ALEKSANDRO STULGINSKIO UNIVERSITETAS

Vaida Jonytienė

ENDOGENINIŲ IR EGZOGENINIŲ VEIKSNIŲ POVEIKIS ŽIEMINIO RAPSO (*BRASSICA NAPUS* L.) UŽSIGRŪDINIMUI IR ATSPARUMUI ŠALČIUI *IN VITRO* IR *IN VIVO*

Daktaro disertacijos santrauka

Žemės ūkio mokslai, agronomija (01A)

Akademija, 2012

Disertacija rengta 2006–2011 m. Aleksandro Stulginskio universitete
2010–2011 m. darbą rėmė Valstybinis studijų fondas

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Disertacija bus ginama viešajame Agronomijos mokslo krypties tarybos posėdyje 2012 m. balandžio 12 d., 11 val. Aleksandro Stulginskio universiteto Centrinių rūmų 261 auditorijoje

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Daktaro disertacijos santrauka išsiuntinėta 2012 m. kovo 12 d.
Disertaciją galima peržiūrėti Aleksandro Stulginskio universiteto ir Lietuvos agrarinių ir miškų mokslų centro bibliotekose

INTRODUCTION

Winter rapeseeds (*Brassica napus* L.) industrial value as well as competitiveness in Lithuania reduces its insufficient adaptability to winter acclimation and cold tolerance. This problem is especially highlighted in the phone of temperate climate and changable conditions of wintertime. Under the influence of adverse environmental factors physiological processes of plants are disrupted. The environmental stress effect on plants depends on the duration, strength and genetics qualities of plants (Alexieva et al., 2003).

Temperature is one of the most important environmental factor which influences physiological processes of plants. Plant cold acclimation is an active biochemical process resulting plant preparation for the tolerance of winter freezing. Plant cells accumulate plastic and preventive substances, the level of water in cells declines, the structure and characteristics of protoplasm changes. Acclimated plants become significantly more resistant not just to cold but also to other negative factors of the winter time. The processes of plant acclimation follow two set stages. During the first stage, when the average daily temperature is from 6 ºC to 0 °C, plants slow the processes of development and growth but photosynthesis occurs in plants. During the second stage of acclimation which happens when the average daily temperature reaches –2 – –6 °C the processes of photosynthesis stops. Low positive temperature activates internal plant reactions and genes which change the conductions of membrane and increase cold acclimation (Anisimovienė et al., 2006).

In the process of evolution plants growing in natural environment have adapted in order to survive in various climatic conditions. The capability of particular plant species to survive in extreme weather conditions and some genetic and molecular aspects of cold acclimation that were researched under investigation of pattern cultures (*Arobidopsis*, *Nicotiana tabacum* and etc.) show that the plant potentialities of adaption are high and not nearly used in cultivated plants. Winter rapeseed generally is affected at the end of the winter time or at the same beginning of the spring time when plants are in a forced not in organic state. Rapeseed acclimation declines under the influence of considerably high fluctuations of temperature – from higher temperature which stimulates growth to low freezing temperature. During the process of de-acclimation plant vegetation might be renewed and they lose their capability to re-acclimate resulting plant cold tolerance when the temperature falls to negative. Scientific studies claim that reaction of winter crop to low temperature in winter – spring seasons depends on different biochemical compounds (proline, sugars, phytohormone and etc.) level in the tissues of plants (Patton et al., 2007; McClinchey, Kott, 2008; Dörffling et al., 2009; Pociecha et al., 2009; Gothandam et al., 2010; Novickienė et al., 2010).

Hypothesis. The content of endogenous proline and soluble sugars which determines the cold tolerance of winter rapeseed changes under fluctuating temperature.

Aim of the work – to determine the effect of endogenous and exogenous factors on the cold tolerance of winter rapeseed *in vitro* and *in vivo*.

The following tasks were set:

1. To evaluate the accumulation of proline and soluble sugars during rapeseed acclimation *in vitro* and *in vivo*.
2. To investigate the effect of exogenous additives in medium on winter rapeseed cold tolerance *in vitro*.
3. To determine the genotype effect on rapeseed cold tolerance *in vitro* and *in vivo*.
4. To evaluate the acclimation stability of winter rapeseed under fluctuating temperature *in vitro* and *in vivo*.

Proposition to be defended:

1. Cold tolerance of winter rapeseed mainly determining by content of endogenous proline but not by soluble sugars.
2. Exogenous additives (abscisic acid, proline, amino acids) in nutrient medium increases the cold tolerance of rapeseed shoots *in vitro*.
3. In *in vitro* system the abscisic acid, proline and amino acids restore the reduced acclimation which occurs during de-acclimation period.
4. Endogenous proline content is the marker of membrane stability and cold tolerance and may be used to create cold-tolerant genotypes.

Originality of the research work. Cold tolerance of winter rapeseed mainly determining by content of endogenous proline in plant tissues – with increasing of proline content electrolyte leakage consistently decreased. Effect of soluble sugars on electrolyte leakage in most case was slight statistically insignificant.

Practical relevance. Research results to allow preconditions for increasing the cold tolerance of winter rapeseed by technological implements. Abscisic acid, amino acids, L-proline (or its analogues) may be effective in increasing the cold tolerance of winter rapeseed under Lithuanian climatic conditions. Proline can be used as a marker in creating cold tolerant winter rapeseed genotypes *in vitro*.

Approbation of research results. The main results have been published in 4 scientific articles which are included in referred ISI WOS with citation index scientific journal "*Journal of Food, Agriculture & Environment*" (2010–2012). The results have also been presented in 3 international and Lithuanian scientific conferences.

Volume of the dissertation. The dissertation is written in Lithuanian. It consists of: introduction; overview of literature; work methods and materials; experimental results; discussions; conclusions; lists of author's publications; references. The dissertation contains 93 pages, including 12 tables, 32 figures. 234 literature references have been used.

MATERIALS AND METHODS

Investigation place, time and object. Investigation *in vitro* and *in vivo* under controlled conditions was carried out during 2007–2011 in the Laboratory of Agrobiotechnology, Department of Crop Science and Animal Husbandry of Aleksandras Stulginskis University, with four winter rapeseed (*Brassica napus* L.) varieties 'Siska', 'Insider', 'Valesca' and 'Sunday'. The experiments *in vivo* under field conditions were carried at the Experimental Station of Aleksandras Stulginskis University during the period 2008–2011. A total plot size was 12 m^2, the plots were arranged in a randomised block design with three replications.

Investigation *in vitro*. 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) without growth regulators, supplemented with 10.0 g l^{-1} sucrose and 8.0 g l^{-1} agar. Media adjusted to pH 5.5 prior to autoclaving at 115 ºC for 30 min. Culture media (20 ml) were dispensed into 90 mm diameter Petri dishes and sealed with parafilm. Sterilization of explants and transfer of the culture was carried out under aseptic conditions. Seeds were incubated at 22 ± 2 ºC temperature, under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night). Apical meristem was transferred to MS medium supplemented with 30.0 g l^{-1} sucrose and 8.0 g l^{-1} agar for shoots formation and maintained for 3 weeks under the same growing conditions before the treatments began. Explants were cultivated at 22 ± 2 ºC temperature, under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night).

Influence of acclimation in rapeseed shoots amount of proline and soluble sugars. Shoots of varieties 'Siska', 'Insider', 'Valesca' and 'Sunday' were acclimated at 4 °C under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night) for 7, 14, 21 and 28 days.

Proline and soluble sugars contents were determinated after 7, 14, 21 and 28 days of acclimation. There were three replications for each treatment.

Influence of freezing temperature on rapeseed shoots cold tolerance *in vitro*. Shoots of varieties ‘Siska’, ‘Insider’, ‘Valesca’ and ‘Sunday’ were used. Shoots were acclimated in a vernalization chamber at 4 ºC temperature under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night) for 21 days. The membrane injury for leaf discs was estimated by the electrolyte leakage test. Discs cut from the youngest leaves were frozen at –6, –10 and –16 ºC temperatures. There were three replications for each treatment.

Influence of de-acclimation and re-acclimation treatments on rapeseed shoots cold tolerance *in vitro*. Shoots of variety ‘Sunday’ were acclimated at 4 °C under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night) for 14 days and subjected to de-acclimation under temperatures of 18/16 ºC, photoperiod 16/8 h (day/night) for 1, 3, 5 and 7 days. After de-acclimation, plants were subjected to re-acclimation at 4 °C under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night) for 1, 3 and 5 days.

Proline and soluble sugars contents were determinated in shoots after 14 days of acclimation after 1, 3, 5 and 7 re-acclimated days and after 1, 3 and 5 days of de-acclimation. The membrane injury for leaf discs under –10 °C freezing temperature was estimated by the electrolyte leakage test. There were three replications for each treatment.

Effect of abscisic acid on rapeseed shoots cold tolerance *in vitro*. Shoots of variety ‘Sunday’ were grown on MS medium in which 0.5 mM, 1.0 mM and 1.5 mM concentrations of abscisic acid was added. Shoots were acclimated in a vernalization chamber at 4 ºC temperature under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night) for 7, 14, and 21 days. There were three replications for each treatment.

Proline and soluble sugars and photosynthetic pigments contents was determinated after 7, 14, 21 and 28 days of acclimation. The membrane injury for leaf discs under –10 °C freezing temperature was estimated by the electrolyte leakage test. There were three replications for each treatment.

Effect of exogenous proline on rapeseed shoots cold tolerance *in vitro*. Shoots of variety ‘Sunday’ were grown on MS medium in which 5.0 mM, 10.0 mM, 15.0 and 20.0 mM concentrations of L-proline was added. Shoots were acclimated at 4 °C under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night) for 14 days and subjected to de-acclimation under temperatures of 18/16 ºC, photoperiod 16/8 h (day/night) for 1, 3, 5 and 7 days.

Proline and soluble sugars contents were determinated after 14 days of acclimation after 1, 3, 5 and 7 days of re-acclimation. The membrane injury for leaf discs under –10 °C freezing temperature was estimated by the electrolyte leakage test. There were three replications for each treatment.

Effect of exogenous amino acids on rapeseed shoots cold tolerance *in vitro*. Shoots of variety ‘Sunday’ were grown on MS medium in which 0.36 µl l^{-1} of amino acids was added. Rapeseed shoots were acclimated at 4 °C under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night) for 14 days and subjected to de-acclimation under temperatures of 18/16 ºC, photoperiod 16/8 h (day/night) for 1, 3, 5 and 7 days. After de-acclimation, plants were subjected to re-acclimation at 4 °C under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night) for 1, 3 and 5 days.

Proline and soluble sugars contents were determinated after 14 days acclimated shoots after 1, 3, 5 and 7 re-acclimated days and after 1, 3 and 5 days of de-acclimation. The membrane injury for leaves discs under –10 °C freezing temperature was estimated by the electrolyte leakage test. There were three replications for each treatment.

Investigation *in vivo* under controlled conditions. Winter rapeseed were grown in vegetative pots at 22 ± 2 ºC under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night). Substrate of the neutral acid peat (pH 6 to 6.5).

Influence of acclimation duration on rapeseed cold tolerance *in vivo*. Varieties of 'Siska', 'Insider', 'Valesca' and 'Sunday' were acclimated at 4 °C under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night) for 7, 14, 21 27 and 33 days. Proline and soluble sugars contents were determinated after 7, 14, 21, 27 and 33 days of acclimation. There were three replications for each treatment.

Cold tolerance was determinated after 7, 14 and 21 days of acclimation. The membrane injury for leaves discs under –10 °C freezing temperature was estimated by the electrolyte leakage test. There were three replications for each treatment.

Influence of freezing temperature on rapeseed cold tolerance *in vivo*. Rapeseed of varieties 'Siska', 'Insider', 'Valesca' and 'Sunday' were used. Plants were acclimated in a vernalization chamber at 4 ºC temperature under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night) for 27 days. The membrane injury for leaves discs was estimated by the electrolyte leakage test. Discs cut from youngest leaves were frozen at –10, –16 and –22 ºC temperatures. There were three replications for each treatment.

Field experiments. Four varieties 'Siska', 'Insider', 'Valesca' and 'Sunday' of rapeseed were used for the investigation. Plants for analysis were taken every week. Proline and total soluble sugars content was determined. The membrane injury was determined by the electrolyte leakage, the tissue were frozen at –10 ºC temperature. The treatment for each variety was determinated in three replications.

The weather conditions. In 2008 the air temperature of August and September was close to that of the long-term 17.9 °C and 12.2 °C. In August the moisture was adequate when rapeseed were sowing. The rapeseed was a normal density, vegetation lasted until middle of November. In 2009 the winter was cold with a great snow cover and a deep ground freeze. The plants well ower-wintered and at the beginning of April vegetation resumed. April was warm, the average of air temperature was 8.9 °C (2.8 °C higher than the long-term), and a very dry month (HTC = 0.32). The average temperature of May was 12.7 °C, and the amount of precipitation 42 mm (HTC = 1.07).

2009 autumn and winter weather conditions were extreme. Ranged in August and September of the moisture distribution (HTC = 1.67; HTC = 0.68) inhibited germination and growth of rapeseed. Plant vegetation lasted until the second half of November. November precipitation exceeded the average long-term 29.2 mm. Rainy and cold autumn, December with out snow was unfavourable to the plants. Coldest days of December when temperature had fallen to –14.1 °C. Late in December thick snow cover the fields. The whole January was cold wintry weather. Monthly average daily temperature was –10.2 °C (5.4 °C lower than the average long-term), while the coldest days were –21 and – 22.4 °C. Cold weather was a larger part of the month of February. By the second decade of the end of February the average of snow thickness was 26–42 cm. The second half in March was dominated a negative temperature. The first ten-day of April was cold and rainy, but the second ten-day was completely dry and warmer than the first. Monthly average temperature was 7.4 °C during this month, precipitation was 58.55 mm, the moisture surplus (HTC = 2.62), which had a negative impact of rapeseed growing season renewal.

In September 2010, the temperature was close to the long-term – 12.0 °C. The monthly precipitation of 10.7 mm larger than the long-term average. The rapeseed was of a normal density, vegetation lasted until end of November. On a monthly average temperature was – 3.4 °C (similar to long-term –3.3 °C), precipitation was 63.5 mm, this exceeded 27.4 mm of precipitation a long-term average. In January, the lowest temperature was –12.8 °C, thaw the days when the highest air temperature of 1.8 to 3.2 °C. In January 2011 to compared the long-term temperature (–4.8 °C), the monthly temperature average was below –2.8 °C. On the beginning of temperature rise to 2.9 °C and snowmelt remain unprotected plants from the temperature fall during the month (the lowest temperature –16.6 °C). In March, the average of temperature was 3.7 °C and 10.6 mm of precipitation (precipitation average in a long-term in March 32.5 mm). Rapeseed vegetation resumed in May.

Hydrothermal coefficient (HTC) of G. Selyaninov (Хомяков, 1989) was used for characterisation of climatic conditions.

Experimental and analytical methods. The plant samples were assessed and analysed for: proline content was determined using a revised ninhydrin method (McClinchey, Kott, 2008); total soluble sugars (reducing and non-reducing) were determined by anthrone method (Yemm, Willis, 1954), total soluble sugar values are expressed as glucose equivalents; the cold tolerance was estimated by electrolyte leakage test (Dai et al., 2007).

Mathematical–statistical evaluation of data. The means and the standard errors were calculated using a software package "Selekcija" (Tarakanovas, Raudonius, 2003) and software "Statistica 10" (Čekanavičius, Murauskas, 2002; Hill, Levicki, 2006).

EXPERIMENTAL RESULTS

In vitro investigations

Influence of acclimation in rapeseed shoots amount of proline and soluble sugars. The investigations established the dependence of proline content in the *in vitro* grown rapeseed shoots on the genotype and acclimation time. Non-acclimated shoots of rapeseed accumulated from 20.8 µM g^{-1} ('Insider') to 40.7 µM g^{-1} ('Valesca') of proline (Fig. 1).

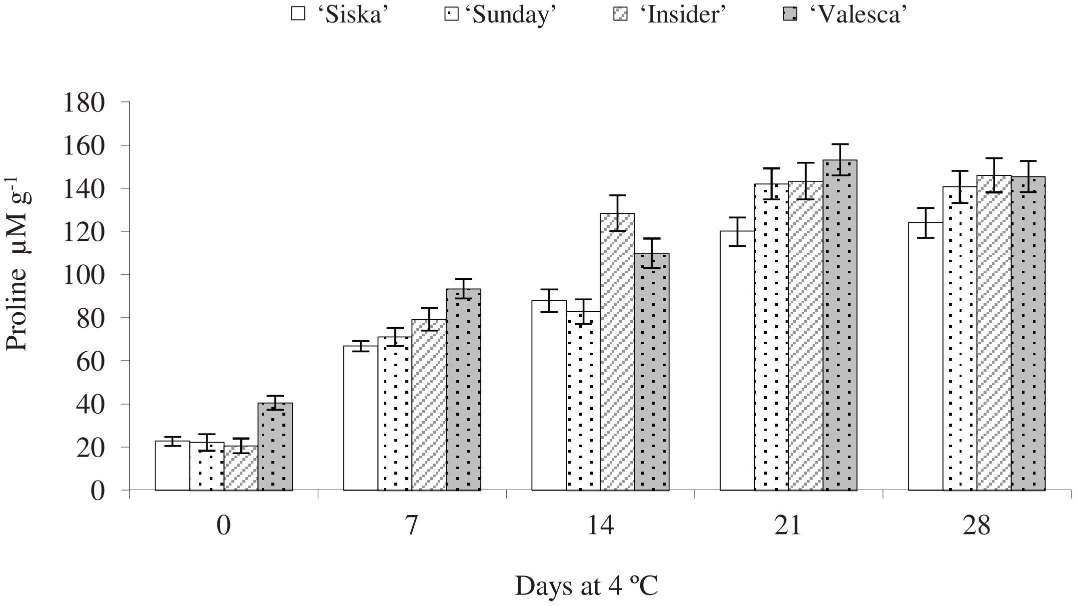

Figure 1. Accumulation of proline during acclimation of rapeseed shoots

After the first week of acclimation the proline content in shoot leaves increased 2.3–3.8 times if compared with that in non-acclimated shoots. During the second week of acclimation the most intensive accumulation of proline was observed in the shoots of 'Insider' variety, and after two weeks of acclimation the amount of proline in the shoots of this variety was significantly higher than that in shoots of other varieties. During the third week of acclimation the most intensive accumulation of proline was observed in the shoots of 'Sunday' variety, the proline content increased by 59.1 µM g^{-1} in comparison to that in the shoots after 2 weeks of acclimation. The proline content in the shoots of other investigated varieties also increased consistently.

The amount of soluble sugars accumulated in non-acclimated shoots of the investigated rapeseed varieties varied from 5.3 mg g^{-1} ('Sunday') to 26.4 mg g^{-1} ('Valesca') (Fig. 2). Soluble sugars content increased markedly under cold acclimation. After 7 days of acclimation the strongest increase of soluble sugars was observed in shoots of 'Insider' and 'Valesca', while this amount in 'Siska' variety shoots exceeded that in non-acclimated shoots only by 2.4 mg g^{-1}. The second week of acclimation was the period of the most intensive accumulation of soluble sugars in 'Siska' shoots, the amount of sugars increased by 81.6 mg g^{-1} in comparison with shoots after 7 days of acclimation. In the second week of acclimation the amount of soluble sugars in shoots of other investigated varieties increased from 8.8 mg g^{-1} ('Insider') to 28.0 mg g^{-1} ('Valesca'). During the third week of acclimation the amount of soluble sugars increased only in shoots of 'Insider' variety, the level of soluble sugars in shoots of other varieties had begun to decrease.

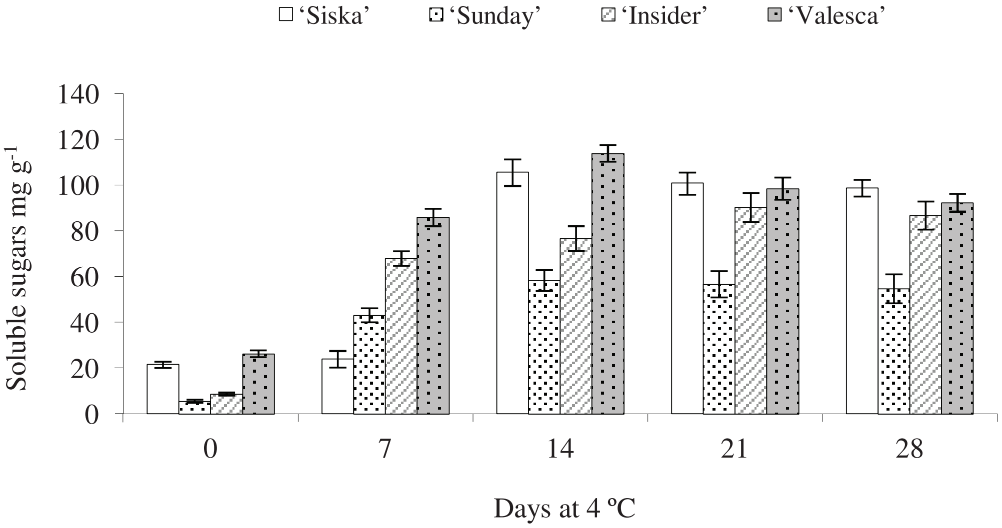

Figure 2. Accumulation of soluble sugars during acclimation of rapeseed shoots

Influence of freezing temperature on rapeseed shoots cold tolerance *in vitro*. Freezing shoots of the investigated varieties at the temperature of –6 °C caused variation of relative injury of leaf tissues from 49.4 % ('Valesca') to 62.9 % ('Siska') (Fig. 3).

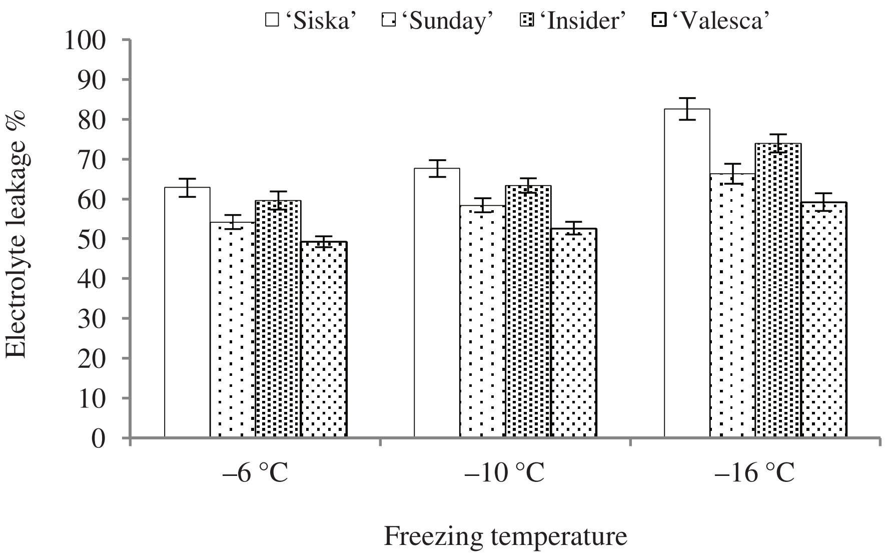

Figure 3. The electrolyte leakage of rapeseed leaves exposed in different freezing temperatures

Reduction of freezing temperature to –10 °C and to –16 °C increased the injury of shoots consistently. ‘Siska’ variety shoots were established to be the most sensitive to the impact of negative temperature, after 24 hours of freezing electrolyte leakage was 67.7 % (at –10 °C) and 82.6 % (at –16 °C). Of all the investigated winter rape genotypes shoots of ‘Valesca’ variety had the best cold tolerance. After freezing at the temperature of –16 °C shoots of this variety had electrolyte leakage by 7.2 % lower in comparison with ‘Sunday’; by 14.8 % lower in comparison with ‘Insider’ and by 23.4 % lower in comparison with ‘Siska’ shoots.

Influence of de-acclimation and re-acclimation treatments on rapeseed shoots cold tolerance *in vitro*. During plant de-acclimation at 18/16 ºC temperature, the remarkable decrease of proline content was observed (Fig. 4a). After 7 days of de-acclimation treatment, proline level decreased by 75.19 µM g^{-1} in comparison with acclimated shoots. Soluble sugars level declined rapidly after shoots were returned to 18/16 ºC, after 1 day of de-acclimation the decrease in soluble sugars was 23.97 mg g^{-1} (Fig. 4b). There was a slight increase in soluble sugars content during third day of de-acclimation before it began to decline.

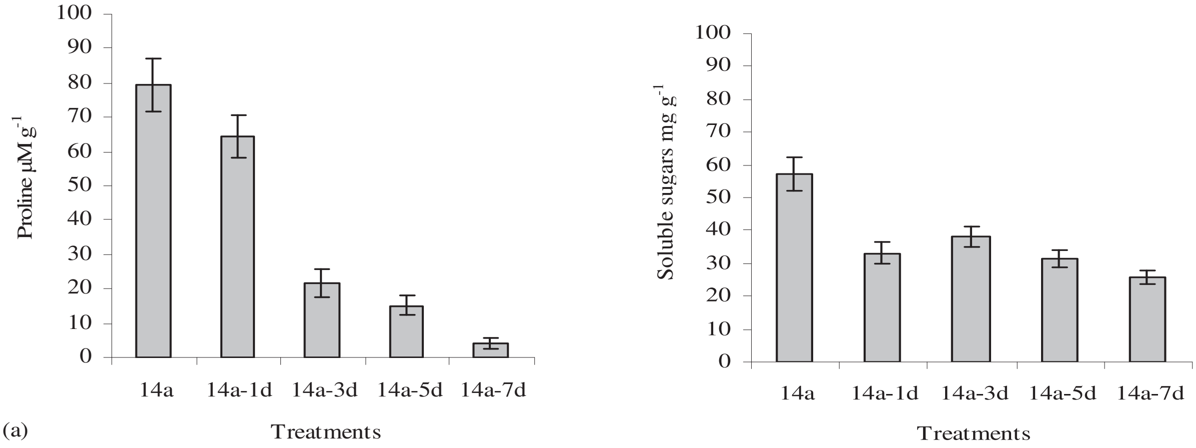

Figure 4. Proline and soluble sugars contents of rapeseed leaves exposed to acclimation temperature and during cold de-acclimation

Treatments were: a – acclimation for days (temperature 4 °C day/night, photoperiod 16/8 h day/night);
d – de-acclimation treatment for days (temperature of 18/16 ºC day/night, photoperiod 16/8 h day/night).

Figure 5 shows cold tolerance of rapeseed leaves exposed to acclimation temperature and during cold de-acclimation. The degree of cold tolerance of leaves (expressed in terms of electrolyte leakage) decreased progressively with increasing duration of de-acclimation treatment. The highest electrolyte leakage value was achieved at 7 days de-acclimation duration.

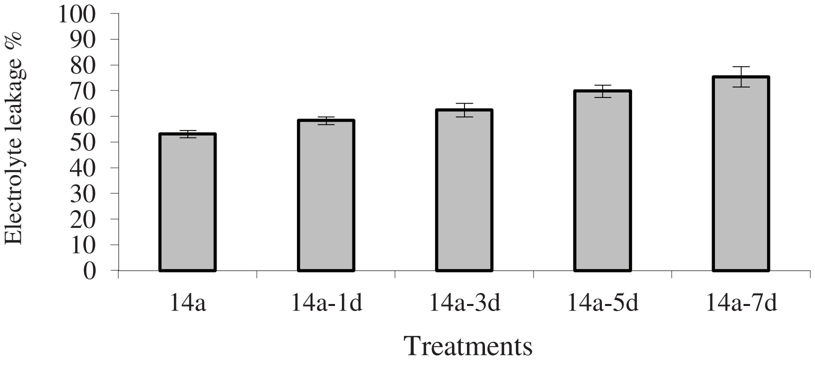

Figure 5. Cold tolerance of rapeseed leaves exposed to acclimation temperature and during de-acclimation

Treatments were: a – acclimation for days (temperature 4 °C day/night, photoperiod 16/8 h day/night);
d – de-acclimation treatment for days (temperature of 18/16 ºC day/night, photoperiod 16/8 h day/night).

Results of the effect of re-acclimation duration on proline, soluble sugars and photosynthetic pigments content in rapeseed shoots cold tolerance during de-acclimation are summarized in Table 1.

Table 1. Accumulation of proline, soluble sugars and electrolyte leakage during re-acclimation

Re-acclimation time days	Proline µM g^{-1}	Soluble sugars mg g^{-1}	Electrolyte leakage %
14a-1d	64.45c	33.23d	58.35a
14a-1d-1r	93.33b	56.44c	52.61b
14a-1d-3r	109.25b	69.64b	49.56bc
14a-1d-5r	146.41a	59.22a	45.73c
14a-3d	21.69c	38.14d	62.42a
14a-3d-1r	37.93b	49.21c	59.71ab
14a-3d-3r	42.15b	57.24b	56.38bc
14a-3d-5r	56.17a	64.82a	53.94c
14a-5d	15.04d	31.21b	69.81a
14a-5d-1r	18.9c	43.15a	65.47ab
14a-5d-3r	34.6b	49.17a	61.48bc
14a-5d-5r	41.96a	53.48a	57.32c
14a-7d	4.11c	25.63b	75.39a
14a-7d-1r	4.49c	41.61a	72.84ab
14a-7d-3r	11.82b	46.84a	67.39bc
14a-7d-5r	35.39a	48.42a	62.85c

Means within a column followed by the same letter are not significantly different, indicated by Duncan's multiple-range ($P \leq 0.01$)

Treatments were: a – acclimation for days (temperature 4 °C day/night, photoperiod 16/8 h day/night);
d – de-acclimation treatment for days (temperatures of 18/16 °C day/night, photoperiod 16/8 h day/night);
r – re-acclimation treatment for days (temperature 4 °C day/night, photoperiod 16/8 h day/night).

Re-acclimation treatment significantly increased proline and soluble sugars accumulation in rapeseed shoots. Re-acclimation treatment for 1 day following 1 day de-acclimation period resulted increasing in proline and soluble sugars by 28.88 µM g^{-1} and 23.21 mg g^{-1}, respectively, and reaching a level near or higher to that of shoots acclimated for 14 days. The highest amount of proline has been observed after 5 days of re-acclimation, while soluble sugars reached maximum content after 3 days of re-acclimation. Re-acclimation treatment following 3, 5 and 7 days of de-acclimation substantially increased proline and soluble sugars content in leaves of rapeseed shoots.

However, rapeseed shoots was able to recover initial level of soluble sugar after 3 days of de-acclimation only and did not recover initial level of proline. Re-acclimation of rapeseed shoots at 4 °C resulted in a gradual increase in the cold tolerance of shoots to extracellular freezing as indicated by changes in electrolyte leakage.

The degree of cold tolerance of leaves increased progressively with increasing duration of cold re-acclimation. Re-acclimation treatment for 5 days decreased electrolyte leakage value to 45.73 % (following 1 day of de-acclimation); 53.94 % (following 3 days of de-acclimation); 57.32 (following 5 days of de-acclimation) and 62.85 % (following 7 days of de-acclimation).

Effect of abscisic acid on rapeseed shoots cold tolerance *in vitro*. Results of the effect of acclimation time and exogenous ABA in culture medium on proline, soluble sugars content and electrolyte leakage in rapeseed shoots are summarized in Table 2.

Table 2. The changes in proline, soluble sugars and electrolyte leakage level during acclimation of rapeseed shoots cultivated with and without exogenous ABA

Acclimation time days	Concentration of ABA mM			
	control (without ABA)	0.5	1.0	1.5
	Proline µM g^{-1}			
0	22.69c	125.50b	160.77a	121.11b
7	59.14d	136.60b	151.39a	122.03c
14	79.28c	232.43a	215.20a	179.17b
21	163.77c	228.93a	214.49a	185.37b
	Soluble sugars mg g^{-1}			
0	32.80c	85.20b	86.60b	119.67a
7	47.20d	82.40c	134.00a	112.40b
14	56.40c	102.40b	139.60a	138.80a
21	58.32d	134.80c	160.80b	192.20a
	Electrolyte leakage %			
0	64.28a	56.39b	53.92b	48.62c
7	58.39a	53.47b	50.34c	47.36d
14	55.14a	51.34b	48.83c	44.52d
21	53.67a	48.51b	47.22b	38.63c

Means within a row followed by the same letter are not significantly different, indicated by Duncan's multiple-range ($P \leq 0.01$)

Proline content in ABA treated shoots was not enhanced during first week at 4 °C, while proline content in control shoots increased by 38.37 %. During the second week of acclimation period, the presence of exogenous ABA increased the accumulation of proline. After 14 days of acclimation the highest proline content has been obtained in shoots cultured on medium supplemented with 0.5 mM ABA. The higher ABA concentrations were less effective. After 21 days of acclimation ABA-treated shoots did not show significant change in proline content compared with shoots at day 14. In contrast, control shoots showed a rapid increase in proline content, reaching a level near to that of shoots cultured on medium supplemented with 1.5 mM ABA.

During the 21 days of acclimation soluble sugar content in rapeseed shoots cultured on medium without abscisic acid increased by 25.5 mg g^{-1} in comparison with non-acclimated shoots. Exogenous ABA significantly increased soluble sugars content in non-acclimated shoots. ABA-treated shoots showed a significant increase in soluble sugar content compared with control shoots during acclimation duration, reaching a highest level after 21 d at 4 °C. A faster change in soluble sugar content caused addition of 1.5 mM ABA.

The highest electrolyte leakage has been obtained from leaves of non-acclimated shoots without ABA treatment. The degree of cold tolerance of the shoots from the different treatment was substantially increased with increasing acclimation duration. The highest cold tolerance for shoots from all tested treatments was achieved at 21 days acclimation duration.

On the media without ABA on the 21th day of acclimation the electrolyte leakage of leaf discs decreased by 10.61 % compared with the non-acclimated shoots. The addition of ABA resulted in significant increase in rapeseed shoots cold tolerance. After 21 days of acclimation electrolyte leakage of ABA-treated shoots was 5.16–15.04 % lower in comparison with non-treated shoots.

Effect of exogenous proline on rapeseed shoots cold tolerance *in vitro*. Results of the effect of de-acclimation duration and proline treatment on endogenous proline, soluble sugars content and electrolyte leakage in rapeseed shoots are summarized in Table 3. After 7 days of de-acclimation treatment, proline level decreased by 75.57 μM g^{-1} in comparison with acclimated shoots.

Table 3. The changes in proline, soluble sugars and electrolyte leakage level during acclimation of rapeseed shoots cultivated with and without exogenous proline

Acclimation time days	Concentation of proline mM				
	control (without proline)	5	10	15	20
	Proline μM g^{-1}				
0	79.68d	91.03c	95.41c	109.54b	148.26a
1	64.45d	86.39c	104.88b	112.03b	152.85a
3	21.36e	58.15d	118.42c	142.44b	171.95a
5	15.04e	55.48d	68.92c	106.97b	124.16a
7	4.11d	30.28c	32.55c	50.65b	64.37a
	Soluble sugars mg g^{-1}				
0	57.21d	69.20c	102.00a	72.39c	89.20b
1	33.24d	35.87d	86.33a	46.33c	67.60b
3	38.14b	23.67c	58.67a	34.40b	31.82b
5	31.23a	8.80c	39.20a	17.60b	22.64b
7	25.62a	14.40b	28.57a	14.32b	16.80b
	Electrolyte leakage %				
0	53.70a	49.67a	42.81b	43.62b	40.51b
1	58.69a	55.41a	48.31b	44.35b	42.94b
3	62.21a	57.53a	51.46ab	44.37b	48.75ab
5	67.14a	60.34ab	64.21a	56.31b	51.66b
7	75.30a	71.10a	65.46ab	62.24b	57.42b

Means within a row followed by the same letter are not significantly different, indicated by Duncan's multiple-range ($P \leq 0.01$)

Exogenous proline significantly increased endogenous proline content in all treatments tested. L-proline-treated shoots showed a significantly higher proline content compared with control shoots during de-acclimation duration. After 7 days at 18/16 °C a highest level of proline have been observed in shoots cultured on medium supplemented with 20 mM L-proline.

Soluble sugars level declined rapidly after shoots were returned to 18/16 °C, after 1 day of de-acclimation the soluble sugars content in shoots grown on medium without exogenous proline decreased by 23.97 mg g^{-1}. There was a slight increase in soluble sugars content during third day of de-acclimation before it began to decline. Addition of proline to the culture medium resulted in an 11.99–44.79 mg g^{-1} increase in soluble sugars content in the acclimated shoots comparing to non-treated shoots. However, during de-acclimation soluble sugars content in proline treated shoots substantially decreased and after 7 d of de-acclimation were similar or even lower in comparison with non-treated shoots. Results of the present experiments show that exogenous proline did not affect the soluble sugars content in the de-acclimated shoots.

In the present study a rapid decrease in cold tolerance during shoots de-acclimation was observed. The degree of cold tolerance of the shoots from the different treatment was substantially decreased with increasing de-acclimation duration. The lowest cold tolerance for shoots from all tested treatments was achieved at 7 days de-acclimation duration. On the media without L-proline on the 7th day of de-acclimation the electrolyte leakage of leaf discs increased by 21.60 % compared with the acclimated shoots.

The addition of exogenous proline to culture medium resulted in significant increase in rapeseed shoots cold tolerance. On the medium supplemented with exogenous proline after

7 days of de-acclimation electrolyte leakage of proline-treated shoots was 4.2–17.88 % lower in comparison with non-treated shoots.

Effect of exogenous amino acids on rapeseed shoots cold tolerance *in vitro*. The current research has showed that the level of proline in de-acclimated shoots and cultivated without amino acids decreased extremely (Table 4.).

Table 4. The changes in proline, soluble sugars and electrolyte leakage level during cold de-acclimation of rapeseed shoots cultivated with and without exogenous amino acids

De-acclimation time days	without amino acids	with amino acids
	Proline μM g^{-1}	
14a	82.23a	80.66a
14a-1d	66.27a	63.92a
14a-3d	23.46b	41.40a
14a-5d	7.44b	12.95a
14a-7d	6.73b	12.78a
	Soluble sugars mg g^{-1}	
14a	62.48b	138.41a
14a-1d	30.16b	49.59a
14a-3d	39.46a	41.22a
14a-5d	29.67a	28.43a
14a-7d	24.79a	20.84a
	Electrolyte leakage %	
14a	53.14a	40.21b
14a-1d	58.35a	46.42b
14a-3d	62.42a	50.64b
14a-5d	69.81a	59.59b
14a-7d	75.39a	68.42b

Means within a row followed by the same letter are not significantly different, indicated by Duncan's multiple-range ($P \leq 0.01$)

Treatments were: a – acclimation for days (temperature 4 °C day/night, photoperiod 16/8 h day/night);
d – de-acclimation treatment for days (temperature of 18/16 °C day/night, photoperiod 16/8 h day/night).

The supplement of amino acids after the 3, 5 and 7 day of de-acclimation in media increased proline essentially by 1,8; 1,7; 1,9 times comparing to rapeseed cultivated without amino acids. The level of soluble sugars in the shoots with the process of de-acclimation declined in comparison to the shoots that were acclimated for 14 days. It was also shown that amino acids in 14 day acclimated shoots positively influenced the level of accumulated soluble sugars (138.41 mg g^{-1}) in comparison to the shoots cultivated and acclimated in media without exogenous amino acids (62.48 mg g^{-1}).

The degree of rapeseed shoots to cold tolerance estimated by electrolyte leakage consistently increased accordingly to prolonging of the duration of de-acclimation. In shoots cultivated without amino acids treatment electrolyte leakage is higher (58.35 %–75.39 %) than in shoots that were cultivated with amino acids treatment (46.42 %–68.42 %).

The present study showed that the exogenous amino acids in medium after the 1, 3, 5 and 7 days of re-acclimation increased the cold tolerance of winter rapeseed (Fig. 6).

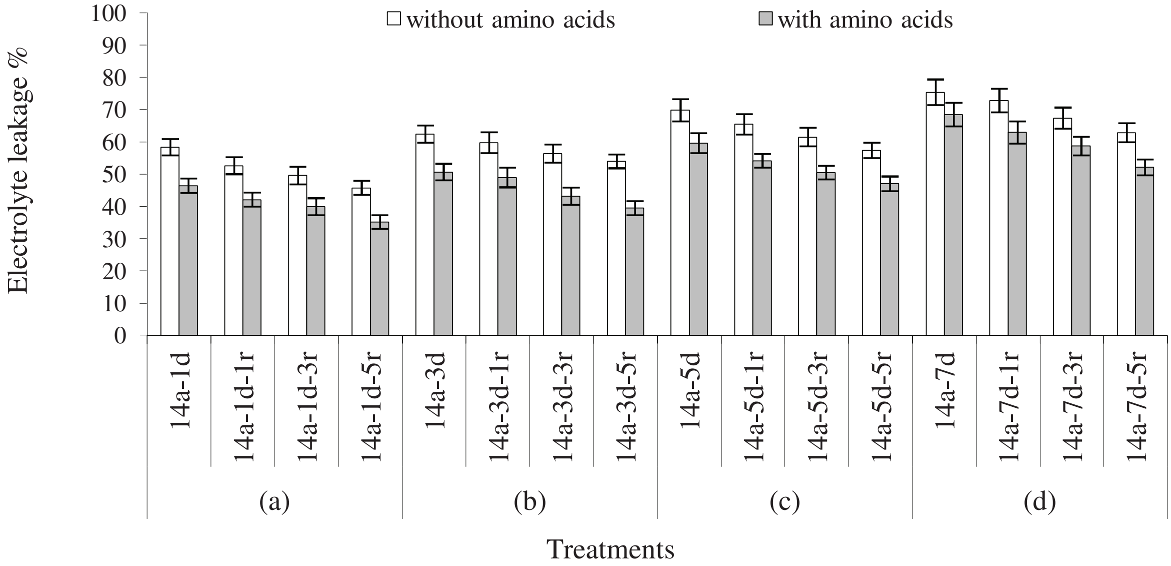

Figure 6. The changes in electrolyte leakage level during re-acclimation of rapeseed shoots cultivated with and without exogenous amino acids

Treatments were: a – acclimation for days (temperature 4 °C day/night, photoperiod 16/8 h day/night);
d – de-acclimation treatment for days (temperatures of 18/16 ºC day/night, photoperiod 16/8 h day/night);
r – re-acclimation treatment for days (temperature 4 ºC day/night, photoperiod 16/8 h day/night).

After de-acclimation and 5 day of re-acclimation exogenous amino acids in medium decreased the electrolyte leakage accordingly by 35.21 % (after the 1 day of de-acclimation); 39.46 % (after the 3 day of de-acclimation); 47,03 % (after the 5 day of de-acclimation) and 52.10 % (after the 7 day of de-acclimation) in rapeseed shoots.

Investigation *in vivo* under controlled conditions

Influence of acclimation duration on rapeseed cold tolerance *in vivo*. In non-acclimated plants of rapeseed proline content had raised from 12.41 μM g^{-1} (‘Siska’) to 52.18 μM g^{-1} (‘Sunday’) (Fig. 7a). After acclimation of the first week the proline content in plants increased from 1.9 to 6.9 times compared with non-acclimated plants. After 14 days of acclimation synthesis of proline increases to 85.46 μM g^{-1} (‘Siska’), 78.23 μM g^{-1} (‘Insider’), 90.88 μM g^{-1} (‘Valesca’) and 99.22 μM g^{-1} (‘Sunday’) in the investigated varieties. After the third week of acclimation the intensive accumulation of proline was determinate in ‘Insider’ (155.73 μM g^{-1}) plants. The highest proline content was determined in plants after 27 and 33 days of acclimation.

Non-acclimated plants of rapeseed accumulated soluble sugars from 32,0 mg g^{-1} (‘Valesca’) to 37,6 mg g^{-1} (‘Sunday’) (Fig. 7b). After 7 days of the acclimation content of soluble sugars increased to 72,11 mg g^{-1} (‘Siska’), 84,02 mg g^{-1} (‘Insider’), 68,83 mg g^{-1} (‘Valesca’) and 80,81 mg g^{-1} (‘Sunday’) compared with non-acclimated plants. Increasing of soluble sugars content in winter rapeseed plants depends on the genotype, after longer acclimation duration. Intensively synthesis of soluble sugars was determinate in fourth and fifth week of acclimation in ‘Siska’ variety, soluble sugars increased from 3.8 to 4.1 times, compared with non-acclimated plants.

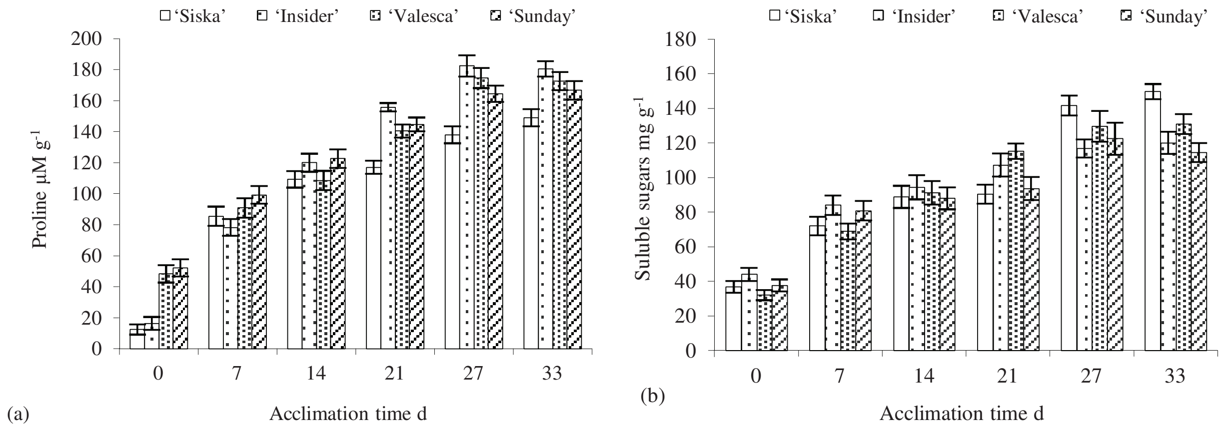

Figure 7. Proline and soluble sugars contents of rapeseed leaves during the acclimation

Winter rapeseed tissues by freezing to –10 °C electrolyte leakage varied from 76.73 % (‘Sunday’) to 85.32 % (‘Siska’) (Fig. 8.). After 7 days of acclimation the electrolyte leakage substantial decrease in varieties of ‘Valesca’ (64.31 %) and ‘Sunday’ (65.25 %).

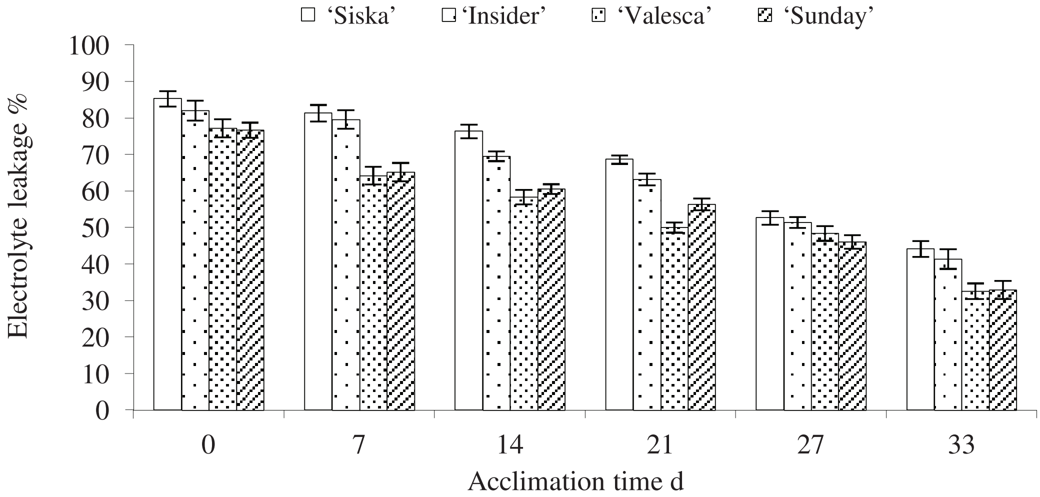

Figure 8. Cold tolerance of rapeseed leaves during the acclimation

It was determinate that after 14 days of acclimation of the investigated varieties of plant tissue electrolyte leakage was reduced to 76.44 % (‘Siska’), 69.59 % (‘Insider’), 58.43 % (‘Valesca’) and 60.63 % (‘Sunday’). Longer than 21 day of acclimation the plant tissue electrolyte leakage consistently decreased. After 27 days of the acclimation, the electrolyte leakage ranged from 46.14 % (‘Sunday’) to 52.73 % (‘Siska’). After 33 days acclimation of plant tissue electrolyte leakage decreased 1.9 (‘Siska’, ‘Insider’), 2.4 (‘Valesca’) and 2.3 (‘Sunday’) times.

Influence of freezing temperature on rapeseed cold tolerance *in vivo*. Plant tissues were frozen at –10 °C temperature, electrolyte leakage varied from 56.15 % (‘Valesca’) to 65.70 % (‘Siska’) (Fig. 9). Reducing the freezing temperature to –16 °C and to –22 °C, the plant tissue damage consistently increased.

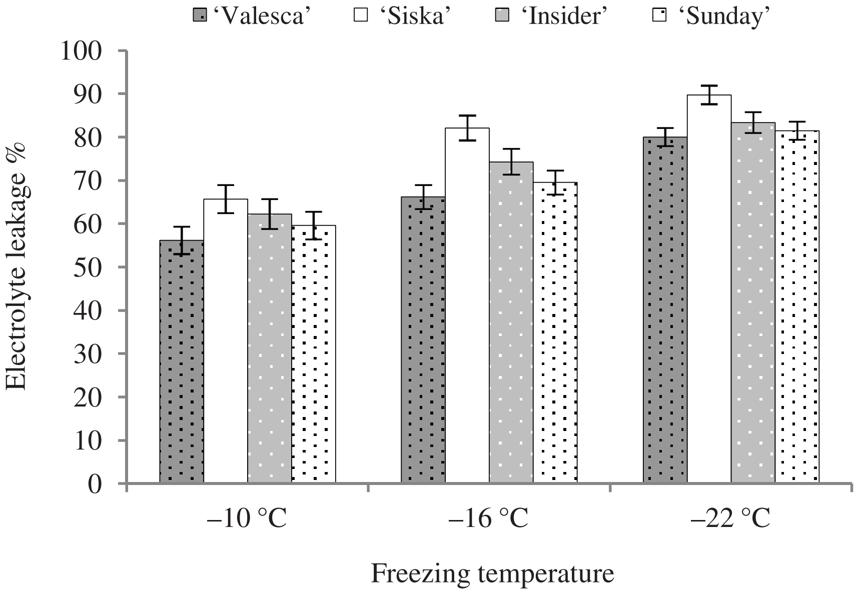

Figure 9. The electrolyte leakage of rapeseed plants exposed in different freezing temperatures

It was determinate that the most sensitive to the negative temperature effect of cultivar 'Siska' plants, after freezing electrolyte leakage was 82.10 % (–16 °C freezing temperature) and 89.73 % (–22 °C freezing temperature). The varieties 'Valesca' plant tissue electrolyte leakage after freezing –16 °C was 66.19 %, and freezing temperature of –22 °C was 80.01 %.

Field experiments

Climate conditions effects of proline and soluble sugars content in rapeseed. After the correlation regression analysis in 2008–2009 was determinate that the temperature and proline content in rapeseed there were reverse, strong and statistically significant at 99 % probability level, relationship was: r = –0.85 ('Siska'), r = –0.83 ('Insider'), r = –0.83 ('Valesca') and r = –0.85 ('Sunday') (Table 5).

Table 5. Changing temperature and proline content correlation in 2008–2011

Varieties	*Year* 2008–2009			Correlation coefficient
	x	y	Regression equation	
'Siska'	Temperature (x_1)	Proline content (y_1)	$y_1 = 94.6778–5.9665x_1$	r = –0.85**
'Insider'	Temperature (x_2)	Proline content (y_2)	$y_2 = 91.8014–6.42x_2$	r = –0.83**
'Valesca'	Temperature (x_3)	Proline content (y_3)	$y_3 = 104.54–7.5574x_3$	r = –0.83**
'Sunday'	Temperature (x_4)	Proline content (y_4)	$y_4 = 93.7656–5.2041x_4$	r = –0.85**
	Year 2009–2010			
'Siska'	Temperature (x_1)	Proline content (y_1)	$y_1 = 78.1148 – 5.6689x_1$	r = –0.85**
'Insider'	Temperature (x_2)	Proline content (y_2)	$y_2 = 79.1292 – 6.1773x_2$	r = –0.83**
'Valesca'	Temperature (x_3)	Proline content (y_3)	$y_3 = 105.7169 – 7.1946x_3$	r = –0.84**
'Sunday'	Temperature (x_4)	Proline content (y_4)	$y_4 = 76.6206 – 5.7231x_4$	r = –0.86**
	Year 2010–2011			
'Siska'	Temperature (x_1)	Proline content (y_1)	$y_1 = 67.908–3.0134x_1$	r = –0.73**
'Insider'	Temperature (x_2)	Proline content (y_2)	$y_2 = 56.2833–2.1971x_2$	r = –0.57**
'Valesca'	Temperature (x_3)	Proline content (y_3)	$y_3 = 59.4706–2.2238x_3$	r = –0.55**
'Sunday'	Temperature (x_4)	Proline content (y_4)	$y_4 = 66.5494–2.008x_4$	r = –0.44*

* – 95 % probability level, ** – 99 % probability level

The results were evaluated by the regression correlation method between temperature and soluble sugars content in winter rapeseed plants exist in reverse, on average strong and statistically significant relationship was r = –0.58 (‘Siska’), r = –0.70 (‘Insider’) and r = –0.72 (‘Valesca’), which reflected the mathematical expression of the linear regression equation (Table 6).

In 2009–2010 determinate the linear inverse, strong and statistically significant correlation between temperature dependence and the accumulation of proline content r = –0.85 (‘Siska’), r = –0.83 (‘Insider’), r = –0.84 (‘Valesca’), r = –0.86 (‘Sunday’) between temperature and soluble sugars content in rapeseed exists in reverse, on average strong and statistically significant relationship was r = –0.64 (‘Siska’), r = –0.61 (‘Insider’), strong and reverse and statistically significant relationship was r = –0.74 (‘Valesca’), r = –0.73 (‘Sunday’) (Table 5).

In 2010–2011 the correlation regression analysis showed that the temperature and proline content in winter rapeseed there were reverse existence, strong and statistically significant at 99 % probability level, relationship was r = –0.73 (‘Siska’), on average strong and statistically significant r = –0.57 (‘Insider’) and r = –0.55 (‘Valesca’) (Table 5).

Table 6. Changing temperature and soluble sugars content correlation in 2008–2011

Varieties	*Year* 2008–2009			Correlation coefficient
	x	*y*	Regression equation	
‘Siska’	Temperature (x_1)	Soluble sugars content (y_1)	$y_1 = 144.0908–4.5231x_1$	r = –0.58**
‘Insider’	Temperature (x_2)	Soluble sugars content (y_2)	$y_2 = 153.4154–5.5295x_2$	r = –0.70**
‘Valesca’	Temperature (x_3)	Soluble sugars content (y_3)	$y_3 = 167.3387–6.9368x_3$	r = –0.72**
‘Sunday’	Temperature (x_4)	Soluble sugars content (y_4)	$y_4 = 66.5494–2.008x_4$	r = –0.44**
	Year 2009–2010			
‘Siska’	Temperature (x_1)	Soluble sugars content (y_1)	$y_1 = 141.3734–7.9884x_1$	r = –0.64**
‘Insider’	Temperature (x_2)	Soluble sugars content (y_2)	$y_2 = 133.7408–7.6832x_2$	r = –0.61**
‘Valesca’	Temperature (x_3)	Soluble sugars content (y_3)	$y_3 = 146.617–9.9637x_3$	r = –0.74**
‘Sunday’	Temperature (x_4)	Soluble sugars content (y_4)	$y_4 = 143.3183–8.9383x_4$	r = –0.73**
	Year 2010–2011			
‘Siska’	Temperature (x_1)	Soluble sugars content (y_1)	$y_1 = 112.22–3.6334x_1$	r = –0.41*
‘Insider’	Temperature (x_2)	Soluble sugars content (y_2)	$y_2 = 81.067–1.6873x_2$	r = –0.35
‘Valesca’	Temperature (x_3)	Soluble sugars content (y_3)	$y_3 = 107.9822–3.9446x_3$	r = –0.54**
‘Sunday’	Temperature (x_4)	Soluble sugars content (y_4)	$y_4 = 82.9854–2.2163x_4$	r = –0.42*

* – 95 % probability level, ** – 99 % probability level

Effect of climatic conditions on cold tolerance in rapeseed. The research results was evaluated by correlation regression analysis in 2008–2009 (Table 7). During that period we found that between the proline content and electrolyte leakage of rapeseed plants existing in reverse, on average strong and statistically significant at 99 % probability level relationship was r = –0.62 (‘Siska’), r = –0.60 (‘Insider’), r = –0.65 (‘Valesca’) and r = –0.58 (‘Sunday’), which reflected the mathematical expression of the linear regression equation. In 2009–2010 the results of regression correlation analysis between proline content and electrolyte leakage in plants existing a reverse, strong to moderate statistically significant relationship was r = –0.76 (‘Siska’), r = –0.77 (‘Insider’), r = –0.70 (‘Valesca’), r = –0.68 (‘Sunday’), which reflected the mathematical expression of the linear regression equation.

Table 7. Proline content and electrolyte leakage correlation in 2008–2011

Varieties	*Year* 2008–2009			Correlation coefficient
	x	*y*	Regression equation	
‘Siska’	Proline content (y_1)	Electrolyte leakage (y_1)	$y_1 = 66.0662 – 0.1498x_1$	r = –0,62**
‘Insider’	Proline content (y_2)	Electrolyte leakage (y_2)	$y_2 = 59.3472 – 0.1478x_2$	r = –0.60**
‘Valesca’	Proline content (y_3)	Electrolyte leakage (y_3)	$y_3 = 53.3204 – 0.1371x_3$	r = –0.65**
‘Sunday’	Proline content (y_4)	Electrolyte leakage (y_4)	$y_4 = 56.116 – 0.1815x_4$	r = –0.58**
		Year 2009–2010		
‘Siska’	Proline content (y_1)	Electrolyte leakage (y_1)	$y_1 = 73.7775 – 0.1366x_1$	r = –0.76**
‘Insider’	Proline content (y_2)	Electrolyte leakage (y_2)	$y_2 = 68.319 – 0.1333x_2$	r = –0.77**
‘Valesca’	Proline content (y_3)	Electrolyte leakage (y_3)	$y_3 = 62.3324 – 0.1037x_3$	r = –0.70**
‘Sunday’	Proline content (y_4)	Electrolyte leakage (y_4)	$y_4 = 56.4184 – 0.1401x_4$	r = –0.68**
		Year 2010–2011		
‘Siska’	Proline content (y_1)	Electrolyte leakage (y_1)	$y_1 = 75.8287 – 0.2403x_1$	r = –0.72**
‘Insider’	Proline content (y_2)	Electrolyte leakage (y_2)	$y_2 = 72.8893 – 0.2464x_2$	r = –0.68**
‘Valesca’	Proline content (y_3)	Electrolyte leakage (y_3)	$y_3 = 65.2212 – 0.2072x_3$	r = –0.64**
‘Sunday’	Proline content (y_4)	Electrolyte leakage (y_4)	$y_4 = 68.4354 – 0.1373x_4$	r = –0.45*

* – 95 % probability level, ** – 99 % probability level

In 2010–2011 the results of the regression correlation method showed that the proline content and electrolyte leakage in plants existed reverse, strong to moderate statistically significant relationship was r = –0.72 (‘Siska’), r = –0.68 (‘Insider’), r = –0.64 (‘Valesca’), which reflected the mathematical expression of the linear regression equation.

SUMMARY OF RESULTS

In preparation for winter biochemical, physiological and anatomical changes in plant cells occur. Plants retain less water thus increasing the cell structure to cold tolerance and destructive effects of ice crystal growing. Plants that are going in to winter accumulate osmotically active matter (proline, sacharides, phospholipids, organic acids) and this material prevents intracellular freezing and reduces the osmotic potential of the cells (Tretiakov, 1998). Different biochemical compounds increase in plant cells during cold acclimation (Korn et al., 2008) and some of these compounds are important for cold tolerance (Chen et al., 2006; Trischuk et al., 2006).

We found that acclimated *in vitro* shoots of rapeseed has increased proline in the first three weeks of the acclimation time. After 21days of acclimation proline increased from 3.8 to 5.4 times, compared with non-acclimated shoots, while the accumulation of proline was the greatest in the variety ‘Valesca’, the least amount of proline accumulation occurred in the variety of ‘Siska’.

Soluble sugars influence as osmoregulators, higher content of dissolved sugars reduces the freezing temperature of fluid in cells, thus increasing cell tolerance to a negative temperature (Jacobsen et al., 2007). Sugars content in plant affects the freezing of tissues and is used as a nutrient and energy reserve, changing membrane characteristics and performs the functions of creoprotector to preserve the protein structure. Accumulation of sugars at low temperatures has been extensively studied in many plants, including cress, cabbage, spinach, raspberries (Guy et al., 1992; Sasaki et al., 1996; Wanner, Junttila, 1999; Polonen et al., 2000). After 11days of acclimation sugar content in barley increased 8 times (Bravo et al., 1998), in goosefoot 10 times (Jacobsen et al., 2007). Our studies have shown that the accumulation of soluble sugars *in vitro* was most intensive during the first two weeks of acclimation. After 21 day of acclimation, soluble sugar content increased only in a variety of ‘Insider’. Cultivating of winter rapeseed plants *in vivo* under controlled conditions, the synthesis of proline and soluble sugars was the most intensive in the first four weeks. Sasaki and colleagues (1996) reported that sugar content is not always determined by the degree of cold and cold tolerance can be affected by other factors such as lipids, soluble proteins and free proline (Bravo et al., 1998; Hincha, 2002). Petcu and Terbea (1995) determined that the free proline content in non-acclimated winter wheat was very low, but after the two weeks of acclimation proline increased in tested plants. The investigation of several different plants showed the increase of free amino acids, especially proline, during acclimation, which correlated with cold tolerance (Petcu, Terbea, 1995; Petcu et al., 2000; Xin, Browse, 2000; Patton et al., 2007).

Affection of freeze is usually caused by dehydration of cells (Xin, Browse, 2000), which might unbalance the cell functions. During the cold stress changes the structure and function of membrane changes. It was determinated in early 1912 that the cold affects the plasma membrane (Li et al., 2004; Szabados, Savoure, 2009). Accumulation of dissolves in cells increases cell osmolarity, which regulates the balance of extracellular and intracellular solution. This raises the turgor, which is essential for cells. Under the osmotic or dehydration stress conditions the membrane must remain solid in order to prevent protein denaturation. Membrane damage mostly occurs due to dehydration in the cycles of acclimation and de-acclimation. Changes of electrolyte leakage show the membrane damage induced by temperature stress (Bertin et al., 1996).

Prolonging the duration of cold acclimation caused the consistent decrease of electrolyte leakage in the tissues of winter rapeseed. After 21days of acclimation *in vitro* and tempering after 27 days *in vivo* under controlled conditions the lowest electrolyte leakage determinated in cultivar ‘Valesca’ plant tissues, the highest – ‘Siska’.

The plant development period determines the resistance to low temperatures. Hume and Jackson (1981) found that soybean plants were more resistant to negative –3.8 °C temperature in the stages of the cotyledons and the first stage of three-leaves than in the first true leaves stage. Our studies have shown that the damage by cold of winter rapeseed shoot tissues of the ‘Siska’ variety depended on the acclimation time and leaf age. Prolonging the duration of acclimation electrolyte leakage consistently decreased. Regardless of the acclimation time the youngest (upper) leaf electrolyte leakage was significantly lower compared with the oldest (lower) leaves. The obtained results confirmed the study done by Wanner and Junttila (1999) that cold tolerance is not the same in all parts of the plant.

Plant de-acclimation sustainability under the changeable temperature conditions is very important for plant resistance in late winter and early spring when the plants are especially damaged by cold. De-acclimation sustainable reduction in both the wild and under controlled conditions occurs much rapidlier (from several days to several weeks) than in acclimation (from several weeks to several months) (Kalberer et al., 2006). The differences of loss of acclimation and de-acclimation kinetics sustainability may be related to different energy needs. Acclimation is related to changes of structure and functions that need high levels of energy (Browse, Lange, 2004). The decrease of de-acclimation sustainability may relatively require less energy, because this process depends more on regulation of gene expression and biosynthesis. The decrease of de-acclimation sustainability can bedetermined by metabolites, often synthesized and stored in cold acclimation during catabolism (Kalberer et al., 2007). Ogren (1996) found that the passive blueberry de-acclimation sustainability depended on the content of osmolyte decrease, including soluble sugars. It was established that under the effect of the low positive temperatures soluble sugars decreased to 54 % in the needles of scoots pine, it was also shown that a linear correlation between the soluble sugars content and cold tolerance (Ogren, 1997). It is expected that large amounts of accumulated proline under stress can be very useful for recovery after stress, because proline decomposes and provides plant cells with carbon and nitrogen (Hare, Cres, 1997). The ability to re-acclimate after de-acclimation is typical for many hibernating plants, how ever, the degree of resistance acquired during initial acclimation is hardly achieved (Repo, 1991). Temperature fluctuations, such as day and night cycles, are more effective for acclimation than constant temperature, because it influences de-acclimation and re-acclimation cycles. Resistance depends on the temperature variation of the frequency and duration (Kalberer et al., 2006). The relationship between the duration and sustainability loss of de-acclimation is not always linear. Some plants can react to de-acclimation rapidly, immediately after the heat exposure and is characterized by a gradual decline of resistance (Solecki et al., 2008), while others are stable during the de-acclimation and need a relatively longer period to lose the acclimation (Vega et al., 2000).

Modelling conditions of de-acclimation and re-acclimation *in vitro* we found that longer duration of de-acclimation proline and soluble sugars content in winter rapeseed shoots decreased. The minimum amount of these compounds in 4.11 μM g^{-1} and 25.62 mg g^{-1} was determinated after 7 days of de-acclimation. Decline in proline content during the de-acclimation has also been established in white clover stolons (Svenning et al., 1997). Re-acclimation stimulated synthesis of proline and soluble sugars in shoots, the highest levels of these compounds determined after 5 days of re-acclimation. A large content of proline under stress conditions can be very useful under repetitive stress conditions (Hare, Cres, 1997). Konstantinova and colleagues (2002) found that resistance to cold of transgenic tobacco enhances the expressionof p5cs gene. Hughes-Robert and colleagues (2003) found that the investigation of rapeseed leaf discs under osmotic stress intensively accumulated proline.

We suggest that longer duration of de-acclimation of rapeseed shoot tissue increased electrolyte leakage – cold tolerance decreased. During re-acclimation the cold tolerance of winter rapeseed increased.

Scientists estimated that the ABA is involved in plant acclimation process. Many plant species accumulate endogenous ABA in response to changing environmental conditions, especially in the low temperature and drought (Davies, Zhang, 1991). Along with the increase of ABA concentration protein synthesis, metabolism and cell ultrastructure changes occur, suggesting that ABA acts as a signal transporter involved in the acclimation process (Loik, Nobel, 1993). The relationship between ABA and proline content changes in operating hyper- and hypo- osmose rapeseed leaf discs (Trotel-Aziz, 2000). Recovery after the stress when a subjected plant was affected by light, ABA intensified accumulation of proline, however, the impact of ABA in the dark had no significant effect on the proline content.

The results of this investigation indicate that exogenous ABA in medium increased the cold tolerance of shoots. The lowest conductivity of the electrolyte was determinated in shoots cultured on medium which had been supplemented with 1.5 mM ABA after 21 days of acclimation. After 14 days of acclimation intensive accumulation of proline was noticed in shoots cultured on medium supplemented with 0.5 mM ABA enhancement, compared with non-acclitated shoots. The scientific literature states that exogenous ABA also increased cold tolerance in cactus (Loik, Nobel, 1993), rape (Wilen et al., 1994), barley (Bravo et al., 1998), tomato (Kim et al., 2002), chickpea (Kumar et al., 2008).

The structural and functional integrity of the membrane is supported by proline M4 lactate dehydrogenase (Chadalavada et al., 1994). Ozturk and Demir (2002) found that exogenous proline had a protective function of catalase, peroxidase and polyphenol oxidase in spinach cultured *in vivo* and *in vitro* concerning the impact of NaCl. Chadalavada and colleagues (1994) estimate that proline effected the formation of hydrophilic colloid in aqueous medium interacting with hydrophobic proteins. Exogenous proline stimulated the growth of rice callus *in vitro* (Kavi Kishor, 1989).

In vitro investigation determined that exogenous proline increased significantly during the de-acclimation of endogenous proline content in all treatments, but the accumulation of soluble sugars had no significant influence. Exogenous proline in medium increased the cold tolerance of rapeseed shoots during de-acclimation. In the nutrient medium supplemented with exogenous proline, after 7 days of de-acclimation, electrolyte leakage of shoot tissues was 4.2 to 17.88 % lower compared to shoots cultured without proline enhancement.

Dromantiene with collegues (2009) found that exogenous amino acids affect plant photosynthesis, respiration, metabolism and other processes, and increase the yield of winter wheat in both quantitative and qualitative indicators. Proline leads to activate plant defense mechanism against osmotic stress caused by cold. Researches in various plants showed that proline content increased during acclimation and correlated with cold tolerance (Xin, Browse, 2000; Patton et al., 2007; Burbulis et al., 2008).

It can be stated that the amino acid in medium substantially increased the proline content in winter rapeseed shoots after 3, 5, 7 day de-acclimation, compared with shoots grown without the enhancement. Proline and soluble sugars synthesis was much more intensive in shoots cultured on medium supplemented with amino acids after 1 day of de-acclimation and for 5 days of re-acclimation.

In the processes of de-acclimation and re-acclimation, exogenous amino acids in medium increased the cold tolerance of rapeseed shoots. The lowest electrolyte leakage of rapeseed shoots was determinated after 1 day of de-acclimation and 5 days re-acclimation.

Proline is related to the overall response to stress (Kavi Kishor et al., 2005; Kumaret al., 2010; Li et al., 2010; Toka et al., 2010), it also acts as creoprotector which higher level is accumulated during the cold (Patton et al., 2007; McClinchey, Kott, 2008; Dörffling et al., 2009; Pociecha et al., 2009; Gothandam et al., 2010).

In conclusion, the results of *in vivo* studies under field conditions suggest that proline and soluble sugars content in winter rapeseed plants depended on the environmental temperature. Applied correlation regression analysis showed that the descent of temperature, proline and soluble sugar content in the plant increased. Cold tolerance of winter rapeseed is mainly effected by the amount of proline in plant tissues – increasing the proline content the electrolyte leakage consistently decreased. The dependence of electrolyte leakage on the amount of soluble sugars was indeterminate.

Investigation *in vitro* and *in vivo* under controlled conditions and *in vivo* under field conditions determined that the most cold-resistant varietys ‘Valesca’ and ‘Sunday’, the most sensitive – ‘Siska’. The obtained results has confirmed the suggestion of other researchers (Petco, Terbea, 1995; Bravo et al., 1998; Rapacz, Janowiak, 1999; Rife, Zeinali, 2003; Dai et al., 2007) that one of the most important factors that determine cold tolerance is genotype.

CONCLUSIONS

1. During rapeseed shoots acclimation *in vitro* the proline content consistently increased during the first three weeks of acclimation. The content of proline accumulation in shoots depends on the genotype.
2. During the cold acclimation of plants *in vitro* and *in vivo* systems the intensiveness of soluble sugars accumulation differed.
3. Constantly increasing the duration of acclimation in *in vitro* and *in vivo* systems the electrolyte leakage of plant tissues, induced by cold, consistently decreased. Electrolyte leakage is determined by genotypes.
4. It was established that prolonged de-acclimation period caused decreasing of the amounts of proline and soluble sugars. During re-acclimation proline and soluble sugars contents consistencly increased.
5. Under longer durations of de-acclimation the electrolyte leakage in rapeseed shoot tissues increased, thus cold tolerance decreased. During the re-acclimation the cold tolerance of winter rapeseed increased.
6. The additive of exogenous abscisic acid to the medium stimulated the synthesis of proline and soluble sugars, and increased the cold tolerance of shoots.

7. Exogenous proline had no significant effect on the accumulation of soluble sugars, but significantly increased the endogenous proline content and cold tolerance of shoots during de-acclimation treatment.
8. Endogenous proline and soluble sugars content affected by exogenous amino acids varied dependingly on the duration of de-acclimation and re-acclimation. Exogenous amino acids increased the cold tolerance of rapeseed shoots in both de-acclimation and re-acclimation processes.
9. Proline and soluble sugars content in winter rapeseed plants depends on the environmental temperature – the decrease of the temperature caused the more intense proline and soluble sugars synthesis.
10. Cold tolerance of winter rapeseed is mainly determined by content of proline in plant tissues – increased proline content consistently decreased electrolyte leakage. The dependance of electrolyte leakage from the soluble sugars content was not determinated. Cold tolerance determinated genetically, the most resistant cultivars were ‘Valesca’ and ‘Sunday’, the most sensitive – ‘Siska’.

LIST OF PUBLICATIONS

***Articles*:**

1. Burbulis N., **Jonytienė V.**, Kuprienė R., Blinstrubienė A., Liakas V. Effect of abscisic acid on cold tolerance in *Brassica napus* shoots cultured *in vitro*. *Journal of Food, Agriculture & Environment*. 2010. vol. 8 (3&4), p. 698–701.
2. Burbulis N., **Jonytienė V.**, Kuprienė R., Blinstrubienė A., Liakas V. Biochemical and physiological factors related to cold de-acclimation and re-acclimation in rapeseed shoots *in vitro*. *Journal of Food, Agriculture & Environment*. 2011. vol. 9 (1), p. 483–487.
3. Burbulis N., **Jonytienė V.**, Kuprienė R., Blinstrubienė A. Changes in proline and soluble sugars content during cold acclimation of winter rapeseed shoots *in vitro*. *Journal of Food, Agriculture & Environment*. 2011. vol. 9 (2), p. 371–374.
4. **Jonytienė V.**, Burbulis N., Kuprienė R., Blinstrubienė A. Effect of exogenous proline and de-acclimation treatment on cold tolerance in *Brassica napus* shoots cultured *in vitro*. *Journal of Food, Agriculture & Environment*. 2012. vol. 10 (1), p. 327–330.

Conference reports:

1. Burbulis N., **Jonytienė V.**, Kuprienė R. Biochemical and physiological factors related to cold acclimation and de-acclimation in rapeseed shoots *in vitro*. Bastutinių šeimos aliejinių augalų produktyvumo ir atsparumo patogenams didinimas. LŽŪU, Akademija, Spalio 15 d., 2010 m.
2. **Jonytienė V.**, Burbulis N. Carbohydrates and proline levels in cold acclimated winter rapeseed shoots in relation to exogenous amino acids in culture media. Science, business and research, partnership. LŽŪU, Akademija, 5–6 May, 2011.
3. **Jonytienė V.**, Burbulis N., Kuprienė R. Effect of exogenous proline on cold tolerance in *Brassica napus* L. shoots cultured *in vitro*. Climate change: agro- and forest systems sustainability. Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry, Babtai, 21–22 June, 2011.

About the author of the dissertation

Vaida Jonytienė was born in Kelmė, on 12th of August, 1980. After graduating secondary school in Kelmė in 1998. She entered the Lithuanian University of Agriculture (now Aleksandras Stulginskis University), Faculty of Agronomy in 2000. Graduated and gained bachelor's degree in society healthcare in 2004 she became the winner of Aleksandras Stulginskis scholarship in 2005. In 2006 Vaida Jonytiene obtained a master's degree in Agronomy and during the years 2006–2011 was a PhD student at the Department of Crop Science and Animal Husbandry. Since 2011 she has been working as junior researcher in the Laboratory of Agrobiotechnology, Aleksandras Stulginskis University. In 2012 she and her colleagues became laureates of the Aleksandras Stulginskis University Science Award for the Scientific Work set on “Investigation of oilseed plant in *in vitro* cultures”.

REZIUMĖ

Žieminio rapso (*Brassica napus* L.) pramoninę vertę bei konkurencingumą Lietuvoje mažina nepakankamas jo adaptyvumas – ištvermingumas žiemą ir atsparumas šalčiui. Ši problema ypač išryškėja kintančio klimato, nepastovių žiemojimo sąlygų fone. Esant nepalankiems aplinkos veiksniams, sutrinka augalų fiziologiniai procesai. Streso poveikis augalams priklauso nuo jo trukmės, stiprumo ir augalo genetinių savybių (Alexieva et al., 2003).

Temperatūra – vienas svarbiausių aplinkos veiksnių, nuo kurio priklauso viso organizmo fiziologiniai procesai. Augalų grūdinimasis – tai labai sudėtingi biocheminiai procesai, kurių metu augalai ruošiasi žiemos šalčiams. Augalų ląstelėse kaupiasi plastinės ir apsauginės medžiagos, mažėja vandens kiekis ląstelėse, keičiasi protoplazmos struktūra ir savybės. Užgrūdinti augalai tampa atsparesni ne tik didesniam šalčiui, bet ir kitiems nepalankiems žiemos veiksniams. Augalų grūdinimosi procesai vyksta dviem etapais. Pirmojo etapo metu, esant vidutinei paros temperatūrai nuo 6 °C iki 0 °C, sulėtėja augalų augimo procesai, bet dar vyksta fotosintezė. Antrojo augalų grūdinimosi etapo metu, kuris vyksta esant nuo –2 iki –6 °C vidutinei paros temperatūrai, fotosintezės procesai visiškai sustoja. Žema teigiama temperatūra, aktyvuoja vidinę augalo reakciją, aktyvina genus, kurie pakeičia membranų laidumą, didina atsparumą šalčiui (Anisimovienė ir kt., 2006).

Natūraliai augantys augalai evoliucijos eigoje prisitaikė, kad išgyventų įvairiose klimato sąlygose. Atskirų augalų rūšių sugebėjimas išgyventi ekstremaliose klimato sąlygose, kai kurie genetiniai ir molekuliniai atsparumo šalčiui aspektai, išaiškinti tiriant modelinius augalus (vairenį, tabaką ir kt.) rodo, kad augalų prisitaikymo galimybės yra labai didelės ir toli gražu neišnaudotos kultūriniuose augaluose. Žieminiai rapsai dažniausiai būna pažeidžiami žiemos pabaigoje pavasario pradžioje, kai augalai yra priverstinės, o ne organinės ramybės būklėje. Rapso užsigrūdinimo tvarumas sumažėja dėl didelių temperatūros svyravimų – nuo skatinančių augimą teigiamų iki žemų neigiamų temperatūrų. Atlydžių metu gali atsinaujinti augalų vegetacija, jie praranda sugebėjimą pakartotinai užsigrūdinti, o vėl nukritus temperatūrai iki neigiamos reikšmės augalai būna pažeidžiami šalčio. Mokslinėje literatūroje teigiama, kad žiemkenčių reakcija į neigiamą temperatūrą žiemos–pavasario periodu priklauso nuo įvairių biocheminių junginių (prolino, sacharidų, fitohormonų ir t. t.) kiekių augalo audiniuose (Patton et al., 2007; McClinchey, Kott, 2008; Dörffling et al., 2009; Pociecha et al., 2009; Gothandam et al., 2010; Novickienė et al., 2010).

Hipotezė – temperatūros pokyčių poveikyje kinta endogeninio prolino ir tirpiųjų sacharidų kiekiai, kurie apsprendžia žieminio rapso atsparumą šalčiui.

Darbo tikslas – nustatyti endogeninių ir egzogeninių veiksnių poveikį žieminio rapso atsparumui šalčiui *in vitro* ir *in vivo*.

Uždaviniai:

1. Įvertinti prolino ir tirpiųjų sacharidų kiekių kaupimosi pokyčius grūdinant rapsą *in vitro* ir *in vivo.*
2. Ištirti egzogeninių priedų maitinamojoje terpėje poveikį žieminio rapso atsparumui šalčiui *in vitro*.
3. Nustatyti genotipo poveikį rapso atsparumui šalčiui *in vitro* ir *in vivo.*
4. Įvertinti žieminio rapso užsigrūdinimo tvarumą *in vitro* ir *in vivo* kintančios temperatūros sąlygomis.

Ginamieji disertacijos teiginiai:

1. Žieminio rapso atsparumas šalčiui didžiąją dalimi lemiamas endogeninio prolino, o ne tirpiųjų sacharidų kiekio augalų audiniuose.
2. Grūdinant ūglius *in vitro* egzogeniniai priedai (abscizo rūgštis, prolinas, tirtas aminorūgščių kompleksas) maitinamojoje terpėje didina atsparumą šalčiui.

3. *In vitro* sistemoje L-prolinas ir tirtas aminorūgščių kompleksas atstato atlydžio metu sumažėjusį užsigrūdinimo tvarumą.
4. Endogeninio prolino kiekis yra membranos stabilumo atsparumo šalčiui žymuo ir gali būti naudojamas kuriant atsparius šalčiui genotipus.

Darbo mokslinis naujumas. Nustatyta, kad žieminio rapso atsparumas šalčiui didžiąja dalimi lemiamas endogeninio prolino kiekio augalo audiniuose – didėjant prolino kiekiui elektrolitų laidumas nuosekliai mažėja. Tirpiųjų sacharidų poveikis elektrolitų laidumui daugeliu atvejų silpnas ir statistiškai nepatikimas.

Darbo praktinė vertė. Tyrimų rezultatų visuma sudaro prielaidas didinti žieminio rapso atsparumą šalčiams technologinėmis priemonėmis. Abscizo rūgštis, aminorūgštys, L-prolinas (arba jo analogas) gali būti veiksmingi didinant žieminio rapso atsparumą šalčiui Lietuvos klimatinėmis sąlygomis. Kuriant atsparius šalčiams žieminio rapso genotipus *in vitro* sistemoje prolinas gali būti naudojamas kaip žymuo.

Išvados

1. Grūdinant rapso ūglius *in vitro* prolino kiekis nuosekliai didėjo pirmųjų trijų grūdinimo savaičių metu. Prolino kiekio kaupimasis ūgliuose nulemtas genotipo.
2. *In vitro* ir *in vivo* sistemose augalų grūdinimosi metu tirpiųjų sacharidų sintezės intensyvumas skyrėsi.
3. Ilginant grūdinimo trukmę *in vitro* ir *in vivo* sistemose šalčio sukeliamas augalų audinių elektrolitų laidumas nuosekliai mažėjo. Elektrolitų laidumą sąlygojo genotipas.
4. Modeliuojant atlydžio ir pakartotinio grūdinimo sąlygas nustatyta, kad ilginant atlydžio trukmę prolino ir tirpiųjų sacharidų kiekiai mažėjo. Pakartotinas grūdinimas intensyvino prolino ir tirpiųjų sacharidų sintezę.
5. Ilginant atlydžio trukmę rapso ūglių audinių elektrolitų laidumas didėjo – atsparumas šalčiui mažėjo. Pakartotinio grūdinimo metu žieminio rapso atsparumas šalčiui didėjo.
6. Egzogeninės abscizo rūgšties priedas maitinamojoje terpėje skatino prolino ir tirpiųjų sacharidų sintezę bei didino ūglių atsparumą šalčiui.
7. Egzogeninis prolinas neturėjo esminės įtakos tirpiųjų sacharidų kaupimuisi, tačiau ženkliai padidino endogeninio prolino kiekį ir ūglių atsparumą šalčiui atlydžio metu.
8. Endogeninio prolino ir tirpiųjų sacharidų kiekiai egzogeninių aminorūgščių poveikyje varijavo priklausomai nuo atlydžio ir pakartotinio grūdinimo trukmės. Egzogeninių aminorūgščių priedas maitinamojoje terpėje didino rapso ūglių atsparumą šalčiui tiek atlydžio tiek pakartotinio grūdinimo metu.
9. Prolino ir tirpiųjų sacharidų kiekiai žieminio rapso augaluose priklauso nuo aplinkos temperatūros – žemėjant temperatūrai, prolino ir tirpiųjų sacharidų sintezė intensyvėjo.
10. Žieminio rapso atsparumas šalčiui didžiąja dalimi lemiamas prolino kiekio augalo audiniuose – didėjant prolino kiekiui elektrolitų laidumas nuosekliai mažėjo. Elektrolitų laidumo priklausomybė nuo tirpiųjų sacharidų kiekio nenustatyta. Žieminio rapso atsparumas šalčiui determinuotas genetiškai, atspariausios veislės buvo ‘Valesca’ ir ‘Sunday’, jautriausia – veislė ‘Siska’.