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CRYOPRESERVATION OF GENETIC RESOURCES OF CHOSEN SPECIES OF WILD FRUIT TREES AND SHRUBS

Wild fruit trees and shrubs are integral components of forest ecosystems in Europe. They are characterized by high-quality wood and slow growth. Their fruits and seeds are eaten by forest animals. Wild apple (*Malus sylvestris*), wild pear (*Pyrus communis*), wild cherry (*Prunus avium*), and hazel (*Corylus avellana*) carry the valuable genetic material of ancestors of currently cultivated varieties (cultivars) of fruit trees and shrubs. They are rare and endangered in Europe, also in Polish forests, so their seeds need to be conserved ex situ, in gene banks.

This study was aimed to verify 2 hypotheses: (1) seeds of the 4 selected species tolerate severe desiccation (over silica gel) and the temperature of liquid nitrogen (LN, -196°C) within a wide range of seed moisture content; and (2) differences in moisture content during cryopreservation cause differences in global cytosine methylation in DNA of seeds and the seedlings that develop from them.

For each of the studied species, the high-moisture freezing limit (HMFL) was assessed after storage in LN for 24 h. The safe range of moisture content was determined for embryonic axes of *Corylus avellana* and seeds of the 3 fruit tree species after storage in LN for 2 years.

For *C. avellana*, whose seed sensitivity to LN was poorly studied before, the alternative method cryopreservation was developed based on embryonic axes isolated from seeds in this study. Plantlets were next regenerated from embryonic axes on agar media in vitro. Thus it was necessary to identify the optimum growth conditions of embryonic axes (without cotyledons) in vitro: concentrations of growth hormones as well as macro- and micronutrients.

The response of seeds and embryonic axes to the temperature of LN was studied by means of germination tests and seedling emergence tests as well as (for the first time) epigenetic analyses. Thin-layer chromatography was used to assay global cytosine methylation (m^5C) in DNA of seeds, embryonic axes, and 3-month-old seedlings developed from LN-treated seeds.

Results of this study indicate that drying of seeds of *M. sylvestris* to a moisture content of 4.2% did not reduce their germination significantly. However, at such a moisture content, seedling emergence from thawed seeds was significantly lower, as compared to the non-frozen seeds (reduction from 96% to 73%). In contrast to *M. sylvestris*, seeds of *Pyrus communis* and *C. avellana* did not loose the potential for seed germination and seedling emergence, even after desiccation to very low moisture levels (2.5% and 2.7%, respectively). Thus they can be classified as orthodox seeds. Nevertheless, results of this study reveal also that the initial drying of seeds of *Prunus avium*, from 17.8-19.8% to about 11-12%, caused a significant decrease in seedling emergence (for the 2 studied provenances: from 73% to 16% and from 89% to 10%), and can be classified as suborthodox (intermediate) seeds.

The safe range of seed moisture content after freezing in LN for 24 h was 6.2-19.4% for *M. sylvestris*, and 6.7-20.5% for *Pyrus communis*. LN-treated seeds of *Prunus avium* in stones and dehusked seeds of *C. avellana*, after rewarming, had a significantly lower seedling emergence than control seeds. LN-treated seeds of *C. avellana* with a moisture content of 7.3% in shells fully tolerated freezing in LN. This study shows that apart from seeds, also embryonic axes of *C. avellana* can be safely cryostored, and the most effective medium (of the 4 media tested) for their conversion into complete plantlets was the Woody Plant Medium (WPM) with a cytokinin, 6-benzylaminopurine (BAP, 0.8 mg/l).

HMFL reached 19.4% for *M. sylvestris* and 20.5% for *Pyrus communis*. HMFL for *Prunus avium* was 14.9% if the seeds were first dried and next moistened before freezing in LN, or 19.8% if the seeds were only slightly dried after collection. HMFL for *C. avellana* was 9.2% for dehusked seeds and 7.3% for seeds frozen in LN in shells.

Seeds of the 3tree species maintained a high seedling emergence after 2-year storage. Also for embryonic axes of *C.avellana* stored in LN for 2 years, seedling emergence rate in vitro was the same as for non-stored axes.

Epigenetic research showed that global cytosine methylation in LN-treated and control pear seeds did not differ significantly when the moisture content of cryostored seeds fitted within the range of 5.3-16.6%. However, it was significantly lower than m^5C in control seeds when their moisture content during freezing reached 20.5%. Similarly, the safe range of moisture content of pear seeds during cryopreservation could be determined on the basis of an analysis of m^5C level in 3-month-old seedlings developed from the seeds (treated with LN or not).

In this study, for the first time, results of germination tests and seedling emergence tests after cryopreservation were correlated with methylation level in LN-treated seeds and the

seedlings developed from the seeds. The results of epigenetic research showed that seeds of *Pyrus communis* can be cryostored safely at seed moisture content of 5.3-16.6%.

For *C. avellana*, epigenetic research revealed that the very decrease in moisture content of seeds from 37% to 7.3% caused a significant decrease in global cytosine methylation of the embryonic axes isolated from them: from 6.38% to 4.71%. LN treatment of seeds of *C. avellana* with a moisture content of 7.3% did not cause any changes in m^5C in embryonic axes, as compared to control seeds wit the safe moisture content.

The presented dissertation provides evidence that long-term seed cryopreservation in gene banks is possible without any loss of viability for all the studied species. Moreover, for the first time it has been proved that severe desiccation of seeds and their freezing at the temperature of LN, may induce changes in methylation level. In most of the species studied here, moderate desiccation of seeds caused a decrease in the global methylation level in seeds, while severe desiccation over silica gel resulted in a substantial increase in m^5C. By contrast, LN treatment decreased the methylation level. Safe ranges of moisture content of control (non-frozen) seeds, based on seed germination and emergence, were corrected on the basis of changes at the epigenetic level.

Results of the present study, describing seed sensitivity to desiccation also at the epigenetic level, enable assignment of the studied species to a correct seed category. Besides, they form the basis for development of safe methods of long-term ex situ conservation of genetic resources in LN in gene banks to protect the biodiversity of Polish and other European forests.