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TOTAL POLYPHENOLS AND ANTIOXIDANT CAPACITY IN DIFFERENT VARIETIES OF *CORYLUS AVELLANA* L. MICRO-PROPAGATED *IN VITRO*

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ABSTRACT

Forest hazelnuts are very valuable fruits, both for their phytochemical content and economically for cultivation. In this work, the development of some hazelnut varieties, such as Tonda Gentile Delle Longue, Butler, Tonda Romana, Tonda di Giffoni, and Barcelona, has been studied. Morphological characteristics were analysed in two periods, at 30 days and 60 days, "in vitro," by recording the number of roots and leaves as well as their length. From the analyses carried out, it was found that the genetic variation of the five varieties studied had a significant influence on all the morphological characteristics examined and the varieties Tonda di Giffoni and Barcelona show higher plasticity under in vitro conditions compared to the other varieties included in the study. In addition, total polyphenol (TPh) content and antioxidant capacity were analysed using FRAP and DPPH assays and the results reported that the Tonda di Giffoni cultivar recorded the highest TPh content (192.71±0.77 mg GAE/100 g Fw), with high antioxidant capacity (FRAP = 5.9±0.02 and DPPH = 216.31±1.38 mol TE/100 g Fw), compared to the other varieties studied.

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Introduction

The hazelnut (*Corylus avellana L.*), native to Asia Minor, belongs to the Betulaceae family and is believed to be one of the oldest plants cultivated by man, being among the main food components of our ancestors. They were then spread and cultivated in many countries, such as Turkey, Italy, Azerbaijan, USA, Spain, Portugal, Georgia, and France, as well as in northern hemisphere regions with a temperate climate [1, 2]. The interest in growing hazelnut trees is due to the characteristics of their fruit, but also because they are long-lived shrubs, with an average lifespan of between 80 and 90 years [3, 4]. The world's demand for hazelnuts is growing. In the last 10 years, the value of shelled hazelnuts on the world market has been €7.8/kg. Romania imports 90% of its hazelnut requirements, making the hazelnut market an attraction for any fruit grower. The average annual production of hazelnuts in the world is about one million tonnes [5], an increase of 35% compared to 2000. Hazelnuts are oilseed fruits encased in a woody shell and are currently one of the most economically important oilseed crops in the world, which has led to the emergence of over 400 varieties [2, 6]. They have a high germination capacity and can be propagated both by seed and vegetatively. One of the common methods of vegetative propagation is by shoots or cuttings [1, 2]. Also, *in vitro* vegetative propagation of Corylus avellana is a more recently used propagation method in Europe and Romania, this guarantees quality material, and plants that are identical to the original biological material. It is a rapid multiplication method with high production volume that can be practiced throughout the year resulting in a large number of quality offspring with a low level

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of microbial contamination. This method is used to optimize quality and productivity. Historically, most hazelnut orchards are planted from seed or stump material, these methods are slower and have a phytosanitary risk. After the multiplication and *in vitro* growth stage, the plants are transferred for rooting in perlite or special pea and then transferred to greenhouses in pots or pots of various sizes. This stage depends on environmental factors, light, humidity, and temperature, under optimal conditions [7, 8].
The methods of propagation, cultivation, and maintenance of the plants are also important factors for the evolution of bioactive compounds in the fruit, such as polyphenols and flavonoids. Polyphenols are among the most studied classes of antioxidants, widely distributed in the plant kingdom, and play an essential role in our diet. Phenols are products of secondary metabolism in plants, which provide essential functions in plant reproduction and growth, act as defense mechanisms against pathogens, parasites, and predators, and also contribute to plant color. In addition to their role in plants, several epidemiological and clinical studies have demonstrated that phenolic antioxidants occurring in plants or fruits are the main contributors to lowering the incidence of several chronic and degenerative diseases [9-13].
The objective of this study focused on the *in vitro* vegetative multiplication of five varieties of Corylus avellana L. (Tonda Gentile Delle Longue, Butler, Tonda Romana, Tonda di Giffoni, Barcelona), varieties that are frequently cultivated in the north-western area of Romania (Bioplant Arad). In addition, the total polyphenol content and antioxidant capacity of the five hazelnut varieties were investigated. Statistical analysis was used to identify the most valuable hazelnut variety in terms of total polyphenol levels and antioxidant capacity.

Materials and Methods

Description of the Biological Material
Tonda Gentile Delle Longue, originally from the NW area of Italy [14] **(Figure 1a)**, is of medium-high vigour, very productive, with small, spherical fruits, grouped by 2-4, with 42-47% core. The ripening stage is between the end of August and the beginning of September. The shrub is moderately vigorous with a semi-upright crown shape. Pollen is released very early, but flowering is late. Buds open early. The shell is larger than the core and the nut falls free from the shell. Appreciated in the chocolate industry, because of its small, round core, whitening capacity, and excellent taste.
The Butler variety, which comes from the NE area of the USA [15] **(Figure 1b)**, is a vigorous variety, with resistance to bacteriosis, with large fruit that contains 47-49% core and ripens at the end of September. It peels itself, and the fall of the nut takes place over a long period of time. This variety shows sensitivity to mana and large mites. It releases pollen for a long time early in the season, but the female flowers are late. It is highly productive with a biannual production trend.
Tonda Romana is an Italian variety from the V zone [16] **(Figure 1c)**, it has a ripening period towards the end of August. Fruits grouped by 2-4, rarely up to 6, fairly large (2.2-2.7g), spherical-flattened with three angular, prominent ribs, have a pronounced and irregularly convex base, sometimes flat, slightly wavy. The shell is suitably thick and durable. The core represents 44-48% of the weight of the hazelnut, has a light-brown skin, and is valued in the sweets industry (chocolate). The plant is medium-vigorous, with very strong stemming, has frost-sensitive buds, and is precocious and suitably productive.
The variety Tonda di Giffoni [17] **(Figure 1d)**, is a variety with a straight crown, of medium vigour, which comes from the SW area of Italy. The medium-sized, semi-round (1.2g) hazelnut kernel weighs 2.5g and has a high yield when shelled (46%). Productivity is high and constant at 3-3.5t/ha. Ripening takes place at the beginning of September, and suitable pollinators for this variety can be other varieties of linden, such as Tonda Gentile Romana, Camponica, or San Giovanni.
Barcelona variety **(Figure 1e)** comes from the NE area of Spain [18], it ripens at the end of August and the beginning of September. The fruits are grouped in groups of 2-5, large and very large (2.8-4.5g), spherically flattened, with a slightly convex base. The bark, fairly thick, has a chestnut colour, with darker, thin longitudinal stripes and a fine pubescence in the upper half. The core holds 39-46% of the weight of the hazelnut, it is fine, crunchy, intensely aromatic, and superior for the table, and about 10% of the fruits have double seeds. It drains quite strongly, is resistant to frost, and is very productive.

a)

b)

c) d) e)

Figure 1. The appearance of the five hazelnut varieties studied; a) Tonda Gentile Delle Longue; b) Butler; c) Tonda Romana(Roma); d) Tonda di Giffoni; e) Barcelona.

In Vitro Culture

To prepare the culture medium, Murashige and Skoog, 1962 (MS) salts supplemented with 3% sucrose and 0.82% agar (Mermaid TM) were used. The pH was adjusted to 5.8 with NaOH for 20 min. The cultures were maintained in a culture room at an air temperature of 25 ± 1˚ C in the presence of light. The composition of the culture medium is one of the most important factors that contribute to the success of meristem culture. Each species and sometimes even each variety requires a special medium, and the choice of the nutrient medium is based on the results obtained over time, published in the specialized literature, in general, the already existing basic mediums, such as MS, are used [19].

To determine morphological characters, root number (NR), leaf number (NF), root length (LR), and leaf length (LD) were measured at two time periods, 30 days and 60 days. The experiment was conducted in 5 replicates for all 5 varieties.

Phytochemical Characterization

Furthermore, in a recent study, total polyphenol content and antioxidant capacity were determined using DPPH and FRAP assays. The investigations mainly focused on the differences obtained from the core of the 5 hazelnut varieties.

Extract Preparation

In the first stage, the hazelnuts of the 5 varieties were peeled, and the extracted core was grated, obtaining a fine powder. From the obtained powder, one gram of each variety was weighed, over which 10 mL of 80% ethanol was added [20]. The samples were placed on the magnetic stirrer (IKA C-MAG HS) for one hour, after which they were transferred into test tubes and centrifuged (HETTICH Universal 320). Finally, the ethanolic extract was collected, and total polyphenols content and antioxidant capacity were measured

Determination of the Content in Total Polyphenols

The total polyphenol content of hazelnut kernels was determined using the Folin-Ciocalteu method with some modifications [20, 21]. Briefly, the hazelnut kernels ethanolic extract (0.1 mL) was incubated with 1.7 mL of distilled water, 0.2 mL of Folin-Ciocalteu reagent (1:10, v/v), and 1 mL of 7.5% Na_2CO_3 solution for 2 hours in the dark, at room temperature. The absorbance was measured at 765 nm (Shimatzu UV-1700 spectrophotometer) and the results are expressed as milligrams of gallic acid equivalents (GAE) per 100 grams of fresh weight (Fw) using gallic acid as a standard.

Determination of Antioxidant Capacity

The FRAP assay was determined according to the method of Benzie and Strain, 1996 [22]. The ethanolic extract obtained from hazelnut kernels (0.1 mL) was allowed to react with 0.5 mL FRAP working solution and 2 mL distilled water, for 1 h, in the dark. The results were expressed as molTrolox equivalent (TE)/100 g Fw.

The radical scavenging capacity of ethanolic extract obtained from hazelnut kernels using the stable 2-picryl-hydrazyl-hydrate (DPPH) radical was determined according to the method of Brand Williams [23]. A volume of 0.1 mL of hazelnut kernel extract was mixed with 2.8 mL of 80µM DPPH solution and stored at room temperature in the dark. The reduction of DPPH was monitored spectrophotometrically, after 30 minutes, at 517 nm and the antioxidant capacity was expressed as molTE/100 g Fw

Statistical Calculation

For statistical analyses, the ANOVA test was used to determine and analyse the variance for both morphological characteristics and bioactive compounds obtained from the 3 methods (TPh, FRAP, DPPH). Tukey test was also performed to determine the degree of difference between their characteristics, but also between the bioactive compounds and the antioxidant capacities of the hazelnut varieties included in the study. R studio and Microsoft Excel software were used for statistical calculations.

Results and Discussion

The analyses, showed a significant influence of different types of hazelnut varieties on different morphological characters such as root number (NR), leaf number (NL), root size (RD), and leaf size (LD). This showed the impact of genetic variation, which

is manifested by an increase in these values. In the 30-day period, variety has a significant influence on root number and size ($p < 0.05$). In contrast, in this period of *in vitro* culture, there is an insignificant increase in leaf size and number ($p > 0.05$), which are not influenced by variety type.

Also, the interaction between root number (NR) and root size (RD), as well as the interaction between leaf number (NL) and leaf size (LD), shows a significant impact ($p < 0.05$). In contrast, the results indicate a significant interaction between growth factors such as root number and leaf size, as well as between leaf number and root size ($p < 0.05$). This shows the importance of developing an adequate root system for the development of significant leaf area in *in vitro* micropropagation at this stage. On the other hand, no significant interaction was found between leaf number and leaf size, as these morphological characteristics had no reciprocal influence in the alum varieties analyzed ($p > 0.05$). In addition, the analysis of variance shows a high significance in the interaction between all the factors considered in the study (NR*DR*NL*DL), with a significance value of $p < 0.05$. From the results, it can be concluded that there is indeed an influence of varietal variability in *in vitro* micropropagation. The interactions between morphological traits in the results demonstrate how associated these morphological traits are with each other determining the optimization of an efficient *in vitro* breeding protocol by stimulating the growth and development of the most adapted varieties with high genetic potential. Already at this stage of observation (30 days) the growth capacities of the hazelnut varieties can be observed.

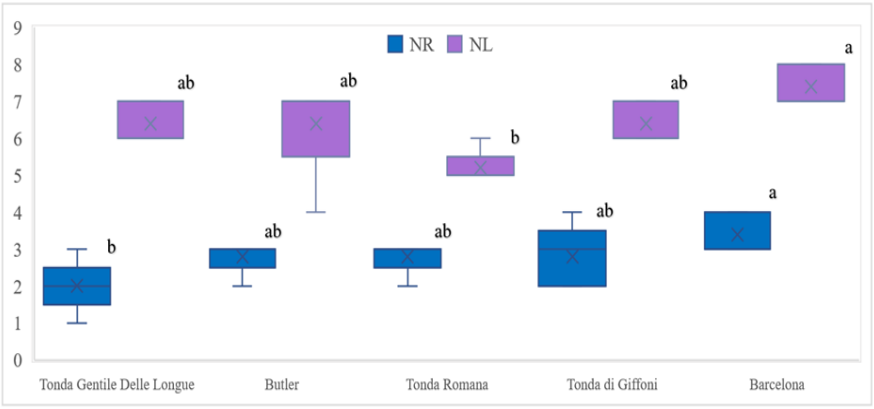

Figure 2. Boxplot representation of the number of roots (NR) and leaves (NL) formed per explant in the *viticulture* of *Corylus avellana* L. varieties after 30 days.

During the 30 days, as can be seen in **Figure 2**, when growing the explants on the "*in vitro*" culture medium, in terms of the characteristic of the number of roots/explant, it was found that the lowest number was recorded in the variety Tonda Gentile Delle Longue and the highest in the variety Barcelona. The values obtained after the first period of 30 days **(Figure 2)** and the statistical calculations place Butler, Tonda Romana, and Tonda di Giffoni varieties as similar in terms of number of roots (NR), the differences between these varieties being insignificant ($p > 0.05$), having significantly higher values than Tonda Gentile Delle Longue ($p < 0.05$) and significantly lower than Barcelona ($p < 0.05$).

Analysing the leaf number (NL) characteristics of explants grown *in vitro* during the first observation period, the lowest values were recorded in the variety Tonda Romana (Roma) and the highest value in the variety Barcelona. Intermediate values, with non-significant differences ($p > 0.05$), were observed in Tonda Gentile Delle Longue, Butler, and Tonda di Giffoni, with the values of these varieties for leaf number (NL) significantly exceeding those of Tonda Romana (Roma) and being below those of Barcelona.

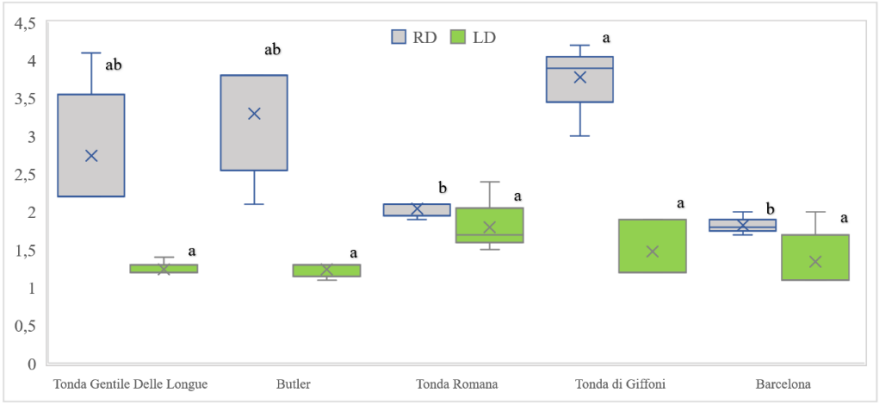

Figure 3. Boxplot representation of the roots dimension (RD) and leaves area (LA) growth per explant in the *in vitro* culture of *Corylus avellana* L. varieties after 30 days;

In terms of both leaf number and root number, Barcelona is significantly larger than the other varieties studied, with the next highest values observed for Tonda di Giffoni and Butler **(Figure 3)**. With regard to the 30-day analysis, it was found that, in terms of root and leaf size, there were no significant differences between the varieties analysed. This can be seen in **Figure 3**, which shows the results for this period. In terms of leaf size (LD), all varieties studied showed significant growth under *in vitro* culture conditions.

On the other hand, in root length (RD), a significant difference was observed between the varieties analysed. The lowest root size values were recorded in the varieties Tonda Romana and Barcelona. In contrast, intermediate values were observed in Tonda Gentile Delle Longue and Butler, and Tonda di Giffoni reported the highest values.

a) b)

Figure 4. Formation of the number of leaves *in vitro* in explants of *Corylus avellana* L (a)-30 days, (b)-60 days

As regards the analyses carried out in the second period (60 days) **(Figure 4)**, statistically significant differences are observed in morphological characteristics. Thus, an influence of varieties can be observed in the interaction between the number of leaves, the number of roots, and their size. These results contribute to the understanding that variety has a significant influence on both 30 and 60 days on morphological characteristics . Therefore, it is essential to consider these aspects in order to optimize a valuable method for *in vitro* propagation of hazelnut varieties and to increase the efficiency of the process. It can already be seen at this stage that some varieties react differently in terms of these characteristics under *in vitro* conditions, with genetic variability playing an important role in exploiting and increasing the resilience of *in vitro* propagation.

In the second period, as reported in **Figure 5**, between the number of roots and the number of leaves, the varieties Tonda Romana and Tonda di Giffoni show the highest values, significantly higher than the other varieties taken in the study. Intermediate values of leaf number and root number were observed in the variety Barcelona. The lowest values were observed in Tonda Gentile Delle Langhe, which also showed the lowest root number values compared to the other varieties. The lowest values were also observed in Butler, which had the lowest number of leaves.

Correlating with the values obtained at 30 days, it can be said that from the point of view of plasticity and adaptability, the variety Tonda di Giffoni has maintained a high capacity for growth and adaptation to *in vitro* conditions, making it the most suitable for these multiplication procedures.

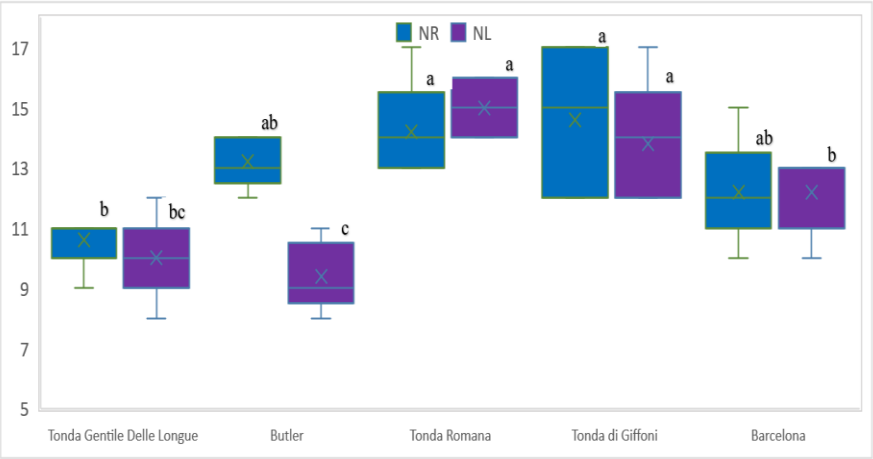

Figure 5. Boxplot representation of the number of roots (NR) and leaves (NL) formed per explant in the *in vitro* culture of *Corylus avellana* L. varieties after 60 days.

In terms of root size and leaf size, the highest values were observed in the varieties Tonda di Giffoni and Barcelona, which were significantly larger during this period **(Figure 6)**. Even if the variety Tonda di Giffoni did not initially show increased plasticity in the first period, it showed increased genetic potential with adaptability for *in vitro* multiplication procedures. The same can be observed in the Barcelona variety, which obtained good results in terms of morphological characteristics throughout the experiment.

On the other hand, a decrease in adaptability can be observed in the varieties Tonda Gentile Delle Langhe and Butler, which, even though they recorded average values in the first 30-day period, showed a decrease in growth rate in the second 60-day period. This demonstrates that adaptability to *in vitro* conditions is significantly influenced by the genetic variability of the varieties studied.

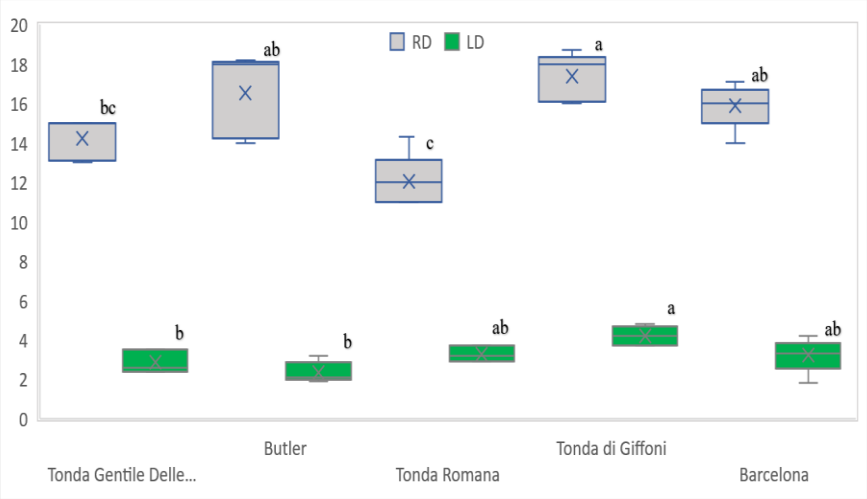

Figure 6. Boxplot representation of the roots dimension (RD) and leaves area (LA) growth per explant in the *in vitro culture* of *Corylus avellana* L. varieties after 60 days.

Phytochemical Composition

The results indicate a high degree of significance in terms of total phenolic compound content. Among the 5 varieties studied, there was a significant difference among them in phenolic compounds with a high degree of significance ($p < 0.001$). Thus, the results suggest the influence of genetic variation on phenolic compounds (TPh) in the hazelnut varieties studied.

Following spectrophotometric analysis, the results obtained using the FRAP (Ferric Reducing Antioxidant Power) method revealed that there was a significant difference ($p < 0.001$) in the antioxidant capacity of the extracts obtained from the varieties analysed. The highest antioxidant capacity was observed in extracts from the Tonda di Giffoni variety and the lowest in the Butler variety. The fact that some varieties have a higher antioxidant capacity by reducing free radicals reflects the potential for genetic variation. The same antioxidant effect influenced by the variety of types studied can also be observed in the DPPH determination method, which is significantly influenced by genetic variation.

In addition to the fact that hazelnuts are rich sources of protein and have a significant content of vitamin E, magnesium, and thiamine, as well as a complex of phytosterols that play an important role in the prevention of cardiovascular diseases, hazelnuts also have a very rich content in phenolic acids with an increased antioxidant capacity [20, 24, 25]. The results regarding the TPh content, determined by the Folin-Ciocalteu method, and the antioxidant capacity determined by the FRAP and DPPH methods, from five hazelnut varieties, are presented in **Table 1**.

Table 1. The bioactive compounds of hazelnut varieties

Genotip	TPh (mg GAE/100 g Fw)	FRAP (moli TE/100 g Fw)	DPPH (moli TE/100 g Fw)
Tonda Gentile delle Langhe	184.21±0.51 ^{c}	5.74±0.01 ^{b}	188.54±1.35 ^{c}
Butler	180.95±1.02 ^{d}	5.67±0.01 ^{c}	180.76±1.35 ^{d}
Tonda Romana	183.12±1.02 ^{c}	5.72±0.03 ^{bc}	186.32±1.35 ^{cd}
Tonda di Giffoni	192.71±0.77 ^{a}	5.9±0.02 ^{a}	216.31±1.38 ^{a}
Barcelone	189.64±0.51 ^{b}	5.85±0.02 ^{a}	205.21±1.38 ^{a}

Results are presented as means ± SD, with different letters indicating significant differences in the same column ($p < 0.001$). Total phenol content (TPh) is expressed in milligrams (mg) of gallic acid equivalent (GAE) per 100 g fresh weight (Fw). FRAP (plasma iron reduction ability) is expressed in Trolox equivalent (TE) moles per 100 g Fw, and DPPH (2,2-diphenyl-picryl-hydrazyl) is expressed in TE moles per 100 g Fw.

From the core of the five studied varieties, Tonda di Giffoni and Barcelona recorded the highest TPh content and high antioxidant capacity, a fact that differentiates them from the other varieties, from a statistical point of view. Also, the results of the study demonstrate that there is a close correlation in terms of the content of total polyphenols and antioxidant capacity, namely, the higher the content in TPh, the higher the antioxidant capacity.

Regarding the TPh content and antioxidant capacity obtained in hazelnut kernels, comparing our findings with previous results, it was shown that it varies greatly depending on the area, climatic conditions, cultivar, extraction solvents, extraction time, but also by the methods used and the basis used for measurement [9, 20, 26-28]. Jakopic *et al*. (2011) reported the TPh content in

methanolic extracts of 20 hazelnut varieties, and the values obtained were between 70 and 478 mg GAE/Kg of hazelnut kernels [25]. In another study, Delgado *et al*. (2010) studied six conditions, involving different solvents (water, methanol, and aqueous acetone) and contact times, for the determination of TPh content in hazelnut kernels, and the highest TPh content was reported in extracts obtained with boiling water for of 30 min, 44.3 ± 7.7 mg GAE/g and that with aqueous acetone solution (80% v/v) for 24 h, 36.2 ± 8.8 mg GAE/g [29].

Because antioxidant compounds in plants are chemically diverse and structurally complex, there is no single appropriate test to accurately determine antioxidant activity for all compounds. For this reason, antioxidant activity was determined in our study using two different antioxidant tests, namely FRAP and DPPH. Methods for evaluating antioxidant activity, such as FRAP, and DPPH, are part of the category of methods based on single electron transfer (SET) [11, 30-32].

In recent years, various studies have demonstrated that the high level of natural antioxidants identified in hazelnuts have multiple benefits for human health, thus considerably increasing the interest in the cultivation and development of as many varieties as possible [11, 20, 28, 31]. Among the important beneficial effects are the anticancer, antimicrobial, antifungal, anthelmintic, and protective effects against neurochemical changes in Alzheimer-type neurodegeneration [11, 20, 28, 31, 33].

As future perspectives, in-depth studies on the phytochemical composition of the new hazelnut varieties introduced in Romania are needed, in order to identify the bioactive compounds responsible for the antioxidant capacity. In addition, new biological effects of these valuable fruits can be exploited.

Conclusion

The examinations revealed significant differences in morphological characteristics, phenolic compound content, and antioxidant capacity between the five hazelnut varieties analysed. Under *in vitro* test conditions, during the 30-day period, a significant influence of variety type on root number and size was identified, while leaf size and number were not significantly affected. In contrast, during the second 60-day observation period, leaf number and size were significantly influenced by variety type. Tonda di Giffoni and Barcelona varieties showed the best adaptability and plasticity under *in vitro* culture conditions, also showing the highest content of phenolic compounds (TPh) and increased antioxidant capacity (FRAP, DPPH). The results highlight the importance of genetic variation in influencing the morphological characteristics and phytochemical composition of hazelnuts. Therefore, proper selection and breeding of varieties are essential to obtain hazelnuts with desirable characteristics in terms of developmental plasticity under *in vitro* conditions as well as antioxidant quality.

The results of the study suggest the importance of introducing varieties with increased adaptability, such as Tonda di Giffoni and Barcelona, to obtain higher-quality products in terms of antioxidant compounds content. These varieties can bring considerable benefits in increasing resistance to viroid through the use of *in vitro* multiplication, but also significant benefits in the food industry, contributing to the promotion of a healthy diet.

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Ethics statement: None

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