

Article

Opportunities for the Early Diagnosis and Selection of Scots Pine with Potential Resistance to Root and Butt Rot Disease

Valentyna Dyshko ^{1,*} , Ivan Ustskiy ¹, Piotr Borowik ²  and Tomasz Oszako ^{2,*} 

¹ Ukrainian Research Institute of Forestry and Forest Melioration Named after G. M. Vysotsky, 61024 Kharkiv, Ukraine; uski1950@ukr.net

² Forest Protection Department, Forest Research Institute, Sękocin Stary, Braci Leśnej 3, 05-090 Raszyn, Poland; pborow@poczta.onet.pl

* Correspondence: valya_dyshko@ukr.net (V.D.); t.oszako@ibles.waw.pl (T.O.)

Abstract: Pine stands affected by root and butt rot (*Heterobasidion annosum* s.l.) contain pines (*Pinus sylvestris* L.) that can survive for a long time without showing external symptoms of the disease ('conditionally resistant' refers to trees that survive without symptoms despite infection). The establishment of stands from the seeds of such trees can significantly increase the effectiveness of artificial afforestation. Since the growth and development of pine trees is determined to a certain extent by the number of cotyledons after seed germination, this article examines this trait in the progeny of trees that are potentially resistant and those that have already been attacked by root pathogens. The number of cotyledons and the resilience of trees is fascinating and not generally known. Presumably, the number of cotyledons can be linked to disease resistance based on increased vigour. Biologically, a larger area for carbon assimilation leads to better photosynthetic efficiency and the production of more assimilates (sugars) necessary to trigger defence processes in the event of infection. From an ecological point of view, this can give tree populations in areas potentially threatened by root system diseases a chance of survival. The aim of this study was to analyze the potential of using the number of cotyledons and other seedling characteristics to predict the resistance of trees to root and butt rot disease. The collected data show that the seedlings from the group of diseased trees exhibited lower growth rates and vigour. However, the seedlings from the group of potentially resistant trees are similar to the control, meaning the trees that show no disease symptoms because they have not come into contact with the pathogen. Our observations suggest that monitoring germinating cotyledons could serve as an early diagnostic tool to identify disease-resistant pines, although further research is needed.

Keywords: *Heterobasidion* spp.; resilience; tolerance; seedlings; cotyledons; priming; early diagnosis; seedling traits; afforestation



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1. Introduction

The main way to combat massive pathological processes in the world is preventive measures, including the selection and introduction of tree species that are resistant to certain massive diseases, as well as the development of methods for their mass propagation. Pine plantations are mostly monocultures. Both artificial and natural monocultures are prone to massive decline and premature failure. The main reason for the failure of such plantations is a violation of the water balance. Pine monocultures, especially in non-forested areas, are the background against which specific and widespread pathological processes develop. In areas with decline, the differentiation of trees is significantly accelerated depending on their condition under the influence of various pathological processes, of which root rot is considered the dominant one. The death of trees in the plantation is caused not only by root rot caused by *H. annosum*, which suppresses the development of the tree, but also by insects, including stem borers, nematodes, bacteria and other fungi. Therefore, the

resistance of pine to desiccation by a complex of negative factors under the conditions of a gap created by the continuous, long-term decline of trees should be better studied in pine monocultures, which are the best model for research for this purpose.

A characteristic feature of the disease caused by *H. annosum* is the death of groups of trees and the formation of gaps [1]. From year to year, the trees around the gap gradually die, and the gap widens. Along the gap, the active edge of the disease centre, there is a constant differentiation of trees according to their condition—from healthy trees with external signs to diseased dying and dead wood. Ukraine's forests are made up of more than 30 species of trees, with pine (*Pinus sylvestris*) dominating [2]. It accounts for 33% of the total forest area. This species is the most economically important tree species in Europe, too. One of the diseases affecting *P. sylvestris* is root rot, which is caused by the fungus *H. annosum* and causes major economic losses. Due to climate change, the impact of this pathogen may increase, so the issues of ensuring sustainable development and protecting forests from pathogenic factors in forestry remain important.

Heterobasidion annosum (Fr.) Bref. sensu lato (s.l.) is a dangerous forest pathogen that causes root and butt rot on most conifers in the northern hemisphere. The pathogen is most widespread in the forests of Europe and North America. In some pine plantations with long-lasting pockets of disease, there are individual trees, and in rare cases groups of trees, that remain viable against a background of continuous massive tree mortality. The economic impact on forestry related to tree mortality, reduced timber yields, and wood decay is calculated in millions of dollars and euros [1]. Given the extent of the economic and ecological damage caused by *H. annosum*, it is crucial to understand the mechanisms of resistance to the disease, such as the activation of plant defence mechanisms. In Scots pine (*Pinus sylvestris* L.), the activation of defence mechanisms could play a key role in its survival in forests affected by root and butt rot.

Priming initially triggers a small part of a defense response that increases the plant's ability to defend itself against future antagonists, for example, herbivores or pathogens [3]. Once a plant has been primed, it will defend itself faster, more strongly, and/or more durably against subsequent threats [4]. Therefore, plant defence priming is a form of plant immunological 'memory' in which an external stimulus is perceived by the plant as a warning signal that leads to a slight activation of the induced defence mechanisms [5]. The external stimulus in question could be chemical, biotic (from a pathogen or beneficial microorganism) or abiotic (from an environmental stressor) [6,7].

The hypothesis that trees 'remember' exposure to pathogens is intriguing, and to ensure that 'memory' is not just a metaphorical term in plant science, an explanation of the biological basis of this memory concept can be invoked, based on molecular mechanisms such as acquired systemic immunity (SAR), which are well recognised in plant–pathogen interactions. This form of 'memory' could be mediated by molecular pathways similar to acquired systemic immunity, in which plants boost their defences after an initial encounter with a pathogen.

In dying pine stands affected by root and butt rot (*H. annosum* s.l.), there are pines (*P. sylvestris*) that survive in 'disease centres' for a long time, which is why they are considered 'conditionally resistant' [8]. In other words, these 'conditionally resistant' pines survive without showing any external symptoms of disease, even though they are exposed to *H. annosum*. Afforestation using seedlings from the seeds of such trees can reduce the risk of the stand being lost in the first generation. Thus, we hypothesised that when pine trees were exposed to a pathogen that did not kill them, they were able to remember this stress. This is likely to lead to a faster and/or stronger defense response to the next pathogen attack [9]. Despite the known impact of *H. annosum* on pine mortality, little is known about the phenotypic traits that may characterise the progeny of asymptomatic pines in disease-prone areas. This study investigates whether the progeny of asymptomatic pines exhibit certain phenotypic traits that could indicate potential resistance.

2. Conception of the Experiment

It may be interesting to the readers if we briefly explain the main concept leading to the design of the experiment that we report in the manuscript. In Figure 1, we present a diagram of reasoning that could help us in that task.

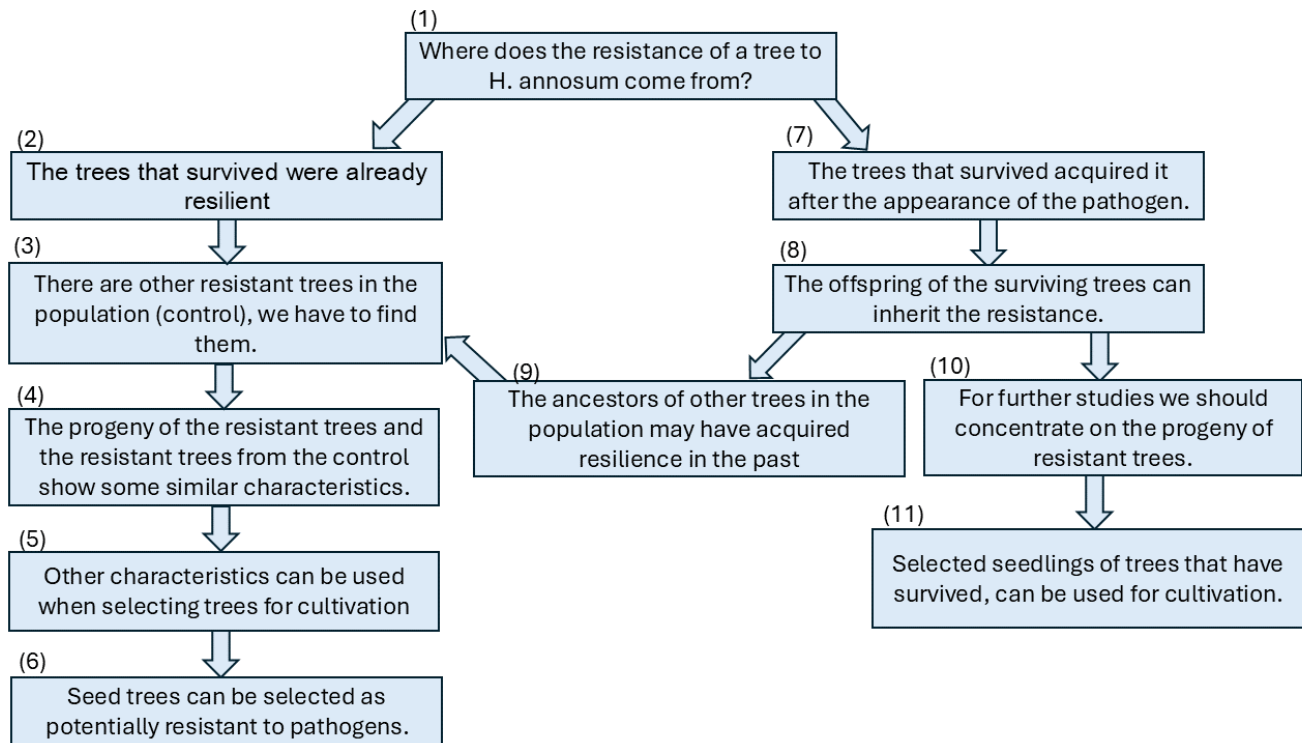


Figure 1. Conceptual diagram of the idea of the reported experiment.

- (1) As is widely known, some trees survive in the disease centers of *H. annosum*, which exhibit some factors of resilience to that pathogen. When we ask what about the origins of such resilience, we can basically go in two directions.
- (2) The first explored path is that the surviving trees had some genetic characteristics making them already resilient to the pathogen.
- (3) Taking that into account, we can expect that in the pine population, there are other trees that also share the characteristics of resilience to *H. annosum*, and our goal consists of searching for a method to select the resilient trees.
- (4) To achieve this goal, we had the idea to observe characteristics of seedlings cultivated from three categories of trees: (i) RESILIENT, which are trees surviving in the disease centers, (ii) CONTROL, which are trees from regions not infested by *H. annosum*, and (iii) DISEASED, trees with symptoms of infection. When seedlings were cultivated in the same conditions, we can assume that observed differences were mainly due to the ancestor's genetic heritage and not due to other environmental characteristics. The goal is to find a measure and find patterns in characteristics of seedlings differentiating these groups.
- (5) The resilience of trees to *H. annosum* is a characteristic that cannot be easily directly observed until the exposure of the seedling to the presence of a pathogen and after that, the observation of tree conditions, especially the potential development of disease. But we expect that the other characteristics of seedlings that can be observed are correlated with resilience, thus allowing for differentiation between more and less resilient trees.
- (6) If we find such characteristics we will be able to propose a method of selection of seed trees to find those which are inherently resilient to the pathogen, thus producing seeds, which may be used for further cultivation, with a hope that the offspring will inherit the resilience. A remark is worth noting, which is that even if we find such

trees, their progeny may not inherit the resilience, as, for example, we cannot control from which tree came the pollen used to the pollinate seeds. However, we can expect that the proportion of resilient trees in the progeny will be higher.

- (7) As we have drawn in the diagram, there can be another decision path, indicating that the trees were not resilient to the pathogen by their genetic heritage, but acquired such resilience after the appearance of the pathogen, or by other means.
- (8) However, even in such a situation, the resilience may be transmitted to the offspring.
- (9) That leads us to observe that since that process happens in this generation of trees, a similar process could happen in the past, and in the whole population, there are other trees resilient to the pathogen. This led our reasoning to the first branch of the diagram that we already discussed.
- (10) An option to consider for further studies is an examination of the progeny of resilient trees and basing on them cultivating new generations of resilient trees.
- (11) An application equivalent to that proposed in item (6) is usage for further cultivation, seeds collected from trees that survived the presence of *H. annosum* in their environment.

The presented manuscript reports an experiment on stage (4) of research presented in the diagram in Figure 1. As described below, we identified some of the characteristics of seedlings that differentiate resilient trees. Further studies are still needed to achieve the ultimate goal of the proposed experiment.

In the conceptual diagram of the experiment, we noticed two possible applications of the concept, which are the final states (6) and (11). In our opinion, there are some advantages of the approach presented as (6). The seed trees, from which we can select the resilient ones, are selected as exhibiting characteristics of wood demanded by the foresters. When we take into account option (11), the surviving trees are rare and may not be selected based on such characteristics important for the economic perspective.

An additional note is worth mentioning. The considered concept consists of supplementary criteria for the selection of seed trees and not seedlings or seeds. The selection of seedlings is laborious, and some of the possible selection criteria may be difficult to implement in practice. The selection of seed trees can be carried out once and then seeds used in further years. However, this does not exclude other paths of research that may be worth investigating.

3. Materials and Methods

3.1. Preparation of Seeds and Seedlings

The experiment consisted of cultivating 30-day-old pine seedlings grown under laboratory conditions from the seeds of trees of known origin and condition.

3.1.1. Collection and Selection of Seeds

Three variants (experimental groups) of trees were selected (Table 1) to compare the characteristics of the seedlings germinated from their seeds.

Genetic studies of pathogenic fungi have not been conducted. In the areas affected by root and butt rot, the search was primarily for fruiting bodies of *H. annosum*, and when these were found, the pines were categorised as damaged by root rot. Wood samples were taken from dead and living trees at the edges of the resulting gaps to monitor the presence of the fungus. Near the standing trees, we dug up the soil at a distance of 10–50 cm with a shovel, looked for roots, and cut off pieces 10–15 cm long with an axe. For lying trees, the roots were cut off in the part adjacent to the soil. The samples for the laboratory tests were taken at random from dead and often fallen trees. Individual pieces of root and wood were placed in sealed bags and incubated at 20 °C. After a three-week incubation period at room temperature, the samples were analysed for the presence of mycelium, conidia and conidia formation, which are characteristic of *H. annosum*. If mycelium, conidiophores and conidia characteristic of *H. annosum* were found on the wood samples, the trees were categorised as damaged by this fungus.

Table 1. Characteristics of the treatment groups of the trees from which the seeds were collected that were included in the studies.

CONTROL	Healthy trees at sites where no trees with symptoms of <i>H. annosum</i> infection were found nearby (at least 20 m). It was assumed that these trees had no direct contact with the pathogen. However, they had similar conditions in terms of soil, climatic environment, and genetics as the other trees analyzed.
DISEASED	Diseased trees are characterised by visible symptoms of infection, the presence of drying tops and branches, pale green needles, traces of resin on the trunk, and an unpleasant odor in the rhizosphere. According to the Sanitary Rules, they are characterised by indicators of categories 3 or 4 of sanitary conditions.
RESILIENT	The trees are characterised by an indicator that corresponds to the 1st category of sanitary conditions and have a densely covered crown of green colour without signs of drying out, are located in the foci of <i>H. annosum</i> infection (disease), and remain viable for a long time against a pathological background. According to external signs, such trees belong to the 1st category of sanitary conditions [10], and they may be inferior in height to more susceptible trees but not in diameter.

Examples of photos of trees in conditions of the studied variants are presented in Figure 2.

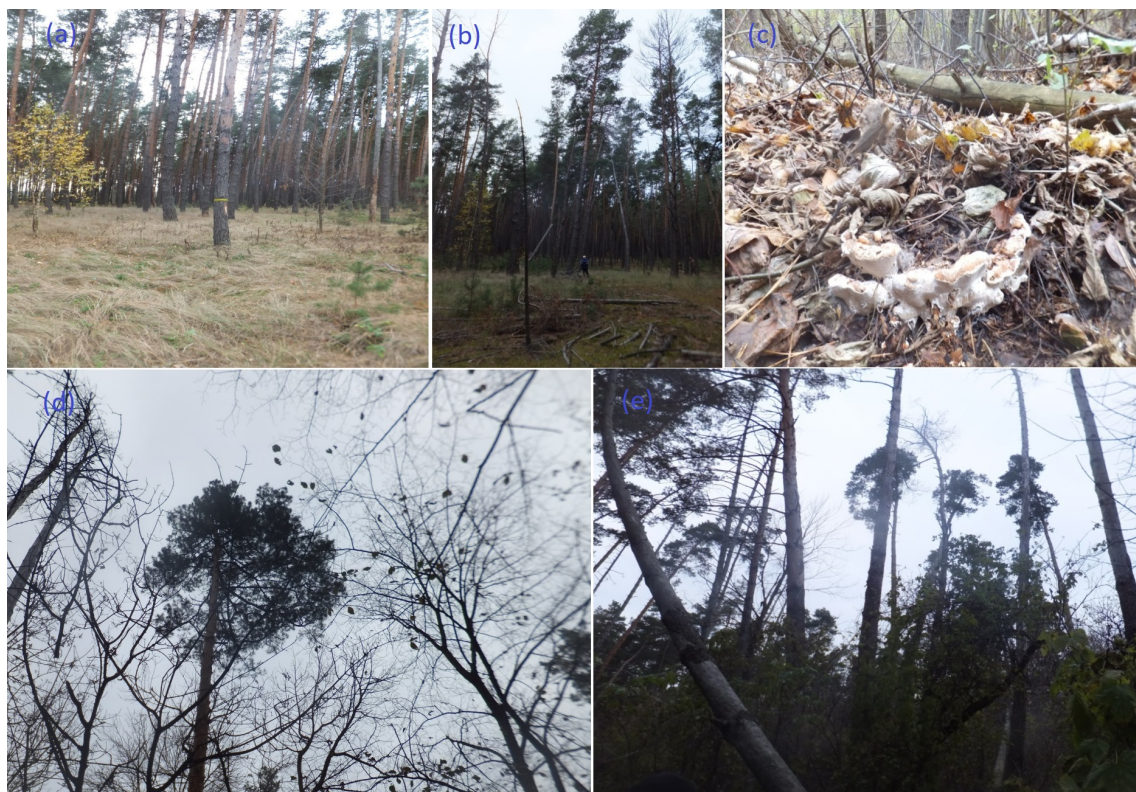


Figure 2. Often one (a–c) or a group (d) of living, asymptomatic trees remains in the gap that has arisen in the stand. The cause of the death of the other trees is the fungus *Heterobasidion* spp., whose fruiting bodies grow on the remaining stumps (c). The dead trees initially remain standing (b) and are then blown over by the wind (c,e).

3.1.2. Germination of Seedlings

Cones were collected from different-aged stands of Scots pine (50–90 years) affected by root and butt rot growing in the Kharkiv region (Ukraine). The cones were collected from felled and standing trees of Scots pine. Cones of other pine species were not studied. The cones were collected from standing trees using a crane with a cradle. To obtain the seeds, the selected cones were dried at a temperature of no more than 40 °C until they had fully opened.

Samples of 100 seeds from each of the selected trees were prepared. The seeds were sterilised for 20 min with a 0.5% KMnO_4 solution (international non-proprietary name: potassium permanganate, manufacturer Istok-Plus LLC, Zaporizhzhia, Ukraine).

The seeds were then rinsed with distilled water, dried, and transferred to sterile Petri dishes for germination. The filter paper was placed on the bottom of each Petri dish. The seeds were sprinkled evenly over the entire Petri dish and moistened. Distilled water (5 mL) was pipetted into each sample. The Petri dishes were placed in an organic glass chamber for germination. The temperature in the chamber was kept between +23 and +25 °C and controlled with a mercury thermometer. The same lighting conditions were created for all samples: light during the day and darkness at night.

The seeds were moistened once a week, and 5 mL of sterile water was added to all samples. The conditions for germination were set so that the seeds were not affected by various environmental factors.

The number of germinated seedlings of each variant and each tree of the experiment are listed in Table 2.

Table 2. Number of trees and seedlings in the experiment. One hundred seeds were prepared per tree, so the number of seedlings represents the proportion of germinated seeds.

VARIANT	No. of Trees	No. of Seedlings	No. of Seedlings per Tree
CONTROL	7	517	82, 76, 75, 67, 79, 69, 69
DISEASED	16	1126	73, 52, 84, 63, 76, 62, 83, 88, 64, 71, 64, 82, 54, 67, 68, 75
RESILIENT	16	1271	96, 84, 77, 80, 87, 81, 81, 89, 66, 93, 77, 64, 78, 71, 80, 67

Examples of photographs of seeds and seedlings, taken during the experiment, are presented in Figure 3.

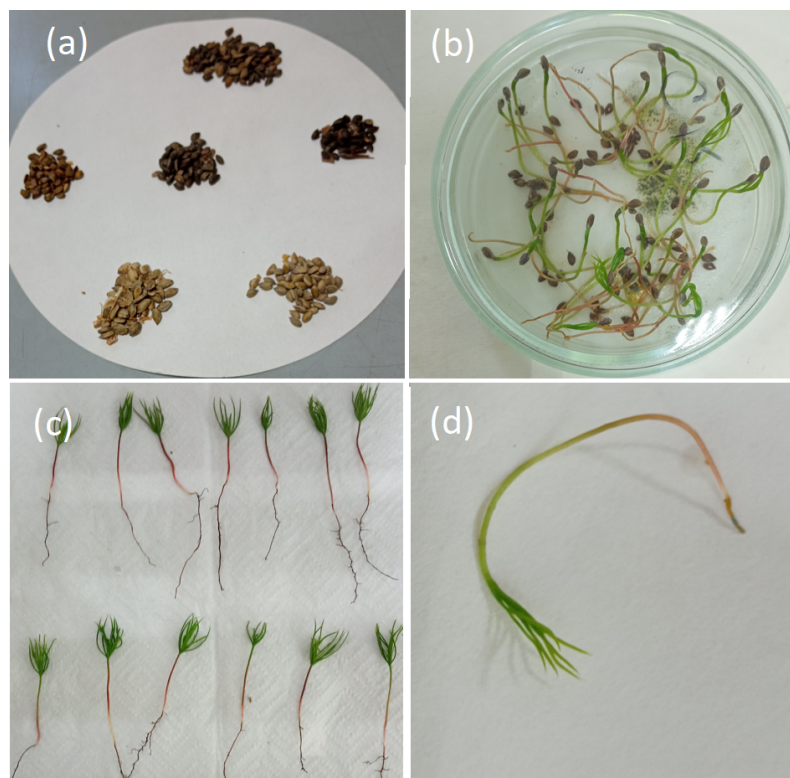


Figure 3. Photos of seeds and seedlings taken during the experiment. (a) Seeds counted and prepared for weighing. (b) Germinated seedlings in a Petri dish. (c) Seedlings prepared for measurements. (d) A single seedling.

3.2. Measurements

The list of characteristics measured in the experiment is shown in Table 3.

Table 3. List of parameters measured in the experiment.

COTYLEDONS	Number of cotyledons counted for each seedling.
WEIGHT	Seeds weight of the seeds was measured in two lots per tree, each lot containing 50 seeds.
ROOTS	Length of roots of each seedling.
STEM	Length of the stem of each seedling.
NEEDLE	Length of the needles of each seedling.
STEM/ROOT	Ratio of stem and root length calculated for each seedling.
SEEDLINGS	Number of germinated seedlings from each seed lot of a tree.

3.2.1. Weighting of the Seeds

Two samples were collected from each tree, with 50 seeds in each sample. All seed samples were weighed on an electronic balance (Axis Electronics, New Delhi, India) to the nearest 1 mg. For each tree, the arithmetic mean was calculated by adding the weights of two samples and dividing them by two. The balance was calibrated automatically, if necessary.

3.2.2. Measurements of the Length of the Seedlings

For each seedling, the lengths of roots stems were measured, and needles were measured. Before measurement, the seedlings were washed with distilled water, straightened (if necessary) and dried on filter paper. To measure the seedling length, a line was drawn on millimetre paper, and the beginning of the start of countdown (0 mm) was marked. Every 10 mm, marks were made, and the distance from the starting point was indicated. The end of the roots of the seedlings was applied to the place from which the countdown began, and three points were recorded: (1)—the length of the entire seedling; (2)—the length of the root, i.e., the distance from the end of the root to the beginning of the stem (the point at which the seedling changed color from brown to green); (3)—the distance from the beginning of the stem to the place where the needles began. The length of the stem and needles was determined by subtracting the registered values from the total length of the seedling.

3.2.3. Cotyledons Counting

The number of cotyledons in the samples was counted by eye (from 3 to 9). The obtained values were noted in a notebook and then entered into an electronic document (Excel), filtered and seedlings with the same number of cotyledons were counted. The proportions of seedlings with different numbers of cotyledons and their average number in the samples were determined.

3.3. Statistical Analysis

3.3.1. Mixed Models

The statistical analysis of the collected data was carried out within the framework of the theory of linear models [11].

There are two factors that classify the collected data. First, the treatment groups (Table 1) are the main factor of interest and the independent variable of modeling.

The observations used for the analysis are measurements of the characteristics of the seedlings (Table 3). However, we can assume that the observations should be independent within each group, since the seedlings germinated from seeds of a given tree share some common characteristics. The lack of independence violates one of the assumptions of the standard ANOVA method. This means that the TREE index should be included as an additional grouping factor in the statistical model. It should be noted that the TREE factor is embedded in the VARIANT factor, as each tree is only observed in conjunction with

one level of the variant. The hierarchy of such embedding of factors should be included in the model. Also, we should note that the collected data are not balanced. The number of trees selected for the collection of seeds differs between the treatment groups, but number of seedlings germinated from each of the trees also varied, and that factor of data imbalance could not be controlled by the experiment settlement. It is also important to note that the trees from which the seeds were collected are, of course, only random samples of trees with the analyzed traits, which signifies that the TREE factor is not a fixed effect but a random effect, which requires a dedicated approach in model building.

These remarks lead to the conclusion that an approach that could be used to statistically analyze the experimental data used belongs to the category of mixed models [11]. The equation of the nested model used in such a case has the form

$$Y_{ijk} = \mu_i + b_{j(i)} + \epsilon_{ijk},$$

where Y is the dependent variable, μ_i is the fixed factor of the model, which is the VARIANT in the case under consideration, and the i -index runs over the three groups under consideration (Table 1). $b_{j(i)}$ is the random factor of the model, which in this case is the TREE. The $j(i)$ notation of the index indicates that the j i.e., the index of the TREE variable, is nested in the i VARIANT index. The error term ϵ_{ijk} and $b_{j(i)}$ are both independent of each other. $\epsilon_{ijk} \propto N(0, \sigma)$, and $b_{j(i)} \propto N(0, \sigma_{b(A)})$.

In simpler terms, the mixed model allows us to account for differences between trees while still assessing the overall effect of the treatment groups on seedling characteristics.

Within the framework of the applied theory of mixed models, we were able to obtain least squares estimates of the means of the analysed characteristics of interest as well as the differences between them with the corresponding confidence limits and p -values. Since there are comparisons between three treatment pairs in the model, we used the Tukey method, which is required for the case of multiple comparisons. The confidence level $\alpha = 0.05$ (95%) was used in the calculations. However, the unadjusted estimates are also presented for comparison.

The statistical models were created for all measured characteristics (Table 3) as dependent variables. An attentive reader may notice that there is an exception when we consider the number of seedlings as a dependent variable. Since there is no nested random effect, the model simplifies to a classic ANOVA analysis.

3.3.2. Data Visualisation

A short remark considering the visualisation of the data presented in the manuscript is needed. As one can notice in Table 2, there is variability in the numbers of seedlings germinated from various trees. For that reason, the presentation of data based on original detailed observations may be biased towards the characteristics of trees with higher seed germination percentages.

That issue of unbalanced data is automatically taken into account in the mixed model's calculations of estimations.

For the visualisation of the data presented in the manuscript, we propose an approach consisting of the aggregation of data to the tree level. Then, the mean values of each tree are visualised. Such an approach is consistent with the concept of selection of treatment groups, in which the trees from which the seeds were collected are the main units. When we examine the mixed model equation presented in Section 3.3.1, the used approach of presentation of data aggregated to the TREE level can be viewed as a presentation of the first two terms of the equation: μ_i , which represents the effect of the treatment group, and $b_{j(i)}$, which represents the mean of each tree. The within-tree variability of data represented by the term ϵ_{ijk} is not presented in our visualisations.

3.3.3. Software Packages and Parameters of Analysis and Results

The statistical modelling presented in this study was performed using SAS 9.4 (SAS on Demand for Academics) software (SAS Institute, Cary, NC, USA). The PROC MIXED procedure [12] was used.

The software packages used enabled the diagnosis of the statistical models. The built-in basic tests proposed by the software were used. The verification of the normal distribution of the residuals was performed using quantile plots and histograms of the distribution the residuals. Also, the heteroscedasticity of residuals was verified by the application of residual versus predicted plots. These results are not presented in the manuscript and were only used to ensure validity. In the performed analysis, we did not observe significant deviations that could suggest that the applied models could not be justified.

In all of the calculations presented in the Results section, we estimated the confidence intervals using a 95% threshold. Such results are presented in all the tables and discussed. However, for some results, we would like to additionally draw the reader's attention, and we note that if using a less restrictive approach, for example, a 90% threshold, the differences could be found to be statistically significant. Such additional notes are explicitly stated in the description of the results.

The presented results of statistical analysis are prepared in the following schema. A pair of tables is presented for each of parameters listed in Table 3. First of all, we include a table reporting the estimates of the mean of parameters measured in the experiment. These estimates are evaluated by the statistical model of each treatment group. Also, the standard error of this estimate is presented. Also, the lower and upper confidence limits of the mean, calculated at a 95% confidence interval, are included in that table. A visual presentation of differences between means of the studied experimental parameters is also presented in the form of a boxplot chart, demonstrating the means of the parameter aggregated at the TREE level. In the second table, we present the estimates of differences of means between each pair of treatment groups. The pairs of treatments are indicated in the first two columns of the tables. The estimates of difference and standard error of the estimate are presented in the following columns. Then, the magnitude of the t -value statistics is presented. We omit from the presentation in the table the degrees of freedom since in all presented results, it is equal to 36, and we do not want to repeat it. The p -value of the estimation is presented as $\text{Pr} > |t|$ as the following column. Also, the lower and upper intervals, calculated at the threshold of 95%, are presented. However, as we already mentioned, since the statistical test involves multiple comparisons (between 3 pairs of treatments), the p -value and confidence limits should be adjusted. The p -value adjusted by the Tukey–Kramer method is presented in the column $\text{Adj } p$, and the adjusted confidence limits at the overall 95% threshold are presented as the Adj Lower and Adj Upper columns. In the following discussion of the results, when we report the magnitudes of the p -value or confidence intervals, we always use the adjusted ones, since they are appropriate for the studies. In a few cases, when we would like to draw the readers' attention to the unadjusted p -value, we mention that explicitly.

Some other analyses are presented only in the form of visualisation and are not supported by the results of statistical tests.

The visualisation of the results was created with the ggplot2 package for R [13]. Default settings of `geom_boxplot()` were used for calculations of the characteristics used for the construction of boxplot charts.

4. Results of Measurements

4.1. Seeds

4.1.1. Seeds Weight

In Figure 4, we show the comparison of the weight of batches of 50 seeds collected from the trees of the different treatment groups of the experiment. The numerical data of the mean weight estimates are presented in Table 4.

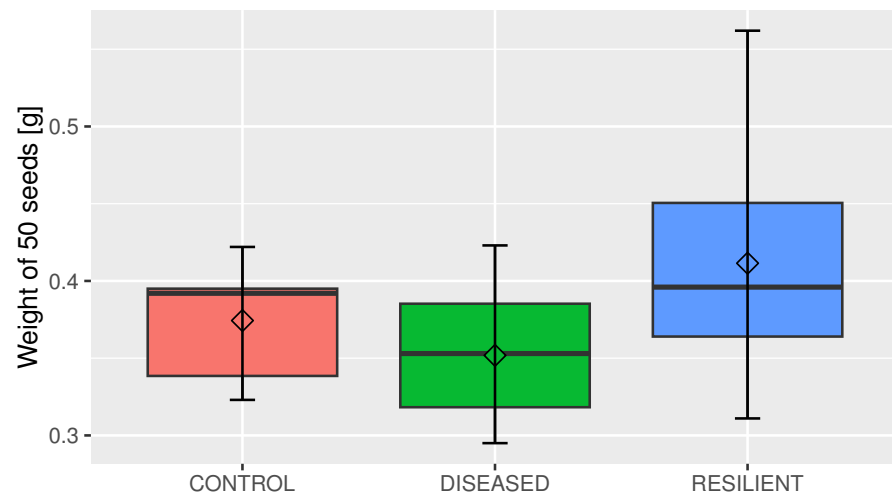


Figure 4. Weight of batches of 50 seeds compared to the treatment variant.

Table 4. Estimates of the mean weight of a batch of 50 seeds collected from trees from each treatment variant. The lower and upper confidence limits are calculated for $\alpha = 0.05$.

VARIANT	Estimate	Std Error	Lower	Upper
CONTROL	0.3744	0.02029	0.3332	0.4155
DISEASED	0.3518	0.01342	0.3246	0.3790
RESILIENT	0.4115	0.01342	0.3843	0.4387

We are also interested in estimating the differences in seed weight between the treatment groups studied. The main statistics of these estimates are presented in Table 5. As we can notice, the difference between the DISEASED and RESILIENT groups is statistically significant at a 95% confidence level, $p = 0.091$. If we compare the results from Table 5 with Table 4, we see that the difference in seed weight between the DISEASED and RESILIENT treatments is estimated to be 15.7%. That magnitude of the effect size is calculated, taking into account that the difference in means between DISEASED and RESILIENT is equal to -0.05977 (95% CI: -0.1060 to -0.01327), which we compare to the average of estimates of mean weight of these groups $(0.3518 + 0.4115)/2 = 0.3816$ (Table 4).

Table 5. Estimates of the differences between the means of the weights of batches of 50 seeds calculated for all pairs of treatment groups. p -value and confidence limits were calculated for $\alpha = 0.05$. Adjustments for multiple comparisons for p and confidence limits were calculated using the Tukey–Krammer method.

VARIANT	_VARIANT	Estimate	Std Error	t Value	Pr > t	Adj p	Lower	Upper	Adj Lower	Adj Upper
CONTROL	DISEASED	0.02254	0.02432	0.93	0.3601	0.6271	−0.02678	0.07187	−0.03691	0.08199
CONTROL	RESILIENT	−0.03711	0.02432	−1.53	0.1358	0.2910	−0.08644	0.01222	−0.09656	0.02234
DISEASED	RESILIENT	−0.05966	0.01898	−3.14	0.0033	0.0091	−0.09814	−0.02117	−0.10600	−0.01327

4.1.2. Seeds Germination

About 75% of the seeds used in the experiment germinated. The comparison of the percentage of germinated seeds from the lots of each tree with the treatment category is shown in Figure 5, and the numerical results of the estimates are shown in Table 6.

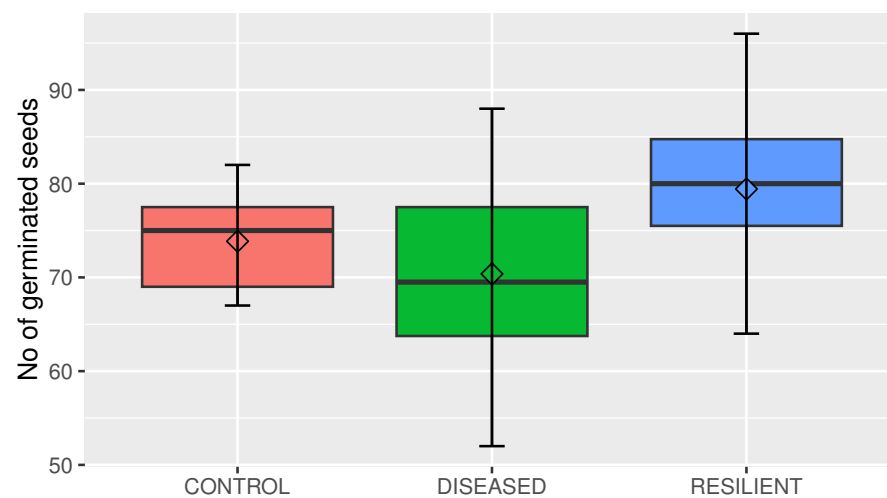


Figure 5. Number of germinated seeds in lots of 100 from each of the considered trees versus the treatment variant.

Table 6. Estimates of the mean of the proportion of germinated seeds from batches of 100 seeds collected from trees of each treatment variant. Lower and upper confidence limits are calculated for $\alpha = 0.05$.

VARIANT	Estimate	Std Error	Lower	Upper
CONTROL	73.8571	3.5437	66.6702	81.0441
DISEASED	70.3750	2.3439	65.6213	75.1287
RESILIENT	79.4375	2.3439	74.6838	84.1912

The estimates of the differences in the average number of germinated seeds between all treatment pairs are shown in Table 7. We can notice here that the only statistically significant difference at the 95% threshold is between the RESILIENT and DISEASED groups. The difference is 9.0625 percentage points more seeds germinated from the seed lots of the RESILIENT trees (95% adjusted CI: -17.1649% to -0.6901 , and p -value of 0.0255).

Table 7. Estimates of the differences between the means of the number of germinated seeds from batches of 100 seeds collected from each tree. Calculations for all pairs of treatment groups. p -value and confidence limits were calculated for $\alpha = 0.05$. Adjustments for multiple comparisons for p and confidence limits were calculated using the Tukey–Krammer method.

VARIANT	_VARIANT	Estimate	Std Error	t Value	Pr > t	Adj p	Lower	Upper	Adj Lower	Adj Upper
CONTROL	DISEASED	3.4821	4.2487	0.82	0.4179	0.6935	−5.1347	12.0990	−6.9030	13.8673
CONTROL	RESILIENT	−5.5804	4.2487	−1.31	0.1974	0.3971	−14.1972	3.0365	−15.9655	4.8048
DISEASED	RESILIENT	−9.0625	3.3148	−2.73	0.0096	0.0255	−15.7853	−2.3397	−17.1649	−0.9601

It may be interesting to compare the results of average weight of seeds collected from a tree with the proportion of seeds germinated from a lot. In Figure 6, we present visualisation of such comparison in the form of a scatter plot.

Similar to the results already presented, we can see that the clusters of data points overlap. We can also notice a shift between the DISEASED and RESILIENT categories, as shown in Figures 4 and 5. However, we can also notice that there is no visible trend or correlation between these two features. (The calculated correlation coefficients are not statistically significant, and their absolute value is on the order of 0.02.)

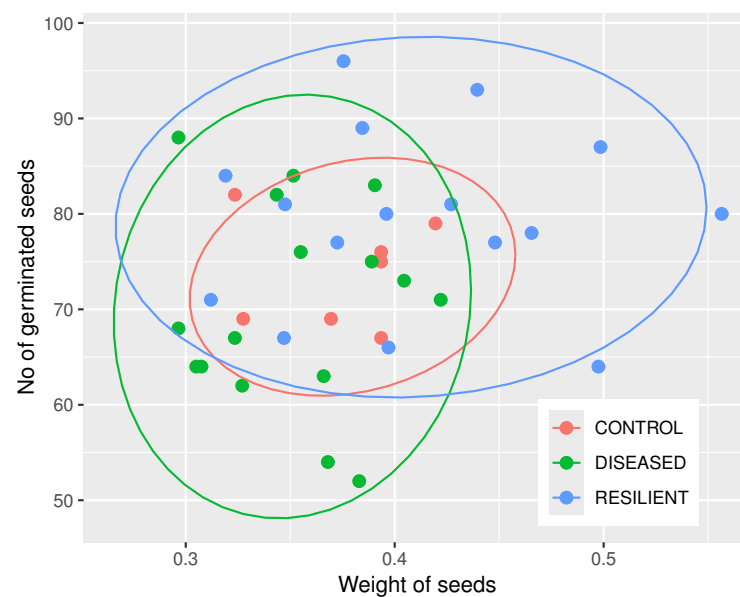


Figure 6. The average weight of a batch of 50 seeds collected from a tree compared to the proportion of germinated seeds. Ninety percent confidence ellipses are plotted as a guide.

4.2. Measurements of Seedling Characteristics

4.2.1. Number of Cotyledons

In Figure 7, we have presented the average number of cotyledons in the seedlings that germinated from the seeds of the analysed trees. The numerical values of the mean values estimated by the statistical model are shown in Table 8.

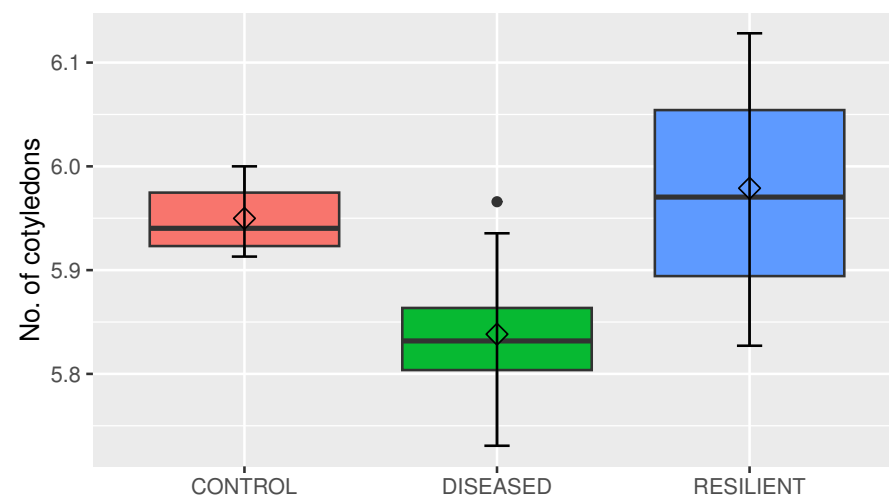


Figure 7. Mean number of cotyledons in seedlings germinated from trees from different experimental treatment categories. Visualisation of the variability of the mean values by tree from which the seeds were collected.

Table 8. Estimates of the average number of cotyledons in germinated seedlings compared to the treatment groups. The lower and upper confidence limits are calculated for $\alpha = 0.05$.

VARIANT	Estimate	Std Error	Lower	Upper
CONTROL	5.9516	0.03242	5.8859	6.0174
DISEASED	5.8419	0.02197	5.7974	5.8865
RESILIENT	5.9819	0.02068	5.9400	6.0238

The objective of the studies is to determine if there is a difference in the studied characteristics between the treatment groups. Statistical estimates of such differences in the mean number of cotyledons in seedlings are presented in Table 9. As one can notice, there is a statistically confirmed difference between seedlings germinating from seeds collected from DISEASED and two other groups of trees. The average number of cotyledons formed by the seedlings is slightly below six (Table 8), and seedlings from the DISEASED treatment group somewhat more frequently formed fewer cotyledons.

The estimated difference between the CONTROL and DISEASED groups is 0.1097 (95% CI: 0.01399 to 0.2055, p -value of 0.0217). The estimated difference between RESILIENT and DISEASED is 0.14 (95% CI: 0.06624 to 0.2137 with a p -value of 0.0001).

Table 9. Estimates of the differences between the means of the number of cotyledons in the seedlings. Calculations for all pairs of treatment groups. p -value and confidence limits were calculated for $\alpha = 0.05$. Adjustments for multiple comparisons for p and confidence limits were calculated using the Tukey–Krammer method.

VARIANT	_VARIANT	Estimate	Std Error	t Value	Pr > t	Adj p	Lower	Upper	Adj Lower	Adj Upper
CONTROL	DISEASED	0.1097	0.03917	2.80	0.0081	0.0217	0.03029	0.1892	0.01399	0.2055
CONTROL	RESILIENT	−0.03026	0.03846	−0.79	0.4365	0.7134	−0.1083	0.04774	−0.1243	0.06374
DISEASED	RESILIENT	−0.1400	0.03017	−4.64	<0.0001	0.0001	−0.2012	−0.07879	−0.2137	−0.06624

4.2.2. Stem Length

In Figure 8 and Table 10, we compare the average stem length of the seedlings germinated from the seeds collected from the trees of the three experimental treatment factor groups considered.

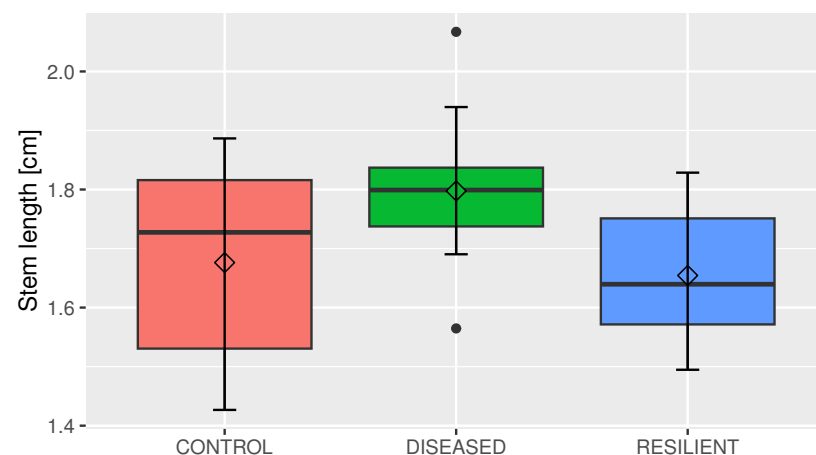


Figure 8. Mean stem length of the germinated seedlings of trees belonging to different experimental treatment categories. Visualisation of the variability of the mean values according to the tree from which the seeds were collected.

Table 10. Estimates of mean stem length of germinated seedlings compared to treatment groups. The lower and upper confidence limits are calculated for $\alpha = 0.05$.

VARIANT	Estimate	Std Error	Lower	Upper
CONTROL	1.6757	0.04740	1.5795	1.7718
DISEASED	1.7977	0.03143	1.7340	1.8615
RESILIENT	1.6546	0.03128	1.5912	1.7180

The comparison of differences between mean stem lengths in treatment group pairs is shown in Table 11, along with estimates of the uncertainty of these estimates. As can

be seen there, the DISEASED treatment group has a higher stem length than the other two groups. The statistically significant difference at the 95% level was found for one pair from the DISEASED and RESILIENT groups. In this case, the difference is 0.14 cm (95% CI: 0.03472 to 0.2515 with a p -value of 0.0073). If we compare this with an overall mean of 1.7 cm (calculated from the values in Table 10), this gives about 8% of the difference.

If we consider the difference between the CONTROL and DISEASED treatment groups, it is not statistically significant at a threshold of 95%, when we take into account the Tukey–Kramer adjustment. The p -value without adjustment for multiple comparison is of 0.0387. In such a case, we would like to draw the reader’s attention to the fact that, if we use less restricted confidence level of 90%, we could also conclude that there is a statistically significant difference between the CONTROL and DISEASED treatment groups. For this pair of treatment groups, the difference is 0.122 cm ($p = 0.0948$), which is of 7% of the length if we compare it to the results in Table 10.

Table 11. Estimates of the means of the differences between the stem lengths of the seedlings. Calculations for all pairs of treatment groups. p -value and confidence limits were calculated for $\alpha = 0.05$. Adjustments for multiple comparisons for p and confidence limits were calculated using the Tukey–Kramer method.

VARIANT	_VARIANT	Estimate	Std Error	t Value	Pr > t	Adj p	Lower	Upper	Adj Lower	Adj Upper
CONTROL	DISEASED	−0.1220	0.05687	−2.15	0.0387	0.0948	−0.2374	−0.00668	−0.2610	0.01699
CONTROL	RESILIENT	0.02108	0.05679	0.37	0.7127	0.9270	−0.09410	0.1363	−0.1177	0.1599
DISEASED	RESILIENT	0.1431	0.04434	3.23	0.0027	0.0073	0.05318	0.2330	0.03472	0.2515

4.2.3. Roots Length

The results of the comparison of root length between the treatment groups are shown in Figure 9. The numerical estimates for the mean root length of the seedlings are shown in Table 12.

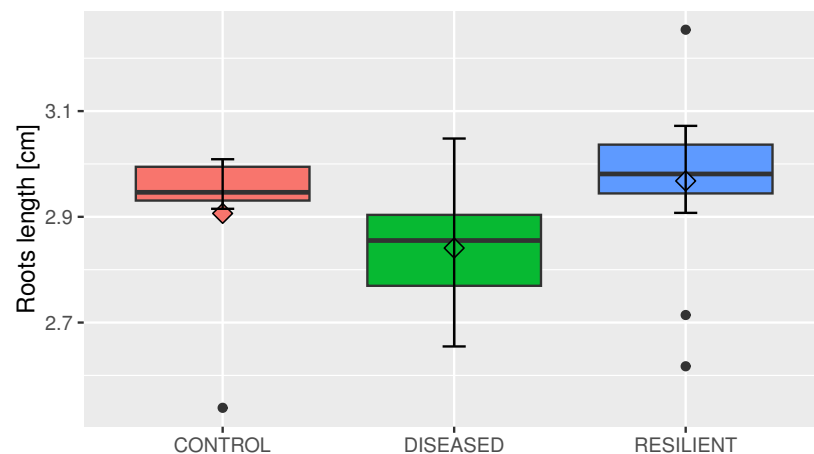


Figure 9. Mean root length of germinated seedlings of trees belonging to different experimental treatment categories. Visualisation of the variability of the mean values depending on the tree from which the seeds were collected.

Table 12. Estimates of the mean root length of germinated seedlings compared to the treatment groups. The lower and upper confidence limits are calculated for $\alpha = 0.05$.

VARIANT	Estimate	Std Error	Lower	Upper
CONTROL	2.9060	0.05138	2.8018	3.0102
DISEASED	2.8407	0.03405	2.7716	2.9097
RESILIENT	2.9681	0.03392	2.8993	3.0369

As can be seen in Table 13, a statistically significant difference in root length between the treatment groups was only found for the comparison between the DISEASED and RESILIENT pair. The seedlings that germinated from the seeds of diseased trees developed about 4% shorter root systems compared to other seedlings. The estimated difference in length of roots for this pair is -0.1274 cm (95% CI: -0.2449 to -0.00984 , $p = 0.0311$). The above-mentioned 4% difference is obtained by taking relation to estimated mean of the root lengths presented in Table 12.

Table 13. Estimates of the means of the differences between the root lengths of the seedlings. Calculations for all pairs of treatment groups. p -value and confidence limits were calculated for $\alpha = 0.05$. Adjustments for multiple comparisons for p and confidence limits were calculated using the Tukey–Krammer method.

VARIANT	_VARIANT	Estimate	Std Error	t Value	Pr > t	Adj p	Lower	Upper	Adj Lower	Adj Upper
CONTROL	DISEASED	0.06532	0.06164	1.06	0.2964	0.5449	−0.05970	0.1903	−0.08535	0.2160
CONTROL	RESILIENT	−0.06206	0.06157	−1.01	0.3202	0.5767	−0.1869	0.06281	−0.2126	0.08843
DISEASED	RESILIENT	−0.1274	0.04807	−2.65	0.0119	0.0311	−0.2249	−0.02990	−0.2449	−0.00989

4.2.4. Needles Length

The last characteristic measured was the length of the seedlings' needles. A visual comparison between treatment groups is shown in Figure 10, and the numerical estimates by the statistical model are shown in Table 14. For these characteristics, the mean differences were not found to be statistically significant, as can be seen from the results of the analysis presented in Table 15.

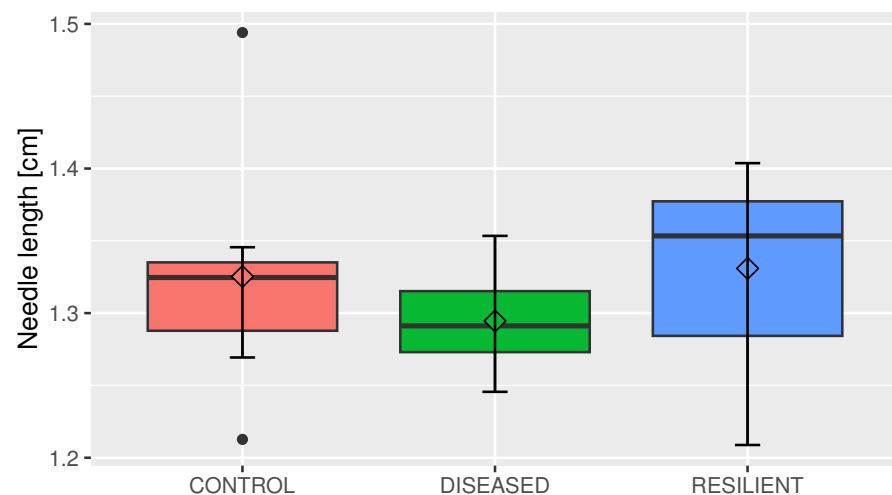


Figure 10. Mean needle length of germinated seedlings of trees belonging to different experimental treatment categories. Visualisation of the variability of the mean values according to the tree from which the seeds were collected.

Table 14. Estimates of the mean value of needle length of germinated seedlings versus treatment groups. The lower and upper confidence limits are calculated for $\alpha = 0.05$.

VARIANT	Estimate	Std Error	Lower	Upper
CONTROL	1.3251	0.02244	1.2796	1.3706
DISEASED	1.2947	0.01487	1.2645	1.3248
RESILIENT	1.3309	0.01481	1.3009	1.3610

Table 15. Estimates of the means of the differences between the needle lengths of the seedlings. Calculations for all pairs of treatment groups. p -value and confidence limits were calculated for $\alpha = 0.05$. Adjustments for multiple comparisons for p and confidence limits were calculated using the Tukey–Kramer method.

VARIANT	_VARIANT	Estimate	Std Error	t Value	Pr > t	Adj p	Lower	Upper	Adj Lower	Adj Upper
CONTROL	DISEASED	0.03041	0.02692	1.13	0.2661	0.5024	−0.02418	0.08499	−0.03538	0.09619
CONTROL	RESILIENT	−0.00586	0.02688	−0.22	0.8286	0.9741	−0.06038	0.04866	−0.07157	0.05985
DISEASED	RESILIENT	−0.03627	0.02099	−1.73	0.0925	0.2088	−0.07883	0.006297	−0.08757	0.01503

4.2.5. Stem/Root Length Proportion

In the results presented above, we found that there was an observed difference between the DISEASED and the other treatment groups for the stem and root length trait. However, it is interesting to note that this difference goes in a different direction. The seedlings that germinated from the seeds of diseased trees had a shorter root system but, on the contrary, longer stems.

Considering this, we can define a parameter that defines the formation of a seedling as the ratio between the length of the stem and the length of the roots (Table 3) and check how this characteristic can be used as a factor to differentiate between the treatment groups. The visualisation of these features is shown in Figure 11 and the numerical estimates in Table 16.

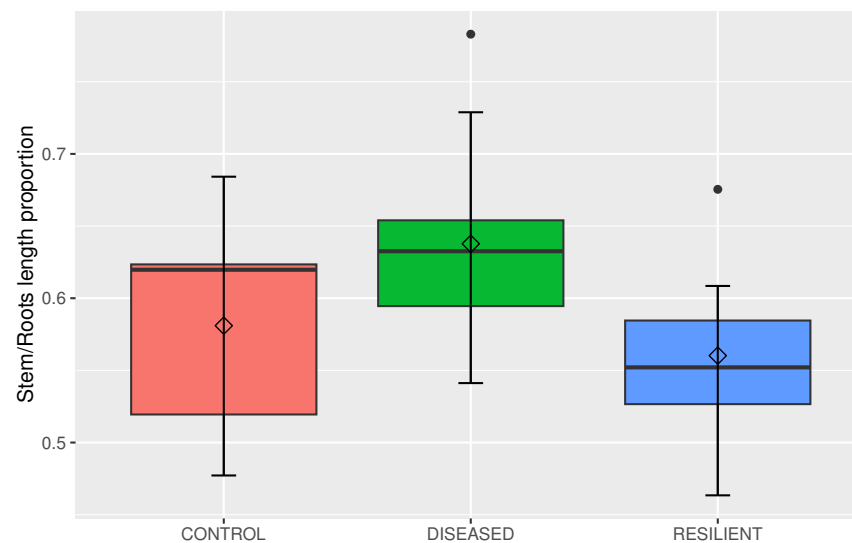


Figure 11. Mean ratio of stem/root length proportion of germinated seedlings of trees belonging to different experimental treatment categories. Visualisation of the variability of the mean values by tree from which the seeds were collected.

Table 16. Estimates of the mean of the proportion of stem/root lengths of germinated seedlings compared to the treatment groups. The lower and upper confidence limits are calculated for $\alpha = 0.05$.

VARIANT	Estimate	Std Error	Alpha	Lower	Upper
CONTROL	0.5809	0.02217	0.05	0.5360	0.6259
DISEASED	0.6378	0.01468	0.05	0.6080	0.6675
RESILIENT	0.5602	0.01465	0.05	0.5305	0.5899

The estimates of the differences in this parameter between pairs of treatment groups with statistical estimates of confidence are shown in Table 17. For the DISEASED and RESILIENT treatment group pairs, we found the difference to be statistically significant at a 95% level. The estimated difference of this parameter is 0.0755 (95% CI: 0.02686 to 0.1282,

$p = 0.0018$). If we compare the estimates of stem/root values (Table 16) with the differences between the pair estimates (Table 17), we can notice that the difference is 13.1%.

The difference between CONTROL and DISEASED is not statistically significant at a confidence level of 95%. But we would like to draw the reader's attention to the fact that if we use a less strict threshold of 90% ($p = 0.0965$), difference between this pair of treatments would be found statistically significant. Also, it can be noticed that in that case, the p -value not adjusted for multiple comparisons is equal to 0.0394. If we compare the stem/root values (Table 16) with the differences between the pair estimates (Table 17), we can notice that the difference is 9.6% of this parameter.

Table 17. Estimates of the mean of the stem/root length ratio of the seedlings. Calculations for all pairs of treatment groups. p -value and confidence limits were calculated for $\alpha = 0.05$. Adjustments for multiple comparisons for p and confidence limits were calculated using the Tukey–Kramer method.

VARIANT	_VARIANT	Estimate	Std Error	t Value	Pr > t	Adj p	Lower	Upper	Adj Lower	Adj Upper
CONTROL	DISEASED	−0.05683	0.02659	−2.14	0.0394	0.0965	−0.1108	−0.00290	−0.1218	0.008163
CONTROL	RESILIENT	0.02072	0.02657	0.78	0.4406	0.7177	−0.03317	0.07460	−0.04422	0.08566
DISEASED	RESILIENT	0.07755	0.02074	3.74	0.0006	0.0018	0.03549	0.1196	0.02686	0.1282

4.2.6. Measurable Traits Compared to the Number of Cotyledons

In the results presented above, we found that the mean number of cotyledons was statistically significantly different between treatment groups. In Figure 12 we plot the distribution of the proportion of cotyledons that developed in seedlings that germinated from seeds that originated from a tree. To avoid any confusion about the meaning of this visualisation, we would like to describe the method of data preparation in more detail. Firstly, for each tree from which the seeds were collected, the percentage of seedlings in relation to the number of developed cotyledons was calculated. Then, an averaging was performed for the treatment groups. Thus, each treatment group summed across the cotyledons equals 100%. This allows the proportions between the treatment groups to be compared based on the number of developed cotyledons.

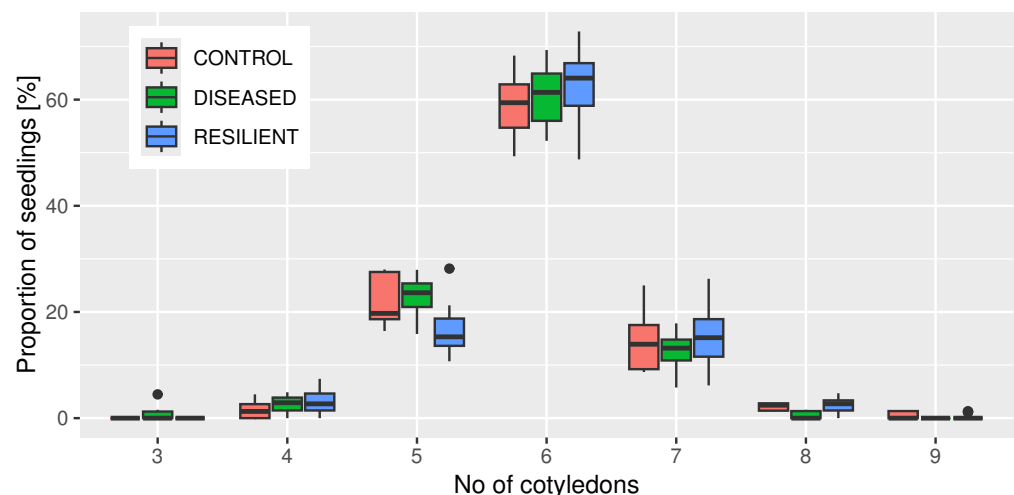


Figure 12. Proportion of seedlings from seeds of a given tree compared to the number of developed cotyledons. Comparison between treatment groups.

As we observed above (Figure 7, Tables 8 and 9), it was found that the DISEASED group formed, on average, a smaller number of cotyledons. Examining the data presented in Figure 12 allows us to see this pattern on a more detailed level and notice that such a difference is caused by observations at the extremes. Seedlings with three cotyledons were found only in the DISEASED group; on the contrary, seedlings with nine cotyledons were not found at all in the DISEASED group. Also, there was a high proportion of seedlings

with eight cotyledons in the DISEASED group. For the range of four to seven developed cotyledons, proportions between the treatment groups did not exhibit any clear pattern.

Another finding that we noticed was the difference between the DISEASED treatment group and the two other groups in terms of average lengths of seedling stems and roots. From these two characteristics of seedling formation, we defined a parameter of proportion, which captured the difference found between the treatment groups. Thus, it may be interesting to explore both of these characteristics of seedlings in one visualisation and observe patterns in the measurement data when we group the seedlings by the number of cotyledons that they had developed (Figure 13).

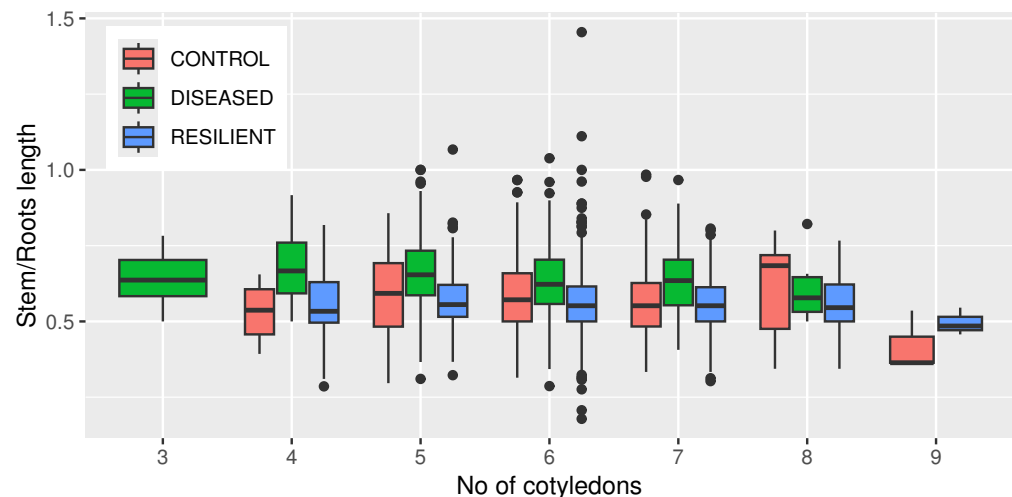


Figure 13. The ratio between stem and root length compared to the number of developed cotyledons in the seedling. Comparison between the treatment groups.

An interesting pattern that can be observed in Figure 13 is the upward shift in the boxes representing the DISEASED treatment group compared to two others. This can be observed in seedlings that develop up to seven cotyledons. Only in extreme cases, when the number of cotyledons developed was very high (eight or nine), this parameter, which represents the shape of the seedling, does not differ from the DISEASED group.

4.3. Main Findings of the Experimental Results and Data Analysis

In the previous subsections, we presented the results of experimental measurements and statistical analysis of these data. It may be helpful to the readers for us to summarise the main findings of these analyses, which are scattered in the description above.

- There is a statistically confirmed difference in the average weight of seeds collected from the RESILIENT compared to the DISEASED treatment group. The difference between the two other treatments is very small.
- A similar pattern was observed for the proportion of germinated seedlings, as the only statistically confirmed difference was between DISEASED and RESILIENT treatments.
- The number of cotyledons in the DISEASED treatment group is smaller than the two other groups, which was a statistically significant result.
- The mean stem length was found to be longer in the DISEASED treatment group than in the two other treatments. However, this could be statistically confirmed at a 95% confidence level only for comparison between DISEASED and RESILIENT. The difference for comparison between DISEASED and CONTROL was found to be statistically significant only at a 90% confidence threshold. Also, the difference between this pair of treatments could be confirmed at a 95% confidence level if the correction for multiple comparisons had not been used. The size of this effect is 7%–8% of the length of the stem.

- Roots in the DISEASED treatment group were found to be short compared to RESILIENT, which could be statistically confirmed at a 95% confidence level with the size of the effect of 4% of the length of roots.
- There was no observed difference in needle length between the treatment groups.
- The stem/root length proportion was found to be statistically significantly different at a 95% confidence level between the DISEASED and RESILIENT pair of treatments, with an effect size of 13%. For comparison between DISEASED and CONTROL, the difference was not statistically significant at a 95% confidence level but was at a 90% level, with an effect size of 9.6%.
- Seedlings with a very small number of developed cotyledons (three) were found only in the DISEASED treatment group, and a large number of cotyledons (above seven) was not found at all in the DISEASED group. For that finding, statistical tests were not performed.
- The stem/root length proportion, which can be treated as a single parameter characterising the shape of the seedling, differs for the DISEASED treatment group, compared to other treatments, for seedlings developing up to seven cotyledons. For that finding, statistical tests were not performed.

From the above experimental results, it is clear that there is a difference between the DISEASED trees and the other groups. This difference was observed with a larger effect size compared to the RESILIENT group and was statistically confirmed with a better confidence level. The parameters analysed for the CONTROL group lie between the parameters measured for the other two groups. We can hypothesise that the natural selection of trees that have survived in the disease centres and thus belong to the RESILIENT group has also favoured trees whose progeny have characteristics such as a greater number of cotyledons, a longer stem, shorter roots and a seedling shape determined by a lower ratio between stem length and roots. The trees in the RESILIENT group thus exhibited these characteristics to an even greater extent than the trees in the CONTROL group. However, the latter observation was not statistically confirmed.

5. Discussion

The issues presented in the article should be linked and understood together, e.g., the role of climate change and its impact on forest diseases such as root rot. More general global problems currently observed, such as the effects of climate-induced stress, e.g., changing precipitation and temperature patterns, exacerbate the occurrence of diseases in monocultures, which in turn provides a deeper theoretical context for the field of global change biology. However, the aim of diagnostics is to identify potentially resistant trees that could become objects for harvesting seed material with increased resistance to root rot damage. This method not only offers the possibility of the early detection of trees susceptible to the disease, but also the breeding of resistant trees in stands for future generations.

The identification and development of resistant trees was carried out by intensive artificial selection in the following species: interspecific chestnut hybrids resistant to *Cryphonectria parasitica* (pathogen causing chestnut blight) [14], Port Orford cedar resistant to *Phytophthora lateralis* (pathogen of Port Orford cedar root rot) [15] and elm tolerant to *Ophiostoma novo-ulmi* (pathogen of Dutch elm disease) [16]. Unfortunately, breeding programmes face many challenges, in particular the long lifespan of tree species [17], which sometimes require decades for completion [14,16]. Therefore, faster and more cost-effective alternative approaches for screening and phenotyping trees are needed, especially for non-model species. In Ukraine, a method for identifying trees that are susceptible to root rot and those that are not has been patented. This is based on the stability of resin metabolism [18]. Seedlings obtained from seeds of trees in old disease areas and seedlings grown from seeds of seed orchards were used for in vitro experiments [19]. After inoculation with *H. annosum*, the progeny of trees from seed orchards showed a mortality rate almost twice as high as seedlings grown from seeds from old disease areas. This indicates that the progeny

of trees self-sown in old disease areas have a higher genetic resistance to annosum root rot. In Lithuania [20], a comprehensive assessment of pine plantations affected by root rot used the degree of damage to the trees as a criterion for persistence, as well as an index of plantation persistence determined by the ratio of infected to non-infected trees, taking into account the distance of the infected tree from the centre of the disease. According to researchers, the effectiveness of this criterion increases with the lack of close correlations between signs of resistance and growth [20]. In Norway, inoculations with the two fungi *Heterobasidion parviporum* and *Ceratocystis polonica* were carried out in two series of progeny tests, each containing complete sibling families planted at two locations and on parent strains in two seed orchards [21]. In both fungi, significant variation in lesion length was observed between families after inoculation, indicating dominant additive inheritance. Significant variations in lesion lengths between parent clones and within ramets of the same clone were also observed in seed orchards. In one series, a high positive correlation ($r = 0.88$) was observed between lesion lengths of *H. parviporum* in male parents and progeny, but not in female parents and progeny. The results support previous conclusions that genetic variation and heritability are large enough for practical resistance breeding [21,22].

The importance of molecular and biochemical testing should be emphasised. In particular, the potential contribution of molecular techniques such as genome-wide association studies (GWASs) or metabolomics profiling to future efforts to understand tree resilience at the genetic level should be emphasised. Martin et al. [23,24] have described in detail the use of FT-IR spectroscopy in combination with chemometrics to discriminate between resistant and susceptible elm species and clones before and after *O. novo-ulmi* infection. Conrad et al. [25] used the same methods to identify *Quercus agrifolia* resistant to the non-native and invasive pathogen *Phytophthora ramorum*. They are linked to practical aspects of protecting forest stands for future generations. The genetic markers were determined from resistant *P. sylvestris* pines [26]. These markers are used to confirm the increased resistance of pines that have maintained high viability against the background of the massive mortality of other trees. The method of the genetic labelling of pines in natural and artificial plantations with increased resistance to root rot involves the electrophoresis of isoenzymes and the determination of the frequency of genotypes that are significantly more abundant in the trees at the allozyme loci [27]. It is also worth noting that current methods (e.g., isozyme electrophoresis to detect genetic markers) have their limitations and that the development of new techniques, such as next-generation sequencing, will certainly improve our ability to more accurately and effectively identify trees, both resistant trees and pathogens that determine their health [28].

Increasing plant resistance to pests and diseases by priming plant resistance is an attractive plant protection concept as it provides long-term protection against a wide range of pests and diseases [29]. However, this article does not discuss the achievements of molecular [30–33], biochemical [34–37] or epigenetic research [38–41], but focuses on the practical aspect [42–44] in our case for forest managers.

The question we are asking is whether seedlings that germinate from seeds of potentially resistant trees (i.e., that grow in gaps in stands created by the death of other trees due to root damage by *Heterobasidion*) have different characteristics than seedlings that germinate but originate from seeds of trees infected with the pathogen, in other words, whether root pathogens in asymptomatic trees may have caused stress that triggered priming in the seeds so that the germinating seedlings differ from other seedlings (diseased and control seedlings) in terms of their external characteristics (morphological characteristics of the structure).

The roots of the trees growing in a plantation are intertwined and form an underground network [45–47] so that the fungus can spread from diseased to healthy trees through root contact [48–50]. The roots damaged by the pathogen supply the tree crown with less water and mineral salts, which in turn does not ensure the effective production of assimilates [16,51,52]. This is likely to lead to reactions that may affect the quality of the seeds and seedlings and influence the number of cotyledons. It is unlikely that the trees that remain viable in the disease centre created by the death of many trees will not affect each other through root

contact. The lack of visible symptoms may indicate that no roots are affected, allowing the tree to function effectively and possibly be resistant to the pathogen. However, root damage in pine trees can lead to a rapid decline in annual shoot and needle growth [1,8]. It is not known whether they possessed a genetically determined individual immunity or acquired it through prolonged contact with the pathogen. However, it seems likely that this information can be transferred via seeds to seedlings for the next generation [53,54].

An important finding about cotyledons is their role in seedling vigour and resistance to root diseases. Unfortunately, the exact mechanism for the correlation between the number of cotyledons and immunity is not yet fully understood. However, it cannot be overemphasised how cotyledons help to secure seedling energy reserves or immune response pathways. As previously written, the largest proportion of seedlings came from resistant trees with six cotyledons. This can be selected as a quantitative trait in breeding programmes, especially as it is probably heritable, but this needs to be confirmed in further studies. Although the significant differences in the number of cotyledons between the healthy groups are convincing, we decided to describe the biological implications of these results in more detail. It is likely that the diseased group with fewer cotyledons has a less efficient photosystem II, which affects the general condition and even the survival of the seedlings. This is due to the insufficient production of assimilates, which are necessary for all physiological processes of plants, especially for the response to stress or other factors affecting plant development (e.g., root nutrition). Kitajima [55] evaluated the relative importance of cotyledons for seedling survival using a factorial field experiment with three neotropical tree species. We concluded that the number of cotyledons may be beneficial for seedling survival in the first life stage. The supply of carbon from the cotyledons and other carbohydrate reserves apparently improved the seedlings' ability to cope with herbivory and disease. In general, chlorophyll is essential for maintaining the proper efficiency of photosynthesis and the production of sugars necessary for growth and development, as well as for the activity of immune processes. Therefore, the number of cotyledons can be a promising characteristic when selecting trees for planting in areas affected by root and butt rot.

Krinitzky et al. [56] proposed a new approach for the development of a method for the early diagnosis of the growth of offspring of loblolly pine plus trees. They characterised the cytogenetic variability of nucleolar activity of the meristem of plus pine seedlings. According to their data, fast-growing clones are characterised by the highest nucleolar activity. They found close correlations ($r = 0.897$) between the nucleolar activity of seedlings and the growth performance of plus trees and their progeny, which allows them to be used for the development of a new method for early growth diagnosis.

The previous studies [8] confirmed the stability of the level of mitotic activity in the roots of germinating seedlings of 'conditionally resistant' trees and their higher intensity compared to 'affected' trees (MI = 10.7% and MI = 5.6%, respectively, where MI is the mitotic index). It was found that the total percentage of seeds with six or more cotyledons was 84.6 in 'conditionally resistant' trees, while it was lower in 'diseased' and control trees (75.9 and 80.7%, respectively). In the present studies, the examination of the apical meristem cells under the microscope showed that the seedlings of resistant and diseased trees differ in cell distribution in different phases of mitosis (prophase, metaphase, anaphase, telophase) (Figure 14). Most differences between the seedlings of 'conditionally resistant' and 'diseased' trees were found in prophase (36.7 and 42.0%, respectively) and metaphase (32.7 and 25.7%, respectively).

Figure 14 shows the cells of the apical meristems of seedling roots under the microscope. It shows cells in different stages of mitosis (prophase, metaphase, anaphase, and telophase) [8].

We hypothesise that the differences between the root cells of 'resistant' and 'diseased' seedlings in prophase and metaphase may be due to the slow formation of the division spindle in the cells of seedlings grown from the seeds of 'diseased' trees or to the presence of chromosomal mutations and abnormalities in the normal course of mitosis. The observation

that diseased seedlings have longer stems but shorter roots is indeed intriguing and worthy of discussion. We hypothesise that this is related to the plant's response to stress, where plants under sub-optimal conditions invest more in shoot growth at the expense of root development [57]. This is related to the resource allocation of plants under stress conditions [58]. This explains how an imbalance between shoot and root growth occurs. In the long term, this is likely to have a negative impact on plant survival or resilience. For example, in a drought, those trees that have developed a deeper root system and reach the groundwater will survive [59].

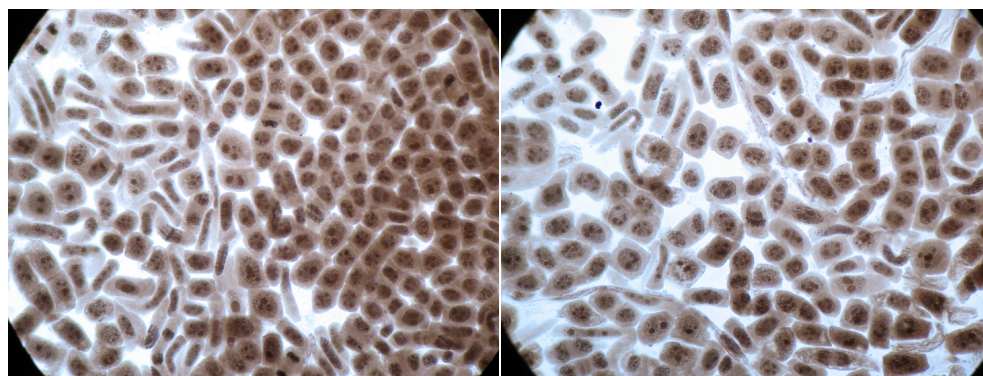


Figure 14. Phases of mitosis in apical meristems of roots of tree seedlings with different resistance to *Heterobasidion* under a light microscope (100× magnification).

The results of these studies indicate that the resistance potential of seedlings grown from ‘conditionally resistant’ seeds is relatively higher. The observed trends require further investigation, in particular, the study of the relationship between the number of cotyledons of the progeny and their morphometric parameters, especially the vigour of the root systems.

Balabushka [60] suggested using the density of seedling needles for the early diagnosis of conifer species. In his opinion, specimens with larger, denser needles are characterised by higher growth energy.

The analysis of the results of this study showed that trees classified as resistant are characterised by a slightly higher average number of cotyledons in the progeny and longer roots, produce heavier seeds, and germinate in greater numbers (better seed quality class), especially compared to seedlings from diseased trees. This is probably due to the fact that infected trees expend a lot of energy defending themselves against pathogens. Plants and pathogens have developed a dynamic interaction. While the plants try to survive after the attack of the pathogens with different mechanisms, the latter try to maximise food consumption to ensure their reproduction and dispersal [61,62]. In this context, the synthesis of photosynthesis—the energy source for both plants and pathogens—and its availability is a topic of the struggle for survival [63].

In the previous studies [64,65], it was found that biometric parameters of the reproductive organs of pines with different degrees of resistance to root rot were resistant trees that had a higher average cone weight than infected and control trees in a healthy closed forest stand. In addition, the mass of their 1000 seeds in the intercellular space was close to the mass of the healthy trees. These values were lowest in diseased trees. The biometric characteristics of one-year-old pine seedlings grown in a nursery from seeds of ‘diseased’ trees located mainly at the edge of the gap, ‘healthy’ trees located in the intercellular space outside the disease centre, and ‘conditionally resistant’ trees located in the gaps of the gaps showed that a characteristic feature of the one-year-old progeny of ‘conditionally resistant’ trees grown within the disease centre of the root rot is a much longer seedling root system, which may allow it to survive under conditions of high disease infestation [64–66].

The results obtained by Koba [67] show that with an increase in limiting factors (air temperature and precipitation), the survival rate of seedlings of *Pinus pallasianu* D. Don.

decreases due to seedlings with an increased number of cotyledons (more than 8). The best proportions of seedlings are those of resistant trees and those with 6 cotyledons. Neither a lower nor a higher number of cotyledons improves the proportion of survival. The production of seven to nine cotyledons probably leads to a consumption of seed reserves, which is not yet compensated by an increased photosynthesis due to a larger assimilation apparatus.

Similarly, the seedlings of resistant trees have longer needles and roots. Photosynthetic processes are more effective in these trees as they are not exposed to the stress of an infection of the root system [68].

The early diagnosis of heritable traits of trees potentially resistant to root rot that may be present in their seeds opens up the prospect of creating sustainable and productive plantations against a pathological background [69]. It is known that the peculiarities of the growth and development of pine seedlings depend to a certain extent on the number of cotyledons in the seed. It was found that pine seedlings with 6–8 cotyledons were relatively dense in number and had a better developed conduction and resin system, and after 20 years, they exceeded the stem volume by 12%–38% [70]. Other studies [71] indicate that seedlings with a minimal number of cotyledons are usually stunted, and seedling size is more related to the activity of the seedling apical meristem. The results of our previous studies, which confirm the conclusions of other scientists, indicate that individuals with increased resistance may be inferior to susceptible individuals in terms of growth intensity [72].

In the case of correlation analysis (e.g., seed weight and germination), the lack of correlation is interesting, but it is difficult for us to speculate on the possible causes of this phenomenon. However, there was a statistically significant difference in seed weight between the groups. We hypothesise that other factors (e.g., seed quality, environmental conditions) might be more decisive. This situation requires new hypotheses and further research. Perhaps seed weight does not matter in the first stages of germination until nutrients are depleted before the seedling is well rooted in the soil and takes over the physiological functions of absorbing water and mineral salts from the soil required for adequate photosynthetic efficiency.

Although the interaction between pathogens, insects, nematodes and other stressors that occur in pine forests has already been discussed somewhat, it is worthwhile to relate the overall picture of forest health management back to multitrophic interactions. For example, root rot fungi (mainly from the genera *Heterobasidion* and *Armillaria*) weaken the trees and release volatile substances that attract bark beetles. The synergy of the occurrence of pathogenic fungi and harmful insects (secondary pests) thus affects the health of the forest, including diseases, even in a so-called healthy forest.

As for the mention of FT-IR spectroscopy and chemometrics, we have already made the first attempts to use modern phenotyping tools on a larger scale, such as remote sensing or hyperspectral imaging, to assess the condition and resilience of forests [28]. We hope that this will open up new possibilities for the scalable, non-invasive monitoring of resilient trees.

It should be noted that the ecological consequences of promoting disease-resistant trees in monocultures need to be discussed. A healthy forest is a forest in which there are also diseased and dying trees that, decomposed by fungi, release nutrients into the soil that enter the carbon or nitrogen cycle. Compromises must therefore be made with regard to forest biodiversity and ecosystem services. One of these compromises is to leave a few percent of the trees (4%–7%) in the stands to decompose naturally. Selection for resistant genotypes can also undoubtedly have a negative impact on biodiversity. *H. annosum* has been found to produce substances that we can use to treat colon cancer. Therefore, its complete removal from the forest environment would be unfavourable for us from this point of view. From the point of view of the forest ecosystem, the situation is similar: the gaps in the monocultures created by the activity of the root pathogens are filled by other tree species (birch, maple, or oak), which has a positive effect on the genetic diversity (pool) at the organism level and thus on the resilience of the pine population.

Understanding the mechanism that prepares the pine tree for interaction with pathogens can be used for integrated pest management (IPM) programs in the future [73], which is why a quicker method to determine tree resilience would be useful. This is particularly important at a time when the use of pesticides is being phased out to maintain good environmental quality and human health [74,75]. By identifying early phenotypic markers for Scots pine resistance, this study aims to contribute to IPM strategies that minimise pesticide use while maintaining forest health.

6. Conclusions

Seedlings from a group of diseased trees infected with the root pathogen *Heterobasidion annosum* are of poor quality, which will have a long-term negative impact on the health, regeneration, and sustainable development of forests. If they create a stand susceptible to *H. annosum*, especially on former agricultural land, the first generation of pines must be replanted as the continuity of ground cover with tree crowns is lost. The forest will become patchy and will need to be cut prematurely to restore the forest area to productivity. This will lead to cascading changes in the forest ecosystem, including in terms of biodiversity, which should be avoided.

It turns out that the seedlings from the group of potentially resistant trees did not differ statistically from the control trees, which showed no symptoms of disease. Therefore, the resulting forest should be stable and long-lived and survive for future generations. Therefore, we believe that the proposed potential early diagnosis method has important aspects of practicability and long-term survivability. Therefore, it should be included in forest management programmes. The idea is to include this method in foresters' programmes for large-scale forest management in the future.

It has also been shown that resistant trees produce heavier seeds and occur in greater numbers (a higher-quality class). Resistant tree seedlings have longer needles and roots, shorter stems, and a lower stem-to-root ratio. Most seedlings come from resistant trees and those with six cotyledons. Observing the characteristics of germinating pine seeds could in the future serve as a method for the early diagnosis of pines that may be resistant to root diseases. We therefore believe that it is worth continuing this research in the long term to observe the long-term behaviour of the progeny of resistant and susceptible trees under different environmental conditions (e.g., drought, pathogen load).

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