HEAT AND CHILLING/FREEZING STRESS

Assessment of Cold and Heat Tolerance of Winter-grown Canola (*Brassica napus* L.) Cultivars by Pollen-based Parameters

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Abstract

Winter-grown canola (*Brassica napus* L.) production is limited mostly by frost and winter kill in the southern canola-growing regions of the United States. Tolerance to cold and heat were assessed by studying percentage of pollen viability (PV), *in vitro* pollen germination (PG) and pollen tube length (PTL) for 12 field-grown cultivars. Freshly collected pollen from all cultivars were incubated on artificial solid growth media at a constant temperature ranging from 10 to 35 °C at 5 °C interval for 30 h to determine PG and PTL. A modified bilinear model best described the temperature response functions of PG and PTL. Canola cultivars showed significant variability ($P < 0.001$) for PV (61.3 % to 89.7 %), PG (29.0 % to 48.2 %) and PTL (463 to 931 μm). The average cardinal temperatures, T_{min}, T_{opt} and T_{max}, for PG and PTL were 6.4, 24.3 and 33.7 °C, respectively. Principal component analysis revealed that maximum PG, PTL, T_{min} and T_{opt} of both PG and PTL were the most important factors in determining cold tolerance, whereas T_{max} of PG and PTL, and maximum PG and PTL were more responsible in separating the cultivars for heat tolerance. The canola cultivar, KS3077, was the most cold tolerant with the lowest T_{min} and the widest temperature adaptability range, and the cultivar Kadore was the most heat tolerant with the highest T_{max} for the PG. The identified cold- and heat-tolerant cultivars may be useful in canola-breeding programmes to develop cultivars suitable for a niche environment.

Introduction

Introduction and cultivation of new crops in a given environment require management practices and trait selection that enable the crop species to perform to its potential. Canola is an important oilseed crop (Downey 1990, Zhang et al. 2003) and its cultivation is expanding, particularly in the western world (FAO 2006), because of its importance as both an oilseed and a bio-diesel crop. Winter-grown canola has attracted attention from both producers and researchers since its introduction in the 1980s (Rife and Zeinali 2003). Canola is cultivated both during winter and spring seasons in United States that expose the crop to winter kill and frost and high temperatures, respectively, during the reproductive period. The temperatures during winter and spring are known to influence all the crucial steps of the reproductive cycle including gametogenesis, pollination, fertilization and embryogenesis (Lardon and Triboi-Blondel 1994, Angadi et al. 2000).

Temperature stress events, which are being experienced now, are expected to intensify because of an increase in emission of greenhouse gases and associated changes in climate. Climate models project an increase in the surface temperature of the Earth by 1 to 11 °C by 2100 (Houghton et al. 2001, Stainforth et al. 2005). Additionally, short episodes of extreme climatic events including low and high temperatures are predicted to occur more frequently in the near future (Meehl and Tebaldi 2004). Studies have shown that these projected changes in climate drastically

reduce crop yields when they coincide with the reproductive stage of plant growth (Hall 1992, Reddy et al. 1992, 1997).

The yield of canola crop grown both in winter and spring is reduced on exposure to low and high temperatures, respectively. Winter-grown canola blooms in March and April in the US mid-south and temperatures <10 °C are not uncommon during that period (Reddy et al. 1995). JinLing (1997) observed that low temperatures result in fewer mature seeds in canola because of the reduced fertilization potential of pollen grains. This could be due to the sensitivity of the binucleate stage of pollen grains to short periods of freezing temperature (−3 °C for 4 h; Lardon and Triboi-Blondel 1994). Similarly, high temperatures inhibit reproductive success. Angadi et al. (2000) reported that exposing *Brassica* species to 35/15 °C day/night temperatures for 7 days during early flowering rather than during early pod development caused greater yield reduction. The yield reduction was attributed to flower abortion caused by pollen infertility. Consequently, the early stages of anther development in *Brassica oleracea* var. *italica* L. showed high sensitivity to the temperature treatment of 35 °C for 7 days compared with the same stress during later growth stages (Björkman and Pearson 1998). Therefore, knowledge of the canola pollen germination processes, cardinal temperature and temperature adaptability range (TAR, T_{max}–T_{min}; Reddy and Kakani 2007) will help us to design breeding strategies to sustain canola production in extreme climatic conditions expected in the future.

Evaluation of crop species for superior traits that can help the crops withstand harsher climatic conditions is the essence of trait-based breeding programmes across the world (Singh et al. 2007). A suitable and relatively faster selection method will enhance the breeding process. Selection for thermotolerance has been obtained either on a whole-plant basis or by evaluation of physiological processes such as photosynthesis and chlorophyll fluorescence (Hall 1992, Fracheboud et al. 1999), and leaf electrolyte leakage of plant tissue subjected to a range of temperatures (Levitt 1980, Ismail and Hall 1999). However, traditional methods of selection for thermotolerance are very time consuming and the physiological parameters are limited for two reasons. First, most of the physiological processes such as photosynthesis are temperature dependent, and secondly, there are evidences that suggest the lack of correlation between physiological processes and final yield. ur Rahman et al. (2004) found a positive correlation between cotton seed yield and leaf electrolyte leakage in the presence of heat stress; however, this correlation was negative when the test was carried out with the leaves obtained from optimum temperature-grown plants. Evaluation techniques that include reproductive structures such as pollen in the selection process enhance the efficacy of the breeding programmes.

Male gametophyte selection has been widely considered an early genotypic selection strategy in plant breeding programmes (Hormaza and Herrero 1996). Pollen grains have been used as a selection tool for thermotolerance in many important crops such as maize (Lyakh and Soroka 1993), tomato (Zamir et al. 1982, Lyakh 1992), cotton (Rodriguez-Garay and Barrow 1988) and canola (Lyakh et al. 1998). In particular, *in vitro* pollen germination (PG) and pollen tube length (PTL) have been extensively used to assess genotypic variability for thermotolerance in several agronomic crops including groundnut (Kakani et al. 2002), cotton (Kakani et al. 2005), *Primula* spp. (McKee and Richards 1998), pepper (Reddy and Kakani 2007) and soybean (Salem et al. 2007). In canola, pollen grain viability is vital in the selection for both low-temperature (Lardon and Triboi-Blondel 1994, Lyakh et al. 1998) and high-temperature (Angadi et al. 2000, Young et al. 2004) stresses. Lyakh et al. (1998) reported that pollen stored at 3 and/or 10 °C for 10 days showed a high percentage of viability with a reduced fertilizing ability, indicating the possible role of PTL in the fertilization process at low temperatures. Additionally, a more than 50 % reduction in yield because of pollen mortality was reported by both Young et al. (2004) and Angadi et al. (2000) when canola was exposed to 35 °C for a week during early flowering. Both PG and PTL are essential traits as *in vitro* PG and PTL were found to be significantly correlated with fruit set in cotton and groundnut (Prasad et al. 1999, Liu et al. 2006) and have been used as selection criteria in tomato breeding programmes for low temperature tolerance (Domínguez et al. 2005). The evidence mentioned above suggests that pollen-related processes could be valuable traits in identifying the genetic variability for thermotolerance, which can be used for cultivar selection in crop breeding.

Studies utilizing the male gametophyte selection technique in oilseed crops such as canola are limited. Canola is highly allogamous (70 %); therefore, pollen sterility/fertility is of great concern because of the restriction of the pollen source due to the restriction in cross-pollination and cytoplasmic male sterility (Denis et al. 1993). Therefore, developing a germination medium to understand canola PG and PTL will be an excellent tool to study the influence of various abiotic stresses on canola pollen behaviour that may represent the reproductive potential of the whole plant. To date, no studies have used pollen germination and tube growth as selection criteria for thermotolerance of canola cultivars. The objectives of this study were to: (a) evaluate *in vitro* PG and PTL responses of 12 canola cultivars to a range of temperatures and (b) develop a

screening tool for both cold and heat tolerance in winter-grown canola cultivars.

Materials and Methods

Growing conditions

Twelve cultivars of winter canola (*Brassica napus* L.) that have either been introduced or commercially grown in the southern United States were used for this study (Table 1). A field experiment was conducted in the winter growing season of 2006 at R. R. Foil Plant Science Research Center, Mississippi State University, Mississippi State (33°28′N, 88°47′W), Mississippi, USA on a Leeper sandy loam. The experiment was of randomized complete block design with three replicated plots, each 18.4 m^2. The seed of the cultivars were drilled into the rows spaced 18 cm apart, 12 g of seed per plot (6.7 kg ha^{-1}) on 1 September 2006. Fertilizer application was performed according to the soil test recommendations provided by the Soil Testing Laboratory at Mississippi State University. Potassium was applied in the form of 67 kg KCl ha^{-1} just before planting. Two separate applications of nitrogen, 22 kg urea ha^{-1} on 20 November 2006 and 40 kg $(NH_4)_2SO_4$ ha^{-1} on 7 March 2007 were applied as top-dressing. To control weeds, the herbicide Treflan (760 g a.i. ha^{-1}) was applied before planting and incorporated with field cultivator in the soil, and no crop injury was observed because of herbicide application. The environmental variables were normal except for a few very cold freezing spells during the beginning of the months of March and April. The maximum and minimum average daily temperatures during the 20 days prior to flower collection were 20 ± 5.5 and 7 ± 5 °C, respectively.

Pollen collection and standardization of solid growth media

More than 150 inflorescences for each genotype were randomly cut from the appropriate field plots and immediately brought to the laboratory around 8:30 hours. Collecting pollen on a single day minimized the effects of variable weather conditions on pollen-related processes that may occur if they were collected over several days. All the inflorescences from an individual genotype were kept in a glass jar filled with water to avoid wilting. The inflorescences were held for 2 to 3 h at room temperature to ensure complete dehiscence of fresh flowers. This time frame resulted in the maximum percentage of germination of pollen grains as determined by a time-series analysis (data not shown). Thereafter, only fresh and dehisced flowers from each genotype were gently tapped in a sterilized Petri dish with the help of a clean fine paint brush. All pollen grains of a single genotype were mixed and divided equally into 21 Petri dishes and stored immediately at −20 °C. The PG and PTL did not differ significantly between fresh pollen and pollen stored at −20 °C for 20 days (data not shown; Sato et al. 1998). The stock solution for pollen growth media consisted of 1 kg sucrose ($C_{12}H_{12}O_{11}$), 250 mg of boric acid (H_3BO_4), 900 mg of calcium chloride ($CaCl_2$), 500 mg of potassium nitrate (KNO_3) and 1000 mg of TRIS (Trizma, Sigma-Aldrich Co., St. Louis, MO, USA) buffer dissolved in 5000 ml of de-ionized water by warming on a hot plate

Table 1 Pollen viability (PV), maximum pollen germination (PG_{max}), modified bilinear equation constants and cardinal temperatures of twelve canola cultivars in response to temperature

			Equation constants				Cardinal temperatures (°C)		
Cultivar	PV (%)	PG_{max} (%)	a	b_1	b_2	R^2	T_{min}	T_{opt}	T_{max}
NPZ0591RR	89.69	48.05	58.4	−1.14	−5.13	0.99	8.61	23.33	32.63
Kadore	86.74	37.15	40.2	−0.59	−3.53	0.97	9.71	23.62	33.55
Kronos	61.34	34.49	35.1	−1.02	−3.37	0.99	9.56	24.47	32.52
DSV05102	76.37	32.26	38.3	−0.76	−3.33	0.98	8.35	23.36	32.78
DSV06200	74.96	28.98	33.9	−0.80	−2.87	0.98	7.40	23.82	33.08
ARC98007	75.56	31.29	34.4	−0.94	−3.02	0.98	7.94	24.47	33.14
KS4085	72.35	31.49	39.2	−0.85	−3.32	0.97	7.28	23.15	32.56
Plainsman	73.91	37.23	45.3	−0.73	−3.90	0.98	8.80	23.22	33.00
KS4002	71.98	31.15	36.5	−0.87	−3.08	0.98	6.45	23.31	32.61
Ceres	79.64	37.73	45.9	−1.01	−3.97	0.97	7.42	22.93	32.21
DSV05101	78.49	47.81	52.9	−1.40	−4.42	0.99	6.14	23.77	33.05
KS3077	83.81	48.18	55.5	−1.77	−4.65	0.98	4.27	23.55	32.45
Mean	77.06	37.15	43.0	−0.99	−3.72	0.98	7.66	23.58	32.80
LSD	8.76***	8.38***	–	–	–	–	1.24***	1.60^{NS}	0.43***

***P < 0.001; NS = non-significance (P > 0.05).

and then stored in the dark at room temperature (Roberts et al. 1983). A fresh solid germination medium was prepared for each temperature treatment by adding 1 % agar to the appropriate amount of stock medium and slowly heated on a hot plate. After the agar was completely dissolved, 10 ml of the medium was poured into each of the three replicated Petri dishes for each genotype in each treatment. After the solidification of the agar, the Petri dishes were covered and incubated at predetermined temperatures.

Pollen culture and temperature treatments

The stored pollen grains were kept at room temperature for 20 min to allow the Petri dishes back to the room temperature. With the help of clean, fine bristle paint brush, a homogenous layer of pollen grains was spread on the surface of the germination medium in Petri dishes and immediately returned to the incubator at the respective temperature treatments from 10 to 35 °C at 5 °C intervals. As all cultivars failed to germinate at 5 °C and most of the cultivars showed less than 5 % PG at 10 °C, we did not test the responses of PG and PTL below 10 °C. An additional temperature treatment of 32.5 °C was included, in order to have more data at the high temperature end, as pollen grains failed to germinate at 35 °C. All genotypes were tested on a given day at a given temperature. As the temperature of the incubator and that of the growth medium did not show a significant difference as determined by using the method of Kakani et al. (2005), the set incubator treatment temperatures were used for further data analyses.

Measurement of PG and PTL

A pollen grain was considered germinated when the tube length equalled or exceeded the grain diameter. No further increment of PTL was observed after a 30-h period of incubation. Therefore, at this time, a thin layer of a fixing solution (3 % glacial acetic acid, 5 % formaldehyde, 20 % glycerin and 72 % water; Feng et al. 2000) was sprayed on the surface of pollen growth medium and the Petri dishes immediately stored at 4 °C until PG and PTL could be scored and measured. The mean percentage PG and PTL per Petri dish was used as a replication and was calculated by counting the total and the number of pollen grains germinated in each of four microscopic fields of 2.4 mm^2 containing more than 100 pollen grains (Nikon Alphaphot YS microscope; Nikon Instrument, Kangava, Japan). The length of 20 pollen tubes was measured by an ocular micrometer fitted into the eyepiece of the microscope and converted into micrometre (μm) units after calibration with a stage micrometer. The replicated mean values of all the variables were analysed using the one-way ANOVA procedure in SAS (SAS Institute Inc. 2004).

Curve-fitting procedures and determination of cardinal temperatures

Response of PG and PTL to the range of temperatures was analyzed and cardinal temperatures, minimum (T_{min}), optimum (T_{opt}) and maximum (T_{max}), were determined with nonlinear curve-fitting procedures as described by Kakani et al. (2002). The best curve-fitting model was obtained using a modified bilinear equation (Eq. 1) that provided the highest coefficient of determination (R^2) with the least root mean squared deviation. The nonlinear regression procedure PROC NLIN was used to derive parameter estimates and T_{opt} by using a modified Newton–Gauss iterative method. T_{min} and T_{max} were calculated by the derivative equations (Eqs 2 and 3, respectively) using T_{opt} and parameters of the bilinear equations.

$$\text{Mean PG or mean PTL} = a + [b_1(T - T_{opt})] + [b_2(ABS(T_{opt} - T))] \quad (1)$$

$$T_{min} = \frac{-a + T_{opt}(b_1 - b_2)}{b_1 - b_2} \quad (2)$$

$$T_{max} = \frac{-a + T_{opt}(b_1 + b_2)}{b_1 + b_2} \quad (3)$$

where a, b_1 and b_2 are the parameter estimates of the bilinear equation, T is the actual temperature at which PG and PTL were determined, and T_{opt} is the optimum temperature for PG and PTL.

Pollen viability

Pollen grains were collected as described for the PG procedure; however, the pollen viability (PV) test was conducted on the same day without storing the pollen grains. Acetocarmine and 2,3,5-triphenyltetrazolium chloride (TTC) were tested for their ability as vital pollen stains in canola. Acetocarmine stained those pollen grains that were heat-killed at 70 °C for 48 h, while TTC stained only fresh live pollen grains. Therefore, only TTC was used for further studies. The canola PV test protocol was standardized according to the procedure described by Aslam et al. (1964). After a series of tests with different concentrations, 0.5 % (w/v) TTC with de-ionized water provided the best staining and properly distinguished the viable from non-viable pollen grains after 12 h (data not shown). No sugar was necessary in the solution because

pollen grains did not burst in either water or in the TTC solution. Pollen grains were gently dusted by tapping them with an artist brush on glass microscopic slides containing a drop of TTC. Then, they were gently agitated with a needle to ensure a homogenous mixture of pollen grains and to avoid air bubbles in the solution. Transparent nail polish was applied to the edge of the cover slips to avoid desiccation of pollen grains through evaporation of solution water. The preparations were stored in the dark at room temperature. The counts of the total number of pollen grains and the number of stained pollen grain were made in two microscopic fields of 2.4 mm^2 containing more than 100 pollen grains from each microscopic field.

PCA and classification of canola cultivars

Principal component analysis (PCA) is a technique for screening multivariate data and is quite useful in the separation of experimental units into subgroups so that similar experimental units belong to the same group (Johnson 1998). PCA was performed on the correlation matrix of 12 genotypes and nine response variables, i.e. maximum percentage of pollen germination (PG_{max}) and pollen tube length (PTL_{max}), T_{min}, T_{opt} and T_{max} for both PG and PTL, and PV using the PROC PRINCOMP procedure (SAS Institute 2004). The PCA produced loadings for these response variables termed as eigenvectors, principal component (PC) scores for each cultivar, and eigenvalues for each PC. A superimposed biplot with the PC scores and corresponding eigenvectors was developed with the same scale units along the abscissa and ordinates having same the physical length as illustrated by ter Braak (1983). The eigenvectors derived from the PC analysis were used to identify the variables that tend to have a strong relationship (i.e. have elements larger in absolute value than the other elements in the same eigenvector) with a particular PC. This criterion was used to classify canola cultivars for both cold and heat tolerance by using the biplot values of PC scores of PC1 vs. PC2 and PC1 vs. PC3, respectively. For cold tolerance, the cultivars were classified as tolerant (−PC1 and −PC2), moderately tolerant (−PC1 and +PC2), moderately susceptible (+PC1 and −PC2) and susceptible (+PC1 and +PC2). However, for heat tolerance, the cultivars were classified as tolerant (+PC1 and +PC3), moderately tolerant (+PC1 and −PC3), moderately susceptible (−PC1 and +PC3) and susceptible (−PC1 and −PC3).

Results

Response of PG to temperature

No bursting of pollen grains of the canola cultivars was observed in the solid medium and significant

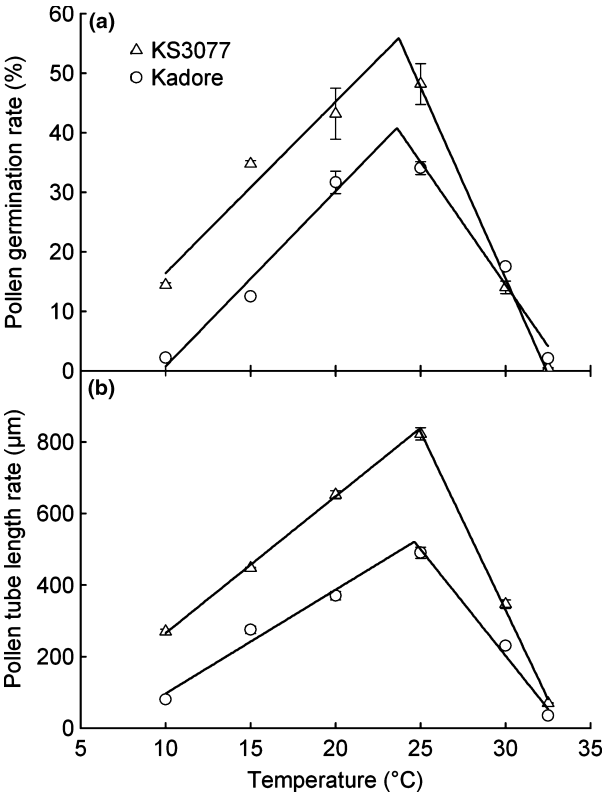

Fig. 1 Pollen germination rate (a) and pollen tube length (b) in response to temperature (symbols) and their fitted lines derived from the modified bilinear equations of two canola cultivars (KS3077 and Kadore). For clarity, data and regression lines for two canola cultivars are presented. Error bars indicate ± S.E.

($P < 0.001$) variation for PG ranging from 29 % (DSV06200) to 48.2 % (KS3077), with a mean of 37.1 % was recorded (Table 1). Temperatures below or above T_{opt} caused a significant linear reduction in the percentage of PG in all cultivars (Fig. 1a, only two cultivars are shown for clarity). Therefore, the modified bilinear equation provided the best fit for the PG response to temperature with a high coefficient of determination ($R^2 > 0.97$). The cardinal temperatures for mean PG, T_{min} and T_{max} differed significantly ($P < 0.001$), whereas T_{opt} did not differ significantly ($P < 0.05$) among the canola cultivars studied. The magnitude of T_{min} ranged from 4.3 (KS3077) to 9.7 °C (Kadore) with the mean value of 7.7 °C. T_{max} ranged from 32.2 (Ceres) to 33.5 °C (Kadore) with a mean of 32.8 °C. The mean value for T_{opt} was 23.6 °C. A wide TAR (T_{max}–T_{min}) was observed for PG that was negatively correlated ($r = -0.97$; $P < 0.001$) with T_{min} (Table 2). However, no significant correlation was observed between the TAR and PG T_{max} (Table 2). The cultivar KS3077 exhibited the widest TAR, 4.3 to

Table 2 Pearson's correlation matrix showing the relationship among maximum percentage of pollen germination (PG_{max}), maximum pollen tube length (PTL T_{max}), cardinal temperatures, pollen viability (PV) and temperature adaptability range (TAR; T_{max}–T_{min}) for pollen germination (PG TAR) and pollen tube length (PTL TAR) of 12 canola cultivars

	PG T_{min}	PG T_{opt}	PG T_{max}	PG_{max}	PTL T_{min}	PTL T_{opt}	PTL T_{max}	PTL_{max}	PV	PG TAR
PG T_{opt}	0.20									
PG T_{max}	0.37	0.36								
PG_{max}	−0.35	−0.15	−0.15							
PTL T_{min}	0.64*	0.02	0.18	−0.17						
PTL T_{opt}	−0.31	0.29	0.15	−0.32	−0.35					
PTL T_{max}	0.36	0.15	0.14	−0.16	0.01	−0.59*				
PTL_{max}	−0.63*	−0.11	−0.31	0.30	−0.18	0.52	−0.66*			
PV	−0.16	−0.39	0.21	0.61*	0.04	−0.45	−0.03	0.01		
PG TAR	−0.97***	−0.12	−0.14	0.33	−0.64*	0.37	−0.34	0.59*	0.22	
PTL TAR	−0.57*	0.01	−0.15	0.14	−0.98***	0.24	0.16	0.06	−0.04	0.57*

***P < 0.001; *P < 0.05; the numbers without asterisks are not significant (P > 0.05). PG T_{min}, PG T_{opt} and PG T_{max} are the minimum, optimum and maximum temperature for pollen germination, respectively. PTL T_{min}, PTL T_{opt} and PTL T_{max} are the minimum, optimum and maximum temperature for pollen tube length, respectively.

32.4 °C (Δ = 28.2 °C), whereas Kronos recorded the lowest range, 9.6 to 32.5 °C (Δ = 23.0 °C) for PG (Table 1).

Response of PTL to temperature

Similar to PG, the PTL also exhibited a bilinear response to temperature (Fig. 1b, only two cultivars are presented for clarity) with a significant (P < 0.001) variability among the cultivars that ranged from 463 (DSV05102) to 931 μm (KS4085) with a mean value of 660 μm (Table 3). However, no significant (P > 0.05) correlation was observed between maximum PTL and maximum PG (Table 2). Pollen tube elongation was observed even after 16 h of incubation and the length remained intact in the *in vitro* solid growth medium. The cardinal temperature for PTL, except T_{max}, exhibited significant (P < 0.001) variation among the canola cultivars (Table 3). PTL T_{min} varied from 1.9 (KS4002) to 7.3 °C (Plainsman) with a mean of 5.2 °C and was positively correlated (r = 0.64, P < 0.05) with PG T_{min} (Table 2). PTL T_{max} ranged from 32.8 to 33.9 °C with a mean value of 33.3 °C and was negatively correlated (r = −0.63, P < 0.05) with PG T_{min} (Table 2). T_{opt} ranged from 23.2 (NPZ0591RR) to 26.8 °C (KS4085) with a mean of 25.0 °C. T_{opt} was negatively correlated (r = −0.59, P < 0.05) with T_{max} (Table 2). The TAR for PTL (T_{max}–T_{min}) varied from 26.0 (Ceres) to 31.4 °C (KS4002) (Table 3). Similar to

Table 3 Maximum pollen tube length (PTL_{max}), modified bilinear equation constants, and cardinal temperatures of 12 canola cultivars in response to temperature

		Equation constants				Cardinal temperatures (°C)		
Cultivar	PTL_{max} (μm)	a	b_1	b_2	R^2	T_{min}	T_{opt}	T_{max}
NPZ0591RR	685	847.4	−17.2	−65.9	0.97	5.65	23.23	33.44
Kadore	491	530.4	−18.3	−47.2	0.98	6.63	25.01	33.27
Kronos	601	651.8	−26.9	−60.0	0.99	5.84	25.58	33.45
DSV05102	463	525.6	−11.3	−38.7	0.97	3.99	23.39	33.91
DSV06200	730	793.6	−40.8	−79.0	0.99	5.57	26.39	33.49
ARC98007	598	671.7	−24.5	−60.9	0.97	6.34	25.00	33.31
KS4085	931	1025.0	−60.8	−110.1	0.99	5.94	26.77	32.77
Plainsman	591	689.1	−14.6	−56.4	0.98	7.34	23.83	33.56
KS4002	601	640.5	−36.3	−62.4	0.99	1.87	26.43	33.24
Ceres	611	706.5	−16.8	−59.1	0.99	7.04	23.78	33.08
DSV05101	798	850.3	−47.7	−84.5	0.99	3.28	26.39	32.93
KS3077	823	852.0	−29.6	−69.9	0.99	3.51	24.73	33.33
Mean	660	732.0	−28.7	−66.2	0.99	5.25	25.04	33.32
LSD	78.5***	–	–	–	–	1.51***	1.67***	0.72^{NS}

***P < 0.001; NS, non-significance (P > 0.05).

the PG, TAR for PTL was also negatively correlated with T_{min} ($r = -0.98$, $P < 0.001$) of PTL (Table 2).

Pollen viability

The percentage PV differed significantly ($P < 0.001$) among the cultivars and ranged from 61.3 % (Kronos) to 89.7 % (NPZ0591RR), with an average of 77.1 % (Table 1). Percentage of PV showed a significant positive correlation ($r = 0.61$, $P < 0.05$) with percentage of maximum PG; however, no such significant correlation ($P > 0.05$) was observed with PTL (Table 2).

Assessment of cold and heat tolerance using PCA

The PCA is a statistical tool that produces the loadings (numbers in absolute values) for the response variables (termed as eigenvectors, Table 4), PC scores for each cultivar and eigenvalues for each principal component. Based on the scree plot of the eigenvalues, three PC were selected (Johnson 1998), that explained 71 % of the variability among the canola cultivars for pollen-based parameters. The eigenvector of PC1 had high to moderate positive loadings for T_{min} and T_{max} of both PG and PTL, and negative loadings for PG_{max}, PTL_{max} and PTL T_{opt} (Table 4). Therefore, cultivars with low scores for PC1 tend to have low values for T_{min} and T_{max} with higher percentage of PG, PV and PTL. The optimum temperature for PTL (PTL T_{opt}) also had lower value for PC1. For instance, the cultivar KS3077 with the lowest score for PC1 (Fig. 2) also had the lowest values for PG T_{min}, relatively low PTL T_{min} and T_{max} for PG and PTL and, PTL T_{opt} in addition to the highest PG and relatively high PTL and PV (Tables 1 and 3). PC2 contrasted the cultivars from T_{opt} (positive loadings) for PG and PTL with the PG_{max} and PV (negative loading). Therefore, cultivars with low scores for PC2 should have lower T_{opt} for PG and PTL with high percentage of PG and PV. Thus, the scores of PC1 and PC2 together revealed the importance of the variables studied in the cultivar separation for cold tolerance (Fig. 2). Consequently, the scores of PC1 with PC3 showed much inclination towards the separation of cultivars for heat tolerance because of the positive loadings for all the elements in either eigenvector of PC1 and/or of PC3 (Table 4, Fig. 3).

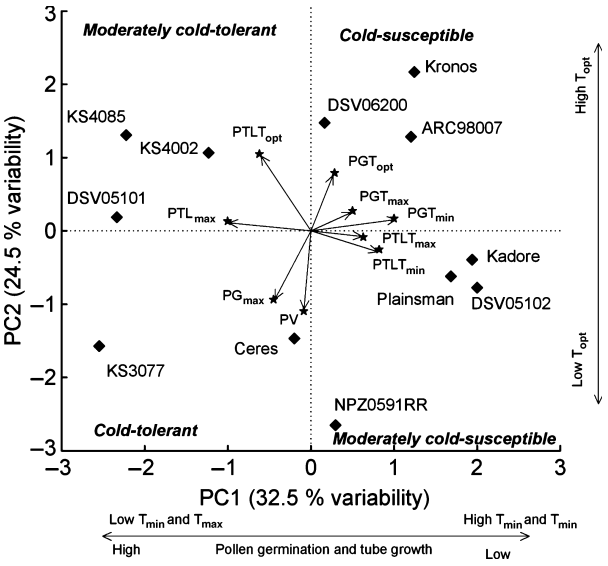

Fig. 2 Principal component analysis (PCA) biplot for the first two principal component (PC) scores, PC1 vs. PC2, related to the classification of twelve canola cultivars (solid diamond symbols) for cold-tolerance. The eigenvectors (PC1 and PC2) for the variables (solid stars) are super-imposed with the PC biplot scores at the similar scale that reflects the contribution of the parameters (variables) in determination of cold tolerance. The arrows radiating from the centre of the figure indicate the direction (angle) and relative magnitude (length) for the parameters. The eigenvectors were multiplied by 2 in order to obtain clear and superimposed figure. The arrows along the right *y*-axis and the bottom *x*-axis indicate the interpretation of the PCs.

Table 4 Principal component analysis eigenvectors of PC1, PC2 and PC3 of 12 canola cultivars for maximum pollen germination (PG_{max}), maximum pollen tube length (PTL_{max}) and their respective cardinal temperatures (T_{min}, T_{opt} and T_{max}), and pollen viability (PV) and the variation accounted for by each eigenvector

	Principal component eigenvectors		
Parameter	PC1	PC2	PC3
PGT_{min}	0.50	0.08	0.16
PGT_{opt}	0.14	0.40	0.20
PG T_{max}	0.25	0.14	0.62
PG_{max} (%)	−0.22	−0.47	0.23
PTL T_{min}	0.32	−0.04	0.34
PTL T_{opt}	−0.31	0.53	0.19
PTL T_{max}	0.41	−0.13	−0.38
PTL_{max} (μm)	−0.50	0.07	0.18
PV (%)	−0.04	−0.55	0.40
Variation (%)	32.5	24.5	14.0

Discussion

The plant reproductive processes leading to flower formation and seed development are more responsive to abiotic factors such as temperature and water deficits. Pollen germination and pollen tube growth are considered as the ability of the pollen grains to perform their function of delivering the sperm cell to the embryo. The current study reveals significant cultivar differences for *in vitro* PG and PTL not only at the optimum temperature but also at the range of temperatures from 10 to 32.3 °C

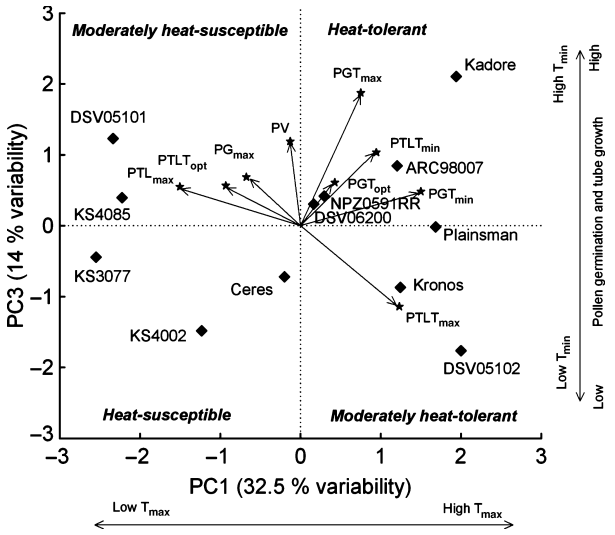

Fig. 3 Principal component analysis biplot for principal components (PC) scores of PC1 vs. PC3 related to the classification of 12 canola cultivars (solid diamond symbols) for heat tolerance. The eigenvectors (PC1 and PC3) of the variables (solid stars) are superimposed with the principal component biplot scores at the similar scale reflecting the contribution of the parameters in determination of heat tolerance. The arrows radiating from the centre of the figure indicate the direction (angle) and relative magnitude (length) for the parameters. The eigenvectors were multiplied by three in order to obtain clear and superimposed figure. The arrow along the right *y*-axis and the bottom *x*-axis indicate the interpretation of the PCs.

(Tables 1 and 3, Fig. 1). The PG_{max} found in this study (29–48 %) was comparable with *in vitro* PG studies of other species such as 35 % in sorghum (Tuinstra and Wedel 2000), 67 % in rice (Khatun and Flowers 1995), 66 % in tall fescue (Wang et al. 2004), and 44 % in cotton (Kakani et al. 2005). *In vitro* pollen germination has also been reported in other *Brassica* species including *B. rapa* L. (10–80 %, Sato et al. 1998), *B. oleracea* L. (20–30 %, Ferrari and Wallace 1975), *B. juncea* L. (60 %, Rao et al. 1992), field-grown *B. napus* L. (57 %, Lyakh et al. 1998) and greenhouse-grown *B. napus* L. (87 %, Shivanna and Sawhney 1995). To the best of our knowledge, this is the first study of canola PG and PTL responses to a range of temperatures that ascertain the cardinal temperatures for these processes. The reported PTL in this study was comparatively higher than that previously reported: 115 μm (Ferrari and Wallace 1975) and 511 μm (Shivanna and Sawhney 1995) for *Brassica* species. The higher mean percentage of PV (77 %) when compared with PG (37 %) obtained in this study (Table 1) is in agreement with previous studies which showed higher PV than PG in artificial media (Rao et al. 1992, Young et al. 2004). A reasonably positive correlation (r = 0.64) between PV and PG indicates the consistency of the evaluated procedure for *in vitro* PG (Table 2). Considerable variation in PV and the role of pollen in the fertilization process have been observed in previous studies with *Brassica*. In a freezing injury test for the reproductive organs in canola, Lardon and Triboi-Blondel (1994) demonstrated that the freezing temperature of −3 °C for 4 h, 4 days before anthesis, resulted in 20 % PV (flurochromatic reaction). However, when the same pollen were used on a non-stressed pistil, fertilization of all ovules was observed. This indicates that 20 % PV may be enough to achieve sufficient fruit set in canola. However, this needs to be confirmed because the intensity and duration of the temperature has variable effects on pollen depending on the developmental stage.

The cardinal temperature (T_{min}, T_{opt} and T_{max}) for growth and development of a crop species are process-dependent (Kakani and Reddy 2007). A temperature stress could be anything below and/or above the T_{opt}, which influences the functionality and success of the biochemical pathway which may reduce efficiency of the particular phase of development, resulting in loss in economic yield. The bilinear response of *in vitro* PG and PTL to the temperature in the current study is not uncommon. A similar response of *in vitro* PG and PTL to temperatures have also been reported and quantified using regression models in other crop species such as peppers (Reddy and Kakani 2007), cotton (Kakani et al. 2005, Liu et al. 2006) and groundnut (Kakani et al. 2002). In canola, linear relationship between yield and temperature has been reported in other studies. In a field experiment utilizing five canola cultivars sown at four different times in Australia, Walton et al. (1999) recorded a negative linear correlation of post-anthesis daily mean temperature above 10 °C with both canola yield and seed oil content. The average cardinal temperatures for PG and PTL reported in this study (6.4 °C – T_{min}, 24.3 °C – T_{opt} and 33.0 °C – T_{max}) are within the expected range estimated for flowering and seed development of *Brassica* species. Hodgson (1978) determined a base temperature of 6.1 °C for field-grown *B. napus* in Australia, which is similar to the 4.7 ± 1.5 °C base temperature calculated in a growth chamber study in Canada (Morrison et al. 1989). Previous studies indicate that the temperature thresholds for flower and seed development in *Brassica* species do not vary to a greater extent, and have a lower limit of daily mean temperature less than 10 °C (JinLing 1997) and upper limit as low as 25 °C (Morrison et al. 1989) to the day time temperature of 30 to 32 °C (Polowick and Sawhney 1988). Thus, the cardinal temperatures for PG and PTL in the current study are in accordance with the previous studies reporting the critical temperatures for reproductive development. However, none of these studies clearly state the optimum temperature for

reproductive development. The cardinal temperature showed highly significant variations in the canola cultivars studied (Tables 1 and 3, Fig. 1) except T_{opt} for PG and T_{max} for PTL. In addition, the cultivar differences were larger for T_{min} than for either T_{opt} or T_{max}. The reduced variability for T_{max} might be attributed to selection and/or the traditional breeding methods used for cold tolerance since the start of the winter-grown canola cultivation. Therefore, traditional breeding selection methods might have limited the exploitation of genetic variability for two opposite extreme ends, e.g. cold and hot (Limin and Fowler 1991). T_{min} values for PG and PTL were correlated, indicating the consistency of genetic variation present in winter-grown canola for cold tolerance (Table 2). The difference between T_{max} and T_{min} of a species or process should reflect the TAR of that species which confer the survival range (Reddy and Kakani 2007). Cultivars KS3077 (4.3–32.4 °C) and KS4002 (1.9–33.2 °C) had the widest survival range for PG and PTL, respectively (Tables 1 and 3), with a T_{min} value very close to the colder side. There was a strong negative correlation of TAR with the T_{min} of PG and PTL (Table 2), which indicates that the genotypes with higher TAR had lower T_{min}. Thus, the survival range of winter-grown canola cultivars is mostly contributed by the large variation for T_{min}.

Canola being a cool-season crop is sensitive to high temperature in most of the spring-canola growing regions. However, winter-grown canola is much more susceptible to the low temperature stress. In the current study, the T_{min} (6.4 °C) and T_{max} (33.0 °C) values observed for the PG and PTL also reflect the critical minima and maxima temperature stresses of 10 °C (JinLing 1997) and 29.9 °C (Morrison and Stewart 2002), respectively, during flowering. Temperatures beyond these thresholds caused severe reduction in PG and PTL in the current study, similar to previous studies that also reported pollen mortality and failure to set fruit in canola (Polowick and Sawhney 1988, Rao et al. 1992, Morrison 1993, JinLing 1997, Angadi et al. 2000, Morrison and Stewart 2002, Young et al. 2004). Furthermore, Wood et al. (2006) reported that the reduction in grain set and harvest index followed a similar trend with that of pollen viability in sorghum plants subjected to chilling treatment for 5 days at the pre-meiotic stage of anther development. This indicates that the ability of pollen germination and pollen tube growth under the temperatures below 10 °C and above 30 °C could be useful in canola genotype selection for cold and heat tolerances, respectively.

In this study with solidified pollen germination media, improved *in vitro* PG and PTL were observed compared with previous studies carried out with liquid pollen growth medium (Rao et al. 1992, Shivanna and Sawhney 1995, Lyakh et al. 1998, Young et al. 2004). Pollen tube growth appeared to be normal at the temperature extremes imposed in this study. In the current study, a constant incubation temperature of 10 and 32.5 °C severely reduced both PG and PTL; additionally, pollen failed to germinate at 35 °C. Contrary to this, Rao et al. (1992) found that *B. juncea* pollen grains were able to germinate after 4 or 24 h at 45 or 60 °C heat treatment prior to incubation in germination media. Once the pollen grains are matured and released, they act as independent functional units that must survive for successful fertilization. The PG of even a high temperature-treated (45 and or 60 °C, Rao et al. 1992) pollen indicates that dehydration may be one of the mechanisms behind thermotolerance in the pollen grains of *Brassica*. Young et al. (2004) also supported that mature *Brassica* pollen grains heat-shocked for 30–60 min at 35 °C were unable to synthesize a subset of heat-shock protein transcripts, suggesting that the desiccated nature of pollen probably protected mature pollen from high temperature while making it unnecessary to produce heat-shock proteins for thermotolerance. However, more studies are needed to understand the mechanisms of thermotolerance in *Brassica* pollen grains.

The PCA is considered an effective statistical procedure to express multidimensional data in a way that highlights their similarities and differences. Through orthogonal linear transformation, PCA creates a new coordinate system for the data sets, generating PC scores or latent vectors capable of explaining the systematic behaviour of the observed variables in a reduced dimension. The biplot derived from the PCA effectively separated and grouped the canola cultivars based on their responses to temperature. The biplot is a simply and specially scaled combination of PC scores and loadings (eigenvectors) that allow the approximate similarities and differences of the cultivars (the scores) to be displayed simultaneously and allow the different response variables (eigenvectors) to be associated with cultivars (Figs 2 and 3). The magnitude of the elements in the absolute values and the sign of the first two eigenvectors (Table 4) revealed that the cultivars with small scores of PC1 and PC2 are cold tolerant. Conversely, the higher scores are more susceptible to cold. This is because in the eigenvectors of PC1 and PC2, the magnitude and the sign of the elements that are important components of cold-tolerance mechanisms, such as T_{min} and T_{max} for both PG and PTL are highly positive, whereas, PTL T_{opt}, PG_{max}, PTL_{max} and PV are highly negative (Table 4). Therefore, the cultivars that have small values for T_{min} and T_{max} will also have lower scores and cultivars with small T_{opt}, PG_{max}, PTL_{max} and PV have larger scores for the first two PC scores and vice versa. As a result, the biplot of PC1 vs. PC2 (Fig. 2) grouped the

cultivars that have relatively lower T_{min} and T_{max}, and higher T_{opt}, PG_{max}, PTL_{max} and PV. The cultivar KS3077, for example, is classified as highly cold tolerant because it showed the lowest score for PC1 and relatively lower score for PC2. In contrast, Kronos with relatively higher scores is classified the most cold-susceptible cultivar (Fig. 2).

Similarly, almost all the elements in the eigenvectors of PC1 and/or PC3 recorded high positive loadings (Table 4). Therefore, the cultivars that have large values for the original variables will also have large scores for PC1 and PC3, and vice versa. The high values of the variables and derivatives of the PG and PTL have already been used for classification of crop species for their heat tolerance (Kakani et al. 2002, Liu et al. 2006). Thus, a biplot of PC1 vs. PC3 should separate the cultivars that have higher values for cardinal temperatures, maximum PG, PV and PTL from those with the lower values. For instance, the cultivar Kadore is considered as the most heat tolerant because of its relatively higher score for PC1 and the highest score for PC2 (Fig. 3). In contrast, KS 4002 is classified as the most heat-susceptible cultivar.

Finally, two distinct classifications, one for cold tolerance and the other for heat tolerance, were generated based on the relative position in the quadrants of the biplot of PC1 vs. PC2 (Fig. 2). The 12 canola cultivars were classified into four groups as cold tolerant (KS3077 and Ceres), moderately cold tolerant (KS4085, DSV05101 and KS4002), moderately cold susceptible (NPZ0591RR, DSV05102, Plainsman and Kadore) and cold susceptible (Kronos, DSV06200 and ARC98007). Similarly, based on the biplot of PC1 vs. PC3 (Fig. 3) the classification for heat tolerance was made as heat tolerant (Kadore, ARC98007, NPZ0591RR and DSV06200), moderately heat tolerant (Plainsman, Kronos and DSV05102), moderately heat susceptible (DSV05101 and KS4085) and heat susceptible (KS4002, Ceres and KS3077) cultivars.

Conclusions

The *in vitro* PG and PTL of canola showed a typical bilinear response to temperature. The cardinal temperatures for PG and PTL reported for the first time in this study were similar to the cardinal temperatures for flower and seed set in canola. The T_{min} exhibited larger variability among the cultivars when compared with the T_{max} for PG and PTL. The canola cultivar KS3077 was the most cold tolerant, with the lowest T_{min} and the widest TAR and the cultivar Kadore was the most heat tolerant with the highest T_{max} for the PG. Therefore, pollen-based parameters could be used to screen winter-grown canola cultivars for their cold and heat tolerance. However, an extended study in controlled environments of several aspects including fruit set will be desirable to completely understand the mechanisms of heat and cold tolerance in winter-grown canola.

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