SEED PHYSIOLOGICAL RESPONSES IN SOME DICOTYLEDONOUS PLANTS FOLLOWING LIQUID NITROGEN (-196ºC) EXPOSURE

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ABSTRACT. Seeds cryopreservation is one of the modern methods used to preserve Plant Germplasm Resources. We followed the seed germination of alfalfa, *(Medicago sativa) and* pea, *(Pisum sativum),* after seed cryopreservation by immersion in liquid nitrogen (LN) (-196 ºC) for five minutes, one hour, one day, one week and one month. The germination haven't been affected negatively anyone was the duration of immersion. In case of alfalfa the seed germination increased after seed cryopreservation.

Keywords: cryopreservation, dicotyledonous plants, alfalfa, pea, germination, seed hardness, abnormal seedlings

INTRODUCTION

Biodiversity is a source of significant economic, aesthetic, health and cultural benefits which form the foundation for sustainable development. However, there is general scientific consensus that the world is rapidly becoming less biologically diverse in terms of genes, species and ecosystems.

Genetic resources are disappearing at unprecedented rates. It is estimated that up to 100 000 plants, representing more than one-third of the world's plant species, are currently threatened or face extinction in the wild (BGCI, 2005). Experts estimate that as much as 8 percent of all plant species could disappear in the next 25 years. Over the past 50 years, new uniform crop varieties have replaced many thousands of local varieties over huge areas of production (Bioversity International, 2006).

The scale of human impacts on biological diversity has been increasing because of world-wide patterns of consumption, production, trade; agricultural, industrial and settlements development; and human population growth. Reducing the rate of biodiversity loss and conserving still existing biodiversity forms the basis of sustainable development and remain major global challenges.

Preservation of the plant biodiversity is essential for classical and modern (genetic engineering) plant breeding programmes. Moreover, this biodiversity provides a source of compounds to the pharmaceutical, food and crop protection industries.

It is now widely accepted that plant biodiversity conservation can be done on-site (*in situ*), and off-site (*ex situ*). An appropriate conservation strategy for a particular plant genetic resource combines the different *ex situ* and *in situ* conservation techniques available in a complementary manner (Engelmann and Engels 2002). *In situ* and *ex situ* strategies, includes a range of classic and relatively new alternative methods and techniques for the different genetic resources (i.e. cultivated species, including landraces and modern varieties, wild relatives, weedy types, etc.). Selection of the appropriate method or techniques should be based on a range of criteria, including the biological nature of the species in question, practicality and feasibility of the particular method chosen (which depends on the availability of the necessary infrastructure) as well as the cost-effectiveness and security afforded by its application (Maxted *et al.* 1997).

Since the 1970, large numbers of landraces and wildrelatives of cultivated crops have been sampled and stored in *ex situ* gene banks. It is estimated that six million samples of plant genetic resources are held in national, regional, international and private gene bank collections around the world (IPGRI, 2004). Traditional approaches to genetic resources conservation have relied on the *ex situ* storage of seeds, where the seeds are dried and stored at low temperatures in genebanks. Nowadays cryopreservation technologies are used in many of them. Important progress in cryobiology was achieved in the second half of the previous century. Over the past decades, plant cryopreservation technologies have been evolving rapidly, giving the possibility of long-term storage of valuable genetic resources of many crop and species. Cryopreservation allows virtually indefinite storage of biological material without deterioration over a time scale of at least several thousands of years (Mazur, 1985).

Cryopreservation, or freeze-preservation at ultra-low temperature in of liquid nitrogen (LN)(-196 °C), is a sound alternative for the long-term conservation of plant genetic resources since biochemical and most physical processes are completely arrested under these

conditions. Therefore, plant material can be stored for unlimited periods. Moreover in addition to its use for the conservation of genetic resources, cryopreservation proved to be extremely useful for the safe long-term storage of plant tissues with specific characteristics, such as medicinal- and alkaloid-producing cell lines, hairy root cultures, and genetically transformed (Elleuch *et al.*, 1998) and transformation-competent culture lines (Gordon-Kamm *et al.*, 1990). Recently, it was also proven that cryotherapy can be successfully applied to eradicate viruses from plum, banana and grape (Brison *et al.*, 1997; Helliot *et al.*, 2002; Wang *et al.*, 2003). Cryopreservation in liquid nitrogen can reduce cost, space, and labor while maintaining genetic integrity.

Specific procedures have been proposed in time for the cryopreservation of a wide range of tissues and organs, such as cell suspensions, embryogenic callus, meristematic tissues, pollen, seeds and embryo axes.

However, despite the fact that cryogenic procedures are now being developed for an increasing number of recalcitrant seeds and *in vitro* tissues/organs, the routine utilization of cryopreservation for the preservation of plant biodiversity is still limit and new researches are needed in order to find species and cultivars characteristic responses to LN exposure.

MATERIAL AND METHODS

The aim of the present work was to explore the feasibility of seed cryopreservation as a tool for alfalfa *(Medicago sativa),* pea *(Pisum sativum),* genetic resources conservation. Therefore, seeds of present varieties of these *dicotyledonous plants* (Ardelean and Mohan, 2006) were subjected to desiccation and immersion in liquid LN.

The seeds of these species are considered orthodox (Hong *et al*., 1996).They can be dehydrated to low water content and can be stored at low temperature for extended periods (Roberts, 1973).

Seed drying conditions were as follows: at 25ºC and 10-15% relative humidity in drying cabinet.

Moisture content (MC) was determined gravimetrically, based on two replicates maintained during 1 hour in an oven at 130ºC and presented on a fresh weight basis (%MC). The seeds of alfalfa, *(Medicago sativa)* were desiccated to 6, 1% MC and those of pea, *(Pisum sativum),* to 7 % MC.

Dried seeds samples were set into cryo-tubes and immersed directly in LN for five minutes, one hour, one day, one week and one month. Frozen seeds were thawed in a water bath at 37-40°C for 1 min (fast rewarming).

Their responses to LN were evaluated based on 2 characters: germination energy and germination capacity. Before and after cryopreservation, seed germination in each species, in control and cryopreserved samples, was studied.

Alfalfa, *(Medicago sativa)* germination tests, according to the ISTA Rules (2006), were performed at 20±1°C, between paper (BP), 10 days, using a germination cabinet. Pea, *(Pisum sativum,)* germination conditions were: 20±1°C, 8 days, BP.

The germination energy was determined after 4 day in case of alfalfa,*(Medicago sativa)* and after 5 day in case of pea *(Pisum sativum).*

Observations were carried out to determine germination capacity as percentage of normal seedling developed at the end of germination test. Each of the individual essential structures that have developed during the above mentioned test period and the seedling as a whole were evaluated according to the ISTA (2003).

Normal seedling, hard seed, dormant seed, abnormal seedling and dead seeds were recorded.

RESULTS AND DISCUSSIONS

As a result of alfalfa *(Medicago sativa)* cryopreservation in LN, (-196 ºC), germination energy increased in all cryopreserved samples with 9-16% (Fig. 1).

The stimulatory effect of cryogenic temperature on germination capacity was also observed in cryopreserved samples which varied between 86 and 88 %. The highest level of germination capacity was recorded in case of sample immersed in LN for 5min. Germination capacity increased with 11-13 % due to germination of hard seeds (Fig. 1).

No significant differences were found in their level of dead seeds comparing to that of control samples.

The intensity of seed hardness varies among the legume species and within the seeds of the same sample, possibly due to the differences in their seed coat structures, physical and chemical properties (Kelly et. al., 1992; Morrison et. al., 1998). These differences might be reflected in seed response to LN exposure. As verified in this work, species control samples (non immersed seeds) seed hardness was 13% and reduced in immersed samples to 1% or 0 %.(Fig. 1). Repeated cycles of cooling in LN and rewarming have been also reported to remove seed hardness in *Trifolium arvense* (Pritchard et all, 1988).LN exposure significantly enhanced germination in *Bowdichia virgilioides* and *Pterodon emarginatus* (Fabaceae) and *Apeiba tibourbou* (Tiliaceae) with variable numbers of hard seeds in the samples (Salomão, 2002). Resultes in criopresercation of alfalfa germplams were also reported by Cachita and Craciun (1995)

No significant differences in germination energy or germination capacity of pea, *(Pisum sativum)* samples after seed criopreservation by immersion in liquid nitrogen (LN) (-196 ºC) for five minutes, one hour, one day, one week and one month were found comparing to that of control samples (Fig. 2).

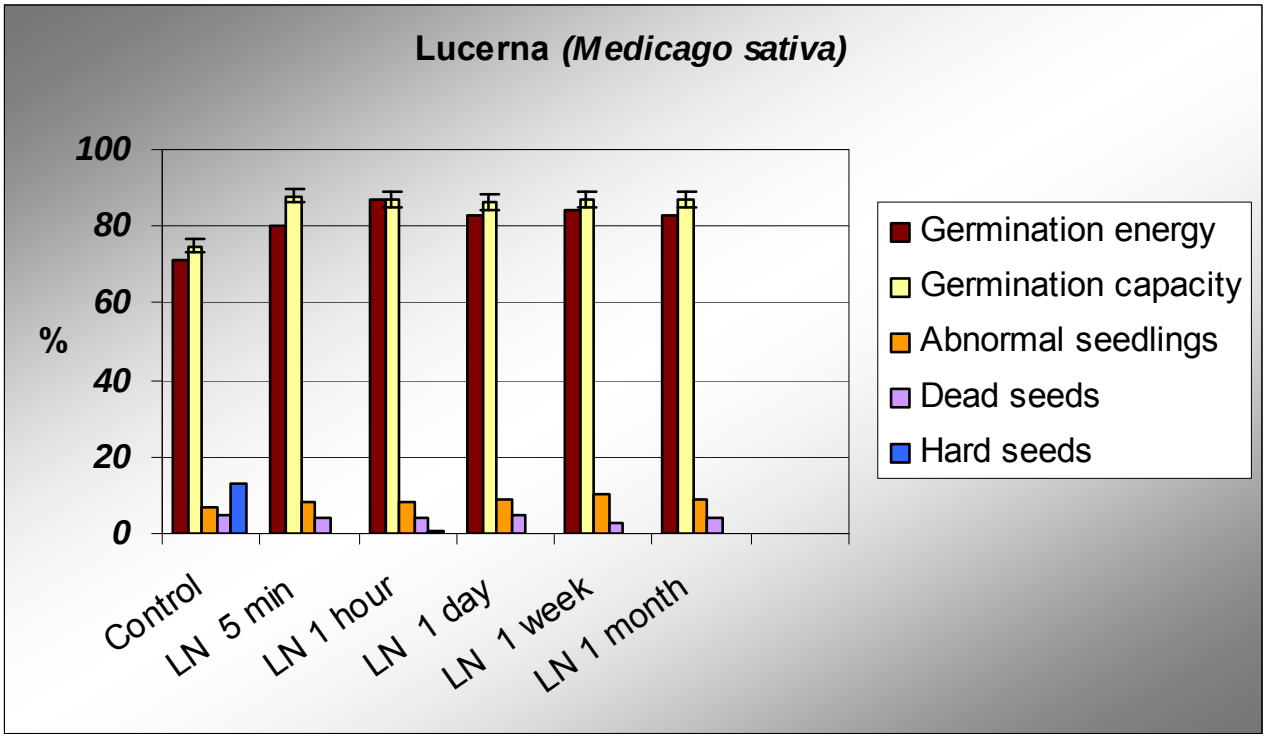

Fig. 1 Effect of cryopreservation in LN (-196 ºC) for five minutes, one hour, one day, one week and one month on alfalfa *(Medicago sativa*) seed samples germination. Control = seeds not cryopreserved

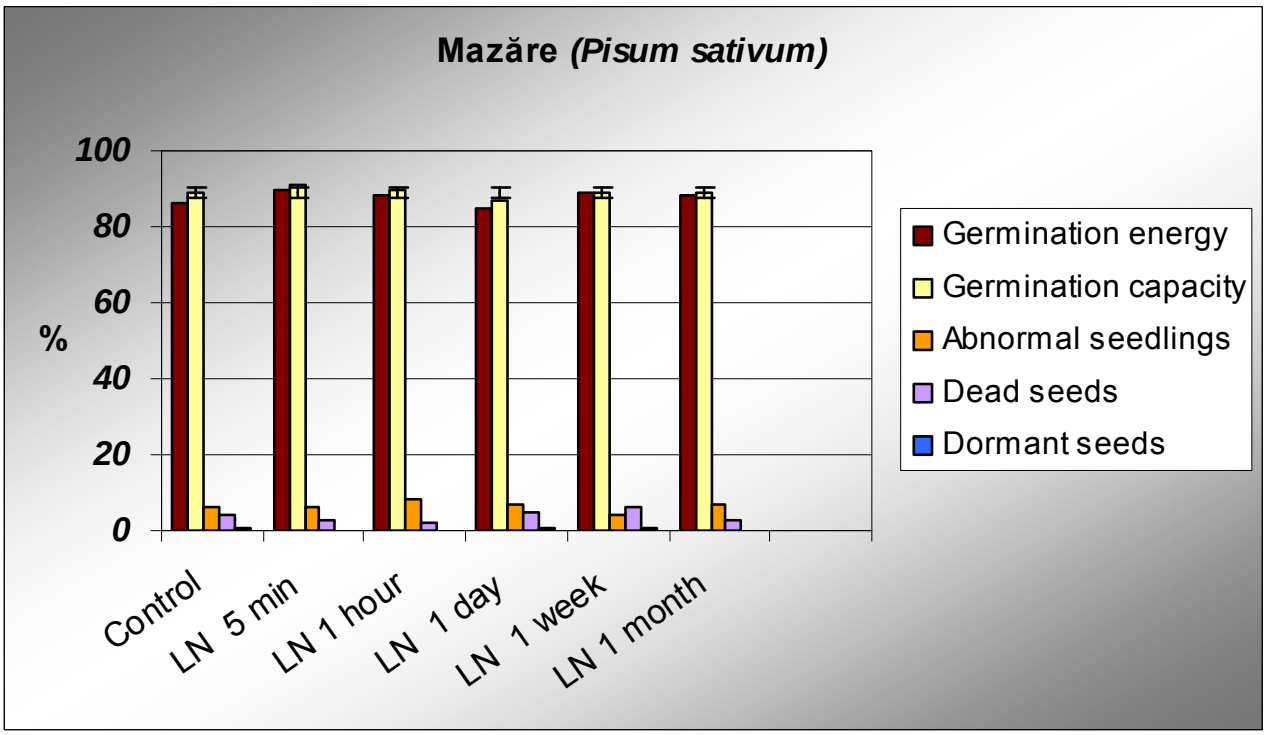

Fig. 2 Effect of cryopreservation in LN (-196 ºC) for five minutes, one hour, one day, one week and one month on pea, *(Pisum sativum),* seed samples germination. Control = seeds not cryopreserved

The seeds of pea survived after storage in LN (-196 °C) with germination of 85-90% comparable to that of the desiccation control.

The highest level of germination capacity was recorded in case of sample immersed in LN for 5min.The same situation was found in alfalfa germination test.

No significant differences were also found in their level of abnormal seedlings and dead seeds.

The percentage of dormant seeds in case of this pea varietes was too low and its variation in immersed samples was not relevant.

Grout and Crisp (1994) suggested that if cryopreservative techniques are to be evaluated for the storage of recalcitrant seeds, false impressions of efficacy might be gained by reliance solely on germination testing. No loss of germination following LN (-196 °C) cryopreservation was recorded but seedlings grown to maturity after the slow cooling treatment showed a greater proportion of morphological abnormalities than a control sample or those that had been rapidly cooled.

Percentage and type of abnormal seedlings were studied in our experiment in both alfalfa *(Medicago sativa)* and pea, *(Pisum sativum*) cryopreserved samples.

No significant differences in their level of abnormal seedlings comparing to that of control samples were found (Fig1-2).

Examples of abnormal seedlings in control and cryopreserved samples at the end of germination test are done below (Fig.4-6)

Fig. 3 Abnormal seedlings (necrotic cotyledons- left and stunted root-right) in alfalfa, *(Medicago sativ,)* control sample at the end of germination test

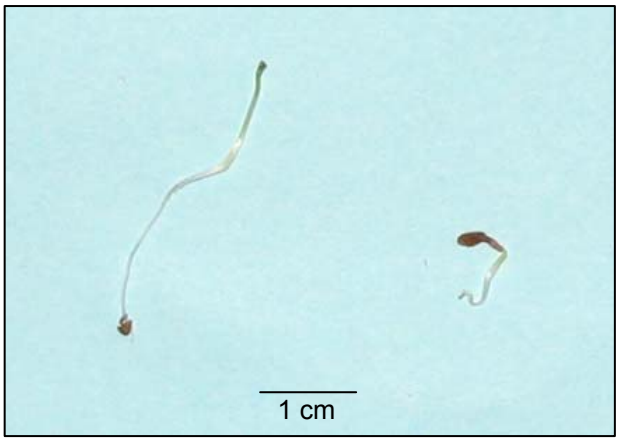

Fig. 4 Abnormal seedlings (detached cotyledons- left, necrotic cotyledons and stunted root-right) in alfalfa, *(Medicago sativa)* cryopreserved samples (LN) (-196 °C) at the end of germination test

Fig. 5 Abnormal seedlings (the root and the shoot system are missing) in pea, *(Pisum sativum*), control sample at the end of germination test

Fig. 6 Abnormal seedlings (the shoot system is missing and the primary root is retardet) in pea, *(Pisum sativum*), cryopreserved samples (LN)(-196 °C) at the end of germination test

CONCLUSIONS

The germination haven't been affected negatively anyone was the duration of cryopreservation in LN (-196 °C). No significant difference in pea seed germination energy or in germination capacity was recorded. In case of alfalfa seed the stimulatory effect of cryogenic temperature on germinability was observed. The germination capacity increased after cryopreservation due to the hard seeds germination.

No significant differences in the level and type of abnormal seedlings comparing to that of control samples were found.

The results obtained suggest that cryopreservation is a sound and feasible alternative for storing both of the seed species tested.

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