COLD TOLERANCE STUDIES IN MAIZE

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ABSTRACT

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Maize grown in North America is often subjected to chilling temperatures at planting time, leading to disruption of seedling development. Laboratory protocols (cold germination, thermogradient plate germination and coleoptyle growth) were performed on 95 inbred lines in 1999 and 2000. Results from these tests were compared with field emergence data obtained from the same seed lots in Michigan and Illinois over two planting dates each year. In 1999 and 2000, cold germination had the highest correlation with early field emergence (r=0.78*** and 0.68***, respectively). Coleoptyle growth (r_{1999}=0.51* and r_{2000}=0.51**) and thermogradient plate germination (r_{1999}=-0.63* and r_{2000}=-0.48**) were also correlated with field emergence. Stepwise regression analyses indicated that the combination of cold germination and thermogradient plate germination was the best predictor of field emergence (r_{1999}=0.64** and r_{2000}=0.70***) when inbred lines are planted into cold, wet soils.

A BC1F2 population (self-pollinated progeny of BC1 individuals) with 147 families was created from the cross of two inbred lines, 1111 (cold tolerant) and 2222 (cold susceptible). A linkage map was constructed with 89 SSR markers spanning 1570 cM

and encompassing the 10 maize chromosomes with an average marker spacing of 30 cM. By means of interval mapping, a total of 21 QTLs, accounting for 8 to 76% of the variability, were identified and mapped. Eight QTLs controlled coleoptyle length, six QTLs controlled germination under cold temperatures and seven QTLs were associated with field emergence under cold soils. Most of these QTLs were clustered in three linkage groups and were consistently associated with field emergence and either coleoptyle growth or cold germination.

Genetic variation exists for some of the major physiological processes or developmental stages that are affected by suboptimal temperatures. Thus, specific physiological processes or developmental stages that are affected during growth at suboptimal temperatures were identified. These traits should be useful to phenotypically characterize large populations for their ability to germinate, grow and develop at chilling temperatures. The cold germination test, the thermogradient plate and coleoptyle growth at chilling temperatures represent inexpensive, reliable, repeatable and easy-to-standardize methods to search for genetic variability for chilling tolerance during germination and early growth stages in corn. Moreover, these protocols have proven successful in the identification of superior donors for cold tolerant traits.

To the loving memory of my mother

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Screening maize inbred lines for cold tolerance

ABSTRACT

Genetic variation exists for some of the major physiological processes or developmental stages in maize that are affected by suboptimal temperatures. Thus, specific physiological processes or developmental stages that are affected during growth at suboptimal temperatures were identified. These traits should be useful to phenotypically characterize large populations for their ability to germinate, grow and develop at chilling temperatures. These protocols have proven successful in the identification of superior donors for cold tolerant traits. The cold germination test, the thermogradient plate and coleoptyle growth at chilling temperatures represent inexpensive, reliable, repeatable and easy-to-standardize methods to search for genetic variability for chilling tolerance during germination and early growth stages in maize. Moreover, these protocols can be used to identify inbred lines as sources for chilling tolerance in a breeding program or in mapping experiments, to phenotype mapping populations and combined with genotypic data used to identity quantitative trait loci for seed and seedling cold tolerance. Eventually, these results have the potential to help in understanding the molecular basis for seed and seedling cold tolerance.

INTRODUCTION

Maize grown in temperate regions is often subjected to chilling conditions before and after emergence that can lead to disruption of seedling development and poor stand establishment (Hope, 1992). The problem is especially serious with the increasing acreage planted in more northerly areas and under no-till conditions. Adoption of conservation tillage results in a slower warm-up of seedbed temperatures in the spring and further increases the need for maize hybrids with superior cold tolerance during germination and early growth (Hayhoe et al., 1996). These damaging effects are often translated into a lack of seed germination or rapid death of young seedlings (Fig. 1-1). Consequently, conservation tillage results in more variable emergence rates and reduced stand establishment of maize compared to conventional tillage.

Uneven stand establishment in maize under low temperatures is a recurring problem, especially in Northern Corn Belt areas. Cold tolerance in maize is defined as the ability to germinate, emerge, and grow under chilling temperatures, ranging from 4 to 12°C. Sensitivity to such cold conditions is an important factor affecting growth reduction of maize in cool climates, especially at the germination and seedling stage. Increased capacity for seed germination and emergence under cold conditions has been recognized as a valuable attribute in maize for several decades (Neal, 1949; Haskell and Singleton, 1949). Heritable variation in seedling response to cold temperatures has been identified in several crops, including maize (Mock and Eberhart, 1972; Mock and Bakri, 1976; McConnell and Gardner, 1979; Greaves, 1996).

Generally, cold tolerance ratings are based on percent germination or emergence. However, to take advantage of the early growing season, cold tolerant maize must not

only germinate and emerge well, but it must also grow under cold stress similar to growth under warmer temperatures (Mock and Eberhart, 1972). It becomes imperative then to identify genotypes with lower thermal thresholds that are able to maintain higher rates of growth and faster emergence at lower temperatures.

Seed and Seedling Vigor

Seedling vigor has been defined as the potential for uniform and rapid seed/seedling emergence under a wide range of soil conditions (McDonald, 1980). High seed and seedling vigor is a desirable trait in most, if not all, crops. It is widely evaluated as an attribute of many crop species, especially maize.

One of the most complete reviews about seed vigor and vigor testing is found in the 'Seed Vigor Testing Handbook' published by the Association of Official Seed Analysts (AOSA, 1983). This handbook is divided in two parts. The first contains a historical perspective of the meaning and importance of vigor testing and vigor test results. It serves also as a reference for most of the literature related to seed vigor. The second part of this handbook provides procedures for several vigor tests such as the accelerated aging, cold test, tetrazolium and conductivity tests.

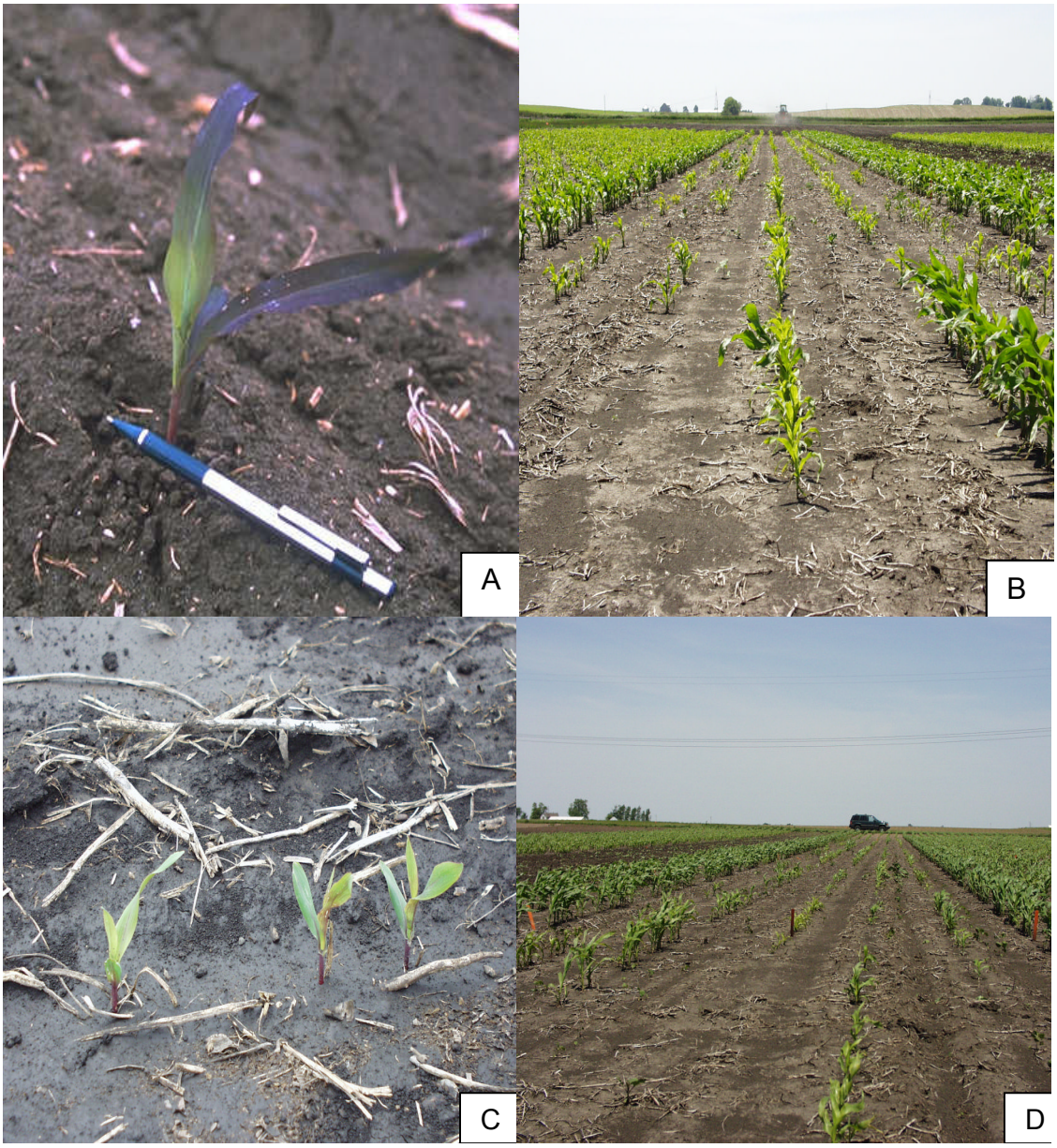

Fig. 1-1. Maize seedlings representing different stages of chilling stress. A) Blue seedling as a result of exposure to chilling stress. B) Row of maize with several missing plants, due to lack of germination in cold soils. C) Uneven growth, blue seedlings in wet, cold fields. D) Complete lack of germination and growth in a susceptible inbred line.

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation).

A large problem in defining or conceptualizing vigor is its varied manifestations in the testing laboratory and the field (speed of germination, emergence and seedling growth, uniformity in emergence, and sensitivity to environmental stresses during germination and stand establishment). It is difficult to account for all these possible manifestations in a short uniform definition. Definitions and concepts of vigor have been reviewed by Heydecker (1972), Perry (1973a), Woodstock (1973) and McDonald (1975, 1980).

Seed vigor was initially defined as the total sum of all seed attributes which favor stand establishment under *unfavorable field conditions* (Isely, 1954). This definition was criticized by Delouche and Caldwell (1960) for placing emphasis on "under *unfavorable field conditions,*" even though in the same publication, Isely wrote "a vigorous seed lot is one most likely to succeed under a wide variety of field conditions."

Seed vigor has also been described as those physiological properties determined by the genotype and modified by the environment, which governs the ability of a seed to produce a seedling rapidly in the soil, and the extent to which the seed tolerates a range of environmental stresses (Perry, 1973b). The influence of seed vigor may or may not persist through the life of the plant and affect yield.

In 1977, the ISTA (International Seed Testing Association) Vigor Test Committee proposed a definition of seed vigor that was adopted by the ISTA Congress (Perry, 1978) as 'the sum of those properties which determine the potential level of activity and performance of the seed or seed lot during germination and seedling emergence'.

AOSA adopted its own definition in 1980 as ‘those properties which determine the potential for rapid, uniform emergence and development of normal seedlings under a wide range of field conditions’ (McDonald, 1980).

Physiological basis for vigor

Biologically, seed vigor is dependent on the genetic constitution of seeds, which establishes their maximum physiological potential, and on the physiological state of the seed at initiation of growth. Seed deterioration begins at physiological maturity and proceeds until all of the seed tissues are dead. The rate of deterioration, including loss of vigor, is determined not only by heredity, but also by environmental and mechanical events occurring during seed development, harvesting, conditioning, and storage. The general strategy in determining seed vigor is to measure some aspects of seed performance or condition that reflect the stage of deterioration or genetic potential.

The changes that occur in seeds as they deteriorate and the consequences in terms of performance potential have been extensively reviewed. Heydecker (1972) developed a diagrammatic scheme which illustrates the rise and fall in vigor or performance potential of a seed or seed lot during maturation and deterioration. Seed vigor is at its highest when the seed reaches physiological maturity, after which germination potential is progressively lost. Initial deterioration occurs due to membrane degradation, followed by impaired biosynthesis, slower growth and development, and finally, loss of germination before eventual seed death.

It is generally accepted that the most important factor affecting seed quality is genetic potential. Thus, it is very important that plant breeders recognize that the

factors that dictate seed vigor are closely related to the inherent capability of the seed to resist both mechanical and environmental stresses. Differences in the level of mechanical damage have been shown for several species and cultivars (Atkin 1958; Davies 1964; Burris 1979), indicating that there is a genetic basis for resistance to mechanical damage. However, mechanical damage to the seed during harvest and handling, along with poor storage conditions can cause rapid seed deterioration. The effect of mechanical damage depends on the extent of the damage, with higher levels of damage resulting in lower germination (Tekrony et al., 1987).

Seedling vigor is influenced by many factors. At the biochemical level, vigor involves the biosynthesis of energy and metabolic compounds that control cellular and membrane integrity and the transportation and utilization of food reserves. It also involves the speed and totality of germination, as well as the exerted force of the emerging seedling. Finally, it involves the tolerance of seedlings to environmental stresses, including disease organisms (AOSA, Seed Vigor Testing Handbook, 1983).

The assessment of seed and seedling vigor has important implications for the seed industry and seed consumers. Among the practical uses of vigor tests is their use in plant breeding programs to develop cultivars with improved seed performance. Equally important, vigor tests are used to reveal where losses in seed quality may have occurred during harvesting, cleaning, drying, storing, bagging, etc., in order to assure that the highest vigor/quality seed reaches the customer.

Methods of testing

The challenge of seed and seedling vigor testing has been to identify measurable parameters associated with seed deterioration or an otherwise impaired quality and performance potential. Many different methods have been proposed for measuring vigor (AOSA, 1983; McDonald, 1999). Only those tests considered to be more practical will be described here.

Standard warm germination test. The standard warm germination test is the most common test to determine the quality of a seed lot prior to planting, and reveals much about seed and seedling vigor. However, this test often fails to predict the performance of a seed lot under actual field conditions since, by definition, it is conducted under optimum conditions for growth and development of seedlings, conditions rarely found in the field. The standard germination test only measures the seed's ability to germinate and does not detect other consequences of seed deterioration. Delouche and Baskin (1973) found that the loss of germinability is the last event before death of the seed.

Cold test. It is often desirable to plant crops early in the season, which increases the risks of inadequate stands because of poor field emergence. Poor field emergence, in temperate climates, is usually associated with very wet soils, low soil temperatures, and/or microbial activity. The cold test was designed to measure the ability of seeds to germinate under adverse conditions. It is the most widely used vigor test for maize in the US and was originally developed to predict the performance of seed lots of maize

planted in early spring in cold wet soils (Grabe, 1976). The cold test has also been found to be an effective vigor test in species such as soybeans, cotton, onion, carrot, sorghum and field and garden beans, as well as many other species (AOSA, 1983).

The moisture and temperature conditions provided in the cold test simulate adverse conditions that can be found in the field, especially during the early spring. The cold test usually represents the lowest germination that would be expected from a seed lot under field conditions, while the standard warm germination test represents the highest germination that could be expected from a seed lot; actual field germination would normally fall between those two values.

Accelerated aging test. The accelerated aging test is second to the cold test in popularity among seed testing laboratories. Its popularity is due to five attributes: 1) the test is simple and inexpensive; 2) the test is performed rather rapidly; 3) the interpretation of results is similar to that of the warm germination test; 4) the test is applicable to all species since all suffer deterioration during natural aging; and 5) the test is easily standardized. Accelerated aging was originally developed as a test to estimate the longevity of seed in warehouse storage. The test functions by exposing seeds to the two most important variables which affect seed deterioration: high temperatures and high relative humidity for short periods of time. The decline in germination following accelerated aging is proportional to the initial physiological potential of the seed (Delouche and Baskin, 1973). Thus, high vigor seed lots show a smaller relative decrease in germination after accelerated aging while low vigor seed lots will show larger reductions. Also, Delouche and Baskin (1973) suggested that

germination after accelerated aging correlated with field performance under a wide range of environmental conditions. However, Martin (1997) indicated that the accelerated aging test was a poor predictor of seedling vigor and field emergence for the environmental conditions usually found in the US Corn Belt.

Conductivity test. The conductivity test utilizes the measurement of electrolytes leaking from plant tissues. It was first adapted in seed testing to measure cotton seed viability (Presley, 1958). Cell membranes lose their integrity as seeds dry after physiological maturity, but during imbibition, membrane integrity is re-established. Vigorous seeds re-establish membranes at a faster rate with less leakage than less vigorous seeds (Powell, 1988). In general, seed lots with high values (>150 μmhos/cm/g) are low in vigor and, in addition, may lose vigor more rapidly during storage (International Seed Testing Association, 1985).

Seedling vigor and field performance

Germination under early spring field conditions and crop yield may be influenced by seedling vigor. Seed vigor greatly influences stand establishment by an interaction with the field conditions present at planting time and during emergence (Johnson and Wax, 1978). The literature of vigor abounds with reports that support this type of response (Burris, 1976; Burris, 1979; Egli et al., 1979; Johnson et al., 1978; Martin et al., 1988; Perry, 1973; Powell, 1988).

Several reports about the effect of seed and seedling vigor on yield are not nearly as conclusive as those affecting seedling emergence. Low vigor seed lots

primarily result in reduced field performance and stand establishment (Edje and Burris, 1971). Reduction in yield due to low vigor seed lots has been associated with low plant stand density (Perry, 1980), although Egli and Tekrony (1979) showed that lower seed vigor had no influence on emergence, stand or yield under optimum field conditions.

Significant associations between laboratory protocols and field emergence studies are common. However, those associations are not consistently correlated from year to year, test to test and location to location. Although some of the laboratory protocols described above (i.e. cold test) have consistently shown significant correlations with field emergence, difficulties in standardization exist due to variations in soil type, moisture content, Pythium activity and concentration, etc.

Thus, there is a need to develop or standardize protocols that will aid in prediction and selection of materials with superior abilities to germinate, emerge and develop under chilling stress.

OBJECTIVES

The framework of this research was to identify a set of traits important for germination, early seedling growth and stand establishment under cold temperatures in maize. The goals were to develop simple, inexpensive and accurate tools to aid in the screening and selection of maize inbred lines and hybrids that have the ability to germinate, grow and develop under chilling stress.

Specifically, the objectives were to screen a diverse pool of maize germplasm for their ability to germinate and tolerate chilling stress (below 10°C) and identify important physiological characteristics associated with early germination and stand establishment.

MATERIALS AND METHODS

Seed Source: All seed materials used in these experiments were proprietary lines from Monsanto Company (St. Louis, MO), including both inbred lines and hybrids (Table 1-1). Although a large set of inbred lines and hybrids were available, only a few seed lots representing cold tolerant and cold sensitive groups were included in these first studies. All seed lots were previously tested for germination and cold vigor test performance and only those lines with high values were selected for further testing (data not shown). These seed lots were selected because they represent a broad range of genetic backgrounds, were produced under the same growing conditions in the same year, and all had excellent seed quality (defined by their levels of germination and cold test results). This is an essential step since all other sources of variability should be reduced to a minimum. Environmental factors during seed development play an important role in seed vigor; consequently, all seed lots were exposed to a similar set of conditions prior to testing to minimize misleading results. Finally, the set of inbred lines and hybrids used in these experiments, do not share parentage. That is, not all parents conforming the hybrids used herein were part of these studies.

Bulk Conductivity: The objective of this experiment was to determine if temperature of water imbibition affected membrane integrity or its permeability. Prior to initial use, the conductivity meter was calibrated using a potassium chloride solution. To calibrate the dip cell of the conductivity meter, 0.745 g of pure analytical grade potassium chloride (dried at 150◦C for 1 h and cooled in a desiccator before weighing) was dissolved in 1 L of deionized water to make a 0.01 M KCl solution.

Initial seed moisture was measured for each seed lot and determined to be within an acceptable range (11 to 15%). Forty ml of double distilled water were placed in a 50 ml beaker for each replication of every seed lot, covered with aluminum foil and allowed to equilibrate for 24 h at 5, 10 and 23◦C (temperature treatments). A control flask with 40 ml of double distilled water was also prepared for each temperature treatment to monitor its conductivity, which is later subtracted from the total bulk conductivity measured for each seed lot. Two replications of 20 kernels each were weighed and added in the 40 ml of water that had been equilibrating for 24 hours. Each beaker was gently swirled for a few seconds to ensure that all kernels were completely immersed in water. All beakers were covered with aluminum foil again, placed in their respective temperature treatment and allowed to equilibrate (and kernels to leach) for another 24 h. Bulk conductivity and the weight of the 20 kernels were measured for each replicate at every temperature treatment.

Table 1-1. Description of seed lots used.

Genotype	Heterotic Group	Type
1001	European Flint	Inbred
1002	LH82	Inbred
1003	B73	Inbred
1004	LH82	Inbred
1005	S35 x S35	Inbred
1006	Iodent	Inbred
1007	Stiff Stalk	Inbred
1008	Stiff Stalk	Inbred
1009	C103	Inbred
1010	Oh43	Inbred
1011	Mexico	Inbred
1012	Argentina	Inbred
1101	RX490	Hybrid
1102	RX355	Hybrid
1103	RX530	Hybrid
1104	RX601	Hybrid
1105	RX697	Hybrid
1106	RX843	Hybrid
1107	RX670	Hybrid

Thermogradient plate germination: The first step in identifying materials with a greater capability to withstand cold conditions is based on the threshold temperature at which each inbred line begins to germinate. A thermogradient table, which was generously

provided to us by Dr. Greg Welbaum at Virginia Tech University, was used to determine the lowest temperature at which each inbred line and hybrid began to germinate (Figs. 1-2 and 1-3). The thermogradient plate consisted of an aluminum base (108 cm long and 81 cm wide), with a water-cooling system underneath. Thick Styrofoam and a wood siding completed the tight insulation. The table is 5 cm deep and has a plexi-glass cover that is sealed. Two water sources, one set at room temperature and the other at 4◦C, are connected at each end of the table, generating a temperature gradient. This gradient (from minimum to maximum, or the space in between each degree unit) can be easily regulated by adjusting the temperature in the water circulators. In order to reduce the temperature gradient from one end of the table to the other, the 'warm' circulator could be turned off. Thus, the temperature gradient is only originated by the 'cold' circulator and is extended through the length of the table, generating a larger distance between each degree of temperature, allowing for the identification of small temperature differences (up to 0.1◦C). The temperature gradient is very stable both along and across the table. Several thermocouples were strategically fixed to the table and the temperature monitored several times a day for 6 weeks. No changes larger than 0.5◦C were detected at any point during the duration of the preliminary study, as long as the water circulators were operating.

The thermogradient plate created a well-defined temperature gradient that helped identify the minimum temperature at which germination occurred after 7 and 14 days. A layer of blue blotter germination paper (at the bottom) and brown heavy-duty germination paper (on top) are used as the moisture source to insure adequate and

homogeneous imbibition (Fig. 1-2 and Fig. 1-3). Kernels were considered germinated once the radicle reached 1 cm in length.

Cold Germination: Two replications of 10 kernels each were planted on petri dishes with moist filter paper (two layers between kernels) and placed in growth chambers at constant 5, 10 and 23°C. Five ml of distilled water were added at the beginning of the experiment and every two days during the duration of the study. Evaluations were made every other day. Kernels were considered germinated once the radicle reached 1 cm in length.

Coleoptyle growth: The objective of this assay was to make detailed observations of the germination sequence. Two replicates of eight kernels each were planted in a flat tray and covered with soil. These trays were then placed in chambers at 23°C for 36 hours to induce sprouting without the imbibitional injury that would occur by chilling at 10°C (McDonald, 1999). Trays were then transferred to 5 and 10°C rooms where coleoptyle length was measured at several intervals during 3 weeks.

Fig. 1-2. Thermogradient plate planted. The blue blotter paper and the heavy duty brown germination paper used as moisture sources can be seen covering several rows of planted maize inbred lines.

Fig. 1-3. Two views of the thermogradient plate, with and without cover.

Field emergence study: Two replications, each consisting of two 5 m rows of twenty five kernels each were planted for each seed lot in three planting dates at the Michigan State University Crops Research Farm in 1999. The space between rows was 0.76 cm. Planting dates were April 8 (Planting date 1 = PD1), April 20 (PD2) and May 3, 1999 (PD3), representing a very early, a moderately early and a normal planting date for the region. After planting, the number of seedlings emerged was counted every five days. Counts were continued until most plants in a row reached the V3 stage (Ritchie, 1992).

Analyses of data: Data were analyzed using the PROC GLM and PROC MIXED protocols in SAS (SAS Institute Inc., 1999). Least significant differences at the 5% level were calculated by the PROC MEANS procedure, applying the Fisher's LSD option. All data were first analyzed to obtain regression curves for each genotype in each experiment by means of the PROC REG procedure. Linear models best described the response of genotypes for the laboratory protocols while a polynomial model of second order best fitted the response in the field study. Cold germination, coleoptyle growth and field emergence data were transformed from daily observations (percent germination, cm of coleoptyle growth and number of plants emerged at days after planting) to calculate 'days to 50% germination', 'days to 3-cm coleoptyle' and 'days to 50% field emergence' by using the PROC PROBIT procedure.

RESULTS

Analyses of variance of *bulk conductivity* measurements reflected highly significant differences among seed lots (within inbred lines and hybrids) at all 3 temperature treatments ($p<0.001$; Appendix Table A2). Among inbred lines, 1003 and 1006 had the lowest conductivity readings when the solution was kept at 23°C (Table 1-2), while inbred 1011 had the highest value. No significant interactions for bulk conductivity readings were measured among seed lots and temperature treatment (Table A2).

Among hybrids, RX530 had the lowest bulk conductivity, at any temperature treatment, while RX490 had the highest value at 5°C and RX355 had the highest values at 10 and 23°C. Conductivity values ranged from 11.9 to 33.4 $\mu S.cm^{-1}.gr^{-1}$ at 5°C and were as high as 47.3 $\mu S.cm^{-1}.gr^{-1}$ when the temperature increased to 23°C.

There was a highly significant positive correlation ($p<0.0001$; Table A3) between the conductivity values measured at 23°C and those measured at 10°C ($r=0.81^{***}$) and 5°C ($r=0.74^{***}$), indicating that although the temperature of the solution had an effect on the results, it is not necessary to use cooler temperatures to make effective observations or determinations on the cold tolerance or susceptibility of maize genotypes. The correlation between bulk conductivity readings at 5 and 10°C was also significantly correlated ($r=0.80^{***}$, $p<0.001$).

Differences in *water uptake* for each seed lot after 24 hr of imbibition at the three temperature treatments were also recorded. Although significant differences among seed lots were measured ($p<0.001$, Table A1), none of the correlations between bulk conductivity measurements and water uptake were significant (Table A3). Weight gain

due to water uptake increased significantly with temperature (Table 1-2). No significant associations were identified between water uptake at any temperature treatment and the cold tolerance screens described here, probably due to the fact that moisture imbibition is a physical process and it is not under genetic control.

The minimum temperature for *germination in a thermogradient plate* was recorded at 7 and 14 days after planting (DAP). After 7 DAP, inbred lines 1002 and 1004 germinated at the lowest temperatures, while inbred 1009 germinated only at very warm temperatures of 17°C (Table 1-3). After 14 DAP, inbred 1004 germinated at temperatures of 8°C, while 1009 still failed to germinate at temperatures below 15°C. The germination of inbred line 1004 at 8°C was very notable, since growth in maize had not been reported to occur at temperatures below 9°C (Blacklow, 1972).

Table 1-2. Bulk conductivity and water uptake of 19 maize genotypes tested at 3 temperatures.

Genotype	Conductivity			Water uptake		
	----------ųS/cm^{-1}gr^{-1}--			----------Grams----------		
INBREDS	**5ºC**	**10ºC**	**23ºC**	**5ºC**	**10ºC**	**23ºC**
1001	38.3	30.4	48.7	2.00	2.70	2.75
1002	19.1	19.9	28.9	2.75	2.75	2.30
1003	17.0	18.2	20.2	2.20	2.70	3.05
1004	19.9	24.2	30.9	1.30	1.70	1.80
1005	29.0	23.9	40.6	2.75	2.70	2.35
1006	11.8	9.8	22.6	1.15	1.30	1.80
1007	31.0	35.0	48.8	1.45	1.45	2.05
1008	16.3	17.9	23.7	2.20	2.75	3.15
1009	28.2	26.5	38.4	2.20	2.25	2.95
1010	19.2	17.2	26.4	2.75	2.30	2.25
1011	41.4	45.1	54.4	2.25	2.35	3.10
1012	51.1	40.3	49.1	1.90	2.20	2.00
HYBRIDS						
RX490	33.4	28.2	38.1	2.30	2.15	2.80
RX355	31.1	34.3	47.3	2.65	2.10	2.75
RX530	11.9	11.9	15.7	2.85	3.05	3.65
RX601	19.9	21.2	32.9	2.05	3.50	3.25
RX697	29.5	22.4	40.2	1.90	2.20	2.70
RX843	22.2	19.6	34.9	2.80	3.10	3.25
RX670	13.5	18.1	19.6	2.50	3.05	3.75
Mean	**25.2**	**29.0**	**38.7**	**2.21**	**2.44**	**2.72**
$LSD_{0.05}$	9.82	9.23	13.4	0.61	0.38	0.39
C.V. (%)	50.8	45.8	39.4	26.9	25.2	22.8

$LSD_{0.05\ across\ temperatures\ for\ conductivity}$= 6.4 ųS/cm^{-1}gr^{-1}

$LSD_{0.05\ across\ temperatures\ for\ water\ uptake}$= 0.15 grams

Similar patterns of germination were observed at 7 and 14 days after planting (14 DAP), although temperatures for germination were always lower after 14 DAP. Highly significant differences among seed lots for minimum temperature for seed germination were recorded at both 7 and 14 DAP.

In the thermogradient plate, hybrid RX670 germinated at the lowest temperatures, both at 7 and 14 DAP (Table 1-3). Hybrid RX843 had the highest temperature (13.5°C) for germination at 7DAP, while RX601 required the warmest temperature to germinate after 14 days (11.7°C).

Highly significant differences in percent *cold germination* were measured when seed lots were placed at constant 10°C (Tables 1-4 and A2). This temperature is normally used in the cold vigor test. Germination first occurred at 5 to 7 days after planting (DAP) for hybrid RX670. Inbred lines 1004, 1005 and 1006 germinated 75% or higher within 10 days, reaching almost 100% germination by the end of the experiment. Inbred lines 1007, 1012, 1009 and 1011 did not reach acceptable germination levels even after the 3-week experiment.

Data for the cold germination test were transformed using PROC PROBIT (SAS Inc, 1999) to obtain the mean number of days to 50% germination for each seed lot (Fig. 1-4 and Table 1-7). Inbred lines 1004 and 1005 had the fastest rates of germination, reaching 50% only 2.5 days after planting (DAP). Conversely, 1009 and 1012 reached 50% germination at 10°C only after two weeks. These seed lots would be unlikely to produce a good field stand. The range in days for 50% germination was from 2.5 days to 16 days, with the majority of inbred lines ranging from 5 to 10 days.

Table 1-3. Minimum temperature at which germination of 19 maize genotypes was recorded in thermogradient plate experiment.

Genotype	Time after planting	
	7 days	14 days
1001	12.4[§]	9.7
1002	11.7	9.1
1003	14.5	11.4
1004	11.9	8.1
1005	11.7	9.4
1006	12.6	10.0
1007	13.6	12.7
1008	12.7	12.3
1009	16.9	15.4
1010	13.6	10.8
1011	14.6	11.7
1012	14.6	11.6
RX490	12.5	10.0
RX355	13.3	10.9
RX530	12.1	9.5
RX601	13.1	11.7
RX697	12.8	10.2
RX843	13.5	9.4
RX670	12.0	9.8
Mean	13.1	10.7
$LSD_{0.05}$	1.6	2.2
C.V. (%)	5.8	9.8

[§] Minimum temperature (in ºC) at which germination occurred

Table 1-4. Percent seed germination of 19 genotypes at 10ºC.

Genotype	Days after planting					
	4	7	10	14	19	22
1001	0.0[§]	8.3	58.3	66.7	66.7	66.7
1002	0.0	25.0	41.7	66.7	83.3	83.3
1003	0.0	16.7	50.0	75.0	75.0	83.3
1004	0.0	41.7	75.0	91.7	91.7	91.7
1005	0.0	33.3	83.3	91.7	100.0	100.0
1006	0.0	0.0	75.0	83.3	100.0	100.0
1007	0.0	0.0	8.3	41.7	41.7	41.7
1008	0.0	8.3	41.7	83.3	91.7	100.0
1009	0.0	0.0	0.0	0.0	16.7	41.7
1010	0.0	0.0	25.0	50.0	75.0	83.3
1011	0.0	0.0	0.0	33.3	33.3	41.7
1012	0.0	0.0	8.3	8.3	33.3	41.7
RX490	0.0	16.7	50.0	83.3	91.7	91.7
RX355	0.0	0.0	58.3	91.7	91.7	91.7
RX530	0.0	66.7	75.0	91.7	100.0	100.0
RX601	0.0	8.3	25.0	41.7	58.3	66.7
RX697	0.0	0.0	25.0	33.3	58.3	66.7
RX843	0.0	16.7	66.7	83.3	91.7	91.7
RX670	0.0	77.8	100.0	100.0	100.0	100.0
Mean	0.0	13.4	42.6	62.0	72.2	76.9
$LSD_{0.05}$	0.0	25.4	23.7	12.5	19.4	18.0

[§] Percent germination

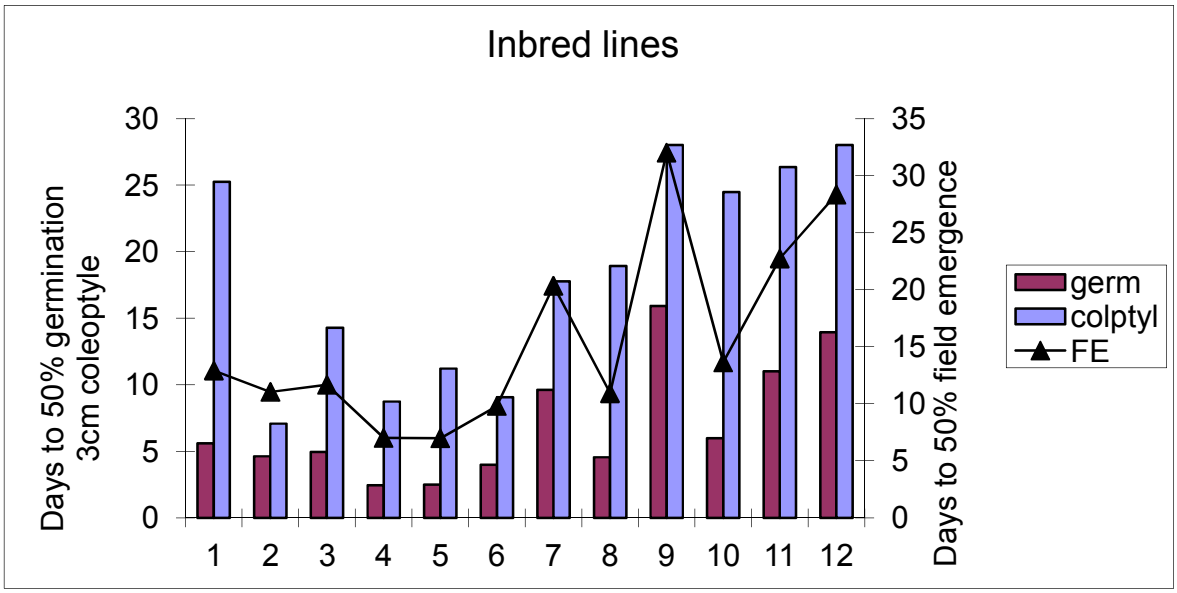

Fig. 1-4. Mean response to germination, coleoptyle growth and field emergence (FE) for all inbred lines.

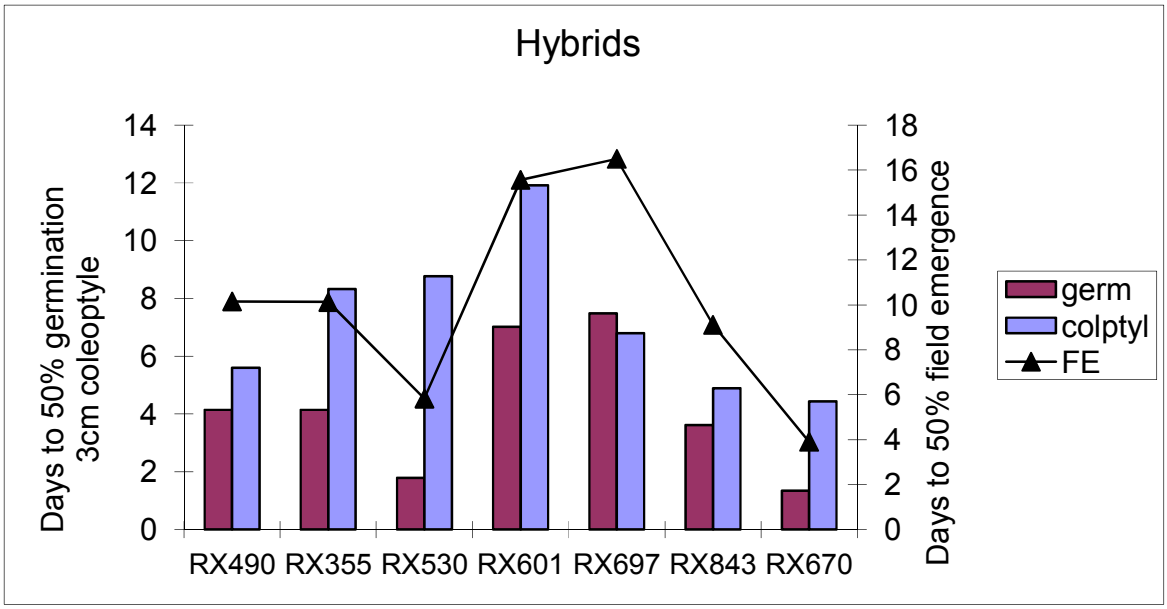

Fig. 1-5. Mean response to germination, coleoptyle growth and field emergence (FE) for all hybrids.

As with inbred lines, highly significant differences were observed within hybrids when subjected to cold germination. At 7 DAP, RX670 and RX530 reached 77.8 and 66.7% germination respectively, while all other hybrids had just began germination or had not germinated at all (Tables 1-4 and 1-7). Differences were reduced at 14 DAP, but RX601 and RX697 still had low and commercially unacceptable germination values. Among hybrids, RX530 and RX670 attained 50% germination in less than 2 days, whereas RX697 needed almost 8 days to attain 50% germination (Fig. 1-5). The range among hybrids was significantly less when compared to inbred lines, from 1.8 days to 7.8 days.

Seeds were germinated at 23◦C for 36 hr and then transferred to growth chambers with temperatures set at 10◦C or 23◦C. At 23◦C, all seed lots grew well, once again demonstrating good initial seed quality. Significant differences in *coleoptyle growth* at 10◦C were observed among seed lots, for both inbred lines and hybrids. Within inbred lines, mean days to 3 cm coleoptyle ranged from 7.1 days for inbred line 1002 and more than 27 days for 1009 (Table 1-7). Inbred lines 1004, 1005 and 1006 also had means less than 10 days. Conversely, inbred 1012 took more than 27 days to grow a 3 cm coleoptyle, suggesting that it would not survive under cool field conditions (Table 1-7; Fig. 1-4).

The differences in rate of coleoptyle growth among hybrids were smaller than those observed for inbred lines (Tables 1-4, 1-5 and 1-7; Fig. 1-5). Differences among hybrids were still highly significant, with RX670 having fastest germination to 3 cm coleoptyle (4.4 days), whereas RX601 had the slowest growth, taking almost 12 days to reach the same coleoptyle length.

Field emergence data was recorded by counting the number of emerged seedlings per row, at several intervals after each of three planting dates. For each planting date, data were transformed using PROC PROBIT to calculate the mean number of days to 50% field emergence. Due to severe rains, the portion of the field where the second planting date study was planted was flooded and a thick crust prevented normal seedling evaluations. For that reason, those plots were abandoned.

When data for PD1 (early planting) were analyzed within inbred lines, significant differences were observed for days to 50% emergence (Fig. 1-4 and Table 1-7). Temperatures at planting time were approximately 9°C and increased very slowly over time (Table A5). Inbred lines 1004 and 1005 had the fastest emergence rates; each had emerged by 7 days (Table 1-7), whereas inbred lines 1009 and 1012 were the slowest emerging at approximately 30 days.

Planting date 3 (PD3) coincided with the normal planting time for maize in the central areas of the state of Michigan in 1999. Soil temperatures were approximately 15°C. Cold stress conditions did not occur after planting in 1999. Consequently, few differences in rate of emergence were measured among inbred lines or hybrids, indicating the high quality of seed lots under evaluation.

In the field emergence experiment, hybrids RX670 and RX530 had the fastest times to 50% emergence among hybrids at 4 and 6 days respectively, while RX697 had the slowest at 16.5 days. The range in both laboratory and field performance indicates that genetic variation exist for these traits and could be exploited through breeding.

Table 1-5. Measurements of coleoptyle length of 19 genotypes at 10°C collected at different time intervals after planting.

Genotype	Days after planting				
	6	9	12	16	19
1001	0.01[§]	0.08	0.11	0.20	0.58
1002	0.13	0.58	0.92	1.46	2.10
1003	0.12	0.33	0.47	0.70	1.03
1004	0.11	0.58	0.82	1.18	1.67
1005	0.13	0.33	0.54	0.89	1.29
1006	0.14	0.30	0.68	0.99	1.65
1007	0.05	0.20	0.38	0.53	0.77
1008	0.10	0.27	0.31	0.43	0.84
1009	0.00	0.00	0.00	0.00	0.03
1010	0.02	0.13	0.15	0.24	0.61
1011	0.02	0.06	0.13	0.26	0.52
1012	0.00	0.00	0.01	0.06	0.08
RX490	0.38	1.16	1.57	2.03	2.70
RX355	0.08	0.38	0.68	1.19	1.73
RX530	0.03	0.36	0.73	0.98	1.67
RX601	0.28	0.42	0.70	0.95	1.29
RX697	0.27	0.67	1.11	1.66	2.18
RX843	0.26	0.93	1.32	2.21	3.31
RX670	0.58	0.94	1.94	2.40	3.06
Mean	0.12	0.38	0.59	0.89	1.34
$LSD_{0.05}$	0.17	0.23	0.20	0.35	0.33

[§] Data in cm

Table 1-6. Field emergence (plants emerged per plot) for 19 maize genotypes at 25 days after planting.

Genotype	PD1[§]	PD3[§§]
1001	10.5[‡]	19.3
1002	1.0	17.3
1003	13.3	18.8
1004	12.5	18.5
1005	15.8	18.5
1006	11.3	18.0
1007	7.0	18.3
1008	15.0	17.8
1009	12.0	11.5
1010	ND[‡‡]	12.0
1011	8.0	ND
1012	9.8	18.5
RX490	11.8	21.3
RX355	17.0	19.5
RX530	17.8	19.0
RX601	11.3	18.3
RX697	14.8	19.8
RX843	14.0	17.9
RX670	17.5	20.3
Mean	12.2	18.0
$LSD_{0.05}$	4.20	2.28

[§] PD1 = planting date number 1, on April 8th, 1999.
[§§] PD3 = planting date number 1, on May 3rd, 1999.
[‡] Data in plants emerged per plot. A total of 25 kernels per plot were planted
[‡‡] ND= no data available, plots abandoned due to soil crusting.

Table 1-7. Mean number of days to 50% germination, 50% 3cm coleoptyle and 50% field emergence for 19 maize genotypes.

Genotype	50% germ	3cm coleoptyle	50% emergence
1001	5.6[§]	25.3	16.9
1002	4.6	7.1	15.0
1003	4.9	14.3	14.7
1004	2.5	8.7	11.0
1005	2.5	11.2	11.0
1006	4.0	9.1	13.8
1007	9.6	17.8	24.3
1008	4.5	18.9	14.9
1009	15.9	>27.0	36.0
1010	6.0	24.5	17.6
1011	11.0	26.4	26.7
1012	13.9	>27.0	32.3
RX490	4.1	5.6	14.2
RX355	4.1	8.3	140.1
RX530	1.8	8.8	9.8
RX601	7.0	11.9	19.6
RX697	7.5	6.8	20.5
RX843	3.6	4.9	13.1
RX670	1.3	4.4	7.9

[§] Average number of days

DISCUSSION

Poor stand establishment of maize under low temperatures is a recurring problem, especially with the increasing acreage planted under no-tillage conditions and the lower soil temperatures associated with them. Sensitivity to cold stress is a critical factor affecting growth reduction of maize in cool climates, especially critical at germination and seedling stages.

Chilling injury occurs in sensitive species at temperatures that are too low for normal growth and development but not low enough to induce ice formation. Plant responses to chilling stress appear to be controlled by more than one genetic system (Hodges et al., 1997) and are highly influenced by the environment. In addition, cold tolerance at one stage of plant development does not guarantee tolerance at other developmental stages. For this reason, individual stages throughout the ontogeny of the plant such as germination, emergence, seedling survival, and growth were evaluated separately for assessment of tolerance and identification of useful genetic components.

Protocols were developed to facilitate screening of inbred lines and hybrids for the ability to germinate, emerge and grow under cold conditions. Availability of new technologies and equipment allows for earlier planting, that often includes no-tillage systems. These advances have lead to a situation where growers plant maize as early as field access is possible. As a consequence, an increasing number of growers will select hybrids that can be planted when soil temperatures range between 5 and 10◦C. For this reason, the best adapted hybrids need to be screened to provide growers with information on low temperature germination. Care should be taken not to misinterpret

data since hybrids that don't have the ability to germinate at colder temperatures may have as good or even better yield potential than their cold tolerant counterparts.

Genetic variation exists for some of the major physiological processes or developmental stages of maize that are affected by suboptimal temperatures. Specific developmental stages that were affected during growth at suboptimal temperatures were identified. These traits should be useful to phenotypically characterize a large number of inbred lines for their ability to germinate, grow and develop at chilling temperatures. These protocols have successfully proved that the identification of superior donors for cold tolerant traits is possible.

Poor membrane structure and "leaky" cells are usually associated with deteriorating seeds. This results in a greater loss of electrolytes (measured by a bulk conductivity meter) such as amino acids and organic acids from imbibing seeds, subsequently increasing the conductivity of the imbibing solution. Theoretically, seeds would loose more electrolytes when imbibed at chilling temperatures than at warmer ones. However, bulk conductivity readings were higher at 23◦C and decreased as the temperature of the water solution also decreased to 10 to 5◦C (Table 1-2). This was due to a decrease in ion activities as temperatures were lowered.

The coefficient of variation for bulk conductivity readings decreased sharply as temperatures increased from 5 to 10 to 23◦C. This could be due to the sensitivity of the measuring cell to chilling temperatures. Fewer resources are needed for tests at 23◦C (room temperature), while cold chambers or refrigerators are needed to run the experiments at 5 and 10◦C. There was significantly less variation within the

measurements at 23◦C compared to the other two temperature treatments. Thus, tests at 23◦C should be preferred over those at lower temperatures.

Several limitations exist for the application of the conductivity test as a valuable screening tool for cold tolerant maize lines on a commercial scale. The difficulty of having all the seed lots produced under the same set of conditions (year, location, planting and harvesting dates, and mechanical processes such as harvesting, drying, shelling, bagging, etc.) every year restricts comparisons. It is essential to assure that differences in test results are the consequence of genotype and not due to adverse physiological or environmental conditions, and mechanical treatments of the seed.

Just as in the thermogradient plate germination experiment, no obvious differences in pattern of emergence or temperature for lowest germination appear to exist between inbred lines and hybrids. Previous evidence that heterosis does not play a major role in germination of maize seed is substantiated. This statement should be examined very cautiously since large differences in emergence occur regularly in the field when hybrids and inbred lines are planted together early in the spring under cold and wet soil conditions. Perhaps, heterosis plays a role in helping hybrids 'grow out' of those stressful conditions more rapidly. These differences observed in the field may occur by impacting root growth and development. In the laboratory, root growth and development appear to be the largest morphological differences when growing both inbred lines and hybrids in petri dishes at cold temperatures.

It is noteworthy that some genotypes are able to germinate at temperatures that ordinarily would cause chilling injury. Also, the rates (given by the slope of their germination curve) at which some inbred lines germinate even though they are being

exposed to cold stress are also noteworthy. Only two hybrids (RX670 and RX530) showed a significantly faster rate of germination than most inbred lines. All others hybrids responded similar to the inbred lines, indicating that hybrid vigor/heterosis may not play an important role in germination. A chilling tolerant inbred line appears to germinate at the same temperatures and at the same 'rate' as most hybrids (Fig. 1-4 and 1-5), which suggests that inbred performance cannot be used to predict hybrid performance.

Although differences in germination temperature were statistically significant, the range in temperatures for germination was relatively small, 1.5◦C after 7 days and 2.4◦C after 14 days. This would indicate that there is little genetic variation for minimum germination temperature among hybrids for this trait, especially when compared to the larger range for germination that was recorded for inbred lines: 5.25◦C after 7 days and 7.25◦C after 14 days. Improving minimum germination temperature with improved inbred lines seems to be a valuable approach, since significant variability appears to exist for the trait.

Based on the protocols described above, efficient determinations can successfully be made to identify superior inbred lines and hybrids with the ability to perform well under chilling stress. These experiments showed that inbred lines 1002, 1004, 1005 and 1006 were the best for cold temperature conditions. These four inbred lines germinated at the lowest temperatures (between 8 and 10◦C), had the fastest rate of germination (between 2.5 and 4.5 days to 50% germination), exhibited the fastest growth rate (between 7 and 10days to 50% 3cm coleoptyle), and showed the fastest rate of seedling emergence (7 to 10 days to 50% field emergence when planted in cold soils). Thus, these lines would be the best candidates for use in early planting schemes. When the same selection

parameters are applied to hybrids, RX530 and RX670 outperformed all the other hybrids tested.

Inbred lines 1002 and 1004 belong to the LH82 heterotic group. These lines are usually characterized as mid-season for maturity and adapted to stressful environments, especially drought. Inbred lines 1005 and 1006 are iodents types, are early flowering, adapted to colder environments and have high yield potential.

Inbred line 1009 is the most sensitive genotype to cold treatments. This inbred is a C103 type, belonging to the Lancaster group, is late maturity and is well known to be adapted to warm temperatures. Inbred line 1012 is a line originating in Argentina and is not adapted to cold, stressful environments. These two inbred lines failed to germinate and grow under most tests, indicating their lack of adaptation to the cold conditions of early planting in the US Corn Belt. Hybrids RX601 and RX697 germinated at the warmest temperature and had the slowest rate of germination, growth, and field emergence. Although they do not seem to be adapted to early planting conditions, they performed very well when planted at later dates.

These laboratory techniques showed that indirect selection for cold tolerance traits in maize inbred lines or hybrids can be successful. The key in a laboratory screening program is to make the test simple, so sources of error and variability can be easily controlled. All these protocols met this criterion. Another advantage of these tests is that they can successfully predict cold tolerance performance of both inbred lines and hybrids, without masking hybrid vigor effects. This is true since these protocols confirmed once again that hybrid vigor does not influence cold tolerance at the germination and early seedling stage.

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CHAPTER 2

Laboratory and Field Tests for Cold Tolerance in Maize Inbred Lines

ABSTRACT

Laboratory protocols (cold germination, thermogradient plate germination and coleoptyle growth) were performed on 95 inbred lines in 1999 and 2000. Results from these tests were compared with field emergence data obtained from the same seed lots in Michigan and Illinois over two planting dates each year. In 1999 and 2000, cold germination had the highest correlation with early field emergence ($r=0.78^{***}$ and 0.68^{***}, respectively). Coleoptyle growth ($r_{1999}=0.51^{*}$ and $r_{2000}=0.51^{**}$) and thermogradient plate germination ($r_{1999}=-0.63^{*}$ and $r_{2000}=-0.48^{**}$) were also correlated with field emergence. Stepwise regression analyses indicated that the combination of cold germination and thermogradient plate germination was the best predictor of field emergence ($r_{1999}=0.64^{**}$ and $r_{2000}=0.70^{***}$) when inbred lines were planted into cold, wet soils. Cluster analyses indicated that all the inbred lines used in these experiments could be placed into one of 7 groups based on their overall cold tolerance ratings.

INTRODUCTION

Maize grown in temperate regions is often subjected to chilling conditions before and after emergence that can lead to disruption of development and poor stand establishment (Hope, 1992). This lack of early vigor in many US inbred lines and hybrids limits their use in locations with wet and cool springs like those of the European Atlantic Coast.

Seedling vigor is usually measured as the weight or size of young seedlings that depend on endosperm reserves (heterotrophic period). However, when these reserves are depleted, seedlings depend upon their own ability to generate assimilates and produce a canopy. Two to three weeks after emergence, a difference in the ability to produce assimilates results in different canopy size, color, and general appearance of the young plants. Collectively, these traits allow the exhibition of what is often referred to as *early vigor.*

Several researchers have studied the relationship between seed size and early seedling growth in maize (Derieux, 1989; Pommel, 1990). Hawkins and Cooper (1979) found significant seed size effects on plant growth during the heterotrophic period. Effects of seed weight had a significant association ($r=0.67^{***}$) with early vigor (Revilla et al., 1999). They indicated that those inbred lines producing heavier seeds should be used as the female parent in a hybrid cross, so the resulting hybrid will have better early vigor and flower earlier.

Burris and Navratil (1979) studied several options of the cold vigor test and found that for inbred lines, much of the cold test response was due to temperature and little to the soil (e.g. microorganisms). They found variable results depending on the method

used, planting date and location. Studies of soil factors usually focused on the standardization of fungal inoculants (Burris, 1976). Some studies show that various *Pythium sp.* concentrations and artificial media can be a suitable substitute for soil in the standard cold test. However, these procedures have rarely shown improved correlations with field emergence (FE) than the standard cold test, although they can potentially be easier to standardize.

Correlation between laboratory and field testing

In these studies, cold tolerance is defined as the ability to germinate, emerge, and grow under low temperatures. Percent field emergence under cold conditions was significantly correlated ($r= -0.59^{***}$) with time of emergence, root and shoot mass, and seed mass in maize (Hotchkiss et al., 1997). The negative correlation implies that as germination and emergence under cold conditions increase, time to emergence decreases.

The minimum temperature for germination, emergence and growth in maize is approximately 9 to 10◦C (Blacklow, 1972; Crevecoeur et al., 1983; Eagles and Hardacre, 1979a). Comparison of time to coleoptyle and root production at two temperatures (11 and 25◦C) revealed no correlation at either temperature (Hope and Maamari, 1994). This indicates that the mechanisms involved during germination at 11◦C are different from those at 25◦C.

There was good correlation between the minimum temperatures in the chilling tests, percent germination and viability with percent field emergence, showing that the most chilling-susceptible maize inbreds in the laboratory corresponded to those

identified as susceptible in the field studies (Hodges et al., 1994). From daily observations on seed germinated (in a germinator) at 11◦C, mean time to 50% production of a 1-cm coleoptyle was a useful screening parameter to select maize hybrids with superior cold tolerance during germination and early growth (Hope et al., 1992). Research in both laboratory and field environments indicate that rapid emergence is associated with high percent germination (Mock and Eberhart, 1972; Eagles and Hardacre, 1979a).

Martin et al. (1988) found that the soil cold test was better than other tests for predicting field emergence for a wide range of inbred lines in a number of environments ($r = 0.74^{**}$), although Burris and Navratil (1979) showed that the cold test is not consistently reliable due to its inherent variability which makes comparison between laboratories difficult.

The conductivity of the electrolytes in the bathing solution leached from the apoplast of imbibing seeds was also highly correlated with field emergence among different sweet maize hybrids in a study by Tracy and Juvik (1988). Conductivity was negatively correlated with field emergence ($r = -0.58^{**}$), while standard laboratory germination was poorly correlated with field emergence ($r = 0.18$). In the same study, when conductivity data was combined with data on dry weight of seedlings from the cold test, the correlation with field emergence improved ($r = -0.80^{**}$). After ten cycles of selection for field emergence and seed weight, conductivity was reduced by indirect selection and thus may be an effective breeding tool to aid in selection for improved field emergence.

Early work in rice by Jones and Peterson (1976) showed good correlations between seedling characteristics (measured in the laboratory by the slantboard test) and seedling vigor (measured by percent emergence) under field conditions. Similar correlations were observed by McKenzie et al. in 1980. Andrew (1982) observed seed size and seed weight to be correlated with root length, root/shoot ratio and germination percentage in shrunken-2 maize.

Results from more than one test for seed and seedling performance and marker data on a wide range of genetic materials (hybrids, inbred lines, etc.) should enable the development of relationships between laboratory and field vigor indices. Wilson et al. (1992), in a search for the highest correlations between vigor test results and final stand count in *sh2* (shrunken-2) maize, concluded that the model with the highest correlation was one that involved the accelerated aging test, leachate conductivity test and embryo weight ($r = 0.78$).

There are several reports about the effect of seed and seedling vigor on yield, but these are not nearly so conclusive as those about seedling emergence. Low vigor seed lots primarily result in reduced field performance and stand establishment (Edje and Burris, 1971). Reduction in yield due to low vigor seed lots has been attributed to low stand density (Perry, 1980). Egli and Tekrony (1979) reported that seed vigor had no influence on emergence, stand or yield under optimum field conditions.

Influence of tillage systems on soil temperature

Low soil temperatures are a major limitation to the direct seeding of many agronomic and horticultural crops, particularly in areas that have relatively short growing

seasons (Herner, 1986). Adoption of conservation tillage has resulted in lower seedbed temperatures in the spring and further increases the need for maize hybrids with superior cold tolerance during germination and early growth (Hayhoe et al., 1996).

Conservation tillage, especially no-tillage, results in more variable emergence rates and reduced stand establishment compared to conventional tillage (Wall and Stobbe, 1983; Gupta et al., 1988; Hayhoe et al., 1993). The physical condition of the seedbed influences seed-soil contact, which can delay and further reduce emergence (Johnson and Wax, 1976; Hayhoe et al., 1996). Percent field emergence and time to emergence are also reduced under no-till conditions. Schneider and Gupta (1985) demonstrated that emergence was delayed for the largest aggregate size because of poor soil-seed contact and for the smallest aggregate size because of high soil penetration resistance.

Tillage system, planting date and soil texture differences often result in a range of seedbed temperature and water conditions (Dwyer et al., 2000). Their results indicate that an interaction between cool soil temperatures (below 12◦C) and high soil water content (field capacity) reduced stand establishment. Tekrony et al. (1989) reported that colder soil temperatures resulted in lower field emergence in non-tilled fields than on conventional tilled soils.

OBJECTIVES

The objectives of this work were to examine the relative performance of cold germination, thermogradient plate germination, and coleoptyle growth to predict field emergence among maize inbred lines. Relationships among inbred lines for cold tolerance were also investigated.

MATERIALS AND METHODS

Seed Source: All 95 inbred lines used are proprietary materials of Monsanto Global Seed Group (St. Louis, MO). Seeds were untreated unless noted for each specific experiment.

Seed lots were subjected to several experiments. A detailed description and the purpose of each test was provided in Chapter 1 (Materials and Methods).

Cold germination: two replicates of 10 seeds each were germinated in petri dishes in a cold chamber at constant 10°C. Moist filter paper was used as media. All seeds were treated with Captan (cis-N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide). Seeds were considered germinated once the radicle reached 1 cm in length. Counts were made at several times after the initiation of the experiment.

Thermogradient Plate: two rows, each representing one replication, were planted for each seed lot in a thermogradient plate as explained in Chapter 1. The minimum temperature at which seeds germinated was recorded at 7 and 14 days after planting (DAP).

Coleoptyle growth: two replications of eight seeds each were planted in inert media in plastic trays. All trays were placed in a 23◦C chamber to allow for imbibition and sprouting of seeds without the damaging effects of imbibitional injury. After 36 hours, all trays were transferred to a 10◦C chamber where they remained until the completion of the experiment. Coleoptyle length was measured at several intervals after planting.

Field emergence: seeds of 95 seed lots were mechanically planted at 20 seeds in a 5.4 m row in all locations. Row spacing was 0.76 m. In 1999, the field experiment was conducted in East Lansing, MI and in Waterman, IL in 2000. For both locations, the first planting date was purposely targeted 3 weeks earlier than normal corn planting conditions for the area (April 8, 1999 and April 12, 2000). A second planting date (for Michigan only) was completed April 25, 1999. The last planting dates were May 3, 1999 and April 25, 2000, respectively. The final planting dates were considered normal planting times for maize in each location. Soil temperatures were monitored with two Hobo Pro Series data loggers (Onset Computer Corporation, 470 MacArthur Blvd., Bourne, MA). In 1999 and 2000, the experimental design was a randomized complete block design with two replications. Stand counts were performed periodically, at an interval of 5 days between counts. Counts started as soon as the first emerged seedling was observed. Counts were continued until most plants in a row reached the V3 stage. In 1999, due to excessive rain and a low area in the field in Michigan, all data from planting date 2 (PD2) were removed from the analyses.

Analyses of data: data were analyzed using the PROC GLM and PROC ANOVA statements in SAS (SAS Institute Inc., 1999). Least significant differences at the 5%

level were calculated by the PROC MEANS procedure, applying the Fisher's LSD option. The data set was subjected to ANOVA and Pearson's correlation coefficients and principal component analyses (PCA). The matrix of raw means (95 inbreds x 19 tests) was converted to an Inbred x Test Interaction (ITI) matrix by subtracting the row, column and grand means from each cell. Individual matrixes containing each laboratory test (cold germination, thermogradient plate germination and coleoptyle growth) and field emergence were generated to analyze the interaction of inbred lines within each test. Eigenvectors and eigenvalues were computed from a variance-covariance similarity matrix derived from the ITI matrix. First and second principal components were obtained from a projection of the ITI effects matrix and the eigenvectors and eigenvalues calculated using NTSYS-pc (Rohlf, 1992). A tree matrix from a UPGMA cluster analysis of distances was computed to study the relationships among inbred lines for overall cold tolerance, including data of all observations for cold germination, thermogradient plate germination, coleoptyle growth and field emergence.

RESULTS

In the Northern US Maize Belt and Europe, maize is often planted early in the spring into soils that are too cold and wet to provide an optimal environment for germination. For this reason, improvement and prediction of seedling emergence and early seedling growth is important to the seed industry and has been the objective of several maize breeding programs (Grogan, 1970; Martin et al., 1988; Greaves, 1996). One of the problems in evaluating cold tolerance has been the lack of consistent results for a large number of genotypes over a period of several years. We report consistent significant associations between laboratory protocols and field emergence for a large number of maize inbred lines, over 2 years and 2 locations.

Soil temperatures at PD1 were 9.2◦C in 1999 and 6.8◦C in 2000 (Appendix Tables A5 and A6). At planting date 3 (PD3), soil temperatures were 13.6◦C in 1999 and 12.3◦C in 2000. Total field emergence increased as planting dates were delayed and as soil temperatures increased (Tables 2-1, A5 and A6). Seedbed conditions in the spring of both years were typical of unfavorable cold, wet soils.

Cold germination ranged from 0 to 97.9%. The minimum temperature for germination determined on a thermogradient plate ranged from 11.9◦C up to 18.3◦C after 7 DAP and from 8.7◦C up to 16.0°C after 14 DAP. Coleoptyle growth varied from 0 cm after 7 days up to 6.0 cm after 14 days. Significant differences within inbred lines were measured for each one of these tests (Tables 2-1 and 2-2). The Coefficient of Variation (C.V.) decreased as the number of days after initiation of the experiments increased (Table 2-1). Thermogradient plate germination had the lowest C.V. (11%)

and the first count of field emergence on PD1 had the highest with 155%. This value was significantly reduced as other field emergence counts were completed at later dates.

Significant differences for cold germination, thermogradient plate germination, and coleoptyle growth were measured among inbred lines in 1999 and 2000 (Tables 2-2 and 2-3). In cold germination and coleoptyle growth experiments, there were no major changes in rank order among inbred lines when comparisons were made at several intervals after the initiation of the experiments. However, a significant interaction was observed between inbred lines and DAP in thermogradient plate germination.

Among inbred lines (Table 2-2), 1111 exhibited the highest cold germination (97.9%), germinated at the lowest temperature in the thermogradient plate (8.7◦C), and had the largest number of plants per row emerged in 2000 (18.5 plants/row). Inbred line 1007 had the largest coleoptyle growth and inbred line 1092 the largest number of seedlings emerged per row in 1999 (15.5 plants/row). Among the most susceptible inbred lines, 1009 showed the lowest cold germination (2.1%). Inbred 2103 germinated at the highest minimum temperature (16◦C), while 1061, 1099 and 1104 did not grow at 10◦C (0 cm of coleoptyle growth). Inbred lines 1099, 1100, 2005, 2078 and 2091 exhibited very poor field performance.

Table 2-1. Summary statistics for thermogradient plate germination, cold germination, coleoptyle growth and field emergence for several inbred lines. Each row, below the experiment name, indicates the number of days after the initiation of the experiment when observations were taken.

Variable	N	Mean	Minimum	Maximum	Range	Std Dev	C.V.
Thermogradient Germination		°C	°C	°C	°C	°C	%
7 days	96	14.8	11.9	18.3	6.3	1.9	12.7
14 days	96	10.9	8.7	16.0	7.3	1.3	11.6
Cold Germination		%	%	%	%	%	%
7 days	94	26.3	0.0	93.8	93.8	1.8	89.2
10 days	94	73.8	0.0	97.9	97.9	1.7	28.3
14 days	94	80.0	6.3	97.9	91.6	1.6	24.1
Coleoptyle Growth		cm	cm	cm	cm	cm	%
7 days	98	0.7	0.0	2.3	2.3	0.7	92.2
10 days	98	1.6	0.0	3.8	3.8	1.1	70.8
14 days	98	2.5	0.0	6.3	6.3	1.6	66.5
Field Emergence[§]		plants	plants	plants	plants	plants	%
PD1C1[†]	98	2.0	0.0	16.5	16.5	3.1	155.3
PD1C2	98	6.3	0.0	18.5	18.5	4.9	77.6
PD1C3	98	7.8	0.0	19.0	19.0	5.3	68.2
PD1C4	98	8.0	0.0	19.5	19.5	5.3	65.8
PD2C1[‡]	98	12.8	0.5	19.5	19.0	4.2	33.1
PD2C2	98	15.4	2.5	20.0	17.5	3.4	22.1
PD2C3	98	15.2	3.5	20.0	16.5	3.3	21.9

[§] Field emergence counts were taken at 5-day intervals after first seedling was observed

[†] PD1C1= planting date 1; emergence counts 1, 2, 3 and 4

[‡] PD2C1= planting date 2; emergence counts 1, 2 and 3

Table 2-2 Cold germination, thermogradient plate germination, coleoptyle growth and field emergence of inbred lines in 1999 and 2000.

Inbred Line	Cold Germ -----%-----	Thermog -----°C-----	Coleoptyle -----cm-----	1999 FE -----plants/row-----	2000 FE -----plants/row-----
1001	52.08	14.1	1.60	7.6	8.3
1004	39.58	13.3	0.50	7.1	9.3
1005	50.00	10.6	0.76	13.3	14.8
1006	41.67	11.2	0.70	6.5	5.3
1007	27.08	12.8	6.20	6.9	6.3
1009	2.08	9.5	3.30	2.4	5.3
1014	66.67	10.3	1.18	14.6	12.8
1015	56.25	9.5	0.00	11.0	13.8
1018	85.42	10.3	5.10	12.0	12.5
1036	81.25	10.2	0.36	7.8	8.3
1045	43.75	11.8	5.00	7.9	8.8
1046	68.75	11.8	0.92	11.3	12.3
1047	93.75	ND	0.60	11.5	15.5
1048	70.83	10.2	4.60	14.5	16.5
1050	58.33	9.2	0.66	9.0	13.8
1052	75.00	9.7	1.94	11.4	13.3
1053	83.33	9.2	3.30	14.4	17.0
1055	35.42	11.1	0.84	7.1	7.5
1058	77.08	10.1	0.50	10.1	12.3
1061	77.08	9.2	0.00	13.4	16.3
1062	72.92	9.7	0.32	11.8	13.5
1082	81.25	9.5	3.12	8.5	15.8
1086	58.33	10.1	2.34	12.8	7.8
1092	66.67	9.7	2.64	15.5	14.8
1096	81.25	10.1	0.40	4.0	4.3
1097	72.92	10.2	3.56	8.1	9.3
1098	66.67	11.7	0.74	6.8	9.8
1099	22.92	12.3	0.00	1.5	2.3
1100	43.75	13.2	2.60	4.6	2.3
1104	83.33	9.7	0.00	11.5	14.3
1107	79.17	10.6	4.82	11.3	13.3
1108	68.75	11.2	1.14	11.9	13.8

Table 2-2 (cont'd)

Inbred Line	Cold Germ -----%-----	Thermog -----°C-----	Coleoptyle -----cm-----	1999 FE -----plants/row-----	2000 FE -----plants/row-----
1110	68.75	10.6	1.30	12.9	16.5
2002	68.75	10.6	4.37	ND	13.3
2005	45.83	12.6	0.60	ND	1.3
2011	68.75	10.8	1.75	ND	5.5
2014	70.83	11.9	3.43	ND	11.0
2015	70.83	11.9	2.15	ND	11.3
2016	60.42	12.1	2.97	ND	11.5
2017	56.25	14.0	2.88	ND	7.5
2018	41.67	12.4	2.73	ND	10.0
2021	51.04	11.6	2.87	ND	7.0
2022	47.92	11.6	4.13	ND	9.0
2025	75.00	10.5	1.37	ND	9.8
2026	75.00	10.9	3.05	ND	9.3
2027	70.83	10.5	2.55	ND	10.8
2028	64.58	10.4	4.32	ND	5.8
2029	52.08	12.1	1.97	ND	4.8
2032	79.17	12.1	4.58	ND	10.5
2033	60.42	11.5	3.40	ND	11.5
2037	72.92	11.3	4.37	ND	11.3
2038	81.25	11.4	3.38	ND	11.8
2043	81.25	10.9	3.72	ND	6.0
2044	54.17	11.2	0.55	ND	8.0
2046	66.67	11.5	1.58	ND	9.0
2051	68.75	10.5	3.80	ND	13.5
2056	68.75	10.2	3.32	ND	12.5
2060	52.08	10.6	3.75	ND	8.8
2061	58.33	9.6	2.93	ND	4.5
2065	54.17	11.3	2.90	ND	11.3
2066	68.75	11.5	2.20	ND	6.3
2067	64.58	10.6	2.27	ND	10.8
2068	52.08	10.5	3.60	ND	8.3
2073	50.00	12.5	0.82	ND	5.3
2074	31.25	11.9	2.80	ND	3.3

Table 2-2 (cont'd)

Inbred Line	Cold Germ -----%-----	Thermog -----°C-----	Coleoptyle -----cm-----	1999 FE -----plants/row-----	2000 FE -----plants/row-----
2076	83.33	9.1	3.35	ND	5.8
2078	50.00	12.6	2.72	ND	1.8
2081	54.17	10.3	3.15	ND	7.5
2082	60.42	10.8	2.87	ND	4.3
2083	41.67	12.3	2.72	ND	4.8
2084	72.92	10.3	3.53	ND	4.8
2089	62.50	10.4	2.77	ND	8.8
2090	83.33	9.5	3.05	ND	7.3
2091	25.00	12.7	0.40	ND	0.3
2103	33.33	16.0	1.07	ND	3.0
2105	43.75	11.4	0.97	ND	9.0
2107	66.67	11.4	2.92	ND	6.5
2110	25.00	11.1	0.62	ND	2.8
2114	58.33	11.0	3.05	ND	10.3
2115	47.92	10.6	4.72	ND	12.0
2116	14.58	12.9	0.68	ND	2.8
2118	58.33	10.0	4.82	ND	9.3
2119	72.92	9.5	2.22	ND	15.8
2122	60.42	9.4	2.37	ND	10.3
2125	62.50	10.8	3.02	ND	9.5
2126	ND	11.8	2.20	ND	6.8
2127	75.00	11.4	2.43	ND	8.5
2128	47.92	9.4	2.50	ND	8.5
2129	77.08	9.4	3.58	ND	12.8
2130	85.42	10.6	1.98	ND	12.8
2131	54.17	11.8	0.15	ND	8.5
1111	97.92	8.7	4.00	ND	18.5
2222	66.67	10.1	2.20	ND	14.5
3333	93.75	9.0	2.05	ND	14.0
$LSD_{5\%}$	21.38	2.0	0.68	2.9	3.4

FE = Field Emergence. Field emergence counts were taken at 5-day intervals after first seedling was observed.

Thermog = Temperate at the Thermogradient Table.

ND = no data available

Table 2-3. Degrees of freedom (df) and mean squares (MS) from analyses of variance for the observed laboratory traits and field emergence.

Source of variation	Germination		Thermogradient		Coleoptyle		Field Emergence	
	df	MS	df	MS	df	MS	Df	MS
Inbred	92	12.4***	92	5.71***	92	8.72***		
Days after planting (DAP)	2	1083.9***	1	1342.9***	2	1167.2***		
Inbred x DAP	184	NS	92	4.77***	184	NS		
Error	285	2.3	188	0.89	368	0.75		
Inbred (I)							97	103***
Planting date (PD)							1	7893***
Year (Y)							1	172***
I x PD							97	154***
I x Y							34	12.6**
I x PD x Y							34	NS…
Error							406	7.36

*** = significant at the 0.001 level of probability
** = significant at the 0.01 level of probability
NS = not significant

There were large significant differences in mean field emergence for inbred lines in both years. In 2000, several inbred lines were included in the studies that were not available in 1999. Although significant interactions between inbred line and year, and inbred line and planting date were observed, there were no interactions between planting date and year. Significant variation occurred due to the effects of inbred lines (I), year (Y) and planting date (PD), as well as the interaction among them (Table 2-3). However, within each planting date, the most important sources of variability for field emergence were differences among inbred lines, and less among environments (location and/or year of test). Results indicate that the average inbred effect was more important than any other treatment effect in determining field emergence response.

There were highly significant correlations between the laboratory protocols and field emergence in 1999 and 2000 (Table 2-4). In 1999, cold germination at 14 DAP was highly correlated with field emergence ($r=0.78^{***}$) whereas thermogradient plate germination and coleoptyle length were also significantly correlated but at lower values, -0.63^{*} and 0.51^{*}, respectively. The negative correlation between thermogradient plate germination and field emergence is due to the lower germinating temperature of an inbred line and the association with better field emergence under cold stress. Stepwise regression analyses indicated that the combination of cold germination and thermogradient plate germination was the best predictor of field emergence ($r_{1999}=0.64^{**}$ and $r_{2000}=0.70^{***}$) when inbred lines are planted into cold, wet soils.

Table 2-4. Pearson's correlation coefficient (r) for cold germination, thermogradient plate germination and coleoptyle growth and field emergence.

Test	**PD1-99**[§]	**PD3-99**	**PD1-00**	**PD2-00**
Germination (7dap)	0.65**	0.66**	0.68**	0.53**
Germination (14dap)	0.65**	0.78**	0.59**	0.51**
Germination (21dap)	0.68**	0.65**	0.64**	0.58**
Thermogradient (7dap)	-0.40[†]	-0.63*	-0.38**	-0.43**
Thermogradient (14dap)	-0.35[†]	-0.51**	-0.48**	-0.38**
Coleoptyle growth (7 dap)	0.51*	NS	0.51**	0.45**
Coleoptyle growth (14 dap)	0.46*	NS	0.45**	0.47**
Germ + Thermog	0.64**	0.68**	0.70**	0.61**
Germ + Therm + Coleop	0.65**	0.68**	0.70**	0.68**

[†] = Significant at the 10% level
* = Significant at the 5% level
** = Significant at the 1% level
NS = not statistically significant
[§] = PD1-99= planting date 1, 1999; PD3-99= planting date 3, 1999; PD1-00= planting date 1, 2000; PD2-00= planting date 2, 2000

Table 2-5. Pearson's correlation coefficients (r) for field emergence in 1999 and 2000.

Test	PD1-00	PD2-00
PD1-99[§]	0.91**	0.55*
PD3-99	0.58**	0.69**

* = Significant at the 5% level

** = Significant at the 1% level

[§] = PD1-99: planting date 1, 1999; PD3-99: planting date 3, 1999; PD1-00: planting date 1, 2000; PD2-00: planting date 2, 2000

In 2000, cold germination had the highest correlation with field emergence (r=0.68***). Thermogradient plate germination (r=-0.48***) and coleoptyle growth (r=-.51***) were also significantly correlated. Data from year 2000 confirmed several results from 1999 while providing substantial information on a different set of inbreds. Correlation coefficients were calculated using 35 inbred lines in 1999 and 95 inbred lines in 2000. There was also a significant correlation (0.91***) between field emergence in 1999 and 2000.

A tree phenogram, calculated from a UPGMA cluster analysis of distances of the phenotypic measurements of cold tolerance is shown in Fig. 2-1. In the phenogram, the horizontal distance at which each point (inbred lines) connects is an indication of the degree of association among them. All 95 inbred lines were grouped into 7 clusters and these relationships of clusters is indicated in the biplots.

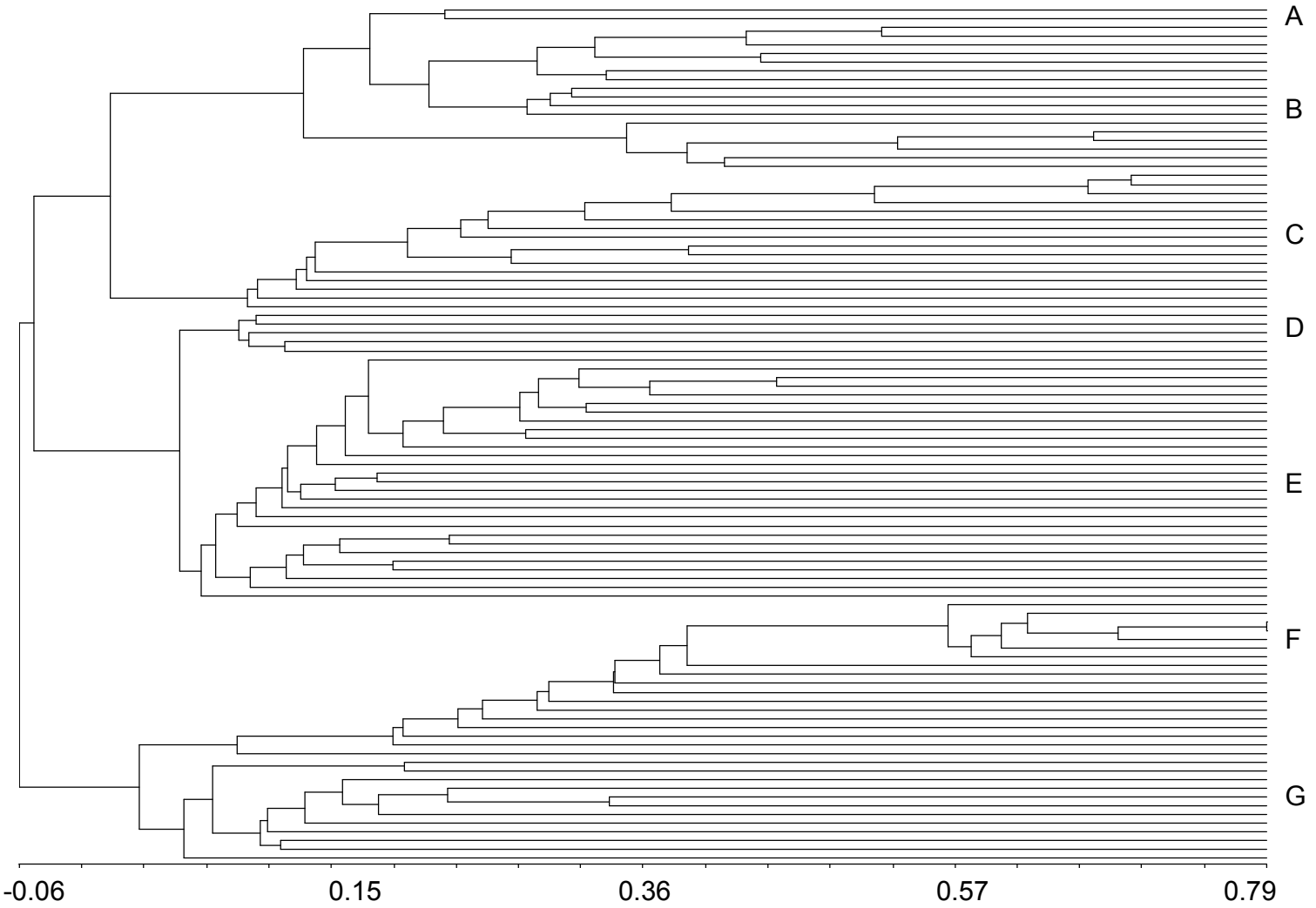

Fig.2-5. Cluster tree describing the degree of relationship among inbred lines for 19 traits for cold tolerance.

DISCUSSION

Maize is often planted into cold and wet soils in template climates, leading to reduced germination, poor stands and lower economic yields. These studies were conducted to determine the merit of several laboratory protocols (cold germination, rate of coleoptyle growth and thermogradient plate germination) in the prediction of field emergence when soil temperatures are considered stressful for optimum maize germination and growth.

Reliable and consistent predictions of field emergence are difficult. However, it is essential to the seed industry if it is to produce and sell high quality maize seed. The ability to identify and select cold tolerant, vigorous inbred lines is an important step in this process, since germination under cold stress in a hybrid is influenced by the maternal parent.

Due to shortage of seeds for experimental purposes, only one location was planted each year. However, significant correlations over two years in two distinct locations and soil types provide a strong indication that the laboratory protocols described herein are valuable and powerful tools in screening a large number of inbred lines for their response to cold soil conditions. These protocols also provided consistent, strong correlations when hybrid seed lots were tested (data not shown).

As proposed by Burris (1976), the cold test will be difficult to standardize as long as soil is a component. They report that significant variability exists when comparing results from different laboratories. This variability among laboratories is unacceptable to private seed companies. A cold germination test that

incorporates only the cold temperature and excludes the confounding factor of soil media was developed and tested. Similar results have been reported (Alessi and Power, 1971; Mandel, 1961). This “sterile” version of the cold test should be easier to conduct and standardize and can be adaptable to most seed testing laboratories. It was especially useful for our purposes of identifying tolerance to low temperatures during germination.

The lowest coefficient of variability in any laboratory protocols was recorded by the thermogradient plate germination. Although differences in minimum temperature for seed germination were small, they were significant. The thermogradient plate can efficiently screen a large number of seed lots for the minimum temperature required to germinate. The only limitation for its adoption on a larger scale is the lack of availability and costs of thermogradient plates. Coleoptyle growth had 3 times more variation compared to cold germination. Since most laboratories already conduct some version of the cold vigor test, the adoption of our proposed test for cold tolerance determinations should be easy.

The cold vigor test was designed to measure seed vigor differences and to determine if physiological problems are present in a seed lot. Such problems are usually due to environmental conditions during seed maturation, mechanical injury during harvesting and processing, and storage. Although the cold germination test described herein has the potential to identify similar problems, it was developed to detect genetic differences in the ability of a seed lot to

germinate at cold temperatures. Thus, it is implied that all inbred lines or seed lots tested by the cold germination test will have high seed quality levels.

Data presented here show significant correlations between our laboratory protocols and field emergence under cold temperatures. Kulic and Yaklich (1982) stated that r values greater than 0.30 represent a significant relationship when studying biological materials. All laboratory protocols developed and tested in these studies had higher correlations with field performance (germination and emergence under cold temperatures) than 0.30. These protocols were designed to aid in predicting germination and emergence of maize seeds under a wide range of conditions. These tests provide an inexpensive, reliable and repeatable means to detect genetic variability for cold tolerance. In addition, inbred lines for use in breeding or mapping experiments as good sources for chilling tolerance were identified.

Results of field screening for performance under early planting are often unreliable because of unpredictable variability in spring conditions (Richner et al., 1997). Traditional evaluation methods have enabled researchers to make significant gains to increase cold tolerance in maize. However, there is a need to develop and evaluate indirect selection criteria for improved germination and early growth of maize at low temperatures. Three laboratory screening protocols that can efficiently and successfully discern between cold tolerant and cold susceptible inbred lines or hybrids are described. These tests should also have value in predicting the performance of a seed lot when planted under early spring conditions of cold and wet soils.

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CHAPTER 3

Identification of quantitative trait loci controlling cold tolerance traits in maize

ABSTRACT

Maize grown in North America is often subjected to chilling temperatures at planting time, leading to disruption of plant development. A BC1F2 population (self-pollinated progeny of BC1 individuals) with 147 families was developed from the cross of two inbred lines, 1111 (cold tolerant) and 2222 (cold susceptible). A linkage map was constructed with 89 SSR markers spanning 1570 cM and encompassing the 10 maize chromosomes with an average marker spacing of 30 cM. Cold germination, coleoptyle growth and field emergence were measured on 147 BC1F2 families. Using interval mapping and single factor analyses a total of 21 QTLs, accounting for 8 to 76% of the variability, were identified and mapped. Eight QTLs were identified linked to coleoptyle length, six QTLs were linked with germination under cold temperatures, and seven QTLs were associated with field emergence in cold soils. All QTLs were generally clustered on three linkage groups and were consistently associated with field emergence and coleoptyle growth or cold germination.

INTRODUCTION

Maize grown in temperate regions is often subjected to chilling conditions (4 to 12°C) before and after emergence. Cold temperatures can prevent germination and disrupt seedling development (Hope, 1992). Genetic and physiological characteristics that improve low temperature stress tolerance during germination and early seedling growth are of interest to maize producers (Prasad, 1997). The exploitation of genetic variation associated with the underlying processes of cold tolerance through either phenotypic or genotypic selection cannot occur until the component traits responsible for stand establishment are understood.

Genetic variation for germination and early seedling growth under suboptimal temperatures appears to exist (Eagles, 1979a and 1988; Eagles and Brookings, 1981; Martin et al., 1988). Inbred lines used in cool temperature regions generally have better heterotrophic and autotrophic shoot growth and faster development than inbred lines adapted to warm tropical conditions (Verheul et al., 1996). This is due to higher rates of relative growth and leaf area expansion, resulting in a higher net assimilation rate at lower temperatures.

Increased capacity for seed germination and emergence under cold conditions has been recognized as a valuable attribute in maize for several decades (Neal, 1949; Haskell and Singleton, 1949). Heritable variation for seedling response to cold temperatures has been reported in several crops and has been considered adequate for the improvement of populations by selection (Mock and Eberhart, 1972; Mock and Bakri,

1976; McConnell and Gardner, 1979; Greaves, 1996). There appears to be limited genetic variation for tolerance to freezing temperatures in maize (Gardner et al, 1987).

The genetic nature of cold tolerance in maize is complex because of significant maternal or cytoplasmic effects associated with the genetics of germination and early growth potential (Pinnell,1949; Helgason, 1953; Grogan, 1970). Significant differences exist between maize lines and cultivars with respect to their capability to germinate at low temperatures (Neal, 1949; Haskell and Singleton, 1949). The genotype of the embryo determines, to a large extent, the behavior of the seed under cool, wet soil conditions. The degree of tolerance to low temperatures (8 to 12◦C) is strongly dependent on the germination potential of the maternal parent of a hybrid (Pesev, 1970). The characteristics of the maternal parent are important in determining not only the percentage of germinated plants, but also the rate of germination and growth of the embryo root and stalk apex.

Chilling sensitivity in maize at vegetative growth stages has been shown to be complexly inherited because of significant maternal effects associated with germination and early seedling growth (Eagles and Hardacre, 1979b; Maryam and Jones, 1983). However, no maternal effects were reported in six reciprocal maize hybrids evaluated at the early growth stage (Aidun et al., 1991). The expression of maternal effects for cold tolerance, therefore, may be restricted to the germination-emergence phase of maize development. Maternal effects were observed for the average time to emergence, in hybrids with CO255 as the maternal parent. These hybrids took significantly less time to emerge than those developed from other inbred lines (Hodges et al., 1997). When CO255 was used as a male, time to emergence was significantly increased. From that

same study, maternal effects had a larger effect at the early seedling growth phase than at the germination-emergence phase.

Similar genetic systems condition cold tolerance in both 'warm' and 'cold' environments (Mock and Eberhart, 1972). These genes were independent of genes controlling stand and maturity under 'normal' planting conditions. Some lines that were initially chilling sensitive or tolerant at the germination stage altered their sensitivity to chilling at the early growth stage (Hodges et al., 1997). These findings suggest that it should be possible to evaluate chilling tolerance of maize by examining plants at both the germination-emergence and early growth stage since the two stages appear to be under the control of different genetic factors.

Epistatic gene effects as well as additive and dominance gene effects have been reported to contribute significantly to the variation observed for germination of maize at 7.2 C in the laboratory and for emergence measured in the field (McConnell and Gardner, 1979). Seedling vigor or growth after emergence in the field appeared to be conditioned predominantly by additive and dominant gene effects. Heritability of seedling dry weight in maize was estimated to be between 0.54 and 0.57, with an expected gain from selection ranging between 7 to 8% per cycle (Martiniello, 1985). Similar results were reported in rice, where genes with additive and/or additive x additive effects controlled a major portion of the phenotypic variability (Li and Rutger, 1980).

Physiology of cold tolerance

Chilling responses occur at temperatures between 4 and 12◦C in chilling-sensitive species, and are frequently associated with changes or degradation in cellular lipid

composition (DeKok and Kuiper, 1977). Chilling induces oxidative stress in 3-day etiolated seedlings, which do not survive 7 days of 4◦C stress unless they are acclimated at 14◦C before 4◦C stress (Prasad et al., 1994; Prasad, 1997). Severe water stress was not observed in maize seedlings when they were exposed to 24 hr of 8◦C stress (Wolfe, 1991). Photosynthesis was reduced between 5% and 30%.

Although acclimation-induced chilling tolerance is developmentally regulated, no tolerance was observed in non-acclimated developing seedling subjected to 7 days of 4◦C stress (Prasad, 1997). Cold acclimation is cumulative with time and involves the synthesis, or increased accumulation, of particular subsets of proteins in plants (Limin et al., 1997). Cold acclimated plants reduce their water content and, upon exposure to freezing temperatures, move water from the cell protoplasm to intercellular ice crystals, causing severe dehydration stress within the cells. The status of oxidative stress and antioxidant defense system determines the extent of chilling injury and tolerance in non-acclimated and acclimated seedlings, respectively (Prasad, 1997). Tolerance to suboptimal temperatures in maize has been improved by manipulating the antioxidant defense system (Foyer et al., 1995).

Chilling tolerance of plants can also be altered by genetically manipulating the fatty acid desaturation by introducing double bonds into fatty acids of membrane lipids. Polyunsaturated fatty acids in the glycerolipids of thylakoids membranes are important for the ability to tolerate low temperatures but not high temperatures (Wada et al., 1994). Transgenic cyanobacterium acquired the ability to introduce a second double bond into palmitoleic acid and oleic acid that enhanced the tolerance of the photosynthetic machinery to chilling stress.

No apparent association between fatty acid composition and response to imbibition or germination has been observed when analyzing the concentration and type (unsaturated vs. saturated) of fatty acids in maize kernels prior to germination (Zemetra and Cuany, 1991). However, Murata et al. (1992) reported that the level of fatty acid unsaturation of phosphatidylglycerol and the degree of chilling sensitivity of *Nicotiana tabacum* can be manipulated by transformation with glycerol 3 phosphate acyltransferases from squash and Arabidopsis. Murata et al. (1992) concluded that the chilling sensitivity of plants is closely correlated with the degree of unsaturation of fatty acids in chloroplast membranes.

Mapping genes conditioning cold tolerance traits

It is well known in the seed maize industry that there is a genetic as well as a physiological basis for differences in vigor in maize. Thus, it should be possible to characterize the potential for different vigor levels in both inbred lines and maize hybrids. Molecular mapping techniques should be used to identify marker-linked QTLs (quantitative trait loci) contributing to chilling tolerance at different stages of germination and seedling development and to improve our knowledge of the genetic control of chilling tolerance and seedling vigor in maize. Although this has not been reported in maize, it has been done for other crops. In tomato three chromosomal regions, accounting for 45% of the phenotypic variance, had significant effects for improving on low temperature germination (Foolad et al., 1998). Graphical genotyping indicated a high correspondence between the phenotypes of the tolerant and susceptible families and their QTL genotypes. Thirteen QTLs (accounting for 7 to 38% of the phenotypic

variance) related to seedling vigor were identified in rice using a restriction fragment length polymorphism's (RFLPs) linkage map (Redona and Mackill,1996b). Four QTLs controlling shoot length, two each for root and coleoptyle lengths, and five influencing mesocotyl length were identified.

The QTLs expressed in the Redona and Mackill (1996a) studies suggest that some type of genotype-by-environment interaction may be involved in the expression of seedling vigor traits in rice at the molecular level. Genotype-by-environment interactions that are of major importance in many quantitative traits can only be studied by separately analyzing the data collected in multiple environments. Multiple QTL mapping (MQM) developed by Jansen et al. (1995) could be very useful, since they accommodate both the mapping of multiple QTLs and the QTL-by-environment interaction that forms part of the models fitted.

Molecular techniques

Restriction fragment length polymorphism (RFLP) markers have been used to find genetic differences among individuals and have overcome many of the constraints associated with the use of morphological or biochemical markers (Tanksley, 1983). RFLPs have proven to be highly polymorphic in maize and useful for assigning maize inbred lines to heterotic groups and to detect pedigree relationships among lines (Dudley et al., 1991; Melchinger et al., 1991; Mumm and Dudley, 1994). However, RFLP analysis is time consuming, labor intensive and relatively expensive.

Random amplified polymorphic DNA (RAPD) markers utilize polymerase chain reaction (PCR) technology, and have proven to be relatively simple, rapid and cost

effective when compared to RFLPs (Staub et al., 1996). RAPDs have some limitations, such as high levels of artifactual variation, limited reproducibility and occurrence of non-parental bands (Ellsworth et al., 1993). These limitations requires experiments to be repeated several times to identify consistent bands.

Simple sequence repeats (SSR), also called microsatellites, are tandem repeat motifs of di-, tri-, or tetra-nucleotides which are abundant in all eukaryotic genomes (Hamada et al., 1982). SSRs have been recognized as good sources of genetic markers in maize (Condit and Hubbel, 1991), soybean (Akayya et al., 1992) and rice (Wu and Tanksley, 1993). Standard PCR analysis of microsatellites requires knowledge of genomic sequences flanking the SSR region and amplification of the microsatellite region to reveal polymorphisms resulting from variation in length of the repeated sequence. The uniqueness and value of SSRs arises from their multiallelic nature, codominant transmission, ease of detection by PCR, relative abundance and the requirement for only a small amount of DNA (Powell et al., 1996).

OBJECTIVES

The objectives of this work were to i) study the inheritance of cold tolerance traits in maize; and ii) identify significant QTL-marker associations that could be used to facilitate indirect selection for cold tolerance in maize breeding.

Research was designed to enable breeders and geneticist to advance the limits of maize's low temperature stand establishment potential to new and economically significant levels.

MATERIALS AND METHODS

Seed Source: Two inbred lines were used as parents in a backcross-mapping population. All inbred lines and derived families used in these studies are proprietary material of the Monsanto Global Seed Group. Seed for the parents and the families in the mapping populations were produced in the same year and location to reduce seed developmental environmental effects to a minimum. The cold tolerant line, designated 1111, was the recurrent parent in the mapping population, whereas the cold susceptible inbred line was designated 2222. Inbred line 1111 is an Iodent type, early in maturity, has high yield potential, and good general combining ability. Inbred line 2222 is an Oh07 type that is late flowering, has excellent grain type and combines well with B73 and B37 types. These parents were selected based on their performance in the screen protocols described in Chapter 2 (inbred lines code 11111 and 22222, respectively). Both lines represent elite inbred lines in Monsanto's breeding programs. Although other inbred lines had significantly lower cold tolerance ratings than 2222, they were very poor in several agronomic aspects of seed maize production. The choice of parents for the population was based on their ability to perform under cold stress. The initial F1 cross, the backcross to generate the BC1F1 generation and the selfing of the BC1F1, which resulted in 147 BC1F2 families used in the mapping population, were carried out in Puerto Vallarta, Mexico. This population was designated the MP1 mapping population.

Phenotyping: Seeds of all families and the two parents were subjected to laboratory and field evaluations as described in detail in Chapters 1 and 2. In all experiments, seeds were treated with Captan (cis-N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide) at a

rate of 2.2 fl oz/100 lb. In the cold germination experiment, seeds were considered germinated once the radicle reached 1 cm in length. Counts were made at 7, 10 and 14 days after the initiation of the experiment. In the coleoptyle growth experiment, seeds were germinated in inert media at 23◦C and later transferred to a 10◦C chamber. Coleoptyle length was measured at 7, 10 and 14 days after planting (DAP). Field experiments were planted in Waterman, IL on April 12, 2000 (Planting date 1 - PD1) and April 25, 2000 (planting date 2 - PD2). Twenty-five seeds were planted in each of two replications in a 5.4 m row. The number of plants emerged per row was recorded at several intervals after planting.

SSR analysis: DNA was extracted from 10 BC1F2 seedlings for each of the 147 families and the two parents. Three leaf disks were collected from the youngest leaf of each seedling and placed in 1.4 ml polypropylene sampling tubes (Screen Mates, Matrix Inc., Hudson, NH) in a 96-well sampling rack. Leaf material was ground and genomic DNA was extracted using a modified CTAB procedure based on the method of Saghai-Maroof et al. (1984).

PCR reactions were performed for each family and the parents. A Master Batch and a Cresol Red Dye Mix were prepared in advance for the PCR reaction. Each Master Batch contained 1.5 μL 10X Gold Buffer (19.5mM Tris–HCl, pH 8.0, 65mM KCl), 1.8 μL 25mM MgCl, 1.2 μL 2.5 mM dNTP, and 3.3 μL ddH_2O. The Cresol Red Dye Mix contained 0.7333 μL Cresol Red Dye and 0.3667 μL Glycerol. The final PCR reaction contained 7.8 μL Master Batch, 1.1 μL Cresol Red Dye Mix, 0.1 μL Taq Gold, 1.0 μL Primer, and 5.0 μL DNA to make a total sample of 15.0 μL.

DNA was amplified with the Perkin Elmer GeneAmp PCR system 9700 (Perkin Elmer, Norwalk, CT) using the following cycles: initial denaturing at 95◦C for 10 minutes, then 35 cycles of denaturing at 94◦C for 45 sec, annealing at 55◦C for 45 sec, extension at 68◦C for 45 sec and a final extension cycle of 5 min at 72◦C. Twenty μL from each sample were loaded into 3% agarose gels and run through electrophoresis. Each loaded gel was placed into a gel tank/box that contained 1X TBE buffer (Tris/Borate/EDTA). Eight 8 μL of Ethidium Bromide were added to the TBE. Gels ran at 160 to 170 volts for 3 to 3.5 hours. DNA bands were photographed under ultraviolet light.

Data analysis: Analysis of variance (ANOVA), mean comparisons and Pearson correlation analysis for all phenotypic traits based on BC1F2 progeny data were performed with SAS programs (SAS Institute Inc, 1999). Heritability estimates were calculated according to Hallauer and Miranda (1988). Linkage analysis and map construction were performed using MAPMAKER/MAPQTL (Lander et al., 1987). Ten linkage groups were assigned to maize chromosomes based on several published maps (Helentjaris et al., 1988; Beavis and Grant, 1991; Shoemaker et al., 1992; Coe et al., 1995 ;Vuylsteke et al., 1999). The exact position and order of all markers was known prior to the initiation of the experiment based on the Maize Genome Database (http://www.maizegdb.org/). The most likely order of markers were determined using the *order, compare, build, place* and *ripple* commands in Mapmaker. In regions were orders were equally likely, order information from previously published maps was selected (Maize Genome Database at http://www.maizegdb.org/. Chi-square tests were used to compute the segregation ratios of individual markers against Mendelian

expectations. Further marker analyses were performed with SAS (SAS Institute Inc, 1999) using the PROC GLM procedure for one-way analysis of variance.

RESULTS

Cold tolerance evaluations

Significant variation for cold tolerance traits occurred in all laboratory and field performance measurements (Table 3-1). In general, coefficient of variation (C.V.) was reduced as days after planting increased. Cold germination CV's ranged from 20% to 129%, coleoptyle growth CV's ranged from 22% to 25%, and field emergence CV's ranged from 5% to 30%. Coefficients of variation were high in the first germination test (germ7d) because most of the families had not germinated, which caused a large dispersion within replications. In the field experiments, as soil temperatures increased, the difference in plants emerged per row decreased. Similar observations on the first (P1C300 and P1C400) and second planting dates (P2C200 and P2C300) were evident.

Highly significant differences among genotypes (families) were observed for cold germination, coleoptyle growth and field emergence (Table 3-2). In the cold germination and coleoptyle growth experiment, no significant changes in rank order were observed as DAP increased. However, a significant interaction between genotype (families) and planting date was noted in the field experiments. Soil conditions at planting time highly influenced the performance of some genotypes, thus altering the performance ranking from planting date 1 (PD1) to planting date 2 (PD2).

Table 3-1. Summary statistics for cold germination, coleoptyle growth and field emergence for 147 BC1F2 families and the corresponding parents (1111 and 2222).

Variable	Mean	Std.Dev.	Minimum	Maximum	Range	C.V.
Cold Germination	%	%	%	%	%	%
7 days	6.1	7.9	0.0	37.5	37.5	128.7
11 days	71.6	20.5	12.5	100.0	87.5	28.6
14 days	84.6	17.1	18.8	100.0	81.3	20.3
Coleoptyle Growth	cm	cm	cm	cm	cm	%
7 days	1.9	0.5	0.8	3.0	2.2	24.3
10 days	2.5	0.6	0.0	4.0	4.0	25.4
14 days	3.4	0.8	0.0	5.1	5.1	22.3
Field Emergence	plants	plants	plants	plants	plants	%
P1C100[+]	9.7	3.0	2.0	17.0	15.0	30.9
P1C200	14.0	2.7	6.5	19.0	12.5	19.1
P1C300	15.5	2.5	6.5	19.5	13.0	15.9
P1C400	15.4	2.4	8.0	20.0	12.0	15.7
P2C100[§]	18.5	1.1	15.0	20.0	5.0	6.2
P2C200	18.6	1.0	15.0	20.0	5.0	5.5
P2C300	18.7	1.0	15.5	20.0	4.5	5.1

+ P1C100 (planting 1, count 1), P1C200 (count 2), P1C300 (count 3), P1C400 (count 4).

§ P2C100 (planting 2, count 1), P2C200 (count 2), P2C300 (count 3).

Table 3-2. Degrees of freedom and mean square errors for cold germination, coleoptyle growth and field emergence for 147 BC1F2 families and the corresponding parents (1111 and 2222).

Source of variation	Germination		Coleoptyle		Field Emergence	
	Df	MS	Df	MS	Df	MS
Genotype (G)	148	6.8***	148	4.3***		
DAP	2	3373.4***	2	354.9***		
G x DAP	296	NS	296	NS		
Error	447	2.71	1341	0.64		
Genotype (G)					149	29.0***
P. Date (PD)					1	13634.5***
G x PD					149	17.2***
Error					1050	4.49

*** = significant at the 0.001 level of probability
** = significant at the 0.01 level of probability
NS = not significant
DAP = days after planting
PD = planting date
Df = degrees of freedom
MS = mean square error

Inbred line 1111, the cold tolerant parent, had significantly better performance than inbred 2222 for most traits (Table 3-3). Overall, families having high scores in one trait (e.g. field emergence) were also the most cold tolerant for other traits such as cold germination and coleoptyle growth. Conversely, the most susceptible families generally had lower cold tolerance values for all traits.

Narrow sense heritability (h^2) for cold germination, coleoptyle growth and field emergence were moderate in the BC1F2 population (Table 3-3). Heritability estimates for coleoptyle growth ranged from 0.58 to 0.63, field emergence heritability estimates ranged from 0.45 to 0.56, and heritability for 14 days cold germination (14 DAP) was 0.50. The heritability for 7-day cold germination (7 DAP) was not significant ($h^2 = 0.07$), indicating low levels of genetic variability for this trait.

Representative frequency distribution charts are presented in Fig. 3-1 to 3-4. Continuous variation was observed over the range of cold germination, coleoptyle growth and field emergence scores for this population. As time from the initiation of experiments increased, the frequency distributions tended to skew to the right, due to a low number of families with low phenotypic scores (i.e. field emergence p1c300 and p2c100; cold germination ger14d; and coleoptyle growth gro14d).

Pearson coefficient correlations between laboratory and field evaluations were significant for all tests (Table 3-4), except coleoptyle growth measured at 14 days and field emergence in the first planting date. For the cold germination test, correlation coefficients (r) ranged from 0.19 up to 0.55. Significant positive correlations were measured between coleoptyle growth measured at 7 days and field emergence (first and second planting dates), with correlation coefficients ranging from 0.14 to 0.17.

Marker evaluations

A BC1F2 population (self-pollinated progeny of BC1 individuals) with 147 families was created from the cross of two inbred lines, 1111 (cold tolerant) and 2222 (cold susceptible). A linkage map was constructed spanning 1570 cM and encompassing the 10 maize chromosomes with an average marker spacing of 30 cM and covering 88% of the maize genome. The position of the SSR markers and map length were consistent with previously published *Zea mays* maps (Vuylsteke et al., 1999).

Eighty-nine SSR (simple sequence repeat) markers were selected because they shared at least one polymorphic band between both parents. This set was analyzed against each derived family in the population. Markers were analyzed by MAPMAKER/QTL to determine linkage relationships. The exact position and order of the markers was known prior to the initiation of the experiment (Maize Genome Database at www.agron.missouri.edu). Several SSR markers were significantly associated with at least one of the phenotypic measurements, either in the laboratory or in the field, or both (Tables 3-5 and Appendix Table A7).

A total of 21 QTLs (quantitative trait loci) were identified for all traits. Seven were associated with early field emergence, eight were associated with coleoptyle growth, and six were associated with cold germination (Table 3-5; Fig. 3-1 through 3-11). These QTLs were located in 7 different linkage groups. Coefficients of determination (r^2), which determine the amount of variation explained by a given marker, varied significantly and ranged from as low as 8% (for 7-day cold germination) up to 76% (for 14-day coleoptyle growth). A cluster of markers in linkage group 8 (LG8) appeared to

be strongly associated both with early field emergence and coleoptyle growth. Two other clusters of markers were identified in LG2 and LG7. Other significant associations were identified in LG1, LG3, LG9 and LG10 (Table 3-5).

The QTL with the largest effect for early field emergence (PD1) was located in LG7 (interval between SSR markers A1792 and A1808; http://www.maizegdb.org/) and explained 35% of the phenotypic variance (Table 3-5). The same marker also explained 10% of the variance in cold germination. Several markers in LG7 were strongly associated with coleoptyle growth. Interval marker A1380-A1792 explained 76% of the variation for this trait and is located adjacent to interval A1792-A1808. These types of associations were common.

Four interval markers in LG1, LG2, LG8 and LG10 were associated with all 3 phenotypic traits (field emergence, coleoptyle growth and cold germination), one interval marker (in LG9) was linked with field emergence and coleoptyle growth, and one marker (in LG7) was linked with field emergence and cold germination (Table 3-5). Interval marker A1037-PHI050 in LG10 had the largest effect for cold germination, explaining 17% of the variation (Table 3-5). This marker also explained 26% of the variation in field emergence and 64% of the variation in coleoptyle growth. Similarly, other markers significantly associated with all three phenotypic traits were A1014-1007 (in LG1), A1092-A2248 (in LG2) and A1067-A1863 (in LG8).

In general, SSR marker intervals were significantly associated with field emergence and one or more laboratory screens. However, some markers only had significant associations with one laboratory test. This was the case of A1909- NC003 (in LG2, associated only with coleoptyle growth), A1496-A1754 (LG3, cold germination),

A1380-A1792 (LG7, coleoptyle growth) and A1812-A1031 (LG8, coleoptyle growth). Considering the higher correlation between phenotypic means of cold germination and field emergence ratings, it was unexpected to identify a greater number of SSR markers associated between field emergence and coleoptyle growth and not between field emergence and cold germination.

Single marker analyses confirmed the presence of all the QTLs identified by interval mapping. Furthermore, it detected additional loci for all scored traits not previously identified (Table A7). However, the variation explained for each trait was lower than that detected by interval mapping, suggesting that these additional QTLs had minor effects. Interval mapping, which estimates values for missing data (Lander et al., 1989), is usually recommended to properly estimate values for marker-trait associations of quantitative characters. This is more effective with long stretches of linked segregating markers. Based on our results and those of others (Koester et al., 1993; Stuber et al., 1992), interval mapping and single marker analysis yield essentially the same results.

Stepwise multiple regression analyses using combinations of significant markers and their interactions revealed that the combination of markers PHI050 and A1067 explained 46% and 41% of the phenotypic variation for field emergence and cold germination, respectively. Also, the combination of markers A1792, PHI050, A1092 and A1808 explained 89% of the phenotypic variation for coleoptyle growth.

Table 3-3. Cold tolerance rankings for the top and bottom five families of the mapping population and their parents based on the field emergence evaluations. Numbers in parentheses indicate the rank of that genotype in each experiment.

Genotype	Field emergence		Cold Germ.		Coleoptyle growth	
	P1C100[+]	P1C200[§]	7 DAP	14 DAP	7 DAP	14 DAP
	-----plants/m-----		-----%-----		-----cm-----	
1-145	17.0 (1)	19.0 (1)	12.5 (19)	100.0 (1)	1.63 (101)	3.00 (101)
1-109	16.0 (2)	17.5 (11)	12.5 (20)	100.0 (2)	1.75 (89)	3.25 (102)
1-083	15.5 (3)	17.0 (17)	0.0 (77)	93.8 (46)	1.40 (127)	2.75 (122)
1-088	15.5 (4)	17.0 (16)	18.8 (10)	100.0 (3)	2.05 (53)	3.88 (42)
1-103	14.5 (5)	18.0 (5)	12.5 (21)	93.8 (47)	1.85 (76)	3.50 (64)
1-036	4.0 (143)	7.5 (145)	0.0 (144)	68.8 (124)	1.60 (107)	2.50 (138)
1-102	4.0 (144)	11.0 (129)	6.3 (76)	100.0 (45)	1.35 (135)	2.63 (136)
1-051	3.5 (145)	8.0 (144)	0.0 (145)	50.0 (140)	2.26 (34)	4.50 (16)
1-063	2.5 (146)	9.0 (142)	0.0 (146)	56.3 (137)	1.88 (75)	2.88 (121)
1-043	2.0 (147)	6.5 (147)	0.0 (147)	37.5 (145)	1.90 (72)	3.13 (99)
1111	15.5	17.5	6.3	100.0	2.05	4.00
2222	3.0	13.5	6.3	75.0	1.28	2.20
Mean	9.7	14.0	6.1	84.6	1.89	3.43
$LSD_{5\%}$	6.3	3.9	NS	21.8	0.63	1.36
CV (%)	30.9	19.1	128.7	20.3	24.3	22.3
h^2	0.45	0.56	0.07	0.50	0.63	0.58

\+ P1C100 (planting 1, count 1)
§ P1C200 (planting 1, count 2)
h^2 narrow sense heritability
NS not significant

Table 3-4. Pearson correlation coefficients between means of cold tolerance protocols and field emergence.

Test	PD1C100	PD1C200	PD1C300	PD2C100
Germ (7dap)	0.44**	0.52**	0.53**	0.23**
Germ (10 dap)	0.44**	0.54**	0.55**	0.19**
Germ (14dap)	0.39**	0.53**	0.55**	0.25**
Coleop. Growth (7 dap)	0.14♣	0.14♣	0.16*	0.17*
Coleop. Growth (10 dap)	0.14♣	0.15♣	0.15♣	0.15♣
Coleop. Growth (14 dap)	NS	NS	NS	0.14♣

♣ = Significant at the 10% level
* = Significant at the 5% level
** = Significant at the 1% level
NS = not statistically significant

Table 3-5. Coefficients of determination (r^2) from interval mapping for a group of SSR markers associated with mean cold tolerance scores of 147 families in the MP1 population. The linkage group (LG) is given and corresponds to those linkage arrangements described in Fig. 3-5 to 3-11.

Marker		Field emergence		Coleoptyle growth		Cold Germ.	
Interval	LG♠	Peak LOD	Variance (%)‡	Peak LOD	Variance (%)	Peak LOD	Variance (%)
A1014-1007	1	4.00	0.14*	8.29	0.65**	4.90	0.10*
A1092-2248	2	5.47	0.28*	3.54	0.54*	8.70	0.12*
A1909-NC003	2			4.08	0.64*		
A1496-A1754	3					3.29	0.12*
A1380-A1792	7			5.20	0.76**		
A1792-A1808	7	7.56	0.35**			3.59	0.10**
A1305-BNGL339	7	3.44	0.30*				
A1067-A1863	8	9.38	0.11*	3.04	0.58*	5.22	0.10*
A1812-A1031	8			3.64	0.63*		
PHI022-PHI027	9	4.87	0.28*	3.03	0.76*		
A1037-PHI050	10	4.48	0.26*	6.35	0.64*	5.01	0.17**

‡ * and ** Significant at the 0.05 and 0.01 level of probability, respectively.
Peak LOD = LOD score (logarithm (base 10) of odds)
♠ Linkage group

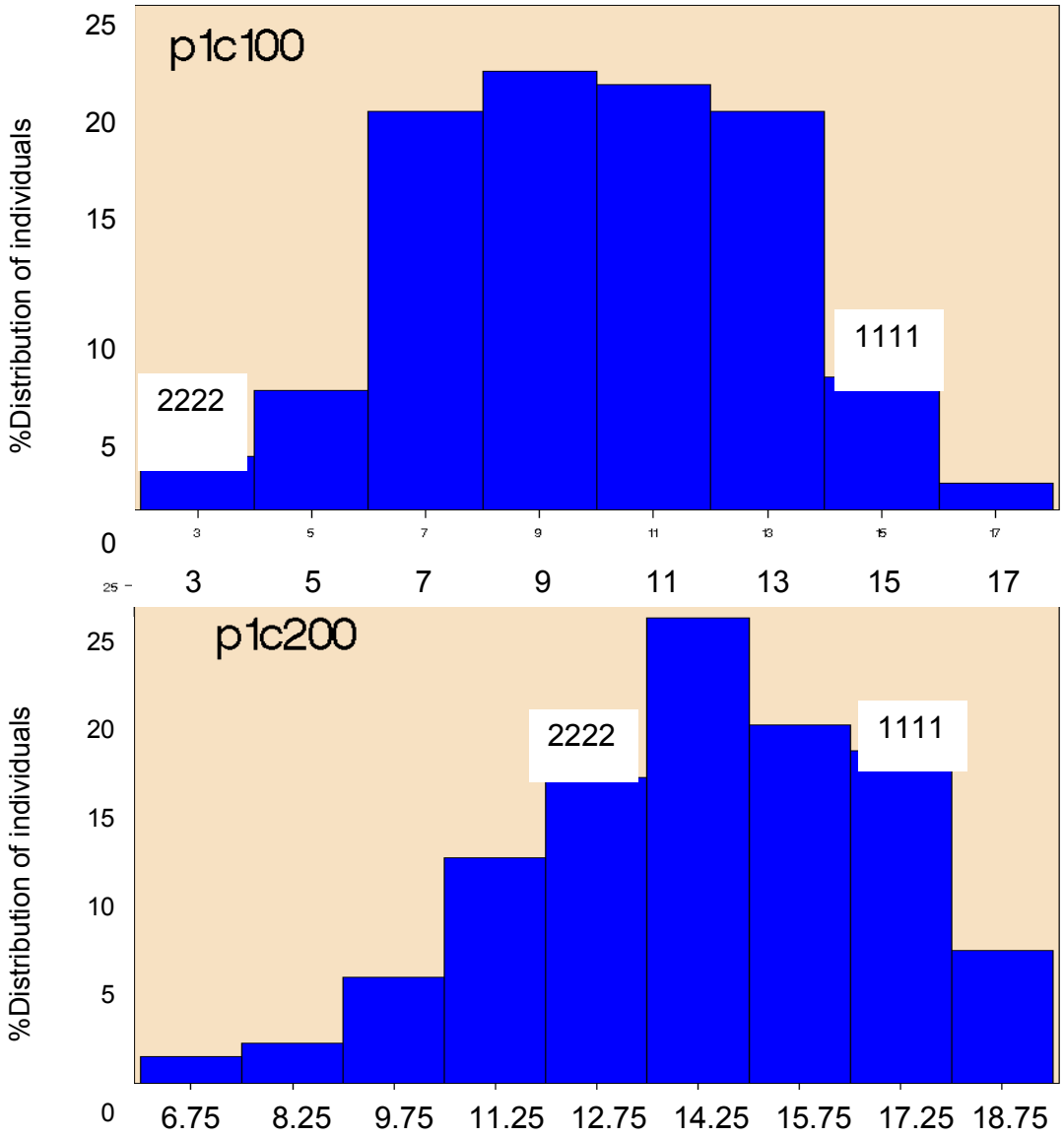

Fig. 3-1. Histograms representing the distribution of field emergence scores for the mapping population MP1 and the parents, 1111 and 2222. A) P1C100 (planting date 1, count 1). B) P1C200 (planting date 1, count 2).

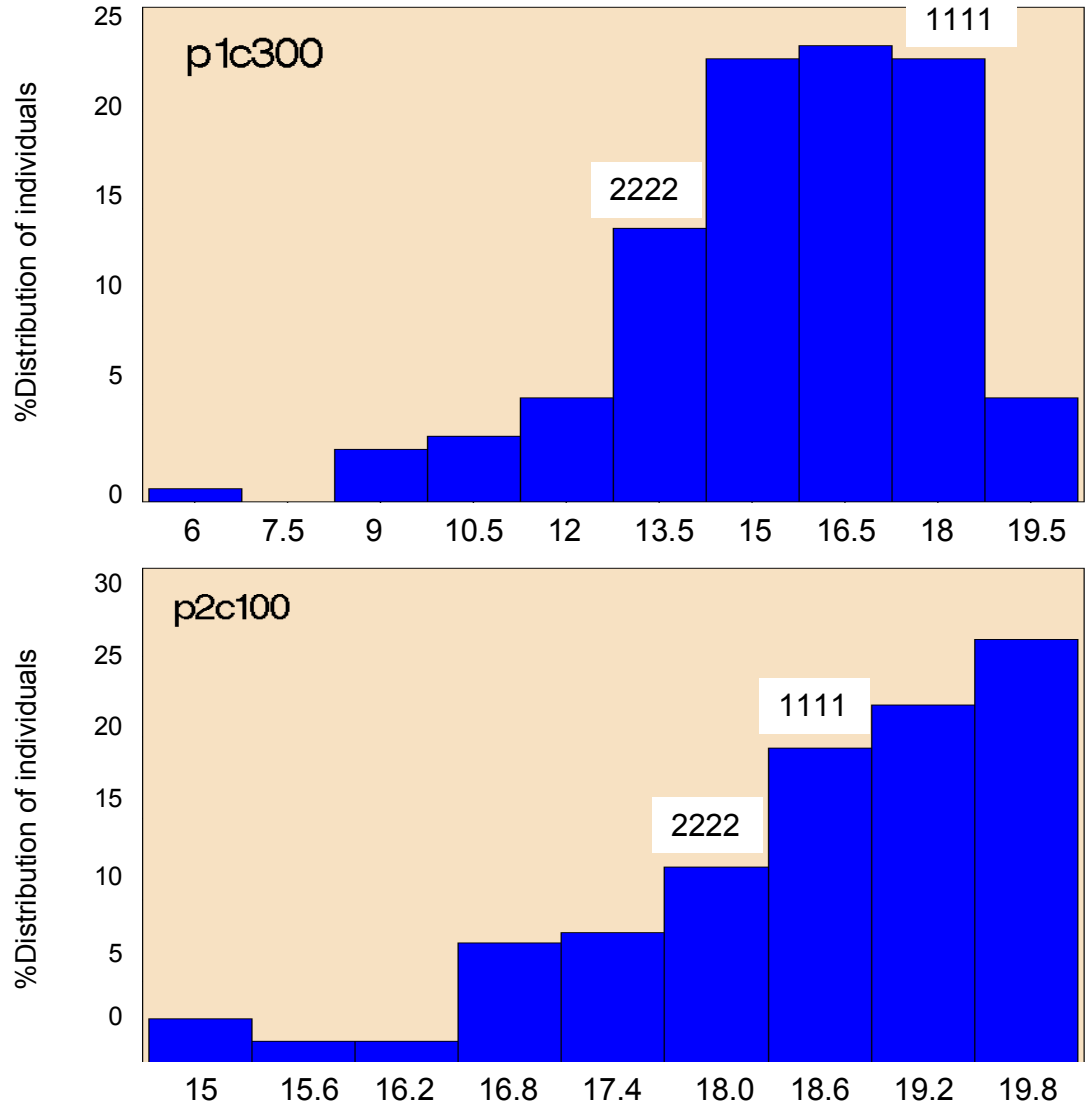

Fig. 3-2. Histograms representing the distribution of field emergence scores for the mapping population MP1 and the parents, 1111 and 2222. A) P1C300 (Planting Date 1, count 3). B) P2C100 (Planting Date 2, count 1).

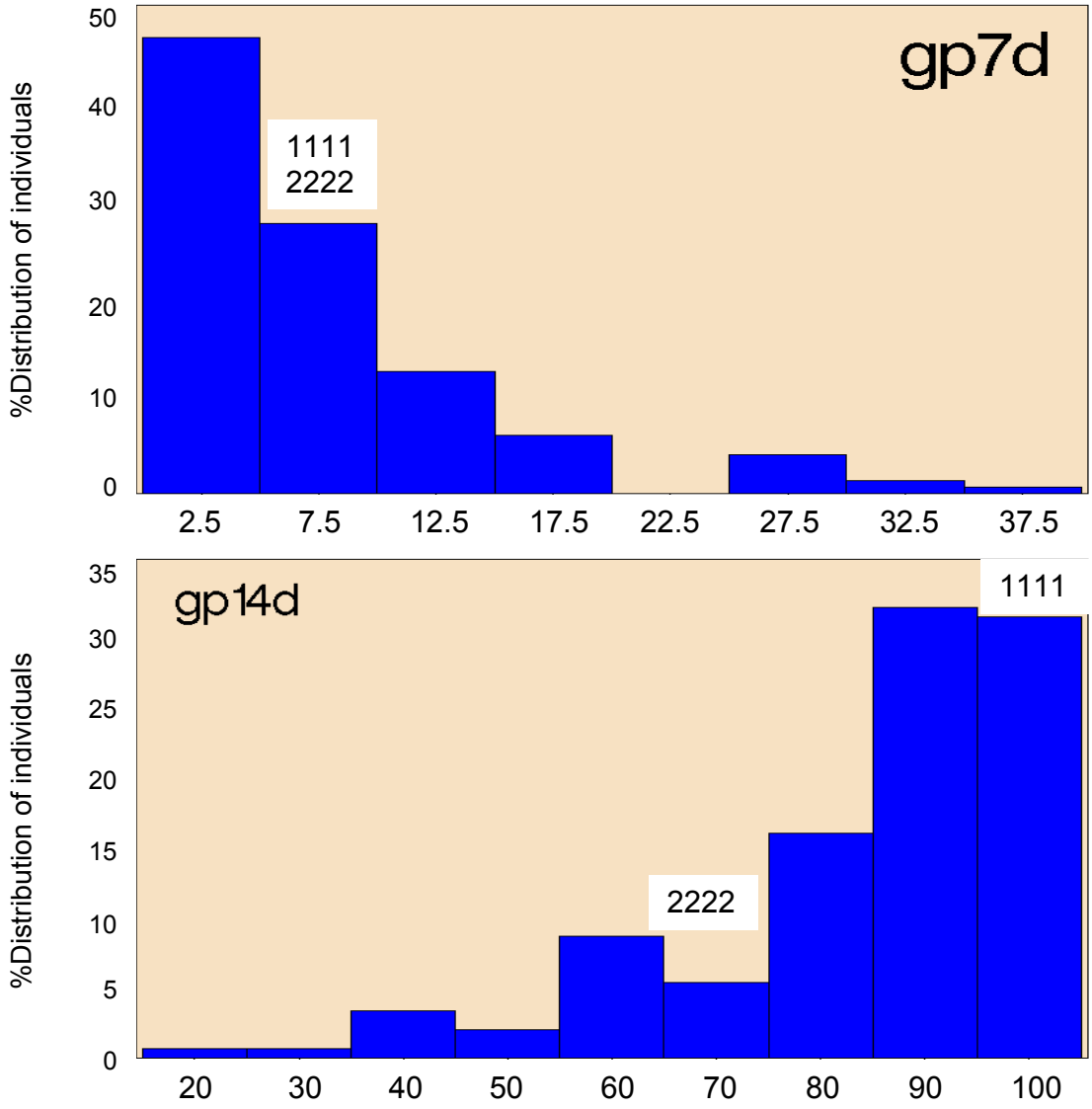

Fig. 3-3. Histograms representing the distribution of cold germination for the mapping population MP1 and the parents, 1111 and 2222. A) gp7d = cold germ at 7 days. B) gp14d = cold germ at 14 days.

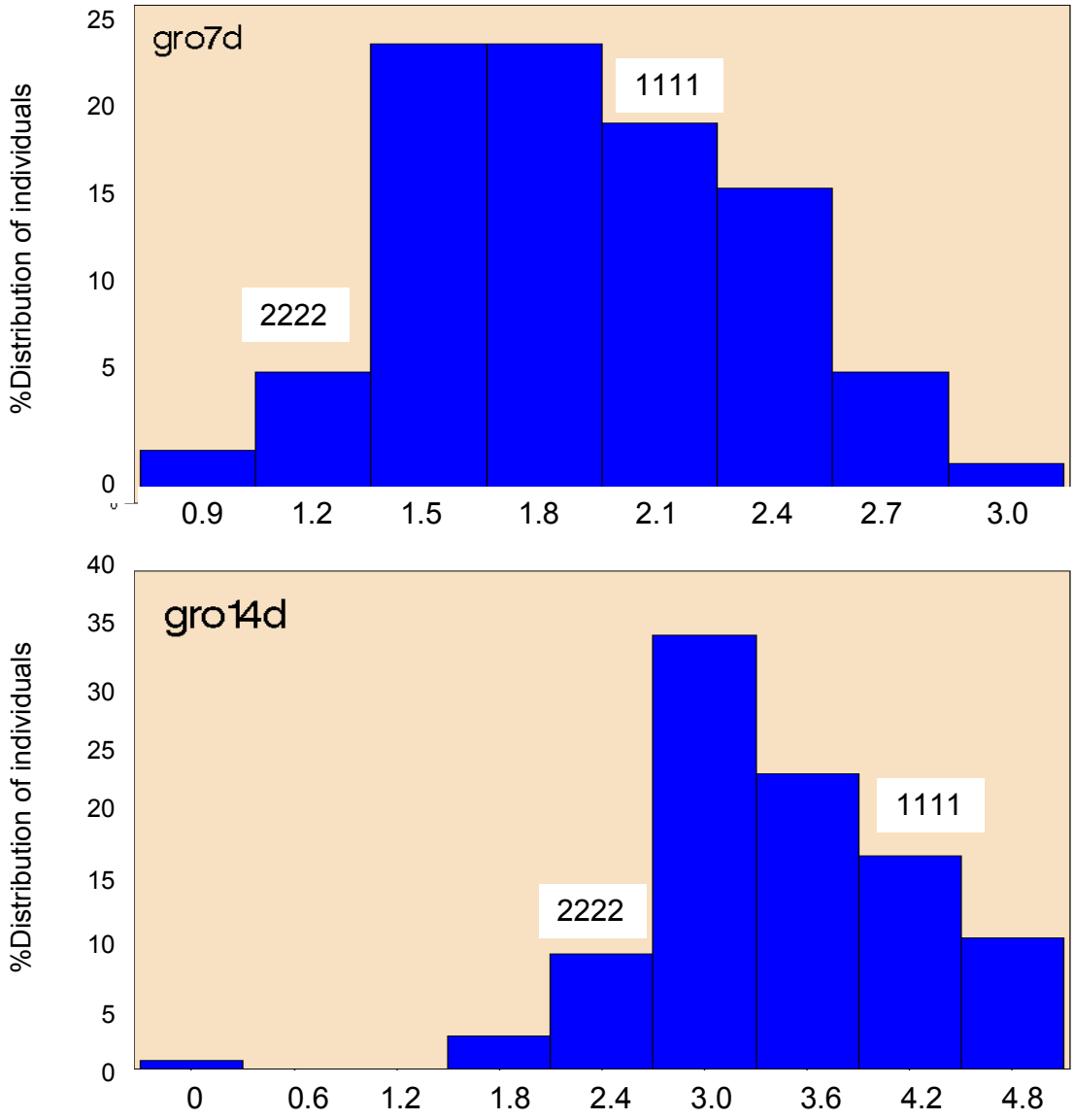

Fig. 3-4. Histograms representing the distribution of coleoptyle growth scores for the mapping population MP1 and the parents, 1111 and 2222. A) gro7d = coleoptyle growth at 7 days after planting in cm. B) gro14d = coleoptyle growth at 7 days after planting in cm.

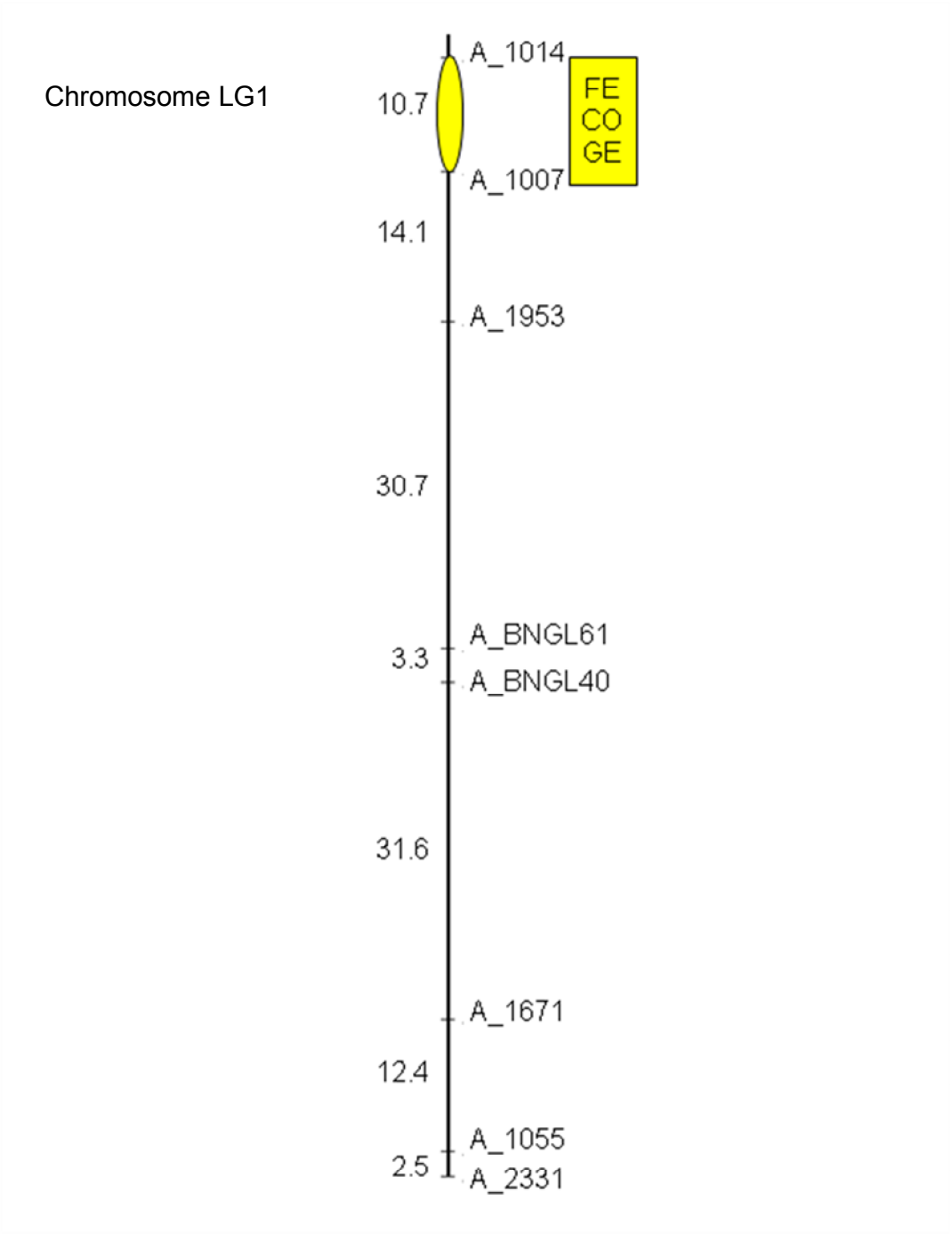

Fig. 3-5. SSR linkage group 1 (LG1) and putative location of markers used in these studies. The ovals indicate the most likely position of cold tolerant QTLs. Traits for which QTLs were significantly associated are indicated with legends in the box (FE=field emergence; CO=coleoptyle growth; GE=cold germination).

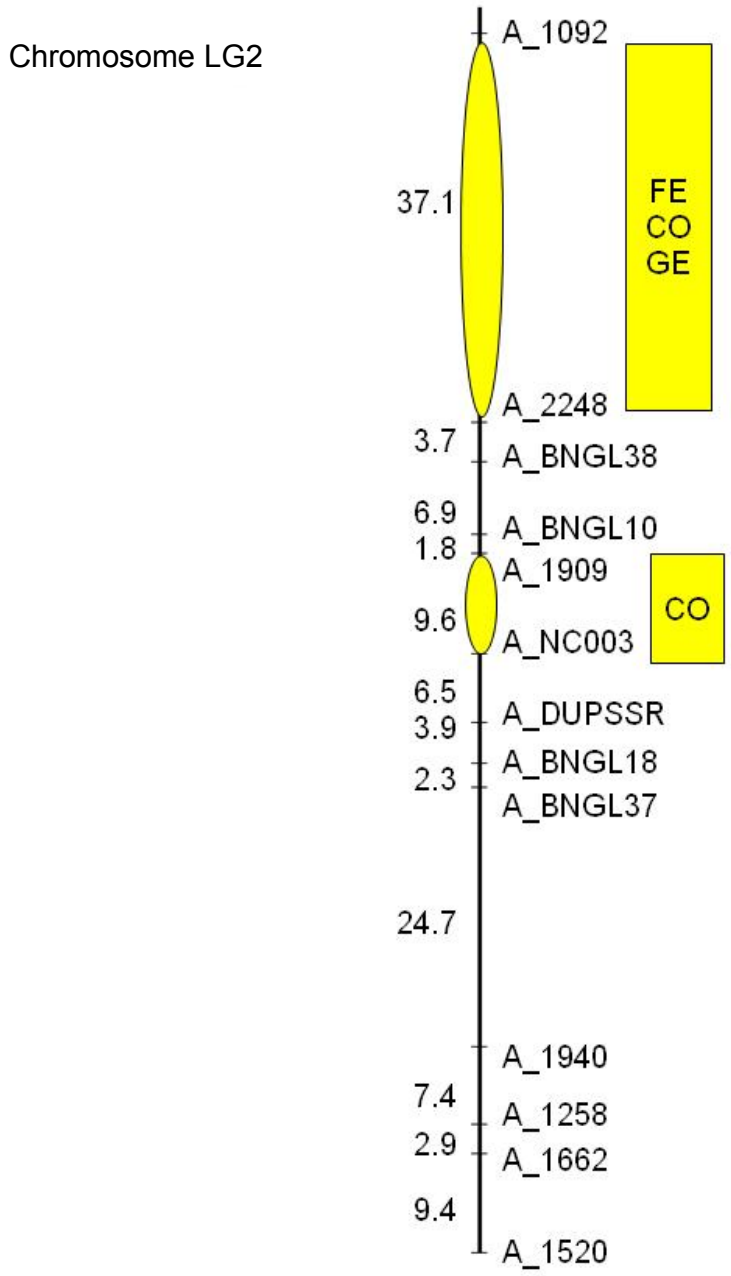

Fig. 3-6. SSR linkage group 2 (LG2) and putative location of markers used in these studies. The ovals indicate the most likely position of cold tolerant QTLs. Traits for which QTLs were significantly associated are indicated with legends in the box (FE=field emergence; CO=coleoptyle growth; GE=cold germination).

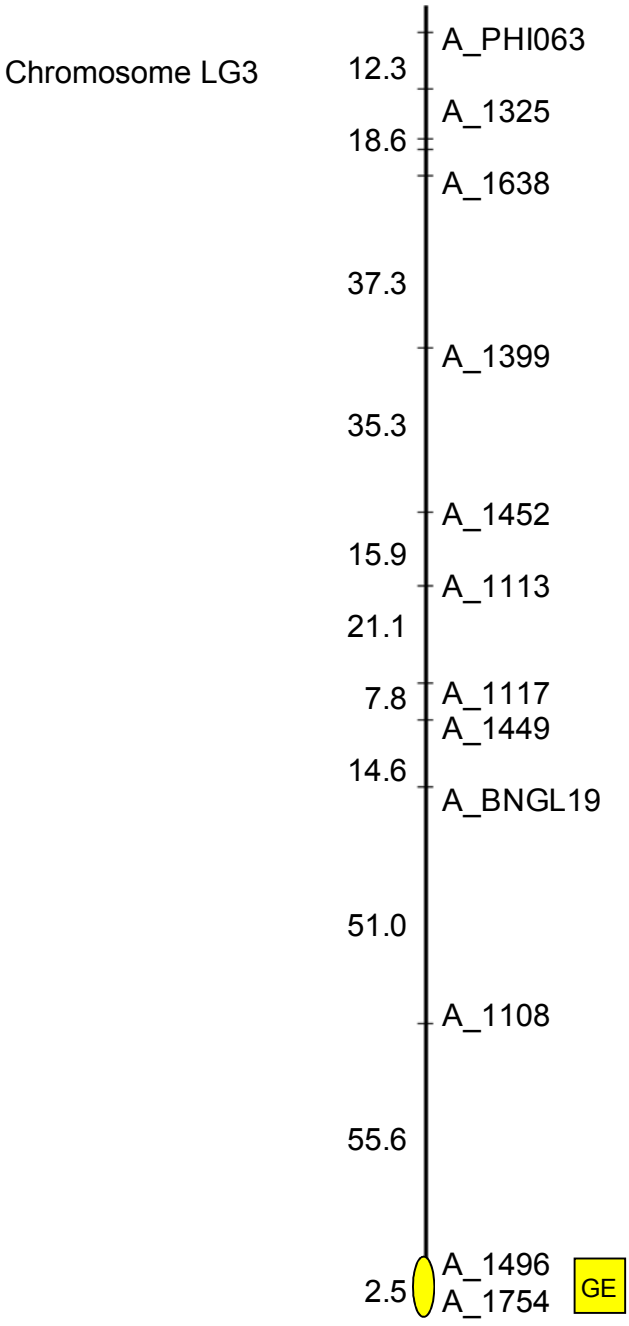

Fig. 3-7. SSR linkage group 3 (LG3) and putative location of markers used in these studies. The ovals indicate the most likely position of cold tolerant QTLs. Traits for which QTLs were significantly associated are indicated with legends in the box (FE=field emergence; CO=coleoptyle growth; GE=cold germination).

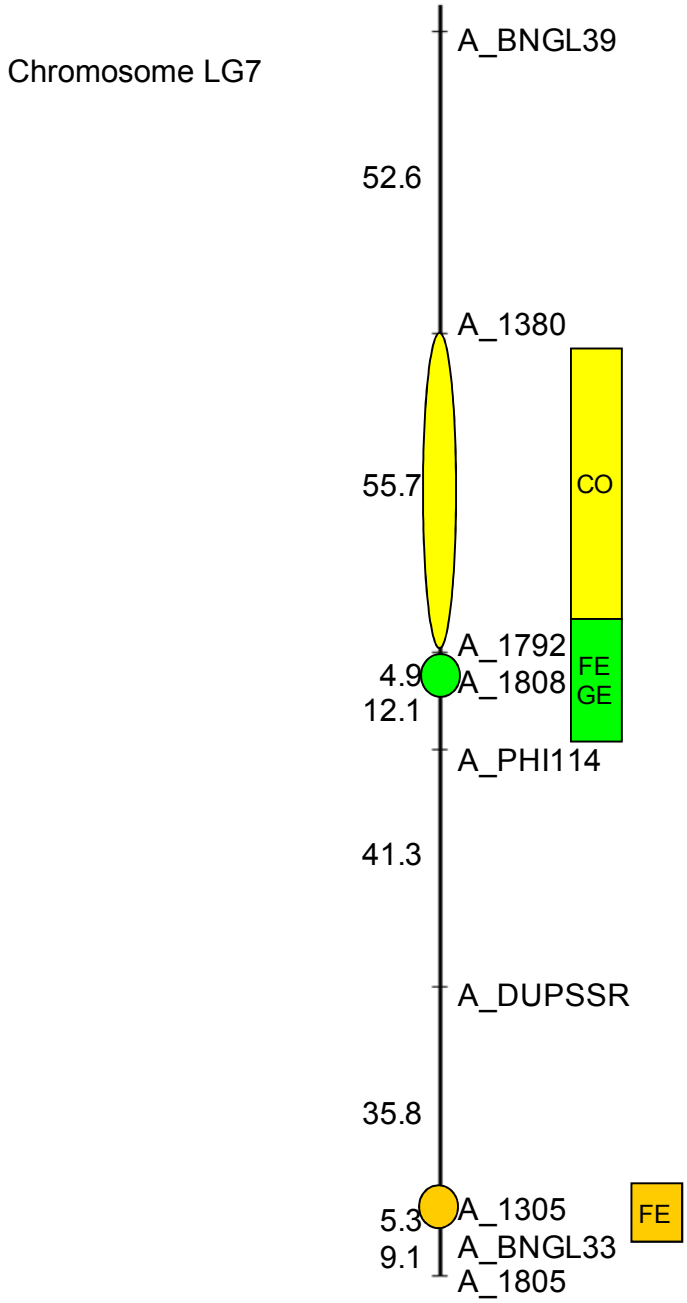

Fig. 3-8. SSR linkage group 7 (LG7) and putative location of markers used in these studies. The ovals indicate the most likely position of cold tolerant QTLs. Traits for which QTLs were significantly associated are indicated with legends in the box (FE=field emergence; CO=coleoptyle growth; GE=cold germination).

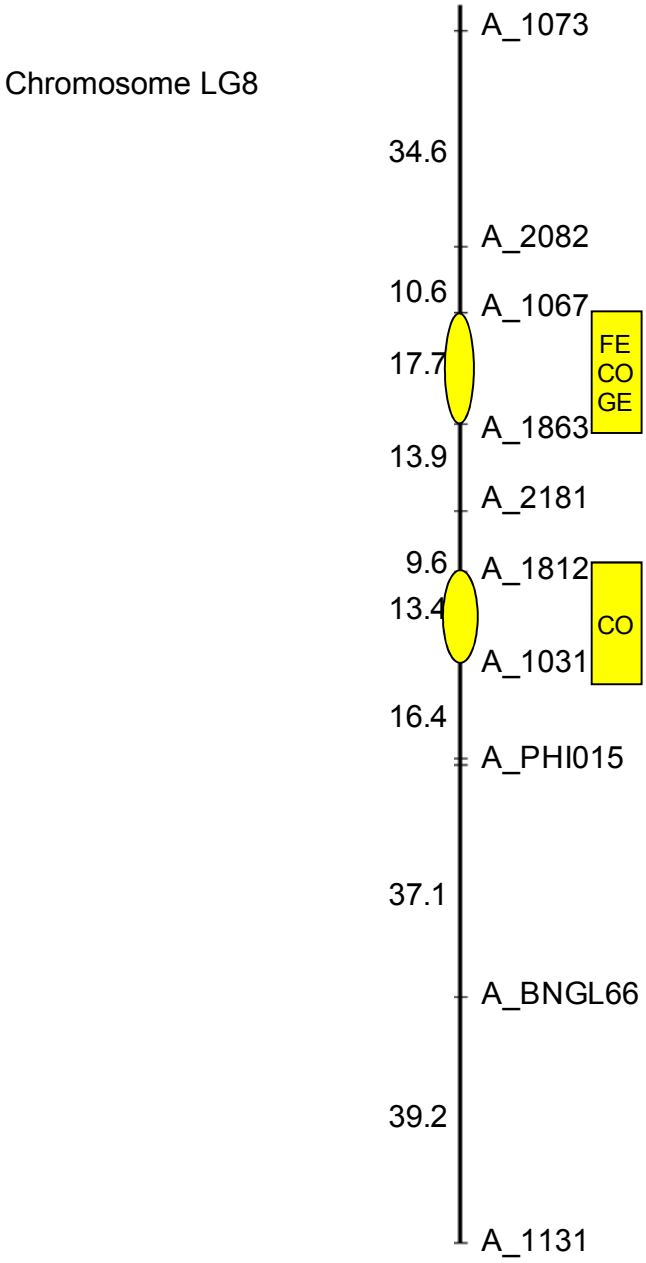

Fig. 3-9. SSR linkage group 8 (LG8) and putative location of markers used in these studies. The ovals indicate the most likely position of cold tolerant QTLs. Traits for which QTLs were significantly associated are indicated with legends in the box (FE=field emergence; CO=coleoptyle growth; GE=cold germination).

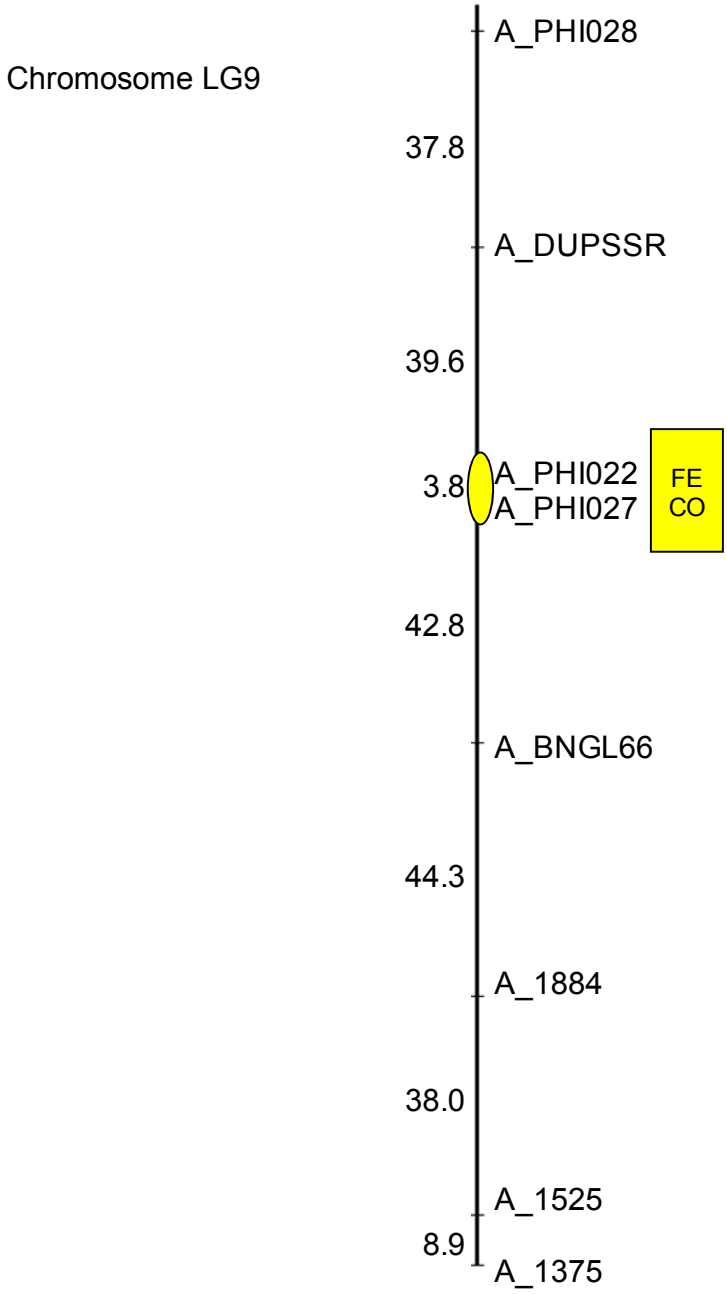

Fig. 3-10. SSR linkage group 9 (LG9) and putative location of markers used in these studies. The ovals indicate the most likely position of cold tolerant QTLs. Traits for which QTLs were significantly associated are indicated with legends in the box (FE=field emergence; CO=coleoptyle growth; GE=cold germination).

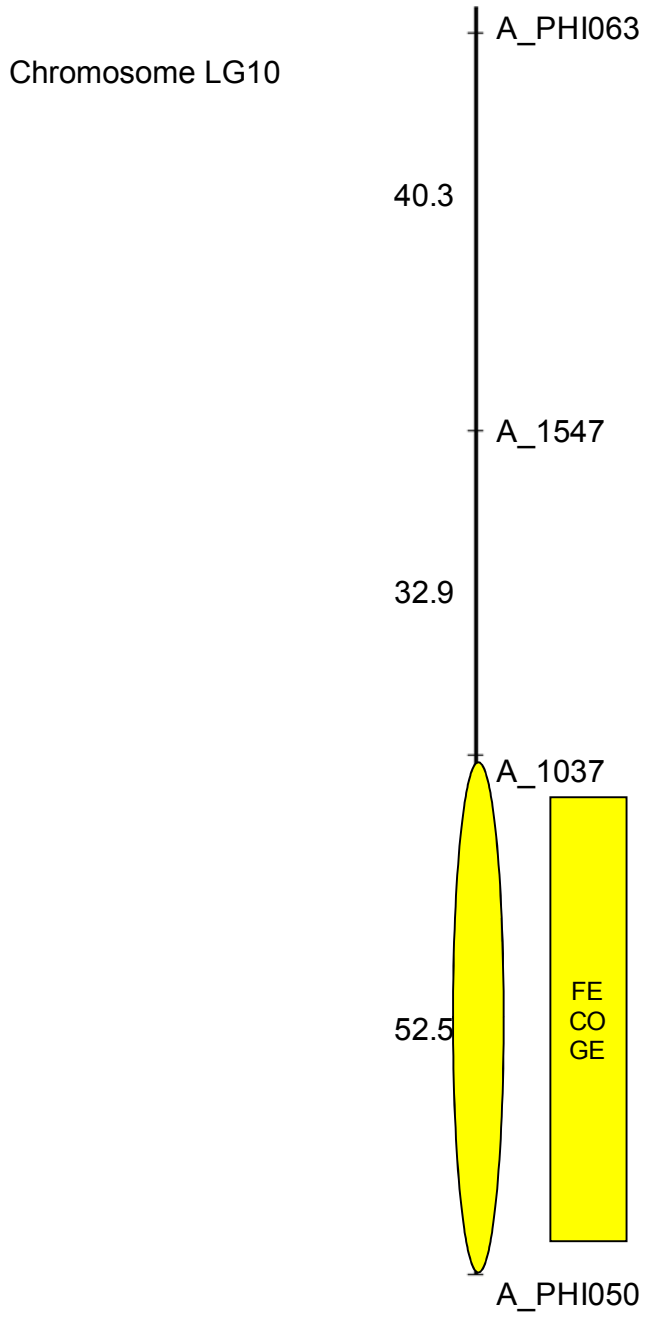

Fig. 3-11. SSR linkage group 10 (LG10) and putative location of markers used in these studies. The ovals indicate the most likely position of cold tolerant QTLs. Traits for which QTLs were significantly associated are indicated with legends in the box (FE=field emergence; CO=coleoptyle growth; GE=cold germination).

DISCUSSION

Germination and early seedling growth are major determinants of stand establishment and are particularly important for commercial production of maize. Recent trends in agronomic practices in the US Maize Belt have been to plant maize earlier in the spring to take advantage of the more optimal summer rainfall and temperatures and to avoid hot, dry periods during pollination and fertilization. Accompanying earlier plantings, several forms of conservation tillage have been adopted, which may result in a slower warming of soils in the early spring. Physiological characteristics that improve germination and early seedling growth at low temperatures are becoming increasingly important to maize producers.

Maize planted and grown under chilling conditions often leads to reduced germination, uneven plant stands and unequal competition between plants within the planted row. Genetic variability for chilling tolerance in maize exists but has not been characterized. Here, we report on the identification of several marker-QTL associations that could potentially be useful in the selection and improvement of maize inbred lines and hybrids for cold tolerance traits. In doing so, information is provided that could allow for the identification of specific clusters of genes involved with the tolerant phenotype.

Of the 10 maize chromosomes, 7 contained at least 1 QTL identified by interval mapping and by one-way analyses of variance. Some areas of the genome contained more than one trait. For example, QTLs for coleoptyle length, cold germination and field emergence mapped to the same or adjacent intervals

on linkage groups 1, 2, 8 and 10. This phenomenon has also been described in other mapping experiments on quantitative traits in rice (Redona and Mackill, 1996a), maize (Schon at al., 1993) and common bean (Nodari et al., 1993).

One QTL found in this study (in maize linkage group 7) maps to a similar location in the rice genome (LG9) where QTLs for coleoptyle and root growth have been identified (Redona and Mackill, 1996b). In a similar location, Ranjhan (1991) found genes of the α-amylase family, which have been implicated in seedling vigor. Gale and Devos (1998) generated a consensus synteny map for grasses. The largest QTLs identified in this study map to LG2, LG7, LG8 and LG10 of maize and correspond to rice linkage groups 7, 9, 1 and 4, respectively. Redona and Mackill (1996b) developed a RFLP rice linkage map and identified QTLs for coleoptyle growth and seedling vigor to map (among others) to rice linkage groups 1, 7 and 9, which broadly coincide with the location of the QTLs found in these studies for maize.

Breeding for increased seedling vigor and cold tolerance using conventional strategies has not been very successful. In part, this may be due to the trait's association with undesired characteristics such as tall plants and lodging susceptibility (Li and Rutger, 1980) that are selected against during the breeding process. Likewise, other factors such as maternal inheritance and quality of seed produced in different environments, highly impacts the evaluations of both inbreds and hybrids, and confounds lack of cold tolerance with either poor vigor or poor seedling emergence. QTL analysis to identify superior donors and marker

assisted breeding (MAB) strategies may be useful in breeding maize for seedling vigor and cold tolerance at early stages of germination and development.

Near isogenic lines combined with a saturated marker map provide a powerful means for the identification of quantitative trait loci in maize (Koester et al., 1993). The abundance of lines created by plant breeders using backcross schemes represents a rich source for these studies. Investigating the genomic regions maintained during selection has a high probability of identifying useful loci for the improvement of specific traits. Selective phenotyping, using only the top and bottom 10% of the phenotypic extremes, can be effective in detecting the most important marker-trait associations. In these studies, the same QTL-marker associations were identified when analyzing the full data set or using selective phenotyping. The major differences consisted in the amount of variance explained by those QTLs, with the selective genotyping data set always yielding higher coefficients of determination (r^2). This should result in a significant reduction in labor and time and at the same time allows the screening of a larger population.

Novel technologies which may aid in the identification of genes or areas of the genome linked to the traits of interest, in our case the ability to germinate and grow under cold temperatures, should also be pursued. Of particular interest, association mapping studies consisting on fine mapping and complete genome sequencing of an array of inbred lines which would describe pools of distinct heterotic backgrounds, maturities and even adaptation, such as northern European flints to tropical pools. Association mapping of such groups may allow the opportunity to identify germplasm groups which may carry genomic regions of

interest, allowing us to narrow the search of such genes into smaller, better defined regions of the genome. Once identified, breeding techniques assisted with marker technologies, such as Advanced Back-cross Breeding, should enable plant breeders to rapidly deploy novel genes into their germplasm of interest.

The ability of corn inbreds and hybrids to germinate, emerge and grow under sub-optimal temperatures continues to present a significant challenge in many corn producing regions around the world, mainly the northern areas of the U.S, Canada and Europe. The adoption of reduced tillage or no-tillage practices contributes to a slower warm up of soils and has created a strong demand for hybrids that germinate and emerge uniformly under these strenuous conditions. In response to this demand, the corn seed industry continues to invest significant resources in developing products and models which will better predict and more effectively respond to this rapidly changing environmental landscape.

The objectives of this work were to study the genetics of cold tolerance during germination and early seedling growth. Cold germination, coleoptyle length and field emergence under cold conditions had moderate heritability values ranging from 0.50 to 0.63, indicating that improvements by selection can be successful. Plant responses to chilling stress appear to be controlled by more than one gene and are influenced by the environment. Twenty-one QTLs for cold tolerance traits were identified, and four of them were associated with all three phenotypic traits. These clustering and strong phenotypic correlations indicate that selecting for one of these traits could potentially improve the performance of maize seeds and seedlings under cold conditions.

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APPENDIX

Table A1. Analysis of variance for the effect of temperature on Water Uptake
Dependent Variable: Water Uptake

Source	DF	Mean Square	Pr > F
Model	56	0.69	0.0001
Error	57	0.11	
Corrected Total	113		

Source	DF	Mean Square	Pr > F
SEED LOT	18	1.47	0.0001
TEMPERATURE	2	2.49	0.0001
SEED LOT * TEMP	36	0.20	0.0273

Table A2. Analysis of variance for the effect of temperature on Bulk Conductivity
Dependent Variable: Bulk Conductivity

Source	DF	Mean Square	Pr > F
Model	56	400.28	0.0001
Error	57	62.24	
Corrected Total	113		

Source	DF	Mean Square	Pr > F
SEED LOT	18	968.85	0.0001
TEMPERATURE	2	1846.25	0.0001
SEED LOT * TEMP	36	35.66	0.9615

Table A3. Pearson correlation coefficients (n=38) between bulk conductivity and weight gain at the three temperature treatments.

	Conductivity 5°C	Conductivity 10°C	Conductivity 23°C
Conductivity 5°C	1.00		
Conductivity 10°C	0.80***†	1.00	
Conductivity 23°C	0.75***	0.81***	1.00
Weight Gain 5C	-0.07^{ns}	-0.11^{ns}	-0.21^{ns}
Weight Gain 10C	-0.09^{ns}	-0.05^{ns}	-0.15^{ns}
Weight Gain 23C	-0.37^{ns}	-0.15^{ns}	-0.18^{ns}

† = Significant at the 0.0001 level
ns = not significant

Table A4. Coleoptyle growth (in cm.) at 23°C

Seed lot	Days after planting		
	6	**9**	**19**
99-1001	5.49	17.95	27.93
99-1002	7.37	17.04	23.04
99-1003	5.82	16.64	26.09
99-1004	7.95	16.80	25.53
99-1005	8.05	20.94	30.05
99-1006	6.37	16.82	21.67
99-1007	5.51	17.75	29.22
99-1008	7.76	20.09	29.17
99-1009	1.48	8.82	19.64
99-1010	4.33	14.85	28.08
99-1011	4.78	15.80	30.92
99-1012	2.24	11.95	21.88
RX490	9.98	24.94	35.67
RX355	8.63	23.51	32.92
RX530	8.74	22.49	33.67
RX601	5.59	20.61	30.56
RX697	5.27	19.85	27.14
RX843	9.52	25.30	34.06
RX670	5.59	24.5	37.4
Mean	**6.4**	**18.6**	**28.3**
$LSD_{0.05}$	**1.66**	**2.46**	**3.91**

Data in cm

Table A5. Means of two soil temperature determinations at East Lansing, MI in 1999.

Date	Minimum Temp (°C)	Maximum Temp (°C)	Average Temp (°C)
31-Mar	8.7	11.3	10.5
1-Apr	9.6	11.4	10.5
2-Apr	10.2	13.7	11.7
3-Apr	11.7	19.6	13.6
4-Apr	9.5	13.5	11.0
5-Apr	7.6	11.0	9.2
6-Apr	8.0	10.2	8.8
7-Apr	6.2	12.0	9.2
8-Apr	7.1	12.6	9.2
9-Apr	7.0	12.2	9.2
10-Apr	4.6	9.4	7.1
11-Apr	5.8	8.2	6.9
12-Apr	4.2	10.2	6.9
13-Apr	5.0	12.9	8.1
14-Apr	5.8	13.3	9.8
15-Apr	8.2	14.5	10.1
16-Apr	7.8	10.9	8.7
17-Apr	6.6	11.4	8.3
18-Apr	6.2	14.5	8.4
19-Apr	5.8	15.2	8.3
20-Apr	11.2	14.5	12.6
21-Apr	9.2	13.8	10.6
22-Apr	9.1	11.1	9.9
23-Apr	6.0	9.1	8.1
24-Apr	3.8	11.8	7.3
25-Apr	5.4	14.1	9.6
26-Apr	7.2	15.9	11.2
27-Apr	8.3	14.3	11.3
28-Apr	8.0	15.9	11.6
29-Apr	8.5	16.0	12.4
30-Apr	8.7	15.7	13.4
1-May	10.2	15.7	12.2
2-May	11.2	16.4	13.8
3-May	11.0	16.9	13.6

Table A6. Means of two soil temperature determinations in Waterman, IL in 2000.

Date	Minimum Temp (°C)	Maximum Temp (°C)	Average Temp (°C)
10-Apr	4.8	5.9	5.6
11-Apr	4.3	5.5	4.9
12-Apr	3.4	8.6	5.5
13-Apr	4.9	9.7	7.0
14-Apr	6.4	12.9	9.2
15-Apr	7.2	12.8	9.8
16-Apr	10.0	12.9	11.2
17-Apr	6.9	10.4	8.0
18-Apr	6.5	10.3	8.1
19-Apr	8.4	10.5	9.1
20-Apr	9.8	14.3	11.5
21-Apr	6.7	11.5	9.1
22-Apr	5.5	15.5	10.3
23-Apr	8.8	12.3	9.8
24-Apr	7.3	14.5	9.9
25-Apr	4.8	12.5	8.6
26-Apr	5.5	16.2	10.7
27-Apr	8.1	17.3	12.3
28-Apr	9.1	19.1	13.5
29-Apr	8.7	17.4	12.6
30-Apr	9.5	18.9	14.2
1-May	13.3	17.4	14.9
2-May	11.1	18.5	15.1
3-May	12.3	19.6	16.2
4-May	16.0	21.7	18.8
5-May	16.0	22.6	19.6
6-May	17.4	23.3	20.3
7-May	19.2	22.4	20.4
8-May	18.6	23.7	21.1
9-May	14.2	20.5	17.4
10-May	11.7	16.5	14.5

Table A7. Coefficients of determination (r^2) from one-way analyses of variance for a group of SSR markers associated with mean cold tolerance scores of 147 families in the MP1 population.

		Field emergence	Coleoptyle growth	Cold Germ.
Marker	LG♠	Variance (%)╫	Variance (%)	Variance (%)
A2331	1			0.05*
DUPSSR12	1	0.13*		
A1520	2	0.17*	0.11*	
BNGL180	2		0.30*	
PHI036	3	0.09*	0.07*	0.07*
A1904	3			0.09*
A1496	3	0.07*		
A1113	3		0.14*	
A1879	5	0.11*	0.17*	
A1879	5			0.06*
PHI087	5			0.05*
A2305	6	0.35**	0.14*	0.12*
NC013	6	0.15*	0.18*	
A1131	8		0.14*	
A1073	8		0.08*	
BNGL469A	9	0.16*		
A1375	9	0.07*		0.06*
A1547	10			0.06*

╫ * and ** Significant at the 0.10, 0.05 and 0.01 level of probability, respectively.
♠ Linkage group