DEBORAH DE SOUZA VIDIGAL

EFFECT OF THE PARENTAL ENVIRONMENT ON *Arabidopsis thaliana* SEED QUALITY

Thesis presented to University Federal of Viçosa, as part of requirements of Department of Fitotecnia, to obtain the title of *Doctor Scientiae*.

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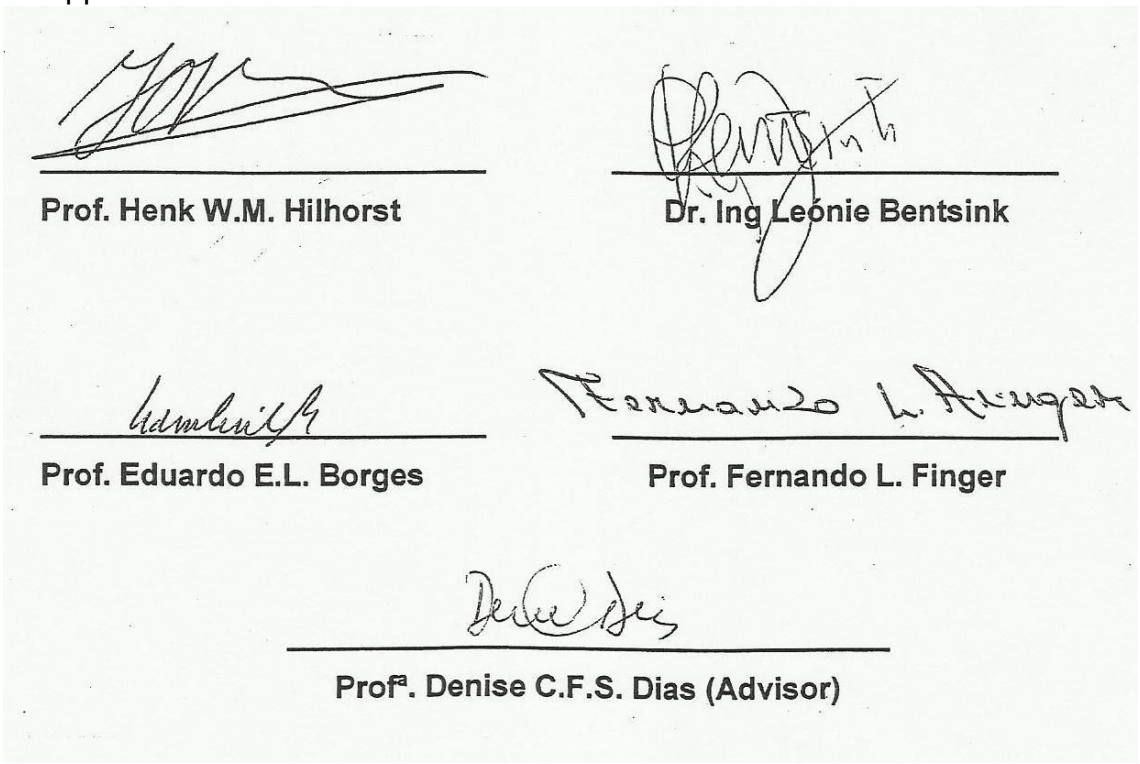

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Dedicated to my parents, Zeni and José Ulisses; my sister, Isabella; my nephew, Bernardo and my love Joachim

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CONTENTS

RESUMO

VIDIGAL, Deborah de Souza, D.Sc., Universidade Federal de Viçosa, março de 2012. **Efeito do ambiente parental na qualidade de sementes de *Arabidopsis thaliana*.** Orientadora: Denise Cunha Fernandes dos Santos Dias.

A qualidade de sementes é determinada pela combinação de fatores gentéticos, físicos, viabilidade, vigor, uniformidade, dormência e longevidade. É adquirida durante o desenvolvimento e maturação da semente, no entanto, condições ambientais desfavoráveis durante esta fase, temperaturas inadequadas, deficit hídrico, falta de nutrientes e luz, podem reduzir a qualidade da semente. O trabalho teve como objetivo analisar a influência do ambiente parental na qualidade da semente, através da aplicação de diferentes concentrações de fosfato e temperaturas, durante o crescimento da planta de Arabidopsis. Para obter uma inicial visão da regulação genética pelos quais o ambiente afeta a qualidade da semente, foi utilizado um conjunto de diferentes genótipos. Primeiro, sementes de genótipo do tipo selvagem Landsberg *erecta* foram produzidas em sete diferentes concentrações de fosfato (0, 12.5, 50, 500, 2500, 5000 e 50000 µM) a fim de determinar a mais efetiva concentração de fosfato para a produção de sementes. Após esse experimento, as sementes de todo o conjunto de genótipos foram produzidas em três concentrações de fosfato (12.5 µM, 500 µM (condição padrão), 3000 µM) em combinação com duas diferentes temperaturas de crescimento (20°C - condição padrão e 25°C). Os resultados mostraram que tanto o fosfato como a tempetura influenciam a qualidade da semente. Para Columbia background, alta concentração de fosfato durante o desenvolvimento da semente diminuiu a dormência e a longevidade das sementes, determinada pelo teste de deterioração controlada.

Alta temperatura de crescimento da planta (25°C) diminuiu a dormência das sementes para os genótipos NIL*DOG1*, NIL*DOG3* e NIL*DOG6*. Em geral, altos níveis de fosfato durante o desenvolvimento da semente proporcionaram melhor germinação em NaCl, manitol e ABA. A combinação de alta concentração de fosfato e baixa temperatura durante o crescimento da planta resultou em maior acúmulo de fitato e fosfato na semente. Entretanto, estudos adicionais são requeridos para melhor esclarescimento das complexas interações entre fatores ambientais e genéticos que regulam a dormência e a qualidade das sementes.

ABSTRACT

VIDIGAL, Deborah de Souza, D.Sc., Universidade Federal de Viçosa, March, 2012. **Effect of the parental environment on *Arabidopsis thaliana* seed quality.** Adviser: Denise Cunha Fernandes dos Santos Dias.

Seed quality is determined by a combination of genetic homogeneity, physical appearance, viability, vigor, uniformity, dormancy and longevity. Seed quality is acquired during seed development and maturation, therefore unfavorable environmental changes during these stages, including the change of temperature, drought stress, lack of nutrients and light, can reduce seed quality. The work presented here analyzes the influence of the parental environment on seed quality, in order to predict seed quality according to growth conditions. To obtain an initial view on the genetic regulation of the environment on seed quality we have used set of different genotypes (standard laboratory genotypes, near isogenic lines and mutants). These plants have be grown in different phosphate concentrations and temperatures. First seeds of wild type Landsberg *erecta* were grown at seven different phosphate concentrations (0, 12.5, 50, 500, 2500, 5000 and 50000 µM) in order to determine the most effective phosphate concentrations. After this experiment, the whole set of genotypes was grown at three phosphate concentrations (12.5 µM, 500 µM (standard condition), 3000 µM) in combination with two different temperatures (20 ºC (standard condition) and 25 ºC). The results show that phosphate concentration and temperature both influence seed quality. For the Columbia background, high phosphate levels during seed development decrease seed dormancy and seed longevity as determined by the controlled deterioration test. High parental growth temperature (25°C) decrease dormancy seeds for genotypes NIL*DOG1*, NIL*DOG3* and NIL*DOG6*. In general, high phosphate levels during seed

development provided a better performance of germination in NaCl, mannitol and ABA. The combined effects of high phosphate concentration and low parental growth temperature resulted in the highest phytate and phosphate content in seeds. However, further investigations are required to fully understand the complex interactions between environmental and genetic factors regulating seed dormancy and seed quality.

1. INTRODUCTION

1.1. The Model Plant Arabidopsis

Arabidopsis thaliana was the first plant and third multicellular organism after *Caenorhabditis elegans* and *Drosophila melanogaster* to be completely sequenced (The Arabidopsis Genome Initiative, 2000). Arabidopsis contains a fully sequenced small genome (125Mbp) made up of only five chromosomes and approximately 26.000 genes (The Arabidopsis Genome Initiative, 2000). In spite of that, the function of a great part of the functional genes still does not have a known function (Bevan and Walsh, 2006). The accumulation of knowledge, biological resources and available molecular tools adds up to attractiveness of Arabidopsis as a model system (Alonso and Ecker, 2006).

Advantages of model organisms are: small size, short generation time, high accessibility, easy manipulation, small genome and important economic potential (Meinke et al., 1998). *A. thaliana* has these properties with exception of the important economic potential. But knowledge of Arabidopsis can be transferred to important crops that are of commercial interest as e.g. tomato (Zhang and Blumwald, 2001).

Many studies on *A. thaliana* contributed to the understanding of the process of seed development, including the regulation of germination and dormancy and the acquisition of desiccation tolerance (Reviewed by Bradford and Nonogaki, 2007 and Kermode, 2011).

1.2. Seed Quality

Seed quality is determined by a combination of genetic homogeneity, physical appearance, viability, vigor, uniformity, dormancy and longevity (Basra,

2006). The quality of seeds is acquired during seed development and maturation, therefore unfavorable environmental changes during these stages, including the change of temperature, drought stress, lack of nutrients and light, can reduce seed quality (Bewley and Black, 1994; Basra, 1995; Basra, 2006).

Seed dormancy prevents germination even though conditions are suitable for germination (relating to water, light, temperature and gaseous conditions) (Fenner and Thompson, 2006). Seed dormancy contributes to the adaptation of plants to their environment by optimizing the germination to the right period of the year. The level of dormancy (primary dormancy) in seeds usually is determined by several factors such as genetic origin and the environmental factors operating during development and maturation (Gutterman, 2000; Bewley and Black, 1994; Fenner and Thompson, 2006). Essentially, the breaking of physiological dormancy is a reversible process by which seeds can become fully dormant again (secondary dormancy) when the conditions for germination are not favorable (Fig. 1) (Hilhorst, 1995; Cadman et al., 2006).

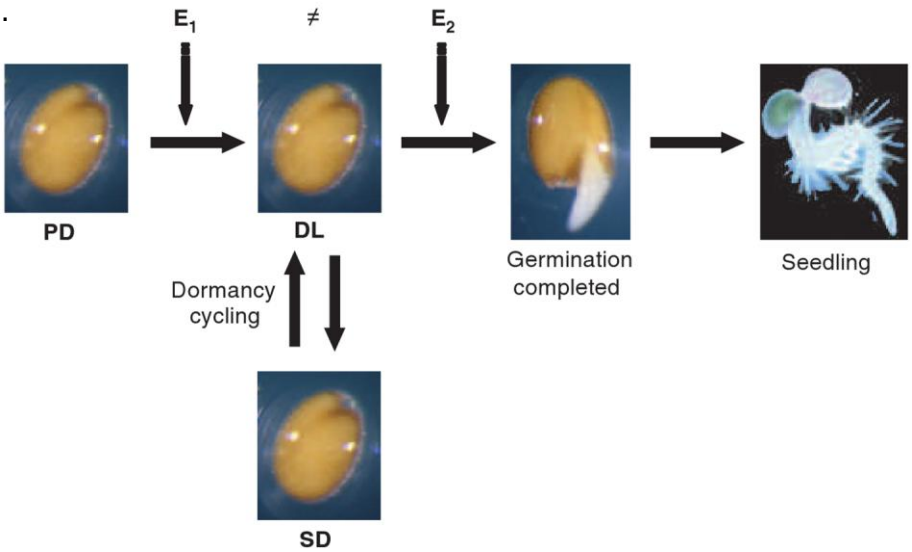


Figure 1. Model for transitions between dormant and after-ripened states in physiologically dormant Arabidopsis accession Cvi seeds. (PD) Primary dormancy; (E_1 and E_2), environmental conditions; (DL) after-ripened seeds and (SD) secondary dormancy. By Cadman et al., 2006.

In natural environments seed dormancy can be released by after-ripening (AR) during several months in a mild/hot temperature conservation of dry seeds or by stratification during low-temperature treatment (cold stratification) of imbibed seeds (Bewley and Black, 1994; Bewley, 1997) inducing GA biosynthesis which in turn promotes seed germination (Yamauchi et al., 2004). However, the molecular mechanisms of dry after-ripening are not understood yet (Finch-Savage et al., 2007) but we know that ABA content decreases.

The phytohormone abscisic acid (ABA) is derived from carotenoids and is a key hormone in the seed germination and dormancy (Kendall et al., 2011). ABA induces dormancy during seed maturation and also maintains the dormant state in imbibed seeds (Hilhorst, 1995; Bewley, 1997; Holdsworth et al., 2008).

ABA is also involved in the induction of stress-responsive genes, stomatal closure and seed development (Seo et al., 2009). Analysis of mutant and transgenic plants of Arabidopsis has provided strong evidence that ABA biosynthesis and responses to this phytohormone are clearly involved in the onset and maintenance of dormancy (Gubler et al., 2005). Endogenous ABA levels can be changed drastically during seed development, germination, and postgermination growth in response to developmental and environmental cues (Okamoto et al., 2006; Bentsink and Koornneef, 2008).

1.3. The Effect of the Environment

Seed dormancy is controlled by multiple genes, that are affected by environmental factors during seed development, seed storage and seed germination (Bentsink and Koornneef, 2011). However, further clarification is

required to fully understand how complex interactions between environmental and genetic factors regulate seed dormancy.

The role of temperature, light quality, day length, water and nutrients in determining the degree of dormancy have been investigated in a wide range of species (Fenner, 1991; Hilhorst, 1995; Baskin and Baskin, 1998). Temperatures during seed development and maturation can affect seed germinability or seed dormancy in many species (Fenner, 1991; Biddulph et al., 2007; Llorens et al., 2008; Javaid et al., 2010). Higher temperature can reduce seed yield and increase protein content of *Lupinus angustifolius* (Jansen, 2008). Seeds that are develop at warmer temperatures are generally less dormant at maturity than those that develop at cooler temperatures as described for many species including *Beta vulgaris*, *Lactura sativa*, *Amaranthus retroflexus*, wild oat and *Avena fatua* by Fenner (1991). To what extent parental growth temperatures promote dormancy is unknown. However, it is known that ABA and GA are involved with temperature changes.

Low temperature can increase the ABA level during seed development (Fenner, 1991), as observed by Goldbach and Michael (1976) in barley grains, plants grown at 26°C compared to those grown at 18°C, had an earlier ABA maximum followed by a sharper decrease in ABA levels. For barley low temperatures during seed-drying phase reduced the rate of gibberellin.

Another important key factor for vigorous germination and successful seedling establishment is the concentration of seed mineral nutrients. The accumulation of minerals in seeds is a complex phenomenon and depends on both, environmental and genetic factors. (Papdi et al., 2009; Ding et al., 2010).

The identification of genes related to plant nutrition will increase our understanding of the mineral uptake and distribution process and may facilitate the improvement of plant nutrient content and use efficiency with potentially beneficial effects on crop yield and quality (Ghandilyan et al., 2009). Matakiadis et al. (2009) reported for Arabidopsis that nitrate treatment could lead to metabolic changes enabling the seed to overcome the inhibition imposed by ABA levels. Alboresi et al. (2005) showed that the depth of seed dormancy of Arabidopsis is inversely correlated to seed nitrate content. Higher nitrate nutrition (50mM) led to the production of less dormant seeds than those produced under standard nitrate nutrition (10mM).

Phosphorus (P) is a major mineral nutrient required by plants, but is one of the most immobile, inaccessible and unavailable nutrients present in soils (Holford, 1997). Phosphorus is essential for all plants for growth, development and reproduction because it is required for the formation of cell membranes (phospholipids), nucleic acids and as a component of energy storage compounds such as ATP (adenosine triphosphate) (Marschner, 1995; Poirier and Bucher, 2002). These compounds are used in germination, photosynthesis, respiration, active uptake of soil nutrients and synthesis of various organic compounds such as carbohydrates, proteins and lipids (Marschner, 1995; Blevins, 1999; Raghothama, 1999).

During the late stages of plant reproductive growth when young seeds are being formed, P is remobilized from older leaves to developing seeds (Blevins, 1999). The major storage for phosphorus in seeds is myo-inositol-1,2,3,4,5,6-hexakisphosphate, commonly referred to as phytic acid (InsP6) or

phytate (Lott et al., 2000; Zhao et al., 2008). Additionally phytate acts as chelate for iron, magnesium, zinc and calcium (Lott et al., 2000).

Inorganic phosphate (Pi) concentration regulates enzymes critical to starch and protein metabolism (Raboy, 1997). ADP-glucose pyrophosphorylase, a key enzyme in starch biosynthesis, is inhibited by Pi (Nakamura and Kawaguchi, 1992). Phosphatases hydrolyze phosphorylated substrates to supply a source of Pi during a shortage of nutrients. The production of these enzymes is linked to Pi deficiency (Dick et al., 2011).

There are many studies linking to P availability with development of root and shoot. These responses of higher plants to phosphate starvation can be morphological and architectural changes in the root system, as formation of lateral root and root hair initiation (Lynch, 1995; Lynch and Brown, 2001; Ma et al., 2001; Péret et al., 2011). The development of the root and shoot in response to P deficiency are controlled by plant growth regulators, like ethylene, auxin, cytokinin (Lynch and Brown, 2001; Ma et al., 2001; Zhang et al., 2003; Chiou and Lin, 2011; Péret et al., 2011). Remarkably, the relationship between parental phosphate nutrition and seed quality has never been investigated.

1.4. Genetic Variation for Seed Quality

1.4.1. Natural Genetic Variation: Seed dormancy is a quantitative trait that is controlled by multiple loci. To identify the loci controlling a quantitative trait one can use quantitative trait loci (QTL) analysis (Koornneef et al., 2002; Paran and Zamir, 2003). QTL analysis has been the tool of choice for the dissection of dormancy, a trait that shows great variability among natural

populations of many plant species (Gubler et al., 2005) and including Arabidopsis (Bentsink and Koornneef, 2011).

QTL analysis reveals the regions on the genome where a gene or several closely linked genes are located and their contribution to the total variance of the trait in that experiment (Reymond et al.; 2007). Populations of recombinant inbred lines (RILs) have been the most frequently used in Arabidopsis due to the advantage derived from their homozygosity (Bentsink and Koornneef, 2011). RILs are obtained by single-seed descent from F2 plants until the F9 or further generation. These populations can be used to map QTL because the influence of the environment on the quantitative trait can be much reduced by assessing multiple individuals of the same genotype instead of just a single plant (Bentsink and Koornneef, 2011). For validation/confirmation of the presence and the effect of a QTL, near isogenic lines (NILs) are used. NILs contain an introgression of one parent's alleles at a QTL position into the genetic background of the other (recurrent) parent (Reymond et al., 2007).

The approach of QTL mapping is a valuable method in elucidating the genetics but also the physiological background of traits involved in seed quality. The natural variation for seed dormancy has been studied in Arabidopsis in many different populations using a quantitative trait loci (QTL) mapping approach. Van der Schaar et al. (1997), evaluated 98 RILs derived from the cross between the two accessions, Landsberg *erecta* (L*er*) and Columbia (Col), both showing a low level of seed dormancy. Fourteen loci were identified by QTL analysis. Alonso-Blanco et al. (2003) used L*er* with low dormancy and the Cape Verde Islands (Cvi), the strong-dormancy accession, identified seven QTL named *Delay of Germination* (*DOG*). Clerkx et al. (2004b) located four QTLs

controlling seed dormancy in RIL populations derived from a cross of L*er* x Shakdara (Sha). The QTLs explained by 54,9% of variance. Bentsink et al. (2006) identified *DOG1* gene involved in the control of seed dormancy in three of the analyzed population.

RIL progeny of a cross between Bay-0 and Sha, was analyzed by Meng et al., 2008. They identified six distinct QTLs controlling seed germinability in response to cold and dark. Where three of which were major loci, each responsible for 17-25% of the phenotypic variability in this trait.

Bentsink et al. (2010) identified 11 *DOG* QTL linked to dormancy in a set of RILs derived from a cross between wild accessions and L*er*. Seven of these QTLs have been confirmed by NIL carrying specific Cvi introgression fragments in a L*er* genetic background. These authors concluded that natural variation for seed dormancy presents different genetic and molecular pathways control seed dormancy.

1.4.2. Induced Genetic Variation: Genes with relatively large effects on phenotype (or trait value) have been identified in Arabidopsis using mutant approaches, often accompanied with overexpression of the genes of interest (Reymond et al., 2007).

Molecular genetic analyses indicated that NCED enzymes (9-*cis*-epoxycarotenoid dioxygenase) are the first step specific to ABA biosynthesis and NCED genes may be a key elements in the control of ABA levels in seeds (Tan et al., 2003; Lefebvre et al., 2006). The NCED genes regulate of physiological processes such as seed development, maturation, desiccation and germination by affecting the ABA concentration in seeds (Tan et al., 2003;

Lefebvre et al., 2006). Lefebvre et al. (2006) showed that ABA levels were reduced in the seeds of *Atnced6* and *Atnced9* mutants and that these mutant seeds were resistant to paclobutrazol, an inhibitor of gibberellin synthesis. Although seeds of single mutants were still dormant reduced dormancy was observed in the *Atnced6 Atnced9* double-mutant seeds (Lefebvre et al., 2006).

Members of the *CYP707A* family, which belong to the ABA catabolic pathway play a prominent role in regulating endogenous ABA levels during seed development and germination (Okamoto et al., 2006). According to Saito et al. (2004) the transcripts of *CYP707A* genes increased in response to abiotic stress, dehydration and exogenous ABA treatment. T-DNA insertion mutants of *CYP707A2* have higher ABA content in seeds and exhibit increased dormancy compared to wildtype plants (Kushiro et al., 2004). The *CYP707A1* is expressed predominantly during mid-maturation and is down-regulated during late-maturation and *CYP707A2* transcript levels increase from late-maturation to mature dry seed, indicating that *CYP707A2* plays a major role in reducing ABA content in after-ripened Arabidopsis seeds or during early seed imbibition (Kushiro et al., 2004; Okamoto et al., 2006; Matakiadis et al., 2009).

The work presented here analyzes the influence of the parental environment on seed quality, in order to predict seed quality according to growth conditions by assessing different phosphate concentrations and growth temperatures and try to identify genetic pathways by which the environment is affecting seed quality. The use of different genotypes (i.e. aba mutants) will allow us to determine which pathways are affected.

2. MATERIALS AND METHODS

The work was conducted in the Laboratory of Plant Physiology, Wageningen Seed Laboratory, of Wageningen University in The Netherlands, from March 2010 until October 2011.

2.1. Plant Material and Growth Conditions

Different genotypes of *Arabidopsis thaliana* plants were used. Two wild types: Landsberg *erecta* (L*er*) and Columbia (Col), five Near Isogenic Lines (NILs) in the L*er* background (NIL*DOG1*, NIL*DOG2*, NIL*DOG3*, NIL*DOG6*, NIL*DOG22*), four mutants in the Col background: *cyp707a1, cyp707a2*, *dog1-2* and *Atnced6-Atnced9* (double mutant = dm) and one mutant in the L*er* background, *dog1-1*, this is a NIL*DOG1* in which *DOG1* is mutated, resulting in loss of dormancy.

Seeds were sown in petri dishes on water soaked filter paper and incubated for three days in cold room at 4 ºC in the darks to break dormancy (seed stratification). Subsequently, the petri dishes were transferred to germinator at 22 ºC (16 hours light per day) for three days before planting. Germinated seedlings were transferred to a climate chamber (20 ºC, 16 h of light at a relative humidity of 70%) on 4 x 4 cm rockwool plugs and watered 3 times per week with a standard solution containing 500 µM of phosphate (Table 1). After flowering, the plants were submitted to different treatments according to Table 2 and Table 3 for experiment 1 and experiment 2 respectively.

Table 1. The standard solution containing 500 µM of phosphate

	N	K	Ca	Mg	SO_4	P	Fe	Mn	Zn	B	Cu	Mo
µM/L	2500	2950	1340	400	940	500	7500	2500	1500	5000	250	250

Table 2. Plant growth conditions: phosphate levels before flowering (vegetative state) and after start flowering (inflorescence emergence).

Vegetative state	**Inflorescence emergence**
500 µM	0 µM
500 µM	12.5 µM
500 µM	50 µM
500 µM	500 µM
500 µM	2500 µM
500 µM	5000 µM
500 µM	50000 µM

Table 3. Plant growth conditions: phosphate levels and temperature before flowering (seedling) and after start flowering (inflorescence emergence).

Vegetative state	**Inflorescence emergence**
500 µM, 20 ºC	0 µM, 20 ºC
500 µM, 20 ºC	12.5 µM, 20 ºC
500 µM, 20 ºC	3000 µM, 20 ºC
500 µM, 20 ºC	0 µM, 25 ºC
500 µM, 20 ºC	12.5 µM, 25 ºC
500 µM, 20 ºC	3000 µM, 25 ºC

2.2. Measurement of Plant Characteristics

Repetition of three plants per treatment were evaluated for plant height (cm), number of siliques per plant, number of seeds per siliques, seed size (mm^2) and weight of 1000 seeds (1000 seed weight (mg)).

2.3. Seed Quality Analyses

To measure the degree of dormancy, $DSDS_{50}$ (Days of seeds dry storage until 50% germination) germination test were performed weekly until dormancy for all treatments had been released. The germination experiments were performed in plastic (15 x 21 cm) trays containing 47 ml water and two layers of blue filter paper. Six samples of approximately 50–200 Arabidopsis seeds were dispersed on the filter paper using a mask to ensure an accurate and reproducible spacing. Clustering of seeds was prevented as much as possible. These trays were kept in an incubation at 22 ºC and constant light, during five days. Twice a day, photos were taken and they were analyzed by GERMINATOR package (Joosen et al., 2010) and some parameters like *maximum percentage of germination* (*gMAX(%)*) and *time to reach 50% germination* (t_{50} *- hours*) during after-ripening were calculated by this program.

2.4. Germination Tests under Stress Conditions

Germination tests at 10 ºC (low temperature) and at 27 ºC (high temperature; 30°C in experiment 2) were performed. Seed storability was determined as viability (germination) after the CDT (Controlled Deterioration Test) at 40 ºC, 85% RH for 0, 2, 4, 6 and 8 days (5 days in experiment 2). Other germination tests were conducted in 0.5 µM of solution of ABA (0.2 µM in

experiment 2) with and without seed stratification, germination at 125mM of sodium chloride (NaCl; 100 mM in experiment 2) or -0.8MPa of mannitol (experiment 2).

2.5. Phytate and Phosphate Measurements (experiment 2)

Dry seeds (1-2mg) were put in an eppendorf with 250 µL of 0.5N HCl and 50mg/L of trans-aconitate (internal standard). Cis-aconitate was used as an internal standard, because it was undetectable in the samples, and its behaviour during the extraction procedure was expected to be similar to other anions present in the samples. After a hole was made in the lid the eppendorfs were boiled for 15 minutes at 100 ºC. The extracts were centrifuged at 13000 rpm for 3 min. 100µM of supernatant was used for analyses in the Dionex ICS2500 HPLC system (Dionex Corporation, Sunnyvale, CA, USA). Anions were separated on an AS 11-HC column, preceded by an AG 11-HC guard column and detection by suppressed conductivity. The elution profile was 0–15 min linear gradient at 5-100 mm of NaOH, followed by a 15–20 min linear gradient with 500 mM of NaOH. After each run, the column was washed for 20-35 min with 5 mM NaOH. Flow rates were 1 mL min-1 throughout the run. Contaminating anions in the eluents were removed using an ion trap column (anion trap column) installed between the pump and the sample injection valve. Anions were determined by conductivity detection. Background conductivity was decreased using an anion self-regenerating suppressor, with water as a counterflow (5 mL min-1), operated at 248 mA. Peaks were identified and quantified by co-elution with known standards.

2.6. Data Analyses

All studies and evaluations were done in three replications, using a factorial design (12 genotypes x 3 phosphate concentration x 2 temperatures). The collected data were analyzed using Analysis of Variance (ANOVA) and the significance of the treatment means was determined using a t-test at a significance level of 0.05. All analyses were carried out using Assistat 7.6 beta software package.

Correlation coefficients between genotypes, applied treatments and seed or parental plant tests (Plant height, Seed Size, Number of siliques per plant; Number of seeds per silique, 1000 seed weight , $DSDS_{50}$, Gmax at 22°C, Gmax at 10°C; Gmax at 30°C (5 months of after-ripening (AR)), Gmax at 30°C (7 months of AR), Gmax in ABA (5 months of AR), Gmax in ABA (7 months of AR), Gmax after CDT, Gmax in Mannitol, Gmax in NaCl, Phosphate and Phytate Content) were calculated using Pearson's correlation coefficient. Only correlations with a significance level with a p-value lower than 0.05 were taken into consideration (experiment 2).

In experiment 1, the means of the different treatments were compared using Tukey's test (P=0.05) and in experiment 2, the data of phosphate concentrations were compared by student's test (P<0.05). On both experiment the error bars represent SE from three measurements.

Contour plots graphs were estimated using linear interpolation of the used phosphate concentration, temperature and the measurement of the respective seed test.

The Software Tool: Neurofuzzy Logic

A neurofuzzy logic approach was applied using FormRules v3.31 (Intelligensys Ltd, UK), a hybrid system that combines the strength and the adaptive learning capabilities of neural networks with the ability to generalize rules from fuzzy logic. It is the implementation of the ASMOD (Adaptative Spline modeling of data) algorithm (Kavli and Weyer, 1994). This method uses global partitioning that involves splitting the model into smaller submodels. Various models and submodels were examined, starting from a set of the simplest modes. The models are sums or products of the basic functions, producing submodels that depend only on a subset of the inputs (Gago et al., 2010; Gallego et al., 2011).

The neurofuzzy logic application finds a predictive model for each parameter measured, named here as output, and generates a set of "IF….THEN" rules with different values of membership degree. Complex models are intensely simplified to make them as simple as possible and perform easily understandable rules.

This neurofuzzy logic application contains various statistical fitness criteria but the best results were found when Structural Risk Minimisation (SRM) was used. The training process was conducted as reported by Shao et al. (2006). Minimization parameters are summarized in Table 4.

The accuracy of the neurofuzzy logic model was further evaluated using the ANOVA correlation coefficient (R^2) for each output.

$$R^2 = 1 - \frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{n} (y_i - \bar{y}_i)^2}$$

Where $\bar{y}$ is the mean of the dependent variable, and $\hat{y}$ is the predicted value from the model. The larger the value of the Train Set R-squared, the more the model captured the variation in the training data. Values between 70-99.9% are indicative of a reasonable model accuracy (Colbourn and Rowe, 2005). Values of ANOVA *f*-test statistic higher than upper critical values of the *f* distribution for the degrees of freedom used for each parameter indicate no significant differences between experimental and predicted data (α<0.05) and, therefore, high model predictabilities.

Table 4. The training parameters setting with FormRules v3.31.

Minimization parameters
Ridge Regression Factor: 1 e^{-6}
Model Selection Criteria
Structural Risk Minimization (SRM)
C1 = 0.74 – 1.00 C2 = 4.8
Number of Set Densities: 2
Set Densities: 2, 3
Adapt Nodes: TRUE
Max. Inputs Per SubModel: 4
Max. Nodes Per Input: 15

3. RESULTS

3.1. Experiment 1. Pilot experiment to determine which parental phosphate concentrations affect seed quality in *Arabidopsis thaliana*.

3.1.1. The Effect of Parental Phosphate on Plant Development

Seeds of wild type Landsberg *erecta* (L*er*) were grown at seven different phosphate concentrations (0, 12.5, 50, 500, 2500, 5000 and 50000 µM of phosphate; Table 2) in order to analyze the effect of parental phosphate on seed quality and to select the best treatments for future experiments.

The environment can affect seed quality direct or indirectly, therefore we have next to seed quality also investigated plant traits like plant height and number of siliques per plant (Fig. 2A). High phosphate levels (50000 µM) led to production of shorter plants and fewer siliques per plant. Thus, we did not get enough seeds to perform all tests. The small plants and the low production of seeds could be explained by a toxic effect of a high phosphate concentration on Arabidopsis.

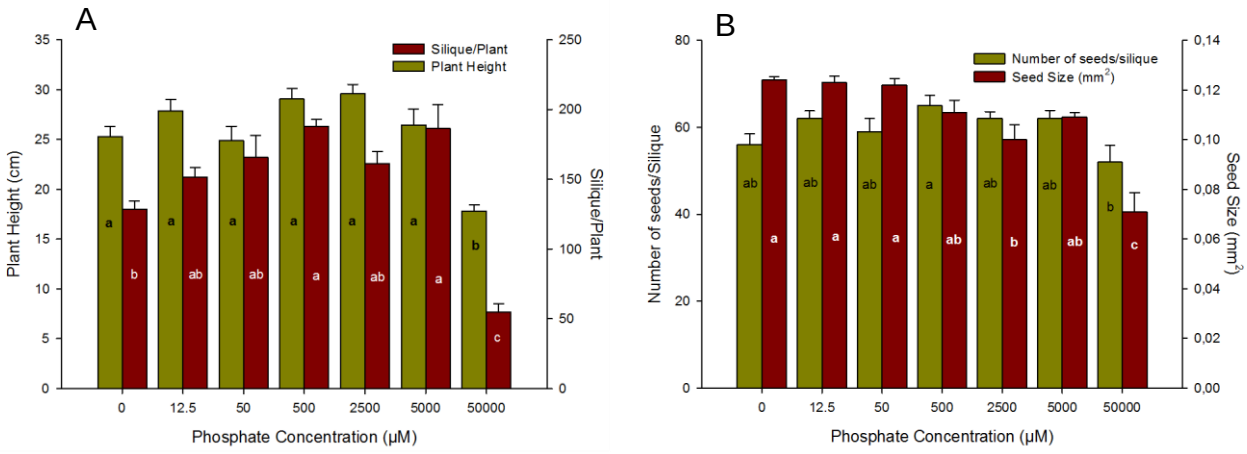


Figure 2. Effect of parental phosphate on plant and seed development (A) Plant Height (cm) and number of siliques per plant; (B) Number of seeds per silique and seed size (mm^2). Each bar represents the mean of three replicates, and error bars represent SE. Significance between treatments are indicated by letters. Means followed by the same letter were not significantly differed by Tukey's test (P=0.05).

For the phosphate treatments, between 0 µM to 5000 µM, no significant differences were found in plant height. Plants that grew without any phosphate (0 µM) produced fewer siliques per plant than the standard level (500 µM) (Fig.2A). Seeds developed in lower than standard concentration show proportional decrease of the number of siliques per plant given a decrease phosphate level.

Seed size (Fig. 2B) was inversely correlated to phosphate levels. Plants exposed to higher phosphate levels had smaller seed size. The highest phosphate concentration (50000 µM) produced fewer seeds per silique than the standard concentration (500 µM) (Fig. 2B).

3.1.2. The Effect of Parental Phosphate on Seed Dormancy

To analyze the effect of parental phosphate levels on seed dormancy, we made weekly germination tests until all seeds released dormancy. The number of days of seed dry storage (after-ripening) necessary to obtain 50% germination ($DSDS_{50}$) was estimated for each treatment. On the first week after harvest, seeds from plants of 50000 µM phosphate had almost 70% of germination while the other treatments had less than 20% of germination (Fig.

3A). Phosphate deprivation had a clear effect on dormancy. Seeds developed under phosphate levels higher than the standard condition (500 µM) germinated more than 90% after 4 weeks after-ripening. While seeds from plant grown at lower phosphate levels required at least 10 weeks of seed dry storage (after-ripening) to do so (Fig. 3A). The $DSDS_{50}$ values (Fig. 3B) of seeds developed under low phosphate concentrations were at least twice higher than $DSDS_{50}$ values of seeds developed under high phosphate concentrations. $DSDS_{50}$ values of higher phosphate levels varying from 10 to 15 days and $DSDS_{50}$ values of lower phosphate levels ranged from 25 to 30 days.

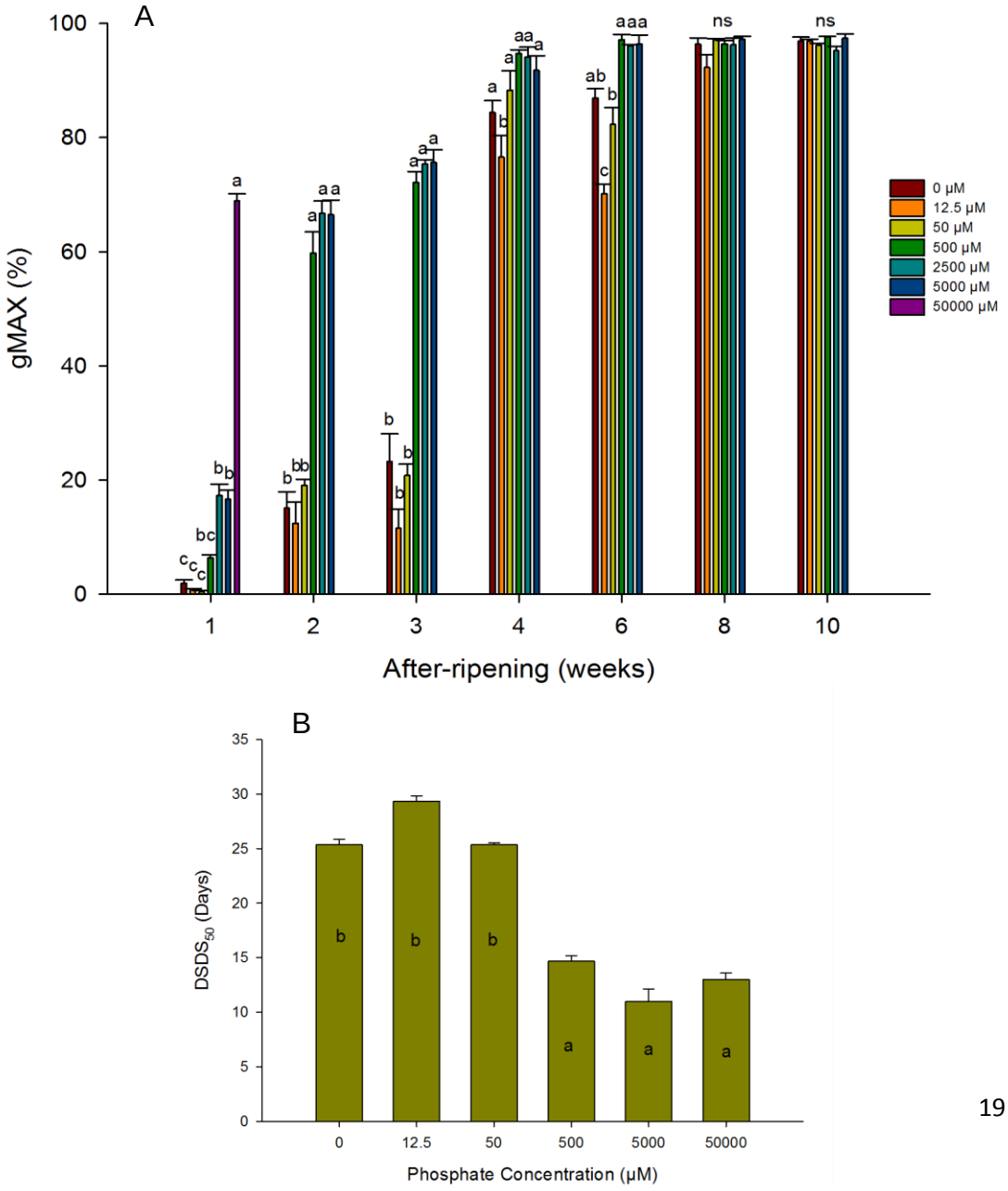


Figure 3. Effect of phosphate during seed development (A) Germination percentage (gMAX) during 10 weeks after-ripening; (B) $DSDS_{50}$ (Days of seed dry storage required to reach 50% germination) under different phosphate concentration. Each bar represents the mean of three replicates, and error bars represent SE. Significance between treatments are indicated by letters. Means followed by the same letter were not significantly differed by Tukey's test (P=0.05).

3.1.3. The Effect of Parental Phosphate on Seed Storability

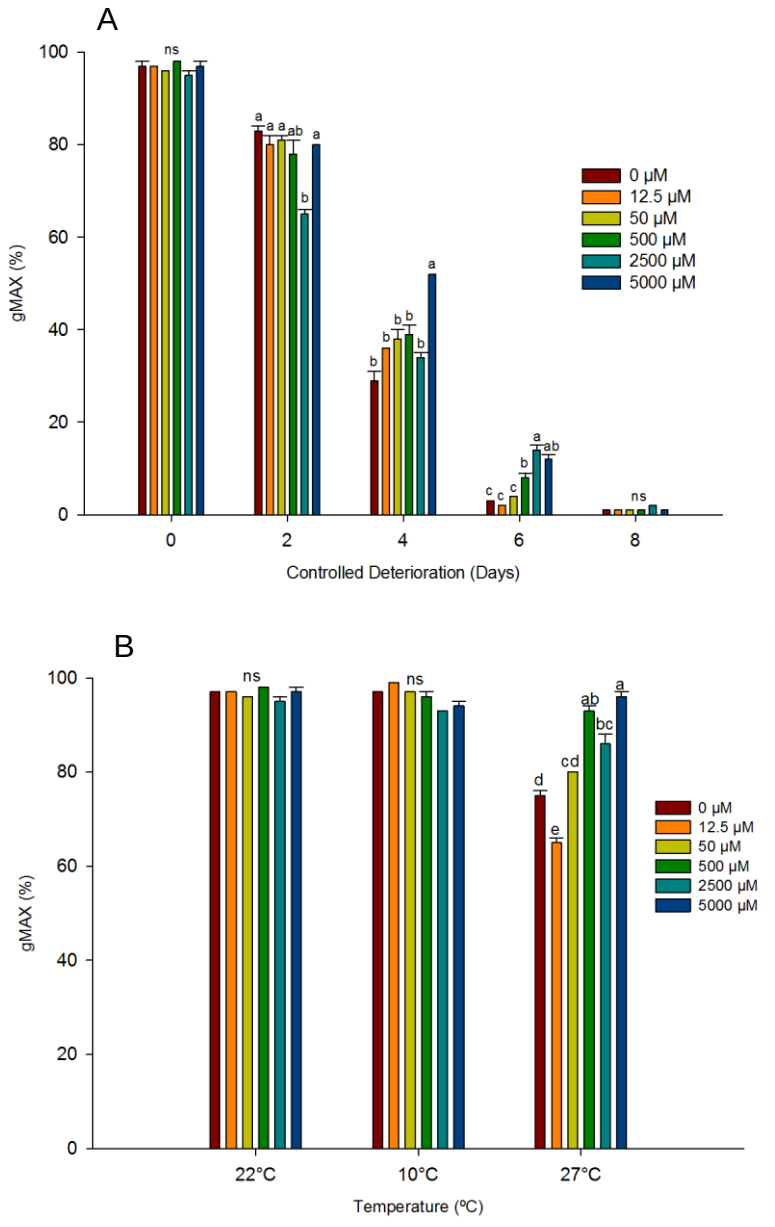
Arabidopsis seeds in general have a life span of approximately 5-10 years under dry conditions. In order to evaluate whether the seed life span was affected by parental phosphate we decided to analyze this trait. To avoid long waiting times we used artificial ageing, by means of a controlled deterioration test (CDT) to get a measure for the storability of the seeds. For this experiment we have exposed the seed for 0, 2, 4, 6 and 8 days to the CDT (Fig. 4A). The CDT treatment of 4 and 6 days led to significant differences between the different phosphate treatments. Higher phosphate levels had a better germination performance after CDT of 4 and 6 days. Phosphate levels, dormancy and storability may be related. Higher phosphate levels produce less dormant seeds and these seeds have a better performance of germination after controlled deterioration, meaning that they have a better storability.

3.1.4. The Effect of Parental Phosphate on Germination under Stress Conditions

Germination was evaluated under high temperature (27°C), low temperature (10°C) and standard temperature (22°C) conditions (Fig. 4B). Low

and standard temperature did not affect germination of L*er* seeds. However, seeds developed by higher phosphate levels resulted in a higher germination (96%) at 27°C.

Phosphate treatment during seed development also affected the sensitivity of Arabidopsis seed to exogenous ABA and germination in salt solution (NaCl) (Fig. 4C and D respectively). The germination of seeds from plants grown under lower phosphate levels were reduced by ABA concentration or NaCl as compared to the ones grown under higher phosphate levels.



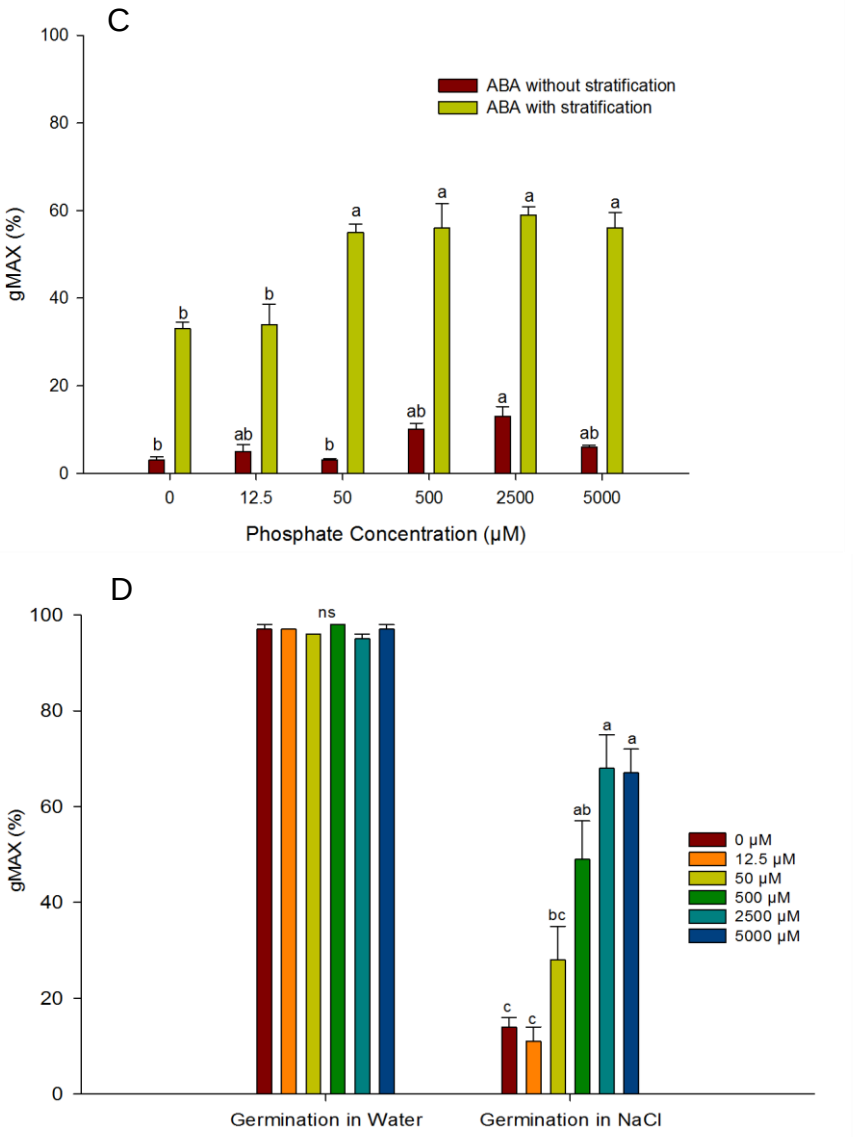


Figure 4. Effect of phosphate during seed development on germination under stress. (A) Controlled deterioration test (CDT) for 0, 2, 4, 6 and 8 days; (B) Germination under different temperatures: 22 ºC (standard condition); 27 ºC and 10 ºC; (C) ABA sensitivity test: germination of seeds with and without stratification in 0,5 µM ABA; (D) Salt treatment: 125mM NaCl. Each bar represents the mean of three replicates, and error bars represent SE. Significance between treatments are indicated by letters. Means followed by the same letter were not significantly differed by Tukey's test (P=0.05).

In summary, 50000 µM phosphate decreased the plant height and the production of siliques per plant (Fig. 2A), resulting in a low total number of seeds. Therefore most germination characteristics (Fig. 3 and 4) could not be determined for this concentration. An increase of phosphate decreased the seed size, although the number of seeds per silique was only influenced by the highest phosphate concentration (50000 µM) (Fig. 2B). Phosphate deprivation during seed development had a clear effect on dormancy. $DSDS_{50}$ values for seeds developed under low phosphate concentrations were higher as compared to the control (Fig. 3B). Lower concentrations of phosphate during seed development resulted in a lower germination at higher temperature (27 ºC) (Fig. 4B). Also germination under other stress conditions, e.g. germination after a CDT, germination on ABA and salt showed a better performance of the seeds developed under higher phosphate levels as compared to the ones developed under lower phosphate concentrations (Fig. 4A, C and D). These results show a clear effect of phosphate concentrations during seed development on the final seed quality.

We have decided to use 12.5 µM, 500 µM (standard condition) and 3000 µM phosphate for the following experiment, since these concentration led to significant difference for seed dormancy and germination under stress conditions and did not negatively affect plant architecture like the 50000 µM phosphate did.

3.2. Experiment 2. The effect of parental phosphate and temperature on plant development and seed quality in different genotypes of *Arabidopsis thaliana*.

3.2.1. The Effect of the Parental Environment on Plant Development

Different genotypes of *Arabidopsis thaliana* (L*er,* Col, NIL*DOG1*, NIL*DOG2*, NIL*DOG3*, NIL*DOG6*, NIL*DOG22*, *cyp707a1*, *cyp707a2*, *dog1-1, dog1-2* and *Atnced6-Atnced9* (dm)) were grow on a standard solution containing 500 µM of phosphate until the beginning of flowering (Table 1). After that, the plants were transferred and grown in different stress condition: two different temperatures: 20 ºC (standard condition) and 25 ºC and three different nutrition solutions: 12.5 µM, 500 µM (standard condition) and 3000 µM phosphate (Table 3). Seeds were harvested and storage at 20 ºC in paper bags until use.

The parental growth temperature affects the life cycle of all Arabidopsis genotypes. Plants grown at 25°C needed 8 weeks to complete their life cycle whereas plants grown at 20°C needed 10 weeks. The phosphate concentration during seed development did not influence the duration of the life cycle.

Data analyses of a large set of traits can be complicated. In order to in general describe the results we used artificial intelligence technology, as a modelling tool (Gago et al., 2011). Neurofuzzy logic has been employed to simultaneously model the effect of the 12 genotypes, 2 different temperatures, 3 phosphate concentrations and 8 stress conditions on seed germination, in total 576 combinations. Eight sets of straightforward rules (Table 5) extracted from the neurofuzzy logic analyses allow us explain the cause-effects and interactions between those variables (inputs) and the parameters measured (outputs).

These rules tell us that plant height and seed dormancy ($DSDS_{50}$) are mostly affected by the genotype (Table 5). Although plant height was most

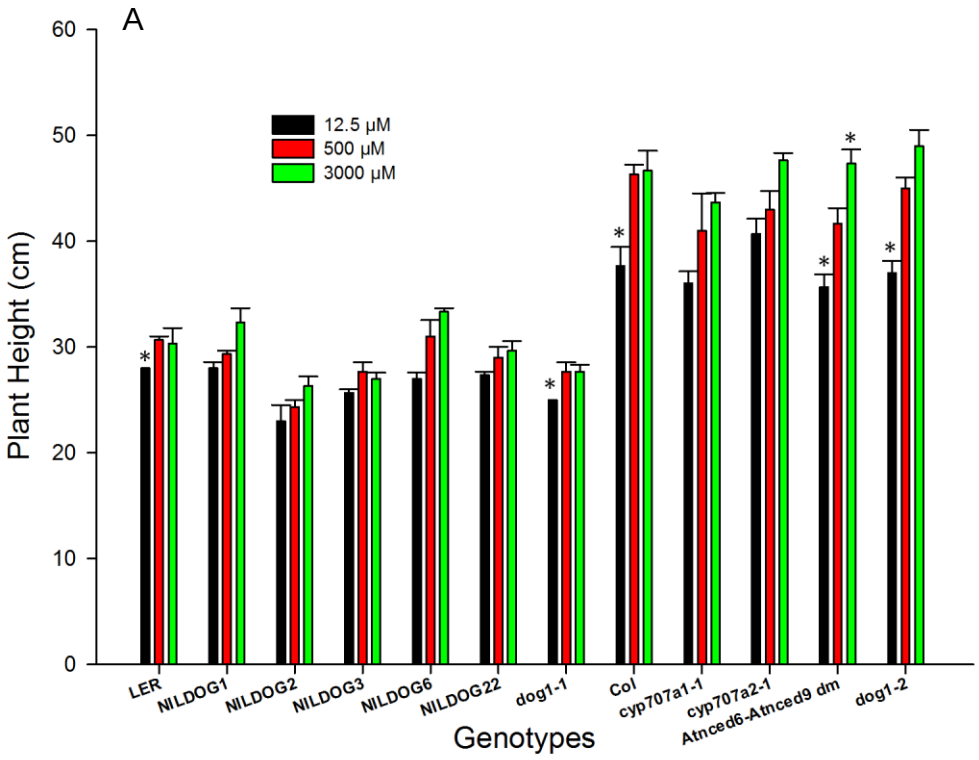
strongly affected by genotype, plant height was greatest at the highest phosphate levels (3000 µM) intermediate in standard condition (500 µM) and lowest in low phosphate level (12.5 µM) in both parental growth temperatures, 20°C and 25°C (Fig. 5A). The influence of phosphate level is indicated by the high correlation between plant height and genotypes, exceptions for L*er*, NIL*DOG3*, NIL*DOG22*, *dog1-1* and Col (Fig.6).

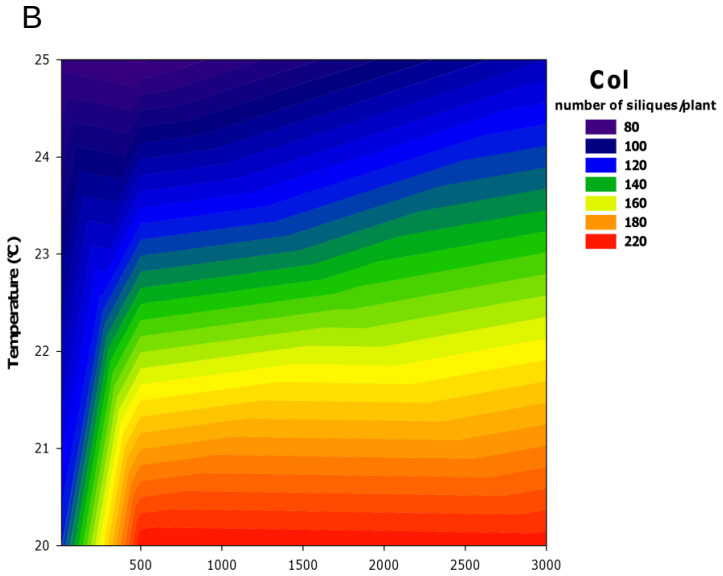
Table 5. Significant inputs from neurofuzzy logic submodels and training R^2 with *f* value, freedom degrees and p value (99 %) in the ANOVA for each output. Inputs: G, genotype; P, phosphate; T, temperature and S, stress conditions.

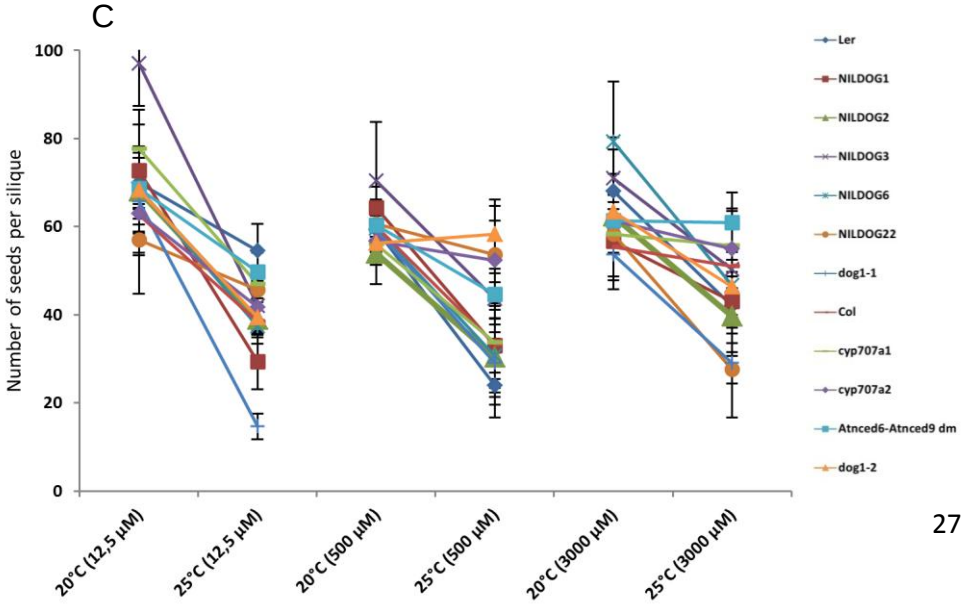
	Submodel	Significant inputs and interactions	R^2	*f* value	df1,df2*	α value
Plant height	1 2 3	G P T	94.4852	63.96	15, 71	<0.01
Number silique plant-^{1}	1 2	T x P G	92.99	42.14	17, 71	<0.01
Number seed silique-^{1}	1 2 3	T G x T P	75.9749	5.47	26, 71	<0.01
1000 seed weight	1 2	T x P G	82.0818	14.55	17, 71	<0.01
$DSDS_{50}$	1 2	G T	91.5898	48.59	13, 71	<0.01
Phosphate content	1	T x P x G	90.4449	4.53	48, 71	<0.01
Phytate content	1 2	P x T G	91.4328	33.90	17, 71	<0.01
Gmax	1 2 2	S x T x P G x S G x T	93.5368	51.05	127, 575	<0.01

Number silique per plant is affected by an interaction between temperature and phosphate (T x P) and to a lower extend by the genotype (Table 5).

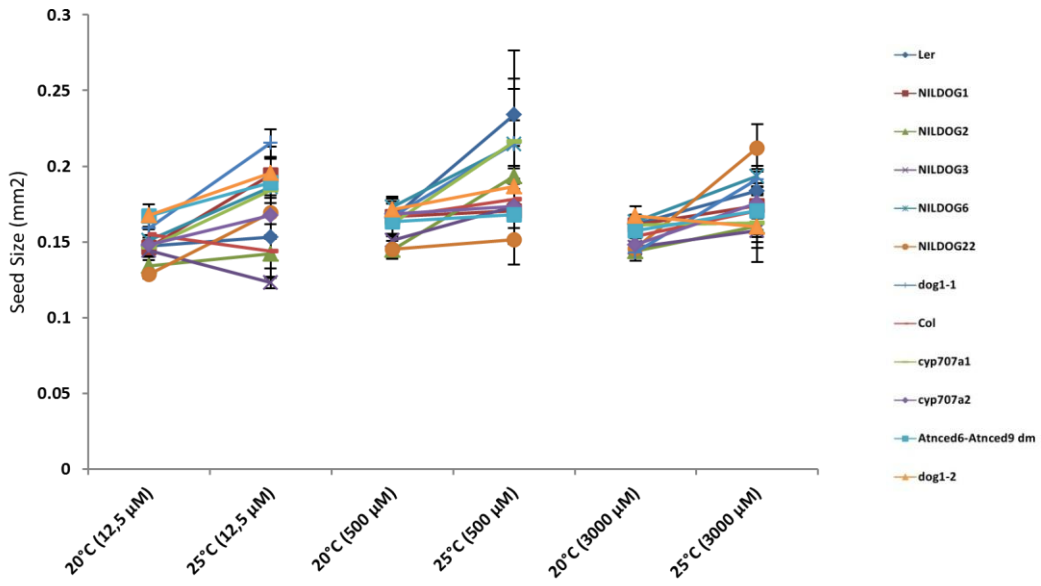
The number of siliques per plant, of all genotypes of Arabidopsis, decreased with decreasing phosphate concentration and increasing parental growth temperature (Fig. 5B). The number of siliques per plant was positively correlated with the parental phosphate concentration for the genotypes *dog1-1*, *cyp707a1* and *Atnced6-Atnced9* (dm), whereas all other genotypes had negative correlation with parental temperature (Fig. 6). Despite of the correlation between 1000 seed weight and temperature, there are exceptions (L*er*, NIL*DOG6* and *Atnced6-Atnced9* (dm)) that do not show this positive (Fig. 6). No correlation was detected between phosphate concentrations and the number of seeds per silique, seed size and 1000 seed weight (Fig.6). The neurofuzzy logic analyzes showed the effect of temperature and phosphate (T x P) on 1000 seed weight (Table 5). For wild type L*er*, higher maturation temperature combined with standard phosphate level (500 µM) provided the highest 1000 seed weight, 23 mg (Fig. 5F). While the wild type Col, was not influenced by phosphate concentration. Higher parental temperature led to heavier seeds (Fig. 5G). Only the double mutant *Atnced6-Atnced9* had an opposite effect for the interaction between temperature and phosphate. Higher temperature of growth produced the lightest seeds in all phosphate concentration (Fig. 5H).


A
Plant Height (cm)
12.5 µM
500 µM
3000 µM
LER
NILDOG1
NILDOG2
NILDOG3
NILDOG6
NILDOG22
dog1-1
Col
cyp707a1-1
cyp707a2-1
Atnced6-Atnced9 dm
dog1-2
Genotypes

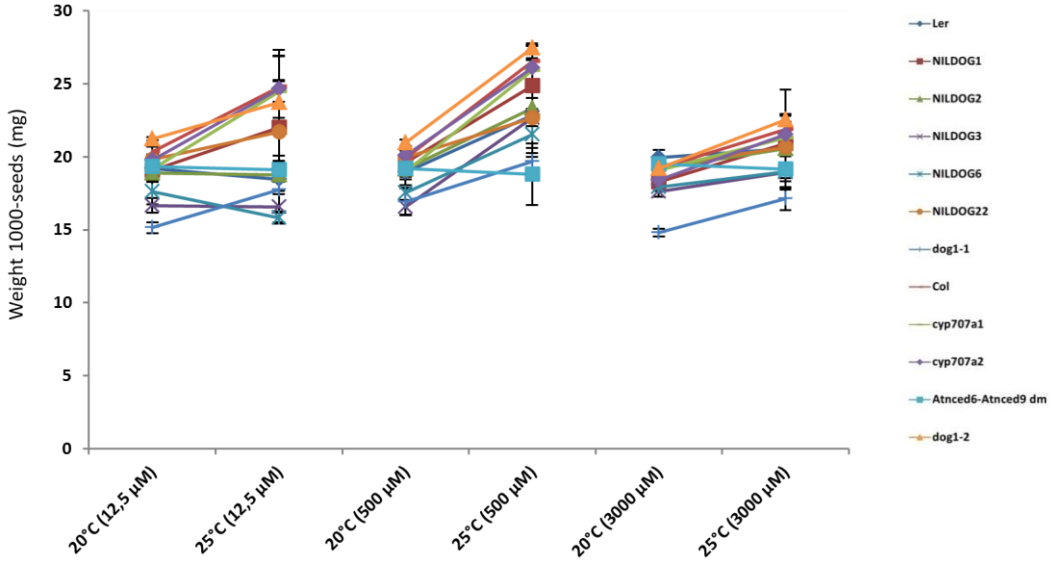

B
Col
number of siliques/plant
80
100
120
140
160
180
220
Temperature (°C)


C
Number of seeds per silique
Ler
NILDOG1
NILDOG2
NILDOG3
NILDOG6
NILDOG22
dog1-1
Col
cyp707a1
cyp707a2
Atnced6-Atnced9 dm
dog1-2
20°C (12,5 µM)
25°C (12,5 µM)
20°C (500 µM)
25°C (500 µM)
20°C (3000 µM)
25°C (3000 µM)

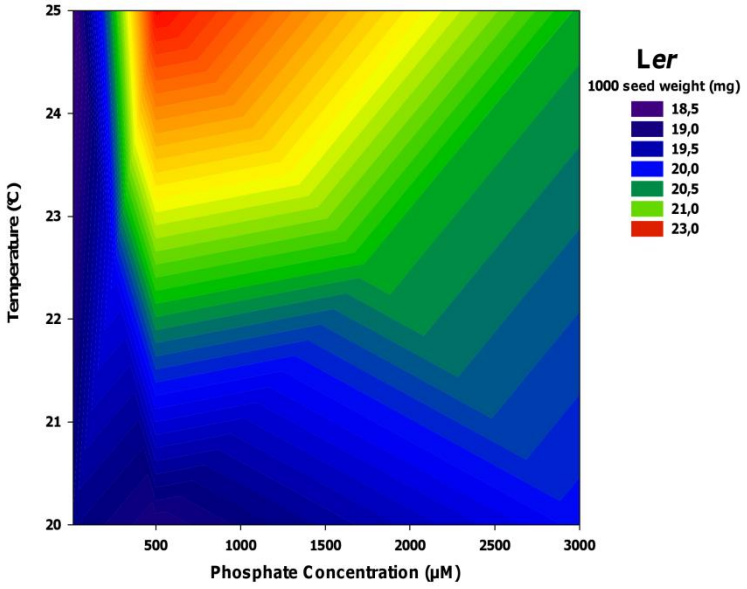
D


Seed Size (mm2)
0.3
0.25
0.2
0.15
0.1
0.05
0
20°C (12,5 µM)
25°C (12,5 µM)
20°C (500 µM)
25°C (500 µM)
20°C (3000 µM)
25°C (3000 µM)
Ler
NILDOG1
NILDOG2
NILDOG3
NILDOG6
NILDOG22
dog1-1
Col
cyp707a1
cyp707a2
Atnced6-Atnced9 dm
dog1-2

E


Weight 1000-seeds (mg)
30
25
20
15
10
5
0
20°C (12,5 µM)
25°C (12,5 µM)
20°C (500 µM)
25°C (500 µM)
20°C (3000 µM)
25°C (3000 µM)
Ler
NILDOG1
NILDOG2
NILDOG3
NILDOG6
NILDOG22
dog1-1
Col
cyp707a1
cyp707a2
Atnced6-Atnced9 dm
dog1-2

F


Temperature (°C)
25
24
23
22
21
20
500
1000
1500
2000
2500
3000
Phosphate Concentration (µM)
Ler
1000 seed weight (mg)
18,5
19,0
19,5
20,0
20,5
21,0
23,0

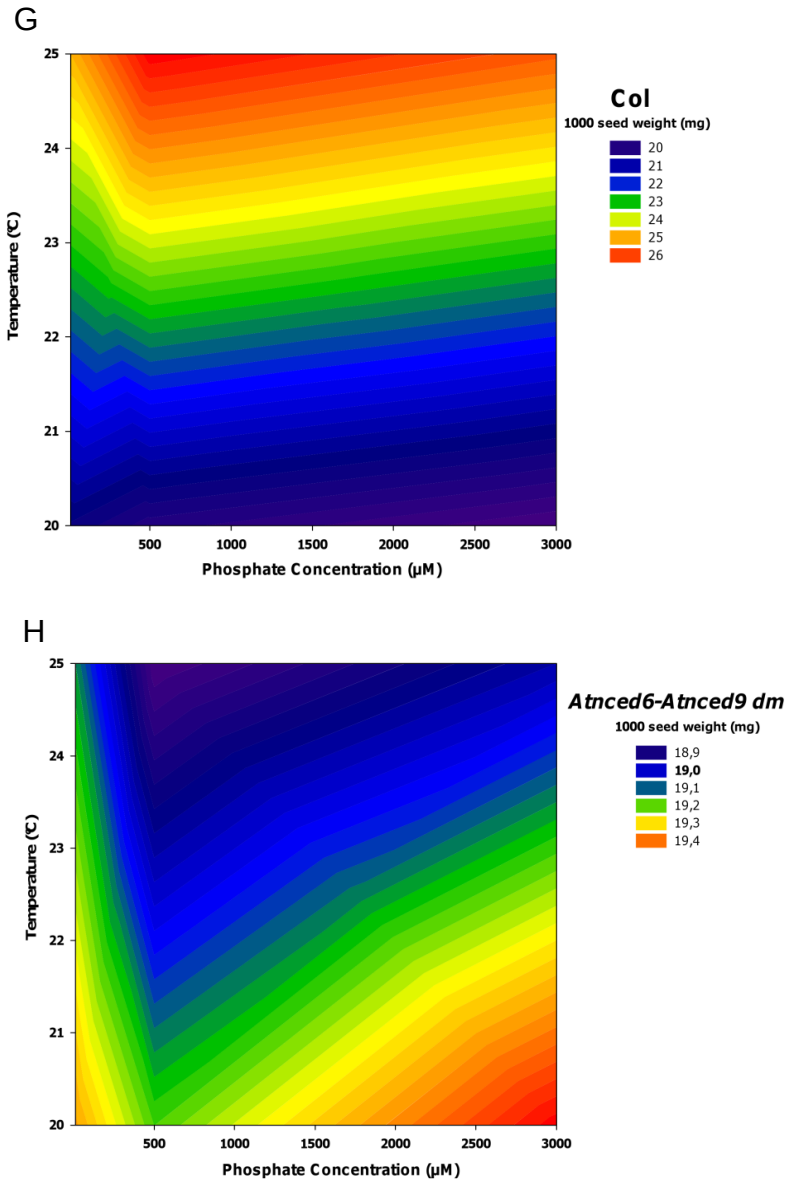


Figure 5. Effect of the parental environment on plant and seed development: (A) Influence of phosphate concentration in plants grown at 20°C and 25°C; (B, F, G, H) Interaction of phosphate concentration and temperature during seed development; (C, D and E) Influence of temperature during seed development in three different phosphate concentration. (A) Plant Height (cm); (B) Number of siliques per plant for wild type Col; (C) Number of seeds per siliques; (D) Seed size (mm^2); (E) 1000 seed weight (mg); (F) 1000 seed weight for wild type L*er;* (G) 1000 seed weight for wild type Col; (H) 1000 seed weight for genotype *Atnced6-Atnced9* (dm). Each bar represents the mean of three replicates, and error bars represent SE. The significance of effect of the treatment in graphic A were determined by a student's test ($P<0.05$).

Number of seeds per silique is most strongly affected by temperature during seed development (Table 5, Fig. 5C). Higher maturation temperature (25°C) led to fewer seeds per silique when compared to lower temperature (20°C) (Fig. 5C). Although these seeds produced at 25°C are larger and heavier (Fig. 5D and E, respectively). *Cyp707a2* was the only exception and did not show correlation between parental temperature and number of seeds per silique (Fig.6). Although all plants with Col background are taller than plants with L*er* background (Fig. 5A), the number of siliques per plant, number of seeds per siliques, seed size and 1000 seed weight did not differ.

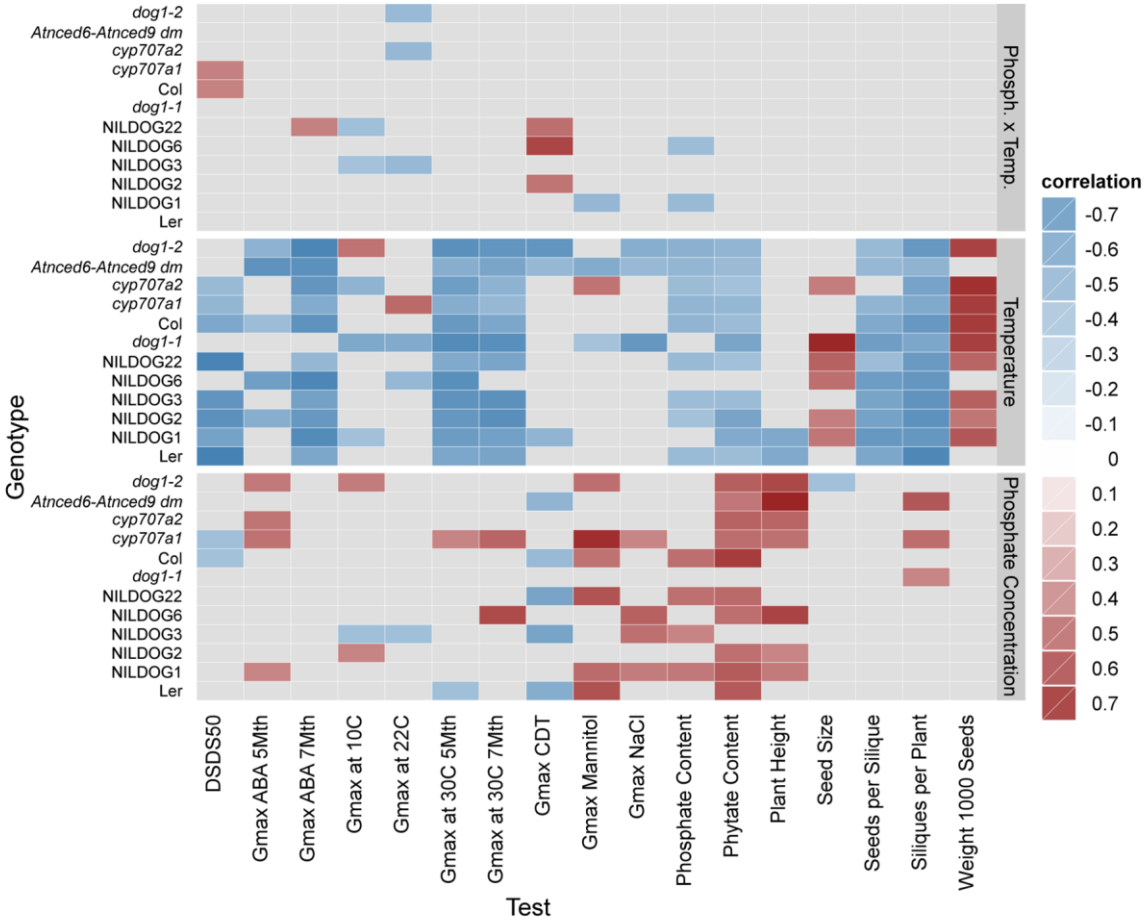


Figure 6. Correlation coefficients between genotypes, applied treatments and seed or parental plant tests (see material and methods). The correlation matrix is divided into three different groups, phosphate concentration, temperature and phosphate concentration x temperature. Positive correlation (red), negative correlation (blue) and no correlation (*p*-value higher than 0.05 (grey))

The gMAX is dependent on the stress condition under which the germination occurs and the temperature and the phosphate under which the seeds have been developed (S x T x P) (Table 5). Details of the different stress conditions are being discussed below.

3.2.2. The Effect of the Parental Environment on Seed Dormancy

The selection of the genotypes used in this experiment was based on their differences in dormancy levels. These dormancy levels could be confirmed when growing the lines under standard conditions (22°C, 500 µM Phosphate). NIL*DOG1*, NIL*DOG3*, NIL*DOG6*, *cyp707a1* and *cyp707a2* present a strong dormancy, whereas *dog1-1*, *dog1-2* and *Atnced6-Atnced9* (dm) do not have dormancy at all. The remainder of the lines (Ler, Col, NIL*DOG2* and NIL*DOG22*) has an intermediate dormancy level. The temperature effect was only visible in the NIL*DOG1*, NIL*DOG3* and NIL*DOG6* (L*er* background). NIL*DOG1*, NIL*DOG3*, and NIL*DOG6* grown at standard phosphate level (500 µM) at 25°C were less dormant than these plants grown at the same phosphate level at 20°C (Fig.7A).

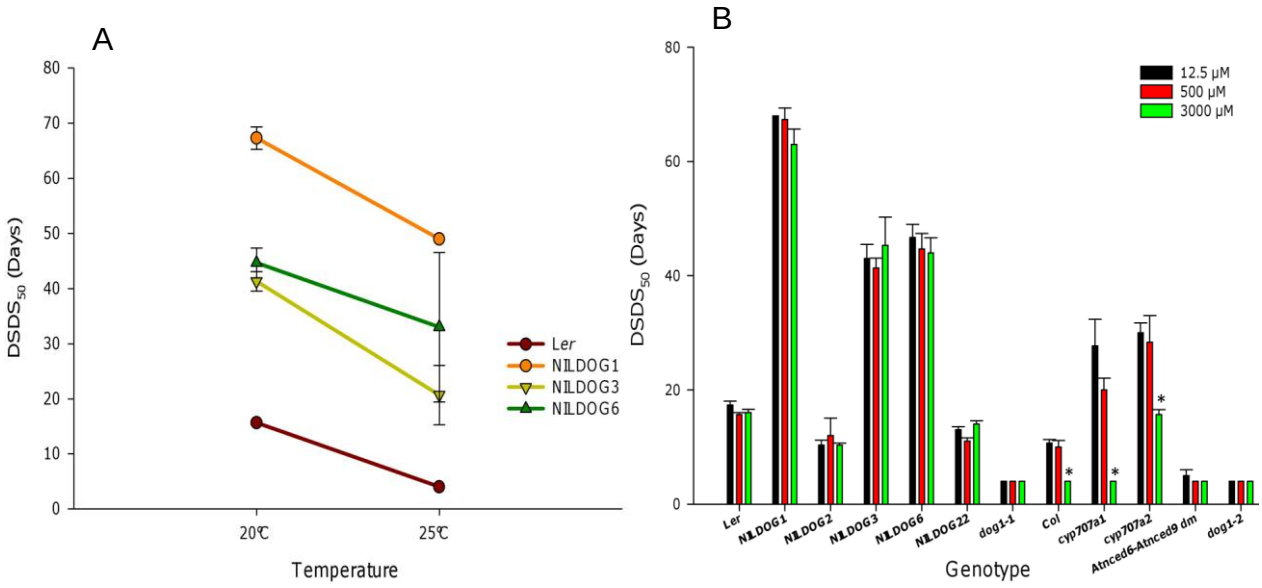


Figure 7. Effect of environment during seed development (A) Comparison of $DSDS_{50}$ of L*er*, NIL*DOG1,* NIL*DOG3* and NIL*DOG6* in different temperatures during seed development.; (B) $DSDS_{50}$ (Days of seed dry storage required to reach 50% germination) under different phosphate concentrations at 20°C. Each bar represents the mean of three replicates, and error bars represent SE. The significance of effect of the treatment in graphic B was compared by student's test ($P<0.05$).

The influence of phosphate concentration was observed only in plants with Col background (Col, *cyp707a1* and *cyp707a2*) (Fig. 7B). In all cases higher phosphate reduced the dormancy level.

3.2.3. The Effect of the Parental Environment on Germination under Stress Conditions

The genotypes differed in their ability to germinate under stress conditions depending on the phosphate concentration and temperature in which the seeds were development. Foolad et al. (1999) worked with tomato, showed that stress conditions such as extreme temperatures, salt stress, and water deficit delayed or completely inhibited germination depending on the stress intensity and the genetic background.

Germination at Low Temperature

The effect of phosphate on low germination temperature was only visible in seeds that were developed under high temp (25°C). Germination at 10°C was lower at 3000 µM phosphate at 25°C for the *dog1-1* mutant, however this was

not significantly different from the gMAX in the standard condition (500 µM). For L*er*, no difference was found in germination at 10°C (Fig.8).

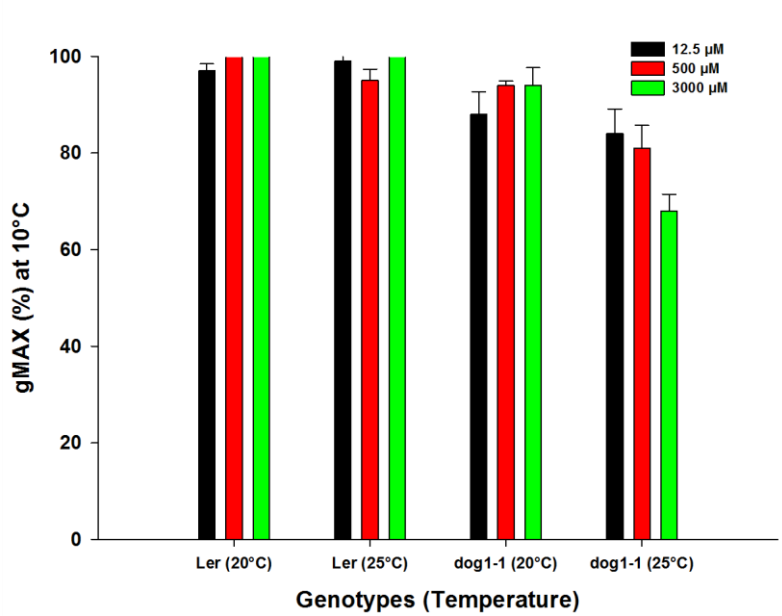


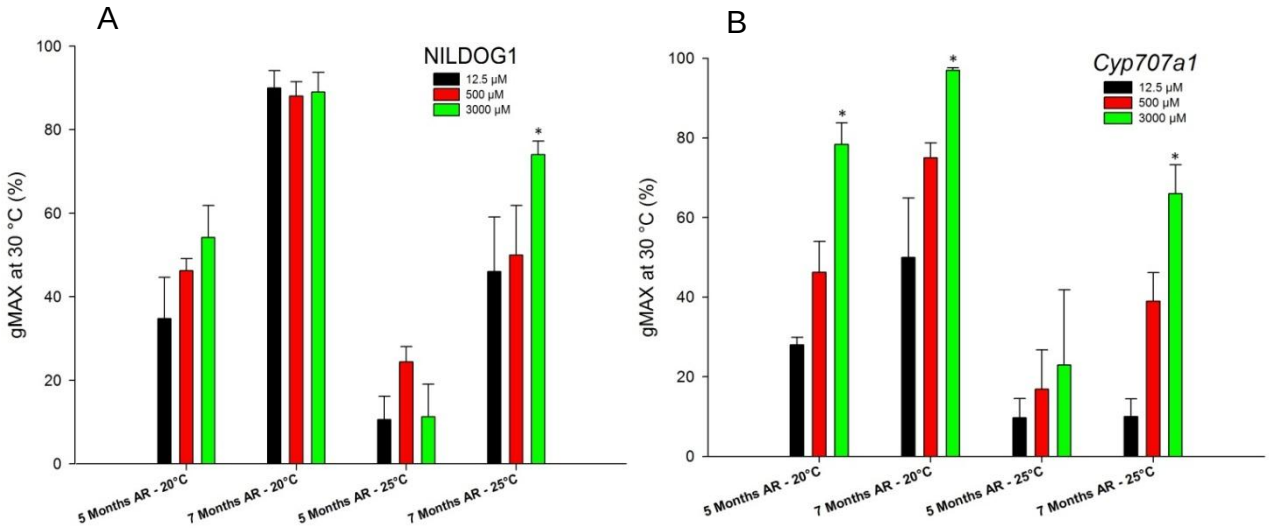
Figure 8. Effect of environment during seed development on germination at 10°C for genotypes L*er* and *dog1-1*. Each bar represents the mean of three replicates, and error bars represent SE.

Germination at High Temperature

Germination tests at 30°C were performed twice, at 5 and 7 months of after-ripening (AR), because of the rather low germination after 5 month of AR. In order to investigate whether germination would improve after longer AR the experiment was repeated after 7 months of AR. Different behavior in the two experiments was seen especially for the more dormant genotypes (NIL*DOG1* and cyp707a1). On average the germination percentage for NIL*DOG1* is two times (mean 2.04; stdev 0.48) higher after 7 months of AR at 20°C compared to the 5 months of AR. At 25°C, the average germination percentage increases up to 4-fold for 7 months of AR (mean 4.32; stdev 2.26) (Fig.9A).

For germination at 30°C after 5 months of AR, the phosphate concentration during seed development had only a significant effect on the germination percentage for the *cyp707a1* mutant grown at 20°C. However, this influence could not be observed anymore for *cyp707a1* grown at 25°C (Fig. 9B). Whereas germination at 30°C after 7 months, the influence of phosphate concentration during seed development had a significant effect for the *cyp707a1* mutant grown in both temperature (20°C and 25°C) (Fig.9B).

The Figure 9C shows the comparison between germination at 22°C (standard condition) and germination at 30°C for plants grown at 20°C (5 months of AR). The wild type genotypes L*er* and Col do not differ between germination in normal condition and in stress condition. Whereas NIL*DOG1* (L*er* background) and *cyp707a1* mutant (Col background) differ among the two germination temperatures, showing decrease of germination at 30°C. Whether or not the effect of phosphate is significant seems to depend mostly on the germination percentage, when the gMAX of the standard condition is around 50% the window seems to be large enough to see the phosphate effect. In general high phosphate leads to a higher gMAX at 30°C.



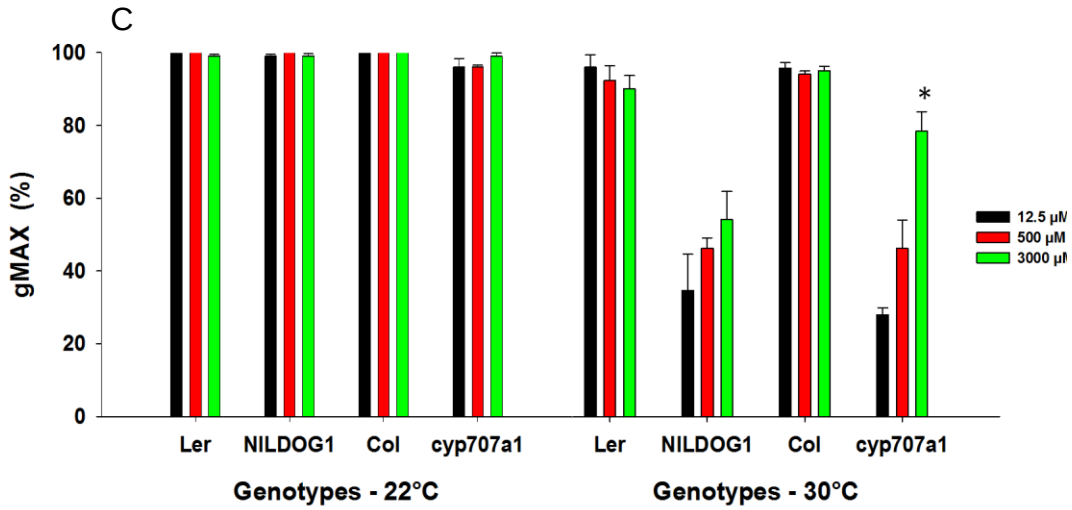


Figure 9. Effect of environment during seed development on germination at 30°C in two different times of germination (5 and 7 month of after-ripening (AR)). (A) Germination at 30°C of genotype NIL*DOG1* (B) Germination at 30°C of genotype *cyp707a1*; (C) Comparison between germination at 22°C and germination at 30°C for genotypes L*er*, NIL*DOG1*, Col and *cyp707a1* for plants grown at 20°C (5 months AR). Each bar represents the mean of three replicates, and error bars represent SE. The significance of effect of the treatment was tested by a student's test ($P<0.05$).

Germination at High Salt

Overall high salt decreased the germination ability of all genotypes. L*er* and Col had better germination performance when developed at high phosphate concentration and low temperature during seed development (Fig. 10A and C, respectively). For Col, a high phosphate level combined with high temperature resulted in lower germination, like was the case for *dog1-1* (Fig 10B). Whereas for *cyp707a2* the opposite was observed, the worst performance was at low temperature and low phosphate concentration (Fig. 10D).

The germination percentage decreased in NaCl (Figure 10E). The deprivation of phosphate levels on the plants provided a lower germination percentage in NaCl.

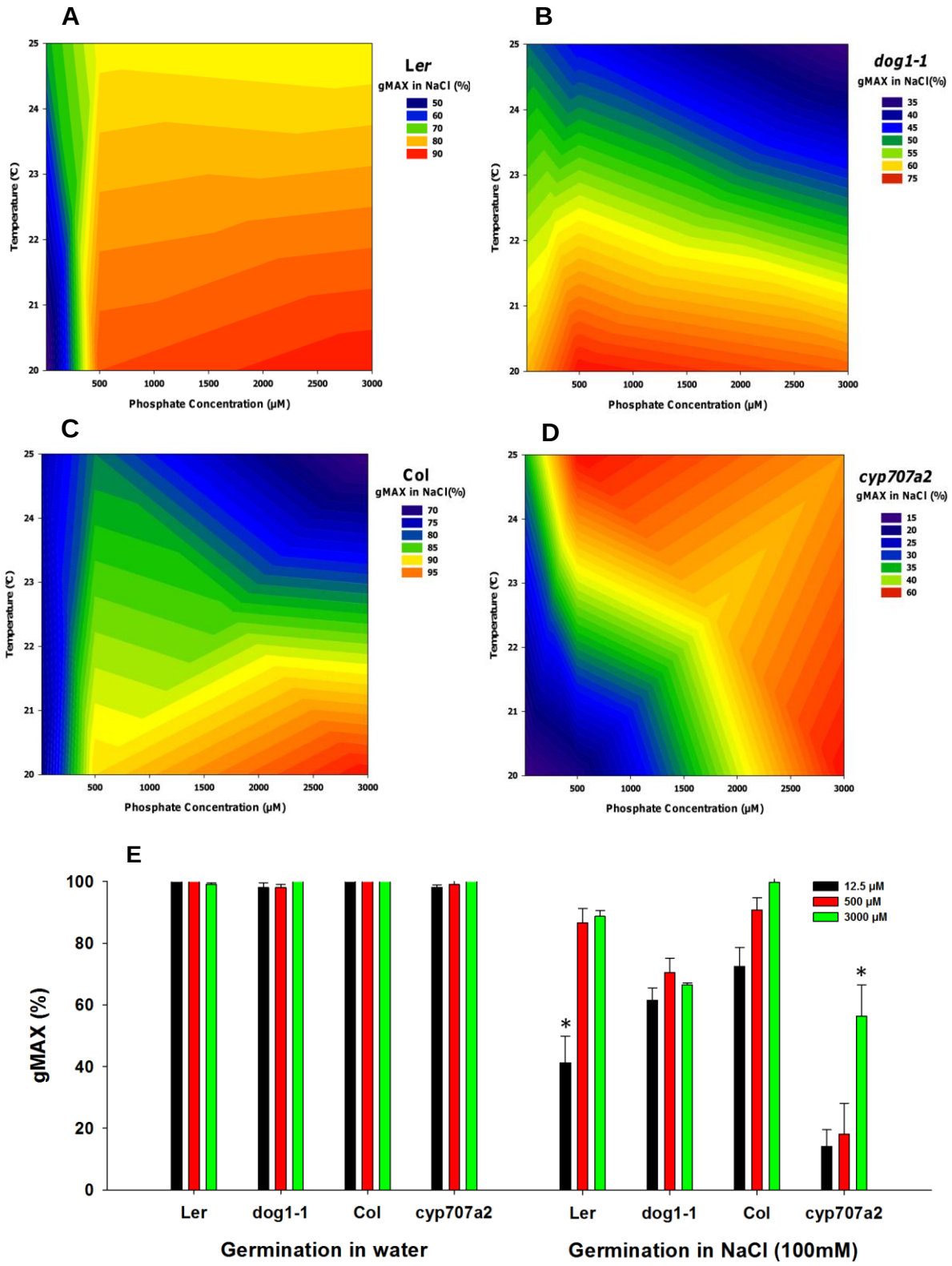


Figure 10. Germination in NaCl (100mM). Interaction of phosphate concentration and temperature during seed development in different genotypes: (A) L*er*; (B) *dog1-1*; (C) Col; (D) *cyp707a2*; (E) Comparison between germination in water with germination in NaCl for genotypes L*er*, *dog1-1*, Col and *cyp707a2* for plants

grown at 20°C and 25°C. Each bar represents the mean of three replicates, and error bars represent SE. The significance of the treatment was analyzed by a student's test (P<0.05).

Germination at Mannitol

In order to determine whether the salt effect on germination is a true salt effect or whether it is an osmotic effect we have germinated the seeds at mannitol. Mannitol is a putative osmoprotectant contributing to salt tolerance in several species (Chan et al., 2011). Overall mannitol decreased the germination ability of all genotypes, with the exception of the *Atnced6-Atnced9* (dm) which seems to be insensitive to mannitol, when grow at 20°C (Fig 11E). Only combination of high temperature and low phosphate level during seed development seems to inhibit the germination percentage of the *Atnced6-Atnced9* (dm) to a gMAX of 65% (Fig. 11B). Col and L*er* had better germination performance in mannitol when grown in high phosphate concentrations and low temperature during seed development (Fig. 11A and C, respectively), similar as was seen for the germination at high salt. There are no influences of temperature in lower phosphate level for both genotypes. The germination behavior of NIL*DOG22* is very remarkable, since it has a better performance in high phosphate level and high temperature during seed development (Fig. 11D).

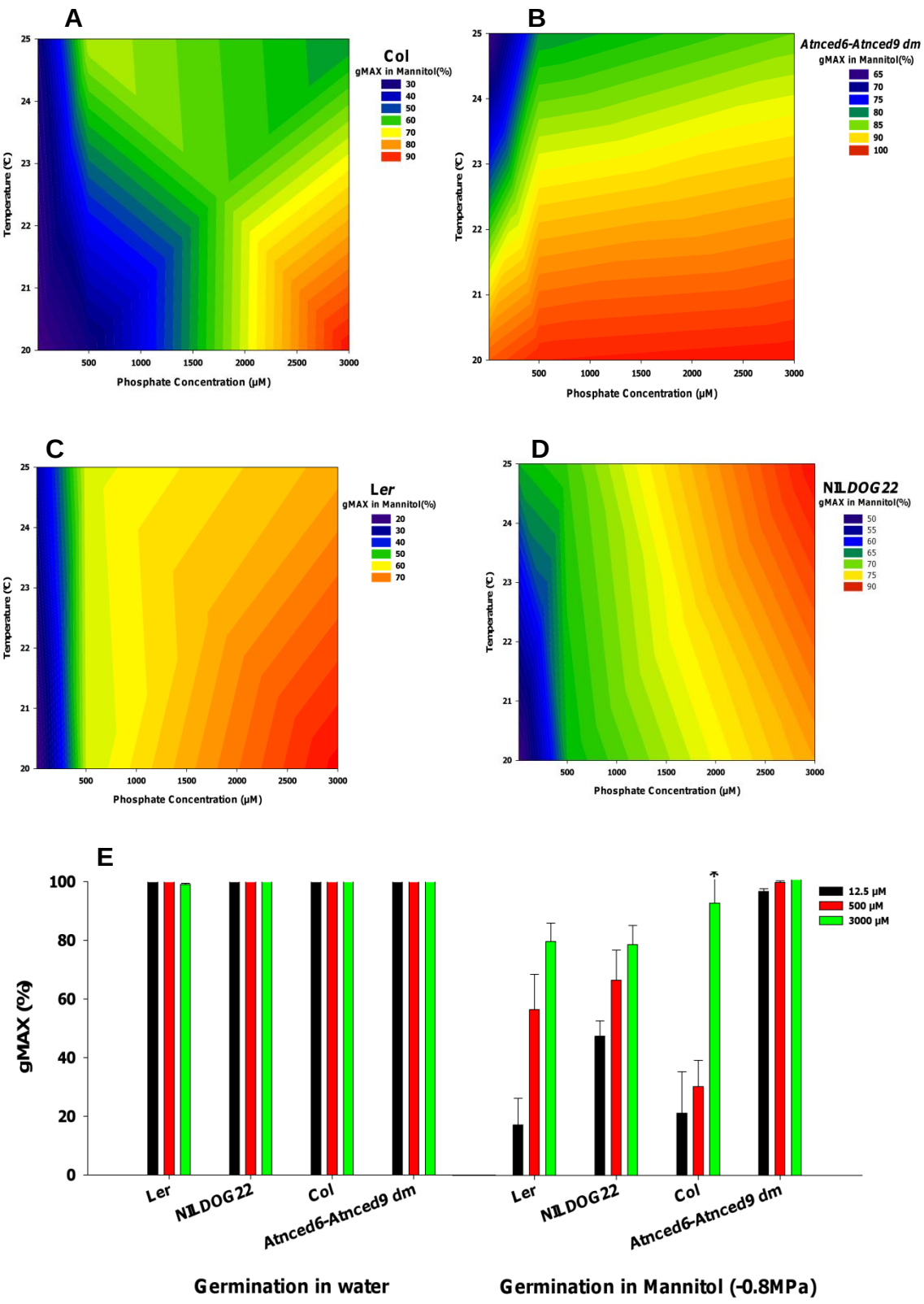


Figure 11. Germination in Mannitol (-0.8MPa). Interaction of phosphate concentration and temperature during seed development in different genotypes: (A) Col; (B) *Atnced6-Atnced9* (dm); (C) L*er*; (D) NIL*DOG22*; (E) Comparison between germination in water and germination in mannitol (-0.8MPa) for genotypes L*er*, NIL*DOG22*,

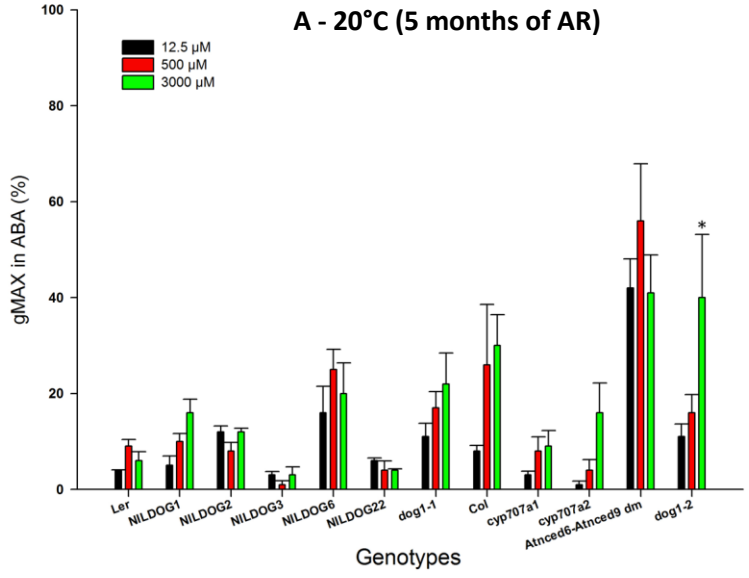
Col and *Atnced6-Atnced9* (dm) for plants grown at 20°C. Each bar represents the mean of three replicates, and error bars represent SE. The significance of effect of the treatment in graphic E were determined by a student's test ($P<0.05$).

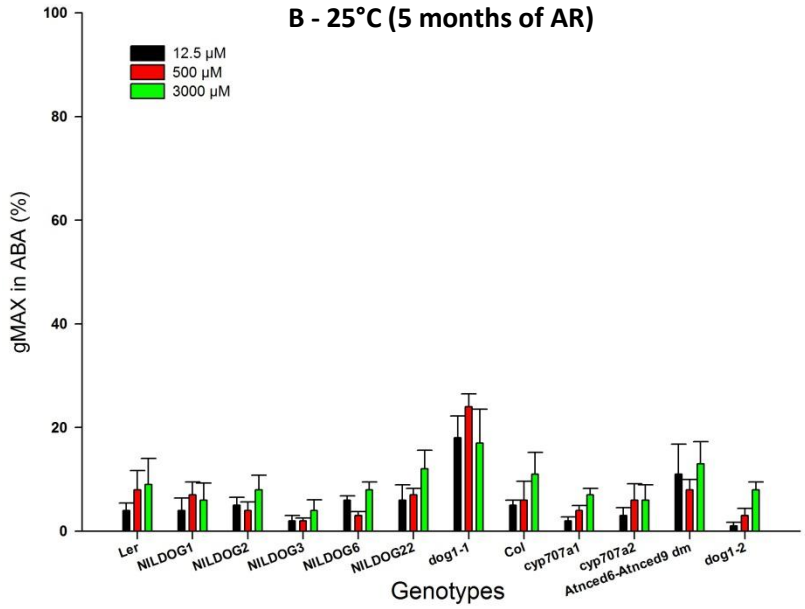
Germination at ABA

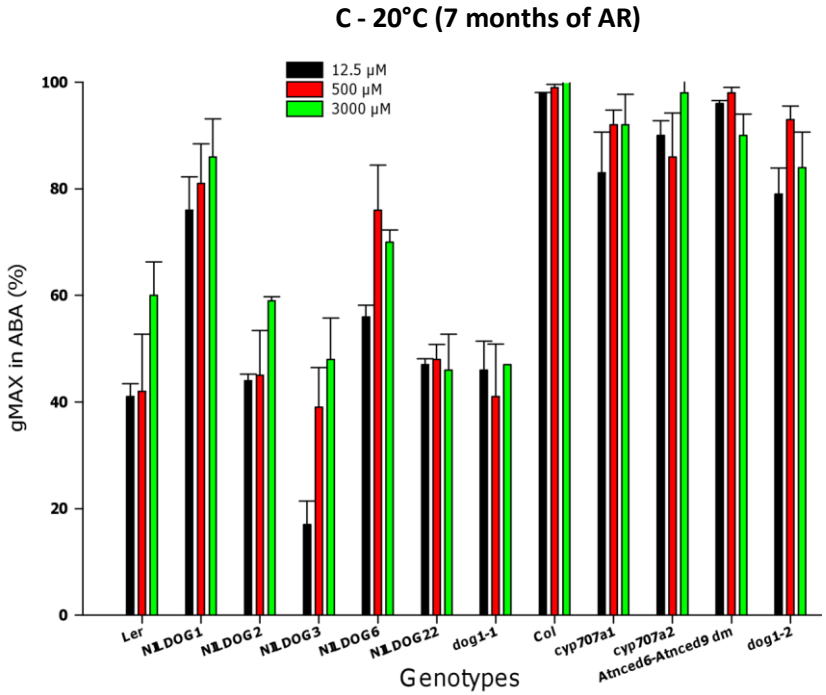
Like the other stress conditions, ABA decreased the germination capacity of all genotypes. Seeds were germinated in ABA after 5 and 7 of months of AR, respectively, with the same reason as mentioned for germination at 30°C. For germination in ABA, a stronger effect AR was observed (Fig. 12). The germination percentage in ABA increases after 7 months of AR (Fig. 12 C and D) compared with 5 months of AR (Fig. 12 A and B). The germination percentage in ABA from seeds development at 25°C had the worst performance (Fig. 12 B and D).

The effect of phosphate concentration on germination was only observed in plants grown at 25°C and after 7 months of AR (Fig. 12D). Seeds developed in higher phosphate concentration had a better performance of germination in ABA than seeds developed in standard phosphate conditions.

After 7 months of AR seeds at 20°C, genotypes of Col background showed a better performance of germination than L*er* background (Fig. 12C).




B - 25°C (5 months of AR)
12.5 µM
500 µM
3000 µM
gMAX in ABA (%)
Genotypes
Ler
NILDOG1
NILDOG2
NILDOG3
NILDOG6
NILDOG22
dog1-1
Col
cyp707a1
cyp707a2
Atnced6-Atnced9 dm
dog1-2


C - 20°C (7 months of AR)
12.5 µM
500 µM
3000 µM
gMAX in ABA (%)
Genotypes
Ler
NILDOG1
NILDOG2
NILDOG3
NILDOG6
NILDOG22
dog1-1
Col
cyp707a1
cyp707a2
Atnced6-Atnced9 dm
dog1-2

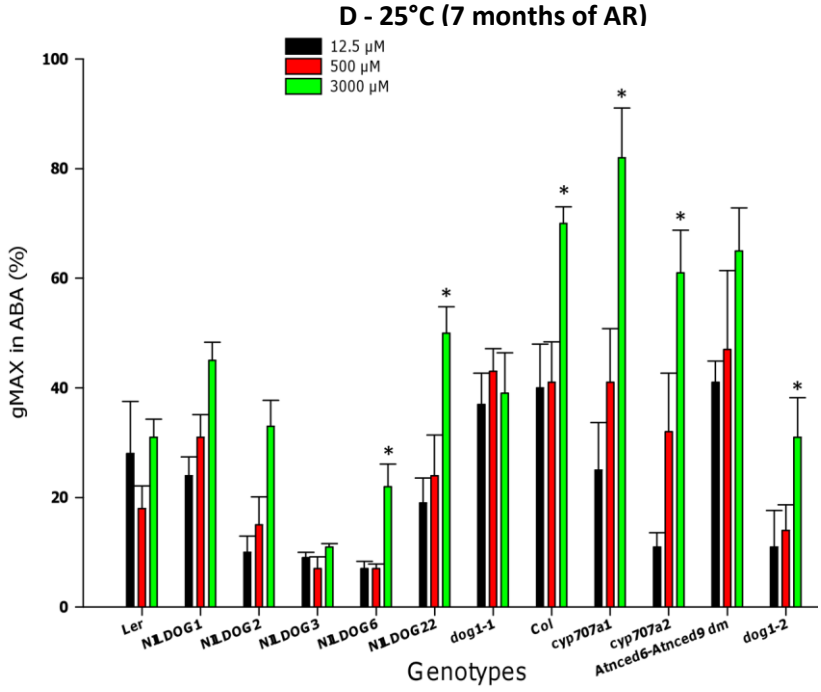

D - 25°C (7 months of AR)
12.5 µM
500 µM
3000 µM
gMAX in ABA (%)
Genotypes
Ler
NILDOG1
NILDOG2
NILDOG3
NILDOG6
NILDOG22
dog1-1
Col
cyp707a1
cyp707a2
Atnced6-Atnced9 dm
dog1-2

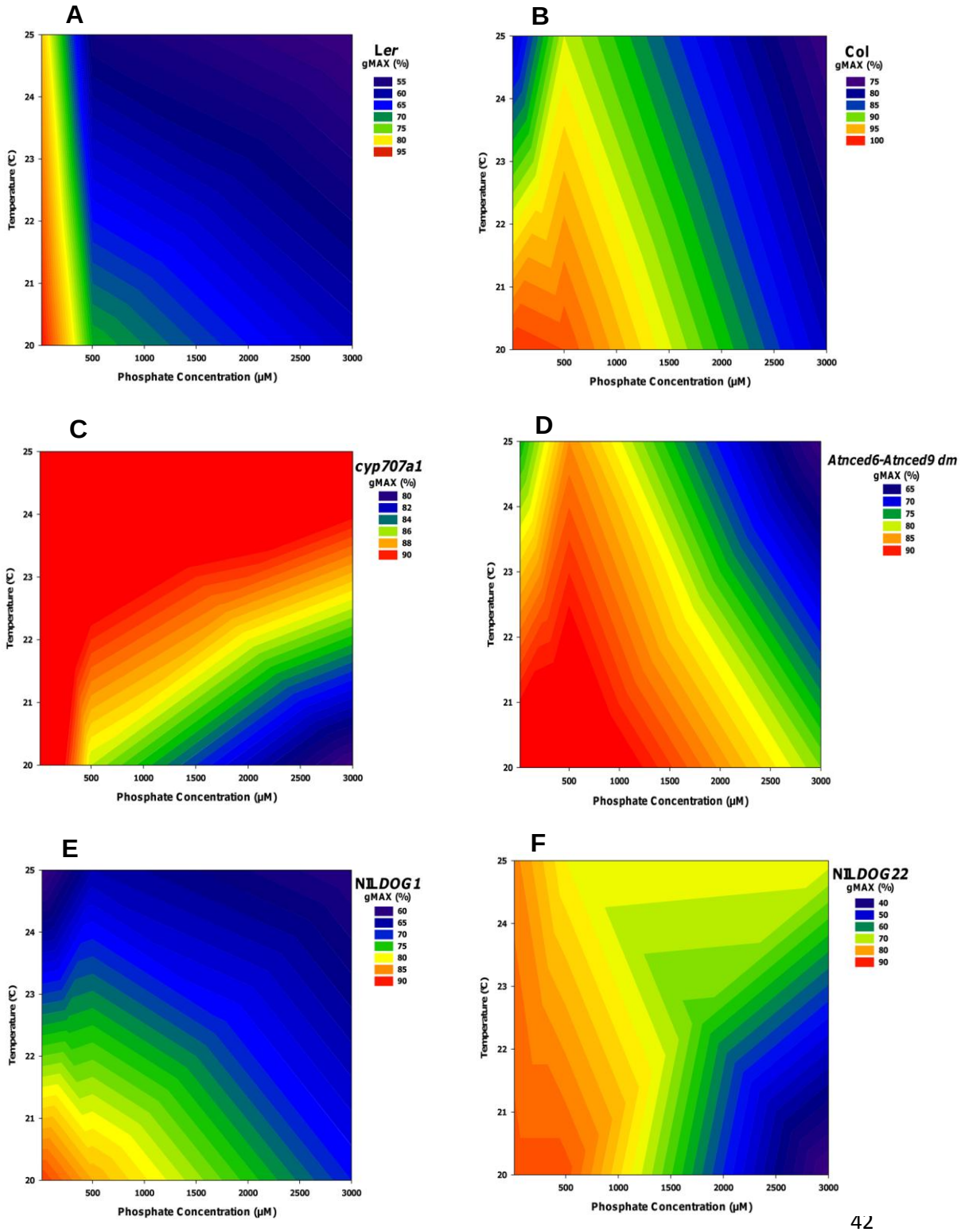
Figure 12. Germination in ABA (0.2 µM). (A) Seed development at 20°C and 5 months of after-ripening (AR); (B) Seed development at 25°C and 5 months of AR; (C) Seed development at 20°C and 7 months of AR; (D) Seed development at 25°C and 7 months of AR. Each bar represents the mean of three replicates, and error bars represent SE. The significance of effect of the treatment was tested by student's test ($P<0.05$).

3.2.4. The Effect of the Parental Environment on Seed Storability

In order to determine the effect of the parental environment on the storability of seeds we determined the germination ability after a controlled deterioration test (CDT). There was a lot of genetic variation among the Arabidopsis genotypes after the CDT (Fig. 13). All genotypes grown at 20°C in higher phosphate concentration were more sensitive to CDT than the ones grown at low phosphate concentration. The genotypes: L*er*, Col, *Atnced6-Atnced9* (dm) and NIL*DOG1* (Fig. 13A, B, D and E, respectively), showed worst performance of germination after CDT for plants grown in higher phosphate levels at 25°C. For genotypes *cyp707a1* (Fig. 13C) and NIL*DOG22* (Fig. 13F), the worst germination percentage was for plants grown in higher phosphate levels at 20°C. These results showed that plants grown in highest phosphate concentration produce seeds that are more sensitive to CDT.

Dog1-1 is a NIL*DOG1* in which the DOG1 gene is mutated resulting in loss of seed dormancy and this genotype cannot be stored as long as NIL*DOG1* wild-type seeds at room temperature (Bentsink et al., 2006). This is confirmed by it bad germination performance after CDT independent of the phosphate concentration and parental growth temperature (Fig. 13G).

In summary, plants of Columbia background, grown at high phosphate concentration (3000 µM) and 20°C presented significantly less dormancy than those plants grown at low phosphate concentrations (Fig. 7A). Plants with less dormancy (seed produced at 3000 µM) were more sensitive to CDT (Fig. 13H). These results suggest us that dormancy and CD tolerance may be linked.



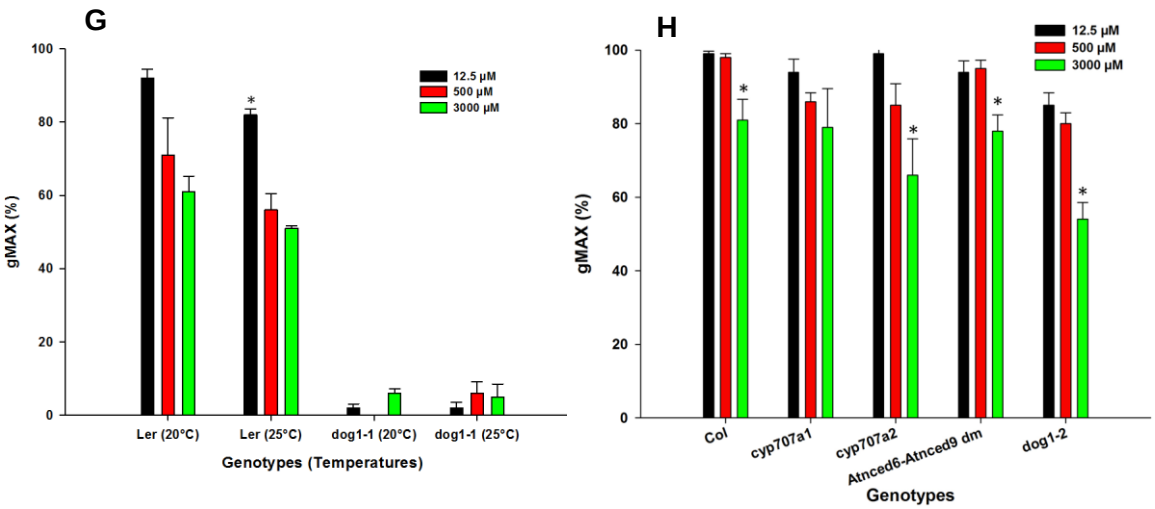


Figure 13. Interaction of phosphate concentration and temperature during seed development on germination after controlled deterioration test (CDT) for 5 days at 40°C. (A) L*er*; (B) Col; (C) *cyp707a1*; (D) *Atnced6-Atnced9* (dm); (E) NIL*DOG1*; (F) NIL*DOG22*; (G) Effect of environment during seed development on germination after CDT for genotypes L*er* and dog1-1 and (H) plants with the Col background grown at 20°C in three different phosphate concentrations. Each bar represents the mean of three replicates, and error bars represent SE. The significance of effect of the treatment in graphic G and H were determined by a student's test ($P<0.05$).

3.2.5. The Effect of the Parental Environment on Phytate and Phosphate Content

The major form in which phosphorus is stored in seed is phytic acid (InsP6) or phytate (Zhao et al., 2008) and the accumulation of phytate in seeds can account for up to several percent of dry weight and about 65-85% of seed total phosphorus (Raboy et al., 2001). In order to investigate whether the phosphate concentration during plant growth would affect the phosphate composition in the seeds of these plants we have analyzed seed phytate and phosphate contents. In this experiment, all genotypes evaluated show the same

trend for phytate accumulation in seeds. The combined effects of high phosphate concentration and low temperature resulted in highest phytate accumulated in seeds, as shown for the genotypes L*er* and Col in Fig. 14 A and B, respectively.

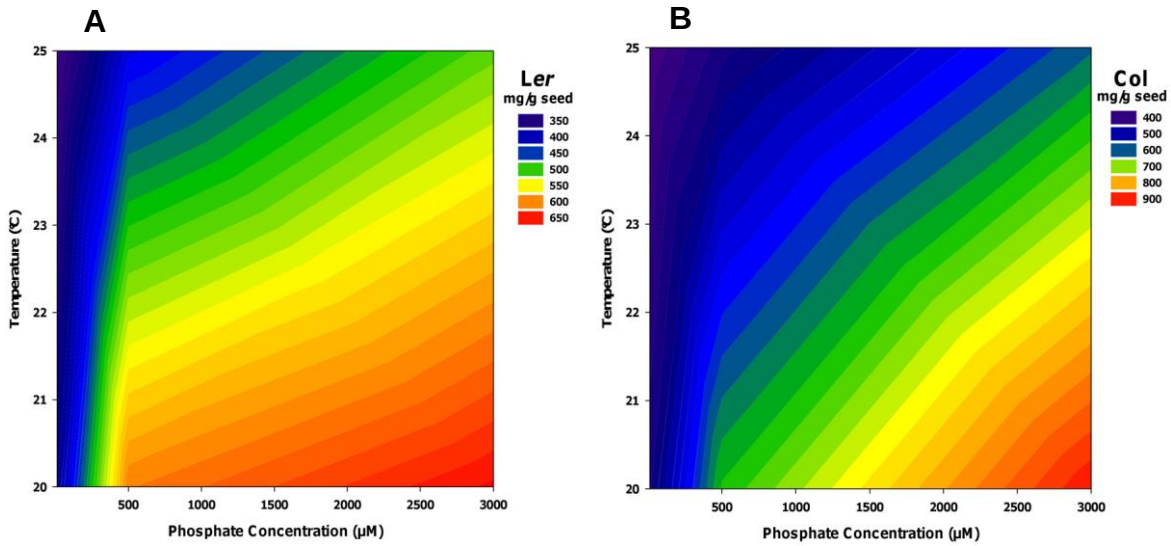
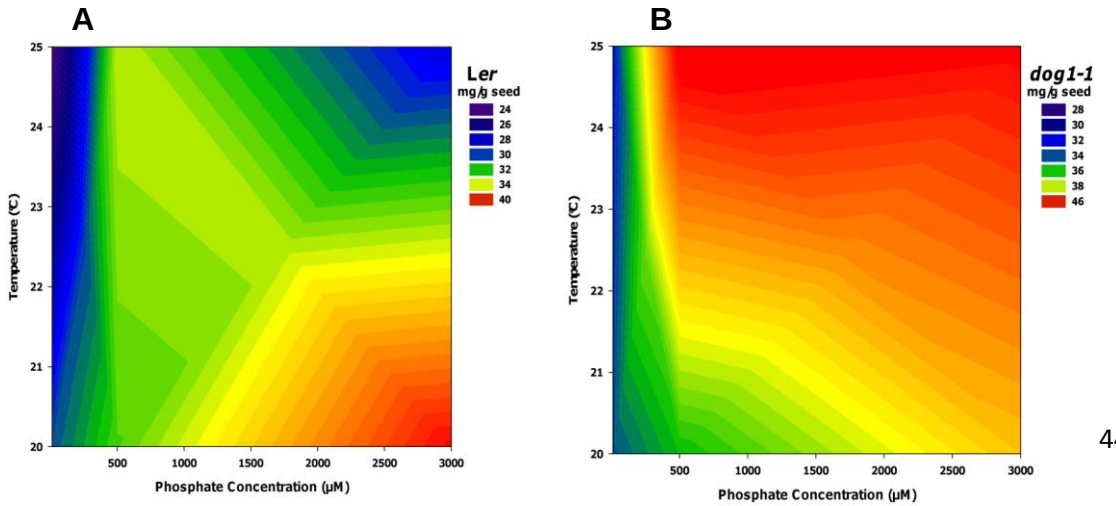


Figure 14. Phytate content (InsP6) in seeds. Interaction of phosphate concentration and temperature during seed development in different genotypes: (A) L*er*; (B) Col.

Although in low quantities, seeds also accumulate inorganic phosphate (Pi). The interaction of phosphate levels and parental growth temperature was observed for Pi content (Fig. 15). But the behavior of interaction was changed among genotypes.



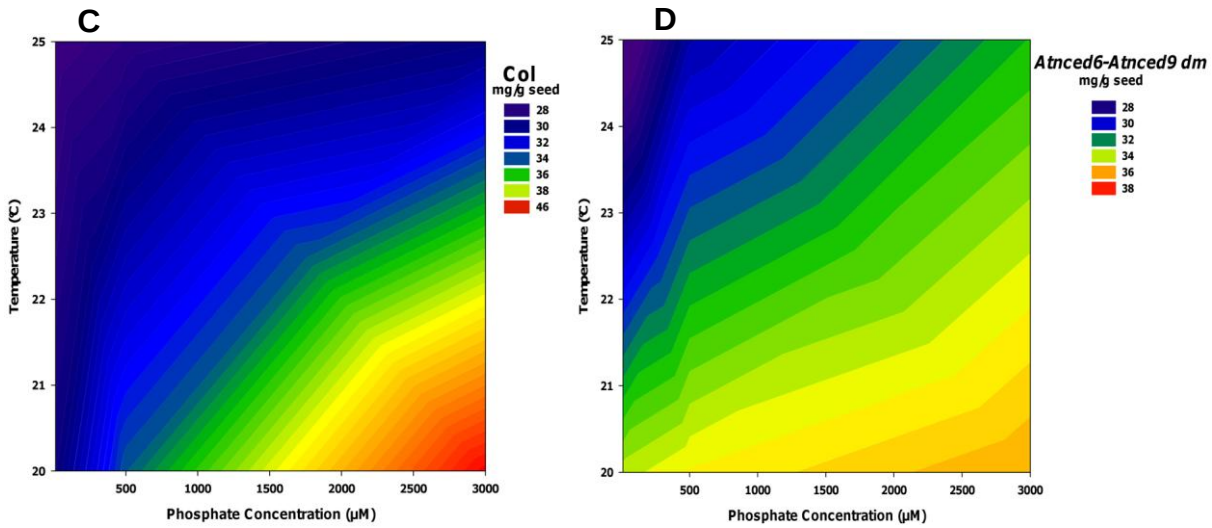


Figure 15. Phosphate content in seeds. Interaction of phosphate concentration and temperature during seed development in different genotypes: (A) L*er*; (B) *dog1-1*; (C) Col; (D) *Atnced6-Atnced9* (dm).

The seeds of the wild types L*er* and Col had highest phosphate content developed in high phosphate concentration (3000 µM) and low temperature during seed development (20°C) (Fig. 15 A and C, respectively). The double mutant *Atneced6-Atnced9* (Col background) also accumulated more phosphate in seed developed in high phosphate concentration (3000 µM) and low temperature (20°C), but the amount of phosphate content in the seeds was 20% less than the wild type Col (Fig. 15D). Only *dog1-1* increased the phosphate content in high temperature (Fig. 15B).

4. **DISCUSSION**

Seed quality is a combination of genetic homogeneity, physical appearance, viability, vigor, uniformity, dormancy and longevity (Basra, 2006). The result of poor quality of the seeds is related with the environmental

stresses, like the change of temperature, drought stress, lack of nutrients and light (Fenner, 1991; Bewley and Black, 1994). There are several reports about the effects of the parental environment on different aspects of seed quality, including germinability, dormancy, size, and composition (Fenner, 1991, 1992; Hilhorst, 1995; Baskin and Baskin, 1998; Contreras et al., 2008a, b; Contreras et al., 2009a, b).

Our pilot experiment showed us that a concentration of 50000 µM phosphate had toxic effect to the plants, and because of that, produced small plants and the low production of seeds (Fig. 2). Phosphate deprivation during seed development had effect on dormancy and a clear effect of phosphate concentrations during seed development on the final seed quality were observed (Fig. 3 and 4).

Plant height was strongly affected by the genotype (Fig. 5A). Large differences where seen between the genotypes in the L*er* background when compared to the genotypes that have the Col background, the Col genotypes being significantly taller. This effect could fully be explained by Landsberg *erecta* carries the erecta mutation and therefore has a short and erect stature, Columbia has the wild-type gene and is therefore taller and less compact. (Torii et al., 1996; Yokoyama et al., 1998; Shpak et al., 2004).

Our data indicated that the number of siliques per plant decreased with decreasing phosphate concentrations and increasing temperatures (Fig. 5B). The phosphate effect is in agreement with data published by Modi (2002). This author reported that high P concentration in wheat plants increased the production of seeds per plant and seed mass (g/plant), possibly by seed abortion. The effect of temperature and nutrition on plant development and seed

production seem to be rather general. For *Senecio vulgaris* temperature changes from warm to cold, decreased plant height and increased the number of inflorescences per plant (Figueroa et al., 2010). In addition, plants of *Plantago cunninghamii* from cool conditions were bigger, had low aborted flowers, increased number of spikes and number of seeds per capsule (Kochanek et al., 2011). For plants of *Senecio vulgaris* it was shown that maternal soil nutrient level affected too, i.e. plants grown in highest soil nutrients produced greater plant height, greater shoot and total biomass, and also high number of inflorescences and seed with high individual mass (Aarssen and Burton, 1990). This effect could be because of the longer life cycle of plants grown in lower temperatures, meaning that plants have more time to produce seeds.

The effect of the parental environments on seed dormancy is only visible in the most dormant lines (NIL*DOG1*, NIL*DOG3*, NIL*DOG6*, *cyp707a1* and *cyp707a2*). High temperature decreases seed dormancy as do high phosphate levels (Fig. 7A and B, respectively). Induction of dormancy in the seed as it develops on the parent plant is very sensitive to temperature as well as the persistence of the primary state of dormancy is strongly influenced by temperature (Simpson, 1990). In general, the duration of dormancy in mature seeds of grasses is enhanced by low temperatures during seed development and diminished by high temperatures (Simpson, 1990). It has been reported that seeds produced in warm conditions are less dormant, whereas those produced under cold conditions are mostly dormant (Roach and Wulff, 1987; Fenner, 1991; Figueroa et al., 2010; Kendall et al., 2011).

The parental temperature effect on seed dormancy is in agreement with work published by Kendall et al. (2011) who showed that the seed maturation transcriptome in *Arabidopsis thaliana* is highly temperature sensitive and revealed that low temperature during seed maturation induces several genes associated with dormancy, including *DOG1* (*Delay of Germination 1*). Although elimination of the synthesis of ABA in Arabidopsis, by the use of ABA mutants, has resulted in the elimination of dormancy induced by cold temperature during seed maturation, *DOG1* expression predicted dormancy was better than expression of genes involved in ABA metabolism (Chiang et al., 2011).

In general, the addition of nutrient fertilizer to parental plants decreases dormancy in the seeds of several species (Fenner, 1991). Benech-Arnold et al. (1995) observed that reduced potassium nutrition in developing seeds of *Sorghum bicolor* increased the germinability, because the ABA content in seeds was reduced. Conditions favoring nitrate accumulation in mother plants of Arabidopsis lead to a lower dormancy of Arabidopsis seeds (Alboresi et al., 2005). This is in contrast with data published for barley, where the effects of soil N availability on dormancy release, were not found (Gualano and Benech-Arnold, 2009). This could have to do with low differences in N, Malagoli et al., (2005) showed that 73% of the total N in silique is remobilized from vegetative parts which apparently can buffer for shortage of N in the soil.

Matakiadis et al. (2009) reported that high nitrate concentrations releases seed dormancy in Arabidopsis in part by reducing abscisic acid levels. Furthermore, these authors stress the central role of the CYP707A2 gene in controlling seed dormancy in response to nitrate concentration. A similar effect was found by Modi and Cairns (1994) who observed that molybdenum

deficiency in wheat resulted in lower seed dormancy by decreasing abscisic acid concentration. It is likely that a deficiency of molybdenum, a co-factor of nitrate reductase, reduces nitrate reductase activity, resulting in higher nitrate levels of the seeds. Little is known about how phosphorus can influence the seed quality. Jain et al., (1982), Quick et al., (1983) and Quick et al., (1997) suggest an inverse relationship between seed dormancy and the amounts of inorganic phosphorus (Pi) available in wild oat. Wild oat seeds produced from phosphorus-stressed plants contained less phosphate were more dormant than seeds from unstressed plants of the same line grown in the same environment chamber (Quick et al., 1983). According to Saluja et al. (1987), exogenous Pi mimics the specific action of GA_3 in regulation the activity of certain enzymes in embryo-less half-seeds of wheat. In our experiment, plants with the Col background grown in highest phosphate concentration produced seeds with lowest dormancy levels. These results support the hypothesis that seed dormancy is influenced by phosphate level, but its degree of influence is dependent on the genotype.

Our experiment showed that the genotypes differed in their ability to germinate under stress conditions, and that the germination depends on the parental environment during seed development. Germination tests at 30°C were performed twice, at 5 and 7 months of AR. Especially the more dormant genotypes NIL*DOG1* and *cyp707a1*, showed that treatment after-ripening, besides releasing seed dormancy also has an influence on germination at 30°C (Fig. 9A and B). Germination at 30°C decrease the germination percentage for all genotypes, but in general higher phosphate concentration during seed development leads to higher germination percentage at 30°C when compared

with standard phosphate concentration (Fig. 9C). ABA levels can be significantly affected by temperature, increase the temperature of germination can decrease the percentage of germination for some species (Walker-Simmons, 1988; Bewley and Black 1994; Hilhorst, 1995). High temperature inhibits germination of lettuce seeds, by maintaining high endogenous ABA contents without affecting endogenous GA contents (Gonai et al., 2004). According to Toh et al. (2008) high temperature stimulates ABA synthesis and represses GA synthesis and signaling through the action of ABA in Arabidopsis seeds. In this experiment, plants grown at high phosphate concentration produced less dormant seeds (Fig. 7B) with a better performance in germination at high temperature (Fig. 9C). These results suggest that phosphate may be involved in ABA synthesis or catabolic pathways and probably prevents the ABA synthesis. Whether this is indeed the case in our experiment will be determined in a future study in which we will analyze the ABA content of the same seeds for which we now have analyzed the germination behavior.

Salinity stress decreased the germination ability of all genotypes (Fig. 10). High phosphate concentration and low temperature during seed development provided a better germination performance in high salt of genotypes L*er* and Col (Fig. 10A and C, respectively). Whereas high phosphate level combined with high temperature decreased the germination ability for *dog1-1* mutant (Fig. 10B) and the opposite, low temperature and low phosphate concentration provided a worst performance for the genotype *cyp707a2* (Fig.10D). High salt conditions in Arabidopsis induce high levels of ABA and inhibit germination (Xiong and Zhu, 2002). Phosphate starvation induced phosphatases that play a role in an organism's adaptation to stress (Dick et al.,

2011). In agreement with these authors, our results showed that the increase of phosphate levels could have prevented the synthesis of ABA, increasing the sensibility the Arabidopsis seeds on germination in salt stress.

Mannitol has a function in osmotic stress tolerance by serving as a compatible solute or osmoprotectant, contributing to salt tolerance (Chan et al., 2011). With the exception of the double mutant *Atnced6-Atnced9*, all genotypes decreased the germination ability (Fig. 11E). Temperature and lower phosphate did not affect the germination behavior of the wild types Col and L*er* (Fig. 11A and C). For the others genotypes the effect was dependent on the genotype (Fig. 11B and D). According to Lefebvre et al. (2006), the *Atnced6-Atnced9* (dm) seeds have a mutation in genes that are involved ABA biosynthesis during seed development, therefore this mutant does not produce ABA in the seeds and is not dormant. Apparently ABA is required for the response to mannitol. In figure 11E, the germination percentage in mannitol for *Atnced6-Atnced9* (dm) was higher than the wild type Col. In agreement with this Clerkx et al. (2004b) showed that the dormant Arabidopsis accession Shakdara decreased the germination percentage in mannitol comparing to the L*er* (less dormant) accession, although they could not confirm the relationship between germination in mannitol and dormancy by locating QTLs.

The germination behavior in ABA was performed after 5 and 7 months of after-ripening (AR), like was done for the germination at 30°C. Similar to the germination at 30°C and effect of AR in seed quality was observed (Fig. 12). The germination percentage in ABA increased after 7 months of AR compared with 5 months of AR. The germination percentage in ABA from seeds development at 25°C had a worst performance (Fig. 12 B and D), contradicting

the results found by Contreras et al. (2009b), where seeds produced under high temperature had lower sensitivity in exogenous ABA.

The seeds from higher parental phosphate had a better germination performance in ABA than seeds developed in standard phosphate concentration when grown at 25°C, after 7 months of AR (Fig. 12D). There is no literature on phosphate in respect to germination under stress however, according to Alboresi et al. (2005), the maternal nitrate concentration did not influence Arabidopsis seeds germinated in ABA solution. Although high nitrate concentrations lead to low dormant seed. This result support the hypothesis that nitrate effect on seed dormancy probably did not involve a change in sensitivity to ABA, but nitrate accumulation in seeds is correlated with a lower requirement of gibberellin for germination (Alboresi et al., 2005).

The controlled deterioration test (CDT) was developed as an alternative to analyze quickly and efficiently the seed storability (Tesnier et al., 2002). We used this test in order to investigate if the parental environment can influence the seed storability. Our result showed a lot of genetic variation among the Arabidopsis genotypes after the CDT (Fig. 13). Plants grown in highest phosphate concentration produce seeds that are more sensitive to CDT, especially for plants grown at 20°C.

Overall plants with the Col background, grown at high phosphate concentration and at 20°C were less dormant (Fig. 7B) and more sensitive to CDT (Fig. 13H). In agreement with data published by Tesnier et al. (2002), where they checked the genetic variation in Arabidopsis by controlled deterioration test and concluded that genotypes with stronger seed dormancy may have been responsible for the greater tolerance of germination to CDT.

According to Clerkx et al. (2004a), the dormancy metabolism is low, hence the seed has a low production of detrimental products, which may indicate that this might improve longevity. By QTL mapping, more dormant Arabidopsis accession Cape Verde Islands (Cvi) and Shakdara (Sha) had a greater survival after a CDT (Bentsink et al. (2000); Clerkx et al. (2004b). Although these QTL analysis showed that the traits for dormancy and CDT tolerance are not genetically linked (Bentsink et al., 2000).

We only find a link between dormancy and seed storability for the lines with the Col background. L*er*, the NILs and mutant with the L*er* background show an effect for seed storability, more phosphate during seed development and maturation leads to worse storable seeds. However, phosphate during maturation does not affect seed dormancy ($DSDS_{50}$; Fig 7B), at least not in the concentrations that were tested in experiment 2. In experiment 1, figure 3B it is shown that concentration over 500uM result in a reduced dormancy level. Higher levels of phosphate also result in less storable seeds in Col and the mutants in this background (Fig. 13H). For these genotypes this correlates with the effect that phosphate has on seed dormancy, higher phosphate also reduces seed dormancy.

Overall it is interesting to see that higher phosphate leads to a better germination performance (less dormancy, better germination under diverse stress conditions) however a reduced storability.

The highest phytate content in seeds was found in seeds development in high phosphate level combined with low temperature during seed development (Fig. 14). Whereas the inorganic phosphate content the behavior of interaction of phosphate levels and parental growth temperature were changed among

genotypes (Fig.15). The accumulation of Phytate and Phosphate on seeds in our experiment is consistent with observation of Lott et al. (2000) and Bentsink et al. (2003). InsP6 and Pi concentrations in seeds may vary because of many factors including climatic factors (Lott et al., 2000) and among natural accessions of Arabidopsis (Bentsink et al., 2003).

5. **CONCLUSIONS**

These results suggest that phosphate concentration and temperature both play a role in plant development, influencing seed quality.

For Columbia background, high phosphate levels during seed development decrease seed dormancy. And theses seeds were more sensitive to CDT, so effect of phosphate in seed development, seed dormancy and seed longevity could be linked.

High parental growth temperature (25°C) decrease dormancy seeds for genotypes NIL*DOG1*, NIL*DOG3* and NIL*DOG6*.

In general, high phosphate levels during seed development provided a better performance of germination in NaCl, mannitol and ABA.

The combined effects of high phosphate concentration and low parental growth temperature resulted in the highest phytate and phosphate content in seeds.

However, further investigations are required to fully understand the complex interactions between environmental and genetic factors regulating seed quality.

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Appendices

Table 1. Analysis of variance (ANOVA) for Plant height, Seed Size, Number of siliques per plant; Number of seeds per silique, 1000 seed weight, $DSDS_{50}$, Phosphate and Phytate Content, evaluated with 12 genotypes, 3 phosphate concentrations and 2 temperatures during seed development of *Arabidopsis thaliana*.

		F Value							
SOURCE	**d.f**	**Plant Height**	**Silique/Plant**	**Seeds/Silique**	**Seed Size**	**1000 seed weight**	**$DSDS_{50}$**	**Phosphate Content**	**Phytate Content**
Genotypes (G)	11	143.6703 **	35.0928 **	2.7174 *	4.9998 **	11.2584 **	38.3414 **	4.1269 **	10.5865 **
Phosphate (P)	2	93.7583 **	117.6071 **	5.2532 **	9.8142 **	12.9075 **	1.4628 ns	30.1186 **	178.6184 **
Temperature (T)	1	18.3524 **	901.7239 **	1.4896 ns	1.8945 *	1.0666 ns	2.3230 **	1.1934 ns	1.8057 *
G x P	22	3.3250 **	1.2395 ns	158.5702 **	58.4917 **	124.0542 **	168.1394 **	86.1148 **	241.5171 **
G x T	11	3.9000 **	5.0197 **	1.9445 *	1.7150 ns	3.4619 **	9.7169 **	4.1868 **	1.7262 ns
P x T	2	0.3254 ns	40.0105 **	4.7808 *	0.3712 ns	7.3935 **	10.7675 **	18.8967 **	14.8864 **
G x P x T	22	1.1129 ns	1.5452 ns	0.9894 ns	1.2643 ns	0.6678 ns	1.3672 ns	1.5943 ns	0.6769 ns
ERROR	71	-	-	-	-	-	-	-	-

** significant at 1% ($p<0.01$)
* significant at 5% ($0.01 \leq P < 0.05$)
ns not significant ($p \geq 0.05$)

Table 2. Analysis of variance (ANOVA) for Gmax at 22°C, Gmax at10°C; Gmax at 30°C (5 months of after-ripening (AR)), Gmax at 30°C (7 months of AR), Gmax after CDT, Gmax in Mannitol, Gmax in NaCl, Gmax in ABA (5 months of AR), Gmax in ABA (7 months of AR). evaluated with 12 genotypes, 3 phosphate concentrations and 2 temperatures during seed development of *Arabidopsis thaliana*.

		F Value								
SOURCE	**d.f**	**Gmax 22°C**	**Gmax 10°C**	**Gmax 30°C 5Mth AR**	**Gmax 30°C 7Mth AR**	**Gmax CDT**	**Gmax Mannitol**	**Gmax NaCl**	**Gmax ABA 5Mth AR**	**Gmax ABA 7Mth AR**
Genotypes (G)	11	6.2862 **	33.6130 **	19.6135 **	10.8302 **	113.1678 **	20.0706 **	20.2237 **	14.4026 **	27.9033 **
Phosphate (P)	2	1.1611 ns	1.4939 ns	6.0295 **	15.6909 **	41.9326 **	47.8562 **	46.0021 **	18.0127 **	61.0515 **
Temperature (T)	1	1.1045 ns	2.0894 *	2.3720 **	2.5769 **	2.0765 *	2.3140 **	2.1132 *	1.9376 *	1.7755 *
G x P	22	14.8730 **	11.0091 **	448.2038 **	255.0812 **	32.2243 **	0.0043 ns	5.3594 *	55.2151 **	530.1439 **
G x T	11	5.7653 **	7.9867 **	4.5849 **	2.5006 *	9.1174 **	3.8631 **	7.4027 **	10.9876 **	10.2989 **
P x T	2	4.7017 *	3.1793 *	6.5989 **	4.2526 *	20.3280 **	6.4343 **	15.3082 **	1.9737 ns	8.7083 **
G x P x T	22	0.8240 ns	1.5663 ns	0.8763 ns	0.4949 ns	1.5875 ns	1.6429 ns	2.0197 *	1.1372 ns	1.6810 ns
ERROR	71	-	-	-	-	-	-	-	-	-

** significant at 1% ($p<0.01$)
* significant at 5% ($0.01 \leq P < 0.05$)
ns not significant ($p \geq 0.05$)