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Original scientific paper

SCREENING OF CAMELINA (*Camelina sativa* L.) DOUBLED HAPLOID LINES FOR FREEZING TOLERANCE IN THE SEEDLING STAGE

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Freezing stress is an important abiotic stress that limiting the yield and the spatial distribution of many important crops. This study was undertaken to screen 136 doubled haploid (DH) lines of camelina (*Camelina sativa* L.) along with four canola (*Brassica napus*) cultivars (Hyola 401, Lord, Roska and Cascade) as experimental control under freezing stress conditions (-14 °C for 6 h) to identify lines with high or low level of tolerance to freezing stress for further studies. First, a protocol was developed for large scale screening of camelina germplasm under freezing stress conditions. For this purpose, an experiment with different freezing temperatures (-5, -10, -15 and -20 °C) was conducted to find an appropriate temperature that discriminated best between genotypes (i.e. the LT_{50} temperature). The LT_{50} values for camelina lines were varied between -10.2 and -17.1 °C with an average of -13.94 °C for all of the camelina lines. Therefore, we selected the -14 °C exposure for 6 h as an appropriate temperature to screening of camelina lines. The principal components of measured parameters (LT_{50}, survival percentage, relative conductivity and scoring) was using principal component analysis that determine freezing-tolerant and freezing-sensitive lines. Among 136 doubled haploid lines, some lines (58, 62 and 101) had higher level of freezing tolerance and some of them (8, 16, 32, 91 and 107) were freezing sensitive. The selected lines in a preliminary freezing screening are useful for further evaluations.

Keywords: biplot, canola, LT_{50}, principal component, relative conductivity

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Abbreviations: DH: Doubled Haploid; LT_{50}: Lethal Temperature at 50%; PCA: Principal Component Analysis

INTRODUCTION

Camelina sativa (L.) Crantz is a member of the Brassicaceae family that receives a remarkable attention as a reemerging oil seed crop (MURPHY, 2016). One of the key issues is that camelina is highly adaptable to adverse environmental conditions such as drought and chilling stress (GUGEL and FALK, 2006; ZUBR, 1997). Camelina has large potential in the cold climates of the world such as northern portions of the United States and Canada as an alternative oil seed crop with a significant benefit to cold tolerance and short season (PLESSERS *et al*., 1962; MURPHY, 2016). Forthermore, camelina is adaptable to many other environmental stresses such as chilling, drought, heat and cockroaches and pollen-eating pests that is common in oilseeds (KAHRIZI *et al.*, 2015) and the only true limitation of camelina is heavy clay soils and organic soils (GUGEL and FALK, 2006).

Freezing temperatures (< 0 °C), as an abiotic stress, adversely impairs the growth and yield of crops and significantly affect the geographical distribution of plants and agricultural productivity (CHINNUSAMY *et al.*, 2007). Many major crops such as maize, rice, soybean, cotton and tomato are freezing sensitive and unable to tolerate ice formation within their tissues (ANDERSON *et al.*, 1994). In contrast, some crop plants have a higher level of freezing tolerance. For example, camelina is a northern climate crop that is not susceptible to low temperatures as the plant can easily bare temperatures to -11°C (PLESSERS *et al*., 1962).

Obtaining camelina lines with different level of tolerance to freezing stress is a prerequisite for understanding of freezing tolerance mechanism in species similar to this plant and improve crop yield in cold climate regions of the world where geographical distribution of species is limited due to freezing temperatures. Also, a protocol for large scale screening of camelina germplasm under frost-simulating conditions has not yet been developed. Therefore, the goal of this study was to develop a protocol for large scale screening of camelina germplasm and screening of camelina doubled haploid lines to freezing stress to identify lines with high or low level of freezing tolerance.

MATERIALS AND METHODS

Determine LT_{50} temperature

In order to determine an appropriate temperature that discriminated best between genotypes (i.e. the LT_{50} temperature) in *C. sativa*, 136 DH lines derived from anther culture of different F_1 hybrids (Table 1), were tested. The seeds were grown in the greenhouse for 14 days at 22±1 °C. Ten seeds were germinated in plug trays (32.5×52×6.7 cm; 40 plugs) filled with Peat moss in a temperature-controlled greenhouse. The freezing experiments were conducted on two-week-old plants at -5 °C, -10 °C, -15 °C and -20 °C for 6 hours with three replications. Before freezing experiments, twelve-day-old plants were transferred to the plant growth chamber for acclimation at 4 °C with a 12-h photoperiod for two days. At the beginning of each treatment, the temperature in the freezer (Jal Tajhiz Co, Iran) was set on the freezing treatment temperatures. Two days after freezing stress, the samples were scored for survivorship. According to mean survival scores of the lines, -14 °C was chosen as the optimum temperature for testing LT_{50}.

Table 1. List of cultivars used as parents in crosses for production of doubled haploid lines

Cross No.	Seed parents (♀)		Pollen parents (♂)		DH lines No.
	Cultivar	Origin	Cultivar	Origin	
1	Voronezskij 349	Russian	Kirgizskij 1	Kyrgyzstan	1, 2, 5, 37, 51, 62, 70, 73, 77, 86, 87, 93, 96
2	Omskij Mestnyj	Russia	Irkutskij Mestnyj	Irkutsk Region	3, 6, 9, 32, 38, 43, 46, 60, 71, 79, 89, 113, 118, 129
3	Przybrodzka	Poland	Hoga	Denmark	7, 14, 17, 20, 22, 104
4	Saratouskij	Russia	Bronowska	Poland	8, 24, 40, 96, 115, 120
5	Chulymskij	Russia	Omskij Mestnyj	Russia	11, 25, 29, 41, 42, 58, 68, 116, 121, 125, 131
6	Krupnosemjannyj	Russia	Brzybrodzka II	Poland	26, 63, 78, 106, 110
7	Came	Germany	Volynskaja	Former Soviet Union	31, 69, 76, 90, 98, 119, 123
8	Boha	Denmark	Volynskaja	Poland	18, 19, 23, 97
9	Came	Germany	Omskij	Former Soviet Union	4, 34, 35, 82, 88, 91, 94, 95, 100, 108, 111, 112, 124, 132
10	Svalöf	Sweden	Ukrajinskij	Former Soviet Union	27, 28, 50, 52, 56, 103, 105, 117, 130
11	Calena	Germany	Blaine Greek	Greece	15, 30, 36, 47, 80, 83, 84, 101, 114, 134, 135, 136 137
12	Zavolzskij	Former Soviet Union	Sortandinskij	Former Soviet Union	21, 39, 44, 45, 59, 67, 81, 99, 102, 126
13	VNIIMK 17	Former Soviet Union	Borowska	Poland	48, 57, 74, 75, 85, 127
14	Voronezh 349	Former Soviet Union	Czestochowska	Poland	10, 16, 49, 54, 55, 61, 109, 133
15	Lindo	Germany	Ukrajinskaja	Former Soviet Union	12, 13, 33, 53, 64, 65, 72, 92, 107, 122, 128

Screening test

In the screening test, a set of 136 camelina DH lines and four *Brassica napus* cultivars named Hyola 401 (spring), Lord (winter), Roska (winter) and Cascade (winter) were tested in -14 °C for 6 hours. The seeds were germinated in the plug trays filled with peat moss and irrigated with Hoagland nutrient solution daily and were grown in a temperature and light-controlled greenhouse for 14 days at 20±1 °C. Two-week-old acclimated plants (4 °C for two days) were transferred to the freezer. For freezing treatment, the temperature was set on the -14 °C and seedlings were incubated at -14 °C for 6 h. After freezing test, seedlings were transplanted to the growth chamber at 4 °C for 24 h before returning to the greenhouse for scoring seedling damage. Scoring was performed three days after the freezing treatment according to FIEBELKORN and RAHMAN (2016) protocol. So, the DH lines were scored individually using a 0 to 5 scale were scored in the greenhouse, where 0 denoted dead, 5 denoted no damage, and 1–4 scores were based on visual estimation of freezing damage. The electrolyte conductivity of the treated sample was measured after 24 h, by transferring 4 ml of the bathing solution into the assay well of a calibrated Portable Conductivity/TDS/Temp meter 8301 at RT or room temperature (EL_1). The solution was returned to the vial containing the sample and boiled for 1 h to kill all cells causing complete leakage of electrolytes. When the temperature of the resultant

solution reached room temperature, the total electrolyte content (EL_2) was measured. Relative conductivity (RC) was calculated as:

$$RC\% = (EL_1/EL_2) \times 100$$

Statistical analysis:

The LT_{50} of the camelina DH lines and correlation matrix of measured traits in the screening test (i.e. LT_{50}, survival percentage, relative conductivity and scoring) were calculated with Microsoft Excel version 2016 and XLSTAT version 2016.02.28451. The XLSTAT was used to compare freezing tolerance between the camelina DH lines and canola cultivars using Principal Component Analysis (PCA) and Biplot graph.

RESULTS AND DISCUSSION

This is the first study providing data to assess freezing tolerance of camelina. Knowledge of freezing tolerance of breeding lines will be valuable for manipulating breeding strategies for yield improvement and for developing or selecting suitable breeding lines for early season planting in many camelina-growing areas. Forthermore, because of the limited germplasm of pre-commercial lines of camelina (SAINGER *et al.*, 2017), conventional breeding is not a very promising approach; hence, we produced 136 DHs drived from 15 different crosses between camelina cultivars, which can be produced lines with high level of freezing tolerance or other envionmental stresses.

Determining LT_{50} values

A preliminary experiment was conducted by freezing 136 DHs of camelina at -5, -10, -15 and -20 ºC for 6 h with three biological replicates. This approach simulated a sudden cold event, when seedlings are non-acclimated or poor acclimated in the fall (HORTON *et al.*, 2016). Based on the results of this preliminary work, the freezing temperature that discriminated best between genotypes (i.e. the LT_{50} temperature) were determined. Visual differences could be observed on the plants after freezing stress. The sensitive seedlings tended to be darker green and wilted, whereas the tolerant plants were lighter green and stood upright three days after freezing treatment (Figure 1a). The scoring of plants was performed in such a way as to avoid bias as much as possible. A score of 0 indicated that the plant was completely or overwhelmingly dead (Figure 1b). A score of 5 indicated that the plant had healthy leaves and was showing no sign of damage (Figure 1c). When the two-week camelina plants exposed to -20 °C, the lines exhibited severe leaf tissue damage within 6 h, while at -5 °C, there was no of significant freezing damage.

Figure 1. Camelina lines with medium (a), low (b) and high (c) level of freezing tolerance.

The LT_{50} values for camelina lines ranged from -10.2 to -17.1 °C with an average of -13.94 °C for all of the camelina lines (Figure 2, and data not shown). Therefore, we selected the -14 °C exposure for 6 h as an appropriate temperature to screening of camelina lines in order to determine the freezing tolerance of the lines. As shown in Figure 2, most of the DH lines had a LT_{50} between -14 and -15 °C, a number of lines had a lower LT_{50} and some of them had a high LT_{50}. These results demonstrated that there is a significant genetic diversity between the camelina lines for freezing tolerance.

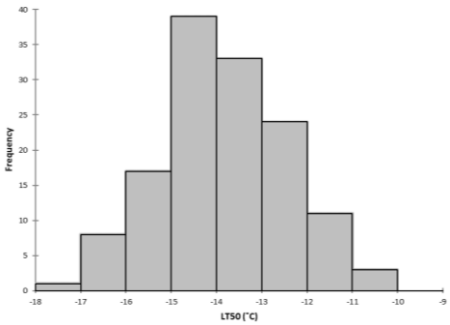

Figure 2. The frequency of camelina doubled haploid lines for LT_{50}

Screening test

To determine freezing tolerance of *C. sativa*, 136 doubled haploid lines were screened at -14 °C in the controlled condition. Four (one spring and three winter) *B. napus* cultivars were applied as control to confirmed the tolerance level of camelina lines. The LT_{50} for 140 genotypes ranged from -8.3 (Hyola 401) to -17.1 °C (DH line No. 58) (Table 2). Visually, tolerance differences appeared between genotype. These differences were confirmed by measuring traits such as scoring of plants, relative conductivity, LT_{50} and survival percentage (Table 2). The LT50 = -14.6 °C was resulted for Cascade cv. in current study. However, it already had been reported as -15.5 °C by HAWKINS *et al*., 1997. This conflict may be due to acclimation or growth stage of the plants.

Table 2. Summary data of 136 camelina DHs and 4 canola cultivars subjected to -14 °C.

Variable	Observations	Minimum	Maximum	Mean	Std. deviation
LT_{50}	140	-17.100	-8.300	-13.885	1.454
Relative conductivity	140	16.000	82.023	49.240	13.034
Survival percentage	140	0.000	92.308	48.640	16.702
Scoring values	140	0.000	5.000	2.507	0.902

Principal component analysis reduces the dimensionality of a data set while retaining most of the variations by producing numbers in absolute values for the response variables (WIJEWARDANA *et al*., 2015). Therefore, the parameters that best describe the freezing tolerance can be represented by selecting relatively few numbers of traits instead of many variables. Then, the data can be plotted, making it conceivable to visually assess similarities and differences and accordingly categorize them into distinct groups (SINGH *et al.*, 2008; WIJEWARDANA *et al*., 2015). In the present study, principal component analysis was used to reduction of the data set and to

identify the principal components of measured parameters (LT_{50}, survival percentage, relative conductivity and scoring) that best described the response to freezing stress, and, hence, to identify freezing-tolerant and freezing-sensitive lines. Among 136 doubled haploid lines, some lines such as 58, 62 and 101 were freezing tolerance and some of them such as 6, 8, 32, 91 and 107 were freezing sensitive (Figure 3). These lines were selected in a preliminary freezing test for further evaluation. The Hyola401 is a spring canola hybrid variety that showed a low level of freezing tolerance in comparison of Lord, Roska and Cascade cultivars. The success and sustainability of any plant breeding programme largely relies upon some key factors in the plant population such as the existence of genetic diversity, the efficiency of selection and heterosis amounts (ESACK *et al.*, 2015). Hence, the selected freezing-tolerant or sensitive lines may be useful for breeders to develop new camelina cultivars that can withstand harsh freezing environments of the world.

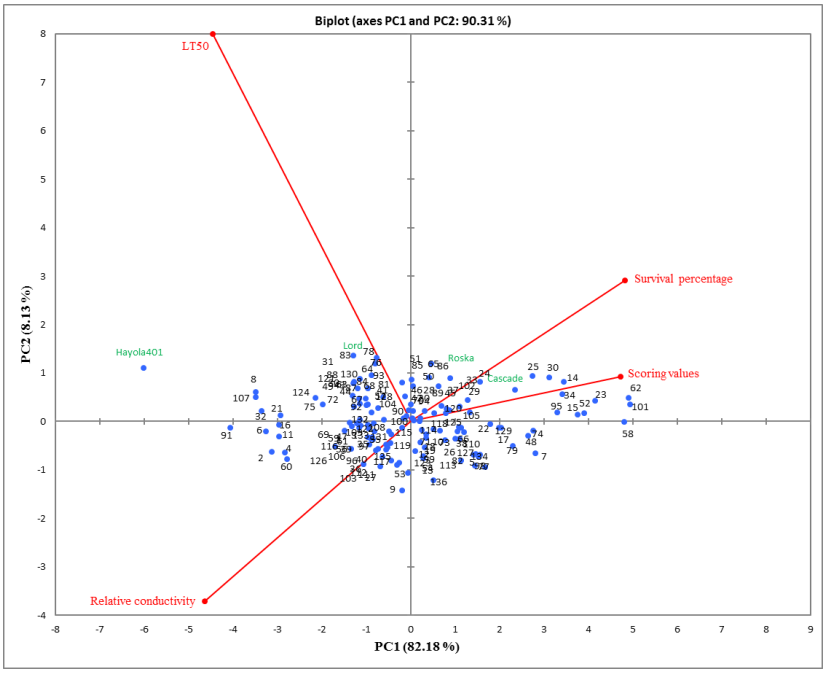

Figure 3. Principal component biplot of the 136 doubled haploid lines of camelina and four cultivars of canola.

As has been shown in Figure 3 and Table 3, scoring values was highly correlated with survival percentage (0.841). Also, relative conductivity had a high negative correlation with survival percentage (-0.820) and plant scoring values (-0.751). Also, the LT_{50} value had a positive correlation with relative conductivity (0.7) and a negative correlation with survival percentage (-0.725) and scoring values (-0.734).

Table 3. Correlation coefficients among measured parameters of 136 camelina DH lines and four canola cultivars under freezing stress condition.

Variables	LT_{50}	Relative conductivity	Survival percentage	Scoring values
LT_{50}	**1**			
Relative conductivity	0.700	**1**		
Survival percentage	-0.725	-0.820	**1**	
Scoring values	-0.734	-0.751	0.841	**1**

Since some of the camelina genotypes have recognized tolerance to harsh freezing environments of the northern United States (GESCH and CERMAK, 2011), producing high tolerant genotypes and understanding the underlying mechanism of freezing tolerance should provide important clues for breeding programs aimed at enhancing winter hardiness in other economically important Brassicaceae species such as winter canola (BERTI *et al.*, 2016).

CONCLUSIONS

The results demonstrated the reliability of the protocol in identifying freezing-tolerant germplasm in camelina. Also, the tolerance of DH lines derived from 15 crosses between different cultivars of camelina was determined. This provides evidence of useful genetic variability and potential choice of freezing tolerant genotypes. It is concluded from screening experiment that some of the camelina lines were more tolerant than cascade, a highly tolerant cultivar of canola. The identified freezing-tolerant lines may perform better than other cultivars planted early in growing conditions with occurring freezing stress. However, these results should be validated under field conditions to evaluate their performance before recommending them to the farmers and breeders to achieve the benefit of area with freezing stress. We recommend the freezing-tolerant camelina DHs for cold climates of Iran such as Ardebil, Hamedan, Sonqor and Shahrekord.

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SKRINING LINIJA DUPLIH HAPLOIDA *Camelina sativa* L. ZA TOLERATNOST NA MRAZ U STANJU KLIJANACA

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Izvod

Stres mržnjenja je važan abiotički stres koji ograničava prinos i prostornu distribuciju mnogih važnih useva. Cilj istrživanja je skrining 136 DH linija (*Camelina sativa* L. zajedno sa četiri kultivara uljane repice (*Brassica napus*) (Hyola 401, Lord, Roska i Cascade) kao kontrola u uslovima mraza (-14 °C for 6 h) da se identifikuju linije sa visokim ili niskim nivoom toleratnosti na stres mraza za buduća ispitivanja. Prvo, razvijen je protocol za skrining većeg broja genotipova pri niskim temparaturama. Za to ogled sa različitim temparaturama (-5, -10, -15 i -20 °C) je sproveden da se pronađu odgovarajuće temparture koje najbolje razdvajaju genotipove (t.j. LT_{50} temperatura). Vrednost LT_{50} je varirala između -10.2 i -17.1 °C sa prosekom -13.94 °C za sve linije. Zato smo izabrali -14 °C izlaganje tokom 6 h kao odgovarajću temparaturu za skrining linija. PCA je korišćena za merenje parametara (LT_{50}, procenat preživljavanja, relativne provodljivosti) da se odrede linije koje su tolertne i osetljive na mraz. Između 136 DH linija, neke linije (58, 62 i 101) su imale viši nivo toleratnosti a neke od njih (8, 16, 32, 91 i 107) su bile senzitivne. Izabrane linije na osnovu primarnog skrininga su korisne za dalju evaluaciju.

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