Cold tolerance in two large maize inbred panels adapted to European climates

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Abstract

Maize (*Zea mays* L.) for northern growing areas requires cold tolerance for extending the vegetative period. Our objectives were to evaluate two large panels of maize inbred lines adapted to Europe for cold tolerance and to estimate the effects of cold-related traits on biomass production. Two inbred panels were evaluated for cold tolerance *per se* and in testcrosses under cold and control conditions in a growth chamber and under field conditions. Comparisons of inbreds and groups of inbreds were made taking into account the SNP-based genetic structure of the panels, and the factors affecting biomass production were studied. Eight flint and one dent inbreds with diverse origins were the most cold tolerant. The most cold tolerant dent and flint groups were the Iodent Ph207 and the Northern Flint D171 groups, respectively. The relationships between inbred *per se* and testcross performance and between controlled and field conditions were low. Regressions with dry matter yield in the field as dependent variable identified plant height (R^2=0.285) as the main independent variable, followed by quantum efficiency of photosystem II (R^2=0.034) and other traits with minor contributions. Cold tolerance-related traits had low and negative effects on dry matter yield. Models intending the prediction of final performance from traits scored in early developmental stages are not expected to be precise enough for breeding. For improving cold tolerance, inbreds released from crosses among the No Iodent group and the Northern Flint group may show high combining ability, as well as between both groups and the Northern Flint D171 group.

Key words: maize, cold tolerance, abiotic stress, germplasm.

Maize (*Zea mays* L.) originated in the tropical highlands of America and is currently grown in northern growing areas all over the world, where temperatures are well below the optimum for this crop. Such wide adaptation is the result of either selection for a short vegetation period in order to escape cold stress or improvement of cold tolerance for surviving under cold conditions. The first strategy is associated with low yields, while the second should allow long vegetation periods that potentially increase yield (Revilla et al., 2005; Strigens et al., 2012). Indeed, early sowing of maize allows for a longer vegetation period that can potentially increase yield and stability, and the probability of escaping summer drought stress (Kucharik, 2006). This is particularly true in some temperate areas, where springs are cold and rainy and summers are hot and dry. But early sowing in temperate areas requires cold tolerance and, consequently, the interest of breeders in cold tolerance is increasing (Darkó et al., 2011; Frascaroli and Landi, 2013; Revilla et al., 2005; Strigens et al., 2012, 2013). In the northwest of Spain, a breeding goal would be to advance maize sowing two weeks, i.e. from May to mid April, because there are no late frosts. However, in northern areas the gain could be just a few days.

The main handicap for breeding programs intending to improve cold tolerance in maize has been the narrow genetic base for this trait (Greaves, 1996; Revilla et al., 2005). Rodríguez et al. (2010) evaluated for cold tolerance the largest collection of germplasm so far published, the European Union Maize Landrace Core Collection (EUMLCC). Their results were not encouraging because most cold tolerant populations from the EUMLCC were not more cold tolerant than the checks. Actually, they were similar to the improved populations and checks already known, including commercial checks and the best cold tolerant hybrids. Furthermore, their agronomic performance was not at the level of commercial standards.

Apparently classical maize breeding for cold tolerance has reached a ceiling (Revilla et al., 2005). The incorporation of new techniques, such as molecular markers, has not

solved the problem so far (Leipner et al., 2008). Although several studies have identified QTLs for cold tolerance, most of them were not reliable enough for marker-assisted selection and were associated with secondary traits such as chlorophyll content or function (Jompuk et al., 2005; Rodríguez et al., 2008). Actually, the main effect of cold temperatures is the reduction of chlorophyll synthesis (Rodríguez et al., 2013). However, some QTLs were consistent when clearly distinct parents of the segregating population under study were used (Presterl et al., 2007). Genome selection has recently been suggested by Strigens et al. (2013), who carried out genome-wide association mapping for cold tolerance in a collection of maize inbred lines. They obtained 19 highly significant association signals, explaining between 5.7 and 52.5% of the phenotypic variance for early growth and chlorophyll fluorescence. They propose the use of whole genome prediction approaches rather than classical marker assisted selection to improve the chilling tolerance of maize.

Other major obstacles for breeders are that cold tolerance has large experimental errors, a strong genotype by environment interaction, and a complex genetic regulation (Revilla et al., 2000, Strigens et al., 2013). Moreover, evaluations for cold tolerance are not accurate enough for a precise discrimination of cold tolerance. This is due to two facts. First, field trials are not reliable because the occurrence of cold temperatures in a concrete year is not guaranteed, and second, controlled conditions in growth chambers are not clearly associated to real conditions in the field. Frascaroli and Landi (2013) pointed out that breeding programs for cold tolerance are efficient for this trait, but may also yield some undesirable associated response in other agronomic traits. As an example, high cold tolerance is generally associated with early flowering, short plants or fewer leaves. Also, several authors have reported a poor relationship between cold tolerance and agronomic traits, e.g. early vigor is neither positively associated with grain yield (Revilla et al., 2000) nor with dry matter accumulation (Leipner et al., 2008). Strigens et al. (2012) found weak associations between early growth and dry matter accumulation, although the association

was larger with biomass accumulation before flowering. The associations between early traits and final yield depend both on the circumstances of the experiments and the germplasm involved. Therefore, large sets of genotypes thoroughly evaluated should provide more reliable estimates of the relationships between cold tolerance traits under controlled and field conditions.

Cold tolerance is an important challenge that must be faced with the powerful tools available nowadays. First of all, the breeding base should be enlarged as much as possible by screening larger collections of germplasm; besides, the evaluation methods should be more precise, involving cold and control conditions as well as field trials, and the traits for which to select should be carefully chosen in order to accurately discriminate tolerant from susceptible genotypes. Breeders generally assume that European Flints are more cold tolerant than Corn Belt Dents, a belief that has been experimentally demonstrated to some extent (Frascaroli and Landi, 2013; Strigens et al., 2013). Several efforts for searching sources of cold tolerance have been carried out in limited collections of European germplasm (Lee et al., 2002; Mosely et al., 1984; Revilla et al., 2000; Rodríguez et al., 2010; Semuguruka et al., 1981; Verheul et al., 1996). Given that most previous reports have faced the problem by using limited resources, we believe that a global evaluation of large collections of genotypes for cold tolerance is still lacking. Therefore, the objectives of this research were to thoroughly evaluate cold tolerance in two large panels of maize inbred lines adapted to European conditions *per se* and in testcrosses, and to estimate the effects of cold tolerance-related traits on biomass production.

Material and Methods

Plant material: inbred lines of the flint and dent panels and their genetic structure

Two panels of maize inbred lines adapted to European conditions, consisting of 306 dent and 292 flint inbred lines, were evaluated *per se* (Appendix 1) and in testcrosses (Appendices 2 and 3). The panels were built from the collections of Spanish, French, and German breeders involved in this research. They come from Western Europe and the USA. The inbreds are public and have been released throughout the history of maize breeding. The inbreds were chosen based on diversity and adaptation to the range of European conditions. Although the range of environmental conditions is wide, the European area represented here has some common characteristics; namely it is temperate with short growing cycles and cold springs. Seed of the inbreds *per se* was multiplied by each station and sent to Pontevedra (Spain) for evaluations. The dent inbreds were crossed to the flint tester UH007 and the flint inbreds to the dent tester F353 in a winter nursery in 2010.

We used the genotyping, diversity, and relationship matrices of Rincent et al. (2012). These authors genotyped the same diversity panels with the Illumina MaizeSNP50 BeadChip described by Ganal et al. (2011) that includes 49,585 SNPs. These authors did not use the data from individuals or markers with a missing rate above 0.1 and 0.2 respectively, or with a heterozygosity above 0.05 and 0.15, respectively. In total, 261 flint lines and 261 dent lines passed the genotyping and phenotyping filter criteria after removing possible contaminations.

Growth chamber trials

The cold chamber was built inside a laboratory with modulated panels, isolated with injected polyurethane. The 598 inbreds *per se* from the flint and dent panels, six checks (C105, CO109, D152, EA1027, F816, FP1) repeated in both panels, and their testcrosses were evaluated for cold and for control conditions in consecutive runs of the cold

chamber. In each trial entries were grown in a single 20 m^3 growth chamber following a randomized complete block design with six replications. Each panel (*per se* or in testcrosses) was evaluated in the chamber for each treatment, but the confounding effects for blocks were limited because the six repetitions were together. Confounding effects are always present in this kind of trials because it is not possible to evaluate under cold and warm conditions in the same space and time. Confounding effects increase the error term and reduce the power to identify significant effects.

Maize seeds were planted in seedbeds filled with sterilized peat (Gramoflor GmbH & Co. KG, Vechta, Germany) with one kernel per plot (altogether six plants per inbred or testcross were used in each growth chamber trial). Each seed was sown in a cell with a surface of 3 cm x 2.5 cm and 5 cm depth; therefore, average distances were 3 cm between seedlings within each column and 2.5 cm between seedlings within each row. The experiments were watered after planting; afterwards the trials were watered as needed. Temperature conditions were set up at 14 ºC/14 h light and 8 ºC/10 h dark for the cold experiments and 25 ºC/14 h light and 20 ºC/10 h dark for the control experiments. The cold conditions were chosen for screening for cold tolerance alone, removing any other stress that the seed can find in the field. Cool light was provided by seven VHO (very high output) fluorescent lamps per shelf with a photosynthetic photon flux (PPF) of 228 μmol m^{-2} s^{-1}. Distance between shelf and fluorescent lamps was 0.5 m.

In both the inbred *per se* and the testcross trials, data were recorded at the three-leaf (V3) stage to assure that plants were at the same developmental stage. Four cold-tolerance related traits were recorded: number of days from sowing to emergence, relative leaf chlorophyll content (SPAD) using a hand-held CCM-200 Chlorophyll Content Meter (Opti-Sciences, Tyngsboro, Massachusetts, USA), quantum efficiency of photosystem II (ΦPSII) recorded using an OS-30p Chlorophyll Fluorometer (Opti-Sciences, , Tyngsboro, Massachusetts, USA), and dry weight of the testcrosses. For inbreds *per se*, instead of dry

weight we scored early vigor using a visual scale from 1=weak plants to 9=vigorous plants. For the traits recorded at the three-leaf stage, each trait was taken simultaneously for all plants. Indeed, simultaneous measurements of a trait in several plants produce distortions as does the measurements of the same trait in different days. We choose the day for measurements based on the average development of the trial as a whole, as most breeders do because it is more precise to carry out simultaneous harvests than picking plants from the plots individually as they reach the appropriate stage.

Field trials

The main trials were carried out in the growth chamber, with field trials as references for testing the performance of the flint and dent panel testcrosses in the field. These field trials were used also for comparing the evaluation under control conditions with a real control in the field under normal conditions.

The 595 testcrosses (306 dents and 289 flints) were evaluated in field trials during two years (2010 and 2011) in Pontevedra, Spain (42º 24' N, 8º 38' W, 20 m above sea level). This location has a humid climate with an annual rainfall of about 1600 mm. The soil is a humic cambisol with a sandy loam texture (53% sand, 28% silt, 18% clay). A previous analysis showed that the soil had 12.3% moisture, 9% organic matter, and pH=6.6. The weather in this location during these years was favorable for maize growth; therefore, field trials were carried out under optimum conditions. The field trials were planted on 19 May 2010 and 12 May 2011. The flint and dent panels were evaluated in adjacent trials following a modified augmented design. The experimental design for evaluations of the 289 flint testcrosses involved 17 blocks (eight included the early testcrosses and nine included the late testcrosses) with 20 entries per block and a total of 340 experimental plots; in each block 17 entries were unrepeated and three were repeated once elsewhere throughout the other blocks; the 51 replicated testcrosses were used for estimating the experimental error.

Likewise, the 306 dent testcrosses were evaluated in 18 blocks (nine included the early testcrosses and nine included the late testcrosses) with 20 entries per block and a total of 360 experimental plots; in each block 17 entries were unrepeated and three were repeated once elsewhere throughout the other blocks; the 54 replicated testcrosses were used for estimating the experimental error.

Each experimental plot consisted of one row with 27 hills per row and two grains per hill. Rows were spaced 0.80 m apart and hills were spaced 0.14 m apart. Hills were thinned to one plant, achieving a final plant density of approximately 90,000 plants ha^{-1}. Currently accepted management and cultural practices were used in both trials and trials were harvested at physiological maturity. We measured percentage of emergence, early vigor, dry weight of 5-week old plants, percentage of dry matter in 5-week old plants, relative leaf chlorophyll content and ΦPSII, plus the vegetative traits days to silking, days to pollen shedding, plant height, dry matter yield, and dry matter content. Data were recorded on 3 plants per plot for traits measured on individual plants. Leaf chlorophyll content and ΦPSII were taken in the central hours of the morning in sunny days.

Statistical analysis

To analyze growth chamber trials, analyses of variance over control and cold conditions were performed for dent inbreds, flint inbreds, dent testcrosses, and flint testcrosses separately. The combined analysis of dent and flint inbreds *per se* was made considering repetitions and inbreds as random effects, while the population effect (flint or dent) was fixed. The analysis of each panel under a given condition considered repetitions as random effects while inbreds or testcrosses were considered fixed effects in order to compare the performance of inbred lines for identifying cold tolerant inbreds. Least squares means for inbreds and testcrosses were calculated for each trait. The analyses of variance were made using the Mixed procedure of SAS (SAS Institute, 2008). Mean comparisons were made for each trait individually with independent cutting levels for

global comparisons of cold tolerance; in other words, the inbreds that did not differ significantly from the best inbred for all traits were considered the most cold tolerant and conversely, the lines that were not significantly different from the worst inbred for all traits were considered the most cold susceptible.

Analyses of variance for the field trials over years were performed for each trait. The testcrosses of the flint and dent panels were analyzed separately; the source of variation 'years' was considered random and 'genotype' fixed. Least square means were estimated for each trait. The analyses were made using the GLM procedure of SAS (SAS Institute, 2008).

Correlation analyses between traits were calculated by using the CORR procedure of SAS. Besides, regression analyses were made by using dry matter yield at harvest or dry matter yield five weeks after planting (both in the field) as the dependent variables; all other previously recorded traits were included as the independent variables. For these regression analyses, we used the REG procedure of SAS with the stepwise method.

In order to find a comprehensive method for classifying genotypes as tolerant or susceptible to cold conditions, principal component analyses were performed for inbreds *per se* in cold conditions using the least squares means of days to emergence, ΦPSII, chlorophyll content, and early vigor. These analyses were made for both flint and dent panels after standardizing the traits. In order to use the principal components for classifying the inbreds as cold tolerant or susceptible, we calculated an index of susceptibility by modifying PC2, i.e. combining only days to emergence and early vigor. Therefore, inbreds with scores on PC1 ≥ 0 and PC2 ≤ 0 are cold tolerant.

Rincent et al (2012) used the markers developed from sequences of the founder lines of the US nested association mapping population (PANZEA SNPs; Gore et al., 2009) to estimate Nei's index of diversity (Nei, 1978) and relationship coefficients. Nei's index of diversity of each SNP was calculated and averaged over the genome for estimating

genotype diversity in the two panels (Appendix 4). Rincent et al (2012) characterized the panels using molecular data with the structure analysis with 'admixture' (Alexander et al., 2009) from K=2 to K=8. One inbred was classified in a group if $Q_K > 0.60$ for any cluster for K = 2 and if $Q_K > 0.50$ for K=3, 4, 5, 6, 7, and 8; the other inbreds were classified as "mixed" (Appendix 4) (Q=estimated membership coefficients for each inbred in each cluster, K=number of clusters). Both dent and flint inbreds were classified in K=2, 3, 4, 5, 6, 7, and 8 groups based on the genetic structure and pedigree knowledge (Rincent et al., 2012), with several possible alternative classifications of inbreds. Differences between group means were calculated for all classifications. This analysis was conducted only for inbreds *per se* and cold and control conditions were analyzed separately.

Results and discussion

Evaluation of inbreds per se: flints vs. dents

In the present study the analyses of variance over cold and control environmental conditions in the growth chamber confirmed that environments were significantly different and that the genotype × environment interactions were significant ($P < 0.05$) for the four traits in both flint and dent panels (results not shown). In the combined analyses, differences among inbreds were significant ($P < 0.05$) for chlorophyll content and early vigor, and not significant for days to emergence or ΦPSII both in the flint and dent panels. The analyses of variance by panel and growth condition showed that the differences among dent inbreds were highly significant ($P < 0.001$) for all traits under both cold and control conditions. The differences among flint inbreds were also significantly different ($P < 0.001$) for all traits except days to emergence with $P < 0.01$ under cold conditions and $P < 0.05$ under control conditions.

The dent inbreds evaluated *per se* germinated earlier and had a higher early vigor than the flints, but the flints had more chlorophyll and a higher ΦPSII than the dents (Table 1, Appendix 1). When performances under control and cold conditions were compared, flints had a larger increase in days to emergence and smaller reductions in chlorophyll content and ΦPSII than dents, while the decrease in early vigor was similar between dents and flints (Table 1). Flints and dents did not behave consistently as differentiated groups for cold tolerance. However, according to the literature, European flints and dents have diverse origins and history, and they are two clearly distinct genetic groups. Both panels evaluated contain inbreds that are adapted to European conditions; most inbreds from the dent panel or their parents originated from the US Corn Belt and were introduced in Europe during the second half of the 20th century, while the ancestors of the flint inbreds probably were introduced along the four preceding centuries. Previous studies have shown that within the flints, there are at least two main origins of European

genotypes (Rebourg et al., 2003; Revilla et al., 2003). First, maize from Central America was introduced through the south of Spain and was the origin of the Mediterranean maize; second, several North American flint populations were introduced through the European Atlantic coast and were the origin of the European flints. While the Mediterranean maize is not expected to be cold tolerant, the European flints are believed to be more tolerant to cold conditions because they are more adapted to northern latitudes than the Corn Belt Dents. Rodríguez et al. (2010) concluded that the European flints had some potential value for improving cold tolerance of maize. Other authors have found results supporting this conclusion because the flint kernel phenotype was associated to cold tolerance (Frascaroli and Landi, 2013). Strigens et al (2013) concluded that flint and dent inbreds adapted to European conditions have diverse mechanisms underlying that adaptation. Revilla et al. (1998) pointed out that the origin of a variety in a cold region does not warrant cold tolerance, because genotypes with short growing cycle escape cold temperatures when planted late.

Evaluation of inbreds per se: variability within panels

The genetic diversity and the genomic relationship matrix showed that the diversity was higher in the dent than in the flint panel (Rincent et al. 2012). Most of the coefficients of similarity between inbreds were low, but there were some pairs of closely related inbreds. In the present study we made sets of inbreds with close genetic relationships within each group (see below). There was no consistency for cold tolerance within each set except for those sets with few inbreds that were all cold susceptible. However, most inbreds were cold susceptible and, therefore, most sets were also cold susceptible, except one mixed set that had four cold susceptible inbreds (UHF084, UHF105, UHF070, and UHF082) and six cold tolerant inbreds (UHF093, UHF098, UHL058, UHF023, UHF091, and UH006).

Based on the genetic structure and pedigree knowledge, several alternative classifications of inbreds are possible (Appendix 4). Assignments of flint and dent inbreds to the different groups for scenarios with K=2 to K=8 are shown in Appendix 4. In the dent panel, the best discriminating ability was obtained for K=6 groups which were designated as Iodent Ph207, Iodent UH4068, Lancaster Oh43, No Iodent, other No Iodent F252, and Stiff Stalk. Among these, the Iodent Ph207 group was the most cold tolerant and the Stiff Stalk group the most cold sensitive (Table 2). Cold tolerance was similar for the Iodent UH4068 and the Lancaster Oh43 groups.

For flint inbreds the results are more clear for K=6 groups which were designated as FV7, Northern Flint (NF), NF D171, No NF, Southern EC18, and Southern Flint from open pollinated varieties (Table 2). The most cold tolerant group was NF D171 (except for chlorophyll content), and the most cold susceptible was No NF; the Southern EC18 group had the highest chlorophyll content and ΦPSII under cold conditions.

When looking at individual inbreds, the nine most cold tolerant inbreds (those that were simultaneously not significantly different from the best inbred for the four traits) were EV18, UHL058, CH34, UHP024, EC51, F364, FV71, F471, and UHF043 (Table 3). Eight of them were flint and one was dent, supporting the conclusion that European Flints are more cold tolerant than Corn Belt Dents. In this outstanding group three inbreds were from Germany, three from France, two from Northern Spain, and one from Switzerland. Therefore, European flint material is at large promising for finding sources of tolerance to cold conditions. Among the 92 inbreds that were not significantly different from the best inbred for three traits, flints and dents were similar as there were 47 flints and 45 dents. The inbreds with less than nine days from sowing to germination under cold conditions were D09, EV23, EZ53, F922, A310, and UHF018, although many others were not significantly different (Appendix 1). Inbreds with a chlorophyll content above 10 were EV18, EP1, FV75, EC237, and EP66. ΦPSII was over 0.650 for EC237, UN2065, EC248,

UHP042, EV18, EC242C, PB57, and F471. Finally, the early vigor score was higher than 5 for EV18, FV353, UH2551, FV335, PHT77, F816, D06, UH6145, CH16.1-295, UHP033, CH113-379, EC326A, B111, FC1571, ML606, F922, EP74, EP27, F362, C105, and EC237. Even though the inbreds come from a wide range of latitudes from Spain to Germany, there are no clear patterns of geographical variability. However, if we classify the inbreds of Appendix 1 in five groups (those with high performance for 4, 3, 2, 1, and 0 traits) most of the Spanish or French inbreds are in the group with 1 outstanding trait while most of the German inbreds are in the group with 2 outstanding traits, which suggests natural selection for adaptation to cold environments in Germany during inbred development. On the other hand, most inbreds with high chlorophyll content or high ΦPSII were from Spain, perhaps as a consequence of the traditional focus on selection for early vigor and dark green color in northern Spain.

Evaluation of Iibreds per se: principal component analysis

The principal component analyses for both panels evaluated under cold conditions identified two principal components (PC) explaining 50% and 29% of the variability, respectively. PC1 had a negative contribution for days to emergence (eigenvector = -0.33) and a positive contribution for chlorophyll (0.55), ΦPSII (0.56), and early vigor (0.53). PC2 had a positive contribution for days to emergence (eigenvector = 0.73), chlorophyll (0.42), and ΦPSII (0.38), and a negative effect for early vigor (-0.38). Therefore, PC1 is an index of cold tolerance while PC2 represents plants that grow less and slower in cold conditions than in normal conditions, but with more chlorophyll and a higher photosynthetic efficiency.

In the principal components, inbreds with scores on PC1 ≥ 0 and PC2 ≤ 0 are cold tolerant (Figure 1, Appendix 5). Considering inbreds with PC1≥ 2 and PC2 ≤ -1, the most cold tolerant flint inbreds were EV18, UHL058, F471, UHL048, EC51, F364, CO255, CH34, UH006, H113-379, UHF093, and UHF091, and the most cold tolerant dent inbreds

were UHP024, LH85, EP74, FV335, PHT77, UH2551, EC140, UHP033, EZ19, and Pa374. These selected groups based on the principal component analysis agreed reasonably with the previous selection based on mean comparisons among groups (K=6), although the agreement between both criteria was better for the flints than for the dents. Furthermore, the dent inbreds had on average lower scores in PC1 than the flint inbreds; therefore, we expect that dents would be more cold susceptible than flints.

Among the inbreds of these panels, some were included in large flint and dent half sib panels (Bauer et al., 2013). Some of the parents of the dent half sib panel were cold tolerant based on our present data (Table 3), namely the dent inbreds D06, Mo17, and UH304, and the flint inbreds UH007, D152, and UH006. Considering that the common parent of the flint half sib panel (UH007) was cold tolerant, the half sib panels provide valuable material for studying the genetics of cold tolerance in segregating populations.

Combining the information from the groups and the individual inbreds, we found that among the nine best inbreds (Table 3) the most cold tolerant dent inbred (UHP024) belongs to the most cold tolerant group (Iodent Ph207) (Appendix 4). On the other hand, among the eight most cold tolerant flint inbreds, only two (UHL058 and UHF043) belong to the most cold tolerant group (Northern Flint D171), while CH34 and F364 are Northern Flint, EV18, FV71, and F471 come from southern open pollinated varieties, and EC51 belongs to the Southern EC18 group. However, differences among groups for cold tolerance are not very clear and both cold tolerant and non-tolerant lines exist in most groups. Considering the most cold tolerant flint and dent inbreds based on the principal component analyses, five out of the 12 flint inbreds belong to the Northern Flint D171 group (UHL058, UHL048, UH006, UHF093, and UHF091), three to Northern Flint (F364, CH34, and CH113-379), two to the Southern open pollinated varieties (EV18 and F471), one to the Southern EC18 group (EC51) and one to the group FV7. Among the dent inbreds, only two (UHP024 and UHP033) of the most cold tolerant 10 inbreds belong

to the most cold tolerant group (Iodent Ph207) while the other eight cold tolerant inbreds (LH85, EP74, FV335, PHT77, UH2551, EC140, EZ19, and Pa374) belong to the cold susceptible group No Iodent. The concordance between the different analyses is better for the flint than for the dent inbreds. These lacks of agreement suggest that the most efficient way for identifying cold tolerant genotypes is the comparison among genotypes per se itself.

Evaluation of testcrosses

The analysis of variance for testcrosses combined over panels and environments in the growth chamber revealed significant differences among testcrosses for chlorophyll and biomass in the V3-stage. The genotype × environment interaction was not significant for days to emergence and for ΦPSII in both dent and flint panels (results not shown). For chlorophyll content, both the differences between testcrosses and the genotype × environment interaction were significant in both panels. Finally, for biomass in the V3-stage, differences among testcrosses were significant but the genotype × environment interaction was not significant in both panels.

Separate analyses of variance for each panel and environmental condition showed significant differences for chlorophyll content and for biomass in the V3-stage in all cases (results not shown). Differences were significant for ΦPSII in all cases except for the flint panel in control conditions. For days to emergence, differences were significant only under cold conditions for both panels. Differences between inbreds *per se* were more often significant than between testcrosses, probably because of reduced genetic variance in testcrosses compared to inbreds. Besides, the flint tester UH007 was also evaluated as inbred *per se* showing a good performance under cold conditions except for chlorophyll content. Therefore, it was difficult to find differences between testcrosses in the dent panel. The dent tester F353 was not evaluated *per se* so we do not know its performance

under cold conditions. Furthermore, as both panels were crossed to different testers, comparisons between flints and dents are not possible.

In the cold chamber, the testcross with highest cold tolerance was EC35G × F353 and did not significantly differ ($P < 0.05$) from the best testcrosses for any of the four traits (Table 4). EC35G was also among the inbreds with high cold tolerance *per se* (Table 3). There were other inbreds that were cold tolerant both *per se* and in testcrosses, namely CH34, CH16.1-295, UHP017, and F670 (Tables 2 and 3). However, most inbreds with high cold tolerance *per se* did not produce a cold tolerant testcross and vice versa. There has been some controversy on the issue of predicting hybrid cold tolerance from inbred performance. Maryam and Jones (1983) stated that hybrid performance could be predicted from their parents, Hodges et al. (1997) found that it is not possible to reliably predict hybrid cold tolerance from the parents' performance, and Revilla et al. (2000) stated that their results partially support the notion that hybrid cold tolerance can be predicted from the performance of the inbred parents. Presterl et al. (2007) found consistent QTLs for cold tolerance in inbreds and their testcrosses, suggesting that cold tolerance of inbreds and hybrids was genetically associated. This strongly depends on the genotypes evaluated, the testers, and the methods being used. Previous reports have shown that it is not always possible to reliably predict hybrid cold tolerance from inbred performance (Revilla et al., 2005). The inheritance of cold tolerance is complex and variable. For instance, McConnell and Gardner (1979) found epistatic, additive, and dominance gene effects for germination under cool conditions, and mainly additive and dominance effects for seedling vigor in crosses among three warm-season and three cool-season inbreds. Eagles (1982) found additive and dominance effects for rate of seedling growth, and Revilla et al. (2000) concluded that the genetic regulation of cold-tolerance traits conformed to an additive-dominance model in a diallel among European flints.

We evaluated testcrosses from the flint and the dent panels in separate but adjacent field trials for two years in the field. In the flint panel, differences were not significant among testcrosses and the year × testcross interaction was not significant for emergence, early vigor, dry weight of 5-week old plants, dry matter content in 5-week old plants, ΦPSII, days to silking, and days to pollen shedding (data not shown). Differences between flint testcrosses were significant for leaf chlorophyll content, plant height, dry matter yield, and dry matter content. The year × testcross interaction was only significant for plant height in the flint panel. In the dent panel, differences between testcrosses were not significant for percentage of emergence, dry weight of 5-week old plants dry matter content in 5-week old plants, ΦPSII, days to silking, and days to pollen shedding. Differences between dent testcrosses were significant for early vigor, leaf chlorophyll content, plant height, dry matter yield, and dry matter content. The year × testcross interaction was not significant for dent testcrosses. All testcrosses were evaluated under favorable conditions in the field. The weather conditions were fine for growth at early stages in both years and as a consequence, testcrosses did not show significant differences for early traits such as percentage of emergence or dry weight of 5-week old plants. Although field conditions are unpredictable, evaluations for cold tolerance could be more informative when sown earlier or in cooler environments.

Relationships among traits

Simple correlations between traits were calculated using means of inbreds and testcrosses separately for both panels and all environments. Most correlations were low and only the significant correlations with values above 0.5 are shown and discussed here. Highly significant ($P < 0.01$) correlations above 0.5 were detected for chlorophyll content and ΦPSII of inbreds in cold conditions (0.61), early vigor and dry matter in 5-week old plants of testcrosses in the field (0.52), days to pollen shedding and silking of testcrosses in the field (0.95), and plant height and dry matter yield of testcrosses in the field (0.53).

Analyzing separately flint and dent panels, correlations were above 0.5 for chlorophyll content and ΦPSII of inbreds in cold conditions (flint: 0.56, dent: 0.64), early vigor and dry matter in 5-week old plants of testcrosses in the field (flint: 0.55, dent: 0.50) and also for testcrosses under control conditions but only in the flint panel (0.58), days to pollen and silking of testcrosses in the field (flint: 0.94, dent: 0.92), chlorophyll content and dry matter content in 5-week old plants of testcrosses in control conditions (0.58 in flint) and in cold conditions (0.50 in flint), and dry matter content in 5-week old plants of testcrosses in cold and in control conditions (0.51 in flint). Within the dent panel, the only noteworthy correlation was between early vigor and percentage of emergence of inbreds in cold conditions (-0.52). None of the correlations for any testcross trait measured in the field and under cold conditions was above 0.5, showing that the evaluations in the growth chamber were not clearly associated with performance in the field. However, evaluations in the field were closer to optimum than to cold conditions and the evaluation of testcrosses provides limited opportunities for differentiating genotypes in the cold chamber due to reduced genetic variance. Several authors have shown that correlations between performance under cold conditions and in the field were positive when growing conditions in the field were colder than in our experiments (Hodges et al., 1995; Bhosale et al., 2007).

In order to check the effect of the early traits on dry matter yield, regression analyses were carried out considering dry matter yield of testcrosses in the field as the dependent variable and the traits recorded in the growth chamber as the independent variables. Most of the latter had significant effects on dry matter yield and were consistent over panels in the field, and also in the control and under cold conditions, although only chlorophyll content explained more than 5% of the variability (data not shown). When the dependent variable was early dry weight, the only trait with a relevant significant effect was early vigor that explained around 27% of the variation (data not shown).

Regression analyses were carried out considering dry matter yield of testcrosses in the field as the dependent variable and the rest of traits as the independent variables. Multiple regression with a stepwise selection method for both panels and considering inbreds and testcrosses as independent variables revealed that the main factor affecting dry matter yield was plant height (R^2=0.285) followed by ΦPSII (R^2=0.034) and six other traits with minor contributions (Table 5). All significant variables had a positive coefficient of regression on dry matter yield, except dry weight at 5 weeks and days to emergence under cold conditions. When the same analysis was made for the dent panel, plant height (R^2=0.200) was again the main factor affecting dry matter yield, the number of significant variables was smaller and the signs of the coefficients were the same for the common variables. The analysis of the flint panel showed a similar result concerning the predominance of plant height (R^2=0.220) and some other variables with smaller effects; one of which was early vigor under cold conditions, that had a negative coefficient of regression on dry matter yield. The effect of plant height on dry matter yield is well known and the other significant traits were quite consistent over the two panels. Among the cold tolerance-related traits, only days to emergence and early vigor under cold conditions were included in the final model. Both, as well as dry weight at 5 weeks in the field, had a negative coefficient of regression. The other cold tolerance traits (ΦPSII and chlorophyll content) had significant positive effects on dry matter yield, but the proportion of variance explained was very low (Table 5). According to other authors, the effects of cold temperatures on final yield is due to leaf size rather than to leaf function (Louarn et al., 2008), which is not in agreement with our results because early vigor in cold conditions had a negative effect on dry matter yield, and high ΦPSII values had a positive effect. Negative effects of cold tolerance-related traits on dry matter yield are not surprising because our experience shows that when we improve either early growth or early vigor, we obtain smaller plants with less dry matter yield. Based on this, both size and function should be

taken into consideration as cold-related factors affecting plant growth, as the same authors concluded later (Louarn et al., 2010). Our results indicate, however, that predictive models based on plant performance under cold conditions cannot explain large proportions of the variance. This might be due to the low differentiation among testcrosses in the field under optimal conditions. When more clearly distinct genotypes are used and biomass is measured under cold conditions, the results can differ (Presterl et al., 2007). Contrarily, Frascaroli and Landi (2013) concluded that inbred performance could be used to predict testcrosses germination measured as the difference between cold and control conditions, although most previous studies have stated that the ability to predict hybrid performance from inbred value was limited. Certainly, the relationship between inbreds and testcrosses for cold tolerance depends on the genotypes, the testers used, and the environments involved.

Conclusions

There is large variability for cold tolerance among the inbred lines adapted to European environments. Some of the inbreds investigated in our study can be used as sources of cold tolerance in breeding populations for improving cold tolerance and for further genetic studies. On the other hand, some of the traits related to the performance of young plants had significant negative, though small, effects on dry matter yield of adult plants.

For breeding purposes, two groups of cold tolerant inbreds can be suggested as base germplasm, namely the groups Northern Flint and No Iodent, particularly the Northern Flint D171 group with UHF043, UHL058, UHL048, UH006, UHF093, and UHF091, and the No Iodent group with LH85, EP74, FV335, PHT77, UH2551, EC140, EZ19, and Pa374. These two groups could yield second cycle inbreds with high combining ability that could also combine favorably with the cold tolerant inbreds UHP024, UHP033, and D171.

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Figure 1. Principal component analyses for maize inbreds *per se* in cold conditions using the least squares means of days to emergence, ΦPSII, chlorophyll content, and early vigor after standardizing the traits. A) Cold tolerant dent inbreds with PRIN1 > 2 and PRIN2 <-1. B) Cold tolerant flint inbreds with PRIN1 > 2 and PRIN2 < -1. C) Dent panel. D) Flint panel.

Table 1. Mean comparisons between flint and dent maize inbred panels *per se* for four traits recorded in cold and control conditions in a growth chamber according to an F test (*p* = probability of significant differences)

	Days to emergence		Relative chlorophyll content[†]		ΦPSII[‡]		Early vigor[§]	
Type	cold	control	cold	control	Cold	control	cold	control
Dent	11.42	3.39	4.61	11.82	0.39	0.72	4.00	5.07
Flint	11.79	3.39	5.54	12.63	0.43	0.71	3.86	4.93
p	0.001	0.856	<0.0001	0.010	0.0023	<0.0001	0.007	0.003

[†] Relative chlorophyll content (SPAD) recorded using a hand-held Chlorophyll Content Meter, CCM-200 (Opti-Sciences, Tyngsboro, Massachusetts, USA)
[‡] Recorded using an OS-30p Chlorophyll Fluorometer (Opti-Sciences, Inc., USA)
[§] Subjective score from 1=weak plants to 9=vigorous plants

Table 2. Comparisons among groups of maize germplasm in the dent and flint panels for cold tolerance-related traits under cold and control conditions evaluated in a growth chamber

	Days to emergence		Chlorophyll content[†]		ΦPSII[‡]		Early vigor[§]	
Germplasm group at K=6 (Number of inbreds in the group)	Cold	Control	Cold	Control	Cold	Control	Cold	Control
Dent panel								
Iodent Ph207 (42 inbreds)	11.31 b	3.34 b	5.72 a	13.59 a	0.47 a	0.71 c	4.07 a	5.26 a
Iodent UH4068 (16)	10.81 b	3.39 ab	4.01 bc	13.88 a	0.42 a	0.73 a	4.19 a	5.07 abc
Lancaster Oh43 (12)	12.18 a	3.42 a	4.82 b	10.15 c	0.44 a	0.72 ab	4.25 a	5.01 bc
No Iodent (80)	11.80 a	3.40 a	4.35 b	12.01 b	0.34 b	0.72 b	3.87 b	5.05 b
Other No Iodent F252 (21)	10.74 b	3.34 ab	4.63 b	12.61 a	0.47 a	0.73 ab	4.25 a	4.86 c
Stiff Stalk (36)	11.01 b	3.38 ab	3.65 c	12.41 b	0.24 c	0.73 ab	3.92 b	5.12 ab
Flint panel								
FV7 (24)	12.11 b	3.4 ab	5.09 ab	11.73 bc	0.44 b	0.70 b	3.85 b	4.78 b
Northern Flint (44)	11.85 b	3.41 b	5.85 a	14.18 a	0.42 b	0.72 a	3.91 b	5.01 a
Northern Flint D171 (43)	11.30 a	3.35 a	5.79 a	13.91 a	0.50 a	0.70 b	4.14 a	5.12 a
No Northern Flint (36)	12.27 b	3.39 a	4.74 b	12.4 b	0.37 c	0.71 ab	3.58 c	4.83 b
Southern EC18 (16)	12.59 b	3.46 b	6.49 a	11.89 bc	0.51 a	0.73 a	3.85 bc	5.01 ab
S. Open Pollinated Varieties (19)	11.48 ab	3.35 a	4.45 b	9.9 c	0.34 c	0.72 ab	3.66 bc	5.14 a

[†] Relative chlorophyll content (SPAD) recorded using a hand-held Chlorophyll Content Meter, CCM-200 (Opti-Sciences, Tyngsboro, Massachusetts, USA)

[‡] Recorded using an OS-30p Chlorophyll Fluorometer (Opti-Sciences, Inc., USA)

[§] Subjective scale from 1=weak plants to 9=vigorous plants

Means followed by the same letter, within the same column and panel, were not significantly different

Table 3. List of 101 inbreds of maize with the highest cold tolerance, i.e. those that were not significantly different from the best inbred for four (in bold) or three traits when evaluated *per se* in cold conditions in a growth chamber. Ranking goes from top to bottom and from left to right, with EV18 being the first and UHP017 the last in the ranking.

Type	Inbred	Type	Inbred	Type	Inbred	Type	Inbred	Type	Inbred
Flint	**EV18**	Flint	PB57	Dent	PH207	Flint	CH31A	Dent	UHS002
Flint	**F364**	Flint	EP71	Flint	FV70	Flint	PLS41	Flint	UHF098
Flint	**F471**	Dent	F7025	Flint	UH5250	Dent	PHV78	Flint	UHF023
Flint	**EC51**	Dent	EV30	Flint	F02803	Flint	UHF091	Flint	UHL038
Flint	**UHL058**	Dent	UHP074	Dent	EP72	Dent	UH6132	Dent	UH6102
Flint	**CH34**	Flint	F591	Dent	C105	Dent	UHP033	Dent	B99
Dent	**UHP024**	Flint	CH16.1-295	Dent	EP74	Dent	Pa31	Dent	UH304
Flint	**FV71**	Flint	CH4.2	Dent	LH85	Flint	F362	Flint	PP87
Flint	**UHF043**	Flint	EA1349	Dent	UHP042	Dent	UH2551	Flint	UH007
Flint	EP1	Flint	UH1494	Dent	FV335	Dent	UH8513	Dent	F816
Flint	FV75	Flint	UHF035	Flint	UH006	Dent	EZ19	Dent	D06
Flint	EC237	Flint	EZ16A	Flint	UHL048	Flint	FV65	Dent	Pa35
Flint	PLS6	Flint	FC1571	Dent	Pa374	Dent	H99	Dent	Mo17
Flint	EC35G	Flint	UH5231	Flint	EP45	Dent	EC151	Flint	FV344
Flint	UH5113	Dent	UH6148	Dent	FC1890	Flint	CH113-379	Flint	PB6R
Dent	NQ508	Dent	EC242C	Flint	EZ53	Dent	FV277	Dent	F908
Flint	D152	Flint	FV79	Flint	CO255	Flint	RT9	Dent	EC326A
Flint	PB268	Flint	FV131	Flint	UHF093	Dent	W602S	Dent	F838
Flint	UHF106	Dent	F7028	Flint	FV355b	Flint	EZ21	Dent	FV113
Dent	LP325	Dent	W604S	Dent	FC1852	Dent	LH82	Dent	UHP017
Dent	F670								

Table 4. List of 23 testcrosses† from maize inbreds with the highest cold tolerance, i.e. those that were not significantly different for four (in bold) or three traits from the best when evaluated in cold conditions in a growth chamber. Ranking goes from top to bottom and from left to right, with EC35G being the first and EZ11A the last in the ranking.

Type	Inbred	Type	Inbred
Flint	**EC35G**	Flint	FV66
Flint	CH34	Dent	F7059
Flint	CH8.7	Dent	NS701
Flint	CH27-12	Flint	FV72
Flint	PP85	Dent	UHP017
Dent	B37	Dent	ML606
Flint	F47	Dent	B14A
Flint	IL101	Flint	UH1291
Flint	CH16.1-295	Flint	EC50
Flint	FV345	Dent	F670
Dent	FC1819	Dent	EZ11A
Flint	CH28-2		

† The dent inbreds were crossed to the flint tester UH007 and the flint inbreds to the dent tester F353

Table 5. Multiple stepwise regressions for biomass yield of testcrosses from two panels of flint and dent maize inbred lines evaluated in the field, and in a growth chamber under control and cold conditions (only significant variables are shown)		
Significant independent variables	Cumulated R^2	Coefficient
Dry matter yield of testcrosses from both panels evaluated in the field		
Plant height of testcrosses (field)	0.285	0.056±0.004
ΦPSII of testcrosses (field)	0.319	0.011±0.002
Days to pollen of testcrosses (field)	0.334	0.113±0.032
Dry early weight of testcrosses (control conditions)	0.348	6.830±2.080
Chlorophyll content of testcrosses (field)	0.356	0.037±0.015
Dry weight at 5 weeks of testcrosses (field)	0.363	-0.060±0.017
Early vigor of testcrosses (field)	0.372	0.307±0.115
Days to emergence (cold conditions)	0.376	-0.101±0.050
Dry matter yield of testcrosses from the dent panel evaluated in the field		
Plant height of testcrosses (field)	0.200	0.055±0.006
Days to emergence (cold conditions)	0.217	-0.180±0.064
Dry early weight of testcrosses (control conditions)	0.230	6.330±3.013
ΦPSII of testcrosses (field)	0.242	0.007±0.003
Dry weight at 5 weeks of testcrosses (field)	0.255	-0.040±0.019
Dry matter yield of testcrosses from the flint panel evaluated in the field		
Plant height of testcrosses (field)	0.220	0.053±0.006
ΦPSII of testcrosses (field)	0.258	0.013±0.004
Dry weight of testcrosses (control conditions)	0.276	9.048±2.789
ΦPSII of inbreds (cold conditions)	0.293	0.003±0.001
Early vigor of inbreds (cold conditions)	0.316	-0.453±0.180
Dry weight at 5 weeks of testcrosses (field)	0.330	-0.093±0.027
Early vigor of testcrosses (field)	0.349	0.453±0.168