SHORT COMMUNICATION

Freezing Tolerance and Proline Content of *in vitro* Selected Hydroxyproline Resistant Winter Oilseed Rape

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Abstract: Twelve doubled haploid (DH) winter oilseed rape plants with altered levels of proline and/or freezing tolerance were obtained by *in vitro* selection for resistance to *trans*-4-hydroxy-L-proline (Hyp) in five segregating microspore populations. No significant response to selection either in proline content or in freezing tolerance, compared with the non-selected control populations, was observed. When data from all examined materials were combined, a weak correlation between proline content and freezing tolerance was observed.

Keywords: abiotic stress; *Brassica napus;* microspore culture; proline

Low temperature is a major abiotic stress factor affecting rapeseed production in the Czech Republic. To survive this stress, plants need to acquire an increased freezing tolerance. During the acclimation of the plants, extensive changes are induced in gene expression and metabolic pathways (Iba 2002; Wang *et al.* 2003).

The chilling and freezing tolerance of many plants is supported by the accumulation of osmotically active agents, called compatible solutes. Among them, one of the most widespread is the amino acid proline. Thanks to its amphiphilic nature, it is able to retain water within the cells under conditions of osmotic stress. And also to stabilize the membrane and cytoplasmic proteins, as well as the membranes themselves. The metabolism of proline *per se* is also of importance, because of the regulation of both the synthesis and the degradation of this amino acid it comes into the cycle process between proline and its intermediates under various conditions. Proline can be a means of reducing the cytoplasm acidity, as well as being a compound that is able to scavenge reactive oxygen species. After recovering from stress, proline becomes the source of carbon, nitrogen, energy, and reducing equivalents, which makes the recovery of normal plant metabolism easier (Hare *et al.* 1998; Wang *et al.* 2003).

Selection for improved freezing tolerance with Hyp was successfully used for callus cultures of potato (Van Swaaij *et al.* 1987; Anjum & Villiers 1998) and for both the cell lines and the embryogenic calluses of cereals (Dörffling *et al.* 1993, 1994, 1997a, b; Tantau *et al.* 2004). Recently, McClinchey and Kott (2008) successfully used combined UV mutagenesis and

in vitro selection in microspore cultures with several proline analogues for the improvement of freezing tolerance in Canadian spring oilseed rape. Free Hyp is a phytotoxic analogue of proline for plants. The negative effect of Hyp applied to the microspore or callus cultures can be reduced by an increased level of proline inside the cells. Thus, it is suggested that regenerants which survived on a Hyp medium, might have an elevated level of proline and thus probably also improved freezing tolerance.

The aim of our study was to obtain DH winter oilseed rape plants with increased levels of proline in leaves, and consequently with higher freezing tolerance by *in vitro* selection in microspore cultures with Hyp as the selecting agent. A protocol for *in vitro* selection in microspore cultures was established and used for the selection of Hyp resistant (HypR) regenerants in the collection of Czech breeding rapeseed materials. The determination of the proline content in the leaves of regenerated plants was carried out after two-month acclimation at 4°C, following the freezing tests. The other goal was to find the relationship between the level of proline in the leaves and the degree of freezing tolerance achieved.

Five F_1 hybrids (breeding materials of winter oilseed rape, highly embryogenically responsive in microspore culture) originated from two breeding organizations: Oseva Pro, Ltd. – Research Institute of Oilseed Crops at Opava (OP22, OP24, OP25), and Selgen, a.s. – Breeding Station at Chlumec nad Cidlinou (CH993, CH2008). Two open-pollinated breeding materials (lines) of winter rapeseed HP3 and HP8, with contrasting freezing tolerances from the Research Institute of Oilseed Crops at Opava, were used in the experiments (Table 1). Donor plants for microspore cultures were vernalized for two months (4°C ± 2°C), and then grown under a controlled environment in a growth chamber (light intensity 84 µmol/m^2/s, 22/20°C day/night, and photoperiod 16/8 h).

Flower buds 2.5–3.5 mm in size were selected and collected from donor plants. The isolation and culture of microspores were carried out according to Klíma *et al.* (2004); however, immediately after isolation, Hyp was added to the NLN liquid medium (Lichter 1985). The concentration of microspore suspension was adjusted to 100 000 per 1 ml of the culture medium. The concentrations of Hyp in the medium were: 0 (control), 1, 4, 10, 16, and 24mM. Two Petri dishes, each with 10 ml of the microspore suspension, were prepared for each Hyp concentration and genotype. All experiments were replicated three times. For further analysis, regenerants from media with 10 and 16mM Hyp were selected, where only a few globular embryos developed to the torpedo stage. Less concentrated media appeared to be ineffective for selection (more than one third of embryos developed) and the concentration 24mM was toxic (no globular embryos developed to the torpedo stage). For the mean number of developed torpedo embryos per Petri dish after 30 days of cultivation in NLN medium see Table 2. For chromosome doubling, the plantlets from microspore-derived embryos were treated with 50 mg/l colchicine solution for 24 h, according to Vyvadilová *et al.* (1993) before transfer to soil (horticulture substrate). Plantlets were grown in a greenhouse, and then cold acclimated at 4°C ± 2°C, 8 h light/16 h dark. After 60 days of cold acclimation (control plants and respective HypR regenerants together), leaf samples were collected for the determination of proline content and freezing tolerance.

Table 1. Characterisation of initial breeding materials used for *in vitro* selection

Donor plant	Origin (A × B)
OP22	OP-1901/02 × OP-1101/02
OP24	OP-BN-07 × OP-1101/02
OP25	OP-1611/02 × OP-1012/02
CH993	I 60/4-85 × I 77/8-193
CH2008	A 4-37 × I 61/2
HP3	frost sensitive line OP-4032
HP8	frost tolerant line OP-BN-07

A – freezing tolerant parent; B – freezing sensitive parent

Proline content was determined in freeze-dried leaf material, according to Bates *et al.* (1973). Calibrations were made with L-proline (Sigma-Aldrich Chemie, GmBH, Steinheim, Germany) as a standard. The concentration of proline was calculated according to the formula [(ml toluene) × (µg proline/ml)]/(g sample × 2/3) = µg proline/g dry weight. Two independent proline measurements of each variant were done. For evaluation, the *t*-test was used. The degree of freezing tolerance was determined after a laboratory freezing test via the electrolyte leakage method (according

Table 2. Mean number of developed torpedo embryos per Petri dish after 30 days of cultivation in NLN medium

Genotype	Mean number of torpedo embryos per Petri dish					
	control	1mM Hyp	4mM Hyp	10mM Hyp	16mM Hyp	24mM Hyp
OP22	226.3	205.5	169.7	7.7	1.8	0.0
OP24	255.3	220.0	138.8	15.3	2.8	0.0
OP25	106.0	75.3	57.5	3.2	1.2	0.0
CH993	80.8	59.8	48.5	8.0	2.2	0.0
CH2008	107.2	87.0	68.2	3.2	1.0	0.0
HP3	243.8	225.0	112.5	8.7	4.5	0.0
HP8	114.0	97.3	56.7	4.2	0.3	0.0

to Prášil & Zámečník 1998) on leaf discs after gradual freezing from –3°C to –30°C. The software package developed by Janáček and Prášil (1991) was used to calculate LT_{50} values (the temperature producing freezing injury in 50%).

Proline accumulates in plants as a response to abiotic stresses such as low temperature, drought, and high salinity (Hare *et al.* 1998). From this aspect proline is known to protect plant tissues in the osmotic stress pathway. As the participation of proline in the cold stress tolerance of rapeseed is not fully understood, the goal of our study was to find out regenerants with elevated proline content (selected *in vitro* with the analogue hydroxyproline) having a higher degree of freezing tolerance.

Nine hydroxyproline-resistant (HypR) DH regenerants derived from five F_1 hybrids and three HypR DH regenerants derived from two open-pollinated breeding materials of winter oilseed rape were obtained via *in vitro* selection in the presence of Hyp. Efficient concentrations of Hyp for selection of HypR microspores were 10 and 16mM, which is in concordance with concentrations used in spring canola by McClinchey and Kott (2008). Regenerated plants were treated first with colchicine, cultivated in *ex vitro* conditions, and then evaluated for their freezing tolerance and proline content in the leaves.

Two HypR DH regenerants (CH993/3, CH2008/1) showed significantly higher amounts of proline in their leaves than did the respective control plants (i.e. regenerants without Hyp treatment) after cold acclimation (Table 3).

Many studies have shown a positive correlation between the resistance of tissue cultures to Hyp and the content of proline in their tissues, particularly in cereals (Tantau & Dörffling 1991; Dörffling *et al.* 1993, 1994, 1997 a, b; Tantau *et al.* 2004) and in potatoes (Van Swaaij *et al.* 1987; Anjum & Villiers 1998). In contrast, there are also studies in which this correlation was not confirmed (e.g. in HypR cell lines of lucerne the constitutively increased proline level was not found (Petrusa & Winicov 1997)). In spite of this, the plants showed an increased resistance to high salinity. The difference between the control and HypR variants was the more rapid accumulation of proline in the roots of the HypR plants after exposure to high salinity. For this reason, it should be interesting to study proline accumulation dynamics in the case of selected HypR plants – not only during acclimation, but particularly after exposure to temperature shock. Maggio *et al.* (1997) described another mechanism in carrot cell lines, where no significant correlation between the resistance to Hyp and the proline content was observed. This could be due to some changes in the membrane transport system that could block the uptake of Hyp. The osmotolerance of the carrot cells changed with the developmental stage; therefore, plants accumulating higher amounts of proline might only be more tolerant to osmotic stress during a specific stage of development. Another possible reason is the enzymatic conversion of some Hyp to proline (Varner 1980). In conclusion, while proline is known to protect plant tissues in osmotic stress, the pathways of proline participation in the cold stress tolerance of oilseed rape are not fully understood. Considering all these possibilities, it is clear that more comprehensive studies of proline dynamics during cold acclimation and freezing temperatures in winter oilseed rape plants are necessary.

Higher freezing tolerances, observed in four HypR plants (Table 3 – OP22/2, OP24/1, CH993/4

Table 3. Proline content and freezing resistance (LT_{50} values) in the leaves of control and hydroxyproline resistant (HypR) oilseed rape regenerants

Regenerant		Proline (mg/g DW)		LT_{50} (°C)	
Control	HypR	control	HypR	control	HypR
OP22	OP22/1	69.0	57.0	–17.7	–15.8*
	OP22/2		72.9		–18.2
OP24	OP24/1	57.9	53.2	–15.0	–15.3*
OP25	OP25/1	40.1	27.2*	–15.7	–12.9*
CH993	CH993/1	36.2	30.6	–15.0	–14.3*
	CH993/2		34.5		–13.4*
	CH993/3		48.2*		–13.6*
	CH993/4		48.1		–15.8
CH2008	CH2008/1	44.6	93.7*	–15.6	–14.6*
HP3	HP3/1	35.3	52.4	–14.5	–12.9*
	HP3/2		38.2		–13.1
HP8	HP8/1	39.2	43.9	–13.7	–14.0

Significant differences between control and Hyp-resistant regenerants are marked with (*) for $P \leq 0.05$

and HP8/1), were statistically significant in only one DH regenerant (OP24/1). The freezing tolerance in the other HypR regenerants was lower than in the initial donor (mother) plants. Microspore cultures of *Brassica napus* L. are a valuable contribution to breeders, not only for the shortening of the breeding processes but also for the prospect of uncovering mutations or recessive phenotypes. Variability in freezing tolerances in the progeny of DH plants obtained from a single mother plant (OP22/1, OP22/2 and CH993/1, 2, 3, 4) was observed (Table 3).

Many previous studies that dealt with the problems of cold stress showed a positive correlation between the accumulated proline in plant tissues or cell cultures and the plant's freezing tolerance, particularly in cereals (Charest & Phan 1990; Dörffling *et al.* 1993, 1994, 1997a, b; Chen & Li 2002; Tantau *et al.* 2004). However, in yet other studies this relationship was not so explicit (e.g. Van Swaaij *et al.* 1985, Fahl *et al.* 1994). Our study showed that there existed a significant correlation between the freezing tolerance and the proline content in leaves of winter oilseed rape, although selection in this case was not successful (Figure 1). However, three DH regenerants (Table 3 – OP22/2, CH993/4 and HP8/1) had both the higher levels of proline and the increased freezing tolerance. Since we did not use any mutagenic agents, the selection of DH lines with elevated proline exploited the natural variability of F_1 hybrid progeny. Therefore, control plants with higher accumulation of proline or improved freezing tolerance were, in fact, randomly selected resistant variants of this progeny.

Two of the *in vitro* selected regenerants with increased proline content (CH993/3 and CH2008/1) had significantly lower freezing tolerance (Table 3). Therefore, it can be concluded that the *in vitro* selection method used in the chosen breeding materials did not lead to increased proline contents, combined with higher freezing tolerance. Nevertheless, it cannot be concluded that the role of proline in plant protection against low temperatures is trivial. It is possible that more important than an elevated proline level is the dynamics of its increase and/or the turnover of proline *per se*; and that by influencing the redox potential of the cell, it contributes to a decrease in oxidative injury (Hare *et al.* 1998). Although these preliminary results do not show so clear conclusions, they afford a methodical tool that could be tested by breeders in larger amounts of samples.

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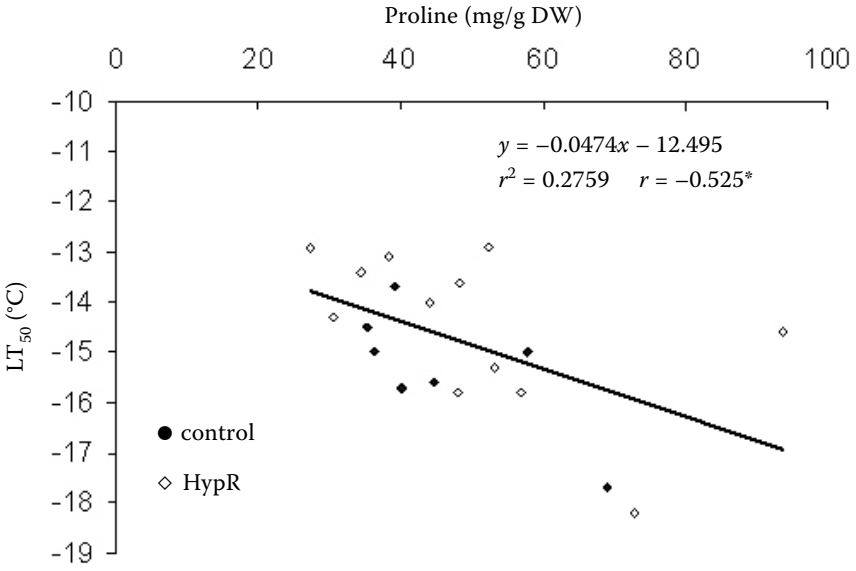

Figure 1. Correlations between proline content and freezing resistance (LT_{50} values) in the leaves of control and hydroxyproline resistant (HypR) oilseed rape regenerants

*correlation coefficient significant at $P \leq 0.05$, $n = 19$

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