

**Portofoliu de lucrări considerate relevante de către candidat,
elaborate în domeniul de doctorat vizat**

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3. Bratu, I.A.; Câmpu, V.R.; **Budău, R.**; Stanciu, M.A.; Enescu, C.M. Subsidies for Forest Environment and Climate: A Viable Solution for Forest Conservation in Romania? Forests 2024, 15(9), 1533;
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In Vitro Propagation of Several Valuable Selections of *Robinia pseudoacacia* L. as a Fast and Sustainable Source for Wood Production

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Abstract: There is an increasing trend in forest production towards planting rapid-growing trees as attractive, environmentally friendly energy sources. This study aimed to establish an alternative to the traditional propagation of a number of selections of *Robinia pseudoacacia* L. by developing an in vitro culture protocol. This study's topic is of great importance, and it reflects an ongoing concern at the University of Oradea's Faculty of Environmental Protection's sustainable research program. The explants from four forms (called S1, S2, S3, and S4), selected for their phenotypic characteristics, were inoculated on four culture media (Murashige–Skoog (MS), Anderson, Chée–Pool, and Driver and Kunyuki Woody (DKW)) with the same phytohormonal balance. DKW medium proved to be the better support of morphogenic activity, and it was further tested under different phytohormonal balances. Different results were observed depending on the hormone content in the DKW environment. In the presence of 0.5 mg/L benzylaminopurine (BAP) and 0.04 mg/L aminoisobutyric acid (AIB), 91.5% of the explants developed an average of 4.45 ± 0.18 shoots, whereas the average upper shoot height (3.82 cm) was recorded on DKW medium with 0.5 mg/L BAP and 0.04 mg/L α -naphthaleneacetic acid (NAA). Auxin, 0.05 mg/L AIB, promoted root production (5.27 ± 0.15 roots/explant), while 0.1 mg/L NAA promoted root length. In conclusion, the S4 selection produced the greatest outcomes of all environmental variables in terms of both the number of shoots and their heights.

Keywords: black locust; *Robinia pseudoacacia* L.; in vitro propagation; tissue cultures; phytohormones



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1. Introduction

Woody plants are usually resistant to in vitro propagation. However, studies to establish in vitro propagation protocols are needed to obtain high-quality micropropagated plants in a short amount of time. Micropropagation techniques are also important when the preservation of genetic resources is desired, when somaclonal variation is exploited, or when genetic engineering techniques are used.

Robinia pseudoacacia L. is a fast-growing, multi-purpose nitrogen-fixing tree that is used for timber and firewood as an important melliferous species and with very good results in the conservation and fight against soil erosion [1–3]. This species is mainly propagated via seeds. This method of propagation has the disadvantage that the plants obtained in this way are significantly uneven. Additionally, some Robinia plants produce few seeds. With a low yield for propagation, root or stem cuttings can also be used, but these methods are difficult and require tremendous efforts and a long time to grow, seeing as, in the case of root cuttings, the radicular biological material is limited, as it is collected in small quantities during the tree's rest period to avoid injury. The propagation of valuable biotypes in

culture involves their identification and selection followed by in vitro micropropagation that ensures a very good uniformity of propagules. Several papers have been published that addressed the problem of in vitro multiplication of this species, with emphasis on the type of explants and the type and concentration of plant growth regulators [4–6]. Plants that are regenerated in vitro form roots more easily than cuttings taken directly from mature trees and can also be produced in large numbers [7,8]. The results that have been reported so far have described a series of difficulties related to the in vitro micropropagation of some Robinia selections [5,9,10]. As approaches to the in vitro regeneration of this species, the literature has outlined propagation from nodal explants and axillary buds [11–13], the formation of adventitious shoots from the callus, leaves, and hypocotyl fragments, cotyledons [14,15], and embryogenesis [16,17].

Optimizing mineral nutrients in the culture medium represents an important solution for enriching the micro-propagation protocol of plants, as these have a substantial influence on morphogenesis and organogenesis [18,19]. The ingredients which comprise culture mediums (organic and non-organic nutrients, such as carbohydrates, vitamins, and plant growth regulators) are determining factors for the quality of the final product obtained in any culturing protocol of plant cells [20]. Multiple studies have reported physiological disturbances and/or toxicity due to their lacking or excessive presence in the un-organic composition of the culture medium [21,22]. The most widely used culture medium for plant tissues is the MS medium [23,24]. Its composition makes it apt for elaborating new medium compositions, although it is often inadequate as it generates physiological disturbances, such as the necrosis of shoot tips and/or vitrification [25–27]. The first study relating to this topic, which focused on the in vitro propagation of acacia, was initially conducted in 1985 by Enescu and Jucan [10] in Romania. In the same year, Balla and Vértessy (1985) [9] reported the successful micropropagation of acacia in Hungary. In subsequent years, these studies have reported that the efficiency of regeneration is influenced both by the genotype and the composition and concentration of micro- and macro-elements, growth regulators, and other components of the culture medium. Initially, the MS culture medium was used, with other compositions being subsequently tested, with the predominant goal of obtaining well-rooted cuttings [28,29]. In Romania, research was not focused on multiplication for the purposes of production of certain cultivars or selected acacia clones, with studies focusing foremost on establishing micropropagation protocols [2].

This study aimed to perfect the in vitro culture techniques used to multiply several valuable forms of acacia, emphasizing, as a novelty element, the influence that the composition of the culture medium has on the morphogenesis processes.

2. Materials and Methods

2.1. Acacia Selections

The four acacia selections were chosen in the O.S. Săcueni of the Bihor Forestry Directorate (latitude: 47°32'38" north; longitude: 22°10'23" east; altitude 140 m). Each selection was chosen by comparing it to at least four other trees with particular qualities and situations in its immediate area (on or within 25–30 m), with the condition that the age difference between them does not exceed ten years.

The main aim of biotype selection is productivity [30]; however, this is directly related to a complex of characteristics, such as tree height, diameter, branching tendency, adaptability to winter frosts, and resistance to diseases and pests.

The trunk/spindle shape in the biotypes investigated was defined via remarkable dimensions, the productivity of woody mass, straightness, and a height of at least 10 m from the ground level.

The phenotypic differences among the acacia selections (labeled as S1, S2, S3, and S4) are shown in Table 1.

Table 1. Phenotypic characterization of the acacia selections.

Acacia Selection	Height (m)	Diameter at a Height of 1.3 m	Volume (m ³)	Height at Which the First Lateral Branch Appears (m)
S1	24	52	2.103	17
S2	22	50	1.780	15
S3	19	46	1.310	13
S4	20	48	1.494	14

2.2. Culture Conditions

The biological material consisted of nodal explants obtained from the selected specimens (labeled as S1, S2, S3, and S4). After the shoots were collected from the selected specimens, they were placed in the growth chamber with the bases in water until the commencement of active growth. We collected the tips of the growing shoots from which the explants were shaped for inoculation.

Nodal explants were sterilized by washing using a tap water jet for 15 min, following which they were disinfected with 70% ethyl alcohol for 30 s and with 0.1% mercuric chloride for 5 min. Treatments were followed by 5 rinses with sterile distilled water, each lasting 5 min. The inoculation was carried out in a sterile hood (Microflow, Tamil Nadu, India) in 30 mL tubes with 8 mL of medium. Following inoculation, the tubes were placed in the Sanyo growth chamber, which was set to 20 °C at night and 25 °C during the day, with a photoperiod of 8 h in the dark and 16 h of light (3000 lx). We made 14 repetitions for each variant. The following aspects were evaluated after 28 days: shoot regeneration, callus formation, number of adventitious roots, and number of shoots formed per explant. The experimental design from which the stages that were completed in this investigation emerge is presented in Figure 1.

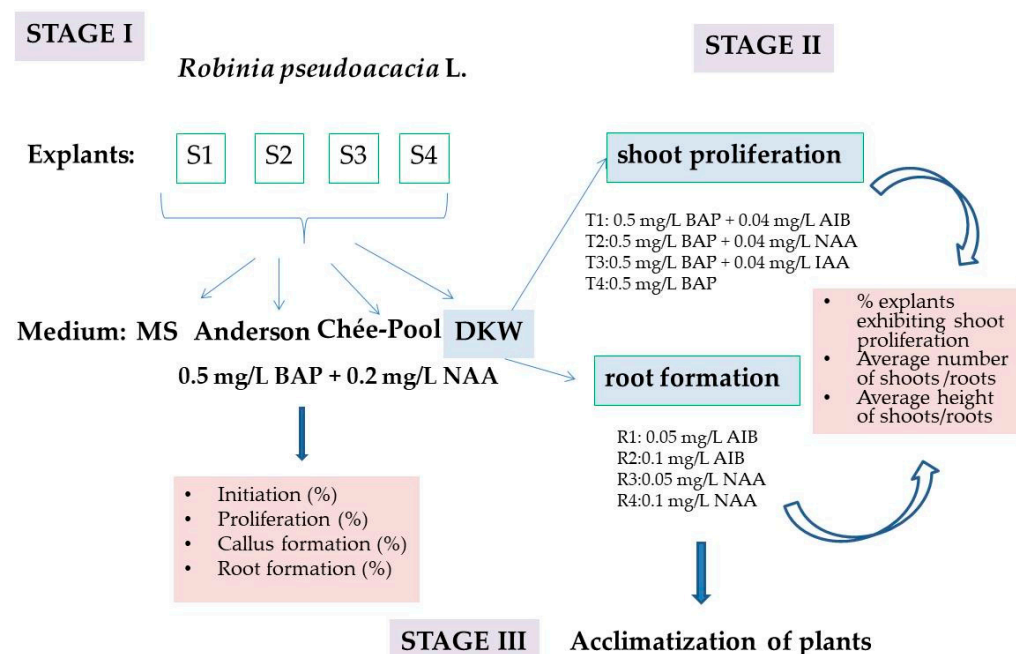


Figure 1. Experimental design. S1, S2, S3, and S4 acacia selections described in Table 1. MS—Murashige–Skoog medium, DKW—Driver and Kuniyuki Woody medium, BAP—benzylaminopurine, NAA— α -naphthaleneacetic acid, AIB— α -aminoisobutyric acid, and IAA—indole-3-acetic acid.

2.3. Shoot Proliferation and Root Formation

The influence of the composition in macro- and micro-elements of some culture media in supporting morphogenesis processes was analyzed using the culture media developed

by Murashige Skoog (1962), Anderson (1975), Chée and Pool (1987), and DKW (Driver and Kuniyuki, 1984) (Table 2). All culture media used in this study were purchased from Duchefa Biochemie (Haarlem, the Netherlands).

Table 2. The chemical composition of the culture media used in the micro-multiplication stage of the species *Robinia pseudoacacia* L.

Culture Media Composition				
Micro-Elements (mg/L)	Murashige–Skoog	Chée–Pool	Anderson	DKW
CoCl ₂ ·6H ₂ O	0.025	0.025	0.025	
CuSO ₄ ·5H ₂ O	0.025	0.025	0.025	0.25
FeNaEDTA	36.70	36.70	73.40	44.63
H ₃ BO ₃	6.20	6.20	6.20	-
KI	0.83	-	0.30	4.80
MnSO ₄ ·H ₂ O	16.90	0.85	16.90	33.80
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25	0.25	0.39
ZnSO ₄ ·7H ₂ O	8.60	8.60	8.60	17.0
Macro-Elements (mg/L)				
Ca(NO ₃) ₂	-	492.30	-	-
KH ₂ PO ₄	170.00	170.00	-	265.0
KNO ₃	1900.00	1900.00	480.0	-
MgSO ₄	180.54	180.54	180.54	361.49
NH ₄ NO ₃	1650.00	1650.00	400.0	1416.0
CaCl ₂	332.02	-	332.02	112.50
NaH ₂ PO ₄	-	-	330.60	-
Ca(NO ₃) ₂ ·2H ₂ O	-	-	-	1664.64
K ₂ SO ₄	-	-	-	1559.0

Culture media were brought to pH 5.6 before autoclaving at a pressure of 1.2 kgf/cm² for 20 min. After sterilization, 0.5 mg/L BAP and 0.2 mg/L NAA were added to each medium recipe through a 0.22 µm sterile filter (ISOLAB, Laborgeräte GmbH, Eschau, Germany).

2.4. Acclimatization

In order to acclimatize, the tubes with rooted plants were opened gradually, over several days, with the duration of opening increasing in order to reduce the relative humidity. This acclimatization stage lasted 14 days, after which the plants were removed from the culture medium and the agar on the roots was removed under running tap water. The culture substrate, consisting of a mixture of sand, peat, and perlite (1:1:1), was previously disinfected by spraying with 0.3% KMnO₄ [2]. The moisture provided to the substrate is a critical factor for plant survival. High humidity results in low aeration; the roots can be infected by mold and other pathogens; a lower substrate humidity dehydrates the leaves, and the seedlings die due to drought stress. During this stage, the relative humidity was maintained in the range of 70–80%.

2.5. Statistical Analysis

The results represent the means and standard deviation (SD). For each acacia selection, 14 repetitions were made in triplicate. Statistical significance between groups was determined with the one-way ANOVA test followed by Tukey's multiple comparison test, using GraphPad Prism. A value of $p < 0.05$ was considered statistically significant. Different letters for each sample indicate statistically significant differences. Data obtained on the proliferation and rhizogenesis of acacia selections were subjected to principal component analysis (PCA) using the statistical analysis software PAST, version 4.09, in order to establish the optimal in vitro culture medium for the development of acacia selections.

3. Results and Discussion

The type of inoculum, the culture conditions, and the ingredients of the culture media (inorganic and organic nutrients, vitamins, and plant growth regulators) are determining factors in any plant cell culture protocol [20].

Murashige–Skoog (MS) medium is the culture medium widely used in tissue culture laboratories [18], although relatively recent studies have revealed that it generates physiological disturbances, such as necrosis of the tip of the shoot and/or hyperhydricity [25,31]. It was hypothesized that the deaths of explants of some species inoculated on MS medium is due to the high concentrations of mineral salts, especially NH_4NO_3 . For many plants, the high content of ammonium nitrogen (NH_4^+) in the MS medium can increase the stress level in explant tissues, with a toxic effect on tissue proliferation. As a result, vitrified micro-shoots appear, and the regeneration potential of the culture is reduced [18]. Decreasing the concentration of NH_4NO_3 in the MS medium by 2–3 times lowers hyperhydricity in *Prunus avium* [32], *Phoenix dactylifera* [33], and *Aloe polyphylla* [34].

Compared to the Murashige–Skoog medium, in the composition of the Anderson medium, we have a reduction to about 1/4 of the concentrations of NH_4NO_3 and KNO_3 . In the medium defined by Chée and Pool, the concentrations of chlorine and manganese are lower, and calcium chloride is replaced with calcium nitrate. Shoot multiplication was improved by excluding iodine and decreasing the manganese concentration. Compared to the Murashige–Skoog medium, the DKW medium lacks the micro-elements $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and KI, and $\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$ is added to the macro-elements as a source of calcium and nitrogen, while K_2SO_4 is replaced with KNO_3 . When compared to MS basal salts, DKW media contains a similar ammonium/nitrate ratio but less total nitrogen [35].

Recent studies have revealed the potential of the DKW medium and that it may represent an alternative to MS in terms of multiple shoot induction from axillary buds and callus-mediated morphogenesis [36,37].

The composition of the culture medium is one of the recognized factors, with a significant influence on the evolution of the morphogenesis processes in the in vitro cultures of plants [35,38]. Under the conditions of the same phytohormonal balance, the morphogenesis processes evolved in different directions depending on the genotype and the chemical composition of the tested medium. The initiation of organogenesis was triggered in all environmental variants and in all selected genotypes, with values between 78–92% and with insignificant differences between variants (Table 3).

On DKW medium, 85–92% of explants produced between 4 and 5.6 shoots, while 50–64% of explants produced between 3.2 and 4.4 shoots on Chée–Pool medium. As a result, the culture medium $\times$ genotype interaction had a significant effect on the number of shoots produced. We did not record significant differences between the number of proliferated shoots from the explants of the selections inoculated on MS and Anderson media ($p = 0.89935$) (Figure 2a). The differences, determined with Tukey's multiple comparison test, were significant in terms of the average number of proliferated shoots/explant between MS and Chée–Pool ($p = 0.00151$), MS and DKW ($p = 0.00000$), Anderson and Chée–Pool ($p = 0.00024$), Anderson and DKW ($p = 0.00000$), and Chée–Pool and DKW ($p = 0.00896$).

The Chée–Pool medium, with an average of 3.9 shoots/explant, and DKW, with 4.5 shoots/explant, are the most advantageous to support the multiplication process (Figure 2b). The Tukey's multiple comparison test revealed statistically significant differences between MS and Chée–Pool ($p = 0.00248$), MS and DKW ($p = 0.00001$), Anderson and Chée–Pool ($p = 0.02901$), and Anderson and DKW ($p = 0.00014$).

The biotype had significant effects on the rate of initiation. There were significant differences between selections S3 and S1 ($p = 0.00433$), S4 and S1 ($p = 0.0004$), and S4 and S2 ($p = 0.0433$). Significant changes in proliferation capacity were found between S2 and S1 ($p = 0.0016$), S3 and S1 ($p = 0.0007$), and S4 and S1 ($p = 0.0002$). Several investigations have demonstrated that there are significant differences in the ability to initiate morphogenesis processes both between the different formulations of culture media and between the

biotypes studied [39–42]. The biotype had a significant impact on the rate of initiation. Similarly, Juncker and Favre [43] showed that the genotype had a significant effect on micropropagation capabilities in a study on *Q. robur*, as some individuals perished during the initial culture phase, while others showed a gradual decline in vitality, and most of the youths displayed rapid growth over time.

Table 3. The effect of different types of culture media, under the conditions of the same phyto-hormonal balance, on the percentage of initiation in organogenesis, proliferation, callus formation, and rhizogenesis.

Medium	Acacia Selection	Initiation (%)	Proliferation (%)	Callus Formation (%)	Root Formation (%)
Murashige–Skoog	S1	85 ± 1.84 ^a	28 ± 2.73 ^{bc}	71 ± 3.26 ^a	-
	S2	85 ± 2.34 ^a	21 ± 1.23 ^b	71 ± 2.24 ^a	7 ± 0.23 ^{bc}
	S3	85 ± 3.42 ^a	21 ± 1.24 ^b	64 ± 1.82 ^a	14 ± 1.12 ^{ac}
	S4	92 ± 2.24 ^a	42 ± 2.36 ^b	42 ± 2.24 ^a	7 ± 0.14 ^{bc}
Anderson	S1	78 ± 2.11 ^a	28 ± 3.09 ^b	50 ± 2.38 ^a	-
	S2	85 ± 3.12 ^a	28 ± 2.12 ^b	64 ± 2.12 ^a	-
	S3	92 ± 2.16 ^a	21 ± 2.02 ^b	71 ± 3.24 ^a	-
	S4	92 ± 4.14 ^a	42 ± 3.46 ^b	64 ± 2.46 ^a	7 ± 0.48 ^{bc}
Chée–Pool	S1	78 ± 2.53 ^a	50 ± 2.97 ^b	35 ± 2.77 ^b	21 ± 2.12 ^{ac}
	S2	85 ± 3.18 ^a	57 ± 1.46 ^b	28 ± 1.86 ^b	21 ± 1.60 ^{ac}
	S3	85 ± 1.26 ^a	42 ± 3.21 ^b	35 ± 2.44 ^{bc}	28 ± 1.60 ^{ac}
	S4	92 ± 4.42 ^a	64 ± 2.48 ^a	42 ± 3.12 ^{ac}	21 ± 1.22 ^{ac}
DKW	S1	85 ± 3.45 ^a	78 ± 2.11 ^a	42 ± 3.45 ^b	21 ± 2.08 ^{ac}
	S2	85 ± 2.62 ^a	85 ± 2.08 ^a	21 ± 3.42 ^b	28 ± 0.68 ^{ac}
	S3	92 ± 4.20 ^a	78 ± 1.68 ^a	64 ± 4.12 ^a	35 ± 1.68 ^{ac}
	S4	92 ± 3.26 ^a	92 ± 2.82 ^a	21 ± 2.42 ^b	42 ± 2.24 ^a

Results are expressed as the mean ± SD (n = 14). Different lowercase letters indicate significant differences within the same column ($p < 0.05$).

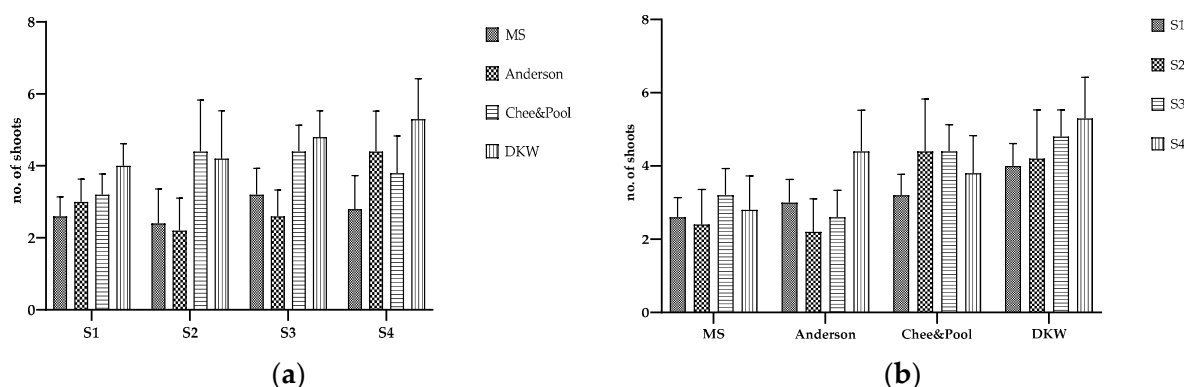


Figure 2. The average number of shoots formed on the tested media after 21 days after inoculation (a) genotype influence; (b) the influence of the culture environment.

The best callus initiation, with an average of 62% on the four inoculated genotypes, was recorded on the MS and Anderson media. MgSO_4 , CaCl_2 , and MnSO_4 are the essential nutrient macro-elements for explant growth in tissue culture and for callus formation [44,45]. Calcium chloride is the form of calcium commonly used in the composition of in vitro

culture media, with roles in cellular pH, carbohydrate translocation, and callus induction [46]. The concentration of CaCl_2 is 332 mg/L in both MS and Anderson culture media. A significant reduction in the induction of calluses formed when Mg^{2+} is missing from the medium has been observed [47,48]. On the DKW medium, 34% of the inocula formed an organogenic callus (after 21 days of inoculation) at the level of the node immersed in the medium. After 5 weeks, calli were formed on this callus from which the shoots were later generated. The presence in the composition of the DKW medium of larger amounts of MnSO_4 (33.80 mg/L) and MgSO_4 (361.49 mg/L) than in the other tested environments favored the formation of this morphogenic callus.

With the exception of the S1 selection, between 7% and 14% of the inocula on the MS formed roots. On the Anderson medium, only 7% of the S4 explants initiated the rhizogenesis process, and in the other selections this process did not commence. On the Chée–Pool and DKW media, all selections generated roots. The average percentage of rooting in all four selections on the Chée–Pool medium was 22.75%, and on the DKW medium the average on the selections was 31.5%. The following selections with a rooting percentage above the average were observed: S3 on Chée–Pool (28%) and S4 on DKW (42%). Regarding the number of roots formed (Figure 3a), no significant differences between the number of roots formed on the explants inoculated between MS and Anderson ($p = 0.11923$) and between Chée–Pool and DKW ($p = 0.45856$) were recorded, as well as significant differences between the other media (MS and Chée–Pool, MS and DKW, Anderson and Chée–Pool, and Anderson and DKW). The average number of roots on explants inoculated on MS and Anderson was 1.4 ± 0.19 , on Chée–Pool media 3.0 ± 0.54 , and on DKW media 3.4 ± 0.47 .

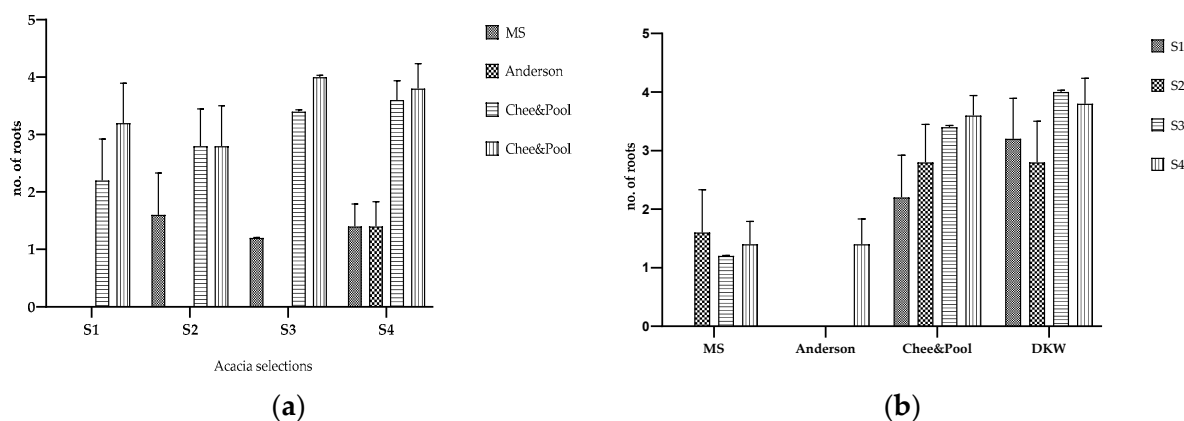


Figure 3. Average number of roots formed on the media tested after 21 days after inoculation (a) genotype influence; (b) the influence of the culture environment.

In S1 inoculums, the rhizogenesis process was only initiated on the Chée–Pool and DKW media, with an average of 2.7 ± 0.69 and 2.4 ± 0.64 roots/explant, respectively (Figure 3b). S2 and S3 did not form roots on Anderson medium. DKW medium is the only medium tested that stimulates the initiation of rhizogenesis in all four selections, ensuring an average number of 3.8 ± 0.56 roots/explant. Suggestive aspects regarding organogenesis on the culture media taken in the study are presented in Figure 4.

Based on the results obtained at this stage, we continued to pursue the possibility of modulating the morphogenesis processes using only the DKW medium with different phytohormonal balances. We carried out several experimental variants with the additions of BAP, NAA, AIB, and IAA. (Table 4). The effect of these combinations on shoot proliferation and root formation is presented in Tables 5 and 6.

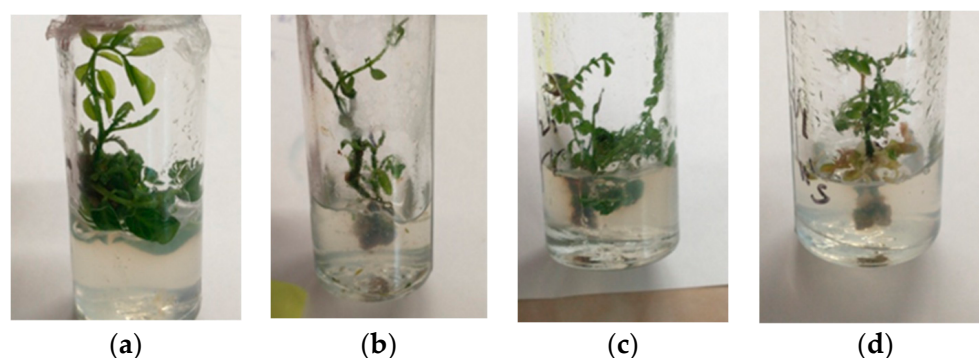


Figure 4. Morphogenesis on the tested media. (a) Multiplication on DKW medium; (b) regeneration of shoots with basal callus on Chée–Pool medium; (c) formation of adventitious shoots with basal callus on Anderson medium; (d) the formation of shoots with friable, green basal callus on MS medium.

Table 4. The phytohormonal balances of the species *Robinia pseudoacacia* L. investigated in vitro on DKW medium.

Variant DKW		AP(mg/L) ¹	AIB (mg/L) ²	NAA(mg/L) ³	IAA (mg/L) ⁴
Multiplication	T1	0.5	0.04	-	-
	T2	0.5	-	0.04	-
	T3	0.5	-	-	0.04
	T4	0.5	-	-	-
Rooting	R1	-	0.05	-	-
	R2	-	0.1	-	-
	R3	-	-	0.05	-
	R4	-	-	0.1	-

¹ BAP—benzylaminopurine; ² AIB—indole-3-butyric acid; ³ NAA—1-naphthaleneacetic acid; ⁴ IAA—indole acetic acid.

The explants consisted of nodal segments of shoots, obtained from the previous experiment. This type of explant exhibits juvenile characteristics, are poor in endogenous contaminants, and give the possibility of ensuring a high homogeneity of the explants. The inoculation was performed in 30 mL tubes with 8 mL of medium. The tubes that were inoculated were incubated at a temperature of 24 ± 1 °C, with a photoperiod of 16/8 h using a white, cold, fluorescent light of 3000 lx. Eighteen replicas of each variant were made. After 5 weeks, the culture was evaluated. The results are presented in Table 5.

The highest percentages of regeneration were obtained in the T1 and T3 environmental variants, with statistically assured differences for $p < 0.05$ compared to the T2 and T4 variants. The largest number of shoots was generated by explants placed on DKW medium with the combination of the phytohormones BAP (0.5 mg/L) and AIB (0.04 mg/L). The most intense shoot growth was ensured by the combination of the phytohormones BAP (0.5 mg/L) and NAA (0.04 mg/L), with statistically ensured differences compared to the other environmental combinations. Our results confirm the observations of Barghchi, M. (1987) and Salem et al. (2022) [1,49] regarding the positive role of NAA in the elongation of shoots grown in vitro in *Robinia pseudoacacia* [1]. The best results, in all environmental variants, in terms of the number of stems and their heights, were recorded in the S4 selection. On average, this selection produced between 10–35% more shoots and 9–14% more roots than the other selections (Table 5). The better morphogenic responses of S4 is consistent with the studies that have stated that the genotype has a large impact on micropropagation ability [43]. The organogenic responses of inoculums to in vitro cultures are determined via the interaction between endogenous phytohormones and those added to the culture medium [50]. Exogenous cytokinins are essential for shoot formation during in vitro culture, but the use of synthetic cytokinins, such as 6-benzylaminopurine (BAP),

can have a long-term residual effect, interfering with subsequent subcultures [51]. Auxin has a decisive role in regulating the spatial and temporal aspects of plant growth and development, being involved in the mechanism of the orientation of the cell division plane, before lateral root initiation, and in the formation of meristems [52,53].

Table 5. Shoot proliferation from stem nodal explants under the influence of different concentrations of phytohormones in DKW medium.

Phytohormonal Variant (mg/L)	Selection	% of Explants Exhibiting Shoot Proliferation	Average Number of Shoots	Average Height of Shoots (cm)
T1 BAP (0.5 mg/mL) + AIB (0.04 mg/mL)	S1	94 ± 3.30	3.8 ± 0.12	2.6 ± 0.8
	S2	89 ± 2.42	4.7 ± 0.28	2.8 ± 0.2
	S3	92 ± 2.02	4.1 ± 0.13	2.4 ± 0.2
	S4	91 ± 3.32	5.2 ± 0.18	3.5 ± 0.12
Mean T1		91.5 ± 2.24 ^a	4.45 ± 0.18 ^a	2.82 ± 0.42 ^b
T2 BAP (0.5 mg/mL) + NAA (0.04 mg/mL)	S1	80 ± 2.40	2.8 ± 0.34	3.8 ± 0.08
	S2	86 ± 2.37	3.4 ± 0.32	3.8 ± 0.3
	S3	88 ± 3.82	3.2 ± 0.22	3.5 ± 0.22
	S4	90 ± 2.02	3.6 ± 0.21	4.2 ± 0.13
Mean T2		86 ± 1.86 ^b	3.25 ± 0.06 ^b	3.82 ± 0.58 ^a
T3 BAP (0.5 mg/mL) + IAA (0.04 mg/mL)	S1	92 ± 2.32	1.5 ± 0.08	2.7 ± 0.08
	S2	90 ± 3.12	2.2 ± 0.22	2.4 ± 0.16
	S3	91 ± 2.32	1.8 ± 0.16	2.6 ± 0.2
	S4	90 ± 1.39	2.8 ± 0.14	3.2 ± 0.03
Mean T3		90.75 ± 1.92 ^a	2.07 ± 0.12 ^c	2.72 ± 0.08 ^{bc}
T4 BAP (0.5 mg/mL)	S1	78 ± 2.22	1.5 ± 0.15	1.8 ± 0.2
	S2	74 ± 2.80	1.2 ± 0.16	2.1 ± 0.12
	S3	80 ± 2.12	1.4 ± 0.14	2.4 ± 0.23
	S4	82 ± 2.79	1.6 ± 0.04	2.4 ± 0.14
Mean T4		78.5 ± 2.02 ^c	1.42 ± 0.02 ^d	2.17 ± 0.12 ^d

Results are represented as means ± SD (n = 14); different lowercase letters indicate significant differences within the same column ($p < 0.05$).

The shoots obtained were shaped to lengths of 2.5–3.0 cm and placed on the variants of the culture medium for rooting. After 4 weeks, the culture was evaluated. The results are presented in Table 6.

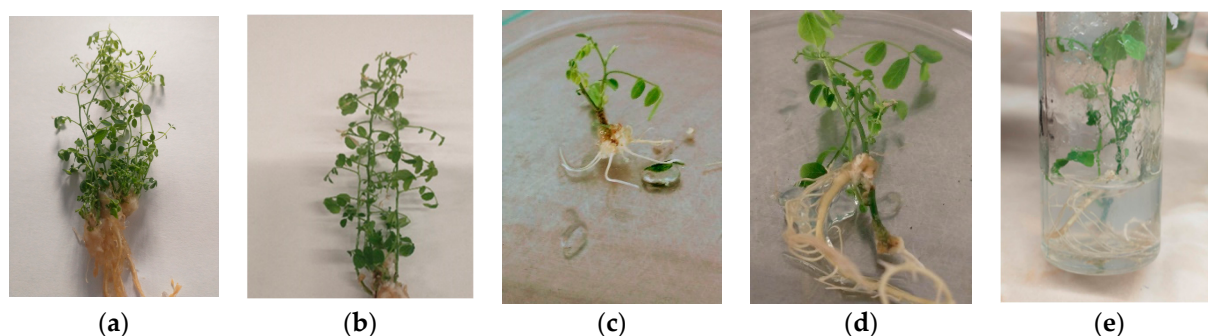
For the significance threshold $p < 0.05$, the Tukey's HSD test did not reveal significant differences between the variants in terms of the percentage of explants that generated roots (Table 6).

The medium variants T1 and T3, with 0.05 mg/L AIB and NAA, ensured a higher number of roots per explant (Figure 5), statistically assured, than the variants T2 and T4 with 0.1 mg/L. It is worth noting that the variant T1, with 0.05 mg/L AIB, provided 35.1% more roots/explant than T2 (statistically assured $p = 0.0001$), and 17.8% more roots than T4 ($p = 0.02715$). The lengths of the roots were stimulated by their presence in the culture medium of NAA (0.05–0.1 mg/L). The selection S4 on the medium with 0.05 mg/L AIB provided the highest number of roots (5.8 ± 0.11) and the longest length of roots on the medium with 0.1 mg/L NAA.

Table 6. Plant rooting in vitro under the influence of different combinations of auxins in DKW medium.

Phytohormonal Variant (mg/L)	Selection	% of Explants Exhibiting Shoot Proliferation	Average Number of Roots/Explant	Average Length of Roots (cm)
R1 AIB (0.05 mg/L)	S1	70 ± 2.02	4.5 ± 0.16	3.8 ± 0.16
	S2	68 ± 3.12	5.0 ± 0.15	4.6 ± 0.14
	S3	82 ± 2.22	5.8 ± 0.18	3.8 ± 0.05
	S4	90 ± 2.80	5.8 ± 0.11	4.2 ± 0.25
Mean R1		77.5 ± 2.54 ^a	5.27 ± 0.15 ^a	4.1 ± 0.15 ^b
R2 AIB (0.1 mg/mL)	S1	67 ± 2.02	4.6 ± 0.16	2.2 ± 0.06
	S2	60 ± 3.32	3.8 ± 0.15	2.8 ± 0.17
	S3	86 ± 1.32	3.4 ± 0.28	2.5 ± 0.14
	S4	72 ± 3.62	3.8 ± 0.14	2.9 ± 0.25
Mean R2		71.25 ± 2.57 ^a	3.9 ± 0.18 ^b	2.6 ± 0.15 ^c
R3 NAA (0.05 mg/mL)	S1	68 ± 2.38	4.7 ± 0.26	4.5 ± 0.05
	S2	71 ± 2.52	4.6 ± 0.05	4.7 ± 0.15
	S3	67 ± 2.82	5.8 ± 0.19	4.2 ± 0.2
	S4	76 ± 1.62	5.6 ± 0.09	4.8 ± 0.24
Mean R3		70.5 ± 2.33 ^a	5.17 ± 0.14 ^a	4.55 ± 0.12 ^a
R4 NAA 0.1 (mg/mL)	S1	68 ± 2.02	4.4 ± 0.18	4.5 ± 0.14
	S2	66 ± 3.12	3.8 ± 0.04	4.9 ± 0.25
	S3	69 ± 1.02	4.6 ± 0.12	4.4 ± 0.05
	S4	68 ± 1.82	5.1 ± 0.13	5.0 ± 0.15
Mean R4		67.75 ± 2.32 ^a	4.47 ± 0.11 ^b	4.7 ± 0.14 ^a

Results are represented as means ± SD (n = 14); different lowercase letters indicate significant differences within the same column ($p < 0.05$).

**Figure 5.** Micromultiplication and rhizogenesis (a,b) and rooting (c–e) of *Robinia pseudoacacia* L. S4 inoculums on DKW medium.

3.1. Acclimatization of Plants Obtained In Vitro

An in vitro micropropagation system can only be considered successful after establishing the conditions of transfer and acclimatization to field conditions. For plants obtained in vitro, under rigorously controlled conditions, transfer to the greenhouse or field environment represents a great challenge due to a lower relative humidity level, higher light level, and septic environment [54]. Plants grown in vitro often show a low rate of photosynthesis and incomplete autotrophy, and these may be the reasons for the low survival rates of plants during the acclimation stage [55]. In addition, the nutrients in the culture media cause deviations in development, and repress or modulate several metabolic pathways differently than soil conditions [56]. As a result, plants grown in vitro develop small juvenile leaves, with a weak cuticular layer, defective stomata, and reduced photosynthetic capacity,

while the roots have no or very few absorbent hairs [57]. The acclimatization of *Robinia pseudoacacia* plants obtained in vitro to an ex vitro environment by gradually exposing them to the relative humidity of the environment and to different light levels better facilitate the survival of young and physiologically sensitive plants when transferred to the soil. The conditions that are ensured at this stage help the plants to develop a fully functional root system and to better control their stomatal and cuticular transpiration [56].

It has been demonstrated by the authors of [57–62] that this approach improves stomatal physiology and increases the production of epicuticular wax. The same authors recommended that the level of relative humidity should not drop below 80% in order to permit good aeration of the culture. *Robinia pseudoacacia* L. adapted to ex vitro conditions is shown in Figure 6. In our study, the average percentage of survival after 4 weeks was 68%.



Figure 6. Plants of *Robinia pseudoacacia* L. adapted to ex vitro conditions.

3.2. Principal Component Analysis

Principal component analysis is a useful statistical technique for determining the correlations between variables. The PCA plot was used to analyze the following in vitro culture medium variables: MS, Anderson, Chée–Pool, and DKW. A 2D plot PCA (Figure 7) revealed the differences in the acacia selections based on the proliferation and rhizogenesis processes. The covariance matrix eigenvalues revealed that the set of the two principal components (PC1 and PC2) accounted for 88.919% of the total variance in the dataset in terms of the organogenesis process (Table 7). PC1 accounted 77.867% of the variance, with PC2 explaining the remainder of the variance, 11.052%. PC1 had an eigenvalue of 3.115, while PC2 had an eigenvalue of 0.442.

Table 7. The percentage of variance explained by each successive principal component.

PC	Eigenvalue	Cumulative Eigenvalues Explained by Each PC Percentage of Variance	Percentage of Variance Explained by Each PC (%)	Cumulative Percentage of Variance (%)
1	3.115	3.115	77.867	77.867
2	0.442	3.557	11.052	88.919
3	0.332	3.889	8.2959	97.215
4	0.111	4.000	2.7848	100.00

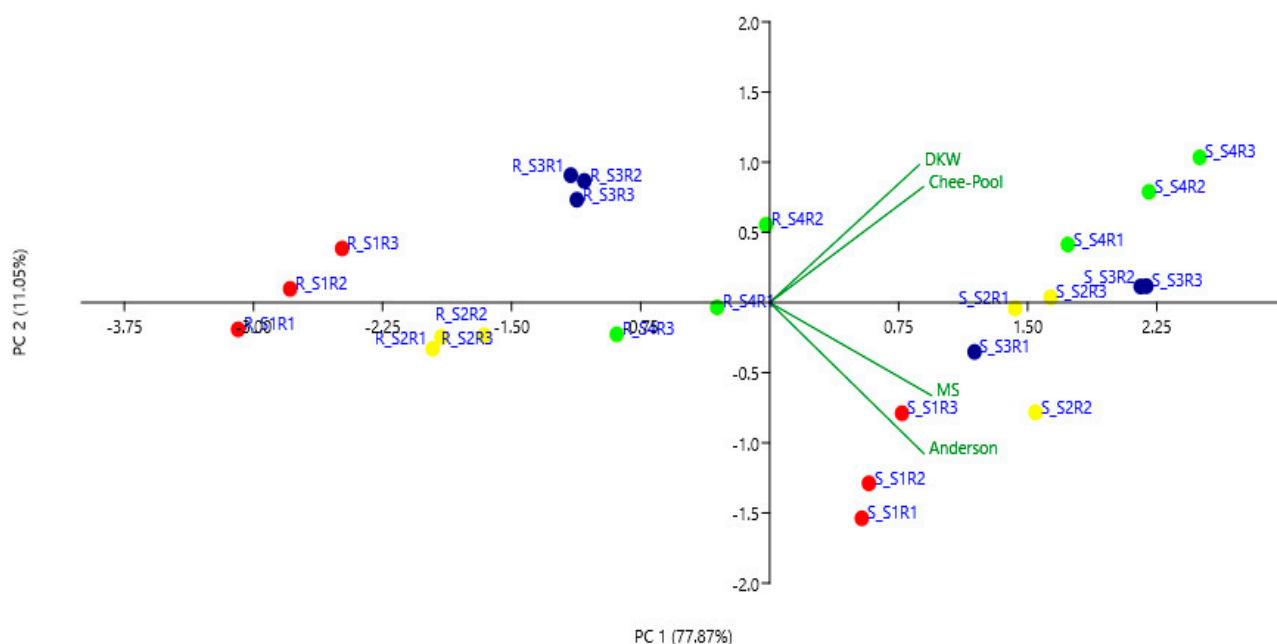


Figure 7. Principal component analysis (PCA) of the organogenesis process of four acacia selections. The red dots represent the percentage of S1 acacia selections' root production (samples with R as the first letter) and proliferation (samples with S from shoots), while the yellow dots represent S2, the blue dots represent S3, and the green dots represent S4.

4. Conclusions

The goal of this research was to test media, with specifications for woody plants, appropriate for large-scale cloning of acacia genotypes reluctant to traditional methods of vegetative multiplication. The vegetative response of the plants varied between the four selections, with a preference for the DKW basal salts. This tendency was manifested both in terms of callogenesis and rhizogenesis and led us to the conclusion that the DKW medium is more suitable for the in vitro multiplication of the *Robinia pseudoacacia* species L. than the other tested media. Considering the current climate context, the *Robinia pseudoacacia* L. species may become more significant due to its strong adaptation to poor and eroded soils, fluctuating temperatures, and positive economic impacts. In addition to providing a starting point for future research on the selection and evolution of this species, the current study can yield important insights for forestry practice and science.

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Article

Expert-Based Assessment of the Potential of Agroforestry Systems in Plain Regions across Bihor County, Western Romania

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Abstract: Agroforestry systems are gaining increasing attention worldwide due to their several benefits both for landowners and also for the environment. Even though Romania has a great potential for adopting these systems on a large scale, only a few examples exist. The aim of this research was to highlight the main agroforestry systems that could be introduced in plain regions across Bihor County, Western Romania. A selection of the most suitable woody species and cereals was carried, based on available data and information. In order to select the most suitable combination, a set of eight criteria was considered and an Analytical Hierarchy Process Analysis was performed, with the aid of the Expert Choice Desktop (v. 11.5.1683) software package. The combinations that had the black locust as the main tree species scored better in comparison with the ones that had pedunculate oak as a main species. This research should be regarded as a first and important step in the analysis of several combinations of agroforestry systems that could be implemented across plain regions of Bihor County. Lastly, this proposed model could be replicated in similar studies aimed at selecting the most suitable agroforestry systems for certain sites. Future research should also consider criteria that account for various aspects, including the functional relationships of these future green spaces with nearby areas.

Keywords: Analytical Hierarchy Process; black locust; green spaces; land management; maize; pedunculate oak; sunflower



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1. Introduction

Agroforestry (AF) systems include several land-use practices in which agricultural crops or pasture are integrated with woody plants (both tree and shrub species), which are regarded as an important component of agricultural land development [1,2].

There are several categories of common agroforestry practices worldwide and also in Europe [3–5] such as wood pastures, riparian buffer strips, hedgerows, windbreaks, grazed forests, intercropped and grazed orchards, forest farming and more novel silvoarable and silvopastoral practices and systems such as alley coppice, alley cropping and woodland

chicken [6]. All these practices have the combination of trees and shrubs with crops and/or livestock in common, offering an integrated land-use system to landowners [7,8].

As this study is focused on agroforestry systems in Bihor County (Western Romania), an overview of the current practices across Europe is required to emphasize its relevance. The development of AF systems in Europe did not take off until now because of the different regulatory systems of the member states and a lack of knowledge on this issue that could enforce more EU regulation. In the European Union, agroforestry is mainly supported through the Common Agriculture Policy (CAP) [9]. The practices of the CAP in the 20th century discouraged agroforestry practices as CAP payments for crops or pastures were often reduced for parcels with scattered trees [10]. But as time passed, land-users and policymakers needed to adapt the CAP to the EU's climate agenda and to the growing enthusiasm in agroforestry around the world, including North America. Therefore, there has been a remarkable turn-around in the perception of the role and importance of trees on farmlands during the past two decades [10].

According to Burgess and Rosati [11], there are two CAP mechanisms for farm support in the EU: direct payments in Pillar I, which is completely financed by the European Commission, and payments that support rural development in Pillar II, whose measures have to be co-financed by the member states. However, different national legal constraints are slowing down the fund uptake for agroforestry systems. For examples, German farmers claiming Pillar I payments need to subdivide agroforestry areas into area containing trees and areas which do not have trees [12]. The possibility of registering the land as agroforestry would reduce bureaucracy and would allow farmers to operate more efficiently. There are also issues regarding the support for the maintenance of lone trees and hedgerows within rural development programs. The difficulties in monitoring the extent and quality of lone trees and hedgerows make payments in Pillar I difficult [13]. Within Pillar II there is one specific agroforestry measure in which the funds are dedicated for this kind of projects, and other 27 measures that may support agroforestry systems to some extent. Therefore, the recognition of agroforestry would be increased if the measures were collated together in one place.

Recently, due to its several benefits as many scientific studies have revealed, agroforestry has gained an increasing interest worldwide, including in Europe [14–17]. For example, within the recently adopted Common Agricultural Policy 2023–27, which entered into force on the 1 January 2023, agroforestry will be supported directly by four countries, namely Portugal, Germany, Greece and the Czech Republic, and indirectly through the support of landscape features from seventeen European countries, including Romania [18]. The higher importance of agroforestry in the CAP 2023–27 can also be understood as a mechanism to reach the objectives of the European Green Deal as agroforestry could increase carbon sequestration alongside cover crops. Therefore, the recent developments within the EU Green Deal show that the European Commission is realizing the potential of agroforestry and tries to take advantage of it.

Considering the fact that AF systems are a sustainable land management option that delivers market and non-market goods and services and the increasing support from the CAP programs, governments need to develop policies and actions that foster agroforestry within an EU policy framework [19]. In addition, a European AF strategy is needed to provide a proper framework that is recognized by the member states, although, the implementation of such strategy would require knowledge of these types of practices at the European level [19].

Besides the Common Agricultural Policy, agroforestry is a key element in other EU policy areas as it is reflected in the following strategies: the Farm to Fork Strategy, 2030, and the Biodiversity Strategy and EU Forest Strategy for 2030. The Farm to Fork Strategy (F2F) addresses the challenges of sustainable food systems, setting ambitions such as reducing the use and risk of chemical pesticides by 50%, decreasing the use of fertilizers by 20% and decreasing nutrient losses by 50%, having at least 25% of the EU's agricultural land under organic farming, or reducing EU sales of antimicrobials for farmed animals and in

aquaculture by 50%, all of which aim to be achieved by 2030 [20]. All these objectives will need nature-based solutions and, along with storing carbon in soil, emphasize agroforestry as one of the most important tools.

The 2030 Biodiversity Strategy aims towards the protection and restoration of nature and putting the biodiversity on the path to recovery by 2030. The objectives of this strategy are to halt and reverse the decline of farmland birds and insects, halt soil degradation and to plant 3 billion trees by 2030 [21]. Thus, the 2030 Biodiversity Strategy strongly focus on making agriculture more nature-inclusive and nature-friendly, and mentions agroforestry as being a key tool [21]. The EU Forest Strategy for 2030 is more action-oriented and tries to accelerate the roll out of carbon farming practices through eco-schemes on agroforestry or rural development interventions and to boost research and innovation on agroforestry systems and other trees outside the forests [22].

Considering the new European developments on agroforestry, such as the CAP 2023–27 or the Forest and Biodiversity strategies, Romania has the potential to become one of the most important actors on the continent. Forest shelterbelts, grasslands with sparse trees, alley cropping, orchard meadows for both fodder and fruit production or even home-gardening are among the most common agroforestry systems worldwide and also in Romania [23–28].

These combinations of agricultural crops and forest plantations generate a broad range of benefits, such as: the development of rural communities and farms due to a sustainable production and livelihood improvement [29–33], increased biodiversity and biological control of the pests [34,35], improved and healthier soils [36,37], food production [38,39], carbon sequestration [1,40] and a veritable strategy to fight against changing climatic conditions. It was recently reported [41] that most of these benefits could be also provided by promoting agroecology techniques. In all these cases, special attention should also be given to water resource management which is critical for sustainable development of any type of social–ecological system [42].

In Romania, with the exception of field-protective forest shelterbelts that had their highest expansion during the middle of the last century, agroforestry systems represent a new concept, that is not perceived, for example, as an independent science [43]. In regards to the forest sector, the focus is on the natural regeneration of the stands and the maintenance of the composition of the stands as close as possible to the composition of natural forest types; by limiting the introduction of allochthonous tree or shrub species, for example [44]. In this context, in the last two decades, the areas afforested at national level decreased significantly [45]. But, by taking also into consideration the circular-bioeconomy transition which interferes with the forest sector [46], and the uneven distribution of forest lands across Romania, with the lowest presence in plain regions, of about 6.5% [47], we consider that agroforestry systems will play a significant role in rural development in several counties across Romania, especially in plain regions.

The aim of this study was to highlight one of the most important agroforestry systems that could be implemented in plain regions across Bihor County.

2. Materials and Methods

2.1. Study Design and Case Description

Bihor County (Figure 1) is situated in the north-western part of Romania, it has all the types of relief units, with an altitude ranging from 90 m a.s.l. (Ateaș-Cefa area) to 1849 m a.s.l. in the Bihorului Mountains. In this relief context, in regard to the forest land, Luvisols and Cambisols are the representative soil classes. Luvisols (25%), eutric cambisols (22%), dystric cambisols (20%), preluvisols (17%) and entic podzols (5%) represent the most common forest soil types in Bihor County [48]. Across the county, 205,800 hectares are occupied by forests (around 27% of the total area of Bihor), of which 85% are occupied by deciduous forests. The National Forest Administration ROMSILVA, through the Bihor Forestry Department, manages an area of 115,260 hectares, of which 61,170 hectares are the property of the Romanian state [49].

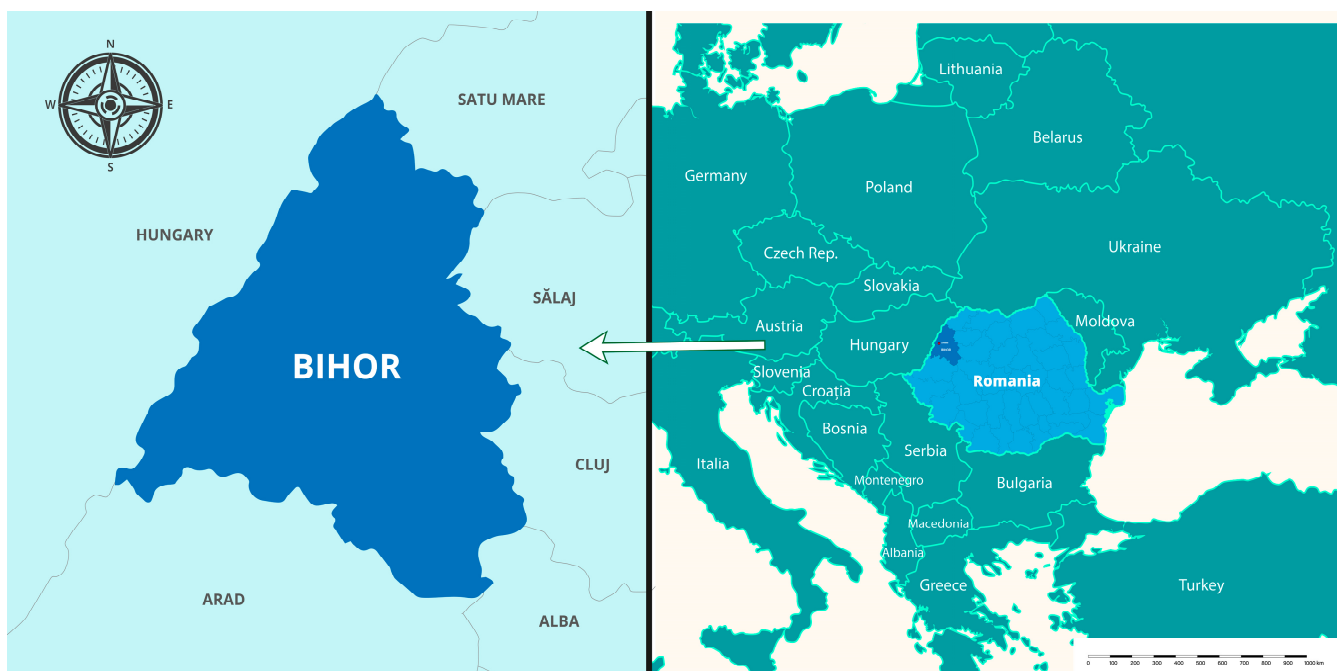


Figure 1. Location of Bihor County.

Bihor County belongs to the historical region of the Crișurilor Plain, which represents almost one quarter of the West Plain [50]. As the second largest plain by surface in Romania, the West Plain is bordered on the east by the West Hills and Occidental Carpathians, on the west by Hungary and Republic of Serbia, on the north by the Tur River, and on the south by the Republic of Serbia [51,52]. The altitude is generally low, predominantly under 100 m, and the groundwater level generally ranges between 0.5 and 3 m [53]. The region has a moderate, temperate continental climate, with strong oceanic influences generated by the dominant westerly winds. According to the meteorological data from the last five decades, the climate is characterized by average rainfall values of 620.0 mm, with a varying between 411.0 mm and 889.8 mm. The average air temperature values is 10.7 °C, with a minimum of 8.9 °C and a maximum of 12.45 °C, respectively [54]. The monthly average values ranged from −1.4 °C in January at Chișineu Criș meteorological station to 21.5 °C in July at Salonta meteorological station. The maximum and the minimum absolute temperature values recorded at Oradea meteorological station were 40.4 °C (in July 2007) and −22.5 °C (in January 1987), respectively [55]. All three meteorological stations are located along the studied area. Pedunculate oak (*Quercus robur* L.), Turkey oak (*Q. cerris* L.), Hungarian oak (*Q. frainetto* Ten.), European ash (*Fraxinus excelsior* L.), black locust (*Robinia pseudoacacia* L.), hornbeam (*Carpinus betulus* L.) and sessile oak [*Q. petraea* (Matt.) Liebl] represent the most common tree species across the Western Plain [51,52,56,57].

2.2. Case Study: Implementation of the Expert Model

To select the most suitable agroforestry system for plain regions across Bihor County, an Analytical Hierarchy Process (AHP) was performed.

AHP represents a multi-criteria decision analysis which is based on a theory of measurements focused on pairwise comparisons. Its aim is to decompose complex decision problems (i.e., the aim of this study: choosing the most suitable agroforestry system for the plain regions across Bihor County) into a hierarchy of sub-problems (i.e., the considered set of criteria), which can be deeply and independently analyzed. Thus, the alternatives (i.e., the proposed agroforestry systems) are compared one to each other and a scoring system is used [58–60].

Being simple to use, flexible and cost effective [61], AHP was widely used in several research fields in the last five decades. For example, in Romania, AHP was used to

choose different tree and shrub species for the establishment of field shelterbelts [62] or to propose the most suitable solution for afforestation of sandy soils in Oltenia Plain and Carei Plain [47].

In this study, eight agroforestry systems were proposed, each of them being composed of a main tree species, a cereal, a secondary tree species and a shrub species. A 50×200 m rectangle, divided into 4 equal squares, was considered (Figure 2). We proposed this standardized model in order to be easy to assess the concrete values regarding the yield, wood production, etc.

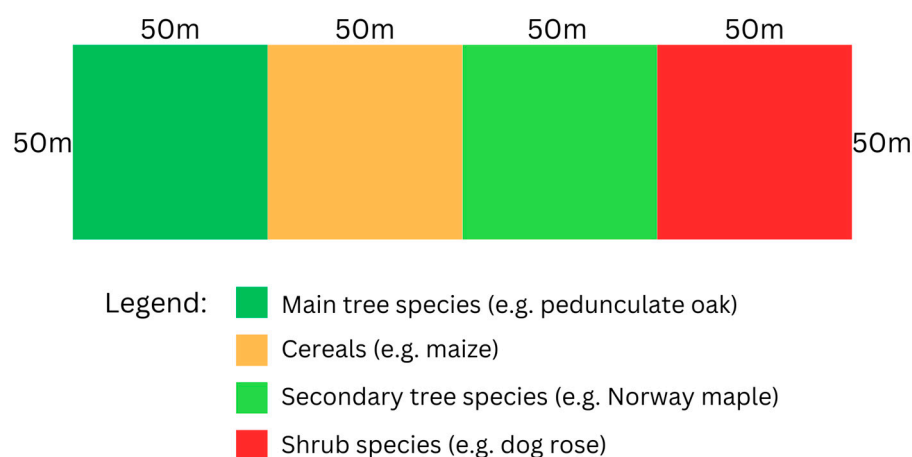


Figure 2. Design of the experimental plots.

Based on experts' opinions, eight alternatives of agroforestry systems were proposed (Table 1). The considered species were the following ones: pedunculate oak (*Quercus robur* L.)—Q.r., common sunflower (*Helianthus annuus* L.)—H.a., European ash (*Fraxinus excelsior* L.)—F.e., saskatoon berry (*Amelanchier alnifolia* Nutt.)—A.a., maize (*Zea mays* L.)—Z.m., Norway maple (*Acer platanoides* L.)—A.p., dog rose (*Rosa canina* L.)—R.c., common wheat (*Triticum aestivum* L.)—T.a., blackthorn (*Prunus spinosa* L.)—P.s., black locust (*Robinia pseudoacacia* L.)—R.p. and honey locust (*Gleditsia triacanthos* L.)—G.t., respectively. The selected woody species are also among the most often used in the afforestation of several categories of degraded terrains across Romania [63].

Table 1. The eight considered alternatives of agroforestry systems.

Alternative	Species			
1	Q.r.	H.a.	F.e.	A.a.
2	Q.r.	Z.m.	A.p.	R.c.
3	Q.r.	T.a.	F.e.	P.s.
4	Q.r.	H.a.	A.p.	A.a.
5	R.p.	Z.m.	G.t.	P.s.
6	R.p.	T.a.	G.t.	R.c.
7	R.p.	H.a.	F.e.	A.a.
8	R.p.	P.s.	F.e.	R.c.

In the case of tree and shrub species seedlings, according to the technical norms, a distance of 2 m between the rows and a distance of 1 m between the seedlings on the same row were adopted, which means that 1250 seedlings were considered to be planted in each square.

For cereal crops the average sowing density was 55,000–80,000 plants/ha for maize, 50,000–65,000 plants/ha for sunflower, using the 20×70 cm sowing scheme in both cases,

and 450–550 plants/m² (180–280 kg/ha) in the case of wheat that is sown at an average distance of 12.5 cm between rows.

Moreover, in the case of the three selected cereals, concrete data regarding the yields/hectare and the costs/kilogram are available from the Romanian Institute of Statistics for the timeframe 2013–2022 [64] (Table 2).

Table 2. Yields and costs for common wheat, maize and common sunflower crops for the timeframe 2012–2022.

Species	Year									
	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022
Yield/hectare [kg/ha]										
<i>Triticum aestivum</i>	3985	3763	3783	2935	4110	4176	4361	4072	4173	3833
<i>Zea mays</i>	4781	4276	3106	4217	6117	7503	7519	6582	6300	2934
<i>Helianthus annuus</i>	1822	2003	1567	1978	2505	2069	2887	2369	2132	1620
Cost/kilogram [Euro/Kg]										
<i>Triticum aestivum</i>	0.16	0.14	0.15	0.12	0.12	0.13	0.14	0.15	0.20	0.30
<i>Zea mays</i>	0.15	0.11	0.11	0.12	0.11	0.12	0.12	0.13	0.20	0.28
<i>Helianthus annuus</i>	0.23	0.21	0.29	0.26	0.25	0.24	0.24	0.31	0.52	0.56
Cost/hectare [Euro/ha]										
<i>Triticum aestivum</i>	654	542	552	340	493	551	628	627	826	1150
<i>Zea mays</i>	698	479	342	506	697	900	887	882	1247	810
<i>Helianthus annuus</i>	419	421	454	522	636	492	699	739	1104	901

In order to select the most suitable agroforestry system for plain regions across Bihor County, a set of 8 criteria was proposed as follows:

1. The cost for planting/sowing (1—the highest cost . . . 8—the lowest cost). This criterion considers the price of seedlings, the number of seedlings per hectare and the needed operations for planting manually and/or mechanized. According to the National Recovery and Resilience Plan, in a plain region, the cost for planting 1 ha of oak-dominated culture is 6379 EUR, while in the case of a black locust plantation, the cost is 5060 EUR, respectively [65]. In the case of cereals, there are minor differences, with the cost being 1100 EUR per hectare for wheat and 1200 EUR per hectare for maize and sunflower, respectively. These costs include plowing, land preparation, herbicide, seed cost, treatments, weeding in the case of maize and sunflower, fertilization and harvesting.
2. Yearly maintenance costs for the first 3 years (1—the highest cost . . . 8—the lowest cost). This criterion includes the cost of grass cutting between the rows with trees/shrubs, soil mobilization around the tree/shrub seedlings and applying fertilizers/pesticides, where needed. In the case of an oak-dominated culture, the cost for the first year accounts for 2025 EUR/ha, which is similar to that of a black locust plantation. Differences between the two plantations appear in the second and third years: with 4089 EUR/ha and 2854 EUR/ha, in the case of an oak-dominated culture, and 2310 EUR/ha and 1226 EUR/ha for black locust, respectively.
3. Woody biomass production after 5 years (1—the lowest quantity . . . 8—the highest quantity). This criterion is also correlated with the speed of growing of the woody species considered within this research.
4. Fruit and cereal production after 5 years (1—the lowest price . . . 8—the highest price). In order to create the hierarchy, the number of fruits produced by the considered shrub species and the yield of the three cereal crops were taken into account.
5. Honey production (1—the lowest quantity . . . 8—the highest quantity). In regard to this criterion, concrete data about honey production exist in the Romanian literature for the woody species, respectively: between 10–20 kg/ha for dog rose, 20 kg/ha in

the case of European ash and pedunculate oak, between 25–40 kg/ha for blackthorn, up to 250 kg/ha in the case of honey locust and around 1000 kg/ha for black locust plantations [66,67].

6. End product diversity (1—the lowest diversity . . . 8—the highest diversity). This criterion takes into account the number and the diversity of derived products which may be obtained from certain morphological parts of the plants (e.g., leaf extracts, juice, etc.).
7. Resistance to abiotic/biotic threats (1—the lowest resistance . . . 8—the highest resistance). The resistance to a broad spectrum of abiotic and biotic threats was assessed (e.g., drought, frosts, bugs, fungi, etc.).
8. Level of biodiversity (1—the lowest level . . . 8—the highest level). This criterion takes into consideration the number of flora and fauna species that could appear and live in the environment generated by the proposed combinations of agroforestry systems.

2.3. Modeled Scenarios

In this study, like in the one used to assess the potential of certain non-wood forest products in six European Regions [68], the Expert Choice Desktop (v. 11.5.1683) software package was used and three scenarios were proposed.

In the first scenario, all 8 criteria received equal shares (i.e., 12.5%), meaning that they have an equal contribution in selecting the most suitable agroforestry system for plain regions across Bihor County.

In the second scenario, criterion “the cost for planting/sowing” and criterion “yearly costs for maintenance in the first 3 years” received a share of 20% each, while the remaining 6 criteria only 10% each, respectively.

Within the third scenario, criterion “fruits and cereal production” and criterion “level of biodiversity” received a share of 25% each, while the remaining 6 criteria received a share of 8.33% each, respectively.

One of the shortcomings of this method consists of the fact that, instead of dictating the ‘right’ choice, AHP assists decision-makers in identifying the option that aligns most effectively with their objectives and their perception of the issue. Therefore, AHP offers a logical structure for organizing a decision challenge, and the outcomes are associated with the expertise of the individuals who devised the hierarchy.

3. Results and Discussion

By summarizing the information from specialized manuals and studies, a brief description of the considered species was performed in accordance with the eight considered criteria. The following information is crucial for creating the hierarchy, by taking into consideration the considered criteria in different scenarios.

Pedunculate oak is one of the main hardwood species across Bihor County, covering one fifth of the forests managed by the Bihor Forestry Department. In the last few decades, it has been intensively studied across Europe and also Romania thanks to its great economic, ecological and social importance [69–71], being one of the most valued tree species in temperate forests [72]. It is a light-demanding species and it can tolerate a broad spectrum of site conditions, being resistant to dry winds and droughts [73,74]. In particular, its seedlings can grow in a wide range of shade levels, from heavy shade to direct light [75]. They also have a very good drought stress adaptive mechanism, by increasing their root system or by restricting their growth [76]. Pedunculate oak is also very appreciated in forest farming, being a key element for the production of truffles. Last but not least, it has been demonstrated that the content of soil organic matter was higher in a chernozem from an oak plantation in comparison with the same soil from a black locust plantation [77].

European ash is widespread across Central Europe, being a species which occurs in various types of broadleaf mixed forests [78]. In Bihor County it appears in mixed hardwood forests dominated mainly by oak species. It is a fast-growing species, which is able to grow in various sites with mean year temperatures ranging from 6.4 to 10.7 °C

or annual amount of precipitation ranging from 400 to 760 mm, for example [79]. In regard to soil conditions, it prefers soils with pH values from 5 to 7.5, rich in moisture and well drained [80]. It was recently reported that, in mesic sites with acidic soils and lower content of soil organic matter, the crown defoliation was lower [81]. In several European countries, in the last few decades, crown defoliation was caused by the invasive pathogen *Hymenoscyphus fraxineus* [81]. Ash is sensitive to late spring frosts, severe winters and long-term drought events [82]. It was successfully used for the afforestation of several categories of degraded lands [83–86].

Norway maple is a widespread tree species across Central and Northern Europe [87–89]. It was introduced in North America where it became invasive, being a serious threat to native forests [87,90]. It has a vigorous juvenile growth rate, it is a shade- and drought-tolerant species and it is able to grow across a broad range of soil conditions, including soils rich in carbonates [87,89–93]. Across its natural distribution range, Norway maple is a good companion for pedunculate oak, where it creates a continuous secondary layer [73]. In the current context of climate change, which generates large-scale vitality losses and dieback in some of the main timber species, the importance of this species is expected to increase [94].

Black locust is native to North America, being the most planted allochthonous species in Romania, especially in sandy soils across south-western part of the country, but also across Bihor County [95]. It is regarded worldwide as a multipurpose tree, mainly due to its great adaptability to face different kinds of environmental stresses [96]. It has a very fast juvenile growth rate, a high annual production of fast-decomposing leaves which generate a high quantity of organic matter and a very good vegetative propagation system. It is a very shade intolerant and a thermophilous tree species [95,97–100]. Moreover, black locust plantations offer several ecosystem services, such as landscape rehabilitation, fuel wood and carbon sequestration [101].

Honey locust is also native to North America. In Romania, it is usually planted in association with black locust, as is the case in the plantations installed in sandy soils in southern Romania, for example, or in the composition of field protective forest shelterbelts. It can grow in differently degraded lands, including salt-affected terrains. It prefers direct exposure to sunlight and a mild climate [102,103], but it requires deep soils, with a moderate humus content [104]. In comparison to black locust, the honey locust also provided good results in carbonated soils [85].

Saskatoon berry is also a species originating from North America, which recently received an increasing amount of attention for cultivation in Romania, especially across western parts of the country [105]. This species can tolerate a broad range of site conditions, being able to grow in different soil types, with a pH ranging from 5.6 to 8, for example [106]. Moreover, it can easily be propagated both in vegetative and generative ways [107]. An adult shrub is able to produce between 4500 and 10,000 berries [108], which represent a good source of vitamins, nutrients, bioactive components and other micro- and macro-elements [105].

Blackthorn is one of the most common shrub species across Romania, including Bihor County. It is a light-demanding species, and it has a slow growing rate, usually reaching a height of 2–3 m. It can reproduce in both generative and vegetative ways, and its roots are deeply developed in depth, with numerous lateral branches [109]. It can grow in several types of soils [109], including acid soils [110]. Its fruits are very appreciated, due to their rich content of vitamins, sugars, minerals, organic acids, polyphenols, tannins, etc. [111].

Dog rose is a light-demanding and a drought-tolerant shrub species, with low requirements for soil conditions [62,112]. It is also very appreciated thanks to its reddish fruits, which have high content of vitamin C, carotenoids and polyunsaturated fatty acids [113]. In Romania, its berries are mainly used to produce juice, jelly, jam, wine and tea [114].

Maize is one of the most widely cultivated cereals worldwide, being famous for its economic value and superior nutritional properties [115,116]. It was introduced to Europe from Central America at the end of 15th century [117]. Particularly, in Romania, maize is of great interest, being a strategic cereal crop both for internal and foreign markets [118,119].

For example, more than 96,000 hectares were cultivated in 2022 across Bihor County [64]. Even if its cultivation is dependent on fertilizers [120], maize has several agrotechnical and agrobiological properties, such as: high yield, high resistance to drought and also to some pests and diseases, high economic benefits and total mechanization of agrotechnical and harvesting works [121,122].

Common wheat plays an important role in human nutrition worldwide [123,124], being the second or the third largest crop in the last two decades [125]. Thanks to its geographical location, climate and soils, Romania is cultivating wheat on approximately one quarter of its arable land [126], being one of the most important wheat producers in the European Union [127] after countries with higher areas (i.e., France, Germany and Poland) [128]. In Bihor County, the area cultivated with common wheat accounted for more than 83,000 hectares in 2022 [64]. In the current context of increasing temperatures, water deficit represents a major challenge to wheat productivity [129]. In this context, in order to protect the crops against drought, several techniques have recently been experimented, with arbuscular mycorrhiza—wheat association or applying biochar amendments—being among them [130,131]. However, nitrogen fertilization is mandatory to increase wheat yield [132].

Common sunflower crop is the third largest agricultural crop, after maize and wheat in Romania [133], being also one of the main crops across the West Plain [57]. A total area of 31,000 hectares was cultivated in 2022 across Bihor County [64]. In recent years, among the European Member States, Romania ranked first in terms of both production and cultivated area [134,135], and particularly in regards to the organic production area [136]. It is very appreciated as a honey plant and also due to its several industrial and food uses, such as the production of edible oil, bio-fuel and fodder [136,137].

An alternative AHP ranking for the eight criteria in the case of the eight alternatives (agroforestry systems), based on the information available in specialized manuals, scientific papers and authors' expertise, is given in Table 3.

Table 3. AHP alternative ranking.

Criterion No.	Alternative							
	Q.r.H.a. F.e.A.a.	Q.r.Z.m. A.p.R.c.	Q.r.T.a. F.e.P.s.	Q.r.H.a. A.p.A.a.	R.p.Z.m. G.t.P.s.	R.p.T.a. G.t.R.c.	R.p.H.a. F.e.A.a.	R.p.P.s. F.e.R.c.
1	2	3	4	1	7	8	5	6
2	2	3	4	1	6	5	7	8
3	4	1	3	2	6	5	8	7
4	6	2	7	4	3	8	5	1
5	4	1	2	3	8	7	6	5
6	2	6	8	3	7	5	1	4
7	1	4	2	3	8	7	5	6
8	6	8	7	5	1	2	3	4

According to the AHP results, when all eight criteria received an equal share, the combination R.p.Z.m.G.t.P.s. (*Robinia pseudoacacia* + *Zea mays* + *Gleditsia triacanthos* + *Prunus spinosa*) proved to be the best alternative in terms of agroforestry systems that could be adopted in plain regions across Bihor County, accounting for 18.9% of the total global priority with respect to the goal (Figure 3, right part of the graph).

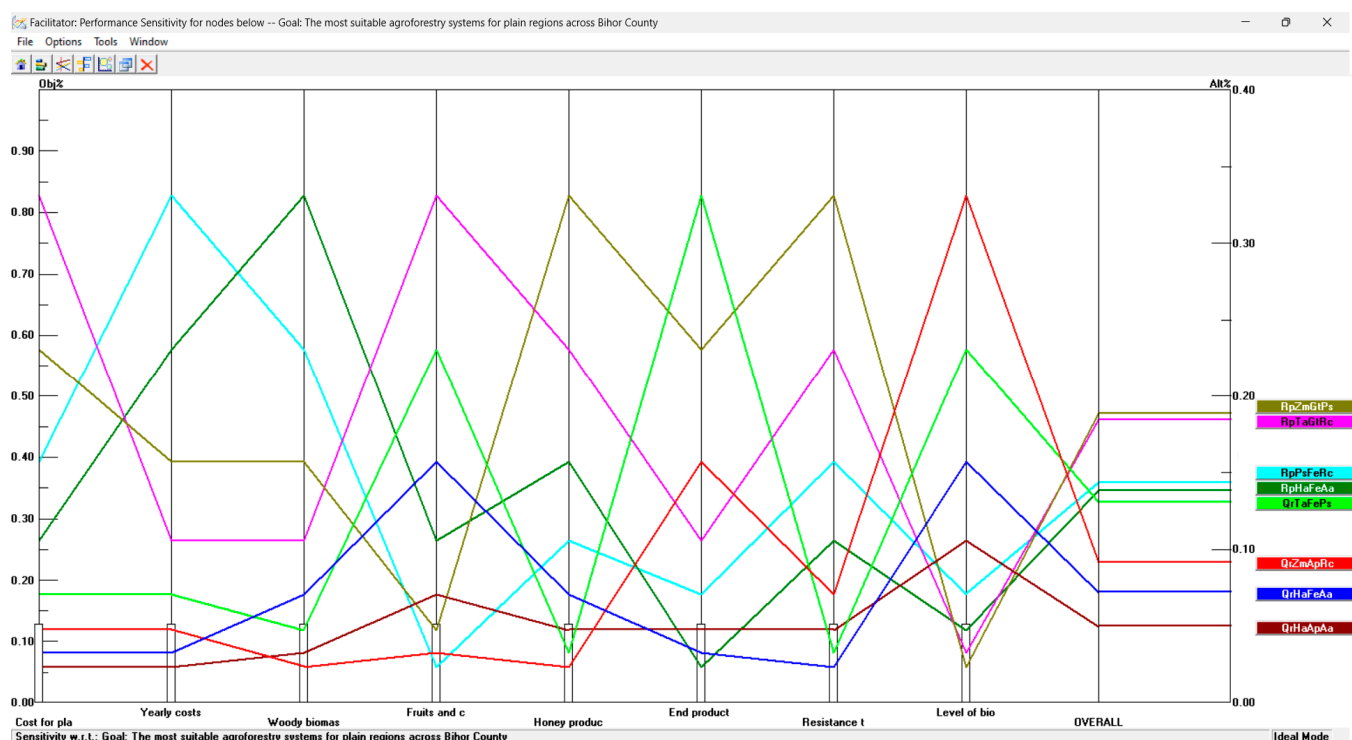


Figure 3. The ranking of the eight proposed agroforestry systems in the first scenario.

The second option was R.p.T.a.G.t.R.c. (*Robinia pseudoacacia* + *Triticum aestivum* + *Gleditsia triacanthos* + *Rosa canina*), which accounted for 18.4% of the total global priority with respect to the goal. Moreover, the four combinations with black locust as the main species scored in the first positions, while the agroforestry systems having pedunculate oak as main tree species situated in the last positions.

Within the second scenario, a switch in the first two proposals from scenario 1 was recorded, namely the combination R.p.T.a.G.t.R.c. (*Robinia pseudoacacia* + *Triticum aestivum* + *Gleditsia triacanthos* + *Rosa canina*) ranked first (Figure 4, right part of the graph), accounting for 19.1% of the total global priority with respect to the goal. However, the four combinations with black locust as main tree species were ranked in the first positions, meaning that the costs for planting and maintenance of these agroforestry systems are cheaper in comparison with the combinations based on pedunculate oak as main tree species. The combination Q.r.H.a.A.p.A.a. (*Quercus robur* + *Helianthus annuus* + *Acer platanoides* + *Amelanchier alnifolia*) was ranked in the last position.

In the third scenario, with two criteria accounting for 50% of the decision of choosing the most suitable agroforestry system, R.p.T.a.G.t.R.c. (*Robinia pseudoacacia* + *Triticum aestivum* + *Gleditsia triacanthos* + *Rosa canina*) was ranked first, accounting for 18.4% of the total global priority with respect to the goal, being followed by Q.r.T.a.F.e.P.s. (*Quercus robur* + *Triticum aestivum* + *Fraxinus excelsior* + *Prunus spinosa*) and R.p.Z.m.G.t.P.s. (*Robinia pseudoacacia* + *Zea mays* + *Gleditsia triacanthos* + *Prunus spinosa*), respectively (Figure 5).

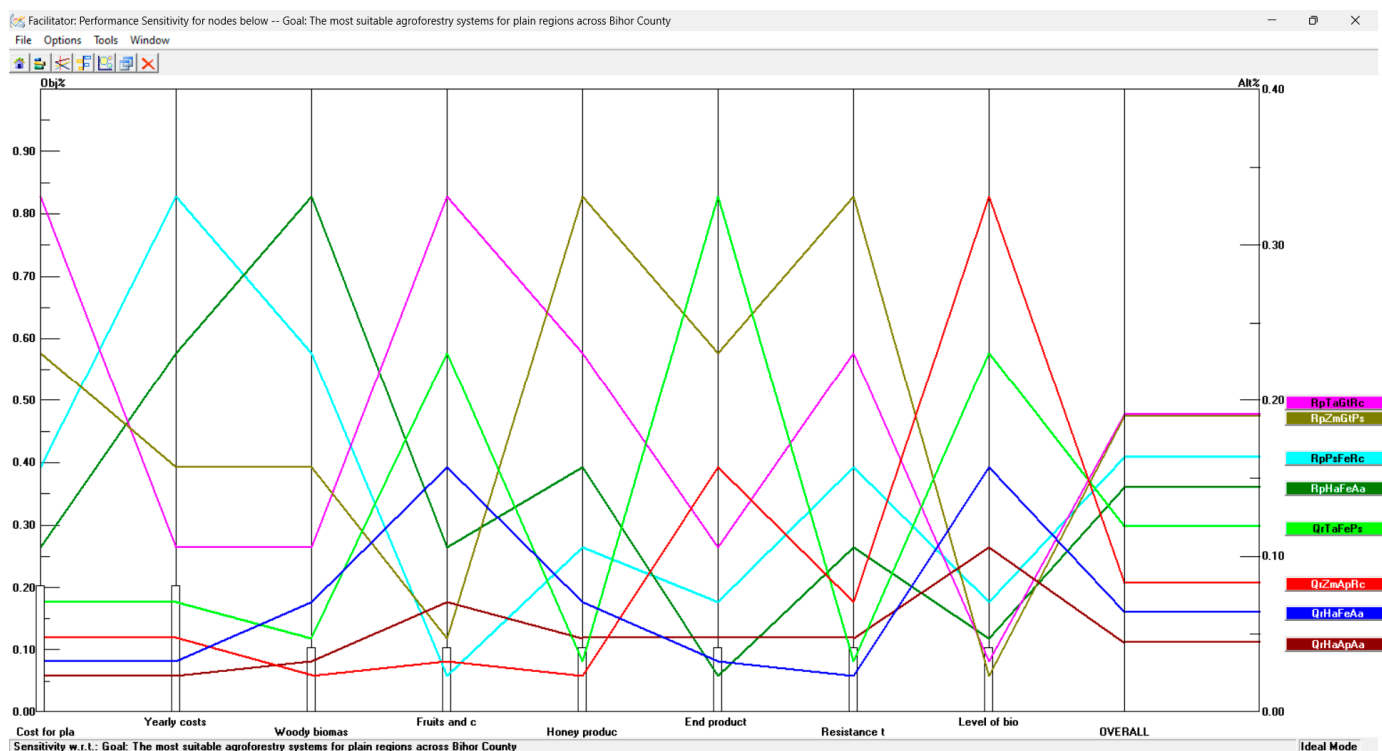


Figure 4. The ranking of the eight proposed agroforestry systems in the second scenario.

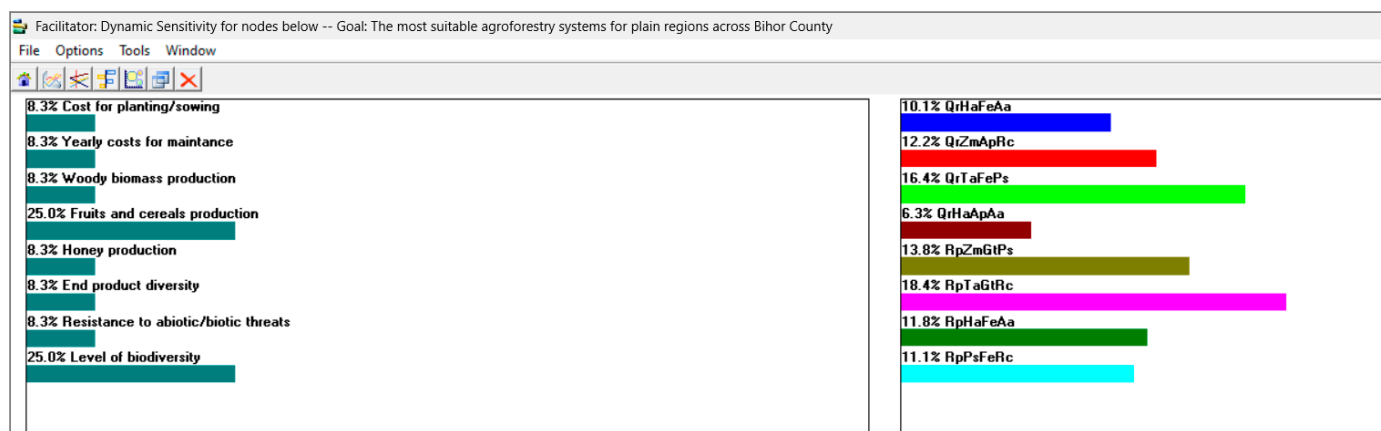


Figure 5. The ranking of the eight proposed agroforestry systems in the third scenario.

4. Conclusions

According to the literature review presented above, corroborated with the results of this study, we conclude that the plain region across Bihor County has a great potential for agroforestry systems. This potential could be better capitalized upon, for example, if more landowners will be aware of the benefits of these systems, on one hand, and if a favorable normative framework existed, on the other hand.

With respect to establishing agroforestry systems across the plain regions of Bihor County, combined with the goal of diversification, and addressing the effects of climate change, as well as addressing food crises or simply enhancing green spaces near localities, there is a pressing need for flexibility in choosing the species to be planted. However, this selection should be science based. Future research should also consider criteria that account for various aspects, including the functional relationships of these future green spaces with nearby areas. This holistic approach is crucial for integrated and sustainable development, with a primary focus on the development of rural communities.

Finally, it can be concluded that the combination of the Analytic Hierarchy Process and the Expert Choice Desktop (v. 11.5.1683) software package has proven to be a viable solution for selecting an agroforestry system that meets all the necessary criteria.

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Article

Subsidies for Forest Environment and Climate: A Viable Solution for Forest Conservation in Romania?

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Abstract: The conservation of biodiversity and forest ecosystems from the perspective of increasingly pronounced climate changes represents a priority objective for present and future society. In recent decades, alongside traditional conservation methods, innovative tools such as PES (Payment for Ecosystem Services) have been introduced to support biodiversity conservation. The aim of this study is to analyze the impact of financial mechanisms for forest ecosystem conservation from the perspective of voluntary adoption by forest owners of additional conservation measures beyond those required by national environmental legislation. Through the conceptual literature review, 6069 articles were analyzed, which described the general context of the application of PES, the aims and objectives related to climate and environmental issues, the beneficiaries, as well as the legislative conditions, and the research direction in the last 7 years. The study aims to provide relevant information regarding the viability and potential impact of PES on achieving the goal of forest ecosystem conservation in Romania, during the period 2017–2023. We identified 904 cases involving 887 entities that have committed to the program for the voluntary adoption of additional measures. It has been stated that a PES approach can lead to increased efficiency in conservation actions. Following the temporal analysis of the number of applications, it was observed that in the first sessions there was a low number of applications, which means very high reluctance of the potential beneficiaries of PES, but once the first program was implemented, their interest increased. The study found a strong correlation between payments and the areas and number of plots under commitment, while there was a less strong correlation with the geographical region. Based on the data obtained, the potential impact of PES in Romania was evaluated at the social level, quantified in the number of applications submitted, and at the economic level, with the amount of money paid to applicants. The environmental impact was measured by analyzing the areas included in the subsidy mechanism and identifying the minimum 20% of the eligible areas, totally exempt from forestry interventions, which produce effects on forests, soil, water and environment.

Keywords: payment for ecosystem services; forest subsidies; biodiversity; forest conservation management



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1. Introduction

Since ancient times, forests have been a shelter and a source of indispensable materials for the economic development of the human species [1]. The relationship between forests and humans has undergone continuous evolution, starting from a civilization dominated by forests to a society that views the forest as an ecosystem capable of providing ecosystem services [1–4]. If an important function of the forest was as a provider of products, such

as wood, berries and mushrooms, the concept has evolved today to a functional and organizational unit of the ecosphere.

Recent extreme climatic events have profoundly affected forest ecosystems and ecological stability [5–7]; thus, the involved stakeholders (e.g., foresters, environmentalists, policymakers, forests owners, and public) are interested in identifying solutions to these problems [8–10]. Forest management is carried out through forest management plans, which are based on principles including “adaptation of forests to climate change” [11]. In Romania, forests are managed in compliance with the forestry regime, which is defined as a “unified system of technical, economic, and legal forestry norms regarding the planning, cultivation, exploitation, protection, and guarding of the forest fund, with the aim of ensuring sustainable management”. In this regard, according to the norms and guidelines, forest owners have several options available for forest management.

Also, forest management must address the challenges related to biodiversity conservation [12]. The implementation of active forest management aims to maintain and increase the forested area, in accordance with Romania’s National Forest Strategy (NSF), which stipulates that the forested area will increase by 5% [13]. As a solution to ensure the future of forest biodiversity in the face of climate change, habitat loss, and fragmentation, protected natural areas are increasingly adopted, especially in Europe but also globally [14–16]. Another instrument in achieving biodiversity conservation objectives is PES (Payments for Ecosystem Services). These have the advantage of directly impacting the local economy and can be viewed as a self-financing mechanism for the management of biodiversity and forest ecosystem conservation [17]. In Romania, a PES program has been implemented in the form of state aid under Measure 15—Forest-Environmental Services, Climate Services, and Forest Conservation, managed by the Agency for Payments and Intervention in Agriculture (APIA) [18].

1.1. Purpose of Study, Objectives and Research Niche

The subject of Payment for Ecosystem Services (PES) has not been previously addressed in Romania. Currently, there are nine articles in the Web of Science that present policy design solutions to guide forest management [19–24] and issues relating to the payment terms for packages 1 and 2 of agri-environmental measures regarding the pastures [25,26].

The aim of this study was to analyze the impact of financial mechanisms for forest ecosystem conservation from the perspective of voluntary adoption by forest owners of additional conservation measures beyond those required by national environmental legislation. The study aims to provide relevant information regarding the viability and potential impact of PES on achieving the goal of forest ecosystem conservation.

The identified research problem is as follows: What is the viability and impact of Measure 15 in Romania?

The potential impact of PES in Romania refers directly to the social and economic impact in the period 2017–2023, quantified in the number of applications submitted and in the amount of money paid to applicants. The environmental impact is measured by analyzing the areas included in the subsidy mechanism and identifying the minimum 20% of the eligible areas, totally exempt from forestry interventions.

The indirect impact on the environment is translated into the conservation of forests for a period of at least 5 years, in which those 20% of the surfaces are treated as areas similar to those of nature reserves, in which any wood harvesting activities are prohibited, with effects on forests, soil, water and environment.

The objectives of the study are as follows:

Objective 1—Identification of the general context of PES application through conceptual literature review.

Objective 2—Analysis of the areas included in Measure 15 in the period 2017–2023.

Objective 3—Study of the financial results produced by Measure 15 in the period 2017–2023.

Objective 4—Description of the temporal evolution of the impact of Measure 15 at the level of surfaces and financial results.

Objective 5—Statistical correlation of data according to areas and financial results.

Figure 1 shows the graphic abstract of the paper, as well as the steps taken in the research.

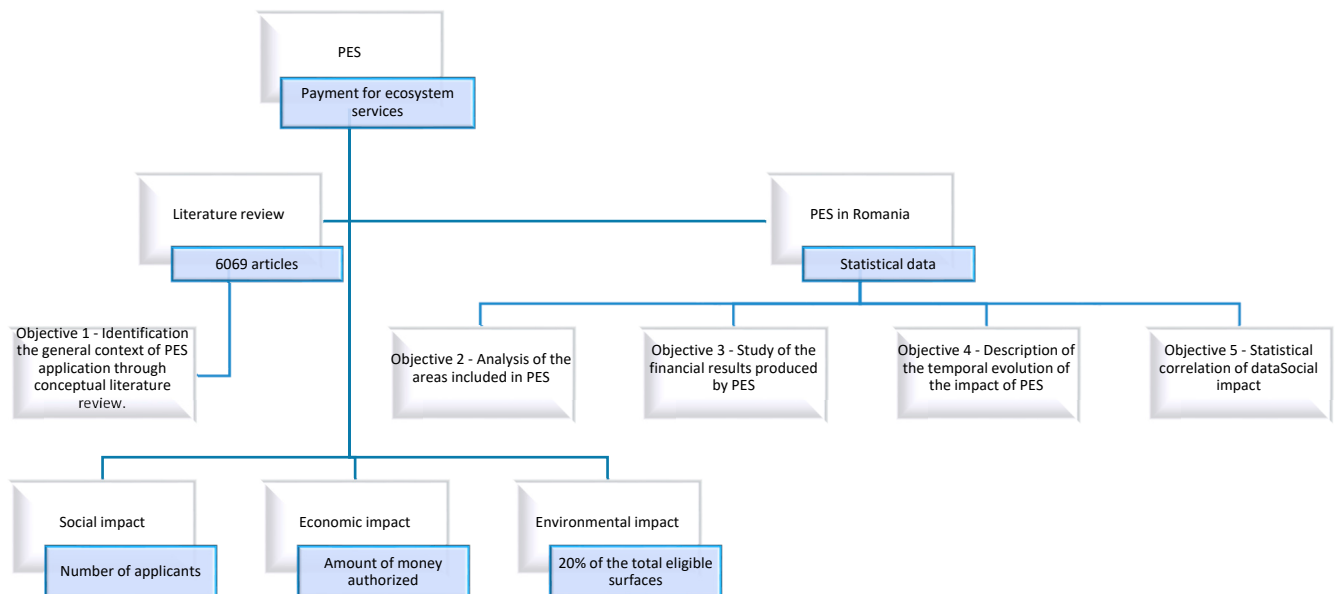


Figure 1. Graphical abstract of the paper.

1.2. Literature Review

To analyze the topic “Subsidies for silvo-environmental services, climate services, and forest conservation”, a search was conducted in the Web of Science that returned a total of 79 articles. The search for each individual topic—payments for environmental services, payments for climate services, and payments for forest conservation—returned a total of 6069 articles. Both the general topic and the individual subjects were analyzed using VOSviewer, a useful tool for visualizing, organizing and analyzing specialized literature [27–33]. The latest versions of the tool offer text mining functionalities, allowing for the construction and visualization of networks of co-occurrence of terms or specific themes in the scientific literature of one or more fields [27,32,33]. A bibliometric analysis was conducted using the VOSviewer version 1.6.18 software tool, which is “a freely available computer program developed for constructing and viewing bibliometric maps”. VOSviewer is increasingly being utilized, either independently or in conjunction with other tools, by researchers in the fields of business and management to conduct systematic reviews [28–30].

The search for the article subject “Payments for environmental services, climate services and forest conservation” in the WoS returned 190 articles, written between 2000 and 2024, of which 62% were authored in countries that are part of the European Union (92 articles). Analyzing the articles with VOSviewer (Figure 2), four clusters were identified, organized around four key terms: service, program, REDD and value.

The four clusters visible in Figure 2A describe four areas of focus for the topic “Payments for environmental services, climate services and forest conservation”: red cluster—service, challenge, ecosystem, forestry, governance, framework, sustainability; green cluster—value, production, environmental service, model, species, carbon sequestration; blue cluster—program, household, incentive, reduction, participation, factor; yellow cluster—REDD, emission, project, carbon. The table in Figure 2B presents the terms with the highest relevance scores: forest carbon, participant, connectivity, protected area, intervention, household, mangrove, species, environmental services, water, interaction. In

Figure 2C, it is observed that, on the temporal axis, subsidies began in 2016 with a general theoretical framework—with REDD, emission, forestry, and forest cover—and then continued with the green area in 2018—with services, value, and production—delving deeper into the significance of these services, followed by the establishment of their impact in 2020, as shown in the yellow area, through income, household, efficiency, and factor. Figure 2D illustrates the areas with the highest density of clusters: service, value, production, program, project and REDD.

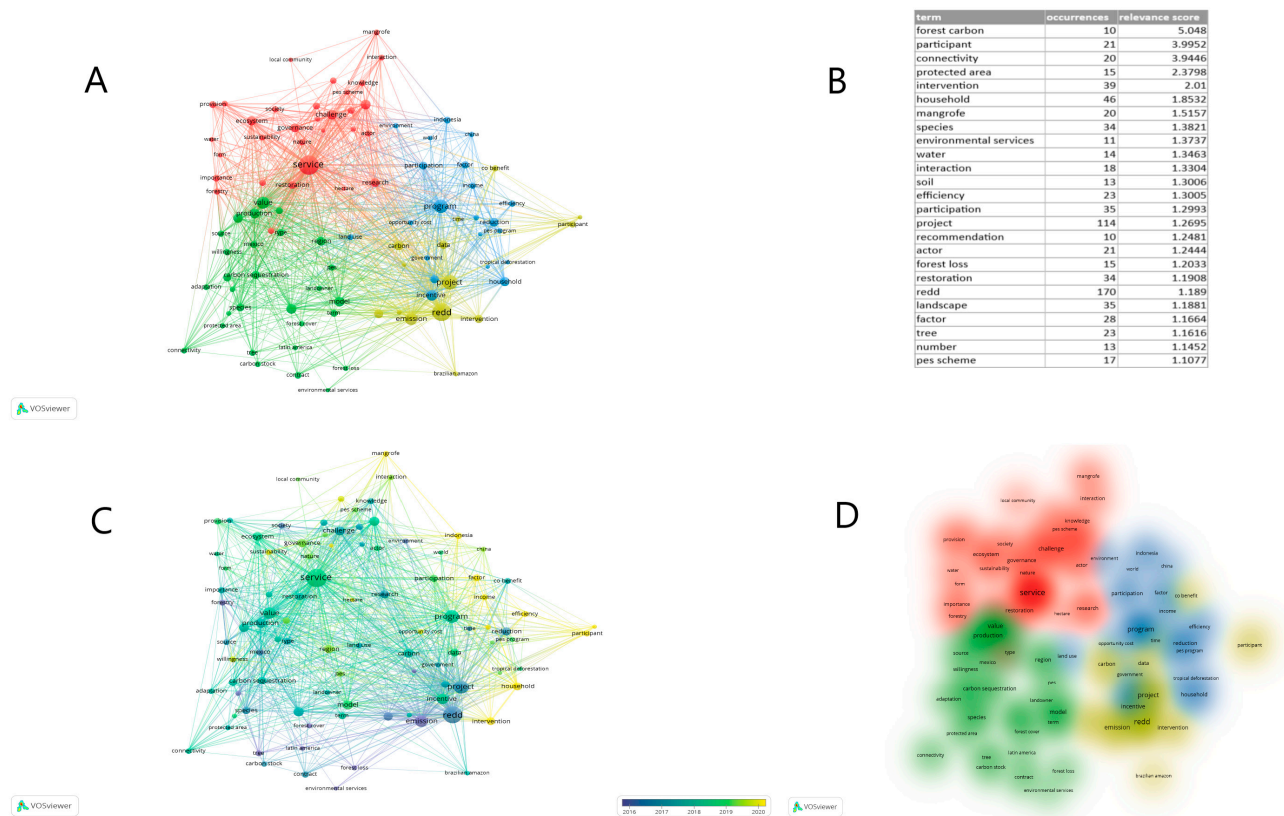


Figure 2. Analysis of the topic “Payments for environmental services, climate services and forest conservation”. (A) VOSviewer visualization map of co-occurrence keywords. (B) Relevance of keywords. (C) Timeline view of clusters of co-cited references. (D) Cluster density visualization.

Following the analysis of scientific articles from Web of Science using two applications, VOSviewer and CiteSpace, it was observed that, alongside the existing legislative framework at both the European Union and national levels [11,13,18,34–39]—which outline the goals and objectives related to climate and environmental issues, the beneficiaries, and the legislative context—the research direction over the past 7 years presents a much broader spectrum of the implications of subsidies, encompassing economic, political, social, legislative, and environmental aspects.

At the economic level, the subsidies provided support for the beneficiaries (forest owners and managers); however, the costs associated with forest conservation have a negative impact on the economic agents in those areas [40–42], as well as on the labor market [43–45]. Some argue that financial transfers supporting “carbon cultivation” neglect social concerns and fail to consider the full spectrum of goods and services provided by forests [46].

There are spatial synergies between existing policies, such as national parks and the payment scheme, and the conservation of ecosystem services [47]; national parks and areas receiving payments offer more services compared to other regions [48]. Accurate cost information is crucial for evaluating trade-offs in land management choices, which is essential for enhancing the effectiveness of forest conservation policies and market

mechanisms. The choice of evaluation method can significantly influence the valuation estimates of the costs related to forest conservation, especially for heterogeneous rural households in underdeveloped markets. Empirical evidence indicates a disparity between market prices and contingent valuation estimates concerning the costs of local forest access restrictions. This evidence is derived from household surveys employing quantitative evaluation methods [41,49]. Additionally, it is crucial to anticipate potential changes in ecosystem services and biodiversity that may result from the climate adaptation behaviors of forest owners. Focusing on maximizing carbon sequestration might not always align with the habitat needs of species [41,50], particularly in dominant Douglas-fir forests, and could encourage more hardwood logging on private forest lands [51]. There are complex interactions between factors, such as the rate of disease spread and its impact on the value of both harvested timber and non-timber benefits. The presence of the disease influences the optimal rotation period for a single-age, single-rotation forest. When private forest owners receive payments for timber benefits to partially account for the social values of forest management, the disease affects both the production and growth of the forest [52].

At the social level, empowering farmers to understand environmental strategies is essential. The introduced measures support the beneficiaries' potential for self-fulfillment by accepting restrictions and receiving financial compensation [53]. Additionally, the migration of populations from rural to urban areas has led to the abandonment of farms and agricultural lands. This phenomenon has resulted in spontaneous forest expansion and an increase in forested areas [54]. Social equity and poverty reduction are additional effects produced by environmental subsidies [50,51,55–57]. A vicious cycle exists where poverty leads to increased environmental destruction, which in turn exacerbates poverty. Education is crucial in breaking this cycle, as it helps individuals and communities access funding and resources necessary for sustainable development and environmental conservation. The analysis of influencing factors reveals that attitudes, behavioral control and awareness are significant determinants in the effective acquisition and utilization of environmental subsidies [58]. Perceived knowledge about forests has the most positive and significant impact on individuals' willingness to apply for subsidies. This indicates that greater awareness and understanding of forest-related issues encourage more people to seek financial support for forest conservation and management activities [59]. While enhancing technical capacities can help overcome some learning limitations, other challenges, particularly those stemming from ideological thinking and institutional imperatives, are more difficult to address. These deeper, more ingrained issues often require substantial cultural or structural changes to overcome [60–62].

Payment for Ecosystem Services (PES) programs are often used as a tool to reduce deforestation and forest degradation [63–66], with major environmental impacts. Subsidies for forest environments and the climate become effective tools for forest protection either directly, by changing forest management, or indirectly, by increasing biodiversity. Action-result hybrids are frequently recommendable. Agri-environmental PES have piloted results-based/hybrid schemes in Europe [67], which have led to an increase in tree species diversity, structural diversity and uneven-aged forestry [68].

PES can help maintain sustainable forest management and simultaneously protect forests. Sustainable forest management means using forests and forest land in a way—and at a rate—that maintains their biodiversity, productivity, regeneration capacity, vitality and their potential to fulfill—now and in the future—relevant ecological, economic and social functions—at local, national, and global levels—and that does not cause damage to other ecosystems [10].

1.3. Definition of Key Concepts

Measure 15—Forest-Environmental Services, Climate Services, and Forest Conservation.

Programs for Payment For Ecosystem Services are being increasingly adopted worldwide to improve sustainability outcomes [69]. Sustainability is a concept applied across

various fields, referring to development that meets present needs while ensuring that future generations can also meet their own needs [10,70–72].

The management of ecosystem services provided by forests is carried out through functional zoning, which categorizes forests into specific functional groups. As per the Romanian Forestry Code, forests are classified into two functional groups based on their main functions: group I consists of forests with special protection functions, while group II includes forests that have both production and protection functions. Within the total area of the National Forest Fund (FFN), forests with special protection functions constitute 53.3%, whereas those with production and protection functions make up 46.7% [37].

In Romania, the Rural Investment Financing Agency (AFIR) is the public institution under the Ministry of Agriculture and Rural Development, which ensures the technical and financial implementation of the European Agricultural Fund for Rural Development (FEADR), as well as the Special Accession Program for Agriculture and Rural Development (SAPARD). The Agency for Payments and Intervention in Agriculture (APIA) is the governmental agency operating under the Ministry of Agriculture and Rural Development, through which European funds are allocated for implementing support measures financed by the European Agricultural Guarantee Fund (EAGF), as well as specific measures 211, 212, 214, 215 and 611.

The funding for Measure 15—Forest-Environmental Services, Climate Services, and Forest Conservation—with sub-measure 15.1—Payments for Forest-Environmental Commitments, which is implemented through APIA—was provided by the National Rural Development Program (PNDR) 2014–2020. This program was approved by the European Commission through Decision C (2015) 3508/26.05.2015, intended to promote a balance between economic growth and the sustainable utilization of natural resources. The support provided through the measure for Forest-Environmental Services, Climate Services, and Forest Conservation aims to complement biodiversity conservation efforts and protect soil resources on forest lands within the National Forest Fund (FFN). This is implemented through the existing regulatory framework for forest management in Romania by encouraging voluntary commitments that enhance sustainable forest land management. Additionally, it contributes to biodiversity conservation and enhancement, soil erosion reduction, and climate change mitigation efforts [37]. The principle underlying sub-measure 15.1 “Payments for Forest-Environmental Commitments” is the reduction in the number of silvicultural interventions in forests and the promotion of wood exploitation technologies with reduced impact on the soil (low-impact forestry). This is accomplished by promoting voluntary commitments that exceed the mandatory requirements set by national forestry management legislation [18,35]. Sub-measure 15.1 (Table 1) falls under intervention areas 4A—Restoration, conservation, and development of biodiversity, including in Natura 2000 areas, areas facing natural or other specific constraints, and in high nature value farming activities, as well as the state of European landscapes (DI 4A)—and 4B—Prevention of soil erosion and improvement of soil management (DI 4B). It contributes to achieving the European Union’s rural development objectives related to these intervention areas [39].

The support provided under this scheme is compensatory, granted annually as a fixed amount per hectare. It compensates forest landowners for income losses, additional costs and transaction costs incurred when they undertake voluntary commitments for a period of five years.

Under the scheme, two types of commitments are compensated, with their specific requirements categorized into the following two packages: Package 1, Provision of Quiet Zones, and Package 2, Use of Draught Animals for Timber Extraction from Thinnings (Package 2 can only be implemented in conjunction with Package 1). The level of non-repayable public support provided through this scheme is set at 100% [18,35].

Table 1. The purpose and objectives of sub-measure 15.1 [73].

The purpose of the sub-measure 15.1	Enhance the stability of forest ecosystems and improve the environmental services they offer;
	Reduce the frequency of silvicultural interventions;
	Maintain increased stand density;
	Compensation for income losses and additional costs incurred by forest landowners who undertake voluntary commitments for a duration of 5 years.
The objectives of the sub-measure 15.1	Promotion of carbon sequestration;
	Adaptation to the effects of climate change;
	Reduction in soil erosion;
	Development of fauna specific to forest ecosystems;
	Restoration and conservation of local biodiversity;
	Improvement in water retention capacity.

The area eligible for the scheme must be included in an active forest management plan. The scheme is applicable to forest lands, except for those classified as functional type I (T I). According to Order 766/2018, Classification of forests into groups, subgroups and functional categories, Group I contains forests with special protection functions, and the functional type T I includes junipers, groves included in natural or scientific reserves, groves declared monuments of nature, groves from virgin and quasi-virgin forests, stands in national and natural parks included—through management plans—in a strictly or fully protected area, and stands in biosphere reserves included in a strictly protected area. Usually, this type of forest is classified as a natural protected area, and forestry works are prohibited [74]. However, areas designated for afforestation or reforestation must not exceed 15% of the total committed area. The minimum area required for making a commitment is 100 hectares [36].

Thus, through Package 1, Provision of Quiet Zones under DI 4A, the primary objective is to create optimal conditions for shelter, nesting and feeding, thereby supporting the growth of unique fauna within forest ecosystems. Additionally, by leaving dead wood (dry but standing trees) in the forest to decompose naturally, rather than utilizing it economically, the aim is to preserve a diverse range of xylophagous insects (wood-feeding insects). This strategy will contribute to maintaining a consistent population of insectivorous birds within the forest. Additionally, by maintaining a higher stand density, the forest's capacity to reduce greenhouse gas concentrations through carbon sequestration and its ability to adapt to the effects of climate change will be enhanced. This also increases the forest's capacity to retain surface runoff, particularly in the event of floods.

As a mandatory requirement, each forest owner must have a written forest management plan prepared by a professional forester and must have entered into a management contract with an authorized forestry entity. Additionally, each owner voluntarily commits to include the entire area they own within a production unit. The minimum required area is 100 hectares. Each applicant must then provide a “quiet zone” of at least 20% of the total area they register, within which no silvicultural activities aimed at timber harvesting are permitted. Only afforestation, ecological restoration and the care of young forests, including thinning and cleaning operations, may be carried out in these areas. In accordance with national legislation, it is mandatory to ensure adequate phytosanitary conditions in the “quiet zones,” with trees affected by wind or snow breakage and uprooting, classified as accidental products, being obligatorily extracted. On the remaining areas that are not part of the “quiet zone,” all activities outlined in the forest management plan are permitted, provided that each management unit is only intervened in once.

The intensity of the non-reimbursable public support granted under this scheme is 100%. The compensatory payment values are as follows: EUR 38 per year per hectare for the area committed under Package 1 and EUR 137 per year per hectare for the area annually requesting support under Package 2. For areas ranging between 100 hectares and 500 hectares (inclusive), financial support is provided at 100% of the compensatory payment value. For areas larger than 500 hectares, a degressive financial support scheme applies, with payment levels adjusted as follows: 85% of the compensatory payment value for areas between 500.01 hectares and 1000 hectares; 65% of the compensatory payment value for areas between 1000.01 hectares and 5000 hectares; and 50% of the compensatory payment value for areas exceeding 5000 hectares. Degressively applies to committed areas larger than 500 hectares for both packages [39].

In Package 1, income losses refer to the volume of wood from sanitation products that is not harvested over a period of five years in the quiet zone and four years in the rest of the committed area of management units where thinning and sanitation cutting operations are planned. Additionally, it includes the volume of products from conservation cuts over a five-year period across the entire committed area designated for such operations.

In Package 2, extra costs arising from using draught animals instead of machinery for thinning operations are compensated. Additionally, the biodiversity conservation measures supported through sub-measure 15.1 contribute to the objectives of Natura 2000 areas that overlap with the committed zones. Notably, around 38.6% of the National Forest Fund is part of the Natura 2000 protected areas network [75,76].

In the same state aid measure, through Package 2—Use of Draught Animals for Thinning Operations under DI 4B, the aim is to protect forest soil resources by promoting extensive, low-impact timber extraction technologies, specifically the use of draught animals for thinning operations, as opposed to mechanized methods, which are currently widely applied. The specificity of this requirement is that the collection operations must include at least the gathering operation and either the extraction or forwarding operation, or both. The eligible collection distance for support must be between 100 m and a maximum of 2000 m, as specified in the Timber Harvesting Operation Documents. The collection distance is calculated graphically as the arithmetic mean of the shortest and longest collection distances from a management unit to the primary platform of the harvesting site (the loading ramp for timber). Draught animals will be used on the condition that the average volume of timber collected does not exceed 0.3 cubic meters. An important aspect concerns the eligibility of interested parties. This state aid scheme targets all forest owners other than the state, who have a valid forest management plan, with a minimum area requirement of 100 hectares. This includes local public administration, individuals and legal entities, owner associations, and religious institutions. The total eligible area is approximately 52% of the total forested area, which amounts to 3.436 M hectares.

This measure, which primarily benefits biodiversity and soil components, addresses the need to maintain biological diversity and the environmental value of forested lands. It supports efficient and sustainable forest management while also protecting and enhancing soil resources, as illustrated in Figure 3.

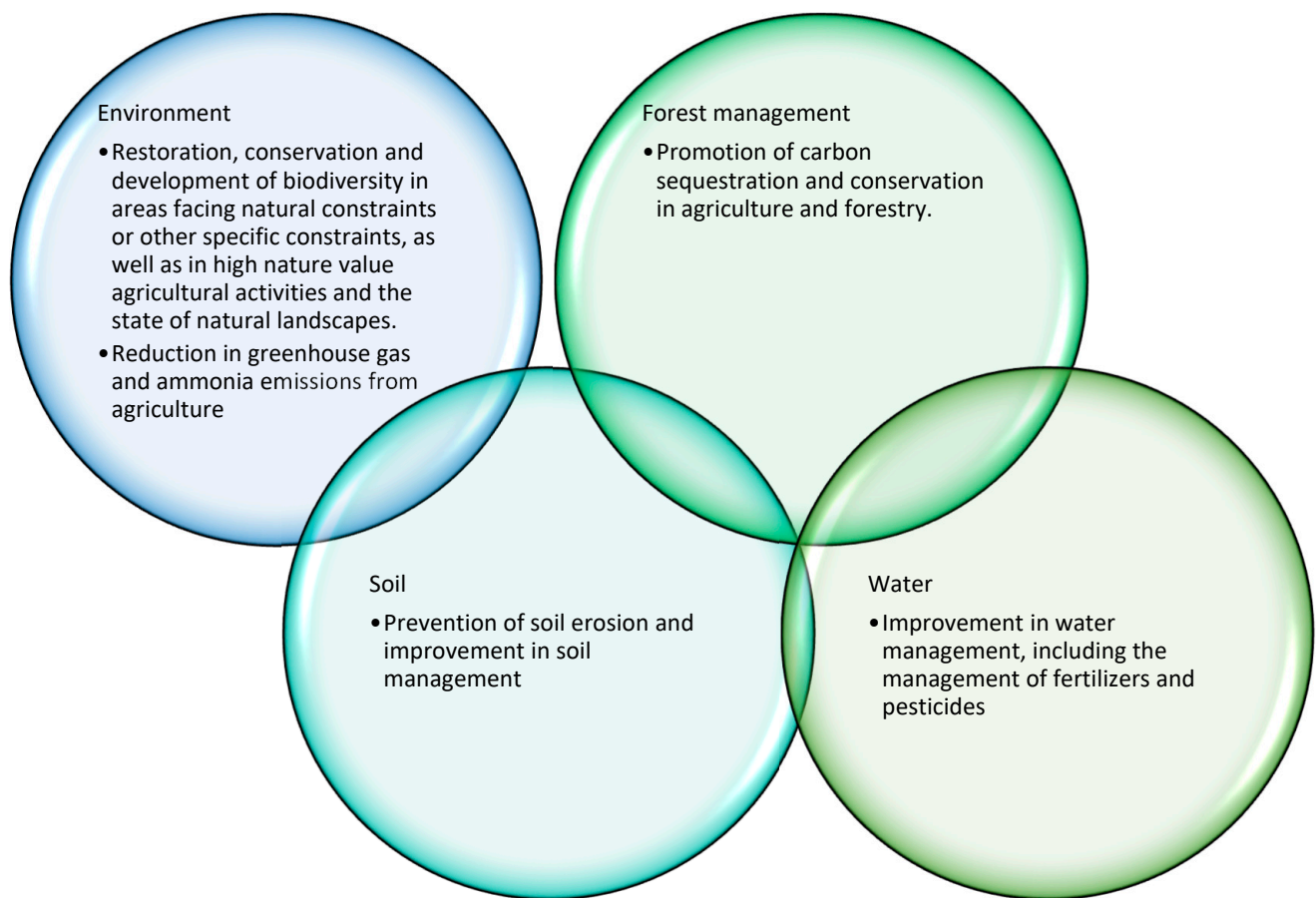


Figure 3. The effects of sub-measure 15.1 [37].

2. Materials and Methods

2.1. Study Area

This study considered forest areas privately or publicly owned in Romania, a country located in southeast central Europe, situated in the northern part of the Balkan Peninsula, along the lower course of the Danube River, and on the northeastern shore of the Black Sea (Figure 4) [38].

There is an uneven distribution of forested areas relative to the relief conditions. It can be observed that in the mountainous region, forests are most prominently represented, covering 59.1% of the area. In the hilly region, forested areas account for 34.2%, while in the plains, forests represent only 6.7% [74]. The significant differences in the distribution across major landforms are due, on the one hand, to agricultural practices historically requiring large flat areas and, on the other hand, to the road network, which makes access to timber resources in the mountainous region challenging.

Moreover, in terms of ownership, out of the total of 6,613 M hectares of forested area, 48.1% are in the public ownership of the state, 16.1% are public property of local administrations, 1.5% are private property of local administrations, and 34.3% are private properties (including religious institutions, individuals or legal entities, and owner associations) [77].

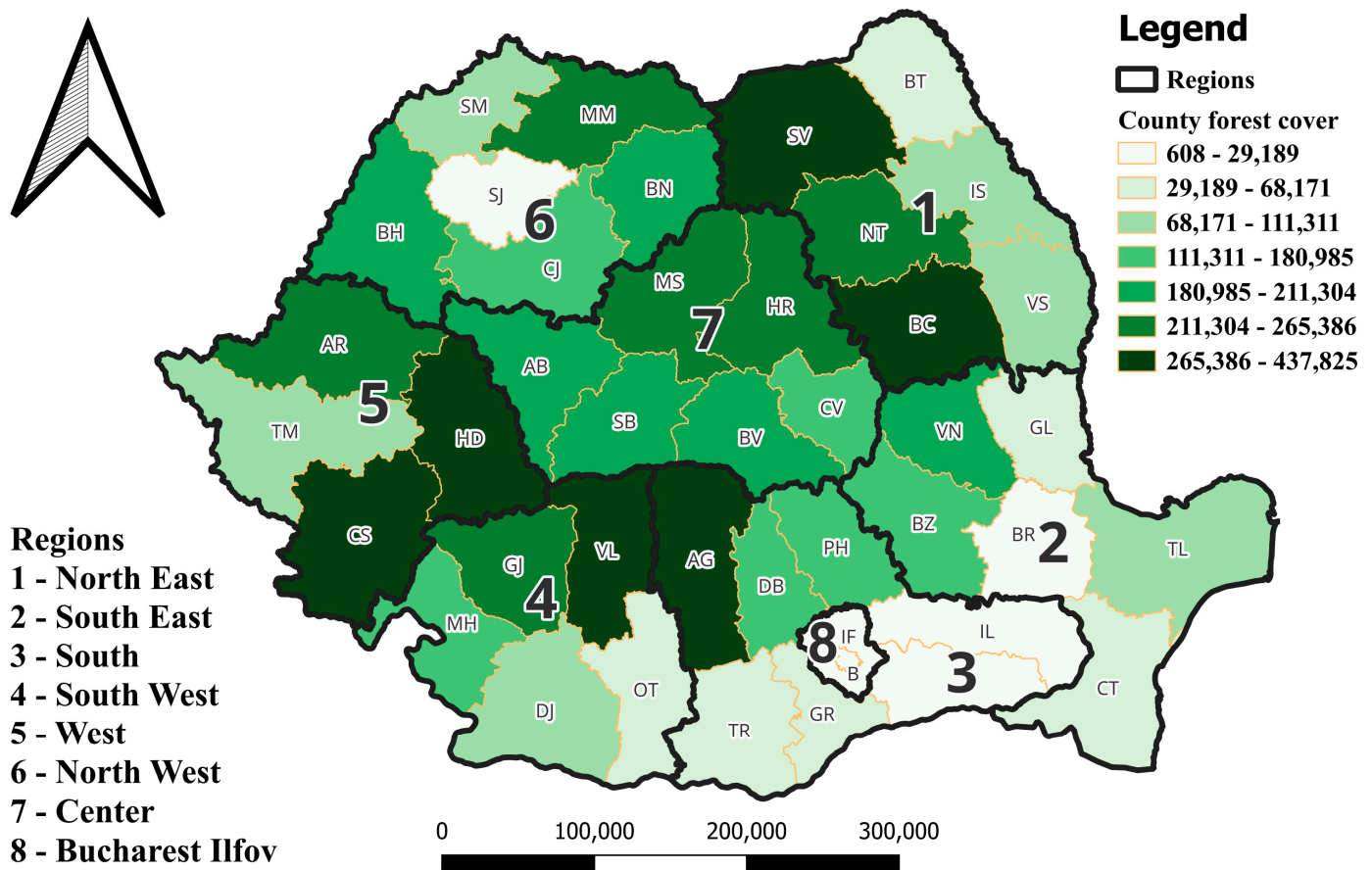


Figure 4. Study area includes 6,6 M Ha of forest-land [78].

2.2. Survey Design and Data Collection

The study design focused on the data collected by the authority responsible for implementing Measure 15. After submission of applications for voluntary participation in the ongoing program, APIA processed the files and collected the information. In accordance with open access to public data, the authors of the study requested that these data be provided to them in an electronic, editable format.

Data related to the implementation of the state aid scheme were requested, in compliance with GDPR, concerning the region, type of forest ownership, form of organization, application submission session, number of parcels, area, authorized amount and any penalties imposed. This study does not include personal data or any information related to individuals.

The data were received in tabular form and subsequently coded to enable processing with specialized statistical software, adhering to the structure outlined in Table 2. The variables were extracted from the database provided by the public authority managing M15. These variables are either numerical (ID, Session, Year, NoPlots, Area_P1, Area_P2, SA, and AA) or categorical (County, Region, FORG). AA (Authorized_Amount) was chosen as the response variable, while the other variables were considered independent variables.

Table 2. Program attributes and attribute levels used. Database structure.

Program Attribute	Attribute Type	Attribute Level Description
AA (Authorized_Amount)	Response variable	The total amount that was paid in year x to the account of the holder of the Support Request
County	Factorial	There are 42 counties in Romania with abbreviation (i.e., ALBA—AB, ARAD—AR)
Region	Factorial	There are 7 regions in Romania (NE, north-east, SE, south-east, S, south, SW, south-west, W, west, NW, north-west, C, center)
ID	Numeric	Anonymized ID of applicants
FORG	Factorial	This study contains 7 forms of organization for forest owners: Economic operators (EOs); Educational institutions (EIs); NGO; Churches (PWs); Private individuals (PIs); Forest owners' associations (PAs); Local administration (LA)
Sessions	Numeric	M 15 had 4 sessions for submitting Support Applications: 2017, 2019, 2020 and 2021
Year	Numeric	M15 is implemented over a period of 5 years, having 4 sessions for submitting Support Requests
NoPlots	Numeric/counts	The number of parcels is shown here. Forestry management plans are organized on plots
Area_P1	Numeric/quantitative	The total area for which support was requested
Area_P2	Numeric/quantitative	The total area for which support was requested
SA (Sanctions_Amount)	Numeric/quantitative	The amount with which the holder of the Support Request was penalized for breaching the commitment

2.3. Data Analysis

The data analysis was carried out starting from the review of the conceptual literature of the subject, which indicated three clear directions of approach to PES: the social interpretation of the data, through the number of participants; the economic impact, through the amount of money authorized; and the impact on the environment, through analysis of the surfaces and calculation of the minimum 20% of the areas that will not be subjected to forestry interventions.

The data set analyzed included 2,680 records, of which there were 887 unique applicants. Some applicants submitted applications for additional areas in subsequent sessions (social impact analysis). Initially, the data from the first year of the five-year commitments were examined, as these contain the initial commitment data, unaffected by any subsequent penalties. The analysis primarily investigates the amounts received by each applicant and whether and to what extent these amounts are influenced by other factors.

To determine if the payments (Authorized_Amount) followed a normal distribution, the Shapiro–Wilk test was applied (economic impact analysis). The result of the test showed that the *p*-value is very low (much lower than the common threshold of 0.05), indicating strong evidence against the null hypothesis that the data followed a normal distribution. Graphs were constructed and histograms and Q-Q charts were created for the other data

(number of plots, total area, area for parcel 2, authorized amount and penalty amount) (Figure 5).

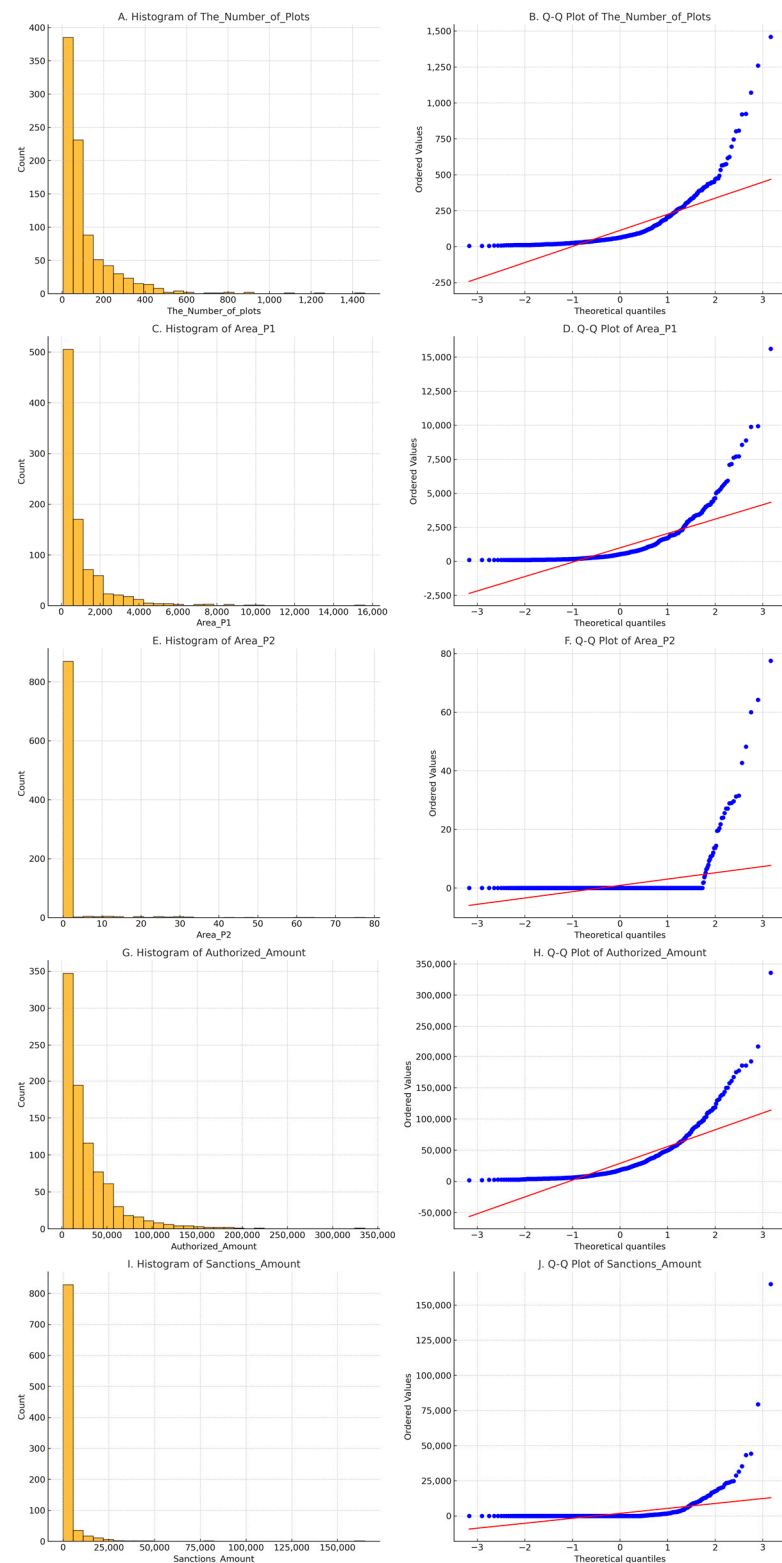


Figure 5. Histograms and Q-Q plots for all data.

As can be observed, the distribution was strongly skewed; thus, Spearman's correlation was subsequently applied using IBM SPSS Statistics version 28 [79,80] and RDA (multivariate linear redundancy analysis) was conducted using Canoco 5 software [81].

This statistical method helps in understanding the relationships between multiple response and explanatory variables, often used in ecological and environmental studies [82], in the second stage of the study.

In RDA, the numerical response variable (AA) was influenced by one or more predictors, which can be of various types (explanatory or independent variables) (numerical: Year, Session; numerical/quantitative: total area—Area_P1, Area_P2; or categorical: County, Region, Organization). These predictors were combined into constrained independent ordination axes (orthogonal), typically represented in the first two dimensions of ordination diagrams. These axes explained the greatest variation in the response variable, with their explanatory power decreasing gradually. Within the ordination diagram, the response variable (AA) is shown as an arrow, with the length of the arrow indicating the extent to which the predictor (independent variables) explains the response variable. A longer arrow suggests a stronger explanation. The direction of the arrowhead relative to the axes indicates the degree of dependence of the dependent variable on the constrained axis. In the ordination diagram, categorical predictors are represented by the centroids of their respective data points. The projection of these centroids onto the axes indicates their effect on the response variable. Numerical predictors are depicted as arrows, with their projection onto the axes suggesting their contribution to explaining the response variable. The significance of the ordination axes was assessed using the Monte Carlo permutation test, employing 999 unrestricted permutations per test to ensure robust statistical validation of the results [81]. Regression analysis was used to determine the significance of the responses, allowing for the assessment of relationships between the response variable and the predictors. This analysis helps in identifying significant factors and understanding how they influence the response variable.

In the third stage, a post hoc test was conducted to evaluate the effects of predictors within an RDA, under the same testing conditions.

3. Results

The statistical analysis began with the establishment of statistical indicators for the characteristics of the data set under consideration, based on the premise that AA (authorized amount) represents the dependent variable relative to the other variables.

The statistical indicators (mean, minimum and maximum values, standard deviation, and coefficient of variation) of independent variables that influence the variation in the authorized amount are presented in Table 3.

Table 3. Statistical indicators of the dependent and independent variables studied.

Variable	Mean	Minimum	Maximum	Standard Deviation	Coefficient of Variation	Spearman's Rho	p-Value
AA	29,311.35	1374.14	335,712.46	31,247.46	106.60	-	-
County	15.11	1	51	10.98	72.70	−0.073	**
Region	5.39	1	7	1.32	24.50	0.117	**
FORG	3.69	1	7	1.25	33.90	0.012	>0.05
Session	2.75	1	4	0.82	29.70	−0.040	*
Year	2.11	1	5	1.00	47.60	0.028	>0.05
NoPlots	113.42	5	1459	137.85	121.50	0.888	**
Area_P1	994.24	100.02	15,603.83	1326.83	133.50	0.983	**
Area_P2	1.25	0.00	210.51	8.12	650.60	0.044	*
SA	1131.62	0.00	164,881.94	5160.15	456.00	0.174	**

Signification code: 0.001 < ** < 0.01 < * < 0.05.

The existence of a correlation between the authorized payment amount (AA), considered as the dependent variable, and the other variables considered as independent was tested by using Spearman rank correlation (Spearman's rho), as shown in Table 4.

Table 4. Coefficient of correlations between AA (authorized amount) and other variables. Only significant ($p < 0.05$, shown in bold) or marginally significant ($0.05 < p < 0.1$) results are shown.

Variable	AA	County	Region	FORG	Session	Year	NoPlots	Area_P1	Area_P2	SA
AA		−0.073 **	0.117 **		−0.040 *		0.888 **	0.983 **	0.044 *	0.174 **
County	−0.073 **		0.081 **	0.091 **	0.104 **	−0.047 *	−0.054 **	−0.077 **	−0.058 **	−0.087 **
Region	0.117 **	0.081 **			0.197 **	−0.068 **	0.113 **	0.125 **		0.117 **
FORG		0.091 **			0.223 **	−0.072 **	0.056 **		0.066 **	
Session	−0.040 *	0.104 **	0.197 **	0.223 **		−0.361 **	−0.038 *	−0.044 *	−0.073 **	
Year		−0.047 *	−0.068 **	−0.072 **	−0.361 **					−0.126 **
NoPlots	0.888 **	−0.054 **	0.113 **	0.056 **	−0.038 *			0.905 **	0.063 **	0.258 **
Area_P1	0.983 **	−0.077 **	0.125 **		−0.044 *		0.905 **			0.255 **
Area_P2	0.044 *	−0.058 **		0.066 **	−0.073 **		0.063 **			0.117 **
SA	0.174 **	−0.087 **	0.117 **			−0.126 **	0.258 **	0.255 **	0.117 **	

Signification code: $0.001 < ** < 0.01 < * < 0.05$.

By applying multiple regression analysis method with all the EVs as predictors, the results showed a highly significant regression. The test on the first axis results in pseudo- $F = 1729$, $p = 0.001$, with the adjusted explained variation being 96.93%.

The tested effects of predictors on the AA (authorize amount) response (dependent) variables are presented in Table 5. The red–orange–gray color code uses a divergent palette. A large number of libraries and tools follow these conventions, including Seaborn and Matplotlib in Python, to create heatmaps.

Table 5. Test effects (left simple and right conditional effects) of predictors on the AA response (dependent) variables. The color codes are related to the adjusted- p (by FDR—false discovery rate) value significance: red means significant ($p < 0.05$), orange stands for marginally significant ($0.05 < p\text{-adj} < 0.1$), and black stands for not significant).

Analysis ‘Test Effects RDA AA~EV All’					Analysis ‘Test Effects RDA AA~EV All’				
Simple Term Effects:					Conditional Term Effects:				
Name	Explains %	Pseudo-F	p	p (adj)	Name	Explains %	Pseudo-F	p	p (adj)
Area_P1	95.834	61,599	0.001	0.0034	Area_P1	95.834	61,599	0.001	0.0102
NoPlots	86.567	17,257	0.001	0.0034	SA	0.923	762	0.001	0.0102
SA	11.91	362	0.001	0.0034	FORG.PI	0.049	40.9	0.001	0.0102
County.BN	6.087	174	0.001	0.0034	FORG.EI	0.001	0.8	0.001	0.0102
FORG.LA	5.223	148	0.001	0.0034	Region.NW	0.047	40.2	0.001	0.0102
County.SB	3.881	108	0.001	0.0034	FORG.LA	0.018	15	0.002	0.017
FORG.PI	2.913	80.3	0.001	0.0034	FORG.EO	0.014	12	0.004	0.0255
Area_P2	2.34	64.2	0.001	0.0034	Region.W	0.01	9	0.003	0.02186
County.SM	1.162	31.5	0.001	0.0034	Region.C	0.005	4.2	0.001	0.0102
Region.C	1.005	27.2	0.001	0.0034	County.CS	0.016	13.6	0.001	0.0102
FORG.FA	0.904	24.4	0.001	0.0034	County.SM	0.008	7.3	0.013	0.07367
County.BV	0.756	20.4	0.001	0.0034	Area_P2	0.006	4.8	0.031	0.1581
County.SJ	0.733	19.8	0.001	0.0034	FORG.PW	0.005	4.5	0.044	0.19125
County.HD	0.61	16.4	0.001	0.0034	County.SJ	0.004	3.5	0.066	0.2414
Region.W	0.554	14.9	0.001	0.0034	County.B	0.004	3.5	0.045	0.19125
County.AR	0.478	12.9	0.002	0.00638	FORG.FA	0.002	1.7	0.109	0.327
County.CS	0.297	8	0.004	0.012	County.VL	0.004	3.4	0.071	0.2414
Region.S	0.251	6.8	0.017	0.04563	County.BN	0.004	3.3	0.067	0.2414
County.VL	0.247	6.6	0.008	0.02267	Year	0.003	2.6	0.102	0.32513
County.IL	0.229	6.1	0.022	0.051	County.BR	0.002	1.7	0.109	0.327
FORG.PW	0.214	5.7	0.023	0.051					
County.MM	0.205	5.5	0.026	0.05525					
County.CV	0.193	5.2	0.022	0.051					
County.AG	0.188	5	0.03	0.06081					
County.DB	0.183	4.9	0.02	0.051					
County.PH	0.182	4.9	0.046	0.08379					
County.TM	0.176	4.7	0.031	0.06081					
Region.SE	0.16	4.3	0.037	0.06989					
County.CL	0.128	3.4	0.056	0.09848					
County.BH	0.1	2.7	0.112	0.168					
Region.SW	0.089	2.4	0.108	0.16691					
County.DJ	0.082	2.2	0.105	0.16691					
County.BT	0.072	1.9	0.102	0.16691					
FORG.EO	0.07	1.9	0.158	0.21778					

Table 5. Cont.

Analysis ‘Test Effects RDA AA~EV All’					Analysis ‘Test Effects RDA AA~EV All’				
Simple Term Effects:					Conditional Term Effects:				
Name	Explains %	Pseudo-F	p	p (adj)	Name	Explains %	Pseudo-F	p	p (adj)
County.OT	0.065	1.7	0.091	0.1547					
County.IF	0.06	1.6	0.149	0.21108					
County.TR	0.06	1.6	0.123	0.17923					
County.BC	0.052	1.4	0.195	0.26171					
County.MH	0.048	1.3	0.245	0.31237					
County.VS	0.045	1.2	0.229	0.29946					
County.VN	0.044	1.2	0.26	0.32341					
County.AB	0.038	1	0.276	0.33514					
County.BR	0.038	1	0.283	0.33565					
County.GJ	0.026	0.7	0.413	0.46807					
County.GL	0.023	0.6	0.394	0.45668					
County.B	0.021	0.6	0.438	0.47528					
FORG.EI	0.021	0.6	0.426	0.4723					
Region.NW	0.019	0.5	0.474	0.49335					
County.HR	0.018	0.5	0.453	0.48131					
County.BZ	0.007	0.2	0.688	0.688					
Year	0.006	0.2	0.67	0.6834					
Region.NE	0.004	0.1	0.741	0.741					
Session	0.003	<0.1	0.772	0.772					
County.CJ	0.001	<0.1	0.845	0.845					

Starting with the number of applicants, it is observed that there were 887 applicants. Among them, 871 had concluded a single commitment, 15 had concluded two commitments, and 1 had concluded three commitments.

Observing the interest of forest owners in PES, starting with the first application submission session, where there is a strong reluctance towards this type of subsidy, applicants, by forms of organization, have experienced an evolution or regression, as depicted in Figure 6.

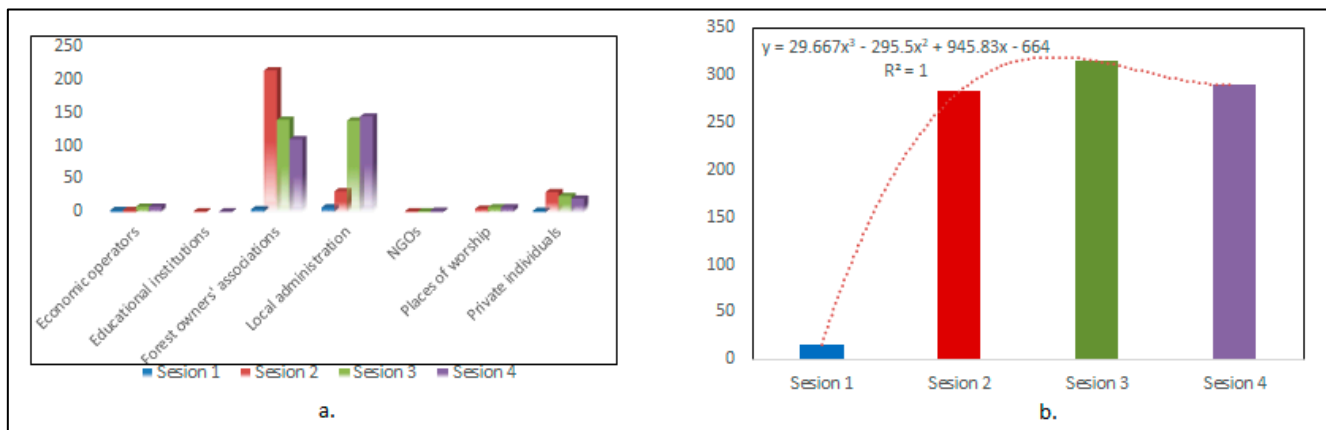


Figure 6. Time series of applicants: (a) grouped by organizational forms; (b) total applicants.

As observed in Figure 6a, private individuals were very hesitant in session 1, before reaching a peak in session 2, and then the number of applicants began to decline. Church applicants experienced a positive trend, with the number of applicants increasing steadily, while the numbers of NGOs and educational institutions remained consistent. Local administration showed a significant increase in participation, peaking in sessions 3 and 4, respectively. A continuous upward evolution also had economic operators, even if the latter are in very small numbers, while Forest Owners' Associations had the highest participation, with a peak in session 2 and a subsequent decline in sessions 3 and 4, respectively. It is observed that the largest number of individuals interested in this type of subsidy were the Forest Owners' Associations, followed by the local administration. Other forms of

ownership have shown a lower level of interest. Regarding the applicants' interest over time (Figure 6b), it can be observed that in the first session, the total number of applicants was extremely low. Subsequently, in the following sessions, interest increased, peaking in session 3. It can be asserted that measures leading to forest conservation and carbon sequestration generate significant interest among forest owners [83,84].

4. Discussion

4.1. The Environmental Impact of PES in Romania

The total area for which voluntary commitments have been made was 0.897 million hectares, representing 26% of the total eligible area. This constitutes a significant portion of the entire eligible area, especially considering that a substantial percentage is located within protected natural areas [17].

By applying multivariate linear redundancy analysis (RDA) [85], considering the simple term effects (as if each of the predictors acts alone, regardless of all the others), the Area_P1 (total area under commitment) and the number of plots were the most important (explaining 96 and 87% of the variation in AA (authorized amount), followed distantly by SA—sanction amount—(roughly $R^2 = 12\%$)), and only some counties and some forms of organization (levels of the corresponding factorial variables) were significant predictors. Among the counties, the most important and significantly linked were Bistrița-Năsăud (BN, coefficient of determination 6%), Sibiu (SB, 4%), and Satu Mare (SM, 1.2%), respectively, while Braşov (BV), Sălaj (SJ), Hunedoara (HD), Arad (AR), Caraş-Severin (CS) and Vâlcea (VL) explained less than 1% (R^2), with all the rest being insignificant.

Among the FORG (form of organization), the significant predictors were LA—local administration (explaining 5% in AA variation), PI—private individuals (3%) and FA—forest owner association (0.9%). The predictor Area_P2 (the area covered with thinnings in package 2 of the commitment) is also significant but weakly linked to the dependent variable ($R^2 = 2.3\%$). Among the regions, the best predictors, but with low levels of coefficient of determination, were C (1%), followed by W and S (less than 1% variance explained), with all the rest being insignificantly linked to the dependent variable.

The conditional effects showed a parsimonious model, from which the highly correlated predictors have been deleted, since it implies the effect of each new predictor selected after accounting for the effect of the previous predictors. For instance, after selecting and accounting for the effect of Area_P1 (96% variance explained), there was a low level of variation that still might be explained by some unique effects of other predictors.

4.2. The Economical Impact of PES in Romania

Only a few explanatory variables explained the residual variation, and all had an R^2 of less than 1%, namely the SA (sanction amount), some FORG (levels PI, EA, EO), some regions (NW, W) and a few counties (CS, and marginally significant CS). The RDA diagram with selected predictors is given in Figure 7.

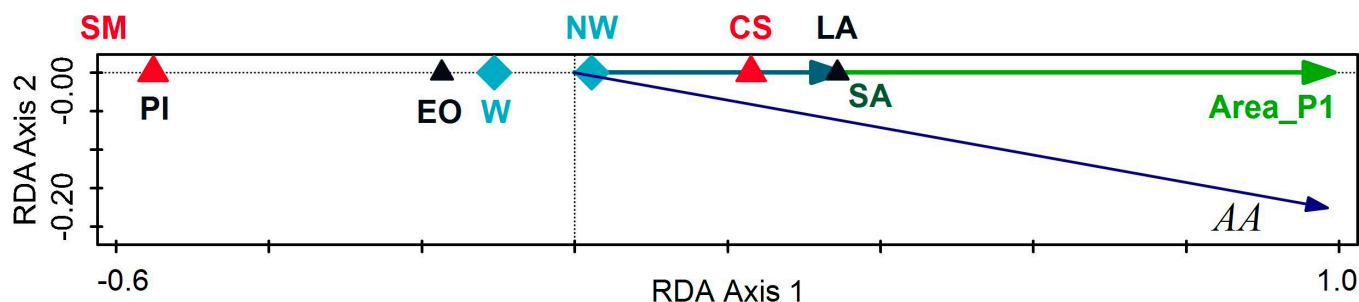


Figure 7. RDA ordination diagram with AA as response variable and the chosen significant predictors by interactive forward selection procedure. The selected predictors explained 96.91% adjusted variation in AA. The symbols used in the graph are FORG—black triangle, County—red triangle, Region—blue rhomb, Area_P1—green arrow, AA—blue arrow.

In Figure 7, the response variable AA is positively related to the predictors Area_P1 and SA and is also related to increased values of FORG.LA and County.CS, while it is linked at a mean value to Region.NW, followed by Region.W, and to reduced values with the county SM and the FORG.PI and FORG.EO.

Related to the time (both year and, separately, session) as a predictor, there are no significant models, meaning that time is not a significant predictor, or there are no significant differences in time related to the dependent variable.

In other countries, Italy for example, Payment For Ecosystem Services (PES) was implemented in protected natural areas such as Natura 2000 sites [17]. In this case, the contracts have a one-year duration, while in the present case, the duration of the contracts was five years.

In our model of spatiotemporal analysis, the evolution of authorized amount was explained by the area and number of plots. The other variables had a marginal or no impact.

In the countries of the European Union, there are examples of good practices regarding payment schemes for forest ecosystem services. In Finland, forest owners can voluntarily offer their forest sites for protection in the METSO program, and they receive financial compensation equivalent to the full value of timber at the protected site. In Croatia, since 2020, all persons that are engaged in economic activities and that have an income greater than HRK 7.5 million (approximately EUR 1 million) are obliged under the Forest Act to pay a fee for the use of forest ecosystem services that amounts to 0.024% of their total annual revenues. Label Bas Carbone 62 is a French standard that focuses on the certification of carbon offset projects in afforestation, reforestation of destroyed or impacted forests, and conversion of coppices to high stands in forests. Each project type lasts for 30 years, and ex ante credits are generated in either year 0 or year 5. Co-benefits are estimated for the socio-economic, water, soil protection and water dimensions. The scheme is open to all entities that want to offset CO₂ emissions, including private firms, public bodies, administrations and citizens. In total, 173 forestry projects had been certified by 2022. The Forest Stewardship Council (FSC) promotes environmentally sustainable, socially responsible and economically viable forest management and has been widely adopted in developed countries, particularly in the EU. The FSC has developed private ecosystem service procedure 63, which is applied globally and implemented in different EU Member States (e.g., Denmark, Estonia, France, Italy, Latvia, Lithuania and the Netherlands) as an add-on to the FSC forest management certification for the quantification of the impact of ecosystem services on land so that positive changes can be quantified, valued and sponsored [69].

5. Conclusions

This study aimed to provide relevant information regarding the viability and potential impact of PES on achieving the goal of forest ecosystem conservation, by analyzing 904 commitment contracts implemented in Romania, totaling 2680 payments. As noted in the case study, Payment For Ecosystem Services (PES) mechanisms offer insight for addressing environmental issues and providing new tools and arguments in favor of biodiversity conservation, particularly within forest ecosystems. These mechanisms incentivize landowners and stakeholders to engage in conservation practices by compensating them for maintaining or enhancing ecosystem services [86,87]. It is thus demonstrated that PES leads to the improvement of ecosystem services through the main objective of conserving forest ecosystems. Through the PES mechanism, a significant percentage of the area (minimum 20%) is designated and treated as an area similar to natural reserves, where activities related to timber harvesting are prohibited. In the remaining areas, the number of forestry interventions should be minimal, with an emphasis on those that have a nature-friendly impact, such as thinning operations conducted with horse-drawn equipment. Under these conditions, the implementation of PES has resulted in the commitment of 0.897 M hectares, which represents 26% of the total eligible forest areas in Romania. Even though forest

management plans include conservation measures, the voluntary adoption of additional measures, such as maintaining a timber volume equivalent to half of the volume intended for extraction through specific conservation activities; maintaining at least one-fifth of the area where only planting and care of forest seedlings will be carried out; and adopting a minimum number of interventions on the remaining area, specifically a maximum of one intervention in areas not designated as quiet zones, contribute to achieving the overall objective of forest ecosystem biodiversity conservation.

The study data reflect a growing spatiotemporal trend. While the first session recorded a small number of interested parties applying (less than 2% of the total applicants), in the subsequent three sessions, the number of applicants remained balanced and almost constant (31%, 35%, 32%). This demonstrates that PES presents an interest to forest owners, establishing itself as a viable tool for the conservation of forest ecosystems. Although from a financial perspective PES might seem like an unsustainable cost for society, they are rather an investment in the well-being of both current and future generations. Given the increasingly adverse effects of climate change, and the necessity for forest resilience to be a strategic priority both nationally and Europe-wide, PES could serve as a practical instrument for forest conservation readily available to policymakers.

In conclusion, the results mentioned above indicate the social impact of PES, quantified by the number of applications submitted; the economic impact, by the amount of money authorized; and the impact on the environment, by calculating the minimum area in the quiet zone (minimum 20%), which is exempt of forestry works.

The spatiotemporal multivariate assessment of PES in Romania offers a comprehensive overview of a tool intended to lead to forest conservation. The model proposed is a potential approach to environment and climate programs that can be optimized in the perspective of continuous PES implementation, as is the intervention DR-07 “Silvo-environment and climate” launched in the summer of this year.

A limitation of the study is that it analyzed the period 2017–2023, and at that time, the intention of the authority regarding the initiation of new sessions for submitting voluntary membership applications to the SPO was not clear, as it happened in the second half of the current year.

Future research should focus on new financial mechanisms that lead to achieving the objectives of forest ecosystem conservation. Additionally, studies are needed to document the reasons behind the passivity of potential beneficiaries in adopting PES, so that the voluntary participation rate can approach its optimum.

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THE ORIGIN OF THE WOODY SPECIES OF PINES CULTIVATED IN THE SYLVA ARBORETUM IN GURAHONȚ, ROMANIA

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RESEARCH ARTICLE

Abstract

The Sylva Arboretum, located in Gurahonț, Romania, serves as a key site for the research and conservation of tree species, including pines. This arboretum boasts a remarkable assortment of tree species from around the world, with a particular focus on the origins of pine species, which are the subject of this study. The present work delves into the origins of the pines planted here, highlighting their ecological diversity. Through a meticulous analysis of the collected data, it was established that the majority of these species originate from Europe, North America, and Asia, with a particular emphasis on native species from the Carpathian Mountains. Furthermore, the research underscores the importance of introducing and acclimatizing exotic species, which play a crucial role in enhancing biodiversity and promoting ecological stability. The findings of this study have significant implications for the sustainable management of forest resources and biodiversity conservation amidst the challenges posed by climate change. In summary, the Sylva Arboretum exemplifies an effective model for the conservation and study of pine tree species, illustrating the critical need to integrate traditional knowledge with contemporary scientific approaches in the management of forest ecosystems.

Keywords: arboretum sylva, pinus, forest ecosystem, Biodiversity, Forest resources

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INTRODUCTION

The distinguished forestry engineer Eusebiu Ștefan took on the significant task of identifying and utilizing the geographical area known to the inhabitants of that period as the "old park," located within the picturesque boundaries of the Hontîșor Valley, in the Gurahonț commune of Arad County. Both the Monograph of the Park, written by the esteemed engineer Eusebiu Ștefan in 1992, and the SYLVA Arboretum 1983, explicitly state that the "Garden Journal" contains a comprehensive account detailing the origins of this notable park. This documentation clarifies that between 1882 and 1889, significant efforts and steps were taken toward establishing a unique dendrological park, designed to encompass a layout defined by trees and flowers, accompanied meticulously by detailed planting instructions and cultivation guidelines. Furthermore, the aforementioned journal reveals that the initial plantings within the limits of Gurahonț began as early as 1750, with various botanists making observations starting in 1883.

During this crucial period, the construction of the Arad-Brad railway was also underway, a project that began in 1885. It is noteworthy that the land upon which the "Sylva" Arboretum now stands was once part of the estate owned by Benjamin Boroș, who, with great foresight, developed this area into a space for recreation, cultivating both native and exotic plant species and maintaining the land with the diligence characteristic of a true nature enthusiast.

Upon the unfortunate death of Benjamin Boroș, the estate, which had been lovingly cared for, was inherited by his son, who, being a lawyer and lacking the necessary knowledge, made the decision to sell the property. Following the sale of this historically significant land, it fell into neglect and degradation, resulting in numerous destructive actions, including the removal of trees, acts which could be considered criminal, particularly cases of abusive grazing that further exacerbated the deterioration of the ecosystem.

In light of these unfortunate circumstances, in 1965, forestry engineer

Eusebiu Ștefan, visiting the site once known as the park, found it in a completely deplorable state, characterized by destruction, overcrowding, and extensive grazing, with parts of the area being used by certain individuals as an improvised refuge and recreational spot.

Subsequent to 1965, initial efforts were launched with the aim of transforming this neglected land area into a dendrological botanical garden, commonly referred to as an Arboretum, covering an area of 4.6 hectares, which represented the historical extent of the old park. It was also here that engineer Eusebiu Ștefan succeeded in expanding this park by an additional 8 hectares, ultimately recording the presence of 2,578 taxa with 3,465 specimens.

In the period after 1990, the efforts of engineer Eusebiu Ștefan to keep the entire area 12.6 ha were in vain because optimal solutions were not found to fairly reconcile the former owners of the land. Due to the resulting situation, in the period that followed only the initial area of 4.6 ha benefited from adequate care, to the detriment of the collection made on 8 ha. As argued by F. Morozan (2018): The negative effect of the authority of *res judicata* refers to the party who lost the trial, as he can no longer call into question his right in another trial. Thus, a solution agreed at the local level was found, and in 2003, by a decision of the Gurahonț local council, the Sylva Arboretum entered the custody of the University of Oradea for a period of 49 years. Since 2015, the University of Oradea, the West University of Timisoara and the University of Agricultural Sciences and Veterinary Medicine of Banat, have joined a consortium for the management of the park.

In a broad and comprehensive examination of the climatic variable of temperature in the specified geographical region over the analyzed time period, it can be conclusively stated that:

- The annual average temperature fluctuates between 8.8°C and 12.2°C, which is considered extremely favorable for the vigorous and healthy growth of various plantation species. Moreover, the conclusions outlined above also apply to observations regarding the recorded annual minimum and maximum average temperatures during the same period. Analytical evaluation suggests that the soil composition in this region is predominantly classified as sandy clay, characterized by a significant presence of particles ranging from 0.2

to 1.002 mm in size, constituting approximately 57.2% of the total soil composition.

- Furthermore, the soil exhibits a slightly acidic reaction, as indicated by the low pH value of 5.65, which suggests an acidity level that may influence the biological and chemical processes occurring in this soil ecosystem..

MATERIAL AND METHOD

The SYLVA Arboretum is geographically located at 46°15'59" N, 22°20'47" E (Google Earth, 2024).

For determining the results, all pine specimens cultivated in the Sylva Arboretum were identified from the archive, with specifications regarding their origin.

Field data were collected to verify the presence or absence of these specimens, as well as their characteristics (height, number of existing specimens, health status). It should be noted that data were collected only for the 19 plots currently managed in this study.

The data were collected during the 2023-2024 period. The biomorphological analysis of the woody plants in the SYLVA Arboretum was conducted according to the criteria set by Sokolov S.Ya. and Sviazeva O.A. (1965), and more recently utilized by E., A. Parakhina et al. (2021), as follows:

- D1 – tree size I (height greater than 20 m);
- D2 – tree size II (height 16-20 m);
- D3 – tree size III (height 11-15 m);
- D4 – tree size IV (height up to 10 m);
- K1 – shrub grade I (height greater than 6 m);
- K2 – shrub grade II (height 4-6 m);
- K3 – shrub grade III (height 2-4 m);
- To4 – shrub grade IV (height up to 2 m).

RESULTS AND DISCUSSIONS

Arboretums represent meticulously curated botanical gardens primarily dedicated to the extensive collection, careful cultivation, and comprehensive study of a diverse range of trees and shrubs. These remarkable sanctuaries fulfill multiple functions, encompassing scientific research, conservation efforts, educational initiatives aimed at both students and the general public, as well as recreational opportunities that allow individuals to engage with nature.

The significance of arboretums lies not only in their ability to conserve biodiversity by providing living collections that serve as essential resources for ecological and evolutionary research, but also in their capacity to offer invaluable educational experiences to

learners of all ages, thereby enhancing public understanding of botanical sciences. Moreover, these vital landscapes contribute to broader urban greening initiatives and play a crucial role in environmental conservation efforts aimed at mitigating the impacts of urbanization and promoting ecological sustainability.

In essence, arboretums are not merely repositories of plant life but dynamic centers of

knowledge that foster a deeper appreciation for the natural world and highlight the importance of conserving the diverse ecosystems of our planet. Ultimately, the multifaceted contributions of arboretums to society underscore their significance as both scientific institutions and community resources in the pursuit of environmental management and education.



Figure 1 Specimens of *Pinus sylvestris* L., remaining from the old park of Benjamin Boroş



Figure 2 Specimen of *Pinus pinaster* Aiton and *Pinus armandii* Franch, Sylva Arboretum 2024

Table 1

The Origin and Characteristics of Pine Trees in the Sylva Arboretum

No.	The Name	Size				Identification / Plot number	The number of specimens	The health conditi on	The origin
		D1	D2	D3	D4				
1	<i>Pinus banksiana</i> Lamb			*		146/IV	1	Good	Montreal Canada
2	<i>Pinus nigra</i> var. <i>Poiretiana</i> (Ant) Asch et Graebn		*			17/V	1	Good	Kamoni Ungaria
3	<i>Pinus sylvestris</i> L.		*			21/V 102/VI	1 1	Good	Gurahonț Romania
4	<i>Pinus strobus</i> L.		*			44/V	1	Good	Simeria România
5	<i>Pinus dalmatica</i> Ait			*		77/V	1	Broke n tree top	Kiev (Foemina) Ucraina
6	<i>Pinus ponderosa</i> Laws		*			81/V 83/V	1 1	Good	Bazos Romania
7	<i>Pinus Armandi</i> Franch		*			96/V	1	Good	Kamoni Ungaria
8	<i>Pinus koraiensis</i> SZ		*			103/5	1	Good	Potsdam Germania
9	<i>Pinus resinosa</i> Ait		*			116/V	1	Good	Bazos Romania
10	<i>Pinus peuce</i> Griseb			*		11/VI	1	Good	Kamoni Ungaria
11	<i>Pinus sylvestris</i> „ <i>Glauca</i> „			*		19/VI	1	Good	Kamoni Ungaria
12	<i>Pinus pinaster</i> L.		*			57/VI	1	Good	Nancy Franta
13	<i>Pinus pinaster</i> Ait „ <i>Magrebiana</i> „		*			85/VI	1	Good	Antibes Franta
14	<i>Pinus sylvestris</i> var. <i>hamata</i> Stev		*			128/VII 138/VII	1 1	Good	Chorog Tadjikistan
15	<i>Pinus nigra</i> var. <i>Poiretiana</i> (Ant)Aschers			*		147/VII	1	Good	Kamoni Ungaria
16	<i>Pinus nigra</i> var. <i>caramanica</i> (Loud)Rehd			*		166/VII 196/VII	1 1	Good	Soci Rusia
17	<i>Pinus monticola</i> Lamb			*		77/XI 78/XI	1 1	Good	Liege Belgia
18	<i>Pinus rigida</i> Mill		*			96/IX	1	Good	Otawa Canada
19	<i>Pinus sylvestris</i> L. var. <i>mongolica</i> Litvin		*			85/XIII	1	Good	Snagov Romania
20	<i>Pinus Thunbergii</i> Parl			*		89/XIII	1	Good	Snagov Romania
21	<i>Pinus armandi</i> Franch				*	90/XIV	1	Good	Alba Iulia Romania
22	<i>Pinus sylvestris</i> L. var. <i>mongolica</i> Litv			*		96/XIV	1	Good	Snagov Romania
23	<i>Pinus mugo</i> Turra		*			118/XVI	1	Good	Iasi Romania
24	<i>Pinus kokiana</i> Klotz ex C. Koch		*			96/XVIII	1	Good	Pamir Tadjikistan
25	<i>Pinus sosnowskii</i> Nakai		*			105/XVIII	1	Good	Tbilisi Georgia

CONCLUSIONS

The origin and characteristics of the pine specimens meticulously cataloged in the esteemed Sylva Arboretum are notable for the fact that a substantial majority, constituting a significant 50%, originates from Romania. This can be easily explained by referring to the comprehensive monograph expertly authored by the distinguished engineer Eusebiu Ștefan. This situation is contextualized within the historical framework of the Communist regime, which predominated in earlier periods, a time when establishing correspondence and maintaining communication with other institutions and similar entities in the same field of study was exceedingly difficult. It appears that a remarkable collaboration existed between engineer Eusebiu Ștefan and several entities located within the geographical confines of Hungary, as evidenced by the interesting statistics indicating that the percentage of pine specimens cultivated in the arboretum is an impressive 17% of the total pine species cultivated in this sanctuary.

The provenance of pine taxa, accounting for 7%, is deduced to be from both Canada and France based on the empirical data collected in the current investigation. The provenance of pine taxa, comprising 4%, has been established for Ukraine, while a uniform percentage of 3% is recorded for each of the following nations: Germany, Russia, Belgium, Tajikistan, and Georgia.

Debazac E.F. (1964) argues that pines, except for those species whose native habitats are specifically mountainous or subalpine regions, have not withstood the harsh conditions of the Hort de Dieu. ("Thus, large reforestation species such as Scots pine, Austrian black pine, and Corsican pine yielded poor or mediocre results. The failure of American-origin pines (*P. strobus*, *P. rigida*, *P. ponderosa*, *P. banksiana*) has been more or less complete. Only mountain pines, such as *Pinus cembra*, *P. pence*, *P. leucodermis*, *P. uncinata*, and *P. pumilio*, are vigorous, though all grow slowly.")

The various species within the genus *Pinus*, when situated within the confines of an arboretum, play an extremely crucial role in enhancing the local ecosystem, not only by increasing biodiversity but also by facilitating processes associated with ecological restoration. These species simultaneously exert a significant influence on the interactions between the various species within that ecosystem. These multifaceted contributions can be viewed as

encompassing a wide range of direct and indirect ecological functions that are vital for maintaining ecological balance. More specifically, the presence of different *Pinus* species serves to create and support suitable habitats for a multitude of diverse organisms, aids in the restoration of degraded areas, and influences the complex dynamics of plant communities through intricate mechanisms involving both interspecies competition and their potential for coexistence in a shared environment.

With regard to biodiversity enhancement, *Pinus* species can support a diverse range of plant and animal species by providing habitat and resources. For instance, in Portugal, *Pinus pinaster* forests are associated with various taxonomic groups, and management decisions can enhance biodiversity conservation in these areas (Maia et al., n.d.). In Indonesia, *Pinus merkusii* forests support a variety of tree species, with 83 species recorded in different growth stages, indicating their role in maintaining species diversity (Supartono et al., 2023).

Another aspect is species diversity, where *Pinus* species can both support and inhibit biodiversity. In some cases, they facilitate the growth of native species by providing a habitat that mimics natural succession processes, as seen in the facilitation of endangered cloud forest species under *Pinus patula* plantations (María Luz et al., 2016).

Habitat provision: Pine forests can serve as important habitats for various species, including some that are endangered. They offer structural diversity that supports different life forms, although this can vary depending on management practices and site quality (Ponce et al., 2017).

Arboretum Sylva, currently appreciated and recognized as a vast and well-organized open-air laboratory, fundamentally dedicated to the rigorous study and systematic observation of a diverse range of plant life forms, serves as a vital component of the practical training and experiential learning of students enrolled in the Department of Forestry and Forest Engineering of the University of Oradea, with various educational activities and exercises carried out within its limits. The remarkable and diverse collection of pine species, together with a significant multitude of other tree species that have been carefully cultivated, carefully organized and strategically positioned in this special location, is poised to exert a profoundly

beneficial influence on ecological and environmental outcomes in the future.



Figure 3 Practical activity of students from the Department of Forestry and Forest Engineering, University of Oradea

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IMPROVEMENT BY SELECTION OF ACACIA (*ROBINIA PSEUDOACACIA* L.) IN ROMANIA

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Abstract

In the broadest sense, the selection involves choosing from a plant population, based on well-established criteria, a certain number of plants that will participate in obtaining the next generation of the respective population, but which similarly follow towards the objective principle of improvement in the case of acacia, that of productivity of wood biomass. Certainly, besides the productivity of woody biomass, there are also other criteria that are worth remembering such as: resistance to pedoclimatic factors, nectar productivity, resistance to pollution, resistance to diseases and pests or the quality of wood.

There are a large number of selection variants, differentiated according to a number of methodological criteria. The present work presents a synthesis of the evolution in time of the improvement works regarding the acacia, with references from the most extensive theses elaborated by researchers of Romanian origin, theses that provided for the selection of trees plus and the creation of stands seed sources, up to current data on the selection and productivity of current varieties of acacia.

Key words: black locust, plus trees, selection, improvement by selection

INTRODUCTION

The ways of penetration of the acacia tree in the areas of Europe located in the south-east of the Carpathians, namely in the eastern part of the Balkan Peninsula, in European Turkey, in Macedonia, Bulgaria, old Romania, up to Bukovina and Bessarabia, are not from the west, from France, through Austria and Hungary, but vice versa, from the south-east, from Constantinople, the intercessors being, very probably, the Turks, and the period when this happened, it was a few decades before 1777. Constantinople seems to have been, thus, in the 18th century, the center from where the acacia tree spread in South-Eastern Europe (Drăcea, 2008;1926).

In Romania, the acacia culture has received special attention, especially since the second half of the Nineteenth century, being cultivated throughout the country, as an ornamental tree, but also in forest crops, in the lands with a lot of summer heat. It became naturalized, becoming a subspontan, from the plains to the lower mountain area. The first acacia forest crops were established in 1852, in Băilești Dolj, so that, subsequently, the species to be planted on larger and larger areas, especially in Oltenia, on lands with moving sands. Besides that area, it was also used in other resorts with continental

sands, such as those in the north-west of the country, between Oradea and Carei, or those in Moldova, at Hanul Conachi etc. (Doniță, 1999).

In Europe, by far, the largest acacia grower is Hungary, with 345,000 ha covered with this species (Németh and Molnár, 2005). According to the data published by DeGomez and Wagner (2011) as well as Keresztesi (2013), at the end of the 80's, large areas cultivated with acacia were also found in the USSR (144,000 ha, mainly in Ukraine and Moldova), Romania (120,000 ha), France (100,000 ha), Bulgaria (58,000 ha), Yugoslavia (50,000 ha), Czechoslovakia (28,000 ha). In Asia, large acacia cultivators are the Republic of Korea (1.22 million ha) and China (1.0 million ha).

Today in Romania, acacia has an area of over 255 thousand hectares forested with acacia, representing a percentage of about 3.71% of the total area of forests reported in the forest system.

In all crop plants, selection is the main method of exploiting the natural or artificial variability existing, at a time, in the species with which they work. In forest species, selection, as a method of improvement, has many peculiarities imposed by the particular biology of each species. The fundamental principles of selection, for the forest species, were enunciated by Wright (1963) and are discussed in detail in valuable treaties of forestry improvement from our country (Stănescu, 1983; 1997; Enescu, 1975; 1985; 2002; Savatti, M. and Savatti Jr., 2005).

MATERIAL AND METHOD

The research was carried out between 2010 and 2021 in Bârzești, Arad County, Romania.

The main objective pursued in the improvement programs for acacia as well as for other crop species is the productivity of woody biomass. The character is very complex and depends on a fairly large number of elements of which the most important are: the height of the trees, their diameter, the tendency to infestation, the growth rate, the resistance to winter frosts, the resistance to diseases and pests.

The researches afferent to the present study aim to evaluate the behavior of the selections of *the Oltenian variety* compared to *the rectissima variety* in the pedoclimatic conditions of the submontane area of the Apuseni Mountains (Bârzești, jud. Arad), following the ways of growing, acclimatizing and manifesting the respective variety in the installation period and the next period.

RESULTS AND DISCUSSION

The data obtained by measurements made on 50 individuals selected from each acacia variety taken in the study show that, in the period 2010-2021, the variety *rectissima* presented average values of diameter growth between 1.26 and 3.1 cm, values recorded at the height of 1.30 meters from the ground (fig.1).

For the *Oltenian variety*, the variability limits of this character were equally extended, with values between 1.19 – 2.8 cm and a coefficient of variability very close to that obtained in the *rectissima variety*.

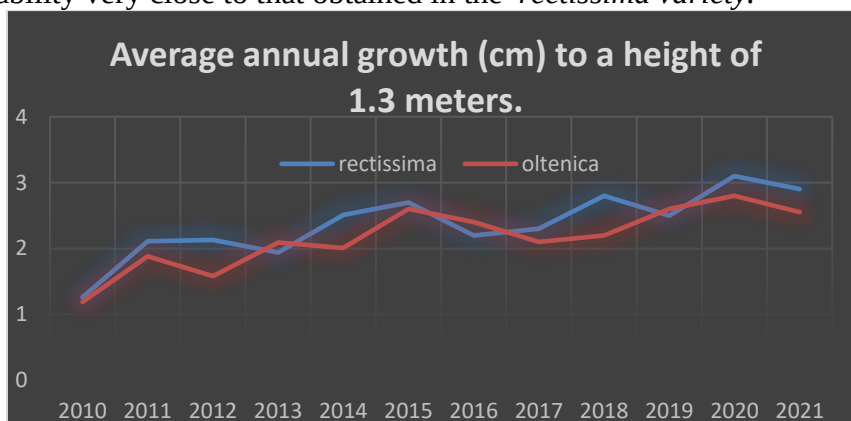


Fig. 1. Average annual growth (cm) to a height of 1.3 meters.

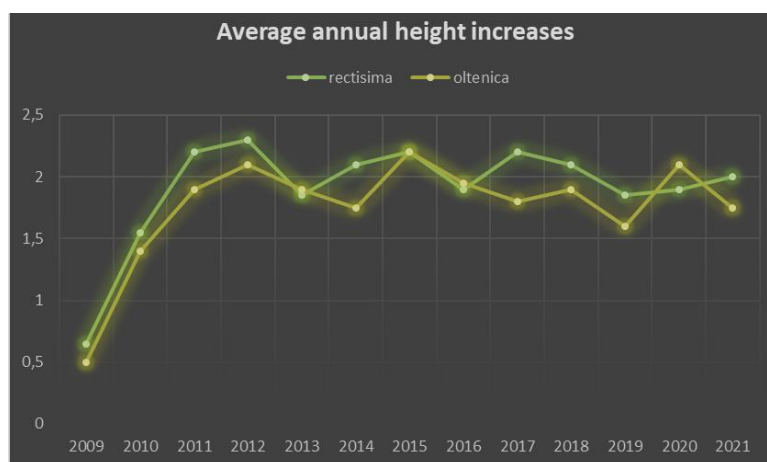


Fig. 2. Average annual height increases

The results obtained in Bârzești, regarding the average annual increase in height (fig.2) of the plants selected by acacia 1 of the two varieties tested, are consistent with those from the specialized literature which states that, in general, the vigor of the Oltenian variety is lower than that of the *rectissima* variety. Obviously, there are real differences between the two varieties at least from two points of view regarding the height of the plants:

- of the speed of growth in height of plants;
- of the dynamics of the average annual increases of this character.

According to the way of assessing the selection criteria, it can be:

- **Phenotypic selection**, based on the visible and eventually measurable manifestation, in the chosen individuals, of the favorable characteristics pursued by the breeder.
- **Genotypic selection** is based not only on the phenotypic aspect of the chosen individuals but also on the performance of the offspring of these individuals. In most forest species this type of selection is quite difficult to apply due to the long life periods of the respective species.

According to the direction in which the selection is oriented, this can be:

- **Positive selection**, in the sense that only elected individuals, who have desired characteristics, are retained for the perpetuation of the population. This type of selection is specific to the improvement and production of seedlings.
- **Negative selection** occurs when all individuals who do not meet the characteristics desired by the breeder are eliminated from the population. This type of selection is commonly used in cultural works applied to stands of different ages.

According to the way in which the performances of the chosen individuals are traced, in the lineage, two fundamental types of selection are distinguished, namely:

- **Individual selection** involves assessing the lineage of each individual chosen and retaining those descendants in which all or most individuals have the characteristics desired by the breeder. The retained descendants will be multiplied (seminal or vegetative) and tested in comparative cultures (plantations) executed with the most accurate measurement of the characteristics pursued and the comparison of the population averages.
- **Mass selection** occurs when all the descendants of the chosen individuals are tested together, in the mixture, in terms of the characteristics pursued. Effectively, through mass selection, the initial population in which the selection was made is replaced by a new population, made up of the descendants of the most valuable individuals of the initial population.

Acacia, being a strictly alogam species, lends itself better to mass selection than to individual. Individual selection, by forcing the descendants of a single individual to pollinate each other, inevitably leads to the manifestation, to varying degrees, of the depression of inbreeding. Mass selection presents three distinct variants, depending on the number of choices made and the ways of grouping the seeds of the chosen individuals:

- *Simple mass selection with one choice;*
- *Repeated mass selection;*
- *Mass selection by groups of plants.*

Regardless of the variant, mass selection efficiently exploits the natural and/or artificial variability of tree populations with an obvious genotypic and phenotypic polymorphism. The efficiency of mass selection is expressed by the so-called genetic gain (CG) which, mathematically, is given by the relationship (Dudley and Moll, 1969):

$$CG = K \cdot h^2 \cdot i \text{ in which:}$$

K is an index of the selection differential whose value is inversely proportional to the selection pressure;

h² = heritability of the character pursued;

i = selection deviation calculated as follows;

I = the average character of the chosen elites: the average of character in the population under selection.

In acacia, the positive mass selection, applied to free fertilized plus trees (fig.3) or to trees plus artificially pollinated, is followed by negative mass selection applied in the form of thinings (eliminations) in the populations of seedlings resulting from seeds. The plantations thus obtained may be used as seed producers necessary for the production of forest saplings intended for afforestation. Depending on the effects on the genetic structure of the populations, the following types of mass selection are distinguished:

Stabilizing selection involves the elimination of extreme variations and the maintenance of genotypes with the highest representativeness in the population, which have the greatest capacity for adaptation and survival.

Directional selection favors the group of extreme genotypes (plus variants or minus variants) which leads to the displacement of the average population towards the favored group.



Fig 3. Trees Plus, Săcuieni Forest District, Bihor County.

Disruptive selection favors one or two optimal phenotypes over intermediate ones. Gradually, the population splits into subpopulation, some very close to the optimal, others very close to the minimum.

In forest species, including acacia, mass selection, positive and negative, also takes place in the form of cultural selection. The works of care for the acacias by applying cleanings and thins along with favoring the development of some trees from the most typical and vigorous drajons are, in fact, forms of mass selection. The mass selection, at the acacia, beyond its value in the improvement of this species, has an equally high value in the realization of the stands (orcharges) seed sources. In Romania, the identification of the seed source stands or the creation of new stands of this kind, started after 1960, reaching that, after a few years, to have about 65,000 ha of such stands (Bumbu and Catrina, 1982).

Clonal selection involves the multiplication of valuable individuals, chosen in the selection process, by organs of vegetative propagation (cuttings, drajons, altoaie, etc.). The resulting clones are tested in comparative crops, the most valuable being used in the establishment of seed-producing stands (plantations). In acacia this type of selection is very common due, first of all, to the ease with which this species can multiply vegetatively. At the level of all forest species, the application of clonal selection has led to so-called clonal forestry. (Libby and Ahuja (1993), Enescu (2002), Savatti and Savatti Jr. (2005) analyzed, in detail, the characteristics of clonale forestry, the benefits and disadvantages of this type of forestry, mentioning the fact that, in species such as acacia, where vegetative propagation is easy to achieve, at moderate costs, the benefits are much higher than the disadvantages.

The assisted selection of molecular markers allows the acceleration and efficiency of the selection process by diminishing its probabilistic character (GALLAIS, 1990). In principle, this type of selection involves the "binding" of a phenotypic character to a certain molecular marker, easy to highlight, at any age of the plant, by analyzing proteins, enzymes, DNA, RNA, etc. For forest trees this aspect is vital if one takes into account the very long time required to carry out genetic analysis using conventional methods. In addition, for the trees where it is possible to obtain intraspecific hybrids, the selection assisted by markers allows the choice of the combinations with the best chances to exteriorize a positive trans-heteroside of wood production and wood quality. It is also not to be neglected that, unlike conventional selection, whose efficiency is directly proportional to the size of heritability (h^2), the selection assisted by markers allows significant genetic gains in characters with small and very small heritability (Baptism, 1994).

Discussing the selection methods applicable in the improvement of acacia does not mean that all these methods have been and are used with the same frequency. In this respect, we consider that the presentation of a few examples can be edifying. As a rule, clonal, individual or mass selection was applied, depending on the characteristics that had to be phenotypically exteriorized by the new cultivar (Bongarten, 1992; DeGomez, 2011). A number of over 30 monoclonal and multiclinal cultivations were homologated and launched into culture during that period. Subsequently, nracs (National Resources Conservation Service, USDA) concentrated acacia breeding in four universities located in areas with great extent of these species: Georgia, Michigan, Kentucky and Maryland, for the common acacia, red acacia and viscous acacia, and Arizona, for the Mexican acacia.

The improvement methods used were diversified (the selection of the half-sib and full-sib families, the selection of artificially induced mutations and polyploids, the exploitation by selection of somatic variability, the transfer of genes mediated by biological, physical and chemical vectors), the results materializing in an impressive number of new cultivation (over 70), not only at *Robinia pseudoacacia* but also at the other three species of acacia related to the common acacia: *R. hispida* (Wandel, 1989), *R. viscosa* (Isley and Peabody, 1984), *R. neomexicana* (Isley and Peabody, 1984).

In Hungary, the first acacia improvement programme was initiated in 1960 and had as its main objective the selection of new clones with higher characteristics in terms of quantity and quality of production of industrially usable wood (Rédei et al., 2008). In several plantations, previously obtained from seed, three groups of individuals have been identified with these characteristics, in each group being nominated trees plus, resulting in a total of 40 such trees (Keresztesi, 2013). Vegetative material from these trees was used for their multiplication by grafting, the clones obtained being tested, in comparative cultures, at the Gödölfi resort.

At the age of exploitation, five of the most productive clones were homologated as new cultivation under the names: Jászakisári, Kiscsalai, Nyírsági, Úllői and Szajki (Keresztesi, 1994). Because, in Hungary, the areas with optimal pedoclimatic conditions for the acacia culture are quite limited, the acacia has come to occupy large areas in areas with suboptimal conditions.

The production of wood and its quality, in those areas, is far below what the new acacia clones produced in the areas of maximum favorability, therefore, the next improvement program, at the acacia, had as main objective the selection of new productive clones, with quality wood and which are able to tolerate changes in ecological conditions. The result of this new selection cycle resulted in 12 new clones, recommended for approval and introduction into culture (Rédei et al., 2002).

The acacia improvement programs, in the following stages (1983; 1996), used as initial material for selection inter and intraspecific hybrids, populations obtained from cuttings irradiated with Co60, 136 polyploid forms. The comparative orientation cultures (6-10 years), with the best half-sib and full-sib families, with mutants and/or tetraploid forms were organized in eight forest districts, with different pedoclimatic conditions, on a total area of 120 ha. The comparative long-term competition cultures (15-20 years) occupied over 300 ha and were organized in three research stations placed in totally different areas in terms of the degree of favorability for the acacia culture.

The results of these programmes resulted in the homologation of seven new acacia cultivation (until 1996) and the submission of homologation proposals for five other valuable new clones (after 1996) (OSVÁTH-BUJTÁS and RÉDEI, 2007). Unfortunately, in the specialized literature in Romania there is little data on the improvement programs carried out at the acacia tree and their results.

The results of short-term programs (1972-1975) were published by BÎRLĂNESCU et al. (1977) and, fortunately, the authors also refer to the results of previous periods, without delimiting these periods in time. One thing is certain about these programmes: they were part of a National Programme on forest conservation and development between 1976 and 2010. According to the authors quoted, in the period before 1972, some notable results were obtained in our country in the improvement of the acacia tree, among which they mention:

- identification and selection of 29 plus trees that have been vegetatively multiplied, the respective clones being used in the creation of the first 15 ha of acacia plantations
- identification of a new variety of acacia for the flora of Romania, *R. pseudoacacia*, var. *oltenica*, differentiated both morphologically and productively from the common acacia;
- the creation of 11 intraspecific acacia hybrids in which var. *oltenica* was one of the parental forms;
- selection of acacia forms of beekeeping interest.

The lack of large-scale provenance studies was compensated, in our country, by studies of behavior of acacia stands in different resorts. In this regard, it is worth mentioning the 16 experimental blocks with acacia stands of different ages installed by ICEF in 1955; 1956, in six forest districts: Ianca, Săcuieni, Calafat, Lehliu, Miteeni and Craiova. The observations and measurements made with a periodicity of three years, in these experimental blocks, allowed the formulation of some interesting conclusions regarding the increases in height, in basic diameter and the peaks of the increases (Armășescu et al., 1969). It was found that, at the same age of the stand, the

respective characters varied significantly from one resort to another, which reveals the importance of the environment and interaction, ***genotype × the environment*** in the phenotypic manifestation of the respective characters.

In recent years, in most countries with large acacia areas (China, USA, Hungary), the study in comparative cultures of the provenances of different origins is increasingly used to highlight clones / families with superior characteristics for one or more improvement objectives. We mention, in this respect, the studies of interfamilial variability of the intensity of photosynthesis and the growth of trees made in the USA (Mebratu and Hanover, 1989), the regional comparative crops in China with acacia clones for fodder (Zhang et al., 2013) and those from Canada and Hungary, for determining the variability of the quality of wood at the different acacia origins (Stringer, 1992; Rédei, 2008).

Probably one of the most interesting studies of origin is the one reported by Liesebach et al, (2004) in which, at the Institute of Genetics and Improvement of Forest Trees in Germany, 18 seminal descendants (families) of acacia, coming from the USA, Germany, Slovakia and Hungary, were characterized with the help of enzymatic markers.

The results were surprising by the way they differentiated these origins: a high variability, at the enzymatic level, was reported within the six populations from Hungary, combined with a very low interpopulation genetic variability. On the contrary, the eight German populations showed a low intra-population genetic variability at the enzymatic level, while the interpopulation variability showed very high values.

The authors conclude that, for Europe, a general valid model of evolution of variability cannot be given at the acacia tree, because it certainly depended, in each country, on the way of multiplication predominantly used in obtaining seedlings for afforestation made with this species.

The analysis of these results indicates that, in the early period of the acacia improvement in Romania, it was started, as in the USA and Hungary, with the clonal individual selection, which implied the identification of plus trees in the stands with an obvious variability of the traced characteristics, their vegetative multiplication followed by the testing of clones in plantings specially established for this purpose to obtain the seed source stands. Also from the work of the authors quoted above we learn that, in the period before 1972, intraspecific hybrids had already been obtained in which, certainly, the selection was applied in the descendants of F1 half-sib and full-sib followed by the vegetative multiplication of elites and the testing of clones in comparative cultures.

According to the data presented by Enescu (1975), by 1960, 79 plus trees had been identified, whose clonal descendants were tested in comparative cultures at the Experimental Station Craiova. According to the

same author, in our country, the first artificial hybridizations, at the acacia, were made by Bîrlănescu et al. in 1969, and the first works of artificial induction of polyploidy, through treatments with colchicine and/or with physical mutagenic agents, were carried out by Leandru et al., in 1971. The most significant results, from the point of view of acacia improvement, of the short-term program 1972-1975, reported by Bîrlănescu et al. in 1977, are:

- Identifying and choosing new plus trees, which increased the number of plus trees introduced as clones, in plantations, to 66 of which 59 were clones obtained in our country.
- The choice of 50 new clones of beekeeping interest, which were to be tested alongside similar clones from Hungary and Bulgaria.
- Performing the first tests with clones from half-sib (maternal) descendants that proved that, due to hybrid vigor, as early as the age of 4-5 years, the clones of hybrid offspring significantly exceeded the control (common acacia).

In the current conditions, wooden resources must be well preserved. In rural areas, for the afforestation of degraded lands, acacia would be a solution to cover the needs of the population with firewood, along with other environmental advantages. In this regard, European funds can be accessed or the relationship between the citizen and the public institution can be encouraged at the level of the administrative decision. In certain situations, environmental protection in the name of the proper functioning of the community justifies restrictions on the exercise of certain rights, such as that relating to the protection of property. (Timofte et al.,2010)

In Romania, very little research has been done related to the genetic determinism of these characteristics on which the productivity of the acacia depends, to the greatest extent. A special mention deserves the phd thesis elaborated by Ciuvăţ (2013) which, based on the analysis of a large number of acacia stands, aged 1-4 years, from south-western Oltenia, elaborates allometric equations for the estimation of the total biomass and its components according to the biometric characteristics of the measured copies. Beyond the value of these data for the needs of the respective doctoral thesis, they could constitute a good database for calculating the heritability of the respective characters characteristic of the respective populations or as a resultant of the individual measurements.

CONCLUSIONS

Unfortunately, so far, in the literature I have not found any paper that attests to an oligogenoic genetic determinism for any of the elements of productivity or quality, in acacia. The small number (1-3)/large number (11-23) of leaflets in the leaf seems to be determined by a single major gene, and this finding can, indirectly, serve to improve productivity. If the parental

forms had as a genetic marker the large/small number of leaflets in the leaf, the hybrid individuals can be easily identified and then processed by selecting the hybrids with the highest level of trans-heterosis.

If it is accepted that var. *rectissima* would be more correct to be called **cultivar** and not **variety**, we do not see why the same rule would not apply in the taxonomic framing of var. *oltenica*, given that it was originally extracted as a subpopulation of lime. *rectissima*.

Regardless of the taxonomic category in which var. *oltenica* is or will be classified, we consider that it has superior productivity and quality characteristics, very similar to those of lime. *retissima* and a good adaptability to the pedoclimatic conditions of the premontane area in the south-west of the Apuseni Mountains. Based on these considerations, we recommend the extension of the Oltenian variety into *culture, along with the rectissima variety* in the above-mentioned area.

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ASPECTS REGARDING THE ACACIA CROP IN THE AGROFORESTRY SYSTEM FOR THE PRODUCTION OF DENDROMASS

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Abstract

One of the main uses of dendromass from the oldest times has also been the production of energy, but its high consumption at global level has raised serious issues in the society lately, so that solutions are permanently sought in order to generate resources which would be capable of energy production. The dendromass obtained in agroforestry systems, for the production of wood pellets, in short production cycles, may be a sustainable alternative for the European Union states, in search of such energy production alternatives.

According to EUROSTAT 2016, EU-28 has been the largest worldwide producer of wood pellets, its production reaching a value estimated at 13,1 million tonnes in 2014; the production in EU-28 has increased by 97% overall in the period 2009-2014; EU-28 is also a net importer of wood pellets: the level of the imports from the member states of the EU has increased to 8 million tonnes in 2014, a global increase of 364% compared to the year 2009. The main suppliers of the EU have been The United States of America and Canada; a lot less is supplied by Russia and other countries such as Belarus and Ukraine.

The production and use of renewable sources such as the dendromass obtained in the energy crops, may significantly contribute in the field of energy security of the states which dedicated mandatory targets regarding the renewable energy sources. At global level it joins the other species of plants capable of storing atmospheric carbon in trunk, branches and root and the soil on which it is cultivated is also enriched by the input of atmospheric nitrogen which the plant fixates in the soil via roots, forming nodosities of the Azotobacter type. It is known that in Romania acacia generally prefers the warm regions, with gentle, long autumns, safe from early frosts. The specialized literature as well as the experience of the past years confirmed the fact that acacia is a capable and available species, a species which presents impressive advantages from the standpoint of biomass production in agroforestry systems.

Key words: energy plantations, agroforestry, short rotation forestry, black locust

INTRODUCTION

The weakly productive agricultural lands from the point of view of the yield or the abandoned agricultural lands may be successfully capitalized by the creation of energy plantations for obtaining dendromass, and the correct choice of the species based on the pedoclimatic conditions may bring a considerable contribution to the production plans of the dendromass obtained in the energy plantations. The dendromass growth rate of plants, the harvesting, humidity, contents of ash and the management complexity may represent the main selection criteria for the implementation of such projects at local, regional or European level.

Thus, a level of access to public information is required, which implies transparency that can be seen from two perspectives: the prospect of the economy, transparency leads to increased performance and, at the same time, earnings, as well as from an institutional point of view the level of public administration for attracting funds. Decisional transparency implies that the means by which public administration provides citizens with all the necessary elements of their own activity (Timofte, 2016).

The results obtained in Lugoj, in an interval of 3 years, in which acacia (*Robiniapseudacacia L.*) was cultivated in an agroforestry system, we may say, are considerable and correspond similarly to researches in the specialized literature; (Erik et al.) states that the former agricultural lands, which have been abandoned due to optimization and intensification of agriculture, allows the use of lands for other purposes, by planting some forestry species for the production of alternative energy, and (Rédei, 2011) says that in Hungary acacia is the most appropriate forestry species for wooden biomass production by establishing energy crops in an agroforestry system.

In general, acacia has been very much used for forestations of some degraded lands, on eroded coasts, on crude soils, being a good soil fixer. The ecologic plasticity it displays, made it to be cultivated sometimes even in improper conditions, because acacia still has some ecologic plasticity limits, well delineated and known in the specialized literature as well as from numerous experimentations carried out in the approximately 150 years of cultivation, in our country.

In Romania (Ciuvăț et al., 2015) mentions that in the south of Oltenia, acacia offers all types of forest ecosystem services in an area characterized by one of the smallest percentages of forestation at national and European level. The forestation and stabilization of the flying sands in the South of Oltenia have had a double advantage: by saving both the agricultural crops and the local villages exposed to desertification and offering wood and wooden products, thus contributing to the durable development of the region by also protecting it from an economic standpoint.

Acacia has proven from the very beginning to be an almost perfect species for this purpose, because it has some properties fully compatible with the necessities of energy production, such as (Halupa, 1992; Rédei, 2000; Hernea et al., 2009):

- ❑ a very rigorous growth in the juvenile phase;
- ❑ a great capacity of tillering;
- ❑ the acacia wood has a great density;
- ❑ increased production of dry substance;
- ❑ excellent wood combustibility;
- ❑ a relatively short duration for the drying of the biomass;

- ❑ easy and well known technologies for biomass harvesting and processing.

Planting short-lived *Robinia* plantations for renewable bioenergy production is currently fashionable (Sádlo et al., 2017). In the last two decades, more and more agricultural land surfaces have started to be cultivated with species producing significant quantities of vegetal biomass, usable in the manufacturing of bio-fuels.

MATERIAL AND METHODS

The researches included in the present paper have been carried out in the period 2015-2017, on the private property of the economic operators, interested in the production of dendromass for the purpose of using it in the production of pellets and finally in the production of electrical and thermal energy.

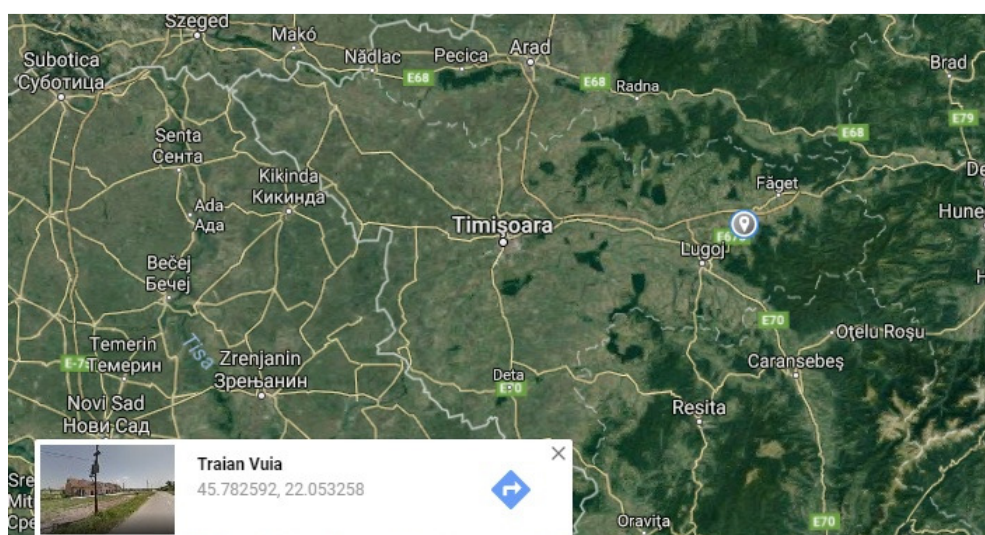


Fig.1. The site for the study (source: google maps)

The establishment of the energy acacia plantations was carried out at the end of 2014, the land was well prepared by works on the whole surface through a scarification at an average depth of 0,6 metres, followed by a soil ploughing at a depth of 0,4 metres, and the breakage of the soil with the disk harrow. The planting process was done in a mechanized manner, by using a plough specialized for this purpose. The productivity at the planting of the acacia seedlings by using a tractor of 110 horse power and the specialized device on two rows was of circa 10 ha/day/12 hours. The seeding material using upon planting was according to the valid Romanian Standard, of first quality.

The number of plants was 7800 pieces/ha upon the establishment of the energy plantation.

In the vegetation period after planting, the interval between the rows of seedlings was maintained over the whole period of vegetation in the form of a black ploughed field, and for the elimination of the weeds which also constituted a competition for the optimum development of the acacia seedlings one used the total herbicidation of the crops.



Fig.2. Overview from the acacia energy plantation in the 2nd year of vegetation

The data collection consisted in:

- the mechanized harvesting of 3 lots of 500 sqm, at the beginning of spring in the period March-April, for each year separately,
- the harvested dendromass was deposited for drying in a natural state,
- in the month of September for each of the three years considered in the study one carried out the weighing of the dendromass which at that moment had 8% humidity.

RESULTS AND DISCUSSION

From the centralization and carrying out of the averages between the obtained values in t/kg, one obtained the following date, represented in Figure 3.

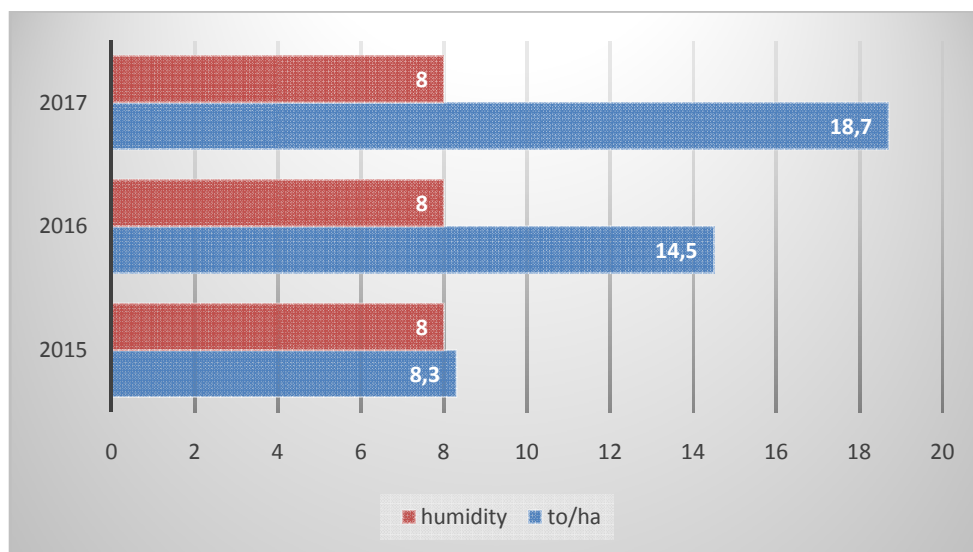


Fig. 3. The quantity of obtained dendromass in to/year/ha, at the humidity of 8%

From the results of these measurements but also from the experience of the three years of cultivation of acacia as energy plantation in order to obtain dendromass based on the number of plants/hectare, as well as by studying the specialized literature, several aspects start to take shape, regarding the most efficient method for establishing and exploiting an acacia plantation for the production of dendromass, so that we may draw the following conclusions:

1. The optimum moment for harvesting the dendromass is in the period February-March.
2. In the first year of harvesting after tillering, as they are thinner, they allow an easier and less expensive harvesting with light machinery which does not cause problems in case of excessive moisture of the plantation soil, the harvesting becomes faster and without risks of losing the optimum cutting moment.
3. The root system increases in volume in each year of vegetation, it is stimulated with each cutting, and an eventual average scarification on top of the harvested acacia row, could have a positive effect on the growth of the offshoots from the new generation, producing a soil oxygenation and easing the activity of the symbiotic bacteria by the extraction of nitrogen from the atmosphere, allowing a natural, cost free fertilization.
4. It is interesting that the acacia bark has a caloric power upon burning greater than that of the acacia wood itself, having the highest caloric power upon burning of the soils used in the energy plantations (Fehér, 2013).

5. For the average production of dendromass we have NOT taken into account:
 - 5.1. The management of the slurry from the zootechnical farms, which may bring a contribution of minimum 20-30% to the dendromass production
 - 5.2. The tillering capacity of acacia together with the formation of the root system, after the age of 5 years after establishment, which is much stronger.

CONCLUSIONS

The results obtained after the three years of experimentations indicate that the annual cutting in an energy acacia plantation for the production of dendromass is very efficient, thus in the third year one obtained an average production of 20,7 tonnes/ha, 14,5 tonnes/ha for the second year and 8,3 tonnes/ha for the first year, dendromass with a humidity of 8%.

We have identified potential beneficiaries of such energy plantations for the production of dendromass established at regional level so:

- the environment, by storing carbon in the biomass and atmospheric nitrogen in the soil; the reduction of the greenhouse effect;
- the fauna;
- the human factor, by the creation of new work places at local level, welfare;
- the local councils of communes and towns which earn own incomes without the need to request help from the government;
- the schools, hospitals, town halls or economic operators from the rural environments of small/medium towns, which can consume thermal agent based on wood/wooden biomass;
- one of the major advantages is *the reduction of the pressure* at local and national level on the forest by the introduction in the local/national circuit of the wooden biomass for consumption for: heating, production of electrical energy, product manufacturing;
- the owners' associations (farmers, tenant farmers);
- the stations for the production of energy;
- the roads and localities (communes and towns) by creating protective forestry curtains.

Pellet manufacturing:

- Due to the high caloric capacity of the acacia dendromass, only CLASS 1 pellets may be obtained;
- The production cost for the pellets is circa 60-70 Euro/tonne;

- 1 cubic metre of methane gas = 10 kWh energy, the equivalent of 2 Kg of acacia pellets;
- 1 kWh methane gas costs between 0,1-0,12 Lei;
- 1 tonne of pellets is the equivalent of 500 kWh;
- 500 kWh x 0,12 = 60 Lei; (140 Euro).....60 Euro.
- **Final conclusion:**
- **500 kWh methane gas acquisition cost 140 Euro**
- **500 kWh from pellets made of energy acacia, cost 60 Euro**

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ASPECTS REGARDING THE ACACIA CULTURE IN AGRO-FORESTRY SYSTEM FOR THE PRODUCTION OF WOOD BIOMASS

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Abstract

The pronounced development of society in the last century at a global level favored high pressures on the environment especially by high consumption of natural resources, energy such as gas, liquid and solid fuels, and now the current challenge for humanity is to produce on a short, medium and long term alternative fuels to generate power at cost as low as possible. In addition to modern techniques of obtaining energy through complex wind, solar or hydrological systems, wood biomass produced in a short production cycle is of particular importance for achieving energy. This paper presents experimental data are based on the way of production and quantitative values of acacia wood biomass grown in an agro-forestry system.

Key words: acacia, wood biomass, agro-forestry system, alternative energy, energy crop, acacia biomass.

INTRODUCTION

In general, acacia has been greatly used for the forestation of degraded lands, on eroded slopes, raw soils, being a good ground fixer. Its ecological plasticity determined its cultivation, sometimes even in improper conditions, because acacia has yet certain limits of ecological plasticity, that are well defined and known both in literature and in numerous experiments made during the approximately 150 years of culture in our country (Budău R., 2014).

The results obtained in Lugoj, within three years, due to which the acacia (*Robinia pseudacacia* L.) was grown in agro-forestry system, are really promising and correspond to similar studies in the literature. (Erik Temeșvar et.al) states that former agricultural lands that have been abandoned due to optimization and intensification of agriculture, can be used now for other purposes, by planting some forest species to produce alternative energy. A crucial element is the right choice of plant species to coincide with the pedo-climatic factors of the area where the energy crops are installed. (McKendry, 2001) states that the main criteria for selection for biomass are the growth rate, ease of management, harvesting and some of its properties such as, moisture, ash, alkali content, and (Rede, 2011) says that

in Hungary, acacia is the most suitable species for wood biomass production through the establishment of energy crops in agro-forestry system.

According to (www.resource-ce.eu, 2010) other major advantage is that acacia species has the ability to fix nitrogen from the atmosphere and thus to improve long-term soil fertility, recommending short cycle crops production.

MATERIAL AND METHODS

The research was conducted on the private lands of the business operators interested in wood biomass for its use in the production of thermal energy from the neighborhood of Lugoj, Timis county, during 2012-2014.

For the establishment of plantations, the land has been well prepared with works throughout the concerned area by scarification to an average depth of 0.6 meters, soil plowing to a depth of 0.4 meters, soil shredding with a disc harrow, and respectively mechanized planting of acacia seedlings (Figure 1). The material used for plantation was purchased from Bărzani Farm, and it was a first quality material according to the Romanian standards it.



Fig.1 Establishment of culture through mechanized planting
(planting scheme 2x0.5 meters)

According to Table 1, three planting schemes were used with a minimum effective of 6250 plants/ha, average effective of 10,000 plants/ha and respectively maximum effective of 16,000 plants/ha.

Table 1

Planting scheme and number of plants / ha		
Planting scheme		
2 x 0.3	2 x 0.5	2 x 0.8
Number of plants /ha		
16000	10000	6250

During the growing season after planting, the interval between seedlings rows was maintained throughout the period of vegetation under the form of black fallow and for the removal of weeds which were also a competitor for the optimal development of acacia seedlings, the total culture herbiciding was made.



Fig.2 Overview of acacia energy plantation after the first 30 days of vegetation from planting

Data collection consisted in random manual harvesting of 30 pieces for each used sample, respectively for each scheme used in the study and the period in which measurements were made was at the end of the active vegetative growth, 15 to 20 October, then, the sample mass was determined by direct weighing in kg, the collected data being pooled for the three planting schemes as it is shown in Table 2.

Table 2

Determination of average increases in kg versus planting scheme

Planting scheme		
2 x 0.3	2 x 0.5	2 x 0.8
1) 0.85	1) 4.24	1) 3.7
2) 0.85	2) 2.3	2) 2.4
3) 1.15	3) 2.47	3) 2.3
4) 1.35	4) 2.2	4) 2.3
5) 1.17	5) 2.2	5) 1.25
6) 1.48	6) 1.3	6) 2.8
7) 1.08	7) 1.3	7) 1.8
8) 1.77	8) 1.81	8) 1.3
9) 2.48	9) 2.5	9) 7.7
10) 1.52	10) 2.6	10) 2.2
$\bar{x} = 1.37\text{kg/plant}$	$\bar{x} = 2.382\text{ kg/plant}$	$\bar{x} = 2.775\text{ kg/plant}$
$1.37 \times 16000 = 21920\text{kg/ha}$ 21.92 t/ha	$2.962 \times 10000 = 23820\text{kg/ha}$ 23.82 t/ha	$2.775 \times 6250 = 17343.75\text{kg/ha}$ 17.34 t/ha

Out of the centralization and making the averages between/among the values obtained in t/kg, it can be concluded that the planting scheme 2 x 1, for which an average value of 23.82 t/ha was achieved, is the recommended scheme for acacia wood biomass production in energy plantations. Following the research, average chips amount (20% moisture) was obtained per ha, ranging in the first year between 8 and 9 tons/year/ha, 14 and 15 t/year/ha in the second year, 20 and 22 t /year/ha in the third year, 19 and 20 t /year/ha, resulting a total average of approx. 400 tons/ha/20 years.

After the three years of study it can be concluded that the optimal planting scheme for acacia energy crop is 2 x 0.5 meters, with a total of 10,000 pieces / ha (Figure 1). This scheme has also been adopted taking into account the best way of mechanized harvesting at a reduced price of wood biomass.



Fig.3 Overview of acacia energy plantation after the first 130 days of vegetation from planting (planting scheme 2x0.5).

Similarly, in a comparative lot in which the plantation is not cut for two-year, on an area of ten hectares, and that was planted in 2012, we have a

lower density due to the setting percent of approx. 65-70%, which was influenced by several reasons: inappropriate planting machine, poorer seeding material.

The measurements were determined by harvesting 30 subsequent plants in the same row. The average on this plot was 3,503kg / plant, and the production was at the time of the measurements of about 24.5 t / ha of wood biomass.

In terms of harvesting wood biomass, it is harvested mechanically by a cutting device (in the stage of patenting) foreseen with a runnout conveyor /palletization system of the wood biomass (Figure 4, 5, 6). The lots of acacia that were not harvested and had at the second and third year of production cycle, the diameter, when packed, presented significant increases of 4-7 centimeters. The harvesting and chopping process of wood biomass requires appropriate devices to resist to the efforts needed for these two processes; the greater the wood material in the stem diameter, the much more effort to harvest in optimal conditions.

The costs of the harvesters are hundreds of thousands of dollars, which in a first phase of investment has a huge cost and the annual harvest by a relatively simple and affordable device, greatly reduce the production costs of wood biomass.

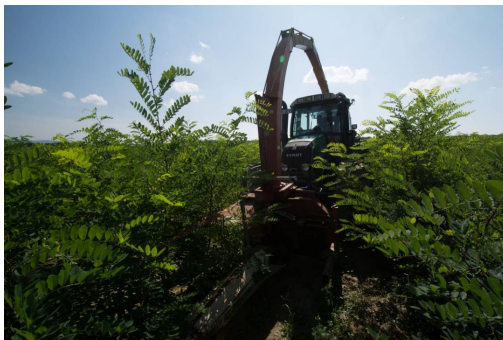


Fig.4 Mechanized harvesting of acacia wood biomass – front view



Fig.5 Mechanized harvesting of acacia wood biomass - side view



Fig.6 Mechanized harvesting of acacia wood biomass - rear view

RESULTS AND DISCUSSIONS

From the results of these measurements, the three-years experience of growing acacia under energy plantation system and planting schemes, the density of plants / ha, and from the study of literature, several aspects of the most efficient method of establishing and exploiting a acacia plantation begin to take shape for the production of wood biomass in agro-forestry system, so that:

I. Being a light demanding variety, the growth of timber is directly proportional to the existing growing space (light), being the highest in the first year after cutting, decreasing every year without cutting because of the self-shading effect. Moreover, the root system increases in volume each year of vegetation, is stimulated by each cutting, and an eventually average scarification near the harvested acacia row could have a positive effect on the growth of shoots of the new generation, producing a soil oxygenation and facilitating the work of symbiotic bacteria by extracting the nitrogen from the atmosphere, allowing a natural free fertilization, etc;

II. In the first year after cutting, the shoots, being thinner, allow an easier and less expensive harvesting with light equipment that does not create problems in case of excessive soil moisture, the harvesting becoming faster and without the risks of losing the optimal cutting timing.

III. Due to the small diameters of shoots, after the leaves fall, the relative humidity will decrease rapidly.

IV. It is interesting that acacia bark has a higher calorific value than the acacia wood itself, having the highest calorific combustion value among the varieties used in the energy plantations (Fehér, 2013).

V. The more the number of shoots per plant, the highest the percentage of bark and implicitly of heating power.

CONCLUSIONS

The results of three years of experience indicate that the annual cutting in energy acacia plantation is the most effective and inexpensive method of biomass exploitation, therefore it is recommended the annual harvest of wood biomass from the energy plantations of acacia, the most efficient method being the plantations with a total of 10,000 plants / ha, using the scheme 2x0.5 plants.

The costs of this method depend on several aspects according to parameters of the field that was chosen to establish the energy crop:

a) the amount of mechanical work necessary to prepare the soil for planting;

b) geometry of the chosen plot (slope, soil texture, existing vegetation, degree of prior usability, etc.;

c) the position of the plot with respect to the nearest infrastructure for the deployment of logistics for plantation setting up works;

Depending on these studied issues, the total costs of an energy acacia plantation vary from 1500 €/ha to 1800 €/ha without VAT, including all the mechanical works, herbicide, labor force until the first cutting. Cutting, chopping, packaging and transport to the storage place adds an extra cost of approx. € 200 /year/ha.

The average amounts of chips (20% moisture) obtained per ha range between 8 and 9 tons /year/ha in the first year, 14 and 15 t /year/ha in the second year, 20 to 22 t /year/ha in the third and a simulation calculation indicates an average production of approx. 19 - 20 t /year/ha in the twentieth year after planting, resulting a total of approx. 400 tons / ha/20 years.

In terms of average cost /ha, we conclude the following:

- € 1,650 (cost to establish the plantation);
- € 4,000/20 years (harvesting and maintenance costs);
- € 5,650/20 years (total expenses for the production of acacia wood biomass);

If the production costs are compared to the amount of acacia wood biomass that can be obtained through a short cycle of production (annual), but over at least two decades installation period of the acacia culture in agro-forestry system, a total production cost of € 14,125 / t results.

The comparison of the current price of natural gas and acacia biomass production cost: 1 m³ methane gas = 2kg acacia chips as calorific power; 500 m³ methane gas = 1 t acacia chips; 500 m³ methane gas = 500 lei at the current price of natural gas for state subsidized consumers!; 1 t chips= 14,125 EUR = 63.8 lei direct expenditures on energy plantation.

We may conclude that the current price of natural gas is significantly higher (800%) than the price of acacia biomass production which clearly demonstrates the economic efficiency of acacia energy plantations in agro-forestry system.

In the current context, we suggest further research in this area and the adoption of some legislative measures for the potential capitalization of producing wood biomass for the species that have a significantly higher growth on a short-term as well as the subsidized support of the investment in this area.

According to (www.europe-aliens.org) acacia is recorded as one the top 100 species, the most invasive in Europe, but all the same specific source and planting species that modern systems and control, is a renewable energy source.

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Article

Bioactive Compounds and Antioxidant Capacity of Several Blackberry (*Rubus* spp.) Fruits Cultivars Grown in Romania

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Abstract: Blackberry fruit (*Rubus* spp.) has a powerful antioxidant capacity due to the high levels of anthocyanins and other phenols it contains. In this work, we investigated the physico-chemical characteristics, the bioactive compounds (total phenols, flavonoids, and monomeric anthocyanins), and the antioxidant capacity of seven blackberry cultivars belonging to the *Rubus fruticosus* L. and *Rubus laciniatus* L. genera growing in the NW region of Romania. In addition, the wild blackberry cultivar from the same area was also evaluated. Anthocyanins from the blackberry fruit were extracted using SPE (Solid Phase Extraction), and the anthocyanin profile was identified and quantified using HPLC-PDA analysis. In terms of polyphenol content and antioxidant capacity, two of the cultivars examined stood out. The majority of anthocyanin found in blackberries was cyanidin-3-glucoside, with the highest amount recorded in the 'Thorn Free' cultivar at 329.26 ± 9.36 mg/g dw. Comparatively, 'Loch Ness' and 'Thorn Free' fruits exhibited total phenol contents of 1830.98 ± 13.55 and 1687.14 ± 62.41 mg GAE/100 g dw, respectively. Antioxidant capacities varied significantly among the eight blackberry cultivars, with cultivars 'Loch Ness' and 'Thorn Free' achieving high values in comparison to the others. The findings of the multivariate analysis also supported the experimental results. Knowing the phytochemical composition and antioxidant potential of different blueberry cultivars, one can use them as fresh, functional foods or for commercial purposes to produce products derived from fruits with a high concentration of bioactive components.

Keywords: *Rubus fruticosus* L.; *Rubus laciniatus* L.; anthocyanins; total phenols; flavonoids; antioxidant capacity; multivariate analysis



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1. Introduction

Blackberry is a shrub belonging to the genus *Rubus* of the family *Rosaceae*. The family *Rosaceae* is the 19th largest family of plants. *Rubus*, with almost 700 species, is the largest genus in this family. *Rubus* comprises 12 subgenera, with few domesticated species. It is a perennial plant believed to have originated in Armenia, with rapid growth distributed and cultivated mainly in Europe, Asia, and North America, but its worldwide popularity is constantly growing. It grows spontaneously in the northern parts of Pakistan, where it is known by various local names: Karwara, Ach, Akhara, and Baganrra. Although the fruit is widely accepted in Pakistan, it is not grown on a commercial scale. There were 7692 ha of blackberries commercially grown in Europe in 2005, with Serbia leading as a producer, followed by Hungary, the United Kingdom, Romania, Poland, Germany, and Croatia [1]. In recent years, Europe has become one of the world leaders in the production of blackberries (*Rubus fruticosus*); the largest regions with blackberry production are Serbia and Hungary [2–6].

Because blackberries naturally grow in relatively vast regions in Romania and the country's primary source of blackberries comes from this flora, blackberries are only grown there on small plots of land. However, cultivating blackberries has attracted particular attention in Romania in recent years. Thus, blackberries harvested from spontaneous flora are of great interest among consumers, and the fruits are consumed both fresh and in the form of juices, jams, compote, syrup, or in the preparation of desserts.

In 2005, the area of wild blackberries in Romania was approximately 2400 ha, and the cultivated organic blackberry area was only 10 ha. Of the cultivated varieties, Loch Ness is the main variety cultivated in Romania [1]. The blackberry of the spontaneous flora produces edible fruits and is spread all over the world, but is mainly concentrated in the northern hemisphere. Blackberries were among the first fruits of the wild flora used for medicinal purposes. Blackberry juice has been used in Europe since the 16th century to treat various oral and eye infections, and the cultivation of blackberry species began in the 17th century [2,7]. The global area under blackberry cultivation expanded by nearly 45% between 1997 and 2007 [5].

Favorable weather and soil conditions, as well as the experience of growers, promote the development of crops. Farmers have developed a variety of *R. fruticosus* varieties using traditional breeding procedures, which differ in firmness, shape, size, aroma, color, weight, yield, ripening season, nutritional content, and pest resistance. The most popular varieties include Jumbo, Chester, Bartin, Ness, Bursa 1, Bursa 2, Bursa 3, Arapaho, Navaho, Thornfree, Chester Thornless, Dirksen Thornless, Cacanska Bestrna, Loch Ness, Cherokee, and Black Satin [3,6].

Fruits from the *Rubus* genus are among those rich in bioactive compounds. Their nutritional profile shows that they contain dietary fiber, vitamins, minerals, and carbohydrates [2,8,9]. In general, fruits have a high level of dietary fiber (cellulose, hemicellulose, pectin) but are low in calories, very low in fats, and high in carbohydrates such as glucose and fructose. Organic acids are present in low concentrations and are responsible for the flavor of the fruit. Certain minerals, some vitamins and the presence of phenolic compounds give *Rubus* fruits the status of functional foods [4,10,11]. Blackberries have a variety of health benefits due to their phytochemical composition, which helps prevent and treat metabolic disorders and chronic diseases such as cancer, diabetes, hypertension, cardiovascular disease, gastrointestinal diseases, atherosclerosis, aging, Parkinson's disease, and Alzheimer's disease [6,12,13].

Anthocyanins, in particular, provide a considerable contribution to blackberries' antioxidant potential and are responsible for a number of benefits for both human and animal health, according to recent literature data on their phytochemical composition [8,9,11,14,15]. Along with flavonoids—such as various glycosylated forms of quercetin, kaempferol, luteolin-3-O-glucuronide, and apigenin-3-glucuronide—cyanidin-3-O-glucoside is the major anthocyanin that predominates among the compounds found in *Rubus* fruits. Various other anthocyanins are also detected in blackberry fruits, such as cyanidin-3-O-arabinoside, cyanidin-3-O-xyloside, cyanidin-3-O-malonylglucoside, cyanidin-3-O-dioxalylglucoside, cyanidin-3-O-diglucoside, cyanidin-3-glucosylrutinoside, cyanidin-3-O-rutinoside, cyanidin-3-(3'-malonyl)glucoside, and cyanidin-3-(6'-malonyl)glucoside. In addition, anthocyanins, a water-soluble pigment, have been found in fruit cell vacuoles [13,15]. Anthocyanins are known to be powerful antioxidants that have the ability to fight oxidative stress and eliminate free radicals from the body, and supplementing the diet with natural antioxidants obtained from fruits could be more effective than consuming an individual antioxidant obtained from other sources [16–18]. The daily intake of anthocyanins in humans in the United States was estimated by Kuhnau, 1976, to be between 180 and 215 mg/day [19]. Blackberries also contain appreciable amounts of flavonols that appear in the glycosylated form and are found exclusively in the fleshy part of the fruit [20].

The objective of this study was to characterize the physical and chemical properties, and total bioactive compound content of blackberry fruits cultivated on a farm in northwest Romania. In addition, the blackberry antioxidant capacity was also investigated. Beyond

these blackberry cultivars, the wild blackberry variety was investigated. Multivariate statistical analysis was used to analyze the results and identify the most valuable blackberry cultivar in terms of its bioactive compounds level and antioxidant capacity. The novelty of this study lies in the fact that blackberry fruit cultivars were characterized in the literature for the first time in terms of their bioactive compounds and antioxidant capacity. Our research focused on the *Rubus fruticosus* L. ('Cester,' 'Triple Crown,' 'Navaho,' 'Loch Ness,' 'Thorn Free,' and 'Ouachita,' including the wild variant) and *Rubus laciniatus* L. ('Thornless Evergreen') genus.

2. Materials and Methods

2.1. Chemicals

The HPLC reference standards of cyanidin chloride (purity $\geq 95\%$ HPLC) were purchased from Sigma Aldrich (St. Louis, MO, USA). The water for the HPLC analysis was purified using a Milli-Q system (Merck Millipore, Burlington, MA, USA). For spectrophotometric methods, quercetin, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Folin and Ciocalteu's reagent were purchased from Sigma Aldrich (St. Louis, MO, USA), and 2,4,6-Tris(2-pyridyl)-s-triazine and aluminum chloride were purchased from Fluka (Charlotte, NA, USA). Neocuproine Hemihydrate $\geq 99\%$, p.a. was purchased from Roth. Ethanol, methanol, copper chloride, and sodium hydroxide were of analytical grade.

2.2. Plant Materials

Seven different cultivars of blackberries were harvested in 2021 from the Bărzani farm in Arad County, located in northwest Romania (coordinates: 46°29'7" N 22°7'57" E), while wild blackberry fruit was harvested from the region's (northwest Romania) native vegetation. Blackberries naturally grow in Romania in plain areas, on steep slopes, and in forested areas. Blackberries from the spontaneous flora (WILD) are thorny shrubs and have very few fruits in comparison to those from cultivated plants [21].

The cultivated blackberries used in this study are used for human consumption and belong to both the genus *Rubus fruticosus* L. ('Chester', 'Triple Crown', 'Navaho', 'Loch Ness', 'Thornfree' and 'Ouachita') and *Rubus laciniatus* ('Thornless Evergreen'), and are described in Table 1.

Table 1. Horticultural characterization and the appearance of blackberry cultivars.

Cultivars	Aspect of Fruits	General Aspects
CHEST		Is a vigorous shrub, semi-erect and fast-growing: ❖ Height of the plants: 3–4 m; ❖ Ripening: August–September; ❖ Frost resistance: of all the thornless varieties, it is the most resistant; ❖ The fruits: produced on shoots of the second year; they are large and sweet, with an aromatic taste and a uniform ripening; ❖ Productivity: approximately 5–6 kg/plant or 15–18 t/ha.
T_C		Is a semi-erect blackberry variety and self-fertile: ❖ Height of the plants: max. 3 m; ❖ Ripening: 30 days between the months of July and August; ❖ Frost resistance: $-20\text{ }^{\circ}\text{C}$ (The frost resistance is average, compared to 'Cester'); ❖ The fruits: large, firm, and with extremely high productivity; ❖ Productivity: approximately 15 t/ha.

Table 1. Cont.

Cultivars	Aspect of Fruits	General Aspects
NAV		Is an erect shrub, and prefers sunny areas and less wet and cold soils: ❖ Height of the plants: approximately 2 m; ❖ Ripening: 30 days, in June; ❖ Frost resistance: -9°C; ❖ The fruits: produced on second-year shoots, large, firm, and juicy; ❖ Productivity: highly productive.
L_NESS		Is a vigorous shrub with semi-erect shoots, prefers very sunny areas and is particularly suitable for smaller gardens as it is compact and does not produce suckers: ❖ Height of the plants: 3–4 m; ❖ Ripening: starts from the end of July and ends in September; ❖ Frost resistance: -25°C; ❖ The fruits: medium-sized, firm, cone-shaped, and ripen evenly; ❖ Productivity: 3.6 kg of fruit/plant.
THRNFR		Is a very vigorous shrub, and during the vegetation period, they produce side shoots; the inflorescences are long, with a large number of flowers: ❖ Height of the plants: 4–8 m; ❖ Ripening: is prolonged and staggered, beginning in mid-August and extending to early September; ❖ Frost resistance: has good resistance to frost; ❖ The fruits: very large fruits, pleasant and slightly aromatic taste, and appear only in the 2–3 years after planting; ❖ Productivity: approximately 20 t/ha.
OCHIT		Is a very vigorous, fast-growing, upright, and erect shrub, and is a sun lover and self-pollinating: ❖ Ripening: begins at the end of June and extends over a period of 5 weeks; ❖ Frost resistance: moderate; it prefers areas with a milder climate; ❖ The fruits: produced on shoots of the second year, are medium to large in size, and extremely sweet and juicy, rivaling the NAV variety; ❖ Productivity: approximately 3 kg/plant.
EVRG		Is a semi-vigorous and productive variety, vulnerable to various diseases, very resistant to drought conditions, and its leaves remain green even in winter: ❖ Height of the plants: the stems are creeping and long—3–6 m; ❖ Ripening: from July to September; ❖ Frost resistance: is sensitive to frost but tolerates cold; ❖ The fruits: have a juicy pulp with a sweet taste in adulthood, firm, and resistant to transportation; ❖ Productivity: 12–14 t/ha.

CHES—'Chester'; T_C—'Triple Crown'; NAV—'Navaho'; L_NESS—'Loch Ness'; THRNFR—'Thornfree'; OCHIT—'Ouachita'; EVRG—'Thornless Evergreen'.

All the fruits were collected at optimum full ripeness. Berries were handpicked and transported (ca. 2 h) in cooled containers to the Food Engineering Laboratory of the University of Oradea. The blackberry fruits were divided into two groups, one group being used for physico-chemical analyses (fruits size and weight, total soluble solids, pH, and titratable acidity), and another group was dried at 40 °C in the oven with ventilation (Nitech Pol Eko oven, model CLN 53, Wodzisław, Poland) for a long time (a few days, depending on the variety) until a constant weight was reached. The dried blackberries were ground to a fine powder, packed in plastic bags and stored in the dark before extraction for the following analysis described below. According to the literature, dehydrating fruit at a temperature of 40 °C has been shown to prevent the deterioration of the compounds that are present in fruits, particularly the anthocyanin content [22].

2.3. Determination of Fruit Weight, Dimensions, Moisture and Firmness of Blackberries

Thirty fruits from each variety were used for the determination of weight and size. Fruit weight was measured with an analytical balance with a sensitivity of 0.0001 g, and length and width were measured with a 150 mm digital caliper with a sensitivity of 0.01 mm (RS PRO, China).

By monitoring the weight loss of oven-dried blackberries at 40 °C (until they reached a constant weight), the moisture content was determined using Equation (1) [23].

$$\% \text{Moisture} = \frac{W_i - W_f}{W_i} \times 100 \quad (1)$$

where W_i is the weight of fresh fruits; W_f is the weight of dried fruits.

Firmness was measured on two opposite sides of 30 fruits per treatment using a portable penetrometer (Fruit penetrometers tester, FT 327) with a 3 mm diameter probe, and the average value was expressed in Newton (N). The firmness of the blackberries was measured immediately after harvest.

2.4. Determination of Total Soluble Solids Content (TSS), pH and Titratable Acidity of Blackberries

TSS was determined from homogenized fruits at 20 °C using a digital Abbe refractometer. After diluting 10 g of the homogenized material with 100 mL of deionized water, the pH was measured using a digital pH meter. Titratable acidity was determined by the titrimetric method using 0.1 N NaOH solution, and the results were expressed as g citric acid/100 g fw.

2.5. The Bioactive Compounds Determination

2.5.1. Preparation of Blackberry Extracts

1 g of the powder obtained from dehydrated blackberries in the oven (Nitech Pol Eko oven, model CLN 53, Poland) at 40 °C was extracted with acidified methanol (trifluoroacetic acid (TFA), 0.1%) in a ratio of 1:10 (w/v), using the Heidolph homogenizer, Silent Crusher M at a speed of 12,000 rpm, for one minute. The homogenized samples were then centrifuged (NÜVE NF 200 BENCH TOP CENTRIFUGE, Ankara, Turkey) at 5000 rpm for 20 min. The pellet was re-extracted until the solvent became colorless, then supernatants were combined, and the methanol was removed by vacuum rotary evaporator (Heidolph Rotary Evaporator, Laborota 4000 rotavapor, Schwabach, Germany) at 40 °C, according to Bunea, et al., (2011), and the dry residue was dissolved in acidified water (0.1% TFA) [24,25]. Finally, bioactive compounds-rich extract was produced, and total polyphenols, flavonoids, monomeric anthocyanins content and antioxidant capacity were measured.

2.5.2. Determination of Total Phenols (TPh), Flavonoids (TFlav) and Total Monomeric Anthocyanins (MAP) Content

The total phenols content in the blackberry fruits was determined using the Folin–Ciocalteu method with some modification [26,27]. Briefly, the aqueous blackberry extract (100 µL) was incubated with 1700 µL of distilled water, 200 µL of Folin–Ciocalteu reagent

(freshly prepared, dilution 1:10, *v/v*) and 1000 μL of 7.5% Na_2CO_3 solution for 2 h in the dark, at room temperature. The absorbance was measured at 765 nm (Shimatzu miniUV-Vis spectrophotometer, Kyoto, Japan), and the results are expressed as milligrams of gallic acid equivalents (GAE) per 100 g dry weight (dw) using gallic acid as a standard.

The total flavonoid content was determined by the colorimetric method of aluminum chloride. Aqueous blackberry extract (1 mL), 4 mL distilled water, and 0.3 mL 5% NaNO_2 were combined in a 10 mL volumetric flask and allowed to react for 5 min. After that, 0.3 mL of 10% AlCl_3 was added, and the mixture was homogenized before a 6 min break. A final volume of 10 mL was obtained by adding 2 mL of 1 M NaOH and distilled water, and after 15 min of pause, a spectrophotometer was used to measure the absorbance at a wavelength of 510 nm. The results were expressed in mg QE (quercetin equivalents)/g dw [28].

Total monomeric anthocyanin pigments content in blackberry fruits was determined by the pH-differential method [29,30], and the results were expressed as cyanidin-3-glucoside equivalent (C3glu)/100 g dw.

2.6. Solid Phase Extraction and HPLC-PDA Analysis of Anthocyanins

As schematically illustrated in Figure 1, the solid phase extraction (SPE) of blackberries anthocyanins was carried out. The blackberry extract was passed through SPE C18 column (200 mg/3 mL) (Finisterre by Teknokroma, Barcelona, Spain). The remaining compounds in the sorbent were washed with water acidified with 0.01% HCl, and then the anthocyanins were eluted with 10 mL of acidified methanol (0.01% HCl). The rotavapor was used to evaporate the methanol from the anthocyanin extract. The aqueous extract obtained was dissolved with 0.01% HCl acidified water to give the anthocyanin-rich extract (R.A.E.) [31].

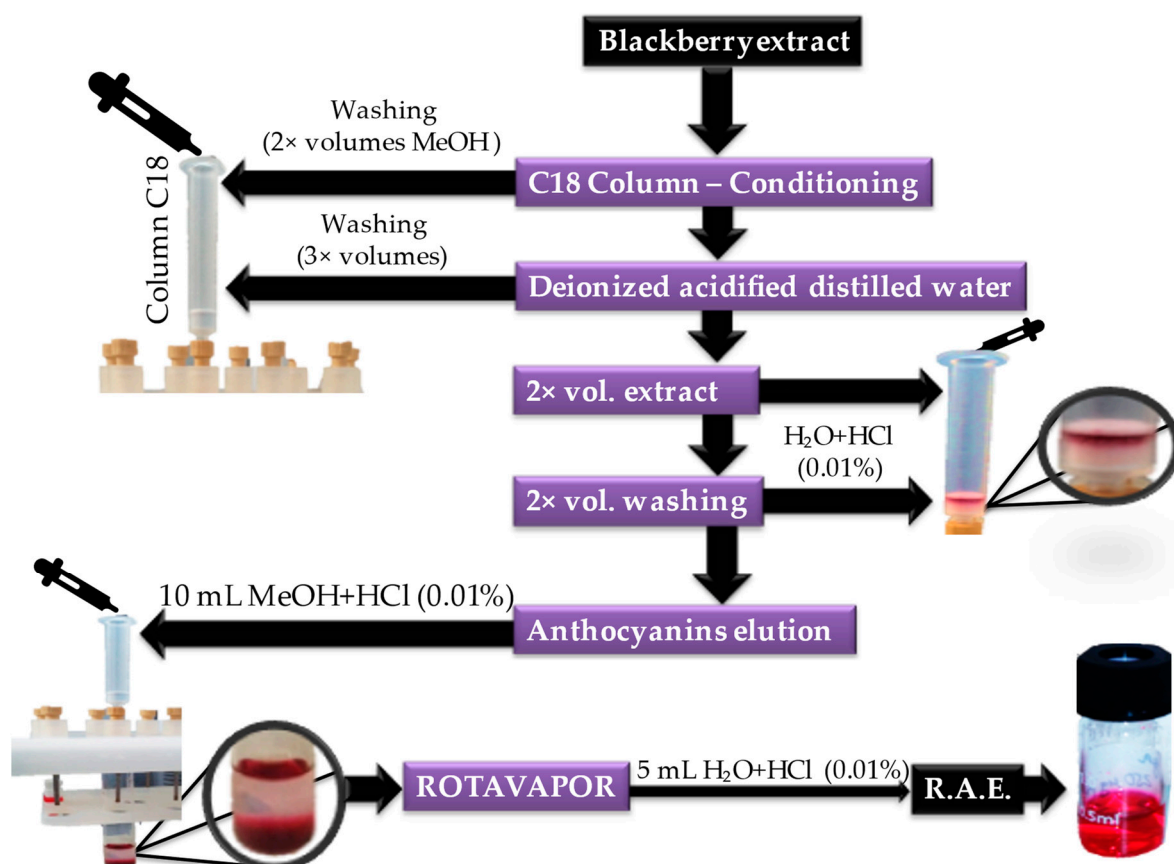


Figure 1. Extraction and purification of anthocyanins from blackberry cultivars using SPE. R.A.E. (rich anthocyanin extract).

Chromatographic analysis of anthocyanins from blackberry fruits was carried out using an HPLC system (Shimadzu Corporation, Scientific Instruments, Kyoto, Japan) equipped with a CBM-20A controller, an LC-20AD pump, a DGU-20A degasser, a SIL-20AC, a CTO-20AC column oven, and a photodiode array detector (SPD-M20A, PDA). The chromatographic column used was Luna (C18) (Phenomenex, Torrance, CA, USA) 100 Å, (250 mm × 4.6 mm, 5 µm), at 30 °C, using a flow rate of 0.5 mL/min and a sample injection of 20 µL. The obtained data were processed by the Labsolution program version 5.10.153 (Shimadzu). Mobile phases were made up of eluent A (4.5% formic acid solution) and eluent B (acetonitrile) using the following gradient schedule: 0–9 min 10% B; 10–17 min linear gradient up to 12% B; 18–30 min linear gradient up to 25% B; 31–50 min linear gradient up to 90% B; and 51–55 min linear gradient up to 10% B [31].

Anthocyanins were detected by monitoring the absorbance at 520 nm. Both the retention time and the UV spectrum of each obtained peak were compared with that of the standards and with the Polyphenol Explorer database (<http://phenol-explorer.eu/compounds/classification>, accessed on 16 September 2021).

A calibration curve was created using cyanidin chloride (purity ≥ 95% HPLC, Sigma) as the standard at concentrations ranging from 15 to 500 µg/mL in order to quantify anthocyanin compounds. Results are expressed as mg cyanidin equivalent/g dw. The regression equation was $y = 3681.7x + 1654.3$ ($R^2 = 0.9967$).

2.7. Determination of Antioxidant Capacity of Blackberries

Antioxidant capacity of blackberry samples was determined using four spectrophotometric assays, DPPH (2,2, diphenyl-picryl-hydrazyl), FRAP (Ferric Reducing Antioxidant Power) and CUPRAC (Cupric Reducing Antioxidant Capacity).

Radical scavenging capacity of blackberry extract using the stable DPPH radical was determined according to the method of Brand Williams, et al., 1995, with minor modifications [27,32]. A volume of 100 µL of blackberry extract was mixed with 2800 µL of 80 µM DPPH solution and stored at room temperature in the dark. After 30 min, the absorbance of the samples was measured using a Shimadzu mini UV-VIS spectrophotometer at a wavelength of 517 nm. The results were expressed as mmol Trolox equivalent (TE)/100 g dw. The regression equation was $y = 970.07x + 0.6222$ ($R^2 = 0.998$).

The FRAP assay tested the antioxidant power of the blackberry samples based on the extract's ability to reduce Fe^{3+} from tripyridyltriazine $\text{Fe}(\text{TPTZ})^{3+}$ complex to the blue-colored complex- $\text{Fe}(\text{TPTZ})^{3+}$ in an acidic medium [33,34]. Blackberry extract (100 µL) was allowed to react with 500 µL FRAP working solution and 2 mL distilled water, for 1 h, in the dark. The results were expressed in mmol Trolox equivalent (TE)/100 g dw. The regression equation was $y = 13,588x + 0.046$ ($R^2 = 0.9977$).

The CUPRAC method consists of the combination of 1 mL copper (II) chloride solution (1×10^{-2} M), 1 mL neocuproine (2,9-dimethyl-1, 10-phenanthroline) alcoholic solution (7.5×10^{-3} M), 1 mL ammonium acetate aqueous buffer (pH 7), and 100 µL blackberry extract, followed by water such that to make the final volume 4.1 mL. The samples' absorbance was measured at 450 nm following a 30-min pause in the dark. The results were expressed in mmol TE/100 g dw. The regression equation was $y = 3.826x + 0.0088$ ($R^2 = 0.9988$) [35,36].

2.8. Statistical Analysis

All tests were performed in triplicate, and the values shown are the means and standard deviations (SD). The univariate statistic test, one-way ANOVA ($p = 0.05$), combined with Tukey's multiple comparisons tests, was used to assess the physico-chemical characteristics, the bioactive compound content, and the antioxidant capacity of blackberry cultivars. Univariate statistical analysis was performed with Stata 17SE statistical software (StataCorp LLC, 4905 Lakeway Drive, College Station, TX, USA). Principal component analysis (PCA), linear discriminant analysis (LDA), multivariate analysis of variance (MANOVA) ($p = 0.05$), and hierarchical cluster analysis (HCA) were the multivariate statistical methods that

were used to classify the analyzed blackberry cultivars in accordance with the parameters examined. Multivariate statistical analysis was performed with MATLAB v2022b CWL (MathWorks, 1 Apple Hill Drive, Natick, MA, USA).

3. Results and Discussion

3.1. Physico-Chemical Parameters of Blackberries

Blackberries are perishable fruits due to their external structure and the very high percentage of moisture in the pulp [37,38].

Table 2 shows the average fresh fruit weight (GrP), height and diameter, and average fruit weight after dehydration (GrD) of the eight different blackberry cultivars as growth and development parameters. Additionally, the moisture content in each blackberry variety was determined (Table 2) by measuring the fruits after they were dried completely out in a 40 °C oven until they stabilized in weight.

Table 2. Moisture content (%), fresh fruit weight (GrP), height, diameter, and dried fruit weight (GrD) for the eight blackberry cultivars (results are presented as means $\pm$ SD).

Samples Parameters	CHES	T_C	EVRG	NAV	L_NESS	THRNFR	OCHIT	WILD
Moisture content (%)	68.8 $\pm$ 0.6 ^a	75.6 $\pm$ 0.02 ^b	73.1 $\pm$ 0.05 ^c	71.8 $\pm$ 0.08 ^d	68.3 $\pm$ 0.02 ^{a,c}	82.7 $\pm$ 0.02 ^f	64.1 $\pm$ 0.1 ^g	79.9 $\pm$ 0.03 ^h
GrP (g)	5.89 $\pm$ 0.14 ^a	6.67 $\pm$ 0.52 ^b	5.87 $\pm$ 0.14 ^a	7.14 $\pm$ 0.12 ^b	4.55 $\pm$ 0.08 ^c	9.11 $\pm$ 1.16 ^d	5.55 $\pm$ 0.18 ^a	1.72 $\pm$ 0.001 ^e
Height (cm)	2.33 $\pm$ 0.35 ^a	2.4 $\pm$ 0.26 ^a	2.47 $\pm$ 0.11 ^a	2.43 $\pm$ 0.49 ^a	2.33 $\pm$ 0.38 ^a	2.77 $\pm$ 0.15 ^a	2.30 $\pm$ 0.26 ^a	1.03 $\pm$ 0.47 ^b
Diameter (cm)	6.67 $\pm$ 0.25 ^{a,d,e}	6.97 $\pm$ 0.23 ^{a,d,e}	6.93 $\pm$ 0.11 ^{a,d,e}	6.77 $\pm$ 0.32 ^{a,d,e}	5.67 $\pm$ 0.51 ^b	7.20 $\pm$ 0.26 ^d	6.17 $\pm$ 0.30 ^e	3.60 $\pm$ 0.37 ^c

Results are represented as means $\pm$ SD; different letters indicate significant differences within the same line ($p < 0.05$); GrP—average weight of a fresh fruit, expressed in g.

Based on the results in Table 2, the percentage of moisture differs according to the variety of fruit studied. The highest moisture percentage was recorded by THRNFR and the lowest by OCHIT. All blackberry varieties showed high moisture percentages; however, the percentage of moisture was lower than in other research in the literature [37,38]. According to the US Department of Agriculture, in addition to other nutrients present in blackberries, a high-water content of approximately 88.2% has been reported [37]. In another study, the moisture content of blackberries was 87.92 ± 0.59 [38].

Agricultural techniques, climate (high temperatures), fruit development stage, size, surface-to-volume ratio, or external structure are only a few of the many factors that could influence fruit moisture. Even amongst cultivars of the same species, all the mentioned factors might result in significant humidity differences [39,40].

From the fresh juice obtained for each variety, the physico-chemical properties of blackberries, including pH, titratable acidity (%), and the amount of total soluble solids (TSS) expressed in °Brix, were determined and are shown in Table 3. Blackberries obtained from the spontaneous flora (WILD) and those grown in cultivation were both assessed for firmness immediately after harvest.

Table 3. The physico-chemical parameters (pH, acidity, firmness, and TSS content of eight different blackberry varieties).

Samples Parameters	CHES	T_C	EVRG	NAV	L_NESS	THRNFR	OCHIT	WILD
pH	3.27 $\pm$ 0.014 ^f	3.65 $\pm$ 0.012 ^d	2.85 $\pm$ 0.17 ^g	3.01 $\pm$ 0.012 ^e	3.84 $\pm$ 0.012 ^{b,c}	3.98 $\pm$ 0.008 ^{a,b,c}	4.066 $\pm$ 0.018 ^a	3.95 $\pm$ 0.014 ^{a,c}
Acidity (% malic acid)	6.21 $\pm$ 0.60 ^b	4.37 $\pm$ 0.30 ^d	7.86 $\pm$ 0.30 ^a	7.46 $\pm$ 0.6 ^a	3.18 $\pm$ 0.44 ^e	2.55 $\pm$ 0.44 ^e	2.53 $\pm$ 0.054 ^{e,f}	3.83 $\pm$ 0.12 ^{d,e}
Firmness	5.28 $\pm$ 1.06 ^a	5.33 $\pm$ 1.49 ^a	4.94 $\pm$ 0.58 ^a	8.56 $\pm$ 2.36 ^b	7.17 $\pm$ 1.85 ^a	5.23 $\pm$ 0.91 ^a	6.06 $\pm$ 1.22 ^a	5.78 $\pm$ 1.88 ^a
TSS	14.13 $\pm$ 0.05 ^d	14.33 $\pm$ 0.1 ^{c,d}	14.4 $\pm$ 0.08 ^c	13.85 $\pm$ 0.05 ^e	11.18 $\pm$ 0.09 ^f	12.2 $\pm$ 0.08 ^f	14.7 $\pm$ 0.18 ^b	18.18 $\pm$ 0.1 ^a

Results are represented as means $\pm$ SD; different letters indicate significant differences within the same line ($p < 0.05$); TSS—total soluble solids.

The cultivars EVRG and NAV had the lowest pH values, which were 2.85 ± 0.17 and 3.01 ± 0.01 , respectively, whereas THRNFR, WILD, and L_NESS had the highest pH values. The titratable acidity of blackberry fruits has been recorded to range from $2.53 \pm 0.44\%$ to $7.86 \pm 0.3\%$, depending on the cultivar. No significant differences were found in terms of firmness, with the exception of the NAV cultivar. Regarding the TSS content, statistically significant differences between the wild variety (WILD) and the cultivated ones were found (Table 3).

De Souza, et al., 2014, reported Brazilian blackberries' pH values of 2.99 ± 0.04 , acidity of $1.51 \pm 0.04\%$ and TSS of 10.17 ± 0.29 °Brix [38]. Petkovsek, et al., 2021, reported pH values between 2.7 and 3.2 for 'Loch Ness', 'Navaho' and 'Thornfree' blackberries (Slovenia), and TSS recorded values between 10.58 and 14.4 °Brix, for 'Loch Ness', between 10.74 and 13.30 °Brix for 'Navaho' and between 7.68 and 9.72 °Brix. Furthermore, the reported weight for the "Loch Ness" variety ranged from 4.85 to 6.9 g, while the weights of the "Navaho" and "Thornfree" varieties were respectively 4.84 and 7.45 g, and 5.26 and 7.07 g [14]. In a recent study, Garazhian, et al., 2020, investigated changes and interactions for fruit weight, total solid solubility, and titratable acidity in four species of Iranian-origin blackberries from the *Rubus* genus over the course of two years. Analysis has indicated that there was no significant difference between the data collected over a two-year period and the berries' weight, TSS, and titratable acidity values, which ranged from 0.14 to 1.30 g, 7.9 to 17.8 °Brix and 0.36 to 0.83%, respectively [41].

3.2. The Content of Total Phenols (TPh), Total Flavonoids (TFlav) in Blackberry Fruits and Monomeric Anthocyanins (MAP)

Blackberries are highly rich in phenolic acids, anthocyanins, and flavonoids [4,38,42], in addition to the typical components such as vitamins and minerals [43].

Figure 2a–c shows the results from the content of TPh, MAP, and TFlav of the blackberry taken in this study.

L_NESS and THRNFR fruits had the highest TPh content among the eight varieties tested (Figure 2a). They exhibited TPh contents of 1830.98 ± 13.55 and 1687.14 ± 62.41 mg GAE/100 g dw, respectively. The result with the lowest value was EYE 847.25 ± 54.26 mg GAE/100 g dw. According to Figure 2c, which compares the TFlav content of samples from eight different varieties of dehydrated blackberries, THRNFR had the highest content (1545.73 ± 77.15 mg QE/100 g dw), followed by sample L_NESS (1271.38 ± 25.03 mg QE/100 g dw), and CHES (1094.4 ± 62.98 mg QE/100 g dw). The OCHIT sample, with a reported value of 638.65 ± 8.84 mg QE/100 g dw, was found to have the lowest TFlav level. THRNFR recorded the highest MAP content (670.03 ± 29.98 mg cyanidin-3-glucoside (C3glu)/100 g dw.) among blackberries (Figure 2b). The lowest MAP content was recorded in NAV, with a reported value of 61.86 ± 1.32 mg C3glu/100 g dw. There were no statistically significant MAP differences ($p < 0.05$) between the WILD, EVRG, and NAV varieties, respectively (Figure 2b).

The TPh, TFlav, and MAP values recorded for dried blackberries were found to be higher than those reported for fresh fruit when compared to research in the literature. For instance, de Souza, et al., (2014) reported that the TPh content in blackberries was 850.52 ± 4.77 mg GAE/100 g f.w, the TFlav was 87.03 ± 4.85 mg CE/100 g f.w, and the MAP content was $58, 61 \pm 2.19$ mg C3glu/100 g of f.w [38].

The anthocyanin and total phenolic content of thornless blackberry varieties such as "Chester" and "Triple Crown" were reported by Wang and Lin in 2000. In comparison to unripe fruits (3.9 ± 1.1 mg C3glu/100 g dw and 7.1 ± 1.7 mg C3glu/100 g dw, respectively), the number of anthocyanins reported in the case of "Chester" was 912.5 ± 27.3 mg C3glu/100 g dw whereas that of "Triple Crown" was 794.6 ± 12.5 mg C3glu/100 g dw [42].

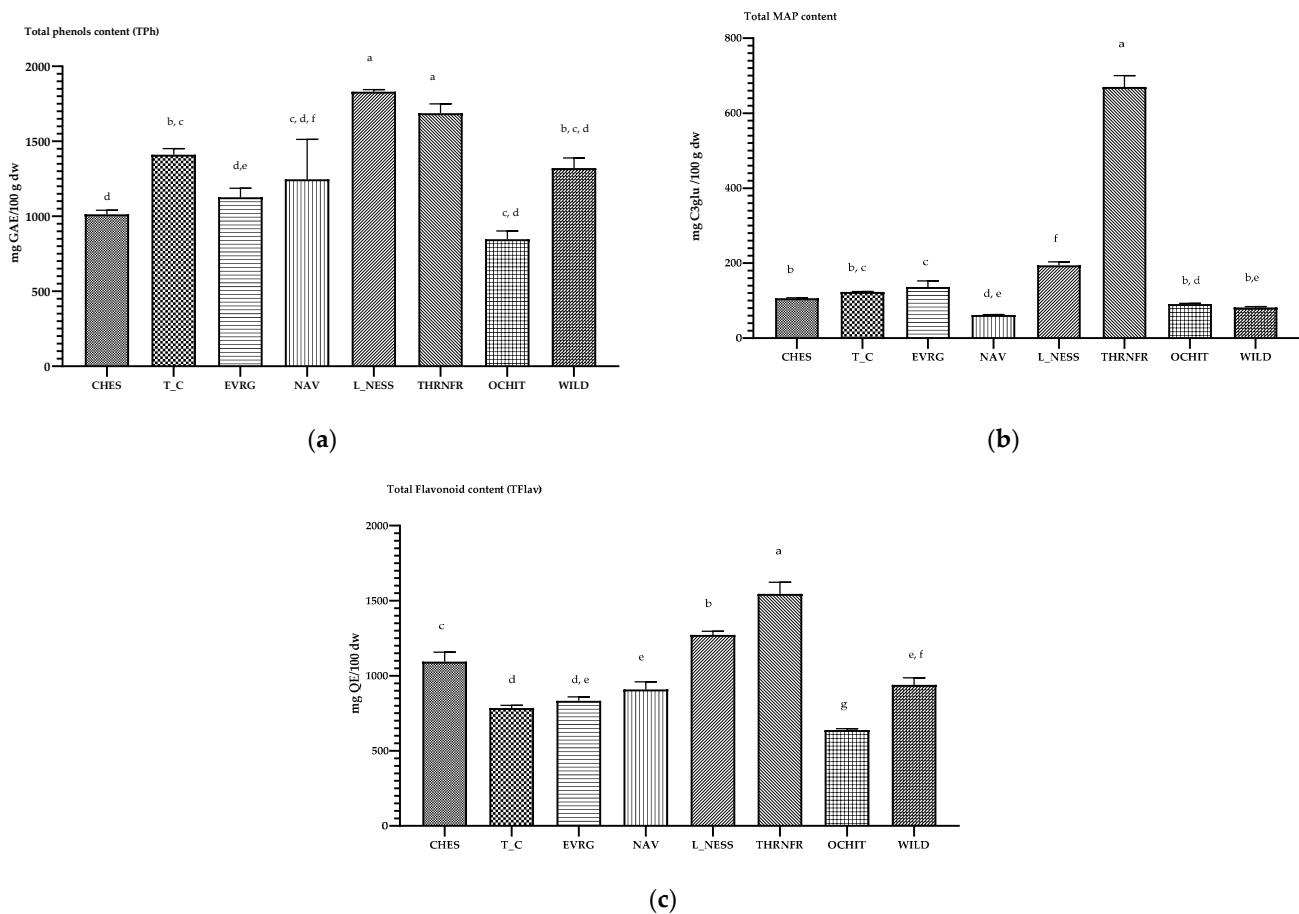


Figure 2. The bioactive compounds of blackberry varieties. (a) Total phenols content (TPh) expressed as milligram gallic acid equivalent (GAE)/100 g dry weight (dw); (b) total monomeric anthocyanin content (MAP) expressed as cyanidin-3-glucoside (mg C3glu/100 g dw.); and (c) total flavonoid content (TFlav) expressed as milligrams of quercetin equivalent (QE)/100 g dw. All results represent the mean $\pm$ SD of triplicate analysis. Different letters indicate a significant difference ($p < 0.05$) between varieties.

Also, Kolniac-Ostek, et al., 2015, studied five thornless blackberry cultivars and five thorny blackberry cultivars, reporting that thornless blackberry fruits had higher anthocyanin content (mean = 171.23 mg/100 g fw.) compared to spiny varieties [44].

In another study, Sariburun, et al., 2010, reported the number of polyphenols (2786.8 ± 21.9 mg GAE/100 g fw.) and flavonoids (82.2 ± 1.3 mg CTE/100 g fw.) from the methanolic extract from fresh fruits of *R. fruticosus* [45]. Our findings are consistent with those in the literature [20] regarding the total amount of phenolics and anthocyanins in *R. laciniatus* ('Evergreen').

There are currently limited investigations of the phytochemical profile and information on the antioxidant capacity of blackberry fruits grown from the *R. fruticosus* and *R. laciniatus* species, with significant differences in their TPh, TFlav, or MAP contents [14,17,20,46–49]. Differences in the content of bioactive compounds present in fruits obtained for the same cultivar or for varieties belonging to the same genus can arise from various factors, such as cultivation techniques, the difference in climatic conditions (temperature, solar radiation, precipitation), soil composition, and the different methods of extraction of bioactive compounds [50–52].

3.3. Anthocyanins Identified from Blackberry Fruits by HPLC-PDA

Anthocyanin pigments are responsible for the color of the fruit; specialized studies have also highlighted the role of these compounds in the prevention and treatment of

numerous ailments [37,48,53]. Therefore, the anthocyanins from the eight varieties of dehydrated blackberry fruits were isolated by SPE and analyzed by HPLC-PDA; the quantitative results are presented in Table 4.

Table 4. Anthocyanins from eight blackberry cultivars separated and quantified (mg cyanidin equivalent/g dw) by HPLC-PDA.

SAMPLES	CHES	T_C	EVRG	NAV	L_NESS	THRNFR	OCHIT	WILD
Cyanidin-3-glucoside	7.51 ± 0.17 _a	58.73 ± 4.34 _{b,c}	54.48 ± 4.01 _{c,f}	13.54 ± 0.88 _{a,f}	87.76 ± 5.74 _d	329.26 ± 7.64 _e	21.48 ± 1.71 _a	43.99 ± 3.15 _f
Cyanidin-3-O-arabinoside	1.45 ± 0.1 _{a,f}	1.02 ± 0.1 _a	5.69 ± 0.19 _{b,e}	2.34 ± 0.02 _f	8.04 ± 0.60 _c	17.40 ± 0.09 _d	1.54 ± 0.01 _{a,f}	5.87 ± 0.39 _e
Cyanidin-3-O-(malonyl)glucoside	0.64 ± 0.04 _f	1.21 ± 0.01 _e	2.04 ± 0.02 _c	1.69 ± 0.01 _d	3.46 ± 0.02 _b	12.83 ± 0.09 _a	nd	nd
Cyanidin-3-O-(dioxalyl)glucoside	0.75 ± 0.001 _g	1.81 ± 0.01 _e	3.39 ± 0.02 _c	nd	5.58 ± 0.03 _b	17.98 ± 0.13 _a	1.05 ± 0.01 _f	2.37 ± 0.04 _d
Cyanidin-3-rutinoside	0.93 ± 0.0001 _{a,g}	2.89 ± 0.02 _b	4.34 ± 0.02 _c	3.33 ± 0.02 _d	17.76 ± 0.13 _e	14.92 ± 0.11 _f	0.84 ± 0.002 _g	2.13 ± 0.01 _h
TOTAL	11.28 ± 0.07 _a	65.67 ± 1.93 _{b,c,f}	69.95 ± 1.80 _{c,f}	20.84 ± 0.39 _a	122.60 ± 2.49 _d	392.42 ± 3.37 _e	24.92 ± 0.76 _a	54.36 ± 1.37 _f

Values are presented as mean ± SD. Different lowercase letters indicate a significant difference ($p < 0.05$) between harvest stages. Rt—Retention time; dw—dry weight; nd—not detected.

Cyanidin-3-glucoside was the main anthocyanin found in all blackberry cultivars (Table 4); THRNFR cultivar detected the greatest quantity, 86.64%, more than in the wild blackberry (WILD), and CHES found the lowest amount, 7.51 ± 0.21 mg cyanidin equivalent/g dw. These findings are consistent with previous research that indicate cyanidin 3-glucoside is the main anthocyanin in blueberry fruits [13,15]. The cultivar L_NESS reported the highest level of cyanidin-3-rutinoside, contributing to 14.51% of all the anthocyanins found in this cultivar and being 88.01% greater than the WILD, while cultivar OCHIT recorded the lowest concentration. Anthocyanins such as cyanidin-3-O-(dioxalyl) glucoside were not found in the cultivar NAV, while cyanidin-3-O-(malonyl) glucoside was not found in the cultivar OCHIT or wild blackberries (WILD). All known blackberry varieties included cyanidin-3-O-arabinoside, although T_C had the lowest concentration (82.62% less than the wild variety).

Blackberries contain anthocyanins in glycosylated form, which are responsible for the purple, blue, or red colors [15,37]. Approximately 94% of the anthocyanins present in blackberries are presented in non-acylated form, of which approximately 90% are monoglycosides, while approximately 10% are found as diglycosides [37,54,55]. Different studies in the specialized literature have demonstrated that the anthocyanins present in blackberry fruits are derived from cyanidin, and anthocyanins such as cyanidin 3-glucoside, cyanidin-3-O-arabinoside, cyanidin-3-O-(malonyl)glucoside, cyanidin-3-O-(malonyl)glucoside, and cyanidin-3-rutinoside, have been identified [56–59]. Blackberry cyanidin derivatives may vary according to the variety, environment, cultivation region, and fruit age, but primarily due to genetic variations [37,57,60]. According to research by Cho, et al., (2004), the distribution of cyanidin aglycones differed with variety, ranging from 75% to 84% for cyanidin 3-glucoside, 1% to 12% for cyanidin 3-rutinoside, 4% to 8% for cyanidin-3-O-(dioxalyl)glucoside, 3% to 8%, and 2% to 3% for cyanidin-3-O-(malonyl)glucoside [59].

Many studies have shown that blackberry anthocyanins are powerful antioxidants [4,13,61]. The color of anthocyanins depends on their structure, the acidity of the environment, and the presence of copigments [37,62]. Blackberries produce more anthocyanin pigments when they ripen, and the color of these pigments varies depending on the class to which they belong [15,48,63–65]. Many variables—including the variety, the agronomic techniques used in cultivation, the maturity stage of collection, as well as the geological and climatic conditions of the area from which the fruits are harvested—affect the color or

the natural pigments present in blackberries; therefore, these parameters are important to study [15,63–65].

3.4. Antioxidant Capacity of Blackberry Fruits

Blackberries are a good source of natural antioxidants. The total amount of phenols, anthocyanins, and flavonoids in blackberries influences their antioxidant capacity [4,38]. Numerous methods have been developed for in vitro measurement of the antioxidant capacity of natural products [16,46]. Three distinct assays were used in this work to measure the antioxidant capacity of extracts from seven cultivated blackberry cultivars and one from the wild flora. The results are presented in Figure 3a–c.

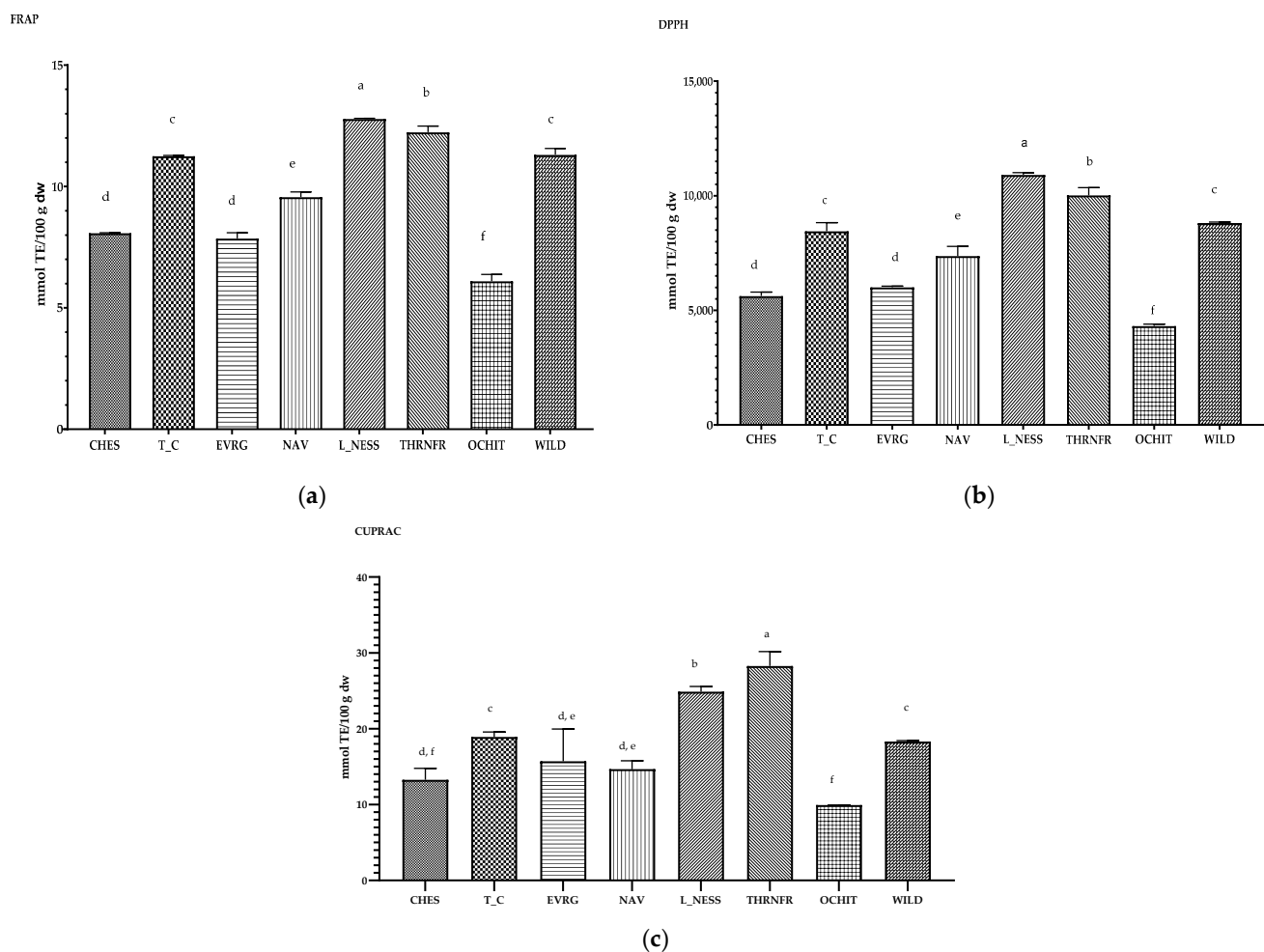


Figure 3. The antioxidant capacity of eight blackberry varieties determined by three different methods; (a) FRAP (ferric reducing ability of plasma) expressed in mmol Trolox Equivalents (TE)/100 g dw; (b) DPPH (2,2, diphenyl-picryl-hydrazyl) expressed in mmol TE/100 g dw; and (c) CUPRAC (Cupric Ion Reducing Antioxidant Capacity) expressed in mmol TE/100 g dw. All results represent the mean $\pm$ SD of triplicate analysis. Different letters indicate a significant difference ($p < 0.05$) for each variety.

Methods based on single electron transfer (SET), including FRAP, DPPH, and CUPRAC, are used to measure antioxidant activity [24,66,67].

Significant differences were obtained between the eight blackberry varieties in terms of antioxidant capacity. The highest value obtained in the case of the FRAP method, and the DPPH test, was in the case of the L_NESS blackberries (12.79 ± 0.02 and, respectively, $10,962.65 \pm 88.96$ mmol TE/100 g dw), followed by THRNFR and WILD. The antioxidant

capacity of blackberries from the spontaneous flora (WILD) determined, both by the FRAP test (11.32 ± 0.26 mmol TE/100 g dw) and DPPH (8818.5 ± 35.6 mmol TE/100 g dw), recorded significantly higher values, compared to other varieties presented in Figure 3a,b. On the other hand, the CUPRAC method's results showed that the THRNFR blackberry cultivar had the maximum antioxidant capacity (28.27 ± 1.89 mmol TE/100 g dw), followed by L_NESS (24.88 ± 0.69 mmol TE/100 g dw) (Figure 3c). The OCHIT blackberry cultivar has the lowest antioxidant capacity, as measured by FRAP (6.1 ± 0.28 mmol TE/100 g dw), DPPH (4321.11 ± 80.06 mmol TE/100 g dw), and CUPRAC (9.93 ± 0.03 mmol TE/100 g dw.).

The FRAP method was initially used to evaluate the antioxidant capacity of plasma, and later, it was tested on fruits and successfully used to evaluate the antioxidant-reducing power of different extracts [33]. Siriwoharn and Wrolstad 2004, tested the antioxidant capacity by the FRAP method, both from the whole fruits of *R. laciniatus*, variety 'Evergreen', as well as from seeds and seedless fruits. The highest antioxidant capacity was reported in the seeds (197 ± 163 μ mol TE/g), about three times higher than in the whole fruits of the cultivar 'Evergreen' [20]. The antioxidant capacity of blackberry fruits varies greatly depending on the area, climatic conditions, variety, fruit maturity stage, extraction solvents, and methods used [4,20,42].

In recent years, various studies have demonstrated that the high level of natural antioxidants in blackberry fruits have multiple benefits for human health, thus considerably increasing the interest in the cultivation and development of as many varieties as possible [3,11,48,68–70].

Heinonen, et al., 1998, demonstrated, in vitro, that berries of various varieties, including blackberry, have remarkably high scavenging activity against chemically generated superoxide radicals [71]. Additionally, the antioxidant capacity measured for the fresh fruits of *R. fruticosus* by the DPPH method recorded values between 7.19 ± 0.06 μ mol TE/g fw. [47] and 177.11 ± 3.17 μ mol TE/g fw. [45]. Another study by Suriburum, et al., (2010) investigated the antioxidant capacity of blackberries using the CUPRAC method, and the results showed a value of 127.15 ± 0.57 mol TE/g fw. [45].

The seven blackberry cultivars that were harvested from the same region were characterized by a varied number of bioactive compounds from the polyphenol class and, in particular, by a different profile and content of anthocyanins. There are several factors that could be responsible for these variations, but genetic factors are highly relevant for the content of these secondary metabolites [72].

3.5. Multivariate Analysis

Table 5 shows the correlation matrix between physico-chemical and biochemical parameters, including the content of total phenols, flavonoids, and monomeric anthocyanins. Physical parameters correlation exhibits very low values (i.e., weak correlation) and only a limited value of statistically significant correlations ($p = 0.05$). A strong correlation is encountered between pH and Acidity parameters (an expected result) and is statistically significant. From these results, pH with Acidity presents statistically significant strong and negative correlations; also, MAP with TPh, TFLAV and all antioxidant capacities present statistically significant average and positive correlations. Furthermore, between MAP and C3glu, C3Oara, C3Omal, C3Oodio, and C3Orut, there are statistically significant strong and positive correlations. However, statistically significant strong correlations are performed between all biochemical parameters.

Table 5. Correlation matrix for physico-chemical and biochemical parameters, as Pearson coefficient. Boldface numbers denote statistically significant correlations ($p = 0.05$).

R	pH	Acid	Firm	TPh	TFlav	TSS	MAP	FRAP	DPPH	CUPRAC	Moisture	C3glu	C3Oara	C3Omal	C3Odio	C3Orut
pH	1.000	−0.961	−0.124	0.290	0.228	0.022	0.357	0.341	0.349	0.446	−0.176	0.394	0.342	0.254	0.389	0.313
Acid	−0.961	1.000	0.107	−0.347	−0.288	0.124	−0.441	−0.351	−0.374	−0.499	0.086	−0.469	−0.403	−0.344	−0.467	−0.413
Firm	−0.124	0.107	1.000	0.128	0.092	−0.178	−0.154	0.213	0.210	0.059	0.009	−0.145	−0.072	−0.068	−0.150	0.168
TPh	0.290	−0.347	0.128	1.000	0.696	−0.505	0.549	0.916	0.952	0.920	0.323	0.603	0.648	0.596	0.605	0.849
TFlav	0.228	−0.288	0.092	0.696	1.000	−0.560	0.804	0.662	0.691	0.818	0.704	0.780	0.833	0.823	0.813	0.798
TSS	0.022	0.124	−0.178	−0.505	−0.560	1.000	−0.494	−0.281	−0.356	−0.472	−0.526	−0.447	−0.396	−0.567	−0.469	−0.749
MAP	0.357	−0.441	−0.154	0.549	0.804	−0.494	1.000	0.468	0.497	0.746	0.783	0.988	0.919	0.983	0.987	0.680
FRAP	0.341	−0.351	0.213	0.916	0.662	−0.281	0.468	1.000	0.984	0.913	0.173	0.541	0.573	0.503	0.525	0.709
DPPH	0.349	−0.374	0.210	0.952	0.691	−0.356	0.497	0.984	1.000	0.934	0.235	0.568	0.629	0.539	0.563	0.793
CUPRAC	0.446	−0.499	0.059	0.920	0.818	−0.472	0.746	0.913	0.934	1.000	0.431	0.797	0.806	0.765	0.795	0.862
Moisture	−0.176	0.086	0.009	0.323	0.704	−0.526	0.783	0.173	0.235	0.431	1.000	0.743	0.785	0.845	0.770	0.553
C3glu	0.394	−0.469	−0.145	0.603	0.780	−0.447	0.988	0.541	0.568	0.797	0.743	1.000	0.939	0.977	0.993	0.700
C3Oara	0.342	−0.403	−0.072	0.648	0.833	−0.396	0.919	0.573	0.629	0.806	0.785	0.939	1.000	0.923	0.963	0.780
C3Omal	0.254	−0.344	−0.068	0.596	0.823	−0.567	0.983	0.503	0.539	0.765	0.845	0.977	0.923	1.000	0.975	0.729
C3Odio	0.389	−0.467	−0.150	0.605	0.813	−0.469	0.987	0.525	0.563	0.795	0.770	0.993	0.963	0.975	1.000	0.738
C3Orut	0.313	−0.413	0.168	0.849	0.798	−0.749	0.680	0.709	0.793	0.862	0.553	0.700	0.780	0.729	0.738	1.000

C3glu—Cyanidin-3-glucoside; C3Oara—Cyanidin-3-O-arabinoside; C3Omal—Cyanidin-3-O-(malonyl)glucoside; C3Odio—Cyanidin-3-O-(dioxalyl)glucoside; C3Orut—Cyanidin-3-rutinoside.

In this study, sixteen parameters were measured, which can be gathered for each sample as a multivariate profile. The profiles were compared with a multivariate statistical sequence, with the purpose of extracting the multivariate correlations between the parameters and the samples and the grouping of the samples (i.e., the clustering). The multivariate statistical sequence consisted of principal component analysis (PCA), linear discriminant analysis (LDA), multivariate analysis of variance (MANOVA) ($p = 0.05$), and hierarchical cluster analysis (HCA). Among these statistical methods, only MANOVA can perform results with statistical significance ($p = 0.05$); in consequence, all the multivariate results and the corresponding conclusions will have 95% accuracy.

Results of the PCA are shown in Table S1 and Figure 4. The total variance of principal components with an eigenvalue higher than unity are PC1–PC3, with a value of 87.27%. For biological systems, this value is satisfactory for drawing the statically proper conclusions. However, the analysis that follows will consider all the principal components for the calculation of the LDA and MANOVA results. Principal component results represented in the PC1–PC3 principal axis frame as 2D and 3D biplots contain both spatial distribution of the variables and samples (Figure 4). The variables distribution is represented with vectors starting from the principal axes' frame center point. The vectors end points out the direction with the highest content of the corresponding variable. The opposite direction points out the direction with the lowest content of the corresponding variable. In consequence, the samples that present their geometric projection on one vector end will perform high content of the corresponding variable, and vice versa.

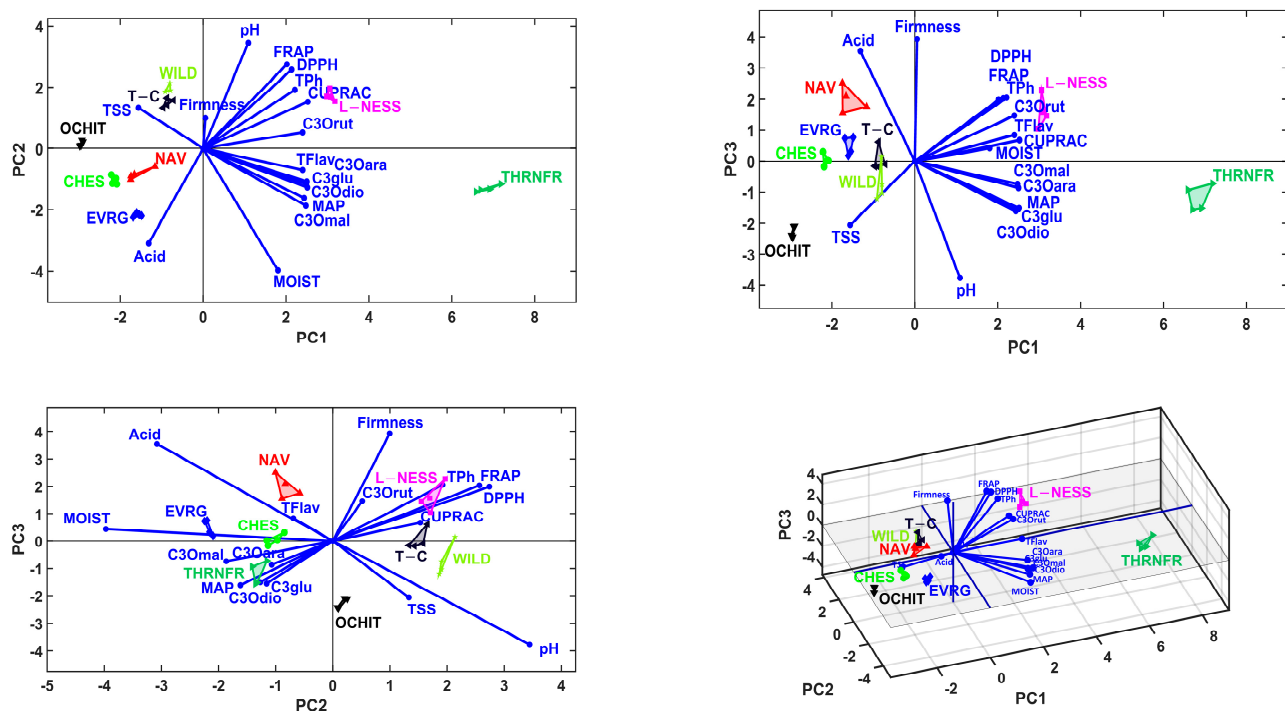


Figure 4. Principal component analysis (PCA) results represented as 2D and 3D biplot graphs.

Geometrical variable vector arrangement can prescribe the multivariate correlations between the variables and, furthermore, the variable grouping. Biplots from Figure 4 suggest five singleton groups: Firmness, Moisture, Acidity, TSS pH, and C3Orut, and two multiple variable groups. The first multiple variables group consists of FRAP, DPPH, TPh, and CUPRAC; the second group consists of TFlav, MAP, C3glu, C3Oara, C3Omal, and C3Odia. These variable groups determine the samples' spatial distributions. Cultivar samples THRNFR and L_NESS have high levels of antioxidant capacity (FRAP, DPPH, TPh, and CUPRAC) together with TFlav, TPh, MAP, C3glu, C3Oara, C3Omal, and C3Odia, also for Moisture, pH, and medium level for Firmness. Cultivars OCHIT, WILD, and T_C

perform high levels of TSS and pH, but with the lowest levels of the other variables. Cultivar samples EVRG, CHES, and NAV perform high levels for variables Acidity, Firmness, and Moisture, but with the lowest levels of the other variables (see Figure 4). In order to determine that this kind of samples grouping (i.e., based on PCA results) generates the same clusters, the LDA, MANOVA ($p = 0.05$) and HCA statistical multivariate methods were applied to the principal coordinates of the samples (i.e., the PC of the samples consisted as input data for mentioned methods).

The results of the effective clustering method are shown in Table S2 and Figure 5. The LDA method generates the canonical coordinates that generally maximize the Euclidean distances between the samples (Figure 5). These canonical coordinates are used by MANOVA ($p = 0.05$) to calculate the statistical significance values from sample profiles pairwise comparisons (Table S2). As it can be noticed, all these values are higher than the $p = 0.05$, a fact that validates each sample to be a singleton cluster.

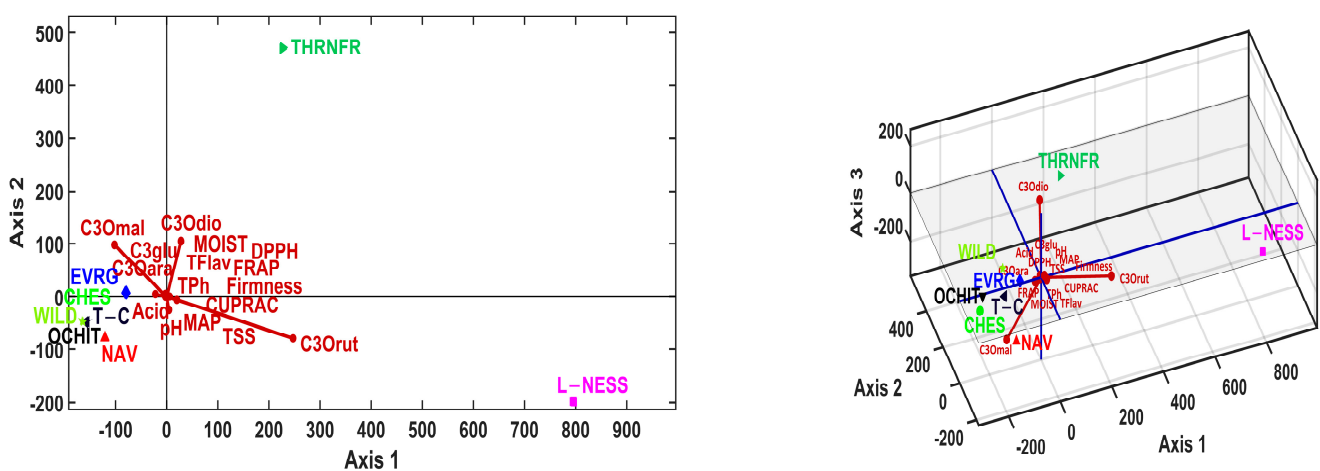


Figure 5. Linear discriminant analysis (LDA) results represented as 2D and 3D biplot graphs.

The heatmap emphasizes that by choosing only the first three canonical axes (e.g., the first three principal axes), the clustering conclusions are still valid (Figure 6, from Axis 4, the black color means null contributions to the clustering process).

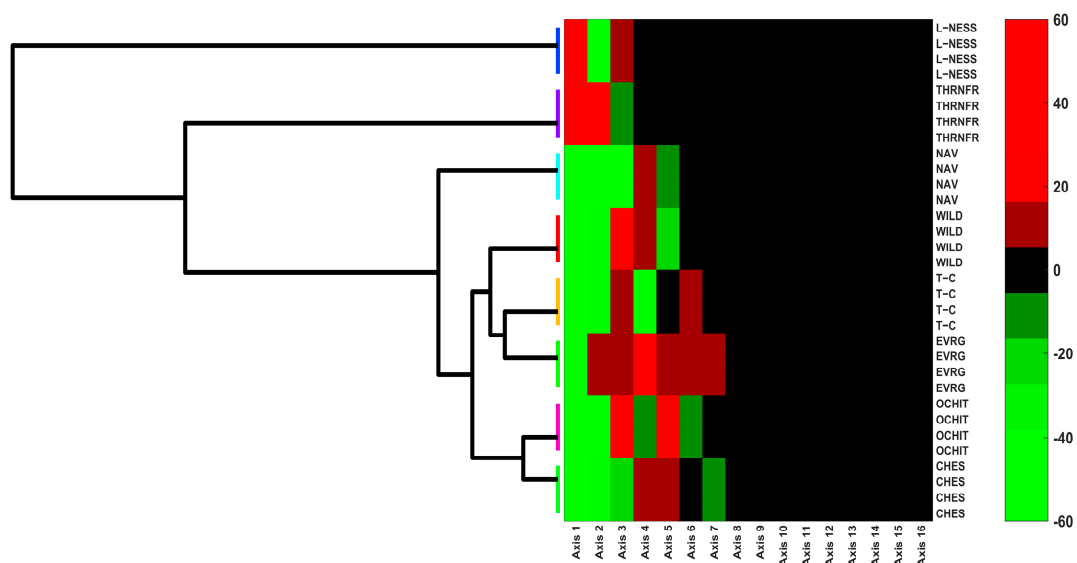
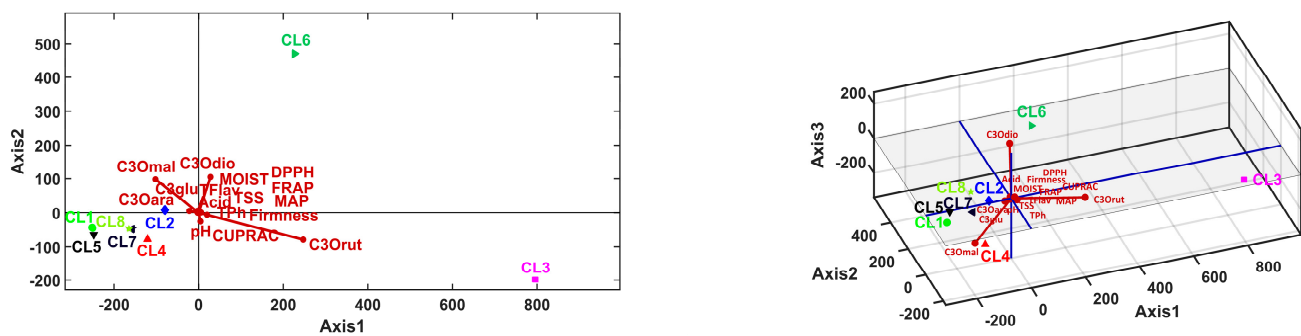


Figure 6. Graphical clustering information of analyzed samples organized as a heatmap.

The graphical LDA biplots with clustering information are displayed in Figure 7.



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