz (2003) on 14 enzyme systems with 28 loci. He studied silver fir from the Western Beskid Mts. (southern Poland), which is characterised by a special orographic formation causing differentiation in plant populations and limiting the number of trees. He discovered that the Western Beskid Mts. had a slightly smaller genetic variability and diversity in comparison to the Eastern Beskid Mts. Both were characterised by an excess of heterozygotes ($F_{IS} = -0.017$ for Eastern and $F_{IS} = -0.064$ for Western Beskid), but the Eastern Beskid Mts. had a greater heterozygosity and a greater number of effective alleles.

Higher genetic diversity as a buffer to the potential threat of dramatic change in climatic conditions was indicated by Longauer et al. (2003) on 11 enzyme systems with 18 loci. They described this phenomenon as the result of vulnerability (lower adaptability). They compared populations from northern and southern regions of fir range and detected that northern populations were characterised by a slight excess of homozygotes, a lower total number of alleles, a lower mean number of alleles per locus and a lower grade of variation than populations from southern regions, which are more exposed to rapidly changing environmental factors.

**Conclusions**

Tisovik is a small, isolated population that has limited number of individuals to crossing. In such population, the higher number of homozygotes is expected as a result of inbreeding. In addition, studied firs were 15-year-old (in juvenile phase), where the selection on homozygotes takes place (which was detected by SSR markers). On the other hand, progeny of Tisovik showed high level of differentiation as an offspring of small, isolated stand. Distinctiveness of some progeny lines and the presence of the private alleles may be the result of pollination from Polish part of Białowieża Forest. Another reason of high variation may be such that some paternal trees may take part into pollination with more frequency than others. So the substructuring of population is observed (dendrograms), and the deficit of heterozygotes detected may be ostensible as a result of Wahlund effect. Besides, isozyme markers detected excess of heterozygotes what may confirm this thesis and be caused by the bottle neck effect postulated by Mejnartowicz (1996a, 1996b). Such pattern of genetic structure can also be an effect of harsh environmental conditions exerting selection pressure and modifying the genetic composition of this population.

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**References**


Genetic variation of silver fir progeny from Tisovik Reserve population...


Longauer R. 1994. Genetic differentiation and diversity of European silver fir in Eastern part of its natural range. EDER, In. (Zusammengest.): 7 IUFRO
Tannensymposium vom 31.10.-4.11. 1994. in Altensteig, Deutschland.


Assessment applicability of selected models of multiple discriminant analyses to forecast financial situation of Polish wood sector enterprises

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ABSTRACT

In the last three decades forecasting bankruptcy of enterprises has been an important and difficult problem, used as an impulse for many research projects (Ribeiro et al. 2012). At present many methods of bankruptcy prediction are available. In view of the specific character of economic activity in individual sectors, specialised methods adapted to a given branch of industry are being used increasingly often. For this reason an important scientific problem is related with the indication of an appropriate model or group of models to prepare forecasts for a given branch of industry. Thus research has been conducted to select an appropriate model of Multiple Discriminant Analysis (MDA), best adapted to forecasting changes in the wood industry. This study analyses 10 prediction models popular in Poland. Effectiveness of the model proposed by Jagiello, developed for all industrial enterprises, may be labelled accidental. That model is not adapted to predict financial changes in wood sector companies in Poland.

The generally known Altman model showed the greatest effectiveness in the identification of enterprises at risk of bankruptcy. However, that model was burdened with one of the greatest errors in the classification of healthy enterprises as sick. The best effectiveness in the identification of enterprises not threatened with bankruptcy was found for forecasts prepared using the Prusak 2 model. However, forecasts based on those models were characterised by erroneous classification of sick companies as healthy. The model best fit to predict the financial situation of Polish wood sector companies was the Poznań model

\[ Pz = 3.562 \cdot X_1 + 1.588 \cdot X_2 + 4.288 \cdot X_3 + 6.719 \cdot X_4 - 2.368 \]

where:

- \( X_1 \) – net income/total assets;
- \( X_2 \) – (current assets – stock)/current liabilities;
- \( X_3 \) – fixed capital/total assets
- \( X_4 \) – income from sales/sales revenue).

KEY WORDS

prediction models, wood sector, Multiple Discriminant Analysis
**INTRODUCTION**

Globalisation and strong pressure imposed by market competition are trends observed in all branches of industry. This also pertains to the wood industry. Implementation of liberal market principles in Poland brought numerous positive economic and financial changes, accompanied at the same time by adverse phenomena, unfortunately also negative, resulting e.g. from overdue payment of receivables or impossibility of their recovery. Competitiveness of wood sector companies on the local, domestic or world markets to an increasing extent depends on the ability to supply customised ranges of products, individual products and services sufficiently efficiently and fast (Adamowicz et al. 2016). An additional element of good management is connected with access to reliable information. In economy we are observing increasing demand for information concerning bankruptcy in the wood sector. This presents an impulse for the scientific community to search for methods and mechanisms making it possible to monitor changes taking place on the timber market. This problem has gained in importance in relation with the recent economic crisis. Additionally, Brexit may have a negative effect on European markets.

Ribeiro et al. (2012) stated that in the last three decades prediction of enterprise bankruptcy has become an important and difficult task, thus becoming an impulse for many studies. This opinion has been shared by many other researchers e.g.: Abellán and Mantas (2014), Adamowicz and Noga (2014), Afik et al. (2016), Brezigar-Masten and Masten (2012), Chen et al. (2013), Delen et al. (2013), Ribeiro et al. (2012) and others.

Prediction of the financial situation of enterprises is crucial for managers, creditors and investors, since it makes it possible to make appropriate business decisions and reduce potential economic losses. Prediction of insolvency and rating predictions are essential in the world of finances. Inaccurate predictions may result in erroneous decisions and as a result lead to huge financial losses (Obermann & Waack, 2015). For this reason in practice systems forecasting the financial situation of enterprises are applied (Early Warning Systems – EWS). EWS is a system used to predict both the level of enterprise success and decline (Ozgulbas and Koyuncugil 2010). In practice there are many bankruptcy prediction models. Due to the specific character of individual branches of industry not all EWSs may be successfully applied in practice. For this reason studies have been undertaken to indicate the most adequate EWS for Polish wood sector enterprises.

**Methods**

Most models that are designed by financial institutions or by scholars to forecast corporate bankruptcy are usually built using a limited number of financial ratios that are measured once (Balcaen and Ooghe 2006). For this reason Multiple Discriminant Analysis (MDA) was the primary research method applied in this study. Failure models are usually designed using financial ratios calculated with data from balance sheets and income statements. The use of ratios is as much due to their predictive power as to their availability and standardization. They generally allow for good discrimination between failed and non-failed firms (Altman 1968), are easily available and are homogeneous, because they are calculated in the same way within a given regulatory framework (du Jardin 2016). In view of the above, forecasts based on discriminant models utilise prognostic properties of financial indexes. These indicators have been constructed based on reporting data from 142 Polish enterprises from the wood sector.

The research process was conducted using data coming from enterprises solely from the region, where timber has been the basis for the production process. Classification of enterprises is consistent with the Polish Classification of Economic Activity. Analyses were based on source materials from sections: 16 – Production of wood products; 17 – Production of paper and paper products, and 31 – Production of furniture.

**Experimental material**

The primary criterion in the collection of source data was connected with availability of financial information. The opinion of Gruszczynski (2005), that a key element in the prediction of bankruptcy is related to the selection of the research sample, was followed in this study. Prior to empirical studies source data were accumulated and catalogued in order to construct a comprehensive, reliable and cohesive database of financial information for individual wood sector companies. Wood companies, for which a motion for bankruptcy and/or
a motion for the initiation of debt conciliation were filed, the co-called failed businesses (FB), were identified and catalogued. Information was also gathered on enterprises with a stable financial situation, the so-called healthy firms (HF). Following the recommendation by Hołda (2001), only companies publishing their complete financial statements were selected for analyses.

Financial statements of those enterprises came from Regional Courts and credit information agencies. For all enterprises in the analytical sample data were collected, based on which their financial and economic standing could be established. The primary criterion identifying a given enterprise as bankrupt was connected with the submission of a bankruptcy motion to a respective regional Economic Department of the National Court Register. Wood sector companies in an advantageous economic and financial situation were identified based on general financial information contained in balance sheets as well as profit and loss accounts, focusing primarily on total debt, profitability and liquidity indexes.

Gathered source materials were comprehensive and selected in terms of the organisational structure, considering the volume of financial assets of analysed companies. The sample of non-bankrupt enterprises comprised only those companies, which did not go bankrupt throughout the entire period of analysis.

Enterprises, which financial data were used in this study, came from all regions of Poland and represented varied legal forms of economic activity. They included stock corporations, limited liability companies, registered partnerships and cooperatives publishing financial statements.

Collected data were analysed to verify whether they have a normal distribution. As a result, the analytical sample comprised a total of 90 companies, which declared their bankruptcy in the years 2006–2012, as well as 52 enterprises, which during that period continued their economic activity. Potential explanatory variables were financial indexes applied in prediction models presented in the further part of this study. After collection and verification of required financial data the reliability of prediction was assessed for the selected multiple-branch models.

**Prediction models**

The following models were selected for tests: Altman (A), Gajdka and Stos (GS), Hadasik (Ha), Hołda (H), Jagiełło (J), Maćczyńska (M), Poznań (Pz), Prusak 1 (P1), Prusak 2 (P2) and Wierzba (W). Selection of these models was connected with their earlier application in Poland.

In order to verify EWS applicability in forecasting of bankruptcy prediction in the wood sector, forecasts were made based on the enterprise classification matrix. Analyses were conducted on EWSs popular in Poland:

\[
A = 0.717 \cdot X_1 + 0.847 \cdot X_2 + 3.107 \cdot X_3 + 0.420 \cdot X_4 + 0.998 \cdot X_5
\]

where:

- \(X_1\) – working capital/total assets;
- \(X_2\) – retained profit/total assets;
- \(X_3\) – EBIT/total assets;
- \(X_4\) – equity/total debt;
- \(X_5\) – sales revenue/total assets.

\[
GS = 0.7732059 - 0.0856425 \cdot X_1 + 0.0007747 \cdot X_2 + 0.9220985 \cdot X_3 + 0.6535995 \cdot X_4 - 0.594687 \cdot X_5
\]

where:

- \(X_1\) – sales revenue/total assets (mean annual value);
- \(X_2\) – current liabilities x 360/costs of goods sold;
- \(X_3\) – net profit/total assets (mean annual value);
- \(X_4\) – gross profit/net sales revenue;
- \(X_5\) – total liabilities/total assets.

\[
Ha = 2.36261 + 0.3654259 \cdot X_1 - 0.765526 \cdot X_2 - 2.40435 \cdot X_3 + 1.59079 \cdot X_4 - 0.00230258 \cdot X_5 - 0.0127826 \cdot X_6
\]

where:

- \(X_1\) – current assets/current liabilities;
- \(X_2\) – current assets – stock/current liabilities;
- \(X_3\) – total liabilities/total assets;
- \(X_4\) – (current assets – current liabilities)/total liabilities;
- \(X_5\) – receivables/sales revenue;
- \(X_6\) – stock/sales revenue.

\[
H = 0.605 + (6.81 \cdot 10^{-1}) \cdot X_1 + (1.96 \cdot 10^{-2}) \cdot X_2 + (1.57 \cdot 10^{-1}) \cdot X_3 + (9.69 \cdot 10^{-3}) \cdot X_4 + (6.72 \cdot 10^{-4}) \cdot X_5
\]

where:

- \(X_1\) – operating assets/current liabilities;
X2 – total liabilities x 100/total property;
X3 – total revenue/mean annual total property;
X4 – net profit (loss) x 100/average total property;
X5 – average current liabilities x 360/costs of sold products, goods and materials.

\[ J = -1.8603 + 12.296 \cdot X_1 + \\
+ 0.1675 \cdot X_2 + 1.399 \cdot X_3 \]

where:

X1 – gross income from sales/costs of operating activity;
X2 – total income/total assets;
X3 – equity/total liabilities.

M = 1.5 \cdot X_1 + 0.08 \cdot X_2 + 10.0 \cdot X_3 + \\
+ 5.0 \cdot X_4 + 0.3 \cdot X_5 + 0.1 \cdot X_6

where:

X1 – (gross profit + depreciation)/total liabilities;
X2 – balance sheet total/total liabilities;
X3 – gross profit/balance sheet total;
X4 – Gross profit/sales revenue;
X5 – Stock/sales revenue – inventory turnover index;
X6 – Sales revenue/balance sheet total – asset turnover index

\[ P_z = 3.562 \cdot X_1 + 1.588 \cdot X_2 + \\
+ 4.288 \cdot X_3 + 6.719 \cdot X_4 - 2.368 \]

where:

X1 – net income/total assets;
X2 – (current assets – stock)/current liabilities;
X3 – fixed capital/total assets
X4 – income from sales/sales revenue

\[ P_1 = 6.524 \cdot X_1 + 0.148 \cdot X_2 + \\
+ 0.406 \cdot X_3 + 2.176 \cdot X_4 - 1.568 \]

where:

X1 – operating profit/balance sheet total;
X2 – operating cost/current liabilities;
X3 – operating assets/current liabilities;
X4 – operating profit/sales revenue

\[ P_2 = 1.438 \cdot X_1 + 0.188 \cdot X_2 + \\
+ 5.023 \cdot X_3 - 1.871 \]

where:

X1 – (net income + depreciation)/total liabilities;
X3 – operating profit/sales revenue

\[ W = 3.26 \cdot X_1 + 2.16 \cdot X_2 + 0.3 \cdot X_3 + 0.69 \cdot X_4 \]

where:

X1 – (profit from operating activity – depreciation)/total assets;
X2 – (profit from operating activity – depreciation)/sales of products;
X3 – operating assets/total liabilities;
X4 – operating capital/total assets.

In literature on the subject we may find e.g. the GS model presented by Godlewska 2010.

Selection of companies

Using the above-mentioned MDAs forecasts were prepared, which were next verified with the actual economic situation. Analyses were performed based on financial data for the population of healthy firms, i.e. HF, and failed businesses (FB). Forecasts were based on the collected retrospective data from the period of 1-, 2- and 3-year periods preceding the year of forecast, which was at the same time the year of its verification.

The use of data from FB and HF in the analyses was connected with the search for a method facilitating an effective prediction of bankruptcy of enterprises under typical economic conditions, in which successful companies operate next to companies going bankrupt.

After forecasts had been made, they were verified with the actual financial situation of the analysed enterprises. Forecast of bankruptcy was conducted separately for 1 year before, for 2 years before and for 3 years before bankruptcy of that firm.

It needs to be stressed that some multi-branch models assume an interval of the explained variable, within which we may not definitely verify the financial situation of a firm (the so-called grey zone). For the needs of this study this area was eliminated by dividing the interval of values corresponding to the grey zone into halves. In the interpretation of the obtained empirical values using the multi-branch models assuming the existence of a grey zone, i.e. A, H, P1, P2 and M, it was assumed that values found in the lower half of the grey zone interval correspond to enterprises at risk of bankruptcy, while values from the upper half – healthy companies. In the other models boundary values were retained in the original form. This approach made possible a direct comparison of individual models.
General economic efficiency indexes as well as type I and type II errors were used in order to assess applicability of individual forecasts prepared using the analysed models in the assessment of wood sector enterprises.

**Enterprise Classification Matrix**

In order to determine which model to a greater degree identifies the risk of bankruptcy for wood sector firms, a test verifying the accuracy of the assessments was performed, i.e. Enterprise Classification Matrix (ECM). ECM is a tool, using which the accuracy of indications of an estimated model is assessed (Card 1982). Using ECM the effectiveness of appropriate classification of enterprises to one of the two groups (healthy vs. failing companies) was evaluated.

Following the recommendations of Prusak (2004), measurement results were interpreted using type I (EI) and type II errors (EII) as well as general efficiency index (SP).

Type I error, which defines what percentage of bankrupt companies was erroneously classified as healthy companies, was calculated using the following formula:

$$EI = \frac{NP1}{(P1 + NP1)} \times 100\%$$

where:

- $P1$ – forecasted number of bankrupt companies correctly classified to the population of failed businesses;
- $NP1$ – forecasted number of non-bankrupt companies erroneously classified to the population of failed businesses.

Type II error, based on which it was determined what percentage of enterprises with a stable financial situation was erroneously classified as bankrupt companies, was calculated according to the following formula:

$$EII = \frac{NP2}{(P2 + NP2)} \cdot 100\%$$

where:

- $P2$ – forecasted number of non-bankrupt firms correctly classified to the population of non-bankrupt firms;
- $NP2$ – forecasted number of bankrupt companies erroneously classified to the population of non-bankrupt firms.

The general efficiency index, using which the relative size of correctly classified enterprises was established, was calculated according to the formula:

$$SP = \frac{(P1 + P2)(P1 + NP1 + P2 + NP2)}{(P1 + NP1 + P2 + NP2)} \cdot 100\%.$$
which averaged SP exceeded 80%. The lowest averaged SP was obtained for forecasts prepared using the J model J (54%). When applying that model EI (29%) was lower than the averaged value, but EII reached the record value of 62%.

In view of 1-, 2- and 3-year forecasts it was found that in all the cases the highest SP was obtained at the application of the Pz model, while it was lowest for the J model. SP for the Pz model was 86% for 1-year forecasts, while EI was 6% and EII – 20%, for 2-year forecasts SP was 76%, EI – 33% and EII – 13%, for 3-year forecasts SP was 83%, EI – 20% and EII – 13%, respectively. For comparison, in the model considered the worst, i.e. the J model, these values were as follows: 1-year forecasts SP – 56%, EI – 33%, EII – 53%, 2-year forecasts SP – 53%, EI – 33%, EII – 60% and 3-year forecasts SP – 53%, EI – 20% and EII – 73% (tab. 1). It needs to be stated that the forecast prepared using that model was comparable to random classification.

**Discussion**

Discussions and assessment of effectiveness of prognostic models focused on the identification of risk of bankruptcy have been conducted by researchers for a long time. Relationships and research trends in the prediction of business failure were discussed in detail e.g. by Dimitras et al. (1996). While since the 1980s the use of MDA has decreased it still remains a generally accepted standard method and it is frequently used as a baseline method for comparative studies (Altman and Narayanan 1997). MDA has been replaced by less demanding statistical techniques such as logit analysis, probit analysis and linear probability modelling (Balcaen and Ooghe 2006). Nevertheless, MDA may be used to predict bankruptcy of enterprises.

There is a need to adapt prognostic models to current domestic conditions, dependent on political and economic changes as well as the modified concept of enterprise management (Juszczyk and Balina 2014). Stawicki and Sojak (2001) as well as Juszczyk and Balina (2014) stressed the need to conduct studies for specific branches of the economy. Variation and dynamics of economic processes and their multifaceted character are so high that it is difficult to consider all significant factors affecting operations of individual economic entities within one model. This proposal may be met by construction of new models or indication of an appropriate model from among those already existing.

Economic forecasting makes it possible to predict the course of trends for free-market economy. However, we need to remember of the selection of an appropriate prediction method. It is crucial that even at the application of adequate methodology economic forecasts may not be treated as the only indicator when making economic decisions (Kocel 2010). However, it does not mean that scientific forecasts of market changes e.g. connected with the number of prospective customers fulfilling their liabilities, is not an important issue in the management process in all sectors, including the wood sector. Prediction of bankruptcy for industrial enterprises in the wood sector is fundamental in view of the multifaceted risk of financial insolvency in free-market economy. It may be an important element supporting the decision making process.

Obviously making decisions in all enterprises is closely connected with the adopted strategy within the framework of the profile of company operations. Adequately effective models (in terms of the accuracy of forecasted market changes) support the decision making process, which makes it possible to reduce the risk of loss of receivables, resulting from the previously executed commercial transactions. We need to agree with the opinion by Kocel (2010) that forecasts should be as accurate as it is possible, particularly in view of the potential financial losses incurred in the case of erroneous forecasts, thus it is advisable to conduct studies indicating appropriate methods to predict bankruptcy in wood sector companies. An additional argument suggesting an urgent need to undertake studies aiming at the identification of an appropriate prediction method for the financial situation of enterprises was connected with an increasing impact of bankruptcy of wood sector companies. This problem affected both manufacturing and commercial enterprises.

Despite the currently stable financial situation in Polish forestry, companies are going bankrupt in the associated wood sector. It seems that deficit of timber assortments in the market of round wood is one of the causes for such a situation. When analysing problems with access to the Polish raw material base it needs to
be stressed that demand on the timber market is a consequence of demand for timber-derived products and it depends on the buying power of households as well as related sectors, e.g. construction industry. Additionally, the current situation in the wood sector is influenced, similarly as the entire economy, by the conditions for operating on the common European market. It pertains first of all to the open market of goods and services as well the labour market, transfer of subsidies, influx of foreign capital in direct foreign investments. They coincide with globalisation processes and business trends in world economy, particularly crisis in financial markets and collapse of major economies both in Europe and worldwide at the end of 2008 and in 2009, followed by the decline and unstable political and economic situation in Greece and Italy in 2011, as well as the current consequences of Brexit.

Analyses of literature on the subject of bankruptcy of firms in the wood sector provide grounds for a thesis on the necessity to search for economic instruments supporting the decision making process in the performance of marketing objectives. One of such instruments is provided by models predicting bankruptcy of enterprises. Their practical importance is considerable due to the potential adoption of appropriate prevention measures based on these forecasts. It needs to be remembered that preventive actions make it possible to avoid negative financial consequences connected with the erroneous selection of contractors. Obviously indication of an appropriate prediction model based on sector predictors is of importance both for science and commercial practice. A sector-based bankruptcy prediction model should be applied both in the decision-making system of forest administration supplying timber for industry and in enterprises converting timber. This model may be particularly important for listed companies. Economic forecast is a basic tool in the assessment of the future financial standing of an enterprise, which affects quotations of a given company. Discriminant models may be a source of information for market contractors concerning the future financial situation and potential bankruptcy of enterprises. Accurate economic inferences based on results of prediction analyses may be made only thanks to selecting an appropriate prediction model for a given sector.

In order to satisfy the formulated thesis on the necessity to construct a prognostic sector-based model and estimate theoretical models, studies were undertaken to identify this problem in the wood sector. When conducting the research process presented in this study the currently existing prediction models for company bankruptcy were also considered.

A significant element affecting reliability of forecasts prepared using early warning models is their static nature. This problem was indicated e.g. by Grice and Dugan (2001) and Balcaen and Ooghe (2006). They stated that static values used in calculation of specified financial indexes investigated in a given period do not consider a dynamic approach to company bankruptcy. This problem has become increasingly important especially recently. For this reason in this study in order to include the dynamics of changes taking place in the timber market in terms of enterprise bankruptcies, we adopted the principle of performing analyses in a step-wise system and the prepared forecasts (1-, 2- and 3-year) were compared with actual market events occurring in the successive years. Thanks to such an approach we could consider the dynamics of occurring economic changes in our investigations.

The next element taken into consideration in this study was the period of analysis. Based on a review of literature it was found that a frequent objection to models forecasting enterprise bankruptcy is their becoming obsolete as well as an insufficient period of analysis, thus producing an inadequately small number of data. This has prevented a reliable interpretation of the situation of enterprises or bankruptcy forecasting. As it was indicated by Matuszyk (2003) and Agarwal and Taffler (2007), testing of efficiency of a prognostic model should cover a period from four to six years. Taking into consideration the above observations this study was based on financial data from the period of 2006–2012.

Within this study we identified wood sector companies threatened with bankruptcy. It was established that the effectiveness of forecasts obtained from individual models varied. Averaged general efficiency of these forecasts ranged from 54% (the J model) to 82% (the Pz model). Taking into consideration the classification of companies to the populations of healthy and failed enterprises it was stated that the Pz model was best adapted to the Polish wood sector. Forecasts provided by this model were characterised by the highest SP. However, other models had lower errors EI (model A) or EII (models P2, GS, H and P1) (tab.1.) Forecasts prepared using
model A classified the lowest number of FB to the class of healthy companies, but as many as 35% HF were incorrectly classified as FB. A worse result was obtained only using the J model.

As it was mentioned earlier, a lower error of classification of HF to FB was observed for forecasts prepared using the P2, GS, H and P1 models. A particularly low EII was recorded in models P2, GS and H. In two former models, i.e. GS and H, in 2- and 3-year forecasts no enterprise with a stable financial situation was incorrectly classified. Unfortunately, FB were also classified to the HF group (a high EI). On average in the P2 model 55% and in both the GS and the H model 40% firms which went bankrupt were classified in the forecasts as companies with a good financial standing. This means that although these models did not identify HF as FB, they identified FB as HF. Forecasts prepared using this model classified a vast majority of firms as not threatened with bankruptcy even if it was not the case. For this reason the authors of this study are of an opinion that these models need to be rejected in forecasting financial changes in wood sector enterprises.

It needs to be stressed that the greatest stability of obtained forecast results based on 1-, 2- and 3-year data was obtained when applying the P2 model. Prediction using this model in each of the three analysed periods of source data collection (1-, 2- and 3-year) was characterised by a uniform SP amounting to 70%. However, apart from the J model, this result was one of the worst. For this reason, despite stability of this model, in the opinion of the authors it should not be applied in the wood sector.

It needs to be stressed that the J model was constructed for the needs of small and medium-sized enterprises in the industrial sector. Thus it would seem that this model should be best adapted to the evaluation of wood sector companies. In reality the accuracy of a forecast prepared using this model was very low.

Testing of prediction models under Polish economic conditions was the first attempt at the search for adequate forecasting methods for financial changes in the wood sector. The authors hope that in view of the specific character of the sector and the specific nature of economic changes taking place in individual countries, applicability of individual models will be verified by researchers from other countries.

**Conclusions**

1. When assessing effectiveness of estimated theoretical prognostic models the highest SP values in the identification of bankruptcy threat were observed for forecasts in the Pz model.
2. Effectiveness of the J model, which was developed for the total population of commercial enterprises, needs to be considered accidental. This model is not suited for the prediction of financial changes in wood sector companies in Poland.
3. The lowest EI level, and thus the lowest effectiveness in the identification of enterprises at risk of bankruptcy were found for forecasts prepared using the A model. However, this model had one of the greatest errors of classification of HF as FB.
4. The lowest EII and thus the lowest effectiveness in the identification of enterprises not threatened by bankruptcy were found in relation to forecasts prepared using models P2, GS and H. However, forecasts made using those models exhibited the highest EI, i.e. erroneous classification of a FB as a HF.
5. The Pz model was best adapted to predict the financial situation of Polish wood sector companies.

**References**


Basic assumptions for forest management and nature conservation from axiological, legal, and economic perspective

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Every man is obliged to refrain from such initiatives or actions, which could bring harm to the natural environment, and since the whole flora plays an irreplaceable role in maintaining the balance of nature, which is indispensable for life in all dimensions, its conservation and respect becomes a particularly urgent need for people.

John Paul II, Zamość, June 12, 1999

ABSTRACT

The subject matters of this article are mutual relationships between nature conservation and forest management, considered from the axiological and legal point, as well as the economic and social conditions of forest management.

INTRODUCTION

The subject matter of this article is the study of mutual relationships between nature conservation and forest management, considered from the axiological and legal point, as well as the economic and social conditions of forest management. Since the legal regulations related to the above subject matters are distributed throughout numerous legislative acts, and they are also the subject of interest of law enforcement authorities, the analysis has been limited to the issue of values in legal norms, related to nature conservation and forest management. Regarding the close association of presented problem with economic and social aspects of forest management, conditions accompanying practical performance of specified matters have also been presented.

Forest related regulations stipulate the specific manner of forest management, the purpose of which is to use thereof in such a manner so that there was no depletion of resources for future generations. The presented manner of forest management was recognized in the 18th century by Hans Carl von Carlowitz in his work ‘Sylvicultura oeconomica, oder hausswirthliche Nachricht Und Naturmäßige Anweisung zur Wilden Baum-Zucht’, who indicated the sustainable forest management, in which as many trees are harvested as may grow again (Bukowski 2011). In Forest Act (Act of… 1991), such use of forests was specified as the permanently sustainable forest management, where an important place is given to nature conservation. The laws of Forest Act regulate the protection of forests (art. 1), although to a limited extent, as well as based on other values than
the laws of Nature Conservation Act (Act of… 2004). The latter assumes that forests constitute the resource, on the preservation of which the existence of life on Earth depends in the long term, including the quality of man’s life environment and health. In addition, Forest Act is the law that regulates economic aspects of forest functioning, which are necessary for maintaining anthropogenic forest resources with the participation of man who conducts forest management.

The work includes presentation of economic conditions accompanying the idea of nature conservation performed in forest areas, which should be taken into account with regard to axiological and legal arguments. The existing laws of Forest Act (art. 7 item 1), from the point of axiology and the regulations of the Constitution of the Republic of Poland, indicate proper sequencing of sustainable forest management purposes. However, business practice shows that their performance depends on the well-balanced use of forests, which does not exceed their production capabilities (art. 13 item 1 point 5), including wood harvesting, which enables its optimal functioning. The above regulation becomes particularly important in view of art. 50 item 1 of Forest Act, which states that State Forests run their activity based on the financial autonomy, and they incur their operational costs from their own revenues. The mentioned provision indicates that State Forests National Forest Holding (Państwowe Gospodarstwo Leśne Lasy Państwowe – PGLLP), could be treated as an enterprise, however, the circumstances in which PGLLP performs the multi-purpose management, not receiving any special purpose grant from the budget or any subsidy, make PGLLP constitute a specific public corporation with multiple obligations imposed on it by the legislator (Hausner and Żylicz 2014). The above does not change the fact that state forests were considered as an enterprise based on the provisions included in the regulation by the Minister of Forestry of December 27, 1949 on the organization and the scope of operation of state forests’ enterprises (Journal of Laws No. 63, item 510) (Dz.U. nr 63, poz. 510), and further based on regulation by the Minister of Forestry and Wood Industry of December 17, 1959, on the organization and the scope of operation of state forests’ enterprises (Journal of Laws of 1960, No. 2, item 13) (Dz.U. z 1960 r., nr 2, poz. 13). It should also be noted that the term ‘enterprise’ is also used nowadays in the context of State Forests – the example of which is the parliamentary draft law on amendment of the Constitution of the Republic of Poland (print no. 2374), which states that ‘forests which constitute the property of the State Treasury are managed based on self-financing by “State Forests” enterprise, pursuant to purposes set forth in art. 5 of the Constitution’ (Report…). The above issues (used more or less consciously) do not change the real extent of public tasks performed by PGLLP, mainly related to non-productive functions of forests (public tasks financed from own funds are not performed by ‘enterprises’, which are stricte business entities).

One of the most important public functions is the preservation of nature; this idea is widely incorporated in programs of forest education in the society, mainly among children and young people. Both PGLLP’s activities relate to the performance of the state’s constitutional responsibilities, included in the performance of policy that ensures the ecological safety for today’s and future generations (art. 74 item 1 of the Constitution of the Republic of Poland). The necessity for active forest management in the mentioned scope is confirmed in the social feeling expressed mostly in a declarative manner in public opinion surveys, and, which is unfortunately still rare, in real attitudes and behaviours.

**LEGAL, AXIOLOGICAL, AND ECONOMIC BASES FOR NATURE CONSERVATION AND FOREST MANAGEMENT**

Issues relating to the preservation of nature seen in the context of forest related problems have been regulated in multiple legal acts, mainly in Nature Conservation Act and Forest Act. In addition, the above issues are governed by the provisions of the following acts: Environment Protection Act (Act of… 2001), Act on the access to information on the environment and its protection, on the participation of society in environment protection, and on the environmental impact assessment (Act of… 2010), Act on the protection of agricultural and forest land (Act of… 1995), tax legislation, civil code, and in implementing acts to the mentioned regulations, as well as indirectly in other legal regulations (Act of… 2003). Frequently, EU directives and the provisions of international conventions constitute the basis for the introduction (transfer) of regulations in domestic provisions of
law. In the context of EU regulations, it is necessary to emphasize that the EU acquis related to the environment protection is very abundant as it includes over 800 legislative acts of different status, out of which over 200 are directives. Some of them play a fundamental role in the enforcement of nature conservation in forests, in particular Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora (OJ EU L 206, 22.7.1992 as amended) and Council Directive 79/409/EEC on the conservation of wild birds (OJ EU L 103, 25.04.1979 as amended).

Based on the above directives, the shape and size of Natura 2000 regions has been settled, where forests constitute 50% of area. In Poland, forest areas in NATURA 2000 sites constitute 33% (Czerepko et al. 2013). At the same time, in Natura 2000 areas, Member States must warrant non-deterioration of species and habitats’ protection because of human activity. It is important that every member state individually decides the manner of respecting these conditions. All nature protection activities must be carried out with regard to economic, social, and cultural, as well as regional and local conditions. Most frequently, minor changes need to be introduced to allow the coexistence of present day use of areas with the protection of present species and habitats. In all cases, it is important that local societies within Natura 2000 regions are involved in decisions related to the long-term management of these areas, beginning from private owners and users of lands, industry and associations aiming at nature conservation, through local self-governments, ending on the government administration. These issues are regulated in various legislative acts.

With such widespread legislative distribution of investigated issues, it is natural that not all regulations are fully consistent and compatible. However, legislators cannot be accused of not taking measures to standardize these issues related to nature conservation and forest management, which might seem difficult to reconcile. The example here are the following regulations: so-called assessment act (Act on access to… 2008), Regulation of the Council of Ministers of November 9, 2010 on projects which may significantly affect the environment, as well as new regulations regarding the nature conservation. A connection link between the nature conservation and forest management is undoubtedly the constitutional regulation related to ensuring the environment protection with regard to the principle of sustainable development (art. 5 of the Constitution of the Republic of Poland). The above principle constitutes an example of a program framework setting the state’s objective to be the environment protection in correlation with the permanently sustainable forest management. Art. 74 of the Constitution of the Republic of Poland is also the binder between forest management and nature conservation, since it relates to the environment protection and the principle of subsidiarity. The above regulation imposes an obligation on public authorities to protect environment through ‘pursuing of the policy which ensures ecological safety for current and future generations, and supporting citizens’ actions in order to protect and improve the condition of environment. As a result, the public authorities are obliged to support actions by single citizens, established forest associations, and other organizational structures operating in the forestry, provided that those entities carry out forest management focusing particularly on the protection objective. At the same time, it results from the above rule of law that every single person is obliged to comply with the rules of nature conservation, including in-forest areas. Words by Pope John Paul II, uttered during one of his visits to Poland, included at the beginning of this article are the essence of the above principle.

The issues of nature conservation and forest management were combined in the provisions of the Act on preservation of the national character of strategic natural resources of the country (Act on the preservation… 2001), classifying state forests and nature resources of national parks as strategic natural resources of the country (art. 1 item 3 and 5). The provisions of the above act indicate that the management of strategic natural resources is carried out in compliance with the principle of sustainable development in the interest of general (society) good (art. 3) and, in order to achieve the above objective, relevant public authorities and other entities, performing, based on separate provisions of law, the management activities on specified natural resources, are obliged to maintain, increase, and improve renewable resources, pursuant to the principle of sustainable development. The above regulation must not surprise, if values provided by forest ecosystem are taken into account. Undoubtedly, all protective roles need to be mentioned here, including water, soil, air, or nature resources. Such broad approach to many various
usability features provided by forest enables the definition of so called intrinsic value of forest (Hausner and Żylicz 2014). This value cannot be defined in economic terms, but it comprises various use values of forests, both measurable and quantifiable material goods, as well as non-material services, for which the results of evaluation and value pricing still cause controversy, and there is still no consent as to their inclusion in macro-economic indicators, of the country, continent, and the whole world (green economy).

The improvement of quality and quantity, and even the maintenance of various values of forests and forest management require relevant financial outlays (costs), which are most frequently not related to any revenues. This is because the specified utilities are predominantly positive external effects of forest management with common good features. Due to this, the foresters’ work is described as public service, although it is financed through the sale of wood raw material. The mentioned obligations related to forest management must be performed in such a manner that revenues from the sale of wood should enable payments of not only the direct costs of nature conservation, but also alternative costs or the reimbursement of losses caused by the use of natural resources by man.

The financing of social and protective features, including nature conservation by forest management, is not only a necessity related to the performance of legal obligations, which were established due to the common intrinsic value of forest ecosystem, but also the response to expectations expressed by the society towards entities dealing with the management of natural resources. This is confirmed by the results of the public opinion survey regarding Natura 2000 areas. The determined level of awareness regarding the matter differed significantly among investigated countries. The percentage of people, who had never heard about Natura 2000, ranged from 19% in Finland to 97% in Ireland and Great Britain. The awareness of what Natura 2000 exactly is was the maximum in Finland (41%) and Bulgaria (38%). Among the countries where over half of the citizens had heard about Natura 2000 were Estonia (59%), Slovenia (55%), Greece (53%), and Poland (51%). In almost all investigated countries, apart from Finland and Bulgaria, there were more people who had just heard about Natura 2000 than those who could

**Table 1.** Social preferences regarding selected non-productive functions of forests determined in research by Forest Research Institute

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<td>1) air protection</td>
<td>27.9</td>
<td>29.7</td>
<td>27.9</td>
<td>29.0</td>
<td>27.2</td>
<td>21.6</td>
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<td>2) forest as a living environment of plants and animals</td>
<td>24.3</td>
<td>20.2</td>
<td>21.1</td>
<td>23.1</td>
<td>24.7</td>
<td>26.8</td>
<td>30.4</td>
</tr>
<tr>
<td>3) forest as a place of recreation and relaxation</td>
<td>11.7</td>
<td>15.1</td>
<td>13.6</td>
<td>15.5</td>
<td>15.3</td>
<td>12.9</td>
<td>15.9</td>
</tr>
<tr>
<td>4) water conservation</td>
<td>11.5</td>
<td>13.2</td>
<td>12.7</td>
<td>12.0</td>
<td>13.8</td>
<td>13.5</td>
<td>8.1</td>
</tr>
<tr>
<td>5) forest vs. climate</td>
<td>13.4</td>
<td>12.3</td>
<td>14.8</td>
<td>10.8</td>
<td>10.0</td>
<td>–</td>
<td>9.4</td>
</tr>
<tr>
<td>6) soil conservation</td>
<td>10.9</td>
<td>9.3</td>
<td>9.5</td>
<td>9.2</td>
<td>8.7</td>
<td>9.0</td>
<td>7.3</td>
</tr>
<tr>
<td>7) forest as a supplier of wood raw material</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>8.9</td>
</tr>
<tr>
<td>8) forest as a place of collecting fruit and fungi</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7.2</td>
</tr>
<tr>
<td>7) other, what?</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
<td>0.4</td>
<td>0.3</td>
<td>–</td>
<td>0.3</td>
</tr>
</tbody>
</table>

explain what this term exactly referred to. The lowest level of awareness about Natura 2000 was amongst English, Irish, and Italian people. The European people’s understanding of the role of protected areas is also worth mentioning. Most respondents (53%) thought that the most important role of protected areas is the conservation of endangered plant and animal species. Countries with the strongest support for this viewpoint were Luxemburg and Germany. The next most important role mentioned was the prevention of destruction of valuable aquatic and terrestrial areas (43%) – the most significant one for Finland and Great Britain, as well as the role of nature in ensuring access to clean water and air (38%), which was indicated by Hungarians and Lithuanians. Spanish and Slovenian citizens mainly indicated the promotion of environment friendly use of land (24% of the entire EU). Only 11% of respondents indicated environment friendly tourism and recreation, and these were mainly citizens of Germany, Belgium, and Ireland (Report 2010). Similar social surveys conducted at the Forest Research Institute (Instytut Badawczy Leśnictwa – IBL) on sample groups of people relaxing in forests indicate similar elements which are in the centre of social interest. The function of forest as an environment for plants and animals’ living was indicated as the second most important forest benefit by different sample groups, and air protection role being mentioned as the most important benefit.

**PLACE OF FOREST MANAGEMENT AND NATURE CONSERVATION REGULATIONS IN THE LEGAL SYSTEM**

Both, regulations related to nature conservation, and forest management regulations are a part of the broader field, i.e., the environment protection law. Forest regulations mainly include the specific manner of forest ecosystems’ management; its main objective is to control the use of forest ecosystem and see to it that overuse does not cause their depletion, which might lead to a situation that there is less forest ecosystem available for the future generations or it is available in worse quality. The provisions of Forest Act describe such a model of use as the permanently sustainable forest management (art. 6 item 1 point 1a) (Act of... 1991). A similar approach was applied to the management of hunting resources (Act of... 1995), fishing resources (Act on fisheries... 1985), or water resources (Water Law Act 2001). This means that the laws of Forest Act, similar to the laws of abovementioned acts, govern the management of resources of one kind – forest resources (Geszprych and Borowiak 2011). In the doctrine related to the environment protection law, the provisions of the abovementioned acts are described as ‘sectional’. Therefore, the environment protection act should be named as ‘integrating’ and ‘bonding’ the ‘sectional’ regulations in the scope of protection of all the environment components (land, water, forests, fish, animals, etc.), since it deals with them ‘however from a certain angle of their particular values’ (Radecki 2008). Another reason for such an approach is the concept of environment protection law and the nature conservation law presented by J. Sommer (2006). According to the author, ‘the environment protection law refers to the rules of law that regulate human’s impact on the environment in order to prevent negative effects, and in particular, a threat to human’s health and life. The environment protection law is the rule of law governing human’s behaviours to prevent unfavourable alterations of nature. However, the unfavourable alteration does not have to relate to the protection of human’s health and life’. As regards the so called ‘sectional’ rules of law, another role may be noticed. Namely, the provisions of the nature conservation act formulate the conservation rules of such nature resources which are distinguished for their unique natural features, including the ones endangered with extinction.

In conclusion, it should be indicated that the nature conservation law may be relatively separated from the environment protection law. This approach is additionally supported by a distinction made in the legal doctrine of separate institutions dealing with nature conservation law in the scope of institutions dealing with the environment protection law (Habuda and Radecki 2010). Adopting, as the principal point of reference, the preservation of natural heritage, including forest assets, the norms included in the provisions of the nature conservation act are the special provisions with reference to other regulations in the scope of the environment protection law, mainly so called ‘sectional’ rules of law, including the forest act. As it is indicated in the subject literature, should there be a concurrence of the above regulations, the provisions of the nature conservation law should prevail (Geszprych and Borowiak 2011).
The above considerations are appropriate, if we assume that the primary objective is preservation of natural assets. However, the preservation of natural assets is not always the primary objective, which shall be discussed in the following part of this article.

**Convergence of nature conservation and forest management objectives**

Many aspects of provisions of the forest act converge with the environment protection objectives set forth in the environment protection act. These common objectives include, among others: preserving biodiversity, ensuring the continuity of plant, animal, and fungi species, along with their habitats, through their maintenance or restoration to the proper protection level, and maintaining or restoring the proper protection of nature habitats, as well as other resources, formations, and nature components – specified in the nature conservation act (art. 2 item 1 of Nature Conservation Act) (Act of… 2004). The above scope includes the rules of maintenance, preservation, and extension of forest resources, and the principles of forest management in relation to other elements of the environment (art. 1 of Forest Act). Forest management principles also refer to the nature conservation (art. 8 of Forest Act). These are the following principles: general protection of forests, durability of forest maintenance, continuity and sustainable use of all forest functions, as well as the extension of forest resources. The convergence of nature conservation and forest management objectives was outlined in the provisions regarding the performance of permanently sustainable forest management, and considering the terms set forth in regulations on nature conservation in forests by the forest management, which constitute nature reserves, and forests comprising national parks (art. 7 of Forest Act).

The nature conservation objectives are also referred to in the obligations of forests’ owners related to the general protection of forests specified in art. 9 item 1 of Forest Act, as well as in the scope of durable maintenance of forests and ensuring the continuity of their use set forth in art. 13 item 1 of Forest Act. The above scope also includes fighting and protective interventions, should harmful organisms occur, set forth in art. 10 of Forest Act. The above purposes are also reflected in the rules for the recognition of forests as protective ones, set forth in art. 15–17 of Forest Act, establishing promotional forest areas as referred to in art. 13b of Forest Act, as well as obligations related to the permanently sustainable forest management, included in art. 13a of Forest Act. It should be mentioned that State Forests, in the performance of the above tasks, initiate, coordinate, and carry out the periodical evaluation of the condition of forests and forest resources, and forecast changes in forest ecosystems; they develop periodical large-area inventories of forests condition, update forest resources’ condition, and they also hold a database of forest resources and forests’ condition.

Similar measures to ensure general and specific conservation of nature have been included in the nature conservation act (see art. 15, 18–22 of Nature Conservation Act). These are additional restrictions in forest resources management in active protection areas, in national parks, and nature reserves (the use of such areas should be subject to protective interventions described in the conservation plan or conservation tasks) until the complete withdrawal from the use of strict protection areas. Additionally, in order to ensure the protection of these areas, the legislator introduced an obligation to agree with the regional director for the environment protection of, among others, the project of forest management plan and a simplified forest management plan in a part related to the buffer zone, as relates the settlements of those plans or tasks, which may have a negative impact on the conservation of nature in the national park or on the nature reserve preservation purposes. Drafts of the last two documents must also be agreed in a part related to a national park (see art. 10 item 7 and 8, and art. 13 item 3b of Nature Conservation Act). Similar restrictions are also applied with reference to the species conservation.

In addition, in the nature conservation act, the legislator formulated detailed praxeological directives (set forth in art. 2–4 of Nature Conservation Act) considering the specifics of this regulation, based on settlements included in Nature Conservation Strategy (Sommer 2006). These are, among others, obligatorily nature protection in business activity, and the common obligation of compliance therewith by all law entities, which means that a forest management plan should include a nature conservation program. This is conformed in legal regulations related to the nature conservation,
which indicate the nature protection program to be a part of a forest management plan including an overall description of nature condition, tasks related to its conservation, and measures to achieve them, covering the territorial range of forest division (art. 6 item 1 point 11 of Nature Conservation Act). These programs fulfil different objectives in forestry, such as: ‘the improvement of preservation conditions, enrichment of natural resources, and maintaining biodiversity in forests (level of species, population, ecosystem, and landscape)’; ‘documenting and imaging natural features and threats to nature in the division’, ‘prioritizing groups of functions of individual forest areas’; ‘the indication of further sites to be covered by particular forms of protection and provisional settlement of subjects and purposes, as well as the protection methods’, and ‘the improvement of forest management and nature conservation with the full use of soil and habitat works (Miś 2002).

It should also be noted that the State Forests receive special purpose subsidies from the state budget, among others, for the development and protection of forests in case of any threat to their durability, as well as for the development of conservation plans for nature reserves under the State Forests’ management, their execution, species-specific protection of fauna and flora, and the supervision over the areas comprising Natura 2000 sites (art. 54 item 3 and 5 of Forest Act). The performance by State Forests of forest education tasks (specified in art 54 item 6 of Forest Act), which refer to establishing and running forest promotion centres, and creating nature and forest trails, also contributes to the nature conservation.

The mentioned actions result in specific economic consequences for forest management, particularly in circumstances in which subsidies from the state budget have decreased since 2008 from 20–30 million PLN annually to only 4–5 million PLN, with the increase of forest management expenditure for nature conservation, or the constant increase of spending on the maintenance of education centres, and the costs of forest cleaning from PLN 21 million in 2010 to over PLN 31 million in 2013 (Financial Statement… 2008–2013). Based on IBL’s research in the years 2005–2009, in RDSF (RDLP) in Katowice, the total unit costs of the execution of certain non-production functions of forest ranged from 120 PLN/ha, and almost 14 PLN/m³ of wood harvested as per the plan, including the nature conservation costs respectively as above 10 PLN/ha and 1 PLN/m³ of harvested wood.

The convergence of nature conservation and forest management objectives was also outlined in penal provisions related to certain behaviours in forests includ-

<table>
<thead>
<tr>
<th>Interventions related to bird conservation and nature conservation</th>
<th>Costs in years [in thousands PLN]</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2005</td>
<td>2006</td>
</tr>
<tr>
<td>Hanging nesting boxes</td>
<td>170</td>
<td>151</td>
</tr>
<tr>
<td>Maintenance of nesting boxes</td>
<td>215</td>
<td>221</td>
</tr>
<tr>
<td>Bird feeding</td>
<td>no data</td>
<td>159</td>
</tr>
<tr>
<td>Total costs of bird conservation</td>
<td>385</td>
<td>531</td>
</tr>
<tr>
<td>Animal species protection in State Forests</td>
<td>257</td>
<td>361</td>
</tr>
<tr>
<td>Fencing anthills and fencing maintenance, costs of interventions in other protected sites</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>Establishing and maintenance of tree clusters, interventions in reserves, hanging and maintenance of bat shelters</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Other nature conservation costs</td>
<td>284</td>
<td>396</td>
</tr>
<tr>
<td>Total nature conservation costs</td>
<td>567</td>
<td>792</td>
</tr>
<tr>
<td>Total</td>
<td>952</td>
<td>1323</td>
</tr>
</tbody>
</table>

ed in the Penal Code (art. 181, 187, and 188) (Code… 1997) and the Violations Code (Section 19, Forest, field, and garden damaging (art. 148–166) (Code 1971). The above objectives were also regulated in many other material provisions, which are all difficult to mention in this paper.

**Conflict of values and divergence of interests – deadlock or action?**

The above considerations pertained only to the points of convergence, not the splitting points between forest management and nature conservation. However, the situation is not always so clear as to prove the primacy of nature related purposes over non-nature objectives. Sometimes, other out-of-environment values turn more important, and their execution is important for the achievement of common good. Such situation results in mutual collisions of norms and values between nature conservation and forest management. This is the case of necessity to remove trees or shrubs which prevent the visibility of signalling and trains or the operation of railway equipment or cause snowdrifts, referred to in the provisions of the railway act (art. 56). Similarly, if a forest tree stand, which grows in the border zone, prevents the visibility of the country border or border signs, it should be removed, which is referred to in the provisions of act on the protection of state border (art. 11). Pursuant to the provisions of the act on developing and implementing nuclear energy projects and related facilities – in a permit for the construction of nuclear energy facility, the governor consents for the removal of trees or shrubs growing on properties covered by a decision on the determination of location for the investment in the scope of building a nuclear energy facility (art. 16–17). Based on the provisions of the act on special terms for the preparation and implementation of public road investments, laws regarding the protection of agricultural and forest grounds do not apply to the agricultural and forest lands covered by decisions permitting the performance of road investment (art. 21 item 1). Further, pursuant to the provisions of the act on supporting the development of telecommunications networks and services, in a permit for the construction of regional broadband network, the governor allows – in the scope necessary for the performance of investment – for the removal of trees or shrubs growing on properties covered by a decision on the determination of location for the regional broadband network investment (art. 61).

Other cases relating to actions, which do not lead to the performance of permanently sustainable forest management, have an economic and business basis. For instance, pursuant to the binding legal regulations and the settled course of court and administrative decisions, a farmer whose farm was completely damaged and who is now in a very difficult financial situation, may request the head of the local authority supervising the forest management in the forest to issue a decision authorizing the farmer to harvest wood contrary to the simplified plan of forest development or an inventory decision (referred to in art. 19 item 3 of Forest Act).

The best course of action that should be taken in the circumstance when the whole tree stand is dominated by root-rot fungus or insects, such as bark beetle, is questionable. From the point of nature, the invasion of root-rot fungus or harmful insects in a tree stand may be beneficial, because they contribute to the functioning and maintaining the continuity of natural processes (e.g., they stimulate natural renewals or contribute to the increase of biodiversity) (Sokołowski 2002). However, for forest management, they constitute serious damage and involve the risk of destroying large areas in a tree stand. The removal of the above tree stand will indirectly contribute to maintaining permanently sustainable forest management in the long term.

The conflict of values in the forestry mainly refers to the choice between the value of business activity freedom and the environment protection. For example, PGLLP manages in the area where the resources of economic goods determine the execution of many public benefit goods. The first mentioned above are governed by the market, the second are outside the market, which may cause conflicts due to the necessity to cover the first ones with protective actions and to decrease their market potential. The second mentioned above benefit as a result of this, which does not have anything in common with the market mechanism. As far as it is possible to settle the amount and structure of demand and supply, they can no longer be the subject of market exchange, and they are not the source of forest management revenues. Balancing these relations to retain the interest of an individual entity (including forest enter-
prises), as well as the society, is a significant problem of the current direction of forest management. The law is attempting to find methods to reconcile these discrepancies by giving an explicit priority to certain values at the expense of the other (which does not bring good results most often), or by trying to assign such meaning to the values, due to which discrepancies are brought to the minimum level or fade away (e.g., the introduction of forest management durability principle), or by creating methods to reach a compromise (the principle of proportionality). An important issue is that arising conflicts should always be solved with the priority given to more valuable aspects (Cieślak 2010).

The above arguments indicate that the thesis regarding the specific character of nature conservation regulations in relation to the provisions of Forest Act, emphasized at the beginning of the subject paper in the context of nature, is not always obvious. At the same time, an important matter is to find an appropriate balance between nature conservation, as the national heritage, and the rules of economy, which do not always have to or should give way to falsely adopted assumptions related to the conservation. In order to undertake effective decisions in the above scope, an owner (manager) should have the knowledge of what values are at his disposal. As far as there is no great difficulty in settling the value of market goods (it is commonly accepted that it is a market price), the problematic issue is still the utilization and the acceptability of results of non-market goods’ pricing. The recognition of impartial values of the full set of forest goods and benefits, as well as forest management, determines not only the appropriateness of business decisions, but it is also the basis for shaping forest related policy, whose law is a basic tool.

**Summary and Final Comments**

Forests, as the public good, should be used in a manner that corresponds to social, cultural, and aesthetic values acknowledged in a given society. Laws must be established along with their enforcement methods to ensure ethical use of forests, which is concisely reflected in sustainable development paradigms: the form of social and economic development, an intergenerational concept, a process integrating all human’s actions, leading to the highest possible equalization of possibilities related to the satisfaction of all people on the planet. It is therefore significant in the activity of all entities operating in the forest sphere to mitigate conflicts arising out of attempts by individuals, local societies, and other groups of interest, regarding the intention to locate residential buildings, perform priority investments of great economic and political importance, in the areas where nature is attractive, and as a result of formulating extreme and unrealistic demands by environmentalists.

At the same time, it must not be forgotten that the implementation of permanently sustainable forest management constitutes the value which is to contribute to the satisfaction of current and future generations’ needs. It is the man, who is the recipient of all goods and benefits of forest and forest management, and who, by ‘cooperation’ with nature, makes these values real. This fact should be taken into account in forest management planning, because such management must not be carried out in isolation from the surroundings in which the forest is operating. Nevertheless, it should be noted that although the Council of Ministers adopted the Strategy for the sustainable development of rural areas, agriculture and fisheries, in April 2012 for the years 2012–2020, a few months later the National Development Strategy 2020 (Strategy… 2012), and then the Action Plan for the execution of the National Spatial Development Concept 2030 (Concept… 2013), none of the above documents indicate forests as a strategic element or a development tool of the country (where forests of different ownership cover almost 1/3 of surface), or even as a tool in spatial planning or development. Neither of them contain separate tasks for forest management and forests (Degórski 2014). The above proves that even though the value of forest conservation leading to the preservation of common good is extremely important, not all are aware of this significane. The lack of understanding for the role and importance of forest management (due to multiple public benefits) is confirmed by not a very prudent decision on the necessity to pay a contribution to the State budget in the amount of PLN 1.6 billion in the years 2015–2016, as well as an obligation to pay 2% of revenues from the sale of wood (art. 58a of Forest Act). The above unfavourable events, which fortunately did not manage to destabilize the PGLLP’s financial situation, and therefore negative social or nature consequences
were avoided (due to the maintenance of constant demand for wood), resulted in the more extensive search for solutions consolidating the public character of the state forests.

**REFERENCES**


**Source**

Konstytucja Rzeczypospolitej Polskiej z dnia 2 kwietnia 1997 r. (Dz.U. nr 78, poz. 483 ze zm.).


Ustawa z dnia 20 maja 1971 r. Kodeks wykroczeń (Dz.U. z 2015 r., poz. 1094 ze zm.).

Ustawa z dnia 18 kwietnia 1985 r. o rybactwie śródlądowym (Dz.U. z 2015 r., poz. 652).

Ustawa z dnia 12 października 1990 r. o Straży Granicznej. (Dz.U. z 2016 r., poz. 1643).
Ustawa z dnia 28 września 1991 r. o lasach (Dz.U. z 2015 r., poz. 2100 ze zm.).
Ustawa z dnia 13 października 1995 r. Prawo łowieckie (Dz.U. z 2015 r., poz. 2168 ze zm.).
Ustawa z dnia 27 kwietnia 2001 r. Prawo ochrony środowiska (Dz.U. z 2016 r., poz. 672 ze zm.).
Ustawa z dnia 6 lipca 2001 r. Prawo łowieckie (Dz.U. z 2015 r., poz. 2168 ze zm.).
Ustawa z dnia 27 kwietnia 2001 r. Prawo ochrony środowiska (Dz.U. z 2016 r., poz. 672 ze zm.).
Ustawa z dnia 27 marca 2003 r. o transporcie kolejowym (Dz.U. z 2016 r., poz. 1727).
Ustawa z dnia 10 kwietnia 2003 r. o szczególnych zasadach przygotowania i realizacji inwestycji w zakresie dróg publicznych (Dz.U. z 2015 r., poz. 2031 ze zm.).
Ustawa z dnia 28 marca 2003 r. o transporcie kolejowym (Dz.U. z 2016 r., poz. 1727).
Ustawa z dnia 10 kwietnia 2003 r. o szczególnych zasadach przygotowania i realizacji inwestycji w zakresie dróg publicznych (Dz.U. z 2015 r., poz. 2031 ze zm.).
Ustawa z dnia 3 października 2008 r. o udostępnianiu informacji o środowisku i jego ochronie, udziale społeczeństwa w ochronie środowiska oraz o oczyszczaniu oddziaływania na środowisko (Dz.U. z 2016 r., poz. 353 ze zm.).
Ustawa z dnia 7 maja 2010 r. o wspieraniu rozwoju usług i sieci telekomunikacyjnych. (Dz.U. z 2016 r., poz. 1537 ze zm.).
Ustawa z dnia 29 czerwca 2011 r. o przygotowaniu i realizacji inwestycji w zakresie obiektów energetyki jądrowej oraz inwestycji towarzyszących (Dz.U. nr 135, poz. 789 ze zm.).
Phosphite fertilisers as inhibitors of *Hymenoscyphus fraxineus* (anamorph *Chalara fraxinea*) growth in tests in vitro

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**Abstract**

This study is designed to test the potential for reducing the growth of the mycelium of the fungus *Hymenoscyphus fraxineus* (anamorph *Chalara fraxinea*) by using phosphate preparations at various concentrations in vitro. The study shows that adding pure phosphate to potato dextrose agar media inhibits the development of the fungus, but if the preparation is applied in the form of ammonium phosphate (Actifos), the growth of fungus will be accelerated. Probably the addition of nitrogen contained in the product Actifos has positive effect on the mycelial growth, but pure phosphate restricts its development. These studies are preliminary and only show the potential use of phosphate to reduce the development of *H. fraxineus*; however, to completely confirm its operation, further research is needed in this area.

**Key words**

ash, Actifos, phosphite, *Chalara fraxinea*, mycelium, development

**Introduction**

Until recently, ash (*Fraxinus excelsior* L.) in Poland was considered to be a species resistant to biotic as well as abiotic factors (Grzywacz 1995). In the past decade of the twentieth century, this situation changed and ash stands began to decline in all age classes. Initially, this phenomenon was observed only in the north-eastern part of Poland, but now, it has spread within the whole country. The cause of ash dieback was prescribed to *Hymenoscyphus fraxineus* (anamorph *Chalara fraxinea*) – a newly described fungus (Kowalski 2006). The study conducted by Kowalski (2009) showed that in all areas of Poland, ash dieback was very common. Currently, this phenomenon occurred in many other European countries, and much research concentrated on the growth inhibition of the pathogen and improving the vitality of ash trees. One of the methods proposed in this paper is the application of phosphites. These compounds being a part of commercial fertilisers are recognised as stimulants of tree resistance, in consequences leading to a reduction...
of activity of pathogens (Tkaczyk et al. 2016). Such studies have been conducted on a large scale in horticulture, where apart from improving the health status of trees, these fertilisers stimulated their growth (Orlikowski 2006; Muszyńska and Orlikowski 2010; Wieczorek et al. 2010; Schroetter et al. 2006; Tkaczyk et al. 2014). The goal of our research was to verify this hypothesis in the experiment in vitro.

**Methodology**

An attempt to inhibit the growth of the fungus *H. fraxineus* by using products containing ammonium phosphite (Actifos) and potassium phosphite (Kalex) was investigated in the Department of Forest Protection of the Forest Research Institute in Sękocin Stary. This experiment was carried out in vitro on potato dextrose agar medium amended with (NH₄)₂PO₃ (0.6%; 1.2%) and K₃PO₃ (0.6%; 1.2%). On such medium, the fungus *H. fraxineus* originating from the FRI collection (KY613993) was implanted on 10 Petri dishes for each variant. Over a period of 20 days, its daily mycelial growth was measured, and for statistical analysis, the nonparametric Kruskal–Wallis test was used.

**Results**

Potassium phosphate (K₃PO₃) inhibited the growth of fungus *H. fraxineus*. Its average radial mycelial growth in Petri dishes after 20 days was significantly lower than that in the control (without the addition of K₃PO₃). The difference between variants of potassium phosphate concentrations (0.6% and 1.2%) was not noticed, which means that the protective treatment performed at a lower concentration (0.6%) is already sufficient to slow down the growth of the fungus. The use of the preparation Actifos (0.6% and 1.2%) shows statistically significant differences between both the concentrations but do not differ from the control (Tab. 1).

In the case of Actifos, the stimulation of the growth of *H. fraxineus* was noticed. The highest pathogen mycelial growth was found in the case of 0.6% Actifos (Fig. 1). This phenomenon may be related to the presence of nitrogen (10.2%) and other microelements (B, 0.02%; Cu, 0.008%; Fe, 0.06%; Mn, 0.04%; Mol, 0.004%; Zn, 0.02%) in Actifos being sold as a fertiliser.

**Table 1.** The Kruskal–Wallis test probability values values of fungal growth on media amended with phosphites

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>K₃PO₃ (0.6%)</th>
<th>K₃PO₃ (1.2%)</th>
<th>Actifos (0.6%)</th>
<th>Actifos (1.2%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.002</td>
<td>0.000</td>
<td>0.118</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>K₃PO₃ (0.6%)</td>
<td>0.002</td>
<td>0.000</td>
<td>0.000</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>K₃PO₃ (1.2%)</td>
<td>0.000</td>
<td>1.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Actifos (0.6%)</td>
<td>0.118</td>
<td>0.000</td>
<td>0.000</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Actifos (1.2%)</td>
<td>1.000</td>
<td>0.030</td>
<td>0.000</td>
<td>0.006</td>
<td></td>
</tr>
</tbody>
</table>

**Conclusions**

Our study shows that the use of phosphorus in the form of potassium can limit the development of the fungus. Moreover, the higher concentration of the preparation significantly decreases the growth of the mycelium. However, the application of Actifos formulation (phosphate in the ammonium form) resulted in the stimulation of mycelial growth. In the concentration variant of 0.6%, Actifos stimulated the growth of the mycelium of *H. fraxineus*.
compared to the control. In double concentration (1.2%), the growth of the mycelium was similar to the control.

This experiment proved that the potassium form of phosphate limits the fungal growth in the test in vitro. In contrast, ammonium form of phosphite stimulates the growth of mycelium, probably, because of the action of nitrogen and other microelements. In the next step, the effect of potassium phosphate should be checked in tests in planta. Possible successful results of the product performance on the plants infected with H. fraxineus can be proposed in order to control the ash dieback phenomenon observed across Europe.

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**References**


Kowalski T. 2006. Chalara fraxinea sp. nov. associated with dieback of ash (Fraxinus excelsior) in Poland. *Forest Pathology*, 36, 264–270.


