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Importance and maintenance of *Nicotiana* genetic resources

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Abstract. The collection and maintenance of genetic resources of plants is the basis for protecting biodiversity of sources of genetic variation used in breeding. Genebanks collect and store plant material in a viable state and also conduct its evaluation. The most critical factor in maintaining viability is assessing the germination capacity of seeds, which depends on the plant species as well as on the conditions and duration of seed storage. The rate of viability loss varies between species but always increases with seed age. Reduced germination capacity indicates the need for seed regeneration of stored samples. Understanding the period during which seeds of a given species remain viable under specific storage conditions allows for optimizing the regeneration of samples in the collection. This study presents data on seed viability of species of the genus *Nicotiana* under different storage conditions. They indicate the significant influence of air temperature, seed humidity and oxygen content in the air on long-term seed storage.

The aim of this study was to present information on the conservation of genetic resources of the *Nicotiana* genus from different countries and their potential utilization in breeding programs. Many species of the *Nicotiana* genus are a valuable sources of resistance to viral, bacterial, and fungal diseases, as well as pests. Numerous resistance traits have been successfully transferred to cultivated tobacco (*N. tabacum*), frequently resulting in the development of new valuable varieties and breeding materials.

Keywords: genetic resources, *Nicotiana*, genebank, storage, seed viability

INTRODUCTION

The conservation and utilization of plant genetic resources are of critical importance for the sustainable agricultural production. The diversity of plant genetic resources provides the basis for adapting agriculture to changes, including ongoing climate change, natural disasters, and socio-economic developments such as urbanization and industrialization. According to the Convention on Biological Diversity, plant genetic resources are defined as genetic material that possesses not only actual but also potential value for food and agriculture (FAO, 2007; Gryziak et al., 2016). Systematic breeding efforts aim to improve resistance and quality traits by utilizing existing forms of cultivated plants and related species as sources of genetic variability. Genetic resources therefore represent great potential for developing new varieties enhanced with valuable traits, resulting into both economic and environmental benefits. The Food and Agriculture Organization of the United Nations (FAO) estimates that approximately 7.4 million accessions are stored in 1750 genebanks around the world. Genebanks collect, store, and maintain plant material for long periods of time in a genetically pure and viable state. They also protect against so-called genetic erosion, which leads to reduced biodiversity. The genebanks are also responsible for characterizing collected objects in terms of

traits that are useful for the creation of biological progress (e.g., quality improvement, resistance to biotic and abiotic stresses) and providing access to collection samples for scientific and breeding purposes (Gryziak, 2020; Salgotra, Chauhan, 2023). Over 90% of accessions maintained in the collection are stored as seeds. The main task of genebanks is to maintain these accessions in a viable state. The handling of samples in genebanks is guided by international standards, which cover seed drying and storage, monitoring their viability during storage, as well as regeneration, characterization, evaluation, documentation and distribution of collection samples (FAO, 2010). It is recommended that the seeds of each accession should be stored in two types of collections (Chojnowski et al., 2022; Czembor et al., 2017). The first is the base collection – the genebank reserve. Seeds in this collection are designated for long-term storage and are not intended for distribution. They are packed in airtight, moisture- and oxygen-impermeable containers made of glass, metal or aluminum foil and stored at -18 °C (Chojnowski et al., 2022; Czembor et al., 2017; Michałowska, Białoskórska, 2022). The second type is the active collection, which contains seeds intended for regeneration, evaluation and distribution to interested users, such as breeders, scientists and farmers. According to FAO recommendations, seeds from the active collection can be stored at temperatures between 5–10 °C and a relative air humidity of 15% ± 3%. Additional protection of valuable genetic material in the eventuality of loss of seed deposited in national genebanks is provided by so-called safe duplicates. These duplicates should be stored in a location away from the genebank holding the original samples. The quantity of seeds to be stored as a safe duplicate should be sufficient for the replication of a sample in the event of its loss in the primary genebank (FAO, 2024). Safety duplicates of crop seeds can also be deposited in the Global Seed Vault (Svalbard Global Seed Vault) located in Svalbard (https://www.croptrust.org/work/svalbard-global-seed-vault/). This facility houses duplicates of 1.3 million seed samples from nearly all countries and has the capacity to store up to 4.5 million samples. Permafrost and rock formations ensure that the seed samples remain safe at sub-zero temperatures and can survive disasters, whether natural or caused by human activity.

THE IMPACT OF STORAGE CONDITIONS ON SEED VIABILITY

The conditions under which seeds are stored are crucial for maintaining their quality and preserving germination capacity at an optimal level. In addition to ensuring safe storage, it is important to maximize the longevity of seed. Several factors determine how long seeds remain viable, including genetic and morphological traits specific to the species, as well as environmental and climatic conditions during the growth and development of the mother plant. Storage conditions, particularly temperature, humidity, and oxygen content in the air, also play a significant role (Nadarajan et al., 2023; Solberg et al., 2020; Rao et al., 2017).

Seed viability and vigor

The factors influencing the physiological potential of seeds are viability and vigor, which regulate the ability of seeds to express their life functions under both favorable and unfavorable environmental conditions (Marcos-Filho, 2015). Seed vigor, as defined by ISTA (International Seed Testing Association), is the sum of seed properties that determine the level of activity and performance of seeds during germination and seedling emergence under a wide range of environmental conditions. Thus, it is not a single measurable trait but a concept related to seed quality, encompassing the speed and uniformity of seed germination and seedling growth, the ability to germinate under adverse environmental conditions and the preservation of germination capacity after prolonged storage (ISTA, 2024). Seed viability is defined as the ability to germinate, expressed as the percentage of normally germinated seeds after a specified time, which leads to the development of a plant capable of reproduction, i.e., producing seeds (Rao et al., 2017; Sano et al., 2016). Germination capacity is fundamental in seed evaluation, both for assessing sowing value and for evaluating materials stored in genebanks (Fu et al., 2015; Hay et al., 2013). The germination test is the method recommended by ISTA and FAO and is widely used for assessing seed quality in genebanks, as it is accurate and reliable (Fu et al., 2015).

Depending on their tolerance to desiccation, seeds are divided into three categories: orthodox seeds (desiccation-tolerant), recalcitrant seeds (desiccation-sensitive), and intermediate seeds (moderately desiccation-tolerant) (Boczkowska et al., 2018; Roberts, 1972; Walters et al., 2013; Zhang et al., 2021). It is estimated that approximately 75–80% of angiosperms produce orthodox seeds, which can tolerate drying to a moisture content below 10%, typically lower than naturally occurring levels. Due to this low water content, orthodox seeds can be stored long-term at -18 °C, significantly extending their viability period (Ellis et al., 1991; Walters, 2015; Walters et al., 2013). Most cultivated plant species in temperate climate zones produce orthodox seeds (Boczkowska et al., 2018).

Seed germination capacity decreases with age, and the rate of viability loss varies among species, even under identical storage conditions (Nagel et al., 2010; Walters et al., 2005). Optimizing storage conditions allows seeds to remain viable for extended periods. However, the aging process cannot be completely inhibited, only slowed down, what requires seed regeneration in genebanks (Boczkowska et al., 2018). It is recommended to conduct regeneration as infrequently as possible due to its associated costs and the need to maintain genetic purity of the samples. This

underscores the importance of monitoring seed viability. While frequent viability testing results in some seed loss, it is important to note that a significant drop in viability may not be detected if monitoring is delayed. Consequently, advanced seed aging can lead to genetic changes or even the loss of the sample from the collection (Pathirana, Carimi, 2022). Regeneration should be performed when viability falls below 85% of its initial value or when the number of seeds remaining in storage is insufficient for three sowings of a representative population of the given sample. The assessment of viability changes in seeds from individual plant species subjected to long-term storage should be conducted in a precise and reliable manner (FAO, 2024; ISTA, 2024).

Seed longevity and aging

Seed longevity is defined as the maximum period during which seeds remain viable (Nadarajan et al., 2023; Probert et al., 2007). The viability of crop seeds stored long-term depends on storage conditions and varies between species (Nagel, Börner, 2010; Priestley et al., 1985; Steiner, Ruckenbauer, 1995; Walters et al., 2005). Seeds can retain viability for just a few days, several months, a few or several years or even decades (Zhang et al., 2021). Such significant differences in seed viability among species are linked to the rate of aging, which is influenced by molecular (Puchta et al. 2021), biochemical (Sano et al., 2015), physiological (Bewley, Black, 2014), and metabolic processes (Buitnik, Leprince, 2008). Slower germination and an increasing proportion of abnormally germinating seeds are symptoms of seed aging (Roberts, 1972; Sano et al., 2016; Walters et al., 2005). Despite extensive research on the mechanisms responsible for seed aging, the extent to which individual factors determine its rate has not yet been established (Boczkowska et al., 2018; Nadarajan et al., 2023; Sano et al., 2015). Seeds have developed two main defense strategies against stress factors: protection and repair systems. The protective mechanism includes the formation of glassy cytoplasm, which reduces the metabolic activity of cells by limiting molecular mobility (Buitnik, Leprince, 2008). Another mechanism involves the production of antioxidants, which prevent the accumulation of oxidized macromolecules. Meanwhile, the repair system removes damage accumulated in DNA, RNA, and proteins through the activity of enzymes activated during imbibition (Sano et al., 2015). Research indicates that LEA (late embryogenesis abundant) proteins play a role in stabilizing the glassy state of cytoplasm and in seed desiccation tolerance. Most *in vivo* studies show the protective function of LEA proteins in relation to osmotic stress. These proteins likely protect the embryo from desiccation and prevent protein aggregation. Reduced levels of transcripts encoding LEA proteins negatively affected seed longevity in *Arabidopsis* (Hundertmark et al., 2011) and pea (*Pisum sativum*) (Dehaye et al., 1997).

It is believed that one of the main causes of seed aging is reactive oxygen species (ROS) and oxidative stress resulting from their accumulation in cells. Reactive oxygen species cause damage to proteins, nucleic acids, sugars and lipids, disrupting cellular functions. DNA methylation also influences seed aging as demonstrated in studies on the aging process of wheat seeds (Singh et al., 2013) and *Arabidopsis* (Cho et al., 2012).

In recent years, research has focused on the role of small RNA molecules in the processes of seed aging and germination. A significant number of miRNA families have been shown to participate in seed germination, suggesting that they may also play a role in regulating the aging process during long-term storage (Puchta, 2020). Studies on the levels of most known and newly identified miRNAs in barley seeds stored long-term revealed that their levels remained stable regardless of whether the seeds were fully viable or exhibited very low germination capacity. It can thus be concluded that miRNAs are the only class of RNA that does not degrade in aging seeds and remains exceptionally stable (Puchta et al., 2021).

CONSERVATION OF *NICOTIANA* GENETIC RESOURCES

The genus *Nicotiana* is the fifth largest in the family Solanaceae, comprising over 80 species native to North and South America, Australia, certain Pacific islands and Africa (Knapp, 2020). The most well-known and widely cultivated species of the *Nicotiana* genus worldwide is cultivated tobacco (*Nicotiana tabacum* L.), which includes numerous varieties belonging to different types. The second species is Aztec tobacco (*Nicotiana rustica* L.), currently grown on a small scale in several countries (Popova et al., 2020). Some wild species (*N. alata*, *N. forgetiana*, *N. sandera*, *N. sylvestris*) are cultivated as ornamental plants (Depta, Doroszewska, 2016; Doroszewska et al., 2009). It is important to emphasize that *Nicotiana* collections hold a significant position in many genebanks worldwide. The largest such collection, comprising over 5300 accessions, is located in China at The Tobacco Research Institute (TRI) of the Chinese Academy of Agricultural Sciences (CAAS). This collection also includes the largest number of tobacco mutants. The genetic resources preserved at the institute are used in research on tobacco genetics and breeding, functional genomics and sequencing, among other topics (https://tric.caas.cn/en/index.htm; TRI, 2016). The second largest *Nicotiana* collection is maintained at the Central Tobacco Research Institute (CTRI) in India. It comprises over 3300 accessions belonging to *Nicotiana tabacum* and *Nicotiana rustica* as well as 60 wild *Nicotiana* species. According to the authors of the CTRI report (2024), this collection is evaluated and utilized in breeding programs aimed at developing varieties resistant to diseases and pests and those with desirable agronomic and quality

traits. The third largest collection in the world is housed at the Oxford Research Station in North Carolina, USA. This collection contains over 2100 accessions, including wild *Nicotiana* species, cultivated varieties, breeding lines and tobacco mutants (Lewis, 2020, 2021).

In Europe, *Nicotiana* collections are maintained in several centers. One of them is the National Center for Plant Genetic Resources (NCPGR) (https://bankgenow.edu.pl/) located at the Institute of Plant Breeding and Acclimatization – National Research Institute in Radzików, which houses over a thousand samples (Czembor et al., 2017). The active *Nicotiana* collection is stored at the Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy (Czubacka, 2022; Depta, Doroszewska, 2023), where the accessions are regenerated and evaluated (Czubacka, 2022).

A *Nicotiana* collection is also maintained in Germany at the largest genebank in Europe, the Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben (https://www.ipk-gatersleben.de/en/), which houses 590 accessions of this genus. Many *Nicotiana* collections are also maintained by private companies. These include Bergerac Seeds, Breeding, which holds the collection previously housed at the Tobacco Institute in Bergerac, France (https://www.bergeracsb.com/en/), and NiCoTa, located in Baden-Württemberg, Germany (Depta et al., 2023).

Numerous studies have focused on proper long-term storage of collections to protect preserved samples from viability loss and to prevent the limitation of genetic variability in the *Nicotiana* genus. These studies have particularly examined the effects of storage temperature and relative air humidity. Kincaid (1943) reported that tobacco seeds lost their germination capacity after three years when stored in containers in a laboratory. However, seeds stored in a freezer in sealed vials with various types of adsorbents retained their germination ability for 11 years. One of the earliest studies on seed viability involved storage in soil. Toole and Brown (1946) published the results of a long-term experiment examining the viability of seeds from different plant species buried at three different soil depths. Germination capacity was assessed over the 39-year duration of the experiment. Seeds of *N. tabacum* retrieved from all soil depths retained their germination capacity throughout the experiment. Among 107 species tested, tobacco was one of 36 species whose seeds retained viability in soil for 39 years. After 10 years, tobacco seeds showed the highest germination rates (40–75%), and after 39 years, they still germinated (17–22%), except for seeds stored in the shallowest soil depth. The first information on seed viability under off-field storage conditions was presented by Priestley et al. (1985), including data on tobacco. Seeds were stored in open systems at 13 different locations. The experiments used seed samples with high initial germination capacity (above 98%). The time required for the initial germination rate to decline to 50% (P50) was calculated. For *N. tabacum*, this time was 10 years.

The first studies on seed viability under controlled storage conditions began with the establishment of dedicated storage facilities in genebanks (Walters et al., 2005). These studies included 276 plant species stored for 16–81 years, including tobacco seeds. The germination capacity of *N. tabacum* seeds stored for 25 years at a temperature of 5 °C was 89%, with a P50 value of 81 years. In contrast, for *N. tabacum* seeds stored for 19 years at +5 °C and then for 26 years at -18 °C, the P50 value was 31 years, and germination capacity was 13%, compared to an initial germination capacity of 91%. Based on her own research and data available in the literature, Walters characterized tobacco as a species with variable viability.

An evaluation of the viability of *Nicotiana* seeds stored under room conditions in the active collection of IUNG-PIB and the genebank at IPK in Gatersleben was carried out (Agacka et al., 2013). Seeds of *N. tabacum* and *N. rustica* were stored for periods ranging from 2 to 12 years. Significant factors included differences in temperature and humidity during storage at the two locations. At IUNG-PIB, storage conditions were not constant; the air temperature in the room with seed storage cabinets ranged from 18 to 22 °C, and relative humidity was between 45% and 60%. At IPK, storage conditions were controlled, with an air temperature of 20.3±2.3 °C and relative humidity of 50.5±6.3%. The proportion of accessions with a germination capacity higher than 75% against seed storage time was calculated. After 2 years, this proportion was 90% in both storage facilities. However, a significant difference emerged after 12 years: the germination capacity of seed accessions stored at IUNG was below 75%, while the constant temperature and humidity conditions at IPK ensured that 40% of the tested accessions maintained germination rates above the desired storage threshold of 75% (Agacka et al., 2013).

At the Central Tobacco Research Institute in India, the viability of tobacco seeds stored under room conditions was also assessed, including storage with and without the use of desiccants. Seeds with an initial moisture content of 5–6% retained viability for 24 years when stored with a moisture-absorbing agent, with the seed moisture content remaining below 4.5%. In contrast, seeds stored without a desiccant lost viability within 24 months, with the seed moisture content reaching 6–7% (Rao et al., 2003).

The viability of *N. tabacum* seeds stored at 0 °C at National Center for Plant Genetic Resources (KCRZG) of IHAR-PIB in Radzików was also examined. After 10 years of storage at 0 °C, 70% of the tested accessions maintained germination rates above 75%. However, after 33 years, this number dropped to less than 10% (Agacka et al., 2014). Seeds stored in the genebank at -18 °C retained germination capacity much longer at levels that did not require regeneration. Similar studies were conducted on the *Nicotiana* collection maintained at the Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben, Germany. The viability of *Nicotiana* accessions was assessed

based on the percentage of seeds with germination rates above 75% against seed storage time. The study material included 226 *Nicotiana* accessions stored in a long-term facility for 14 to 39 years in hermetically sealed glass jars at -18 °C with a seed moisture content of 6%. The study demonstrated that over 90% of *N. tabacum* and *N. rustica* samples maintained germination rates above 75% after 20 years, and about 60% of samples after 38 years (Agacka et al., 2014). High germination capacity was also demonstrated by seeds of 117 *Nicotiana tabacum* accessions stored in a genebank in Bulgaria (Desheva, 2016). After 13 years of storage at -18 °C and a seed moisture content of 3.3%, germination capacity decreased slightly, from 97% to 93%. Even after 22 years, the tested samples still exhibited very high germination rates of 90%.

It is of great importance to conduct research on the maintenance of seed viability, including those of the *Nicotiana* genus, in order to establish the optimal conditions and duration for long-term storage. Knowledge about the collected genetic resources enables their protection against loss of viability and genetic purity. Furthermore, it permits the utilization of available genetic diversity in both breeding programs and fundamental research. Species of the *Nicotiana* genus are utilized in breeding as sources of resistance to diseases or as a source of cytoplasmic male sterility (Berbeć, 2001; Berbeć, Doroszewska, 1992; Berbeć, Laskowska, 2005). They are often studied as model plants in phytopathological research (Bui et al., 1992) and in genetic transformation in higher plants (Baulcombe, 1999; Lewis, 2011).

COLLECTION AND UTILIZATION OF *NICOTIANA* GERMPLASM RESOURCES

The *Nicotiana* collection housed in the Department of Biotechnology and Plant Breeding at IUNG-PIB in Puławy, overseen by the National Center for Plant Genetic Resources (KCRZG) at IHAR-PIB, is one of the largest in the world. The Polish collection of *Nicotiana* genus was created by professor Lucjan Kaznowski, who initiated the collection of the first cultivars and wild species for breeding purposes in the 1920s. Over the years, the collection has been systematically expanded (Czubacka, 2022). Currently, it comprises 1008 samples from various regions of the world, including 780 varieties of cultivated tobacco (*Nicotiana tabacum* L.), 83 varieties of Aztec tobacco (*Nicotiana rustica* L.), and 145 samples of wild *Nicotiana* species, polyploid forms, and valuable breeding lines. These collection samples were gathered through seed exchanges with numerous research institutions from over 30 countries. More than 31% of *N. tabacum* varieties originate from Polish breeding centers (Laskowska, 2007). The varieties and breeding lines of *N. tabacum* represent genetic material with high diversity, covers all types of tobacco. Wild *Nicotiana* species has a wide geographic range, and are native to North and South America, Australia, Pacific islands, and Africa. They exhibit significant variation in morphological traits, chromosome numbers, chemical composition, disease resistance, and other traits (Depta et al., 2023; Doroszewska et al., 2009). Accessions of the *Nicotiana* genus stored in the genebank are systematically characterized in terms of their morphology, genetics and resistance to viral and fungal diseases (Doroszewska et al., 2009). This allows their effective use in tobacco resistance breeding programs (Berbeć, Doroszewska, 2020; Depta et al., 2023).

Many species of the *Nicotiana* genus are valuable sources of genetic resistance to viruses, including potato virus Y (PVY) (Czubacka, 2022; Depta et al., 2020; Doroszewska, Czubacka, 2008; Doroszewska, Depta, 2011; Korbecka-Glinka et al., 2017), tomato spotted wilt virus (TSWV) (Depta et al., 2021; Laskowska, Berbeć, 2006; Laskowska et al., 2013) and tobacco mosaic virus (TMV) (Depta et al., 2018). Samples from the *Nicotiana* genus have also been studied for resistance to the most significant fungal disease in Poland, black root rot, caused by the fungal pathogen *Berkeleyomyces basicola* (formerly *Thielaviopsis basicola*) (Berbeć, Trojak-Goluch, 2001; Doroszewska, Przybyś, 2007). Resistance to this pathogen has been successfully transferred from *Nicotiana debneyi* and *Nicotiana glauca* into cultivated tobacco varieties. As a result of these breeding efforts, new resistant varieties have been developed (VRG 1, VRG 2, VRG 4, HTR 2, HTR 3, VRG 5TL, Wigola) (Berbeć, 2006; Berbeć, 2007; Trojak-Goluch, 2014). Genetic resources from the *Nicotiana* genus are widely used in tobacco resistance breeding conducted at IUNG-PIB in Puławy. For example, resistance to potato virus Y was transferred from the wild tobacco species *N. africana* into the cultivated tobacco variety BP-210 (Doroszewska, 2010), resulting in lines tolerant to the most virulent strains of this virus. *Nicotiana alata*, as the only source of resistance to tomato spotted wilt virus, has been utilized in breeding resistant varieties (Gajos, 1981) as well as breeding lines (Korbecka-Glinka, 2019; Laskowska, Berbeć, 2006).

The examples of the utilization of genetic resources from the *Nicotiana* genus described above highlight their great importance in the preservation of biodiversity and in breeding programs. In the context of a narrowing gene pool, it is crucial to establish protocols for the optimal storage condition of collection samples, ensuring seed viability at the desired level.

SUMMARY

Plant genetic resources are of particular importance for preserving diversity in the natural environment, especially in view of the systematic reduction in the gene pool, among others, due to breeding work. In the current situation, the primary task of genebanks is to protect genetic diversity

through proper storage and regeneration of samples as needed, while maintaining genetic purity. Factors influencing the physiological potential of seeds are their viability and vigor, which regulate their ability to express life functions. Seed viability is determined by genetic, morphological, and habitat factors of the mother plant. During storage, temperature, humidity and oxygen content in the air are critical. The most significant parameter in seed evaluation is germination capacity, which changes with age, while the rate of seed aging is linked to molecular, physiological, and metabolic processes.

The evaluation of seed viability in *Nicotiana* species stored under varying conditions at different locations (IUNG-PIB in Puławy, IHAR-PIB in Radzików, and IPK in Gatersleben, Germany) revealed that storage temperature was a significant factor influencing seed viability. Decreasing the temperature from 20 °C to 0 °C extended the average seed storage period from 10 to 30 years. Further reducing the temperature from 0 °C to -18 °C increased the storage period by an additional 20 years, assuming a germination capacity threshold of above 75%. Seasonal fluctuations in air humidity also had a substantial impact on the reduction of tobacco seed viability.

Proper seed storage in collections and knowledge about the preserved genetic resources allows the utilization of available germplasm in breeding programs as well as in basic research.

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