Examining the physiological and genetic response of maize to low temperature conditions

Mauro Di Fenza

(B.Sc. Biotechnology, University of Naples Federico II, Italy)

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School of Biology and Environmental Sciences

Head of School: Dr. Thomas Gallagher

Principal Supervisor: Dr. Thomas Gallagher

Co-supervisor: Dr. Susanne Barth

UNIVERSITY COLLEGE DUBLIN, DUBLIN

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Statement of Original Authorship

I hereby declare that I am the sole author of this thesis and that the research work in it has not previously been submitted to obtain a degree elsewhere. I authorise University College Dublin to lend or photocopy the thesis in whole or in part to other institutions or individuals for the purpose of scholarly research.

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Mauro Di Fenza

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Abstract

Maize (*Zea mays*) is an emerging forage crop in Ireland, originating in warmer climates. Under Irish climate conditions establishment can be problematic due to low soil temperatures at early stages of establishment. Maize varieties with improved chilling tolerance have been developed and are on the market, but maize in Ireland is still established under plastic and further varietal improvements are required to make this crop more economically viable. To date, varieties are selected principally by phenotypic traits rather than genetic response. Investigation of the physiological and genetic response of maize towards cold/chilling stress at early developmental stage, in particular the response of developing maize roots to cold stress, can make a contribution towards the understanding of the molecular mechanisms conferring plant cold tolerance.

The objectives of this study were aimed to create, at first, an experimental design to test the physiological response to low temperatures, under controlled environmental conditions, of various commercial maize cultivars adapted to grow in temperate climates. Responses to abiotic stresses such as cold involve changes in gene expression, therefore, once indentified the hybrids showing contrasting degrees of cold tolerance, these were profilied to examine gene expression and identify possible cold regulated genes.

The physiological experiments on twelve maize varieties identified four genotypes with contrasting cold tolerance.

Microarray analysis profiling these varieties was used to identify up and down regulated genes under cold/chilling conditions. The stress induced by the cold temperature in the genotypes Picker, PR39B29, Fergus and Codisco was reflected only on the expression profiles of the two varieties with superior cold tolerance, Picker and PR39B29. No significant changes in expression were observed in Fergus and Codisco in response to cold stress. The overall number of genes up and down regulated in the two cold tolerant varieties amounted to 69, which were, however, divided in a group of 39 genes in PR39B29 and 30 genes in Picker, as the two varieties exhibited two different trancriptomic patterns in which only four genes (RNA binding protein, pathogenesis-related protein 1 and two unknown proteins) were shared, although not all with the same degree of regulation. No cold regulated genes ware detected.

The gene expression of the four-shared genes was further investigated with qRT-PCR in order to estimate the expression pattern over time. Five time points were used to analyse the expression trend of the genes. The gene expression was not maintained over the five time points, but it was subjected to fluctuation. However, with the exception of the RNA binding protein gene, the expression pattern was similar between the two varieties, indicating a common response to chilling stress.

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Abbreviations

$(NH_4)_2SO_4$	ammonium sulphate
°C	degree Celsius
ABA	abscisic acid
ABRE	ABA-responsive elements
Adh	alcohol dehydrogenase
ATP	adenosine triphosphate
NAD^+	nicotinamide adenin dinucleotide
Ca^{2+}	calcium
CBF	c-box binding factors
cDNA	complementary deoxyribonucleic acid
cm	centimetre
COR	cold regulated
cRNA	complementary ribonucleic acid
CRT	C-repeat
CTP	cytosine triphosphate
Cy	cyanine
D	delta
DMI	dry matter intake
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DRE	Drought responsive elements
DREB	drought responsive elements binding
dT	deoxy-thymine nucleotide

DTT	dithiothreitol
E. Coli	*Escherichia Coli*
E_{amp}	amplification efficiency
Ef1-a	elongation factor-1-α
EST	expressed sequence tag
g	grams
GAPDH	glyceraldeyde-3-phosphate dehydrogenase
GR	glutatihone reductase
GTP	guanosine triphosphate
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
Hmgp	high motility group protein
Inv	invertase
KCl	potassium chloride
l	litre
m	micro
M	molar (mol l^{-1})
$MgCl_2$,	Magnesium chloride
miRNA	microRNA
mm	millimetre
mRNA	messenger ribonucleic acid
n	nano
NTP	nucleoside triphosphate
PCR	polymerase chain reaction
PR-1	patogenesis related protein-1
qRT-PCR	quantitative Real-Time polymerase chain reaction
QTL	quantitative trait locus

RBP	RNA binding protein
RNA	ribonucleic acid
RNase	ribonuclease
RNI	RNA Integrity Number
ROS	reactive oxygen species
rpm	revolutions per minute
SSC	saline-sodium citrate
SDS	sodium dodecyl sulphate
T7	T7 bacteriophage
Tua5	tubulin alpha-5 chain
Ukw-P	uknown protein
UTP	uracil triphosphate
ZmUBI	*Zea mays* ubiquitin

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1 Introduction

1.1 Maize

Origin

Maize *(Zea mays)* belongs to the family *Poaceae,* formerly known as *Gramineae* (Hochholdinger et al., 2004) and has its origin in Mexico and Central America, where the genus *Zea*, a group of grasses of those regions, is native. The genus *Zea* includes the wild taxa, known collectively as teosinte (*Zea ssp.*), and domesticated corn, or maize (*Zea mays L. ssp. Mays*) (Beadle, 1939).

Flint and dent corn

The most widespread germplasms of maize around the globe are the dent, commonly grown in North America, and the flint, widely grown in Asia, Europe and in Central and South America (Dickerson, 2003). The difference between the two types lies in the type of kernel, which is harder in the outer layer of flint corn than dent corn, which, on the contrary, has high content of soft starch. Flint germplasm is better suitable than dent corn to chilling conditions and is used as a source of cold/chilling tolerance while dent corn grows better in warmer climates and it has a high yield potential when grown near optimal temperature conditions (Reimer, 2010).

Growth conditions

Maize is a C_4 tropical plant whose growth range of temperature extends from 30°C to 35°C (Miedema, 1982) with optimum at 25-28°C and therefore is arguably sensitive to low temperature, particularly in the early growth stages (Presterl et al., 2007). Nevertheless, hybrids derived from highland maize can adapt to lower temperatures than the optimal range (Bennetzen, Hake, & Ebrary, 2009). However, temperatures below the optimum cause a steady decline of growth of maize, which definitely stops around 6-8°C. Prolonged exposure to low temperatures involves irreversible cellular and tissue injury (Greaves, 1996), and the effect is mainly marked in the early growth stages as it impairs several developmental and physiological processes (Greaves, 1996; Marocco et al., 2005). Chilling is responsible for yield losses and lower metabolisable energy content (starch, sugar) in maize (Frei, 2000); in particularly affects photosynthesis due to an over excitation of the PSII reaction centres and a concomitant production of oxygen radicals (ROS), which are demonstrated to produce injurious effects to the photosynthetic apparatus (Nie, 1992).

Nitrogen fertilisation has allowed an extensive production of maize in Europe, thank to its low cost compared to other fertilisers and its capability to ensure a good harvest. In temperate regions, where soil warming is slow (Arshad & Azooz, 1996; Tollner et al., 1984), so is the establishment of the crop and blanket application of nitrogen fertilisers can result in a mismatch of the availability of nitrogen for a rapid uptake (Shanahan et al., 2008). Moreover, an extensive use of nitrogen fertiliser can contribute to the ground water pollution

by nitrate leaching (Cassman et al., 2002). Nitrate leaching is also increased when cattle manure is used, a practise in regions where maize is cultivated for the production of silage maize (Gallais & Coque, 2005). The European Union directive (91/676/EEC) limits the use of nitrogen fertilizers.

The introduction of new varieties with improved nitrogen use efficiency and a greater management in the use of nitrogen supply can lead to a compromise between profit and risk of pollution (Gallais & Coque, 2005). Maize hybrids with improved nitrogen stress tolerance have made progress in this direction, showing a good performance at low concentration of nitrogen (Duvick, 1984).

Distribution

Maize is the major cereal product in the world (www.fao.org) and Europe is third largest producer of maize grain after the United States and China. In the UE, the major producers in 2012 are Romania, France, Hungary, Italy, Germany, Bulgaria, Spain, Poland, Greece, Austria, Slovakia and Portugal (Figure 1-1).

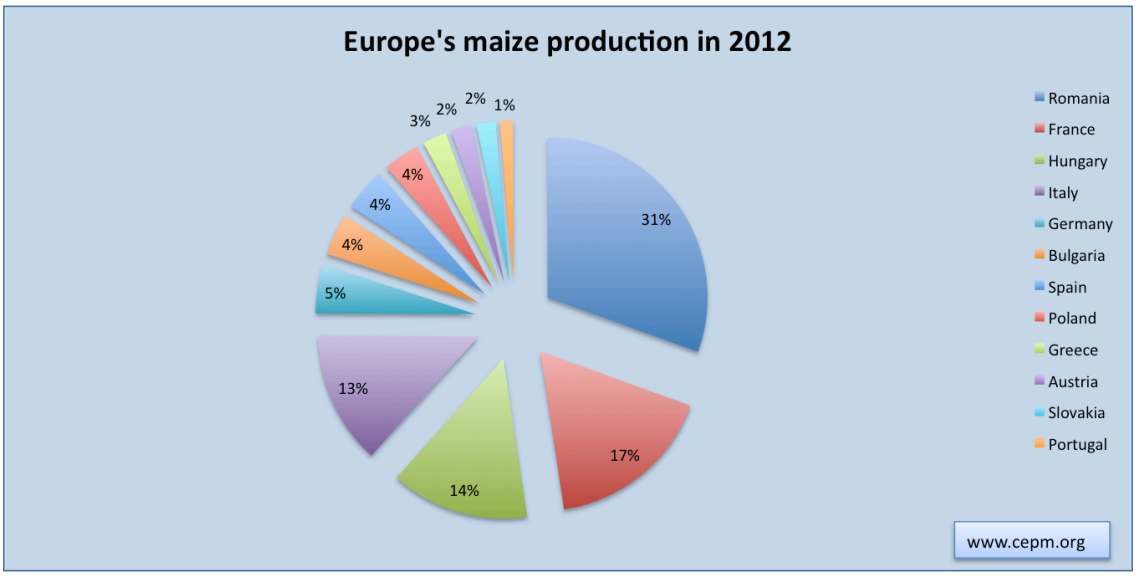


Figure 1-1 Major producers of maize in Europe in 2012. The countries are listed in descending order of contribution.

About 83% of maize grown worldwide is used for compound feed and the remainder for human food. However, in Ireland, maize is mainly used as winter forage for cattle and sheep, for which the whole plant above about 12-15 cm is used. The beneficial effects on forage dry matter intake (DMI) characteristics of maize silage improve cattle and sheep performance with regard to high meat and milk production when compared to grass silage (Keane, 2002; Kirkland & Patterson, 2006). Maize is also appealing thanks to its capability to give high yield in a single harvest and to be easily stored and preserved. As previously said, in Ireland maize is an emerging forage crop. The initial attempts to introduce this crop in the country date back to 1970 with unsuccessful results, because of the low temperature of the Irish climate that are problematic at early stages of

establishment. All the countries of Central and Northern Europe where the establishment of the maize plant is limited belong to those regions defined marginal areas for maize production.

In the next paragraphs, the main biotic and abiotic stresses influencing plant growth with special regards to maize, the main factors playing a significant role in response to chilling/cold stress as well as the strategies applied in Ireland to deal with low temperatures will be discussed.

1.2 General overview of abiotic stress factors

Plants are subjected to various biotic stresses, mostly represented by pathogens (Balachandran et al., 1997) and abiotic stresses, such as water availability, salinity, temperature and light (Farooq, 2009). To cope with environmental stresses, plants have evolved mechanisms consisting of molecular and cellular changes, as well as the expression of genes, that favour adaption and tolerance. Each of the pathways activated during plant adaptation in response to a specific stimulus is part of a more complex signalling network whose elements can be involved in response to different environmental cues. This means that some key common elements are induced by more than one stimulus. However, different pathways can be triggered to deal with the same stress (Janská et al.,

2010; Theocharis et al., 2012) and signalling cross-talk occurs between the various abiotic stress pathways (Shinozaki et al., 2000).

Low temperatures, along with water availability, represent one of the major impediments for plant productivity and geographical distribution in the world. However, some plant species have the ability to adapt to cold/chilling environments, through a series of molecular and physiological changes known as cold acclimation (Theocharis et al., 2012). This adaptation, in response to cold/chilling temperatures, is not completely constitutive, but requires an initial exposure to low temperatures in order to confer cold tolerance to plants (Zhu et al., 2007).

1.2.1 Chilling, cold and freezing temperatures

Definition

Low temperatures are defined as such when they range from 0-15°C, below which they are considered freezing (Theocharis et al., 2012). Temperatures below 5°C are defined cold, while temperatures between 5-15°C are defined chilling (Nguyen et al., 2009).

Freezing injury

Freezing injury is mainly due to ice formation, which leads to dehydration and, subsequently, to membrane damage (Thomashow, 1999). The effects are similar to those provoked by drought, since the osmotic disequilibrium caused by the ice formation in the intercellular space reduces water availability for cellular processes. Once ice thaws, water moves back to the cytoplasm and becomes available for use again, but cells are not able to contain the new volume, which causes membrane disruption (Uemura & Steponkus, 1989). Freezing tolerance (FT) is mainly constitutive, but it can be enhanced through gradual exposure of plants to low temperatures (Thomashow, 1999). However, tolerance to freezing temperatures is very limited in maize and genetic variability is minimal Greaves, 1996); the plant is seriously damaged when the temperature drops to -2 to -3°C (Dhillon et al., 1988).

Cold and chilling injury

Low temperature causes injuries that are manifested at both cellular and molecular levels, nevertheless without ceasing cellular functions. The effects of low temperatures on plants have been associated with visible symptoms like chlorosis or necrosis (Ruelland & Zachowski, 2010) as well as modifications at cellular level, in particular in the lipid composition of the plasma membrane (Matteucci et al., 2011) and several ultrastructural changes involving plastids and mitochondria (Zhang et al., 2011). In particular, membranes are considered

the primary site affected by low temperatures and probably the cellular component capable to sense cold (Theocharis et al., 2012). It is proposed that the cascades of reactions that lead to the molecular and biological changes necessary for the acclimation are initiated at the membrane level. However, little evidence exists that membranes play such a role. It is known that the change in fluidity of the cellular and chloroplast membranes in acclimated plants lowers the threshold of temperature for cellular damage (Uemura & Steponkus, 1989). The higher fluidity is due to the increase of unsaturated fatty acid content (Vogg et al., 1998) and it is maintained due to the activity of membrane-associated enzymes (Matteucci et al., 2011). It has been suggested that specific membrane receptors are responsible for detecting and reporting different ranges of temperature, because evidence has been found in mammalian cells (Bali et al., 2009). Therefore, it is possible that similar mechanisms can be involved in higher plants as well. In a recent review on temperature sensing in plants (McClung and Davis 2010) are discussed a range of possible temperature sensing mechanisms in plants. These included membrane based mechanisms such as fluidity or lipid rafts that modulate membrane–protein interactions, altered Ca^{2+} channels, and movements of metabolites. Other mechanisms proposed are alterations in chromatin state; RNA mediated mechanisms based either on altered folding of transcripts, generation of splice variants, or altered microRNA (miRNA) kinetics and a range of protein based mechanisms such as temperature-sensitive protein stability, and alterations in chaperone-dependant protein folding.

Cytoskeletal rearrangements (Orvar et al., 2000), MAP-kinase cascades (Teige et al., 2004), calcium elevations and accumulation of carbohydrates are some of the principal responses to low temperatures (Theocharis et al., 2012). In particular, soluble sugars show the higher sensitivity to low temperatures than other components of photosynthesis (Fernandez et al., 2012) and play a significant role as cryoprotectants, osmoregulators and signalling molecules (Welling & Palva, 2006). They are indeed involved in reducing water depletion for ice formation and preserve, this way, water availability inside the cells (Ruelland et al., 2009). They also represent a useful source of scavengers of reactive oxygen species (ROS), reducing membrane injury and, consequentially, ensuring its stability (Bohnert & Sheveleva, 1998).

It has also been documented that cold stress increases the biosynthesis of photosynthesis-related pigments, which help protect the photosystems, like flavonoids (Crifo et al., 2011), which accumulate in stems and leaves and xanthophylls (Ivanov et al., 2006).

Tropical and sub-tropical plant species such as maize usually experience chilling stress when temperatures drop below 10°C, although it can be also experienced when the temperature is higher than that (Levitt, 1980). In maize, the effects caused by cold stress include alteration of cell membrane composition (increase of unsaturated fatty acids) and its fluidity (Farooq et al., 2009), reduced cellular respiration, elevated abscisic acid (ABA) and cryoprotectants levels, increased production of reactive oxygen species and consequent enhanced production of antioxidants. The cellular composition is one the main changes

observed, with alteration in lipid composition, increases in soluble protein content and accumulation of simple sugars, proline and organic acids (Levitt, 1980; Sakai & Larcher, 1987).

When low temperatures are considered, it must be stated that all plants are capable to detect not only the absolute temperature, but also the duration of it and its rate of change. Plants can sense temperatures as low as 1°C (Heather Knight & Knight, 2000), but that is not always interpreted as an environmental stress. Fluctuations in temperature occur several times throughout the day. Therefore, plants must be able to distinguish harmless changes in temperatures from the injurious ones so as defense mechanisms can be activated and plants can eventually engage cold acclimation (Knight & Knight, 2012). Cold or chilling acclimation involves molecular and physiological adaptation than can be accompanied by changes in gene expression (Theocharis et al., 2012). It is known that the circadian clock and the quality of light play a crucial role in determining the type of response when plants experience a drop in temperature and the effects of circadian factors on the expression of cold regulated genes (COR) have been described (Bieniawska et al., 2008). It is probable that the detection of multiple signals is necessary in order to induce an appropriate response when plants are exposed to low temperatures (Huang et al. 2012).

1.2.2 Cold regulated genes (COR) and drought responsive elements (DRE)

Cold regulated genes can be involved in the direct protection against cold/chilling stress or in the regulation of gene expression, which guides the biochemical and physiological changes for the adaptation to low temperatures (Thomashow, 2010). Drought responsive elements (DRE), also know as CRT (C-repeat), are a motif contained in the promoters of many cold-regulated genes, which are bund by transcriptional factors belonging to the CBF (c-box binding factors) or DREB (drought responsive elements binding factors) family (Knight & Knight, 2012). The positive regulation of the CBF/DREB genes is maintained by ensuring protein stability of specific CBF transcription factors (Park et al, 2011), while negative regulation is also crucial in order to manage the effects of the CBF/DREB gene expression, which, if sustained, can impair and slow down plant growth (Jaglo-Ottosen et al., 1998).

1.2.3 Water deficiency

Many cold-regulated genes are regulated as a consequence of dehydration (Shinozaki & Yamaguchi-Shinozaki, 2000); in maize, low temperature has a significant effect on plant water relations (Farooq et al., 2009). Chilling is responsible for reduced root hydraulic conductance and loss of stomata control followed by considerable decrease in leaf water and turgor potential (R. Aroca et

al., 2003; Fennel & Markhart, 1998). All the effects mentioned above are an indirect consequence of a reduced water uptake by the root system, whose cells are seriously impaired in their structure and function (Shabala & Shabala, 2002).

1.2.4 Role of abscisic acid (ABA) in plant stress tolerance

Another key-role is played by the phytohormone abscisic acid (ABA), which is involved in many physiological processes in plants (Bonetta & McCourt, 1998). Cellular levels of ABA are increased by several stress conditions such as drought, cold, light and temperature. The action of ABA involves modification of gene expression (Swamy & Smith, 1999). The protective function of ABA is provided through stabilisation of membranes (Prasad, et al., 1994), stomata closure (Downton et al., 2006) and protection against oxidative stress reducing the accumulation of ROS (Liu et al., 2011). ABA-responsive elements (ABRE) have been found in many promoters of cold-regulated genes (Suzuki et al., 2005) and ABA has been shown to play a role in inducing the expression of the DREB genes, although to a lesser level than that directly produced by low temperatures (Knight et al., 2004). The role of ABRE is quite unclear, as a study has demonstrated that they are not involved at all in response to cold (Narusaka et al., 2003) and that ABA accumulation is mediated by DRE, although ABRE plays a crucial role in those cold-regulated genes where DRE is lacking (Mikkelsen & Thomashow, 2009). The accumulation of ABA in chilling-tolerant maize genotypes occurs faster and the amount is up to four times higher compared to the amount in the chilling-sensitive genotypes (Janowiak et al., 2002).

1.2.5 ROS as indicator of oxidative tissue damage and stress in plants

Another harmful effect of low temperature is an enhanced production of reactive oxygen species (ROS), which cause extensive cellular damage by reacting with proteins, lipids and nucleic acid, finally leading to death of cells (Foyer & Fletcher, 2001). An efficient antioxidant system, capable to adjust to the changes in ROS concentration is therefore crucial for a plant to survive under cold and chilling stresses (Kocsy et al., 2001). Antioxidants play an important role in preventing the toxic accumulation of ROS, which are mainly represented by superoxide radical, hydrogen peroxide and lipid peroxides (Kellos et al., 2008). Low levels of ROS are normally produced (Nakashima et al., 2002), but their production is strongly enhanced under stress conditions. The increase in ROS concentration in turn activates antioxidants, which scavenge ROS (Kellos, Timar, et al., 2008). The antioxidant system can be represented by the ascorbate-glutathione cycle, which involves the glutathione reductase (GR), and the enzymes ascorbate peroxidase, glutathione S-transferase and catalase. The relationship between the levels of these antioxidants and stress tolerance has been demonstrated in previous works (Kocsy et al., 2001) and also in transgenic plants (Noctor et al., 1998).

Several studies have reported similar results in maize, where stress-tolerant varieties show a higher activity of GR and the antioxidant enzymes, compared to the stress-sensitive varieties (Aroca et al., 2003).

Among the antioxidant enzymes, thioredoxins represent a key factor in response to the oxidative stress in maize (Kocsy et al., 2001). Thioredoxins are

ubiquitous disulfide reductases, which play a key role in regulating the oxidative status of plant cells by maintaining the redox status of target proteins. They give plants tolerance of oxidative stress by detoxifying lipid hydroperoxides or repairing oxidized protein and acting as regulators in signalling pathways and scavenging mechanisms (Dos Santos & Rey, 2005). Chilling tolerant maize shows increased levels of thioredoxins, which allow the plant to cope with low temperatures and reduce the oxidative stress caused by the ROS accumulated in the cells (Kocsy et al., 2001).

A recent research has established that ROS play a considerable role in plant stress acclimation (Suzuki et al., 2011) by acting as signalling molecules that regulate genes encoding for antioxidant enzymes and modulators of the intracellular levels of the reactive oxygen species H_2O_2 (Gechev et al., 2003; Neill, Desikan, Clarke, Hurst, & Hancock, 2002; N. Suzuki et al., 2011). Fowler and Thomashow (2002) also reported the role of ROS as mediators of gene expression, indicating that transcriptional changes are necessary for chilling tolerance.

1.2.6 Role of calcium (Ca^{2+}) in cold acclimation

A number of studies conducted on Ca^{2+} have revealed that the fluctuation of its concentration inside the cell, known as Ca^{2+} signature, represents one of the earliest events in response to low temperatures as well as abiotic stresses in

general (Knight & Knight, 2012). The Ca^{2+} response occurs upstream the activation of the CBF machinery and it is conserved in different species regardless their degree of tolerance (Knight et al., 1996).

It has been demonstrated that Ca^{2+} is involved in the regulation of the promoters containing the motifs CRT and ABRE (Whalley et al., 2011) and, therefore, it is responsible for the expression of cold induced genes (Knight et al., 1996; Sangwan et al., 2001), whose expression pattern, in turn, controls the Ca^{2+} signature (Knight et al., 1998) when plants detect low temperatures. The levels of Ca^{2+}, in fact, differ according to whether or not plants have experienced cold stress previously (Knight et al., 1996), a phenomenon known as cold or stress memory. It is probable that cytoskeletal configuration may play a significant role in conferring a stress memory, since it is strongly influenced by environmental stresses, and along with it, the Ca^{2+} levels (Knight & Knight, 2012).

Calcium plays also an important role in the activation of Ca^{2+}-binding proteins such as Ca^{2+}-responsive protein calmodulin (CaM), Ca^{2+}-dependent protein kinases (CPKs) and mitogen-activated proteins (MAPs) that are induced by low temperatures (Knight & Knight, 2012).

1.2.7 Post-transcriptional regulation

Special attention must be addressed to splicing variants created when plants are exposed to low temperatures, since recent studies have shown that

low temperatures induce the creation of new mRNA variants that, in turn, encode for new protein isoforms, which regulate the splicing of genes involved in the control of the circadian clock (James et al., 2012; Marquez, Lang, & Palva, 2012).

Post-transcriptional regulation in plant stress response is also mediated through miRNAs, which are short nucleotides that act as repressors of mRNAs to which they hybridise promoting their degradation or reducing their translation (Sunkar et al., 2012). A specific miRNA in Arabidopsis has been found to improve plant response to chilling-induced oxidative stress by down regulating a target gene, whose repression allowed the expression of genes encoding for antioxidant proteins (Sunkar & Zhu, 2004).

1.3 Effect of low temperature on maize growth at early developmental stages

As previously cited, maize is particularly susceptible to low temperatures at early phases of growth (Prestrel et al. 2007, Figure 1-2). Maize root growth generally occurs between 9°C and 40°C, but it has been demonstrated that the critical lower temperature threshold for maize growth is dependant on the soil temperature

Figure 1-2. Maize seedlings at early stages of development.

during the early phases of development (Richner et al., 1996) and ranges from 10°C to 17°C according to the trait and variety (Blacklow, 1972; Bowen, 1991; Haldimann et al., 1996). Roots can survive at lower and near freezing temperatures (Blum, 1988). As the temperature decreases, it is possible to observe morphological changes in the root system; roots become swollen behind the tip (Barlow & Adam, 1989) and thicker, with a higher number of seminal roots (Kiel & Stamp, 1992). The effect of low temperature on roots may be indirectly reflected on shoot elongation and leaf formation. Shoot development, in fact, may be affected by a reduced nutrient supply through roots (Hund et al., 2007), which, as said above, is strongly dependent on soil temperature. It is also probable that shoot and leaf growth are affected by a direct effect of temperature on shoot meristem activity (Engels & Marschner, 1990; Farooq et al., 2009). However, an appropriate root system development is crucial for a successful plant growth, since it provides the uptake of all the necessary nutrients, including phosphorus, potassium and nitrogen (Hund et al., 2007). It has been observed that, among all the studied root traits, there is a strong correlation between the length of lateral roots and photosynthesis and shoot dry weight (Hund et al., 2007).

In contrast to air temperature, soil temperature plays a most significant role in maize early growth, since it influences the development of meristematic tissues until shoot emergence. It has been demonstrated that maize phenology, canopy development, biomass and yield are more affected by soil temperature than by air temperature (Stone et al., 1999). At early stages of development there

is little correlation between growth under field and controlled conditions (Stamp et al., 1985). Plants, in fact, show a different vigour under cold conditions, which is more pronounced under field conditions, because cold stress is unpredictable and may occur for several reasons (Stamp et al., 1985). Yet, it is still possible to predict the growth potential of maize genotypes by physiological and biochemical characteristics of seedlings grown under controlled conditions in growth chambers (Stamp, 1986).

1.3.1 Root development

The root system has a very important role in plant development and its function can be considered duplex. Its major tasks are the anchorage of the adult plant in the soil and water and nutrients uptake (Aichen & Smucker, 1996). In order to fulfil these functional requirements, a complex root architecture, properly established, is necessary (Hochholdinger et al., 2004). The maize root system is very complex and very different from that of other monocot families (Hochholdinger et al., 2004, Figure 1-3).

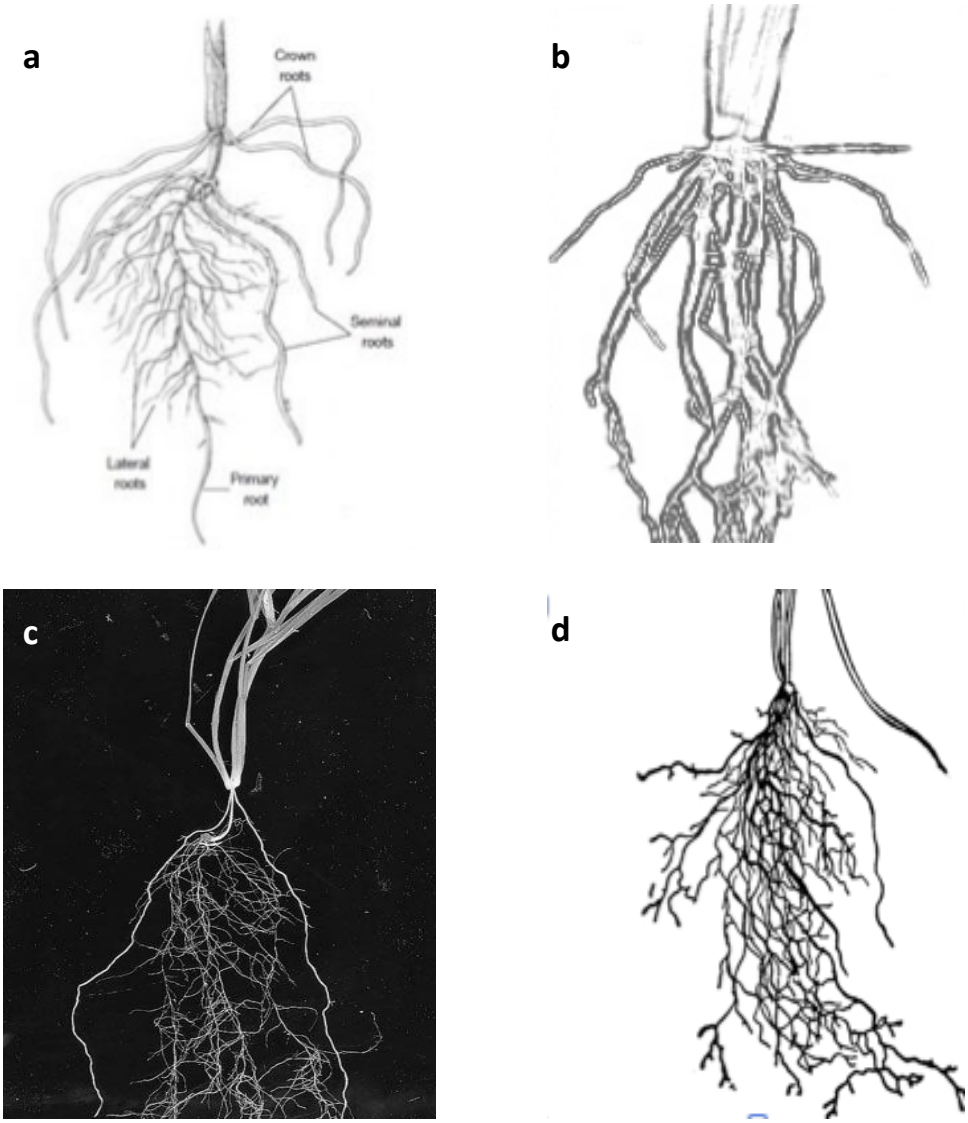


Figure 1-3. Root architecture in different monocot plants. a) Maize (*Zea mais),* b) Rice (*Oryza sativa*), c) Wheat (*Triticum spp.*), d) Oat (*Avena sativa*). The root system of maize is quite different from the ones of the other three monocts. Photos taken from a) http://www.hochholdinger-lab.uni-bonn.de/research/the-maize-root-system, b) http://www-plb.ucdavis.edu/labs/rost/Rice/roots/rtarch.html, c) http://plantsinaction.science.uq.edu.au/edition1//?q=figure_view/102, d) http://etc.usf.edu/clipart/23300/23362/oat_plant_23362.htm.

For this reason it has been needed to introduce new terms referring to and describing the organization and structures which form during plant development. Maize and monocot cereals in general display a root structure composed by several root types (Feix, 2002), some of which are developed early and belong to the embryonic root system, whilst others only develop later on and belong to the post-embryonic root system (Aibbe & Stein, 1954).

1.3.2 Embryonic root system

The roots emerging at this stage of development are represented by the primary root and seminal roots in variable numbers (Hochholdinger et al., 2004). The former emerges from the basal pole of the seedling and it is formed endogenously inside the maize embryo during the early embryonic development and it is visible about 10-15 days after pollination (Yamashita & Ueno, 1992; Yamashita, 1991). Seminal roots are also formed during embryogenesis but emerge from the scutellar node and are only visible in the embryo after 22-40 days after pollination (Erldeska & Vidovencova, 1993; Feldman, 1994; Sass, 1977) These two kinds of roots can undergo two fates: they either persist and function for the entire life cycle of the maize plant (McCully & Canny, 1985), or die after the formation of the shoot-borne roots in the post-embryonic growth phase (Feldman, 1994).

1.3.3 Post-embryonic root system

The post-embryonic root system arises from differentiated pericycle cells (Esau, 1965) of the primary and seminal roots. Two other types of roots develop: lateral roots and the shoot-borne roots (Hochholdinger et al., 2005). Lateral roots basically originate from all the main roots previously formed and are also known as branch roots, because of their branching arrangement in lateral roots of multiple orders (Hochholdinger et al., 2004). These roots are principally very short (Varney & McCully, 1991), have structural function (Lynch, 1995) and in mature maize play a fundamental role in the acquisition of water and nutrients (Wang et al., 1994). This is due to presence of open vessels in the late metaxylem for most of their length that are responsible for water transport (Hochholdinger et al., 2004; Wang et al., 1995). The shoot-borne root formation occurs at consecutive nodes of the stem and they are divided into brace or crown roots according to their above or underground site of origin. These roots become the dominant structure in the post-embryonic growth phase, resulting in the establishment of dense rootstock, which is responsible for lodging resistance and water uptake (Feldman, 1994; McCully & Canny, 1988).

Additional roots can develop at atypical sites of the maize plant, in case of wounds or when other exogenous stimuli apply. These roots are referred to as adventitious (Hochholdinger et al., 2004).

1.3.4 Biotic and abiotic influences on root architecture

Environmental conditions have strong effects on the root architecture (Hochholdinger, 2004); plants respond to environmental changes in availability of water and nutrients, but also to the presence of microorganisms surrounding the roots. The exchange of information between the environment and roots is mediated by the rhizosheath (Figure 1-4), a structure that guarantees efficient root-soil interactions (McCully, 1999). The rhizosheath is constituted of tightly bound soil particles associated with root hair and rhizobacteria (McCully, 1995).

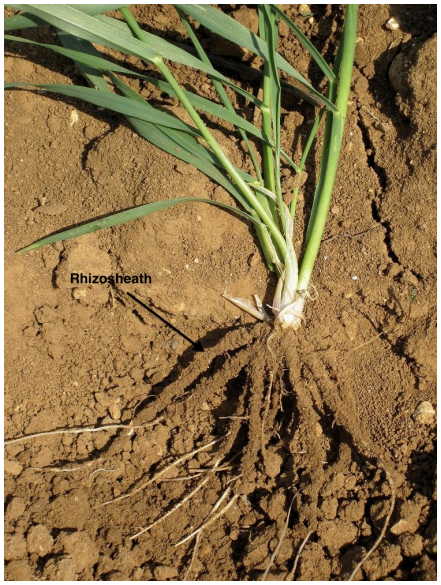


Figure 1-4. Rhizosheath. The structure guarantees efficient root-soil interactions. Photo taken from *www.euroot.eu.*

Because root architecture is susceptible to external signals, analysis of seedling at early developmental stage becomes necessary under standardized conditions. Besides, very young maize seedlings, about 10-14 days post germination, already present almost the whole set of root types, particularly the primary and seminal roots. The nutrients stored in the endosperm are sufficient to guarantee an adequate growth until the major root types develop (Hochholdinger et al., 2004).

1.3.5 Effect of low temperature on seed germination

Poor germination has been noticed in some genotypes due to imbibitional injury caused by chilling temperatures (Miedema et al., 1987), although that is not believed to be the major cue responsible for the low germination (Greaves, 1996).

From a study on germination of four tropical species, *Echinocla utilis*, *E. frumentacea, Pennisetum americanum and Zea mays,* the last one showed a better performance at temperature as low as 10/15° (Hughes et al., 1984).

1.3.6 Importance of early vigour in cold environments

Early vigour represents the capacity for plants to perform early autotrophic growth, which consists in quickly producing assimilates after endosperm reserves are exhausted (Revilla et al., 1999). Basically there are three stages of early plant development: germination, heterotrophic growth and early autotrophic growth (Prestrel et al., 2007). Early vigour is of high importance for adaptation of maize varieties to regions with sub optimum temperatures like in parts of Central and throughout Northern Europe (Prestrel et al., 2007) where soil warming is known to be slow (Arshad & Azooz, 1996; Tollner et al., 1984). This aspect is very important for maize development as it was demonstrated that

both the root and shoot meristems are directly influenced by the soil temperature (Hund et al., 2007).

Moreover, in such regions sowing is dependent on anticipated weather conditions so that the earliest and most delicate stage of development occurs when the soil temperature is still warm enough to not impair plant tissues and jeopardize the subsequent growth phases and final yield (Keane et al., 2003).

Maize varieties with improved early vigour reach maturity much quicker, show a better ground cover and less leakage of nitrate from root at the beginning of the maize growing season. As a result of classical plant breeding, traits of interests have been spread out over the Central Europe resulting in a constant improvement of the early plant vigour (Frei, 2000). Further progress towards this direction can be possible with the marker-assisted selection (MAS), through which the traits of interest are genotypically detected and the varieties containing those traits are then quickly selected and used in the production of new improved lines (Collard et al., 2005; Collard & Mackill, 2008).

Genotypic differences in cold tolerance exist for the development of the root (Stamp, 1986) and shoot (Lee, Staebler, & Tollenaar, 2002), which are manifested in photosynthesis and with strong evidence at the threshold air temperatures of around 15^0C (A Hund et al., 2007). First QTL studies on chilling tolerance in maize related to the effects on photosynthesis were carried out recently (Presterl et al., 2007).

Early vigour ensures successful transition to the autotrophic growth stage (Hund et al., 2004), although the other two growth stages, germination and heterotrophic growth, can influence plant performance significantly by ensuring a uniform germination, plant establishment and an appropriate carbohydrate supply. This results in the development of a functional photosynthetic apparatus in the autotrophic stage, which is of extreme importance, especially under cold stress (Hund et al., 2004).

1.4 Maize in Ireland

In the European Union, maize is used for grain in less than 50% of the area cultivated for the crop, while the rest is used for the production of silage and alternatively as biofuel. In marginal areas the adoption of early maturing varieties still represents a compromise between the risk of yield failure and acceptable level of yield gain (Stamp, 1984).

In Ireland, maize growth is constrained because of the short growing season due to the air frost occurring in spring and autumn. In recent years, the development and the introduction of early maturing varieties have made maize production possible in Ireland mainly in the East, Southeast and South of the country. In Ireland, where environmental conditions are marginal for the crop, the area devoted to forage maize production increased from approximately 200 ha in 1989 to 4500 ha in 1995 (Keane et al., 2003). Over the years, in fact, one of

the physiological aspects that breeders and growers have directed their attention to is the time maize varieties take to achieve the target maturity.

In Ireland maize is generally sown in the last week of April, when soil temperature is warm enough for seeds to germinate, and harvested in autumn before the first air frost occurs damaging the crop with temperatures below -2°C. The date of sowing and the date of harvest determine the length of the growing season and therefore the level of maturity and quality of the crop. Early maturing varieties reach maturity earlier; this means that the development of the canopy occurs earlier and so does its closure reducing, this way, the risk of yield losses than can be caused by the first autumn air frost at the end of the growing season. However, despite the improvement in crop quality and yield, these cultivars are still dependent on suitable soil temperatures for the initial establishment of the seedlings, a crucial stage in the development of the crop. Plus, they still benefit from a longer growing season that, in Ireland, can be extended by establishing the plants earlier (Crowley & Park, 1998).

By the use of biodegradable polythene films laid on the soil surface, it has been possible to solve the soil temperature issue giving maize growth a significant enhancement (Keane, 2002). These films act as a miniature greenhouse that raise the soil temperature aiding germination and plant development. Nevertheless despite the benefits achieved by the use of this technique, the results in terms of crop yield and quality are inconsistent and the impact on the harvest is not well characterised, therefore further varietal improvements are required to make the maize crop more economically viable.

1.5 Microarray analysis

Introduction

The development of maize hybrids able to grow with high performance at low temperatures can result in an improved establishment of the crop in cool climate regions like Ireland. Varieties included in the Irish Recommended List show a high yield performance under Irish climate conditions in specific trials, but they are mostly grown under plastic films or, when they are not, they are still dependant on suitable soil temperatures for the initial establishment of the seedlings and early maturing varieties still benefit from a longer growing season, that, in Ireland, can be extended by establishing the plants earlier (Crowley & Park, 1998). Several works on cold tolerance in cereals have led to the identification of QTLs (Szira, 2006). In maize, QTLs were associated to cold tolerance in photosynthesis (Fracheboud, 2002) and roots, where morphological traits of root development were associated to resistance to cold stress (Hund et al., 2004; Presterl et al., 2007). Another approach is aimed to the detection and identification of gene expression patterns, activated when the maize plant is grown at low temperatures, where specific genes are regulated to cope with the environmental stress (Janská et al., 2010). The activation of these patterns may be more or less intense, which results, in turn, in a lower or higher response to low temperatures; the maize genotypes can be therefore distinguished in cold tolerant and cold sensitive varieties (Ricardo et al., 2001; Farooq et al., 2009). While low temperatures seriously injure the cold sensitive varieties (Richner et

al., 1996), the cold tolerant genotypes adjust their metabolism to adapt to the environmental conditions in which they grow through the activation of metabolic mechanisms that increase the content of specific molecules, like cryo-protective compounds and antioxidants, but they also involve the down-regulation of some other gene products (Janská et al., 2010). The activation of these metabolic pathways is the result of an increased number of mRNA transcripts, whose levels can be detected.

The detection of the transcripts and the identification of the genes associated to them will lead, with an appropriate breeding programme, to the transfer of the traits of interest to new hybrids with an improved tolerance to low temperatures.

The detection of gene expression can be made possible by the employment of technologies like microarray and qRT-PCR capable to screen a large set of transcripts or even the entire transcriptome. Thanks to the microarray technology, it is nowadays possible to profile gene expression patterns of tens of thousands of genes in a single experiment.

A microarray consists of an array of DNA molecules fixed on a glass slide at specific locations called spots or features. The spots on the array act as probes that hybridise with the complementary nucleic acid sequences, represented by cDNA molecules synthesised from mRNA by reverse transcription. The extent of hybridisation is then estimated by detecting fluorescent dyes (fluorophores) with which cDNA is labelled.

According to the size and the number of the spots on the slide, the array can be distinguished into macro, with larger and fewer spots, and into micro, where spots are smaller in size and higher in number. The DNA molecules fixed on the slide can be either cDNA or oligonucleotide strands that correspond to specific genes. Arrays with cDNA are generally composed of PCR-amplified cDNA sequences that are spotted onto the slide; oligonucleotide arrays (oligoarrays) are instead composed of short (25-30 bp) or long (60-75 bp) oligomers synthesised directly on the slide, representing a single gene or gene family. The Agilent (30 oligomers) and Affymetrix (60 oligomers) systems represent examples of short and long oligoarray respectively.

The widest use of the microarray technology consists in comparing the expression of a set of transcripts from a reference sample with the transcripts from a sample in a given condition, e.g. cold treatment (Smyth & Speed, 2003).

There are two main types of array, according to the use of one or two fluorescent dyes employed in labelling the cDNA samples. In the one-colour (or one-channel) microarray, only one fluorophore is used, generally Cy3, which is used for both the reference/control and treatment. Samples are hybridised to separate slides. Hence, this approach requires two separate single-dye hybridizations in order to compare two conditions and the array provides data by comparing the intensity of fluorescence of the two samples. The strength of the single-dye system lies in the fact that a flaw in a chip, such as an aberrant sample or a failed hybridisation, will affect only that chip and will not influence the raw data of the other samples, since every chip is only hybridised with one

sample. On the other hand, the large number of slides required to carry out the analysis represents a drawback. Examples of one-channel microarray analysis are given by the Agilent and Affymetrix systems.

Microarray can also be competitive when it is carried out with transcripts labelled with two different fluorophores, such Cy3 and Cy5. This is sometimes referred to as a two-colour (or two-channel) microarray. This method relies on the use of one dye to label the control sample and the other dye to label the treated sample. The two labelled cDNA samples are then mixed and hybridised to the array and the relative intensity of the fluorescence is measured to quantify up and down regulated genes. In this method the extent of hybridisation of an individual labelled transcript is dependant on its relative abundance. In order to obtain reliable data from microarray analysis it is requested high quality RNA, considered in terms of integrity and absence of contamination.

The way samples are compared, dye labelling and direction of comparison (i.e. control vs. treatment) define the microarray experimental design. There are two main ways to compare samples in a two-colour microarray, by indirect or direct comparison. The first method is based on the use of a common control sample to which the treated sample is compared. Here, each treated sample is hybridised on a single slide along with the standard reference sample, e.g. the same genotype under low temperature and control conditions. The direct method consists in directly comparing the samples of interest without the use of a common control sample. One of the major disadvantages of the two-channel microarray is the dye-bias consequential to the different incorporation of the two

chemical dyes. Dye bias can be partially compensated with a dye swap, consisting in labelling the control and the treated samples by alternating the two dyes across biological or technical replicates. The dye swap must result in a dye balance, where every sample is equally labelled with each fluorophore. In a direct design, all the samples of interest are compared to each other and are dye-balanced, so as there is no need for a dye swap. The best implementation of a direct design is described by the loop design, where all the samples are dye balanced and evenly represented in the arrays. Although biological replicates are still advised, a loop design requires half of the slides needed for the common reference design and it is more efficient when more factors, such as genotype and treatment, are included in the experimental design (Vinciotti et al., 2005). The loop design can be also represented in a more powerful way when it is built with further internal loops, where more connections across samples are described, resulting in an interwoven loop design (Kerr & Churchill, 2001).

1.6 Quantitative Real Time PCR (qRT-PCR)

Real Time PCR is, to date, the most sensitive method for the detection of smallest changes in gene expression, even for low quantity of mRNAs (Wong & Medrano, 2005).

Relative quantitative Real Time PCR describes the change in expression of a target gene, when a treatment is applied, relative to its expression under control conditions assumed as a reference. The detection of the product is made possible by the use of fluorescence, which indicates the level of amplification at each cycle of the PCR reaction. The change in expression is practically defined as the difference in the number of cycles needed for the fluorescent signal to cross a fixed threshold, where the amplification of the specific target begins. The threshold cycles are named Ct values and are inversely proportional to the abundance of the target. In order to avoid bias, the expression values obtained form the Real Time PCR are normalised against internal controls, usually housekeeping genes, whose expression should not fluctuate between control and treatment (Dheda et al., 2004; Vandesompele et al., 2002).

1.7 Objectives of the work

The present work was structured in multiple objectives consisting in:

1) Selecting a sufficient number of maize varieties with a known degree of tolerance to low temperature and therefore already adapted to marginal areas. The use of these varieties was a prerequisite for applying a more intense cold treatment so as to unveil possible more resistant varieties.

2) Choosing the temperature regimes to be adopted for control and treatment growth conditions.

3) Planning an experimental design that would establish number and arrangement of seeds for the measurement of the physiological response to the different temperature cycles applied.

4) Gene expression profiling. Once indentified varieties showing contrasting cold resistance, these were used for gene expression profiling. The final goal of this thesis aimed, therefore, to identify cold regulated genes controlling the physiological response of maize to low temperatures.

2 Material and Methods

2.1 Plant material

The seed companies Caussade, Pioneer (France) and Codisem (Ireland) provided the maize seeds used for the experiments, for a total of twelve varieties. Algans, Justina and Picker were included in the Irish Recommended List 2008 of the cultivars that have shown a high yield performance under Irish climate conditions in specific trials (www.goldcrop.ie). The varieties also differ in the type of the kernel (flint, dent and flint-dent) and time to maturation (Table 2-1).

Delivery of the seeds was spaced out hence the first experiments were conducted with the varieties available at the time.

Table 2-1. Maize varieties info. The maize varieties were orderd from different seed companies and differed in the type of kernel and time to reach maturity. Some varieties are included in the Irish Recommended List 2008 for showing high performance under Irish climate conditions in specific trials. * Varieties included in the Irish Recommended List 2008 suitable for growing in the open/without plastic. ** Varieties included in the Irish Recommended List 2012 suitable for growing covered/with plastic.

Variety	Maturing Time	Type of Kernel	Breeding Company
Fergus*	Early	Dent	Caussade
Picker	Very early	Flint-Dent	Caussade
Lakti	Early	Flint-Dent	Caussade
Huski	Very early	Flint-Dent	Caussade
Crazi	Mid-early	Flint-Dent	Caussade
Clariti	Mid-early	Flint-Dent	Caussade
Algans*	Mid-early	Flint-Dent	Caussade
Codisco	Mid-early	Flint-Dent	Codisem
Codifar	Early	Flint-Dent	Codisem
PR39B29	Very early	Flint	Pioneer
Justina**	Early	Flint-Dent	Pioneer
PR39D60**	Early	Flint	Pioneer

Seeds were arranged on a surface of capillary matting lying over two layers of blotting paper soaked with 100ml of distilled water to keep the environment moist without excess free water. Trays were daily watered with 100ml of distilled water. The blotting paper and capillary matting were

placed in 52cm x 42cm x 9cm seed trays, which were covered by another inverted seed tray in order to reduce water loss (Figure 2-1). The blotting paper was placed below the single layer of capillary matting. Seeds were used directly from the seed bag and placed under controlled growth conditions.

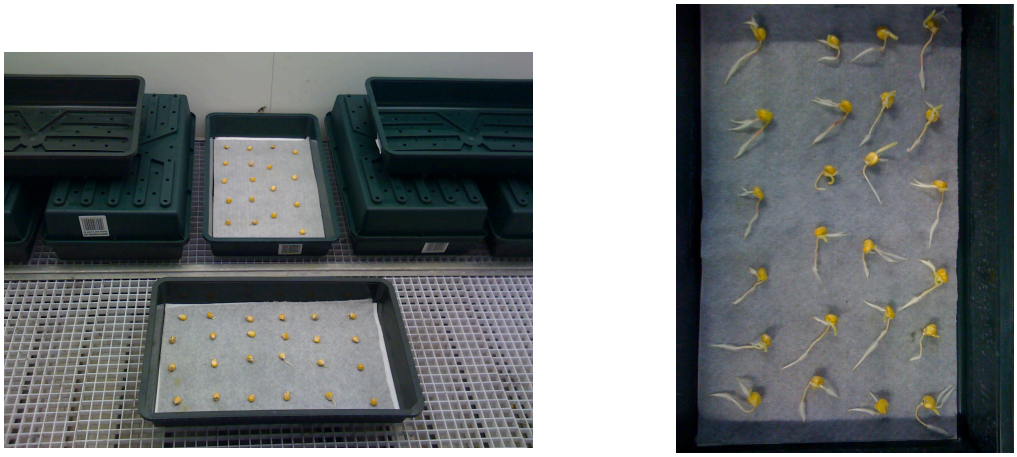


Figure 2-1. Arrangement of maize seeds. Maize seeds were arranged on a layer of capillary matting and two layers of blotting paper. The three-layered 'bed' was fitted in a seed-tray covered with another inverted tray.

Different temperature regimes were tested to define the control and treatment conditions.

1) Growth conditions for testing temperature regimes

High temperature cycle: 16 hours light/ 8 hours dark; 22°C in the light, 18°C in the dark; 400 µmol m^{-2} s^{-1}, 40% relative humidity.

Medium temperature cycle: 16 hours light/ 8 hours dark; 18°C in the light, 12°C in the dark; 400 µmol m^{-2} s^{-1}, 40% relative humidity.

Low temperature cycle: 16 hours light/ 8 hours dark; 10°C in the light, 4°C in the dark; 400 µmol m^{-2} s^{-1}, 40% relative humidity.

Each variety was represented by a group of 45 seeds, which were placed as sub-groups of 15 in three separate seed trays.

2) Growth conditions for modified temperature regimes

Medium temperature cycle: 16 hours light/ 8 hours dark; 18°C in the light, 12°C in the dark; 400 µmol m^{-2} s^{-1}.

Low temperature cycle: 16 hours light/ 8 hours dark; 12°C in the light, 6°C in the dark; 400 µmol m^{-2} s^{-1}.

Each variety was represented by a group of 45 seeds, which were placed as sub-groups of 15 in three separate seed trays.

3) Growth conditions for the full-set experiment

Control temperature cycle: 16 hours / 8 hours; 18°C / 12°C in the dark.

Chilling temperature cycle: 16 hours / 8 hours; 12°C / 6°C in the dark.

Each variety was represented by a group of 45 seeds, which were placed as two sub-groups of 21 and 24 in two separate seed trays.

Seeds were germinated and grown in environmental growth chambers (Snijders Microclima 1750, Snijders, The Netherlands). The three layers of blotting paper and capillary matting were kept constantly moist by the addition of 100ml of water every 24 hours.

2.2 Measurements of physiological parameters

Germination

Germination was scored on a daily basis over an eight-day period. Seed germination was classified as such when the radicle emerged from the meristem and was at least 1 mm long. Varietal germination was classified as such when at least the 5% of seeds was germinated.

Roots and Shoots elongation

The length of primary roots and shoots were measured, after germination, with a Staedtler® digital calibre at 24-hour intervals for a period of eight days post-germination. Seeds were removed from the trays for measurements and placed back to their previous position. Average growth was calculated with two methods:

1) Both germinated and non-germinated seeds at a specific time point were taken into account in the calculation of the average growth of the corresponding variety as well as late germinated seeds.

2) Average growth was calculated on germinated seeds from day 4 post-germination of the corresponding variety till day 8 post-germination. Late germinated seeds were excluded from calculation.

2.3 Isolation of total RNA from maize roots

Total RNA was isolated from 3cm maize roots harvested, in 3 biological replicates, on days 4, 5, 6, 7, 8 days post-germination. Roots were excised and snap frozen in liquid nitrogen and stored at -80^{0}C until required. Frozen root samples were homogenised while still frozen in 1.5 ml microcentrifuge tubes with the use of tube pestles (Sigma-Aldrich®, USA) and used as a starting material for RNA extraction. The isolation of total RNA was carried out using the Qiagen RNeasy© Plant Mini Kit (Cat. No.: 74104, Qiagen, UK) according to the protocol recommended by the manufacturer. The concentrations and the quantity of the RNA obtained were determined using the Qubit® 1.0 fluorometer (Invitrogen, UK) and repeated on the Agilent 2100 BioAnalyzer (Agilent Technologies, USA). The quality of the RNA was determined using the Agilent 2100 BioAnalyzer and the kit and reagents were used according to the manufacturer's instructions.

For microarray analysis total RNA was first extracted and column purified, then quantified and run through a quality check with the Agilent 2100 BioAnalyzer platform. Microarray analysis is a demanding technology in terms of RNA quantity and quality; hence RNA extraction and purification represent a crucial step to get a reliable interpretation of the data. At least 1μg of RNA is required for the hybridisation and the quality is determined in relation to both RNA integrity and the extent of contamination (Nolan et al., 2006). The most reliable method to establish RNA integrity is the use of instruments such as the Agilent BioAnalyzer. This instrument separates RNA by size and reports the extent of RNA degradation based on the relative proportions of ribosomal RNA bands.

Eliminating genomic DNA contamination

During the isolation process total RNA was retained on the membrane of an RNeasy© spin column provided with the kit. The sample was treated with a DNase incubation mix (80μl) for 15 min to remove genomic DNA contamination. The DNase incubation mix was composed of 10μl DNase I stock solution and 70μl Buffer RDD provided with the Qiagen RNeasy© Plant Mini Kit (Qiagen, UK). The DNase I stock solution is prepared by dissolving the lyophilized 150 units of DNase I (Qiagen, UK) in 550μl of RNase-free H_2O provided with the kit.

The DNase incubation mix was added directly to the RNeasy© spin columns of the extraction kit and allowed to act for 15 minutes at room

temperature (20-30°C). 350µl Buffer RW1 of the extraction kit was added to the columns that were centrifuged for 15s at ≥ 8000 x *g* (≥ 10000 rpm). Flow-through was discarded and total RNA was extracted according to the protocol recommended by the manufacturer.

2.4 Preparing material for qRT-PCR

cDNA sythesis for quantitative Real Time PCR (qRT-PCR)

500ng total RNA was used for the synthesis of cDNA. RNase-free H_2O was added to bring the volume to 11µl of RNA solution. 1µl 10mM dNTP (Bioline, UK) and 1µl of oligo-$dT_{(20)}$ primers (0.5µg µl-1; Cat. No.: SKU#18418-020; Invitrogen, UK) were added to the solution before incubating at 65°C for 5min to unfold the secondary structure and then chilled on ice for 1 minutes. Subsequently, 4µl of 5X first strand (FS) reaction buffer (supplied with the enzyme), 1µl 0.1M DTT (supplied with the enzyme), 1µl RNase-free H_2O and 1µl SuperScript® III reverse transcriptase (Cat. No.: 18080-044, Invitrogen, UK) were added to the reaction mixture bringing the volume to 20µl. The reaction was incubated in Biometra thermocycler at 50°C for 45-60 minutes before being inactivated at 70°C for 15 minutes. At the end of the inactivation period, the final volume was made up to 50µl by adding 30µl RNase-free H_2O provided with the Qiagen RNeasy© Plant Mini Kit.

2.5 Preparing material for custom macro array

Table 2-2. List of reagents and enzymes used for custom macro array.

Reagents and enzymes
5X First Strand (FS) Buffer (250mM Tris-HCl, pH 8.3, 375mM KCl, 15mM $MgCl_2$)
$MgCl_2$-βNAD$^+$ (17.5mM $MgCl_2$, 0.75mM βNAD$^+$)
10X Second Strand (SS) Buffer (200mM Tris-HCl pH 6.9, 900mM KCl, 46 mM $MgCl_2$, 1.5mM βNAD$^+$, 100mM $(NH_4)_2SO_4$)
0.1 M DTT
dNTPs (10mM each)
NTPs (100mM each)
Oligod$T_{(20)}$ primers (50μM, 500ng μl^{-1})
Random hexamers (7μM, 200ng μl^{-1})
RNase Inhibitor
RNase-free H_20
DNA polymerase I
RNase H
E. Coli DNA ligase
T7 RNA polymerase
Pyrophosphatase
SuperScript® II
ATP/UTP/GTP-mix stock solution (33mM each)
CTP-nucleotide stock solution (50mM)
Cy3/Cy5-UTP (5mM)
2X SCC, 0.5X SCC, 0.05X SSC, 1%SDS, 0.5% SDS

The material used for the custom macro array was prepared by amplifying the RNA samples through two rounds of double stranded cDNA synthesis and the generation of a cRNA, which was then Cy-labelled. The Cy-labelled cRNA was used for the hybrisation on the array.

RNA amplification and cRNA synthesis

Total RNA was amplified by using the reagents included in the two-colour Quick-Amp Labeling kit (Cat. No.: 5910-0424, Agilent Technologies, USA) and following the protocol recommended by the manufacturer with some modifications as follows.

First strand (FS) cDNA

Three µl of 10-100ng of total RNA were used for the synthesis of the first strand cDNA. 2µl 5µM oligodT-T7 primers were added before incubating the reaction at 5°C for 5 minutes and chilling on ice for 2 minutes. Subsequently, 2µl of 5X FS reaction buffer, 1µl 0.1M DTT, 0.5µl 10mM dNTP (Bioline, UK), 0.5µl RNase inhibitor, 0.5µl RNase-free H_2O and 0.5µl Superscript® II reverse transcriptase were added to the reaction before incubating the 10µl volume at 42°C for 1 hour. After the incubation, the reaction was heated in a Biometra thermocycler at 70°C for 10 minutes to inactivate the reverse transcriptase and cooled down at 4°C for 2 minutes.

Second strand (SS) cDNA

The following reaction was assembled on ice. 4µl $MgCl_2$-βNAD$^+$ (17.5mM $MgCl_2$, 0.75mM β-NAD$^+$), 0.4µl 10mM dNTP (Bioline, UK), 0.5µl RNase H, 0.6µl DNA polymerase I, 0.2µl *E. Coli* DNA ligase and 4.3µl RNase-free H_2O were added to the reaction before incubating the 20µl volume at 16°C for 2 hours in a water bath in a cold room according to the protocol. The reaction was then inactivated at 75°C for 10 minutes. After the denaturation, the reaction was chilled at 4°C for 2 minutes.

cRNA synthesis

The following reaction was assembled at room temperature to prevent precipitation of cDNA by spermine in the 5X FS reaction buffer. 10µl 5X FS reaction buffer, 4.5µl 100nM of ATP, 4.5µl 100nM of GTP, 4.5µl 100nM of CTP, 4.5µl 100nM of UTP, 2µl T7 RNA polymerase, 0.5µl pyrophosphatase, RNase-free H_20 were added to the reaction before brief centrifugation in a beaker with a magnetic stir bar. Subsequently, the 50µl reaction was incubated at 37 °C for 16 hours. The cRNA (complementary RNA) was column purified by using the RNaesy© Mini Kit (Cat. No.: 74104, Qiagen, UK) according to the protocol recommended by the manufacturer.

Second round of first strand cDNA synthesis

Nine µl of the eluted cRNA were used for the second round of single strand cDNA synthesis. 2µl 7µM random 6mer (Cat. No.: SKU# 48190-011,

Invitrogen, UK) primers were added to the sample before incubating in thermocycler with heated lid at 70°C for 10 minutes and chilling on ice for 2 minutes. The volume of the reaction was made up to 20µl by adding 4µl 5X FS reaction buffer, 10mM dNTPs, 2µl 0.1 M DTT, 1µl RNase inhibitor, 1µl SuperScript® II reverse transcriptase before incubating at 42°C for 1 hour and cooled down at 4°C for 2 minutes. The enzyme RNase H was added to the samples for degrading the RNA and incubated at 37 °C for 20 minutes. Subsequently, the samples were heated at 95°C for 5 minutes and chilled at 4°C for 2 minutes.

Second round of second strand cDNA synthesis

For the second round of SS-cDNA, 16.5µl 10X second strand (SD) reaction buffer (200mM Tris HCl pH 6.9, 900mM KCl, 46mM $MgCl_2$, 1.5mM B-NAD^+, 100mM $(NH_4)_2SO_4$), 5µl 10mM dNTPs, 2.5µl DNA polymerase I, 1µl *E.Coli* DNA ligase and 79µl RNase-free H_2O were added to the samples before incubation at 16°C for 2 hour and chilling at 4°C for 2 minutes. Double strand cDNA was isolated through PCR cleanup column (Qiagen, UK) and ethanol precipitation. Samples were resuspended in a volume of 10µl.

Labelling double strand (DS) cDNA

1.25µl of 50-150ng of DS-cDNA were used for labelling. 2 µl 5X FS reaction buffer, 2µl of a stock solution containing 33mM of ATP/UTP/GTP

each, 1µl 50mM CTP-nucleotide stock solution, 1µl T7 RNA polymerase, 0.25µl Pyrophosphatase and 2.5µl 5mM Cy3-UTP/Cy5-UTP were added to the DS-cDNA samples before incubating at 37°C for 16h in the dark. The ratio Cy-UTP/UTP was 1:4. The labelled DS-cDNAs were used for macroarray hybridisation.

2.6 Construction of cold/chilling genes database

The 'Gene Hunting' method was used to detect genetic sequences involved in response to abiotic stress in maize. *Arabidopsis thaliana* was chosen as the reference plant. The Arabidopsis database (*www.arabidopsis.org*) was searched for the keywords *chilling stress*, *cold stress* and *cold tolerance* in order to find the genes and their sequences involved in the response to abiotic stress factors. Subsequently, the protein sequence was searched in the *Maize Genetic Database (www.maizedgb.org)* with the previously obtained Arabidopsis abiotic stress nucloetide sequences. Finally, the protein sequences were translated into nucleotide sequences by using the *Maize GDB Blast Service* (*www.maizegdb.org/blast.php*). From the nucleotide sequences, only the EST (Expression sequence tags) sequences were used to construct a custom macro array.

2.7 Custom macro array and microarray platforms

Macroarray

The array was constructed with 257 ESTs of the genes involved in response to cold. It was spotted on glass superamine 2 slides using a GeneMachines™ OmniGrid Accent™ Arrayer (Digilab® Genomic Solutions, USA). The array consisted of three replicate sub-arrays per slide and each spot was 150nm from the next with 300nm between the sub- arrays. The ESTs were in size between 1kb and 3kb. However, most of the sequences were around 1.5-2Kb and spotted at an average concentration of 200ng μl^{-1}.

Microarray

Microarray hybridisation was conducted in the Institute of Genomic Research in Arizona with the use of a 46K 70-mer oligos. Each array was printed using 48 pins and comprised 4 metarows and 12 metacolumns. The 46K array had subarrays, which had 31 columns and 31 rows. The selection of the oligos was determined by the analysis of the expression profiles generated for sixteen diverse maize tissues and eliminating oligos from the original two slide set (57K oligos printed over two slides) that had not reported any significant expression in any of the sixteen tissues. These oligos may represent very weakly expressed genes or not perform well during hybridisation due to design issues. RNA samples were sent from Teagasc over the Institute in Arizona in RNAstable® microfuge tubes (Biometrica,

USA) according to the manufacturer's instructions. Image acquisition was conducted with a GenePix® scanner (Axon, USA).

2.8 Construction of a custom macro array

Microarray immobilisation

The long oligonucleotides were deposited onto specially modified glass surfaces during the print process. Slides were rehydrated over a 60°C water bath for 10 seconds paying attention that spots did not merge together. Slides with their label-side up were quickly dried on a 65°C heating block for 2 seconds and cooled down for 1 minute at room temperature. Subsequently, slides were UV cross-linked to irreversibly bind the DNA to the glass surface by exposing the slides to the Stratalinker® (Stratagene, UK) cross-linker. Slides were then washed in 1% SDS for 5 minutes at room temperatures on a shaker or agitated by hand before denaturing the probes on the microarray slides at 95-100°C by immersing the slides in boiling milliQ water for 2 minutes. SDS was removed by dipping the slides into nano-pure H_2O 10 times and in 100% ethanol 20 times. Slides were placed in 50ml Falcon tubes and centrifuged at 1000rpm for 4 minutes to dry them out.

Microarray Hybridisation

The labelled targets were denaturised by incubation in boiling water for 2 minutes and placed on ice. Subsequently, the pre-heated targets were placed in an ArrayIt® Hybridisation Cassette (ArrayIt® Corporation, USA) for hybridisation at 55°C for 12 hours.

Microarray Washing

Slides were washed at room temperature with: 2X SCC, 0.5% SDS for 5 minutes, with 0.5X SCC for 5 minutes and with 0.05X SSC for 5 minutes by immersing the slides in a glass slide-staining jar. Slides were then spin dried in 50ml Falcon tubes in a centrifuge at 1000 rpm for 4 minutes and stored in a light proof box at room temperature under dry conditions.

2.9 Primers

All primers were designed using Primer3 primers designer tool (http://simgene.com/Primer3) and synthesised by Metabion (Germany). Lyophilised primers were re-suspended in nuclease-free water to a final concentration of 100pmol μl^{-1} (mM/L). Re-suspended primers were diluted to the working solutions of 10pmol μl^{-1}. Table 2-2 lists the primers used for quantitative Real Time polymerase chain reaction (qRT-PCR).

Table 2-3 Primers used for qRT-PCR. Primers were re-suspended in nuclease-free water to a final concentration of 100pmol μl^{-1}. Working solutions were diluted to 10pmol μl^{-1}.

Name	Forward sequence	Reverse sequence
Actin	TCTGCTGAACGCGAAATTGT	ACAGATGAGCTGCTCTTGGCA
GAPDH	ACTGTTCATGCCATCACTGC	GAGGACAGGAAGCACTTTGC
ZmUBI	GGTGGTATGCAGATCTTT	GTAGTCTGCTAGGGTGCG
Adh1	CGTCGTTTCCCATCTCTTCCTCC	CCACTCCGAGACCCTCAGTC
Hmgp	TTGGACTAGAAATCTCGTGCTGA	GCTACATAGGGAGCCTTGTCCT
Invr1	CGCTCTGTACAAGCGTGC	GCAAAGTGTTGTGCTTGGACC
Zein	GCCATTGGGTACCATGAACC	AGGCCAACAGTTGCTGCAG
Cyclophilin	TGTGAACCGATTTAGGCACA	CGCTGTCGTCAACTTATCCA
Ef1-α	GGCTGGCACTATCAGAGAGG	CACGACGTACTTTGCTCTCG
γ-tubulin	ACATCATTCAAGGGGAGGTG	CTTGCAGGTGCCCAGTCTAT
Thioredoxin	ACCCCGACATTCTTCTTCCT	CGGCTGCTAGCACTTTCTTC
Tua5	GTCGACCTTGAACCCACTGT	GTTAGCTGCGTCTTCCTTGC
MZ00003507	TTCGGATCCTGTCTTCCATC	ACTCCACGAAAGCATCAAGG
MZ00004486	GAGCAACGTGATTGGACAGA	GCATATGCGGGGAGAACATA
MZ00022876	GGAAGGTGTGTTCTGCGTTT	TATGAAGCTGACTGGCGTTG
MZ00041708	TTGCTGCTCTTCACCTTCCT	ACAACAATGGCTTCCCTGAA
MZ00023411	GCACCACGAGAAGAAGAAGG	CACAAGCAAGCAGCTACCAA
MZ00026737	AGAAGGCCATGGTCAAGATG	GCTTGCAGGTCATGAACAAA
MZ00029223	CGAAGATCAAGGACGAGGAG	ACGACTGCTCTGCAACAGAA
MZ00026029	GCTACATCAACAGCCCCAAC	TACACGACAACACGACACGA
MZ00037140	AAGTAAGGCCACGCGTTTTA	CCTGGACTACGAGACGGTGT

2.10 Quantitative Real Time polymerase chain reaction (qRT-PCR)

Relative Real Time PCR reactions were conducted using Sybr® Green as fluorescent reporter. The LightCycler® 480 Sybr® Green I Master Mix (Cat. No.: 04887352001, Roche Applied Science, Germany) and the Fast Sybr® Green Master Mix (Cat. No.: 4385612, Applied Biosystems, USA) were used for PCR reactions. PCR reactions were performed using the Roche LightCycler® 480 and the Applied Biosystems 7500/7500 Fast Real-Time PCR System according to the manufacturer's instructions, except that the reagents were used in half the quantity in a reaction volume of 10µl (to save costs).

LightCycler® 480 Real-Time PCR reactions

The volume of 2.5µl cDNA was used for PCR reactions. 5µl Sybr® Green I Master Mix, 1.5µl H_2O, 0.5µl 10µM/L forward primer and 0.5µl 10µM/L reverse primer were added to the reaction before incubating at 95°C for 5 minutes with heated lid at 90°C. Subsequently, 40 thermal cycles were repeated with the following profile: 95°C for 10 seconds as a melting/denaturation temperature, 60°C for 20 seconds as annealing temperature, 72°C for 30 seconds as elongation/extension temperature.

7500/7500 Fast Real-Time PCR System reactions

2.5µl cDNA was used for PCR reactions. 5µl Sybr® Green I Master Mix, 1.5µl H_2O, 0.5µl 10µM/L Fw primer and 0.5µl 10µM/L reverse primer were added to the reaction before incubating at 95°C for 10 minutes with heated lid at 90°C. Subsequently, 40 thermal cycles were repeated with the following profile: 95°C for 15 seconds as a melting/denaturation temperature, 60°C for 1 minute as annealing/elongation temperature.

2.11 Statistical analysis

Physiological experiment

The experiment was carried out according to a randomised block design. Each experiment was conducted in three replications. A multifactorial ANOVA (Analysis of variance) was used to calculate three effects on growth: genotype, treatment and time point. The replication of the experiment was interpreted as a blocking factor. The analysis was performed with the GenStat statistical software package (VSN International) and the Statistical Analysis System (SAS) software (SAS Institute Inc.)

Microarray analysis

The analysis of microarray was performed with the MapMan Robin software package (http://mapman.gabipd.org/web/guest/home) and the web-based application CARMAweb (Comprehensive R-based Microarray Analysis web service, https://carmaweb.genome.tugraz.at/carma/). Both the applications are based on the R (http://www.r-project.org/) programming language and environment for statistical computing. The analysis was performed using the Bioconductors limma (Linear Model for Microarray Data) package integrated in the Robin and CARMAweb software. Microarray data were quality checked, adjusted and normalised before analysis.

Before the analysis, the microarrays need to be pre-processed to remove the technical variance and systematic errors without altering the biological variance within the data. It is also possible to carry out a quality check to detect the presence of artefacts (printing, dust contaminants or wash defects) on the chips, caused by poor hybridisation, which results in bad spots. This quality check is usually carried out when the chips images are scanned and therefore it is not necessary in the data pre-processing. The pre-processing of the microarray consists in the correction of the background optical noise related to non-specific binding of the fluorophores within each array and in the normalisation of the expression values of the data. Background correction and normalisation can be performed with different methods, depending on the type of the array, the image scanning software or the platform used. The background signal can be defined as the optical noise of the hybridisation that needs to be subtracted from the foreground signal,

which corresponds to true level of hybridisation. The *minimum* method was used in this case, which consists in giving the half the minimum positive corrected intensities for the array to any intensity that is equal to zero or negative. This method is particularly recommended for arrays scanned by the GenePix® scanner according to the manufacturer's instructions.

The pre-processing steps include two normalisations: in the first one, the data is adjusted within each array (*within-array* normalisation), while, in the second one, the data is adjusted across the arrays (*between-array* normalisation). The *within-array* normalisation was performed with the *print-tip loess* method; spots on the chips are generally printed in batches (print-tip groups) and this can cause variation in the level of purity of the probes among the print-tip groups and so of the intensities of the spots after hybridisation and scanning. Technical variation among the groups was then removed with the print-tip loess normalisation method, which normalises the log-ratios intensities in each print-tip group, relying on the fact that each spot shares the same spatial and technical characteristics (recommended for GenePix® scanners).

For the between-array normalisation, the expression values were *scaled* so that the log-ratios had the same median-absolute-deviation (MAD) across the arrays (Yang et al, 2002; Smyth and Speed, 2003).

Within-array and between-array normalisation were also carried out to remove dye bias and to adjust the expression intensities so that the log ratios have similar distribution across the arrays and become, therefore, comparable.

The pre-processing analysis was performed with the MapMan Robin software package and the web application CARMAweb. Both the applications can generate graphs and plots showing the quality of the chips and the distribution of the expression values before and after the background correction and normalisation. Robin, in particular, is able to generate a warning for some of the quality assessment functions whether any of the quality processes fails or the results do not fall within a threshold of reliability. By loading the data spreadsheets obtained from Arizona, the user has to specify the array format or simply the software used by the scanner to read the chips; it is also possible to choose the columns to be used to read the signal intensities of the Cy-dyes and, in case, to exclude all the bad spots flagged out by the GenePix® scanning software. The median intensities were chosen for the quality check and the microarray analysis, because median distributions are less affected by the presence of possible artefacts (Yang et al., 2002).

False colour images were then generated by Robin to assess the presence of artefacts indicating poor quality spots, which should be then discarded from the analysis. Similar images were obtained from the Arizona Institute of Genetic Research along with a spreadsheet containing the values of the fluorescent intensity for each spot (in Arizona, the false colour images were generated from the chip scanning and the results reported in the spreadsheet; in Robin the false colour images were generated from the reading of the spreadsheet). Flags were assigned to bad spots as previously described. Robin is not able to assign flags to bad spots, but it only detects the

presence of artefacts in the chips, which can be discarded if they may bias the microarray analysis.

In microarray analysis values are transformed by taking the logarithm in base 2 (log_2), because, this way, the data becomes roughly normally distributed and it can be used for statistical tests. A difference of 1 unit between the two fluorescent intensities for a gene represents, thus, a 2-fold change in expression and it is described as M value. M is the log_2 ratio of Cy_3 and Cy_5 intensities and describes the measure of the treatment effect. Another value that is considered is the average of the expression level of each gene that is defined as A. M and A values are plotted against one another so as to generate MA-plots, which are indicative of the data distribution of the microarray. MA-plots are generated before and after background correction and normalisation.

For the analysis of the data, the number of comparisons is defined and the dyes assigned, then the control and the treated samples must be selected for each comparison. The type of analysis to be carried out is dependent on the type of the microarray. In case of a small number of arrays the simple fold change analysis can be performed, where the change in expression of the genes is defined by the difference of the M-value between the treated sample and its control. The user has to define cut-off values for the M and A values so that only the genes falling within the selected criteria, regarding fold-change and average expression, are considered differentially expressed. In the specific case of the present work, the analysis was performed using the limma paired moderated t-test statistics, which is based on the empirical

Bayesian approach. The test is particularly recommended for small group sizes (up to 8 arrays) and when each treated sample has its own control to be compared to.

Quantitative Real Time PCR (qRT-PCR)

The housekeeping genes for the relative qRT-PCR were selected using the geNorm algorithm (Vandesompele et al, 2002) according to the user guide's instructions. The application geNorm is an algorithm that selects the optimal pair of reference genes out of a larger set of control genes. The algorithm is based on the assumption that the candidate genes are not co-regulated and that the expression ratio of two genes is identical in all the samples, regardless the experimental conditions. So, the variation of the ratio is indicative of a variation in stability of expression. Two parameters are defined: M (average expression stability) and V (pair wise variation). An M-value describes the variation of a gene compared to all other candidate genes, in particular, a low M value indicates a more stable expression, so a better suitability for a gene to be used as a reference. The gene with the highest M-value is eliminated, and the process is repeated until there are only two genes left. The last pair of candidates remaining is recommended as the optimum pair of reference genes. A V-value describes the pair wise variation of a control gene with all the others. The V-value of 0.15 is considered as a threshold, below which the use of an additional gene is not requested. In other words, it tells how many reference genes are needed (Vandesompele et al, 2002).

Primer pairs were designed for all the genes picked for the qRT-PCR (Table 2-4). Once primers were designed, they were tested for amplification efficiency in qRT-PCR. Amplification efficiency describes the rate at which a PCR product (amplicon) is amplified and it is commonly expressed as a percentage value. A PCR amplicon is theoretically doubled during the amplification phase (also called log phase or exponential phase) and the efficiency is equal to 100%. The slope of a standard curve is usually used to estimate the PCR amplification efficiency. A standard curve is graphically represented as a semi-log regression line where the natural log of a serially diluted template (in this case cDNA) is plotted against the Ct values generated from the reaction. A slope of -3.32 indicates a 10-fold increase of the amplicon every 3.32 cycles during the amplification phase and a PCR reaction with 100% efficiency (Livak & Schmittgen, 2001; Pfaffl, 2001). However, in practice, several factors may influence PCR amplification (primer dimer formation, poor quality of the samples, carry-over chemicals), therefore the efficiency can be distant from 100%. The amplification efficiency (E_{amp}) of qRT-PCR is calculated by the following formula:

$$E_{amp} = (10^{-1/slope} - 1) \times 100$$

Differences in efficiencies define the type of analysis that can be performed to determine the relative fold change of the treated samples when compared to the control samples. Livak's method as well as the comparative

Ct-method can be used when the amplification efficiencies of all the primers are near 100% and the difference is within 5% of each other (Livak & Schmittgen, 2001).

In the present work, the analysis of the quantitative Real Time PCR was carried out with the Livak's method (Livak & Schmittegen, 2001), which can be described by the formula:

Expression ratio (fold change) = 2^-ΔΔCt

Ct is the threshold cycle at which amplification of the target is detected. The ΔΔCt in the equation is obtained from the difference between the normalised target gene under control ($\Delta Ct_{control}$) and the normalised target gene under treatment ($\Delta Ct_{treatment}$). Each ΔCt is calculated by subtracting the Ct value of the reference gene from the Ct value of the target gene. The '2' in the equation is based on the assumption that the target doubles at each amplification cycle and it is equal to ($10^{-1/slope}$ -1) when the slope of the standard curve is -3.32 and the efficiency is 100%.

Since two reference genes were used in the analysis, the Ct of the reference is calculated with the squared geometric mean of the Ct values of the two housekeeping genes.

The relative fold change was calculated for each gene at each time point for the 3 biological replicates.

Table 2-4 Primers designed for the genes of interest.

Primer Sequence ID	Gene product	Forward sequence	Reverse sequence
MZ00003507	RNA binding protein	TTCGGATCCTGTCTTCCATC	ACTCCACGAAAGCATCAAGG
MZ00004486	Patogenesis related proteint 1 (PRP-1)	GAGCAACGTGATTGGACAGA	GCATATGCGGGGAGAACATA
MZ00022876	Unknown protein 1(Ukw-1)	GGAAGGTGTGTTCTGCGTTT	TATGAAGCTGACTGGCGTTG
MZ00041708	Unknown protein 2 (Ukw-2)	TTGCTGCTCTTCACCTTCCT	ACAACAATGGCTTCCCTGAA
MZ00023411	Draught inducible protein (DIP)	GCACCACGAGAAGAAGAAGG	CACAAGCAAGCAGCTACCAA
MZ00026737	Peroxidase	AGAAGGCCATGGTCAAGATG	GCTTGCAGGTCATGAACAAA
MZ00029223	Putative heat shock protein hsp22 precursor (HSP)	CGAAGATCAAGGACGAGGAG	ACGACTGCTCTGCAACAGAA
MZ00026029	Probable lipid transfer protein (LTP)	GCTACATCAACAGCCCCAAC	TACACGACAACACGACACGA
MZ00037140	Glucose starvation-induced protein precursor (GSIP)	AAGTAAGGCCACGCGTTTTA	CCTGGACTACGAGACGGTGT

3 Results

3.1 Physiological experiment

The aim of the experiment was to evaluate the cold tolerance of maize at very early stages of establishment in terms of percentage of germination and root and shoot growth. According to Stamp (Stamp, 1986) it is possible to predict the growth potential of maize genotypes by physiological and biochemical characteristics of seedlings grown under controlled conditions in growth chambers.

3.1.1 Preliminary experiments to apply cold treatment

3.1.1.1 Growth conditions for testing temperature regimes

Three varieties were used for the experiment (Algans, Fergus and Picker) and three different light/dark temperature regimes were set up. A high temperature cycle was set at 22°C for 16 hours in the light and 18°C for 8 hours in the dark, a medium temperature cycle at 18°C for 16 hours in light and 12°C for 8 hours in dark, and a low temperature cycle at 10°C for 16 hours in light and 4°C for 8 hours in dark.

The higher temperature light/dark cycle resulted in an early occurrence of germination (between day 1 and day 2) with a quick root and shoot emergence and development. Under medium temperature conditions seeds germinated after two/three days of the experiment, whilst no germination occurred under the low temperature conditions applied for a period of up to 10 days (Figure 3-1).

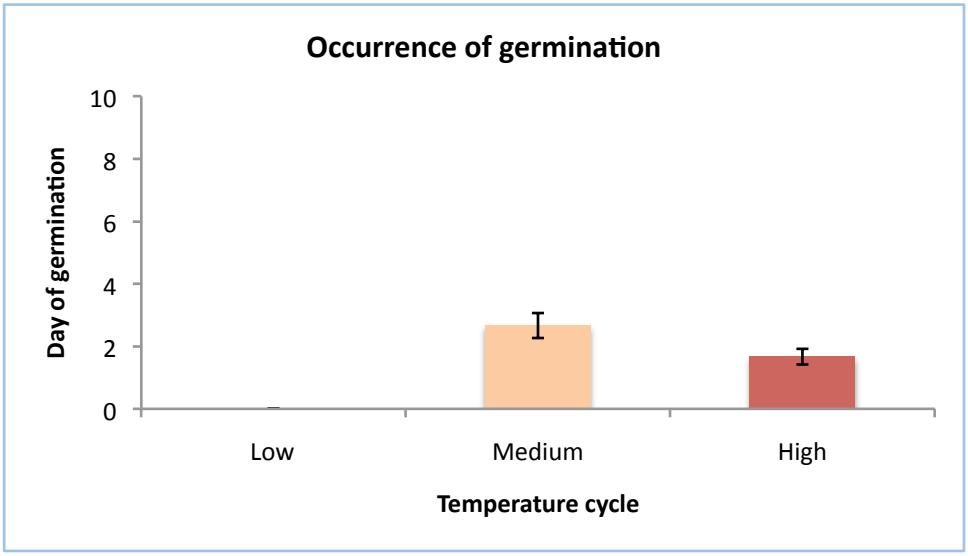


Figure 3-1. Occurrence of germination under different temperature cycles. Germination was considered to occur when at least 5% of seeds of every variety germinated. Results are mean ± SE (error bars; n=135). Three 12h light/8h dark temperature cycles were used: Low (12°C/4°C), Medium (12°C/6°C), High (18°C/12°C).

3.1.2 Growth conditions for modified temperature regimes

A second trial was then carried out, with an altered low temperature day/night cycle, which was shifted to 12°C for 16 hours under light and 6°C in the dark. The setup of the experiment was left unchanged and was conducted in three replications.

The new low temperature regime resulted in a positive response from maize seeds in terms of germinations, which occurred after 6 days of the experiment (Figure 3-2).

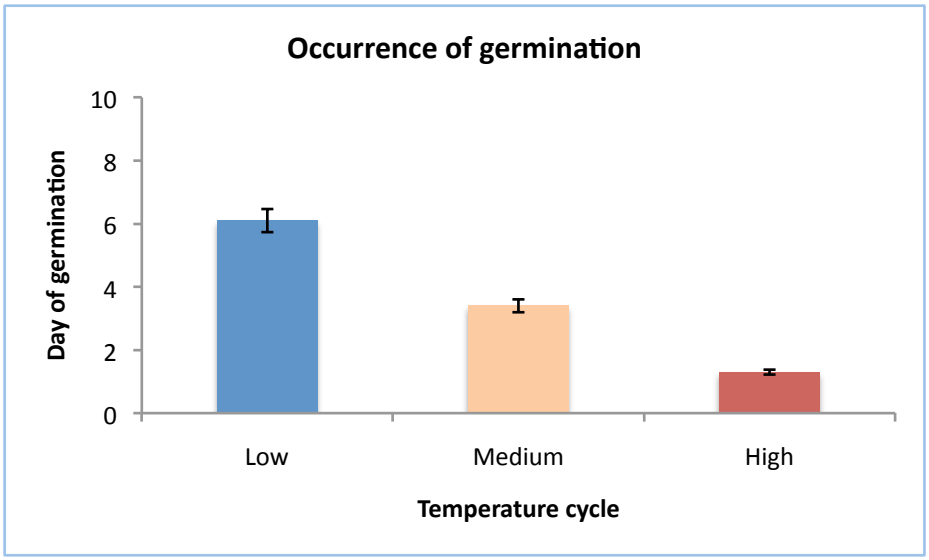


Figure 3-2. Occurrence of germination under modified temperature cycles. Germination was considered to occur when at least 5% of seeds of every variety germinated. Results are mean ± SE (error bars; n=135). Three 12h light/8h dark temperature cycles were used: Low (12°C/6°C), Medium (12°C/6°C), High (18°C/12°C).

The medium and the new low temperature regimes were picked for the next run of the experiment, as control and cold treatment respectively. The aim of the experiment was to determine the germination rate and the growth performance of the genotypes, in terms of primary root and shoot elongation, under the cold treatment.

The high temperature conditions proved to be unsuitable for the purpose of the experiment, since root elongation was so high that individual roots overran the growth area of the surrounding seeds, evolving in a complex and interwoven root system, making it difficult to score individual root growth at later stages of the experiment. In many cases, because of these impediments, root would pierce the layer of capillary matting continuing to extend underneath the same for several millimetres. Moreover, secondary roots were produced along the primary root, contributing in a major way to the interwoven root system. Root hairs, in a similar way, would weave with the hairy surface of the capillary matting, practically making the roots attached with the layer. All these events made it actually impossible to take individual measurements without breaking the samples from removing them off the trays or simply assess how far a root extended before it twisted around another one.

The high temperature would have been useful to examine the response of the commercial varieties used in the experiment to higher temperatures than the ones they were adapted to. In fact, since the hybrids were already known to have a degree of tolerance to cold (and for this reason

on the market), the high temperature regime was not strictly necessary, since the medium temperature cycle was used as the control.

3.1.2.1 Germination test

After the occurrence of germination, percentage of germination was calculated and scored on 24-hour intervals over a period of 8 days post-germination. EU legislation requires for maize a germination of 90% for commercial use. However, as a rule of thumb for germination test, germination of 70% is considered the lowest threshold for a good ability of seeds to break dormancy and germinate under the experimental conditions. Therefore, the 70% threshold was set for the purposes of this experiment. The time required for estimating the percentage of germination was eight days after the first varietal germination observed. Figure 3-3 shows the percentage of germination on day 8 post-germination under control and cold treatment.

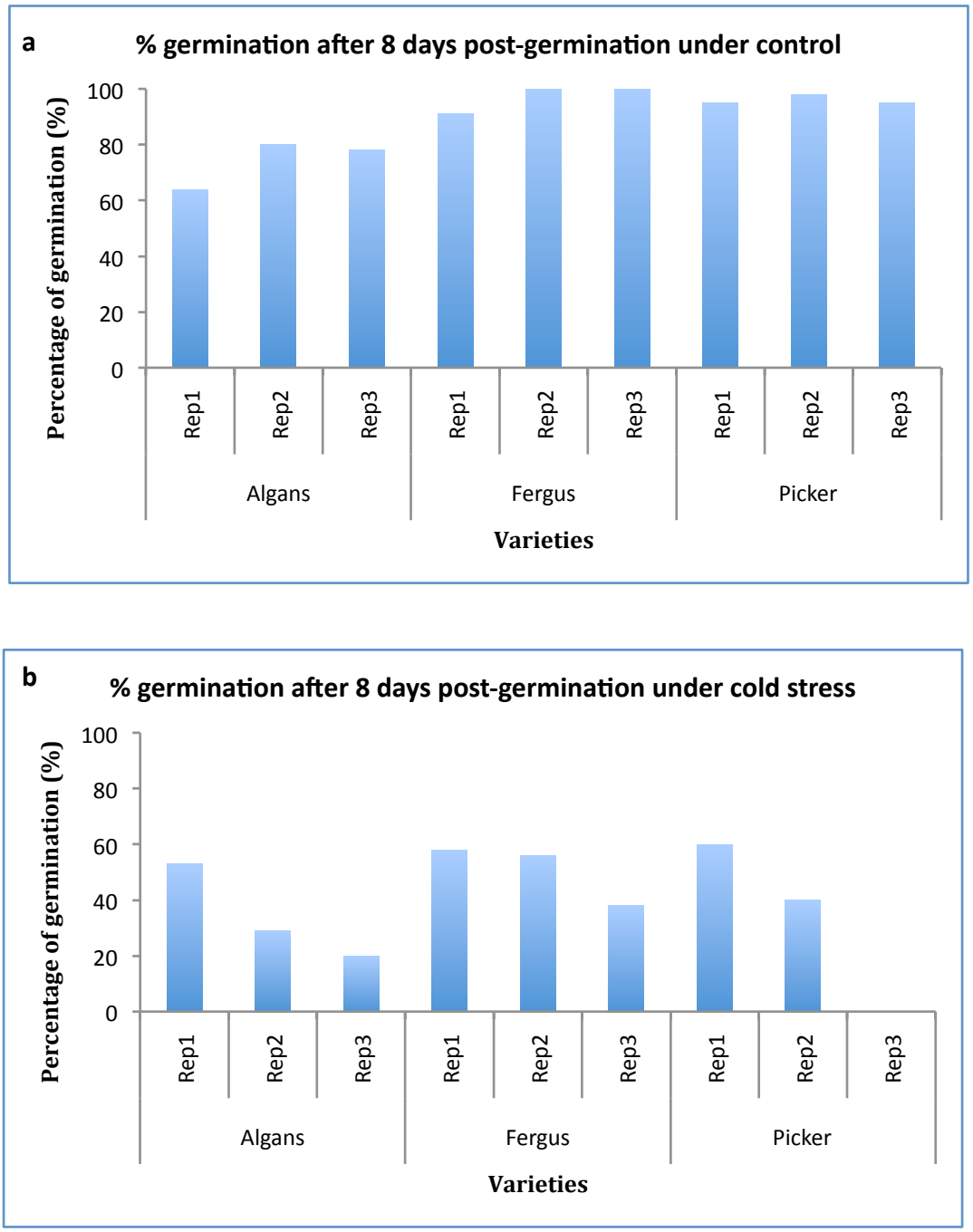


Figure 3-3 Percentage of germination of the three varieties under (a) control conditions and (b) cold stress. Rep1, Rep2, Rep3 represent replicates. Percentage of germination was calculated for each replicate (n=45).

Under control conditions, Fergus and Picker showed an average percentage of germination above 90%, while Algans showed an average germination of 70%. The temperature regime was therefore considered suitable for being adopted as the control, since all the varieties showed a percentage of germination within the set threshold. The percentage of germination of the three varieties under cold stress was below 60%; therefore the temperature regime produced a treatment effect on germination.

3.1.2.2 Growth test

After the occurrence of germination, the length of primary roots and shoots were measured after 8 days post-germination. All the growth measurements were started after seed germination, which occurred on different days for seeds under control or low temperature conditions. The late/non-germinated seeds, when measurements were made, were taken into account in the calculation of the average growth of the corresponding variety, as well as seeds that have germinated late in the experiment. The experiment was carried out in three replications. Primary root and shoot elongation were measured in millimetres (mm).

The growth response to cold stress estimated on day 8 was interpreted as a percentage of growth reduction or growth ratio, obtained by dividing the length of a measured variable (root or shoot) under cold treatment by the length of the same variable under control (Figure 3-4).

Growth ratio = Measured variable$_{(low\ temperature)}$*/ Measured variable*$_{(control)}$

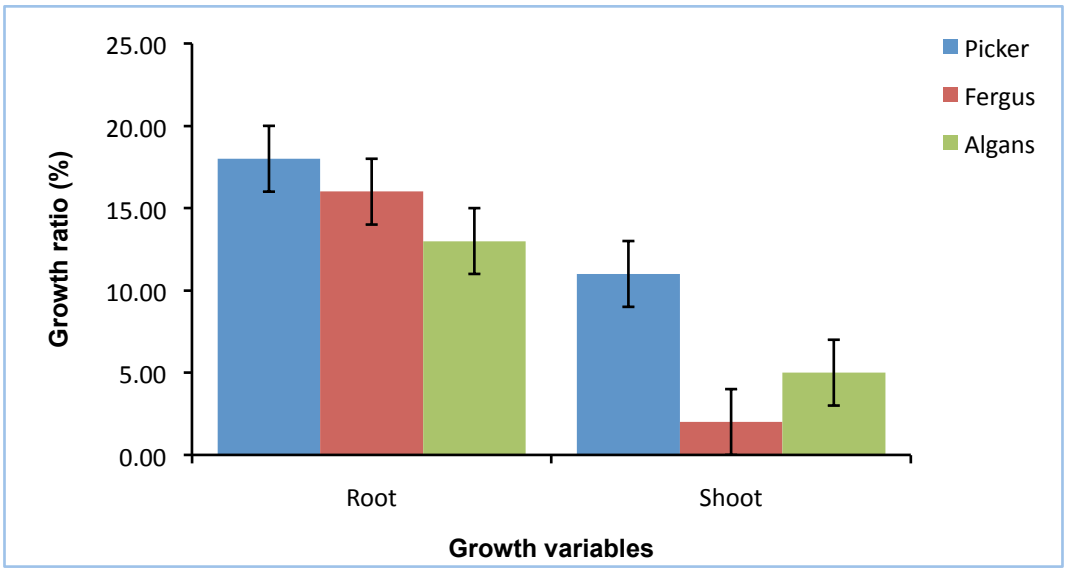


Figure 3-4. Root and shoot response to cold stress expressed as the growth ratio. The growth ratio for roots and shoots was obtained by relating the average length of a root and shoot of a treatment to the length of root and shoot of control, respectively. Seeds, which had germinated late or had not germinated at all, were included into the calculation of the average length. Results are mean ± SE (error bars; n=135). Difference between varieties was significant at $P<0.05$.

Picker, Fergus and Algans presented a similar response to cold stress in terms of root growth due to the high standard deviation (SD), which was representative of the high variability within the genotype. Fergus and Algans showed the lowest shoot tolerance, while Picker showed the higher shoot tolerance to cold stress.

3.1.3 Growth conditions for the full-set experiment

The experiment was subsequently extended to more varieties, when these became available for use. For practical reasons this experiment was carried out in the dark while retaining the 16/8-hour temperature regimes. This was mainly driven by practical considerations. Due to the large number of varieties and replicated it was not possible to accommodate the trays in the available growth chambers so that they would receive all light. Growing the seeds in the dark enabled the trays to be stacked and be arranged in the cabinets.

3.1.3.1 Germination test

The experiment in constant dark conditions was conducted in three replications, in each of which 12 varieties were used and tested with a representative group of 45 seeds each. Each group was split in 2 sub-groups of 21 and 24 seeds and sorted in two different trays. Trays were stacked on three different levels and randomly distributed in the cabinets according to a randomised block design as for the other experiments. Percentage of germination was calculated and scored as previously described.

Figures 3-5 and 3-6 display the percentage of germination of the varieties from day 1 to day 8 post-germination. In the control all the genotypes on day 4 post-germination showed a high percentage of

germination, which is very similar across the three replicates and there was little or no difference in the next days post germination. On day 4 post-germination, most of the varieties under control conditions presented a mean percentage of germination around 90% (Figures 3-7a). After 8 days post-germination, the varieties presented little variation in the mean germination (Figure 3-7b). Under cold treatment, on day 4 post-germination, the mean germination of the varieties Algans and Justina did not exceed 50% in any of the three replications of the experiment (Figure 3-8a). On day 8 post-germination for these varieties germination was still low with a mean percentage less than 50% (Figure 3-8b).

3.1.3.2 Growth test

After the occurrence of germination, the length of primary roots and shoots were measured at 24-hour intervals over a period of 8 days post-germination (Figure 3-9a, b, c, d). Measurements were taken by removing the seeds from the trays and placed back afterwards. All the growth measurements were started after seed germination, which occurred on different days for seeds under control or low temperature conditions. The non-germinated and late germinated seeds were taken into account for the calculation as previously described. Besides, the growth ratio was also calculated without including the non-germinated seeds in the calculation and considering as germination the seeds germinated after 4 days of varietal

post-germination. This allowed having a sufficient number of observed germinated seeds. The growth response to cold stress estimated on day 8 post-germination. The measurements scored over time were used to identify the earliest time point and the time span where the effect of cold treatment was significant (*Par. 3.1.4)*. These time points were then chosen for collection of root samples to be used for microarray analysis and qRT-PCR.

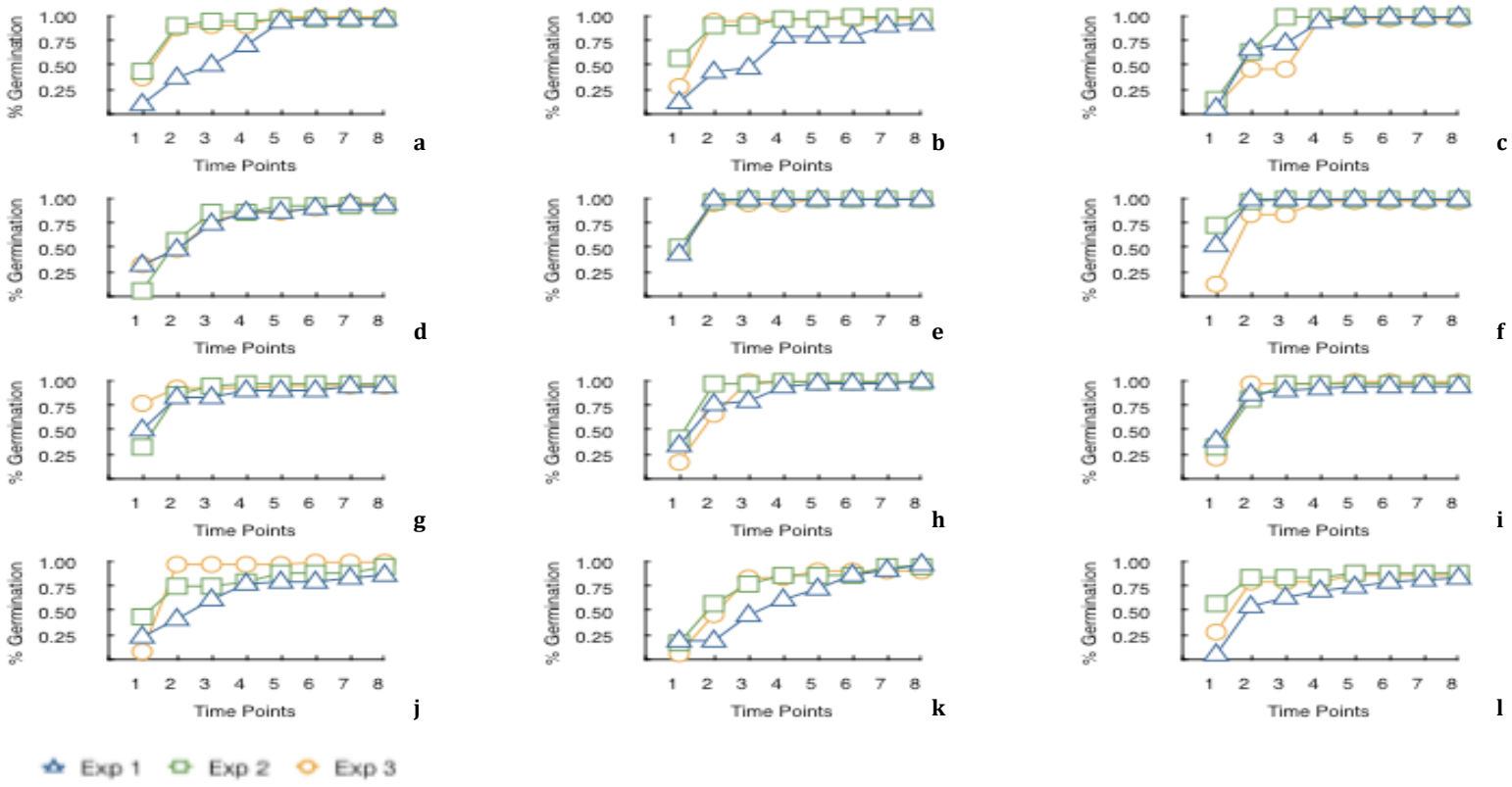


Figure 3-5 Percentage of germination across 8 days post-germination under control temperature regime. Percentage of germination was calculated for 8 days (time points). Exp1, Exp2, Exp3 represent the experiments (n=45 each). a) Crazi, b) Huski, c) Lakti, d) Algans, e) Picker, f) Fergus, g) Codisco, h) Codifar, j) Justina, k) PR39B29, i) PR39D60.

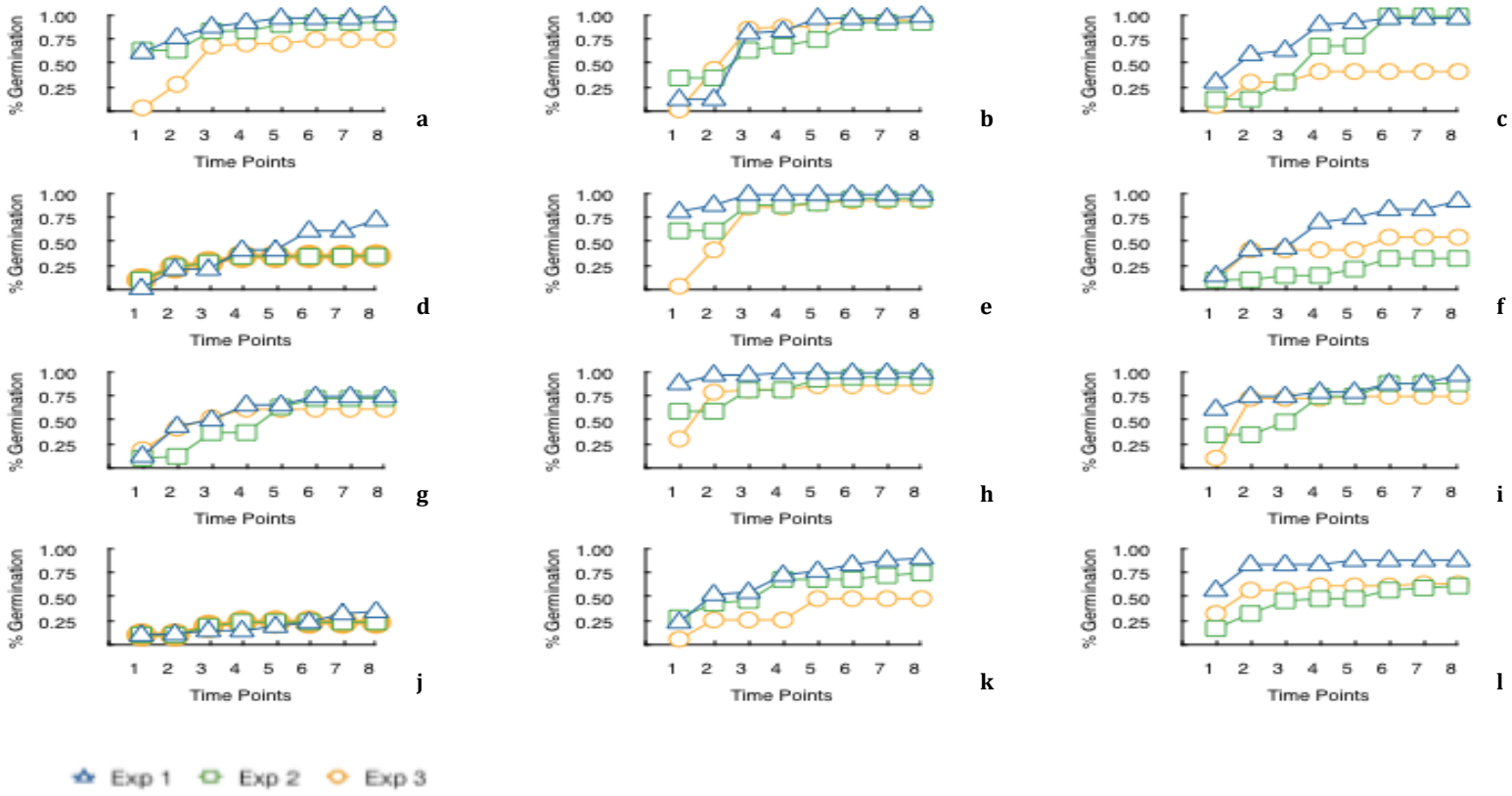


Figure 3-6 Percentage of germination across 8 days post-germination under cold stress. Percentage of germination was calculated for 8 days (time points). Exp1, Exp2, Exp3 represent the experiments (n=45 each). a) Crazi, b) Huski, c) Lakti, d) Algans, e) Picker, f) Fergus, g) Codisco, h) Codifar, j) Justina, k) PR39B29, i) PR39D60.

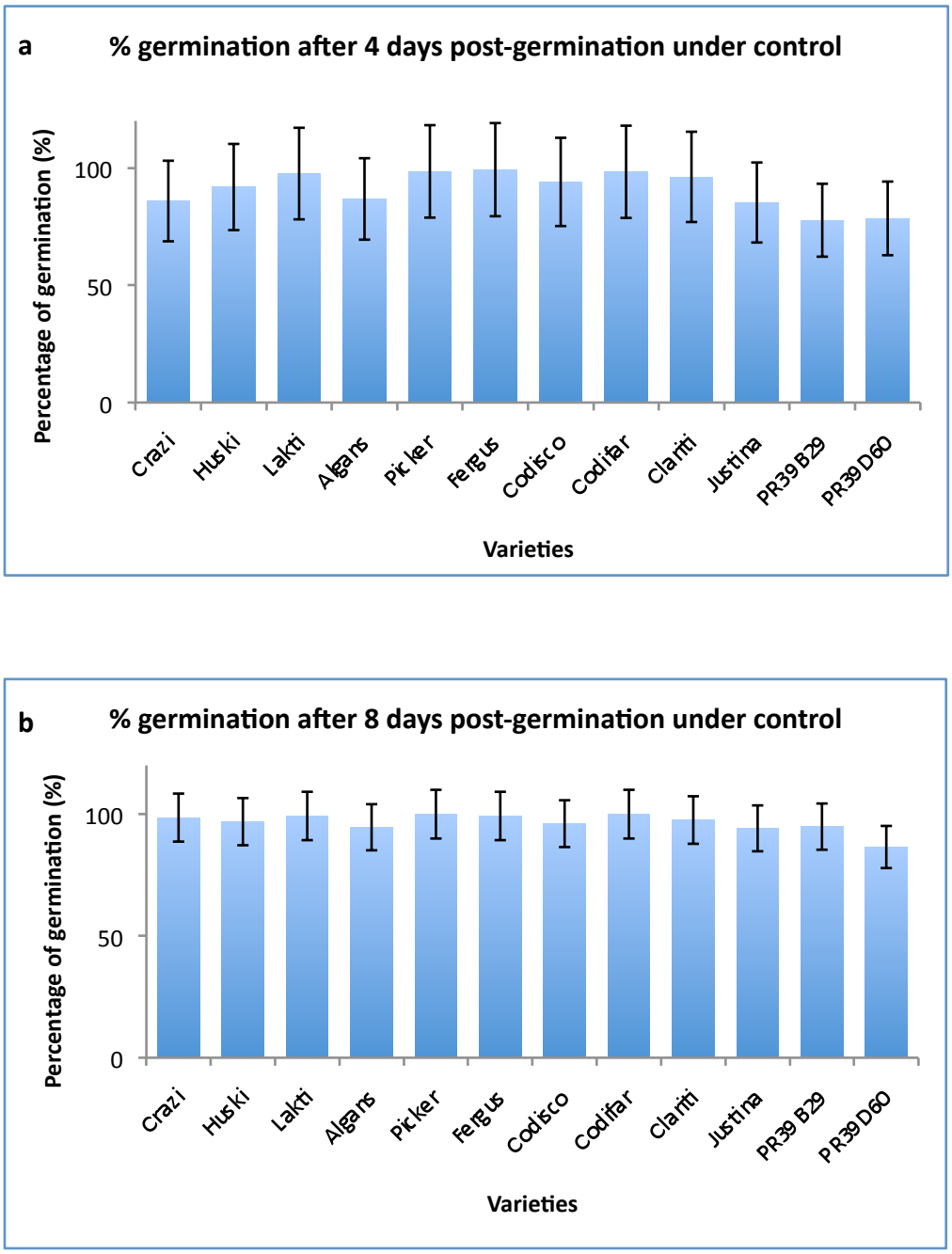


Figure 3-7 Percentage of germination on day 4 (a) and on day 8 (b) post-germination under control temperature regime. Results are mean ± SE (error bars; n=135). Percentage of germination was calculated by dividing the number of germinated seeds by the total number of seeds.

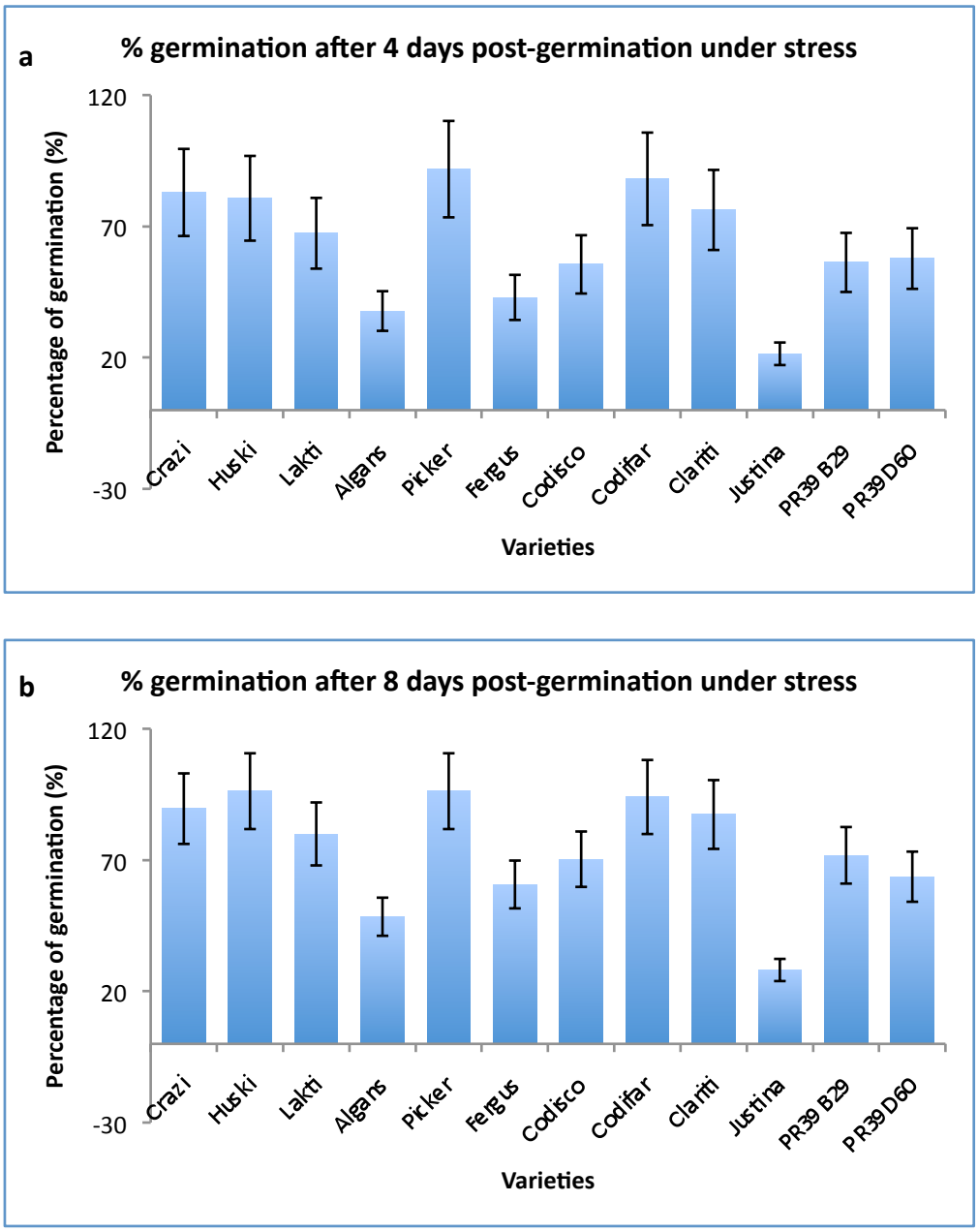


Figure 3-8 Percentage of germination on day 4 post-germination under cold stress. Results are mean ± SE (error bars; n=135). Percentage of germination was calculated by dividing the number of germinated seeds by the total number of seeds.

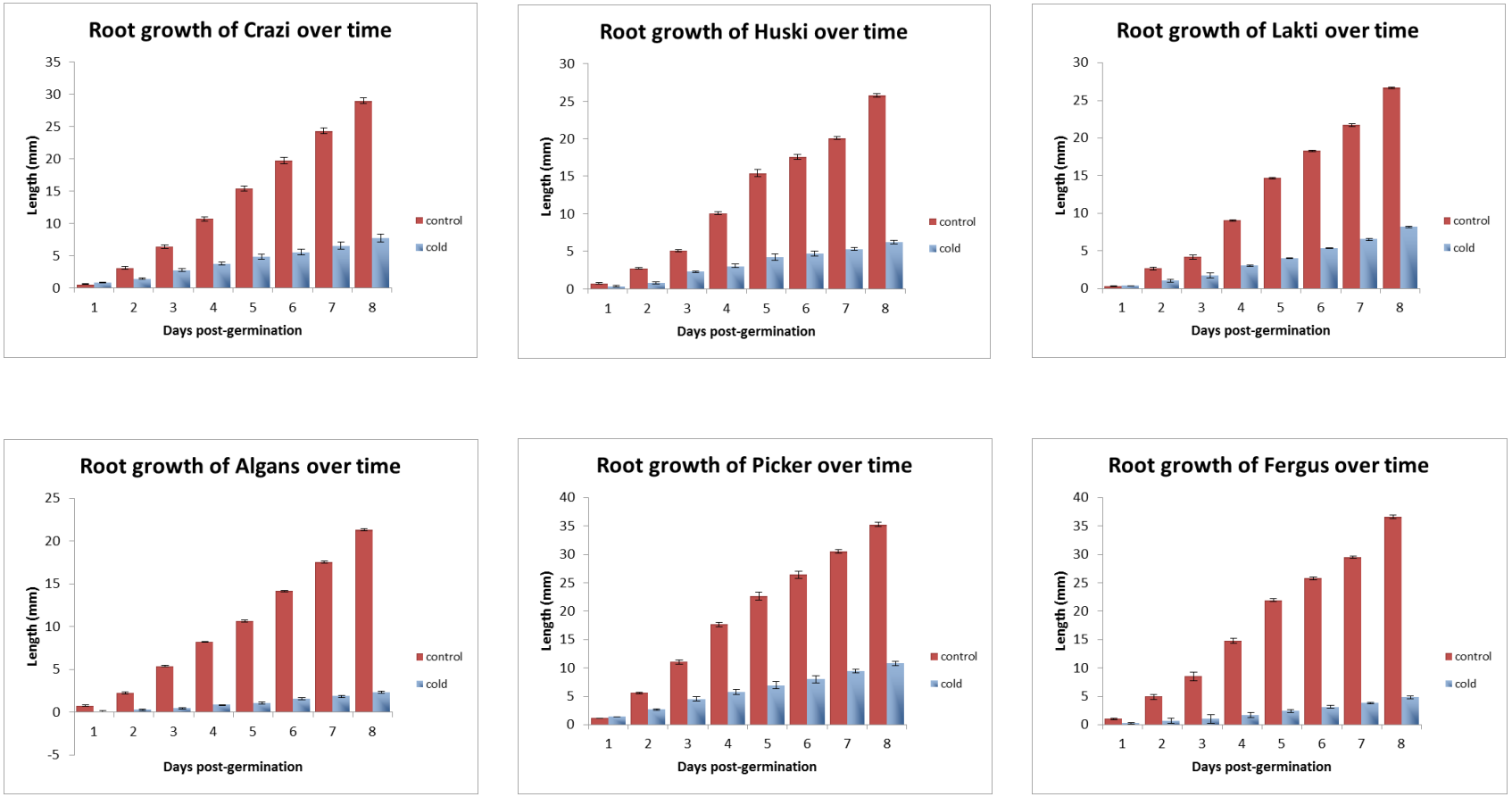


Figure 3-9a. Root growth over 8 days post-germination. Root growth was measured daily under control and cold stress conditions for eight days post-germination. Results are mean ± SE (error bars; n=135). Mean vas calculated including seeds which had germinated late or had not germinated at all. Continues on next page.

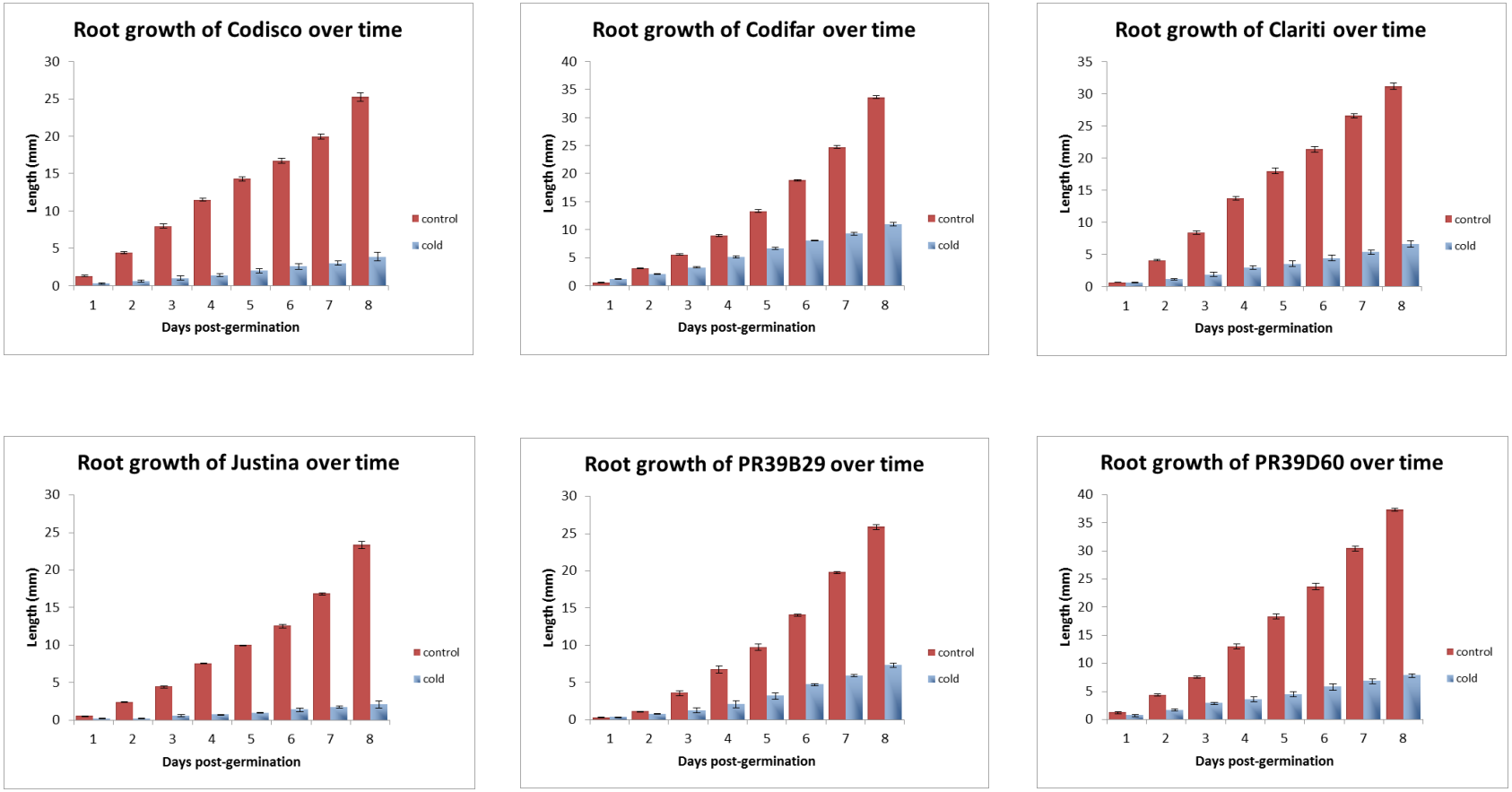


Figure 3-9b. Root growth over 8 days post-germination. Continued from previous page. Root growth was measured daily under control and cold stress conditions for eight days post-germination. Mean vas calculated including seeds which had germinated late or had not germinated at all. Results are mean ± SE (error bars; n=135). Continues on next page.

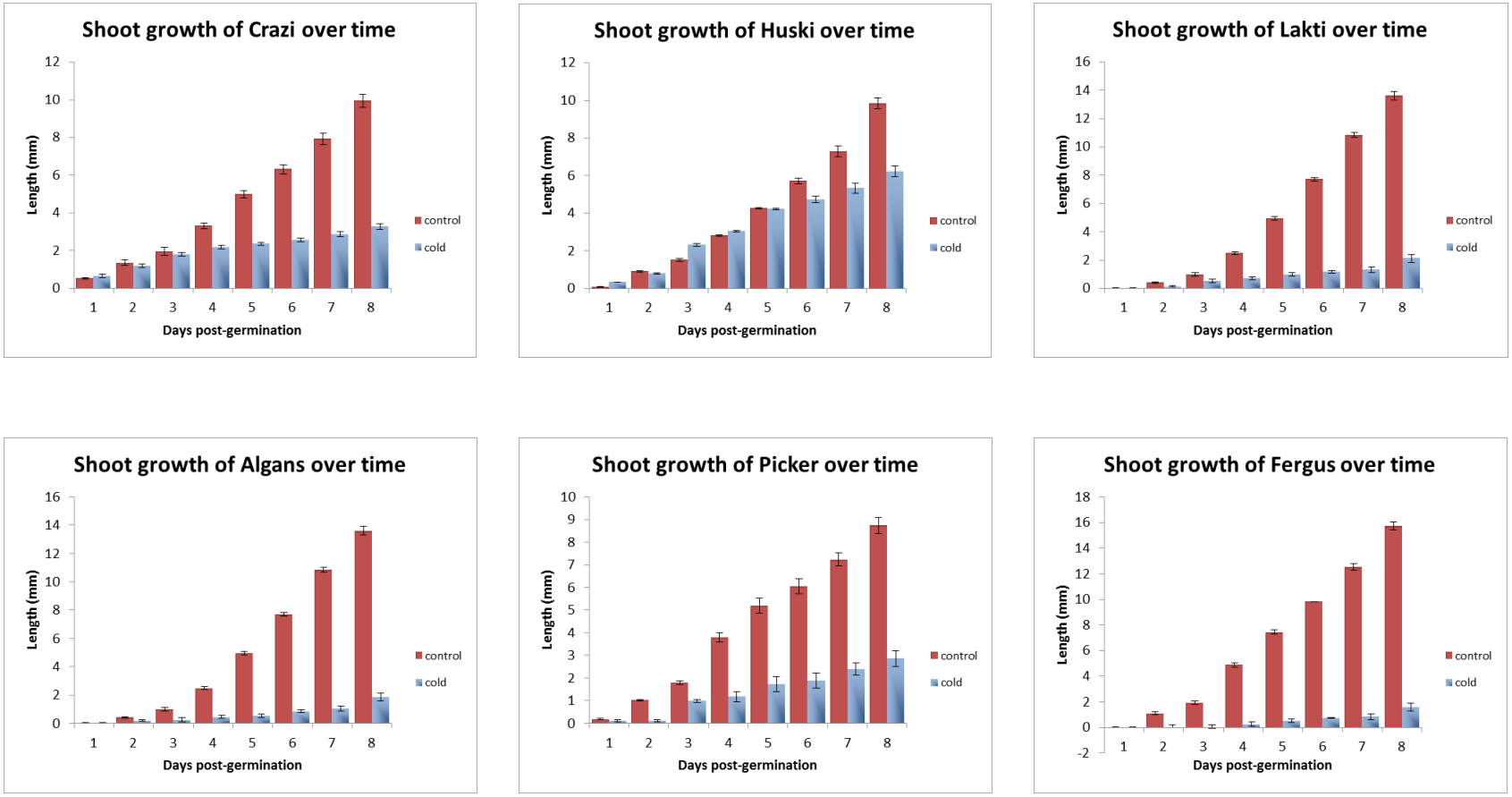


Figure 3-9c. Shoot growth over 8 days post-germination. Continued from previous page. Shoot growth was measured daily under control and cold stress conditions for eight days post-germination. Mean vas calculated including seeds which had germinated late or had not germinated at all. Results are mean ± SE (error bars; n=135). Continues on next page.

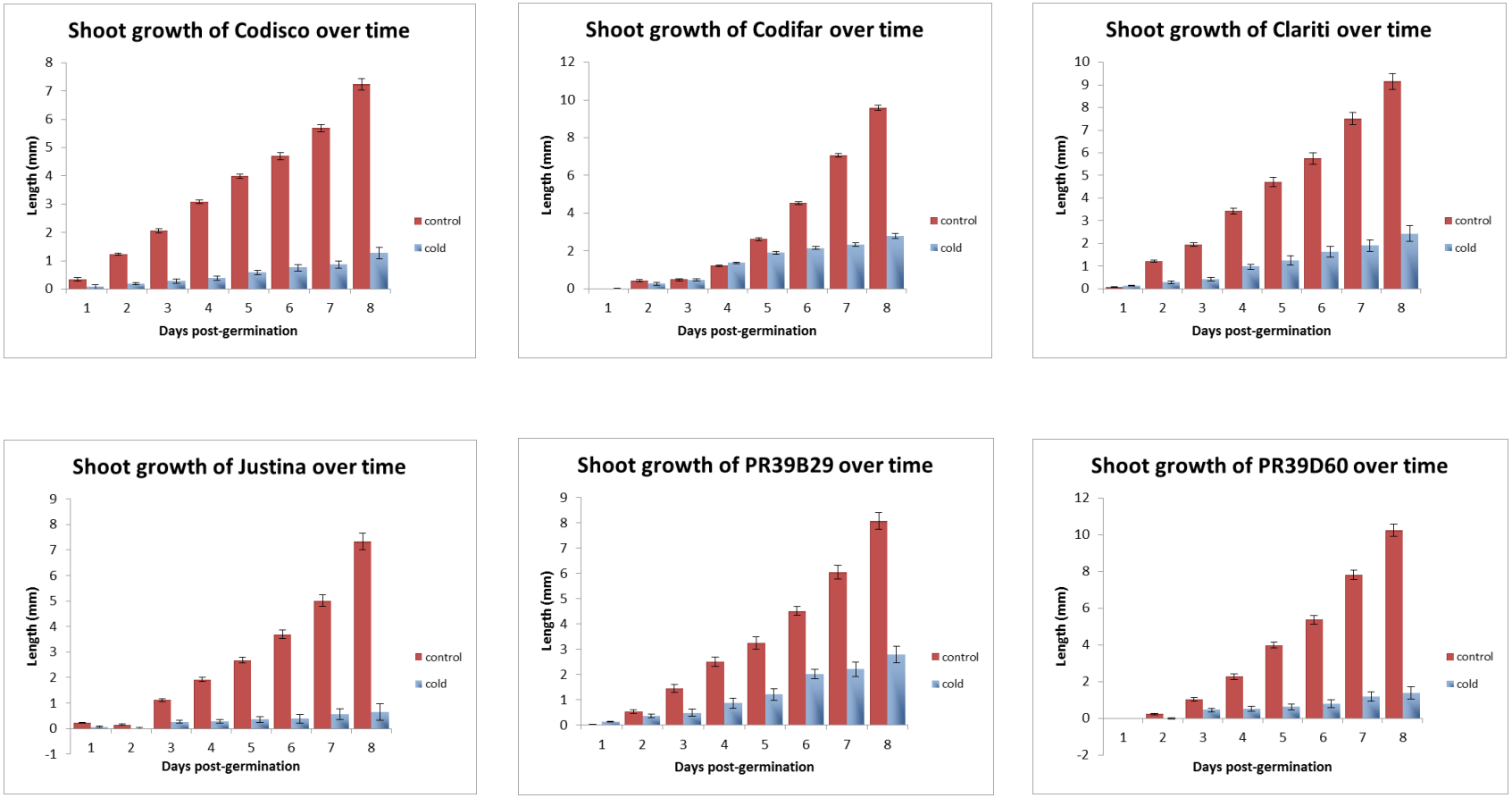


Figure 3-9d. Shoot growth over 8 days post-germination. Continued from previous page. Shoot growth was measured daily under control and cold stress conditions for eight days post-germination. Mean vas calculated including seeds which had germinated late or had not germinated at all. Results are mean ± SE (error bars; n=135).

3.1.4 Statistical analysis

The statistical significance effect of cold treatment was evaluated with a three-way ANOVA. The analysis of variance took into account three effects (genotype, treatment, time point) and a blocking factor (replication). Table 3-1 provides all the effects and all the interactions of interest.

Table 3-1. Tests of genotype, treatment and time point on growth. Three-way ANOVA was performed and all the possible interactions of interest were made. P value <0.05 was taken as significant. There was a significant Variety *x* Treatment *x* Time Point effect, which means that the effect of name (the difference between two varieties) varies depending on whether or not the treatment was cold or control, and that the variation of the name effect with treatment further depended on the time point at which the measurements were made.

Effect	Root P value	Shoot P value
Replicate	<.0001	<.0001
Variety	<.0001	<.0001
Treatment (control or cold)	<.0001	<.0001
Veriety *x* Treatment	<.0001	<.0001
Time Point	<.0001	<.0001
Variety *x* Time Point	<.0001	<.0001
Treatment *x* Time Point	<.0001	<.0001
Variety *x* Treatment *x* Time Point	<.0001	<.0001

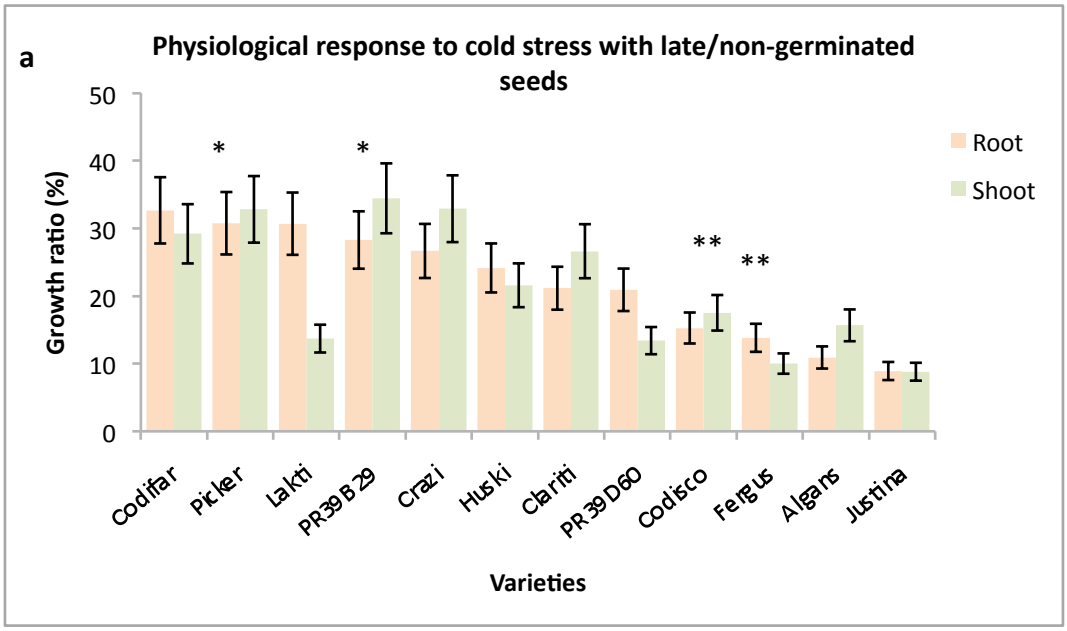
P<0.05 was taken as significant. The results were natural logged to correct the non-constant variance indicated in the residual of the analysis of the untransformed data (Appendix). Measurements over time were correlated as they were repeated at each time points on the same samples.

Both for root and shoot results, there was a significant Variety *x* Treatment *x* Time Point interaction. This means that the effect of Variety (the difference between two varieties) varies depending on whether or not the treatment was cold or control, and that the variation of the name effect with treatment further depended on the time point at which the measurements were made. So it is not possible to talk about a difference between two varieties independently of the treatment and time. In the table all the other interaction and main effects are not singularly interpretable, that is, it cannot be stated that the significant name effect is interpretable as a straightforward comparison of means for all the varieties, because the treatment and time must be taken into account. This is due to the fact that the genotypes are different from one another under the same control experimental conditions.

Moreover, It is not possible to interpret the blocking factor *Replicate* as significant (even though a P-value is given), because there was no randomisation possible. Nevertheless, the size of the F-value (not shown) was considerably greater than 1 (1 indicates no effect of blocks), so this can be interpreted as evidence that results vary from replication to replication and that account must be taken of this for a precise measurement of the other effects. The high variance among replications can be justified by the high

standard deviation of the samples examined. The results in the table of **Tests of Effect Slices** (Appendix) provide additional information; the most meaningful pattern here appears to be that there tends to be no treatment effect (difference between control and cold) for the first or possible first two time points for most levels of Variety.

Although the blocking factor had a significant effect on the experiment, this was taken into account when further analysis was carried out in order to make an assessment in terms of cold tolerance. The response to the cold treatment was estimated on day 8 post-germination as two growth ratios (in percentage), one including non-germinated/late germinated seeds and the other excluding non-germinated/late germinated seeds in the calculation (Figure 3-10). Although the response to cold stress was different in terms of absolute values, with some exception (Justina), the overall trend between the two growth ratios was similar. Algans and Justina showed a very low percentage of germination (Figure 3-8), therefore they were discarded for the gene expression analysis. Picker and PR39B29 were still in the top group of the resistant varieties, while Codisco and Fergus in the bottom group.



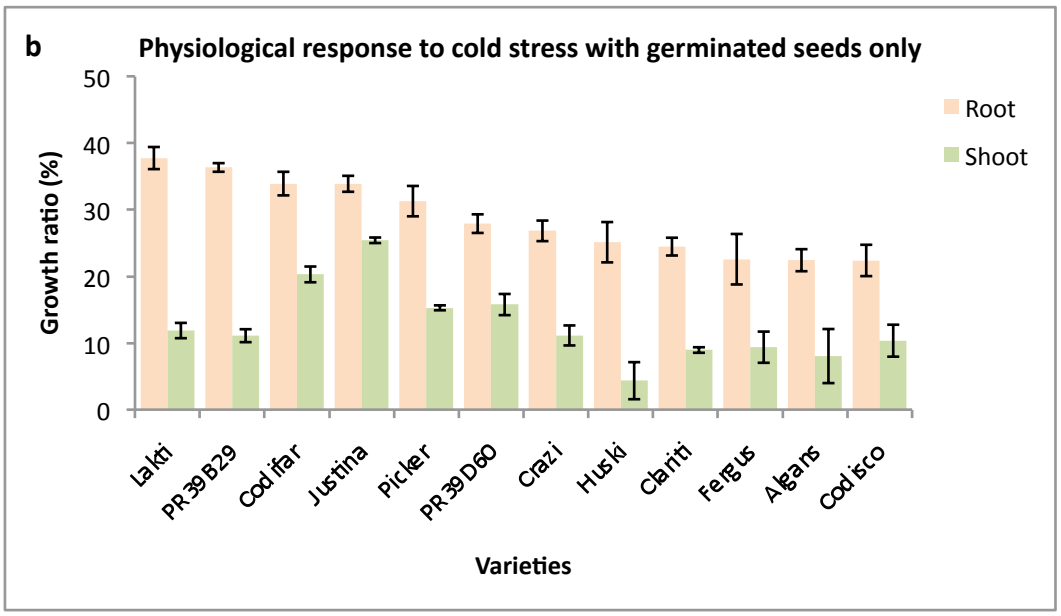


Figure 3-10 Growth ratio as response to cold stress. The growth ratio for roots and shoots was obtained by relating the average length of a root and shoot of a treatment to the length of root and shoot of control, respectively. (a) Seeds, which had germinated late or had not germinated at all, were included into the calculation of average length. (b) Only seeds that had germinated were included in the calculation of average length. The Tukey's range test was carried out to determine the two varieties with the highest combined root and shoot response to cold stress, as indicated by the * ($p<0.05$). ** Varieties with the lowest combined root and shoot tolerance to cold stress. Results are mean ± SE (n=135).

The varieties that showed the highest tolerance to cold treatment in terms of root growth did not present the same performance as shoot ratio. In order to classify the varieties, according to their combined root and shoot developmental performance to cold stress, the Tukey's range test (Armitage P, Berry G, 2002), a multiple comparison method, was carried out.

The growth ratios calculated in light/dark conditions and constant dark conditions were also compared (Figure 3-11).

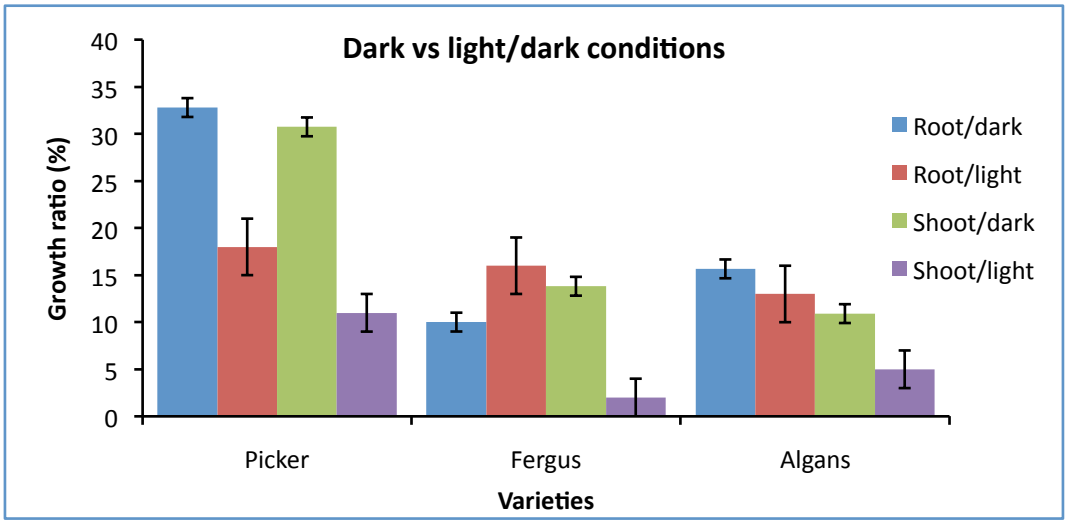


Figure 3-11 Cold response in maize seedlings grown under constant-dark and light/dark conditions. The growth ratio for roots and shoot was obtained by relating the average length of a root and shoot of a treatment to the length of root and shoot of control, respectively. Seeds, which had germinated late or had not germinated at all, were included into the calculation of average length. Results are mean ± SE (n=135). Difference between growth ratios was significant at $P<0.05$.

The graph shows that the response to low temperatures changed when the varieties were grown under the light/dark cycle or in constant dark. The response of the three varieties was significantly different when they were grown in light conditions compared to dark conditions, with the exception of Algans, whose root response presented no significant difference. Moreover, the SD of the growth ratio in light/dark conditions was high, showing high variability across the replicates.

For the gene expression profiling purposes of this work and to provide a relevant picture of the gene pattern involved in response to cold stress, the two varieties that showed the highest level of tolerance and the two varieties that showed the lowest were selected for the microarray analysis (Figure 3-12).

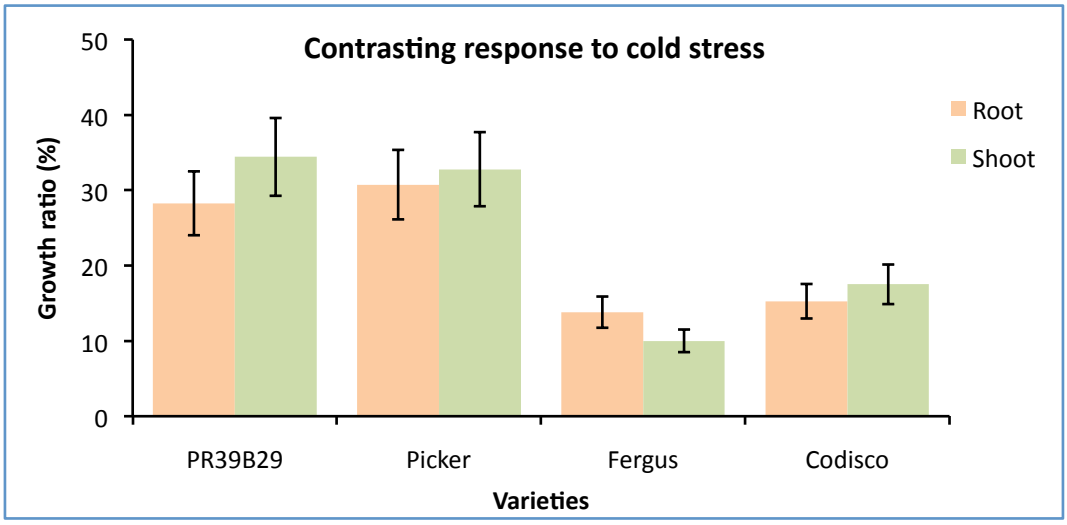


Figure 3-12 Maize varieties showing the highest and lowest level of cold tolerance. The growth ratio for roots and shoots was obtained by relating the average length of a root and shoot of a treatment to the length of root and shoot of control, respectively. Seeds, which had germinated late or had not germinated at all, were included into the calculation of average length. Results are mean ± SE (error bars; n=135). Difference between growth ratios was significant at $P<0.05$.

The varieties PR39B29 and Picker showed the least reduction in growth in response to the cold treatment, while Fergus and Codisco resulted significantly affected by the low temperature.

3.2 Microarray analysis

Macroarray analysis was initially conducted, on cDNA of known maize cold resistance genes, in order to test if it was possible to detect changes, between the two experimental conditions, in the gene expression pattern of

the varieties included in the Irish Recommended List, before the samples of interest were employed for microarray analysis. The macroarray analysis was performed by preparing a custom array with a set of testing probes and the hybridisation was successful, but a true comparison between control and cold stress conditions, was not carried out.

3.2.1 Root sample collection

The four varieties (Picker, PR39B29, Fergus, Codisco) selected from the physiological experiment on the basis of the percentage of growth reduction, were used for the microarray analysis. Roots were collected from seedling grown under the following experimental conditions: 18°C/16 hours for the control temperature regime and 12°C/8 hours for the low temperature regime, in constant dark conditions. Root samples were harvested at day 4, 5, 6, 7, and 8 post-germination. At each time point, three root samples per variety were collected as biological replicates. The three biological replicates from day 4 post-germination were used for microarray analysis. A 3cm section from the root tip of primary roots was excised, placed in 1.5ml centrifuge tubes, flash frozen in liquid nitrogen and stored at -80^{0}C until required.

3.2.2 RNA extraction and quality check

Roots samples collected on day 4 post-germination were used for microarray analysis. The results are displayed as images of pseudo agarose gels (virtual gels), where band sizes are defined (Figure 3-13), quantitation is performed and the RNA integrity number (RIN) is given (Table 3-2). The integrity number is a measure of RNA quality and is a value based on a numbering system from 1 to 10, assigned to the RNA, with 1 being the most degraded and 10 the most intact (Schroeder, 2006). The number is generated by an algorithm, which is based on different features extracted from an electrophoretic trace, as signal areas, intensities and 28S/18S ratios. For a successful hybridisation RINs of 7 or above are required to be used for microarray analysis. The relation of the RNA quality and the RIN is visible in the images of the pseudo gels, where, for example, the lane E (Figure 3-13, b), whose RIN is 8.8, presents a slight smear. Similarly, samples with a 10 RIN present no smear on the gel.

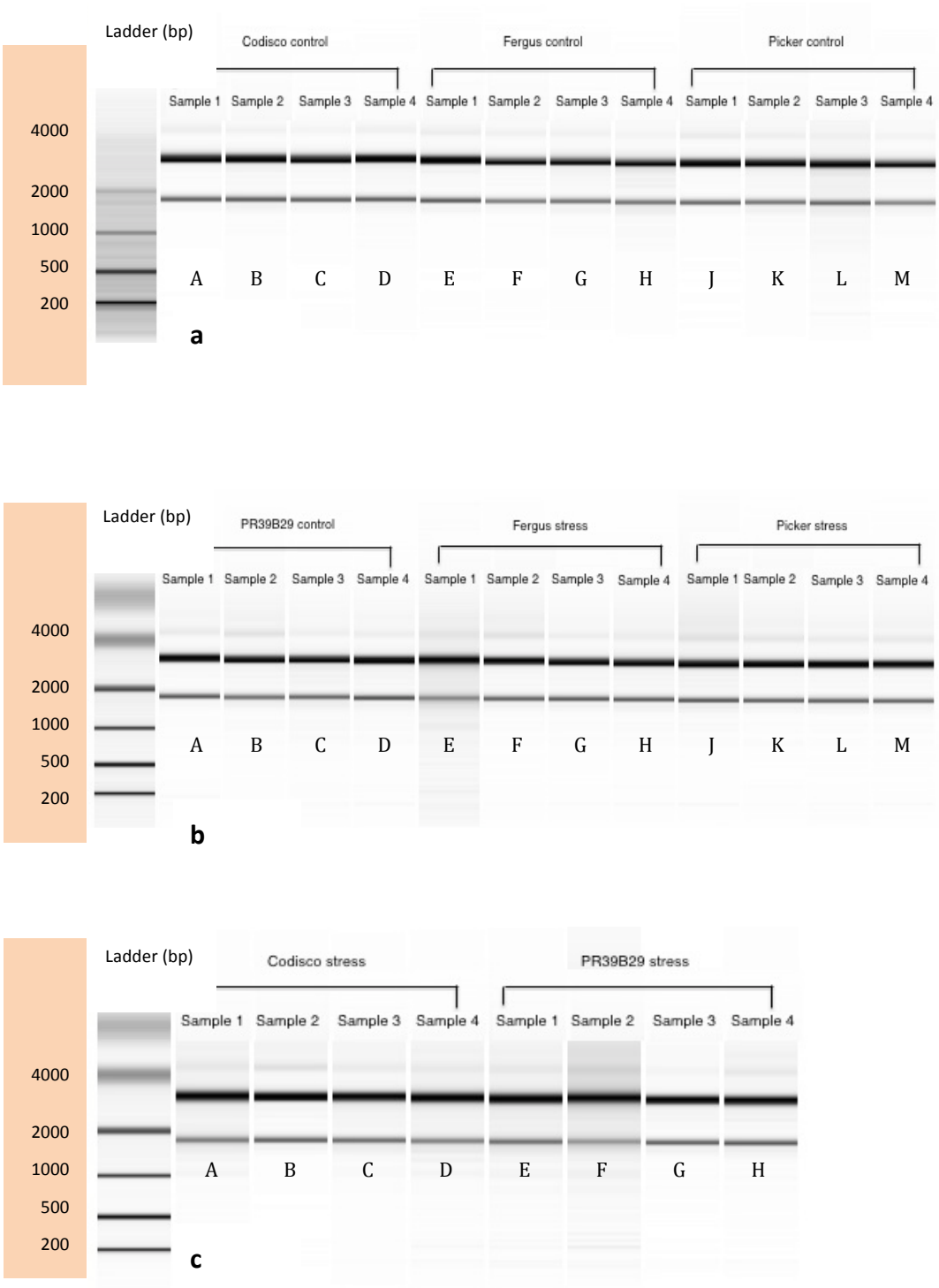


Figure 3-13. Pseudo gels displaying the quality of RNA samples. Two clear, sharp separate 28S and 18S rRNA bands are shown, the former twice as intense as the latter, according to a 2:1 ratio as the requested indicator for RNA integrity. Letters are lanes. a) Codisco control, Fergus control, Picker control; b) PR39B29 control, Fergus stress, Picker stress; c) Codisco stress, PR39B29 stress.

Table 3-2. RNA quantitation and RIN are displayed for each sample. RIN represent the RNA integrity number, which shows the quality of the RNA.

Variety	Treatment	Rep	RNA yield (ng)	RIN
Fergus	Control	1	3,891.0	10.0
Fergus	Cold stress	1	3,374.0	8.8
Picker	Control	1	3,808.0	10.0
Picker	Cold stress	1	4,046.0	10.0
PR39B29	Control	1	3,127.0	10.0
PR39B29	Cold stress	1	5,194.0	9.5
Codisco	Control	1	4,442.0	10.0
Codisco	Cold stress	1	4,115.0	9.6
Fergus	Control	2	4,809.0	9.7
Fergus	Cold stress	2	4,332.0	10.0
Picker	Control	2	3,868.0	9.9
Picker	Cold stress	2	4,967.0	10.0
PR39B29	Control	2	3,715.0	10.0
PR39B29	Cold stress	2	4,016.0	9.0
Codisco	Control	2	3,312.0	10.0
Codisco	Cold stress	2	4,761.0	10.0
Fergus	Control	3	4,089.0	9.7
Fergus	Cold stress	3	4,406.0	10.0
Picker	Control	3	4,222.0	9.5
Picker	Cold stress	3	3,570.0	10.0
PR39B29	Control	3	3,984.0	10.0
PR39B29	Cold stress	3	5,389.0	10.0
Codisco	Control	3	4,477.0	10.0
Codisco	Cold stress	3	3,499.0	9.4

3.2.3 Microarray experimental design

The RNA samples from the four varieties and the two treatments were compared to one another according to an interwoven loop design (Figure 3-14).

In the diagram, maize varieties under a specific temperature regime (control and cold stress) are compared. Two names of variety represent a single array and the orientation of the arrows describes how the samples were compared and labelled with the two Cy-dyes; the tail of the arrows refers to the samples labelled with the Cy3 dye and the tip the samples labelled with Cy5 dye. All the samples represented in the loop design are dye balanced and present in equal number. The experimental design involves one biological replicate at a time and was extended to three biological replicates in total.

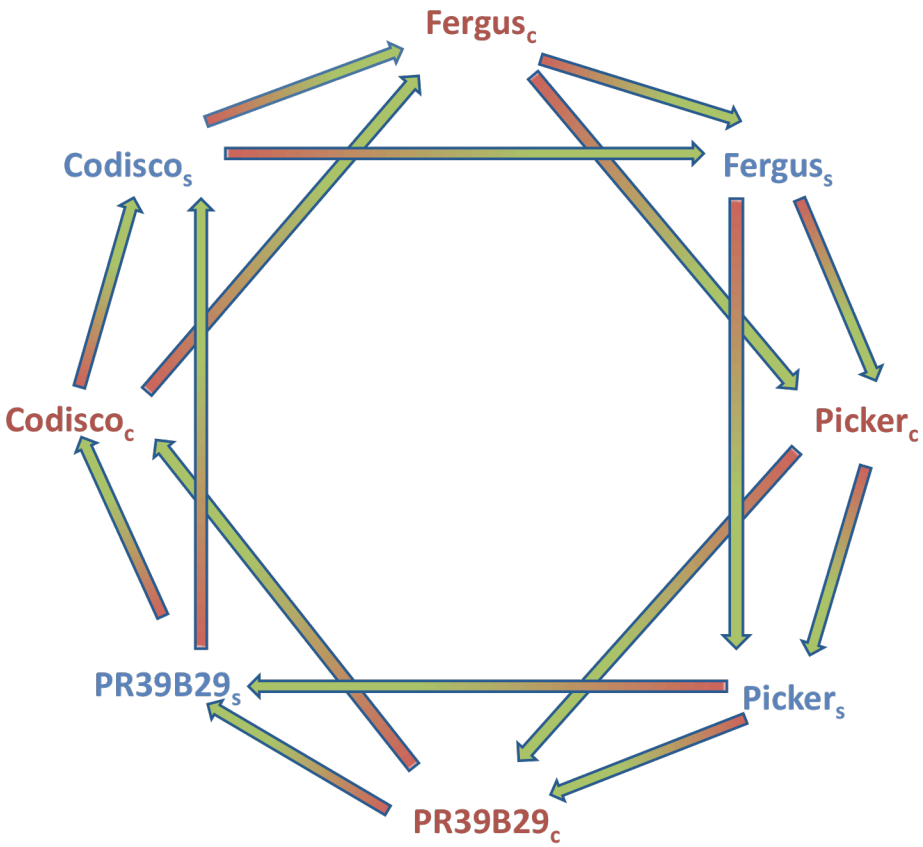


Figure 3-14. Interwoven loop design for microarray analysis. The varieties under control (subscript 'c') and cold stress (subscript 's') are shown in the diagram. Two names of variety represent a single array. The arrows represent the comparisons or connections; the tails and the tips the direction of the comparisons and the Cy-labelling: tail with Cy3 and tip with Cy5.

Once the samples were sent over to the Institute of Genomic Research on Arizona, labelling and hybridisation were carried out. The signals emitted from the hybridisation were read by the GenePix® image scanner, which generated a spreadsheet containing the level of fluorescence detected for each spot on the array. The image scanning software also tested the quality of

hybridisation and so, the reliability of the data; the software, so as to be possibly discarded from analysis, flagged spots with poor hybridisation. In addition spots on the arrays were manually inspected to identify aberrant spots not picked up by the software.

3.2.4 Microarray quality check

The quality of the chips was good and none of them was discarded from the analysis (Figure 3-15).

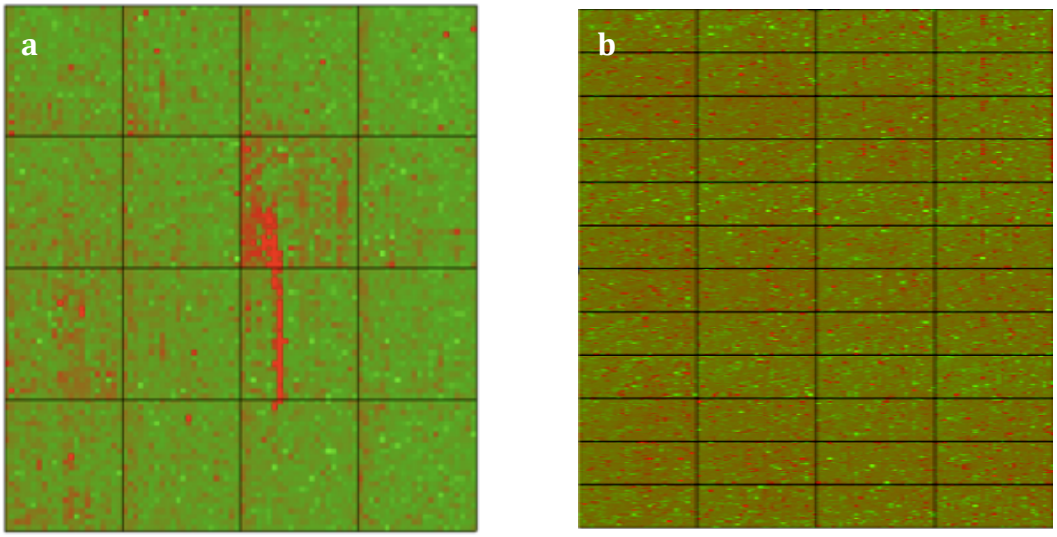


Figure 3-15 Hybridisation quality check. Green and red dots represent the Cy3 and the Cy5 fluorescence of each spot. a) The false colour image shows artefacts on the array (image taken from the Linear Models for Microarray Data User's Guide). b) The false colour image was generated by Robin and shows no artefacts on the array.

Nevertheless, bad spots were flagged in the GenePix® spreadsheet and therefore not included in the subsequent analysis. Their lack was compensated, where possible, by the use of biological replicates.

MA-plots were drawn for the microarrays, before and after the background correction, in order to assess the distribution of the data. Figure 3-16 shows an example of MA-plot before and after normalisation.

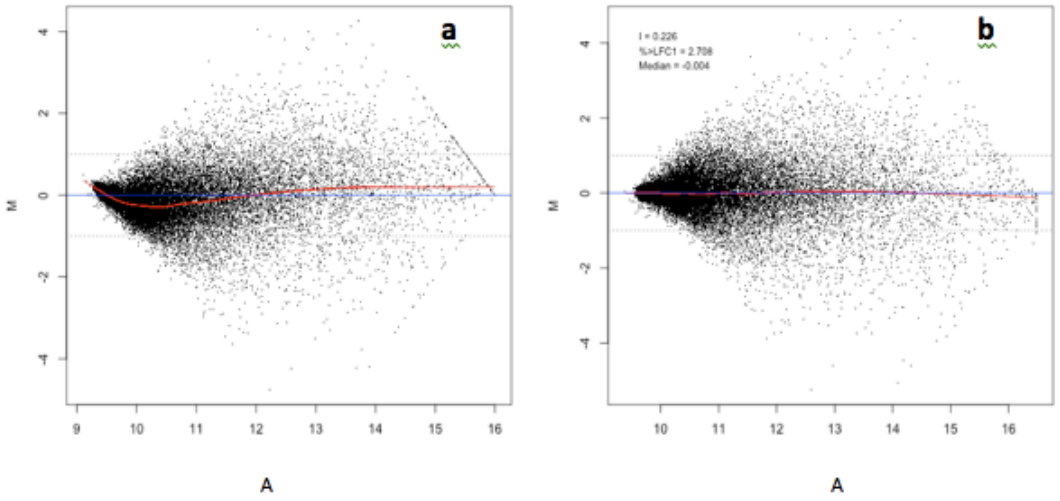


Figure 3-16 MA-plots before and after normalisation. a) Values are un-normalised data; b) values are normalised data. The normalised data show the percentage of genes (%LFC) with a log-fold change higher than 1 (meaning a two-fold change in expression). Robin issues a warning if more than 5% of the genes show a 2-fold or larger change in expression. The red line shows the deviation of the expression values from a normal distribution. If I > 1 an artefact is found and a warning is generated.

In an ideal experiment the assumption is that most of the genes are not differentially expressed (McCormick et al., 2011) hence most of the data points should lie on the M=0 line of the MA-plots, where, as previously described, M indicates the measure of the treatment effect. The values shown for the normalized data indicate the percentage of genes (%LFC) showing a log fold change higher than 1 (meaning a two-fold change in expression). The MapMan Robin software issues a warning if more than 5% of the genes show a 2-fold change in expression. In the specific case of the present work no warning was issued, indicating that corrections made fit the data and did not reject the initial assumption (null hypothesis).

The biological replicates were handled by merging the corresponding slides into a single one; the normalised intensities of the means of the replicated spots were averaged across replicated arrays. The same method was also used to average the expression values of the replicated spots within each array excluding the bad flagged spots.

3.2.5 Microarray data analysis

The analysis of the samples was carried out on the comparisons between each genotype from the control and the same genotype from the cold treatment.

When a large number of genes are compared simultaneously within one microarray experiment it is necessary to apply adjustment methods to reduce the loss of power of the statistical test, which may generate a high

false discovery rate, where some genes may be defined as differently expressed when they are actually not or vice versa. For this purpose a non-specific pre-filtering of the data was performed in order to remove from consideration all genes that are not significantly expressed under any comparison. This means that genes were filtered on the basis of their variance across the samples, regardless the type of variety or treatment. The pre-filtering ruled out the 60% of the genes and the analysis was hence performed on the 40% of the genes with the biggest variance across the samples. Differently expressed genes were determined according to their average M-values; the average M-value describes the level and type of gene regulation (up or down) for each gene and it is calculated by subtracting the average M-value of the gene in the control from the average M-values of the gene under treatment. Adjusted p-values were then generated according to the Benjamini and Hochberg method to correct for multiple testing in an experiment (Benjamini & Hochberg, 1995). The top one hundred genes with the smallest p-values were saved in a file along with the average M-value (regulation) and A-values (expression). MA and volcano plots were drawn to illustrate the average regulation of the genes if genes are differentially expressed between the treatments and sample groups. The volcano plot scatters the p-values (more precisely the $-\log_{10}$ of the p-values) on the y-axis against the average regulation value (biological significance) on the x-axis.

The results of the microarray analysis showed that the cold tolerant varieties Picker and PR39B29 have, each, a set of differentially expressed genes (up and down regulated, p-value <0.05), while no genes were listed for

the cold sensitive varieties Fergus and Codisco, indicating that no significant change in expression was found in any of the genes analysed (Tables 3-3 and 3-4).

As shown in Tables 3-3 and 3-4, the two groups of genes identified by the microarray analysis showed differences in the number and identity of genes with the exception of four genes. Nevertheless, these four genes displayed a different or even opposite level of regulation in the different varieties. The Gene Ontology (GO) functions of the genes were available in the probe dataset. However, information about MZ00003507 was not available and the nearest match obtained for this sequence was an RNA binding protein in *Arabidopsis thaliana*.

Table 3-3 List of the most significant regulated genes in the cold tolerant maize variety PR39B29. ID and Name are annotation of the NSF Maize Oligonucleotide Array Project. BH stands for Benjamini and Hochberg and represents the adjusted p-value used as confidence interval. P-values <0.05 indicates a significant change in expression. MeanM and MeanA describe the average regulation and the average expression of each gene, resulting from the mean of the values of the biological replicates.

The IDs marked with * are the genes present in both the cold tolerant varieties.

ID	Name	BH	meanM	meanA	Gene Product
MZ00003507*	BE130044	0.01	-2.79	10.81	RNA binding protein {Arabidopsis thaliana;}
MZ00004486*	TC253575	0.05	-2.42	9.35	pathogenesis related protein-1 - maize {Zea mays;}
MZ00022876*	TC254729	0.02	-2.71	9.71	hypothetical protein {Oryza sativa (japonica cultivar-group);}
MZ00041708*	TC193433	0.02	-2.68	8.61	unknown protein {Oryza sativa (japonica cultivar-group);}
MZ00023411	TC258611	0.05	2.46	11.87	22 kDa drought-inducible protein {Saccharum hybrid cultivar;}
MZ00026737	TC272703	0.03	2.55	11.62	peroxidase {Zea mays;}
MZ00029223	TC253998	0.05	-2.47	11.38	putative heat shock protein hsp22 precursor {Oryza sativa (japonica cultivar-group);}

Table 3-4 List of the most significant regulated genes in the cold tolerant maize variety Picker. ID and Name are annotation of the NSF Maize Oligonucleotide Array Project. BH stands for Benjamini and Hochberg and represents the adjusted p-value used as confidence interval. P-values <0.05 indicates a significant change in expression. MeanM and MeanA describe the average regulation and the average expression of each gene, resulting from the mean of the values of the biological replicates.

The IDs marked with * are the genes present in both the cold tolerant varieties.

ID	Name	BH	meanM	meanA	Gene Product
MZ00003507*	BE130044	0.01	2.43	10.59	RNA binding protein {Arabidopsis thaliana;}
MZ00004486*	TC253575	9.70E-07	-3.66	9.86	pathogenesis related protein-1 - maize {Zea mays;}
MZ00022876*	TC254729	0.00	2.61	8.66	hypothetical protein {Oryza sativa (japonica cultivar-group);}
MZ00041708*	TC193433	0.04	2.18	10.16	unknown protein {Oryza sativa (japonica cultivar-group);}
MZ00026029	TC251197	0.04	-2.17	9.82	probable lipid transfer protein - rice {Oryza sativa;}
MZ00037140	BM381350	6.23E-06	-3.45	11.24	glucose starvation-induced protein precursor (clone pZSS2) - maize {Zea mays;}

3.2.6 Validation of microarray data

Microarray data should be validated by another gene expression profiling technique in order to ensure the reliability of the results. Quantitative Real Time PCR is the technique that is commonly used to confirm microarray data (Dallas et al., 2005). Validation is recommended because of the significant variation of the quality of the expression data obtained from microarray analysis according to the type of platform and procedures used (Morey et al., 2006). It was also demonstrated that several factors, such as p-value, fold-change and the type of regulation (positive or negative) might influence the correlation between oligoarray microarrays and Real Time PCR (Morey et al., 2006). Quantitative Real Time PCR was also used to detect and describe the expression trend of each of the shared genes across the five time points at which root samples were collected and highlight possible variations in the biological replicates.

3.3 Quantitative Real Time PCR (qRT-PCR)

A set of 11 candidate housekeeping genes previously used in cold stress studies on maize were tested to validate their stability of expression in the different experimental conditions (Table 3-5).

Table 3-5 Housekeeping genes info.

Name	Accession No.	References
Actin	AY104722	Nguyen H T, Leipner J, Stamp P, Guerra-Peraza O (2009) Low temperature stress in maize (*Zea Mays* L.) induces genes involved in photosynthesis and signal transduction as stuided by suppression subtractive hybridization. Plant Physiology and Biochemistry **47**, 116-122
GAPDH	X75326	Luo M, Liu J, Lee R D, Guo B Z (2008) Characterization of gene expression profiles in developing kernels of maize (*Zea mays*) inbred Tex6. Plant Breeding **127**, 569-578
ZmUBI	S94496	Simonovic A D, Anderson M D (2007) Analysis of methionine oxides and nitrogen-transporting amino acids in chilled and acclimated maize seedlings. Amino Acids **33**, 607-613
Adh1	X04050	Hernandez M, Duplan M N, Berthier G, Vaitilingom M, Hauser W, Freyer R, Pla M, Bertheau Y (2004) Development and comparison of four Real-Time Polymerase Chain Reaction Systems for specific detection and quantification of *Zea Mays* L. J. Agric. Food Chem. **52**, 4632-4637
Hmgp	AJ131373	Hernandez M, Duplan M N, Berthier G, Vaitilingom M, Hauser W, Freyer R, Pla M, Bertheau Y (2004) Development and comparison of four Real-Time Polymerase Chain Reaction Systems for specific detection and quantification of *Zea Mays* L. J. Agric. Food Chem. **52**, 4632-4637
Invr1	U16123	Hernandez M, Duplan M N, Berthier G, Vaitilingom M, Hauser W, Freyer R, Pla M, Bertheau Y (2004) Development and comparison of four Real-Time Polymerase Chain Reaction Systems for specific detection and quantification of *Zea Mays* L. J. Agric. Food Chem. **52**, 4632-4637
Zein	X07535	Hernandez M, Duplan M N, Berthier G, Vaitilingom M, Hauser W, Freyer R, Pla M, Bertheau Y (2004) Development and comparison of four Real-Time Polymerase Chain Reaction Systems for specific detection and quantification of *Zea Mays* L. J. Agric. Food Chem. **52**, 4632-4637
Cyclophilin	X68678	Brunner A M, Yakovlev I A, Strauss S H (2004) Validating internal controls for quantitative plant gene expression studies. BMC Plant Biology **4**:14
Ef1-α	CV071927	Brunner A M, Yakovlev I A, Strauss S H (2004) Validating internal controls for quantitative plant gene expression studies. BMC Plant Biology **4**:14
γ-tubulin	X78891	Ding D, Zhang L, Wang H, Liu Z, Zhang Z, Zheng Y (2009) Differential expression of miRNAs in response to salt stress in maize roots. Annals of Botany **103**(1):29-38
Thioredoxin	AF435816	Dembinsky D, Woll K, Saleem M, Liu Y, Fu Y, Borsuk L A, Lamkemeyer T, Fladerer C, Madlung J, Barbazuk B, Nordheim A, Nettleton D, Schnable P S, Hochholdinger F (2007) Transcriptomic and proteomic analysis of pericycle cells of maize primary root. Plant Physiology **145**:575-588
Tua5	EB822881	Santi S, Locci G, Monte R, Pinton R, Varanini Z (2003) Induction of nitrate uptake in maize roots: expression of a putative high-affinity nitrate transporter and plasma membrane H^+-ATPase isoforms. Journal of Experimental Botany **54**:389

These published works also provided the sequences for the specific primers. Once the primers were obtained they were tested for their suitability as reference genes. Total RNA was extracted from the root samples and then converted into cDNA for the Real Time PCR. Total RNA was extracted from roots samples of Algans, Picker and Fergus, at day 4, 5, 6, 7, 8 post-germination under control and cold treatment conditions. Two sequential screening processes were employed to select the most suitable reference genes.

All the different cDNAs were pooled together to make a unique template to be initially used with each pair of primers in conventional PCRs. Pooling the cDNAs together ensures that the gene on interest (in this case a reference gene) is expressed at least in one sample (and so it is in the reaction template) and provides validation that the corresponding primers work properly. The PCR products were checked on agarose gel. Once verified that the products were amplified and that the primers gave the predicted band sizes on the gel, these were then tested in a Real Time PCR. The best performing primers were those that amplified the target gene at earlier cycles in a range between 20 and 24 amplification cycles (Table 3-6).

Table 3-6 Primers of housekeeping candidate genes and their Ct values. The best performing primers were those that amplified the target gene at earlier cycles in a range between 20 and 24 amplification cycles. The housekeeping genes in blue are the ones with the lowest Ct value and so with the highest expression levels.

Housekeeping genes	Ct values
Actin	24
GAPDH	27
ZmUBI	33
Adh1	24
Hmgp	34
Invr1	34
Zein	28
Cyclophilin	31
Ef1-α	22
γ-tubulin	29
Thioredoxin	28
Tua5	24

Actin, Adh, Tua5 and Ef1-α had the lowest Ct values (highest expression levels) and their stability of expression was calculated with the Vandesompele statistics by an Excel spreadsheet software application called geNorm (Vandesompele et al., 2002).

The algorithm in geNorm uses the expression values obtained from the conversion of the Ct values as explained in the user manual. The test was carried out on samples collected from Algans, Picker and Fergus on day 4, 5, 6, 7, 8 post-germination under control and cold temperature conditions. The different cDNA templates were not pooled together, in order to detect variation across the samples.

The most stable reference genes are shown in Figure 3-17, which is a graph generated by geNorm and which shows the most and the least stable housekeeping genes.

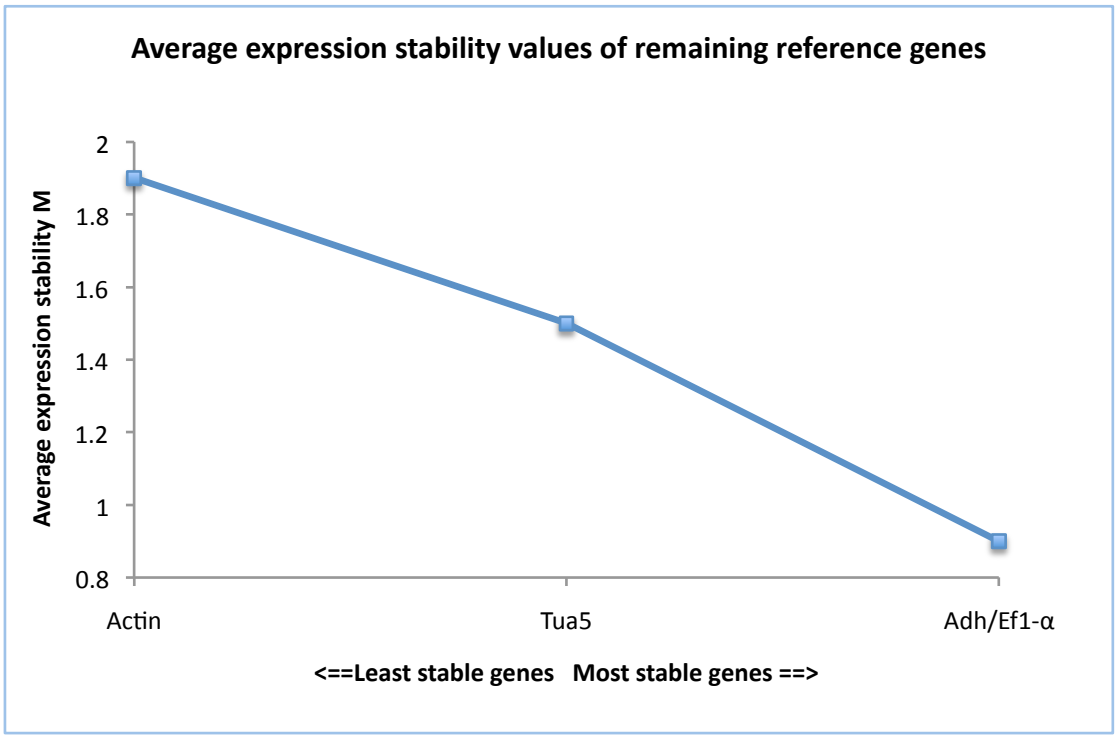


Figure 3-17 Average expression stability of the housekeeping genes. The genes with the lowest M value (Adh, Ef1-α) are the most stable ones. Average expression for each housekeeping gene was calculated by the geNorm alghoritm (n=10).

Adh and Ef1-α resulted to be the most stable reference genes, since they have the lowest M-values. The bar chart displayed in Figure 3-18 was generated by geNorm and describes how many reference genes are requested for normalisation.

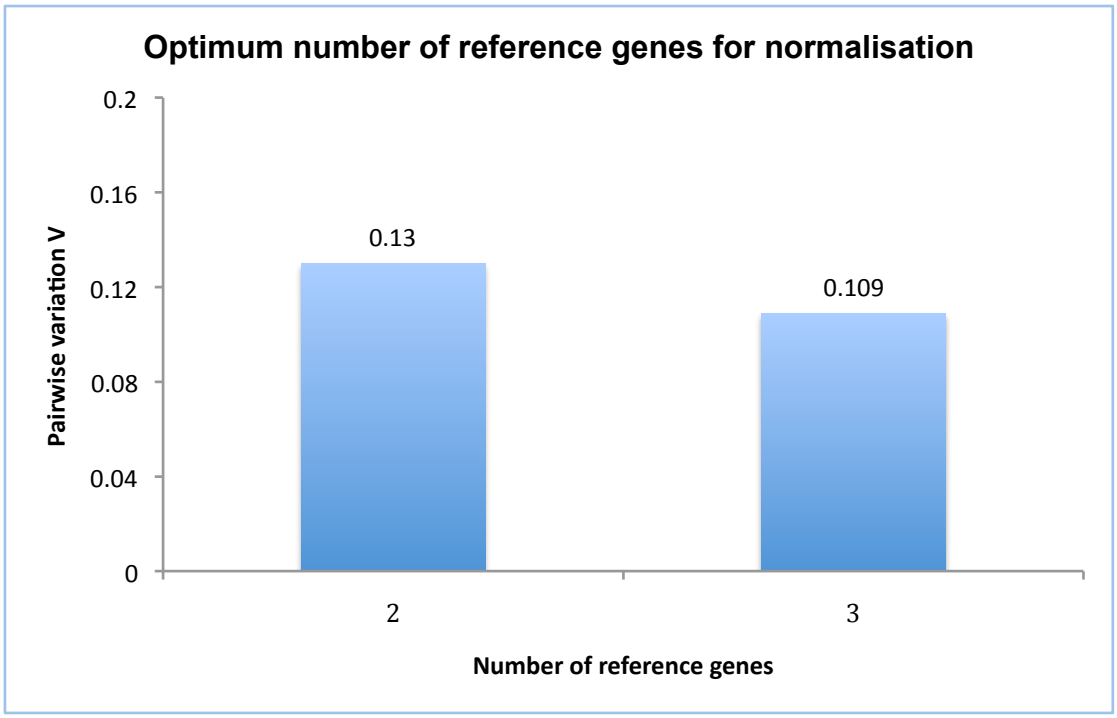


Figure 3-18 Determination of the optimal number of reference genes for normalization. The graph was generated by geNorm and shows the pairwise variation of the housekeeping genes resulting in a V-value displayed on top of each column. V < 0.15 on the first column indicates that two reference genes are sufficient for relative expression normalisation. The V-values were calculated for four housekeeping genes with n=10 each.

The first column presented a V-value of 0.13, which indicates that a pair of reference genes is sufficient for normalisation and a third reference gene is not required, since the threshold value for the pair wise variation is 0.15. The second column indicates that the use of a third control gene would be still possible (V-value lower than 0.15) and would actually improve the normalisation, as the V-value was lower than the first column. Nevertheless, the use of three reference genes is not strictly necessary.

The selected pair of reference genes was tested on two cold inducible (COI) genes, ZmACA1 and ZmDREB2, whose expression is up regulated in leaves (Nguyen et al., 2009). Two pools of cDNA were made: one containing the templates of Algans, Picker and Fergus on day 4, 5, 6, 7, 8 post-germination in control conditions and the other one made with the same varieties and time points, but from the cold treatment. The expression values of ZmACA1 and ZmDREB2 were normalised against the two reference genes (Figure 3-19) and their expression was calculated according to the comparative Ct method (Pfaffl, 2001). Both ZmACA1 and ZmDREB2 were up regulated under cold stress as expected (Table 3-7).

Table 3-7 Relative gene expression. The expression values show the fold-change in the expression of the cold regulated genes ZmACA1 and ZmDREB2 under cold stress.

	Expression	Regulation
ZmACA1	4.8	UP regulated
ZmDREB2	5.9	UP regulated

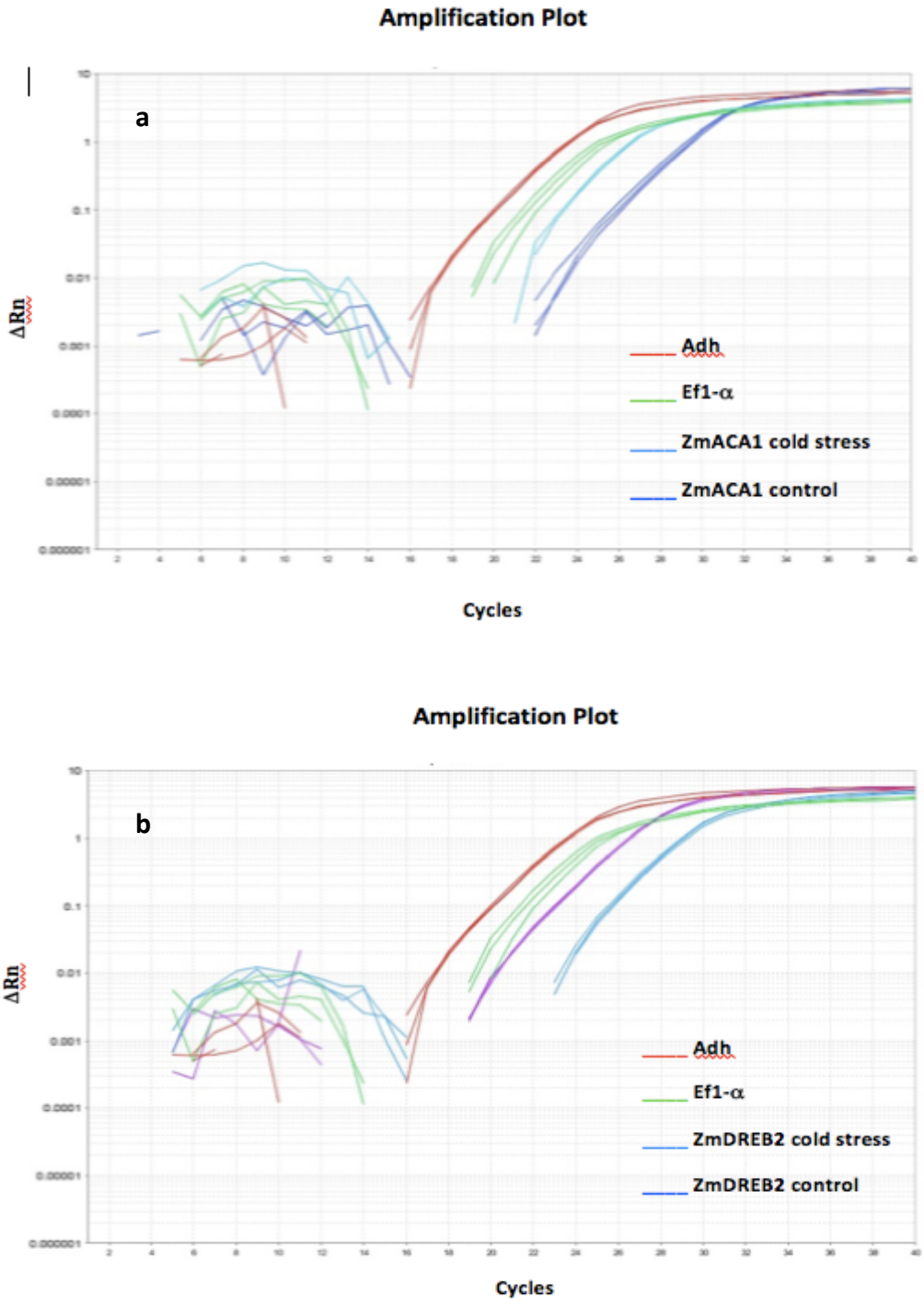


Figure 3-19 Relative quantification analysis. Amplification plots show the Ct at which the target genes were amplified. The comparative Ct-method was used to normalise the Ct values and calculate the expression values. ΔRn is the magnitude of the fluorescence signal generated during the PCR. a) Amplification plot of ZmACA1. b) Amplification plot of ZmDREB2. The amplification plots of the housekeeping genes Adh and Ef1-α are also shown.

3.3.2 Relative quantification Real Time PCR

The efficiencies calculated for all the primers displayed a slight variation (Table 3-8), although they are close to 100% and the difference between the different genes was less than 5%. Therefore the Livak's method was used to calculate the gene expression.

Table 3-8 qRT-PCR primer efficiencies and slopes. $E_{amp} = 10^{-1/slope}$

Gene	E_{amp} (%)	Slope
RNA binding protein	99%	-3.3
Patogenesis related proteint (PRP)	98%	-3.3
Unknown protein (Ukw-1)	100%	-3.3
Unknown protein (Ukw-2)	97%	-3.4
Draught inducible protein (DIP)	100%	-3.2
Peroxidase	100%	-3.2
Putative heat shock protein hsp22 precursor (HSP)	100%	-3.3
Probable lipid transfer protein (LTP)	97%	-3.4
Glucose starvation-induced protein precursor (GSIP)	100%	-3.3
Adh	95%	-3.3
Ef1-α	96%	-3.4

The most significantly regulated genes selected from the microarray analysis were used in qRT-PCR for data validation. The log_2 average expression values of the qRT-PCR at time point 4 were correlated to the log_2 values (M-values) of the microarray analysis for validation (Mattiello et al. 2010; Morey et al., 2006) as shown in Figure 3-20.

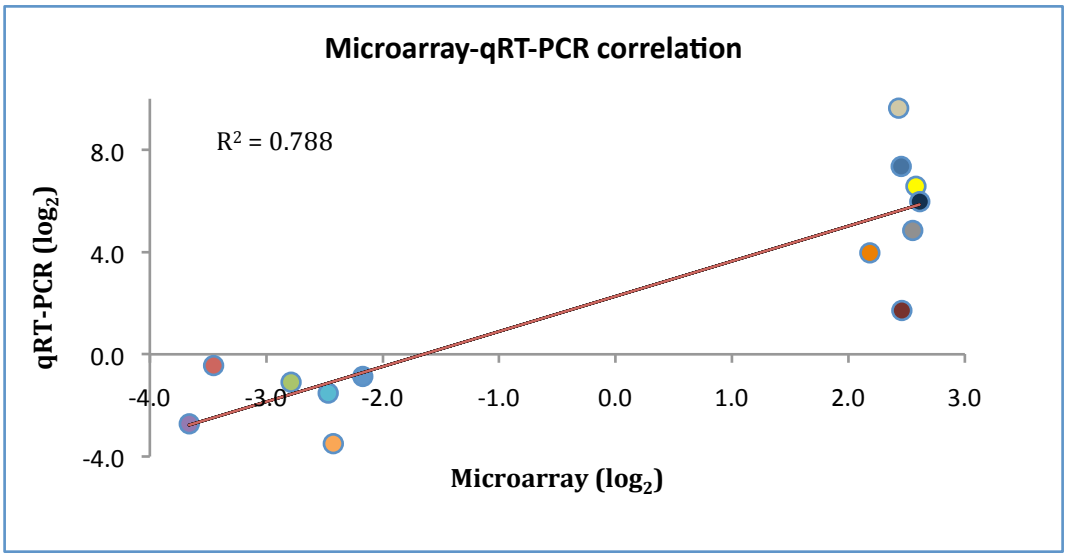


Figure 3-20 Microarray-qRT-PCR correlation. The most significantly regulated genes in the macroarray analysis are shown in the figure. The log_2 expression values of the qRT-PCR were plotted against the M-values of the microarray. Each colour represents a different gene. The red line represents a regression line. R^2 = Correlation coefficient. Spearman's correlation = $\sqrt{R^2}$ = 88%.

Spearman's correlation (rho) for non-parametric distribution was derived from the square root of R^2, which was 88%. This strong correlation between the two expression profiling techniques assessed that microarray data were successfully validated by qRT-PCR.

The use of qRT-PCR was also aimed at estimating the expression pattern of the genes of interest across the five time points in the two varieties Picker and PR39B29. As shown in Figure 3-21, the gene expression was not maintained over the time, but it was subjected to fluctuation. Except the RBP, the gene expression pattern was similar between the two varieties, in particular for PRP-1.

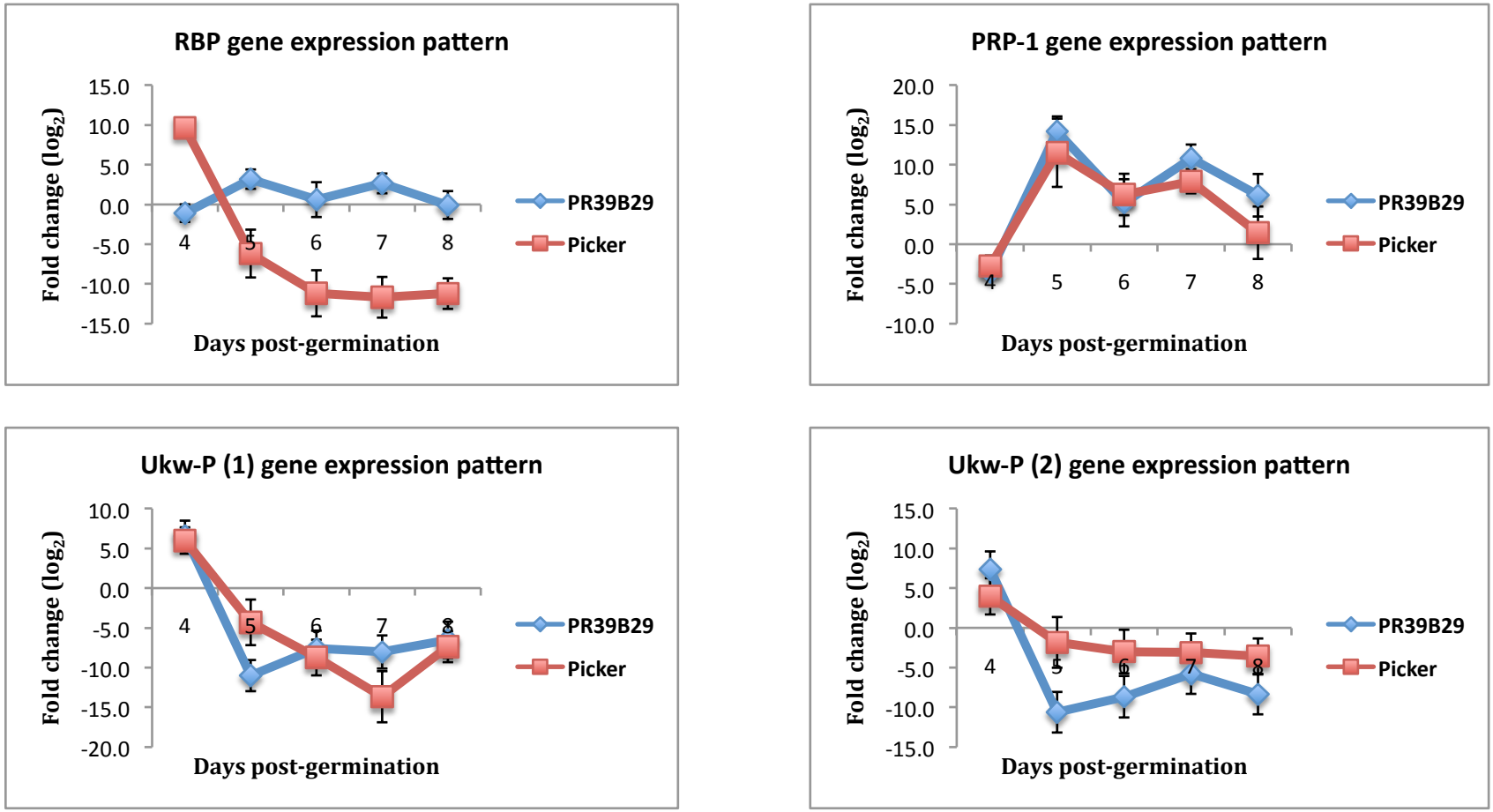


Figure 3-21 Gene expression pattern of the four genes shared between the two varieties Picker and PR39B29. The gene expression pattern was examined over five days post-germination, from day 4 to day 8. Results are mean ± SE (error bars; n=3).

4 Discussion

4.1 Physiology and genetics of maize roots grown at low temperatures

Investigation on the cold tolerance in maize has mainly focused on the early phases of growth, as it is known that plant establishment is fundamental for the crop to reach maturity and maximum development. The photosynthetic apparatus is susceptible to low temperatures (Baker & Nie, 1994; Fracheboud, 1999; Hayden & Baker, 1989; Stamp, 1984), therefore most of the attention has been focused on the effect of cold on leaf development and on photosynthesis. When maize is grown at sub-optimal temperatures impaired chloroplast function through photoinhibition as well as altered pigment composition and chlorophyll deficiency result in damaged photosynthetic apparatus (Greaves, 1996; Marocco et al., 2005). Responses to cold/chilling stress were detected by the chlorophyll fluorescence method. However, germination and the heterotrophic phase of development are also known to be considerably impaired when soil temperatures are too low for a functional root system to establish. Besides, the photosynthetic performance in maize seedlings is influenced by the different cold tolerance in root and shoot development (Stamp, 1984; Tollenaar & Lee, 2002). QTL analysis on maize has associated different root traits such as number, weight and thickness, with low temperature tolerance (Hund et al., 2004). However, investigation on molecular level has not been carried out in chilling stressed maize roots, therefore the goal of the present study was to detect the

expression patterns of specific genes controlling the first developmental stages from radicle and shoot emergence.

Studies on roots are usually performed on field grown maize, although this approach is not free from flaws, mostly due to the destruction of root material during sampling. The analysis is usually done by image acquisition, which can be biased because of the background noise due to soil (Dong et al., 2003).

Over the years, hydroponic cultivation (Sanguineti et al.1998) or the use of sand columns (Rut et al., 2010) have been adopted to grow roots under controlled environments. Nevertheless, although these techniques improve labour, time and accuracy, they still present disadvantages when it comes to root sampling.

The acquisition of data on root traits can be carried out in several ways, from recording roots using a simple camera or photocopier (Collins et al., 1987; Liedgens & Richner, 2001), a scanner (Dong et al., 2003; Hund et al., 2009; Manschadi et al., 2008) to X-ray imaging techniques (Gregory et al., 2003). Root image analysis has progressed to the development of advanced software for the quantitative analysis or root growth and the architecture of complex root systems (Lobet et al., 2011). However, these techniques can be invasive and damage the root samples as well as reduce the sample size under consideration. It is therefore necessary to use techniques that are not destructive and take repetitive measurements of the traits of interest, giving temporal information about root growth over a certain period of time.

The growth of maize seedlings on blotting paper for root development analysis used in this work has been used previously, with the specific aim to develop a phenotyping platform for non-destructive and repeated measurements of root growth for mapping studies of QTLs (Hund et al., 2009). Nevertheless, in Hund's work seedlings were grown in pouches and roots extend gravitropically. It has been demonstrated that growth in pouches is highly correlated with growth in sand and soil pots (Bonser et al., 1996) and to some extent consistent with field trials (Liao et al., 2004). In the present work, the experimental setup did not involve the use of pouches, because this system does not allow the use of a high number of seeds.

4.2 Experimental conditions

Plants grew in controlled environmental chambers under a range of temperature regimes to optimise the temperature conditions for a cold stress experiment. The three temperatures regimes used in the *Growth conditions for testing temperature regimes* were in the range of previous studies (Blacklow, 1972; Bowen, 1991; Farooq et al., 2009; Marocco et al., 2005; Richner et al., 1996). However, the 22°/18°C photoperiod was unsuitable for the purposes of this work. The main reason is because fast growth of primary roots at the higher temperature resulted in an overlapped and interwoven root structure; measurement of root length in such state was then unfeasible.

Another reason for that may be explained with the impedance of gravitropic elongation of the primary root due to the experimental setup and the angle of root growth (angle from the horizontal plane and the primary root). In such conditions, roots were forced to grow horizontally, along the surface of the three moist layers rather than downward, as it would occur in soil. This resulted in a curved root elongation along the horizontal plane that was more pronounced at the higher and lower temperature regimes, probably because the angle of root growth becomes bigger the more the temperature departed from 17°C (Onderdon & Ketcheso, 1973). However, at low temperatures growth is retarded, therefore the scenario of the higher temperature treatment did not arise.

The failure in germination under the 12°/4°C photoperiod is not completely unexpected, probably because germination requires a minimum temperature of 10°C (Levitt, 1980) and cellular and tissue damage occur when temperature is below 5°C (Greaves, 1996). Prolonged exposure below this temperature can seriously injure seedlings that are no longer able to recover (Theocharis et al., 2012). Several studies have reported that brief exposure to low temperatures do not impair the photosynthetic apparatus (Leipner, 2009). Moreover, it has been demonstrated that a short period of sub-optimal temperatures does not cause irreversible damage in maize seedlings at early developmental stages as seedlings maintained at low temperatures for three days subsequently recovered (*Bridget Hogg, personal information*). Therefore, the 12°/6°C temperature regime used in the *Growth conditions for modified temperature regimes* was sufficient to favour

germination when temperature was at 12°C and not detrimental when it was at 6°C.

The two final temperature regimes used for germination and root/shoot growth experiments both represent chilling conditions, as, in the literature, chilling temperatures range is defined as temperatures between 5-15°C (Nguyen et al., 2009). The 18/12°C temperature cycle represented the control conditions under which seeds were germinated and grown, while the 12/6°C cycle represented the cold treatment. It is important to emphasise that the genotypes used for the experiment all possess a certain degree of cold tolerance and for this reason they are commercially available. However, defining these varieties cold tolerant is technically incorrect; it would be more appropriate and advised to use the term cold avoidance rather than cold tolerance, because the genetic basis conferring cold tolerance remains unknown. Therefore, which specific genes are up and down regulated in response to low temperatures has yet to be defined particularly in relation to root growth. The lower temperature used to induce cold stress was, therefore, likely to show differences in the degree of tolerance among the varieties and detect new cold-regulated genes whose expression changes when temperature drops below a certain threshold.

Germination occurring in constant dark conditions, as it would occur if seeds were placed in soil, is not unusual (Sowiński et al., 2005; Yu-qin & Song-quan, 2008) since light is not considered crucial for seed germination in maize, but has a huge effect on seedling during the transaction from the heterotrophic to the photoautotrophic phase (Chory, 1997). It has also been

reported that when light hits the the root cap, growth is retarded, while no significant difference to growth in dark conditions has been seen when light hits the root elongation zone (Pilet & Ney, 1978). Nevertheless, differences in root growth were observed between seedlings grown under light/dark and only dark conditions. This discrepancy can be due not necessarily to the use of light, although it may play a role in stimulating germination and break seed dormancy (Bewleyl, 1997). Nevertheless, trays were covered with inverted trays, which somehow prevented light from hitting the seeds, although light could still penetrate the miniature growth tray-chamber through the orifices in the back of the trays. However, the differences in germination and growth were more likely due to the employment of a different experimental design. More varieties were, in fact, used in the *Growing conditions for the full-set experiment* and seed trays were differently distributed in the growth chambers. Also, the water uptake may have played a role, because of the different number of seeds accommodated in the seed trays across the physiological trials, while the quantity of water supplied was unchanged.

The physiological experiment was conducted until eight days post-germination, a time range that is consistent with the fact that nutrients stored in the endosperm are sufficient to guarantee an adequate growth of maize seedlings for about 10-14 days post-germination (Hochholdinger, 2004). Seeds were germinated without any nutrient solution unlike other studies, where a pre-germination test is usually carried out with different temperature and growth conditions (Bhosale et al., 2007).

Growth conditions for modified temperature regimes and *Growth conditions for the full-set experiment* showed a similar germination rate, but a different response was obtained as regards to root and shoot growth. In *Growth condition for modified temperature regimes*, the low temperature considerably affected all the three genotypes, Algans, Picker and Fergus, which, however, did not show a significant difference in response among each other. In *Growth conditions for the full-set experiment* where 12 varieties were employed, it is possible to notice the effects of the new experimental layout when the cold response of Algans, Picker and Fergus is analysed. Picker showed indeed a clear and significant tolerance compared to the other two varieties.

The poor germination rate displayed by Algans and Justina in any of the experimental conditions used may be due to imbibitional chilling injury, since it has been reported that chilling stress during imbibition reduces germination in some genotypes (Miedema et al., 1987). However, imbibition impair is not believed to be the primary cause for reduction in germination (Greaves, 1996).

4.3 Diverging response to low temperatures

The physiological response to low temperatures has led to the identification of two groups of genotypes with contrasting chilling tolerance.

The varieties Picker and PR39B29 showed the highest resistance to cold stress in terms of both root and shoot growth. Codisco and Fergus presented the lowest degree of tolerance, right after the varieties Algans and Justina, which, because of their poor ability to germinate under the stress conditions applied, were excluded for the microarray analysis. Therefore, the four final genotypes used for the gene expression profiling were Picker, PR39B29, Codisco and Fergus.

The root and shoot response to cold stress has also been investigated as a possible kernel-related result or on the basis of the earliness of each variety to reach maturity. The sample of genotypes at disposal was indeed quite heterogeneous and cold tolerance might be due to multiple genetic traits. All the info relative to the different genotypes is listed in the Table 2-1.

4.3.1 Kernel type

The difference in chilling tolerance between the varieties Fergus and PR29B29 seemed to be compatible with their types of kernel, respectively flint and dent. The sensitivity to low temperatures of the photosynthetic apparatus in dent genotypes is well known in literature (Kellos et al., 2008; Kocsy et al., 2001; Richner et al., 1996) and this effect could also be exhibited at very early developmental stages on root and shoot growth. Nevertheless, the other two varieties, Codisco and Picker, although they are both flint-dent

genotypes, showed contrasting level of chilling tolerance, which is therefore, in this case, not dependant on a particular kernel type. Moreover, the flint genotype PR39D60 presented a level of tolerance that is closer to the one of the two cold sensitive Fergus and Codisco, rather than the cold tolerant PR39B29 and Picker. In particular, the relative root growth of PR39D60 was somewhere in between the two extreme levels of tolerance, although slightly tending towards a higher tolerance, while the relative shoot growth certainly place PR39D60 in the pool of the lowest chilling tolerant varieties. It can be inferred from this that the type of kernel itself does not grant tolerance to low temperatures. This is not surprising since all the varieties examined are adapted in the climatic marginal areas and some of them also included in the Irish Recommended List. Nevertheless, the response of Fergus was consistent with the type of kernel, but it was also the only dent variety in the study; it would be so necessary to extend the experiment to other dent genotypes to validate this data.

4.3.2 Maturing time

Although selection of maize genotypes commercially available with superior cold tolerance is mainly based on visual evaluation rather than on the knowledge of the genetic mechanisms, it is known that early vigour plays a key role in cold tolerance during emergence and post-emergence stages, under both controlled and field conditions. This makes early vigour a

reference trait for selecting the most suitable genotypes (Rodríguez et al., 2007). All the varieties in the study were early varieties as they are all adopted in Central and Northern European countries and established without plastic mulch. However, they slightly differer in vigour, which may be relevant for improved tolerance to chilling temperatures.

The two most chilling tolerant varieties, Picker and PR39B29, are both very early maturing genotypes; the only other very early maturing variety, Huski, presented an intermediate level of tolerance. However, a conflicting scenario was presented when the mid-early and early maturing varieties were compared. The mid-early maturing varieties Algans and Codisco showed low resistance to chilling stress compared to the other two mid-early maturing Crazi and Clariti, which, instead, expressed an intermediate level of tolerance.

The five early maturing varieties, Fergus, Justina, PR39D60, Codifar and Lakti, displayed contrasting root and shoot responses to low temperature. Fergus and Justina were more affected by the chilling stress, while PR39D60 showed an intermediate response. Codifar and Lakti presented a higher tolerance in terms of root response, while shoot growth was significantly impaired in Lakti.

4.3.4 Interaction of kernel type and maturing time

The varieties Algans and Codisco share the same flint-dent kernel and mid-early maturing time as Crazi and Clariti but presented a diverging overall response to chilling stress. Consideration must be taken when the very early maturing varieties PR39B29, Picker and Huski are compared. The three genotypes also present a flint component in their kernel and showed a high or intermediate response to cold stress.

The physiological results showed that very early maturing varieties tend to be more able to cope with low chilling temperatures, while the type of kernel cannot grant the same response. The combination of the kernel and early vigour did not prove to be relevant in cold acclimation for the mid-early and early varieties, but this was not entirely true for the very early varieties, which were totally or partially flint genotypes. This may indicate, therefore, a probable better suitability to cope with chilling temperatures for those varieties showing both the flint and very early maturing phenotypes.

The better response to chilling temperatures by Picker and PR39B29 indicates that the two varieties possess a lower thermal threshold, which is the genetic capability to grow at temperatures lower than the one for optimum growth (Keeling & Greaves, 1990). As a result, and for the specific case of the present work, Picker and PR39B29 can be defined as chilling tolerant, while Fergus and Codisco chilling sensitive.

4.3 How plants detect changes in temperature

The use of contrasting genotypes increased the probability to identify differences in gene expression and therefore highlight the genes that may play a role in response to chilling temperatures. Nevertheless, cold or chilling acclimation does not always involve changes in gene expression, which can occur as an additional mechanism to physiological and molecular adaptations to cope with low temperatures (Theocharis et al., 2012). Moreover, the abundance of mRNAs is not always correlated with the abundance of the corresponding proteins, because of changes in protein turnover rates, which make it hard to simply predict protein expression levels from the number of mRNA transcripts (McKay et al., 2004; Pradet-Balade, Boulme, Beug, Mullner, & Garcia-Sanz, 2001; Tian et al., 2004). Therefore, the absence of significant changes in the transcriptome observed in the two low tolerant varieties, Fergus and Codisco, may indicate that the effect of root and shoot acclimation is not reflected on the gene expression in these two varieties.

Another reason for the absence of changes in gene expression could be explained by the fact that plants do not initiate a full cold acclimation every time fluctuations in temperature are experienced (Knight & Knight, 2012), although plants can detect variations of as little as 1°C (Knight & Knight, 2000). Under natural conditions plants are continuously exposed to small changes in temperature. Therefore, changes in gene expression describe the way the genotypes interpret the exposure to low temperatures, which cause a cascade of events that will eventually lead to cold acclimation.

Since all the varieties used in this study are adapted to grow in cool climate regions, they all possess a degree of cold tolerance, which is reflected on the gene expression pattern. None of the genes that are normally differentially expressed in response to cold were differentially expressed in the microarray study. As a result, cold acclimation at the lower chilling temperature regime is also dependant on the ability of the genotypes to distinguish between the two different temperature conditions and trigger a series of downstream reactions to gene regulation. Given the considerably small relative root growth in Codisco and Fergus, the two varieties were likely to be significantly impaired by chilling stress. Picker and PR39B29, instead, showed a lower thermal threshold compared to Fergus and Codisco that was reflected on the regulation of genes.

4.5 Gene expression profiles of maize genotypes with contrasting chilling tolerance

The microarray analysis of the four genotypes, selected on the basis of their physiological response to chilling stress after four days post-germination, was conducted in order to detect differentially expressed genes under the two different temperature regimes. Therefore, the gene expression profile of each variety was defined as the comparison of each genotype under control conditions and the same genotype under chilling stress.

The stress induced by the chilling temperature in the genotypes Picker, PR39B29, Fergus and Codisco was reflected only on the expression profiles of the two varieties with superior chilling tolerance, Picker and PR39B29. No significant changes in expression were observed in Fergus and Codisco when exposed to the lower temperature regime.

From the analysis of microarray, no cold-regulated genes were found. This might be probably due to the fact that COR are regulated on initial phases of cold adaptation, while the time point chosen for the microarray analysis was at a later stage. However, as described in the following paragraphs, unknown genes possibly involved in response to cold stress were found.

The overall number of genes up and down regulated in the two chilling tolerant varieties amounts to 69, which are, however, divided in a group of 39 genes in PR39B29 and 30 genes in Picker, as the two varieties exhibited two different trancriptomic patterns in which only four genes were shared, although not all with the same degree of regulation.

The relative small number of genes detected from the microarray analysis is in agreement with other microarray studies conducted in roots at low temperatures (Melkonian et al., 2004) or in other environmental stresses (Mattiello et al., 2010). Besides, the number of genes tested was reduced to the 40% of genes that had the biggest variance between the temperature regimes. This analysis was then conducted on this 40%, which led to a reduction of the false discovery rate, therefore contributing not only to detect

the most significantly expressed genes, but also to increase the probability that those genes were truly significantly expressed.

The majority of both the up and down regulated genes in the two varieties was mainly involved in molecular functions based on the Gene Ontology (GO) annotations. In particular, the number of up regulated genes in PR39B29 was considerably higher compared to the down-regulated ones, in contrast with Picker, which presented an opposite trend. Of the four shared genes (probe ID: MZ00003507, MZ00004486, MZ00022876, MZ00041708), three genes (MZ00004486, MZ00022876 and MZ00041708) were regulated in the same manner in Picker and PR39B29: two (MZ000022876 and MZ00041708) were up regulated and encoded for unknown proteins, while one (MZ00004486) was down regulated (with a higher degree in Picker) and encoded for a pathogenesis related protein-1 (PR-1). The fourth shared gene (MZ00003507) was down regulated in PR39B29 but up regulated in Picker. The function relative to this gene was not available in the probe dataset. However, the nearest match obtained for this sequence is an RNA binding protein in *Arabidopsis thaliana*.

The two opposite directions of regulation could describe a different biological/physiological state at which roots were undergoing when samples were collected. In fact, the heterotrophic growth phase actually involves several distinct biological processes such as imbibition, breakdown of kernel reserves, cell division, cell extension, that are controlled by different quantitative traits, each equally contributing to resistance to low temperatures (Greaves, 1996). Therefore, if any of the various heterotrophic

processes is impaired or retarded by the low temperature, so will the overall phase and the physiological status of the seedlings at a specific time point will reflect the degree of the temperature effect on a particular biological process.

It is interesting to notice that MZ00003507 was the only gene shared by Picker and PR39B29 whose regulation was differently oriented and in agreement with the overall expression trend of the respective varieties. The other three-shared genes presented instead the same (up) regulation, suggesting a common way of the two varieties to respond to cold stress. The only information available is concerning the RNA binding protein (RBP) and the PR-1 protein. In particular, the role of PR-1 as a constitutive barrier against pathogens and abiotic stresses was already known.

4.5.1 Pathogenesis related protein (PR-1)

PR-1 belongs to a group of PR-families of low-molecular weight proteins (6-43 kDa) that are present in all plant organs, like leaves, stems, flowers and roots (Van Loon, 1999). PRs are constitutively expressed in plants and play a major role as preformed natural defence barrier against fungi, as seen in several pathosystems (Gau et al., 2004; Lawrence et al., 2000; Vleeshouwers et al., 2000) In particular, maize PR-1 genes, along with the genes of the PR-5 family, are involved in induced resistance responses

against pathogen attack (Morris et al., 1998). However, PRs are also induced in response to several abiotic stressful environmental stimuli, including wounding and low temperatures (Van Loon, et al., 2006). These data are in agreement with other studies that have discovered association between stress tolerance and resistance to pathogens in corn (Chen, et al., 2004) and *Arabidopsis* (Rizhsky et al., 2004), indicating that biotic and abiotic stress alone can induce expression of genes involved in response to both biotic and abiotic stresses (Chen et al., 2004).

For a better understanding of the meaning of the expression of the PR-1 gene under cold stress in this work, it is necessary to consider that the data obtained from the microarray analysis only show a picture of the gene regulation occurring in a particular time of root development. The time-course expression profiling carried out by the qRT-PCR, illustrated that gene expression was not constant over the time, but continuously fluctuated, probably to adapt the seedlings to the external and internal stimuli perceived by root cells. Therefore, it is important to consider the entire expression pattern of the genes for a more reliable interpretation of their response to cold stress.

The expression pattern of the PR-1 gene was not maintained over the five days post-germination examined in either of the genotypes. In particular, the expression switched from down regulation of the day 4 post-germination to up regulation of the remaining days post-germination, although with a fluctuation in expression. It is interesting to note that the trend of the gene expression is similar in PR39B29 and Picker, suggesting a common defence

mechanism, which is regulated in the same way in the two varieties. Actually, for their general protective role against any type of stressful cue, PRs are recognised as markers of systemic acquired resistance or SAR (Ward et al., 1991). Nevertheless, studies in *Arabidopsis* (Pieterse et al., 1996) and crop plants (Reitz et al., 2001; Siddiqui, & Shaukat, 2004) have established that PRs are not always induced as a primary defensive mechanism against stressful symptoms. Therefore, it can be assumed that PRs are only a fraction of multiple means activated by plants against environmental cues and/or that may represent an ultimate attempt of defence, suggesting that their presence is related to the severity of the stress. This can be an explanation of why the PR-1 gene was under-expressed on day 4 post-germination until it was eventually up regulated when the stress became too injurious. However, variations in the gene expression pattern of these proteins in different tissues, cells, organs and developmental stages, suggest for them functions beyond defence against pathogens (Edreva, 2005). PRs are induced in seed germination in tobacco by weakening the endosperm and favouring radicle emergence (Vögeli-Lange et al., 1994); they are also involved in embryogenesis in carrots (Kragh et al., 1996) and accumulate in plants during the transition to flowering and senescence (Fraser, 1981; Hanfrey et al., 1996). These findings are supported by the fact that the sequence of the PR-1 type protein is highly conserved in monocots and dicots plants (Morris et al., 1998) as well as in other organisms (yeast, insects and vertebrates), suggesting a common evolutionary origin and a fundamental function for surviving of living organisms (Van Loon, 2001) that has probably evolved in an additional protective role (Van Loon & Van Strien, 1999).

4.5.2 RNA binding protein gene (RBP-gene)

MZ00003507 was the only gene within the four genes identified in both the varieties whose expression pattern over time was different between the two genotypes. Once more, however, the regulation on the day 4 post-germination was oppositely oriented to the one from day 5 to day 8 post-germination. RBPs are known to be involved in the post-transcriptional regulation of RNAs, modulation of the expression pattern during development and in the adaptation of plants in response to environmental stresses (Lorković, 2009). The different gene regulation observed in the two varieties could be explained by the way the RBP gene was regulated. However, there is no evidence that links the regulation of MZ00003507 with that of other genes.

4.4.3 Unknown protein genes (Ukw-genes)

Each of the two genes (MZ000022876 and MZ00041708) coding for the unknown proteins showed a similar response in the two varieties and to some extent also between them. As seen for the PR-1 and the RBP genes, the two Ukw-genes switched from a regulation state on day 4 post-germination to the opposite one from day 5 to day 8 post-germination. In this case, the

level of expression was oriented towards down regulation. Unfortunately, there is no information available as regards to the protein function of two genes and therefore no assumption can be made about their exact role without further investigation, which was not done due to time constraint.

5 Conclusions and future perspectives

5.1 Conclusions

Cold tolerance is a topic of great interest because, after water availability, low temperature is the major limitation to plant productivity and distribution around the world (Theocharis et al., 2012). Over the years, outstanding progresses have been made in understanding the molecular mechanisms involved in the response to low temperature in plants. Nevertheless, the exact dynamics of the events and the way plants sense the low temperatures are still not fully understood. To date, the criteria of selection of maize varieties used in the breeding programmes are still based on visual ratings rather than on a genetic approach. In Ireland, the introduction of early maturing maize varieties has significantly improved the establishment of the crop, in particular in the East, Southeast and South of the country. However, despite the improvement in crop quality and yield, early maturing varieties are still dependent on suitable soil temperatures for the initial establishment of the seedlings and they still benefit from a longer growing season that can be extended by establishing the plants earlier. The use of plastic mulch for plant establishment has led to further improvements, but the benefits of this technique on the harvest are inconsistent (Keane, 2002) and in any case still related to an approach aiming to manage with low temperatures rather than developing a true cold tolerance at the genetic level. The main goal of this project was therefore directed to the detections

and identification of specific genes in maize primary roots involved in cold tolerance that are capable to enable maize plants to continue growing at lower temperatures. By identifying genes involved in cold tolerance, a traditional breeding selection is still possible and with an appropriate breeding programme the traits of interest can be transferred to new improved hybrids. However, the breeding process can be accelerated through marker-assisted selection (MAS). The traits of interest are detected and target genotypes are then more effectively selected, resulting in quicker line development and variety release (Collard & Mackill, 2008).

By the means of controlled environmental growth chambers it was possible to apply a cold treatment on different early maturing maize varieties that are on the market, evaluate their performance in root and shoot development and finally detect the genes involved in response to cold.

The analysis of microarray data has led to the identification of genes whose expression was regulated by the stress treatment, but none of the cold-regulated genes was found. However, a study aimed to the identification and characterisation of the two-uknown proteins is still possible and could reveal a possible role in cold adptation.

5.2 Future perspectives

Several approaches can be taken next:

1) The identification of the amino acidic motifs of the two unknown proteins to understand whether they share a common role or they are involved in two different molecular functions. Besides, this will help understand the cellular localisation of the two proteins and if they can be associated to known cold-regulated genes.

2) Extending the investigation of the gene expression pattern to the two chilling sensitive genotypes. As previously described, the expression trend was studied only in the two chilling tolerant varieties (for time constraint), the only ones that showed a significant difference in gene expression in response to chilling stress. An expression trend of the two-unknonw genes non-consistent with the one of the cold tolerant varieties would confirm that the regulation of those genes is a distinctive trait for cold tolerance.

3) Extending the expression profiling to late maturing maize cultivars to detect differences between these genotypes and the early maturing ones.

4) Extending the study to the multiple and distinct biological processes occurring during root elongation such as water uptake, cell division and cell extension, each of which contribute at some extent to the final adaptation of the seedling to low temperature. This study will detect new genes related to specific biological processes and associated to specific traits that might improve the overall response to cold stress at the early phases of

establishment.

5) The use of different profiling technique to overcome the limitations of the microarray technology. Microarray analysis is a very powerful tool in screening an entire transcriptome, but it can also be rather expensive and limited. The maize oligonuclotide array used in the present work has provided a relative low cost microarray but it was designed before the whole maize genome sequence was released. Therefore it is essentially constructed with expression tag sequences (ESTs), which cannot ensure precise information about the expression of all the genes. Alternative splicing products, multiple gene copies and cross-hybridisation were indeed not taken into consideration and the expression measured could be biased without all that information. With the availability of the maize genome sequence a re-annotation of the maize oligonucleotide array probe set has been recently conducted (Seifert et al., 2012), but that information has not been released yet. Besides, microarray analysis can only profile the information conveyed in the transcripts that are represented by existing genomic sequence probes on the array and is constrained by the unspecific high background levels (Marioni et al., 2008). RNA-seq is an alternative and recently developed sequencing profiling technique that has shown enormous advantages in transcriptome profiling, capable to overcome the limitations of the microarray analysis by exploiting the principles of the high-throughput sequencing technology (Wang et al., 2009). This technology basically consists in the conversion of RNA transcripts into cDNA fragments, which are then sequenced. However, despite its advantages, this technology is still an

emerging alternative to microarray and is not bias-free, like in the accuracy of the estimates of absolute transcript levels (Fu et al., 2009).

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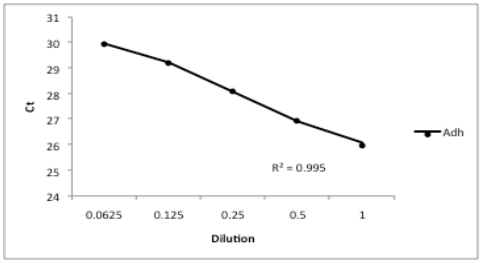
Appendix


Ct
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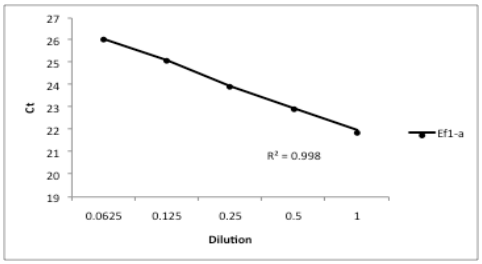
0.0625
0.125
0.25
0.5
1
R² = 0.995
Adh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0.25
0.5
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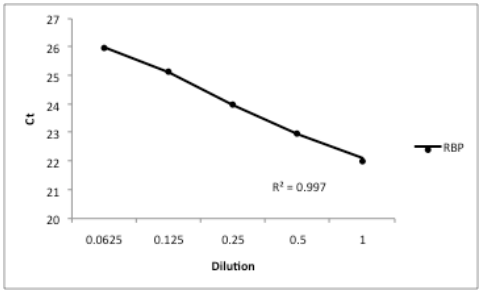
R² = 0.998
Ef1-a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0.125
0.25
0.5
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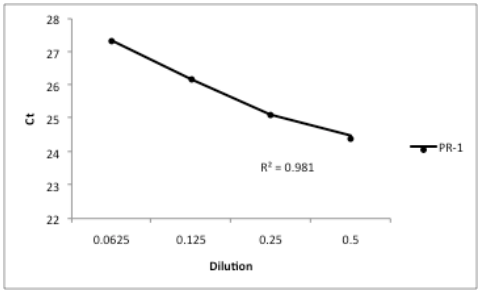
R² = 0.997
RBP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Dilution
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0.25
0.5
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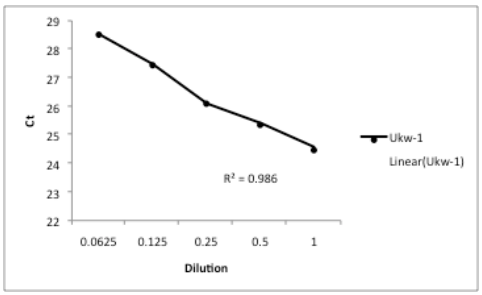
PR-1


Ct
Dilution
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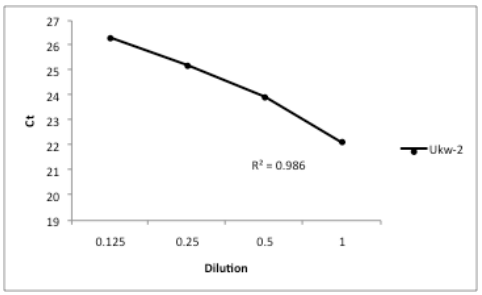
0.25
0.5
1
R² = 0.986
Ukw-1
Linear(Ukw-1)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Dilution
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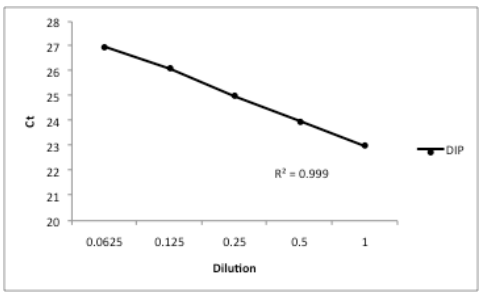
0.25
0.5
1
R² = 0.986
Ukw-2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Dilution
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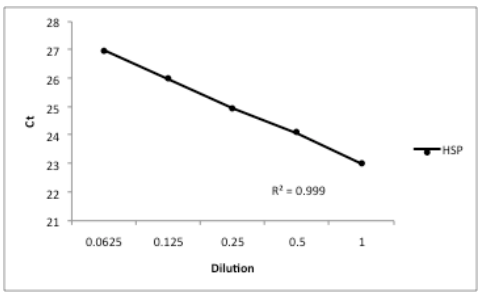
0.125
0.25
0.5
1
R² = 0.999
DIP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Dilution
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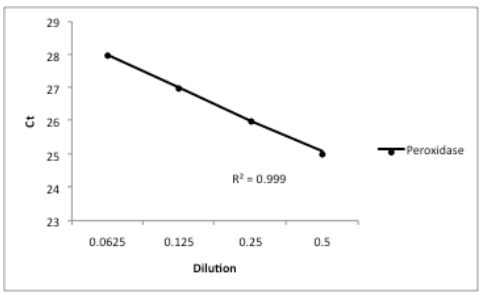
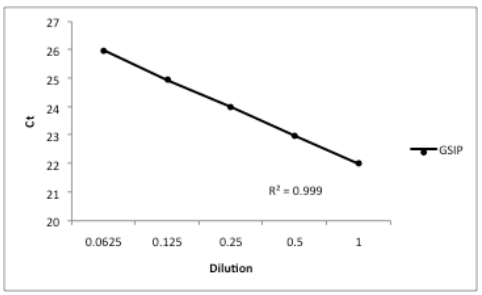
0.125
0.25
0.5
1
R² = 0.999
HSP


Ct
Dilution
0.0625
0.125
0.25
0.5
R² = 0.999
Peroxidase


Ct
Dilution
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0.125
0.25
0.5
1
R² = 0.999
GSIP

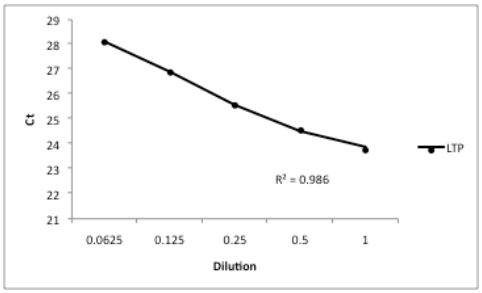


Figure 1 Standard curves to calculate the efficiency of qRT-PCR primers.

Standard curves were generated plotting Ct values of each primer pair against $\log_{10}$ RNA concentration in serial (1:2) dilution of cDNA in qRT-PCR.

Table 1 Tables of data generated by the Statistical Analysis Software.

Model Information	
Data Set	WORK.COLD
Dependent Variable	logroot
Covariance Structure	Unstructured
Subject Effect	REP*NAME*TREAT*EXP
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	*Levels*	*Values*
REP	2	1 2
NAME	12	ALGANS CLARITI CODIFAR CODISCO CRAZI FERGUS HUSKI JUSTINA LAKTI P329D60 PICKER PR29B29
TREAT	2	cold control
EXP	3	1 2 3
TIME	8	1 2 3 4 5 6 7 8

Dimensions	
Covariance Parameters	36
Columns in X	354
Columns in Z	0
Subjects	144
Max Obs Per Subject	8

Number of Observations	
Number of Observations Read	1152
Number of Observations Used	1152
Number of Observations Not Used	0

Iteration History			
Iteration	*Evaluations*	*-2 Res Log Like*	*Criterion*
0	1	907.44444779	
1	2	-1019.48058925	0.00000366
2	1	-1019.48573183	0.00000000

Convergence criteria met.

*Estimated R Correlation Matrix for REP*NAME*TREAT*EXP 1 ALGANS cold 1*								
Row	*Col1*	*Col2*	*Col3*	*Col4*	*Col5*	*Col6*	*Col7*	*Col8*
1	1.0000	0.7398	0.5485	0.4579	0.3828	0.3041	0.2389	0.1973
2	0.7398	1.0000	0.8807	0.7326	0.6022	0.5117	0.4194	0.3593
3	0.5485	0.8807	1.0000	0.8680	0.7586	0.6555	0.5533	0.4871
4	0.4579	0.7326	0.8680	1.0000	0.9472	0.8660	0.7813	0.7214
5	0.3828	0.6022	0.7586	0.9472	1.0000	0.9593	0.8854	0.8248
6	0.3041	0.5117	0.6555	0.8660	0.9593	1.0000	0.9687	0.9243
7	0.2389	0.4194	0.5533	0.7813	0.8854	0.9687	1.0000	0.9807
8	0.1973	0.3593	0.4871	0.7214	0.8248	0.9243	0.9807	1.0000

Covariance Parameter Estimates		
Cov Parm	*Subject*	*Estimate*
UN(1,1)	REP*NAME*TREAT*EXP	0.09942
UN(2,1)	REP*NAME*TREAT*EXP	0.08012
UN(2,2)	REP*NAME*TREAT*EXP	0.1180
UN(3,1)	REP*NAME*TREAT*EXP	0.06093
UN(3,2)	REP*NAME*TREAT*EXP	0.1066
UN(3,3)	REP*NAME*TREAT*EXP	0.1241
UN(4,1)	REP*NAME*TREAT*EXP	0.04748
UN(4,2)	REP*NAME*TREAT*EXP	0.08274
UN(4,3)	REP*NAME*TREAT*EXP	0.1006
UN(4,4)	REP*NAME*TREAT*EXP	0.1081
UN(5,1)	REP*NAME*TREAT*EXP	0.03750
UN(5,2)	REP*NAME*TREAT*EXP	0.06428
UN(5,3)	REP*NAME*TREAT*EXP	0.08305

Covariance Parameter Estimates		
Cov Parm	*Subject*	*Estimate*
UN(5,4)	REP*NAME*TREAT*EXP	0.09679
UN(5,5)	REP*NAME*TREAT*EXP	0.09656
UN(6,1)	REP*NAME*TREAT*EXP	0.02993
UN(6,2)	REP*NAME*TREAT*EXP	0.05487
UN(6,3)	REP*NAME*TREAT*EXP	0.07210
UN(6,4)	REP*NAME*TREAT*EXP	0.08890
UN(6,5)	REP*NAME*TREAT*EXP	0.09306
UN(6,6)	REP*NAME*TREAT*EXP	0.09747
UN(7,1)	REP*NAME*TREAT*EXP	0.02379
UN(7,2)	REP*NAME*TREAT*EXP	0.04549
UN(7,3)	REP*NAME*TREAT*EXP	0.06156
UN(7,4)	REP*NAME*TREAT*EXP	0.08112
UN(7,5)	REP*NAME*TREAT*EXP	0.08688
UN(7,6)	REP*NAME*TREAT*EXP	0.09550
UN(7,7)	REP*NAME*TREAT*EXP	0.09973
UN(8,1)	REP*NAME*TREAT*EXP	0.02013
UN(8,2)	REP*NAME*TREAT*EXP	0.03993
UN(8,3)	REP*NAME*TREAT*EXP	0.05552
UN(8,4)	REP*NAME*TREAT*EXP	0.07674
UN(8,5)	REP*NAME*TREAT*EXP	0.08293
UN(8,6)	REP*NAME*TREAT*EXP	0.09336
UN(8,7)	REP*NAME*TREAT*EXP	0.1002
UN(8,8)	REP*NAME*TREAT*EXP	0.1047

Fit Statistics	
-2 Res Log Likelihood	-1019.5
AIC (smaller is better)	-947.5
AICC (smaller is better)	-944.6
BIC (smaller is better)	-840.6

Null Model Likelihood Ratio Test		
DF	*Chi-Square*	*Pr > ChiSq*
35	1926.93	<.0001

Type 3 Tests of Fixed Effects

Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
EXP	2	118	17.38	<.0001
NAME	11	118	10.91	<.0001
TREAT	1	118	585.40	<.0001
*NAME*TREAT*	11	118	4.44	<.0001
TIME	7	118	688.76	<.0001
*NAME*TIME*	77	118	3.62	<.0001
*TREAT*TIME*	7	118	87.55	<.0001
*NAME*TREAT*TIME*	77	118	2.60	<.0001

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	ALGANS	cold	1	0.07492	0.1287	118	0.58	0.5617
*NAME*TREAT*TIME*	ALGANS	cold	2	0.2420	0.1402	118	1.73	0.0870
*NAME*TREAT*TIME*	ALGANS	cold	3	0.3639	0.1438	118	2.53	0.0127
*NAME*TREAT*TIME*	ALGANS	cold	4	0.5977	0.1342	118	4.45	<.0001
*NAME*TREAT*TIME*	ALGANS	cold	5	0.6930	0.1269	118	5.46	<.0001
*NAME*TREAT*TIME*	ALGANS	cold	6	0.8968	0.1275	118	7.04	<.0001
*NAME*TREAT*TIME*	ALGANS	cold	7	0.9994	0.1289	118	7.75	<.0001
*NAME*TREAT*TIME*	ALGANS	cold	8	1.1246	0.1321	118	8.51	<.0001
*NAME*TREAT*TIME*	ALGANS	control	1	0.4732	0.1287	118	3.68	0.0004
*NAME*TREAT*TIME*	ALGANS	control	2	1.1356	0.1402	118	8.10	<.0001
*NAME*TREAT*TIME*	ALGANS	control	3	1.8373	0.1438	118	12.77	<.0001
*NAME*TREAT*TIME*	ALGANS	control	4	2.2120	0.1342	118	16.48	<.0001
*NAME*TREAT*TIME*	ALGANS	control	5	2.4398	0.1269	118	19.23	<.0001
*NAME*TREAT*TIME*	ALGANS	control	6	2.6942	0.1275	118	21.14	<.0001
*NAME*TREAT*TIME*	ALGANS	control	7	2.8990	0.1289	118	22.49	<.0001
*NAME*TREAT*TIME*	ALGANS	control	8	3.0917	0.1321	118	23.41	<.0001
*NAME*TREAT*TIME*	CLARITI	cold	1	0.4533	0.1287	118	3.52	0.0006
*NAME*TREAT*TIME*	CLARITI	cold	2	0.7277	0.1402	118	5.19	<.0001
*NAME*TREAT*TIME*	CLARITI	cold	3	1.0180	0.1438	118	7.08	<.0001
*NAME*TREAT*TIME*	CLARITI	cold	4	1.3275	0.1342	118	9.89	<.0001
*NAME*TREAT*TIME*	CLARITI	cold	5	1.4816	0.1269	118	11.68	<.0001
*NAME*TREAT*TIME*	CLARITI	cold	6	1.6341	0.1275	118	12.82	<.0001

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	CLARITI	cold	7	1.7796	0.1289	118	13.80	<.0001
*NAME*TREAT*TIME*	CLARITI	cold	8	1.9712	0.1321	118	14.92	<.0001
*NAME*TREAT*TIME*	CLARITI	control	1	0.4907	0.1287	118	3.81	0.0002
*NAME*TREAT*TIME*	CLARITI	control	2	1.6199	0.1402	118	11.55	<.0001
*NAME*TREAT*TIME*	CLARITI	control	3	2.2191	0.1438	118	15.43	<.0001
*NAME*TREAT*TIME*	CLARITI	control	4	2.6744	0.1342	118	19.92	<.0001
*NAME*TREAT*TIME*	CLARITI	control	5	2.9219	0.1269	118	23.03	<.0001
*NAME*TREAT*TIME*	CLARITI	control	6	3.0861	0.1275	118	24.21	<.0001
*NAME*TREAT*TIME*	CLARITI	control	7	3.2978	0.1289	118	25.58	<.0001
*NAME*TREAT*TIME*	CLARITI	control	8	3.4500	0.1321	118	26.12	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	1	0.7321	0.1287	118	5.69	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	2	1.0631	0.1402	118	7.58	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	3	1.3733	0.1438	118	9.55	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	4	1.7363	0.1342	118	12.93	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	5	1.9456	0.1269	118	15.34	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	6	2.0760	0.1275	118	16.29	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	7	2.1866	0.1289	118	16.96	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	8	2.3321	0.1321	118	17.66	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	1	0.4120	0.1287	118	3.20	0.0018
*NAME*TREAT*TIME*	CODIFAR	control	2	1.3767	0.1402	118	9.82	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	3	1.8418	0.1438	118	12.81	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	4	2.2680	0.1342	118	16.90	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	5	2.6503	0.1269	118	20.89	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	6	2.9869	0.1275	118	23.43	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	7	3.2492	0.1289	118	25.20	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	8	3.5455	0.1321	118	26.84	<.0001
*NAME*TREAT*TIME*	CODISCO	cold	1	0.2530	0.1287	118	1.97	0.0517
*NAME*TREAT*TIME*	CODISCO	cold	2	0.4671	0.1402	118	3.33	0.0012
*NAME*TREAT*TIME*	CODISCO	cold	3	0.7099	0.1438	118	4.94	<.0001
*NAME*TREAT*TIME*	CODISCO	cold	4	0.8611	0.1342	118	6.41	<.0001
*NAME*TREAT*TIME*	CODISCO	cold	5	1.0899	0.1269	118	8.59	<.0001
*NAME*TREAT*TIME*	CODISCO	cold	6	1.2326	0.1275	118	9.67	<.0001
*NAME*TREAT*TIME*	CODISCO	cold	7	1.3356	0.1289	118	10.36	<.0001
*NAME*TREAT*TIME*	CODISCO	cold	8	1.5214	0.1321	118	11.52	<.0001
*NAME*TREAT*TIME*	CODISCO	control	1	0.8125	0.1287	118	6.31	<.0001
*NAME*TREAT*TIME*	CODISCO	control	2	1.6792	0.1402	118	11.97	<.0001

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	CODISCO	control	3	2.1812	0.1438	118	15.17	<.0001
*NAME*TREAT*TIME*	CODISCO	control	4	2.5228	0.1342	118	18.79	<.0001
*NAME*TREAT*TIME*	CODISCO	control	5	2.7216	0.1269	118	21.45	<.0001
*NAME*TREAT*TIME*	CODISCO	control	6	2.8647	0.1275	118	22.48	<.0001
*NAME*TREAT*TIME*	CODISCO	control	7	3.0367	0.1289	118	23.55	<.0001
*NAME*TREAT*TIME*	CODISCO	control	8	3.2578	0.1321	118	24.66	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	1	0.5437	0.1287	118	4.22	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	2	0.8369	0.1402	118	5.97	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	3	1.2737	0.1438	118	8.86	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	4	1.5142	0.1342	118	11.28	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	5	1.7039	0.1269	118	13.43	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	6	1.8177	0.1275	118	14.26	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	7	1.9415	0.1289	118	15.06	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	8	2.0924	0.1321	118	15.84	<.0001
*NAME*TREAT*TIME*	CRAZI	control	1	0.4147	0.1287	118	3.22	0.0016
*NAME*TREAT*TIME*	CRAZI	control	2	1.3243	0.1402	118	9.44	<.0001
*NAME*TREAT*TIME*	CRAZI	control	3	1.9110	0.1438	118	13.29	<.0001
*NAME*TREAT*TIME*	CRAZI	control	4	2.4125	0.1342	118	17.97	<.0001
*NAME*TREAT*TIME*	CRAZI	control	5	2.7739	0.1269	118	21.87	<.0001
*NAME*TREAT*TIME*	CRAZI	control	6	3.0105	0.1275	118	23.62	<.0001
*NAME*TREAT*TIME*	CRAZI	control	7	3.2177	0.1289	118	24.96	<.0001
*NAME*TREAT*TIME*	CRAZI	control	8	3.3890	0.1321	118	25.66	<.0001
*NAME*TREAT*TIME*	FERGUS	cold	1	0.2080	0.1287	118	1.62	0.1088
*NAME*TREAT*TIME*	FERGUS	cold	2	0.4484	0.1402	118	3.20	0.0018
*NAME*TREAT*TIME*	FERGUS	cold	3	0.6312	0.1438	118	4.39	<.0001
*NAME*TREAT*TIME*	FERGUS	cold	4	0.8492	0.1342	118	6.33	<.0001
*NAME*TREAT*TIME*	FERGUS	cold	5	1.0805	0.1269	118	8.52	<.0001
*NAME*TREAT*TIME*	FERGUS	cold	6	1.2228	0.1275	118	9.59	<.0001
*NAME*TREAT*TIME*	FERGUS	cold	7	1.3716	0.1289	118	10.64	<.0001
*NAME*TREAT*TIME*	FERGUS	cold	8	1.5438	0.1321	118	11.69	<.0001
*NAME*TREAT*TIME*	FERGUS	control	1	0.6368	0.1287	118	4.95	<.0001
*NAME*TREAT*TIME*	FERGUS	control	2	1.6536	0.1402	118	11.79	<.0001
*NAME*TREAT*TIME*	FERGUS	control	3	2.1229	0.1438	118	14.76	<.0001
*NAME*TREAT*TIME*	FERGUS	control	4	2.7415	0.1342	118	20.42	<.0001
*NAME*TREAT*TIME*	FERGUS	control	5	3.1257	0.1269	118	24.64	<.0001
*NAME*TREAT*TIME*	FERGUS	control	6	3.2844	0.1275	118	25.77	<.0001

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	FERGUS	control	7	3.4146	0.1289	118	26.49	<.0001
*NAME*TREAT*TIME*	FERGUS	control	8	3.6240	0.1321	118	27.44	<.0001
*NAME*TREAT*TIME*	HUSKI	cold	1	0.2737	0.1287	118	2.13	0.0355
*NAME*TREAT*TIME*	HUSKI	cold	2	0.5403	0.1402	118	3.85	0.0002
*NAME*TREAT*TIME*	HUSKI	cold	3	1.1530	0.1438	118	8.02	<.0001
*NAME*TREAT*TIME*	HUSKI	cold	4	1.3682	0.1342	118	10.19	<.0001
*NAME*TREAT*TIME*	HUSKI	cold	5	1.6154	0.1269	118	12.73	<.0001
*NAME*TREAT*TIME*	HUSKI	cold	6	1.7204	0.1275	118	13.50	<.0001
*NAME*TREAT*TIME*	HUSKI	cold	7	1.8223	0.1289	118	14.13	<.0001
*NAME*TREAT*TIME*	HUSKI	cold	8	1.9609	0.1321	118	14.85	<.0001
*NAME*TREAT*TIME*	HUSKI	control	1	0.4937	0.1287	118	3.84	0.0002
*NAME*TREAT*TIME*	HUSKI	control	2	1.2574	0.1402	118	8.97	<.0001
*NAME*TREAT*TIME*	HUSKI	control	3	1.7401	0.1438	118	12.10	<.0001
*NAME*TREAT*TIME*	HUSKI	control	4	2.3784	0.1342	118	17.72	<.0001
*NAME*TREAT*TIME*	HUSKI	control	5	2.7803	0.1269	118	21.92	<.0001
*NAME*TREAT*TIME*	HUSKI	control	6	2.9168	0.1275	118	22.88	<.0001
*NAME*TREAT*TIME*	HUSKI	control	7	3.0467	0.1289	118	23.63	<.0001
*NAME*TREAT*TIME*	HUSKI	control	8	3.2850	0.1321	118	24.87	<.0001
*NAME*TREAT*TIME*	JUSTINA	cold	1	0.1679	0.1287	118	1.30	0.1945
*NAME*TREAT*TIME*	JUSTINA	cold	2	0.1823	0.1402	118	1.30	0.1961
*NAME*TREAT*TIME*	JUSTINA	cold	3	0.4410	0.1438	118	3.07	0.0027
*NAME*TREAT*TIME*	JUSTINA	cold	4	0.5133	0.1342	118	3.82	0.0002
*NAME*TREAT*TIME*	JUSTINA	cold	5	0.6430	0.1269	118	5.07	<.0001
*NAME*TREAT*TIME*	JUSTINA	cold	6	0.8271	0.1275	118	6.49	<.0001
*NAME*TREAT*TIME*	JUSTINA	cold	7	0.9489	0.1289	118	7.36	<.0001
*NAME*TREAT*TIME*	JUSTINA	cold	8	1.0421	0.1321	118	7.89	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	1	0.3866	0.1287	118	3.00	0.0033
*NAME*TREAT*TIME*	JUSTINA	control	2	1.1742	0.1402	118	8.37	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	3	1.6330	0.1438	118	11.35	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	4	2.0752	0.1342	118	15.46	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	5	2.3495	0.1269	118	18.52	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	6	2.5696	0.1275	118	20.16	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	7	2.8570	0.1289	118	22.16	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	8	3.1683	0.1321	118	23.99	<.0001
*NAME*TREAT*TIME*	LAKTI	cold	1	0.2953	0.1287	118	2.29	0.0235
*NAME*TREAT*TIME*	LAKTI	cold	2	0.6252	0.1402	118	4.46	<.0001

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	LAKTI	cold	3	0.9145	0.1438	118	6.36	<.0001
*NAME*TREAT*TIME*	LAKTI	cold	4	1.2404	0.1342	118	9.24	<.0001
*NAME*TREAT*TIME*	LAKTI	cold	5	1.4362	0.1269	118	11.32	<.0001
*NAME*TREAT*TIME*	LAKTI	cold	6	1.6558	0.1275	118	12.99	<.0001
*NAME*TREAT*TIME*	LAKTI	cold	7	1.8340	0.1289	118	14.23	<.0001
*NAME*TREAT*TIME*	LAKTI	cold	8	2.0364	0.1321	118	15.42	<.0001
*NAME*TREAT*TIME*	LAKTI	control	1	0.2435	0.1287	118	1.89	0.0610
*NAME*TREAT*TIME*	LAKTI	control	2	1.2316	0.1402	118	8.78	<.0001
*NAME*TREAT*TIME*	LAKTI	control	3	1.5866	0.1438	118	11.03	<.0001
*NAME*TREAT*TIME*	LAKTI	control	4	2.3057	0.1342	118	17.18	<.0001
*NAME*TREAT*TIME*	LAKTI	control	5	2.7502	0.1269	118	21.68	<.0001
*NAME*TREAT*TIME*	LAKTI	control	6	2.9586	0.1275	118	23.21	<.0001
*NAME*TREAT*TIME*	LAKTI	control	7	3.1231	0.1289	118	24.22	<.0001
*NAME*TREAT*TIME*	LAKTI	control	8	3.3210	0.1321	118	25.14	<.0001
*NAME*TREAT*TIME*	P329D60	cold	1	0.4757	0.1287	118	3.70	0.0003
*NAME*TREAT*TIME*	P329D60	cold	2	0.9349	0.1402	118	6.67	<.0001
*NAME*TREAT*TIME*	P329D60	cold	3	1.3118	0.1438	118	9.12	<.0001
*NAME*TREAT*TIME*	P329D60	cold	4	1.4864	0.1342	118	11.07	<.0001
*NAME*TREAT*TIME*	P329D60	cold	5	1.6572	0.1269	118	13.06	<.0001
*NAME*TREAT*TIME*	P329D60	cold	6	1.8819	0.1275	118	14.77	<.0001
*NAME*TREAT*TIME*	P329D60	cold	7	2.0043	0.1289	118	15.55	<.0001
*NAME*TREAT*TIME*	P329D60	cold	8	2.1018	0.1321	118	15.91	<.0001
*NAME*TREAT*TIME*	P329D60	control	1	0.6933	0.1287	118	5.39	<.0001
*NAME*TREAT*TIME*	P329D60	control	2	1.6568	0.1402	118	11.81	<.0001
*NAME*TREAT*TIME*	P329D60	control	3	2.1233	0.1438	118	14.76	<.0001
*NAME*TREAT*TIME*	P329D60	control	4	2.6182	0.1342	118	19.50	<.0001
*NAME*TREAT*TIME*	P329D60	control	5	2.9431	0.1269	118	23.20	<.0001
*NAME*TREAT*TIME*	P329D60	control	6	3.1907	0.1275	118	25.03	<.0001
*NAME*TREAT*TIME*	P329D60	control	7	3.4322	0.1289	118	26.62	<.0001
*NAME*TREAT*TIME*	P329D60	control	8	3.6289	0.1321	118	27.47	<.0001
*NAME*TREAT*TIME*	PICKER	cold	1	0.7025	0.1287	118	5.46	<.0001
*NAME*TREAT*TIME*	PICKER	cold	2	1.1937	0.1402	118	8.51	<.0001
*NAME*TREAT*TIME*	PICKER	cold	3	1.6087	0.1438	118	11.18	<.0001
*NAME*TREAT*TIME*	PICKER	cold	4	1.8203	0.1342	118	13.56	<.0001
*NAME*TREAT*TIME*	PICKER	cold	5	1.9623	0.1269	118	15.47	<.0001
*NAME*TREAT*TIME*	PICKER	cold	6	2.0768	0.1275	118	16.29	<.0001

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	PICKER	cold	7	2.2144	0.1289	118	17.18	<.0001
*NAME*TREAT*TIME*	PICKER	cold	8	2.3602	0.1321	118	17.87	<.0001
*NAME*TREAT*TIME*	PICKER	control	1	0.6984	0.1287	118	5.43	<.0001
*NAME*TREAT*TIME*	PICKER	control	2	1.8342	0.1402	118	13.08	<.0001
*NAME*TREAT*TIME*	PICKER	control	3	2.4435	0.1438	118	16.99	<.0001
*NAME*TREAT*TIME*	PICKER	control	4	2.8917	0.1342	118	21.54	<.0001
*NAME*TREAT*TIME*	PICKER	control	5	3.1335	0.1269	118	24.70	<.0001
*NAME*TREAT*TIME*	PICKER	control	6	3.2885	0.1275	118	25.80	<.0001
*NAME*TREAT*TIME*	PICKER	control	7	3.4417	0.1289	118	26.70	<.0001
*NAME*TREAT*TIME*	PICKER	control	8	3.5807	0.1321	118	27.11	<.0001
*NAME*TREAT*TIME*	PR29B29	cold	1	0.2748	0.1287	118	2.14	0.0348
*NAME*TREAT*TIME*	PR29B29	cold	2	0.5620	0.1402	118	4.01	0.0001
*NAME*TREAT*TIME*	PR29B29	cold	3	0.7747	0.1438	118	5.39	<.0001
*NAME*TREAT*TIME*	PR29B29	cold	4	1.0578	0.1342	118	7.88	<.0001
*NAME*TREAT*TIME*	PR29B29	cold	5	1.3945	0.1269	118	10.99	<.0001
*NAME*TREAT*TIME*	PR29B29	cold	6	1.6997	0.1275	118	13.34	<.0001
*NAME*TREAT*TIME*	PR29B29	cold	7	1.8805	0.1289	118	14.59	<.0001
*NAME*TREAT*TIME*	PR29B29	cold	8	2.0460	0.1321	118	15.49	<.0001
*NAME*TREAT*TIME*	PR29B29	control	1	0.2404	0.1287	118	1.87	0.0642
*NAME*TREAT*TIME*	PR29B29	control	2	0.6904	0.1402	118	4.92	<.0001
*NAME*TREAT*TIME*	PR29B29	control	3	1.4166	0.1438	118	9.85	<.0001
*NAME*TREAT*TIME*	PR29B29	control	4	1.9642	0.1342	118	14.63	<.0001
*NAME*TREAT*TIME*	PR29B29	control	5	2.3261	0.1269	118	18.34	<.0001
*NAME*TREAT*TIME*	PR29B29	control	6	2.6830	0.1275	118	21.05	<.0001
*NAME*TREAT*TIME*	PR29B29	control	7	3.0069	0.1289	118	23.32	<.0001
*NAME*TREAT*TIME*	PR29B29	control	8	3.2662	0.1321	118	24.73	<.0001

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*	ALGANS			15	118	27.47	<.0001
*NAME*TREAT*TIME*	CLARITI			15	118	35.40	<.0001
*NAME*TREAT*TIME*	CODIFAR			15	118	38.48	<.0001
*NAME*TREAT*TIME*	CODISCO			15	118	30.51	<.0001
*NAME*TREAT*TIME*	CRAZI			15	118	33.14	<.0001

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*	FERGUS			15	118	42.18	<.0001
*NAME*TREAT*TIME*	HUSKI			15	118	39.51	<.0001
*NAME*TREAT*TIME*	JUSTINA			15	118	33.86	<.0001
*NAME*TREAT*TIME*	LAKTI			15	118	42.39	<.0001
*NAME*TREAT*TIME*	P329D60			15	118	34.64	<.0001
*NAME*TREAT*TIME*	PICKER			15	118	33.52	<.0001
*NAME*TREAT*TIME*	PR29B29			15	118	36.21	<.0001
*NAME*TREAT*TIME*		cold		95	118	15.25	<.0001
*NAME*TREAT*TIME*		control		95	118	48.53	<.0001
*NAME*TREAT*TIME*			1	23	118	2.45	0.0009
*NAME*TREAT*TIME*			2	23	118	11.99	<.0001
*NAME*TREAT*TIME*			3	23	118	17.21	<.0001
*NAME*TREAT*TIME*			4	23	118	28.55	<.0001
*NAME*TREAT*TIME*			5	23	118	37.42	<.0001
*NAME*TREAT*TIME*			6	23	118	38.29	<.0001
*NAME*TREAT*TIME*			7	23	118	40.65	<.0001
*NAME*TREAT*TIME*			8	23	118	41.57	<.0001
*NAME*TREAT*TIME*	ALGANS		1	1	118	4.79	0.0307
*NAME*TREAT*TIME*	ALGANS		2	1	118	20.30	<.0001
*NAME*TREAT*TIME*	ALGANS		3	1	118	52.47	<.0001
*NAME*TREAT*TIME*	ALGANS		4	1	118	72.30	<.0001
*NAME*TREAT*TIME*	ALGANS		5	1	118	94.80	<.0001
*NAME*TREAT*TIME*	ALGANS		6	1	118	99.44	<.0001
*NAME*TREAT*TIME*	ALGANS		7	1	118	108.54	<.0001
*NAME*TREAT*TIME*	ALGANS		8	1	118	110.89	<.0001
*NAME*TREAT*TIME*	CLARITI		1	1	118	0.04	0.8376
*NAME*TREAT*TIME*	CLARITI		2	1	118	20.24	<.0001
*NAME*TREAT*TIME*	CLARITI		3	1	118	34.87	<.0001
*NAME*TREAT*TIME*	CLARITI		4	1	118	50.33	<.0001
*NAME*TREAT*TIME*	CLARITI		5	1	118	64.45	<.0001
*NAME*TREAT*TIME*	CLARITI		6	1	118	64.90	<.0001
*NAME*TREAT*TIME*	CLARITI		7	1	118	69.34	<.0001
*NAME*TREAT*TIME*	CLARITI		8	1	118	62.68	<.0001
*NAME*TREAT*TIME*	CODIFAR		1	1	118	3.09	0.0813
*NAME*TREAT*TIME*	CODIFAR		2	1	118	2.50	0.1166
*NAME*TREAT*TIME*	CODIFAR		3	1	118	5.31	0.0230

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*	CODIFAR		4	1	118	7.84	0.0060
*NAME*TREAT*TIME*	CODIFAR		5	1	118	15.43	0.0001
*NAME*TREAT*TIME*	CODIFAR		6	1	118	25.54	<.0001
*NAME*TREAT*TIME*	CODIFAR		7	1	118	33.97	<.0001
*NAME*TREAT*TIME*	CODIFAR		8	1	118	42.20	<.0001
*NAME*TREAT*TIME*	CODISCO		1	1	118	9.44	0.0026
*NAME*TREAT*TIME*	CODISCO		2	1	118	37.36	<.0001
*NAME*TREAT*TIME*	CODISCO		3	1	118	52.32	<.0001
*NAME*TREAT*TIME*	CODISCO		4	1	118	76.61	<.0001
*NAME*TREAT*TIME*	CODISCO		5	1	118	82.71	<.0001
*NAME*TREAT*TIME*	CODISCO		6	1	118	81.98	<.0001
*NAME*TREAT*TIME*	CODISCO		7	1	118	87.05	<.0001
*NAME*TREAT*TIME*	CODISCO		8	1	118	86.41	<.0001
*NAME*TREAT*TIME*	CRAZI		1	1	118	0.50	0.4800
*NAME*TREAT*TIME*	CRAZI		2	1	118	6.04	0.0154
*NAME*TREAT*TIME*	CRAZI		3	1	118	9.82	0.0022
*NAME*TREAT*TIME*	CRAZI		4	1	118	22.39	<.0001
*NAME*TREAT*TIME*	CRAZI		5	1	118	35.57	<.0001
*NAME*TREAT*TIME*	CRAZI		6	1	118	43.79	<.0001
*NAME*TREAT*TIME*	CRAZI		7	1	118	49.00	<.0001
*NAME*TREAT*TIME*	CRAZI		8	1	118	48.18	<.0001
*NAME*TREAT*TIME*	FERGUS		1	1	118	5.55	0.0202
*NAME*TREAT*TIME*	FERGUS		2	1	118	36.93	<.0001
*NAME*TREAT*TIME*	FERGUS		3	1	118	53.79	<.0001
*NAME*TREAT*TIME*	FERGUS		4	1	118	99.35	<.0001
*NAME*TREAT*TIME*	FERGUS		5	1	118	129.95	<.0001
*NAME*TREAT*TIME*	FERGUS		6	1	118	130.82	<.0001
*NAME*TREAT*TIME*	FERGUS		7	1	118	125.56	<.0001
*NAME*TREAT*TIME*	FERGUS		8	1	118	124.01	<.0001
*NAME*TREAT*TIME*	HUSKI		1	1	118	1.46	0.2293
*NAME*TREAT*TIME*	HUSKI		2	1	118	13.08	0.0004
*NAME*TREAT*TIME*	HUSKI		3	1	118	8.33	0.0046
*NAME*TREAT*TIME*	HUSKI		4	1	118	28.31	<.0001
*NAME*TREAT*TIME*	HUSKI		5	1	118	42.15	<.0001
*NAME*TREAT*TIME*	HUSKI		6	1	118	44.06	<.0001
*NAME*TREAT*TIME*	HUSKI		7	1	118	45.10	<.0001

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*	HUSKI		8	1	118	50.25	<.0001
*NAME*TREAT*TIME*	JUSTINA		1	1	118	1.44	0.2320
*NAME*TREAT*TIME*	JUSTINA		2	1	118	25.02	<.0001
*NAME*TREAT*TIME*	JUSTINA		3	1	118	34.34	<.0001
*NAME*TREAT*TIME*	JUSTINA		4	1	118	67.69	<.0001
*NAME*TREAT*TIME*	JUSTINA		5	1	118	90.47	<.0001
*NAME*TREAT*TIME*	JUSTINA		6	1	118	93.45	<.0001
*NAME*TREAT*TIME*	JUSTINA		7	1	118	109.52	<.0001
*NAME*TREAT*TIME*	JUSTINA		8	1	118	129.56	<.0001
*NAME*TREAT*TIME*	LAKTI		1	1	118	0.08	0.7763
*NAME*TREAT*TIME*	LAKTI		2	1	118	9.35	0.0028
*NAME*TREAT*TIME*	LAKTI		3	1	118	10.92	0.0013
*NAME*TREAT*TIME*	LAKTI		4	1	118	31.49	<.0001
*NAME*TREAT*TIME*	LAKTI		5	1	118	53.64	<.0001
*NAME*TREAT*TIME*	LAKTI		6	1	118	52.24	<.0001
*NAME*TREAT*TIME*	LAKTI		7	1	118	50.00	<.0001
*NAME*TREAT*TIME*	LAKTI		8	1	118	47.29	<.0001
*NAME*TREAT*TIME*	P329D60		1	1	118	1.43	0.2344
*NAME*TREAT*TIME*	P329D60		2	1	118	13.25	0.0004
*NAME*TREAT*TIME*	P329D60		3	1	118	15.92	0.0001
*NAME*TREAT*TIME*	P329D60		4	1	118	35.55	<.0001
*NAME*TREAT*TIME*	P329D60		5	1	118	51.37	<.0001
*NAME*TREAT*TIME*	P329D60		6	1	118	52.72	<.0001
*NAME*TREAT*TIME*	P329D60		7	1	118	61.34	<.0001
*NAME*TREAT*TIME*	P329D60		8	1	118	66.83	<.0001
*NAME*TREAT*TIME*	PICKER		1	1	118	0.00	0.9819
*NAME*TREAT*TIME*	PICKER		2	1	118	10.43	0.0016
*NAME*TREAT*TIME*	PICKER		3	1	118	16.84	<.0001
*NAME*TREAT*TIME*	PICKER		4	1	118	31.85	<.0001
*NAME*TREAT*TIME*	PICKER		5	1	118	42.62	<.0001
*NAME*TREAT*TIME*	PICKER		6	1	118	45.19	<.0001
*NAME*TREAT*TIME*	PICKER		7	1	118	45.31	<.0001
*NAME*TREAT*TIME*	PICKER		8	1	118	42.69	<.0001
*NAME*TREAT*TIME*	PR29B29		1	1	118	0.04	0.8505
*NAME*TREAT*TIME*	PR29B29		2	1	118	0.42	0.5188
*NAME*TREAT*TIME*	PR29B29		3	1	118	9.96	0.0020

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*	PR29B29		4	1	118	22.80	<.0001
*NAME*TREAT*TIME*	PR29B29		5	1	118	26.96	<.0001
*NAME*TREAT*TIME*	PR29B29		6	1	118	29.76	<.0001
*NAME*TREAT*TIME*	PR29B29		7	1	118	38.17	<.0001
*NAME*TREAT*TIME*	PR29B29		8	1	118	42.67	<.0001
*NAME*TREAT*TIME*	ALGANS	cold		7	118	8.68	<.0001
*NAME*TREAT*TIME*	ALGANS	control		7	118	38.91	<.0001
*NAME*TREAT*TIME*	CLARITI	cold		7	118	15.84	<.0001
*NAME*TREAT*TIME*	CLARITI	control		7	118	55.21	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold		7	118	16.10	<.0001
*NAME*TREAT*TIME*	CODIFAR	control		7	118	64.75	<.0001
*NAME*TREAT*TIME*	CODISCO	cold		7	118	15.50	<.0001
*NAME*TREAT*TIME*	CODISCO	control		7	118	40.43	<.0001
*NAME*TREAT*TIME*	CRAZI	cold		7	118	16.17	<.0001
*NAME*TREAT*TIME*	CRAZI	control		7	118	52.29	<.0001
*NAME*TREAT*TIME*	FERGUS	cold		7	118	14.88	<.0001
*NAME*TREAT*TIME*	FERGUS	control		7	118	63.92	<.0001
*NAME*TREAT*TIME*	HUSKI	cold		7	118	21.70	<.0001
*NAME*TREAT*TIME*	HUSKI	control		7	118	58.73	<.0001
*NAME*TREAT*TIME*	JUSTINA	cold		7	118	7.00	<.0001
*NAME*TREAT*TIME*	JUSTINA	control		7	118	54.80	<.0001
*NAME*TREAT*TIME*	LAKTI	cold		7	118	19.49	<.0001
*NAME*TREAT*TIME*	LAKTI	control		7	118	68.05	<.0001
*NAME*TREAT*TIME*	P329D60	cold		7	118	15.41	<.0001
*NAME*TREAT*TIME*	P329D60	control		7	118	52.73	<.0001
*NAME*TREAT*TIME*	PICKER	cold		7	118	17.47	<.0001
*NAME*TREAT*TIME*	PICKER	control		7	118	51.60	<.0001
*NAME*TREAT*TIME*	PR29B29	cold		7	118	22.00	<.0001
*NAME*TREAT*TIME*	PR29B29	control		7	118	53.11	<.0001
*NAME*TREAT*TIME*		cold	1	11	118	2.64	0.0047
*NAME*TREAT*TIME*		cold	2	11	118	4.94	<.0001
*NAME*TREAT*TIME*		cold	3	11	118	7.41	<.0001
*NAME*TREAT*TIME*		cold	4	11	118	10.05	<.0001
*NAME*TREAT*TIME*		cold	5	11	118	11.85	<.0001
*NAME*TREAT*TIME*		cold	6	11	118	10.97	<.0001
*NAME*TREAT*TIME*		cold	7	11	118	11.04	<.0001

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*		cold	8	11	118	10.92	<.0001
*NAME*TREAT*TIME*		control	1	11	118	1.95	0.0399
*NAME*TREAT*TIME*		control	2	11	118	5.19	<.0001
*NAME*TREAT*TIME*		control	3	11	118	4.42	<.0001
*NAME*TREAT*TIME*		control	4	11	118	4.28	<.0001
*NAME*TREAT*TIME*		control	5	11	118	4.54	<.0001
*NAME*TREAT*TIME*		control	6	11	118	3.34	0.0005
*NAME*TREAT*TIME*		control	7	11	118	2.52	0.0069
*NAME*TREAT*TIME*		control	8	11	118	1.89	0.0476

Model Information	
Data Set	WORK.COLD
Dependent Variable	logshoot
Covariance Structure	Unstructured
Subject Effect	REP*NAME*TREAT*EXP
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	*Levels*	*Values*
REP	2	1 2
NAME	12	ALGANS CLARITI CODIFAR CODISCO CRAZI FERGUS HUSKI JUSTINA LAKTI P329D60 PICKER PR29B29
TREAT	2	cold control
EXP	3	1 2 3
TIME	8	1 2 3 4 5 6 7 8

Dimensions	
Covariance Parameters	36
Columns in X	354
Columns in Z	0
Subjects	144
Max Obs Per Subject	8

Number of Observations	
Number of Observations Read	1152
Number of Observations Used	1152
Number of Observations Not Used	0

Iteration History			
Iteration	*Evaluations*	*-2 Res Log Like*	*Criterion*
0	1	590.09044887	
1	2	-661.25426832	0.00026940
2	1	-661.60673864	0.00000510
3	1	-661.61300458	0.00000000

Convergence criteria met.

*Estimated R Correlation Matrix for REP*NAME*TREAT*EXP 1 ALGANS cold 1*								
Row	*Col1*	*Col2*	*Col3*	*Col4*	*Col5*	*Col6*	*Col7*	*Col8*
1	1.0000	0.3693	0.3308	0.1593	0.1027	0.09491	0.1390	0.05840
2	0.3693	1.0000	0.7658	0.6194	0.4914	0.4406	0.3790	0.3628
3	0.3308	0.7658	1.0000	0.8095	0.6710	0.6023	0.5836	0.5011
4	0.1593	0.6194	0.8095	1.0000	0.8538	0.7898	0.7102	0.6393
5	0.1027	0.4914	0.6710	0.8538	1.0000	0.9283	0.8616	0.7533
6	0.09491	0.4406	0.6023	0.7898	0.9283	1.0000	0.9537	0.8722
7	0.1390	0.3790	0.5836	0.7102	0.8616	0.9537	1.0000	0.9126
8	0.05840	0.3628	0.5011	0.6393	0.7533	0.8722	0.9126	1.0000

Covariance Parameter Estimates		
Cov Parm	*Subject*	*Estimate*
UN(1,1)	REP*NAME*TREAT*EXP	0.02368
UN(2,1)	REP*NAME*TREAT*EXP	0.01373
UN(2,2)	REP*NAME*TREAT*EXP	0.05840
UN(3,1)	REP*NAME*TREAT*EXP	0.01562
UN(3,2)	REP*NAME*TREAT*EXP	0.05678
UN(3,3)	REP*NAME*TREAT*EXP	0.09412
UN(4,1)	REP*NAME*TREAT*EXP	0.007566
UN(4,2)	REP*NAME*TREAT*EXP	0.04620
UN(4,3)	REP*NAME*TREAT*EXP	0.07665
UN(4,4)	REP*NAME*TREAT*EXP	0.09526
UN(5,1)	REP*NAME*TREAT*EXP	0.004374
UN(5,2)	REP*NAME*TREAT*EXP	0.03286
UN(5,3)	REP*NAME*TREAT*EXP	0.05697

Covariance Parameter Estimates		
Cov Parm	*Subject*	*Estimate*
UN(5,4)	REP*NAME*TREAT*EXP	0.07292
UN(5,5)	REP*NAME*TREAT*EXP	0.07657
UN(6,1)	REP*NAME*TREAT*EXP	0.004164
UN(6,2)	REP*NAME*TREAT*EXP	0.03036
UN(6,3)	REP*NAME*TREAT*EXP	0.05268
UN(6,4)	REP*NAME*TREAT*EXP	0.06950
UN(6,5)	REP*NAME*TREAT*EXP	0.07324
UN(6,6)	REP*NAME*TREAT*EXP	0.08129
UN(7,1)	REP*NAME*TREAT*EXP	0.006400
UN(7,2)	REP*NAME*TREAT*EXP	0.02741
UN(7,3)	REP*NAME*TREAT*EXP	0.05359
UN(7,4)	REP*NAME*TREAT*EXP	0.06561
UN(7,5)	REP*NAME*TREAT*EXP	0.07136
UN(7,6)	REP*NAME*TREAT*EXP	0.08138
UN(7,7)	REP*NAME*TREAT*EXP	0.08958
UN(8,1)	REP*NAME*TREAT*EXP	0.002781
UN(8,2)	REP*NAME*TREAT*EXP	0.02713
UN(8,3)	REP*NAME*TREAT*EXP	0.04758
UN(8,4)	REP*NAME*TREAT*EXP	0.06107
UN(8,5)	REP*NAME*TREAT*EXP	0.06451
UN(8,6)	REP*NAME*TREAT*EXP	0.07697
UN(8,7)	REP*NAME*TREAT*EXP	0.08454
UN(8,8)	REP*NAME*TREAT*EXP	0.09579

Fit Statistics	
-2 Res Log Likelihood	-661.6
AIC (smaller is better)	-589.6
AICC (smaller is better)	-586.7
BIC (smaller is better)	-482.7

Null Model Likelihood Ratio Test		
DF	*Chi-Square*	*Pr > ChiSq*
35	1251.70	<.0001

Type 3 Tests of Fixed Effects

Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
EXP	2	118	3.24	0.0426
NAME	11	118	5.52	<.0001
TREAT	1	118	446.64	<.0001
*NAME*TREAT*	11	118	4.76	<.0001
TIME	7	118	491.43	<.0001
*NAME*TIME*	77	118	3.07	<.0001
*TREAT*TIME*	7	118	88.93	<.0001
*NAME*TREAT*TIME*	77	118	2.83	<.0001

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	ALGANS	cold	1	0.04458	0.06282	118	0.71	0.4793
*NAME*TREAT*TIME*	ALGANS	cold	2	0.1008	0.09865	118	1.02	0.3092
*NAME*TREAT*TIME*	ALGANS	cold	3	0.3119	0.1252	118	2.49	0.0142
*NAME*TREAT*TIME*	ALGANS	cold	4	0.4014	0.1260	118	3.19	0.0018
*NAME*TREAT*TIME*	ALGANS	cold	5	0.4498	0.1130	118	3.98	0.0001
*NAME*TREAT*TIME*	ALGANS	cold	6	0.6539	0.1164	118	5.62	<.0001
*NAME*TREAT*TIME*	ALGANS	cold	7	0.7340	0.1222	118	6.01	<.0001
*NAME*TREAT*TIME*	ALGANS	cold	8	0.8910	0.1263	118	7.05	<.0001
*NAME*TREAT*TIME*	ALGANS	control	1	0.2339	0.06282	118	3.72	0.0003
*NAME*TREAT*TIME*	ALGANS	control	2	0.4909	0.09865	118	4.98	<.0001
*NAME*TREAT*TIME*	ALGANS	control	3	1.1960	0.1252	118	9.55	<.0001
*NAME*TREAT*TIME*	ALGANS	control	4	1.4857	0.1260	118	11.79	<.0001
*NAME*TREAT*TIME*	ALGANS	control	5	1.7017	0.1130	118	15.06	<.0001
*NAME*TREAT*TIME*	ALGANS	control	6	1.9286	0.1164	118	16.57	<.0001
*NAME*TREAT*TIME*	ALGANS	control	7	2.1247	0.1222	118	17.39	<.0001
*NAME*TREAT*TIME*	ALGANS	control	8	2.3334	0.1263	118	18.47	<.0001
*NAME*TREAT*TIME*	CLARITI	cold	1	0.1259	0.06282	118	2.00	0.0474
*NAME*TREAT*TIME*	CLARITI	cold	2	0.2035	0.09865	118	2.06	0.0413
*NAME*TREAT*TIME*	CLARITI	cold	3	0.3397	0.1252	118	2.71	0.0077
*NAME*TREAT*TIME*	CLARITI	cold	4	0.6565	0.1260	118	5.21	<.0001
*NAME*TREAT*TIME*	CLARITI	cold	5	0.7935	0.1130	118	7.02	<.0001
*NAME*TREAT*TIME*	CLARITI	cold	6	0.9363	0.1164	118	8.04	<.0001

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	CLARITI	cold	7	1.0336	0.1222	118	8.46	<.0001
*NAME*TREAT*TIME*	CLARITI	cold	8	1.2066	0.1263	118	9.55	<.0001
*NAME*TREAT*TIME*	CLARITI	control	1	0.06683	0.06282	118	1.06	0.2896
*NAME*TREAT*TIME*	CLARITI	control	2	0.7893	0.09865	118	8.00	<.0001
*NAME*TREAT*TIME*	CLARITI	control	3	1.0675	0.1252	118	8.52	<.0001
*NAME*TREAT*TIME*	CLARITI	control	4	1.4646	0.1260	118	11.62	<.0001
*NAME*TREAT*TIME*	CLARITI	control	5	1.7040	0.1130	118	15.08	<.0001
*NAME*TREAT*TIME*	CLARITI	control	6	1.8675	0.1164	118	16.04	<.0001
*NAME*TREAT*TIME*	CLARITI	control	7	2.1038	0.1222	118	17.22	<.0001
*NAME*TREAT*TIME*	CLARITI	control	8	2.2773	0.1263	118	18.02	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	1	0.03466	0.06282	118	0.55	0.5821
*NAME*TREAT*TIME*	CODIFAR	cold	2	0.2034	0.09865	118	2.06	0.0414
*NAME*TREAT*TIME*	CODIFAR	cold	3	0.3743	0.1252	118	2.99	0.0034
*NAME*TREAT*TIME*	CODIFAR	cold	4	0.7452	0.1260	118	5.91	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	5	0.9873	0.1130	118	8.74	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	6	1.0657	0.1164	118	9.16	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	7	1.1236	0.1222	118	9.20	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold	8	1.2352	0.1263	118	9.78	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	1	-174E-17	0.06282	118	-0.00	1.0000
*NAME*TREAT*TIME*	CODIFAR	control	2	0.3242	0.09865	118	3.29	0.0013
*NAME*TREAT*TIME*	CODIFAR	control	3	0.3641	0.1252	118	2.91	0.0044
*NAME*TREAT*TIME*	CODIFAR	control	4	0.7754	0.1260	118	6.15	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	5	1.2717	0.1130	118	11.26	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	6	1.7003	0.1164	118	14.61	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	7	2.0872	0.1222	118	17.08	<.0001
*NAME*TREAT*TIME*	CODIFAR	control	8	2.3549	0.1263	118	18.64	<.0001
*NAME*TREAT*TIME*	CODISCO	cold	1	0.06617	0.06282	118	1.05	0.2944
*NAME*TREAT*TIME*	CODISCO	cold	2	0.1709	0.09865	118	1.73	0.0858
*NAME*TREAT*TIME*	CODISCO	cold	3	0.2179	0.1252	118	1.74	0.0846
*NAME*TREAT*TIME*	CODISCO	cold	4	0.3079	0.1260	118	2.44	0.0160
*NAME*TREAT*TIME*	CODISCO	cold	5	0.4430	0.1130	118	3.92	0.0001
*NAME*TREAT*TIME*	CODISCO	cold	6	0.5447	0.1164	118	4.68	<.0001
*NAME*TREAT*TIME*	CODISCO	cold	7	0.6006	0.1222	118	4.92	<.0001
*NAME*TREAT*TIME*	CODISCO	cold	8	0.7814	0.1263	118	6.18	<.0001
*NAME*TREAT*TIME*	CODISCO	control	1	0.2525	0.06282	118	4.02	0.0001
*NAME*TREAT*TIME*	CODISCO	control	2	0.7927	0.09865	118	8.04	<.0001

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	CODISCO	control	3	1.1029	0.1252	118	8.81	<.0001
*NAME*TREAT*TIME*	CODISCO	control	4	1.3937	0.1260	118	11.06	<.0001
*NAME*TREAT*TIME*	CODISCO	control	5	1.5894	0.1130	118	14.07	<.0001
*NAME*TREAT*TIME*	CODISCO	control	6	1.7238	0.1164	118	14.81	<.0001
*NAME*TREAT*TIME*	CODISCO	control	7	1.8750	0.1222	118	15.35	<.0001
*NAME*TREAT*TIME*	CODISCO	control	8	2.0834	0.1263	118	16.49	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	1	0.4406	0.06282	118	7.01	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	2	0.7552	0.09865	118	7.66	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	3	1.0172	0.1252	118	8.12	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	4	1.1450	0.1260	118	9.09	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	5	1.1988	0.1130	118	10.61	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	6	1.2572	0.1164	118	10.80	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	7	1.3305	0.1222	118	10.89	<.0001
*NAME*TREAT*TIME*	CRAZI	cold	8	1.4315	0.1263	118	11.33	<.0001
*NAME*TREAT*TIME*	CRAZI	control	1	0.4011	0.06282	118	6.39	<.0001
*NAME*TREAT*TIME*	CRAZI	control	2	0.7821	0.09865	118	7.93	<.0001
*NAME*TREAT*TIME*	CRAZI	control	3	0.9831	0.1252	118	7.85	<.0001
*NAME*TREAT*TIME*	CRAZI	control	4	1.4125	0.1260	118	11.21	<.0001
*NAME*TREAT*TIME*	CRAZI	control	5	1.7670	0.1130	118	15.64	<.0001
*NAME*TREAT*TIME*	CRAZI	control	6	1.9609	0.1164	118	16.85	<.0001
*NAME*TREAT*TIME*	CRAZI	control	7	2.1594	0.1222	118	17.67	<.0001
*NAME*TREAT*TIME*	CRAZI	control	8	2.3631	0.1263	118	18.70	<.0001
*NAME*TREAT*TIME*	FERGUS	cold	1	0.03450	0.06282	118	0.55	0.5839
*NAME*TREAT*TIME*	FERGUS	cold	2	0.07377	0.09865	118	0.75	0.4561
*NAME*TREAT*TIME*	FERGUS	cold	3	0.05608	0.1252	118	0.45	0.6552
*NAME*TREAT*TIME*	FERGUS	cold	4	0.1796	0.1260	118	1.43	0.1567
*NAME*TREAT*TIME*	FERGUS	cold	5	0.3625	0.1130	118	3.21	0.0017
*NAME*TREAT*TIME*	FERGUS	cold	6	0.4740	0.1164	118	4.07	<.0001
*NAME*TREAT*TIME*	FERGUS	cold	7	0.5315	0.1222	118	4.35	<.0001
*NAME*TREAT*TIME*	FERGUS	cold	8	0.7962	0.1263	118	6.30	<.0001
*NAME*TREAT*TIME*	FERGUS	control	1	0.03155	0.06282	118	0.50	0.6165
*NAME*TREAT*TIME*	FERGUS	control	2	0.6925	0.09865	118	7.02	<.0001
*NAME*TREAT*TIME*	FERGUS	control	3	1.0035	0.1252	118	8.01	<.0001
*NAME*TREAT*TIME*	FERGUS	control	4	1.7515	0.1260	118	13.90	<.0001
*NAME*TREAT*TIME*	FERGUS	control	5	2.1201	0.1130	118	18.77	<.0001
*NAME*TREAT*TIME*	FERGUS	control	6	2.3738	0.1164	118	20.39	<.0001

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	FERGUS	control	7	2.5901	0.1222	118	21.20	<.0001
*NAME*TREAT*TIME*	FERGUS	control	8	2.7998	0.1263	118	22.16	<.0001
*NAME*TREAT*TIME*	HUSKI	cold	1	0.03165	0.06282	118	0.50	0.6154
*NAME*TREAT*TIME*	HUSKI	cold	2	0.1033	0.09865	118	1.05	0.2973
*NAME*TREAT*TIME*	HUSKI	cold	3	0.3933	0.1252	118	3.14	0.0021
*NAME*TREAT*TIME*	HUSKI	cold	4	0.4738	0.1260	118	3.76	0.0003
*NAME*TREAT*TIME*	HUSKI	cold	5	0.6197	0.1130	118	5.49	<.0001
*NAME*TREAT*TIME*	HUSKI	cold	6	0.6983	0.1164	118	6.00	<.0001
*NAME*TREAT*TIME*	HUSKI	cold	7	0.7651	0.1222	118	6.26	<.0001
*NAME*TREAT*TIME*	HUSKI	cold	8	1.1360	0.1263	118	8.99	<.0001
*NAME*TREAT*TIME*	HUSKI	control	1	0.09491	0.06282	118	1.51	0.1335
*NAME*TREAT*TIME*	HUSKI	control	2	0.6101	0.09865	118	6.18	<.0001
*NAME*TREAT*TIME*	HUSKI	control	3	0.8781	0.1252	118	7.01	<.0001
*NAME*TREAT*TIME*	HUSKI	control	4	1.3141	0.1260	118	10.43	<.0001
*NAME*TREAT*TIME*	HUSKI	control	5	1.6582	0.1130	118	14.68	<.0001
*NAME*TREAT*TIME*	HUSKI	control	6	1.8935	0.1164	118	16.27	<.0001
*NAME*TREAT*TIME*	HUSKI	control	7	2.0973	0.1222	118	17.16	<.0001
*NAME*TREAT*TIME*	HUSKI	control	8	2.3709	0.1263	118	18.76	<.0001
*NAME*TREAT*TIME*	JUSTINA	cold	1	0.05002	0.06282	118	0.80	0.4275
*NAME*TREAT*TIME*	JUSTINA	cold	2	0.01889	0.09865	118	0.19	0.8485
*NAME*TREAT*TIME*	JUSTINA	cold	3	0.2114	0.1252	118	1.69	0.0940
*NAME*TREAT*TIME*	JUSTINA	cold	4	0.2190	0.1260	118	1.74	0.0848
*NAME*TREAT*TIME*	JUSTINA	cold	5	0.2820	0.1130	118	2.50	0.0139
*NAME*TREAT*TIME*	JUSTINA	cold	6	0.2990	0.1164	118	2.57	0.0114
*NAME*TREAT*TIME*	JUSTINA	cold	7	0.4171	0.1222	118	3.41	0.0009
*NAME*TREAT*TIME*	JUSTINA	cold	8	0.4723	0.1263	118	3.74	0.0003
*NAME*TREAT*TIME*	JUSTINA	control	1	0.1919	0.06282	118	3.06	0.0028
*NAME*TREAT*TIME*	JUSTINA	control	2	0.1328	0.09865	118	1.35	0.1808
*NAME*TREAT*TIME*	JUSTINA	control	3	0.6762	0.1252	118	5.40	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	4	0.9988	0.1260	118	7.93	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	5	1.2325	0.1130	118	10.91	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	6	1.4625	0.1164	118	12.56	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	7	1.7048	0.1222	118	13.95	<.0001
*NAME*TREAT*TIME*	JUSTINA	control	8	2.0504	0.1263	118	16.23	<.0001
*NAME*TREAT*TIME*	LAKTI	cold	1	0.01889	0.06282	118	0.30	0.7642
*NAME*TREAT*TIME*	LAKTI	cold	2	0.1519	0.09865	118	1.54	0.1262

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	LAKTI	cold	3	0.2057	0.1252	118	1.64	0.1032
*NAME*TREAT*TIME*	LAKTI	cold	4	0.3319	0.1260	118	2.63	0.0096
*NAME*TREAT*TIME*	LAKTI	cold	5	0.3947	0.1130	118	3.49	0.0007
*NAME*TREAT*TIME*	LAKTI	cold	6	0.5702	0.1164	118	4.90	<.0001
*NAME*TREAT*TIME*	LAKTI	cold	7	0.6547	0.1222	118	5.36	<.0001
*NAME*TREAT*TIME*	LAKTI	cold	8	0.9752	0.1263	118	7.72	<.0001
*NAME*TREAT*TIME*	LAKTI	control	1	0.04511	0.06282	118	0.72	0.4742
*NAME*TREAT*TIME*	LAKTI	control	2	0.3124	0.09865	118	3.17	0.0020
*NAME*TREAT*TIME*	LAKTI	control	3	0.5986	0.1252	118	4.78	<.0001
*NAME*TREAT*TIME*	LAKTI	control	4	1.2252	0.1260	118	9.72	<.0001
*NAME*TREAT*TIME*	LAKTI	control	5	1.7668	0.1130	118	15.64	<.0001
*NAME*TREAT*TIME*	LAKTI	control	6	2.1450	0.1164	118	18.43	<.0001
*NAME*TREAT*TIME*	LAKTI	control	7	2.4523	0.1222	118	20.07	<.0001
*NAME*TREAT*TIME*	LAKTI	control	8	2.6631	0.1263	118	21.08	<.0001
*NAME*TREAT*TIME*	P329D60	cold	1	-333E-18	0.06282	118	-0.00	1.0000
*NAME*TREAT*TIME*	P329D60	cold	2	-333E-18	0.09865	118	-0.00	1.0000
*NAME*TREAT*TIME*	P329D60	cold	3	0.3346	0.1252	118	2.67	0.0086
*NAME*TREAT*TIME*	P329D60	cold	4	0.3788	0.1260	118	3.01	0.0032
*NAME*TREAT*TIME*	P329D60	cold	5	0.4581	0.1130	118	4.06	<.0001
*NAME*TREAT*TIME*	P329D60	cold	6	0.5626	0.1164	118	4.83	<.0001
*NAME*TREAT*TIME*	P329D60	cold	7	0.7095	0.1222	118	5.81	<.0001
*NAME*TREAT*TIME*	P329D60	cold	8	0.7615	0.1263	118	6.03	<.0001
*NAME*TREAT*TIME*	P329D60	control	1	-666E-18	0.06282	118	-0.00	1.0000
*NAME*TREAT*TIME*	P329D60	control	2	0.1910	0.09865	118	1.94	0.0553
*NAME*TREAT*TIME*	P329D60	control	3	0.6381	0.1252	118	5.09	<.0001
*NAME*TREAT*TIME*	P329D60	control	4	1.0864	0.1260	118	8.62	<.0001
*NAME*TREAT*TIME*	P329D60	control	5	1.5640	0.1130	118	13.84	<.0001
*NAME*TREAT*TIME*	P329D60	control	6	1.8184	0.1164	118	15.62	<.0001
*NAME*TREAT*TIME*	P329D60	control	7	2.1497	0.1222	118	17.59	<.0001
*NAME*TREAT*TIME*	P329D60	control	8	2.3900	0.1263	118	18.92	<.0001
*NAME*TREAT*TIME*	PICKER	cold	1	0.09411	0.06282	118	1.50	0.1368
*NAME*TREAT*TIME*	PICKER	cold	2	0.09783	0.09865	118	0.99	0.3234
*NAME*TREAT*TIME*	PICKER	cold	3	0.6649	0.1252	118	5.31	<.0001
*NAME*TREAT*TIME*	PICKER	cold	4	0.7623	0.1260	118	6.05	<.0001
*NAME*TREAT*TIME*	PICKER	cold	5	0.9946	0.1130	118	8.80	<.0001
*NAME*TREAT*TIME*	PICKER	cold	6	1.0472	0.1164	118	9.00	<.0001

Least Squares Means

Effect	*NAME*	*TREAT*	*TIME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
*NAME*TREAT*TIME*	PICKER	cold	7	1.1851	0.1222	118	9.70	<.0001
*NAME*TREAT*TIME*	PICKER	cold	8	1.3197	0.1263	118	10.45	<.0001
*NAME*TREAT*TIME*	PICKER	control	1	0.1380	0.06282	118	2.20	0.0300
*NAME*TREAT*TIME*	PICKER	control	2	0.6543	0.09865	118	6.63	<.0001
*NAME*TREAT*TIME*	PICKER	control	3	0.9742	0.1252	118	7.78	<.0001
*NAME*TREAT*TIME*	PICKER	control	4	1.4928	0.1260	118	11.85	<.0001
*NAME*TREAT*TIME*	PICKER	control	5	1.7535	0.1130	118	15.52	<.0001
*NAME*TREAT*TIME*	PICKER	control	6	1.9066	0.1164	118	16.38	<.0001
*NAME*TREAT*TIME*	PICKER	control	7	2.0862	0.1222	118	17.07	<.0001
*NAME*TREAT*TIME*	PICKER	control	8	2.2469	0.1263	118	17.78	<.0001
*NAME*TREAT*TIME*	PR29B29	cold	1	0.1145	0.06282	118	1.82	0.0709
*NAME*TREAT*TIME*	PR29B29	cold	2	0.2940	0.09865	118	2.98	0.0035
*NAME*TREAT*TIME*	PR29B29	cold	3	0.3855	0.1252	118	3.08	0.0026
*NAME*TREAT*TIME*	PR29B29	cold	4	0.5975	0.1260	118	4.74	<.0001
*NAME*TREAT*TIME*	PR29B29	cold	5	0.7791	0.1130	118	6.90	<.0001
*NAME*TREAT*TIME*	PR29B29	cold	6	1.0847	0.1164	118	9.32	<.0001
*NAME*TREAT*TIME*	PR29B29	cold	7	1.1538	0.1222	118	9.44	<.0001
*NAME*TREAT*TIME*	PR29B29	cold	8	1.3108	0.1263	118	10.37	<.0001
*NAME*TREAT*TIME*	PR29B29	control	1	0.01128	0.06282	118	0.18	0.8579
*NAME*TREAT*TIME*	PR29B29	control	2	0.3566	0.09865	118	3.61	0.0004
*NAME*TREAT*TIME*	PR29B29	control	3	0.8103	0.1252	118	6.47	<.0001
*NAME*TREAT*TIME*	PR29B29	control	4	1.1885	0.1260	118	9.43	<.0001
*NAME*TREAT*TIME*	PR29B29	control	5	1.3806	0.1130	118	12.22	<.0001
*NAME*TREAT*TIME*	PR29B29	control	6	1.6616	0.1164	118	14.27	<.0001
*NAME*TREAT*TIME*	PR29B29	control	7	1.9037	0.1222	118	15.58	<.0001
*NAME*TREAT*TIME*	PR29B29	control	8	2.1619	0.1263	118	17.11	<.0001

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*	ALGANS			15	118	23.03	<.0001
*NAME*TREAT*TIME*	CLARITI			15	118	24.47	<.0001
*NAME*TREAT*TIME*	CODIFAR			15	118	33.03	<.0001
*NAME*TREAT*TIME*	CODISCO			15	118	16.32	<.0001
*NAME*TREAT*TIME*	CRAZI			15	118	19.25	<.0001

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*	FERGUS			15	118	34.74	<.0001
*NAME*TREAT*TIME*	HUSKI			15	118	27.64	<.0001
*NAME*TREAT*TIME*	JUSTINA			15	118	19.44	<.0001
*NAME*TREAT*TIME*	LAKTI			15	118	35.12	<.0001
*NAME*TREAT*TIME*	P329D60			15	118	28.95	<.0001
*NAME*TREAT*TIME*	PICKER			15	118	26.27	<.0001
*NAME*TREAT*TIME*	PR29B29			15	118	24.11	<.0001
*NAME*TREAT*TIME*		cold		95	118	9.43	<.0001
*NAME*TREAT*TIME*		control		95	118	39.42	<.0001
*NAME*TREAT*TIME*			1	23	118	3.65	<.0001
*NAME*TREAT*TIME*			2	23	118	7.70	<.0001
*NAME*TREAT*TIME*			3	23	118	7.60	<.0001
*NAME*TREAT*TIME*			4	23	118	14.45	<.0001
*NAME*TREAT*TIME*			5	23	118	25.15	<.0001
*NAME*TREAT*TIME*			6	23	118	28.59	<.0001
*NAME*TREAT*TIME*			7	23	118	32.28	<.0001
*NAME*TREAT*TIME*			8	23	118	32.18	<.0001
*NAME*TREAT*TIME*	ALGANS		1	1	118	4.54	0.0351
*NAME*TREAT*TIME*	ALGANS		2	1	118	7.82	0.0060
*NAME*TREAT*TIME*	ALGANS		3	1	118	24.91	<.0001
*NAME*TREAT*TIME*	ALGANS		4	1	118	37.02	<.0001
*NAME*TREAT*TIME*	ALGANS		5	1	118	61.40	<.0001
*NAME*TREAT*TIME*	ALGANS		6	1	118	59.96	<.0001
*NAME*TREAT*TIME*	ALGANS		7	1	118	64.77	<.0001
*NAME*TREAT*TIME*	ALGANS		8	1	118	65.16	<.0001
*NAME*TREAT*TIME*	CLARITI		1	1	118	0.44	0.5076
*NAME*TREAT*TIME*	CLARITI		2	1	118	17.63	<.0001
*NAME*TREAT*TIME*	CLARITI		3	1	118	16.88	<.0001
*NAME*TREAT*TIME*	CLARITI		4	1	118	20.57	<.0001
*NAME*TREAT*TIME*	CLARITI		5	1	118	32.48	<.0001
*NAME*TREAT*TIME*	CLARITI		6	1	118	32.01	<.0001
*NAME*TREAT*TIME*	CLARITI		7	1	118	38.36	<.0001
*NAME*TREAT*TIME*	CLARITI		8	1	118	35.90	<.0001
*NAME*TREAT*TIME*	CODIFAR		1	1	118	0.15	0.6971
*NAME*TREAT*TIME*	CODIFAR		2	1	118	0.75	0.3886
*NAME*TREAT*TIME*	CODIFAR		3	1	118	0.00	0.9541

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*	CODIFAR		4	1	118	0.03	0.8655
*NAME*TREAT*TIME*	CODIFAR		5	1	118	3.17	0.0776
*NAME*TREAT*TIME*	CODIFAR		6	1	118	14.86	0.0002
*NAME*TREAT*TIME*	CODIFAR		7	1	118	31.09	<.0001
*NAME*TREAT*TIME*	CODIFAR		8	1	118	39.27	<.0001
*NAME*TREAT*TIME*	CODISCO		1	1	118	4.40	0.0382
*NAME*TREAT*TIME*	CODISCO		2	1	118	19.86	<.0001
*NAME*TREAT*TIME*	CODISCO		3	1	118	24.97	<.0001
*NAME*TREAT*TIME*	CODISCO		4	1	118	37.13	<.0001
*NAME*TREAT*TIME*	CODISCO		5	1	118	51.49	<.0001
*NAME*TREAT*TIME*	CODISCO		6	1	118	51.31	<.0001
*NAME*TREAT*TIME*	CODISCO		7	1	118	54.39	<.0001
*NAME*TREAT*TIME*	CODISCO		8	1	118	53.09	<.0001
*NAME*TREAT*TIME*	CRAZI		1	1	118	0.20	0.6577
*NAME*TREAT*TIME*	CRAZI		2	1	118	0.04	0.8477
*NAME*TREAT*TIME*	CRAZI		3	1	118	0.04	0.8475
*NAME*TREAT*TIME*	CRAZI		4	1	118	2.25	0.1361
*NAME*TREAT*TIME*	CRAZI		5	1	118	12.65	0.0005
*NAME*TREAT*TIME*	CRAZI		6	1	118	18.28	<.0001
*NAME*TREAT*TIME*	CRAZI		7	1	118	23.01	<.0001
*NAME*TREAT*TIME*	CRAZI		8	1	118	27.19	<.0001
*NAME*TREAT*TIME*	FERGUS		1	1	118	0.00	0.9735
*NAME*TREAT*TIME*	FERGUS		2	1	118	19.67	<.0001
*NAME*TREAT*TIME*	FERGUS		3	1	118	28.61	<.0001
*NAME*TREAT*TIME*	FERGUS		4	1	118	77.81	<.0001
*NAME*TREAT*TIME*	FERGUS		5	1	118	121.03	<.0001
*NAME*TREAT*TIME*	FERGUS		6	1	118	133.19	<.0001
*NAME*TREAT*TIME*	FERGUS		7	1	118	141.92	<.0001
*NAME*TREAT*TIME*	FERGUS		8	1	118	125.74	<.0001
*NAME*TREAT*TIME*	HUSKI		1	1	118	0.51	0.4778
*NAME*TREAT*TIME*	HUSKI		2	1	118	13.20	0.0004
*NAME*TREAT*TIME*	HUSKI		3	1	118	7.49	0.0072
*NAME*TREAT*TIME*	HUSKI		4	1	118	22.24	<.0001
*NAME*TREAT*TIME*	HUSKI		5	1	118	42.26	<.0001
*NAME*TREAT*TIME*	HUSKI		6	1	118	52.72	<.0001
*NAME*TREAT*TIME*	HUSKI		7	1	118	59.44	<.0001

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*	HUSKI		8	1	118	47.76	<.0001
*NAME*TREAT*TIME*	JUSTINA		1	1	118	2.55	0.1129
*NAME*TREAT*TIME*	JUSTINA		2	1	118	0.67	0.4158
*NAME*TREAT*TIME*	JUSTINA		3	1	118	6.89	0.0098
*NAME*TREAT*TIME*	JUSTINA		4	1	118	19.15	<.0001
*NAME*TREAT*TIME*	JUSTINA		5	1	118	35.40	<.0001
*NAME*TREAT*TIME*	JUSTINA		6	1	118	49.96	<.0001
*NAME*TREAT*TIME*	JUSTINA		7	1	118	55.53	<.0001
*NAME*TREAT*TIME*	JUSTINA		8	1	118	78.00	<.0001
*NAME*TREAT*TIME*	LAKTI		1	1	118	0.09	0.7684
*NAME*TREAT*TIME*	LAKTI		2	1	118	1.32	0.2524
*NAME*TREAT*TIME*	LAKTI		3	1	118	4.92	0.0284
*NAME*TREAT*TIME*	LAKTI		4	1	118	25.13	<.0001
*NAME*TREAT*TIME*	LAKTI		5	1	118	73.76	<.0001
*NAME*TREAT*TIME*	LAKTI		6	1	118	91.52	<.0001
*NAME*TREAT*TIME*	LAKTI		7	1	118	108.22	<.0001
*NAME*TREAT*TIME*	LAKTI		8	1	118	89.23	<.0001
*NAME*TREAT*TIME*	P329D60		1	1	118	0.00	1.0000
*NAME*TREAT*TIME*	P329D60		2	1	118	1.87	0.1737
*NAME*TREAT*TIME*	P329D60		3	1	118	2.94	0.0892
*NAME*TREAT*TIME*	P329D60		4	1	118	15.77	0.0001
*NAME*TREAT*TIME*	P329D60		5	1	118	47.91	<.0001
*NAME*TREAT*TIME*	P329D60		6	1	118	58.20	<.0001
*NAME*TREAT*TIME*	P329D60		7	1	118	69.46	<.0001
*NAME*TREAT*TIME*	P329D60		8	1	118	83.05	<.0001
*NAME*TREAT*TIME*	PICKER		1	1	118	0.24	0.6220
*NAME*TREAT*TIME*	PICKER		2	1	118	15.91	0.0001
*NAME*TREAT*TIME*	PICKER		3	1	118	3.05	0.0834
*NAME*TREAT*TIME*	PICKER		4	1	118	16.80	<.0001
*NAME*TREAT*TIME*	PICKER		5	1	118	22.57	<.0001
*NAME*TREAT*TIME*	PICKER		6	1	118	27.26	<.0001
*NAME*TREAT*TIME*	PICKER		7	1	118	27.19	<.0001
*NAME*TREAT*TIME*	PICKER		8	1	118	26.93	<.0001
*NAME*TREAT*TIME*	PR29B29		1	1	118	1.35	0.2477
*NAME*TREAT*TIME*	PR29B29		2	1	118	0.20	0.6546
*NAME*TREAT*TIME*	PR29B29		3	1	118	5.75	0.0180

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*	PR29B29		4	1	118	11.00	0.0012
*NAME*TREAT*TIME*	PR29B29		5	1	118	14.17	0.0003
*NAME*TREAT*TIME*	PR29B29		6	1	118	12.28	0.0006
*NAME*TREAT*TIME*	PR29B29		7	1	118	18.83	<.0001
*NAME*TREAT*TIME*	PR29B29		8	1	118	22.69	<.0001
*NAME*TREAT*TIME*	ALGANS	cold		7	118	7.22	<.0001
*NAME*TREAT*TIME*	ALGANS	control		7	118	38.32	<.0001
*NAME*TREAT*TIME*	CLARITI	cold		7	118	10.34	<.0001
*NAME*TREAT*TIME*	CLARITI	control		7	118	41.57	<.0001
*NAME*TREAT*TIME*	CODIFAR	cold		7	118	13.00	<.0001
*NAME*TREAT*TIME*	CODIFAR	control		7	118	57.58	<.0001
*NAME*TREAT*TIME*	CODISCO	cold		7	118	4.85	<.0001
*NAME*TREAT*TIME*	CODISCO	control		7	118	26.46	<.0001
*NAME*TREAT*TIME*	CRAZI	cold		7	118	7.89	<.0001
*NAME*TREAT*TIME*	CRAZI	control		7	118	32.79	<.0001
*NAME*TREAT*TIME*	FERGUS	cold		7	118	7.26	<.0001
*NAME*TREAT*TIME*	FERGUS	control		7	118	63.72	<.0001
*NAME*TREAT*TIME*	HUSKI	cold		7	118	15.48	<.0001
*NAME*TREAT*TIME*	HUSKI	control		7	118	41.72	<.0001
*NAME*TREAT*TIME*	JUSTINA	cold		7	118	2.93	0.0073
*NAME*TREAT*TIME*	JUSTINA	control		7	118	35.45	<.0001
*NAME*TREAT*TIME*	LAKTI	cold		7	118	9.86	<.0001
*NAME*TREAT*TIME*	LAKTI	control		7	118	62.93	<.0001
*NAME*TREAT*TIME*	P329D60	cold		7	118	6.67	<.0001
*NAME*TREAT*TIME*	P329D60	control		7	118	53.01	<.0001
*NAME*TREAT*TIME*	PICKER	cold		7	118	17.84	<.0001
*NAME*TREAT*TIME*	PICKER	control		7	118	36.79	<.0001
*NAME*TREAT*TIME*	PR29B29	cold		7	118	13.90	<.0001
*NAME*TREAT*TIME*	PR29B29	control		7	118	37.73	<.0001
*NAME*TREAT*TIME*		cold	1	11	118	3.49	0.0003
*NAME*TREAT*TIME*		cold	2	11	118	4.06	<.0001
*NAME*TREAT*TIME*		cold	3	11	118	3.97	<.0001
*NAME*TREAT*TIME*		cold	4	11	118	4.85	<.0001
*NAME*TREAT*TIME*		cold	5	11	118	6.94	<.0001
*NAME*TREAT*TIME*		cold	6	11	118	6.64	<.0001
*NAME*TREAT*TIME*		cold	7	11	118	5.93	<.0001

Tests of Effect Slices

Effect	*NAME*	*TREAT*	*TIME*	*Num DF*	*Den DF*	*F Value*	*Pr > F*
*NAME*TREAT*TIME*		cold	8	11	118	5.30	<.0001
*NAME*TREAT*TIME*		control	1	11	118	3.97	<.0001
*NAME*TREAT*TIME*		control	2	11	118	5.96	<.0001
*NAME*TREAT*TIME*		control	3	11	118	3.85	<.0001
*NAME*TREAT*TIME*		control	4	11	118	4.32	<.0001
*NAME*TREAT*TIME*		control	5	11	118	4.69	<.0001
*NAME*TREAT*TIME*		control	6	11	118	4.06	<.0001
*NAME*TREAT*TIME*		control	7	11	118	3.75	0.0001
*NAME*TREAT*TIME*		control	8	11	118	2.93	0.0019

Model Information	
Data Set	WORK.COMBINE
Dependent Variable	ratio
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information

Class	*Levels*	*Values*
EXP	3	1 2 3
NAME	12	ALGANS CLARITI CODIFAR CODISCO CRAZI FERGUS HUSKI JUSTINA LAKTI P329D60 PICKER PR29B29

Dimensions	
Covariance Parameters	1
Columns in X	16
Columns in Z	0
Subjects	1
Max Obs Per Subject	36

Number of Observations	
Number of Observations Read	36
Number of Observations Used	36
Number of Observations Not Used	0

Covariance Parameter Estimates

Cov Parm	*Estimate*
Residual	0.003105

Fit Statistics	
-2 Res Log Likelihood	-47.6
AIC (smaller is better)	-45.6
AICC (smaller is better)	-45.4
BIC (smaller is better)	-44.5

Type 3 Tests of Fixed Effects

Effect	*Num DF*	*Den DF*	*F Value*	*Pr > F*
EXP	2	22	61.61	<.0001
NAME	11	22	6.33	0.0001

Least Squares Means

Effect	*NAME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
NAME	ALGANS	0.1079	0.03217	22	3.35	0.0029
NAME	CLARITI	0.2112	0.03217	22	6.56	<.0001
NAME	CODIFAR	0.3189	0.03217	22	9.91	<.0001
NAME	CODISCO	0.1533	0.03217	22	4.77	<.0001
NAME	CRAZI	0.2717	0.03217	22	8.44	<.0001
NAME	FERGUS	0.1322	0.03217	22	4.11	0.0005
NAME	HUSKI	0.2425	0.03217	22	7.54	<.0001
NAME	JUSTINA	0.09462	0.03217	22	2.94	0.0076
NAME	LAKTI	0.3050	0.03217	22	9.48	<.0001
NAME	P329D60	0.2119	0.03217	22	6.59	<.0001
NAME	PICKER	0.2975	0.03217	22	9.25	<.0001
NAME	PR29B29	0.2932	0.03217	22	9.11	<.0001

Differences of Least Squares Means

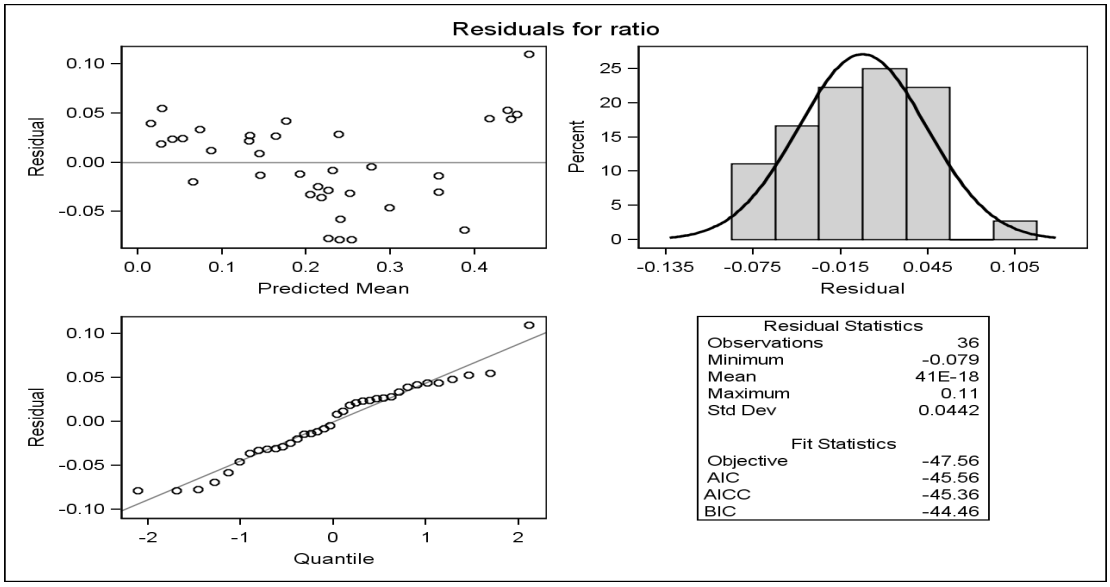
Effect	*NAME*	*_NAME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*	*Adjustment*	*Adj P*
NAME	ALGANS	CLARITI	-0.1033	0.04550	22	-2.27	0.0334	Tukey	0.5226
NAME	ALGANS	CODIFAR	-0.2110	0.04550	22	-4.64	0.0001	Tukey	0.0055
NAME	ALGANS	CODISCO	-0.04541	0.04550	22	-1.00	0.3291	Tukey	0.9961
NAME	ALGANS	CRAZI	-0.1638	0.04550	22	-3.60	0.0016	Tukey	0.0542
NAME	ALGANS	FERGUS	-0.02430	0.04550	22	-0.53	0.5987	Tukey	1.0000

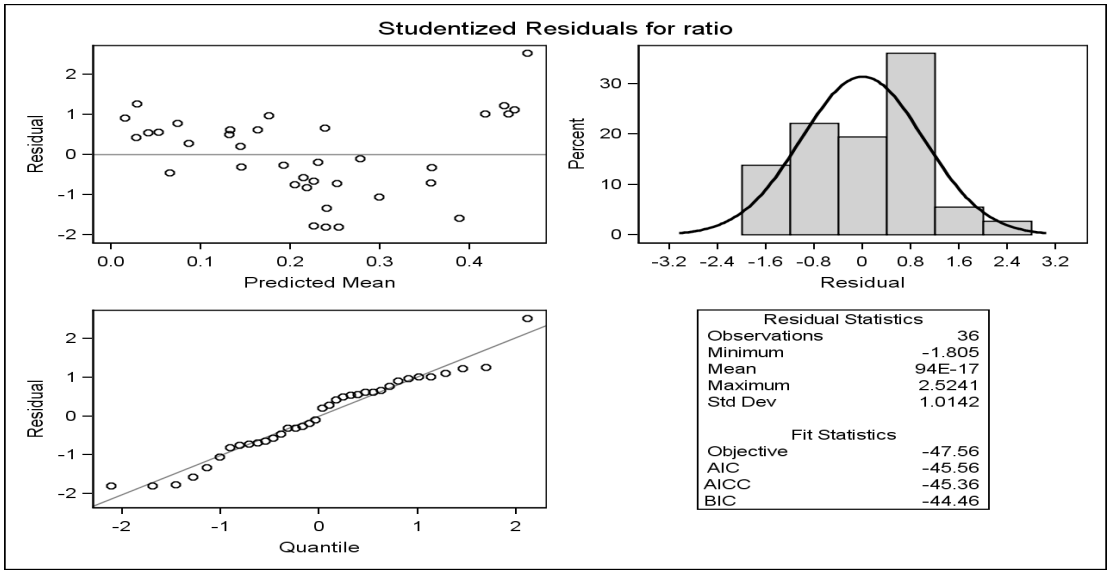
Differences of Least Squares Means

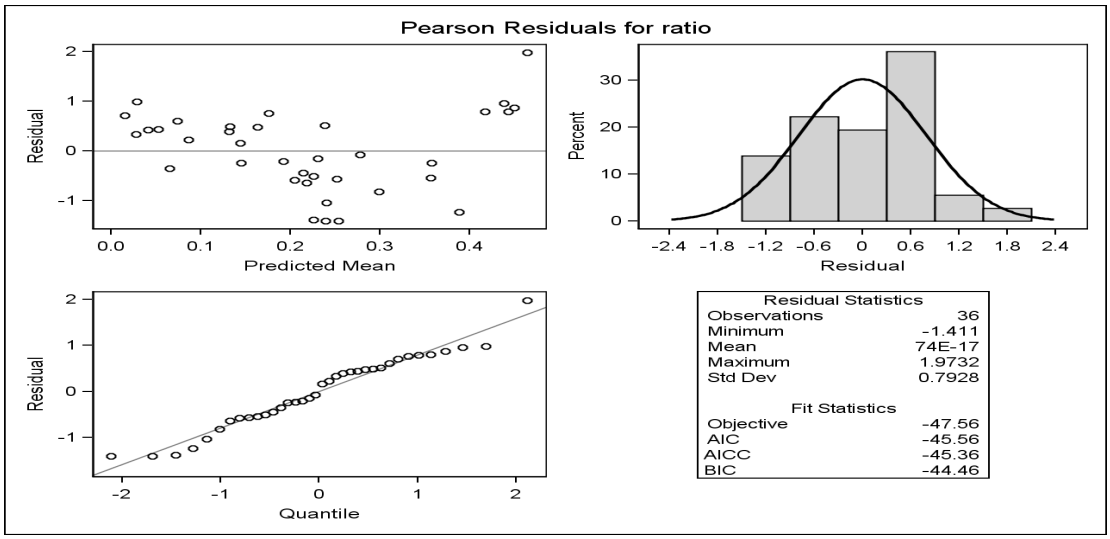
Effect	*NAME*	*_NAME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*	*Adjustment*	*Adj P*
NAME	ALGANS	HUSKI	-0.1346	0.04550	22	-2.96	0.0073	Tukey	0.1873
NAME	ALGANS	JUSTINA	0.01331	0.04550	22	0.29	0.7726	Tukey	1.0000
NAME	ALGANS	LAKTI	-0.1970	0.04550	22	-4.33	0.0003	Tukey	0.0110
NAME	ALGANS	P329D60	-0.1039	0.04550	22	-2.28	0.0323	Tukey	0.5134
NAME	ALGANS	PICKER	-0.1896	0.04550	22	-4.17	0.0004	Tukey	0.0159
NAME	ALGANS	PR29B29	-0.1852	0.04550	22	-4.07	0.0005	Tukey	0.0197
NAME	CLARITI	CODIFAR	-0.1078	0.04550	22	-2.37	0.0271	Tukey	0.4632
NAME	CLARITI	CODISCO	0.05786	0.04550	22	1.27	0.2168	Tukey	0.9747
NAME	CLARITI	CRAZI	-0.06049	0.04550	22	-1.33	0.1973	Tukey	0.9655
NAME	CLARITI	FERGUS	0.07897	0.04550	22	1.74	0.0966	Tukey	0.8337
NAME	CLARITI	HUSKI	-0.03132	0.04550	22	-0.69	0.4985	Tukey	0.9999
NAME	CLARITI	JUSTINA	0.1166	0.04550	22	2.56	0.0178	Tukey	0.3552
NAME	CLARITI	LAKTI	-0.09377	0.04550	22	-2.06	0.0513	Tukey	0.6520
NAME	CLARITI	P329D60	-0.00068	0.04550	22	-0.01	0.9882	Tukey	1.0000
NAME	CLARITI	PICKER	-0.08630	0.04550	22	-1.90	0.0711	Tukey	0.7495
NAME	CLARITI	PR29B29	-0.08197	0.04550	22	-1.80	0.0853	Tukey	0.8011
NAME	CODIFAR	CODISCO	0.1656	0.04550	22	3.64	0.0014	Tukey	0.0498
NAME	CODIFAR	CRAZI	0.04726	0.04550	22	1.04	0.3102	Tukey	0.9946
NAME	CODIFAR	FERGUS	0.1867	0.04550	22	4.10	0.0005	Tukey	0.0183
NAME	CODIFAR	HUSKI	0.07644	0.04550	22	1.68	0.1071	Tukey	0.8591
NAME	CODIFAR	JUSTINA	0.2243	0.04550	22	4.93	<.0001	Tukey	0.0028
NAME	CODIFAR	LAKTI	0.01398	0.04550	22	0.31	0.7615	Tukey	1.0000
NAME	CODIFAR	P329D60	0.1071	0.04550	22	2.35	0.0280	Tukey	0.4720
NAME	CODIFAR	PICKER	0.02145	0.04550	22	0.47	0.6419	Tukey	1.0000
NAME	CODIFAR	PR29B29	0.02578	0.04550	22	0.57	0.5766	Tukey	1.0000
NAME	CODISCO	CRAZI	-0.1183	0.04550	22	-2.60	0.0163	Tukey	0.3354
NAME	CODISCO	FERGUS	0.02111	0.04550	22	0.46	0.6472	Tukey	1.0000
NAME	CODISCO	HUSKI	-0.08917	0.04550	22	-1.96	0.0628	Tukey	0.7130
NAME	CODISCO	JUSTINA	0.05872	0.04550	22	1.29	0.2102	Tukey	0.9719
NAME	CODISCO	LAKTI	-0.1516	0.04550	22	-3.33	0.0030	Tukey	0.0929
NAME	CODISCO	P329D60	-0.05854	0.04550	22	-1.29	0.2116	Tukey	0.9725
NAME	CODISCO	PICKER	-0.1442	0.04550	22	-3.17	0.0045	Tukey	0.1276
NAME	CODISCO	PR29B29	-0.1398	0.04550	22	-3.07	0.0056	Tukey	0.1523
NAME	CRAZI	FERGUS	0.1395	0.04550	22	3.07	0.0057	Tukey	0.1545
NAME	CRAZI	HUSKI	0.02917	0.04550	22	0.64	0.5280	Tukey	0.9999
NAME	CRAZI	JUSTINA	0.1771	0.04550	22	3.89	0.0008	Tukey	0.0291

Differences of Least Squares Means

Effect	*NAME*	*_NAME*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*	*Adjustment*	*Adj P*
NAME	CRAZI	LAKTI	-0.03328	0.04550	22	-0.73	0.4722	Tukey	0.9998
NAME	CRAZI	P329D60	0.05981	0.04550	22	1.31	0.2022	Tukey	0.9681
NAME	CRAZI	PICKER	-0.02581	0.04550	22	-0.57	0.5763	Tukey	1.0000
NAME	CRAZI	PR29B29	-0.02148	0.04550	22	-0.47	0.6416	Tukey	1.0000
NAME	FERGUS	HUSKI	-0.1103	0.04550	22	-2.42	0.0240	Tukey	0.4308
NAME	FERGUS	JUSTINA	0.03761	0.04550	22	0.83	0.4174	Tukey	0.9992
NAME	FERGUS	LAKTI	-0.1727	0.04550	22	-3.80	0.0010	Tukey	0.0357
NAME	FERGUS	P329D60	-0.07965	0.04550	22	-1.75	0.0939	Tukey	0.8266
NAME	FERGUS	PICKER	-0.1653	0.04550	22	-3.63	0.0015	Tukey	0.0505
NAME	FERGUS	PR29B29	-0.1609	0.04550	22	-3.54	0.0019	Tukey	0.0616
NAME	HUSKI	JUSTINA	0.1479	0.04550	22	3.25	0.0037	Tukey	0.1091
NAME	HUSKI	LAKTI	-0.06245	0.04550	22	-1.37	0.1837	Tukey	0.9573
NAME	HUSKI	P329D60	0.03064	0.04550	22	0.67	0.5077	Tukey	0.9999
NAME	HUSKI	PICKER	-0.05498	0.04550	22	-1.21	0.2397	Tukey	0.9825
NAME	HUSKI	PR29B29	-0.05065	0.04550	22	-1.11	0.2776	Tukey	0.9906
NAME	JUSTINA	LAKTI	-0.2103	0.04550	22	-4.62	0.0001	Tukey	0.0057
NAME	JUSTINA	P329D60	-0.1173	0.04550	22	-2.58	0.0172	Tukey	0.3475
NAME	JUSTINA	PICKER	-0.2029	0.04550	22	-4.46	0.0002	Tukey	0.0083
NAME	JUSTINA	PR29B29	-0.1985	0.04550	22	-4.36	0.0002	Tukey	0.0102
NAME	LAKTI	P329D60	0.09309	0.04550	22	2.05	0.0529	Tukey	0.6611
NAME	LAKTI	PICKER	0.007471	0.04550	22	0.16	0.8711	Tukey	1.0000
NAME	LAKTI	PR29B29	0.01180	0.04550	22	0.26	0.7977	Tukey	1.0000
NAME	P329D60	PICKER	-0.08562	0.04550	22	-1.88	0.0732	Tukey	0.7579
NAME	P329D60	PR29B29	-0.08129	0.04550	22	-1.79	0.0878	Tukey	0.8087
NAME	PICKER	PR29B29	0.004332	0.04550	22	0.10	0.9250	Tukey	1.0000


Residuals for ratio
Residual
Predicted Mean
Percent
Residual
Residual
Quantile
Residual Statistics
Observations 36
Minimum -0.079
Mean 41E-18
Maximum 0.11
Std Dev 0.0442
Fit Statistics
Objective -47.56
AIC -45.56
AICC -45.36
BIC -44.46


Studentized Residuals for ratio
Residual
Predicted Mean
Percent
Residual
Residual
Quantile
Residual Statistics
Observations 36
Minimum -1.805
Mean 94E-17
Maximum 2.5241
Std Dev 1.0142
Fit Statistics
Objective -47.56
AIC -45.56
AICC -45.36
BIC -44.46


Pearson Residuals for ratio
Residual
Predicted Mean
Percent
Residual
Residual
Quantile
Residual Statistics
Observations 36
Minimum -1.411
Mean 74E-17
Maximum 1.9732
Std Dev 0.7928
Fit Statistics
Objective -47.56
AIC -45.56
AICC -45.36
BIC -44.46