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GENETICALLY COPYING POLAND'S OLDEST OAKS

Typically, a plant's genotype can be replicated through vegetative propagation. Oak trees, however, cannot be reproduced by this method. So how can we preserve the ancient genotypes of monumental oaks, many of which are dying?

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The ancient oak trees (*Quercus robur* L.) growing in Poland are up to about 800 years old. Unfortunately, their lives eventually reach their ends – for natural, or increasingly for human-caused reasons. For instance, the Napoleon oak, which until recently grew in the Lubusz region and had a circumference of 1052 cm (measured at a height of 1.3 m), died in 2010 as a result of arson. Our team noticed firsthand the rapid pace at which ancient oaks are now dying when we collected material during a five-year research project concerning the possibility of cloning monumental oaks using the micropropagation method. We discovered that several of these centuries-old trees died within the short period of the project or shortly thereafter. For example, the monumental Chrobry oak from the Szprotawa Forest District, which was estimated to be about 800 years old and from which we collected material in 2013, fell victim to arson the following year. It survived for another six years before finally succumbing in 2020. Another tree, the approximately 400-year-old Bolesław oak in the village of Bagicz in the Ustronie Morskie commune, was felled by a storm in 2016. The Mieszko I oak, the oldest oak tree in the Mazowieckie Voivodeship,

estimated at nearly 600 years old, was set on fire in June 2019. Among the three oldest oaks in the Rogalin Landscape Park (which has the largest cluster of centuries-old pedunculate oaks in Europe, with a total of about 1400 trees), one, the Czech oak, is now dead, while another, the Lech oak, is experiencing the disintegration of its main trunk.

Old oaks are certainly more vulnerable than younger trees to acts of vandalism. However, there are also other reasons why these ancient trees are dying. These include climate change, deteriorating water conditions, the presence of insect pests, pathogenic fungi, and improperly performed conservation work. Moreover, because old trees have a huge, sprawling crown, a cracked and hollow trunk, and at the same time a very small root mass compared to the above-ground part, they are increasingly susceptible to toppling over.

Copying genotypes

The monumental pedunculate oaks growing in Poland possess many valuable traits involving resistance to changing environmental factors, which have been shaped over hundreds of years. Furthermore, for local communities, these ancient trees are more than just an important element of the landscape. This means there is a desire to protect these trees, or at least their genes, which gives rise to the question of whether it is possible, using vegetative methods, to cultivate a seedling that possesses a genetically exact copy of the genotype



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The 800-year-old oak Rus, one of the oldest in the Rogalin National Park – which is home to Europe's largest cluster of such trees

of the mother tree. At the same time, preserving the genotypes of monumental oaks through cloning is a potential way of preserving biological diversity.

Vegetative reproduction is a technique in which a new plant is grown from a fragment or cutting of an old one. It can be used with many species of horticultural plants, as well as forest trees, poplars, or willows, and ensures the preservation of all the traits (genotype) of the mother plant. However, it is not possible in the case of oaks. Unfortunately, the non-woody shoots of these plants do not form roots. Neither are they formed by woody stem cuttings, layerings, or live stakes taken from oaks. In addition, the process of rooting oak shoots is particularly ineffective in the aged trees we studied. The method of grafting an oak sapling can be used, but it does not ensure

that a complete copy of the mother tree's genome is obtained, since in grafting the rootstock comes from another plant of the same species. Therefore, obtaining a copy of the entire oak genome appeared to require *in vitro* micropropagation techniques. In our research, we sought to answer the question of whether it is possible to initiate *in vitro* cultures of shoots from approximately 800-year-old oaks, to maintain them by multiplying the shoots in sterile jars, and then to root them also in *in vitro* cultures.

Micropropagation in glass

The method of growing plant tissue in sterile *in vitro* cultures utilizes a phenomenon called *totipotency* – the predisposition of even a single plant cell to

regenerate into a new, complete organism with stem and root. The starting material taken from the mother plant for such cultivation – known as an *explant* – can be a fragment of seed containing the growth meristem or a piece of leaf or shoot with a bud. To guide cell growth in a desired way, appropriate stimuli need to be supplied in the form of growth regulators, cytokinins or auxins, as well as macro- and microelements, vitamins, and sugar, which under normal conditions is produced entirely during photosynthesis.

In successive stages of *in vitro* cultivation, such guidance seeks to trigger leafy shoots to produce roots. They are placed in a medium containing growth regulators in appropriate concentrations, in the presence of activated carbon, with light exposure, and at room temperature. The final, difficult stage involves the acclimatization of seedlings from *in vitro* cultures to *ex vitro* conditions.

The process of acclimatization to *ex vitro* conditions should be gradual, as the shoot and root experience a shock from the sudden shift out of their previous environment. The leaves must learn to close and open their stomata, the roots to properly develop in solid substrate. The stomata of plants grown *in vitro* are unable to close, and so during acclimatization to *ex vitro* conditions, excessive transpiration occurs through the constantly open stomata of the delicate structure of leaves that developed back in the sterile jar. Furthermore, these leaves have been observed to have a different shape in plants grown in tissue cultures compared to those grown in greenhouses: generally, plants from *in vitro* cultures have thinner leaves, a poorly developed palisade mesophyll layer, large air spaces in the mesophyll, and fewer conducting bundles. Moreover, *in vitro* cultivation disturbs the synthesis of protective substances (such as wax), whose intense production only begins during the acclimatization process. Another important aspect of the acclimatization process involves changing the

feeding strategy from mixotrophic (sugar is taken from the agar medium and also produced during photosynthesis) to autotrophic, when sugar comes entirely from photosynthesis.

All this means that during acclimatization, the plant must quickly develop new leaves, or the leaves formed *in vitro* culture must quickly restructure their mesophyll tissue before they dry out due to an insufficient wax layer of the epidermal cell cuticle. The roots, too, change environment from soft agar to the hard reality of the potting soil. An effective acclimatization process must be gradual over a sufficiently long period of time, allowing the micro-seedlings to adapt to natural atmospheric conditions and undo the changes caused by *in vitro* cultivation. This is an extremely difficult period in the life of the plants, but if it is divided into stages during which the humidity is decreased gradually, if the substrate and containers are properly selected, and if the physiological condition of the plants is good, then about 80–90 percent of them survive.

Preserving the oldest oaks

In our study, we used woody shoots of pedunculate oaks – obtained from 21 of the most impressive and oldest oaks growing in Poland, having the status of monumental trees – as material for initiating at first vase cultures, and then *in vitro* cultures. The breast-height girth of the mother trees ranged from 470 to 1036 cm, and their approximate age, determined by dendrochronological studies, ranged between about 300–800 years (the ages of most of the monumental trees studied are estimated roughly in 50-year increments). The woody shoots were placed in vase culture at high humidity and a temperature of 20°C. Over four weeks, sprouts of some 2–20 cm grew from the epicormic (dormant) buds under the cork the woody shoots (the first stage of tissue rejuvenation). Small fragments of these shoots, about 2 cm long, containing 1–2 buds, were then used to initiate *in vitro* cultures. Initiating sterile *in vitro* cultures before placing explants on sterile agar medium requires that microorganisms (spores of fungi, bacteria) found on the surface or inside the explants must be eliminated. In our study, an appropriately concentrated solution of mercury chloride was used, followed by four rinses in sterile water. The sprouts were grown under *in vitro* conditions – facilitated using Woody Plant Medium (WPM), a mixture of chemical compounds containing essential macro- and microelements needed for plant growth – with cyclic transfers to fresh medium of the same composition (the second stage of rejuvenation). The medium was also supplemented with vitamins, amino acids, sugar (as an energy source), and growth regulators, mainly 6-benzylaminopurine (BAP). Appropriately developing shoots were induced to grow on a medium

Oaks in *in vitro* cultures



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supplemented with growth hormones, mainly auxin and cytokinin, and activated carbon. In the case of pedunculate oaks, well-rooted plants from *in vitro* cultures, which reached a height of about 8 cm and root length 10–15 cm, were transplanted into tall containers while straightening out the root twisted during growth in the jar, and into appropriately prepared solid substrate. The seedlings were placed conditions similar to those *in vitro*, with temperature about 20°C, light exposure and high humidity, which was then gradually reduced.

In our research, alongside the main goal of cloning approximately 800-year-old oaks using *in vitro* methods, we strove to answer the question: what was crucial for the survival of shoots in the first month of *in vitro* culture and their preservation in subsequent months? Was it the age of the ancient oaks from which they derived, or more the individual genotype of the particular mother tree? Our findings were somewhat surprising, as they showed that for trees of various ages, the genotype of the mother tree was the decisive factor. At the same time, even the oldest plants we studied also showed the highest predisposition for micropropagation. Moreover, we found that not all ancient oaks could be reproduced using the *in vitro* method. However, the developed method made it possible to propagate (obtain a complete seedling with shoot and root) half of the monumental trees tested. However, the developed method allowed for half of the tested monumental trees to be propagated successfully (defined as obtaining a complete seedling with shoot and root).

Happy ending

The oak clones that propagated well and formed rooted in *in vitro* cultures, well enough that we could grow a full two-meter-tall seedling, were planted next to their mother trees or in other places in Poland to preserve for future generations their original, centuries-old genotypes.

On 12 April 2019, the first clone of a monumental oak obtained by the *in vitro* method was planted. This was a four-year-old seedling of one of the Rogalin oaks, one of the oldest oaks in Poland at approximately 800 years, which found a home near the Palace Museum in Rogalin, the former residence of the Raczyński family and now a branch of the National Museum in Poznań. The next tree – a clone of the Chrobry oak (the mother tree is already dead) with a genotype that formed around 1000 years ago, around 1025, when Bolesław Chrobry was crowned – was planted on 7 October 2022, near the Museum of the First Piasts at Lednica.

Cones of the Wybicki oak – which is about 500 years old and grows on the former estate where Józef Wybicki, the author of the lyrics to Poland's national



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A seedling from the 800-year-old Rus oak tree, cloned in *in vitro* culture

anthem, was born – have been planted in several sites in Poland affiliated with Wybicki. The first two were planted next to the mother tree on the estate in Będomin (in 2019 and 2020), followed by another (in 2022) in Manieczki, where Wybicki died. A year later another was planted in Brodnica, which had been the site of Wybicki's grave for 100 years (before his ashes were moved to Poznań). In 2022, another clone of the Wybicki oak was planted in a park in Dobrzyca, the former estate of Augustyn Gorzeński, a friend of Wybicki's, followed by two more in 2023, near the Śrem Museum and Józef Wybicki Agricultural Schools in Grzybno.

Thanks to the collection of material and application of the micropropagation method under this project, clones of monumental oaks are also now growing on the premises of the PAS Institute of Dendrology in Kórnik and in the Arboretum of the Kostrzyca Forest Gene Bank. The authors of the project wish to thank the owners of the properties where we have been able to plant these oaks, the carriers of precious genetic heritage. ■

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Further reading:

Chmielarz P., Kotlarski S., Kalemba E.M., Martins J.P.R., Michalak M., Successful *In Vitro* Shoot Multiplication of *Quercus robur* L. Trees Aged up to 800 Years, *Plants* 12/2023, doi: 10.3390/plants12122230

Kotlarski S., Michalak M., Chmielarz P., Klonowanie najstarszych dębów pomnikowych rosnących w Polsce z wykorzystaniem metody *in vitro*, *Rocznik Polskiego Towarzystwa Dendrologicznego* 2019.

Martins J.P.R., Wawrzyniak M.K., Kalemba E.M., Ley-Lpez J.M., Mendes M.M., Chmielarz P., Calcium silicate mitigates the physiological stress induced by 6-benzylaminopurine during the *in vitro* multiplication of *Quercus robur*, *Industrial Crops and Products* 2023, doi: 10.1016/j.indcrop.2023.116377