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Development of a protocol for frost-tolerance evaluation in rapeseed/canola (*Brassica napus* L.)

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ABSTRACT

Spring frost can severely damage or even kill rapeseed/canola (*Brassica napus* L.) seedlings. A protocol for large scale screening of rapeseed germplasm under frost-simulating conditions has not yet been developed. Accordingly, the present study was conducted to develop a protocol for screening rapeseed germplasm under artificial frost-simulation conditions in a plant growth chamber and in a greenhouse. Nine rapeseed varieties, including three commercial hybrids, three spring types, and three winter types were used. Cold acclimation at 4 °C was applied for 0, 7, or 14 days to two-week old seedlings. The seedlings were treated with four freezing temperatures (−4 °C, −8 °C, −12 °C, and −16 °C). The length of the freezing period was 16 h, including the ramping of temperature down from 4 °C and up from the respective freezing temperature to 4 °C. Plants were allowed to recover at 4 °C for 24 h before they were moved back to the greenhouse. Frost damage was scored on a 0–5 scale, where 0 denotes completely dead and 5 denotes no damage. Seedling survival from the freezing treatment increased from the non-acclimation to the cold acclimation treatment. However, no significant differences ($P < 0.05$) were found between 7 and 14 days of acclimation. Frost treatment at −4 °C resulted in significant differences in seedling damage relative to the other three temperatures, with the −16 °C treatment resulting in the highest overall seedling damage. Significant differences were found between the spring type and the other two types (hybrid and winter). However, no significant differences were found between the hybrid and winter types. The suggested protocol for the assessment of frost tolerance is acclimation of two-week old seedlings for 7 days at 4 °C followed by frost treatment at −4 °C for 16 h.

1. Introduction

Rapeseed/canola (*Brassica napus* L.) is an important crop for the U.S. state of North Dakota (ND), which produces about 84% of the U.S. crop. It is grown primarily in the northeast and north central parts of the state. Canola is considered to be a healthy oil for human consumption compared to other vegetable oils because of favorable combinations of the essential fatty acids in seeds [1].

Frost susceptibility is an abiotic stress that impairs plant growth and crop production [2]. Frost at the seedling stage of rapeseed can be harmful and may destroy the whole crop. The

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frost-free date in North Dakota is generally considered to be May 25, but the date can vary from northern to southern regions of the state and also from year to year. Given that canola is grown in the northern part of the state, the frost-free period tends to start later. The average air temperatures for Langdon, ND in April and May are 4 °C and 11 °C, respectively [3]. However, the minimum temperatures during the same time period are −2 °C and 4 °C, respectively. The severity of frost injury depends on moisture condition, plant growth stage, cold severity, duration of cold temperature, and other factors. Canola seedlings are not affected by a light spring frost that causes leaf wilting but not browning. Frost damage can be seen on leaves and symptoms can include wilting, bleaching, or in extreme cases, plant death. Bleaching occurs owing to phyto-oxidation of pigments in leaves [4]. Wilting is caused by a loss of water from cells. Resistance to chilling by frost is complex and may be difficult to incorporate. Canola growers usually look for blackened cotyledons and/or leaves as an indicator of frost damage necessitating replanting. It is necessary to wait for 5–10 days to confirm whether the plants are recovering by generating green shoots at the growing point of apical meristems in the center of the frozen leaf rosette. Canola is more susceptible to frost at the cotyledon stage than at the three- to four-leaf stage. When early spring-seeded canola is exposed to cold temperature, the defense mechanism allows the plant to withstand cold temperature via gradual hardening of plant tissue. Slow-growing seedlings are harder and less susceptible to cold than rapidly growing seedlings. In spring canola, the process of unhardening the plants to initiate active growth is rapid [5]. Usually, winter type canola is capable of hardening faster, can tolerate cold temperatures for a longer time, and is unhardened slower, reducing frost damage [6]. However, variation in frost hardiness is also available within winter- and spring-type germplasm.

Identifying frost tolerance in canola would be beneficial for growers, especially in North Dakota, but also in other places where early planting poses the threat of frost damage. Screens for frost tolerance in canola using artificial growing conditions have not been established. Field testing of frost tolerance relies heavily on weather conditions each year and these cannot be predicted. Thus, screening for frost tolerance under controlled environmental conditions may help to identify frost-tolerant germplasm and can also be performed multiple times in a year, increasing screening capacity over that by field testing.

Canola displays different growth habits. The winter type is grown mainly in western Europe and part of the USA. Vernalization is required for flowering of winter-type rapeseed. The spring type is grown in Canada, USA, Australia, India, eastern Europe, and other countries. China grows mainly a semi-winter type. Owing to its severe winters, North Dakota grows only spring-type canola.

This study aimed to identify a protocol for screening frost tolerance in canola under artificial frost-simulation conditions.

2. Materials and methods

Nine canola varieties chosen from two growth types, were planted in a randomized complete block design (RCBD) with three replicates and eight plants per line per replicate were grown in the greenhouse for two weeks at 20 °C. The photoperiod was 16 h of light and 8 h of dark and the average humidity was 47.3%. The varieties grown included three commercial hybrids (DKL 70–07, Pioneer 45H26, and Sprinter), three spring lines (NDSU151000, Hi-Q, and Kanada), and three winter lines (Fashion, ARC 2180–1, and Galileo). The hybrids are commercial varieties commonly grown in North Dakota and were chosen for this reason. These winter and spring type varieties are commonly used in the North Dakota State University canola breeding program. Because the varieties represented two growth habit types and are commonly used in the breeding program, we chose these winter- and spring-type varieties for this study.

After two weeks of growth, plants were moved to the plant growth chamber for acclimation at 4 °C with a 12-h photoperiod provided by GE Ecolux F32T8 SP35 Eco (32 W T8) style bulbs (General Electric Company). Three acclimation times (0, 7, and 14 days) were used. A total of 216 seedlings (9 varieties × 8 seedlings/variety × 3 acclimation times) per replication per freezing treatment were used. Seedlings were fertilized with 20–20–20 water-soluble fertilizer prior to cold acclimation.

An ESPEC BTU-433 freezing chamber (ESPEC North America, Inc.) was used for frost simulation. Four freezing temperatures were tested: −4 °C, −8 °C, −12 °C, and −16 °C. The total time for frost simulation was 16 h, including the lowering and raising of the temperature from and to 4 °C, along with holding at the minimum temperature. Sixteen h of treatment was chosen, based on overnight freezing temperatures in North Dakota.

In the −4 °C treatment, the temperature started at 4 °C and was lowered at −2 °C h^{-1} over 4 h to reach the treatment temperature. The seedlings were kept at −4 °C for 8 h. The temperature was raised again to 4 °C at a rate of 2 °C h^{-1}, requiring another 4 h. In the −8 °C treatment, the temperature started at 4 °C and was lowered at −2 °C h^{-1} for 6 h to reach the treatment temperature. The seedlings were kept at −6 °C for 4 h. The temperature was raised again to 4 °C at 2 °C h^{-1} over another 6 h. In the −12 °C treatment, the temperature started at 4 °C and was lowered at −3 °C h^{-1} over 5.33 h to reach the treatment temperature. The seedlings were kept at −12 °C for 5.34 h and the temperature was again raised to 4 °C at 3 °C h^{-1} over another 5.33 h. Finally, in the −16 °C treatment, the temperature started at 4 °C and was lowered at −3 °C h^{-1} for 6.66 h to reach the treatment temperature. The seedlings were kept at −16 °C for 2.67 h. The temperature was again raised to 4 °C at 3 °C h^{-1} over another 6.66 h (Table 1).

After frost simulation, seedlings were placed in the growth chamber at 4 °C for 24 h before being moved back to the greenhouse for scoring seedling damage and evaluations. Scoring was performed every three days starting three days after the frost treatment. Each plant was scored individually using a 0 to 5 scale, where 0 denoted dead, 5 denoted no damage, and scores of 1–4 were based on visual estimation of frost damage. Notes on general plant color were also taken. The experiment was replicated three times. A total of 2,592 seedlings (9 varieties × 8 plants/variety × 3 acclimations × 4 frost treatments × 3 replications) were scored in the greenhouse and in the growth chamber.

Table 1 – Treatment times in the freezing chamber.

Treatment (°C)	Starting temp (°C)	Temp ramp-down rate (°C h^{-1})	Time required to reach the treatment temp (h)	Treatment length (h)	Temp ramp-up rate (°C h^{-1})	Time required to reach at 4 °C (h)
−4	4	−2	4	8	+2	4
−8	4	−2	6	4	+2	6
−12	4	−3	5.33	5.34	+3	5.33
−16	4	−3	6.66	2.67	+3	6.66

The means of seedling damage from all plants within hybrid, spring-type, and winter-type were used. SAS 9.3 (SAS Institute Inc., USA) was used to calculate the analysis of variance. The analysis was performed for an RCBD and run as a split–split–plot arrangement where A was temperature, B was acclimation time, and C was genotype. LSDs were calculated for significant factors. All data were combined with SAS to conduct this calculation (e.g. N = 36, which is four temperatures × three acclimation times × three genotypes).

3. Results

The ANOVA table indicated that all three factors were significant (Table 2). Some of the interactions were also highly significant. These interactions included temperature × time, and temperature × genotype. The ANOVA was calculated using all the data from the experiment. LSDs were calculated for the individual factors (A, B, and C). Genotypes showed different reactions across different temperatures and acclimation times.

The means of seedling damage for the frost-simulating temperatures were significantly different (Table 3). The warmest temperature (−4 °C) had the highest overall mean (3.1963), corresponding to the lowest seedling damage, and the coldest temperature (−16 °C) had the lowest overall mean (1.4271), corresponding to the highest seedling damage. Different temperatures affected the canola differently. The coldest temperatures caused bleaching (−12 °C and −16 °C) and seedling death, whereas the warmest temperature (−4 °C) did not cause as much damage and some seedlings showed no damage.

Cold acclimation time had an effect between 0 and 7 days, but no significant differences were observed between acclimations for 7 and 14 days (Table 4). Seven or 14 days of acclimation did not change the overall survival of the genotypes. Thus, the optimum acclimation time that should be used is 7 days in the growth chamber.

Genotype differences in response to frost were observed. Frost damage to spring genotypes was significantly different from that to hybrid and winter genotypes (Table 5). The observed differences between the genotypes were somewhat expected. The genotypes used in this study were chosen based on their spring or winter types.

Visual differences could be detected on the plants after frost. The plants that underwent frost simulation tended to be darker green and wilted, whereas the control was lighter green and stood upright (Fig. 1). The initially observed frost damage did not imply later visual damage, as plants could still recover the appearance of a healthy non-frost exposed plant.

Scoring of plants was performed in such a way as to avoid bias as much as possible. A score of 5 indicated that the plant had grown past the initial shock and was showing no sign of damage (Fig. 2-a). A score of 0 indicated that the plant was completely dead (Fig. 2-b). Dead plants were usually white in color, a phenotype called bleaching that can occur when cells are ruptured by freezing. A score of 1 meant that the plant was almost dead, a score of 4 meant that the plant had very little

Table 2 – ANOVA from plants scored 3 days after frost simulation.

Source	df	Sum of squares	Mean square	F	P-value
rep	2	4.240	2.120	91.46	0.0001
A	3	48.260	16.087	694.02	0.0001
rep × A	6	4.437	0.739	31.9	0.0001
B	2	38.229	19.114	824.65	0.0001
A × B	6	8.220	1.370	59.11	0.0001
rep × A × B	16	10.700	0.669	28.85	0.0001
C	2	0.245	0.122	5.28	0.0085
A × C	6	0.659	0.110	4.74	0.0007
B × C	4	0.130	0.033	1.4	0.2466
A × B × C	12	0.289	0.024	1.04	0.4294

N.B.: A = freezing temperature, B = acclimation time, C = rapeseed variety.

Table 3 – Effect of different freezing temperature on seedling damage using α = 0.05 and scored 3 days after the frost treatment.

Temperature (°C)	Mean (seedling damage score)	t grouping*	N
−4	3.20	A	27
−8	2.17	B	27
−12	1.74	BC	27
−16	1.43	C	27

LSD = 0.5727.
* Means accompanied by the same letter are not significantly different.

Table 4 – Effect on seedling damage of different lengths of frost acclimation period using α = 0.05 and scoring 3 days after frost treatment.

Acclimation time (days)	Mean (seedling damage score)	t grouping*	N
14	2.71	A	36
7	2.37	A	36
0	1.31	B	36

LSD = 0.4186.
* Means accompanied by the same letter are not significantly different.

Table 5 – Response of genotypes on seedling damage using $\alpha = 0.05$ and scoring 3 days after the frost treatment.

Genotype	Mean (seedling damage score)	t grouping*	N
Hybrid	2.18	A	36
Winter	2.15	A	36
Spring	2.07	B	36

LSD = 0.0722.
* Means accompanied by the same letter are not significantly different.

damage, and scores of 2 and 3 were estimated on the basis of level of seedling damage after respective treatments. Visually, differences appeared between acclimation, temperature, and genotype. These differences were confirmed by analysis.

4. Discussion

Frost tolerance of cultivars would allow growers to plant canola earlier with less concern for damage to the crop. Early-planted canola, which usually flowers early and can thereby avoid high temperatures during flowering, could use early-season moisture and better compete with warm-season weeds, resulting in higher seed yield. In North Dakota, early-planted canola often suffers frost damage that severely affects the crop stand. For this reason, it is important for canola growers to have genetically frost-tolerant canola varieties with rapid-germination capacity that can grow well at low temperature and tolerate early spring freezing and thawing, thereby overcoming problems posed by early spring planting [6].

We have developed a protocol for frost treatment in a controlled environment, where the cold acclimation and the freezing temperature can be maintained as required. The naturally acclimated plants tend to experience temperature changes that are more variable. Plants grown in controlled environments are not exposed to this variation. Although naturally acclimated plants may be exposed to cooler temperatures, they are also exposed to varying temperatures, which may affect their frost tolerance. To get the variation of response of seedlings to freezing temperature, it is best to use a controlled environment where the optimum freezing temperature can be simulated to screen germplasm for frost-tolerant cultivar development. Moreover, because we do not have control over the field sites where seedlings often exhibit either complete survival or complete frost kill, it is difficult to screen germplasm under natural conditions. A strong correlation between field survival and growth chamber studies has been reported [7].

Plant growth stage is an important criterion for screening frost tolerant germplasm. Plants are more susceptible to freezing at the cotyledon stage. We accordingly used two-week-old seedlings, which were expected to show the most variation in response to different freezing temperatures.

Various methods have been used to screen freezing tolerance in plants, such as plant tissue water content [8], ion leakage from cold-stressed plant cells [9], and changes in luminescence [10]. A laboratory freezing tolerance screening at the meristem regrowth stage was performed to assess the viability of damaged seedlings [11]. These authors used various freezing temperatures to determine whether the plant tissues were alive or dead after exposure to cold temperature. Evaluation of freezing-tolerant plants is usually performed by placing plant tissue in distilled water and measuring the electrical conductivity of the resulting liquid solution [12,13]. Higher electrolyte loss is an indication of tissue damage by freezing. Another method, chlorophyll fluorescence, can also be used to screen plants for freezing tolerance [14]. However,

Fig. 1 – Plants exposed to frost (left) compared with the control plants (right).

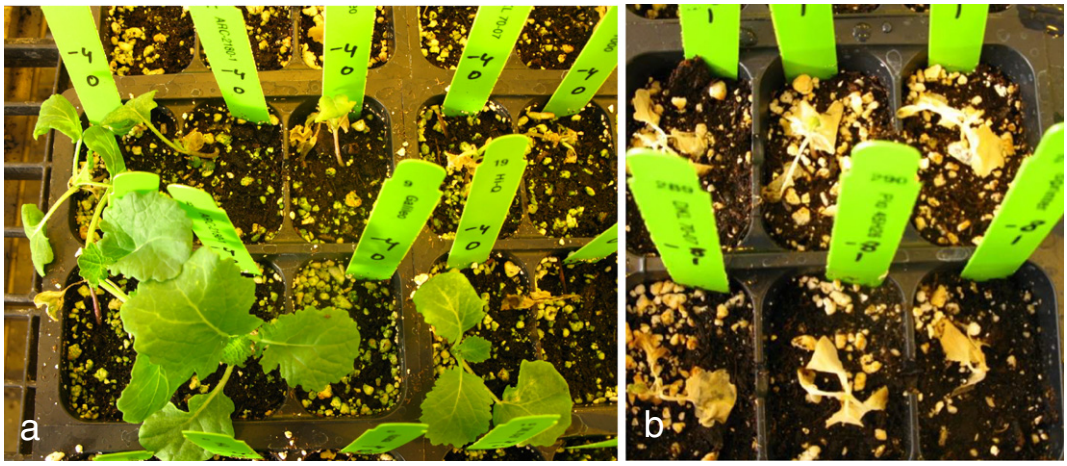

Fig. 2 – a) A score of 5 showing no damage. The plant looks healthy and is growing normally even after frost exposure. b) A score of 0; the plant is completely dead and plants are bleached.

none of these studies were conducted on seedlings under frost-simulating conditions. We have developed a new method for screening a large number of germplasm entries at the two-week-seedling stage under frost-simulating conditions in a plant growth chamber.

Cold acclimation is one step that is necessary for frost tolerance. Cold acclimation is the process of introducing the plant to cool temperatures to improve their survival at freezing temperatures. Usually, plants are naturally acclimated before exposure to natural freezing temperatures. For this reason, we acclimated seedlings before freezing treatment in the plant growth chamber. Many species show increased frost tolerance when cold acclimation is applied before frost exposure [15,16]. The optimum acclimation procedure must also be used for artificial conditions [16]. The optimum acclimation procedure can be tested and determined before frost tolerance studies are initiated. Different species may have different optimum acclimation procedures, but a starting point should be established. For this reason, we used different lengths of time for acclimation before frost treatment. Cold acclimation activates cold-induced genes associated with several physiological and biochemical alterations in the plants to protect cell membranes against freezing-induced injury [17]. Cold acclimation and freezing showed a strong positive correlation in frost tolerance in both winter- and spring-type rapeseed [18]. Our study showed a significant difference between acclimation and non-acclimation to cold temperature before exposure to freezing temperature.

Another factor that may affect frost tolerance is water content in leaves and stems. Higher tissue water content has been shown to be associated with lower hardiness and cold tolerance in plants [19–25]. High water content in tissues could decrease the survivability; however, drought-stressed plants should also have decreased survivability. In artificial conditions, the amount of water received by plants can be controlled and a frost tolerance study can be conducted. This is not the case in the field.

The genetic composition of plants plays a vital role in cold tolerance. The aim of the study was to develop a protocol to identify frost-tolerant germplasm in a wide collection of accessions for use in breeding programs. Freezing tolerance of wheat is a genetically complex trait and complementary gene action may be involved in freezing-tolerance genetics [26]. However, it may be possible to develop genetically tolerant germplasm for growers [26]. Winter survival of barley has been studied and complex inheritance is suspected [27,28]. Different combinations of genes could control winter hardiness in different varieties and winter hardiness is controlled by both recessive and dominant genes [27,29]. Accordingly, in this study, representatives of different growth habit types including winter-type, spring-type, and hybrid cultivars were used to reveal genetic variability in the germplasm.

We have developed a protocol for frost tolerance evaluation under controlled environmental conditions. In this protocol, seedlings were grown for two weeks in the greenhouse, acclimated at 4 °C for seven days, exposed to frost at −4 °C, allowed to recover at 4 °C for 24 h, and scored in the greenhouse for frost damage three days after treatment.

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