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IDENTIFICACIÓN Y CARACTERIZACIÓN DE FUENTES DE RESISTENCIA A SEQUÍA EN GUISANTE

DOCTORANDA

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PhD THESIS

IDENTIFICATION AND CHARACTERIZATION OF DROUGHT RESISTANCE SOURCES IN PEA

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Que el trabajo "IDENTIFICACIÓN Y CARACTERIZACIÓN DE FUENTES DE RESISTENCIA A SEQUÍA EN GUISANTES" realizado por Rebeca Iglesias García bajo su codirección se considera ya finalizado y puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Genética de la Universidad de Córdoba.

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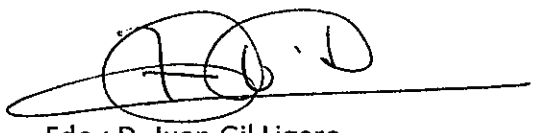

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A mi director de tesis, el **Dr. Diego Rubiales Olmedo** por darme la oportunidad de trabajar en su grupo, por la confianza y el respaldo depositados en mí durante estos años. Gracias por seguir creyendo.

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.

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SUMMARY

Pea (*Pisum sativum* L.) is the first grain legume in Europe and the second in the world. Major constraints for its cultivation include abiotic complex stresses such as drought. Both, the availability of resistance sources and a comprehensive understanding of the mechanisms through which pea can tolerate stress are necessary for successful breeding programmes. In the present work, by different approaches we aimed to search and characterize new sources of drought tolerance in pea and further understand mechanisms underlying water stress responses. In a preliminary screening we sought drought tolerance pea genotypes using different physiological parameters related with drought stress under controlled conditions and compared their drought responses with a susceptible check. In addition, their adaptation to different Mediterranean environments was assessed in field trials during four seasons. Further, interaction with resistance to *Fusarium oxysporum*, an important biotic stress causing wilting of peas, was investigated. In order to ease breeding we identified quantitative trait loci related with tolerance over a recombinant inbred line population and based on model plant databases we developed a genetic model for drought tolerance using bioinformatics tools and verified experimentally by gene expression assays.

RESUMEN

El guisante (*Pisum sativum* L.) es la primera leguminosa-grano en Europa y la segunda en el mundo. Entre los principales problemas que afectan a su cultivo se encuentran estreses abióticos complejos como la sequía. La disponibilidad de fuentes de resistencia, así como la comprensión de los mecanismos de tolerancia a estrés en guisante son necesarias para desarrollar programas de mejora exitosos. En el presente trabajo nos propusimos buscar y caracterizar fuentes de tolerancia a la sequía en guisante y profundizar en el conocimiento de los mecanismos implicados en la respuesta a estrés hídrico mediante distintas aproximaciones. En un análisis preliminar buscamos genotipos de guisante tolerantes a la sequía y comprobamos su respuesta frente a un control susceptible. Además evaluamos su adaptación a distintos ambientes Mediterráneos durante cuatro campañas agrícolas. Por otra parte, investigamos la interacción con la resistencia a *Fusarium oxisporum*, un importante estrés biótico causante de la marchitez del guisante. Por otra parte, identificamos loci de caracteres cuantitativos asociados con la tolerancia sobre una población de líneas recombinantes congénitas, destinados a facilitar el proceso de mejora, y . desarrollamos un modelo genético para la tolerancia a la sequía basado en bases de datos de plantas modelo utilizando herramientas informáticas y lo verificamos experimentalmente mediante ensayos de expresión génica.

OBJECTIVES

The main objectives of this work were:

- Identification and characterization of novel sources of resistance to drought in pea.
- Assessment and evaluation of pea genotypes under multi-environment field conditions.
- Refinement of suitable methodology to evaluate the simultaneous action of biotic and abiotic stress over pea.
- Identification of pea genotypes with resistance/tolerance to Fusarium wilt and drought
- Identification of quantitative trait loci (QTL) associated to drought tolerance.
- Analyse the genetics of drought tolerance through the use of *Arabidopsis* as a model plant.

INDEX

General introduction

LEGUMES

The history of legumes (Family Leguminosae) starts with human civilization and their evolution throughout many different regions of the world. Legumes, accompanied by cereals, were the first plants cultivated in the south-west of Asia, where ancient agriculture evolved. Legumes are traditionally used in crop rotations due to their property of fertilizing the soil. In fact, the legumes which evolved in the Mediterranean basin played an important supporting role to that of the cereals in sustaining the development of the classical civilisations (Cubero, 1983).

With around 20000 species, legumes are the third largest family of higher plants. The Gramineae has only some 10000 species and the Brassicaceae 3500 species. The Leguminosae are second to cereal crops in agricultural importance based on area harvested and total production (Gepts *et al.*, 2005).

Traditionally, Leguminosae family has been divided into three subfamilies: Caesalpinieae, Mimosoideae, and Papilionoideae. The latter subfamily has the most agronomical interest because the seeds and pulses of its herbal species are edible for human and animals (Cubero, 1983). On this subfamily we have to highlight some species know as grain legumes, which first utility are the seeds, opposite to other species cultivated as forage (e.g. alfalfa, clover, *Stylosanthes* sp., *Desmodium* sp.) in temperate and tropical regions, or the horticultural ones such as green legumes, cultivated for the collection of their green pods and soft seeds (Moreno, 1983) .

Other diverse roles of legume plants are often overlooked. Grain legumes provide about one-third of all dietary protein nitrogen and one-third of processed vegetable oil for human consumption (Graham and Vance, 2003). Seeds of grain legumes contain at least 20% to 40% of protein. In many places of the world,

legumes complement cereals or root crops, the primary source of carbohydrates, in terms of amino acid composition. Whereas cereal seed proteins are deficient in lysine, legume seed proteins are deficient in sulfur-containing amino acids and trypthophan (Wang *et al*., 2003). This situation may explain why in most centers of crop domestication, legumes and cereals have been domesticated together (Gepts, 2004).

Legumes also provide essential minerals required by humans (Grusak, 2002a) and produce health promoting secondary compounds (Grusak, 2002b; Madar and Stark, 2002) that can also protect the plant against the onslaught of pathogens and pests (Ndakidemi and Dakora, 2003; Dixon *et al*., 2002). In addition to their blood cholesterol-reducing effect (Andersen *et al*., 1984), grain legumes generally also have a hypoglycaemic effect, reducing the increase in blood glucose after a meal and, hence, blood insulin. Legumes are, therefore, included in the diet of insulin-dependent diabetics (Jenkins *et al*., 2003). Furthermore, legume crops are of great significance because they produce substantial amounts of organic nitrogen fertilizer resulting from a symbiosis between the plant and bacterial symbionts (Hirsch, 2004; Jensen and Hauggaard-Nielsen, 2003).

Certain legumes as peas (*Pisum sativum*), faba beans (*Vicia faba*), and lupins (*Lupinus* spp.) however, produce antinutritional factors, such as trypsin inhibitors and phytohemagglutinins (Gupta, 1987) or allergens, the latter being a severe problem in peanut (Spergel and Fiedler, 2001). Genomics approaches, including metabolomics and proteomics, are essential to understand the metabolic pathways that produce these antinutritional compounds and to eliminate these factors from the plant.

The European Union (EU) faces the challenge of providing high quality protein for both animal and human consumption. Europe currently imports about 75% of its plant protein yet much of this could be derived from EU grown Grain Legumes. Furthermore, legume use in arable crop rotations reduces the need for fertiliser application and acts as a break-crop, reducing the need for pest and disease control. Together this is a unique combination of benefit to the environment. Despite all these advantages, the absence of varieties adapted to the specific conditions in a region together with the diseases and pests, yield inconsistency and low quality of the seeds are the main problem of these crops.

In these last years, the EU has developed some strategies to increase grain legumes use by European farmers, investing in researching programs to solve the main problems of yield inconsistency and quality of the seeds. In this sense, pea, one of the main legumes in Europe and the world, has been one of the most studied crops and would play an important role to solve these problems.

PEA

The pea crop was known in the prehistoric age in Europe. Peas dating from the Stone Age have been discovered in the excavations at Aggetelek in Hungary and in lake-dwellings in Switzerland (Fourmont, 1956). Erksine *et al.* (1994) and Smartt (1990) indicated that peas date back to 7000-5400 B.C., being the main legume in a Neolithic site at Erbaba in Turkey. In France, peas exhumed from dwellings in the Bourget lake belong to the Bronze Age (1000-2000 B.C) and are assumed to have been grown by Aryan people (Gibault, 1912).

Probably originated in Abysinia and Afghanistan, some areas in the Mediterranean area were colonized later by pea. The range of wild representatives of *P. sativum* extends from Iran and Turkmenistan through Anterior Asia, northern Africa and southern Europe (Maxted *et al*., 2010; Maxted and Ambrose, 2000; Makasheva, 1979). From these areas the pea spread to other parts of Europe and Asia (Cousin, 1997). Vavilov (1926) distinguished four original cores, today considered as diversity cores: the Abyssinian (Ethiopia), the Mediterranean (Turkey, Greece, Yugoslavia, Lebanon) and Central Asia (Northwest India, Pakistan, Afghanistan, Russia). However, due to the early cultivation of pea it is difficult to identify the precise location of the centre of its diversity, especially considering that large parts of the Mediterranean region and Middle East have been substantially modified by human activities and changing climatic conditions.

Thanks to its climate adaptability, pea is one of the most common crops. Dry peas are mainly used for animal feeding, whereas green peas are used in fresh, canned or frozen for human food. Only since XVI century did it begun to be consumed by the man as green grain, firstly in England and then spreading to France, as before was only used as dry grain or forage crop. Only a low percentage around 3% from total production of dry pea is used in human consumption (Maroto, 1995). Its composition of 50% slowly digestible starch, 23-25% proteins, 5% sugars, 2% oil, minerals and vitamins (Bastianelli *et al*., 1998) made pea useful for simple-stomach animals feed, which is the main use for the compound feed containing legumes.

Pea is also one of the grain legumes highly interesting in research activity.

Botanical description

Pea is enclosed in the genus *Pisum*. There has been some agreement in the literature over the number of taxa included in this genus, but much dispute over their rank. Traditionally the classification proposed by Davis (1970) which recognised two species and multiple subspecific taxa within *P. sativum* has largely been adopted, see Table 1. However, this classification was produced for a national flora and does not include taxa found outside of the Middle East and so it would be not comprehensive (Maxted and Ambrose, 2001).

Table 1. Traditional classification of the *Pisum* genus proposed by Davis in 1970.

Species	Subspecies	Varieties
Pisum sativum L.	*sativum* L.	*sativum* L.
		arvense (L.) Poiret
	elatius (M.Bieb) Aschers y Graebn	*elatius* (M. Bieb) Alef
		brevipedunculatum Davis and Meikle
		pumilio Meikle (syn P. *humile* Boiss. and Noë)
P. abyssinicum A. Br		
P. fulvum Sibth. and Sm.		

Pisum is very diverse and its diversity is structured, showing a range of degrees of relatedness that reflect taxonomic identifiers, ecogeography and breeding gene pools.

Nowadays, the Kew database (http://epic.kew.org) lists 82 different species of *Pisum*, although not all names are "valid" according to the International Plant Names Index (http://www.ipni.org).

In the USDA-GRIN database (http://www.ars-grin.gov), the names of 13 species are listed, 3 of which correspond to other genera, and of the remaining 10 "*arvense*" and "*commune*" are considered synonymous with "*sativum*". This leaves

us with the names *P. abyssinicum*, *P. elatius*, *P. fulvum*, *P. humile*, *P. jomardii*, *P. sativum*, *P. syriacum* and *P. transcaucasicum*. Several of these names also occur in the ILDIS database and in both some also appear as subspecies or varieties of *P. sativum*. The ILDIS names follow Maxted and Ambrose (2001) and recognize *P. sativum* ssp. *sativum*, *P. sativum* ssp. *elatius*, *P. abyssinicum* and *P. fulvum*.

The cultivated pea (*P. sativum* ssp. *sativum*) is a plant with compound leaves of a variable number of leaflets, from one to seven, and terminal tendrils. Although, there are some varieties identify as afila with all the leaflets turned into tendrils (Laguna *et al*., 1997). Two stipules are at the base of each leaf. The stem has an angled section and its appearance is variable, being possible to find single stem and highly branched stems plants. Pea plants exhibit an indeterminate growth habit. The first nodes, some of which give rise to branches are vegetative, while subsequent nodes are reproductive. Generally two flowers, from which the pods develop, are present at each reproductive node. The fruit is a legume or pod, with variable shapes and sizes, from 3 to 15 cm length. The seeds can be round or cubic, smooth or rough. The number of seeds per pod depends on the variety and on the environmental conditions, from 4 to 12 seeds.

The wild species and some subspecies often have tall (more than 2 meters) slender and branched stems, purple or pink flowers and small pods producing a small quantity of seeds with colored coat. *P. sativum* ssp. *elatius* and *P. abyssinicum* have distinct toothed leaflets and stipules. *P. sativum* ssp. *elatius* has colored flowers, lilac-blue standards, dark purple wings and maroon veiny brown seeds. *P. abyssinicum* has pink flowers and dark purple seeds. *P. fulvum* may have two fructification types, a normal one located in the upper part of the plant, the other

very peculiar with very short basal branches which push the pods slightly into the ground. *P. sativum* ssp. *elatius* var. *humile* is characterized as a medium sized climbing species with dentate leaf margins and light blue flowers. This species is strict autogamous by a mechanism of cleistogamy (self polinization before the flower is open) (Maroto, 1995) although it is known some cases of natural hybridization.

Requirements of the crop

Pea is a crop adapted mainly to humid or temperate climates. The optimum growing temperature is between 16 and 18ºC. Although a huge part of the varieties are sensitive to frost, some cultivars can show a moderate resistance (to -2 or -3ºC) or even a high resistance (-9ºC). Thus, we can distinguish between winter and spring varieties depending on the level of resistance to cold (Maroto, 1995).

Regarding the soil type, pea grows well in slightly acidic or alkaline soils, supporting a range of pH between 5,5 and 8,8. The optimum value is located between 6 and 6,6 (Guerrero, 1998). Respect to the texture, it is better suited to light or mean soils, but well-drained. Otherwise problems may appear due to the lack of breathability.

The fertilization with nitrogen is not normally needed because of its symbiosis with *Rhizobium leguminosarum* as this bacterium is commonly found on Spanish soils. According to Guerrero (1998) the average requirements for fertilization are 40-70 UDF/ha of P_2O_5 and 22UDF/Ha of K_2O

Economical importance

Dry pea currently ranks second only to common bean as the most widely grown grain legume in the world with primary production in temperate regions.

FAOSTAT registered 94 countries growing it in 2010, being the cultivated area about 6.5 million hectares (Fig. 1).

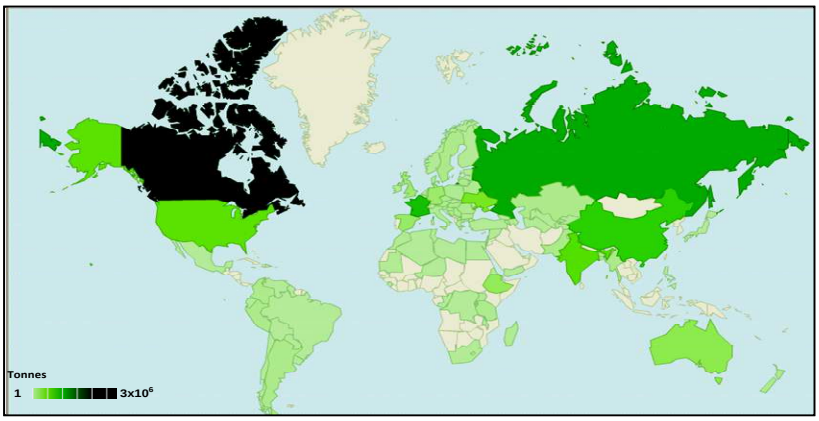

Fig. 1. Global production of dry pea in 2010.

Canada has remained the leading pea producing country in the world over the last decade. The continents with the bigger areas harvested in 2010 were Europe, with 1897 x 10^3 Ha and Asia with 1786 x 10^3 Ha (Fig. 2).

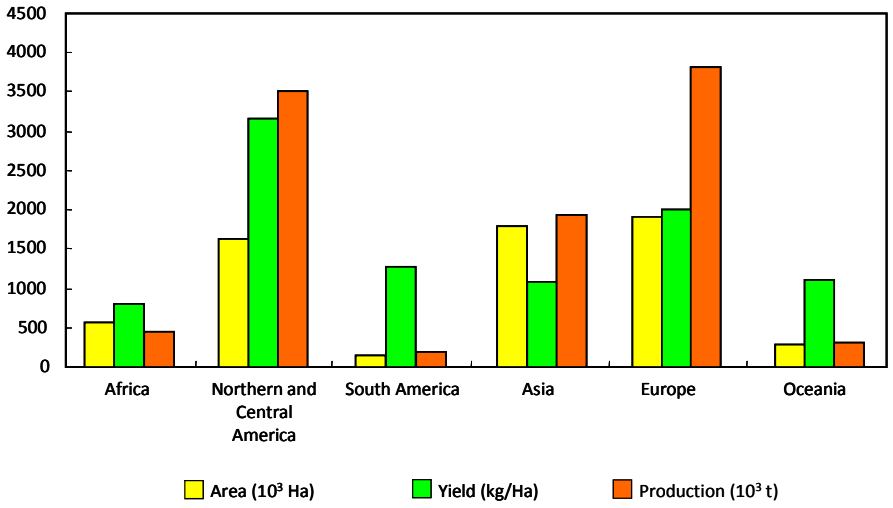

Fig. 2. Distribution by continents of the harvest area, production and yield of dry pea in 2010.

However, the most productive continents were Europe (3815 x 10^3 t), where it is the first grain legume produced, and North America (3507 x 10^3 t). The reasons why these continents have bigger production levels than Asia rely in the yield differences. In fact, whereas in Asia the yield mean is of 1079 kg/Ha, the highest yields of 1700-2100 kg/Ha were achieved in Northern America and Europe.

Dry pea production in Europe declined during the period from 2001 to 2007 (Fig. 3) while the opposite trend was recorded for the Russian Federation, India and USA. The reasons for these changes include economic, biological, physical, sociological and technical factors (Smýkal *et al*., 2012). However, in the last four years there has been a slight increase in the production, probably because of the favourable environmental conditions.

The yields were variable within the years being the maximum values reached in 2004 (2,7 x 10^3 Kg/Ha) and the minimum (1,8 x 10^3 Kg/Ha) in 2007. Since then, there have been annual fluctuations and a slight increase as well.

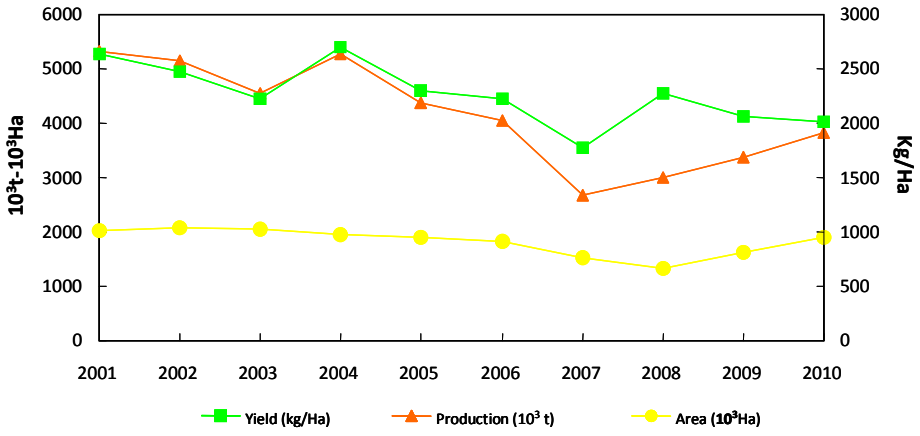

Fig. 3. Evolution of the harvested area, yield and production of dry pea in the EU from 2001 to 2010.

However, the countries with higher production in Europe in 2010 are not necessarily the ones with better yields, as it can be observed in Table 2. Although

the production in France has decreased dramatically in the last decade, it is still the main producer of dry pea in the UE.

The other countries which most contribute to the whole production are Germany and Spain, which increased its production in the last decades, and the United Kingdom, which decreased its production since the 90´s.

Table 2. Top ten producer countries and top ten countries with the best yields in the UE.

	Production (10^3 t)		Yield (Kg/Ha)
France	1098	**Netherlands**	5000
Spain	194	**France**	4393
Germany	177	**Belgium**	4182
United Kingdom	147	**Switzerland**	4171
Sweden	54	**Ireland**	3923
Czech Republic	48	**United Kingdom**	3868
Lithuania	40	**Norway**	3260
Romania	40	**Denmark**	3218
Hungary	37	**Germany**	3010
Poland	33	**Luxembourg**	2977

The bigger yield levels are reached in the Netherlands, France, Belgium and Switzerland, with more than 4 t/Ha. Although Spain is among the main producers of dry pea the yield obtained, 963 Kg/Ha, is the lowest value in all the EU countries.

In Spain, from the 30´s to the 80´s the cultivation area for dry pea was decreasing from 67000 Ha to 5200 Ha harvested. The reasons for this decline were mainly the low yields and their instability upon the cereals, the lack of integration into the feed market, the low prices compared with alternative crops, the lack of support for research in grain legumes and the strong competition of the soy as a protein source in the compound feed industries (Caminero, 2002). This trend continued till 1994, when there was a big increase in the cultivated area and the

production levels, motivated by the PAC support, recovering in a few years what was lost during the last decades. In 2004 was detected the highest peak in harvested area and production (Fig. 4)

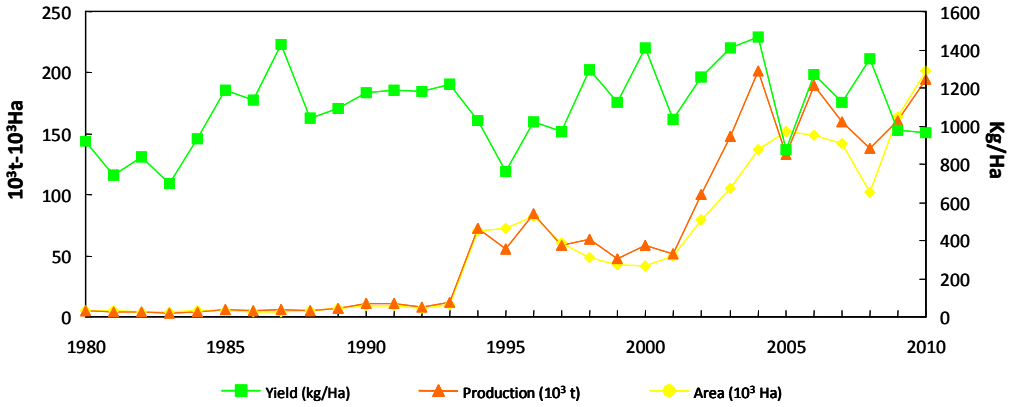

Fig. 4. Evolution of yield, production and harvested area of dry pea in the last years in Spain

However, in 2005 the production was dramatically decreased, despite the increase in the harvested area, probably due to the drought suffered that year. In general, the yields have been quite variable during the last five years, despite the production levels and harvested areas were increasing.

According to the statistics of 2010, the most productive communities for dry pea were Castilla y León with a 47% of the total production, Castilla la Mancha with a 27%, Extremadura with an 8% and Andalucía with a 7% (Fig.5). The harvested area in Andalucía was strongly reduced after the years 1995 and 1996, when the crops were severely affected by broomrape (*Orobanche crenata* Forsk.) (Rubiales *et al*., 1999) The attacks were so strong that most of the 80% of the cultivated surface remained unharvested, being even impossible to sown again in subsequent years in such areas (Rubiales *et al*., 2003). Despite all these difficulties of the crop, the production levels are increasing every year.

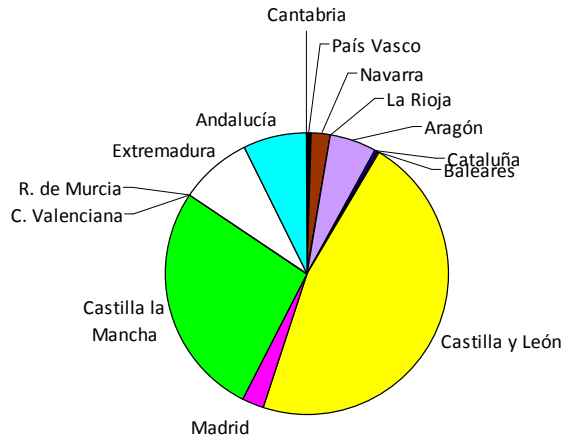

Fig.5. Production of dry pea in Spain in 2010.

In Andalusia the harvested area for dry pea has also been increased in the last years, as well as the production (Table 3), being the grain legume most cultivated. Regarding the harvested area, the provinces of Córdoba, Málaga and Sevilla were the ones with bigger crop surface in 2008. Furthermore, Almería, Córdoba, Huelva, Jaén and Granada have increased this harvested area, as well as their production levels in the last three years.

Table 3. Harvested area and production of dry pea in Andalusia provinces from 2008 to 2010. Source: Ministry of Agriculture, Fisheries and Environment.

Province	2008		2009		2010	
	Ha	t	Ha	t	Ha	t
Almería	1	1	28	11	475	**238**
Cádiz	857	618	865	900	380	**315**
Córdoba	1398	1798	1556	1867	1863	**2236**
Huelva	428	203	760	335	2820	**576**
Jaén	198	143	115	80	467	**403**
Granada	428	200	760	228	2820	**2424**
Málaga	2528	3921	2385	3816	1520	**2200**
Sevilla	**1584**	**1393**	**1500**	**1605**	**1550**	**1381**

Córdoba, Huelva and Málaga have the highest yields in 2010, being around 1000 Kg/Ha, whereas the other provinces had lower medium yields.

The dry pea crop is increasing in Spain, as well as in Andalucía, however it has the lowest yield in the EU. The main reason for this lack can be the absence of cultivars specifically adapted to the particular environmental conditions of this country (Rubiales *et al.*, 2009).

DROUGHT AND FUSARIUM STRESS IN PEA

Legumes are sensitive to abiotic stresses, such as water deficit and soil salinity. Drought is the major abiotic stress factor limiting crop productivity worldwide. In Mediterranean countries water deficit is encountered not only in arid and semiarid regions, but also in areas where total precipitation is high but not evenly distributed during the growing season. In a context of increasing limitations to water use due to climate change and increased population, improving water use efficiency of crops is an important goal. On the other hand, diseases are considered the most important causes of reduced biomass production and seed yields in pea (Ali *et al*., 1994; Rubiales *et al*., 2011; Smýkal *et al*., 2012). Thus, the interaction between abiotic and biotic stresses can have devastating effects on crop yield. In this sense *F. oxysporum* f. sp. *pisi* (*Fop*) is an important and destructive pathogen of field pea, that has been reported in every country where pea is grown (Kraft and Pfleger, 2001).

Drought stress

Climate change can be expected to exacerbate climate unpredictability and to result in unprecedented levels of heat and drought stress during the reproductive phase in agricultural areas of the temperate, sub-tropical zones worldwide, especially in the sub-Sahara and north central India. One of the predictions is that summers will be drier in Europe, and one of the impacts of this climate change is likely to be an increase in drought affected spring crops. Peas generally require temperate conditions and drought during the flowering and pod filling period of spring varieties of combining peas can severely reduce yield. Targeted utilization of selected landraces and wild relatives for adaptation to climate change will almost certainly be an urgent priority during this century.

Drought is a meteorological event which implies the absence of rainfall for a period of time, long enough to cause moisture-depletion in soil and water-deficit with a decrease of water potential in plant tissues (Kramer, 1980). But from agricultural point of view, its working definition would be the inadequacy of water availability, including precipitation and soil-moisture storage capacity, in quantity and distribution during the life cycle of a crop plant which restricts the expression of full genetic potential of the plant (Sinha, 1986).

In agriculture, drought resistance refers to the ability of a crop plant to have an acceptable yield with minimum economical losses in a water-deficit environment. In this sense, the plant develops various strategies to avoid water losses.

The mechanisms of drought resistance can be grouped into three categories: drought escape, drought avoidance and drought tolerance (Levitt, 1972. Drought escape is defined as the ability of a plant to complete its life cycle before serious soil and plant water deficits develop. This mechanism involves rapid

phonological development (early flowering and early maturity), developmental plasticity (variation in duration of growth period depending on the extent of water-deficit) and remobilization of preanthesis (Turner, 1979) assimilates to grain. Drought avoidance is the ability of plants to maintain relatively high tissue water potential despite a shortage of soil-moisture, whereas drought tolerance is the ability to withstand water-deficit with low tissue water potential. Thus, the responses of plants to tissue water-deficit determine their level of drought tolerance (Mitra, 2001).

Drought avoidance is performed maintaining turgor through increased rooting depth, efficient root system and increased hydraulic conductance and by reduction of water loss through reduced epidermal conductance (stomatal and lenticular), reduced absorption of radiation by leaf rolling or folding (Begg, 1980; O´Toole and Moya, 1987) and reduced evaporation surface (leaf area) (Turner, 1986; Passioura, 1976). Plants under drought condition survive by a balance between maintenance of turgor and reduction of water loss (Shashidhar *et al*., 2000). The mechanisms of drought tolerance are maintenance of turgor through osmotic adjustment (a process which induces solute accumulation in cell), increase in elasticity in cell and decrease in cell size and desiccation tolerance by protoplasmic resistance (Ugherughe, 1986; Sullivan, 1979).

Unfortunately, most of the adaptations to drought have disadvantages. A genotype of short duration usually yields less than one of normal duration because the mechanisms that confer drought resistance by reducing water loss usually result in reduced assimilation of carbon dioxide. Osmotic adjustment increases drought resistance by maintaining plant turgor, but the increased solute concentration

responsible for osmotic adjustment requires extra energy supply (Turner, 1999). Consequently, crop adaptation must reflect a balance among escape, avoidance and tolerance while maintaining adequate productivity.

There have been developed different breeding approaches for drought resistance. For example, one would be to breed for high yield under optimum conditions. The idea was that a superior genotype under optimum levels will also yield relatively well under drought conditions because it has the maximum genetic potential. However, the genotype-environment interaction was the limiting factor for this approach, as it may restrict the highyielding genotype to perform well under drought. Another approach would be trying to breed under drought conditions. The problem with this relied on the high variability in the intensity of drought from year to year. As a consequence of environmental selection pressure, breeding materials would change drastically from generation to generation. Other way would be to improve drought resistance in high-yielding genotypes through incorporation of morphological and physiological mechanisms of drought resistance. However, transferring drought resistance in high-yielding genotypes is complicated due to lack of understanding of the physiological and genetic basis of adaptation in drought condition.

Nowadays, improving the yield potential of an already resistant material may be a more promising approach, provided there is genetic variation within such a material (Smýkal *et al*., 2012; Mitra, 2001; Yunus, 1982). Simultaneous selection in non-stress environment for yield and in drought condition for stability may be done to achieve the desired goal of evolving drought-resistant genotype with high yield. Thus, the success of any breeding programme depends on the availability of the

screening technique, especially for drought resistance. Such an effort relies primarily on the identification of relevant physiological traits and their use within breeding schemes that combine crop modeling, genetic and environmental dissections (Schoppacha, 2012).

Fusarium oxysporum f.sp*. pisi*

The genus *Fusarium* comprises a number of fungal species producing characteristically shaped fusoid macroconidia, that are widely distributed in soil and on organic substrates and have been isolated from permafrost in the arctic to the sand of the Sahara. *Fusarium* species have for a long time been known as important plant pathogens or as mycotoxin-producing contaminants of human and animal food (Moss and Smith, 1984).

Fusarium wilts are widespread diseases caused by many forms of the soil-borne pathogen *Fusarium oxysporum* (*Fo*), the most common species of the genus, affecting many agricultural crops, including most legumes.

As in other *Fusaria*, its identification has generally been based on morphological criteria such as the shape of micro- and macroconidia, structure of microconidiophores and formation and disposition of chlamydospores (Moss and Smith, 1984). When grown in culture, *Fo* initially produces colourless to pale yellow mycelium that turns pink or purple with age. The species includes nonpathogenic, plant pathogenic and human pathogenic strains. With the exception of grasses and most tree crops, few of the widely cultivated crops are not hosts to a pathogenic form of *Fo*. Isolates have been divided into more than 120 different formae speciales according to their host range (Armstrong and Armstrong, 1981). A particular forma specialis can be further subdivided into physiological races based on a characteristic

pattern of virulence on differential host cultivars. A classical gene-for-gene relationship has been proposed to mediate the interaction between *Fo* races and host cultivars, based on dominant monogenic resistance traits against known races (Di Pietro *et al*., 2003).

Some members of the genus *Fusarium*, e.g. *F. solani* (*Nectria haematococca*) or *F. graminearum* (*Gibberella zeae*), can complete the sexual life cycle under natural and laboratory conditions, whereas others like *F. oxysporum* have not known sexual stage.

As a soil inhabitant, *Fo* can survive extended periods in the absence of the host, mainly in the form of thick-walled chlamydospores. Indeed, once an area becomes infected with *Fo*, it usually remains so indefinitely (Agrios, 1997). The proximity of roots induces the dormant propagules to germinate and initiate infection. After germination, infection hyphae adhere to the host roots (Bishop and Cooper, 1983a; Di Pietro *et al*., 2001) and penetrate them directly (Rodriguez-Gálvez and Mendgen, 1995). The mycelium then advances intercellularly through the root cortex until it reaches the xylem vessels and enters them through the pits (Bishop and Cooper, 1983a). At this point, the fungus remains exclusively within the xylem vessels, using them to rapidly colonize the host (Bishop and Cooper, 1983b). Thus, the characteristic wilt symptoms appear as a result of severe water stress, mainly due to vessel clogging (Di Pietro *et al*., 2003). As long as the plant is alive, the vascular wilt fungus remains strictly limited to the xylem tissues and a few surrounding cells. Only when the plant is killed by the disease does the fungus invade the parenchymatous tissue and sporulate on the plant surface. *Fo* occupies a

highly specific ecological niche, shared by only a few other fungal pathogens (Agrios, 1997).

Fusarium oxysporum f. sp. *pisi* (*Fop*) is an important and destructive pathogen of field pea. There are four different races of *Fop* isolates according to their capacity to induce disease in a set of differential lines. Among them, race 1 and 2 occur worldwide (Infantino *et al*., 2006). In addition, *Fop* is continually evolving, with new variants of the pathogen emerging (Bodker *et al*., 1993; Kraft and Pfleger, 2001). Thus, control of this disease is achieve mainly by the integration of different disease management procedures, including agronomic and farming practises (Navas-Cortes *et al*., 1998), soil disinfection (Momma *et al*., 2010), biocontrol (Alabouvette *et al*., 2009) and breeding for resistance (Sharma *et al*., 2010). The use of resistant cultivars of plants is the preferred approach among these methods, being the only practical measure for controlling the disease in the field (Lebeda *et al*., 2010). Thus, a recent study described the existence of sources of resistant to *Fop* race 2 in pea that may be useful for a breeding programme (Bani *et al*., 2012), detecting a wide variety of responses in a *Pisum* germplasm collection.

Conventional breeding methods have been successful in improving pea germplasm towards development of superior cultivars through introduction of novel traits from wild germplasm and landraces as well as pyramiding multiple positive alleles in adapted genetic backgrounds (Rubiales *et al*., 2011). However, the improvement of several important agronomic characters such as disease resistance, abiotic stress tolerance and stability of seed yield and composition is a difficult task. Breeding success will depend on availability of consistent resistance genes or quantitative trait loci (QTLs) within or outside the species (Rubiales *et al*., 2011) as

well as on the availability of molecular and physiological tools to characterize the resistance mechanisms.

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Chapter 1

Identification and characterization of drought tolerance sources in pea (*Pisum sativum* L.)

INTRODUCTION

Genetic breeding is the most appropriate approach to obtain genotypes adapted to environmental stresses providing us with high yield cultivars adapted to different locations. Identifying and characterizing sources of resistance in germplasm collections, is a priority with the anticipated increase in the frequency of weather extremes associated to climate change and affecting agricultural production (Upadhyaya *et al.*, 2011) and will be crucial for a successful breeding. With a wide range of approaches now available for genotyping and declining cost for whole genome sequencing, the greatest limitation for gene banks is phenotyping, not only for descriptive traits but agriculturally relevant quantitative traits related to expression of yield, crop growth and disease resistance. One approach is to use core collections that have been developed based on geography or using molecular marker diversity or developed based on priority traits (Bhullar *et al.*, 2009). This has led to use climatic site descriptors for characterization of natural selection and hence abiotic stress response and to provide lists of prospective germplasm with potential tolerances to heat, frost and drought (Bhullar *et al.*, 2009). The sources of resistance to abiotic stresses are frequently found in wild accessions (Ali *et al.*, 1994), although they are also present in high yielding cultivars. Thus, several molecular diversity studies in recent years have had a significant impact on our understanding of the nature of the diversity within pea germplasm, highlighting the importance of ecogeographical factors (Burstin *et al.*, 2001; Baranger *et al.*, 2004; Tar´an *et al.*, 2005; Kosterin and Bogdanova, 2008; Zong *et al.*, 2009; Kosterin *et al.*, 2010).

Drought stress is a major constraint to the production and yield stability of pea (*Pisum sativum* L.). The study of drought tolerance in different accessions is

crucial for a properly characterization and selection of sources of resistance for a breeding program, through the adoption of standard phenotypic evaluation methods (Mitra, 2001). Any effort for genetic improvement in drought resistance utilizing the existing genetic variability requires an efficient screening technique, which should be rapid and capable of evaluating plant performance at the critical developmental stages and screening large populations using only a small sample of plant material. However, the lacks of effective selection criteria as well as the variation of the target traits within species are considered to be a major impediment to breeding for drought-prone environments (Araus *et al.*, 2002; Ouk *et al.*, 2006; Venuprasad *et al.*, 2007).

Modern breeding strategies attempt to include physiological, biochemical and molecular characteristics which may better reflect lineage productivity and responses to environmental stress (Araus, 2002; Mitra, 2001; Richards, 1996; Slafer and Araus, 1998), enabling a better understanding of drought tolerance mechanisms. Key features may be the capacity to maintain cell/tissue water and to avoid oxidative damage through antioxidant machinery (Farooq *et al.*, 2009; Jones, 2007). Thus, water related features such as relative water content (RWC), stomatal conductance and water use efficiency (WUE) have been studied in different species under drought stress (Xin *et al.*, 2008; Merah, 2001; Singh and Patel, 1996. Water deficit also affect vital processes as the photosynthetic capacity and is directly related with the osmotic adjustment (Cattivelli, 2008) through the accumulation of specific compounds such as sugars (i.e. from the raffinose family oligosaccharides), sugar alcohols (such as mannitol), amino acids (such as proline) and amines (such as glycine, betaine and polyamines). In recent years, attention has been focused on the

role of polyamines () in plant defence against abiotic and biotic stresses, as it had been observed that they can alter their titres in response to various types of environmental stresses (Yang *et al*., 2007 and references within).

All these mechanisms described are closely related. In this sense, drought-resistant genotypes will be capable of maintaining higher leaf water potentials and lower stomatal conductance than susceptible genotypes as a result of a lower leaf water potential threshold for stomata closure (Itoh and Kumura, 1986; Ristic and Cass, 1991). Osmotic adjustment under drought stress enables leaf turgor maintenance for the same leaf water potential thus supporting stomatal conductance under lower leaf water status (Ali *et al.*, 1999; Sellin, 2001). Soil drought occurring during plant growth inhibits photosynthesis, reduces dry matter and induces changes in the distribution of assimilates (Muller *et al.*, 1986).

In pea cultivars, different responses to drought have been observed, but the physiological basis of drought susceptibility or tolerance is far from being understood. A better understanding of drought tolerance mechanisms is important to further define targets in germplasm screenings. In this chapter we identify and characterise drought tolerant sources for its use in breeding.

MATERIALS AND METHODS

Preliminary screenings under controlled conditions of the pea germplasm available in our group were made in order to find possible sources of resistance. Among the genotypes screened were wild species, parental lines of recombinant inbred lines (RILs) populations as well as commercial varieties (Table 1.1).

Table 1.1. Genotypes used in preliminary screenings for drought resistance and main agronomical traits.

Species	Accession	Characteristic	Bibliography
P. sativum ssp.*sativum*	Messire	Susceptible check to *Erisiphe pisi* and *Micosphaerella pinodes* /Parental line	Fondevilla *et al.*(2005)
	P245 P238	Parental lines	Irzykowska *et al.*(2002)
	P1123	Resistant to *Uromyces pisi*/ Parental line	Barilli *et al.*(2009)
	Frisson Ballet Solara Kebby HR-1 Desso Polar ZP-108	High turgor maintenance and osmotic adjustment	Sánchez *et al.* (1998)
	406N	Normal-wax, *Wel/Wel*	Marx (1969)
	406G	Glossy, wax mutant *wel/wel*	
	Dark Skin Perfection Little Marvell 74SN New era New season 902131 WSU28	Lines with diferential resistance to *Fusarium oxysporum* fs. *pisi*	Hagedorn (1984) Haglund and Anderson (1987)
	Radley	Susceptible to *Erisiphe pisi* and moderately to *Mycosphaerella pinodes*	Fondevilla *et al.*(2005)
	Puget	Moderate tolerance to salt stress, susceptible to *Aphanomyces euteiches*	Gómez *et al.*(2004) Kraft and Boge (1996)
P. fulvum	P660	Resistant to *Erysiphe pisi*/Parental line	Fondevilla *et al.*(2005)
P.sativum ssp. *syriacum*	P651	Resistant to *Erysiphe pisi* and *Orobanche crenata*.	Fondevilla *et al.*(2005), Pérez-de-Luque *et al.*(2004)
	P665	Resistant to *Mycosphaerella pinodes*	Fondevilla *et al.*(2005)

Plant material and growth conditions

Pea cvs. Polar, Messire and Kebby and *P. sativum* ssp. *syriacum* accession P665 were used in the experiments. P665 derives from accession IFPI-3280, kindly provided by ICARDA, Syria, and was previously characterized as *Mycosphaerella pinodes* resistant (Fondevilla *et al.*, 2005). Seeds were pregerminated in Petri dishes

with moistened filter papers in the dark for 48 h in a cold chamber at 4ºC and then placed for another 48 h in a growth chamber at 65% relative humidity and 20ºC.

Seedlings were planted individually in 0,5 L pots filled with peat: sand (3:1) for the polyamine and WUE experiments and returned to the chamber, growing under a of 200 μmol m^{-2} s^{-1} photons flux density (PPFD) supplied by high output white fluorescent tubes. For the rest of the experiments, pots were filled with compost (Levington F2+S, The Scotts Company, Ipswich, UK) and placed in a glasshouse. In the glasshouse the temperatures fluctuated during the experimental periods (October–December 2010/ September–October 2011) with a mean temperature of 20 ± 2.5 °C. Lighting was maintained at a minimum threshold PPFD of 1800 μmol m^{-2} s^{-1} for a 12 h day, and supplementary lighting was switched on if light intensity fell below this threshold. The PPFD at plant level was 200 μmol m^{-2} s^{-1}. A minimum number of 5 plants per assay and evaluation were used. Plants were watered and positions changed every two days during their 21 days growing period.

Visual scale

Plants were well watered until the beginning of the drying episode. From this time, the genotypes were evaluated daily by a visual scale. The visual scale had been used previously in our group to discriminate susceptible and tolerant genotypes to drought in a quickly and accurate way in oat plants (Sanchez-Martin *et al*., 2012). According to previous assays the scale was adapted to pea behaviour (Iglesias-García *et al*., 2012). Thus, we used the 4^{th} pair of leaves instead of the whole plant to evaluate drought symptoms on each genotype uniformly.

Five physiological status were established, numbered from 5 to 0, with which we could assess the temporal evolution of water stress symptoms (Fig. 1.1)

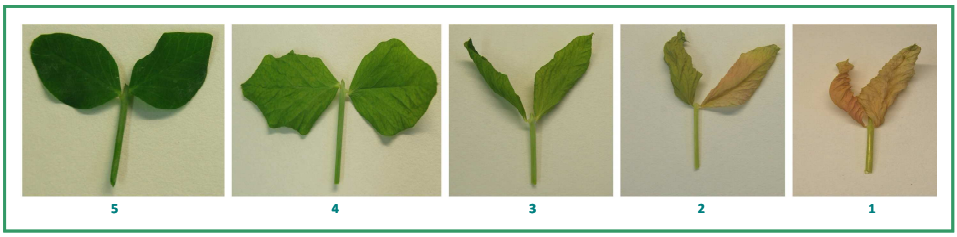

Fig.1.1. Representation of the visual scale and the different status of the leaves along the water stress period from the 5 status (pair of leaves without symptoms) to 1 (pair of leaves completely wilted).

Each status in the scale corresponds with the following characteristics: 5) No symptoms observed in the pair of leaves; 4) General softening of the pair of leaves; 3) Curved leaves with marked ribs; 2) Necrosis observed (0-50% of the pair of leaves); 1) Complete wilting of the pair of leaves.

Relative water content

Relative soil water content (rSWC) was calculated daily according to the methodology used by Bechtold *et al*. (2010). Briefly: pots were filled with identical amount of substrate and kept well watered until the beginning of the drying episode. At the same time, three control pots were used to determine 100 and 0% soil water content, named saturated weight (SW) and dry weight (DW), respectively. Five plants per genotype were maintained at well-watered conditions and water was withdrawn from other five plants. Pot weight was determined daily and relative soil water content (rSWC) was calculated, according to the formula rSWC=(FW-DW)/(TW-DW). Pots were left to dry until 15-20% rSWC was reached.

To determine relative leaf water content (rLWC), leaves segments were collected 0, 5, 10 and 15 days after watering withdrawal. Segments were weighed (fresh weight, FW), then left saturated in water for 24 hours and their turgid weights (TW) were calculated. The samples were then dried in an oven at 60°C for 72 hours

and weighed (DW). The rLWC was determined as follows: rLWC = (FW - DW)/(TW - DW) x 100 (Cabrera-Bosquet *et al*., 2007).

Gas exchange and Carbon fixation measurements

"Snapshot" readings of gas exchange variables and Carbon fixation were made using a CIRAS portable infrared gas analyser (PP Systems, Hitchin, UK), as set in Lawson and Weyers (1999). Records of rates of gas exchange (stomatal conductance) and carbon fixation were taken every 2 minutes during 24 hours in three control plants and three 8-days-droughted plants per genotype.

Water use efficiency

Water use efficiency (WUE) expressed in g of plant production per Kg of water consumed was measured gravimetrically in at least 4 plants per genotype and processed according to Xin *et al.* (2008). Briefly, pots were watered until water dripped from the bottom and covered from both ends with two polythene bags that were fixed to the pot with elastic bands. A small slit was made in the top bag to allow the plant to grow through. Control pots without plants showed minimum water loss. The initial and final (after 4 weeks) pot weights were taken and water used was calculated by subtracting the final pot weight from the initial weight. Roots were collected by washing the potting mix core on a wire mesh. Dry weight measurements of roots and shoots were taken after a minimum of 72 h of drying at 60°C when the samples reached a constant weight. WUE was calculated by dividing the total dry biomass by the amount of water transpired.

Polyamine analysis

Samples from the 3rd and the 4th pair of leaves were collected 0, 7 and 14 days after watering withdrawal and kept frozen at -80ºC. Tissues were extracted in

5% cold $HClO_4$ at a ratio of about 100 mg/ml $HClO_4$. After extraction for 1 h in an ice bath, samples were pelleted at 48,000g x 20 min, and the supernatant phase, containing the 'free' polyamine fraction, was stored frozen at -20°C in plastic vials according to the recommendations and protocol from Flores *et al*. (1982). One ml of 2N NaOH was mixed with 250 to 500 µl of $HClO_4$extract. After addition of 10 µl benzoyl chloride, vortexing for 10 s and incubation for 20 min at room temperature, we added 2 ml saturated NaCl. Benzoyl-polyamines were extracted in 2 ml diethyl ether anhydrous (Sigma). After centrifugation at 1500g x 5 min, 1 ml of the ether phase was collected, evaporated to dryness under a stream of warm air, and redissolved in 100 pl methanol (Sigma; HPLC grade). Standards were treated in a similar way, with up to 50 nmol of each polyamine in the reaction mixture.

The standards from PAs putrescine (Put), spermidine (Spd), spermine (Spm) and the intermediate agmatine (Agm) were obtained from Sigma as their hydrochlorides. Standards and plant extracts were benzoylated according to Redmond and Tseng (1979). HPLC analysis of benzoyl-PAs was performed using an Agilent 1200 series liquid chromatograph. Derivatized PAs were injected into a fixed volume 20 μl and chromatographed at ambient temperature through a 4,6 x 250 mm, 5 μm particle size C18 reverse-phase column (Trader Excel 120 ODSB, Tecknokrome). PAs and Agm were eluted at a flow rate of 1,0 ml/min using the water (solvent A)/MeOH (solvent B) stepped gradient program followed by a column cleaning/regeneration: 50 to 65% B in 7 min/65 to 80% B in 6 min/80%B for 5 min/80 to 100%B in 6 min/ 100%B for 5 min/100 to 50% B in 4 min/50% B equilibration for 7min. The PAs were separated according to their retention times and their UV

spectrums (254 nm). The levels of the main polyamines were calculated according to a calibration curve of standards.

Measurement of chlorophyll fluorescence under high light

Samples from the 4th pair of leaves were collected after 7 days of watering withdrawal. To apply the high light stress (HLS) treatment, 9 detached pea leaves per genotype, 3 per treatment (control, droughted and high lighted) were placed in 20cm^2 paper dishes wet with distilled water and then half of them were exposed to a white light pulse of 2000 μmol photons $m^{-2}s^{-1}$ through a glass filter filled with cold water (Hernandez *et al.*, 2004). After 15 min leaves were dark adapted for 10 minutes in the growing chamber and then the measures of maximum quantum efficiency of PSII, $F_v/F_{m,}$ as well as the non-photochemical quenching, (NPQ) were done, using a fluorescence imaging instrument (Fluorimager, Technologica, Colchester UK; Barbagallo *et al.*, 2003).

Statistical analysis

All experiments were designed in a randomized complete block design. Means of raw percentage data are presented in tables and figures. Standard analysis of variance was performed using GenStat 11th edition, after which residual plots were inspected to confirm that data conformed to normality. The significance of differences between means was determined by contrast analysis (Scheffe's).

RESULTS

After preliminary screenings we selected the genotypes Polar, Messire and P665 to study their possible tolerance to drought and the genotype Kebby as susceptible check.

Visual scale and relative water content

The visual scale allowed a relatively fast and easy discrimination among accessions, with Polar and P665 being ranked as the most tolerant accessions (Fig.1.2.A). Measurement of *rSWC*, which reflect the water content on soil (Fig.1.2.B) indicated that water was reduced in all the genotypes, although Kebby exhibited a more rapid decline compared with P665, Polar and Messire. In these three genotypes the water content of the soil during the water stress period was reduced slower than in the most susceptible genotype according to the visual scale.

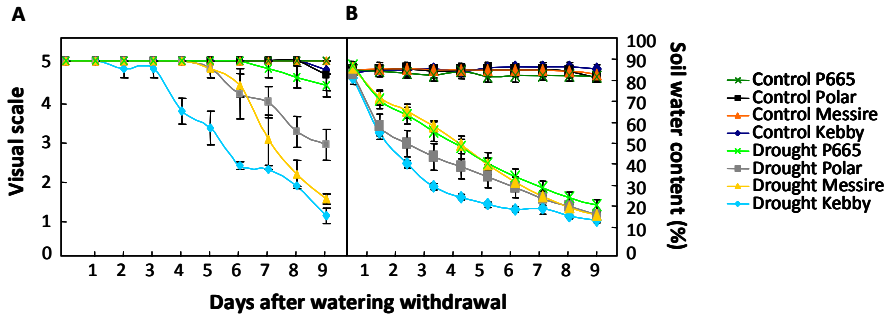

Fig.1.2. Visual scale (A) and soil water content (B) assessment of the pea genotypes P665, Polar, Messire and Kebby along a water stress period of 10 days. Points represent the mean values for each observation and bar the standard error values.

Assessment of the *rLWC* showed values of approximately 90% in the four genotypes before withholding water with no significant differences between accessions (Fig.1.3) after watering withdrawal that the genotypes P665 and Polar maintained higher water content in leaf tissue till the 15th day, when a significant reduction of the water level in the genotype Kebby was produced.

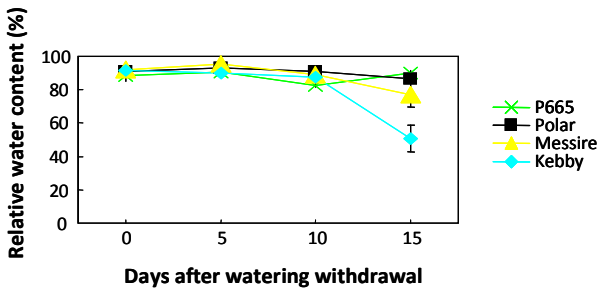

Fig.1.3. Temporal evolution of *rLWC* after watering withdrawal expressed in percentage. Points represent the average value for each genotype and bar the standard error on each time point.

According to the visual scale and the water relative content, Kebby were dramatically affected by watering withdrawal and could be classified as susceptible whereas P665 would be the tolerant genotype.

Gas exchange and Carbon fixation measurements

The stomatal conductance was measured during 24 h in control and droughted plants (Figure 1.5.A). P665, Messire and Kebby showed similar levels of stomatal conductance in control conditions whereas Polar levels were significantly lower ($p<0,05$). In drought conditions, P665 and Kebby halved their stomatal conductance whereas Messire showed a significant reduction of the 75% ($p<0,05$) with respect to the controls. Polar maintained the same conductance levels both in control and droughted plants.

Changes in carbon fixation during 24 hours were closely related to those on stomatal conductance. Well watered control plants from all the genotypes showed similar levels of carbon fixation during 24 hours (Fig 1.5.B) and the genotypes reduced their rates under water stress conditions.

The genotype P665 maintained the highest carbon fixation rate under stress conditions ($p>0,05$) compared with Messire and Kebby.

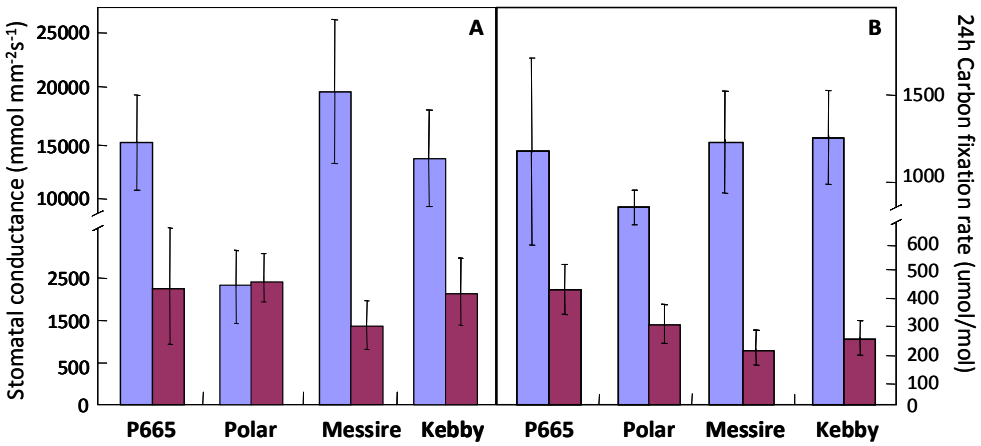

Fig.1.5. Mean values of stomatal conductance (A) and carbon fixation rate average (B) during 24 hours. Measures were taken 0 and 8 days after water withdrawal. Blue and purple bars are for control and droughted plants, respectively.

No significant differences were found in the carbon fixation rate under drought stress conditions between Polar and the rest of the genotypes. However, while the reduction in the carbon fixation rate under water stress conditions was of approximately of a 50% in the genotypes P665 and Polar, the rate in Messire and Kebby is decreased a 75% with respect to the controls. Thus, the genotypes P665 and Polar would be more conservative with their efficiencies under droughted conditions than Messire and Kebby.

Water use efficiency (WUE)

WUE expressed in terms of dry biomass (g) per Kg of water consumed ranged between 3,00 (Kebby) and 3,75 (Polar) respectively (Table 1.2).

Table 1.2. Average water use efficiency (WUE) and water use (WU) based on shoot dry biomass and root dry biomass of the pea genotypes P665, Polar, Messire and Kebby.

	Shoot WUE (g/Kg)	Root WUE (g/Kg)	Total WUE (g/Kg)	Use of water (Kg)
P665	1,64 c	0,62 b	2,68 c	0,06 a
Polar	2,23 ab	1,37 a	3,75 a	0,15 b
Messire	2,31 a	0,80 b	3,29 b	0,13 b
Kebby	2,08 b	0,99 b	3,00 b	0,15 b

Messire and Polar showed the highest shoot WUE, being significantly different to P665 ($p<0,01$). The genotype Polar had significant ($p<0,01$) higher values of total WUE and root WUE with respect to the other genotypes.

P665 showed lower values of shoot and total WUE than the other genotypes ($p<0,01$), accordingly to its use of water during the assay, which was half than the other genotypes along the assessment period ($p<0,05$).

Polyamine patterns

Differences in the polyamine patterns of the genotypes P665, Polar, Messire and Kebby during the drought treatment were found at a highly significant level ($p>0,001$) for the polyamines Put, Spd, Agm and Spm (Fig.1.4).

The genotype P665 shows higher levels of Put in the basal status as well as at the terminal water stress treatment than the rest of the genotypes ($p<0,05$) (Fig.1.4.A). However, the cv. Polar showed higher levels of putrescine than the rest of the genotypes 7 days after the watering withdrawal ($p<0,05$).

No Spd was detected constitutively in Polar (Fig.1.4.B)., Genotype P665 showed higher ($p<0,05$) levels of Spd than the rest of the genotypes. Nevertheless, both P665 and Polar showed higher levels of Spd in the middle of the water stress period as well as in the terminal period. Despite the higher or the lower levels of Spd, all the genotypes experimented a significant ($p>0,01$) decrease of this polyamine at the end of the water stress period.

The cv Polar showed lower constitutive levels of Agm than the cvs. Kebby and Messire ($p<0,05$) and no significant differences with Agm content on genotype P665 (Fig. 1.4.C). However, after seven days of water stress, Polar and P665 showed higher levels of Agm than Kebby and Messire ($p<0,01$). At the end of the water stress

period the levels of Agm rise again to or up to 12000 nmol/gfw (grams of fresh weight), showing equal levels on the different genotypes.

Regarding the Spm (Fig.1.4.D), the highest constitutive levels were found in P665 ($p<0,01$). After 7 days of watering withdrawal the cv. Polar and also genotype P665 showed higher levels than Messire and Kebby ($p<0,01$). The lowest levels in this time point were observed in Messire ($p<0,05$).

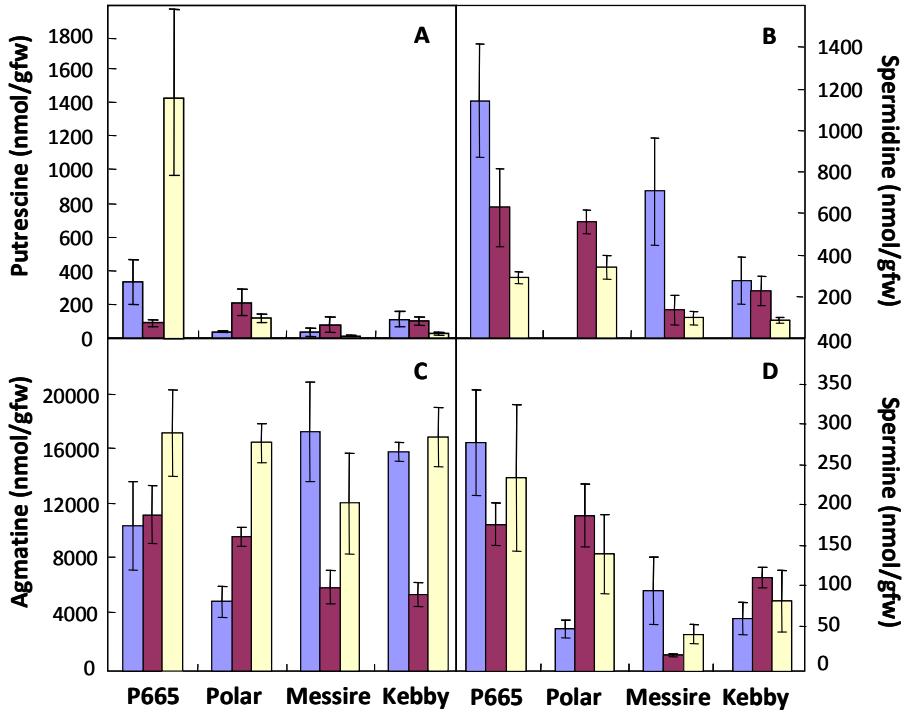

Fig.1.4. Levels of polyamines (A: Putrescine; B: Spermidine; C: Agmatine and D: Spermine levels).in pea leaves from the genotypes P665, Polar, Messire and Kebby. Samples were taken 0 (blue), 7 (purple) and 14 (yellow) days after water withdrawal.

At the end of the water stress period, the genotype P665 showed higher levels than cvs. Polar, Messire and Kebby ($p<0,05$) whereas Messire showed the lowest levels of all genotypes ($p<0,05$).

Measurement of chlorophyll fluorescence under high light

Plants exposed to drought conditions often are subjected to high light stress. When the leaf is transferred from darkness into light, PSII reaction centres are progressively closed giving to an increase in the yield of chlorophyll fluorescence during the first second of illumination. Following on from this, after the saturation of the PSII reaction centres there is a decrease on the fluorescence level over a time-scale of a few minutes. This phenomenon is termed fluorescence quenching and involves an increase in the rate at which electrons are transported away from PSII (photochemical quenching) as well as an increase in the efficiency with which energy is converted to heat (non-photochemical quenching or NPQ). On the other hand, decreases in the Fv/ Fm ratio can be due to development of slowly relaxing quenching process and photo damage to PSII reaction centres, both of which reduce the maximum quantum efficiency of PSII photochemistry (Baker *et al*., 2004). Then, we explored the behaviour of the 4 genotypes after applying to them high light conditions by taken measurements of these two parameters in dark adapted leaves.

As it is shown in Figure 1.6, whereas control leaves ratios remained above 0.75, a significant decrease in Fv/Fm was observed in all genotypes after 15 minutes of HLS ($p<0,01$). This decline was higher in P665 than in the rest of genotypes.

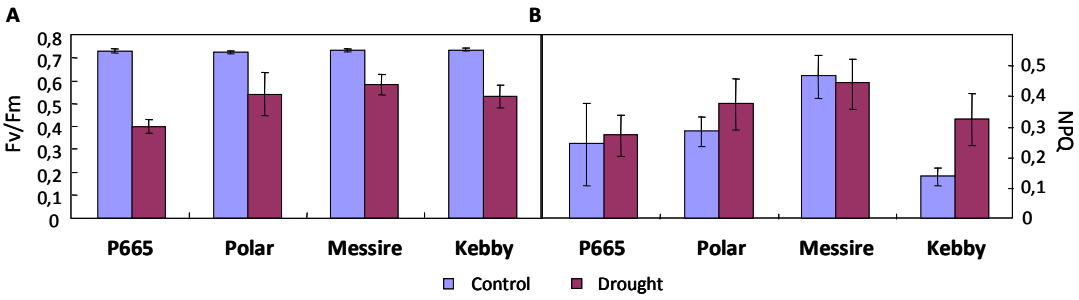

Figure 1.6. Effect of HLS on Fv/Fm (A) and *NPQ* (B) values in pea leaves of four pea genotypes.

A significant increase in NPQ values after the treatment was observed only in the genotype Kebby ($p<0,01$), whereas the others remained with NPQ levels similar to their controls. Furthermore, Kebby was the genotype with the lowest levels in the control plants. Messire showed the highest levels of NPQ even in the control leaves ($p<0,01$). These results indicated for P665 a relative susceptibility to HLS, whereas Kebby would be more tolerant.

DISCUSSION

The purpose of these studies was to find new sources of tolerance to drought in pea genotypes and to characterize them in base of their underlying resistance response with the final aim of using these genotypes in plant breeding programmes. Thus, after previous screenings, we selected two apparently tolerant genotypes (P665 and Polar), one genotype which seemed moderately tolerant (Messire) and one genotype looking as a susceptible check (Kebby). Since P665 was a different subspecies than the other pea genotypes we also choose Polar as tolerant *P. sativum* subsp. *sativum* cv.

In general, water related parameters, together with the visual scale, pointed out P665 and Polar as those genotypes able to maintain the highest levels of turgor in the plant tissues along the water withdrawal period.

The visual scale allowed discrimination between genotypes. In addition, this evaluation method can be taken quickly in the field providing information for evaluation and selection that could not otherwise be obtained due to time or cost constraints of quantitative techniques. Confirming preliminary screenings, our

results showed genotype P665 and cv. Polar were more tolerant to drought than Messire and Kebby according to the visual assessment of the symptoms.

However, precise definition of water status in different parts of the soil–plant system was also required for the formulation and testing of any hypotheses and to define the conditions (both in terms of the treatments applied and in terms of the effects on the plants) and as a first step in facilitating repetition of the experiment (Jones, 2007). For this reason, we also assess the rSWC and the rLWC together with the visual scale. As expected rSWC and rLWC declined in all the genotypes after watering withdrawal, but the evolution of these parameters was different within the genotypes. P665 was the genotype which showed lesser looses of water, agreeing with the visual assessment of wilting symptoms whereas Kebby showed significant water looses in both soil and plant. Soil water deficits are usually considered as the underlying stresses in the system. Thus, the leaf water status would be a result of the soil water deficit. Indeed the leaf water status is modulated by plant responses so it uniquely does not describe the experimental treatment, although it is an appropriate measure of plant water status in terms of the physiological consequence of cellular water deficit (Kramer, 1988; Jones, 2007). Other parameters such as water potential as an estimate of plant water status are useful in dealing with water transport in the soil–plant–atmosphere continuum (Kramer, 1988). Nevertheless, only rLWC takes into account the possible effect of both leaf water potential and osmotic adjustment.

Initial responses to stress occur at the leaf level (Iriti *et al.*, 2009; Flexas *et al.*, 2004; Haldimann and Feller, 2004). As a result from turgor differences between guard cells and the surrounding subsidiary or epidermal cells, stomata close rapidly

under drought conditions (Meidner *et al*., 1968). Thence, we have observed a decrease in stomata conductance in the genotypes P665, Messire and Kebby after 8 days of watering withdrawal. However, the behaviour of the cv. Polar was different from the rest maintaining, surprisingly, the same levels of stomatal conductance in both control and droughted plants. This reduction in the stomata transpiration under water stress is closely related with a decrease in the chloroplast CO_2. Accordingly, we observed a decrease in the carbon fixation rate measured under drought conditions in these genotypes. Lower reductions of the carbon fixation rate under drought stress were observed for the genotypes Polar and P665, being closely related with the WUE data. Polar was the most efficient genotype regarding total WUE, whereas P665 used less than half the water than the rest of the genotypes producing less biomass. However, in terms of proportion, if P665 would have used the same amount of water than the other genotypes it would have produced more biomass than the others. Therefore, P665 and Polar could be considered more efficient photosynthetically than the cvs. Kebby and Messire.

Cellular hydration is also preserved through powerful mechanisms such as osmotic adjustment or osmoregulation, enabling plants to maintain water absorption and cell turgor pressure under drought conditions, through the accumulation of specific compounds (Blum, 1989; Beck *et al.*, 2007; Farooq *et al.*, 2009). PAs are one of these osmoregulators contributing to improved tissue water status through variations in their accumulation patterns. Accordingly, we have observed differences in the PA patterns of the genotypes P665, Polar, Messire and Kebby during the drought treatment with respect to the controls.

It has been reported than stress-tolerant plants increase their endogenous *PA* levels to a much greater extent than sensitive ones (Lee, 1997). Consequently, the tolerant genotype P665 showed an accumulation in all the studied polyamines both under mild and terminal drought stress. Transgenic plants overproducing PAs possess greater stress tolerance (Galston *et al.,* 1997) and exogenous application of PAs confers protection from a variety of abiotic stresses (Nayyar *et al.*, 2005; Nayyar and Chander, 2004; Basra *et al.*, 1997). Thus, the quantification of changes in the polyamine levels has been a helpful tool to evaluate the ability of plants to maintain cell and tissue turgor under drought stress, contributing to their drought tolerance.

The diamine Put, the triamine Spd and the tetramine spermine Spm are the main PAs found in all living cells, being also believed to protect plants against water deficit (Groppa and Benavides, 2008; Capell *et al.*, 2004). In plants, Put is synthesized by the decarboxylation of either arginine or ornithine catalyzed by arginine decarboxylase (ADC) or ornithine decarboxylase (ODC) (Fig.1.6). Agm is an intermediate synthesized by ADC, being one of the precursors of the Put. Spd and Spm are formed by the subsequent addition of an aminopropyl moiety onto Put and Spd, respectively, in reactions catalysed by the enzymes Spd synthase (SPD*S*) and Spm synthase (SPM*S*).The aminopropyl moiety results from the decarboxylation of S-adenosylmethionine (SAM) by the enzyme S-adenosylmethionine decarboxylase (SAMDC) (Slocum, 1991). P665 showed a high content of constituve PAs with levels similar of those found in rice (*Oryza sativa* L) (Yang *et al.*, 2007) whereas the rest of the genotypes showed an amount of similar to those found in chickpea (*Cicer arietinum* L.) (Nayyar *et al*., 2004).

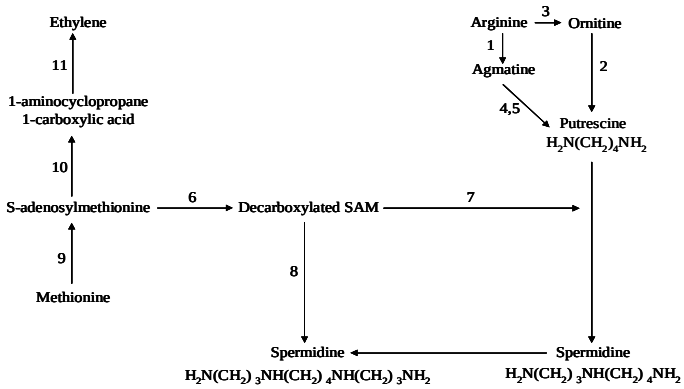

Fig.2.1. Pathways of biosynthesis of the major plant polyamines (Putrescine, Spermidine and Spermine) and relationship with ethylene biosynthesis. 1, Arginine decarboxylase (ADC); 2, Ornithine decarboxylase (ODC); 3, Arginase; 4, Agmatine iminohydrolase; 5, *N*-carbamoyl putrescine amidohydrolase; 6, SAM decarboxylase (SAM DC); 7, Spermidine synthase; 8, Spermine synthase; 9, SAM synthase; 10, ACC synthase; 11, ACC oxydase.

Till date, no studies of PA accumulation patterns under drought stress have been developed about pea. The general trend in the genotypes but P665 was a decrease in the levels of Put, Spd and Spm. This decline in PA levels with the ageing of the leaves has also been observed in rice (Yang *et al.*, 2007), soybean and chickpea (Nayyar *et al*., 2004) and could be due to their reduced biosynthesis when leaves become older (Lazcano-Ferrat *et al*., 1999). It has been reported for different rice cvs. (Yang *et al.*, 2007) that the content of free PAs in water stressed leaves increased and exhibited one peak for each cultivar before decreasing with plant ageing. Accordingly to our results, the peak values and time of appearance varied greatly with cultivar, being higher and sooner in time those peaks for the tolerant cultivars. In the case of Polar, this could explain the trend in the obtained accumulation pattern of Put, Spd and Spm, reaching the highest peak after 7 days of watering withdrawal. Also, it has been reported that polyamines regulate stomatal responses by reducing their aperture and inducing closure (Liu *et al.* 2000; An *et al.*

2008), thus, polyamine pattern is concordantly with the odd stomata conductance data obtained for Polar. The low levels of free PAs in drought susceptible and intermediate tolerant genotypes such as Kebby and Messire, respectively, could be due to a slower mechanism of response compared with the tolerant one. In fact, it is reported that these peaks of free PAs appear at a latter stage accordingly to the susceptibility of the cultivars in rice, (Yang *et al.*, 2007). However, more detailed time courses would be necessary to extract further conclusions about these genotypes as well as P665, which showed the highest basal levels.

Changes in polyamines under single or combined stresses have been extensively investigated (Tiburcio *et al.*, 1994; Liu *et al.* 2006b ; Urano *et al.*, 2003; Kuthanová *et al.*, 2004; Camacho-Cristóbal *et al.* 2004; Mo *et al*., 2002; Shen *et al.*, 2000;Nam *et al.*, 1997; Santa-Cruz *et al.*, 1997b; Scalet *et al.*, 1995; Rowland-Bamford *et al.*,1989). The phenomenon that Put accumulates in plants under abiotic stress has been observed for more than 50 years. However the physiological role of Put in abiotic stress responses remains a matter of controversy (Bouchereau *et al.*, 1999; Chen *et al*., 2000; Capell *et al.*, 2004; Kuehn *et al*., 2005).

The physiological meaning of the accumulation of the intermediate Agm is closely related with the ADC pathway that would allow the conversion of Agm into Put. Our results support an increase of the ADC activity during the first period of the drought treatment for P665 as well as for Polar in the middle of the water stress, but not for the other genotypes.

It is described that transgenic plants expressing *Datura ADC* produced much higher levels of Put under stress, promoting Spd and Spm synthesis and ultimately protect the plants from drought (Capell *et al.*, 2004). Accordingly, P665 showed the

highest values of Spd and Spm in all the time points whereas Polar had also higher values than Messire and Kebby after seven days of watering withdrawal and in the terminal water stress period. Looking into the polyamines biosynthesis, the fact that non detectable values of Spd were found constitutively in Polar is probably related also with the low values of Put found for this genotype. An increase in Spd and Spm drought tolerant cultivars under water stress has been reported in wheat and groundnut while the sensitive ones only experienced an increase in Put (Liu *et al.*, 2007). Thus, our data support the activation of the polyamines metabolism in Polar and P665 as one of the responses involved in the drought tolerance observed in these genotypes.

Plants exposed to drought conditions often are subjected to high light stress. Then, we have assessed the behaviour of the genotypes P665, Polar, Messire and Kebby under high light conditions by chlorophyll fluorescence measurements. According to our results, all the genotypes showed a reduction on the Fv/Fm ratio being more affected the genotype P665. This reduction implies the development of slower relaxing quenching process than the other genotypes and higher photodamage of PSII reaction centres (Baker, 2008). Changes in the maximum quantum yield of PSII (Fv/ Fm) provide an estimate of the maximum quantum efficiency of PSII photochemistry (Butler, 1978) and have been widely used to detect stress-induced perturbations in the photosynthetic apparatus (Valladares and Pearcy, 1997). Polyamines also play a role in preventing photo oxidative damage (Løvaas, 1997; Groopa *et al*., 2008), thus the less oxidative damage in Polar with respect to P665 could also be related with its PA pattern accumulation after 7 days

of drought, providing this genotype with an additional protection against other abiotic stresses.

On the other hand, any change in NPQ measures a change in the efficiency of heat dissipation relative to the dark-adapted state. Broadly, an increase in the NPQ levels can occur as a result either of processes that protect the leaf from light-induced damage or of the damage itself (Maxwell and Johnson, 2000). In this sense, the genotype Kebby would experience a quicker recovery from high light stress, being less susceptible to photo oxidative damage than the other genotypes and being highly efficient dissipating heat after suffering HL stress. Altogether, these changes are thought to be associated with protecting cellular functions or with maintaining the structure of cellular components (Seki *et al.* 2007).

To conclude, according to our observations, genotypes Polar and P665 seemed to be the most interesting drought tolerant sources. Each one showed a high ability to maintain tissue turgor during the water stress period. However, the mechanisms that mediate their response seem to be different. In the genotype P665 all the studied traits point out to a multi factorial resistance response mediated by different mechanisms. Whereas in the cv. Polar, polyamine-based osmoregulation is one of the main factors involved in its tolerance to drought stress. The fact that Polar is not as sensible to HL stress as P665 would make of this genotype a better one for its use in semiarid regions, where plants are subjected to both water and HL stress during their growth. However, as P665 is a non cultivated species that already have shown resistance to different stresses, mainly biotic (Fondevilla *et al.*, 2008; 2011) and also seems to be tolerant to drought stress, it would also be a genotype of interest for a breeding program.

Given its interest, studies on resistance in the RIL (Recombinant Inbreed Line) of the genotype P665 crossed with Messire are being conducted to determine QTL involved in the drought tolerance, and Polar is currently being included in field studies to check yield under different environments.

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Chapter 2

Multi-environment assessment of yield, growth, phenology and natural biotic and abiotic stress in ten pea (*Pisum sativum* L.) genotypes

INTRODUCTION

Pea (*Pisum sativum* L.) is a cool season legume grown worldwide as a source of protein both for human food and animal feed. Pea is the most widely grown grain legume in Europe and the second-most in the world (FAOSTAT 2011) and represents a versatile and inexpensive protein source for animal feeding. As a grain legume, pea crops are useful to conserve the soil, add organic matter, fix nitrogen, save soil nitrogen, and help in controlling cereal diseases. Furthermore, the contribution of legumes, such as pea, to soil fertility is one of the key factors in sustaining the production of cereal crops in rainfed dry areas in the developing world (Jacobsen *et al*., 2012).

Yield variability is a major problem for field pea crops both within and between sites and seasons due to poor pollination, drought stress and diseases (White, 1987; Moot *et al*., 1995; Jacobsen *et al*., 2012). Significant efforts have been made in pea breeding for adaptability in continental and oceanic conditions where it is mainly spring sown (Cousin, 1997) and plenty of cultivars are adapted to those conditions. However, pea cultivation is strongly hampered in Mediterranean and Middle East farming systems by the occurrence of biotic and abiotic stresses, still causing important yield losses, partly due to the absence of varieties specifically adapted to these conditions. In these areas, with mild winters and dry springs, spring pea types are autumn sown and the main problems of the crop are the broomrape (*Orobanche crenata* Forks.), followed by water stress (Rubiales *et al*., 1999).

Therefore, the key breeding objectives for pea involve increasing yield potential and select genotypes that produce high and stable yields, being adapted to diverse environments and with improved biotic and abiotic stress resistance (Smýkal

et al., 2012; Moot *et al*., 1995). Thus, proper management, selection of cultivars, and breeding, are necessary tools to improve productivity, and the use of appropriate selection criteria is an important element in meeting this challenge.

Furthermore, the stability of the cultivars should be checked through time and space, hence the importance of carrying out multi-location and multi-year experiments. The need for multi-environmental testing arises from the fact that genotype x environment (GxE) interactions are common in field trials of diverse crops (Carson *et al*., 2002; Hess *et al*., 2002; Pinnschmidt *et al*., 2002; Brancourt-Hulmel *et al*., 2003; Forbes *et al*., 2005; Zinsou *et al*., 2005). This is of great importance in breeding programs, since large GxE interactions bring about discrepancies between expected and realized responses to selection due to an higher stimation of genetic variances (Haussmann *et al*., 2001). This makes it difficult to predict the behaviour of the accessions in situations where they have not been tested before, thus reducing their adaptability to different environments (Dixon *et al*., 2002). The Genotype plus Genotype by Environment interaction (GGE) biplot statistic methodology applied over multi-environment field trials can help to overcome these difficulties, allowing the decomposition of the interaction GxE (Yang *et al*., 1999; 2000) into two main components.

MATERIALS AND METHODS

Plant material and experimental design

A Pea Network consisting on 8 commercial varieties and 2 improved lines (gently supplied by the ITACyL, Valladolid, Spain) were evaluated over four crop

seasons (2008-2009 to 2011-2012) at 5 contrasting locations (Table 2.1). An environment was defined as the combination of a year and a location. The cultivars studied were: Ballet, Desso, Frisson, HR-1, Kebby, Messire, Polar, Solara and ZP-108.

Table 2.1. Description of the environments (combination of location and season) of the trials for the multi-environment study. Climatic data are provided for the growing season.

						Weather during growing season		
Environm.	Location	Lat.	Long.	Altitude (mASL)	Growing season	Absolute Max. T (ºC)	Absolute Min. T (ºC)	Rain (mm)
BEJA09	Beja, Tunisia	36° 44' N	9° 13' E	222	2009-10	44,5	-10,8	598
BEJA10	Beja, Tunisia	36° 44' N	9° 13' E	222	2010-11	43,7	-0,4	458
BEJA11	Beja, Tunisia	36° 44' N	9° 13' E	222	2011-12	43,7	0,3	644
CAR08	Carmona, Spain	37° 28' N	5° 38' O	253	2008-09	22,0	3,6	463
CORD08	Córdoba, Spain	37° 50' N	4° 50' W	90	2008-09	34,5	-4,1	445
CORD09	Córdoba, Spain	37° 50' N	4° 50' W	90	2009-10	33,3	-3,9	741
CORD10	Córdoba, Spain	37° 50' N	4° 50' W	90	2010-11	29,7	-2,9	432
CORD11	Córdoba, Spain	37° 50' N	4° 50' W	90	2011-12	35,7	-5,9	175
ESC08	Escacena, Spain	37° 25' N	6° 15' W	88	2008-09	34,4	-2,0	240
ESC09	Escacena, Spain	37° 25' N	6° 15' W	88	2009-10	33,3	-2,4	924
ESC10	Escacena, Spain	37° 25' N	6° 15' W	88	2010-11	30,8	2,8	614
ESC11	Escacena, Spain	37° 25' N	6° 15' W	88	2011-12	24,6	-2,8	224
VILLA10	Villamor, Spain	41° 19' N	6° 6' W	777	2010-11	35,8	-7,6	515
VILLA11	Villamor, Spain	41° 19' N	6° 6' W	777	2011-12	37,2	-9,3	209

The selection of these cultivars was based on previous bibliographic research of pea varieties apparently tolerant to water stress (Grzesiak, 1997, Manzanares *et al.*, 1998). These environments are characterized by mild and moderately rainy winters and warm and dry springs (Table 2.1), being winter sowing of spring crops a common practice.

At each location a randomized complete block design with two to six replications was used. Each replicate consisted in independent plots consisting in three 1m-long rows bordered by lentils. Within each plot, the rows were separated from each other by 30 cm. Sowings took place between the end of November and the beginning of January, according to local practice, at a sowing density of around 30 seeds m^2. Infections occurred naturally in all the locations.

AUDPC coverage date, biomass and yield assessments

The coverage was visually assessed as the percentage of the plot covered by the plants. Observations were made monthly from two months after the sowing date until four months, where the plants were grown. This allowed calculation of the Area under the disease progress Curve (AUDPC) according to Wilcoxson *et al.*, (1975). At the end of the season, plots were harvested and whole-plants and seeds weighted together and separately to obtain the total biomass as well as the yield per plot. Additionally, 100 grains per plot were weighted. AUDPC coverage, biomass values and yield were referred to the number of plants in the plot.

Flowering and fruiting assessments

To determine the date of flowering and fruiting, the number of days from the sowing date until 50% anthesis and fructification were considered, respectively.

Infection and disease assessments

The number of crenate broomrapes (*Orobanche crenata* Forks.) per plant was obtained immediately after harvesting, counting all the broomrapes per plot and dividing it by the number of pea plants within the plot. Powdery mildew (*Erisiphe pisi*) was assessed once time per season when the symptoms were observed. as a visual estimation of the percentage of whole plant tissue covered by mycelium.

Frost symptoms assessment

The percentage of yellowing in the plants was estimated visually. Observations were made just once, when the symptoms were observed.

Statistical analysis

Data of each trait were submitted to a combined analysis of variance (ANOVA) with genotype and location-year environment as fixed factors (Table 2.2).

The GGE biplot method (Yan *et al*. 2000) was employed to study the genotype by location-year environment interaction of pea mildew symptoms, AUDPC coverage, date of flowering and fruiting, biomass, crenate broomrape infection and seed yield. This methodology used a two-dimensional biplot, constructed using the first two principal components (PC1 and PC2) derived from subjecting the environment-centered data to singular value decomposition. Singular value partitioning was achieved by providing a scaling factor *f* to obtain alternative cultivars and environment scores. We chose the most straightforward variant called symmetric scaling (*f* = 0.5) since it beard most of the properties associated to other scaling methods (Yan, 2002).

Cultivars and environments were displayed in the same plot. This GGE biplot allowed identifying broadly adapted cultivars that offer stable performance across all sites, as well as cultivars that perform well under specific sites and putative different mega-environments (a group of environments that consistently share the same best cultivar or cultivars). To compare genotypes by their performance and stability we used an Average Tester Coordinate (named ATC by Yan 2001).

An ATC is a virtual environment whose first and second principal components scores are equal to the average of the first and second principal components scores, respectively, across all environments (Yan 2001). The ATC X-axis passes through the biplot origin and the marker of the PC1 average across the environments, called ATC_a. The ATC Y-axis passes the plot origin and is perpendicular to the average tester coordinate X-axis, called ATC_o.

The contribution of each genotype to a specific trait was approximated by their projections to the ATC X-axis and the stability was measured by their projection

to the ATC Y-axis. Thus, the genotypes could be distributed in increasing order along the X-axis from left to right, according to their development for a specific trait, and the greater the absolute length of the projection of a cultivar in the Y-axis, then less stable it is. In this sense, ideal test environments should have a large average tester coordinate X-axis score (more discriminating of the cultivars in terms of the genotypic main effect) and small (absolute) average tester coordinate Y-axis score (more representative of the overall environment). An environment near the center of the biplot did not discriminate the genotypes, which could mean that all genotypes performed similarly, and it is less informative.

To study the traits frost symptoms and 100 grains weight a one-way ANOVA was developed, followed by a Tuckey mean comparison, as they were not affected by Genotype by Environment interactions.

All the analyses were made with a SAS® 9.3 (SAS Institute Inc.) program for graphing GGE biplots developed by Burgueño *et al*. (2003).

RESULTS

Variance results for pea data (table 2.2) indicated that genotype (G), environment (E) and genotype by environment (GE) interactions showed significant ($p<0,0001$) differences among pea genotypes tested for AUDPC coverage, biomass, date of flowering, date of fruiting, yield, crenate broomrape per plant and mildew.

This result showed that these traits were significantly influenced by E which accounted for 39% to 78% of the total variation, whereas G and GE interaction explained from 1% to 57% and 7% to 26%, respectively (Table 2.2). The mean and standard error values of the evaluated traits: “AUDPC coverage”, “biomass”, “date of

flowering", "date of fruiting", "grain yield", "powdery mildew" and "crenate broomrape per plant" is showed in tables 2.3, 2.4, 2.5, 2.6, 2.7, 2.8 and 2.9.

Table 2.2 Genotype (G), location-year environment (E) and genotype by location-year environment interaction (GE) terms for AUDPC coverage, Biomass (Kilograms per hectare), date of flowering, date of fruiting, yield (Kilograms per hectare), broomrape per plant and percentage of mildew, weight of 100 seeds and percentage of frost for the pea performance trials, from 2008 to 2011.

Trait	Source of variation	df[a]	Mean Squares[b]	Explained variation % of G, E and GE[c]	% of PC1 + PC2[d]
AUDP coverage	E	13	608,29***	40	
	G	8	51,22**	3	25 + 62
	GE	104	57,94***	26	
Biomass	E	10	9158205, 82***	44	
	G	8	199357, 39	1	80 + 10
	GE	80	421992, 51**	16	
Date of flowering	E	13	6851,85201***	77	
	G	8	1266,48429***	9	73 + 11
	GE	104	76,19468***	7	
Date of fruiting	E	12	7426,06***	78	
	G	8	711,97***	5	57 + 15
	GE	96	86,24***	7	
Grain Yield	E	11	2234327,28***	43	
	G	8	49513,01	1	9 + 84
	GE	88	99419,82**	15	
Powdery mildew	E	3	27809,36***	61	
	G	8	1273,20***	7	58 + 37
	GE	24	996,80***	17	
Broomrape per plant	E	6	26,14***	39	
	G	8	6,74***	14	79 + 10
	GE	48	1,98***	24	
Weight 100 seeds	E	8	103,65***	14	
	G	8	405,00***	57	
	GE	64	7,86	9	
Frost symtoms	E	1	2216,96	8	
	G	8	1303,62**	36	
	GE	8	552,34	15	

[a] degrees of freedom
[b] **, *** Significant at the 0.001 and 0.0001 level of probability, respectively.
[c] Percentage sums of squares respect from the total sums of squares
[d] Proportions of the first two Principal Components derived from singular value decomposition of the environment-centered data.

The partitioning of G and GE interaction through GGE biplot analysis showed that the first two principal components were significant factors for the first group of traits ("yield", "biomass", "date of flowering", "date of fruiting", "AUDPC coverage", "crenate broomrape per plant" and "mildew") explaining from 9% to 84% of total G and GE interaction sum of squares (Table 2.2).

However, we did not find any GE interaction for the traits “100 seeds weight” and “frost symptoms”. Only significant differences between E and G were found ($p<0,0001$) for the first one, whereas for the frost symptoms differences were only found in G ($p<0,001$).

AUDPC coverage

In GGE biplot analyses, a polygon is formed with the most extreme genotypes or vertex cultivars. Perpendicular lines from the origin to each side of the polygon determine different sectors where the cultivars are included.

Figure 2.2 shows the GGE biplot for the AUDPC coverage. This trait is an indirect estimation of the rate of growth of the cvs. The vertex cultivar for each sector had the maximum or minimum value AUDPC coverage trait compared with the others in all environments that fall in the sector. Therefore, the best cultivar would be that with the highest grain yield (positive projection on ATC_a) and the highest stability, which is defined by a projection on ATC_o close to zero.

Cultivars with a high positive projection on ATC_a-axis had higher AUDPC coverage and the cultivars with a projection on ATC_o-axis close to zero showed high stability for this trait. The projection of perpendicular lines from the biplot origin to each of these sides determined 5 sectors, some of them containing one or more environments. Therefore, a five-sided polygon was derived from the most extreme genotypes, which were the cvs. Polar, Kebby, Ballet, ZP108 and Messire, clockwise.

The cultivars were ranked along the ATC axis abscissa (ATC_a), with an arrow pointing to a greater value based on their mean performance across all environments. The double arrowed line (ATC_o) separated entries with below–average means from those with above-average means (in Fig. 2.2, those cultivars

that are on the left of the ATC_0-axis had a low biomass value), and either direction away from biplot origin indicated greater genotype by environment interaction effect and reduced stability.

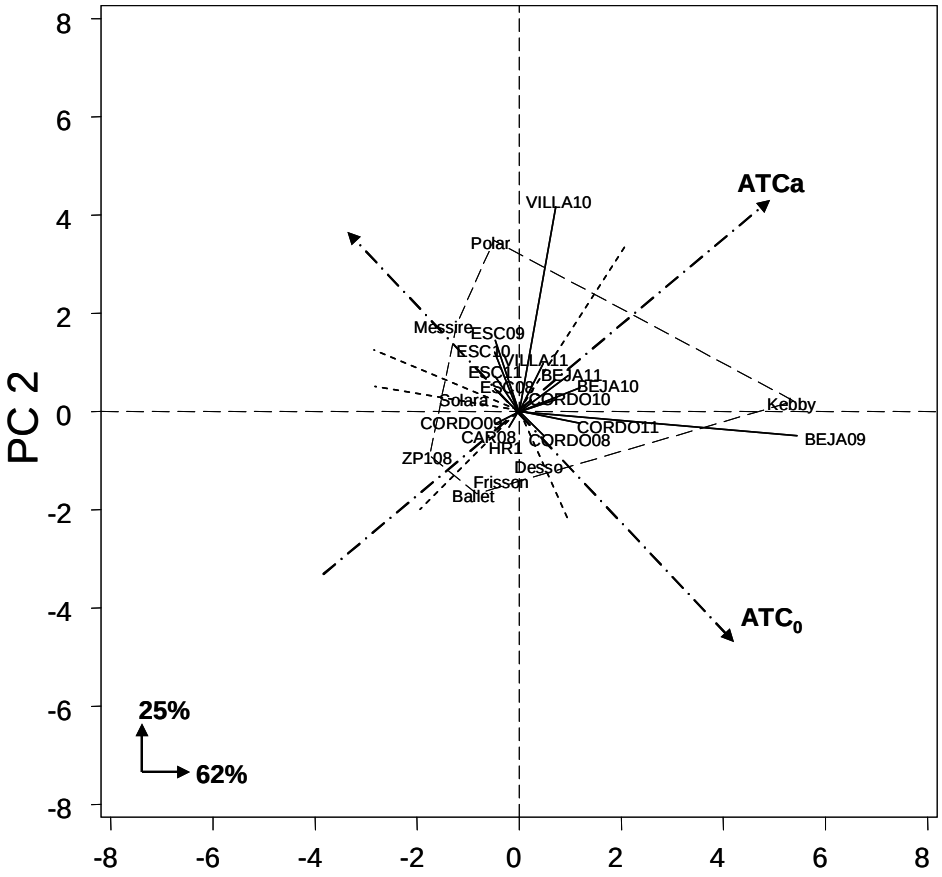

Fig. 2.2. GGE biplot based on the AUDPC coverage data of 9 pea cultivars grown at 14 location-year environments, from 2008 to 2012. The cultivar markers located away from origin were connected with straight lines to form a polygon. Lines perpendicular to the side of the polygon are drawn. PC, principal component. ATCa, ATC_o average tester coordinate abscissa and ordinate, respectively.

Polar and Messire were the best genotypes in the environments ESC08, ESC09, ESC10, ESC11, VILLA10 and VILLA11, which were all located in the first sector. Polar showed a higher growing rate compared to Messire. However, these genotypes were not very stable for this trait in any of the environments.

The cultivar Kebby was the vortex in the second sector, enclosing the environments BEJA09, BEJA10, BEJA11, CORDO08 and CORDO10. Nevertheless, it was the most instable of all the genotypes for this trait. On the contrary, the most stable genotype for this trait was HR-1.

The genotypes Desso, Solara, HR-1 and Messire showed medium AUDPC coverage values, indicating a normal growth rate. Frisson was slightly delayed with respect to the medium rates. Solara also showed a moderate stability for this trait.

Ballet was the winner cultivar in the third sector, which included the environment CAR08, and ZP-108 was the vortex in the fourth sector, in which the environment CORDO09 was included. These last two genotypes showed the slowest growing rates compared with the other genotypes, as well as a moderate stability.

The small angle between the tested environments indicated that they were closely associated and the same information about the genotypes could be obtained from fewer test environments. CORDO09, CORDO10 CAR08 and the Tunisian environments BEJA09 and BEJA10 were the ones with smaller angles than the other environments with the ATC_a-axis, so that they would be more representative than other test environments. On the other hand, the most discriminant environment would be VILLA11 and BEJA09, as they showed longer vectors than the rest. However, due to their angles with the ATC abscissa axis they were not very representative.

Biomass

Those cultivar markers of the biomass (Fig. 2.3) being farthest from the biplot origin (cvs. Messire, ZP108, Polar, Kebby and Desso, clockwise) formed the corners of the polygon divided into 5 sectors.

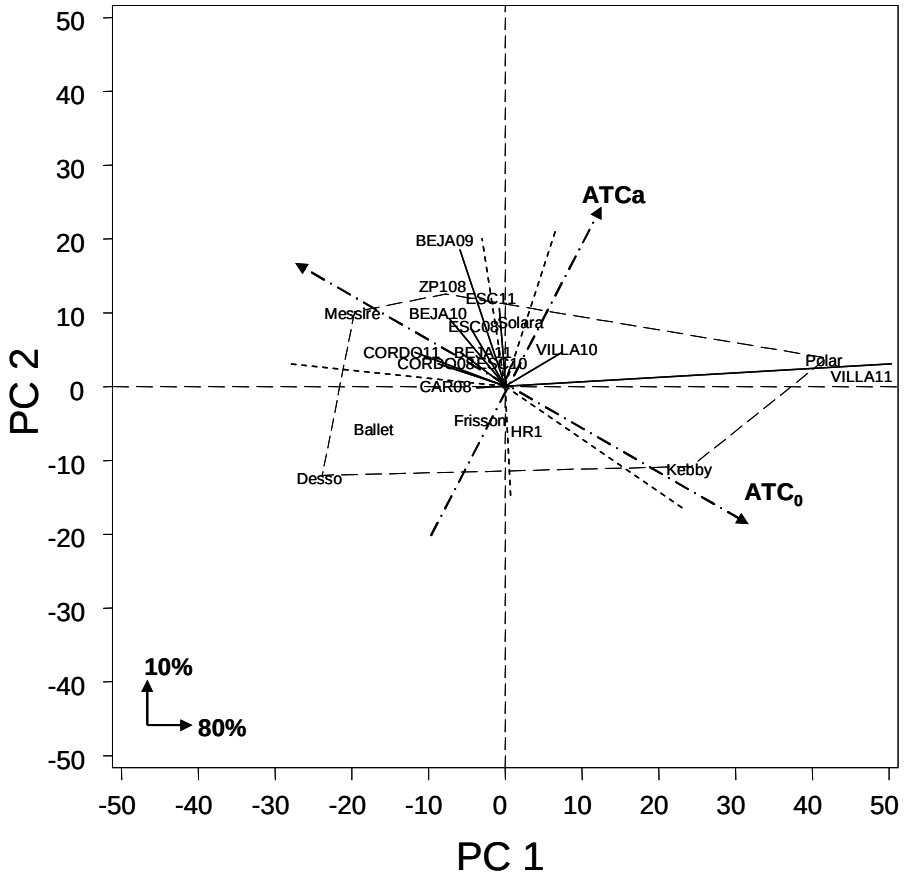

Fig. 2. 3. GGE biplot based on the biomass data per plant of 9 pea cultivars grown at 11 location-year environments, from 2008 to 2012. The cultivar markers located away from origin were connected with straight lines to form a polygon. Lines perpendicular to the side of the polygon are drawn. PC, principal component. ATCa, ATC_o average tester coordinate abscissa and ordinate, respectively.

The genotypes were ranked along the ATC_o-axis, with an arrow pointing to a greater value based on their mean performance across all environments. Those genotypes on the left of the perpendicular had a low mean biomass value.

Messire was the winning cultivar in most environments, showing the highest biomass on all of them, but not much stability for this trait. ZP108 was the winning cultivar in the second sector, including the environment ESC11. Polar showed the highest biomass in VILLA10 and VILLA11 both included in the third sector, although

it also showed the lowest stability. Kebby was also included in this sector and showed a biomass close to the mean values, but also was quite instable for this trait.

Genotype HR1, included in the fourth sector, showed low values of biomass and a moderate stability. Finally, Desso, Ballet and Frisson would be the genotypes which produced lower biomass, all of them included in this sector. Desso was the winning cultivar in the fifth sector, which included the environment CAR08 thus showing the lowest biomass values of all the genotypes. Frisson was the most stable among the genotypes for this trait.

The environment VILLA11 was the most discriminatory for this trait, showing the longest vector. However, due to the big angle formed with the ATC_a-axis, this environment was not representative for this trait. Again, the small angle between the tested environments, except from VILLA 10 and VILLA 11, indicated that they were closely associated and the same information about the genotypes could be obtained from fewer test environments.

Date of flowering

The GGE biplot for days to flowering (Fig. 2.4) showed a four-sided polygon formed by the union of the vertex genotypes ZP-108, HR-1, Messire and Kebby, clockwise. The ATC_o-axis leaved on its right the genotypes with a longer flowering time.

Little or less variability among most of the tested cultivars was found and none of the genotypes showed a consistent response across the environments, apart from HR-1 and Polar. Besides, HR-1 was delayed in flowering with respect of the rest of the tested genotypes, as indicated the positive value in the ATC_o axis, but ZP-108, whereas Polar showed earlier flowering than the rest. Kebby also stood out for its short flowering time,

although its marker was not associated to any environment. The genotype ZP-108 was the one with the longest growing period till flowering was reached.

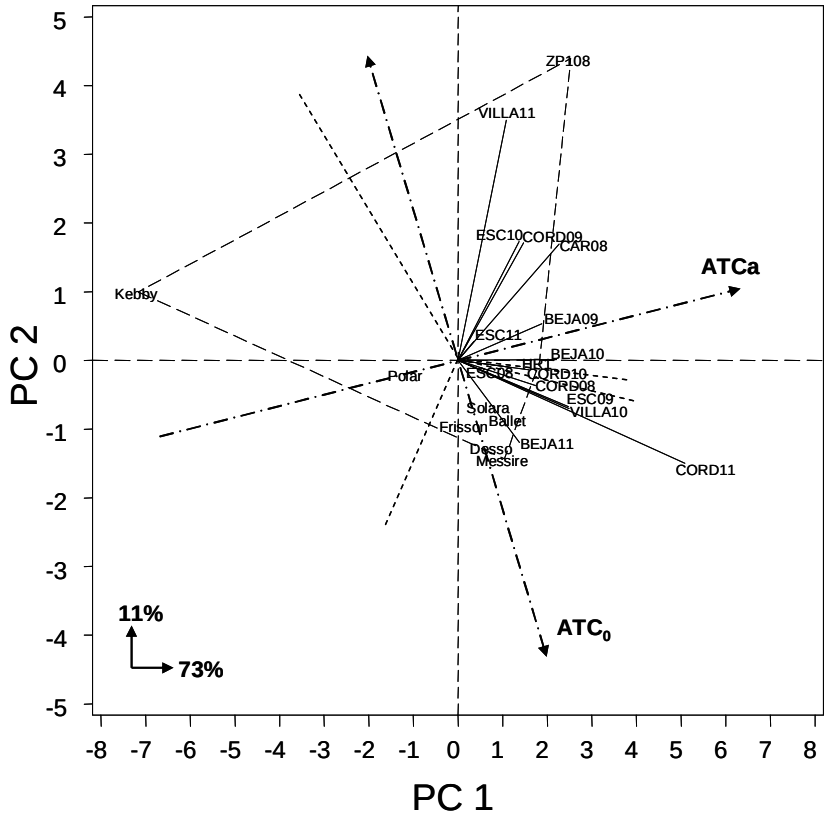

Fig. 2. 4. GGE biplot based on the date of flowering data of 9 pea cultivars grown at 14 location-year environments, from 2008 to 2012. The cultivar markers located away from origin were connected with straight lines to form a polygon. Lines perpendicular to the side of the polygon are drawn. PC, principal component. ATCa, ATC_o average tester coordinate abscissa and ordinate, respectively.

No discriminatory and representative environment was found for this trait, which was strongly affected by the genotype by environment interaction. However, VILLA11 and CORD11 would be discriminant environments for inferior genotypes, given their long vectors and their large angles with the ATC_a-axis. Close associations among test environments BEJA10, CORD10, CORD08, ESC09 and VILLA10 were found, defined by acute angles.

Date of fruiting

The vertex cvs. in the GGE biplot for date of fruiting were Frisson, Ballet, ZP-108, Polar and Kebby, clockwise (Fig. 2.5).

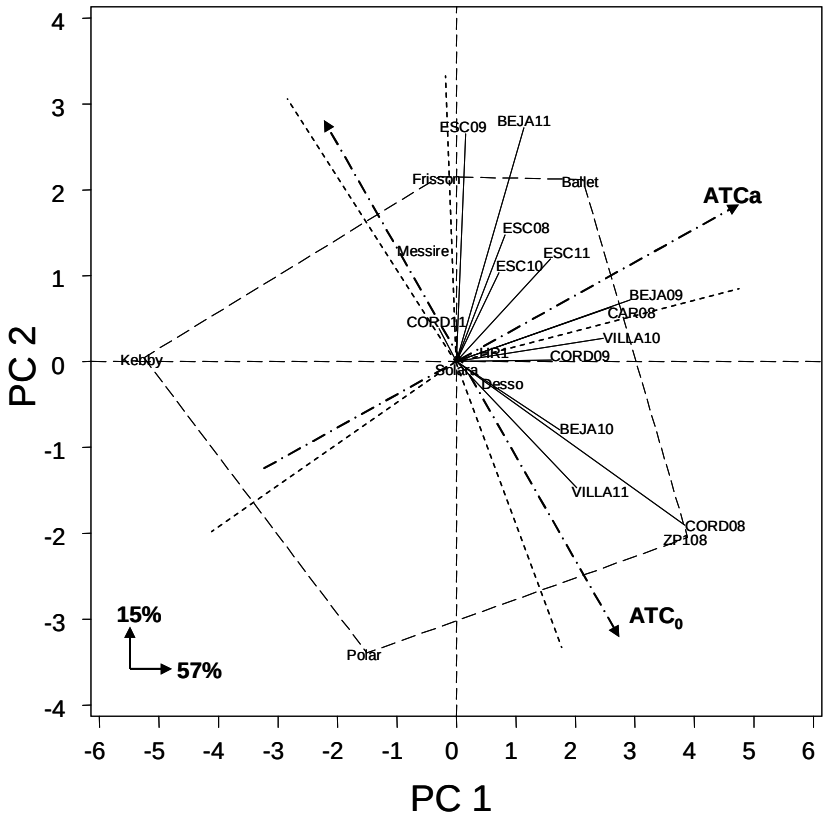

Fig. 2.5. GGE biplot based on the date of fruiting data of 9 pea cultivars grown at 13 location-year environments, from 2008 to 2012. The cultivar markers located away from origin were connected with straight lines to form a polygon. Lines perpendicular to the side of the polygon are drawn. PC, principal component. ATCa, ATC_o average tester coordinate abscissa and ordinate, respectively.

The perpendicular lines from the origin of the biplot to the polygon sides divided it into five sectors. The markers for Polar and Kebby did not fell into a sector which any environment. Polar was less stable for days to fruiting than for days to flowering, whereas Kebby was quite unstable, as happened with the days to flowering.

Ballet, Frisson and ZP-108 were the genotypes which showed a higher delay in fruiting with respect to the others. This behaviour was maintained in most

environments, although these genotypes showed not much stability for this trait, according to their distances to the ATC_a-axis. The cvs. Desso, HR-1, Messire and Solara showed similar periods till fruiting, being all around the mean values. However, Messire was the one with less stability for this trait.

The environments BEJA09 and CAR08 were both representative and discriminating for selecting widely adapted cultivars.

On the other hand, the environments settled in Escacena (ESC08, ESC10 and ESC11) showed a similar pattern in several years, so these environments were not really explicative of the GxE interaction.

Grain Yield

The GGE biplot (Fig. 2.6) for this trait showed a six-sided polygon formed by the vertex cultivars (cvs. Solara, Polar, Kebby, ZP108, Desso and Messire, clockwise).

Neither any genotype nor environment was included in the first sector, defined by the cultivars Solara and Messire. Polar was the winning cultivar in the second sector, which included the environments VILLA10, VILLA11 and CAR08. The cultivars Kebby and Solara were also included in this sector and showed higher yields than the other genotypes. However, these three genotypes with the highest yields were quite instable for this trait, according to the high size of their projections over the ATC_o-axis.

The cultivar HR1 was the one with more stability for this trait enclosed in this second sector, showing an intermediate yield compared with the rest of the genotypes. ZP108 was the winning cultivar in the third sector, showing the lowest yield in the environments ESC08, ESC09 and ESC10.

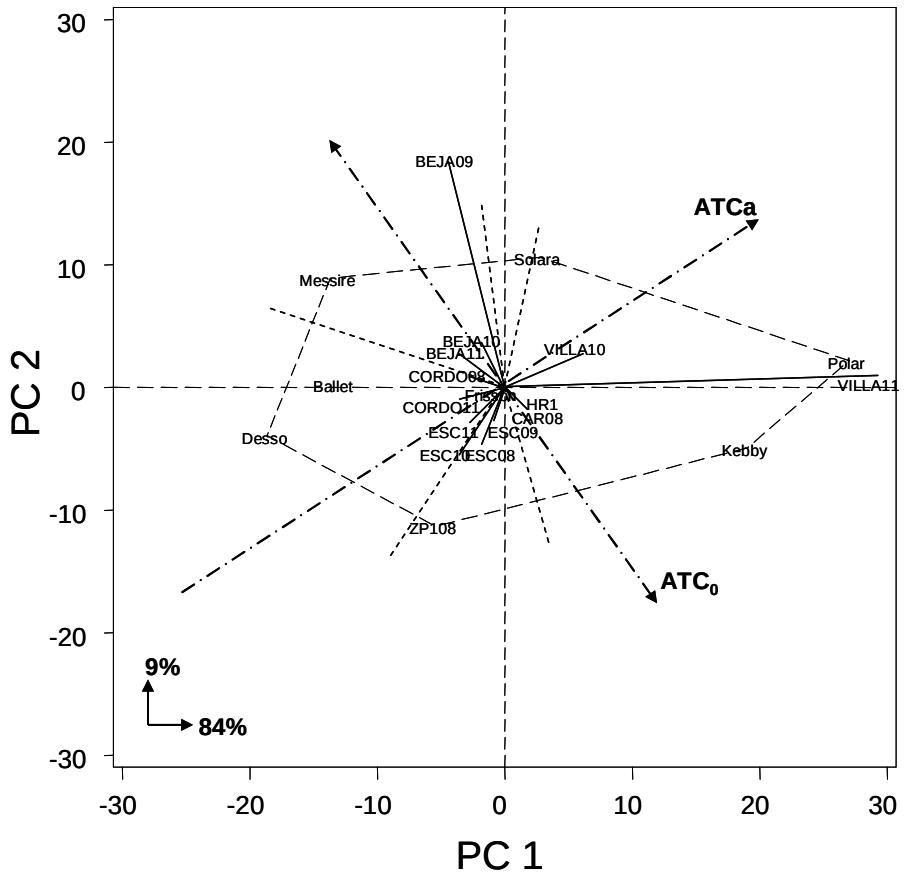

Fig. 2.6. GGE biplot based on the grain yield data per plant of 9 pea cultivars grown at 12 location-year environments, from 2008 to 2012. The cultivar markers located away from origin were connected with straight lines to form a polygon. Lines perpendicular to the side of the polygon are drawn. PC, principal component. ATCa, ATC_o average tester coordinate abscissa and ordinate, respectively.

Cultivar Desso was the one with the lowest yield among all the genotypes, being the winner cultivar in the environments CORDO08, CORDO11 and ESC11, included in the forth sector. Ballet, also included in this sector, showed low yield compared with the other genotypes. However, these two genotypes showed low stability for this trait. Cultivar Frisson was also included in this sector, showing a slightly lower yield value compared with the rest, but also being the genotype with the highest stability for yield.

Finally, cv. Messire, included in the fifth sector was the winner cultivar in the Tunisian environments (BEJA09, BEJA10 and BEJA11), although the mean values for this trait were lower than in the other genotypes and the genotype showed low stability for this trait.

VILLA 11 and BEJA09 were the most informative environments as indicated by the largest distance between their marker projection on the ATC_a -axis and the origin. However, due to the moderate secondary score on ATC_o-axis of these environments, cultivar differences observed might not exactly reflect the cultivar differences in average yield over all environments, thus being not much representative for this trait.

Powdery mildew

The GGE biplot for powdery mildew showed a 5 sided polygon formed by the vertex genotypes Polar, Messire, Kebby, Ballet and Frisson (Fig. 2.7).

Cultivars Ballet and Frisson showed fewer symptoms than those with positive projections on the ATC_a-axis, such as Messire, HR1 and Kebby. Furthermore, the genotypes Solara, Polar Desso and ZP108 showed an intermediate symptom rate. Regarding the test environments, the vectors for BEJA09 and BEJA10 formed a right angle, which means that they showed no correlation as they had gave us very different results.

These two environments were the most discriminant, but the angles of their vectors with the ATC_a-axis pointed out that they were not representative at all, as happened to ESC11. In the case of ESC08, there is no projection over the ATC_o-axis, which made of it a non discriminating environment.

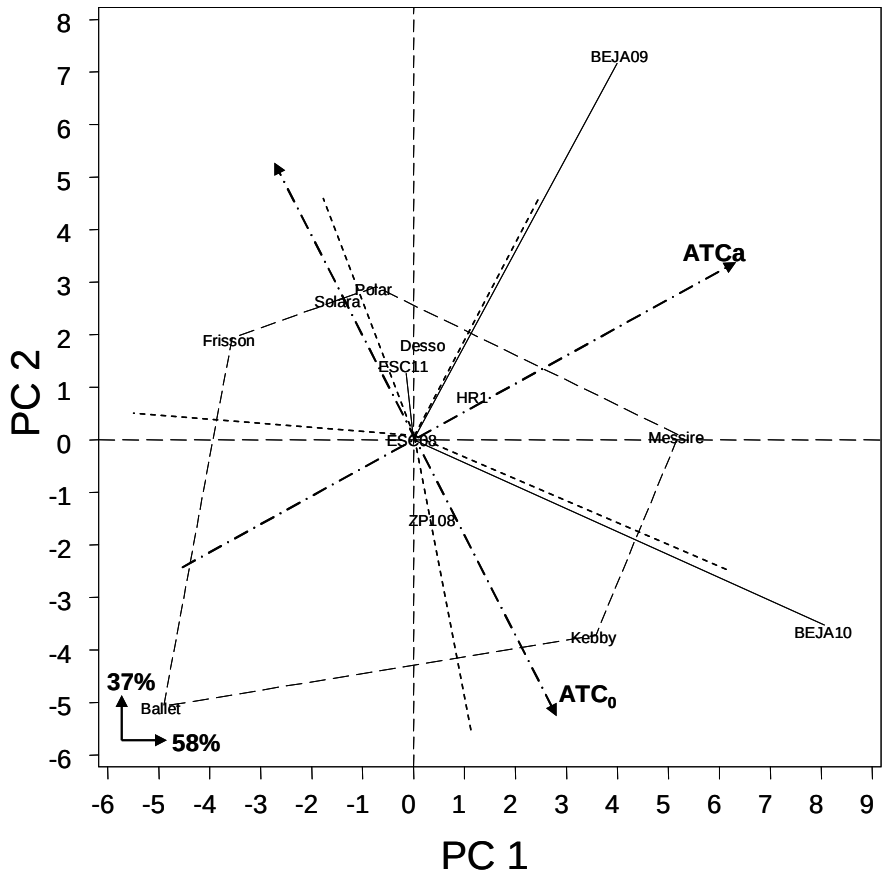

Fig. 2.7. GGE biplot based on the powdery mildew data of 9 pea cultivars grown at 4 location-year environments, from 2008 to 2012. The cultivar markers located away from origin were connected with straight lines to form a polygon. Lines perpendicular to the side of the polygon are drawn. PC, principal component. ATCa, ATC_o average tester coordinate abscissa and ordinate, respectively.

Crenate broomrape per plant

Kebby, Polar and Ballet were the genotypes less affected by *O. crenata* according to their negative projections in the ATC_a-axis (Fig. 2.7).

The resistance of Kebby and Ballet was more stable, as their markers were the ones with lower projections on the ATC_a-axis.

On the contrary, cvs. Messire, ZP108 and Desso were the genotypes most affected, whereas the rest showed an intermediate response. Frisson was the cv.

which showed most stability in this response, regarding the proximity of its marker with the ATC_a-axis.

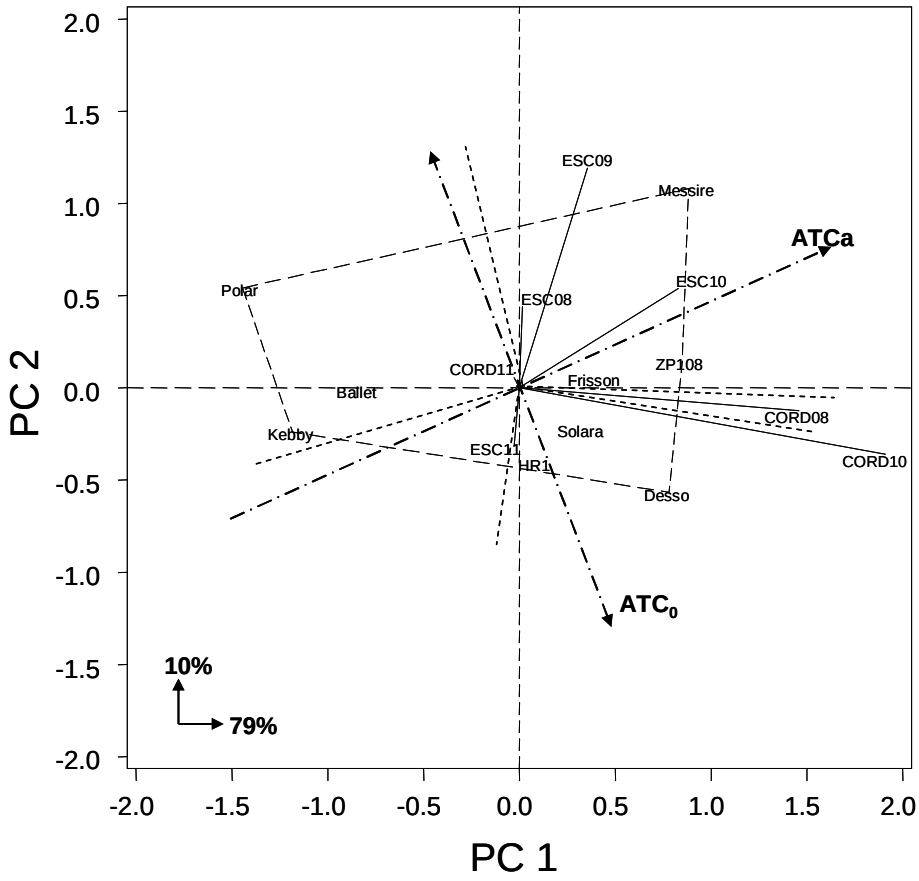

Fig. 2.7. GGE biplot based on the broomrape per plant data of 9 pea cultivars grown at 7 location-year environments, from 2008 to 2012. The cultivar markers located away from origin were connected with straight lines to form a polygon. Lines perpendicular to the side of the polygon are drawn. PC, principal component. ATCa, ATC_o average tester coordinate abscissa and ordinate, respectively.

With respect to the tested environments, the most discriminant environments would be ESC09, ESC10, CORD08 and CORD10. ESC10 would be also a representative environment, given the small angle formed with the ATC_a-axis and the higher proximity of its projection with the ATC_o-axis compared with the other environments.

100 seeds weight and frost symptoms

The 100 seeds weight ranked among 11,5 g of the cv. Frisson to 22,4 g of Solara (Table 2.10). Mean value for seed weight was around 17 g. The genotypes could be divided into five groups according to these values.

Table2.10. Tuckey mean comparison of the effects of environment and genotype in the 100 seeds weight (left) and the percentage of frost symptoms (right) of the 9 genotypes of *P.sativum* at 9 or 2 location-year environments, respectively. Estimates with the same letter are not significantly different.

	100 seeds weight (g)	Frost symptoms (%)
Ballet	19,0 b	10 abc
Desso	13,5 d	4,2 bc
Frisson	11,5 e	1,2 c
HR1	18,9 b	1,8 c
Kebby	19,4 b	41,7 a
Messire	20,3 b	12,5 abc
Polar	13,3 de	37,5 ab
Solara	22,4 a	13,3 abc
ZP108	16,6 c	12,8 abc

The mean comparisons for the frost symptoms divided the genotypes in three groups according to the percentage of damage by frost (Table 2.10). Most of the genotypes showed an intermediate low response to frost stress, ranking from 10% to 13% the percentage of the symptoms observed.

However, genotypes as Kebby and Polar were the most affected by frost whereas Frisson and HR1 showed fewer symptoms. The intensity of the frost stress experienced was not very high and it was early in the growing season, thus all the plants could recover and finished their growing cycle.

DISCUSSION

Genotype (G), environment (E) and genotype by environment (GE) interaction of 10 pea varieties were tested for yield, biomass, date of flowering, date of fruiting, AUDPC coverage, crenate broomrape per plant and mildew in field trials.

The partitioning of G and GE interaction through GGE biplot analysis showed that the first two principal components were significant factors for all traits but “100 seeds weight” and “frost symptoms”. This suggested that a biplot with the first two principal components adequately approximated the environment-centered data, whereas the other two traits were genetically determined and could be analyzed trough a mean comparison. As expected, growth and development related along with phenological traits were strongly affected by environment.

The GGE biplot analysis allows us to appreciate and determine differences among the genotypes which could serve to select interesting varieties according to farming necessities (Yang *et al*., 1999; 2000). GGE biplot analysis is first of all an agricultural issue rather than a statistical one (Yang *et al*., 2007). Therefore, it is important to understand how cultivars are selected and recommended in the real world to have a realistic assessment about gains from model diagnosis. Breeders do not select cultivars on the basis of only a single trait (e.g., yield), because superior cultivars must meet requirements for multiple breeding objectives. Breeders do not select just one genotype with respect to a single trait, because breeding objectives are often negatively associated, and it is rare to find a genotype that is best for everything (Yan *et al*., 1995).

In general, Ballet would be the worse genotype for all the traits evaluated but broomrape resistance and mildew symptoms. This genotype showed slower growing rates, lower biomass production and yield, delayed phenology, average seed size and frost damaging, but also had low stability in all the environments tested.

The cultivar Desso did not show any interesting agronomical characteristic in the tested environments apart from low frost symptoms. Messire was quite susceptible to broomrape and mildew, in agree with previous reports (Fondevilla *et al*., 2007; Rubiales *et al*., 2003). This cultivar also showed low yield and was instable for all the studied traits in the different environments. However, it showed a high seed weight compared with Frisson, Polar and ZP-108. Solara showed good markers for yield, also showing the highest seed weight of all the studied genotypes. However, it was moderately unstable for all the studied traits but fruiting and biomass.

Frisson showed less mildew symptoms than the rest of the genotypes but Ballet, as well as and one of the less affected by frost together with HR-1, although it was affected by broomrape and had the lowest seed size. Furthermore, it was moderately fast in the pod filling and the most stable one for yield. These characteristics would make of it an interesting cultivar to use for cold and wet environments.

HR-1 was the genotype more stable for all the evaluated traits. It showed low growing rate and biomass, being slightly delayed on its phenology with respect to the other cultivars and one of the less affected by frost. These facts along with the moderately high grain weight would make this cultivar especially interesting for cold environments.

ZP-108 showed a high stability only for AUDPC coverage, indicating a regular growing rate, although it was delayed with respect to all the other genotypes. This variety was also quite affected by frost and broomrape, but showed fewer mildew symptoms, together with Frisson. Phenologically it was also quite instable, being

delayed as well with respect to the other genotypes but Ballet. However, this variety showed the highest biomass in the Tunisian environments, indicating a possible suitability for crop rotations, due to higher soil enrichment in nitrogen, or thermal power generation (Kaperstein-Machan *et al.*, 2000; Huang *et al*., 2011) in these environmental conditions. Finally, it was the one with the lowest yield in Escacena along the different years, which indicated that it would be better to orientate the use of this variety towards crop rotations in Mediterranean region.

Kebby and Polar showed a faster growth and phenological development compared with the other genotypes, although Kebby seemed to grow faster when annual rainfall was lower and temperatures were warmer, whereas Polar grew faster in colder environments. These two genotypes were also less affected by broomrape, and showed the fewest symptoms of mildew, although they were moderately affected by frost. Unlike for Polar, grain weight for Kebby was also among the highest, but these two varieties were quite instable for most of the traits.

Polar was the genotype with the highest yield and biomass along the different years in Villamor, a location which would correspond to a Continental Mediterranean climate, with colder winters with respect to the warm Interior Mediterranean climate of the other locations. This would indicate that Polar behaviour was improved by colder temperatures. On the other hand, its earliness and faster growth rate, along with the ones from Kebby would allow these genotypes to be sown either in winter or spring, as they would be able to finish their development before the drought period characteristic of the Mediterranean climate. In warmer regions their use would also be appropriate for winter sown as they seemed to have a good behaviour under low temperatures and scarce rainfalls.

According to the genotypes behaviour, we could distinguish between two mega-environments. One will be formed by the locations with an Interior Mediterranean climate (Córdoba, Escacena and Beja) along the different years assessed and the other would be constituted by VILLA10 and VILLA11. Furthermore, an "ideal" test environment for broomrape would be found in ESC10, as it has the longest vector of all test environments and is located almost on the AEC abscissa (most representative) (Yan, 2001). However, the lack of consistence with the data collected in other years made it impossible to define it as an ideal location to discriminate genotypes over the years.

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Table 2.7. Mean, total (T) and standard error (SE) values of the date of AUDPC coverage of 9 cultivars of *P.sativum* at 14 location-year environments from Pea Network.

	BEJA 09	BEJA 10	BEJA 11	CAR 08	CORD 08	CORD 09	CORD 10	CORD 11	ESC 08	ESC 10	ESC 11	ESC 09	VILLA 10	VILLA 11	T. Mean	T. SE
Ballet	11,20	33,67	60,33	10,73	12,43	47,33	30,00	27,67	71,33	32,50	37,00	24,33	89,50	87,67	65,08	9,0
Desso	18,10	72,67	59,33	75,67	11,33	43,33	65,67	26,33	39,33	27,33	41,50	22,00	12,10	62,00	72,60	1
Frisson	14,03	63,00	55,33	97,00	11,00	53,00	64,00	27,67	41,00	27,67	71,67	18,00	96,67	77,00	69,27	8,7
HR1	14,17	62,67	47,33	11,37	11,30	53,67	67,67	28,33	40,67	29,67	46,33	24,33	13,82	52,67	71,73	7,9
Kebby	46,67	56,00	55,00	97,33	13,23	63,00	58,00	28,67	35,00	33,50	89,67	24,00	22,44	10,47	11,21	8,8
Messire	70,67	44,00	11,33	69,00	68,33	62,00	87,00	25,00	61,33	51,33	73,33	22,00	22,53	70,33	84,56	5,5
Polar	99,67	53,67	74,00	94,67	11,80	39,67	55,00	26,33	59,00	90,50	10,83	24,67	31,65	62,50	93,05	7,8
Solara	85,33	81,33	47,33	95,33	12,10	59,67	75,00	36,00	54,00	41,67	69,00	22,67	17,45	73,00	80,69	8,7
ZP108	52,00	30,33	31,00	13,77	15,17	66,00	76,33	41,33	81,33	53,33	77,67	28,67	12,77	17,20	81,64	8,7
T. Mean	14,99	55,26	60,33	98,63	11,68	54,19	67,04	29,70	53,46	41,92	69,31	23,41	16,80	83,78	81,20	2,9
T.SE	11,20	33,67	60,33	10,73	12,43	47,33	30,00	27,67	71,33	32,50	37,00	24,33	89,50	87,67		

Mean, total (T) and standard error (SE) values of the biomass (Kg per hectare) of 9 cultivars of *P.sativum* at 11 location-year environments from Pea Network.

	VILLA 10	VILLA 11	BEJA 09	BEJA 10	BEJA 11	CAR 08	CORD 08	CORD 11	ESC 08	ESC 10	ESC 11	VILLA 10	VILLA 11	T. Mean	T. SE
Ballet	1267,30	364,26	913,67	426,33	93,33	99,33	49,67	215,00	59,33	84,67	673,00	220,80	1267,30	364,26	805,5
Desso	821,00	292,91	864,67	356,00	104,00	86,00	61,33	213,00	83,50	258,50	605,33	166,17	821,00	292,91	592,1
Frisson	2146,00	378,51	879,67	339,33	234,00	97,00	64,67	181,00	28,00	134,00	640,67	193,50	2146,00	378,51	840,6
HR1	2386,70	469,88	741,33	321,67	120,00	36,33	65,67	208,00	39,00	184,00	666,67	345,50	2386,70	469,88	841,8
Kebby	3690,30	485,44	544,67	91,67	65,67	10,50	35,67	66,00	24,00	153,00	421,00	241,60	3690,30	485,44	664,1
Messire	1164,00	464,66	1357,70	369,00	141,00	180,00	200,67	185,00	381,00	180,50	656,33	333,00	1164,00	464,66	869,0
Polar	4804,00	605,33	657,00	198,00	37,33	28,00	50,00	136,67	38,00	152,33	597,67	332,00	4804,00	605,33	785,8
Solara	2458,00	506,75	1181,30	335,67	129,67	47,00	88,67	264,00	25,00	238,33	687,33	313,00	2458,00	506,75	889,0
ZP108	1875,00	496,28	1093,70	603,33	228,33	20,00	145,67	323,00	117,00	36,00	963,00	286,00	1875,00	496,28	997,2
T. Mean	2335,00	448,54	914,85	337,89	128,15	76,73	85,64	199,07	91,65	151,71	656,78	266,08	2335,00	448,54	276,1
T.SE	1267,30	364,26	913,67	426,33	93,33	99,33	49,67	215,00	59,33	84,67	673,00	220,80	1267,30		

Table 2.3. Mean, total (T) and standard error (SE) values of **grain yield** (KG per hectare) of 9 cultivars of P.sativum at a 11 location-year environments from Pea Network.

	VILLA 10	VILLA 11	BEJA 09	BEJA 10	BEJA 11	CAR 08	CORD 08	CORD 11	ESC 08	ESC 09	ESC 10	ESC 11	VILLA 10	VILLA 11	T. Mean	T. SE
Ballet	52,50	601,67	140,92	346,33	187,67	42,67	21,67	13667,00	94000,00	23,00	12,50	22,00	319,00	52,50	601,67	140,92
Desso	64,50	365,00	114,66	300,33	171,00	54,67	14,67	15,67	107,00	20,00	96,67	125,50	317,00	64,50	365,00	114,66
Frisson	63,50	1130,00	150,08	282,67	134,67	64,67	12,00	13,00	87,67	12,00	90,00	66,00	292,00	63,50	1130,00	150,08
HR1	113,25	1302,30	197,42	244,33	150,33	39,00	12,33	10,33	79,33	20,67	21,33	57,00	300,00	113,25	1302,30	197,42
Kebby	73,60	1961,30	215,61	103,00	39,33	44,00	85,00	76,67	21,33	50,00	0,00	70,00	207,33	73,60	1961,30	215,61
Messire	122,67	605,67	175,89	527,67	181,33	65,00	13,67	44,33	80,67	40,67	14,00	135,50	319,33	122,67	605,67	175,89
Polar	113,00	2329,50	266,32	211,67	91,33	10,67	26,00	40,00	63,33	11,00	0,00	74,00	317,33	113,00	2329,50	266,32
Solara	121,50	1313,00	219,64	485,33	161,00	56,33	18,33	11,67	122,67	73,33	83,33	99,67	328,67	121,50	1313,00	219,64
ZP108	97,33	932,50	185,51	128,33	224,00	86,33	26,00	33,00	158,00	105,00	10,67	218,67	410,33	97,33	932,50	185,51
T. Mean	89,90	1204,60	183,20	292,19	148,96	51,48	15,77	18,08	90,44	25,52	10,68	95,29	312,33	89,90	1204,60	183,20
T.SE	52,50	601,67	140,92	346,33	187,67	42,67	21,67	13667,00	94000,00	23,00	12,50	22,00	319,00	52,50		

Table2.8. Mean, total (T) and standard error (SE) values of mildew symptoms percentage in 9 cultivars of *P.sativum* at 4 location-year environments from Pea Network.

	BEJA 09	BEJA 10	ESC 08	ESC 11	T. Mean	T. SE
Ballet	6,67	36,67	0,00	5,00	12,08	5,24
Desso	77,33	53,33	0,00	8,33	34,75	11,29
Frisson	61,33	23,33	0,33	20,00	26,25	7,94
HR1	71,00	66,67	0,00	30,33	42,00	10,66
Kebby	50,67	100,00	0,00	2,00	38,17	12,76
Messire	84,00	100,00	0,66	10,00	48,67	13,35
Polar	81,33	41,67	0,00	8,33	32,83	9,89
Solara	77,00	36,67	0,67	6,67	30,25	9,84
ZP108	54,00	66,67	0,67	5,00	31,58	9,69
T. Mean	62,59	58,33	0,26	10,63	32,95	3,44
T. SE	4,95	6,46	0,08	3,20		

Table2.9. Mean, total (T) and standard error (SE) values of the number of crenate broomrape per plant of 10 cultivars of *P.sativum* at 7 location-year environments from Pea Network.

	COR D08	COR D10	COR D11	ESC 08	ESC 09	ESC 10	ESC 11	T. Mean	T. SE
Ballet	2,07	1,00	0,83	0,53	0,55	0,60	0,77	0,92	0,18
Desso	3,87	4,67	0,23	0,57	0,40	1,83	1,13	1,81	0,40
Frisson	3,17	3,80	0,47	0,77	0,87	1,87	0,40	1,62	0,31
HR1	2,63	3,43	0,60	0,13	0,53	1,37	1,10	1,40	0,29
Kebby	0,53	1,17	0,37	0,43	0,15	0,27	0,53	0,51	0,10
Messire	3,37	4,70	0,40	1,17	2,63	2,50	0,40	2,17	0,10
Polar	0,00	0,21	0,50	1,00	0,75	1,07	0,77	0,61	0,39
Solara	2,43	4,03	0,30	0,43	0,50	2,20	0,90	1,54	0,35
ZP108	4,03	4,23	0,33	0,53	0,93	2,87	0,53	1,92	0,30
T. Mean	2,39	3,22	0,43	0,66	0,83	1,57	0,76	1,41	0,44
T. SE	0,30	0,36	0,06	0,08	0,21	0,20	0,08		

Chapter 3.

Effects of the interaction between *Fusarium oxysporum* f.sp. *pisi* infection and drought stress over seven pea (*Pisum sativum* L.) genotypes

INTRODUCTION

Legumes are sensitive to abiotic stresses, most significantly water deficit and soil salinity. Drought is currently a major factor limiting crop productivity worldwide. In Mediterranean countries, water deficit occurs not only in arid and semiarid regions but also in areas where total precipitation is high but not evenly distributed during the growing season. In a context of increasing limitations to water use due to climate change and increased population, improving water use efficiency of crops is a necessary goal.

Among the legumes, pea is well established as a valuable break crop in arable rotations. It offers the potential to reduce mineral nitrogen in the rotation due to bacterial nitrogen fixation from their symbiosis with *rhizobia*. Peas have a zero requirement for soil nitrogen during the growing period and healthy crops produce a nitrogen residue immediately available to autumn planted cereals.

Biological nitrogen fixation is an extremely complex process very sensitive to environmental stresses (Zahran, 1999). In general, drought is a mayor constrain to the production and yield stability of pea (*Pisum sativum* L.) (Mitra, 2001). Although peas generally require temperate conditions and are suited to medium to light soil types, drought during the flowering and pod filling period of spring varieties of combining peas can severely reduce yield.

Apart from abiotic stresses, such as drought or salinity, pea crops are often exposed to biotic stresses in the field. Diseases are considered the most important causes of reduced biomass production and seed yields. Many diseases and pests affect pea (Kraft *et al*., 2001) being the fungal and viral pathogens responsible for the most severe damages. Among the fungus, *Fusarium* species that cause root rot

(*Fusarium solani* f.sp. *pisi* and *F. avenaceum*) or wilt (*F. oxysporum* f.sp. *pisi*) are one of the most important diseases affecting pea crops throughout the world (Kraft *et al*., 1998).

Fusarium wilts are widespread diseases caused by many forms of the soil-borne pathogen *F. oxysporum*, affecting many agricultural crops, including most legumes, cucurbits, tomato, potato, pepper, strawberry, asparagus, cotton, banana, etc. These soil borne pathogens can survive as thick-walled chlamydospores, which remain viable in the soil for many years.

F. oxysporum f. sp. *pisi* (*Fop*) is an important and destructive pathogen of field pea, that has been reported in every country where pea is grown (Kraft *et al*., 2001). Control is problematic because *F. oxysporum* can grow saprophytically in the absence of a susceptible crop, making it difficult to remove once it is established. The only effective response is soil sterilization, which is far too expensive for most farmers. Crop rotation is the most affordable way to maintain safe levels of inoculum, requiring the frequency of pea cropping in a field to be limited. In some regions, this is often no more than once in five years. Some control can be achieved with fungicides but the use of resistant cultivars of plants is the preferred approach (Lebeda *et al*., 2010).

Sources of resistance in peas are rather limited and difficult to estimate, but single genes which have been identified and used in breeding, are rapidly overcome by new races of the pathogen (Infantino *et al*., 2006). A continuous search for novel resistance sources to complement and strengthen the resistance of elite cultivars is thus essential. Recently, potential sources of quantitative resistance to race 2 of *Fop* within a *Pisum* spp. germplasm collection were identified (Bani *et al*., 2012), pointing

out this necessity of identifying resistance sources based on quantitative and polygenic mechanisms.

Successful plant infection by *F. oxysporum* is a complex phenomenon that requires a series of highly regulated processes (Di Pietro *et al*., 2001). The characteristic wilt symptoms appear as a result of severe water stress, mainly due to vessel clogging. Wilting is most likely caused by a combination of pathogen activities, such as the accumulation of fungal mycelium and/or toxin production and host defence responses, including production of gels, gums and xyloses and vessel crushing by proliferation of adjacent parenchyma cells (Beckman, 1987; Di Pietro *et al.*, 2003). This mode of infection means that under warm and drought conditions the wilt symptoms will be more severe due to the decreasing of water available for the plant. Thus, the simultaneous action of drought and *Fop* over pea crops could be particularly dangerous in arid and semi-arid zones where both kinds of stresses are likely to concur.

Whereas there is an extensive literature on the response of plants to single stresses under laboratory and field conditions, the study of multiple stresses is hardly beggining (Mittler *et al*., 2010). Furthermore, there still is not developed any suitable methodology to analyze their effects simultaneously over the plants. Most of the information at the gene level comes from bioinformatics and experimental studies in which it is noted that lots of genes show responses to a diverse range of biotic and abiotic stresses suggesting a role for them in response to combined stresses (Swindell *et al*., 2007).

The reason for this confluent response could rely in the cellular level, where the response to diverse environmental stimuli may be the same. The best example

of a common cellular response is the triggering of oxidative stress which is possibly associated with induction of common sets of defence networks (e.g. antioxidants or chaperones). This has been known for many years to elicit the so-called cross-protection (Foyer *et al*., 1994) and recent examples include the exposure of *Arabidopsis* to excess light which elicits the production of micro-lesions associated with subsequent resistance to biotrophic pathogens (Muhlenbock *et al*. 2008). However, no attempt has been made at defining the response of the plants (nearly always *Arabidopsis*) to multiple stresses and determining how it differs from application of the individual stresses. Works about combined stresses are mostly descriptive, being confined to causes rather than effects, e.g. how changes in humidity, salinity or temperature affect resistance to pathogens (Yoshioka *et al*., 2001; Bechtold *et al*., 2005; AbuQamar *et al*., 2009) or lists of genes from microarray experiments (Rizhsky L, *et al* (2004)).

The objective of this work was to develop a suitable methodology to analyze the simultaneous and separate effects of drought and *Fop* stress over pea genotypes previously characterized as tolerant or resistant to those stresses separately, with the final aim of identify sources of resistance to both stresses that could be useful in a breeding program for arid or semi-arid environments.

MATERIALS AND METHODS

Fungal isolates and cultural conditions

F. oxysporum f. sp. *pisi* race 2 strain R2F42 was kindly provided by Dr W. Chen (USDA-ARS, Pullman, USA) for use in all the experiments. The fungal strain was stored as microconidial suspensions at -80ºC in 30% glycerol. For microconidia

production, cultures were grown in potato dextrose broth (PDB; Difco) at 28ºC in a shake culture set at 170 rpm (Di Pietro *et al*., 1998).

Plant material and growing conditions

The homogeneity of the resistant or susceptible responses to *Fop* was tested previously in separate experiments with five seedlings of the *Pisum* spp. accessions Dark Skin Perfection, JI1412, Kebby, Little Marvel, Messire, New Era, New Season, 902131, Polar, P665, 74SN5, WSU31 and WSU28 (data not shown). According to the symptoms showed 20 days after inoculation (dai), seven *P. sativum* cultivars with resistant and tolerant responses were selected by visual assessment to be used in this study.

Regarding the origin and characteristics of the selected cultivars, Polar and Kebby were characterized as drought tolerant and susceptible, respectively. The cv. Marlin was identified as JI1412 by the John Innes Institute and characterized as high resistant to *Fop* race 2 by Bani *et al*., 2012. Finally, the cvs. New Era, New Season, 74SN5 and WSU28, from the USDA core collection of the differential set for the four races of *Fop*. 74SN5 is described as resistant to the four races of *Fop*, whereas New Era is reported as resistant to race 1 and 2, New Season is described as resistant to races 1, 2 and 6. WSU28 is reported as resistant to races 1 and 5, but susceptible to race 2 (Grunwald *et al*., 2003), and was used as susceptible check for the fungus.

Pea seeds were surface-sterilized for 20 min in a 20% solution of sodium hypochlorite and then rinsed with sterile water. The seeds were pregerminated in Petri dishes with moistened filter papers in the dark for 48 h in a cold chamber at 4ºC and then placed for another 48 h in a growth chamber at 65% relative humidity and 26± 2ºC. Once germinated, the seedlings were transferred to pots (6x6x8 cm)

containing sterile vermiculite (1–3 mm diameter) and grown in a controlled environmental chamber under a 12 ⁄ 12 h light-dark photoperiod at 26± 2ºC . Lighting was maintained at a minimum threshold PPFD of 1800 μmol m^{-2} s^{-1}. The PPFD at plant level was 200 μmol m^{-2} s^{-1}. A minimum number of 5 plants per assay and evaluation were used. Plants were watered and positions changed every two days during their growing period. After two previous independent experiments, the optimal conditions to evaluate both *Fop* and water stress were determined as follows.

Plants Inoculation

At least five replicates of seven-day-old seedlings (2-3 node stage) per genotype were inoculated according to the protocol described in Bani *et al*., 2012. Briefly, vermiculite was removed from the roots which were trimmed by a third (Lichtenzveig *et al*., 2006) in order to help the penetration of the fungus and immersed for 5 min in a suspension containing $5x10^6$ microconidia ml^{-1} of water. Control plants were treated in the same way and were immersed in sterile water. Seedlings were planted in individual pots containing sterile vermiculite and maintained in the same growth chamber. Control and droughted plants were treated in the same way but immersed in sterile water. Plants were watered every two days along the assessed period.

Application of water stress

Water stress was applied to five replicates per genotype by withdrawing water 48 hours after inoculation with *Fop*. Controls for the water stress were half of the plants previously treated with sterile water. Roots were cut in all the treatments and the controls.

Disease and water stress assessment

Both disease and water stress symptoms were assessed daily from the beginning of the water stress period till 22 days post inoculation (dpi). We also assessed both stresses jointly using a symptom-based approach, observing the percentage of the plant wilted by drought or *Fop* stress and assigning a visual index ranging from 1 (0% plant affected) to 9 (100% plant affected) and reporting these values for each individual plant (Table 3.1).

Table 3.1. Visual scale applied for drought and *Fop* symptoms.

Scale value	Percentage of the plant affected
1	0
2	0-25
3	25
4	25-50
5	50
6	50-75
7	75
8	75-100
9	100

Data obtained were used to calculate the area under the disease progression curve (AUDPC) using the formula:

$$AUDPC = \Sigma\ [(x_i + x_{i+1})/2] * (t_{i+1}-t)$$

Where x_i = estimated proportion of disease severity at date i, x_{i+1} = estimated proportion of disease severity at date $i+1$, and $ti+1-ti$ = number of days between scoring dates i and $i+1$. To classify accessions as resistant or susceptible, their disease symptoms were compared to those of accessions New Season and WSU28 used as resistant and susceptible controls for *Fop*, respectively. On the other hand, the symptoms were compared with those of the genotypes Polar to classify the accessions as tolerant and Kebby to determine if they were susceptible to water stress.

Finally, we subtracted the mean AUDPC of each genotype controls from the individual AUDPC data of each genotype and treatment. Then we calculated the mean AUDPC value for the genotypes to obtain stress data referred to the controls. Mean data were statistically compare using Sheffes´ mean comparisons and the software GenStat 11th edition.

RESULTS

Genotypes assessment

Daily visual assessment of the genotypes showed that the initial wilting symptoms appeared on the primary leaves around 4-7 dai in the case of *Fop* inoculated plants and around 4-6 days after watering withdrawal (daw) (which means 6-8 dai) for water stressed plants (Fig.3.1). The symptoms reached sequentially the later-formed leaves until the whole plant withered and died.

As expected, there was a rapid increase in the symptoms observed on the susceptible genotypes Kebby (Fig. 3.1. B) and WSU28 (Fig. 3.1. F) since the beginning of the time course. However, the biggest differences among treatments were observed in the last part of the time course. During the whole time course, all the genotypes were more affected by the combination of both *Fop* and drought stress than by the other treatments except for JI1412 (Fig. 3.1.G), which showed equal symptoms of combined stresses than of *Fop* infection. The response of this genotype on the first days of the time course was also different from the others, being less affected by the combination of drought and *Fop* than by *Fop* infection alone.

Generally, most of the genotypes seemed to be less affected by drought stress than by *Fop* or *Fop* and drought combination in the middle of the time course.

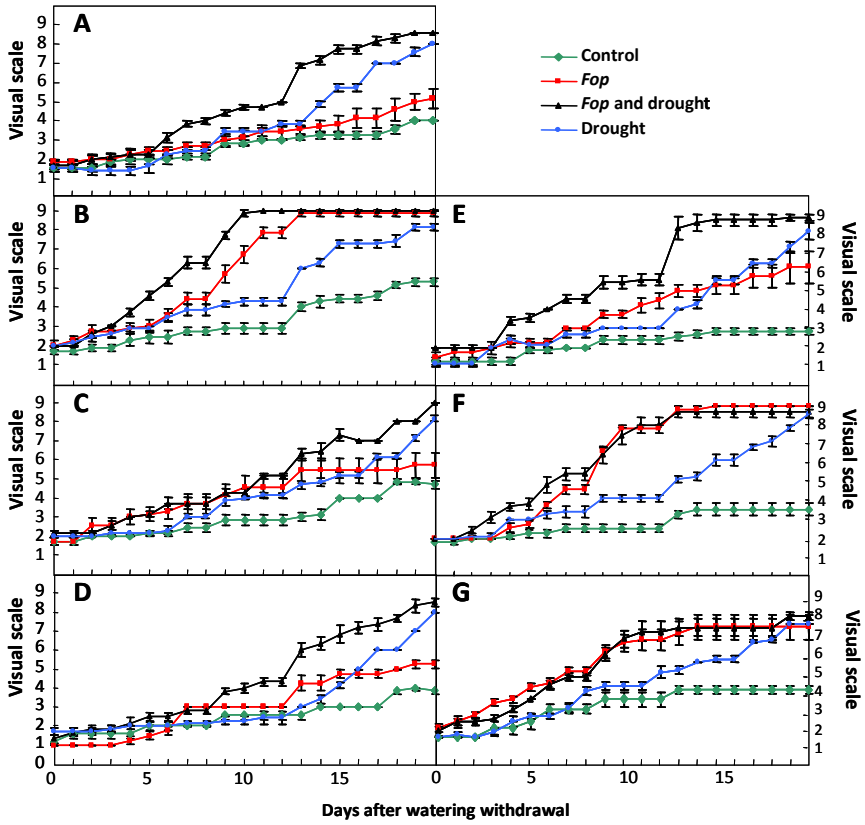

Fig. 3.1. Visual assessment of wilt symptoms in well watered controls, *F. oxysporum* (*Fop*) inoculated plants,drought stressed plants and the combination of both *Fop* and drought stress in the genotypes Polar (A), Kebby (B), New Era (C), New Season (D), 74SN5 (E), WSU28 (F) and JI1412 (G). Points represent the mean values of five observations in each time point.

Surprisingly, the genotype Polar, previously characterized as drought resistant, was more affected by drought than by *Fop* infection, which could mean this genotype to be more resistant to *Fop*. The opposite fact occurred with the genotype JI1412, previously characterized as *Fop* resistant, which was less affected by drought than by *Fop*.

To summarize, the genotypes Polar (Fig. 3.1.A), New Era (Fig. 3.1.C) and New Season (Fig. 3.1.D) were the less affected by the stresses. 74SN5 (Fig. 3.1.E) showed a resistance response pattern to *Fop* similar to that one for drought, but was

highly susceptible to the combination of both stresses. The genotype JI1412 (Fig. 3.1.G) showed more tolerance to drought than resistance to *Fop*, and a resistance to both stresses similar to that for *Fop*. Finally, the genotypes Kebby (Fig. 3.1.B) and WSU28 (Fig. 3.1.F) were highly susceptible to all the treatments.

Fop stress assessment

The response of the cultivars to *Fop* stress is illustrated in Fig. 3.2.A. The genotypes Polar, New Era and New Season showed the strongest resistance response against the pathogen if compare with the rest of the genotypes.

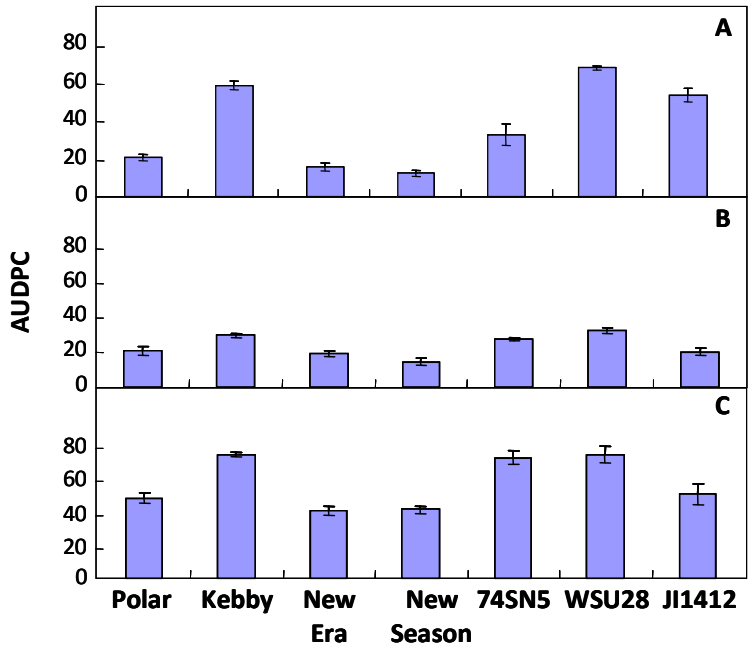

Fig.3.2. AUDPC values calculated for each genotype from the medium values of the evaluation of *Fop* infection (A), water stress (B) or *Fop* infection and water stress (C) symptom percentage in each individual plant.

The genotype 74SN5 showed an intermediate resistance response ($p<0,05$) compared with the susceptible genotypes Kebby and WSU28. JI1412 showed moderate susceptibility, as it was different from WSU28 ($p<0,05$).

Kebby and WSU28 were the genotypes most affected by *Fop.* Kebby was differently affected by *Fop* than the rest of the genotypes ($p<0,05$) except WSU28, which was different from the rest of the genotypes ($p<0,05$).The genotypes JI1412 as well as the genotype New Season, although being resistant if compared to WSU28 ($p<0,01$) showed lower values of disease incidence than those described per Bani *et al*. (2012).

Water stress assessment

All the genotypes showed a moderate response to water stress (Fig 3.2.B). As expected, Kebby was one of the genotypes most affected by drought. WSU28, susceptible check for *Fop* infection, showed also high susceptibility to water stress. The differential line 74SN5, although being resistant to *Fop*, was quite affected by drought, not showing significant differences with the sensitive genotypes Kebby and WSU28.

The genotypes New Era, Polar and JI1412 showed an intermediate tolerance against water stress, being different from the susceptible genotypes previously mentioned ($p<0,05$). Finally, New Season was the genotype less damaged compared with the others ($p<0,01$).

To summarize, Polar, New Era and New Season were found as tolerant whereas 74SN5, Kebby and WSU28 were susceptible to water stress.

Drought and Fop stress assessment

The conjunction of both *Fop* and water stress increased the symptoms observed in all the genotypes (Fig. 3.2.B), allowing us to separate the genotypes in four groups according to their mean values. The genotypes most affected, and therefore most susceptible to both stresses, would be Kebby, 74SN5 and WSU28. On

the other hand, JI1412, New Era and Polar would be similar. Thus JI1412 and Polar can be considered as moderately susceptible. Finally the resistant genotypes would be New Era and New Season, showing fewer symptoms than the others in the combination of both stresses.

Genotypes comparison according to AUDPC values

In order to compare genotypes and observe to which stress were more resistant, we calculated the percentage of symptoms of each genotype with respect to the most sensitive, the one with the highest mean AUDPC value of all the treatments, which was the one observed for Kebby and the combination of both stresses. (table 3.2) Thus, the values of these percentages would be close to 100 if the genotypes were highly affected and close to 0 if they were scarcely affected for the different stresses.

Table 3.2. Comparative percentage for the AUDPC mean values observed in the pea genotypes.

Genotype	*Fop*	Water stress	*Fop* and water stress
Polar	28	30	64
Kebby	81	43	100
New Era	22	28	55
New Season	17	21	56
74SN5	45	40	97
WSU28	95	47	100
JI1412	74	29	68

The genotypes Polar, New Era and New Season were slightly less affected by *Fop* (28%, 22% and 17%, respectively) than by water stress (30%, 28% and 21%, respectively). When both stresses applied together, the increment of the percentage in Polar and New Era was approximately double of their individual values, or the sum

of both individual stresses. This was not the case for New Season, which was the most resistant to *Fop* and the most tolerant to water stress as well as the less affected by the combination of both stresses. However, New Season showed and increment on the percentage of more than double of their individual values, either the sum of both individual stresses. Even so, this genotype was still the most resistant of all the genotypes.

The differential line 74SN5 was more resistant to *Fop* than tolerant to water stress, and when both stresses applied together its percentage was close to the maximum. Finally, Kebby and WSU28 were the genotypes most affected by *Fop* stress than by water stress, being their percentages maximum under both stresses.

DISCUSSION

In the field, plants are often exposed to various environmental factors, including biotic and abiotic stresses. Whereas there is an extensive literature about the effects of abiotic and biotic stresses separately, little or less about simultaneously applied stresses under control conditions can be found apart from molecular studies which deal most with the causes than with the effects over the plant. In the present work, separate and simultaneous effects of drought and *Fop* stress over tolerant/resistant and susceptible genotypes of pea were assessed for the first time.

One of the limiting factors when working with two stresses simultaneously relies in the methodology. In this sense, the former and most important decision to take was which one of the stresses should be applied first. This decision relied in two

main factors: the moment for symptoms to appear in the plant and the inoculation system.

In order to determine the onset of the symptoms of each stress, we analyzed the results from previous screenings for each stress separately. We observed that the symptoms produced by *Fop* usually appeared later than the symptoms of drought in the plants. However, we should take into account that the perception of the pathogen by the plant happened nearly simultaneously to that of the absence of water, at it was reported for each stress (Di Pietro *et al*., 2001; Bani *et al*., 2012; Hsiao, 1970). Furthermore, the inoculation protocol for *Fop* was based in the resuspension of the spores in water with a previous cut in the root system (Haglund, 1979), which could them be more damaging if the plant was first subjected to water stress. Also, given that the spores needed water to germinate, it was clear that the fungal stress should be applied first.

The next question was when we should apply water stress over the plants, as they should have enough time to recover from the inoculation in order we could assess the symptoms in a time course. We made previous screenings, applying water stress simultaneously and 48 hours after the inoculation. In the first case, plants were not watered after the inoculation and all of them were death soon enough to perceive differences between stresses or genotypes. In the second case, with plants watered after the inoculation, we could observe differences between the genotypes along the time course and the symptoms were comparable with those of each stress separately. Besides, the relative standards of resistance or susceptibility were maintained: the resistance and susceptibility to *Fop* observed for the differentials and the genotypes was preserved as previously described by Bani *et al*., 2012, being

WSU28 the susceptible check. Furthermore, the genotypes Polar and Kebby were found resistant and susceptible to drought, respectively, despite the growing conditions were different from those used to characterize drought resistance (chapter 1). All these agreements with previous reports showed that the methodology employed in this study was suitable for the desired objective of discriminate genotypes according to their resistance/tolerance or susceptibility.

In accordance with their different responses to each treatment, New Season would be the genotype with higher resistance and tolerance, followed by Polar, New Era and JI1412. The genotype 74SN5, although being moderately resistant to *Fop* showed high susceptibility to water stress, whereas Kebby and WSU28 showed high susceptibility for all the stresses. This criterion should suffice the objective of selecting genotypes for a breeding program, but it did not reveal the inner ability of each genotype in resist or tolerate each stress.

Similar profiles on the symptoms were observed for all the genotypes under the different stresses. Although no previous reports about biotic and abiotic stresses applied simultaneously could be found in the literature, it has been observed an increase when two abiotic stresses, such as drought and cold were applied over chickpea (Nayyar *et al*., 2004). Therefore, it was expected that the intensity of the symptoms would increased if the stresses would be combined. This happened for most of the genotypes, except for JI1412, which was slightly less affected by both stresses than by *Fop* stress alone.

The singular response of the genotype JI1412 points to the existence of defence mechanisms that could somehow increase the resistance of this genotype when both stresses were applied simultaneously.

Combined stresses have been known for many years to elicit the so-called cross-tolerance, a phenomenon whereby a plant acclimates to a range of different stresses after exposure to one specific stress (Foyer *et al*., 1994; Pastori *et al*., 2006). Several studies have indicated that plant responses to environmental stresses could have some effects on their responses to pathogens. In *Arabidopsis*, a short period of drought stress significantly increased the growth of the avirulent bacterium *Pseudomonas syringae* pv *tomato* relatively to its growth in unstressed plants (Mohr *et al.*, 2003; 2007). In rice (*Oryza sativa*) plants, low temperature suppressed the resistance to infection by *Magnaporthe grisea* (Koga *et al.*, 2005). However, very little is known about the molecular mechanisms underlying these phenomena (Mauch-Mani *et al.*, 2005). One of the physiological consequences of cross-tolerance could be the "primed" state of the plant, a physiological situation in which plants re-exposed to biotic or abiotic stress are able to "recall" the previous infection, root colonisation or chemical treatment, a feature frequently associated with enhanced disease resistance (Goellner *et al*., 2008). Because stress sensors are not well known and most of the signalling intermediates have not yet been identified, there is little definitive information regarding cross-talk between different stress signal transduction pathways in plants mechanisms (Chinnusamy *et al*., 2004). Observations made in *Arabidopsis* have suggested that the exposure to excess light elicits the production of micro-lesions associated with subsequent resistance to biotrophic pathogens (Muhlenbock *et al*, 2008). Priming might be a common component that mediates cross-talk between pathogen defence reactions on one hand and responses to abiotic stress, such as wound or osmotic stresses, on the other (Kohler *et al*., 2002). Thus, both abiotic and biotic stresses can prime and

induce plant defence responses towards a large range of pathogens (Feys *et al.*, 2000; Pieterse *et al.* 2001).

Priming phenomena could explain the behaviour of the genotype JI1412. Besides, it is described in the literature that overlapping molecular responses to biotic and abiotic stresses can lead to a detectable cross-protection (Francia *et al*., 2007). We should remind here that *Fop* inoculation was mediated through root wounding, and water stress began 48 hours after *Fop* inoculation. Normally, costs related to wound repair do not generally decrease the plant fitness if a pathogen attack follows. According to our results, it seems that wounding the roots did not have any effect over the other genotypes to each stress, because their patterns of resistance or tolerance were preserved, so the different response of the genotype JI1412 could also be due to the infection of the fungus instead of wounding. In any case it seems acceptable that priming should have a slightly effect on its response. Differences in stress tolerance between genotypes may arise from differences in signal perception and transduction. Thus, differential perception of stress could lead to a different response in each genotype.

It could also be argued that signalling pathways sharing common components may not necessarily cross-talk if the common components are scaffolded into distinct protein complexes (Park *et al*., 2003) and that would be the reason of not having found the same results in the other genotypes. However, it is clear that drought and *Fusarium* stress responses separately share several intermediates in their pathways and are closely related. Necrotrophic fungi as *Fop* produce toxins, cell-wall degrading enzymes and reactive oxygen intermediates that determine the severity of disease (Edlich *et al.*, 1989; Tiedemann, 1997;

Muckenschnabel *et al.*, 2002). These disease factors cause electrolyte leakage, changes in ion fluxes, cell death and other stress responses, underlining the similarities in plant responses to microbial necrotrophy and abiotic stresses.

Also, there is strong evidence that plant hormones ethylene, salicylate, jasmonate and abscisic acid (ABA) act synergistically or antagonistically to regulate plant responses to pathogens and abiotic stress factors (Rao *et al.*, 2000, 2002; Borsani *et al.*, 2001; Turner *et al.*, 2002; Xiong *et al.*, 2002). For instance, ABA can act as a positive or negative regulator of disease resistance, depending on the nature of the host-pathogen interaction (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004; Mauch-Mani *et al.*, 2005). Increased endogenous levels of ABA were observed in response to infection by viruses, bacteria, and fungi (Steadman *et al.*, 1970; Whenham *et al.*, 1986; Kettner *et al.*, 1995). ABA deficiency in tomato and impaired ABA responses in *Arabidopsis* result in increased resistance to necrotrophic pathogens, as a result of the reduced ABA signaling but increased jasmonate or ethylene responsive gene expression (Audenaert *et al.*, 2002). This last antagonistic action could explain the fact that the genotype New Season showed and increment of the symptoms when subjected to *Fop* and drought stress of more than double of their individual values. Maybe in different genotypes for the same plant species the signals could act differentially, although no literature has been found within this respect.

Regarding the common components of the signalling pathways, regulators such as reactive oxygen intermediates, secondary messengers (i.e. Calcium) and transcription factors are required to modulate plant responses to biotic and abiotic stress (Bowler *et al*., 2000;Mengiste *et al*., 2003; AbuQamar *et al.,*2009), highlighting the close relationship between both stresses. Also, the fact that plant resistance to

drought and *Fusarium* is determined separately by multiple host and environmental factors seems to require the contributions of multiple loci for full resistance, thus implying the effect of different genes and different proteins non specific for one single stress (Bani *et al.*, 2012; Sánchez *et al.*, 2002).

To summarize we conclude that we have developed a suitable method to analyze the simultaneous effects of drought and *Fop* stress in pea genotypes, testing the stresses together and separately. The genotypes showed different responses and sources of resistance were identified that could be useful in a breeding program for arid or semi-arid environments, when both *Fop* and drought stress could occur together. Thus, the recommended genotypes for breeding in such atmosphere would be New Season, New Era, Polar and JI1412.

On the other hand, understanding the genetic control of pathogen and abiotic stress responses has a bearing on rational crop breeding. Plants could be bred to resist specific or non specific stress conditions based on the knowledge of the molecular regulation of physiological responses. In this sense, the genotypes JI1412 and New Season would probably be useful to develop molecular studies based on cross-tolerance phenomena and thus help with the understanding of stress physiology in pea.

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Chapter 4

Mapping quantitative trait loci associated to relative water content in pea (*Pisum sativum* L.)

INTRODUCTION

Dry pea (*Pisum sativum* L.) is one of the most widely grown grain legumes in the world with primary production in temperate regions. One of its advantages as a legume crop relies on its capacity for symbiotic nitrogen fixation, allowing the reduction in the use of fertilizers in crop rotations. However, this process is highly sensitive to environmental stresses such as drought and salinity (Zahran, 1999) which are major constraints to the production and yield stability of pea, especially during the flowering and pod filling (Doré *et al.*, 1998).

Water deficit induces a range of physiological and biochemical responses within the plant which include stomatal closure, activation of respiration, repression of cell growth and photosynthesis. At the cellular level, plant responses to water deficit may result from cell damage, whereas other responses may correspond to adaptive processes. Dehydration in plant tissues induces changes in cells membranes stability and permeability, which finally lead to changes in the cell functions.

One of the plant strategies to preserve cell functions and maintain cell membrane stability under water stress is to maintain high relative water contain (RWC) within the tissues, which can be achieved by an increase in water transport through the plant (O´Toole *et al.*, 1987; Ludlow, 1989) or a reduce in water looses (Turner, 1979; Ludlow *et al.*, 1990). However, when water is a limiting factor, come into action the mechanisms for osmotic adjustment, allowing the plant to maintain cell turgor through the accumulation of specific compounds which will finally produce water passive transport inside the cells to balance the proportion of solutes (Turner *et al.*, 1980; Morgan, 1983; Nguyen *et al.*, 1997; Cattivelli, 2008). All these different mechanisms of response and physio-biochemical changes at both cellular

and whole plant level induced by drought make of it an abiotic complex stress. Therefore, to overcome the study of drought stress, one of the options is to divide the complex trait into different components that are highly heritable and easy to measure.

It is reported that traits such as flowering date (Medway 1972; van Schaik *et al.*, 1993; Brearley *et al.*, 2007; Forres *et al.*, 2010), deep rooting (Ekanayake *et al.*, 1985; Lilley *et al.*, 1994; Pantuwan *et al.*, 1996; Wade et al; 1996; Lanceras *et al.*, 2004) and osmotic adjustment (Ludlow *et al.*, 1990; Jonggdee *et al.*, 1998; Zhang *et al.*, 2001) are associated with drought perception and tolerance. Besides, traits allowing the indirect assessment of the water amount in the plants, such as RWC, may reflect the ability to maintain cell turgor when measured under drought stress, providing an idea of the tolerance capacity of the plant. Thereby, RWC have been widely used as physiological index for the evaluation of drought and temperature tolerance (Hunt *et al.*, 1987; Tripathy *et al.*, 2000; Siddique *et al.*, 2000).

The potential value of RWC for breeding under drought stress conditions was demonstrated by Schonfeld *et al.* (1988) in winter bread wheat. These authors showed that RWC is inherited quantitatively and controlled by genes with additive effects. A wide variation was observed by Tahara *et al.* (1990) and Martin *et al.* (1997) in bread wheat, by Merah (2001) in durum wheat, by Peltonen-Sainio and Makela (1995) in oat and by Matin *et al.* (1989), Arnau and Monneveux (1995) and Teulat *et al.* (1997) in barley. In addition, a positive correlation between grain yield and RWC has been observed in durum wheat (Merah, 2001), in bread wheat (Schonfeld *et al.*, 1988; Tahara *et al.*, 1990; Singh and Patel, 1996) and in oat (Peltonen-Sainio and Makela, 1995).

Although RWC could be a suitable index for water stress, its measurement in large segregating populations can be tedious and expensive. Since the genes which directly control this trait are unknown (Keurentjes *et al.*, 2008), molecular tools can be a good approach to overcome these problems and facilitate the identification of the genes or QTLs controlling this trait. Till date, these technologies have been widely employed to identify and map traits related with water stress. Among others, phenological and root traits have been studied in different crops such as wheat (Dhanda *et al.*, 1998), rice (Tripathy *et al.*, 2000; Lanceras *et al.*, 2004), and legumes like chickpea (Serraj *et al*., 2004; Lichtenzveig *et a*l., 2006), soybean (Virginia *et al.*, 2012), faba bean (Díaz *et al*., 2009; Torres *et al*., 2011) and pea (Tar´an *et al*., 2003, 2004; Burstin e*t al*., 2007; Fondevilla *et al*., 2011). Nevertheless, genes controlling these traits and many others, such as RWC, still remain widely unknown. Curiously, scarce works reported molecular markers associated with traits related to plant water-status or drought resistance in legumes (Nayak, 2010, Badri *et al*., 2011) but none of them is about pea.

All these works illustrate the major breakthrough in the characterization of quantitative traits which supposed the development of genetic maps and QTL analyses, enabling the identification of associated genomic regions and their contribution to the phenotypic variation (St Clair, 2010). Quantitative approaches based on phenotypic evaluations can estimate the heritability and the weight of dominance and additive effects in the control of the trait. In this sense, polygenic regulation of a complex phenomenon such as drought tolerance can be overcome by the technology of QTL analyses (Price *et al.*, 2002). Mapping QTLs is a useful tool to identify molecular markers linked to the tolerance genes that could be used to assist

breeding. As and advantage, the identification of quantitative resistance or tolerance, usually governed by multiple minor genes, is expected to be more durable than monogenic resistance, which supposes stability in the cultivars improved by marker assisted selection (MAS).

The objective of this work was to identify QTLs controlling drought tolerance in pea. With this aim we assessed RWC, under water stress conditions, in the Recombinant Inbred Line (RIL) population P665 x Messire. The parental lines of this RIL population were found to be tolerant and susceptible to drought, respectively in previous experiments (Chapter 1).

MATERIALS AND METHODS

Plant material and growth conditions

The population used in the study consisted of 103 $F_{7:8}$ RILs families from a cross between P665 and cv. Messire. P665 (derived from the ICARDA accession IFPI3280) is a *Pisum sativum* subsp. *syriacum* full-leafed accession previously reported as partially resistant to different isolates of *M. pinodes* (Fondevilla *et al*., 2005) as well as to *O. crenata* (Fondevilla *et al*., 2010) that showed wild traits such as late flowering, creeping growht habit and violet flowers. Messire is a *P. sativum* subsp. *sativum* full-leafed, early-flowering and white-flowered pea cultivar that is susceptible to both *M. pinodes* and *O. crenata* and shows an erect growth habit. Parents P665 and Messire have been described as tolerant and moderately susceptible to water stress, respectively, in our previous experiments (Chapter 1).

Seeds from all the RILs families along with the parents were pregerminated in Petri dishes with moistened filter papers in the dark for 48 h in a cold chamber at

4ºC and then placed for another 48 h in a growth chamber at 65% relative humidity and 20ºC.

Seedlings were planted individually in 0,5 L pots filled with peat: sand (3:1) and placed into a growth chamber in a complete block design with 3 replicates. Plants were growth at 21 °C, under a photons flux density (PPFD) of 200 μmol m^{-2} s^{-1} supplied by high output white fluorescent tubes. Plants were watered and positions changed every two days during their 21 days growing period. Plants were well watered until the beginning of the drying episode. A minimum number of 3 plants per time point and line were used for the measurements.

Relative water content measurements

Relative water content (RWC) was determined according to (Cabrera-Bosquet *et al.*, 2007). Briefly, the fourth leaf of each plant was collected 0, 4, 8, 12 and 16 days after watering withdrawal. Leaf segments were weighed (fresh weight, FW), then saturated in water for 24 hours and their turgid weights (TW) were calculated. The samples were then dried in an oven at 60°C for 72 hours and weighed (DW). RWC was determined as follows:

RWC = (FW - DW)/(TW - DW) x 100.

As RWC was assessed along a time course, we calculated the area under the disease progression curve (AUDPC) using the formula:

$$AUDPC = \Sigma [(x_i + x_{i+1})/2] * (t_{i+1} - t)$$

Where x_i = estimated proportion of water stress severity at date *i*, x_{i+1} = estimated proportion of water stress severity at date *i+1*, and *ti+1-ti* = number of days between scoring dates *i* and *i+1*. This trait was called AUDPC and represented the evolution of the RWC along the time course for each RIL and the parents.

Statistical analysis and heritability estimation

Statistical analyses were performed using SAS ver. 9.2 (SAS Institute Inc. 2004). Standard analysis of variance (ANOVA) was performed using PROC GLM to determine variation in AUDPC and RWCT16 (which represented the value of RWC for each RIL 16 days after watering withdrawal). Variance components were estimated using PROC VARCOMP.

Broad sense heritability (h^2) that represents the part of genetic variance in the total phenotypic variance was calculated using the formula: $h^2=\delta^2_g/ (\delta^2_{g+} \delta^2/r)$, where δ^2_g is the genotypic variance, δ^2 is the error variance and r is the number of replications. Normality of residual distribution was checked using the Kolmogorov-Smirnov test. Pearson correlation coefficient between traits was estimated using PROC CORR in SAS.

Map Construction

A previous genetic map developed using the RILs of the cross P665 x Messire (Fondevilla *et al.*, *et al*. 2011) and improved by adding 119 additional SNPs markers (Deulvot *et al*., 2010), was used for QTL analysis. This map covered 1119,46 cM and contained 414 markers: 3 morphological traits, 1 isozyme, 222 RAPDs (Random Amplified Polymorphic DNAs), 59 SSRs (Simple Sequence Repeats), 12 ESTs (Expressed sequence tags), 117 SNPs (Single nucleotide polimorfisms) and 21 SSRs (Simple sequence repeats) distributed in 7 linkage groups (LGs).

The linkage map was constructed by MAPMAKER Version 3.0b (Lander *et al.* 1987) using a LOD score of 5.0 as the threshold for significant linkage. The marker orders were established using MSTMap (Wu *et al.*, 2008) by finding the minimum spanning tree of a graph for each linkage group. MAPMAKER was used to confirm

marker order determined by MSTMap. Recombination fractions were converted to centiMorgans (cM) using the mapping function of Kosambi (1944).

QTL analysis

QTL analysis was conducted using composite interval mapping (CIM) and multiple interval mapping (MIM) in Windows QTL Cartographer V2.5 (Wang *et al.*, 2011). Markers to be used as cofactors for CIM were selected by forward-backward stepwise regression. The number of markers controlling the genetic background in CIM was set to five. The thresholds for the detection of QTLs were estimated by permutations analysis (Churchill *et al.*, 1994) using 1,000 permutations. One- and two-LOD support intervals for the position of each QTL were calculated as described by Darvasi *et al.* (1997).

To obtain more precise information on QTL effects and positions and to evaluate for the presence of digenic epistatic interactions across the QTL pairwise combinations, multiple-interval mapping (MIM) (Kao *et al.*, 1999; Zeng *et al.*, 1999), as implemented in WinQTL Cartographer, was used by considering as initial QTL models the CIM results obtained for the trait. The initial CIM-derived QTL model was subjected to a search for significant epistatic interactions among QTLs. Both main additive effects and their epistatic interactions were tested for significance using the Bayesian information criterion (BIC) with the penalty function c(n) = log(n), with n (sample size) = 111 (Zeng *et al.*, 1999). The final main additive and epistatic QTL effects and the R^2 values of the model were then estimated.

RESULTS

Relative water content assessment

The AUDPC values represented the evolution of the RWC along the time course, as they were calculated using the RWC data in the different time points, including the last one (RWCT16). The parental P665, which is considered drought tolerant (Chapter 1) showed high RWC values along the time course, displaying an average AUDPC value of 311,28 and a final mean RWC of 71,76 %. On the other side, cv. Messire which show a moderate susceptibility to drought, displayed an average AUDPC value of 257,26 and a final mean RWC of 47,65 % (Table 4.1). The final RWC mean, obtained 16 days after watering withdrawal, represented the time point in which more differences were observed among the RWC of the genotypes.

Table 4.1. Descriptive statistics No: number of RILs; SD: standard deviation; CV: coefficient of variation (%).

		Trait	
		AUDPC	RWCT16
Parentals	P665	311,28	71,76
	Messire	257,26	47,65
RILs population	No.	103	103
	Average	257,23	56,09
	SD	44,98	19,71
	Min	117,65	13,04
	Max	335,26	88,69
	CV	17,49	35,14

The ANOVA revealed that the variation in AUDPC and RWCT16 among the RIL families was highly significant ($p<0,001$). Transgressive RIL lines with increased tolerance and susceptibility were identified for both traits (Fig. 4.1). Broad sense heritabilities (h^2) were high for both traits (h^2 = 0,719 for AUDPC and h^2 = 0,674 for RWCT16).

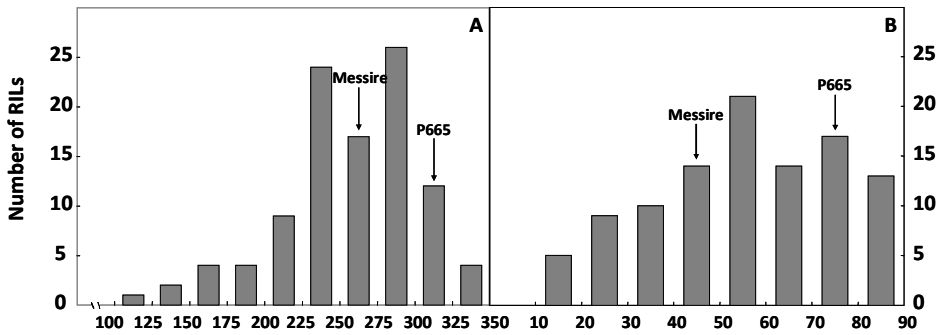

Fig. 4.1. Frequency distributions for AUDPC (A) and RWCT16 (B) in P665 × 'Messire' RIL population. Values for both parents are indicated by arrows.

Correlation between AUDPC and RWCT16 was significant ($p<0,05$) but low ($r = 0.248$).

QTL analysis

Quantitative trait loci analysis revealed a total of three genomic regions (*rwct16-1*, *rwct16-2* and *rwct16-3*) associated RWCT16 along LGsIII and VII and another QTL (*audpc*) associated with AUDPC on LG II (Fig.4.2). Genomic position, additive effects and LOD scored of each QTL are shown in Table 4.2.

Table 4. QTLs for AUDPC and RWCT16 in the RIL population derived from cross P665 × Messire.

TRAIT[a]	QTL	Linkage group	Flanking marker(s)	LOD[b]	Add[c]	R^{2d}
AUDPC	*audpc*	II	$OPJ14_{713}$ / $OPX20_{1111}$	3,47	17,34	13,87
RWCT16	*rwct16-1*	III	$OPAI14_{1353}$ / $OPAI14_{1273}$	13,62	12,34	34,73
RWCT16	*rwct16-2*	III	$OPW5_{387}$ / $OPAE5_{538}$	4,37	-5,92	9,37
RWCT16	*rwct16-3*	VII	tRALs_SNP1	3,49	5,10	6,43

[a] Traits: AUDPC, Area under disease progress curve calculated for the mean values of RWC along a time course; RWCT16, mean value of the RWC after 16 days of watering withdrawal.
[b] LOD peak LOD score.
[c] Add:additive effect from CIM (for *audpc*) and MIM (for *rwct16*).
[d] R^2 (%):proportion of phenotypic variance explained by the respective QTL (%) from CIM (for *audpc*) and MIM (for *rwct16*).

The QTL associated with AUDPC explained near 14% of the phenotypic variation. The QTLs associated with RWCT16 explained individually from 6% to 35% of the phenotypic variation and altogether 50%.

Alleles conferring higher RWC or AUDPC, and thus tolerance to water stress, were originated from P665 in the case of the the QTLs *audpc*, *rwct16-1* and *rwc16-3,* and from Messire in the case of *rwct16-2*, according to the additivity signs, which reflected parents assignations in the data matrix to build the map.

LOD threshold derived from 1,000 permutations at α=0,05 was equal to 3.27 for AUDPC and 3.04 for RWCT16. No significant pairwise epistatic interactions among the three QTLs for RWCT16 were found in multiple-interval mapping (MIM).

DISCUSSION

Differences among the RILs parental genotypes P665 and Messire in tolerance to drought were described in previous studies (Chapter 1). These differences made it possible to analyze segregation of RWC as a reliable parameter associated to this trait along the RILs originated from their cross. Correlation observed between the traits AUDPC and RWCT16 was due to both derived from RWC measurement. However, the continuous and wide distribution of these traits and the fact that the parent lines values are well separated indicated that these parameters could be useful to find QTLs in our map from pea.

Polygenic nature of drought tolerance has been previously reported (Bartels *et al.*, 2005) as well as the polygenic mechanisms which are related with RWC (Schonfeld *et al.*, 1988). The continuous distribution observed following assessment of RWC traits in the RIL population and the identification of four QTLs associated to different regions in the genome, explaining phenotypic variation, suggested the polygenic control of RWC in pea.

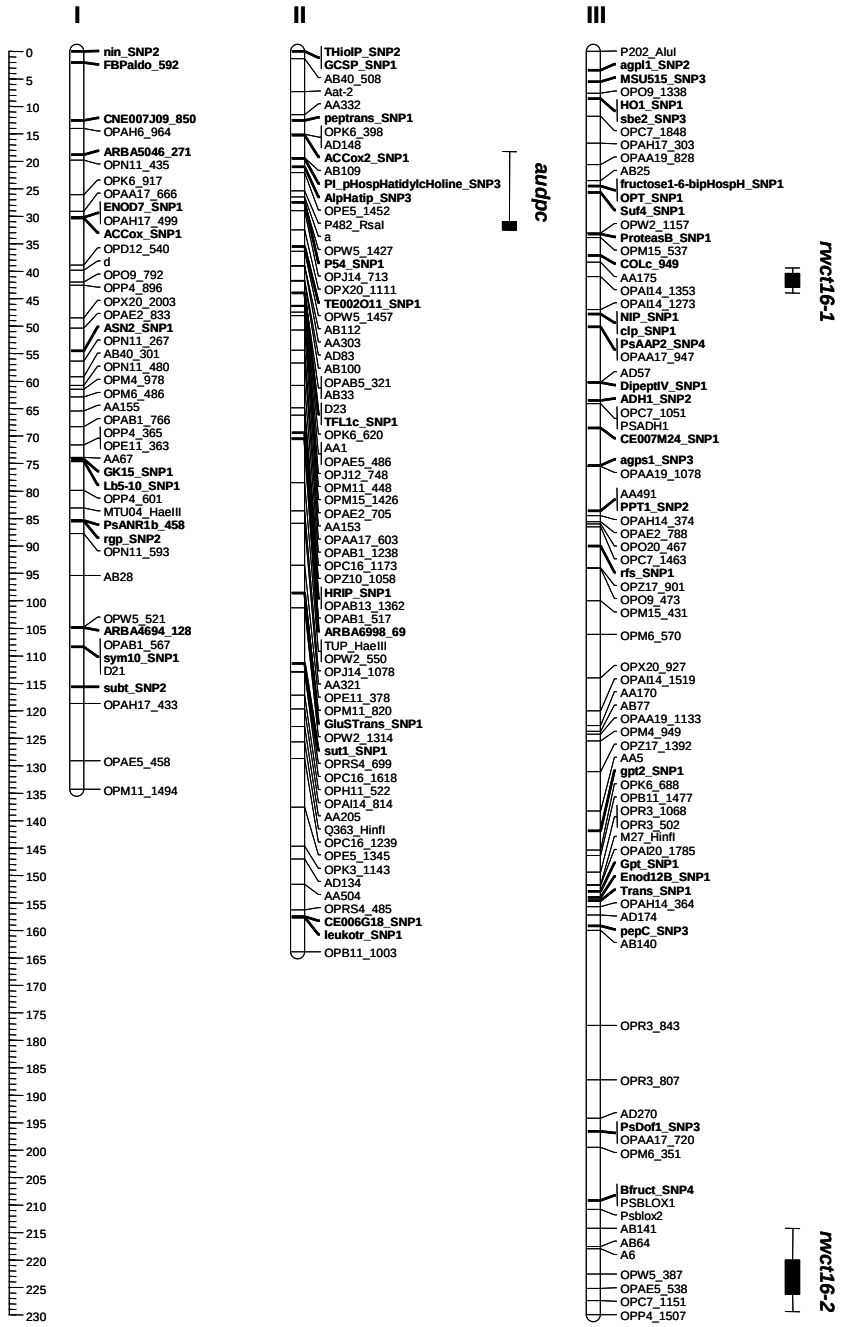

Fig. 4. 2. Pea genetic linkage map costructed from a population formed by 111 $F_{6:7}$ recombinant inbred lines (RILs) derived from the cross between the P. sativum subsp. syriacum accession P-665 and the P. sativum subsp. sativum cv. Messire. Bar positions indicate locations of quantitative trati loci: outer and inner interval corresponding to 1-LOD and 2-LOD support interval are indicated as a full box and a single line, respectively. SNP markers are shown in bold.

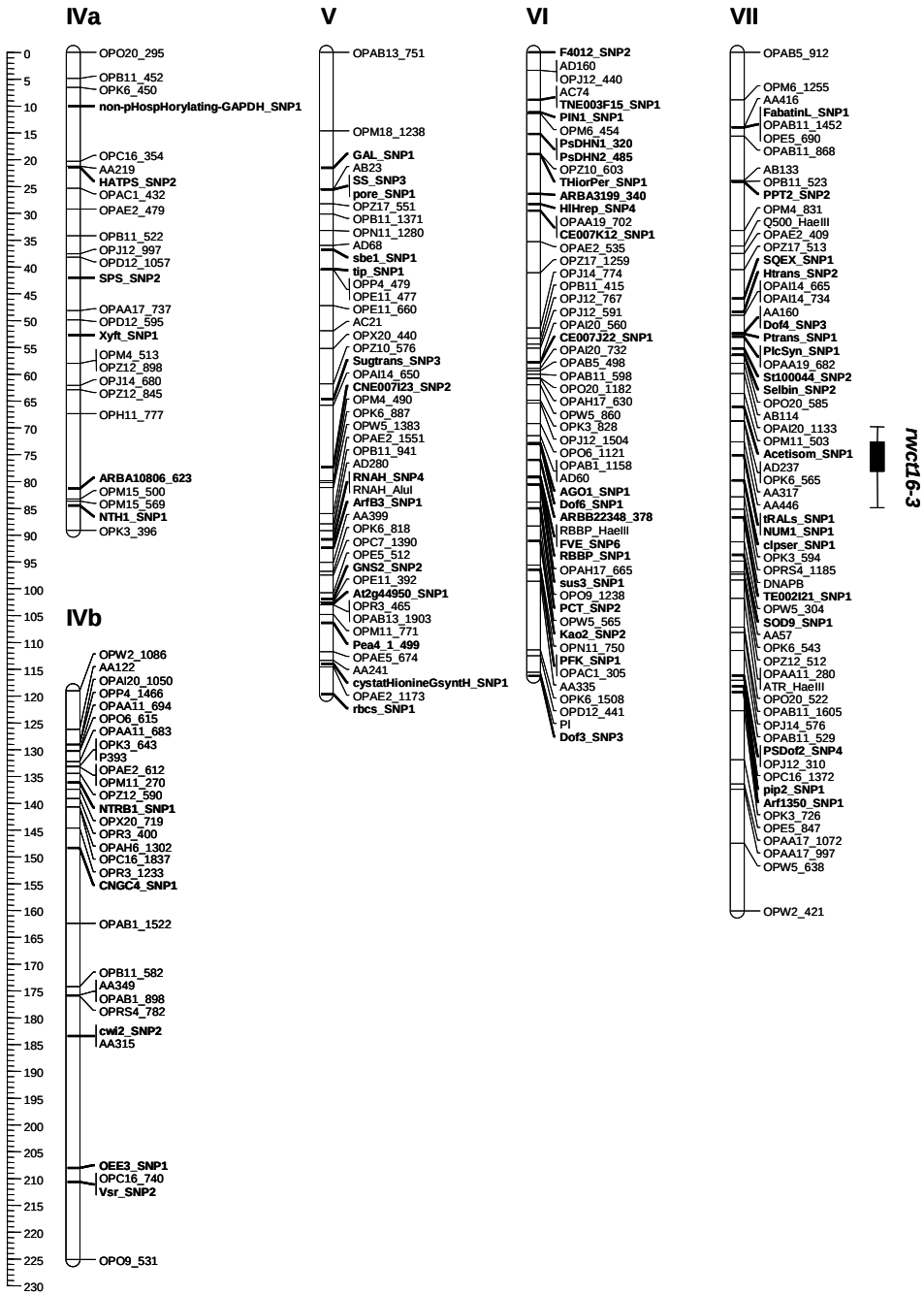

Fig.4. 2. Continued.

RWC has been previously reported as a valuable and appropriate index to assess drought tolerance (Hunt *et al.*, 1987; Tripathy *et al.*, 2000; Siddique *et al.*, 2000), being closely related with the visual scale that we have developed for pea (Chapter 1). In pea subjected to drought stress, sensitive genotypes have been found to be more affected by the decline in RWC than tolerant ones (Upreti *et al.*, 2000). Furthermore, a decrease in RWC was found to be a main factor resulting in reduced growth in response to osmotic stress in pea (Alexieva *et al.*, 2001).

P665 showed higher RWC during the water deficit treatment and also a higher AUDPC value than cultivar Messire. However, the distribution of these traits in the RIL population was skewed towards lower RWC, and many families showed lower values of RWC than Messire, suggesting the existence of some alleles promoting high RWC under water stress in Messire. In agreement with this, one of the QTLs associated with high RWC (*rwct16-2*) derived from Messire. In fact, previous studies (Chapter1) showed that Messire preserved a moderately high RWC under water stress when compared with genotypes highly affected by drough such as Kebby, indicating that Messire was moderately susceptible. This result suggested that genes conferring higher RWC in Messire and P665 were different. According to that, these more susceptible lines in this RIL population would be those lacking some or all of these genes. Also some transgressive RIL families with higher RWC values than both parents were identified. The RIL families showing a higher RWC than P665 may possessed all the tolerance genes.

As drought stress is a complex trait in which lots of genes are implied, it was expected that the phenotypic variance observed could not be explained by the QTLs identified in this study. The narrow differences in RWC between the two parental

lines of this cross may have resulted in the identification of only the main QTLs conferring higher RWC in this cross, hampering the identification of smallest QTLs also contributing to a high RWC. In addition, these parents may possess tolerance genes in common that could also not be identified in this study. A QTL analysis in a population between P665 and a more susceptible line may allow the detection, in the resulting segregating population, of additional genes governing high RWC. Furthermore, the use of different parameters and assessment tools for other traits related with drought tolerance applied over these RILs will allow us to unravel the complexity of the genetic networks implied on the physiological responses in the plant.

QTLs have been mapped for a wide range of agronomic traits in pea, including biotic and abiotic stresses. QTLs for partial and complete resistance have been detected for the most important diseases affecting pea crops such as *Aphanomyces euteiches* (Pilet-Nayel *et al.*, 2002;2005), Ascochyta blight (Dirlewanger *et al.*, 1994; Tar´an *et al.*, 2003; Timmerman-Vaughan *et al.*, 2002; 2004; Prioul *et al.*, 2004; Fondevilla *et al.*, 2008), Fusarium root rot (Weeden *et al.*, 2007), *Pseudomonas syringae* (Fondevilla *et al.*, 2012) and *Orobanche crenata* (Valderrama *et al.*, 2004; Fondevilla *et al.*, 2010). Furthermore, QTLs have been mapped for winter frost tolerance and frost damage (Lejeune-Hénaut *et al.*, 2008; Dumont *et al.*, 2009). However, nothing is known about the genomic regions implicated in the tolerance to drought in pea, and as far as we know, this is the first study addressing this aspect.

Among the QTLs associated with RWC derived from P665, *rwct16-1* (LGIII) explained the 34,7 % of the phenotipic variability for this trait, being the most

explicative of all. Interestingly, this QTL was located in the same genomic region as a QTL (*rl3*) associated with root length in a previous study (Fondevilla *et al.*, 2011). Selection for deep and extensive root system has been advocated to increase productivity of food legumes under moisture deficit conditions as it can optimize the capacity to acquire water. Turner *et al.* (2001) identified rooting depth and density as a main drought avoidance trait in grain legumes for use in terminal-drought environments. Grzesiak *et al.* (1997) showed that drought resistant pea cultivars had extensive and prolific root systems (O´Toole *et al.*, 1987; Ludlow, 1989). The fact that rooth lenght and RWC were related support the importance of this agronomic trait in pea drought tolerance

Two other QTLs asociated with RWC (*rwct16*-2 and audpc) were in the same genomic region as two QTLs controlling resistance to *O. crenata* in this same RIL population (Fondevilla *et al.*, 2011). Thus, *rwct16*-2 mapped exactly in the same region of LGIII as the QTL *n°br03_2*, which is a QTL for *O. crenata* incomplete resistance based on the broomrape shoots emerged per pea plant under field conditions. Similarly, the QTL *audpc* was in the same genomic region in LGII as *n°t2*, a QTL controlling the number of broomrapes per root length. The parasitic plant *O. crenata* obtains nutrients, but also water from its pea host. Therefore, symptoms produced by drought and *O. crenata* are similar and resistance to these two different stresses could be controlled by the same genomic region

Furthermore, the QTL *rwct16-2* was located in the confidence interval of the QTL *dfII.1*, associated to earliness in Messire (Fondevilla *et al.*, 2011), which would also allow avoiding seasonal drought stress (Forres *et al.*, 2010). Also other QTLs such as *MpIII.1_DRst_05*, associated with incomplete resistance to *M. pinodes* and

Psy1, associated with *Pseudomonas syringae* pv.*syringae* resistance, are located in this region (Fondevilla *et al.*, 2011; 2012). Therefore, this distal part of LG III may contain genes involved in broad spectrum resistance to pathogens or genes involved in other processes showing pleiotropic effects. As we have previously mentioned (Chapter 3), biotic and abiotic stresses are frequently related. Hormones such as ethylene, salicylate, jasmonate and abscisic acid (ABA) act synergistically or antagonistically to regulate plant responses to pathogens and abiotic stress factors (Rao *et al.*, 2000, 2002; Borsani *et al.*, 2001; Turner *et al.*, 2002; Xiong *et al.*, 2002).In this sense, it has also been reported the presence of the ''*Le*'' gene in this region (Lester *et al.*, 1997). This gene encodes a Gibberelin 3P-hydroxilase, an enzyme related with activation of the plant hormones Gibberellins (GAs), traditionally associated with growth regulation (Lange *et al.*, 1999), but also found to be implied in stress protection (Vettakkorumakankav *et al.*, 1999) and modulation (Alonso-Ramírez *et al.*, 2009).

The profile of molecular markers $OPAI14_{1353}$ and $OPAi14_{1273}$ (flanking QTL *rwct16-1*) as well as $OPW5_{387}$ and $OPAE5_{538}$ (flanking QTL *rwct16-2*) could be used as a first step to discard probable susceptible individuals in segregating populations derived from Messire. To enhance the efficiency of these markers in MAS, the conversion of these RAPD (Random Amplified Polymorphic DNA) markers into more reproducible ones, as SCARs (Sequence Characterized Amplified Region) is desirable. Thus, the development of more efficient molecular markers associated to this region would allow the selection for a set of agronomical interesting traits providing biotic and abiotic stress protection. In addition, QTL *rwct16-1* is in the vicinity of the SSR marker AA175 (Fondevilla *et al.*, 2011), what allows the use of this marker as a

marker to detect QTL *rwct16-1*. SSR markers are locus-specific, easier to score due to the absence of similar sized interfering fragment, less sensitive to reaction conditions and more reproducible. Therefore, SSR markers are more suitable than RAPDs for MAS and comparative mapping.

Regarding the QTL *rwct16-3*, it has been found to be associated to the SNP marker tRALs_SNP1, encoding a Cytosolic tRNA-Ala synthetase reported to be expressed in root caps and induced during early moments of cellular differenciation in pea, *Medicago truncatula* and *Picea abies* (Wen *et al*., 2009). Furthermore, these enzymes are related with regulation of translation and could be involved in the rapid control of the expression of other proteins involved in responses to stress or other environmental changes (Scheper *et al*., 2007).

In addition, Feng *et al*. (2011) identified a QTL related with Fusarium root rot resistance flanked by the microsatellite markers AA416 and AB60 reported by Loridon (2005). Marker AA416 appears consecutively to tRALs_SNP1 in our map, which could indicate the association of both markers with the two traits. SNP genotyping is easily automated, cost effective, and low in error rate (Xing *et al.*, 2005). Thus, if both QTLs could be related to the tRALs_SNP1, this marker could efficiently be used as a first step to discard probable susceptible individuals in segregating populations derived from Messire.

The knowledge of the genetic system controlling tolerance to drought in accession P665 and cv. Messire would facilitate gene transfer to pea cultivars, through the use of this information in MAS schemes. The introgression of tolerance to drought from P665 into elite cultivars will be facilitated by the low effect of the

environment in the trait, as shown by the high heritability value, and the absence of epistatic interactions between the genes controlling resistance.

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Chapter 5

Modelling gene networks for drought stress related genes from pea (*Pisum sativum* L.) and experimental validation

INTRODUCTION

Dry pea is one of the most widely grown grain legumes in the world with primary production in temperate regions. One of its advantages as a legume crop is its capacity for symbiotic nitrogen fixation as it allows the reduction in the use of fertilizers in crop rotations. However, this process is highly sensitive to environmental stresses such as drought and salinity (Zahran, 1999), being major constraints to the production and yield stability of pea (*Pisum sativum* L.).

Drought or soil water deficit can be chronic in climatic regions with low water availability due to changes in weather conditions during the period of plant growth. The effects of drought are expected to increase with climate change and growing water scarcity. Thus, an understanding of drought stress and water use in relation to plant growth is an important fact for sustainable agriculture. Drought is an abiotic complex stress which induces a range of physiological and biochemical responses, including stomatal closure, activation of respiration, repression of cell growth and photosynthesis. All these processes are controlled at both the cellular and molecular levels and this allow plants to respond and adapt to water deficit, accumulating osmolytes and proteins specifically involved in stress tolerance.

For several years, a wealth of molecular information has been generated on the response of plants to drought stress. It has been reported the induction of both regulatory and functional sets of genes (Ingram and Bartels, 1996; Ramanjulu and Bartels, 2002; Bartels and Sunkar, 2005) and the early events in the perception of stress signals (Urao *et al.*, 1999; Ueguchi *et al.*, 2001; Wohlbach *et al.*, 2008). Understanding gene regulation is particularly important in the case of a multigenic traits like drought because different regulatory pathways determine the expression

of a whole set of genes. In model plants, powerful tools such as microarray technology employing cDNAs or oligonucleotides have been developed to analyse gene expression profiles exposed to abiotic stresses such as drought, high salinity, or cold (Seki *et al*., 2001, 2002; Kreps *et al*., 2002). An assortment of genes with diverse functions are induced or repressed by these stresses (Shinozaki *et al.*, 2003; Bartels *et al.*, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005). Most of their gene products may function in stress response and tolerance at the cellular level. Currently, these genes are identified but analysing the functions of these genes is critical to further understand the molecular mechanisms governing plant stress response leading to enhancement of stress tolerance in crops.

Most of the tools to analyze gene expression and functions have been worked out for and from *Arabidopsis thaliana* (L) Heynh., which is by far the quintessential model plant. One of the most important technical innovations in *Arabidopsis* genetics was the discovery by Bechtold *et al*. (1993) that vacuum infiltration produced independent transformants in the progeny at a high frequency. In this process, plants are immersed in a culture of *Agrobacterium* subjected to a vacuum for a few minutes and then grown to maturity. Transformation has become such an easy and rapid aspect of the process of gene characterization that, until other plants with fully sequenced genomes can be transformed with comparable ease, *Arabidopsis* is unlikely to be displaced as the plant of first choice for experimental molecular geneticists (Somerville *et al*., 2002). Insertion mutants have contributed to demonstrate the ease with which various processes could be disrupted by mutation, such as the response of plants to phytohormones and to light (Koornneef *et al*., 1980a, b; Koorneef *et al*., 1982) or to understand gene regulation

in *Arabidopsis* in physiological processes such as flowering and seed dormancy (Rédei, 1992) or stress conditions (Shinozaki *et al.*, 2000). Despite all these advances, much of the revolution in molecular genetics, especially genomics-based approaches, has yet to have an impact in pea, although some progresses have been made in the last years (Ellis, 2011). For instance, the development of a systematic mutant population for reverse genetics (Dalmais *et al*., 2008), the bacterial artificial chromosome libraries (Coyne *et al*., 2007) and an effective gene silencing system (Constantin *et al*., 2004) as basic resources for pea. Together with the availability of high-throughput sequencing and genotyping methodologies, these resources hold promise for a resurgent interest in basic genetic studies in pea (Ellis, 2011).

Mutational approaches have been widely exploited in breeding and basic research. However, most methods are still mainly based on *Agrobacterium* T-DNA vectors and thus rely on the ability of plants like *Arabidopsis* to be transformed. To standardize the results obtained in model plants to crop species recalcitrant to *Agrobacterium*-based transformation, like pea, it is necessary to employ different approaches such as TILLING (targeting induced local lesions in genomes). TILLING uses chemical mutagenesis, based on alkylating agents, coupled with gene-specific detection of single-nucleotide mutations (McCallum *et al*., 2000; Henikoff *et al*., 2004; Comai *et al*., 2006). Nowadays, the development of this technology will allow us to obtain pea mutants for target genes and thus to clarify gene functions in pea in a similar way as it was done in *Arabidopsis*.

The objective of the present work was to identify interesting genes related with drought in pea using bioinformatics tools and based on the genetics resources of the model plant *Arabidopsis thaliana.*

MATERIALS AND METHODS

Analysis and selection of sequences

Five hundred sequences of expressed sequence tags (ESTs) under drought stress from a library of cDNA clones from pea (*Pisum sativum* L. cv. Puget) as well as the microarray datasets for drought related genes from *Arabidopsis* were kindly provided by Professor Phil Mullineaux. The library of cDNA clones was developed by Markus Klennel (unpublished data).

The BLASTN (nucleotide query-nucleotide database search) and TBLASTX (6 frames translated nucleotide query-6 frames translated nucleotide database search) tools from the database TAIR (www.arabidopsis.org) were used to obtain one homologue sequence of Arabidopsis per each pea EST. After the BLAST search we selected only those sequences with scores among 50%-100% and the Expected (E) value closer to 0 to ensure maximum similarity among the sequences. On a second step we selected those sequences which showed the same homologue in the BLASTN than in the TBLASTX search.

The following analyses (plotting, clustering and modelling) were done with informatics tools developed in the Universities of Warwick, Essex and Exeter, within the project PRESTA (Plant Responses to Environmental Stress in *Arabidopsis*).

-Plotting tools

To visualize expression profile of each gene we used the Gene Viewer (GV) tool, based in the *Arabidopsis* microarray for drought related gene expression. GV generated a plot for each gene sequence illustrating its expression profile over a drought time course with respect to a non droughted controls. Using this tool we selected visually those genes whose expression patterns were different at the

beginning, 0-4 days after water withdrawal (DAW), middle (4-8 DAW) and final stress period (8-14 DAW). To separate the groups we observed the patterns where at least two successive time points were different according to their standard error bars.

-Clustering tools

The statistical tool chosen to group genes according to their different responses to water stress was the TCAP-2 (Temporal Clustering by Affinity Propagation, version 2) tool (Kiddle *et al.*, 2010). This tool applied the algorithm AP (Affinity Propagation) (Frey *et al*., 2007) to group the genes according to their expression patterns along the time series, allowing us to see if they were aligned with the dominant pattern or if their responses were delayed or forwarded in time. We used this tool with the groups obtained after the GV selection.

Another clustering analysis was developing after the TCAP-2 with the tool Spline Cluster (SC) (Heard *et al*., 2005). This tool allowed to generate a heat map where the different groups of genes were shown according to their differential response along the time series withred colour indicating an induction in the gene expression and green indicating a repressive response.

-Modelling

On the last step of the sequences analysis, the genes selected after the clustering analysis were subjected to a Variational Bayesian State Space Modelling (VBSSM) which allowed us to determine the interactions between the different genes (Beal *et al.*, 2005; Breeze *et al.*, 2011). Modelling was developed with the Matlab software (The MathWorks) and the results obtained were visualized with the Cytoscape software (Maere *et al.*, 2005), with which the schemes for the genetic models were made selecting the maximum number of interactions to determine (20

seeds) as well as 20 state dimensions. The F-value was automatically calculated at each interaction to determine its strength.

Plant material and growing conditions

In order to biologically validate the model obtained in *Arabidopsis*, 2 homozygous mutant lines of the *Arabidopsis* Columbia 0 (Col0) genotype for the gene AT4G32940, central node of the model, were ordered to the NASC (Nottingham *Arabidopsis* Stock Centre) to check their differential expression of 7 genes in the model as well as their behaviour under drought stress. These mutants, called N681987 and N672354, were SALK lines, segregating flank-tagged T3-generation T-DNA lines generated by vacuum infiltration of Columbia (Col) plants with *Agrobacterium tumefaciens* vector pROK2 (http://arabidopsis.info/). Figure 5.1 represent the insertion zone for each mutant.

At least ten replicates of all insertion mutant lines were grown in 0,3 lpots in compost (Levington F2+S, The Scotts Company, Ipswich, UK). Plants were kept in an 8/16 h light/dark cycle at a photosynthetically active photon flux density (PPFD) of 120 mmol m-2 s-1 at 60% RH and 23 °C.

Molecular analyses

Molecular analyses were applied in order to validate the genotype and gene expression of the mutant lines.

-DNA extraction

Five weeks after the sowing, 150 mg of fully expanded leaves of four plants per mutant were collected, immediately frozen in liquid nitrogen and stored at -80ºC.

ATGGCCACAACGATGACACGTGTCTCCGTCGGCGTCGTCCTCTTTGTTCTCTTAGTC
TCGCTGGTTGCCGTCTCCGCCGCGAGAAGCGGTCCTGATGATGTTATCAAACTCCCT
TCGCAGGCTTCTCGCTTCTTCCGTCCTGCTGAAAACGACGACGATTCTAACTCCGGT
ACTAGGTGGGCTGTTCTAGTCGCCGGATCTAGCGGATATTGGAATTACAGGCATCAG
gtactgttacgactagtctctgtttatttgactctttttctctaatcggaaattttg
aatttgcctccgattgctgctatgtggtgttttggtgtgattctatatactaaagtt
gcggtgtagtttgataggatcagctgaatctactcatattttgaatttttcatgtag
agtttagatacgtttggcatctgatgaactaaggatagtagtagagtttttgtatta
gatactaatgagtttgttaacttttagGCTGATATATGCCATGCCTATCAACTTCT
GAGGAAAGGTGGATTGAAAGAGGAGAATATTGTGGTATTCATGTATGATGATATTGC
TAACAATTACGAGAATCCAAGGCCTGGAACCATTATCAACAGCCCTCATGGAAAAGA
TGTCTATCAAGGAGTTCCCAAGgtcttttgctttcttacgttttgattgattctct
caatatgtgtttactcatttggttgggattattcttcgtatggcagGATTATACTGG
AGATGATGTCAATGTTGATAATCTATTTGCTGTGATCCTTGGAGACAAAACTGCTGT
TAAAGGGGGAAGTGGGAAGGTTGTGGATAGTGGTCCTAATGATCATATCTTCATATT
CTACAGTGACCATGGTGGTCCTGGAGTTCTTGgtgagttccgttatacacacagaaa
ttgaatggttttggaccaacatttttgatgattctgtctttttatttgcagGGATGC
CAACTTCTCCTTACCTATATGCAAATGATCTCAATGATGTCTTGAAGAAGAAACATG
CTTTAGGAACATATAAAAGCTTGgtataaatcgtgaaggttcctctgaaatactttt
gttgttggtgctgcctatgagtaatactaaaaggcaaacctgcagGTGTTTTATCTC
GAAGCTTGCGAATCTGGAAGTATCTTTGAAGGGCTTCTTCCTGAGGGTTTGAACATC
TATGCCACAACTGCATCAAACGCCGAAGAAAGCAGTTGGGGTACCTATTGCCCTGGA
GAGGAACCCAGTCCTCCACCGGAGTATGAAACTTGTTTAGGTGACTTGTACAGTGTT
GCTTGGATGGAAGATAGgtaagctaaagaatccagatacgccaataagagttgcagt
gttctgtttctcatgtttatgaatctctatggatgtcccttatattgattctgattt
gtttaaaatgcaacagTGGTATGCACAATTTACAGACTGAGACTCTGCACCAGCAAT
ATGAACTTgtaagtttctagtttttgtggtttgcctgtaatgatggagctaaaactt
tttcaactgtaaattagattgttagcaaagatgtgcatcataaagtgtg[illegible]
[illegible]GTGAAAAGGAGGACTGCACCTGTTGGGTACTCTTATGGTTCTCATGTCAT
GCAATATGGCGATGTAGGAATTAGCAAGGATAATCTCGATCTTTATATGGGAACAAA
CCCTGCCAATGACAATTTTACCTTTGCGGATGCGAATTCACTAAAGCCACCTTCAAG
AGTTACAAACCAGCGTGATGCAGATCTTGTTCATTTTTGGGAAAAGgttattttcct
ttcttgtttgcatcttttggatacttaatagcttcttggattgctaaatacaatttc
gtttgcatgttattgttttcatgaagTACCGAAAAGCACCAGAAGGTTCAGCAAGAA
AAACAGAAGCTCAGAAGCAAGTACTTGAAGCCATGTCTCACAGACTTCATATTGACA
ATAGCGTGATACTCGTCGGAAAAATCTTGTTTGGCATTTCGAGAGGTCCTGAAGTGC
TAAACAAAGTACGGTCTGCTGGGCAACCTCTAGTCGATGACTGGAACTGCCTTAAAA
ATCAGgtaaataaataggccacttgccccttaagactttgttgtgtcaatcttatca
tcagcccttgtggttagtattgaaaaacgtattggtatagtcatgtcctcatgggat
aacatcaatcatcgtaatggaagatggaaaaaaatctcagatagattgtggagccca
gcactctattattaggacgcaggggctgattgtgacgcaggactctatttactctt
atccttttaggacgtttcagattatgaactcattgactgatgtttcatatagagaag
aactgcataaccttcattgtatgttcagGTGAGAGCTTTCGAGAGGCACTGTGGATC
GCTGTCTCAGTACGGTATCAAGCACATGAGGTCTTTTGCAAACATCTGCAATGCAGG
GATTCAAATGGAGCAAATGGAGGAGGCAGCTTCACAGGCTTGTACCACACTGCCAAC
TGGTCCTTGGAGCTCGCTTAACCGTGGATTCAGTGCATAG

Fig.5.1. Sequence of the *Arabidopsis* gene AT4G32940. Red cases are for introns, the blue and the grey ones for the insertion zone on the mutant lines N681987 and N672354, respectively. Primers positions are coloured green (fordward) and purple (reverse). Transcription origin (ATG) / end (TAG) are marked in yellow.

In order to check if the Salk lines were insertion mutants, total genomic DNA (gDNA) was isolated according to the cetylmethylammonium bromide (CTAB) extraction procedure (Murray *et al*., 1980). After the extraction, samples were resuspended on distillate water and treated with RQ1 RNase-Free Dnase (Promega, Madison, USA) according to manufacture's protocols, to avoid any RNA contamination. The mutations were checked by PCR with specific primers: Fw: 5'-3': CTGAGGGTTTGAACATCTATG /Rv 5'-3': CGCATCCGCAAAGGTAAAAT, situated in the regions where the insertions were supposed to be. Figure 5.1 illustrates the gDNA sequence of the AT4G32940 gene as well as the position of all the primers designed.

-ARN extraction and cDNA synthesis

To check the mutants genotype Total RNA for PCR was isolated using TriZol reagent (Invitrogen, Karlsruhe, Germany) according to manufacture's protocols. Integrity of total RNA was checked on agarose gels and its quantity, as well as purity, was determined using NanoDrop ND1000 (NanoDrop Technologies, Inc., Wilmington, USA). RNA was further purified and concentrated to 1 µg/µl using the RNeasy MinElute Cleanup Kit (Quiagen, Hilden, Germany). Total RNA (5 µg) was reverse-transcribed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Karlsruhe, Germany) using Oligo(dT) as priming method. The absence of genomic DNA was checked by PCR using specific primers situated at the end of the sequence that amplify and exon-intron-exon sequence Fw: 5'-3': ACAAAGTACGGTCTGCTGGG/Rv 5'-3': AGTGTGGTACAAGCCTGTGAA (Fig. 5.1).

PCR reactions were set as follow: polymerase activation (94°C for 3 min), amplification and quantification cycles repeated 20 times for the cDNA samples and 30 for the gDNA samples (94°C for 30 seconds of denaturising, 60°C for 30 seconds

of annealing and 72°C for 1 min of extension) and a final step of 72°C for 6 min before ending (4°C).

-*Quantitative real time PCR* (qPCR) *analysis*

The expression of the two hub genes of the model and four other nodes we checked by *Quantitative real time PCR* (qPCR) in control and droughted plants.

The primers for each sequence were specifically designed for qPCR using the Primer3 v3.0 freeware (primer3.sourceforge.net) and are listed in table 5.2. In the case of the AT4G32940 gene the primers are the same than those used for checking the cDNA contamination.

Table 5.2. Primers sequences the genes selected to analyze the mutant lines.

Gene	Sense	Primer sequence 5´-3´
AT3G10985	Fw	CGTCGTTTCCTTCGGATCTA
	Rv	TCGTCCAGCAACAACGTTAC
AT2G31380	Fw	CTCTAAACCGCCAACTCAGC
	Rv	GGAATGAGCATGAGCCAAAT
AT2G19830	Fw	TGCTTTGAGGACTGGAGCTT
	Rv	CTCAGCGCCTTCTAGTTCGT
AT1G77180	Fw	ACTCTGGTTTCGCTGCTGAT
	Rv	CTCTGAAGCCCCTGTGAAAG
AT5G48220	Fw	GGGGAGCAGCATGTCTTAGT
	Rv	ACTGCATCTGCGCCTTTACT

RNA for qPCR was extracted from 5 replicates per mutant line and controls Col0 leaf material using TriReagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA was treated with RNase-free DNase1 (Ambion) and the absence of contaminating genomic DNA confirmed as described above. RNA (3 µg) of the 3 best quality samples was used to make random-primed cDNA as described by Galvez-Valdivieso *et al*., 2009. Quantitative real-time PCR was performed for at leas two technical replicates in a 96-well plate with a CFX96 Real-

Time System (Bio-Rad, Hercule, CA, USA), using a cybergreen fluorescence based procedure with the Bioline SensiFAST^{TM} SYBR No-ROX One-step Kit (London, UK) according to manufacture instructions. The following standard thermal profile was used for all RT-qPCR reactions: polymerase activation (95°C for 2 min), amplification and quantification cycles repeated 53 times (95°C for 5 seconds of denaturising, 60°C for 10 seconds of annealing and 72°C for 5 seconds of extension) and dissociation curve (65°C to 90°C; ΔT: 0,5ºC s^{-1}).

Relative cDNA levels between two sets of threshold cycle (Ct) values were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalized with respect to relative cDNA levels for CYCLOPHILIN. This reference gene was chosen because it shows unchanging transcript levels under drought stress conditions (Rossel *et al*., 2006).

Phenotyping of the mutant lines N672354 and N681987

We set 2 different experiments under control and water stress conditions in order to evaluate the response of the mutant lines with respect to the control Col0. For all the experiments, plants were transferred into individual pots (filled with identical amount of compost) 2 weeks after the sowing date and were kept well watered until the beginning of the drying episode.

-Rosettes weight assessment

Five rosettes per mutant line and controls were cut from their roots, placed on a disc paper in the growing chamber and weighted every half hour during seven hours to determine the water losses (Bouchabke *et al*., 2008).

-Soil water content assessment

At least 20 replicates per genotype were sown in the conditions previously described. At the same time, three control pots were used to determine 100 and 0% soil water content, named saturated weight (SW) and dry weight (DW), respectively. Five weeks after sowing, half the plants were maintained at well watered conditions, while for the remaining half, water was withdrawn and pot weight was determined daily. Pot weight was determined daily and relative soil water content (rSWC) was calculated, according to the formula rSWC=(FW-DW)/(TW-DW) (Bechtold *et al.*,2010). Pots were left to dry until 15-20% rSWC was reached, at which point the whole rosettes were harvested, immediately frozen in liquid nitrogen and stored at -80°C.

RESULTS

Analysis and selection of sequences and modelling

Following the search across TAIR database a total amount of 557 Pea ESTs related to drought were used to find homologous sequences in *Arabidopsis*. We used the tools blast and tblastx in order to have only one homologous sequence of *Arabidopsis* per EST from pea. Finally, we selected 222 sequences of *Arabidopsis* which were both related to drought stress and homologous to pea.

The 222 *Arabidopsis* CATMA sequences were screened using the GV tool from the PRESTA group. GV profiles clustered all genes in four groups: 54 genes with differential expression at the beginning of the time course, 77 genes with differential expression for the medium stress period, 136 genes which expression was different from the control at the end of the stress period and finally 16 genes which expression was different from the control during most of the time course. Figure 5.2 illustrates the main expression patterns observed for CATMA genes.

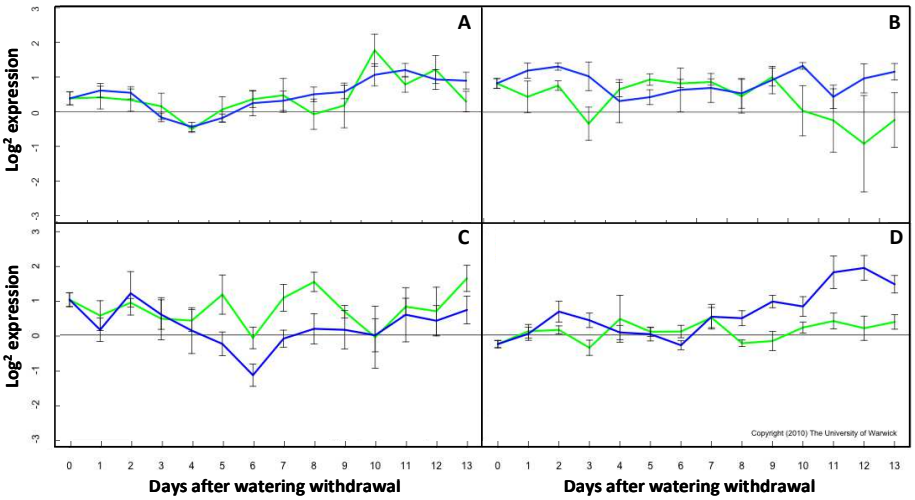

Fig. 5.2. Examples of the different expression patterns observed with the GV tool. Green lines were for the watered controls and blue for the droughted plants. A) No differences observed between control and droughted plants expression patterns (gene CATMA3A40830). B) Differences at the beginning and at the end of the time course (gene CATMA4A20340) C) Differences between the control and droughted plants in the middle of the time course (gene CATMA3A05625). D) Differences at the end of the water stress period (gene CATMA2A18350).

Following GV analysis, the TCAP-2 analytical tool was performed on each group of genes. The algorithm allowed us to group the genes according to their expression pattern along the time course, as well as to determine if the genes were aligned with the dominant expression pattern or if their responses were delayed or forward in time (Fig. 5.3).

Following TCAP2 analysis we obtained different clusters of genes per group and selected those that included more genes with similar expression patterns within each group. The first cluster (Fig. 5.3.A) was made from the genes grouped with GV as early differentially expressed and encompassed 17 of the initial 54 genes.

One of these genes showed an expression pattern delayed with respect to the others and less than a half of the 17 genes were anti-regulated with respect to the main pattern.

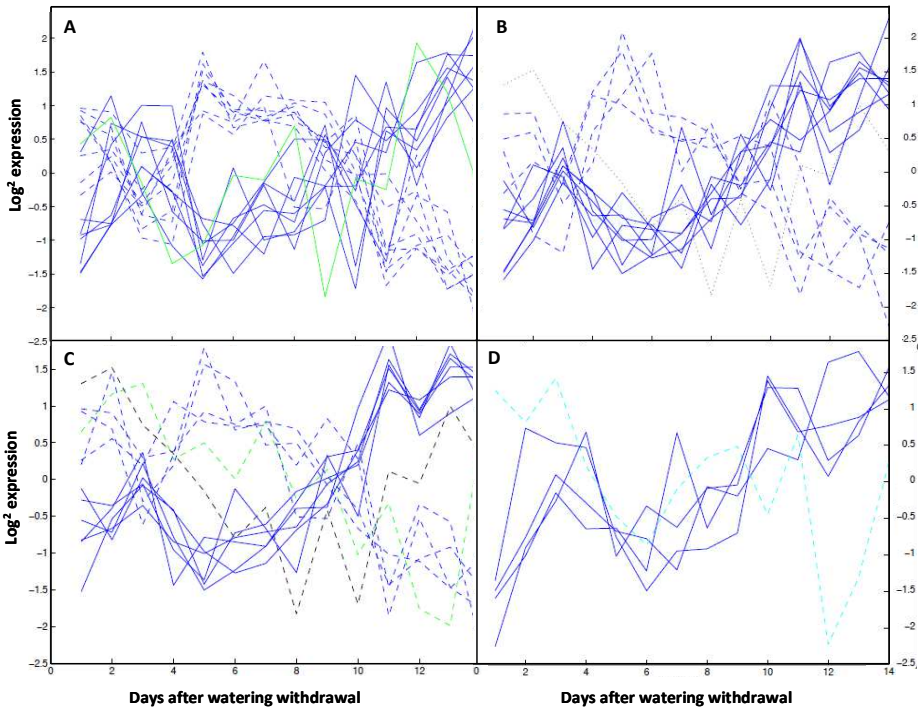

Fig. 5.3. Expression pattern of the grouped genes selected with altered expression under drought stress conditions after the TCAP-2 analysis. Here the clusters selected per group are represented; continuous lines mean co-regulation and discontinuous lines anti-regulation respect controls. Blue colour indicates dominant expression in the group, green lines mean negative delay of gene expression along the time course and the dotted line indicates forward gene expression with respect to the group (only in C).

The second cluster of genes was made from the 77 genes previously grouped as differentially expressed during the middle of the water stress period, selecting 9 co-regulated genes and 5 anti-regulated, one of them forward in time (Fig.5.3. B).

The 136 genes differentially expressed at the end of the water stress period (Fig. 5.3. C), were grouped as 6 co-regulated and 6 anti-regulated genes, one of them delayed in time.

Finally, 5 of the 16 genes with an expression pattern different along the whole time course that were grouped after TCAP as co-regulated and one as anti-regulated (Fig.5.3 D). After TCAP test, we maintained 58 CATMA sequences from the

initial 222. The GV analyses as well as the T-CAP tests were made with CATMA data, but the affymetrix genes (named as AT genes) could correspond with more than one CATMA. Thus, we continued working with AT genes in order to avoid duplicates. EST codes from pea as well as AT homologue sequences and CATMAs from *Arabidopsis* are showed in table 5.2. After the change in nomenclature, the number of sequences was reduced to 33 AT genes.

To corroborate the similarity of response of the selected genes a heat map was generated using the SC tool from the PRESTA group (Fig 5.4). The 33 genes were grouped according to their response to drought stress during 14 time points (each time point for a day after water withdrawal). Red or green coloured blocks indicated an induction or a repression of the gene expression, respectively.

The heat map showed genes from 1 to 16 (Fig.5.4) were induced during most of the time course respect to the controls. Gene 17 was completely repressed with respect to the controls. The third group of genes, 18 to 33 was characterized for a strong repression at the beginning and final period of the drought time course.

These clusters were not in agreement with the groups established through the GV tool, although data came from the same dataset, because the program did not distinguish between the groups those genes whose expression was different in the end than in the beginning of the stress or during the entire temporal course.

As an additional control of the GV selection, the SC tool was also used with all the 222 sequences initially selected and a t-test analysis was applied over the groups separated by the dendrogram in order to find those genes with a maximum expression response.

Table 5.2. Equivalence of the sequences analyzed and selected: 230 ESTs , 222 CATMAs and 206 ATs.

EST	AT	CATMA
RE01_F06	AT5G45010	CATMA5c64764
RE03_H14	AT5G45900	CATMA5A41900
PL02_B02	AT1G50250	CATMA1A41325
RE02_L04	AT1G53020	CATMA1A44050
RE01_A12	AT1G53850	CATMA1A44936
RE03_A08	AT1G55500	CATMA1A46600
PL04_B01	AT1G55510	CATMA1a46603
PL03_B05	AT1G55680	CATMA1A46760
PL05_F11	AT1G59820	CATMA1A48870
		CATMA1A48880
PL03_G02	AT1G61150	CATMA1A50200
PL05_C04	AT1G62250	CATMA1A51320
RE03_P07	AT1G62750	CATMA1A51870
RE01_F19	AT1G63010	CATMA1A52180
RE03_P24	AT1G64040	CATMA1A53285
RE02_F12	AT1G64720	CATMA1A54040
		CATMA1A54045
RE03_L11	AT1G66670	CATMA1A55946
RE01_F12	AT5G58250	CATMA5A54010
RE03_B04	AT5G60600	CATMA5A56310
RE02_E12	AT2G41790	CATMA2A40170
RE02_B01	AT2G42490	CATMA2A40930
PL03_E03	AT2G44100	CATMA2A42525
		CATMA2A42530
RE02_B08	AT2G46290	CATMA2A44670
RE01_D16	AT2G46820	CATMA2A45270
PL01_B01	AT3G03380	CATMA3a02320
RE02_E21	AT3G05970	CATMA3a05020
RE02_O09	AT3G06483	CATMA3a05625
RE03_I13		
PL03_C12	AT3G07700	CATMA3a06960
PL01_F01		
RE02_D02	AT3G09980	CATMA3a08860
RE01_G16	AT5G56290	CATMA5A52095
PL02_E09	AT5G57030	CATMA5A52776
RE01_M16	AT5G48220	CATMA5A44190

EST	AT	CATMA
RE01_C11	AT5G47860	CATMA5A43860
RE02_A07	AT5G47760	CATMA5A43740
RE02_D16	AT2G20990	CATMA2A19620
PL03_D08	AT2G21150	CATMA2a19800
RE03_L14	AT2G24580	CATMA2a22915
		CATMA2A22973
PL05_H09	AT2G26250	CATMA2A24610
RE02_C23	AT2G26280	CATMA2A24640
RE01_M20	AT2G28250	CATMA2A26640
RE01_J14	AT2G29630	CATMA2A28000
		CATMA2A28010
PL05_B09	AT2G31380	CATMA2A29610
RE01_P17	AT2G32080	CATMA2A30355
PL01_H02	AT2G33430	CATMA2A31585
PL03_H10	AT2G33450	CATMA2A31610
PL04_G02	AT2G33800	CATMA2A32000
RE01_B19	AT2G34840	CATMA2A32960
RE01_A09	AT2G39730	CATMA2A37967
RE03_D17		
RE03_N09		
PL01_A03	AT3G58030	CATMA3A51040
PL01_E12	AT3G59020	CATMA3A52050
		CATMA3A52060
PL04_F12	AT3G59780	CATMA3A52800
RE03_J15	AT3G61110	CATMA3c57897
RE01_L18	AT3G61470	CATMA3A54606
RE03_K15	AT3G62030	CATMA3a55155
PL03_D05	AT3G62910	CATMA3A56080
		CATMA3A56090
PL05_D04	AT3G63140	CATMA3A56330
PL03_H02	AT4G01370	CATMA4a01565
RE01_C21	AT4G02080	CATMA4a02367
PL05_F10	AT4G02770	CATMA4a03133
RE01_A01	AT5G54270	CATMA5A50147
RE03_D12	AT5G50100	CATMA5A46020
RE03_F16	AT1G68020	CATMA1A57410

Table 5.2. Equivalence of the sequences analyzed and selected: 230 ESTs , 222 CATMAs and 206 ATs.

EST	AT	CATMA
PL04_H04	AT4G36250	CATMA4A37900
RE03_M03	AT3G10360	CATMA3a09360
PL05_D06	AT3G10985	CATMA3A10050
PL04_E03	AT3G15190	CATMA3A14550
RE01_A19	AT3G16080	CATMA3a15500
RE02_M19	AT3G16290	CATMA3A15690
		CATMA3A15700
RE03_C01	AT3G23800	CATMA3A23750
PL01_F08	AT3G23920	CATMA3A23850
PL04_E05		
RE02_P15	AT3G24190	CATMA3A24120
PL05_G04	AT3G24570	CATMA3A24540
RE01_J23	AT3G25530	CATMA3A25290
RE03_J05	AT3G26710	CATMA3A26460
RE01_F05	AT3G43230	CATMA3A35590
RE01_M19	AT3G44160	CATMA3d01270
PL01_E06	AT3G46780	CATMA3A39870
RE01_N21	AT3G46970	CATMA3A40050
RE01_H17		
RE03_M19	AT3G47810	CATMA3A40830
RE01_K05	AT3G47850	CATMA3A40890
RE01_D17	AT3G49620	CATMA3c57669
		CATMA3c57709
		CATMA3c57834
PL05_D01	AT3G51880	CATMA3A44795
RE02_C03	AT3G53090	CATMA3A46056
RE03_F04	AT3G55140	CATMA3A48150
RE03_H23	AT3G55440	CATMA3A48405
RE01_L20		
PL01_F04	AT3G55610	CATMA3A48595
PL01_C07	AT3G55800	CATMA3A48770
PL04_C09		
PL01_B04	AT3G56940	CATMA3A49925
RE01_N07	AT3G56990	CATMA3A49970
		CATMA3A49980
RE03_N13	AT4G33790	CATMA4A35550

EST	AT	CATMA
RE02_A10	AT5G62790	CATMA5a58375
RE03_C04	AT4G03280	CATMA4a03586
RE01_C05	AT4G04340	CATMA4a04940
RE01_L23	AT4G04770	CATMA4a05320
PL01_D10	AT4G08390	CATMA4a08153
		CATMA4a08156
RE02_N21	AT4G09180	CATMA4a09150
RE01_D13	AT4G09010	CATMA4a08970
RE02_L18		
PL04_G07	AT4G09350	CATMA4a09340
		CATMA4a09350
RE02_O08	AT4G10340	CATMA4A10365
RE01_O11	AT4G11600	CATMA4A11745
RE01_G09	AT4G12230	CATMA4A12320
RE02_O05	AT4G15560	CATMA4A16296
RE03_I01		
PL02_G11	AT4G16450	CATMA4A17300
RE03_H12	AT4G19170	CATMA4A20340
RE03_F19	AT4G19710	CATMA4A20940
PL05_E01	AT4G20330	CATMA4A21580
PL04_F07	AT4G21585	CATMA4A23240
RE03_M16	AT4G21960	CATMA4A23655
RE03_H08	AT4G22100	CATMA4c42445
RE02_L09	AT4G22220	CATMA4A23920
RE01_D06	AT4G22850	CATMA4A24620
PL03_F04	AT4G24190	CATMA4A25920
RE02_F05		
RE02_B10		
RE01_G19	AT4G28750	CATMA4A30435
PL02_A01	AT4G31040	CATMA4A32720
RE03_M09	AT4G31390	CATMA4A33070
PL05_C12	AT4G32410	CATMA4A34150
RE01_L19		
RE02_J10	AT4G32940	CATMA4A34690
RE01_H10	AT4G33070	CATMA4A34810
PL04_A03	AT5G61670	CATMA5A57270

Table 5.2. Equivalence of the sequences analyzed and selected: 230 ESTs , 222 CATMAs and 206 ATs.

EST	AT	CATMA
PL03_B10	AT4G36130	CATMA4A37790
PL04_G11		
RE03_K07	AT4G39710	CATMA4A41065
PL04_F10	AT5G01530	CATMA5a00590
RE03_N08	AT5G03340	CATMA5c64041
RE03_G05	AT5G03900	CATMA5a03090
RE01_B17	AT5G04140	CATMA5a03320
PL01_C12	AT5G05200	CATMA5a04400
RE03_F07	AT5G06060	CATMA5a05250
RE01_B03	AT5G06290	CATMA5a05500
RE02_L08	AT5G09810	CATMA5a08615
RE03_N07	AT5G09830	CATMA5c64147
PL02_H02	AT5G13630	CATMA5A11860
RE02_N01		
RE02_A19	AT5G17920	CATMA5A16195
RE01_B08	AT5G19140	CATMA5A17550
PL01_H01	AT5G19440	CATMA5A17870
RE03_L03	AT5G20490	CATMA5A18970
RE01_N08	AT5G22060	CATMA5c64363
RE02_I12	AT5G25360	CATMA5c64430
RE02_A23	AT5G43330	CATMA5A39190
RE02_L16	AT1G01320	CATMA1a00310
RE03_A23	AT1G06690	CATMA1a05730
RE02_F06	AT1G07180	CATMA1a06230
RE03_N01	AT1G08450	CATMA1a07425
PL02_E02	AT1G08510	CATMA1a07475
RE03_O10	AT1G11860	CATMA1A10870
PL01_D12	AT1G15820	CATMA1A14860
RE03_G12	AT1G16470	CATMA1A15446
RE02_L14	AT1G17220	CATMA1A16230
RE02_K12	AT1G18260	CATMA1c71273
PL01_G05	AT1G19150	CATMA1A18195
RE01_A17	AT1G19800	CATMA1A18810
RE01_C07	AT1G21600	CATMA1A20670
RE02_E16	AT1G23740	CATMA1A22630
PL01_D06	AT1G30580	CATMA1A28620

EST	AT	CATMA
RE02_N24	AT5G63570	CATMA5A59100
RE01_J20	AT5G64170	CATMA5A59630
RE02_H05	AT5G66090	CATMA5A61480
RE02_K18	AT5G66420	CATMA5A61750
RE02_J17	AT5G67030	CATMA5A62480
RE03_K03		
RE01_P19	AT5G58110	CATMA5A53890
RE01_E23	AT1G68520	CATMA1A57900
PL04_B06	AT2G40100	CATMA2A38335
PL04_H07	AT1G68010	CATMA1A57406
RE01_D11	AT5G13650	CATMA5A11880
RE02_K11		
RE01_I17	AT5G27280	CATMA5A24640
RE03_F23	AT5G28840	CATMA5A26920
PL04_E06	AT5G34850	CATMA5A29650
PL02_H11	AT5G35360	CATMA5A30475
RE03_E10	AT5G35410	CATMA5A30530
RE01_J15	AT5G38820	CATMA5A34420
RE01_O16	AT5G42270	CATMA5A37990
RE01_H13	AT1G50200	CATMA1A41290
RE01_F07	AT1G68590	CATMA1A57970
		CATMA1A61020
RE02_C14	AT1G71810	CATMA1A61030
PL03_B09	AT1G72020	CATMA1A61240
RE01_O23	AT1G73030	CATMA1A62260
PL03_F09	AT1G74470	CATMA1A63880
RE02_A06		
RE01_L09	AT1G76080	CATMA1A65310
RE01_O17		
PL04_B02	AT1G76940	CATMA1A66170
PL01_C01	AT1G77090	CATMA1A66300
RE03_M08	AT1G77180	CATMA1A66400
RE02_J13	AT1G77490	CATMA1A66671
		CATMA1A66682
RE03_A20	AT1G78630	CATMA1A67690

Table 5.2. Equivalence of the sequences analyzed and selected: 230 ESTs , 222 CATMAs and 206 ATs.

EST	AT	CATMA
RE01_E12	AT1G31170	CATMA1A29400
PL02_D12	AT1G31420	CATMA1A29670
PL01_D05	AT1G32550	CATMA1a30942
PL03_B03	AT1G32700	CATMA1A31040
RE01_O15	AT1G35670	CATMA1A33863
RE02_B18	AT1G36240	CATMA1a34350
PL01_G06	AT1G42960	CATMA1A36290
PL02_H10	AT1G43670	CATMA1A37020
PL05_C10	AT1G49380	CATMA1A40500
RE01_P04	AT1G49970	CATMA1A41065
PL01_A05		
PL03_C09	AT2G19830	CATMA2A18350
PL01_A08	AT5G54680	CATMA5A50510
PL02_D02	AT4G34350	CATMA4A36180
RE02_N13	AT1G30880	CATMA1A29030

EST	AT	CATMA
RE01_P23	AT1G79920	CATMA1A69100
PL01_C11	AT1G80600	CATMA1A69800
RE02_I22	AT2G13360	CATMA2A11975
PL01_B10		
PL04_D05		
RE02_G09		
RE03_E07	AT2G17972	CATMA2a16640
RE01_O10	AT2G14740	CATMA2c47181
		CATMA2c47257
RE02_D05	AT2G18020	CATMA2c47258
RE02_C17	AT2G18950	CATMA2a17540
RE01_L02	AT5G13770	CATMA5A12010
RE02_F24	AT4G35760	CATMA4A37420
RE03_F24	AT1G79040	CATMA1A68150

Finally, a second SC analysis was made over these groups of genes to corroborate their responses. The results obtained with the SC analysis were similar to those obtained with the GV, so we determined that the gene selection from GV expression patterns was correct.

A number of genes from this interaction model seemed to be potentially interesting for drought stress studies. The annotation and function of each node is described in Table 5.3.

For the last step, the 33 genes selected were subjected to a VBSSM analysis, which allowed us to determine the interactions among the different genes. The F values obtained were of 100% for all the interactions indicating strong relationships between the genes.

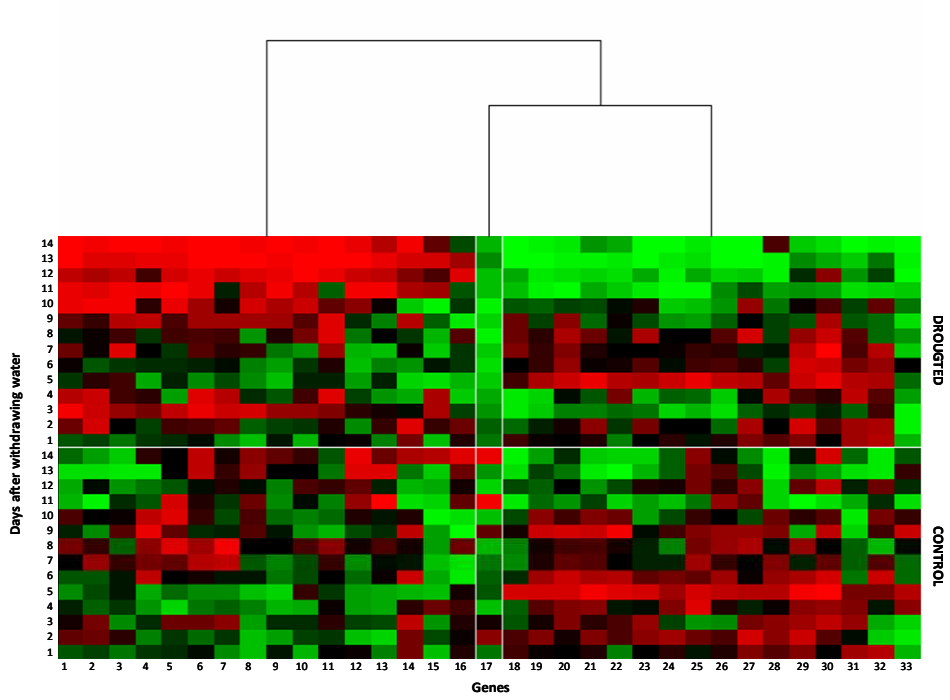

Fig.5.4. Dendrogram of the 33 gene selected expression under water stress and control conditions during a time course of 14th days. Red colour is for induction and green for repression. SC divided the genes in three groups (genes 1 to 16, gene 17 and genes 18 to 33) according to their expression.

Data obtained from Matlab software were visualized with the Cytoscape free software, with which the schemes of the models were generated. Three models were generated and the same structure was obtained for the three of them (Fig. 5.5).

The main node corresponded to the GAMMA-VPE (AT4G32940) gene. The secondary node was the gene SNF7.2 (AT2G19830), encoding a vesicle-mediated transport related protein.

Among the interesting genes, we could distinguish the APX4 gene (AT4G09010), which encodes an ascorbate peroxidase, enzyme with a protective function under high light and drought stress (Mittler *et al*., 1994; Rossel *et al*., 2006) the putative transcription factor SKIP (AT1G77180), involved in responses to abscisic

acid, salt and osmotic stress (Lim *et al*., 2010) and the SAG20 (AT3G10985), which expression is induced in response to necrosis and codes for a senescence-associated protein and STH transcription factor (AT2G31380).

Thus, although all genes from the derived model seemed to be related with drought stress, some of them might have a direct physiological function directly implied in drought tolerance.

Table 5.3. Annotations of the genes represented in the model. Data obtained from the TAIR database (www.arabidopsis.org).

ATGs	Gene name	Function of the protein encoded
AT4G32940	GAMMA-VPE	Vacuolar processing enzyme cysteine-type endopeptidase.
AT2G19830	SNF7.2	Vesicle-mediated transport.
AT4G09010	APX4	Microsomal ascorbate peroxidase .
AT5G54270	LCHB3	Component of the main light harvesting chlorophyll a/b-protein complex of PS II.
AT5G48220	TIM barrel family protein	Indole-3-glycerol phosphate synthase.
AT1G77180	SKIP	Putative transcriptional factor.
AT2G31380	STH	Salt Tolerance Homologue (STH) transcription factor of the zinc ion binding type.
AT2G33800	PSRP-3/Ycf65	Ribosomal protein S5 family protein, structural constituent of ribosome.
AT3G43230	RING/FYVE/PHD-type zinc finger family protein	Phosphatidylinositol binding, zinc ion binding, metal ion binding.
AT3G10985	SAG20	Senescence-associated, gene expression induced in response to necrosis.
AT3G62030	ROC4	Related with response to ABA in *Arabidopsis thaliana* seeds.
AT3G23920	BAM1	Chloroplast beta-amylase1, necessary for leaf starch breakdown in the absence of BAM3.
AT3G24570	MOB24.15	Peroxisomal membrane family protein.
AT1G63010	F16P17.18	General substrate transporter.
AT3G63140	CSP41A	Protein with ribonuclease activity that is involved in plastid rRNA maturation.
AT4G39710	FKBP16-2	Involved in protein folding.
AT4G04770	LAF6	Involved in Fe-S cluster assembly and the regulation of iron homeostasis. Interacts with AtNAP7 inside the chloroplast.
AT2G33450	F4P9.22	Structural constituent of ribosome involved in translation.
AT5G35360	CAC2	Fatty acid biosynthetic process.

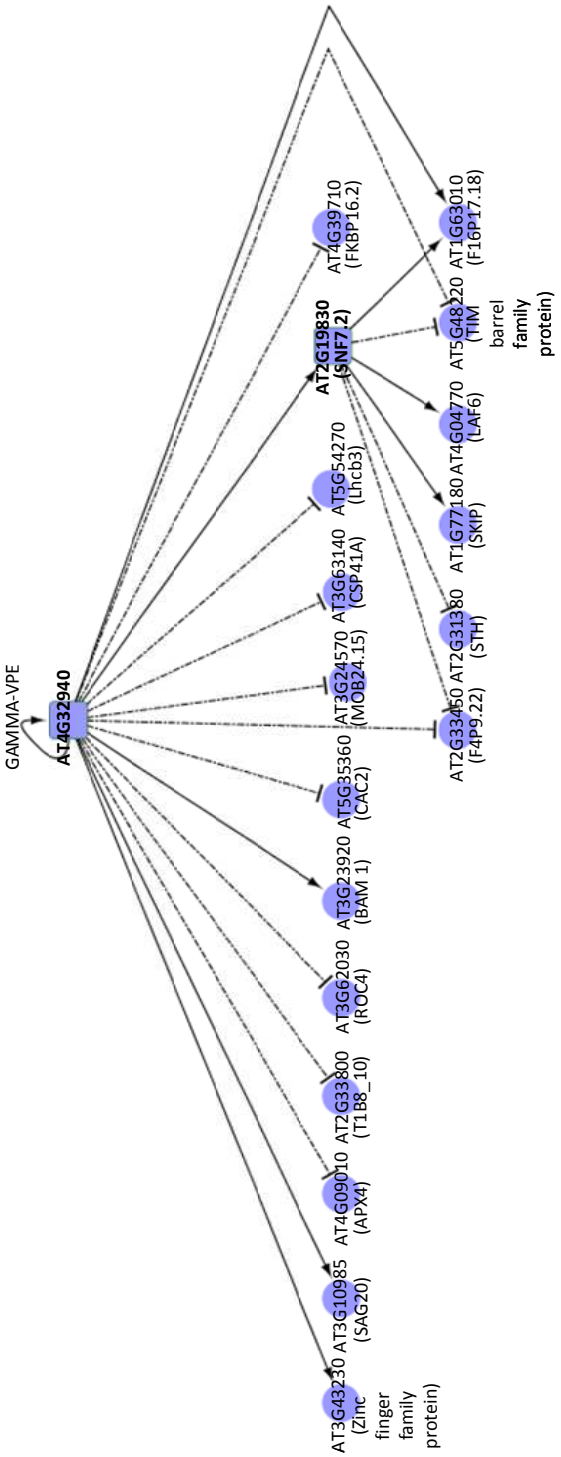

Fig 5.5. Genetic net work model generated with the drought stress related *Arabidopsis* sequences homologues to pea sequences and the *Arabidopsis* under drought stress microarray data from the PRESTA group. The dash and dot lines represent negative interactions, continuous lines positive interactions between two genes.

This result, along with the stability of the model revealed following 3 replicates of the VBSSM analysis suggested the validation of the model with mutant lines.

Mutant phenotypes

Two homozygous Salk lines from *Arabidopsis* (N672354 and N681987) were found in the NASC database (http://arabidopsis.info/), both mutants for the GAMMA-VPE gene, the main node of the derived model. Unfortunately, only a heterozygous mutant line was found for the secondary node, corresponding to the SNF7 gene, so we decided not employ it by the moment.

We observed some phenotypic differences between the mutant lines and the wild type (Fig.5.6). Surprisingly the 2 mutant lines showed a different phenotype, different also from the wild type.

Fig 5.6. Phenotype of the *Arabidopsis* mutant lines N681987 and N672354 opposite to the wild type Col0.

The genotype N681987 grew slower than Col0, whereas the N672354 line grew slightly faster than the wild type. However, these differences were not so clear in older plants.

Check of mutations

To check if the mutations were truly present in the mutant lines N672354 and N681987, we designed specific primers located within their insertion zones and try to amplify each region using cDNA (Fig. 5.5 A) and gDNA (Fig. 5.5 B).

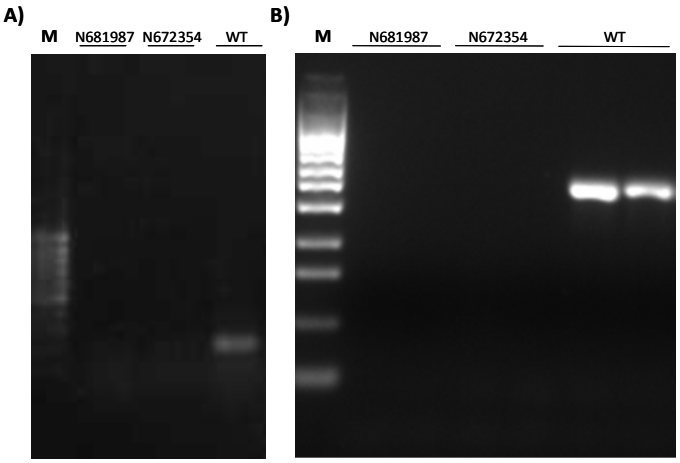

Fig.5.5. Agarose gel electrophoresis of cDNA (A) and gDNA (B) of the *Arabidopsis* N681987 and N672354 mutant lines, using the wildtype Col0 (WT) as a control for the amplification with primers specifics for the insertion zones. M: molecular markers.

cDNA corresponding to the GAMMA-VPE was amplified in the wild type line but no in the mutant (Fig. 5.5.A). This indicated that the insertions were present and in the mutants since primers were located in the terminal part of the sequence (Fig. 5.1).

In addition, no amplification of gDNA was observed for the mutants when we tried to amplify the genomic region where the insertions were supposed to be, whereas, a gDNA band of the expected size on the lines corresponding to each pair of primers was observed in the wild type (Fig. 5.5. B).

This indicated that the insertions were located within the tested gene sequence. Thus, we concluded that as expected, both lines mutants were Knockout for the GAMMA-VPE gene.

Rosettes weight and Soil Water Content (SWC) assessment under drought

The behaviour of the mutant lines N672354 and N681987 was explored by two different drought experiments.

On the first one, the rosettes were weighted every half hour along 7 hours and differences (p<0,05) from the wild type during the first time points were showed (Fig 5.7. A). The second one consisted on a temporal course of 9 days during which the SWC was assessed (Fig 5.7.B). Interestingly, the mutant lines showed significant differences (p<0,05) from the wild type after nine days from water withdrawal.

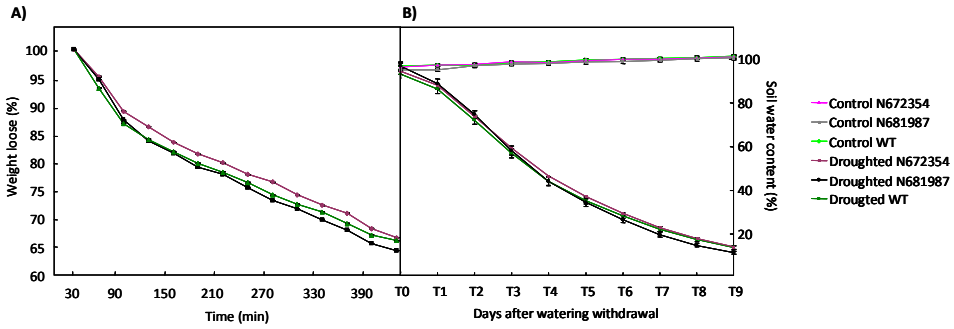

Fig 5.7. Assessment of the rosettes weighted every half hour during 7 hours (A) and soil water content during 9 days after withholding water (B) of the *Arabidopsis* mutant lines N672354 and N681987 and the control wild type (WT) Col0.

Water looses in the rosettes of the mutant line N672354 were significantly lower with respect to the Col0 genotype during most of the studied period (p<0,01), whereas the line N681987 showed higher water looses than both N672354 and Col0. SWC assessment did not confirm this trend for the mutants, not showing significant differences from the wild type.

Analysis of gene expression patterns under drought

To determine whether some of the water stress responsive genes of the genetic model were transcriptionally affected we chose some of them and check their expression by qPCR analyses in the mutant lines and the wild type in droughted and control plants. Fig.5.8 illustrates the gene expression patterns of the mutants with respect to the wild type Col0. A gene was considered induced when its expression relative to the wild type was higher than 1,5 and repressed when the

value for its expression was lower than 0,5. Thus, expression values between 0,5 and 1,5 were considered equal to the wild type.

As expected, the GAMMA-VPE (AT4G32940), main node of the model, was repressed in control and droughted plants of both mutant lines respect to the wild type, confirming them as knockout mutants for the GAMMA-VPE.

The SNF7.2 (AT2G19830) gene, secondary node of the model was supposed to have a direct relationship with the GAMMA-VPE (Fig. 5.5).

SNF7.2 was slightly induced in control conditions in the mutant line N681987 and not different from the wild type in the mutant line N672354. On the contrary, in drought conditions it was equal to the wild type in the mutant line N681987 and slightly repressed in drought conditions in the line N672354, showing expression levels lower than 0,5 but close to that value.

The gene SKIP (AT1G77180) followed a similar pattern than the SFN7.2 and was slightly induced in control conditions in the line N681987 and slightly repressed in drought conditions in the mutant N672354. TIM barrel family protein (AT5G48220) and SAG20 (AT3G10985) were both repressed under control and drought conditions in the mutant line N672354 and only in drought conditions in the line N681987. The gene SAG20 was more repressed in the line N672354. No significant changes in the expression of these genes were found in the mutants in control conditions.

Finally, the gene AT2G31380, encoding a STH transcription factor, showed high levels of transcript in both mutant lines. This gene was inducted with respect to the wild type in control and drought conditions, but the induction was attenuated in the mutant N681987 under drought conditions.

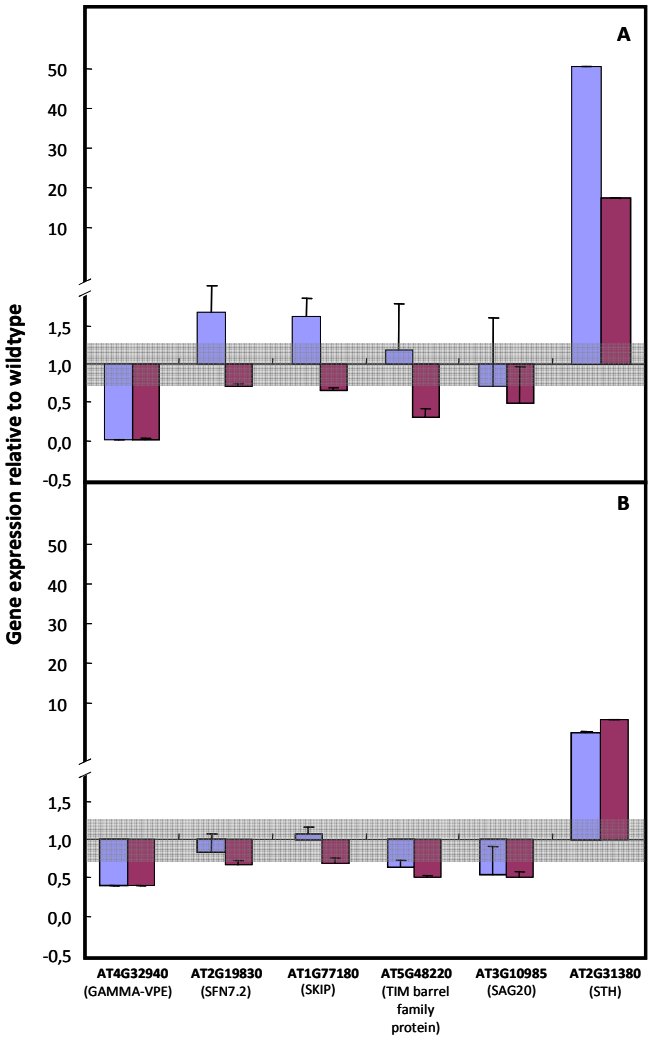

Fig. 5.8. Transcripts levels in 6 drought related genes in rosettes of the *Arabidopsis* mutants (A) N681987 and (B) and N672354 respect to the wild type levels in control (blue bars) and droughted (purple bars) conditions. The coloured grey zone indicates the levels of gene expression with no significant changes in their induction or repression respect to the wild type. Transcript levels were normalized with respect to CYCLOPHILIN and referred to the wild type.

Although both mutants were knockout, the mutant line N681987 showed a pattern of gene expression more altered than the mutant N672354 with respect to the wild type both in control and drought conditions. However, the differences between mutant lines and wild type clearly indicated an effect of the mutation over the gene expression patterns and underlined the relationship between the two nodes of the model as well as the genes studied.

DISCUSSION

Genomic resources available in pea are scarce and the use of model plants is thus necessary to unravel the molecular complexity of gene expression under complex plant-stress interaction. From model plants, genetic information can be moved to crops exploiting genome synteny and taking advantages of conserved molecular pathways, including those controlling stress tolerance (Cativelli *et al*., 2008). Molecular analysis of the model plant *Arabidopsis* has sketched the complex network constituting cell communication during drought response. Thus, in the present work, starting from a cDNA library of pea expressed under drought stress, we have searched and selected highly homologue sequences of *Arabidopsis*. This allowed filtering them from a drought gene expression dataset of a microarray by using informatics approaches. Microarray technology employing cDNAs or oligonucleotides has been proof as a powerful tool for analysing gene expression profiles of plants exposed to abiotic stresses such as drought, high salinity, or cold (Seki *et al*., 2001, 2002; Kreps *et al*., 2002). In our work, this technology along with clustering bioinformatics tools allowed us to group the homologue sequences according to their differential expression patterns under drought and control conditions.

The sequences grouped and selected after clustering analysis were used to infer the network whereby the genes they represented were linked. Deduction of gene networks correctly from gene expression measurements can lead to a better understanding of cellular processes and therefore have applications to stress studies. Bayesian networks are a widely used approach to model gene networks in biological systems, especially in ecological studies and medicine (Livak *et a*l., 2001; Smith *et al*.,

2002; Imoto *et al*., 2003; Ott *et al*., 2004). Recent studies also applied Bayesian probability in microarray gene expression (Liu *et al*., 2009; Wrzaczek *et al*., 2010; Bonett *et al*., 2010) and transcriptomics studies (Ruckle *et al*., 2012). In Bayesian networks, the behaviour of the gene network is modelled as a joint probability distribution for all genes, allowing a very general modelling of gene interactions (Ott *et al*., 2004). The presence or absence of a directed edge from one gene to another indicates the states of those genes are dependent or independent, respectively. This implies their regulatory relationship, and the regulatory interactions among genes and their directions are derived from expression data. Thus, when the expression data are given, we can use the Bayesian structure learning to capture interactions (Liu *et al*., 2009).

Therefore, our model predicted direct interactions between the main node, the GAMMA-VPE gene and the genes RING/FYVE/PHD-type zinc finger family protein, SAG20, BAM1, F16P17.18 and SNF7.2, which was the secondary node. It also predicts negative interactions between the GAMMA-VPE and the genes APX4, PSRP-3/Ycf65, ROC4, CAC2, MOB24.15, CSP41A, TIM barrel family protein, LCHB3 and FKBP16-2.

Regarding the secondary node, our model predicted a positive interaction between it and the F16P17.18, SKIP and LAF6 genes as well as a negative interaction with the STH, F4P9.22 and TIM barrel family protein. It should be noted that F16P17.18, TIM barrel family protein and F4P9.22 were co-dependent of the main and secondary node and only the F16P17.18 gene, encoding and structural constituent of ribosome involved in translation showed a positive interaction with both of them.

Gene annotations provided us further information about the network based in previous experimental evidences which could be useful to validate biologically the model (Álvarez-Buylla *et al*., 2007). For instance, the VPEs are a family of enzymes up-regulated in association with various types of cell death and under stressed conditions, mediating the susceptible response of toxin-induced cell death (Grudkowska *et al*., 2004; Kuroyanagi *et al*., 2005). This gene has been reported to increase its expression under treatments as wounding, ethylene and salicylic acid (Kuroyanagi *et al*., 2005) as well as senescence, necrosis and several stress conditions (Weaver *et al*., 1998; Kinoshita *et al*., 1999; Keates *et al*., 2003).

Interestingly, the GAMMA-VPE was not the only gene studied related with senescence. The TIM barrel family protein gene was possible indirectly related with the auxin metabolism, as part of the Tryptophan-dependent synthesis pathway (Ouyang *et al*., 2000). The phytohormone auxin regulates many biological processes, from cell division, elongation and differentiation to root initiation, tropistic responses, flowering, fruit ripening and senescence (Davies, 1995). The interaction between these two genes would be negative, according to the model prediction. The biological sense of this interaction would rely in a possible reduction of the growing processes triggered by water stress.

Also, the SNF7.2 family of proteins is involved in protein sorting and transport from the endosome to the vacuole/lysosome in eukaryotic cells. This gene would have a positive interaction with the GAMMA-VPE, which biologically made sense as vacuoles/lysosomes play an important role in the degradation of both lipids and cellular proteins. In order to perform this degradative function, vacuoles/lysosomes contain numerous hydrolases which have been transported in

the form of inactive precursors via the biosynthetic pathway and are proteolytically activated upon delivery to the vacuole/lysosome (Babst *et al*., 2002). Vacuole/lisosome transport is a mechanism highly important in many plant physiological processes, including stress responses (Mazal *et al*., 2004; Valluru *et al*., 2008).

Interestingly, genes described as directly related with stress showed negative interactions with the main nodes of the model. For instance the STH gene, which is a transcription factor previously described to be induced under cold, salt and drought stress (Kreps *et al*., 2002). Also the LCHB3, which has been reported as photoprotection implied (de Bianchi *et al*., 2011) and the APX4, antioxidant enzyme reported in pea with a protective function and expressed under drought stress (Mittler *et al*., 1994). This allowed us to launch the hypothesis that, as senescence and drought stress regulation seemed to have opposite regulation, and that knockout mutants for the main node of our model could be resistant to drought and experiment lower senescence than the wild type. The consistency and the strong interactions between the genes of our model pointed out the high probability of these genes within it to be related, albeit the model should be further validated biologically to confirm the interactions between the genes and improve our knowledge of drought gene networks.

In order to get this aim, two homozygous mutant lines of *Arabidopsis* for the main node of our model, the GAMMA-VPE gene were used. The two mutant lines showed a different pattern of growth between them and respect to the wild type. Differences between the two mutants might be caused by the vacuum infiltration method, as the T-DNA could be inserted in more than one place within

the *Arabidopsis* genome (Krysan *et al*., 1999). Due to the nature of the mutants, the predicted direct and indirect relationships proposed in the model between the genes should not be taken literally when we were trying to validate it biologically, as possibly there was not only a single one mutation affecting to the genome. Therefore, it was necessary to identify if the mutations on these lines had effects on their drought tolerance or susceptibility phenotype, in order to prove the relationships between the genes predicted by the model to be truth.

The mutant lines also showed opposite responses in the first moments of desiccation with respect to the wild type when cut rosette weight was measured. We observed that the mutant line N672354 suffered lower water looses than the N681987. As they were mutants for the GAMMA-VPE gene and this gene is closely related with the senescence we would expect a phenotype of tolerance with respect to the wild type, being thus the mutant N672354 the one that pointed towards it. However, the methods we employed did not allow a clear discrimination between susceptible or tolerant lines. Possibly, an increased drought time course would allow us to establish such differences.

The lack of a phenotype for *Arabidopsis* knock-out mutants is a common problem and several reports have shown that is presumably caused by the ability of higher plants to adapt their physiology to various stresses without undergoing morphological changes and by our inability to detect slight physiological alterations and/or weak reductions in fitness and partial or complete functional redundancy (Bouché *et al*., 2001). Therefore, the evaluation of other traits related with drought such as relative water content in leaves (Bechtold *et al*., 2010) or the assessment of stomata conductance or aperture (Mustilli *et al*., 2002; Bouchabke *et al*., 2008) used

in previous reports will also might be useful in discriminating between the mutant and the wild type, clarifying the role of the GAMMA-VPE gene in drought response. However, we should take into account that if the mutant phenotypes were too similar to the wild type could be due functional redundancies, as the mutated gene function would be necessarily important for the plant (Bouché *et al*., 2001). Gene expression analysis would thus be useful to understand the effects of the mutations as well as to determine their effects over the genes in the model.

Expression analysis confirmed no expression of the GAMMA-VPE in any of the mutants under control or drought conditions, confirming both mutants as knockouts for this gene.

Regarding the SNF7.2 gene, secondary node of our model, the expression pattern of this gene with respect to the wild type was in accordance with the predictions of the model, although it was different in both mutants. Thus, this gene was repressed under drought conditions in the mutant N681987, and not expressed at all in the mutant N672354. This pointed towards the mutant line N681987 as being only effectively affected by the mutation in the GAMMA-VPE. At any rate, this expression pattern highlights the relationship between the GAMMA-VPE and this secondary node.

The most determinant evidence of the GAMMA-VPE mutation was observed throughout the alteration of the STH expression. In agreement with previous reports we observed an induction of this gene under drought conditions, being the expression rates much higher in the mutant N681987 than in the N672354. The alterations on the expression patterns in the mutants with respect to the wild type

confirmed a deregulation with a common origin in the GAMMA-VPE, as well as underlined the differences between the two mutant lines.

The mutants showed differences in the pattern expression of the studied genes not only with the wild type, but also within themselves. These differences could also be related with those observed while phenotyping. Regarding the rest of the genes analyzed, most of them showed and altered expression in the mutants with respect to the wild type. This fact agreed with the direct effect of the mutation over the rest of the genes in the model and thus with the existing relationship between them. The mutant line N672354 seemed to be affected by more than one mutation, whereas N681987 was likely to be a knockout just for the GAMMA-VPE. However, as both mutations are supposed to affect the same gene, further studies should be carried with both mutants together in order to clarify the way their mutations are affecting their drought tolerance or susceptibility.

While the model has been validated in *Arabidopsis*, work is in progress to validate the model in pea. To this aim the sequences from the GAMMA-VPE and the SNF7.2 genes are being used to isolate pea TILLING mutant lines for genetic and physiological characterisation under drought. Future prospects will lead us to analyze to what extent the results obtained with *Arabidopsis* were valid, finding pea mutants for these target genes in the TILLING platform and thus intending to verify the gene function.

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CONCLUSIONS

Chapter 1. Identification and characterization of drought tolerance sources in pea (*Pisum sativum* L.)

1- Water related parameters and visual scale highlighted P665 and Polar as those genotypes able to maintain the highest levels of turgor in the plant tissues during the water stress period.

2- The physiological studies pointed out to multi factorial tolerance response mediated by different mechanisms in genotype P665, whereas for cv. Polar, polyamine-based osmoregulation is one of the main factors involved in its tolerance to drought stress.

3- According to our analyses, genotypes Polar and P665 would be sources of drought tolerance and thus interesting for its use in breeding programs.

Chapter 2. Multi-environment assessment of yield, growth, phenology and natural biotic and abiotic stress in ten pea (*Pisum sativum* L.) genotypes.

1-The partitioning of genotype (G) and genotype-by-environment (GE) interaction through GGE biplot analysis showed that the first two principal components were significant factors for most of the studied traits, justifying the use of this analysis with data from multi-environment trials

2- Environmental grouping indicated the presence of two mega-environments for pea growing within the Mediterranean basin defined by temperature and altitude differences.

3- Field data revealed differences among genotypes. Thus, HR-1 was the genotype more stable for all the evaluated traits, showing slow phenology and low biomass. Cultivars Frisson and HR-1 were the less affected by frost. ZP-108 showed the highest biomass in the Tunisian environments. In addition, Solara showed a good yield, although with low stability, and the highest seed weight of all the studied genotypes.

4- Despite their performance was highly influenced by the environments, cultivars Polar and Kebby were the highest yielding cultivars. These cultivars were less affected by broomrape, and showed the fewest symptoms of powdery mildew, although they were moderately affected by frost.

5- Field studies together with the high drought tolerance levels observed under controlled conditions define Polar as the most interesting cultivar to be used in breeding.

Chapter 3. Effects of the interaction between *Fusarium oxysporum* f.sp. *pisi* infection and drought stress.

1- A suitable method to assess the simultaneous effects of drought and *Fusarium oxysporum* f.sp. *pisi* (*Fop)* stress in pea was developed.

2- Sources of resistance to simultaneous *Fop* infection and water deficit were identified. Thus, New Season would be the genotype with higher resistance and tolerance to both *Fop* and drought stress, followed by Polar, New Era and JI1412.

4- The genotype JI1412 was slightly less affected by both stresses than by *Fop* stress alone, pointing towards the existence of tolerance responses to drought that could mediate in a slight increase of *Fop* resistance when both stresses were applied simultaneously.

Chapter 4. Mapping quantitative trait loci associated to relative water content in pea (*Pisum sativum* L.).

1- Differences among the parents Messire and P665 on the relative water content (RWC) allowed the search of QTLs associated to RWC over a recombinant inbred line population (RIL).

2- Four QTLs associated to different regions in pea genome and explaining phenotypic variation of RWC were identified, suggesting the polygenic control of RWC in pea.

3- The existence of transgressive RILs showing lower RWC values than Messire and the detection of one QTL related with high RWC associated to this genotype suggested the existence of some alleles promoting high RWC under water stress in Messire.

4- Among the identified QTLs, *rwct16-1* (LGIII) derived from P665 was co-located with a QTL previously associated with root lenght. QTL *rwct16*-2 was located in a genomic region involved in broad spectrum resistance and other physiological processes. QTL *audpc* was in the same genomic region as *n°t2*, a QTL controlling the number of broomrapes per root length. QTL *rwct16-3*, was associated to the SNP marker tRALs_SNP1, which could efficiently be used for marker assisted selection in segregating populations derived from Messire.

Chapter 5. Modelling gene networks for drought stress related genes from pea (*Pisum sativum* L.) and experimental validation.

1- 206 sequences from *Arabidopsis* homologues to 230 sequences from pea related with water stress were identified.

2- Bioinformatics tools developed for *Arabidopsis* allowed to infer a genetic model network whereby the genes they represented were linked. This model predicts direct interactions between the GAMMA-VPE gene and genes related with senescence and stress in *Arabidopsis*.

4- Interactions predicted by the model were confirmed by gene ontology and gene expression analysis in *Arabidopsis* mutants for the GAMMA-VPE gene.

6- Expression analysis confirmed both mutants as knockouts for the GAMMA-VPE gene. The expression patterns of the genes tested were in agreement with the predictions of the model.

CONCLUSIONES

Capítulo 1. Identificación y caracterización de fuentes de tolerancia a la sequía en guisante (*Pisum sativum* L.)

1- Los parámetros relacionados con el agua así como la escala visual señalaron P665 y Polar como genotipos capaces de mantener mayor turgencia en sus tejidos durante el periodo de estrés hídrico.

2- Los caracteres estudiados indicaron una tolerancia multi factorial en el genotipo P665, mientras que en el cultivar Polar, la osmorregulación basada en las poliaminas sería uno de los principales factores implicados en su resistencia al estrés hídrico.

3- Según nuestros análisis, los genotipos Polar y P665 serían fuentes de tolerancia a la sequía y por lo tanto interesantes para su introducción en un programa de mejora.

Capítulo 2.Evaluación en múltiples ambientes del rendimiento, crecimiento, fenología y estrés biótico y abiótico natural en diez genotipos de guisante (*Pisum sativum* L).

1- La partición de la interacción Genotipo (G) y Genotipo-por-ambiente (GE) mediante el análisis GGE biplot mostró que las dos componentes principales fueron factores significativos para la mayoría de caracteres evaluados, justificando el uso de este análisis con los datos obtenidos en múltiples ambientes.

2- El agrupamiento de los ambientes indica la existencia de dos mega-ambientes dentro de la cuenca Mediterránea para el cultivo de guisante definidos por distintas temperaturas y altitud.

3- Los datos de campo revelaron diferencias entre los genotipos. Así, HR-1 fue el genotipo más estable para todos los caracteres evaluados, mostrando lenta fenología y baja biomasa. Los cultivares Frisson y HR-1 fueron los menos afectados por heladas. ZP-108 mostró la mayor biomasa en los ambientes tunecinos. Además, Solara tuvo buen rendimiento, aunque baja estabilidad, y también el peso de sus semillas fue el mayor entre todos los genotipos estudiados.

4-A pesar que su comportamiento estuvo fuertemente influido por los ambientes, los cultivares Polar y Kebby tuvieron el máximo rendimiento. Estos cultivares fueron menos afectados por jopo y mostraron los menores síntomas de oidio, aunque fueron moderadamente afectados por heladas.

5- Los estudios de campo unidos a los altos niveles de tolerancia a la sequía observados en condiciones controladas definen Polar como el cultivar más interesante para ser utilizado en mejora.

Capítulo 3. Efectos de la interacción entre la infección por *Fusarium oxysporum* f.sp. *pisi* y estrés hídrico en 7 genotipos de guisante (*Pisum sativum* L.).

1- Se ha desarrollado un método apropiado para evaluar los efectos simultáneos del estrés por sequía y *Fusarium oxysporum* f.sp. *pisi* (*Fop)* en guisante.

2- Se han identificado fuentes de resistencia a la acción simultánea de *Fop* y sequía. Así, New Season fue el genotipo con mayor resistencia y tolerancia a ambos estreses, seguido por Polar, New Era y JI1412.

3- El genotipo JI1412 fue ligeramente menos afectado por ambos estreses que cuando sólo fue infectado por *Fop* indicando la posible existencia de respuestas de tolerancia que podrían mediar en un ligero aumento de la resistencia de este genotipo a *Fop* cuando ambos estreses se aplicaron simultáneamente.

Capítulo 4. Mapeo de loci de caracteres cuantitativos asociados al contenido relativo de agua en guisante (*Pisum sativum* L.)

1- Las diferencias entre los parentales Messire y P665 en el contenido relativo de agua en condiciones de estrés hídrico permitieron la búsqueda de loci de caracteres cuantitativos (QTLs) asociados al contenido relativo de agua (RWC) en una población de líneas recombinantes congénitas (RIL).

2- Se han identificado cuatro QTLs asociados a diferentes resgiones del genoma del guisante y explicativos de la variación fenotípica del RWC, lo que sugiere el control poligénico de este carácter en guisante.

3- La existencia de RILs transgresoras con menores valores de RWC que Mesire y la detección de un QTL relacionado con alto RWC asociado a este genotipo sugirió la existencia de algunos alelos que promueven un alto RWC en Messire.

4- Entre los QTLs identificados, *rwct16-1* (LGIII), procedente de P665 estaba situado en el mismo lugar que un QTL asociado con longitud de raíces. El QTL *rwct16*-2 se localizó en una región genómica implicada en resistencia de amplio espectro y también en otros procesos fisiológicos. El QTL *audpc* estaba en la misma región genómica que *n°t2*, un QTL que controla el número de jopos por longitud de raíz. El QTL identificados, *rwct16-3* está asociado al marcador molecular SNP marker tRALs_SNP1, que puede

utilizarse eficientemente como primer paso para la identificación de individuos susceptibles en poblaciones segregantes procedentes de Messire.

Capítulo 5. Modelado de redes génicas para genes relacionados con sequía en guisante (*Pisum sativum* L.) y validación experimental.

1- Se han identificado 206 secuencias de *Arabidopsis* homólogas de 230 secuencias de guisante relacionadas con estrés hídrico.

2- Distintas herramientas bioinformáticas desarrolladas para *Arabidopsis* permitieron inferior un modelo genético de red en el que los genes incluidos se encontraban asociados.

3- El modelo predice interacciones entre el gen de la GAMMA-VPE y genes relacionados con senescencia y estrés en *Arabidops*is.

4- Las interacciones predichas por el modelo fueron confirmadas por ontología génica y análisis de expresión génica en mutantes de Arabidopsis para el gen de la GAMMA-VPE gene.

5- Los análisis de expresión génica confirmaron ambos mutantes como knockouts para el gen de la GAMMA-VPE. Los patrones de expression de los genes analizados coincidieron con las predicciones del modelo.