The effect of de-acclimation and re-acclimation treatments on winter rapeseed cold resistance *in vitro*

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Winter rapeseed (*Brassica napus* L.) is one of the most important crops in Lithuania. Winter hardiness is a complex trait limiting cultivation of winter rapeseed to the regions of temperate climate. Cold resistance of four winter rapeseed cultivars *in vitro* and their ability to recover after de-acclimation and re-acclimation periods were investigated at the Laboratory of Agrobiotechnology of Lithuanian University of Agriculture. Shoots were acclimated at 4 °C for 14 days and transferred to freezing temperatures. For study influence of de-acclimation period on rapeseed cold resistance acclimated shoots were de-acclimated and subjected to freezing temperature with and without repeated acclimation.

A significant decrease in frost resistance of shoots after 14 days de-acclimation period for all tested cultivars has been obtained. Repeated acclimation followed after de-acclimation period resulted increasing the shoot ability to cold re-acclimation. Exogenous sucrose has positive effect on winter rapeseed cold resistance, but repeated acclimation is more important for shoot recovery after de-acclimation.

Key words: winter rapeseed, shoot injury, de-acclimation, re-acclimation.

Introduction. Low temperature is a major environmental stress for many crops worldwide. One of the most important agronomic traits in winter rapeseed (*Brassica napus* L.) is winter-hardiness or freezing tolerance, which can be enhanced by cold acclimation (Rife, Zeinali, 2003). During cold acclimation, several physiological changes occur, including alteration of lipid composition in plasma membranes, accumulation of protecting compounds, such as carbohydrates, free amino acid or other osmolytes and induction of new gene activity (Hughes, Dunn, 1996).

Many important cultivated plants, such as potato, tobacco, and maize, have a limited capacity to survive temperature below freezing. In contrast, leaves of plants such as cabbage, lettuce, and spinach can develop tolerance to below-freezing temperatures in response to low but above freezing temperatures. Significant differences in freezing tolerance can be seen even after one day at low temperature, and some plants reach maximum tolerance after only a few days. Enhanced freezing tolerance is also rapidly reversible; it can be lost within a few days after plants are returned to a higher temperature (Wanner, Junttila, 1999).

Several reports suggest that soluble sugars and oligosaccharides combined with
proteins are involved in frost tolerance. There is a relationship between carbohydrates and cold hardiness in many crops (Palonen, Buszard, 1997; Dionne et al., 2001). Further, sugar solutions in association with heat stable proteins function in concert to protect plants against stress (Gusta et al., 1996).

Frost tolerance field studies are difficult to replicate due to the extreme environmental variability. Variability in factors associated with the effect of temperature makes it more difficult to decide how much damage occurs from low temperature alone. Factors such as crop species, growth stage, duration of freezing temperature, soil moisture, soil type, hardening, freezing and thawing sequences, occurrence of pathogens and insect pests contribute to a highly complex pattern that determines frost tolerance of a particular species (Badaruddin, Meyer, 2001). Winter field trials may not experience heavy frost or in severe cases completely kill all plants. Thus, a rapid, affordable, repeatable indoor cold prediction test that correlated with survival in field trials is extremely important (McClinchey, Kott, 2008). Cold de-acclimation has not been as comprehensively studied as acclimation, although resistance to de-acclimation and the ability to re-acclimate may be of greater importance for winter survival particularly in the fluctuating winter conditions (Rapacz, 2002).

The aim of the present study was to evaluate ability of winter rapeseed shoots to restore frost resistance after de-acclimation and re-acclimation periods.

**Objects, methods and conditions.** The investigation was carried out with four winter rapeseed cultivars: ‘Banjo’, ‘Elvis’, ‘Silvia’ and ‘Valesca’. Seeds were surface sterilized with 10% sodium hypochlorite for 10 min, washed with sterile water and placed for germination and growth in vitro on basal MS medium (Murashige, Skoog, 1962), supplemented with 4, 6 and 8% sucrose, solidified with 8 g l\(^{-1}\) Difco-Bacto agar. Media adjusted to pH 5.5 prior to autoclaving at 115 °C for 30 min. Explants cultivated under 22 ± 2 °C temperature, illumination 50 µmol m\(^{-2}\) s\(^{-1}\), photoperiod 16/8 h (day/night).

Apical meristem was transferred to the same medium for shoot formation. Shoots were grown till three true leaves stage under conditions described above.

Shoots were acclimated in a vernalization chamber at 4 °C for 14 days. After acclimation treatment, shoots were placed randomly in a low-temperature incubator for the freezing treatment. Initial temperature in the incubator was +2 °C. The temperature was subsequently lowered to -1 °C over a period of 2 h, and held at -1 °C for 12 h, to ensure that nucleation occurred evenly. The temperature then was decreased 1 °C h\(^{-1}\) until the desired minimum temperature was reached. Freezing temperature was -5, -6, and -7 °C, duration of freezing was 24 h. After freezing, temperature was gradually returned to +2 °C at a rate of increasing -1 °C h\(^{-1}\) and then incubated for 15 h. The shoots were placed into growth chambers with normal conditions as described above. The number of injured shoots was determined for each treatment in 30 days after freezing.

For study influence of de-acclimation period on rapeseed cold resistance, acclimated shoots were de-acclimated and subjected to freezing temperature with and without repeated acclimation. Experiments consisted of three de-acclimation treatments. Treatment 1 was de-acclimated for 14 days at 18 °C and placed for the freezing treatment without repeated acclimation. Treatment 2 was de-acclimated for 14 days at 18 °C and repeatedly acclimated for 14 days at 4 °C. Treatment 3 was de-acclimated
for 21 days at 18 °C and repeatedly acclimated for 14 days at 4 °C. At the end of each de-acclimation and re-acclimation periods, these treatments were subjected to the freezing procedure. The numbers of injured shoots were determined for each treatment at 30th day after freezing.

Experiments were set up in a completely randomized design and repeated three times, with at least 40 explants per treatment.

The data of the investigations were calculated using the computer programme STAT 1.55 from “SELEKCIJA” (Tarakanovas, 1999) and ANOVA for EXCEL, vers. 2.1.

**Results.** The effect of freezing treatment on cold tolerance of winter rapeseed shoots after 14 days of acclimation is summarized in Figure 1.

Shoots subjected to low temperature showed a wide range of frost resistance, percent of injured shoots varied with freezing temperature and sucrose level in the medium as well as cultivar. Generally, increasing the sucrose level to 6 % increased amount of uninjured ‘Elvis’ and ‘Valnes’ shoots.

**Fig. 1.** The effect of 14 days acclimation treatment on freezing tolerance of winter rapeseed cultivars

The medium supplemented with 8 % sucrose resulted in significant increasing of cold tolerance in ‘Silvia’ shoots. While it was not significant differences in ‘Banjo’ shoots injury on medium with 4 % and 6 % of sucrose.

Following a 14 days de-acclimation period, all cultivars suffered a significant reduction in cold tolerance (Fig. 2).
Fig. 2. The effect of 14 days de-acclimation treatment on freezing tolerance of winter rapeseed cultivars

Fig. 3. The effect of 14 days re-acclimation treatment followed by 14 days de-acclimation period on freezing tolerance of winter rapeseed cultivars
A significant loss of cold tolerance was detected in all tested freezing treatments, but especially sensitive were shoots subjected to -7 °C temperature. Percent of uninjured shoots decreased from 35.3 % (‘Elvis’ on medium supplemented with 8 % sucrose) to 49.3 % (‘Valesca’ on medium supplemented with 4 % sucrose) in comparison with acclimated shoots on the same media. Increasing sucrose level from 4 to 6 % resulted in increasing of uninjured shoots in ‘Banjo’, ‘Elvis’ and ‘Valesca’, while medium supplemented with 8 % sucrose was most suitable for ‘Silvia’ shoots.

Repeated acclimation of shoots after 14 days de-acclimation period had substantial positive effects on shoot injury over the de-acclimated shoots at a particular temperature across tested cultivars (Fig. 3). After the lowest (-7 °C) temperature treatment, percent of uninjured shoots was increased from 4.1 % (‘Elvis’ on medium supplemented with 8 % sucrose) to 26.1 % (‘Valesca’ on medium supplemented with 4 % sucrose) by re-acclimation treatment following 14 days de-acclimation period. Relatively sensitive cultivar ‘Silvia’ showed high cold tolerance to -5 °C temperature treatment on medium with 8 % sucrose, however, decreasing temperature by 1 °C resulted in rapid decreasing of uninjured shoots percent on the same medium.

A significant reduction in cold tolerance was observed in all tested genotypes after re-acclimation followed by 21 days of de-acclimation treatment (Fig. 4).

**Fig. 4.** The effect of 14 days re-acclimation treatment followed by 21 days de-acclimation period on freezing tolerance of winter rapeseed cultivars

Comparison of the results from Figures 3 and 4 indicated that longer (21 days) period of de-acclimation decreased the ability of plants for further cold acclimation and their freezing tolerance; however, decreasing degree strongly depended on genotype and sucrose level interaction. On average, medium supplemented with 6 %
sucrose resulted in increased ‘Banjo’ and ‘Elvis’ sensitivity to cold treatment, while highest amount of injured ‘Valesca’ and ‘Silvia’ shoots was obtained on medium with 8 % sucrose.

Discussion. De-acclimation is primarily a response to increasing temperatures, and the rate of de-acclimation positively correlates with temperature (Svenning et al., 1997). The de-acclimation period limits the effectiveness of cold acclimation by reducing the amount of energy available for this process (Rapacz, Janowiak, 1998). On the other hand, little is known of the potential mechanisms of temperature sensing involved in cold de-acclimation. Cold de-acclimation, especially when leading to elongating growth promotion, may strongly modify the plant’s ability to cold re-acclimation and thus freezing tolerance (Rapacz, 2002). A possible role of photosynthetic apparatus during cold de-acclimation was studied in spring and winter cultivars of *Brassica napus* (Rapacz, 2002). Acceleration of elongation growth rate during cold de-acclimation and re-acclimation resulted in a deeper loss of frost resistance and in a slower re-acclimation rate, respectively. Spring rapeseed started to elongate from the second week of the de-acclimation. Following re-acclimation, tested cultivar was neither able to recover frost resistance or re-acclimate its photosynthetic apparatus to cold (Rapacz, 2002).

In the present study, there was a remarkable decrease in frost resistance of shoots after 14 days de-acclimation period for all tested cultivars. A similar effect of almost simultaneous decrease in frost resistance was shown for non-prehardened winter rapeseed (Rapacz, 1998).

Accumulation of soluble carbohydrates is one of the best-known responses of plants to cold. It begins early during the response to cold, whereas the time-scale of increase in freezing tolerance and stress-gene expression is much longer, which indicates that changes in sugar supply may precede acclimation and cold-induced gene expression (Tabaei-Aghdaei et al., 2003). An increase in carbohydrate composition and in the concentration of sugar-metabolism enzymes has been found during cold acclimation in several species (Revilla et al., 2005). During winter, annual species maximize the production of sugars with a possible cryoprotective function and accumulate sufficient carbohydrate reserves to support basal metabolism and regrowth in the spring. Some researchers have found that in cell cultures sugars without cold could induce freezing tolerance (Travert et al., 1997; Tabaei-Aghdaei et al., 2003). In contrast, the current results showed that addition of sucrose in culture medium in the absence of re-acclimation period slightly increased winter rapeseed cold resistance but percent of uninjured shoots was very low.

Most previous studies that have indicated a role of sugars in acclimation to cold, found that cold was also necessary (Wanner, Juntila, 1999). Presented results clearly demonstrate the strong positive effect of re-acclimation followed after de-acclimation period. However, any of tested cultivars did not recover its initial level of frost resistance even after shortest (14 days) de-acclimation period. Influence of different sucrose level on shoots cold resistance depended from genotype and freezing temperature.

Conclusions. 1. De-acclimation period resulted in significant loss of winter rapeseed shoot cold tolerance.

2. Exogenous sucrose has positive effect on winter rapeseed cold resistance, but
repeated acclimation is more important for shoots recovery after de-acclimation.

3. Shoots of tested cultivar are partially able to recover cold resistance after repeated 14 days acclimation, but percent of uninjured shoots is significantly lower, in comparison with shoots without de-acclimation.

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References


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„Atlydžio“ ir pakartotinio grūdinimo įtaka žieminų rapsų atsparumui šalčiui in vitro

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Santrauka


Reikšminiai žodžiai: žieminiai rapsai, ūglių pažeidimas, „atlydis“, pakartotinis grūdintimas.

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