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Preparation of oilseed rape for over-wintering according to autumnal growth and cold acclimation period

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Abstract

A field experiment on different sowing dates of the two winter rape cultivars ‘Sunday’ and ‘Kronos’ (hybrid) was carried out during 2008–2009 at the Experimental Station of the Lithuanian University of Agriculture (54°53′N + 23°50′E). The soluble protein assays were done at the Laboratory of Plant Physiology, Institute of Botany. The study was designed to explore the effect of autumnal growth and cold acclimation period on winter rape (*Brassica napus* L.) cultivars’ preparation for over-wintering.

According to biometric parameters (number of leaves per plant, root collar diameter and height of apical bud), rape sown in the second half of August (20 08) and at the end of August (30 08), whose autumnal growth and cold acclimation period was 64–76 days, was best prepared for wintering. The duration of autumnal growth and cold acclimation period significantly influenced biometric parameters of both winter rape cultivars. A consistent increase in the amount of crude protein in apical bud of both winter rape cultivars of later sowing dates was estimated.

Upon completion of cold acclimation (preparation for wintering) period, the composition of soluble protein fractions derived from the cells significant for rape wintering organs – apical buds – was analyzed. The differences or/and peculiarities in protein composition in these cells of both tested cultivars ‘Kronos’ and ‘Sunday’ as influenced by the duration of their autumnal growth and cold acclimation period were revealed. It was established that a higher content of individual protein components and proteins having molecular masses less than 66 kDa were accumulated in ‘Kronos’ apical bud cells. A longer autumnal growth and cold acclimation period had a more distinct effect on the increment of protein component number in ‘Kronos’ compared to cv. ‘Sunday’, while a shorter (about 60 days) period exerted a negative effect on the total soluble protein component number in apical buds of both tested cultivars, especially ‘Kronos’.

Key words: winter rape, sowing date, over-wintering, cold acclimation, proteins.

Introduction

Sowing date, being determinant for growth stage, may play a decisive role in optimizing freezing resistance of winter annual plants (Crosatti et al., 2008). Meyer and Badaruddin (2001), Lecomte et al. (2003) reported that freezing tolerance was higher in younger plants of legumes and cereals. All bi-annual plants have the inherent genetically determined period for preparation for wintering, generally termed as “cold acclimation” (Thomashow, 1999) during which they acquire frost tolerance or resistance and dormancy maintenance characteristics. The duration of the preparation period for over-wintering of winter rape is about 60–80 days (Brazauskienė, Šidlauskas, 2001).

Biometric parameters of plants and their ability to accumulate nutrients depend on sowing

time (Velicka et al., 2006). Results of some investigations show that successful over-wintering of rape plants is observed after they develop 6–8 leaves, a root collar diameter of 8–10 mm and a height of apical bud not exceeding 3 cm in autumn before wintering (Cramer, 1990; Маковски, 1990). The chemical composition of apical bud of winter rape in autumn is very important for over-wintering. A negative correlation was established between rape over-wintering and content of crude protein in apical bud: $r = -0.79$ (Velička et al., 2005). The lower content of proteins in apical bud, the better over-wintering of rape was noticed.

The main environmental factors inducing plant adaptive cold acclimation processes to start are low non-freezing temperature (+2–+10°C), short day-length, light/dark duration ratio (Hughes, Dunn, 1996; Jeknič, Chen, 1999; Dionne et al., 2001). Only fully cold acclimated plants are frost resistant and able to maintain dormancy (Ивонис и др., 1984; Merkys, Anisimovienė, 1998; Horvath et al., 2003). There are numerous data showing that during the period of plant cold acclimation the changes in the expression of various genes, physico-chemical peculiarities of membranes, chemical structure of the cells, accumulation of osmoprotectants, antioxidants, alteration in photosynthetic capacity, plant growth and developmental processes, up to temporary suspension of visible growth of any plant structures containing a meristem occur (Thomashow, 1999; Horvath et al., 2003; Rapacz et al., 2008). Despite these genetic, biochemical and physiological studies of plant adaptation processes, perception and subsequent transduction of environmental signals, primary biochemical events in the cell, changes of metabolism and metabolic routes leading to different responses have not been discovered yet.

Moreover, cold acclimation of introduced plant species, particularly from countries with warmer climate, is problematic. This is also true for economically significant oilseed plant – winter rape (Velička et al., 2005). In Lithuanian climate conditions with frequently alternating cold and thaws, and circadian temperature fluctuation, winter rape crops are often damaged in winter (Anisimovienė, Novickienė, 2003; Anisimovienė et al., 2006).

Therefore, an understanding of the molecular basis of such plants cold acclimation – preparation for dormancy period is significant from the theoretical point of view and has a potential practical application. Our experimental data (Ивонис и др., 1984; Merkys, Anisimovienė, 1998; Anisimovienė et al., 2006), as well as that of other researchers (Hudges, Dunn, 1996; Jeknič, Chen, 1999; Svensson et al., 2002; Welling et al., 2002; Stupnikova et al., 2003; Bentem et al., 2006), demonstrate that protein synthesis and/or metabolism during plant cold acclimation period, as well as protein composition in the cells of plant organ, significant for wintering, upon completion of cold acclimation period are closely related to plant cold acclimation degree and dormancy maintenance. It was reported that an increase in the number of dehidrins – proteins with mol wts of 65, 60, and 14.4 kDa was related to plant cold acclimation and acquired frost resistance (Jeknič, Chen, 1999; Velička et al., 2005).

The objective of this study was to analyse the effect of autumnal growth and duration of cold acclimation (preparation for wintering) period on biometric parameters and physiological-biochemical peculiarities, namely protein composition transformation, in the two winter rape cultivars ‘Sunday’ and ‘Kronos’.

Material and methods

A field experiment on different sowing dates of two different winter rape (*Brassica napus* L. spp. *oleifera biennis* Metzg.) cultivars ‘Sunday’ and ‘Kronos’ (hybrid) was carried out in 2008–2009 at the Experimental Station of the Lithuanian University of Agriculture. The soil of the experimental site is *Endocalcari-Epihypogleyic Cambisol* (*CMg-p-w-can*). Experimental treatments: Factor A – winter rape cultivars ‘Sunday’ and ‘Kronos’ (hybrid); Factor B – sowing dates: 1) August 10, 2) August 20, 3) August 30, 4) September 10. The experimental plots were laid out in a randomised design with each plot replicated four times. The size of each plot was 30 m^2. Conventional soil cultivation practices were applied. The field before winter oilseed rape sowing was kept under black fallow. Plant fertilization ($N_{120}P_{60}K_{90}$) was performed as follows: P and K were applied in autumn prior to sowing, and N was supplied in spring. After sowing the rape was sprayed with a herbicide Butizan 400 (2.5 l ha^{-1}). During the growing season the crops were sprayed with insecticides three times: Karate Zeon (0.10–0.15 l ha^{-1}), Fastac (0.10–0.15 l ha^{-1}), Bulldock (0.10–0.15 l ha^{-1}), and with a fungicide Folicur (1.0 l ha^{-1}) once – at the end of flowering.

In autumn when average air temperature dropped below +2ºC and stayed such for three successive days, that is when vegetation of winter rape is over (Упманис, 1972; Cramer, 1990), ten plants were sampled randomly from each plot for measurement of biometric parameters and chemical analyses. Rape apical buds were analysed by near

infrared spectroscopy (Shenk, Westerhous, 1995) using a PSCO/ISI IBM-PC 4250 infrared spectrometer (Rimkevičienė, 2000). Crude protein content in apical bud was predicted by equation developed at the Analytic Laboratory at the Experimental Station of the Lithuanian University of Agriculture. The database of NIR spectrometer was composed of rape samples of different cultivars grown in various areas of Lithuania during 1998–2008. Chemical composition of rape plants for calibration and prediction sets was determined by reference methods – crude protein by Kjeldahl method.

Analyses of protein composition were done on the apical buds of winter rape cultivars 'Kronos' and 'Sunday', sown at four different dates (10 08, 20 08, 30 08 and 10 09), sampled upon completion of cold acclimation period – in the first half of November. The cold acclimation period for oilseed rape usually ends when the air temperature drops below +2°C (Hughes, Dunn, 1996), which commonly occurs in Lithuania in the first half of November. The test material was frozen immediately at −80°C and preserved at −33°C until used.

The soluble protein fraction from squashed apical buds has been extracted employing the procedure prepared to obtain the protein preparations from vegetative plant tissues suitable to their characterization by non-denaturing 1D electrophoretical approach (Laemmli, 1970; Merkys, Anisimovienė, 1998). Amount of protein in fractions extracted from test material by 100 M TRIS-HCl buffer, containing additives – 1 mM EDTA, 1 mM PMSF, 1 mM DTT (Velička et al., 2005; Anisimovienė et al., 2006) was evaluated according to Bradford (1976). The bovine serum albumin (monomer, 66 kDa) was used as a standard. In all cases the 90 µg protein was introduced into the line. The molecular masses of proteins were calculated according to localization of protein standards, having mol masses 545, 272, 132, 66, 45, 29, 14 kDa, using a molecular weight marker kit for non-denaturing polyacrylamide gel electrophoresis ("Sigma").

Statistical significance of differences between treatments was evaluated using Fisher's criterion and protected least significant difference test at $P_{(level)} < 0.05$ and correlation-regression analyses were performed using *Systat 10* (SPSS Inc., 2000).

Results and discussion

It is estimated, that sowing date and duration of autumnal growth and cold acclimation period influence preparation for over-wintering of winter rape (Velička, 2002). Our further findings are consistent with those. Sowing date, autumnal growth and cold acclimation period significantly influenced biometric parameters of both cultivars 'Sunday' and 'Kronos'. Meteorological conditions in the autumn of 2008 were favourable for winter rape growth and development, as warm and humid weather dominated. Even winter rape sown at the beginning of September formed the number of leaves which was optimal for over-wintering – 6.2 leaves per plant ('Kronos') and 7.6 leaves per plant ('Sunday') (Table). The highest leaf number (8.3–11.7 per plant) and the thickest root collar (11.6–12.3 mm) in the autumn were recorded for winter rape of both varieties sown on the first date (10 08). It was estimated that the root collar of winter rape of both cultivars 'Sunday' and 'Kronos' sown at the latest date (1009) was too thin (4.4–4.8 mm) for good wintering, and the apical bud of winter rape sown on the first date (10 08) was elongated (10.6–11.1 cm).

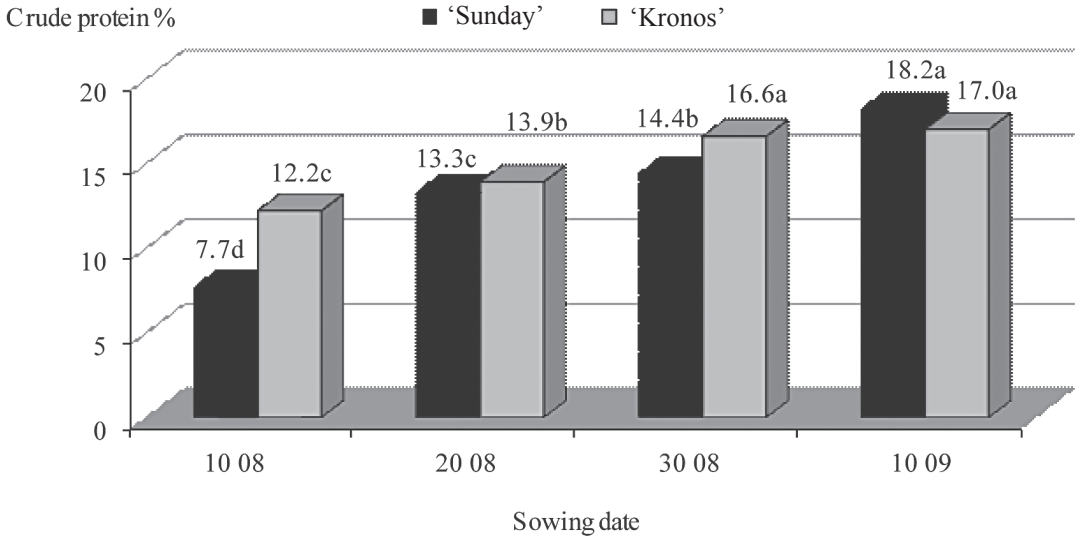
No significant differences of biometric parameters between winter rape cultivars 'Sunday' and 'Kronos' were noticed in 2008.

Table. Biometric parameters of rape prepared for wintering
LUA Experimental Station, 2008–2009

Sowing date	Autumnal growth and cold acclimation period (in days)	Winter rape cultivars					
		'Sunday'			'Kronos'		
		number of leaves per plant	height of apical bud cm	diameter of root collar mm	number of leaves per plant	height of apical bud cm	diameter of root collar mm
10 08	88	11.7a	10.6a	11.6a	8.3a	11.1a	12.3a
20 08	76	8.5b	5.4b	9.3b	7.3b	5.3b	9.4b
30 08	64	8.5b	1.6c	8.7b	7.4b	1.9c	8.6b
10 09	49	7.6c	0.6c	4.8c	6.2c	0.7c	4.4c

Note. Means not sharing a common letter (a, b, c) are significantly different ($P < 0.05$).

A close and inverse relationship determines the accumulation of organic matter in plant. The higher the sugar accumulation, the lower the protein concentration (Третьяков, 1998). Accumulation of crude protein is more intensive in young plants. The concentration of crude protein in apical bud of winter rape sown at the latest date (10 09) was the highest – 17.0–18.2% compared with that of winter rape sown at earliest date (10 08) – 7.7–12.2% (Figure 1). Protein content in apical bud of winter rape of cultivar 'Kronos' was higher compared with that of cultivar 'Sunday', except for the plants with the shortest period of autumnal growth and cold acclimation (sowed on 10 09). Investigation showed that winter rape of hybrid cultivar 'Kronos' sown at the latest date was better adapted for over-wintering compared with the cultivar 'Sunday'.



Note. Means not sharing a common letter (a, b, c, d) are significantly different ($P < 0.05$).

Figure 1. Influence of sowing date on protein content in apical buds of winter rape cultivars

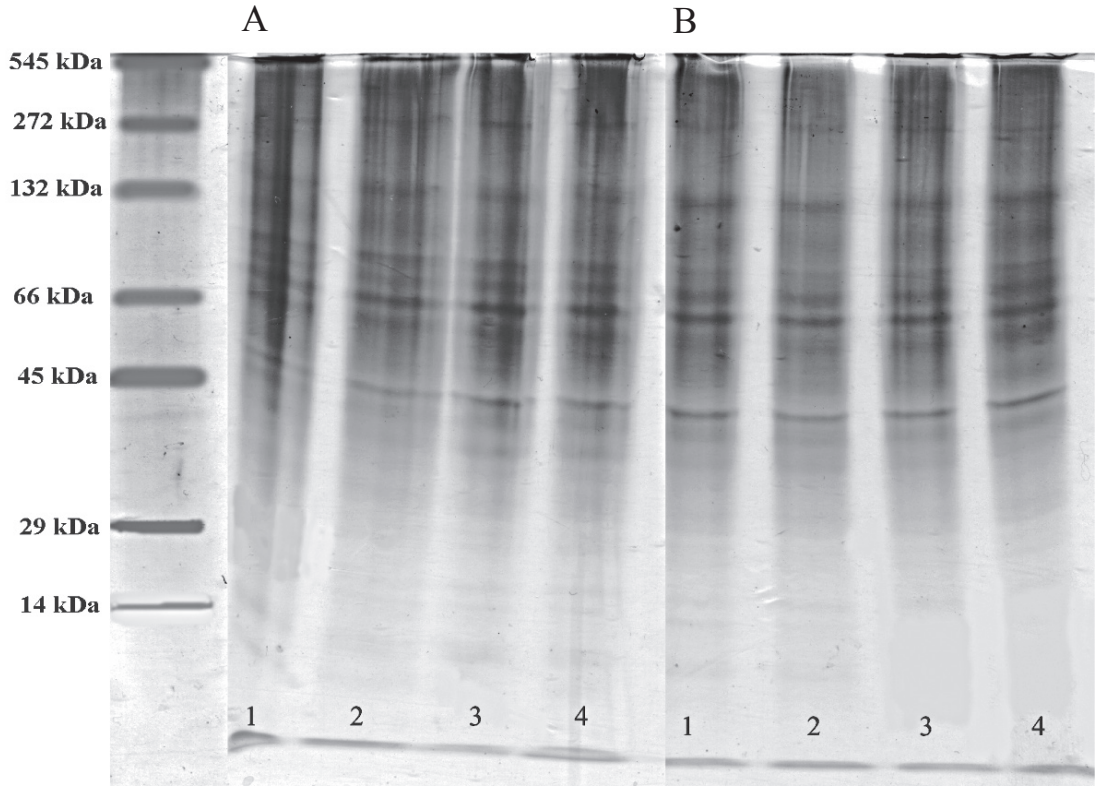
Significant, negative and very strong correlation was established between protein content in apical buds of winter rape cultivars 'Sunday' and 'Kronos' and total rainfall during period of autumnal growth and cold acclimation: $y = 26.44344 - 0.12558$ x, $r = -0.99$, $P < 0.01$; $y = 21.63074 - 0.06308$ x, $r = -0.93$, $P < 0.01$, accordingly. The correlation between sum of temperatures above +2°C during the period of autumnal growth and cold acclimation and protein content in apical buds of both cultivars was very strong and significant: $r = -0.96$, $P < 0.01$. Duration of autumnal growth and cold acclimation period correlated with protein content in apical buds of both cultivars: 'Sunday' – $y = 30.66846 - 0.24778$ x, $r = -0.95$, $P < 0.01$; 'Kronos' – $y = 23.88567 - 0.12804$ x, $r = -0.94$, $P < 0.01$.

Cold acclimation, from physiological-biochemical point of view, is a complex process. To study the mechanism of this adaptive process various approaches are possible. Such as: ice formation in their cells, changes in lipid composition and membranes fluidity, accumulation of osmoprotectants, particularly sugars, LT_{50} magnitude, and admittedly, expression of separate families of genes (for example, *COR*, *LEA*, *ELIP*) and either their products-proteins (Hudges, Dunn, 1996; Thomashow, 1999; Dionne et al., 2001; Svensson et al., 2002; Rapasz et al., 2008). We consider the investigation of proteins more informative (Velička et al., 2005; Anisimovienė et al., 2006).

Having in mind, that: a) one of the aims of our study was to determine the composition of soluble proteins fraction in apical buds of the two introduced genetically different winter rape cultivars upon completion of cold acclimation period and to reveal the possible differences between them in their protein fractions composition, depending on the different duration of their autumnal growth and cold acclimation period, and b) experimental procedures may have effect on the results of proteins composition assays, at the first stage of investigations the optimal conditions (i.e. fresh weight and buffer ratio, tandem of extraction procedures, etc.) for soluble protein fraction preparation were picked experimentally. The most effective procedure accepted was three stages one by one extraction at fresh weight / buffer ratio 1:5, 1:3 and 1:3, during 60 min at +4°C. The supernatant from residue was separated by centrifugation at 10000 g x 15 min.

Thus, the choice of these procedures enables us to have the highly concentrated soluble protein fraction specimens from both cultivars and separate their variants depending on to the duration of autumnal growth and cold acclimation periods. The amount of soluble proteins extracted from the same weight material in separate tests does not differ significantly – only 7–8%, whereas greater differences were established in the fresh weight and development of individual apical buds of both tested cultivars, depending on their autumnal growth and cold acclimation duration.

The comparative study of soluble protein fraction composition by obtained electrophoregrams (Figure 2) revealed a different number of their components (individual proteins) in apical bud cells, which depends on cultivar and cold acclimation duration (Figure 3).

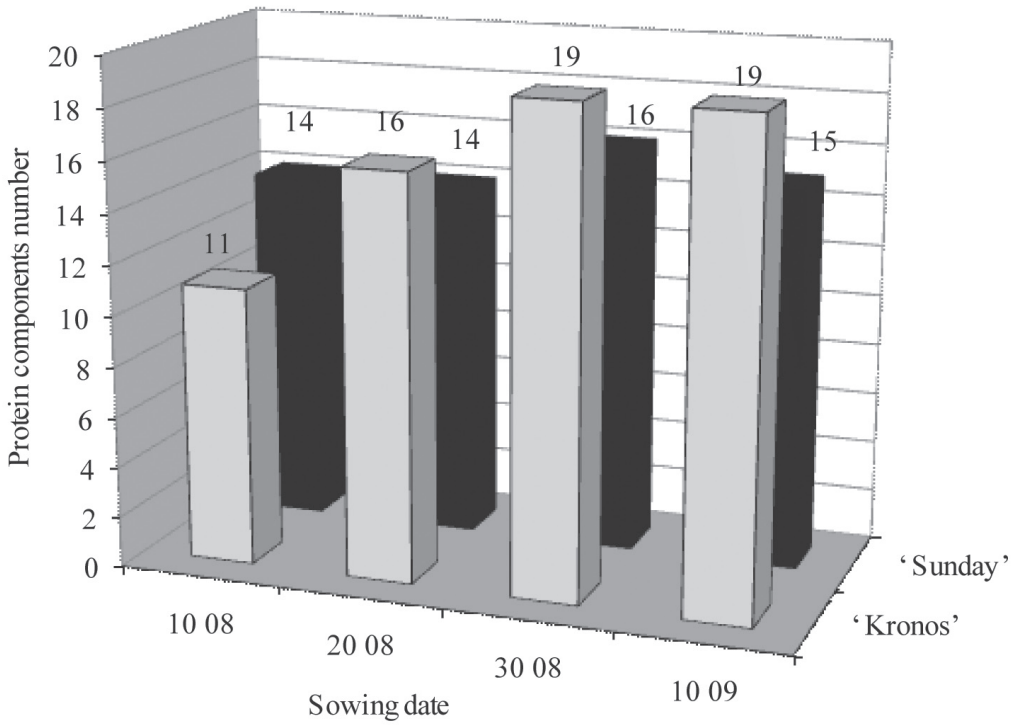


Note. 1–4 different sowing treatments: 1 – autumnal growth and cold acclimation period about 60 days, 2 – about 70 days, 3 – about 80 days and 4 – about 90 days.

Figure 2. Electrophoregram of total soluble protein fraction components from winter rape cultivars 'Kronos' (A) and 'Sunday' (B) depending on their autumnal growth and cold acclimation duration

Data analysis of the soluble protein fraction composition in apical buds of different cultivars showed several differences. Our findings suggest that 'Kronos' may be characterized as a cultivar with a higher number of individual proteins, particularly in plants which have longer autumnal growth and cold acclimation period (sown on 10 08 and 20 08) (Figure 2). This cultivar also has a greater number of proteins of higher electrophoretical mobility, with medium and low molecular masses (less 66 kDa), in comparison to the cultivar 'Sunday' (Figure 2). In 3–4 treatments the number of such proteins in 'Sunday' ranges about 7–9, while in 'Kronos' – 12. The composition of high molecular weight proteins is modified less.

Increase in protein component number, particularly in ranges 66–14 kDa, is a typical characteristic for winter rape cold acclimation-preparation for wintering and correlates to their over-wintering success or degree (Anisimovienė, Novickienė, 2003; Anisimovienė et al., 2006). It was demonstrated by model trials designed to search for possibilities to improve over-wintering of various cultivars of these plant as well as by analysis of the composition of soluble and membranous proteins fractions in prepared for wintering apical bud cells of the cultivars 'Valesca' and 'Casino' differing in winter hardiness (Янкаускене, Анисимовене, 2010). It was revealed, that in Lithuanian climate conditions over-wintering of winter rape cultivar 'Valesca' is better than that of 'Kronos' (Velička, 2002).



Note. 1–4 different sowing treatments: 1 – autumnal growth and cold acclimation period about 60 days, 2 – about 70 days, 3 – about 80 days and 4 – about 90 days.

Figure 3. Comparison of protein component number obtained in soluble protein fraction extracted from terminal buds of different winter rape cultivars after various duration of autumnal growth and cold acclimation

The formation of medium and low molecular masses proteins is the feature inherent to cold acclimation and preparation for wintering of various biennial and perennial plants (Jeknič, Chen, 1999; Welling et al., 2002; Stupnikova et al., 2003; Bentem et al., 2006). Three of low mol masses thermostabile proteins-dehydrins 65, 60 and 14 kDa, products of *LEA II* group genes are recognized as specific protein-markers for plant cold acclimation (Jeknič, Chen, 1999; Svensson et al., 2002; Stupnikova et al., 2003; Anisimovienė, Novickienė, 2003).

Comparison of protein composition of both tested cultivars evidenced that four or three low molecular masses proteins having mol masses below 29 kDa are specific to all treatments of 'Kronos', while in apical buds of the cultivar 'Sunday' they may be not formed, or: a) their accumulation is inhibited, b) increased disturbance (metabolism, utilization), especially in the cases when they have longer periods to prepare for wintering (sown on 10 08 and 20 08). In protein specimens derived from buds of these treatments we did not identify the protein component having mol masses about 132 kDa, although it occurs in bud cells of plants having shorter autumnal growth and cold acclimation period.

Thus, these results concur with our own conclusion (Merkys, Anisimovienė, 1998; Anisimovienė et al., 2006; Янкаускене, Анисимовене, 2010), as well as the one made by other investigators (Jeknič, Chen, 1999; Svensson et al., 2002; Stupnikova et al., 2003) that protein composition is one of significant biochemical factors related to perennial and biennial plants cold acclimation, preparation for wintering – frost tolerance and dormancy maintenance. Dormancy regulation in vegetative buds is a complex process necessary for plant survival and the role of internal signals such as proteins, hormones, sugars as well as external signals as light or temperature is not questionable (Ивонис и др., 1984; Jeknič, Chen, 1999; Horvath et al., 2003).

Conclusions

1. Sowing date, autumnal growth and cold acclimation period significantly influenced biometric parameters (number of leaves per plant, root collar diameter and height of apical bud) of both cultivars 'Sunday' and 'Kronos' (hybrid). No significant differences of these parameters were estimated between the investigated cultivars in 2008.

2. A consistent increase in the amount of crude proteins in apical bud of both winter rape cultivars of later sowing dates was observed.

3. At the phase of cold acclimation period, in both rape cultivars the soluble protein fractions compositions in apical buds were not identical. The cultivar 'Kronos' had more individual protein components in comparison with 'Sunday', particularly in the cases of longer autumnal growth and cold acclimation period.

4. The longer autumnal growth and cold acclimation period (88 days) had a more positive effect on soluble protein components number in cultivar 'Kronos' compared with 'Sunday'.

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Rapsų pasiruošimas žiemoti, priklausomai nuo augimo ir grūdinimosi laikotarpio trukmės

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Santrauka

Rapsų rudeninio augimo bei grūdinimosi ir pasiruošimo žiemoti lauko bandymas darytas 2008–2009 m. Lietuvos žemės ūkio universiteto bandymų stotyje, augalų tirpių baltymų analizės – Botanikos instituto Augalų fiziologijos laboratorijoje. Tyrimų tikslas – įvertinti sėjos laiko įtaką linijinių ir hibridinių veislių žieminio rapso (*Brassica napus* L.) pasiruošimui žiemoti.

Pagrindiniai biometriniai rodikliai (skrotelės lapų kiekis, šaknies kaklelio skersmuo ir viršūninio pumpuro aukštis) parodė, kad geriausiai pasiruošė žiemoti rapsai, sėti rugpjūčio antroje pusėje (08 20) ir pabaigoje (08 30), tai yra tie, kurių rudeninio augimo ir pasiruošimo žiemoti laikotarpis buvo 64–76 dienos. Rudeninio augimo ir pasiruošimo žiemoti laikotarpio trukmė turėjo esminės įtakos linijinių ir hibridinių veislių rapsų biometriniams rodikliams. Žalių baltymų kiekis rapsų viršūniniame pumpure didėjo vėlinant jų sėją.

Pasibaigus grūdinimosi (pasiruošimo žiemoti) laikotarpiui, išanalizuota rapsų žiemojimui svarbaus organo – viršūninio pumpuro – ląstelių tirpių baltymų frakcijų sudėtis. Atskleisti veislių ‘Kronos’ ir ‘Sunday’ rapsų viršūninio pumpuro ląstelių baltymų sudėties skirtumai ir jų baltymų sudėties ypatybės, priklausomai nuo rudeninio augimo ir grūdinimosi laikotarpio trukmės.

Nustatyta, kad didesnis baltymų tam tikrų komponentų kiekis ir baltymų, turinčių mažesnę nei 66 kDa molekulinę masę, kiekis susikaupė veislės ‘Kronos’ rapsų viršūninio pumpuro ląstelėse. Ilgesnis rudeninio augimo ir grūdinimosi laikotarpis ryškesnį poveikį turėjo rapsų veislės ‘Kronos’ baltymų komponentų kiekio didėjimui, palyginti su veislės ‘Sunday’. Trumpesnis rudeninio augimo ir grūdinimosi laikotarpis (apie 60 dienų) turėjo neigiamą poveikį rapsų abiejų veislių bendram tirpių baltymų komponentų kiekiui, ypač veislės ‘Kronos’.

Reikšminiai žodžiai: žieminis rapsas, sėjos laikas, žiemojimas, grūdinimasis, baltymai.