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ORIGINAL PAPER

Divergent selection in a maize population for germination at low temperature in controlled environment: study of the direct response, of the trait inheritance and of correlated responses in the field

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Abstract Improving cold tolerance in maize (*Zea mays* L.) is an important breeding objective, allowing early sowings which result in many agronomic advantages. Using as source the F_2 population of B73 × IABO78 single cross, we previously conducted four cycles of divergent recurrent selection for high (H) and low (L) cold tolerance level, evaluated as the difference (DG) between germination at 9.5 °C and at 25 °C in the germinator. Then, we pursued the divergent selection in inbreeding from S_1 to S_4. This research was conducted to study (1) the direct response to selection (by testing ten S_4 L and ten S_4 H lines), (2) the trait inheritance (in a complete diallel scheme involving four L and four H lines), (3) the associated responses for cold tolerance in the field (at early and delayed sowings) and (4) the responses for other traits, by testing the ten L and the ten H lines at usual sowing. Selection was effective, leading to appreciable and symmetric responses for DG. Variation among crosses was mainly due to additive effects and the ability to predict hybrid DG based on parental lines DG was appreciable. Associated responses for cold tolerance traits in the field were noticeable, though the relationship between DG and these traits was not outstanding. High tolerance was also associated with early flowering, short plants, less leaves, low kernel moisture, red and thin cob, and flint kernels. These divergently selected lines can represent valuable materials for undertaking basic studies and breeding works concerning cold tolerance.

Introduction

Early sowing in maize (*Zea mays* L.) can result in several agronomic advantages such as (1) greater yield obtained by growing later and more productive genotypes, (2) better yield stability, achieved by anticipating flowering before the hottest and driest period of the year and (3) the possibility to harvest the crop in advance, or with a lower kernel moisture. Indeed, a general trend to anticipate the sowing date has been widely noted in the last 30 years (for the US Corn Belt regions, see Kucharik (2006)), but this trend is expected to continue in the future, due to the need to further increase both grain yield and its stability, and to reduce the cropping costs. However, to allow an early sowing, maize genotypes should be characterized by a high level of cold tolerance, i.e., the ability to steadily germinate and grow even under adverse thermic conditions.

Therefore, improving maize cold tolerance has been and still is a very important breeding objective and, given the complex genetic basis of the target traits (Grogan 1970; McConnell and Gardner 1979b; Eagles 1988) it was often pursued by means of recurrent selections (Mock and Bakri 1976; Mosely et al. 1984; Hoard and Crosbie 1985; Sezegen and Carena 2009). Responses to such selections were appreciable only in some cases, at least partly because of important genotype by environment interactions due to the vagaries of the climatic trends in the early growing season (Mock and McNeill 1979; Rodríguez et al. 2008). In fact, cold stress in the field is unpredictable, being determined

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by several factors, such as the values of low temperatures, the duration of exposure to such temperatures and the growth stages in which they occur (Miedema 1982).

Alternatively, selection for cold tolerance at germination and/or subsequent growth stages could be conducted in controlled environment (e.g., McConnell and Gardner 1979a), hence reducing the negative role of the genotype by environment interaction. In controlled environment, the cold stress conditions can be easily reproduced, so that selection for cold tolerance traits can be performed throughout the breeding process regardless of the year-to-year climatic variations. Moreover, the genotypes' testing can be made during the off-season, thereby making the subsequent breeding work in the field less demanding because only selected materials can be grown. However, selection for cold tolerance in controlled environment should involve genes also affecting cold tolerance in the field so as to have associated responses consistent with the direct responses. Positive relationships between the cold tolerance level observed in the controlled environment and in the field were noted in several studies (Haskell and Singleton 1949; Pinnell 1949; Pešev 1970; Mock and Eberhart 1972; Menkir and Larter 1987; Hope et al. 1992; Hodges et al. 1995; Bhosale et al. 2007), thus encouraging the undertaking of such an indirect selection.

Based on these premises, Landi et al. (1992) undertook a divergent recurrent selection for germination at low temperature in controlled environment (germinator), using as source an F_2 population, and after four cycles of selection they achieved appreciable direct and associated responses. However, a clear tendency to level off was noted for germination traits, with the mean values of the divergently selected populations approaching either one or the other limit. According to Falconer and Mckay (1996), such a tendency to level off for traits expressed as percentage should not necessarily imply the exhaustion of the genetic variability and many loci controlling the selected trait may not be fixed yet. Therefore, we decided to continue the selection work on these materials, but following a different breeding procedure, i.e., a pedigree selection on subsequent selfing generations. In fact, selfing increases the homozygosity level and, hence, the additive component of the genetic variability while reduces the component due to dominance (Falconer and Mckay 1996), thus enhancing the selection effectiveness. Selection was carried out for three selfing generations (i.e., at S_1, S_2 and S_3 level). The present study was conducted to analyse: (1) the direct responses obtained throughout the selection process for germination traits in the germinator; (2) the inheritance of these traits and the ability to predict the crosses performance based on the parental lines performance, because for most maize breeding programmes the final use of lines is in hybrids production; (3) the associated responses obtained for cold tolerance traits evaluated in the field at both early and delayed sowings; (4) the associated responses for plant, ear and kernel traits, evaluated in field trials sown at usual dates.

Materials and methods

Brief description of the previous recurrent selection

The source population of the recurrent selection was the F_2 of the single cross between the inbred lines B73 and IABO78. B73 is a wellknown inbred line of the US Corn Belt characterized by red cob, dent kernel and shows a level of cold tolerance higher than that of other public inbred lines of the US Corn Belt (Mock and McNeill 1979). On the other hand, B73 can also show unsatisfactory field germination in case its seeds mature during the rainy periods in the late summer (Landi et al. 1992). IABO78 is an inbred line derived from Italian germplasm, characterized by white cob, flint kernel and that usually shows satisfactory field germination and emergence across various growing conditions (Landi et al. 1992). For these reasons and especially because of their different origins, these two inbreds were chosen as parents of the source population. Throughout the recurrent selection, the germination ability was assessed at 25 ± 1 °C (favourable temperature) and 9.5 ± 0.5 °C (unfavourable temperature), and were designated as G25 and G9.5, respectively. The difference (DG) between the mean value of G9.5 and of G25 was used as selection criterion, to emphasize selection for reaction to cold rather than for germination per se. An intrapopulation full-sib family recurrent selection was adopted (for details on the procedure see Hallauer and Miranda (1988)) and to get a better insight of the selection effects, a two-way (or divergent) procedure was followed by selecting for both low (L) and high (H) DG. The breeding materials herein described were always grown at the Bologna University Experimental Farm of Cadriano (close to Bologna, northern Italy; 44°33′N latitude 11°24′E longitude), in a loam-clay soil. Sowing in the nursery was always made at the end of April-beginning of May (i.e., a slightly delayed sowing) to minimize the chance of having cold stress in the field, as selection was based only on data obtained in the germinator. At the end of the fourth selection cycle (C4), the two divergently selected populations for low and high DG value (C4-L and C4-H, respectively) were reproduced for two generations by crossing more than 100 pairs of random plants to favour the recombination processes without altering the allelic frequencies.

Description of the present selection work and evaluation of responses throughout the selection process for germination traits (germinator)

In 2006, the two divergently selected populations C4-L and C4-H were grown as previously described. For each population about 120 S_0 random plants were selfed and 96 well-fertilized S_1 ears were used to start the selection in inbreeding. Ears were hand-shelled to avoid possible pericarp injures. For each S_1 line, seed germination was evaluated in two experiments: one to estimate G9.5 and one to estimate G25, as made in the previous selection work. In particular, seeds were sterilized for 15 min in a solution of sodium hypochlorite (2 %), carefully rinsed, transferred to Petri dishes lined with wet filter paper. During germination water was added as needed. Each experiment was conducted as randomized complete block design with two replications. The experimental unit was a Petri dish containing 40 seeds. G9.5 and G25 were evaluated 20 and 7 days after sowing, respectively, considering as germinated those seeds with both radicle and coleoptile at least 1 mm long. Then, for each S_1 line, DG was calculated as G9.5–G25. Twenty S_1 lines with the lowest DG and 20 S_1 lines with the highest DG were selected within L and H populations, respectively.

In the subsequent year (2007), six to seven random plants per S_1 line were selfed and four well-fertilized ears were obtained in most instances, thus giving a total of 77 and 80 S_2 ears for L and H populations, respectively. Then, for each S_2 line, G9.5 and G25 were evaluated and DG was used for selection. In particular, in the L population, we first identified the 12 groups of S_2 lines (i.e., derived from the same S_1 line) with the lowest overall mean for DG; then we selected within each group the two S_2 lines with the lowest DG, thus giving a total of 24 S_2 selected lines. The same procedure was followed in the H population and 24 S_2 lines were selected for the highest DG.

In 2008, six random plants per S_2 line were selfed and three well fertilized S_3 ears were obtained in most instances, thus giving a total of 66 and 65 ears for L and H populations, respectively. Then, for each S_3 line, G9.5 and G25 were evaluated and DG was calculated. In the L population, we first identified the 10 groups of S_3 lines (i.e., derived from the same S_1 line) with the lowest mean value for DG; then, within each group we selected the sub-group of S_3 lines (i.e., derived from the same S_2 line) with the lowest mean value for DG and finally we selected the S_3 line with the lowest DG within each sub-group, thus giving a total of 10 selected S_3 lines of different origins (i.e., descending from different S_1 lines). The same procedure was followed in the H population and ten S_3 lines were selected for the highest DG.

In the subsequent year (2009), eight random plants per S_3 line were selfed and four well-fertilized S_4 ears were saved. The seeds of these four S_4 ears were bulked since no further selection for DG was made within each S_3 line, given the expected very low level of their genetic variability. Therefore, the subsequent evaluation in the germinator at S_4 level was made with the only objective of investigating the final response to selection. For each S_4 line, G9.5 and G25 were evaluated. Each experiment was a randomized complete block design with four replications and each experimental unit was a Petri dish containing 25 kernels. As controls, we included the two parental inbreds B73 and IABO78 and their single cross (as bulk of the two reciprocal crosses). These controls were investigated to analyse whether: (1) the selection responses were symmetric (selected lines vs. parents); (2) dominance effects were important (parents vs. their cross). The seed of the controls was produced in the same year (2009). In each replication, controls were included two times. Data for G25, G9.5 and DG were obtained for all genotypes as previously reported.

For G9.5 and G25, the analysis of variance (ANOVA) was conducted on the mean value of each experimental unit, according to the randomized complete block design. For DG, the error variance was obtained as sum of the error variances of the two components G9.5 and G25. For both G9.5 and G25, the ANOVA was also conducted on data subjected to angular transformation (arcsine). However, given the consistency between the results of the analyses on transformed and untransformed data as well as to have consistency with DG data, only the results concerning the untransformed data are herein presented.

Inheritance of germination traits in germinator (diallel mating design) and ability to predict the crosses performance based on the parental lines performance

In 2009 four S_3 lines of the L group and four S_3 lines of the H group (identified as lines no. 2, 7, 11 and 14 for the L group, and lines no. 104, 106, 110 and 111 for the H group) were crossed according to the diallel mating design, giving rise to 56 combinations, i.e., all the possible 28 single crosses and their 28 reciprocals. The eight parental lines were selfed and advanced to S_4. The 56 crosses were evaluated, following the testing procedures already seen, together with the eight parental lines, to have a total of 64 genotypes. The partition of the genotypes' source of variation (63 degrees of freedom, *df*) was as follows: (a) among the eight parental lines per se (7 *df*), (b) between the mean value of the eight parental lines and the mean value of the 56 crosses (1 *df*) and (c) among the 56 crosses (55 *df*). Details on this partition are presented in Supplemental Table S1. Because the eight parental lines belonged to two groups divergently selected (L and H), the 7 *df* were partitioned into within group (3 *df* within L and 3 *df* within H) and between groups (L vs. H, 1 *df*). Then, the source of variation for crosses was analysed according to Griffing (1956), Model I (fixed set of parents), Method 3. This method does not involve the parental lines per se in the calculation of the combining ability effects, to avoid possible biases due to large differences between lines and crosses. In the analysis, concerning crosses we considered: (1) the effects due to general combining ability (GCA, 7 *df*), (2) the effects due to specific combining ability (SCA, 20 *df*) and (3) the reciprocal effects (28 *df*). The GCA effects were then partitioned as were the lines per se. Moreover, the reciprocal effects were partitioned in general reciprocal (or maternal) and specific reciprocal effects (7 and 21 *df*, respectively) following Cockerham (1963).

Based on the distinction of parental lines into L and H groups, the 56 crosses could be also distinguished as L × L (12 crosses), L × H (16 crosses), H × L (16 crosses) and H × H (12 crosses). Therefore, the 55 *df* for crosses were also partitioned following this alternative approach, i.e., among four groups of crosses (3 *df*) and within each group (52 *df* on the whole). The among groups of crosses was then partitioned into the three components: (L × L) versus (H × H), (L × H) versus (H × L) and (L × L and H × H) versus (L × H and H × L) (with 1 *df* for each component); the first component is mainly due to additive effects, the second to reciprocal effects and the third to dominance effects.

Finally to study the ability to predict the crosses' performance based upon the parental lines' performance, the mean value of each single cross was regressed on the corresponding mid-parent value.

Associated responses for cold tolerance traits (emergence and early growth) evaluated in the field at early and delayed sowings

The ten S_4 L lines and ten S_4 H lines, together with the controls (B73, IABO78 and the two populations C4-L and C4-H), were also investigated in field trials conducted at Cadriano for 2 years (2010 and 2011). Before sowing, kernels of all genotypes were subjected to a fungicide treatment (based on Fludioxonil and Metalaxil). In each year, two trials were conducted; one trial was sown as early as possible (16 March 2010 and 21 March 2011) to likely subject the germinating kernels and the developing seedlings to some temperature stresses, whereas the other trial was sown 2 months later (17 May 2010 and 23 May 2011), to likely have more favourable temperature conditions. The experimental design for each trial was a randomized complete block with two replications. Controls were included two times in each replication. The experimental unit (plot) was a single row of 12 hills; two kernels per hill were hand sown at a depth of 5–6 cm. In all trials, the number of emerged seedlings (whose coleoptile was at least 1 cm outside the soil) were detected every day from the beginning to the end of emergence; these data were then used to calculate the average time to emergence (TE, d) as $\Sigma(n_i d_i)/\Sigma n_i$, where n_i represents the number of seedlings emerged in day i, whereas d_i represents the number of days from sowing to time i. The whole number of emerged seedlings was also used to calculate the percentage of field emergence (FE, %). Moreover, at the second leaf stage (i.e., during the heterotrophic growth phase) the plot fresh weight (PFW, g) was investigated by harvesting the above ground part of the seedlings; finally the seedling fresh weight (SFW, g) was calculated as ratio between PFW and the whole number of emerged seedlings. Daily maximum and minimum temperatures in the soil at sowing depth (5–6 cm) were detected by an automatic data logger; the growing degree days (GDD) were calculated by subtracting from the means between daily minimum and maximum values the base temperature of 10 °C (when the minimum was lower than 10 °C it was equated to 10 °C; (Mock and Bakri 1976)).

The ANOVA for the four traits was conducted on each plot value within each trial and then combined across trials; a mixed model ANOVA was adopted having considered both lines and sowing times (early vs. delayed) as fixed and the years as random factors. Concerning FE, the ANOVA was also conducted on data subjected to angular transformation; also in this case there was a consistency of the analyses on transformed and untransformed data, thus only the data of the analysis on the untransformed data are presented (for conciseness and for consistency with G9.5 and G25). Moreover, to have an insight of the association between data collected in the germinator and in the field, regression analyses were performed, considering as independent variables the mean values of each of the 20 selected lines for DG and G9.5 and as dependent variables the respective mean values for FE, TE, SFW and PFW for the early sowings across the 2 years (G25 and field traits in delayed sowings were ignored as they were characterized by a low level of variation).

Associated responses for plant, ear and kernel traits evaluated in the field at usual sowings

The ten S_4 L and ten S_4 H lines were also tested for plant, ear and kernel traits together with the two parental inbreds and the two source populations C4-L and C4-H (controls). Trials were conducted at Cadriano for 2 years at the usual sowing dates adopted in the nurseries throughout the selection work (29 April 2010 and 3 May 2011). The experimental design was a randomized complete block with two replications. Controls were included two times in each replication. Plots were single rows for lines and two rows for populations, each row including 13 plants at a density of 4.5 plants m^{-2}. Border rows were used to separate populations and lines given the expected differences for plant vigour traits. Plants were grown according to the usual field techniques (not described for conciseness) and data were collected for the following traits: days to pollen shedding (PS, day), as interval from field emergence to PS date (50 % of plants with anthers extrusion); plant height (PH, cm) measured at the base of the tassel; number of leaves per plant (LP, no.); cob diameter (CD, mm) measured at the base of the cob; cob colour (CC) as red (homozygous for the *P1-wr* allele), white (homozygous for the *P1-ww* allele) and pink (heterozygous); kernel type (KT, as visual score from 1, dent, to 5, flint); kernel moisture (KM, %); kernel weight (KW, mg). For the S_4 and parental lines, data collection at the single plot level concerned five plants for LP and PH, five ears for CD, 100 kernels for KW, while PS, CC, KT and KM were taken at the whole plot level. For the two populations C4-L and C4-H, the data per plot were collected on a doubled number of plants or ears for LP, PH, CD and on a doubled number of kernels for KW.

The ANOVA was conducted for all traits except CC on the mean values of each plot of each trial and combined across trials. A mixed model was used, having considered the genotypes (lines and populations) as fixed and trials (years) as random factors. For CC, the trait was investigated considering the number of copies of the *P1-wr* and of the *P1-ww* alleles in each L and H groups (lines and populations); a Chi-square test, with the Yates' correction for continuity (Steel and Torrie 1980) was applied to test the null hypothesis that the two divergently selected groups (S_4 L vs. S_4 H and C4-L vs. C4-H) had the same allelic frequencies.

Results

Responses throughout the selection process for germination traits (germinator)

The results obtained for DG, G9.5 and G25 throughout the selection work are presented in Table 1. For DG, the mean values of L lines showed a marked linear decline throughout selection (−11.0 % per cycle; $r^2 = 0.975$, as regression analysis weighted for the no. of ears per cycle); on the contrary, the mean values of H lines changed mildly from one generation of selection to the other, showing a negligible average response per cycle. As to the divergence between the two selected groups, the difference was 17.1 % at S_1 level and then increased linearly up to S_4 at a rate of 12.1 % per cycle ($r^2 = 0.952$, as weighted regression analysis), with a final divergence of 48.8 % at S_4 level. The different trends shown by L and H lines throughout selection are in contrast with the substantial similarity (as absolute values) of the selection differentials cumulated from S_1 to S_4, which were −68.0 % for L and 62.4 % for H (data not shown). Regarding the two parental inbreds, B73 was nonsignificantly superior to IABO78, whereas the mean value of their cross was markedly superior to the parental mean (−25.0 vs. −40.9 %, respectively), thus indicating dominance for high DG. On the other hand, the mean value of the two parents was rather close to the mean value of the 20 S_4 lines (−40.9 vs. −42.2 %), thus indicating that, when referred to the mean of the two parental inbreds, the whole response to the divergent selection was substantially symmetric, i.e., −25.7 for L and 23.1 % for H. Interestingly, also the ratios between such responses and the cumulated selection differentials (providing an estimate of realized heritabilities) were rather similar, i.e., 0.38 for L and 0.37 for H. Moreover, our results indicate that the large divergence finally obtained between L and H S_4 lines was due to both contributions of the previous recurrent selection (as confirmed by the superiority exhibited by the H lines at S_1 level) and of the subsequent selection in inbreeding herein presented. The DG data of the S_4 lines are also illustrated in Fig. 1a and b, showing, in particular, no overlap among L and H lines, since the worst performing H line exhibited a DG value (−33.8 %) superior to that exhibited by the best performing L line (−55.0 %). Moreover, a marked transgressive segregation was observed, because all the H lines exceeded the best parental inbreds (B73) as well as all the L lines were exceeded by the worst parental inbred (IABO78).

The trends noted for G9.5 were similar to the ones of DG; in particular, from S_1 to S_4 there was a marked decline for the L lines, whereas changes were much milder for the H lines. Consequently, the divergence between H and L lines increased linearly at a sizeable rate (17.4 % per cycle; $r^2 = 0.817$, as weighted regression analysis). As referred to the parental mean, the responses were again symmetric and the cumulated response was −30.3 % for L and 32.6 % for H; considering that the cumulated selection differentials were −51.6 and 58.5 %, respectively, the response/differential ratios were again rather similar (0.59 for L and 0.56 for H). Assuming that these ratios can provide reliable estimates of realized heritability also for G9.5, it follows that these estimates are much higher than the ones for DG, a finding to some extent expected, given the greater complexity of DG versus its component G9.5. A clear distinction without overlaps was found between the H and L groups of selected S_4 lines for G9.5 (data not shown), similarly to that found for DG.

Table 1 Mean values for germination traits of L and H groups of selected maize lines (from S_1 to S_4) and of controls (parental inbreds and their single cross)

Selected lines and controls	DG[a] (%)	G9.5[b] (%)	G25[c] (%)
S_1 L[d]	−36.9	40.2	77.1
S_1 H	−19.8**[e]	56.4**	76.2 ns
S_2 L	−45.2	26.3	71.5
S_2 H	−11.1**	75.7**	86.8**
S_3 L	−59.9	21.0	80.9
S_3 H	−17.5**	72.8**	90.3**
S_4 L	−66.6	3.1	69.8
S_4 H	−17.8**	66.0**	83.8**
Mean (S_4 lines)	−42.2	34.6	76.8
B73	−36.9	35.0	71.9
IABO78	−45.0 ns[f]	31.9 ns	76.9 ns
Mean	−40.9 ns[g]	33.4 ns	74.4 ns
B73 × IABO78	−25.0**[h]	66.3**	91.3**

ns not significant

** Significant at $P \leq 0.01$

[a] Difference between germination traits as G9.5 - G25

[b] Germination at 9.5 °C

[c] Germination at 25 °C

[d] Lines selected for low (L) or high (H) DG

[e] Comparison between L and H selected lines at the same level of inbreeding

[f] Comparison between the two parental inbreds

[g] Comparison between the mean of S_4 L and H selected lines and the mean of the two parental inbreds

[h] Comparison between the mean of the two parental inbreds and their single cross (as bulk of the two reciprocal crosses)

As to G25, the trends observed from S_1 to S_4 were much milder than those previously seen, with a slight tendency to decline, when selecting for L, and to increase, when selecting for H. Hence, a certain tendency to diverge between L and H lines was also noted for this trait, with the H lines being superior to the L lines starting from the S_2 level.

Inheritance of germination traits in germinator (diallel mating design) and ability to predict the crosses performance based on the parental lines performance

With reference to DG, highly significant differences were detected for the majority of the investigated sources of variation (see Supplemental Table S1 for the ANOVA and Table S2 for the mean value of each genotype). The differences among the eight parental lines per se were highly significant and mainly due to the comparison between the two groups of parents L and H (−67.5 vs. −22.2 %; Table 2). The comparison between the overall mean of the eight lines per se and the overall mean of the 56 crosses was not significant, the two means being very close one another. Most of the variation detected among the 56 crosses was due to GCA effects (73.2 %, as ratio between the sum of squares due to GCA and the sum of squares of the 56 crosses; these sums of squares can be evinced from Supplemental Table S1). In turn, the variation determined by GCA effects was mainly ascribable to the component L versus H; in fact, the distinction among parental lines based on GCA effects was rather clear with no overlap (in accordance with lines per se), with all the L lines showing

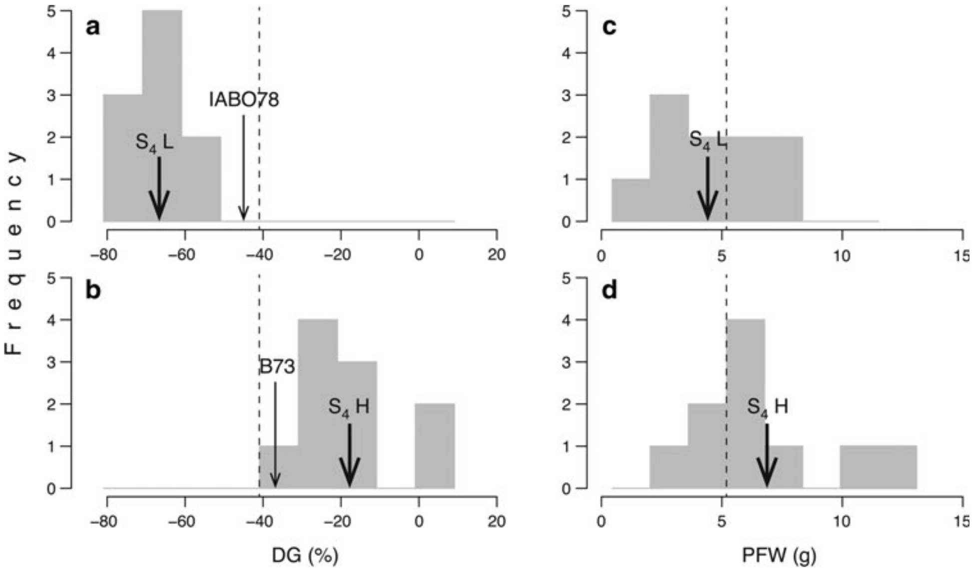

Fig. 1 Distributions of the ten maize S_4 L lines, panel (**a**), and 10 S_4 H lines, panel (**b**), for DG (%) and distributions of the 10 S_4 L lines, panel (**c**), and 10 S_4 H lines, panel (**d**), for PFW (g). *Thick arrows* indicate the means of L and of H selected lines, *thin arrows* indicate (only for DG) the mean of parental inbreds IABO78 and B73, and the *dashed lines* indicate the mean of the two parental inbreds. The class interval is defined as the standard error of the difference (for details on L and H lines see "Materials and methods")

Table 2 Mean values for germination traits of groups of maize parental lines (four selected for L and four for H) and of groups of their crosses

Groups	DG (%)	G9.5 (%)	G25 (%)
Parental lines			
L	−67.5	3.1	70.6
H	−22.2**[a]	58.1**	80.3**
Mean	−44.8	30.6	75.5
Crosses			
L × L	−76.5	4.4	80.8
H × H	−11.5**[b]	75.4**	86.9**
Mean	−44.0	39.9	83.9
L × H	−52.7	33.9	86.6
H × L	−33.0**[c]	47.3**	80.4**
Mean	−42.9 ns[d]	40.6 ns	83.5 ns
All crosses mean	−43.3 ns[e]	40.3**	83.7**

ns not significant

*, ** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively

[a] Comparison between mean value of L and mean value of H groups of parental lines

[b] Comparison between L × L and H × H groups of crosses

[c] Comparison between L × H and H × L groups of crosses

[d] Comparison between mean value of (L × L and H × H) and mean value of (L × H and H × L) groups of crosses

[e] Comparison between mean value of all parental lines and mean value of all crosses

negative effects and all the H showing positive effects (Supplemental Table S3). The variations due to SCA effects and reciprocal effects were significant, too, but of smaller importance (accounting for 5.9 and for 20.9 %, respectively, of the whole variation among crosses). In particular, reciprocal effects were mainly due to crosses involving L × H versus H × L, as the variance between these reciprocal crosses was, on average, ca. four times greater than the variance of the L × L reciprocal crosses and ca. three times greater than the variance of the H × H reciprocal crosses (data not shown). The general reciprocal, or maternal, component was more important than the specific reciprocal component in accounting for the whole reciprocal effects (Supplemental Table S1). Consistently with the presence of significant maternal effects, differences among means of crosses sharing the same female parent were larger than differences among means of crosses sharing the same male parent (Supplemental Table S2). In particular, the maternal effects were always negative for the L lines and always positive for the H lines (Supplemental Table S3), with a high relationship with GCA effects ($r = 0.954^{**}$).

With respect to the alternative analysis for crosses, the comparison L × L versus H × H groups crosses was highly significant and was largely the most important component in accounting for the variation among the four groups of crosses (89.1 %, as ratio between the corresponding sums of squares; these sums of squares can be evinced from Supplemental Table S1). The differences L × L versus H × H crosses (−76.5 vs. −11.5 %; Table 2) was even larger than the difference between L and H lines, owing to the fact that the L × L crosses performed slightly worse than their L parents and the H × H crosses performed slightly better than their H parents. Moreover, the H × L were superior to L × H (consistently with the significance of reciprocal effects), whereas the L × H and H × L crosses were, on average, intermediate between L × L and H × H.

For G9.5, the data were highly associated with those of DG ($r = 0.941^{**}$, considering all 64 genotypes), thus indicating that most of the variation observed for DG could be accounted for by its linear relationship with G9.5. Moreover, G9.5 revealed a significant comparison lines versus crosses, which was not detected for DG (Supplemental Table S1). Concerning G25, no association was found with DG; moreover, a much lower variation than for DG and G9.5 was observed among lines and among crosses (Supplemental Table S1). Nonetheless, most of the sources of variation still proved to be significant, at least partly because the experimental error of G25 was much smaller than the experimental error of G9.5 (Supplemental Table S1); this finding is in accordance with expectation, given the lower level of stress conditions and, hence, of uncontrolled variation for seeds germinating at 25 °C. Also for G25, the mean of the H lines exceeded the mean of the L lines, the mean of the H × H crosses exceeded the mean of L × L and the mean of all crosses exceeded the mean of all parental lines (Table 2).

With respect to the ability to predict the crosses performance based on parental lines performance, for DG (Fig. 2) the linear relationship was highly significant revealing a determination coefficient (r^2) of 62.0 %. This value resulted from the high association between GCA effects and the performance of each line per se ($r^2 = 84.8$ %), and from the fact these GCA effects accounted for 73.2 % of the whole variation among crosses. Some overlaps among the values of crosses of different groups were observed (Fig. 2), with the best L × L crosses exceeding the worst L × H crosses and some H × L crosses exceeding some H × H crosses; however, even the best L × L cross was not able to exceed the worst H × H cross. The regression coefficient was superior to 1 % ($b = 1.36 \pm 0.14$ %), owing to the greater variation among crosses than among mean parental values. As compared to DG, a slightly greater association of the crosses versus the mid-parent performance was noted for G9.5 ($r^2 = 75.7$ %) as a result of both a higher relationship between the GCA effects and the lines per se performance ($r^2 = 97.1$ %) and of a greater importance of GCA in affecting the variation among the 56 crosses ($r^2 = 78.0$ %, as previously seen). In contrast, for G25 the ability to predict the crosses performance based on parental lines

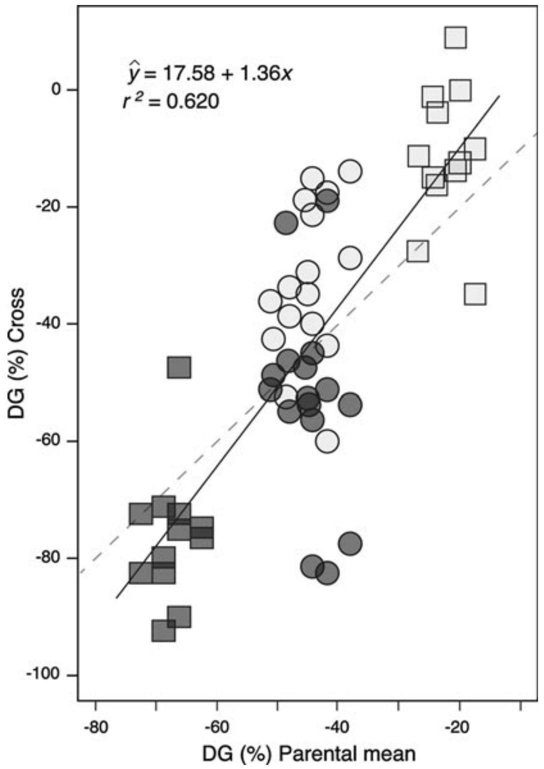

Fig. 2 Regression analysis of each maize single cross on the parental mean for DG (%). *Dark squares* represent L × L crosses and *pale squares* H × H crosses. *Dark circles* represent L × H crosses and *pale circles* H × L crosses. The *dark line* represents the regression line and the *pale dashed line* has slope equal to one

performance was negligible, as a result of both a modest relationship between GCA effects and lines per se and of the modest role played by GCA effects in accounting for the whole crosses' variation.

Associated responses for cold tolerance traits (emergence and early growth) evaluated in the field at early and delayed sowings

For the early sowings, the soil temperatures from sowing to field emergence were on average of 15.6 °C in 2010 and 16.4 °C in 2011, whereas, for the delayed sowings they were 19.5 and 22.6 °C, respectively. Moreover, for the early sowings the minimum temperatures were generally around 11 °C–13 °C and below the threshold of 10 °C for three times in 2010 and once in 2011, whereas for the delayed sowings the minimum temperatures were generally around 17 °C–18 °C and never below 10 °C. Nonetheless, the GDD cumulated from sowing to emergence for the early sowings (91.9 °C as average across the 2 years) was quite similar to that of the delayed sowings (83.8 °C). The ANOVA conducted across the four trials (2 years × two sowing dates) pointed out that differences between the 2 years and the interactions involving them were significant only in few instances (data not shown). In contrast, for all traits there were highly significant differences between sowing dates (Table 3), as the early sowings exhibited a lower mean value for FE (52.6 vs. 73.4 %), SFW (0.47 vs. 0.51 g, despite we tried to harvest seedlings at approximately the same growth level) and PFW (6.27 vs. 9.11 g), whereas showed a longer TE (15.4 vs. 7.7 day). Moreover, highly significant interactions genotypes × sowing dates were detected and for this reason the mean values of the genotypes groups are analysed separately in the two sowing dates across the 2 years (Table 3).

In early sowings, the H lines were agronomically superior to L lines for all traits as they showed a higher mean for FE (56.7 vs. 44.6 %), SFW (0.50 vs. 0.39 g) and PFW (6.87 vs. 4.42 g) and a shorter TE (15.3 vs. 15.6 day). Such associated responses were always symmetric, as pointed out by the non-significance of the comparison between the mean value of all selected lines and of the two parental inbreds. The superiority of the H over the L lines was notable for PFW (54.4 %), because of the positive contributions of both components FE and SFW. Nonetheless, the distinction between the L and H lines even for PFW was not as clear as that for DG and G9.5 and changes of rank among selected lines were noted, with the best performing L lines exceeding the worst H lines (Fig. 1 c, d). The comparison between C4-L and C4-H was not significant for any trait indicating that the previous FS recurrent selection did not lead to appreciable correlated responses for cold tolerance traits in the field.

In the delayed sowings, the differences among the investigated materials were of much smaller size and not significant in most instances; in particular, the comparison between L and H groups of lines was not significant for any trait (Table 3).

The regression analysis of the lines' performance in early field sowing on the mean values for germination traits in controlled environment was significant in all instances (data not shown). However, the ability to predict the field performance based on the linear relationship with the trait observed in germinator was not remarkable, as pointed by the fact that the determination coefficient between G9.5 and TE (showing the strongest relationship) was of just 46 %.

Associated responses for plant, ear and kernel traits evaluated in the field at usual sowings

The difference between years and the line × year interaction were not significant in most instances and, therefore, the mean values across the 2 years were considered. Highly significant differences among the S_4 lines were detected for all investigated traits (Table 4). On the average, the L lines were later, taller and with a greater number of LP; moreover, they showed a greater CD and a lower frequency of

Table 3 Mean values across 2 years of the two groups of selected maize S_4 lines, of the two parental inbreds and of the two populations C4-L and C4-H (as S_0) evaluated in the field at early and delayed sowings

Mean values	FE[a] (%)		TE[b] (day)		SFW[c] (g)		PFW[d] (g)	
	Early	Delayed	Early	Delayed	Early	Delayed	Early	Delayed
S_4 L	44.6	71.6	15.6	7.7	0.39	0.45	4.42	7.83
S_4 H	56.7**[e]	71.9 ns	15.3**	7.7 ns	0.50**	0.47 ns	6.87**	8.24 ns
Mean	50.7	71.8	15.5	7.7	0.44	0.46	5.64	8.03
B73	47.1	73.7	15.2	7.6	0.44	0.54	5.11	9.63
IABO78	48.8 ns[f]	76.6 ns	15.4 ns	7.7 ns	0.44 ns	0.46 ns	5.27 ns	8.46 ns
Mean	47.9 ns[g]	75.1 ns	15.3 ns	7.7 ns	0.44 ns	0.50 ns	5.19 ns	9.04 ns
C4-L	64.6	81.2	15.0	7.6	0.66	0.78	10.28	15.07
C4-H	70.0 ns[h]	79.0 ns	15.1 ns	7.7 ns	0.64 ns	0.74	10.65 ns	14.07 ns
Overall mean	52.6	73.4**[i]	15.4	7.7**	0.47	0.51**	6.27	9.11**

ns not significant

*, ** significant at $P \leq 0.05$ and $P \leq 0.01$, respectively

[a] Field emergence

[b] Average time to emergence

[c] Seedling fresh weight evaluated at the two-leaf stage

[d] Plot fresh weight evaluated at the two-leaf stage

[e] Significance of the comparison between mean values of L and H lines

[f] Significance of the comparison between mean values of parental inbreds B73 and IABO78

[g] Significance of the comparison between mean value of selected lines (L and H) and mean value of the two parental inbreds

[h] Significance of the comparison between mean values of C4-L and C4-H populations

[i] Significance of the comparison between mean values of early and delayed sowings

the *P1-wr* allele, whereas showed lower means for KT score and KW and a higher KM. It is noteworthy that for most of these traits, differences between L and H materials were already significant at S_0 level (i.e., between C4-L and C4-H) and were even increased at S_4 level. In contrast, for KT the difference between L and H materials was notable at S_0 and remained almost the same at S_4, whereas for KW, the difference was negligible at S_0 and highly significant at S_4.

The comparison between the mean value of all selected lines and the mean value of the two parental inbreds was not significant for PS, PH, LP and CC, thus indicating that the associated responses were symmetric. For CD and KM, the comparison was significant even if the parental mean was still included between the L and the H mean values, thus revealing associated responses slightly asymmetric, whereas for KT and KW the parental mean was not included, showing marked asymmetric responses.

Discussion

Responses throughout the selection process for germination traits (germinator)

Different trends for DG were shown throughout selection by L and H lines (i.e., markedly negative for the former group and negligible for the latter), thus suggesting asymmetry of responses. However, responses proved to be symmetric, when the S_4 selected lines were compared to the two parental inbreds, i.e., materials having similar inbreeding levels (as suggested by Falconer and Mckay (1996)). Therefore, the observed asymmetry should be largely ascribed to inbreeding depression, which emphasised the decline of DG when selecting downward (L) and hindered the increase of the trait when selecting upward (H). Also other factors such as the genotype by year interaction (from one cycle of selection to the other) or the presence of limits inherent to the expression of the selected trait, might have contributed to the observed response trends. The divergence at S_4 level between the two groups of selected L and H lines was rather marked; an important contribution to such a divergence was provided by both the former FS recurrent selection and by the subsequent selection in inbreeding. In all likelihood, such a divergence has to be mainly ascribed to the presence of a large genetic variability in the source F_2 population, in turn connected with the rather diverse origins of the two parental inbreds. Moreover, the final symmetric responses (at least when referred to the parental inbreds) indicate that the same effectiveness was attained when selecting for L and for H. Since the selection work was started using a population with intermediate allelic frequencies (0.5), these symmetric

Table 4 Mean values across 2 years for associated plant, ear and kernel traits of the two groups of selected maize S_4 lines, of the two parental inbreds and of the populations C4-L and C4-H (as S_0) evaluated in the field as usual sowing dates

Selected lines and controls	PS[a] (day)	PH[b] (cm)	LP[c] (no.)	CD[d] (mm)	CC[e] (*P*)	KT[f] (score)	KM[g] (%)	KW[h] (mg)
S_4 L	57.3	179	19.9	28.6	0.25	3.6	26.3	253
S_4 H	53.3**[i]	152**	19.0**	23.5**	0.70**	4.5**	20.3**	270**
Mean	55.3	166	19.5	26.0	0.48	4.1	23.3	262
B73	57.5	184	20.5	29.7	1.00	1.0	26.5	269
IABO78	54.5**[j]	154**	18.7**	25.2**	0.00	5.0	22.1**	278 ns
Mean	56.0 ns[k]	169 ns	19.6 ns	27.5*	0.50 ns	3.0**	24.3*	273**
C4-L	51.7	242	20.2	30.5	0.43	2.9	21.6	291
C4-H	50.6 ns[l]	225**	19.7*	29.3*	0.71**	3.9**	20.4*	288 ns

ns not significant

* and ** significant at $P \leq 0.05$ and $P \leq 0.01$, respectively

[a] Pollen shedding as interval from field emergence

[b] Plant height

[c] Leaves per plant

[d] Cob diameter

[e] Cob colour expressed as frequency (p) of the *P1-wr* allele

[f] Kernel type, as visual score from 1 (dent) to 5 (flint)

[g] Kernel moisture

[h] Kernel weight

[i] Significance level of the comparison between the means of the two groups of selected S_4 lines. In case of CC, the significance level was analyzed by Chi-square test, assuming (as null hypothesis) that L and H groups of lines have the same allelic frequencies

[j] Significance level of the comparison between the mean of the two parental inbreds

[k] Significance level of the comparison between the mean of the two groups of selected S_4 lines and the mean of the two parental inbreds

[l] Significance level of the comparison between the sources C4-L and C4-H populations

responses can be accounted for by assuming that most of the available genetic variability was additive (Falconer and Mckay 1996). Another important aspect of the final evaluation (involving S_4 lines and the two parental inbreds) was the detection of a rather clear transgressive segregation, indicative of both the involvement of several loci controlling the selected trait and of the dispersion of the favourable alleles in the two parents. This latter finding is noteworthy as emphasizes the importance of the local parental inbred IABO78 (besides the other well-known parent B73) as donor of favourable alleles for cold tolerance at germination.

Divergent selection affected also the two DG components, as the H lines outperformed the L lines for G9.5 and, though to a smaller extent, for G25. In the FS recurrent selection of Landi et al. (1992), the H populations outperformed the L populations only for G9.5, whereas did not for G25; that substantial lack of difference for G25 between L and H populations was also confirmed in this study when selection in inbreeding began, i.e., when the two groups of S_1 lines were compared. Overall, these findings thus suggest that while the FS recurrent selection acted mainly on adaptive genes (affecting germination only at 9.5 °C), the subsequent selection in inbreeding acted also on constitutive genes, affecting germination both at 9.5 °C and, though at a smaller extent, at 25 °C.

Inheritance of germination traits in germinator (diallel mating design) and ability to predict the crosses performance based on the parental lines performance

The diallel analysis suggested that for DG the largest part of the differences among crosses was due to additive effects, consistently with the importance of GCA and of the large difference between the two groups of crosses L × L and H × H (for details on the genetic interpretation of the comparison among groups of crosses of L and H lines divergently selected from the same source see Lamkey and Hallauer (1986)). This is also consistent with the genetic theory, being expected that intrapopulation selection procedures, as the ones we adopted, are particularly effective in changing allelic frequencies at loci with important additive effects (for details on this issue see Hallauer and Miranda (1988)). The importance of additive effects for DG is also consistent with the findings of a number of other studies on the inheritance of cold tolerance traits at germination all emphasising the prominent role of these effects (Grogan 1970; McConnell and Gardner 1979b; Eagles 1988; Revilla et al. 2000). On the contrary, non-additive effects proved to be of much lesser importance because of the modest contribution of the SCA to variation among crosses and because the comparisons between parental lines versus crosses and (L × L and H × H) versus (L × H and H × L) were negligible. Reciprocal effects proved to be appreciable, too, as pointed out, in particular, by the clear superiority of the H × L crosses versus the L × H. An important contribution to such reciprocal effects was due to the maternal effects. It is interesting to note that the maternal effects for DG were associated with the KT scores of the parental lines per se ($r = 0.756^*$), indicating that the higher the score the higher the maternal effect (i.e., mother plants with flint kernels produced seeds more tolerant to cold). This finding is noteworthy in that it stresses the importance of the flint kernel type for tolerance to low temperature at germination in the material herein investigated. Significant reciprocal effects for germination under cold conditions were observed in many other studies (Pinnell 1949; Grogan 1970; Pešev 1970; Eagles and Hardacre 1979; Maryam and Jones 1983; Kollipara et al. 2002). In particular, Pinnell (1949) and Pešev (1970) found that tolerant × susceptible crosses were more tolerant than the reciprocal susceptible × tolerant crosses. Therefore, our findings are consistent with these previous findings, further emphasising that to have crosses with high cold tolerance at germination both parents should be tolerant, or, in case a susceptible line was involved, it should be used as male parent.

The ability to predict the crosses performance on the basis of the parental lines' performance was of a certain importance for both DG and especially for G9.5, and can be ascribed to the already mentioned great importance of additive effects in determining the differences among the investigated crosses. These findings compare favourably with those of other studies, which showed from poor to moderate predicting capacity for most of the investigated cold tolerance traits (Maryam and Jones 1983; Aidun et al. 1991; Hodges et al. 1997; Bhosale et al. 2007) likely due to the greater importance of non-additive gene actions in those studies. Assuming that the results provided by the four L and the four H parental lines can be extended to the other L and H lines, an important outcome for breeding could arise, implying that selection for cold tolerance at germination on lines per se can also lead to an improvement detectable at the hybrid level. However, since the predicting capacity was not outstanding, the findings also suggest that for assessing the hybrid potential of these lines the final evaluation should be conducted in hybrid combination.

Associated responses for cold tolerance traits (emergence and early growth) evaluated in the field at early and delayed sowings

The early and delayed sowings allowed the attainment of different growing conditions which, based on the recorded soil temperatures, can be considered of moderate cold stress for the former and rather favourable for the latter; this assumption is corroborated by the lower FE percentages and by the longer TE revealed by the early sowings. In the early sowings, associated responses to selection were detected for all the four cold tolerance traits; moreover, these associated responses were symmetric, consistently with the responses obtained for DG and G9.5. These findings are noteworthy considering that both temperature and moisture were kept constant in the germinator during selection whereas were variable in the field; furthermore, in the field we examined traits connected not only with kernel germination but also with the subsequent seedling growth both before and after emergence. To provide a possible explanation for these associated field responses, it should be first considered that throughout the selection work data for G9.5 were taken 20 days after sowing, i.e., when the overall germination was not completed yet, at least in case of the slowest germinating families (Landi et al. 1992). Therefore, it is reasonable to assume that such an approach allowed selection of genotypes having not only lower or higher germination but also slower or faster germination and, hence, lower or greater early vigour. Moreover, a favourable association between percentage germination (or field emergence) at low temperatures and early seedling growth was demonstrated in several studies conducted on different materials grown in different environmental conditions (Mock and Eberhart 1972; Mock and McNeill 1979; Eagles and Brooking 1981; Maryam and Jones 1983; Menkir and Larter 1987). These findings thus suggest that genes which determine a higher germination under stress conditions can also determine a faster seedling growth, hence a higher seedling weight and finally a higher plot production. Hence, such a pleiotropic action can likely result from a developmental relationship due to genes first affecting kernel germination and then these associated traits. Consequently, the pleiotropic control can have further contributed to the achievement of the correlated responses that we observed in the field. At least partly, the associated responses could have also resulted from random genetic drift and/or from linkage between genes controlling DG and genes controlling the associated traits (Falconer and Mckay 1996). In all likelihood, random genetic drift should have not played an important role throughout all selection process, except for the last two inbreeding generations, when the number of selected lines was markedly reduced. Moreover, drift effects are expected to be erratic (because of sampling effects caused by restricted population size), whereas the associated responses for cold tolerance traits in the field were symmetric. Regarding linkage effects, they likely played an important role during the first cycles of our previous FS recurrent selection (as the source population was an F_2) but just a minor role in the subsequent selection in inbreeding, because of the previous six generations of intermating. Therefore, the pleiotropic action of the genes involved in the selection process should be the most important cause for the associated responses herein observed. These field responses compare favourably with the ones of McConnell and Gardner (1979a); they conducted a recurrent selection for cold tolerance based on germination data in controlled environment but then did not get appreciable associated responses in the field, likely because the environmental conditions were not sufficiently severe to allow the detection of correlated responses.

However, the distinction between the L and H lines in the field was not as clear as for DG and the capacity to predict the field performance on the basis of the germinator performance was not remarkable. Hence, we can hypothesize that the variation for cold tolerance in the field was also affected by genes that control the reaction to fluctuating temperatures and moisture levels and thus were not involved by selection for DG. On the whole, these findings indicate that selection for DG had a merit in this breeding project not only because selection could be conducted every year under easily reproducible growing conditions, but also because it led to appreciable responses in the field. However, because the predicting capacity for field performance based on the germinator performance was not remarkable, the suggestion is that the best performing genotypes should be finally identified on the basis of field trials conducted at early sowing.

Associated responses for the four investigated traits were not detected in the delayed sowings characterized by favourable conditions; these findings thus suggest that the associated responses observed in the early sowings were due to the action of genes having mainly adaptive effects.

Associated responses for plant, ear and kernel traits evaluated in the field at usual sowings

Divergent selection for DG also affected several plant, ear and kernel traits whose selection was carefully avoided throughout the selection work. As these responses were generally symmetric or just slightly asymmetric, they should be likely ascribed to selection effects (because of pleiotropy and/or linkage) rather than to random genetic drift. In particular, the H lines were earlier at flowering and at harvesting, with lower LP and smaller PH (these two traits usually tend to be associated with earliness). Moreover, the H lines exhibited a smaller CD, a feature that can have further contributed to a reduced kernel moisture, as ears with thin cobs tend to dry faster (Reid et al. 2010). Interestingly, all these differences tended to be already appreciable at the end of FS recurrent selection and were further emphasised in the subsequent selection in inbreeding. One possible hypothesis accounting for these associated changes is that the greater earliness of H lines allowed them to escape at least partly adverse events in the last part of the ripening process (such as unfavourable meteorological trends and ear rot attacks) worsening the kernel quality and, thereby, germination traits, especially at low temperatures. Corroborating this hypothesis, several studies conducted across different materials and growing conditions consistently revealed that selection for cold tolerance at germination led to an increase of earliness (i.e., Mock and Bakri 1976; Hoard and Crosbie 1986; Sezegen and Carena 2009) and to a reduction of ear diameter (Sezegen and Carena 2009). Moreover, Pinnell (1949), Grogan (1970) and Menkir and Larter (1985) found a negative relationship between lateness and cold tolerance level of the investigated materials.

With regard to CC, selection for L was accompanied by an increase of the allele determining white cob and vice versa with selection for H; the trend was already significant in the two populations C4-L and C4-H, and even clearer at S_4 level. Therefore, this result suggests that there might be a tight linkage between *P1* gene (in bin 1.03) and one or more QTL which, either directly or indirectly, affect germination at low temperature. In this respect, it is noteworthy that Jiang et al. (2011) pointed out that bin 1.03 harbours a QTL for coleoriza length, i.e., a trait connected with seed vigour and playing an important role in promoting germination. Bin 1.03 also proved to harbour QTL affecting several traits for which associated responses were observed, i.e., PS, PH, LP, KM (Landi et al. 2008). Moreover, in the adjacent bin 1.02 Veldboom and Lee (1996) detected a QTL for CD, whereas in bin 1.04 Wang et al. (2007) detected genes for the superoxide dismutase enzyme, which is known to exert a protective action during germination against stress factors (Revilla et al. 2009).

In contrast with the other traits, KT and KW clearly revealed asymmetric responses, thus suggesting an involvement of random genetic drift. It should be noted that in the previous FS recurrent selection the responses for KT followed a symmetric trend, suggesting an involvement of selection effects (Landi et al. 1992). In all likelihood, the initial response was due to loose linkage effects, which vanished as a result of intermating, and/or to the approaching to fixation of one to few major genes affecting both KT and DG; hence, the subsequent changes observed for KT were likely determined by drift. As to KW, an erratic trend of the selection responses was already noted in the previous FS recurrent selection (Landi et al. 1992).

Future prospects

The obtaining of the ten H and ten L lines can represent an important starting point for undertaking further studies on cold tolerance and concerning both basic and applied aspects. With respect to basic studies, an important objective could be the identification of the biochemical and physiological mechanisms involved in the genetic variation for cold tolerance at germination and subsequent growth stages. Selected H and L lines, being divergently developed from the same source, are materials particularly suitable for this objective. In fact, they can be assumed to differ only for those genes controlling the selected trait (besides the ones tightly linked), while sharing the same genetic background. In this respect, it is noteworthy that studies conducted on populations C4-L, C4-H and on the source F_2 (De Santis et al. 1999, 2011; Tampieri et al. 2011) pointed out that the FS recurrent selection brought about associated changes for several mitochondrial properties especially in seedlings grown at 14 °C (i.e., near to the lower growth limit), whereas changes were much less appreciable at 25 °C. The associated changes observed in these studies were always symmetric, thus suggesting the main role of selection in determining such changes.

Moreover, the ten H and ten L lines could be used for further studies aimed at detecting the chromosome regions (quantitative trait loci, QTL) affected by selection, by analysing these lines using molecular markers well distributed throughout the genome. Interestingly, the analysis herein conducted on the genetic marker cob colour (as determined by *P1* gene in bin 1.03) suggests that this chromosome region harbours one or more important QTL affecting either directly or indirectly cold tolerance at germination. Moreover, the six cycles of intermating before selfing generations should increase the genetic resolution of polymorphism in the 20 selected lines. Knowing the chromosome regions affected by selection could be also helpful for the development of new breeding strategies (such as marker assisted selection) aimed at enhancing the efficiency for cold tolerance selection.

Future applied studies on breeding for cold tolerance could be conducted just as unidirectional selection aimed at improving the trait performance and could involve the H lines as source of important genes for improving elite inbred lines and/or populations. Moreover, the selected trait could be G9.5 rather than the more complex DG, whose utilization was functional to the divergent selection procedure. Using just G9.5 should simplify selection as well as improve its effectiveness due to the higher heritability of the trait and to the higher degree of association with cold tolerance traits in the field as compared to DG.

Conclusion

The accomplishment of the present study allows us to draw the following conclusive remarks.

1. Selection in inbreeding was effective, leading to an appreciable direct response for DG in both L and H directions (symmetric response). This selection in inbreeding acted not only on adaptive genes but also on constitutive genes affecting germination both at low temperature and, though more mildly, at the higher temperature.
2. For both DG and G9.5, differences among crosses involving L and H selected lines were largely due to additive effects. The reciprocal effects were also of some importance, as indicated by the superiority of the H × L crosses in comparison with the corresponding L × H. The ability to predict the hybrid performance based upon the parental lines performance was appreciable even if not remarkable.
3. Appreciable associated responses to selection were observed in the field at early sowings, but not at delayed sowings, thus indicating that selection for DG involved genes exerting an adaptive action in the field. The relationship between the data collected in germinator and in the field at early sowing, however, was not outstanding, thus suggesting that the final selection for cold tolerance should be made in early sown field trials.
4. Several associated responses were also found for plant, ear and kernel traits. Most of such responses were likely attributable to selection effects, and ascribable either to pleiotropic actions (as for earliness, plant size and cob diameter) or to tight linkage (as for cob colour).
5. Finally, the L and H lines developed in this study can represent a valuable material to undertake both basic and applied studies. The basic studies can be aimed at improving our knowledge on the biochemical and physiological bases of cold tolerance traits and at detecting the chromosome regions affecting such traits. The applied studies can concern unidirectional selections, involving the H lines as donor of important genes and using only G9.5 as selection criterion.

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