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Long-Term Monitoring Studies of the Mycorrhizal Colonization of *Aesculus hippocastanum* L. Roots and the Vitality of Soil Microorganisms in Urban and Non-Urban Environments

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Abstract: Stress factors typical in urban environments adversely affect the productivity and vigor of trees and may limit the development of tree roots with associated soil fungi and bacteria. Studies on mycorrhizal symbiosis and the activity of soil microorganisms are important in monitoring the adaptation of trees to urban conditions. We compared the symbiotic activity of arbuscular mycorrhizal fungi (AMF) living in the rhizosphere of mature white chestnut trees (*Aesculus hippocastanum* L.) in an urban ecosystem at sites with varying degrees of anthropopressure and in a rural area. We used two methods: (1) direct analysis of the root samples, (2) assessment of the colonization of trap plants grown in the soil taken from under selected white horse chestnut trees. The seasonality of mycorrhizal colonization and soil microbial respiration based on the enzyme activity of nonspecific dehydrogenase (DHA) was studied over several growing seasons. The concentrations of macronutrients in the soil from all study sites were in the ranges accepted as normal for the upper soil layer. However, the C/N ratio indicated carbon limitation in the soil at urban and rural study sites. The results showed that arbuscular mycorrhizal fungi indigenous in the rhizosphere of *A. hippocastanum* developed a functioning mycorrhizal symbiosis at all research sites, including at highly disturbed urban locations, and that the mycorrhizal colonization varied between the study sites and the research term. The trap culture method confirmed the high biological potential of the soil microbial community, including AMF in urban ecosystems, which was comparable to that at the reference site in the rural environment. Soil moisture strongly affected the overall soil microbial vitality. This research showed that the mycorrhizal status of *A. hippocastanum* is more strongly influenced by climatic conditions and seasonal rhythms of trees than by urban/non-urban locations.

Keywords: arbuscular mycorrhizal fungi; *Aesculus hippocastanum* L.; soil nonspecific dehydrogenase; urban environment

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1. Introduction

Horse chestnut (*Aesculus hippocastanum* L., Hippocastanaceae) is a large, magnificent deciduous tree native to mountain areas of the Balkan Peninsula in south-eastern Europe. The species was successfully introduced to other parts of Europe during the 16th and 17th centuries. It is cultivated for shade and as an ornamental city and park tree, and is often planted along streets [1]. The best growth of *A. hippocastanum* occurs in moist, fertile soils in full sun and in areas protected from wind [2,3]. In urban environments, especially along transport routes, trees are exposed to unfavorable living conditions, such as limited free space around the trunk, a small root space, insufficient access to water and minerals, excessive heat, and air and soil pollution with unwanted chemical compounds. Harmful factors change the physiology of trees, their productivity and their vigor [4], and the average lifespan of trees in cities is significantly shorter than that of trees of the same species in natural conditions [5]. *A. hippocastanum* has been reported to grow well in almost any urban soil with an acidic to neutral pH with a very restricted root zone [6], and is relatively

tolerant to shade and low winter temperatures [7]. On the other hand, *A. hippocastanum* is considered to be quite sensitive to air pollution factors typical in urban ecosystems [6] and to drought [8,9]. Its tolerance to salinity is unclear because of inconsistent reports indicating that this species can be highly sensitive [10–12] or resistant to this stress factor [13] and it is quite tolerant to soils with a high calcium content [14].

Under natural conditions, the fine roots of most plant species develop symbiotic associations (ectomycorrhiza and endomycorrhiza) with specialized soil fungi. Mycorrhizae enhance plant growth, water and nutrient uptake and photosynthetic activity; stimulate nutrient cycling; modify plant metabolites; and increase the tolerance of plants to pathogens and toxic substances [15–17]. In return, the plant provides the mycorrhizal fungi with carbohydrates produced during photosynthesis. The most widespread mycorrhizal type, associated with over 80% of higher plant species, is arbuscular mycorrhiza (AM), a type of endomycorrhizae [16,18]. AM fungi are obligate biotrophs belonging to the phylum Glomeromycota [19].

Chestnut trees develop mycorrhizal symbiosis with arbuscular mycorrhizal fungi (AMF) [20]. Arbuscular mycorrhiza (AM) was reported in the roots of seedlings of *A. hippocastanum* and *A. pavia* [12], in the roots 2- to 3-year-old *A. hippocastanum* plants [21,22] and in the roots of mature trees of *A. hippocastanum* L. [23–26].

Properly functioning mycorrhizal symbiosis helps to maintain the vitality and health of trees in often difficult urban conditions. Mycorrhizal formation and functioning are strongly affected by soil properties such as soil type, chemistry, moisture, temperature, cation exchange capacity, pH and organic matter [27–32]. Any environmental factors, natural or anthropogenic, that decrease carbon assimilation and allocation to the root system may influence the mycorrhizal symbiosis negatively [33]. Anthropogenic biotic and abiotic stresses such as nitrogen deposition, toxic metals and ozone have been shown to decrease AM colonization [34–36]. Monitoring of the mycorrhizal symbioses of urban trees is important for urban ecosystem management.

The few studies performed on the mycorrhizal status of trees growing in urban environments compared to trees from natural forests or rural habitats have shown a generally lower intensity of mycorrhizal colonization in urban compared to non-urban conditions for ectomycorrhizal (ECM) fungi [24], as well as for arbuscular mycorrhiza (AM) [37,38]. The mycorrhiza of mature *A. hippocastanum* was observed by Karliński et al. (2014) [25], who reported a similar range of AM colonization in tree roots growing both in urban and rural environments. Research by Bainard et al. (2011) [23] on the mycorrhiza status of 26 tree species, including *A. hippocastanum*, growing in urban and rural environments showed that some species had lower and others higher colonization of roots by AM and ectomycorrhizal fungi in urban habitats, and in the roots of several species, the difference between urban and rural areas was not significant. The results of the above studies were based on plant material collected at one time, so the impact of seasonal changes in environmental conditions on mycorrhiza was not taken into consideration. So far, no long-term observations (lasting for several growing seasons) of mycorrhizal colonization of mature *A. hippocastanum* roots have been conducted. The seasonality of mycorrhizal colonization of *A. hippocastanum* roots was only investigated in a field experiment with 2–3-year-old seedlings of this species in two growing seasons [22].

Studies conducted over several seasons, taking into account the changing environmental conditions controlled by seasonal climate fluctuation and the impact of local anthropogenic factors, are important for a better understanding of the plant–mycorrhizal fungi interactions and the importance of mycorrhizal fungi in the adaptation of trees to specific urban conditions. This is why the research presented here was undertaken.

The objectives of this research were (1) to study, over several growing seasons, the arbuscular mycorrhizal status of mature trees of *Aesculus hippocastanum* L. grown in urban environments with different degrees of anthropopressure at a reference site (an old arboretum located in a countryside), (2) to assess the mycorrhizal activity of AM fungi indigenous in urban and non-urban soils using the trap culture method, (3) to evaluate

the vitality of soil microorganisms. We expected that the unfavorable conditions of urban habitats, such as the compacted soil, drought and low availability of minerals, would limit the development of arbuscular fungi in urban soil and mycorrhizal colonization of chestnut roots. *Plantago lanceolata* was used as the trap plant. To measure the activity of the soil microorganisms, we determined the activity of the nonspecific dehydrogenase in the soil samples. We expected that unfavorable soil conditions typical in urban environments and the reduced water content in the soil may have a negative impact on the activity of the soil microorganisms.

2. Materials and Methods

2.1. Study Sites

The study was conducted in the city of Bydgoszcz (358,000 inhabitants), which is situated in northern Poland, on the Brda and Vistula rivers (53°07′ N, 18°00′ E), and in Kórnik Arboretum (52°15′ N, 17°04′ E), located in a rural area 25 km from metropolitan Poznań. The urban habitats included: Site 1—a street-side place located in the city center in a narrow green belt surrounded on four sides by transport routes with heavy car traffic. Site 2—the campus of Kazimierz Wielki University. The chestnut trees grow in the vicinity of university buildings, 30 m from a street with heavy traffic on one side and a large lawn on the other. Site 3—the Botanical Gardens of Kazimierz Wielki University, covering 2.33 ha, located in the city center between the university campus and three streets with motor traffic. Site 4—"Five Lakes Valley", a city park in the vicinity of a housing development and supermarket with compacted soil containing many pieces of concrete materials (the remains of construction of neighboring buildings). Site 5—the rural site at Kórnik Arboretum, an old countryside park covering an area of 38 ha, built in the 17th century and containing about 3500 species and cultivars of woody plants. Trees growing in urban conditions were affected to varying degrees by abiotic stress factors generated by the urban environment. The rural site in the Kórnik Arbotetum was free from the indirect influence of anthropogenic stresses. Among the examined chestnut trees, those growing at site 1 were exposed to the most stress factors, affecting both the above-ground part (direct access of toxic gases and dust) and the underground part (limited free space near the trunk, soil compaction, lack of soil cover with vegetation).

2.2. Sample Collection

Root and soil samples were taken under two selected mature *A. hippocastanum* trees at each study site (three root and three soil samples from each *A. hippocastanum* L. tree). Test samples were collected in autumn (November 2008, 2009, 2013) and spring (April 2009, 2011, 2014) with a spade to a depth of 10 cm under each horse chestnut tree, about 70–100 cm away from the tree trunk. The roots were traced back to the stem to ensure that the sampled roots had been connected with a given tree and were isolated for mycorrhizal assessment. Soil samples for the analysis of soil parameters were collected only once in November 2008. The samples were stored in plastic bags at −18 °C until analysis.

2.3. Root Colonization Assessment

Root samples were carefully cleaned of soil and debris with tap water. Fine roots (<2 mm in diameter) were detached from coarse roots and cut into 1 cm segments, and subsamples of 0.5 g moist weight each were collected for visualization of fungal structures. The clearing and staining of roots were performed following the method by Kormanik and McGraw (1982) [39], modified specifically for *A. hippocastanum* L. roots. For this, root samples were placed in 10% KOH for 1.5–2 h (depending on the thickness and color of the roots) and subsequently rinsed with water. After this, the root segments were placed for 1 h in alkaline hydrogen peroxide at room temperature and then stained with 0.05% trypan blue in lactoglycerol for 8 min at 90 °C in a water bath. The roots were stored in glycerol/lactic acid/water (1:1:1) as the conservation solution. Root segments were then mounted on slides in glycerol/lactic acid (5:1) and analyzed using a Zeiss Axiostar

Plus light microscope at ×200 magnification. Mycorrhizal colonization assessment was carried out according to the method proposed by Trouvelot et al. (1986) [40]. At least 50 cm of fine roots for each subsample was assessed for the presence of AM fungal structures. The mycorrhizal parameters (frequency of mycorrhizal presence, intensity of mycorrhizal colonization in the root system, and arbuscule richness in the root system) were calculated using the 'Mycocalc' computer program [40]. An estimate of the mycorrhizal frequency (F%) is given as the ratio between root fragments colonized by AMF mycelium and the total number of root fragments analyzed. The relative mycorrhizal root length (M%) is an estimate of the amount of root cortex colonized by AMF relative to the entire root system investigated. The relative arbuscular richness (A%) is an estimate of arbuscule richness in the entire analyzed root system [40].

2.4. Chemical Analyses of Soil

Soil pH was determined using a soil suspension in water (1:2.5 (*w*/*v*) soil/H_2O) and in 1 M potassium chloride (1:2.5 (*w*/*v*) soil/KCl). The soil water content was estimated on a thermogravimetric basis with soil samples dried at 105 °C for 24h and expressed as (%). Prior to chemical analyses, soil samples were sieved and air dried. Concentrations of nutrients and the conductivity of the soil were determined at a licensed laboratory. The carbon and nitrogen content in the soil was measured using an Elemental Combustion System 4010. The C/N ratio was calculated on a mass basis. Macronutrients were extracted in 0.03 N acetic acid. Micronutrients and Pb were extracted using 1 dm^3 of modified Lindsey solution, which contained 5 g $EDTAH_4$, 9 ml 25% ammonia, 4 g citric acid and 2 g $(CH3COO)_2Ca{\cdot}2H_2O$ [41,42]. Available P (P_2O_5) was determined via the colorimetric method with ammonium molybdate. K, Ca and Na were examined via flame photometry; chlorides were assayed nephelometrically; Mg and Pb were assayed via atomic absorption spectroscopy; and S-SO_4 was analyzed via an ion-selective electrode. The soil's electrical conductivity (EC) was measured using the conductometric method in solution created by centrifuging a mixture of soil/water (1:5, *w*/*w*). An MP-2 ether conductor made in Poland was used for measurements.

2.5. Estimation of Dehydrogenase Activity

The soils were sieved to remove roots, and the activity of nonspecific dehydrogenase was measured using the tetrazolic method developed by von Thalmamm (1987) [43] and modified by Rossel et al. (1997) [44]. Samples of 2.5 g were incubated in 5 cm^3 of 0.5 M Tris buffer, pH 8.0, containing 1% 2,3,5-triphenyltetrazolium chloride (TTC) as an electron acceptor, for 24 h at 30 °C in the darkness. Suitable reference samples were analyzed to eliminate any influence of nonenzymatic absorbance: (1) soil + Tris buffer and (2) TTC + Tris buffer. Ethanol was used to extract the colored reaction product formazan from the incubation mixtures. The extract was measured spectrophotometrically at 480 nm. Enzyme activity was expressed as nanomoles of 2,3,5-triphenyltertazolium formazan (TTF) per gram of dry soil 24 h^{-1}.

2.6. Establishment of Trap Cultures

Soil samples for establishing the trap cultures were excavated once in spring 2011 from the rhizosphere of all investigated chestnut trees. Soil samples were mixed with autoclaved sand (grains 1–4 mm) (1:1 (*v*:*v*)) and placed into 11 × 11 × 12 cm plastic pots with a capacity of liter. Six pots were prepared for each study site. *Plantago lanceolata* (L.) seedlings cultured in sterile sand were used as host plants. Six seedlings were planted in each pot. Each pot was placed into a plastic bag to protect it from biological contamination and to maintain the moisture level. The trap cultures were cultivated in a growth chamber under controlled conditions for 6 months. The plants were watered with sterile water every three days and with Rorison's nutrient solution for arbuscular mycorrhizal fungi [45] every two weeks. After finishing the cultivation, the plant roots were washed with tap water, cut into 0.5–1.0 cm segments, cleaned in 5% KOH and stained with 0.05% trypan blue

in lactoglycerol. The mycorrhizal colonization percentage was determined as previously described. The activity of the nonspecific dehydrogenase in the soil in which the *Plantago lanceolata* (L.) plants grew was measured as previously described.

2.7. Statistical Analysis

Statistica version 13.3 (StatSoft, Cracow, Poland) was used for processing the statistical data. A two-way ANOVA was used to examine the levels of significance ($p < 0.05$) of the factors (site, time and their interactions) with regard to mycorrhizal colonization, soil dehydrogenase activity, pH values and water content in soil. Mean values of root and soil parameters were separated using Tukey's honest significant difference test. In order to determine the relationship between mycorrhizal colonization and water content and activity of nonspecific dehydrogenase and water content, the Pearson correlation coefficient was calculated. Before the analysis, the data were checked for normality (Shapiro–Wilk test) and homogeneity (Bartlett's test), and the proportional data were transformed according to the Bliss formula [46]: $x = \arcsin\sqrt{}(n\%/100) \times 180/\pi$, where n% is the percent value. The tables and figures present untransformed data.

3. Results

3.1. Chemical and Physical Characteristics of the Soil

Soils at all study sites were slightly alkaline, with a pH_{H2O} in the range of 7.11–7.77 and a pH_{KCl} in the range of 7.01–7.30. The conductivity values (EC) were between 0.15 and 0.26 (mS cm^{-1}) (Table 1).

Table 1. Concentration of assimilable forms of macro- and micronutrients and pH and conductivity (EC) values in soils under *Aesculus hippocastanum* in Bydgoszcz (site 1—street-side place, site 2—campus of the Kazimierz Wielki University (KWU), site 3—Botanical Garden of KWU, site 4—city park) and Kórnik (site 5—Kórnik Arboretum). Mean values of soil parameters were separated using Tukey's honest significant difference test. Data are means ± SE (n = 6). Significant differences between sites are indicated by different letters.

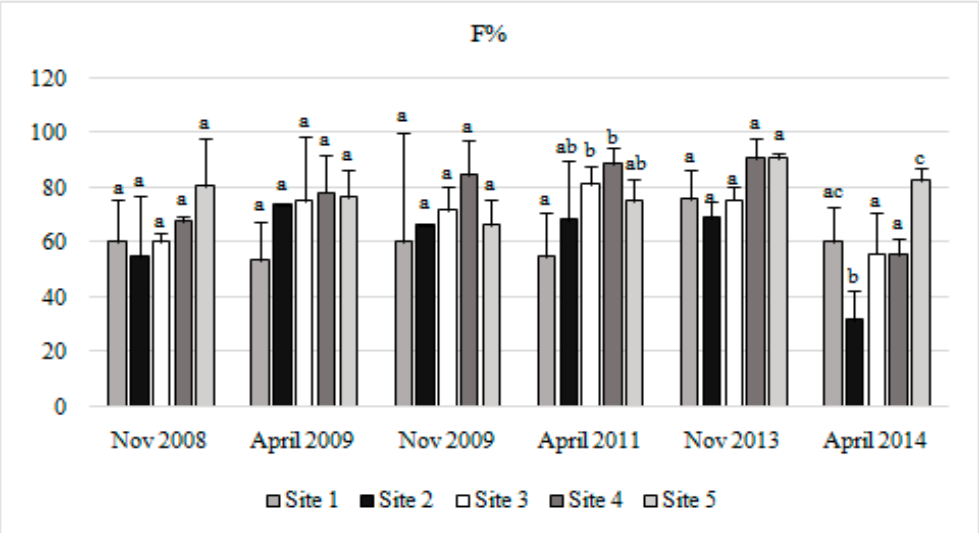
	Site 1	Site 2	Site 3	Site 4	Site 5
pH (H_2O)	7.77 ± 0.52	7.11 ± 0.60	7.73 ± 0.12	7.35 ± 0.29	7.62 ± 0.13
pH (KCL)	7.30 ± 0.40	7.01 ± 0.81	7.20 ± 0.01	7.29 ± 0.02	7.08 ± 0.09
P (mg 100 g^{-1})	3.77 ± 2.14 a	13.90 ± 0.68 ab	3.36 ± 1.77 a	11.40 ± 2.69 b	5.40 ± 1.48 ab
K (mg 100 g^{-1})	15.00 ± 1.84 ab	13.52 ± 5.22 ab	21.99 ± 12.70 a	20.10 ± 0.85 a	7.33 ± 2.36 b
Ca (mg 100 g^{-1})	196.30 ± 17.34 a	78.00 ± 29.88 b	170.10 ± 70.32 a	117.50 ± 33.66 a	117.94 ± 15.41 a
Mg (mg100 g^{-1})	13.50 ± 0.71 a	7.00 ± 0.47 a	20.12 ± 1.60 ab	11.05 ± 2.48 a	32.96 ± 4.12 b
S-SO4 (mg 100g^{-1})	1.05 ± 0.92	0.69 ± 0.33	1.73 ± 1.68	0.95 ± 0.35	1.42 ± 0.89
Cl (mg 100 g^{-1})	1.90 ± 0.56	2.03 ± 0.57	1.69 ± 0.11	2.25 ± 0.35	1.42 ± 0.11
Na (mg 100 g^{-1})	1.75 ± 0.49	1.42 ± 0.07	2.85 ± 0.01	2.05 ± 0.78	1.78 ± 0.69
Pb (mg kg^{-1})	12.92 ± 2.89	12.97 ± 2.97	24.00 ± 0.09	25.23 ± 9.80	27.99 ± 25.95
C (%)	0.40 ± 0.32 ab	0.20 ± 0.32 ab	0.77 ± 0.20 b	0.15 ± 0.00 a	0.70 ± 0.27 b
N (%)	0.10 ± 0.13 a	0.11 ± 0.20 a	0.44 ± 0.11 b	0.13 ± 0.05 a	0.28 ± 0.01 ab
C/N	4.00 ± 0.14	1.77 ± 1.67	1.69 ± 0.17	1.11 ± 0.05	2.49 ± 0.10
EC (mS cm^{-1})	0.18 ± 0.04	0.15 ± 0.01	0.22 ± 0.11	0.15 ± 0.05	0.26 ± 0.79

No significant differences between the urban study sites and the rural site in Kórnik Arboretum were found in concentrations of $S\text{-}SO_4$, Cl, Na and Pb or the C/N ratio. Significant differences were found in the concentrations of P, K, Ca, Mg, N and C (Table 1). The P content was significantly higher in the soil from site 4 (city park) than that from site 1 (street-side place) and site 3 (Botanical Garden). Soils from site 3 (Botanical Garden of KWU) and from site 4 (city park) were characterized by significantly higher contents of K. Significantly lower concentrations of Ca were present in the soil from site 2 (campus of the Kazimierz Wielki University) than in the soil from other study sites. Soils from the

urban study sites in Bydgoszcz were characterized by significantly lower concentrations of available Mg than soil from the rural study site (Kórnik Arboretum). Soil samples from site 3 (Botanical Garden) and site 5 (Kórnik Arboretum) were characterized by higher values of C% than soil samples from site 4 (city park). Significantly higher N% values were recorded in the soil from site 3 (Botanical Garden) than in the soil from the other study sites in Bydgoszcz (Table 1).

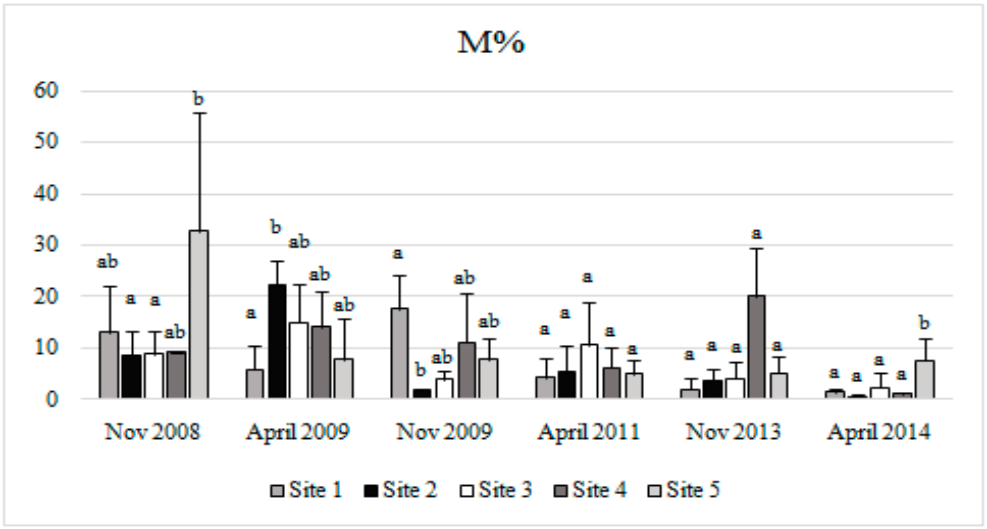
3.2. Mycorrhizal Colonization

The observations showed that the roots of mature trees of *Aesculus hippocastanum* L. on all study sites were colonized by arbuscular mycorrhizal fungi, with typical symbiotic structures: arbuscules, vesicles, intramatrical mycelium, spores (Supplementary Figure S1a). The presence of arbuscules in the roots indicates the functioning of the symbiosis. Figure 1a–c shows the share of mycorrhizal structures in the colonization of chestnut roots on particular research dates. The frequency of mycorrhizal colonization (F%) ranged from 32% (KWU campus) in April 2014 to 91% (Kórnik Arboretum) in November 2013 (Figure 1). The study term, study site and the term and study site together significantly influenced the mycorrhizal frequency in the root system of *Aesculus hippocastanum* L. (Table 2). The most influencing factor for F% was the study site—F = 11.884 (Table 2). However, the significant differences between the sites concerned two spring dates, and no significant differences were found in the three autumn dates and one spring date (Figure 1a, Table 2). The intensity of mycorrhizal colonization in the root system (M%) of mature chestnut trees was highly differentiated between the sites and dates of the research and ranged between 0.5% at the KWU university campus in April 2014 and 33% at Kórnik Arboretum in November 2008 (Figure 1b). The study term, study site and both the term and study site together had a significant influence on the intensity of mycorrhizal colonization of root systems (M%). The most influencing factor for M% was the study term: F = 9.121 (Table 2). Arbuscules were observed in the roots of each of the trees examined, although the temporal absence of arbuscules at certain urban sites (the street-side place, KWU campus, the city park and KWU Botanical Garden) was noted in three of six study terms (two fall terms and one spring term) (Figure 1c). A significant influence on the arbuscule abundance in the root system (A%) was found linked to the study term, and study site and the interaction between these two factors. The factor with the greatest influence on A% was the study term (F = 6.628) (Table 2). The highest arbuscule abundance in the root system (A%) was observed in November 2008 at Kórnik Arboretum (14%) (Figure 1c).

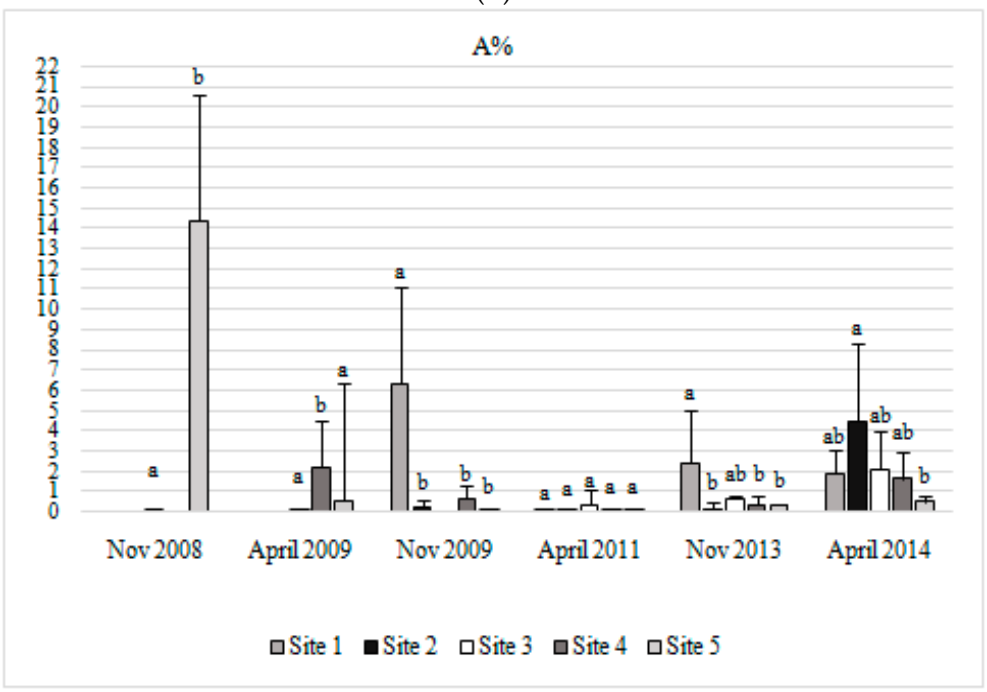


(**a**)

Figure 1. *Cont.*



(**b**)



(**c**)

Figure 1. Mycorrhizal colonization of roots of *Aesculus hippocastanum* L. trees at six sampling times in Bydgoszcz (site1—street-side place, site 2—campus of the Kazimierz Wielki University (KWU), site 3—Botanical Garden of KWU, site 4—city park) and Kórnik (site 5—Kórnik Arboretum). (**a**)—frequency of mycorrhiza in the root system F% (**b**)—intensity of mycorrhizal colonization in the root system M%, (**c**)—arbuscule abundance in the root system. Mean values of root parameters were separated using Tukey's honest significant difference test. Data are means ± SE (n = 6). Significant differences between sites are indicated by different letters.

Table 2. Results of an ANOVA testing the influence of study term and site on mycorrhizal colonization of roots of *Aesculus hippocastanum* (L.): frequency of mycorrhiza in root system (F%), intensity of mycorrhizal colonization in the root system (M%), arbuscule abundance in the root system (A%) for overall study terms and for each study term separately.

	F%		M%		A%	
	F	***p***	***F***	***p***	***F***	***p***
Overall						
term						
study site	7.183	0.000 *	9.121	0.000 *	6.628	0.000 *
term and study	11.884	0.000 *	2.484	0.046 *	1.905	0.112
site	1.888	0.018 *	3.219	0.000 *	5.134	0.000 *
In each study						
term						
term 1	2.633	0.058	3.141	0.032 *	3.013	0.037 *
study site						
term 2	2.417	0.075	5.690	0.002 *	6.170	0.001 *
study site						
term 3	1.218	0.328	2.783	0.048 *	18.244	0.000 *
study site						
term 4	5.281	0.003 *	1.325	0.287	0.649	0.632
study site						
term 5	3.144	0.032 *	2.551	0.064	5.228	0.003 *
study site						
term 6	13.727	0.000 *	11.688	0.000 *	4.199	0.009 *
study site						

* significant effect.

3.3. Activity of Nonspecific Dehydrogenase and Water Content

A higher water content was observed at Kórnik Arboretum and the urban green areas than other urban sites (Tables 3 and 4). The study term, study site and the interaction between these factors influenced the water content and activity of nonspecific dehydrogenase in the soil samples taken from all study sites. The most influential factor both for the activity of nonspecific dehydrogenase and the water content was the study site: F=60.533 (for activity of nonspecific dehydrogenase) and F= 34.780 (for water content) (Table 4).

Table 3. Water content (%) and enzyme activity of nonspecific dehydrogenase (μmol TTF g^{-1}s.m 24 h^{-1}) in the soil taken under *Aesculus hippocastanum* L. trees at the study sites (site 1—street-side place, site 2—campus of the Kazimierz Wielki University (KWU), site 3—Botanical Garden of KWU, site 4—city park, site 5—Kórnik Arboretum) in April 2009, November 2009, April 2011, November 2013, April 2014. Mean values of the water content (%) and activity of nonspecific dehydrogenase were separated using Tukey's honest significant difference test. Data are means ± SE (n = 6). Significant differences between sites are indicated by different letters.

	Water Content (%)				
Study Term	**Study Site**				
	1	**2**	**3**	**4**	**5**
April 2009	7.69 ± 0.27 a	10.06 ± 3.20 a	30.33 ± 1.97 b	5.27 ± 0.59 a	39.58 ± 26.82 b
November 2009	9.23 ± 1.04 ab	9.80 ± 3.18 ab	21.79 ± 3.22 b	4.77 ± 1.10 a	45.90 ± 22.07 c
April 2011	7.78 ± 0.33 a	8.08 ± 0.00 a	28.72 ± 0.02 b	8.08 ± 0.00 a	8.07 ± 0.03 a
November 2013	1.99 ± 0.55 a	1.70 ± 0.64 a	7.37 ± 1.31 b	2.33 ± 1.53 a	35.52 ± 0.75 c
April 2014	5.66 ± 2.28 a	5.36 ± 1.35 a	14.72 ± 6.26 b	5.65 ± 0.66 a	32.46 ± 4.36 c

Table 3. *Cont.*

Water Content (%)					
Study Term	**Study Site**				
	Activity of nonspecific dehydrogenase (μmol TTF g^{-1}s.m 24 h^{-1})				
	1	**2**	**3**	**4**	**5**
April 2009	0.225 ± 0.00 a	0.349 ± 0.17 a	0.815 ± 0.22 b	0.207 ± 0.11 a	0.680 ± 0.29 b
November 2009	0.309 ± 0.11 a	0.367 ± 0.03 a	0.725 ± 0.08 b	0.102 ± 0.05 a	0.134 ± 0.48 a
April 2011	0.264 ± 0.04 ac	0.497 ± 0.13 ab	0.599 ± 0.09 b	0.349 ± 0.16 a	0.360 ± 0.16 a
November 2013	0.075 ± 0.05 a	0.089 ± 0.05 a	0.169 ± 0.10 a	0.067 ± 0.02 a	0.630 ± 0.15 b
April 2014	0.387 ± 0.24 a	0.215 ± 0.07 a	0.620 ± 0.09 b	0.310 ± 0.06 a	0.620 ± 0.17 b

Table 4. Influence of study term and site on activity of nonspecific dehydrogenase and water content in the soil taken under *Aesculus hippocastanum* L. trees in study sites in Bydgoszcz (site 1—street-side place, site 2—campus of the Kazimierz Wielki University (KWU), site 3—Botanical Garden of KWU, site 4—city park) and in Kórnik (site 5—Kórnik Arboretum) for overall study terms and for each study term separately.

Two-Way Analysis of Variance				
	Activity of Nonspecific Dehydrogenase		**Water Content (%)**	
	F	***p***	***F***	***p***
Overall				
Term	19.277	0.000 *	15.862	0.000 *
study site	60.533	0.000 *	34.780	0.000 *
term and study site	8.573	0.000 *	8.024	0.000 *
In each study term				
term 1				
study site	12.955	0.000 *	12.915	0.000 *
term 2				
study site	27.472	0.000 *	22.123	0.000 *
term 3				
study site	7.080	0.000 *	121.666	0.000 *
term 4				
study site	42.720	0.000 *	216.666	0.000 *
term 5				
study site	10.156	0.000 *	3.961	0.018 *

* significant effect.

The results showed a statistically positive correlation between the water content in the soil and the activity of soil nonspecific dehydrogenase (Table 5).

Table 5. The relationship between the water content in soil at research sites and the activity of nonspecific dehydrogenase, the frequency of mycorrhiza in the root system F%, the intensity of mycorrhizal colonization in the root system (M%), the arbuscule abundance in the root system (A%) at the study sites in Bydgoszcz and Kórnik Arboretum.

Factor	**Pearson Correlation Coefficient**			
	F%	**M%**	**A%**	**Activity of Nonspecific Dehydrogenase**
Water content %	0.057	0.153	−0.238 *	0.843 *

* statistical significance with $p < 0.05$.

The results of a Pearson correlation analysis showed a significant negative correlation between the water content in the soil (%) and the arbuscule abundance in the root system

(A%). Soil water content was not correlated with frequency of mycorrhiza in root systems (F%) or the intensity of mycorrhizal colonization in the root system (M%) (Table 5).

3.4. Trap Cultures

The mycorrhizal activity of the soil taken from study sites in Bydgoszcz and Kórnik was assessed on the basis of the degree to which the roots of the trap plant *Plantago lanceolata* were colonized by indigenous arbuscular fungi when grown under the same cultivation conditions. The presence of symbiotic structures (arbuscules, vesicles, hyphal coils, intramatrical mycelium) produced by AM fungi was observed in roots of the trap plants growing in soils taken from all urban study sites and in soil probes from Kórnik Arboretum (Table 6, Supplementary Figure S1b).

Table 6. Mycorrhizal colonization of roots of the trap plant *Plantago lanceolata* growing in soil taken from the study sites in Bydgoszcz (site 1—street-side place, site 2—campus of the Kazimierz Wielki University (KWU), site 3—Botanical Garden of KWU, site 4—city park) and Kórnik (site 5—Kórnik Arboretum) and the results of the influence of the study sites on the mycorrhizal colonization of roots of *Plantago lanceolata*. Mean values of root parameters were separated using Tukey's honest significant difference test. Significant differences between sites are indicated by different letters. F%—frequency of mycorrhizal colonization of roots, M%—the intensity of mycorrhizal colonization in root system, A%—the arbuscular abundance in the root system. Data are means ± SE (n = 6).

Mycorrhizal Colonization	Origin of Soils in Trap Cultures					One-Way Analysis of Variance	
	Site 1	Site 2	Site 3	Site 4	Site 5	*F*	*p*
F%	41.66 ± 14.72 a	82.22 ± 7.50 b	58.33 ± 2.43 ab	52.77 ± 17.81 ab	34.44 ± 14.71 a	4.604	0.006 *
M%	0.48 ± 0.24 a	3.80 ± 3.54 b	4.23 ± 4.70 b	0.73 ± 0.42 a	0.48 ± 0.31 a	3.740	0.016 *
A%	0.15 ± 0.12 ab	0.33 ± 0.12 ab	1.80 ± 0.40 a	0.09 ± 0.06 ab	0.02 ± 0.02 b	3.296	0.026 *

* significant effect.

The origin of the soil significantly affected the parameters of AM colonization the mycorrhizal frequency (F%), the intensity of mycorrhizal colonization in the root system (M%) and the arbuscular abundance in the root system (A%) (Table 6). The highest mycorrhizal frequency (F%) was detected in the roots of *P. lanceolata* growing in the urban soil taken from the KWU university campus (F = 82.22%) and a significantly lower frequency was found in the roots of plants growing in the soil taken from the street-side place (F = 41.66%) and from the non-urban Kórnik Arboretum F = (34.44%). The intensity of mycorrhizal colonization in the root system (M%) was significantly lower in the roots of trap plants growing in the soil from the street-side place (M = 0.5%), Kórnik Arboretum (M = 0.48%) and from city park (M = 0.7%) than in the roots of plants growing in the soil from the university campus (M = 3.8%). The arbuscule abundance in the root system (A%) was significantly lower in the roots of *P. lanceolata* growing in the soil taken from Kórnik Arboretum (A = 0.03%) than in the soil taken from the Botanical Garden in Bydgoszcz (A = 1.8%) (Table 6). Table 7 shows the results of the activity of the nonspecific dehydrogenase (DHA) in the soil. The origin of the soils used in the trap cultures significantly affected the activity of the DHA in the soil. The enzyme activity was significantly higher in the soil from Kórnik Arboretum (0.208 µmol TTF g^{-1}s.m 24 h^{-1}) than in the soil taken from the street-side place in Bydgoszcz (0.043 µmol TTF g^{-1}s.m 24 h^{-1}) (Table 7).

Table 7. Activity of nonspecific dehydrogenase (μmol TTF g^{-1}s.m 24 h^{-1}) in the soil of trap cultures with *Plantago lanceolata* and the interaction between the site of soil origin and the enzyme activity. Soil for establishing the trap cultures was taken under canopies of *Aesculus hippocastanum* L. trees growing in the study sites in Bydgoszcz (site 1—street-side place, site 2—campus of the Kazimierz Wielki University (KWU), site 3—Botanical Garden of KWU, site 4—city park) and Kórnik (site 5—Kórnik Arboretum). Mean values of the activity of nonspecific dehydrogenase were separated using Tukey's honest significant difference test. Significant differences between sites are indicated by different letters. Data are means ± SE (n = 6).

Factor	Site 1	Site 2	Site 3	Site 4	Site 5	One-Way Analysis of Variance	
						F	*p*
The activity of nonspecific dehydrogenase	0.043 ± 0.02 a	0.124 ± 0.06 ab	0.128 ± 0.2 ab	0.105 ± 0.08 ab	0.208 ± 0.14 b	0.338	0.026 *

* significant effect.

4. Discussion

When undertaking the research presented here, we expected that the unfavorable conditions of urban habitats would limit the development of arbuscular mycorrhizal colonization of chestnut roots and that the harsh environmental conditions in urban locations would be important factors limiting the chestnut mycorrhizal symbiosis. However, the results of our research showed that mature trees of *Aesculus hippocastanum* L. form functional arbuscular mycorrhizal symbiosis even in extremely unfavorable environmental conditions, such as sites located in the center of large city, where trees grow in a very limited root space and are surrounded by busy transport routes (Figure 1). Moreover, the results demonstrated that the term of mycorrhiza assessment had a significant impact on root colonization by AMF (Table 2). The symbiotic activity of the arbuscular fungi native to the soils investigated was confirmed by trap cultures (Table 6). At the same time, the respiratory activity of soil microorganisms from the rhizosphere soil of trap plants did not differ between rural and urban soils, with the exception of roadside soil (site 1) (Table 7).

As far as we know, this is the first study of *Aesculum hippocastanum* mycorrhiza in several growing seasons. The few previously published works on the mycorrhiza of mature *A. hippocastanum* trees reported results concerning only one sampling of roots for mycorrhiza analysis [23,25] or two sampling dates in one growing season [24,26]. Bainard et al. (2011) [23] found that the degree of mycorrhizal colonization in roots of *A. hippocastanum* in urban and rural environments in Canada did not differ significantly between urban and extra-urban sites. The authors performed their research on one date only, collecting root samples in late spring between May and June, and they assessed only the total colonization of roots by mycorrhizal fungi, without distinguishing individual mycorrhizal structures. Our study showed variability in the frequency and intensity of AMF colonization of horse chestnut roots between particular growing seasons and between spring and autumn dates, and only in two out of six study terms was the level of mycorrhizal colonization lower in urban areas than in rural environments (Figure 1, Table 2). Although the study time and the related climatic conditions determined the status of mycorrhizal colonization, no clear trend was observed. Our results prove that assessing the mycorrhizal status on one date only may lead to incorrect conclusions.

As in our study, Karliński et al. (2014) [25] found mycorrhizal colonization of *A. hippocastanum* L. roots in all urban sites investigated. Unlike Bainard et al.'s (2011) [23] research, Karliński et al. (2014) [25], in a single investigation carried out in November, observed higher average AM colonization in urban sites than in agricultural ecosystems, with a statistically significant difference between one urban site and one rural site. Karliński et al. (2014) [25] did not find arbuscules in the roots of horse chestnut trees in any of the urban and rural sites they examined. Arbuscules are the sites of nutrient transfer between the plant and the fungus. They are created in cells of fine roots when carbohydrates produced

during photosynthesis are transported from the above-ground to the below-ground part of the plant [47]; therefore, they can be absent in November after tree leaves fall. In deciduous trees, the occurrence of arbuscular mycorrhiza is related to the phenology, which depends on temperature and humidity. At low temperatures, the process of substance exchange between the plant and the mycorrhizal fungus becomes ineffective [48], and the activity of arbuscular fungi is inhibited at ±7 °C [49]. However, some plant–endophytic fungus combinations function effectively at lower temperatures, as evidenced by the arbuscular mycorrhiza of plants in arctic regions and alpine sites [50]. The development of structures of AMF is also influenced by the availability of water in the soil. The research presented here did not show any correlation between the water content in the soil (%) and the frequency of mycorrhizal colonization (F%) and intensity of mycorrhizal colonization in the root system (M%). However, in our study, we observed a significant negative correlation between the water content in the soil and the arbuscule abundance in colonized root systems (A%) (Table 5). The impact of water conditions on mycorrhizal colonization varies and depends on the fungal genotype [51,52]. Schellenbaum et al. (1998) [53] did not observe any effect of water stress on mycorrhizal colonization of maize roots or on arbuscule abundance. Kyriazopoulos et al. (2014) [54] also did not observe any effect of water stress on colonization of *Dactylis glomerata* roots but they indicated an increased number of arbuscules.

Soils in urban agglomerations may have altered contents of mineral nutrients and pollutants and their availability to plants [55]. In our study, the concentrations of macronutrients (P, K, Ca, Mg, S-SO_4, Na) were found, both in the urban and non-urban soil substrates, in ranges accepted as normal for the upper soil layer [56]. Among the macronutrients analyzed, the concentrations of P, Mg, C, K, Ca and N varied significantly between the study sites. We observed higher values of P, Mg, C and N in the urban green areas (Botanical Garden, city park in Bydgoszcz) and the rural site (Kórnik Arboretum). Adopting the criteria given in the literature [57], the content of phosphorus in the soils studied in the present study was at all sites in the range indicated as normal [56]. The content of calcium observed in the soils from all research sites was higher than in forest soils [58], but lower than in urban soils studied by Karliński et al. (2014) [25].

Often among the factors causing the dieback of trees along transport routes are high concentrations of $Cl^-$and Na^+ ions in the substrate. The source of these elements in the urban soil is salt, used in winter as the cheapest and most effective de-icing agent for roads and sidewalks. A high degree of salinity contributes to the degradation of soils and to an increase in their pH, as well as to disturbing the ionic balance [59], and causes physiological drought, because the water strongly bound in soil colloids is inaccessible to plants [60,61]. The concentrations of Na and Cl in soil, which may limit the health of trees, were determined as 2.7 mg 100 g^{-1} DM of soil for Na and 3–3.5 mg 100 g^{-1} DM of soil for Cl [62–64]. In the present study, the concentration of Na slightly exceeded the limit at one urban site (the Botanical Garden—2.85 mg 100 g^{-1} DM of soil) and the concentration of Cl, ranging from 1.42 to 2.25 mg 100 g^{-1} DM of soil, was below the dangerous level in both the urban and rural habitats. Additionally, the electrical conductivity of the soils at all sites investigated was much below the value of 1.0 mS cm^{-1} considered critical for non-irrigated soils [65]. One of the most common and harmful heavy metals to plants and microorganisms is Pb, derived from gasoline and lead paints used until just recently. In the tested soils, the concentration of available Pb was 3–5 times lower than the average concentration of available Pb in a dozen or so urban soils in Poland [66].

The enzyme activity of non-specific dehydrogenase (DHA) in the soil indicated a significantly higher vitality of soil microorganisms in the urban green site (Botanical Garden of KWU) and in the rural environment than in soil from the remaining urban sites. The activity of nonspecific soil dehydrogenase was positively correlated with the water content in the soil (Table 5). Lower DHA levels in soils in urban sites located in Upper Silesia and in southern and eastern Poland, compared to non-urban sites, were also shown by Bielińska et al. (2011) [67]. Lower DHA levels in urban areas, especially along busy streets, may be due to the pollution caused by toxic gases emitted by cars. A negative correlation between

the activity of soil DHA and the level of air pollution was reported by Li et al. (2015) [68] in soils from areas located along transportation routes in urban localities in Beijing, China, compared to non-urban Yanqing areas. Toxic gases can damage leaves and reduce their photosynthetic activity, which in turn limits the pool of carbohydrates transported to the roots and secreted into the soil. Soil microorganisms use carbohydrates in root exudates as respiration substrates; therefore, reducing the amount of carbohydrates can reduce the viability of microorganisms and their respiration activity [69]. Reducing the viability of soil microorganisms may reduce the rate of decomposition of organic matter and thus limit the level of nutrients available for plants and the soil microbial community [68].

The neutral and slightly alkaline soil pH found in soil under canopies of *A. hippocastanum* trees in this study was reported as beneficial for dehydrogenase activity [70–72]. Trevors (1984 b) [73] observed a decrease in dehydrogenase activity with a decrease in soil pH, and a soil acidity between 1.5 and 4.5 units resulted in a strong inhibition of the enzyme's activity compared to neutral soils [74]. A significantly different result from the above-mentioned findings was reported by Fernandez-Calviño et al. (2010) [75], who noted the highest dehydrogenase activity at pH 4.1. The reason for such varied results may be the fact that the DHA in the soil consists of the activity of all dehydrogenases produced by various groups of soil microorganisms that may have different soil pH optima. Bacteria are highly dependent on soil pH, whereas fungi are generally only weakly pH-dependent and can grow over a wider pH range than bacteria [76,77]. The biomass of microorganisms in soils and the activity of dehydrogenase are also affected by soil moisture [78]. Both the deficiency of water in the soil and its excess inhibit the activity of nonspecific dehydrogenase [69].

5. Conclusions

This multi-season research study showed that *Aesculus hippocastanum* L. has the ability to establish and maintain an effective and functional mycorrhizal symbiosis with native arbuscular fungi at all study sites, even in unfavorable urban habitats.

The mycorrhizal status of *Aesculus hippocastanum* is more strongly influenced by climatic conditions and seasonal rhythms of trees than by urban/non-urban locations.

Under controlled conditions (trap cultures), the mycorrhizal infectivity of the arbuscular mycorrhizal fungi native to urban habitats is similar to the infectivity of the arbuscular fungi in non-urban soil. The results suggest that the growth and functioning of native arbuscular mycorrhizal fungi and associated soil microorganisms in urban environments can be improved through management practices, especially by increasing water availability.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15010084/s1, Figure S1 a: Arbuscular mycorrhizal colonization of *Aesculus hippocastanum* L. roots, b: Arbuscular mycorrhizal colonization of *Plantago Lanceolata* roots.

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